

RESEARCH ARTICLE

Bioconversion of Hg and Molecular Characterization of *merA* and *merB* Gene in a Clone Constructed by Culture Independent Technique

Gaurav Jaiswal¹, Rajni Singh¹, Nahar Singh² and Shalini Porwal^{1*}

¹Amity Institute of Microbial Biotechnology, Amity University, Sector-125, Noida - 201 303, India.

²CSIR-National Physics Laboratory, Dr KS Krishnan Marg, Pusa, New Delhi, Delhi - 110 012, India.

Abstract

The anthropogenic use of mercury (Hg) has led to the wide outlook to the problem and health concerns caused by it. Metagenomic approach has opened the galore to the ease of access to the gene pool. The permissible limit of Hg is 0.001ppm but there are some sites in India having very high concentration of Hg (Ulhas estuary, Mumbai, having 107 ppm Hg). Library of clones containing mer operon, was constructed using culture independent technique. Out of 150 clones studied, UC07 has shown growth at 70ppm Hg. It has also shown antibiotic resistance towards vancomycin (30 mcg), Kanamycin (30 mcg) and Norflaxin (10 mcg). Clone has the capability to volatilize 97.94% of 5ppm Hg which gradually decreased to 47% with the increase in Hg to 70ppm. Validation study using actual site (Ulhas soil) showed the effective conversion of Hg up to 99.9% by UC07. The clone contains both *merA* and *merB* genes, so it can be effectively utilized for organic and inorganic contaminated site. This is the first study of validation of clone using Hg contaminated site. The results are quite promising which shows that UC07 can be utilized effectively for bioconversion of both organic as well as inorganic form of Hg contamination.

Keywords: Mercury, metagenomic, mercury resistance, mer operon, antibiotic resistance, mercury volatilization.

*Correspondence: sporwal@amity.edu, +91120-4392900

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INTRODUCTION

Mercury (Hg) is naturally occurring element found in environment and amongst the top most toxic elements in the plethora of 6 million substances and found in earth crust at varied array from 21 (lower crust) to 56 (upper crust) ppb¹ in the elemental form and mixture of minerals (hypercinnabar, metacinnabar and cinnabar etc.)² It is one of the most widely used heavy metal due to its characteristic feature to form amalgams with other metals (dental amalgams or gold extraction) and also in agriculture as bactericides, fungicides, insecticides and herbicides.

These anthropogenic usages of mercury have led to pollute the water bodies and surrounding soil by direct and indirect discharge of mercury, which in turn has turn hazardous to the organisms living in such habitat and is also one of the major concerns to human health as it could lead to neurotoxic diseases. The need to study on mercury elevated because of its usage as topical disinfectant and antiseptics in the community and hospitals. It has been estimated that 800 tons of Hg(II) is released in environment due to weathering of 1010 tons of rocks globally every year, furthermore 200 million tons of mercury is estimated to be present in oceanic reservoir. The concentration of dissolved Hg (II) in water varies from 0.03 to 2.0 sg/liter (i.e., up to 10⁻⁸ M), in the midst of such surroundings, it is not astonishing that bacteria have evolved with the mechanism to overcome such toxic compound. The cluster of genes responsible for mercury resistance was discovered as mercury operon (*mer* operon) consisting of two major genes *merA* and *merB*. *merA* genes, encoded in structural gene found on *mer* loci or transposon 501. Also, evolutionary studies depict the presence of *merA* gene in some microorganism due to horizontal gene transfer. Gene *merA* encodes for enzyme known as "mercury reductase" that converts the inorganic form of mercury (Hg²⁺) to volatile and less toxic form of mercury (Hg⁰) and *merB* genes encodes for organomercury lyase that breaks down organic form of mercury (CH₃ Hg⁺) to Hg²⁺. These enzymes are found in the cytoplasm of the cell. In addition, other genes are also present like genes for transport (*merT*)³. Depending upon the presence of gene in bacteria two type of resistance mechanisms are found, termed as Broad spectrum-

where the organism carries both the genes *merA* and *merB* conferring resistance against HgR and the other as Narrow spectrum: where only *merA* gene is present providing resistance to bacteria against only Hg²⁺. As per various researches it has been found that *merB* gene is present upstream to *merA* gene conferring resistance to both organic and inorganic form of mercury^{3,4}. The increase in Hg pollution has augmented antibiotic resistance⁵. The presence of antibiotic resistance is thought to be linked with the presence of mercury found in environment⁶.

There are few sites in India like Ulhas estuary, Kodai kanal and Panipat industrial area which have surpassed the permissible limit of Hg (1x10⁻³ ppm) as per Indian standards and WHO and has entered the food chain. One of such site is Ulhas estuary containing 107ppm of Hg concentration⁷. This study was undertaken with the objective to obtain a clone (containing *mer* operon) which has the ability to withstand high concentration of Hg as well as efficiently convert its organic form to in-organic toxic form (Hg²⁺) and further transformation into non-toxic volatile form (Hg⁰).

MATERIALS AND METHODS

Sample collection and analysis of Hg concentration in soil

Soil sample was collected from Ulhas estuary, Thane, Maharashtra. The soil was taken from 10 cm beneath the surface in the clean container and was immediately stored at 4°C till further use. CV-AAS analysis of the soil was done by digesting it by mixing it with equal volumes of water and nitric acid and heated till the volume reduced to one third of original volume. Later the content was filtered through Whatman filter paper and reading was taken for Hg volatilization.

Metagenomic library construction of (*mer* operon)

Metagenomic DNA was isolated from the soil sample by using MOBIO kit by given protocol. DNA quantification was done by diluting the DNA up to 500 folds and taking UV absorption at 260nm. Partial restriction digestion was performed to get the DNA in range of 5-10kb for cloning. 100µl digestion mixture was setup in sterile micro-centrifuge tubes which had 80µl of DNA, 9µl of sterile double distilled water, 10µl of 10X buffer

solution and 1µl of Sau 3A1 restriction enzyme. The reaction was setup in the ice and immediately transferred to 16°C cold water bath for 4h. Reaction was stopped by heating the mixture at 70°C. The partially digested metagenomic DNA was subjected to gel electrophoresis in 1% agarose and viewed under UV light. DNA fragments in the range of 5-10kb were eluted using HiYield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan). Partially digested DNA (400ng) in range of 5-10kb was ligated to pUC 19 vector (predigested with BamHI) and dephosphorylated using T4 DNA ligase enzyme at 4°C for 16h in micro-centrifuge tubes the reaction was terminate by heating the reaction mixture at 70°C. The ligated DNA product was introduced into the host bacteria i.e. *E.coli* DH10B by electroporation at 200Ω, 25µf and 2.5 kV, which was carried out by Micropulser II (Bio-Rad). The reaction mixture was mixed with 1ml of Luria Broth (LB), 100µl was plated on Luria Bertani (LB) agar plates supplemented with ampicillin, X-Gal and IPTG. The plates containing transformants were kept in Incubator at 37°C for 16h.

Screening and Selection of mercury resistant clones

White colonies were picked and streaked on the LB agar plates supplemented with 5ppm of Mercury Chloride (HgCl₂) and kept in incubator at 37°C for 18-72h. The clones which were able to grow at 5ppm of Hg were inoculated in LB supplemented with 5-90ppm of HgCl₂ and the ones showed growth at maximum Hg concentration were chosen for acclimatization. The cultures were inoculated in LB with 5ppm HgCl₂ and incubated at 37°C for 48h. It was further transferred to 10, 20, 30 till 80ppm of HgCl₂. OD was taken at 600nm.

The clone which has shown growth at highest concentration of HgCl₂ was selected for further studies. Since the clone was able to grow in presence of HgCl₂, indicated that it carries *merA* gene. It was also examined for presence of *merB* gene by inoculating it in LB with CH₃HgCl₂ (5ppm and 10ppm) and incubating at 37°C at 180rpm for 24 - 48 h. OD was taken at 600nm. The clones were also inoculated in LB supplemented with 5ppm-80ppm HgCl₂ respectively for cfu count and incubated at 37°C for 18-24h. Overnight grown culture (100µl) was plated onto LB agar and incubated at 37°C for 16h. CFU was calculated by using online CFU at <http://openCFU.source-forge.net/>.

net/⁸.

Quantification and validation of Hg volatilization

Since the selected clone indicated the presence of both *merA* and *merB* genes, it was checked for its ability to convert in-organic toxic form of Hg to non-toxic volatile form. The clone was grown in LB supplemented with 10, 20 up to 80 ppm of HgCl₂ and, incubated at 37°C at 180rpm. The culture was withdrawn on 2nd, 4th and 6th day. Culture was centrifuged at 6000rpm for 10 minutes and supernatant was used for quantification of Hg volatilization by CV-AAS (Analytical Jena make (Vario-6) cold vapor HG-AAS).

For validation, the Ulhas soil sample (without autoclaving) was mixed with sterile soil (3:2wt/wt) to make 10 kg of mixture with Hg concentration to approx. 60 ppm. 500ml (1 OD) of overnight grown culture of selected clone was sprayed on the diluted soil. Samples were withdrawn on 2nd, 4th and 6th day respectively. CV-AAS was done to check the ability of clone to transform toxic form of mercury to non-toxic form as an application to original site.

Antibiotic resistance

Antibiotic resistance was checked through disk diffusion method against Streptomycin (10 and 25 mcg), Norflaxin (10mcg), Tetracyclin (30mcg), Ampicillin (10, 20 and 30mcg), Kanamycin (30mcg), Vancomycin (30mcg), Gentamycin (30mcg). Zone of inhibition (in cm) was measured after 18h of incubation.

Identification of the mercury determinants

(a) Genomic DNA Isolation

Culture was inoculated into 10 mL of nutrient broth and incubated overnight at 37°C. Culture was centrifuged at 6,000rpm at room temperature (RT) for 10 minutes followed by subsequent washing of pellet 2-3 times by 1X TE buffer. Pellet was thoroughly mixed with 40µl of 10% SDS and 400µl of 1X TE buffer and incubated at 37°C for 30 minutes. Centrifugation was done at 7000rpm for 10 minutes at RT and supernatant was collected into fresh micro-centrifuge tubes and then phenol chloroform-based extraction was done. DNA was concentrated using absolute ethanol by leaving it at -20°C overnight and was thoroughly centrifuged at 10,000 rpm for 15 minutes at RT followed by subsequent washing with 70% ethanol and air dried. Finally the DNA was re-suspended in 50µl of 1x TE buffer.

(b) Amplification of *merA* and *merB* genes

Amplification of *merA* and *merB* genes were done using *merA* F; R and *merB* F; R primers sets and PCR Master Mix (Fermentas Life Sciences, USA) in 50µl reaction using an automated thermocycler (Eppendorf AG 22331). The amplified PCR product along with 10kb and 1kb DNA ladder (Merck millipore, India) was visualized on agarose gel. The PCR products ~1.6kb for *merA* and ~500bp for *merB* were eluted and purified using HiYield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan) and sequencing was done.

Construction of phylogenetic tree

For phylogenetic analysis, *merA* and *merB* gene sequences were compared with *merA* gene and *merB* sequences respectively, available in Gene Bank using BLAST Search tool. While studying the genes, ten best sequences showing maximum similarity (~99%) with individual gene were selected for the construction of phylogenetic tree. Multiple sequence alignment was done using CLUSTAL OMEGA⁹. The statistical testing was done by resampling the dataset for 1000 times in program SEQBOOT. To estimate evolutionary distance between the sequences DNADIST program was used to calculate pairwise distance. The resultant distance matrix was used to draw tree with neighbor joining method using program NEIGHBOR¹⁰. Finally, the phylogeny tree was viewed in Tree View, version 1.6.6.

RESULTS

Metagenomic DNA isolation and restriction digestion

The concentration of the DNA isolated from Ulhas estuary site was 700ng/µl (260nm). DNA fragment in the range of 5 to 10Kb was gel eluted and ligated onto dephosphorylated plasmid digested with BamH1 restriction enzyme and metagenome was digested by using Sau3A1 (Fig. 1).

Metagenomic Library construction, screening and selection of clones

More than 150 clones were selected and screened for Hg tolerance (Fig. 2). Ninety-eight of them were able to grow on the LB agar plates supplemented with 5ppm of HgCl₂.

Amongst all the screened clones, maximum have shown growth at 5ppm, 84 were

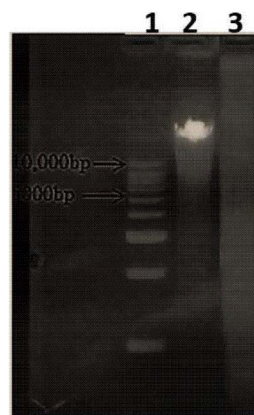


Fig. 1. Metagenome of Ulhas soil sample. Lane 1: 10 kb DNA ladder, 2: Metagenomic DNA (Ulhas); 3: Restricted digested with Sau3A1

able to grow at 10ppm and a drastic decrease was seen in the number of clones at 20ppm till 40ppm (beyond which no growth was seen) (Table 1). Only 4 clones were able to grow at 40ppm, which were used for acclimatization study.

The clones (UC07, UC26, UC79, UC126) were grown overnight at different concentrations of HgCl₂ (5 to 80 ppm) and number of viable cells were calculated (Table 2). The clones were acclimatized gradually by growing them at higher Hg concentration, only UC07 was able to grow till 70ppm Hg conc. hence selected for further studies (Table 3). UC07 was able to grow in existence of HgCl₂, indicated the clone carrying *merA* gene. It was also checked for presence of *merB* gene by using CH₃HgCl₂ as a substrate. UC07 was able to grow at 5ppm and 10ppm CH₃HgCl₂ showing

Table 1. Ulhas clones showing the growth at different concentrations of Hg

S.No.	Concentration of HgCl ₂	Number of tolerant clones
1	05	98
2	10	84
3	20	32
4	30	16
5	40	4
6	50	-
7	60	-
8	70	-
9	80	-
10	90	-

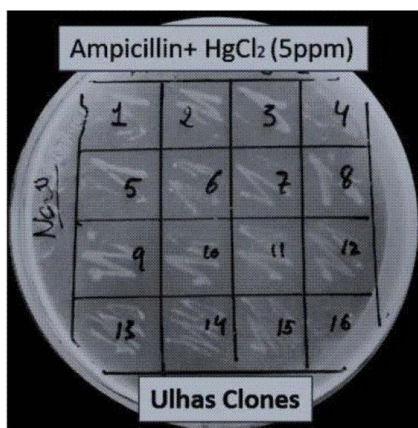


Fig. 2. Clones (containing mer operon) from Ulhas soil showing growth on 5ppm Hg

Table 2. CFU count of Ulhas clones at different Hg concentration

S. No.	Mercury Chloride Conc (ppm)	CFU			
		UC07	UC26	UC79	UC126
1	Control (0)	802	604	508	617
2	5	1024	965	722	712
3	10	921	567	684	525
4	20	662	410	312	338
5	30	427	368	293	216
6	40	415	307	301	267
7	50	397	-	63	52
8	60	274	-	-	-
9	70	131	-	-	-
10	80	-	-	-	-

the OD, 1.67 and 1.45 at 600nm respectively, confirming the presence of *merB* gene as well.

Quantification and validation of Hg volatilization

The quantification of Hg volatilization was done by CV-AAS for the samples withdrawn on day 2nd, 4th and 6th. It was observed that UC07 was able to effectively volatilize Hg supplemented in media. The conversion was found to be maximum at 5ppm (97.94%) on day 6th whereas on day 4th (93.23%) and day 2nd (86.34%) (Fig. 3). In all the Hg conc. (ppm) taken, the maximum bioconversion can be considered on 6 th day.

To check the bioconversion ability of UC07 at actual site, Ulhas soil sample was treated with it. The volatilization of Hg was checked on 2nd, 4th and 6th day, and the maximum was

Table 3. Acclimatization of Ulhas clones at different Hg concentration

S. No.	Mercury Chloride Conc (ppm)	Clones			
		UC07	UC26	UC79	UC126
1	10	√	√	√	√
2	20	√	√	√	√
3	30	√	√	√	√
4	40	√	√	√	√
5	50	√		√	√
6	60	√			
7	70	√			
8	80				

(√) showing growth of clones

CV-AAS of UC07

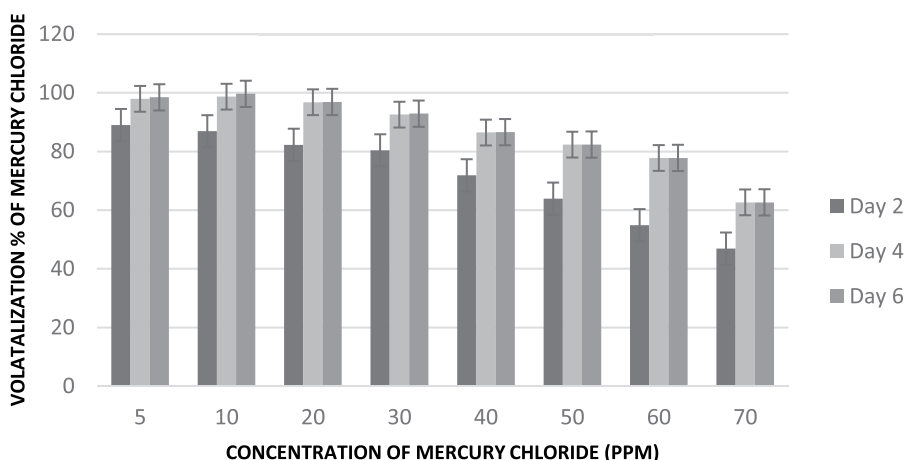


Fig. 3. Mercury bioconversion (%) by UC07 clone on day 2, 4 and 6

observed in sample withdrawn on 6th day which is 99.97% (Fig. 4) as compared to 2nd and 4th day which corresponds to 95.40% and 98.35% of volatilization.

Antibiotic resistance

Antibiotic activity of UC07 was checked by disk diffusion method. On the basis of the

measurement of the zone of inhibition (in cm) it was observed that UC07 was resistance to Vancomycin (30 mcg), Kanamycin (30 mcg) and Norflaxin (10 mcg), whereas it has shown susceptibility towards Streptomycin (25 mcg) (Table 4). 3.5 Identification and deposition of *merA* and *merB* gene sequences: The *merA* gene that

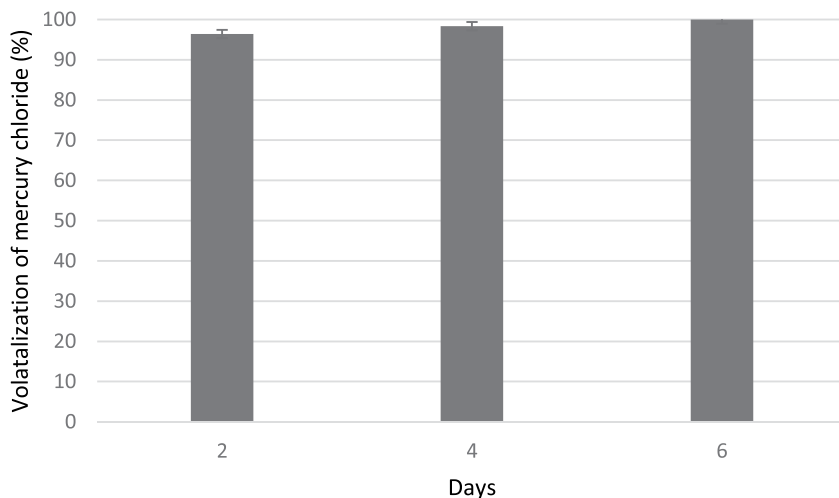


Fig. 4. Mercury bioconversion (%) by UC07 clone on day 2, 4 and 6 on Ulhas soil (actual site)

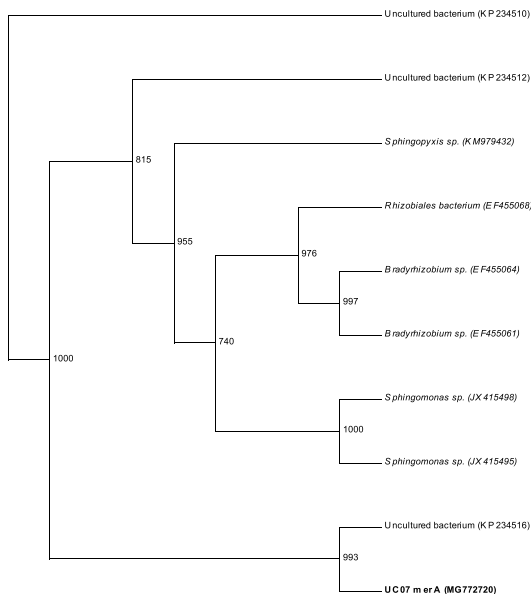


Fig. 5. phylogenetic tree based on partial sequences of *merA* genes was produced applying the Neighbor-Joining method. The bootstrap values, calculated from 1000 replicates, show the percentage of replicate trees in which the associated ratio clustered together.

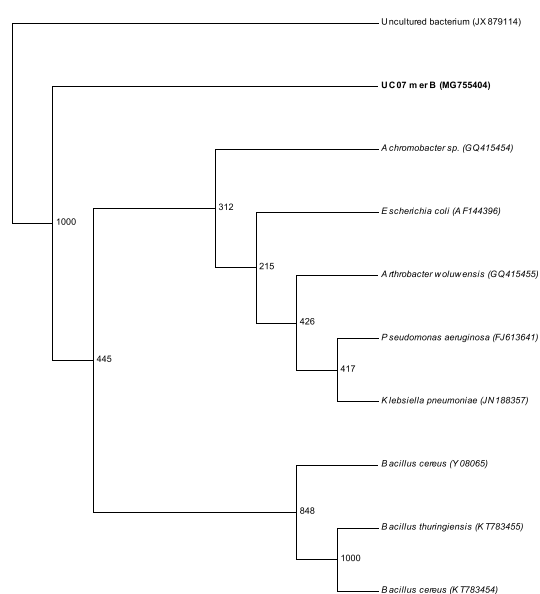


Fig. 6. phylogenetic tree based on partial sequences of *merB* genes was produced applying the Neighbor-Joining method. The bootstrap values, calculated from 1000 replicates, show the percentage of replicate trees in which the associated ratio clustered together.

Table 4. Antibiotic sensitivity assay of UC07

Antibiotics	Disc Conc.	Resistant	Intermediate	Susceptible
Vancomycin	30 mcg	+		
Kanamycin	30 mcg	+		
Norflaxin	10 mcg	+		
Getamycin	30 mcg		+	
Tetracyclin	30mcg		+	
Streptomycin	10 mcg		+	
	25 mcg			+

*Resistant (0-4mm), Intermediate (5-8mm) and Suseptible (9-12mm)

codes for mercury reductase enzyme and *merB* gene coding for methylmercury lyase of UC07 were sequenced. The partial sequences were checked for chimera using DECIPHER's Find Chimeras web tool (<http://decipher.cee.wisc.edu/FindChimeras.html>). The presence of both the genes *merA* (Accession No: MG772720) and *merB* (Accession No: MG755404) gave the indication of mercury volatilization which was supported by further experiments.

Phylogenetic analysis of *merA* and *merB* genes

The sequence obtained for *merA* and *merB* genes were globally aligned using BLAST and top ten matches showing similarity $\approx 99\%$ were used for phylogenetic tree construction (Fig. 5 and Fig. 6). The presence of *merA* and *merB* genes within the phylogenetic tree with high boot strap value (993-1000) confirms that UC07 contain both the genes.

DISCUSSION

Extensive use of mercury by various industries has become major cause of concern as it is dumped without proper discard procedure has found to enter the water bodies and as well as human food chain. Various researchers have found that there are many bacteria which can convert toxic form of (Hg^{2+}) to non-toxic form of mercury (Hg^0). These microorganisms are of great use as they play important role in biological degradation of mercury in its habitat³. Metagenomic approach gives the new insight of isolating the genomes of the bacteria which are un-cultivable i.e. cannot be cultured in lab^{11,12}. Among various clones, UC07 was found to be the best as it can grow in 70ppm of $HgCl_2$. UC07 can be used for broad spectrum volatilization as it was able to utilize

both $HgCl_2$ and CH_3HgCl_2 . The gene product converts the dangerous methylmercury and other organic mercury derivatives to ionic mercury; which can then be reduced to Hg^{013} . It has shown antibiotic resistance towards vancomycin (30 mcg), Kanamycin (30 mcg) and Norflaxin (10 mcg). Hg^0 loci and antibiotic resistance are commonly found on the same plasmids and transposons¹⁴, also microorganisms found in mercury pollutant sites have been discovered with antibiotic resistance⁵. Results indicated that maximum volatilization in case of media study was seen on day 4 (97.94%) whereas it was $\sim 100\%$ on day 6 in validation study using Ulhas soil. The Hg concentration in Ulhas soil was reduced down to 60ppm which may have favored the conditions for the autochthonic microbes enhancing the volatilization. The PCR and sequencing confirmed the presence of *merA* and *merB* genes within UC07 and the tree constructed using these sequences revealed the close relationship of both *merA* and *merB* already present in database. Since the clone can be used for broad spectrum bioconversion of Hg, it has also shown efficient volatilization on actual site (Ulhas soil). This clone can thus be maintained and explored for efficient volatilization of Hg.

Hg is one of the sixth most toxic elements found among the plethora of different element found on the earth. Its exposure can give rise to serious health problems and sometimes even death. Due to alarming situation it is important to find new strategies for its control. Bio conversion is one of the most widely chosen approaches in order to reduce the toxic waste. Ulhas soil, Mumbai, India was used to construct metagenomic library of *mer* operon. The clone obtained, was able to grow at 70ppm Hg. It has shown promising results

in bioconversion of Hg in actual site and can be used effectively for volatilization of both organic and inorganic form of Hg.

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CONFLICT OF INTEREST

The author declares that there are no conflict of interest.

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