

*Full Length Research Paper*

# Isolation and characterization of *Pseudomonas* resistant to heavy metals and poly aromatics hydrocarbons (PAHs) from Persian Gulf sediments

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Accepted 17 January, 2012

The Persian Gulf is the most strategic waterway in the world due to its importance in the global oil transportation. Heavy metals and poly aromatics hydrocarbons (PAHs) are the most important pollutants in the Persian Gulf and other marine ecosystems. Our goal in this study is to isolate high resistant degrading species for copper and phenanthrene from Khur Mousa sediments (located in the North of the Persian Gulf, Iran). Among 10 bacterial species isolated from marine sediment, one strain represented high potential to grow in medium supplemented with copper and phenanthrene. Isolated bacterium was identified as *Pseudomonas* sp. by biochemical tests. Over 70% of copper sorbed on *Pseudomonas* sp. within 150 min. In addition, about 96.52% of initial concentration of phenanthrene was degraded during 120 h. Based on the capacity of isolate in degradation of phenanthrene and biosorption of copper, this species is introduced as an appropriate microorganism for bioremediation of contaminated environments.

**Key words:** *Pseudomonas* sp, bioremediation, biosorption, Persian Gulf, phenanthrene, copper.

## INTRODUCTION

Marine pollution is the major environmental issue that has been increased through industrial development, land-based activities and pollutants all over the world. It has negative influences on animal and plant organisms, ecosystems, and mineral resources. Furthermore, marine pollution could lead to decrease or extinction of marine species and biodiversity loss (Malatove, 2005; Vieira and Volesky, 2000).

Heavy metals and poly aromatic hydrocarbons (PAHs) are the most important pollutants in marine environments. They have been recognized to be potentially toxic with high durability which causes accumulation of these compounds in marine organism's tissues (Jarup, 2003). They reduce the rate of biodiversity and reproduction as well as destroying coastal regions, many sensitive

habitats such as mangrove forests, coral reefs and seaweed sanctuaries (Floodgate, 1995; Hetzer et al., 2006).

Nowadays, many methods are being used for treatment of contaminated water environments. However, bioremediation is an effective technology with a range of advantages rather than traditional methods. Bioremediation of waste materials which contain hydrocarbons and heavy metals is based on the ability of microorganisms such as bacteria and fungi to absorb or degrade them to non-toxic products. The microorganisms could be indigenous to a polluted area or isolated from another place and transferred to the contaminated site (Park et al., 2005; Price, 2000; Lyer et al., 2004).

Bioremediation is the natural process that can be used in the Persian Gulf which is located between the Arabian Peninsula and Iran. It sits on top of the largest hydrocarbon reserve in the world that makes this region extremely important for oil production. Water evaporation, extended drilling and oil extraction are significant factors which increase water pollution alarming in this sensitive

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marine ecosystem (Zolgharnein et al., 2007). Therefore, in the present study, copper and phenanthrene resistant bacteria were isolated from the Persian Gulf. Moreover, morphological properties and the ability of the strain in sorption of copper and degradation of phenanthrene have been analyzed.

## MATERIALS AND METHODS

### Sampling

Samples of contaminated sediments were collected by Van Veen Grab from three different places at a depth of 3 m. The Grab was sterilized by 75% ethanol before sediment collection and washed with seawater. Sediment samples were transferred to sterile capped tubes and stored in ice.

### Isolation of bacteria by enrichment method

Sediment samples were mixed in a sterile laboratory environment. Then, 5 g of each sample was added to 10 ml sterile distilled water separately. Sediment suspensions were centrifuged at 2000 rpm for 10 min and supernatants were collected for isolation of heavy metal resistance bacteria (Coral and Karagoz, 2005). Bushnell-Haas broth (BH) and nutrient broth supplemented with 50 ppm phenanthrene and copper were used in the enrichment technique. BH medium contained  $\text{MgSO}_4$  0.2 g/L,  $\text{CaCl}_2$  0.02 g/L,  $\text{KH}_2\text{PO}_4$  1 g/L,  $(\text{NH}_4)_3\text{PO}_4$  1 g/L,  $\text{KNO}_3$  1 g/L and  $\text{FeCl}_3$  0.05 g/L, pH 7.2. Nutrient broth media contained 5 g/L peptone, 3 g/L yeast extract and pH 7.2. The supernatants were inoculated into 100 ml BH media and nutrient broth containing 50 ppm phenanthrene and copper respectively (The inoculum's size was 0.1 ml). The samples were shaken at 170 rpm and 28°C for 7 days. 1 ml of primary enrichment was transferred to fresh BH and nutrient broth. The incubation was done in prior conditions. After the second enrichment, 0.1 ml of media was screened on BH and nutrient agar and incubated at 28°C (Dzairi et al., 2004; Malatova, 2005). Among 10 bacteria isolated, one specie that could tolerate both copper and phenanthrene concentration was selected.

### Biochemical tests

Morphological identification of the bacterium was done by gram staining. Biochemical test like oxidase-catalase test, methyl red - voges proskauer (MR-VP), citrate, triple sugar iron (TSI), partial discharges (PD), nitrate reduction, indole test etc., were prepared according to laboratory manual and workbook in Microbiology (Morello et al., 2002). The species of the isolate was identified based on Bergey's Manual of Systematic Bacteriology (Garrity et al., 2002).

### Growth measurements

The isolate was inoculated in 10 ml of PC medium (tryptone 5.0 g/L, yeast extract 2.5 g/L and glucose 1.0 g/L, pH 7.2) in 125 ml flask and incubated for 48 h at 28°C on rotary shaker. After incubation, 3 ml of cells suspension were added to 20 ml of BH broth containing 0.48 ml phenanthrene and then inoculated in Luria-Bertani (LB) broth containing 50 ppm copper. The flasks were incubated at 28°C for 12 days, non-shaking. Bacterial growth was monitored every 24 h for 12 days through measuring the optical density (OD) of cultures using spectrophotometer at 600 nm (Malatova, 2005; Taoufik et al.,

2004).

### Phenanthrene biodegradation

About 500 µl of cell suspension were inoculated into 100 ml of BH medium, which had been already supplemented with phenanthrene. The solutions were incubated at 28°C for 6 days on rotary shaker at 150 rpm. 2 ml of each solution were taken for chromatographic analysis at 24 h intervals. Then 2 ml of hexane were added to 5 ml of BH medium in screw cap glass tubes and shaken several times. 1 ml of upper phase (hexane) was transferred to clean tubes and evaporated on laboratory temperature. The remaining pellets were dissolved in 2 ml of high-performance liquid chromatography (HPLC) mobile phase. 20 µl of each sample were injected into HPLC analytic system. The concentration of phenanthrene was detected by UV absorbance at 254 nm according to Coral and Karagoz (2005).

### Biosorption experiments

1 ml of bacterial suspension was inoculated into 100 ml of copper solution (50 ppm). Samples were incubated at 160 rpm for 150 min. The remaining copper concentration was measured at 30 min intervals by the atomic absorption spectroscopy (SavantAAS) (Kim et al., 2007).

## RESULTS

In order to obtain the most resistant marine bacteria, we isolated 10 different species and exposed them to copper and phenanthrene. The one that could grow on media containing both copper and phenanthrene was selected for further study. An isolated bacterium was gram negative and belongs to *Pseudomonas* sp. Other results are shown in Table 1.

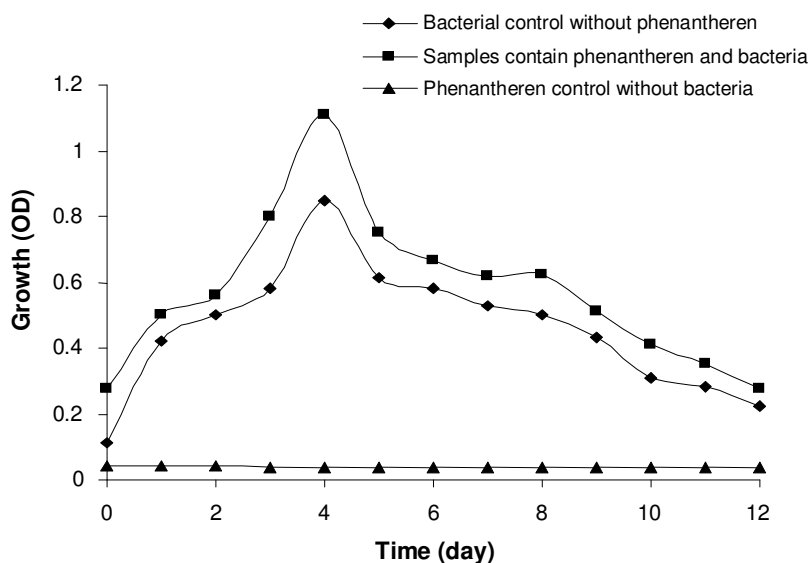
The species grew strongly in LB and BH broth supplemented with copper and phenanthrene respectively. The growth curve of *Pseudomonas* sp. revealed a rapid initial phase within 24 h of incubation (Figure 1). The maximum optical density was observed on 4th day. Comparison of samples contained phenanthrene with the control samples showed that an isolated bacterium consumed phenanthrene as the sole carbon source.

The growth rate of *Pseudomonas* sp. in presence of copper is displayed in Figure 2. The bacterium did not show any lag phase and it began to grow after inoculation. The optical density of bacterium was decreased after three days while the bacterial growth in the control sample continued till the fifth day.

The copper biosorption capacity of the isolate was 70.3% while the bacteria in media supplemented with phenanthrene showed higher tolerance and was able to degrade 96.52% of initial concentration of phenanthrene (Table 2). The ability of *Pseudomonas* sp. to decrease phenanthrene concentration was examined in BH media within 6 days. Maximum degradation of phenanthrene was observed within 48 h of incubation (Figure 3). The stain 1248 could reduce phenanthrene concentration from 43.41 ppm to less than 10 ppm.

**Table 1.** Biochemical characteristics of isolated bacteria.

Biochemical test	Bacterial reaction
MR test	+
PD test	+
VP test	-
NaCl test	+
Lysine test	+
Indole test	-
Nitrate test	+ (Gas production)
Urea test	-
SIM test	+ (H <sub>2</sub> S production)
OF (Glucose test)	Oxidative
Maltose test	Oxidative
Catalase test	+
MacConkey test	+
Lactose test	-
Oxidase test	+
TSI test	+
Citrate test	+

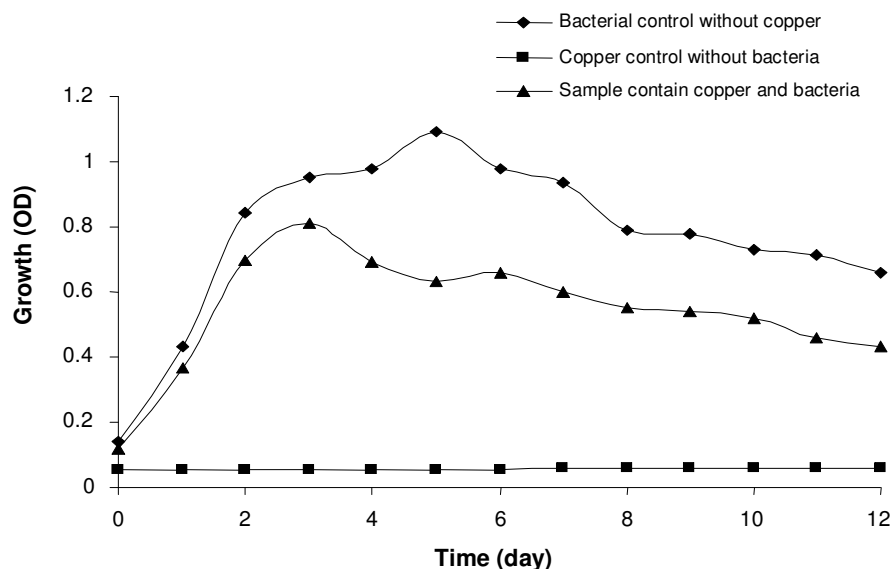
**Figure 1.** Growth curve of *Pseudomonas* sp. using BH broth supplemented with 50 ppm phenanthrene.

The copper removal by *Pseudomonas* sp. within 150 min is presented in Figure 4. The intended bacterium absorbed copper with high rate in 30 min after incubation and decreased the amount of copper to  $16.16 \pm 2.03$  ppm.

## DISCUSSION

*Pseudomonas* sp. was identified as a high resistant bacterium to copper and phenanthrene. The ability of

*pseudomonas* species in degradation of hydrocarbon compounds and heavy metals was confirmed in different researches. Taoufik et al. (2004) isolated the species of *Pseudomonas* sp. from hydrocarbons contaminated river in Morocco. It appeared that it has the ability to use benzene, toluene and naphthalene as the sole source of carbon and energy. Also Badar (2003) isolated *Pseudomonas* species from industrial areas in Pakistan that had a high resistance to copper because they could produce suitable enzymes which are compatible to



**Figure 2.** Growth curve of *Pseudomonas* sp. using LB broth supplemented with 50 ppm copper.

**Table 2.** Removal of phenanthrene and copper by *Pseudomonas* sp.

Contaminated substrate	Initial concentration (ppm)	Final concentration (ppm)	Amount of decrease (ppm)	Removal percentage
Phenanthrene	43.41 ± 1.247	1.51 ± 0.91	41.9 ± 0.91	96.52
Copper	50	14.87 ± 2.97	35.13 ± 2.97	70.30

copper. The activity of CAT, POX and APOX, which are antioxidant enzymes of *Escherichia coli* ASU 3, increases in different concentrations of copper and protect the cells against stress conditions and toxic metals such as Cu, Cd and Zn (Abskharon et al., 2010)

In this study, the growth of *Pseudomonas* sp. in BH broth showed the log phase within 24 h of incubation. It means that the bacterium was able to degrade and used phenanthrene as the sole source of carbon and energy by applying special enzymes such as TpbA. Therefore, the bacterial population increases through the cell division and it was recognized by measuring the optical density. When *Pseudomonas* sp. reached to maximum OD (> 0.8), bacterial reproduction and growth reduced regularly due to the carbon source and nutrients reduction (Kader et al., 2007).

Maximum optical density of the strain in LB broth containing 50 ppm copper was observed on the third day. Comparison of bacterial activity in presence of copper and the control sample showed that metal has a negative effect on growth of bacteria. The results supported the findings of Filali et al. (2000) which reported the growth of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* and their tolerance to heavy metals and PAHs. The growth curves indicated a slight inhibitory

action of mercury, copper, and zinc. The study also investigated the bacterial growth in presence of aromatic compounds. The results demonstrated that these bacteria could be good candidates for remediation of some heavy metals and PAHs in polluted sites.

The study of *Pseudomonas* sp. ability in degrading of phenanthrene showed that this bacterium could degrade 96.52% of phenanthrene within 120 h. This result agrees with the findings of Thavasi et al. (2007) reporting that *Pseudomonas* sp. was able to degrade 85.15% of crude oil. The results displayed that this species had the highest ability in removal of oil compounds because of two plasmids it has. Furthermore, Coral and Karagoz (2005) reported that strains ARP26 and ARP28 belong to the genus *Pseudomonas* and could degrade up to 93 and 98% of oil compounds respectively. The findings prove the capacity of *Pseudomonas* sp. in bioremediation of PAHs.

The uptake of copper by *Pseudomonas* sp. was impressive and almost 70.3% of copper was absorbed within the initial 30 min. This is probably due to the availability of sorption sites on cell wall surfaces. In fact, the strain is metabolically active, the cell wall charged negatively and metal cations can make a complex with chemical groups such as carboxyl, hydroxyl and carbonyl

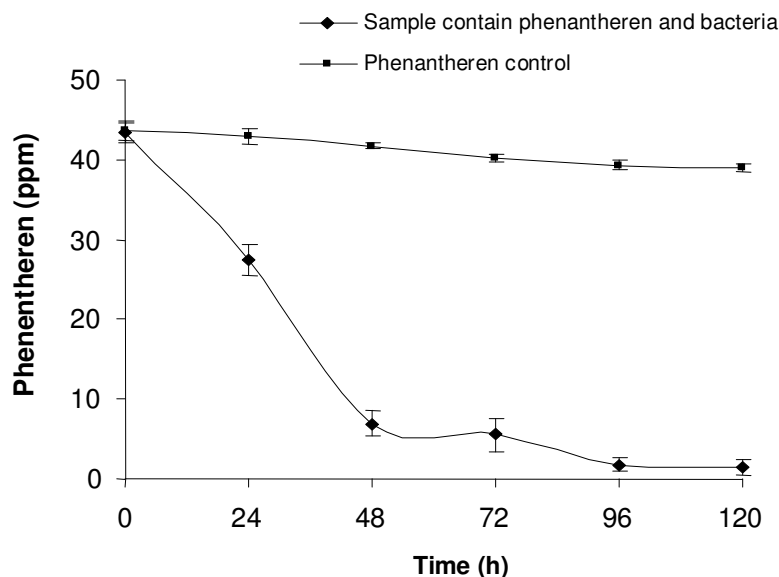


Figure 3. Phenanthrene reduction at 50 ppm initial concentration over 120 h.

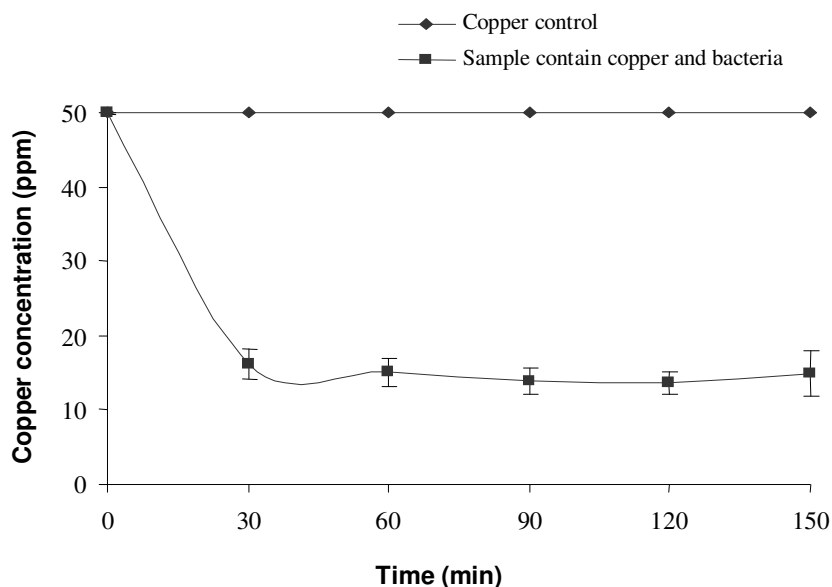


Figure 4. The removal of copper at 50 ppm initial concentration over 150 min.

(Vieira and Volesky, 2000; Joo et al., 2010). Similar study was done on *Pseudomonas putida* and *P. fluorescens* species in Egypt. The percentage of Cu (II) removal ranged between 50 and 93% (Hussein et al., 2004).

## Conclusions

The present study showed removal of 96.52 and 70.3% of phenanthrene and copper, respectively. High diversity and production of necessary enzymes by *Pseudomonas*

sp. led to degradation of hydrocarbon and biosorption of heavy metals. In addition, *Pseudomonas* sp. has a natural ability in adaptation with different environmental conditions. Consequently, this bacterium is introduced as an appropriate organism for bioremediation of contaminated environments especially the Persian Gulf.

## ACKNOWLEDGEMENT

This study was supported by Khorramshahr University of Marine Science and Technology, Iran.

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