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MOLECULAR CHARACTERIZATION AND BIOREMEDIATION ASSESSMENT OF A LEAD-RESISTANT BACTERIUM-DERIVED FROM INDUSTRIAL WASTE USING INDUCTIVELY COUPLED PLASMA OPTCAL EMISSION SPECTROSCOPY

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ABSTRACT

The environmental pollution due to the lead discharge from industrial effluent is a major concern owing to its non-biodegradable and toxic nature. The present study was aimed to isolate, screen, and molecularly characterize a lead-resistant bacterium isolated from the industrial effluents in Pune (India). The study was conducted in the years 2022-2024. The effluent samples were analysed for physicochemical characteristics and lead concentrations, both before and after bioremediation treatment. The lead concentration in the samples was quantified by inductively coupled plasma optical emission spectroscopy during a lead reduction study over 48 h. Of the 30 bacterial isolates from the samples collected, five isolates *viz*., S_1, S_4, S_8, S_{12} and S_{14} exhibited resistance up to 1000 ppm. Isolate S_8 showed maximum lead reduction (14.80-6.78) in 48 h. Based on the morphobiochemical and molecular characterisation the isolate was identified as a *Bacillus cereus* (Accession No. PZ327497). The 16S rRNA sequencing and phylogenetic analysis were performed using MEGA11 software. Further, the physicochemical parameters of industrial effluent such as BOD, COD, TSS, and TDS were considerably reduced after treatment. The study demonstrated that *B. cereus* strain S_8 is a potential isolate for the bioremediation of lead polluted industrial effluent.

Keywords: *Bacillus cereus*, bioremediation, ICP-OES, lead, physico-chemical parameters

INTRODUCTION

Heavy metals are naturally occurring elements present on the Earth, exhibiting high density and elevated toxicity even at trace levels. Expedited urbanisation, industrialisation, and modernisation contaminate water and soil quality, resulting in hazardous effects for living organisms. Consequently, heavy metal pollution is emerged as a global environmental concern (Van Deuren *et al*., 2002). Due to non-biodegradable nature of heavy metals several complex ecological and public health issues occur (Senthil Kumar, 2014). Their capacity to biomagnify in the food chain across multiple trophic levels intensifies their toxicity and necessity of their effective removal (Kadirvelu *et al*., 2001). Among various heavy metals, lead (Pb) is considered as toxic and widely distributed pollutant known for its carcinogenic effect (Dekhil *et al*., 2011). As a result, the permissible limits of lead in drinking water are specified by regulatory agencies, including the WHO, ICMR, and ISI,

at 0.05, 0.05, and 0.10 ppm, respectively. In contrast, the CPCB does not permit relaxation of lead levels in drinking water (Kumar and Puri, 2012). Lead contamination mainly originates from industrial processes such as smelting, mining, plating, storage battery production, ammunition manufacturing, the glass and ceramic industries, and tetraethyl lead manufacturing (Held and Don, 2000). Lead is a primary toxicant that can be exposed to humans through inhalation and ingestion of food and water and affects various physiological systems including nervous, cardiovascular, reproductive, gastrointestinal, and hematopoietic systems (Sood and Prakash, 1998). Various methods have been employed for the removal of heavy metals, including ion exchange, reverse osmosis, electro-chemical treatment, evaporation, and precipitation (Marin *et al.*, 2010; Lezcano *et al*., 2011). However, these methods are relatively expensive, environment-unfriendly, produce sludge as secondary pollutant and ineffective in removing low concentrations of heavy metals. These constraints emphasize on the necessity to develop eco-friendly and cost-effective alternatives.

Bioremediation is the most effective alternative to detoxify and eliminate heavy metals from the polluted environment (Marin *et al*., 2010; Lezcano *et al*., 2011). Microorganisms have evolved several strategies to immobilise, sequester, and mitigate the effects of toxic metals, converting them into a less harmful form (Donald, 2003). Bioremediation is a persistent tool for cleaning up polluted environments by utilising the biochemical capabilities of microorganisms and plants (Kleijntjntens and Luyben, 2000). The bioremediation approach includes bioaccumulation, biosorption, bioventing and phytoremediation. These methods are influenced by environmental factors such as pH, temperature, nutrient availability, and oxygen requirements, as well as the presence of aerobic or anaerobic conditions (Kang *et al.,* 2016). Bioremediation techniques aid in the removal of various xenobiotic compounds, heavy metals, oil spills, sludge, and marine sediments (Zhiqi *et al*., 1999).

Although bioremediation of heavy metals has been extensively studied, there remains a specific gap on isolation and molecular characterisation of lead resistant bacteria from industrial effluent in particular location. Additionally, the quantitative evaluation of lead removal using inductively coupled plasma optical emission spectroscopy (ICP-OES) is still underexplored. Hence, the present study was aimed to isolate and identify lead-resistant bacteria from industrial waste and assess their heavy metal resistance and effectiveness in removing lead quantitatively by using ICP-OES. Therefore, the research focused on identifying the best bacterial candidate for lead bioremediation.

MATERIALS AND METHODS

Sample collection

The effluent water samples were collected from three different sites from the industrial area of Pune (India) specifically from battery manufacturing, metal processing industries and electroplating which are known sources of lead contaminated environment. They were labelled as S_1, S_2 and S_3, respectively. The samples were collected during March, May and August 2022 using grab sampling method from different points within each industrial site in pre-sterilised and labelled bottles. Sample representiveness was ensured by triplicate sampling and subsequently a composite sample was prepared by pooling all the samples from one industry. They were transferred immediately to the laboratory, where it was stored in a refrigerator at 4°C to maintain its integrity.

Physicochemical characteristics

The collected samples were assessed for their physicochemical characteristics *viz*., colour, odour, pH, turbidity, biological oxygen demand (BOD), chemical oxygen demand (COD), and total solids (TS). All characteristics were measured both before and after bioremediation treatment, to determine efficiency of lead removal by the isolated bacteria. In this context treatment refers to the application of lead resistant bacterial isolates under controlled laboratory conditions. All the experiments were carried out as per the protocol provided by (APHA 1995).

Isolation and screening of bacterial strains

Initially, 10 mL of industrial effluent was enriched in 90 mL of Luria Bertani broth supplemented with 10 ppm of lead acetate, and incubated for 48 h at 30°C on a rotary shaker at 150 rpm. The enriched samples were then serially diluted from 10^{-1} to 10^{-7} and a loopful sample (0.1mL) streaked and spread on a Luria agar plate containing 10 ppm of lead acetate. The plates were incubated for 24 h at 37°C. The same procedure was repeated several times until isolated colonies with different morphologies were obtained. The purified colonies with distinct morphologies were selected and sub-cultured on the same media. Further screening of isolates for lead resistance was performed by culturing them on different media including LB agar, nutrient agar, Mueller-Hinton agar, and minimal agar supplemented with lead acetate, to avoid false positive results. Control plates were maintained without lead acetate. The growth was compared on control plate and lead containing test plates. All the experiments were performed in triplicate.

Minimum inhibitory concentration (MIC)

The isolates were cultured on Luria Bertani (LB) agar plates containing varying concentration of lead acetate *viz.*, 100, 200, 500, and 1000 ppm. Simultaneously control plates of LB agar medium without lead acetate were also maintained for comparison. All the isolates were spot inoculated on both the plates control and lead containing plates and incubated at 37°C for 24 h. All the experiments were carried out in triplicate to ensure reproducibility. Bacterial growth was assessed by colony formation compared with control. MIC was determined as the highest concentration of lead acetate showing visible growth while inhibition indicated by no colony formation.

Morphobiochemical characterisation

The screened isolates were further studied for their morphological and biochemical characterisation. Colony characters, Gram staining, and motility tests were performed as per the standard protocol used for bacterial identification (Bergey's Manual of Systematic Bacteriology, 1984). Various biochemical tests have been performed, including the indole test, methyl red test, Voges-Proskauer test, citrate test, oxidase test, catalase test, starch hydrolysis test, and sugar fermentation test. All tests were performed on the screened isolates according to the standard protocol (Ma *et al.,* 2015).

Lead reduction studies

The screened isolates were processed for inductively coupled plasma optical emission spectroscopy (ICP-OES) using Agilent 5110 ICP-OES model to assess quantitative reduction of lead. The analysis was carried out at a wavelength 220.353 nm using plasma flame and argon gas. The isolates were inoculated in 100 mL LB broth containing different concentration of lead, S_1, S_4 and S_8 were ~30 ppm while S_{12} and S_{14} were exposed to higher lead concentrations ~500 and ~750 ppm, respectively. All the flasks were incubated at 37°C for 48 h. A control setup containing LB broth with lead acetate without bacterial inoculation was maintained under identical condition. The culture medium was withdrawn every 6 h, and the initial period started at 0 h. At 6 h intervals, 10 mL medium was taken from the flask and centrifuged for 20 min at 4000 rpm. Then supernatant was collected in a separate test tube and digested with double volume of nitric acid. The cooled digest was filtered through Whatman filter paper 1. The clear filtrate obtained was diluted with double-distilled water and stored in a sterile plastic vial for further analysis (Marzan *et al.,* 2017).

$$\text{Lead reduction (\%)} = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \text{ X } 100$$

Molecular identification

The most efficient bacterial strain was selected for molecular identification using the 16S rRNA technique. The method used for genomic DNA isolation was the Macherey-Nagel Nucleospin microbial DNA kit Ref. No.740952.250 as per the manufacturer's protocol. Amplification of the 16S rRNA gene was carried out using universal primers 16S1 5′AGTTTGATCCTGGCTCAG 3′ and 16S4 5′AGGCCCGGGAACGTATTCAC 3′ approximately 1500 bp fragment was targeted.

Polymerase chain reaction (PCR) was performed using reaction volume of genomic DNA (5 µL), 10X PCR buffer (2.5 µL), $MgCl_2$ (0.75 µL), dNTP mix (0.5 µL), primers (1 µL each), DMSO (1.25 µL), Taq DNA polymerase (0.2 µL), and nuclease-free water. PCR cycling steps were followed as initial denaturation temperature was given 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min with an annealing temperature of 54°C for 1 min further extension at 72°C for 1 min and with a final extension at 72°C for 10 min. The amplified products were amplified using 1% agarose gel electrophoresis and the bands were detected by UV transilluminator. The amplified PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent. Then, sequencing was performed using the ABI BigDye Terminator V3.1 kit Applied Biosystems, USA. SeqA software was used for sequence analysis and identified using the BlastN tool at the NCBI database (Altschul *et al.*, 1990). The phylogenetic tree was constructed using MEGA11 software using Neighbour joining method with bootstrap analysis (1000 replicates) [Tamura *et al*., 2021].

Statistical analysis

All experiments were performed under controlled laboratory condition and by using completely randomised design and carried out in triplicate and average observed values were reported (Zar, 2010). Statistical analysis was carried out using Microsoft excel. Lead reduction over the time was checked and percentage reduction was calculated (Montgomery, 2017). Experimental data analyses were conducted following the standard biostatistical method (Zar, 2010).

RESULTS AND DISCUSSION

Physicochemical characteristics of industrial wastewaters

The physicochemical characteristics and the lead content of collected industrial wastewaters from different sites analysed for their quality revealed that the sample 1 (from battery manufacturing site) and sample 3 (from electroplating site) had higher lead content (0.87 and 0.56, respectively) than the permissible limit (0.1 ppm), so were selected for further treatment. Sample 2 had lead content < 0.1 ppm, so was not proceeded further. The samples 1 and 3 were hazy and grey, putrid in odour and turbid with pH within the permissible range, but BOD values significantly higher than normal (Table 1). The observed DO values were lower than the normal, due to the increase in suspended solids. TDS and COD values were higher. The physicochemical characteristics and lead concentration, assessed before and after bioremediation treatment, revealed that BOD, COD, TSS, and TDS values were significantly decreased, while DO increased after treatment. The lead content quantitatively assessed by ICP-OES, decreased substantially.

Table 1: Comparative analysis of physicochemical parameters before and after treatment

Parameters	Sample 1		Sample 3		WHO (2017)
	Untreated	Treated	Untreated	Treated	
Colour	Hazy	Hazy	Grey	Grey	Transparent
Odour	Putrid	Putrid	Putrid	Putrid	Agreeable
Turbidity	Present	Present	Present	Present	Agreeable
Ph	8.5	7.0	8.0	7.0	6.5-8.5
BOD (mg L^{-1})	80	28	55	30	15
DO (mg L^{-1})	4.5	6.1	4	5.6	5-6
COD (mg L^{-1})	320	95	450	50	40
TSS (mg L^{-1})	460	55	280	40	20
TDS (mg L^{-1})	650	160	800	200	500
Lead (mg L^{-1})	0.87	0.17	0.56	< 0.10	0.10

BOD: Biological oxygen demand, COD: Chemical oxygen demand, DO: Dissolved oxygen, TSS: Total suspended solids, TDS: Total dissolved solids. The values are mean values. WHO limits are given for comparison,

Bioremediation is an ecofriendly technique that involves the use of microbes to treat waste-water and reduce heavy metals. A significant decrease in physico-chemical parameters was observed after treating wastewater with lead-resistant bacteria.

Table 2: Changes (%) in the biochemical characteristics of wastewater samples after bioremediation

Chemical parameters (mg L^{-1})	Lead reduction (%)	
	Sample 1	Sample 3
Biological oxygen demand	65	45
Chemical oxygen demand	70	89
Total suspended solids	88	85
Total dissolved solids	75	75
Lead	80	82

BOD in sample 1 was reduced from 80 mg L^{-1} to 28 mg L^{-1} showing a 65% reduction after treatment. Other parameters such as COD, TSS and TDS also exhibited remarkable reduction. Similarly, sample 3 showed 89% reduction in COD (from 450 mg L^{-1} to 50 mg L^{-1}) [Table 1 and 2]. Previously Rana *et al*. (2013) have reported significant improvement in seed germination and plant growth in diary waste water after chemical/biological treatment.

Isolation, screening and minimum inhibitory concentration (MIC) of bacterial isolates

A total of 30 different bacterial isolates, designated S_1-S_{30}, were selected based on their morphology and cultural characteristics on LB agar supplemented with lead acetate as a source of lead. A preliminary screening was performed on various media, including LB agar, nutrient agar, Mueller-Hinton agar, and minimal agar. Moreover, the MIC values were determined based on primary treatment. The screening of isolates for tolerance to the increasing concentrations of lead (100, 200, 500 and 1000 ppm) revealed growth of five isolates, *viz.*, S_1, S_4, S_8, S_{12}, and S_{14}, in all test concentrations. Since the growth was observed even at 1000 ppm, the MIC for these isolates was considered to be higher than 1000 ppm. A similar study was conducted on the bacteria isolated from endophytic roots of *Tridax procumbens* and of the 5 bacterial isolates selected the strain RM showed highest MIC (450 mg L^{-1}) for lead (Govarthanan *et al.*, 2016).

Morphological and biochemical characterisation

The morphobiochemical characterisation of selected 5 isolates revealed that all isolates produced moist, smooth and circular colonies, with the exception that the colonies in S_4 were moist smooth and irregular and in S_8 were irregular fuzzy and rough. The isolates S_4 and S_{12} were Gram-negative rods and motile, isolates S_8 and S_{14} Gram-positive rods and motile, while isolate S_4 was Gram-positive rod and non-motile. All the isolates were negative for indole test (except S_{12}), methyl red test (except S_{12} & S_{14}), oxidase test (except S_8 & S_{12}) and sugar fermentation (except S_{12} & S_{14}). All isolates were positive for catalase test, Vogus Proskaeur test (except S_4 & S_{12}), citrate test (except S_{12}) and starch hydrolysis (except S_{12}). A study carried out on lead bioremediation by lead-resistant bacteria isolated from industrial effluent observed that the isolated bacteria were Gram-positive, rod-shaped, endospore-forming bacteria, showing biochemical results as indole and amylase positive while in absence of lead it was indole negative and another isolated bacteria was also Gram positive rod shaped and endospore forming bacteria with biochemical results as amylase and protease positive in presence of lead acetate (Chatterjee *et al.*, 2012).

Lead reduction by isolates

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to determine the quantitative reduction of lead by screened isolates over 48 h, among 30 isolates selected based on the screening results. The ICP-OES was performed on five isolates: S_1, S_4, S_8, S_{12}, and S_{14}. Table 3 shows the reduction in lead concentration by isolates. According to the results obtained from AAS, the lead bio-reduction percentage was calculated using the formula provided in the methodology section. The isolate S_1 concentration was recorded as 28.84 ppm at 0 h and 17.1 ppm at 48 h, which showed a 40.7% lead reduction. Similarly, the lead concentration of other isolates, such as S_4, S_8, S_{12}, and S_{14}, was recorded and which showed lead reductions of 39.72, 54.18, 12.34, and 30.23%, respectively, in 48 h. Although a standard reference strain was not included in this study, the comparative performance of isolates was assessed under identical experimental conditions. However, in future studies a standard metal-resistant strain need to be included for validation.

All the bacterial isolates showed lead reduction (Fig. 1). The maximum lead reduction was shown by isolate S_8, causing 54.18% in 48 h incubation. A study conducted on lead biosorption by *Bacillus*

Table 3: Lead reduction by various bacterial isolates

Time (h)	Lead reduction by different isolates (ppm)				
	S_1	S_4	S_8	S_{12}	S_{14}
0	28.84	33.43	14.80	491.91	758.40
6	26.54	30.55	13.15	491.70	622.25
12	25.20	28.32	12.23	490.23	601.14
18	21.11	24.95	11.51	478.49	580.23
24	19.62	24.66	09.49	450.66	554.06
30	19.00	23.65	08.30	434.26	541.78
36	18.15	22.87	07.89	436.30	539.89
42	17.62	21.12	07.10	432.21	532.24
48	17.10	20.15	06.78	431.10	529.12

A decrease in lead concentration indicates a low lead concentration in the media after a specified period of incubation

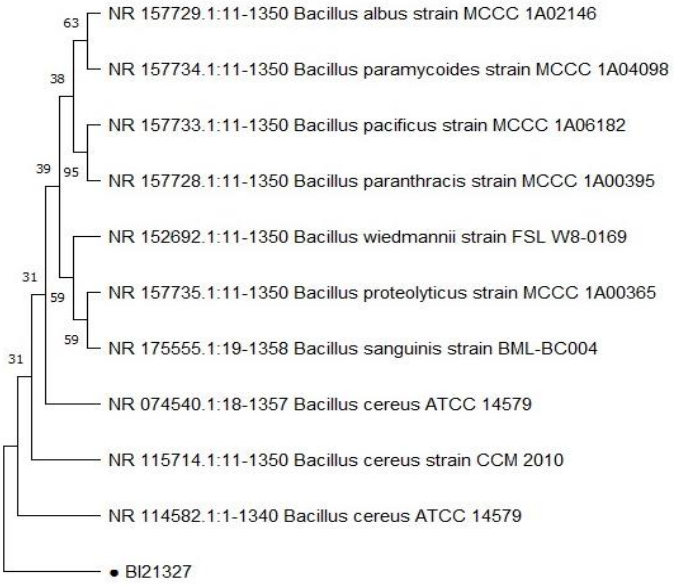

Fig. 1: Phylogeny tree of *Bacillus cereus* isolate S8 (BI21327)

licheniformis has shown 89.39% reduction in lead, determined by AAS (Gayatri *et al.*, 2017). Another study on lead adsorption onto polysaccharides, produced by *Bacillus firmus*, revealed that *B. firmus* could remove 98.3% lead (Salehizadeh and Shojaosadati, 2003). Four bacterial strains, namely *Escherichia coli*, *Salmonella typhi*, *B. licheniformis*, and *Pseudomonas fluorescens*, isolated from textile industry dye effluent, exhibited a lead removal of 86.73, 92.15, 89.45, and 94.32%, respectively (Basha and Rajaganesh, 2014). The findings indicated that *Bacillus* sp. effectively remediate lead contents, even at high levels.

Molecular identification

Thirty isolates were obtained from the different lead containing industrial effluents. Based on the highest reduction efficiency, the isolate S_8 was selected for phenotypic characterisation and molecular identification via 16S rRNA sequencing. The sequence was deposited in NCBI GenBank database under the Accession No. PZ327497. The phylogenetic tree was built using the Neighbour-joining method and the Mega XI version of the distance matrix alignment tool (Tamura *et al.*, 2021) with bootstrap 1000 replicates. In phylogenetic tree, BI21327 is positioned in proximity to *Bacillus cereus* strains; which confirms that isolate S8 is closely related to the *B. cereus* clade (Fig. 1).

In present study the isolated bacteria showed maximum lead uptake revealing them to possess the enzyme required for lead bioremediation. Earlier studies also have shown that *Bacillus* sp., isolated from an extreme habitat, has good lead biosorption capacity (Idrees *et al.*, 2023). *Bacillus pumilus* isolated from marine environment demonstrated 98% of lead removal (De *et al.*, 2008).

Conclusion: The current study isolated and screened lead-resistant bacteria from industrial effluent. The physicochemical analysis revealed the industrial effluent to be highly polluted, as evidenced by significantly reduced DO levels and increasing BOD values. This suggested an elevated level of biodegradable matter. The screening of lead-resistant bacterial isolates showed growth at 1000 ppm lead acetate indicating high lead tolerance, as growth was observed at the highest tested concentration, the MIC was considered to be greater than 1000 ppm. The resultant lead-resistant bacteria LRB, *Bacillus cereus*, isolated from industrial effluent, were more efficient in reducing lead at 48 h incubation. The identity of *B. cereus* was confirmed by morpho-biochemical tests and molecular profiling, while AAS confirmed lead reduction. The values of BOD, COD, TSS and TDS

were decreased and DO levels increased after treatment of industrial effluent with *B. cereus*. Revealing its strong bioremediation potential.

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