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Research Article

Separation and Molecular Identification of Resistant Bacteria to Lead from Behbahan Bidboland Gas Refinery Wastewater (Iran)

Azam Mehrbakhsh[1], Monir Doudi[1*] and Hossein Motamedi[2]

[1]Department of Microbiology, Falavarjan Branch,
Islamic Azad University, Falavarjan, Isfahan, Iran.
[2]Department of Biology, Faculty of Science,
Shahid Chamran University, Ahvaz, Iran.
Corresponding Author: Monirdoudi@yahoo.com OR Doudi@iaufala.ac.ir

ABSTRACT

Heavy metals are one of the pollution sources in environment. The pollution due to these metals is the problem that could have negative impact on water. Human is faced with these poisons effects due to occupational reasons. The lead is regarded as heavy metal whose industrial applications cause environmental pollution in high rate.The aim of this project was Separation and Molecular Identification of Resistant Bacteria to Lead from Behbahan Bidboland Gas Refinery Wastewater (Iran).

For this aim, 6 samples of water and refinery output wastewater is provided after sampling, pH, COD, BOD rates and lead metal primary concentration was determined in wastewaters, after this phase the concentration and separation of resistant bacteria was done to separate them from lead. In this phase the minimum inhibitory concentration was defined from heavy metal growth (MIC). To determine the bacteria identity first the evaluation method of bacteria morphologic and gram staining and then biochemical tests and finally colony–PCR molecular method was used and then separated bacteria 16S rRNA gen was identified. The obtained results from this research showed that among the separated bacteria from Behbahan Bidboland gas refinery wastewater, the *Providencia* genous had the most resistant toward Pb. The MIC rate for this bacteria was calculated at 1140 mg/l. the result of this study showed that separated bacteria in this wastewater seemed to have high resistant toward heavy metals due to long encountering to pollution sources and hence they could be proper factor for bioremediation process of heavy metals

Key terms: Separation, Identification, Bacteria, Resistance, Pb, 16S rRNA.

INTRODUCTION:

Heavy metals generally are called to metals that have density more than 5g/cm3, actually the heavy metal density is 5 times more than water. The most important of these elements include: Ni, Hg, Cu, Cd, Pb, Va, Zn, Sn, and other metals which are in contact to poisonous pollutions, Samanta, 2012. These metals are consierable due to their irresolvable feature and their physiological effects on life creatures in low concentrations.

From the metal connecting mechanism to the cell external surface we can point out to the electrical absorption, physical absorption or absorption

with covalent forces interference and chemical absorption, Rehman et al, 2007.

Today due to industrial development and entering polluted wastewaters from many different industries as leather making factories, mines excavation and industrial factories to environment, the environment around the factories and surface waters and underground waters is under the pollution danger that this issue will effect harmful effects in short and long run on living creatures as soil, plants and living beasts in such regions, Rehman et al, 2007. The chemical elements pollution is one of the main factors in environmental destruction. In the chemical pollution, heavy metals have special importance due to ecological and biological and sanitary effects, Gupta and Kumar, 2012.

Around the refineries and their surroundings there are many agricultural lands that their products are consumed by human and beasts and birds. Hence it is essential to study this pollution elimination, Krishna et al, 2012. The evaluations have shown that physical and chemical method as oxidization and redusing, chemical sedimentation, filtration, electrochemical treatment, vaporization, ion exchanging and reverse osmosis process have limitations and they are not economical, Lucius et al, 2013.

Microorganisms absorb some metals which are poisonous intensively from the environment and change them none-linear and none-poisonous and then save them and again release them to the external environment. For this aim, they use different mechanism that includes metal methylation, sulphureting and poisoning metal sedimentation indirectly, Ahemad, 2012.

Because metals are not cleaned due to their poisoning identity as heavy metals feature in environment, therefore the elemental identity is not remain the same because metals are not destructive neither by temperature nor through microbiological process.

As the result the metal elimination is difficult from environment in addition to the metal concentration that in environment essentially is not reversing the metal biological poisoning. Due to poisoning and metal extensive presence of metals in microbes environment reveals exclusive ways to come up with metals, Verma et al, 2000, Fillali et al, 2000.

Some of microorganisms have some mechanism for stabilization and fixing metals while some other in reality increase the metal solvability, Mair et al, 2000.

MATERIAL AND METHODS:

1-Sampling

In order to separate bacteria samples resistant to Pb from water and deep sediments which come from wastewater of a gas refinery in Behbahan Bidbolan, the sampling was performed in 500 meter distance from each other.

2-Wastwater and sediment analysis

After sampling of wastewater and sediments, analysis was done and below factors is evaluated, pH, TDS, TH, BOD, COD, and temperature

3-Lead measuring in wastewater and sediment phase

To determine the Pb concentration in wastewater and sediment samples, the atomic absorption measuring method was used. To run this test, first the sample was harmonized completely and transferred to the digesting 200 ml capacity balloon in 50 to 100 ml rate and 5 ml of nitric acid was added to the sample and it was evaporated slowly on the vapor bath. The digestion process continued to the phase of clarification as during digestion phase, it was not dried, when the sample rate reduced to 10 to 20 ml, the balloon wall was washed with water and the sample arrived to the intended volume. Then by the use of atomic observing system made by Fars Abshenasan Zagrous Company and the results were written for heavy metals.

4- Enrichment with resistant bacteria to Pb

First 4 Erlenmeyer flask were chosen in 250 ml capacity that 2 of them were for sediments samples and 2 of them were used for wastewater

samples of 1 and 2. In each Erlenmeyer flask 90 ml of NB environment was prepared and then 10 ml of wastewater samples were added to Erlenmeyer flask 1 and 2 and also to Erlenmeyer flask contained 1 gr of sediment sample, then 10 times more than the primary metal concentration which had been observed through atomic absorption will be added to each of them. It should be noted that other Erlenmeyer flask without the sample mixing will be considered as witness for the environment darkness comparison. To enrich resistant bacteria to metal flasks are kept for 3 to 4 days in 28 to 30 of centigrade inside the shaking incubator.

5- Microbial culture and purification of resistant bacteria to Pb (primary separation)

After 3 to 4 days when the environment is become dark, a sterile loop from each flask was grown on the (NA;Merck) environment 10 times of metal primary concentration in linear form then each pilot was incubated for 48 hours in 28 to 30 centigrade.

The grown colonies were compared based on the shape, size, surface, smell and brink and different colonies were grown in (NA;Merck) different environment and temperature was incubated for 48 hours in 28 to 30 °c. After above procedure each of samples were grown in (NA;Merck) environment including 50 to 1100 mg/ml concentration of metal with 100 distance in unit and also they were grown n growing environment including above metal, after the colony growth on one environment, samples were transferred to the higher concentration growing environment and there were cultivated again.

6-Selecting optimized strains for resistant Bactria to Pb

To determine the most resistant bactria stains to Pb, the MIC and MBC methods were use, Abouzeid et al, 2009.

7- Evaluation of metal absorption rate by resistant samples in experimental plan

In order to evaluate the capability of resistant samples to eliminate Pb from environment, first samples were grown in (NB;Merck) medium and after arriving to darkness to 1 ml rate in this environment, they were added to the next phase nutrient broth. For each of samples the NB growing medium was prepared about 100ml and then Pb was inserted to the samples which were resistant about. After adding 1 ml of grown bacteria, the growing medium was incubated for 3 days at 28 °c. Then the medium was rotated at 4000 rpm centrifugally for about 10 minutes and the surface liquid was removed and then it was measured by the atomic metal concentration absorption system.

8-Identification resistant bacteria to Pb

Identification of samples was done through frequent methods of microbiologic methods including staining and evaluation of colonies morphologies and biochemical tests based on the source book and also colony- PCR molecules method of 16S rRNA gen of separated bacteria were identified.

RESULTS:

Table 1: the wastewater and sediment chemical analysis from Behbahan and Bidboland gas refinery

Table 1: The sediment and wastewater analysis in Behbahan and Bidboland gas refinery

Temperature(c°)	COD(mg/l)	BOD(mg/l)	TH (mg/li $caco_3$)	pH	TDS(mg/l)	EC (ds/m)	Sewage type
28	134.50	66.10	1050	8.75	2700	4151	Wastewater
28	200.50	105	940.00	8.20	3675	5256	Sediment

Table 2: shows the available lead rate in wastewater

Table 2: The available lead rate in wastewater and sediment (mg/l) in Behbahan and Bidboland gas refinery

Pb(mg/l)	**Sewage type**
0.0030	Wastewater
0.0050	Sediment

Table3-Resistant Bactria strains to lead and evaluation resistance of each bacterium toward different concentration has been shown

The resistant tolerance rate toward lead in separated bacteria from sediments and wastewater in Behbahan and Bidboland gas refinery

BGR_{14}	BGR_{7}	BGR_{3}	BGR_{1}	BGR_{4}	BGR_{11}	BGR_{5}	BGR_{9}	BGR_{12}	BGR_{16}	BGR_{15}	**Isolates name**
500	500	500	500	500	500	600	700	700	900	1100	**concentration of (metal** mg/l)

Table 4- These bacteria resistance results has been shown about the lead different concentration and MIC and MBC different rates.

Bacteria resistant results to different lead metal concentration and different rate of MIC and MBC in (mg/l) in Behbahan and Bidboland gas refinery

BGR_{14}	BGR_{7}	BGR_{3}	BGR_{1}	BGR_{4}	BGR_{11}	BGR_{5}	BGR_{9}	BGR_{12}	BGR_{16}	BGR_{15}	**Solates name**
520	**550**	**530**	**530**	**550**	**520**	**630**	**730**	**750**	**920**	1140	MIC(mg/l)
570	**580**	**570**	**570**	**590**	**590**	**660**	**780**	**780**	**950**	**1170**	MBC(mg/l)

Table 5-Lead heavy metal reduction rate is shown by the most resistant sample in laboratory condition in highest concentration in which the bacteria has been resistant to after 72 hours.

The lead metal reduction rate by the most resistant bacteria sample in Behbahan and Bidboland gas refinery

metal concentrations at 72 h (ppm)	**initial concentration of metal (ppm)**	**Metal name**	**Isolates name**
441.12	1100	Pb	BGR_{15}

Table 6- The primary results of bacteria realization has been shown by the use of biochemical tests and using Bergy´s categorization.

The bacteria primary realization by the use of biochemical tests and Bergy´s categorization application(- =Not reaction;+ =Positive reaction; *=No reaction value)

BGR_{16}	BGR_{15}	BGR_{14}	BGR_{12}	BGR_{11}	BGR_{9}	BGR_{7}	BGR_{5}	BGR_{4}	BGR_{3}	BGR_{1}	**Test Name/Isolate Name**
*	+	+	*	*	*	+	*	*	*	+	Catalase
-	-	+	+	-	+	+	-	-	-	+	Oxidase
*	+	-	+	*	+	-	*	*	*	*	Acid Fast
+	+	-	+	*	+	-	-	-	*	*	Hydrolysis of Gelatine
*	*	+	*	*	*	-	*	*	*	+	Acid from -Froctose
+	-	-	+	+	-	-	+	-	+	+	Acid from -Lactose
*	*	*	*	*	*	-	*	*	*	-	Acid from -Galactose

+	-	-	*	*	*	*	+	*	*	*	Acid from -Arabinose
-	+	-	*	+	*	-	-	-	*	-	Urease
*	+	-	*	+	*	-	+	-	-	-	Formation of Indol
*	+	-	*	+	*	-	*	*	*	-	Groth on MaC Conky
*	*	+	-	*	+	-	-	*	*	+	Utilization of Citrate
+	-	-	*	*	*	-	*	*	*	-	Reduction of Nitrate
+	-	-	*	*	*	-	-	-	+	+	Motility
+	-	-	*	*	*	-	*	*	*	-	Production of H_2S
-	+	+	*	+	*	-	+	+	-	+	Methyle Red test
-	K/A+ گاز	*	*	-	*	+	-	-	+	-	Voges-Proskauer test
A/A-گاز+	+	*	Alk/Aگاز-	A/Aگاز-	Alk/Alkگاز-	A/Aگاز-	Alk/Aگاز-	A/A-گاز-	*	*	TSI test
-	*	*	-		-		+	-	+	*	Lysin Carboxylase
*	*	O^+/F^-	*	*	*	*	+	+	+	*	Ornitin De carboxylase
-	-	-	-	-	-	-	-	-	-	*	Coagolase
-	-	-	-		+	-	+	+	-	*	Acid from -Mannitol
*	*	*	*	*	*	*	*	*	*	*	Hydrolysis By Sculin
-	*	*	*	*	*	*	*	*	*	*	Sencitivity to Novobiocine
*	*	*	*	*	*	*	*	*	-	*	Production of Pigment
-	-	-	-	-	-	-	-	-	-	-	Growth in NaCl 6.5%
*	*	*	*	*	*	+	*	*	*	*	Hydrolysis of Starch
*	*	*	*	*	*	-	*	*	-	+	Formation of Spore
Citrobacter freundii	*Providencia .sp*	*Flavobactr .sp*	*S.fluorecence*	*K.oxytoca*	*S.aeruginosa*	*E.coli*	*Sh.sonnei*	*E.aerogenes*	*B.macerans*	*B.sphaericus*	Resalts

After realization of samples by biochemical method, the most resistant sample toward lead was recognized by genetic method. The PCR reaction for resistant sample was done in order to reproduce 16S rRNA in sectional format. The results related to some isolated electrophoresis which have had the most absorption of lead have been shown in figure 1.

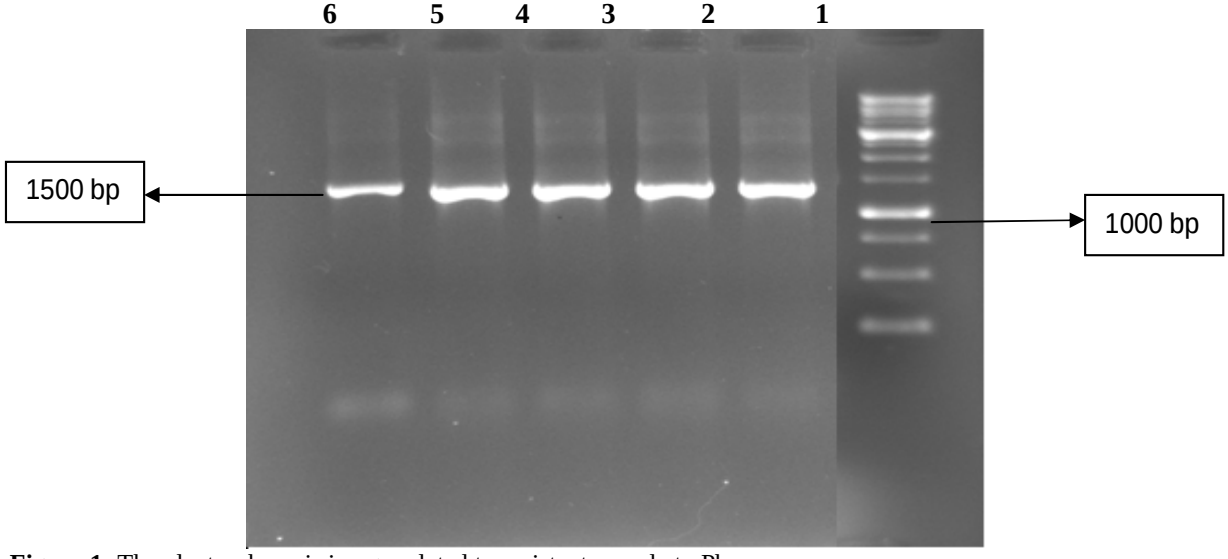


Figure 1- The electrophoresis image related to resistant sample to Pb

From left to right respectively: **Lin1**: Weight marker (1000 bp); **Lin2:** *Providencia .sp* (BGR_{15}); **Lin3:** *Citrobacter freundii* (BGR_{16});

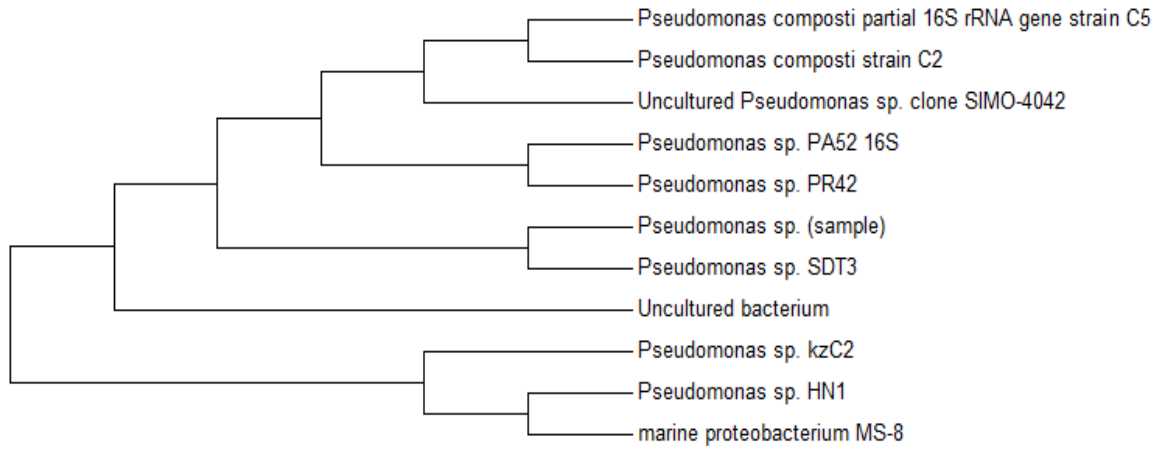
Lin4:*Pseudomonas fluorecens*(BGR_{12}); **Lin5:** *Pseudomonas aeruginosa* (BGR_9) ; **Lin6:** *E.coli* (BGR_7).

Based on obtained results from 16S rRNA gen frequency, the most resistant sample to Pb was identified in NCBI that the BGR15 strain is belonged to *Providencia .sp* . The sequencing of gen has been shown in figure 2 and Phylogenetic tree in figure 3.

Figure 2- The obtained results from generic status product ordering of 16S rRNA in BGR15 sample which has been reproduced with general primers and determined in sequence.

TTTTTATGGCCCCTTCTGGGTCGCATGGCGGCAGGCATAACACA
TGCAAGTCGACGGGGTTTTTGTAAGGAACAGATTTAATCAGTCATGATCTGAGTGGTAGCGCCCTGTTT
GCGTACCCACCCTTGGTTCGCACTGTTGTCCCAGGAGCGCGGAGGACAAGGGACGGCGCGTGTCATGG
CTGCACGGCATTTCCGATTACTACTGATTTATGGTTCAGAGTTAACAGTGTTAACTCTGTCCTCACTAA
GCCCCATGATGCACCTGACGCCGCACGAGAATTCCGAGGATAACTATGGTGTTGTGCCCTAACGTGGA
TGAAGTTGGTGGATAGGCCTGACGGTGTTAGTATGAAGTTCTAAAGATTTGGTTGGCGAGACTCATTT
AAACTCCCCATGTGGATACTGAGAACACTTTTGGGTTTATGATTTGCTCTCACTCTGACTGTTGATCGC
TCTATTTTCCCACCCTTAGGCGGCTGGTCCCCTGTCCTTGTTCCCAAAGGAATTTTGCGTCCCTTGGGTT
TCTGATCACTTTGGATGATGTTATGGGAAACCATTCCGTGAAGGTTAATGAAAACAAATCGGCGGTCC
CCTCAAAGATGGTCTGTCTCTTCTGAACCGGCCCGCCGACACCTTTGCCACACTGATTGAGCTGTATGT
CACAAGCTGCATCTGGATCCTGAGAACCTCAGGGTGAGTCTATGGGACCTTTAATGCG

Figure3- BGR15 sample Phylogenetic tree



DISCUSSION AND CONCLUSION:

Researchers are hopeful that they could be able to reduce the industrial wastewaters poisoning rate in high extend and by consuming low costs, bacteria have high industrial capability to recycle metals by having ion specifications on their surface and producing cellular external polymer and absorption of enzyme.

According to the increasing growth of industry in our country and region, keeping environment and reducing pollution due to them is of items that should be considered by many factories and related industries all around the country.

In this research at first the refineries Bidboland gas refinery separated bacteria samples resistant to Pb were assessed and from separated samples two of them had the most resistance which had

the capability to observe leads and both of selected samples were the gram negative bacteria.

One of the measured factors in this research was pH that is the most important parameter in evaluation of wastewater and natural water evaluation and hydrogen ion concentration is one of the effective parameters in absorption process. It has been observed that in the intended wastewater in our research the PH was alkaline. Generally according to the pH rate, many of metal compositions are changed to liquid and the presence of liquid metal in environment has effect on the bacteria resistance. Sabin during his research pointed out to the PH role and the bacteria resistance rate. In low pH rates, the absorption rate reduction could be due to high movement of hydrogen ions about metal ions. The higher moving of hydrogen leads that such ions reflect more than metal ions to absorption active soluble, Sabin and Pefister, 1990. In a research which performed by Nath and et al in 2012, the pH rate of water soluble was about 7 that was lower recent research. In Lucius and and et al in their study at 2013 observed pH rates more than 6 for metal soluble has been sediment and it is out of Bactria accessibility, therefore the pH range was chosen between 2-6. In Samanta et al research in 2012 it was observed that pH rate is increase between 3 and 4 fast and in 5 pH rate s fixed. By reduction of pH, the magnesium ions absorption is increased 9 times more than primary rate and cupper ions absorption has increased 19 times more than primary rate.

The proper pH for most of bacteria is at neutral level and when pH in one environment take distance from natural and tolerable limit, it is obvious that the microbe population available in that environment is under the effect and it is followed by reduction, therefore the metal absorption rate is reduced, Durve and et al, 2012, according to obtained results from other researches, pH in under studying wastewater is alkaline. BOD shows the low load of water chemical contain and also the low rate of microorganisms or available microorganisms in water does not need the oxygen consumption or it is dead or near to die.

In recent research the COD=134.50 mg/l and BOD=66.10 mg/l for wastewater and BOD=105 mg/l and COD= 1200.20 mg / l for sediment.

The research which was done by Alboghbeysh et al in 2013 on 3 types of industrial wastewater showed the reverse relation of BOD and COD of wastewaters. Also in wastewaters that have the maximum rate the COD=3458 mg/l and the minimum rate is BOD=169.5 mg / l and the maximum concentration of heavy metal has been observed. In their research the wastewater which included the maximum BOD rate, there were more resistant bacteria and in reverse. Therefore the COD and BOD in refinery wastewater have been lower compared to above research.

In the same study on 9 wastewater sample from industrial factories and coating metals, maximum and minimum BOD was in order 18 and 120 mg/l and also the minimum and maximum rate of COD was 70 and 45400 mg/l orderly.

While the COD rate in this research is more than COD rate reported before. In this research the COD and BOD rate comparison were shown for two water and sediment sample that by increasing these factors rates, the heavy metal rat also increased and by studying resistant microorganism also we find this result that sometimes by increasing the metal rate related to the bacteria type, the resistance will be reduced but in some conditions by increasing the metal type, special kind of resistant bacteria which has the resistant mechanism to that special metal will remain and other will be eliminated and it shows the positive relation. Therefore it is understood that by increasing BOD and COD that represent the pollution augmentation, the heavy metal rate also increased and according to the wastewater power categorization in pollution BOD and COD rate of refinery has been put in weak wastewater group in this research.

Samanta et al in 2012 used resistant bacteria for separation that lead concentration in this wastewater was lower than 10 mg/l.

In another research made by Alboeghobeysh in 2013, the heavy metal rate as lead, nickel, cadmium which exists in cupper coating wastewaters had been orderly equal to 0.022, 0.418 and 0.151 respectively.

Therefore according to researches made by researchers about the studied wastewater, heavy metal rate was low and this wastewater s categorized as the low pollution wastewaters.

Shamima and Rehman in 2012 and Hussein and et al in 2003 considered strains from whose growth with concentration of 1 mM from Cd ، Pb ،Zn and Ni and 100 µg/ml had not been avoided and the rate resistant has been regarded.

Edvard Raji et al in 2009 assumed strains whose growth had not been avoided as the resistant with concentration of 10 µg/ml from Pb metal was considered as resistant, while recent research the *Providencia .sp* in which Pb metal was assumed as resistant with 1100 mg/l that compared to other research samples showed more resistance. In the research which was done by Malik from the microbe samples which are resistant to metal that had been derived from industrial soil, the MIC rates for lead and nickel metals were more than 2400 µg/l that is lower than present research.

In the same study the MIC rates was equal to 1600 µg/l for Pb between the separated bacteria from such soil.

In Edvard et al research in 2009 from gathered samples about wastewater inside and around Madura around India among the samples the highest MIC related to the lead was equal to 6 mM/l which has been observed the same for 100% of samples.

In researches made by Durve et al in 2012 about external wastewater samples from textiles coloring industries the highest MIC lead was 625.8 ppm related to the bacteria and Pseudomonas.

Nath et al in 2012 made a research about industrial wastewater, garage and pump station in India Asam region, the MIC highest for Pb obtained at 1200 µg/ml.

In an evaluation made on artificial textile factory external wastewater after resistant bacteria separation to heavy metal, the highest MIC rate was obtained for Pb metal at 8 mM/l that has been observed in 100% of strains, Alboeghobeysh and et al, 2013.

Finally the researchers' studies have shown that the heavy metal biological absorption has been introduced as the potential method for elimination of these metals from industrial wastewaters in the world, Lucius et al, 2013.

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