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Identification, purification and characterization of mercury resistant bacteria from Chlor-alkali industrial effluent

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ABSTRACT

Heavy metal contamination near chlor-alkali industry was mainly due to mercury as it was used as an electrolyte in the electrolysis of brine for preparation of caustic soda & chlorine. Mercury, a liquid heavy metal, is most toxic as it remains in the body of the organisms affecting the biota. Mercury remains in the environment for years and can be a potent contaminant even after years of its discharge from industry. Bacteria survive and grow in the effluent and contaminated areas showing a possible way of reclamation. Effluent sample was tested for such mercury resistant bacteria surviving in the effluent canal. A group of 5 bacteria were isolated from the effluent out of which 2 bacterial isolates has shown promising role in mercury resistance at 30mM and 40 mM of $HgCl_2$. Biochemical characterization confirmed that these bacterial species belonging to the genus *Vibrio*.

Keywords*: Mercury resistance, Bacteria, Chlor-alkali industry, Effluent, $HgCl_2$.*

Introduction

Global industrialization has put every single life on earth into a detrimental risk of pollution. With deviation from the zero discharge policy, industries have created contaminated sites in and around the industry by altering the chemical composition of the air, water and soil. Though industrialization provides a better life to human with maximum comforts, it has also degraded the environment significantly. Heavy metal pollution has drastically affected living organisms. Industrial releases of mercury (Hg), cadmium (Cd), lead (Pb), arsenic (As), and chromium (Cr) are considered to be the most toxic heavy metals (Alloway, 2013) affecting the environmental segments. Mercury is a liquid heavy metal mostly released from chlor-alkali industries. It was used as a cathode in electrolysis of brine (salt water) for production of caustic soda and chlorine. Mercury is a neurotoxin (Chang, 1977) and persistent chemical banned by European Union in chlor-alkali industrial use in 2015 (Crook and Mousavi, 2016). In nature, mercury generally exists in three valence states, i.e., Hg^0 (metallic mercury), Hg^{2+} (mercuric mercury), and Hg_2^{2+} (mercurous mercury). These three forms of mercury maintain equilibrium among themselves by the processes of chemical dismutation. Hg_2^{2+} ! $Hg^0 + Hg^{2+}$ (Robinson and Tuovinen, 1984). The inorganic mercury is methylated by bacteria in the environment mostly by anaerobic bacteria like sulphur reducing bacteria (Robinson and Tuovinen, 1984; Clarkson and Magos, 2006; Eagles-Smith *et al*., 2018). Methylation and demthylation of mercury is mediated naturally by microorganisms (Gilmour *et al*., 2013). Methyl mercury (CH_3-Hg) is the most toxic form that can

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easily enter into the living systems and stored in their tissue. Animals like fishes, crab, birds, and human show bioaccumulation of mercury (Rodriguez *et al*., 2009). Bacteria can grow on long term basis in metal contaminated areas (Silver and Phung, 1996; Chen *et al*., 2018) and developed resistance towards heavy metals (Pepi *et al*., 2013; Dash and Das, 2015). Mercury resistant bacteria (MRB) contain *mer* genes located on plasmid that encode enzyme mercuric reductase (Nies, 1999). This enzyme converts most toxic ionic mercury (Hg^{2+}) to less toxic volatile metallic mercury (Hg^{0}) (Vetriani *et al*., 2004; Kafilzadeh *et al*., 2013). Bacteria were used a tool for bioremediation of mercury pollution (Lima de Silva *et al*., 2012). The present studies aims at identifying resistant bacteria from effluent of chlor-alkali industry and characterize the bacteria for possible use in reclamation process to decontaminate a contaminated environment.

Materials and Methods

Collection of samples

Effluent was collected in sterile and pre-autoclaved collection bottles from the contaminated sites near a chlor-alkali industry M/s. Jayashree Chemicals (P) Ltd., Ganjam, Odisha (19°22'48"N & 85°03'10"E). This industry disposes its liquid wastes into a large open effluent stocking pond just nearer to the Rushikulya River. The effluent stocking pond is just 1.5 km away from the estuary of Bay of Bengal. Samples were immediately transported to laboratory for bacterial analyses.

Isolation and Culturing of bacteria

Effluent sample was serially diluted with 0.89% normal saline solution (0.89 g sodium chloride in 100 ml distilled water) up to 10^{-5} dilution to reduce the load of bacteria from the sample. 20µl from 10^{-5} serially diluted sample was spread plated on Nutrient Agar (NA) media (peptone– 5g/l, beef extract– 3g/l, sodium chloride– 5 g/l, agar– 18g/l, distilled water– 1000 ml, pH– 7.2) and incubated at 37 °C for 24-48 h. Spread plate was observed for number of CFUs, colony morphology, etc. To isolate pure cultures from the spread plate, streak culture method was followed.

Biochemical characterization

Oxidase Test: Oxidase test is to detect the presence of terminal enzyme system in aerobic respiration called cytochrome C oxidase or cytochrome a3. A filter paper is soaked in 1% solution of tetramethyl-p-phenylenediamine dihydrochloride. The filter paper was moistened with a drop of sterile distilled water. A distinct colony from the culture was taken and smeared on the wet filter paper. A colour change to deep blue or purple within 10-30 secs is tested positive test. No change in colour indicated negative test.

Glucose Fermentation: Microbes under anaerobic condition do fermentative degradation of glucose. Bacteria which use glucose as food will produce acid as the end product of metabolism. Fermentative degradation of glucose is carried out on a fermentation broth in a fermentation tube containing a Durham tube placed inverted in it. The fermentation broth contains ingredients of nutrient broth (peptone, beef extract, sodium chloride), glucose and pH indicator phenol red. It is red at neutral pH 7 and turns yellow at pH less than 6.8 due to production of acid. Bacterial isolates capable of fermenting glucose produce an organic acid thus the colour of medium changes to yellow at low pH showing positive test result. No change in medium colour is negative test result.

Salt tolerant Test: It is to determine salt tolerant bacteria. Bacterial isolates were streaked on culture medium slants with NaCl 65g/l. The inoculated slants were incubated at 37 °C for 24 hours. Then observed for growth of the colonies by examining turbidity of the culture tubes.

Voges – Proskauer Test: This test is to detect Acetoin in bacterial broth culture. The test was performed by adding alpha naphthol and potassium hydroxide to the VP broth inoculated with bacteria. Digestion of glucose to acetyl methylcarbinol reacts with alpha naphthol and potassium hydroxide to form red colour indicated positive test result. Yellow brown colour of the medium otherwise indicates negative test results.

Pigment Test: Overnight grown nutrient broth culture was subjected to centrifuge at 8000 rpm for 15 minutes. Supernatant and bacterial cell pellet were extracted using 95% methanol in ratio 1:5 until pellet was colourless. The extract was then analysed for absorbance at 400-600 nm using UV visible spectrophotometer.

Growth at 37 °C: The bacterial culture was spreaded on plates and incubated at 37 °C for 24 – 48 hours.

Molecular Approach

Next Gen Sequencing

The effluent sample from the contaminated site was subjected for metagenomics study to find out the bacteria species and their diversity. Complex bacterial and other microflora community structures were detected from the sample using molecular approach targeting the 16S rRNA gene sequencing. Phylogenetic tree and taxonomical annotations were done to support the presence of bacterial diversities and the isolated bacterial species.

Gene Analysis

The functional predictions of all the 16S amplicon V3-V4 sequences were performed using Picrust and QIIME software. All the predicted genes from the samples were analyzed.

Results

Effluent water from the industry was collected and serially diluted up to 10^{-5} with 0.89% normal saline solution (0.89 g NaCl in 100ml distilled water) and spread plated on nutrient agar media amended with 20mM $HgCl_2$, incubated at 37 °C for 48 h. Five bacterial colonies were isolated showing mercury resistance at 20mM $HgCl_2$.

Morphological characterization

All the isolated colonies were observed for morphological characterization given in Table 1.

Gram Staining

All the bacterial isolates were subjected to gram staining. The bacterial isolates C1, C2, C3 and C5 were gram negative and were rod shaped while colony C4 was gram positive and spherical in shape.

Distribution of mercuric content around the industry were analyzed and recorded in table 3 showing the extent of mercury pollution from the source. Even after closure of the mercury cell technology, mercury availability in the sediments of the vicinity.

Table 2. Gram staining off bacterial isolates.

Colony no.	POSITIVE/ NEGATIVE	SHAPE
C1	Gram Negative	Rods
C2	Gram Negative	Rods
C3	Gram Negative	Rods
C4	Gram Positive	Spherical
C5	Gram Negative	Rods

Screening for bioremedial activity

Bacterial colonies were grown at different concentrations of mercuric chloride. The growth was measured at 600 nm (Table 4). Colony C1 and C5 has shown highest growth at 40mM $HgCl_2$ and 30mM $HgCl_2$ concentrations respectively. These two isolates have also shown maximum growth at the highest concentration of mercury 110mM showing the maximum resistance towards mercuric chloride. Colonies C2 and C3 were also showing maximum growth of 1.02 and 1.03 at 40mM and 30mM of $HgCl_2$ respectively but unable to survive at higher mercury concentration. Hence colonies C1 and C5 were the potent isolates that are mercuric resistant and can be a possible way towards mercury bioremediation.

Biochemical Characterization

The two potent isolates were further analysed for their biochemical characterization (Table 5).

The above chemical characterization indicated the bacterial species to be *Vibrio orientalis* for colony C1 and *Vibrio splendidius* for colony C5.

Next Gen Sequencing

The effluent sample was subjected for metagenomics study to list out the total bacterial diversity. Complex bacterial and other microflora

Table 1. Colony morphology of isolates.

Colonyno.	SIZE	COLOR	MARGIN	ELEVATION	TEXTURE
C1	Irregular	Off white	Undulate	Raised	Smooth
C2	Regular	Transparent	Entire	Raised	Smooth
C3	Filamentous	Off white	Undulate	Raised	Rough
C4	Punctiform	Off white	Curled	Flat	Rough
C5	Regular	Off white	Entire	Raised	Smooth

Table 3. Total mercury (mg/L) content around the industry.

Sites	Upstream River	Downstream River	North Side
0.5 Km	0.072 ± 0.007	0.062 ± 0.004	0.132 ± 0.028
1.0 Km	0.063 ± 0.002	0.064 ± 0.004	0.101 ± 0.013
1.5 Km	0.052 ± 0.004	0.080 ±0.012	0.054 ± 0.007
2.0 Km	0.029 ± 0.001	0.167 ±0.021	ND

ND- Not detected

Table 4. Bacterial growth at different concentration of $HgCl_2$.

Colony	Blank	30 mM $HgCl_2$	40 mM $HgCl_2$	50 mM $HgCl_2$	60 mM $HgCl_2$	70 mM $HgCl_2$	80 mM $HgCl_2$	90mM $HgCl_2$	100mM $HgCl_2$	110mM $HgCl_2$
C1	0.21	0.99	1.15	0.68	0.89	0.71	0.53	0.56	0.25	0.25
C2	0.14	1.00	1.02	0.78	0.92	0.65	0.56	0.66	0.23	0.16
C3	0.11	1.03	0.91	0.76	0.87	0.66	0.35	0.55	0.14	0.19
C4	0.13	1.00	1.00	0.73	0.85	0.62	0.35	0.44	0.15	0.21
C5	0.11	1.03	1.01	0.78	0.85	0.72	0.37	0.43	0.10	0.25

community structures were detected form the sample using molecular approach targeting the 16S rRNA gene sequencing. Using the data of molecular analysis of the sample phylogenetic tree was done (Figure 2). Taxonomical annotation of the varied species from the sample was also done (Figure 1). These analyses supports occurrence of bacterial species belonging to *Vibrio* genera.

Gene Analysis

The functional predictions of all the 16S amplicon V3-V4 sequences were performed using Picrust and QIIME software. All the predicted genes from the samples were analyzed. A total of 6538 genes were

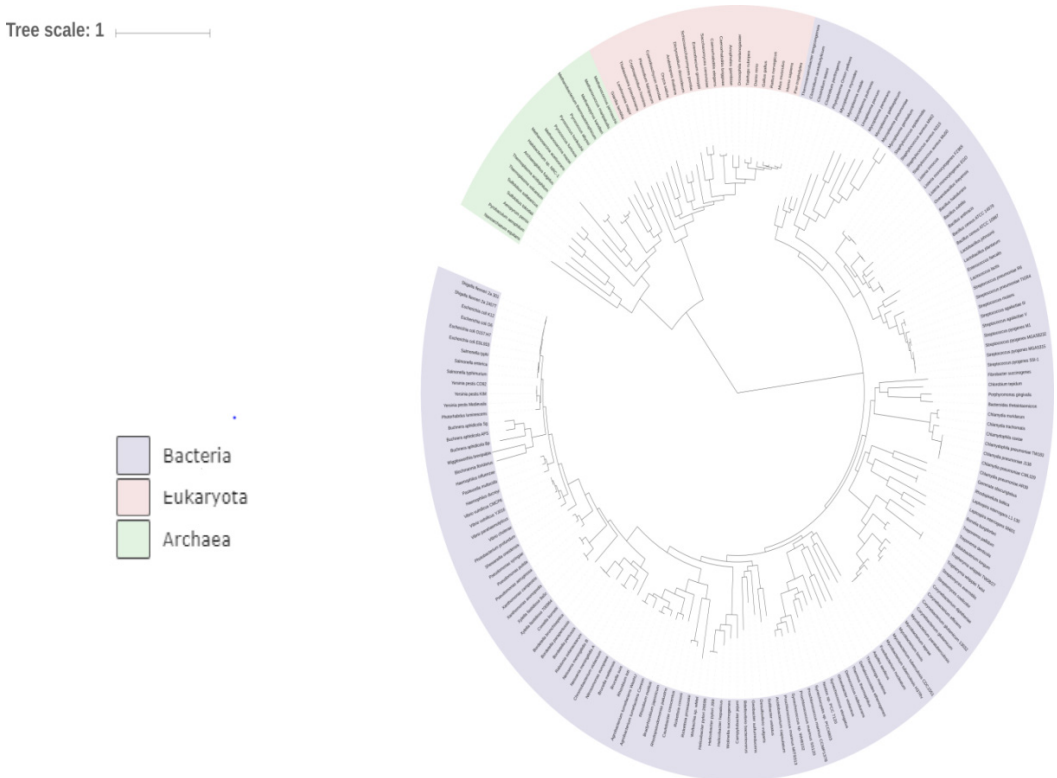

Fig. 1. Taxonomical annotation showing varied species of archaea and eubacteria.

predicted with their functional aspects. Ten perspective genes and proteins (Table 6) were detected from the bacteria collected from the effluent that are associated with mercury resistance in bacteria. The genes are alkylmercuric lyase (merB), mercuric reductase (merA), MerR family transcriptionl regulator (mercuric resistance operon regulatory protein), mercuric ion transport protein (merF / merT), periplasmic

Fig. 2. Phylogenetic tree showing varied bacterial species.

mercuric ion binding protein, MerR family transcriptional regulator_redox-sensitive transcriptional activator SoxR, MerR family transcriptional regulator_heat shock protein HspR, MerR family transcriptional regulator_Zn(II)-responsive regulator of zntA, MerR family transcriptional regulator_copper efflux regulator and MerR family transcriptional regulator_glutamine synthetase repressor.

Alkylmercuric lyase (merB) also known as organomercurial lyase carry out protonolysis of organic mercury into mercury ion (Hg^{2+}) by cleaving the carbon-mercury bonds of organic mercury. Mercuric reductase (merA) converts toxic mercury ions into relatively inert elemental mercury (Hg^0). MerR family transcriptional regulator is associated with regulation of transcription by binding to the DNA. Mercuric ion transport protein (merF / merT) allows uptake of Hg^{2+} and prepares reduction by mercuric reductase. Periplasmic mercuric ion binding protein (merP) acts as a mercury scavenger.

Table 5. Biochemical characterization of mercury resistant isolates.

Biochemical Tests	Results	
	C1	C5
Oxidase test	+	+
Glucose fermentation	+	+
6.5% NaCl test	+	-
Voges-Proskauer test	-	-
Pigment test	-	-
Growth at 37 °C	+	-

+ Positive result; - Negative result

Discussion

The bacterial colonies isolated from the effluent of a chlor-alkali industry showed mercury resistance. The colony C1 was resistant to $HgCl_2$ at 40mM showing maximum growth of 1.03. Colony C2 has maximum growth 1.02 at 40mM $HgCl_2$. Colony C3 has maximum growth 1.03 at 30mM $HgCl_2$. Colony C4 has maximum growth 1.00 at both 30mM and 40mM $HgCl_2$. Colony C5 has maximum growth 1.03 at 30mM $HgCl_2$. Colony C2, C3 and C4 were seen to be weakly growing at higher concentrations of $HgCl_2$. This may be due to the differences in composition of culture media from that of environmental soil and water composition. Colony C1 and C5 were of high growing as compared to other bacterial colonies. Due to high resistance of the two bacterial colonies they may be used as a tool for bioremediation of mercury contamination. From the morphological and biochemical characterization the bacterial isolates were identified as species of *Vibrio*. Further identification was done and the species expected to be *Vibrio orientalis* (C1) and *Vibrio splendidius* (C5). The possible resistance of these bacterial communities is due to mercuric reductase enzyme activity (Giovanella *et al*., 2016). Mercuric reductase shows optimal activity at pH 8 and temperature 35-37°C. The isolated bacteria colonies have mercury resistance that can be used for bioremediation. Further research in this area is to find out the use and promising role of *Vibrio sp*. in bioremediation of mercury in contaminated areas and to carry out the impact of these mercury resistance bacteria on the microbial diversity.

It is a fact that metals play a key role in different metabolic processes of bacteria, plants and animals. This allowed bacterial cells to grow even in the presence of toxic heavy metals. Bacteria collected and isolated from mercury contaminated sites were the

Table 6. Gene analysis of the effluent sample showing mercury resistant genes.

Sl. No.	KEGG Orthology	KEGG Descriptions	DNP Sample
1	K00520	mercuric reductase [EC:1.16.1.1]	46712
2	K00221	alkylmercury lyase [EC:4.99.1.2]	656
3	K08365	MerR family transcriptional regulator, mercuric resistance operon regulatory protein	9083
4	K08363	mercuric ion transport protein	5341
5	K08364	periplasmic mercuric ion binding protein	3773
6	K13639	MerR family transcriptional regulator, redox-sensitive transcriptional activator SoxR	14307
7	K13640	MerR family transcriptional regulator, heat shock protein HspR	5867
8	K13638	MerR family transcriptional regulator, Zn(II)-responsive regulator of zntA	3416
9	K11923	MerR family transcriptional regulator, copper efflux regulator	2213
10	K03713	MerR family transcriptional regulator, glutamine synthetase repressor	313

best representatives for using them in bioremediation process. Hence these resistant and tolerant bacteria can be used for bioremediation of heavy metals as a eco-friendly solution (Nanda *et al*., 2019).

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