

Short Communication

## Characterization of Bacteria Degrading Petroleum Derivatives Isolated from Contaminated Soil and Water

M. Mashreghi<sup>1,\*</sup> and K. Marialigeti<sup>2</sup>

<sup>1</sup> Department of Biology, Faculty of Sciences, Ferdwosi University of Mashhad, Mashhad, Islamic Republic of Iran

<sup>2</sup> Department of Microbiology, Faculty of Science, Eotvos Lorand University, Budapest, Hungary

### Abstract

Due to widespread use of petroleum products, the number of petroleum-contaminated sites has abounded. Natural attenuation, which relies on in situ biodegradation of pollutants, has received a large amount of attention, especially for petroleum contamination. Therefore in this research two types of samples, natural and enriched, from two different sources, soil and water were chosen and oil degrading microorganisms were isolated using different gasoline containing mineral media supplemented with yeast extract and or glucose. Fifty-five isolated strains were selected according to their simultaneous good growth on mineral medium with oil and nutrient agar. Several micromorphological and biochemical characteristics of isolated oil-degrading strains were determined. 73% of them were gram negative, 42.5% oxidase negative, 40.7% catalase positive, 59% showed oxidative glucose metabolism. The dominant portion of the strains could possibly belong to the family Pseudomonadaceae, while an other dominant group was members of the Coryneform taxa. By checking the biodegradative ability of our selected oil-degrading strains on individual hydrocarbon derivatives we showed that 10% of our strains could decompose n-dodecane easily and very fast. The utilization of 1-dodecene, naphtalene, benzol, and cyclohexane, respectively seems to be characteristic for always less and less strains. The speed of growth of 1-dodecene was lower than other compounds.

**Keywords:** Oil-degrading bacteria; Petroleum derivatives; Enrichment culture; Isolation and purification

### Introduction

Oil pollution has been one of the most important environmental problems in the last 60 years. Explosion of oil tanks by rockets or other war equipments, using

old tankers for oil transport, damaging of oil pipes and other catastrophic events could together lead to disastrous oil distribution into natural environment [3]. This inevitably has hazardous effects on human life and safety. Many studies have been planed to either prevent

\* E-mail: mmashreghi@yahoo.co.uk

the contamination or clean up the polluted sites after the contamination [5,6,9,12]. Physical, chemical and biological methods can be used for cleaning up the polluted sites. It also shows that microorganisms have broad range of enzymes that enable them to degrade many chemicals [4,7,14]. The application of microorganisms in oil biodegradation has been shown at different environmental conditions [8,15]. Mishra [10] used a bacterial consortium amended with nutrient for in situ bioremediation of oil polluted soil in a refinery plant. Although petroleum persist evaporation and could remain within environment for long period due to presence of aromatic derivatives in its composition, changes in microbial population within the polluted site would speed up the process of degradation of these recalcitrant compounds [1]. This study focused on isolation of degrading bacteria using enrichment method and further characterization of these isolates in order to get better knowledge of using indigenous microflora for in situ bioremediation of contaminated soil and water.

## Materials and Methods

### Sampling

Four top layer (0-10 cm) soil samples were collected aseptically from oil polluted sites in different regions of Budapest, including a petrol station, suburban railway and rail station. Because of huge amounts of hydrocarbon wastes in sewage, water sampling was focused on sewage and sewage sludge. Therefore wastewater has been taken from a treatment plant and from the Danube, at the entrance of a sewer. Also a soil microcosm containing 1000 g of good compost soil has been filled into 21 beakers, wetted with tap water to approximately Potential Factor 2 (PF<sub>2</sub>) water potential, and to each beaker 5 g of gasoline has been added and mixed till even distribution. Then the soil microcosm, were weighed, covered by aluminum foil and incubated for one month at 10-12°C in refrigerator, or and at room temperature (22-25°C).

### Isolation of Degrading Bacteria

Environmental samples were either plated for isolates after being diluted, or used for enrichment cultures with gasoline. A mineral base medium (NH<sub>4</sub>Cl 0.5 g, NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 0.5 g, KH<sub>2</sub>PO<sub>4</sub> 0.5g, Mg SO<sub>4</sub>·7H<sub>2</sub>O 0.5 g, NaCl 4.0 g, Trace element solution 1 ml, Distilled water 1 L) has been chosen and supplement with different organic compound (medium A: Mineral medium + gasoline, medium B: Mineral medium + gasoline + yeast extract, medium C: Mineral medium + gasoline +

yeast extract + glucose). 1 g of each soil sample and 1ml of each water sample were inoculated in 5 parallels into the different media, and incubated for a week by continuous shaking with 250 rpm at 10-12°C and 28°C, respectively.

Soil and water samples, as well as samples from the soil enrichment cultures were serially diluted and plated. The parallel enrichment cultures were finally unified to give a composite sample. From the different composite samples 10 ml were taken, serially diluted and plated on different agar media. For the even distribution of gasoline in the agar media an emulsifying agent Teepool 410 was added in 1ml/l concentration. The seeded media were incubated at two different temperatures: 10°C and 28°C.

After one week incubation, colonies were counted and one full loop of each of randomly selected seemingly non-cross contaminated colonies was transferred to agar slants. Clearing of oil droplets around single colonies could be detected, the colony was definitely picked up and isolated. Where necessary, re-isolation was also made.

### Basic Tests for Identification

Several basic morphological and biochemical tests were performed in our investigation including: colony morphology, cell micromorphology, Gram reaction, Motility tests, oxidation and fermentation of Glucose, acid and gas production from glucose, oxidative activity and catalase test.

### Biodegrading Capability of Strains Tested with Individual Chemical Compounds

Five hydrocarbon compounds (n-Dodecan, 1-Dodecane, Cyclohexan, Benzol and Naphthalene) were selected as representative of each organic compound groups and to check whether the isolated strains are able to grow on hydrocarbon compounds, mineral base medium was used and supplemented with filter sterilized petroleum derivatives to give a final concentration of 10 g/l, excepting naphthalene, which was added in the form of powder directly to agar medium. Isolated strains were grown on slanted medium and then medium was incubated up to three weeks, examined regularly for growth.

## Results and Discussion

### Germ Count Estimations

Direct plating from different soil and water samples

resulted in colony counts which were not suitable for statistical evaluation. Most possibly source of the samples were affected by novel, or extremely concentrated hydrocarbon spills, or by other inhibitive environmental factors. Accordingly germ counts were low and plating was made from very diluted suspensions. As can be seen from the data tabulated (Table 1), in case of medium A, enrichment soil inoculation produced higher final germ counts than sewer samples. This seems to be connected with possibly higher initial germ counts of inocula in case of soil, as compared with sewage. On the other hand there are no significant differences in germ counts estimated on different plating media. Degradation capability of 55 isolated strains was also checked on nutrient Agar. Results showed that all isolated strains could grow on nutrient agar.

**Basic Morphological and Biochemical Tests**

The results of colony morphological characterization of selected strains are summarized at Table 2.

The biochemical results expressed in percentile values are also tabulated (Table 3). Micromorphological results showed that 49% of strains were bacillary shape, 24% were cocci, 18% of them were coccobacilli, while 9% showed the characteristics of pleomorphisms. Most of selected strains were gram negative, catalase positive with no fermentative capabilities. The results indicate that all enrichment and isolation methods favored the aerobic organisms. On the other hand the description of oil degrading genera also corroborate our results, since the dominant portion of genera enumerated were also oxidative. Results reveal the dominant presence of

Pseudomonads and coryneforms among our selected strains. Representatives of these groups are otherwise well known, characteristic oil degraders, often isolated all over the world [2,11]

**Biodegrading Ability of Isolated Strains**

According to Table 4, results of tests for utilization of individual hydrocarbon compounds indicated by direct observation of growth of isolates are summarized in two statements. First of all the order of utility of different compounds among the strains proved to be:

n-Dodecan>1-dodecen>Naphthalin>Benzol>Cyclohexan  
(11) (7) (6) (4) (4)

on the other hand, the speed of growth on (or adaptation) the medium gave the following order:

n-Deodecan>Cyclohexan>Benzol>Naphthalene>1-Dodecen  
(8) (4) (3) (1) (0)

Comparing our results with literature data [13,15], a high similarity can be established according to the biodegrading capability and growth characteristic of similar strains on the same compounds. The cyclohexane molecules are similarly stable and biodegradation resistant because of the presence of the benzene ring. On the other hand it is a bit surprising that naphthalene and 1-dodecene were utilized by basically equal number of strains. It is also of interest that in case of cyclohexan seemingly no adaptation could be detected.

**Table 1.** Oil degrading bacterial count values estimated per ml of enrichment culture volume

Sample	Medium A enrichment				Medium B enrichment			
	Medium A		Medium B		Medium B		Medium C	
	10°C	28°C	10°C	28°C	10°C	28°C	10°C	28°C
Sewage	1.1 × 10 <sup>6</sup>	2.6 × 10 <sup>6</sup>	2.8 × 10 <sup>6</sup>	8.5 × 10 <sup>7</sup>	1.7 × 10 <sup>8</sup>	3.6 × 10 <sup>8</sup>	3.2 × 10 <sup>8</sup>	5 × 10 <sup>9</sup>
Soil	4.7 × 10 <sup>7</sup>	3.7 × 10 <sup>8</sup>	5.3 × 10 <sup>8</sup>	1.2 × 10 <sup>9</sup>	8.3 × 10 <sup>8</sup>	3 × 10 <sup>8</sup>	2.2 × 10 <sup>8</sup>	3.2 × 10 <sup>9</sup>
Average	2.4 × 10 <sup>7</sup>	3.7 × 10 <sup>8</sup>	5.3 × 10 <sup>8</sup>	1.2 × 10 <sup>9</sup>	5 × 10 <sup>8</sup>	3.3 × 10 <sup>8</sup>	2.7 × 10 <sup>8</sup>	4.1 × 10 <sup>9</sup>

**Table 2.** Colony morphological characteristics of selected oil degrading strains

	Colony characteristics					
	Edge	Form	Elevation	Surface	Transparency	Color
Number of isolates	31 Cir.	29 entire	30 Convex	27 Smooth	11 Tp.	7 Whitish
	19 Irr.	4 curled	10 Raised	28 Rough	27 Op.	26 Yellowish
	4 Rhixoid	22 loate	14 Flat		17 Tl.	11 Brown
			1 Umbonate			1 Red

Cir= Circular, Irr = Irregular, Tp= Transparent, Tl = Translucent, Op = Opaque

**Table 3.** Percentile results of biochemical tests and micro-morphological characters\* of oil degrading selected strains

Test	Percent	
	Positive	Negative
Gram staining	27	73
Oxidase**	37	42.5
Catalase	96.3	3.7
Glucose Oxidation***	60	38.2
Glucose Fermentation***	1.8	96.4
Acid from Glucose	13	87
Gas from Glucose	1.8	98.2
Motility	38	62

\*49% of strains showed bacillary shape, 24% were cocci, 18% of them were coccobacilli and 9% showed the characteristics of pleomorphisms.

\*\* 20% of the strains gave doubtful results

\*\*\*1.8 % of the strain gave doubtful results

## Conclusion

In this studies different culture media and broad range of bacterial species (near to 200 colonies) were used. our results were complied with the findings of other studies in case of dominant biodegrading strains which were Pseudomonad. Slide of degradation and its speed reconfirmed the ease of degradation of non-cyclic compound compare to cyclic chemical compounds. Isolated strains could be used for in situ bioremediation of polluted sites as indigenous bacteria. Further studies on biochemical and genetic characteristics of these kinds of strains will also help environmental protection agencies for proper applications of such agents.

**Table 4.** Comparison of growth of 55 selected oil degrading bacteria on 5 representative individual chemical compounds as sole source of carbon

Growth Time	Chemical compounds									
	n-Dodecan		1-Dodecene		Cyclohexan		Naphthalin		Benzol	
	(+)	(++)	(+)	(++)	(+)	(++)	(+)	(++)	(+)	(++)
24 h	8 strains	1 strain	0	0	4 strains	0	2 strains	0	3 strains	0
48 h	9 strains	1 strain	6 strains	0	4 strains	0	2 strains	0	4 strains	0
1 week	10 strains	1 strain	7 strains	0	4 strains	0	6 strains	0	2 strains	2 strains
Total Growth	11 strains		7 strains		4 strains		6 strains		4 strains	

## References

- Abed R.M.M., Safi N.M.D., Kster J., Beer D.D., El-Nahhal Y., Rullkttter J., and Garcia-Pichel F. Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. *Appl. Environ Microbiol.*, **68**: 1674-1683 (2002).
- Atlas R.M. Microbial degradation of petroleum hydrocarbons: An environmental perspective. *Microbiol. Rev.*, **45**: 180-209 (1981).
- Boehm P.D., Page D.S., Gilfillan E.S., Bence A.E., Burns W.A., and Mankiewicz P.J. Study of the fates and effects of the Exxon Valdez oil spill on benthic sediments in two bays in Prince William Sound, Alaska. 1. Study design, chemistry, and source fingerprinting. *Environ. Sci. Tech.*, **32**: 567-5576 (1998).
- Chen W., Brühlmann F., Richins R.D., and Mulchandani A. Engineering of improved microbes and enzymes for bioremediation. *Curr. Opin. Biotech.*, **10**: 137-141 (1999).
- Head I.M. and Swannell R.P.J. Bioremediation of petroleum hydrocarbon contaminants in marine habitats. *Ibid.*, **10**: 234-239 (1999).
- Jones W.R. Practical applications of marine bioremediation. *Ibid.*, **9**: 300-304 (1998).
- Kanaly R.A. and Harayama S. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.*, **182**: 2059-2067 (2000).
- Margesin R. and Schinner F. Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. *Appl. Environ. Microbiol.*, **67**: 3127-3133 (2001).
- Margesin R. and Schinner F. Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in alpine soils. *Ibid.*, **63**: 2660-2664 (1997).
- Mishra S., Jyot J., Kuhad R.C., and Banwari La. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Ibid.*, **67**: 1675-1681 (2001).
- Prince R.C. Petroleum spill bioremediation in marine environments. *Crit. Rev. Microbiol.*, **19**: 217-242 (1993).
- Sayler G.S. and Ripp S. Field applications of genetically engineered microorganisms for bioremediation processes. *Curr. Opin. Biotech.*, **11**: 286-289 (2000).
- Sharon A., Churchill S.A., Harper J.P., and Churchill P.F. Isolation and characterization of a mycobacterium species capable of degrading three- and four-Ring aromatic and aliphatic hydrocarbons. *Appl. Envir. Microbiol.*, **65**: 549-552 (1999).
- Watanabe K. Microorganisms relevant to bioremediation. *Curr. Opin. Biotech.*, **12**(3): 237-241 (2001).
- Whyte L.G., Bourbonniere L., and Greer C.W. Biodegradation of petroleum hydrocarbons by psychrotrophic Pseudomonas strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways. *Appl. Envir. Microbiol.*, **63**: 3719-3723 (1997).