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Biodegradation of Used Engine Oil Using Mixed and Isolated Cultures

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ABSTRACT: It is known that native oil-degrading microorganisms are ubiquitous. They can be isolated from contaminated soils. In this study, biodegradation experiments were carried out to evaluate the efficiency of pollutant removal by adding the selected microorganisms. Five mixed cultures and 3 single bacteria strains, Pseudomonas sp., Arthrobacter sp. and Mycobacterium sp. were isolated from hydrocarbon-contaminated soils by enrichment on either crude oil or individual hydrocarbons, as the sole carbon sources. The strains were selected based on their ability to grow in medium containing crude oil, used engine oil or both. Their ability to degrade hydrocarbon contamination in the environment was investigated using soil samples contaminated with used engine oil. The mixed starter culture #1 degraded 66 % of aliphatic compounds in the engine oil, after 60 days of incubation. The mixed starter culture #5 removed 47 % of aromatic compounds during 60 days of incubation, which is the maximum efficiency among the starter cultures, in this study.

Key words: Biodegradation, Waste Engine Oil, Mixed Culture, Decontamination, Contaminated Soils, Hydrocarbons

INTRODUCTION

Large amounts of lubricating oils, composing long-chain saturated hydrocarbons (base oil) and additives are used in car engines. The main components of the base oil are cyclic alkanes (c-alkanes). Long-chain hydrocarbons and c-alkanes are known as recalcitrant to microbial degradation. The base oil contains C_{16}-C_{36} hydrocarbons, and more than 75% c-alkanes. The rings number of c-alkanes in the base oil is from 1 to 3 and any ring contains 5 or 6 members. Most of the c-alkanes in the base oil have long alkyl side chains (Koma *et al.*, 2003). Table 1 shows the components of car engine base oil (Koma *et al.*, 2001).

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Since the degradation of long chain hydrocarbons, which are solid at temperatures less than 10 °C, is hindered by their limited bioavailability, the waste oil is hardly degraded by microorganisms in nature (Gough and Rowland, 1990; Sorkhoh *et al.*, 1995; Ijaha and Antaib, 2003). In addition, the recalcitrance of hydrocarbons and/or the inhibition of microorganisms by minor ingredients in waste oil, also hinder the degradation of waste oil (Gough and Rowland, 1990). Susceptibility of a

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hydrocarbon to microbial degradation varies with type and size of the hydrocarbon molecule. Alkanes of intermediate chain length (C_{10}–C_{24}) are often degraded rapidly, while very long chain alkanes are increasingly resistant to microbial degradation (Ijaha and Antaib, 2003).

Biodegradation of complex hydrocarbons; particularly pollutants that are made up of many different compounds, such as crude oil or petroleum; usually requires the cooperation of more than one single species. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so the mixed cultures with overall broad enzymatic capacities are required to increase the rate of petroleum biodegradation. Several microbial populations have been detected in petroleum-contaminated soils or water. This phenomenon, strongly suggests that each strain or genera has its role in hydrocarbon transformation processes. The degradation capacity of any microbial culture is not necessarily the result of merely adding the capacities of the individual strains (Ghazali *et al.*, 2004). Rambeloarisoa *et al*. (1984) reported a mixed culture, containing 8 strains of 6 genera, which could effectively degrade crude oil. Interestingly, only 5 strains between them could grow as pure cultures, on different hydrocarbons. However, when the other 3 strains were removed from the culture, the effectiveness of the mixed culture was remarkably reduced. These results showed that each member in a microbial community has a significant role and may be dependent on the presence of the other species or strains for surviving.

Table 1. Chemical composition of car engine base oil (Koma *et al.*, 2001)

Component	(%)
Saturated fraction	90.9
Normal paraffin	15.5
Cyclic paraffin	75.4
Aromatic fraction	9.1
Naphthalene	1.7
Fluorene	1.2
Benzene	1.1
Dibenzofuran	1.0
Dinaphthenebenzene	0.8
Dibenzanthracene	0.6
Naphthobenzothiophene	0.3
Perylene	0.2
Benzothiophene	0.2
Chrysene	0.1
Unknown	1.9

Syoko *et al*. (1996) reported the sequential degradation of Arabian light crude oil by two different genera. *Acinetobacter* sp. *T4* biodegraded alkanes, then *Pseudomonas putida PB4* began to grow on the metabolites and finally degraded aromatic compounds of crude oil. It can be attributed to the effects of synergistic interactions among the members of the association, although the mechanisms may be complex. It is possible that one species can remove the toxic metabolites of the other species, or degrade some compounds better than others (Alexander, 1999).

In this study, the biodegradation of used engine oil components were investigated under equal condition. To our knowledge, this is the first experimental study which investigates biodegradation of used engine oil components.

MATERIALS & METHODS

The contaminated soil samples were prepared from the garage area of heavy vehicles in Tehran-Saveh road, Iran and transferred to Biotechnology Laboratory of Chemical Engineering Department, Tarbiat-Modares University.

Bacterial strains were isolated after enriching the extract of different contaminated soils. One ml of the enriched samples was added to 100 ml of the basal mineral medium and 1 ml of used engine oil. The used engine oil was obtained from Behran Oil Engine Company, Tehran, Iran and then filtered and sterilized prior to addition to the soil. The liquid basal medium (BM) was composed of (g/L) K_2HPO_4, 0.5; NH_4NO_3, 2.0; KNO_3, 2.0 and $MgSO_4$ 7 H_2O, 0.4. The growth was monitored by observation the medium turbidity (at 540 nm), during 30 days incubation at 28 °C. A medium without carbon source, served as control.

Five mixed cultures, developed on agar, with 0.1 g engine oil as carbon source were selected.

Also the bacterial colonies, developed on agar containing engine oil, were randomly picked up and purified by sub-culturing on fresh agar plates. Isolated colonies were transferred to the nutrient agar slants, properly labeled and stored as stock cultures. Three hydrocarbon-degrading bacteria were characterized to genus level; based on the morphology and pigmentation of the colony, gram staining and biochemical tests; and identified according to the report of Persian Type Culture Collection (PTCC).

The isolated strains were selected based on their ability to grow in a medium containing crude oil, individual hydrocarbon compounds (o-xylene, eicosane, dodecane and fluorene) or both. Octacosane ($C_{28}H_{58}$) was used as an index for biodegradation of car engine oil. For this purpose, mineral salt medium was dispensed in 100 mL quantities, into 500 mL flasks; and 1 mL of 50mg/L index hydrocarbon (prepared in hexane) was added to each flask. After sterilization, the bacterial isolates and microbial mixed cultures were separately inoculated into the flasks, at 30 °C, while one flask was considered as the blank. After 100 hrs of incubation, the chloroform-methanol (3:1) extraction was applied. Thirty mL chloroform-methanol was added to 100 mL of the cultures, to extract the residual oil. The extracted samples were used for GC analysis and measurement of the waste oil degradation rate. All the measurements were performed in triplicate (Koma *et al.*, 2001).In order to obtain standard inocula, the individual bacteria were grown for 18 hrs on Tryptone Soy Broth at 37 °C, on an orbital shaker at 150 rpm. The cells were then harvested by centrifugation and rinsed 3 times in sterile saline medium. After inoculation, the measured absorbance of the medium at 540 nm should be equal to 0.5 (Ghazali *et al.*, 2004). Mesocosm experiments were conducted in polyethylene containers with diameter of 12.5 cm and height of 15.5 cm. After removal of plant residues and soil aeration, 1.2 kg of soil was put in each mesocosm. The artificially contaminated soil was prepared in the ratio of 1.5 mg used engine oil/g soil dry mass. One hundred ml of the prepared standard inocula was sprayed on the soil and well mixed. The mesocosms were incubated in the dark, under aerobic conditions, for 60 days. They were homogenized twice a month and the samples were collected from the surface layer of the soil (Coulon *et al.*, 2005). Seventy gram of each soil sample was dried at 60 °C for 12 h. and Soxhlet-extracted with 100 mL CCl_4 for 24 h. The solvents were evaporated for 24 h. and each fraction was weighed (Del Arco and de Franc, 2001). The residue was re-suspended in hexane and filtered through a Whatman GF/A filter. Asphaltenes (hexane-insoluble) were retained on the filter and weighed. The hexane-soluble compounds (maltenes) were fractionated into saturated, aromatic and resin compounds, by liquid-solid chromatography column. The column (1.5 cm × 15 cm) was loaded by 100-200 mesh activated silica-gel and eluted by 60 mL hexane, 60 mL toluene and 45 mL methanol for separating the saturated, aromatic and resin compounds, respectively (Oudot, 1984; Chaineau *et al.*, 2005). The solvents were evaporated for 48 hrs. Each fraction was weighed and the saturated fraction was analyzed by GC. The analyses were done by GC-PU 4410 Phillips (Analytical Laboratory, Faculty of Engineering, TMU), fitted with a flame ionization detector and SPB1 dimethyl polysiloxane capillary column (30 mm × 0.32 mm) (Supelco). The injector and detector were maintained at 290 °C, the oven temperature was programmed to rise from 100 °C to 280 °C at a rate of 8 °C increment/min and held at 280 °C for 15 min. The initial holding time at 100 °C was 5 min. Hydrogen (H_2), compressed air and He were kept at pressures of 60, 90 and 80 lb/in^2, respectively (Ghazali *et al.*, 2004). This study was conducted at room temperature and monitored on days 0, 15, 30, 45, and 60, after incubation. For monitoring the cell number, the biodegradation rate and pH, 10 g of the soil was removed from each container at the same time and re-suspended in 10 mL of sterile distilled water. The mixture was vigorously shaken on a vortex mixer for 5 min. Then, the soil particulates were allowed to settle for 1 min before sampling. About 0.1 mL samples were taken for CFU (Colony Forming Unit) counts, by the most probable number (MPN) method (Atlas and Bartha, 1972) and pH measurement

Table 2. The results of the isolated pure microorganisms and mixed cultures, characterized using various carbon sources

Isolate identification	Substrate					
	Crude oil	Used-engine oil	Oxylene	Eicosane	Dodecane	Fluorene
Pseudomonas sp.	+	+	+	+	+	-
Arthrobacter sp. (I)	+	+	+	-	-	+
Mycobactrium sp.	+	+	+	+	+	-
Arthrobacter sp. (II)	+	+	+	+	-	-
Mixed culture #1	+	+	+	+	+	-
Mixed culture #2	+	+	+	+	+	-
Mixed culture #3	+	+	+	+	+	+
Mixed culture #4	+	+	+	+	+	+
Mixed culture #5	+	+	+	+	+	-

(Okerentugba and Ezeronye, 2003). Ten gram of soil was removed from each container and put in 100 °C oven for 24 hrs, weighed again and its humidity was determined.

RESULTS & DISCUSSION

The bacterial samples were isolated based on their capability to grow on the crude oil and/ or individual hydrocarbons (oxylene, eicosane, dodecane and fluorine) as their sole carbon source. Three bacterial isolates; *Pseudomonas sp*., *Arthrobacter sp*., *Mycobacterium sp*.; and five bacterial mixtures were isolated and used in this study (Table 2).

Table 2 shows that substrate specificity of *Arthrobacter sp*. is restricted for the aliphatic hydrocarbon compounds, while *Pseudomonas sp*. and *Mycobactrium sp*. strains can grow on more substrates of examined hydrocarbons, in this study.

The isolates were examined for their ability to degrade octacosane ($C_{28}H_{58}$) and the results are shown in Table 3. The remaining hydrocarbon was extracted by the chloroform-methanol extraction method and then the degradation rate was determined from the peak area of GC analysis. The isolates were examined for their ability to degrade octacosane. Mixed cultures #1 and #2 contain three different bacterial strains; mixed cultures #3 and #4 contain two bacterial strains and one fungus; and the mixed culture #5 contains two species of *Pseudomonas sp*. strain. It is important to note that the degradation capacity of any

Table 3. The biodegradation rate of octacosane by different isolates

Isolated microorganisms	% Octacosane biodegradation
Pseudomonas sp.	51%
Arthrobacter sp. (I)	34.3%
Mycobacterium sp.	45%
Arthrobacter sp. (II)	42.8%
Mixed culture #1	72%
Mixed culture #2	71%
Mixed culture #3	68%
Mixed culture #4	65%
Mixed culture #5	63%

microbial mixed culture is not necessarily the result of merely adding the capacities of the individual strains. The advantages of mixed cultures in bioremediation have also been widely demonstrated (Surzhko *et al.*,1995; Syoko *et al.*, 1996; Koma *et al.*, 2001; Ijaha and Antaib, 2003). It could be attributed to the effects of synergistic interactions of the different microorganisms. Some species are able to remove the toxic metabolites, which hinder the activities of the other species. Then, it is possible that the other species degrade complex compounds totally (Ghazali *et al.*, 2004).

The inoculated used engine oil contaminated soil samples were containing 7.0×10^7 cfu/g of microorganisms, at the first day. The control soil sample did not have any microbial culture, to compare the effects of microorganisms. However, the microbial number decreased from the time of inoculation up to 20 days. The cell counting remained lower than the amount of inocula at 20 days sampling time. For pure

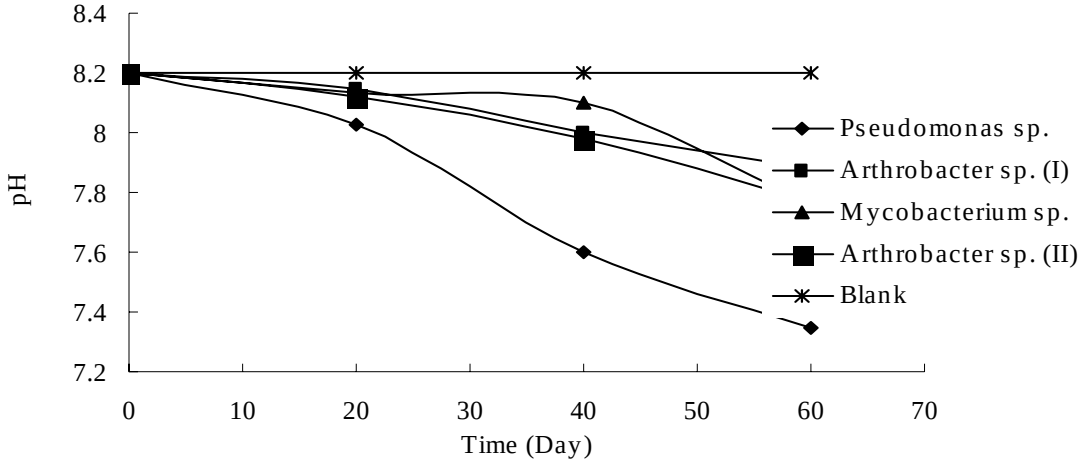

Fig. 1. Variation of pH, during 60 days biodegradation by the isolated bacterial species

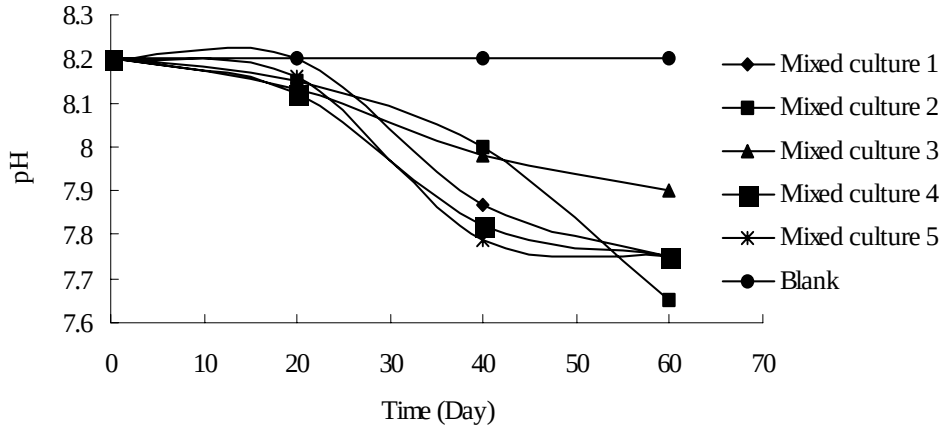

Fig. 2. Variation of pH during 60 days biodegradation by the mixed consortiums

cultures, the CFU counts decreased to 10^7 and in mixed cultures were about 3×10^7 after 20 days. It increased after 60 days and remained constant at 10^8.

The results of pH monitoring are shown in Figs. 1 and 2. Nutrient addition increased the pH of the samples from 7.0 to 8.2, in the first day. This pH increment can be attributed to the addition of inorganic salts to the soil. It is possible that after prolonged time of soil contamination, the ideal ratios of inorganic to organic essential nutrients, for microbial activities have been changed. Thomas *et al*. (1992) suggested that the ratio of C:N:P should be maintained at 120:10:1 to promote microbial growth and activity. In the soil samples used in this study, the ratio of total petroleum hydrocarbons to nitrogen was 105:1. The amount of nitrogen in the soil was at least 10 times less than the levels that Thomas *et al*. (1992) recommended for microbial growth. The pH values remained unchanged in the blank flask during incubation period. In this study, pH of all samples was measured every 20 days, and a continuous reduction of pH was seen in the results. The pH of the soil samples decreased to 7.4 in flask, during 60 days incubation with *pseudomonas sp*.

In this study, the oil engine was put at room temperature for 15 days to consider any losses due to the evaporation of the lightest compounds (naphtha). The results of the weighed residues are shown in Figs. 3-8. The maximum loss of the engine oil was observed in the soil treated

by the mixed culture #1. During 60 days of experiments, 70 % of the engine oil was degraded by the mixed culture #1. Aliphatic compounds are degraded as linear aliphatic compounds > branched aliphatic compounds > cyclic aliphatic compounds, respectively (Sorkhoh *et al.*, 1995). According to this sequence, 66 % of aliphatic compounds were degraded by mixed culture #1.

Aromatic compounds of the samples were extracted by toluene and weighed after toluene evaporation. The experiments were repeated 3 times and the results are shown in Figs. 7 and 8. The most significant reduction in the aromatic compounds was seen in the soil seeded by the mixed culture #5.

The degradation rate of the aromatic compounds was less than that of n-aliphatic, and depends on the number of benzene rings in their structure. About 47 % of aromatic compounds were effectively removed by mixed culture #5. The biodegradation rate of some desired compounds were measured by HPLC, and the results showed that the biodegradation of aromatic compounds increased after 3 weeks (Data has not shown).

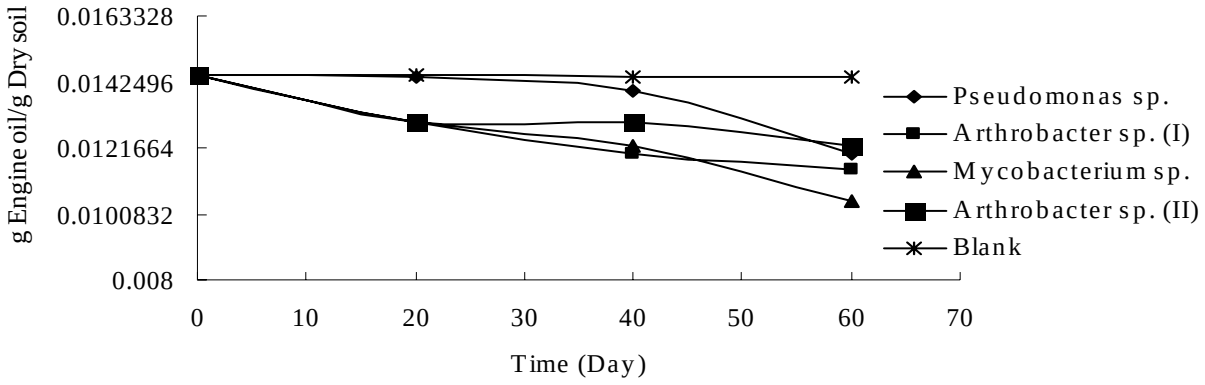

Fig. 3. Gravimetric experiments of engine oil treated by the isolated bacterial species during 60 days

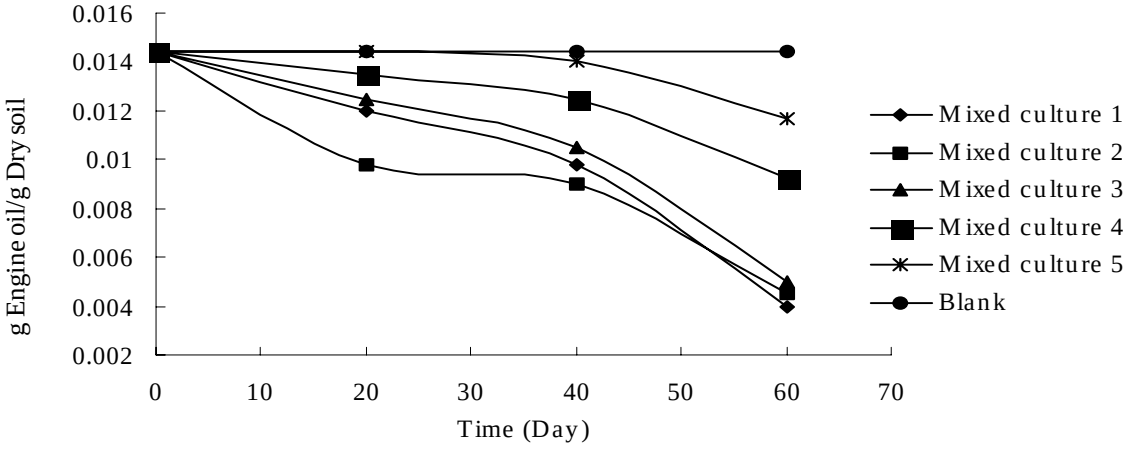

Fig. 4. Gravimetric experiments of engine oil treated by the mixed consortiums during 60 days

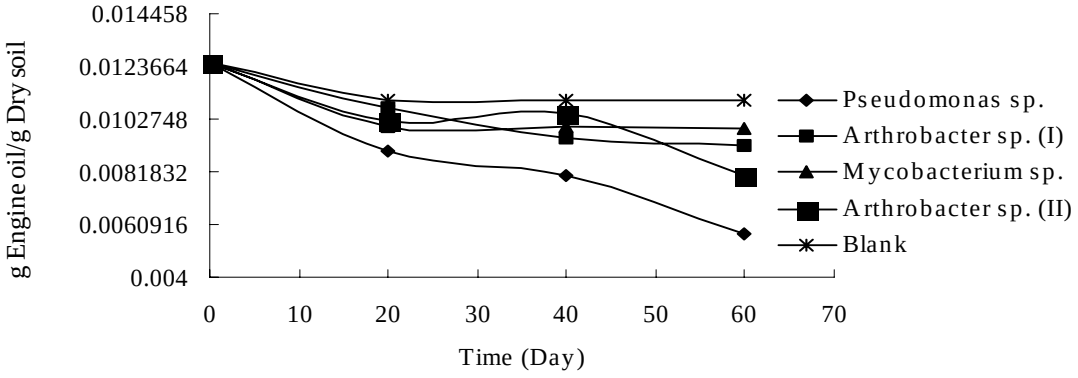

Fig. 5. Gravimetric experiments of aliphatic engine oil compounds treated by the isolated bacterial species during 60 days

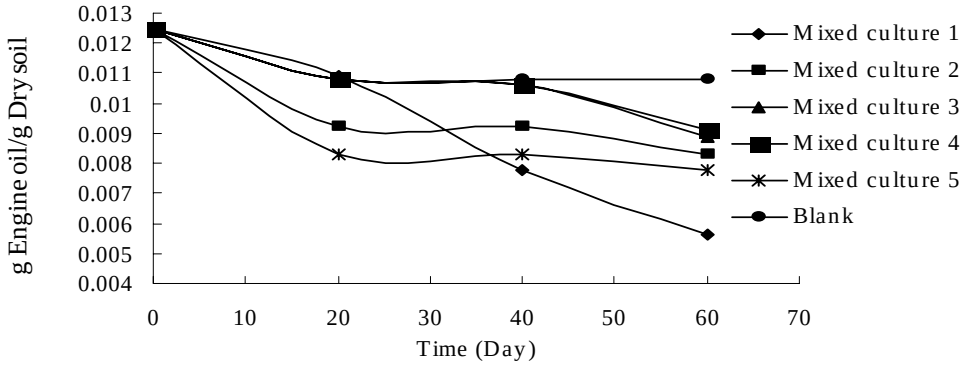

Fig. 6. Gravimetric experiments of aliphatic engine oil compounds treated by the mixed consortiums during 60 days

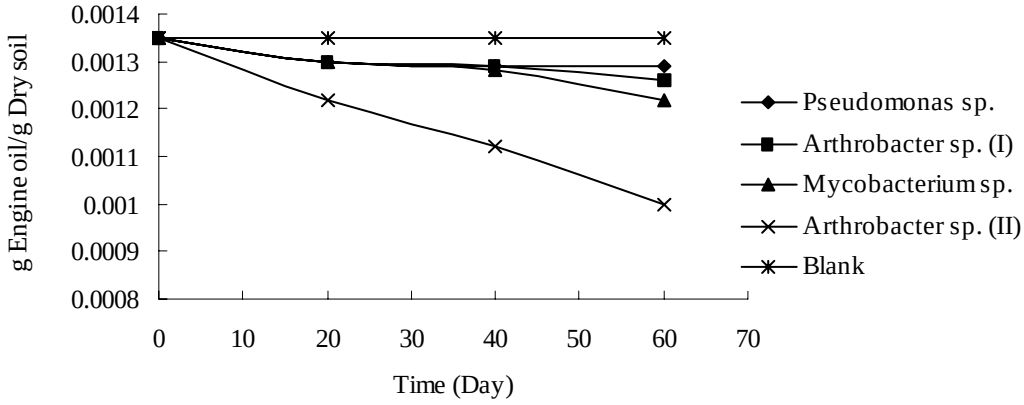

Fig. 7. Gravimetric experiments of aromatic engine oil compounds treated by the isolated bacterial species during 60 days

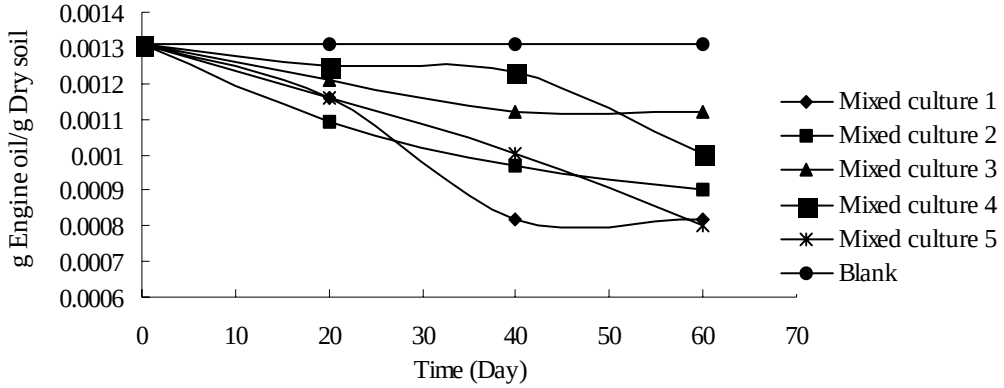

Fig. 8. Gravimetric experiments of aromatic engine oil compounds treated by the mixed consortiums during 60 days

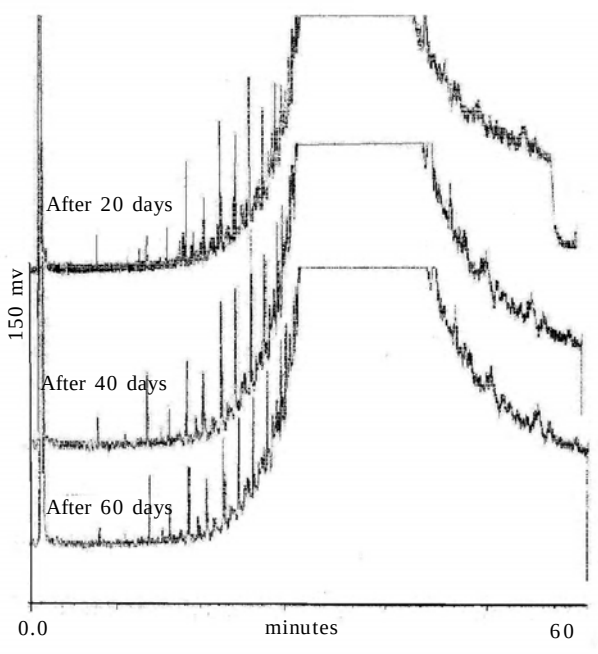

Fig. 9. GC chromatogram for samples extracted from contaminated soil before biodegradation

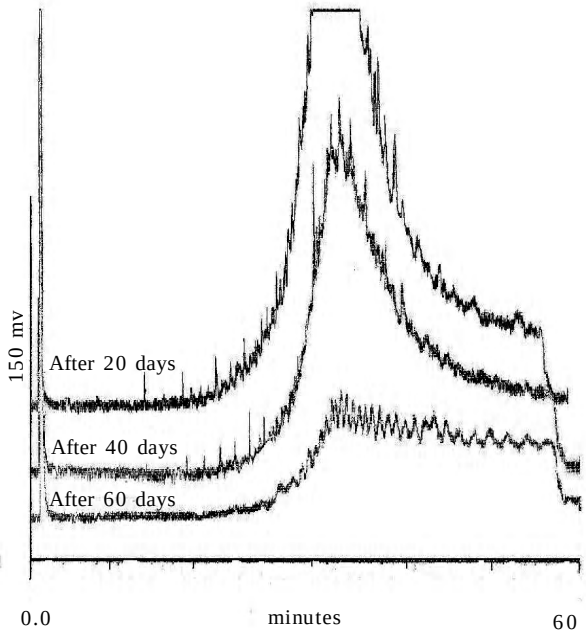

Fig. 10. GC chromatogram for samples extracted from contaminated soil treated by the mixed culture #1

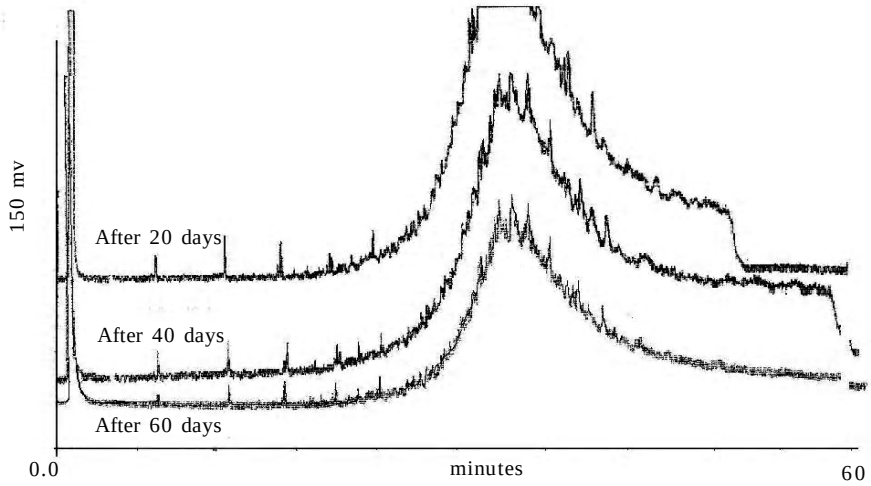

Fig. 11. GC chromatogram of sample extracted from contaminated soil treated by pure *pseudomonas sp*.

Asphaltenes, consisting of phenols, carboxylic acids, ketones, esters, and porphyrins, decreased during early log phase; increased during late log phase and early stationary phase; and decreased during the late log phase.

Resins, comprising pyridines, quinolenes, carbazoles, sulfoxides, and amides, decreased after 1 and 2 weeks and increased steadily after the second week. Resins and asphaltenes are the most likely products of weathering and biodegradation (Atlas and Bartha, 1972; Chaineau *et al.*, 2005). Asphaltenes and resins are complex

compounds with hard biodegradation.The GC analysis of the hydrocarbon extracted from the soil, at the beginning of the study, is shown in Fig. 9. It shows the blank samples at days 20, 40 and 60. Also, comparisons between the hydrocarbon compositions of soil after 20, 40 and 60 days are shown in Figs. 10 and 11 (as some examples) to show the degradation pattern of the compounds treated by different cultures. Addition of the microbial mixed and pure cultures resulted in enhanced degradation of the middle- and long-chain aliphatic compounds in the soil, compared to the soil that was not supplemented by any microbial culture. The peaks show aliphatic compounds of used car engine oil.

The peak reduction is a strong reason for degradation, as it shows a significant reduction in aliphatic compounds under equal condition. The most significant reduction is related to the soil seeded by the mixed culture #1 (Fig. 10), which could degrade 66% of aliphatic compounds during 60 days. More reduction of the hydrocarbons was seen with *Pseudomonas sp.* (Fig. 11), among 4 pure cultures that were investigated. It is obvious that mixed cultures are more effective. Also, the biodegradation of short- and middle-chain aliphatic compounds were more extensive, compared to the long-chain hydrocarbons. Analysis repeated 3 times for each sample, under equal condition.

CONCLUSION

Biodegradation of multi-component used engine oil was studied by both pure and mixed cultures. In this study, the biodegradation of the used engine oil by four different strains and five mixed cultures was evaluated. The results showed that the mixed cultures are more effective in the biodegradation. However, pure *Pseudomonas sp.* culture could degrade high percent of aliphatic compounds. Among the different investigated mixed cultures, the mixed culture #1 degraded 66 % of aliphatic compounds and the mixed culture # 5 degraded 47 % of aromatic compounds in 60 days.

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