Original Article

Isolation and characterization of potential microbe for bio-remediating heavy metal from Mithi river

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Abstract

Background: Heavy metals contamination is common problem in the current time in river water and other natural resources. Mithi River receives the different kind of industrial and domestic wastes, which also includes toxic heavy metal pollutants. Bioremediation of the heavy metal pollutants can be a promising option for their removal.

Methods: Isolation and characterization of the heavy metal resistant microorganisms obtained from the Mithi River was performed using basic biochemical test and confirmed by 16S rDNA technique. SEM analysis, MIC and flocculation index were carried out to detect the efficiency of bacteria for bioremediation.

Results: Significant populations of heavy metal resistant microorganisms were found in Mithi River. Microorganisms of Mithi River studied in the current research were gram negative as well as gram positive.

Conclusion: Microorganisms having the potential of bioremediation of heavy metals were isolated successfully from Mithi River and their identity was confirmed. The potential of the microorganism for their capacity of bioremediation of heavy metals was also confirmed.

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1. Introduction

Environmental contamination owing to the anthropogenic activities and the natural resources is increasing progressively on account of an unabated increment in population, industrialization and urbanization ([1]). Heavy metal(s) are widespread pollutants of great concern as they are non-degradable and thus persistent ([2]). Extensive industrialization and improper disposal are attributed to be a prime factor responsible for the release of heavy metals into the ecosystems ([3]).

"Heavy metal" is a general collective term, which applies to the group of metals and metalloids with atomic density greater than 4000 kg m-3, or 5 times more than water ([4], [5]). The different sources of heavy metals like Pb, Cd, Hg, Cr, As, Cu and Ni are lead acid batteries, paints, E-waste, Smelting operations, coal- based thermal power plants, ceramics, bangle industry, chlorine-alkali plants, electroplating and thermal power plants etc. ([6]).

Some of these metals are useful to the body in low concentration like arsenic, copper, iron, nickel, etc. but are toxic at high concentration ([7]). Heavy metals contamination impose various health problems like headache, irritability, abdominal pain and various symptoms related to the nervous system, anxiety, bladder and kidney cancer ([8]) either by displacing the vital nutritional minerals from their original place, thereby, hindering their biological function or accumulating, thereby disrupt function in vital organs and glands such as the heart, brain, kidneys, bone, liver, etc. ([9]).

Conventionally, heavy metals remediation can be classified under three categories. Chemical, Physico-Chemical and Biological/Biochemical/Biosorptive Technologies ([5]).Bioremediation is superior to other remediation processes as these processes generate additional pollution, expensive and often do not permanently alleviate the pollution hazard ([10], [11], [12]).

Bioremediation is a collective term used to describe the use of biological systems such as microorganisms to decontaminate polluted soil, water or air. Bioremediation is the process of breaking down or transforming hazardous materials into simple nontoxic substances by biological treatments ([13]). Application of microorganisms for heavy metals remediation is considered as a natural, stable and economical solution ([14]).

Generally, the higher concentration of these metals above threshold levels has deleterious impact on the functional activities of microbial communities. Microorganisms inhabiting in metal polluted areas have evolved various mechanisms to resist themselves against metal stress when exposed to higher concentration of toxic waste ([15]). These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state ([16]) Various microorganisms such as *Pseudomonas* Spp., *Closteridium* Spp. *Bacillus* Spp. *Escherichia* Spp. etc. have been reported as efficient candidates for bioremediation of heavy metals ([17, 18]).Microorganisms and microbial products can be highly efficient bioaccumulators of soluble and particulate forms of metal especially dilute external solutions ([19]).

Mithi River home of diverse micro flora originates at Powai and meets Arabian Sea at Mahim Creek flowing through residential and industrial complexes of Powai, Saki Naka, Kurla and Mahim over a distance of about 15 km ([20]). The river receives raw sewage, industrial waste and garbage of all types. There are many illegal industrial activities along the bank of the river ([21]). Themicroflora of Mithi River can be used for bioremediation purpose of heavy metals as the contamination level of the river make the flora of the river resistance to the heavy metals.

The purpose of present study is to isolate and characterize the microbes from Mithi River which can be further used as a potential tool for bioremediation and removal to toxic metals from the heavy metal polluted areas.

2. Materials and Methods

2.1 Microbial load of the Mithi River- Microbial load of water is also a very important factor in the quality of water. The number of bacteria present in the Mithi River is determined by diluting the water sample (Mithi River) till 10^{-5} and plating dilutions on the nutrient agar containing 0.5% peptic digest, 0.5% sodium chloride, 0.15% beef extract, 0.15% yeast extract and 1.5% agar. The total CFU/ml was calculated by using the number of colonies observed on nutrient agar plates.

2.2 Isolation and purification of heavy metals resistant microorganisms from Mithi river-
Bacteria resistant against lead and mercury were isolated from water sample collected from Mithi River at different sampling points (Kalanagar, BKC area).Water sample was plated nutrient agar medium containing 10 ppm ofPb and Hg as salt of $Pb(NO_2)_2$ and $HgCl_2$ (Pb and Hg added to separate plates). After incubation at 37°C for overnight, isolated colonies were re-streaked again and again till single type of cells are observed in gram staining. Glycerol stock of purified culture were prepared in triplicate and stored at -20°C for further study.

2.3 Characterization and Identification- Isolated bacterial cultures were characterized by biochemical analysis. Bacterial cultures were analysed by indol, methyle red, Voges–Proskauer, citrate utilization, triple sugar iron, catalase, motility, gelatinase test and sugars like glucose, lactose, sucrose, maltose, mannitol and xylose.

2.4 Molecular identification by 16S rRNA PCR-The genomic DNA were extracted from cells by heating the single colony in 100 µl of sterile distilled water for 10 minutes in boiling water bath. The supernatant was collected and used as template DNA. 27F 5' AGA GTT TAG TCCTGG CTC AG 3' and 1492R 5' GGTTAC CTTGTTACGACT T 3' (Merck Millipore, India) were used as forward and reverse primers. Reaction mixture of 40 µl was prepared by 2x of master mix (Genei), nuclease free water, and 20pmol/µl of forward and reverse primer. PCR amplification was carried out on thermocycler (Applied biosystems). PCR cycle was as follow initial denaturation was at 94˚C for 7 minutes, 38 cycles of denaturation at 94˚C for 30 seconds, primer annealing at 54˚C for 35 seconds, DNA extension at 72˚C for 45 seconds and final extension was at 72˚C for 10 minutes. The amplified PCR product was checked on agarose gel electrophoresis and given for sequencing toxcelris Labs Ltd., Ahmedabad. DNA sequences were compared with already submitted sequence in database using BLAST software.

2.5 Determination of heavy metal tolerance of resistant isolate- Maximum tolerance limit of isolated bacteria against Lead was determined. The isolated bacteria were grown in nutrient broth in presence of lead at concentration of 10, 30, 50, 100, 200, 300, 400, 500, 700 ppm. The tube were incubated at 37°C for 24 hours.

2.6 SEM analysis of heavy metal resistant isolates- In order to study the effect of heavy metals of bacterial surface, isolated bacteria were subjected to scanning electron microscopy (FEI quanta FEG 250). Culture without metals was used as control and culture with metals was taken as test. Cells were centrifuged at 10,000 rpm for 5 minutes. The pellet was washed with sterile PBS and re-centrifuged at 10,000 rpm for 5 minutes. This step is repeated two times to remove the traces of media. Re-suspend the pellet in 500 µl of PBS. Mix the pellet homogeneously with PBS than load 50 µl of mixed culture on glass coverslip. Fix the culture by putting 3.7% formaldehyde on coverslip. Mix it by gently moving coverslip and keep it for 5 minutes. Dehydrate the culture in oven at 80°C for one hour. The bacteria was viewed at low vacuum.

2.7 Flocculation index of the isolate- Flocculation index in absence and presence of lead was determined for isolated bacteria.One was control without any lead and other media tubes containing 20, 40, 80 ppm of lead. 1 x 10-7 cells/ ml was inoculated in each nutrient broth tube. The tubes were incubated at 37°C for 24 hours. After 24 hours OD_{600} was determined after mixing the media (F_0). The tubes were kept undisturbed at 37°C for 2 hours to let the cells settle down. Supernatant were taken into new tubes without shaking the media and $OD_{600}$was taken of each tubes (F_t). The floc-

culation index was determined using the formula given below (Sannasi P. *et. al*.2009);% Flocculation index = $\frac{F_0 - F_t}{F_0} X\ 100$

3. Results and Discussion

3.1 Microbial load of the riverwater sample of obtained from Mithi River was cultured on culture media for isolation. The number of colonies grown on the plates is given in table 1. The CFU count of the river was found to be $4.2x10^5$ cells/ml and the CFU count of river water in presence of 10 ppm of lead was $1.7x10^5$ cells/ml. Decrease in CFU of river water in presence of lead indicate that not all the microbes of the river are able survive at 10 ppm of lead. 40.48% of the microbe from water sample were found to grow in the presence of lead. This indicates that the significant population of micro flora of the river is acclimatized to the stress condition exerted by the metal present in the river on the bacterial cells. Further, the microorganisms isolated from the Mithi River could be used as the potential candidate for the bioremediation of heavy metals.

Table 1: Number of colonies observed after plating the dilutions on the nutrient agar plates without any lead and in presence of 10 ppm of lead.

Dilution	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
CFU (without lead)	Confluent	Confluent	148	26	3	1
CFU (with lead)	Confluent	Confluent	113	22	2	0

3.2 Isolation of heavy metals resistant microorganisms from Mithi River-Organisms was carried out in the culture media containing lead. Five organisms which had shownthe significant resistance towards lead were isolated successfully and purified. Thesemicroorganisms were selected on the basis of their differential colony characteristics and were given a code of WPb-1, WPb-2, WPb-3, WPb-4 and WPb-5. Majority of the lead resistant microorganisms that were isolated showed the gram negative character.Various investigations have reported the gram negative character of the lead resistant microorganisms which corroborates with the current investigation ([22]).

Table 2: Colony characteristics of isolates**.**

Sr. No.	Media used	Characters	WPb-1	WPb-2	WPb-3	WPb-3	WPb-5
1	NA	Size(mm)	1	3	2.5	3	2
2	NA	Shape	Circular	Circular	Circular	Circular	Circular
3	NA	Color	White	White	Creamish	Creamish	Creamish
4	NA	Margin	Entire	Entire	Entire	Entire	Entire
5	NA	Opacity	Translucent	Opaque	Translucent	Opaque	Translucent
6	NA	Elevation	Convex	Convex	Raised	Convex	Convex
7	NA	Consistency	Smooth	Mucoid	Smooth	butyrous	Mucoid
8	NA	Gram's Nature	Gram + Bacilli	Gram –ve coccobacilli	Gram –ve coccobacilli	Gram –ve coccobacilli	Gram –ve coccobacilli

NA-Nutrient agar

3.3 Characterization and Identification- Different colony characteristics were observed for each isolate. Colony characteristic are mentioned in the table no. 2. The biochemical analysis of five isolate are presented in Table 3.

Table 3: Biochemical test of five isolated bacteria.

Test	WPb-1	WPb-2	WPb-3	WPb-3	WPb-5
Indol test	Negative	Negative	Negative	Negative	Negative
Methyl red	Positive	Negative	Positive	Positive	Negative
Voges–Proskauer	Negative	Negative	Negative	Negative	Negative
Citrate utilization	Negative	Positive	Negative	Negative	Positive
Triple sugar iron	Red/Red	Red/yellow, gas production	Yellow/Yellow	Yellow/Yellow	Red/Red
Catalase	Negative	Negative	Negative	Negative	Negative
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Gelatinase	Negative	Negative	Negative	Negative	Negative
Glucose	Negative	FG	Negative	F	Negative
Sucrose	Negative	FG	F	F	Negative
Lactose	G	F	Negative	Negative	Negative
Mannitol	Negative	FG	F	Negative	Negative
Maltose	Negative	FG	FG	F	Negative
Xylose	Negative	G	Negative	Negative	Negative

F:Sugar fermented, G: gas production

3.4 Molecular identification by 16S rRNA- PCR amplification of 16S rDNA of all the isolates produced 1.5 Kb products. The distinct bands were seen adjacent to 1.5 kb band of DNA ladder on electrophoresing the DNA on 1.5% agarose (fig. 1). The bacteria identified were *Bacillus thuringenesis* strain KPWP-1, *Enterobacter cloacae*, *Acinetobacter barumanii* strain AIEB-2, *Pseudomonas fluorescens* and *Acinetobacter johnsonii*. *Bacillus* sp. and *Pseudomonas* sp. are also commonly found in industrial effluent and many researchers have isolated and studied heavy metal bio sorption competence of this group of microorganisms ([23]). This indicates that the microorganisms isolated and selected in the current investigation have the potential for the bioremediation of heavy metal which is also supported by their growth in presence of heavy metals.

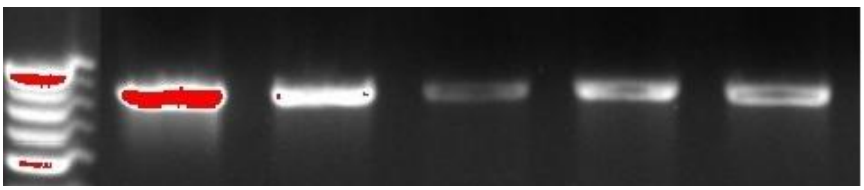

Figure 1: AGE of 16S rDNA PCR product. Band of 1.5 kb were observed for WPb-1, WPb-2, WPb-3, WPb-4 and WPb-5 isolates.

3.5 Determination of heavy metal tolerance of resistant bacterialisolate- Metal tolerance of all the bacterial isolates was found to be very high. The growth of four bacterial isolates out of five was seen till 500 ppm which indicates that the isolated bacteria can survive in the high concentration of metal and can be used as the potential candidate for the bioremediation. The growth of bacteria at different metal concentration was shown in the table 4. The MIC of WPb-1, WPb-2, WPb-3, WPb-4 and WPb-5 for lead was found to be 500 ppm, 600 ppm 600 ppm, 600 ppm and 600 ppm respectively. Four out of five isolate showing MIC of 600 ppm of lead. High tolerance of the lead by the bacterial isolates of the current investigation indicates their potential for the bioremediation of heavy metals.

Table 4: Tolerance limit of the isolated bacteria.

Sr. No.	Concentration of lead (ppm)	WPb-1	WPb-2	WPb-3	WPb-4	WPb-5
1	10	+	++	++	++	++
2	25	+	++	++	++	++
3	50	+	++	++	++	++
4	100	+	++	++	++	++
5	200	+	+	++	++	+
6	300	+	+	++	++	+
7	400	+	+	+	++	+
8	500	-	+	+	++	+
9	600	-	-	-	-	-

+ Sign indicates growth and ++ sign indicates dense growth & - no growth.

3.6 SEM analysis of heavy metal resistant bacterial isolates- Difference in the image of the WPb-2 was seen after SEM analysis. The surface of bacteria shown accumulation of lead when grown in the presence of lead. The SEM analysis indicates that the bacteria inhibit influx of metal in the bacterial cell. This mechanism may be used by the microorganism at the initial stages when exposed to the metal. Some morphological and physiological changes in bacteria have been observed when exposed to metals. The production of exopolymers or biopolymers is sometimes related to the cell's defense mechanisms as it immobilizes toxic heavy metal ions thus inhibiting them from entering the cell as reported by Sannasi P. ([24]).

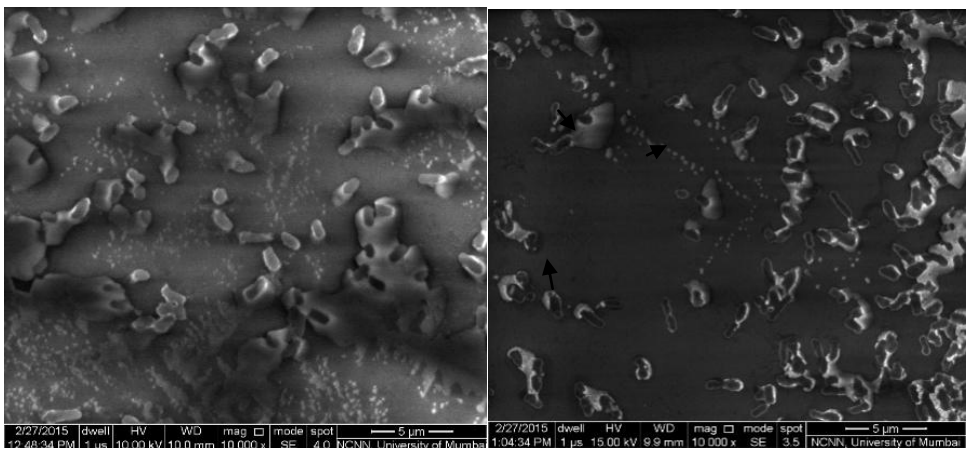

Figure 2:SEM images of the WPb-2. (a)SEM image of WPb-2 without lead and (b)SEM image of WPb-2 in presence of lead magnified to 10,000X. Arrow showing the accumulated lead on the surface of WPb-2 isolate.

3.7 Flocculation index of the isolate- The flocculation index of the bacterial isolates in the current investigation increased with the increase of the lead concentration. Control group of bacteria showed low flocculation index as compared to the bacterial isolates growing in presence of heavy metals. This may be due to the non-availability of the metal ions on the surface of the bacterial isolates containing tube as there is no metal ions to bind on the surface on bacteria make floccules of the cells which settle down faster than unfloculated cells. This result confirms the binding and accumulation lead on the surface of the bacteria.

Table 5: The flocculation index of WPb-2 isolate at different concentration of lead

Growth tube	Flocculation index (%)
Control tube	5.1
Metal tube (40ppm)	6.0
Metal tube (80ppm)	6.7
Metal tube (100ppm)	7.0

Conclusion:

Heavy metal resistant microorganisms were isolated from the Mithi River. Both gram negative and positive microorganisms studied in the current investigation have shown the capability of bioremediation of heavy metals. Microorganisms, viz *Bacillus thuringenesis* strain KPWP-1 and *Pseudomonas fluorescens*, isolated and studied under the current investigation have been earlier reported for their bioremediation characteristics. While the microorganisms such as *Enterobacter cloacae*, *Acinetobacter barumanii* strain AIEB-2 and *Acinetobacter johnsonii* have not been reported so far for their bioremediation characteristics. Some new species was also identified that are not extensively found in bioremediation process. On the basis of results of MIC, SEM and flocculation, it has been observed in the current investigation that these microorganisms have the potential to grow in the natural sites containing the toxic heavy metal. Further, these organisms may possess the mechanism for the bioremediation of heavy metals. Exopolymer secretion by the microorganisms studied in the current investigation may be one of the mechanisms used by them to survive in the metal contaminated sites and remediate the heavy metals. The isolated microorganisms of the present study can be used for bioremediation of heavy metal.

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Competing Interests:

None declared

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