Biodegradation
DOI 10.1007/s10532-011-9483-z

ORIGINAL PAPER

Mercury bioremediation by mercury accumulating *Enterobacter* sp. cells and its alginate immobilized application

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Received: 29 November 2010 / Accepted: 14 May 2011

Abstract The effective microbial remediation of the mercury necessitates the mercury to be trapped within the cells without being recycled back to the environment. The study describes a mercury bioaccumulating strain of *Enterobacter* sp., which remediated mercury from the medium simultaneous to its growth. The transmission electron micrographs and electron dispersive X-ray analysis revealed the accumulation of remediated mercury as nano-size particles in the cytoplasm as well as on the cell wall. The *Enterobacter* sp. in the present work was able to accumulate mercury, without being engineered in its native form. The possibility of recovering the accumulated mercury from the cells is also indicated. The applicability of the alginate immobilized cells in removing mercury from synthetic and complex industrial effluent in a batch mode was amply demonstrated. The initial load of 7.3 mg l^{-1} mercury in the industrial effluent was completely removed in 72 h. The cells immobilized in calcium alginate were similarly effective in the complete removal of 5 mg l^{-1} $HgCl_2$ of mercury from the synthetic effluent in less than 72 h. The immobilized cells could be reused for multiple cycles.

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Keywords *Enterobacter* sp. · Mercury bioremediation · Mercury bioaccumulation · Immobilized cell

Introduction

Mercury is the third most toxic element occurring in the biosphere (Nies 1999). Its discharge into the environment leads to the deposition in soil and water bodies from where it enters into the food chain and gets biomagnified. In human and other living systems, it binds to cysteine residues of proteins and nitrogen atom of nucleic acids causing extreme toxicity and physiological disorders. Mercury is taken up from the periphery by all nerve endings and rapidly transported inside the axon of the nerves to the spinal cord and brainstem causing neurological disorders (Brodkin et al. 2007; Holmes et al. 2009). Chlor-alkali, electronic industries, power plants, metal mining and refining are some major anthropogenic sources of mercury discharge in the atmosphere and surface water. Unavoidable natural processes, like volcanic eruption, geothermal activities and wild fires further add to the total mercury concentration in the environment. The anthropogenic activities contribute about 2190 t of mercury in the environment (Li et al. 2009). Global mercury emission is projected to reach the level of 2390–4860 Mg by the year 2050 (Streets et al. 2009). The long atmospheric residence time has made its transport a global phenomenon causing its

Published online: 24 May 2011

presence even in the areas with very little or no anthropogenic activities. Not surprisingly, increasing level of mercury in the environment remains a major concern worldwide. Efforts are underway to reduce mercury level in industrial wastewaters using various technologies. Conventionally, carbon absorbents, ion exchange, reverse osmosis, precipitation and electrochemical treatment are used (Akpor and Muchie 2010; Okoronkwo et al. 2007; Wang et al. 2009). In a recent work, even the use of gold and manganese oxide nanoparticles have been found to be promising (Lisha et al. 2010; Kamarudin and Mohamad 2010). However, these processes are in their early stage of development. Nevertheless, most of these techniques are expensive, generally non-specific, often less efficient at low metal concentration and generate hazardous by-products (Chiarle et al. 2000; Manohar et al. 2002; Zhang et al. 2005). In this context, microbial bioremediation seems a potential approach because of low cost and better efficiency at low metal concentration (Wagner-Döbler 2003). Many attempts have been made to exploit the living (Bafana et al. 2010; Barkay and Wagner-Döbler 2005; De et al. 2006; Pepi et al. 2011), dead (Das et al. 2007; Green-Ruiz 2006; Sari and Tuzen 2009) and immobilized microbial cells (Bayramoğlu et al. 2006; Chen et al. 2005; Oehmen et al. 2009; Sugio et al. 2003) to remediate mercury. Yet, the unique property of mercury to enter into vapor stage at room temperature (from Hg^{2+} to Hg^0) poses a challenge for the microbial remediation process. The remediated mercury is often recycled back into the atmosphere in form of mercury vapor. Thus the ideal process for mercury decontamination will be the one wherein it can be trapped as Hg^{2+} or as Hg^0 (without letting it evaporate back to the environment). Efforts have been made to engineer the microbial strain which can accumulate mercury in their cytoplasm (Deng et al. 2008; Kiyono and Pan-Hou 2006; Zhao et al. 2005).

We have previously reported the isolation of a mercury accumulating *Enterobacter* sp. strain (Gupta et al. 2006). The current work describes mercury remediation vis-à-vis its bioaccumulation by free and alginate immobilized *Enterobacter* sp. cells. The strain was able to remediate mercury from the medium during its growth. The remediated mercury is accumulated inside the cytoplasm as well on the cell wall. Although the *mer* operon and mercury reductase activity have been observed in Enterobacteriaceae (Essa et al. 2003), the accumulation inside the cytoplasm is observed for the first time to the best of our knowledge.

Materials and methods

Materials

$HgCl_2$ was purchased from Merck Ltd. (Mumbai, India). $MgSO_4{\cdot}7H_2O$ was a product of Qualigen Fine Chemicals, Glaxo Laboratories Ltd. (Mumbai, India). The media components were procured from Hi Media Laboratories (Mumbai, India). All other chemicals used were of analytical grade. All the glasswares were soaked in 10% (v/v) HNO_3 overnight prior to the experiments.

Culture and inoculum

Enterobacter sp. isolate having mercury resistance property (Gupta et al. 2006), was used in the present study. It was maintained on agar slants at 4°C and sub-cultured at 15 days interval.

Mother culture was prepared by transferring loopful of culture from the stock slant into the culture medium containing (g l^{-1}): peptone, 5.0; yeast extract, 3.0; NaCl, 2.5 and pH 7.0. The incubation was carried out at 30°C with constant shaking at 120 rpm in an orbital shaker (Orbitek, Scigenics Biotech, India). Twenty-four hour grown culture (OD $\simeq$ 1) was used as inoculum.

Growth and mercury bioremediation

Enterobacter sp. cells were grown in culture medium (NB) containing (g l^{-1}): yeast extract, 3.0; peptone, 5.0; glucose, 5.0; NaCl, 2.5; $MgSO_4{\cdot}7H_2O$, 0.5; (pH 7.0) and amended with 5.0 mg l^{-1} of $HgCl_2$ (no $HgCl_2$ was added in the control). The media was seeded with 1% (v/v) mother culture and incubated at 30°C with constant shaking at 120 rpm in the orbital shaker. The samples were withdrawn aseptically at different time intervals. Growth in the culture medium was monitored by recording absorbance at 660 nm spectrophotometrically (Specord 200, Analytikjena, Germany). Part of the withdrawn sample was centrifuged at 14,000$\times g$ for 10 min at 4°C to monitor the residual mercury in the supernatant. The mercury was estimated by atomic absorption spectrophotometer

using sodium tetrahydroborate according to the recommended conditions provided by the manufacturer (Perkin-Elmer MHS-15 Mercury/Hydride System, USA). Another set of flask containing the culture media amended with 5.0 mg l^{-1} of $HgCl_2$, but not inoculated with *Enterobacter* sp. cells was subjected to similar treatments simultaneously, to monitor any abiotic reduction in mercury.

Transmission electron microscopy (TEM)

Cells grown for 96 h in the absence or presence of mercury (5 mg l^{-1} $HgCl_2$) were harvested and washed thrice with sodium phosphate buffer (0.1 M, pH 7.4). The washing was done by suspending the cells in 5 ml sodium phosphate buffer (0.1 M, pH 7.4) followed by vortexing for 2 min. The vortexed cell mass was centrifuged at 8,000×*g* for 10 min at 4°C. Washed cells were collected as pellet and fixed in modified Karnovsky's fluid overnight at 4°C. Post fixation was done with 1% OsO_4 for 1 h at 30°C, followed by dehydration with 30, 50, 70, 90 and 100% acetone for 30 min at each concentration and further processed as per the procedure of David et al. (1973). Transmission electron micrographs were recorded in high resolution transmission electron microscopy (HRTEM) equipped with energy dispersive X-ray (EDAX) analyzer (Technai G^2, 200 kV, USA) without the regular double staining. EDAX was done on the same bacterial thin film used for taking TEM micrographs in nanoprobe mode.

Energy dispersive X-ray (EDAX) analysis of sonicated cells

Cells grown for 96 h in culture medium containing 5 mg l^{-1} $HgCl_2$ were harvested by centrifugation at 8,000×*g* for 10 min at 4°C. Pellets were washed thrice with Milli Q water and sonicated at a frequency of 24 kHz for 10 min. The lysate was filtered through 0.45 μm Millipore filter. One drop of filtered lysate was loaded on carbon coated grid, dried at room temperature and subjected to TEM/EDAX analysis.

Immobilization of *Enterobacter* sp. cells

Pellets of 48 h grown cells (0.25 g of wet weight) were resuspended in 2 ml saline and mixed with 50 ml of 3% (w/v) sodium alginate solution until it become homogeneous. The mixture was extruded dropwise in ice cold 0.2 M $CaCl_2$ solution, forming uniform sized beads. The beads were allowed to harden in 0.2 M $CaCl_2$ solution at 4°C for 24 h.

Mercury bioremediation in synthetic effluent

The synthetic effluent media consisting of (g l^{-1}) glucose, 5.0; NaCl, 0.1; KCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.02; $(NH_4)_2SO_4$, 0.1; $CaCl_2 \cdot 2H_2O$, 5.8 (pH 7.0) was used to check the efficiency of mercury bioremediation by immobilized cells. Thirty gram (wet weight) of immobilized beads were added to 60 ml of synthetic effluent containing 5 mg l^{-1} $HgCl_2$ and incubated at 30°C with constant shaking at 120 rpm.

The samples were withdrawn at regular time intervals and centrifuged at 14,000×*g* for 10 min at 4°C. The supernatants were analyzed for residual mercury content using atomic absorption spectrophotometer. Similar experiment was performed with (0.25 g of wet weight) free cells and without *Enterobacter* sp. cells.

Reusability of immobilized cells

Mercury remediation was performed as described in "Materials and methods" section. However, for reusing the beads, these were washed thrice with Milli Q water and used for the next experiment.

Mercury bioremediation in effluent samples

This study was performed to check the efficiency of remediation by immobilized cells under complex effluent situation. Sample was collected from a local industrial discharge nearby Delhi, India. Since the mercury content in the sample was below detectable level, these were amended with 7.3 mg l^{-1} of mercury (final concentration). The 30 g (wet weight) of immobilized cells was added into 60 ml of sample and incubated at 30°C with constant shaking at 120 rpm. The aliquots were withdrawn at different time intervals and centrifuged at 14,000×*g* for 10 min at 4°C. The supernatants were analyzed for residual mercury content by atomic absorption spectrophotometer. A control without alginate beads was run simultaneously.

All the experiments were carried out in triplicates and the difference in the individual results in each set of experiments was less than ±5%.

Results

Growth and mercury bioremediation

The isolation of *Enterobacter* sp. and its mercury resistance property was reported previously (Gupta et al. 2006). The present study was aimed to evaluate mercury bioremediation potential of this isolate. Cells were grown in culture media enriched with 5 mg l^{-1} $HgCl_2$ and residual mercury was estimated at different time intervals. Figure 1 clearly shows decrease in mercury concentration simultaneous to the growth of *Enterobacter* sp. cells. The presence of mercury in the medium caused the increase in the lag phase. This pertains to the phase wherein the cells acclimatize towards the toxic effect of mercury.

Mercury bioaccumulation its localization and characterization

In order to understand whether the mercury was volatilized or remediated, transmission electron micrographs of the cells grown in absence (Fig. 2a) and presence (Fig. 2b) of 5 mg l^{-1} $HgCl_2$ were recorded. The accumulation of mercury on the cell surface and in the cytoplasm was distinctively visible in the cells grown in mercury containing media. To confirm the nature of accumulated particles, the energy dispersive X-ray was performed on selected electron dense area on the cell wall (Fig. 3a) and cytoplasm (Fig. 3b). Both the spectra exhibited a conspicuous peak of mercury, thus confirming the bioaccumulation of remediated mercury by the strain. The possibility of recovery of bioaccumulated mercury was explored by sonicating the mercury containing cells. Figure 4a shows the corresponding transmission electron micrograph of the accumulated mercury in the sonicated and filtered cell lysate. The EDAX profile (Fig. 4b) further confirmed

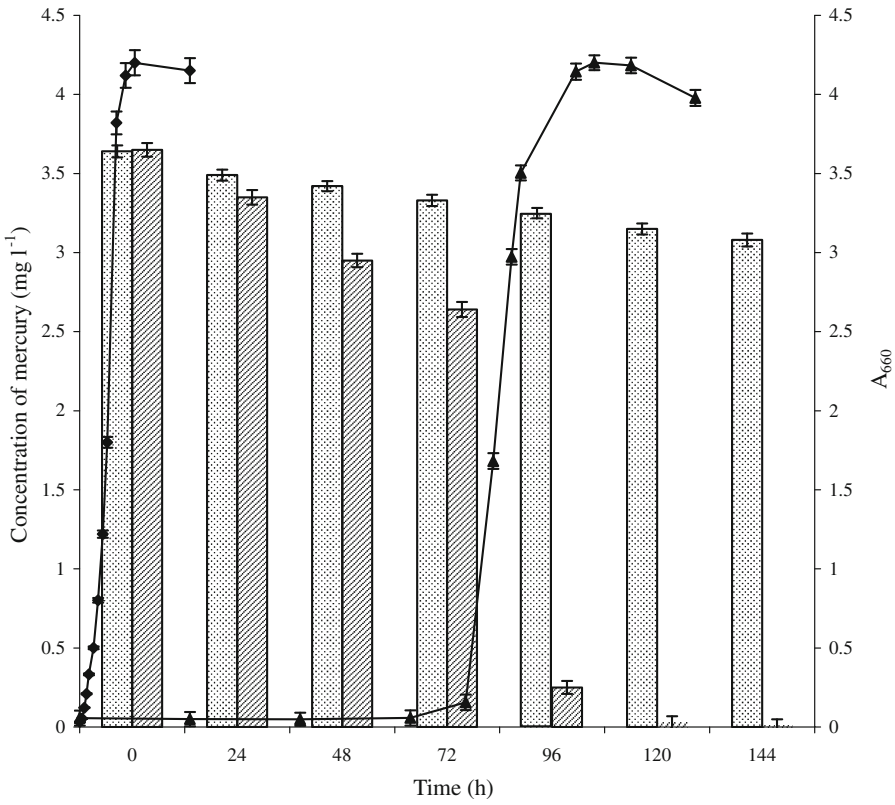

Fig. 1 Growth and mercury bioremediation profile of *Enterobacter* sp. cells. *Enterobacter* sp. cells were grown in NB medium in presence or absence of 5 mg l^{-1} $HgCl_2$ (pH 7.0) as described in "Materials and methods" section. Samples were aseptically withdrawn at different time intervals. Growth was monitored by recording absorbance at 660 nm. Mercury was estimated by atomic absorption spectrophotometer. Bacterial growth (A_{660}) in presence of 5 mg l^{-1} $HgCl_2$ (*filled triangle*); absence of $HgCl_2$ (*filled diamond*); residual mercury concentration in culture media in presence of *Enterobacter* sp. cells (*square with upper right to lower left fill*); in absence of *Enterobacter* sp. cells (*squared dots operator*)

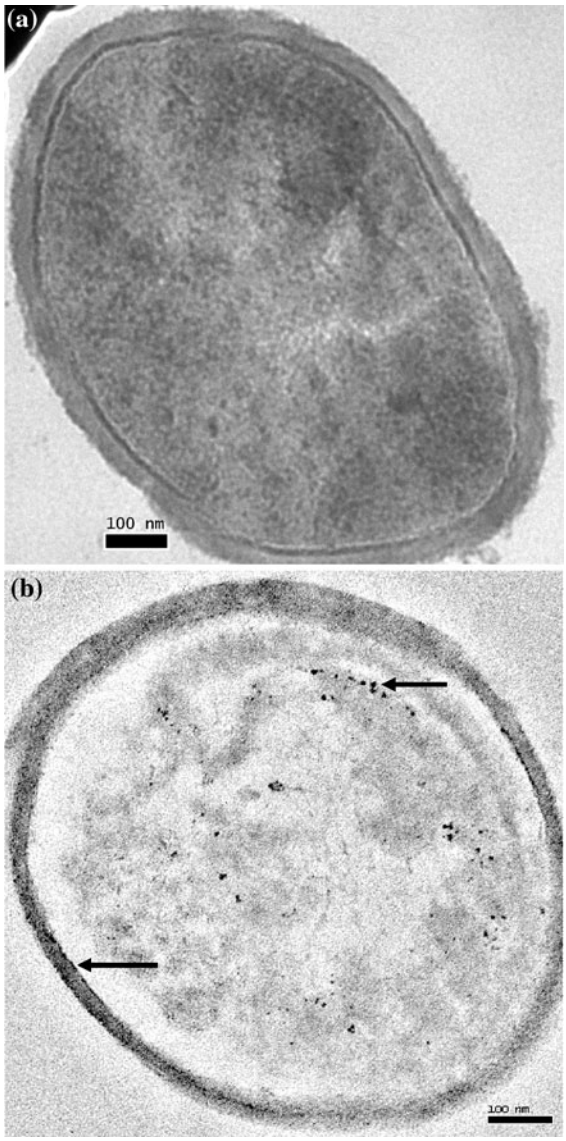

Fig. 2 Transmission electron micrograph (TEM) of *Enterobacter* sp. cells. Cells were grown in absence and presence of 5 mg l^{-1} $HgCl_2$ and processed for transmission electron microscopy as described in "Materials and methods" section: **a** absence of mercury; **b** presence of mercury. *Arrow head* shows mercury accumulation. *Bar scale* 100 nm

these to be mercury nanoparticles and support the possibility of their recovery.

Mercury bioremediation in synthetic effluent and reusability

The bioaccumulating traits in *Enterobacter* sp., makes it a potentially useful strain for in situ mercury remediation. For assessing the viability of the process, remediation was attempted by using immobilized form of *Enterobacter* sp. The cells were immobilized in 3% (w/v) calcium alginate gel and used for mercury remediation from the synthetic effluent medium. For comparison, similar experiment was performed with free cells. The immobilized cells were found to be more efficient in removing mercury from the mercury enriched media as compared to free cells (Fig. 5). The complete removal of 3.65 mg l^{-1} mercury was achieved in less than 72 h by immobilized cells. The free cells remediated only 69% in the same time period. While similar experiment was performed with alginate beads without immobilized *Enterobacter* sp. cells, the beads alone reduced 19% of the initial mercury concentration. The complete remediation by alginate immobilized cells is thus attained by alginate adsorption and biological process by the cells.

The reusability of the immobilized beads were studied for four consecutive cycles (Fig. 6). While complete removal of mercury was achieved in Ist cycle, the removal efficiency decreased from 97 to 49% in the subsequent cycles.

Mercury bioremediation in effluent samples

To check the mercury removal efficacy of immobilized *Enterobacter* sp. cells in the complex nature of effluent, samples from local industrial discharge were taken as model system. The initial turbidity, total dissolved solid and pH of the water sample were 2.75 NTU, 204 mg l^{-1} and 7.2 respectively. Experiment was performed after enriching the water sample with 7.3 mg l^{-1} of mercury. The immobilized *Enterobacter* sp. cells were similarly effective in removing mercury from the complex effluent sample and the mercury was completely remediated in 72 h (Fig. 7).

Discussion

Mercury bioaccumulation, its localization and characterization

Enterobacter sp. cells studied in present work showed not only high resistance to mercury but also good mercury remediation potential. It remediated 5 mg l^{-1} of mercury in 72 h from the synthetic effluent. Major

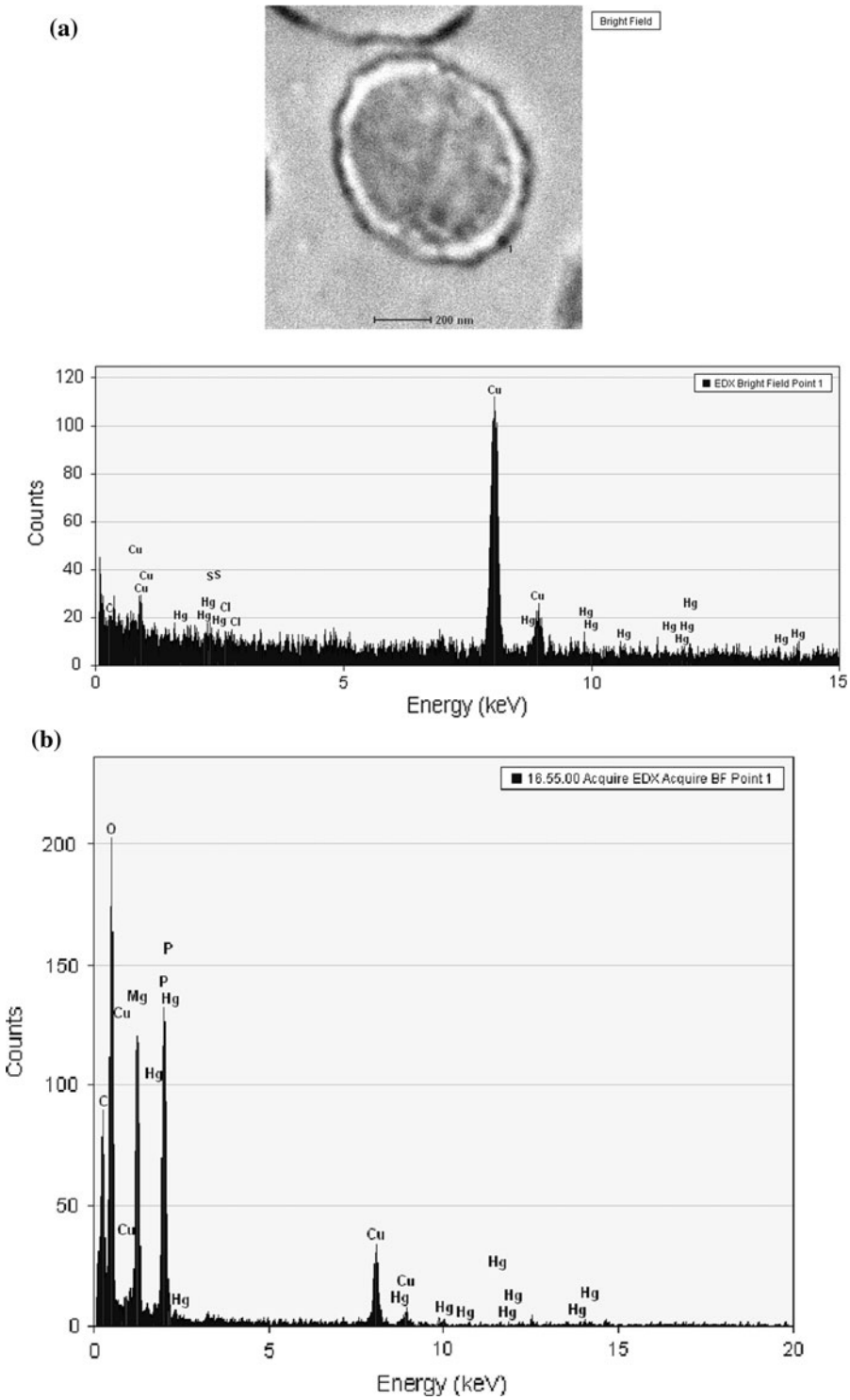

Fig. 3 Energy dispersive X-ray (EDAX) profile of *Enterobacter* sp. cells. Cells were grown in presence of 5 mg l^{-1} $HgCl_2$, EDAX was recorded at selected electron dense area: **a** cell wall; **b** cytoplasm. EDAX was performed on the same bacterial thin films which were used for recording TEM micrographs

advantage of the strain lies in its capacity to accumulate the remediated mercury inside the cytoplasm and not allowing it to recycle back to the environment. The bioaccumulation of mercury was clearly seen in transmission electron micrographs. The energy dispersive X-ray profile recorded at electron dense area on

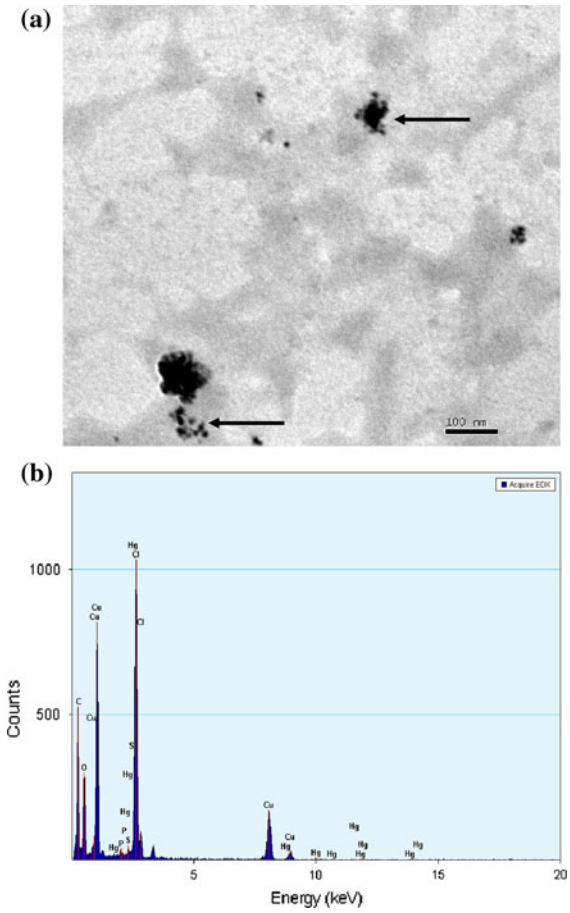

Fig. 4 Energy dispersive X-ray (EDAX) and TEM micrograph of recovered mercury after cell sonication. *Enterobacter* sp. cells were grown for 96 h in culture medium containing 5 mg l^{-1} $HgCl_2$ and harvested by centrifugation at 8,000×*g* for 10 min at 4°C. Pellets were sonicated at 24 kHz for 10 min. The lysate was processed for TEM/EDAX as described in "Materials and methods" section: **a** TEM of cell lysate showing mercury nanoparticles recovered after sonication (*arrow head* shows mercury nanoparticles); **b** EDAX analysis of recovered nanoparticles

cell wall and cytoplasm confirmed them to be mercury particles. Signals in EDAX at 9.98, 11.92 and 2.19 keV can be attributed to mercury (Hg) Kα and the secondary (Hg) Kβ peaks and (Hg) M peaks respectively, which prove that the accumulated particles were indeed mercury.

Recovery of bioaccumulated mercury

It was possible to recover accumulated mercury by cell lysis. The recovery of remediated mercury is another major advantage for sustainability of *Enterobacter* sp.

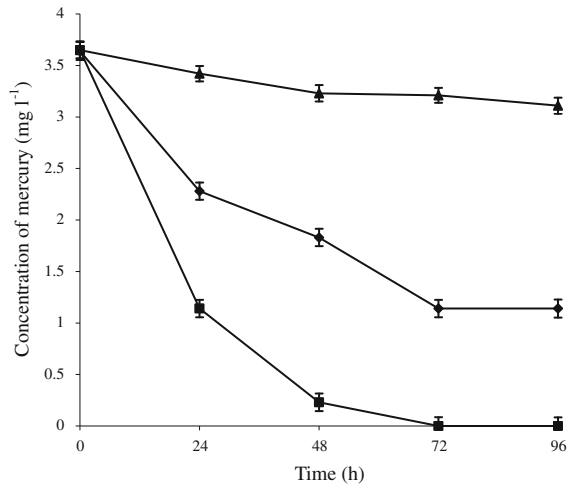

Fig. 5 Mercury bioremediation by free and alginate immobilized *Enterobacter* sp. cells. Thirty gram of immobilized cells or 0.25 g of free cells were incubated separately in 60 ml synthetic effluent containing 5 mg l^{-1} $HgCl_2$. The inoculated effluents were incubated at 120 rpm and 30°C. The samples were withdrawn at regular time intervals and residual mercury content was determined by atomic absorption spectrophotometer. The control was simultaneously run under similar condition without free or immobilized cells. Control (*filled triangle*); free cells (*filled diamond*); alginate immobilized cells (*filled square*)

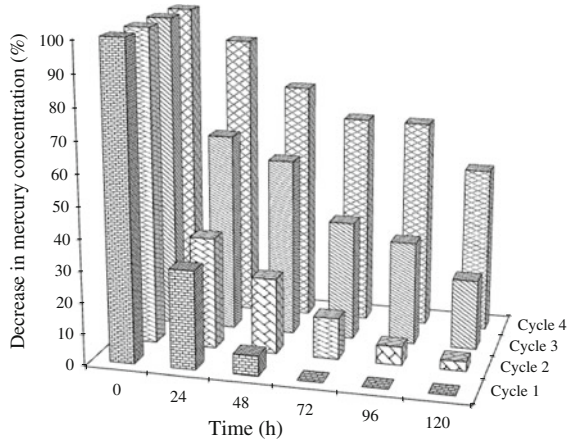

Fig. 6 Reusability of alginate immobilized *Enterobacter* sp. cells. The experiment was performed as described in "Materials and methods" section. After each cycle the immobilized cells were harvested by decanting the medium. The harvested cells were washed with Milli Q water three times and used for the next cycle. The mercury content was monitored by atomic absorption spectrophotometer. (▦) Ist cycle; (▨) IInd cycle; (▨) IIIrd cycle; (▨) IVth cycle

mediated mercury detoxification process. The size of the recovered mercury particles was in the range of 2–5 nm. It is seen that the average size of recovered

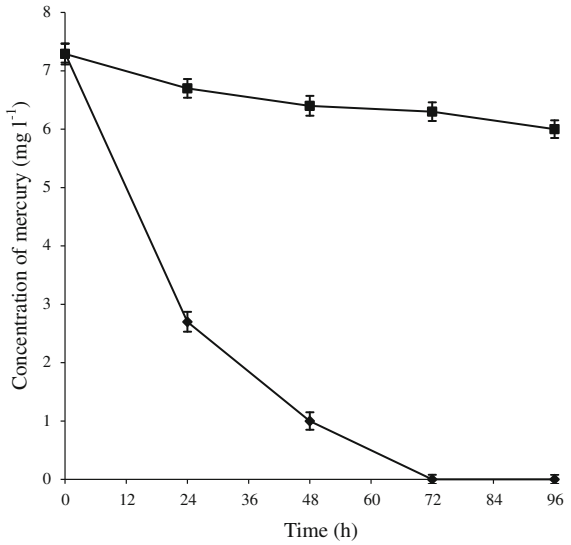

Fig. 7 Mercury bioremediation in industrial effluent by alginate immobilized *Enterobacter* sp. cells. Thirty gram of immobilized cells were incubated in 60 ml industrial effluent amended with 7.3 mg l^{-1} mercury. The inoculated effluents were incubated at 120 rpm and 30°C. The samples were withdrawn at regular time intervals and residual mercury content was determined by atomic absorption spectrophotometer. The control was simultaneously run under similar condition without free or immobilized cells. Control (*filled square*); alginate immobilized cells (*filled diamond*)

nanoparticles was 3.75 ± 0.03 nm. The bioaccumulation of heavy metals is a known phenomenon in certain bacterial species when challenged with the toxic environment (Gadd 1990; Thakkar et al. 2010; Valls and de Lorenzo 2002). Metal uptake is generally a preceding step to metal detoxification by microbial cells. Because microbial metal uptake and detoxification process being species specific, different mechanism of uptake and detoxification could operate simultaneously or individually. The mercury resistance has been previously noted in Enterobacteria (Essa et al. 2003). However, mercury remediation by *Enterobacter* sp. has never been attempted. Pseudomonads have rather been used for mercury bioremediation. The mercury remediation by Pseudomonads has been accorded to the presence of well defined *mer* operon consisting of a predominant mercury reductase (*mer* A) responsible for the conversion of Hg^{2+} to Hg^0 (Barkay et al. 2003; Yamaguchi et al. 2007). Other associated proteins are mercury binding protein (*mer* P) and a transporter protein (*mer* T) responsible for facilitating entry of mercury inside the cell wherein organomercurial lyase (*mer* B) or mercury reductase (*mer* A) further act on it. The resultant Hg^0 finally diffuses out of the cell.

The accumulation of mercury within the cytoplasm in the present study indicated that either the process of Hg^{2+} reduction to Hg^0 is impaired or Hg^0 is converted back into mercury particles through some cellular proteins or alternate mechanism. In a preliminary experiment, we have also detected presence of mercury reductase (*mer* A) in the *Enterobacter* sp. It ranged 2–4 IU ml^{-1}. Thus, the mercuric ions might have been transported to cytoplasm via known *mer* P and *mer* T transporters (Barkay et al. 2003; Hobman et al. 2002).

Kiyono and Pan-Hou (2006), have genetically engineered an *Escherichia coli* to express a *mer* transport system (*mer* P and *mer* T), organomercurial lyase (*mer* B) and polyphosphate kinase (*ppk*). The kinase phosphorylated the Hg^{2+}, so that it was unable to escape or diffuse out of the cell and accumulated into the cytoplasm. The *Enterobacter* sp. in present case was able to accumulate mercury (without being engineered) in its native form. This makes it a novel isolate endowed with unique property of mercury accumulation and disallowing its escape.

Application of alginate-immobilized cells in mercury bioremediation from effluent

Use of alginate immobilized *Enterobacter* sp. cells offers viability to bioprocess in terms of reusability, better operational controls and easy scale-up. Immobilized cells exhibited better remediation efficiency as compared to free cells. This can possibly be attributed to the polymeric nature of the alginate, which might non-specifically capture metals and further facilitate its entry into the cells. The initial drop in the mercury concentration in the solution within 24 h may thus be due to quick adsorption of mercury on the immobilization support.

Reusability of immobilized cells was tested in repetitive batch mode, wherein 100 and 97% mercury was removed in two consecutive cycles. Removal efficiency in the next successive cycles decreased further. The decrease in efficiency of alginate immobilized cells may be because of loss of viability of cells and progressive saturation of the adsorption sites. The loss of viability could also be due to the washing of the cells with Milli Q water. The applicability of the alginate immobilized cells in

removing mercury from complex effluent was amply demonstrated. The initial load of 7.3 mg l^{-1} mercury was completely removed in 72 h. A marginal decrease in mercury in the effluent being used as control (without cells) was observed and may be attributed to abiotic factors.

Conclusions

The results in the present study demonstrate the mercury remediation and bioaccumulation by *Enterobacter* sp. cells. The novel features were: (i) that the remediated mercury remained confined to the cells without vaporizing back to the environment and (ii) the possibility of recovering the bioaccumulated mercury. The feasibility of mercury bioremediation from complex industrial effluent adds to the merit of the isolate. The cells could be used in immobilized form with more efficacy. The use of immobilized cells enlarges the scope of the work at a scale-up level. Thus, *Enterobacter* sp. based mercury remediation has a potential to develop into a green viable process.

Acknowledgments The financial support provided by the Department of Biotechnology (Govt. of India) is gratefully acknowledged. Authors gratefully acknowledge the guidance and facilities for EDAX provided by Prof. B.R. Mehta, Department of Physics, IIT Delhi. The kind help given by Dr. V. Singh in recording and analyzing TEM micrographs is also gratefully acknowledged. Author A. Sinha also acknowledges the fellowship provided by the University Grants Commission (Govt. of India), New Delhi.

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