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Bioremediation of Gasoil by Indigenous Bacterial Strains Isolated from Oil Contaminated Soils

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Abstract

Iran an oil-rich country and encounters oil pollution of soil and water. Bioremediation of these pollutants is an appropriate solution compared to the physical and chemical remediation methods. Therefore, the aim of this study was to determine the rate of gasoil bioremediation by two indigenous bacterial isolates (from oil contaminated soils in south of Tehran oil refinery) in two different media including soil and soil-sawdust mixture. The isolation of two superior indigenous bacteria was conducted thorough three steps: i) Isolation and purification of indigenous oil degrading bacteria in a soil extract agar as a selective media, ii) The study of efficiency of the isolates in a liquid mineral based media with 7% (V/V) of gas oil. iii) The comparison of the respiration rates of the isolates in a media containing 3% (W/W) gas oil. The results indicated that in optimal environmental conditions (temperature, 27±2 °C, humidity 60% Water Holding Capacity and daily manual aeration), bacterial isolates were able to degrade, about 78.87% and 93.53% of gasoil during the period of 45 days in soil and soil-sawdust mixture media, respectively. These results imply the role of sawdust in improving of aeration, water holding capacity and consequently, increasing bioavailability of gasoil to bacteria.
Keywords: Biodegradation, Hydrocarbon Compounds, Oil-degrading Bacteria, Oil contaminated soil.

Introduction

Soil contamination with petroleum hydrocarbons is a worldwide problem. Hydrocarbon contamination is one of the most general types of contaminations in dry as well as aqueous ecosystems (Schaefer et al. 2005). Among the sources of PHC[1], petroleum-refining results in the generation of large quantities of oil sludge, consisting hydrophobic and refractory substances (Caouto et al. 2010). Concentration of these chemicals in environment causes a serious threat to human health, organisms, and bio-ecosystems (Mirsal Ibrahim 2004).
Therefore, one of the most important challenges, which Environmental Protection Agency tries to solve, is to remediate contaminated sites. For soil cleanup, physico-chemical treatments can be applied. However, they are extremely expensive (Ouyang et al. 2005) and, in addition, they damage soil structure and/or utilize organic solvents, which are harmful for the environment. In contrast, biological remediation treatments are cost-effective approaches that rehabilitate soil structure (Caouto et al. 2010). One of the best approaches to remedy contaminated soil is to use microorganisms which are able to degrade those toxic compounds (Bento et al. 2005).

Bioremediation is a method that uses the microorganism's potential to increase the rate and amount of contaminants degradation. Ideally, the contaminant is the sole source of energy, ensuring that only those microorganisms that could consume contaminant, will grow in the media (Devinny and Chang 2000). Degradation of hydrocarbon contaminants can be enhanced by the inoculation of contaminated soils with microbial consortia or single isolates known to be able to degrade hydrocarbons (Richard and Vogel 1999; Bento et al. 2005). Many laboratory and field tests have demonstrated that the biological methods for soil remediation could be a cost-effective and environment friendly technology to treat organic contaminants, particularly petroleum hydrocarbon contaminated soils (Mathew et al. 2006). Biodegradation could be carried out either by the autochthonous or allouchthonous organisms or both,through seeding (Ajayi et al. 2008). New methods to amend contaminated soils by certain bacteria have been established such as inoculation of soils with indigenous bacteria of the same regions which is isolated and purified during subsequentional steps. Adapted microbial communities usually have high proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants. Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of bacterial growth conditions. For example; nutrients amendment, manual

[1] Poly Hydro Carbon

aeration etc. Degradation of petroleum-based pollutants can be enhanced by the provision of nutrients to stimulate the activity of indigenous microorganisms (Seklemova et al. 2001; Bento et al. 2005; Ueno et al. 2006; Jayamani and Cupples 2013). The introduction of hydrocarbon to soil environment creates an imbalance in the nutrient ratio in the soil because of the high level of carbon supplied (Atagana et al., 2003). The addition of N and P to soil contaminated with hydrocarbons is known to stimulate the biodegradation of such compounds and increase the abundance of microbial species (Bossert and Bartha 1984; Baker and Herson 1994; Alexander 1999). Hence, the objective of this study was to investigate the ability of two indigenous bacteria isolated from oil-rich areas in order to remediate gasoil in two various media (soil and soil-sawdust).

Materials & Methods

Isolation of indigenous bacteria for remediation of hydrocarbon contaminated soils; evaluation of their gasoil degradation efficiency; and finally selection of superior strains.

Having absorved the oil contaminated soils in south of Tehran refinery, six soil samples were selected from the sites which were likely to be contaminated. Hydrocarbon contamination was clearly visible on the soil surface in these sites. The samples were kept in labeled closed jars in a refrigerator and transferred to the laboratory. Then gasoil degrading superior bacteria were isolated and selected thorough three steps of growth tests.

1- Isolation in solid selective culture media of Contaminated Soil Extract/Agar which had petroleum hydrocarbons as the sole source of carbon (Bardi et al. 2000; Ilyina et al. 2003).

2- Study of bacterial OD[2] variations using spectrophotometer in liquid mineral media with gasoil as the source of carbon (Pontecorvo 1949; Bardi et al. 2000; Ilyina et al. 2003; Marquez-Rocha et al. 2000; Shukor et al. 2009; Idise et al. 2010; Karamalidis et al. 2010)

3- Evaluation of respiration amount of the superior strains in a media with gasoil carbon source (Alef and Nannipieri 1995; Sabaté et al. 2004). At the end of these 3 steps, 2 bacterial species were isolated and purified. For typing and grouping isolated bacteria, macroscopic and microscopic experiments such as Oxidize test, Catalaze test, Mobility and Grams staining test, were fulfilled (Table 1). All these experiments were done based on microbiological standard methods (Gerhardt 1981).

Preparation of soil

A soil with sandy clay loam texture was sampled from typical areas around Tehran. Soils were air-dried, and sieved by 2 mm sieve. Then some Physicochemical properties were determined as described: soil pH, soil moisture (field capacity percent), soil salinity (electrical conductivity), soil organic matter and soil organic carbon, total nitrogen, plant available phosphorous and also soil particle size distribution. All of the methods were standard (Page 1983). Based on determined standards of soil P:N:C ratio, to optimum growth of bacteria for bioremediation operations (1:5:100), deficiency of these elements were evaluated in the soil and it was supplied by adding K_2HPO_4 andNH_4NO_3 (USEPA 2000). The nutrients were added and dissolved in water to facilitate the entrance/dissolution on N and P in the soil matrix (Caouto et al. 2010).

Measuring the degradation of gasoil by two superior strains

In this step, 12 plastic containers of the same size were chosen. Then the containers were filled up as follow; to the six of the containers 600 gr soil and to the rest of them 540 gr soil and 60 gr sawdust were added. There were three replications for each of the media. The media were contaminated with 4% gasoil (w/w). As far as gasoil density is 0.89, so 4% (W/W) of gasoil for 600 gr experimental media equals to 24 gr or 26.7 ml gasoil. Then 600 gr experimental media well-homogenized with 26.7 ml gasoil to final concentration of about 4 gr contaminant per 100 gr media.

Finally, the contaminated media were inoculated by 11 ml suspension of bacteria with the population of 3×10^9 bacterial cell/ml and the wetness of media were kept 60% WHC[3] during the experiment because it was experimentally observed that higher amount of the mentioned wetness causes a muddy medium that would prevent appropriate aeration.

The experimental units were incubated in temperature equal to 27±2 °C for 45 days and the following two factors were daily controlled:

1- Soil manual aeration were done using a garden hoe to provide the factor of optimum aeration for bacterial growth (Caouto et al. 2010).
2- Addition of bacterial essential water by an atomizer, to keep humidity.

The containers were differently treated as described in Table 1. After 45 days, 10 gr sample of contaminated media was weighted and the residual gasoil amount was measured in experimental units. In this

[2] - Optical Density
[3] - Water Holding Capacity

research, "n-Hexane" was used as gasoil extraction solvent and for each 10 gr sample of the media, 50 ml n-Hexane was used Then it was shaken for 2 hours in 200 rpm and then it was transferred to centrifuge tube and centrifuged for 10 minutes in 500 rpm. Then the amount of residual gasoil in samples was measured. (EPA 413.1) (USEPA 2000, Eaton and Franson 2005).
This experiment was performed by both of the bacterial strains and a control treatment (three treatments) in two different media (soil and soil-sawdust mixture) (Table 2). The experiment was carried out in triplicate as a Completely Random Design (CRD). Mean comparison of the treatments was carried out by Duncan's multiple range test ($P<0.01$).

Results and Discussion

In the first step, to study the ability of oil-degrading bacteria to degrade gasoil, 41 isolates were purified in solid selective media of Soil Extract/Agar upon their growth rate and maximum colony diameter during 20 days.

Then in the second step, four species were selected based on the turbidity of their culture as an indicator of the growth in liquid mineral media (with proportion of 7% volume gasoil concentration as a source of carbon) during 15 days.

In the third step, upon the mineralization method (CO_2 a production and measurement the respiration related to each species of bacteria) two species during six weeks were selected as the superior and more efficient species to degrade gasoil in contaminated soils of southern Tehran refinery. Characteristics of these two isolates are illustrated in Table1.

Some physical and chemical characteristics of soil are introduced in Table 3 and the results of particle size distribution are illustrated in Fig 1. The soil texture was "sandy clay loam" and the particles with 0.1 mm in diameter of less comprised minor portion of soil particles. If otherwise the soil couldn't supply optimum condition for bacterial growth.

After measuring residual gasoil amount in each experimental unit, and subtracting it from the first amount of gasoil contamination (4% [w/w]), removal rate, indeed "Biological Elimination" of gasoil was calculated. The residual amount of gasoil in treatments had a significant difference. Comparison of the means by Duncan's multiple range test illustrated that there was a significant difference between the inoculated treatments and the control (*P>0.01*). Significant differences were also seen between the soil and the soil-sawdust media (*P>0.01*). Table 4 and Fig 2 shows the residual of gasoil in the experimental media after the 45 days. Inoculation of both bacterial strains could decrease the amount of contamination from 0.4 gr as preliminary gasoil to 0.0823 and 0.0253 gr in soil and soil-sawdust mixture media respectively. The amount of residual gasoil in control treatment (without bacteria) was approximately the same as the preliminary gasoil. According to the Table 4, the amount of gasoil was also decreased in control treatment after 45 days probably due to spontaneous degradation. This amount was subtracted from the other treatments for final determination of gasoil biological elimination efficiency.

Also, previous studies (Marquez-Rocha et al. 2000) showed that the amount of oil contaminants with bacterial inoculation could be decreased to 15 % of its prior amount during 5 weeks. The same results were obtained in research doing by Salanitro (2001), Xu & Obbard (2003), Ferguson et al. (2003) and Yang et al. (2009) as the results of current study about degradation and elimination of hydrocarbon contaminants by bacterial species. This efficiency could be explained by the autochthonous adaptation with their contaminant hydrocarbon habitat that allows microorganisms to be physiologically compatible to digest and degrade the contaminant (Bento et al. 2005).

The effectiveness of gasoil bioremediation in the different treatments is illustrated in Fig 3. The gasoil elimination in inoculated treatments is due to the bacterial consumption of gasoil as a carbon source for their growth. The results illustrate that at the mentioned environmental condition (the temperature of 27±2 °C, wetness of 60% WHC and also daily manual aeration) bacterial strains could degrade approximately 78.87% and 93.53% of gasoil in soil and soil-sawdust mixture media, respectively.

Table 1: The characteristics of superior strains

Strain	Macroscopic characteristics	Microscopic characteristics	Mobility test	Grams staining test	Catalase test	Oxidase test
BJ.1	smooth edge, mucoid & milky color	Cocco bacil	Negative	Negative	Negative	Negative
BM.1	smooth edge, mucoid & milky color	Small cocci	Negative	Negative	Negative	Negative

Table 2: Experimental design

Experimental units*	Inoculation of both bacterial strains
SM	+
SMC	-
SSM	+
SSMC	-

Growth + and no Growth -
* SM: Soil Media
SMC: Soil Media Control
SSM: Soil/Sawdust Media
SSMC: Soil/Sawdust Media Control

Table 3: Some physicochemical characteristics of the soil used as a media

Characteristics	Value
Electrical Conductivity (ds/m)	0.223
Organic Matter (%)	0.160
Organic Carbon (%)	0.097
Field Capacity Moisture (%)	32.2
Nitrogen (%)	0.004
Phosphorous (mg/kg)	13.2
pH	8.20

Table 4: Residual gasoil amount (gr gasoil in 10 gr of contaminated soil, after 45 days)

Mean residual gasoil amount	Treatment
0.082^{b}	Inoculation on soil media
0.025^{c}	Inoculation on soil-sawdust media
0.389^{a}	Control-soil media
0.386^{a}	Control-soil-sawdust media

The values with different alphabets are significantly different in $P<0.01$

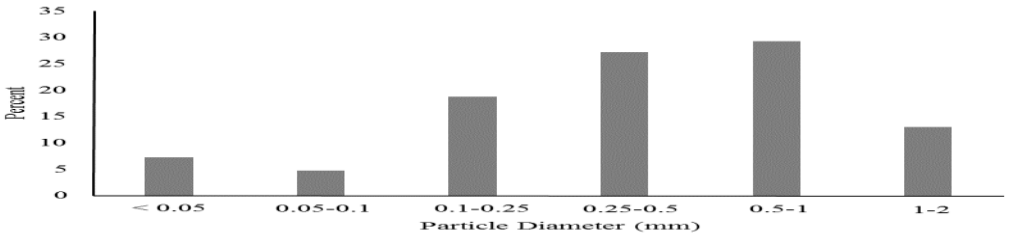

Fig.1: Soil particle size distribution

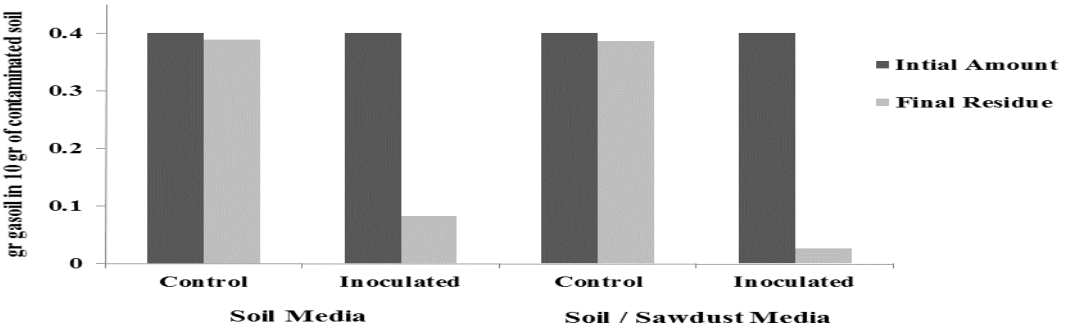

Fig. 2: The amount of gasoil decrease by bacterial species after 45 days

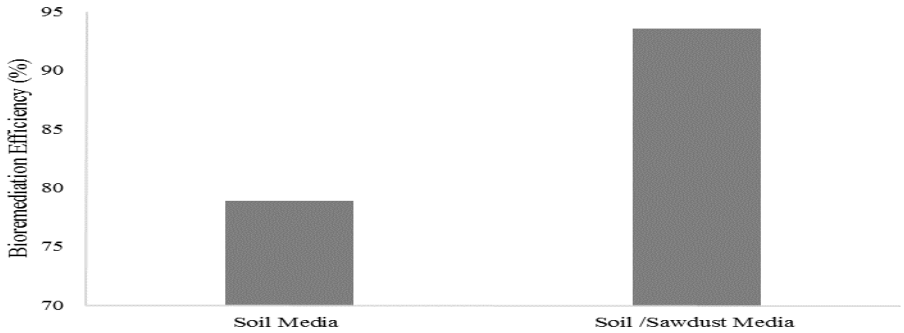

Fig. 3: The efficiency of gasoil bioremediation by both of the bacteria

Conclusion

Regarding to the experiment time (45 days), it seems to be an acceptable result. Gasoil contamination is decreased during 45 days to less than ½ amounts of preliminary contamination amount in the media inoculated with oil-degrading bacteria. Also soil-sawdust media is more appropriate than the soil media in gasoil bioremediation. It is probably because of the impact of sawdust on improving the environmental conditions for bacterial growth. These results imply that sawdust may be involved in the degradation by: i) improving aeration, ii) increasing water holding capacity (WHC) and iii) increasing bioavailability of gasoil to bacteria. Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of the environmental conditions for bacterial growth. Bioremediation using indigenous microorganisms is one of the most effective methods that has no harmful environmental effects. It is also the most effective method in degradation of hydrocarbon contaminants in a short time.

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