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Full Length Research Paper

Occurrence and growth potentials of hydrocarbon degrading bacteria on the phylloplane of some tropical plants

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The surface of leaf samples from ten tropical plants, *Anthocleista, Sarcophrynium, Canna, Colocassia, Musa, Cola, Citrus, Mangifera, Terminalia* and *Annona* were cultured for the estimation of total heterotrophic and hydrocarbon utilizing bacteria. The total heterotrophic bacteria ranged from 0.75 x 10^7 to 0.98 x 10^7 (cfu/cm^2) while the hydrocarbon utilisers ranged from 0.86 x 10^6 to 2.12 x 16^6 (cfu/cm^2). The percentages of hydrocarbon degraders were highest on *Mangifera* and lowest on *Colocassia.* Hydrocarbon degraders identified as *Acinetobacter, Flavobacterium,* and *Micrococcus* were obtained. Most of the organisms grew well on diesel. *Pseudomonas* sp. grew luxuriantly on diesel and kerosene while *Bacillus* sp. did not grow on kerosene. Optimal growth on the hydrocarbon occurred between the 8^{th} and 14^{th} day. It was therefore concluded that bacteria with ability to utilize hydrocarbons could be obtained from leaf surfaces. Such organisms could serve as seeds for bioaugmentation during remediation of polluted environments.

Key words: Phylloplane, bacteria, hydrocarbon, degradation, kerosene, diesel, lubricant.

INTRODUCTION

Plant surfaces and interior are important habitats for microorganisms. Indeed, some microorganisms are able to grow only in association with plants. Organisms found on the aerial parts of plants are sometimes called epiphytic microbes. Unlike the epiphytic plants that derive no nutrient from the plants surfaces they inhabit, epiphytic microbes actively utilize compounds exuded from leaves. Although noncolonising microbes frequently can be found in the phylloplane, there is substantial evidence of defined microbial succession and the development of specific phylloplane communities (Dandurand and Knudsen, 2002).

Numerous plants synthesized hydrocarbon in their tissues; such plants are variously referred to as fuel plants, biocrude producing plants or petrocrops (Swaminathan and Kochlar, 1989). The waxes that cover the leaves of plants contain hydrocarbons derived from mixtures of essential oils, cyclohexane and alkanes. Hydrocarbons found on plant surfaces are end or by products of metabolism. The amounts of wax present on the leaf surface of different plants are variable (Schlegel, 1995).

The ability of diverse microbial population to synthesize hydrocarbons has been known since the 19^{th} century (Rose, 1979). More than 100 species representing about thirty genera have been identified. These include mostly bacteria, actinomycetes, yeasts and filamentous fungi (Atlas, 1992). Microorganisms which oxidize various hydrocarbons are widely distributed in soil, water and on aerial surfaces. Arrival of most microorganisms or spores on leaf surface is mediated by air, rain splash, vector or brush contact. The presence of hydrocarbon on leaves thereafter results in the selective increase in the populations of hydrocarbon degraders (Atlas, 1978).

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Many of the phylloplane flora, especially bacteria, are able to degrade the n-alkane components of waxes. Chromogenic and Gram negative bacteria including species of *Xanthomonas*, *Flavobacterium*, *Pseudomonas* and *Erwinia* are common in this respect. Gram positive bacteria such as *Corynebacterium*, *Lactobacillus* and *Bacillus* also occur (Lynch and Hobbie, 1988). The phylloplane is therefore a training ground for many organisms with hydrocarbon degradation ability. Hydrocarbon degraders on leaves would augment hydrocarbon assimilatory capacity of an environment, especially pristine, after leaf defoliation. Presently, information and identity of such organisms existing on tropical plants is non-existent to the best of our knowledge. In this paper, we report the types of hydrocarbon degrading bacteria associated with common tropical phylloplane and the potential of the isolates to degrade some hydrocarbon substrates.

MATERIALS AND METHODS

Leaf samples

Leaf samples of ten plants located in various parts of the Lagos metropolis were used for the study. They include *Cola acuminata, Annona muricata, Citrus sinensis, Mangifera indica, Terminalia catappa, Colocasia* sp., *Musa sapientum, Anthocleista* sp., *Sarcophynium* sp., and *Canna* sp.

Media

Nutrient agar (Oxoid, UK) was used to isolate total heterotrophic bacteria from the leaf samples. Hydrocarbon utilizing bacteria were isolated using the minimal salts medium described by Mills et al. (1978). Sterilization was carried out by autoclaving at 121ºC for 15 min.

Isolation of total heterotrophic and hydrocarbon utilizing bacteria from leaf samples

A 12 mm sterile cork borer was used to punch each leaf sample over a surface area of 2.545 cm^3. The leaf discs were immediately placed inside McCartney bottles containing sterile distilled water (9 ml). The mouth of each bottle was covered tightly and then shaken vigorously in order dislodge the microorganisms on the leaf discs. The content of the bottles were serially diluted and aliquots (0.1 ml) plated on sterile nutrient and minimal salts agar in triplicates. Crude oil (Escravos light) which served as the only carbon source in the minimal medium was introduced by vapour-phase transfer by placing filter paper discs impregnated with the oil into the lids of the Petri dishes as described by Amund and Akangbou (1993). The plates were incubated at room temperature (30ºC ± 2) for 48 and 144 h for nutrient and minimal salts agar, respectively. After the incubation period, the microbial load of the different leaf samples on nutrient agar and minimal salts were compared and recorded. The cultural characteristics of isolates were observed while pure cultures were stored on nutrient agar slants at 4ºC.

Characterization of the isolates

Each isolate was examined many times for its size, shape, margin, consistency, opacity, elevation, pigmentation, Gram reaction and cell morphology as described by Cowan (1974). The isolates were characterized as described by Holt et al. (1994). Diagnostic properties used include motility, production of cytochrome oxidase, catalase, indole and urease, gelatin liquefaction, starch hydrolysis, oxidation/fermentation of sugars, methyl red test, Voges Proskaeur test, and growth at 42ºC and 5ºC.

Growth potential of hydrocarbon utilizing bacteria on diesel, kerosene and lubricating oil

Minimal salts medium (100 ml, pH 1.0) supplemented with trace elements solution (2.5 ml per litre) of Bauchop and Elsden (1960) were put into 500 ml Erlenmeyer flasks. The hydrocarbon substrates (0.1%, v/v); diesel, kerosene and fresh lubricating oil (SAE 40) were used as sole carbon sources. The media were inoculated with cells previously grown for 24h in nutrient broth and washed four times in phosphate buffer (pH 7.0) to remove traces of nutrient. Incubation was carried out at room temperature (30ºC ± 2). Growths of the organisms were assayed after 48 h by optical density (0D) measurement at 600 nm. Inoculated minimal salts medium without hydrocarbon served as control. Cultures without any increase in turbidity over the initial OD of test and control were scored as no growth (-), cultures with slight increase over initial OD but significantly greater than the control OD were scored as poor growth (+). Cultures with growth well above the initial were scored as moderate (++) while cultures with luxuriant growth were scored as heavy growth (+++). The time-course growth was monitored by total viable count on nutrient agar from the day of inoculation and subsequently after 48 h for 14 days.

RESULTS AND DISCUSSION

The populations of microorganisms on leaf surfaces will vary in size and diversity depending on the influence of numerous biotic and abiotic factors which affect their growth and survival. These factors include leaf age, external nutrients, interactions between populations of different microorganisms, temperature, relative humidity, duration of leaf wetness, light intensity, wind speed and the presence of air pollutants and pesticides (Bakker et al., 2002). The leaves of different plants studied harboured various heterotrophic bacteria. As shown in Table 1, *Anthocleista* sp. had the highest heterotrophic population ($0.98 \pm 0.3 \times 10^7$ cfu/cm^2) while *Colocassia* had the lowest ($0.71 \pm 0.2 \times 10^7$ cfu/cm^2). Hydrocarbon degrading bacteria were most abundant on the leaves of *Annona muricata* and least on the leaves of *Colocassia* sp. while the leaves of *Mangifera indica* had the highest percentage of hydrocarbon degraders. The hydrocarbon degraders were in the range of 12.1 – 26.3%. Hydrocarbon degraders in most pristine environment often constitute less than 10% of the total population. High populations of hydrocarbon degraders found on the leaves studied indicate prior exposure of these organisms to hydrocarbons. The hydrocarbons would certainly be of biogenic origin and mainly from the leaves. The sources of the organisms, however, could be the dust, air current and rain splashes. Their establishment on the leaf surface is aided by the presence of nutrients such as exudates or waxes from the cuticle (Rao, 1977). The leaves of *Mangifera indica* probably contained the highest amount of readily utilizable hydrocarbons.

Table 1. Populations of total heterotrophic and hydrocarbon-utilizing bacteria associated with selected tropical surfaces.

Leaf Sample	Total heterotrophic bacteria ($\times 10^7$ cfu/cm^2)	Hydrocarbon-utilizing bacteria ($\times 10^6$ cfu/cm^2)	% Hydrocarbon utilizers
Anthocleistra spp.	0.98 ± 0.3	1.53 ± 0.3	15.6
Sarcophrynium spp	0.75 ± 0.1	0.94 ± 0.1	12.5
Canna spp.	0.88 ± 0.1	1.38 ± 0.2	16.6
Colocassia spp	0.71 ± 0.2	0.71 ± 0.2	12.1
Musa sapientum	0.79 ± 0.2	0.79 ± 0.2	13.4
Cola acuminate	0.79 ± 0.3	1.45 ± 0.3	18.4
Citrus sinensis	0.91 ± 0.1	1.57 ± 0.2	17.3
Magnifera indica	0.79 ± 0.5	2.08 ± 0.2	26.3
Terminalia catappa	0.97 ± 0.1	1.77 ± 0.2	18.3
Annona muricana	0.85 ± 0.4	2.12 ± 0.1	24.9

Table 2. Growth potentials of isolates on some hydrocarbons.

Organism	Hydrocarbons		
	Diesel	Kerosine	Lubricating oil
Acinetobacter sp.	+++	+	+
Flavobacterium sp	++	+	+
Bacillus sp.	++	-	+
Pseudomonas sp	+++	+++	++
Alcaligenes sp	+++	++	++
Corynebacterium sp	++	++	++
Micrococcus roseus	+++	++	+
Micrococcus sp I	+	++	+
Micrococcus SP II	+	++	++

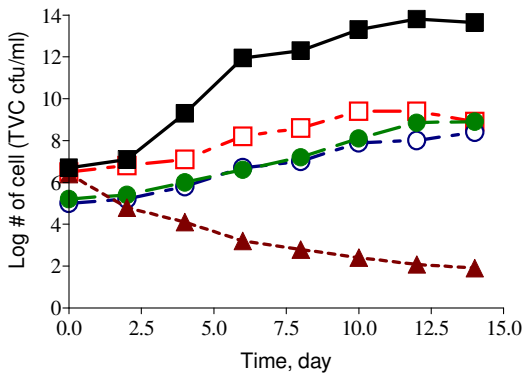

Figure 1. Growth dynamics of isolates on Kerosine. ■, *Pseudomonas aeruginosa*; □, *Alcaligenes* sp.; ●, *Micrococcus* sp. I; ○, *Micrococcus* sp. II; ▲, *Bacillus* sp.

Hydrocarbon utilisers belonging to seven genera were isolated from the various leaf surfaces and subsequently identified as *Acinetobacter*, Flavobacterium, *Bacillus, Pseudomonas, Alcaligenes* and *Corynebacterium*. Three of the isolates were *Micrococcus* sp., one of which was identified as *Micrococcus roseus*. The results of the biodegradation potentials of the isolates are presented in Table 2 and Figures 1 - 3. *Pseudomonas* sp. also grew luxuriantly on kerosene; its peak of growth on the hydrocarbon occurred on the 12th day (Figure 1). Moderate growth on kerosene was recorded by cultures of *Alcaligenes* sp., *Corynebacterium* sp. and *Micrococcus* sp. The hydrocarbon could, however, not be utilized by *Bacillus* sp. Kerosene had been reported to have the capacity to disrupt the membrane properties of many microorganisms (Betts, 1993). The organisms utilized diesel oil to varying degrees. Luxuriant growth was recorded by cultures of *Acinetobacter* sp., *Pseudomonas* sp., *Alcaligenes* sp. and *Micrococcus roseus*. The 10th day was the peak of growth of *Pseudomonas* sp., *Bacillus* sp. and *Micrococcus* sp. 1 on diesel. *Alcaligenes* sp. and *Micrococcus* sp. however had their optimal growth on the hydrocarbon on the 12th day (Figure 2). Moderate growth was obtained on the lubricating oil by some of the organisms as shown in Table 2. *Alcaligenes* sp. grew best on the lubricant; its peak of growth was on the 10th day (Figure 3). The poorest growth on the lubricant was by *Micrococcus* sp. I.

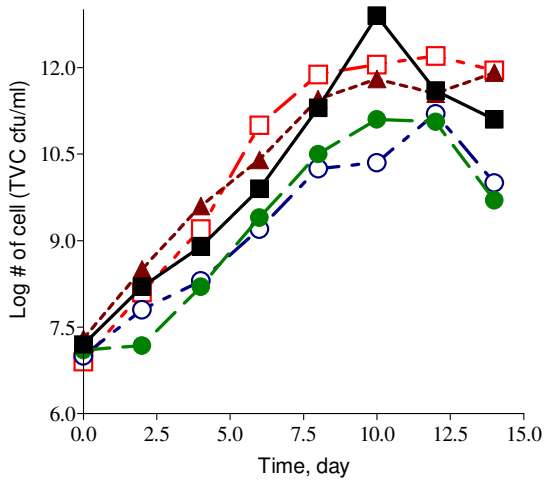

Figure 2. Growth dynamics of isolates on Diesel. ■, *Pseudomonas aeruginosa*; □, *Alcaligenes* sp.; ●, *Micrococcus* sp. I; ○, *Micrococcus* sp. II; ▲, *Bacillus* sp.

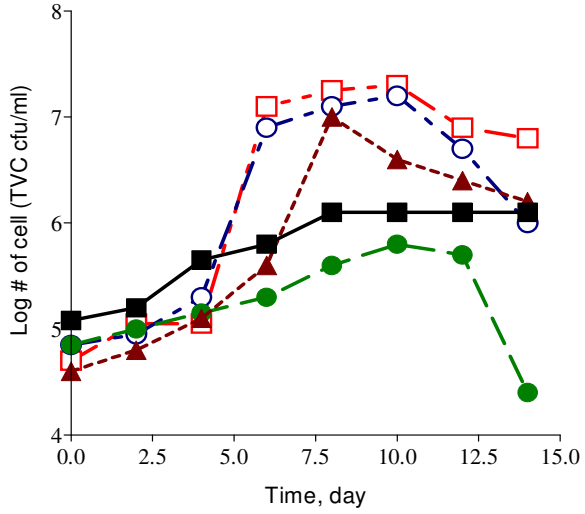

Figure 3. Growth dynamics of isolates on Lubricating oil SAE 40. ■, *Pseudomonas aeruginosa*; □, *Alcaligenes* sp.; ●, *Micrococcus* sp. I; ○, *Micrococcus* sp. II; ▲, *Bacillus* sp.

Petroleum hydrocarbons play important roles in every community as sources of energy for lighting, cooking and for lubrication. Bulk carriages from point of production to vending stations often involve accidental discharge and pollution of lands and bodies of waters. Sources of organisms with potential to degrade the pollutants are of utmost importance in any environmental pollution contingency plans. In conclusion, the results of this work have shown that hydrocarbon degrading bacteria could readily be isolated from the leaves of tropical plants for possible deployment as seed organisms in biore-mediation processes.

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