Study of Some Physiological Activities for Multimetal Tolerant Bacteria Under Metallic Stress

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Abstract: Electroplating effluents contain heavy metals besides nitrate, which can be harmful to aquatic and terrestrial life if released into the environment. Bioremediation is an imperative task to remove these contaminants from aquatic surfaces. Bacterial species that are tolerant to multiple metals were isolated from electroplating effluents and studied for their ability to withstand high metal concentrations and perform physiological activities. These activities included the production of carotenoid pigments and the reduction of nitrate during exponential and stationary growth phases after exposure to high concentrations of single and quaternary metals. The influence of heavy metals on these activities and bacterial behavior was evaluated. The results showed that the production of carotenoid pigments increased over time, with the highest quantities produced at 48 hours with Zn, Cu, and quaternary. Pigments produced vary with the different types of metals and their concentrations. The ratio of pigment extract to unladen metal cells was higher compared to laden metals cells. The isolates were also capable of reducing nitrate and producing nitrite, but high metal concentrations hindered the conversion of nitrite to nitrogen gas and accumulated great amounts of nitrite between the end of the exponential phase and the start of the stationary phase. The production of carotenoids allowed the bacteria to tolerate high metal concentrations and continue reducing nitrate. This study suggests that pigmented bacteria and aerobic denitrifiers have the potential for bioremediation and nitrate reduction, which can help clean and recover contaminated environments.

Keywords: Bioremediation, Heavy metals, physiological activities, nitrate reduction, carotenoids pigment, metallic stress.

1. INTRODUCTION

The Harnessing microbial activities and metal–microbe interactions in favor of treating metallic pollutants may offer practical solutions to metallic contamination problems. They also offer very specific, potentially cost-effective alternatives. This can be utilized for cleaning contaminated water, which can be widely used in both situ and ex-situ. The action and dynamics of some of the physiological activities of metal-tolerant bacteria under metallic stress are still not fully known and should be studied further, such as denitrification and pigmentation production. Identifying inhibitory or stimulating rates for substrates that are involved in respiratory processes during bioremediation of metals will highlight the level of physiological activity of metal-tolerant bacteria and its relationship to metal remediation. Consequently, one should try to investigate the activity level to overcome the limitations of their efficient use for successful bioremediation. That is because it was reported that heavy metals have toxic impacts on the microbial population by affecting key microbial metabolic processes such as respiration, denitrification, and enzymatic activity

(Li *et al*., 2020; Bai *et al*., 2021; Shuaib *et al*., 2021). A change was noticed in the morphology and physiology of the cell when the concentration of metal ions was increased for accumulation (Pham *et al*., 2022).

Pigment production is one of the physiological activities of bacterial cells and occurs predominantly within the cytoplasm as a responsive factor to antagonistic environmental conditions that present many environmental functions (Pagano and Dhar, 2015). However, bacteria may encounter different stress conditions, and to deal with these hostile conditions, preventive strategies are adapted, including morphological changes and the production of different metabolites that possess important biotechnological and industrial applications. Physically and chemically harsh environments provide a suitable setting for microorganisms to produce pigment with unique qualities and applications (Sajjad *et al.,* 2020). It was found that the pigments could enhance the ability of bacteria to compete and survive in unfavorable environmental conditions (Zerrad *et al*., 2014). It also acts as a protective agent against unfavorable conditions such as exposure to chemicals (oxidizing agents and heavy metals), which grant antibacterial and heavy metal resistance to bacteria (Azman *et al*., 2018). Moreover, pigmented bacteria have also been used as biological sensors to detect environmental pollution like oil spills, pesticides, and heavy metals. One such change is the rate of pigment production or the change in the color of specific pigments in pigmented bacteria, which can be effectively used as bioindicators. For instance, indigoidine pigment production by *Vogesella indigofera* is suppressed by Cr^{6+} in a concentration-dependent manner, which is an indication of chromium concentration and toxicity in the environment (Sajjad *et al.,* 2020).

Pigments can provide valuable benefits through their cells. For example, carotenoids safeguard microbial cells from photo-oxidative injury and other environmental stresses by inhibiting ROS generation. In some cases, the ability to produce pigment may be directly related to metal tolerance. Bacterial pigmentation also protects the cell from photo-oxidative damage caused by toxic metal ions (Hayat *et al*., 2012). Also, prevent severe damage such as inhibition of metabolic processes and cellular repair mechanisms (Sajjad *et al.,* 2020). They assist in maintaining the safety and constancy of the membrane and are also essential in respiratory or photosynthetic processes; carotenoid, for example, may play a role in the modulation of membrane fluidity to survive and gain energy by photosynthesis (Azman *et al*., 2018). The presence of metals may improve the production of pigments in microbial cells when they stimulate the formation of the enzyme responsible for the production of pigments, but increasing the metal concentrations, such as divalent metal ions, slows down the production of pigments (Sharma *et al*., 2018).

Wastewaters that contain a high content of heavy metals often contain high quantities of nitrate and nitrite (Rajta *et al*., 2020). These metal ions are toxic and typically affect bacterial metabolism activity by reducing or preventing the reduction of these compounds (Liu *et al*., 2018b; Thorgersen *et al*., 2019). Cellular activity measurements can reveal the role of individual and quaternary metals in stimulating or inhibiting metabolic activity. These tested metals have a physiological function in bacteria that produces affirmative effects (Fashola *et al*., 2016).

With increased industrial activity nowadays, a large amount of nitrogenous compounds are emitted into the environment, leading to the spread of nitrate pollution in addition to the presence of heavy metals. Bioremediation ensures a cost-effective and environmentally friendly method to treat this problem. The process of aerobic denitrification can reduce nitrate compounds to innocuous nitrogen gas (Rajta e*t al*., 2020). Nitrate, which is reduced to nitrogen gas, is an essential component of the nitrogen cycle, which has been detected to take place in aerobic conditions, and a NAP appears to be the major enzyme and may be the proof of aerobic denitrification (Ji *et al*., 2015). On the contrary, it was reported that denitrification of microbial cells is anaerobic respiration, which enables bacteria to use nitrate, nitrite, and nitrous oxide as final electron acceptors. As well as being additional respiration, it is activated in response to oxygen diminution and the presence of NO_3^-, NO_2^- or N_2O^- (Cabezas *et al*., 2022). Nitrates can be removed from contaminated areas by microbial denitrification, but the presence of heavy metals may hamper denitrification. Heavy metal toxicity affects the microbial metabolic activity associated with nitrogen removal, resulting in a reduced rate of nitrogen removal. These metal ions are toxic and must be effectively removed (Liu *et al*., 2018b; Thorgersen *et al*., 2019). In contrast, it was found that several heterotrophic aerobic denitrifiers are able to tolerate various metal ions and have higher growth rates, resulting in more efficient denitrification (Rajta *et al*., 2020; Cai *et al*., 2019).

This study investigates the behavior of multimetal- tolerant bacteria over intervals of metallic stress and their ability to produce pigmentation and reduce nitrate in the presence of high concentrations of a mixture of metals in highly polluted environments. The bacterial species can serve as models for understanding how pigmentation rates occur

and how nitrate contamination can be mitigated during periods of metallic stress and growth phases, especially during exponential and stationary phases. In addition, evaluation of the cellular activities of these species, their susceptibility to stressful conditions, and their efficacy for the successful application of a bioremediation approach using multimetal-tolerant bacteria.

2. MATERIAL AND METHODS

2.1 Bacteria and Culture Media

The bacterial isolates employed in the present study were isolated from electroplating industrial effluents. The bacterial isolates were obtained from nutrient agar plates (Oxoid, Lab-Lemco Powder) incorporated with a concentration of copper, zinc, nickel and chromium as individual metals and as a quaternary metals solution at 37º C for 24h. The isolates selected to examine some of their physiological processes under metallic stress were those that could withstand and grow at high concentrations. In this study, three isolates were used after they identified and confirmed as *Bacillus megaterium* (NR_117473.1), *Sphingobacterium ginsenosidimutans* (NR_117473.1), *Kocuria rhizophila* (NR_026452.1) and based on 16S rDNA data.

2.2 Pigment Production Under Metallic Stress

The inoculums (OD=0.6) of five pigmented isolates (*M. paraoxydans, M . arabinogalactanolyticum, S. ginsenosidimutans, K. rhizophila* and *S. detergens* were grown in 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth supplemented with 10, 50, and 100 mg/L concentrations of metals. The growth conditions were 37 °C and 150 rpm for 24, 48, and 72 hr. Extraction of the pigment from the media broth was done by the solvent extraction method (Goswami and Bhowal, 2014). The isolates were harvested by centrifuging at 6,000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in acidified ethanol (4% of 1 M HCl in 96 ml of ethanol). The mixture was vortexed (Vortex- MIXER UZUSIO, LMC. Tokyo, Japan (VTX-3000L) and the suspension was centrifuged at 6000 rpm for 10 min. The concentration of clear supernatant was determined by measuring absorbance under a spectrophotometer at 450 nm.

Calculations of the mass of carotenoid pigments extracted from isolates:

$$\mathbf{X} = \frac{AY}{A_{1Cm}^{1\%}} \quad (1)$$

Where: x is the mass of total carotenoids (g), A is the absorption spectrophotometric reading at 450nm, y is the volume of solvent (2 mL), and $A_{1Cm}^{1\%}\ equal$ 2500 constant value (O'Donnell and Goodfellow, 1994).

The ratio of bacterial growth to carotenoids production from bacterial isolates was calculated as the ratio of the OD of carotenoid to the OD of the growth (Boontosaeng *et al*., 2016):

$$\textbf{The degree of cell pigmentation} = \left(\frac{\text{ODλmax}}{\text{OD660}}\right) \quad (2)$$

The results were analyzed to determine significant differences in pigment production between means, which were identified by a two-way ANOVA and Fisher LSD test. Means at $p \leq 0.05$ were considered significantly different. The data were expressed as the mean ± standard deviation (SD) of the values obtained experimentally.

2.3 Denitrification Assay Under Metallic Stress

It is crucial to comprehend the impact of heavy metals on microbial physiology. Heavy metals, both single and multiple, can be used as tools to study microbial activity under metallic stress, which is critical to understanding the impact of metals on microbial processes in the aquatic environment, such as denitrification. The current study looks into the effect of a mixture of four metals (quaternary include Cu, Zn, Ni, and Cr) as well as individual metals on metal-resistant bacteria isolated from electroplating effluent and compares nitrate reduction rates in the presence of different levels of the single and quaternary metals.

2.3.1 Method of Rapid Colorimetric Determination of Nitrate

Estimation of leftover nitrate under metallic stress was carried out using four isolates (*B. paramycoides*, *B. megaterium*, *S. ginsenosidimutans*, and *K. rhizophila*) (2% v/v) that were inoculated in nitrate broth supplemented with initial concentrations (10, 50, and 100 mg/L) of Cu, Zn, Ni, Cr, and quaternary. The cultures were incubated at

intervals 2-24 hr at 37°C under agitating conditions at 120 rpm. After each time intervals, 5 mL samples were withdrawn and harvested at 8609 × g for 15 min, and the supernatants or cell-free extracts were taken for determination of left-over nitrate. Aliquot 200 µl of salicylic acid was added to 40 µl of each supernatant and vortexed each tube. Tubes were incubated in the dark for 10 min. The reaction was stopped by adding 2 mL of 2N NaOH and vortexed until the contents were clear. Then the optical density of the reaction mixture was measured after 20 min at 420 nm and compared to the standard curve prepared with known concentrations of KNO_3 (100-1000 mg/L) to determine the concentration of remaining nitrate amounts in the medium (Cataldo *et al*., 1975; Sharma and Dwivedi, 2017).

2.3.2 Estimation of Formed Nitrite

Four isolates (*B. megaterium, B. paramycoides, S. ginsenosidimutans*, and *K. rhizophila*) (2% v/v) were inoculated in nitrate broth supplemented with initial concentrations (10, 50, and 100 mg/L) of Cu, Zn, Ni, Cr, and quaternary. The cultures were incubated at intervals (2-24 hr) at 37 °C under agitating conditions at 120 rpm. Aliquots 5 mL samples were drawn after each period and centrifuged at 8609 × g for 15 min, and cell-free extract (CFE) or supernatant was taken to determine the amount of nitrite formed (Sharma and Dwivedi, 2017). The Griess method was used for the determination of the formed nitrite (Wany *et al*., 2017). In the supernatants, the amount of nitrite was measured using a Griess reagent. The Griess reaction is used to assess the denitrification rates of bacterial isolates under metallic stress. It is a common test as a result of its cost and simplicity of use, and it can assess denitrification rates in different environments. It is formed from sulphanilamide, and N-(1-naphthyl) ethylenediamine (NED or NEDA) is used to determine the nitrite (Tsikas, 2007; Braissant *et al*., 2020). In the detection method, a volume of 50 µL of the supernatant was mixed with 50 µL of a Griess reagent (1% sulfanilamide, 2.0 % H_3PO_4, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, and then the mixture was incubated at room temperature for 10 min. The absorbance was measured at 540 nm, the free metal medium was used as a blank, and the amount of nitrite was determined from a standard curve for sodium nitrite (Rao *et al*., 2016).

The data obtained on denitrification of different metals at different concentrations and time intervals by individual isolates (*B. megateriumm*, *S. ginsenosidimutan*, and *K. rhizophila*) were subjected to statistical analysis using the SPSS program (SPSS Version 25). The relationship between formed nitrite, left-over nitrate in isolated bacteria, and variable parameters (time and metal concentration) was correlated using multiple regression analysis and ANOVA to detect the correlation and statistical significance of time and metal concentrations on the denitrification activity of bacteria, thus determining significance differences ($p \leq 0.05$) between means.

2.3.3 Calculations of Leftover Nitrate and Formed Nitrite

The amount of nitrite in the supernatants was determined using a Griess reagent mixture that was vortexed until homogeneous. The mixture was then diluted with sterilized distilled water, added to the marked line of the volumetric flask, and shaken until homogeneously mixed (dilution factor 10.0 times). Absorbance was measured at 540 nm. Lastly, the concentration of nitrite (x) was calculated by substituting the obtained absorbance (Y) for the regression equation. Nitrite levels in the sample were calculated by multiplying by volume and a dilution factor. A statistical analysis of the variance of formed nitrite amounts with time between single bacterial isolates was performed using multiple regression analysis and ANOVA. For determining leftover nitrate, the concentration of nitrate (x) was calculated by replacing the absorbance (Y) obtained in the regression equation. Nitrate levels in the sample were calculated by multiplying the total sample volume by the dilution factor and dividing by the volume of the supernatant used in measurement (Nerdy and Putra, 2018).

2.3.4 Standards for Standard Curve of Nitrate

Stock solution 0.25 g/L NO_3-N, which is equivalent to (250 mg/L, 250 µg/mL) was prepared in a 1.0L standard flask containing approximately 600 mL of distilled water. 1.805g of potassium nitrate (Bendosen; Bendosen in Holtålen, Norway) were well dissolved. Then, the mixture was made up to the mark with distilled water, mixed, and stored in a suitably labeled plastic container. Preparation of standards containing 0 to 60 µg NO_3-N in a 0.25 mL aliquot. Table 1 shows the amounts of KNO_3 and the volume of stock and extractant.

Table 1: Preparation of KNO_3 standards

Amount of NO_3-N	Volume stock (mL)	Volume extractant (or H_2O)
62.5	0.25	0.00
50	0.20	0.05
37.5	0.15	0.10
25	0.10	0.15
12.5	0.05	0.20
0.0	0.0	0.25

The blank (0.25 mL) prepared with reagent consisted of 0.8 mL of concentrated H_2SO_4 (minus salicylic acid) and 19 mL of 2 N NaOH (Cataldo *et al*., 1975). An aliquot of each extract or standard was pipetted into a 50-mL Erlenmeyer flask. The extracts were mixed thoroughly with 0.8 mL of 5% (w/v) salicylic acid (Bendosen-Laboratory Chemical, Bendosen in Holtålen, Norway) in concentrated (H_2SO_4 98%; R and M Chemical; Himachal Pradesh, India). After 20 minutes at room temperature, 19 mL of 2N NaOH were added to raise the pH above 12, and the samples were cooled to room temperature. The absorbance was measured spectrophotometrically at 420 nm.

2.3.5 Preparation of nitrite stock solution for standard curve of nitrite

Sodium Nitrite (Bendosen Laboratory Chemical, Bendosen in Holtålen, Norway) was dried at 110◦C for one hour, cooled in a desiccator, and weighed at 15.0 mg of sodium nitrite in a separate 100 mL volumetric flask containing 10.0 mg of nitrite. The flask was then filled with 60.0 mL of distilled water, shaken until dissolved, diluted with distilled water to the marked line, and shaken until homogeneously mixed (a stock solution with a nitrite concentration of 1000 µg/mL was obtained). Five mL of stock solution was pipetted and transferred into a separate 100.0 mL volumetric flask, diluted with distilled water to the marked line, and shaken until homogeneously mixed (obtaining a solution with a nitrite concentration of 50 µg/mL).

The amounts ranged between 0.002 and 15.0 mL of nitrite solution. 50 µg/mL were transferred into a 100.0 mL volumetric flask. Each flask received 2.5 mL of sulfanilic acid solution (HmbG Chemicals; , Hamburg, Germany), which was shaken until homogeneous and left for 5 minutes before adding 2.5 mL of N-(1-Naphthyl)- ethylenediamine dihydrochloride solution (Sigma- Aldrich; Burlington,USA). The flasks were shaken until homogeneous, diluted with distilled water to the marked line, and shaken until homogeneously mixed. The obtained nitrite solutions ranged from 0.001 µg/mL to 7.0 µg/mL. At 540nm, absorbance was measured. The calibration curve was performed by plotting the absorbance (Y-Ordinate) versus concentration (X-Axis) for each solution. The regression equation was calculated, and the correlation coefficient was determined (Nerdy and Putra, 2018).

3. RESULTS AND DISCUSSION

3.1 Assay of the physiological activities under metallic stress

The physiological activities in bacteria that are related to bioremediation may contribute in interactions with metals and form less toxic forms. These activities include bacterial pigmentation, denitrification processes, and the activities of enzymes such as catalase and dehydrogenase, which are essential components of the redox-system within oxidative phosphorylation in the cellular respiration process. It is appropriate to assess the potential interactions and the physiological and metabolic changes derived from this interaction under metallic stress. Studying these changes can provide significant information on bacterial activities and reflect alterations in the nutritional and respiratory activities of bacteria in response to changes in the environment under stressful conditions, proving their viability and tolerance, which characterize them.

3.2 Pigment production under metallic stress

In this study, five of the total nine isolates were metal-laden individually and quaternarily, and metal-unladen biomasses were used to assess the amounts of carotenoid produced. The results showed that the mass of carotenoid pigments increased over time, and the biggest quantities of carotenoid pigments were produced at 48 h with Zn, Cu, and quaternary. More carotenoid was produced at low metal concentrations than at high concentrations. This

confirmed the fact that it had been reported that an increase in the concentration of divalent metal ions inhibits pigment production, which means that a high concentration may produce toxic effects (Sharma *et al*., 2018). Although bacteria were exposed to high metal concentrations, pigment production continued, but the quantities produced were less in comparison to 10 mg/L, which produced more carotenoids than the other concentrations. The lowest carotenoid produced was with Ni and Cr; generally, bacteria may be affected by physical and chemical stress, and exposure to different concentrations of metals may influence the color of the pigment produced by bacteria, which was confirmed as well by Bhadekar *et al*. (2017) and Pote *et al*. (2014).

The results of the mass of carotenoid pigments and the ratio of bacterial growth to the carotenoids produced at different levels of heavy metals are shown in Tables 2 and 3, respectively. The amounts of carotenoid produced in metal-unladen isolates (control) are shown in table 4. The isolates capable of producing the largest amounts of carotenoid at 10 and 50 mg/L of Cu, Zn, and Ni for 48 h were *M. paraoxydans, M. arabinogalactanolyticum*, *S. detergens*, and *K. rhizophila*. While *S. ginsenosidimutans* and *S. detergens* were able to produce more carotenoid with quaternary at 10, 50, and 100 mg/L. *M. paraoxydans* and *M. arabinogalactanolyticum* produced more carotenoid at 10 mg/L for 48 h with Zn. The ratio of pigment extract under stressful conditions to cell growth metal-free was elucidated; the highest ratio with Zn, Cu, and quaternary at 10 mg/L compared to Ni and Cr.

Table 2: The mass of total carotenoid produced at different concentrations of heavy metals (*p*<0.05)

Metal Levels	Isolates	BMA1			DMA-3			A6MA-7			MIC-8			RMA-9			∗Means±SD *P*≤ 0.05
	Time(hr)	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	
Copper	10 mg	0.696	0.928	0.680	0.692	0.736	0.522	0.904	0.952	0.832	0.920	0.964	0.640	0.928	0.944	0.816	0.810±0.139 a
	50 mg	0.672	0.684	0.656	0.680	0.648	0.371	0.632	0.612	0.456	0.912	0.800	0.596	0.528	0.776	0.520	0.636±0.136b
	100 mg	0.624	0.674	0.648	0.576	0.584	0.352	0.560	0.568	0.392	0.696	0.748	0.552	0.480	0.492	0.451	0.559±0.111c
Zinc																	
	10 mg	0.408	1.096	0.688	0.720	1.080	0.920	0.792	0.927	0.520	0.816	1.008	0.984	0.776	0.800	0.636	0.811±0.198a
	50 mg	0.368	0.832	0.456	0.681	0.672	0.432	0.552	0.624	0.468	0.802	0.848	0.720	0.760	0.778	0.624	0.641±0.155d
	100 mg	0.352	0.608	0.416	0.456	0.384	0.401	0.480	0.488	0.465	0.731	0.816	0.648	0.656	0.452	0.342	0.513±0.144e
Nickel	10 mg	0.384	0.560	0.502	0.536	0.520	0.432	0.372	0.488	0.292	0.600	0.588	0.354	0.376	0.472	0.408	0.459±0.093f
	50 mg	0.280	0.536	0.398	0.489	0.496	0.424	0.348	0.440	0.264	0.548	0.552	0.348	0.348	0.411	0.328	0.414±0.095g
	100 mg	0.248	0.488	0.227	0.464	0.420	0.346	0.304	0.336	0.232	0.504	0.550	0.272	0.299	0.408	0.244	0.356±0.109h
Chromium	10 mg	0.480	0.580	0.512	0.576	0.548	0.472	0.548	0.540	0.284	0.632	0.684	0.564	0.560	0.588	0.401	0.531±0.096i
	50 mg	0.408	0.448	0.400	0.484	0.540	0.448	0.464	0.472	0.268	0.584	0.680	0.368	0.520	0.561	0.272	0.461±0.110 j
	100 mg	0.344	0.464	0.400	0.480	0.376	0.328	0.312	0.376	0.261	0.472	0.541	0.336	0.436	0.264	0.188	0.372±0.096k
Quaternary																	
	10 mg	0.588	0.856	0.592	0.672	0.888	0.552	0.928	0.940	0.744	1.016	0.840	0.736	0.840	1.001	0.832	0.802±0.149l
	50 mg	0.456	0.576	0.460	0.568	0.684	0.648	0.480	0.856	0.600	0.648	0.660	0.644	0.500	0.872	0.612	0.618±0.125m
	100 mg	0.400	0.567	0.446	0.564	0.580	0.152	0.448	0.744	0.420	0.512	0.640	0.416	0.496	0.832	0.392	0.507±0.162n

∗Means followed by different superscripts are significantly different at an alpha level of (p=0.05) according to an LSD test

Where: x= mass of total carotenoid at 24hr, 48hr and 72hr.

(**BMA-1**. *M.paraoxydans*, **DMA-3**. *M . arabinogalactanolyticum*, **A6MA-7**. *S. ginsenosidimutans*, **MIC-8**. *K.rhizophila* and **RMA-9**. *S. detergens*)

Table 3: The Ratio of Carotenoids Produced in Cells to Their Growth at Different Concentrations of Heavy Metals

Metals	Cu			Zn			Ni			Cr			Quaternary		
Bacterial isolates	r_{24h}	r_{48h}	r_{72h}	r_{24h}	r_{48h}	r_{72h}	r_{24h}	r_{48h}	r_{72h}	r_{24h}	r_{48h}	r_{72h}	r_{24h}	r_{48h}	r_{72h}
10 mg/L															
BMA-1	0.094	0.182*	0.115	0.055	0.215*	0.117	0.052	0.110*	0.085	0.065	0.114	0.087	0.079	0.168*	0.087
DMA-3	0.093	0.118	0.076	0.097	0.135*	0.134	0.072	0.079	0.063	0.078	0.135*	0.069	0.091	0.107	0.078
A6MA-7	0.115	0.156*	0.109	0.100	0.116	0.086	0.049	0.080*	0.038	0.069	0.089	0.037	0.118	0.154*	0.097
MIC-8	0.118	0.087	0.060	0.105	0.091	0.092	0.077	0.053	0.052	0.081	0.062	0.053	0.131	0.085	0.075
RMA-9	0.120	0.129	0.132	0.100	0.110	0.133	0.049	0.065	0.066	0.105	0.125*	0.104	0.120	0.142	0.134
50 mg/L															
BMA-1	0.090	0.100	0.098	0.049	0.100	0.063	0.038	0.073	0.045	0.055	0.069	0.049	0.061	0.085	0.052
DMA-3	0.092	0.078	0.057	0.091	0.081	0.065	0.066	0.059	0.056	0.065	0.065	0.054	0.077	0.083	0.069
A6MA-7	0.092	0.093	0.062	0.079	0.080	0.064	0.047	0.056	0.036	0.053	0.060	0.036	0.074	0.122*	0.109
MIC-8	0.105	0.117*	0.103	0.093	0.109*	0.105	0.063	0.071*	0.051	0.067	0.087	0.054	0.094	0.076	0.069
RMA-9	0.073	0.130*	0.091	0.1	0.131*	0.109	0.044	0.069	0.058	0.067	0.095*	0.048	0.107	0.109*	0.077
100 mg/L															
BMA-1	0.084*	0.082	0.074	0.047	0.091*	0.051	0.033	0.064*	0.034	0.046	0.054	0.045	0.054	0.069	0.037
DMA-3	0.090*	0.076	0.045	0.082	0.050	0.052	0.036	0.055	0.051	0.054	0.049	0.053	0.060	0.076	0.024
A6MA-7	0.064	0.078	0.051	0.055	0.079	0.061	0.035	0.055	0.030	0.036	0.062	0.034	0.051	0.109*	0.055
MIC-8	0.080	0.068	0.052	0.084	0.074	0.059	0.058*	0.050	0.038	0.055*	0.049	0.031	0.059	0.058	0.039
RMA-9	0.068	0.052	0.065	0.098*	0.048	0.049	0.046	0.043	0.035	0.066*	0.028	0.027	0.061	0.104*	0.049

Where: r= ratio of pigment extract to cell growth

(**BMA-1**. *M.paraoxydans*, **DMA-3**. *M . arabinogalactanolyticum*, **A6MA-7**. *S. ginsenosidimutans*, **MIC-8**. *K.rhizophila* and **RMA-9**. *S. detergens*)

Table 4: The results of mass of total carotenoid produced by metals unladen bacterial cultures (control)

Carotenoid amounts Isolates bacterial	X_{24h}	X_{48h}	X_{72h}
M. paraoxydans **BMA-1**	2.016	1.828	1.800
	2.168	1.884	1.664
	2.109	1.744	1.844
M. arabinogalactanolyticum **DMA-3**	1.984	1.952	1.936
	2.064	2.061	1.992
	1.908	2.144	2.056
S. ginsenosidimutans **A6MA-7**	1.001	1.718	1.541
	1.087	1.784	1.616

	1.093	1.775	1.580
***K. rhizophila* MIC-8**	1.481	1.836	1.888
	1.512	1.825	1.822
	1.472	1.830	1.936
***S. detergens* RMA-9**	1.064	1.728	1.608
	1. 560	1.720	1.532
	1.068	1.724	1.612

Where: X= mass of total carotenoid at 24h, 48h and 72h.

To detect the pigment color under metallic stress, the color of *K. rhizophila* biomass on nutrient agar that was loaded with nickel, chromium, and quaternary at different concentrations was compared with nickel, chromium, and quaternary-free (unladen) *K. rhizophila* biomass. Figures 1 and 2 showed the effects of single metals on the produced carotenoid amounts, which were less than quaternaries, and with increased of metal concentrations. However, carotenoid mounts in biomasses loaded with single and quaternary metals were lower than those of metal-free biomass. This is consistent with the reported facts that metals have an impact on pigment production, depending on the type of metal. The amount of pigment produced varies according to minerals and their concentrations. (Sharma *et al*., 2018). The ability of bacterial isolates to produce pigments is one of the physiological activities that can determine the level of cell activity and viability, especially under extreme conditions. The bacteria showed resistance to heavy metals and the ability to produce pigments under metallic stress, which helped the bacteria adapt to survive in polluted environments.

The results of the statistical analysis are presented as mean values and standard deviations, as shown in Table 2. The results showed that concentrations of metals, time, and interference between them had a significant effect on carotenoid production in bacterial isolates. There was also a significant difference in pigment production between the control and all isolates ($p \leq 0.05$).

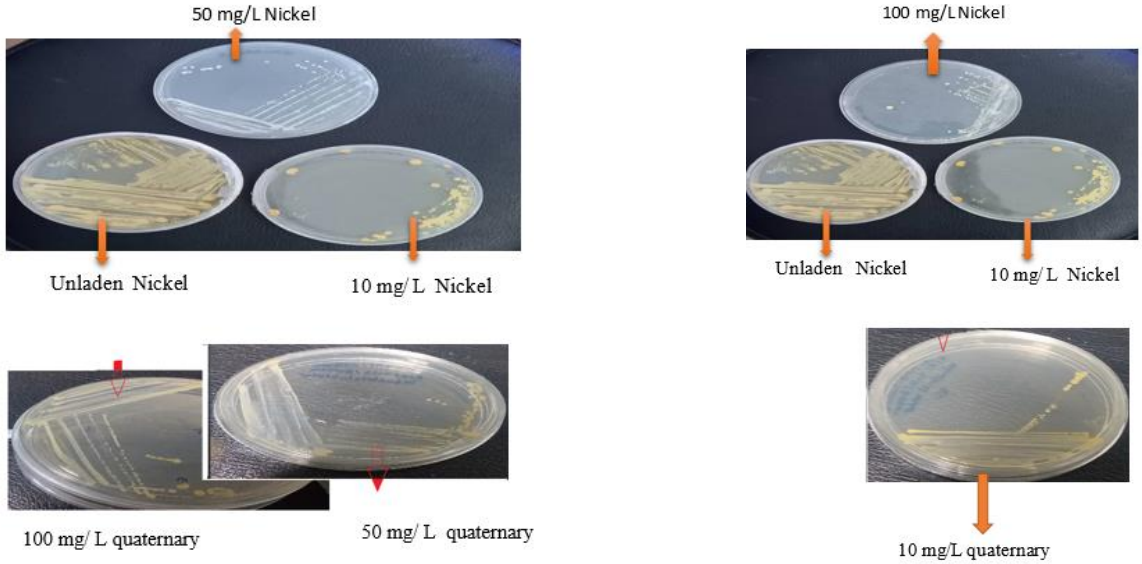


Figure 1: The Pigment Color Under Metallic Stress (nickel stress)

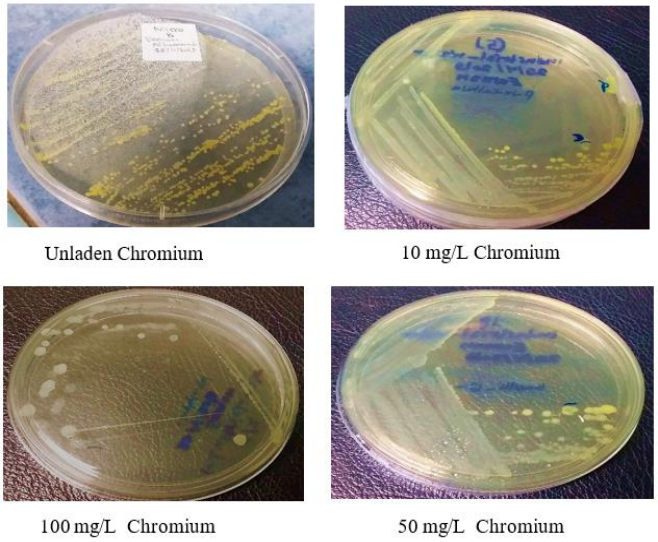


Figure 2: The pigment colour under chromium stress

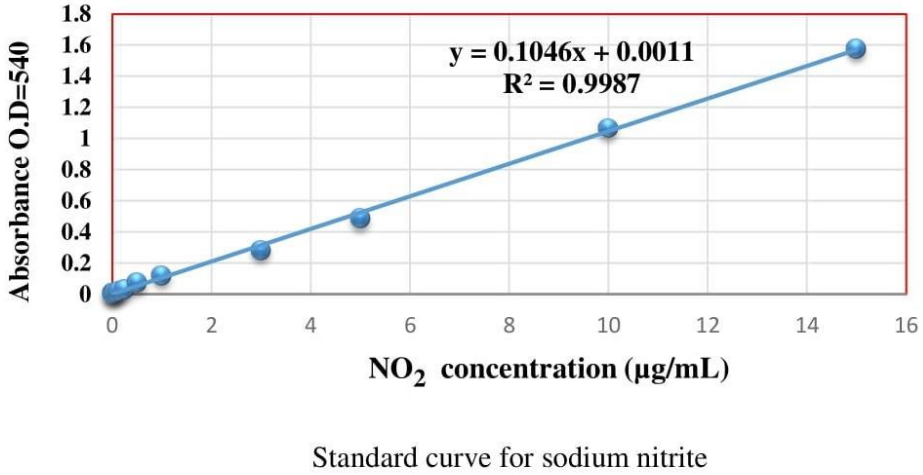
This is consistent with the reported facts that metals influence on pigment production depending on the type of metals and pigment produced, which vary depending on difference minerals and their concentrations (Sharma *et al*., 2018). The ability of bacterial isolates to produce pigments is one of the physiological activities that can determine the level of cell activity and viability, especially under extreme conditions. The bacteria showed resistance to heavy metals and the ability to produce pigments under metallic stress, which helped the bacteria adapt to survive in polluted environments. The results of the statistical analysis are presented as mean values and standard deviation, as shown in Table 2. The results showed that concentrations of metals, time, and interference between them had a significant effect on carotenoid production in bacterial isolates. There were also significant difference in pigments production between control and all isolates ($p \leq 0.05$).

3.3 Denitrification Assay under Metallic Stress

Denitrification is one of the physiological activities of metal-tolerant bacteria that can take place under natural conditions and metallic stress. It is one of the primary objectives for studying bacterial metabolic behavior and the extent to which it responds to stressful conditions. The bacterial activity for nitrate reduction was determined in the presence of single metals and quaternary. Denitrification was studied along with metallic stress intervals, specifically through logarithmic and stationary growth phases, to determine the effect of exposure and the potential bacterial response.

3.3.1 Estimation of formed nitrite

Bacterial isolates that achieved high denitrification under constant conditions and those that achieved the highest tolerance and performance indexes were utilized to evaluate denitrification under metallic stress. The ability of nitrate-reducing bacteria was evaluated with individual metals and quaternary at six time points in the growth curve: the 2.0 hr lag phase, exponential onset 4.0 hr, the mid of exponential phase 6.0 hr, the late of exponential phase 10.0 hr, the early-stationary phase 18 hr, and mid-stationary phase 24 hr. The formed nitrite was determined using Griess reagent in the supernatants of bacterial isolates. Four metal-resistant and nitrate-reducing isolates were examined for a period of 2- 24h to determine the potential and efficiency of these bacteria for denitrification. The amount of formed nitrite was determined from a standard curve for sodium nitrite Figure (3).



Standard curve for sodium nitrite

Figure 3: Standard curve for sodium nitrite

The results showed that all isolates were able to reduce nitrate at similar rates at 10 mg/L using individual and combination of metals. The isolates, both in the presence and absence of the single metals and quaternary were capable of reducing nitrate and producing large amounts of nitrites. The maximum amount of nitrite formed occurred between 10 and 18 hr after the end of the exponential phase and the start of the stationary phase, and it gradually decreased as it entered into the mid-stationary phase. It was observed that the greatest amount of nitrite accumulation at the end of the exponential-phase and onset stationary, compared to the control was at the onset and mid-stationary phase. Formed nitrite at 10 mg/L was less than control in *B. paramycoides* and *K. rhizophila*, while it was much more in *B. megaterium* and *S. ginsenosidimutans*.

When the metal concentrations increased to 50 and then 100 mg/L, large amounts of nitrite were accumulated, especially in *B. megaterium* and *S. ginsenosidimutans*, in comparison to *B. paramycoides* and *K. rhizophila*. The lowest accumulation of formed nitrite was at 10 mg/L concentrations with *B. paramycoides* and *K. rhizophila*. Whereas high concentrations of metals led to the accumulation of nitrite largely and hindered or slowed down the rapid conversion of nitrite as an intermediate in this process to the final product as nitrogen gas. It was indicated that metal ions could cause the accumulation of denitrification intermediates (NO_2 and N_2O) (Ramrez *et al*., 2018). In addition, it was shown that various metal ions in sufficiently high concentrations inactivate oxidoreductase enzymes, such as nitrate reductase, and respiratory pathways, such as denitrification. The metals that were used in this study were among the metals that were reported to be inhibited (Spain and Krumholz, 2011; Thorgersen *et al*., 2019).

The results as shown tables 5 and 6 indicated as shown a decrease in the amounts of formed nitrite in the early and mid-stationary phases in *B. megaterium* and *S. ginsenosidimutans;* this is a relatively faster reduction compared to other isolates. In *B. paramycoides* and *K. rhizophila* isolates, nitrite amounts that formed were the highest during the early and mid-stationary phases. Thus, they were slower in completing the reduction process that lasted until the stationary phase, despite the fact that the quantities of formed nitrite were not large. High concentrations of soluble metals in the polluted environment may form sediments that mix with the components of the surrounding environment and affect the pH of the media to be closer to acidity or form an acidic precipitate. This sediment incorporates and absorbs molybdate, the soluble form of Mo, which is required for the action of the nitrate reductase enzyme, making it unavailable to microbes to reduce nitrate. Mo can incorporate and adsorb to insoluble metals such as iron and aluminum hydroxides in acidic, polluted environments and in the presence of heavy metals, where its concentration decreases as the pH increases. The nitrate reductase enzyme requires molybdenum (Mo) as a cofactor (Ge *et al*., 2019). Thus, high concentrations of metals are another factor that can hinder nitrate reduction, as in polluted environments.

Mo can incorporate and adsorb on insoluble metal surfaces such as iron and aluminum hydroxides in acidic, polluted environments and in the presence of heavy metals, where its concentration decreases as the pH increases. The nitrate reductase enzyme requires molybdenum (Mo) as a cofactor (Ge *et al*., 2019). Thus, high concentrations of metals are another factor that can hinder nitrate reduction, as in polluted environments. However, all isolates retained

denitrification activity, which indicated that nitrate reductase activity continued when they were grown in the presence of multiple minerals and at high concentrations.

However, excessive amounts of nitrites formed with increasing metal levels were clearly visible, which has a negative effect on microbial activity. That is because the activity of the nitrite reductase enzyme (Nir) will be inhibited if the nitrite concentration is high due to its toxicity to microbial cells, which will minimize the rate of nitrite reduction to other nitrogen compounds and then to N_2 nitrogen gas (Onley *et al*., 2018). The analysis of variance of formed nitrite amounts with time among single isolates was performed using multiple regression analysis and ANOVA. The results of the analysis of variance showed that there were significant differences ($p \leq 0.05$, $p=0$) in the amount of formed nitrite in all isolates

Table Error! No text of specified style in document.**:** The Amounts of Formed Nitrite (NO_2- µg/mL) from Denitrification by Metal Resistant Bacterial Isolates and Regression Statistics Analysis

Metals / Time intervals	Cu (mg/L)			Zn (mg/L)			Ni (mg/L)			Cr (mg/L)			Quaternary (mg/L)			Control (mg/L)		
	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100	1	2	3
Bacillus paramycoides																		
2h	0.030	0.225	0.417	0.032	0.309	0.392	0.104	0.252	0.334	0.032	0.078	0.307	0.028	0.304	0.289	0.210	0.231	0.240
4h	0.882	0.817	0.438	0.991	0.329	0.434	0.273	0.526	0.428	0.035	0.507	0.338	0.949	0.583	0.432	1.279	1.120	0.888
6h	1.149	1.152	0.654	1.204	0.517	0.440	0.277	0.685	0.524	0.691	0.609	0.574	1.353	0.744	0.543	1.773	1.331	1.214
10h	1.359	1.422	7.302	1.225	1.593	8.076	0.252	0.809	12.909	1.085	1.597	1.415	1.551	1.260	11.206	2.229	2.059	2.049
18h	1.363	1.445	18.411	1.235	1.311	18.850	0.208	1.116	10.984	1.323	2.085	18.906	1.555	2.085	14.227	2.239	2.254	2.298
24h	1.369	1.121	12.574	1.153	0.888	15.670	0.198	0.516	7.867	1.507	1.573	26.381	1.505	1.403	40.126	2.265	2.279	2.277
***R**	0.992	0.914	0.864	0.939	-	0.881	-	-	0.869	0.883	0.948	-	0.991	0.895	-			
R^2	0.985	0.836	0.746	0.881	-	0.775	-	-	0.756	0.780	0.899	-	0.982	0.801	-			
Adj-R^2	0.981	0.794	0.682	0.852	-	0.719	-	-	0.695	0.725	0.874	-	0.977	0.751	-			
t	15.994	4.508	3.426	5.451	-	3.716	-	-	3.517	3.769	5.968	-	14.716	4.011	-			
P	0.000*	0.011	0.027	0.006	>0.05	0.021	>0.05	>0.05	0.025	0.020	0.004	>0.05	0.000	0.016	>0.05			
Kocuria rhizophila																		
2h	0.078	0.116	0.119	0.114	0.109	0.221	0.053	0.118	0.151	0.045	0.093	0.164	0.089	0.196	0.173	0.137	0.127	0.166
4h	0.133	0.212	0.281	0.116	0.244	0.275	0.248	0.217	0.237	0.097	0.309	0.177	0.194	0.321	0.189	0.198	0.191	0.206
6h	0.143	4.359	4.396	0.210	1.227	0.463	1.087	1.221	0.649	0.154	0.915	0.445	0.139	1.191	0.553	0.639	0.648	0.618
10h	0.714	3.662	4.007	0.336	1.723	21.444	1.028	1.503	22.950	0.620	1.070	20.565	0.125	1.229	13.704	4.191	4.202	4.438
18h	1.583	3.365	3.505	0.371	1.919	8.725	0.890	1.884	12.553	0.576	1.488	17.720	0.118	1.555	9.595	2.001	1.976	1.999
24h	1.630	2.327	3.336	0.419	2.667	6.225	0.846	2.271	6.009	0.562	2.480	4.817	0.087	2.827	2.788	1.744	1.739	1.733
***R**	-	-	-	-	-	0.994	-	-	0.981	0.896	-	0.899	-	-	0.940			
R^2	-	-	-	-	-	0.988	-	-	0.963	0.755	-	0.808	-	-	0.884			
Adj-R^2	-	-	-	-	-	0.985	-	-	0.953	0.694	-	0.760	-	-	0.855			
t	-	-	-	-	-	18.384	-	-	10.148	3.514	-	4.108	-	-	5.524			
P	>0.05	>0.05	>0.05	>0.05	>0.05	0.000	>0.05	>0.05	0.001	0.025	>0.05	0.015	>0.05	>0.05	0.005			

*The parameters of statistical regression analysis include the values of coefficient, t-test values and p-values for the variables affecting nitrite formation

Table 6: The Amounts of Formed Nitrite (NO_2- µg/mL) from Denitrification by Metal Resistant Bacterial Isolates and Regression Statistics Analysis

Metals / Time intervals	Cu (mg/L)			Zn (mg/L)			Ni (mg/L)			Cr (mg/L)			Quaternary (mg/L)			Control (mg/L)		
	10	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**	**1**	**2**	**3**
	Bacillus megaterium																	
2h	0.172	0.339	1.982	0.403	0.332	2.074	0.878	0.459	2.137	1.310	0.284	3.323	0.604	0.311	1.012	2.604	2.673	2.417

4h	29.666	26.610	19.896	30.105	25.482	10.042	24.059	28.913	20.335	32.322	20.168	18.389	38.891	27.489	20.984	32.385	33.222	30.523
6h	33.076	28.819	33.912	34.958	27.783	26.779	35.732	32.489	24.582	29.687	36.214	40.649	36.758	39.539	30.607	27.302	26.967	22.762
10h	33.933	32.281	33.138	10.315	36.526	37.866	10.126	32.302	31.088	10.336	31.904	32.531	11.370	29.959	33.159	10.273	10.524	11.967
18h	15.043	21.423	31.297	9.499	33.954	36.967	9.519	26.465	26.590	9.624	31.046	22.322	10.210	29.101	30.003	8.955	10.210	9.975
24h	12.616	20.712	30.147	8.662	33.619	35.168	7.281	24.206	26.109	9.519	27.846	21.402	9.917	28.578	27.657	8.808	8.955	9.666
*R	-	-	-	0.938	-	-	0.908	-	-	0.996	-	-	0.995	-	-			
R^2	-	-	-	0.922	-	-	0.825	-	-	0.993	-	-	0.991	-	-			
Adj- R^2	-	-	-	0.968	-	-	0.781	-	-	0.991	-	-	0.988	-	-			
t	-	-	-	7.757	-	-	4.335	-	-	23.715	-	-	20.693	-	-			
P	>0.05	>0.05	>0.05	0.001	-	-	0.012	>0.05	>0.05	0.000	>0.05	>0.05	0.000	>0.05	>0.05			
	Sphingobacterium ginsenosidimutans																	
2h	4.252	0.587	0.289	2.938	0.606	0.407	2.668	0.284	0.406	2.915	0.563	0.300	2.616	0.656	0.350	0.608	0.601	0.572
4h	12.281	4.398	11.612	10.440	3.249	15.168	7.972	24.979	15.168	8.223	3.319	13.076	7.511	23.308	16.465	8.934	8.829	8.599
6h	12.407	17.574	18.787	16.339	17.970	32.155	15.126	25.691	32.155	13.766	17.386	21.883	9.541	16.883	24.687	10.168	9.938	10.085
10h	15.335	16.758	31.005	15.460	18.055	34.038	14.499	16.904	31.046	14.415	14.771	33.055	10.168	14.561	31.737	10.670	10.817	10.566
18h	15.126	15.712	31.067	12.407	14.792	32.489	13.662	16.632	29.289	12.699	14.561	30.816	11.089	12.387	30.063	10.252	10.189	9.813
24h	13.327	13.934	29.874	8.682	10.544	31.276	13.327	14.373	28.473	10.481	14.457	29.226	12.574	11.361	28.557	9.707	9.101	8.996
R	0.972	0.828	0.827	0.860	-	0.919	0.915	0.819	0.931	0.918	-	0.872	0.901	-	0.935			
R^2	0.945	0.685	0.684	0.740	-	0.845	0.838	0.672	0.866	0.843	-	0.761	0.813	-	0.873			
Adj- R^2	0.932	0.606	0.605	0.675	-	0.806	0.797	0.589	0.833	0.804	-	0.702	0.766	-	0.842			
t	8.323	2.950	2.944	3.377	-	4.665	4.544	2.860	5.093	4.635	-	3.571	4.164	-	5.251			
P	0.001	0.042	0.042	0.028	>0.05	0.010	0.010	0.046	0.007	0.010	>0.05	0.023	0.014	>0.05	0.006			

*The parameters of statistical regression analysis include the values of coefficient, t-test values and p -values for the variables affecting nitrite formation

The Plackett–Burman design was utilized to determine the most significant independent variables to perform denitrification. The multiple linear regression coefficient of the model was analyzed by Statistical Package for the Social Sciences SPSS (version 25) via the student's t-test. Tables 5 and 6 elucidated the coefficient of each variable and its effect on the amount of formed nitrite, as well as their p-values, which indicate the significance of each independent variable in the design.

The ANOVA of the Plackett-Burman design indicated that the model was highly significant, as was evident from Fisher's F-test with a very low p-value and the t-stat. The larger the quantity of the t-value and the smaller the p-value mean, the greater the significance and effect of the corresponding coefficient term on the response of the formed nitrite. Based on the calculated p-value, the highest and lowest initial concentrations of Cu, Cr, and quaternary (10 and 50 mg/L), in addition to 100 mg/L of Cu, Zn, and Ni, were significantly influenced in the formation of NO_2 in *B. paramycoides. B. megaterium* at 10 mg/L Zn, Ni, Cr, and quaternary were more significant than other concentrations. While (10 and 100 mg/L) Cu, quaternary, Ni, and Zn had a significant effect in *S. ginsenosidimutans* and 100 mg/L Zn and Ni in *K. rhizophila*. In addition, the total performance of the model was evaluated by the coefficient of determination (R^2) and the adjusted R^2 value (R^2), which should be in sensible agreement with the R^2 value. The strength of the model and its validity occur when R^2 is close to 1, which was achieved with metals and in the concentrations mentioned earlier. It has been reported that a high R^2 value of a regression model greater than 80% indicates model fit (Eltarahony *et al*., 2021). The previous results of the regression model of metal concentrations showed values of adj-R^2 that were close to R^2, which seems to be in good agreement between them, reflecting perfect coordination between the predicted and observed values of formed nitrite amounts under metallic stress.

3.3.2 Determination of leftover nitrate

At the same time, the supernatant of bacterial isolates, which was utilized for determining the formed nitrite, was also used to assess the leftover nitrate after six periods of time. The salicylic acid method was the one adopted for calculating reduction rates, and the standard curve was prepared with known concentrations of KNO_3 (100–1000 ppm) to determine the concentration of remaining nitrate amounts in the medium, as shown in Figure 4.

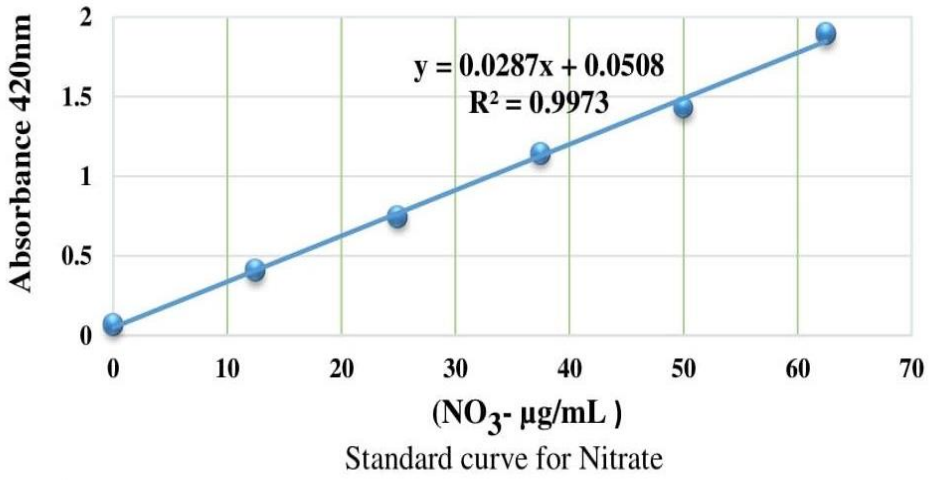


Figure 4: Calibration curve of Nitrate

The results showed that leftover nitrate reduced over time; the lowest amount was in the middle of the stationary phase, the leftover nitrate was close to control at 10 mg/L with single and quaternary. Through the obtained results, it appeared that the isolates were able to obtain the energy needed for growth by reducing nitrate in the presence of multiple metals. However, the leftover nitrate was relatively higher at high concentrations (50-100 mg/L) than at the lowest concentration (10 mg/L) over time intervals. Overall, the results were a reflection of their ability to reduce nitrate under metallic stress, and as shown, there were some differences in rates of reduction between the isolates. The details of the leftover nitrate are presented in Tables 7 and 8. More importantly, these are largely unaffected by high concentrations of multiple metals. The bioremoval capabilities of nitrates using heterotrophic and aerobic denitrification, which may combine more than one characteristic as denitrifiers and metal removal, have gained great advantage and are receiving wide attention in the study (Ge *et al*., 2014; Sun *et al*., 2014; Van De Hende, 2014). That is because of the desirable properties they contain for cleaning the environment and the integrated treatment of pollutants.

The results of the statistical analysis showed the levels of the significant variables and the interference effects between metal concentration and time intervals that influence denitrification by determining the amount of leftover nitrate after the completion of this process by the bacteria. The design of the Plackett-Burman was used to determine the most significant variables affecting leftover nitrate from aqueous solutions using bacterial isolates. The summary of the linear regression model and overall fit statistics are shown in Tables 7 and 8.

Table 7: The Concentration of Leftover Nitrate (NO_3- µg/ml) After Denitrification Activity by Metal Resistant Bacterial Isolates and Regression Statistical Analysis

Metals / Time intervals	Cu (mg/L)			Zn (mg/L)			Ni (mg/L)			Cr (mg/L)			Quaternary (mg/L)			Control (mg/L)		
	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100	1	2	3
Bacillus paramycoides																		
2h	1.597	1.733	1.725	1.759	1.712	1.696	1.644	1.701	1.785	1.594	1.779	1.800	1.709	1.772	1.642	1.747	1.730	1.754
4h	1.482	1.708	1.712	1.570	1.677	1.689	1.461	1.682	1.685	1.531	1.774	1.789	1.432	1.696	1.640	1.430	1.715	1.622
6h	1.455	1.597	1.709	1.448	1.672	1.667	1.434	1.438	1.649	1.456	1.699	1.779	1.420	1.609	1.624	1.416	1.386	1.598
10h	1.246	1.433	1.665	1.344	1.643	1.653	1.242	1.409	1.614	1.257	1.624	1.684	1.260	1.529	1.619	1.228	1.257	1.336
18h	1.222	1.422	1.615	1.339	1.535	1.649	1.199	1.405	1.591	1.249	1.611	1.633	1.250	1.522	1.593	1.199	1.231	1.234
24h	1.189	1.384	1.579	1.248	1.401	1.629	1.176	1.370	1.580	1.227	1.584	1.603	1.213	1.517	1.586	1.174	1.166	1.156
***R**	0.957	0.976	0.965	0.974	-	0.956	0.984	0.988	0.918	0.933	0.984	0.986	0.999	0.973	0.945			
R^2	0.915	0.952	0.930	0.948	-	0.914	0.968	0.976	0.842	0.871	0.969	0.973	0.999	0.946	0.894			

Adj-R^2	0.894	0.940	0.913	0.935	-	0.893	0.960	0.969	0.803	0.839	0.961	0.966	0.999	0.933	0.867			
t	6.573	8.903	7.317	8.548	-	6.525	10.940	12.621	4.620	5.196	11.165	12.018	59.321	8.399	5.795			
P	0.003	0.001	0.002	0.001	>0.05	0.003	0.000	0.000	0.010	0.007	0.000	0.000	0.000	0.001	0.004			
Bacillus megaterium																		
2h	1.642	1.729	1.778	1.555	1.626	1.785	1.799	1.781	1.779	1.618	1.721	1.768	1.679	1.742	1.725	1.759	1.783	1.764
4h	1.394	1.585	1.400	1.382	1.489	1.508	1.572	1.453	1.363	1.351	1.411	1.380	1.393	1.529	1.363	1.250	1.380	1.380
6h	1.342	1.330	1.376	1.329	1.334	1.395	1.309	1.341	1.338	1.228	1.402	1.333	1.231	1.322	1.317	1.162	1.305	1.304
10h	1.278	1.323	1.364	1.263	1.288	1.335	1.299	1.331	1.314	1.225	1.382	1.276	1.223	1.289	1.277	1.155	1.160	1.271
18h	1.269	1.308	1.314	1.208	1.269	1.323	1.253	1.289	1.304	1.221	1.345	1.254	1.220	1.282	1.267	1.102	1.119	1.104
24h	1.209	1.295	1.250	1.159	1.265	1.261	1.212	1.255	1.267	1.216	1.241	1.348	1.197	1.225	1.255	1.091	1.104	1.094
***R**	0.973	0.934	0.973	0.923	0.966	0.977	0.932	0.983	0.946	0.987	0.959	0.915	0.980	0.967	0.957			
R^2	0.946	0.872	0.947	0.852	0.934	0.954	0.869	0.967	0.895	0.975	0.920	0.837	0.961	0.936	0.915			
Adj-R^2	0.933	0.841	0.934	0.815	0.918	0.943	0.837	0.959	0.869	0.969	0.900	0.796	0.951	0.920	0.894			
t	8.399	5.230	8.487	4.793	7.524	9.128	5.158	10.848	5.836	12.505	6.772	4.526	9.878	7.648	6.564			
P	0.001	0.006	0.001	0.009	0.002	0.001	0007	0.000	0.004	0.000	0.002	0.011	0.001	0.002	0.003			

*The parameters of statistical regression analysis include the values of coefficient, t-test values and p -values for the variables affecting nitrite formation

Table 8: The Concentration of Leftover Nitrate (NO_3- µg/ml) After Denitrification Activity by Metal Resistant Bacterial Isolates and Regression Statistical Analysis

Metals	**Cu (mg/L)**			**Zn (mg/L)**			**Ni (mg/L)**			**Cr (mg/L)**			**Quaternary (mg/L)**			**Control (mg/L)**		
Time intervals	**10**	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**	**1**	**2**	**3**
Sphingobacterium ginsenosidimutans																		
2h	1.539	1.626	1.635	1.630	1.709	1.569	1.638	1.678	1.622	1.677	1.843	1.676	1.451	1.676	1.678	1.628	1.593	1.650
4h	1.538	1.592	1.627	1.616	1.705	1.544	1.635	1.487	1.546	1.629	1.636	1.539	1.224	1.636	1.623	1.522	1.507	1.545
6h	1.441	1.477	1.454	1.581	1.471	1.529	1.517	1.400	1.396	1.622	1.410	1.453	1.118	1.404	1.496	1.456	1.488	1.449
10h	1.098	1.463	1.446	1.092	1.385	1.409	1.311	1.379	1.386	1.282	1.343	1.400	1.064	1.295	1.425	1.235	1.232	1.219
18h	1.091	1.380	1.438	1.083	1.165	1.382	1.261	1.308	1.339	1.212	1.286	1.345	1.056	1.228	1.358	1.157	1.182	1.185
24h	1.050	1.369	1.384	1.079	1.132	1.366	1.146	1.249	1.336	1.199	1.275	1.318	1.050	1.182	1.353	1.121	1.117	1.121
R	0.977	0.918	0.910	0.964	0.940	0.986	0.980	0.886	0.926	0.981	0.881	0.961	0.873	0.940	0.979			
R^2	0.956	0.842	0.829	0.929	0.884	0.973	0.961	0.785	0.857	0.962	0.777	0.923	0.762	0.883	0.957			
Adj-R	0.944	0.803	0.786	0.911	0.855	0.966	0.951	0.732	0.822	0.953	0.721	0.903	0.703	0.854	0.947			
t	9.268	4.625	4.396	7.224	5.526	11.967	9.953	3.824	4.906	10.096	13.732	6.910	3.582	5.503	9.492			
P	0.001	0.010	0.012	0.002	0.005	0.000	0.001	0.019	0.008	0.001	0.020	0.002	0.023	0.005	0.001			
Kocuria rhizophila																		

2h	1.506	1.557	1.693	1.507	1.556	1.678	1.499	1.515	1.684	1.501	1.544	1.691	1.614	1.688	1.704	1.503	1.517	1.552
4h	1.503	1.498	1.615	1.354	1.348	1.480	1.386	1.464	1.467	1.342	1.465	1.633	1.342	1.591	1.604	1.327	1.330	1.391
6h	1.418	1.449	1.549	1.337	1.343	1.469	1.327	1.358	1.436	1.305	1.314	1.593	1.324	1.529	1.599	1.242	1.328	1.382
10h	1.133	1.141	1.513	1.124	1.151	1.433	1.127	1.174	1.420	1.180	1.181	1.522	1.119	1.227	1.482	1.100	1.106	1.154
18h	1.177	1.133	1.502	1.115	1.123	1.429	1.117	1.163	1.415	1.164	1.150	1.509	1.107	1.135	1.477	1.087	1.100	1.102
24h	1.084	1.114	1.482	1.106	1.110	1.378	1.109	1.122	1.412	1.127	1.098	1.501	1.102	1.109	1.475	1.077	1.080	1.083
R	0.911	0.966	0.944	0.983	0.998	0.879	0.984	0.966	0.811	0.994	0.958	0.986	0.991	0.968	0.992			
R^2	0.830	0.933	0.890	0.967	0.995	0.773	0.968	0.933	0.658	0.988	0.918	0.973	0.981	0.938	0.983			
Adj-R	0.788	0.917	0.863	0.958	0.994	0.716	0.960	0.916	0.572	0.984	0.898	0.966	0.977	0.922	0.973			
t	4.421	7.484	5.695	10.757	29.606	3.686	10.992	7.435	2.771	17.791	6.694	11.979	14.523	7.752	15.381			
P	0.012	0.002	0.005	0.000	0.000	0.021	0.000	0.002	0.050	0.000	0.003	0.000	0.000	0.001	0.000			

*The parameters of statistical regression analysis include the values of coefficient, t-test values and p-values for the variables affecting nitrite formation

The results showed that the statistical regression analysis parameters included the determination coefficient (R^2) value, the adj-R^2 value, the t-value, and the p-value. The values of R^2 were greater than 0.9 for most metals and isolates, with the exception of concentrations of 100 mg/L Zn and Ni in *K. rhizophila* and 50 mg/L Ni and Cr in *S. ginsenosidimutans*, which were less. While the insignificant effect in *B. paramycoides* was only 50 mg/L Zn, it was reported that a regression model with a determination coefficient (R^2) value higher than 0.9 had a very high correlation. The value of R^2 should not be less than 0.75 until the model is suitable (El-Naggar *et al*., 2018). The largest correlation was found with 10 mg/L quaternary in *B. paramycoides*; R^2 was 0.999, indicating that 99.9% of the variations in the amounts of leftover nitrate were assigned to the independent variables and the model could only describe 0.1% of the total variables. In addition, the regression model for R^2 was 99.9%, which appears to be in good agreement with R^2, reflecting a perfect coordination between the predicted and observed values of the leftover nitrate amounts under mineral stress. Generally, the data of this experiment were more in agreement with this model than the previous experiment for estimating the amount of formed nitrite.

A study of denitrification and pigment production in isolated bacteria showed that the production of carotenoids made the bacteria able to tolerate high concentrations of metals and continue to reduce nitrate. It has been revealed that natural pigments are crucial for microorganism physiology, adaptation, protection, and photosynthesis, enabling adaptation to extreme environments (Sutthiwong *et al*., 2014; Celedón and Díaz, 2021). That is because of their role in antioxidants activities in the cell (Ramesh *et al*., 2019). Since pigments in bacteria absorb light, results showed that using yellow light at 590 nm accelerates denitrification by 35.4% in industrial wastewater, affecting microbial activity and microbial physiology. This effect is supported by antioxidant potential, microbial community shifts, metabolic flux redirection, and viability (Liao *et al*., 2023). It can be concluded that pigmented bacteria and aerobic denitrifiers were able to persist in their physiological activities under metallic stress.

4. CONCLUSION

In this study, bacterial species obtained from electroplating effluents were used for evaluating their physiological activities along with intervals of metallic stress. These species were tested for their ability to produce carotenoid pigment and obtain energy for growth by nitrate reduction in the presence of high concentrations of single and quaternary metals, namely Cu, Zn, Ni, and Cr. The potential of these isolates to produce carotenoids and act as aerobic denitrifiers in bioremediation was also examined at intervals of exponential phase and stationary phase after exposure to high concentrations of heavy metals. The production of carotenoids and the nitrate-reducing activities of the bacteria were shown to be higher during the exponential phase than the stationary phase. Furthermore, as metal

concentrations increased, the activities decreased, which had a negative effect on carotenoid production and the accumulation of intermediate compounds rather than the rapid transformation of nitrite. However, all isolates retained the production of carotenoids in yellow and orange colors and nitrate reduction activities even with decreased amounts produced when grown in the presence of both single and multiple metals and at high concentrations of metals. It can be concluded that the carotenoids produced enabled the bacteria to tolerate high metal concentrations while continuing to reduce nitrates. Thus, these isolates are promising with nitrate removal and metals potential and can be used for bioremediation of nitrate-metals contaminated sites, leading to environmental restoration and clean-up.

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CONFLICT OF INTEREST

The author declares that there are no conflict of interest.

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