ChemBioNexus

ISSN: 2024-2025
ISSN: 2024-2026

Eco-friendly Copper Remediation through Optimized Biosorption by Industrial Effluent Bacteria

Fatima Mustafa[*], Minahil Fatima, Qamar Abbas
Department of Biology, Lahore Garrison University, Faculty of Basic Sciences, Lahore

[*]**Correspondence**
Email: fatimamustafa024@gmail.com

DOI:
Received: 12th May, 2025
Revised: 16th July, 2025
Accepted: 6th August, 2025

Keywords:
Minimum inhibitory concentration
Biosorption
Wheat seeds
Pseudomonas
Salmonella

Abstract
Wastewater samples collected from the industrial area of Kot Lakh pat, Lahore, exhibited temperatures ranging from 35°C to 45°C and pH values between 8 and 9. A total of seven morphologically distinct bacterial isolates (ISO1-ISO7) demonstrating copper resistance were obtained from these samples. Minimum inhibitory concentration (MIC) analysis revealed variable tolerance levels, with ISO7 showing the highest resistance at 450 µg/ml, followed by ISO3 (410 µg/ml) and ISO6 (400 µg/ml). These three isolates were selected for further characterization. Physiological studies indicated optimal growth at 37°C and pH 9 for ISO3, and 45°C and pH 7 for ISO6 and ISO7. Biosorption assays demonstrated that temperature, pH, and incubation time significantly influenced copper uptake. Maximum Cu^{+2} biosorption occurred at 25°C for ISO7 and at 37°C for ISO3 and ISO6, with peak uptake observed at pH 9 for ISO3 and ISO6 and at pH 7 for ISO7 after 48 h incubation. Biochemical profiling identified ISO3 as *Enterococcus*, ISO6 as *Pseudomonas*, and ISO7 as *Salmonella*. Growth promotion studies on wheat seeds revealed that all three isolates enhanced root and shoot lengths compared to controls, while co-application with copper mitigated the inhibitory effects of metal stress on germination. Overall, these results highlight the potential of copper-resistant bacteria for biosorption applications and plant growth promotion under metal stress, offering a sustainable approach for bioremediation of industrial effluents.

Introduction

Copper (Cu) is a vital element for numerous biological functions, ranging from small bacteria to humans. It plays a crucial role in regulating harmful substances and supporting essential cellular processes, such as oxidative phosphorylation and photosynthesis[1,2]. Historically, copper has been extensively utilized in plumbing, manufacturing, and the preservation of perishable goods[3]. However, excessive copper presence can lead to toxicity, prompting regulatory guidelines to be established. In 1991, the Environmental Protection Agency (EPA) set a maximum contaminant level goal (MCLG) of 1.3 mg/L for copper in drinking water[4]. The environmental impact of copper, especially in agricultural practices, has resulted in its infiltrioninto water bodies, posing detrimental consequences[5].

Apart from causing nutritional imbalances, soil copper contamination poses ecological risks, impacting plants, edaphic fauna, microorganisms, and overall ecosystem functions[6,7]. Excessive copper in topsoil from anthropogenic sources can induce toxicity in non-tolerant plants, affecting growth or causing death. Studies have reported a strong tendency for copper accumulation in plant roots, known as excluder plants, which can mitigate herbivore risks and human poisoning, though not eliminate all risks[8,9]. Hyper accumulator plants, found in environments with high copper soil levels, can accumulate

copper levels exceeding 300 mg kg^{-1} in leaves[10]. Because copper may infiltrate water bodies, bio accumulate in fish and other aquatic animals, and migrate up the food chain, potentially affecting higher trophic levels, copper contamination also poses a threat to aquatic ecosystems[11,12]. Chronic copper poisoning has been observed in copper-sensitive species such as sheep, where a progressive accumulation of copper in the liver (usually from eating soil particles loaded with copper on grass) causes serious health problems such as hemolysis, anemia, jaundice, and hemoglobinuria[13–16].

Chemical, physical, and biological treatments have all been suggested and used in a variety of ways to remediate heavy metal contamination in various contexts[17]. Precipitation, ion exchange, electrolysis, chemical extraction, leaching, hydrolysis, polymer microencapsulation, excavation, and landfilling are examples of conventional technologies. Despite being widely employed, these chemical techniques' toxicity and potential for mutagenesis effects present serious concerns to human health and the environment. In order to remove heavy metal ions from contaminated sites, remediation approaches such as vapor extraction, stabilization, and solidification, vitrification, and membrane technology have been investigated[16,18]. However, the implementation of many of these technologies on a large scale is hindered by their considerable expense and the challenges associated with continuous monitoring and control, as they may not eliminate heavy metalcontaminants[17,19]. Recognizing the need for innovative treatment technologies, Medfu *et al*., (2020)[19] emphasized the demand for alternative methods to remove heavy metal ions from wastewater. To address this,bioremediation emerges as a wise solution, employing living organisms, particularly microorganisms, to activate metabolic networks and mitigate the presence of hazardous pollutants. This environmentally sustainable approach is recognized for its potential in cleansing contaminated environments[2]. Microbes, with their widespread presence and rapid growth, demonstrate adaptability to heavy metal ions in contaminated sites. Through interactions with metal ions, microbes develop tolerance and resistance strategies. Understanding the genetic and resistance mechanisms against heavy metals has contributed significantly to addressing heavy metal pollution[20].

The objectives of the current study was to isolate and distinguish copper-resistant bacteria from industrial effluents. The research aimed to determine the minimum inhibitory concentration of copper for the isolated bacteria. Additionally, the study seeked to optimize copper biosorption in culture media by exploring various parameters, including temperature, pH, and incubation time. Furthermore, the heavy metal stress alleviation ability of the isolated bacteriaon the germination of wheat seeds was also studied.

Materials and Methods

Sample Collection

Wastewater samples were collected from the industrial area of Kot Lakh pat, Lahore, Punjab. All the samples were obtained in sterile autoclaved containers and were safely moved to the laboratory for processing. The physicochemical characteristics (temperature and pH) of the wastewater samples were noted at the time of sampling by using a thermometer and litmus paper. These samples were stored at 4°C until processed for isolation of Copper-resistant bacteria.

Isolation of Copper-resistant bacteria

The wastewater samples were directly spread on the $CuSO_4.7H_2O$ supplemented nutrient agar plates. Wastewater samples were directly spread on the plates. The plates were kept in the incubators for 24 hours at 37ºC. Morphologically different colonies were selected and purified on $CuSO_4.7H_2O$ supplemented plates through quadrant streaking.

Morphological Characterization of bacterial isolates

The morphological characteristics including colony, cell and spore morphology was studied following Cappuccino and Sherman, 2013[21].

Estimation of Minimum Inhibitory Concentration for Copper (Cu^{+2})

The lowest concentration of Cu^{+2} known as minimum inhibitory concentration (MIC) to inhibit the visible growth of bacterial isolates was determined by using an Agar-Plate method. MIC experiment was performed on nutrient agar plates. Nutrient agar plates with varying concentrations (25 to 1000 µg/ml) of Cu^{+2} supplementation were prepared. Fresh cultures of bacterial isolates were streaked under the aseptic condition on the Cu^{+2} supplemented plates. The streaked plates were incubated for 24 hours at 37ºC in to determine the MIC values of Cu^{+2} for each bacterial isolate[22].

Determination of optimum temperature and pH for bacterial growth

The optimum temperature of each isolate was investigated. The three media sets were prepared and

autoclaved. Overnight bacterial cultures were prepared and the optical density of all the bacterial cultures was adjusted to 0.5 (OD_{600} = 0.5) to standardize the bacterial cultures. The inoculum of each isolate was given in the tubes and kept in the shaking incubator at 100 rpm for 24 hours set to various temperatures (20, 37, and 45ºC). For the determination of optimum pH, the nutrient broth was prepared with three different pH values (*i.e.,* 5, 7, and 9). The media was added into the tubes and autoclaved. Overnight bacterial cultures were prepared and the optical density of all the bacterial cultures was adjusted to 0.5 (OD_{600} = 0.5) to standardize the bacterial cultures. The inoculum of each isolate was given in the tubes and kept in the shaking incubator at 100 rpm for 24 hours at the optimum temperature. Growth was observed using spectrophotometer [22].

Optimization of different Physiological parameters for Copper biosorption[23]

Different physiological parameters (temperature, pH, and incubation time) were optimized for copper biosorption in nutrient broth.

Effect of Temperature on Cu^{+2} biosorption

The temperature of the environment has a great impact on bacterial growth and physiology. Any fluctuation in temperature gradually affects both. Three different temperatures like 25, and 37 and 45ºC were selected to determine the optimized temperature for the biosorption of Cu^{+2} by bacteria. Nutrient broth was prepared (100 ml per flask) and autoclaved. Overnight bacterial cultures were prepared and the optical density of all the bacterial cultures was adjusted to 0.5 (OD_{600} = 0.5) to standardize the bacterial cultures. The nutrient broth in each flask was supplemented with 100 µg/ml Cu^{+2} stress and inoculated with standardized bacterial cultures in their respective flasks. 1% bacterial inoculum was added in each experimental flask and no inoculum was added in control flasks. After inoculation, the flasks were incubated at three different temperatures (25 and 37 and 45ºC) at 100 rpm. After 24 hours of incubation, growth was observed in flasks and then cultures were centrifuged (*HERMLE, Z 216 MK*) at 5000 rpm for 25 minutes. After 25 minutes, supernatants were collected and stored in a refrigerator at 4ºC. These samples were further used for the estimation of the Cu^{+2} through Atomic Absorption Spectrophotometry (AAS).

Effect of pH on Cu^{+2} biosorption

The pH value is essential for microbial growth. It can affect the biosorption ability of bacterial isolates. To determine the effect of pH on the copper biosorption ability of each bacterial isolate, the experiment was performed to evaluate the optimum pH for biosorption potential of bacteria. To find the best pH for the biosorption of Cu^{+2} by the selected bacterial isolates, three different pH values 5, 7, and 9 were selected. After preparing the nutrient broth, media was poured into flasks (100 ml per flask), and pH values were adjusted through a pH meter (*Inno Tech*). All flasks were properly covered and then autoclaved. Overnight bacterial cultures were prepared and the optical density of all bacterial cultures was adjusted to 0.5 (OD_{600} = 0.5) to standardize the bacterial cultures. Nutrient broth-containing flasks were supplemented with 100 µg/ml Cu^{+2} stress and then inoculated with the standardized bacterial cultures in their respective flasks. 1% bacterial inoculum was added in each experimental flask and control flasks were without any inoculum. After inoculation, the flasks were incubated at their optimized temperatures in a shaking incubator at 100 rpm for 24 hours. After incubation time, growth was observed in flasks and then cultures were centrifuged at 5000 rpm for 25 minutes. The same procedure was done for AAS, after the centrifugation, as described above.

Effect of Incubation time on Cu^{+2} biosorption

The increase in incubation time primarily affects the biosorption ability and bacterial growth. The experiment was carried out to evaluate the optimum time period for the growth of bacteria. For the biosorption of Cu^{+2} by the selected bacteria, two time periods were selected (24 hours and 48 hours), to determine the optimized incubation time. For this experiment, nutrient broth was prepared poured in flasks (100 ml per flask), and autoclaved after adjusting the pH at optimum value. Overnight bacterial cultures were prepared in test tubes and the optical density of all the bacterial cultures was adjusted to 0.5 to standardize the bacterial cultures. The nutrient broth in the flasks was supplemented with 100 µg/ml of Cu^{+2} stress and then inoculated with standardized bacterial cultures in each flask. 1% bacterial inoculum was added in each flask and no inoculum was given in the control flask. After inoculation, all flasks were incubated at their optimum temperature in a shaking incubator at 100 rpm. Bacterial culture was poured into 50 ml falcon tubes for centrifugation, cultures were centrifuged at 5000 rpm for 25 minutes after the completion of their incubation time periods. Supernatants were collected and stored in a refrigerator at 4°C. To estimate the Cu biosorption

potential of all bacterial isolates these supernatants were further used in AAS.

Estimation of Cu^{+2} biosorption through Atomic Absorption spectrophotometry

Absorption Spectrophotometry is an analytical technique that is used for detecting trace metals quickly, it is predicated on ground state atoms in a flame or an electro thermal graphite furnace absorbing light with wavelengths peculiar to a given element. The Atomic Absorption Spectrophotometry was performed at the School of Biochemistry and Biotechnology, University of the Punjab, Lahore. Five different calibrating standard stock solutions of different concentrations (5 ppm to 25 ppm) were prepared by using $CuCl_2$ salt under sterile conditions to perform the AAS. All the experimental samples were arranged to analyze the concentration of Cu^{+2} in the samples. A hollow cathode lamp of Cu^{+2} was used in the Atomic Absorption Spectrophotometer (*GBC Xplor AA*) to detect the concentration of Cu^{+2} in samples. Absorbance values of the samples were taken and were further used to calculate the concentration of Cu^{+2}.

Biochemical characterization of selected bacterial isolates

The biochemical characterization of the bacterial isolates was performed following the Cappuccino and Sherman, 2013[21]

Cu^{+2} stress alleviation ability of bacterial isolates on wheat seeds germination

Viability of wheat seeds

Wheat seeds were purchased from a local market and checked for their viability. To assess the viability of the wheat seeds, a seed germination test was performed according to the methodology of Afzal *et al.,* (2015)[24]. Wheat seeds were thoroughly rinsed in 70% ethanol for one minute before being treated in 20% commercially available bleach for 10 minutes. The seeds were then rinsed ten times with autoclaved distilled water to thoroughly eliminate the leftover chemicals. Wheat seeds were dipped for 30 minutes in a 0.03 M solution of magnesium sulphate after washing. With the aid of sterile forceps, seeds were put in petri plates lined with sterilized filter paper and supplemented with 5 ml autoclaved distilled water. Petri plates were kept in the dark at room temperature for 7 days to observe for seeds germination.

Evaluation of the combined effect of Copper and bacteria on wheat seed germination

Following the procedure of Afzal *et al.,* (2015)[24], the combined impact of Cu^{+2} and bacterial isolates was investigated. The bacterial isolates were cultured in nutrient broth for 24 hours, and placed in a shaking incubator. After incubation, bacterial cells were extracted via centrifugation. The pellet was washed twice with 0.03 M solution of $MgSO_4$ and the supernatant was discarded. The washed pellet was re-suspended to an O.D of 0.5 at 600 nm (OD_{600}=0.5). The suspensions were then applied to sterilized seeds. Following washing, the seeds were submerged for 20-30 minutes in bacterial solutions. Autoclaved soil was taken in the pots. A total of 8-10 wheat seeds were added to the pot soil. Wheat seeds treated only with $MgSO_4$ were used as a control. Autoclaved distilled water and copper sulphate at a concentration of 25 µg/ml were used for watering these seeds for 5-7 days in the dark. The experiment was run in replicates. The seeds were germinated and the length of the root and shoot, no of leaves, and no of roots were observed (table 1).

Statistical analysis

All the experiments were carried out in replicates and results were analyzed by applying the statistical analysis. Results were determined by calculating the average values and arranged in tabulated form. Standard deviation was determined for plotting of graphs and represented with error bars in graphs. The error bars were added by using Microsoft Excel.

Results

Physicochemical characteristics of wastewater samples

Wastewater samples were collected from the industrial area of Kot Lakhpat, Lahore, Punjab. The physicochemical characteristics (temperature and pH) of the wastewater samples were noted at the time of sampling. It was found that the temperature for wastewater samples was from 35 to 45°C and the pH ranged from 8 to 9 as given in Table 2 below

Isolation of Copper-resistant bacteria

A total of 07 bacterial isolates that showed resistance against copper were selected based on the basis of differences in the morphology of bacterial colonies (Table 3).

Morphological characteristics of bacterial isolates

The colony characteristics of the selected bacterial

isolates have been given in table 4 below.

Table 1: Experimental design of copper, bacterial isolates, and wheat seeds germination

Sr. No.	Experimental groups	Seeds + bacterial combination	Dousing of seeds
1	Group 1	Seeds	Sterile distilled water
2	Group 2	Seeds	$CuSO_4.7H_2O$ solution (25 µg/ml)
3	Group 3	ISO3 +Seeds	Sterile distilled water
4	Group 4	ISO6 +Seeds	Sterile distilled water
5	Group 5	ISO7 + Seeds	Sterile distilled water
6	Group 6	ISO3 +Seeds	$CuSO_4.7H_2O$ solution (25 µg/ml)
7	Group 7	ISO6 +Seeds	$CuSO_4.7H_2O$ solution (25 µg/ml)
8	Group 8	ISO7 + Seeds	$CuSO_4.7H_2O$ solution (25 µg/ml)

Table 2: Physicochemical characteristics of wastewater samples

Sample codes	pH	Temperature (ºC)
K1	8	35
K2	9	37
K3	9	45

Table 3: Selected bacterial isolates along with their codes and number

Sample codes	No. of the colonies selected	Codes for the Colonies
K1	3	ISO1, ISO2, ISO3
K2	2	ISO4, ISO5
K3	2	ISO6, ISO7

Table 4: Morphological characteristics of bacterial isolates

Isolates	Form	Consistency	Size	Pigments	Margin	Elevation
ISO1	Circular	Dry	Moderate	Orange	Entire	Convex
ISO2	Irregular	Dry	Moderate	White	Entire	Convex
ISO3	Irregular	Dry	Large	Off white	undulate	Umbonate
ISO4	Irregular	Buttery	Moderate	yellow	Entire	Convex
ISO5	Circular	Dry	Moderate	Off white	Entire	Convex
ISO6	Circular	Buttery	Moderate	Yellow	Entire	Convex
ISO7	Circular	Buttery	Moderate	Off white	Undulate	Convex

Minimum inhibitory concentration (MIC) of Cu^{+2} for bacterial isolates

The MIC values for Cu^{+2} have been shown in figure 1. MIC value for each bacterial isolate was completely different from each other. ISO7 showed the highest MIC value which is equal to 450 µg/ml. The bacterial isolates ISO1, ISO2, ISO4, and ISO5 showed values equal to 290 µg/ml, 350 µg/ml, 380 µg/ml and 395 µg/ml respectively. The bacterial isolates ISO6 and ISO3 showed values equal to 400 µg/ml and 410 µg/ml respectively. Based on MIC values, three bacterial isolates, namely ISO3, ISO6, and ISO7 were selected for further studies due to their highest resistance ability against Cu^{+2}.

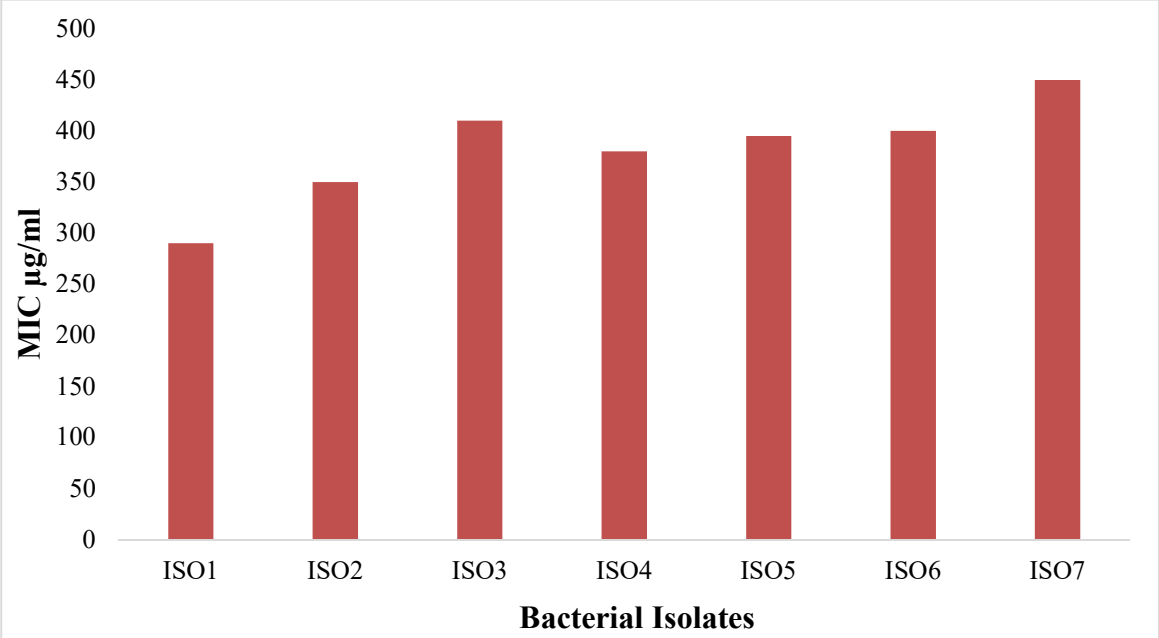

Figure 1: Minimum inhibitory concentration of bacterial isolates for Cu^{+2}

Physiological Characterization of bacterial isolates

The pH 7 was found to be the optimum pH for the selected two bacterial isolates (ISO6 and ISO7) and the optimum pH for isolate ISO3 was 9. Similarly, 45°C was found to be the optimum temperature for the selected two bacterial isolates (ISO6 and ISO7) and the optimum temperature for isolate ISO3 was 37°C (figures 2 and 3).

Physiological optimization of bacterial isolates for Cu^{+2} biosorption

Effect of temperature on Cu^{+2} biosorption

The copper biosorption potential of all the bacterial isolates was studied at different temperatures *i.e.,* 25°C, 37°C, and 45°C. According to the results, ISO3 absorbed 73.54 µg/ml, 77.71 µg/ml, and 69.83 µg/ml at 25°C, 37°C, and 45°C respectively. ISO6 absorbed 74.82 µg/ml, 76.73 µg/ml, and 72.72 µg/ml, while, ISO7 absorbed 80.87 µg/ml, 76.73 µg/ml, and 72.62 µg/ml at 25°C, 37°C, and 45°C respectively. The results showed that ISO3 and ISO6 showed maximum copper biosorption potential at 37°C while ISO7 showed maximum copper (Cu^{+2}) biosorption at 25ºC (Figure 4).

Effect of pH on Copper biosorption:

After checking the copper biosorption at different temperatures, the biosorption potential of bacteria at different pH (5, 7, and 9) was also studied. This was done to determine the optimum pH for bacterial isolates for copper biosorption. After incubation for 24 hours at optimum temperatures (37°C for ISO3 and ISO6, and 25°C for ISO7), Cu^{+2} biosorption was estimated using AAS. According to the results, ISO3 absorbed 82.84 µg/ml, 84.14 µg/ml, and 86.56 µg/ml of Cu^{+2} at pH 5, 7, and 9 respectively. The bacterial isolate ISO6 absorbed 76.29 µg/ml, 80.79 µg/ml, and 84.22 µg/ml of Cu^{+2} at pH 5, 7, and 9 respectively while, the bacterial isolate ISO7 absorbed 88.36 µg/ml, 84.03 µg/ml and 80.87 µg/ml of Cu+2 at pH 5, 7, and 9 respectively. The results revealed that ISO3 and ISO6 showed maximum copper biosorption at a pH 9 while ISO7 at pH 7 (Figure 5).

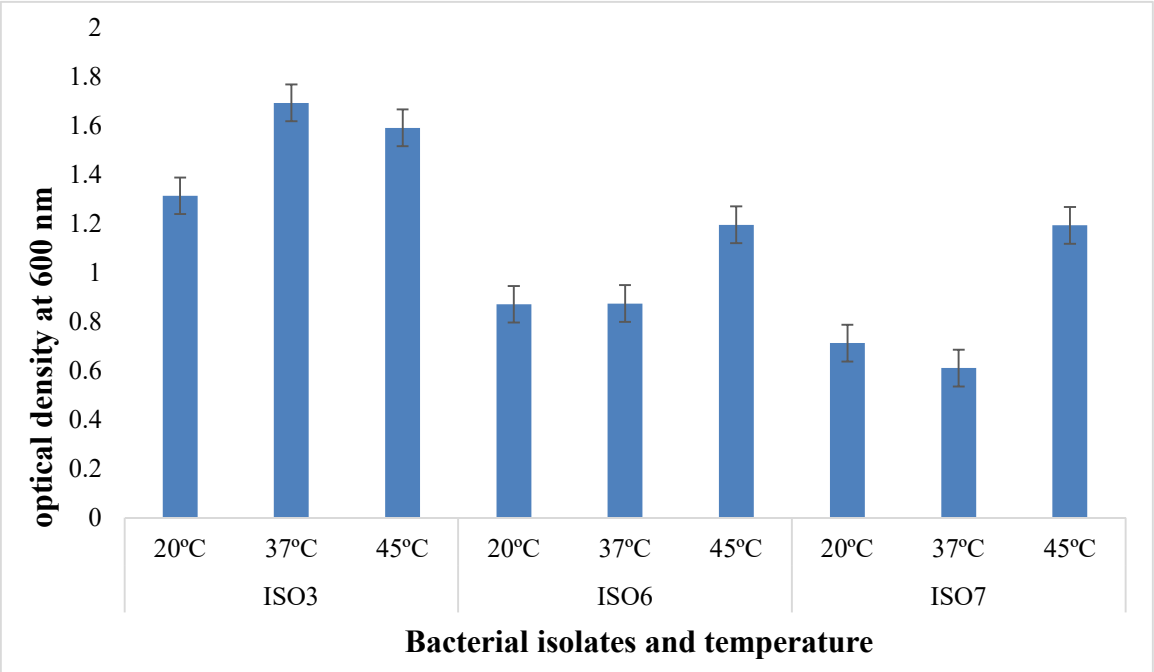

Figure 2: Optimum growth temperature for the bacterial isolates, ISO3, ISO6 and ISO7

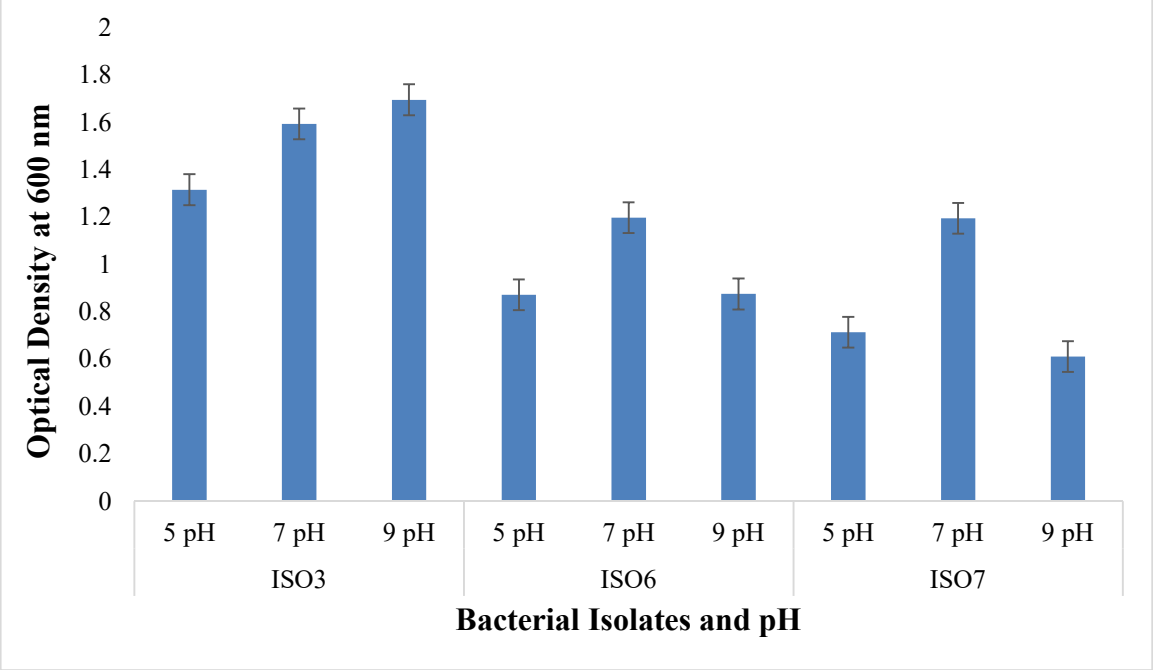

Figure 3: Optimum pH for the growth of bacterial isolates, ISO3, ISO6 and ISO7

Effect of Incubation time on copper (Cu^{+2}) biosorption:

Incubation time significantly affects growth as well as the copper (Cu^{+2}) biosorption potential of the bacterial isolates. AAS was done to estimate the absorbed concentration of lead from the media after two incubation times *i.e.,* 24 hours and 48 hours. The bacterial isolates were incubated at the optimized temperature and pH values. The results showed that ISO3 absorbed 92.09 µg/ml and 94.42 µg/ml at 24 hours and 48 hours respectively. The bacterial isolate ISO6 showed copper biosorption equal to 94.52 µg/ml and 96.75 µg/ml at 24 hours and 48 hours respectively. The bacterial isolate ISO7 showed copper biosorption equal to 93.13 µg/ml and 96.13 µg/ml at 24 hours and 48 hours respectively According to the results, all the

bacterial isolates showed maximum biosorption of copper at 48 hours of incubation. Out of all the bacterial isolates, ISO6 showed the highest biosorption i.e., 96.75 µg/ml, and ISO7 showed the highest biosorption *i.e.,* 96.13 µg/ml which is the second highest value after ISO6. ISO3 showed 94.42 µg/ml biosorption which is the lowest but still significant biosorption valve (Figure 6).

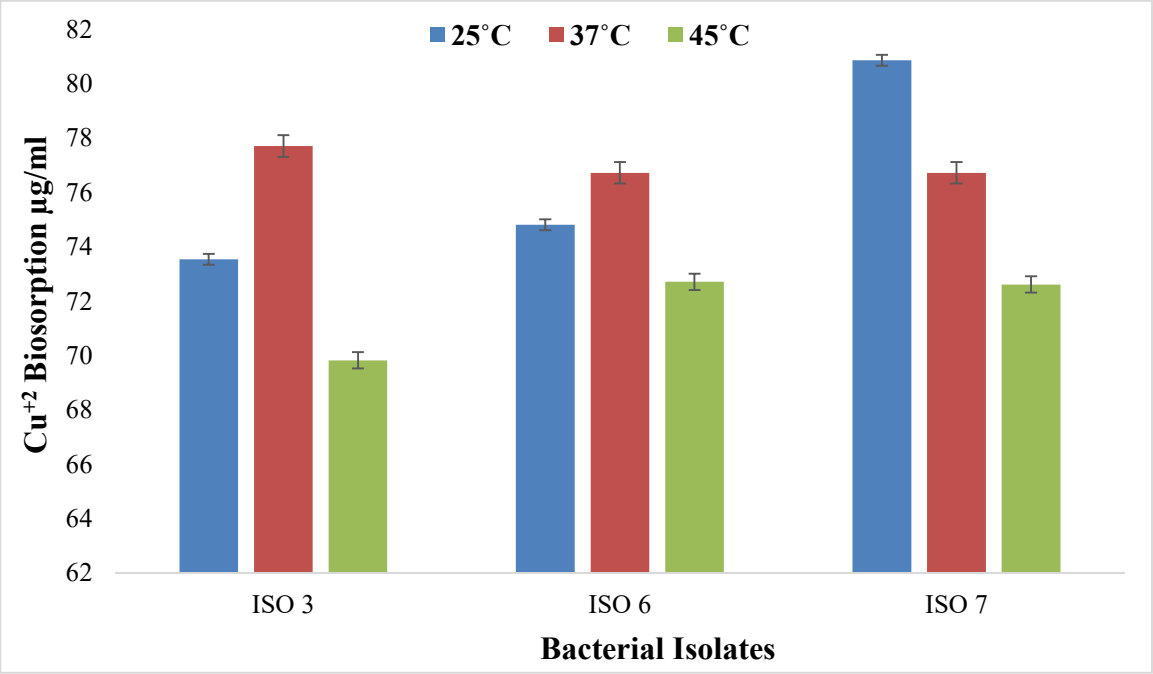

Figure 4: Biosorption potential of bacterial isolates for copper (Cu^{+2}) at different temperatures

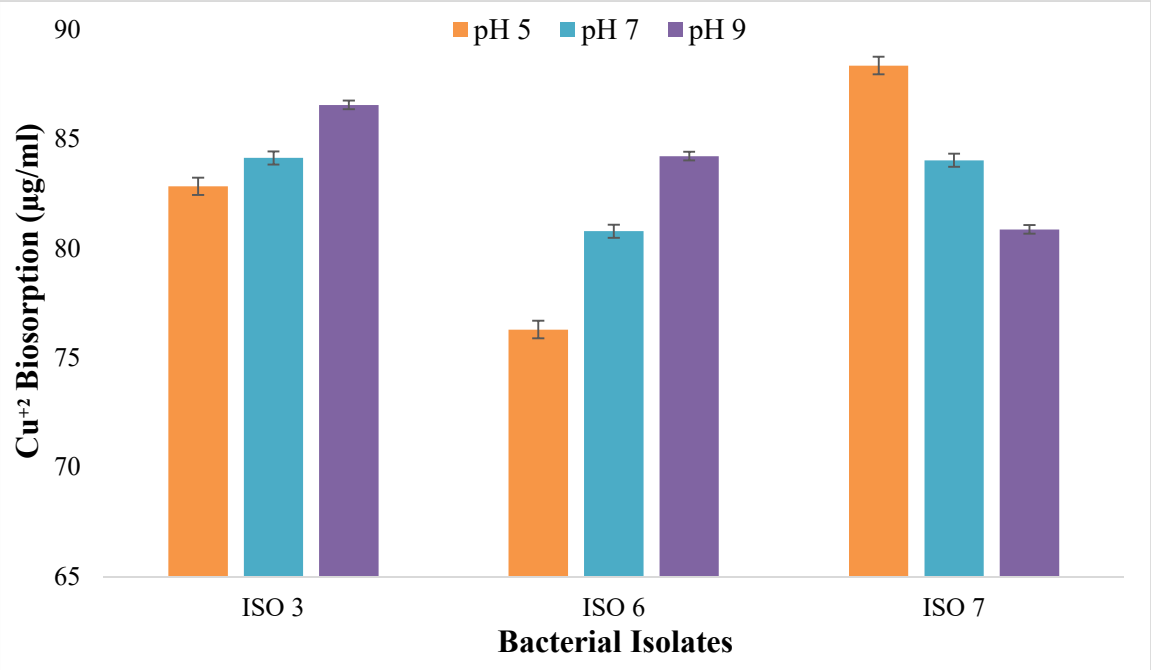

Figure 5: Biosorption potential of bacterial isolates for copper (Cu+2) at different pH

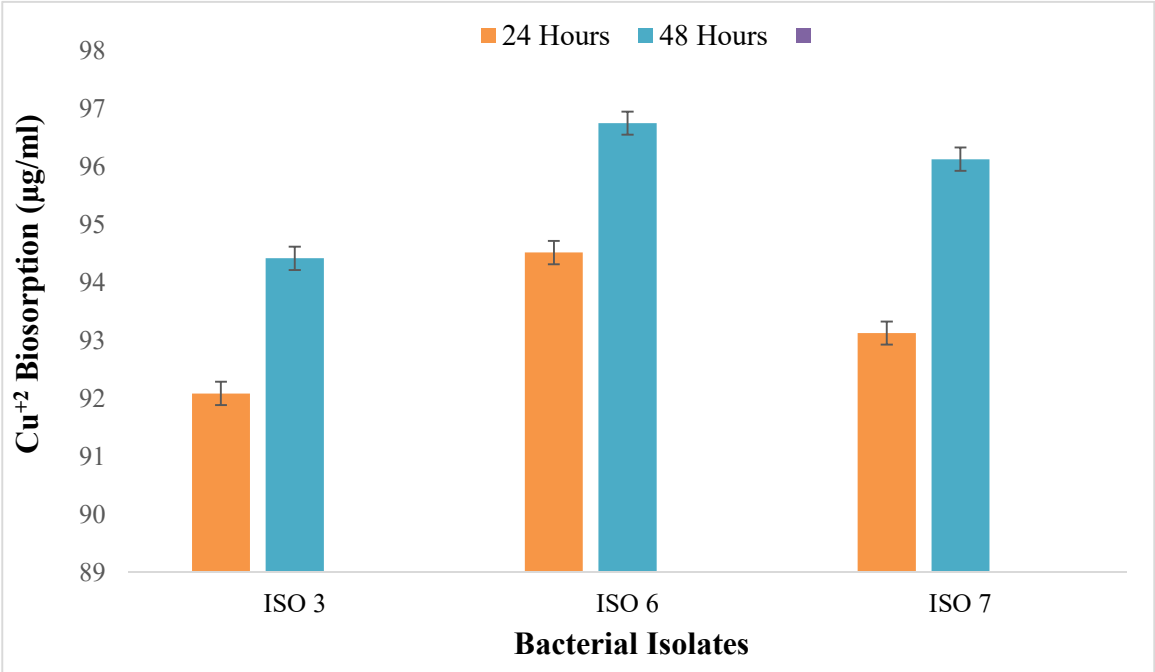

Figure 6: Biosorption potential of bacterial isolates for copper (Cu^{+2}) at different periods

Biochemical characterization of bacterial isolates

The biochemical tests were performed to determine the genus of the bacterial isolates. The results of each biochemical test performed have been mentioned in table 5.

Growth promoting impact of bacterial isolates on wheat seeds

Viability of wheat seeds

A seed germination test was performed to assess the viability of wheat seed. It was found that 90% of the seeds were viable since 9 out of 10 seeds germinated under normal conditions (Figure 7).

Table 5: Biochemical characterization of bacterial isolates

Biochemical Tests	Bacterial Isolates		
	ISO3	ISO6	ISO7
Gram staining	+	-	-
Spore staining	-	-	-
Oxidase Test	-	+	-
Catalase Test	-	+	+
Methyl Red- (MR)	+	-	+
Voges Proskauer Test (VP)	-	-	-
Indole Test	-	-	-
Citrate Utilization	-	+	+
Urease Test	-	-	-
Hydrogen Sulphide Production Test	-	-	+
Motility	Non motile	Motile	Motile
Triple sugar iron(TSI)	No sugar fermented	No sugar fermented	Glucose fermented only

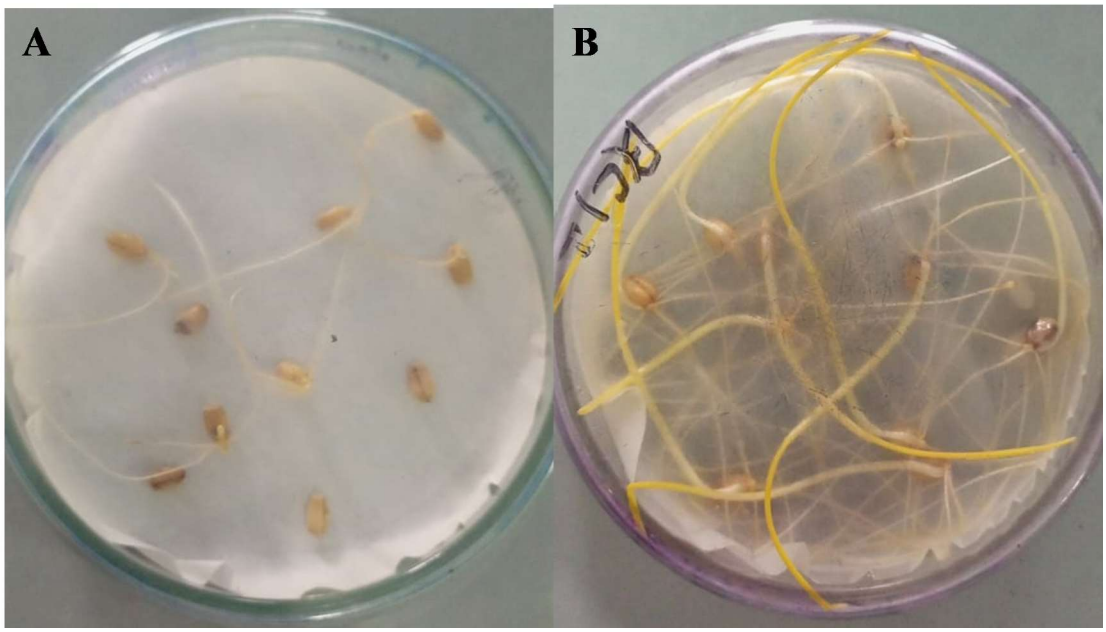

Figure 7: Viability test of wheat seeds A (day 3) and B (day 7)

Impact of copper stress on wheat seeds

To determine the stress of copper on wheat seeds, seeds were doused with a Cu^{+2} concentration of 25 µg/ml. Cu^{+2} concentration of 25 µg/ml was shown to reduce the root and shoot length and the number of leaves of wheat plants. The root length for the control group was equal to 10 cm while for the experimental group (seeds wetted with copper solution) was equal to 8.25 cm. Similarly, the shoot length for the control group was equal to 7 cm, while for the experimental group, it was reduced to 5.75 cm. The Number of leaves was also reduced as for the control group it was equal to 4 leaves while for the seeds dosed with lead solution, it was equal to 3 leaves (figure 8).

Growth promotion effects of bacterial isolates on wheat seeds

The growth promoting impact of bacterial isolates ISO3, ISO6, and ISO7 was investigated on wheat seeds. The results revealed that in the presence of bacterial isolates, the root length and shoot length increased compared to the root length and shoot length of the control group (seeds without bacterial treatment). At the same time, the number of leaves remained the same in both the control and experimental pots. The root length for the control group was equal to 10 cm. The maximum root length equal to 12.75 cm was observed for the seeds treated with ISO7 followed by 11.85 and 10.75 cm for ISO3 and ISO6 respectively. The shoot length for the control group was equal to 7 cm and for the seed treated with bacterial strains, the shoot length equal to 9.25, 8.75 and 7.35 cm were observed for ISO3, ISO7 and ISO6 respectively. The number of leaves equal to 4 were observed for both control and experimental seeds (figure 9).

The combined effect of bacterial isolates and copper stress on wheat seeds germination

Effect of Copper with ISO3, ISO6 and ISO7 on wheat seeds germination

The combined impact of bacterial isolates ISO3, ISO6, and ISO7 and copper on wheat seed germination revealed an increase in root length, shoot length, and leaves number when compared to wheat seeds dampened with copper solution. This mean root length (8.25 cm) was recorded when wheat seeds were wetted with copper concentration (25µg/ml). The seeds treated with ISO3 and wetted with copper solution (25 g/ml) had a mean root length equal to 10.65 cm, similarly, the mean root length of wheat seeds treated with ISO6 and ISO7 and copper solution was 9.5 cm and 9.85 cm respectively. The mean shoot lengths equal to 6.75, 6.25 and 6.95 cm were observed for the seeds treated with ISO3, ISO6 and ISO7 and doused with copper solution, respectively, compared to the shoot length of the seeds equal to 5.75 cm when doused with copper solution without bacterial treatment (figure 10). The no. of leaves for the seeds treated with ISO3, ISO6 and ISO7 and doused with copper solution were equal to 3, 4 and 3 respectively (figure 10).

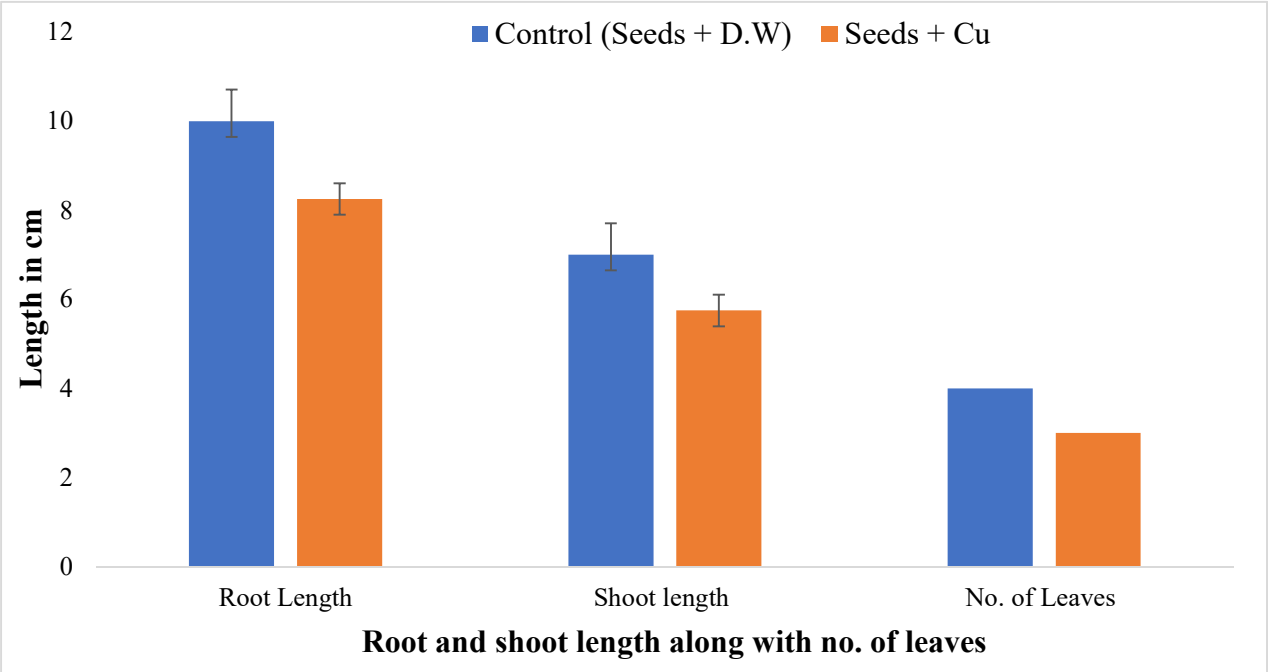

Figure 8: Effect of copper (Cu^{+2}) stress on the root length, shoot length, and no of leaves of wheat plant

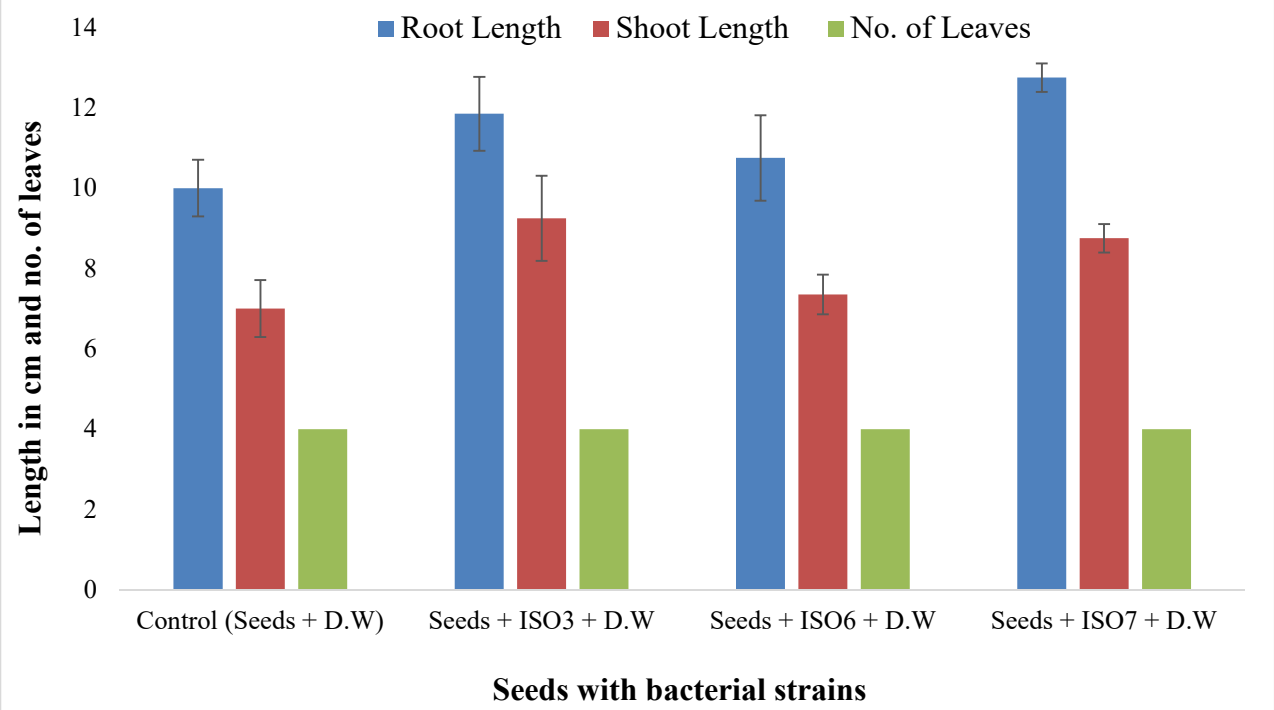

Figure 9: Impact of bacterial isolates on the root length, shoot length and no. of leaves of wheat seeds during germination

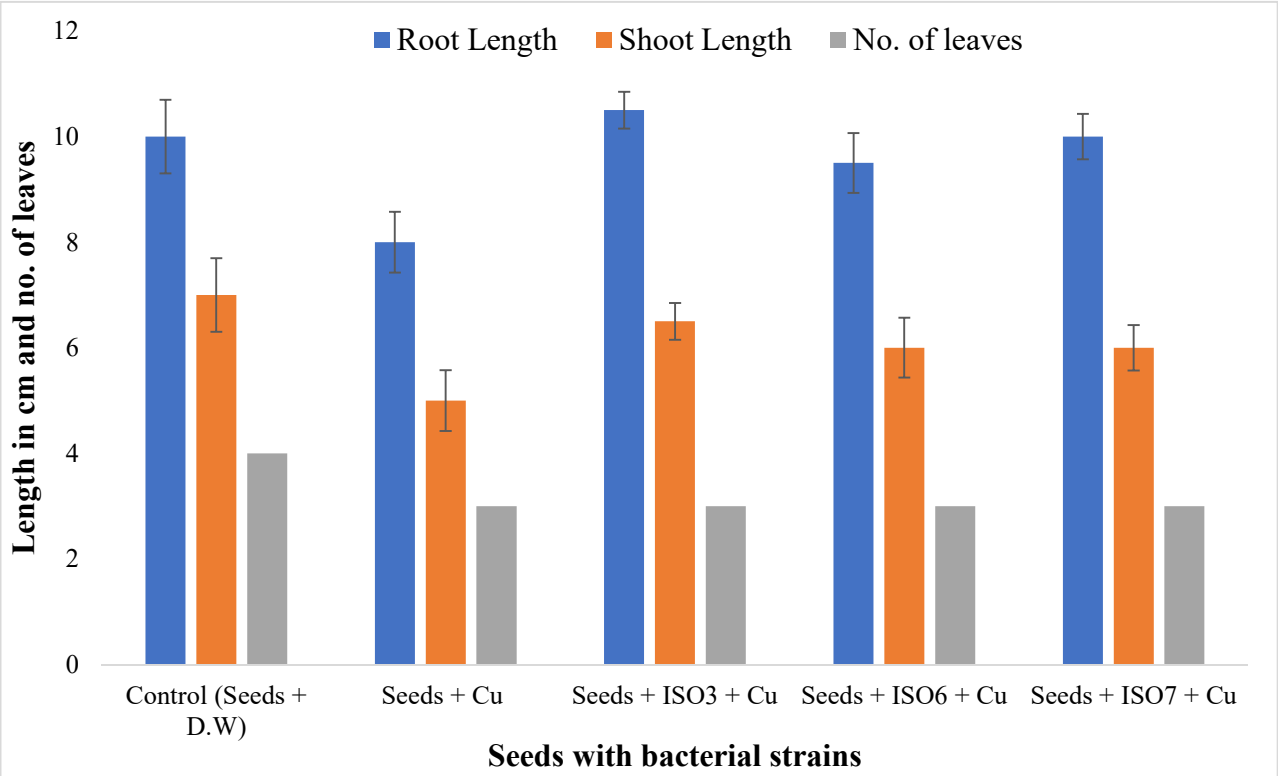

Figure 10: Impact of bacterial isolates and Cu^{+2} stress on root length, shoot length, and no. of leaves of the wheat plant

Identification of bacterial genera on the basis of biochemical characterization

According to the biochemical test, ISO3 belongs to the genus *Enterococcus*, ISO6 belongs to genus *Pseudomonas* and ISO7 belongs to the genus *Salmonella*.

Discussion

Bioremediation stands to be among the most effective ways of remediating heavy metal contamination. The optimum conditions that would enable these bacteria further to efficiently remove copper from polluted effluents, thereby reducing the metal concentration affecting ecosystems and human health, have been the main objectives of the present work. This research contributes further to the biosorption capacity of the bacterial strains regarding the feasibility of bioremediation as an eco-friendly technology for the uptake of industrial pollutants. The results are not only a testament to the effectiveness of microbial biosorption but also hint at the optimization of environmental conditions for the enhanced mitigation process. The subsequent discussion will tear into the results obtained in this study, compared to available literature, to appraise its potential for practical applications under industrial scenarios.

The presence of 12 copper-resistant bacterial isolates in the diverse samples of polluted water underlines the capability of microbial communities to adapt to the polluted environment. Seven such isolates showing distinct morphological features, were selected for further characterization, thus, extending further the view of microbial life variety in industrial effluents. It is some of the descriptions that are normally used in microbial species: colony features such as color, shape, and texture. Most are due to environmentally toxic metals, that is, toxic metals, and the response by bacteria towards the same[25].

These diversities in the bacterial isolates give an impression of various species depending on different strategies for survival in conditions with high concentrations of copper. The various strategies include the active production of extracellular polymeric substances, which may interfere with heavy metals; involvement of metal efflux pumps, which actively pull metals out of the cell; and the transformation of ions of metals into less injurious forms[26,27].

The various values obtained for MIC in this work are useful in showing the extent of resistance to copper by the bacterial isolates. ISO7 is the most resistant, its resistance depicting an MIC value of 450 µg/ml. This

suggests probable very efficient detoxification mechanisms against copper. This high resistance could be related to the upregulation of metal-resistance genes, such as copper-binding protein, efflux pumps, or enzymatic activity responsible for the change in Cu^{+2} to a less toxic form, Cu^{+1}, among others. However, on the contrary, no one was significantly more resistant to ISO1, which can suggest either less effective detoxification mechanisms or simply fewer numbers. These differences in the MIC values among the various isolates may also be due to differences in the genetic makeup, metabolic pathways, and ecological niches of the isolates. For example, resistant strains may be growing in a wastewater microenvironment where copper levels happen to be high, allowing a phenotype with a higher magnitude to this environmental condition to emerge[28].

The recorded MIC values from the present study were on par with those previously reported. Kang *et al.,* (2023)[29], recorded the bacterial isolates from copper-contaminated soils showing MIC values of between 300 and 500 µg/ml, while the most resistant had resistance levels equal to that of ISO7. This test result shows the fact that bacteria from several environments are likely to develop strategies of resistance that are approximately similar to a comparable level of stress by the potentially toxic metals[29,30].

That the pH optimum for copper biosorption was pH 9 in ISO3 and ISO6 and pH 7 in ISO7 would suggest that charge properties of the surface of bacterial cells can play an important role in the speciation of metal ions. It may, however, be observed that with higher pH than optimum, all the cations of copper Cu^{+2} will be observed to further tend to be converted to more hydroxide complexes, hence making adsorption on bacteria surfaces easier. This is in agreement with a study that supports one by Ayangbenro *et al*., (2017)[31]. There is also an increase in the electrostatic attraction between metal cations and the bacterial cell wall, with the cell-surface charge becoming more negative at high pH 8-12[32] . All these results are in good agreement with the previously reported literature where the pH effect is noticed in the metal biosorption processes. For example, Rabia *et al*., (2023)[33] reported the peak at pH 7 in the biosorption of copper by *Pseudomonas aeruginosa*, falling into what was achieved for ISO7 in this work. The improved biosorption efficiency of all metals experimented upon, including copper at an elevated pH up to an optimum point, beyond which further increases resulted in decreased efficiency due to metal precipitation.

The copper biosorption capability of these bacterial isolates was heavily dependent on the incubation time, being higher in the samples incubated for 48 hours. Such a trend suggests that increased incubation time is of huge interest, since it permits the increase in copper-binding sites on the surface of the bacterial cell wall and may induce mechanisms for metal resistance[34]. High biosorption capacities in ISO6 (96.75 µg/ml) and ISO7 (96.13 µg/ml) after 48 hours probably indicate intensified chelation activity of these isolates toward copper ions and an increased ability to sequester high amounts of metal over time. This increase in biosorption upon increasing the incubation time could be related to the phase of bacterial growth, during which multiplication and cell metabolism become active enough to produce more biosorption sites[35]. During this exponential increase phase, bacteria may synthesize more EPS, which can complex metals and over-express genes controlling metal transport and detoxification[26]. This 'exponential' phase of increase in the metabolic rate is important for the optimization of bio-sorption efficiency since the bacteria are optimally poised to interact with metal ions. One of the important factors, that has gained previous recognition, is the incubation time for studies related to metal biosorption. In this regard, Redha *et al.,* 2020[36], have described that an increase in biosorption capacity concerning higher incubation times had taken place due to the extended interaction time of metals with the cell surface. This allows for tighter interaction of metals with the surface of the cells, and internalization of metal ions into cells. As a result, there takes place an overall net gain in biosorption efficiency.

This was highly expressed in the seed viability test, where the germination percentage was high, averaging 90% of the seeds under normal conditions during the study. In prime, such high live values provide a sound footing in the estimation of copper stress and growth promotion by bacterial isolates. Since seed viability is among the features important to agriculture for measuring quality and viability, it directly impacts the final crop yield and rate of productivity[37]. The high viability recorded above in this study ensures that effects on seed growth flow from the experimental treatments and not from the seeds themselves.

Copper is an essential trace element in plants, but in large rates of application, it acquires a toxic level in

them. Wheat seeds exposed to 25 µg/ml of copper in the present experiment reveal that a considerable growth inhibition may occur under this metal concentration. The fall in the length of both the root and shoot with a reduction in the number of leaves indicates the negative impact of copper stress on the growth of the plant body. The explant of the experiment group has decreased to 8.25 cm from 10 cm, and root length to 5.75 cm from 7 cm. In addition, the number of leaves has reduced to 3 from 4. Copper toxicity in plants occurs due to the interference or inhibition of particular physiological functions such as photosynthesis, respiration, or other enzyme activities[38]. Copper stress induces the production of reactive oxygen species in many plants, which, in turn, causes oxidative damage to major cellular constituents such as lipids, proteins, and DNA[39]. The shortening of root length may be due either to a toxic effect of copper on cell division and elongation in the root meristem or to impaired nutrient and water uptake by roots[40]. Thus, the decrease in shoot length and leaf number reflects copper's effect on photosynthetic efficiency and the general metabolism of the plant. Inhibition in chlorophyll synthesis will finally limit photosynthesis, which must be an expected cause for limiting growth by functional inhibition of chloroplasts due to copper ions as learned[41]. On the other hand, data from the present experiment agree with those of previous studies that stated that high levels of copper seriously restrict plants in their growth and development, causing dwarfism and poor yield, among others [41]. ISO3, ISO6, and ISO7 bacterial isolates through direct inoculation on the wheat seeds had a highly effective growth induction impact. The results indicated that all these bacterial treatments supported longer roots and shoots than the control without bacteria[42].

Among them, the longest root recorded was 12.75 cm in ISO7 seeds, followed by 11.85 cm in ISO3 seeds and 10.75 cm in ISO6 seeds. Also, it accumulated more shoot length up to 9.25 cm in the ISO3 treatment, then 8.75 cm in the ISO7, and 7.35 cm in the ISO6 treatment, compared with a 7 cm length in the control. The implication that can be drawn from the results is that the bacterial isolates possess plant growth-promoting traits since there was an increment in the root and shoot margin length. These PGPTs are capable of elaborating phytohormones such as indole-3-acetic acid, which promotes shoot and root elongation and the development of branching root systems, and gibberellins, which promote shoot elongation[39,43].

They can also solubilize and take up essential nutrients like phosphorus and iron, among others that are closely linked with host growth promotion. The better performance of the isolate ISO7 as the best root and shoot growth inducer compared to all the isolates may thus be due to a higher number of PGPTs in this isolate or more efficient nutrient-solubilizing mechanisms in this isolate[41]. More so, as the number of leaves formed was expressed hardly differently between the control and experimental groups, this is indicative that though bacterial treatment significantly stimulates root and shoot elongation, it may have a minor effect at this stage in the induction of leaf formation.

Further, bacteria were also co-inoculated with the same bacteria isolates for copper stress at the time of germination of wheat seeds. It was noted that the bacterial isolates reduced the deleterious effect of copper and the average root and shoot lengths were found to be much more than seeds treated with only copper. Specifically, seeds subjected to ISO3 root growth treatment along with copper yielded a mean root length of 10.65 cm, which would provide that ISO6 and ISO7 produced a mean root length of 9.5 cm and 9.85 cm, respectively. Conversely, under conditions where no bacterial treatment was subjected, yet the seeds were permitted to be exposed to copper, the said seeds developed to grow their roots up to the mean length of only 8.25 cm. The shoot length also increased with bacterial treatment, being most pronounced after application for ISO3 at 6.75 cm, followed by ISO7 at 6.95 cm and ISO6 at 6.25 cm, while being solely 5.75 cm in seeds treated only with copper. However, the second parameter, the number of leaves, reacted differently, where the seedlings treated with ISO6 resulted in four leaves; while the ISO3 and ISO7 resulted in three leaves, the same response as to the control not copper stressed reaction. The presentation here relates that the bacterial isolates are capable of not only improving the usual overall growth but also preventing the copper-induced stress in the wheat seeds. One such mechanism may be the sequestration and immobilization of copper ions by bacteria, which, in turn, reduces bioavailability and, therefore, the toxicity of copper ions to plants[44]. Moreover, bacteria can induce systemic resistance in plants through which the plants increase their potential to tolerate abiotic stresses, one of which is heavy metal toxicity. These divergent activities of the bacterial isolates concerning root and

shoot growth under copper stress indicate the importance of selecting well-defined bacterial strains for bioremediation and plant growth promotion in polluted soils. In particular, ISO3 appears to provide the most effective protection under copper-stressed conditions and hence seems very promising in studies directed toward the formulation of agricultural bio inoculants.

Conclusion and Future Prospective

The present study has been conducted with an emphasis on illustrating bacterial isolates, isolated from industrial effluents for copper, one of the most ecologically relevant metals that creates a serious threat to ecosystems and human health. This study optimized the conditions through which these bacteria were subjected to remove copper from the culture media; therefore, it lays the foundation for the provision in important insights into the use of bioremediation as a sustainable approach toward industrial pollution. In the results, it has been established that the copper uptake potentials of the bacterial isolates are essential and performance-dependent parameters due to general environmental conditions such as temperature and pH. The study also enumerates the importance of the incubation period for the boosting effect of the biosorption relied upon since longer time periods are most likely to increase the capacity at which the bacteria will be able to capture the copper ions. This work underlined the application of such bacterial isolates for wheat seed growth under copper stress and their further use in agricultural applications, especially for the relieving of negative effects due to heavy metal contamination in soils. Further studies regarding the genetic and molecular mechanisms underlying the observed biosorption and plant growth-promoting characteristics are encouraged for such bacterial strains. It is desirable that in the future, research tries to transfer such types of bacteria to practical use in industry and agriculture on a more extensive basis to test their efficiency in large-scale bioremediatory operations and their possible inclusion into the system of ecologically clean agriculture on the principles of sustainability.

Acknowledgement

Authors acknowledge the facilities provided by the Department of Biology, Lahore Garrison University, Lahore for providing the lab facilities for the conduct of this research work.

Author Contribution

Fatima Mustafa and Minahil Fatima performed the experimental work of this project and Qamar Abbas supervised the overall research work and helped in the preparation of this manuscript.

Conflict of Interest

Authors declare no conflict of interest.

References

1. Festa R A, Thiele D J. Copper: An essential metal in biology. Curr Biol. 2011; 21(21): 877-883.
2. Nurlaila I, Irawati W, Purwandari K, Pardamean B. K-Means Clustering Model to Discriminate Copper-Resistant Bacteria as Bioremediation Agents. Procedia Computer Science. 2021: 804-812.
3. Staehlin B M, Gibbons J G, Rokas A, O'Halloran T V, Slot J C. Evolution of a heavy metal homeostasis/resistance island reflects increasing copper stress in enterobacteria. Genome Biol Evol. 2016; 8(3): 811-826.
4. Santo C E, Morais P V, Grass G. Isolation and characterization of bacteria resistant to metallic copper surfaces. Appl Environ Microbiol. 2010; 76(5): 1341-1348.
5. Gautam R K, Sharma S K, Mahiya S, Chattopadhyaya M C. Contamination of Heavy Metals in Aquatic Media: Transport, Toxicity and Technologies for Remediation. In: *Heavy Metals In Water: Presence, Removal and Safety*. The Royal Society of Chemistry. 2014: 1-24.
6. Karimi B, Masson V, Guilland C, Leroy E, Pellegrinelli S, Giboulot E, Ranjard L. Ecotoxicity of copper input and accumulation for soil biodiversity in vineyards. Environ Chem Lett. 2021; 19(3): 2013-2030.
7. Kumar V, Pandita S, Sidhu G P S, Sharma A, Khanna K, Kaur P, Setia R. Copper bioavailability, uptake, toxicity and tolerance in plants: A comprehensive review. Chemosphere. 2021; 262:127810.
8. Napoli M, Cecchi S, Grassi C, Baldi A, Zanchi C A, Orlandini S. Phytoextraction of copper from a contaminated soil using arable and vegetable crops. Chemosphere. 2019; 219: 122-129.
9. Coelho D G, Marinato C S, de Matos L P, de Andrade H M, da Silva V M, Neves P H S, de Oliveira J A. Evaluation of Metals in Soil and Tissues of Economic-Interest Plants Grown in

Sites Affected by the Fundão Dam Failure in Mariana, Brazil. Integr Environ Assess Manag. 2020; 16(5): 596-607.

10. Lange B, van Der Ent A, Baker A J M, Echevarria G, Mahy G, Malaisse F, Faucon M P. Copper and cobalt accumulation in plants: a critical assessment of the current state of knowledge. New Phytol. 2017; 213(2): 537-551.
11. Abril S I M, Costa P G, Bianchini A. Metal accumulation and expression of genes encoding for metallothionein and copper transporters in a chronically exposed wild population of the fish *Hyphessobrycon luetkenii*. Comp Biochem Physiol Part - C Toxicol Pharmacol. 2018; 211: 25-31.
12. Perlatti F, Martins E P, de Oliveira D P, Ruiz F, Asensio V, Rezende C F, Ferreira T O. Copper release from waste rocks in an abandoned mine (NE, Brazil) and its impacts on ecosystem environmental quality. Chemosphere. 2021; 262: 127843
13. Shen X L, Yu J H, Zhang D F, Xie J X, Jiang H. Positive relationship between mortality from Alzheimer's disease and soil metal concentration in mainland China. J Alzheimer's Dis. 2014; 42(3): 893-900.
14. Patel R, Aschner M. Commonalities between copper neurotoxicity and Alzheimer's disease. Toxics. 2021; 9(1): 1-11.
15. Squitti R, Ventriglia M, Simonelli I, Bonvicini C, Costa A, Perini G, Rongioletti M. Copper imbalance in alzheimer's disease: Meta-analysis of serum, plasma, and brain specimens, and replication study evaluating *atp7b* gene variants. Biomolecules. 2021; 11(7): 11070960.
16. Poggere G, Gasparin A, Barbosa J Z, Melo G W, Corrêa R S, Motta A C V. Soil contamination by copper: Sources, ecological risks, and mitigation strategies in Brazil. J Trace Elem Miner. 2023; 4: 100059.
17. Siddiquee S, Rovina K, Azad S A. Heavy Metal Contaminants Removal from Wastewater Using the Potential Filamentous Fungi Biomass: A Review. J Microb Biochem Technol. 2015; 7(6): 000243.
18. Qazilbash A A. Isolation and Characterization of Heavy Metal Tolerant Biota from Industrially Polluted Soils and Their Role in Bioremediation. 2004. (Doctoral dissertation, Quaid-i-Azam University Islamabad, Pakistan).
19. Medfu Tarekegn M, Zewdu Salilih F, Ishetu A I. Microbes used as a tool for bioremediation of heavy metal from the Environment. Cogent Food Agric. 2020; 6(1): 1783174.
20. Volarić A, Svirčev Z, Tamindžija D, Radnović D. Microbial bioremediation of heavy metals. Hem Ind. 2021; 75(2): 103-115.
21. Cappuccino J G, Sherman N. 2013. Microbiology: a laboratory manual. 10th Edition, Pearson Education Inc., San Francisco.
22. Wadood H Z, Sabri A N. Screening, characterization and biofilm formation of nickel resistant bacteria isolated from indigenous Environment. Polish J Microbiol. 2013; 62(4): 411-418.
23. Choińska-Pulit A, Sobolczyk-Bednarek J, Łaba W. Optimization of copper, lead and cadmium biosorption onto newly isolated bacterium using a Box-Behnken design. Ecotoxicol Environ Saf. 2018; 149: 275-283.
24. Afzal I, Shinwari Z K, Iqrar I. Selective isolation and characterizationnof agriculturally Beneficial endophytic bacteria from wild hemp using canolaa. Pakistan J Bot. 2015; 47(5): 1999-2008.
25. Harpke M, Kothe E. Biofilm formation in Gram-positives as an answer to combined salt and metal stress. J Basic Microbiol. 2023; 63(8): 888-896.
26. Nies D H. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev. 2003; 27(2-3): 313-339.
27. Abbasi M, Khan I, Rehman A, Hayat A, UR REHMA M, Shah T A, Albekairi T H. Bioremediation of heavy metals contaminated soil by using indigenous metallotolerant bacterial isolates. Appl Ecol Environ Res. 2024; 22(2): 1623-1648.
28. Rebelo A, Almeida A, Peixe L, Antunes P, Novais C. Unraveling the Role of Metals and Organic Acids in Bacterial Antimicrobial Resistance in the Food Chain. Antibiotics. 2023; 12(9): 12091474.
29. Kang Y R, Chung D R, Ko J H, Huh K, Cho S Y, Kang C I, Peck K R. Genetic alterations in methicillin-susceptible *Staphylococcus aureus* associated with high vancomycin minimum

inhibitory concentration. Int J Antimicrob Agents. 2023; 62(6): 106971.

30. Wallner A, Antonielli L, Mesguida O, Rey P, Compant S. Genomic diversity in *Paenibacillus polymyxa*: unveiling distinct species groups and functional variability. BMC Genomics. 2024; 25(1): 720.
31. Ayangbenro A S, Babalola O O. A new strategy for heavy metal polluted environments: A review of microbial biosorbents. Int J Environ Res Public Health. 2017; 14(1): 14010094.
32. Zhu Z, Bao L, Pestov D, Xu P, Wang W N. Cellular-level insight into biointerface: From surface charge modulation to boosted photocatalytic oxidative disinfection. Chem Eng J. 2023; 453:139956.
33. Rabia H, Ould Hamou M, Kasperkiewicz K, Krzykawski T, Malicka M, Potocka I, Augustyniak M. Native Bacteria Isolated from Phosphate Deposits Reveal Efficient Metal Biosorption and Adhesion to Ore Particles. Minerals. 2023; 13(3): 388.
34. Hossain S, Morey J R, Neville S L, Ganio K, Radin J N, Norambuena J, Kehl-Fie T E. Host subversion of bacterial metallophore usage drives copper intoxication. *MBio*. 2023; 14(5): e01350-23.
35. Long S, Hamilton P B, Wang C, Li C, Xue X, Zhao Z, Xu F. Bioadsorption, bioaccumulation and biodegradation of antibiotics by algae and their association with algal physiological state and antibiotic physicochemical properties. J Hazard Mater. 2024; 468: 133787.
36. Ali Redha A. Removal of heavy metals from aqueous media by biosorption. Arab J Basic Appl Sci. 2020; 27(1): 183-193.
37. Pagano A, Pagano P, Dueñas C, Griffo A, Gaonkar S S, Messina F, Macovei A. Seed quality assessment and improvement between advancing agriculture and changing environments. Ansari M W, Singh A K, Tuteja N (Eds.) In: Global Climate Change and Plant Stress Management. 2023: pp 319-334. Wiley.
38. Hafeez A, Rasheed R, Ashraf M A, Qureshi F F, Hussain I, Iqbal M. Effect of heavy metals on growth, physiological and biochemical responses of plants. Azamal Husen A. (Eds) In: Plants and Their Interaction to Environmental Pollution: Damage Detection, Adaptation, Tolerance, Physiological and Molecular Responses. 2022: pp139-159. Elsevier Inc.
39. Bhadrecha P, Singh S, Dwibedi V. 'A plant's major strength in rhizosphere': the plant growth promoting rhizobacteria. Arch Microbiol. 2023; 205(5): 165.
40. Franco A, Buoso S, Zanin L, Pinton R, Tomasi N. Copper toxicity in maize: the severity of the stress is reduced depending on the applied fe-chelating agent. J Plant Grow Regul. 2023; 42(3):1567-1581.
41. Li Y, Shi S, Zhang Y, Zhang A, Wang Z, Yang Y. Copper stress-induced phytotoxicity associated with photosynthetic characteristics and lignin metabolism in wheat seedlings. Ecotoxicol Environ Saf. 2023; 254: 114739.
42. Rashid A, Schutte B J, Ulery A, Deyholos M K, Sanogo S, Lehnhoff E A, Beck L. Heavy Metal Contamination in Agricultural Soil: Environmental Pollutants Affecting Crop Health. Agronomy. 2023; 13(6): 13061521.
43. Hasan A, Tabassum B, Hashim M, Khan N. Role of Plant Growth Promoting Rhizobacteria (PGPR) as a Plant Growth Enhancer for Sustainable Agriculture: A Review. Bacteria. 2024; 3(2): 59-75.
44. Rob M M, Akhter D, Islam T, Bhattacharjya D K, Khan M S S, Islam F, Chen J. Copper stress in rice: Perception, signaling, bioremediation and future prospects. J Plant Physiol. 2024; 302: 154314.