Isolation of oil- degrading microorganisms from soil contaminated with petroleum hydrocarbons

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ABSRTACT

The extensive use of petroleum products leads to the contamination of almost all compartments of the environment. This problem must be mentioned by countries which produce or use this type of energy. Petroleum pollution is a major concern for Tehran refinery in Iran. Oil spills as a result of pipeline ruptures, tank failures and various production storage and transportation accident caused the pollution of soil and groundwater in this region. Bioremediation is a new method which has been proposed for remediation of petroleum pollutants using of indigenous or allochthonous microorganisms to detoxify and degrade environmental contaminants. Thus this study conducted to identify and isolate of microorganisms which have the potential for degradation of petroleum pollutants in soil media. Soil samples were taken from soil surface (0-30cm) at 8 points in Tehran refinery,Iran and kept at 4 ^{o}C until analysis. Ten grams of each sieved soil (<2mm) added to 25 ml oil broth media for isolation of hydrocarbon adopted bacteria. This media involves KH_2PO_4 1gl^{-1}, K_2HPO_4 1gl^{-1}, $(NH_4)_2SO_4$ 1gl^{-1}, $MgSO_4$,7H_2O 0.04 gl^{-1}, $FeCl_2$,6H_2O 0.004 gl^{-1} and 0.1 of solute containing 100 mg/l H_3BO_4, $MnSO_4$, $ZnSO_4$, $CuSO_4$ and $COCl_2$. All samples incubated in a shaker incubator at 30^{o}C and 140 rpm for 2 days. In next stage, 25 ml of each flask was removed and added to new flask which involved oil broth media and incubated for 2 days. This stage repeated for 2 weeks. Finally 0.1 ml of last flasks spread on Petri dishes that contained nutrient agar (NA). Fungi were isolated with serial dilution of soils in 10 ml sterile distilled water. 0.1 ml of each dilution being spread on Petri dishes that contained potato dextrose agar (PDA). After growing the fungi and bacteria in growth media, they were purified. The morphological characterization of each isolate was first performed, including color, size and colony characteristics. Gram strain test was performed for each isolate. The following biochemical tests were used in the bacterial identification process: oxidase. catalase, glucose fermentation and gas production. Bacterial strains were identified based on Bergey's manual of systematic bacteriology. Fungi were identified according to general principles of fungal classification, using selective media and macro and microscopic examination of morphological characters. Results showed that bacterial strains belong to the genus *Micrococcus,Rhodococcus,Nocardia,Corinebacterium, Acinetobacter, Pseudomonas* and *Alcaligenes*. Fungi which have been identified were *Aspergillus, Fusarium* and *Zygopus*. Growing these strains in oil broth media with 400 micro liter crude oil, proved their potential for degradation of petroleum pollutants.

INTRODUCTION

Petroleum hydrocarbons are wide spread in our environment as fuel and chemical compounds. The uncontrolled realease of petroleum hydrocarbons negatively impacts many of our soil and water resources. The contamination can result from leaking underground storage tanks, petroleum refineries and bulk storage facilities, broken oil pipelines, spills of petroleum products in chemical plants and transportation processes (Atlas,1981). It is believed that accumulation of contaminants in the environment constitutes a serious to ecological and human health (Sullivan et al., 2001). Bioremediation is an effective measure in dealing with such contaminations particularly those from petroleum hydrocarbon sources more ever bioremediation is emerging as a promising technology for the treatment of soil and ground water contaminations (Robert, 1992). Microorganisms survive in

contaminated habitats because they are metabolically capable of utilizing its resources and can occupy a suitable niche. Contaminants are often potential energy sources for microorganisms (Capelli et al., 2001, Chen et al., 1999). Bioremediation a process that exploits the catalytic abilities of living organisms to enhance the rate or extent of pollutant destruction is an important tool in attempts to mitigate environmental contamination (Leahy and Clowell, 1990). Bioremediation achieves contaminant decomposition or immobilization by exploiting the existing metabolic potential in microorganisms with catabolic functions derived through selection or by the introduction of genes encoding such functions. The effectiveness of bioremediation is often a function of the extent to which a microbial population or consortium can be enriched and maintained in environment. When few or no indigenous degradative microorganisms exist in a contaminated area and potentially dose not allow time for the natural enrichment of suitable population, inoculation may be a convenient option(Margesin and Schinnur, 1997). In Iran, an endless number of contaminated sites exist as a result of more than 50 years of oil petroleum activity. in recent years this problem has motivated researches to recover those contaminated sites. The goal of the present work is to isolate the bacterial and fungal strains from hydrocarbons contaminated soils in Tehran refinery, Iran to assess their potential for bioremediation and develop a byproduct useful for soil inoculation.

MATERILAS AND METHODS

Soils contaminated by different petroleum hydrocarbons were collected from 8 different places(Table 1), in 0-30 cm depths. Enrichment process was carried out for increasing the microorganism population. Ten grams of each sieved soil (<2mm) added to 25 ml oil broth media for isolation of hydrocarbon adopted bacteria. This media involves 400 micro liter crude or white oil, KH_2PO_4 $1gl^{-1}$, K_2HPO_4 $1gl^{-1}$, $(NH_4)_2SO_4$ $1gl^{-1}$, $MgSO_4,7H_2O$ 0.04 gl^{-1}, $FeCl_2,6H_2O$ 0.004 gl^{-1} and 0.1 of solute containing 100 mg/l H_3BO_4, $MnSO_4$, $ZnSO_4$, $CuSO_4$ and $COCl_2$ (Churchill, 1999). The autoclaved flasks without any oil were used as controls. All samples incubated in a shaker incubator at 30^oC and 140 revolutions per minute (rpm) for 2 days. In next stage, 25 ml of each flask was removed and added to new flask which involved oil broth media and incubated for 2 days. This stage repeated for 2 weeks. A visible increase of turbidity in a flask was used as an indication of an isolated colony's ability to grow using oil as the carbon source. Finally 0.1 ml of last flasks spread on Petri dishes that contained nutrient agar (NA). Fungi were isolated with serial dilution of soils in 10 ml sterile distilled water. 0.1 ml of each dilution being spread on Petri dishes that contained potato dextrose agar (PDA). After growing the fungi and bacteria in growth media, they were purified. The morphological characterization of each isolate was first performed, including color, size and colony characteristics. Gram strain test was performed for each isolate. The following biochemical tests were used in the bacterial identification process: oxidase. catalase, glucose fermentation and gas production. Bacterial strains were identified based on Bergey's manual of systematic bacteriology (Palleroni, 1984). Fungi were identified according to general principles of fungal classification, using selective media and macro and microscopic examination of morphological characters.

The potential for bioremediation can be assessed in two ways. The principle developed in this study is to test weather or not an isolated colony can grow when oil is used as the soil carbon source. If bacteria can grow under this condition it means that the colonies can use the oil for their metabolism and biodegradation is likely to happen. In a first experiment the growth potential of each colony was determine in a flask by providing nutrients and oil. A second test was conducted by using a plate which was only made of mineral salts and agar. Each colony was spreaded on the surface of the plate. Crude and white oil were used to provide the carbon source in the experiment. Plate was placed upside down so that the bacteria can obtain carbon from oil. Pates were put in an incubator at 30 ^0C until visible growth was observed.

RESULTS AND DISCUSSION

Soils characteristics are shown in table1. The amount of total petroleum hydrocarbons(TPH) in soil samples was in the range of 8.5-23.4% which affected some soil properties such as pH and organic carbon(OC%). It seems that the microorganism which have isolated from this soils must tolerance high amount of petroleum hydrocarbons in soil media. A total of 40 bacterial strains belong to the genus *Micrococcus,Rhodococcus,Nocardia,Corinebacterium, Acinetobacter, Pseudomonas* and

Alcaligenes. Fungi which have been identified were *Aspergillus, Fusarium* and *Zygopus*. Chailan et al., 2004 showed that these microorganisms have the biodegradation potential of tropical soils.
Isolated colonies can grow when oil was used as the soil carbon source it means that these microorganisms can use the oil for their metabolism. All colonies could grow in flasks which containing 0.5, 1, 5 and 10% v/v crude and white oil. The measurement of optical density (OD) at 540 nm during one week proved that isolated microorganisms can grow in the present of oil. Adding the surfactant (Tween 80) with oil did not change these results (Figure 1).All the microorganisms could grow in the plates which contained mineral salts, agar and 400 micro liter crude oil. Some colonies which are grown on the surface of plate are shown in figure 2. The results of these study showed that the isolated microorganisms can be used as a microbial consortium in bioremediation of contaminated soils with petroleum hydrocarbons.

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	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7	Soil 8
PH	5.71	6.58	6.91	6.82	6.12	6.45	6.21	6.18
EC (ds/m)	1.25	1.97	5.52	7.28	8.29	1.37	4.38	4.31
% OC	7.429	2.269	1.878	7.945	7.686	6.588	6.514	5.050
% N	0.771	0.248	0.202	0.817	0.789	0.679	0.673	0.529
P (ppm)	33.2	24.0	30.0	47.8	50.0	182.0	38.2	98.6
% sand	83	71	89	64	79	63	41	83
% silt	10	18	6	23	12	24	36	8
% clay	7	11	5	13	9	13	23	9
% TPH	18	11.7	8.5	20.1	23.4	15.3	16.7	13.6
Global position	N 35° 32′ 49.5″ E 51° 25′ 28.3″	N 35° 32′ 49.5″ E 51° 25′ 28.3″	N 35° 32′ 43.6″ E 51° 25′ 26.7″	N 35° 32′ 29.7″ E 51° 25′ 31.7″	N 35° 32′ 10.7″ E 51° 25′ 28.3″	N 35° 31′ 54″ E 51° 25′ 35.2″	N 35° 22′ 28.5″ E 51° 25′ 36.4″	N 35° 22′ 23.4″ E 51° 35′ 35.5″

Table 1- The characteristics of soil samples

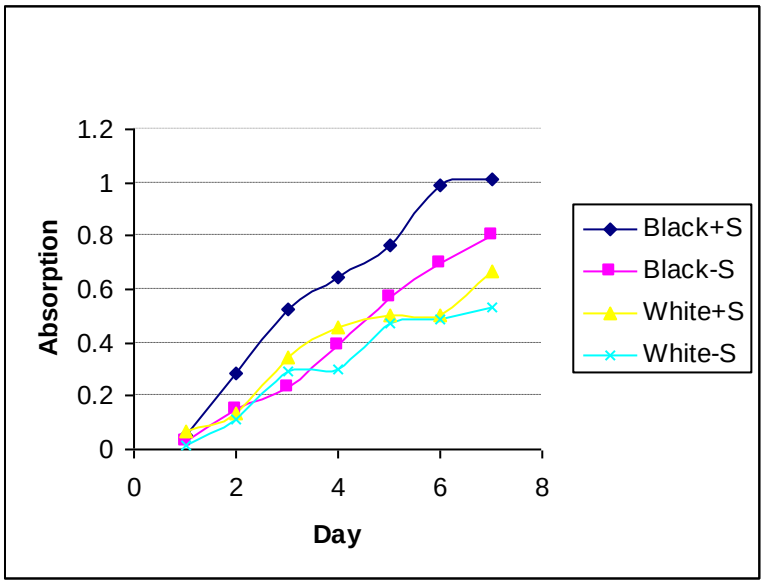

Figure 1. Optical Density (OD) of isolated microorganisms in present of crude and white oil.(Black=crude oil, White= white oil, S= surfactant)

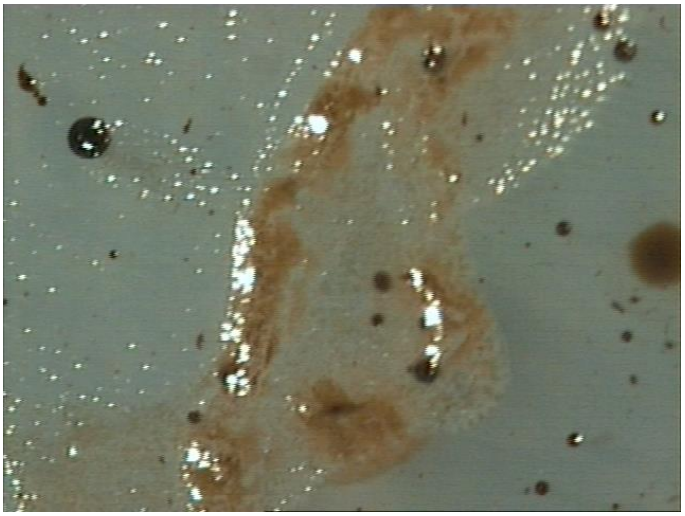

Figure2. microbial colonies in surface of agar plates in present of crude oil.