Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i18.3200

ISOLATION, CHARACTERIZATION, AND IDENTIFICATION OF MULTIPLE HEAVY METAL AND ANTIBIOTIC-RESISTANT BACTERIA FROM WASTEWATER

Muhammad Bakir Hussain[1], Saba Abbas[2*], Hafiz Khawar[3], Muhammad Raza[2], Hubba Chaudhary[3], Hafiza Nida Shehzadi[2], Noor Fatima[4], Zeemal Seemab Amin[5], Muhammad Hamza[6]

[1]Laboratory manager, Horizon hospital Lahore,
[2*]School of Medical Laboratory Technology, Faculty of Allied Health Sciences, Minhaj University Lahore,
[3]Institute of Industrial Biotechnology, Government College University, Lahore,
[4]University of Central Punjab, Lahore.
[5]School of Biochemistry, Faculty of Applied Sciences, Minhaj University Lahore.
[6]Quality Assurance Officer CSH pharmaceutical Lahore.

[*]**Corresponding Author:** Saba Abbas
Email: sabaabbas786786@gmail.com

ABSTRACT

Heavy metals are the most persistent pollutant in wastewater and cause several health and environmental hazards, contaminate soil, and reduced plant growth and productivity. Novel biotechnological approaches through inoculating bacterial strains might be adopted for the remediation of wastewater containing heavy metals. The study was conducted to reduce the toxicity of heavy metals in wastewater through inoculation of bacterial strains. The wastewater samples were collected under aseptic conditions from wastewater canals in Lahore. Bacterial strains were isolated by using nutrient agar media amended with 50 µg mL^{-1} of heavy metals e.g. Zn, Cd, Co, and Hg. The multiple heavy metal-resistant bacterial strains were screened for different biochemical and morphological characteristics. Furthermore, selected multiple-heavy metals tolerant strains were evaluated resistance for multiple antibiotics resistance under *in vitro* conditions. A total of eighty multiple heavy tolerant bacterial strains were isolated from industrial effluents. The biomass of these multiple heavy metals-resistant bacterial strains was recorded in terms of optical density (OD). The strains with heavy maximum OD in 50 µg mL^{-1} were selected for antibiotics resistance and revealed five bacterial strains were most resistant against antibiotics. The best-performing strains were identified as *Klebsiella sp*. strain BH49 and *Salmonella sp*. BH67 heaving accession numbers of MT074326 and MT074327, respectively, through 16S rRNA partial gene sequencing. Those identified strains might be used as a bioremediation agent for the efficient removal of heavy metals in contaminated wastewater.

Keywords: Bioremediation, heavy metals, minimum inhibitory concentration, phylogenetic tree, wastewater treatment

1.0 Introduction

Water is crucial to supporting all forms of life on the earth's crust. It has a dipolar nature because it can dissolve almost all solvents. It acts as a solvent to dissolve the solutes found in the bodies of living organisms. It is helpful to carry out all the metabolic activities (Ishak *et al.,* 2011). Water constitutes found in the human body is 71 % and all the biochemical and biological processes are carried out with the help of water. All living organisms need water to live, and it plays a key role in maintaining the balance of the ecosystem (Sharpe, 2003).

Pakistan has the best canal irrigation system, but the demand for water for domestic use and agriculture is greater than its availability. Pakistan has an intensive cropping system, and the population is also increasing day by day, so domestic demand for agricultural produce is increasing abruptly (Ghafoor, 1999). Arid and semiarid regions in Pakistan are more humid and rain-fed. Annual rainfall is approximately 7–25 cm in Arid and semiarid areas, which is not enough to attain maximum agriculture production efficiency. In the USA, the canal system provided one cusec of water to irrigate 70 acres in 1995, while in Pakistan, this quantity of water is used to irrigate almost 350 acres (Ansari, 1995). The water shortage and its increasing demand are big problems in Pakistan and Saudi Arabia. Farmers are using brackish water and wastewater to irrigate their lands. This water contains heavy metal contamination and a heavy amount of dissolved salts(Al-Rashed and Sherif, 2000)and the brackish water is being used at domestic levels and for agricultural purposes to get maximum crop production. The wastewater contains heavy metals and carries a huge amount of nutrients. Thus, it is good for crop production, but it also contaminates the soil and plants with carcinogenic metals (Ertek *et al.,* 2002).

Water quality at the domestic and agricultural levels is very important. Plants use contaminated water and accumulate all the elements in their bodies, including heavy metals and salts, which in one way or another affect human health. Production quality is greatly affected by the quality of the water (Bauder *et al*., 2004). Some chemicals that contain heavy metals are highly carcinogenic and are not good for animals or human health as well. Government bodies are working against the use of such types of chemicals as Hg, Pb, and Cd, and public health authorities are worried about the presence of these toxic chemicals in rivers and seafood (Khaniki *et al.,* 2005).

When water is contaminated with a variety of contaminants, as in the case of sewage water or wastewater, it may become the place for the growth of different types of microorganisms, which may have the potential to spread a variety of diseases to animals and humans. Sewage water is unprocessed water collected from different sources, such as domestic sources, hospitals, and industries (Sharpe, 2003). Microorganisms are viewed as most valuable in the expulsion of overwhelming metal particles from defilement zones. Remembering the significance of substantial metal-safe microbes in bioremediation, the present examination was arranged with destinations of segregation and distinguishing proof of substantial metal-resistant bacterial strains, assurance of least inhibitory focus, and anti-infection affectability designs against various anti-toxins (Cismasiu, 2001). Organisms' decomposers regularly convert dangerous substances into simple forms, which they can use in their metabolic procedures for their development. Microorganisms and parasites act as decomposers, which convert macromolecules into items that they can consolidate in their digestion (Pattanapipitpaisal *et al*., 2002).

Some of the modern procedures result in the arrival of heavy metals into water bodies, which is alarming about the impact of poisonous heavy metals as ecological toxins. This sort of pollution introduces a test, as the presence of overwhelming metals in soils and fluid effluents prompts major issues since they can't be biodegraded. In contrast to numerous poisons, overwhelming metals are hard to expel from the earth (Ren *et al.,* 2009). The nearness of high concentrations of lethal heavy metals in wastewater straightforwardly prompts both tainting of accepting water bodies and injurious effects on sea life(Moten *et al*., 1998). Some heavy metals are hazardous with no known job; different metals are crucial for life at a low level and turn out to be poisonous at high levels (Shi *et al*., 2002). These heavy metals include zinc (Zn), lead (Pb), chromium (Cr), cadmium (Cd), silver (Ag), arsenic (As), iron (Fe), mercury (Hg), copper (Cu), and platinum (Pt). Soil and water pollution

increase with high levels of these metals. The concentration of these metals increases when the polluted material is dumped in the sea, lakes, and rivers without proper treatment (Gadd, 1992; Badar *et al*., 2000; Franke *et al.,* 2003).

Every element has a specific quality. Heavy metals are characterized by some qualities, like an atomic weight from 63.5 to 2006.6 with a density greater than 5 g/cm³ (Fu and Wang, 2011). Some heavy metals are very important even in very small amounts; most of them are very toxic to human health at high concentrations (Adarsh *et al*., 2007). Heavy metals are naturally present in the environment, and the main source of these metals is volcanic eruptions (MacKenzie and Canil, 2008) or other sources are pesticides, human activity, paints, batteries, dying colors for clothes, different coal industries, and coal combustion (Wuana and Okieimen, 2011). Industrial waste contains different types of toxic pollutant carbons and hydrocarbons (Diya'uddeen *et al*., 2011), heavy metals, and toxic compounds such as CR, Cd, Ni, Pb, Cu, As, and Zn(Barakat, 2011).

The heavy metal contents can be diminished by the industries up to an acceptable level in the sewage water (Dabrowski, 2004). Various methods are being used to remove heavy metals from the waste of industries, such as adsorption (Mohan and Pittman, 2007; Ngah and Hanafiah, 2008), precipitation,ion exchange,electrodialysis, and membrane filtration (Fu and Wang, 2011). Plant and microbial biomass, along with substantial metal particles, could be an economical and eco-accommodating choice for heavy metal remediation (Ahluwalia and Goyal, 2007). A few microbes can proficiently lessen heavy metal defilement from wastewater by creating extracellular polysaccharide containers in which metal particles are chelated (Nies, 1999).

Natural procedures are considered financially and ecologically benevolent strategies for the remediation of overwhelming metal-tainted soils (Congeevaram *et al*., 2007). Microbes that can make substantial metals can be utilized as operators of bioremediation, through which immobilization and distinctive change procedures can be performed. The procedure of bioaccumulation is effectively performed, which depends on the fuse of metals inside the biomass that ingests the metal particles at the cell surface through different instruments (Raghavan and Sang, 2008). Microscopic organisms, *viz*. *Bacillus* sp., *Pseudomonas* sp., and *Klebsiella* species can be detached through the use of substantial metals, and metals fluctuate. Microbes that develop on metals play a significant role in the biogeochemical cycling of metal particles (Haferburg and Kothe, 2010). It was studied that there is a connection between anti-toxin opposition in microbes and metal resilience as two opposing qualities that are firmly related to the plasmid of the microbes or on the DNA chromosome of microscopic organisms (Piddock, 2006). Numerous researchers separated and identified various types of metal-safe microbes from different water sources and soil, and these accounted for heavy metal absorption (Abo-Amer *et al*., 2014).

Some steps were taken towards expelling metal particles from the fluid arrangement by utilizing innovative approaches that comprise physical, synthetic, and natural advances. Regular techniques like concoction precipitation, flocculation, layer filtration, particle trade, and electrodialysis are expensive or ineffective for evacuating or lessening toxic fixation (Wang and Chen, 2009). Bioremediation of substantial metals by using microorganisms has gained extraordinary consideration, particularly for its potential industrial application. This is a result of their non-destructive characteristics, shoddy utilization, and prudent utilization (Rehman *et al.,* 2012). Substantial metal-tolerant microbes may have a critical job in the dirt treatment of metal poison. The potential of these microbes to detoxify the metal poison can be controlled for bioremediation purposes, particularly for evacuating the overwhelming metal sulfide in both wastewater and soil. Effluents containing heavy metals can be treated with these microbes by adopting a few procedures, including biosorption, bioaccumulation, and bio-precipitation.(Rajbanshi, 2008). Biosorption utilizing microbes had been concentrated to sequester metal particles from the watery arrangement, which was known as the shabby elective technique compared with traditional procedures. This was because of the utilization of easy sorbent material in the biosorption process(Nameni *et al.,* 2008). Microscopic organisms having the potential to collect metal can be utilized in metal remediation by evacuating, thinking about, and recuperating metals from mechanical effluents (Chowdhury *et al.,*

2008). A few reports have appeared on indigenous microbes that could endure overwhelming metal fixations in various ways and may assume a huge job in the rebuilding of a polluted site (Ge *et al.*, 2009). Disengagement of microbes from metal-contaminated conditions ought to be done to locate the metal-safe strain applicants that could be utilized for overwhelming metal evacuation and bioremediation purposes (Malik, 2004).

Mining activities have been identified as a major contributor to soil sulfation with substantial metals. Mining tasks regularly create vast amounts of waste materials, which contain high concentrations of overwhelming metals, *viz*., Cu, Zn, Fe, Mn, Ni, Pb, and Cd (Monica *et al*., 2008) and can result in far-reaching pollution of soils and water bodies. Today, soil pollution, groundwater, silt, surface water, and air with substantial metals speak to a genuine risk to the earth and the soundness of every single living life form since most metals are profoundly dangerous and can't be debased like carbon-based atoms and accordingly persevere in the earth indefinitely (Navarro *et al*., 2008). Consequently, the cleanup of metal-polluted materials is important for natural and human well-being and safeguarding. In such a way, a few physiochemical techniques, for example, precipitation, particle trade, switch assimilation, electrodialysis, and ultrafiltration, are usually used to expel metal particles from fluid media (Hashim *et al*., 2011).

Bacterial biomasses can be utilized *in situ*, are more efficient, don't produce synthetic compounds or organic slop, offer the possibility of metal recuperation, are cost-effective and can be effectively incorporated with various other remediation advancements (Malik, 2004). It has been discovered that metal-tainted situations more often contain microbes that display a cluster of biochemical and hereditarily encoded systems to defeat the dangerous impacts of overwhelming metals in their environment (Lee *et al*., 2006). These may incorporate efflux frameworks that expel metal particles from the cell by methods for transport frameworks, intracellular sequestration of the metal by specific metal ion restricting proteins, extracellular precipitation into complex mixes, and enzymatic change of metal particles to less-lethal animal categories (Yan and Virarghavan, 2000).The present study was designed to isolate, identify, and characterize the heavy metals in brackish water. To identify the antibiotic-resistant bacteria, present in brackish water and also check the role of bacteria in reclaiming or poisoning the water.

3.0 Materials and Methods

3.1 Study Area and samples collection

The water samples were collected aseptically from sewage water from different waste canals. Water samples were collected in a sterile bottle and brought to the laboratory at a temperature of 4 °C. These water samples were stored in the Laboratory at the recommended temperature (2–6 °C). The 100 mL of water samples were used for isolating the bacteria through serial dilution and agar plate culture techniques.

3.2 Isolation of bacteria

The heavy metal-tolerant bacterial isolates were isolated based on the principle that reduced bacterial colony numbers could be obtained by taking a water sample containing a microbial population. The wastewater samples were diluted through the serial dilution method up to 10^{-5} (Rajbanshi, 2008). The 100 µL of wastewater samples from each of the dilutions were poured on nutrient agar plates and incubated at 37 ºC for 24 h. Bacterial colonies with variable morphology were selected for purification (Collins *et al*.,1989). The resulting bacterial colonies were purified through the strike plate method and preserved in 40% glycerol stock at -20 ºC.

3.3 Screening of heavy metal resistance

The isolated bacterial strains, including Zn, Cd, Co, and Hg, were screened for heavy metal resistance. The Luria Bertani (LB) agar medium amended with 300 µg mL^{-1}, Zn was prepared, autoclaved, and inoculated through the strike plate method. The plates were incubated at 3°C for 48 h, and results were recorded in terms of resistance to Zn toxicity after observing growth in culture

plates. The Zn-resistant isolates were screened for Cd resistance. The LB agar medium amended with 300 μg mL^{-1} Cd was prepared, autoclaved, and isolates were inoculated. The plates were incubated at 37 ºC for 48 h. The appearance of bacterial growth on Cd-amended agar media was considered as Cd-resistant isolates. Furthermore, such isolates were inoculated on Co amended LB media, and after incubation, bacterial growth was considered Co resistant. The Zn, Cd, and Co-resistant bacterial strains were screened for Hg tolerance. The 300 μg mL^{-1} amended LB agar plates were autoclaved and inoculated with Zn, Cd, and Co-resistant isolates. After 48 h of incubation, the bacterial growth on Hg-amended LB media was considered Hg-resistant isolates. The control plates each for Zn, Cd, and Co amended LB media were also run simultaneously without inoculating bacterial isolates to check the microbial contamination (Haq and Shakoori, 1998). The Zn, Cd, Co, and Hg resistant bacterial isolates were termed as multiple heavy metal resistance and were purified and preserved at -20 ºC until further experiments.

3.4 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of heavy metals including Zn, Cd, Co, and Hg at various concentrations starting from 300 μg mL^{-1} up to 1000 μg mL^{-1} The LB agar media amended separately with Zn, Cd, Co, and Hg were prepared to have respective heavy metal concentrations and screened for a minimum inhibitory concentration of these selected heavy metals. The bacterial strains were streaked on respective heavy metal-amended media and incubated for 48 h at 37 ºC. The MIC concentration was identified by identifying the gentle incline in the growth of bacterial strains.

3.5 Characterization of bacterial strains

To differentiate the bacteria and determine whether they belong to gram-positive or gram-negative groups, gram staining was performed. A thin smear of culture was made on glass slides, the smear was dried and heat fixed, and covered one by one with crystal violet (60 seconds), gram's iodine (60 seconds), 95% C_2H_5OH (20 seconds), and safranin (40 seconds). Air-dried the slides after washing them with distilled water and observed them under a microscope. Morphological characteristics such as shape, size, and color were studied by microscopic observation. The shape of the colony can be studied by observing its margin and elevation, and the colonies may fall into either a round, rod, or coccid shape. The size of the bacteria was investigated by microscopic observation, and it can be calculated in millimicrons. The size of the colony was measured by the scale. The color of the bacteria was identified by observing the colony under a microscope. The unknown cultures were performed more to clarify the organism by Biochemical tests, which include an amylase test, catalase test, citrate test, indole test, methyl red test, urease test, and Voges-Proskauer test. These tests were performed according to standard methods (Collins *et al*.,1989).

3.7 Antibiotic resistance assay

Antimicrobial Sensitivity testing was performed on Mueller-Hinton agar. It was first evenly implanted throughout the plate with the desired isolate that was diluted at a standard concentration (approximately 1 to 2 x 10^8 colony-forming units per ml). Commercially prepared discs, each of which is pre-seeded with a standard concentration of a required antibiotic (ampicillin, azithromycin, doxycycline, cefuroxime, cefixime, ceftriaxone, ceftazidime, ciprofloxacin, erythromycin, gentamycin, tetracycline, imipenem, and meropenem), were dispensed and impregnated on the agar surface. The test antibiotic immediately begins to diffuse outward from the disc, creating a gradient of antibiotic concentration in the agar such that the highest concentration was found close to the disc with decreasing concentrations further away from the disc. Growth around each disc was examined after overnight incubation at 37 °C.Tested isolates were susceptible to a particular antibiotic, and a clear area of "no growth"was observed aroundthatparticulardisc. The zone around an antibiotic disc that has no growth was referred to as the zone of inhibition since this approximates the minimum

antibiotic concentration sufficient to prevent the growth of the test isolate. Plates without antibiotics were also used as controls(Cooper, 1995).

3.8 Identification through 16S rRNA sequencing

Sequence analysis of the 16S rRNA gene is used as a powerful mechanism for identifying new pathogens for routine identification of bacterial isolates (Patel, 2001). Sequence identification was useful for slow-growing, unusual, and fastidious bacteria as well as for bacteria that are poorly differentiated by conventional methods. The strains were identified by using a partial sequence of the 16S rRNA gene on MEGA 7.0.14 software and BLASTn searches on NCBI servers. Sequences of closely related, validly published type strains (n = 15) were used for constructing the phylogenetic tree and retrieved from the MEGA database. The phylogenetic and molecular analyses were performed with selected closely related taxa according to the procedure described previously (Roohi *et al*., 2012) using MEGA version 7.0.14 (Kumar *et al*., 2016). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and presented in the units of the number of base substitutions per site (Mumtaz *et al*., 2017).

4.0 Results

4.1 Isolation of bacteria strains

A total of eighty bacterial strains were isolated on nutrient agar (NA) medium by serially diluting the water samples. Bacterial cultures from the mixed cultures were purified using the single colony method. Bacterial strains were purified by picking up discrete bacterial colonies aseptically and inoculating them on the NA-containing Petri plates. All the bacterial strains inoculated on plates were incubated at 26 ± 2 ºC for 48 hours. Pure bacterial colonies were preserved in 20% glycerol-containing sterilized vials and stored at -80 ºC until further use in the experiments. A total of 80 bacterial isolates were recovered from the samples and preserved at -80 ºC.

4.2 Screening of bacteria strains for multiple heavy metal tolerance

A total of 80 bacterial strains coded BH1, BH2, BH3, . . . BH80 were subjected to multiple heavy metal tolerance tests, and results are presented in Table 4.1. Pure bacterial strains were inoculated on Zn-amended LB agar medium, and out of 80 tested bacterial strains, 71 strains were found to be resistant to Zn metal, while nine isolated bacteria were susceptible to Zn metal. In the Cd-amended medium, out of 71 tested bacterial isolates, 56 strains displayed a resistance response against the Cd metal, while 15 bacterial isolates were found sensitive. Out of 56 bacterial isolates tested in a Co-amended growth medium, 39 showed resistance responses against Co metal. A total of 39 bacterial isolates displaying resistance responses against Zn, Cd, and Co metals were further screened against Hg metal. Out of all the tested bacterial strains, five bacterial strains, including BH 1, BH18, BH27, BH49, and BH67, showed resistance responses against Hg heavy metal, and these five bacterial isolates were declared multiple heavy metal strains. These resistant bacterial strains were further subjected to morphological studies, MIC detection, and antimicrobial resistance studies.

Table 4.1: Screening of bacterial isolates for Resistant and Sensitive response against heavy metals.

Bacterial Isolates	Zn	Cd	Co	Hg
BH1	R	R	R	R*
BH2	R	R	R	S
BH3	S	NT	NT	NT
BH4	S	NT	NT	NT
BH5	R	R	R	S
BH6	R	R	R	S
BH7	R	S	NT	NT
BH8	R	R	S	NT

BH9	R	R	S	NT
BH10	R	R	R	S
BH11	R	R	R	S
BH12	R	R	R	S
BH13	R	S	NT	NT
BH14	R	R	R	S
BH15	R	R	S	NT
BH16	R	S	NT	NT
BH17	R	R	R	S
BH18	R	R	R	R*
BH19	R	R	R	S
BH20	R	R	S	NT
BH21	R	S	NT	NT
BH22	R	S	NT	NT
BH23	R	R	S	NT
BH24	R	R	S	NT
BH25	R	R	R	NT
BH26	R	S	NT	NT
BH27	R	R	R	R*
BH28	R	S	NT	NT
BH29	R	S	NT	NT
BH30	R	R	R	S
BH31	R	R	R	S
BH32	S	NT	NT	NT
BH33	R	R	S	NT
BH34	S	NT	NT	NT
BH35	R	R	R	S
BH36	R	R	R	S
BH37	S	NT	NT	NT
BH38	R	R	R	S
BH39	R	R	R	S
BH40	R	R	S	NT
BH41	R	S	NT	NT
BH42	R	R	S	NT
BH43	R	R	R	S
BH44	R	R	R	S
BH45	S	NT	NT	NT
BH46	R	R	R	S
BH47	R	R	R	S
BH48	R	R	R	S
BH49	R	R	R	R*
BH50	R	R	R	S
BH51	R	R	S	NT
BH52	R	S	NT	NT
BH53	R	R	S	NT
BH54	R	R	S	NT
BH55	R	R	R	NT
BH56	R	R	R	S
BH57	R	R	R	S
BH58	S	NT	NT	NT
BH59	R	R	S	NT
BH60	R	R	R	S
BH61	R	R	R	S
BH62	R	R	S	NT

BH63	S	NT	NT	NT
BH64	R	S	NT	NT
BH65	R	R	R	S
BH66	R	R	R	S
BH67	R	R	R	R*
BH68	R	R	R	S
BH69	R	R	S	NT
BH70	R	S	NT	NT
BH71	R	R	R	S
BH72	S	NT	NT	NT
BH73	R	R	R	S
BH74	R	R	S	NT
BH75	R	S	NT	NT
BH76	R	R	R	S
BH77	R	R	S	NT
BH78	R	R	R	S
BH79	R	R	R	S
BH80	R	S	NT	NT

R = resistant, S = sensitive, and NT = not tested

4.3Determination of Minimum Inhibitory Concentration (MIC) of heavy metals against bacterial strains.

The results of bacterial strains on minimum inhibitory concentration (MIC) against heavy metals are shown in Table 4.2. Data showed that in the case of the Zn-amended medium, the MIC value ranged between 2 and 6 µg/ml against all the tested bacterial isolates. In the case of Cd treatments, MIC values ranged from 0.8 to 2 µg/ml against all the tested bacterial strains. The maximum MIC value was recorded at 2 µg/ml against BH 27, while the lowest was observed at 0.8 µg/ml against BH 67. The MIC in the case of Co metal varied from 1 to 4 µg/ml for all the bacterial strains. In the treatments of Hg heavy metal amended medium, MIC varied between 0.7 and 1.4 µg/ml against all the bacterial strains.

Table 4.2. Minimum Inhibitory Concentration (MIC) of heavy metals against bacterial strains.

Heavy Metal	**BH1**	**BH18**	**BH27**	**BH49**	**BH67**
Zn	33 mM	66 mM	22 mM	99 mM	99 mM
Cd	33 mM	22 mM	11 mM	66 mM	66 mM
Co	22 mM	22 mM	22 mM	66 mM	66 mM
Hg	33 mM	22 mM	11 mM	33 mM	33 mM

4.4 Bacterial Strains and morphological characteristics

Five bacterial isolates, viz., BH1, BH18, BH27, BH49, and BH67, were subjected to morphological and biochemical studies, and results are given in Table 4.3. In the case of the Gram reaction, out of five tested bacterial strains, BH18 showed a positive reaction towards the Gram staining reaction, while the rest of the bacterial isolates were Gram Negative. Morphological studies under a microscope have confirmed that all the bacterial strains were rod-shaped, and except for BH49, all the bacterial strains were motile, while BH49 was a non-motile bacterium. The colony color of BH1 was pink or red, while BH18 was creamy in appearance. BH 27 was green to brown in colony color, and BH 49 was pinkish in appearance. BH 67 was opaque or yellow in colony color. When cultured on MacConkey agar media, all the bacterial isolates showed positive test results. Maximum colony growth in BH 1 was observed at a pH range of 5.7–8.0, while BH 18 displayed maximum colony growth at a pH range of 4.5–8.5. In the case of BH 27, the pH range was observed at 4.0–8.0, while for BH 49, the pH ranged from 7.0–7.2, and for BH 67, the pH range was recorded at 6.5–7.5.

All the bacterial strains showed variations at different temperatures. BH 1 strain showed maximum

growth at 37 ℃, followed by BH 18 at 35 ℃ and BH 27 at 32 ℃, while BH 49 and BH 67 showed maximum growth at 35–37°C. In the Indole test, BH 1 showed positive test results, while all other bacterial agents showed negative test results. In the methyl red test, out of 5, two bacterial isolates, BH 1 and BH 67, showed positive test results. When cultured on Voges-Proskauer medium, only one bacterial strain, BH 49, showed positive test results. Two bacterial strains, BH 1 and BH 49, displayed positive test results, while in the case of the Citrate test, all the bacterial strains except BH 67 showed positive responses.

Of the five bacterial strains, three, BH 18, BH 27, and BH 49 showed positive test results for the urine test. All the tested bacterial strains except BH 69 showed positive responses for the Catalase test, while BH 18 and BH 27 were positive for the oxidase test. In the ONPG test, one bacterial strain, BH 49, showed a positive test result, while BH 27 and BH 67 were positive for H_2S production. Based on morphological and biochemical features, BH 1 was identified as *E. coli,* while BH 18 belonged to *Bacillus*. BH 27 was identified as *Pseudomonas*, BH 49 as *Klebsiella,* and BH 67 as *Salmonella*.

Table 4.3. Morphological and Biochemical characteristics of Metal Resistance Bacterial strains.

Characteristics	BH1	BH 18	BH 27	BH 49	BH 67
Gram Reaction	-	+	-	-	-
Cell Type	Rod	Rod	Bacilli	Rod	Bacilli
Motility	+	+	+	-	+
Colony Color	Pink/Red	Creamy	Green, Lt Brown	Pinkish	Pale
On MacConkey Agar	+	+	+	+	+
Growth at PH	5.7 - 8.0	4.5 - 8.5	4.0 - 8.0	7.0 - 7.2	6.5 - 7.5
Temperature	37 ℃	35 ℃	32°C	35–37°C	35–37°C
Indole Test	+	-	-	-	
Methyl Red Test	+	-	-	-	+
Voges-Proskauer	-	-	-	+	-
Lactose	+	-	-	+	-
Citrate	+	+	+	+	-
Urease	-	+	+	+	-
Catalase	+	+	+	+	-
Oxidase	-	+	+	-	-
ONPG	-	-	-	+	-
H2S Production	-	-	+	-	+
Strain Type	*E. coli*	*Bacillus*	*Pseudomonas*	*Klebsiella*	*Salmonella*

4.5 Sensitivity and Resistance Response of Bacterial Strains against Antibiotics

In vitro sensitivity and resistance responses of bacterial strains against antibiotics such as ampicillin, azithromycin, doxycycline, cefuroxime, cefixime, ceftriaxone, ceftazidime, ciprofloxacin, erythromycin, gentamycin, tetracycline, imipenem, and meropenem were tested, and results are given in Table 4.4. Results showed that BH 1 was sensitive to cefixime, ampicillin, amikacin, trimethoprim + sulfamethoxazole, chloramphenicol, ceftriaxone, cefuroxime, and ceftriaxone while showing resistance against ciprofloxacin, amoxicillin, and clavulanic acid, as displayed in Figure 4.1. BH 27 showed sensitivity against tazobactam/piperacillin, imipenem, meropenem, levofloxacin, polymyxin B, cotrimoxazole, aztreonam, tobramycin, cefepime, cefdinir while showing resistance against trimethoprim-sulfamethoxazole and ceftazidime. Bacterial strain BH 49 displayed sensitivity against Imipenem, Meropenem, Ertapenem, Azithromycin, and Tazobactam/Piperacillin and a resistance response against Trimethoprim-sulfamethoxazole and cefalexin. BH 67 was found sensitive towards amikacin, ampicillin, cefuroxime, cefixime, ceftriaxone, tobramycin, and chloramphenicol while exhibiting resistance responses towards ciprofloxacin, amoxicillin + clavulanic Acid, Azithromycin, and Tetracycline.

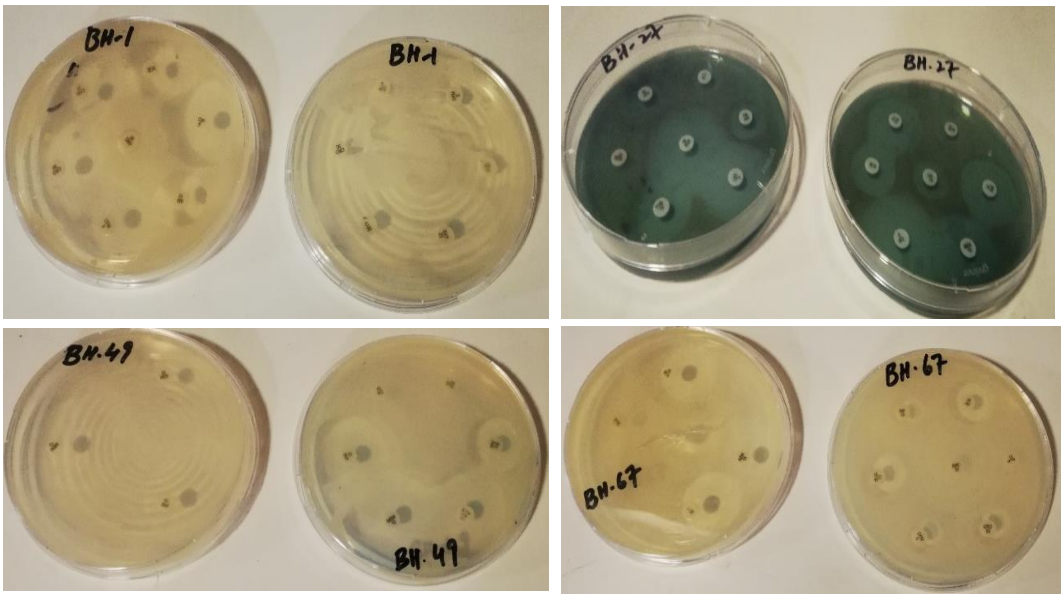

Figure 4.1. Sensitivity and Resistance Response of Bacterial Strains against Antibiotics.

Table 4.4. Sensitivity and Resistance Response of Bacterial strains against antibiotics.

S. No.	Bacterial strain	Sensitive	Resistant
1	BH 1	Cefixime, Ampicillin, Amikacin Trimethoprim + Sulfamethoxazole, Chloramphenicol, Ceftriaxone, Cefuroxime, Ceftriaxone,	Ciprofloxacin, Amoxicillin + Clavulanic Acid
2	BH 18	NA	NA
3	BH 27	Tazobactam/Piperacillin, Imipenem, Meropenem Levofloxacin, Polymyxin B Cotrimoxazole, Aztreonam Tobramycin, Cefepime, Cefdinir	Trimethoprim-sulphamethoxazole, ceftazidime
4	BH 49	Imipenem, Meropenem Ertapenem, Azithromycin, Tazobactam/Piperacillin	Trimethoprim-sulphamethoxazole, Cefalexin
5	BH 67	Amikacin, Ampicillin, Cefuroxime, Cefixime, Ceftriaxone, Tobramycin, Chloramphenicol	Ciprofloxacin, Amoxicillin + Clavulanic Acid, Azithromycin, Tetracycline

4.6 Multiple Sequence alignment of Bacterial strains

Many commercial packages, e.g., the GCG package (Wisconsin Package, Genetics Computer Group, Madison, WI) and its X Window graphical user interface, SeqLab, were used to study genomic sequence alignments. The sequence analysis was performed using Clustal X software. Four genomic sequences of four bacterial strains (BH-49, BH-67, BH-27, and BH-18) were aligned, and the results are presented in Figure 4.2. The results demonstrate that there are five colors in the sequence blast, and highly identical residues were recorded among all sequences, which are denoted by the * sign. Furthermore, the sequences showed a highly conserved column among the species (denoted by --), and in a few places, the test sequences showed a weakly conserved column (denoted by -).

4.7 Phylogenetic analysis of Bacterial strains

The phylogenetic analysis of 16S rRNA was performed by using Parsimony (PAUP; Swofford, 2002) and Molecular Evolutionary Genetics analysis (MEGA) version 7 (Kumar et al., 2016). The

software PAUP has not been updated for many years and does not have the accompanying graphic documents. Furthermore, PAUP requires more time (in hours or even many days) for computing to run a maximum likelihood analysis. So, the phylogenetic analysis was done using the software MEGA7. The Phylogenetic analysis is presented in Figure 4.3, and it shows the ancestral relationship among the bacterial isolates based on the maximum likelihood method. The tree showed the most similarity among the BH-49 and BH-67, displaying an evolutionary distance of 0.12, while the BH-18 and BH-27 are not linked with this and do not share the maximum similarity with the other tested sequences.

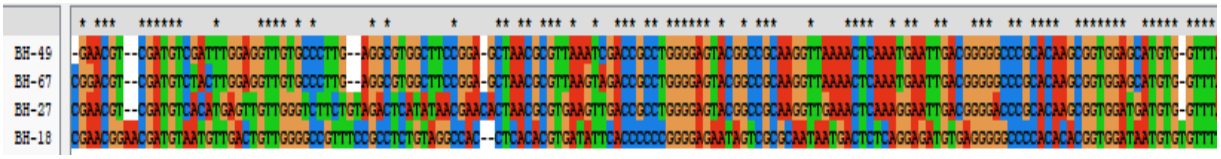

Figure 4.2. Multiple alignments of four BH binding domain protein sequences. Residues are colored according to the following criteria: AVFPMILW are shown in red, DE is blue, RHK is magenta, STYHCNGQ is green and all other residues are grey. The residue range for each sequence is shown after the sequence name.

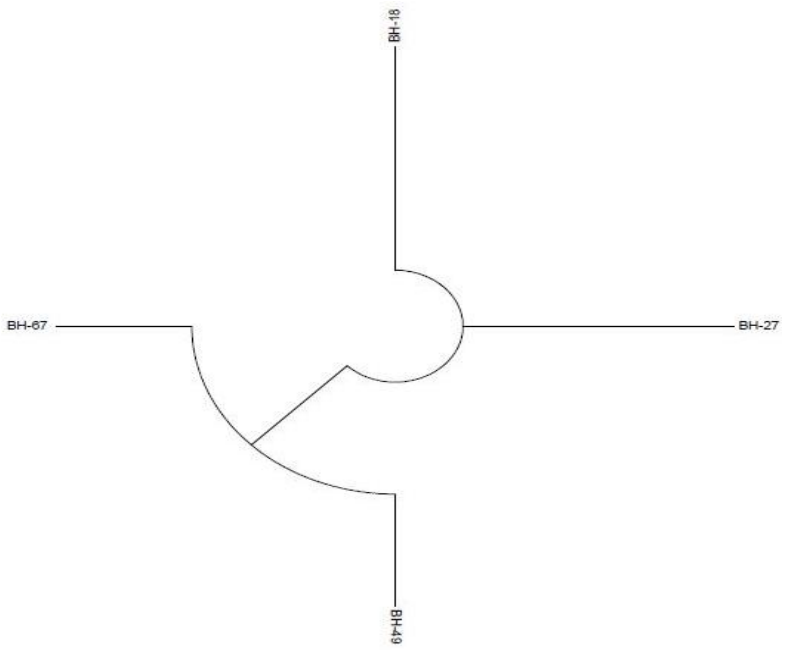

Figure 4.3. Phylogram for "BH COATING" showing distances and the placement of LD2 in the order. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model.

5.0 Discussion

Based on the obtained results, five bacterial strains were screened for further studies on the basic degree of heavy metals and antibiotic resistance. Previously, various studies have been conducted highlighting the valuable properties of bacterial strains and how these properties could be improved through cutting-edge hereditary design. In various cases, information on metal-related responses catalyzed by different microbial agents permits the improvement of the ideal procedure by modifying the physicochemical states of the contaminated areas. The mix of genetic improvement of the bacterial isolates impelled sensible eco-building of the polluted areas, which should be used in future bioremediation methodologies based on resistance patterns, and these bacterial strains were identified as belonging to *Enterobacter* sp. (Cu1), *Enterobacter* sp. (Cu2), *Stenotrophomonas* sp. (Cd1), *Providencia* sp. (Cd2), *Corynebacterium* sp. (Co1), *Comamonas* sp. (Co2), *Ochrobactrum*

sp. (Cr), and *Delphia* sp. (M1). The four heavy metal-resistant bacterial strains were tested in mixture forms to remove high concentrations of heavy metals and reduce the organic load of wastewater effluent. Obtained results showed that the utilization of activated sludge along with resistant bacteria helps remove the heavy metal as compared to activated sludge alone. It was concluded that the utilization of activated sludge and a resistant bacteria mixture could be very effective in removing heavy metal contamination from industrial effluents (Bestawy *et al*., 2013). Zouboulis *et al*., (2004) investigated the removal of heavy metals from contaminated wastewater by using biological agents, which include *Bacillus laterosporus* and *B. licheniformis* under laboratory conditions.

Bacterial strains were isolated from polluted (metal-laden) soil. The bacterial strains showed tolerance against heavy metals and showed the best survival percentage even at high doses of metals. The bacterial strains BH1, BH18, BH27, BH49, and BH67 were resistant to Zn, Cd, Co, and Hg metals. Gikas (2008) reported that Ni and Co at high frictions act as microbial growth inhibitors. Aka and Babalola (2017) isolated eleven bacterial strains from soil samples and tested them for heavy metal tolerance against Cr, Cd, and Ni *in vitro*. All the bacterial strains showed varying levels of tolerance, while BCr3, BCd33, and BNi11 showed the highest tolerance against heavy metals. BH 18 was Gram-positive, while BH 1, BH 27, BH 49, and BH 67 were found Gram-negative in biochemical reactions.

Bacterial strains were isolated on heavy metal-incorporated LB agar medium. The bacterial strains were individually inoculated on MacConkey agar medium. On MacConkey agar, two types of growth patterns were observed in which lactose-fermented and non-lactose-fermented bacterial strains were separately isolated. Fulthorpe *et al*. (1993) screened bacterial isolates for substrate utilization patterns, taxonomic characters, plasmid content, and resistance to antibiotics like ampicillin, streptomycin, kanamycin, tetracycline, nalidixic acid, Hg, Ni, Cu, Co, Cd, and Zn. The 16S rRNA gene sequence analysis proved that bacterial strains with the highest As resistance belonged to the genera *Enterobacter asburiae* and *E. cloacae*. It was found in a research study that both the bacterial strains encode for the arsenite oxidising gene aoxA and the arsenate reducing gene, and this gene characterization helped develop efficient bioremediation strategies (Slevi *et al*., 2014). Aka and Babalola, (2017) isolated eleven bacterial strains from the soil samples and tested for heavy metal tolerance against Cr, Cd, and Ni *in vitro*. All the bacterial strains showed varying levels of metal tolerance, while BCr3, BCd33, and BNi11 showed the highest tolerance against all the tested heavy metals. Belimov *et al*. (2005) isolated Cd-tolerant bacterial strains from the rhizosphere of Indian mustard (Brassica juncea L. Czern.) cultivated in soil supplied with Cd and Cd-contaminated sewage sludge and mining waste. Bacterial strains belonging to *Variovorax paradoxus*, *Rhodococcus* sp., and *Flavobacterium* sp. showed tolerance to Cd and additionally showed tolerance to Zn, Cu, Ni, and Co and promoted root elongation of *B. juncea* seedlings.

The multisequence alignment results demonstrated that there are five colors in the sequence blast analysis, and high levels of identical residues were recorded among all bacterial genetic sequences. Furthermore, the sequences showed a highly conserved column among the species (denoted by --) and a few showed a weakly conserved column (denoted by -). He, *et al*., (2009) isolated a bacterial strain CSCr-3 with high Cr (VI)-reducing ability under alkaline conditions and identified it as *Ochrobactrum* sp. based on 16S rRNA gene sequence analysis. Phylogenetic analysis showed the ancestral relationship of the bacterial strains present in the phylogenetic tree made using the maximum similarity method. The tree showed sequence homology among the BH-49 and BH-67, having a distance of 0.12, while the BH-18 and BH-27 are not linked with this and do not share the common bacterial family. The bioballs used in the bioreactor were proven to be efficient attachment surfaces for biofilm development and metal accumulation. In a previous research study, microbes, which include *Pseudomonas* sp., *Sphingomonas* sp., and *Bacillus* sp., with potential metal-tolerant ability were also identified based on phylogenetic analysis as presented byJackson *et al*., (2009). Based on heavy metal and antibiotic resistances, four bacterial isolates were selected and subjected

to morphological, biochemical, 16S rDNA gene sequencing, and phylogenetic analysis (Marzan *et al.*, 2017).

The results demonstrated a significant amount of variability in the tested bacterial strains. The bacterial strains differ in genomic and phylogenetic analysis. The sequence analysis showed that the bacterial strains have more differences at many loci. The heavy metal-tolerant strains showed the most variability as compared to the heavy metal-sensitive strains.

6.0 Summary

Water is crucial to supporting all forms of life on the earth's crust. It has a dipolar nature because it can dissolve almost all solvents. It acts as a solvent to dissolve the solutes in living organisms and is also helpful to carry out all metabolic activities. The water shortage and its increasing demand are big problems in Pakistan, like in many other countries around the world. Farmers are using brackish water and wastewater to irrigate their agricultural lands. This water contains heavy metal contamination and a heavy amount of dissolved salts. Keeping this in mind, the present study was designed to isolate and screen the bacterial strain for heavy metal tolerance. Two hundred colonies were screened on heavy metal-supplemented LB agar medium. From the obtained results, twenty bacterial isolates were selected for further screening. Finally, five bacterial strains were selected based on their high levels of heavy metals and antibiotic resistance. The bacterial strains BH1, BH 49, and BH 49 were Gram-negative, rod-shaped bacteria, and BH 27 and BH 67 were Gram-negative, *bacilli*-shaped, motile bacteria. On the other hand, BH 18 was found Gram-positive and motile. The bacterial strains isolated from sewage water showed optimum growth at 30 °C and pH 7.0. In heavy metal tolerance testing, bacterial strains BH1, BH18, BH27, BH49, and BH67 were resistant to Zn, Cd, Co, and Hg metals. The isolated bacterial strains were individually inoculated on a MacConkey agar medium. Zn and Co were observed to be less toxic at MIC heavy metal resistance than Hg and Cd. All the bacterial strains showed varied levels of sensitivity against Imipenem, meropenem, azithromycin, and levofloxacin while showing resistance responses against Doxycycline and ceftriaxone.

In the sequence blast analysis, highly identical residues were recorded among all the bacterial genomic sequences (denoted by *). Furthermore, the sequences showed highly conserved columns among the species (denoted by --) and a few showed weakly conserved columns (denoted by -). The phylogenetic tree showed a close association among the BH-49 and BH-67 having a distance of 0.12, while the BH-18 and BH-27 did not share common ancestors and belonged to different bacterial species.

7.0 Conclusion

The results demonstrated a significant amount of variability among all the tested bacterial strains. The strains showed variations in their genetic makeup in phylogenetic analysis. The sequence analysis showed that the bacterial strains displayed more differences at many loci. The heavy metal-tolerant strains showed high variability among all the tested bacterial strains as compared to the heavy metal-sensitive strains. These bacterial strains may be used in fields with high heavy metal toxicity to enhance the productivity of these contaminated soils. These heavy metals that tolerated bacterial strains also showed resistance to many tested antibiotics.

Ethics approval and consent to participate

Ethics approval

Not applicable.

References

1. Abbas SZ, M Rafatullah, N Ismail and J Lalung (2014). Isolation, identification, and characterization of Cd resistant *Pseudomonas* sp. M3 from industrial wastewater. *Journal of Waste Management*.

2. Abou-Shanab RAI, PV Berkum and JS Angle (2007). Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of Alyssum murale. *Chemosphere***68(2):360-367**.
3. Ahluwalia SS and D Goyal (2007). Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresource technology***98(12):2243-2257**.
4. Akpor OB and M Muchie (2010). Remediation of heavy metals in drinking water and wastewater treatment systems: Processes and applications. *International Journal of Physical Sciences* **5(12):1807-1817**.
5. Akpor OB, GO Ohiobor and TD Olaolu (2014). Heavy metal pollutants in wastewater effluents: sources, effects and remediation. *Advances in Bioscience and Bioengineering* **2(4):37-43**.
6. Al-Garni SM (2005). Biosorption of Pb by Gram-ve capsulated and non-capsulated bacteria. *Water Sa***31(3):345-350**.
7. Alloway BJ (2012). Heavy metals in soils: trace metals and metalloids in soils and their bioavailability. *Springer Science & Business Media.*
8. Anderson CK, Pederson & AM Jakobsson. (2006). Autoradiographic comparisons of radionuclide adsorption between subsurface anaerobic biofilms and granitic host rocks. *Geomicrobiology Journal***23(1)15–29.**
9. Bååth E (1989). Effects of heavy metals in soil on microbial processes and populations. *Water Air Soil Pollut***47(3-4):335–379.**
10. Badar U, N Ahmed, AJ Beswick, P Pattanapipitpaisal and LE Macaskie (2000). Reduction of chromate by microorganisms isolated from metal contaminated sites of Karachi, Pakistan. *Biotechnology Letters* **22(10):829-836**.
11. Bartlett L, FW Rabe and WH Funk (1974). Effects of Cu, Zn and Cd on Selanastrum capricornutum. *Water Research***8(3):179-185**.
12. Battin TJ, WT Sloan, S Kjelleberg, H Daims, IM Head, TP Curtis and L Eberl (2007). Microbial landscapes: new paths to biofilm research. *Nature Reviews Microbiology***5(1):76–81.**
13. Belimov AA, N Hontzeas, VI Safronova, SV Demchinskaya, G Piluzza, S Bullitta and BR Glick (2005). Cd-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (Brassica juncea L. Czern.). *Soil Biology and Biochemistry* **37(2):241-250.**
14. Bestway EE, S Helmy, H Hussien, M Fahmy and R Amer (2013). Bioremediation of heavy metal-contaminated effluent using optimized activated sludge bacteria. *Applied water science***3(1):181-192.**
15. Bjarnsholt T, PØ Jensen, M Burmølle, M Hentzer, JAJ Haagensen, HP Hougen, H Calum (2005). *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology* **151(2):373-383.**
16. Boles BB, M Theondel & PK Singh (2004). Self-generated diversity produces insurance effects in biofilm communities. *Proceedings of the National Academy of Sciences USA***101(47):16630–16635.**
17. Borriello G, E Werner, R Frank, AM Kim, D Garth, Ehrlich and SP Stewart (2004). Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother***48(7):2659–2664.**
18. Borsetti F, F Francia, RJ Turner and D Zannoni (2007). The thiol: disulfide oxidoreductase DsbB mediates the oxidizing effects of the toxic metalloid tellurite (TeO_3^{2-}) on the plasma membrane redox system of the facultative phototroph Rhodobacter capsulatus. *J. Bacteriol***189(3)851-859.**
19. Çelo VD, B Babi, Baraj & A Çullaj (1999). An Assessment of heavy metal pollution in the sediments along the Albanian Coast. *Water Air Soil Pollut***111(1-4): 235–250.**

20. Chang WC, GS Hsu, SM Chiang & MC Su (2006). Heavy metal removal from aqueous solution by wasted biomass from a combined AS-biofilm process. *Bioresource Technol***97(13)1503–1508**.
21. Chen M, P Xu, G Zeng, C Yang, D Huang and J Zhang (2015). Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides,
22. Chlorophenols and heavy metals by composting: applications, microbes and future research needs. *Biotechnology Advances* **33(6):745-755**.
23. Collins CH, PM Lyne and JM Grange (1989). *Microbiological Methods*, 6th ed., Butterworth, London.
24. Congeevaram S, S Dhanarani, J Park, M Dexilin and K Thamaraiselvi (2007). Biosorption of Cr and Ni by heavy metal resistant fungal and bacterial isolates. *Journal of hazardous materials***146(1-2):270-277.**
25. Cooper KE (1995). Theory of antibiotic inhibition zones in agar media. *Nature***176:510-511**.
26. Costerton JW, PS Stewart & EP Greenberg (1999). Bacterial biofilms: a common cause of persistent infections. *Science***284(5418):1318–1322.**
27. Davies JA, JJ Harrison, LL Marques, GR Foglia, CA Stremick, DG Storey, RJ Turner, ME Olson, H Ceri (2007). The GacS sensor kinase controls phenotypic reversion of small colony variants isolated from biofilms of *Pseudomonas aeruginosa* PA14. *FEMS microbiology ecology* **59(1):32-46.**
28. Diels L, PH Spaans, RS Van, L Hooyberghs, A Ryngaert, H Wolters, E Walter, J Winters, L Macaskie, J Finlay, B Pernfuss (2003). Heavy metals removal by sand filters inoculated with metal sorbing and precipitating bacteria. *Hydrometallurgy***71(1-2):235-41.**
29. Dixit R, D Malaviya, K Pandiyan, U Singh, A Sahu, R Shukla, B Singh, J Rai, P Sharma, H Lade and D Paul (2015). Bioremediation of heavy metals from soil and aquatic environment: an overview of principles and criteria of fundamental processes. *Sustainability***7(2):2189-2212.**
30. Drancourt M, C Bollet, A Carlioz, R Martelin, JP Gayral and D Raoult (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of clinical microbiology***38(10)3623-3630.**
31. Foulkes MJ, BR Sylvester, RK Scott (1998). Evidence for differences between winter wheat cultivars in acquisition of soil mineral nitrogen and uptake and utilization of applied fertilizer nitrogen. *The Journal of Agricultural Science***130(1):29-44.**
32. Fulthorpe RR, SN Liss and DG Allen (1993). Characterization of bacteria isolated from a bleached kraft pulp mill wastewater treatment system. *Canadian journal of microbiology***39(1):13-24.**
33. Gadd GM (1992). Metals and microorganisms: a problem of definition.*FEMS Microbiology*
34. Geslin C, J Llanos, D Prieur & C Jeanthon (2001). The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. *Research in microbiology***152(10)901-905.**
35. Gikas P (2008). Single and combined effects of Ni (Ni (II)) and Co (Co (II)) ions on activated sludge and on other aerobic microorganisms: a review. *Journal of hazardous materials***159(3):187-203.**
36. Glick BR (2003). Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnology advances***21(5):383–893.**
37. Haagensen JA, M Klausen, RK Ernst, SI Miller, A Folkesson, TT Nielsen, S Molin (2007). Differentiation and distribution of colistin-and sodium dodecyl sulfate-tolerant cells in *Pseudomonas aeruginosa* biofilms. *Journal of bacteriology***189(1):28-37.**
38. Hall SL, JW Costerton & P Stoodley (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature Rev. Microbiol***2(2):95–108.**

39. Haq RU and AR Shakoori (1998). Microbiological treatment of industrial wastes containing toxic Cr involving successive use of bacteria, yeast and algae. *World Journal of Microbiology and Biotechnology* **14(4):583-585**.
40. Harrison JJ, H Ceri, J Yerly, M Rabiei M, Y Hu, R Martinuzzi, RJ Turner (2007). Metal ions may suppress or enhance cellular differentiation in Candida albicans and Candida tropicalis biofilms. Appl. *Environ Microbiol***73(15):4940-9.**
41. Harrison JJ, H Ceri, NJ Roper, EA Badry, KM Sproule, RJ Turner (2005). Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiology***151(10):3181-95.**
42. Harrison JJ, M Rabiei, RJ Turner, EA Badry, KM Sproule, H Ceri (2006). Metal resistance in Candida biofilms. *FEMS microbiology ecology***55(3):479-91.**
43. Hasegawa, M., H. Kishino and T. Yano (1985). Dating the human-ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution.***22: 160-174.**
44. He Z, F Gao, T Sha, Y Hu and C He (2009). Isolation and characterization of a Cr (VI)-reduction *Ochrobactrum sp*. strain CSCr-3 from Cr landfill. *Journal of hazardous materials***163(3):869-873.**
45. Hrynkiewicz K and C Baum (2014). Application of microorganisms in bioremediation of environment from heavy metals. *In Environmental deterioration and human health Springer Dordrecht***215 – 227.**
46. Huang CT, KD Xu, GA McFeters & PS Stewart (1998). Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. *Appl Environ Microbiol***6(4):1526–1531**.
47. Hunter RC & TJ Beveridge (2005). Application of a pH sensitive fluoroprobe (C-SNARF-4) for Ph microenvironment analysis in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol***71(5):2501–2510**.
48. Igiri BE, SI Okoduwa, GO Idoko, EP Akabuogu, AO Adeyi and IK Ejiogu (2018). Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: a review. *Journal of toxicology*.
49. Jackson VA, AN Paulse, AA Bester, JH Neethling, S Khan and W Khan (2009). Bioremediation of metal contamination in the Plankenburg River, Western Cape, South Africa. *International Biodeterioration & Biodegradation***63(5):559-568.**
50. Jaishankar M, T Tseten, N Anbalagan, BB Mathew and KN Beeregowda (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary Toxicology* **7(2):60-72.**
51. Jefferson B, JE Burgess, A Pichon, J Harkness and SJ Judd (2001). Nutrient addition to enhance biological treatment of greywater. *Water Research***35(11):2702-2710.**
52. Kelly CJ, N Tumsaroj and CA Lajoie (2004). Assessing wastewater metal toxicity with bacterial bioluminescence in a bench-scale wastewater treatment system. *Water Research***38(2):423-431.**
53. Kelly JJ and RL Tate (1998). Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a Zn smelter. *Journal of environmental quality***27(3):609-617.**
54. Kessi J & KW Hanselmann (2004). Similarities between the abiotic reduction of selenite with glutathione andthe dissimilatory reaction mediated by *Rhodospirillum rubrum* and *Escherichia coli. The Journal of Biological Chemistry***279(49): 50662-50669.**
55. Kim DW, DK Cha, J Wang and CP Huang (2002). Heavy metal removal by activated sludge: influence of *Nocardia amarae*. *Chemosphere***46(1):137-142.**
56. Kumar S, G Stecher and K Tamura (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33(7):1870- 1874.**
57. Lafleur MD, CA Kumamoto & K Lewis (2006). Candida albicans biofilms produce antifungal tolerant persister cells. *Antimicrobial Agents Chemother* **50(11):3839-3846.**

58. Lawrence JR, MR Chenier, R Roy, D Beaumier, N Fortin, GD Swerhone, TR Neu, CW Greer (2004). Microscale and molecular assessment of impacts of Ni, nutrients, and oxygen level on structure and function of river biofilm communities. *Appl Environ Microbiol***70(7):4326-4339.**
59. Lewis K (2007). Persister cells, dormancy and infectious disease. *Nature Rev. Microbiol***5(1):48-56.**
60. Silva AAL, MA Carvalho, SA de Souza, PMT Dias, RGD Silva Filho, CS Saramago, CA Bento and E Hofer (2012). Heavy metal tolerance (Cr, Ag and Hg) in bacteria isolated from sewage. *Brazilian Journal of Microbiology***43(4):1620-1631.**
61. Lohmeier VEM, S Ung & RJ Turner (2004). In vivo 31P nuclear magnetic resonance investigation of tellurite toxicity in *Escherichia coli*. *Appl Environ Microbiol***70(12):7342-7347.**
62. Malik A (2004). Metal bioremediation through growing cells. *Environment International* **30(2):261-278.**
63. Marzan LW, M. Hossain, SA Mina, Y Akter and AMA Chowdhury (2017). Isolation and biochemical characterization of heavy-metal resistant bacteria from tannery effluent in Chittagong city, Bangladesh: Bioremediation viewpoint. *The Egyptian Journal of Aquatic Research***43(1):65-74.**
64. Mathew M and JP Obbard (2001). Optimization of the dehydrogenase assay for measurement of indigenous microbial activity in beach sediments contaminated with petroleum. *Biotechnology letters***23(3):227-230.**
65. Merroun ML, M Nedelkova, JJ Ojeda, T Reitz, ML Fernández, JM Arias, M Romero-González and S Selenska-Pobell (2011). Bio-precipitation of uranium by two bacterial isolates recovered from extreme environments as estimated by potentiometric titration, TEM and X-ray absorption spectroscopic analyses. *Journal of Hazardous Materials***19(7):1-10.**
66. Moges F, M Endris, Y Belyhun and W Worku (2014). Isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non- hospital environment. *Northwest Ethiopia. BMC Research Notes **7(1):215.***
67. Moten AM and A Rehman (1998). Study on heavy trace metal ions in industrial waste effluents in Pakistan.
68. Mumtaz MZ, M Ahmad, M Jamil and T Hussain (2017). Zn solubilizing *Bacillus* spp. potential candidates for biofortification in maize. *Microbiological Research* **20(2):51- 60.**
69. Muñoz R, MT Alvarez, A Muñoz, E Terrazas, B Guieysse, B Mattiasson (2006). Sequential removal of heavy metal ions and organic pollutants using an algal-bacterial consortium. *Chemosphere***63(6):903-911.**
70. Nameni M, MRA Moghadam and M Arami (2008). Adsorption of hexavalent Cr from aqueous solutions by wheat bran." *International Journal of Environmental Science and Technology***5(2):161-168.**
71. Aka RJN and OO Babalola (2017). Identification and characterization of Cr-, Cd-, and Ni-tolerant bacteria isolated from mine tailings. *Bioremediation journal* **21(1):1-19.**
72. Nweke CO, JC Okolo, CE Nwanyanwu and CS Alisi (2006). Response of planktonic bacteria of New Calabar River to Zn stress. *African Journal of Biotechnology***5(8):653-658.**
73. Pacarynuk LA and HC Danyk (2004). Biochemical Tests. In: Principles of Microbiology. *Laboratory Manual, spring, TX, USA* **28-34.**
74. Patel JB (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis* **6(4):313-321.**
75. Petzow G (1999). Metallographic etching: techniques for metallography, ceramography, plastography. *ASM international.*
76. Pomposiello PJ & B Demple (2002). Global adjustment of microbial physiology during free radical stress. *Adv Microb Physiol* **4(6):319-341.**

77. Pringault O, E Epping, R Guyoneaud, A Khalili & M Kuhl (1999). Dynamics of anoxygenic photosynthesis in an experimental green sulphur bacteria biofilm. *Environ Microbiol***1(4):295-305.**
78. Purevdorj B, WJ Costerton & P Stoodley (2005). Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. *Microbiology***151(5):1569–1576.**
79. Raja CE, K Anbazhagan and GS Selvam (2006). Isolation and characterization of a metal-resistant *Pseudomonas aeruginosa* strain. *World Journal of Microbiology and Biotechnology***22(6):577-585.**
80. Rajbanshi A (2008). Study on heavy metal resistant bacteria in Guheswori sewage treatment plant. *Our Nature* **6(1):52-57.**
81. Ramage G, SP Saville, PD Thomas & JL Ribot (2005). Candida biofilms: an update. *Eukaryotic Cell***4(4):633-638.**
82. Rani SA, B Pitts, H Beyenal, VR Aeluchamy, Z Lewandowski, WM Davison, MK Buckingham, PS Stewart (2007). Spatial patterns of DNA replication, protein synthesis and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J. Bacteriol***189(11):4223-4233.**
83. Rawlings DE & DB Johnson (2007). The microbiology of biomining: development and optimization of mineral oxidizing microbial consortia. *Microbiology***153(2):315–324.**
84. Rehman J, HJ Zhang, PT Toth, Y Zhang, G Marsboom, Z Hong, R Salgia, AN Husain, C Wietholt and SL Archer (2012). Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. *The FASEB Journal* **26(5):2175-2186.**
85. Roohi A, I Ahmed, M Iqbal and M Jamil (2012). Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. *Pakistan Journal of Botany***44(1):365-370.**
86. Sahlström L (2003). A review of survival of pathogenic bacteria in organic waste used in biogas plants. *Bioresource Technology* **87(2):161-166.**
87. Saitou N and N Masatoshi (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4(4):406-425.**
88. Selvi MS, S Sasikumar, S Gomathi, P Rajkumar, P Sasikumar and S Govindan (2014). Isolation and characterization of As resistant bacteria from agricultural soil, and their potential for As bioremediation. *International Journal of Agricultural Policy and Research* **2(11):393-405.**
89. Shakoori AR and B Muneer (2002). Cu-resistant bacteria from industrial effluents and their role in remediation of heavy metals in wastewater. *Folia Microbiologica* **47(1):43-46**.
90. Siddiquee S, K Rovina, SA Azad, L Naher, S Suryani and P Chaikaew (2015). Heavy metal contaminants removal from wastewater using the potential filamentous fungi biomass: a review. *J Microb Biochem Technol***7(6):384-395.**
91. Singh R, D Paul & RK Jain (2006). Biofilms: implications in bioremediation. *Trends Microbiol* **14(9):389-397.**
92. Southey PCJ, DG Davies & K Sauer (2005). Characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol* **187(23):8114-8126.**
93. Spoering A & K Lewis (2001). Biofilm and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol***183(23):6746-6751.**
94. Stewart PS (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol***292(2):107-113.**
95. Stohs SJ & D Bagchi (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med***18(2):321-336.**
96. Tamura K, M Nei and S Kumar (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences.* **101(30):11030-11035**.

97. Tchounwou PB, CG Yedjou, AK Patlolla and DJ Sutton (2012). Heavy metal toxicity and the environment in Molecular, Clinical and Environmental Toxicology. *Springer Basel***133-164**.
98. Teitzel GM & MR Parsek. (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol***69(4):2313–2320.**
99. Thomson JM and RA Bonomo (2005). The threat of antibiotic resistance in Gram-negative pathogenic bacteria: β-lactams in peril. *Current Opinion in Microbiology* **8(5):518- 524**.
100. Tremaroli V, S Fedi & D Zannoni (2007). Evidence for a tellurite-dependent generation of reactive oxygen species and absence of a tellurite-mediated adaptive response to oxidative stress in cells of *Pseudomonas pseudoalcaligenes* KF707. *Arch. Microbiol***187(2):127-135.**
101. Turner RJ, Y Aharonowitz, JH Weiner & DE Taylor (2001). Glutathione is a target of tellurite toxicity and is protected by tellurite resistance determinants in *Escherichia coli*. *J. Microbiol* **47(1):33-40.**
102. Valls M and VD Lorenzo (2002). Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS microbiology Reviews***26(4):327-338.**
103. Vilchez R, C Pozo, MA Gomez, B Rodelas & JG Lopez. (2007). Dominance of sphingomonads in a Cu exposed biofilm community for groundwater treatment. *Microbiology***153(2):325–337.**
104. Walters MC, F Roe, A Bugnicourt, MJ Franklin & PS Stewart. (2003). Contributions of antibiotic penetration, oxygen limitation and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrobial agents and chemotherapy***47(1):317–323.**
105. Werner E, F Roe, A Bugnicourt, MJ Franklin, A Heydorn, S Molin, B Pitts and PS Stewart (2004). Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol* **70(10):6188-6196.**
106. Wuana RA and FE Okieimen (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *International Scholar Research Notes.*
107. Xu KD, GA McFeters & PS Stewart (2000). Biofilm resistance to antimicrobial agents. *Microbiology***146(3):547-549.**
108. Xu KD, PS Stewart, F Xia, C Huang & GA McFeters (1998). Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol***64(10):4035-4039.**
109. Yuncu B, FD Sanin and U Yetis (2006). An investigation of heavy metal biosorption in relation to C/N ratio of activated sludge. *Journal of hazardous materials***137(2):990-997.**
110. Zannoni D, F Borsetti, JJ Harrison & RJ Turner (2007). The bacterial response to the chalcogen metalloids Se and Te. *Adv. Microb. Physiol***5(3):1-71.**
111. Zaved HK, MM Rahman, A Rahman and SMY Arafat (2008). Isolation and characterization of effective bacteria for solid waste degradation for organic manure. *Current Applied Science and Technology* **8(2):44-55.**
112. Zhang GL, FG Yang, YG Zhao, WJ Zhao, JL Yang & ZT Gong (2005). Historical change of heavy metals in urban soils of Nanjing, China during the past 20 centuries. *Environ. Int***31(6):913-919.**
113. Zouboulis AI, MX Loukidou and KA Matis (2004). Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. *Process Biochemistry***39(8):909-916.**