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The Role of *Pseudomonas putida* in Bioremediation of Naphthalene and Copper

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Abstract: Over the past decade Poly Aromatic Hydrocarbons and heavy metal compounds caused serious problems for marine environments owing to their persistent and stability. The objectives of the present study were to find best marine bacteria capable of decreasing naphthalene and copper to the normal levels. This research resulted in an identification of resistant bacteria as a *Pseudomonas putida* strain 1389. The mentioned bacterium showed well growth rate in presence of naphthalene and copper and also could degrade %91.48 of naphthalene during 120 hours and adsorbed almost 59% of copper in 120 minutes. Generally experiments proved the potential of *P. putida* strain 1389 in bioremediation of copper and naphthalene so we recommend using of *P. putida* strain 1389 as an appropriate micro-organism for decreasing of water pollution.

Key words: Bioremediation · Naphthalene · Copper · *Pseudomonas putida*

INTRODUCTION

Over the past decade industrial, urban, petrochemical and agricultural wastewaters have been increased in marine ecosystems through industrialization, population growth and human activities. These effluents were discharged into marine ecosystems contain high level of heavy metal and PAHs [1]. PAHs are a group of aromatic compounds consist two or more benzene rings. They are toxic, mutagenic and carcinogenic that have been documented by many studies [2-4]. PAHs are stable in the marine environments and not degrade easily due to their hydrophobic character and low solubility in water. These substances cause serious problems and several detriments for environment owing to their persistent and stability that make them as a recalcitrant pollutant for a long period of time [5].

Heavy metals are also a serious environmental problem due to their stability, bioaccumulation and biomagnification in the food chains [6, 7]. Although some heavy metals are essential like copper that is used in various enzymes such as cytochrome oxidase and superoxide dismutase. Low level of this heavy metal is necessary for organism's survival [8, 9]. However, it can be toxic and effects on growth, reproduction potential and physiological behaviors of marine organisms in high concentrations [10].

Nowadays various physicochemical standard methods have been recommended to eliminate PAHs and heavy metals from contaminated sites but they may be non-effective or expensive. The ideal process is microbial removal, a natural technique utilizes microorganisms capacity [11, 12]. It is an economical and efficacious option for removal of dissolved aromatic hydrocarbons and heavy metals from marine environments and efficient strategy to speed up cleaning processes for the benefit of humankind. Some bacteria, fungi, algae and yeast have special properties and functional groups in their cell walls that can decrease metal ions concentrations from ppt to ppb with high efficiency. Also hydrocarbon degrading microorganisms' usage PAHs as a carbon source and degrade them to non-toxic products [13].

This natural method can be used in the Persian Gulf, one of the most contaminated areas that located in the west south of Asia between Iran and Arab peninsular. It was estimated almost 20000 to 350000 tankers pass through the Persian Gulf annually that contains 40% of all the world tankers traffic. These tankers discharge several million tons of ballast water in this region. In addition high rate of water evaporation, extended drilling, oil extraction and petrochemical plants along this naval district intensify PAHs and heavy metals pollution [14, 15]. So the present study aimed to find the best marine bacteria resistant to copper and naphthalene and evaluate bacterial growth in presence of these pollutants.

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MATERIALS AND METHODS

Sediment samples were collected by Van Veen Grab from Persian Gulf located in the south of Iran. Samples were plated separately on nutrient agar containing copper (100 ppm) and MSM agar containing naphthalene (60 ppm) as a sole carbon source and incubated at 30°C for 72 h. One common dominant colony in two different plates was selected and purified [16].

Identification of isolated bacteria was done by biochemical tests [17] and 16S rRNA analysis (Sinagen co, Esfahan, Iran).

After inoculation of isolated bacterium in LB and MSM broth medium supplemented with copper and naphthalene respectively, the bacterial growth was monitored every 24 h for 7 days by measuring the optical density using spectrophotometer [18].

For biosorption studied, the bacterial colony was suspended in 100 ml solutions containing 100 ppm copper. The control sample without copper was considered and the pH of metal solutions was adjusted on 6. The flasks were shacked at 160 rpm for 6 h [19] and finally the copper concentration was measured by Atomic Absorption (SavantAAΣ) every 30 minutes.

Also the ability of isolated strain to degrade naphthalene as a sole source of carbon and energy was assessed by determining the amount of remained naphthalene. Five ml of each culture was picked up in specific times and mixed with 2 ml of hexane using vortex. Upper liquid was transferred to the clean flasks and after evaporation of hexane; 5 ml of acetonitril was added to residual pellet [20]. Samples were injected to HPLC by particular syringe. Flow rate was regulated at 1 ml min^{-1} and detection of naphthalene was done by UV absorbance at 254 nm. Comparison of pick area of standards and unknown samples determined naphthalene degraded concentration.

RESULTS AND DISCUSSION

Bioremediation and biosorption are technological methods for removal of heavy metals and biodegradation of PAH compounds. These biological processes are based on microorganisms potential to tolerate high levels of toxic materials [21,22,23]. The present study resulted in an identification of resistant bacteria as a *Pseudomonas putida* strain 1389. The results of biochemical tests are shown in Table 1.

Table 1: Results of microbiological and biochemical tests of isolated bacteria

Biochemical Tests	Isolated bacteria
colony character	Round and cream color
Colony size	Medium
Cell type	Rod
Gram reaction	-
KOH test	+
MR test	+
VP test	-
NaCl test	+
Indole test	-
Urea test	-
SIM test	+(H_2S production)
OF (glucose test)	Oxidative
Maltose test	Oxidative
Catalase test	+
Oxidase test	+
Lactose test	-
MacConkey test	+

Pseudomonas putida is a saprotrophic bacterium with adaptability to various environments. This bacterial species has diverse aerobic metabolisms for bioremediation of aromatic compounds (naphthalene, toluene, xylene and etc) and biosorption of heavy metals [24-27]. The ability of *pseudomonas* species in degradation of hydrocarbon and heavy metal materials has been reported by many researchers. Thavasi *et al.* [28] isolated four bacterial species from oil polluted marine environment. Results indicated that *P. aeruginosa* with 85.15% oil degradation, had the best ability to remove oil hydrocarbons among the others. In another research *Pseudomonas putida* 5a1 and *Pseudomonas aeroginosa* DHT-GL were isolated from oil polluted soils, from Guanaco Asphalt Belt in Venezuela. The mentioned species were tested for their ability to degrade naphthalene and some other oil hydrocarbons. The results of oil reduction in MSM medium inoculated with *P. putida* 5a1 and *P. aeroginosa* DHT-GL were 44% and 24%, respectively [29]. The ability of *Pseudomonas fluorescens* and *P. Putida* to tolerate Cr, Cu, Ni and Cd was reported by Hussein *et al.* [30]. They also mentioned that the species are capable to grow in presence of heavy metals.

Growth curve of *P. putida* strain 1389 in presence of naphthalene is shown in Fig.1. Schematic growth curve of isolated bacteria had no lag phase and indicated a rapid growth in presence of naphthalene. The maximum optical density was monitored at 3^{th} day and after that the bacterial growth rate was decreased as a result of

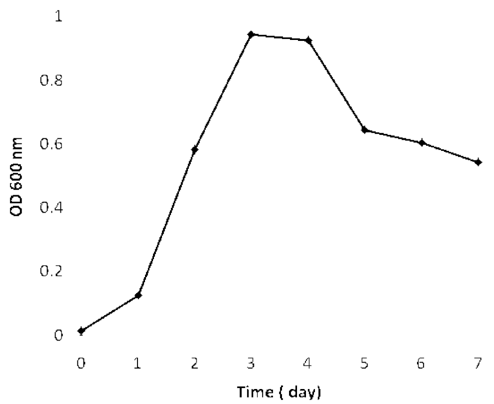

Fig. 1: Optical density of *P. putida* strain 1389 in presence of naphthalene (60 ppm)

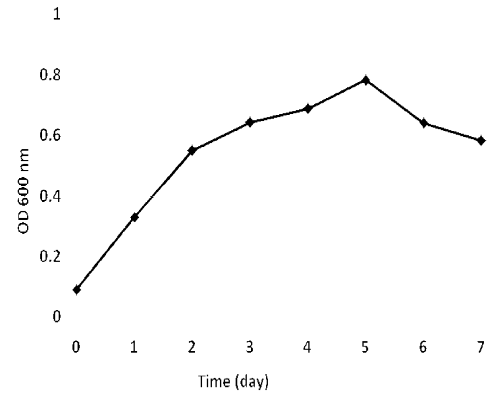

Fig. 2: Optical density of *P. putida* strain 1389 in presence of copper (100 ppm)

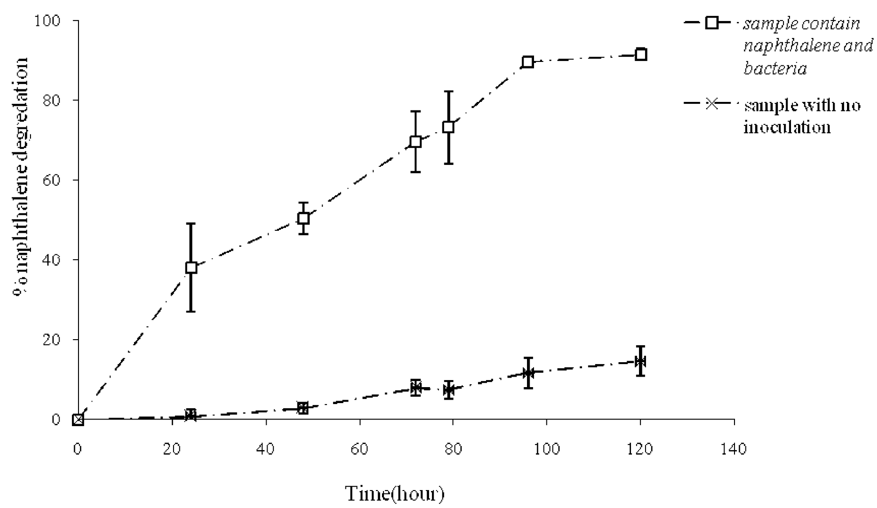

Fig. 3: Percentages of naphthalene bioremediation by *P. putida* strain 1389 within 120 hours

naphthalene depletion and also increasing the toxicity of secondary metabolic substances. The results supported the findings of Nnamchi *et al.* [31], which succeeded to isolate 24 bacteria utilize naphthalene as a sole carbon source from three polluted stations of Nsukka soil in Nigeria. Spectrophotomrtric experiments at 600 nm showed that *Pseudomonas* and *Borkholderia* species have highest OD value and the best growth in presence of naphthalene.

Fig. 2 showed the growth of *P. putida* strain 1389 in presence of copper. Mentioned bacteria started to grow after inoculation with 0.09 optical density and reached to its maximum growth (OD= 0.78) in 5th day. In fact, the cell division occurred and bacterium increased in size and number. However, medium nutrients were decreasing and the toxic products accumulated in the intracellular or extracellular parts of bacteria [32, 33]. Current research showed the change of optical density from 0.78 to 0.58 between 5th and 7th days. Actually the rate of bacterial growth and number of viable cells declined [34].

As shown in Fig.3, naphthalene bioremediation activity was increased dramatically and almost %40 of naphthalene was removed in 24 hours. Total naphthalene degradation (%91.48) demonstrated the high capacity of *P. putida* strain 1389 to reduce PAHs concentration in marine regions. The findings of Coral and Karagoz [20], confirmed the obtained results of this study. They examined fifty strains belonging to the genus *Pseudomonas* were achieved from crude oil polluted soil at a petroleum refinery in Mersin, Turkey. Results showed that Strains ARP26 and ARP28, which grew best on MM9 medium, were able to degrade 93% and 98% of oil respectively.

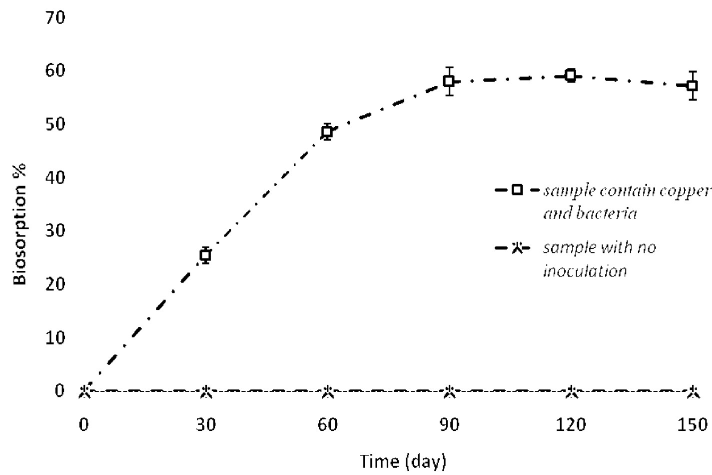

Fig. 4: Percentages of copper biosorption by *P. putida* strain 1389 within 150 minutes

The percentage of copper removal is shown in Fig 4. The graph indicates that *P. putida* strain 1389 began to uptake copper immediately after inoculation and almost 59% of copper was adsorbed in 120 minutes. This suggested that the copper removal could be related to resistant metabolism of the isolated strain which has complexing agents to bind metal ions [6].

The pH of the culture medium was adjusted on 6. Effect of pH on metal biosorption is an important factor. Previous investigations reported that the removal of heavy metals increases till the pH of 6. Also biosorption studied were not performed at higher pH values because the heavy metals precipitate at pH above 6 and the experiments don't show the correct results [35, 6, 30].

CONCLUSION

In general, these experiments proved the ability of *P. putida* strain 1389 in biosorption of copper and degradation of naphthalene. So we can suggest it as an appropriate organism for removing toxic pollutants from marine environments.

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