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Full Length Research Paper

Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria

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***Acinetobacter anitratus*, *Alcaligenes faecalis*, *Acinetobacter mallei* and *Micrococcus varians* were isolated from polycyclic aromatic hydrocarbon polluted soils by enrichment culture using chrysene as sole carbon and energy source. Physiochemical evaluation revealed that these isolates grew well at a temperature range of 20 - 40°C, pH 6.0-8.0 but less tolerable to various salt concentrations except *Micrococcus varians* which grew at 1.0 to 7.5% NaCl. These organisms utilized chrysene, anthracene, naphthalene, crude oil, kerosene, diesel and engine oil as sole carbon source. None could utilize benzene, hexane, xylene, phenol and toluene as carbon sources. Growth study of the isolates on 0.1% (w/v) chrysene resulted in highest cell density of $8.8x10^7$, $7.9x10^7$, $6.3x10^6$, $6.3x10^6$ cfu/ml for *A. anitratus*, *Alc. faecalis*, *A. mallei* and *M. varians*, respectively. There was statistical significant difference ($P< 0.05$) in the growth of these organisms on chrysene as sole carbon and energy source when compared with non-chrysene control. This study indicates the potential of these hitherto unreported tropical bacterial strains as chrysene degraders and their use in biodegradation processes involving petrochemical products.**

Key words: High molecular weight, polycyclic aromatic hydrocarbons, crude oil, tropical bacteria, chrysene, biodegradation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in soils, sediments and water, and are of environmental concern because of their mutagenic and/or carcinogenic effects. They are produced during fossil fuel combustion, waste incineration, or as by-products of industrial processes, such as coal gasification, production of aluminum/iron/steel and petroleum refining, component of wood preservatives, smoke houses and wood stoves (Shuttleworth and Cerniglia, 1995; Jones et al., 1989). While low-molecular-weight PAHs (composed of two or three fused benzene rings) are readily degraded by bacteria, high-molecular-weight PAHs (HMW PAHs) consisting of four rings or more are recalcitrant to biodegradation and persist in the environment (Wilson and Jones, 1993; Cerniglia, 1992). The chemical properties, and hence the environmental fate, of a PAH molecule are dependent in part upon both molecular size, i.e., the number of aromatic rings, and molecule topology or the pattern of ring linkage. Generally, an increase in the size and angularity of a PAH molecule results in a concomitant increase in hydrophobicity and electrochemical stability. PAH molecule stability and hydrophobicity are two primary factors which contribute to the persistence of HMW PAHs in the environment. Consequently, they have been detected in numerous aquatic and terrestrial ecosystems at concentrations high enough to warrant concern about bioaccumulation.

Chrysene is one of the high-molecular-weight PAHs consisting of four fused benzene rings and among PAHs classified as priority pollutants by the U.S. Environmental Protection Agency (Smith et al, 1989). Microbial

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biodegradation of HMW PAHs has been found as possible way to clean up polluted soils and water systems (Alexander, 1999; Atlas, 1981). However, relatively little information is available on microbial metabolism HMW PAHs (Kanaly and Harayama, 2000). A number of bacterial isolates capable of chrysene metabolism have been described; *Rhodococcus* sp. Strain UW1 (Walter et al., 1991), *Sphingomonas yanoikuyae* which oxidized chrysene (Boyd et al., 1999) while *Pseudomonas fluorescens* utilised chrysene and benz[a]anthracene as sole carbon sources (Caldini et al., 1995). Chrysene oxidation occurs by incorporation of an oxygen molecule in an aromatic ring. This is catalyzed by dioxygenase to a *cis*-dihydrodiol intermediate, which undergoes further metabolism via pyridine nucleotide dependent dehydrogenation reaction to produce catechols. These are substrates for ring cleavage enzymes, which lead to the complete mineralization (Hinchee et al., 1994).

The search for suitable degraders of recalcitrant HMW PAHs for use in bioremediation strategies has warranted this work. In this study, four bacterial isolates isolated from crude oil and wood processing site in Nigeria were evaluated for chrysene degradation potential. To the best of our knowledge, this is the first report of these tropical bacteria strains as chrysene degraders.

MATERIALS AND METHODS

Chemicals and media

Solid hydrocarbons used in this study; chrysene, anthracene and naphthalene were purchased from Sigma (Germany). Liquid hydrocarbons were benzene, hexane, xylene, phenol and toluene (BDH Laboratory supplies, England). Kerosene, diesel and engine oil were from Petrochemical Marketer (Nigeria), while crude oil was obtained from (Escravos light, Nigeria). Nutrient agar, bacteriological agar and nutrient broth were purchased from Sigma (Germany).

Soil samples

Samples were taken from crude oil polluted and wood processing sites in Nigeria, placed into sterile bottles and transported immediately in cold storage containers to the laboratory for further work.

Isolation and identification of chrysene degraders

Chrysene utilizing bacteria were isolated from soil samples by enrichment culture on minimal salt agar medium as described by Kästner et al. (1994) using chrysene as sole carbon and energy source. The medium contained per liter: 2.13 g Na_2HPO_4, 1.3 g KH_2PO_4, 0.5 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$ and trace elements solution (1 ml per liter) as described by Bauchop and Flsiden (1960). Both were sterilized separately by autoclaving at 121^0C for 15 min. 0.2 ml ethyl acetate solution (containing 0.1% w/v) chrysene) was aseptically pipetted and uniformly spread on the agar surface as described by West et al. (1984). Ethyl acetate was allowed to evaporate under sterile condition before inoculation. 0.1 ml diluted soil samples were inoculated onto the agar surface, plates were covered with foil and black polyethylene bag, and then incubated in the dark at room temperature for 14 days. Control minimal salt plates free of chrysene were included. Colonies on the control plates were counted and taken as oligothrophs able to grown on the medium components without any further carbon source. Colonies that formed crystal cleared zones on the chrysene coated plates were replicated onto fresh chrysene coated agar plates and incubated for 14 days. Isolates that grew on this plate were selected as chrysene degraders. Chrysene degraders were identified as described by Cowan (1974) and Holt et al. (1994). Morphological and biochemical tests carried out include Gram reaction, motility, colonial morphology, methyl red and Voges-Proskauer tests, production of oxidase, catalase, indole, gelatin liquefaction, starch hydrolysis and sugar utilization.

Physiochemical properties of isolated chrysene degraders

Growth at different pH was determined by adjusting the pH values of different nutrient broth media to pH 2.0 - pH 9.6 using 0.1 N HCL and 0.1 N NaOH solution. 10 ml of the medium were distributed into test tubes before autoclaving. After autoclaving, the pH value were rechecked, inoculated with the test cultures and incubated at 30°C for 48 -120 h. Turbidity of the broth compared with the uninoculated controls was used as indicator of growth of the culture. Tubes containing sterilized 5 ml of nutrient broth were inoculated with 18 h old cultures and incubated in water baths set at 10 - 65°C to determine growth of isolates at different temperatures. Growth of isolates in 1.0 - 10% NaCl was determined by inoculating isolates into 30 ml sterilized peptone - salt medium and incubated at 30°C for 48 – 120 h. Increased turbidity of medium was recorded as positive for growth. Uninoculated tubes served as control.

Assay for liquid hydrocarbon degradation

Growth of the different organisms on liquid hydrocarbon were tested by growing each organism in sterile 250 ml Erlenmeyer flasks containing 99 ml minimal salt minimal salt and various sterile substrates (1% v/v) which include hexane, xylene, toluene, phenol, benzene, diesel, kerosene, crude oil and engine oil (Ilori and Amund, 2000). Incubation was carried at 28°C for 5 days. Cultures without increase in turbidity over initial optical density and noninoculated control were scored as no growth (-) while cultures with increased turbidity significantly greater than the control were scored as growth (+), their OD readings were above 0.2.

Assay for solid hydrocarbon degradation

Spray plate technique was followed for this purpose as described by Kiyohara et al. (1983). Hydrocarbon solutions (0.1% w/v ethyl acetate) were sprayed onto the surface of minimal salt agar. The ethyl acetate was evaporated leaving behind a thin film of hydrocarbon on the agar surface. The overnight cultures were spotted on the minimal salt agar plates. The plates were incubated at 30°C for 14 days. The appearance of growth on the plates indicated positive test.

Utilization of chrysene as sole carbon and energy source

Cells grown for 14 days on chrysene coated minimal salt were harvested and suspended in 10 ml sodium phosphate buffer (50 nm, pH 7.2), and then centrifuged at 4°C for 10 min at 10,000 x g. Pelleted cells were washed twice with same buffer, resuspended in

Table 1. Morphological and biochemical properties of the bacteria isolated.

Parameter	A. anitratus	Alc. faecalis	A. mallei	M. varians
Gram reaction	-	-	-	+
Cellular morphology	Coccoid rods	Rods	Rods	Cocci
Catalase	+	+	+	+
Oxidase	-	+	-	+
Indole	-	-	-	-
Motility	-	+	-	-
Methyl red	-	-	-	-
Voges-Proskauer	-	-	-	-
Citrate	+	+	+	+
Urease	+	-	-	-
Starch hydrolysis	-	-	-	-
Gelatin hydrolysis	-	-	-	+
NO_3 reduction	-	+	+	+
Coagulase test	-	-	-	-
Spore test	-	-	-	-
Mannitol	-	-	+	-
Glucose	+	+	+	+
Xylose	+	-	-	+
Lactose	+	-	-	-
Sucrose	-	+	-	+
Raffinose	-	-	-	-
Arabinose	-	-	-	-
Maltose	-	-	-	+
Galactose	-	+	-	-
Salicin	-	-	-	-

minimal salt medium (10 ml) to a final population of about 10^4 per ml of the medium and used as bacteria starter culture for chrysene growth experiment. 3 ml of chrysene (0.1%, w/v ethyl acetate) were added to 250 ml Erlenmeyer flask, covered with foil and allowed to stand for a day to evaporate ethyl acetate after which minimal salt media (90 ml) were dispersed into the various flasks. The media were sterilized and inoculated with 10 ml of each starter culture. Incubation was carried out in the dark at 28°C with interval shaking for 35 days in triplicate. This set-up was designated "Experimental (E)". Two controls (C1 and C2) were also set up to evaluate the role played by the test bacteria in utilizing chrysene for growth. The first, C1, consisted of the same materials present in E but without chrysene and was designed to establish utilization of chrysene in E. The second, C2, contained all the materials in E with no test bacteria inoculation and was designed to evaluate the role played by contaminants picked from the laboratory environments where the study was conducted. Growth on chrysene by the organisms was assayed by measuring optical density ($OD_{600\ nm}$) and total viable count (TVC) at interval of 5 days.

RESULTS AND DISCUSSION

Acinetobacter anitratus, *Alcaligenes faecalis*, *Acinetobacter mallei* and *Micrococcus varians* strains were identified as chrysene degraders based on the ability to form clearing zones and subsequent growth on chrysene as sole carbon and energy source. The morphological and biochemical properties of these isolates are shown in Table 1. Growth profile of the organisms at varying temperature, pH and sodium chloride solution as shown in Table 2 revealed that the four organisms could grow well at temperature range between 20 - 40°C, pH 6.0 - 8.0 but generally not tolerable to various salt concentrations except for *M. varians* which grew at 1.0 to 7.5% NaCl.

The range of aromatic hydrocarbon utilized by the organism as shown in Table 3 indicated that the organisms could utilize crude oil and its petrochemical products, which include diesel, kerosene and engine oil for growth. Pollution of the environment due to accidental seepages, rupture of pipelines, blow out of terrestrial oil wells and sabotage has been reported (Awobajo, 1983; National Academy of Science, 1975). The resulting spillages have brought about economic losses as well as contamination of the aquatic and terrestrial ecosystem. Therefore, the degradability property of the evaluated organisms could be explored in bioremediation strategies for oil-impacted agricultural soils in oil producing countries of the world, including Nigeria. Many bacteria and fungi isolates from crude oil polluted soil have been

Table 2. Physiochemical properties of bacteria isolates.

Organism	Growth at varying pH					Growth in varying NaCl (%)					Growth at varying temperature (T^0C)							
	2.0 - 4.9	5.0 - 5.9	6.0 - 7.0	7.1 - 8.0	8.1 - 9.6	1.0 - 4.0	4.1 - 5.0	5.1 - 6.4	6.5 - 7.5	7.6 - 10	10	20	30	35	40	45	55	65
A. anitratus	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-
Alc. faecalis	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	w+	-	-
A. mallei	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-
M. varians	-	-	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-

+ = Growth - = No growth w+ = weak growth

Table 3. Growth test on different liquid and solid hydrocarbon.

Substrate	*A. anitratus*	*Alc. faecalis*,	*A. mallei*	*M. varians*
Crude oil	+	+	+	+
Kerosene	+	+	+	+
Diesel oil	+	+	+	+
Engine oil	+	+	+	+
Hexane	-	-	-	-
Xylene	-	-	-	-
Phenol	-	-	-	-
Toluene	-	-	-	-
Benzene	-	-	-	-
Chrysene	+	+	+	+
Anthracene	+	+	+	+
Naphthalene	+	+	+	+

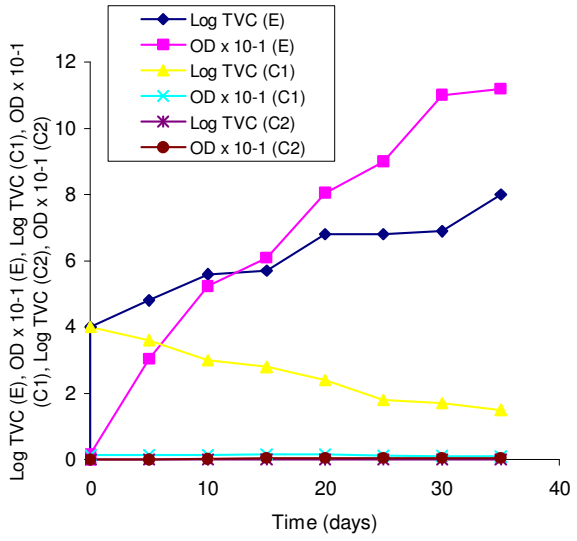

Figure 1. Growth profile of *Acinetobacter anitratus* on chrysene.

found to degrade crude oil and spent oil. They include *Pseudomonas putida, Pseudomonas aeruginosa, Bacillus subtilis, Alicaligenes eutrophus, Micrococcus luteus, Acinetobacter iwoffi, Proteus* sp. *Aspergillus* sp., *Penicillium* sp. and R*hizopus* sp. (Okerentugba and Ezeronye, 2003; Nwachukwu et al, 2000; Ilori and Amund, 2000 and Amund et al, 1993). However, the isolates could not utilize benzene, hexane, xylene and phenol. Membrane toxicity and non-possession of the necessary enzymes may be the reason for inability to utilize these hydrocarbons. Lipophilic hydrocarbons accumulate in the membrane lipid bilayer, affecting the structural and functional properties of these membranes. The resulting accumulation of hydrocarbon molecules leads to membrane loss of integrity, increase in permeability to protons and consequently, dissipation of the proton motive force and impairment of intracellular pH homeostasis (Sikkema et al., 1995).

Measuring the success of bioremediation of oil spills is based on several parameters, among them the degradation of polycyclic aromatic hydrocarbons in the crude oil. The four organisms in this study were found to utilize chrysene, anthracene and naphthalene. The ability to utilize both low and high molecular weight PAHs by all the isolates is an indication of the possession of the ring fission enzymes (Ilori and Amund, 2000). The growth profile of the organisms on chrysene as sole carbon and energy source is presented in Figures 1 to 4. *A. anitratus* was the best chrysene degrader; it had the highest cell density of 8.8×10^7 cfu/ml after 35 days of incubation.

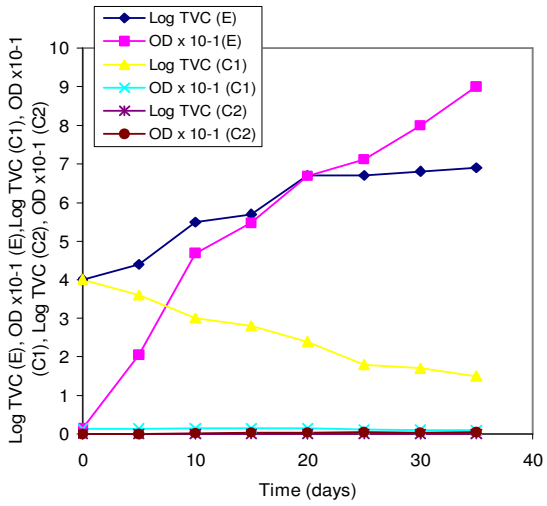

Figure 2. Growth profile of *Alcaligene faecalis* on chrysene.

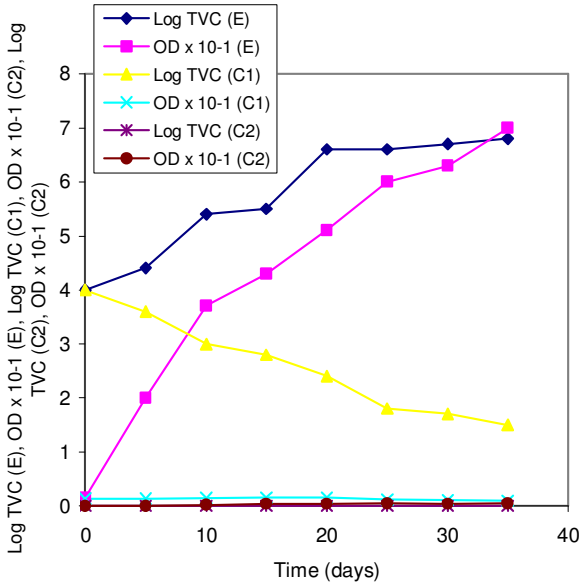

Figure 3. Growth profile of *Acinetobacter mallei* on chrysene.

Highest cell densities of 7.90 x 10^7, 6.30 x 10^6 and 6.30 x 10^6 were observed for *Alc. faecalis*, *A. mallei* and *Mi. varians*, respectively. Previous studies have shown the degradation of three HMW PAHs; pyrene, benz[a]anthr-acene and benzo[a]pyrene by *Mycobacterium sp.* strain RJGII-135 (Schneider et al., 1996). Strain EPA505 has been found to cometabolically metabolise radiolabeled chrysene to nearly 42% $^{14}CO_2$ in 48 h (Ye et al., 1996). In a six-component synthetic mixture of three-, four-, and five –ring PAHs, *Mycobacterium* PYR-1 degraded all of the components to various degrees, with the exception of chrysene (Kelley and Cerniglia, 1995). As shown in Figures 1-4, there was slow growth of the organisms at the onset between days 0-5 after which significant growth

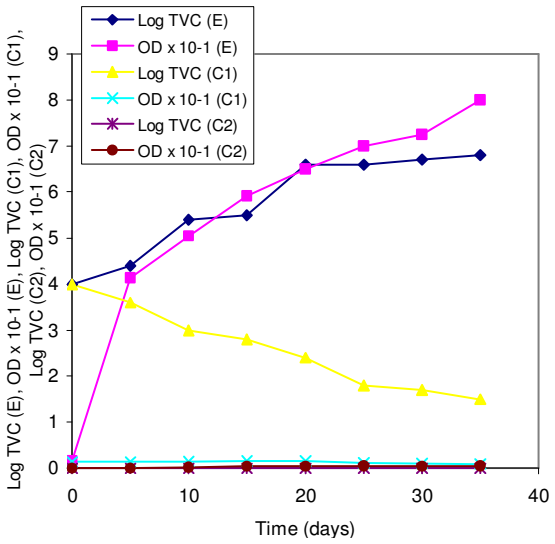

Figure 4. Growth profile of *Micrococcus varians* on chrysene.

rate were observed. This could be attributed to the adaptation period of the isolates to chrysene. Between days 20 - 25, there was barely any change in bacteria growth and may signify time when nutrient supplementation may be necessary to enhance biodegradation.

From the cell densities observed for the organisms, using student t-test, there was no statistical significant difference ($P>0.05$) in the utilization of chrysene for growth by the various isolates. However, there was statistical significant difference ($P>0.05$) between the experimental and control. The non-growth of laboratory organisms in C2 could be related to the toxicity of chrysene to non-adapted bacteria; this is in accordance with the fact that oil pollutants persist in some environment for many years undegraded (Yveline et al., 1997; Teal et al., 1992; Atlas, 1991). Investigation into the regulatory interactions within PAH-degrading consortia and the mechanisms by which HMW PAH biodegradation occur are underway and will prove helpful for predicting the environmental fate of these compounds and for developing practical PAH bioremediation strategies in the future.

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