Study of Acenaphthene Biodegradation by Indigenous Bacteria Isolated from Crude Oil Contaminated Soil around Abadan Refinery (Iran)

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PAHs are non-polar organic compounds which consist of fused aromatic rings and do not contain heteroatom or carry substituent. They are one of the most common contaminants which are present in soil. Among these compounds, acenaphthene is a multi-ring hydrocarbon that is extensively existed in nature. In this study acenaphthene degrading bacteria were isolated and identified. Then biodegradation of acenaphthene by these bacteria were studied in contaminated soil around Abadan refinery. Samples were enriched in mineral salt medium then bacterial colonies were isolated and purified in the same solid medium. Bacteria were identified by biochemical diagnosis tests. Bacteria were cultivated in culture medium that consisted 0.5 to 0.8 g/l acenaphthene. Their growth rate was measured by spectrophotometer. In conclusion, acenaphthene biodegradation was evaluated by HPLC. In the present investigation, *Bacillus* sp, Micrococcus luteus, Corynebacterium sp, Staphylococcus epidermidis, Pseudomonas sp were isolated and identified. Bacillus and Micrococcus had the most growth rate in different concentrations of acenaphthene. The obtained data showed that 92.543±2.302% and 89.124±3.145% from initial concentration of acenaphthene (500 ppm) were degraded by Bacillus and Micrococcus luteus within 168 hours after incubation respectively.

Key words: Acenaphthene, Degrading bacteria, Bacillus, Micrococcus, Abadan refinery.

Environmental pollution has increased by development of industry all over the world .One of the most important pollutants is hydrocarbon compounds, many of these hydrocarbons considered to be a potential health hazard. Some of hydrocarbon compounds pollutants are polycyclic aromatic hydrocarbons¹. Polycyclic aromatic hydrocarbons (PAHs), also known as poly-aromatic hydrocarbons are potent atmospheric pollutants that consist of fused aromatic rings and do not contain heteroatom or carry substituent². PAHs are found in oil, coal and tar deposits, and are produced as byproducts of fuel burning (whether fossil fuel or biomass). As a pollutant, they are of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic. Studies have shown that high levels of PAHs are found, for example, in meat cooked at high temperatures such as grilling or barbecuing, and in smoked fish³.

Polycyclic aromatic hydrocarbons are lipophilic, meaning they mix more easily with oil

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than water. The larger compounds are less watersoluble and less volatile (i.e. less prone to evaporate). Because of these properties, PAHs in the environment are found primarily in soil, sediment and oily substances, as opposed to in water or air. However, they are also a component of concern in particulate matter suspended in air natural crude oil and coal deposits contain significant amounts of PAHs, arising from chemical conversion of natural product molecules, such as steroids to aromatic hydrocarbons4. PAHs toxicity is very structurally dependent, with isomers (PAHs with the same formula and number of rings) varying from being nontoxic to being extremely toxic. Thus, highly carcinogenic PAHs may be small or large. One PAH compound, benzo[*a*]pyrene, is notable for being the first chemical carcinogen to be discovered (and is one of many carcinogens found in cigarette smoke). Seven PAH compounds have been classified as probable human carcinogens: benz[a]anthracene, indeno(1,2,3-cd)pyrene, benzo[b]fluoranthene, chrysene, benzo[k] fluoranthene, , dibenz(a,h)anthracene, and benzo[a]pyrene⁵. The simplest PAHs, as defined by the International Union of Pure and Applied Chemistry (IUPAC) are phenanthrene and anthracene, which both contain three fused aromatic rings. PAHs are classified according to the number of rings, the type of ring and the atom composition. The low molecular weight (LMW) PAH contains two or three aromatic rings and the high molecular weight (HMW) contain more than three. It is known today that LMW PAHs are actually toxic and HMW PAHs are considered genotoxic⁶. This group of compounds is also used as raw material for producing pharmaceuticals, polymers, explosive, chemical fertilizer, paints, plastics, cleaners, anti fungi and insecticides and some are even used for medical purposes.

During usage or transporting them, contamination by the surrounding environment is unavoidable^{7,8}. These environmental pollutants are found in urban areas and industrial centers in large quantity⁹. They have relatively low solubility in water, but are highly lipophilic⁷. One of the primary factors believed to limit the extent of PAH degradation is the low water solubility of these compounds¹⁰. Acenaphthene is three c aromatic rings hydrocarbon that is found in high concentrations in polycyclic aromatic

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hydrocarbons contaminated sediments, surface soils and waste sites. This hydrophobic contaminant is widely distributed in the environment⁷. The potential use of microorganisms to clean up contaminated soil, sediments and water can provide efficient, inexpensive and environmentally safe clean up of waste material. Addition of microorganisms with known metabolic capabilities to contaminate materials has been used as a remediation technology to enhance the degradation rates in recently contaminated soil and sediments⁶.

Some research have been done on bio reform phenomenon include: bio analysis and vital access PAHs (naphthalene and pyrene) by microorganism in sediments polluted by PAHs of river in Iceland¹¹, separation of multi-chain aromatic hydrocarbons degrading bacteria in soil polluted by crude oil in Iran¹⁰, isolation of multi-chain aromatic hydrocarbons degrading bacteria in soil polluted by heavy oil by⁶ and separation and identification of hydrocarbon degrading bacteria¹². In this study acenaphthene degrading bacteria were isolated and identified then biodegradation of acenaphthene by these bacteria were studied in contaminated soil around Abadan refinery.

MATERIALS AND METHODS

Soil sampling

To isolate acenaphthene degrading bacteria, oil polluted soil samples from Abadan refinery, which is one of the most important oil areas of Iran and a centre for refinement and desalination of crude oil, were collected from a depth of 0 to 10 cm from 3 different stations in the oil polluted area. Then they were placed in sterile plastic containers and kept in ice to be transferred to the lab immediately^{11,13}. Bacterial analysis was carried out 24 to 48 hours after sample collection⁵. **Isolation method of acenaphthene degrading bacteria**

To separate the bacteria from the polluted soil samples, 5gr of soil from each station was added to 10ml of sterile water and was centrifuged for 10 minutes at 2000 rpm. Then, 500 μ l supernatant was added to 100ml mineral salt medium (MSM) culture. The culture is composed of (g/l): MgSO₄(0.1), NH₄NO₃ (0.1), KH₂PO₄ (2), Na₂HPO₄ (2.4), FeSO₄.7H₂O (0.1), NaCl (0.1), ZnSO₄.7H₂O(2.4),

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 $CaCl_{2}2H_{2}O(0.013), CuSO_{4}.7H_{2})(1.4), MnSO_{4}.7H_{2}O(1.2), CuSO_{4}.5H_{2}O(0.25), yeast extract (0.1), acenaphthene (500 ppm) in 1000 ml distilled water.$

After a week turbidity was observed in the culture medium. 10% of acenaphthen was added to a new inorganic culture medium for more degradation by microorganism. Presence of acenaphthene in the culture medium caused to increase bacterial growth. Under this new condition, renewal of culture medium continued for 3 weeks¹⁴. Then 1ml of liquid culture that obtained from the last enrichment by pour plate method was cultured on solid environment surface MSM. For purifying bacteria, colonies that were different in appearance, were cultured continuously on inorganic base solid culture medium containing acenaphthene in a linear manner¹³.

Identification of acenaphthene degrading bacteria

The first distinctive character of bacteria is its appearance. Therefore the appearance of bacteria such as size, form, color, edge, surface and transparency were studied. For more accurate identification, the usual microbiological and biochemical tests were carried out¹⁵.

Investigation of bacterial ability to degrade acenaphthene

In this method bacteria are chosen based on the turbidity that was conformed because of their growth in the culture. It means that more turbidity was the result of more bacterial growth. By comparing the tubes containing bacteria with McFarland's 0.5 standard, about 10⁸ bacteria were inseminated to 250 ml erlen containing 100 ml inorganic culture medium. After 3 days, turbidity of culture medium at 600nm was measured by a spectrophotometer. The turbidity was also observed by naked eye and the results were noted¹⁴.

Evaluation of isolated bacteria's growth in various acenaphthene concentrations by spectrophotometer

In this method the best acenaphthene degrading strains were separated from acenaphthene degrading bacteria by studying their absorption by spectrophotometer. In separated arlen was added 150 ml culture broth containing acenaphthene (with different concentration of acenaphthene), then equal to half McFarland standard concentration was prepared from growth of bacteria and 1.5 ml of this concentration was added to the culture medium for conducting kinetics of growth. Four erlens were used for each bacterium and in each erlen 0.5 to 0.8 g/l acenaphthene was added. An erlen was considered for each bacterium as a control sample. In this erlen there was only culture medium without acenaphthene with specific strain of bacterium. Cultures were incubated for 24 hours at 30°C and then their absorption was read at 600 nm. This was continued for a week and every 12 hours ¹³.

Investigation of acenaphthene biodegradation by bacterial species

100 ml inorganic culture medium containing 500 ppm acenaphthene in 250 ml erlen was prepared. Then about 10⁸ bacteria were inseminated into erlen. For each bacterium this test was done in triplet. Also, three other erlens in equal conditions to original samples but without bacterial insemination were considered as a control. The control sample removed the effect of biological factors such as absorption, evaporation and photooxidation in reduction of acenaphthene concentration. Erlens were heated at 30±2°C and 150 rpm. Periodically, 5 ml from each culture medium with 2 ml hexane were added to indoor test tubes and mixed by high velocity vortex, and then they were centrifuged for 10 minutes with 3000 rpm to transfer acenaphthene from aqueous phase to organic phase (hexane). After that supernatant was transferred to the sterile beaker and evaporated the solvent. Then 5 ml acetonitrile was added to the organic phase containing acenaphthene to be ready for analyzing by HPLC system¹³.

High efficiency liquid chromatographic analysis

Quantity analysis of acenaphthene in culture medium was done by high-performance liquid chromatography (HPLC). The aqueous component of the mobile phase included mixture of water and acetonitrile in the volume ratio of 30 to 70 and rate of 1ml/min. Quantity analysis of acenaphthene was done in 254 nm. For using HPLC first of all solutions with different concentrations of acenaphthene in acetonitrile were prepared and injected into the device. It had two results at first recovery time of acenaphthene was achieved and the second, area under the curve of acenaphthene was defined thus the standard curve was Completion time of each determined. chromatogram was 5 minutes and recovery time of acenaphthene was 1.8 minutes. Obtained samples from extraction stage were injected by syringe at 60 μ l into the device to determine concentration. The software calculated concentration of the unknown sample by comparing the standard curve and injected sample curve. After measuring the remaining acenaphthene in each erlen it was deducted from the initial acenaphthene quantity so acenaphthene biological deletion was obtained¹⁶.

RESULTS

In this research after sampling and enrichment process, 5 species of acenaphthene degrading bacteria from soil around Abadan refinery were separated and purified based on morphological differences. Most isolated bacteria were gram-positive from different genuses such as *Bacillus* sp, *Micrococcus luteus*, *Corynebactrium* and *Staphylococcus epidermis*. In addition a gram-negative bacterium from *Pseudomonas* genus was also isolated in this research. The highest percentage of separated bacteria was *Bacillus* sp (36%) and the lowest percentage was *Pseudomonas* (4%) (Fig. 1).

Turbidity measurement of culture medium 3 days after insemination of these bacteria by spectrophotometer in 600 nm showed that the highest absorption was related to culture medium containing *Bacillus* sp and *Micrococcus luteus* 0.761 and 0.646 respectively. These two genuses were selected for continuing of biodegrading tests because of their high ability in decomposing acenaphthene (Table 1).

Studying growth of Bacillus sp in

inorganic culture medium containing various acenaphthene concentrations (800 ppm, 700 ppm, 600 ppm, 500 ppm) showed this bacterium grew slowly 12 hours after insemination. Due to this tread absorption in 600 nm increased gradually. Growth curve of Micrococcus luteus in inorganic culture medium containing these acenaphthene concentrations showed the bacterium grew slowly 24 hours after insemination but it had little growth until 36 hours. Comparison of growth curves of both bacteria in various acenaphthene concentrations showed that Bacillus sp started its growth quicker than Micrococcus luteus and its absorption was more than *it* in all stages of the test. Their logarithmic stage continued until 96 hours after insemination so their growth curve had ascending behavior. After that their growth rate reduced. Both bacteria in comparison with the control sample had the highest growth in 500 ppm and the lowest in 800 ppm of acenaphthene (Fig. 2,3).

Results of quantity analysis of acenaphthene in culture medium containing above bacteria by HPLC demonstrated perceptible reduction of acenaphthene.

Percentage of reduction in initial acenaphthene concentration in culture medium of *Bacillus* sp and *Micrococcus luteus*, 168 hours after inseminating was 92.543 ± 2.302 and 89.124 ± 3.145 respectively. Results showed that the most decomposition rate of acenaphthene was 37.438 ± 12.095 percentage that was achieved by *Bacillus* sp, 24 hours after inseminating in culture medium. *Micrococcus luteus* only decomposed 22.895 percentage of acenaphthene, 24 hours after insemination which in comparison to *Bacillus* genus showed weaker performance (Fig. 4,5).

Name of bacteria	Time (hours)					Color of the medium	The results of
	24	31	48	55	72	after 72 hours	optical absorptio
Control						Transparent yellow	0.00
Micrococcus luteus		+	++	+++	+++	Yellowish brown	0.646
Pseudomonas Sp		+	+	++	+++	Brown	0.332
Corynebacterium Sp	+	+	+	++		Opaque	0.311
Bacillus sp	++	++	+++			Opaque	0.761
Staphylococcus epidermidis		+	+	+	+	Opaque	0.276

Table 1. Time and approximate amount of the growth of bacteria degrading acenaphthene in mineral medium base

+: Weak growth ++: Medium growth +++: High growth

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Fig. 1. Frequency of bacteria isolated from different stations



Fig. 2. The growth curve of *Bacillus* sp in different concentrations of acenaphthene as carbon source



Fig. 3. The growth curve of *Micrococcus luteus* in different concentrations of acenaphthene as carbon source



Fig. 4. Acenapthene reduction in medium containing *Bacillus* sp and *Micrococcus luteus* and incubated without bacteria



Fig. 5. Percentage of acenaphthelen reduction in medium containing the *Bacillus* sp and *Micrococcus luteus* and incubated without bacteria

DISCUSSION

The techniques for cleaning contaminated soil included burning furnace and chemical treatment. In recent decades, biodigradation has been a suitable replacement for cleaning soil contamination¹⁷.

Biodigradation is an effective and cheap technique for antisepticising or cleaning soil contaminated by PAHs. This technique is based on specific species of microorganisms which metabolize PAHs completely or partially¹⁸. Generally, various microorganisms are able to degrade aromatic hydrocarbons but bacteria that have various degrading enzymes are more important^{19,20}. Zhuang *et al*.,(2003) have shown that gram-positive bacteria have an important role in degrading of oil hydrocarbons so that biodegradation of aromatic compounds by Micrococcus and Bacillus have been reported by various scientists. They isolated three grampositive bacteria from Micrococcus, Bacillus and Staphylococcus genuses which degraded phenanthrene in contaminated sediments of tropical tidal areas by oil compounds. Results showed that these three isolated genuses formed more than 69% of phenanthrene degrading bacteria population in that area²¹. Bacillus subtilis that degraded pyrene and benzoalphapyrene was separated from contaminated soil²². 14 strains of gram- positive bacteria were isolated (3 strains Micrococcus, 11 strains Bacillus) from contaminated soil²³. Oil degrading indigenous bacteria were isolated from polluted ecosystem in Nigeria. These bacteria were coded from DDV, to DDV, and were identified as, Micrococcus roseus, Pseudomonas aeruginosa and also a yeast such as Saccharomyces cerevisiae24. In current research Bacillus and Micrococcus genuses as acenaphthene degrading bacteria isolated from Abadan refinery soil which correlated with findings of other researches. In current research Spectrophotometer was used at 600 nm to evaluate the growth of isolated bacteria in various acenaphthene concentrations. This method was used also by others²⁵⁻²⁹. Results were obtained by this method correlated with findings of other researchers. In current research HPLC was used to study acenaphthene biodegradation by different bacteria and measured remaining acenaphthene in culture medium. This method was used by whom isolated Burkholderia cepacia, Bacillus firmus, Pseudomonas alcaligenes, Bacillus licheniformis, Bacillus subtilis and Micrococcus lylae from oil contaminated soil. Degradation rate that was measured by HPLC from all PAHs' compounds in culture medium by bacteria mentioned above were 88.32%, 37.14%, 57.989%, 66.068% and 81.551% ppm respectively⁶. It was showed that *Bacillus* subtilis 4 days after starting incubation, degraded $20 \,\mu g/ml$ of pyrene and benzoalphapyrene by 40%and 50% respectively²². Present research showed better results than these two recent research. 50 species of phenanthrene degrading bacteria were isolated and purified which all belonged to Pseudomonas genus, from contaminated soil at

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Mersin Oil Refinery in Turkey. Two species of ARP28 and ARP26 which had the best growth in MM9 culture medium containing phenanthrene were selected for the next test. Biodegradation rate by HPLC showed that phenanthrene reduction rate was 93% and 98% after insemination of ARP28 and ARP26 respectively¹³. In current research, Percentage of reduction in initial acenaphthene concentration in culture medium of Bacillus sp and Micrococcus luteus, 168 hours after inseminating was 92.543±2.302 and 89.124±3.145 respectively that was studied by HPLC. Results were almost similar to others^{30,31}. Pseudomonas aeruginosa and Bacillus subtilis were isolated from oil contaminated soil and studied their ability for comparison of biodegradation of crude oil hydrocarbons in soil and rotary flasks. These bacteria could use oil hydrocarbons as the only source of energy³². Also, other researchers evaluated oil compounds which were degraded by bacteria that confirmed results of present findings³³⁻³⁹.

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