Isolation, Identification and Bioaccumulation of Multi Metal Resistant Bacteria from Mirza Khan Pond of Darbhanga, India.

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ABSTRACT: *In the present study multi heavy metal resistant bacterium was isolated from polluted water of Mirza Khan Pond of Darbhanga, India. On the basis of morphology, Biochemical test, Acid production test and 16S rRNA gene sequencing analysis, the isolate was identified as Pseudomonas aeruginosa. The The minimal inhibitory concentration (MIC) of isolates against cadmium (Cd), Zinc (Zn), Iron (Fe) and lead (Pb) was determined on solid medium. The isolated strain Pseudomonas aeruginosa showed resistance against all the four heavy metals up to 1000 mg/l. The percentages of Bioaccumulation of Cd, Zn, Fe and Pb by Pseudomonas aeruginosa were determined 22.14%, 64.59%, 67.26% and 38.05% respectively. The results of present study showed that the multi metal resistant bacterium Pseudomonas aeruginosa can be used for heavy metal bioremediation.*

Key Words: *Bioaccumulation, Metal resistance, Heavy metals, Pseudomonas aeruginosa,*

1. INTRODUCTION

Any type of Pollution whether it is air, water, soil *etc*. have become major cause of life thread not only for human being but also for all living organism. Increasing population, urbanization, industrialization and some natural variations, result in the increase in contamination of every types of ecosystem. Causes of water pollution are one of the major fields to concern. And the major causes of water pollution are discharge of untreated wastewater into the river, ponds and lakes. Wastewaters contain different types of toxic heavy metals, synthetic dyes and hazardous substances (Das *et al*., 2011; Saratale *et al*., 2011; Sheikh *et al*., 2017). Some of the heavy metals (cobalt, chromium, nickel, iron, manganese and zinc) at low concentration are essential for metabolic processes of living organism and some of the heavy metals (Lead, Cadmium,) even at very low concentration are harmful for the same. At high concentration all essential and non nonessential metals are toxic to the organisms (Rathnayake *et al*., 2010). In water pollution, metal pollutants are great concern as it accumulate in human, animals, plants and microorganism (Ogbo *et al*., 2011; Hashem *et al*., 2002) and causes several metabolic and physiological disorders (Rani and Goel, 2009; Matyar *et al*., 2010). Heavy metals are stable and persistent in environment, since they cannot be degraded or destroyed therefore it involves in food chain (Sevgi *et al*., 2010). Bioaccumulation is an active process dependent upon metabolic energy of microorganisms (Rani and Goel, 2009). It is one of the microbial processes to remove heavy metals from their surrounding (Zolgharnein *et al*., 2010).

Therefore, this study was designed with the objective to isolate the multi metal resistant bacteria from heavy metal contaminated water of Mirza Khan Pond and to determine their capability on the removal of metals (Cd, Zn, Fe and Pb) through bioaccumulation process.

2. MATERIALS AND METHODS:

2.1 Collection of samples

The Mirza khan pond is one of the major pond of Darbhanga situated at ***26^{0}07'42.5"N 85^{0}53'54.2"E.*** It is surrounded by several hospitals, medical shops, small industries and some resident. Because of not strong management of medical waste, washing of infected clothes of hospitals and direct flowing of sewage from that area the water of the pond became polluted harshly. Water samples from the pond for bacteriological analysis were collected in the autoclaved glass bottles. After keeping the samples into the ice box it was immediately transferred to the laboratory.

2.2. Isolation of heavy metals resistant Bacteria

Water sample were serially diluted from 10^{-1} to 10^{-7} labeled dilution tubes. Samples of 100µl from 10^{-5} and 10^{-6} were taken and spread onto the different Nutrient Agar plates. Different isolates were given the name as MK01 to MK20 when randomly selected from the plates and streaked onto the separate Petri plate containing 50 mg/l of each metal. The enumerations of bacterial colonies were done by the method

as described by Reddy *et al.* (1986). Pure cultures were taken from the Petri plate using 10 µl sterile loops and then suspended on the sterile cryopreservation media. These suspensions were then stored at -80°C.

2.3. Determination of minimal inhibitory concentrations (MIC) for Cd, Zn, Fe and Pb

The minimum inhibitory concentration (MIC) of Cd, Zn, Fe and Pb of bacteria were determined by disc diffusion method. The concentrations of all the four heavy metals were taken between 250 to 1000 mg/L. Filter paper discs were saturated with heavy metals for 30 min, and then placed on nutrient agar plates and incubated for 24 h at 30°C. 6 out of 20 bacteria could grow between the range 250 to 1000 mg/L of different heavy metals. And out of 6, one bacterium showed resistivity against all the four heavy metals up to 1000 mg/L.

2.4. **Bacterial characterization**

For identification of multi metal resistant bacteria, physical, biochemical, and acid production test were performed along with morphological studies.(Barrow and Feltham, 1993, Bergey et al., 1974)

S16 rRNA gene based identification

For the sequencing of 16S rRNA gene and identification of isolate, DNA extraction, PCR amplification of 16S rDNA and purification of the product was carried out as described by Rainey *et al. (*1996). Genomic DNAs were isolated and 16s rRNA gene was amplified by using universal primers F27 (5'-GAGTTTGATCCTGGCTCAG-3') and R1492 (5'- TACCTTGTTACGACTT-3') (Sigma, India). Then the strain was sent for sequencing along with primer to Xcelris Labs, Ahmadabad. Obtained sequence was compared with sequences available online in a GenBank database (http://www.ncbi.nlm.nih.gov). Homology was also performed online by BLASTn search tool (https://blast.ncbi.nlm.nih.gov)

2.5. Bioaccumulation of heavy metals by isolated strain

To check the ability of the isolated strain (*Pseudomonas aeruginosa*) to resistance heavy metal salt it was inoculated on liquid nutrient media supplemented with known concentration of different heavy metals. All the cultures were incubated at 30°C for 48 hours. After incubation cultures were centrifuged at 4000 rpm for 15 minute. Final concentration of heavy metals in supernatant was measured with the help of inductive plasma coupling mass spectrometer **(** iCAP RQ ICP-MS**)**.

3. RESULTS AND DISCUSSION

3.1. Enumeration and isolation:

The total viable cell count from the 10^{-5} dilution agar plate was found 1.3 x10^{8} CFU/ml. The bacterial counts on nutrient agar supplemented with different heavy metals were found from 1.2 x10^{6} CFU/ml to 4.4 x10^{6} CFU/ml. 20 bacterial isolates were randomly selected from different nutrient agar plates supplemented with different metals (5 from each plate) and given the name MK01 to MK20.

Minimum inhibitory concentration of the bacterial isolates against heavy metals

6 out of 20 bacteria could grow between the range 250 to 1000 mg/L. And out of 6, one bacterium MK02 showed resistivity against all the four metals up to 1000 mg/L. Table -1

Table -1 MIC of the bacterial isolates against different heavy metals.

Strains	Cd(mg/l)				Zn(mg/l)				Fe(mg/l)				Pb(mg/l)				Mixed
	250	500	750	1000	250	500	750	1000	250	500	750	1000	250	500	750	1000	
MK01	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
MK02	++	++	+	+	+++	++	+	+	++	++	+	+	+++	+++	+	+	+
MK04	++	+	-	-	+	+	-	-	+	+	-	-	++	+	-	-	+
MK08	-	-	-	-	++	+	-	-	+	-	-	-	+	-	-	-	-
MK12	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
MK15	+	-	-	-	++	+	-	-	+	+	-	-	++	-	-	-	+

3.2. Characterization and molecular identification of isolated bacteria

Multi metal resistant strain MK02 was characterized and identified by morphological, physical, biochemical and acid production tests (Table-2). It was presumptively identified as Pseudomonas species. After submitting 16S rRNA gene to Blastn database 16S ribosomal RNA sequences it shown very close relation with rDNA sequence of *Pseudomonas aeruginosa.*

Table -2 Morphological, Biochemical and Acid production test of bacterial isolates MK02 (*Pseudomonas aeruginosa)*.

Morphological characteristics	
COLONY COLOUR	White
CELL MORPHOLOGY	Rod shape
GRAM REACTION	-
OPTIMUM pH	7
OPTIMUM TEMPERATURE(^{0}C)	30°C
Biochemical test	
Growth in MacConkey agar	+
Indole test	-
Nitrate reduction	+
Catalase test	+
Oxidase test	+
Acid production test	
Glucose	-
Maltose	-
Arabinose	-

3.3. Bioaccumulation of heavy metals by isolated strain

Isolated strain **(*Pseudomonas aeruginosa)*** was determined for accumulation of different heavy metals. It was found that the ***Pseudomonas aeruginosa*** removed 22.14% cadmium, 64.59% zinc, 67.26% iron and 38.05% lead from medium.

Table: 3 Table showing results of percentage accumulation of different heavy metals from medium by MK02 (*Pseudomonas aeruginosa)*

Parameters(mg/100ml)	Initial Concentration	Final Concentration	% Accumulation
Cadmium	2.98	**2.32**	**22.14**
Zinc	1.61	**0.57**	**64.59**
Iron	2.78	**0.91**	**67.26**
lead	3.18	**1.97**	**38.05**

3.4. Discussion

These days contamination of water with heavy metals became a serious problem for aquatic life, other animals as well as for us. So there is need of cost effective and environmental friendly strategies for removal of heavy metals from water. The ability of microorganisms to grow in heavy metals contaminated site and their bioaccumulation can be use for waste water treatment and environmental cleaning. When microorganisms are exposed to high heavy metal concentration, they developed different resistance mechanisms for survival. These mechanisms can be used for detoxification of heavy metals from polluted site. Keeping these problems in mind, isolation, identification and bioaccumulation of multi metal resistant bacteria from the polluted water of Mirza Khan Pond were carried out.

Initial screening of the samples for heavy metal resistance ability showed that few randomly isolated samples were grown in Cd, Zn, Fe and Pb at 50mg/l concentration. Out of 20 bacteria MK01, MK02, MK04, MK08, MK12 and MK15 could show MIC between the range 250 to 1000 mg/L against different heavy metals. Among them only one MK02 strain showed resistivity against all the four heavy metals up to 1000 mg/l. MK02 bacterial strain was then characterized by morphological, biochemical and Acid productin test. Identification of MK02 strain by Bergey's Manual of Determinative Bacteriology (Barrow and Feltham, 1993; Bergey et al., 1974) and 16S rRNA gene sequencing analysis confirm Pseudomonas aeruginosa. Bioaccumulation of different heavy metals by Pseudomonas aeruginosa strain was determined in lab condition and it was observed that Pseudomonas aeruginosa strain removes 22.1% Cadmium, 64.59% Zinc, 67.26% Iron and 38.05% Lead. The accumulation of heavy metals from medium was studied with the help of inductive plasma coupling mass spectrometer. The present study evaluates that isolated Pseudomonas aeruginosa strain can be used for removal of different heavy metals from polluted water bodies because it can easily survive in contaminated conditions and shows efficient accumulation of heavy metals from contaminated site .

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