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Characterization and Evaluation of the Nickel-Removal Capacity of *Kluyvera cryocrescens* M7 Isolated from Industrial Wastes

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ABSTRACT

Heavy metal contamination poses grave risks to all kinds of life. The fastest growing automotive, electroplating, and battery industries release the most common heavy metal, Nickel, into the environment, which has lethal impacts on human health. Our research aims to find Ni-resistant bacteria in the metal-contaminated soil that have a great potential for removing Ni from the environment. Attempts have been made to extract and characterize Ni-resistant bacteria from automobile and electroplating industry waste-contaminated soil using serial dilution, streak plating, and various morphological, biochemical, and genetic techniques. The maximum tolerable concentration of Ni and other heavy elements, such as cadmium, lead, and aluminium for the selected isolate, was investigated using the UV-Vis spectrophotometric method. Additionally, the bacterial strain's ability to remove Ni was assessed using an atomic absorption spectrophotometer. The current research reveals a novel strain of *Kluyvera cryocrescens* that could withstand Ni, Cd, Pb, Al, and combinations of these heavy metals. The maximum tolerance concentration of *K. cryocrescens* M7 for Ni, Cd, Pb, and Al was found to be 150 ppm, 200 ppm, 1000 ppm, and 150 ppm, respectively. Additionally, it was also observed that the bacterial strain could remove Ni by 29.57%, 35.36%, 48.41%, 46.91%, and 44.88% after 12, 24, 48, 72, and 96 hours, respectively. The strain has also exhibited resistance to vancomycin, ampicillin, carbenicillin, and streptomycin. This research discovered a novel bacterial strain, *K. cryocrescens* M7 that may be beneficial for removing heavy metals, particularly Ni, from metal-contaminated soil.

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INTRODUCTION

The 21st century is seeing the onset of the Industrial age, and as a result, numerous pollutants are being discharged into the air, water, and land, which is having an adverse effect on our ecosystem (Ashraf et al., 2019). Heavy metals, such as such as nickel (Ni), cadmium (Cd), lead (Pb), aluminium (Al), mercury (Hg), arsenic (As), copper (Cu), lithium (Li), zinc (Zn), and silver (Ag) are the most menacing ones as these are extremely toxic to the environment and they keep on accumulating in our food chain (Raimi et al., 2022; Adiama et al., 2022). A well-known heavy metal, Ni, is found in the environment at low concentrations but is extremely toxic. Environmental pollution has, however, been greatly exacerbated by the extensive industrial uses of Ni during its manufacture, recycling, and disposal (Gupta et al., 2016; Raimi et al., 2022; Olalekan et al., 2022). Ni is released into the environment either by the volcanic eruption, Ni mining, or from various industries such as stainless-steel manufacturing plants, electroplating industries, rubber and plastic industries, power plants or incinerators, and Ni-Cd battery industries (Meshram and Pandey, 2018; Suhani et al., 2021).

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Heavy metals, such as Ni produce reactive oxygen species, which comprise superoxide, hydrogen peroxide, and hydroxyl radicals (Tietze et al., 2017). The free radical formed causes DNA damage, lipid peroxidation, and alteration in protein conformation, which results in various Ni-induced pathophysiological changes in human beings and plants (Sharifi-Rad et al., 2020). Chronic exposure to Ni metal can cause a variety of negative effects in humans, like bronchitis, immunotoxicity, neurotoxicity, genotoxicity, infertility, and skin diseases (Genchi et al., 2020; Raimi et al., 2022; Olalekan et al., 2022). High-level inhalation of the Ni metal is carcinogenic to humans and causes lung, nasal, kidney, and liver cancer (Raimi and Sawyerr, 2022). It also accumulates in different plant tissues and affects their growth and development by interfering with many metabolic functions, such as inhibition of respiration and photosynthesis, and degeneration of main cell organelles that cause stunted growth, chlorosis, delayed germination, senescence, loss of enzyme activities, and reduced crop yield (Jamla et al., 2021; Raimi et al., 2022).

Elevated concentrations of many toxic metals in the environment lower crop production and soil microbial activity, putting human health at risk via the food chain (Yaashikaa et al., 2022). In light of the adverse effects of metal poisoning, prompt action is required to address the detoxification of heavy metals (Afolabi and Raimi, 2021). To address these issues, various techniques including physical methods (filtration, ion exchange, adsorption, magnetic separation, flocculation, and coagulation) and chemical methods (metal precipitation, reverse osmosis, neutralization, solvent extraction, and electrochemical treatment) are used (Torres, 2020). However, most of these have their own drawbacks because they generate a lot of hazardous sludge, are typically quite expensive, and are ineffective when metal concentrations are low (Mathivanan et al., 2021). Bioremediation is generally recognized as the most widely used technique for removing heavy metal toxicity from the environment since it is more economically advantageous and eco-friendly than conventional chemical and physical techniques (Shome, 2020; Bisht and Kumar, 2022). Numerous studies demonstrated the bioremediation potentiality of various bacteria, including *Klebsiella* sp., *Escherichia coli*, *Bacillus* sp., *Staphylococcus* sp., and *Pseudomonas* sp. against different heavy metals, such as Hg, Pb, Cd, Al, As, Ni, Zn, and Cu (Nwagwu et al., 2017; Bukowski et al., 2019; Nath et al., 2019; Vélez et al., 2021; Khan et al., 2022; Feruke-Bello et al., 2022). There are very limited studies on metal resistance in *Kluyvera* species and their application in the bioremediation of metal-contaminated soil. The genus *Kluyvera* is comprised of four species: *Kluyvera cryocrescens*, *Kluyvera georgiana*, *Kluyvera ascorbata*, and *Kluyvera cochleae*. *Kluyvera* spp. have a similar phenotype to *E. coli* but are citrate positive (Watson et al., 2018). Burd et al. (2000) claimed that *K. ascorbata* is capable of removing heavy metals from the environment and is resistant to Ni and other heavy metals. The bacteria *K. intermedia*, which is extremely resistant to Pb, was isolated from soil samples and demonstrated resistance to a number of other metals, including Ni, Cd, Ba, Cu, Zn, Cr, and Pb (Boechat et al., 2018).

K. cryocrescens is known for its capability to remove oil and 2,4,6-trinitrotoluene, but its capacity to remove Ni has not yet been tested (Gumuscu et al., 2015; Gao et al., 2019). Therefore, the Ni tolerance and removal capacity of *K. cryocrescens* M7 were examined in the current study. The bacteria was also investigated for its resistance capacity to Ni in combination with other metals (Pb, Cd, and Al). As metal resistance is frequently associated with antibiotic resistance, the organism's antibiotic resistance profile was examined too.

MATERIALS AND METHODS

The research methodology included the collection of samples, the isolation of pure bacterial colonies, the screening for Ni-tolerant bacterial isolates, the identification of isolates using morphological and biochemical tests, the analysis of the 16S rDNA sequence, as well as further characterization of the strain for Ni-metal tolerance and removal. The entire research

project was completed over a period of four years and seven months. For isolating Ni resistant bacteria, the soil sample was retrieved from a metal-contaminated waste disposal site near an electroplating and automobile workshop in Punjab, India. The sample was taken and sent to the lab in sterilized plastic bags, where it was stored at 4°C for further examination. Bacterial strains were isolated using the serial dilution technique on Luria agar (LA) plates supplemented with Ni in the form of their salts, $NiCl_2.6H_2O$. Incubation was done in the incubator shaker (Lab-Therm) for 24 hours at 37°C. Plate streaking was carried out repeatedly until morphologically distinct colonies were observed and acquired as pure cultures. All isolates were further screened by observing their tolerance (growth) in Luria-bertanii (LB) media supplemented with different concentrations of Ni (25 ppm, 50 ppm, and 75 ppm). The selected bacterial strains were kept as glycerol stocks at -80°C for future characterization.

Selected bacterial isolates underwent morphological, biochemical, and molecular tests to determine their genus and species. The biochemical analysis included the catalase test, oxidase test, gelatin utilization test, starch hydrolysis test, glucose utilization test, and citrate utilization test. The morphological analysis included the genus identification based on morphology, surface, color, motility, and gram staining. The bacteria were identified at the species level using molecular techniques, such as 16S rRNA gene sequence analysis. Genomic DNA from the isolates was extracted using the modified Rapid One-Step Extraction technique (Steiner et al., 1995). The 16S rRNA gene region of DNA was amplified by using universal primers (8f-5' AGA GTT TGA TCC TGG CTC AG 3' and 1492r-5' CGG TTA CCT TGT TAC GAC TT 3'). The setup of PCR program was as follows: pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, and post extension at 72°C for 7 minutes. The steps from denaturation to extension were repeated 35 times. The Gene AMP PCR System 2720 (Applied BioSystems, California, USA) was used to amplify DNA fragments, and the results were then further analyzed by electrophoresis on a 1.0% w/v agarose gel. The PCR products were sent to Amnion Biosciences in Bangalore, India, for sequencing. The resulting sequences were then checked against existing sequences in the GenBank database using the Seq-Man V 4.1 program (Swindell and Plasterer, 1997). Additionally, MEGA 11.0 software's neighbor-joining method was used to develop the phylogenetic tree (Tamura et al., 2021).

To determine the selected bacterial strain's resistance against Ni and other heavy metals, a stock solution of each metal salt (Ni, Pb, Cd, and Al) was prepared and then diluted to the appropriate concentrations. The selected bacterial strain was streaked from log phase culture on LA plates and incubated at 37°C for 24 hours. The bacterial colonies were then inoculated into separate culture medium (LB) supplemented with different quantities of Ni (50 ppm, 75 ppm, 100 ppm, 150 ppm, and 200 ppm); Pb (250 ppm, 500 ppm, 750 ppm, 1000 ppm, and 1250 ppm); Al (50 ppm, 75 ppm, 100 ppm, 150 ppm, and 200 ppm); Cd (50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm). As a control, the culture medium without metal was used to cultivate the bacterial isolate. Each set, including the control set was incubated at 37°C with agitation (150 rpm). A UV-VIS spectrophotometer (HITACHI U-2900) was used to measure optical density (O.D.) at various time intervals, such as 0, 12, 24, 48, 72 and 96 hours. The maximum tolerance concentration (MTC) of the bacterial strain was observed for Ni, Cd, Pb, and Al. The relative growth (%) of the bacterial strain with respect to the control (bacterial strain without metal) was also investigated. Further research into the effects of other metal stress was conducted using an isolate that had been selected for its resistance to Ni metal. At 37°C, culture was grown in LB media supplemented with various concentrations of heavy metals such as Ni+Cd, Ni+Al, Ni+Pb, NI+Al+Pb, Ni+Al+Cd, Ni+Cd+Pb, and Ni+Al+Cd+Pb. Using a UV-VIS spectrophotometer, OD600 readings were acquired at various time intervals, including 0, 12, 24, 48, 72 and 96 hours.

The accumulation or uptake of Ni in the cellular parts of the selected isolate was determined using the method followed by Mwandira et al. (2020) with few modifications. The overnight

culture was inoculated into LB media with 100 ppm Ni and incubated at 37°C in a shaking flask incubator. 10 ml of sample was taken at 12, 24, 48, 72 and 96 hours and cells were harvested at 8000Xg for 10 minutes. The supernatant was digested with 3 ml concentrated HNO_3, diluted with MilliQ water and analysed in Flame Atomic Absorption Spectrophotometer (FAAS) (Agilent Technologies). The pellet was first washed with STE buffer (5 M NaCl , 1 M TrisCl pH-8.0, and 0.5 M EDTA pH-8.0), then incubated at 90°C for 30 minutes after again adding STE buffer and 10% SDS. Ultrasonication (30 seconds pulse, 30 seconds pause for 3 minutes) was also performed, followed by 15 minutes of centrifugation at 8000Xg to separate cell precipitate (cell debris: cell wall/membrane) and supernatant (cell-free extract: cytoplasm). The control used was LB media + Ni. The Ni content of both digested cellular fractions was measured with FAAS at 232 nm, and air-acetylene flame. Subsequently, the Ni removal efficiency of *K. cryocrescens* M7 was calculated using the formula:

$$\textit{Metal removal efficiency (\%)} = [(Ci - Cf)/ Ci] \times 100$$

where *Ci* and *Cf* are the initial and final concentrations of Ni in LB media, respectively.

The experiment for heavy metals tolerance and removal was reproduced three times under the same circumstances in order to obtain a high degree of reliability on the obtained data. The standard deviation was calculated as the square root of the variance by computing the deviation of each data point from the mean. Microsoft Excel 2016 was used to compute the standard deviation. Data was represented as Mean±Standard deviation.

The disk diffusion method was used to examine antibiotic resistance for the chosen isolate against seven different antibiotics. (Bauer et al., 1966). In this study, antibiotic discs, such as Streptomycin (10 mcg), Vancomycin (30 mcg), Tetracycline (30 mcg), Kanamycin (30 mcg), Carbenicillin (100 mcg), Ampicillin (10 mcg), and Chloramphenicol (30 mcg) were used. These disks were put on fresh lawns of the isolate on LA plates using sterile swabs. Incubation was done at 37°C for 24 hours. The resultant zone of inhibition was measured in millimetres (mm) and classified as resistant (R), intermediate (I), or susceptible (S) according to the Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2018).

RESULTS AND DISCUSSION

The present study identified metal-tolerating bacterial isolates from the soil samples collected from electroplating and automotive waste disposal sites. A total of 29 bacterial isolates were obtained from LA plates inoculated with 25 ppm Ni on the basis of the morphologically distinct colonies. Among the 29 isolates screened in liquid medium (LB) for their growth on different concentrations of Ni (25 ppm, 50 ppm and 75 ppm), only seven bacterial isolates showed tolerance with higher concentration of Ni-75 ppm. Several morphological and biochemical tests were done to characterize these seven isolates. Microscopic examination showed that each isolate was gram-negative, rod-shaped, aerobic, and motile. Having two membrane layers may make gram-negative bacteria more resistant to pollutants than gram-positive bacteria (Murínová and Dercová, 2014). Biochemical analysis revealed a positive reaction for the catalase test, starch hydrolysis, and citrate utilization tests for all the isolates. Except for isolate M3, the glucose utilisation test was also found to be positive. Negative reactions were obtained for the oxidase test and gelatin hydrolysis tests, but isolate M3 showed opposite results (Table 1). The negative oxidase test differentiates the families of *Enterobacteriaceae* from *Pseudomonadaceae* and *Pasteurellaceae.* Therefore, it was concluded that isolates M1, M2, M4, M5, M6, and M7 belong to the *Enterobacteriaceae* family and M3 belongs to the *Pseudomonadaceae* family based on morphological and biochemical tests.

In addition to the conventional phenotypic and biochemical methods, PCR amplification of

Table 1. Morphological and Biochemical characteristics of isolated bacterial strains.

Morphological & Biochemical Tests	Isolates						
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**
Color	Off-white	Off-white	White	White	White	White	Off-white
Surface	Rough	Rough	Glistening	Shiny	Shiny	Shiny	Rough
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram's Staining	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Growth condition	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Motility	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Oxidase	–	–	+	–	–	–	–
Citrate utilization	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Gelatin hydrolysis	–	–	+	–	–	–	–
Glucose utilization	+	+	–	+	+	+	+

Different morphological and biochemical tests were done to identify the bacterial isolates M1-M7. The results were analyzed i-n accordance with the Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). +: positive reaction; - : negative reaction

the 16S rRNA gene and its sequencing is an important method for the identification of bacteria at the species level. Genomic DNA was extracted from all the isolates and used as template for amplification of 16S rRNA gene using 8f and 1492 primers. Around 1.5 kb band was observed on 1.0% agarose gel (Figure 1).

Sequence analysis revealed that isolates M1, M2, M3, M4, M5, M6 and M7 was regarded as *Cedecea davisae* M1, *Citrobacter freundii* M2*, Pseudomonas* sp.M3*, Enterobacter* sp. M4*, Enterobacter* sp. M5, *Enterobacter* sp. M6*,* a*nd Kluyvera cryocrescens* M7, respectively. The 16S rRNA gene sequences of the bacterial strain M1, M2, M3, M4, M5, M6, and M7 have been submitted in the GenBank under accession numbers KF146959.1, KF146956.1, KF146957.1, KF146960.1, KF146961.1, KF146958.1, and KF146962.1, respectively. A phylogenetic tree was also made based on neighbor-joining method, showing relationships between selected isolates and their closest database relatives with Genbank accession numbers using MEGA 11.0 software (Figure 2). It was also evident from the phylogenetic analysis that isolates M1, M2, M3, M4, M5, M6 and M7 were closely related to *C. davisae* M1, *C. freundii* M2*, Pseudomonas* sp.M3*, Enterobacter* sp. M4*, Enterobacter* sp. M5, *Enterobacter* sp. M6*,* a*nd K. cryocrescens* M7, respectively.

K. cryocrescens M7 strain was chosen for further research among the seven metal-tolerant isolates since there hasn't been much study on its resistance to heavy metals and removal from the environment. The strain was evaluated for its ability to withstand various concentrations of Ni as well as other metals like Cd, Al, and Pb. The *K. cryocrescens* M7 was found to have a maximum tolerance for Ni, Cd, Pb, and Al of 150 ppm, 200 ppm, 1000 ppm, and 150 ppm, respectively (Figure 3).

In case of Ni, as the MTC of *K. cryocrescens* M7 was 150 ppm, the strain showed relative growth of 56.33%, 59.12%, 68.34%, 68.25%, and 67.01% at 12, 24, 48, 72, and 96 hours respectively as compared to the control. When the concentration has increased to 200 ppm of

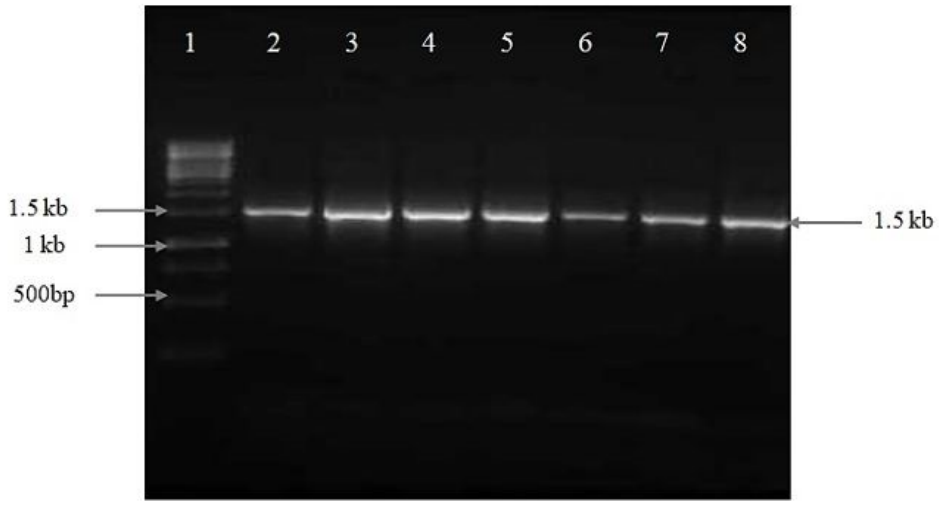

Fig. 1. PCR amplification of 16srRNA gene of bacterial isolates M1-M7
Agarose gel electrophoresis of PCR amplified products of 16 S rRNA gene of the isolates. Lane1: 1kb DNA ladder SM0312 (Thermo scientific); Lane 2, Lane 3, Lane 4, Lane 5, Lane 6, Lane 7, Lane 8: Isolate M1, M2, M3, M4, M5, M6, M7.

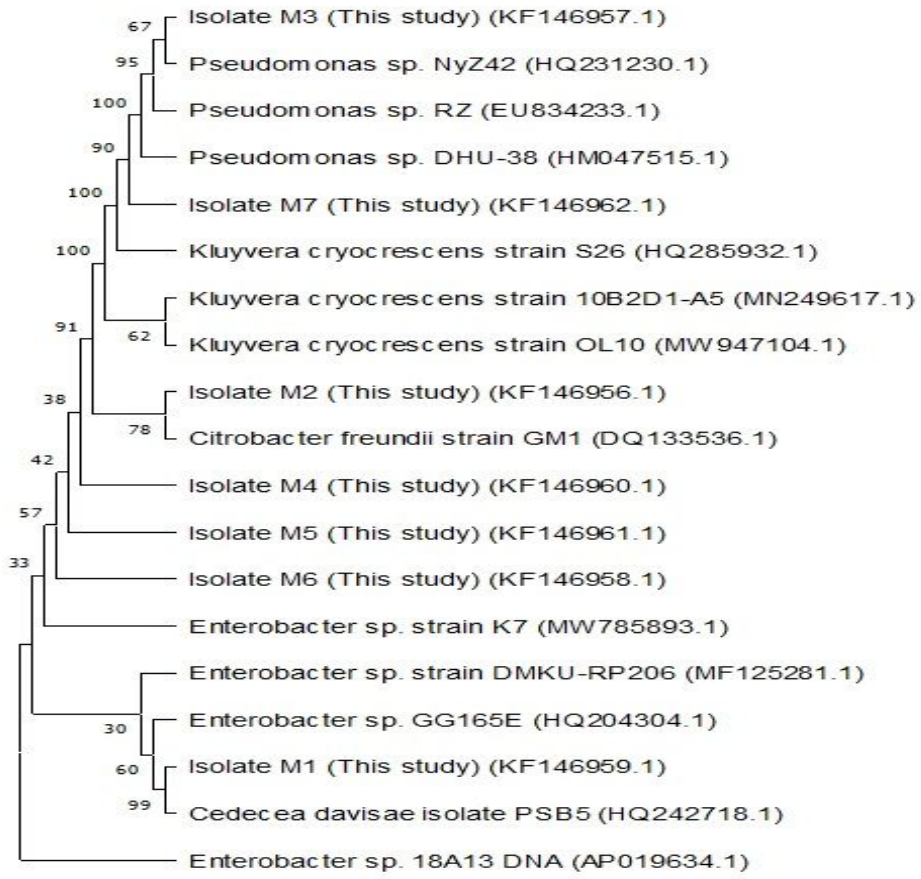

Fig. 2. Phylogenetic neighbor-joining tree of 16S rRNA gene sequence of the isolates M1-M7.
Sequence of 16S rRNA of isolates M1-M7 was analyzed using NCBI BLAST and its phylogenetic relatedness was studied. Dendrogram was constructed based on neighbor-joining method with 1000 replicates.

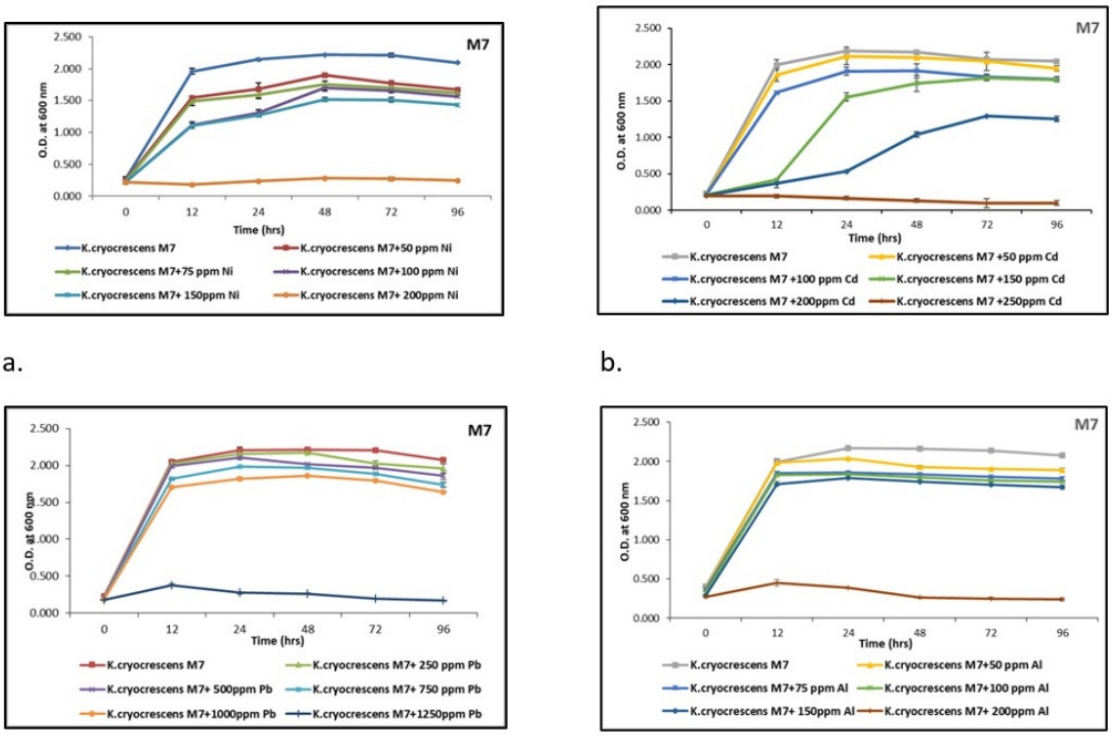

Fig. 3. The growth of *K. cryocrescens* M7 monitered at various concentrations of metals (a.) observed growth in the presence of 0-200 ppm Ni; (b.) observed growth in the presence of 0-250 ppm Cd; (c.) observed growth in the presence of 0-1250 ppm Pb; (d.) observed growth in the presence of 0- 200 ppm Al. The graphs represent mean results from the value of triplicate readings with error bars indicating standard deviations (n=3).

Ni in the culture medium, the relative growth drastically reduced to 9.14%, 10.98%, 12.55%, 12.26%, and 11.60% at 12, 24, 48, 72, and 96 hours respectively. In case of Cd, the relative growth of the strain found to be 18.49%, 24.42%, 47.97%, 62.27%, and 61.27% at 200ppm, which was decreased to 9.78%, 7.66%, 6.14%, 4.77%, and 4.99% at 250 ppm in time intervals of 12, 24, 48, 72, and 96 hours respectively. When the culture medium was supplemented with Pb, the relative growth at 1000 ppm was 83.22%, 82.49%, 83.81%, 81.39%, and 79.02%, which was dropped to 18.41%, 12.46%, 11.97%, 8.84%, and 8.08% at 1250 ppm in 12, 24, 48, 72, and 96 hours respectively. In case of Al, at 150 ppm, the relative growth of the bacterial strain was observed as 85.99%, 82.26%, 80.63%, 79.66%, and 79.60% at 12, 24, 48, 72, and 96 hours respectively, while at 200 ppm, the growth was greatly decreased to 22.57%, 17.72%, 12.31%, 11.81%, and 11.66% at 12, 24, 48, 72, and 96 hours, respectively (Table 2).

Furthermore, the toxic effects of Ni on bacteria were also observed when Pb, Cd, and Al were present in various combinations. In the presence of a higher concentration of two-metal mixture, i.e. 100 ppm Ni + 100 ppm Cd and 100 ppm Ni + 500 ppm Pb, the lag phase was also significantly prolonged and was observed for up to 48 hours. In case of 100 ppm Ni + 100 ppm Al, the strain showed lag phase up to 12 hours as Al is comparatively less toxic. In triple and quadruple combinations of heavy metals such as 75 ppm Ni + 75 ppm Cd + 75 ppm Al; 75 ppm Ni + 75 ppm Cd + 500 ppm Pb; 75 ppm Ni + 75 ppm Al + 500 ppm Pb; and 75 ppm Ni + 75 ppm Cd + 75 ppm Al + 500 ppm Pb, the bacterial strain showed tolerance to lower concentrations and therefore, it exhibited shorter lag phase upto 12 hours. The declining phase of bacterial growth was started after 72 hours (Figure 4).

Table 2. The percentage relative growth of *K. cryocrescens* M7 for different heavy metals

Heavy Metals	Metal conc. (ppm)	Relative growth (%)				
		12 hours	24 hours	48 hours	72 hours	96 hours
Nickel	50	78.70	78.39	85.66	80.19	79.75
	75	76.05	74.15	79.12	76.89	77.13
	100	58.48	60.80	76.45	74.94	73.60
	150	56.33	59.12	68.34	68.25	67.01
	200	9.14	10.98	12.55	12.26	11.60
Cadmium	50	93.17	96.60	96.68	98.55	95.23
	100	80.89	87.32	88.43	88.22	87.94
	150	20.86	71.14	80.42	87.55	87.42
	200	18.49	24.42	47.97	62.27	61.27
	250	9.78	7.66	6.14	4.77	4.99
Lead	250	98.96	97.90	97.97	91.62	94.27
	500	97.18	95.66	90.95	89.19	89.75
	750	88.85	89.89	88.90	85.45	83.64
	1000	83.22	82.49	83.81	81.39	79.02
	1250	18.41	12.46	11.97	8.84	8.08
Aluminium	50	99.58	93.65	89.14	89.27	90.95
	75	92.98	85.42	84.89	84.41	85.97
	100	91.83	84.61	83.02	82.39	83.83
	150	85.99	82.26	80.63	79.66	79.60
	200	22.57	17.72	12.31	11.81	11.66

The growth of *K. cryocrescens* M7 was measured as compared to the control (*K. cryocrescens* M7 without metal) for different heavy metals- Ni, Cd, Pb, and Al at different concentrations in various time intervals- 12, 24, 48, 72, and 96 hours.

When *K. cryocrescens* M7 was subjected to 100 ppm Ni stress, the highest Ni removal efficiency was reported. Bacteria exposed to 100 ppm Ni were observed to eliminate 29.57%, 35.36%, 48.41%, 46.91%, and 44.88% of Ni after 12, 24, 48, 72 and 96 hours, respectively. The maximal effectiveness of elimination was recorded at 48 hours. By analyzing the cellular fractions, it was discovered that at 48 hours, amount of Ni uptake by cytoplasmic fraction was 36.31 ppm and by pellet fraction was 12.10 ppm. After 48 hours, removal efficiency started decreasing (Table 3).

Antibiotic resistance was studied for the bacteria *K.cryocrescens* M7 using the disk-diffusion method. The bacterial strain showed resistance to carbenicillin (100 mcg), ampicillin (10 mcg), streptomycin (10 mcg), vancomycin (30 mcg), and intermediate resistance to kanamycin (30 mcg). It was also found to be susceptible to tetracycline (30 mcg), and chloramphenicol (30 mcg) (Table 4).

Heavy metal pollution of soil and wastewater is a serious environmental issue, which needs to be addressed and resolved. The present study discovered a new strain of *K. cryocrescens* M7, which has tolerated high concentrations of Ni-150 ppm (2.56 mM), Pb-1000 ppm (4.83 mM), Al-150 ppm (5.56 mM), and Cd-200 ppm (1.79 mM) and also showed cross-resistance to heavy metals. At an initial Ni concentration of 100 ppm, the bacterial strain achieved a maximum Ni removal efficiency of 48.41% in 48 hours. The relative growth of *K. cryocrescens* M7 was 68.34% (48 hours), 62.27% (72 hours), 83.81% (48 hours), and 85.99% (24 hours) with Ni, Cd, Pb, and Al respectively, at their highest tolerance concentrations. The current finding demonstrated that Ni and Al were more tolerable at lower concentrations than Cd and Pb. This could be due to *K. cryocrescens* M7 having a high efflux rate or a better adsorption capacity for Cd and Pb at its surface. Because of the aforementioned reasons, Ni was more toxic to the bacterial strain than Pb

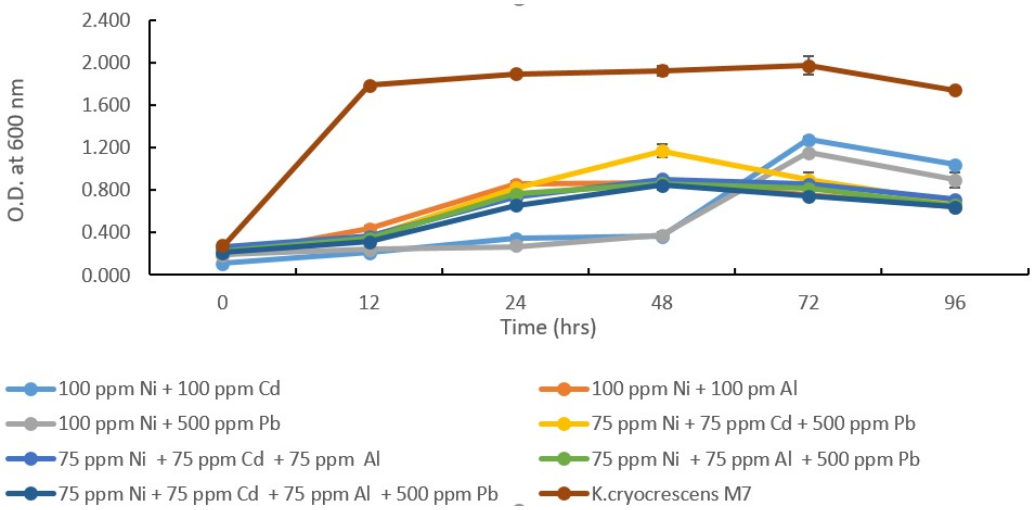

Fig. 4. The growth of *K. cryocrescens* M7 in different combinations of metals
The graph represents mean results from the value of triplicate readings with error bars indicating standard deviations (n=3).

Table 3. Removal efficiency of Ni by Flame Atomic absorption spectrophotometer

Time (hours)	Ni uptake (cytoplasm) (ppm)	Ni uptake (cell wall/ membrane) (ppm)	Removal efficiency (%)
12	23.44±1.14	6.13±0.86	29.57
24	26.22±2.40	9.14±1.23	35.36
48	36.31±1.13	12.10±1.54	48.41
72	35.24±4.06	11.67±0.81	46.91
96	34.07±1.39	10.81±1.38	44.88

The Ni uptake in different cellular fractions of *K. cryocrescens* M7 was estimated by using FAAS. The removal efficiency of Ni at 100 ppm was observed at different time intervals- 0, 12, 24, 48, 72, and 96 hours. Control used was LB+100 ppm Ni. The results were shown as the mean of the triplicate values ± standard deviation. Ni: Nickel; ppm: parts per million

Table 4. Antibiotic resistance profile of *K. cryocrescens* M7

Antibiotics	Zone of Inhibition (mm)	Interpretative criteria for *Enterobacteriaceae* according to CLSI Standard		
		Sensitive (mm or more)	Intermediate (mm)	Resistance (mm or less)
Vancomycin (30 mcg)	**(R)**	17	15-14	13
Tetracycline (30 mcg)	**19 (S)**	15	12-14	11
Kanamycin (30 mcg)	**14 (I)**	18	14-17	13
Ampicillin (10 mcg)	**(R)**	17	14-16	13
Carbenicillin (100 mcg)	**(R)**	-	-	-
Streptomycin (10 mcg)	**10 (R)**	15	12-14	11
Chloramphenicol (30mcg)	**19 (S)**	18	13-17	12

The bacterial strain was analysed to find resistance, intermediate resistance, or susceptibility towards different antibiotics using disk-diffusion method.
Diameter of disks: 6mm; NI: No zone of inhibition; S: Sensitive; I: Intermediate; R: Resistant; mm: millimetres; mcg: microgram; CLSI: Clinical Laboratory Standard Institute; Zone of inhibition: diameter of the zone along with the disk.

and Cd. The justification is supported by the report, which showed that microorganisms utilize a variety of defence mechanisms against heavy metal toxicity (Mathivanan et al., 2021; Bisht and Kumar, 2022). The common mechanisms of resistance to heavy metals are the entrapment of heavy metals by negatively charged groups like hydroxyl, carboxyl, and phosphoryl, as well as extracellular polymers like proteins and polysaccharides on the cell surface, and the presence of efflux transporters at the membrane of microorganisms (Syed et al., 2021).

The MTC of heavy metals by *K. cryocrescens* M7 was found to be greater in comparison to the findings of Jeevaraj et al. (2022), who reported a *Bacillus thuringiensis* strain resistant to Ni, Cd, and Pb at a concentration of 2.0 mM. On the contrary, certain bacteria, such as *Pseudomonas aeruginosa*, showed higher level of tolerance to Ni (5 mM) on solid media plates (Haroun et al., 2017). Due to the polymeric nature of the solid agar medium, the tolerance concentrations of metals by bacterial strains were higher in solid media than in liquid media, according to the research (Paul and Mukherjee, 2016). The removal efficiency of *Pseudomonas aeruginosa* strain RA-14 was found to be 74.3% at an initial Ni^{2+} concentration of only 2.0 ppm (Al-Ansari et al., 2021). In addition, 38% Ni removal efficiency of *Curtobacterium* sp. FM01 at 0.125–0.5 mM initial Ni^{2+} concentration was analyzed and reported (Masoumi et al., 2016). Boechat et al. (2018) reported the tolerance capacity of *K. intermedia* against Pb, Ni, Cu, Zn, Cr, Cd, and Ba heavy metals.

In the metal tolerance experiments, the relative growth was reduced as the level of metal stress in the medium increased. This was consistent with the findings of Babar et al. (2021), who explained that increasing the metal concentration in the medium causes toxicity and cell lysis. Due to the cell damage brought on by metal toxicity, microbes spend energy to repair it. Thus, as the metal concentrations rise, more energy is required for cell maintenance, resulting in less substrate available for microbial growth (Sodhi et al., 2020). In the current study, it was also observed that the growth of the bacterial strain for the different combinations of metals was lower as compared to a single metal when supplemented in LB medium inoculated with the bacterial strain. The lag phase was also relatively long in the presence of a mixture of metals. According to Jeevaraj et al. (2022), increasing concentrations of different metals cause increased toxicity, which results in a sharp decline in microbial activity, reflected in a reduction in the apparent growth rate and the lengthening of the lag time. When the metals were present in the medium separately as opposed to all at once, metal resistance was found to be higher. This is due to increased competition among the metal ions in the medium mixture for the metal-binding sites in the bacteria (Irawati et al., 2015). Multiple tolerances are a typical occurrence among heavy metal-resistant bacteria since the harmful mechanisms of all heavy metals are identical (Oladipo et al., 2018).

The potential of *K. cryocrescens* M7 for Ni removal has also been investigated in the present study. The results showed that the removal of Ni by the bacterial strain from the medium (LB +Ni) at an initial Ni concentration of 100 mg/L, increased slowly with increasing the incubation time (till 48 hours). The removal of Ni was highest in 48 hours, whereas it started decreasing after 48 hours. The decrease in the reduction of Ni could be due to reactive oxygen species causing oxidative damage to the cell in the stationary phase or the organism's metal binding sites becoming saturated (Bhutada and Dahikar, 2017; Oyewole et al., 2019). It was also noted that the amount of Ni in the cytoplasmic fraction was higher as compared to the pellet fraction. This might be due to the high expression of metal binding proteins in the bacterial strain (Heidari et al., 2020).

Antibiotic-contaminated environments promote the growth of antibiotic-resistant microbes, but they can also be found in natural settings when certain non-antibiotic elements, particularly heavy metals, are present (Chattopadhyay and Grossart, 2011). It is well known that heavy metal ions co-regulate genes involved in antibiotic resistance and reduce antibiotic sensitivity (Zhai et al., 2016; Chen et al., 2019). In the current study, it was concluded that *K. cryocrescens* M7 was resistant to vancomycin, ampicillin, carbenicillin, and streptomycin but sensitive to tetracycline

and chloramphenicol. Similarly, Stock (2005) also indicated that strains of *K. cryocrescens* were naturally sensitive to certain antibiotics, such as tetracycline and chloramphenicol, and showed resistance to rifampicin and several macrolides. In *K.cryocrescens*, the chromosome-mediated KLUC-1 showed 85% identity with CTX-M-1, which is responsible for resistance to some antibiotics (Bonnet, 2004). Rodríguez et al. (2021) also discussed chromosomally encoded antibiotic resistance genes in *Kluyvera* species. Mutoh et al, (2019) concluded that *K. ascorbata* was resistant to first- and second-generation cephalosporins and penicillins. Due to the co-regulation of resistance genes, environmental contamination not only causes heavy-metal co-selection processes but also enhances the amount of tolerance to certain antibiotics (Nguyen et al., 2019).

As inadequate industrial waste disposal damages our ecosystem and has unintended negative impacts on human health, heavy metals should therefore be thoroughly processed by bacteria before being released into the environment (Suman et al., 2018). Bioremediation of heavy metals employing bacteria is an efficient, cost-effective, and environmentally friendly alternative to conventional treatment procedures. However, there are constraints due to the little difficulty of extrapolating results from small-scale studies in the lab to the larger-scale deployments at the field level (Kapahi and Sachdeva, 2019). Future research should focus on analyzing the bioremediation capacity of *K.cryocrescens* M7 for other heavy metals and using the bacterial strain in real-world settings for the bioremediation of heavy metals. In addition, the focus should also be on the enhancement of the Ni accumulation capacity of bacteria from the contaminated soil and wastewater by genetically modifying the specific metal transporter genes or simply mutate the bacterial strain by chemical mutagens.

CONCLUSION

Global environmental pollution is growing as a result of industrial development. Compared to physicochemical methods for heavy metal removal, the bioremediation approach is more cost-effective and environmentally friendly. Based on biochemical, morphological, phylogenetic, and 16S rRNA gene sequence analysis, the current study reported the isolation and identification of seven bacterial strains: *C. davisae* M1, *C. freundii* M2, *Pseudomonas* sp. M3, *Enterobacter* sp. M4, *Enterobacter* sp. M5, *Enterobacter* sp. M6, and *K. cryocrescens* M7. The findings of the present study showed that *K. cryocrescens* M7 is a promising bacteria that can withstand high concentrations of heavy metals, such as Ni (150 mg/L), Pb (1000 mg/L), Al (150 mg/L), and Cd (200 mg/L) as well as combinations of these metals. Because there has been limited research on the removal of Ni by *K. cryocrescens* M7, the current study sought to learn about this strain's Ni removal capacity and found 48.41% removal efficiency in 48 hours when the initial Ni concentration was 100 ppm. The bacterial strain also demonstrated resistance to some antibiotics, such as vancomycin, ampicillin, carbenicillin, and streptomycin. The current study discovered the potential of a novel strain of *K. cryocrescens* M7 that can be beneficial for the removal of Ni and other heavy metal toxicity from the contaminated soil and wastewater. This research will also help the researchers in exploring this bacterial strain for the bioremediation of other heavy metals and organic pollutants that many researchers have not been able to explore with this strain in particular.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication &/ or falsification, double publication &/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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