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# Uptake of mercury by a bacterium, *Pseudomonas* sp. AN29, isolated from industrial effluents and its potential use in wastewater treatment

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**Abstract**

The present study is aimed at assessing the ability of *Pseudomonas* sp. AN29 to uptake $Hg^{2+}$ from the metal contaminated environment. The minimum inhibitory concentration (MICs) of $Hg^{2+}$ was 500 µg/ml. *Pseudomonas* sp. AN29 could tolerate $Pb^{2+}$ (600 µg/ml), $Cu^{2+}$ (200 µg/ml), $Cd^{2+}$ (50 µg/ml), $Zn^{2+}$ (50 µg/ml), $Ni^{2+}$ (550 µg/ml) and $Cr^{6+}$ (150 µg/ml). The isolate showed typical growth curve but phases (lag and log) extended in the presence of mercury. Bacterial isolate showed optimum growth at 37ºC and at pH of 8. Metal processing ability of the isolate was determined in a medium containing 100 µg/ml of $Hg^{2+}$. *Pseudomonas* sp. AN29 could decrease 85% of mercury from the culture medium after 40 hours and was also able to remove $Hg^{2+}$ 10%, 40%, 50% and 56% from the growth medium after 8, 16, 24 and 32 hours, respectively. *Pseudomonas* sp. AN29 was also able to uptake 74% of $Hg^{2+}$ (pellet) from the medium after 24 hours of incubation at 37ºC. Excellent metal uptake ability of *Pseudomonas* sp. AN29 indicates the possibility of using the bacterial strain for the removal of mercury from metal-contaminated wastewater.

***Keywords:*** Heavy metal resistance; mercury; *Pseudomonas* sp. AN29; Uptake; wastewater; bioremediation

## 1. Introduction

Heavy metals represent an important environmental problem due to their potential toxic effects, and their accumulation throughout the food chain leads to serious ecological and health problems. Compounds of mercury are toxic to both eukaryotic and prokaryotic cells. These compounds can pass through biological membranes and bind with high affinity to thiol (SH) groups in proteins, thus causing damage to membranes and inactivating enzymes (Flora et al., 2008). Mercury is also genotoxic; inorganic Hg (II) is capable of strong reversible interactions with the nitrogens in purines and pyrimidines, and organic mercury compounds, e.g. methylmercury, also produce irreversible damage to nucleic acids (Sletten and Nerdal, 1997). Environmental contamination with mercury compounds can have devastating effects as mercury toxicity is cumulative, with the highest levels of mercury compounds being found in consumers at the top of the food chain (Glendinning et al., 2005).

Mercury is one such metal that has been reported to produce metabolic disorders in a variety of animals such as fish (Company et al., 2004), rat (Reinhardt et al., 1985), rabbit (Shakoori et al., 2002) and man (Miwa et al., 1987). Various health problems such as pneumonitis, abnormal cramps, bloody diarrhea and suppression of urine, cancer, and hypersecretion of sweat glands are caused by mercurial and mercuric forms of mercury. Romero et al. (2004) studied the toxic effects of mercury chloride in two cell lines of renal origin. The most notable findings in treated cells were the presence of intracytoplasmic inclusion bodies and apoptotic bodies.

Mercury resistant bacteria have been reported by several authors (Chang et al., 1998; Brown et al., 2002; Mindlin et al., 2005; Fortunato et al., 2005). The genus *Rhodosporidium* sp. was also isolated from metallurgical waste by Baldi and Pepi (1995). Ghosh et al. (2006) reported that one yeast strain, *Rhodotorula rubra* $GVa_5$, was resistant to $HgCl_2$ up to 400 nmole/0.05 ml. Mercury is also efficiently removed by algae (Davis et al., 2003; Chojnacka et al., 2004). Mercury accumulation by plants is also reported by many researchers (Zeroual et al., 2003; Bennicelli et al., 2004). Recently, microbial bioremediation has emerged as an alternative technique to such traditional chemical treatments (Brierley, 1990). One of the objectives of this study

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was to evaluate the minimum inhibitory concentration (MIC) of the bacterial isolate against $Hg^{2+}$ and determine its ability to uptake mercury.

## 2. Materials and methods

### *2.1. Sample collection*

Wastewater samples were collected in screw capped sterilized bottles from industrial area of Sialkot (Pakistan). Some physicochemical parameters of wastewater viz., temperature (ºC), pH and mercury (µg/ml) were measured (APH, 1992).

### *2.2. Isolation of mercury resistant bacterium*

For isolation of mercury resistant bacteria, 10 ml of the wastewater sample was spread on Luria-bertani (LB) agar plates containing 50µg of $Hg^{2+}$/ml of the medium. The medium consisted of NaCl 1 g, tryptone 1 g, yeast extract 0.5 g in 100 ml distilled water, pH adjusted from 7.2 to 7.5 and then 1.5 g agar was added in the 250 ml flask and this medium was autoclaved at 121ºC and 15 lb pressure for 15 minutes. The growth of the bacterial colonies was observed after 24 hours of incubation at 37ºC. Isolated colonies were picked up with sterilized wire loop and streaked on LB agar medium plate containing 100µg $Hg^{2+}$/ml. It was again incubated at 37ºC for 24 hours. This process was repeated with successively higher concentrations of $Hg^{2+}$ (150, 200, 250, 300, 350, 400, 450, 500µg $Hg^{2+}$/ml) until the minimum inhibitory concentration (MIC) of bacterial isolate was ascertained.

### *2.3. Physical, biochemical and molecular characterization of the bacterial isolate*

For biochemical characterization the bacterial isolate was tested for oxidase activity, motility, citrate utilization, urease activity, triple sugar iron reaction, indole reaction, and MacConkey agar test. For physical and biochemical characterizations of bacterial isolate the criteria adopted by Benson (1994) and those of Bergey's Manual of Determinative Bacteriology were followed.

For molecular characterization genomic DNA was extracted as described by Carozzi et al. (1991) and the 16S rRNA gene was amplified by PCR using 16S rRNA primers (RS-1; 5′-AAACTC-AAATGAATTGACGG-3′, and RS-3; 5′-ACGGGCGGTGTGTAC-3′) (Rehman et al., 2007). PCR was performed by initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The PCR product of 0.5kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 16S rRNA gene was purified with a Fermentas purification kit (#K0513) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

### *2.4. Determination of optimum growth conditions*

For determination of optimum temperature, 5 ml LB broth was added in 4 sets, each of three test tubes for bacterial isolate, autoclaved and inoculated with 20 µl of freshly prepared culture of isolate. The four sets of tubes were incubated at 25ºC, 30ºC, 37ºC and 42ºC. After an incubation of 12 hours, their absorbance was taken at 600 nm and a graph was plotted with temperature at x-axis and absorbance along y-axis. For determination of optimum pH, test tubes having 5 ml LB broth were prepared in 9 sets, each containing 3 test tubes, for bacterial isolate and their pH was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 then autoclaved. These tubes were inoculated with 20 µl freshly prepared culture of the isolate. After incubation period of 12 hours, their absorbance was taken at 600 nm wavelength and then graph plotted between pH (along x-axis) and absorbance (along y-axis).

### *2.5. Growth curves of bacterial isolate*

Growth curves of bacterial isolate were determined with (50µg $Hg^{2+}$/ml) and without mercury. For bacterial isolate 50 ml LB broth was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 50 µl of the freshly prepared inoculum. These cultures were incubated at optimum temperature in a shaker at 60-80 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, 24, 36, and 48 hours, absorbance was taken at 600 nm wavelength. Growth was plotted graphically.

### *2.6. Cross metal and antibiotic resistance*

The cross heavy metal resistance of bacterial isolate was determined by using stock solutions of 10 mg/ml of different metal salts such as, lead nitrate, cadmium chloride, copper sulphate, potassium dichromate, zinc sulphate and nickel chloride. The cross metal resistance was checked by increasing the concentration of respective metal in a stepwise manner with 50 µg/ml of metal increased

each time. Streaked plates containing metal ions, incubated at 37°C for 24 hours and growth was observed for four days. Antibiotic sensitivity against bacterial isolate was checked by measuring the zone of inhibition. The antibiotics used were ampicillin (10µg), chloramphenicol (30µg), gentamicin (10µg), carbenicillin (100µg), oxytetracyclin (30µg) and penicillin (10µg).

### *2.7. Estimation of $Hg^{2+}$ processing ability of the isolate*

Mercury processing capability of the bacterial isolate was checked by adding $Hg^{2+}$ at a concentration of 100 µg/ml in the culture medium. The control culture medium was also run for mercury containing the same concentration as in the treated one *i.e.*, 100 µg/ml but without the bacterial isolate. The cultures were incubated for 40 hours and from each medium (control and treated) 5 ml culture was taken out under sterilized conditions after 0, 8, 16, 24, 32 and 40 hours, respectively. The cultures were spun down at 3000 rpm for 5 minutes and the supernatants were used for the estimation of $Hg^{2+}$ by Tekran Mercury Vapor Analyzer at wavelength 253.7nm (Møller et al., 2011). Three samples were used for each measurement and the $Hg^{2+}$ concentration was determined against a six-point Hg standard curve. The percentage decrease in the amount of $Hg^{2+}$ in the medium was calculated.

### *2.8. Uptake of mercury by bacterial isolate*

The uptake of mercury by bacterial isolate in LB medium was carried out by acid digestion. $Hg^{2+}$ (50 µg/ml) was added in the culture medium and cells were collected after 24 hours of incubation at 37°C, washed three times in saline solution and acid digested ($H_2SO_4$: $HNO_3$,1:1). Metal content of the digest was measured by Tekran Mercury Vapor Analyzer at wavelength 253.7nm (Møller et al., 2011). Amount of mercury uptake by bacterial cells was calculated in µg/ml by using standard curve.

### *2.9. Statistical analysis*

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

## *3. Results*

### *3.1. Physicochemical characteristics of industrial* wastewater

Some physicochemical characteristics of industrial wastewater, from where metal tolerant bacterium was isolated, were measured. The temperature of different samples ranged between 19.5ºC and 24.0ºC, pH ranged between 7.70 to 8.4, and $Hg^{2+}$ ranging between 0.70±0.08 to 1.50 ±0.08 µg/ml.

### *3.2. Identification of bacterial isolate*

The morphological and biochemical characteristics of bacterial isolate have been shown in Table 1. Based on these characters and the 16S rDNA PCR product from the culture was sequenced and BLAST analysis of the sequences obtained revealed 94% homology with *Pseudomonas* sp. AN29. The nucleotide sequence coding for 16S rRNA gene of *Pseudomonas* sp. AN29 has been submitted to the GenBank database under accession number AB262516.

**Table 1.** Morphological and biochemical characteristics of *Pseudomonas* sp. AN29

| Characteristics | *Pseudomonas* sp. AN29 |
|---|---|
| Gram-reaction | - |
| Morphology | Rods |
| Colour | Off-white |
| Urease production | - |
| Citrate utilization | - |
| Oxidase reaction | + |
| Triple sugar iron reaction | + |
| Indole reaction | - |
| Motility | + |

(+) positive; (-) negative

### *3.3. Optimum growth conditions*

Maximum growth for *Pseudomonas* sp. AN29 was observed at pH 8 (Fig. 1).

The most suitable temperature for the mercury resistant bacterial isolate was found to be 37°C (Fig. 2).

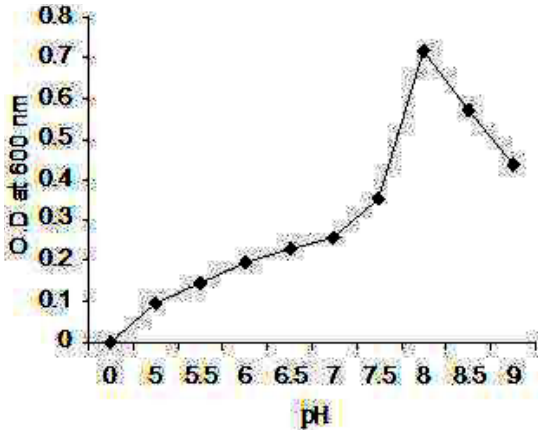


**Fig. 1.** Effect of pH on the growth of *Pseudomonas* sp. AN29, isolated from industrial effluents of Sialkot, Pakistan

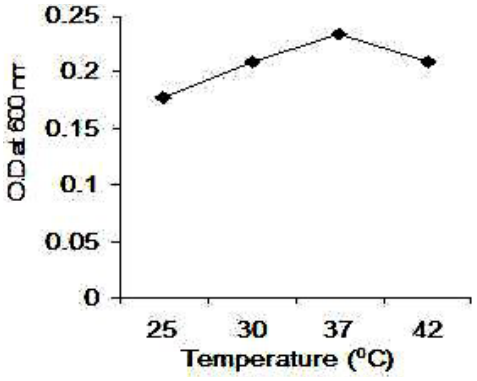


**Fig. 2.** Effect of temperature on the growth of *Pseudomonas* sp. AN29, isolated from industrial effluents of Sialkot, Pakistan

### *3.4. Effect of $Hg^{2+}$ on bacterial growth*

The growth curve pattern was studied by containing 50µg $Hg^{2+}$/ml in the treated medium. Growth of the Hg-treated bacterial isolate was comparable to that of control at 50µg $Hg^{2+}$/ml. It is interesting to note that the lag phase of the isolate has been extended. It is also shown by the growth curve pattern that the isolate has also extended its log phase up to 36 hours. The growth pattern has been shown in Fig. 3.

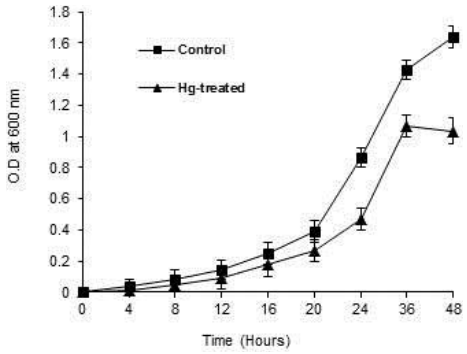


**Fig. 3.** Growth behavior of *Pseudomonas* sp. AN29 in LB medium containing 50µg $Hg^{2+}$/ml after incubation at 37°C

### *3.5. Bacterial resistance to metal ions and antimicrobial compounds*

The bacterial isolate was studied for higher concentrations of mercury and was found to be resistant to mercury at a concentration of 500 µg/ml. The bacterial isolate was also checked for its resistance to various heavy metals, viz., chromium, cadmium, copper, lead, zinc and nickel (Table 2). *Pseudomonas* sp. AN29 showed maximum resistance against $Pb^{2+}$ at a concentration of 650 µg/ml and the order of resistance regarding the metal concentration was, therefore, $Pb^{2+}>Ni^{2+}>Hg^{2+}>Cr^{6+}>Cd^{2+}>Cu^{2+}>Zn^{2+}$. The mercury tolerant bacterium was tested for antimicrobial susceptibility profile and results have been depicted in Table 3. *Pseudomonas* sp. AN29 was found resistant to penicillin (10 µg) and chloramphinicol (30 µg) but was sensitive to gentamycin (10 µg), oxytetracyclin (30 µg), carbenicillin (100 µg) and ampicillin (10 µg).

**Table 2.** Cross resistance of *Pseudomonas* sp. AN29 against different heavy metal ions

| Bacterial isolate | $Cr^{6+}$ (µg/ml) | $Cd^{2+}$ (µg/ml) | $Cu^{2+}$ (µg/ml) | $Pb^{2+}$ (µg/ml) | $Zn^{2+}$ (µg/ml) | $Ni^{2+}$ (µg/ml) |
|---|---|---|---|---|---|---|
| *Pseudomonas* sp. AN29 | 150 | 50 | 200 | 600 | 50 | 550 |

**Table 3.** Antimicrobial susceptibility test profile for *Pseudomonas* sp. AN29

| Antibiotic doses | *Pseudomonas* sp. AN29 Diameter (mm) of inhibition zone |
|---|---|
| Chloramphinicol (30µg) | R |
| Penicillin(10µg) | R |
| Gentamycin(10µg) | S (18) |
| Carbenicillin(100µg) | S (26) |
| Oxytetracyclin(30µg) | S (24) |
| ampicillin (10µg) | S (18) |

S=Sensitive; R=Resistant

### *3.6. Metal uptake ability of the bacterial isolate*

Mercury processing capability of the bacterial isolate was checked by adding $Hg^{2+}$ at 100 µg/ml in the culture medium (Fig. 4). *Pseudomonas* sp. AN29 showed an excellent ability to pick up mercuric ions from the culture medium and could decrease 85% of mercury from the medium after 40 hours of incubation. *Pseudomonas* sp. AN29 was also capable of removing $Hg^{2+}$ (100 µg/ml) 10%, 40%, 50% and 56% from the medium after 8, 16, 24, and 32 hours, respectively. The biosorption term has been used in the present study to indicate that the metal was removed by one or more of these processes. During the present investigation the bioaccumulation of mercury by *Pseudomonas* sp. AN29 was observed 74% after 24 hours of incubation at 37°C (Table 4).

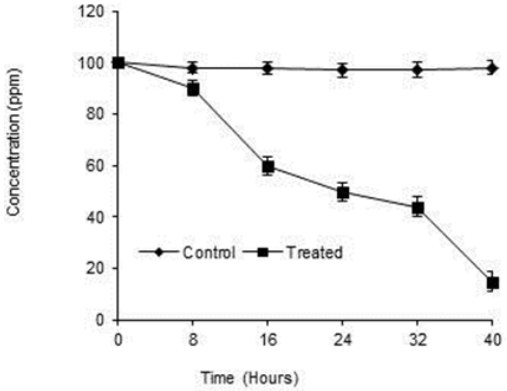


**Fig. 4.** Uptake of $Hg^{2+}$ by *Pseudomonas* sp. AN29 growing in $Hg^{2+}$ containing medium. The control did not contain cells of the isolate.

**Table 4.** Percentage bioaccumulation of $Hg^{2+}$ by *Pseudomonas* sp. AN29 with initial concentration of 50.0 µg/ml of $Hg^{2+}$ in the LB broth medium at 37°C

| Bacterial isolate | Percent bioaccumulation of $Hg^{2+}$ after 24 hours | | |
|---|---|---|---|
| | Supernatant (µg/ml) | Pellet (µg/ml) | % bioaccumulation |
| *Pseudomonas* sp. AN29 | 13 | 37 | 74 |

## 4. Discussion

A number of microorganisms have developed resistance mechanisms to deal with mercury compounds. Mercury resistance was first reported in *Streptomyces aureus* (Moore, 1960) and since then has been described in a number of bacterial species. One of the best defined mercury resistance determinants is the *mer* operon encoded by transposon Tn*501*, found in Gram-negative bacteria. The function of the minimal number of proteins is required to confer full resistance (Hobman and Brown, 1997). Thiomersal biodegrading mercury resistant *Pseudomonas putida* (Fortunato et al., 2005) and growth promoting *Pseudomonas fluorescens* (Gupta et al., 2005) strain have also been isolated and characterized.

*Pseudomonas* sp. AN29 showed resistance to mercury at a concentration of 500 µg/ml and also resisted $Pb^{2+}$ at a concentration of 650µg/ml. The order of resistance regarding the metal concentration was, therefore, $Pb^{2+}$>$Ni^{2+}$> $Hg^{2+}$>$Cr^{6+}$>$Cd^{2+}$>$Cu^{2+}$>$Zn^{2+}$. The industrial wastewater usually contains different metal ions at varying level and the use of such bacterium having multiple resistances is feasible to ameliorate metal ions from the wastewater.

Mercury resistance occurs widely with Gram-negative and Gram-positive bacteria in environmental, clinical and industrial isolates, and frequently mercury resistance genes are found on plasmids and encoded by transposons. Saha et al. (2006) reported that *Pseudomonas* strains UR2, PS4 and UR5 contained plasmid DNA and were highly resistant to mercury. Mercury resistance in these *Pseudomonas* strains may be plasmid determined. Nakahara et al. (1997) reported that in some cases resistance to certain antibiotics and metals is mediated by the same plasmid. In the present study *Pseudomonas* sp. AN29 showed resistance against antibiotics and metal ions and may be the resistance to both is mediated by the presence of plasmid. Like the development of antibiotics resistance, human activities have created environments of high selection for metals. Resistance to metals, however, has been documented before the use of antibiotics (Ji and Silver, 1995).

Uptake of metals by bacterial cells has become one of the most attractive means for bioremediation of industrial wastes and other metal polluted environments. Heavy metal uptake processes by biological cells are known under the general term of biosorption. These phenomena include both passive adsorption of heavy metals to the cell walls and metabolically mediated uptake (Gadd, 1990). Clean-up of mercury containing wastewater by mercury resistant microbes is a simple, environmentally friendly and cost effective alternative to current treatment technologies (Ledin, 2000; Wanger-Dobler, 2003).

Deng and Wilson (2001) reported that genetically engineered *E. coli* cells removed more than 99% of the mercury in the wastewater and the final amount of mercury accumulated was 26.8 mg/g cell dry weight. Mercury detoxification efficiency for *Pseudomonas aeruginosa* PU21 (Rip64) was 54% and less was observed for *E. coli* PWS1 (Chang and Law, 1998). During the present investigation *Pseudomonas* sp. AN29 showed an excellent capability to pick up $Hg^{2+}$ ions from the culture medium and could decrease 85% of mercury after 40 hours of incubation (Fig. 4) and also accumulated 74% ($Hg^{2+}$) after 24 hours of incubation at 37°C (Table 4). Thus high metal uptake ability of bacterial isolate can be used in the development of a strategy for better exploitation of bacteria for the treatment of wastewater released by industries.

Rehman et al. (2007) reported that *Pseudomonas aeruginosa* could decrease 93% of mercury from the medium after 40 hours and was also able to remove $Hg^{2+}$ 35%, 55% 70% and 85% from the medium after 8, 16, 24 and 32 hours, respectively. *Brevibacterium casei* could also efficiently remove 80% mercury from the medium after 40 hours and was also able to remove $Hg^{2+}$ 20%, 40%, 50%, and 65% from the medium after 8, 16, 24 and 32 hours, respectively.

Many bacteria belonging to the genera *Pseudomonas, Bacillus* and *Staphylococcus* have been reported to reduce $Hg^{2+}$ to $Hg^{\circ}$ (Silver and Phung, 2005; Saha et al., 2006). The mercury tolerant bacterium isolated during the present study showed high level of metal tolerance and accumulated substantial amount of $Hg^{2+}$ from the medium. Thus the strain *Pseudomonas* sp. AN29 may be employed for metal removal from metal contaminated wastewaters.

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