



MICROBIAL BIOTRANSFORMATION OF MERCURY USING A MARINE *PSEUDOMONAS PUTIDA*

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ABSTRACT

Industrial revolution resulted in increasing amount of waste materials with the introduction of metal contaminants into the aquatic system. Toxic metals tend to accumulate in fresh and marine water sediments from which they enter into the food chain, reaching human beings and produce acute and chronic ailments. The present work were focused to isolate, identify and optimize potential mercury resistant *Pseudomonas putida* strain against mercury pollution using physical, analytical and molecular level approaches. From our study, we obtained novel insights on mercury resistance *Pseudomonas putida* strain and its benefit regarding contaminated heavy metals bioremediation.

KEY WORDS: *Pseudomonas putida*, SDS – PAGE, RAPD, Atomic absorption spectroscopy.

INTRODUCTION

Industrial revolution resulted in increasing amount of waste materials which is aggravated by the growing population. As industries are around water ways, development of new towns and urbanization transform lakes, rivers and coastal waters as dumping sites of various pollutants and sewage. Experts estimated that at least one million different pollutants enter into natural waters in various regions of the earth. These pollutants harm the aquatic environment at varying degrees, substances such as polycyclic aromatics, pesticides, radioactive elements and heavy metals severely damage the environment affecting aquatic and human life. Toxic metals such as mercury, cadmium, arsenic, copper etc., tend to accumulate in fresh and marine water sediments from which they may be enter into the food chain, there by reaching human beings and produce chronic and acute ailments.^[1]

The introduction of metal contaminants into the aquatic system has various sources, including smelting processes, fuel combustion via atmospheric fall out, pollution from leaks, effluents and dumping activities and run off from terrestrial systems. On the other hand, polluted water bodies led to the metal contamination of terrestrial ecosystems by irrigation, dredging activities and through biota.^[2] Of the various chemical elements, metals make up the largest group.^[3] Microorganisms have co-existed with metals since early history. This is reflected in the wide range of divalent or transition

metals at the active centres of many enzymes. The chemical properties of the metal have been recruited for catalysing key reactions or for maintaining protein structure. These metals are therefore required in minute amounts for normal cell metabolism and their intake is subjected to intricate homeostatic mechanisms that ensure sufficient but not excessive acquisition. Many other metals seem to serve no biologically relevant function. Instead, they cause damage, mostly due to their affinity for the sulphhydryl groups of proteins, which they block and inactivate.^[4]

More recently, anthropogenic mobilisation from metal ores has created novel, metal-loaded niches with a strong selective pressure for metal endurance. Several prokaryotes show specific resistance determinants, tolerating a wide range in concentration of these elements by a variety of mechanisms. This knowledge forms the basis for novel strategies aimed at remediating (or at least mitigating) intensive and extensive pollution caused by heavy metal ions. Virtually all metals can produce toxicity when ingested in sufficient quantities, but there are several which are especially important because either they are so pervasive, or produce toxicity at a lower concentration. Heavy metals such as lead, mercury, iron, copper, cadmium, arsenic and nickel are generally highly toxic.^[5]

In general, heavy metals produce their toxicity by forming complexes or "ligands" with organic

compounds. The most common groups involved in ligand formation are oxygen, sulphur and nitrogen. When metals bind to these groups they may inactivate important enzyme systems, or affect protein structure.^[6] There is a considerable cross-over in many of the toxic manifestations of the mercury, and in the agents used to treat the toxicity. Mercury can combine with a methyl group to become methyl mercury. This form of mercury is found in a variety of environmental pollution conditions and can produce a range of toxicities. Recent estimates calculate the annual global emissions between 4800 – 8300 tons per year. The cost of remediation of each pound of mercury from the environment using current technologies is in the range of tens of thousands of dollars. Therefore, finding alternate remediation approaches is an urgent need.^[7]

There is an increasing interest in bacterial strains that degrade aromatic compounds and tolerate toxic metals. Bacteria and fungi play a fundamental role in the biogeochemical cycles in nature. These microorganisms remineralize organic matter to carbon dioxide, water and various inorganic salts.^[8] The marine organisms that contribute directly to pollutant degradation are principally bacteria, and to a lesser (or indirectly) degree fungi, protozoa, and benthic invertebrates. Microorganisms in contaminated environments have developed resistance to mercury and are playing a major role in natural decontamination. The *mer* operon is widely distributed amongst the natural microbial community possessing resistance to mercury and bacterial adaptation to high concentration of mercury include the induction of the *mer* operon through the action of the *merR* (regulator of *mer* operon).^[9] Organomercurials are highly toxic because of their hydrophobicity, which facilitates their movement across cell membranes and accumulation in membrane bound organelles, inhibiting essential oxidative and photosynthetic pathways.^[10]

Mercury-resistant bacteria are now considered a potential approach to biological remediation. Bioremediation strategies including biotransformation, biosorption and bioprecipitation of mercurials have been developed and rarely been applied to remediation of mercurials in the environment.^[11] Biotransformation through bacterial reduction and volatilization of mercurials mediated by the mercury-resistant bacteria for the environmental remediation of mercury pollution has been one of the most actively studied processes. Bacterial bioremediation using mercury-resistant bacteria has been shown to be useful. There have been several studies on multiple resistances offered by both natural and engineered bacteria. Amine and carboxyl groups of the cell wall of *Bacillus subtilis* were chemically modified individually to neutralize their electrochemical charge for determination of their contribution to the metal uptake process.^[12]

The microbial uptake of mercury is thus a key step both in its methylation and its bioaccumulation. Most metals enter cells via specialized transmembrane cation transporters, or they “leak” through the transporters of other metals. Indeed, at high concentrations, Hg(II) is transported into *mer*-carrying bacteria via a specialized MerT transport protein.^[13] In high concentrations, heavy metals ions react to form toxic compounds in cells. To have a toxic effect, however, heavy metal ions must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems one is quick and unspecific, driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP, and the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. While the first mechanism is more energy efficient, it results in an influx of a wider variety of heavy metals, and when these metals are present in high concentrations, they are more likely to have toxic effects once enter inside the cell.^[14]

MATERIALS AND METHODS

Collection of the samples

Mercury-resistant bacteria were isolated from water samples taken from Uppanar estuary of Cuddalore where industrial effluents of SIPCOT area are mixing with coastal waters.

Bacterial isolation: Marine bacteria were isolated by serially diluting 1 ml of the water sample in sterile 50% sea water and 0.1 ml of the appropriate dilutions were plated by spread plate technique on Zobell Marine Agar plates (Hi-media India) supplemented with 10ppm of mercuric chloride. Later, the plates were incubated at 35°C for 24 h and observed for bacterial growth.

Microbial identification: Identification was done based on morphological, cultural, biochemical and physiological characteristics based on Cappuccino *et al.* (1999) and Schaad *et al.* (2001) and the results were cross checked with Bergey's Manual of Determinative Bacteriology (Buchanan *et al.*, 1974).

Growth study: Optimal growth of potential strain was estimated at various parameters such as temperatures (25, 30, 35 and 40°C), different incubation periods (0-84hr) and pH (5.5, 6.5, 7.5, 8.5, 9.5 and 10.5). The impact of NaCl concentration on biomass production was also evaluated using various concentrations (0.0, 1.5, 2.5 and 3.5%) in NB with 10ppm mercury as substrate.

Growth Media: Nutrient broth was used for the growth and maintenance of the organisms. Cells were grown at pH 7.0 on a NB medium. Mercury (10 ppm-600 ppm) was added to the medium after autoclaving.

Mercury removal/reduction: Mercury removal was done in 3 sets of experiments with increasing concentration of mercury used in mineral medium.

- Mercury removal study with lower concentration – Mercury concentration in the range of 10 ppm-100 ppm.
- Mercury removal study with medium concentration – Mercury concentration in the range of 125 ppm to 200 ppm.
- Mercury removal study with higher concentration (Mercury concentration from 200 ppm to 600 ppm).

The above experiments were carried out in 250ml conical flasks containing 100ml of medium with the respective mercury concentration. The flasks were incubated at room temperature. Based on the turbidity, the frequency of analysis was decided as represented. Thereafter, mercury contents in medium and cells were measured by cold vapour atomic absorption spectrometry (Perkin Elmer AA700) using the procedure of Sadhukhan *et al.* (1997).

SDS-PAGE (protein profile): Protein profile of control and mercury treated cells of *P. putida* was observed in the present study. Control strain originally isolated using 10ppm mercury was transferred to nutrient broth without mercury for 3 consecutive times. Each time log phase cultures were centrifuged, and transferred to nutrient broth which was free from mercury. To study the protein profile of treated culture, the culture obtained through 100 ppm mercury amended broth was used as at this concentration growth was comparatively better.

Protein Separation- SDS-PAGE- (Laemmli, 1973): The proteins were separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein can be determined by comparing its electrophoretic mobility in SDS-PAGE gel with mobility marker proteins of known molecular weight.

Plasmid isolation: The plasmids were isolated from mercury-resistance strain adopting the method of Sambrook and Russel (2001). Cells were pelleted by centrifugation at 5000 rpm for 15 min at 4°C and suspended in 100 µl of solution I and centrifuged for 5 min. Then 200 µl of solution II and 150 µl of solution III were added. The suspension was mixed vigorously and precipitated with ethanol and stored in TE buffer at -20°C. 0.8% of agarose gel was casted by dissolving 0.8 g of agarose in 100 ml of IX TAE buffer and the plasmid DNA was loaded along with the loading buffer in the wells formed on the gel and allowed to run in an electrophoresis tank by providing 50 mA of current, and the DNA bands were viewed under UV trans-illuminator in a Gel-documentation system.

Agarose Gel Electrophoresis of Plasmid DNA: The isolated plasmid was analyzed by agarose gel electrophoresis using 1.5% agarose gel in IX TE buffer and with 2 µl of ethidium bromide was incorporated for plasmid DNA staining. 25 µl of samples were added to each well and was run in IX TE buffer at 100 V for 30

min. Hind III digested DNA was used as the molecular weight marker.

Curing of plasmid DNA: Curing of the plasmids was performed by incubating the isolates overnight at 30°C in LB broth containing 25 mg/ ml of acridine orange. Sample of 0.1 ml from culture broth after appropriate dilution were separately plated on LB agar plates containing various concentration of mercury by spread plate technique and the plates were incubated at 30°C for 24 h. The cultures were inoculated to LB broth also, which contained the same concentration as in plates.

RAPD ANALYSIS

Template DNA Preparation: 1.5 ml of overnight grown cells were taken. The tube was spun at 10000 rpm for 2 min. The supernatant was discarded and dissolved the pellet completely in 250 µl of lysis buffer solution. The tube was incubated on ice for 10 min. Then the tube was again incubated at 65°C for 10 min. and then allowed to cool at room temperature. The tube was again spun at 10000 rpm for 5 min. To the supernatant, double the volume of absolute ethanol (ice cold) and 5 µl of 3 M sodium acetate were added. The tube was incubated on ice for 5 min. and again spun at 10000 rpm for 5 min. The resulting pellet was washed with 200 µl of 70% ethanol. Air dried completely and dissolved in 50 µl of sterile distilled water.

Procedure: Reactions were performed in 50 µl volumes in 0.2 ml optical-grade PCR tubes (Tarsons, India). Each 50 µl reaction volume contained 25 µl Master mix (2X) consisting of 100 pM of random primer, 1.5 U of Taq DNA Polymerase, 1.5 mM MgCl₂ and 100 µM dNTPs (Genei, India). The template DNA was isolated according to the Standard Extraction buffer protocol (Sambrook *et al.*, 2000). The DNA concentration of 100 ng in 2 µl was used per reaction. The RAPD-PCR cycling conditions were - Initial Denaturation at 94°C for 5 min, Denaturation at 94°C for 1 min., primer annealing at 36°C for 1 min. and extension at 72°C for 2 min. for a total of 35 cycles, followed by a final extension at 72°C for 10 min. RAPD was performed in a Thermal cycler.

RAPD analysis was performed using Random primers (Genei, Bangalore). The Sequence of the primers were:
Primer 1: 5'- GGTGCGGGAA - 3'
Primer 2: 5'- CCCGTCAGCA - 3'
Primer 3: 5' - GTTTCGCTCC - 3'.

Agarose Gel Electrophoresis (AGE) Analysis: The RAPD products were detected by subjecting a sample from each reaction tube to 1.5% agarose gel electrophoresis stained with ethidium bromide (1.6 mg /ml) at 11 V cm 1 for 90 min in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA (pH 8.0). The sequences of the amplicons were determined in order to confirm the successful amplification of polymorphism.

Polymorphism percentage: Polymorphism percentage between the control and treated bacterial samples were detected according to Blair *et al.* (1999).

Polymorphism Calculation: $A/B \times 100 = \text{Percent Polymorphism}$

Where, A = Number of polymorphic bands in the test and B = No. of bands in the control.

RESULTS AND DISCUSSION

Isolation of Mercury-resistance bacteria: A number of isolates growing at 35°C on Zobell marine Agar containing mercury were obtained.



Fig. 1: Enrichment of collected samples with mercury.

From **Fig. 1**, Colonies were selected based on varying morphology. 4.1×10^6 CFU/ml was the density of mercury utilizing bacteria. Based on growth in mercury containing broth, most potential strain was selected (i.e.) the one that grew in higher concentrations was selected for further study.



Fig. 2: Isolated colonies of *Pseudomonas putida*.

From **Fig. 2**, mercury removal by a high tolerant marine bacterial strain *Pseudomonas putida* isolated from a polluted environment was attempted. Nutrient broth medium was used to check the biotransformation pattern.

Identification of potential strain: Gram staining was performed on logarithmic phase cultures and confirmed using a positive control. Other tests were as per the Bergey's manual of determinative bacteriology.

Table 1: Biochemical Characterization of *Pseudomonas putida*.

Characters	Results
Gram reaction	-
Shape	Rod
Fluorescent pigment	-
Pyocyanin	-
Carotenoid pigment	-
Growth at 41°C	-
Oxidase	+
Gelatin hydrolysis	-
Starch hydrolysis	-
Tween 80	-
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Glucose	+
Trehalose	-
Inositol	-
Sucrose	+
Lactose	-

From the Table – 1, the strain was identified as *Pseudomonas putida* since the shape is rod and gives positive for Oxidase, Arginine dihydrolase, Glucose, sucrose and negative for Gram reaction, Fluorescent pigment, Pyocyanin, Carotenoid pigment, Growth at 41°C, Gelatin hydrolysis, Starch hydrolysis, Tween 80, Lysine decarboxylase, Ornithine decarboxylase, Trehalose, Inositol and Lactose.

Growth optimization: Maximum growth occurred was observed at 36h under the conditions of pH 8.5, temperature 35°C, salinity 2.5% and mercury concentration 10ppm in shake flask. Growth was measured spectrophotometrically at 600 nm.

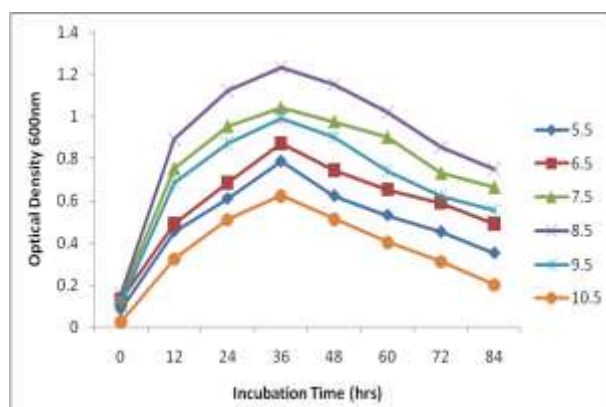


Fig. 3: Effect of pH on growth.

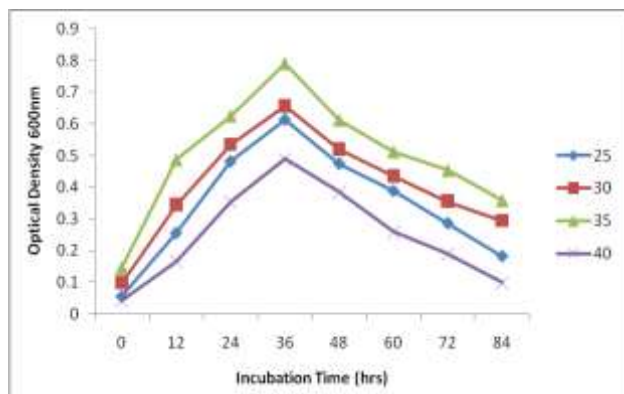


Fig. 4: Effect of Temperature on growth.

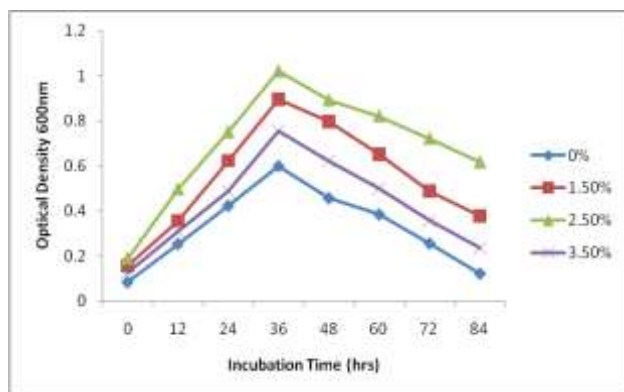


Fig. 5: Effect of Salinity on growth.

From Fig.3, Fig.4 and Fig.5, the potential strain *Pseudomonas putida* growth was estimated at various parameters such as temperatures (25, 30, 35 and 40°C), different incubation periods (0-84 hr) and pH (5.5, 6.5, 7.5, 8.5, 9.5 and 10.5). The impact of NaCl concentration on biomass production was also evaluated using various concentrations (0, 1.0, 1.5, 2.5 and 3.5%) in NB with 10 ppm mercury was used as an additive. Maximum growth was observed at 36 hr at pH 8.5, temperature 35°C and 2.5% salinity. Growth was measured spectrophotometrically at 600 nm.

Mercury Removal/Reduction: The heavy metal mercury removal/reduction study was done in 3 sets of concentration of mercury using Nutrient broth medium.

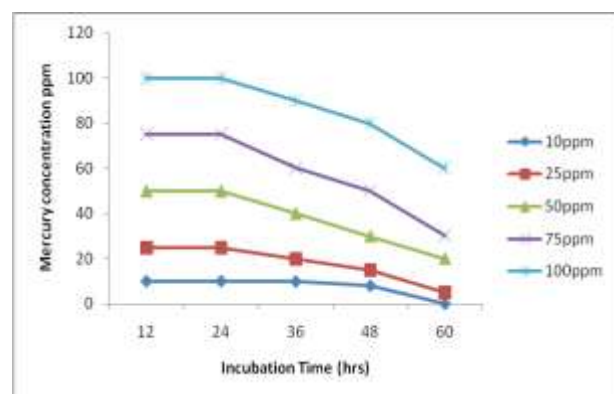


Fig. 6: Mercury removal pattern at lower concentration.

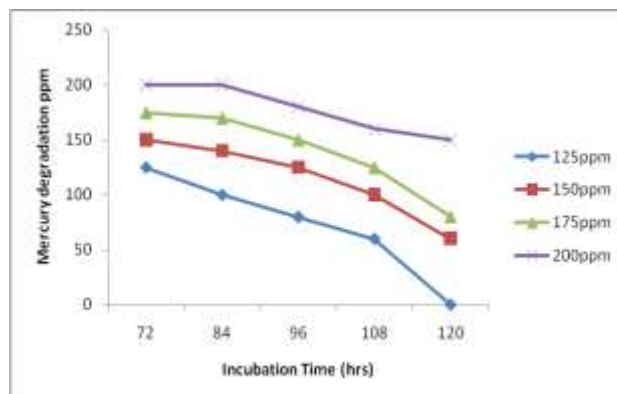


Fig. 7: Mercury removal pattern at medium concentration.

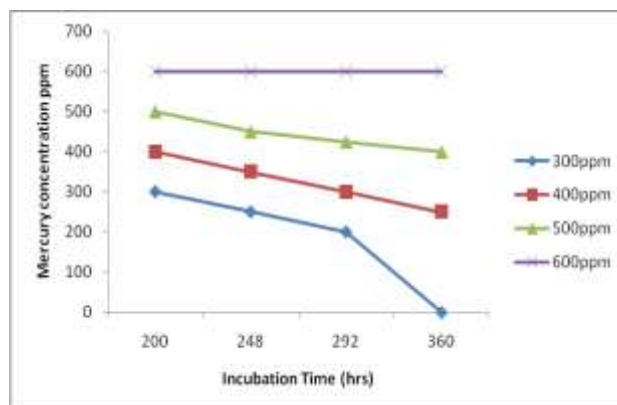


Fig. 8: Mercury removal pattern at higher concentration.

From Fig. 6, Fig.7 and Fig.8, at lower concentration, mercury growth was higher, compared to the growth in higher concentration. The lower concentration of mercury in culture broth was found to be supportive to growth and at 10 ppm of mercury concentration maximum growth was observed at 24 hr itself. Time lag in mercury removal was very much obvious especially at the concentration level of 200-600 ppm.

Mercury concentration was readily decreased within 12 to 60 hr when the concentration was lower. When the concentration was increased to 100-200 ppm, total reduction in concentration was observed around 60-120 hr. In higher concentration (i.e.) 300 ppm to 400 ppm upto 200-360 hr, no visible growth was observed.

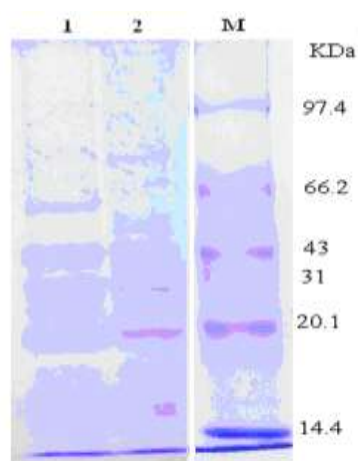
The study addressed many key issues involved in bioremediation of an important pollutant (i.e.) mercury. *Pseudomonas putida* seemed to be an ideal organism for bioremediation of mercury.

Mercury analysis using Atomic absorption spectrometry (AAS)

The concentration of total soluble Hg in the water samples collected at Uppanar estuary was 119.32 µg/L, as determined by AAS. The amount of mercury removed was calculated in cell free extract as well as in cells

based on AAS analysis. Cells didn't contain any mercury.

SDS-PAGE: The protein profile of treated and control strains of *P.putida* were analysed on 12% SDS-PAGE.

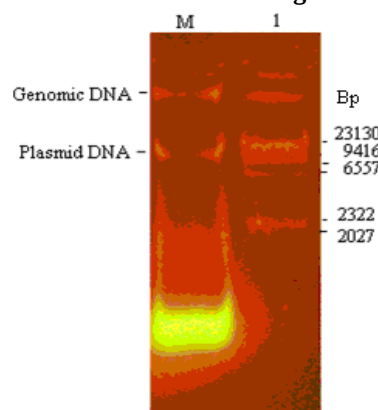


Lane 1 : Control Sample
Lane 2 : Mercury Treated Sample
Lane M : Standard Protein Molecular Weight Marker

Fig. 9: SDS- PAGE Profile of Mercury Resistant *P.putida*.

From **Fig.9**, The protein pattern between higher concentration (100 ppm) grown and the control strains showed significant variations. A 61 KDa protein which was expressed in the control strain was not expressed in the strain treated with higher concentration of mercury. Whereas a new protein of 82 KDa, which was expressed in the treated strain was not found in the control strain. Compared to the control 3 prominent proteins in the treated sample ranged 28, 19 and 16 KDa were expressed higher in the control strain. Further study is needed in this regard to reveal the exact function of these proteins.

Plasmid isolation and curing



Lane 1: Plasmid DNA isolated from Mercury resistant bacteria
Lane M: Lambda DNA+ Hind III Digest Marker

Fig. 10: Plasmid DNA Profile of Mercury Resistant *P.putida*.

From **Fig. 10**, the *P. putida* strain used in the present study possessed a single plasmid of 16.2 Kbps. After curing the plasmid, the strain lost its mercury resistance ability.

RAPD Analysis

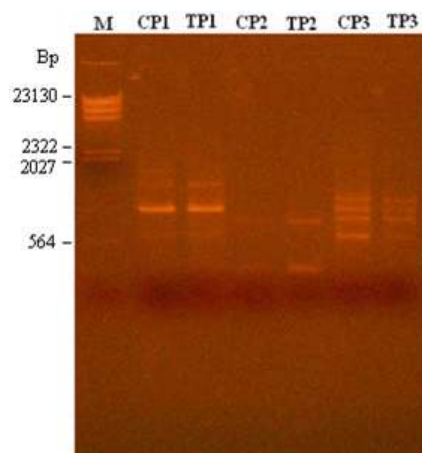
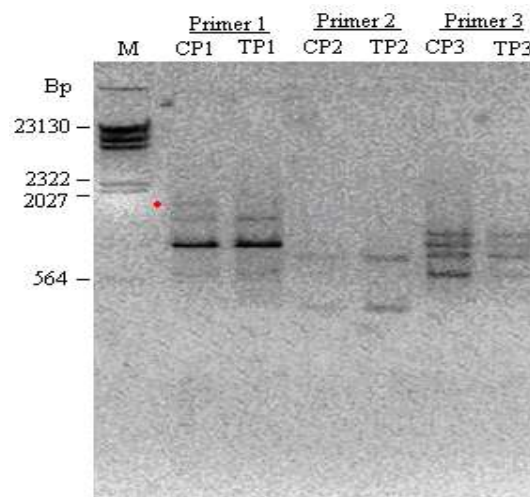


Fig. 11: RAPD Profile of Mercury Resistant *P.putida*.



CP1 : Control Sample for Primer 1
TP1 : Control Sample for Primer 1
CP2 : Control Sample for Primer 2
TP2 : Control Sample for Primer 2
CP3 : Control Sample for Primer 3
TP3 : Control Sample for Primer 3
M : Lambda DNA Hind III digest marker

Fig. 12: RAPD Profile of Mercury Resistant *P.putida*.

From Fig.11 and Fig.12, the Polymorphism percentage are calculated as follows:

Table – 2: Polymorphism percentage.

S. No	Bands Scored	Description	Primer
1	7	Three bands are common to control and treated sample; one band was unique to control sample; No band was unique to treated sample.	1
2	4	Two bands are common to control and treated sample. No polymorphism was found in both control and treated sample.	2
3	8	Four bands were common to control and treated sample; two bands were unique to control sample; one band was unique to treated sample	3

From Table -2, the level of true polymorphism between test and control was evaluated at 15% with primer 1. The level of true polymorphism between test and control was 0% with primer 2. The level of true polymorphism between test and control was evaluated at 0% with primer 3.

The percentage of true polymorphism observed between control and treated sample was around 15%.

Biotransformation (or) mercury removal studies were conducted in three sets viz (i) Lower concentration- 10-100ppm (ii) Medium concentration -125ppm- 200ppm and (iii) Higher concentration -300ppm- 600ppm. From one level to another, acclimatization at the highest concentration of that level was done. This made the strain to adapt to removal of still higher concentration of mercury used.

The next notable observation was lag period. Whenever the culture was introduced to a higher concentration the time delay in starting the removal process seemed to be unavoidable. This lag period increased as the variation between the acclimatized concentration and the newly exposed concentration increased.

At lower concentration (10-100ppm) the mercury removal process started by 12h. When the concentration was in the range of 100-200ppm it took 72h to start the degradation process. In 300-500ppm range it took 200h to observe notable reduction in concentration. Surprisingly when the strain was acclimatized to a particular concentration, the next higher concentration it transformed fully (i.e.) total removal was observed. (i.e.) when the strain was acclimatized to 100ppm for a week time, it transformed 125ppm concentration within a stipulated time (i.e.) 160h. Likewise when the strain was acclimatized to 200ppm 100% removal was observed when it was transferred to 300ppm (within 360h). However further acclimatization at 600ppm was not done in the present study. The results clearly indicated that there is a strong correlation exists between the prevailing concentrations at a particular environment and the exposure of the organisms to it.

Surprisingly though at 600ppm no reduction in concentration was noted, live cells were still present. *Pseudomonas putida* might be tolerating the higher concentration of mercury. The strain might be capable of tolerating other pollutants also.

The study showed a plasmid of 16.2Kb plasmid was responsible for mercury resistance and plasmid curing totally removed the mercury resistant property.

The SDS-PAGE results showed that the protein profile of control (10ppm) and strain grown at 100ppm were different. New protein fragments of 28 KDa, 19 KDa and 16 KDa appeared in the strain when grown at higher concentration. These proteins might be responsible for mercury resistance. However this needs further clarification.

The RAPD analysis showed that the percentage of true polymorphism observed between control and treated sample was 15%, which showed the genetic changes due to mercury exposure.

CONCLUSION

From the present study on mercury removal by a *Pseudomonas putida* strain isolated from Uppanar estuary which is highly polluted, 4.1×10^6 CFU/ml of mercury resistant bacteria were estimated in the present study. The growth study conducted revealed that 35°C, pH 8.5 and 2.5% salinity were ideal for the organism. Maximum density was observed at 36h of incubation. Thus the present study was a good piece of scientific work contributed certain new insights to mercury resistance in *Pseudomonas putida* strain and its usefulness to bioremediation of contaminated heavy metal sites.

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