

Removal of mercury in fixed-bed continuous upflow reactors by mercury-resistant bacteria and effect of sodium chloride on their performance

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ABSTRACT

Urgent need to reduce the amount of toxic mercury compounds in the wastewater of industries and subsequent reuse of metal ions, has led to an increasing interest in microbial bioremediation. Two *Pseudomonas aeruginosa* strains, namely, isolate CHO7 and isolate Bro12, and a genetically engineered strain *P. putida* (KT 2442 *mer::73*) were used to study the kinetics of mercury removal from liquid M9 medium, considering the potential of the bacteria in volatilizing ionic mercury to its gaseous form. The *P. aeruginosa* strains were further used to remove toxic mercury from synthetic wastewater in fixed-bed, continuous upflow reactors and thereafter to recover the toxic metal from the reactor beds. We also studied the effect of sodium chloride on the kinetics of mercury removal by the isolate CHO7 from marine sediment, as well as the other two non-marine bacteria. After a successful run of over a month, the bioreactors were able to retain the toxic metal, which resulted in a recovery of approximately 64% of the influent mercury. No major alteration in the retention capacity of the bioreactors occurred during drastic changes in concentration of inflowing metals or salt concentration.

Keywords: Mercury, bioreactor, bioremediation, sodium chloride, pumice granules

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INTRODUCTION

There are several practical reasons for selectively separating all types of heavy metal ions from aqueous media. A few examples include the remediation of hazardous or radioactive wastes, the remediation of contaminated groundwater, and recovery of precious and/or toxic metals from industrial processing solutions. In modern applications of available techniques, recovery and re-use of the extracted material is becoming more important. This is being driven by tougher environmental regulations, high initial costs of new, more effective and selective extractants, and the need to minimize the volume of waste destined for permanent disposal. Mercury (Hg), deriving from atmospheric deposition, erosion, urban discharges, agricultural materials, mining, and combustion/industrial discharges, including chloralkali, paper and pulp, oil refinery, paint, pharmaceutical and battery manufacturing and industrial wastewater discharges etc., present an ongoing and serious threat to human health and natural water quality.¹⁻³

There are huge variations in the level of toxic mercury in the biosphere and almost no physiological roles for this metal have been documented. Several bacteria have adapted to this highly toxic metal and have developed systems for metabolizing and utilizing the built-in energy of mercuric compounds to drive their own biosynthetic processes.^{4,5} Mechanisms of microbial metal resistance include the precipitation of metals as phosphates, carbonates and sulfides; metal volatilization by methyl or ethyl group addition; physical exclusion by electronegative components in membranes and extracellular polymeric substances; energy-dependent metal efflux systems; and intracellular sequestration with low molecular weight, cysteine-rich proteins.⁶⁻⁸ Comparatively lower costs and higher efficiency at low metal concentrations make biotechnological processes very attractive in comparison with physicochemical methods for heavy metal removal.⁹ Initially interest focused on technologies that could be applied to achieve *in situ* immobilization of metals, later on these shifted towards the actual metal removal, as it is difficult to guarantee metals will remain immobilized indefinitely.¹⁰ The need to reduce mercury ions in wastewater streams of industries and its subsequent reuse, has led to an increasing interest in microbial remediation.^{3,6,7} Enzymatic reduction of Hg^{2+} to Hg^0 has been identified as the major bacterial mechanism to deal with Hg toxicity.¹¹ It has been realized that the *mer* operon that confers both resistance and detoxification capabilities to its possessor, is almost universally distributed in resistant bacterial populations.^{5,12} High concentrations of sodium chloride (NaCl), causing less bioavailability of metal usually inhibit the volatilization of mercury by bacteria. It has been shown that NaCl interferes with the activity of mercuric reductase, probably due to the formation of mercurio-chloro complexes.¹³ Therefore, it was necessary to determine the efficiency of microbial mercury removal in the presence of different salt concentrations. This was important as the isolate CHO7 is from a marine environment, whereas the other bacteria, being non-marine, would prefer lower salt concentrations.

Mercury removal processes utilize physical and chemical approaches that involve either trapping or collecting mercury from the contaminated sites, or the chemical precipitation of mercuric compounds. Such processes are costly and often leave behind hazardous by-products. Remediation technologies based on mercury volatilization have barely been explored beyond laboratory scale as collecting this toxic metal is technically tedious and expensive.¹⁴ Removal of mercury in a laboratory test reactor using mercury-resistant bacteria was first reported nearly three decades ago.¹⁵ However, serious consideration towards the bioremediation of this metal started in the 1990s.¹⁶ Several researchers^{17,18} demonstrated that the elemental mercury formed could be retained in a packed bed bioreactor, consisting of inert porous carrier material that was covered by a biofilm of Mercury-Resistant Bacteria (MRB). The aim of this study was to investigate the reduction potential of three mercury-resistant strains of *Pseudomonas putida* (one isolate) and *Pseudomonas aeruginosa* (two isolates) to high Hg^{2+} loads. These strains of bacteria were tested for their capacity in remediation and retention of mercury from artificial wastewater in fixed-bed continuous upflow bioreactors. We also studied the effect of NaCl on mercury removal rate by the mercury-resistant bacteria.

MATERIALS AND METHODS

Bacterial cultures

Two *P. aeruginosa* isolates namely, isolate CHO7 and isolate Bro12, and a genetically engineered strain *P. putida* KT2442::*mer-73* were used in the experiments. The *P. aeruginosa* CHO7 was isolated from marine sediment and has been deposited with accession number: NRRL B-30604.³ The other strain

P. aeruginosa Bro12 was isolated from chloralkali wastewater and has been described elsewhere.¹⁹ The *P. putida* KT2442::mer73 was a genetically engineered strain, which had a high and constitutive expression of the mer genes.²⁰ The bacteria were grown overnight (16 hrs) in modified M9 minimal medium (10x salts containing Na₂HPO₄·7H₂O, 70 g; KH₂PO₄, 30 g; NaCl, 25 g; NH₄Cl, 10.0 g; distilled water 1 litre) amended with trace elements (MgO, 10.75 g; CaCO₃, 2.0 g; ZnSO₄, 1.44 g; MnSO₄, 1.12 g; CuSO₄, 0.25 g; CaSO₄, 0.28 g; H₃BO₃, 0.06 g; MgSO₄, 120 g; FeSO₄·7H₂O, 10 g; conc. HCl, 51.3 ml; distilled water, 1 litre), glucose (4 g/l) and 1 ppm (mg/l) HgCl₂ at 30°C, on a rotary shaker. The “preculture” was made by adding this overnight grown culture to fresh medium and allowing the bacteria to grow to a desired cell density in the exponential phase. This was examined by frequent measurements of OD₆₀₀. For reliable comparison between the experimental runs, care was taken to transfer the preculture at a specific time to ensure that the bacterial cells always came from the growth phase.

Kinetics of mercury removal

The set up for an online system to measure the kinetics of mercury transformation by bacteria is shown in Figure 1. It comprised a Cold Vapour Atomic Absorption Spectrophotometer (CVAAS), a three-reaction vessels-assembly, one magnetic stirrer, a water bath (with temperature control device), and a motor with timer and channels for carrying air and mercury vapour to the AAS. Gaseous mercury (Hg⁰) was generated by enzymatic transformation caused by the MRB and the accumulated gaseous mercury was blown out into the AAS by airflow through the reaction vessel. A magnetic stirrer was used to mix the liquid bacterial culture and M9 medium amended with mercury. The reaction vessels had a volume of 50 ml and had a screw cap lid. Each lid had a plastic tube for inflow and outflow of air and a needle to couple the syringe. Direction of airflow was always from the reaction vessels directly into the AAS. Air tubes had unidirectional valves above the reaction vessels so that there was no back-flow into the vessels. Therefore, the reaction vessel was airtight when the inflow valve was closed. The whole experiment was carried out at room temperature of approximately 25°C.

Mercury biotransformation assay

For this assay to biotransform mercury, 20 ml of M9 minimal medium (including 4g/l glucose as carbon source) was prepared with defined mercury concentrations (for example 1, 2, 5, 8, 10, 15, 20 ppm). Mercury transformation was allowed to take place in 3 parallel reaction vessels, where 600 µl of culture containing approximately the same cell numbers was added to 5.4 ml M9 medium, amended with defined concentrations of mercury. A magnetic stirrer in each reaction vessel allowed continuous mixing of these solutions. Effects of NaCl concentration on mercury removal were checked with all three isolates in the experiment at a fixed mercury concentration of 8 ppm. Varying NaCl concentrations in a series of 5 g/l to 30 g/l were used for this experiment. A standard solution of 1000 ppm Hg(NO₃)₂ was used to prepare different concentrations to derive a calibration curve for mercury throughout the experiment.

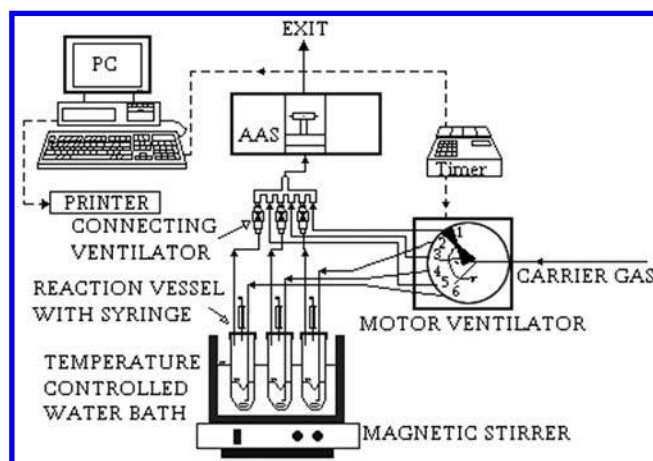


Figure 1. Apparatus for measuring mercury removal kinetics.

Calculation

Transformed gaseous mercury (Hg^0) was blown out to the CVAAS, which was detected as a distinct output peak corresponding to the amount of Hg^0 . The highest peak area resulting from each vessel was taken into consideration and the background level was subtracted from the last cycle of the pre-blowouts to obtain the abstract values. This value was calculated based on the calibration curve into the total amount of mercury in nanograms (ng) per cycle. The kinetics of mercury biotransformation (ν) was expressed as $\text{Hg}^{2+}/\text{cell}/\text{min}$.

$$\nu = \text{Hg}^{2+}(\text{ng})/0.6 \text{ ml culture} * \text{cells (no./ml)} * 3.2 \text{ minutes} = \text{Hg}^{2+}(\text{ng})/\text{cell}/\text{minute}.$$

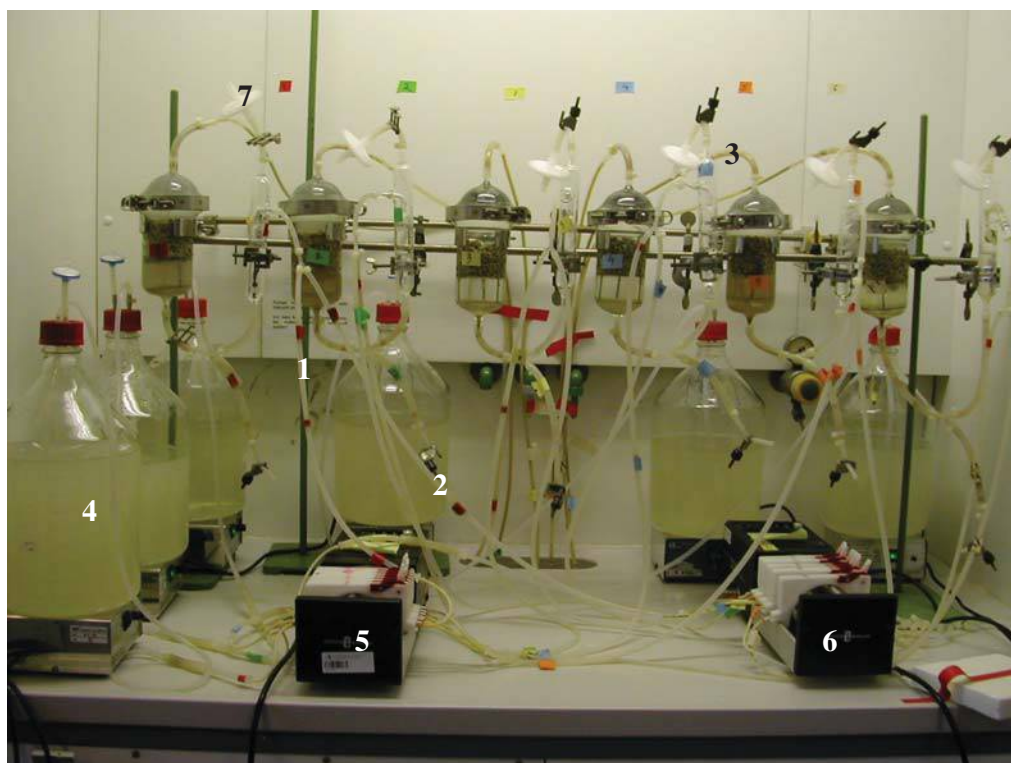
Remediation of mercury by mercury-resistant bacteria in bioreactors

Reactor instillation

The apparatus consisted of six reactors (numbered 1–6) arranged in parallel, with pairs of the reactor vessels containing similar bacterial inocula (reactors 1 & 2 with strain CHO7, 3 & 4 with strain Bro12, and 5 & 6 with mixed cultures of both strains). Each reactor unit consisted of a reactor bed filled with pumice stones (80 ml measured by water displacement), one side-arm glass tube containing provisions for inlet and outlet, along with a bubble trap, and one glass tube for inoculating the reactor with liquid bacterial culture. The complete set up (photograph 1) was steam sterilized by autoclaving and was placed inside a fume hood with provisions for disposal of waste effluent.

Reactor function

The sterilized reactors were operated to measure the efficiency of the bacteria, either singly or in combination, for removal of mercury. The reactors were all connected to individual medium reservoirs and one common wastewater reservoir via separate routes. Filter (0.2 μ) sterilized solution of mercury salt was added to the wastewater under sterile conditions. The mercury-containing waste (with different concentrations of mercury viz., 1, 2, 3.32, 4, 5.08, 6 and 8 ppm containing different salt concentrations ranging from 8 g/l to 16 g/l) and duly sterilized medium (modified M9 medium



Photograph 1. Bioreactor set up showing different reactor vessels and connections to influent and effluent Hg flows and other accessories. 1, channel for medium; 2, channel for wastewater inflow; 3, channel for wastewater outflow; 4, medium; 5, pump for medium; 6, pump for wastewater; 7, bubble trap.

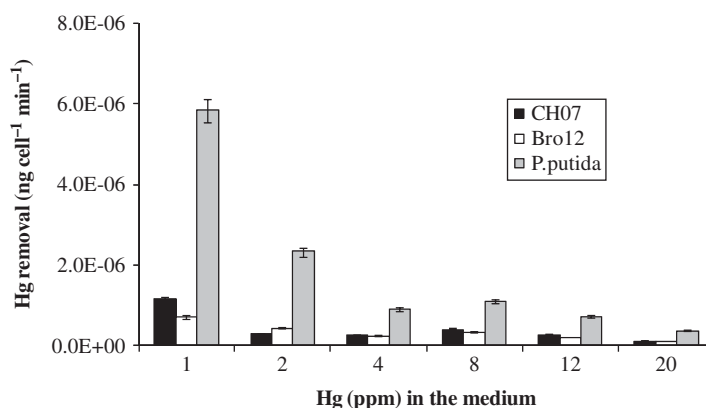


Figure 2. Rates of mercury removal by *P. aeruginosa* CH07, *P. putida* KT 2442 *mer::73*, and *P. aeruginosa* Bro12.

supplemented with 4 g/l glucose) were pumped into the reactors in an up-flow mode at a controlled speed. Mechanical disturbances, such as the blockage of pipes or the occurrence of bubbles inside the bioreactors causing disturbances to the functioning of the reactors, were corrected periodically. Mercury in the inflow and outflow was measured using CVAAS.

Mercury recovery

After the completed run the bioreactors were disassembled and pumice stones collected to recover the transformed metallic and thus, trapped, mercury. The pumice stones from each reactor were separately immersed in a solution of concentrated HNO_3 and H_2SO_4 (1:1) for 48 hrs. Stones were washed with deionized water 5 times, and the washings were collected and pooled together. Concentration of mercury from the washings was measured by CVAAS.

RESULTS

Kinetics of mercury removal

All three isolates were able to remove mercury by means of volatilization from the M9 assay medium containing up to 20 ppm mercury. The mercury removal rate was the highest in the reaction with 1 ppm Hg^{2+} , though the efficiency was relatively good up to 8 ppm Hg^{2+} , in the normal M9 medium (Figure 2). However, with all mercury concentrations tested, the *P. putida* strain achieved the highest removal rate when compared to the other two strains, possibly due to its constitutive expression of the *mer* genes and higher enzyme production. The removal rate at 1 ppm mercury was 1.16×10^{-6} ng/cell/min for CH07, whereas the genetically engineered strain removed mercury at a rate four times higher, 5.84×10^{-6} ng/cell/min. The trend in removal magnitude changed with increasing concentrations of NaCl (Figure 3). The marine *pseudomonas* isolate CH07 was most effective at the salt concentration of 30 g/l. The Bro12 strain was most efficient at the salt concentration of 20 g/l NaCl, whereas the *P. putida* had its best removal potential at moderate salt concentration peaking at 12 g/l NaCl.

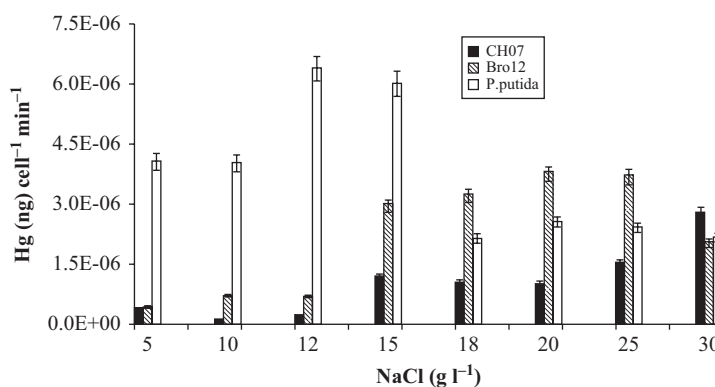


Figure 3. Effect of NaCl on mercury removal rates by different mercury-resistant bacteria.

Remediation of mercury by mercury-resistant bacteria in bioreactors

Both *P. aeruginosa* strains (isolate CH07 and Bro12) reduced mercury concentrations in the bioreactors relatively efficiently over a month (Figure 4). An average of 204.33 mg mercury was passed through each reactor, totaling 1225.99 mg mercury during this operation (Table 1). A total of 786.74 mg was recovered from the pumice stones. The retention and recovery of mercury in one of the replicates (reactor 2) was very high (> 95%) with isolate CH07, whereas the corresponding data with isolate Bro12 were moderate, with a recovery of 56 and 60% metallic mercury in two respective vessels. The two reactors run with mixed cultures of isolates CH07 and Bro12, resulted in a slightly higher recovery of mercury, amounting to 60 and 66% mercury, respectively from reactors 5 and 6 (Table 2). Mechanical disturbances, such as pipe blockage, bubbles in the reactor, differential flow rate, effected mercury retention in the bioreactor by mercury resistant bacteria, however the two strains were able to retain 42 to >95% (totaling 64%) of the influent mercury. Within the concentration ranges studied, the functioning of the reactors was not markedly affected by the change in mercury or NaCl levels. There were some large deviations in the outflow mercury concentrations at times probably due to bubbles in the pipes that may have blocked the flow of medium, as well as wastewater.

DISCUSSION

Technologies for treating mercury-polluted environments have been a major concern over the last couple of decades.^{6,18,8} One of the initial efforts to retain mercury in bioreactors using bacterial strains

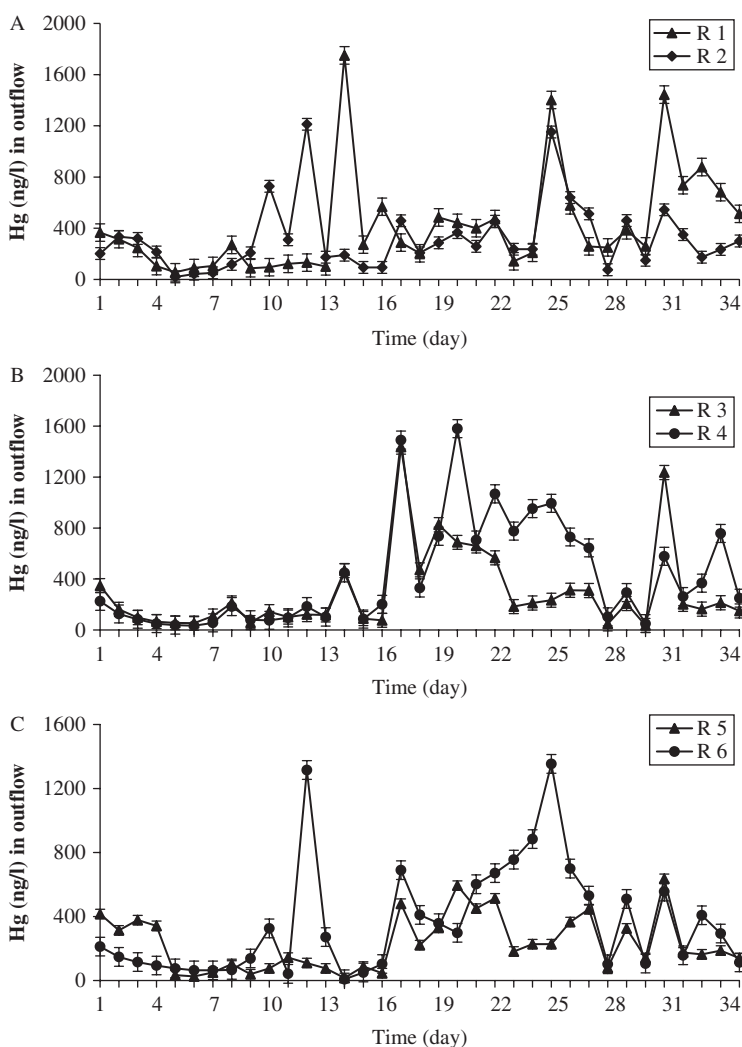


Figure 4. Mercury concentration in waste from reactors using isolate CH07 (A; R1 & R2), and isolate Bro12 (B; R3 & R4), and mixed cultures of both isolates (C; R5 & R6).

Table 1. Mercury retention in the reactors.

| Days of operation | Hg inflow | | ^a Hg retention rate in the reactors (μg/hr) | | | | | | |
|-------------------|-------------|------------------------|--|--------|--------|--------|--------|--------|------------|
| | Conc. (ppb) | R ^b (μg/hr) | r ^{c-1} | r-2 | r-3 | r-4 | r-5 | r-6 | NaCl (g/l) |
| 1–7 | 1000 | 80 | 65.35 | 66.47 | 69.78 | 72.98 | 62.17 | 71.15 | 8 |
| 8–11 | 2000 | 160 | 148.48 | 132.72 | 149.68 | 151.28 | 152.72 | 148.56 | 8 |
| 12–16 | 4000 | 320 | 274.82 | 291.78 | 306.05 | 303.65 | 314.62 | 292 | 8 |
| 17–22 | 3320 | 265.6 | 235.06 | 238.53 | 203.63 | 186.8 | 231.01 | 225.2 | 8 |
| 23–26 | 4000 | 320 | 273.4 | 274.72 | 301.16 | 250.96 | 299.92 | 246.12 | 8 |
| 27–28 | 6000 | 480 | 459.68 | 456.48 | 465.68 | 450.08 | 458.96 | 454.72 | 8 |
| 29–30 | 8000 | 640 | 614.32 | 615.6 | 630.24 | 626.24 | 621.28 | 615.36 | 8 |
| 31–32 | 8000 | 640 | 552.8 | 604.24 | 582.48 | 606.4 | 607.52 | 611.44 | 4 |
| 33 | 4000 | 320 | 249.76 | 249.76 | 249.76 | 249.76 | 249.76 | 249.76 | 8 |
| 34–35 | 4000 | 320 | 272.24 | 298.64 | 305.44 | 279.68 | 306.88 | 303.72 | 16 |

^aReactors 1 & 2 were inoculated with *Pseudomonas aeruginosa* CHo7, 3 & 4 with *Pseudomonas aeruginosa* Bro12, and 5 & 6, with the mix culture of CHo7 and Bro12. ^bR, rate; ^cr, reactor.

was made by Brunke et al.²⁰ Chang and Law²¹ developed a detoxification process using *P. aeruginosa* PU21 in batch, fed-batch and continuous bioreactor systems. Reduction of Hg²⁺ by mercury-resistant bacteria, one of the best mechanisms for its removal from chloralkali waste, has been demonstrated by Canstein et al.^{19,22} using several *P. putida* strains in which nearly 97% of the mercury was recovered from waste containing 3–10 ppm mercury. In such bioreactors, outflow concentrations of mercury did not depend on inflow mercury concentrations.¹⁴ In the bioreactors run with isolates CHo7 and Bro12, more than 60% influent Hg²⁺ rapidly accumulated. Though a consortium of different bacterial strains have been reported to be far more effective,¹⁹ combining CHo7 and Bro12 in this study did not make a significant difference. This might imply that the compatibility of the consortium-candidates is of relevance. Both isolates belonging to the same species might have competed for similar substrate and/or niche in the reactors, instead of sharing the microenvironment.

Biosorption is another biological method, which involves adsorption of metals into the biomass, such as algal or bacterial (either dead or alive), has been inexpensive and promising.^{23,24} Biosorption using natural^{25,8} or recombinant microbial biomass²⁶ has been experimentally successfully. Essa et al.²⁷ reported three mechanisms of mercury detoxification of wastewater in *Klebsiella pneumoniae* M426. These mechanisms are enzyme-mediated reduction, aerobic precipitation of ionic Hg²⁺ as insoluble HgS, as a result of H₂S production, and biomineralization of Hg²⁺ as an insoluble mercury-sulfur complex other than HgS. However, under aerobic condition HgS is methylated to the considerably toxic methylmercury, for this reason anaerobic treatment of mercury-contaminated matrices require extreme safety measures. Recently, it has been claimed that the process “In Situ Mercury Stabilization” (ISMS) can treat and remove mercury contamination from the ground in a cost-effective manner.²⁸

Kiyono et al.²⁹ successfully used bioaccumulation as a measure of bioremediation of Hg using mer-ppk fusion plasmid. They were able to remove mercury at low concentrations (2–4 ppm), but the efficiency was not as good at higher mercury concentrations i.e., 8–16 ppm, due to inactivation of viable cells inside the alginate beads. Chen and Wilson³⁰ constructed a genetically engineered *E. coli* strain for simultaneously expressing an Hg²⁺ transport protein, as well as metallothionein, to improve the limitation of trans-membrane mercury transport. This was effective at low concentrations of mercury as its capacity for Hg²⁺ accumulation was limited by the number of cellular metallothionein. The mer

Table 2. Total mercury recovered from the reactors inoculated with isolates *Pseudomonas aeruginosa* CHo7 (in reactors 1 & 2), *Pseudomonas aeruginosa* Bro12 (in reactors 3 & 4) and of both isolates (in reactors 5 & 6).

| Reactor | Total Hg (mg) inflow | Total Hg (mg) recovered | % Recovered |
|---------|----------------------|-------------------------|-------------|
| 1 | 192.65 | 81.54 | 42.32 |
| 2 | 209.62 | 201.12 | 95.94 |
| 3 | 211.03 | 119.72 | 56.73 |
| 4 | 201.72 | 122.5 | 60.73 |
| 5 | 214.95 | 129.62 | 60.31 |
| 6 | 196.02 | 132.24 | 66.44 |

operon has been expressed in the radio-resistant *Deinococcus radiodurans*, which tolerates extremely high dosages of radiation and could be useful for cleaning up sites contaminated with radioactive wastes.³¹ Phytoremediation expressing *merA* and *merB* genes in plants (especially rhizospheres), have been investigated for the cleanup of contaminated soils.^{32,33} A genetically engineered rice plant³⁴ has been used for the bioremediation of mercury. However, the volatile mercury (Hg^0) produced this way is released into the atmosphere directly, which is again a matter of concern.

Results from the present study clearly demonstrate the possibilities of employing mercury-resistant bacteria for mercury detoxification and recovery of this costly metal under special circumstances. Mercury bioremediation using the resistant bacteria stated here, can principally be applied to bioremediation of most kinds of mercury contamination. Further, leads from this study can be explored to adapt these bacteria for bioremediation under various other situations.

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