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Impact of Hg (0) on Microbial functional diversity with their Enzyme Profile (MerA)

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Abstract: ***Mercury (Hg) is one of the most important toxic pollutants widespread in the environment. It is being extensively used in industrial applications (chlor-alkali electrolysis, fungicides, disinfectants, dental products, etc), resulting in local hot spots of pollution and serious effects on biota and humans. During study we investigated changes in the microbial and functional communities within mercury contaminated effluent sample .The composition and diversity of microbial communities and specific functional groups involved in key pathways in the geochemical cycling of Mercury were characterized using cultivation-dependent and Independent Approach .The most common bacterial mercury resistance mechanism is based on the reduction of Hg^{2+} to Hg^{0}, which is dependent on the mercuric reductase enzyme (merA) activity. Seven Mercury resistant bacteria Isolated, out of them highest resistance 200ppm were given by SG-1 And SG-4 Isolates. It was found that strong correlation may be present between higher metal tolerance and Antibiotic resistance. During Enzyme activity study it was found that SG-1 gave maximum enzyme activity Rnaging between 65-80 unit/ml/mg. The metal uptake ability suggests possibility of using these bacterial strains for removal of mercury from Hg^{2+} contaminated Effluent.***

Keywords: Mercury, Fuctional Diversity, Mercury resistance, Mercury Reductase.

1. Introduction

Functional diversity is quantifying the distribution of functional units in a multidimensional space. Functional diversity could be divided into three primary components – functional richness, functional divergence and functional evenness. Functional richness represents the amount of functional space occupied by a species assemblage [1]. Functional evenness corresponds to how regularly species abundances are distributed in the functional space. Finally, functional divergence defines how far high species abundances are from the centre of the functional space. This decomposition of functional diversity reflects complementary characteristics of the distribution of taxa (or individuals) in functional space. Linking indices to a particular functional diversity component could greatly aid ecologists in deciding on a minimum set of indices from the ever increasing range of options [2].

Industrial Development Generating effluents, untreated effluent become source of soil, water and Air Pollution. In mixture of all pollutant, Heavy metal considered as most powerful Toxin affect human health and Aquatic life. These Heavy metals are recognised to be powerful inhibitors of biodegradation activities[3]. These metals cannot be degraded, and are ultimately indestructible. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes.

Mercury is the most toxic of the heavy metals and occupies the sixth position in the list of hazardous compounds[4]. Major sources of mercury include industries associated with its use, such as those producing , paints, disinfectants, pharmaceuticals, paper and pulp , natural processes such as volcanic eruptions, geothermal activities, soil erosion, hydrological cycle, wild fires, and the reemission of the deposited mercury contribute to the global mercury load through the elemental form of mercury .Sewage treatment facilities also constitute a widespread environmental source of both inorganic and organic mercury compounds such as elemental mercury, mercuric mercury, methyl mercuric chloride (MMC), and dimethyl mercury[5]. Mercury is a major environmental pollutant, classified among those bioaccumulative toxins that persist in the environment for long periods; i.e., its estimated residence time is between 0.5 and 2 yr.

To survive in the presence of mercury in their natural habitats, many microorganisms have evolved an effective resistance mechanism based on the enzymatic transformation of Hg (II) into metallic mercury (Hg (0)). Hg (0) is not toxic for microorganisms and can leave the microbial cell by diffusion. The reductive transformation occurs inside the microbial cell and requires biochemical reduction equivalents ($NADPH_2$). The reaction is catalyzed by the enzyme, mercury reductase, which is the product of the MerA gene. Genes conferring these functions are designated as *mer*T, *mer*P (transport), *mer*A (mercury reduction), *mer*B (cleavage of Hg from organic residue), *mer*R and *mer*D (regulation). Further, more of *mer*genes have been identified recently; *mer*C and *mer*F, both membrane proteins, conferring transport functions . Also *mer*G is known to confer resistance to phenyl mercury. Most mercury resistance operons are inducible, i.e. Hg^{+2} has to be present in order to activate expression of resistance whereas transcription is suppressed in the absence of Mercury .

The best-characterized MerA (encoded by transposon Tn*501*) is from *Pseudomonas aeruginosa[6]* , and its sequence consists of 560 amino acid residues . Three cysteine pairs have been found essential for activity in Tn*501* as well as other MerA proteins. These cysteine pairs are located at the N-Mer A (N-terminal of the Mer A protein which consists of about 90 residues), at the active site, and at the carboxyl terminus. The N-terminal cysteine pair (residues Cys11 and Cys14 according to the Tn*501*

numbering), capture Hg from the environment (e.g., from other Mer proteins) and pass them to the cysteines that are located in the active site (at the catalytic core) . The active site of MerA has a cysteine pair at location of Cys136 and Cys141 [7]. These cysteines have a direct role in reduction of Hg^{+2} by binding to Hg and FAD (flavin adenine dinucleotide) molecules. FAD assists with the transfer of electrons between NAD(P)H and Hg^{+2} molecules. Both NAD(P)H and Hg^{+2} are substrates of MerA, which reduces Hg^{+2} and oxidizes the NAD(P)H (equation 1). Therefore, the activity of the enzyme can be tested in two different ways, *i.e.*, mercury reduction and NAD (P)Hoxidation. The third pair (residues Cys558 and Cys559) are located at the carboxyl terminus and bring the mercury to the active site for reduction .

NAD(P)H + RS-HG-SR + H+ → NAD(P+) + Hg0 + 2RSH--------- :(Equation -1)

$Hg^{+2}$is reduced to Hg0 in an NADPH-dependent reaction. The non-toxic Hg0 is then released into the cytoplasm and volatilizes from the cell. The purpose of above work to determination the physiochemical parameter & concentration of different heavymetal present in effluent, to assess the microbial diversity Index & abundance of bacteria capable of reducing mercury in industrial effluent, Morphological and molecular characterization of mercury resistance isolate and also to determination of enzyme activity and Purification from crude extract which will be helpful for bioremediation purpose.

2. Materials and Methods

a) Sample Collection and Storage
Industrial effluent sample were collected from different Industrial area near south Gujarat region.Samples were collected in sterile autoclaved bottles. Samples were stored at 4^0C for 30 day before analysis.

b) Physicochemical analysis and Metal concentration Estimation
Physicochemical parameter like pH, TS, TDS, TSS ,BOD ,COD ,Chloride were determined by using method Described in APHA (2009). Heavy metal like Mercury (Hg), Arsinate (As) and Nickel (Ni) concentration determined by Atomic Absorption Spectroscopy (AAS) [8].

c) Microbial Diversity Indices calculation
Microbial diversity indices has been measured for Dominance_D,Simpson_1-D, Shannon_H, Evenness_e^H/S, Brillouin, Menhinick, Margalef, Equitability_J, Fisher alpha, Berger-Parker, Chao-1for bacterial,Fugal and Actinomycetes cultivation. Microbial diversity index was calculated by Past 3 software [9].

d) Determination of Minimum inhibitory concentration
Minimal inhibitory concentration (MIC) of Hg+2 was tested by growing the resistant strains on Nutrient broth with Mercury concentrations ranging between 0.78-400 ppm .To have the desired turbidity, the number of bacteria per ml was prepared according to 0.5 MacFarland standard (1.5 × 108 cfu/ml) Then 1 mL of bacteria suspension (1.5 × 108 cfu/ml) was added to each tube and the tubes were incubated at 37°C for 48 h. Optical density was measured at the time of inoculation to 48hours with Uvvis spectrophotometer at 560nm wavelength [10].

e) Effect of pH on growth
The Study of pH on Microbial growth was done by using Nutrient Broth with pH ranging Between 3-12 in Presence and Absence of Mercury. Different pH of Medium was Adjust by using 1N HCL and 1N NaOH. Optical density was measured at 48hours with Uvvis spectrophotometer at 560nm wavelength [11] .

f) Determination of Antibiotic resistance
The antibiotic resistance was done by standard agar disc diffusion method on Nutrient Agar medium using Commercial Disc .100 µl of fresh bacterial cultures were spread on Nutrient Agar medium. Antibiotics Disc was placed Aseptically on the center of medium with the help of sterile forceps . The plates were Incubated at 37 0C for 48 hours.Inhibition zones in diameters were measured in mm surrounded by Individual Disc [12].

g) Extraction and purification of Enzyme
Crude Extract was prepared from centrifugation of Isolates cells Grown in Nutrient broth medium containing 50 ppm mercury concentration grown at 37 0C for 48 hours.Mercuricreductase activity was determined by measuring Hg+2 induced NADPH oxidation spectrophotometrically at 340nm following the standard method by Zachary Freedman [13].Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard. One unit of mercuric reductase activity was defined as the amount of enzyme protein in milligrams that oxidized one micromole of NADPH Per minute in the presence of HgCl2.

Further Purification of Crude extract Was done by using Ammonium sulphate Precipitation as well as by using CMC cellulase column.

3. Result

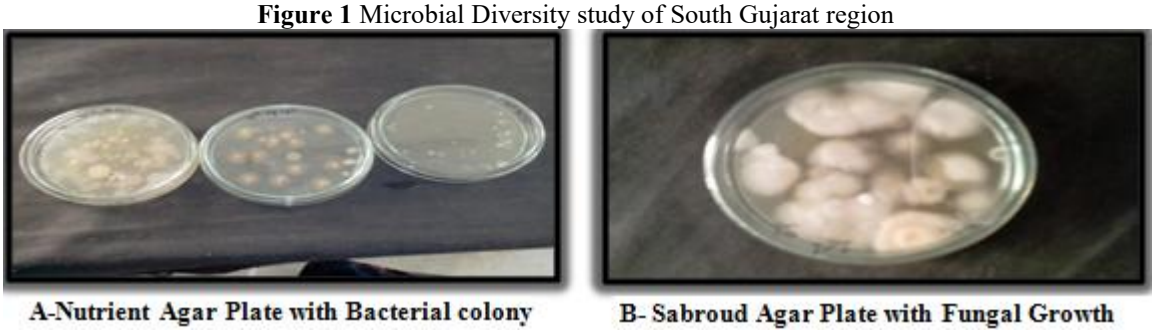
Table 2: Relationship between Effluent parameter

Parameter	*pH*	*TS*	*TSS*	*TDS*	*DO*	*BOD*	*COD*	*Chloride*	*Mercury*	*Arsinate*	*Nickel*
pH	1										
TS	-0.2	1									
TSS	-0.137	0.99	1								
TDS	-0.471	0.065	-0.07	1							
DO	-0.45	-0.243	-0.307	0.465	1						
BOD	-0.38	0.5654	0.558	0.048	0.335	1					
COD	-0.1	0.59	0.594	-0.035	0.117	0.847	1				
Chloride	-0.186	0.665	0.667	-0.021	0.365	0.648	0.576	1			
Mercury	0.1799	-0.232	-0.186	-0.333	-0.105	-0.09	-0.475	-0.08	1		
Arsinate	-0.482	0.058	-0.057	0.853	0.126	-0.201	-0.15	-0.28	-0.5168	1	
Nickel	-0.007	0.664	0.715	-0.378	-0.225	0.752	0.833	0.477	-0.203	-0.382	1

Table 3: Microbial Indices calculation by Past-3 Software

	SGI-1	SGI-2	SGI-3	SGI-4	SGI-5	SGI-6	SGI-7	SGI-8
Taxa_S	6	5	3	5	4	3	4	3
Individuals	50	30	24	30	24	29	26	21
Dominance_D	0.39	0.268	0.33	0.25	0.288	0.3722	0.3166	0.3243
Simpson_1-D	0.6	0.73	0.66	0.74	0.711	0.62	0.68	0.67
Shannon_H	1.23	1.48	1.2	1.52	1.31	1.237	1.24	1.37
Evenness_e^H/S	0.57	0.73	0.82	0.76	0.933	0.574	0.864	0.656
Brillouin	1.089	1.25	1.01	1.29	1.12	1.03	1.064	1.095
Menhinick	0.84	1.09	0.81	1.095	0.8165	1.11	0.784	1.309
Margalef	1.27	1.47	0.94	1.47	0.944	1.485	0.92	1.642
Equitability_J	0.69	0.82	0.865	0.85	0.95	0.693	0.89	0.764
Fisher_alpha	1.78	2.255	1.37	2.25	1.37	2.297	1.32	2.806
Berger-Parker	0.58	0.4	0.41	0.33	0.416	0.51	0.4231	0.4762
Chao-1	6	5	3	5	4	3	4	3

Figure 1 Microbial Diversity study of South Gujarat region



A-Nutrient Agar Plate with Bacterial colony

B- Sabroud Agar Plate with Fungal Growth

Figure-2 Mercury Resistance Isolates (SG-1,SG-2,SG-3)

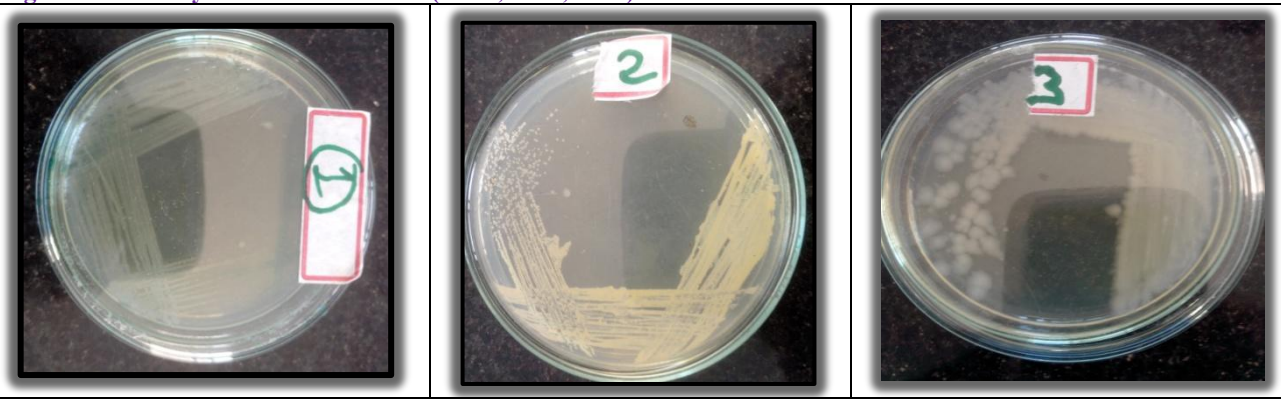


Figure-3 Mercury Resistance Isolates (SG-4,SG-5,SG-6,SG-7)

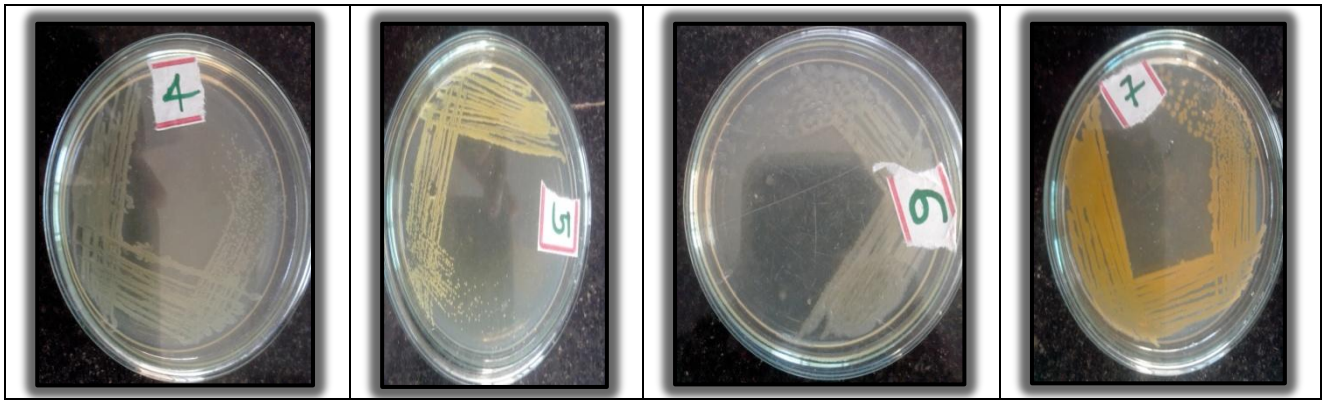


Table 1: Maximum concentration Determination of Isolates

Mercury concentration (ppm)	SG-1	SG-2	SG-3	SG-4	SG-5	SG-6	SG-7
400	-	-	-	-	-	-	-
200	**0.310**	-	-	**0.339**	-	-	-
100	0.30	**0.276**	**0.237**	0.25	-	-	-
50	0.30	0.266	0.22	0.25	**0.259**	**0.477**	**0.56**
25	0.30	0.266	0.22	0.24	0.245	0.45	0.43
12.5	0.30	0.256	0.22	0.24	0.240	0.45	0.43
6.25	0.30	0.256	0.22	0.24	0.23	0.43	0.40
3.125	0.30	0.256	0.22	0.24	0.23	0.43	0.40
0.78	0.30	0.266	0.22	0.24	0.23	0.43	0.40

Table 2: Effect of Antibiotic on Microbial Growth athoteq 'Bio –Disc-12'code No.112 (For Gram Positive Bacteria)

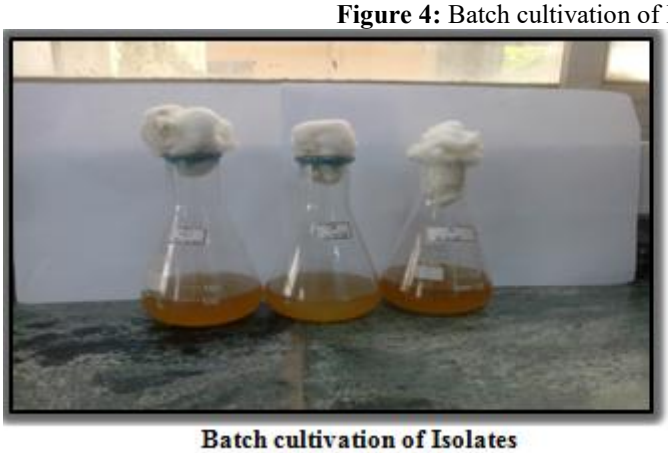
Agent	Symbol	Strength	Zone of Inhibition (mm)		
			SG-2	SG-3	SG-7
Ampicillin	AS	200mcg	-	-	-
Co-Trimoxazole	BA	25 mcg	-	-	-
Cephalexin	PR	30 mcg	26mm	30mm	20mm
Tetracycline	TE	30 mcg	20mm	13mm	20mm
Cefotaxime	CF	30 mcg	-	-	-
Ciprofloxacin	RC	5 mcg	25mm	16mm	16mm
Levofloxacin	QB	5 mcg	24mm	10mm	21mm
Linezolid	LZ	30 mcg	20mm	22mm	27mm
Cloxacillin	CX	1 mcg	22mm	18mm	16mm
Roxythromycin	AT	15 mcg	-	14mm	-
Lincomycin	LM	2 mcg	-	22mm	16mm
Gentamicin	GM	10 mcg	18mm	18mm	12mm

Table 3: Effect of Antibiotic on Microbial Growth Pathoteq 'Bio –Disc-12'code No.212 (For Gram Negative Bacteria)

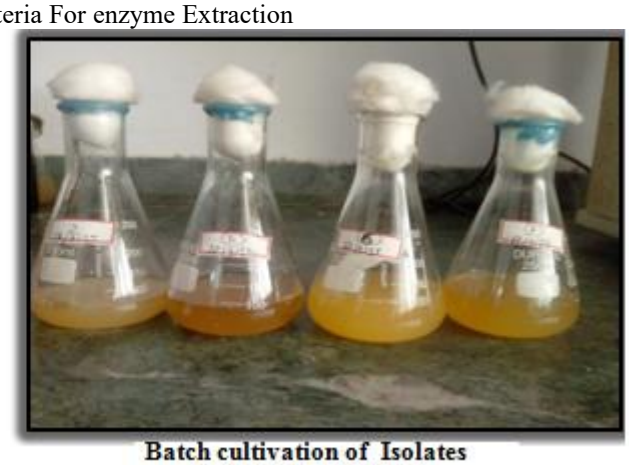
Agent	Symbol	Strength	Zone of Inhibition (mm)	
			SG-1	SG-4
Ampicillin	AS	200mcg	-	-
Co-Trimoxazole	BA	25 mcg	10mm	14mm
Cefotaxime	CF	30 mcg	-	-
Piperacillin	PC	100 mcg	5mm	14mm
Chloramphenicol	CH	30 mcg	-	21mm
Ciprofloxacin	RC	5 mcg	30mm	25mm
Ceftizoxime	CI	30 mcg	-	18mm
Tetracycline	TE	30 mcg	-	5mm
Ofloxacin	ZN	1 mcg	20mm	16 mm
Gentamicin	GM	10 mcg	13mm	12mm
Amikacin	AK	30 mcg	-	14mm
Gatifloxacin	GF	10 mcg	15mm	-

Extraction and purification of Enzyme

Figure 4: Batch cultivation of Bacteria For enzyme Extraction



Batch cultivation of Isolates SG-1,SG-4,SG-3



Batch cultivation of Isolates SG-2,SG-5,SG-6,SG-7


4. Discussion

After Physicochemical analysis correlation Between all Parameter were calculated. Correlation is the mutual relationship between two variables. Direct correlation exists when increase or decrease in the value of one parameter is associated with a corresponding increase or decrease in the value of the other. The correlation is said to be positive when increase in one parameter causes the increase in the other parameter and it is negative when increase in one parameter causes the decrease in the other parameter. The correlation coefficient (r) has a value between +1 and -1. Correlation is characterized as strong, when it is in the range of +0.8 to 1.0 and -0.8 to -1.0, moderate if it is in the range of +0.5 to 0.8 and -0.5 to -0.8 and weak when it is in the range17 of +0.0 to 0.5 and-0.0 to -0.5. The correlation coefficients (r) among various Effluent parameters were calculated and the values of the correlation coefficients (r) are given in Table 1. There is strong positive correlation between most of the parameters. For instance, TS and TSS (0.99),TDS and Arsinate (0.853),COD and Nickel (0.833),BOD and COD (0.847). The correlation coefficients between TS and BOD (0.565),TS and COD (0.590),TS and Chloride (0.665),TS and Nickel (0.664),TSS and Nickel (0.752)were found to be moderate.Very weak correlation between pH and other parameters.

During analysis Physicochemical study it was found thatincrease in Mercury methylation leads to Decrease in pH as well as Increase in total mercury and Heavymetal concentration in Effluent sample[14].The adsorption of Hg(II) increased with pH increasing from 2.5 to 5.5, then significantly decreased at about pH 5.5. For the same Hg(II) concentration and the same pH (range 2.5-5.5 pH), the greater adsorption of Hg(II) was observed for soils and with higher content of organic matter . The observed release of mercury with decreasing pH was the result of a release of humus substances together with the mercury it contained.Chemical oxygen demand of all samples were very higher, indicatesorganic matter can bind up to 95% of the divalent mercury species.Also, methylmercury has a strong affinity to organic matter, where it is strongly bound by sulphur species main sulphur species responsible for binding mercury are those reduced in functional groups such as thiol (R-SH), disulphide (R-SS-R) and disulphane (R-SSH) . Apart from sulphur, oxygen and nitrogen atoms can also bind the mercury in organic matter, but to a significantly smaller degree because they form weaker bonds with mercury[15] .

MIC was defined as lowest concentration of Mercury chloride allowing no visible growth in culture tube. Among all Isolates, Isolate SG-1 and SG-4 were found to be highest resistant. According to their Resistance,Isolates were divided into three categories :(i) Resistance 200ppm .(ii) Resistance 100ppm.(iii) Resistance 50ppm. Antibiotic suceptiblity test indicates that all Gram positive isolates (SG-2,SG-3,SG-7) were highely resistant against Ampicillin, Co-Trimoxazole, Cefotaxime. All Gram Negative isolates (SG-1,SG-4) were highely resistance to, Ampicillin,Cefotaxime. Isolate SG-1 was highly resistant against Ceftizoxime, Tetracycline, Amikacin.

5. Conclusion

The Present study revealed the capacity of bacterial isolates Resist to Higher Mercury concentration and Antibiotic resistance due to mobile genetic elements such as Plasmids or transposons, can carry multiple genes encoding metal and antibiotics resistance. The stability of the activity of this enzyme allows its use in several biotechnological applications specially the conception of an enzyme electrode devoted to mercury analysis. Moreover, the determination of the mercury resistance mechanism has allowed us to develop a fluidized bed bioreactor for mercury removal from mercurial waters using Mercury Resistance Isolates immobilized in alginate gel. The Native Isolates, Which tolerated high concentration, can be effective in remediation strategies for Ecosystem polluted with metals .In addition, mergene expression at the translational level has yet to be examined in the environment. Antibodies to mercuric reductases from bothGram-negative and Gram-positive bacterial strains have been produced , but to our knowledge, have not yet been applied to examining the relationship between rates of mercury volatilization and mercuric reductase expression in the environment. These method ologies and others, as they develop, will contribute to our understanding of bacterial gene expression and its role in mercury cycling..

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