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Research Article

Study of respiratory metabolism for multimetal tolerant bacteria under metallic stress

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Abstract

In aquatic environments, excessive amounts of inorganic nutrients, such as heavy metals, pose a metabolic risk and threaten to halt microbial activity. Metal-resistant bacteria, through various metabolic activities, can treat and detoxify harmful inorganic compounds. Thus, higher energy and electron transport system (ETS) demands may be necessary for metal bioremediation, but cell viability may be affected. Therefore, the metabolic respiration activity of cells in the presence of Cu, Zn, Ni, and Cr individually and in the quaternary is an important aspect of this study that relies on estimating the action of respiration enzymes responsible for metabolic activity and glucose reduction over periods of metallic stress and growth phases. Three species of bacteria, including B. megaterium, S. ginsenosidimutans, and K. rhizophila, were isolated from the electroplating effluent and used to determine the activity level of catalase, dehydrogenase enzymes, and glucose reduction. Their ability to sustain metabolic activity and understand their role in conferring tolerance and bioremediation capabilities to bacteria was evaluated. The findings revealed that metabolic activity was greater during the exponential phase than during the stationary phase. Catalase production was less affected by high metal levels; additionally, sugar reduction was improved but decreased with increased metal levels, and growth progressed, in comparison to dehydrogenase activity, which was more sensitive to high metal levels. Although respiratory metabolism activity decreased with increasing cell age and high metal concentrations, metabolism and viability persisted under metallic stress. This establishes the tolerability of bacteria and recommends them for potential bioremediation of metallic pollutants and environmental clean-up.

Keywords: Respiratory metabolism, Heavy metals, metallic stress, Reactive Oxygen Species (ROS), Antioxidants

Introduction

Cellular respiration begins when electrons are transferred from NADH (nicotinamide adenine dinucleotide) and FADH2 (flavin adenine dinucleotide), which are made in metabolic reactions, through a series of redox reactions to a final inorganic electron acceptor (either oxygen in aerobic respiration or non-oxygen inorganic molecules in anaerobic respiration) (Parker *et al*., 2017). Bacteria that use oxygen as the final electron acceptor and are able to respire oxygen are likely to grow rapidly and outnumber other microbes that lose this ability. In a diffuse, limited environment such as waterlogged soil, oxygen will be used, anaerobic respiration will prevail, and there will also be microbes that can use alternative electron acceptors. In order to obtain most of the available energy, final electron acceptors include nitrate, Mn (IV), Fe (III), and sulfate, which will replace the oxygen. Some of the metalloids and metals in the waste can also act as electron acceptors and are transformed by reducing microbial of metal (Newsome and Falagan, 2021). In a fairly common case, the ability of bacteria to use multiple metabolic pathways to function under different environmental conditions, such as switching from aerobic respiration to nitrate reduction (facultative anaerobes), or H2 oxidizing bacteria to switch to a heterotrophic metabolism when organic compounds are available (facultative chemolithoautotrophs), or fermentation of organic matter when terminal electron acceptors are limited (Pepper & Gentry, 2015), This flexibility allows these bacteria to benefit from more efficient metabolic processes under changing conditions (Newsome and Falagan, 2021).

Microorganisms use chemical pollutants as an energy source through their own metabolic processes. However, excessive quantities of inorganic nutrients cause microbial inhibition (Tarekegn *et al*., 2020; Ahirwar *et al*., 2016). Microbial cells, in particular, have the ability to treat, detoxify, and even accumulate harmful inorganic compounds as well as organic compounds (Tarekegn *et al*., 2020). During various metabolic activities, the microbial cells can absorb and accumulate metals. Many cells have become resistant to heavy metals by using them for detoxification and respiration mechanisms (Singh and Hiranmai, 2021). In contaminated environments, wastes have different and multiple chemical compositions. It was reported that heavy metal contamination predominately occurs as mixtures of various contaminants, including organic and inorganic contaminants, in wastewater (Bind *et al*., 2019). Microbial cells in contaminated environments require more energy to survive stressful or unfavorable conditions. Moreover, the higher ETS(ETS electron transport system) may indicate that, given the great stress to which the cells are exposed, the increasing energy

requirement to maintain cellular components is declining (Santos *et al*., 2014). Therefore, estimation of the ability of metal-resistant bacteria to reduce sugar under stressful conditions combined with metallic resistance gives them further advantages as highly efficient tools for bioremediation of contaminated environments.

Oxidation of organic matter, a total estimate of metabolism (aerobic and anaerobic), can be obtained by measuring the activity of dehydrogenase enzymes. This enzyme belongs to the enzymatic oxidation group that catalyzes the transfer of electrons and hydrogen ions from a donor to an acceptor or from substrates to acceptors (Matyja et al., 2021). The enzyme aids in the biological oxidation of organic matter, and it serves as an indicator of microbial oxidative activities and microbial redox systems (Yeboah et al., 2021). That is because these enzymes are part of the electron transport chain system and their activity can be used to determine the respiratory rate, which is an indicator of the overall activity and state of microorganisms (Maachowska-Jutsz and Matyja, 2019; Matyja et al., 2021).

In contrast, metallic stress caused by heavy metals alters the physiological and biological system within a living cell, leading to inhibition of cellular characteristics such as growth, variety, morphology, and biological activity such as metabolism, enzymatic activity, and oxidative phosphorylation (Fashola et al., 2016; Igiri et al., 2018). It was demonstrated that the metabolic activity of living cells might be affected by the presence of heavy metals, causing functional defects, inhibiting enzyme activity and oxidative phosphorylation (Fashola et al., 2016). The inhibition of the metabolic activity of bacterial cells is due to the binding of metals to the amino sulfides of protein components in the cells. In addition, the chemical affinity of metals for thiol groups (SH) of amino acids can alter enzyme specificity and disrupt cellular functions (Syed et al., 2021).

Heavy metal toxicity to living cells manifest itself in several ways, including an increase in the production of reactive oxygen species such as O2, OH-, and H2O2 (Hussein and Joo, 2013). Reactive oxygen species can destroy a variety of important cellular molecules, including DNA, RNA, proteins, and lipids, and affect the ability of cells to survive. To prevent or reduce ROS-induced oxidative stress, bacteria use different ROS defense mechanisms called antioxidant enzymes, such as superoxide dismutases (SOD), catalases (CAT), peroxides (POD), and ascorbate peroxidase (APX) (Borisov et al., 2021; Benghait and Blaghen, 2019). In addition, heavy metals bind with the enzyme substrate complex, denature the enzyme, or interfere with the active sites of the protein, resulting in reduced enzyme activity (Li et al., 2015; Yeboah et al., 2021). It was confirmed that in polluted environments, exposure of the cell to metal ions

leads to a decrease or inhibition of dehydrogenase activity, which plays a major role in redox reactions in the activities of electron transport systems (ETSAs) (Pennafirme et al., 2015). Therefore, the metabolic activity of living cells in the presence of single and multiple metals is an important aspect of this study, which depends on estimating the action of respiratory enzymes responsible for metabolic activity and sugar reduction along periods of metal stress according to the growth curve of bacterial cells. Tracking these physiological activities of bacteria under the influence of individual and quaternary metals to determine the level of influence and comparison between types of metals, especially the combined effect of metals, is important in assessing the viability and activity of bacteria under metal stress. This is so that it accurately reflects the circumstances that bacteria might encounter along with the extent of their responses to those conditions.

Martial and methods

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 24hr. Three isolates that were able to tolerate and grow at high concentrations were chosen for the respiratory metabolism study under metallic stress. In this study, based on 16S rDNA data, these isolates were identified and confirmed as Bacillus megaterium (NR_117473.1), Sphingobacterium ginsenosidimutans ((NR_108689.1)),Kocuria rhizophila (NR_026452.1).

Determination of the Rate of Hydrogen Peroxide-Splitting Activity

1% of the overnight isolates (B. megaterium, S. ginsenosidimutans, and K. rhizophila) (OD = 0.6) were inoculated in nutrient broth with a concentration of 10, 50, and 100 mg/L Cu, Ni, Cr, and Zn individually and quaternary with shaking at 37 °C and 200 rpm at six time points in the growth curve (4, 6, 10, 24, 30, and 48 hr). Which represent the exponential phase onset (4.0 hr), the exponential phase midpoint (6.0 hr), the exponential phase late point (10.0 hr), the stationary phase onset (24 hr), the stationary phase midpoint (30 hr), and the stationary phase late point (48 hr). In addition, control media without heavy metals were also included for comparison. Catalase activity was determined by the Cohen method (Cohen et al., 1970), in which aliquots of 5 mL were drawn at each interval and centrifuged at 7000 × g for 10 min to obtain the supernatant. One tenth of the supernatant was added to test tubes containing 0.5 mL of 2 mmol hydrogen peroxide and a blank containing 0.5 mL of distilled water. Then, the tubes

were incubated on ice for 3 min, and 6N H_2SO_4 was used to stop the reaction by adding 1 mL in the same fixed time period to the test tubes. Finally, 7 mL of a 0.1 N $KMnO_4$ solution were added within 30 sec and mixed well, and the absorbance was recorded at 480 nm within 30–60 sec (Zhang et al., 2002; Achuba and Clarke, 2008). The rate of hydrogen peroxide-splitting activity was calculated by measuring decomposed hydrogen peroxide that reacted with an excess of potassium tetraoxomanganate, and the remaining $KMnO_4$ was measured spectrophotometrically at 480 nm (Nafiu and Rahman, 2015).

Calculations of catalase enzyme

Preparation Spectrophotometer standard

It was prepared by adding 7 ml of 0.1 N $KMnO_4$ to a mixture of 5.5 ml of 0.05 N phosphate buffer at pH 7 and 1 ml of 6N H_2SO_4. The spectrophotometer was then zeroed with distilled water before taking absorbance readings. In this assay, one unit of catalase enzyme activity equals k / (0.00693) (Aebi, 1974), and an enzyme unit is defined as the enzyme activity that catalyzes the conversion of one µmol substrate into product in one minute.

A spectrophotometric assay is usually applied for this purpose on a selected substrate. Enzyme activity could be calculated using the following equation:

Where: $k = \log (S0/S2) \times (2.3/t)$

S0 = absorbance of standard – absorbance of blank,

S2 = absorbance of standard – absorbance of sample, and t = time interval.

Estimation of Triphenyl Formazan (TF) Yield as an Indicator of Dehydrogenase Enzyme Activity

Bacteria isolates of B. megaterium, S. ginsenosidimutans, and K. rhizophila (OD = 0.6) were inoculated into nutrient broth containing individual and quaternary Cu, Ni, Cr, and Zn concentrations of 10, 50, and 100 mg/L. The cultures were under agitation conditions at 37 °C and 200 rpm for 4, 6, 10, 24, 30, and 48 hr, and control media without heavy metals were also included for comparison. After that, 1 mL of each culture flask was transferred to sterilized test tubes, followed by 1 mL of triphenyl tetrazolium/glucose solution, and the contents were gently swirled to mix. All tubes were incubated at 37 °C for 20 min in the dark under agitating conditions. The resulting formazan was centrifuged at 4000 × g for 3 min, followed by decantation of the supernatants. The pellets obtained were resuspended and vortexed so that they would aid in the extraction of triphenyl formazan from the cells, then centrifuged again in 2.5 mL of methanol, and the supernatant was decanted. Two additional extractions were performed with methanol, and the supernatants from three extractions were collected together for measurement. The absorbance of the obtained red formazan solution from three combined

supernatant extractions was finally measured by a spectrophotometer at 484 nm (Burdock et al., 2011). A standard curve was established to determine the formazan concentration TF (µmol/mL) corresponding to an absorbance measurement at 484 nm (OD484). Thereafter, the TF is calculated by replacing the absorbance (Y) obtained in the regression equation.

Preparation of Triphenyl Tetrazolium Chloride (TTC) for Standard Curve of dehydrogenase enzyme

Triphenyl Formazan (TF)

An aliquot of 1 gm glucose and 2 gm TTC (TCI, Tokyo Chemical Industry) were dissolved in 100 mL distilled water. Then, they were stored in the dark at 4˚C until needed (Burdock et al., 2011).

Preparation of Triphenyl Formazan (TF) Standard Curve

A standard curve was developed to determine the concentration of triphenyl formazan (µmol/mL) corresponding to an absorbance measurement at 484 nm (O.D 484). A stock solution of 0.2 mol/mL was prepared by dissolving 0.015 g Triphenyl Formazan (TF, Sigma-Aldrich) in 250 mL methanol to obtain 0.0002 M Triphenyl Tetrazolium Formazan. 1µmol Triphenyl Formazan = 300.36 µg TF. After dissolving 0.06 g of triphenyl formazan in 1000 mL of methanol, 200 mol of triphenyl formazan was obtained. One, 3, 5-triphenyl tetrazolium formazan, TF, C19C16N4, Molecular Weight: 300.36 µg/mol; 300.36 µg /µg mol. The stock solution was diluted with methanol (CH3OH, HmbG® Chemicals) to produce nine solutions with TF concentrations ranging from 0.004 to 0.080 µmol/mL. The preparation of standard solutions are shown in the following Table (1).

Table 1. Preparation of Standards Solutions of 1, 3, 5-Triphenyl Formazan (TF)

µg/mL	**µmol/mL (TF)**	**Stock (60 µg/mL,0.20 µmol/mL TF) Volume(mL)**	**Diluent (Methanol) Volume(mL)**
0	0.000	0.0	10.0
1.2	0.004	0.2	9.8
3.0	0.010	0.5	9.5
6.0	0.020	1.0	9.0
9.0	0.030	1.5	8.5
12.0	0.040	2.0	8.0
15.0	0.050	2.5	7.5
18.0	0.060	3.0	7.0
21.0	0.070	3.5	6.5
24.0	0.080	4.0	6.0

The absorbance of each solution was measured with a spectrophotometer (Spectronic, Genesys 20) at a wavelength of 484 nm. The absorbance readings (O.D 484) were plotted against the TF concentration (µmol/mL).

The ability to Reduce Sugars

Overnight-grown fresh *B. megaterium, S. ginsenosidimutans*, and *K. rhizophila* were used to study their ability to reduce sugars. The nutrient broth medium (pH = 7.2) in 95 mL was autoclaved into 100 mL Erlenmeyer flasks at 15 lb/sq. in. for 20 min. Thereafter, the sterilized media were supplemented with filter-sterilized glucose stock, consisting of 10 mL of a 10% (w/v) glucose solution, and 1 mL (10, 50, and 100 mg/L, respectively) of heavy metals (Cu, Zn, Ni, Cr, and quaternary) were added from the aqueous stock solution to the media. The control medium without heavy metals was also included for comparison. An aliquot of 1 mL (OD 0.6) of culture was added to each flask. The cultures were incubated under agitation conditions of 200 rpm at 37°C at 4, 6, 10, and 24 hr intervals. Then, 5 mL were collected and centrifuged at 3500 g for 15 min; the output supernatant was collected for residual glucose analysis. The supernatant was introduced into 15 mL sterile centrifuge tubes and wrapped with aluminum foil, then placed in a water bath maintained at 25°C, for 10 min to allow the tubes and solution to reach a constant temperature. A blank solution made using distilled water was also placed in the water bath. One mL of 1 N NaOH was added, vortexed, and returned quickly to the bath for 6 min to allow equilibrium temperature to be reached. Then, 1 mL of a 1% tetrazolium salt solution was added to each tube. The tubes were agitated and returned to the water bath for 30 minutes after the addition of the tetrazolium salt. Two mL of methanol were added, bringing the total volume with methanol to 10 mL. The tubes were well mixed to dissolve the formazan, a clear red solution was produced except in the case of the blank, which is colorless, and the absorbance of the supernatants was recorded at 485 nm. From this value, the sugar content can be determined using a standard curve prepared with known glucose concentrations (0–2 mg) (Mattson and Jensen, 1950; Fairbridge *et al*., 1951).

The data obtained on respiratory metabolism activity with different metals at different concentrations and time intervals by individual isolates (*B. megaterium, S. ginsenomutans*, and *K. rhizophila*) were subjected to statistical analysis using the SPSS program (SPSS Version 25). The relationship between enzymatic activities in isolated bacteria and variable parameters was correlated using multiple regression analysis, thus determining the significance of differences ($p \leq 0.05$) between means.

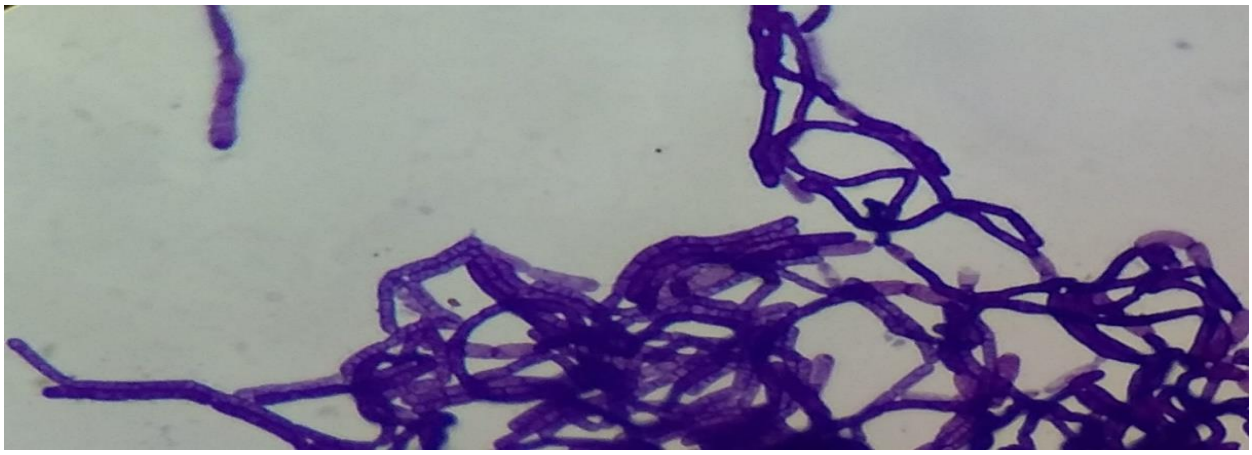
Standard Curve for Estimation of Glucose Reduction with Tetrazolium

Into clean, dry test tubes graduated at 10 mL, dilutions of glucose were pipetted to give a range of 0–2 mg, and the volume was set to 3 mL with distilled water. A blank is included in each series, containing 3 mL of distilled water. One ml of 1% triphenyltetrazolium chloride solution and 2 mL (1N) sodium hydroxide were added to each tube. The tubes were heated in a boiling

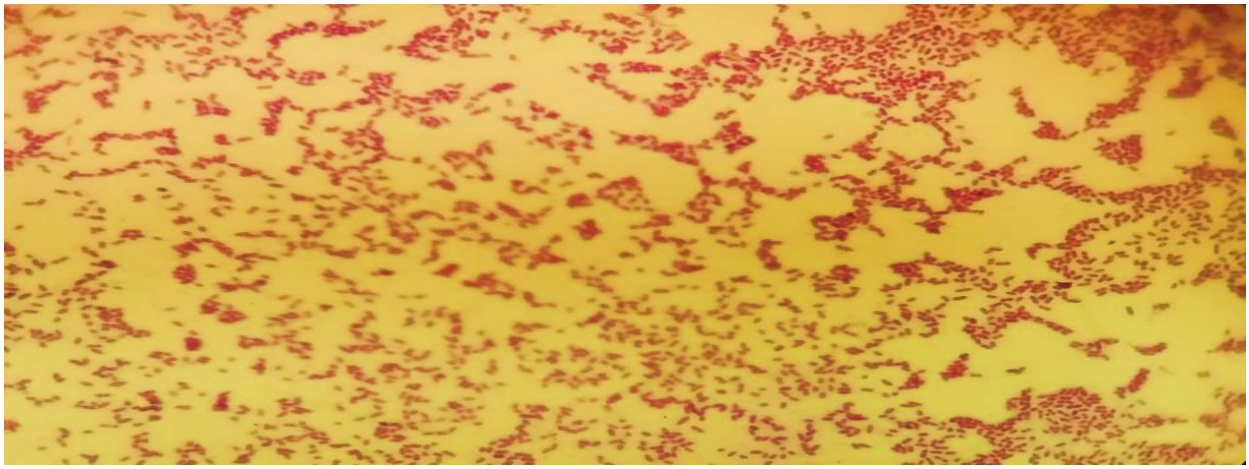
water bath (Memmert) for exactly 3 minutes, and when removed from the bath, approximately 2 ml of N-acetic acid was added, and the tubes were immediately cooled in cold water. The volume is adjusted to 10 mL with methanol, and the mixture is shaken to dissolve the formazan. Two mL of TTC were pipetted into another graduated test tube, and the volume was adjusted to 10 mL with methanol. Color measurement was made by a spectrophotometer at 485 nm (Fairbridge et al., 1951).

Results and discussion

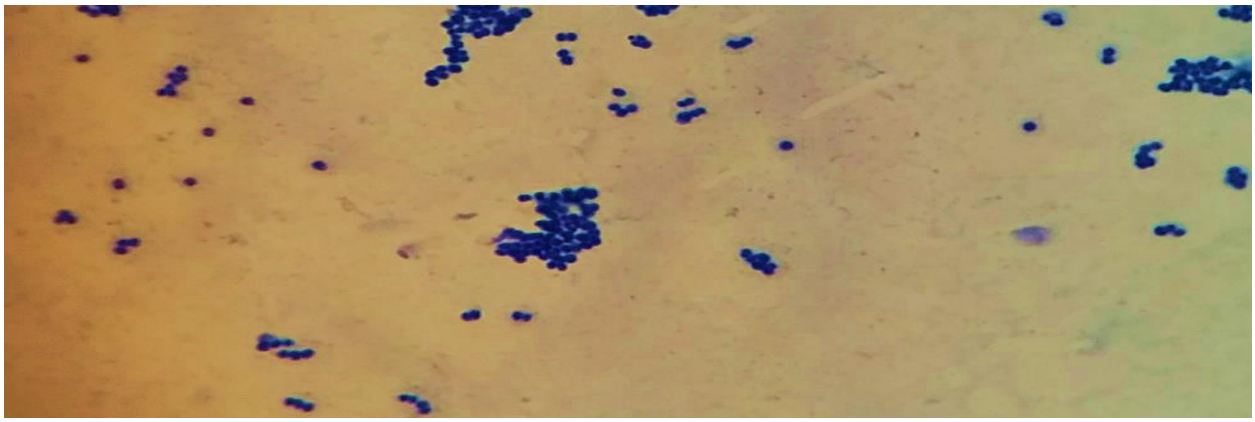
Cellular metabolic activities that take place in the presence of oxygen and redox enzymes, which are in charge of redox reactions through the respiratory chain, are referred to as respiratory metabolism. It is essential for the redox balance and energy production of aerobic bacteria. Metals have an impact on a process that is connected to respiratory metabolism, which hinders development and reproduction. To better understand their function and how they can contribute to conferring bacteria with metal tolerance and bioremediation abilities, it may be helpful to evaluate this process by looking at the agents responsible for its accomplishment in isolated bacteria, which demonstrated the ability to absorb and reduce the metal concentration. Three bacterial species used to examine respiratory activity and metabolism rates are depicted in Figure 1 using the Gram staining technique under a light microscope at 1000X magnification.



B. megaterium



S. gensensidimutans



K. rhizophila

Figure 1. The bacterial isolates used in the study of physiological activities under metallic stress.

Determination of the Rate of Hydrogen Peroxide-Splitting Activity

The enzyme catalase is one of the most important indicators of resistance to heavy metals because these pollutants change the cellular redox state due to the oxidative stress produced in bacteria under metal stress. However, antioxidant enzymes can maintain cellular redox state and reduce damage from reactive oxygen species (ROS) (Kaushal et al., 2018; Achuba and Okoh, 2014). In the current study, the activity of the catalase enzyme was chosen to determine the respiratory metabolism of bacterial isolates for its association with the respiration process and as an indicator of the persistence of nutritional activity under stressful conditions. The activity of the catalase enzyme in *B. megaterium*, *S. ginsenosidimutans*, and K. rhizophila isolates during intervals of stress and within the growth curve in exponential and stationary phases was estimated in the presence of Cu, Zn, Ni, and Cr individually and quaternarily. The results are presented in Tables 2-4.

The results showed that the reaction rate and units of enzyme activity increased with the increase in metal concentration. This is due to the increased oxidative stress that accompanied

the increased concentration of the metal. This requires the cell to counteract it by increasing the activity of the antioxidant enzymes and reducing the damage caused by reactive oxidants. The maximum rates of reaction and enzyme activity units occurred within 10 to 24 hours of the cells entering stationary phase; when the cells entered stationary phase, these parameters decreased across all metal concentrations and bacterial isolates.

In comparison to controls, catalase enzyme activity in tolerant bacteria was less impacted and maintained its functional activity in the presence of metals. According to the results of this study, increased catalase activity was an indicator of the response of microorganisms to metallic stress in the environment (Kharchenko et al., 2013). The results may also confirm that catalase enzyme action is one of the tools that metal-resistant bacteria rely on to resist metal stress. The findings might also imply that metal-resistant bacteria utilize the catalase enzyme as one of their defense mechanisms against metal stress. This safeguard is critical because hydrogen peroxide accumulates and may become fatal to bacteria.

The effect of the metal concentration and time intervals was statistically analyzed using the Plackett-Burman design. The results obtained for the activity units of the catalase enzyme were used for the statistical analysis of the experiment. There were significant differences ($p \leq 0.05$) in the units of catalase enzyme produced by the bacterial species during the logarithmic and stationary phases. The ANOVA and regression model results demonstrate that the model is highly significant, as evident from the high value of t and very low probability value. Values of p less than 0.05 indicate that model terms are significant. The t- and p-values were estimated for each independent variable, and the significance of each coefficient was also determined by the p-value in Table 5. Each factor, as well as their interference, had a significant effect. It was also evident that the interference effect between the metal concentrations and time on enzyme units were synergistic. Their positive coefficients indicate a higher percentage of enzyme units produced upon elevating the concentration and time. The obtained R2 and Adj-R2 values were mostly greater than 0.9, especially at 10 mg/L, which was considered to be very highly correlated. However, at 50 and 100 mg/L, some of them were greater than 0.75 (less than 0.9), which was considered high according to El-Naggar et al. (2018). The regression model with a high value of R2 and adjusted R2 indicates high precision and validity for the model used in predicting the units of catalase enzyme produced by metal-tolerant bacteria under metallic stress.

Table 2. The catalase enzyme activity of *Bacillus megaterium* under metallic stress

Metals	Cu			Zn			Ni			Cr			Quaternary			Control	
Time Intervals	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU
									10 mg/L								
4 h	3.41	493.41	75.97	3.15	454.55	69.98	3.21	463.20	71.32	3.54	512.20	78.86	3.59	519.15	79.93	4.50	649.35
6 h	4.80	692.68	70.08	5.27	760.54	76.94	5.09	734.58	74.32	5.44	786.23	79.54	5.51	795.62	80.49	6.85	988.46
10h	7.47	1077.62	84.08	8.1	1169.36	91.24	7.76	1120.25	87.41	8.16	1177.66	91.88	8.49	1224.56	95.55	8.88	1281.64
24h	6.85	988.54	76.91	7.52	1085.36	84.44	7.48	1079.36	83.98	7.89	1138.52	88.58	8.30	1198.59	96.09	8.90	1285.33
30h	6.59	951.60	76.29	7.24	1044.73	88.76	7.06	1019.15	81.71	7.79	1125.51	90.24	7.98	1151.51	89.59	8.64	1247.30
48h	6.31	911.82	75.49	6.73	972.49	80.52	6.81	983.55	81.43	7.31	1056.13	87.44	7.93	1145.33	94.83	8.36	1207.79
									50 mg/L								
4 h	3.82	552.32	84.32	3.63	524.14	80.03	3.75	541.49	82.67	3.95	570.81	87.15	3.80	549.05	83.83	4.53	654.97
6 h	4.94	713.91	71.59	5.33	769.81	77.20	5.18	748.80	75.09	5.52	797.48	79.97	5.66	816.75	81.91	6.91	997.14
10h	7.89	1138.75	87.54	8.21	1186.01	91.17	8.10	1170.03	89.95	8.34	1204.32	92.58	8.54	1233.50	94.82	9.0	1300.83
24h	7.63	1101.65	84.45	8.02	1157.42	88.73	7.77	1121.64	85.98	8.02	1157.42	88.72	8.38	1209.80	92.74	9.04	1304.47
30h	6.54	944.97	72.90	7.55	1090.47	84.12	7.16	1034.16	79.78	7.82	1128.72	87.08	8.12	1172.10	90.42	8.98	1296.22
48h	6.34	916.06	74.75	6.83	986.19	80.48	6.93	1000.12	81.61	7.49	1081.94	88.29	7.84	1131.69	92.35	8.49	1225.46
									100 mg/L								
4 h	3.91	553.81	85.44	3.78	545.45	84.16	3.87	558.75	86.21	4.00	577.54	89.10	3.93	576.66	87.58	4.49	648.15
6 h	5.03	726.24	72.82	5.39	777.93	78.00	5.27	761.87	76.39	5.61	810.30	81.24	5.76	831.55	83.38	6.91	997.30
10h	8.00	1155.57	89.51	8.25	1191.28	92.27	8.18	1180.72	91.45	8.44	1218.24	94.36	8.59	1240.09	96.05	8.95	1291.07
24h	7.75	1119.29	86.96	8.13	1173.31	91.15	7.88	1138.35	88.44	8.21	1186.01	92.14	8.43	1216.73	94.53	8.91	1287.13
30h	6.44	929.88	72.74	7.77	1121.33	87.72	7.24	1045.39	81.77	7.85	1133.08	88.64	8.29	1196.52	93.60	8.86	1278.32
48h	6.24	899.90	72.69	6.89	994.45	80.32	6.81	984.07	79.48	7.52	1085.36	87.67	8.16	1177.54	95.11	8.57	1238.06

Where: **K** is the reaction rate, **(IU)** Units of enzyme activity (µmol/ml/min), **% (C)** enzyme activity determined relative to control

Table 3. The catalase enzyme activity of *Sphingobacterium ginsenosidimutans* under metallic stress

Metals	Cu			Zn			Ni			Cr			Quaternary			Control	
Time Intervals	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU	% (C)	Kmin^{-1}	IU
									10 mg/L								
4 h	2.96	426.53	56.52	2.78	401.58	53.21	2.86	413.52	54.79	2.98	431.34	57.15	3.10	447.37	59.28	5.23	754.69
6 h	4.52	651.89	66.02	4.71	680.59	68.92	4.66	673.39	68.19	4.76	688.28	69.70	4.92	710.38	71.94	6.84	987.44
10h	8.28	1194.80	97.49	8.15	1176.46	96.00	8.03	1159.34	94.61	8.19	1182.10	96.46	8.26	1192.31	97.29	8.49	1225.46
24h	8.16	1177.66	95.72	8.07	1165.57	94.74	7.95	1147.76	93.29	8.15	1176.46	95.63	8.20	1184.50	96.28	8.53	1230.27

30h	7.70	1112.15	91.43	7.85	1133.08	93.15	7.31	1056.13	86.82	7.93	1145.33	94.15	8.17	1178.93	96.92	8.43	1216.45
48h	6.45	930.81	78.76	6.56	947.19	80.09	6.39	922.32	78.04	6.69	966.22	81.76	6.90	996.03	84.28	8.19	1181.82
50 mg/L																	
4 h	3.09	445.59	57.49	3.16	456.38	60.93	3.06	441.12	58.89	3.22	464.73	62.05	3.33	481.03	64.22	5.19	748.97
6 h	4.66	673.71	66.97	4.91	709.08	94.67	4.71	681.02	67.69	4.86	702.09	69.79	4.98	719.14	71.49	6.97	1005.97
10h	8.23	1188.94	95.80	8.28	1195.13	96.29	8.22	1186.24	95.58	8.26	1192.31	96.07	8.47	1222.87	98.53	8.60	1241.07
24h	7.94	1147.14	92.76	7.98	1151.51	93.11	7.79	1125.51	91.01	7.97	1151.39	93.11	8.32	1201.27	96.79	8.57	1236.65
30h	7.63	1101.76	89.56	7.75	1119.40	90.98	7.58	1094.59	88.97	7.85	1133.08	92.10	8.11	1171.63	95.23	8.53	1230.27
48h	6.34	916.06	77.17	6.50	938.75	79.09	6.26	903.41	76.11	6.50	938.75	79.09	6.82	984.89	82.97	8.23	1187.00
100 mg/L																	
4 h	3.26	471.406	62.46	3.46	499.27	66.16	3.17	458.05	60.69	3.40	491.07	65.07	3.38	487.73	64.63	5.23	754.69
6 h	4.72	681.25	68.03	5.03	725.82	72.48	4.82	695.93	69.49	4.96	715.72	71.47	5.07	731.67	73.06	6.94	1001.44
10h	8.36	1207.12	97.34	8.46	1221.34	98.49	8.32	1201.52	96.88	8.42	1215.70	98.03	8.54	1232.52	99.39	8.59	1240.09
24h	7.87	1136.29	92.19	7.89	1139.22	92.43	7.64	1102.45	89.45	7.90	1140.92	92.57	8.50	1226.95	99.55	8.54	1232.52
30h	7.54	1088.29	89.11	7.76	1120.68	91.76	7.49	1081.43	88.55	7.82	1128.35	92.39	8.17	1180.14	96.63	8.46	1221.34
48h	6.27	905.35	76.22	6.43	928.54	78.18	6.22	897.54	75.57	6.47	934.72	78.69	6.73	972.49	81.88	8.23	1187.71

Where: **K** is the reaction rate, **(IU)** Units of enzyme activity (µmol/ml/min), **% (C)** enzyme activity determined relative to control

Table 4. The catalase enzyme activity of *Kocuria rhizophila* under metallic stress

Metals	**Cu**			**Zn**			**Ni**			**Cr**			**Quaternary**			**Control**	
Time Intervals	**Kmin^{-1}**	**IU**	**% (C)**	**Kmin^{-1}**	**IU**	**% (C)**	**Kmin^{-1}**	**IU**	**% (C)**	**Kmin^{-1}**	**IU**	**% (C)**	**Kmin^{-1}**	**IU**	**% (C)**	**Kmin^{-1}**	**IU**
10 mg/L																	
4 h	3.38	488.57	67.77	3.53	510.11	70.76	3.61	521.35	72.32	3.42	493.50	68.45	3.69	532.61	73.88	4.99	720.95
6 h	5.07	731.77	75.44	5.77	833.09	85.89	5.67	818.82	84.42	5.79	835.98	86.18	5.91	852.99	87.94	6.72	969.99
10h	7.44	1073.59	84.61	8.02	1157.28	91.21	8.18	1181.16	93.09	7.81	1127.38	88.85	8.22	1187.00	93.55	8.79	1268.82
24h	8.08	1167.18	93.77	7.86	1134.53	91.15	7.95	1147.76	92.21	7.98	1151.75	92.53	8.14	1175.28	94.43	8.62	1244.67
30h	7.81	1127.38	93.61	7.75	1119.29	92.93	7.79	1124.83	90.37	7.85	1133.08	94.08	7.89	1138.75	94.56	8.34	1204.33
48h	7.60	1097.55	93.22	7.55	1090.47	92.62	7.64	1102.45	93.64	7.43	1073.15	91.14	7.71	1113.72	94.59	8.15	1177.37
50 mg/L																	
4 h	3.75	541.68	73.61	3.79	547.88	74.45	3.76	543.18	73.81	3.58	517.06	70.26	3.72	537.98	73.11	5.09	735.87
6 h	5.24	756.74	79.25	5.91	853.97	89.44	5.73	828.17	86.73	5.85	845.25	88.52	5.94	857.77	89.83	6.61	954.85
10h	7.78	1122.88	88.37	8.08	1167.18	91.86	8.24	1189.60	93.62	7.92	1143.59	89.99	8.04	1161.34	91.39	8.80	1270.66
24h	7.99	1153.57	92.49	7.79	1124.17	90.14	7.88	1137.60	91.21	8.10	1169.36	93.76	8.20	1183.65	94.90	8.64	1247.19
30h	7.77	1121.64	92.56	7.60	1096.80	90.51	7.68	1109.06	91.52	7.78	1122.88	92.66	7.74	1118.18	92.27	8.39	1211.79
48h	7.53	1087.05	91.99	7.54	1088.29	92.10	7.58	1093.84	92.57	7.31	1056.13	89.38	7.66	1105.33	93.54	8.18	1181.59
100 mg/L																	
4 h	3.85	556.91	78.59	3.82	551.42	77.82	3.88	560.60	79.11	3.95	571.23	80.62	3.84	554.76	78.29	4.91	708.55
6 h	5.52	797.82	81.69	5.96	861.15	88.18	5.83	841.62	86.18	6.04	871.57	89.24	6.14	886.19	90.73	6.76	976.64

10h	7.84	1131.57	89.75	8.19	1182.96	93.83	8.28	1195.88	94.86	8.13	1174.24	95.41	8.10	1170.03	92.81	8.73	1260.74
24h	8.13	1174.00	94.59	7.69	1111.08	89.52	7.74	1117.00	90.00	8.22	1186.24	95.58	8.36	1207.24	97.27	8.60	1241.07
30h	7.73	1115.86	91.71	7.56	1091.74	89.72	7.64	1103.33	90.67	7.65	1105.13	90.83	7.69	1111.08	91.32	8.43	1216.73
48h	7.47	1079.16	90.06	7.39	1067.51	89.08	7.48	1080.02	90.13	7.23	1044.62	87.17	7.56	1091.10	91.05	8.30	1198.33

Where: **K** is the reaction rate, **(IU)** Units of enzyme activity (µmol/ml/min), **% (C)** enzyme activity determined relative to control

Table 5. Regression statistics of the Plackett–Burman design used for determining variables influencing produced catalase enzyme units

Metals			**Zn**		**Ni**		**Cr**		**Quaternary**	
Parameters	**Cu**		**Iso1**	**Iso 2**	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**
	Iso1	**Iso 2**	**Iso 3**		**Iso 3**		**Iso 3**		**Iso 3**	
	Iso 3									
10 mg/L										
R	0.975	0.966	0.987	0.979	0.992	0.974	0.993	0.978	0.990	0.982
	0.977		0.997		0.998		0.993		0.999	
R^2	0.951	0.932	0.974	0.959	0.983	0.949	0.985	0.957	0.980	0.964
	0.955		0.995		0.997		0.987		0.997	
Adj- R^2	0.939	0.915	0.967	0.948	0.979	0.936	0.982	0.947	0.975	0.955
	0.944		0.993		0.996		0.983		0.996	
t	8.832	7.423	12.198	9.627	15.259	8.635	16.367	9.476	14.103	10.313
	9.194		27.242		36.377		17.288		37.179	
P	0.001	0.002	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.000
	0.001		0.000		0.000		0.000		0.000	
50 mg/L										
R	0.933	0.962	0.933	0.970	0.972	0.963	0.986	0.968	0.991	0.970
	0.993		0.995		0.998		0.993		0.994	
R^2	0.871	0.926	0.871	0.941	0.945	0.927	0.973	0.936	0.982	0.941
	0.985		0.990		0.997		0.986		0.989	
Adj- R^2	0.839	0.907	0.839	0.926	0.932	0.908	0.966	0.920	0.978	0.926
	0.982		0.988		0.996		0.983		0.986	
t	5.202	7.058	5.202	7.991	8.320	7.105	11.995	7.660	14.964	7.996
	16.353		20.323		35.087		16.803		18.666	
P	0.007	0.002	0.007	0.001	0.001	0.002	0.000	0.002	0.000	0.001
	0.000		0.000		0.000		0.000		0.000	
100 mg/L										
R	0.908	0.953	0.908	0.957	0.962	0.958	0.983	0.961	0.989	0.965
	0.993		0.996		0.996		0.991		0.994	
R^2	0.825	0.908	0.825	0.917	0.925	0.917	0.966	0.923	0.979	0.930
	0.986		0.993		0.993		0.982		0.987	
Adj- R^2	0.781	0.885	0.781	0.896	0.906	0.896	0.958	0.903	0.973	0.913
	0.982		0.991		0.991		0.977		0.984	
t	4.344	6.281	4.344	6.639	7.033	6.655	10.734	6.910	13.522	7.311
	16.608		23.086		23.668		14.750		17.636	
P	0.012	0.003	0.012	0.003	0.002	0.003	0.000	0.002	0.000	0.002
	0.000		0.000		0.000		0.000		0.000	

Where: **Iso 1** *B megaterium*, **Iso 2** *S ginsenosidimutans* and **Iso 3** *K rhizophila*

Estimation Triphenyl Formazan (TF) Yield as Indicator to Dehydrogenase Activity

Triphenyltetrazolium chloride (TTC) was used as an indicator of electron transport system (ETS) activity and energy conversion under metallic stress. The activity of dehydrogenase is an indicator of the growth and metabolism of living cells under metallic stress and is determined by the reduction of triphenyltetrazolium chloride to formazan. A standard curve was used to determine the concentration of TF (µmol/mL) corresponding to an absorbance measurement at 484 nm as shown in Figure 2. Dehydrogenase activity was evaluated with three isolates at six time points in the growth curve, which have already been used for determining catalase activity.

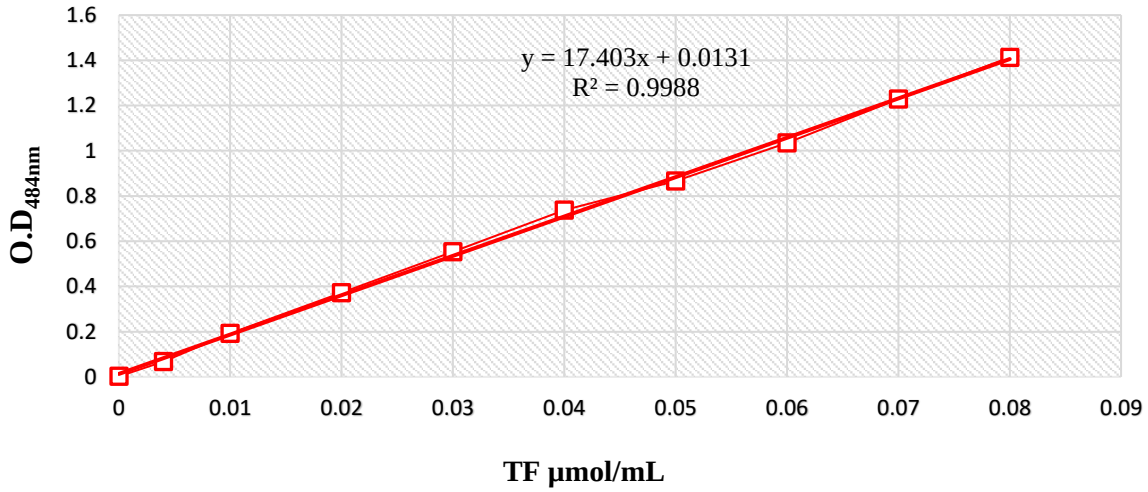


Figure 2. Standard curve of TPF concentration (µmol/mL) versus Absorbance

The results, as shown in Tables 6-8, revealed that the amount of formazan increased during the exponential phase (6-10 hours), but reduced when the experiment entered the stationary phase. At the lowest concentration (10 mg/L), the formazan amount gradually decreased over time, as an indicator of decreased enzyme activity. However, an increase for formazan was observed at the mid- and late-stationary phases, along with an increase in metal concentrations. When the metal concentrations were increased, the amount of formazan produced increased relatively compare to the 10 mg/L at the mid- and late-stationary phases. These results may be attributed to the high metal concentrations being cytotoxic and leading to rapid and increased cell death due to damage to the plasma membrane and leakage of dehydrogenase into the medium. Metals have previously been shown to have a negative effect on cellular components such as the cell membrane (Fashola *et al*., 2016).That is due to metal ion binding of cells surfaces and degradation of membrane functions (Syed *et al*., 2021). In comparison to the control, enzyme activity is normal in the absence of metals, but the activity was reduced when the cells entered the stationary phase. Moreover, the activity of enzymes was better with quaternary than single

metals, and high concentrations did not increase the amounts of formazan at the stationary phase. This may support the previous conclusion that the chemicals may interact and alter the toxicity of each other in a mixture (Nwanyanwu *et al*., 2017). However, stimulation, which takes place through the gradual increase of formazan amounts within 6–10 hr, can be attributed to the role of some individually tested minerals as essential components, participating as cofactors for the enzyme. In general, despite high mineral levels, bacterial metabolism continued under mineral stress and was higher at the logarithmic phase than at the stationary phase

Table 6. The dehydrogenase enzyme activity of *Bacillus megaterium* under metallic stress

Metals	**Cu**		**Zn**		**Ni**		**Cr**		**Quaternary**		**Control**
Time Intervals	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**
						10 mg/L					
4h	0.883	58.824	1.249	83.529	1.075	71.765	0.970	64.706	0.918	61.176	1.492
6h	2.154	66.129	2.345	72.043	2.223	68.279	2.415	74.194	2.711	83.333	3.250
10h	4.207	47.817	4.381	49.802	4.225	48.016	4.242	48.214	5.077	57.738	8.784
24h	4.155	44.653	4.312	46.341	4.138	44.465	4.189	45.028	5.251	56.473	9.289
30h	5.391	64.108	4.551	53.942	4.294	51.037	4.903	58.299	5.162	61.411	8.40
48h	5.965	88.144	4.594	67.784	4.346	64.175	5.356	79.124	3.337	49.227	6.766
						50 mg/L					
4h	0.818	52.809	1.109	74.118	1.022	65.169	0.866	55.056	0.831	55.294	1.562
6h	2.084	62.632	2.171	65.263	2.154	64.737	2.363	71.053	2.624	78.947	3.319
10h	3.128	35.029	4.277	47.945	4.103	45.988	4.172	46.771	5.025	56.360	8.906
24h	2.328	24.675	4.172	44.341	4.015	42.672	4.120	43.785	5.234	55.659	9.393
30h	5.791	68.454	4.712	51.546	4.382	51.753	4.973	58.763	5.095	60.206	8.454
48h	5.982	87.692	4.816	65.385	4.503	66.154	5.425	79.744	3.285	48.201	6.800
						100 mg/L					
4h	0.779	50.00	1.039	67.045	0.883	56.818	0.848	54.545	0.796	51.136	1.545
6h	0.938	66.845	2.101	64.171	1.162	35.294	1.022	31.016	1.057	32.086	3.267
10h	2.589	29.077	4.155	46.758	4.033	45.383	4.103	46.169	4.869	54.813	8.871
24h	2.293	24.214	4.085	43.253	3.981	42.144	3.929	41.589	4.381	46.396	9.428
30h	3.911	45.902	4.799	56.352	4.520	53.074	3.933	46.311	3.093	35.041	8.506
48h	4.085	60.622	5.129	76.157	4.851	72.021	4.068	60.363	2.467	36.528	6.731

Where: **(TF)** produced triphenyl formazan (µmol/ml), (**% of C)** TF yield determined relative to control

Table 7. The results of dehydrogenase enzyme activity of *Sphingobacterium ginsenosidimutans* under metallic stress

Metals	**Cu**		**Zn**		**Ni**		**Cr**		**Quaternary**		**Control**
Time Intervals	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**
						10 mg/L					
4h	0.866	51.579	1.318	78.947	1.092	65.263	0.900	53.684	0.848	50.526	1.666
6h	2.815	87.978	2.954	92.349	2.885	90.164	1.806	56.284	2.449	76.503	3.198
10h	2.537	30.083	4.399	51.037	4.242	50.415	4.022	47.718	5.304	63.071	8.401
24h	2.014	21.739	3.946	42.722	3.894	42.155	3.737	40.454	4.990	54.060	9.219
30h	4.016	66.286	3.476	57.349	3.285	54.179	2.863	47.193	3.902	64.265	6.052

48h	4.172	74.224	3.128	55.590	3.111	55.279	2.798	49.697	3.293	58.385	5.617
					50 mg/L						
4h	0.814	56.790	1.214	75.824	1.022	63.736	0.866	53.846	0.796	49.451	1.597
6h	2.693	82.796	2.885	88.709	2.815	86.559	1.736	53.226	2.415	74.194	3.250
10h	2.049	23.975	4.294	50.407	4.155	48.770	3.998	46.926	4.433	52.049	8.506
24h	1.997	21.739	4.225	46.187	4.085	44.656	3.981	43.511	4.189	45.802	9.132
30h	4.103	66.765	3.998	65.057	3.650	59.375	2.658	43.318	3.842	62.500	6.139
48h	4.277	77.287	3.877	70.032	3.233	58.359	2.554	46.057	3.737	67.508	5.529
					100 mg/L						
4h	0.661	40.860	1.127	68.817	1.249	76.344	0.831	50.538	0.744	45.161	1.632
6h	2.589	77.487	2.745	82.199	2.763	82.723	1.527	45.549	2.293	68.586	3.337
10h	2.014	23.663	4.033	47.531	4.051	47.737	3.981	46.914	4.364	25.000	8.471
24h	1.945	20.219	3.964	41.348	3.929	40.984	3.789	39.526	4.138	43.169	9.567
30h	4.451	72.034	3.476	56.215	2.363	38.136	2.537	40.960	2.711	43.785	6.174
48h	5.286	93.519	2.432	42.901	2.049	36.111	2.466	43.519	2.136	37.654	5.652

Where: **(TF)** produced triphenyl formazan (µmol/ml), **C (%)** TF yield determined relative to control

Table 8. The dehydrogenase enzyme activity of *Kocuria rhizophila* under metallic stress

Metals	**Cu**		**Zn**		**Ni**		**Cr**		**Quaternary**		**Control**
Time Intervals	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**	**C (%)**	**TF**
					10 mg/L						
4h	0.814	47.423	1.214	71.134	1.231	72.165	1.127	65.979	1.092	63.918	1.701
6h	1.475	42.424	2.432	70.202	2.484	71.717	2.624	75.758	2.832	81.818	3.459
10h	4.503	56.579	4.990	62.719	4.451	55.921	4.085	51.316	4.607	57.895	7.949
24h	4.433	46.863	4.590	48.524	4.381	46.309	4.016	42.435	4.642	49.077	9.446
30h	4.642	53.414	4.416	50.803	4.364	50.200	3.964	45.582	4.225	48.594	8.679
48h	4.695	68.622	4.399	64.286	4.329	63.265	3.842	56.122	4.189	61.224	6.823
					50 mg/L						
4h	0.709	42.105	0.918	54.737	1.022	61.053	1.092	65.263	0.988	58.947	1.666
6h	1.353	38.119	2.363	66.832	2.415	68.317	2.502	70.792	2.780	78.713	3.494
10h	4.329	54.148	4.973	62.227	4.346	54.367	3.998	50.000	4.520	56.550	7.984
24h	4.259	44.853	4.416	46.507	4.298	45.404	3.929	41.360	4.573	48.162	9.467
30h	5.043	58.739	4.782	55.691	4.920	57.317	3.842	44.715	4.103	47.764	8.575
48h	5.199	75.443	4.886	70.886	5.025	72.911	3.807	55.189	4.033	58.481	6.887
					100 mg/L						
4h	0.605	36.957	0.761	46.739	0.709	43.478	0.622	38.043	0.901	55.435	1.614
6h	1.039	28.922	1.109	30.882	2.379	66.667	2.449	68.882	2.711	75.980	3.563
10h	4.033	50.217	4.834	60.217	3.981	49.565	3.964	49.348	4.486	55.869	8.018
24h	3.824	58.318	4.346	45.521	3.877	40.585	3.894	40.768	4.364	45.704	9.533
30h	5.321	61.245	4.855	56.024	5.077	58.537	3.742	43.173	4.051	46.586	8.679
48h	5.408	78.283	5.008	72.475	5.164	72.911	3.685	53.283	3.998	57.828	6.905

Where: **(TF)** produced triphenyl formazan (µmol/ml), (**% of C)** TF yield determined relative to control

The Plackett-Burman design was applied to determine the most significant factors for estimating amounts of produced triphenyl formazan from aqueous solutions using bacterial isolates under metallic stress as indicators for the dehydrogenase enzyme. It was also used for the screening of significant variables along with the corresponding amounts of formazan. The relationship between the formed formazan and variables was determined by multiple-regression statistical analysis and the analysis of variance (ANOVA).

The ANOVA of the Plackett-Burman design showed that the model was highly significant, as was evident from a very low p-value and t-stat. The t and p-values were estimated for each independent variable as shown in Table 9, which showed the coefficient of each variable (the effect of that variable on enzyme activity as well as their p-values). In general, the p-value indicated the importance of each variable in the design. Whereas the higher the t-value and lower the p-value, the greater the significance and effect of the corresponding coefficient term on the response model, with a high correlation and significance less than 0.05. The fit of the model was checked by the determination of the coefficient (R^2). The largest R^2 was greater than 0.9 for Cr in *S. ginsenosidimutans* at all concentrations, then quaternary at 10 mg/L, as were at 10 and also 50 mg/L more than 0.75 (less than 0.9). Some isolates had a R^2 greater than 0.75 in *K. rhizophila* at all concentrations of Zn, Cr, and quaternary and at 10 and 50 mg/L of Cu and Ni, while it was less than 0.75 at 100 mg/L. As well, in *B. megaterium*, there was more than 0.75 at all concentrations of Ni, Zn, and quaternary and less than 0.75 at 10, 50 mg/L Cr, and 10 mg/L Cu. The results showed that there was no significant effect of variables on the enzyme activity of *S. ginsenosidimutans* with copper. In addition, in comparison of quaternary with individual metals, the results showed a strong relationship greater than 0.9, especially at 10 mg/L quaternary in *S. ginsenosidimutans*, *K. rhizophila*, and *B. megaterium*, which was considered to be very highly correlated, which means that the variation in the activity of enzymes can be explained by the model. In addition, the adjusted R^2 value was more than 0.8, which was too high to confirm the great significance of the model. The previous adjusted R^2 values greater than 0.75 and consistent with R^2 confirmed that the model is very important and appropriate for explaining the relationship between selected variants and enzyme activity in terms of formazan formation.

Table 9. Regression statistics of the Plackett–Burman design used for determining variables influencing dehydrogenase enzyme activity

Metals	**Cu**		**Zn**		**Ni**		**Cr**		**Quaternary**	
Parameters	**Iso1**	**Iso 2**	**Iso 3**	**Iso1**	**Iso 2**	**Iso 3**	**Iso1**	**Iso 2**	**Iso 3**	**Iso1**	**Iso 2**	**Iso 3**
10 mg/L												
R	0.815	-.946	0	0.944	0.914	0.951	0.949	0.911	0.956	0.862	0.981	0.945	0.969	0.976	0.949
R^2	0.664	-.894	0	0.891	0.836	0.905	0.900	0.830	0.915	0.744	0.963	0.893	0.938	0.953	0.901
Adj- R^2	0.580	-.868	0	0.863	0.795	0.881	0.875	0.788	0.893	0.680	0.953	0.867	0.923	0.941	0.876
t	2.813	-.822	5	5.706	4.511	6.160	5.998	4.426	6.547	3.407	10.138	5.789	7.806	8.966	6.038
P	0.048	> 0.05	0	0.005	0.011	0.004	0.004	0.011	0.003	0.027	0.001	0.004	0.001	0.001	0.004
50 mg/L												
R	-	-					0.922	0.928	0.911	0.846	0.994	0.949	0.971	0.931	0.953
	0.907			0.912	0.904	0.929									

R^2	-	-			0.851	0.860	0.715	0.988	0.943	0.866		
		0.823	0.832	0.817	0.831		0.901		0.907			
			0.863									
Adj- R^2	-	-	ɔoooo00n	0.890	0.814	0.826	0.644	0.986	0.929	0.833		
		0.779			0.788		0.876		0.884			
t	-	-			4.780	4.967	3.169	18.468	8.134	5.088		
		4.317	4.448	4.232	4.430		6.030		6.264			
			5.011									
P	·0.05	> 0.05	0		0.009	0.008	0.034	0.000	0.001	0.007		
		.012	0.011	0.013	0.011		0.004		0.003			
			0.007									
100 mg/L												
R	-	-	0.871	0.911	0.890	0.861	0.945	0.985	0.937	0.943		
		0.855	0.918		0.857		0.939		0.945			
R^2	-	-	0.758	0.829	0.792	0.741	0.893	0.970	0.878	0.889		
		0.731	0.842		0.734		0.882		0.894			
Adj- R^2	-	-	0.698	0.787	0.741	0.676	0.866	0.962	0.847	0.861		
		0.663	0.802		0.668		0.853		0.867			
t	-	-	3.542	4.409	3.908	3.379	5.782	11.367	5.359	5.651		
		3.294	4.616		3.324		5.480		5.805			
P	>0.05	> 0.05	0.024	0.012			0.004	0.000	0.006	0.005		
		0.030	0.010		0.017	0.028	0.005		0.004			
					0.029							

Where: **Iso 1** : *B megaterium*, **Iso 2** : *S ginsenosidimutans* and Iso **3**: *K rhizophila*

Estimation of Triphenyl Formazan (TF) Yield as an Indicator of the Ability to Reduce Sugars

The capacity of three bacterial isolates to decrease carbohydrates under metallic stress was tested at six time points along with the growth curve. Reducing sugars in an alkaline medium, are capable of reducing triphenyltetrazolium chloride, and the amount of formazan produced is proportional to the amount of reducing sugars present. The quantity of formazan generated is used to assess the capacity of bacteria to reduce sugars in the presence of metals. Figure 3 depicts the standard curve used to measure the reducing sugars of three bacterial isolates.

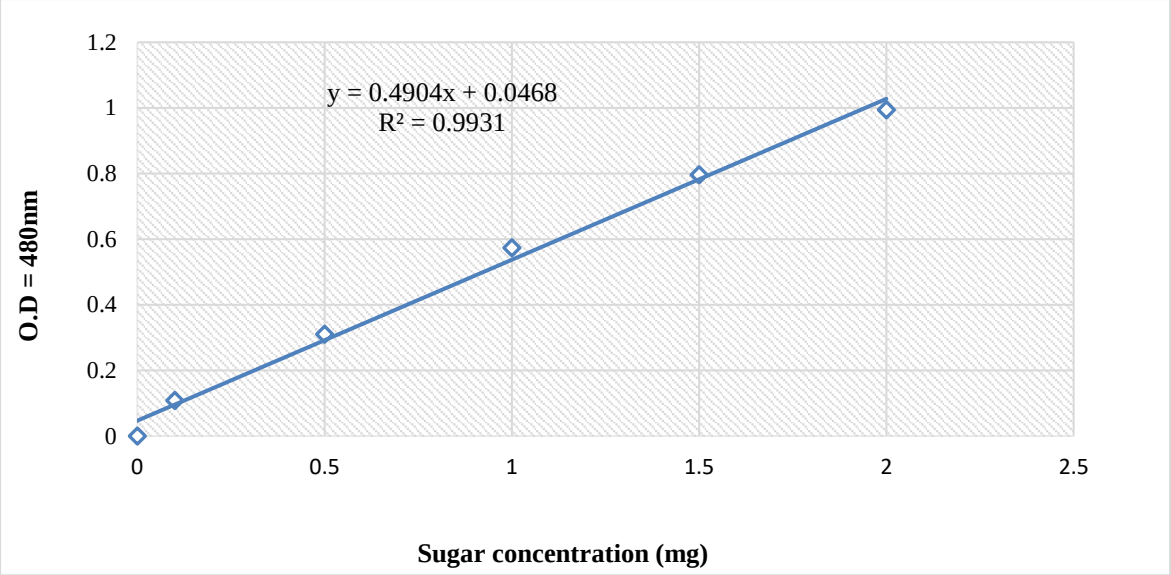


Figure 3. the standard curve for reduced sugar quantities (mg) versus absorbance

Determining the ability of bacterial isolates to reduce sugars under metallic stress was evaluated with three isolates at six time points in the growth curve as used to determine the denitrification process. Reducing sugars in an alkaline medium are capable of reducing triphenyltetrazolium chloride, and the quantity of formazan formed is proportional to the quantity of reducing sugar present. The amount of formazan formed used as an indicator for determining the ability of isolated bacteria to reduce sugar in the presence of metals. The results showed that the reduction power was gradually increasing throughout the exponential phase. The majority of the sugar was consumed within 6-18 hr. Bacterial isolates were able to reduce sugars progressively until entering the stationary phase. The results of reducing sugars under metallic stress are presented in Tables10-12.

Table 10. The amounts of Reduced Sugar with Tetrazolium Salts of *Bacillus megaterium* under metallic stress

Metals	**Cu**			**Zn**			**Ni**			**Cr**			**Quaternary**			**Control**		
Time Intervals	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**
								10 mg/L									
2h	6.298	0.0052	85.37	6.347	0.0054	86.04	5.808	0.0039	78.73	6.396	0.00547	86.71	6.249	0.0051	84.71	7.377	0.0075
4 h	9.289	0.0101	82.56	8.603	0.0093	76.46	10.614	0.0114	94.30	10.957	0.0117	97.39	11.006	0.0117	97.82	11.251	0.0119
6 h	18.656	0.0156	96.45	17.969	0.0151	92.89	16.744	0.0147	86.56	18.804	0.0153	97.19	18.607	0.0153	96.19	19.343	0.0155
10h	30.279	0.0172	63.36	37.389	0.0178	78.24	33.319	0.0175	69.73	31.603	0.0174	66.13	37.046	0.0178	96.92	47.786	0.0184

18h	55.633	0.0187	79.09	56.172	0.0187	79.85	57.888	0.0187	82.29	56.319	0.0187	80.06	58.329	0.0188	82.92	70.345	0.0190
24h	41.46	0.0018	63.79	44.990	0.0183	69.22	44.157	0.0182	67.93	45.579	0.0182	70.12	47.149	0.0184	72.54	64.999	0.0189
								50 mg/L									
2h	5.660	0.0035	78.29	5.857	0.0040	81.00	5.464	0.0029	75.58	6.249	0.00512	86.43	6.151	0.0048	85.08	7.230	0.0072
4 h	8.701	0.0094	77.67	8.162	0.0087	72.86	9.241	0.0100	82.49	9.927	0.0108	88.62	10.760	0.0115	96.05	11.202	0.0118
6 h	17.528	0.0149	92.26	17.283	0.0148	90.97	14.234	0.0137	74.97	17.823	0.0150	93.81	17.578	0.0149	92.52	18.999	0.0153
10h	28.808	0.0170	65.31	33.368	0.0175	75.65	29.004	0.0171	65.76	25.914	0.0167	58.75	32.584	0.0175	94.99	44.108	0.0182
18h	38.616	0.0179	54.93	46.021	0.0183	65.47	36.409	0.0178	51.79	54.358	0.0186	77.33	57.349	0.0187	81.58	70.296	0.0190
24h	26.503	0.0168	40.93	37.586	0.0179	58.04	30.524	0.0173	47.14	43.618	0.0182	67.34	46.266	0.0183	71.45	64.754	0.0189
								100 mg/L									
2h	5.562	0.0032	77.97	5.464	0.0029	76.62	5.170	0.0019	72.49	6.200	0.0050	86.93	5.611	0.0033	78.67	7.132	0.0070
4 h	8.013	0.0087	71.22	8.064	0.0086	71.67	7.230	0.0071	64.26	9.045	0.00984	80.39	9.829	0.0107	87.36	11.251	0.0119
6 h	16.842	0.0147	87.07	14.929	0.0139	77.18	12.772	0.0129	66.03	13.949	0.0135	72.11	15.469	0.0142	79.97	19.343	0.0155
10h	23.953	0.0164	53.07	27.630	0.0169	61.21	23.560	0.0163	52.19	21.403	0.0159	47.42	31.456	0.0174	89.03	45.138	0.0183
18h	37.537	0.0178	53.18	45.432	0.0182	64.36	36.017	0.0177	51.02	36.997	0.0178	52.41	54.995	0.0186	77.91	70.589	0.0190
24h	25.963	0.0167	40.22	35.918	0.0177	55.64	28.906	0.0171	44.78	31.358	0.0173	48.57	40.283	0.0180	62.39	64.558	0.0189

Where: **C** reduced sugar concentration (mg/L), **K** the reaction constant values, **C (%)** TF yield determined relative to control

Table 11. The amounts of Reduced Sugar with Tetrazolium Salts of *Sphingobacterium ginsenosidimutans* under metallic stress

Metals	**Cu**			**Zn**			**Ni**			**Cr**			**Quaternary**			**Control**	
Time Intervals	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**
												10 mg/L					
2h	7.524	0.0077	92.74	7.769	0.0081	95.76	7.671	0.0079	94.55	7.573	0.0077	93.34	7.868	0.0083	96.98	8.113	0.0086
4 h	41.117	0.0180	95.67	42.529	0.0181	98.97	41.656	0.0181	96.92	36.311	0.0178	84.48	37.194	0.0178	75.06	42.980	0.0182
6 h	48.129	0.0184	73.33	40.871	0.0181	62.27	44.648	0.0183	68.02	42.735	0.0181	65.11	60.537	0.0188	92.22	65.637	0.0189
10h	36.017	0.0177	64.63	39.498	0.0179	70.87	38.419	0.0179	68.94	37.144	0.0174	66.64	49.601	0.0185	89.00	55.731	0.0187
18h	16.106	0.0145	31.42	20.029	0.0156	39.07	17.528	0.0149	34.19	18.313	0.0152	35.72	43.519	0.0182	84.89	51.268	0.0185
24h	15.910	0.0144	32.11	16.744	0.0147	33.79	16.204	0.0145	32.70	14.880	0.0139	30.03	41.264	0.0181	83.27	49.552	0.0185
												50 mg/L					
2h	6.985	0.0067	87.15	7.573	0.0078	94.49	7.475	0.0076	93.26	7.328	0.0073	91.43	7.622	0.0079	95.09	8.015	0.0084

4 h	38.125	0.017	89.93	40.675	0.018	95.95	40.283	0.018	95.03	35.575	0.017	83.92	36.899	0.017	74.84	42.392	0.018
6 h	44.942	0.018	68.63	39.842	0.017	60.84	42.048	0.015	64.21	42.342	0.018	64.66	59.114	0.018	90.27	65.489	0.018
10h	33.221	0.017	59.87	35.771	0.017	64.47	35.526	0.017	64.03	32.486	0.017	58.55	47.198	0.018	85.06	55.485	0.018
18h	13.507	0.013	26.47	16.351	0.014	32.05	16.155	0.014	31.66	17.774	0.015	34.84	43.225	0.018	84.72	51.023	0.018
24h	12.772	0.012	25.90	13.458	0.013	27.29	13.311	0.013	26.99	12.968	0.013	26.30	40.822	0.018	82.79	49.306	0.018
												100 mg/L					
2h	6.739	0.006	85.65	7.230	0.007	91.89	7.279	0.007	92.51	7.083	0.006	90.02	7.377	0.007	93.76	7.868	0.008
4 h	37.439	0.017	89.88	40.430	0.018	97.06	39.302	0.017	94.35	33.368	0.017	80.10	36.654	0.017	74.49	41.656	0.018
6 h	44.059	0.018	67.33	39.155	0.017	59.83	41.705	0.015	63.73	39.989	0.018	61.11	58.232	0.018	88.98	65.441	0.018
10h	32.535	0.017	58.48	34.643	0.017	62.27	34.447	0.017	61.92	31.652	0.017	65.65	46.511	0.018	83.60	55.632	0.018
18h	11.791	0.012	23.19	14.145	0.013	27.83	13.654	0.013	26.86	17.185	0.014	33.81	42.882	0.018	84.37	50.827	0.018
24h	11.791	0.012	23.96	12.134	0.012	24.66	12.772	0.012	25.96	11.742	0.012	23.86	39.930	0.018	81.16	49.208	0.018

Where: **C** reduced sugar concentration (mg/L), **K** the reaction constant values, **C (%)** TF yield determined relative to control

Table 12. The amounts of Reduced Sugar with Tetrazolium salts in *Kocuria rhizophila* under metallic stress

Metals	**Cu**			**Zn**			**Ni**			**Cr**			**Quaternary**			**Control**	
Time Intervals	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**	**C (%)**	**C mg/L**	**K**
												10 mg/L					
2h	7.132	0.007	93.57	6.789	0.006	89.07	6.739	0.006	88.42	6.593	0.005	86.49	6.838	0.006	89.71	7.622	0.007
4 h	8.603	0.009	91.64	8.505	0.009	90.59	8.946	0.009	95.29	8.456	0.009	90.07	8.554	0.009	91.12	9.388	0.010
6 h	14.145	0.013	92.61	14.880	0.013	97.43	14.684	0.013	96.14	14.929	0.013	97.75	13.899	0.013	91.05	15.273	0.014
10h	24.002	0.016	49.97	25.473	0.016	53.03	24.149	0.016	50.28	25.179	0.016	52.42	25.718	0.016	53.54	48.031	0.018
18h	60.144	0.018	80.61	66.519	0.018	89.16	64.074	0.018	85.88	65.342	0.018	87.58	66.127	0.018	88.63	74.611	0.019
24h	57.790	0.018	85.50	63.969	0.018	94.70	59.065	0.018	87.44	63.528	0.018	94.05	64.509	0.018	95.49	67.549	0.018
												50 mg/L					
2h	6.887	0.006	90.94	6.642	0.006	87.71	6.543	0.005	86.39	6.396	0.005	84.46	6.691	0.006	88.35	7.573	0.007
4 h	8.456	0.009	91.99	8.309	0.008	90.39	8.750	0.009	95.19	8.211	0.008	89.33	8.358	0.008	90.93	9.192	0.010
6 h	10.025	0.010	64.81	13.262	0.013	85.73	14.291	0.013	92.38	14.389	0.013	93.02	13.409	0.013	86.68	15.469	0.014
10h	23.855	0.016	50.54	23.806	0.016	50.44	23.560	0.016	49.92	24.099	0.016	51.06	25.277	0.016	53.56	47.198	0.018

18h	43.225	0.0182	58.09	47.688	0.0184	64.09	38.468	0.0179	51.69	57.104	0.0187	76.73	61.125	0.0188	82.14	74.415	0.0191
24h	37.144	0.0178	55.11	42.588	0.0182	63.16	37.978	0.0179	56.35	41.215	0.0181	61.15	48.570	0.0184	72.06	67.402	0.0189
												100 mg/L					
2h	6.691	0.0061	88.93	6.593	0.0059	87.63	6.249	0.0051	83.05	6.298	0.0052	83.71	6.543	0.0058	86.96	7.524	0.0077
4 h	8.259	0.0088	90.33	7.966	0.0084	87.13	8.603	0.0093	94.09	7.475	0.0076	81.76	8.064	0.0086	88.19	9.143	0.0099
6 h	9.388	0.0102	61.08	12.821	0.0129	83.41	13.752	0.0135	89.47	12.330	0.0127	80.22	13.164	0.0131	85.64	15.371	0.0142
10h	22.972	0.0162	48.02	23.364	0.0163	48.84	22.629	0.0162	47.31	23.757	0.0163	49.66	24.198	0.0164	50.59	47.835	0.0184
18h	42.441	0.0181	56.92	47.002	0.0183	63.04	38.027	0.0178	51.00	55.584	0.0186	74.54	58.183	0.0188	78.03	74.562	0.0191
24h	36.017	0.0177	53.39	38.860	0.0179	57.61	37.292	0.0178	55.29	40.773	0.0181	60.45	43.618	0.0182	64.67	67.451	0.0189

Where: **C** reduced sugar concentration (mg/L), **K** the reaction constant values, **C (%)** TF yield determined relative to control

Increased metal concentrations reduced sugar levels but did not inhibit them. The reaction constant values were highest at 10 mg/L and reduced with increasing metal concentrations. Reduction rates were affected by increasing metal concentrations and time as a result of a functional defect in the oxidative phosphorylation process responsible for the reduction of sugars (Fashola *et al*., 2016). It was clear that as time passed, reduced sugar amounts decreased while metal concentrations increased. It has been reported that glucose plays a functional role in bacterial viability, benefiting cellular envelope survival and reducing the effects of stressors such as Cu (Zahri *et al*., 2021), and that high levels of metals can impair the decomposition of organic matter (Fashola *et al*., 2016).

The Plackett–Burman Design (PBD) was used to detect the significant variables that influence the amounts or concentrations of reduced sugar and the reaction constant values under metallic stress. The multiple regression analysis of the model and the analysis of variance (ANOVA) are presented in Tables 13 and 14. The results of the multiple regression analysis by the Plackett-Burman Design (PBD) and the analysis of variance (ANOVA) showed that in a regression model with a determination coefficient R^2 value higher than 0.9 and 0.8 for all concentrations of *B. megaterium* and *K. rhizophila*, there was a very high correlation. That R^2 value should not be less than 0.75 until the model is appropriate. While there was no significant effect of variables on the concentration of reduced sugar and no significant differences between individual metals in *S. ginsenosidimutans*, the R^2 value was found to be greater than 0.9 only with quaternary, and Adj-R^2 was also very high, indicating the fitness of the model for the experimental data with quaternary. The greatest values of R^2 were recorded, and adjusted R^2 for the reaction constant values reached 0.999–1.000 with quaternary at all concentrations,

more than 0.9 with Cu, Zn, and Ni, and more than 0.8 with Cr at 50 and 100 mg/L in *K. rhizophila*. As well, *B. megaterium* was greater than 0.9 with Zn, Ni, Cr, and quaternary, whereas there was no significant effect of variables on the reaction constant values with Cu. These values were lower in *S. ginsenosidimutans*; it was greater than 0.75 at 10 mg/L of all individual metals, 50 mg/L of Cr, Ni, and Zn, and 100 mg/L of Cr, but less than 0.75 at 100 mg/L of Cu, Zn, and Ni. Because the R^2 value should not be less than 0.75, for the model to be appropriate, high values of the previous results indicate high significance for the model and good agreement.

Table 13. Regression statistics of the Plackett–Burman design used for determining variables influencing reduced sugar concentration

Metals	**Cu**		**Zn**		**Ni**	**Cr**		**Quaternary**	
	Iso1	**Iso 2**	**Iso1**	**Iso 2**	**Iso1 Iso 3**	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**
Parameters	**Iso 3**		**Iso 3**			**Iso 3**		**Iso 3**	
					10 mg/L				
R	0.976	-	0.992	-	0.986 -	0.986	-	0.993	0.995
	0.962		0.960		0.960	0.960		0.962	
R^2	0.953	-	0.984	-	0.971 -	0.972	-	0.985	0.989
	0.926		0.922		0.921	0.921		0.925	
Adj- R^2	0.942	-	0.980	-	0.964 -	0.965	-	0.981	0.987
	0.907		0.903		0.902	0.902		0.906	
t	9.028	-	15.526	-	11.639 -	11.759	-	16.294	19.229
	7.060		6.899		6.845	6.849		7.018	
P	0.001		0.000		0.000 > 0.05	0.000		0.000	0.000
	> 0.05		> 0.05		0.002	> 0.05		0.002	
	0.002		0.002			0.002			
					50 mg/L				
R	0.935	-	0.983	-	0.976 -	0.980	-	0.992	0.996
	0.993		0.985		0.989	0.964		0.973	
R^2	0.875	-	0.966	-	0.953 -	0.961	-	0.985	0.992
	0.987		0.971		0.978	0.930		0.946	
Adj- R^2	0.844	-	0.958	-	0.942 -	0.951	-	0.981	0.990
	0.984		0.964		0.972	0.912		0.933	
t	5.286	-	10.668	-	9.042 -	9.866	-	16.027	22.710
	17.333		11.606		13.279	7.286		8.383	
P	0.006	0.05	0.000		0.001 > 0.05	0.001		0.000	0.000
	0.000		> 0.05		0.000	> 0.05		0.001	
			0.000			0.002			
					100 mg/L				
R	0.953	-	0.991	-	0.991 -	0.991	-	0.984	0.996
	0.991		0.984		0.987	0.969		0.967	

R^2	0.905 - 0.982		0.983 - 0.968		0.983 - 0.974		0.982 - 0.939		0.969 0.991 0.936	
Adj- R^2	0.885 - 0.978		0.978 - 0.960		0.978 - 0.968		0.978 - 0.923		0.961 0.989 0.920	
t	6.273 - 14.912		15.070 - 10.950		15.096 - 12.286		14.868 - 7.825		11.120 21.273 7.650	
P	0.003 > 0.05 0.000		0.000 > 0.05 0.000		0.000 > 0.05 0.000		0.000 0.05 0.001		0.000 0.000 0.002	

Where: **Iso 1** *B megaterium*, **Iso 2** *S ginsenosidimutans* and **Iso 3** *K rhizophila*)

Table 14. Regression statistics of the Plackett–Burman design used for determining variables influencing the reaction constant values

	Cu		**Zn**		**Ni**		**Cr**		**Quaternary**	
Metals	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**	**Iso1**	**Iso 2**
Parameters	**Iso 3**		**Iso 3**		**Iso 3**		**Iso 3**		**Iso 3**	
				10 mg/L						
R	- 0.991	0.904	0.995 0.991	0.930	0.991 0.988	0.911	0.993 0.992	0.913	0.994 0.994	1.000
R^2	- 0.982	0.817	0.991 0.983	0.865	0.982 0.977	0.830	0.987 0.984	0.833	0.988 0.987	0.999
Adj- R^2	- 0.978	0.771	0.988 0.979	0.832	0.978 0.971	0.788	0.983 0.980	0.792	0.985 0.984	0.999
t	- 14.930	4.226	20.512 15.171	5.072	14.868 12.990	4.420	17.287 15.721	4.474	17.922 17.598	70.446
P	>0.05 0.000	0.013	0.000 0.000	0.007	0.000 0.000	0.012	0.000 0.000	0.011	0.000 0.000	0.000
				50 mg/L						
R	0.990 0.982	0.854	0.995 0.995	0.873	0.996 0.989	0.869	0.995 0.991	0.889	0.995 0.995	1.000
R^2	0.981 0.964	0.730	0.990 0.989	0.762	0.991 0.978	0.755	0.990 0.981	0.790	0.990 0.989	1.000
Adj- R^2	0.976 0.955	0.663	0.987 0.987	0.703	0.989 0.973	0.694	0.988 0.977	0.737	0.987 0.987	1.000
t	14.372 10.364	3.289	19.627 19.207	3.581	21.127 13.459	3.514	19.927 14.456	3.876	19.469 19.282	234.537
P	0.000 0.000	0.030	0.000 0.000	0.023	0.000 0.000	0.025	0.000 0.000	0.018	0.000 0.000	0.000
				100 mg/L						
R	0.992 0.975	0.824	0.999 0.996	0.837	0.997 0.988	0.846	0.997 0.996	0.873	0.996 0.994	1.000
R^2	0.984 0.950	0.679	0.998 0.993	0.701	0.995 0.976	0.715	0.994 0.992	0.762	0.993 0.989	1.000
Adj- R^2	0.980 0.938	0.599	0.997 0.991	0.627	0.993 0.969	0.644	0.992 0.989	0.702	0.991 0.986	1.000
t	15.869 8.757	2.910	43.263 23.210	3.065	27.186 12.622	3.167	25.397 21.670	3.574	23.387 18.560	118.459
P	0.000 0.001	0.044	0.000 0.000	0.037	0.000 0.000	0.034	0.000 0.000	0.023	0.000 0.000	0.000

Where: **Iso 1** *B megaterium*, **Iso 2** *S ginsenosidimutans* and **Iso 3** *K rhizophila*)

Conclusion

Living cells can use both organic and inorganic materials through metabolic activities. In polluted environments, there is usually a mixture of organic and inorganic pollutants. The inorganic pollutants, such as heavy metals, may be used as an energy source through their own metabolic processes. However, excessive amounts of inorganic substances cause inhibition of metabolic activity, which affects viability and impedes the processing of metallic pollutants. Therefore, the metabolic and enzymatic activities of bacteria isolated from the electroplating effluent in the presence of Cu, Zn, Ni, and Cr were tested individually and quaternary during bacterial growth periods. Respiratory enzymes responsible for metabolic activity in bacteria, such as catalase and dehydrogenase enzymes, and sugar reduction activity were estimated. The results showed that respiratory metabolism activity was more in the exponential phase compared to the stationary phase under metallic stress. High metal concentrations had a more detrimental impact on the function of the dehydrogenase enzyme than they did on the catalase enzyme or the reduction of glucose. Dehydrogenase was more sensitive to high metal concentrations that led to damage to cellular components over time. Respiratory metabolism activity associated with bacterial metabolism persisted under metal stress, even as metal concentrations increased. It can be concluded that catalase enzyme action is one of the tools that metal-resistant bacteria rely on to resist metal stress; however, high levels of metal with progressing stages of growth can impair the metabolism in living cells. The isolates that were utilized proved to maintain and sustain metabolic activity under metallic stress, despite increased metal concentrations, which in turn helped the bacteria reduce or mitigate metallic pollutants.

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