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Isolation and Characterization of Mercury Resistant Bacteria from Haldia river sediments

Ankhi.Maiti [1*], Sagarika.Bhattacharyya [2]
[1, 2] *(Chemistry Department, Dr.S .C.Sur Degree Engineering College (JIS) West Bengal University of Technology, Kolkata, India*

Abstract: *The Mercury is known as a toxic heavy metal. The present work is aimed to isolate and characterize mercury resistant bacterial strains from the Haldia dock area river sediment. Bacterial cells are grown in 10 ppm of HgCl2 containing nutrient media. These isolates were resistant up to 150 ppm of mercury salt. The bacterial isolates were identified to belong to the genera: Serratia and Streptococcus or Enterococcus.. Many of the chosen isolates were tested for growth in a variety of antibiotics. Results of this study demonstrate the occurrence of diverse groups of marine bacteria capable of high tolerance to mercury .Mercury tolerant strains from dock area can be used to detect mercury toxicity in this type of polluted area and can be used for bioremediation of mercury toxicity.*

Keywords: *Bioremediation, MRB, Quorum sensing, Serratiae, Streptococcus.*

I. INTRODUCTION

Mercury is known to be the most toxic heavy metal and it exists naturally in small amounts. However, its levels have risen in the environment due to contamination from anthropogenic activities [1]. Mercury binds to enzymes and proteins, thereby inactivates vital cell functions [2]. Even small amounts of mercury can become toxic for all organisms. Mercury pollution released into the environment becomes a serious threat when it settles into waterbodies where it builds up in aquatic organisms that we eat. Its contamination in soil, water can be major problem for human health, industrial process, and defense related sites worldwide.In human body, excessive mercury is a neurotoxin, interfering mostly with the brain and nervous system. Even in low doses, mercury can affect a child's development. In adults, mercury poisoning can adversely affect fertility blood pressure regulation; memory loss, tremors, and vision loss .People affected by mercury pollution are also found to be resistant to several antibiotics [3].

Microbes which are resistant to mercury convert inorganic mercury to methlymercury, which has higher toxicity level. Thus a small environmental concentration of mercury and subsequent presence of MRB (mercury resistant bacteria) at a site increases the chances of accumulation of mercury in higher levels of food chain through biomagnifications.Studies show that many Indian coastal areas are polluted by mercury compounds and the amount ranges from 2 to 15 ng of dissolved mercury. The environments near Haldia dock area has shown very low pH levels (4.6 to 5), thus favoring the production of methyl mercury. The aim of this study is to identify and isolate the mercury resistant bacteria and to utilize them toxic metal bioremediation.

II. MATERIALS AND METHODS

2.1 SEDIMENT COLLECTION AND STORAGE

Sediment samples were collected from three zones of Haldia dock area riverbeds for the isolation of mercury resistant bacteria. The samples were collected with nitric acid pre-rinsed 1 L plastic container for chemical analysis and sterile glass container for microbial culturing. After collection, the samples were placed in cooler boxes with ice bags whilst being transported to the laboratory and kept at about 4°C before chemical and microbial analysis. During sample collection the pH of water was found acidic (pH 4.6- 5) and temperature was 25^0C.The chemical ingredients for the experiment were purchased from Himedia chemicals, India.

2.2 ISOLATION OF MERCURY RESISTANT BACTERIA

Isolation of Hg resistant bacteria was performed by primary enrichment method and directly plating on amended mercury. For the isolation of mercury resistant bacteria, 1g of sample was inoculated in sea water nutrient broth, (composition: peptone 5.0g, beef extract 3.0g, aged seawater 750 ml and deionised water 250 ml; final pH 7.5). To induce selective growth of only mercury resistant bacteria about 10ppm $HgCl_2$ salt was added singly to sterile medium prior to inoculation. Tubes are incubated at 24^0C for 24 hours in shaker. Growth was determined visibly by turbidity and streaking of a loopful of liquid culture on Luria Bertani (LB) agar plates supplemented with $HgCl_2$. MRB were isolated by spreading techniques on agar plates with $HgCl_2$.Single colonies were obtained following enrichment techniques. A simultaneous negative control test was done with available

bacteria on solid media plates containing mercuric chloride**.** The pure cultures of isolated strains were preserved in agar slants containing 10ppm of mercury salt in vials under refrigerated 4^0C conditions.

2.3 ENUMERATION OF VIABLE CELL COUNT

All samples were serially diluted in Phosphate-Buffered Saline (PBS) (2.2 g of NaH_2PO_4 per liter, 6 g of Na_2HPO_4 per liter, 5.8 g of NaCl per liter) .About 0.1 mL of each dilution were spread on Luria Bertani agar (10 g of peptone per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter, 12 g of agar per liter) supplemented with 10 mg of $HgCl_2$ per liter and without $HgCl_2$. The plates were incubated at 24°C for 48 hours. After incubation period, the appeared colonies on both LB agar containing Hg (II) and without Hg (II) were enumerated using total viable plate count method [4].

2.4 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERIZATION OF ISOLATES

2.4.1. COLONY AND CELL MORPHOLOGY

Size, shape, color, elevation and margins of bacterial colonies were observed for 24 hours incubated culture on agar plates containing 10ppm mercury salt. Cells were observed under microscope and motility test done to check presence of flagella.

2.4.2. TEMPERATURE TOLERANCE

To study the effect of temperature on isolated MRB strains about 100µl of overnight grown culture was dispensed into test tubes and incubated at different temperature like 4^0C, 11^0C, 24^0C, 30^0C, 38^0C for 24 hours. Optical density (OD) measured at 600nm using spectrophotometer and results recorded.

2.4.2. METAL TOLERANCE

The viability of cells following exposure to high levels of mercury was studied. Chosen isolates were grown at different concentrations of mercury metal. Resistant strains grown on LB broth amended with $HgCl_2$ and incubating it at 23°C for 24 hr. Six LB media with different concentration of mercury (4ppm, 10ppm, 25ppm, 50ppm, 100ppm and 150ppm) are inoculated with 100µl of bacterial suspension and the tubes were incubated at 23°C for 24 hr. Optical density recorded at 600nm to see growth pattern.

2.5. BIOCHEMICAL CHARACTERIZATION

A Biochemical characterization of isolated MRB strains were done by following the Bergey's manual of determinative bacteriology [5].Himedia Rapid Biochemical Identification kit was used in this study. Single cell isolated colony was picked up and inoculated in 10 ml sea water nutrient broth and incubated at 24ºC for 24 hours. Identification kit wells were inoculated with 50 µl of the above inoculums by surface inoculation method and kept for inoculation at 24°C for 18-24 hours. At the end of the incubation period, the appeared colonies were identified with gram staining and conventional biochemical tests

2.6. ANTIBIOTIC SENSITIVITY

The antibiotic resistance characteristics of mercury resistant bacterial isolates were studied by antibiotic disc diffusion method [6]. The antibiotics used were Streptomycin (4µg/ml), Ampicillin (25µg/ml), Chloramphenecol (25µg/ml), Penicillin (25µg/ml), Gentamicin (4µg/ml), and Rifampicin (4µg/ml), Cephalosporin ,Vancomycin, Carbapenems .The antibiotic discs were placed on nutrient agar plates previously seeded with 18 hr broth culture of the test organisms. The plates were incubated at 25^0C for 24 hr, after which diameter of zones of inhibition and MIC was examined. Earlier; the potencies of all the antibiotics used in the study were confirmed using susceptible E. coli strains.

III. RESULT AND DISCUSSION

Sediments samples of Haldia dock area were directly inoculated onto selective medium supplemented with 10ppm mercury salt. During this direct selection of mercury resistant bacteria using high level of $HgCl_2$ in media, it is possible that some low level MRB growth was inhibitend [7]. On enrichment and streaking to isolate single colonies, two distinct types of isolates (1 & 2) were seen on sea water nutrient media amended with $HgCl_2$. Previous studies suggest that generally the optimal growth temperature of MRB is around 37ºC, but the fact that only two isolates obtained, may be due to low temperature (23^0C) during sample collection and high amonut of mercury salt used in preliminary isolation media.During bacterial enumeration, the total viable counts ranged from 1.7 x 10^7cfu g^{-1} in SC1 sediment samples to 2.1X10^7 cfu g^{-1} in SC2 sediment samples. The frequencies of resistance to Hg varied from 2% in to 22% (Table 1).

Further study on mercury resistant bacteria showed variability in growth pattern with time and concentration of $HgCl_2$.Generally lag phase persisted for 1-2 hours. The all isolated strains could resist upto 150ppm of $Hgcl_2$ in LB agar media (Fig 1).The isolate1 showed more defined growth at 4ppm and 50 ppm of mercury salt

concentrations, while at 150ppm growth was considerable reduced due to the high concentration metal stress (Fig 2).The pH tolerance studies showed preferred pH of the isolates was in acidic range (Fig 3).The preferred pH was 5, which was reflected by the increase in value of optical density at this pH. Growth decreased with higher alkaline pH. Temperature sensitivity test showed optimal temperature of the isolated MRBs was 24^0C. (Fig 4).The strains showed no growth at 37^0C even when incubated for 48 hours.

Morphological studies showed colonies circular, flat and some convex and they had entire margins. Isolate one showed cells with distinct red pigment.The second type isolated colony cells were off white in color (Table 2).All strains could resist Chloramphenecol, Penicillin, Ampicillin, and sensitive to Streptomycin, Gentamicin, and Rifampicin (Table3). Single colonies were taken further to identify the bacteria at generic level [11, 12, and 13].Table 4 shows the biochemical characteristics of the isolate 1 and 2. The isolate 1 had high salt tolerance and had low minimal growth temperature .These isolates were gram negative short rods, having non diffusible red pigments. Isolate 1 was able to grow in CT agar plates. These are facultative anaerobes and give positive catalase reaction. The production of red pigment of isolate 1 was suppressed through quorum sensing at high levels of mercury and antibiotics in media.It is known that Serratia genus produce a distinctive red pigment, prodigiosin**.** From the antibiotic resistance and biochemical characteristic profile study of this isolate1, is affiliated to the genus Serratia [9, 10,] of the family of Enterobactereacea. Based on generic analysis using key characters of genus description it was found isolate 1 is Serratia mercescens. Testing further the isolate 1 in milk agar plate gave a positive casein test. The isolate 2 was off white circular colonies with cells existing in pairs (diplococcal).These being gram positive were assigned to be of genus Streptococcus or Enterococcus based on their biochemical characters.

IV. Figures and Tables

Table 4. 1 Frequencies of Hg resistant.

Sample	Total bacteria (cfu /g)	Hg resistant Bacteria (cfu/g)	Hg resistant Bacteria (%)
SC1	1.7×10^7	3.5×10^5	2.058
SC2	2.1×10^7	4.7×10^6	22.38
SC3	1.8×10^7	2.2×10^6	12.22

Table 4. 2. Colony Morphology

Isolate No.	Color	Shape	Elevation
1	red	circular	flat
2	Off white	circular	convex

Table 4. 3. Antibiotic resistance profile

Antibiotic	Isolate No.			
	$isolate_{11}$	$isolate_{12}$	$isolate_{21}$	$isolate_{22}$
Chloramphenecol	R	R	S	R
Penicillin	R	R	S	R
Streptomycin	S	S	S	S
Gentamicin	S	S	S	S
Rifampicin	R	R	R	R
Ampicillin	R	R	S	S
Clindamycin	R	R	S	R
Erythromycin	R	R	S	S
Cephalosporin	R	R	R	R
Vancomycin	S	S	S	S
Carbapenems	R	R	R	R

R= Resistant, S=Sensitive

Table 4. 4. Biochemical characterization

Characteristics	MRB Isolates	
	isolate 1	isolate 2
Gram reaction	–	+
shape	Short rods	Cocci
Motility	+	–
Color	Red	off white
Oxidase	–	–
Growth at 4% NaCl	+	–
H2S production	–	–
Voges proskar	+	+
Glucose fermentation	+	–
Catalase	+	–
Urease	–	Nd
Nitrate broth	+	–
Citrate	+	Nd
Starch hydrolysis	–	–
Pyrrolidonyl arylamidase	–	+
Growth at37^{0}C	–	–
Growth at 24^{0}C	+	+
Indole test	–	+
Methyl red test	+	–
Lipase	+	+
DNase	+	Nd
Proteinase	+	Nd
Mackoncy agar	+	–
Phenylalanin deaminase	–	–
Inulin	Nd	–
Raffinose	Nd	–

Nd=Not determined, (+) = positive, (–) =negative,

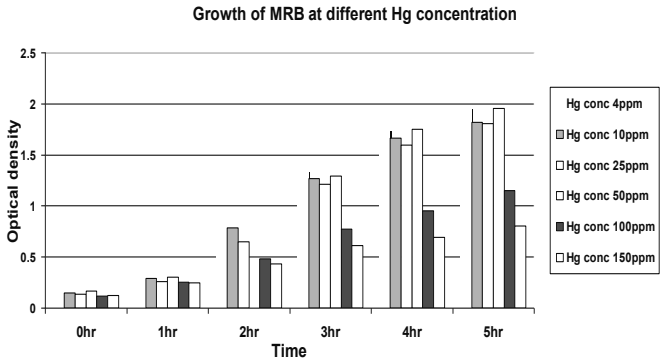

Fig 1: Growth pattern of MRB isolates at various Hg+2 concentrations**.**

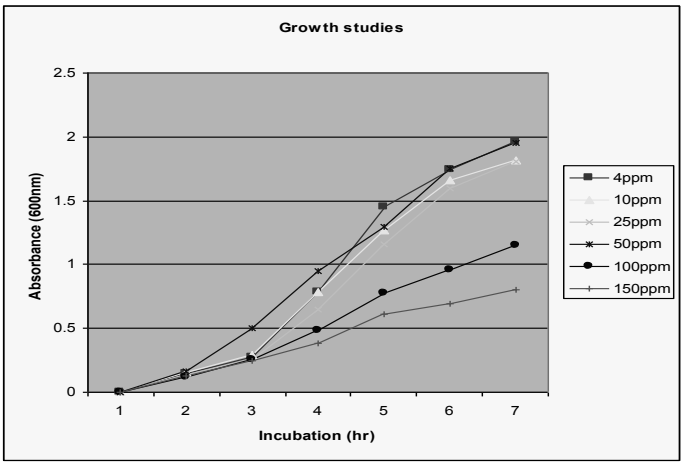

Fig 2: Shows the growth pattern of isolate 1 at various mercury salt concentration

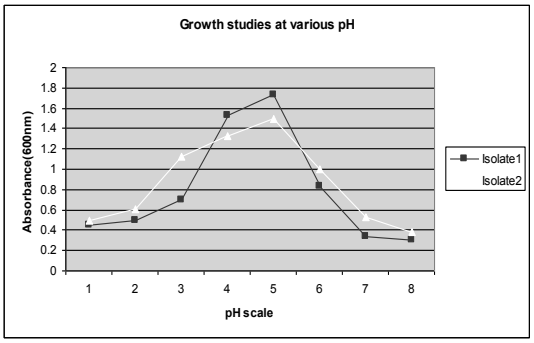

Fig 3: Shows the growth pattern of isolate 1& 2 at various pH

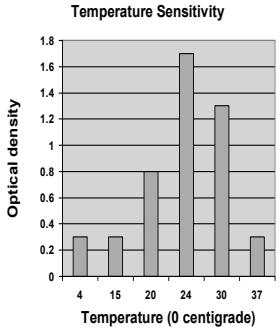

Fig 4: Shows the growth of isolate SC1 at various temperatures

V. Conclusion

The results achieved in the present study show frequencies of mercury resistant bacteria in the contaminated areas (dock) are high and thus a potential threat to the nearby population. The study successfully isolated two mercury resistant bacteria strains. Based on biochemical characteristics they are identified to two different genera: Serratia and Streptococcus orEnterococcus. These isolates have high potential to remove Hg from the dock area (bioremediation) as can tolerate mercury contamination up to 150 ppm which can be further investigatend to check their maximum mercury tolerance level. These can be used in future for detection of mercury toxicity in potential polluted areas. Future investigations can be done at genetic level to know their antibiotic resistance properties of MRB, which may help treatment of mercury affected people.

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