Advanced Journal of Microbiology Research Vol. 2005
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Utilization of petroleum hydrocarbons by *Pseudomonas* sp. and transformed *Escherichia coli*

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Accepted 10 August, 2004

Our previous studies showed that the biodegradation of petroleum oil by a *pseudomonas* isolated from a petroleum -contaminated soil was instable. In this work, it is shown that when the isolates are immobilized on Perlite, they are more stable for oil degradation. Although the isolate did not have any chemotaxis to octadecane, dodecane and octane but utilize octadecane and dodecane better than octane and it did not utilized hexane, benzene, kerosene, pentane, heptane or thiophenol. The generation time for degradation of petroleum oil, dodecane and octadecane was 20, 22, and 25 h respectively. This phenotype was not transformed to *Pseudomonas* by conjugation even with lysozyme treatment, however the petroleum oil and octadecane utilization were transformed to *Escherichia coli* by lysozyme treatment. The transformed *E. coli* lost the ability to use octadecane after three subcultures on nutrient broth and 34 generations.

Keywords: Petroleum, transformation, chemotaxis, biodegradation, immobilization.

INTRODUCTION

Oil pollution from industrial sources and other activities are hazardous to terrestrial and marine ecosystems. Petroleum is a complex mixture of aliphatics, aromatics, resins and asphaltenes. Leahy and Colwell, (1990) have reported biodegradation of petroleum oil by *Achromobacter, Arthrobacter Acinetobacter, Alcaligenes, Bacillus, Flavobacterium, Nocardia, Pseudomonas* and *Rhodococcus.*

Biodegradation of oil by fungi *Rhodotorula*, *Sporobolomyces*, *Aspergillus* and *Penicillium* has also been studied (Head and Swannell, 1999). Previous studies showed that biodegradation of some oil derivatives depends on plasmids.. Plasmid pWWO, pTOM, pNAH, and pOCT are involved in degradation of benzene, toluene naphthalene and alkane (Pemberton, 1983). The degradation capacity of the engineered consortium was studied by Kapley et al. (1999).

So many factors affect degradation of oil, and these include concentration oil, temperature, salinity, pressure and water activity (Leahy and Calwell, 1990). Several studies report the adaptation of microbial communities to hydrocarbons that increases the rates of transformation of hydrocarbons associated with oil-contaminated environment (Korda et al., 1997; Kapley et al., 1999; Del Arco and De Franca, 2001; Barathi and Vasudevan, 2001).

Bioremediation approaches such as seeding with hydrocarbon - utilizing microorganism and fertilization with nutrient may be technically feasible for the coasts but obviously not for the open sea. The major technical problem with the open sea is that any seeded microorganisms would be washed out and diluted. A feasible solution to this problem might thus be seeding with immobilized microorganisms (Radwan et al., 2002). In the previous work, it is shown that pseudomonas has positive chemotaxis to naphthalene and growth on naphthalene or salicylate induced the chemotactic response (Grimm and Harwood, 1997). Several authors have also studied the bacterial chematoxis toward environmental pollutants (Pandey and Pakesh, (2002); Parales et al. (2000); Ortega et al. (2003) studied the

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bacterial chemotaxis toward environmental pollutants. In this work, it is shown that the isolate dose not have any chemotaxis to petroleum oil rather it is stable for oil degradation when it was immobilized.

MATERIAL AND METHODS

The *Pseudomonas* was isolated from contaminated soil. The isolation was carried out in a minimal medium containing NH4Cl (2.5 gl^{-1}), KH2PO4 (5.4 gl^{-1}), Na2HPO4 (4.7 gl^{-1}), MnSO4 (0.20 gl^{-1}), NaCl (0.10 gl^{-1}) and petroleum oil (2 ml) at pH 7. Identification was carried out following Bergey's manual.

The utilization of crude oil by *pseudomonas* was determined by gas chromatography as described by Geerdink et al. (1996).

Detection of spontaneous loss of oil degradation ability

The under test organism was grown in liquid petroleum medium to late exponential phase (starter culture). This culture (0.1 or 1.0 ml) was used to inoculate 100 ml of nutrient broth + Tween80 (Culture A). This was incubated at 27°C in a way to make the colonies visible, these colonies were then counted and 100 colonies were picked off and patched out firstly onto petroleum oil agar and then onto nutrient agar (NA) The plates were examined after incubation at 27°C, and any strain which grew on the NA but failed to grow on the oil agar were counted, sub- cultured on NA and petroleum mineral salt agar and then tested in liquid medium to confirm that they were non degraders.

Reversion experiment

Oil variants (which cannot use oil) of bacterial, strains which were formerly able to degrade petroleum, were tested for reversion to oil phenotype (able to use petroleum) by serially sub-culturing in NB + Tween 80. Before each subculture event, a sample of culture was centrifuged, washed in sterile water, and then diluted and used to inoculate petroleum oil mineral salts agar (neat samples and low dilutions, 10-1 and 10-2 were used). NA was also inoculated so the number of bacteria in the culture could be determined. After incubation (at 27°C) for at least 14 days, the petroleum mineral salts plates were examined for any colonies showing good growth. These possible revertants were sub-cultured onto oil mineral salts agar and NA and then into liquid media to test for petroleum degradation and confirm reversion.

Chemotaxis

Chemotaxis was tested with drop assay (Grimm and Harwood, 1997). For this assay, 40 ml of cells were harvested in the logarithmic phase of growth and resuspended in 12 ml of chemotaxis buffer (100 mM potassium phosphate [pH 7.0], 20 mM EDTA). A small amount of a test attractant was added to the center of a dish (10 ml). A chemotactic response of cells to the added compound resulted in the formation of a ring of turbidity near the center of the petri dish.

Conjugation

Twelve different pseudomonas sp. with white colony and one *Escherichia coli* were grown on NB to the logarithmic phase of growth. 1 ml of each culture was mixed with 1 ml of oil degrader (pink colony) with lysozyme (400 mg/ml) SDS (0.1 %) and EDTA (2 mg/ml) treatments. The transformed white colonies were isolated on petroleum oil agar.

Hydrocarbon degradation

The basal mineral medium was sterilized by autoclaving at 121°C for 15 min. The model petroleum mixture used for biodegradation was a mixture of hydrocarbons hexane, heptane, pentane, octane, dodecane and octadecane (1.5 ml). The model petroleum mixture was added to the sterile mineral medium at 500- ppm concentration of prepared mix. The medium was inoculated to a final optical density of 0.1 at 620 nm. In various intervals the residual model petroleum was extracted with the use of n-hexane (1:1 v/v) and hydrocarbon degradation was monitored on a capillary gas chromatograph (Pepkin Elmer, Auto system equipped with 1022 GC plus). 0.4 µl of the extracted sample was injected into a 25 ml CP-Sil-8Ch column with Flame Ionization Detector. The carrier gas used was hydrogen at 4.5 psi. The injector and detector ports were maintained at 300°C. Oven temperature was programmed from 100°C to 130°C with a gradient of 30°C min^{-1}, then raised to 270°C at the rate of 10°C min^{-1}, and finally held at this temperature for 5 min. All treatments were in triplicates (Kapley et al., 1999).

Immobilization of the isolates

The strain was grown on oil broth for 3 days. The Perlite was sterilized by autoclaving. 100 ml of culture were mixed and dried with 50 g of Perlite. The number of bacteria per gram of dried Perlite was obtained.

RESULTS AND DISCUSSION

In our previous work (Emtiazi and Mirdamadian, 2003), a pure isolate was obtained that grew on petroleum oil as a sole source of carbon and energy. The isolate was motile, gram-negative rod, catalase negative, oxidase positive and oxidative. Although the catalase was negative, this isolates wasidentified as Pseudomonas. The isolate grew well on 2 ml petroleum oil and mineral salts. The isolate could use toluene and catechol but not naphthalene and aniline. To understand the biodegradation of different compound in petroleum oil, different alkanes wereused as chemotaxis and growth substances.

Although the isolate have positive chemotaxis to glucose, peptone and catechol, the chemotaxis to octadecane, petroleum oil, dodecane, hexane, cyclohexane and benzene were negative. Recent results with the biodegradation of carbon tetrachloride and naphthalene have shown the potential of chemotaxis to enhance biodegradation in laboratory- scale microcosms. Indeed chemotaxis have already been studied in bacteria able to degrade a wide variety of organic pollutants such as naphthalene, BTEX (Benzene, Toluene, Ethylbenzene, and Xylene), and pesticides. Parales et al. (2001), showed toluene-degrading bacteria are chemotactic towards the environmental pollutants, benzene, toluene, and trichloroethylene. In addition, Harwood and Hawkins (2002), showed the chemotaxis of Ralstonia to herbicide. Bacterial chemotaxis toward environmental pollutants

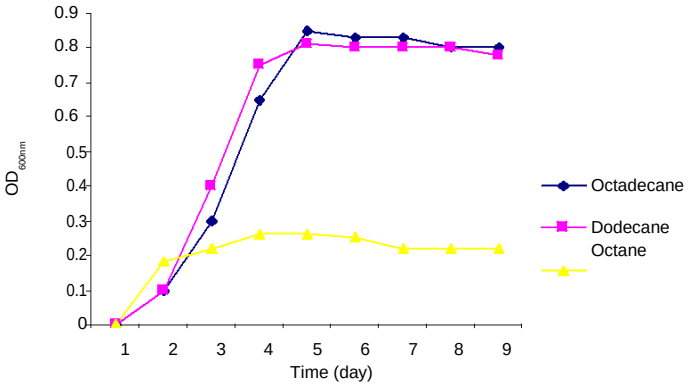

Figure 1. The growth rate of the the isolate on octane, dodecane and octane as the only source of carbon and energy at 30^{o}C

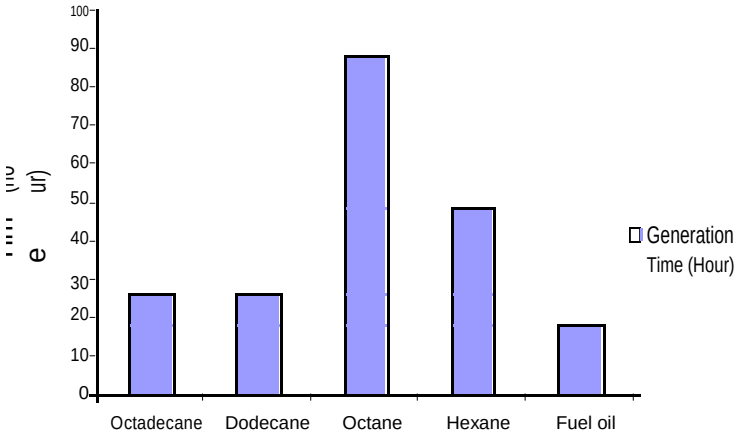

Figure 4. A comparison of the generation time for the isolate growth on different substrate.

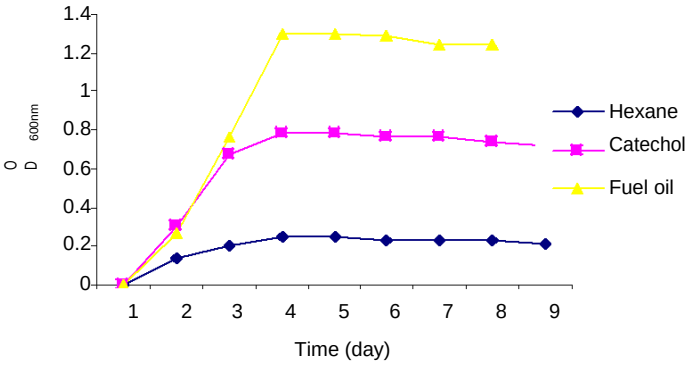

Figure 2. The growth rate of the isolate on hexane, catechol and fuel oil as the only sources of carbon and energy at 30^{o}C.

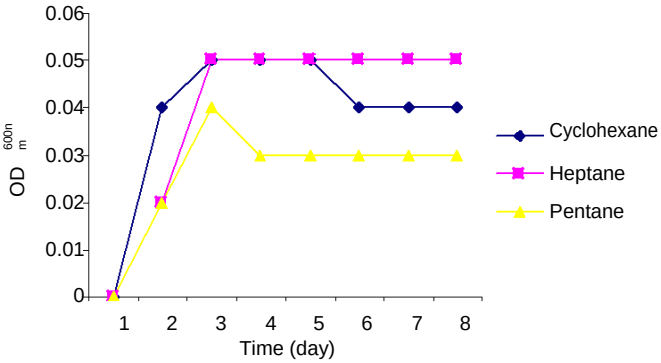

Figure 3. The growth rate of the isolate on cyclohexane, heptane and pentane as the only source of carbon and energy at 30^{o}C.

have important role in bioremediation. Although we did not have any chemotaxis to alkane, the isolate had good growth on octadecane and dodecane with maximum optical density of 0.8 in 5 days. However, maximum growth on octane had 0.28 OD600nm (Figure 1). This isolate did not have good growth on cyclohexane, toluene, thiophenol, heptane and pentane (Figures 2 and 3). The generation time of duplication on octane and dodecane was almost near to generation time on petroleum oil (Figure 4). However, the generation time on octane was more than 80 h. The biodegradation of petroleum oil was confirmed by GC profile. The data on GC also showed than the isolate used octadecane, dodecane and octane more rapidly (Figure 5a and 5b).We (Emtiazi and Mirdamadian, 2003) have showed that this isolate had two plasmid 45 kb and 60 kb, which are not stable in nutrient broth. In this work, it was shown that the phenotype of biodegradation do not transfer to other *Pseudomonas* sp. However, this phenotype transferred to *E. coli*. The frequency of transformation was calculated by this formula:

Frequency= N*/N1

N*= the number of transformed cells
N1= the primary number of cells

Without treatment with SDS, penicillin or lysozyme, there was no transformation of the *E. coli.* The transformated *E. coli* could use petroleum oil and dodecane with maximum 0.3 optical density and 52 hours generation time (Figure 6). However, 76% of this phenotype was lost after 32 generations (Table 1).

The phenotype of oil degradation was not stable in the mutant or wild. To get stable strain, which could degrade oil, the strain was grown on nutrient broth, centrifuged and was mixed with Perlite and dried. Each gram of immobilized Perlite had 2x108 bacteria. This can be use

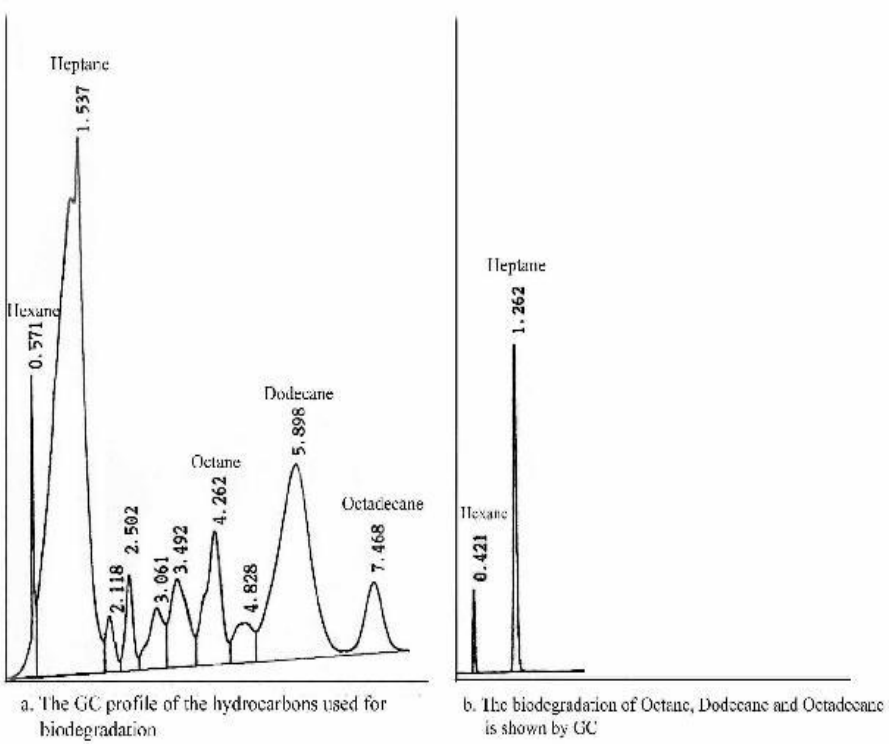

Figure 5. The profiles of GC, before (a) and after (b) biodgradation.

Table 1. The percentage of lost in oil degradation of transformant *E. coli**.

Parameter	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Subculture 5
Maximum OD_{600nm} on dodecane	0.25	0.18	0.06	0.033	0.02
Decreasing OD (%)	0	28	76	86.6	92
Total number of generations	10.08	21.34	32.34	41.34	54.62

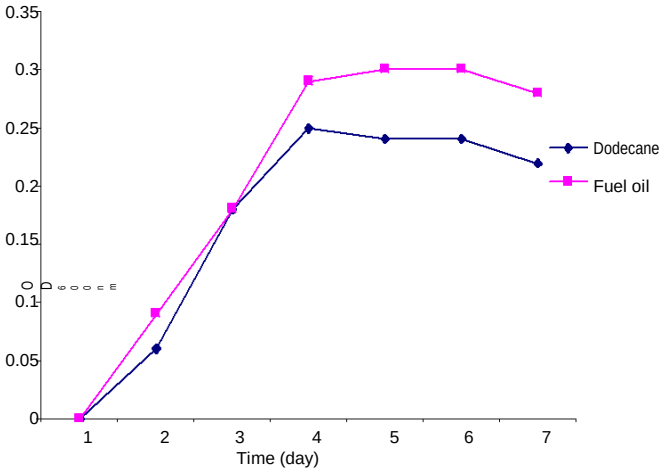

Figure 6. The growth rate of transformant *E. coli* on petroleum oil and dodecane at 30^{o}C.

as biofilm to degrade oil and this biofilm is stable for oil degradation for four months. The isolate can be immobilized on Perlite, Lignite, Cillis and Cilite. However, it was more stable on Perlite. Surface tension measurements indicated the production of biosurfactant during the microbial degradation of hydrocarbon. Barathi and Vasudevan (2001) also observed this phenomenon.

Here it was shown that when the immobilized cells on Perlite were used, biosurfactant is produced. Although the isolate did not have any growth on 5% salts, the immobilized cells could tolerate 5% salts. Kapley et al. (1999) used genetically engineered microbial consortium for degradation of hydrocarbons in marine environments. Radwan et al. (2002), indicated that macroalgae submerged in the seawaters, which coated with biofilms,

are rich in oil utilizing bacteria. These microorganisms were found immobilized in biofilm coating the thalli, which contribute to hydrocarbon attenuation in water. In this work, we showed that biofilm of oil degrader on Perlite could be use for soil contamination with oil.

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