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“Characterization and possibility growth for multifetal resistant bacteria from electroplating effluent to potential metal bioremediation”

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ABSTRACT

Industrialization increased metal-laden wastewater discharges. Electroplating pollutes wastewater with metals. These toxins affect environmental systems and public health due to toxicity, persistence, and water quality degradation. Electroplating effluent-exposed microorganisms minimise metal contamination. Metal-resistant bacteria settle debris in industrial effluents for bioremediation. Current work isolates and identifies naturally occurring bacteria from electroplating effluents, analyses effluent physicochemical properties, and verifies their ability to resist, thrive on various metals, and combined toxicity of quaternary combinations on isolate survival. 60 bacterial isolates were cultivated on agar plates containing copper, zinc, nickel, and chromium. New metal-resistant species and combinations emerge. Secondary screening identified nine 50mg/l-growing isolates. Morphological, biochemical, and 16S rRNA gene sequencing were performed. MIC, growth kinetic, and combined metals toxicity effects are used to determine multi metal resistance and growth at the highest concentration. Microbacterium paraoxydans, Streptomyces werraensis, Microbacterium arabinogalactanolyticum, Staphylococcus haemolyticus, Bacillus paramycoides, Bacillus megaterium, Sphingobacterium ginsenosidimutans, Kocuria rhizophila, and Sphingobacterium detergens were found. All isolates showed multi-metal tolerance and better growth performance with MICs between 200 and 1200 mg/L. First and control had S- and bell-shaped growth curves with lag, log, saturation, and decline phases. Metals impacted stationary more.

Keywords: Heavy metals, metal resistant bacteria, growth patterns, electroplating effluents, Minimum Inhibitory Concentration, bioremediation.

نبذة مختصرة:

أدى التصنيع إلى زيادة تصريف مياه الصرف الصحي المحملة بالمعادن. الطلاء الكهربائي يلوث مياه الصرف بالمعادن. تؤثر هذه السموم على النظم البيئية والصحة العامة بسبب السمية والثبات وتدهور جودة المياه. تقلل الكائنات الدقيقة المعرضة للنفايات السائلة المطلية بالكهرباء من التلوث المعدني. تقوم البكتيريا المقاومة للمعادن بترسيب الحطام في النفايات السائلة الصناعية للمعالجة الحيوية. يعزل العمل الحالي ويحدد البكتيريا التي تحدث بشكل طبيعي من النفايات السائلة بالطلاء الكهربائي ، ويحلل الخواص الفيزيائية والكيميائية للنفايات السائلة ، ويتحقق من قدرتها على المقاومة ، والازدهار على معادن مختلفة ، والسمية المجمعة للتركيبات الرباعية على بقاء العزلة. 60 عزلة بكتيرية زرعت على صفائح الاجار المحتوية على النحاس والزنك والنيكل والكروم. ظهور أنواع وتركيبات جديدة مقاومة للمعادن. حدد الفحص الثانوي تسعة عزلات تنمو 50 ملجم / لتر. تم إجراء التسلسل الجيني المورفولوجي والكيميائي الحيوي و 16 الرنا الريباسي. يتم استخدام تأثيرات MIC وحركية النمو والتأثيرات السمية للمعادن المركبة لتحديد مقاومة المعادن المتعددة والنمو عند أعلى تركيز. الميكروبكتريوم باروكسيدان ، Bacillus ،Staphylococcus haemolyticus ،Microbacterium arabinogalactanolyticum ،Streptomyces werraensis and ،Kocuria rhizopherium ،Sphingobacterium ginsidimutans ،Bacillus megaterium ،paramycoides detergensactan were found.أظهرت جميع العزلات تحمل متعدد المعادن وأداء نمو أفضل مع MICs بين 200 و 1200 مجم / لتر. الأول والتحكم لهما منحنيات نمو على شكل S و bell مع مراحل التأخر والتسجيل والتشبع والانحدار. تأثرت المعادن الثابتة أكثر.

الكلمات المفتاحية: المعادن الثقيلة ، البكتيريا المقاومة للمعادن ، أنماط النمو ، الطلاء الكهربائي ، الحد الأدنى من التركيز المثبط ، المعالجة الحيوية.

Introduction

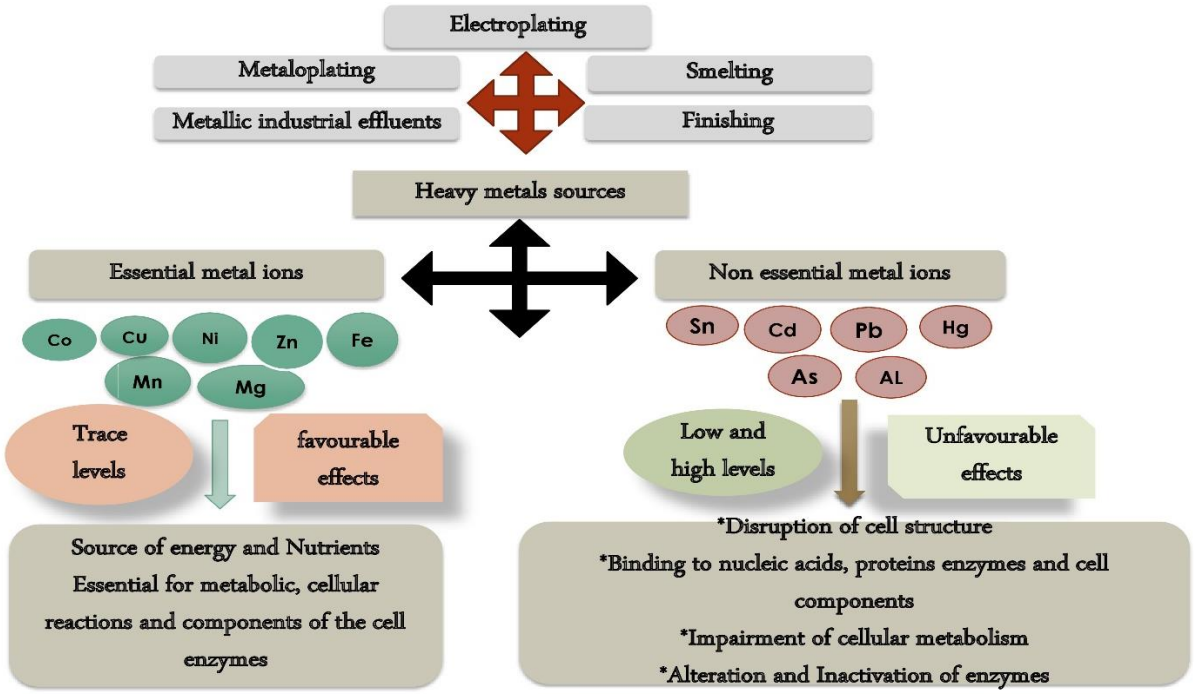
The past decades witnessed rapid industrial developments during which various industrial production activities released huge quantities of effluent containing high level of heavy metals into the natural environments. Such as metal plating, steel production and chemical industries (Zhenggang *et al*., 2019; Bankole *et al*., 2019). Plating is one of the trending industrial activities in which metal is applied to a surface of a material to protect against corrosion by electroplating process. This process includes acid pickling, alkaline cleaning, plating, and rinsing and main metals used for plating are brass, zinc, silver, gold, nickel, copper, iron, aluminium, lead, tin, platinum and chromium plating (Bankole *et al*., 2019).

Specifically, the electroplating industry is considered the most dangerous among the chemical-intensive industries widespread. Beside, high levels of metals, and chemical oxygen demand (COD), other persistent toxic substances are produced at the time of electroplating working as cyanides, nitrates, and sulphate complexes. That is because within the electroplating process it is possible to involve a thin metal on another metal through electro-deposition and the use of different chemicals along with metal salt which eventually leads to pollution troubles. It was found that about 2–20% of the chemicals used along with the valuable metals and cyanide are lost in wastewater which eventually raises the pollutants amount into wastewater (Guan *et al*., 2020; Rajoria *et al*., 2021). Most of the serious metal ions such as copper (Cu), nickel (Ni), Chromium (Cr), and lead (Pb) come from electroplating industry wastewater (Xiao *et al* ., 2019; Rajoria *et al*., 2021).

However, in microbial cells some metals are essential trace elements for biological processes such as Zn, Ni, Cu ,Fe and Mn but become toxic at higher concentrations (Singh and Hiranmai, 2021). Others non-essential and have no known biological role and are toxic even at low concentrations such as Hg, Pb, Cd, and As (Bazzi *et al*., 2020) as shown figure(1).

(Modified from Henao and Ghneim-Herrera,2021;and Sonone *et al*.,2021 and Kahraman, 2020)
Figure(1): The source of heavy metals pollution and its effect on microbial cells
Consequently, purification of industrial electroplating effluent has become a great challenge due to its composition, toxicity, persistence in environment and non-biodegradable which can adversely affect the aquatic life and living organisms (Hoque *et al*., 2019).

Several physical, chemical and biological treatment methods have been used to ensure the effective treatment of polluted water against increase the released amounts of heavy metals into water bodies (Hamdan *et al*., 2021). These



methods include processes such as, thermal treatment, adsorption, chlorination, chemical extraction, ion-exchange, membrane separation, electrokinetics, and filtration etc (Selvi *et al*., 2019). However, despite their success and prevalence they may face certain defects such as low efficiency at low metal concentration, high cost, and toxic by-products (Nugroho *et al* .,2021). Nevertheless, incorporating biological and conventional approaches remains an appropriate strategy for remediation of electroplating effluent and mineral decontamination (Wang *et al*., 2021).

The shortcomings of conventional methods of treating toxic heavy metals in industrial effluent have led to the use of microorganisms in the bioremediation of heavy metals. In order to repair polluted environments and protect the health of the deteriorating environment (Zhenggang *et al*., 2019). It is necessary prompting of effective , economically feasible, and eco-friendly alternative technology for mitigating heavy metal concentrations in industrial effluent to levels environmentally acceptable(Hamdan *et al*., 2021). Microorganisms used for remediation of metals can be isolated from indigenous habitats and applied to the contaminated area. Add to these contaminants can be transferred by living cells through reactions that occur as part of their metabolic processes (Tarekegn *et al*., 2020). Studies of the ability of microorganisms to use chemical pollutants as an energy source through their own metabolic processes throughout the microbiological process are well documented, and it is also well acknowledged that excessive amounts of inorganic nutrients cause microbial inhibition. (Tarekegn *et al*., 2020; Kapahi and Sachdeva, 2019).

Tarekegn et al (Tarekegn *et al*., 2020) reported that microbial cells are able to survive and adapt in metal-contaminated environments and have ability to tolerate heavy metals either being able to remove them from the environment or break them down into benign forms or less toxic then use in their metabolic processes for growth, Table (1) show bioremediation of heavy metals by bacteria and its efficiency. It was confirmed that bacteria have the ability to break down most naturally occurring compounds, in addition manufacturing new ones(Zango *et al*., 2020). As well, the easy availability of microbes into contaminated environments and their natural occurrence assisted to treat huge quantities of heavy-metal-rich industrial effluent (Cai *et al*., 2019). Bioremediation including in- situ and ex-situ process which can be applied to bioremediation of pollutants (Rosanti *et al* 2020), figure(2) show characteristics of bioremediation and its applicability.

Table (1) bioremediation of heavy metals by bacteria and its efficiency .

Bacteria	**Metal**	**Metal concentration (mg/L)**	**Sorption efficiency %**
Bacillus cereus	Cr	1500	80
Bacillus circulans MN1	Cr	1500	96
Bacillus subtilis	Cr	0.57	99.6
Staphylococcus	Cr	4.105	45
***Streptomyces.* sp.**	Pb	0.286	32.5
***Streptomyces.* sp.**	Cu + Ni	0.161	25
Micrococcus.sp	Cu + Ni	50	55
Pseudomonas.sp	Cu+Ni	1	53
Bacillus. firmus	Zn	-	61.8
***Pseudomonas.* sp**	Zn	-	49.8

Tarekegn *et al*., 2021

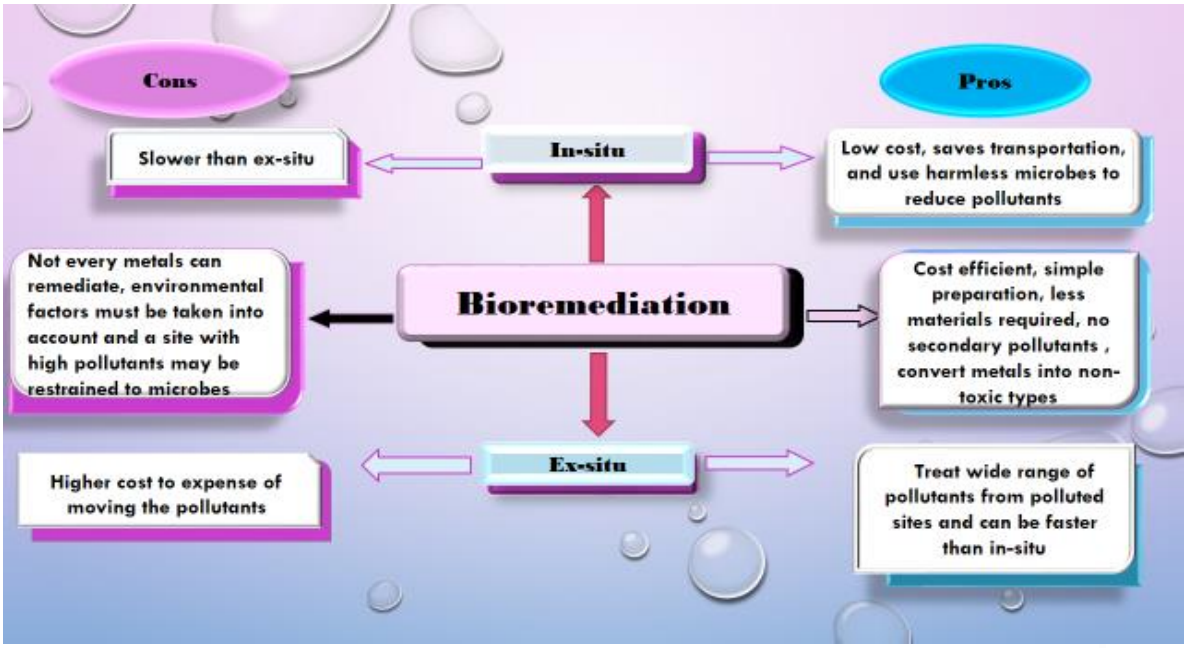


Figure (2): characteristics of bioremediation and its applicability, modified from (Rosanti *et al.*, 2020).

The bioremediation technology can be performed in two ways, on-site or off-site. The place where pollutants can be processed is the main difference between them. Each method has its advantages and disadvantages but the most important matter is that it shows the versatility of this technique and its applicability under different conditions. The efficacy of bioremediation by microbial cells depend on several parameters including bacteria, growth stage, and metal ion concentration and type. Accordingly, for success of the practical application of bioremediation of heavy metals, the optimal parameter should be established (Igiri *et al.*, 2018). Moreover, the concentration of metal ions, their chemical forms and the redox potential of metal ions in microbial cells determine the inhibitory or stimulatory concentration to microbes (Shaifali, and ChethanKumar, 2021).

As a result of the aforementioned and since heavy metal pollution occurs predominately as a mixture of various pollutants. So, bacteria in contaminated sites are usually exposed to a mixture of metals not individual, it is mainly important to consider bacteria capable of treating these contaminants as combined mixtures as well as individual metal solutions. This can lead to a synergistic or antagonistic effect on treating of the synthesized combined mixtures depending on their interactions with each other as well as with the bacteria (Bind *et al*., 2019). Therefore, examination of these interactions can determine the bacterial efficiency to grow and tolerate the composite mixtures of metals.

In general, the effects of the presence of multiple chemicals simultaneous have not received as much attention as the effects of single metals. Those the effects of multiple chemicals more dangerous than single effects should not neglected (Adnan *et al*., 2021). There is not enough data for combined toxicological interaction for heavy metals, and there is not much literature indicating the interactions of heavy metals with each other as well as with the biosorbent in the composite mixtures. As a novelty of this paper pay particular attention to study the combined effect of quaternary mixtures or the combined toxicity of four heavy metals based on optical density (O.D) and colony-forming units (CFU/mL) at time intervals of metal exposure. As well, response or behaviour of the bacteria towards quaternary mixtures, then compared to the individual ones. The expected synergistic or antagonistic interaction between heavy metals will produce significant effects either positively stimulating or negatively inhibiting on bacterial growth and decontamination process of metals mixtures).

This study contributes to identification of some bacterial species that inhabit electroplating effluent through determine the phenotype and genotype characteristics. In addition, the characteristics of effluent and its relationship to the presence of microbes. These species are a source for obtaining potential metal resistant bacteria can assist to study a number of bacterial activities. For example, metals resistance which is expressed in terms of the Minimum Inhibitory Concentration (MIC), growth kinetic and combined toxicity at the highest concentration of metals. The combined toxicity assessment of quaternary mixtures including copper, zinc, nickel and chromium metals and compared to individual ones are performed at the highest concentration initial concentrations of these metals.

2. Material and methods

Electroplating effluent were collected simultaneously and subjected to physicochemical and microbiological analyses in order to isolate and identify the bacteria that were then used in further studies.

2.1. Pre-sampling Quality Assurance and Quality Control (QA/QC)

All laboratory instruments and samplers were washed with 10 % HNO_3 and pre-soaked with distilled water one day before sampling to remove any trace concentrations of metals (Benavides *et al*., 2019). The glassware was enfolded with aluminium foil then sterilized by autoclaved at 121 °C for 15 minutes (El-Sayed *et al*.,2016).

2.2. Collection of effluent samples

The electroplating effluent were collected from the direct and final outlet of one of Metal Electro-Plating Factory. Three samples were collected from three different points in this site, and the fourth sample was a mixture for the entire site, each sample collected in triplicate portions. One separate portion of the effluents destined for determination of heavy metals were acidified with concentrated nitric acid which was added to samples at the time of samples collection-to maintain pH ranging between 1.5 and 2.0 as described by(Davies, 1996) in order to minimize any kinds of precipitation, adsorption and microbial activities. Second separate portion was placed into BOD bottles for determining the Biological Oxygen Demand, The last separate portion for determining Chemical Oxygen Demand (COD) and microbiological study was put into sterile glass bottles. The fourth sample was also divided into three portions, similar to the previous three samples. Samples collected were preserved on ice at 4 °C before transferred to the laboratory and processed, to prevent from contamination and allowing the sample to stay for a longer time (Kumar *et al*., 2011).

Figure (3) Electroplating effluent sampling location

Figure (4): Sampling sites 1, 2, 3

2.2. Physicochemical characteristics of the electroplating effluent

The samples were divided into three portions for chemical and microbiological examinations and determining the characteristics of each sample. The physicochemical parameters were determined included temperature, pH, turbidity, alkalinity, electrical conductivity, Dissolved Oxygen (DO),Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), of electroplating industrial effluent using standard methods (APHA, 2005) and concentration of nickel , copper, zinc and chromium metals were estimated by ICP-MS (Inductively coupled plasma mass spectrometry). Method of Analysis of the electroplating effluent: colors (visual), pH (pH meter), turbidity by YSI 30, salinity conductivity, Dissolved Oxygen (DO) by YSI 52 dissolved oxygen meter and temperature these were measured in situ. The others like alkalinity as ($CaCO_3$) titrimetric and COD by Dichromate reflux were determined in lab, BOD by YSI 52 dissolved oxygen meter was measured the dissolved oxygen (DO) levels of effluents samples prior incubation in situ and after 5 days of incubation measured (D_5) in the lab(Lohchab and Saini, 2017).

2.3. Preparation of Heavy Metal Solutions

Stock solutions of 1000 mg/L concentration of zinc, copper, chromium, and nickel were prepared by dissolving zinc sulphate ($ZnSO_4$), copper sulfate ($CuSO_4$), potassium chromate ($K_2Cr_2O_7$) (R&M Chemical) and nickel chloride ($NiCl_2$) mg/L (HmbG Chemical) in distilled water. Thereafter, the solutions were shaken for 15 min and then left to stand for 24 hr to obtain complete dissolution. Metal solutions were sterilized by membrane filter, and store at 4°C. (Abd El Hameed *et al*., 2015).

2.4 Isolation and identification of metal tolerant bacterial strains from the electroplating effluent

The last separate portion of the collected samples were used to isolate the bacteria. The samples were serially diluted in sterilized phosphate buffer saline solution up to 10^{-9} dilutions. Sample (0.1ml) from 10^{-6} and 10^{-7} dilutions were taken and spread on the nutrient agar plates, which are supplemented with individual and quaternary heavy metals Cr, Zn, Cu, and Ni in the form of their salts, $K_2Cr_2O_7$, $ZnSO_4$, $CuSO_4$ and $NiCl_2$ at concentration (10 – 50 mg/l) respectively. The plates were incubated at 37º C for 24-48 hours (Pandit *et al*., 2013). These selected dilutions gave a significant decrease in biomass concentration which facilitated differentiation and separation of colonies (Talaro and Chess, 2007). Bacteria that were grown were then subsequently sub-cultured, so that pure cultures were obtained on nutrient agar containing the same concentration of heavy metals. The isolates were given the following abbreviations; (BMA-1), (ACM-2), (DMA-3 (STM-4), (BSM-5), (BME-6), (A6MA-7), (MIC-8) and (RMA-9). The bacterial isolates were maintained on Nutrient Agar slants at 4°C for further use (Silva *et al*., 2012).

2.5. Morphological and biochemical characteristics for identification of bacterial isolates

All bacterial isolates were identified through morphological and biochemical characteristics. Colony morphology, Gram's staining and endospores forming were used to differentiate between bacteria and identify the Gram reaction and endospores forming of the given cultures to classify and describe the morphology and phenotype of the isolates. The (IMVIC) tests which include, Iodole Production, Methyl Red reaction, Voges Proskauer reaction, and Citrate Utilization test, respectively used to determine the ability to decompose the amino acid tryptophan, oxidize glucose, produce acetone and use citrate. The efficacy of isolates to produce enzymes such as nitrate reductase, urease, catalase, α- amylase and gelatinase respectively was determined by the following tests nitrate reduction, urease, catalase, hydrolysis starch and gelatin. Also, carbohydrates fermentation test (glucose, lactose, sucrose) were used to test acid and gas production by isolates. The isolates were partially identified according to Bergey's Manual of Systematic Bacteriology was used (Bergey and Holt, 1989).

2.6 Molecular Characterization (extraction of bacteria DNA and amplification of 16S rRNA

The molecular identification or genetype of the isolates from the nutrient agar with metal supplementations was done by the sequencing of 16S rRNA.

The genomic DNA was extracted from isolated bacterial cultures using (Extraction kit: Rapid bacterial genomic DNA isolation kit, (Biobasic.inc) according to the manufacturer protocol. 16s rRNA gene was amplified using universal primer 27F 5' (AGA GTT TGA TCM TGG CTC AG) '3 and reverse primer
1492R 5'(TACGGY TAC CTT GTT ACG ACT T) 3'. The PCR mixture 25 µL consisted of 10.5 µL nuclease free water, 12.5 µL OneTaq. 2X Master Mix (NEB), 0.5 µL 10 µM 27F and 0.5 µL 10 µM 1492R with 1 µL of 50ng DNA as templates. PCR was performed in conditions at 94 ◦C for 4 mins to initiate denaturation follows by 30 cycles of 94◦C for 30 seconds denaturation, 55◦ C for 30 seconds annealing and 68 ◦C for 1 min 30 seconds extension and lastly 68 ◦C for 5 minutes for final extension. 3 µL PCR products were loaded into 1% 0.5 X TBE Agarose Gel and electrophoresed in 0.5 X TBE using 90V electricity for 50 minutes. Agarose gel was documented using Cleaver Scientific ChemiPRO gel documentation system. Sequencing was performed by BioBasic sequencing facility, Sequencing results were analyzed using Sequence Scanner V1.0 (Applied Biosystem), edit and assemble using Chromas. Assembled sequence was then undergo homology search in NCBI nucleotide collection 16s database excluding uncultured/environmental samples sequences using BLAST. Top ten BLAST result were used to construct unrooted neighbour joining phylogenetic tree using BLAST Software.

2.7. Determination of Minimum inhibitory concentration (MIC) of heavy metals

Isolates were inoculated in 10 ml sterile nutrient broth containing (100-1000mg/l) of each metal individually and quaternary. Tubes were incubated at 37◦C for 24 hr. After 24 hr growth of bacteria were measured at 600 nm by Atomic Adsorption Spectrophotometer (AAS). A positive control consisted of a metal deficient medium inoculated with the bacteria was used. All metal salts were added to the medium after autoclaving and cooling to 45-50ºC, from filter sterilized stock solutions (Pandit *et al*., 2013).

2.8. Growth patterns of bacterial isolates

Conventional techniques were used to reveal the bacterial growth. The spread plate method and measurement of optical density were combined. Among the three initial concentrations (10, 50 and 100mg/L) in which all isolates were grown, the highest concentration of 100mg/L was used. For spread plating, overnight cultures of nine bacterial isolates in nutrient broth were diluted serially in physiological salt solution, 0.9% NaCl. Dilutions were repeated till 10^{-8} in duplicate, then 1 mL aliquot for each dilutions were transferred to sterilize nutrient agar plates containing 100 mg/L copper, zinc, nickel chromium metals and quaternary, and spread across the entire surface of the plates. Every spread plate was made in duplicate and incubated at 37°C, thereafter colonies were counted via colony counter at every 24hr period to 72hr and growth was expressed as colony forming units per millilitre (CFU/mL).The seventh dilution (10^{-7}) was chosen and was more appropriate among the dilutions that were performed because the cells numbers could be distinguished and counted, they ranged from 30 to 300 cells.

Optical density was done by taking 1 mL of each culture broth samples every 24hr interval to 72hr and measured (O.D) using Spectrophotometer at a wavelength of 600 nm (Thermo Spectronic Genesis 20, Spectrophotometer). Zero point calibration was performed with a blank nutrient broth (Monballiu *et al*., 2015). At the same time growth of the

isolates on nutrient broth and nutrient agar with no metal supplementation (control) was done. The CFU and OD at 600 nm were recorded for every 24hr interval. To illustrate the bacterial growth, the growth curve of each of isolate was plotted. The control and with media supplemented with metals of both methods which performed were compared by plotting CFU, incubation time and optical density (Chien *et al*., 2013).

2.9. Statistical analysis

The results was of physicochemical parameters were analysed by calculating mean ± SE, analysis of variance (ANOVA), *P* value was calculated to determine the significant results. Results showing P value less than 0.05 were considered as significant ($P < 0.05$). As well, the significance of MIC values between isolates were analysed statistically using ANOVA Honest Significant Difference.

3. Results and Discussion

3.1 The physicochemical characteristics of electroplating effluent

The physicochemical parameters of electroplating were estimated, the results are presented in Table (2). The pH of the electroplating effluent was (5.28 - 6.41) slightly tend to acidity which may effect on bacterial growth where (6.5-7.5) is the optimal pH for bacterial growth. Add to this, when pH levels are low, this may encourage heavy metal solubility resulting in the mineral cations being released into the water instead of being absorbed into the sediments. While alkaline pH prefers the precipitation of most minerals as insoluble solids (Edokpayi *et al*., 2015). The electroplating effluent temperature as average (29.35°C), a high value of electrical conductivity could indicate of the presence of high amount of ionic substances and high total dissolved solids concentration, EC is directly proportional to the total dissolve solids (TDS) which is not investigated in this study.

All samples were (yellowish green) colour with turbidity reading 9.99NTU, and all these measurements were carried out in-situ. The alkalinity ranged between (12-13 mg/L), it is as a stabilizer for pH and a measure of the ability of water to neutralize acids, Alkaline compounds such as bicarbonates, carbonates, and hydroxides remove hydrogen ions and reduce the acidity of water, thus increasing the pH. The results showed that alkalinity was low, consequently, the pH decrease and is a favourable medium for metals solubility and not being deposited. There were no specific standards for the alkalinity levels in industrial effluents.

Dissolve oxygen is an important parameter associated with the aquatic body and provides direct and indirect data such as bacteria activity Photosynthesis, and availability of nutrients, it was found to be higher than 7. Biological Oxygen Demand as indicator to measure microbial oxygen depletion was between (32.58 - 34.52mg/L) while Chemical Oxygen Demand (66.4 - 76mg/L) which is indicator to measure oxygen depletion by chemical pollutants. Previous results reported that Chemical Oxygen Demand (COD) is generally 2 to 3 times that of BOD. This is because some organic substances are resistant to microbial oxidation, but can be easily oxidized chemically and it is almost close to the results obtained in this study, similar results have been reported by other researchers. Higher values of BOD, the greater the extent of oxygen depletion in the water environments, this indicates the reduction of oxygen available for aquatic life, affect aquatic organisms and thus its termination.

The contents of heavy metals in the electroplating effluent were higher with Cr, Zn and in one sample with Cu. Statistically, significant differences were not found between sites (ANOVA, $P > 0.05$) in results the physicochemical parameters of samples. Its appeared that most of the physicochemical parameters were within the permissible limit according to Malaysia's Environmental Law, ENVIRONMENTAL QUALITY ACT, 1974, the Malaysia Environmental Quality (Sewage and Industrial Effluents) Regulations, 1979, 1999, 2000, except for the following parameters turbidity, Ec, pH in one sample, COD, colour, concentrations of chromium, zinc and one copper sample were higher than the standards limits.

Table (3): The results of physicochemical parameters of electroplating effluent

Samples Parameters	S1	S2	S3	S4	Effluent Standard by Environmental Quality ACT, 1974
Temperature	29.4◦C	29.3◦C	29.2◦C	29.5◦C	40◦C
Colour	Yellowish green	Yellowish green	Yellowish green	Yellowish green	Not objectionable*
Turbidity	9.99NTU	9.99NTU	9.99NTU	9.99NTU	< 5*
pH	5.28*	6.12	6.41	6.22	6 - 9
Alkalinity mg/L	12	12.6	12.9	13	-
EC (Electrical Conductivity)	5.18 ms/cm	5.63 ms/cm	2.61ms/cm	2.59ms/cm	1000µS/cm*
Biochemical Oxygen Demand (BOD) mg/L	32.85	34.0	34.52	32.58	20-50 < 50
Dissolved oxygen mg/L	8.33	8.5	8.52	8.42	5-7 >7
Chemical Oxygen Demand (COD)mg/L	76	73.6	70.4	66.4	< 50*
Concentrations of heavy metals :- Copper (Cu) mg/L	0.6799*	0.2181	0.1529	0.2187	0.2
Zinc(Zn)mg/L	1.5724	1.1729	1.2318	1.2177	1.0*
Nickel (Ni)mg/L	0.0095	0.0315	0.0077	0.0372	0.2
Chromium (Cr)mg/L (Hexavalent)	2.209	0.678	0.466	0.414	0.05*

* Higher than the standards limits of Environmental Quality ACT, 1974.

3.2. Isolation and identification of multimetal-resistant bacteria

A total of 60 bacterial isolates were isolated initially, cultured on nutrient agar plates incorporated with a concentration of (10 - 50) mg/L of copper, zinc, nickel and chromium as individual metals and as a quaternary metal solution. Based on the secondary screening nine isolates that were able to grow at the highest concentration of 50mg/l were chosen.

3.2.1 Morphological characteristics of bacterial isolates

Morphological and biochemical characteristics for nine selected bacterial isolates (BMA-1, CMA-2, DMA-3, STM-4, BMS-5, BME-6, A6MA-7, MIC-8 and RMA-9) from electroplating industrial effluents were examined. They were tested on the nutrient agar as showing in Figure (5). The growth media were incorporated with initial concentrations range (10-50)mg/L of cu, zn, ni and cr salts to confirm the ability of growth, resistance and purity, thereafter were recultured and maintained. Primary characterization of the bacterial isolates were determined by colony morphology or phenotype (colour, surface, margin and elevation). The description morphology are as shown Table (3).

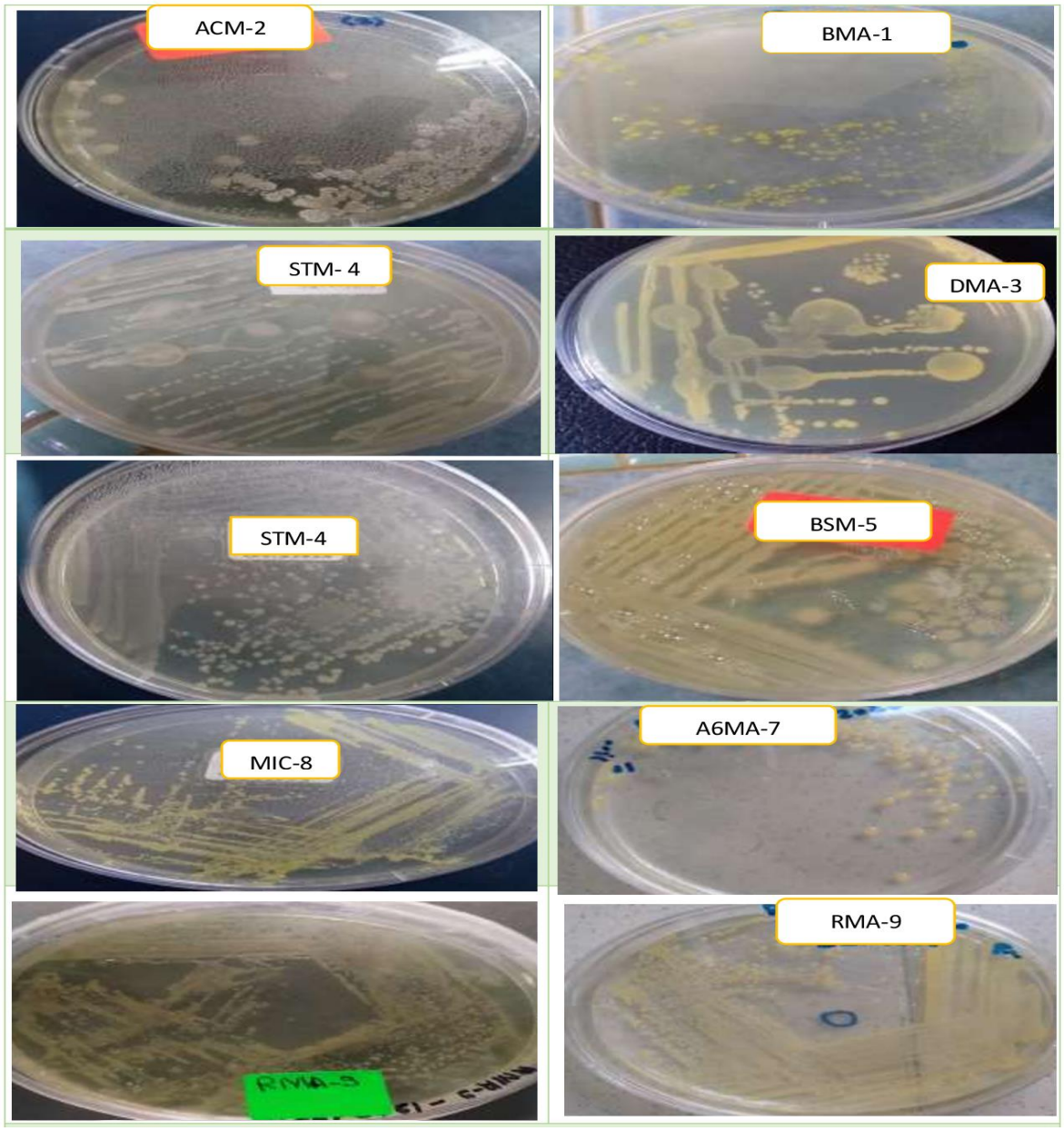


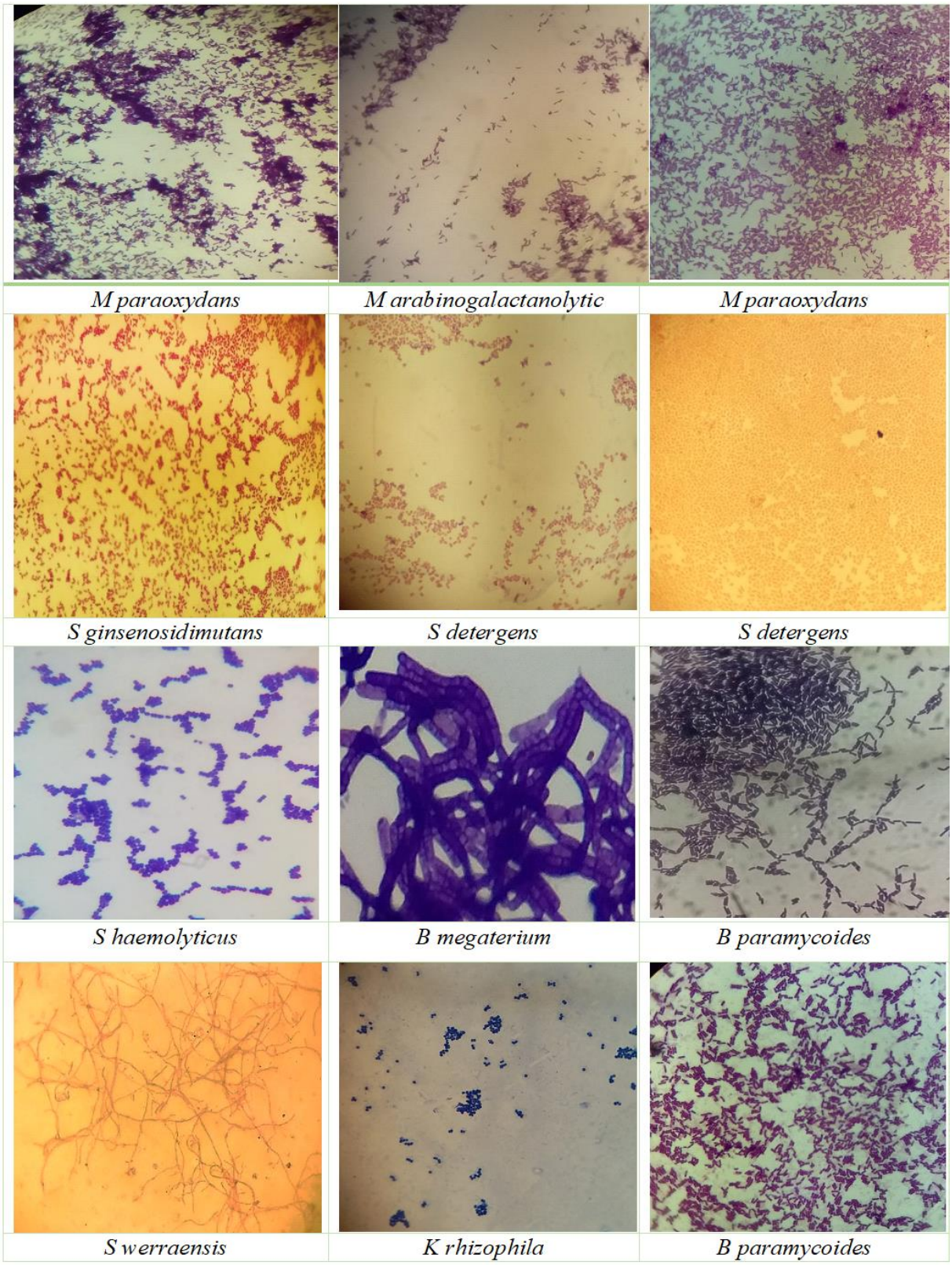
Figure (5): isolates bacterial on the nutrient agar plate

Table (3): The morphological characteristics of the bacterial isolates

Isolates	**Colony colour**	**Motility**	**Gram nature and shape**	**Cell morphology**
BMA-1	Cream to yellow	+	Positive, short rods, coryneform rods single, paired and clumps	Non-spore-forming Undulate, flat and smooth.
ACM-2	White and grey	_	Positive to variable	Mycelia or rough and crumbly in texture.
DMA-3	yellow	+	Positive /Cells were small, short and slender rods and occurred singly or in irregular clusters	Non-spore-forming, translucent, yellow-pigmented, opaque, circular, low-convex, moist, margins and entire
STM-4	White and yellowish	_	Positive/ cocci	Non-spore-forming, glistening, with entire margins, smooth, opaque, graped in pairs, tetrads and clusters.
BMS-5	white	+	Positive/ rods in chains	Spores-forming, opaque, waxy, circular with jagged edges.
BME-6	faint yellow	+	Positive/ rods in pairs and chains	Spores-forming, round, Smooth and flat.
A6MA-7	orange	_	Negative/ coccoid to bacillary	Non-spores-forming, round, smooth, raised, and glistening with entire edges.
MIC-8	yellow	_	Positive/ cocci pairs, tetrads, and irregular clusters,	Non-spores-forming, circular, shiny smooth, convex, entire and small.
RMA-9	salmon-	_	Negative/ coccoid to bacillary	Non-spores-forming, smooth, circular, yellowish in colour, raised, and glistening with entire edges.

*Variable results (positive and negative)

The morphological characteristics of the bacterial isolates demonstrated that the predominant shapes were rods, coccobacilli then cocci, Most of them were gram positive, only two out of nine isolates were gram negative. The isolates screened microscopically as illustrated Figure (6)



Figure(6) The microscopic properties of the most effective isolates

Where : BMA-1 (*Microbacterium paraoxydans*), ACM-2(*Streptomyces werraensis),* DMA-3*(Microbacterium arabinogalactanolyticum),* STM-4(*Staphylococcus haemolyticus)* BMS-5(*Bacillus paramycoides),* BME-6(*Bacillus megaterium),* A6MA-7 (*Sphingobacterium ginsenosidimutans),* MIC-8 (*Kocuria rhizophila),* RMA-9*(Sphingobacterium detergens).*

3.2.2. Biochemical characteristics of bacterial isolates

Biochemical characteristics were conducted in order to determine the physiology of each isolates, the results are shown in Table (4). All isolates were able to produce catalase and nitrate reductase enzymes, but no isolate was able to produce urease. Some of them produced α- amylase and gelatinase enzymes, and utilized lactose and sucrose. Carbohydrate that all isolates used was glucose and they produces acid and gas. IMVIC tests showed bacterial isolates ability to oxidize glucose to organic acids, produce acetone and use citrate, but they could not decompose the amino acid tryptophan. Based on these results they were partially identified according to Bergey's Manual of Systematic Bacteriology was used.

Table (4): The results of biochemical tests of bacterial isolates

Isolates	BMA-1	ACM-2	DMA-3	STM-4	BMS-5	BME-6	A6MA-7	MIC-8	RMA-9
Catalase	+	+	+	+	+	+	+	+	+
Citrate	-	-	+	-	-	+	-	-	-
Gelatin	-	-	-	-	+	+	-	+	-
Starch	-	-	-	-	+	+	+	-	-
Indole	-	-	-	-	-	-	-	-	-
MR	+	+	+	-	+	+	-	-	-
VP	-	-	-	V	-	-	+	+	-
nitrate	+	+	+	V*	+	+	+	+	V*
urease	_	_	_	_	_	_	_	_	_
Utilization of: Glucose	+	+	+	+	+	+	+	+	+
Lactose	-	-	+	V	-	+	-	-	+
Sucrose	+	+	+	+	+	+	+	-	+

3.2.3. Molecular identification of bacterial isolates

Molecular identification of the electroplating effluents isolates by 16S agarose rRNA, according to the alignment and BLAST at NCBI. The DNA of all nine isolates BMA-1, CMA-2, DMA-3, STM-4, BMA-5, BME-6, A6MA-7MIC-

8, and RMA-9 were amplified with primers 1492Rand 27F. The PCR amplified products were detected by 1% gel electrophoresis. This test was done according to database available, after alignment with other 16S rDNA sequences in GenBank, these isolates were registered in the NCBI GenBank and were given the accession numbers as listed in Table (5).

Phylogenetic tree, based on 16S rRNA gene has been considered a fast and accurate method to identify the phylogenic position of bacteria and relationships of nine bacterial species. Partial 16S rRNA of isolates were sequenced and used to construct phylogenetic development trees which clearly determined that the selected isolates belong to the following genera and species: *Microbacterium paraoxydans, Streptomyces werraensis, Microbacterium arabinogalactanolyticum, Staphylococcus haemolyticus, Bacillus paramycoides, Bacillus megaterium, Sphingobacterium ginsenosidimutans, Kocuria rhizophila and Sphingobacterium detergens.*

Table (5) BLAST results of the16SrRNA gene sequences of the isolates at NCBI.

Accession	Description	Query	Query cover%	E.Value	Identity %
NR_025548.1	*Microbacterium paraoxydans*	BMA-1	100	0.0	99.93
NR_112390	*Streptomyces werraensis*	ACM-2	99	0.0	99.65
NR_0449321	*Microbacterium arabinogalactanolyticum*	DMA_3	100	0.0	99.86
NR_036956.1	*Staphylococcus haemolyticus*	STM_4	100	0.0	99.53
NR_1577341	*Bacillus paramycoides*	BSM_5	99	0.0	100
NR_117473.1	*Bacillus megaterium*	BME_6	99	0.0	99.93
NR_108689.1	*Sphingobacterium ginsenosidimutans*	A6MA-7	96	0.0	98.57
NR_026452.1	*Kocuria rhizophila*	MIC_8	100	0.0	100
NR_118238	*Sphingobacterium detergens*	RMA_9	100	0.0	98.01

The results indicated that the high diversity isolates obtained from metals contaminated environments and their ability to grow on all metals. The phylogenetic tree based on 16S rRNA gene sequences, isolate BMA-1 was classified with *Microbacterium pataoxydans* (NR_ 025548.1), isolate ACM-2 with had the great similarity (99.86%) with *Streptomyces capillispiralis* (NR_ 041168.1) compared to *Streptomyces werraensis*(NR-112390.1) was (99.65%), but was classified and had a closer genetic relationship with *S. werraensis* (NR_112390 .1). Thus, this isolate might belong to *S. werraensis*. Isolate DMA-3 was classified with *Microbacterium arabinogalactanolyticum* (NR- 044932.1) similarity ratio (99.86%) and isolate STM-4 was classified with *Staphylococcus haemolyticus* (NR_036955.1) similarity rate(99.93%).

Two isolates were the same genus but of different species were BSM_5 which classified with *Bacillus paramycoides*(NR_ 157734.1) and isolate BME-6 was classified with *Bacillus megaterium*(NR_117473). The previous isolates and the taxonomy were matched due to they showed the closest distance on the phylogenetic tree. Isolate A6MA-7 was classified with *Sphingobacterium ginsenosidimutans* (NR_108689.1) and *Sphingobacterium pakistanense(NR_113311.1),and* had a closer genetic relationship with S. *ginsenosidimutans. So, isolate* A6MA-7 belonged to S. *ginsenosidimutans.* Also, isolate MIC-8 was classified with *Kocuria rhizophila* (NR_026452.1) and *Kocuria arsenatis* (NR_ 148610.1), and had a closer genetic relationship with *Kocuria rhizophila*. Thus, isolate MIC-8 belonged to *K. rhizophila*. Although isolate RMA-9 had the similarity of 98.57% with *Sphingobacterium ginsenosidimutans* (NR_108689.1), it was the closest distance to *Sphingobacterium detergens* (NR_ 118238.1). Thus, isolate RMA-9 might belong to *S. detergens.*

3.2.4 Minimum inhibitory concentration of the bacterial isolates against heavy metals

The results showed high MIC values which indicated to high resistance of the bacterial isolate toward the metal. Although the original sites from which these species were isolated did not contain excessive concentration of metals, all isolates showed multi tolerance to metals to varying levels. It was explicated through another study that non-polluting environments may also contain metal-resistant organisms or organisms that easily adapt to high mineral concentrations. Furthermore, it was reported that occurrence of strains carrying the resistant plasmid is more common in the contaminated sites than in the non-contaminated region (Chellaiah, 2018). The results of heavy metal tolerance study is shown Figure 7 which illustrated multi- metal resistance and high tolerance to Cu, Zn and Ni

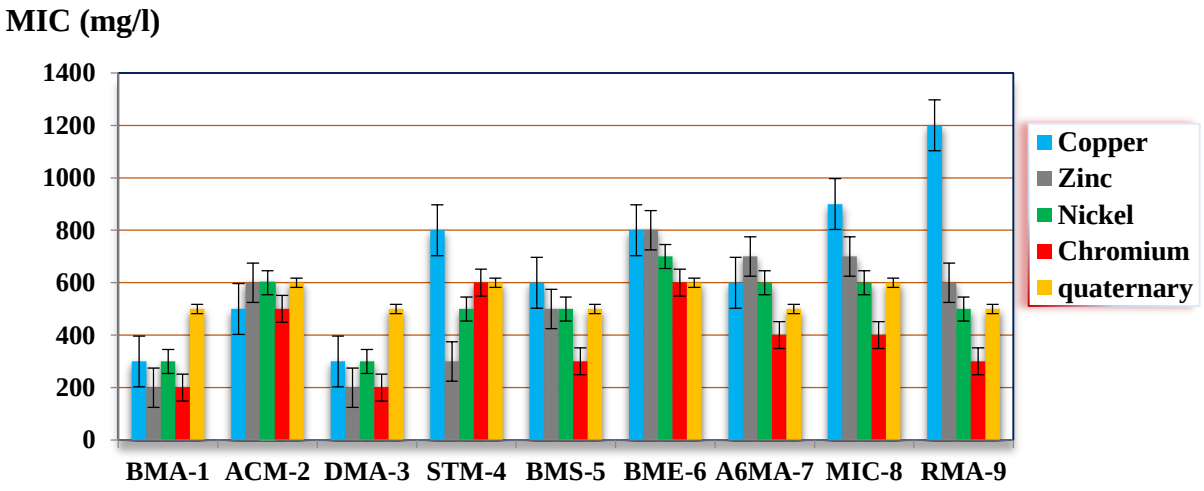


Figure (7) Minimum inhibitory concentration (MIC) of multimetal resistant bacteria

MIC for heavy metals ranging between 200 and 1200 mg/l, resistance values of all isolates and with each bacteria. The range of MIC for each metal were respectively (300-1200 mg/L) Cu, (300-600 mg/L) Ni, (200-800 mg/L) Zn, and (200-600 mg/L) Cr and quaternary. The highest metal tolerance was specifically found with *Kocuria rhizophila*, *Bacillus megaterium*, *Sphingobacterium ginsenosidimutans* and *Sphingobacterium detergens*. The results also *showed that Staphylococcus haemolyticus* and *Bacillus megaterium* were higher metal tolerance to the chromium (600 mg/L) compared to the other bacterial isolates. These results in agreement with much of the researches that indicated that there are prominent bacterial strains of metal resistance isolated from contaminated sites (Banerjee *et al.*, 2018). It was showed previously that *Bacillus megaterium* is able to reduce toxic Cr (VI) to non-toxic Cr (III) (Ingle and Saler, 2021).

It was found that heavy metal resistant bacteria exhibited highly resistance of heavy metals belonging to different genera and species, for example, members of the *Bacillus* genus have high resistance to heavy metals (Huang *et al.*, 2020a). It has been previously reported that *Bacillus megaterium* possesses high levels of resistance to harmful conditions such as exposure to heavy metals like Ni (Rivas-Castillo *et al.*, 2018). It was considered that *Bacillus sp* are the most effective bacterial species in the treating of heavy metals due to the ability to survive and detoxify metals (Alotaibi *et al.*, 2021). The class of actinomycetes that inhabits polluted environments to which *Streptomyces werraensis* belongs has been reported to be resistant to heavy metals compared to those in unpolluted environments (Zabermawi *et al.*, 2021). Genetic analysis of *K rhizophila* revealed that it contained genes related to tolerance of different heavy metals concentrations (50 mg/ L to 500 mg/ L)(Afridi *et al.*, 2021).

In addition, Sphingobacterium was one of isolates which achieved the highest specific growth rates in the presence of the heavy metals and selected as one of the bacterial strains with maximum heavy metal. The results of the present study found that species of Sphingobacterium showed high copper tolerance capabilities, it has been reported that these species of Gram-negative bacteria are more resistant to metals than gram-positive bacteria. The reason was attributed that gram-negative bacteria have a complex three-layered cell wall, which acts to immobilize metals more effectively (Mutiat *et al*., 2018), unlike the gram-positive cell wall which is mainly composed of a thick layer of peptidoglycan within periplasmic space (Ishida , 2018). In one of studies was found that a strain of *Sphingobacterium* isolated from mineral dumps was shown to thrive in high concentrations of copper, cadmium, and lead, and had the ability to grow in 1000 ppm of lead, copper, and chromium (Mgbodile *et al*., 2022).

Microbacterium species shown multimetal resistance, it was confirmed that *Microbacterium isolates* taken from sites contaminated with heavy metals were more resistant to heavy metals and contain more genes related to metal homeostasis (eg, metal regulators). The extensive ability of *Microbacterium strains* to mobilize many metals has been reported (Corretto *et al*., 2020). Many strains of *Microbacterium* can survive in environments contaminated with heavy metals. These strains can reduce certain metals such as hexavalent chromium (Kumar and Saini, 2019; Corretto *et al.*, 2020). The isolated species that showed resistance to metals in this study were previously found to be with antibiotics resistance. Thus, antibiotic resistance may associate with ability of bacteria to resist metals as well previous studies found that total of 16 different strains of *Microbacterium* showed a wide range of tolerances to heavy metals and antibiotics (Learman *et al*., 2019).

The correlation between the acquisition of antibiotic resistance by microbial cells and metal tolerance has been extensively studied by several researchers, and both resistance genes were found to be located on the same mobile genetic elements (Pathak *et al*., 2020; Nath *et al*., 2020). These abilities were also found with *Staphylococcus haemolyticus,* the results appeared high MIC of tested metals. It was reported that *S haemolyticus* contain MCO gene that confers it the property of multiple tolerance to different chemical factors including heavy metals. (Xingxing *et al*., 2020). The results of this study confirmed that the isolated bacteria from polluted electroplating environments have resistance capabilities of heavy metals. The results of statistical analysis were found significant difference in MIC values between species of *Microbacterium* and *B. megaterium,* while no any significant effect between the other species

3.2.5 Growth patterns of bacterial isolates

Exposing the isolates to metals (Zn, Ni, Cr and Cu) individually and quaternary as form metals salts at a high concentration of 100 mg/ L at different times started from 1h then 24, 48, and 72 hours was studied. Hundred mg/L concentration was used that is because the highest concentration which all isolates grew in it according to the results of minimum inhibitory concentration. Growth patterns were carried out for determining the survival of isolated bacteria in different growth phases after exposure to 100 mg/L of Cu^{2+}, Zn^{2+}, $Ni^{2+}$and Cr^{6+}. As well, to verify the distinction of growth among the bacterial isolates in the presence of these metals. The results presented through figures (8-16).

The effects of Cu, Zn, Ni, and Cr were determined based on optical density (O.D) and colony-forming units (CFU/mL) at time intervals of metal exposure. Observation of metals impact on bacterial viability point out that the number of isolates that achieved relatively better growth curves patterns and were closer to the control, respectively it was six out nine for zinc and nickel, four out of nine were for quaternary, chromium and copper. The tested isolates possess equal and much CFU and OD from the others but may showed S-shaped growth curve with lag, log, saturation and decline phases less than control such as *Bacillus megaterium, Staphylococcus haemolyticus and Kocuria rhizophila*.

In addition, the metals may disturb the growth of isolates that is because they almost lose the saturation/stationary phase in the growth curve, becoming bell shaped such as *Bacillus paramycoides, Microbacterium paraoxydans and Microbacterium arabinogalactanolyticum*. This might be due to early bacterial cell death, and it was also consistent with other findings that showed that under the influence of some minerals, bacterial growth may be hindered when they nearly lose the saturation/ stability phase of the growth curve, and become bell-shape (David *et al*., 2016). The

stationary stage was disturbed from the usual straight shape with tested metals as shown growth curves of isolates in the absence and presence metals.

It is also noticeable during the obtained growth patterns with quaternary or copper of some isolates after 24 hours which did not occur the prominent decline in the last stage and was to a lesser level compared to the other metals such as *Streptomyces werraensis*, *Bacillus paramycoides*, *Kocuria rhizophila*, *Sphingobacterium ginsenosidimutans and Sphingobacterium detergens*. This indicates that beside the dead cells there were living cells that are still growing under metal stress and did not stop yet their growth. This can be attributed to the previously mentioned fact that environments rich in heavy metals can improve adaptation bacteria mechanisms (Escamilla-Rodríguez *et al*., 2021).

The results showed that the isolates in some metal-modified liquid media exhibited shape of growth closer to the control medium were included lag, log and decline phases. That is because these stages appeared somewhat clear in some isolates more than others. The growth curves of isolates which are almost similar to the growth curves of the control in some metals, may be more tolerant because of the fact that the metal acts as a catalyst to bacteria and lead to the synthesis of bacterial stimulating proteins, which helps in their growth and tolerance. Bacteria may show the resistance abilities and control mechanisms that they possess to counter the metal stress as inducible proteins aid the growth of metal-resistant bacteria.

It is important to determine whether bacterial growth is related to the type of metal because the tested metals in this study included singles and quaternary. The analysis showed a statistically significant difference between the type of metal and bacterial growth with a value of ($P < 0.05$). A significant effect of copper and chromium appeared to differ from zinc, nickel and quaternary according to Tukey's test there was a significant difference between them. On the contrary, there were not significant difference between zinc with nickel and copper with chromium. The obtained results indicates that the type of metals affects differently on bacterial growth, because metal affect each single specie differently (Ngwewa *et al*., 2022).

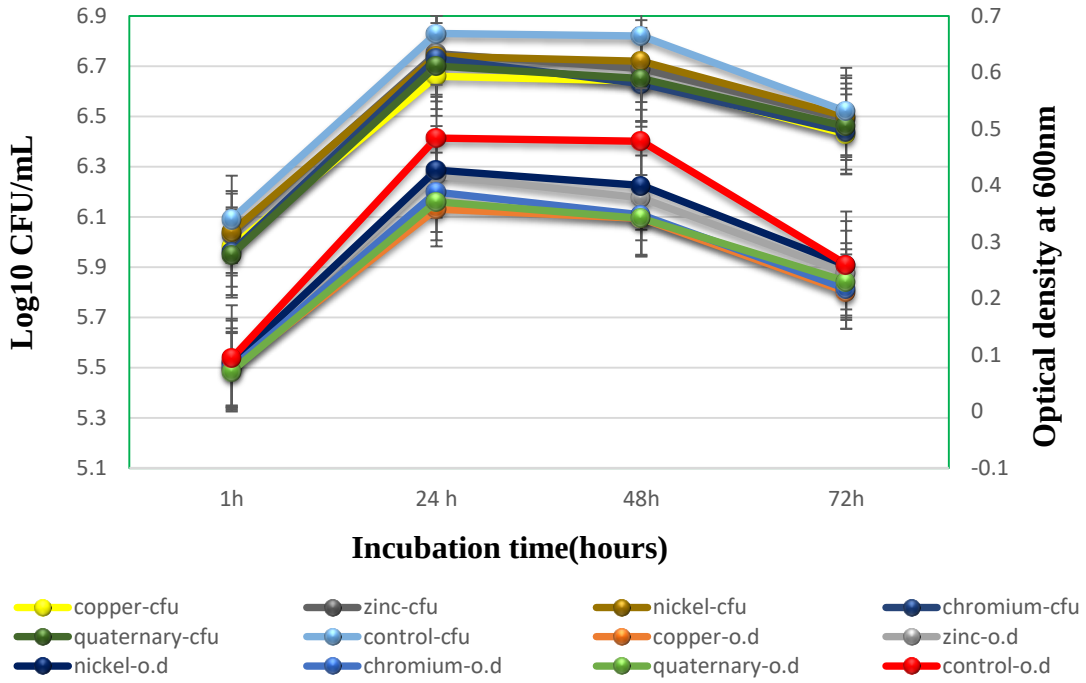


Figure 8: *Microbacterium paraoxydans* isolate viability in presence of different metals

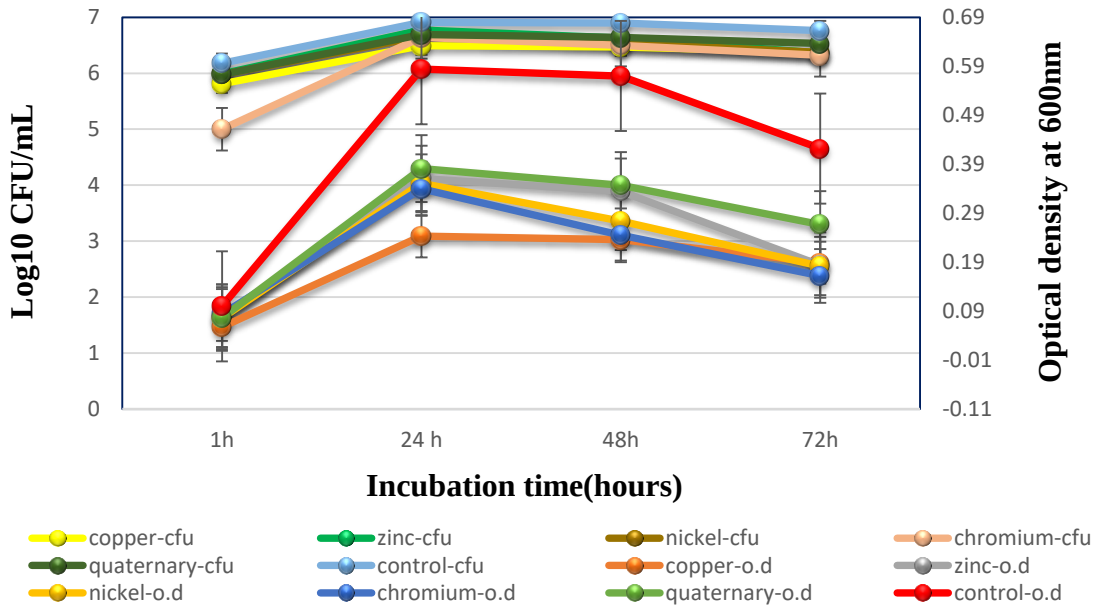


Figure 9: *Streptomyces werraensis* isolate viability in presence of metals

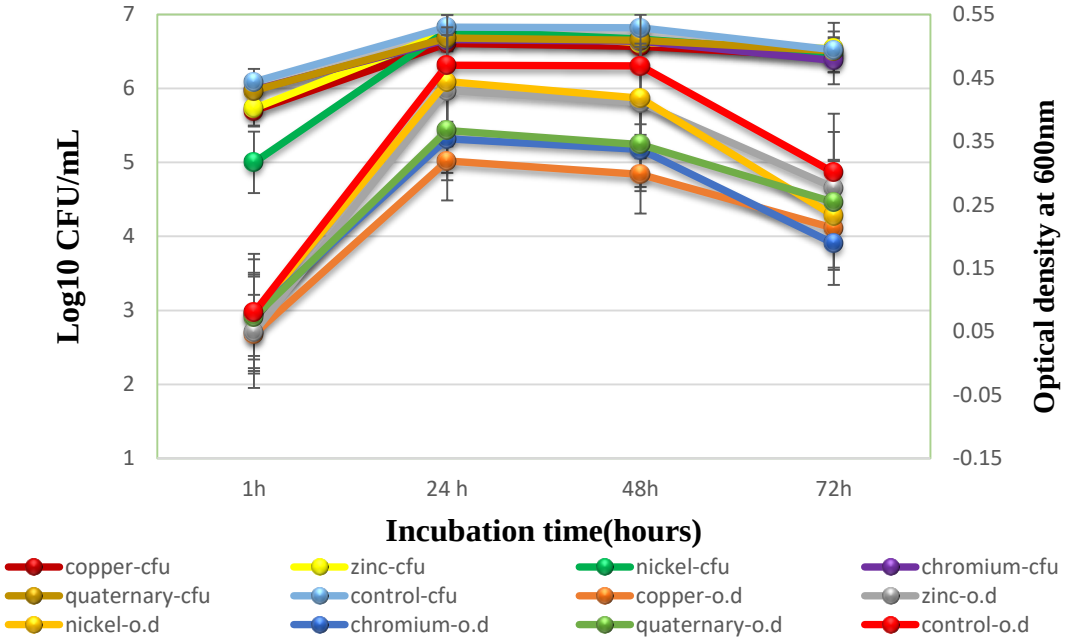


Figure 10 : *Microbacterium arabinogalactanolyticum* isolate viability in presence of different metals

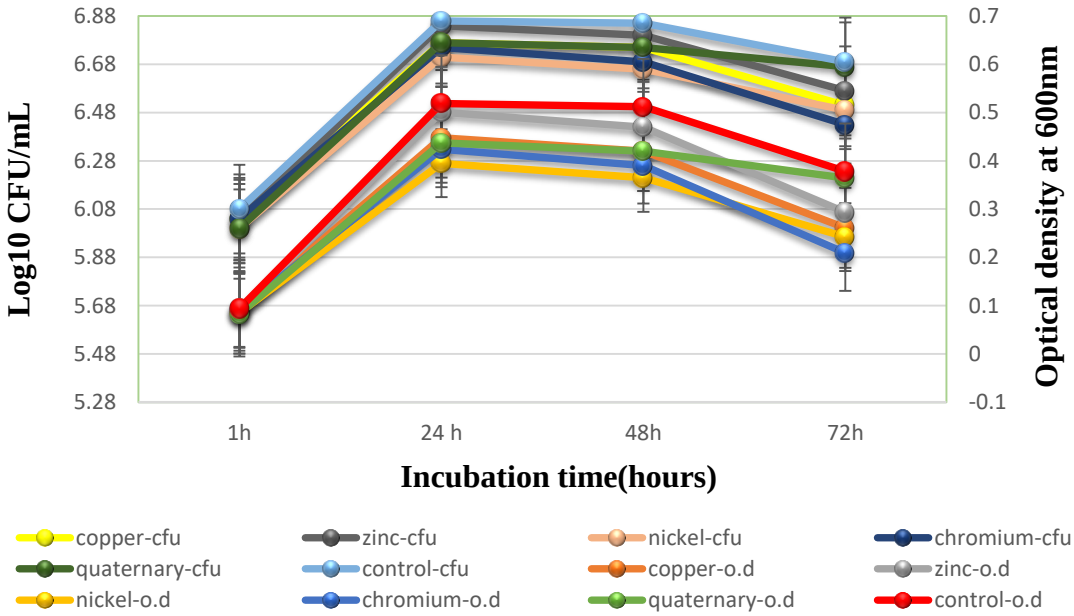


Figure 11 : *Staphylococcus haemolyticus* isolate viability in presence of different metal

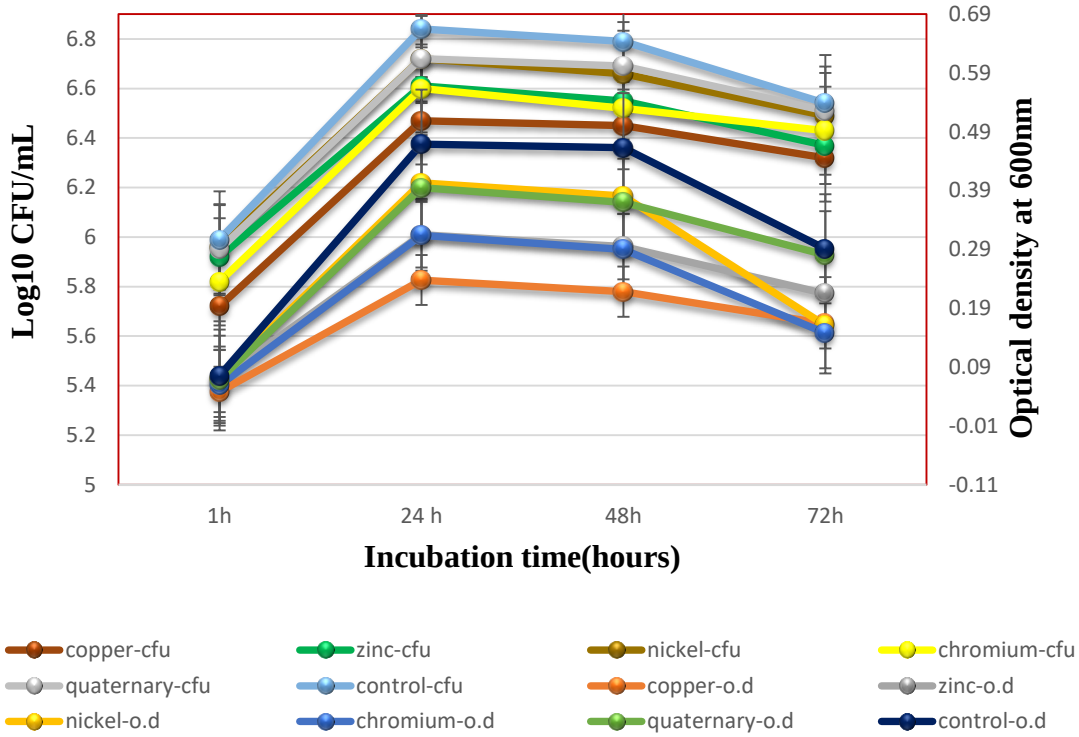


Figure 12: *Bacillus paramycoides* isolate viability in presence of different metals

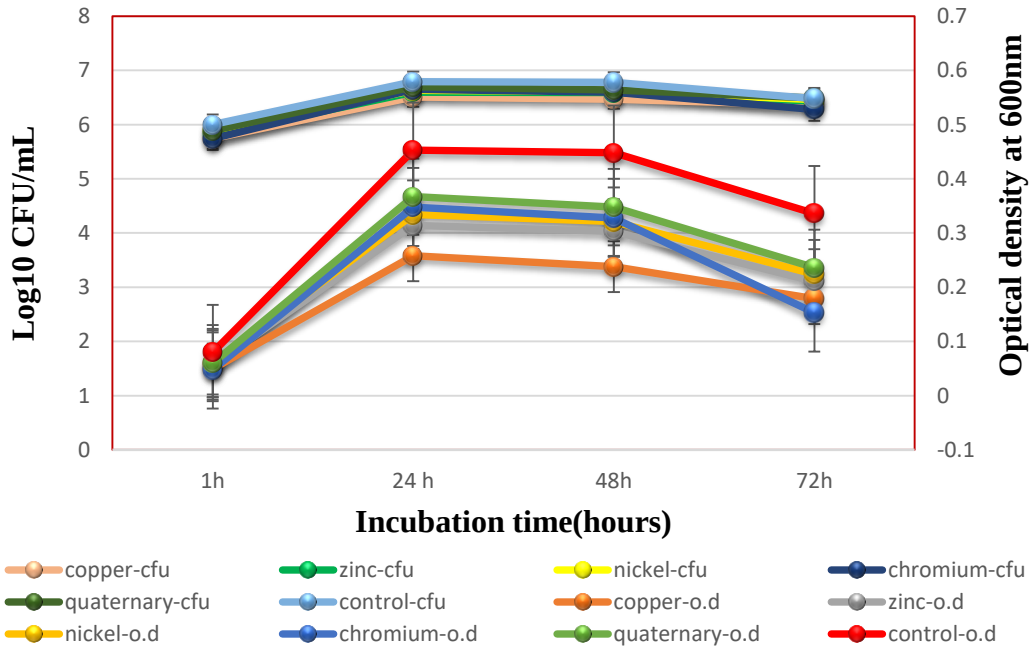


Figure 13: *Bacillus megaterium* isolate viability in presence of different metals

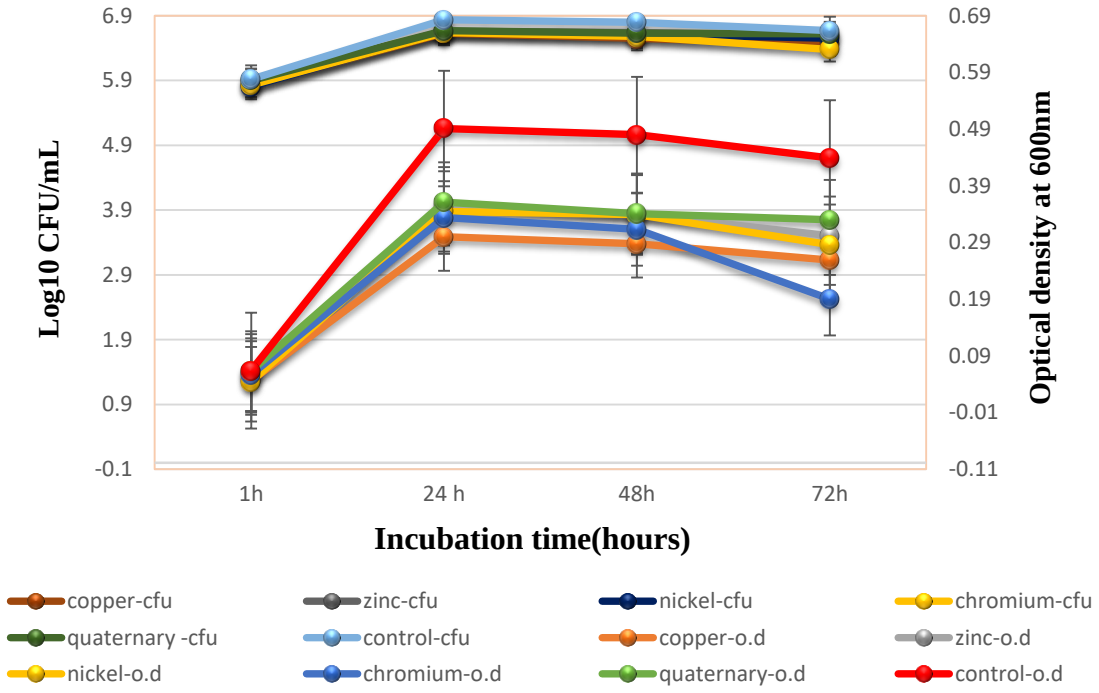


Figure 14: *Sphingobacterium ginsenosidimutans* isolate viability in presence of different metals

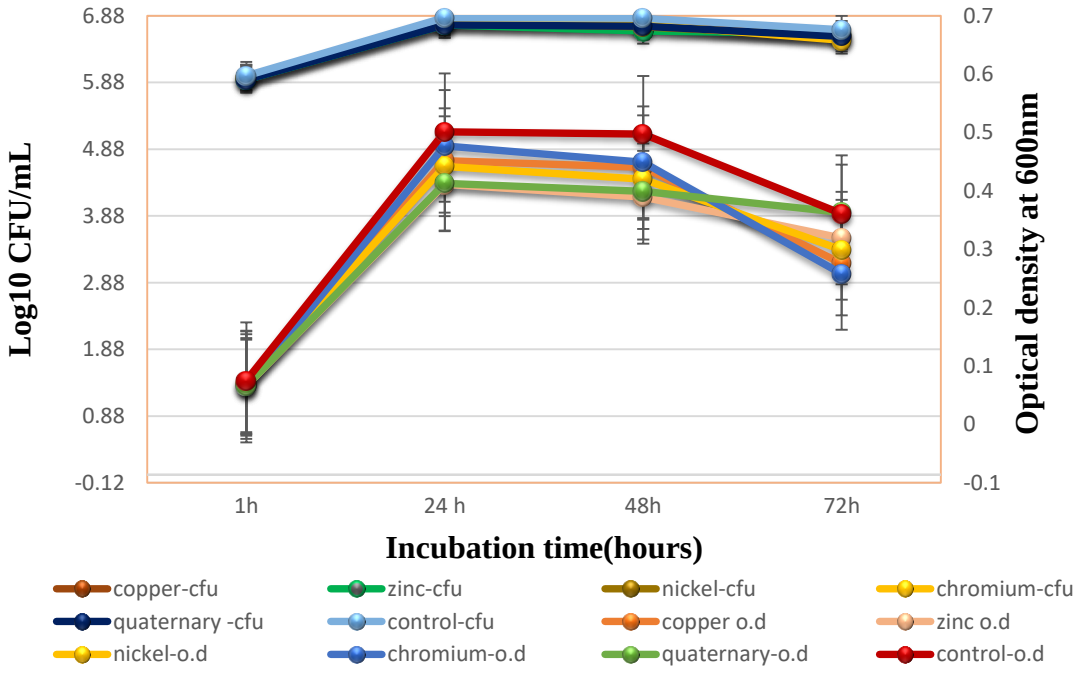


Figure 15: *Kocuria rhizophila* isolate viability in presence of different metals with control

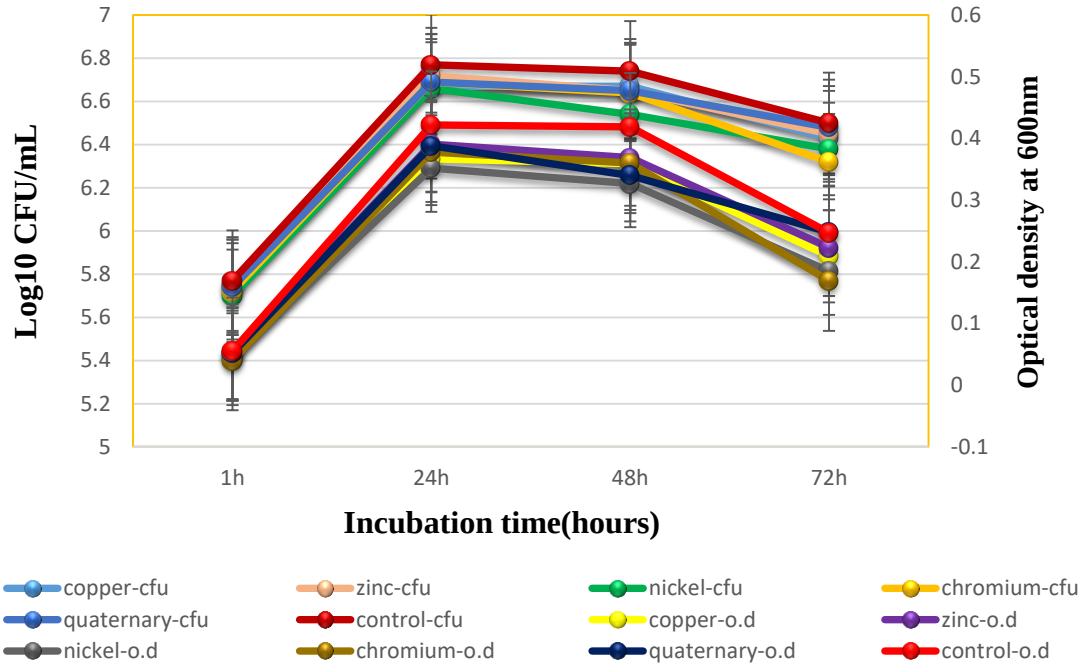


Figure 14: *Sphingobacterium detergens* isolate viability in presence of metals

Conclusion:

Contaminated environments became a habitat of various bacteria having distinctive properties in rendering multiple heavy metals resistance capacity. This study proposed further tolerant bacterial isolates to broaden our knowledge of microorganisms in metallic contaminated environments. In total, nine tolerant bacterial species were identified, and distinguished based on phenotype and genotype. An additional experiment has been conducted to estimate heavy metal tolerance through the determination of the minimal inhibitory concentration (MIC) and the growth kinetics of isolated bacteria. The results demonstrated that the bacteria have the ability to survive on multiple metals and a higher degree of tolerance to heavy metals and thus higher MIC values. As well, the isolates showed greater resistance as indicated by the growth kinetics. Therefore, it can be concluded that minerals act as catalysts leading to the synthesis of catalytic proteins helped bacteria maintain normal growth stages even in the presence of a high concentration of the tested metals. On the other hand, the negative impact of metals was focused on weakening the natural access of bacteria to the stationary phase, which may accelerate their access to the decline phase. These results will provide more scope for future research on the multi-metal resistance pathways and the growth capacity of these isolates, as well as their role in microbial removal of metal ions. Physiological, biochemical and molecular techniques could provide complete information about bacterial identification and characterization, to develop integrated bacteria - based systems can be used to apply bioremediation to contaminated areas.

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CONFLICT OF INTEREST:

The author declares that there is no conflict of interest.

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