Mercury Bioremediation by *Pseudomonas aeruginosa: in vitro* insights

Ghangale S.S.[1]* and Saler R.S.[2]
1. Department of Biotechnology, Changu Kana Thakur Arts, Commerce and Science College, New Panvel, MS, 410206, INDIA
2. Department of Environmental Science, K.T.H.M. College, Nashik, MS, 422002, INDIA
*gssharmi@yahoo.com

Abstract
Since the last few decades, mercury pollution has posed serious health and environmental threats due to its long-term non-biodegradable nature. Bioremediation has the potential to address this concern. This study gives systematic in vitro insights into mercury bioremediation by Pseudomonas aeruginosa. The isolation of mercury-resistant bacterial isolates followed by their optimization to their maximum mercury removal ability, pH and temperature was conducted. Identification and characterization of bacterial isolate having maximum remediation capacity for mercury were conducted with the VITEK 2 compact system. The mercuric reductase enzyme assay was conducted to see the metabolic process of microbial bioremediation. Based on the standard method for identification and characterization, mercury-resistant bacterial isolate Hg-I3 was identified as Pseudomonas aeruginosa.

It was seen that among all the isolated bacteria, isolate Hg-I3 shows the highest potential i.e. 75.72% for mercury bioremediation. Enzyme Mercuric reductase activity was found to be 0.0827 units/ml at 30mins; the bioremediation process was found to increase with the increase in enzyme production. Optimization studies showed that pH (6.0) and temperature conditions (35°C) enhanced bioremediation. These findings underscore significance of microbial bioremediation in addressing mercury contamination and highlight the potential for further research and application in the field of sustainable environmental remediation.

Keywords: Bioremediation, Heavy metal, Mercury, *Pseudomonas aeruginosa*, VITEK.

Introduction

Heavy metals such as mercury, lead and zinc, present a significant threat to human health even at minimal concentrations due to their accumulation in the food chain[23]. Biomass naturally occurring at the contaminated sites including bacteria, fungi, yeast and algae, has historically proven effective in treating heavy metal contaminations below 100 mg L^{-1} [2]. Mercury, a highly toxic heavy metal, poses a substantial environmental challenge, affecting various environmental systems including soil, water and air. Bioremediation offers an economical alternative where the application of mercury-resistant microbes presents a promising opportunity to remove heavy metals from contaminated sources[14,22]. In the case of mercury, complete detoxification is achieved by the reduction of the more toxic Hg_2^+ form to less harmful and volatile Hg^0 form [17]. Several biological techniques have been studied for mercury bioremediation using bacterial sources, including enzymatic reduction (mercuroreductase, organomercuric lyase etc.), biosorption, phytoextraction and phytoremediation, leveraging the synergy of microbes and plant actions[9].

Studies have characterized mercury-resistant bacterial strains and assessed their potential applicability in the bioremediation such as in the contaminated Orbetello Lagoon[17]. In another study, a strain of *Pseudomonas putida*, designated as SP1, was isolated from a marine environment, exhibiting notable resistance to 280 μM $HgCl_2$[12]. However, existing literature on mercury bioremediation primarily focuses on specific backgrounds, including contaminated soil, marine water, estuarine water, or the use of fungi or algae with specific strains, while studies on treating industrial effluents by isolation of multiple bacterial strains are lacking[1,4,5,16,18,20,21].

This study aims to bioremediate mercury from heavy metal-contaminated sites by collecting and identifying resistant bacterial strains, enriching these strains, conducting bioremediation, enzymatic assay and studying the effect of pH and temperature on the bioremediating properties of the resistant bacterial strain. By doing so, this study will offer significant insights into microbial ecosystems around metal-contaminated sites along with their implications for sustainability.

Material and Methods

Collection and Enrichment of Metal-Tolerant Bacteria: Samples were collected in sterile disposable plastic containers from various metal-contaminated sites in and around Nashik, Pune and Mumbai, Maharashtra and stored at 4°C. To enrich mercury-resistant bacteria, the samples were mixed and a 20ppm mercury stock solution was added to 100ml of sterile Luria-Bertani (LB) broth (10g Peptone, 5g Yeast Extract, 10g NaCl dissolved in 950ml of sterile distilled water; pH maintained at 7.0 using 1N NaOH). All ingredients used for preparing the media were obtained from Hi-Media, Mumbai, India. The enrichment process was conducted at room temperature for one week [13]. Enriched mercury-resistant bacterial samples were isolated further by streak plate and serial dilution methods [6] and purified mercury-tolerant bacterial isolates were preserved and stored at 4°C.

Mercury Tolerance study: Bacterial isolates underwent a tolerance study with a mercury stock solution (50 ppm) and were incubated at room temperature for next 48 hours. After the initial screening for heavy metal tolerance, selected isolates were examined to determine their tolerance concentration to mercury. Initially isolated bacterial colonies were inoculated in sterile conical flask containing 100ml LB broth supplemented with increasing mercury concentrations from 10 ppm to 100 ppm to determine the maximum metal tolerance concentration of bacterial isolates for mercury.

Bioremediation: The bioremediation study aimed to determine the maximum metal removal capacity of mercury-resistant bacteria. Tolerant strains were inoculated in liquid culture media having pH 7.0 and incubated for 48 hours at room temperature. The optical density (O.D) of the culture media was recorded at 600nm. Once the O.D reached 1, the suspension culture was supplemented with mercury (20ppm) and incubated on a rotary shaker (120 rpm) at room temperature for 120 hours. At regular intervals (0, 24, 36, 48, 72, 96 and 120 hours), the suspension culture underwent centrifugation at 6000 rpm for 10 minutes. The supernatant was collected and was analysed to assess the mercury removal by the bacterial isolates. The percentage of mercury removal efficiency was determined using Atomic Absorption Spectroscopy[13,15].

Optimization of parameters for metal bioremediation and selection of the best bioremediating isolate: To identify the optimal isolate with the highest mercury bioremediation capability, several factors affecting the bioremediation of mercury were evaluated. Mercury solution (20ppm) was introduced into the liquid culture media and incubated on a rotary shaker (120 rpm) for all subsequent assessments. The evaluations were conducted using Atomic absorption spectroscopy. The effect of pH was examined using buffer solutions with varying pH levels, ranging from pH 5 to pH 8. These solutions were incubated at room temperature for 120 hours. The effects of pH and temperature on the bioremediation of mercury were assessed at the intervals of 24 hours till 120 hours. To investigate the influence of temperature, the bacterial isolate was incubated at different temperature settings (20°C, 25°C, 30°C and 35°C).

The effects of temperature on mercury bioremediation were evaluated from 24 to 120 hours[24]. The best isolate was further subjected to mercury solution (50ppm) in sterile media with the optimized pH and incubated on a rotary shaker (120 rpm) at room temperature for next 48 hours. The growth pattern of the isolate was assessed by measuring its OD at 600 nm using a UV Spectrophotometer (UV-1800, Shimadzu, Japan) at 8 hours intervals from 0 to 48 hours.

Identification and characterization of the isolate: Colony and cell morphology were examined using a dissecting microscope for colony morphology and an oil emulsion lens of a compound microscope for cell morphology. Subsequently, rapid characterization and identification of comparatively best bacterial isolate were carried out using VITEK2 compact system (bioMérieux)[3].

Isolation of enzyme involved in microbial bioremediation of mercury: The crude mercuric reductase enzyme was isolated in liquid media supplemented with bacterial isolates. For the enzymatic assay during the bioremediation study, a 24-hour-old culture of bacterial isolates was utilized and cultured cells were sonicated using a Bio-Era sonicator at 600 watts for 1 minute at 50% amplitude.

Subsequently, the homogenized solution underwent centrifugation (15,000 rpm) for 30 minutes at 4°C. This process was repeated at regular intervals of 24 hours up to 120 hours. The supernatant obtained was collected for further enzyme assay.

Mercuric reductase enzymatic assay: The assay method involved the preparation of a 3.9 ml mercuric reductase assay solution in a dark tube. This solution was composed of 50mM sodium phosphate buffer (pH 7), 0.2 mM $MgSO_4$, 0.1% β-mercaptoethanol and 0.1 ml of NADH (used as the substrate). To this mixture, 1 ml of 80μM $HgCl_2$ and 0.1 ml of crude enzyme were added. The resulting reaction mixture was incubated at different time intervals (30, 60, 90, 120 and 150 min) at 37°C. 200 μM NADH was used to study the mercuric reductase enzyme activity. The amount of residual NADH was measured at 340nm. The enzyme activity of mercuric reductase was defined as one unit when 1 μM of NADH was oxidized per minute at given condition.

Statistical analysis: All experiments were conducted in triplicate. Data collection was performed using Microsoft Excel 2010, which was further employed for calculating descriptive statistical values. All values are represented as mean (SD), specified otherwise. A linear regression model was employed to evaluate the statistical significance of mercuric reductase assay. A two-sided p value of <0.05 was statistically significant.

Results

Samples were collected from 10 different industrial areas located in the State of Maharashtra, India (Supplementary Table S1). The samples were stored at 4°C and subsequently enriched with 20ppm mercury stock solution. Mercury tolerance study was conducted to check the heavy metal tolerance in the isolates. The isolates are labelled in table 1.

Isolation of the mercury resistant bacteria: Overall, 32 mercury tolerant bacterial isolates were isolated out of which, 25 isolates showed growth in liquid media containing 100ppm of mercury solution. Furthermore, bacterial isolate Hg-S1 and Hg-I3 exhibited highest level of mercury resistance after 96 hours. of incubation, with a metal tolerance concentration (++) of 100 mg/L. This shows that isolate Hg-S1 and Hg-I3 displayed minimum inhibitory concentration (Table 1).

Bioremediation studies: Hg-I3 and Hg-S1 isolates were found to have maximum activity against mercury stock solutions to be 100ppm (Table 1). Hence, both isolates were selected for *in vitro* bioremediation studies. During the bioremediation study, isolate Hg-I3 showed superior reduction of mercury (II) (75.72%) as compared to Hg-S1 (65.04%) after 120 hours. Therefore, isolate Hg-I3 was selected for further optimizations (Tables 2 and 3).

Optimization of parameters for selected isolate: Isolate Hg-I3 showed the highest reduction of mercury (77.66%) after 120 hours at pH 6 surpassing the other pH levels.

Supplementary Table S1
Collection of samples from various metal-contaminated sites

Sample ID	Site Type	Site Name
SM 1	Metallurgical Work	MUKUND Metallurgical Work, Ambad MIDC, Nashik
SM 2	Auto Service Station	MILIND Auto Service Station, Satpur Industrial Estate, Satpur, Nashik
SM 3	Petrol pump and Parking locations	Petrol pump and Parking locations In and around Nashik
SM 4	Electroplating Industry	Electroplating Industry, Satpur, MIDC, Satpur, Nashik
SM 5	Metal coating Works	Metal Coating Works, Shirwane, Nerul, Navi Mumbai
SM 6	Zinc Plating Industries	Zinc Plating Industries, Pimpri- Chinchwad Industrial Area, Pune
SM 7	Metal Plating Industries	Metal Plating Industries, Bhosari Industrial Area, Pune
SM 8	Effluent Treatment Plant	Effluent Treatment Plant, MIDC, Pune
SM 9	Petrol pump and Parking locations	Petrol pump and Parking locations in and around Pimpri-Chinchwad Industrial Area, Pune
SM 10	Electroplating Industry	Electroplating Industry, Taloja MIDC, Navi Mumbai

Table 1
Maximum metal tolerance study of bacterial isolates for metal mercury after 96 hrs.

Selected isolates	Mercury solution (ppm)									
	10	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Hg -I1	+++	+++	+++	+++	+++	++	++	++	+	+
Hg -I2	+++	+++	+++	++	++	++	+	+	-	-
Hg -I3	+++	+++	+++	+++	+++	+++	+++	++	++	++
Hg –I4	+++	+++	+++	++	++	++	+	+	-	-
Hg-II1	+++	+++	+++	+++	+++	++	++	++	++	+
Hg -II2	+++	+++	+++	++	++	++	+	+	-	-
Hg -II3	+	++	+	+	-	-	-	-	-	-
Hg -II4	++	+	+	+	+	-	-	-	-	-
Hg -II5	++	+	+	+	+	+	-	-	-	-
Hg -III1	+++	+++	+++	+++	++	++	++	+	+	+
Hg -III2	+++	+++	+++	+++	+++	++	++	++	+	+
Hg-III3	++	++	++	++	+	+	+	-	-	-
Hg –S1	+++	+++	+++	+++	+++	+++	++	++	++	++
Hg -S2	++	+	+	+	-	-	-	-	-	-
Hg -S3	++	++	++	+	+	+	-	-	-	-
Hg -S4	+++	+++	+++	++	++	+	+	-	-	-
Hg -S5	++	++	++	++	+	+	-	-	-	-
Hg -SI1	+++	+++	+++	++	++	+	+	+	-	-
Hg -SI2	+	+	+	+	-	-	-	-	-	-
Hg -SII1	++	++	++	++	+	+	-	-	-	-
Hg –SII2	++	+	+	+	+	-	-	-	-	-
Hg –SIII	+++	+++	+++	+++	++	++	++	+	+	-
Hg-SI (Mix)	+++	+++	+++	+++	+++	+++	++	++	++	++
Hg-SII (Mix)	++	++	++	++	++	+	+	+	+	+
Hg-SIII (Mix)	+++	+++	+++	+++	++	++	++	+	+	+

(+) =Positive growth, (-) =No growth

Table 2
Optimization of parameters for selected isolate

Parameters	Initial concentration of mercury in the medium (ppm)	Mercury remediation concentration at 24 hours (ppm)	Mercury remediation concentration at 48 hours (ppm)	Mercury remediation concentration at 72 hours (ppm)	Mercury remediation concentration at 96 hours (ppm)	Mercury remediation concentration at 120 hours (ppm)
Bioremediation of mercury for selection of best isolate						
Hg-I3	20.6	4.5	8.6	14.2	15	15.6
Hg-S1	20.6	3.3	7	9.3	11.9	13.4
Effect of pH on Hg-I3						
pH= 5	19.7	4.7	8	10.8	13.3	13.8
pH= 6	19.7	5.5	9.6	11.8	14.4	15.3
pH= 7	19.7	4.1	8.6	12	14.1	14.7
pH= 8	19.7	3.5	7	9.8	12.2	13
Effect of temperature on Hg-I3						
20°C	20.4	2.4	5.7	7.1	9.9	10.8
25°C	20.4	3.8	7.1	9.4	12.2	13.6
30°C	20.4	4.9	8.6	11.6	14.5	15.3
35°C	20.4	5.8	9.9	13.2	15.7	16.1

Table 3
Effect of heavy metal mercury on growth pattern of Hg-I3

Time (hrs)	Test Hg-I3 (OD)*	Control (OD)*
0	0.17 (±0.06)	0.17 (±0.05)
8	0.17 (±0.05)	0.21 (±0.04)
16	0.36 (±0.04)	0.91 (±0.05)
24	0.61 (±0.05)	0.97 (±0.04)
32	0.76 (±0.03)	1.06 (±0.05)
40	0.79 (±0.04)	1.01 (±0.03)
48	0.84 (±0.04)	0.93 (±0.03)

*Values in Mean (±SD); OD, Optical Density

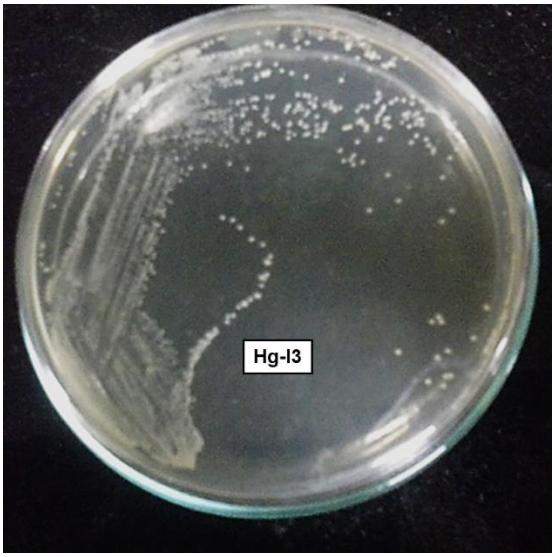

Figure 1: Growth pattern of the best isolate, Hg-I3 i.e., *Pseudomonas aeruginosa*

Besides, the highest reduction of mercury (78.92%) was shown by isolate Hg-I3 at 35°C temperature, over 120 hours. outperforming the other temperature ranges. This indicates that pH 6 and temperature 35°C are the optimal condition for bioremediation and reduction of mercury by isolate Hg-I3.

The growth pattern assessment with the best-selected isolate Hg-I3 showed slower progression in the log phase as compared to the control group. The stationary phase was observed between 24 to 48 hours. Thereafter, decline in growth was evident.

Supplementary Table S2
VITEK identification of Hg-I3

VITEK- 2 system report for identification of the organism			
Identification Information	Card: GN	Lot Number: 241272040	Expires: Feb 20, 2020, 12:00 IST
	Completed: June 8, 2019, 18:53, IST	Status: Final	Analysis Time: 5.00 hours
Selected Organism	99% Probability *Pseudomonas aeruginosa*		
	Bio number: 0043053103500242		
	Confidence: Excellent identification		

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	+			

Installed VITEK 2 System Version: 06:02

Table 4
Characterization of isolates for identification

Tests	**Results**	**Tests**	**Results**
Colony morphology		**Cell Morphology and Staining**	
Configuration	Large	Gram's reaction	- ve
Margin	Irregular	Cell shape	Rod
Elevation	Flat	Size (µm)	Short
Surface	Smooth	Endospore	Absent
Pigment	Bluish Green	Shape	-
Opacity	Translucent	Position	-
Physiological Tests		Sporangial bulging	-
		Motility	+
Growth at temp.(C)		**Acid Production**	
10	-	Glucose	+
20-40	+	Maltose	-
50-60	-	Xylose	-
Growth at pH		Salicin	-
5	-	Mannose	+
6.0-9.0	+	Melibiose	+
10	-	Arabinose	-
Growth in NaCl (%)		Lactose	-
2 to 8	-		
10	-		
Biochemical characteristics			
Growth in Luria-Bertani Agar	+	Starch Hydrolysis	+
Indole Test	-	Nitrate Reduction	+
Methyl Red Test	-	Esculin Test	+
VP Test	-	Catalase Test	+
Citrate Utilization	+	Oxidase Test	+
H2S Production	-	Arginine Dihydrolase	-
Gas Production	+	Tween 20 Hydrolysis	-
Casein Hydrolysis	+	Tween 40 Hydrolysis	-
Gelatin Hydrolysis	+	Tween 80 Hydrolysis	+

(+) =Positive, (-) =Negative

Table 5
Mercuric reductase enzymatic activity for *Pseudomonas aeruginosa*

Time (mins)	Absorbance (λ 340 nm)	Total amount of NADH (µM)	Oxidized NADH (µM/5ml/min)	Oxidized NADH (µM/ml/min)	Mercuric Reductase Enzyme Activity (Units/ml)
30	0.92	187.6	12.4	2.48	0.0827
60	0.87	178.0	9.6	1.92	0.0320
90	0.83	169.0	9.0	1.80	0.0200
120	0.80	163.9	5.1	1.02	0.0085
150	0.78	159.2	4.7	0.94	0.0062

Table 6
Linear regression model for enzymatic assay of mercuric reductase

Coefficients:`				
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.08283	0.01707	4.852	0.0167
time	-0.00059	0.000172	-3.429	0.0416
Residual standard error: 0.01628 on 3 degrees of freedom				
Multiple R-squared: 0.7968, Adjusted R-squared: 0.729				
F-statistic: 11.76 on 1 and 3 DF, p-value: 0.04156				

It was found that the addition of mercury has significant impact on the log phase of growth of bacteria under study. The results are illustrated comprehensively in table 2. The effect of mercury exposure on the growth of isolated Hg-I3 is detailed in table 3.

Identification and characterization of the isolate: Morphologically, single, circular, well isolated and discrete colonies were observed on both streaked and serial dilution petri plates (Figure 1). Based on standard method for identification and characterization of bacterial isolates along with use of VITEK 2 system (bioMérieux), isolate Hg-I3 for the degradation of mercury, was identified and confirmed as *Pseudomonas aeruginosa* (Table 4) (Supplementary Table S2).

Mercuric reductase enzymatic assay: The quantitative NADH estimation showed a linear relationship between decreasing concentration and absorbance. The assay showed rapid enzymatic metabolic degradation of mercury within the first 30 minutes at the rate of 0.0827 units/ml followed by a tapering curve. Linear regression model revealed statistical significance of the enzymatic reduction over the period of 150 minutes (P= 0.0416). The mercuric reductase enzymatic activity for *Pseudomonas aeruginosa* is illustrated in tables 5 and 6.

Discussion

During the study, 32 bacterial strains were successfully isolated from heavy metal-contaminated sites in Maharashtra, India. Among these isolates, only 25 demonstrated growth with respect to 100 ppm mercury. A prior study, which collected similar effluents, reported the isolation of *Bacillus, Klebsiella, Enterobacter* and *Acinetobacter* strains in Mumbai, India [19]. The isolated *Pseudomonas aeruginosa* achieved a mercury (II) reduction of 75.72% after 120 hours. The preliminary bioremediation results highlight the potential of this strain for mercury detoxification.

The optimization of bioremediation parameters is a crucial step in enhancing the efficiency of mercury removal. A study investigating effect of pH on mercury uptake by an aquatic bacterium reporting even a slightest change in pH may lead to larger bacterial uptake of mercury (II)[10]. The study investigated the effects of pH and temperature on bioremediation capacity of isolate Hg-I3. The results indicated that pH 6 and 35°C temperature could be the optimal conditions for mercury reduction.

According to the standard methods used for the morphological, cultural, physiological and biochemical characterization and VITEK 2 compact system, isolate Hg-I3 was identified as *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* exhibited promising results in the bioremediation of mercury with highest reduction potential (75.72%). Previously, 60-62% of mercuric reduction has been reported[7,11]. This finding underscores the potential of this isolate for eco sustainable bioremediation.

Mercuric reductase is a key enzyme involved in the reduction of mercury, converting toxic mercuric ions into less harmful elemental mercury. The enzymatic assay demonstrated rapid degradation of mercury within the first 30 minutes, further validating the strain's potential for efficient mercury bioremediation. This enzymatic assay offers valuable insights into the mechanisms underlying the strain's bioremediation capabilities. A similar study detected the activity of the enzyme mercuric (II) reductase reported 86% removal of mercury (II) by newly isolated *Pseudomonas sp. B50A*. However, this isolate showed optimal activity at pH 8, at temperatures between 37 °C and

45 °C. This observation is slightly different from the study of Giovanella et al[8].

The observed rapid enzymatic metabolic degradation of mercury within the first 30 minutes in our assay aligns with the efficient mercury reduction exhibited by *Pseudomonas aeruginosa* strain Hg-I3 during the bioremediation study. The consistent activity over the 150-minute duration suggests the sustained effectiveness of mercuric reductase in the bacterial isolate. The linear relationship between decreasing NADH concentration and absorbance shows the reliability of the assay in quantifying mercuric reductase activity. *Pseudomonas aeruginosa* strain Hg-I3 has demonstrated a high capacity for removing mercury, under optimized conditions making it valuable in addressing complex heavy metal-contaminated environments.

The characterization of mercuric reductase enzyme activity further adds to the understanding of the strain's mechanisms. This research contributes to the broader field of bioremediation by providing insights into the isolation, characterization and optimization of a bacterium capable of efficiently reducing mercury contamination. These findings have practical implications for environmental cleanup efforts and may pave the way for more ecofriendly sustainable and cost-effective approaches to mitigate heavy metal pollution and the resulting adverse impact on biodiversity.

Conclusion

In conclusion, the study successfully isolated and characterized a mercury-resistant bacterial strain *Pseudomonas aeruginosa.* According to the conducted experimental studies, this bacterium shows noteworthy potential i.e. 75.72% for mercury bioremediation. Significant mercuric reductase activity i.e. 0.0827 units/ml was notable. It was also observed that optimization of pH and temperature conditions could enhance its bioremediation capabilities. The identification and characterization of this strain, along with mercuric reductase enzymatic assay, provided *in vitro* insights of mercury bioremediation by *Pseudomonas aeruginosa.*

In locations with exceptionally elevated mercury contamination levels, this application assumes particular significance. To prevent the escape of metallic Hg^0 into the atmosphere, addressing its recovery becomes imperative. Enhancing the comprehension of these capabilities not only contributes to the understanding of physiological responses but also strengthens our ability to innovate and to develop technologies for remediating wastes contaminated with mercury.

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