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Molecular Characterization of Mercury Resistant Bacteria Isolated from Tannery Wastewater

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ABSTRACT

Mercury resistant (Hg^R) bacteria were isolated from heavy metal polluted wastewater and soil, collected from the proximity of some tanneries from Kasur, Pakistan. Three out of 30 bacterial strains were screened out on the basis of resistance level against various concentrations of $HgCl_2$. Bacterial isolates AZ-1, AZ-2 and AZ-3 showed resistance up to 40 µg/mL of $HgCl_2$ and mercury sensitive (Hg^S) isolate ZA-15 was taken as a negative control. 16S rDNA ribotyping and phylogenetic analysis were performed for the characterization of isolates as *Bacillus* sp. AZ-1 (KT270477), *B. cereus* AZ-2 (KT270478), *B. cereus* AZ-3 (KT270479) and *Enterobacter cloacae* ZA-15 (KJ728671). Phylogenetic relationship on the basis of *mer*A nucleotide sequence confirmed 51-100% homology with the corresponding region of the *mer*A gene of already reported mercury resistant Gram positive bacteria. Restriction fragment length polymorphism (RFLP) analysis was applied to the amplification products of 16S rRNA and *mer*A genes and a specific restriction patterns was successfully obtained after treatment with different endonucleases. A small scale reservoir of Luria Bertani (LB) medium supplemented with 30 µg/mL of $HgCl_2$ was designed to check the detoxification ability of the selected strains. The results demonstrated 83% detoxification of mercury by both *B. cereus* AZ-2 and *B. cereus* AZ-3, and 76% detoxification by *Bacillus* sp. AZ-1 ($p<0.05$).

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Authors' Contribution
AA designed and executed the experiments. ZL designed the experiments and supervised the work. AA executed the experimental work and analyzed the data. MS proofread the manuscript.

Key words
***Bacillus* spp., Hg-detoxification, RFLP, 16S rRNA, *mer*A.**

INTRODUCTION

Mercury (Hg) pollution is a global problem due to its toxicity to human, plants and animals. In the environment, the level of mercury pollution is increasing day by day due to the natural and anthropogenic sources. Natural sources include volcanoes, forest fires, cinnabar (ore) and fossil fuels whereas anthropogenic activities include the release of industrial waste from chlor-alkali industries, metal-mining, emissions from coal-using power plants and incineration of municipal and medical waste (Steenhuisen and Wilson, 2015). Mercury occurs in two forms as ionic and organic in the environment which cause cytotoxicity and neurotoxicity to humans and animals (Santos-Gandelman *et al.*, 2014b).

Bacillus sp. RC607, first time identified to have chromosomal resistance against mercury, was isolated from contaminated site, Boston Harbor, USA (Sedlmeier and Altenbuchner, 1992). In later studies, 74 *Bacillus* spp. isolated from Minimata Bay sediment, Japan and other bacterial isolates from Russian environment showed nucleotide sequences like the strain RC607. It was suggested the possible worldwide distribution of RC607 *mer* operon. Plasmids containing *mer* operon were found in several *Bacillus* strains analyzed in the Russian study (Nakamura and Silver, 1994; Bogdanova *et al.*, 1998).

Microorganisms have evolved mercury detoxification systems against mercury containing compounds due to the existence of mercury in the environment and its toxic effects. Bacteria showed the potential to detoxify the toxic form of mercury (Hg^{+2}) into non-toxic elemental form (Hg^0) through a cytoplasmic enzyme, mercuric reductase which was encoded by *mer*A gene of *mer* operon (Lund *et al.*, 1986). 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 (Moore *et al.*, 1990). 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 (Huang *et al.*, 1999).

Bacterial 16S rRNA possesses mosaic composition

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of phylogenetically conserved and variable regions which make this gene a common target for taxonomic purposes. The molecular technique in which difference in DNA sequences is detected by the presence of fragments of different lengths after digestion of the DNA samples of interest with specific restriction enzymes is known as restriction fragment length polymorphism (RFLP). A 16S rRNA-RFLP method for the identification of *Arcobacter* species has previously been described by Figueras *et al.* (2008). Many researchers have targeted the 16S, 23S rRNA and *mer*A genes for RFLP analysis in order to identify species belonging to a variety of genera and species (Jeng *et al.,* 2001). The effects of stress and genetic parameters like genetic diversity, gene transfer and evolution could be evaluated by mercury resistance determinants. Osborn *et al.* (1997) have employed *mer*A gene to check the diversity in Gram positive mercury resistant determinants because of its key role in mercury detoxification system.

Keeping in mind the available information about Hg-resistant bacteria and their potential to detoxify mercury, the current study is designed to determine the detoxification efficiency of selected Hg-resistant bacteria isolated from mercury-contaminated sites at lab scale. Furthermore, characterization and diversity of selected Hg- resistant bacteria is determined on the basis of RFLP analyses of 16S rRNA and *mer*A genes.

MATERIALS AND METHODS

Isolation and screening of Hg^R bacteria

Mercury resistant bacteria were isolated from wastewater and contaminated soil samples obtained from some tanneries around Kasur city of Pakistan. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were prepared from 1% of the original water and soil samples. For the isolation of individual colonies of Hg-resistant bacteria, LB agar plates supplemented with different concentrations of $HgCl_2$ ranging from 1-40 µg/mL were spread by 100 µL of 10^{-3} and 10^{-4} dilutions. All the plates were placed in an incubator set at 37°C for 24 h. After incubation, the isolated Hg-resistant bacterial colonies were selected and then re-streaked on new LB agar plates without $HgCl_2$ to get purified colonies (Amin and Latif, 2017a). All the purified Hg-resistant bacterial cultures were stored as glycerol stocks at -80°C.

PCR-amplification of 16S rDNA and merA gene

The bacterial isolates AZ-1, AZ-2 and AZ-3, showing high resistance against $HgCl_2$ were characterized by 16S rDNA ribotyping. The bacterial genomic DNA was extracted by Pure Link® Genomic DNA Mini Kit (www.lifetechnologies.com). The universal primers were used for the amplification of 16S rRNA gene; 16S-F (5′AGAGTTTGATCCTGGCTCAG3′) and 16S-R (5′AAGGAGGTGATCCAGCCGCA3′) (Normand, 1995) and for *mer*A gene; merAF: 5'TGGGTGGAACTTGCGTTAA3' and merAR: 5'TTATCCAGCACAGCAAGATA3' using BIOER XP-Thermal Cycler. The amplification conditions for both genes were set as 35 cycles of a denaturation step (94°C, 1 min), an annealing step (55°C, 1 min) and an extension step (72°C, 2 min) with a final 10 min chain elongation at 72°C (Amin and Latif, 2017b; Amin *et al.,* 2019). 16S rRNA gene sequencing was carried out by Macrogen sequencing core facility, Seoul, Korea. The obtained sequences were checked for homology through nucleotide blast and 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 *mer*A 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 were same as used to infer the phylogenetic tree.

RFLP analysis of 16S rRNA and merA genes

PCR products of partially amplified 16S rRNA and *mer*A genes were subjected to RFLP. Two restriction endonucleases *Eco*R1 and *Taq*1 were used for 16S rRNA gene while *Hin*F1 and *Hae*III (*Bsu*R1) were used for *mer*A gene. The reaction mixture contained 3.0 µL of 1X buffer (R-buffer for *Hae*III (*Bsu*RI) and *Hin*F1, and unique-buffer for *Eco*R1 and *Taq*1), 15.0 µL PCR products (approximately 1.0 µg), 1 µL of specific endonuclease and 11 µL of deionized water with total volume of 30 µL. The reaction mixtures were incubated at their specific temperatures as recommended by Fermentas restriction enzymes buffer activity chart (www.fermentas.com). The restriction fragments were separated along with 100bp plus DNA ladder on 1.5% w/v agarose gel.

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

For the detoxification/reduction of toxic ionic mercury (Hg^{+2}) into less toxic elemental Hg^0, 5 mL of LB medium was inoculated with each 5 µL of Hg-resistant bacterial glycerol stock of AZ-1, AZ-2 and AZ-3 and ZA-15 (Hg-sensitive, used as -ve control). After overnight incubation at 37°C, 1.5 mL of each starter culture (O.D. 2.0 at 600 nm) was taken to inoculate four flasks (4^{th}

flask was taken as –ve control), each containing 30 mL of LB medium supplemented with 30 µg/mL of $HgCl_2$ in triplicate. All flasks were incubated at 37°C for 24 h at 120 rpm of agitation. After incubation, the pH of culture medium for each strain was adjusted to 0.4 by adding concentrated H_2SO_4 and then cultures were spun at 12000 × *g* for 15 min. After centrifugation, the supernatant was separated and then transferred to separating funnel. To the cooled supernatant, 4 mL of 6N acetic acid and 2.5 mL chloroform were added and vigorously shaked for 1 min. The phases were allowed to separate completely and took out the chloroform layer and discarded. Freshly prepared 5 mL of 0.001% dithizone solution was added in the remaining solution of each strain and again shaked vigorously for 1 min. The layers were allowed to separate by putting cotton in the tips of separating funnels and eluted the dithizone-Hg^{+2} complex. The elemental mercury (Hg^0) was vaporized due the reduction ($Hg^{+2} \rightarrow Hg^0$) by selected Hg-resistant bacteria through mercuric reductase (encoded by *mer*A gene). On the reduction of all given concentration of Hg^{+2} to Hg^0, no significant color was produced with dithizone. In the presence of Hg^{+2}, an orange color was observed in the organic layer and the color became more pronounced with higher concentration of Hg^{+2}. The optical density (O.D.) of water–free chloroform extracts of each strain was determined at 500 nm against reagent blank (dithizone) to estimate the detoxification of LB medium (Elly, 1973; Humaira *et al.*, 2005).

Statistical analysis

All data of small scale Hg-detoxification experiment were subjected to mean ± standard deviation. The analysis of variance (ANOVA) was performed by using statistical package for social sciences (SPSS) windows V.20 software.

Table I.- Growth of bacterial strains at different concentrations of $HgCl_2$.

Isolates	Growth against $HgCl_2$			
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL
AZ-1	+	+	+	+
AZ-2	+	+	+	+
AZ-3	+	+	+	+
ZA-15	-	-	-	-

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 (Chang and Huang, 1998). 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 resistance to higher $HgCl_2$ concentrations (MIC: upto 1-40 µg/mL) and one strain ZA-15 was taken as a negative control (Table I). In literature, 100 µM, 50 ppm, 100 µg/mL and 20 µg/mL were found to be the MIC for mercury in *Bacillus* spp. (Sathyavathi *et al.*, 2013; Santos-Gandelman *et al.*, 2014a; Dash and Das, 2015; Amin and Latif, 2017).

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), *B. cereus* (KT270478), and *B. cereus* (KT270479), respectively. Other close matches to *Bacillus* sp. (KT270477) included *B. anthracis* HQ200405 and *Bacillus* sp. JN593078 and GU566355. The bacterial isolates also showed similarity (97%) among themselves. In the same clade, *B. cereus* AZ-1 and AZ-2 showed 97% 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 *mer*A gene showed 100% homology with already reported sequences of *mer*A genes in *B. cereus* NG034957, *B. megaterium* NG035056, *Bacillus* sp. LC015492, *Paenibacillus* sp. LC015492. Likewise, *B. macroides* Y09906 showed 99% homology, *Bacillus* sp. RC607 AF138877 and *Clostridium butyricum* AB024961 as 88%, respectively by *B. cereus* AB066362 as shown in Figure 2.

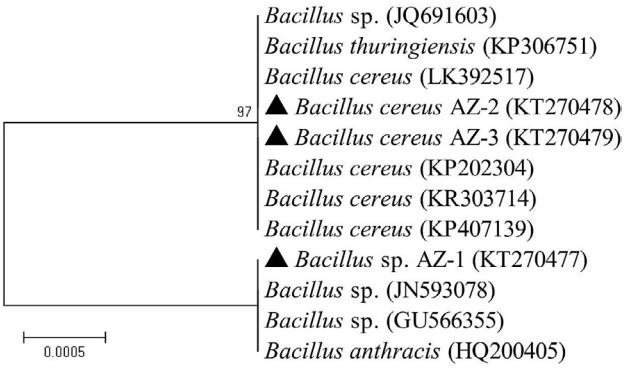

Fig. 1. Evolutionary relationships of different *Bacillus* sp. on the basis of 16S rDNA ribotyping.

16S rRNA gene is an excellent molecular marker that is widely being used for the study of bacterial community composition due to the possession of variable regions in its nucleotide sequence. Many researchers such as Osborn *et al*. (1997), Narita *et al*. (2003) and Scheidegger *et al*. (2009) described PCR-RFLP based analysis of 16S rRNA

and *mer*A genes to find the genetic variability and diversity in bacterial community. In the present study, selected bacteria already characterized by 16S rDNA ribotyping were further characterized and confirmed at species level by RFLP analyses of both 16S rRNA and *mer*A genes. PCR-amplified products of 16S rRNA (≈ 1.5 kb) and *mer*A (≈ 1.3 kb) genes of four bacterial strains were digested by *Eco*R1 and *Taq*I, and by *Hin*F1 and *Hae*III, respectively. Different restricted fragments of 16S rRNA gene showing different sizes of four bacterial strains, AZ-1, AZ-2, AZ-3 and ZN-15 were resolved on 1% agarose by comparing with 100bp plus DNA ladder. Similarly, restriction pattern of *mer*A gene of three bacterial strains, AZ-1, AZ-2 and AZ-3 (except ZN-15 used as a negative control for *mer*A gene) was observed on 1% agarose gel. The number and size of bands of both genes after treatment with restriction endonucleases varied on agarose gel as shown in Figure 3A and 3B. It was resulted from PCR-RFLP experiment that selected Hg-resistant bacteria belonged to same genera as Gram +ve *Bacillus* on the basis of restriction pattern of 16S rRNA gene except *E. cloacae* ZN-15 (Gram -ve). Likewise, RFLP analysis of *mer*A gene amplified from characterized bacterial strains confirmed the presence of Gram +ve *mer* operon due to the similar digestion pattern.

Review of literature showed that certain species such as cyanobacteria, *Klebsiella aerogenes*, *K. aerogenes* NCTC418 and *Pseudomonas putida* were involved in bio-reduction of toxic mercury compounds under laboratory conditions (Glendinning *et al.*, 2005; Oehmen *et al.*, 2009). The species of *Enterobacter*, *Cronobacter* and *Pseudomonas* showed the detoxification potential of mercury up to 95% in yeast extract medium (YEM). Nakamura *et al.* (1999) reported the decrease of mercury from various mercury compounds like mercuric chloride ($HgCl_2$) as 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-chloromercuric benzoate (p-CMB) 92.3% by *Pseudoalteromonas haloplanktis* strain M-1.

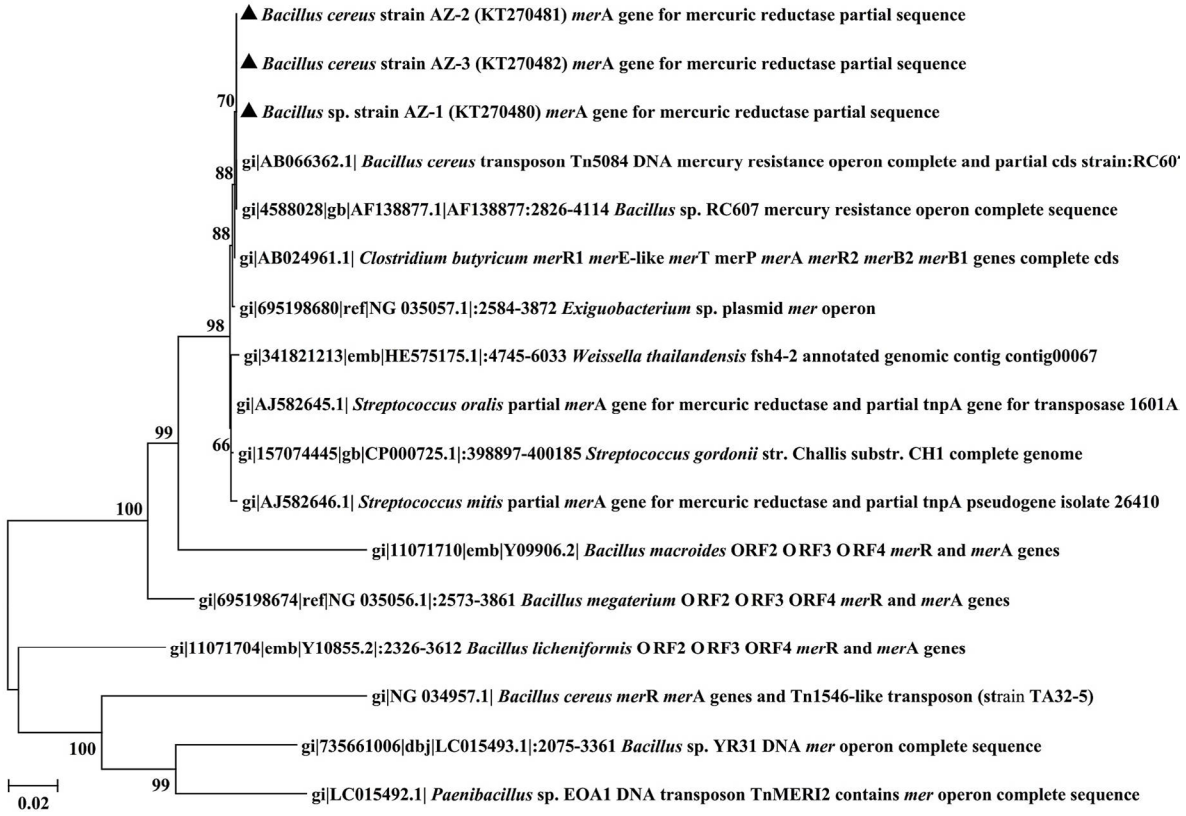

Fig. 2. Evolutionary relationship of different *Bacillus* sp. on the basis of *mer*A gene was inferred using the Neighbor-Joining method with the sum of branch length 0.53623375. The branch lengths and the evolutionary distances used to infer the phylogenetic tree are in the same units with the bootstrap 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 (Tamura *et al.*, 2004).

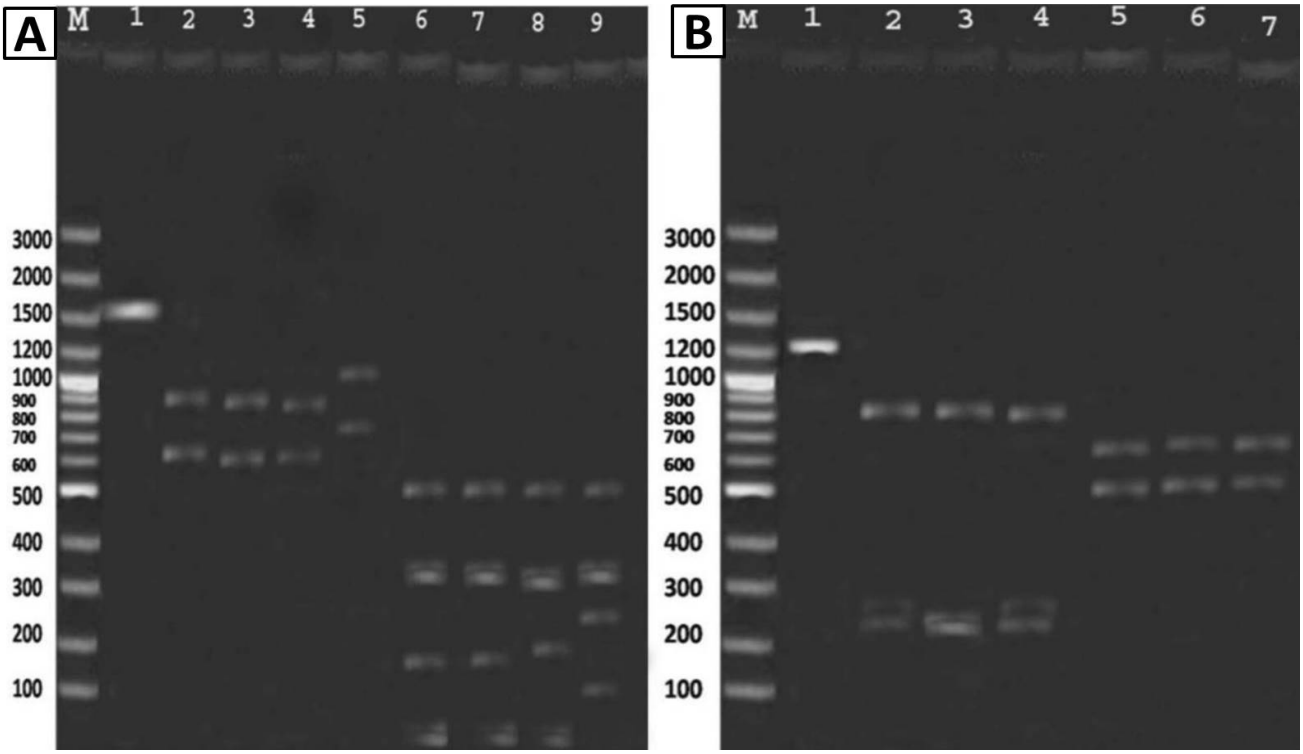

Fig. 3. A, digested patterns for the 16S rDNA fragment from bacterial strains (Hg^R), *Bacillus* sp. AZ-1, *B. cereus* AZ-2, *B. cereus* AZ-3 and *E. cloacae* ZN-15 (Hg^S) (Lane M, size markers of 100 bp plus DNA Ladder; Lane 1, 1543bp PCR product of 16S rDNA fragment; Lanes 2-5, digested patterns of the 16S rDNA fragments by *Eco*R1; Lanes 6-9, digested patterns of the 16S rDNA fragments by *Taq*1); B, digested patterns for the *mer*A gene from bacterial strains (Hg^R), *Bacillus* sp. AZ-1, *B. cereus* AZ-2 and *B. cereus* AZ-3 (Lane M, size markers of 100bp plus DNA Ladder; Lane 1, 1289bp PCR products of *mer*A gene; Lanes 2-4, digested patterns of the *mer*A gene by *Hin*F1; Lanes 5-7, digested patterns of the *mer*A gene by *Hae*III).

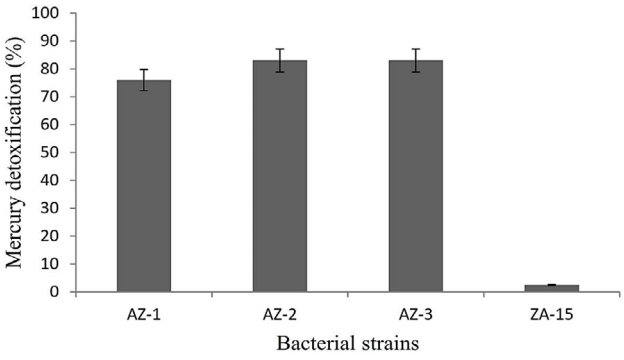

Fig. 4. Detoxification of Hg^{2+} by mercury resistant bacterial strains ($p<0.05$).

The bio-sorption capability of the immobilized *B. cereus* cells was described by Sinha *et al.* (2012) as 104.1 mg/g Hg^{2+} at 30°C and pH 7.0, biomass concentration of 0.02 g/L and contact time of 72 h. In this study, a lab scale experiment was designed to analyze the detoxification potential of mercury by selected bacterial strains. The results indicated 23 (76%), 25 (83%), 25 (83%) and 1 (2.5%) µg/mL detoxification of Hg^{2+} out of 30 µg/mL (100%) by the selected bacterial strains as *Bacillus* sp. AZ-1, *B. cereus* AZ-2, *B. cereus* AZ-3 and *E. cloacae* ZA-15, respectively (Fig. 4).

CONCLUSION

In the present study, the detoxification potential of Hg-resistant bacteria, *Bacillus* sp. AZ-1, *B. cereus* AZ-2 and *B. cereus* AZ-3 was checked (*E. cloacae* ZA-15 used as –ve control) and then their molecular identification and phylogenetic analysis were described on the basis of 16S rDNA ribotyping and merA gene sequence. Furthermore, the nucleotide sequence variations in 16S rRNA and *mer*A genes of selected bacteria were determined by RFLP analyses. The results showed that mercury resistant bacterial strains, *B. cereus* AZ-2 and *B. cereus* AZ-3 isolated from Kasur city of Pakistan can be used for the Hg-detoxification of industrial effluents due to the presence of *mer*A gene and its manipulation into mercuric reductase

expression system of bacteria.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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