African Journal of Biotechnology Vol. 2 (9), pp. 288-292, September 2003
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2003 Academic Journals

Full Length Research Paper

Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria

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Accepted 29 July 2003

The ability of three bacterial isolates (*Bacillus spp*, *Micrococcus spp* and *Proteus spp.*) and some fungal species (*Penicillin spp.*, *Aspergillus spp*. and *Rhizopus spp.*) isolated from two rivers and refinery effluent to degrade two Nigerian Crude oils was studied. The results showed changes in pH, optical density and total viable count for the bacterial isolates after a 17-day period. There was an increase in biomass for the fungal isolates after a 35-day period. It was observed that these organisms were able to utilize and degrade the crude oil constituents, with bacterial isolates showing increase in cell number and optical density as pH decreases. Single cultures were observed to be better crude oil degraders than the mixed cultures (bacteria or fungi). It was also observed that oil degraders could be isolated from a non-oil polluted environment, although those from oil-polluted environments have higher degradation potentials.

Key words: Petroleum, refinery, biodegradation, plasmids, effluent.

INTRODUCTION

Crude oils are composed of mixtures of paraffin, alicylic and aromatic hydrocarbons. Microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon-degrading bacteria and bacterial plasmids encoding hydrocarbon catabolic genes (Leahy and Colwell, 1990). Adapted microbial communities have higher proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants. The measurement of biodegradation rates under favorable laboratory conditions using ^{14}C-labelled hexadecane has led to the estimation that as much as 0.5 – 60 g oil/m^3 seawater convert to carbon dioxide, depending on temperature and mineral nutrient conditions. The principal forces limiting the biodegradation of polluting petroleum in the sea are the resistant and toxic components of oil itself, low water temperatures, scarcity of mineral nutrients (especially nitrogen and phosphorous), the exhaustion of dissolved oxygen and in previously unpolluted areas, the scarcity of hydrocarbon-degrading microorganisms (Atlas, 1981). Low winter temperature can limit rates of hydrocarbon biodegradation increasing resident time of oil pollutant (Bodennec et al., 1987).

Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil. Although some studies have reported their removal at high rates under optimal conditions (Rotani et al., 1985; Shiaris, 1989), high molecular weight aromatics, resins and asphaltenes are generally considered to be recalcitrant or exhibit only low rates of biodegradation. In aquatic ecosystems, dispersion and emulsification of oil

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in slicks appear to be prerequisites for rapid biodegradation. Large masses of mousse, tar balls or high concentrations of oil in quiescent environments tend to persist because of the limited surface areas available for microbial activity. Petroleum and petroleum fractions containing asphalt components are not degraded quantitatively. The residues, along with polymerization products formed from free radical degradation intermediates with each other, forming tar globules. The tar is a partially oxygenated high molecular weight material resistant to further microbial degradation. Floating tar globules are encountered in the marine environment in increasing quantities (Butler et al., 1973).

An ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders of that environment. A number of well-known microorganisms are responsible for the biodegradation of oil hydrocarbons. Bacteria have evolved regulatory systems that ensure the synthesis of enzymes so that the initial attack on these compounds is induced only when required. Thus, for an organism with the genetic information for utilizing benzene as carbon source, the enzyme for degrading benzene is induced when benzene reaches the bacterial environment. Some of these organisms have evolved an additional and highly effective system for responding to a variety of potential growth substrate. The essential genes of bacteria are carried on a single chromosome but genes specifying enzymes required for the catabolism of some of these unusual substrates may be carried on plasmids. Plasmids have been implicated in the catabolism of octane (Chakrabarty et al., 1973), naphthalene (Dunn and Gunsalus, 1973) camphor (Rheinwald et al., 1973) and toluene (Williams and Murray, 1974) as well as a number of other compounds (Chakrabarty, 1976).

Increasing petroleum exploration refining and other allied industrial activities in the Niger Delta have led to the wide scale contamination of most of its creeks, swamps, rivers and streams (Okpokwasili and Amanchukwu, 1988; Okpokwasili and Odokuma, 1990; Odokuma and Okpokwasili, 1992) with hydrocarbons and dispersant products. The contamination of these habitats constitutes public health and socio-economic hazards (Kobayashi and Rittman, 1982; Smith and Dragun, 1984). The xenobiotics so discharged may also pose serious aquatic toxicity problems (Wang, 1984; Bauda and Atlas, 1985). Xenobiotics may affect the microorganisms, physiological processes (Lundahl and Cabridenc, 1978), genetic machinery (Vandermeulen and Lee, 1986) and population (Lal and Saxena, 1982). The pollutant may inhibit some microbial communities that are important in some biogeochemical cycles of that ecosystem and this affects the productivity of such ecosystems (Rhodes and Hendricks, 1990).

Acclimatization of the organism after prolonged exposure to the polluting compound may eventually results in the degradation of the pollutant. Mechanical cleaning of spilled oil is nearly impossible in these “protected” ecosystems. Microbial degradation is the major mechanism for the elimination of spilled oil from the environment (Colwell and Walker, 1977; Atlas, 1981; Ibe and Ibe, 1984). In this study, the petroleum degrading potentials of a single and mixed bacterial culture is examined with the hope of isolating and stocking useful organisms with high crude oil degrading potentials in the Niger Delta of Nigeria.

MATERIALS AND METHODS

Collection of water and effluent samples

Water sample were collected from New Calabar River located in the Niger Delta and Omuihuechi River/Stream located in Omuihuechi village in Aluu, Ikwere, both in Rivers State of Nigeria. The New Calabar River is brackish water being influenced by the marine influx associated with tidal cycles. The river has been subjected to effluent discharges from industries sited along its bank. Omuihuechi River is not associated with oil pollution but may be subjected to domestic discharges, soil erosion, surface run-off and other human activities. The effluent samples were collected from NNPC refinery.

Isolation and enumeration of bacteria cultures

Total viable bacteria were enumerated by spread plate method using 0.1ml of the dilutions 10^{-1} to 10^{-4} onto nutrient agar. All cultures were incubated for 24 h to 48 h at 37^{o}C. The bacterial colonies, which developed on the plates, were randomly picked and purified by sub culturing unto fresh agar plates using the streak-plate technique. Isolated colonies, which appeared on the plates, were then transferred unto nutrient agar slants properly labeled and stored as stock cultures. The bacterial isolates were identified based on their morphology, Gram reaction and their biochemical reactions.

The fungi were isolated from the water samples using Czapek Dox agar unto which sterile streptomycin (50 mg ml^{-1}) had been added to suppress bacterial growth. Pure cultures of the fungi isolates were made and transferred unto Czapek Dox agar slants as stock cultures. The microscopic and macroscopic features of the hyphal mass, morphology of cells and spores, and nature of the fruiting bodies were used for identification.

Growth of Bacterial Isolates In Crude Oils (Bonny Light and Medium).

Mineral salt medium of were dispensed in 99 ml quantities into eleven 250 ml Erlenmeyer flasks. To each flask was added 1ml of the effluent sample (Bonny light and medium). After sterilization, the bacterial isolates namely; *Bacillus spp, Micrococcus spp* and *Proteus spp* were inoculated into the four flasks while the fifth flask was inoculated with a mixture of the bacterial isolates. The flask was inoculated at room temperature with a shaking at intervals for 17 days. The optical density (OD_{560nm}), total viable count (TVC) and pH of the culture were determined.

Growth of fungi in crude oils

The same procedure used for bacteria was repeated using fungal isolates namely *Penicillin spp, Aspergillus spp, Rhizopus spp.* Three flasks were inoculated using these fungal isolates while the fourth flask was inoculated with mixed cultures of the fungal isolates. The procedure was repeated for the Bonny light and medium samples and the growth of the organism estimated by dry weight measurement of the mycelium.

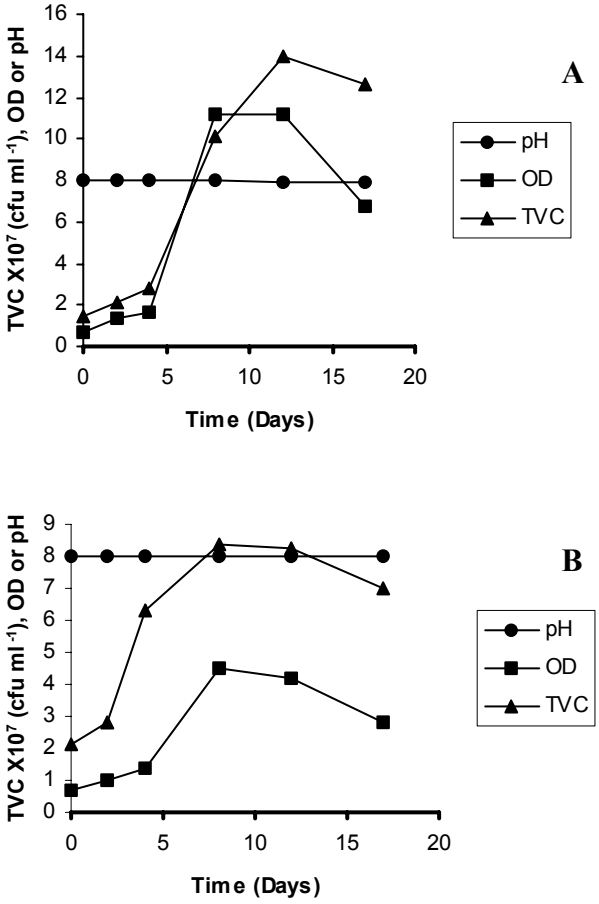


Figure 1. Growth profile of *Bacillus* species in mineral salt medium containing Bonny Light (A) and Bonny Medium (B) crude oil as sole source of carbon and energy.

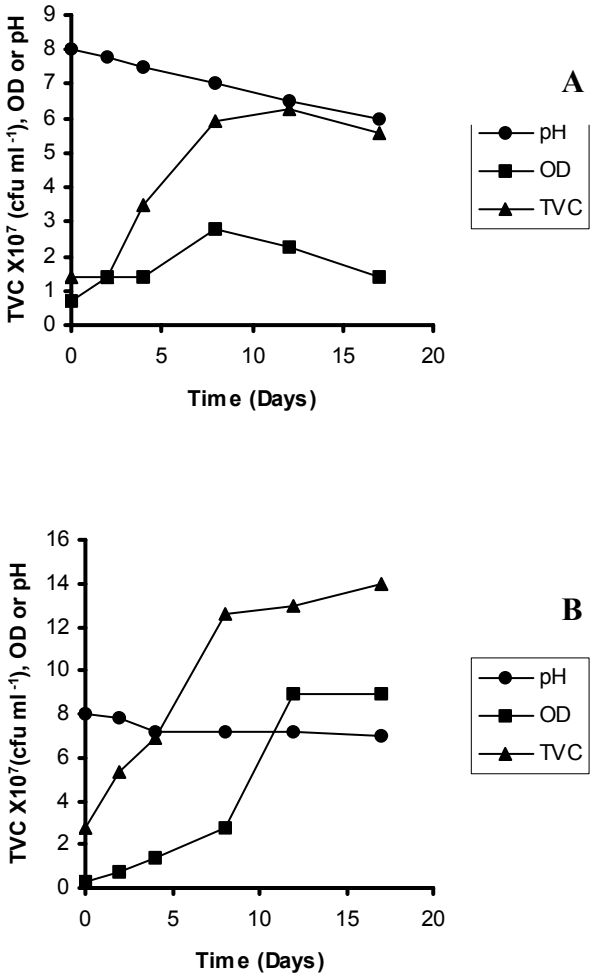


Figure 2. Growth profile of *Proteus* species in mineral salt medium containing Bonny Light (A) and Bonny Medium (B) crude oil as sole source of carbon and energy.

RESULTS AND DISCUSSIONS

The bacterial isolates obtained from the different sites (Omuihuechi River, New Calabar river and the NNPC effluents) were identified to be of the following genera: *Chromobacterium, Flavobacterium, Bacillus, Vibro, Citrobacter, Enterobacter, Micrococcus, Klebsiella, Planococcus, Pseudomonas* and *Camplobacter.* The predominant forms were rod shaped bacteria and a few cocci. Most of the bacilli were motile, indole negative and non-gas producing in TSI agar medium. The fungi isolates were mainly *Aspergillus, Penicillium*, and *Rhizopus* species. These organisms have been associated with petroleum product degradation. Mean weights of the fungal isolates are presented in Table 1. Almost all the bacterial isolates produced turbidity indicating an ability of each to utilize the crude oils. The *Bacillus* species had the highest optical density followed by the *Micrococcus sp* and thirdly by the *Proteus species*.

The growth profile of the selected bacterial isolates determined by the optical densities, total viable count and the pH of the culture medium are shown in Figures 1 to 4. The growth of fungi isolates in Bonny light (A) and Bonny medium (B) crude oil as sole C-source is shown in Figure 5. The *Penicillium* species grew better in Bonny light than the Bonny medium crude oils. The Bonny medium is

Table 1. Mean weight values of some fungal isolates grown on (light and medium) crude oils for 35 days.

Isolates	Mean weight of organism (g)	
	Bonny light	Bonny medium
Control	0	0
Mixed Culture (1,2,7 & 12)	0.311	0.406
Ref_1 (Isolate 1) *Penicillium Species*	0.369	0.364
Ref_2 (Isolate 2) *Aspergillus Species*	0.229	0.440
OM_1 (Isolate 7) *Unidentified Species*	0.315	0.405
NCR (Isolate 12) *Penicillium Species*	0.345	0.415

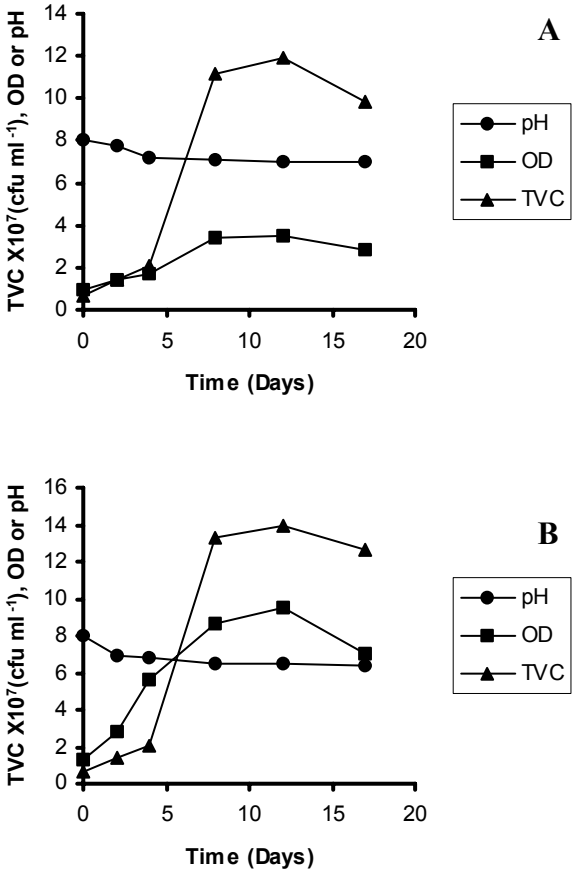


Figure 3. Growth profile of *Micrococcus* species in mineral salt medium containing Bonny Light (A) and Bonny Medium (B) crude oil as sole source of carbon and energy.

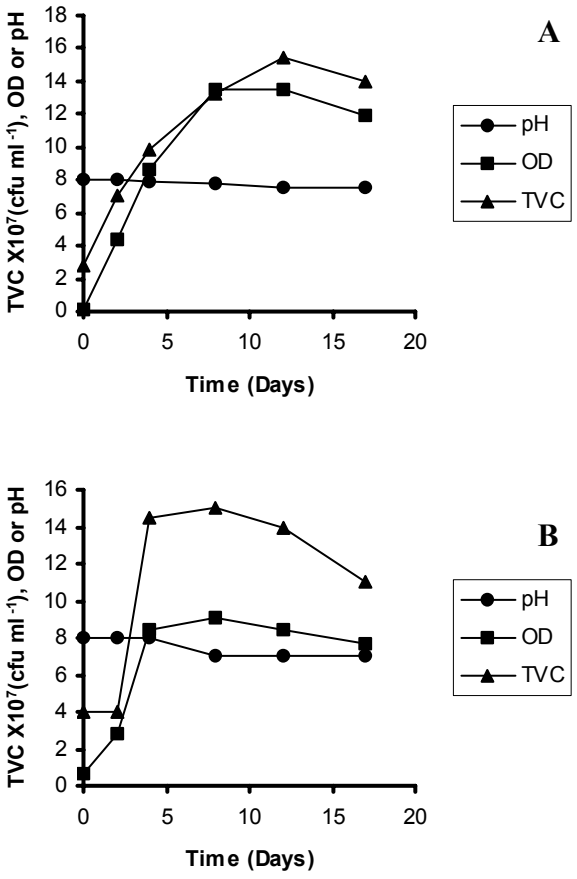
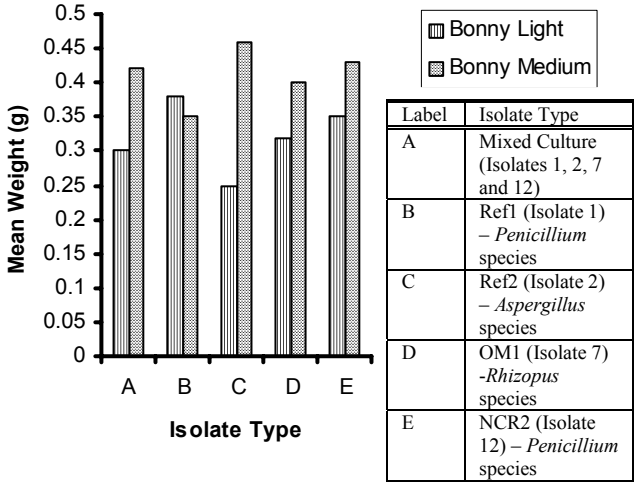


Figure 4. Growth profile of a mixed culture of *Bacillus*, *Micrococcus*, *Proteus* and *Bacillus* species. in mineral salt medium containing Bonny Light (A) and Bonny Medium (B) crude oil as sole source of carbon and energy.



Label	Isolate Type
A	Mixed Culture (Isolates 1, 2, 7 and 12)
B	Ref1 (Isolate 1) – *Penicillium* species
C	Ref2 (Isolate 2) – *Aspergillus* species
D	OM1 (Isolate 7) -*Rhizopus* species
E	NCR2 (Isolate 12) – *Penicillium* species

Figure 5. Growth performance of fungi isolates for 35 days on Bonny Light and Bonny Medium crude oil as the sole carbon and energy source.

heavier than the light sample and the result showed that the ability of the organisms to utilize Bonny light was more than that of the Bonny medium. The lower biodegradation rates exhibited by the heavier oil (Bonny medium) probably reflect its lower content of saturated hydrocarbons and high asphaltene content. Atlas (1975) reported that asphaltenes are known to be more resistant to microbial degradation.

The utilization of the crude oil as sole carbon and energy source by these microorganisms resulted in their growth with a concomitant production of acid. These acidic metabolic products might account for the decrease in pH of the cultures. The growth profile of the respective microorganisms followed the typical Monod kinetics with prolonged lag phases. This can be attributed to the low adaptation rates for the isolates from non-oil polluted waters. The total viable count ranged from 2×10^7 to 1.8×10^8 cfuml^{-1} within the 17 day growth. The bacterial growth reached the stationary phase and moved into the death phase in almost all the cases. This shows that the bacterial isolates utilized and degraded the crude oil but at a slower rate. All the isolates utilized the crude oils as sole C-source equally well, there was no statistical difference ($p>0.05$) in the utilization rate for the organisms from all the three sources namely Omuhuechi river, New Calaber river and refinery effluent. These results reflect the need for further research and intense prospecting for novel organisms and genes with high crude oil (hydrocarbon) degrading potential in the Niger Delta.

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