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Evaluation of Bioremediation of naphthalene using native bacteria isolated from oil contaminated soils in Iran

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are of the group of aromatic compounds having interlocking rings. They enter to environment due to incomplete combustion of fossil fuels. Having the toxic and carcinogen characteristic, PAHs are of the factors which threaten the environment. some of the bacteria with particular mechanism are able to degrade these compounds. The goal of this study is to evaluate the biological modification of naphthalene using native bacteria isolated from oil contaminated soils in Iran and to assess their growth kinetic. Several naphthalene-degrading bacteria were isolated from oil-contaminated soil in a crude oil extraction and desalination center in Omidieh, Ahvaz, Iran. Bacterial strains which had better growth on the enriched medium were isolated and identified by biochemical and morphological tests. The findings of this study show that most of the isolated bacteria were found to belong to Staphylococcus sp, Corynebacterium sp, Pseudomonas sp, Bacillus sp, and Micrococcus sp. These species were significantly able to degrade naphthalene. The efficiency of naphthalene as the only source of carbon and energy was evaluated by High performance liquid chromatography (HPLC) analysis. HPLC analysis showed that the Bacillus sp and Pseudomonas sp, 86% and 80%,Corynebacterium sp and Staphylococcus sp, 77% and 69% and Micrococcus sp, 58% degrade the naphthalene after one week incubation respectively.

Keywords: Naphthalene, Bioremediation, *Bacillus*, *Pseudomonas*, HPLC.

INTRODUCTION

Organic compounds are generally divided into three groups: Aliphatic, Salicylic, and Aromatics. The main structural unit in aromatic compounds is benzene (C_6H_6). Polycyclic aromatic hydrocarbons (PAHs) are the most important environmental pollutants. PAHs are a large group of organic compounds that have two or more aromatic cycles fused with each other in linear, angular, and branching. Polycyclic aromatic hydrocarbons are degraded when exposed to the UV rays of the sun. In addition, PAHs can react with ozone in the atmosphere, which consequently produces some compounds such as nitrogen oxides, sulfur dioxide, nitro- and dinitro-PAHs, and

sulfonic acids [1] . Moreover, due to their hydrophobic characteristics, polycyclic aromatic hydrocarbons are less soluble in water; therefore these compounds settle in the residues of rivers, lakes, and oceans. However, they are soluble in non-polar organic solvents. Thus, the existence of these pollutants in aquatic environments is very toxic and dangerous for humans and other creatures, They are also harmful for useful microorganisms of plants in contaminated soils [2] . since, the oil compounds are very resistant to evaporation, due to having aromatics derivatives in their structures, they can remain in the environment for a long time; however, the microbial population in contaminated sites results in an increase in the degradation rate of these stable compounds [3] . Recently, many physical-chemical and biological methods have been used to clean up the contaminated sites. But these methods are not economic, or they lead to the formation of other toxic compounds in the environment. Therefore, a bioremediation method is considered as an economical and safe approach for the environment [4] . Bioremediation is the use of microorganisms to remove environmental contamination. This method is used for harmful toxic substances and also for oil contamination cleanup. Bacteria convert the pollutant organic compounds into less harmful compounds by aerobic and anaerobic respiratory reactions, fermentation, cometabolism, and dehalogenation; using them as the only source of carbon and energy [5]. there are several studies on Bioremediation of hydrocarbon conducted by Ilyina et al (2003) , by Survery et al (2004) , by Mittal et al (2009) [5, 2, 6] .

Studies have also detected that PAHs are the most important environmental carcinogen factors, impairing the structure of DNA and consequently leading to mutation. The studies show that short term exposure to naphthalene causes eye and skin inflammation [5]. Moreover, studies showed that native microorganisms in oil-compounds contaminated areas are more effective than other organisms for biodegradation of oil pollutants. In addition, biodegradation rates in contaminated areas depend on factors including microbial populations, pollutant compounds type, contamination values and type, and chemical and geological conditions in the contaminated area [4]. The goal of this study is to evaluate the biodegradation rate of naphthalene by native isolated bacteria, and also to assess the growth kinetic of these bacteria.

MATERIALS AND METHODS

Sampling

This experimental and laboratory study was conducted on one of the most important Iranian oil areas, the Maroon II oil field, which is a center for extraction and desalination of crude oil, located in Khuzestan province. The soil samples were conducted from 0 to 10 cm depth of three different sites which were contaminated with crude oil . Samples were collected in sterilized plastic dishes put into flasks containing ice, and carry to the laboratory in less than 24 hours. The experiment was initiated on the soil in the laboratory [3].

Bacterial Counts

The bacteria were counted by the Total Viable Plate Count method. In this method the dilution from 10^{-1} to 10^{-10} was prepared from soil samples by physiological serum, then cultured in nutrient agar medium (produced by Merck Co., Germany), containing 0.4 g/L naphthalene and nutrient agar medium without naphthalene by surface plate method. The cultured plates, then incubated at 30 °C for 48 h. After incubation, the number of colonies was counted in cultures with and without naphthalene [8].

Isolation and Identification of naphthalene-degrading bacteria

To isolate the bacteria from the soil samples, 5 g of samples was mixed with 90 ml of naphthalene broth culture (0.4 g/L), and put into a shaker incubator at 30 °C for one week. After one week, 1 ml of this culture was inoculated to a 90 ml new culture, and put into a shaker incubator with aeration at 30 °C for one week. The passage process was done until the environment became completely turbid. This turbidity probably results from bacterial growth, not because of the turbidity of residues mixed with medium. The samples were further cultured on the naphthalene agar medium. Bacteria were purified and colonies were prepared (1). Then, to identify the naphthalene-degrading bacteria, the colonies were morphologically evaluated. Tests which were conducted on the colonies including gram staining, morphological characteristics study, oxidase and catalase reaction, along with other diagnostic tests (IMVIC) [3].

Potent strains selection method

In this portion of the study, after isolation of bacterial strains, the basal mineral medium with desired substrate and concentration were used to screen the best and the most potent strains. Those bacteria that had begun to grow in the minimum of time and also had the most turbidity were chosen as the sufficient bacterial strains [9].

Growth assessment of isolated bacteria in different concentrations of naphthalene

In order to determine the bacteria growth curve in different concentrations of naphthalene, 20 mL of naphthalene broth medium (with different concentrations of naphthalene) were poured into separate Erlenmeyer flasks. Then, a bacterial suspension was prepared based on the 0.5 McFarland standard and 5mL was added to the medium. Four Erlenmeyers were used for each bacterial suspension. In each Erlenmeyer flask 0.4 to 0.7 g/L concentrations of naphthalene were added. In addition, one Erlenmeyer was used as control for each bacterial suspension. In the control medium, there was only basal medium without naphthalene and a certain strain. Then, all of the Erlenmeyers were incubated at 30 °C. Finally, absorption measurement in 600 nm was daily accomplished by spectrophotometer. This procedure carried on for 7 weeks after incubation [9].

HPLC analysis

High performance liquid chromatography (HPLC) was used to evaluate the naphthalene degradation by degrader bacteria. Naphthalene degradation experiments were conducted in the Erlenmeyer flasks containing 100 ml basal mineral medium and naphthalene in a final concentration of 0.4 g/L soluble in acetone was added to the medium. Bacterial strains in 5 ml of the nutrient broth were incubated in 30 °C for 24 h, then 1 ml of bacterial suspension was added to the medium and placed into the shaker incubator in 30 °C for one week. After incubation, to obtain cell mass, the mediums were centrifuged at 6000 rpm for 15 min and the bacterial cell mass was collected. This process was done in several steps and then they were successively washed 3 times with sterile liquid basal mineral medium. In the next step, 2mL of hexane was added to 5mL of mineral medium and was shaken for several times in the glass tubes with caps. Finally, 1ml of upper phase (hexane) was shifted to the sterile tubes and was used for (HPLC) analyses [10].

The (HPLC) system used in this study was a Waters 600 E (USA) model equipped with a degasifier in-line system with helium gas, a six-part injection valve Rheodyne (7125i, USA) model with 10 ml loop, a multi-wavelength fluorescence detector (2475, Waters, USA), and an analytical column Novapak C_{18} from Waters company (60 Å , 150 x 3.9 mm I.D,4 µm) with a guard column Novapak C_{18} from Waters Co. (60 Å, 20 x 3.9 mm, I.D). In order to control the

system and collect data, the software and interface used from Millennium Workstation (Waters, USA) and Waters SAT/IN Module (USA) were used, respectively.

Statistical analysis

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

RESULTS AND DISCUSSION

The results of bacterial counting showed that the logarithmic average of the number of bacteria in the medium containing naphthalene was 2.631; in compare to the control medium which was 6.588. There was a significant difference at the 5% level between the logarithmic values of the numbers of bacteria in the medium without naphthalene and the medium with naphthalene. Comparing the stations with regard to the logarithmic average of the number of naphthalene-degrading bacteria, station B was found to have the most bacteria degrader, 7.068; and station A was found to have the least bacteria degrader, 5.136. The logarithmic average of the numbers of naphthalene-degrading bacteria in different stations was significantly different at a 5% level. In addition, the abundance percentage of determined gram positive bacteria was more than gram negative bacteria and had significant differences at a 5% level. 67% of isolated degrader bacteria were gram positive and 33% were gram negative. Moreover, the results showed that the most abundance percentage was belonging to *Bacillus* (30%) and the least abundance percentage belonged to *Micrococcus* (10%). The growth of isolated strains was monitored during one week to evaluate the ability of bacteria for naphthalene degradation. The growth curve of the strains on the different concentrations of naphthalene showed that all of the isolated strains have a partially identical growth pattern in 0.4 g/L concentration of naphthalene (Diagrams 1, 2, 3, 4, 5). In addition, the results of (HPLC) showed that the most potent naphthalene degrader strains were *Bacillus* and *Pseudomonas* that degraded 86% and 80% of naphthalene, respectively. Most recent research has been focused on the degradation activity of oil compounds by microorganisms. Naphthalene has higher activity over other polycyclic aromatic hydrocarbons, and the fact that naphthalene degrader enzymes are encoded by plasmid has facilitated the research about naphthalene's biological degradation. In the past, the physicochemical methods were used to degrade aromatic compounds and their derivatives, but today the priority is bioremediation. Thus, oil compounds-contaminated soils can be cleaned up by isolation, purification, and reproduction of the species with higher potency to remove these compounds [5]. Survey et al. (2004) studied the soil near several gas stations in Karachi, Pakistan. They successfully isolated and identified 60 bacterial strains including *Staphylococcus* (11.5٪) *Corynebacterium*(5٪), *Bacillus*(10٪), *Proteus*(21.6٪), *Pseudomonas*(8.3٪), *Escherichia*(33.3٪), *Klebsiella* (10 ٪).These bacteria were capable of degradation of the hydrocarbons[2]. In the same study by Survey et al isolated bacteria were included of *Bacillus*، *Pseudomonas* ،*Corynebacterium*، *Staphylococc*. In Italy, Alquati et al. (2005) studied naphthalene-degrading bacteria in oil-contaminated soils and successfully isolated 60 species of bacteria, which were belong to *Rhodococcus*, *Arthrobacter* and *Nocardia* [11]. In our study ,none of these strains were isolated in Maroon II oil field. Coral et al , successfully isolated 50 bacterial strains that all belonging to *Pseudomonas* and were capable of degrading naphthalene [10]. Since native bacteria of contaminated areas are constantly in contact with aromatic compounds, these bacteria should somehow be able to degrade the materials surrounding them. Walczak et al and Bestett et al have report the degradability of naphthalene by native bacteria [12, 13]. In this study, the most dominant naphthalene-degrading bacteria isolated from the Maroon II oil field belonged to *Pseudomonas* and *Bacillus*. Therefore, the results of this study showed that these bacteria are

native in this area. In addition, they have strong mechanisms to remove the aromatic compounds such as naphthalene. These results are in paralel with other studies. Othman et al , isolated the bacteria capable of degrading naphthalene from soils in Malaysia. They have concluded that the most dominant species in naphthalene degradation is *Micrococcus*. These bacteria were capable of degrading 85% of naphthalene after 6 days' incubation [14].

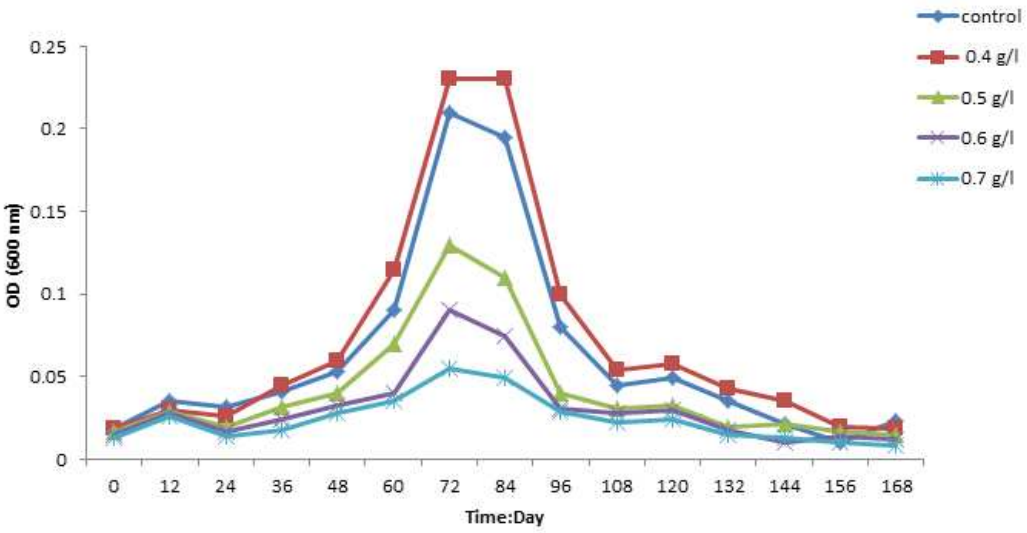

Figure-1:Growth curve of Bacillus sp on naphthalene

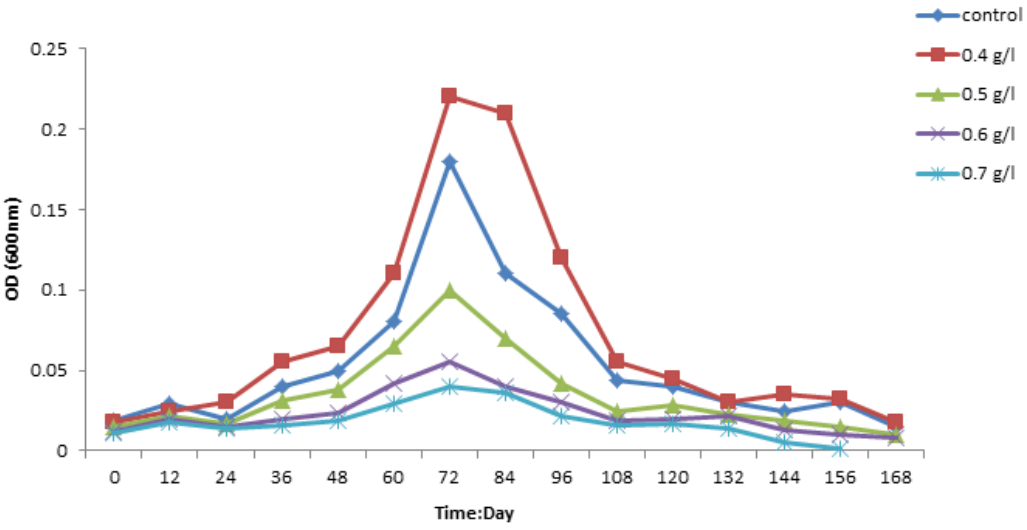

Figure-2: Gtowth curve of Pseudomonas sp on naphthalene

In addition to the above-mentioned strains, Jeon et al, has introduced *Polaromonas naphthalenivorans sp* as the dominant species of naphthalene degrader. This bacteria was also capable to degrade naphthalene as the only source of carbon and energy [15]. Different methods have used by researchers to measure naphthalene degradation by degrader bacteria. For example, Mehrasbi et al used gas chromatography to measure degraded naphthalene values [16]. In addition, other researchers such as Aytkeldiyeva et al and Mittal et al. used this method to measure naphthalene degradation values [17, 7].

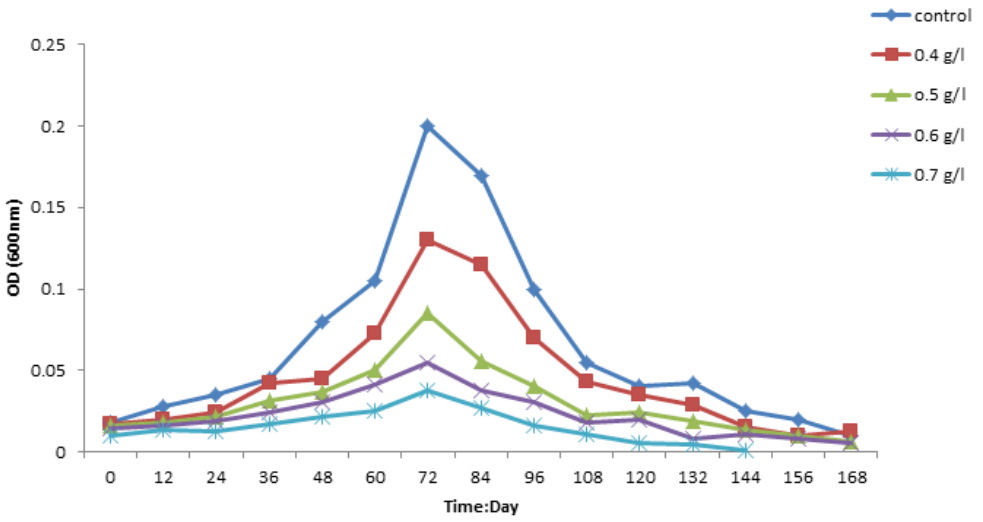

Figure-3:Growt curve of Corynebacterium sp on naphthalene

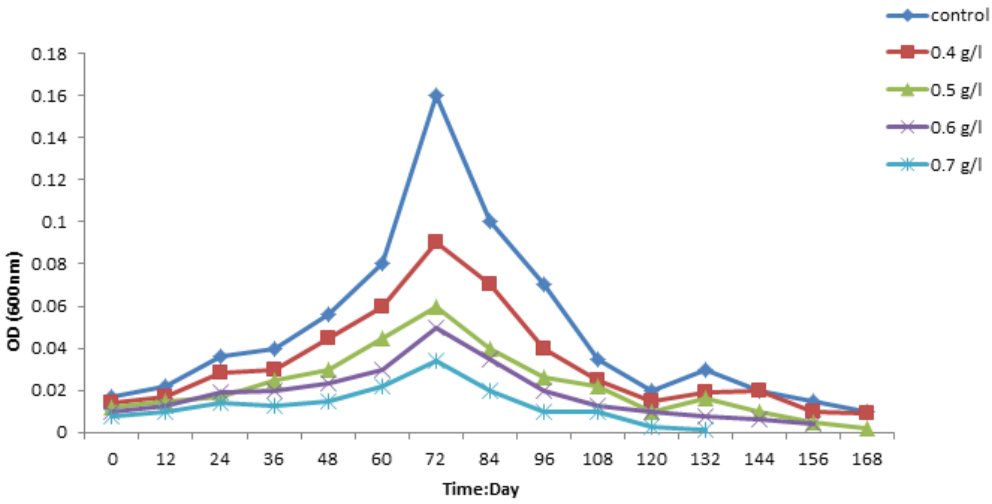

Figure-4: Growth curve of Staphylococcus sp on naphthalene

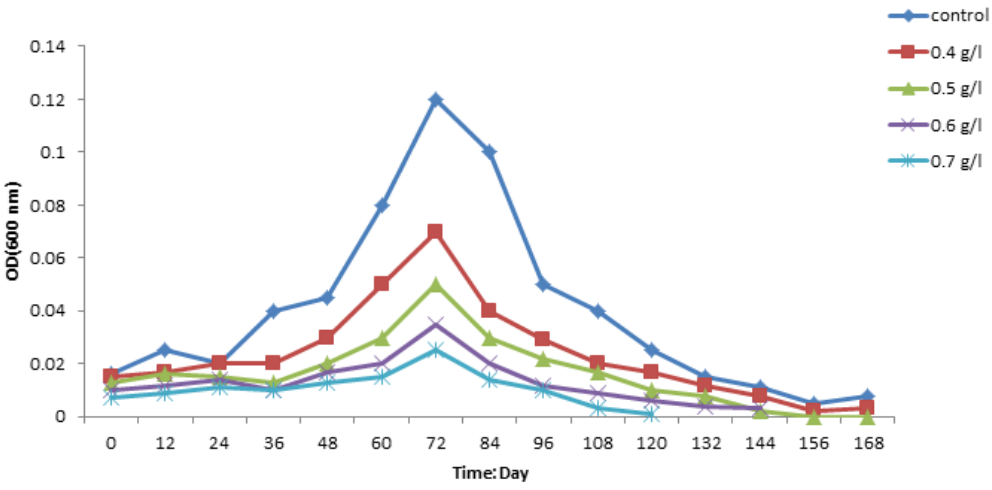

Figure-5: Growth curve of Micrococcus sp on naphthalene

In this study, HPLC was used to measure naphthalene remaining in medium. This method has been used by Tian et al, Seoud et al, Coral et al and Bisht et al [18, 19, 10, 20]. In this study, the results of remained naphthalene measurement by HPLC method were correlated with findings of other researchers.

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