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RESEARCH ARTICLE

Exploring cadmium tolerance in soil microbes: Isolation and identification of resistant strains

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Abstract

Cadmium poses detrimental effects on our surrounding environment and causes various health hazards. In this research, the bacterial diversity of soil samples procured from the contaminated sites of Coimbatore, Tamil Nadu, India was investigated and their cadmium removal potential was assessed. 16S rRna sequencing was followed for the identification of two isolates, which belong to the same genus, *Cupriavidus* sp. This genus was used for assessing the cadmium bioremediation potential. It was found that the strain S1CL (*Cupriavidus necator*) exhibited a high ability to remove cadmium (9 %) compared to S3CL (*Cupriavidus alkaliphilus*), which removed 2 %. The strains were compatible to each other and exhibited 1 2% removal efficiency in combination. The results revealed that *Cupriavidus necator* possessed a great potential for the cadmium bioremediation process.

Keywords

cadmium; bioremediation; *Cupriavidus necator; Cupriavidus alkaliphilus;* compatibility

Introduction

The major obstacle faced by modern human society in the present era is environmental degradation. Because of their capacity to persist and accumulate, heavy metals pose a significant threat to various environmental components (1, 2). Since the 1940s, the rapid expansion of industrialization has dramatically increased the mobilization of heavy metals in the environment. (3). However, heavy metals are present in low concentrations in the soil environment due to both lithogenic and pedogenic processes (4). Various anthropogenic activities are the primary causes of heavy metal accumulation in soil (5, 6).

Heavy metals have been categorized as essential and non-essential based on their biological significance (1). Essential metals such as iron (Fe), zinc (Zn), manganese (Mn) and nickel (Ni) are considered indispensible for development and biological functioning of living organisms in relatively small concentration (7). According to a study, non-essential heavy metals, namely cadmium (Cd), lead (Pb) and silver (Ag), have minimal or no biological activity (8, 9). Cadmium was recognized as a human carcinogen by the Department of Health and Human Services in 2021 (10) and was listed in seventh position as the most hazardous material to human health by the Agency for Toxic Substances and Disease Registry (11). The anthropogenic sources responsible for the emission of cadmium involve mining activities, agricultural processes

and industrial output. Weathering of rocks, forest fires and volcanic activity are considered as the natural sources of cadmium emission in the environment (12). Various environmental components are at high risk from cadmium (Cd) due to its persistent, non-degradable, highly mobilizable, toxic and bioaccumulative nature (13, 14). Traditional physical or chemical remediation of heavy metal -contaminated soil is not only expensive but also has the potential to cause secondary pollution (15). Furthermore, the efficacy of phytoremediation of heavy metal-polluted soil is unstable since enrichment plants have relatively long growth cycles and are easily affected by the growing environment. Numerous microorganisms can be found in the environment, and some of them have shown great potential in removing or neutralizing pollution caused by cadmium. It is inexpensive, practical and efficient to use microbial remediation techniques (16-18). This research paper focuses on the isolation and identification of various cadmium-tolerant organisms from contaminated areas collected along the sewage drains of Coimbatore city.

Materials and Methods

Soil collection and analysis

Eight samples of soil were randomly collected from various contaminated sites of Coimbatore city (Tamil Nadu, India). Samples of contaminated soil (0–10 cm) were taken from the uppermost layer, placed in tagged polythene bags and brought to the laboratory at the Department of Environmental Sciences at Tamil Nadu Agricultural University for analysis. A small portion of each collected sample was ground using a pestle and mortar for physical and chemical examination. The samples were then dried and sieved through a 2 mm screen to remove stone particles, wood fragments and other debris (19).

Determination of soil pH, Electrical conductivity and Cadmium concentration in soil

Each sample of soil was weighed (10 g) and suspended in 25 mL of distilled water for analysis. The contents were allowed to stand for 30 min and the pH of the samples was recorded using a calibrated glass pH meter. All analyses were conducted three times to reduce error. A conductivity meter was used to examine the electrical conductivity of each sample. The concentration of heavy metals in the collected contaminated samples was determined using Microwave Plasma Atomic Emission Spectroscopy (MP-AES). The samples were digested using a microwave digester.

Preparation of Cadmium solutions

The stock solution of cadmium was prepared using $CdCl_2 \cdot 2H_2O$ salt. The required quantity of salt was dissolved in double-distilled water to achieve the required concentration of cadmium solution. This experiment employs only analytical-grade reagents. The purity of $CdCl_2 \cdot 2H_2O$ was 99 %. To prevent metal contamination, all plastic and glassware were thoroughly rinsed numerous times with deionized water after being acid-washed in 2N HNO_3.

Isolation of cadmium resistant bacteria from enriched soil

Contaminated soil samples were enriched with different concentrations of cadmium. The soils were enriched with 100, 200, 300, 400 and 500 ppm of cadmium respectively. The enriched soil was then incubated for 15 days. The incubated soil was used as a source for the isolation of microorganism. Using Luria Bertani medium, bacteria resistant to cadmium was isolated from the contaminated soil sample (LB media). Cadmium resistant/tolerant bacteria were isolated further by amending the LB medium with 100, 200, 300, 400 and 500 ppm of Cd^{2+} solution.

1 g of contaminated soil sample was dissolved in 10 mL of sterile distilled water, vortexed for 30 sec and 1 mL of the mixture was spread out on nutrient agar plates. Petri plates were incubated at 37 °C following inoculation for 24 h. To obtain pure culture, the isolated strain was sub -cultured on the same medium multiple times. Following the incubation time, the bacterial colonies with the highest level of Cd tolerance (200 ppm) were chosen and characterized phenotypically and genotypically.

Bacterial colonial and cellular morphology of isolates

Purified isolates produced in isolated colonies on LB plates were inspected and details on their size, shape and color were recorded (20). Using a conventional method, bacterial colonies were gram stained to examine the cellular shape. Under the light microscope, slides were examined to assess the gram reaction as well as the morphology and arrangement of the cells.

Cadmium-tolerant assay and compatibility evaluation among the isolates

Initially, Nutrient Agar medium enriched with varying doses of cadmium (0, 50, 100, 150, 200 and 250 ppm) was used to test all bacterial isolates for cadmium tolerance. A growth curve experiment was conducted to further evaluate the culture's ability to grow under cadmium free condition (control) and also in cadmium spiked condition (50 ppm). The 48 h old cultures from the broth were transferred with a definite quantity of inoculum to 100 mL NA broth supplemented with 50 ppm of cadmium and to control (0 ppm of cadmium). The OD values at 600 nm were measured for every 4 h for all the isolates until the attainment of stationary phase. The OD values obtained at 600 nm were plotted against the time. By following this process, growth curve was obtained. Fig. 2 and 3 represents the growth curve of isolated strains between control and media spiked with cadmium (50 ppm).

The cross-streaking method utilizing NA medium was used to evaluate the compatibility of the bacterial isolates. The bacterial strain was streaked at one end of the plate and then the other bacterial strain was streaked perpendicular to it. The plate was then incubated for 24 to 48 h at 30 °C. The absence of inhibition zone demonstrated that the cultures were compatible to each other (21).

Identification of isolates through 16S rRNA sequencing

Molecular analysis of the 16S rRNA gene for bacteria

allowed for the identification and confirmation of the bacterial isolates. Bogar Bio Bee Stores Pvt. Ltd.'s EXpure Microbial DNA isolation tool was used for the isolation of DNA from microbial samples. The Qubit fluorometer 3.0 was used to measure the concentrations of DNA. The amplification of PCR was done by addition of 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Primers, 5 µL of deionized water and 12 µL of Taq Master Mix). The DNA template was heated for about 2.5 min to 95 °C and then cooled for 30 sec to 55 °C. The temperature is then increased to 72 °C, which is the ideal level for DNA polymerization. The Montage PCR Cleanup kit (Millipore) was used to remove the dNTPs and unincorporated primers from the PCR products. With universal primers for rRNA below 16s, single-pass sequencing method was followed. The samples underwent electrophoresis on an ABI 3730 x l sequencer after being resuspended in distilled water. The NCBI blast similarity search tool was used to identify the 16s rRNA sequence. Multiple sequence alignment was used to accomplish the phylogenetic analysis with closely associated sequence of NCBI blast findings. Multiple sequence alignments were done using MUSCLE 3.7 (22). Program G blocks 0.91b was used to treat divergent areas and poorly aligned positions (23). Finally, HKY85 was utilized as the substitution model and PhyML 3.0 aLRT was employed for the phylogenetic analysis. For the purpose of drawing trees, Tree Dyn was utilized (24). 16 S rRNA sequences have been deposited in GenBank.

Plant growth promotion assessment

The ability of identified isolates to estimate phosphate, zinc and potash releasing efficiency was evaluated in relation to their promotion of plant growth and development ability. To facilitate assessment, each experiment was run in triplicate.

Phosphate solubilization assay

The capacity to solubilize inorganic phosphate was assessed by spot-inoculating the identified bacterial isolates on modified Pikovskayas agar plates containing tri -calcium phosphate as a substrate (25). After incubating the bacterial colonies for seven days at 28 °C, the development of clear halo zones present near the colonies was anticipated to be a sign of phosphate solubilization activity. The phosphate solubilization efficiency was calculated by the following formula,

Phosphate solubilizing efficiency (%) = (Halozone diameter/ Colony diameter)* 100

Zinc solubilization efficiency

The effectiveness of identified bacterial isolates' zinc solubilization potential was assessed using a plate assay. Spot inoculation (10 µL) (1x 10^7 CFU/mL) was performed on the 24 h old cultures in Bunt and Rovira media with 0.1 % of zinc oxide. For 48 h, the plates were subjected to 28 ± 2 °C for incubation. According to a report, colonies displaying the clearing zone were deemed positive for zinc solubilisation (26). The following formula was used to compute the zinc solubilization efficiency.

Zinc solubilizing efficiency (%) = (Halozone diameter/ Colony diameter)* 100

Potash releasing efficiency

Using Aleksandrov's medium, the isolates' potency in releasing potassium was evaluated. A 10 µL culture of bacterial suspension (10^8 cfu/mL) was cultivated for 24 h and placed on Aleksandrov's medium with 0.3 % potassium alumino silicate added as a supplement. After the incubation period of 48 h at 30 ± 2 °C, colonies that showed a clear zone surrounding them were deemed positive for potassium release. The following formula was used to compute the potassium release efficiency.

Potassium releasing efficiency (%) = (Halozone diameter/ colony diameter) * 100

Cadmium removal potential of isolated organisms

In batch studies, the identified microorganisms' Cd (II) adsorption capability and/or removal efficiency were assessed. The studies were conducted using Erlenmeyer flasks on a rotating shaker at 28 °C and with 20 mL of cadmium working solution at 180 rpm. The following parameters were maintained constant: pH at 6, starting Cadmium concentration of 50 mg/L with the contact period of 24 h. A control was maintained without microbial inoculation. Comparison between the working solutions with and without microbial inoculation was observed. Following adsorption, the contents were centrifuged at 10000 rpm 10 min to extract the supernatant. Inorganic filter membrane was used to filter the contents and microwave plasma atomic emission spectroscopy was used to measure the amount of Cd (II) present in the supernatant. The removal efficiency (% removal) was calculated using Eq. (1) (27).

$$\% \text{ removal} = \frac{Co - Ce}{Co} \times 100 \qquad (1)$$

Where C_o (mg/L) indicates the initial Cd (II) concentration and C_e (mg/L) means Cd (II) concentration at equilibrium or a certain time. All experiments were carried out in triplicate.

Statistical analysis

Statistical analysis of the obtained all data was performed with SPSS and statistical significance was evaluated using one-way ANOVA with least significant differences (LSD). By computing mean values and standard error using the SPSS statistical tool, the physicochemical characteristics of soil samples and the growth curve of identified bacterial isolates that is capable of withstanding metals were statistically examined.

Results and Discussion

Soil characteristics

Various physicochemical characteristics of the contaminated soils investigated in this study are represented in Table 1. The mean pH of soil samples ranged from 5.7 to 8.2 that indicate the samples are slightly acidic to mild alkaline, whereas the electrical conductivity (EC) ranged from 0.3 to 2.0 dSm^{-1}. Important factors that have a significant impact on the chemical behaviour of cadmium

Table 1. Physiochemical characteristics of soil samples collected from contaminated sites of Coimbatore city.

Sl. No.	Sample	pH	EC (dSm^{-1})	Cadmium (ppm)	Chromium (ppm)	Copper (ppm)	Nickel (ppm)
1.	SS_1	6.2	1.1	BDL	1.13	0.28	0.42
2.	SS_2	5.7	1.3	BDL	0.28	0.07	0.01
3.	SS_3	6.4	1.7	BDL	1.05	0.35	0.40
4.	SS_4	7.2	0.8	BDL	0.85	0.25	0.38
5.	SS_5	7.5	2.0	BDL	0.60	0.28	0.21
6.	SS_6	5.7	1.3	BDL	0.97	0.25	0.39
7.	SS_7	6.2	1.4	BDL	1.42	0.23	0.53
8.	SS_8	8.2	0.3	10.0	1.61	0.17	0.50

metal ions found in terrestrial and aquatic environments are soil pH and EC. They have an impact on mobility and solubility of cadmium ions both directly and indirectly as well as their capacity to form chelates with other components of soil (28). For example, metal ions are generally less soluble in any given soil solution when the pH is neutral. It was observed a significant drop in pH value results in increased concentration of cations in the soil solution (29). Their research demonstrated that the pH drop has a specific impact on the solubility and mobility nature of metals like cadmium. Cadmium concentration is often higher in soil samples with high pH and low EC. Soil pH plays a crucial function in regulating Cadmium partitioning and bioavailability (30).

Analysis of soil samples using Microwave Plasma Atomic Emission Spectroscopy revealed a significant high level concentration of heavy metal ions in the following order: Cd > Cr > Ni> Cu with increased concentrations of 10, 1.61, 0.50 and 0.35 ppm respectively and it is represented in Fig. 1.

Isolation and selection of Cd tolerant/ resistant microorganism

High concentrations of heavy metals in soil can be a source of metal-tolerant microorganisms (31). Data obtained from several studies indicate that increasing metal ion concentrations provide a selective environment that promotes the evolution of bacterial communities with lesser diversity with greater tolerance to heavy metals (32-34). In light of this, the goal of the current work is to isolate and characterize bacterial isolates that are tolerant to cadmium.

Potentially metal-tolerant bacteria were found in reasonably considerable numbers on LB medium supplemented with varying doses of $CdCl_2.2H_2O$. The number of viable colony forming units (CFUs) from different plates are selected and cultured. The isolation and characterization of Cd-tolerant bacteria from soil contaminated with metals have also been reported by a number of researchers. In this trend, isolated 27 bacterial isolates from eight soil samples on media amended with cadmium (100 ppm) (35). Only one isolate has a high level of minimum inhibitory concentration (MIC) for cadmium metal. Morphologically unique colonies were chosen at random during the first screening stage and evaluated for tolerance against progressively greater metal concentrations. Two bacterial isolates were ultimately chosen for additional processing. Tables 3 and 4 contain a collection of bacterial codes and phenotypic traits.

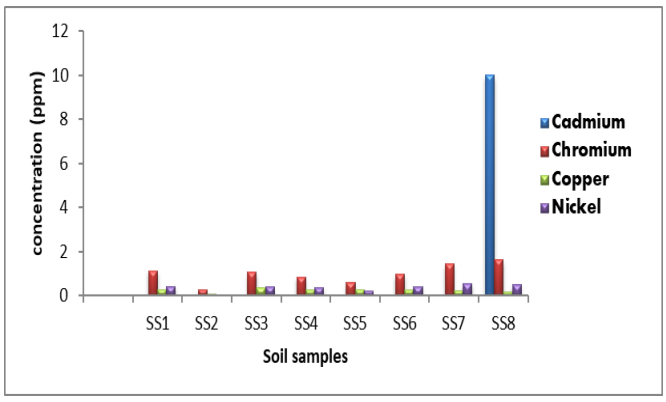

Fig. 1. Concentrations of cadmium, chromium, copper and nickel present in samples collected from the contaminated sites of Coimbatore city.

Colonial and Cellular morphology of isolated bacterial strains

Morphological analysis of the isolates revealed that the bacterial cells were round in shape (Table 2). It exhibits convex and flat elevation. Bacterial margins were entire. The colors exhibited by the 2 bacteria were opaque light yellow and beige respectively. The bacterial cells were rod-shaped and oval shaped respectively (Table 3). Both bacterial isolates were gram-negative. Numerous studies have shown that gram-negative bacteria showed increased resistant to heavy metals than gram-positive bacteria (36-38). Gram-negative bacteria's cell wall is a far more powerful barrier against hazardous metals (39). Additionally, he mentioned that metal ions interact with the surface structures that comprise the cell wall. It was also endorsed it (40). The remediation potential exhibited by the bacterial isolates (S1CL, S3CL) may be rendered due to their gram negative nature of the cell wall.

Table 2. Colonial morphology of isolated bacterial strains.

Sl. No.	Isolate	Colour	shape	Margin	Elevation
1.	S1CL	Opaque light yellow	circular	Entire	Convex
2.	S3CL	Beige	circular	Entire	Flat

Table 3. Cellular morphology of isolated bacterial strains observed under microscope.

Sl. No.	Isolate	Colour	Cell shape	Gram reaction
1.	S1CL	Pale white	circular	Entire
2.	S3CL	Pale white	circular	Entire

Effect of Cd on bacterial growth

Fig. 2 and 3 represents the growth curves for each bacterial isolate in LB medium with 50 ppm of Cd and compare them to the corresponding growth patterns without the addition of heavy metals. In summary, we found that all of the bacterial isolates had lower optical densities when exposed to heavy metals as opposed to when they were in a metal-free media. These findings are consistent with other findings (41, 42). Presence of cadmium in the media affects the growth of the bacterial isolates significantly compared to the control. All of the bacterial isolates grew at a constant pace in the absence of cadmium (Fig. 2 and 3), peaking 24 h before they entered the stationary phase.

16S rRNA–based identification

Microbial DNA (EXpure) extraction produced high-quality DNA efficiently. A set of primers was used to successfully amplify the 16S rRNA genes. Using BLAST, partial 16S rRNA nucleotide sequences from every bacterial strains were compared to related sequences on the NCBI website. The strains were found to be closely related to species of *Cupriavidus, Bacillus, Proteus* and *Burkholderiaceae,* according to the results of the BLAST query. Fig. 4 and 5 represents the identified bacterial isolates together with their phylogenetic tree. The generated nucleotide sequence was registered with the NCBI database and Table 4 provides the GenBank accession numbers.

Table 4. Isolated bacterial strains and their accession number provided from GenBank.

Strain	Best match	Accession number	Match index
S1CL	*Cupriavidus necator*	PQ182265	100 %
S3CL	*Cupriavidus alkaliphilus*	PQ182306	98.44 %

Cadmium removal potential of isolated organisms

Experiments were conducted to determine the identified microorganisms' adsorption capacity and/or removal efficiency for Cadmium. Using the equation (1) the percentage removal efficiency is calculated. It is found that S1CL exhibits 9 % removal efficiency and S3CL exhibits 2 % removal efficiency. S1CL and S3CL in combination exhibited 12 % removal efficiency. Future experiments shall be conducted to assess the impact of varying environmental factors on removal efficiency to determine whether these strains are viable for broader applications. *Citrobacter* sp. JH 11-2 was discovered in soil mining areas near an abandoned electronic company and it was able to successfully remove about 47.7 % of the cadmium (43). Microorganisms frequently use biological absorption and biomagnification processes to remove heavy metals. Cadmium-tolarent bacteria (CdtB) contain cadA and cadB gene systems which is responsible for their Cd-resistant capacity (44). *Cupriavidus necator* not only possess bioremediation potential but also play a vital role in low-cost production of PHB from a realistic lingo-cellulosic biomass feedstock (45).

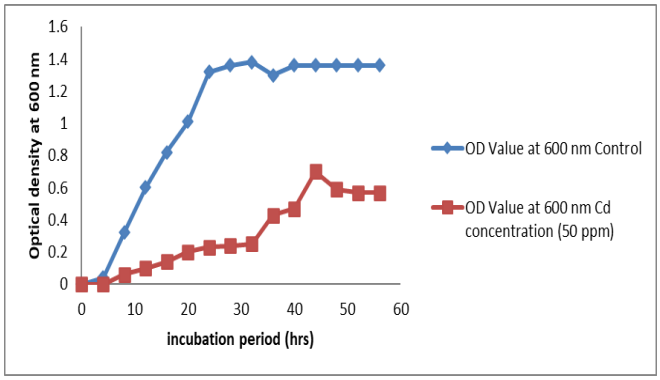

Fig. 2. Growth of *Cupriavidus necator* (S1CL) with and without cadmium concentration.

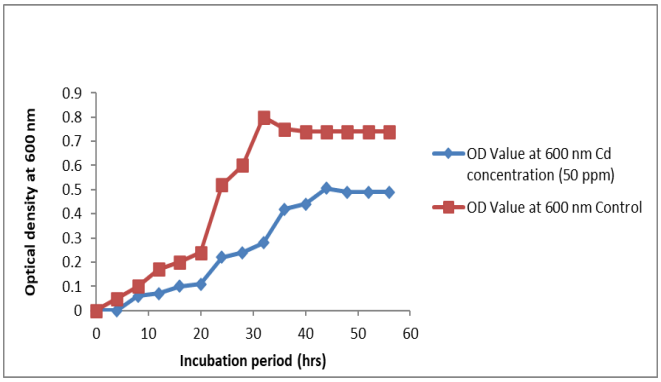

Fig. 3. Growth curves of *Cupriavidus alkaliphilus* (S3CL) with and without cadmium concentration

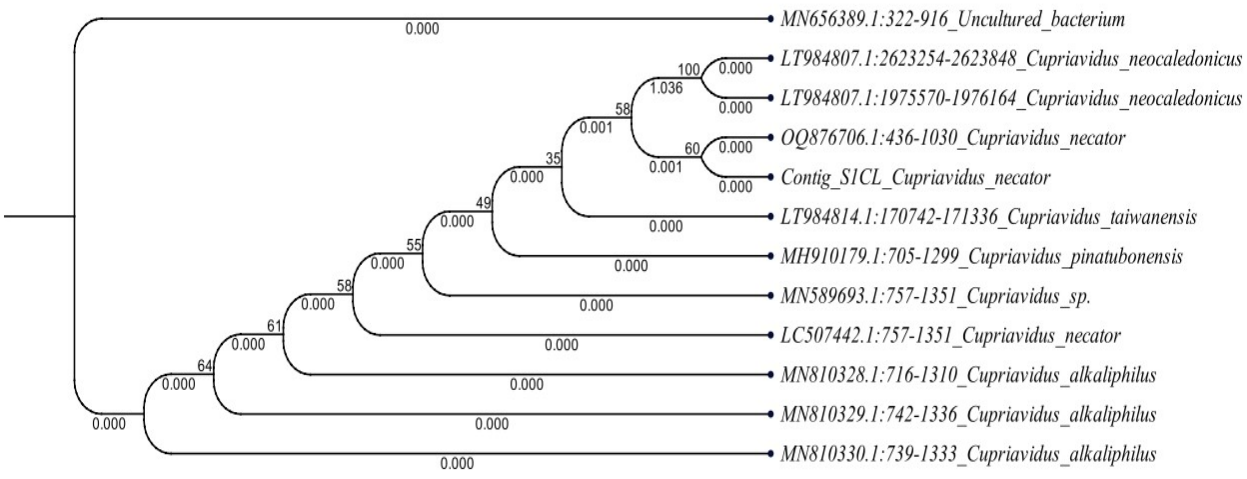

Fig. 4. Phylogenetic tree of S1CL obtained from 16S r RNA sequencing.

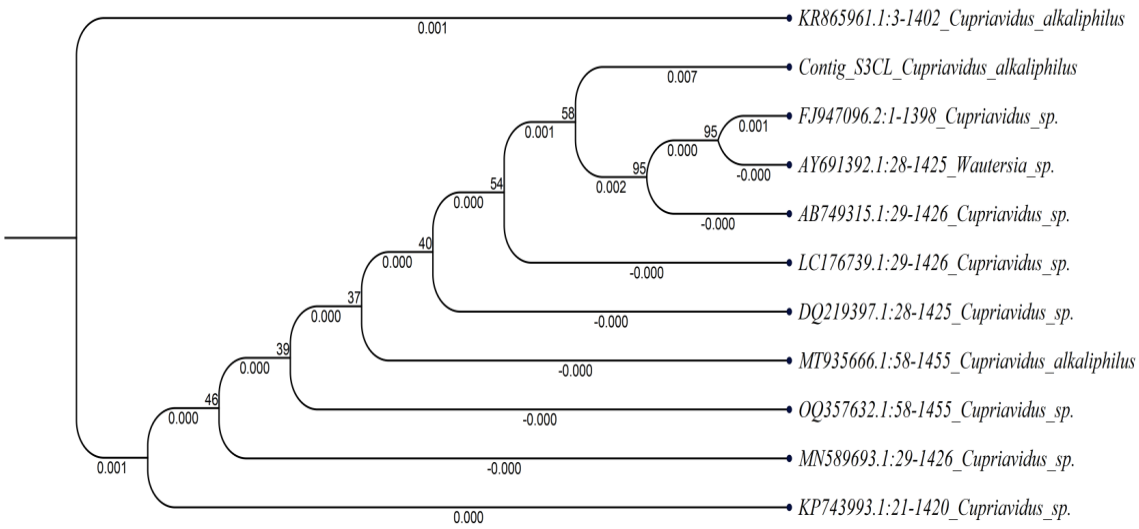

Fig. 5. Phylogenetic tree of S3CL obtained from 16S r RNA sequencing.

Compatibility activity of isolated bacterial strains

Bacterial isolates present in the microbial consortium's needs to be able to multiply in the presence of other bacteria without impeding their ability to grow and develop. A signalling network that the phytohormone builds allows the bacteria to cooperatively regulate a number of metabolic processes. A consortium becomes unstable and fails to achieve its intended purpose when isolates in it have adversarial relationships with one another (46). The isolates were compatible with each other, according to a compatibility examination of 2 cultures using the line streak assay. *Cupriavidus necator* (S1CL) and *Cupriavidus alkaliphilus* (S3CL) were compatible, as shown by the absence of an inhibitory zone (Fig. 6).

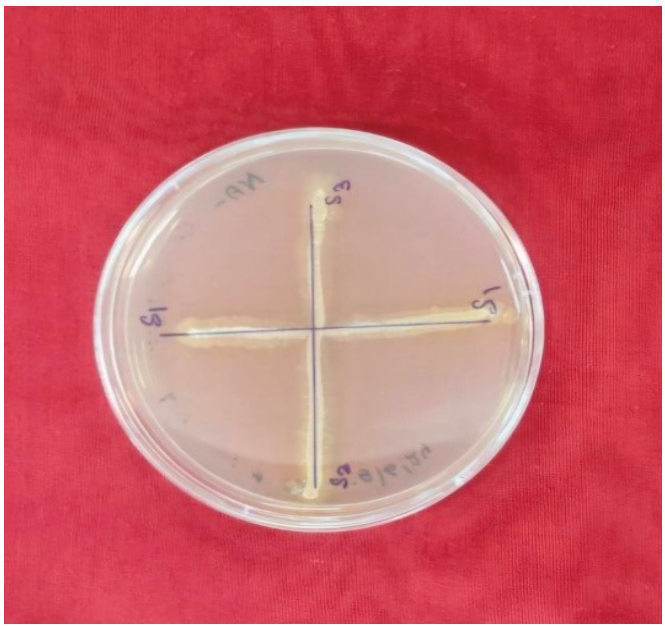

Fig. 6. Compatibility assay between S1CL and S3CL bacterial isolates

Assay for plant growth promotion and regulation assessment

The ability of isolated bacterial strains to solubilize phosphate, zinc and potash was evaluated in relation to their ability to promote plant development. Every experiment was carried out in triplicate and Table 5 presents the results of the plant growth promotion activity assessment.

Phosphate solubilization

One advantage of using microorganisms as biofertilizers to increase crop production and support the development of sustainable agriculture is the presence of P-solubilizers in soils. Phosphate solubilization is primarily caused by the synthesis of microbial metabolites such organic acids, which decrease the pH of the media (47). After three days of incubation in Pikovskaya's medium, the isolates' phosphate solubilization was visualised by the formation of clear zones (Halo-zone) around the bacterial colonies. The clear zones in each isolate represent the phosphate solubilizer out of the 2. *Cupriavidus alkaliphilus* isolates did not pass the phosphate solubilization test. According to a study, microorganisms from the *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* spp. families are the most often used phosphate solubilizers (48). By increasing the amount of phosphorus available in the soil, phosphate solubilizing bacteria can support crop growth. By inhibiting the growth of phytopathogens and producing phytohormones, phosphate solubilization bacteria can occasionally promote plant growth both directly and indirectly (49).

Table 5. Plant growth promoting activity of isolated bacterial strains.

Sl. No	Strain	Isolates	Zinc soilubilization efficiency at 28 ± 2 °C	Phosphate solubilization efficiency 28 ± 2 °C	Potash releasing efficiency 30 ± 2 °C
1.	S1CL	*Cupriavidus necator*	42.5 %	26.3%	-
2.	S3CL	*Cupriavidus alkaliphilus*	25 %	-	-

Zinc solubilization

Zn solubilizers in soils are a good indicator that microorganisms are being used as biofertilizers to increase crop production and support the advancement of sustainable agriculture. Zinc-solubilizing bacteria have the ability to change insoluble forms of zinc into forms that are readily absorbed by plants. This process increases the amount of zinc that is bioavailable in soil and helps to mitigate crop zinc deficiencies (50). After 3 days in bunt and rovira media, the isolates' zinc solubilization potential was visible as the formation of clear zones surrounding the bacterial colonies' strains. Both isolates was zinc solubilizer which is indicated by halozone. The isolates that exhibit Zn solubilizing property could be more promising for being used as Zn bio-fertilizers, in the future.

Potash releasing efficiency

One potential benefit of using microorganisms as biofertilizers to increase crop production and support the development of sustainable agriculture is the presence of bacteria in soil that release potassium. Both isolates showed negative sign for potash releasing efficiency.

Conclusion

The current experimental study aimed to isolate the cadmium resistant/ tolerant bacteria, identify the organism and characterize them from the collected soil samples of cadmium contaminated sites in Coimbatore city, Tamil Nadu, India. Two isolates were recovered as the most tolerant isolates for cadmium. The colonial and cellular morphological features of the isolates were studied. Then these two strains were examined for their compatibility and percentage removal efficiency of cadmium. Results showed that it is compatible to each other and exhibit increased removal efficiency of 12 % when combined together. This proves that the isolates can be significantly used in cadmium bioremediation process. The cadmium bioremediation mechanisms of these newly isolated strains require further study in the future**.** Future research should include transcriptomic or proteomic analyses to identify specific genes or proteins involved in cadmium removal. Additionally, exploring potential genetic modifications to enhance cadmium removal efficiency could be valuable.

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Authors' contributions

CLR carried out isolation works, analysis part and drafted the manuscript. BA conceived of the study and participated in its design and coordination. DP coordinated the work. KT helped in identification of strains and their accession number. VD participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare

Ethical issues: None.

Did you use generative AI to write this manuscript?

No, GenAI tools should only be used to improve language and readability, with caution.

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