Article

Evaluating the resistance of indigenous bacteria to multi-metal contaminant and their co-relation with antibiotic resistance

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Received 4 October 2024; Accepted 10 November 2024; Published online 25 November 2024; *Published 1 June 2025*

Abstract
The accumulation of heavy metals resulting from the immersion of idols in local water bodies can lead to adverse effects, disrupting the growth, metabolism, and reproduction of organisms and impacting the entire trophic chain, including humans. In light of this, the current study investigated the resistance of indigenous bacteria isolated from idol immersion sites to three distinct heavy metals (Mn^{+2}, Pb^{+2}, and Zn^{+2}) and their consortia. Water samples collected from the idol immersion site were utilized to isolate bacteria, for the present study. The relative growth of bacterial isolates was assessed in the presence of heavy metals and their consortia using the agar dilution method, while their antibiotic susceptibility was determined through the Kirby–Bauer Disc Diffusion method. The growth of bacterial isolates varied with the increasing concentration of each heavy metal in the supplemented medium, yet the general order of resistance against the metals was observed as Pb > Zn > Mn. Among the three most robust isolates, two (*Bacillus* spp 1 and *Bacillus* spp 2) exhibited heightened tolerance to the consortia of heavy metals (Mn^{+2}, Pb^{+2}, and Zn^{+2}) up to 400 µg mL^{-1}. Antibiotic investigation revealed that the isolates were resistant to gentamycin and kanamycin, possibly due to mechanisms such as target modification, altered permeability, antibiotic inactivation, and metabolic pathway bypass. As a result, these bacteria hold promise for further exploration in the bioremediation of polluted water containing multiple toxic metals.

Keywords antibiotics; heavy metal; idol immersion; religious practice; bioremediation.

Proceedings of the International Academy of Ecology and Environmental Sciences
ISSN 2220-8860
URL: http://www.iaees.org/publications/journals/piaees/online-version.asp
RSS: http://www.iaees.org/publications/journals/piaees/rss.xml
E-mail: piaees@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

In India, religious practices often involve immersing religious wastes in water bodies, particularly during the annual festivals of Ganesh puja, Durga puja, etc. This tradition has significantly impacted the natural ecosystem and human health by depositing various materials such as flowers, colourful clothes, plastics, pigmented decorations, ornaments, plaster of Paris, bamboo sticks, and other objects into local water bodies.

This practice has led to a decline in water quality, including increased concentrations of heavy metals, hardness, and turbidity (Desai and Tank, 2010; Su et al., 2014; Watkar and Barbate, 2014).

The heavy metals viz., Arsenic, Copper, Calcium, Iron, Magnesium, Manganese, Mercury, Molybdenum, Silicon, Lead, Chromium, Nickel, Cadmium and Zinc are used to prepare synthetic paints of different colours. The concentration of these metals rises in multiple folds after the immersion of idols and persists in the environment posing ecological, evolutionary, nutritional, and environmental challenges due to their non-biodegradable nature (Su et al., 2014; Ceci et al., 2019). Exposure to heavy metals has resulted in issues like decreased plant growth, organ dysfunction in animals and humans, and alterations in the conformation of biological molecules in microorganisms (Su et al., 2014; Häder et al., 2020). While physicochemical methods have been widely explored to control heavy metals in water bodies, these methods are often inefficient and economically impractical when metal concentrations are below 100 mg L^{-1}. Consequently, researchers are increasingly turning to biological methods for the removal of heavy metals from water, focusing on isolating and identifying microorganisms present in contaminated water or wastewater (Su et al., 2014; Zhong et al., 2021).

Microbes need heavy metals in trace amounts but higher concentrations of these metals become toxic (Asitok et al., 2019). Due to accelerating industrialization and growing population, heavy metals accumulate in the ecosystem, resulting in autochthonous microbes developing means (such as efflux pumps) to handle these heavy metals (Gonzalez et al., 2021).

Research indicates that antibiotics and heavy metals co-resistance occurs through similar functional and structural mechanisms, which are carried either on plasmids or chromosomes (Li et al., 2017; Meng et al., 2022). Various pollutants like crude oil, sewage and heavy metal enhance the prevalence of antibiotics in the environment, which naturally occur in low concentrations (Vats et al., 2022). In such situations, antibiotic resistance genes (ARG) can potentially migrate among microorganisms via horizontal and vertical pathways, worsening multi-drug resistance and adding complexity to the clinical prognosis of infectious ailments. Furthermore, these ecosystems can act as reservoirs for ARG crossing over from environmental into clinical settings (Mujawar et al., 2019). Numerous investigations have confirmed the coexistence of antibiotic and heavy metal resistance in contaminated environments, yet research exploring this phenomenon in water bodies remains limited.

This paper specifically addresses the heavy metal resistance of indigenous bacteria isolated from idol immersion site. The objectives include the isolation of naturally occurring bacteria from polluted sites, screening for resistance against manganese (Mn^{+2}), lead (Pb^{+2}), and zinc (Zn^{+2}), evaluating multi-metal tolerance, assessing the antibiotic resistance, identifying bacteria up to the genus level, profiling and curing of the plasmids. This fundamental research aims to establish the basis of bacterial resistance for potential use in a comprehensive heavy metal bioremediation scheme in the near future.

2 Study area and Methodology

2.1 Study site

The study was conducted in the district of Bhopal, Madhya Pradesh, India. The study area includes the idol immersion site of Upper Lake (part of Bhoj wetland) i.e. Khatlapura ghat (23.24946ºN, 77.409574˚E). This wetland system is one of the important ecological zones of Bhopal, Madhya Pradesh, India, and has been recognized for biodiversity and environmental wealth. Being greater than the average size as compared to the Lower Lake, the Upper Lake plays a vital role in water supply and regional ecological balance.

2.2 Data collection

2.2.1 Water samples were collected from selected stations from a depth of 10 -15 cm from the surface in triplicate in clean plastic bottles.

2.2.2 The isolation of bacteria was performed on a nutrient agar medium (NAM) using the spread plate technique. The NAM was inoculated with 100 µl of the sample and incubated in an inverted position for 24 h at 37°C. Individual distinct colonies were subsequently sub-cultured using the streak plate method and microscopically characterized (Cappucino and Sherman, 2014).

2.3 Analysis of data

2.3.1 Bacterial tolerance to manganese (Mn^{+2}), lead (Pb^{+2}) and zinc (Zn^{+2}) was determined by the agar dilution method (Nath et al., 2019), which were added in the form of salts i.e. $MnSO_4$, $(CH_3COO)_2Pb$ and $ZnSO_4$. The initial concentration of these metal salts in nutrient plates was kept at 100 µg mL^{-1}, and then metal salt concentration was progressively increased. The bacterial growth was checked by streaking on the respective plates. The concentration at which bacterial isolates failed to grow, MIC was noted. Each experiment was done in triplicates.

2.3.2 Selected bacterial isolates were tested for their tolerance against heavy metal consortia consisting of manganese Mn^{+2}, lead (Pb^{+2}) and zinc (Zn^{+2}) in the nutrient broth with graded metal concentration from 50 µg mL^{-1} to 500 µg mL^{-1}. Exponentially growing cultures were inoculated in the metal consortia incorporated broth and inspected at 24 h intervals through UV-Vis spectrophotometer (Shimadzu UV 1900i) at 600 nm.

2.3.3 Tests were performed to investigate the biochemical characteristics of the bacterial isolates included indole test, motility, methyl red test (MR), Voges-Proskauer (VP) tests, catalase reaction, citrate reaction, starch hydrolysis, nitrate reduction. For all biochemical tests, 24 h old culture was taken. One control was kept in each case (Cappucino and Sherman, 2014).

2.3.4 To determine heavy metal tolerance index, 1 mL of the exponentially growing culture was transferred to nutrient broth supplemented with heavy metals ranging from 100 µg mL^{-1} to 500 µg mL^{-1}. It was incubated for seven days at 35 °C and inspected each day through UV-visible spectrometry at 600 nm verifying microbial growth (Muñoz et al., 2012).

The Tolerance index (TI) represents the relative growth rate of the bacteria, and it was computed as follows

$$TI = \mu_{ms}/\mu_c$$

where μ_{ms} is the growth rate in a metal-containing solution and μ_c is the growth rate in a control solution at 72 h. TI is commonly used to quantify metal tolerance in organisms, the higher the TI value, the greater the tolerance.

2.3.5 Plasmid was extracted from the screened isolates by alkaline lysis method for plasmid isolation (Green and Sambrook, 2018) and the concentration and purity of DNA were determined by a spectroscopic method (Shimadzu UV1900i). Their molecular size was determined using the Himedia plasmid DNA molecular size determination kit (200bp – 10kb).

2.3.6 To determine whether the heavy metal-resistant gene was plasmid-encoded or chromosomally encoded, plasmid curing was performed. The method described by John and Okpokwasili, (2012) was applied with some modifications. Bacterial isolates were grown in a mineral salt medium overnight. Later, 4 mL of the overnight culture was added to 50 mL MSM broth containing 20 mg mL^{-1} ethidium bromide and incubated at 37°C for 24 hours. Subsequently, the broth was shaken to homogenize it, and 1 mL of the culture was subcultured into 50 mL of MSM medium containing 500 mg mL^{-1} of heavy metal consortia, incubated for 24 hours at 37°C. Finally, optical density measurement was taken. The isolates that did not grow on MSM medium were considered cured.

2.3.7 Kirby–Bauer disc diffusion technique was used to test the isolates for their antibiotic susceptibility using 8 antibiotics (Baccer et al., 1966). Standard Himedia antibiotic discs of concentration 30 µg of Nalidixic acid,

Gentamicin, Tetracycline, Chloramphenicol, Clarithromycin, Kanamycin, Streptomycin and Ofloxacin were used. Antibiotic discs were placed on lawns prepared of each isolate on nutrient agar plates followed by incubation at 37 °C for 24 h. After incubation, diameter of inhibition zone was measured, and the isolates were classified as resistant (R), intermediate (I), or susceptible (S) following the standard antibiotic disc chart.

2.3.8 Statistical evaluations was done using version 20.0 of the SPSS software. The results were shown as the standard error of the mean, and the significance level was specified at ($p < 0.05$; Zhang, 2022).

3 Results and Discussion

3.1 Isolation of bacteria from water sample

From the water samples, a total of sixteen bacterial isolates were isolated. Among them, eight isolates (KTH1, KTH2, KTH3, KTH4, KTH5, KTH6, KTH7 and KTH8) were obtained after initial screening.

3.2 Screening of heavy metal tolerant bacteria and mic study

Heavy metals and bacteria can interact directly or indirectly depending on the microorganisms. The ability to remove hazardous heavy metals from the environment by the bacteria is mainly due to the rapid growth rate and small size of the bacteria (Tarfeen et al., 2022). The uptake of heavy metals by microbes depends on factors such as temperature, pH and metal ions, etc. Based on the MIC study (Table 1), the three bacterial isolates KTH 1, KTH4 and KTH7 exhibited the highest MIC value of 500 µg mL^{-1} of Mn^{+2}, Pb^{+2} and Zn^{+2} respectively. Hence, these three isolates were selected for further studies.

Table 1 (MIC) of the selected morphotypes against Mn^{+2}, Pb^{+2} and Zn^{+2}.

Heavy metal	Concentration (µg mL^{-1})	Morphotypes							
		KTH1	KTH2	KTH3	KTH4	KTH5	KTH6	KTH7	KTH8
Manganese	100	++	++	++	++	++	++	++	++
	200	++	++	++	++	++	++	++	++
	300	+	-	+	-	-	-	+	+
	400	+	-	-	-	-	-	-	-
	500	M	-	-	-	-	-	-	-
Lead	100	++	++	++	++	++	++	++	++
	200	++	++	-	++	-	++	+	-
	300	+	+	-	+	-	-	+	-
	400	-	-	-	+	-	-	-	-
	500	-	-	-	M	-	-	-	-
Zinc	100	++	++	+	++	-	-	++	++
	200	++	++	++	++	++	++	++	++
	300	+	+	-	+	-	-	-	-
	400	-	-	-	-	-	-	+	-
	500	-	-	-	-	-	-	M	-

++ : Extensive growth of bacteria, + : less growth of bacteria, - : no growth of bacteria, M : MIC.

The multi-metal tolerance study indicated that the bacterial population's tolerance property is well spread. KTH1 shows good overall growth across all concentrations of heavy metal composite (Fig. 1). Even at 500 mg L^{-1} moderate growth was observed. KTH4 showed lower OD_{600} values when compared to KTH1 (Fig. 2). A higher concentration of heavy metals slows down bacterial growth. KTH7 shows the poorest growth pattern,

with less overall growth and more sensitivity to the heavy metal mixture (Fig. 3). These graphs tell us that KTH1 bacteria had the strongest growth among the three. It showed the most growth potential and handled the heavy metal mixture best at different concentrations.

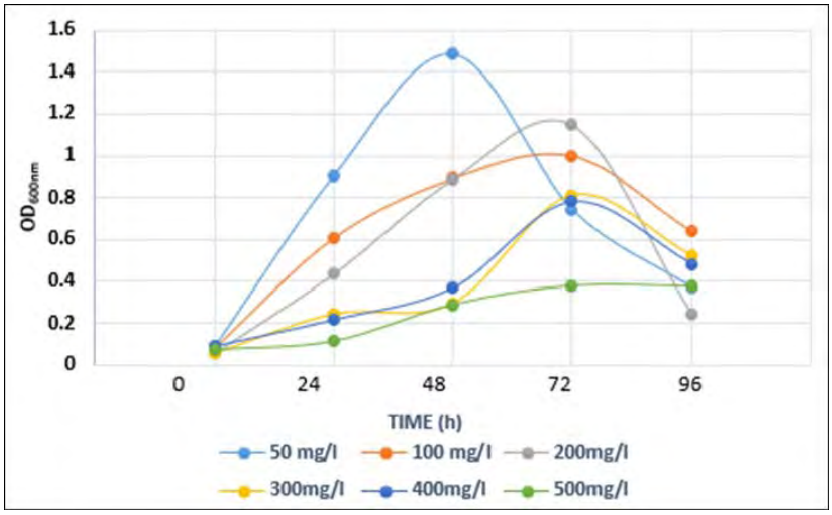


Fig. 1 KTH1 tolerance to different concentrations of heavy metal composites.

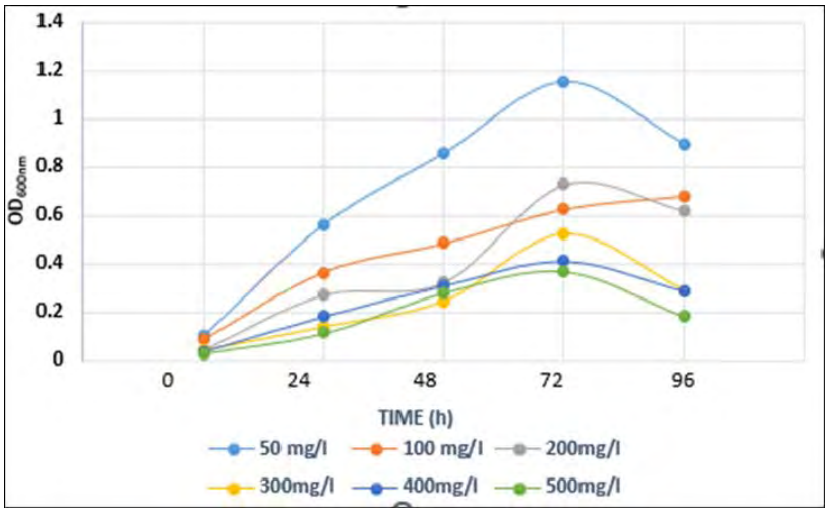


Fig. 2 KTH4 tolerance to different concentrations of heavy metal composites.

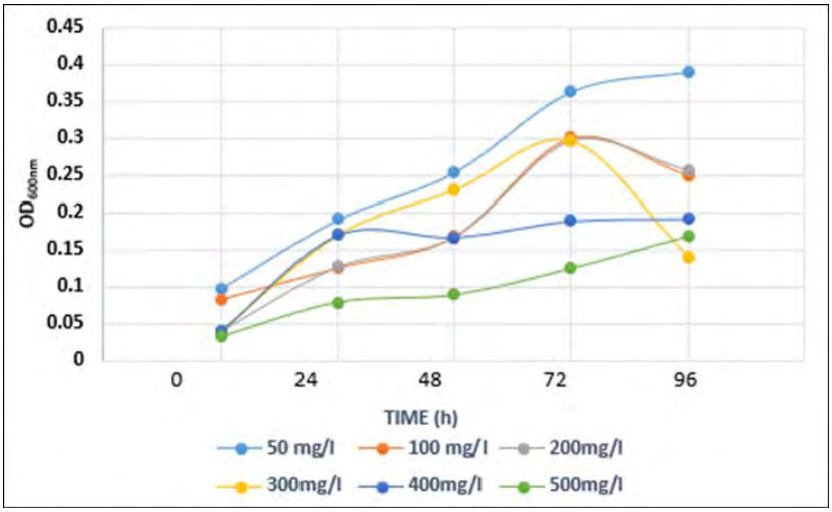


Fig. 3 KTH7 tolerance to different concentrations of heavy metal composites.

Manganese (Mn) is a transition metal, found in several oxidation states, of which the divalent form is found to be the most common as reported (Gadd, 2010). The cell walls of Gram-positive bacteria such as *Bacillus* have a high capacity for metal binding because of their thickness and anionic character (which is mainly due to peptidoglycan, teichoic acid and teichuronic acids) (Beveridge and Murray, 1980). The regulation and removal of manganese by bacteria is mediated by the metal-specific regulators that control the level of Mn inside the cell. Many studies have reported bioremediation of Mn by the *Bacillus* spp (Abioye et al., 2018; Huang et al., 2020). In this study, the isolate KTH1 (*Bacillus* spp 1) was able to tolerate Mn^{+2} upto 400 µg mL^{-1}. Similar results were obtained by studies conducted by Zhenggang et al., (2019) where *Bacillus* cereus strain HM-5 showed that the Mn biosorption capacity of the *Bacillus* strain up to 98.9% at 600 mg L^{-1} initial metal ion concentration. This may be due to the requirement of Mn by the *Bacillus* strain for the growth and modulation of free and labile Mn concentration inside the bacterial cell (Fisher et al., 1973). Bacterial cell regulates the expression of Mn efflux pumps, MneP and MneS when there is an excess of Mn in bacterial cells (Paruthiyil et al., 2020).

Lead (Pb) is a toxic heavy metal that exists in two states: Pb^{2+} and Pb^{4+}. Previous studies have reported Pb-resistant *Bacillus* spp. (Varghese et al., 2012). In an experiment conducted by Chen et al. (2015) *B. thuringiensis* 016 showed highest biosorption capacity of Pb(II) upto 165 mg g^{-1} (dry weight) through batch and microscopic experiments. In this study isolate KTH4 exhibited much higher tolerance to Pb^{+2} upto 400 µg mL^{-1}. *Bacillus* employs the *pbr* operon (Hynninen et al., 2009) and active transport mechanisms to mitigate Pb^{+2} toxic effects (Silver, 1994). Adsorption onto the cell wall is another mechanism used by bacteria. Macromolecules such as polypeptides, polysaccharides, and proteins present on the cell wall adsorb Pb through electrostatic forces, including Van der Waals forces, covalent, or ionic bonds (Qiao et al., 2019). Similar to other Gram-positive bacteria, *Bacillus* species utilize one or several of these methods to remove Pb from contaminated environments (Arifiyanto et al., 2017).

Zinc (Zn) is an essential trace element for all living organisms (Lee, 2018). In bacteria, multiple mechanisms for zinc resistance have been identified. The intracellular regulation of Zn^{2+} ions across the cell membrane involves the Zur/SlyA/MarR family and the MerR (ZntR) and ArsR/SmtB families (Mikhaylina et al., 2018). In *Bacillus subtilis*, the uptake of Zn^{2+} is primarily regulated by the Zur family. However, another uptake system, known as ZosA, a P-type ATPase, becomes active under oxidative stress conditions (Suryawati,

2018). In this study, the isolate KTH7 was able to tolerate Zn^{2+} concentrations up to 400 µg mL^{-1} indicating the presence of some form of zinc resistance mechanism in the bacteria.

3.3 Biochemical characterization of bacterial isolates

From the microscopic and biochemical characterization, all three isolates were Gram-positive bacilli. From the different biochemical tests, it could be speculated that KTH1 is *Bacillus* spp 1, KTH4 is *Bacillus* spp 2 and KTH7 is *Bacillus* spp 3. *Bacillus* is one of the largest bacterial genera and members of this genus show varied results for single biochemical test. Further identification of these isolates by 16S rRNA gene sequencing would allow accurate identification at species level.

3.4 Heavy metal tolerance index of bacterial isolate

The metal tolerance index (TI) is a metric used to identify metal-tolerant organisms (Muñoz et al., 2012). The isolate KTH1 showed the highest tolerance index for 400 µg mL^{-1} and 500 µg mL^{-1}. The KTH7 showed the least tolerance index (Fig. 4). The substantial differences in heavy metal resistance may be attributed to variations in the isolation site and the adaptive mechanisms that the bacteria have developed.

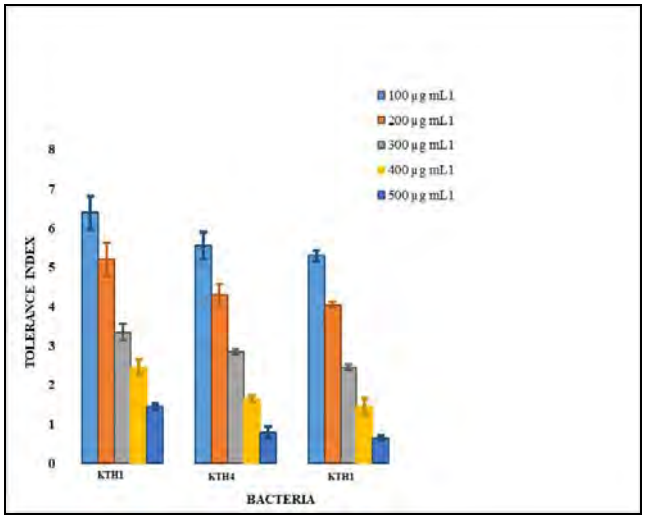


Fig. 4 Tolerance index of selected bacteria to heavy metal tested.

3.5 Plasmid isolation of bacterial isolate

The plasmid profile of the three isolates (Fig. 5) exhibited three bands with the size of 5kb (KTH1), 200bp (KTH4) and 2kb (KTH7).

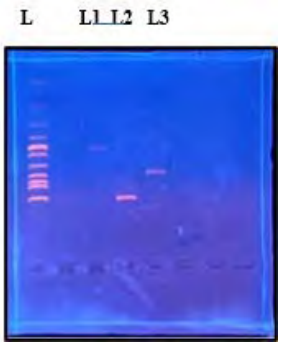
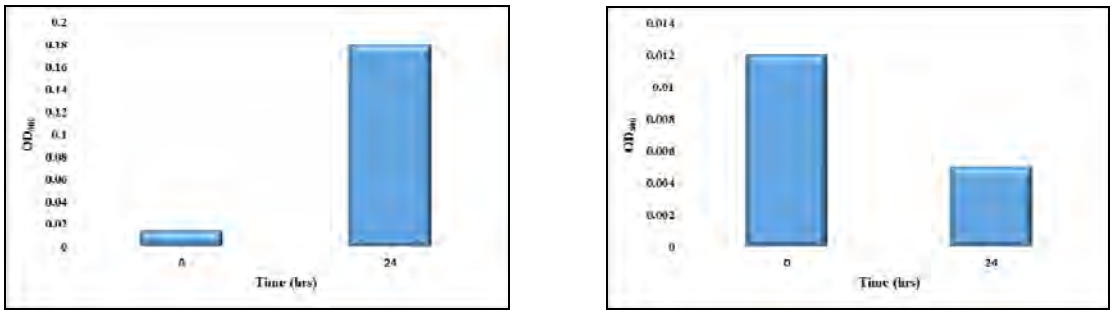


Fig. 5 Electrogram of plasmid profile of the morphotypes (L: Ladder, L1: KTH1, L2:KTH4, L3:KTH7).

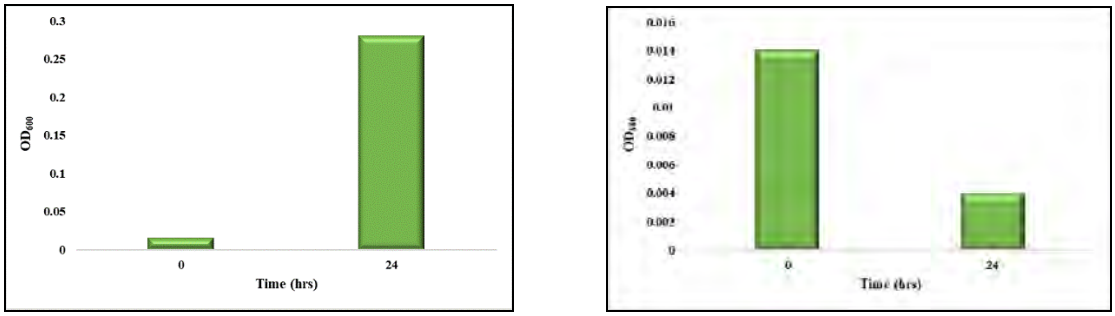
The role of plasmids in conferring resistance to both antibiotics and metals has been previously demonstrated. Each of the three isolates exhibited distinct plasmid bands of varying kb in gel electrophoresis, suggesting that plasmids could potentially carry the resistance mechanisms to heavy metals and antibiotics. Such bacteria could be utilized for detoxification and removal of heavy metals from the contaminated environment (Chen et al., 2015).

To find out the location (chromosomal and extra-chromosomal) of genes responsible for the degradation of heavy metals, we performed a curing experiment with a chemical agent. Based on the plasmid curing experiment, it was found that cured bacterial isolates KTH1, KTH4 and KTH7 could not grow in the presence of 500 mg L^{-1} of heavy metal composite. The growth pattern of KTH1 (O.D. 0.005), KTH4 (O.D. 0.004) and KTH7 (O.D. 0.006) showed a decline phase within 24 hrs of incubation. From Fig. 6a-8a, it is evident that uncured (without EtBr) bacterial isolates KTH1 (O.D. 0.185), KTH4 (O.D. 0.280) and KTH7 (O.D. 0.198) grew in the presence of heavy metal composite and their optical density was increased within 24 hrs. Therefore, curing experiments demonstrated that cured isolates lost their plasmids and corresponded to their heavy metal biodegradation ability (Fig. 6b-8b).



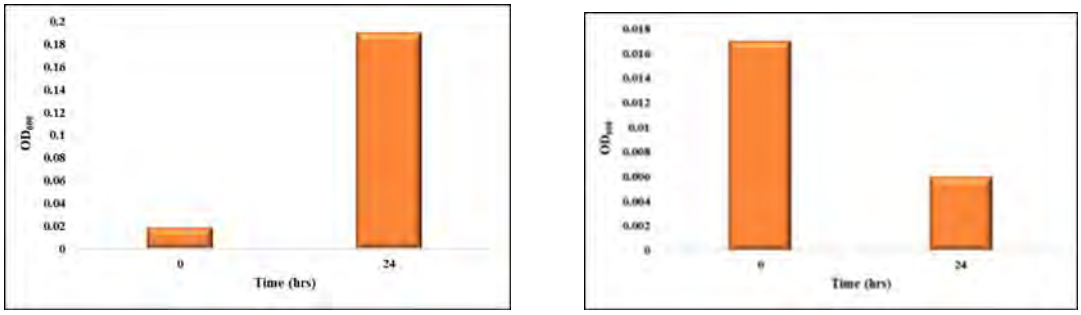
(a) (b)

Fig. 6 Response of KTH1 in presence of heavy metal composite towards (a) uncured and (b) cured plasmid.



(a) (b)

Fig. 7 Response of KTH4 in presence of heavy metal composite towards (a) uncured and (b) cured plasmid.



(a) (b)

Fig. 8 Response of KTH7 in presence of heavy metal composite towards (a) uncured and (b) cured plasmid.

3.6 Antibiotic susceptibility test

The bacterial morphotypes were also subjected to broad-spectrum antibiotic susceptibility tests to decipher the antibiotic resistance profile of heavy metal resistant bacteria. The association between heavy metal and antibiotics resistance was already reported in the literature. Results listed in Table 2 revealed that metal-resistant isolates were also resistant to different antibiotics, which is plasmid-borne.

Table 2 Antibiotic resistance profile of the bacterial morphotypes.

S. No	Antibiotics	Isolates		
		KTH1	KTH4	KTH7
1	PIZ	S	S	S
2	AMC	R	S	R
3	GEN	S	S	S
4	CLR	I	R	S
5	CTX	R	S	I
6	FAR	I	R	R
7	AK	S	S	I
8	NX	S	R	S
9	LZ	R	R	S
10	CIP	S	R	S
11	CD	R	S	R
12	DO	S	I	S
13	LE	S	I	S
14	CFS	S	S	S
15	OF	S	R	S

*R: Resistant, S: Sensitive, I: Intermediate

The antibiotic sensitivity profile of the three bacterial isolates, KTH1, KTH4, and KTH7, showed variable resistance and susceptibility. KTH4 was found to have the highest resistance to 6 out of the 15 antibiotics used in testing: CLR (Clarithromycin), FAR (Fusidic Acid), NX (Norfloxacin), LZ (Linezolid), CIP (Ciprofloxacin) and OF (Ofloxacin). KTH1 resisted 4 antibiotics: AMC (Amoxicillin), CTX (Cefotaxime), LZ (Linezolid) and CD (Clindamycin). KTH7 showed the least resistance to 3 antibiotics: AMC, FAR, and CD. All of the isolates are susceptible to PIZ, GEN, and CFS. The kind of resistance observed, as evidenced in KTH4, may indicate the presence of genes that mediate this resistance on transmissible genetic elements which in some instances may also carry both heavy metal and antibiotic resistance genes. This may pinpoint the role that environmental factors play in the development of antibiotic resistance. The increasing use of antibiotics in therapeutics and agriculture has resulted in the realization of increasing antibiotic-resistant genes (ARGs). However, more evidence has showed that the dissemination of ARGs can also be influenced by heavy metal contamination (Knapp et al., 2017; Tan et al., 2018). As early as in the 1970s, it had been found that heavy metal resistance and antibiotic resistance can be selected simultaneously in the heavy metal contaminant ecosystem due to as co-selection or cross-selection (Seiler and Berendonk, 2012).

Antibiotics resistance is a multifaceted global problem (Iskandar et al., 2021). The misuse of antibiotics has resulted in the emergence of antimicrobial drug resistance and antibiotics resistance genes can be transferred to other hosts in the environment (Bengtsson-Palme et al., 2021). In this study, the test isolates exhibited multidrug resistance to the antibiotics and these isolates are primarily associated with causing diseases in humans within clinical settings. Heavy metal accumulation in the environment triggers a co-selection mechanism with antibiotics (Zhong et al., 2021). Isolation of *Bacillus* spp. from two major rivers in Bangladesh showed resistance to ceftazidime, ceftriaxone, tetracycline, ampicillin and nalidixic acid in various degrees and were also tolerant to heavy metals such as lead, chromium, zinc and copper. Similar to this report, isolates in this study that were tolerant to heavy metal composite were also resistant to gentamycin and kanamycin (Shammi and Ahmed, 2016). This might be due to the presence of genes encoding for heavy metal resistance are located on the same plasmids along with antibiotic resistance genes. Thus, selective pressure from one compound might indirectly promote the selection of the entire set of resistances. Alternatively, bacteria might have unspecific resistance mechanisms common to different substances including heavy metals and antibiotics (Mgbemena et al., 2012). Further investigation of these *Bacillus* isolates, which are resistant to heavy metals and antibiotics, at the molecular level would provide insight into the likely mechanisms involved**.**

4 Conclusions

With the increased concentration of the metal, the toxic effect of the metal also increased. In our study, the order of resistance to heavy metals shown by different isolates was in the order of Pb > Zn > Mn. This hierarchy thus indicates that lead showed the highest toxicity, followed by zinc and then manganese. Amongst the tested isolates, the isolated *Bacillus* spp. 1 and *Bacillus* spp. 2 showed maximum tolerance towards the heavy metal consortia. In these *Bacillus* strains, resistance may occur due to the presence of systems tending to decrease the metal toxicity by efflux systems, metal sequestration, or enzymatic detoxification. Given the resilience of these Bacillus isolates, there is great potential use for them in bioremediation applications within aquatic environments contaminated with multiple toxic metals. That is particularly important, as environmental heavy metal contamination is in complex mixtures rather than single metals. This potential is of importance specifically since *Bacillus* spp 1 and *Bacillus* spp 2 can thrive in and remediate such conditions, hence making them quite useful in the development of suitable strategies toward mitigating heavy metal pollution in aquatic systems

Acknowledgement

The author is thankful to the Department of Biotechnology, Barkatullah University for providing the research infrastructure and financial support respectively.

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