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Full Length Research Paper

Biodegradation of Bonny light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow stations saver pits in Nigeria

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In an effort at developing an active indigenous bacterial consortium that could be of relevance in bioremediation of petroleum contaminated systems in Nigeria, four hydrocarbon degrading bacteria strains were isolated. Partial sequencing of the 16S rDNA of the isolates suggests that they are all strains of *Pseudomonas aeruginosa*. Axenic cultures of the isolates biodegraded Bonny light crude oil in soil microcosm. Amount of crude oil biodegraded in 15 days ranged significantly ($P < 0.05$) from 4.9% to 29.6%. Degradation rates and specific growth rates varied significantly ($P < 0.05$) between 0.049 and 0.351 day^{-1} and 0.017 and 0.028 $hour^{-1}$ respectively. Major peak components of the oil were reduced by between 6.5% and 70.6%. It would appear that oil degradation capability of axenic cultures of at least three of these isolates was not different from that of their consortium. Also, the multiple antibiotic resistance observed in the isolates is an important factor to consider in their eventual use in bioremediation exercises.

Key words: Crude oil, soil microcosm, biodegradation.

INTRODUCTION

Petroleum exploration started in the late 1930s with the first exploration well drilled by shell D`Arcy at Iho, North West of Owerri (SPDC, 1996). However, the first commercial oil field was discovered in 1956 at Oloibiri in the Niger delta region of Nigeria, and ever since, the frontiers of oil exploration in Nigeria has been expanding, producing medium and light (such as Bonny light) crude oil (Amund and Akangbou, 1993).

The development of petroleum industry into new frontiers, the apparent inevitable spillage, which usually occur during routine operations, and records of acute accidents during transportation, has called for more studies into oil pollution problems. These pollution problems have been prevalent in Nigeria since the 1950s (Okoh et al., 2001).

Remediation of polluted systems could be achieved by physical, chemical or biological methods. However, the attendant negative consequences of the physicochemical methods make the biological alternative or bioremediation more attractive.

We have isolated several candidate bacterial strains (Okoh et al., 1996) in our effort at developing an active bacteria consortium that could be of relevance in the bioremediation of crude oil contaminated systems in Nigeria. Four of these isolates have been reported to have tremendous potentials for biodegradation of petroleum hydrocarbon in aqueous system (Okoh et al., 2000, 2001) . This paper reports some attributes and potentials of these bacteria isolates for the biodegration of Nigerian Bonny light crude oil in soil system.

MATERIALS AND METHOD

Bacterial isolation, identification, antibiotic susceptibility and screening for crude oil degradation

The four bacterial isolates used in this study were isolated from some crude oil flow stations' saver pit effluent in the Niger delta area of Nigeria (Okoh et al., 1996). The isolates were coded as OK1, MT1, RQ1 and T2 and preserved in glycerol at $-70^{o}C$. Identification of the bacterial strains (except MT1) was carried out using molecular techniques that exploit the nucleotide sequences of their 16S rRNA genes. Amplification of the 16S rRNA genes were done as described by Wilson (1987) using the 16F27 and 16R1492 primers (Lane, 1991). The amplified products were then purified and partially (approx. 500 bp) sequenced using an automated DNA sequencer (Perkin-Elmer, Applied Biosystems, version 377), and the nucleotide sequences were analysed as described elsewhere (Pearson and Lipman, 1988). The antibiotic susceptibility pattern of the bacteria isolates were analysed using the agar diffusion method (Bouchez et al., 1995), while initial screening for crude oil degradation was done as described before (Okoh et al., 2001).

Soil Physicochemical Properties

The soil used for this study was garden topsoil from Irri town, an oil exploratory locality in the delta region of Nigeria, at latitude 5^{o} 33' and 5^{o} 40' N and longitude 6^{o} 11' and 6^{o} 13' E. Soil pH was determined using a 1:1 soil - water ratio with the aid of a glass electrode pH meter. Soil organic matter, moisture and texture were estimated according to the methods of Gardner (1965) and Nelson and Sommers (1982).

Soil Microcosm Experiment

Biodegradation of Bonny light crude oil (BLCO) by axenic and mixed cultures of the bacterial isolates was assessed as previously described (Hanson et al., 1997) . Ten gram of sterile garden soil artificially contaminated with 4% BLCO, in glass tubes (15 cm X 2.25 cm internal diameter) and bioaugmented with standardised suspension (OD_{546} 0.1) of the pure isolates in sterile Bushnell-Hass mineral medium (BHM) such as to achieve 30% moisture condition. Uninoculated tubes containing only sterile BHM were set up to serve as controls. Sixteen such tubes were set up per isolate and incubated at room temperature (28 ± 2 ^{o}C) for fifteen days. Sampling period was fixed for 0, 5, 10 and 15 days. Four tubes were sampled for each sampling time. One of the tubes was used for the estimation of total viable count, while the other three tubes were harvested for residual crude oil estimation.

The bacterial consortium was prepared by mixing equal volumes of the standardised suspensions (OD_{546} 0.1) of each isolate. The content of the tube selected for viable count estimation was emptied in a sterile petri-dish and mixed well for homogenisation. One gram of the soil sample was aseptically added into 10 ml of sterile normal saline, shaken vigorously to dislodge the cells from the soil particles and allowed to stand for about ten minutes, after the supernatant was serially diluted. The cell densities of the appropriate dilution were determined by standard spread plate technique (Seeley and Vandenmark, 1981).

Total residual crude oil was extracted using chloroform (Okoh et al., 2001) and estimated gravimetrically (Haramaya et al., 1997), while the major peak components (MPC) was identified by gas chromatography (Okoh et al., 2001).

RESULTS

Identification of bacterial strains

The PCR amplification of the 16S rRNA genes of the bacterial isolates yielded the expected DNA amplicon of approximately 1.5 kb (Figure 1) which falls within the size range of prokaryotic 16S rRNA gene. Analysis of the partially sequenced 16S rDNA of the isolates revealed OK1 and RQ1 as having 100% similarity to *Pseudomonas aeruginosa* PA1 (accession number AE004883), while isolate T2 had 98.371% similarity to *Ps. aeruginosa* PA1 . The sequence of MT1 16S rDNA could not be determined due to logistic problems. Sections of the partial nucleotide sequences of the isolated 16S rRNA genes are as shown below.

RQ1:

23…AAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGG
ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGT
CCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGG
GATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGA…200

OK1:

23…AAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGG
ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGT
CCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGG
GGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG…200

T2:

23…GCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTG
ATTCGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG
GGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGG
GAGAAGTGGGGATCTTCNGACCTCACGCTATCAGATGAGC…200

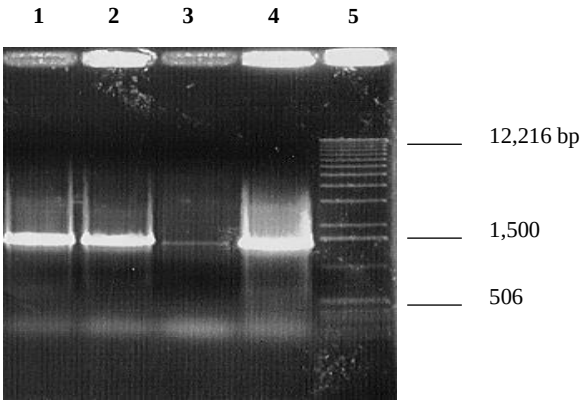

Figure 1. Gel electrophoresis of 16S rDNA amplicons of the bacterial isolates. Lane 1 represents RQ1; Lane 2 represents OK1; Lane 3 represents T2; Lane 4 represents MT1 and Lane 5 represents lambda phage molecular weight DNA marker.

The antibiotic susceptibility patterns of the isolates are as shown in Table 1. Isolates T2, OK1 and RQ1 were resistant to ten of the twelve antibiotics used, while MT1 was resistant to nine antibiotics.

Soil microcosm studies

The physicochemical properties of the soil sample used for the soil microcosm studies are shown in Table 2. The textural qualities suggest that the soil belong to the sandy loam classification. The Bonny light crude oil was degraded to varying extents by the axenic cultures of the bacterial isolates. The amount of total crude oil biodegraded after 15 days of incubation ranged significantly ($P \leq 0.05$) between 4.9% (T2) and 29.6% (RQ1) (Figure 2). Also, a consortium of the four bacterial isolates biodegraded 14.9% of the crude oil. This was less than what was recorded for RQ1, OK1 and MT1. Degradation rates varied significantly ($P < 0.05$) between 0.049 and 0.351 day^{-1} for the axenic cultures, and 0.173 day^{-1} for the consortium (Table 3). The level of reduction

Table 1. The antibiotic susceptibility pattern of the bacterial isolates. R = Resistant, S = Sensitive, I = Intermediate, NF = Nitrofurantoin (300 µg), CF = Cephalotine (30 µg), CRO = Cephtriaxone (30 µg), AM = Ampicillin (10 µg), SXT = Trimetoprin-sulfametoxazol (25 µg), CXT = Cefotaxime (30 µg), NET = Netilmicine (30 µg), PEF = Pefloxacine (5 µg), GE = Gentamicine (10 µg), CB = Carbeniciline (100 µg), CL = Chloramphenicol (30 µg), AK = Amikacine (30 µg). Values in parenthesis represent diameter of zone of inhibition in centimeters.

Test antibiotic	Isolates' responses			
	T2	RQ1	OK1	MT1
NF	R(0)	R(0)	R(0)	R(0)
CF	R(0)	R(0)	R(0)	R(0)
CRO	R(0)	R(14.5)	R(14.7)	R(14.5)
AM	R(0)	R(0)	R(0)	R(0)
SXT	S(24)	R(0)	R(0)	R(0)
CXT	R(9.5)	R(13.3)	R(13.3)	R(12)
NET	R(0)	I(14.3)	I(13.3)	I(13.7)
PEF	R(8.5)	R(7.0)	R(9)	R(0)
GE	R(0)	R(11.0)	R(13)	R(12)
CB	R(10.5)	R(12.3)	R(12)	R(12.7)
CL	S(18.5)	R(0)	R(0)	R(0)
AK	R(0)	I(15.3)	I(16)	I(15)

Table 2. Some physicochemical properties of the soil used for the microcosm studies.

Physicochemical Properties	Value
pH	4.7 ± 0.01
Organic Matter (%)	2.42 ± 0.2
Moisture (%)	1.24 ± 0.8
Sand (%)	69 ± 0.0
Silt (%)	20 ± 0.0
Clay (%)	11 ± 0.0

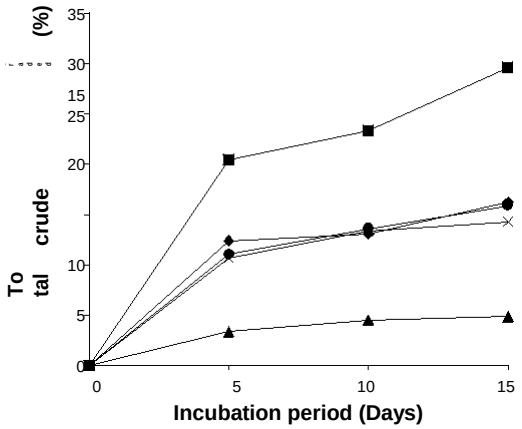

Figure 2. Biodegradation of Bonny light crude oil by the axenic cultures of MT1 ( ), RQ1 (■), T2 (▲), OK1 ( ), and the bacterial consortium (•) in a soil system supplemented with BHM.

Table 3. Degradation rates and specific growth rates profiles during biodegradation Bonny light crude oil in soil microcosm.

Experimental code	Degradation rates (day $^{-1}$)	Specific growth rates (Hour $^{-1}$)
OK1	0.177	0.024
RQ1	0.351	0.028
T2	0.049	0.017
MT1	0.154	0.024
Consortium	0.173	0.019

of the major peak components (MPC) of the crude oil varied significantly ($P \leq 0.05$) among the isolates in compare to the consortium. The MPC reduction for the isolates OK1, RQ1 and MT1 were 70.3%, 69.3% and 70.6% respectively, and 66.6% for the consortium (Figure 3). The lowest MPC value (6.5%) was recorded for T2. The specific growth rates of the axenic cultures were ranged significantly ($P < 0.05$) between 0.017 and 0.028 hour^{-1} in five days, and remained constant at 0.019 hour^{-1} for the consortium.

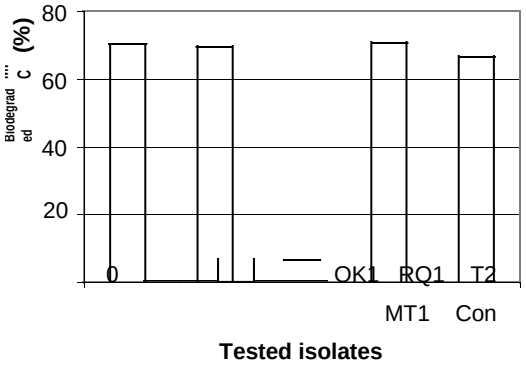

Figure 3. Biodegradation of MPCs (Major Peak Components) of Bonny light crude oil in soil system during 15 days of activity using axenic cultures of isolates OK1, RQ1, T2, MT1 and their consortium (Con).

DISCUSSION

Although, the four bacterial isolates had been tentatively identified in our previous studies (Okoh et al., 1996, 2000, 2001), based on biochemical/enzymatic characteristics, as strains of *Pseudomonas aeruginosa* (OK1 and MT1), *Burkholderia cepacia* (RQ1) and *Stenotrophomonas maltipholia* (T2), analysis of the 16S rDNA sequences suggested OK1, RQ1 and T2 are strains of *Pseudomonas aeruginosa*. Difficulties in resolving the taxonomy of the *Pseudomonas* genus using

a combination of RNA homology and phenotypic characteristics have been reported before (Palleroni, 1992). Besides, several genera of microorganisms have been found to be active in the biodegradation of crude oil, but the genus *Pseudomonas* stand out as most versatile (Miklosovicova and Trzilova, 1991). Also, the multiple antibiotic resistance exhibited by the isolates is a common phenomenon amongst the pseudomonad, and this is an important factor to consider in the use of these organisms in biocontrol measures. These characteristics have been exemplified by the bacterial isolates used for this study and further support their assignments to the *Pseudomonas* genus.

The soil microcosm experiments revealed that the four bacterial isolates were able to biodegrade Bonny light crude oil in the soil system, although the degree of activities varied. Isolate RQ1 was evidently the most active compared to other axenic cultures and the consortium. The isolate had a significantly ($P \leq 0.05$) higher degradation rate compared to the other isolates, while isolate T2 was the least active. The oil degradation rates and total oil metabolised by the consortium culture did not differ significantly from that of isolates OK1 and MT1. Hence, it would appear that oil degradation capability of axenic cultures of at least three of these isolates was not different from that of their consortium. This observation further confirms the close genetic similarity of the isolates in respect of oil degradation capability. This experience would be one of the few cases in which the application of a bacterial consortium for bioaugmentation purposes may be of little effect compared to axenic cultures as previously suggested (Bouchez et al., 1995). In this case all members of the consortium belong to the same genus and they probably carry the same hydrocarbon-degrading gene(s). The composition of a microbial consortium is an important factor, which must be made to ensure synergistic enhancement of catabolic activities. Haramaya et al. (1997) reported that a microbial consortium called SM8 exhibited higher activity than an axenic culture of *Acinetobacter* for the biodegradation of light and heavy crude oils.

The reduction of the major peak components (MPC) of the crude oil appeared to be similar for all test conditions except for isolate T2. This observation corroborates our earlier report (Okoh et al., 2000), which recognised T2 as a weak degrader of crude oil compared to other *Pseudomonas* species. It is suggested that this isolate has lost some important genetic factors related to its catabolic versatility. The elucidation of this factor(s) is the subject of on going investigation. Nevertheless, T2 could be relevant as a partner in a consortium. The findings of these study shows that the test isolates could be useful for use in the bioremediation of soil systems contaminated with Bonny light crude oil.

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