Title: Expression and Purification of Transmembrane Protein MerE from Mercury Resistant Bacillus cereus

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Keywords: 16S rRNA, Ni–NTA chromatography, pHLMerE, Thrombin cleavage, Hg–detoxification
Expression and Purification of Transmembrane Protein MerE from Mercury Resistant 
*Bacillus cereus*

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**Running title:** Expression and purification of MerE

**Author contributions:** Aatif Amin cloned the plasmid, expressed and purified the MerE protein under the supervision of Stanley J. Opella along with executed mercury detoxification experiments and then also wrote the draft. Zakia Latif proofread the manuscript.

**Conflict of interest:** The authors declare that they have no conflict of interest.

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Abstract

Mercury resistant (Hg\textsuperscript{R}) bacteria were isolated from heavy metal polluted wastewater and soil collected near to tanneries of district Kasur, Pakistan. Bacterial isolates AZ-1, AZ-2 and AZ-3 showed resistance up to 40 μg/mL against mercuric chloride (HgCl\textsubscript{2}). 16S rDNA ribotyping and phylogenetic analysis were performed for the characterization of selected isolates as Bacillus sp. AZ-1 (KT270477), Bacillus cereus AZ-2 (KT270478) and Bacillus cereus AZ-3 (KT270479). Phylogenetic relationship on the basis of mer\textsubscript{A} nucleotide sequence confirmed 51-100% homology with the corresponding region of the mer\textsubscript{A} gene of already reported mercury resistant Gram positive bacteria. The mer\textsubscript{E} gene involved in the transportation of elemental mercury (Hg\textsubscript{0}) via cell membrane was first time cloned into pHLV vector and transformed in overexpressed C43(DE3) E.coli cells. The recombinant plasmid (pHLMerE) was expressed and the native MerE protein was obtained after thrombin cleavage by size exclusion chromatography (SEC). The purification of fusion/recombinant and native protein MerE by Ni-NTA column, dialysis and fast protein liquid chromatography (FPLC/SEC) involved unfolding/refolding techniques. A small scale reservoir of wastewater containing 150 μg/mL of HgCl\textsubscript{2} was designed to check the detoxification ability of selected strains. It resulted 83% detoxification of mercury by B. cereus AZ-2 and B. cereus AZ-3, and 76% detoxification by Bacillus sp. AZ-1 respectively (p<0.05).

Keywords: 16S rRNA, Ni-NTA chromatography, pHLMerE, Thrombin, Hg-detoxification
**Introduction**

Mercury pollution is a global problem due to its toxic effects on human and animals. Anthropogenic sources and activities e.g. the release of untreated industrial wastewater from chlor-alkali plants, the metal mining and the incineration of coal, are increasing mercury pollution day by day in the environment [1]. It is evident that the cytotoxic and neurotoxic effects to humans and animals are caused by inorganic (ionic) and organic forms of mercury [2].

In Pakistan, the maximum release of mercury (kg/year) is estimated as 1625.11, 2500, 21120, 1071.79, 5779 and 4637.55 in extraction process, cement industries, chlor-alkali industries, intentional uses of mercury in consumer products, waste deposition/land filling and waste water treatment plants respectively indicating 36898.77 kg total cumulative release of mercury per year [3].

*Bacillus* sp. RC607, first time identified to have chromosomal resistance against mercury, was isolated from contaminated site, Boston Harbor, USA [4]. In later studies, the strain RC607 like sequences were found in 74 *Bacillus* spp. isolated from Minimata Bay sediment, Japan and also in several Russian environmental bacterial isolates. It was suggested the possible global distribution of the RC607 *mer* operon. *Bacillus mer* operon bearing plasmids were found in several strains analyzed in the Russian study [5, 6].

Due to the presence of mercury in the environment and its toxic effects, microorganisms have evolved mercury detoxification systems to deal with mercury containing compounds. Bacteria have ability to detoxify the toxic form of mercury (Hg$^{+2}$) into non-toxic elemental form (Hg$^{0}$) through a cytoplasmic enzyme, mercuric reductase which is encoded by *mer*A gene of *mer* operon [7]. A lot of research analysis was done on this cytoplasmic enzyme, a member of flavin containing NADPH dependent dithiol oxidoreductase and was isolated from Gram positive as well as Gram negative bacteria [8]. Gram positive and some of Gram negative bacteria also possess organomercurial lyase encoded by *mer*B gene of *mer* operon which cleaves the C–Hg bond of many mercury containing compounds. The enzyme, organomercurial lyase confers broad spectrum resistance against mercuric compounds which results the elemental mercury (Hg$^{0}$) diffused out of the cell [9].

Bacterial genes involved in mercury resistance are well-organized and well-characterized into genetic elements known as *mer* operons. The Gram negative bacterial *mer* operons contain a broad spectrum structural gene *mer*E that encode a CH$_3$Hg$^{+1}$ and Hg$^{+2}$ detoxification system. [10].
The merE gene immediately following the merD at the downstream end of the bacterial mer operon, is frequently found in many narrow and broad spectrum mer operons [11]. Kiyono et al., [12] have reported the transcription and translation of merE gene into a membrane protein with molecular mass of ~8 kDa in Gram negative bacteria. Moreover, MerE protein is a methylmercury membrane transporter. This system involved in transportation of mercury ions across the cell membrane [12].

For structural and biochemical studies, the active and proper folded form of soluble and transmembrane proteins is essential during its expression and purification procedures. For rapid screening of soluble and transmembrane protein, the fast high-yield production has been developed by recombinant expression systems. Among bacterial, insect or yeast, mammalian and plant expression systems, the bacterial expression system is less expensive and fast with two major problems. The protein folding machinery is the least complex and the formation of inclusion bodies (IBs) which are insoluble aggregates of recombinant proteins [13].

Refolding/activity of proteins from IBs is affected by several factors such as solubilization of IBs by denaturants, removal of the denaturant, and assistance of refolding by small molecule additives. The methodologies being used now a days for refolding aggregated proteins can be divided into two major categories: chromatographic i.e. solvent exchange by size-exclusion chromatography (SEC), on-column refolding and chaperone-assisted refolding [14] and non-chromatographic for rapid dilution of denaturants from denatured proteins i.e. dialysis, dilution, chemical additives (NH₄)₂SO₄, amino acids (ArgHCl), glycerol and cyclodextrins [15-17].

In this study, screening, molecular identification and phylogenetic analysis of Hg-resistant bacteria were described on the basis of 16S rDNA ribotyping and merA gene sequence and then recombinant plasmid construction, expression and partial purification by Ni-NTA column of transmembrane protein MerE involved in mercury detoxification system were performed. Furthermore, the native MerE protein was purified by size exclusion chromatography (FPLC). Finally, the detoxification potential of Hg-resistant bacteria from Hg-contaminated wastewater was also checked.
Materials and methods

Chemicals and expression vectors

For DNA restriction, the enzymes, HindIII and SacI were purchased from New England Biolabs (www.neb.com) unless otherwise noted, and the oligonucleotides were synthesized by Integrated DNA Technologies (www.idtdna.com). Ligation Kit was purchased from life sciences (www.lifescience.roche.com). For bacterial genomic DNA, GeneJET Purification Kit and E. coli cells (DH5α™ Competent) were obtained from thermo fisher® (www.thermofisher.com). Bacterial competent cell (E.coli) C43(DE3) was obtained from Lucigen® (www.lucigen.com). For plasmid preparations, small scale Gel Extraction Kit (Miniprep Kit) and Ni-NTA resin were obtained from Qiagen® (www.qiagen.com). The PCR reagents were purchased from Agilent Technologies® (www.genomics.agilent.com). Sephacryl S-200 column for size exclusion chromatography was purchased from Pharmacia LKB (Piscataway, NJ).

Isolation and screening of HgR bacteria

Mercury resistant bacteria were isolated from wastewater and contaminated soil samples obtained from the tanneries near Kasur district in Pakistan. The physico-chemical properties such as temperature, soluble salts, organic matter, Hg\(^{+2}\) concentration and soluble N, P and K, of all samples were checked. To this end, serial dilutions of 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\) and 10\(^{-4}\) were prepared from 1% of the initial water and soil samples. For obtaining the isolated colonies of Hg-resistant bacteria, 100 μL from the 10\(^{-3}\) and 100\(^{-4}\) dilutions were spread on Lauria Bertani (LB) agar plates supplemented with HgCl\(_2\) of different concentrations ranging from 1-200 μM. The plates were incubated at 37°C for 24 h. After incubation, the isolated and Hg-resistant colonies were obtained which were re-streaked on new LB agar plates without HgCl\(_2\) for obtaining purified colonies and stored as glycerol stocks at -80°C.

PCR-amplification of 16S rRNA and merA genes

The bacterial isolates AZ-1, AZ-2 and AZ-3 showing high resistance against HgCl\(_2\) were characterized by 16S rDNA ribotyping. Bacterial strain ZA-15 was used as a negative control. The bacterial genomic DNA was extracted by GeneJET Genomic DNA Kit. The universal primers were used for the amplification of 16S rRNA gene; 16S-F (5’AGAGTTTGATCCTGGCTCAG3’) and 16S-R (5’AAGGAGGTGATCCAGCCGCA3’) [18] and for merA gene; merAF : 5’TGGGTGGAACCTTGCGTTAA3’ and merAR : 5’TTATCCAGCAGCAAGATA3’ using thermal cycler. The amplification conditions for
both genes were consisted of initial denaturation at 95°C (5 min) and then 30 cycles of 95°C (1 min), 55°C (1 min), and 72°C (1.5 min), plus an additional cycle at 72°C (5 min) for chain elongation. The amplified products were sent to Macrogen sequencing core facility, Korea and obtained sequence results were checked by NCBI nucleotide BLAST and finally submitted to GenBank.

**Phylogenetic relationship**

The multiple sequence alignment through clustalW was used to check phylogeny among bacterial species of *Bacillus* genera on the basis of 16S rRNA and merA genes through neighbor joining method using MEGA 5 software. The percentage of homology among different clade was checked with bootstrap test at value of 1000 replica as shown next to the branches. The tree is drawn to scale which represent the nucleotide change. The units of branch lengths and the evolutionary distances are same, used to infer the phylogenetic tree.

**Construction and expression of recombinant plasmid pHLMerE**

The merE gene of mercury resistant isolate *Bacillus cereus* AZ-2 was amplified using the primers; F-MerE [5’ATCTATAAGCTTATGAAAAACATAATAAAAAGTTTCAGGTTGTTTCTTGTCG3’], R-MerE [5’ATATATGAGCTCCTCAGCTGACTTGAACCTTCTCCATGCTGCAA C3’]. *Bacillus megaterium* MB1 was used as a positive control for both merA and merE genes [9]. The amplified DNA of 270 bp was cloned in HindIII-SacI-cleaved pHLV expression vector. The designed recombinant plasmid was transformed into DH5α competent cells. The pHLMerE (3842 bp) was confirmed by restricting with same enzymes and visualized on agarose gel electrophoresis. The nucleotide sequence of merE gene was also confirmed by DNA sequencing facility provided by University of California, San Diego (UCSD), USA. The recombinant plasmid pHLMerE was then transformed into *E. coli* competent cells C43(DE3) as described in our previous study [19].

Five μL of clone C43(DE3):pHLMerE from glycerol stock was inoculated in 5 mL of LB medium containing 50 mg/L of cabenicillin and incubated for 5 hours at 37°C. After incubation, 1 mL was taken from 5 mL culture and inoculated 50 mL of M9 minimal medium (Na₂HPO₄ 7.0 g, KH₂PO₄ 3.0 g, NaCl 0.5g, CaCl₂ 0.1 mM, MgSO₄ 1 mM, thiamin 50 mg, d-glucose10 g, (NH₄)₂SO₄ 1 and 50 mg/L of carbenicillin for 1 litre) and kept in shaking incubator at 37°C for
overnight with 300 rpm. An overnight 50 mL culture was poured into 450 mL of same M9 medium and allowed to grow at same growth conditions. When the optical density of culture is reached at O.D$_{600}$ ~ 0.6 and isopropyl-β-D-thiogalactoside (IPTG) with final concentration of 1 mM was added for the production of His-trp∆LE-thrombin site_MerE fusion protein and continued shaking in same incubator for another 7 h. The cell pellet harboring recombinant plasmid was obtained by centrifugation at 7,000 rpm for 30 min at 4°C.

**Purification of the recombinant protein by Ni$^{2+}$-NTA column**

The purification of the recombinant protein by Ni$^{2+}$-NTA column was performed by using method as described in our previous studies with some modifications [20-22]. The cell pellet was re-suspended in 30 mL lysis buffer consisting 50mM Tris hydrochloride, 15% glycerol (v/v), 1mM Na$_3$N with pH 8.0 and incubated at room temperature for 10 min. The collected cell lysate was disrupted by sonication for 5 min on ice (5 sec ON and 10 sec OFF) and spun down at 17,000 rpm for 30 min at 4°C. At this stage, purified inclusion bodies contained presumably 90% of MerE recombinant protein. The inclusion bodies were solubilized in the binding buffer consisting 20 mM Tris hydrochloride, 500 mM NaCl, 6 M GndCl, 5 mM imidazole at pH 8.0 by tip sonication. The cell lysate was centrifuged again at 19,000 rpm for 1 hour to remove protein and lipid-associated cell debris. The Ni$^{2+}$-NTA Histidine binding resin was pre-equilibrated with binding buffer and then the supernatant was loaded on to the column. The resin was extensively washed by washing buffer consisting 20 mM Tris hydrochloride, 500 mM NaCl, 6 M GndCl, 50 mM imidazole with pH 8.0. The polyhistidine-tagged fusion protein was eluted with elution buffer consisting 20 mM Tris hydrochloride, 500 mM NaCl, 6 M GndCl, 500 mM imidazole with pH 8.0. The eluted polyhistidine-tagged fusion protein MerE was monitored by SDS-PAGE and then concentrated to 30 mL by using YM10 filter membrane containing Amicon stirred concentrator cell. The elute was dialyzed against ddH2O in a 10 kDa MWCO dialysis membrane with four water changes until the protein precipitated out of solution and then lyophilized.

**Thrombin cleavage of the fusion protein**

The cleavage of fusion partner by thrombin was performed by following method described by Das *et al.* [23] with some modifications. The lyophilized polypeptide was re-suspended in 30 mL of binding buffer consisting 20 mM Tris HCl, 500 mM NaCl, 1% SDS, 10
mM imidazole, 0.1% TCEP with pH 8.0, and loaded on to Ni$^{2+}$-NTA column which was pre-
equilibrated by 20 bed volumes of thrombin cleavage buffer consisting 20 mM HEPES, 250 mM
NaCl, 0.1% hexadecylphosphocholine, pH with 8.0). The fusion partner trp∆LE was cut-off by
thrombin (1000 units) by incubating for 2 h at room temperature. The column was then washed
with 10 bed volumes of washing buffer consisting 20 mM Tris HCl, 0.5%
dodecylphosphocholine, 50 mM NaCl, 20 mM imidazole with pH 7.3). The native MerE and
fusion partner were eluted with 3 bed volumes of elution buffer consisting 20 mM HEPES, 0.5%
dodecylphosphocholine, 500 mM imidazole, 50 mM NaCl with pH 7.3. Approximately 2-3 mg
of MerE protein was obtained from each 10 mL elute and lyophilized. The cleaved polypeptide
was washed and precipitated by using 3,500 kDa MWCO dialysis membrane against ddH$_2$O and
then lyophilized.

**Purification of MerE by FPLC**

The purification of MerE protein by FPLC was performed as described by Amin and
Latif [22]. The cleaved and lyophilized MerE protein was solubilized in 5 mL phosphate SDS
buffer containing 100 mM Na$_2$HPO$_4$, 20 mM DTT, 4 mM SDS, I mM EDTA, lmM NaN$_3$ with
pH-8.2. The aggregated protein was disturbed by extensive bath sonication which presumably
incorporated SDS into micelles for maintaining stable tertiary structure. For the separation of
leader peptide + His-tag from the pure MerE protein, a Pharmacia FPLC system was used
containing Sephacryl S-200 column. The column was pre-equilibrated by phosphate-SDS buffer
and 5 mL of sample was loaded. Fractions containing protein MerE were pooled and
concentrated down to 30 mL using 3,500 kDa MWCO membrane of Amicon Stirred cell. The
pure MerE protein was then dialyzed against ddH2O with 40 mM β- mercaptoethanol to remove
SDS for 6 12-h changes until the protein was precipitated. The protein was quickly lyophilized
and stored at -20 ºC for further experiments. Approximately 0.5-1 mg of MerE protein per one
litre of cell culture was obtained as measured by spectrophotometer. SDS-PAGE was used at
each step for monitoring the purification of final native MerE protein.

**Detoxification of Hg$^{2+}$ by selected bacteria at a lab scale**

Selected bacterial strains (AZ-1, AZ-2, AZ-3 and ZA-15) were inoculated in four flasks
containing 30 mL of wastewater containing 30 μg/mL of HgCl$_2$ separately. The detoxification
efficiency of mercury was determined by inoculating 1.5 mL of overnight free cell culture (O.D.
2.0 at 600 nm) in four flasks (4$^{th}$ flask was taken as control) in triplicates and incubated at 37°C
for 24 h at 120 rpm of agitation. After incubation, cultures were centrifuged at 14000 rpm for 15 min and the supernatant was separated. The pH of supernatant for each strain was adjusted to 0.4 by adding concentrated H$_2$SO$_4$ and then transferred to separating funnel. To the cooled solution, 4 mL of 6N acetic acid and 2.5 mL chloroform were added and vigorously shaken for 1 min. The phases were allowed to separate completely and took out the chloroform layer and discarded it. Freshly prepared 5 mL of 0.001% dithizone solution was added in the remaining solution of each strain and again shaken vigorously for 1 min. The layers were allowed to separate and placed cotton in the tips of separating funnels and eluted the dithizone-mercury complex. In the absence of mercury (Hg$^{+2}$) no significant color was extracted into dithizone, while in the presence of Hg$^{+2}$, an orange color was observed in the organic layer, the color became more pronounced with higher concentration of Hg$^{+2}$. The optical density (O.D) of water–free chloroform extracts was determined at 500 nm against reagent (dithizone) blank to estimate the detoxification of Hg$^{+2}$ [24, 25].

**Statistical analysis**

All statistical analyses i.e. mean, standard deviation, analysis of variance (ANOVA) of lab scale mercury detoxification experiment were performed by using SPSS V.20 software.
Results and Discussion

The interaction between heavy metals and heavy metal resistant microorganisms has been determined by bacterial transformation and the conversion of metallic ion form to elemental form by reduction [26]. Some of these microorganisms have shown promising activity to decrease mercury from contaminated environments. In the present study, three bacterial strains out of 30 were selected on the basis of resistant to higher HgCl$_2$ concentrations (MIC: up to 1-200 μM) and two strains, ZA-15 and MB1 were used as a negative and positive controls respectively (Table1).

The physicochemical parameters of selected samples at the time of collection were checked as temperature 24°C, pH 8.2, soluble salts 0.7 g/kg, organic matter 8.4 g/kg, Hg$^{+2}$ concentration 10-20 μg/mL, soluble N, P and K 53.5, 3.5 and 45 mg/kg. In literature, 100 μM, 50 ppm and 100 μg/mL were found to be the MIC for mercury in Bacillus spp. [27-29].

Mercury resistant bacterial isolates (AZ-1, AZ-2 and AZ-3) were characterized by 16S rDNA ribotyping (≈ 1.5 kb) and identified as Bacillus sp. (KT270477), Bacillus cereus (KT270478), and Bacillus cereus (KT270479), respectively. Other close matches to Bacillus sp. (KT270477) included Bacillus anthracis HQ200405 and Bacillus sp. JN593078 and GU566355. The bacterial isolates also showed similarity (98%) among themselves. In the same clade, B. cereus AZ-1 and AZ-2 showed 98% similarity with already reported B. thuringiensis KP306751, Bacillus sp. JQ691603 and B. cereus KP407139, KR303714, KP202304 and LK392517 (Fig. 1).

Phylogenetic analysis of selected bacterial strains on the basis of merA gene showed 100% homology with already reported sequences of merA genes in B. cereus NG034957, B. megaterium NG035056, Bacillus sp. LC015492, Paenibacillus sp. LC015492, 98% in B. macroides Y09906, 89% in Bacillus sp. RC607 AF138877 and Clostridium butyricum AB024961 and 88% in B. cereus AB066362 and also with themselves (Fig. 2).

The gene construct containing merE was designed by modifying previously reported pHLVpu plasmid constructed by pHLV_ML vector which is derivative of the commonly used PET vector pET-3a [30]. The vector consisted of 9-His tag- Tryptophan leader-Vpu fusion on plasmid encoding carbenicillin resistance. In modified vector, Vpu gene was replaced by merE gene by designing specific primers as shown in Fig. 3 (A). In the gene construct, His-tag and Trp-leader peptide sequences were inserted for the ease of purification and the formation of inclusion bodies respectively. The recombinant plasmid was transformed into competent DH5α
and amplified in LB medium. The plasmid was then transformed into \textit{E. coli} C43(DE3) cells for producing high yield of MerE recombinant protein. The fusion protein was found non-toxic to C43(DE3) cells and expressed at levels up to approximately 20% of total cellular protein.

After expression, the separation and purification of MerE were performed. Briefly, the inclusion bodies containing recombinant/fusion protein were separated from the total cell lysate by centrifugation. The fusion protein was partially purified by nickel affinity chromatography and dialyzed to decrease gradually the denaturant concentration for efficient protein refolding. The data in Fig. 4 illustrated the expression and isolation of inclusion bodies of full-length recombinant MerE by Ni$^{2+}$-NTA column. During dialysis, the protein refolding activity of chemically denatured protein was increased sufficiently due to the decrease in denaturant concentration. The protein concentrations remained almost constant during the refolding process. The rate of refolding to the native (active) structure was also increased by increasing dialyzing time along with the decrease in concentration of denaturant [15, 31].

After elution by nickel column, the targeted fusion protein was solubilized in thrombin cleavage buffer and again bound to the affinity column for cleaving native MerE from fusion partner (TrpΔLE) and dialyzed. The lane 2 in Fig. 5 (A) showed the cleavage of fusion protein and the lane 3 contained the impurified fraction of MerE while lanes 4-7 showed the purified fractions native MerE after size-exclusion chromatography (FPLC). Fig. 5 (B) showed the peaks of MerE and TrpΔLE in FPLC equipped with Sephacryl S-200 HR column. The fully denatured proteins by GndCl or urea, usually have a high tendency to aggregate due to the exposure of buried hydrophobic residues. The gel filtration column used for SEC/FPLC restricts the aggregation by physically isolating the molecules and allows the successful recovery of biologically active (refolded) proteins. Using this approach, Neely \textit{et al.} [32] successfully obtained refolded/active β$_{1b}$ subunit of a calcium channel. In a similar way, SEC refolding was successfully used to obtain biologically active forms of the α5 subunit of the 20S proteasome from human [33]. In another example, Ouellette \textit{et al.} [34] used denatured inclusion bodies of human interleukin (IL)-7 to load onto a Superdex 200 column and obtained its biologically active (refolded) form.

This was a first study for reporting the plasmid construction, expression and purification of MerE protein from Gram positive \textit{Bacillus cereus} AZ-2. Previously, Sone et al., [35] had
reported the expression and preliminary purification by Nickel agarose column of MerE protein from Gram negative bacteria. Park SH, Opella SJ [36] and Lu et al., [37] had also performed NMR structural studies of purified samples of MerE from Gram negative bacteria. The gene and protein sequences of MerE of both types of bacteria were totally different as shown in Fig.6. In Gram positive bacteria, the polypeptide consisted of 89 amino acids with internal methionine residues at different sites while in Gram negative bacteria, 78 amino acids containing peptide had only single methionine at N-terminal. Due to the presence of internal methionine residues, the gene construction, expression and cleavage of recombinant protein for obtaining the native sequence of MerE varied comparative to previous reports as shown in Fig. 3 (B). Topological alignment of all amino acids of MerE separated according to the predicted hydrophobic (membrane-spanning) elements is shown in Fig. 6.

It was reported that certain species such as cyanobacteria, *Klebsiella aerogenes*, *K. aerogenes* NCTC418 and *Pseudomonas putida* were involved in bioreduction of toxic mercury compounds under laboratory conditions [38, 39]. Nakamura K, Hagimine M, Sakai M, Furukawa K [40] reported the decrease of mercury from various mercury compounds like mercuric chloride (HgCl₂) 88.9%, methyl mercuric chloride (MeHg) 95.4%, ethylmercuric chloride (EtHg) 83.8%, thimerosal 91.9%, fluorescein mercuric acetate (FMA*) 74.6%, phenylmercuric acetate (PMA) 5.7%, and p-chloromericuric benzoate (p-CMB) 92.3% by *Pseudoalteromonas haloplanktis* strain M-1. The biosorption capability of the immobilized *B. cereus* cells was described by Sinha A, Pant KK, Khare SK [41] as 104.1 mg/g Hg²⁺ at 30°C and pH 7.0, biomass concentration of 0.02 g/L and contact time of 72 h. A lab scale experiment was designed to analyze the detoxification potential of mercury by selected bacterial strains. Results indicated 23 (76%), 25 (83%), 25 (83%) and 27 (90%) μg/mL detoxification of Hg²⁺ out of 30 μg/mL (100%) by selected bacterial strains as *Bacillus* sp. AZ-1, *B. cereus* AZ-2, *B. cereus* AZ-3 and *E. cloacae* ZA-15 respectively (Fig. 7). In conclusion, phylogenetically similar and *mer* operon harboring mercury resistant bacterial strains *B. cereus* AZ-2, *B. cereus* AZ-3 can be used for the detoxification of mercury from industrial effluent.
Acknowledgments

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Legends of figures

Fig. 1. Evolutionary relationships of different *Bacillus* sp. on the basis of 16S rDNA ribotyping.

Fig. 2. Evolutionary relationship of different *Bacillus* sp. on the basis of *merA* gene was inferred using the Neighbor-Joining method with the sum of branch length 0.53623375. The branch lengths and the evolutionary distances used to infer the phylogenetic tree are in the same units with the bootstrap test value 500. The analysis involved 17 nucleotide sequences and codon positions included were 1st+2nd+3rd+Noncoding. A total of 1279 positions were in the final dataset with no gaps and missing data. Evolutionary analyses were performed by MEGA 5 software [42].

Fig. 3. (A) The pHLV plasmid; in linear form showing nucleotide and amino acid sequences of His-tag, fusion partner, thrombin site and MerE while in circular form showing T7 promoter, T7 terminator, His-tag_trpΔLE_thrombin site_MerE, *Hind*III and *Sac*I restriction sites, ori and selection marker (B) Amino acid sequences of MerE polypeptides in Gram positive and Gram negative bacteria.


Fig. 5. (A) SDS-PAGE of native protein MerE and fusion partner TrpΔLE after cleavage by thrombin. L: ladder (kDa). C: cleavage by thrombin. F1-F5: fractions of native MerE from Sephacryl S-200 column. (B) SEC chromatogram showing peaks of MerE and TrpΔLE.

Fig. 6. Topological alignment of all amino acids of MerE separated according to the predicted hydrophobic elements generated by TMHMM software.

Fig. 7. Detoxification of Hg^{2+} by mercury resistant bacterial strains (p<0.05).
Table 1 Growth of bacterial strains at different concentrations of HgCl$_2$.

<table>
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<tr>
<th>Isolates</th>
<th>Growth against HgCl$_2$ (µg/mL)</th>
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<td>AZ-2</td>
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<td>ZA-15 (-ve control)</td>
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<td>MB1 (+ve control)</td>
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Fig. 1 Evolutionary relationships of different Bacillus sp. on the basis of 16S rDNA ribotyping.

- Bacillus sp. (JQ691603)
- Bacillus thuringiensis (KP306751)
- Bacillus cereus (LK392517)
- Bacillus cereus AZ-2 (KT270478)
- Bacillus cereus AZ-3 (KT270479)
- Bacillus cereus (KP202304)
- Bacillus cereus (KR303714)
- Bacillus cereus (KP407139)
- Bacillus sp. AZ-1 (KT270477)
- Bacillus sp. (JN593078)
- Bacillus sp. (GU566355)
- Bacillus anthracis (HQ200405)
Fig. 2 Evolutionary relationship of different Bacillus sp. on the basis of merA gene was inferred using the Neighbor-Joining method with the sum of branch length 0.53623375. The branch lengths and the evolutionary distances used to infer the phylogenetic tree are in the same units with the bootstrap test value 500. The analysis involved 17 nucleotide sequences and codon positions included were 1st+2nd+3rd+Noncoding. A total of 1279 positions were in the final dataset with no gaps and missing data. Evolutionary analyses were performed by MEGA 5 software [28].
Fig. 3 (A) Linear and circular forms of nucleotide and amino acid sequences of pHLV plasmid with merE gene. T7_PR, T7 promoter; T7_TT, T7 terminator; His-tag_trpΔLE, fusion partner; HindIII and SaeI, restriction enzyme sites; ori, replication (B) Amino acid sequences of MerE polypeptides in Gram positive and Gram negative bacteria.
Fig. 4 SDS-PAGE of the expressed MerE fusion protein in E. coli strain C43(DE3). L: Molecular weight (kDa) ladder. S: supernatant. IB: Inclusion bodies. FT: Flow through. W: Washing. E1–E5: Elution fractions from Ni-NTA column. Lane 1 was showing AccuRuler pre-stained protein ladder (www.lamdabio.com). After the 7-hour of induction period, the cells were lysed by sonication and centrifuged. After centrifugation, the supernatant containing approximately the total cellular protein except inclusion bodies which was discarded is shown in lane 2 of a 12% SDS-PAGE. Lane 3 contained the inclusion bodies solubilized in binding buffer and lanes 4 and 5 showed the soluble fraction (flow through) and washing of other proteins with washing buffer except targeted fusion protein respectively. The lanes 6–10 showed that the insoluble fraction (inclusion bodies) consists primarily of the fusion protein of interest.
Fig. 5 (A) SDS-PAGE of thrombin cleavage MerE fusion protein and purified native MerE. L: Molecular weight (kDa) ladder. C: cleavage of fusion protein. F1–F5: Purified fractions of MerE from FPLC column. (B) FPLC chromatogram showing peaks of native MerE and fusion partner Trp∆LE.
Fig. 6 Topological alignment of all amino acids of MerE separated according to the predicted hydrophobic elements generated by TMHMM software.
Fig. 7 Detoxification of Hg\textsuperscript{2+} by mercury resistant bacterial strains (p<0.05).