Bulgarian Chemical Communications, Volume 48, Special Issue F, (pp. 14 – 19) 2016

Anthracene biodegradation by *Pseudomonas aeruginosa* isolated from Babolrood River estuary in Mazandaran province

M. Sasani[1], S. Khoramnejadian[1*], R. Safari[2]

[1]*Department of Environment, Damavand branch, Islamic Azad University, Damavand, Iran*
[2]*Ecological academy of Caspian Sea, Department of Aquatic Biotechnology, Sari, Iran*

Received February 17, 2016; Revised December 26, 2016

A wide variety of polycyclic aromatic hydrocarbons (PAHs) is found in the environment as a result of the incomplete degradation of organic substances or by many anthropogenic activities, such as the petrochemical industry and oil refining. In these activities, PAHs are existing in the effluent as complex compounds of low bioavailability that are highly limiting for conventional biodegradation techniques. These compounds are mutagenic and carcinogenic for human and animals. Biodegradation is one of the most important methods for decreasing of environmental pollutants. This process was done using different microorganism particularly bacteria and fungi. Various parameters such as pH, temperature, time, biotic and abiotic factors influence on biodegradation. Anthracene is three rings aromatic and exists in petroleum oil contaminated sites. Some of bacteria, owing to degrader enzymes production, are capable to degrade of anthracene. The aim of this study was isolation of anthracene biodegrading bacteria from Babolrood River estuary and evaluation of the biodegradation process in bench scale. Sampling was done from river estuary sediment and had cultured in Minimal Salt Medium (MSM). *Pseudomonas aeruginosa* was one of the isolated bacteria from river sediment that identified by molecular technique. In next step, influence of pH, temperature and concentration of anthracene were surveyed on anthracene biodegradation by *P. aeruginosa* during zero, 24 and 48 hours. The results showed that the optimized condition for biodegradation included pH= 7.5, 35 ◦C and 150 ppm of anthracene. Bacterial degradation of anthracene was increased with prolong of incubation time. Efficiency of *P. aeruginosa* for decomposition of anthracene was more than 50% during 48 hours. As regards to high ability of *P. aeruginosa* and also its survival in improper condition this bacteria can be used as biological tools for degradation of anthracene in oil contaminated regions.

Keywords: *Pseudomonas aeruginosa*, Anthracene, Babolrood River, PAHs.

INTRODUCTION

Growth population, urbanization and industrial development and enhance the standard levels of living, cause increase needing to new resources and the arrival of new compounds to human life cycle leading to contamination of water resources. High doses of chemical pollutants via industrial wastewater and municipal wastewater and industrial enter to the environment and natural water resources [1]. High volumes of organic waste production as a result of urbanization and necessity to manage different organic waste at low-input must be mentioned as well as eco-friendly basis [2].

Polycyclic aromatic hydrocarbons (PAHs) are recognized as carcinogenic and mutagenic compounds [3]. These compounds are often found as complex mixtures in the environment and rarely seen in single form. These compounds are usually colorless (white or light yellow color) and used in the painting, making plastics, pesticides and asphalt roads [4]. Although some of the PAHs compounds in natural conditions were decayed by the indigenous microbial population but this process usually takes time [5] and requires using of techniques with high performance which is able to carry out this process in a shorter time. Some physical methods such as flotation has a no high efficiency and common chemical methods such as using of surfactants was usually costly and have a lot of limitations. One effective method to reduce the PAHs compounds is use of biological methods that microbes individually or in combination in order to complete decomposition of pollutants were used. Research on biodegradation of petroleum compounds show that this method is one of the most economic and effective methods to hydrocarbon removal from aqueous environments [3]. In the process of biodegradation some microorganisms should be used that are indigenous in the infected area and are not included manipulated organism [6]. Ability of bacteria in

* To whom all correspondence should be sent:
E-mail khoramnejad@damavandiau.ac.ir

soil, water or sediment to biodegradation of PAHs depends on the complexity of the chemical structure of substances and compatibility enzyme producing bacteria in the environment of pollutants. Generally, two-ring PAHs are readily biodegradable and various bacteria are capable of degrading these compounds and used them as a source of carbon and energy [7]. Degradation of compounds containing benzene rings hardly was done and only bacteria have indicator enzymes was able to do this process. Anthracene including tricyclic aromatic compounds is less studied. According to the few studies on indigenous bacteria in Caspian region in northern Iran, the main objective of this study was biodegradation of polyaromatic hydrocarbons (anthracene) by *Pseudomonas aeruginosa*, isolated from sediments of estuaries Babolrood River and identification of secondary metabolites by GC-MS analysis.

MATERIAL AND METHODS

All tests were done based on standard methods described in the Examination of Water and Wastewater [8]. All chemicals were purchased from Merck Company (Germany).

Sampling

In this study, Babolrood estuarine sediment in Mazandaran province was used as a bacteria isolation medium. Samples were conducted using the Grp from sediment Babol River at several points of the estuary. In order to prevent the death of bacteria in the sediment, samples placed on plastic containers and transferred to the central laboratory in the shortest time with low temperature.

Obtain pure samples of anthracene degrading bacteria

Generally, the medium contain the pollution index compound was used to isolate the bacteria with desired properties as a selector agent. For this purpose, after transferring the samples to laboratory and preparation serial dilution, culture was conducted at MSM medium. After adjusting the pH in the range of 7, disinfection process was carried out at 120 ° C for 15 m and after arriving medium temperature to 50 °C, sterilized Anthracene with 0.45 micron filter Millipore as a source of carbon (50 mg/l) was added. After incubation at 30 °C for 5 days and growth of cultured bacteria (the appearance of opacity). Subsequently culture was conducted at MSM agar contain anthracene and samples were incubated at 30 °C for 72 h. After growth of anthracene degrading colonies, to subculture and purifying colonies index was action [9-10].

Molecular study

Polymerase chain reaction (PCR) was applied for identification of isolated bacteria [11]. Two pairs of primers were designed based on the nucleotide sequences of the 16s rRNA gene [11-13] of *P. aeruginosa* include: F: (5′-CCAGTTTGATCMTGGCTCAG –3′) and R: (5′-AGAGTTTGATCMTGGCTCAG –3) in order to identify the *P. aeruginosa*. Primers were synthesized by Cinna Gen Company (Tehran, Iran). DNA extracted according the method described by Samaei et al. (2013) and polymerase chain reaction was repeated in 30 cycles [11] under the following conditions: 30 s min at 94 °C (1 Hz), 1 min at 64 °C, 1 min at 58°C, 1.5 min at 72 °C (35 Hz) and finally, PCR was completed with the final extension step at 72 °C for 8 min. Distilled water was used as a negative control in each PCR reaction.

Sequencing of PCR product

For each sample 30 µl of PCR products and 20 µl of each forward and reverse primers (Concentration of 20 µmol) were transfered in a separate tubes to the BIONEER Company (South Korea). After receiving the results of sequencing using Sanger assay and BLAST in the GenBank NCBI, unknown bacteria were identified [11].

Preparation of bacteria to inoculate the broth media to evaluate the decomposition process

At first, the primary cultured bacteria carried in TSB medium and after incubation at 30 °C for 24 h, medium was centrifuged at 13,000 rpm for 5 min and after discarding the supernatant, 10 ml of saline solution was added to the remaining sediment and after mixing, opacity suspension was compared with McFarland standard tubes (0.5 McFarland equivalents 1.5×10^8 CFU/ ml) [9].

Biodegradation of anthracene in broth media

Anthracene biodegradation test were performed in 500 ml Erlenmeyer flasks containing 200 ml of MSM media. Studied parameters, including concentration of anthracene (150, 200 ppm), incubation time (0, 24, and 48 h), pH (6.5 and 7.5) and temperature (25 and 35 °C). Samples was put in a shaking incubator at 120 rpm and tryptic soy agar (TSA) was used to assess the process of growth bacteria and the number of colonies was determined. The high-performance liquid chromatography device (HPLC, KNAUER,

Germany) was used to measure the concentration of anthracene residue after decomposition by bacteria [5]. Acetonitrile solvent and water with ratio of 90 to 10 as a mobile phase with a flow rate of 1 mL per minute, C18 column and UV detector were used [12-14]. All experiments were conducted With 3 replications.

Statistical analysis

The obtained data were subjected to two-way analysis of variance using SPSS statistical software, release 18.0. Duncan's new multiple range test was performed to determine the significant differences between means at the 5% probability level ($P< 0.05$).

RESULTS

Isolated bacteria

Biochemical tests and PCR assay confirmed *P. aeruginosa.* After PCR assay, DNAs extracted from isolated bacteria gave the expected 357 bp PCR fragment of 16S rDNA sequences, which is specific for *P. aeruginosa* which shown in Fig. 1.

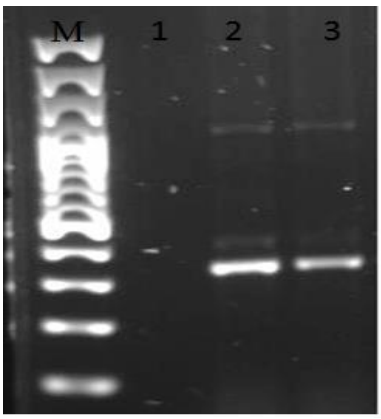

Fig. 1. Electerophoretic analysis (2% agarose gel) of DNA amplified fragments from 3 isolates in this experiment (M: Marker, Lan 1: negative control, Lan 2: positive control, Lan 3: positive isolate).

The effect of initial concentration of anthracene on the removal efficiency

Results of Tables 1 and 2 showed that by increasing the concentration of anthracene process of growth of *P. aeruginosa* was decreased. On the other hand, anthracene reduction in the concentration of 200 mg/l at 24 and 48 hours has been very slowly. Changes in the number of bacteria and concentrations of anthracene was significant after 24 and 48 h ($P <0.05$). The number of *P. aeruginosa* shown in table 1. Concentration changes of anthracene in the presence of *P. aeruginosa* in 150 and 200 mg/l were shown in table 2.

The effect of initial pH on the removal efficiency

The concentration of 150 mg/l has a better effect on bacterial decomposition. Therefore, 150 mg/l concentrations were considered as the optimum concentration for next study. Results of Tables 3 and 4 showed that pH=7.5 had a better bacterial decomposition compared to pH of 6.5. The number of *P. aeruginosa* (log) at pH= 6.5 and 7.5 were shown in table 3. Table 4 shown the Concentration changes of anthracene in the presence of *P. aeruginosa* at pH= 6.5 and 7.5.

Table 1. The number of *P. aeruginosa* (log) in 150 and 200 mg/l concentration of anthracene.

Incubtion time (h)	Concentration (mg/l)	
	200 mg/l	150 mg/l
0	7.00 ±0.2 aC	7.02 ±0.33 aC
24	8.25 ±0.03 bB	8.48 ±0.03 aB
48	8.41 ±0.07 bA	9.25 ±0.07 aA

*Different small and capital superscript letters within each row and column, respectively, represent significant differences ($p < 0.05$).

Table 2. Concentration changes of anthracene in the presence of *P. aeruginosa* in 150 and 200 mg/l concentration.

Incubtion time (h)	Concentration (mg/l)	
	200 mg/l	150 mg/l
0	200.00 ±4.25 A	150.00 ±1.45 A
24	165.00 ±4.89 B	120.00 ±3.26 B
48	150.00 ±2.30 C	73.00 ±4.11 C

*Different capital superscript letters within column, represent significant differences ($p < 0.05$).

Table 3. The number of *P. aeruginosa* (log) at pH= 6.5 and 7.5 (in 150 mg/l concentration of anthracene)

Incubtion time (h)	pH	
	7.5	6.5
0	7.04 ±0.08 aC	7.01 ±0.12 aC
24	8.68 ±0.04 aB	8.52 ±0.03 bB
48	9.52 ±0.07 aA	9.18 ±0.07 bA

*Different small and capital superscript letters within each row and column, respectively, represent significant differences ($p < 0.05$).

Table 4. Concentration changes of anthracene in the presence of *P. aeruginosa* at pH= 6.5 and 7.5

Incubtion time (h)	pH	
	7.5	6.5
0	150.00 ±1.11 aA	150.00 ±1.21 aA
24	121.00 ±4.17 aB	119.00 ±3.42 aB
48	73.00 ±3.26 aC	70.00 ±1.82 aC

*Different small and capital superscript letters within each row and column, respectively, represent significant differences ($p < 0.05$).

The effect of temperature on the removal efficiency

The effect of temperature on decomposition of anthracene are shown in Tables 5 and 6. Most bacterial decomposition at 35 °C was observed. Observed changing in the number of bacteria and 150 anthracene concentrations in both 25 and 35 ° C at 24 and 48 hours was significant (P<0.05).

Table 5. The number of *P. aeruginosa* (log) in 25 Cº and 35 Cº at pH= 7.5 and 150 mg/l concentration of anthracene)

Incubtion time (h)	Temperature	
	25 Cº	35 Cº
0	7.00 ±0.09 aC	6.98 ±0.06 aC
24	8.50 ±0.06 bB	8.73 ±0.09 aB
48	9.35 ±0.20 bA	9.85 ±0.28 aA

*Different small and capital superscript letters within each row and column, respectively, represent significant differences (p < 0.05).

Table 6. Concentration changes of anthracene (150 mg/l concentration) in the presence of *P. aeruginosa* in 25 and 35 ºC at pH= 7.5

Incubtion time (h)	pH	
	25 Cº	35 Cº
0	150.00 ±1.45 aA	150.00 ±1.34 aA
24	120.00 ±2.12 aB	122.00 ±3.20 bB
48	71.00 ±3.25 aC	75.00 ±1.84 aC

*Different small and capital superscript letters within each row and column, respectively, represent significant differences (p < 0.05).

Survey the removal efficiency in optimum condition

Based on the results, the best conditions to decomposition of anthracene was pH=7.5, 35 °C temperature and 150 mg/l of anthracene concentration. In this condition, the removal efficiency was more than 50 percent.

Identification of secondary metabolites producing from the decomposition of anthracene

In figure 2, different secondary metabolites producing from the biodegradation of anthracene was shown. The highest peak of the secondary metabolite was 2,3-dehydroxy naphthalene followed by catechol and phthalate. These metabolites are recognized as intermediate terminal products and represent a relatively complete biodegradation of anthracene by *P. aeruginosa*. isolats. Based on the figure 2, a lot of metabolites with very low values were produced that immediately affected by microbial degradation and turn into the next metabolite. Among the most important of these compounds can be mentioned to Anthracene cis 1,2-dihydroxy DL, 1,2-dihydroxy Anthracene, Cis 4, 2-oxobut enoic acid.

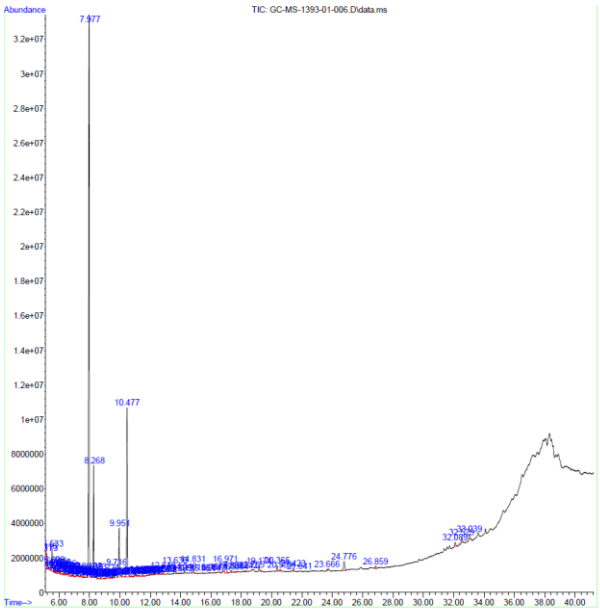

Fig. 2. Chromatograms showing degradation of the PAH by *P. aeruginosa*.

DISCUSSION

Isolated bacteria

In Jacques et al. (2005) study, *P. aeruginosa* was recognized as indicator bacteria isolated from soils contaminated with petroleum which was able to biodegradation of anthracene in high concentrations [15]. In Simarro et al. (2013) survey, *Pseudomonas* sp., was able to biodegradation of anthracene and naphthalene at different concentrations [16]. Prakash et al. (2014) reported that *Pseudomonas* sp., *Bacillus* sp. and *Micrococcus* sp. were the dominant genera isolated from contaminated areas with petroleum that able to biodegradation of benzene, toluene, anthracene, naphthalene and gasoline. Results showed when these bacteria were used in combination forms, significant decline of petroleum compounds were observed [14]. In Safahieh et al. (2010) study, *P. aeruginosa* and *Pseudomonas putida* was recognized as bacteria with high ability in biodegradation of naphthalene in Khoor Moosa and the rate of degradation of naphthalene by these bacteria after 120 h were 96% and 91%, respectively [17]. In these mention study, different species of *Pseudomonas* were able to growth in different conditions and capable to biodegradation of petroleum compounds. These bacteria because of producing the different enzymes and have a spore form have a high efficacy to reduce the different environmental pollutants.

The effect of initial concentration of anthracene on the removal efficiency

The results indicate that by increasing concentrations of anthracene the trend of biodegradation was slower that related to toxicity of PAHs in high concentrations of bacteria [18]. Some works reported that *P. aeruginosa* and *P. putida* isolated from soils contaminated with petroleum capable to biodegradation of anthracene in high concentrations after 120 h. Ability of bacteria to biodegradation of petroleum compounds was depend on the genus and species of bacteria, ability of bacteria to producing of biodegradation enzymes and finally to period time of incubation [15]. In current study the period of incubation time was 48 h which has been relatively little time for biodegradation of bacterial and bacteria need the more time for adaptation to the new conditions and biodegradation of petroleum compounds. In some studied, this time was 5 days. In current study *P. aeruginosa* were able to biodegradation of anthracene at a concentration of 150 mg/l during 48 hours with efficacy more than 50%. Although in other studies, during 6 to 10 days the efficiency of biodegradation was between 55 to 65% [15-17]. Therefore, it is expected that with increasing the incubation time, the efficiency of biodegradation of anthracene was increased by *P. aeruginosa*.

The effect of initial pH on the removal efficiency

Study of Simarro et al. (2013) showed that *Pseudomonas* species capable to biodegradation of Polycyclic aromatic hydrocarbons at pH=7 and relatively high temperature (30 °C), in this study biodegradation of naphthalene was faster than anthracene [16]. Results of Jacques et al. (2005) study indicated that *P. aeruginosa* isolated from contaminated soil able to biodegradation of anthracene at pH 6.5, 7 and 7.5 with 28.2, 32.5 and 34.5 during 5 days which reflects the high efficiency of biodegradation at pH=7.5. These results were similar to our study and confirmed it [15]. Most environmental bacteria has optimum activity at pH=7.5. Also, in lower or higher pH were able to growth and proliferation.

The effect of temperature on the removal efficiency

In other work Research of Jacques et al. (2005) proved that *P. aeruginosa* isolated from contaminated soil able to biodegradation of anthracene at 20, 25, 30, 35 and 40 ° C with 46.8, 59.6 and 64% during 5 days. These results indicated high efficiency of this bacterium to biodegradation of anthracene at 20 and 30 °C, in our study highest efficiency of *P. aeruginosa* to biodegradation of anthracene was observed in 30 °C. Some Studies showed that different species of Pseudomonas capable to biodegradation of naphthalene and anthracene at 35 °C and used them as a source of carbon and energy. Different bacteria depending on their natures (thermophilic, psychrophilic and mesophilic) has been able to grow and reproduce at different temperatures and has a minimum, maximum and optimum temperatures of growth [16].

The removal efficiency in optimum condition

Studies by other researchers confirmed the results of the present study and in most study pH= 7.5 and 35 °C temperature provide the optimum conditions for biodegradation bacteria, So in mention condition some bacterial species able to adapted to higher concentrations of petroleum compounds and eventually biodegradation them. In other studies, the incubation time was between 6 to 10 days or even more, although in present study the incubation time was 2 days. However, in this period time the efficiency was more than 50 percent which reflects the high ability of *P. aeruginosa* to biodegradation of anthracene.

Identification of secondary metabolites producing from the decomposition of anthracene

In our study similar to Arab and Salim (2010) survey, intermediate compounds was produce due biodegradation of anthracene by *P. aeruginosa* isolated from contaminated soil [15]. Various metabolites due biodegradable of aromatic ring compounds was identified and compared with each for quality and quantity properties [18-19]. The presence of phthalates indicating that the bacteria action through the phthalate metabolite and anthracene was converted to phthalate. In this assay the number of generated metabolites was low [20]. The presence of catechol indicated that Anthracene affected by bacterial decomposition was converted to the secondary metabolites and finally to catechol that is different from the phthalate metabolite pathway [21].

CONCLUSION

Results of current study indicated that *P. aeruginosa* isolated from estuarine sediments of Babolrood River had the high potential and ability to biodegradation of Anthracene and in the optimum condition (temperature and pH) able to biodegradation more than 50% of Anthracene in *in vitro* condition. So, after complete and additional studies and confirmed the ability biodegradation of Aromatic compounds by *P. aeruginosa* and achieve

to reasonable results, *P. aeruginosa* for native to the climatic conditions of Mazandaran province could be used to clean up oil spills in polluted areas.

REFERENCES

1. W. Christopher, R. Bentham, *Appl. Environ. Soil Sci.* , **46**, 171 (2011).
2. H, Radnezhad, S. H. Khalili Arjaqy, F, Mortazaeinezhad, M. Foroughi Abari, M. Sadeghi, J. Pure Appl. Microbiol., **9**, 2203 (2015).
3. G. Coral, S. Karagoz, *Ann. Microbiol.*, **55**, 255 (2005).
4. D. Boyle, C. Wiesner, R. A. Ichardson, *Soil Biol. Biochem.*, **30**, 873 (1998).
5. A. Arab, B. Salim, *J. Am. Sci.*, **10**, 1237 (2010).
6. M. Emtiazjo, S. Sedighi, *Int. J. Sci. Environ.* ***Tech.***, **11**, 258 (2009).
7. A. Sharon, L. Churchil, P. Jenniffer, R. Harpe F. Perry**,** *Appl. Environ. Microbiol.***, 65**, 52 (1999).
8. APHA/AWWA/WEF Standard Methods for the Examination of Water and Wastewater, 20 ed. Washington DC, (1998).
9. R. Safari, Z. Yaghobzadeh, *J. Mar. Sci. Tech.*, **3**, 35 (2006).
10. C. Cerniglia, *Biodegredation*, **3**, 331 (1992).
11. M. R. Samaei, S. B. Mortazavi, *Fresen Environ. Bull.*, **22**, 1304 (2013).
12. M. Barret, H. Carre, *Water Res.*, **44**, 3797 (2010).
13. C. Kazunga, M. Aitken, *Appl. Environ. Microbiol.*, **66**, 1917 (2000).
14. A. Prakash, S. Bisht, J. Singh, *Turk J. Eng. Environ. Sci.*, **38**, 41 (2014).
15. R. J. S. Jacques, E. C. Santos, F. M. Bento, M. C. R. Peralba, P. A. Selbach, E. L. S. Sa´, A. O. Flavio Camargo, *Int. Biodeterior. Biodegr.*, 56, 143 (2005).
16. R. Simarro, N. Gonzalez, F. L. Bautista, C. M. Molina, *FEMS Microbiol. Ecol.*, **83**, 438 (2013).
17. S. Bisht, P. Pandey, A. Sood, *Braz. J. Microbiol.*, **41**, 922 (2010).
18. C. Cripps, A. John, B. Steven, D. Aust, *Appl. Environ. Microbiol.*, **56**, 1114 (1990).
19. C. E. Cerniglia, *Adv. Appl. Microbiol.*, **30**, 31 (1984).
20. A. Safahieh, F. Mojodi, H. Zolgharnin, I*nt. J. Environ. Stud.*, **58**, 149 (2010).
21. S. Bajkic, B. Vasiljevic, *Arch. Biol. Sci.*, **63**, 1057 (2011).