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Isolation and molecular identification of fluoranthene degrading bacteria from the mangrove sediments in South of Iran

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Abstract

Mangrove forests are of the wooden plants which are susceptible to contamination by oil. They are like a reservoir which could absorb much of PAH compositions from the oil contaminations. On the other hand, the contaminated sediments contain many types of indigenous microorganisms able to degrade PAH compositions. This study was designed to isolate indigenous microorganisms which are able to degrade biologically fluoranthene from Persian Gulf sediments and analyze their growth kenetics. The isolated bacteria included *Bacillus circulans* , *Alcaligenes faecalis* , *Enterobacter* , *Listeria* and *Staphylococcus*. They showed good power to degrade the fluoranthene. The fluoranthene degraded by them was assessed by HPLC so the *Bacillus circulans* and *Alcaligenes faecalis* were the strongest isolated bacteria and ten days after the enrichment they degraded the fluoranthene 73.4 and 71 percent, respectively. Also the *Enterobacter*, *Listeria* and *Staphylococcus* could degrade the fluoranthene 67.4, 54 and 48.70 percent, respectively .

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Introduction

The polycyclic aromatic hydrocarbons are a class of the organic compounds formed of two or more interrelated benzene rings or five ring molecules arranged in different structures (Bamforth and Singleton, 2005). Polycyclic aromatic hydrocarbons are of the most important organic micro pollutants because of their vast distribution and low degradability in the environment (Arbabi *et al.,* 2004). Such compositions are produced and colonized by two natural and anthropogenic processes and their colonization is dangerous for human life (Hong *et al.*, 2008). Such compositions differ from each other in view of their displacement, distribution and fate in the environment and their effects on the biological system.

United States Environmental Protection Agency (USEPA) has introduced 16 PAH compounds as primary pollutants. Dispersion of some of these compounds in the environment can cause cancer in humans (Seo *et al.,* 2009). Nowadays many researchers and industries are interested in aromatic biodegradation compositions because of their toxicity and late degradation (Mohite *et al.,* 2010). Biodegradation is the metabolic power of the microorganisms to mineralize the organic pollutant or convert them to less harmful and safe materials. This method is an economic, operational technology to clean nondestructively and also it is favorable in view of preparation because it accelerates the natural biological degradation through optimizing limited conditions (Margesin and Schinner, 2001). Mangrove is a type of wooden plants present in tidal, tropical and subtropical regions; specially they are susceptible to oil contaminations and like a reservoir they absorb much of PAH compositions from the oil contaminations, sewage and industrial discharges (Liu *et al.*, 2010); the contaminated sediments contain many types of indigenous microorganisms able to degrade and benefit from PAH compositions (Guo *et al.*, 2005). Often it is useful to use indigenous microorganisms to biodegrade the poisonous contaminations because they may be more effective economically. The indigenous microorganisms may adapt better to the environment so the problems relating to foreign microorganisms release are avoidable (Tam *et al.*, 2008). There is an extensive body of knowledge on mineralization or degradation of hydrocarbons by microorganisms in mangrove sediments. In a recent study, Tian *et al.* (2008) reported the capability of native bacterial population to mineralize phenanthrene, fluoranthene and benzo(a)pyrene from mangrove sediments in China. Similarly, Guo *et al.* (2005) demonstrated that *Sphengonomus, Rhodococcus* and *Parcoccus* isolated from superficial sediments of some mangrove are able to degrade fluoranthene. Virtually all of these studies utilized organisms isolated from contaminated sites and degradation competencies were only tested on hydrocarbons. In this report however, we chose to isolate fluoranthene degraders from the mangrove sediments in South of Iran and assay their growth kinetics and biodegradation ability. The results from this investigation would be useful for prediction of the bioremediation mechanisms of these microbial isolates.

Material and methods

Sampling

In this study the mangrove sediments in South of Iran located in Nayband national park were examined. The park is located in the provinces: Booshehr and Hormozgan. Assalooyeh poisonous installments are in Northwest of the park. The sampling was conducted from superficial sediments to depth of 0 – 3 cm in three different parts in autumn and summer. The samples were put into sterile bottles, then into the containers full of ice and then transferred to lab. for enrichment in less than 24 hours . GPS device was used to define the sampling places. Table 1 shows geographical situation of the sampling stations.

Bacterial count

The bacteria were counted by total viable plate count method. Having gathered the sediments samples they were diluted 10^{-1}–10^{-10}. Then the diluted samples were cultured on agar nutrient fluoranthene

by surface plate method. Then the cultured plates were put into the 22° C oven (Incubation). The colonies were counted after 48 hours (Udeani *et al*., 2009).

Enrichment and Isolation of fluoranthene degrading bacteria

First 10 ml floating sediments (deionized water: sediment; 1 : 10) were added to 90 ml sterile mineral salt medium environment containing 500 mg/lit fluoranthene and put in shaker incubator in 22° C in 150 rpm g revolution in order to microbial enrichment by fluoranthene to be incubated . After two weeks 10 ml was departed from the environments and transferred to a new MSM environment with the same conditions (Tam *et al*., 2008). After 3–4 times repetition the samples on agar MSM medium covered with a layer of fluoranthene were cultured in 500 mg/lit rate. The bacterial colonies producing transparent halos were considered positive (Guo *et al*., 2005). Having purified such bacteria they were identified by gram staining, morphological studies and catalase and oxidase reactions and other diagnostic tests were conducted (Mashreghi and Mashreghi, 2005).

Selecting strong bacteria

after isolation and identification of bacteria they were cultured in MSM medium containing fluoranthene to find the best and strongest fluoranthene degrading strains. Hence the medium with the most turbidity and the bacteria with the most growth beside aromatic hydrocarbon were selected as the strongest bacteria to degrade fluoranthene (Kafilzadeh *et al*., 2009). Furthermore the agar MSM medium was used to designate stranger isolates based on their sooner appearance on the medium to guarantee the results.

Assessing the growth of the isolated bacteria by optical density different concentrations of fluoranthene

Four flasks containing 20 ml of the MSM culturing medium were considered for each isolated bacteria in order to examine the growth of bacteria in different concentrations of fluoranthene. One of the fluoranthene concentrations (500, 600, 700 and 800 mg/lit) was added to each flask and then 5 ml of microbial suspension was added to each flask containing MSM medium. Plus the test containers, control medium without fluoranthene was used and after incubation in 22° C for one week optical density was conducted daily in 600 nm wavelength in 12 hour intervals by spectrophotometer (Kafilzadeh *et al*., 2009).

Fluoranthene degradation study

100 mg fluoranthene per liter was added to the flasks containing 100 ml basic mineral medium in order to examine the isolated bacteria potentiality capable to degrade fluoranthene and then bacterial suspension was provided from each isolated bacteria based on 0.5 McFarland and added to the prepared basic media and incubated in an orbit shaker at 22˚C for 10 days. Than 2 ml hexane was transferred to 5 ml of mineral medium and centrifuged in 6000 rpm for ten minutes. Then one ml of above phase (Hexane) was added to the sterile tubes; the fluoranthene degradation rate through the isolated bacteria was read by high performance liquid chromatography (HPLC) Model: Waters 600 E (U.S.A.) (Coral and Karagoz, 2005).

The 16S rRNA gene sequence of the strong bacteria in fluoranthene degradation

The 16S rRNA gene sequence of two strongest fluoranthene degrading bacteria was defined after measuring their ability to degrade fluoranthene. DNA extraction was conducted by the special kits to extract the bacterial genome DNA (Fermentase K0512) related to the Fermentase Company .

PCR (Polymerase chain reaction)

16S rRNA gene multiplication

The PCR was conducted in the volume of 50 microliter by the thermocycler device (Corbet , Australia). Temperature cyclic program of the PCR reaction are shown in Table 2 (Sanders and Miller, 2010).

Electrophoresis

Having finished the PCR cycles the gel electrophoresis was conducted on one percent agarose (w/v) with 70 voltages for one hour in order to see the PCR product band pattern. The TAE buffer was used to have the gel and also electrophoresis tank buffer was used. The 1 kb ladder (Fermentase Gen Ruller SM 0373) was used to define the PCR products sizes (Sanders and Miller, 2010).

Defining the sequence of the 16S rRNA gene

The 16S rRNA gene multiplication PCR product was sent by Fanavaran Gene Company to Ampliquon Company (South Korea) in order to define the sequence.

Bioinformatics

Sequence modification

The sequence resulted from 16S rRNA gene sequence definition was modified by the software Bioeds (Version 7.0.9.0). Having defined the sequence of two ranges and completed them the probable errors regarding the sequences were controlled and modified. Then the cleaned sequence was saved into FASTA format (Sanders and Miller, 2010).

BLAST Analysis

The strains were identified By BLAST analysis and the sequence from the gene bank (http://www.ncbi.nlm.nih.gov/genebank). By virtue of this analysis the 16S rRNA gene sequence of the strain under examination is compared with all sequences of the gene in the Gene bank and the strains with the most similarity are shown downwardly in the table and the strain under examination was identified by virtue of such findings (Sanders and Miller, 2010).

Drawing the phylogenetic tree

Cluster Analysis

By the software Mega 5 on the basis of the Neighbor – Joining algorithm the Cluster Analysis was conducted on the 16S rRNA gene sequences of the strains under examination and similar bacterial species in order to define the phylogenetic strains under examination; the sequences were obtained from the Gene bank . The Bootstrap values tree obtained from 1,000 probable phylogenetic trees were shown as percent in the division place to test statistically the tree (Sanders and Miller, 2010).

Statistical analysis

The ANOVA test was used to compare the stations and seasons in viewpoint of the differences between the means of the fluoranthene degrading bacteria numbers in the sediment samples. The significance level is one percent ($P<0.01$).

Results

In current research five bacteria including *Bacillus circulans*, *Alcaligenes faecalis*, *Enterobacter*, *Listeria* and *Staphylococcus* from all bacteria which were isolated from Persian Gulf mangrove sediments were identified as the most abundant ones. Also they have more ability to degrade fluoranthene than the other isolates.

Table 1. Geographical situation of the sampling stations.

Station A	**27.464ˈ.N27º 527 E052º40.**
	27.426ˈ.N27º E052º40.516
	27.392ˈ.N27º E052º40.559
Station B	27.536ˈ.N27º 306 E052º39.
	27.542ˈ.N27º E052º39.295
	27.553ˈ.N27º E052º39.284
Station C	27.515ˈ.N27º 83 E052º38.
	27.502ˈ.N27º 814 E052º38.
	27.492ˈ.N27º 804 E052º38.

The counting results have shown that stations had different number of bacteria. The most number of bacteria (5.069) were found in station A whereas station B had the least one (4.721). There were

significant differences at 1% level between all stations. Also the most number of bacteria in the control medium without hydrocarbon was 5.062 and the least number of them in fluoranthene medium was 4.775. There was significant difference between these two groups at 1% level. Also comparison of counted bacteria in autumn and winter has shown that the most number of bacteria was 5.018 in autumn and the least number was 4.826 in winter. There was a significant different between these two seasons at 1% level. Furthermore, the numbers of gram-positive bacteria were more than gram-negative ones, but there was no significant difference between them. Gram-positive and gram-negative bacteria were 54% and 46 % of all isolated bacteria respectively. Also the findings have shown that *Alcaligenes faecalis* were most abundant bacteria with 3.89 % and *Listeria* and *Staphylococcus* bacteria were the least one with 11.1%. The *Bacillus circulans* were 35.30%.

Table 2. The Temperature cycle to have the PCR reaction.

Stage	Time	Temperature	Repeats
Initial denaturation	3.00 min	94 °C	1
Three-step cycling			35
Denaturation	1.00 min	94 °C	
Annealing	30 sec	48 °C	
Extension	1.00 min	72 °C	
Final Extension	7.00 min	72 °C	1
Storage	∞	4 °C	

The results of HPLC have shown that strongest strains for degrading fluoranthene were *Bacillus circulans* and *Alcaligenes faecalis* which degraded 73.4% and 71% of fluoranthene, respectively. Also the *Enterobacter*, *Listeria* and *Staphylococcus* strains could degrade 67.40% , 54% and 48.70% of fluoranthene, respectively .

According to the results of molecular identification based on the 16 S rRNA gene sequence similarity and also based on the phylogenetic tree the two isolated strains with the highest power to degrade fluoranthene belonged to the *Alcaligenes faecalis* IAM12369 and *Bacillus circulans* strains. The 16S rRNA gene of the two selected bacteria were registered in the NCBI Gene bank; their final reg. Nos. are as follows: *Alcaligenes faecalis*: JX486706 and *Bacillus circulans*: JX486705 .

The growth rates of isolates in presence of fluoranthene are displayed in Figures 1-5. The bacteria did not show any lag phase and they began to grow after inoculation. The optical densities of bacteria were decreased after three days of incubation averagely.

Discussion

The mangrove forests which are near human activities are susceptible to variety of pollutions such as PAHs of anthropogenic sources. The present microorganisms in the polluted sediments of the mangroves are able to degrade highly PAH compositions because of their adaptation (Yu *et al.*, 2005). Many PAHs degrading bacteria have been isolated from oil polluted sites. Lors *et al.* in 2012 conducted a laboratory test on the soils polluted by PAH and isolated 13 strains belonged to nine genera including *Pseudomonas*, *Acinetobacter*, *Enterobacter* and *Klebsiella*, *Sinorhizobium* and *Erythromicrobium*. Kafilzadeh *et al.* in 2011 examined the soils polluted by oil in Iran and isolated *Staphylococcus*, *Corynebacterium* , *pseudomonas*, *bacillus* and *micrococcus* bacteria which were able to degrade the naphthalene.

In current research 5 fluoranthene degrading bacteria, *Bacillus circulans*, *Alcaligenes faecalis*, *Enterobacter*, *Listeria* and *Staphylococcus*, were isolated to evaluate their degradation ability via degradation experiments from mangrove sediments of Persian Gulf (Iran). Also some of the bacteria separated in current study were similar to those that isolated by Eduok *et al.* (2010), Arbabi *et al.* (2009), Reda (2009), Zhuang *et al.* (2003) and Servery *et al.* (2004).

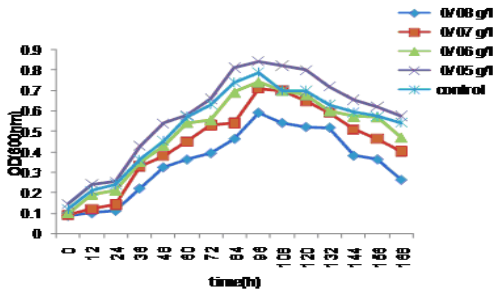

Fig. 1. Growth curve of *Bacillus circulans* on fluoranthene.

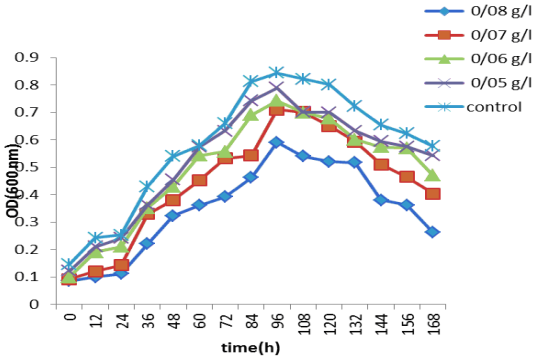

Fig. 2. Growth curve of *Alcaligenes faecalis* on Fluoranthene.

It was observed in present investigation that decomposition of fluoranthene was rapid up to 73.4% and 71% by the strongest strains, *Bacillus circulans* and *Alcaligenes faecalis*, respectively. Also 67.40 %, 54% and 48.70% of fluoranthene was degraded by *Enterobacter*, *Listeria* and *Staphylococcus* respectively. The findings prove high ability of these isolates in bioremediation of PAHs. Many researchers have examined isolated bacteria from different mangrove sediments based on their ability to degrade PAH compositions. For example, Tam *et al.* (2008) isolated PAH degrading microbial consortia from superficial sediments of two mangroves. 89.90–96.70% of phenanthrene and less than 40% of flouranthene were degraded by isolates. Tian *et al.* (2008) measured PAHs degradation rates by the bacteria isolated from mangrove sediments in China and found 100% and 44.20% and more than 20 % of phenanthrene, benzo(a)pyrene and fluoranthene were degraded respectively after ten days of incubation. Yu *et al.* (2005), isolated the mangrove autochthonous bacteria in Hong Kong SAR. after 4 weeks of incubation the bacteria were able to degrade 99 % of fluorene and phenanthrene and 30 % of pyrene. Gue (2005), examined the superficial sediments of some mangrove samples and isolated *Sphingomonas* sp., *Rhodococcus* sp. and *Paracoccus* sp. with ability to degrade 60, 30 and 100 percent of fluoranthene respectively. It seems researchers' findings are in agreement with current study. Also HPLC were used to measure fluoranthene degradation rate which was used by other researchers to measure the PAH compositions degrade rate such as Turlough (1999), Tam *et al.* (2008), Guo *et al.* (2005), Carmela *et al.* (2005) and Tian (2008).

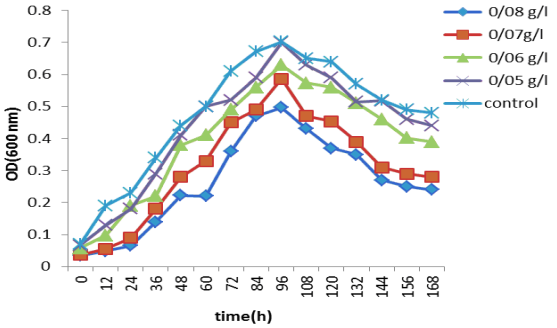

Fig. 3. Growth curve of *Enterobacter* sp. on Fluoranthene.

According to Figures 1-5 bacterial growth fallow bacterial schematic growth curve. There were seen no lag phase that explain good bacterial acclimation to hydrocarbon source and they enter to the logarithmic phase rapidly. It means that the isolates were able to degrade and use fluoranthene as the sole source of carbon and energy by applying special enzymes. Therefore, the bacterial population increases through the cell division and it was recognized by measuring the optical density. *Bacillus circulans* reached to maximum OD (> 0.8), bacterial reproduction and growth reduced regularly due to the carbon source and nutrients reduction (Kader *et al.,* 2007). The high growth of this isolates in presence of fluoranthene could be result of its compatibility and degrading ability. This adaptation can be depends on many factors, such as the induction of specific enzymes for degradation pathways of particular compound or adaptation of existing catabolic enzymes to degradation of novel compounds. Akhavan Sepahi *et al.* (2008), isolated

fifteen crude oil degrading *Bacillus* spp. Two isolated showed best growth in liquid media with 1-3% (v/v) crude oil and mineral salt medium, then they were studied for enzymatic activities on tested media. Typical generation time on mineral salt with 1% crude oil varies between 18-20h, 25-26h respectively for Bacillus S6 and S35. The results demonstrated that *Bacillus* spp. can utilize crude oil as a carbon and energy source.

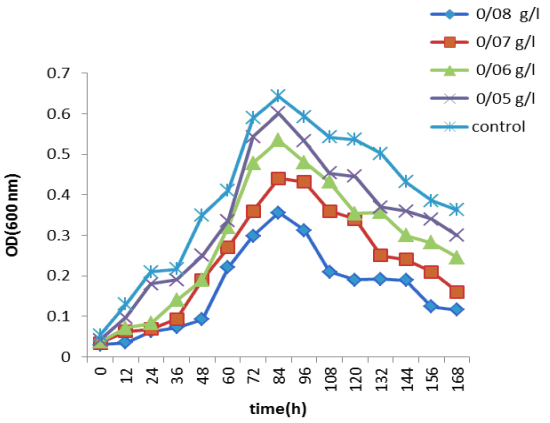

Fig. 4. Growth curve of *Enterobacter* sp on fluoranthene.

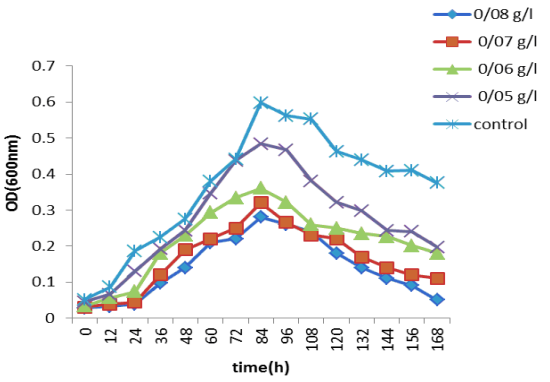

Figure 5. Growth curve of *Staphylococcus* sp. on Fluoranthene.

Conclusion

In present research, some degrading bacteria were isolated and identified as the 'Powerful' one able to degrade fluoranthene from the Persian Gulf mangrove sediments. The most dominant bacteria among the isolated bacteria included *Bacillus circulans* and *Alcaligenes faecalis* which had the most power to degrade fluoranthene. Furthermore results of current study have shown that these two indigenous bacteria were more in autumn than other season because temperature in this season is near the optimal temperature for growing of isolated bacteria from Persian Gulf mangrove sediments which are mesophilic and thermophilic bacteria.

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