Full Length Research Paper

Utilisation of crude oil and gasoline by ten bacterial and five fungal isolates

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This study investigated the abilities of ten bacterial and five fungal isolates indigenous to polluted mechanic soils to utilise and degrade crude oil and gasoline. Of all the bacterial and fungal isolates obtained in this study *Pseudomonas* sp. *Bacillus* sp. and *Aspergillus* sp. were found to be more predominant in the polluted mechanic soils. The growth profiles were determined by monitoring the optical density, total viable counts, dry weights and pH of the culture utilizing crude oil and gasoline as carbon and energy source. Total viable counts increased significantly with optical density and dry weights of fungi as the days of incubation progressed until the 14th day (P<0.001). There was significant difference (P<0.002) in the pH values of the fungal isolates. The pH values decreased significantly (P<0.001) as fungal cells metabolised crude oil and gasoline. Of all the bacterial and fungal isolates used in this study *Bacillus subtilis, Pseudomonas aeruginosa* and *Aspergillus ochraceus* have shown the best abilities to utilise and degrade crude oil and gasoline *in- vitro.* All the organisms used in this study are all indigenous to the environment from which they were isolated and all of those which were tested for biodegradation were able to biodegrade organic contaminants actively. The biodegradation of contaminants is the best means to completely remove oil pollutants.

Key words: Bacteria, fungi, crude oil, gasoline, biodegradation.

INTRODUCTION

Commercially explored since the middle of the 19th century, petroleum has been used for many decades for illumination, and on smaller scale, as lubricant. The invention of the internal combustion engine and its fast adoption in all transport forms enlarged the employment of this natural resource, thus increasing its demand in production, transport, stockpiling and distribution, as well as the raw oil and its bye products. All these activities involve pollution risks that can be minimised, but not totally eliminated, causing several problems for the environment (Pala et al., 2006).

Crude oil consists of four main groups of hydrocarbons including aliphatics, aromatics, resins and asphaltines (Colwell and Walker, 1997). The leakage of crude oil into soil damages the biological systems residing in the soil, including microorganisms and plants (Dariush et al., 2007). Some fractions of crude oil are toxic for living organisms. However various microorganisms are able to

use some crude oil fractions as sole carbon source and change these component to non-toxic materials such as CO_2 and H_2O (Ewis et al., 1998). The contamination of soil and aquifer systems by gasoline hydrocarbons as a consequence of accidental spillage can cause serious environmental problems. The major gasoline constituents (benzene, toluene and xylene- BTX) are relatively soluble in water and are considered human carcinogens (Claudia and Selma, 2000).

One of the best approaches to restore contaminated environments is to make use of the physiological potentials of microorganisms able to degrade the pollutants in a bioremediation process. It is an attractive approach to cleaning up hydrocarbons because it is simple to maintain, applicable over large areas, cost effective and leads to complete destruction of the con-taminant (Bento et al., 2005). Numerous microorganisms are known for their ability to degrade hydrocarbons. The biodegradation capabilities of bacteria have been recognised (Elisane et al., 2008) but fungi have been subject recent research (Colombo et al., 1996; Krivobok et al., 1998; Salicis et al., 1999; Garcia et al., 2000; Garon et al., 2000; Baheri and Meysami, 2002; Romero et al., 2002; Chaillan et al., 2004;

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Santos and Linardi, 2004; Potin et al., 2004), due to their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin degradation, which are capable of degrading high molecular weight, complex or more recalcitrant com-pounds, including aromatic structures. The application of biotechnological processes involving microorganisms, with the objective of solving environmental pollution problems, is rapidly growing, in recent decades, where petroleum and its by products are concerned. Bioreme-diation processes, which take advantage of microbial degradation of organic and inorganic substances, can be defined as the use of organisms to remove environmental pollutants of soil water and sediments (Pala et al., 2006).

Petroleum production began in Nigeria in 1958 and since then, cases of petroleum and refined petroleum spills onto agricultural lands through petroleum production operations have been reported (Odu, 1977; Awobajo, 1981; Grevy, 1995; Moffat and Linden, 1995). Hence the aim of this work is to determine the ability of indigenous bacteria and fungi to utilise crude oil and gasoline as carbon source and for growth thus degrading both petroleum fractions.

MATERIALS AND METHODS

Sample collection

Crude oil samples were obtained from Federal Institute of Industrial Research Oshodi (FIIRO) Lagos State, Nigeria. Contaminated soils were collected from a mechanic workshop in Ago-Iwoye, Ogun State Nigeria. Non-contaminated soil was also collected from a distance not less than 120 m from contaminated sites. All soil samples were collected in triplicates.

Isolation and characterisation of hydrocarbon utilizing bacteria and fungi

The bacterial species indigenous to mechanic soil samples were isolated by pour plate technique using 0.1 ml aliquots of appropriate dilution into nutrient agar plates. Individual cultures were identified by morphological and biochemical techniques using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

The fungi species indigenous to the mechanic soils were isolated using Potato Dextrose Agar (PDA) into which streptomycin (50 mg/ml) had been added to suppress bacterial growth. Fungal isolates were characterized as described by Barnett and Hunter (1998).

The obtained cultures of bacteria and fungi were screened for their ability to utilise crude oil and gasoline using crude oil and gasoline separately as substrate.

Determination of the ability of bacterial and fungal isolates to utilise crude oil and gasoline

A known volume of 150 ml of the basal medium (minimal salt medium, composition 10 g NaCl, .29 g KCl, .42 g $MgSO_4$, .83 g KH_2PO_4, .42 g $NaNO_3$, 1.25 g $NaHPO_4$, 100 ml distilled water, pH 7.2) was dispensed into 250 ml conical flasks and crude oil and gasoline were introduced separately into flasks at 1.0% v/v after

sterilization (Okpokwasili and Okorie, 1988). Overnight broth culture (20 g/L of nutrient broth for bacteria and 35 g/L of malt extract broth for fungi) of each organism was seeded into each flask and incubated in a gyratory shaker incubator (New Brunswick Scientific Incubator Shaker) at 150 rev/min and 30°C. Utilisation of crude oil and gasoline were monitored at two days interval for 14 days by monitoring bacterial and fungal growth measured by viable count on nutrient agar. The optical density was determined at 600 nm wavelength with PG T70 U.V/VIS spectrophotometer and changes in pH, was determined with pH meter (Model Hama microprocessor P211 pH meter). Fungi was harvested on the filter paper by filtration and dried in the oven, the weight was then determined using the digital mettler balance P 163.

RESULTS

The characterisation of bacterial and fungal isolates obtained from contaminated sites have revealed ten species of bacteria which include: *Pseudomonas stutzeri, Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas mallei, Bacillus licheniformis, Bacillus cereus, Bacillus subtilis, Corynebacterium* sp, *Alcaligenes eutrophus* and *Enterobacter aerogenes* and five fungal species which include: *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Aspergillus ochraceus,* and *Trichoderma* sp were isolated from contaminated soil in this study and capable of utilizing crude oil and gasoline as carbon source. In addition, three bacterial species which include: *P. putida, B. cereus* and *B. subtilis* and five fungal species which include: *A. niger, A. flavus, Rhizopus* sp, *Penicillum* sp, and *A. terreus* were isolated from control soil samples.

There was no significant difference in the pH values of bacterial isolates (P=0.450) also no significant difference was observed in the changes in pH values obtained on crude oil and gasoline by bacterial and fungal isolates respectively (P=0.371) (P=0.226) . There was significant difference (P<0.002) in the pH values of the fungal isolates. The pH values dropped significantly (P<0.001) during utilisation of crude oil and gasoline by all bacterial and fungal isolates from 0h to the 14th day of incubation. During the utilisation of crude oil *Corynebacterium* sp produced the highest pH of 6.75 after 14 days of incubation while *E. aerogenes* had the lowest pH of 6.56 after 14 days of incubation while utilising crude oil (Figure 1). *A. terreus* had the lowest pH of 4.9 after 14 days of incubation (Figure 7). *A. ochraceus* had the highest pH value of 5.25 after 14 days of incubation (Figure 7). During the utilisation and degradation of gasoline, *A. eutrophus* had the highest pH of 6.88 after 14 days of incubation while *P. putida* and *P. aeruginosa* produced the lowest pH of 6.34 at the 14th day of incubation (Figure 2). *Trichoderma* sp produced the lowest pH of 5.05 on gasoline while *A. flavus* recorded the highest pH of 5.55 on gasoline (Figure 8).

The interaction between bacterial isolates and days of incubation showed significant effect on the changes in optical density (P<0.005). The optical density values increased significantly (P<0.001) from 0 h to the 14th day
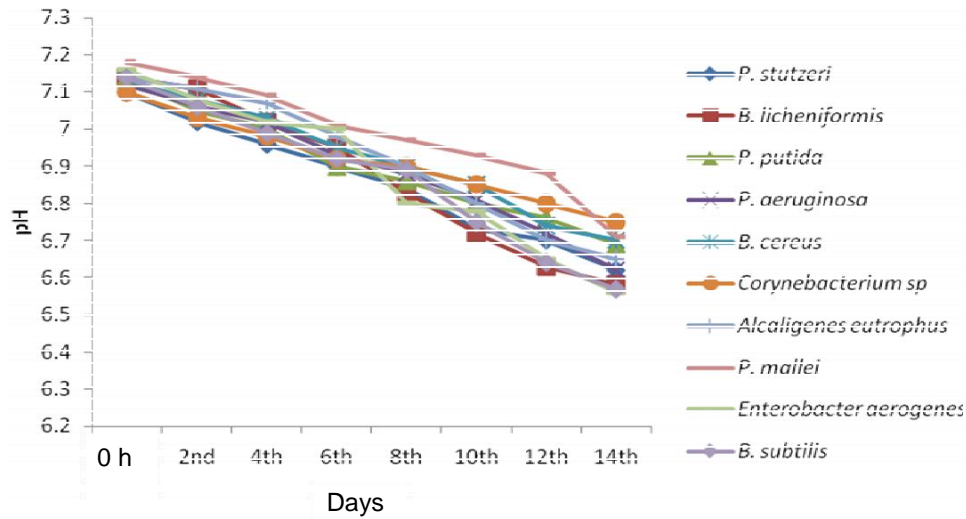
Figure 1. Changes in pH produced by bacterial isolates during utilisation of crude oil.
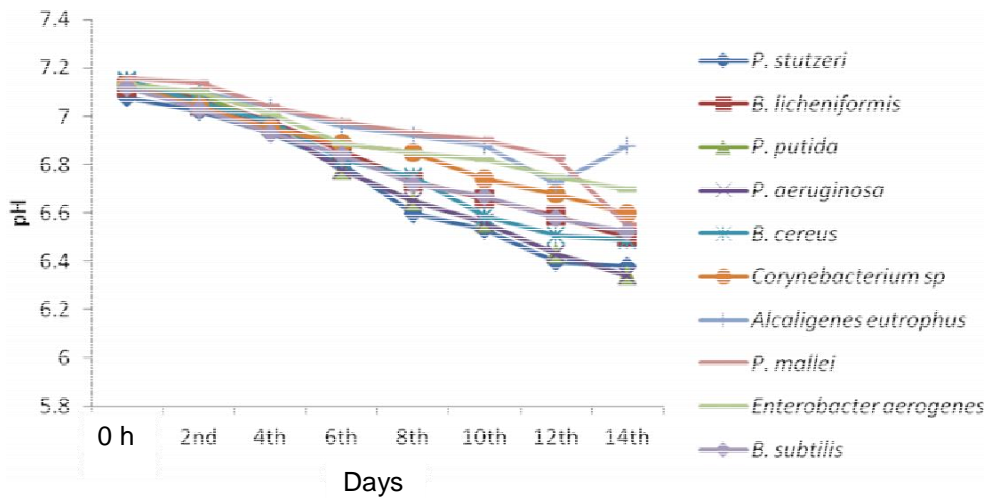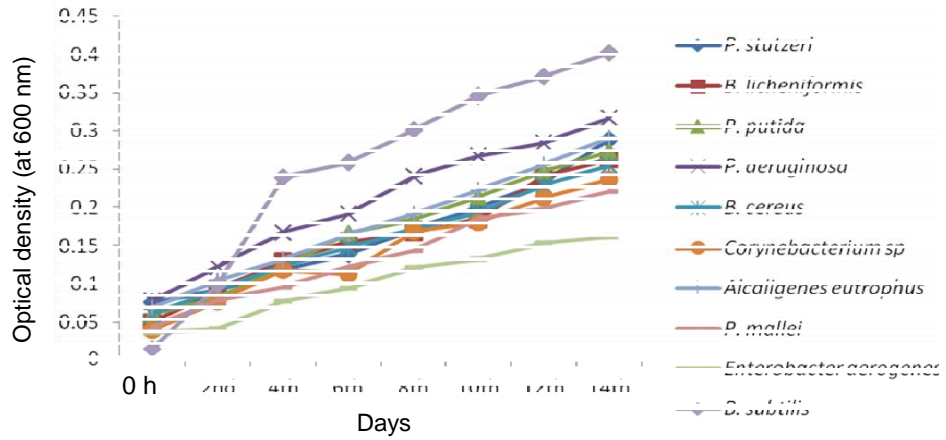

Figure 2. Changes in pH produced by bacterial isolates during utilisation of gasoline.

of incubation during the utilisation of crude oil and gasoline by all the bacterial isolates used in this study. Meanwhile there was no significant difference (P = 0.789) in the optical density values obtained from crude oil and gasoline. *B. subtilis* produced the highest optical density of 0.4 while *E. aerogenes* produced the lowest optical density of 0.16 during the utilisation of crude oil (Figure 3). *P. aeruginosa* recorded the highest optical density of 0.42 on gasoline (Figure 4) while *E. aerogenes* had the lowest optical density of .19 after 14 days of incubation (Figure 4).

The viable counts of all bacterial and fungal isolates increased significantly (P<0.001) from 0 h to the 14th day of incubation during the utilisation of crude oil and gasoline. There was no significant difference observed

for viable counts on crude oil and gasoline for both bacterial and fungal isolates respectively (P = 0.639) (P = 0.289). *B. subtilis* recorded the highest viable count value of 8.25 on crude oil while *E. aerogenes* recorded the lowest viable count value of 8.07 (Figure 5). *A. ochraceus* had the highest viable count value of 6.26 on crude oil after 14 days of incubation on crude oil while *A. flavus* and *A. niger* both recorded the lowest viable count value of 8.11 (Figure 9) after the 14th day of incubation. In Figure 10, *A. ochraceus* recorded the highest viable count value of 6.3 on gasoline while *A. niger* had the lowest viable count value of 6.15. *P. aeruginosa* recorded the highest viable count value of 8.11 during the utilisation of gasoline (Figure 6) while *Corynebacterium* sp had the lowest viable count value of 7.88 after 14 days

Figure 3. Changes in optical density produced by bacterial isolates during utilisation of crude oil.
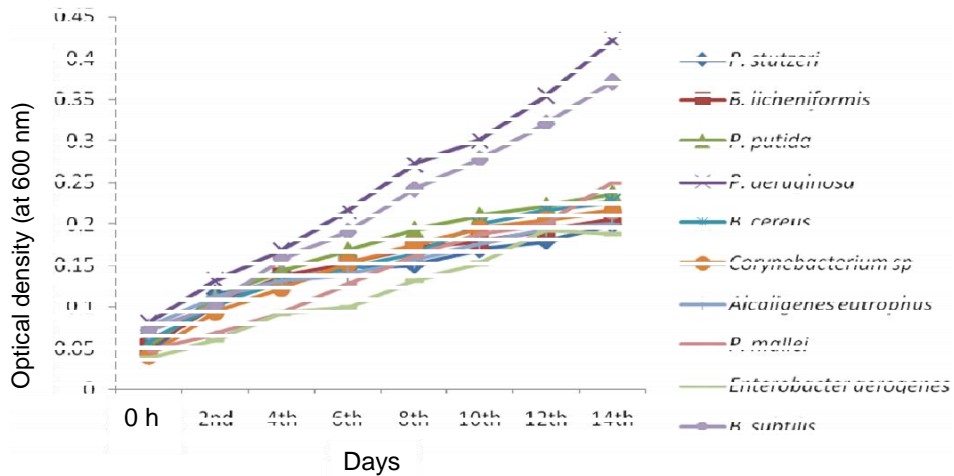
Figure 4. Changes in optical density produced by bacterial isolates during utilisation of gasoline.
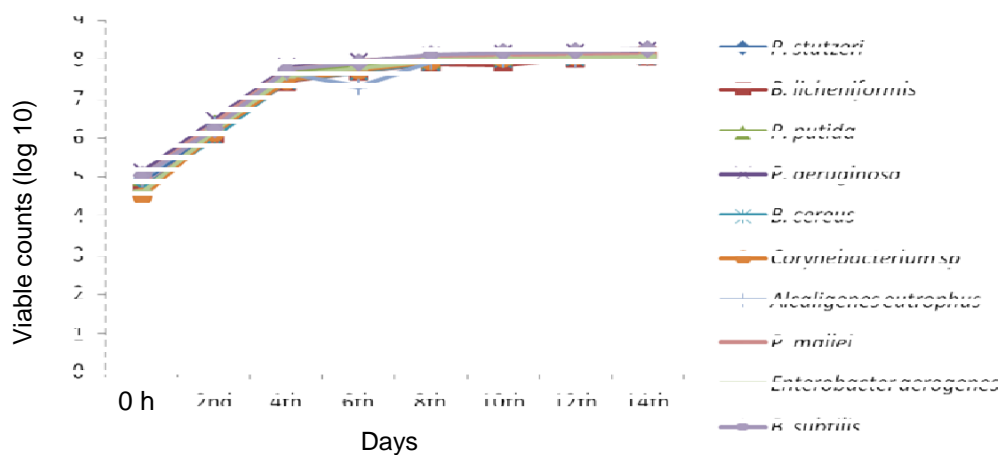
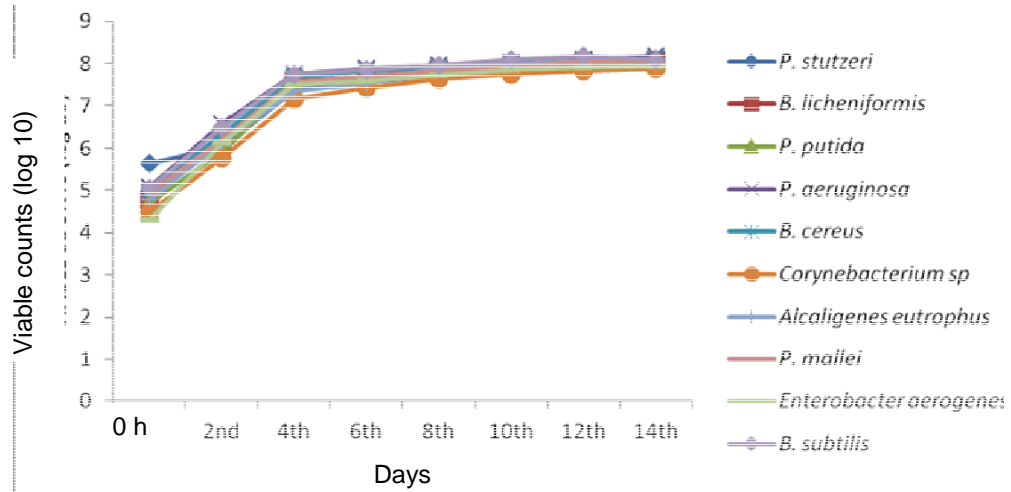**Figure 5.** Changes in viable counts produced by bacterial isolates during utilisation of crude oil.

Figure 6. Changes in viable counts produced by bacterial isolates during utilisation of gasoline.
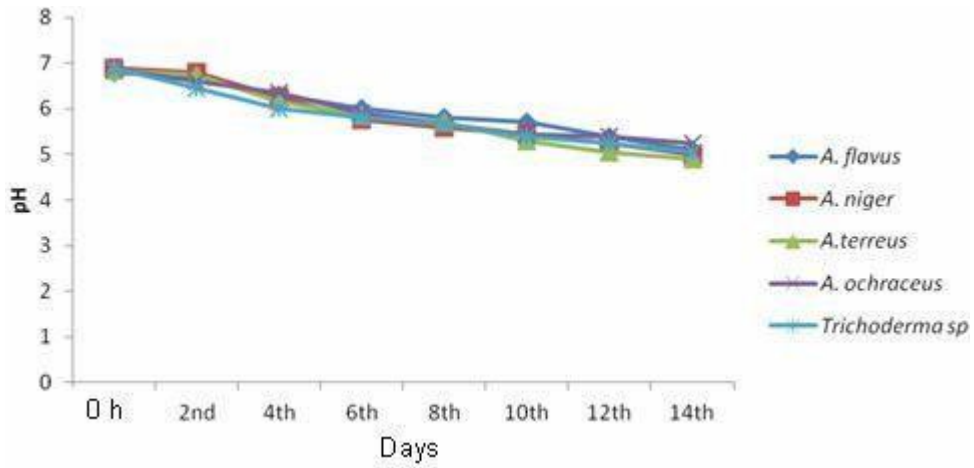

Figure 7. Changes in pH produced by fungal isolates during utilisation of crude oil.
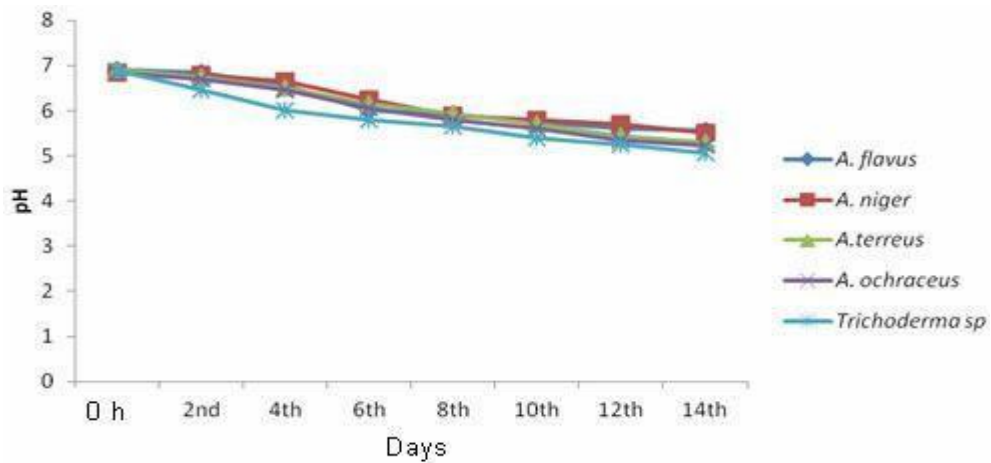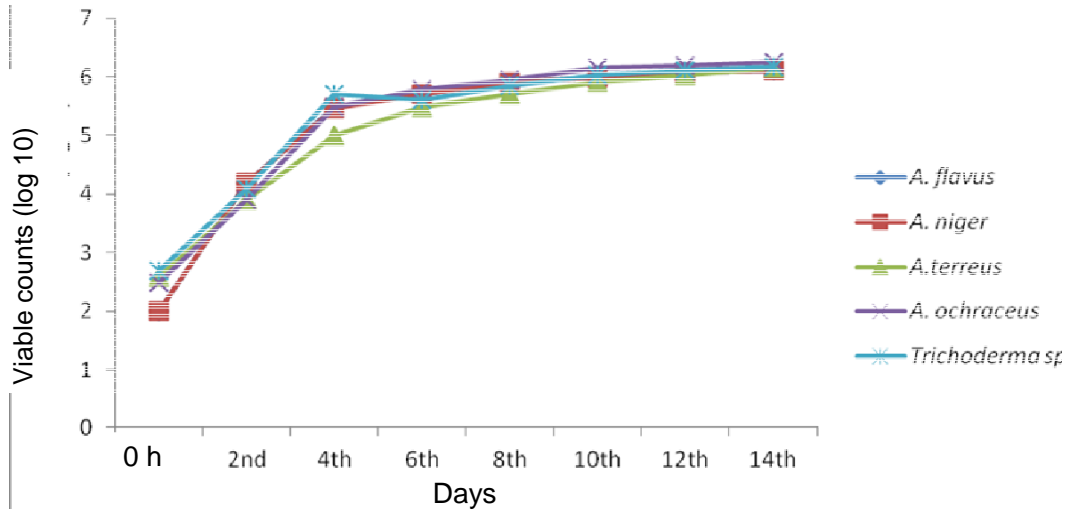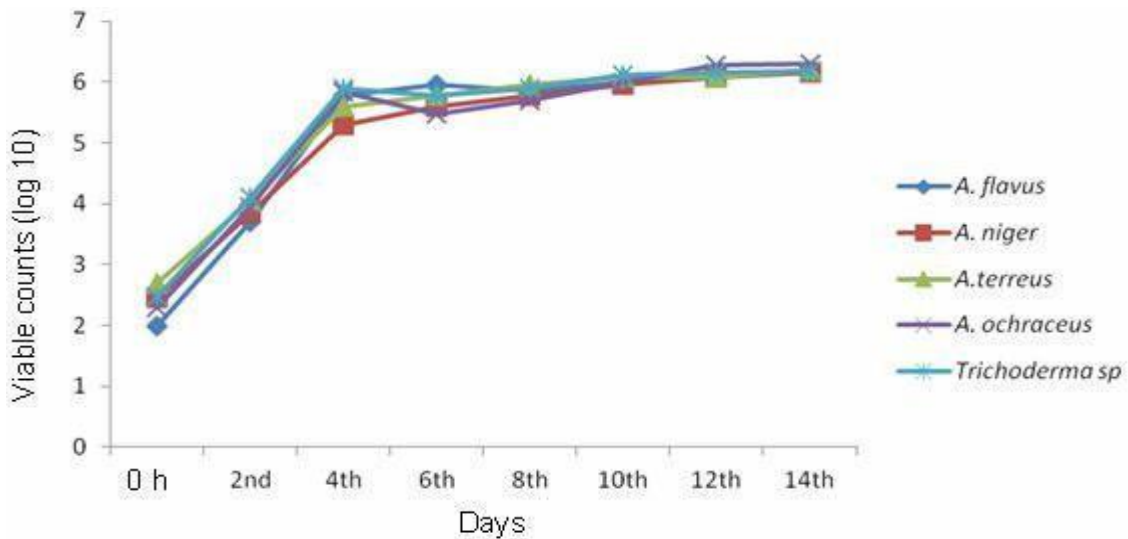

Figure 8. Changes in pH produced by fungal isolates during utilisation of gasoline.

Figure 9. Changes in viable counts produced by fungal isolates during utilisation of crude oil.
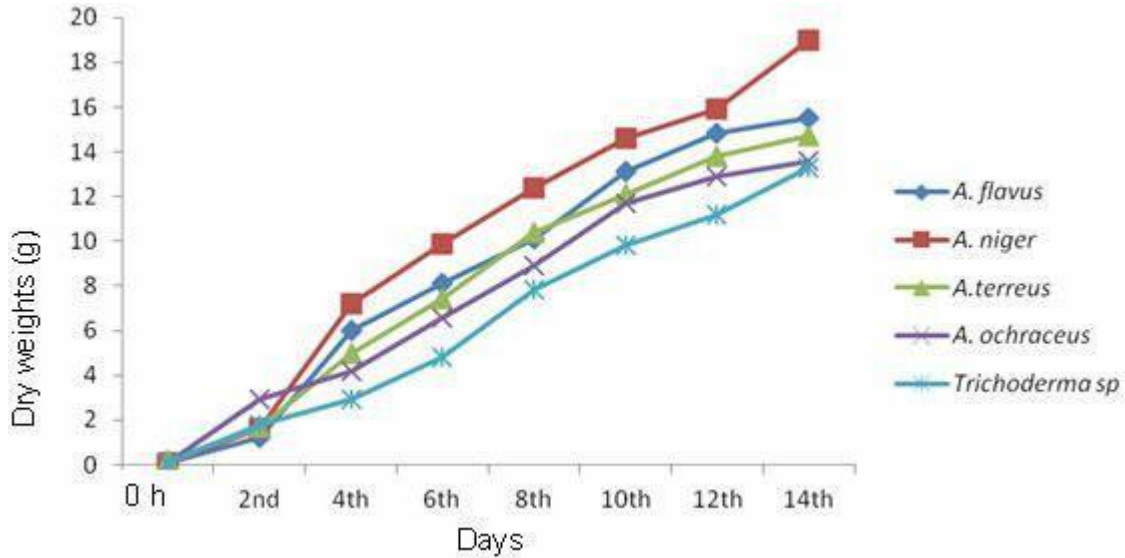

Figure 10. Changes in viable counts produced by fungal isolates during utilisation of gasoline.

of incubation.

Dry weight values increased significantly (P<0.001) from 0 h to 14th day of incubation during the utilisation of gasoline and crude oil by fungal isolates. Meanwhile dry weight values on crude oil and gasoline showed no significant difference (P = 0.212). There was significant difference (P<0.036) in dry weights of fungal isolates. *A. niger* had the highest dry weight value of 19 on crude oil while *Trichoderma* sp. had the lowest dry weight value of 13.3 on crude oil (Figure 11). In Figure 12, *A. terreus* recorded the highest dry weight value of 16.8 while *A. niger* had the lowest dry weight value of 13.3 on gasoline after the 14th day of incubation.

Correlation analysis results of the bacterial isolates showed that there was positive (correlation coefficient value = 0.740) correlation between optical density and viable counts, consequently as optical density increased viable counts also increased concomitantly. There was high negative correlation (correlation coefficient value = 0.798) between optical density and pH signifying that as optical density increased pH decreased. Similarly there was also high negative correlation (correlation coefficient value = -0.718) between viable counts and pH.

Hence as viable counts increased pH decreased. Correlation analysis results of the fungal isolates showed positive (correlation coefficient value = 0.820) correlation

Figure 11. Changes in dry weights produced by fungal isolates during utilisation of crude oil.
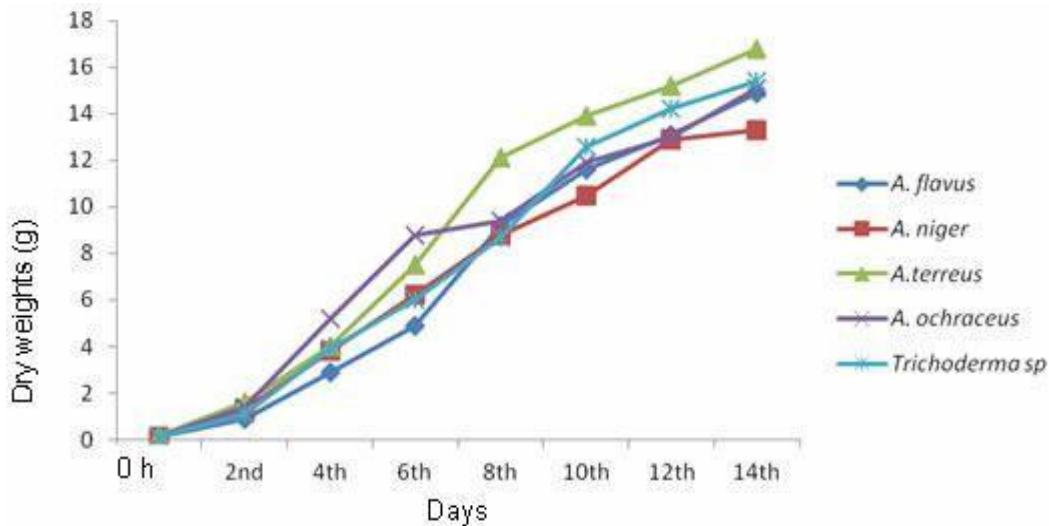
Figure 12. Changes in weights produced by fungal isolates during utilisation of gasoline.

between viable counts and dry weights. Hence dry weights increased with a concomitant increase in viable counts. High negative correlation (correlation coefficient value = -0.844) occurred between viable counts and pH. Similar trend as mentioned above for bacterial isolates was observed. There was also high negative correlation (correlation coefficient value = -0.940) between dry weights and pH, hence as dry weights increased pH decreased concomitantly.

DISCUSSION AND CONCLUSION

The bacteria and fungi isolated from contaminated soils indicated the prevalence of *Pseudomonas* sp, *Bacillus* sp., and *Aspergillus* sp. In this study *B. subtilis*, *P. aeruginosa* and *A. ochraceus* have shown the best abilities to utilise and degrade crude oil and gasoline. Oboh et al. (2006) have reported the abilities of bacterial species; *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Citrobacter* and fungal species which include; *Aspergillus* sp., *Penicillum, Rhizopus* and *Rhodotorula* species to grow on crude petroleum as the sole carbon and energy source when screened for hydrocarbon utilisation. The major genera of bacteria active in polluted soils were *Pseudomonas*, *Bacillus*, *Serratia* and *Acinetobacter*, while fungal genera were *Aspergillus*, *Penicillum* and *Mucor* (Nkwelang et al., 2008). Uzoamaka et al. (2009)

reported that the eight isolates showed potentials for hydrocarbon biodegradation are *A. versicolor*, *A. niger*, *A. flavus*, *Syncephalastrum* spp., *Trichoderma* spp., *Neurospora sitophila*, *Rhizopus arrhizus* and *Mucor* spp. Santos et al. (2008) reported the ability of *Aspergillus* sp. to biodegrade gasoline.

Atlas and Bartha (1972) observed that both water in oil and oil in water emulsions are formed following oil spillage. The two phase liquid medium where the bulk of the carbon and energy source are found is water insoluble and all other minerals nutrients are dissolved in the water phase, microbial growth typically occurs at the interface of the two liquids. The ability of the micro-organisms to lower the interfacial tension will increase the interface and thus accessibility of the hydrocarbon substrate. Similar observations were made in this study as the role being played by agitation during hydrocarbon degradation was visibly observed. While flasks placed on shaker resulted in crude oil and gasoline complete disappearance, those put in the incubator without shaking showed little or no degradative effect. Agitation breaks the hydrocarbon into droplets, thereby providing increased surface area to accelerate biodegradation. The oil in this state is not only made readily available but its movement through water column makes oxygen and other nutrient more readily available to the organism. The physical state of the petroleum hydrocarbons is known to have a marked effect on its biodegradation; the more soluble it is in water the more liable it is to microbial attack. Hydrocarbon degrading organisms act mainly at the oil and water interface. This investigation however shows that gasoline was utilised to a greater extent than crude oil.

The reduction in pH of the culture fluids in flasks within the 14 day incubation period confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes (Atlas and Bartha, 1972). The growth profiles of the fungal isolates on crude oil and gasoline revealed a sharp drop in pH. Hydrogen ion concentration is a major variable governing the activity and composition of fungi. Many species can metabolise over a wide pH range from the highly acidic to alkaline extremes. Thus, the insensitivity of the fungi to high hydrogen ion concentration and narrow pH range of most bacteria account for the sharp drop in pH. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (Nwachukwu and Ugoji, 1995; Okpokwasili and James, 1995). Thus organic acids probably produced account for the reduction in pH levels (Oboh et al., 2006).

The growth profiles showed that none of the bacterial and fungal isolates exhibited lag phases. This observation has been reported previously (Amund, 1991; Okeretungba and Ezeronye, 2003; Oboh et al., 2006) and can be attributed to genetic make-up due to the con-stitutive expression of hydrocarbon catalysing enzymes or physiological owing to previous exposure to exogenous hydrocarbons present in the contaminated soils. This may

be followed by a concomitant development of the ability of the organisms to emulsify petroleum hydrocarbons and which is a major factor in hydrocarbon uptake and assimilation. Ptaek et al. (1987) reported that many of the petroleum degrading bacteria produce extracellular emulsifying agents. *P. aeruginosa* produce rhamonlipids during growth on hydrocarbons which is necessary to emulsify aromatic and aliphatic hydrocarbons.

Utilisation of crude oil and gasoline resulted in increase in cell densities with a concomitant reduction in the oil layer complete disappearance of crude oil and gasoline with prolonged incubation. According to Dariush et al. (2007) increasing crude oil concentration decreased the reduction of crude oil in vegetated and non vegetated soil samples. In all the contaminated vegetated soils, the reduction of the crude oil was higher than non vegetated soils. In the higher concentrations (7 and 10%) the difference of crude oil reduction between the vegetated and non vegetated soil samples was not significant, while the reduction was significant between the vegetated and non vegetated samples in concentrations up to 5% (Dariush et al., 2007). *B. subtilis*, *P. aeruginosa* and *A. ochraceus* have shown the best abilities to degrade both crude oil and gasoline.

It is interesting to note that all the organisms used in this study are all indigenous to the environment from which they were isolated and that all of those, which were tested for biodegradation, are able to biodegrade organic contaminants actively. The biodegradation of contaminants is the best means to completely remove oil pollutants. Biodegradation should be accelerated in order to develop a faster means of cleaning up pollutants. The ultimate success of bioremediation is dependent upon microorganisms staying in close physical contact with the substance to be degraded. The key to increasing the rate of biodegradation of contaminant is to optimise the growth rate of indigenous soil degrading micro flora. Finally increasing biodegradation rates of indigenous microorganisms is the best option to maximise contaminant clean up when all avenues have been exhausted.

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