Assessment of the Effect of Kerosene Spill on Microbial Population of Soil Ten Years after the Spill at Maikunkele, Niger State Nigeria.

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Abstract- Assessment of the effect of kerosene spill on microbial properties of soil ten years after the spill at Maikunkele, Niger State was carried out. The counts of total aerobic heterotrophic bacteria (AHB) ranged from $1.1x10^6$cfu/g to $1.9x10^6$cfu/g of soil in the kerosene polluted soil (KPS) as compared to $1.3x10^6$cfu/g - $2.1x10^6$cfu/g of soil in the kerosene free soil (KFS). However, the spill promoted the growth of kerosene utilizing bacteria (KUB) in the soil. The population of KUB in the KPS ranged from $1.1x10^5$cfu/g to $1.7x10^5$cfu/g as compared to $1.0x10^5$cfu/g-$1.6x10^5$cfu/g in the KFS. The results obtained also revealed that the counts of fungi were higher in KFS ($1.3x10^3$cfu/g-$2.1x10^3$cfu/g) than in KPS ($1.1x10^3$cfu/g-$1.6x10^3$cfu/g). The kerosene utilizing microorganisms were identified as species of *Bacillus, Micrococcus, Acinetobacter, Alcaligenes, Pseudomonas, Aspergillus, Fusarium, Mucor* and *Penicillium.* The amount of carbon dioxide evolved by *Bacillus subtilis* strains (KUB07 and KUB04) was relatively higher compared to the amount of carbon dioxide evolved by other strains and mixed culture after 16 days, meaning that there may be no special benefit in using mixed culture in the bioremediation of kerosene pollute environment. Thus *Bacillus strains* KUB04 and KUB07 can be used in reclaiming the kerosene polluted soil.

Index Terms- Kerosene spill, Soil, Micro-organism, Bioremediation, Carbon dioxide evolution

I. INTRODUCTION

Oil spills on land affect plants, animals, soil microbes and physicochemical properties of soil. Oil spills kill or inhibit the continuous growth of an already established vegetation. It deprives plants of oxygen availability and decreases moisture and air availability in soil for plant use. Such anaerobic conditions may give rise to microbial generation of compounds that are toxic to plants, such as hydrogen sulphide. The exhaustion of oxygen could be due to use of the oxygen by petroleum degrading microorganisms (Ijah *et al.*, 2000; Okoh, 2006). Terrestrial animals suffer much from oil spills due to toxicity of some petroleum components, particularly the low boiling compounds. Oil blocks the respiratory channels of arthropods and interferes with their respiration (Okoh, 2006). When petroleum spill in soil the physicochemical properties of the soil undergo major alterations which affect the growth of plants and microbial activities. A typical microbial growth curve is observed when oil is introduced to soil. A short lag period is followed by a rise in numbers of microorganisms until maximum number of growth is reached. The decrease in microbial number in soil immediately after oil addition is due to the unfavourable condition the oil creates. However, the high microbial numbers, particularly the oil utilizers could be due to nutrients provided by the oil contaminant (Ojo, 2005). Thus, the level of hydrocarbon-utilizing microorganisms may reflect the degree of pollution of the ecosystem (Ijah *et al.,* 2000; Bragg *et al*., 2010). In Nigeria, most of the terrestrial ecosystem in oil producing and non oil producing communities are important agricultural land under cultivation. Any contact with petroleum hydrocarbon results in damage to soil condition of this agricultural lands, microorganisms and plants (Onuoha *et al.,* 2003). Petroleum hydrocarbon polluted soils are of environmental concern because they are unsuitable for agriculture and recreational uses, and are potential sources for surface and ground water contamination. Generally speaking, high concentration of petroleum hydrocarbon in an environment is harmful to soil biota and crop growth. When natural ecosystems are contaminated with petroleum hydrocarbons, the indigenous microbial communities are likely to contain microbial populations of different taxonomic characteristics, which are capable of degrading the contaminating hydrocarbons (Antai, 1990; Ijah and Ukpe, 1992; Ijah *et al*., 2000; Ijah and Antai, 2003). Microbial degradation process aids the elimination of oil spill from the environment after initial removal of large amounts of the oil by various physical and chemical methods. This is possible because microorganisms have enzymatic systems to degrade and utilize petroleum as a source of carbon and energy (Ijah and Antai, 2003). This advantage has been used in the current area of bioremediation of oil spills. Petroleum degrading microorganisms isolated from the Nigerian environment include species of *Bacillus, Pseudomonas, Candida, Streptomyces, Penicillium, Aspergillus and Torulopsis* (Akpor, 2007). The main aim of the study was to assess the microbiological properties of the soil impacted by kerosene at Maikunkele, Niger State ten years after the spill, to ascertain if the impacted area has recovered completely. The specific objectives of the study were to assess the effect of the kerosene on the microbial population of the soil, to isolate and identify microorganisms in the kerosene polluted soil, and to assess the rates and the total extent at which residual kerosene in the soil was degraded.

II. Materials and Methods

Description of Study Site: The study site was kerosene spilled soil at Maikunkele, Bosso Local Government Area of Niger State, Nigeria. The kerosene spill covered an area of 1800m^2. The spill occurred in August 1998, when a tanker carrying several thousands litres of kerosene spilled its content on a field near Airport Junction at Maikunkele, Bosso Local Government Area of Niger State (Ijah *et al.*, 2000). The spillage withered grasses and shrubs for a period of over one year (Ijah *et al.*, 2000). At the time of first sampling in April 2008, the affected site was covered with grasses such as *Ajaratum coinzoides, Cylindrica indica, Sida acuta, Bohavia diffusa* and *Aspilla africana*, similar to the unpolluted control site. The soil particle analysis revealed that the study site is made up of coarse sand, fine sand, and clay. Thus, the soil is a sandy loam soil with a good drainage system.

Collection of Samples: The kerosene polluted site divided into two plots of 900m^2 each. Four soil samples were collected from each plot at random, making a total of eight bulk samples. The kerosene free soil was also divided into two plots of 900m^2 each and the samples were similarly collected. The samples were collected in polythene bags each month, for a period of six months (April-September) and transported to the laboratory for analysis.

Enumeration of Microorganisms: Ten grammes (10g) of the soil samples were serially diluted and plated on Nutrient agar (NA), Sabouraud dextrose agar (SDA) and kerosene agar (KA), for the enumeration of total aerobic heterotrophic bacteria, fungi and kerosene utilizing bacteria respectively. The NA and KA plates were incubated at 30^{0}C for 48 h while SDA plates were incubated at room temperature (28 ± 2^{0}C) for 5 days. Colonies which developed on the plates were counted and recorded as colony forming units per gramme (cfu/g) of soil. The isolates were subcultured repeatedly to obtain pure cultures. The pure cultures were stored on slants in the refrigerator for further characterization and identification. The isolates were characterized using the following biochemical tests: Gram staining, Methyl red-Voges proskauer (MR-VP) tests, indole test, sugar fermentation, coagulase test, catalase test, motility test and spore staining test. The bacteria isolate were identified by comparing their characteristics with those of known taxa using the scheme of Cowan (1970).

The fungi isolates were characterized based on the colour of aerial and substrate hyphae. Furthermore, the nature of hyphae and shape as well as the presence of special structures such as rhizoid were noted. Other structures were examined using the method described below: The characteristics of the fungi isolates were compared with those of known taxa using the scheme of Domsch and Gams (1970) to identify the fungi.

Utilization of Kerosene by Microbial Isolates: Mineral salt medium of Zajic and Supplisson (1972) containing kerosene as the only source of carbon was inoculated with 0.1ml (10^{3}cells) nutrient broth grown culture of kerosene utilizing bacteria isolates and incubated at 30^{0}C for 4 days. Similarly, Bushnel and Haas (1941) medium was inoculated with fungi spores and incubated for 7 days. Turbidity produced as a result of microbial growth on kerosene was monitored visually at the end of the incubation period and assigned (+) to (+++) depending on the intensity of the growth.

Determination of Rates of Kerosene Degradation by Bacteria Isolates: Carbon dioxide ($C0_2$) evolution method of Stolky (1965) was used to measure the rates of kerosene degradation by the bacterial isolates

Two hundred and fifty milliliters (250ml) of Zajic and Supplisson (1972) medium plus 1.25ml of kerosene in twenty four samples of screw capped bottles were sterilized by autoclaving at 121^{0}C for 15minutes. 0.1ml of nutrient broth culture of kerosene utilizing bacteria such as species of *Bacillus* (KUB03, KUB04, KUB07, KUB08) were aseptically inoculated into the kerosene medium. One gramme of Barium peroxide was mixed with 5ml of distilled water in a plastic vial and the mixture was lowered into each of the screw capped bottle and incubated to absorb the carbon dioxide liberated during kerosene degradation. A control (with no organism was equally set up). At the end of each incubation period of four days interval for a period of sixteen days at 30^{O}c, the vials containing barium carbonate and barium hydroxide ($BaCO_3$ and BaOH) were washed with 40ml distilled water into 250ml conical flask and the residual barium hydroxide (BaOH) was titrated 2N HCL using phenolphthalein (1 or 2 drops) as indicator. The amount of carbon dioxide produced by the bacteria that utilized the hydrocarbon was estimated by Stolky's (1965) formula given below:

(B-V)NE

Where;

B = Volume (ml) of acid used to titrate the alkaline in carbon dioxide collectors from control to end point.

V =Volume (ml) of acid used to titrate the alkaline in the carbon dioxide collectors from treatment to end point.

N=Normality of acid.

E=Equivalent weight, if data are expressed as CO_2, E = 22 but if expressed as C, E=6.

III. Statistical Analysis

The microbial counts and physicochemical properties in this study were analyzed statistically using parametric tests involving the Analysis of Variance (ANOVA).

IV. Results

The results showed that the counts of total aerobic heterotrophic bacteria (AHB) in kerosene polluted soil ranged from 1.1 × 10^6 cfu/g to 1.9 × 10^6 cfu/g of soil while in kerosene free soil the counts ranged from 1.3 × 10^6 cfu/g to 2.1 × 10^6 cfu/g of soil. The counts of AHB in kerosene polluted soil decreased rapidly from April to May, and remained unchanged until August when the counts decreased slightly (Fig.1).

Conversely, the counts of AHB in kerosene free soil increased gradually from April till July, when the highest counts (2.1 x 10^6 cfu/g) were recorded. Thereafter the counts decreased gradually (Fig.1). Generally, the counts of AHB were higher in kerosene free soil than kerosene polluted soil (Fig.1). Statistical Analysis using Analysis of Variance ANOVA, revealed that the difference in counts between the two sites was not significant (P>0.05).

The counts of kerosene utilizing bacteria (KUB) in kerosene polluted soil (KPS) ranged from 1.1 × 10^5 cfu/g to 1.7 × 10^5 cfu/g of soil while in kerosene free soil the counts ranged from 1.0× 10^5 cfu/g to 1.6 × 10^5 cfu/g of soil.

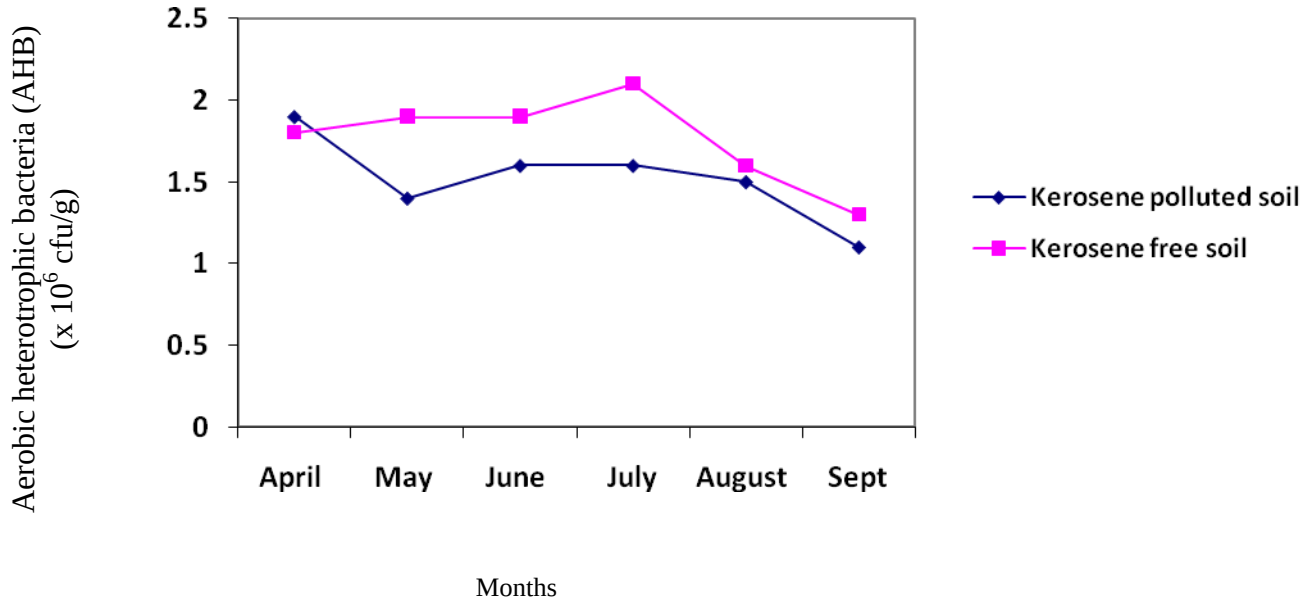

Figure 1. Counts of total aerobic heterotrophic bacteria in kerosene polluted soil.

The counts of KUB in both kerosene polluted soil and kerosene free soil decreased gradually from April to September (Fig.2). It was however, observed that there were higher counts of KUB in kerosene polluted soil than in kerosene free soil (KFS). There was no significant difference ($p>0.05$) in counts between KPS and KFS The counts of fungi in kerosene polluted soil (KPS) ranged from 1.1 ×10^3 cfu/g to 1.6 ×10^3 cfu/g of soil while in kerosene free soil (KFS), the counts ranged from 1.3 ×10^3 cfu/g to 2.1× 10^3 cfu/g of soil. The result (Fig.3) indicated that counts of fungi in KPS soil increased gradually from April to July, after which the counts decreased till the end of the study in September. Similar trend was observed in the kerosene free soil (KFS). Generally, the counts of fungi in KFS were relatively higher than those of KPS. However, statistical analysis of the data revealed that there was no significant difference ($P>0.05$) in counts between KPS and KFS.

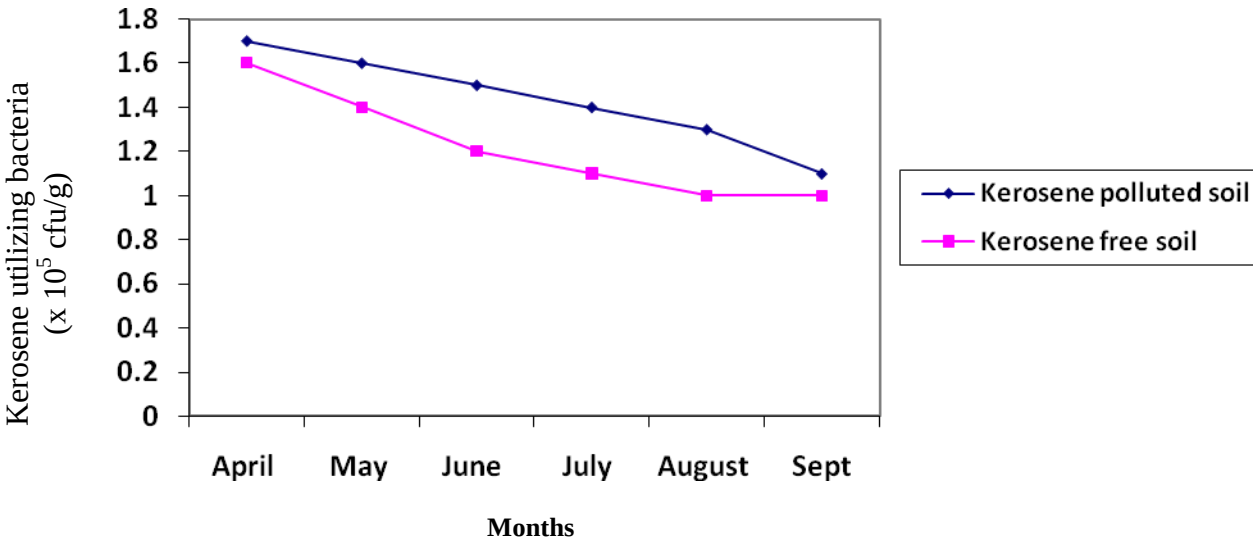

Figure 2. Counts of kerosene utilizing bacteria in kerosene polluted soil.

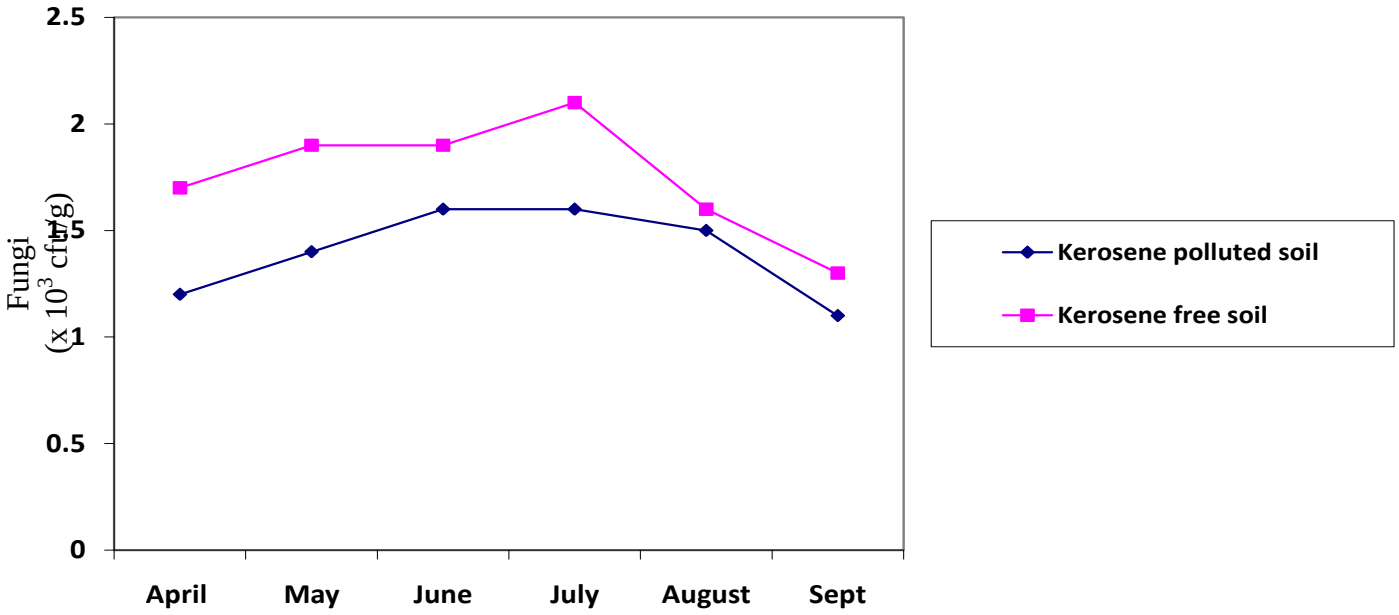

Figure 3. Counts of fungi in kerosene polluted soil.

The bacterial isolates were identified as species of *Acinetobacter, Alcaligenes*, *Bacillus, Micrococcus*, and *Pseudomonas.* The fungi were identified as species of *Aspergillus, Mucor*, *Penicillium* and *Fusarium*.

Table 1. revealed the ability of the microbial isolates to utilize kerosene as a source of carbon and energy. *Bacillus* species exhibited higher capacity of utilization than other isolates tested. The fungi utilized the hydrocarbon at moderate rates like some bacteria. However, one fungus isolate (*Penicillium notatum* KUF05) utilized the incorporated hydrocarbon at a minimal rate.

The results (Table 2) revealed that the amount of carbon dioxide evolution by the *Bacillus* isolates increased gradually from the 4th to the 16th day. It was observed that the amount of carbon dioxide evolved by *Bacillus* isolates (KUB 03) was relatively lower compared to the amount evolved by other isolates and the mixed culture after 16 days. However, statistical analysis (ANOVA) revealed that the difference in carbon dioxide production between KUB03 and KUBMC was significant ($P< 0.05$). Conversely, *Bacillus* strains KUB04 and KUB07 produced more carbon dioxide than the mixed culture, but the difference was not significant ($P> 0.05$). The carbon dioxide that was produced by *Bacillus* strains KUB07 was higher compared to the carbon dioxide evolved by the mixed culture. Statistical analysis (ANOVA) also revealed that the difference in carbon dioxide production between the two organisms was not significant ($P> 0.05$). It was also observed that the mixed *Bacillus* culture produced more carbon dioxide than *Bacillus* strain KUB08 but the difference was no significant ($P>0.05$).

Table 1. Utilization of kerosene by microbial isolates

Coded microorganisms	Growth in kerosene medium after 7 days
Micrococcus sp KUB 01	++
Pseudomonas aeruginosa KUB 02	++
Bacillus subtilis KUB 03	+++
Bacillus subtilis KUB 04	+++
Micrococcus sp KUB 05	++
Pseudomonas aeruginosa KUB 06	++
Bacillus subtilis KUB 07	+++
Bacillus subtilis KUB 08	+++
Acinetobacter sp KUB 09	++
Acinetobacter sp KUB 10	++

Alcaligenes sp KUB 11	++
Alcaligenes sp KUB 12	++
Aspergillus niger KUF 01	++
Mucor sp KUF 02	++
Fusarium sp KUF 03	++
Aspergillus niger KUF 04	++
Penicillium notatum KUF 05	+

+++: maximum growth, ++: moderate growth, +: minimal growth.

Table 2 Carbon dioxide evolved as a result of kerosene degradation by *Bacillus* strains

Time	Carbon dioxide evolved (cm^3)				
	Bacillus strains				
(Days)	KUB03	KUB04	KUB07	KUB08	kUB MC
4	13.20	167.20	122.00	136.40	114.40
8	15.00	184.80	171.60	142.00	161.60
12	18.50	193.60	198.00	185.00	184.80
16	22.00	210.00	228.00	193.60	198.60

MC: Mixed culture of *Bacillus* strains (KUBO3, 04, 07, 08)

V. Discussion

In the present study, the counts of aerobic heterotrophic bacteria (AHB) in kerosene free soil were higher than the counts in kerosene polluted soil probably because of the inhibitory effects on some hydrocarbon components (Okoh, 2006). The results of the study indicated that counts of AHB were less than those obtained by Ijah and Abioye (2003) who studied the same site 30 months after the spill. The difference in counts may be due to changes in the physicochemical properties of the soil. However, in the present study, no significant difference in counts of AHB between kerosene polluted soil (KPS) and kerosene free soil (KFS) was observed probably due to rapid biodegradation of the kerosene in the same soil. The counts of kerosene utilizing bacteria (KUB) in KPS were higher than those of KFS, although the difference was not significant. The reason for higher counts in KPS may be the presence of residual kerosene in KPS which boosts the carbon supply in the soil, hence favour the growth of the bacteria as compared to KFS. However, the counts of KUB decreased gradually in KPS and KFS from April to September. This may be as a result of moderate rainfall during the months of July to September which might have washed away some residual kerosene and nutrients in the soil or hinder oxygen in the soil thus affecting bacteria growth adversely (Ijah and Antai, 2003).
The counts of fungi in KPS increased gradually from April to July, after which the counts decreased till the end of the study in September. Similar trend was observed in the KPS. These changes may be attributed to seasonal variation (Ijah and Antai, 2003). Generally, the counts of fungi in KFS were relatively higher than those of KPS, which is a sharp contrast to the results of Ijah and Abioye (2003). This may be as a result of changes in nutrient status of the soil. It is also possible that the residual kerosene in the soil has some toxic components which do not favour fungal growth.

The rate of Kerosene biodegradation in the soil seems to be rapid. This may be due to the fact that the microorganisms in the soil have efficient ability in utilizing the residual kerosene as a source of carbon and energy. Kerosene utilizing microorganisms isolated from the soil were species of *Bacillus, Acinetobacter, Alcaligenes, Micrococcus, Pseudomonas, Aspergillus, Penicillum, Fusarium* and *Mucor*. *Bacillus subtilis* predominated especially in the kerosene polluted soil. This may be due to the ability of the bacteria to produce spores which may shield them from the toxic effect of the hydrocarbons (Ijah and Abioye, 2003). *Aspergillus niger* also produce spores which may help the fungi to be widely distributed in the soil (Okoh, 2006).

The rates of utilization of kerosene in mineral salt medium by the microorganisms varied. *Bacillus subtilis* manifested higher capacity of utilization than other isolates tested. The greater ability of *Bacillus subtilis* in utilizing the kerosene in mineral salts medium than the other isolates may be due to production of efficient enzymes for the breakdown of the hydrocarbon (Ojo, 2005).

The amount of carbon dioxide evolved by *Bacillus* strains KUB04 and KUB07 was relatively higher compared to the amount of carbon dioxide evolved by other strains and mixed culture after 16 days, meaning that there may be no special benefit in using mixed culture in the bioremediation of the kerosene polluted environment as they are prone to competition for hydrocarbon as carbon and energy sources thereby limiting the competence of the organisms for effective biodegradation. Thus *Bacillus* strains KUB04 and KUB07 can be used in reclaiming the kerosene polluted soil.

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