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Degradation of naphthalene, phenanthrene and pyrene by *Pseudomonas* sp. and *Corynebacterium* sp. in the landfills

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are non-polar organic compounds made up of two or more fused benzene rings, arranged in linear, angular or clustered structures. These compounds are normal products of organic matter combustion. In this study, bacterial strains that utilizes naphthalene, phenanthrene, or pyrene as carbon and energy sources for growth was isolated from landfills in Iran. In addition, Bacterial cell density was monitored by measuring the OD_{600}. Therefore, to assess the amount of PAHs remained in the mineral base medium by strong strains isolated from high-performance liquid chromatography (HPLC) were used. Initial results showed that the ability of *Pseudomonas* sp. and *Corynebacterium* sp. in removing combinations of PAHs is higher than other bacteria . In addition, These two bacteria have the highest OD_{600} at the third to fifth day interval in the presence of the PAH compounds. The results of HPLC evaluation showed that the rate of the phenanthrene degradation by *Corynebacterium* sp. and *Pseudomonas* sp. were 91.5% and 83.2% respectively and the rates of pyrene degradation were 79.4% and 68.2% respectively. While naphthalene was completely consumed by both strains after 10 days of incubation. According to the results obtained, isolated bacteria in the study can be used to improve microbial populations in areas polluted by the PAHs and to perform the bioremediation process.

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Introduction:

Polycyclic aromatic hydrocarbons (PAHs) are non-polar organic compounds made up of two or more fused benzene rings, arranged in linear, angular or clustered structures. They are hydrophobic with very low water solubility and high octanol-water partition coefficient (Bayoumi, 2009). PAHs are released into the environment from the incomplete combustion of fossil fuels and organic matter, the accidental spilling of processed hydrocarbons and oils, run off from asphalt pavements, coal liquefaction and gasification and natural geological processes (Zhang et al., 2004). Many past studies show that PAHs with lower molecular weight are toxic compounds, while high molecular weight compounds are significantly genotoxic (Juhasz and Naidu, 2000). Phenanthrene with three aromatic rings and pyrene with four aromatic rings, are two of the most abundant PAH found in nature. Although these compounds are not genotoxic, they are often used as indicators for monitoring PAH-contaminated wastes; molecular structures of phenanthrene and pyrene are found in potent carcinogenic PAHs (Bishnoi *et al.,* 2009).

Possible fates for PAHs released into the environment include photolysis, chemical oxidation, photo-oxidation, bioaccumulation, adsorption on soil particle and volatilization (Othman et al., 2009). The major decomposition processes for their successful removal are currently believed to be microbial transformation and degradation.

Aromatic with one, two or three aromatic rings (benzene, naphthalene and phenantherene) are also efficiently biodegraded; however, those with four or more aromatic ring (pyrene) are quite resistant biodegradation (Sutiknowati, 2007). Besides, the physical processes are often limited to aquatic environments only. The microorganisms should possess all the necessary enzymes needed to degrade PAHs (Al-Thani *et al.,* 2009).

Extensive studies have been done on the biodegradation of isolated bacteria from the natural environment leading to isolation of some bacteria which have the ability of using PAHs compounds as the sole carbon and energy source (Shafiee *et al.,* 2006; Shafie *et al.,* 2003; Kafilzadeh *et al.,* 2011). Isolating the bacteria with necessary performance for degradation of organic pollutants such as naphthalene, pyrene and phenanthrene in soil and water ecosystems can be the perfect solution for improving the microbial population in areas contaminated by hydrocarbons.

In current research, three combinations of naphthalene, pyrene and phenanthrene were studied by two powerful strains of *Corynebacterium* and *Pseudomonas*. In addition, appropriate concentrations for bacterial growth and chromatographic analysis to evaluate degradation rate of the PAH compounds by two strains of *Corynebacterium* and *Pseudomonas* were studied.

Materials and methods

Landfill soil sampling was done in Shiraz, Iran. Soil samples were collected randomly from depths 5-10 cm beneath the surface using spatula and were packed in sterile polybags. The samples were kept in flasks at 40 C and were transferred to the laboratory within 24 hours (Alam Khan and Rizvi, 2011).

Colony count

The soil samples were passed through a sieve to remove large pieces of debris and vegetation. The bacteria were originally isolated by plating dilutions of soils in saline solution (0.9 % NaCl) on nutrient agar and incubated at 37 ^{o}C for 48 h . The developed colonies were counted in plates and the average number of colonies per plates was determined. The number of total bacteria (CFU) per gram dry weight soil was determined (Bahig 2008).

Enrichment, isolation and identification of naphthalene, phenanthrene or pyrene-degrading bacteria

The minimal basal salts (MBS) medium used for enrichment and further experiments contained per liter:

1.0 g of $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 5 mg of $Fe(NH_4)_2(SO_4)_2$ and 1.0 ml of trace elements solution.

The trace element solution contained per liter: 23 mg $MnCl_2.2H_2O$, 30 mg $MnCl_4.H_2O$, 31 mg H_3BO_3, 36 mg $CoCl_2.6H_2O$, 10 mg $CuCl_2.2H_2O$, 20 mg $NiCl_2.6H_2O$, 50 mg $ZnCl_2$, 30 mg $Na_2MoO_4.2H_2O$.

The basic salt environment and tracing solution for separate elements were autociaved (20 minutes , 121° C). After sterilization, 1 ml tracing solution was poured slowly onto the salt base. Respectively, 0.3 , 0.4 , and 0.4 g/l of naphthalene, pyrene and phenanthrene compounds, were added to the 90 ml mineral base in the erlenmeyer flasks whose heads were covered with cotton. The compounds were dissolved and added to acetone. Flasks were then filled with 10 g soil sample and the culture environment PH was set at 7 using sodium hydroxide. The environment was incubated using a shaker turning on 180 rpm at a temperature of 30° C in the dark for 7 days. After a week, 10 ml of this medium was added to the 90 ml of the new culture medium and put onto shaker at 30° C for a week with proper aeration. Passage was done until the medium was completely opaque.

To prepare solid culture medium, 20 g agar per liter was added to the above medium and was poured into petri dishes. Using spray-plate method, each of the compounds was sprayed onto individual plates. Agar plates were incubated at 30° C for 3-5 days. After the incubation, colonies that showed a precipitation halo were selected for identification and further characterization. The isolates were identified on the basis of morphological , trophic and biochemical traits according to the Bergey·s Manual of Systematic Bacteriology (Coral and Karagoz, 2005).

Selection of powerful strains from among all isolated strains

In order to select the best and strongest degrading strains, the polycyclic aromatic hydrocarbons (PAHs) and the isolated bacteria with the desired substrate for concentration range were cultured in mineral culture base. Bacteria that started growing fast and had a high turbidity in the vicinity of the aromatic compounds were selected as appropriate microbial strains (Gina *et al.,* 1998; Owabor, and Ogunbor, 2007; Walczak *et al.,* 2001).

Determination of the time course of growth of the isolates

Bacterial cell density was monitored by measuring the OD_{600}. For this purpose, different concentrations of 0.3, 0.4, 0.5, 0.6 and 0.7 g/l of naphthalene hydrocarbon were produced and added to the 200 ml mineral base medium of erlenmeyer flasks whose heads were covered with cotton. Pyrene and phenanthrene were also used in these concentrations in separate flasks. Each of the flasks was filled with half McFarland of the *Pseudomonas* sp. and *Corynebacterium* sp. Then they were incubated at 30° C in the dark room. Within 12 hours, 5.0 ml sample was collected from each flask and assayed for OD at 600 nm in a UV spectrophotometer (Nnamchi *et al.,* 2006).

High performance liquid chromatography (HPLC) analysis

To calculate the amount of PAHs remained in the mineral base medium, high-performance liquid chromatography (HPLC) was used. For this purpose, the bacterial strains of *Pseudomonas* and *Corynebactrium* were solved separately in 5 ml of nutrient broth medium and were incubated at 30° c for 24 hours. After incubation, 1 ml of nutrient broth that contained the bacteria was added to 100 ml of mineral base medium in the Erlenmeyer flasks. Then the flasks were filled with pyrene (0.3 g/L). The process was repeated for the naphthalene and phenanthrene compounds with concentration of 0.4 g/L. Then the flasks were incubated for 10 days on a rotary shaker. Two replicates for each bacterial strain were prepared that were used for decomposition experiments. After 10 days, the cultures were centrifuged at 6000 rpm for 15 min and cells collected. This process was done in several times and then washing step was done for three times with

basal mineral medium. Finally cultures stored in 5 ml of the same medium and 2 ml of hexane was added to them in screw cap glass tubes that were shaken several times. 1 ml of the upper phase (hexane) was transferred to clean tubes and evaporate on a rotary evaporator. The remaining pellets were dissolved in 2 ml of HPLC mobile phase and stored at 4 °C for HPLC analysis (Coral and Karagoz, 2005).

High-performance liquid chromatography system model E600 (USA) Waters was used which was equipped with a microliter system, a multiple wavelength detector (2475, Waters, USA) and a Novapak C18 decomposition column from Waters Co (4 µm, 150 × 3.9 mm I.D, 60 Å).

Statistical analysis

The statistical analysis of the results was conducted by ANOVA test and SPSS, at a level of significance of $p< 0.05$.

Results

The results from accounting bacteria showed that the average of bacteria numbers in PAHs medium in comparison with the average of bacteria numbers in controlled medium were lower. There was a meaningful difference 0.05 between the average of the bacteria in medium with and without PAHs. The maximum numbers of bacteria 5.4×10^4 and the minimum numbers of PAHs degrading bacteria 2×10^4 of view. The percentage of PAHs degrading bacteria was 37.03% . Different bacterial strains from the soil around the landfill near Shiraz, Iran were isolated which included both gram-positive and gram-negative bacteria. Of these, 82.8 percent were gram positive and the rest were gram-negative. The isolated and identified bacteria include *Mycobacterium* sp., *Micrococcos* sp., *Bacillus* sp. , *Corynebacterium* sp. *Pseudomonas* sp. , *Rhodococcus* sp. and *Nocardia* sp. Among the strains isolated, two strains of *Corynebacterium* sp. and *Pseudomonas* sp. had the maximum growth rate in the presence of hydrocarbons. These two strains made the medium opaque in less than 30 hours, and were identified as the fastest and most effective bacteria in decomposing PAHs compounds.

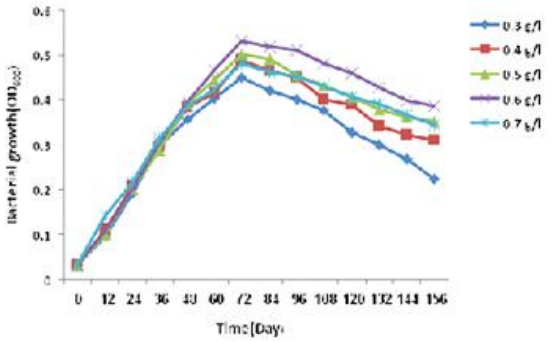

Fig. 1. Growth curve of *Corynebacterium* sp. on naphthalene.

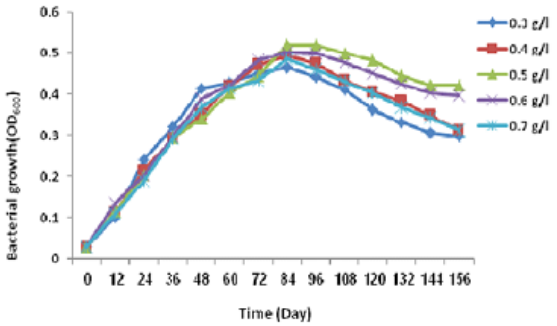

Fig. 2. Growth curve of *Corynebacterium* sp. on phenantherene.

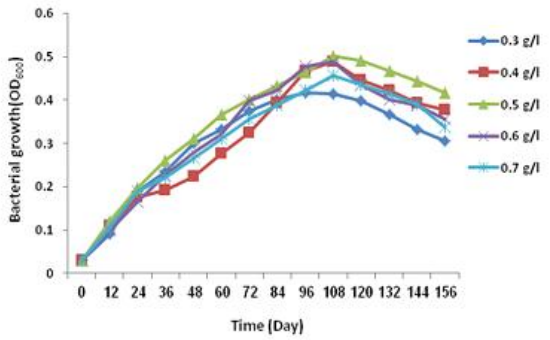

Fig. 3. Growth curve of Corynebacterium sp. on pyrene.

Pseudomonas sp. was gram-negative, motile, rod-like, non-sporulating and grew aerobically. Results of various biochemical tests were: catalase positive, oxidase positive, denitrification-positive and O-F test negative. The bacterial colony on blood agar is smooth and its borders are rough and have a feathery appearance.

Corynebacterium sp. was gram-positive, non-motile, rod-like and non-sporulating. Results of various

biochemical tests were: catalase positive, oxidase negative, nitrate reduction- negative and fermentation-positive.

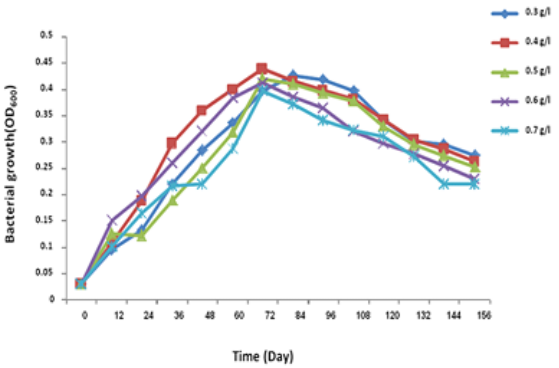

Fig. 4. Growth curve of *Pseudomonas* sp. on naphthalene.

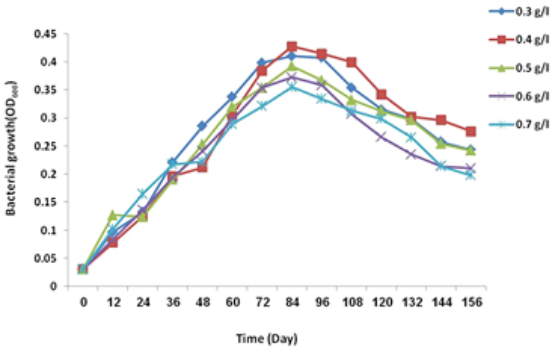

Fig. 5. Growth curve of *Pseudomonas* sp. on phenantherene.

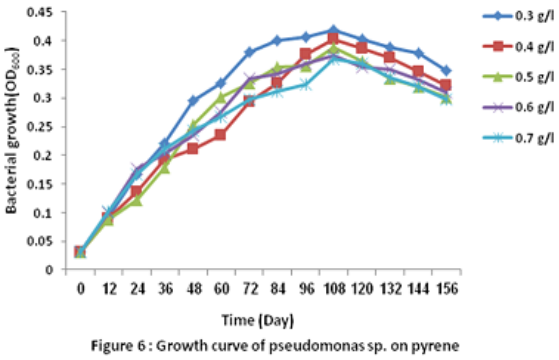

Fig. 6. Growth curve of *Pseudomonas* sp. on pyrene.

Kinetic experiments determined rate of PAHs degradation by these bacteria. Also effect of PAHs concentration on the growth of isolated bacteria was investigated by these experiments. Growth curve of isolated bacteria within 7 consecutive days showed that *Corynebactrium* sp. in the presence of 0.5 g/L pyrene and phenanthrene (curves 2 and 3) and 0.6 g/L naphthalene (curve 1) had high growth rate and *Pseudomonas* sp in the presence of 0.3 g/L pyrene (curve 6) and 0.4 g/L Phenanthrene (curve 5) and naphthalene (curve 4) had the best growth rate. *Pseudomonas* isolated by this method was not capable of tolerating higher concentrations of PAH compounds. It showed that *Corynebactrium* sp. had more ability to degrade PAHs than *Pseudomonas* sp. Moreover, growth curve of the isolated bacteria shows that these bacteria need more time to achieve the highest optical density (OD_{600}) in the presence of higher molecular weight compounds such as pyrene. Pyrene with four aromatic rings had the highest optical density between the fourthe and fifth days. But in case of naphthalene and phenanthrene the highest OD_{600} was observed at the third and fourth days.

Degradation rate of PAH compounds was evaluated by chromatographic analysis during 10 days of incubation. After incubation in optimal conditions (T: 30° C and pH: 7) naphthalene was completely consumed by both strains of *Pseudomonas* sp. and *Corynebactrium*. Degradation rate of phenanthrene by *Corynebactrium* sp. and *Pseudomonas* sp. were 91.5% and 83.2% respectively. Degradation rate of pyrene were 79.4% and 68.2% respectively.

Discussion

Microorganisms play an important role in the biodegradation of chemicals in natural ecosystems. The ability of bacteria in water, soil or sediment to degrade PAHs depends on the complexity of the PAH chemical structure and the extent of enzymatic adaptation by indigenous bacteria in response to chronic exposure to aromatic hydrocarbons. The degradation of xenobiotics may result from catabolism by individual strains of microorganisms or from combined metabolism by microbial communities (Heitkamp *et al.,* 1988). In current study, after the initial enrichment, different bacterial strains were isolated in landfills around Shiraz. Initial tests showed that the ability of *Pseudomonas* sp. and *Corynebacterium* sp. in removal of PAH compounds is more than any other bacteria. Thus these two bacteria were identified as the strongest

strains for degradation of PAH compounds in Shiraz landfill soils. These two bacteria had the highest OD_{600} in the presence of aromatic compounds. Isolated bacteria in the presence of low molecular weight compounds such as naphthalene in comparison with high molecular weight compounds like pyrene and phenanthrene achieved the highest optical density during a short time. Also comparing the growth curves of the two isolated strains showed that *Corynebacterium* sp. is more capable to grow adjacent to high concentration of aromatic compounds than *Pseudomonas* sp. This reflects the higher power of *Corynebacterium* sp. than *Pseudomonas* sp. could not tolerate concentrations higher than those needed for isolation.

In other studies many bio-degrading bacteria were isolated and identified. For example Zhang *et al.* (2004) isolated *Pseudomonas* sp., *Microbacterium* sp., and *Paracoccus* from contaminated soil with different PAHs compounds. In similar studies, Shafiee *et al.* (2006) identified and isolated *Pseudomonas*, *Mycobacterium*, *Dienococcus*, *Eikenella*, *Oligella* and *Corynebacterium*. It is obvious that isolated bacteria in Shafiee et al research are the same as bacteria which were isolated in Shiraz landfill soil project. Nnamchi et al (2006) isolated *Pseudomonas aeroginosa* and *Burkholderia capacia* in Nigeria. The isolated bacteria, respectively, showed the rate of optical density (OD_{600}) in the 3-day period in the presence of naphthalene. Also Al-Thani *et al.* (2009) isolated the bacterial strains of *Pseudomonas geniculate* and *Achromobacter xylosoxidans* which had ability to use naphthalene, phenanthrene and anthracene. Another study was carried out by Othman et al (2009) on the two-ring naphthalene compound. Under optimum conditions, the only strains *Micrococcus divrsus* was isolated which had a high tendency for degradation of this compound. Furthermore, Narasimhulu and Setty (2011) studied the isolation and identification of naphthalene-degrading bacteria in soil and found similar results. Two strains of *Pseudomonas aeruginosa* and *Pseudomonas cepacia* showed a high growth rate that was determined by increasing the optical density at 600 nm.

In recent decades, the ability of bacteria in degrading PAH compounds has been documented by extensive studies. The indigenous bacteria in contaminated areas are continuously in contact with aromatic compounds. These bacteria are able to degrade these substances in their surroundings because they possess all the necessary enzymes which are needed to degrade PAHs (Nnamchi *et al.,* 2006). These enzymes are encoded by plasmids (Al-Thani *et al.,* 2009) that have an essential role in the evolution of biodegradation ability of microorganisms. They enable horizontal gene transfer by bacterial populations that may be important for their survival. They also improve genetic variety as a means to promote genetic recombination (Sho *et al.,* 2004; Obayori and Salam, 2010). The achieved results in present study showed that *Pseudomonas* sp. and *Corynebactrium* sp. had highly ability to decompose PAH compounds in the soil of landfill. The biodegradation of low molecular weight (two-and three-ring) PAHs occurred much more rapidly and extensively than high molecular weight (four-, five- and six-ring) hydrocarbons (Li *et al.,* 2007). Shafiee *et al.* (2006) after analysis of PAHs during the ten-day incubation reported that phenanthrene completely; anthracene 60%, pyrene 80%, fluorene 30% and fluoranthene 20% were decomposed by soil bacteria. The results showed that increase the number of benzene rings in PAH compounds caused to decreases the rate of degradation of them. The biodegradability primarily depends on the complexity of chemical structures and physico chemical properties of PAHs. The results of present study confirmed these findings. In another study, Othman *et al.* (2010) reported 84% phenanthrene degradation by strains of *Corynebactrium urolyticum* after two weeks of incubation. In another similar study by Ping *et al.* (2011), 50% pyrene degradation by strain *Pseudomonas putida PL2* after 6 days of incubation was reported. But the co-presence of pyrene and phenanthrene after two weeks, lead to the same amount of pyrene being

used. In case of co-presence of two aromatic compounds, the bacteria first used the compound with low molecular weight. In addition, low molecular weight PAHs (2-3 benzene rings) were more easily degraded in the environment and therefore exhibit lower toxicity than high molecular weight PAHs.

Conclusions

The results of the regional surveys and soil biological studies indicated the presence of indigenous microorganisms that degraded polycyclic aromatic hydrocarbons. Indigenous bacteria isolated in this study had high ability in removing PAH compounds from landfills. Thus biological stimulation of the bacteria can be chosen as one of the biological treatment methods for the biological cleaning process.

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