Quantification of plasmid isolated resistant *Pseudomonas aeruginosa* present in heavy metal soil as biomarkers

Nadia M. T. Jebril

Department of Biology, College of Sciences for Women, University of Babylon - Iraq

Abstract— **Microorganisms have chromosomal, transposon, and plasmid-mediated resistance systems to survive in contaminated environments. Therefore, this study aimed to isolate *Pseudomonas aeruginosa* from heavy metal-contaminated environments and investigate whether their plasmid's DNA are responsible for the metal resistances, which could be used as biomarkers of heavy metal pollution. Plasmid detection was carried out to confirm whether the genes for resistance were encoded by plasmid DNA or not. Inductively coupled plasma-mass spectrometry (ICP-MS) and Energy X-Ray Fluorescence (EXRF) were used to analyse the heavy metal concentrations in the collected soil. A CHN analyser was used for TC, TOC, and TIC estimates. Procedural blanks, standard solutions and certified reference materials were used to verify precision and accuracy. A total of 28 isolates of *P. aeruginosa* resist to cadmium (*P. aeruginosa* Cd5), lead (*P. aeruginosa* Pb2.3), mercury (*P. aeruginosa* Hg30) and arsenic (*P. aeruginosa* As3.3) were isolated. Their minimum inhibitory concentration (MIC) of Cd, Pb, Hg and As were 5 mM Cd, 30 µM Hg, 2.3 mM Pb and 3.3 mM As, respectively. The concentration of heavy metals collected soil were 93 Pb, 19 As, 1.3 Cd and 0.0 Hg, 16 Mo, 56 Zr, 37 Sr, 13 U, 63 Rb, 17 Th, 78 Zn, 61Mn, 0.0 Cr, 59 V, 76 Sc, 11530 Fe, 532 Ca, 765 K and 2134 S (µg/g soil). There were relationships between MIC abilities of all isolated *P. aeruginosa* apart from three isolates were resist to 30 µM Hg which isolated from soil did not have any concentration of Hg. It is concluded that *P. aeruginosa* isolated from heavy metal-contaminated environments were mainly contained plasmid DNA responsible for resistances and thus can be used as a biomarker for any environmental contamination and monitoring environments.**

Keywords — **Heavy metal, metal resistant plasmid, *P. aeruginosa*, plasmid DNA.**

I. INTRODUCTION

Heavy metals such as cadmium, mercury, arsenic and lead are highly relevant environmental contaminants worldwide due to industrial, agricultural activities leading to water bodies and soils[1],[2]. Due to their toxicity, metals are thriving in the environments and ecosystems. Microorganisms play an important role in the geochemical cycles of heavy metals for transformation and mineralisation naturally. These microorganisms evolved a different mechanism to survive under extreme metal environmental conditions, including efflux system, biosorption, precipitation, extracellular sequestration and sequestration [3]. Microorganisms have many genetic systems for maintaining their resistances against heavy metals through chromosomal, transposon, and plasmid-mediated resistance systems [4]. Genes are always the response of resistances located in an operon, and the operons are generally encoded either on plasmid DNA [5] or normally on genomic DNA. There are differences between chromosomal and plasmid-based metal resistance systems[5]. Essential metal resistance systems are usually chromosome-based and more complex than plasmid systems [6]. Studies of adaptive responses usually contain studying phenotypic characters. A simpler understanding of adaptation uses a molecular technique to aim for a particular genetic determination and useful biomarkers of bacterial responses to metal environmental conditions and reveal the quantification of the plasmid of adaptation [7]. *Pseudomonas* sp. was almost isolated from soils and water contaminated with metals and their resistant mechanisms and system well-known [8] [9] [10]. Cai, Salmon [11] found chromosomal *ars* operon responsible for resistance to arsenic in *P. aeruginosa*, while be found plasmid response of resistance to chromate in *P. aeruginosa* [12]. As plasmids in gram-negative *Bacteria* could contribute resistance to more than one heavy metal [7], using selective media for growing and isolating metal-resistant plasmids from *Pseudomonas* sp. that isolated from metal contaminated soils would be used as a biomarker for metal occurring in an environment. This biomarker gives an idea of the resistance ability of the population living naturally within heavy metal contaminated soils. For this, the study aimed to examine if the isolated metal resistant *P. aeruginosa* had plasmid responsible for the resistance mechanisms to Cd, Hg, Pb and As. Then, to find out the relationship of plasmids with their MIC levels and the concentrations of the heavy metals in the environment as a biomarker tool for heavy metal contamination.

II. MATERIALS AND METHODS

A. Collection of soil sample

For further experiments, one kilogram of surface soil contaminated with heavy metals was collected in sterilised plastic bags and stored in a refrigerator at 4 °C.

B. Sample preparation of all analysing ICP-MS, XRF and CHN analyser

The soil contamination with heavy metals in Dartmoor, Devon, Southwest England, UK, was well-known due to the mining activities. Therefore, the heavy metal levels of this soil were investigated. The samples were homogenised and dried at 50-60 °C for 72 h, crushed using a mortar and pestle, then sieved by 180 µm particles sizes to be ready for analysis using ICP-MS, XRF and elemental analyser. Samples were prepared to be the same species as the certificated reference materials

(CRMs) to certify particle size between the CRMs and samples throughout the measuring analysis.

C. Heavy metal analysis

Fig. 1 shows the typical sample preparation (*aqua-regia* digests). General weighing of soils was put in a glass tube, and HNO_3 1% was added to the soil for the digestion using a microwave digestion system at 60 °C for 120 minutes. The resultant solution of digested soils was transferred into a volumetric flask by filtration and diluted up the glass with HNO_3 1% for measurement [13]. ICP-MS was used to determine the concentration of heavy metals and gold (1 ml) and added to all samples (50 ml) to avoid the volatilisation of mercury. Standard solutions of As, Pb, Cd and Hg were prepared for the range of 0, 10, 40, 100, and 400 ppb using deionised (18MΩ) water were, a mixed standard solution was used to determine the other elements. Reference soils were used to evaluate the digestion the measuring and use procedure blank that did not have soil, just acid digested along with all samples. Also, XRF was used to measure the heavy metals without any preparation of sieved soil by using CRM for low and high values.

Fig. 1. Sample digestion (*aqua-regia*)

D. Total organic carbon analysis

Elemental analyser EA 1110 CHNS was used for total organic carbon (TOC) analysis. To measure TOC, 100 mg of sieved soil in the ceramic crucible was acidified by adding 1 mL of HCl (10%) and leaving for 24 hours to eliminate all carbonates. Then the liquid from the soil was removed using pipettes, and the soil was finally washed with Milli-Q water and dried completely at 60 °C. At the same time, the direct analysis was subjected to the soil without acidification to determine the total carbon (TC). The content was measured depending on the weight of soil in tin capsules (6mmx4mm pressed, OEA laboratories Ltd) before measuring with the minimum 2 mg and maximum. L-Cystine (C= 29.99%, H= 5.03%, N= 26.63% and S= 26.69%, OEA laboratories Ltd) was used as standard. CRM of soil standard, elemental microanalysis OAS, was used as reference soil. All samples in tin capsules were placed in the Elemental analyser for measuring (Fig. 2). Total inorganic carbon (TIC) was determined by the difference between TC and TOC [14].
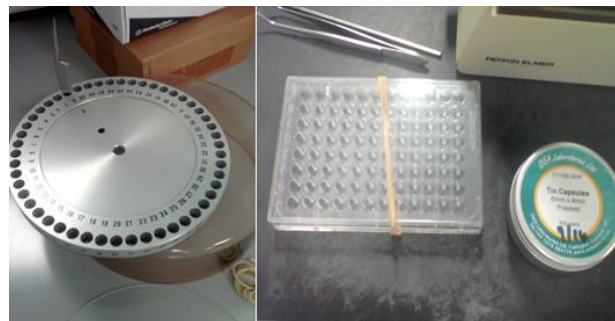

Fig. 2. Sample disc in Elemental analyser.

E. Isolation of P. aeruginosa

P. aeruginosa was isolated from soil separately using a proposed enrichment method. A half gram of soil was dissolved in 50 ml of EBS broth [15] in Erlenmeyer-flasks (250 mL). These flasks were incubated at 44 °C until getting visible turbidity growth. Once bacterial growth was detected, 5 ml of supernatant from each previous incubated soil culture was transferred into 50 mL of EBS broth supplemented with fructose (10 mM) and incubated at 44 °C for 24 hours. After five enriching cyclings, MIC was determined of enrichment culture by growing with Cd, Pb, As, and Hg to isolate *P. aeruginosa* resist Cd, Pb, As and Hg. Concentrations of metals were started from 1 μM until getting no growth at a specific concentration which was obtained. This concentration was considered the maximum concentration that b*acteria* can resist and grow within heavy metals. Cetrimide agar isolated *P. aeruginosa* from cultures resistant to heavy metals. EBS agar supplemented with their MIC concentrations was used to confirm the MIC of each isolate of *P. aeruginosa* . Cetrimide agar was also finally used to isolate and purify *P. aeruginosa* again.

F. Plasmid DNA

Each isolate of *P. aeruginosa* was grown in 50 ml of ceramide broth, and the cells were harvested using centrifugation. Pellets were resuspended in TE buffer (1x concentration) [16] to get the optical density (*OD*) of the cell at 440 to 0.5. Plasmid DNA was extracted according to [2].

G. Observation and quantification of the plasmid DNA

Plasmid DNA was observed via agarose gel electrophoresis, according to [17]. 1 x TAE buffer was used in running an agarose gel and frequently replaced after four gel running times. The loading buffer in the ratio 2:1 was mixed with DNA samples for loading into the gel. Electrophoresis was run at 60 volts for 60 minutes. Gels were visualised under UV Dog using a gel imaging system. The quantity of plasmid DNA was estimated using the plasmid DNA ladder (1 kb) run along with plasmid DNA samples to determine the size of plasmid DNA by lab image software.

III. RESULTS AND DISCUSSION

A. The concentration of heavy metals

The concentrations of heavy metals were 93 Pb, 19 As, 1.3Cd, 0.0 Hg, 16 Mo, 56 Zr, 37 Sr, 13 U, 63 Rb, 17 Th, 78 Zn, 61 Mn, 0.0 Cr, 59 V, 76 Sc, 11530 Fe, 532 Ca, 765 K and 2134 S (µg/g soil) (Fig 3).
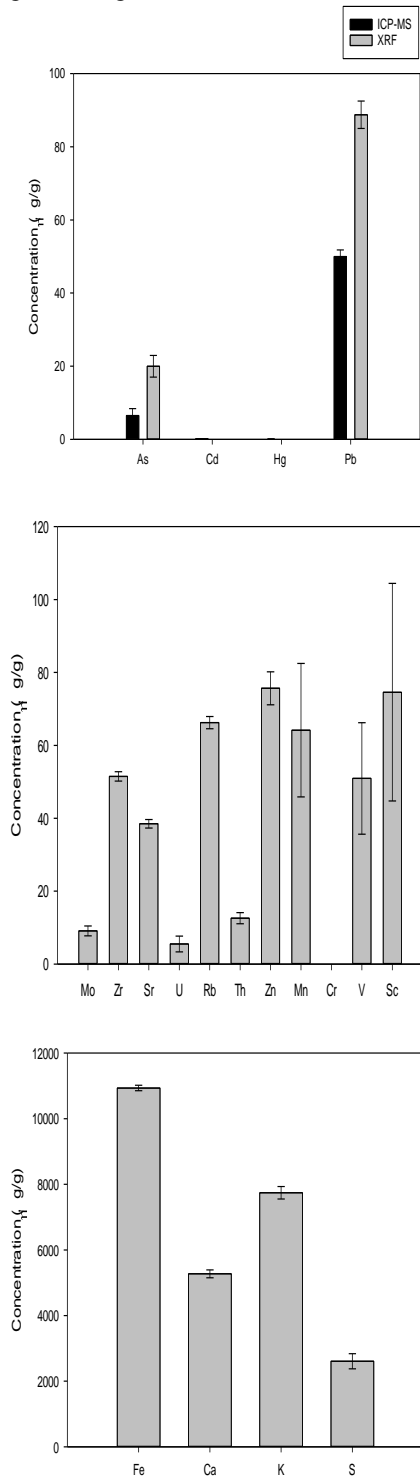

Fig. 3. The concentrations of heavy metals recorded in the soil sample were measured by ICP-MS and XRF. Error bar represents the standard error of the mean ($n = 3$).

B. Organic content in the soil sample

It's important to know the content of organic contamination in samples as an indicator of the level of pollution. Organic contents in soil were measured by CHNS elemental analysis. Soil contains two different forms of carbon: total organic carbon (TOC) and total inorganic carbon (TIC). The results showed an equal amount of TC and TOC, 39% and 37%, respectively. These data gave TIC to 2% (Fig.4).
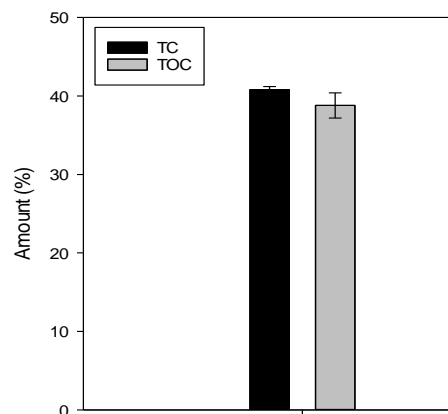

Fig.4. Amounts of TC and TOC were measured by CHNS elemental analysis. Error bar represents the standard error of the mean ($n = 3$).

C. Isolation of P. aeruginosa resistant to Cd, Hg, As and Pb

A total of twenty-eight metal resistant *P. aeruginosa* were isolated from the secondary screening process on EBS agar supplemented with Cd, As, Hg and Pb (Fig. 5). Twelve isolates resist Pb isolated at 2.3 mM, and nine isolates resist to As isolated at 3.2 mM. While fewer isolates, five isolates resist Cd (5 mM) and two isolates resist Hg (30 µM) were isolated from *P. aeruginosa*. These isolates were designated depending on the metal's name, MIC concentration, and isolate's number, as shown in Table 1.
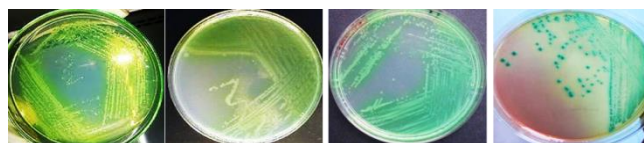

Fig. 5. Minimal inhibitory concentration (MIC) to Hg, Cd, As, and Pb among 28 isolates of *P. aeruginosa* in EBS agar supplemented with heavy metals. MICs were first determined in the broth assay as the forward concentration, which no growth obtained was considered MIC.

D. Using plasmid DNA as a biomarker

As suggested in this study, quantification of plasmid DNA could serve as a tool biomarker study in three expectations. Firstly, the presence of plasmid DNA induction on gel electrophoresis might serve as a biomarker for the response of *P. aeruginosa* to metal contamination in mining soil. This parameter could be used to expect how resistant bacteria

Table 1. The designate of isolated *P. aeruginosa* grown on EBS agar supplemented with 30 µM Hg, 2.3 mM Pb, 3.3 As and 5 mM Cd.

| Mercury resistant P. aeruginosa	Cadmium resistant P. aeruginosa	Arsenic resistant P. aeruginosa	Lead resistant P. aeruginosa
P. aeruginosa Hg1 30	*P. aeruginosa* Cd1 5	*P. aeruginosa* As1 3.3	*P. aeruginosa* Pb1 2.3
P. aeruginosa Hg2 30	*P. aeruginosa* Cd2 5	*P. aeruginosa* As2 3.3	*P. aeruginosa* Pb2 2.3
P. aeruginosa Hg3 30	*P. aeruginosa* Cd3 5	*P. aeruginosa* As3 3.3	*P. aeruginosa* Pb3 2.3
	P. aeruginosa Cd4 5	*P. aeruginosa* As4 3.3	*P. aeruginosa* Pb4 2.3
	P. aeruginosa Cd5 5	*P. aeruginosa* As5 3.3	*P. aeruginosa* Pb5 2.3
		P. aeruginosa As6 3.3	*P. aeruginosa* Pb6 2.3
		P. aeruginosa As7 3.3	*P. aeruginosa* Pb7 2.3
		P. aeruginosa As8 3.3	*P. aeruginosa* Pb8 2.3
		P. aeruginosa As9 3.3	*P. aeruginosa* Pb9 2.3
			P. aeruginosa Pb10 2.3
			P. aeruginosa Pb11 2.3
			P. aeruginosa Pb12 2.3
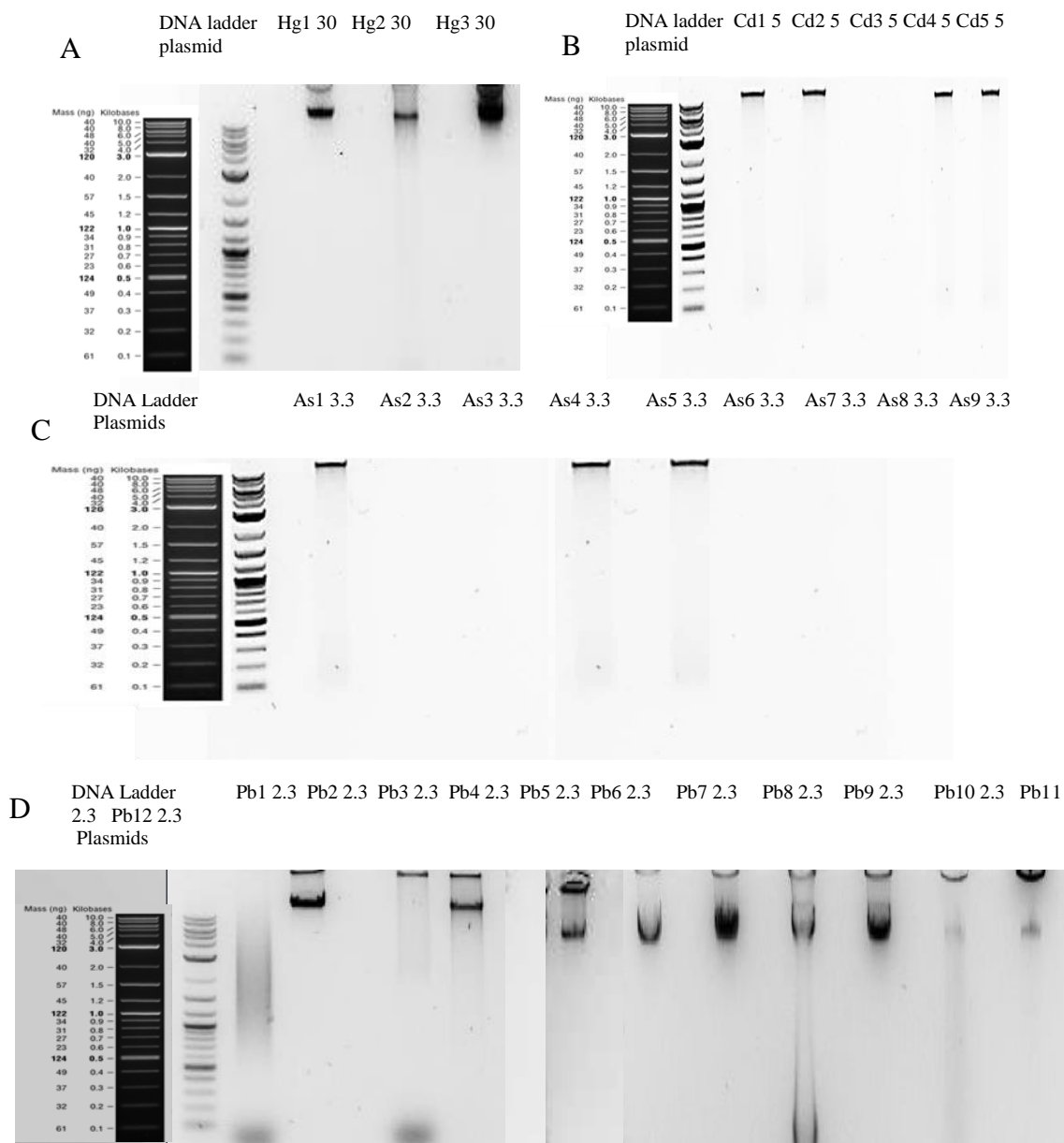
Fig. 6. Observation of plasmid DNAs of different isolated *P. aeruginosa* grown in EBS agar supplemented with (A) 30 µM Hg, (B) 5 mM Cd, (C) 2.3 mM Pb and (D) 3.3 As isolated from heavy metal contaminated soil on agarose gel electrophoresis.

respond to extreme heavy metals concentrations in their environment when subjected to other bacterial species. Second expectation, the observation of plasmid DNA in the gel of *P. aeruginosa* having the metal resistant gene might be used as a biomarker for indicating the level of heavy metal contamination in a given ecosystem, considering that metal resistant genes can be encouraged by these environmental stress factors. Thirdly, the weight of plasmid DNA induction on gel electrophoresis might serve as a biomarker if and via how much the detoxification capacity *P. aeruginosa* is resistant.

E. Plasmid and MIC relationships

All isolates carried one plasmid that responded for adaptations such as all isolates resist to Hg, however; few other resistant isolates for other metals (Cd3 5, As2 3.3, As3 3.3, As6 3.3, As7 3.3, As9 3.3, Pb1 2.3 and Pb3 2.3) did not carry a plasmid. This missing plasmid indicates that the isolates resisted Cd, As and Pb. The plasmids did not have the genes on plasmids for resistant mechanisms. The weight of the majority of plasmids within that range is either 10 to 12 Kb. Few plasmids were estimated to have a Kb less than 10 Kb, and none less than 6 Kbl were observed (fig 6). The MIC abilities of all isolated *P. aeruginosa* were related to the concentrations of heavy metals. However, the mercury concentration was zero, even though three isolates were resistant to 30 μM Hg, and this MIC is considered as good resistant ability as the highest mercury. This might of the volatilisation of mercury from the soil. MIC resistant *Bacteria* recorded is to 100 μM Hg (18). These results were similar to other studies in terms of MIC related to concentrations of heavy metals in soil (19), (7).

CONCLUSION

Soil contains heavy metals cadmium, arsenic, mercury and lead host resistant *P. aeruginosa* and this is used as a biomarker of contamination regarding its MIC and plasmid DNA for resistance. Further work could be done to study and examine the metal-resistant genes of plasmids in metal contaminated soil using molecular techniques such as DNA– DNA hybridisation, polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). This will help in establishing the ecological role of plasmid DNA elements in isolated *P. aeruginosa*: (*P. aeruginosa* Cd5), lead (*P. aeruginosa* Pb2.3), mercury (*P. aeruginosa* Hg30) and arsenic (*P. aeruginosa* As3.3).

REFERENCES

1. Khalil, A.H. (2017). Effectiveness of Carbonate Content on Removal of Cadmium from Contaminated Soil by Soil Washing Process. J of *Babylon Uni Eng Sci*; .(1) (**25**):20-29.

2. Al-Mansori, N.J. (2017). Develop and Apply Water Quality Index to Evaluate Water Quality of Shatt-Al-Hilla River. *J of Babylon Uni Eng Sci*. (2) (**25**):368-374.

3. Naji, H. F., Tawfiq, N. M. & Kuder, A. A. A. (2011) 'Bacteriological and Physiochemical Analysis of Drinking Water in Hilla City, Iraq', *The first international conference on agriculture and natural resources. Razi and Babylon universities*: 4-6.

4. Bruins M.R, Kapil S. and Oehme F.W. (2000). Microbial resistance to metals in the environment. Ecotoxicology and environmental safety;45(**3**):198-207.

4. Chudobova, D, Dostalova, S, Ruttkay-Nedecky B, Guran, R, Rodrigo M.A. M, Tmejova, K, et al. (2015). The effect of metal ions on Staphylococcus aureus revealed by biochemical and mass spectrometric analyses. *Microb Rese*. 170:147-56.

5. Jang , S. (2016). Multidrug efflux pumps in *Staphylococcus aureus* and their clinical implications. *J Microbiol*.;54(**1**):1-8.

6. Trevors, J, Oddie, K, and Belliveau, B. (1985). Metal resistance in bacteria. *FEMS Microbiol Lett*;32(**1**):39-54.

7. Abou-Shanab, R, Van Berkum, P, and Angle, J. (2007). Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of Alyssum murale. *Chemosphere*;68(**2**):360-7.

8. Baligarx, V.C. (2012). Mechanisms of nickel uptake and hyperaccumulation by plants and implications for soil remediation. *Adv Agron*. ;117:117-183.

9. Muneer, B, Iqbal, M.J, Shakoori, F.R, and Shakoori A.R. (2016). Isolation, Identification and Cadmium Processing of *Pseudomonas aeruginosa* (EP-Cd1) Isolated from Soil Contaminated with Electroplating *Industrial Wastewater. Pak J Zool*;48(**5**):231-239.

10. Deote, S, Ingle, A.B, and Magar, S.P.(2018). Study on antibiotic sensitivity of chromium tolerant bacteria isolated from chromite mine area. *J Pharm Res*.12(**3**):352-361.

11. Cai J, Salmon K, and DuBow, M.S. (1998). A chromosomal ars operon homologue of *Pseudomonas aeruginosa* confers increased resistance to arsenic and antimony in *Escherichia coli. Microbiol*;144(**10**):2705-29.

12. Cervantes, C, Ohtake H, Chu L, Misra T, and Silver S. (1990). Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. *J bacterial* ;172(**1**):287-91.

13. McBride M.B, and Spiers G. (2001). Trace element content of selected fertilisers and dairy manures as determined by ICP–MS. *Comm Soil Sci and Pla Ana*.;32(**1-2**):139-56.

14. Schumacher, B.A. (2002). Methods for the determination of total organic carbon (TOC) in soils and sediments. United States Environmental Protection Agency Environmental Sciences Division National .

15. Kelly, D, and Syrett P. (1964). The effect of uncoupling agents on carbon dioxide fixation by a *Thiobacillus. J gen microbiol*. 34(**2**):307-17.

16. Protocol CSH. Tris-EDTA buffer- Recipe. Cold Spring Harb Protocol. 2009.

17. Sambrook, J, and Russell DW. (2001).Molecular cloning: a laboratory manual.. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; 2001.

18. Essa, A.M, Macaskie L.E, and Brown N.L. (2005). A new method for mercury removal. *Biotech lett*.;27(**21**):1649-55.

19. Baya A, Brayton P, Brown ,V, Grimes, D, Russek-Cohen, E, and Colwell R. (1986). Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. *Appl Environ Microbiol*. 51(**6**):1285-92.