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Full Length Research Paper

Determination of petroleum-degrading bacteria isolated from crude oil-contaminated soil in Turkey

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In order to establish an experimental basis for bioremediation of soil contaminated with crude oil, 33 strains of bacteria with hydrocarbon-degrading ability were isolated from the contaminated soil in Adana, Batman and Adiyaman, Turkey. The strains were identified as *Pseudomons* spp., *Paucimonas lemoignei, Stenotrophomonas maltophilia*, *Eschericha* spp., *Enterobacter* spp. *Citrobacter koseri, Acinetobacter* spp.*, Aeromonas caviae, Sphingobacterium multivorum, Klebsiella pneumoniae. Pseudomons aeruginosa, Pseudomonas putida* biotype A, *Citrobacter amalonaticus* GC subgroup A and *Acinetobacter genomospecies,* respectively. The ability to utilize crude oil as carbon source for their growth was ascertained. These bacterial isolates obtained in this study have catabolic capabilities for the biodegradation of petroleum hydrocarbons. Further study under soil conditions may be necessary to determine biodegradation potential of bacterial mixture tested in contaminated soil.

Key words: Soil, crude oil, soil pollution, bacteria, biodegradation.

INTRODUCTION

Turkey has recently been exhibiting an increasing development in terms of spreading oil pipe-line constructions. In a few months, Baku-Tiflis-Ceyhan pipe-line would be carrying 50 million ton crude oil per year from Ceyhan through all over the world. In the future, no doubt that new pipe lines will be connected to our oil transporting network so that Turkey would be much more effective to convert his geographical advantage to the income. However, we lack knowledge on the impacts of oil pollution and its elimination from our lands. As well as its economic and geopolitical contributions, pipe-line transportation has a great risk in terms of oil contamination by spillage, leakage and accidents, etc. on the natural sources.

Crude oil is a complex mixture of hydrocarbons and other organic compounds, including some heavy metals and metallic compounds (Butler and Mason, 1997; Rhykerd et al., 1998). Crude oil contains more metals and heavy polycyclic aromatic hydrocarbons (PAHs) (Keith and Telliard, 1979; Cooney, 1980; Hagwell et al., 1992; Rhykerd et al., 1998; Boonchan et al., 2000). In addition, polycyclic aromatic hydrocarbons (PAHs) have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment (Odu, 1997; Van Hamme et al., 2003). Crude oil contains higher percentage hydrocarbons that would be more of a concern for acute toxicity to organisms (Morgan and Watkinson, 1989; Alvarez et al., 1991; Rhykerd et al., 1998).

Physical and chemical method to reduce hydrocarbon pollution is expensive (Bonnier et al., 1980; El-Nawawy et al., 1987; Rhykerd et al., 1998) and time consuming than biological method (Eckenfelder and Norris, 1993). Bioremediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms (Atlas, 1978), particularly the indigenous bacteria present in the soil. Hydrocarbons, including PAHs have been long recognized as substrates supporting microbial growth (Bushnell and Haas, 1941;

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Speight, 1991).

Bioremediation makes use of indigenous oil-degrading microorganisms by enhancing and fertilizing them in their natural habitats. Microorganisms degrade these compounds by using enzymes in their metabolism and can be useful in cleaning up contaminated sites (Atlas, 1981; Atlas and Bartha, 1992; Steffan et al., 1997; Alexander, 1999).

These microorganisms can degrade a wide range of target constituents present in oily sludge (Barathi and Vasudevan, 2001; Mishra et al., 2001). A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil and aquifers (Ojumu et al., 2005; Peressuttia et al., 2003; Rahman et al., 2002a; Ökmen and Algur, 2000; Johnson et al., 1996; Komukai-Nakamura et al., 1996; Kiyohara et al., 1992; Jack et al., 1985; Fall et al., 1979). Other petroleum hydrocarbon-degraders include *Bacillus subtilis* (DM-04), *Pseudomonas aeruginosa* (Kishore and Ashis, 2007), *Enterobacter sakazakii, Bacillus mycoides, Klebsiella oxytaca, Acinetobacter calcoaceticus*, (Pokethitiyook et al., 2002), *Bacillus megaterium, Pseudomonas diminuta, Gluconobacter cerenius, Pasteurella caballi, Sphingomonas paucimobilis* and *Sphingobacterium multivoru* (Jùrgensen et al., 2000). Bioremediation processes have been shown to be effective methods that stimulate the biodegradation in contaminated soils (McLaughlin, 2001; Swannell et al., 1996). The aim of this study was to find out whether the bacterial isolates obtained have catabolic capabilities for the biodegradation of petroleum hydrocarbons.

MATERIALS AND METHODS

Culture media

Medium A contained in (g l^{-1}): yeast extract, 0.01; NaCl, 0.1; K_2HPO_4, 1; $CaCl_2$, 0.1; KNO_3, 1; (g l^{-1}). The pH of the media was adjusted to 6.3 with 0.1 N HCl. After sterilization of the medium, the crude oil was added at 1% (v/v) emulsified with an aqueous solution of Triton X-100 (1:1) at 1% (v/v). Medium B contained the components of medium A plus agar at 1.5% (w/v). Cultures on agar medium were incubated at 28 ± °C for 72 h. Liquid cultures were shaken at 180 rpm. All media and solutions used were sterilized by autoclaving at 121°C for 15 min (Rojas-Avelizapa et al., 1999).

Enrichment and isolation of bacteria

Crude oil-degrading microorganisms were isolated using the following enrichment procedure: 10 g of soil, collected from Adana, Batman, Adıyaman, was transferred into 500 ml Erlenmeyer flasks containing 100 ml of medium A. Medium A was used as the enrichment media with 1% (v/v) used crude oil: Triton X-100 (1:1) was used as the sole carbon source to isolate crude oil-degrading bacteria. The flasks were shaken for 3 days at 180 rpm and 28 ± °C. After the 3rd day, 10 ml of the supernatant were transferred to 125 ml of fresh medium A. The incubation transfer to the fresh medium was repeated twice. At the third transfer, 0.1 ml sample of the recovered medium was streaked onto plates with medium B. Plates were incubated at 28 ± °C for 24 h. The single colonies were streaked onto nutrient agar plates, tryptic soy agar plates and B medium, were incubated at 28 ± °C overnight for their isolation and purification and were stored at 4°C until further use (Rojas-Avelizapa et al., 1999). For long term preservation, the bacterial isolates were stored in 50% glycerol at -20 and -70°C.

Bacterial identification

Phenotypic identification was carried using identification reactions oxidase (Bactident oxidase Merck 1.13300) after incubation for 16 to 44 h at 28 ± °C. Gram stain test was performed for each isolate; Bergey's manual was used for the identification (Holt et al., 1994). A loopful of growth from a colony of the organism was emulsified on the surface of a glass slide in a suspension of 3% KOH (Suslow et al., 1982, Arthi et al., 2003).

Taxonomic diversity

Whole-cell fatty acid analyses were performed on all of the PAH-degrading isolates by growing the cells at 28°C for 24 h on TSA plates. Cellular fatty acids were saponified, methylated, extracted and analyzed by gas chromatography following the procedures given for the Sherlock microbial identification system (MIDI, Inc., Newark, Del.). Identification and comparison were made to the Aerobe (TSBA version 60) database of the MIDI. The dendrogram program of the MIDI software package was used to produce unweighted-pair matchings based on the fatty acid compositions. Fatty acid methyl ester (FAME) analysis is commonly used by soil scientists as a sole method for identifying soil bacteria (Klement et al., 1990, Paisley, 1995, Matsumoto et al., 1997; Şahin, 1997; Şahin et al., 2003a, b). All experiments were done in triplicate.

Biodegradation of petroleum hydrocarbon in liquid culture

Inoculants were obtained from 500 µl transferred to 25 ml reaction vials with 5 ml of medium A with 1% of crude oil, incubated in the dark at 28 ± °C and shaken at 180 rpm on an orbital for 5 days. Abiotic controls were also included in medium A with 1% of crude oil without inoculums. Abiotic controls were prepared to evaluate the hydrocarbon evaporation. Biotic controls were also included in medium A with 1% of crude oil (0 day). At the end of the 5th day, cultures were filtered through a 0.2 µm millipore membrane and the filtrate was liquid-liquid extraction of DCM (dichloromethane). Cultures, abiotic control and biotic controls were collected, centrifuged at 3000 rpm and extracted three times with 5 ml DCM (2-2-1 ml). Extracts were combined and evaporated at room temperature. The organic phase was collected for analysis by gas chromatography (GC HP 680 series GC system, US90704303) (Olivera et al., 1997; Boonchan et al., 2000). Degradation was estimated as the difference between the initial (0th) and final (5th) concentrations of the total hydrocarbons. All experiments were done in triplicate. This analysis was accepted as a standard biodegradation of petroleum (Boonchan et al., 2000).

Degradation potential and its growth patterns

The bacterial cultures (12 h old, 33 bacteria) were inoculated in medium A with 1% of crude oil. Inoculants obtained (500 µl) from each of the bacteria culture were transferred to micro plate (BIOLOG well). Micro plates were incubated at 28 ± °C. Microbial

Table 1. Results of 3% KOH test, oxidase test, gram staining and MIS applied on the isolated bacteria from Batman soil.

Code	3% KOH	Oxidase test	Gram staining	MIS result
1	(-)	nonenteric	Gr (-) cocobacil	*P. aeruginosa*
2	(-)	nonenteric	Gr (-) rod	*P. lemoignei*
3	(-)	nonenteric	Gr (-) cocobacil	*P. mucidolens*
4	(-)	nonenteric	Gr (-) cocobacil	*P. aeruginosa*
4a	(-)	enteric	Gr (-) rod	*S. maltophilia*
4b	(-)	nonenteric	Gr (-) rod	*P. aeruginosa*
5	(-)	enteric	Gr (-) rod	*Eschericha coli*
6	(-)	enteric	Gr (-) rod	*E. hormaechei*
7	(-)	enteric	Gr(-) rod	*C. koseri*
8	(-)	nonenteric	Gr(-) rod	*P. putida biotype A*
9	(-)	-	Gr(-) rod	*E. hormaechi*
10	(-)	nonenteric	Gr(-) rod	*P. putida biotype A*
11	(-)	nonenteric	Gr(-) rod	*P. putida biotype A*
12	(-)	enteric	Gr(-) rod	*K. pneumoniae*
13 a	(-)	enteric	Gr(-) rod	*S. maltophilia*
13 b	(-)	enteric	Gr(-) rod	*S. maltophilia*
14	(-)	nonenteric	Gr(-) cocobacil	*P. aeruginosa*

Table 2. Results of 3% KOH test, oxidase test, gram staining and MIS applied on isolated bacteria from Adana soil.

Code	3% KOH	Oxidase test	Gram staining	MIS result
15	(-)	-	Gr (-) cocobacil	*A. genomospecies 3*
16a	(-)	nonenteric	Gr(-) rod	*P. putida biotype A*
16b	(-)	nonenteric	Gr (-) cocobacil	*A.caviae*
17	(-)	-	Gr (-) cocobacil	*A. genomospecies 3*
18	(-)	enteric	Gr (-) cocobacil	*A. calcoaceticus*
19a	(-)	nonenteric	Gr(-) rod	*A. caviae*
19b	(-)	nonenteric	Gr(-) rod	*S. multivorum*
20a	(-)	enteric	Gr(-) rod	*C.amalonaticus*
20b1	(-)	enteric	Gr (-) rod	*E. hormaechei*
20b2	(-)	nonenteric	Gr(-) rod	*P. putida biotype A*

growth was determined at different times (from 0 to 120 h) by a spectrophotometer at 620 nm. All experiments were performed in duplicate (Rahman et al., 2002a, b).

RESULTS AND DISCUSSION

Results of 3% KOH test, oxidase test, gram staining and MIS applied on the isolated bacteria

Crude oil degrading bacteria was successfully isolated and identified from the three different soil contaminated with crude oil hydrocarbons. Of the isolated 33 bacterial strains (Tables 1 to 3), 9 were identified as *PseudomonAs* spp.,1 strain as *P. lemoignei,* 3 strains as *Stenotrophomonas maltophilia*, 3 strains as *Eschericha* spp., 6 strains *as Enterobacter* spp., 2 strains as *C. koseri,* 4 strains as *Acinetobacter* spp*.,* 2 strains as *Aeromonas caviae, S. multivorum* and *K. pneumoniae.* It was reported that bacteria from these mentioned genera were involved in crude oil degradation (Shivaji et al., 1992, Takeuchi and Yokota, 1992; Kim et al., 2006).

Jùrgensen et al. (2000) isolated and identified from contaminated soil with crude oil, *E. sakazakii, B. mycoides, K. oxytaca and A. calcoaceticus*, *B. megaterium, P. diminuta, G. cerenius, P. caballi, S. paucimobilis,* and *S. multivorum*. Yabuuchi et al. (1983) isolated and identified 7 strains of *Sphingobacterium* spp.

Determination of bacteria growth patterns in petroleum culture (620 nm)

Bacterial development in food medium was evaluated by

Table 3. Results of 3% KOH test, oxidase test, gram staining and MIS applied on isolated bacteria from Adiyaman soil.

Code	3%KOH	Oxidase test	Gram staining	MIS result
21	(-)	nonenteric	Gr (-) cocobacil	*A. genomospecies*
22	(-)	enteric	Gr (-) rod	*E. hormaechei*
23	(-)	enteric	Gr (-) rod	*E. fergusonii*
24	(-)	enteric	Gr (-) rod	*E. sakazakii*
25	(-)	enteric	Gr (-) rod	*E. fergusonii*
26	(-)	enteric	Gr (-) rod	*E. sakazakii*

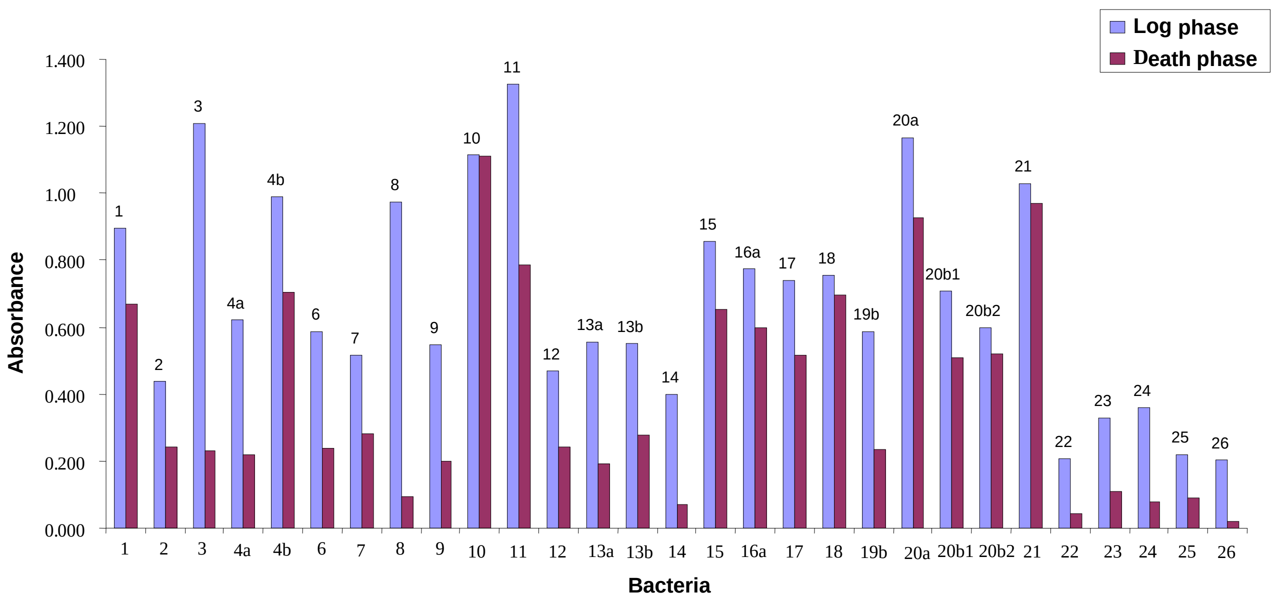

Figure 1. Bacteria growth capabilities in the petroleum culture (620 nm).

measuring the light transmittance in liquid medium spectrophotometrically based on the increase of bacteria. Spectrophotometric reading results (Biologist 620 nm) of the reproduction abilities of the 33 bacteria isolated from the petroleum-contaminated soil in raw petroleum medium are given in Figure 1. As can be seen in Figure 1, the best results were obtained from bacteria no 1, 4b, 10, 11, 20a and 21 from the petroleum (1%) medium and transition of these bacteria from stationary phase to death phase at the end of the 5th day was later than that of others.

A similar study by Leahy et al. (2003) pointed out the hydrocarbon degrading abilities of bacteria by measuring spectrophotometrically the bacteria development in 600 nm. At 620 nm wavelength, bacteria development and hydrocarbon degrading abilities of these bacteria were spectrophotometrically determined by Rahman et al. (2002a). In other studies, hydrocarbon degrading percentages were presented by preliminary and final measurements at 420 nm wavelength (Rahman et al., 2002b). In another study, petroleum degrading kinetics of isolated bacteria was spectrophotometrically calculated at 540 nm (Hyina et al., 2003). Rahman et al. (2002b) presented the crude oil degrading abilities of the bacteria *Micrococcus* spp., *Bacillus* spp., *Corynebacterium* spp., *Flavobacterium* spp. and *Pseudomonas* spp. at 420 nm.

Evaluation of petroleum degradation in liquid culture

Liquid-liquid extraction was made in two sampling periods; day 0 and 5 from feed-lots containing 1% of the raw petroleum+Triton-×100 (1:1) separately on each bacteria, in order to determine the degradation level of raw petroleum by the bacteria (Boonchon et al., 2000). Total fatty acid content in the extracted part was read in GC-MS system and the results were considered as criteria for the biological degradation of petroleum.

The highest petroleum-degradation of the 33 bacteria isolated from soil contaminated with petroleum in liquid

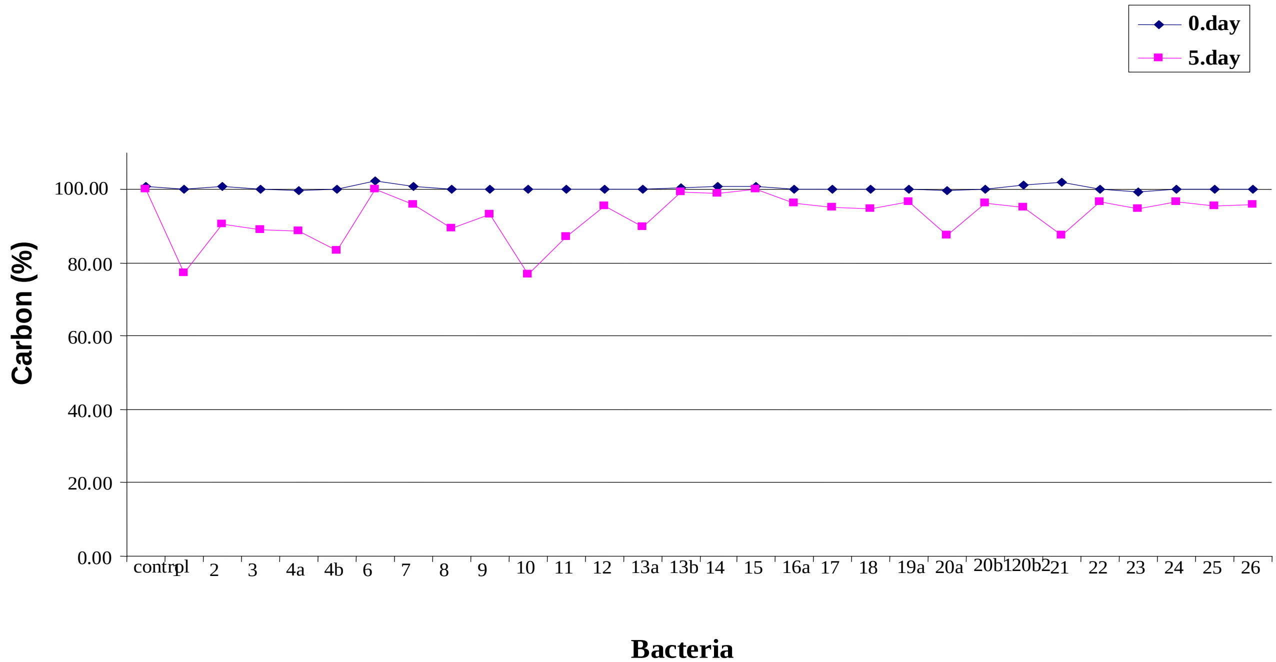

Figure 2. Determination of petroleum degradation in the liquid culture.

culture (in 1% raw petroleum medium with the highest difference between day 0 and 5.) was observed in the bacteria entities numbered 1, 4b,10, 11, 20a and 21 (Figure 2). Bacteria that exhibited the best development in the raw petroleum medium as in biologist measurement degraded the highest amount of petroleum during this development process. The selected bacteria were of the following types and species: 1- *P. aeruginosa* (isolated from Batman soil); 4b- *P. aeruginosa* (isolated from Batman soil); 10- *P. putida biotype A* (isolated from Batman soil); 11- *P. putida* biotype A (isolated from Batman soil); 20a-*C. amalonaticus-GC* subgroup A (isolated from Adana soil); 21- *A. genomospecies* 3 (isolated from Adiyaman soil).

Many researchers have pointed out the petroleum-degrading abilities of bacteria by different chemical substances and different wavelengths. For instance, they measured the degraded hydrocarbon content by TLC (thin layer chromatography) using methanol:chloroform [(2:1); v/v] mixture. They determined the microbial development at 436 nm (Alvarez, 2003). Kapley et al. (1999) presented hydrocarbon degrading capacity in gas chromatography using extraction by hexane. They determined optical density at 620 nm.

One of the main ideas of this research is to determine the existence of microorganism which can eliminate the contamination caused by petroleum and similar substances on the soil and to evaluate its activity in laboratory environment. Rojas-Avelizapa et al. (1999) have defined the bacteria they isolated from an area infected by PCB (polychlorinated bifenyls) as *C. acidovorans, A. calcoaceticus, Achromobacter* spp., *Pseudomonas* spp., *Flavobacterium devorans, Bacillus lentus, Bacillus mascerans* and *Bacillus thuringiensis* and they observed that, this mixed culture degraded 75% of the contaminant with high PCB content in soil-free *in vitro* medium.

In another similar study, Kishore and Ashis (2007) tested *B. subtilis* (DM-04), *P. aeruginosa* (M) and bacteria they isolated from a region contaminated with petroleum in South-eastern India in soil-free *in vitro* and in contaminated soil environments. They observed that, *P. aerugionasa* (M) bacteria was more active than *B. subtilis* (DM-04) in soil-free medium and in the petroleum soil experiment, *P. aerugionasa* (M) a contaminated soil isolate proved a dissociation of up to 100% at the end of the incubation process of 120 days, while *B. subtilis* DM-04 proved a dissociation of 50%. Similar results were also obtained by Porta et al. (1998). Also other researchers reported that complex hydrocarbons, phenols, phenanthrene and benzopyrenes exhibited extensive metabolic abilities in gram positive bacteria in the degradation of oxidation products (Sextone et al., 1978; Song and Barta, 1990).

Conclusions

In the study, changes that occur in the population dynamics of the different petroleum-degrading bacteria were examined. Different bacteria materials were provided under the conditions of Turkey in order to determine their petroleum-degrading ability (33 bacteria strains were isolated from Adana, Batman and Adiyaman regions) and they underwent dissociation tests in the laboratory environment. In raw petroleum medium, 6 bacteria strains that exhibited best development processes and had the highest levels of petroleum-degradation were determined *(P. aeruginosa, P. putida biotype A, C.-amalonaticus-GC subgroup A, A. genomospecies).* It is a significant study that may light the way for bioremediation studies.

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