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Original research paper

Isolation and characterization of bacterial strains with pyrene metabolic functions from cow dung and *Terminalia catappa* phylloplane

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ABSTRACT

Proteus vulgaris strain CPY1 and *Pseudomonas aeruginosa* strain LPY1 exhibiting competent pyrene metabolic functions isolated respectively from animal waste and phyllosphere were identified by analysis of their morphological, physiological and biochemical properties. A batch culture experiment with axenic cultures of both strains on 100 mg l^{-1} pyrene yielded over 88% degradation concomitant with over 3-orders-of-magnitude biomass production. A critical evaluation of the kinetic data showed that over 50% of the PAH was consumed in less than 9 days of cultivation, resulting in an approximate degradation rate of 0.255 mg l^{-1} h^{-1} compared with 0.147 mg l^{-1} h^{-1} determined for days 9–18. Overall, the degradative competence of both strains did not differ significantly ($P < 0.05$) even though strain CPY1 appeared to utilize the substrate better. This study provides new insight into pyrene degradation, with the first evidence for the likely role of *Proteus vulgaris* – an enteric bacterium in pyrene metabolism. It further demonstrates that pyrene metabolic capability may be more widespread than previously believed and that such functionality may not be confined to contaminated systems and soil microorganisms.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants comprising two or more fused aromatic rings. They are produced and released into the environment through natural and anthropogenic pyrolysis of organic material such as forest fires, automobile exhaust, coal-refining processes and activities of the petroleum industry. Owing to their genotoxicity, carcinogenicity, mutagenicity and recalcitrance to microbial attack (Ghosh et al. 2014; Moscoso et al. 2012; Cerniglia, 1992), most PAHs, particularly high molecular weight (HMW) PAHs, are of great environmental and public health concern. As a consequence, many of the HMW PAHs are listed as priority pollutants by USEPA (Keith and Telliard, 1979; Wang et al. 2000) and also by environmental protection agencies of most countries including Nigeria.

Increasing number of aromatic rings and angularity confers greater thermodynamic stability and hydrophobicity to HMW PAHs, hence their persistence in the environment. Their hydrophobic property enables them to adsorb to organic-rich soils and sediments making them available for biological uptake. As a result, they have a high potential for biomagnification through trophic transfers, thus constituting a serious health threat to aquatic and terrestrial ecosystems. In addition, hydrophobicity is also a factor responsible for their non-bioavailability to microorganisms (Das and Mukherjee, 2007).

Pyrene, a four-ring peri-condensed compound, is one of the most abundant HMW PAHs in environmental matrices that has demonstrated striking recalcitrance to microbial degradation. Nevertheless, in spite of its molecular stability and super-hydrophobicity, there have been some reports of microorganisms with remarkable pyrene metabolic functions. *Mycobacterium vanbaalenii* strain PYR-1, a bacterium originally isolated from estuarine sediment near an oil field, was the first organism reported to metabolize pyrene in a medium supplemented with low level of organic nutrients (Heitkamp and Cerniglia, 1988). Since this initial report, several other organisms, mostly Gram-positive actinomycetes, have been shown to degrade pyrene (Heitkamp et al., 1988; Mueller et al., 1990; Cerniglia, 1992; Wang et al., 2000; Vila et al., 2001; Silva et al., 2009; Kanaly and Harayama, 2010; Wen et al., 2011). The pathways for pyrene metabolism in these organisms unequivocally indicated that the PAH was metabolized through β-ketoadipic acid via protocatechuic acid (Kim et al., 2007; Kanaly and Harayama, 2010). Previously, reports of pyrene degradation by non-actinomycete bacteria have been scanty in literature.

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However, in the last fifteen years, new non-actinomycete bacteria that possess metabolic capabilities for pyrene degradation have been isolated from different contaminated systems. For instance, *Paracoccus* sp. strain Ophe1 utilized pyrene as a sole source of carbon and energy in addition to other HMW PAHs (Zhang et al., 2004). An enteric bacterium, *Leclercia adecarboxylata* strain PS4040 obtained from an oily sludge-contaminated soil, was shown to grow on pyrene resulting in nearly 60% degradation of 200 mg l^{-1} pyrene over an incubation period of 20 days (Sarma et al., 2004). In another study, Wang et al. (2008) reported that a deep sea bacterium identified as *Cycloclasticus spirillensus* strain P1 grew excellently well with pyrene. Obayori et al. (2008, 2009) also documented the isolation of pyrene-degrading *Pseudomonas aeruginosa* strains LP5 and LP6 as well as *Pseudomonas* sp. strain LP1 from contaminated soils in Lagos, Nigeria. With the exception of LP6, a poor degrader; the organisms, one of which was a biosurfactant producer, utilized over 66% of 100 mg l^{-1} pyrene in a 30-day incubation period.

Currently, the metabolic functions of microorganisms are being challenged by unquantifiable amounts of xenobiotics indiscriminately released into the environment. The effectiveness of bioremediation technologies will largely be dependent on rigorous sourcing for competent microbial strains that will not only attack the target pollutant but also degrade potential co-contaminants within the vicinity of the environmental matrix. The main objective of the present study was to screen for microorganisms with pyrene metabolic capability from two unusual environmental sources, namely plant leave surfaces and animal fecal material. The standard protocol for isolating microorganisms with ability to degrade environmental pollutants is to source them from contaminated sites through repeated enrichment procedures. This process has been mostly successful for the isolation and characterization of pyrene degraders which mostly are actinomycete bacteria. It appears, therefore, that the gene pool for pyrene degradation, even though scarce, is somewhat restricted to Gram-positive organisms. We reasoned that using alternative environmental sources, we might be able to isolate unusual microorganisms with unique metabolic capabilities. Interestingly, several workers have shown the abilities of some plants to induce metabolic capabilities in microorganisms including those for polychlorinated biphenyl (PCB) degradation (Gilbert and Crowley, 1997). Cow dung (CD) was recently shown to be rich in hydrocarbonoclastic organisms (Akinde and Obire, 2008; Agamuthu et al., 2013; Okoro et al., 2013). Field et al. (1993) established the association of lignin-degrading organisms with degradation of environmental pollutants. According to them, such organisms may be isolated from fecal materials obtained from animals that consume a woody plant diet. In another study, Juhasz and Naidu (2000) obtained multiple microorganisms from both contaminated and uncontaminated plant and animal fecal materials which were capable of degrading a wide range of pollutants such as PCBs, PAHs, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), and organochlorine pesticides. Previously too, we isolated several bacterial species belonging to different genera from the leave surfaces of *Citrus sinensis*, *Mangifera indica*, *Cola acuminata*, *Musa sapiensis*, *Annona muricata*, and *Terminalia catappa* (Ilori et al., 2006). These organisms grew very well on crude petroleum (CP) and petroleum derivatives. Although the bacteria were able to utilize the aliphatic components of the oil, none had the capacity to attack the aromatic fractions and PAHs and to the best of our knowledge, isolation of such organisms from phylloplanes is yet to be demonstrated for pyrene degradation.

2. Materials and methods

2.1. Chemicals

High purity (98%) pyrene, acetone and hexane were purchased from Sigma Aldrich (St. Louis, MO, USA). Escravos light crude petroleum was obtained from Chevron Nigeria Limited. All other chemicals were of analytical grade with high purity.

2.2. Sampling

Fresh leaf samples of *T. catappa* (tropical almond) located behind the Faculty of Science, University of Lagos, Nigeria were collected and placed in a sterile, polyethylene bag. The plant leaves used for this study were those attached to the third and fourth nodes below the terminal bud so as to ensure comparable leaf ages. Fresh CD was collected from cow abattoir in Ikorodu, Lagos and placed in sterile screw-capped bottles. All samples were promptly transported to the laboratory for analysis.

2.3. Enumeration of bacterial populations

Heterotrophic bacterial populations of both leaf and animal waste samples were analyzed by standard plate count techniques as previously described by Ilori et al. (2006) and Adebusoye et al. (2007). Populations of hydrocarbon utilizers were estimated on a mineral salts (MS) medium formulated by Kästner et al. (1994). The pH of the medium was adjusted to 7.2 and fortified with nystatin at 50 μg ml^{-1} to suppress fungal growth. Trace elements solution (1 ml l^{-1}) described by Bauchop and Elsden (1960) was sterilized separately and added aseptically to the medium. Prior to sterilization, the medium was solidified by bacteriological agar and handled as described by Adebusoye et al. (2007). CP served as the sole carbon and energy source and was made available to the cultures through vapor-phase transfer. Unless otherwise stated, all incubations were performed at room temperature (27.0 ± 2.0 °C) for 2–7 days.

2.4. Isolation of pyrene-degrading bacteria

Bacteria able to degrade pyrene were isolated by use of a repeated enrichment technique as described previously (Adebusoye et al. 2007, 2008). Briefly, 2 g of ground dried CD were added into 250 ml Erlenmeyer flask containing 50 ml MS medium. In the case of phyllospheric microorganisms, a sterile cork borer was used to punch a whole sample of *T. catappa* leaf. The leaf discs were then placed in a flask with 50 ml MS medium. The flask was vortexed vigorously to dislodge the phyllospheric organisms. All flasks were supplemented with 100 mg l^{-1} pyrene and incubated with shaking for 30 days. After four successive transfers, aliquots of an appropriate dilution of the enriched cultures were inoculated onto nutrient agar as well as MS agar. The MS agar was subsequently coated with a thin film of pyrene and incubated for 10 days. The colonies that appeared were purified and screened for pyrene and CP utilization before taxonomic characterization using an API 20 E test system (bioMerieux Vitek, Hazelwood, MO, USA).

2.5. Evaluation of CP and pyrene biodegradation

Hydrocarbon degradation was assayed by inoculating replicate 250 ml flasks containing 50 ml MS medium with fresh bacterial culture. Pyrene was added as a sole carbon and energy source at a concentration of 100 mg l^{-1} while CP was supplied at a concentration of 2.0% (v/v). Flasks inoculated with heat-killed cells served as controls. Biodegradation was monitored by determination of pH, total viable count (TVC) and residual hydrocarbon at