

Biosorption of Mercury by Bacteria, Isolated from Industrial Effluents: Potential Use in Bioremediation of Wastewater

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Abstract.- Two mercury resistant bacterial strains-*Pseudomonas aeruginosa* and *Pseudomonas* sp. H1-G1 were isolated from industrial wastewater of Sialkot (Pakistan). The minimum inhibitory concentrations of Hg^{2+} ranged between 400-500 $\mu g/mL$. *Pseudomonas aeruginosa* could tolerate Pb^{2+} (650 $\mu g/mL$), Cu^{2+} (200 $\mu g/mL$), Cd^{2+} (50 $\mu g/mL$), Zn^{2+} (50 $\mu g/mL$), Ni^{2+} (550 $\mu g/mL$) and Cr^{6+} (100 $\mu g/mL$). *Pseudomonas* sp. H1-G on the other hand, showed resistance against Pb^{2+} , Cu^{2+} , Cr^{6+} , Ni^{2+} , Zn^{2+} and Cd^{2+} at a concentration of 650, 350, 150, 550, 50, and 250 $\mu g/mL$, respectively. The isolates showed typical growth curves but lag and log phases extended in the presence of mercury. Both isolates showed optimum growth at 37°C and pH 8-9. Metal processing ability of the isolates was determined in a medium containing 100 $\mu g/mL$ of Hg^{2+} . Both *Pseudomonas aeruginosa* and *Pseudomonas* sp. H1-G1 could reduce 90% of mercury from the medium after 40 hours of incubation at 37°C. Both bacterial strains have shown remarkable ability to uptake metal ions from the culture medium. *Pseudomonas aeruginosa* was observed to uptake 75% and *Pseudomonas* sp. H1-G1 65% of Hg^{2+} from the medium after 24 hours of incubation at 37°C. The isolated strains can be exploited for specific environmental clean-up operations.

Key words: Heavy metal resistance, mercury accumulation, wastewater, bioremediation

INTRODUCTION

Wastewaters discharged by the industries are one of the major causes of environmental pollution, particularly in the developing countries. Heavy metals, particularly in industrial effluents, are constantly contaminating our environments, and pose serious threat to human life. Mercury is one such metals (Fan, 1987), which has been reported to produce metabolic disorders in variety of animals such as rotifer (Ramirez-Perez *et al.*, 2004), fish (Company *et al.*, 2004), rat (Reinhardt and Pelli, 1986), rabbit (Shakoori *et al.*, 2002) and man (Miwa *et al.*, 1987). Various health problems such as pneumonitis, abnormal cramps, bloody diarrhea and suppression of urine, cancer and hypersecretion of sweat glands are caused by mercurial and mercuric forms of mercury. Romero *et al.* (2004) studied the toxic effects of mercury chloride in two cell lines of renal origin. The most notable findings in treated cells were the presence of intracytoplasmic inclusion bodies and apoptotic bodies.

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Biological treatment has been recognized as a promising method for dealing with a wide range of pollutants present in the environment. Bioremediation is environment friendly and less expensive than other physical-chemical methods, which involves the natural processes resulting in the efficient conversion of hazardous compounds into innocuous products. This technology involves suitable microbes undergoing various physical and chemical reactions in the polluted water system, and, during the microbial metabolism, the pollutants are degraded and removed. Recently, microbial bioremediation has emerged as an alternative technique to such traditional chemical treatments (Brierley, 1990).

Mercury resistant bacteria have been reported by several authors (Chang *et al.*, 1998; Brown *et al.*, 2002; Mindlin *et al.*, 2005; Fortunato *et al.*, 2005; Rehman *et al.*, 2007). The genus *Rhodospiridium* sp. was also isolated from metallurgical waste by Baldi and Pepi (1995). Mercury is also efficiently removed by algae (Chojnacka *et al.*, 2004). Five mercury resistant yeast strains (CMBLYHg1-5)

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with minimum inhibitory concentration ranging from 80-350 $\mu\text{g}/\text{mL}$ were isolated from industrial effluents and showed high Hg^{2+} processing ability. CMBLHg1 showed maximum efficiency to remove Hg^{2+} from the medium *i.e.*, 85% in 72 hours (Shakoori and Farooq, 2000).

The present study deals with the isolation and characterization of mercury-resistant bacterial strains isolated from industrial effluents of Sialkot, a district of Punjab in Pakistan. The ability of the bacterial strains to reduce Hg has also been assessed with a view to use them to detoxify industrial wastewaters contaminated mercury.

MATERIALS AND METHODS

Isolation of Hg-resistant bacteria and Hg tolerance of isolates

Wastewater samples were collected in screw capped sterilized bottles from ponds receiving effluent from industrial area of Sialkot (Pakistan). Some physicochemical parameters of wastewater *viz.*, temperature ($^{\circ}\text{C}$), pH and mercury ($\mu\text{g}/\text{mL}$) were measured (APHA, 1992).

One hundred microliters of the effluent were spread on Luria-Bertani (LB) agar plates [NaCl 1g, tryptone 1g, yeast extract 0.5g in 100 mL distilled water, pH 7.2 and 1.5g agar; autoclaved at 121°C , 15lb (6.8kg) pressure for 15 min] containing Hg^{2+} 50 $\mu\text{g}/\text{mL}$ and incubated at 37°C . The bacterial colonies were further grown in LB media containing different concentrations of Hg ranging from 50 $\mu\text{g}/\text{mL}$ to 500 $\mu\text{g}/\text{mL}$, *viz.*, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 $\mu\text{g}/\text{mL}$.

Chemicals

All chemicals used in the present study are of analytical grade.

Characterization and identification of bacterial isolates

The bacterial isolates were stained with Gram-staining and characterized for oxidase activity, motility, citrate utilization, urease activity, triple sugar iron reaction, indole production, and MacConkey agar test according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). For further identification, genomic DNA was

isolated and the 16S rRNA gene was amplified by PCR using two general bacterial 16S rRNA primers (RS-1; 5'-AAACTC-AAATGAATTGACGG-3', RS-3; 5'-ACGGGCGGTGTGTAC-3'). The PCR product of 0.5kb was detected. The fragment was cloned in pTZ57R/T vector and amplified 16S rRNA gene was purified with a Fermentas purification kit (#K0513) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA.

Optimum growth conditions and growth curves of bacterial isolates

For optimum growth of the bacterial isolates, two parameters *i.e.* temperature and pH were considered. For determination of optimum temperature, 5 mL LB broth was added in 4 sets, each of three test tubes, for each isolate, autoclaved and inoculated with 20 μL of freshly prepared culture of isolates. The four sets of tubes were incubated at 25°C , 30°C , 37°C and 42°C . After an incubation of 12 hours, their absorbance was taken at 600 nm.

For determination of optimum pH, test tubes having 5 mL LB broth were prepared in 9 sets, each containing 3 test tubes, for each isolate and their pH was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 then autoclaved. These tubes were inoculated with 20 μL freshly prepared culture of the isolates. After incubation period of 12 hours, their absorbance was taken at 600 nm.

Growth curves of bacterial isolates were determined with (50 μg Hg^{2+}/mL) and without mercury. For each isolate 50 mL LB broth was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 50 μL of the freshly prepared inoculum. These cultures were incubated at their optimum temperature in a shaker at 60-80 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, 24, 36, and 48 hours, absorbance was taken at 600 nm wavelength. Growth was plotted graphically.

Cross metal and antibiotic resistance

The cross heavy metal resistance of

bacterial isolates was determined by using stock solutions of 10 mg/mL of different metal salts such as, lead nitrate, cadmium chloride, copper sulphate, potassium dichromate, zinc sulphate and nickel chloride. The cross metal resistance was checked by increasing the concentration of respective metal in a stepwise manner with 50 µg/mL of metal increased every time. Streaked plates containing metal ions, incubated at 37°C for 24 hours and growth was observed for four days. Antibiotic sensitivity against bacterial isolates was checked by measuring the zone of inhibition. The antibiotics used were ampicillin (10µg), chloramphenicol (30µg), gentamicin (10µg), carbenicillin (100µg), oxytetracyclin (30µg) and penicillin (10µg).

Estimation of Hg²⁺ processing ability of the isolates

The metal processing capability of bacterial isolates was checked by adding Hg²⁺ at a concentration of 100 µg/mL in the culture medium. The control culture medium was also run for mercury containing the same concentration as in treated one *i.e.* 100 µg/mL but was without the bacterial isolates. The cultures were incubated for 40 hours and from each medium (control and treated) 5 mL culture was taken out under sterilized conditions after 0, 8, 16, 24, 32 and 40 hours, respectively. The cultures were spun down at 3000 rpm for 5 minutes and the supernatants were used for the estimation of Hg²⁺ by Atomic Absorption Spectrophotometer (Varian, U.S.A) at wavelength 253.7nm. The amount of metal in the supernatants was determined by using standard curve. The percentage reduction in the amount of Hg²⁺ in the medium was calculated.

Uptake of mercury by bacterial isolates

The uptake of mercury by bacterial isolates in LB medium was carried out by acid digestion. Hg²⁺ (50 µg/mL) was added in the culture medium and cells were collected after 24 hours of incubation at 37°C, washed three times in saline solution and acid digested (H₂SO₄: HNO₃, 1:1). Metal content of the digest was measured by Atomic Absorption Spectrophotometer (AAS) at 253.7nm wavelength. Amount of mercury uptake by bacterial cells was calculated in µg/mL by using standard curve.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken; their mean and standard error of the mean were calculated.

RESULTS

Physicochemical characteristics of industrial wastewater

Table I shows physicochemical characteristics of industrial wastewater of four different ponds, from where mercury tolerant bacteria were isolated. The temperature of different samples ranged between 21°C to 24.46°C, pH ranged between 7.84 and 8.7, and Hg²⁺ ranging between 0.80±0.03 and 2.00±0.04 mg/L.

Table I.- Physicochemical parameters of wastewater collected from Nullah Egg receiving effluents from industrial area of Sialkot, Pakistan.

Parameters	Pond 1 (n=3)	Pond 2 (n=3)	Pond 3 (n=3)	Pond 4 (n=3)
Temperature (°C)	22.68± 0.81	24.46± 0.47	24.00± 0.81	21.00± 0.47
pH	8.70 ± 0.08	8.62 ± 0.04	8.48 ± 0.08	7.84± 0.12
Mercury (µg/mL)	1.20 ± 0.04	2.00 ± 0.08	0.80± 0.04	1.40± 0.08

*Means ± standard error of the mean.

Identification of bacterial isolates

The morphological and biochemical characteristics of bacterial isolates have been shown in Table II. Based on these characters and the 16S rDNA PCR products from the two cultures were sequenced and BLAST analysis of the sequences obtained revealed 94% homology with *Pseudomonas aeruginosa* and 99% homology with *Pseudomonas sp.* H1-G1. The nucleotide sequences coding for 16S rRNA gene of *Pseudomonas aeruginosa* and *Pseudomonas sp.* H1-G1 have been submitted to the GenBank database under accession number AB262514 and AB262515, respectively.

Optimum growth conditions

The most suitable temperature for both the mercury resistant bacterial isolates was found to be 37°C. Maximum growth for *Pseudomonas aeruginosa* was observed at pH 9 while *Pseudomonas* sp. H1-G1 showed maximum growth at pH 8. The growth curve pattern was studied by taking 50 µg Hg²⁺/mL in the treated medium. Growth of these isolates was comparable to that of control at 50 µg Hg²⁺/mL. It is interesting to note that the lag phase of both isolates has been extended. It is also shown by the growth curve pattern that both the isolates have extended their log phase up to 48 hours. The growth pattern has been shown in Figure 1.

Table II.- Morphological and biochemical characteristics of bacterial isolates.

Characters	Strains and their responses	
	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> sp. H1-G1
Gram-reaction	-	-
Morphology	Rods	Rods
Colour	Light orange	Light orange
Urease production	-	-
Citrate utilization	-	-
Oxidase reaction	+	+
Triple sugar iron reaction	+	+
Indole reaction	-	-
Motility	+	+

+, positive; -, negative

Table III.- Cross resistance of mercury resistant bacterial isolates from industrial wastewater against other heavy metals.

Heavy metal (µg/mL)	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> sp. H1-G1
Cr ⁶⁺	100	150
Cd ²⁺	50	250
Cu ²⁺	200	350
Pb ²⁺	650	650
Zn ²⁺	50	50
Ni ²⁺	550	550

Bacterial metal resistance to other heavy metals

The bacterial isolates were studied for higher concentrations of mercury and *Pseudomonas*

aeruginosa was found to be resistant to mercury at a concentration of 400 µg/mL while *Pseudomonas* sp. H1-G1 showed maximum resistance at a concentration of 500 µg Hg²⁺/mL. The bacterial isolates were also checked for their resistance to various heavy metals, viz., chromium, cadmium, copper, lead, zinc and nickel (Table III). *Pseudomonas aeruginosa* showed highest MIC 650 µg/mL for Pb²⁺. The order of resistance regarding the metal concentration was, therefore, Pb²⁺>Ni²⁺>Cu²⁺>Cr⁶⁺>Zn²⁺>Cd²⁺. *Pseudomonas* sp. H1-G1 showed maximum resistance against Pb²⁺ at a concentration of 650 µg/mL. The order of resistance regarding the metal concentration was, therefore, Pb²⁺>Ni²⁺> Cu²⁺>Cd²⁺> Cr⁶⁺>Zn²⁺. The mercury tolerant bacterial isolates were also tested for antimicrobial susceptibility profile and results have been depicted in Table IV.

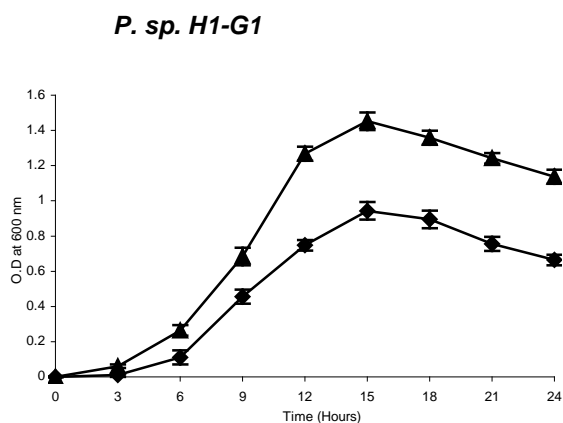
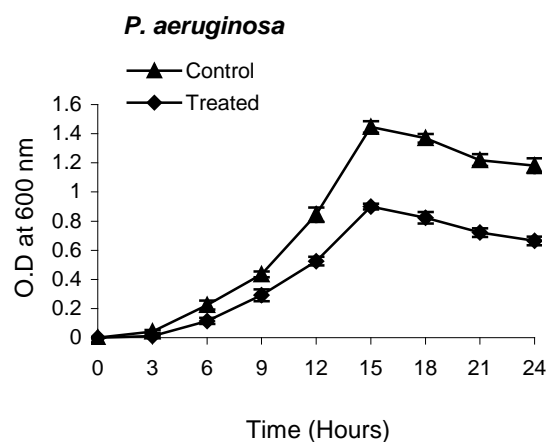


Fig. 1. Growth curves of mercury resistant *Pseudomonas aeruginosa* and *Pseudomonas* sp. H1-G1 in LB medium containing 50µg Hg²⁺/mL after incubation at 37°C.

Table IV.- Antimicrobial susceptibility test profile for mercury resistant bacterial isolates.

Antibiotic doses	Diameter (mm) of Inhibition Zones	
	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> sp. H1-G1
Chloramphenicol (30µg)	S	R
Penicillin(10µg)	R	R
Gentamycin(10µg)	S (18)	S (14)
Carbenicillin(100µg)	S (12)	S (16)
Oxytetracyclin(30µg)	S (20)	S (14)
Ampicillin (10µg)	S (14)	S (12)

S, Sensitive; R, Resistant.

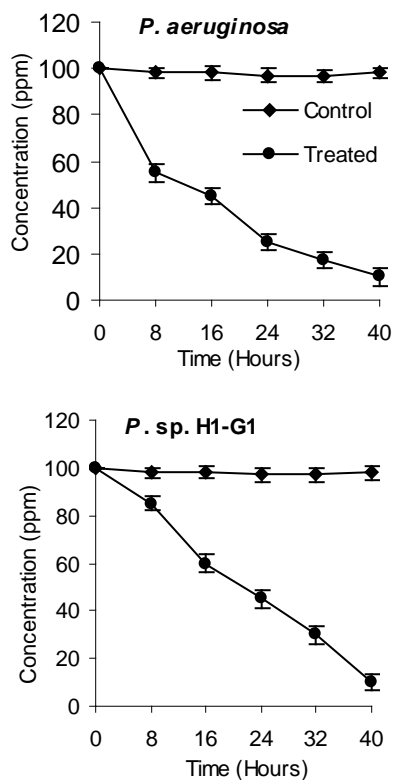


Fig. 2. Uptake of Hg²⁺ by mercury resistant bacterial isolates growing in Hg²⁺ containing medium. The control did not contain cells of the isolates.

Metal processing ability

Mercury processing capability of both the bacterial isolates was checked by adding Hg²⁺ at 100 µg/mL in the culture medium (Fig. 2). *Pseudomonas aeruginosa* showed an excellent ability to pick up mercuric ions from the culture medium and could reduce 90% of mercury from the medium after 40 hours. *P. aeruginosa* was also capable to remove Hg^{□2+} (100 µg/mL) 45%, 55% 75% and 83% from the medium after 8, 16, 24 and 32 hours, respectively. *Pseudomonas* sp. H1-G1 could also efficiently process mercury from the medium and 90% mercury from the medium after 40 hours was removed by *Pseudomonas* sp. H1-G1. The organism was also capable to remove Hg^{□2+} (100 µg/mL) 15%, 40%, 55%, and 70% from the medium after 8, 16, 24 and 32 hours, respectively.

Mercury uptake by bacterial isolates

The biosorption term has been used in the present study to indicate that the metal was removed by one or more of these processes. During the present investigation the bioaccumulation of mercury for *Pseudomonas aeruginosa* was observed 75% and for *Pseudomonas* sp. H1-G1 was 65% (Table V).

Table V.- Bioaccumulation of Hg²⁺ by bacterial isolates with initial concentration of 50 µg/mL of Hg²⁺ in LB broth medium at their optimum temperatures.

Bacterial isolate	Bioaccumulation of Hg ²⁺ after 24 hrs		
	Supernatant (µg/mL)	Pellet (µg/mL)	% bioaccumulation
<i>Pseudomonas aeruginosa</i>	12.5	37.5	75
<i>Pseudomonas</i> sp. H1-G1	17.5	32.5	65

DISCUSSION

Metal resistance is a common process in many microorganisms that deal with toxic compounds in their habitats. In the last few years, metal resistance has increased our knowledge about the cellular mechanisms involved in metal resistance. Mercury resistance has been described in a number of bacterial species (Nakamura and Silver,

1994; Mergeay *et al.*, 2003). One of the best defined mercury resistance determinants is the *mer* operon encoded by transposon Tn501, found in Gram-negative bacteria. The functions of the minimal number of proteins required to confer full resistance (Hobman and Brown, 1997). Thiomersal biodegrading mercury resistant *Pseudomonas putida* (Fortunato *et al.*, 2005) and growth promoting *Pseudomonas fluorescens* (Gupta *et al.*, 2005) strain have also been isolated and characterized.

Microbial cell surfaces are providing important reactive interfaces for the adsorption of contaminants and nutrients. Biological membranes are sites of uptake, exudation and have a range of enzymatic processes (Claessens *et al.*, 2004). Some organisms take up metals, some sequester them for their own use, and some bind them in nontoxic forms. Some organisms chelate metals, some organisms reduce metals; others oxidize them (Morel and Price, 2003).

Silver and Phung (2005) reported that in addition to the MerA-mediated mechanism of Hg reduction other enzymes are also able to reduce Hg²⁺. Essa *et al.* (2000) demonstrated three different mechanisms (Enzymatic reduction to Hg⁰ and volatilization, formation of insoluble HgS and biomineralization of Hg²⁺ as an insoluble mercury-sulphur complex other than HgS) for mercury detoxification in *Klebsiella pneumoniae* M426, which may increase the capture efficiency of mercury. Mercuric reductase reduces Hg²⁺ into Hg⁰ in the presence of NADPH and a sulphhydryl compound. Hg⁰ volatilizes out of the system due to its high vapour pressure (Ghosh *et al.*, 1996).

Mercury resistance occurs widely with Gram-negative and Gram-positive bacteria, in environmental, clinical and industrial isolates, and frequently mercury resistance genes are found on plasmids and encoded by transposons. Saha *et al.* (2006) reported that *Pseudomonas* strains UR2, PS4 and UR5 contained plasmid DNA and were highly resistant to mercury. Mercury resistance in these *Pseudomonas* strains may be plasmid determined. Nakahara *et al.* (1997) reported that in some cases resistance to certain antibiotics and metals is mediated by the same plasmid. Bacterial plasmids encode resistance system for toxic metals including Ag²⁺, As²⁺, Cd²⁺, Cr⁶⁺, Hg²⁺, Ni²⁺ and Pb²⁺

(Silver, 1998). With in various Gram-positive and Gram-negative bacteria Cd²⁺, Cr⁶⁺, Cu²⁺, and Hg²⁺ resistant genes have frequently been found on plasmids or transposable elements (Yureiva *et al.*, 1997).

In the present investigation Gram-negative bacteria, *P. aeruginosa* and *Pseudomonas* sp. H1-G1, were found to be resistant to mercury up to a concentration of 400 and 500 µg/mL, respectively. Metal resistance is defined as the ability of microorganism to continue growing in the presence of an antimicrobial compound whereas tolerance is defined as the ability of a microorganism to survive exposure to, but not grow in the presence of, an antimicrobial (Harrison *et al.*, 2006).

Uptake of metals by bacterial cells has become one of the most attractive means for bioremediation of industrial wastes and other metal polluted environments. Heavy metal uptake processes by biological cells are known under the general term of biosorption. These phenomena include both passive adsorption of heavy metals to the cell walls and metabolically mediated uptake (Gadd, 1990). Deng and Wilson (2001) reported that genetically engineered *E. coli* cells removed more than 99% of the mercury in the wastewater and the final amount of mercury accumulated was 26.8 mg/g cell dry weight.

In the present study both *Pseudomonas aeruginosa* and *Pseudomonas* sp. H1-G1 showed fairly high capability to remove metal from the environment. The percent removal of mercury was 90%. *Pseudomonas aeruginosa* was capable to decrease Hg²⁺ ions (0.1 mg/L) by 45%, 55%, 75%, 83% and 90% from the medium after 8, 16, 24, 32 and 40 hours, respectively. Likewise, *Pseudomonas* sp. H1-G1 was capable to decrease Hg²⁺ ions (0.1 mg/L) by 15%, 40%, 55%, 70% and 90% from the medium after 8, 16, 24, 32 and 40 hours, respectively.

Rehman *et al.* (2007) reported that *Brevibacterium casei* culture grown in the medium containing Hg²⁺ (0.1 mg/L) could reduce 80% of mercury from the medium after 40 hours of incubation. The bacterium was also capable to decrease Hg²⁺ ions by 20%, 40%, 50% and 65% from the medium after 8, 16, 24 and 32 hours, respectively. *Pseudomonas aeruginosa* PU21

(Rip64) and genetically engineered *E.coli* PWS1 were used to detoxify soluble mercuric ions with repeated fed-batch operations. Mercury detoxification efficiency for *Pseudomonas aeruginosa* PU21 (Rip64) was 54% and less was observed for *E.coli* PWS1 (Chang and Law, 1998).

In the present study both *Pseudomonas aeruginosa* and *Pseudomonas* sp. H1-G1 showed good biosorption ability to remove mercury from the medium *i.e.*, 90%. Further work is needed to know that what mechanism do these bacteria employ after uptaking the mercury from the environment. The mercury resistant bacteria isolated during the present study showed high level of metal resistance and accumulated substantial amount of Hg²⁺ from the medium and therefore may be applicable for the treatment of wastewater.

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