MICROBIAL BIODIVERSITY ANALYSIS OF HEAVY METAL CONTAMINATED SOIL: A METAGENOMICS-BASED APPROACH

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Abstract. The current study focused on the exploration of bacterial community in heavy metal contaminated soil using culture dependent and independent approaches that might be applied to the bioremediation process. A total of 150 bacterial colonies were examined from the heavy metal-contaminated soil at initial level, and 25 isolates were chosen for further examination. Finally, twelve strains were chosen for future research based on their high levels of heavy metal resistance. The 10 bacterial strains i.e.,1K-10K were then characterized on the basis of their morphology and microscopic analysis. The morphological and biochemical characteristic relied on the basis of Bergey's manual of bacterial classification that 1K as *Pseudomonas sp*., 2K as *Enterobacter sp.,* 3K as *Streptococcus sp*., 4K as *Staphylococcus sp*.,5K as *Staphylococcus sp*., 6K as *Pseudomonas sp*., 7K as *Micrococcus sp*., 8K as *Staphylococcus sp*., 9K as *Staphylococcus sp*. & 10K as *Staphylococcus sp*. In metagenomics analysis, the most prevalent bacteria present in samples were "*Actinobacteria, Proteobacteria, Acidobacteria, Pseudomonas Cyanobacteria, Bacteriodes,* and *Sphingomonas*. Among the 29 identified phyla, *Chloroflexi* and *Tenericutes* were the most dominant phyla and their relative abundance ranged from 0.75-1.00. While, on the genera level, the most abundant genera were *Blautia, Rhodoplanes* followed by *Prevotella* and *Arenimonas*. The relative abundance of these genera ranged from 0.75% to 1.00%. Depending on the relative abundance of taxons, the results of this study showed *Proteobacteria, Synergistetes* and *Bacteriodes* were most abundant phylum while the least abundant phylum present in heavy metal contaminated soil was *Chlamydia* and *Tenericutes*. Screening of soil-based libraries using functional and sequence-based specifications, which has disclosed information on soil microbial communities, has made it possible to identify new microbial communities. Thus, the culture dependent and independent approaches revealed that even heavy metal contaminated soil compose of diverse group of bacterial community that could be explored for bioremediation purposes.
Keywords: *nanoparticle, Olea europaea, SnO-NPs, SnO dope with $FeCl_2$-NPs, antibacterial applications, dye degradation*

Introduction

Pollution is any human activity that deteriorates or destroys the quality of the natural environment. Environmental pollution is one of the most important factors contributing

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to illness and death in the world. The main cause of pollution is heavy metals. Metals with a density greater than 5 g per cubic centimeter are typically referred to as heavy metals. Due to anthropogenic, industrial, agricultural, and modern industrialized activity, all of which have detrimental effects on human health, there has been an increase in heavy metal exposure. Numerous human organs are affected by HM, such as mercury, cadmium, and chromium (Yu et al., 2024). The negative effects of heavy metals include cancer, gastrointestinal and kidney failure, disorders of the brain, skin blisters, immune system issues, birth defects, and more. HMs are teratogenic and carcinogenic in nature, and they damage organs, disrupt the nervous system, create oxidative stress, and hinder growth and development. A variety of human organs are severely harmed by the harmful environmental pollutant (Gupta et al., 2019).

Technology's rapid growth has drawn attention to the social economy and environmental issues associated with industrial pollution in recent years. The most common and well-known pollutants are heavy metals. They are poisonous, bioaccumulative, and difficult to naturally bioremediate. Studies on the contamination of heavy metals in diverse media, like soil, air, and sediments in rivers or lakes, are expanding throughout the world.

Heavy metal accumulation results in contamination of the soil and water. Water quality is impacted, and the risk of heavy metal contamination in grains and vegetables is increased. A significant threat to people's health is the environment's rising amount of heavy metals. The economic sector known as industry is connected to the production and manufacturing of goods. 23 percent of Pakistan's annual GDP (gross domestic product) is contributed by the industry (Saeed et al., 2010). Karachi is an industrial city with two planned industrial areas: "Sindh Industrial Trading Estate (SITE) and Korangi Industrial Area (KIA)." With a total area of 34.4 km2, the Korangi industrial region in district east contains about 2000 different kinds of small and medium-sized industrial facilities. However, KIA's primary industries are the leather and textile sectors (Saeed et al., 2010). Industry generates a lot of effluents depending on the type of manufacturing. Industrial effluents are frequently divided into three groups: inorganic process waste from the chemical industry, organic process waste, and chemical process waste from the chemical industry (waste from the companies that manufacture fertilizers, pesticides, dyes, acids, and bases) (Xu et al., 2024; Aziz et al., 2023; Samuchiwal et al., 2021).

Effluents from industries have a number of distinct characteristics, such as anomalous turbidity, total suspended solids (TSS), conductivity, and total hardness. Numerous contaminants, such as phosphates, oil and grease, nitrates, and chlorides, are included in these effluents. It has been found that heavy metal concentrations in particulate matter are higher than acceptable and tolerated limits and that industrial effluents contain additional toxicants for humans.

Due to rapid industrialization and the increased demand for the product, the concentration of contaminants has increased. All types of biological forms are negatively impacted, either directly or indirectly, by the discharge of various hazardous industrial wastes into bodies of water. People who ingest certain harmful heavy metals could experience severe health issues (Gaur et al., 2020).

Soils naturally cover the surface of the ground and serve as the interface between solids (such as geological materials and biologically degraded matter), liquids (such as water), and gases (such as air in soil pores). One distinguishing characteristic of soil is the presence of soil minerals (Dominati et al., 2010). Many naturally occurring

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microorganisms with heavy metal resistance could eliminate heavy metal contaminants from the environment or alter them into less hazardous forms (Emenike et al., 2018). The microbial world of soil is remarkably diverse. These microbes can be classified as bacteria, yeast, fungi, algae, or protozoa. Soil microorganisms are a key indicator of soil environmental quality. Microorganisms in the soil carry out almost all biochemical reactions (Hassan et al., 2014).

The best indications of hazardous substances that affect soil are thought to be microorganisms (Bouchez et al., 2016). The harmful HMs' effects on soil microbial populations have been explored in several studies (Wang et al., 2019). Due to their harmful effects on almost all bacteria's development, morphology, and metabolism, as well as their inhibition of vital cellular processes, heavy metals alter the function, activity, and diversity of the soil microbial communities (Xing et al., 2015). The number of bacteria as a whole, actinomycetes, and enzymatic activities are all negatively impacted by the increased heavy metal concentration in soil (Zhang et al., 2016). The majority of individuals recognize that heavy metals interfere with how carbon, nitrogen, and organic matter are transformed, reduce the function of soil enzymes, and reduce the biodiversity and biomass of microorganisms (Jiang et al., 2023; Ayangbenro and Babalola, 2017) causing certain HM-tolerating bacteria to predominate in the soil (Chen et al., 2014). Thus, keeping in view the importance of metallotolerant microbial strains for the bioremediation process, the current study was designed to analyze the microbial biodiversity that is present in heavy metal-contaminated soil by using molecular approaches.

Materials and methods

Sampling sites

Total 10 heavy metals containment soil samples were collected from effluent of Korangi Industrial Area, Karachi, which is located in district east Karachi with a total area of 34.4 km^2, the district east's Korangi industrial sector consist about 2000 different small and medium-sized businesses.

Sample collection

Each soil sample was taken from the top 5 to 15 cm into five equal subsamples, which was kept in separate sterile plastic bags/falcon tubes. Soil samples was taken in falcon tubes and immediately transferred to the laboratory by using the methodology mentioned by Khan et al. (2019b).

Characterization of soil

Different physicochemical soil parameter was analyzed including soil pH and Heavy Metals contamination from industrial effluent of Korangi Industrial Area, by using methodology mentioned by Khan et al. (2019a).

Microbial isolation by culture dependent method

Dilution plating and culture technique was used to study the microbes that are present in the soil (Uesugi et al., 2023). Culturing methods have been used to cultivate soil microbial communities using a range of culture media in an effort to recover as many of

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the different types of soil- microbial species as possible. For each composite, 1 g of soil was completely vortexed after being suspended in 9 ml of sterilized distilled water. A series of dilutions was carried out from this stock solution. Aliquots were smeared on nutrient agar (NA), LB, and MacConkey media.

Isolation of bacterial strains

By using the serial dilution approach, bacteria were isolated. For isolation procedure 1 g of the dried sample was diluted in 10 mL of sterile distilled water. After being diluted up to 10-4 times in sterile physiological salt solution (0.9% NaCl), an aliquot of 0.1 mL was added to Luria-Bertani (LB) media plates. A plate was incubated at 37°C for 24 h. Colonies was isolated after incubation. This technique was repeated until pure cultures were obtained (Gerits et al., 2016).

Growth conditions

The isolated bacterial strains were cultured in media containing 10 g of peptone, 5 g of yeast extract, and 10 g of sodium chloride per liter of LB broth. With the help of 0.01 M HCl and 0.01 M NaOH, the pH was raised to 7. Incubation was carried out at 37°C.

Morphological characterization

For identification purpose, gram staining of isolates was carried out. After gram staining, the morphological characteristic of several isolates was examined under a microscope. Morphological characteristics such as colony, morphology, color, shape, transparency, margin, elevation and surface was observed (Agrawal and Agrawal, 2013).

Gram staining

A gram reagent was produced by using crystal violet, iodine, alcohol, and the counterstain safranin. Using distilled water and a sterile wire loop, isolate was made by spreading pure culture on a slide. A drop of crystal violet was applied, swirled around, and let it dry for 1 min before being washed with sterile water. Iodine was added in a drop form for 1 min, after which acetone was applied for 30 s. Finally, a drop of safranin was added, held for 1 min, and then completely eroded away. After being dried on blotting paper and stained with a drop of immersion oil, the mounts were seen at a magnification of 100X under a microscope (Kholik et al., 2023).

Biochemical characterization

The bacterial strain was subjected to a variety of biochemical assays, including the methyl red, catalase, coagulase, and oxidase tests. Briefly described as follows:

Catalase test

The catalase test was performed on a clean slide, a single colony was gently coated with hydrogen peroxide. The presence of catalase was determined by adding H_2O_2 (3% v/v) to a bacterial culture, and the presence of catalase was proven by free oxygen gas bubbles (Reiner, 2010).

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Coagulase test

For coagulase test, the physiological saline was added as a drop on each end of a slide. The loop, straight wire or wooden stick was used to emulsify a portion of the isolated colony to produce two thick suspensions with each drop. After that, a drop of human or rabbit plasma was added to the initial suspensions, and it was gently integrated. Clumps of the organisms within 10 s indicated positive result. No plasma was added to the second solution in order to distinguish between any granular appearance of the organism and true coagulase clumping. Negative results were indicated with no clots (Katz, 2010).

Oxidase test

All aerobic, oxidase-positive bacteria can utilize oxygen as a terminal electron acceptor during respiration. They may not be actually aerobes. Bacteria that lack oxidase can be facultative, anaerobic, or both. The outcome merely shows that these organisms lack the cytochrome c oxidase required to oxidize the test reagent. During respiration, they may transfer electrons using different oxidases. There are several techniques to do an oxidase test. The filter paper test, the filter paper spot test, the direct plate method, and the test tube method are among them, in current study the filter paper spot test was used to identified the oxidase positive and negative bacteria from the isolates (Shields and Cathcart, 2010).

Methyl-red test

For the analysis of methyl-red test, the test tubes of MRVP broth were prepared. Two loopful of each bacterial culture was aseptically added to the broth to inoculate it. After inoculating an organism into test tubes, they were incubated at 37°C for 48–72 h and label with the name of the organism A few drops of methyl red indicator was added to the incubated tubes after the incubation period. Each tube was observed for a distinct red color indicated positive results whereas negative reaction was noticed in form of yellow color (Tille and Bailey, 2014).

Metagenomics analysis of soil sample

The GJC DNA Purification kit was used to purify metagenomic DNA from 1 g soil samples (heavy metal contaminated soil) in accordance with the company's guidelines. Purified DNA was subjected to gel electrophoresis and DNA estimation by NanoDrop Spectrophotometer (Thermo Inc. USA).

The 16S rRNA fusion primers (V3-V4 region) and a 1 microgram quality DNA template were inserted for the polymerase chain reaction (PCR). All PCR products are labeled to finish library building after being dispersed in elution buffer and purified with Agencourt AMPure XP beads. Library size and quantity was measured with the help of Agilent 2100 Bioanalyzer. A qualified library was sequenced with the aid of next-generation DNA sequencing technology.

Bioinformatics analysis workflow

Clear the reads that may overlap and are merged into tags after being processed to produce high-quality clean data, and then clustered to OTU. The operational taxonomic unit (OTU) is the basic unit in taxonomy. An individual, a species, a genus, or classes are examples of these units. The taxonomic classification of OTU demonstrative sequences

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was done using the Ribosomal Database Project database. Analyses such as alpha variety, beta variety, differential species analysis, network, and prediction of models was carried out based on the OTU profile table and the conclusions of the taxonomic annotations.

Data filtering

To produce clean reads of high quality from raw data, the following step was taken:

1. Reads will be terminated if their average paired quality values fall below 20 across a 25 bp sliding frame. Remove reads whose lengths are shortened to 75% of their original lengths.
2. Remove adapter-contaminated reads (default parameter: maximum 3 bases of mismatch between reads and adapters overlapped by 15 bases).
3. Remove reads with an unclear base (N base).
4. Remove readings with a modest level of complexity (the default is 10 consecutive reads with the same base). Clean readings were allocated to associate samples by alignments (0 base mismatches) against barcode sequences in order to ensure that barcode sequences were removed from pooling libraries.

Tags connection

When paired-end reads crossed paths, FLASH (Fast Length Adjustment of Short reads) produced consensus sequences.

OTU clustering

Operational Taxonomic Units, or OTUs for short, are a unified marker used in molecular phylogenetic analysis to analyze a taxon unit (i.e., a taxonomic level) at seven levels. The sequences must be grouped into OTUs with a 97% similarity in order to calculate the total amount of bacteria in each sample. With USEARCH (v7.0.1090), tags were grouped into operational taxonomic units (OTUs). Details are given below:

1. A 97% threshold was used by UPARSE to classify tags into OTUs, which contain the different OTU representative sequences.
2. The UCHIME (v4.2.40) program filters out chimaeras. The programs mentioned above screen and filter chimaeras in OTU for 16S rDNA and ITS sequences.

Metagenomic NGS data analysis

OTU taxonomy annotation: RDP Classifier (v2.2) was used to align OTU representative sequences against the database for taxonomic annotation.

Optimizing soil metagenomics

Analyzing whether variations in libraries are the result of community composition changes, sampling, or both during library construction, bioinformatics tools that enable statistical comparisons of built libraries (Tariq et al., 2016). Libraries of 16S rRNA genes may be useful. The sequence and functional based screening of soil-based libraries has thrown light on the microbial communities in the soil and enabled the identification of novel biomolecules (Tariq et al., 2016).

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Results

Isolation and identification

Current study shows total 10 bacterial strains i.e., K1-K10 were isolated from soil contaminated with heavy metals i.e., Korangi Industrial Estate Karachi. The sample was initially screened for their capacity to withstand heavy metals, and the results indicated that all samples were positively grown in their culture media. Based on their morphology, serial dilutions of all the samples produce ten (10) different isolates from the heavy metals-contaminated soil (*Fig. 1*).

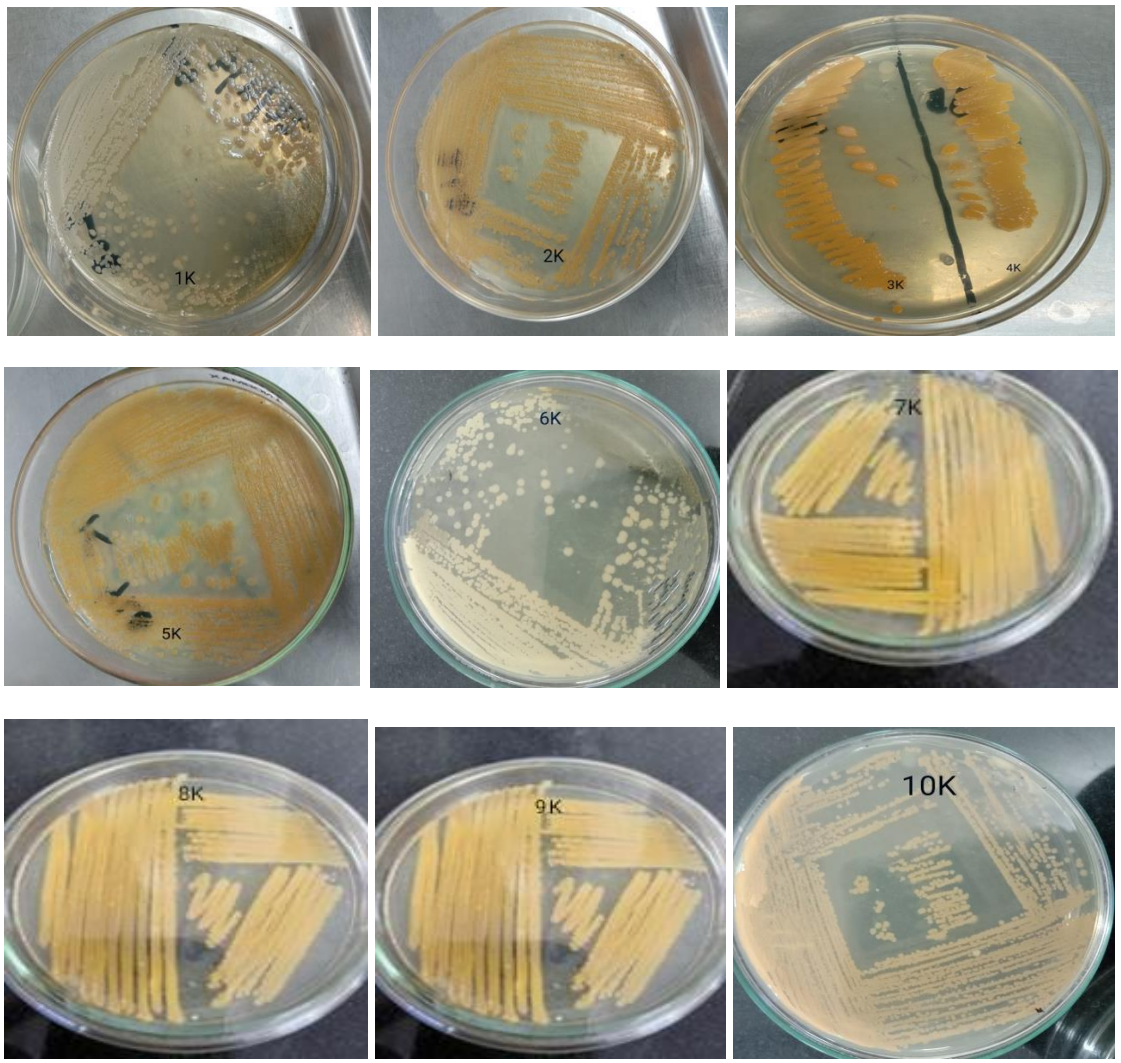

Figure 1. *Morphological characterization of bacterial isolates on LB medium*

Characterization of soil

The pH of the soil was measured by using a multimeter (Model 520) and heavy metals concentration by using atomic absorption spectrometry as mentioned above. In this result pH of the soil was calibrated to 7.40 which was neither too much acidic nor too much basic it was near to neutral and heavy metals concentrating are mentioned in *Table 1*.

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Table 1. *pH measurement*

Code	pH	Cu (mgL^{-1})	Pb (mgL^{-1})	Cr (mgL^{-1})	Zn (mgL^{-1})
S1	7.40	0.759	0.418	8.295	1.940

Gram staining

Depending on how the stain and bacteria interact, the bacteria in a sample will either remain purple or turn pink or red. If the bacteria continue to be purple, they are Gram-positive. If the bacteria turn pink or red, they are Gram-negative. Based on gram staining, 6 isolates 3K,4K,5K,7K, 8K and 10K was identified as gram-positive cocci and the others i.e.,1K,2K, 6K, and 9K was gram negative rods bacteria as shown in results section (*Table 2; Fig. 2*).

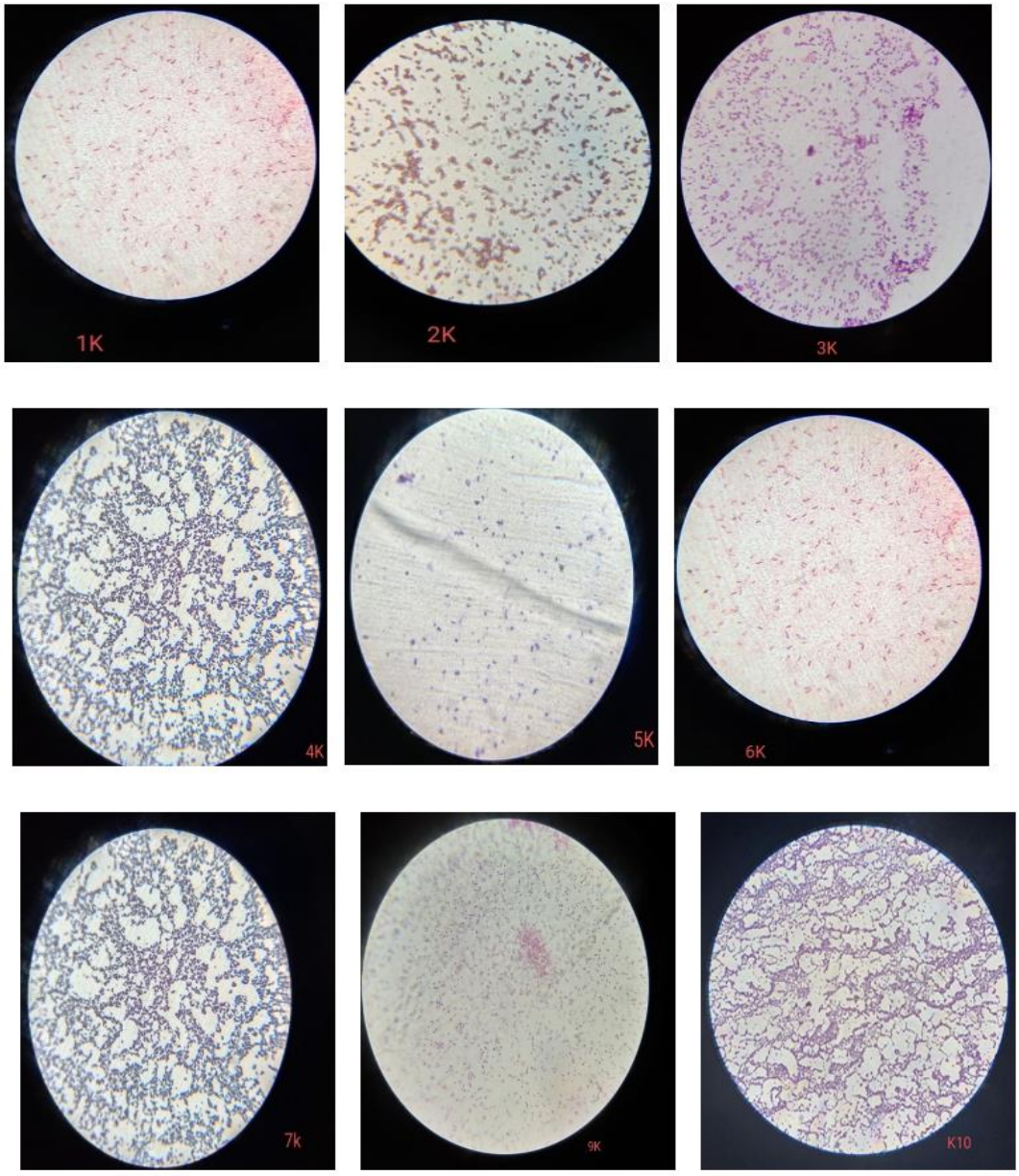

Figure 2. *Microscopy of bacterial isolates*

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Table 2. *Gram's staining and microscopy of bacterial isolates*

Organism	1K	2K	3K	4K	5K	6K	7K	8K	9K	10K
Gram test	-	-	+	+	+	-	+	+	-	+
Shape	Rods	Cocci	Cocci	Cocci	Cocci	Rods	Cocci	Cocci	Rods	Cocci

Biochemical characterization

Bacterial isolates characterization was done by using following standard tests such as catalase, coagulase, oxidase and methyl-red test. Brief description of each test is given in *Table 3*.

Table 3. *Biochemical characterization of bacterial isolates*

No of strains	Gram staining	Shape of isolates	Catalase test	Oxidase test	Coagulase test	Methyl-red test	Proposed genus according to Bergey's manual
1K	Gram negative	Rods	+	+	-	-	Pseudomonas
2K	Gram negative	Cocci	-	-	-	-	Enterobacter
3K	Gram positive	Cocci	+	-	-	-	Streptococcus
4K	Gram positive	Cocci	-	+	+	-	Staphylococcus
5K	Gram positive	Cocci	+	+	+	-	Staphylococcus
6K	Gram negative	Rods	-	+	-	+	Pseudomonas
7K	Gram positive	Cocci	+	+	-	-	Micrococcus
8K	Gram positive	Cocci	+	-	+	+	Staphylococcus
9K	Gram negative	Rods	+	+	+	-	Staphylococcus
10K	Gram positive	Cocci	+	+	+	-	Staphylococcus

Catalase test

After treating the bacteria with some drops of 3% H2O2 on a glass slide, it formed gas bubbles, indicating a positive catalase test. The catalase test result showed 1K positive, 2K negative, 3K positive, 4K positive, 5K positive, 6K negative, 7K positive, 8K positive, 9K positive and 10K positive results for bacterial isolates (*Table 3; Fig. A1* in the *Appendix*).

Coagulase test

The bacterial cells adhering to one another following the addition of plasma indicates a positive test result. When there is no agglutination, the test is negative. Coagulase test

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results showed 1K negative, 2K negative, 3K negative,4K positive,5K positive,6K negative, 7K negative, 8K negative, 9K negative and 10K negative, results for bacterial isolates (*Table 3*; *Fig. A2*).

Oxidase test

Kovac's oxidase reagent (tetra-methyl- p-phenylenediamine dihydrochloride) was used for this purpose. An organism's ability to produce the cytochrome c oxidase enzyme was assessed using the oxidase test. The bacterium was positive to test whether the purple color formed between 30-60 s. Our study's isolates were five oxidase positive and five oxidase negative. Showed 1K positive, 2K negative, 3K negative, 4K positive and 5K positive, 6K positive, 7K negative, 8K negative, 9K negative, 10K positive results for bacterial isolates (*Table 3; Fig. A3*).

Methyl-red test

In this test glucose phosphate broth was used. Methyl red indicator was used for this purpose. If the methyl red indicator is added to an aliquot of culture broth and the red color will appear then it indicates positive result and the capacity of the bacteria to use glucose to produce stable acid methyl red's color changes from yellow to red which indicates positive result. If the color of methyl red does not change it indicates negative results. Methyl red test showed 1K negative,2K negative,3K negative, 4K negative, 5K negative,6K positive,7K negative,8K positive,9 and 10K negative (*Table 3; Fig. A4*).

Classification of isolates on basis of morphological and biochemical characterization

In accordance with Bergey's manual of bacteriology the bacterial classification on the basis of above-mentioned characterization the following were the proposed genus of bacterial isolates such as 1K belongs to genus *Pseudomonas*, 2K belongs to genus *Enterobacter* while 4K, 5K, 8K,10K belongs to genus *Staphylococcus*. Results for bacterial isolates were shown in (*Table 3*).

Metagenomics analysis of soil samples

Metagenomic sequencing data

The heavy metals contaminated soil sample were assigned with paired-end reads (300:300 bp) depending on the unique barcode and raw reads were filtered and cut off, in order to produce clean tags. Then the paired-end reads were combined by using FLASH. Once the reads were merged, 16SrRNA gene sequencing was done and internal transcribed sequences were determined by PEAR (v0.9.6). According to this study the raw data produced during metagenomic sequencing was 38.031 and clean data in mbp was 37.50 as shown in result section (*Table 4*).

Tags connection

When paired-end reads overlapped with each other, FLASH (Fast Length Adjustment of Short reads) produced consensus sequences. According to this study the total pair read number was 67745 and the total connect tag number was 56526 with a connect ratio of 83% during metagenomic analysis of soil sample as shown in *Table 5*.

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OTU clustering

In Molecular Phylogeny, a uniform marker was used to analyze a taxon unit. The sequences must be grouped into OTUs with a 97% similarity in order to calculate the total amount of bacteria in each sample. A sequence was clustered according to their similarity. According to this study 1803 OTUs (operational taxonomic units) with a tag number 40049 were assigned to find out the bacterial diversity present in heavy metal contaminated soil as shown in *Table 6*.

Table 4. *Metagenomic sequence data*

Sample name	Paired-end reads length (bp)	Raw data (in Mbp)	Clean data (in Mbp)
HM contaminated soil	300:300	38.031	37.50

Table 5. *Tags connection*

Sample name	Total pair read number	Connect tag number	Connect ratio (%)	Average length and SD
HM contaminated soil	67745	56526	83.44	415/12

Table 6. *OTU statistics*

Sample name	Tag number	OTU number
HM contaminated soil	40049	1803

Metagenomic NGS data analysis

OTU taxonomy annotation

By using the RDP classifier (v2.2) program, OTU representative sequences was aligned against the database for taxonomic annotation. In this study, 20 OTU (enlisted in *Table 6*) were used for taxonomy annotation. Among which, OTU1218 and OTU 1322 showed the highest abundance and were identified as *Piscinibacter aquaticus* and *Prevotella brevis* species respectively as shown in *Table 7*.

Venn diagram

In this study 2118 bacterial species were identified, out of which 511 were common in both heavy metals contaminated and non-contaminated soil while 1291 bacterial species were present only in heavy metal contaminated soil. In the non-contaminated soil 315 unique bacterial species were identified as shown in *Figure 3*.

Phylum level distribution of bacteria

The metagenomic analysis came up with 29 bacterial phyla. Among the 29 identified phyla, *Chloroflexi* and *Tenericutes* were the most dominant phyla and their relative abundance ranged from 0.75-1.00. Whereas, *Planctomycetes* and *Acidobacteria* were the second most dominant phyla of the heavy metal contaminated soil. *Proteobacteria* was the least common phyla with the relative abundance of 0.00-0.25 as shown in *Figure 4*.

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Table 7. *OTU taxonomy annotation*

#OTUId	Abundance	Taxonomy
Otu1219	4	Bacteria;Bacteroidetes;Sphingobacteriia;Sphingobacteriales
Otu1218	12	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiales_incertae_sedis;Piscinibacter;Piscinibacter_aquaticus
Otu1217	7	Bacteria;Acidobacteria;Acidobacteria_Gp10;Unclassified;Unclassified;Gp10
Otu1216	5	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Glycomycetaceae;Glycomyces;Glycomyces_albus
Otu1215	7	Bacteria;Verrucomicrobia;Unclassified;Unclassified;Unclassified;Unclassified;bacterium_Ellin5102
Otu1214	3	Bacteria;Planctomycetes;Planctomycetia;Planctomycetales;Planctomycetaceae
Otu1213	4	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadales_incertae_sedis;Maricurvus;Maricurvus_nonylphenolicus
Otu1212	6	Bacteria;Verrucomicrobia;Opitutae;Puniceicoccales;Puniceicoccaceae
Otu1211	4	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio_bacteriovorus
Otu1210	9	Bacteria;Firmicutes;Clostridia;Clostridiales
Otu1329	3	Bacteria;Candidatus_Saccharibacteria;Unclassified;Unclassified;Unclassified;Saccharibacteria
Otu1328	5	Bacteria;Candidatus_Saccharibacteria;Unclassified;Unclassified;Unclassified;Saccharibacteria
Otu1323	5	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales
Otu1322	11	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;Prevotella_brevis
Otu1321	3	Bacteria
Otu1320	10	Bacteria
Otu1327	3	Bacteria;Bacteroidetes
Otu1326	5	Bacteria;Proteobacteria;Deltaproteobacteria
Otu1325	8	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales
Otu1324	6	Bacteria;Acidobacteria;Acidobacteria_Gp3;Unclassified;Unclassified;Gp3

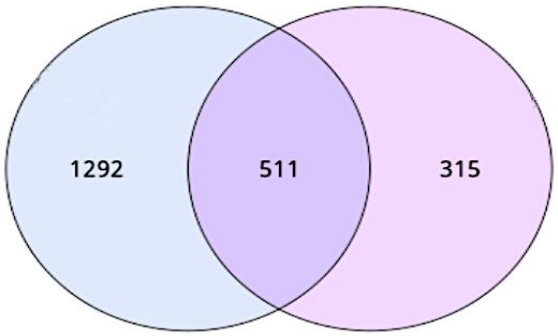

Figure 3. *Venn diagram illustrating number of unique and common bacteria based on OUT of heavy metal contaminated and non-contaminated soil*

Genus level distribution of bacteria

On genera level, most abundant genera in heavy metal contaminated soil were *Blautia* and *Rhodoplanes*, followed by *Prevotella* and *Arenimonas*. The relative abundance of these genera was ranged from 0.75 to 1.00%. In addition to these 26 more genera were also identified as shown in *Figure 5*.

Phylum level heatmap

A uniform marker was used to analyze the sequence. Sequences must be grouped into OTUs with a 97% similarity in order to calculate the total amount of bacteria in each sample. OTUs are grouped together on the vertical axis and ordered in rows. Depending on the relative abundance of taxons, the results of this study showed *Proteobacteria, Synergistetes and Bacteriodes* was most abundant phylum while the least abundant phylum present in heavy metal contaminated soil was *Chlamydia and Tenericutes* as shown in *Figure 6*.

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Genus level heatmap

This study was conducted to characterize the diversity and function of microbial communities present in heavy metal contaminated soil. The results showed that the bacterial communities varied greatly with increase in heavy metal concentration. According to this study *Nocardiodies, Leglonella, Flavobacterium* and *Acidobacter* were dominant bacterial communities while *Sulforum, Clostridium* are least dominant bacterial communities present in heavy metal contaminated soil as shown in *Figure 7*.

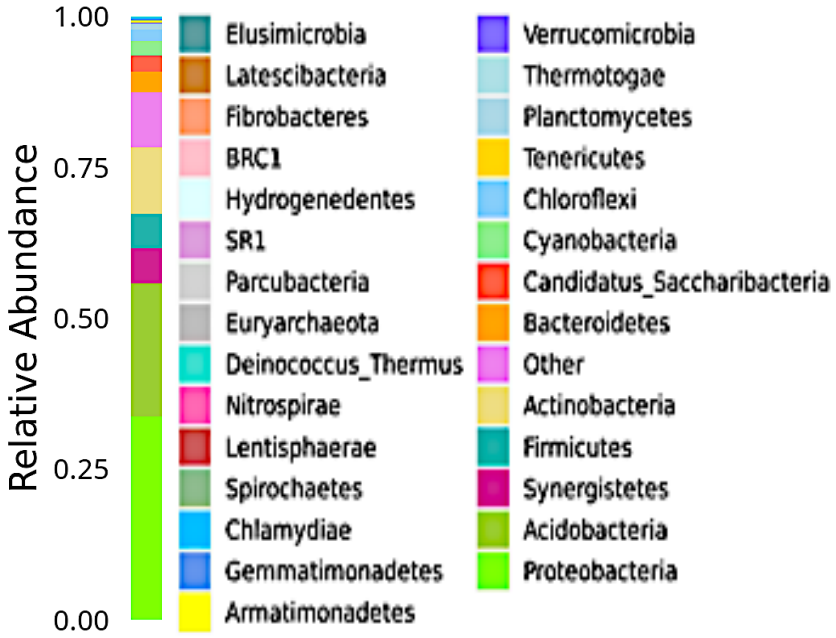

Figure 4. *Metagenomics analysis of bacterial communities at phylum level*

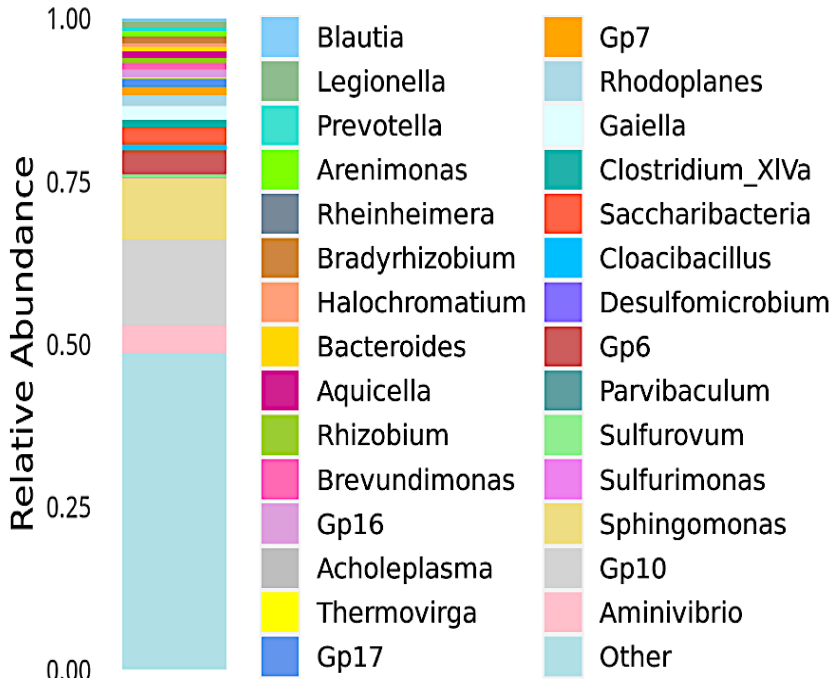

Figure 5. *Metagenomics analysis of bacterial communities at genus level.*

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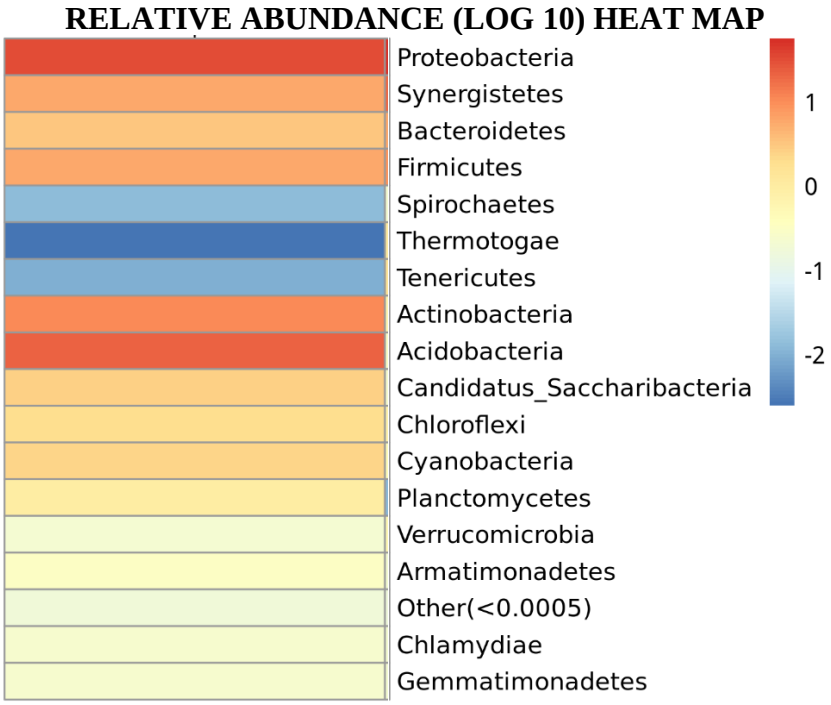

Figure 6. *Heatmap distribution of OTUs at phylum level*

Discussion

The largest city in Pakistan is Karachi, which has two planned industrial areas called Sindh Industrial Estate and Korangi Industrial Estate. Heavy metal contamination of the soil occurs as a result of production processes and stacking practices during industrial production (Khan et al., 2011). Heavy metal exposure at extremely high concentrations can have severe negative effects on living organisms as well as soil microbial communities (Hayat et al., 2024; Khan et al., 2023; Zolfaghari et al., 2018).

Isolation of soil microorganisms contaminated with heavy metals in the current work, bacteria isolated from industrially affected soil are identified and characterized. The initial amount of heavy metal-contaminated soil produced a number of colonies that were screened. In the subsequent screening using soil samples, 15 isolates were selected. Ten strains were ultimately chosen for additional research. In the current investigation, heavy metal-contaminated soil from Korangi Industrial Estate in Karachi revealed a total of 10 bacterial strains, labelled 1K to 10K. The heavy metal-contaminated soil samples were obtained, and preliminary testing revealed that all samples were successfully grown using various culture media such as LB, MacConkey and NA media. Based on their morphology, serial dilutions of all the samples produce ten (10) different isolates from the heavy metal-contaminated soil.

The bacterial isolates were then evaluated for morphological characteristics, by using different medium which showed that a regular, opaque, flat, medium-size colony on LB medium, while some strains produce pale pink to red colonies on the surface of the medium (MacConkey media). 1K was able to grown on MacConkey, *sp.* (2K) produced a regular, short, facultatively anaerobic, non–spore-forming, gram-positive rod that is motile and forms bluish gray colonies on nutrient agar, *Streptococcus* (3K) produced Gray-white, round, opaque, flat, drying, medium-size colony on LB medium, while *Staphylococcus sp.*

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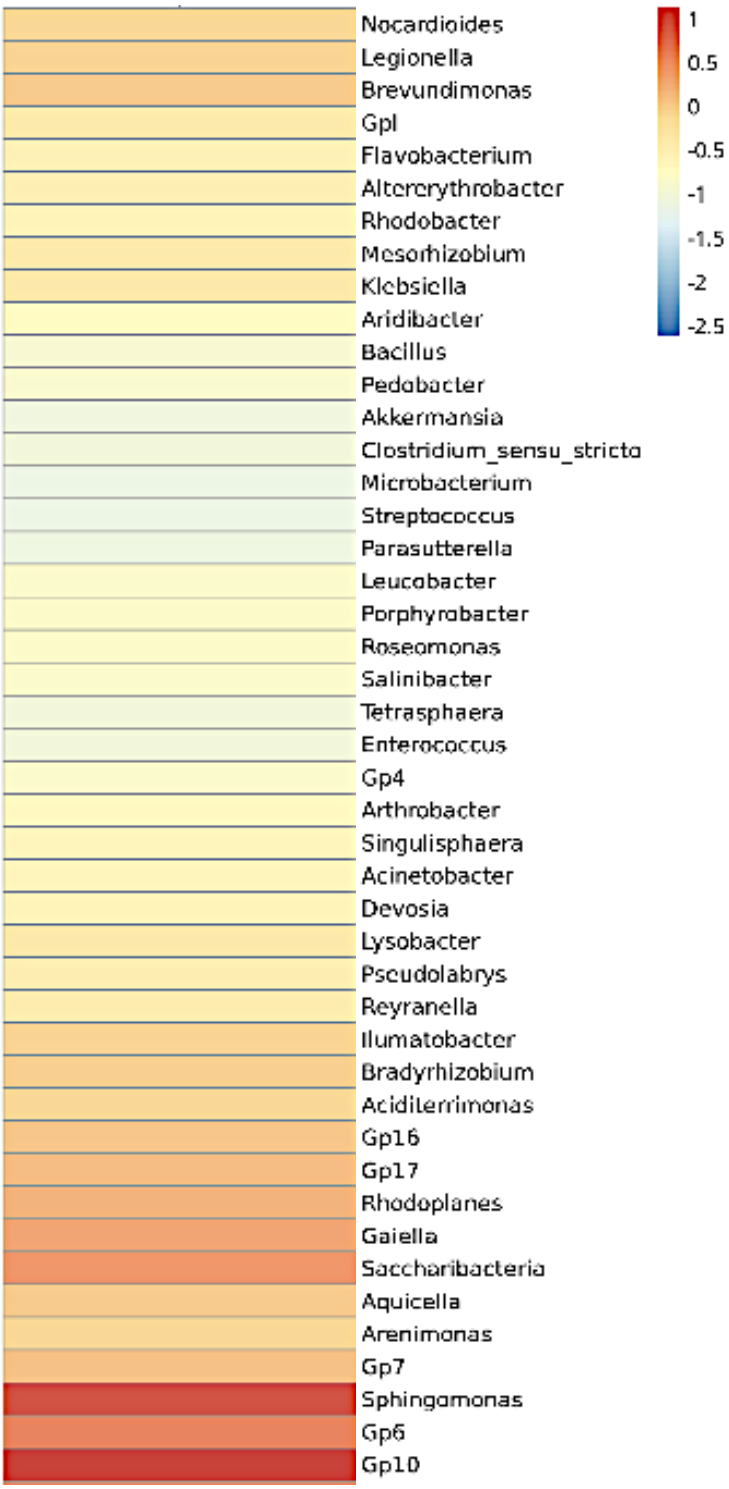
(4K) were colorless and transparent and typically do not alter appearance of the MacConkey medium and Staphylococcus sp. (5K, 6K, 7K, 8K) produce pale pink to red colonies and enterococci produce compact tiny red colonies either on or beneath the surface of the medium. Similar study was also conducted by numerous scientists in according to Bergey's Manual of Determinative Bacteriology which shows that five different strains were also observed on different media and found red colonies on MacConkey media while yellow opaque colonies were formed on LB media.



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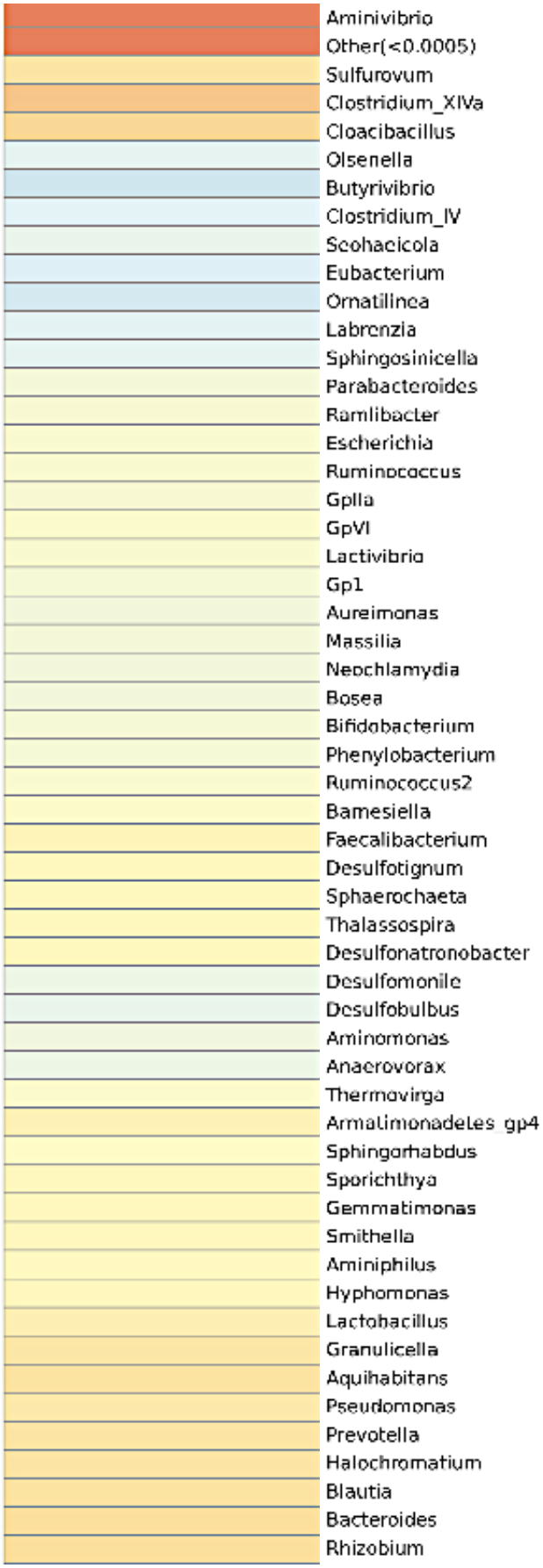

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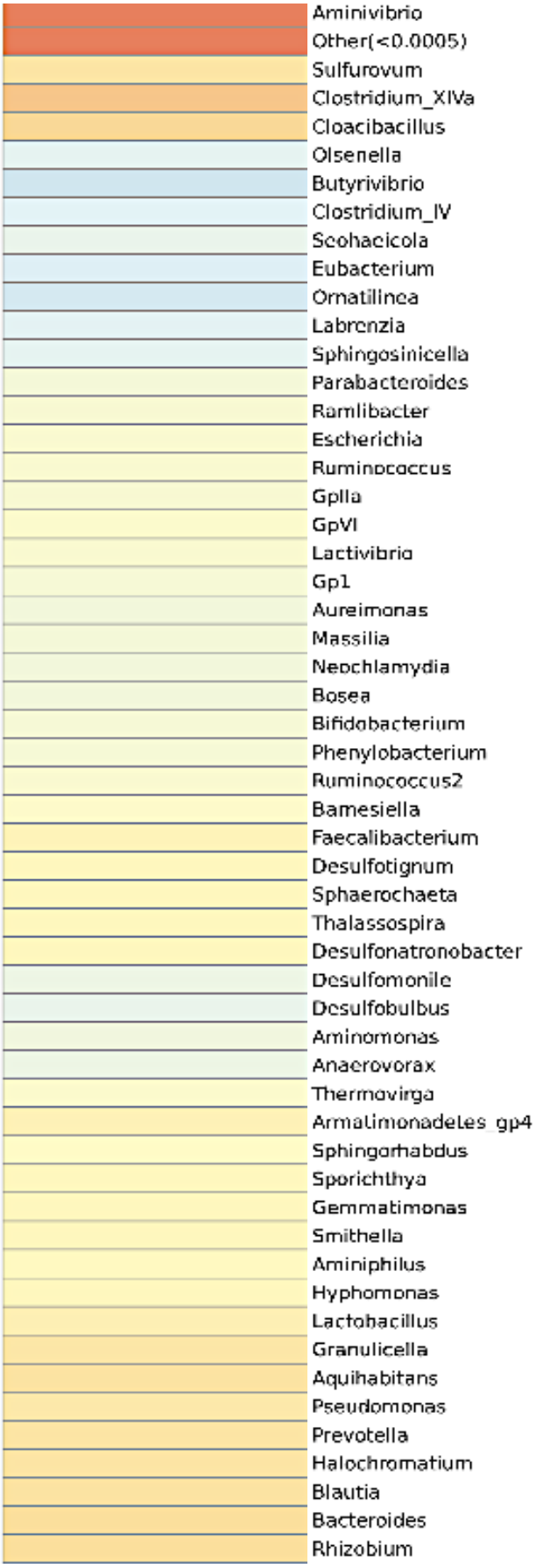

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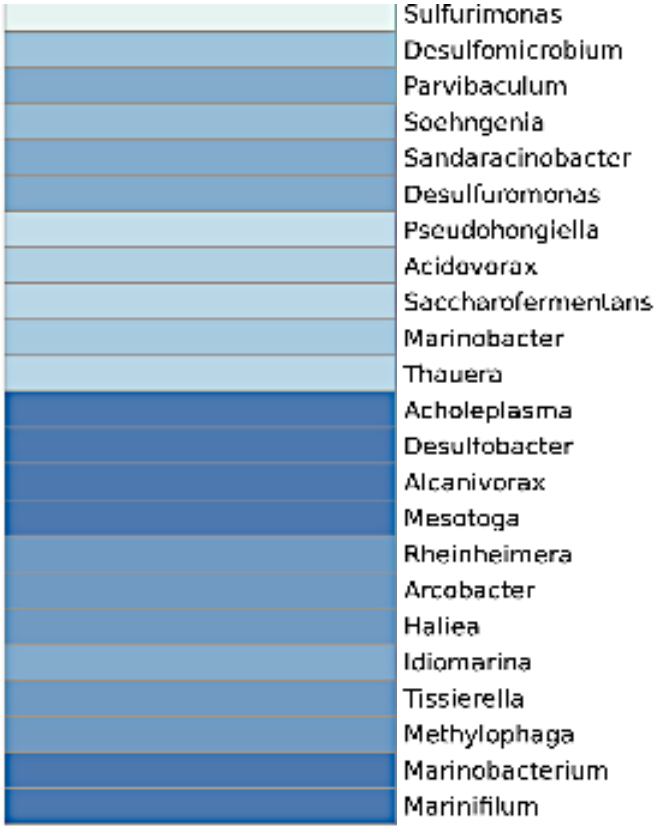

Figure 7. *Heatmap of log10 fold change in relative abundance of bacteria*

On the basis of their form and color, bacterial colonies were identified. In our results we concluded that 60% of bacterial isolates was gram positive while 40% was gram negative. Some bacteria had a round shape in microscopic examination, whereas others had a rod shape. While, depending on gram staining, 36isolates 3K, 4K, 5K, 7K, 8K and 1OK were identified as gram-positive cocci and the others i.e., 1K, 2K, 6K and 9K were gram negative rods bacteria as shown in results section Our findings in this study are in line with those from earlier ones. According to Bergey's Manual of Determinative Bacteriology, several scientists also carried out similar research (Aneja, 2007; Begum et al., 2017) the strains Proteus vulgaris (MR1), Pseudomonas fluorescence (SS4) and Pseudomonas fluorescence (SS5) was Gram-negative, rod shaped motile and Bacillus cereus (MR2), was Gram-positive, rod-shaped motile bacteria by identifying peptidoglycan layer present in bacterial cell.

Targeted isolates (K1- K10) revealed various biochemical characteristics based on morphological analysis and biochemical testing (as shown in *Table 2*). Current studies showed that 7 strains were positive for catalase test and 3 strains were negative for Catalase, whereas 7 strains showed oxidase positive and 3 strains showed oxidase negative. On the other hand, 5 strains were positive for coagulase test and 10 strains were negative for methyl red. Same study was conducted by Udgire et al. (2015) identified some strains by identifying various enzymes that are present in a dense layer of bacteria. Baker (1984), showed similarities of 1K, 3K, 6K and 8K with *Gemella* sp., which is gram negative, and utilizes all carbohydrates and is oxidase positive, catalase negative and coagulase negative (Solomon and Viswalingam, 2013). While 2K, 4K, 10K resembles

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with *Staphylococcus* sp., which are generally oxidase-negative, catalase positive and 5K, 7K AND 8K resembles with *Micrococcus* sp., that was identified as oxidase positive, catalase negative. And methyl red positive.

One of the primary objectives of metagenomics analysis is taxonomic profiling using hypervariable regions of 16S rRNA. Operational taxonomic unit (OTU) clustering techniques are essential tools for completing taxonomic profiling since they categorize 16S rRNA sequence readings into clusters. In our results the dominant bacterial species present in heavy metal contaminated soil was *Bacteriodes, Proteobacteria, Planctomycetes, Firmicutes and Acidobacteria*. Similar study was conducted by Yang et al. (2023), which shows that there is a significant correlation between the abundance of heavy metals and microbial communities. Similar to previous work, in terms of bacterial community composition, *Proteobacteria, Actinobacteria,* and *Acidobacteria* were the dominant bacterial species. In addition, several other dominant bacterial phyla, such as *Acidobacteria* and *Bacteroidetes,* have also been reported.

At the genus level, the results showed that one of the most abundant bacterial genera in the soil while in our study *Blautia, Legionella, Protevella and Arenimonas* were dominant genera. Similar study was also conducted by Hemmat-Jou et al. (2018) and the predominant phylum is *Proteobacteria* and *Actinobacteria* (Tamošiūnė et al., 2019). According to our conclusion heavy metals reduces microbial diversity and this conclusion is supported by earlier investigations (Gołębiewski et al., 2014). *Actinobacteria and Acidobacteria* have also been shown to predominate in heavy metal-polluted soils and in unpolluted areas. As a result, they can effectively adapt to their surroundings, and the current study also found that they were abundant (Kaur et al., 2015). The most abundant bacterial genera were from the following families: *Solirubrobacteriaceae genus Solirubrobacter, Pseudomonadaceae genus Pseudomonas, Nitrosomonadaceae genus Nitrosomonas, Xanthobacteraceae genus Xanthobacter* (Gołębiewski et al., 2014) which shows that, high concentration of Pb and Zn had negative effects of on microorganisms' population and diversity, which is supported by results of other studies. Another study that found that soil bacterial diversity decreased when Pb and Zn concentrations increased and is consistent with our findings (Gołębiewski et al., 2014).

To identify the common species among soil samples, a Venn diagram was examined. The communities' overlaps were also displayed using a Venn diagram. Similar research was done by Bartossek et al. (2012) which shows that Venn figure showed that nearly all genera were present in soil. The heat map shows the distribution of all bacteria genera throughout the different stages of growth (Carrión et al., 2015). This may imply that heavy metals may have an impact on the diversity of species in affected areas. Another study found that soil bacterial diversity decreased when Pb and Zn concentrations increases and is consistent with our findings (Gołębiewski et al., 2014).

A hierarchically clustered heatmap was created to give a broad overview of the connections between the analyzed data. Each bacterial family relative percentage in each sample was displayed on a heatmap plot (X-axis grouping of variables). Color intensity was used to represent each bacterial genus' relative abundance to (Peng et al., 2015) studies. In our results the abundant bacterial genus was *Sphingomonas, Eubacteria, Salinibacter, Bacillus, Nocardiodies* and some other genera are present in long term polluted soil. A similar study was conducted by Gołębiewski et al. (2014), demonstrates that genus *Thiobacillus, Proteobacteria, Firmicutes and Chloroflexi* was most dominant genera present in heavy metals contaminated soil.

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Conclusion

In conclusion, total eight bacterial strains were isolated and identified from soil contaminated with heavy metals in Karachi's Korangi Industrial Estate and microbial community of 1803 operational taxonomic units (OTUs) was found through metagenomic research to be diverse. *Prevotella brevis* and *Piscinibacter aquaticus* were among the dominant species. In polluted and uncontaminated soil, the Venn diagram showed the frequent and uncommon bacterial species. While, 29 bacterial phyla were found by phylum-level analysis, with Tenericutes and Chloroflexi predominating. Blautia, Rhodoplanes, Prevotella, and Arenimonas were found to be abundant genera in the distribution at the genus level. Phylogenomic and genus heatmaps showed the relative abundance of the operational taxonomic units. The results highlight the necessity for environmental management techniques by indicating that heavy metal contamination has a major impact on the variety and composition of bacterial communities in soil.

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Conflict of interests. All the authors declare no conflict of interest.

Availability of data and materials. All data generated in this research work has been included in this manuscript.

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APPENDIX

Figure A1. *Catalase test results for all bacterial isolates*

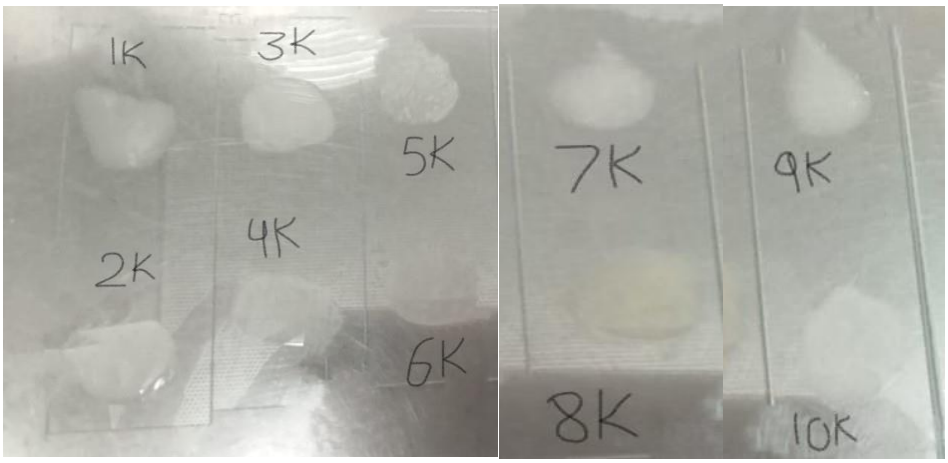

Figure A2. *Coagulase test for all bacterial isolates*

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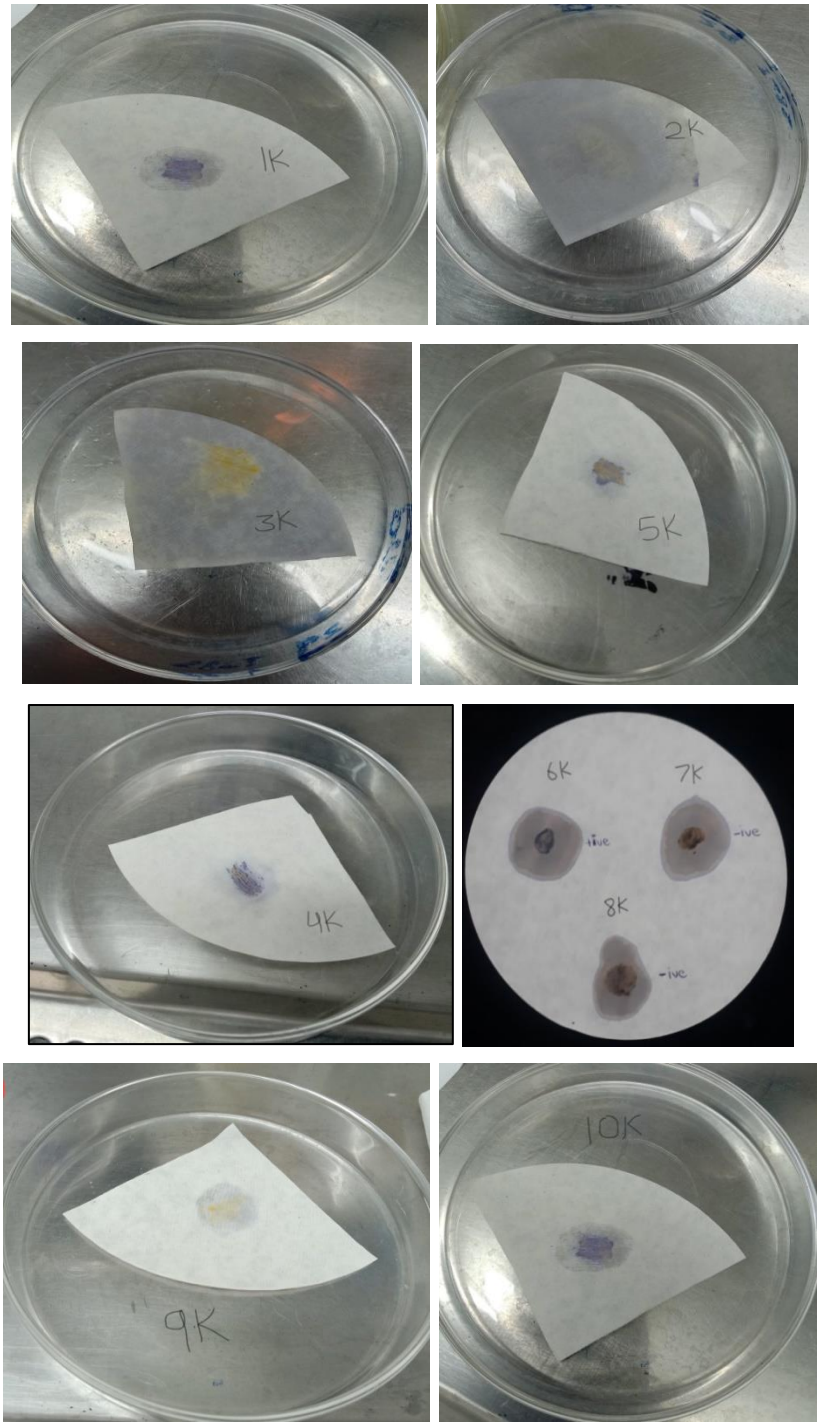

Figure A3. *Oxidase test for all bacterial isolates*

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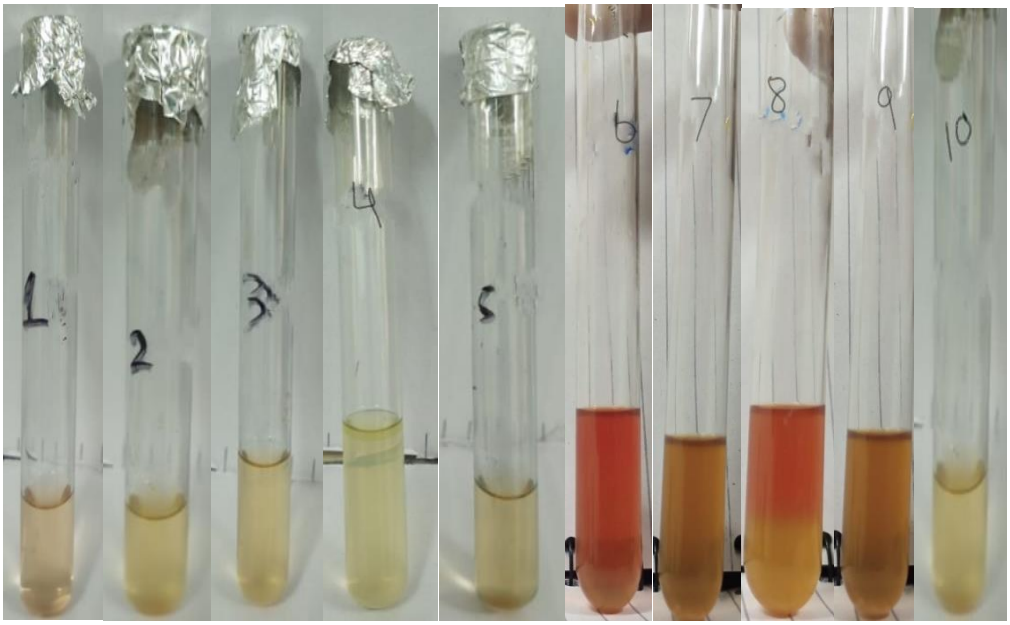

Figure A4. *Methyl red test for all bacterial isolates*

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