Screening and molecular identification of Mercury Tolerant Bacteria from Dairy Waste and Evaluation of their Bioremediation Potential by Atomic Absorption Spectroscopy

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Abstract
The present study aimed to isolate, to screen and to identify molecularly mercury-resistant bacteria from industrial effluents and to evaluate their mercury-reducing potential using Atomic Absorption Spectroscopy (AAS). Effluent samples from the dairy industries in Pune city were collected and enriched in nutrient broth supplemented with $HgCl_2$ for the isolation of mercury-resistant bacterial isolates. The isolates were obtained on different agar media (Nutrient Agar, LB Agar, Minimal Agar and Mueller–Hinton Agar) containing mercury concentrations ranging from 10 ppm to 200 ppm. The morphological and biochemical characterizations were performed for selected isolates. The mercury reduction by selected isolates was evaluated by using AAS over 48 hours.

Molecular identification of potent isolates was performed by 16S rRNA gene sequencing, followed by phylogenetic analysis using MEGA 11 software. Out of 25 isolates, three isolates H1, H6, H7 exhibited growth at 200 ppm $HgCl_2$, while K1 exhibited growth at 100 ppm concentration of $HgCl_2$. Based on AAS analysis, H1 and H6 demonstrated the highest mercury reduction, 7.44 ppm and 16.76 ppm respectively after 30 hours of incubation. Morphological, biochemical and molecular characterization revealed H1 as Pseudomonas aeruginosa and H6 as Bacillus paranthracis. The study demonstrated that Pseudomonas aeruginosa and Bacillus paranthracis isolates possess strong mercury reduction capabilities, highlighting their potential applications in the bioremediation of mercury-contaminated environments.

Keywords: Mercury-tolerant bacteria, Industrial effluent, Atomic Absorption spectroscopy, Bioremediation, $HgCl_2$.

Introduction

The industrial revolution has significantly contributed to the rise in heavy metal contamination, posing serious risks to human health. Various anthropogenic activities transform these metals into highly toxic forms that persist in the environment for extended periods[3]. Industrial effluents from sectors such as steel manufacturing, electroplating, chemical processing and leather tanning serve as major sources of heavy metal pollution, contaminating nearby land and water bodies[27]. While trace amounts of certain metals like iron, zinc, copper and manganese are essential micronutrients for growth, protein stability and enzymatic functions in living organisms, others are highly toxic and lead to severe cellular alterations and excessive concentrations of heavy metals can have toxic effects[9].

The harmful impact of toxic element contamination in soil and aquatic ecosystems was largely due to their non-biodegradable nature, which led to their accumulation in the food chain and posed unavoidable risks[17]. The accumulation of the elements was evident in their biomagnification within living organisms across the food chain[8]. Certain toxic elements, such as Cd, As and Ni, bind to microbial protein molecules, disrupting DNA repair pathways and inhibiting the DNA replication process[19]. The failure of DNA replication consequently prevented further cell division, growth and DNA repair[30]. Similarly, the accumulation of these toxic elements in the human body has been linked to cancer development and organ damage[4].

Additionally, elements such as Cd, As, Cr and Hg were associated with certain types of cancer, even at low concentrations[26]. The presence of these potentially toxic elements in the environment undeniably affects ecosystems, leading to alterations in biomass, significant shifts in microbial communities and disruptions in elemental cycling[14]. Heavy metals are difficult to degrade in the soil, making their complete detoxification a significant challenge for scientists. Despite ongoing efforts to address environmental pollution, the harmful effects of heavy metal contamination remain a global concern. Various physicochemical methods, including filtration, chemical precipitation, electrochemical treatment, oxidation-reduction, ion exchange, membrane technology, reverse osmosis and evaporation recovery, have been developed for heavy metal removal from contaminated water[28].

However, many of these techniques are costly, less efficient, labour-intensive, or lack selectivity in the treatment process[7]. Therefore, innovative solutions are needed to mitigate this issue, with bioremediation emerging as a

promising approach[24]. Bioremediation involves microbial absorption and adsorption of toxic metal ions, reducing their harmful effects[21]. Microorganisms facilitate heavy metal remediation through mobilization or immobilization followed by oxidation-reduction, chelation and biomethylation[22]. Enzymatic catalysis by microorganisms enhanced metal solubility by reducing oxidation states, with aerobic microbes proving more effective than anaerobic ones[2]. Using membrane-associated transport, microbes convert heavy metals into less toxic forms[13].

To survive in metal-contaminated environments, microorganisms employ various strategies such as biosorption, bioaccumulation, biotransformation and bioleaching. These approaches have been widely utilized in bioremediation and environmental clean-up processes[15]. The aim of the present study was the isolation and identification of mercury-tolerant bacteria from industrial effluent, along with the evaluation of their mercury-reducing potential of the most potent organisms through Atomic absorption spectrophotometry.

Material and Methods

Sample Collection: The effluent samples were collected from the dairy industry located in Pune. A clean plastic container was used for the collection to ensure no contamination during the process. The samples were then transferred to the laboratory and stored in a refrigerator to maintain their integrity until it was ready for analysis.

Sample Enrichment and Isolation of Bacteria: A 10 ml aliquot of industrial effluent was inoculated with 90 ml of $HgCl_2$-supplemented nutrient broth and incubated at 30ºC on a rotary shaker at 150 rpm for 48 hrs. The serial dilution of the enriched media was prepared from 10^{-1} to 10^{-7} and streaked on the nutrient agar plates and incubated for 24 hours at 37ºC.

Screening of the Bacteria: The single colony from the nutrient agar was then picked and streaked on the nutrient agar, Mueller–Hinton and Minimal agar and LB agar containing 10 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm concentration of the $HgCl_2$ respectively. The plates were then incubated for 24 hours at 37ºC.

Morphological and Biochemical Characterization: Gram-staining and motility tests for the isolates were performed microscopically as per the standard protocol for the identification of bacteria. The Biochemical tests included IMViC, catalase test, oxidase test, starch hydrolysis test and nitrate reductase test performed as per the standard protocol for all isolated organisms[16].

Atomic absorption spectroscopy: The bacterial cultures were incubated in 100 mL of LB broth supplemented with 0.02 g of mercury (Hg). At regular intervals of every 6 hours, 10 mL of the culture medium was withdrawn and centrifuged at 4000 rpm for 20 minutes to separate the biomass. The supernatant was carefully collected and digested with a double volume of concentrated nitric acid. The digested samples were allowed to cool to room temperature before being filtered through Whatmann filter paper no. 1. The clear filtrate was collected into clean centrifuge tubes and diluted appropriately with double-distilled water. The prepared samples were then analyzed for metal concentration using atomic absorption spectroscopy to measure absorbance[10,18].

Molecular Identification of the isolates: The most potent isolate was selected for molecular identification by amplification and sequencing of 16S rRNA. Genomic DNA was extracted from bacterial isolates using the standard phenol/chloroform extraction method. The 16S rRNA gene was then amplified via polymerase chain reaction (PCR) employing universal primers 16F27 (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) and 16R1492 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). The resulting PCR products were purified through polyethylene glycol (PEG)-NaCl precipitation.

Subsequently, the purified amplicons were sequenced bidirectionally using an ABI 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA), following the manufacturer's protocols. Additional internal primers were utilized to ensure that each nucleotide position was read at least twice, enhancing the accuracy of the sequencing data. The phylogenetic tree was then constructed using the MEGA 11 software[5,29].

Results and Discussion

Isolation and Screening of the isolates: A total of 25 bacterial isolates were obtained on nutrient agar supplemented with $HgCl_2$. All isolates were subjected to preliminary screening for mercury tolerance on nutrient agar, Mueller–Hinton Agar, Minimal Agar and LB agar supplemented with $HgCl_2$ at concentrations of 10 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm. Among these, three isolates demonstrated maximum growth at the highest concentration (200 ppm) of $HgCl_2$ and one at the 100-ppm concentration of $HgCl_2$ on LB agar. These isolates were labelled as H1, H6, H7 and K1 selected for further characterization.

Morphological and Biochemical Characterization: The colony characteristics, morphological and Gram nature studies of the isolated bacteria are shown in table 1. The current study isolated mercury-resistant bacterial isolates from the dairy industry wastewater. After the screening, the streak plate method isolated the three most potent organisms on the LB agar plate. Three isolates exhibited growth on LB agar supplemented with the highest 200 ppm concentration of $HgCl_2$. The H1 and H7 were Gram-negative and non-motile, whereas isolate H6 was Gram-positive and non-motile. A similar study was conducted where they found that the Gram-negative *P. aeruginosa* and Gram-positive *B. casei* had mercury resistance[25]. Another study also found that *P. putida* can degrade thiomersal mercury[11].

Table 1
Morphological properties of the bacterial isolates

Colony Characters	Bacterial Isolates			
	H1	**H6**	**H7**	**K1**
Size	Pin-Point	Large	Large	Small
Shape	Circular	Irregular	Irregular	Circular
Color	Blue-green	Off White	Off White	Off-white
Margin	Entire	scalloped	Entire	Irregular
Elevation	Raised	Flat	umbonate	Slightly Raised
Opacity	Opaque	Translucent	Opaque	Opaque
Consistency	Moist and Smooth	Moist and Smooth	Moist and Smooth	Fuzzy and Rough
Grams Nature	Negative (Rod)	Positive (Rod)	Negative (Rod)	Positive (Rod)
Motility	Motile	Non-Motile	Non-Motile	Non-Motile

Table 2
Biochemical characterization for the isolated bacteria

Sample	Indole	Methyl red	Vogus proskeaur	Citrate	Catalase	Oxidase	Sugar fermentation	Starch hydrolysis test
H1	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve
H6	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
H7	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve
K1	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve

Note: The sign -ve indicates Negative biochemical characterization and +ve indicates Positive biochemical characterization of isolates.

The differences in the Gram nature of the isolates were because the outer membrane of the organism was rich in lipopolysaccharides, which carried a net negative charge and served as a first line of defense by binding Hg^{2+} ions and slowing their entry into the cytoplasm.

They also possessed membrane-embedded efflux pumps and the 'mer' operon, which enzymatically reduced Hg^{2+} to volatile Hg^0, thereby lowering intracellular mercury levels [23]. The structural and biochemical defenses explained that both H1 and H6 emerged as the most potent mercury-removing isolates in the current study despite their differing Gram-staining characteristics. The biochemical characterization for the isolates is mentioned in the table 2.

Atomic Absorption Spectroscopy: Table 3 and figure 1 show the mercury concentrations (in ppm) over different incubation periods, as measured by Atomic absorption spectroscopy (AAS), for three bacterial isolates: H1, H6 and H7. The Atomic absorption spectroscopy was not performed for the K1 bacteria as it does not give significant results in the 200-ppm concentration of $HgCl_2$ as compared to the H1, H6 and H7.

Initially, at 0 hours, the mercury concentrations were recorded as 8.59 ppm for H1, 18.19 ppm for H6 and 15.91 ppm for H7. Over time, the concentrations decreased for H1 and H6, indicating that these isolates effectively removed mercury from the samples. After 24 hours of incubation, H1 and H6 showed substantial reductions, with remaining mercury concentrations of 1.21 ppm and 1.5 ppm respectively. In contrast, H7 maintained a relatively high mercury concentration of 15.56 ppm, suggesting a lower removal efficiency. After 48 hours, H1 and H6 continued to maintain low residual mercury levels (1.40 ppm and 3.15 ppm, respectively), whereas H7 exhibited fluctuating concentrations, with 17.82 ppm. These results indicated that isolates H1 and H6 were more efficient in reducing mercury content over the incubation period compared to H7.

The study indicated similar results to the current study, suggesting that *Pseudomonas stutzeri* demonstrated efficient mercury removal over time, achieving approximately 35% reduction at 10 hours, 60% at 20 hours, 75% at 30 hours and 90% at 40 hours[1]. These isolated bacteria, due to their small size, possess a high surface area-to-volume ratio, which facilitates extensive contact with metal ions. This characteristic enhances bacterial capacity to adsorb and interact with metals, making them effective agents in bioremediation processes[20].

Another study reported that the *Bacillus cereus* demonstrated the ability to remove 60.06% of the initial mercury concentration after 48 hours of incubation under the experimental conditions, with the mercury content in the media, which was 100 ppm[12]. These results were also similar to the present study, where bacteria show the highest reduction of mercury at 30 hrs., but at the high mercury concentration in the present study, which was 200 ppm of mercury in the media by *Bacillus paranthracis.* These findings demonstrate that members of the *Bacillus* genus can efficiently sequester and detoxify mercury from contaminated media, even at elevated concentrations and within shortened time.

Table 3
Reduction of Mercury by Bacterial Isolates by Atomic Absorption Spectroscopy

Time in Hrs.	H1	H6	H7
	Reduction of Mercury in ppm		
0 hr.	8.59	18.19	15.91
6 hr	4.85	13.43	16.72
12 hr.	4.61	12.76	15.88
18 hr.	2.91	10.21	14.23
24 hr.	1.21	1.5	15.56
30 hr.	1.15	1.43	14.78
36 hr.	1.72	2.14	22.17
42 hr.	1.69	2.09	21.73
48 hr.	1.40	3.15	17.82

Note: The lower number means the remaining mercury concentration in the media after the incubation.

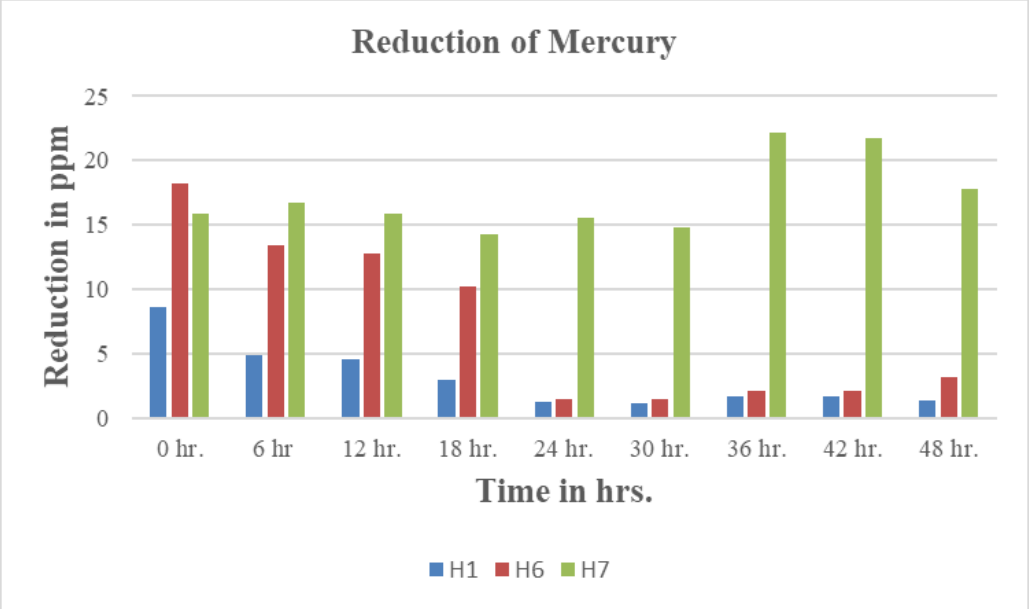

Figure 1: Reduction of Mercury by Bacterial Isolates by Atomic Absorption Spectroscopy

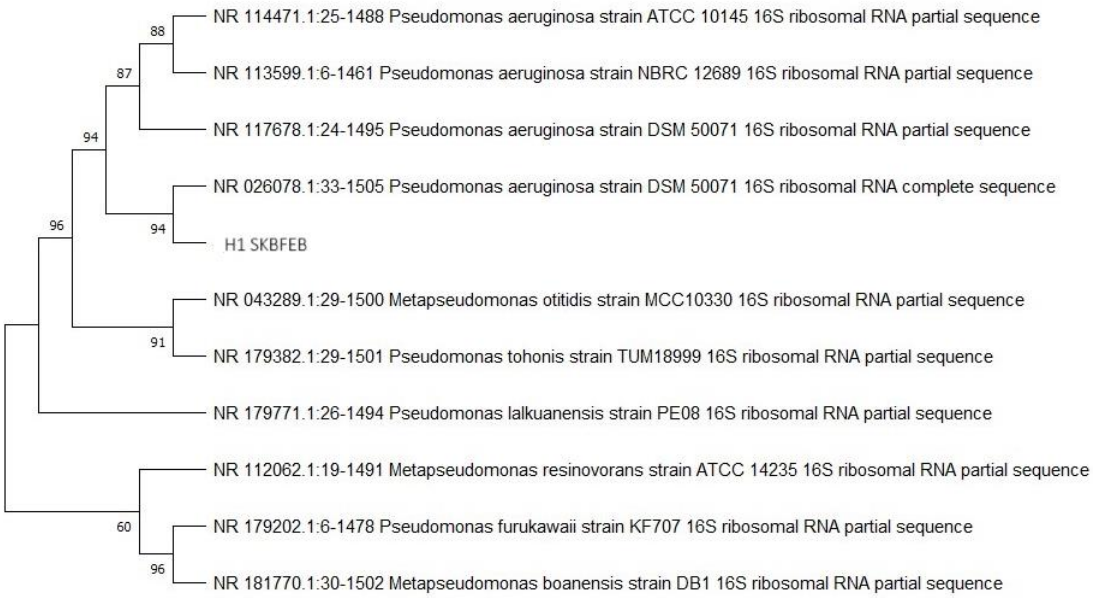

Figure 2: Phylogeny tree for isolate H1

Molecular Identification: As per the results of atomic absorption spectroscopy, the H1 and H6 organism shows the highest reduction of mercury as compared to the H7 over the period. Thus, the H1 and H6 were selected for the molecular identification.

After gene sequencing and sequence editing, the obtained bacterial sequences were compared with 16S rRNA gene sequences available in the NCBI GenBank database using the BLASTN tool. The BLAST analysis retrieved several closely related sequences. These sequences were used in the MEGA 11 software to construct the phylogeny tree for H1 and H6. The phylogenetic tree illustrates the evolutionary relationships among various *Pseudomonas* and *Metapseudomonas* species based on 16S rRNA gene sequences. The isolate labeled H1 SKBFEB, identified in the present study, clustered closely with *Pseudomonas aeruginosa* strains including ATCC 10145, NBRC 12689 and DSM 50071, with strong bootstrap support values ranging from 87 to 96. The close clustering indicates a high level of genetic similarity between H1 SKBFEB and the *Pseudomonas aeruginosa* clade. The phylogenetic analysis confirms that the isolate H1 SKBFEB belongs to the *Pseudomonas aeruginosa* lineage shown in figure 2. The mercury-reducing ability of *Pseudomonas aeruginosa* has been well documented in previous studies, where it has been shown to express key genes of the 'mer' operon, such as *merA* and *merB*, which enzymatically reduce toxic Hg^{2+} to less toxic elemental Hg^{0} [6].

The phylogenetic tree illustrates the evolutionary relationship among various *Bacillus* species based on 16S rRNA gene sequences. The bacterial isolate labeled as (1) H6 SKBFEB was identified in the present study. This isolate clustered closely with *Bacillus paranthracis* Mn5 (MACE01000012), supported by a high bootstrap value of 99.93%, indicating a strong genetic similarity. The clade also includes closely related species such as *Bacillus nitratireducens*, *Bacillus anthracis* and *Bacillus paramycoides*, suggesting phylogenetic proximity within this branch of the *Bacillus cereus* group. This analysis confirmed that the isolate belongs to the *Bacillus paranthracis* lineage shown in figure 3.

The members of the *Bacillus* genus, including *B. cereus* and related species, have been reported to possess mercury resistance mechanisms, either through mer operon expression or the production of hydrogen sulfide, which can precipitate mercury as insoluble HgS[12]. The findings of the current study are consistent with such reports, reinforcing the role of both Gram-negative and Gram-positive bacteria in mercury detoxification. Moreover, the rapid and significant reduction of mercury by H6 at a high initial concentration (18.19 ppm) suggests that *Bacillus paranthracis* may possess robust metal resistance and detoxification pathways, potentially involving both enzymatic reduction and biosorption mechanisms.

In the present study, the isolate H6, molecularly identified as *Bacillus paranthracis*, exhibited significant mercury-reducing ability, with a maximum reduction observed at 30 hours of incubation. This finding is particularly noteworthy, as it suggests that *B. paranthracis* may possess detoxification mechanisms similar to other members of the genus, such as the presence of the 'mer' operon or other metal-binding proteins involved in mercury resistance. The absence of previous studies on *B. paranthracis* in this context highlights the novelty of the current work and suggests the need for further investigation into its genomic and functional traits related to heavy metal resistance.

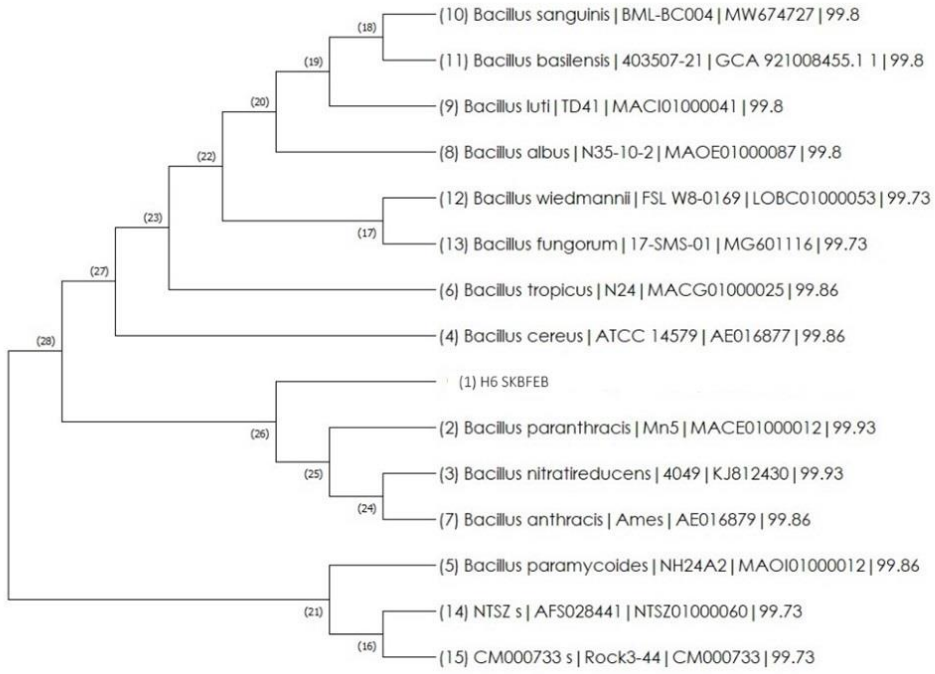

Figure 3: Phylogeny tree for isolate H6

These results expand the known diversity of mercury-resistant *Bacillus* strains and open new avenues for exploring their application in environmental bioremediation.

Conclusion

The present study successfully isolated and screened mercury-resistant bacteria from contaminated sources, identifying *Pseudomonas aeruginosa* (H1) and *Bacillus paranthracis* (H6) as the most efficient mercury-reducing strains. Both isolates demonstrated significant mercury reduction, with H6 showing the highest removal at 30 hours of incubation. Morphological, biochemical and molecular analyses confirmed their identity and mercury tolerance. These findings suggest their strong potential for application in mercury bioremediation.

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