Bull Environ Contam Toxicol (2012) 89:727–732
DOI 10.1007/s00128-012-0796-3

Growth Study and Hydrocarbonoclastic Potential of Microorganisms Isolated from Aviation Fuel Spill Site in Ibeno, Nigeria

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Received: 27 June 2012 / Accepted: 16 August 2012 / Published online: 26 August 2012

Abstract The growth study and hydrocarbonoclastic potential of microorganisms isolated from aviation fuel spill sites at Inua-eyet Ikot in Ibeno, Nigeria were examined using standard microbiological methods. The results of the analysis revealed that the viable plate count of microorganisms in the polluted soil ranged from $2.2 \pm 0.04 \times 10^3$ to $3.4 \pm 0.14 \times 10^6$ cfu/g for bacteria and $1.4 \pm 0.5 \times 10^2$ to $2.3 \pm 0.4 \times 10^4$ cfu/g for fungi while count of biodegraders ranged from $1.2 \pm 0.4 \times 10^3$ to $2.1 \pm 0.8 \times 10^5$ cfu/g. A total of 11 microbial isolates comprising of *Micrococcus*, *Klebsiella*, *Flavobacterium*, *Bacillus*, *Pseudomonas*, *Candida*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Saccharomyces* and *Fusarium* were characterized. The ability of the selected isolates to utilize the pollutant (aviation fuel) as their sole source of carbon and energy was examined and noticed to vary in growth profiles between the isolates. The results of their degradability after 28 days of incubation shows that species of *Cladosporium*, *Pseudomonas*, *Candida*, *Bacillus*, *Micrococcus* and *Penicillium* were the most efficient Aviation fuel degraders with percentage weight loss of 86.2, 78.4, 78, 56, 53 and 50.6 respectively. *Flavobacterium*, *Saccharomyces* and *Aspergillus* exhibited moderate growth with percentage weight loss of 48, 45.8 and 43.4 respectively while *Klebsiella* and *Fusarium* species showed minimal growth with percentage weight loss of 20 and 18.5 respectively. The results imply that the most efficient biodegraders like *Cladosporium*, *Pseudomonas*, *Candida*, *Bacillus* and *Microoccus* could tolerate and remove aviation fuel from the environment.

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Keywords Growth study · Hydrocarbonoclastic potential · Microorganisms · Aviation fuel

The Niger Delta, West Africa, is vulnerable to hydrocarbon pollution. The most common hydrocarbon contaminant source in the Niger Delta is crude oil. This contaminants present serious environmental problem (Vandermeulen and Lee 1986). They may affect physiological processes, population and behavioral profiles of organisms (Okpokwasili and Odukuma 1994). Catalytic cracking of crude oil provides a source for various fuels such as gasoline, jet fuel, domestic kerosene and diesel oil (Murray 1980) which are commonly used in the Niger Delta. Aviation fuel is a mixture of hydrocarbons whose boiling point is below 200°C. It is obtained from distillation of petroleum. It is known to volatilize readily into the air to form a flammable mixture of alkanes (paraffins), hexane, heptane and octane.

The continuous input of petroleum-based pollutants has resulted in an enriched microbial community capable of surviving toxic contamination. Microorganisms are sensitive to fluctuations/changes in their environment. Whenever their chemical or physical environment is suddenly altered, there is a lag period during which the microbial community adapts to the new conditions (Chikere and Okpokwasili 2004; Nweke and Okpokwasili 2004). This lag period is also called acclimatization period and enables the microorganisms to acquire the metabolic repertoire necessary for their survival (Yakimov et al. 2007). This phenomenon has been shown to occur both in terrestrial and aquatic ecosystems (Macnaughton et al. 1999; Margesin et al. 2007).

Several studies have revealed that the microbial community composition in hydrocarbon-polluted soil tend to comprise mostly bacteria and fungi that are specially adapted to use hydrocarbons as carbon source (Engelhardt et al.

2001; Kasai et al. 2002). Information on the composition of the microbial populations in a polluted site is of valuable importance in order to estimate the self-purification capability of the ecosystem and the feasibility of biological decontamination if engineered bioremediation should be considered (Allen et al. 2007; Said et al. 2008). Oil-degrading microorganisms are usually abundant in chronic crude oil-polluted ecosystems and tend to degrade specific petroleum hydrocarbons, aliphatic and aromatic, or both (Essien et al. 2003). In this study, microorganisms associated with aviation fuel were enumerated, characterized identified and their growth and hydrocarbonoclastic potentials determined using aviation fuel supplemented medium.

Materials and Methods

The study area is the Mobil Producing Nigeria's (MPN's) ruptured pipeline. The rupture occurred on 8 August, 2001 along the 30-year-old Qua Iboe Terminal (QIT)-jetty aviation fuel pipeline which resulted in the spillage of about 1,000 bbls of aviation fuel into the environment, at Inua Eyet Ikot, Ibeno Local Government Area of Akwa Ibom State, Nigeria.

Soil samples were taken from aviation fuel spill sites at Inua Eyet Ikot in Ibeno-Nigeria placed into sterile bottles and transported immediately in cold storage containers to the laboratory for further work. International jet A-1 fuel was aseptically collected from aircraft tank at local airport into sterile plastic container. The samples were analyzed within 6 h of collection. Standard plate counts method was used for heterotrophic bacteria using tryptone soya agar (TSA) supplemented with filter-sterilized cycloheximide antibiotics (40 g/mL) (Sigma Chemical Co. USA) to inhibit fungal growth. The mycological count was measured by the spread plate method using potato dextrose agar (PDA) supplemented with 0.5 mg streptomycin/L to inhibit bacterial contaminants. Mineral salts agar (MSA) was used to determine total aviation fuel-degrading microorganisms employing vapour phase transfer (VPT) technique (Okpokwasili and Okorie 1988); in this method, sterile Whatman No. 1 filter papers saturated with sterile crude oil were aseptically placed on the covers of inverted petri dishes immediately after inoculation. Inoculated TSA, PDA and MSA plates were incubated at room temperature (28°C) for 2, 5 and 14 days respectively before enumeration. Pure culture of the microbial isolates obtained from the agar plates were characterized and identified according to the procedures described by Cowan (2003) and Holt et al. (1994) for bacterial identification and Barnett and Hunter (1987); Samson et al. (1984) for fungal identification.

Pure isolates obtained were inoculated onto mineral salts medium supplemented with 1 % v/v aviation fuel and incubated for 14 days at 28°C. Cultures without increase in turbidity over initial optical density and non-inoculated control were scored as no growth (−) while cultures with increased turbidity significantly greater than the control were scored as growth (+) i.e., growth attenuance (optical density OD reading above 0.2). The population dynamics of the inocula in the MSM were determined 48 hourly by cultural technique as an index of their ability to utilize aviation fuel as their sole source of carbon and energy. Growth of the test organisms was also scored as abundant or high (+++), moderate (++) and minimal (+) depending on degree of turbidity. The degradation rates of selected isolates were determined using aviation fuel supplemented medium. Two hundred milliliter of sterile mineral salt medium (MSM) was dispensed into sterile 250 mL capacity Erlenmeyer flasks. Two milliliter of filter-sterilized aviation fuel was added to each of the flasks and 0.2 mL of 18 h old tryptone soya broth culture of test organisms was inoculated also to each of the flasks. Non-inoculated flask served as control for each culture. The cultures were incubated on orbital shaker (SGM-300, Gallenkemp, England) at 120 rev/min for 28 days. During incubation, representative samples were taken 96 hourly for determination of pH, optical density (OD) and the residual aviation fuel. The pH was determined using a pH meter (EIL 7020, Kent Industrial Measurement Ltd). The OD was determined at 560 nm wavelength using the spectrophotometer (Spectronic 20, Milton Roy Co. New York) while the residual aviation fuel was determined gravimetrically using diethyl ether as the extraction solvent. This method has earlier been adopted by many authors (Ijah and Ukpe 1992; Itah and Essien 2005). For each sample, 5 mL of diethyl ether was added and vigorously shaken manually. The mixtures were then separated using a laboratory funnel and evaporated at room temperature to remove the solvent thus leaving the residual aviation fuel. The weights of the aviation fuel residues were then determined using a standard curve. The percentage biodegradation rate of the aviation fuel was determined from the mathematical relationship:

$$\% \text{ Degradation} = \frac{a - b}{a} \times \frac{100}{1}$$

where a is the weight of aviation fuel (control), b is the weight of aviation fuel remaining in each case.

Results and Discussion

The relatively high potentials of microorganisms in aviation fuel spill site at Inua Eyet Ikot in Ibeno, Nigeria were investigated. Table 1 revealed a mean total heterotrophic bacterial and fungal counts range of 2.2 (0.01) $\times$ 10^3–3.4 (0.14) $\times$ 10^6 cfu/g and 1.4 (0.5) $\times$ 10^2–2.3 (0.4) $\times$ 10^4 cfu/g

respectively while the count of oil degraders ranged from 1.2 (0.4) $\times$ 10^3–2.1 (0.4) $\times$ 10^5 cfu/g. This was twice higher than the magnitude of the fungal counts recorded for the aviation fuel spill soil. These findings are in agreement with the report that soil polluted with hydrocarbon product harbor a wide variety of micro-organisms most of which are hydrocarbonoclastic (Hubert et al. 1999; Wilson and Bouwer 1997). The hydrocarbonoclastic microorganisms utilize the aviation fuel for growth and proliferation as depicted by level of turbidity produced in mineral salts medium supplemented with aviation fuel. Table 2 shows the frequency occurrence of the microbial isolates with *Pseudomonas aeruginosa* having the highest frequency of ten while *Klebsiella* sp. was the least among the bacterial isolates. The most frequently occurring fungi were *Candida albicans*, *Cladosporium resinae* and *Candida tropicalis* while the least was *Fusarium semitectum*. However, the ability of the isolates to utilize and proliferate as depicted by the level of turbidity produced in the MSM-supplemented with aviation fuel revealed that *P. aeruginosa*, *Micrococcus varians*, *Bacillus subtilis*, *C. albicans*, *C. resinae* and *Penicillium citrinum* exhibited abundant growth on MSM-supplemented with aviation fuel indicating a strong potential to degrade the fuel. *Flavobacterium oderatum*, *Saccharomyces cerevisiae* and *Aspergillus fumigatus* exhibited moderate growth and ability in utilizing MSM-supplemented with aviation fuel. *Klebsiella* sp. and *F. semitectum* showed minimal growth in utilizing MSM-supplemented with aviation fuel. The varying degrees of aviation fuel utilization and degradation by some isolates could be attributable to their ability to produce elaborate vital hydrogenages enzymes required for degradation of recalcitrant components of the fuel as earlier reported (Itah and Essien 2005). Tables 3 and 4 reveals that some isolates degraded the fuels by causing weight loses of the original fuel in varying degrees and metabolized the fuel as their sole source of carbon and energy for growth and development. The fungus with the highest aviation fuel degrading potential was *C. resinae* which had a high utilization ability (+++) and caused a weight loss of 20.8 %–86.2 % of the original fuel over 24 days incubation period at room temperature. This was closely followed by *C. albicans* 78 %, *C. tropicalis* 70.8 %, *P. citrinum* (50.6 %). Amongst the bacteria *P. aeruginosa* had the highest fuel utilization (+++) and degrading capabilities ranging from 15 %–78.4 % after 24 days of incubation at room temperature. This was closely followed by *B. subtilis* (8 %–56 %), *M. varians* (10 %–53 %), and *F. oderatum* 5 %–48 %. The weak degradability demonstrated by and *Klebsiella* sp. (3 %–20 %) and *F. semitectum* (12.6 %–18.5 %) despite their impressive formation of turbidity or optical density (OD) level suggests that the ability of microorganisms to produce turbidity in culture media might not necessarily be an indication of its hydrocarbon-degrading capability.

Table 2 Frequency of occurrence of microbial isolates from aviation fuel contaminated soil

Microorganisms	Frequency of occurrence	Growth in fuel mineral salt broth
Bacillus subtilis	10	+++
Micrococcus varians	7	+++
Flavobacterium oderatum	6	++
Pseudomonas aeruginosa	10	+++
Klebsiella sp.	5	++
Saccharomyces cerevisiae	6	++
Candida albicans	10	+++
Candida tropicalis	8	++
Cladosporium resinae	10	+++
Penicilliun citrinium	6	++
Aspergillus fumigatus	7	+++
Fusarium semitectum	4	+

+++ High/abundant growth
++ Moderate growth
+ Minimal growth

The growth study of selected aviation fuel degrading bacteria based on their optical density OD at 560 nm wavelength and pH of cultures at 28°C are presented in

Table 1 Mean counts of microorganisms isolated from aviation fuel contaminated soil

No. of sample weeks	Heterotrophic bacteria (cfu/g)	Fungi count (cfu/g)	Aviation fuel degrading microorganism (cfu/g)
1.	$2.2 \pm 0.01 \times 10^3$	$2.46 \pm 0.4 \times 10^2$	$2.24 \pm 0.04 \times 10^2$
2.	$2.42 \pm 0.08 \times 10^3$	$2.48 \pm 0.15 \times 10^2$	$3.56 \pm 0.14 \times 10^2$
3.	$2.32 \pm 0.10 \times 10^3$	$1.4 \pm 1.5 \times 10^2$	$3.42 \pm 0.08 \times 10^2$
4.	$1.28 \pm 0.12 \times 10^4$	$2.28 \pm 0.08 \times 10^3$	$2.45 \pm 0.16 \times 10^3$
5.	$1.68 \pm 1.8 \times 10^4$	$1.62 \pm 0.10 \times 10^4$	$3.16 \pm 0.18 \times 10^3$
6.	$2.14 \pm 0.18 \times 10^4$	$1.48 \pm 0.18 \times 10^4$	$2.86 \pm 0.10 \times 10^3$
7.	$2.58 \pm 1.2 \times 10^5$	$1.24 \pm 1.18 \times 10^4$	$2.54 \pm 1.2 \times 10^3$
8.	$3.4 \pm 0.4 \times 10^5$	$1.28 \pm 0.12 \times 10^3$	$3.66 \pm 1.8 \times 10^4$
9.	$1.84 \pm 1.2 \times 10^5$	$2.44 \pm 0.16 \times 10^2$	$3.82 \pm 1.6 \times 10^4$
10.	$2.96 \pm 1.6 \times 10^4$	$2.32 \pm 1.2 \times 10^3$	$2.18 \pm 0.08 \times 10^3$

Table 3 Weight losses from aviation fuel resulting from growth of hydrocarbonoclastic fungi

Incubation period (days)	*Saccharomyces cerevisae*	*Cladosporium resinae*	*Candida albicans*	*Aspergillus fumigatus*	*Penicillium citrinium*	*Fusarium semitectum*	*Candida tropicalis*
2	15.9	20.8	18.5	16.4	17	12.6	17.8
4	18.6	25.4	22.2	19.2	19	14.2	20.4
6	21.8	30.5	28.6	22.4	24.4	15.8	26
8	28.4	36.8	32.4	26.8	28.2	16.2	30.6
10	36.6	52.6	38.8	32.2	36.6	16.8	48.6
12	40.2	74.8	60.5	42.7	44.4	17.8	56.8
14	45.8	86.2	78	43.4	50.6	18.5	70.8

Table 4 Weight losses from aviation fuel resulting from growth of hydrocarbonoclastic bacteria

Incubation period (days)	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Klebsiella* sp.	*Micrococcus varians*	*Flavobacterium oderatum*
2	8	15	3	10	5
4	12	18	9	14	10
6	16	22	10	18	16
8	20	28	12	22	20
10	26	34	15	32	30
12	40	58	18	44	38
14	56	78.4	20	53	48

$$\text{Weight losses } (\%) = \frac{\text{Wt.of fuel(control)} - \text{Wt.of fuel(degraded)}}{\text{Wt.offuel(control)}} \times 100$$

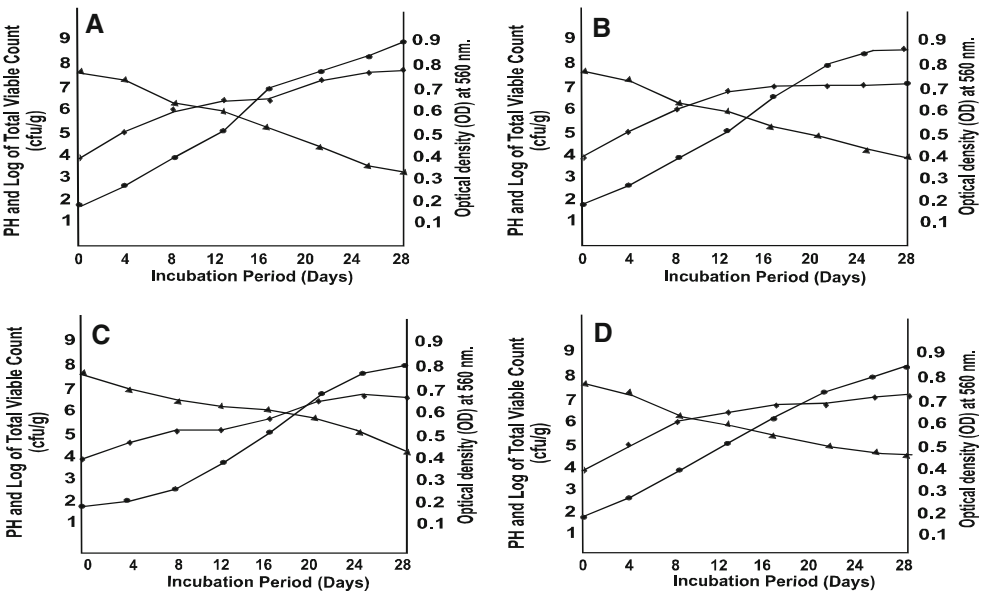

Fig. 1 Growth profile of **a** *C. resinae*, **b** *C. albicans*, **c** *C. tropicalis*, **d** *P. aeroginosa* in mineral salt medium supplemented with aviation fuel. *Filled triangle* pH, *filled diamond* Log Tvc, *filled circle* OD 560 nm

Figs. 1, 2, 3; *C. resinae* recorded the highest OD of 0.9 after 28 days of incubation (Fig. 1a). This was closely followed by *C. albicans* (Fig. 1b) and *P. aeruginosa* (Fig. 1d). Both organisms recorded 0.86 in MSM-supplemented with the fuel after incubation for the same period. *B. subtilis* and *M. varians* also exhibited remarkable levels of attenuance (Fig. 2a, b) while *S. cerevisiae*, *A. fumigatus* (Fig. 2c, d) as well as *F. oderatum* and *P. citrinium* (Fig. 3a, b) recorded moderate attenuance. However, *Klebsiella* sp. and *F. semitectum* (Fig. 3c, d) exhibited very low attenuance at 560 nm over 28 days of incubation. The results revealed a decrease in pH of the growth media over

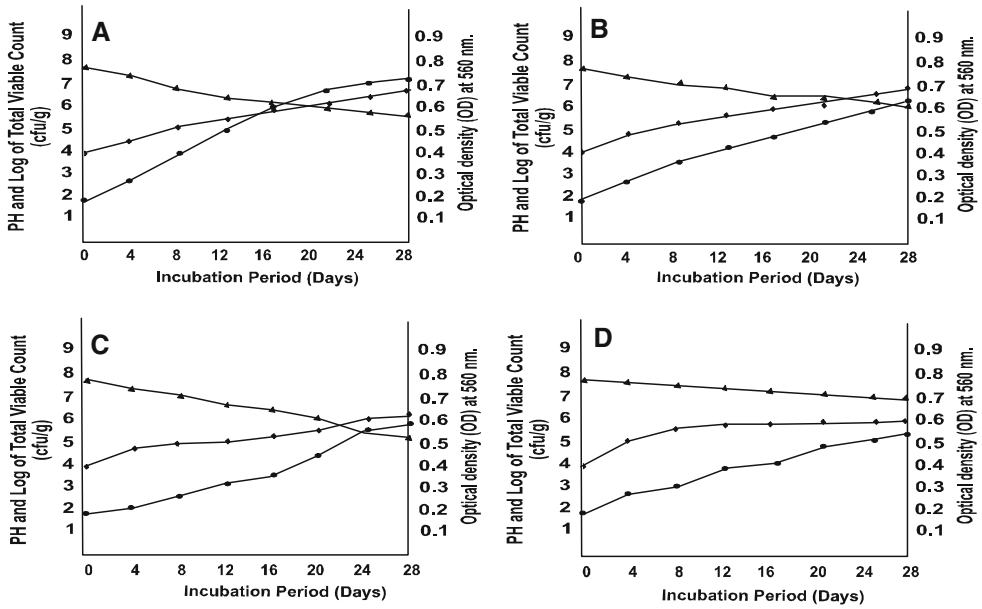

Fig. 2 Growth profile of **a** *B. subtilis*, **b** *M. varians*, **c** *S. cerevisiae*, **d** *A. fumigatus* in mineral salt medium supplemented with aviation fuel. *Filled triangle* pH, *filled diamond* Log Tvc, *filled circle* OD 560 nm

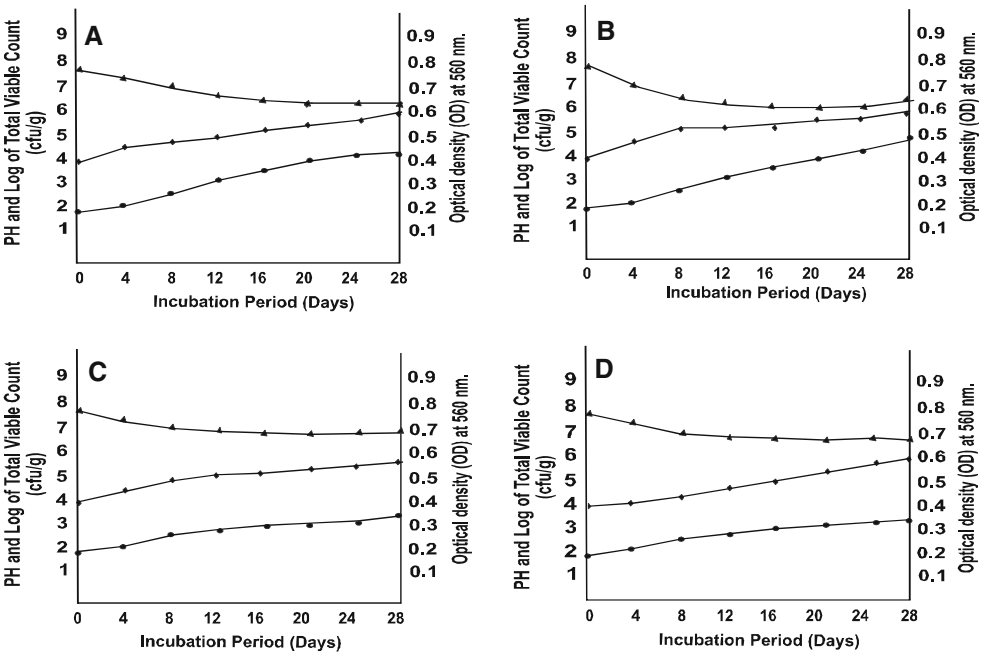

Fig. 3 Growth profile of **a** *F. oderatum*, **b** *P. citrinium*, **c** Klebsiella sp., **d** *F. semitectum* in mineral salt medium supplemented with aviation fuel. *Filled triangle* pH, *filled diamond* Log Tvc, *filled circle* OD 560 nm

time due to production of acidic metabolites and increase in temperature of the fuel supplemented media probably because biodegradation is an exothermic process. This, however, varied depending on the microorganisms involved and their ability to produce relevant enzymes. Okpokwasili and Okorie (1988) reported that bacteria are the principal biodegraders of fuel when pH is near neutral but later taken over by molds and yeast as the pH becomes acidic. However, fungi such as *A. fumigatus*, *Penicillium* spp. and *Candida* spp. which are known to grow best at acidic pH can exhibit some degree of tolerance for bacteriological media or environment of alkaline or near alkaline pH. It is therefore not surprising to find diverse genera and species of yeasts, molds and bacteria in soil contaminated with aviation fuel. It would imply that aviation fuels contaminated soil harbor both acid tolerant bacteria as well as yeast and molds capable of tolerating alkaline pH in alkaline environment. As a result of the synergistic interactions of the microbial consortia in the fuel, degradation of the fuel would be enhanced. This is in consonance with earlier observations by Walker and Colwell (1975) that reported enhanced hydrocarbon fuel degradation when there is associated growth of microorganisms. Some microorganisms possess the special attributes of initiating

degradation of larger molecules to smaller fragments for subsequent degradation by others. A complete biological degradation of aviation fuel microorganisms would yield carbon dioxide, water, sulphates, nitrates and methane as major products. Intermediate products include acids, ketones, aldehydes, alcohols, peroxides and sulphoxides which may be deposited at the bottom of fuel tanks (Itah et al. 2009).

The present study has revealed a relationship between growth profile and aviation fuel degradation. The levels of attenuance and extent of pH recorded reflected the aviation fuel-degrading capacities of most hydrocarbonoclastic microorganisms except *Klebsiella* sp. and *F. semitectum.* It is established that the higher the attenuance of the cultures, the more acidic the growth medium and probably the more capable the organism to degrade recalcitrant hydrocarbons. This relationship is supported by the superior hydrocarbonoclastic potential of *C. resinae*, *C. albicans* and *P. aeruginosa*. These organisms may be the major degraders associated with disappearance of aviation fuel from contaminated environments.

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