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Bioremediation of Manganese by Thermophilic Bacterial Isolates of Tapt Kund, Soldhar, and Gauri Kund Hot Springs of Uttarakhand, India

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ABSTRACT

Manganese (Mn) contamination in groundwater is a global concern due to its harmful effects. The high concentration of Mn^{2+} in humans creates memory issues, decreased fertility, appetite loss, sleeplessness, sperm abnormalities, and 'Manganism'. In this study, the isolation of thermophiles was followed by their assessment for MIC (minimum inhibitory concentration) and Mn bioremediation. We have isolated a total of 11 Mn-resistant bacterial strains of thermophiles with the identification of their bioremediation potential from the Tapt Kund, Soldhar, and Gauri Kund hot springs of Uttarakhand, India. Out of 11 strains, three isolates (TA8, SA9, and GA7) were identified with the highest metal resistance properties for toxic Mn^{2+}. The metal tolerance capabilities of the strains were evaluated through MIC and the metal biosorption rate was estimated by the live cells bioremediation through thermophilic bacteria. ICP-MS (inductively coupled plasma mass spectrometry) was used to assess the Mn^{2+} removal rate of bacterial bioremediation. It turned out that every strain exhibited promising bioremediation potential and proved Mn-resistant. The bacterial strain TA8 exhibits the highest MIC (600 $\mu g.L^{-1}$.) with a bioremediation rate of 98.34% for Mn^{2+}. The bacterial strain SA9 has a MIC value of 525 $\mu g.L^{-1}$, with a biosorption rate of 77.74% for Mn^{2+}. The bacterial strain GA7 has a MIC of 475 $\mu g.L^{-1}$, with an efficiency rate of 61.17% for Mn^{2+} removal. The most promising strain of thermophilic bacteria for Mn^{2+} bioremediation is the TA8, which has demonstrated the highest potential (98.34%) out of all the tested strains. The findings may have public health implications, as reducing manganese levels in groundwater can help mitigate health risks associated with Mn exposure. Also, this research enriches our knowledge of microbial bioremediation and its potential applications in environmental management. Ultimately, this research could offer a novel, economical, and environmentally beneficial approach to managing metal toxicity.

INTRODUCTION

Heavy metals are elements that have an atomic number greater than 20, except actinoids, lanthanoids, alkaline earth, and alkali metals. Whereas the density of heavy metals is more than 5 $g.cm^{-3}$ (Arya et al. 2023, Zeng et al. 2024). At present time, technology and modernization result in an increased amount of metal contaminants in the environment (Vishwakarma et al. 2024). Some of the most toxic and hazardous heavy metal elements for the environment and health of organisms include arsenic, manganese, cobalt, selenium, zinc, copper, iron, lead, chromium, cadmium, and mercury. Manganese (Mn) is a vital trace element for both humans and plants. The presence of Mn supports the preservation of human health, including reproduction, digestion, homeostasis, metabolism, antioxidant defenses, and brain functions (Ao et al. 2024, He et al. 2024).

In addition to being a fundamental and necessary nutrient for all living things, Mn is utilized in a wide range of industries to produce steel, electrode materials, non-ferrous metallurgy, batteries, and catalysis. Therefore, excessive atmospheric concentrations of Mn^2 may be produced by industrial activities. The maximum amount of metal that is permitted to be present in drinking water, according to the World Health Organization (WHO), is 0.05 $mg.L^{-1}$. Since Mn is not a metallic element that degrades naturally, it might be dangerous. In biological systems, Mn exists in eleven distinct oxidation states, but it mostly resides in two states: soluble (Mn^{2+}) and insoluble (Mn^{4+}) (Patil et al. 2024).

According to Wu et al. (2022) and Vishwakarma et al. (2024), Mn is a complex mineral that is involved in respiration, photosynthesis, pathogen defense, detoxification of reactive oxygen species (ROS), and hormone signaling in plants. However, Mn is an essential component needed by all living things. Amorim et al. (2018) and Patil et al. (2024) stated that a variety of industrial operations may lead to elevated hazardous Mn^{2+} concentrations that are harmful to the environment and living beings. Mn^{2+} is a migratory element that is difficult to control, hard to metabolize, and bioaccumulates in the environment (Amorim et al. 2018, Queiroz et al. 2024). Due to the risk of memory loss, insomnia, sperm abnormalities, decreased fertility, and appetite loss, exposure to high concentrations of Mn^{2+} is harmful to human health (Vishwakarma et al. 2024, He et al. 2024). "Manganism" is a disorder that emerges when the brain's central nervous system (CNS) possesses an abnormally high concentration of Mn^{2+} (Vishwakarma et al. 2024, Siddha & Kumar 2024).

To mitigate the adverse effects of heavy metals on the environment, the World Health Organization (WHO) sets maximum permissible limits for the availability of heavy metals in the environment. For this purpose, to keep the balanced concentration of Mn in the environment, a healthy treatment method is required. Common techniques for Mn^{2+} detoxification include physical, chemical, and biological techniques (Idris et al. 2023, Krishnan et al. 2024). Although these chemical and physical processes result in hazardous byproducts, are difficult to observe for a prolonged amount of time, and ultimately introduce more contaminants into the treated effluent (Krishnan et al. 2024). Therefore, biological treatment such as bioremediation is considered an eco-friendly, economically feasible, and safe technique for metal detoxification (Vishwakarma et al. 2024, Latif et al. 2024). For this purpose, the utilization of bacteria to detoxify hazardous Mn^{2+} metal into its less complex form is a significant approach. Bacterial bioremediation is a popular alternative for the biological treatment of toxic metal ions (Latif et al. 2024). Biosorption, bioaccumulation, bioprecipitation, and biological oxidation are the main components of the mechanism of bacterial bioremediation. Several distinct features of bacteria, such as their genetic and structural makeup, extra polymeric substances (EPS), and cell wall composition, are important to accelerate the process of bioremediation. Therefore, bacteria can evolve the genetic resistance mechanisms to handle the metal stress conditions (Vishwakarma et al. 2024). In comparison to bacteria, thermophiles are considered to be more resistant to heavy metals due to their lack of stable thermozymes (Vishwakarma et al. 2024, Singh et al. 2023). The facility of enzyme-mediated catalysis, solubilization, and precipitation of heavy metals make thermophilic bacteria more potent to detoxify heavy metal ions efficiently (Wu et al. 2022, Xu et al. 2024).

The importance of studying heavy metal bioremediation continues to rise as a result of the adverse impacts of toxic metal pollution on the ecosystem. By exploring the bioremediation potential of metal-resistant strains of thermophiles that were isolated from Taptkund hot spring (Chamoli), Soldhar hot spring (Chamoli), and Gaurikund hot spring (Rudraprayag), this study seeks to understand the effects of Mn^{2+} on these strains and their biological accumulation capacities. Inductively coupled plasma mass spectroscopy, or ICP-MS, was applied to investigate the metal bioremediation of potential thermophilic strains. Through the use of thermophiles, this investigation could offer an effective and novel approach to regulating Mn^{2+} toxicity and draw attention to the ecological importance of the hot springs located in Garhwal Himalaya, which is among Uttarakhand's most prominent pilgrimage destinations.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A total of 11 Mn-resistant bacterial isolates (Tapt Kund: TA8, TA9, Soldhar: SA7, SA8, SA9, SA10, SA11, SA12, Gauri Kund: GA7, GA8, GA9) were isolated from three distinct hot springs named Taptkund (Chamoli), Soldhar (Chamoli), and Gauri Kund (Rudraprayag) (Fig. 1) of Uttarakhand, India. The samples of water were taken from thermal springs in sterile plastic vials and kept in sterile thermos flasks to maintain the appropriate temperature during the transportation. The sampling was done in October, although the temperature and pH of the hot spring remained the same throughout the year. The bacterial colonies were obtained by the pour plate method (Aneja 2007) that helps to culture

Mn-resistant strains on Mn-containing Tryptone Soya Agar (TSA) medium plates. 1 liter of TSA medium consists of 15g Casein peptone (pancreatic), 5g Soya peptone (papainic,) 5g Sodium chloride, and 15g agar with pH 7.3 +/- 0.2 to provide nutritious favorable conditions for the growth of bacterial colonies. The inoculated plates were incubated at 55°C for 24-48 h. Each distinctive colony was subsequently streaked on TSA plates to obtain a pure culture.

MIC of Mn-Resistant Bacterial Strains

The agar dilution method was used to estimate the minimum inhibitory concentration (MIC) of Mn that inhibits bacterial growth. Agar plates were prepared with 50 $\mu g.L^{-1}$ of sterilized metal as the initial concentration. After inoculating the plates with various bacterial strains, they were incubated for twenty-four hours at 55°C. The bacterial isolates were transferred using the streak plate method onto different agar plates with progressively higher metal concentrations until the isolates stopped growing. This experiment was done in triplicates to ensure the accuracy of this work. The concentration of heavy metals that halted the growth of bacterial cells in the medium was referred to as the minimum inhibitory concentration (MIC) (Srinath et al. 2002).

Bioremediation of Mn^{2+} by Live Bacterial Cell

Bacterial bioremediation employs live cells to bioremediate Mn^{2+} and to evaluate the metal removal capabilities of bacterial strains (Özdemir et al. 2013, Vishwakarma et al. 2024). ICP-MS analysis of the samples revealed a decrease in the concentration of Mn ions throughout a 24-hour incubation period of the bacteria with the metal. For this purpose, Mn ions were generated as a heavy metal solution (1000 $\mu g.L^{-1}$) by dissolving its 100% pure chloride salt ($MnCl_2 \cdot 4H_2O$) with sterilized distilled water (Kang et al. 2016). To prevent precipitation, a few drops of concentrated HNO_3 were added to the further filtered metal solution. The heavy metal solution was diluted successively until it reached the appropriate concentration.

The overnight-cultivated bacteria inoculum was inoculated with 100 micrograms per liter ($\mu g.L^{-1}$) in 20 mL of broth medium. The mixture was then incubated for 48 h at 55°C at 150 rpm. An additional 24 h were spent incubating the medium after adding 100 $\mu g.L^{-1}$ of sterilized Mn^{2+} solution (except control). To extract the bacterial cells from the broth, centrifugation was performed for 15 min at 10,000 rpm. After collecting the supernatant, it was digested with 2% conc. HNO_3, which was subsequently utilized to determine the rate at which bacteria biosorb metals using ICP-MS (Özdemir et al. 2013, Vishwakarma et al. 2024). The remaining concentration of control samples identified through ICP-MS was used as the standard to calculate the bioremediation percentage. All the experiments were performed in triplicates to ensure the accuracy of the results.

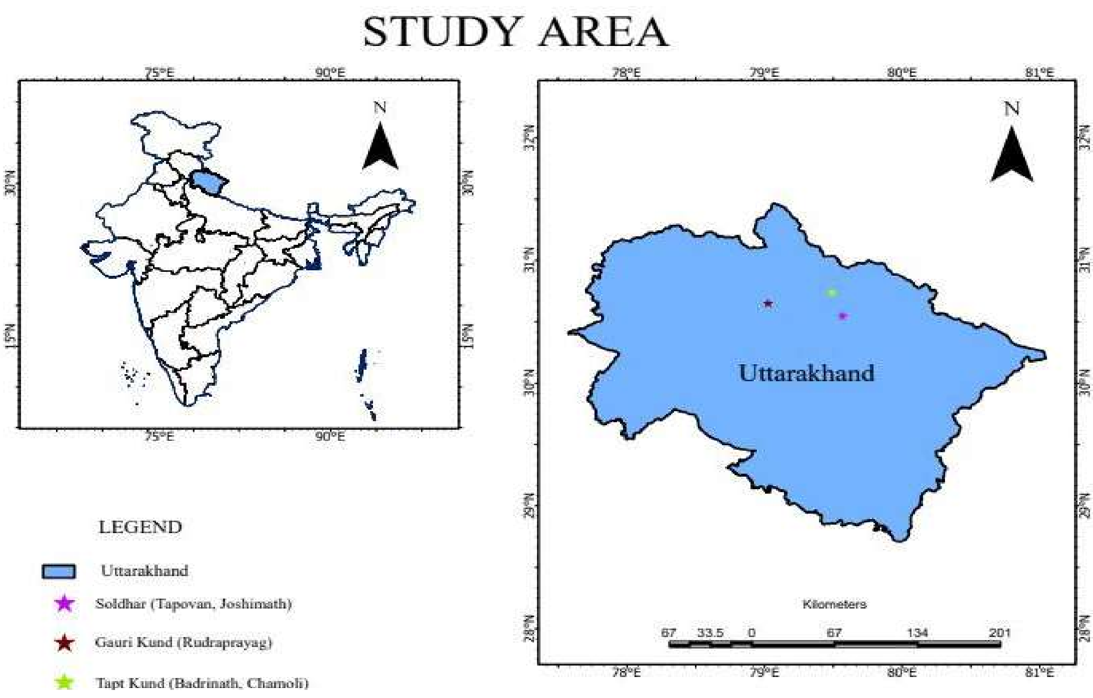

Fig. 1: Geographical locations of hot springs.

Data Analysis and Calculations

Dilutions of the 1 g.L^{-1} stock solution for ICP–MS were prepared to calculate the concentration of total Mn using the PerkinElmer Pure Plus instrument calibration standard 2 solution. To prepare the Mn standard solutions, the 1 g.L^{-1} stock solution of instrument calibration standard 2 was diluted. ICP-MS was used to evaluate the bacterial isolates' capacity for heavy metal biosorption. The concentration of metal ions ingested by bacteria was measured using the Perkin-Elmer Nexlon 300x ICP-MS Model. Every sample has been processed sequentially with their standards. Using an appropriate calibration curve or, if available, simply reading the concentration provided by the instrument, the metal ion concentration was measured in micrograms per liter (μg.L^{-1}). To determine the heavy metal degrading capability (%), the final result was compared to the control (Vishwakarma et al. 2024) as mentioned in equation (1). The initial concentration is represented as $C_{i,}$ the final concentration is represented as $C_{f,}$ and the live cell bioremediation absorption % is represented as $B_{(L)}$%.

$$B(L)\% = \frac{Ci - Cf}{Ci}\ x\ 100 \qquad \ldots(1)$$

RESULTS AND DISCUSSION

Bacterial Strains and Culture Conditions

The importance of the bacterial diversity found in the hot springs of Uttarakhand is made clear by the study of thermal springs located in the Garhwal region of the Uttarakhand state (Vishwakarma et al. 2024, Kaur et al. 2023). A total of 11 Mn-resistant bacterial isolates (Tapt Kund: TA8, TA9, Soldhar: SA7, SA8, SA9, SA10, SA11, SA12, Gauri Kund: GA7, GA8, GA9) were isolated from three distinct hot springs of Uttarakhand, India.

The optimal temperature and pH for the growth of thermophilic strains have been reported as 55°C and 7.0, respectively (Vishwakarma et al. 2024). Similarly, Kumar et al. (2020) found comparable values for temperature and pH. Generally, most thermophilic strains thrive within a temperature range of 45°C to 80°C, and those capable of growing at 80°C are classified as thermophilic bacteria, as noted by Brock (1978). Pandey et al. (2015) indicated that the temperature range of the Soldhar hot spring is between 90°C and 95°C. The most favorable pH conditions for bacterial growth are typically neutral. Previous studies have suggested that the temperature and pH parameters are relevant to the hot springs in Manikaran, Himachal Pradesh (Devi & Kanwar 2016), and Surya Kund, Yamunotri, Uttarakhand (Vishwakarma et al. 2024).

Additionally, Ortiz-Cortés et al. (2021) observed that the optimal growth of *Alicyclobacillus* species of thermophiles occurs at 65°C and within a pH range of 3 to 5. Temperature is a key factor influencing the composition of microbial communities in any given ecological niche. It impacts the morphological, physiological, and molecular traits of these communities, resulting in shifts both in composition and function. In this study, the bacterial isolates showed a remarkable capacity to survive across a temperature range of 20 to 70°C, surpassing other colonizers. Furthermore, these isolates displayed tolerance to a broad range of pH levels and moderate salt concentrations, which are also important traits.

MIC of Mn-Resistant Bacterial Strains

The Mn metal tolerance potential of bacterial strains was determined through MIC. MIC is the lowest concentration of metals at which bacterial growth was not observed, and it was used to confirm the Mn^{-}resistant behavior of the bacterial strains. Table 1 displays the MIC values of all the bacterial strains against toxic Mn ions**.** All the bacterial strains were found capable of growing in the presence of Mn. The observed Mn-resistant average count (CFU.mL^{-1}) of Gauri Kund is 3.5×10^4, Tapt Kund is 3.6×10^4, and Soldhar is 7.1×10^4.

TA8 has the highest MIC (600 μg.L^{-1}.) among all the thermophilic strains isolated from the Tapt Kund hot spring, whereas SA9 has the highest MIC value of 525 μg.L^{-1}. Among all the strains of the Soldhar spring, GA7 was 475 μg.L^{-1}, showing the highest MIC value among all the bacterial strains of the Gauri Kund hot spring.

The similar findings of some previous research increase the significance of this study. According to the previous study by Hou et al. (2015), *Klebsiella* species can withstand doses of metals up to 80 mM for Mn (II), among other

Table 1: MIC of Mn^{2+} tolerated by the bacterial strains.

Hot spring	Strains	Metal concentration [μg.L^{-1}]
Tapt Kund	TA8	600
	TA9	350
Soldhar	SA7	500
	SA8	425
	SA9	**525**
	SA10	300
	SA11	475
	SA12	475
Gauri Kund	GA7	**475**
	GA8	400
	GA9	400

metals. *Marinomonas* sp. *S11-S-4*, isolated from arctic ocean sediment, grew at up to 100 mM (Noszczyńska et al. 2020) of Mn^{2+}, while strains isolated from Sanindipur mines in Odisha, India, showed discernible growth at up to 500 mM of Mn. Interestingly, at 1000 mM $MnCl_2$ concentration, *Acinetobacter* sp. MSB 5 from the Sanindipur mines was still viable (Mohanty et al. 2017). *Bacillus thuringiensis* HM7, on the other hand, was able to grow at 31.78 mM after being isolated from Mn ore in Xiangtan, China. Minimal inhibitory concentrations (MIC) of *Alteromonas macleodii* ASC1 (El-Moselhy et al. 2013) isolated from Hurghada harbor in the Red Sea were examined, and resistance to multiple heavy metals was noted at 300 ppm for Mn (El-Moselhy et al. 2013). According to Özdemir et al. (2023), the thermophilic strain *Bacillus cereus* SO-16 was shown to be Mn resistant, with MIC values of Mn (II) 380 $mg.L^{-1}$. Similar to this, Vishwakarma et al. (2024) isolated eight thermophilic metal-resistant strains of *Firmicutes* and *Proteobacteria* from the Surya Kund Hot Spring in Yamunotri, Uttarakhand, and found that the strains' minimum inhibitory concentrations (MICs) varied from 300 to 375 $mg.L^{-1}$.

Bioremediation of Mn^{2+} by Live Bacterial Cell

The 24-hour incubation of metal and bacterial isolates interacting solution was resulting the degradation of Mn ions concentration in ICP-MS measurement. Table 2 contains information about heavy metal and tested strains used for the bioremediation assay. The Mn concentration in the control, as determined by ICP-MS analysis, was 25.82 $\mu g.L^{-1}$. All the strains are found capable of absorbing the toxic Mn^{2+}. The highest percentages of metal biosorption among all the Mn-resistant strains, including the Tapt Kund strains, have

Table 2: Metal absorption percentage of live bacterial isolates.

Metal	Isolate	Initial Metal concentration [$\mu g.L^{-1}$]	Final metal concentration [$\mu g.L^{-1}$]	Bioremediation [%] with live bacterial cells	Mean (Average)	Standard Deviation
Mn^{2+}	TA8	25.82	0.43	98.35%	13.13	17.953
	TA9		6.96	73.04%	16.39	13.336
	SA7		8.14	68.49%	16.98	12.502
	SA8		11.0	57.02%	18.46	10.409
	SA9		5.75	**77.74%**	15.79	14.192
	SA10		16.05	37.84%	20.94	6.908
	SA11		10.06	61.02%	17.94	11.144
	SA12		9.24	64.21%	17.53	11.724
	GA7		10.02	**61.17%**	17.92	11.172
	GA8		11.72	54.60%	18.77	9.970
	GA9		13.27	48.62%	19.55	8.874

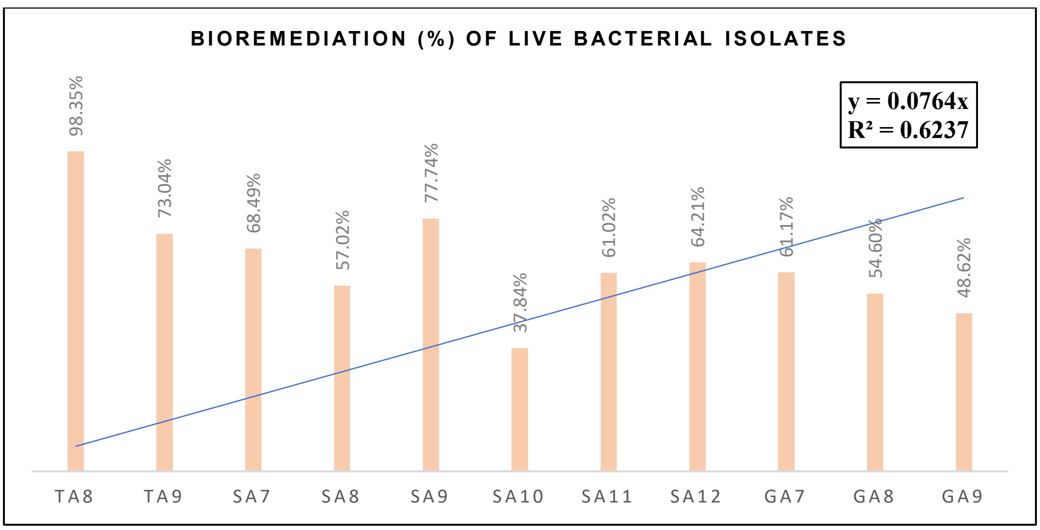

Fig. 2: Rate of Mn^{2+} absorption through live cells of bacterial isolates [X axis: Isolates, Y axis: Bioremediation%].

been observed in the TA8 isolate (98.34%). Among Gauri Kund strains, the highest percentage of Mn observation has been observed in GA7 (61.17%), and among Soldhar strains, the highest metal absorption percentage has been observed in SA9 (77.74%) respectively (Fig. 2). SA10 isolate of the Soldhar spring has observed with the least percentage (37.84%) of Mn absorption.

This finding highlights the differences in metal absorption capabilities among various strains from the same ecological niche. The SA10 isolate from the Soldhar spring showed the lowest Mn absorption rate at 37.84%, raising questions about the factors that may be contributing to its reduced effectiveness. Possible explanations could include genetic variations, differences in metabolic pathways, or adaptations to environmental conditions. Overall, these results reveal the diverse capacities of different bacterial strains to absorb manganese, suggesting that certain isolates could be effectively utilized in bioremediation efforts. Further investigation into the mechanisms driving these differences in biosorption rates could provide valuable insights for enhancing bioremediation strategies.

The R^2 value is less than 1 with a straight line, indicating that the data of results are significant (Fig. 2 and Table 2). To validate the accuracy of the results, a standard deviation was calculated. This measure indicates the extent of variability within the data set. By comparing each data point to the overall average, standard deviation provides a value that reflects how closely the points cluster around the mean. A low standard deviation suggests that the data is closely aligned with the average, indicating higher accuracy, while a high standard deviation points to significant variation from the mean. In this analysis, the observed R^2 value for standard deviation is notably low at 0.87, which underscores the reliability of the bioremediation outcomes (Fig. 3). Consequently, the isolates have shown promising potential for Mn metal bioremediation.

Each isolate demonstrated robust resistance to the Mn^{2+} and demonstrated the ability to proliferate on the plates containing metal. All of the bacterial isolates used in the bioremediation experiment were able to biosorb Mn^{2+} by using live biomass. It has been demonstrated by the current study and references to other research that thermophilic bacteria are capable of bioremediating Mn^{2+} from the environment. Hussein et al. (2005) examined the bioaccumulation of heavy metals in mesophilic environments by using two strains of *Pseudomonas*. For that, two distinct metal ions were gathered for the bioaccumulation of Cu (II) (151.42 $mg.L^{-1}$) and Ni (II) (54 $mg.L^{-1}$) effectively. Vishwakarma et al. (2024) discovered that *Acinetobacter* sp. *LSN10* has the highest potential for eliminating Mn^{2+} ions in both live (41.202%) and dead (64.721%) environments. According to Majumder et al. (2015), *Acinetobacter guillouiae* and the *Enterobacter* genera were discovered to be capable of bioaccumulating harmful metal ions and of having an efficient removal mechanism for Cu^{2+} bioremediation. Firmicute members can use bioremediation to lower environmental concentrations of iron and sulfate when conditions are favorable (Banerjee et al. 2015, Vishwakarma et al. 2024). According to Imron et al. (2021), *V. damsela, P. fluorescens, P. stutzeri,* and *P. aeruginosa* are the four bacterial strains that have been isolated from leachate and exhibit a high resistance for Cu, Fe, and Mn.

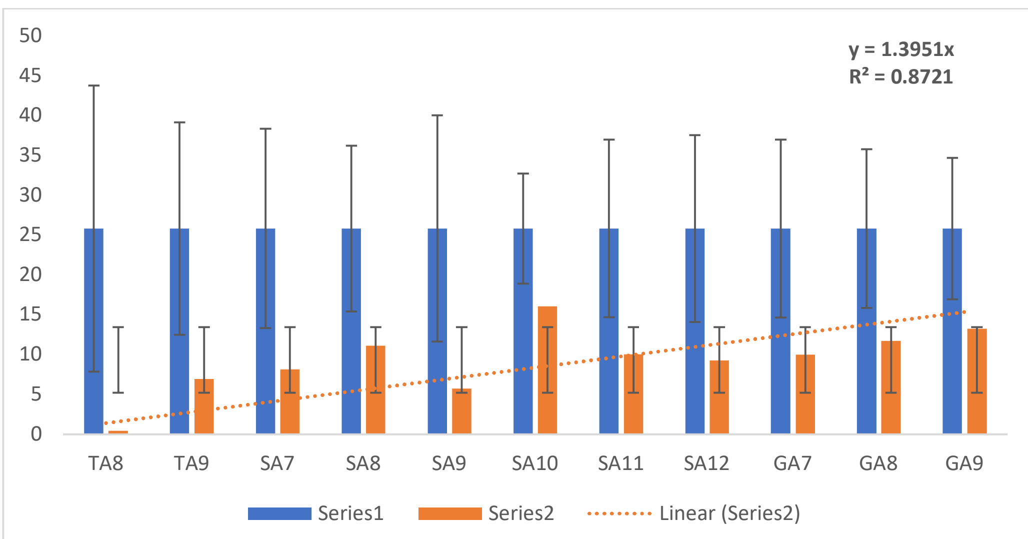

Fig. 3: Standard deviation of Mn resistant isolates.

The thermophilic bacteria *Bacillus thuringiensis* has been reported by Huang et al. (2020) to be able to withstand 4,000 mg.L^{-1} of Mn (II) and to have a maximum clearance rate of 95.04% at 400 mg.L^{-1}.

According to these previous studies, the most effective bacterial strains for bacterial bioremediation are thermophilic strains, which are also highly resistant to heavy metals. Additionally, it is observed that a mixture or consortium of bacterial species exhibits a significant influence on the rate of metal biosorption. The potential of the novel manganese-oxidizing bacteria (MOB) consortium AS for the removal of Mn^{2+} was investigated by Wan et al. (2020). The MOB consortium was able to remove 98%, 91%, 99%, and 76% of metal ions by performing Mn (II) and Fe (II), Ni (II), Cu (II), and Zn (II) biosorption assay. As a result, it can also be said that the rate of Mn (II) of MOB consortium AS is increased by the presence of different organics and other metals. Therefore, a bioremediation assay through the consortium of some bacteria has been considered for further studies (Patil et al. 2024, Bee et al. 2024, Patil et al. 2024). The dead biomass of bacteria also has a significant role in heavy metal bioremediation (Patil & Arya 2024). Therefore, these strains will further explore the dead biomass bioremediation to check the comparative potential of live and dead biomass of thermophilic bacterial strains.

This research is significant for several reasons. Firstly, it deepens our understanding of bioremediation strategies for managing toxic metal contamination in groundwater, particularly manganese, which is often overlooked. Secondly, identifying effective thermophilic strains offers a sustainable and eco-friendly approach to reducing Mn^{2+} toxicity in contaminated environments. By leveraging the natural capabilities of these bacteria, this study paves the way for the development of innovative bioremediation techniques that are both cost-effective and environmentally beneficial.

CONCLUSION

Manganese is an important metal that is necessary for the accomplishment of several biological processes, but excessive amounts of it can be harmful. The environment and human health are more at risk due to the elevated concentration of Mn^{2+} contaminants in the environment. As a result, it's necessary to investigate several natural remediation processes that can effectively and economically extract Mn^{2+} from the environment. Through this study, we were able to isolate some Mn-resistant strains of bacteria from the thermal springs of Uttarakhand's Himalaya range. Among all the bacterial strains, TA8 has the highest Mn^{2+} bioremediation rate (98.34%) with the highest minimum inhibitory concentration (MIC) of 600 μg.L^{-1}., therefore this strain can be considered for further in-depth study. It had been proven that the bacterial strains could bioabsorb and detoxify Mn^{2+} from their systems when the conditions (pH, temperature, and incubation time) were ideal. This study has important implications for environmental policies and industrial practices. It can help policymakers refine regulations on manganese contamination in groundwater and establish permissible levels. Moreover, the identification of thermophilic strains with high Mn^{2+} absorption supports industries in adopting sustainable bioremediation techniques as alternatives to chemical methods, reducing environmental impact. However, more research is needed on the comparative analysis of consortia biosorption of heavy metals and dead biomass bioremediation by utilizing different potent bacterial strains. However, molecular identification of these strains has been included in another part of the study. The results of this study could provide new optimism for managing metal toxicity in the future. The findings of this study might provide an economical, sustainable, and effective substitute for Mn^{2+} metal biosorption.

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