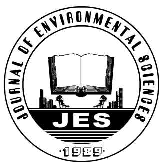

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Differential degradation of crude oil (Bonny Light) by four *Pseudomonas* strains

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Abstract

Four hydrocarbon degraders isolated from enriched oil- and asphalt-contaminated soils in Lagos, Nigeria, were tested for their petroleum degradation potentials. All the isolates were identified as species of *Pseudomonas*. *Pseudomonas putida* P11 demonstrated a strong ability to degrade kerosene, gasoline, diesel, engine oil and crude oil while *P. aeruginosa* BB3 exhibited fair degradative ability on crude oil, gasoline, engine oil, anthracene and pyrene but weak on kerosene, diesel and dibenzothiophene. *Pseudomonas putida* WL2 and *P. aeruginosa* MVL1 grew on crude oil and all its cuts tested with the latter possessing similar polycyclic aromatic potentials as P11. All the strains grew logarithmically with 1–2 orders of magnitude and with generation time ranging significantly between 3.07 and 8.55 d at 0.05 level of confidence. Strains WL2 and MVL1 utilized the oil substrate best with more than 70% in 6 d experimental period, whereas the same feat was achieved by P11 in 12 d period. BB3 on the other hand degraded only 46% within 6 d. Interestingly, data obtained from gas chromatographic analysis of oil recovered from the culture fluids of MVL1 confirmed near-disappearance of major peaks (including aliphatics and aromatics) in the hydrocarbon mixture.

Key words: biodegradation; crude oil; hydrocarbons; pollution; *Pseudomonas* strains

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Introduction

Nigeria is a major producer of petroleum. Petroleum production and operations produce serious ecological problems. Pollution of environment due to accidental oil spillage, seepage and ruptured pipelines has been well reported (Okerentugba and Ezeronye, 2003). This problem has been further compounded by sabotage and vandalisation of pipelines in restive communities, particularly in the Niger-Delta area of the country. Microbiological degradation is the major process that results in the decontamination of surface soils and sediment with little or no impact to the environment (Cerniglia, 1992).

Bacteria and fungi make the major contribution to mineralization of oil pollutants (Abed *et al*., 2001). Bacteria most commonly encountered are the Gram-negative of the alpha-proteobacteria group such as species of *Pseudomonas*, *Sphingomonas*, *Moraxella*, *Acinetobacter*, *Alcaligenes*, and *Proteus*. Also important are the low G+C Gram-positives such as *Bacillus*, *Micrococcus* and the high G+C Gram-positives, particularly the actinomycetes (Amund, 2000; Wackett and Hershberger, 2001; Parales *et al*., 2002). *Pseudomonas* species are often isolated from hydrocarbon contaminated sites and hydrocarbon-degrading cultures. Members of this genus have broad affinity for hydrocarbon and can degrade selected alkanes, alycyclics, thiophenes and aromatics (Vankateswaran *et al*., 1995; Allen *et al*., 1997). Polycyclic aromatic hydrocarbons (PAHs) are among the most recalcitrant components of crude oil (Kanaly and Harayama, 2000). Microbial degradation of crude oil often occur by attack on alkanes or light aromatic fractions, while the high molecular weight aromatics, resins and asphalthenes are considered recalcitrant (Lal and Khanna, 1996). Low molecular weight PAHs such as naphthalene and phenanthrene are degraded rapidly in sediments, whereas higher molecular weight PAHs such as benzo[a]anthracene, pyrene, chrysene, benzo[a]pyrene are quite resistant to microbial attack (Cerniglia, 1992). It is uncommon to find organisms which could degrade both aliphatics and aromatics effectively. However, such findings portend well for the future of bioremediation. The difficulty may not be unconnected with the difference in the pathways for biodegradation of the two classes of hydrocarbons. But long periods of exposure to mixture of hydrocarbon and preponderance of enabling intrinsic and extrinsic factors could lead to acquisition of such rare ability.

Bacterial species with broad substrate specificity for

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both alkanes and polycyclic aromatic hydrocarbons have been reported by Amund *et al*. (1987). It is fair guess, therefore, that given the ubiquitous nature of hydrocarbon degraders and the reckless pollution of the environment with oils and PAHs, organisms capable of degrading wide range of hydrocarbons could abound and only need to be sought out and properly identified and characterized. Here, we report the degradative potentials of four *Pseudomonas* species isolated on a mixture of polycyclic aromatic hydrocarbons on crude oil and common petroleum products including gasoline, diesel, engine oil, and kerosene.

1 Materials and methods

1.1 Sampling site

Soil samples were collected from four contaminated sites in Lagos, Nigeria, namely PWB (an asphalt polluted soil adjacent to Lagos State Ministry of Works Asphalt Production Plant Yard along Lagos-Ibadan Highway), PS (a crude oil polluted farmland), WL (a diesel tanker park at Wilmar, Apapa), and MVL (a mechanic workshop opposite Lagos State University, Ojo). The samples were collected at a depth of 10–12 cm with sterile trowel after clearing debris from the soil surface.

1.2 Enrichment and isolation of hydrocarbon degrading bacteria

Bacterial being able to degrade PAHs were isolated on PAH mineral medium (MM) by continual enrichment method. The medium described by Kästner *et al*. (1994) was used. After the pH was adjusted to 7.2 it was fortified with nystatin (50 mg/L) to suppress fungal growth. Trace elements solution (1 mL/L) described by Bauchop and Elsden (1960) was sterilized separately and added aseptically to the medium.

Contaminated soil sample (5 g) was added to 45 mL of MM containing mixture of phenanthrene, anthracene, and pyrene at 50 mg/L each. Enrichment was carried out with intermittent shaking at room temperature (27 ± 2°C) for 3 months in the dark until there was growth (turbidity). After 5 consecutive transfers at shorter intervals (4–5 weeks), hydrocarbon degraders were isolated by plating out the final flask on Luria-Bertani (LB) agar. Several colonies appeared were further purified by subculturing once on LB agar. Ability to degrade PAHs and other hydrocarbons was confirmed by inoculating LB broth-grown pure cultures (18 h) into fresh MM flask containing the PAH mixture as sole carbon sources. Colonies were then transferred individually on MM broth containing crude oil (Escravos blend) at 1% (*V/V*), incubated in the dark with shaking at room temperature for two weeks. The crude oil consumption was indicated by turbidity variation.

1.3 Maintenance and identification of isolates

Organisms were maintained in glycerol nutrient broth (1:1, *V/V*). Colonies growing on LB agar with significant low percentage of PAH (0.005%) were harvested with sterile inoculating loop, pooled and transferred to the medium. The mixture was shaken well to homogenize and kept at –20°C.

Isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to Cowan and Steel's Manual (Barrow and Feltham, 1995). This was complemented using the API 20E rapid test kit phenotypic typing.

Salt tolerance was tested in LB broth containing varying concentrations (1%–10%, *W/V*) of NaCl. Incubation was carried out at room temperature for two weeks with intermittent shaking.

1.4 Substrate specificity

Abilities of the isolates to grow on varieties of hydrocarbon substrates were evaluated in liquid media supplemented with 50 mg/L of respective solid hydrocarbons or 0.1% (*V/V*) in case of liquid substrates as a sole carbon and energy source. Degradation was monitored by cell increase with reference to uninoculated flasks coupled with disappearance of the oil slick in case of petroleum cuts. The hydrocarbons tested include pyrene, naphthalene, phenanthrene, anthracene, and biphenyl phenol, toluene, hexane, crude oil, diesel, petrol, engine oil and kerosene. Inocula were 24-h LB-grown cultures and inoculation was carried out to achieve initial optical density of 0.05 (OD_{500}). Incubation was performed at room temperature in the dark for 21 d.

1.5 Time course of isolate on crude oil

Replicate flasks containing 50 mL of MM with 0.5 mL (1%, *V/V*) of crude oil were prepared. Isolates were inoculated to achieve an initial concentration of the total viable count (TVC) of about 2.0×10^7 cfu/mL. Control flasks were inoculated with heat inactivated cells. Total viable counts were determined at 3 d interval by plating out appropriate dilutions of the cultures into nutrient agar. Residual oil also determined. The isolates were also grown in the same medium at different pH level (6.5, 7.0, 7.5) to determine the effect of pH on versatility.

Residual oil was extracted by adding 20 mL of hexane to broth culture in flask and shaking thoroughly. After removing the aqueous phase by separating funnel, the organic phase was evaporated to constant weight in a pre-weighed Petri dish. Residual oil was determined gravimetrically and expressed as percentage of values obtained for respective controls as previously described by Yveline *et al*. (1997).

1.6 Gas chromatographic analysis

The residual oil was extracted twice from the culture fluid with equal volume of *n*-hexane as described by Adebusoye *et al*. (2007). The hexane extract (1.0 μL) was subsequently analyzed with Hewlett Packard 5890 Series II gas chromatograph (USA) equipped with flame ionization detector (FID) and 30 m long HP-5 column (i.d., 0.25 mm; film thickness, 0.25 μm). The carrier gas was nitrogen. The injector and detector temperatures were maintained at 300 and 350°C, respectively. The column was programmed at an initial temperature of 70°C, held for 2 min, then ramped at 10°C/min to 320°C and held for

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10 min.

1.7 Statistical analysis

Data were analysed using Microsoft Excel Toolpak and GraphPad Prism 5.

2 Results

2.1 Isolation, identification and characterization of hydrocarbon degraders

Enrichment resulted in the isolation of several hydrocarbon degraders. The best degraders from each of the four-soil sample were used in this study. The four isolates were Gram-negative, motile rods, oxidase positive and failed to ferment any sugars (glucose, galactose, fructose, maltose, sucrose, mannose mannitol, inositol, adonitol, sorbitol, trehalose, rhamnose, cellobiose, and palatinose). P11, obtained from PS, had round smooth edges on the agar with a slightly raised colony which did not produce pigment in the medium. It was ornithine decarboxylase negative. It was *L*-arginine positive, urea negative, did not grow at 42°C, did not reduce nitrate to nitrite and was thus identified as *P. putida*. The colony of strain BB3, obtained from PWB, had irregular rough edges spreading on the line of inoculation and produced yellow pigment. It was ornithine decarboxylase negative, did not deaminate phenylalanine, *o*-nitrophyl-N-acetyl-β D-glucosamine negative. It grew at 42°C, thus identifying it as *P. aeruginosa*. WL2 and MVL1 could not be differentiated on the basis of biochemical tests, most of which they shared with BB3. For both WL2 and MVL1 produced yellow pigment and grew at 42°C, confirming their identity as *P. aeruginosa*. All four isolates grew well at NaCl concentration of 2.5% (*W/V*) in nutrient broth. P11 tolerated 10% NaCl, while the others only tolerated 9%. The favorable pH level for P11, BB3 and MVL1, and WL2 grew was 7, 7.5, and 6.5, respectively.

2.2 Growth on hydrocarbons

The substrate specificities of the isolates are shown in Table 1. P11 degraded crude oil and various components well; grew fairly on naphthalene, phenanthrene and biphenyl, but not on pyrene and anthracene. BB3 degraded petrol, engine oil and crude oil well, but kerosene and diesel only slightly. However, BB3 only grew slightly on all PAHs tested, except anthracene on which the growth was appreciable. WL2 and MVL1 grew on crude oil and all its cuts tested. Both also grew on anthracene and dibenzothiophene. Additionally MVLI grew on phenanthrene and pyrene.

Figure 1 shows the growth profiles of isolates on crude oil. None of the organisms displayed lag phases. Growth was generally slightly more than two-orders-of-magnitude especially for strain P11. This organism grew from an initial cell density of 2.8×10^7 cfu/mL to peak at 2.8×10^9 cfu/mL on day 15, and then dropped sharply. The other two strains demonstrated similar growth patterns since growth continued until the end of the experiment. The least growth was observed in flask inoculated with strain BB3. The kinetic data obtained from the growth curves are summarized in Table 2. MVL1 and WL2 utilized oil best with degradation rate of 4.95%/d and 4.64%/d, respectively. Interestingly, these two strains displayed the least growth rates (0.08–0.09). They degraded about 70% of the oil in 6 d, whereas the same feat was achieved by P11 in 12 d. BB3 on the other hand degraded only 46% within 12 d. By the end of the 28 d cultivation period, nearly 90% of the oil had been consumed by WL2 and MVL1 whereas amounts of oil recovered from flasks inoculated with other strains was in the range of 30%–42% of the initial concentration.

Table 1 Substrate susceptibility of isolates on various components of crude oil

Substrate	Isolate			
	P11	BB3	WL2	MVL1
Crude oil	+++	++	+++	++
Gasoline	++	++	++	++
Kerosene	++	+	++	++
Diesel	+++	+	+++	+++
Engine oil	+++	++	++	++
Hexane	–	–	–	–
Benzene	+	–	–	–
Naphthalene	+	+	–	–
Phenanthrene	++	+	–	–
Anthracene	–	++	++	++
Pyrene	–	+	–	++
Phenol	–	–	–	–
Dibenzothiophene	–	–	++	+
Biphenyl	++	+	–	–

+++: luxuriant growth; ++: very good growth; +: poor growth; –: no growth.
Cultures were incubated for 7–21 d.
All substrates were supplied at a concentration of 50 mg/L.

Qualitative changes in profiles of hydrocarbon compounds inherent in the recovered oil from culture fluids of strains P11 and MVL1 are shown in Fig. 2. The GC fingerprints obtained in heat inactivated cells (Fig. 2a) were similar to those recovered from uninoculated tubes (data not shown), suggesting that the reduction and disappearances obtained in Fig. 1 panels B and C were essentially due to biological action and not physicochemical losses. The data showed almost 100% depletion of all components in the MVL1. Significantly, the results validated those obtained from the gravimetric method described above. The fingerprints showed that all the components of the hydrocarbon mixture were consumed albeit to different extent. Other peaks of major importance that did not belong to the *n*-alkanes were also degraded although at slower rates than the former with the same retention

Table 2 Growth kinetics of bacterial strains on crude oil

Isolate	M (d^{-1})	T_g (d)	R^2	D_6 (%/d)	D_{18} (%/d)
P11	0.21	3.27	–0.898	4.4	3.9
BB3	0.23	3.07	–0.854	3.66	3.0
WL2	0.09	7.73	–0.754	12.52	4.64
MVLI	0.08	8.55	–0.909	10.97	4.95

M: specific growth rate; T_g: mean generation time; R^2: correlation between total viable count population and residual pyrene for respective isolates; D_6 and D_{18}: degradation rate during the first 6 d and 18 d of incubation, respectively.

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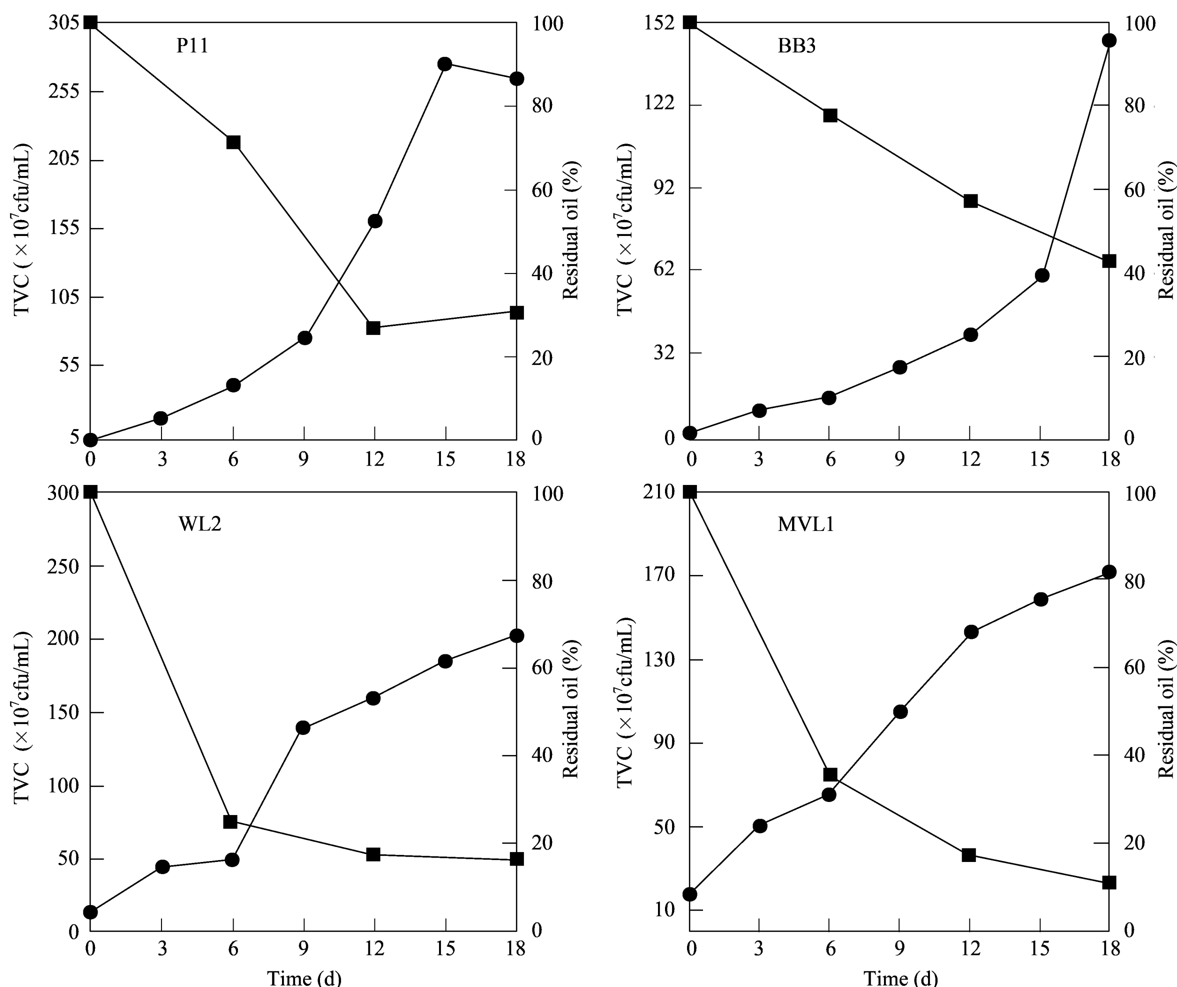

Fig. 1 Growth profiles of *Pseudomonas* species on 1% Bonnyl Light crude oil. Crude oil was not degraded in flasks inoculated with heat inactivated cells. Data values represent averages of three replicate determinations.

time. As depicted in Fig. 2, the degradation observed shows that the lighter part of the crude oil (C1–C13) was nearly completely utilized compared with the heavier part, although it was not completely eliminated.

3 Discussion

Biodegradation is one of the major means by which hydrocarbon pollutants can be removed from the environment (Lal and Khanna, 1996). A wide range of organisms are involved in this process, often acting as consortia. The polycyclic aromatic hydrocarbons, especially the high molecular weight PAHs, are regulated contaminants at sites polluted with crude oil (Kazunga and Atkins, 1999). It is often difficult to find organisms that will individually degrade all the fractions of crude oil (aliphatics, alicyclic, and aromatic). The four organisms isolated in this study which were identified as species of *Pseudomonas* possess the ability of grow on both aromatic and aliphatic fractions of petroleum. This is of great interest because previous findings have demonstrated broad substrate spectra of the genus not only on hydrocarbons but also on diverse range of xenobiotic compounds (Amund and Adebiyi, 1991; Wackett and Hershberger, 2001; Parales *et al.*, 2002). Similarly, Christopher and Christopher (2004) have demonstrated the importance of *Pseudomonas* species in the early stage of petroleum land treatment unit.

It is noteworthy that the four strains used in this study showed higher order of population increase within a shorter period than reported by Okerentugba and Ezeronye (2003) for Gram-positive strains from tropical waters. The ability of the four *Pseudomonas* isolates to degrade the various components of crude oil and re-emphasized the broad specificity of their oxygenases. The fact that P11, WL2, and MVL1 generally had a better degradative ability on the various oil cuts than BB3 (Table 1) might not be unconnected with the fact that these organisms, even though enriched on a mixture of PAHs, were actually isolated from a soil collected from crude oil-polluted site, while BB3 was isolated from asphalt-contaminated soil. It is doubtless that they must have acquired the ability to degrade crude oil components during a long period of exposure to the pollutants. This inference is further corroborated by the fact that strain P11 was mainly able to degrade the lower fractions of PAHs, as well as the limited ability of BB3 to degrade those lower fractions.

The data obtained from the growth experiment correlate well with the general trend on susceptibility tests as strain

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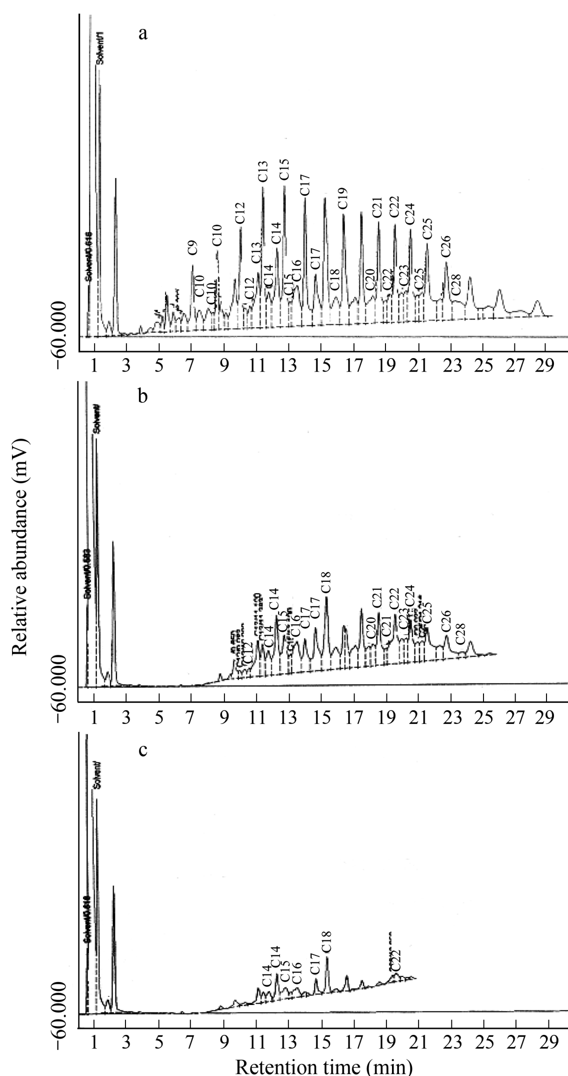

Fig. 2 Gas chromatographic fingerprints of Bonny Light crude oil recovered from flasks inoculated with heat inactivated cells (a), P11 (b), and MVL1 (c) after 18 d of incubation. The oil substrate was supplied at a concentration of 1% (*V/V*) and separated on 30 m long HP-5 column.

P11 had a general higher cell density than BB3. This was further confirmed by the fact that P11 grew rapidly without a lag phase within the first 6 d before declining, while BB3 showed a partial lag phase between day 0 and 2. The fact that P11 and BB3 had higher growth rates and shorter generation times (Table 2) and exhibited lower degradation rate may be due to the inability of these organisms to fully process the degraded component for biomass production. Another reason could also be that the organisms did more of mineralization than biomass production, concluded that only a fraction of carbon source used by degraders is actually converted to biomass in some organisms (Vila *et al*., 2001; Das and Mukherjee, 2007). GC fingerprints showed that peaks of major importance aliphatics and aromatics were degraded. The sharp peaks shown in Fig. 2 generally represent the aliphatics while the tiny short peaks in between them comprise mainly the naphthenes and aromatics having similar molecular weights to the adjacent aliphatics. The degradation showed that nearly total disappearance of the aliphatic and non-aliphatic peaks in flasks inoculated with strain MVL1, thus reinforcing the ability of this organism to utilize both aliphatics and aromatics as sole source of carbon and energy.

The ability of our isolates to thrive well when salt concentrations above 2.5% and tolerate up to 9% lead them as possible candidates for bioaugmentation. It has been shown that salinity of the inoculating medium could be crucial in the survival of allochtonous strains during bioremediation (Kastner *et al*., 1998). From the foregoing, it is fair enough judgment that these *Pseudomonas* isolates are potential candidates for bioremediation of hydrocarbon-polluted sites and certain fractions of PAHs. Thereby, bioremediation has become one of the major ways of reclaiming polluted environments. What needs to be done is to determine the environmental factor favourable for the application of the organisms for this and, of course, a study of genetics of the organisms as a leeway to their amenability to genetic engineering.

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