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Full Length Research Paper

Studies on the bioutilization of some petroleum hydrocarbons by single and mixed cultures of some bacterial species

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The ability of four bacterial species (*Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp. and *Proteus* sp.) to utilize some petroleum hydrocarbons (kerosene, engine oil and automotive oil) was studied. Laboratory experiments were conducted over a five day period in Erlenmeyer flasks containing mineral salt medium (MSM) under aerobic conditions using the hydrocarbons as carbon sources. A bacteria consortium capable of utilizing the hydrocarbons was prepared from the bacterial cultures. All the bacterial species utilized the hydrocarbons as sole carbon and energy sources showing increases in cell number and optical density with decreases in pH of the culture media, and carbon dioxide (CO_2) evolution. Data from statistical analyses showed significant difference ($p<0.05$) in the utilization competence of the bacterial cultures on the three hydrocarbon substrates. A significant variation was observed between the efficiency of single and mixed bacteria cultures. Mixed bacterial cultures showed the most appreciable growth on the substrates tested. It was also observed that *Pseudomonas* sp. utilized the three hydrocarbons better than the other bacterial species. This study has shown that the mixed bacterial cultures have potential application in the bioremediation of sites polluted with kerosene, engine oil and automotive oil and can assist researchers in developing strategies for removing hydrocarbon pollutants from the environment.

Key words: Biodegradation, bioremediation, mixed bacterial cultures, hydrocarbons, bacterial consortium.

INTRODUCTION

Hydrocarbons are very simple organic compounds. They are composed only of two elements-carbon and hydrogen-hence their name. Petroleum, which means *rock oil* in Latin, occurs as a dark, sticky, viscous liquid. Petroleum products such as gasoline, kerosene, and fuel oils are complex mixture of organic compounds basically of paraffinic, oleiffinic and aromatic hydrocarbons and in small of molecules containing sulphur, nitrogen, metals, oxygen etc. (Viera et al., 2006). They have been found useful in automobiles and in petrochemical industries. One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Accidental releases of petroleum products are of particular concern in the environment and this has led to a concerted effort in studying the viability of using oil-degrading micro-organisms for bioremediation (Sebiomo et al., 2010). Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants. Currently accepted disposal methods of incineration or insecure landfills can become prohibitively expensive when amounts of contaminants are large. Mechanical and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive. Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. Bioremediation functions basically on biodegradation, may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other

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simpler organic compounds by biological agents like microorganisms (Nilanjana and Preethy, 2010). Several researchers have studied the use of microorganism to decompose petroleum products and have shown this to be a promising technological alternative (Gogoi et al., 2003; Townsend et al., 2004; Kaluarachchi et al., 2000; Bielicka et al., 2002; Diaz et al., 2000). Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi.

The reported efficiency of biodegradation ranged from 6% (Jones et al., 1970) to 82% (Pinholt et al., 1979) for soil fungi, 0.13% (Jones et al., 1970) to 50% (Pinholt et al., 1979) for soil bacteria, and 0.003% (Hollaway et al., 1980) to 100% (Mulkins et al., 1974) for marine bacteria. Many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil (Bartha and Bossert, 1984), fresh water (Cooney, 1984) and marine environments (Atlas, 1985). Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in the environment (Rahman et al., 2003; Brooijmans). Several bacteria are even known to feed exclusively on hydrocarbons (Yakimov et al., 2007). Floodgate (1984) listed 25 genera of hydrocarbon degrading bacteria and fungi each which were isolated from marine environment. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye et al. (2007). Nine bacterial strains, namely, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus* sp*., Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium* sp., *Micrococcus roseus, and Corynebacterium* sp. were isolated from the polluted stream which could degrade crude oil.

Microbiological activities are affected by a number of environmental factors including energy sources, donors and acceptors of electrons, nutrients, pH, temperature and inhibition by the substratum or metabolites (Catallo and Portier, 1992; Atlas, 1995; Dubey, 2009). These parameters influence how quickly microorganisms adapt to the available substratum. Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Ulrici, 2000).

Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Atlas and Bragg, 2009). In order to improve the natural tendency of soil microorganisms to decompose hydrocarbons from crude oil, many techniques of land farming that is, mineral fertilization, organic amendments, (Turgay et al., 2010) cropping systems have been proposed and tested (Sims and Sims, 1999). As no single microbial species is capable of degradating all components of crude oil, complete oil degradation requires simultaneous action of different microbial populations (Erdogan and Karaca, 2011). Microbial degradation is the major and ultimate natural mechanism by which one can cleanup the petroleum hydrocarbon pollutants from the environment (Atlas, 1992; Amund and Nwokoye, 1993; Lal and Khanna, 1996).

This study was aimed at assessing the hydrocarbon utilizing potentials of single and mixed cultures of hydrocarbon utilizing bacterial species in an effort to develop active microbial species with characteristics that could be of relevance in bioremediation of petroleum contaminated systems in Nigeria.

MATERIALS AND METHODS

Source of micro-organisms

Four bacterial species (*Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp. and *Proteus* sp.) capable of utilizing hydrocarbons as sole carbon and energy source were obtained from the Culture Collection Center, Department of Microbiology, University of Nigeria, Nsukka.

Bioutilization experiment

Growth of the bacterial species on hydrocarbon substrates was carried out by the inoculation of each of the bacterial cultures into a 250 ml Erlenmeyer flask containing 99 ml of mineral salt medium with a composition of (g/L): 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 0.2 Mg $SO_4.7H_2O$; 0.3 KNO_3; 1 $(NH_4)_2SO_4$.as described by Vecchioli (1990). The pH of the mineral salt medium was 7.0. Kerosene, engine oil and automotive gasoline oil (AGO) were obtained from a filling station and added to this medium in the proportion of 1:1:1 (v/v), as the only carbon source. In addition, control samples on hydrocarbon free basis were run in parallel. The culture was incubated for 48 h at room temperature with continuous agitation. The optical density (OD_{600nm}), total viable count (TVC) and pH of the culture fluids were monitored at determined time intervals.

Carbon dioxide measurement

Mineralization studies were based on measuring the time-dependent release of carbon dioxide by bacterial cultures associated with the utilization of different hydrocarbons as carbon sources as described by Alvarez (2003). Fresh cells of bacterial species were grown aerobically overnight at 25°C in 10 ml nutrient broth (w/v). After growth, cells were harvested and then washed once with sterile NaCl solution (0.85%, w/v) and resuspended in sterile NaCl solution with an absorbancy of 1 (A_{436}). Then 0.5 ml of the suspensions was used to inoculate sixteen 250 ml Erlenmeyer flasks containing 50 ml MSM supplemented with 1% of each of the hydrocarbons as a sole carbon source. Each of the flasks was designed with delivery tubes to receive vials containing 2 ml of 1 M NaOH (4% w/v), an alkaline trap to trap CO_2 produced by the cells. These vials were removed every 24 h and replaced by new vials containing a fresh NaOH solution. The flasks were tightly sealed with rubber stoppers and incubated for 24 h at 37°C with shaking. The alkaline solution traps carbon dioxide by transforming it into bicarbonate (HCO_3-) form. The bicarbonate$^-$ was then precipitated with 2 ml of 1 M barium chloride solution and then titrated with 1 M HCL acid in the presence of phenolphthalein (an indicator). The amount of CO_2 trapped was calculated using the formula:

$$\frac{M_A V_A}{M_B V_B} = \frac{N_A}{N_B}$$

where: M_A is the molarity of the acid, M_B is the molarity of the base, V_A is the volume of the acid (obtained from burette readings), V_B is the volume of the base, N_A is the number of moles of the reacting species of the acid, N_B is the number of moles of the reacting species of the base. N_A and N_B where obtained from balanced equation of the reaction.

Bacterial consortia preparation

The four bacterial species were grown on nutrient broth and incubated for 96 h to allow the cells to fully grow to the mid log phase. The cells were harvested and mixed in equal proportion with a corresponding cell density of 10^8 CFU /ml for each.

Bioutilization experiment

This experiment was conducted to examine the ability of the bacterial consortium in utilizing the hydrocarbons. The laboratory tests were carried out in duplicates under aerobic conditions in Erlenmeyer flasks (250 ml). The hydrocarbon utilizing efficiency of different strains and consortium was screened on a mineral salt medium previously described. Flasks were incubated for 5 days. The CO_2 evolved was determined as earlier described. The optical density (OD_{600} nm) and total viable count (TVC) of the culture medium were also determined.

Statistical analyses

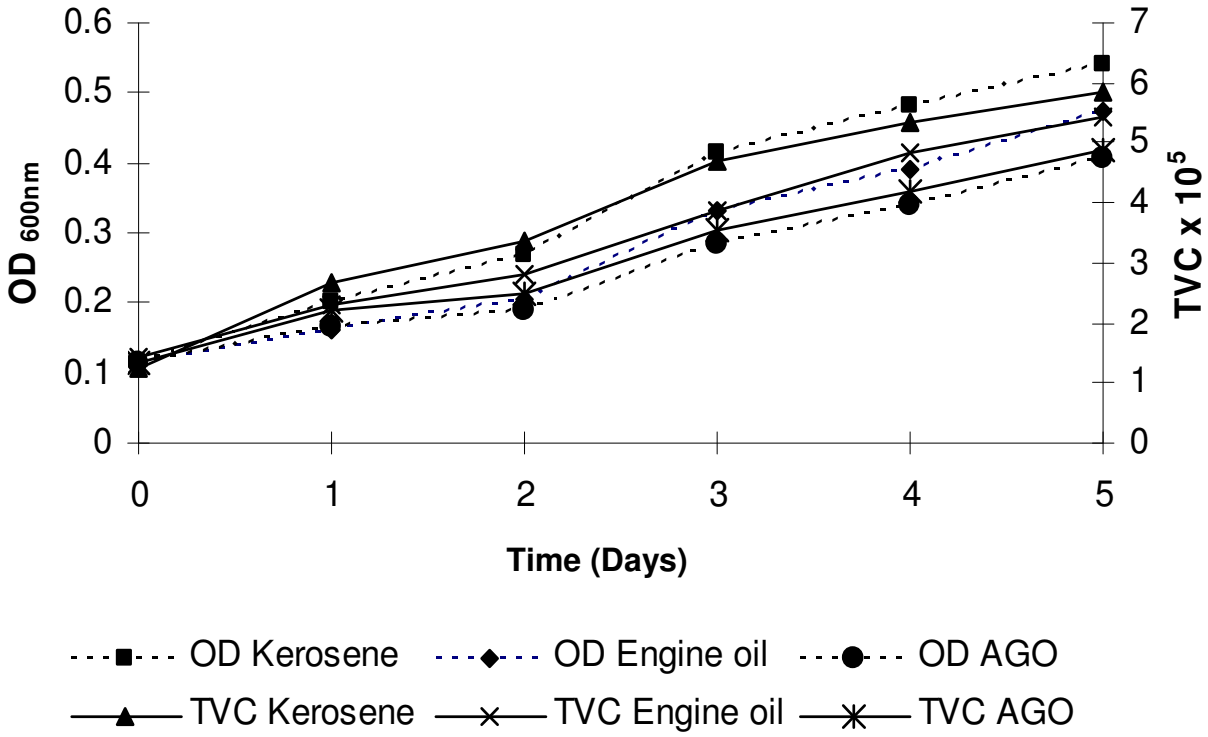
Two way analysis of variance (ANOVA) and LSD tests were used to determine whether hydrocarbon utilization differed significantly according to type of inoculum. *P* value of less than 0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

The bacterial species of the following genera: *Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp., and *Proteus* sp. were assayed for their hydrocarbon-utilizing potential. The results obtained in this study revealed that the four bacterial species used in this work exhibited different levels of hydrocarbon degradation. Figures 1 to 4 show the utilization potentials of *Pseudomonas* sp., *Bacillus* sp, *Klebsiella* sp. and *Proteus* sp. respectively on kerosene, engine oil and AGO under aerobic condition. The growth dynamics of the organisms were determined by the optical density, total viable counts and amount of CO_2 evolved. All bacterial cultures tested were able to grow on kerosene, engine oil and AGO as the sole source of carbon and energy when screened for hydrocarbon utilization except in the negative control. Significant ($P < 0.05$) increase in numbers of the species occurred mainly towards the end of the cultivation period. Interestingly, these organisms especially *Pseudomonas* sp. and *Bacillus* sp. have been implicated in hydrocarbon degradation (Amund and Adebiyi, 1991; Atlas, 1992; Nwachukwu and Ugoji, 1995; Nwachukwu, 2001). *Pseudomonas* sp are frequently found at sites polluted by petroleum and petroleum derivatives (Foght et al., 1999; Richard and Vogel, 1999; Oboh et al., 2006) suggesting that the genus effectively metabolizes hydrocarbon molecules and may dominate bacterial populations in soils where petroleum pollution has occurred. *Bacillus* sp. has also frequently been reported as an effective agent for the degradation of hydrocarbons (Benkacaker and Ekundayo, 1997; Diaz et al., 2000). Ghazali et al. (2004) isolated *Pseudomonas* sp. and *Bacillus* sp. from hydrocarbon polluted soils and studied these genera relative to their potential to biodegrade crude oil, benzene, ethylbenzene, 0-xylene, n-tetradecanol, octanol and decanol. Okerentugba and Ezeronye (2003), have studied the potential of *Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp. and *Proteus* sp. isolated from rivers polluted by hydrocarbons and refinery effluent to degrade petroleum.

Direct counts as a measure of total microbial activity are problematic because they include dormant and moribund cells. As a result of these problems, the measurement of microbial metabolic activities in the samples was focused more on the quantification of metabolic activity such as respiration which was calculated based on CO_2 concentration value at days 1 and 5 (Figures 1b, 3b and 4b). Carbon utilization from the substrates was at different rates, suggesting different metabolic capabilities for the bacterial species. This agrees with the findings of other authors (Hinchee and Ong, 1992; Alvarez, 2003) who reported the transformation of different hydrocarbon mixture into carbon dioxide by mineralization. Data from statistical analysis (LSD test) showed that the growth of *Pseudomonas sp.*, *Bacillus sp.*, *Klebsiella sp.*, was enhanced more significantly ($P < 0.05$) by kerosene and engine oil, while the growth of *Proteus sp.* was enhanced more significantly ($P < 0.05$) by kerosene and AGO. The rates of utilization of different hydrocarbon fraction by the bacterial cultures were found to be related to the molecular complexity of the hydrocarbons involved. The high utilization rates exhibited by microbial species on kerosene are probably connected with its molecular weight. Raina et al. (2000) reported that mid size straight chain aliphatic (*n*-alkane C_{10} to C_{18} in length) are utilized more readily than *n*-alkane with either shorter or longer chain.

Utilization of hydrocarbons by the mixed bacterial consortium is shown in Figure 5. The growth of mixed bacterial consortium during the cultivation was signifycantly ($P < 0.05$) higher in kerosene as compared with engine oil and AGO. The amount of CO_2 evolved by bacterial consortium increased appreciably in the media containing the three hydrocarbons till the end of the monitoring. A high significant variation ($P < 0.05$) was observed between the efficiency of single and mixed bacterial cultures in utilizing the hydrocarbon substrates. The advantages of using mixed cultures instead of pure cultures in biodegradation processes have been amply demonstrated (Ghazali et al., 2000; Diaz et al., 2000).



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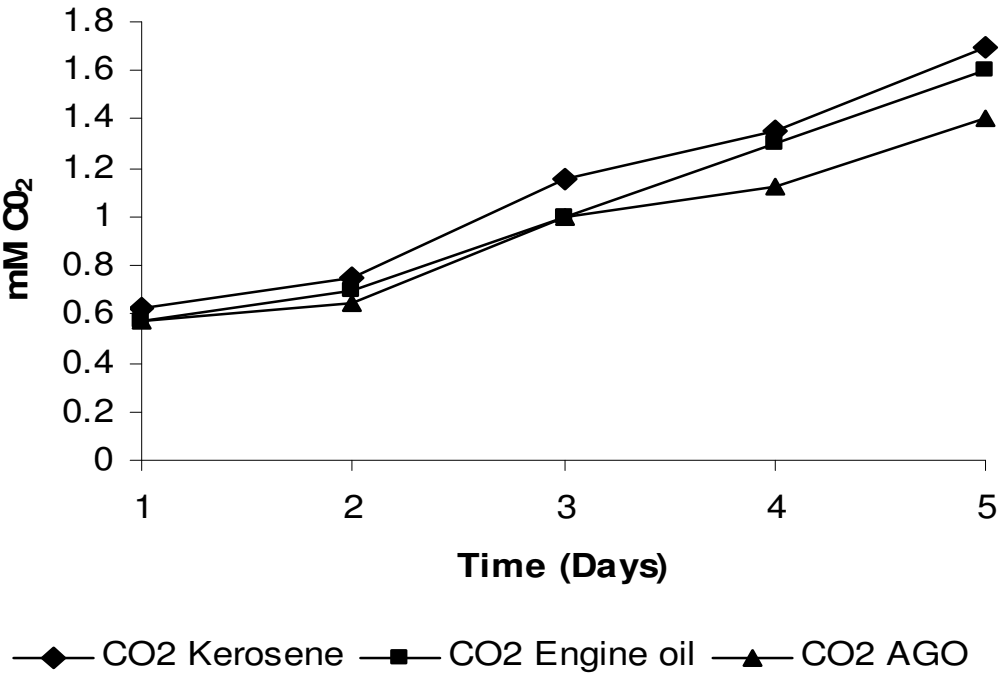
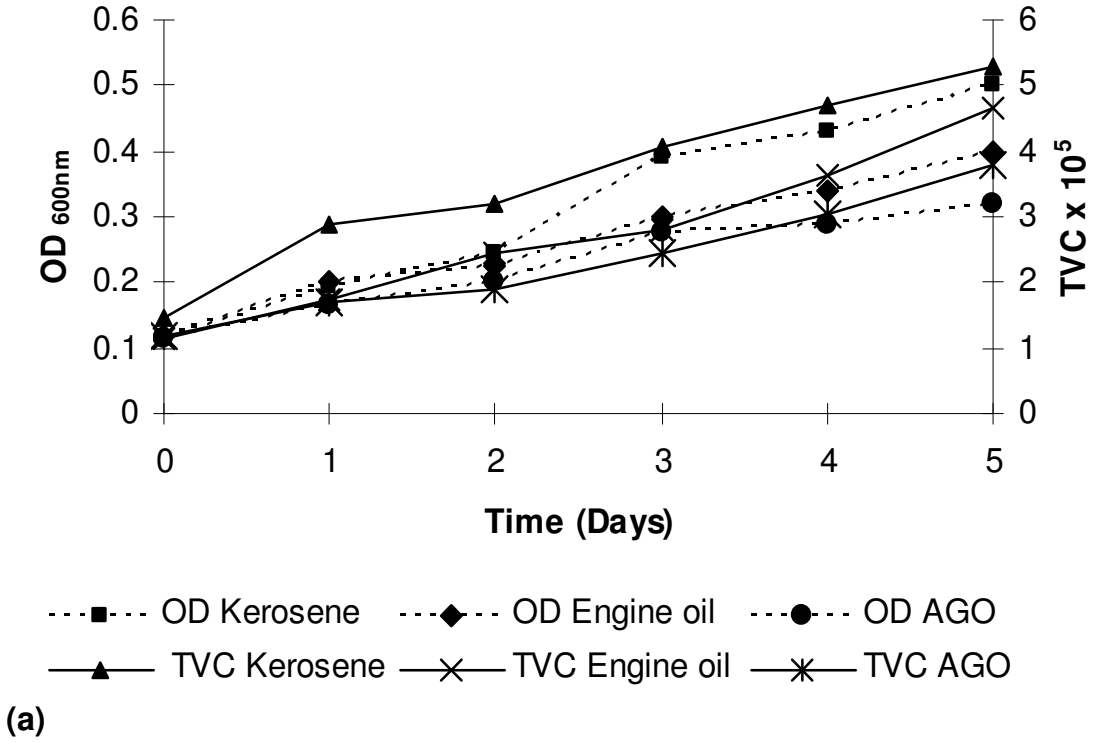


(b)

Figure 1. Growth pattern of *Pseudomonas* sp. on kerosene, AGO and engine oil.

These advantages could be attributed to synergistic interactions among the members of the association, which can be complex and favor petroleum degradation mechanism. This is possible when one species removes



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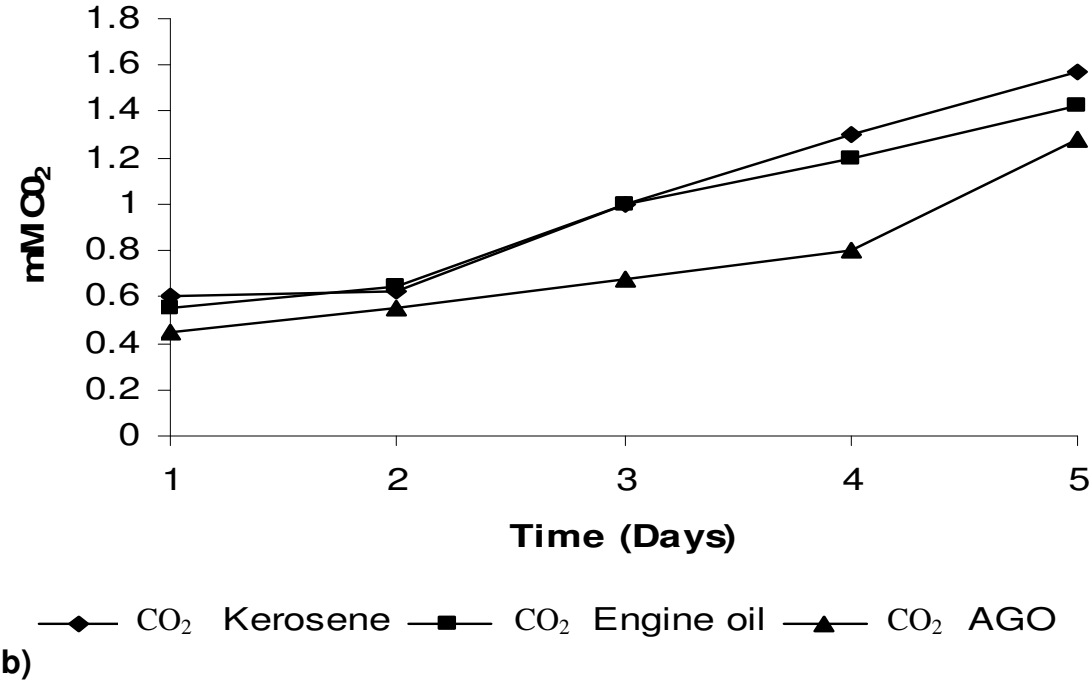
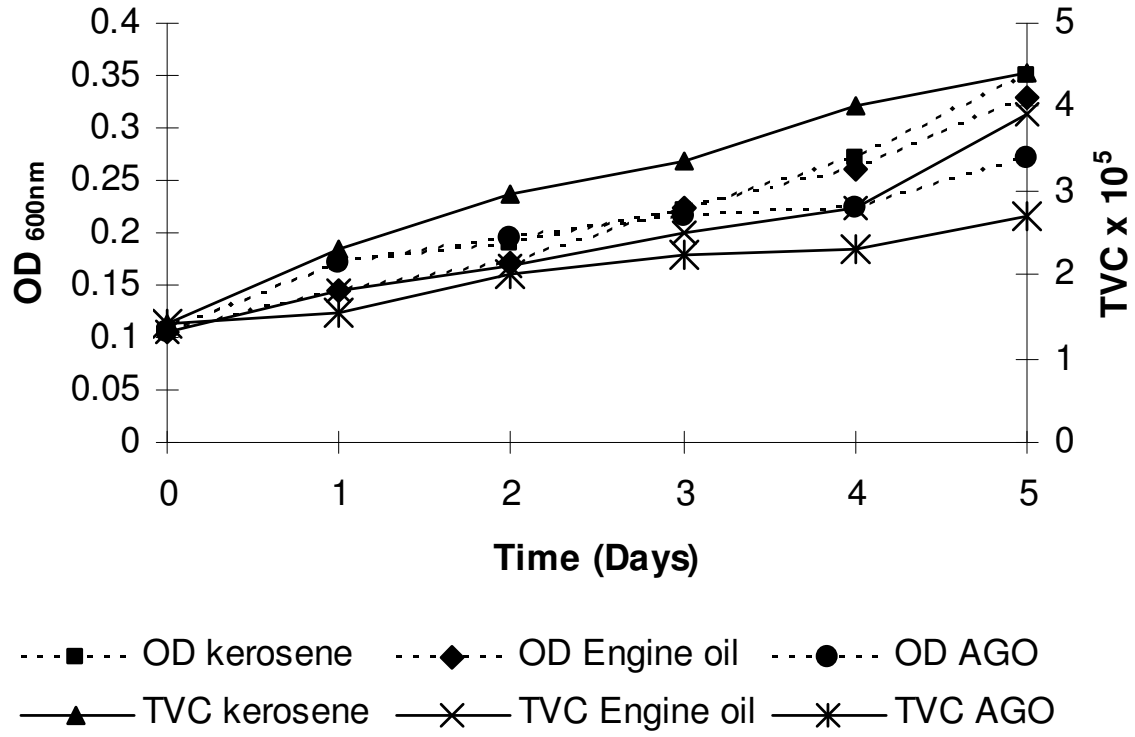


(b)

Figure 2. Growth pattern of *Bacillus* sp. on kerosene, AGO and engine oil.

toxic metabolites of another species that began the biodegradation process or when two species work in succession with the first partially degrading compounds and the second finishing the job.

Cunha et al. (2000) studied the biodegradation of gasoline by single and mixed cultures. The mixed culture studied presented the following degradation indices: 51.4% for toluene, 46.3% for the ethylbenzene, 49.4% for *n*-C9, 53.4% for *n*-C11, 50.4% for *n*-C12 and 50.0% for *n*-C13. Richard and Vogel (1999) in their study of the



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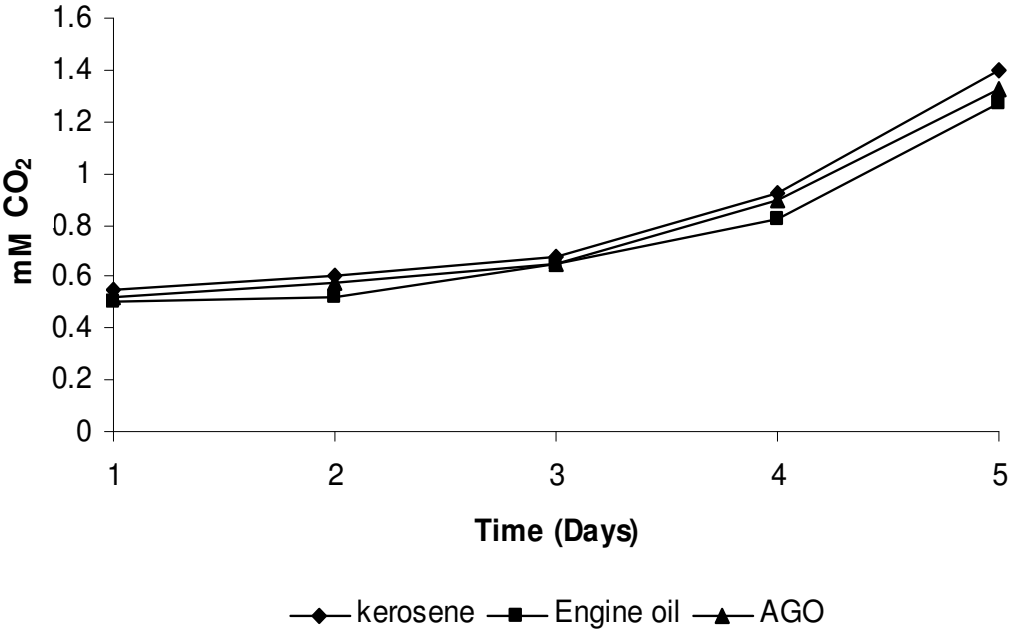
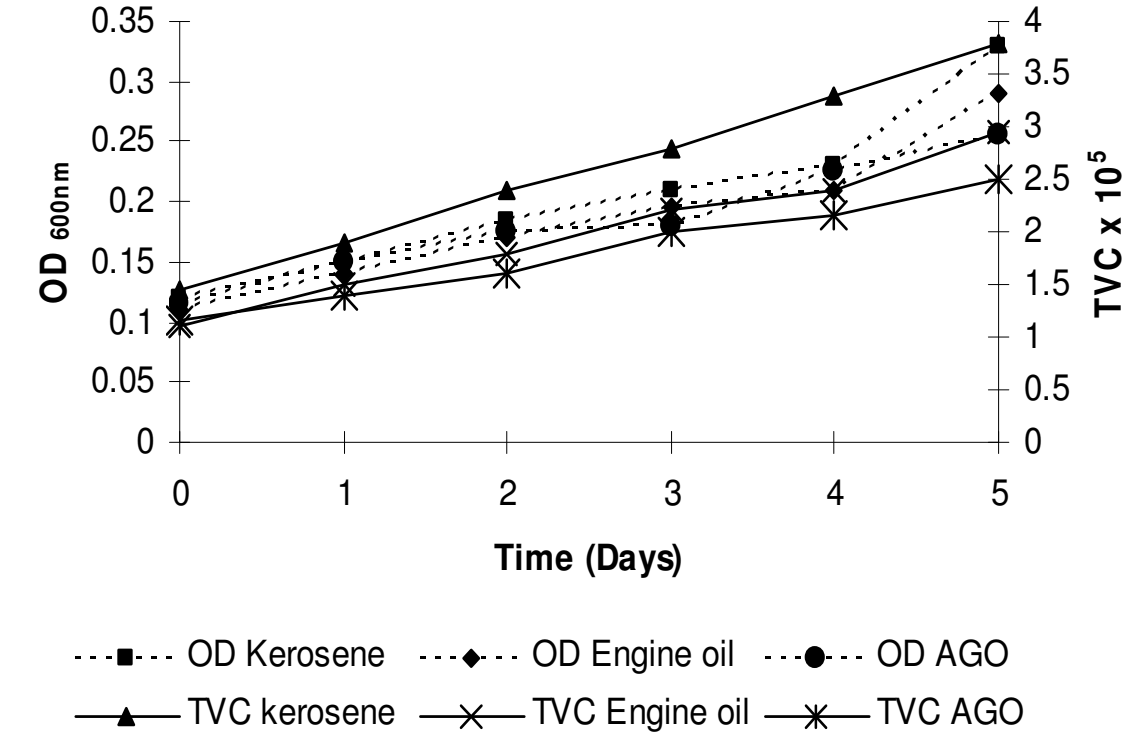


(b)

Figure 3. Growth pattern of *Klebsiella* sp. on kerosene, AGO and engine oil.

kinetics of diesel oil biodegradation by a consortium of microorganism reported a maximum value of 64.1% of TPH (%) removal for an isolated *Acrombacteria anthropi* culture and 90 for a consortium (at the end of 50 days of process, using a mineral medium and an initial diesel oil concentration of 1% v/v). The lowest level of TPH



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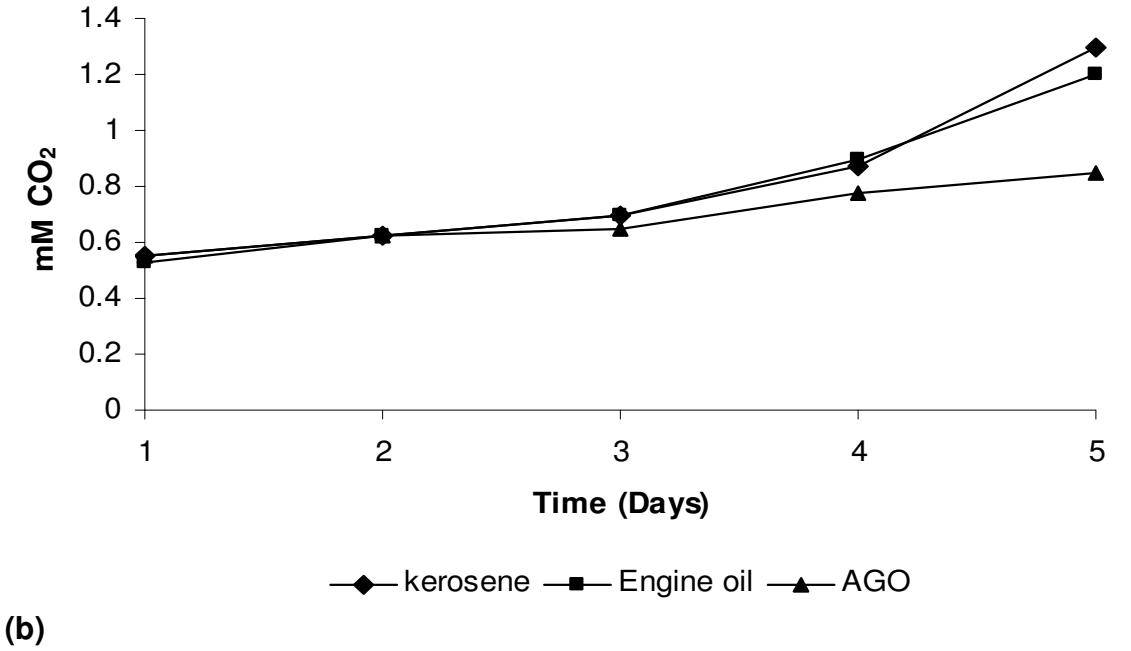
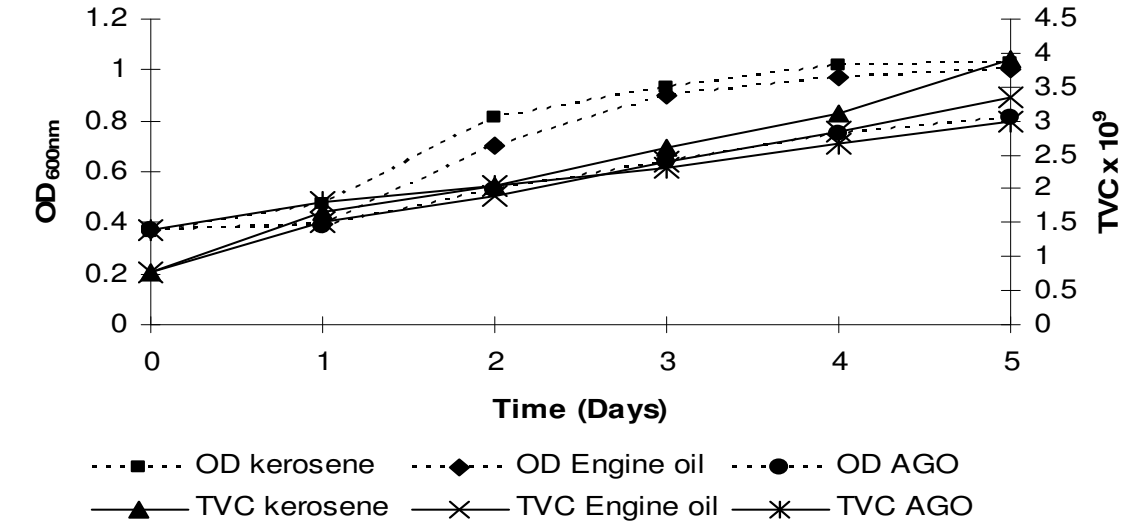


(b)

Figure 4. Growth pattern of *Proteus* sp on kerosene, AGO and engine oil.

removal was identified when isolated microorganisms such as *Pseudomonas fluorescence* (named P2 and P25) were used, resulting in TPH removal of less than 20% after 50 days. A similar observation was also reported by other researchers (Bassam and Mohammed, 2005; Vieira et al., 2006) where removal of crude oil degradation by mixed microbial consortium isolated from oil-contaminated soil samples was observed. Other



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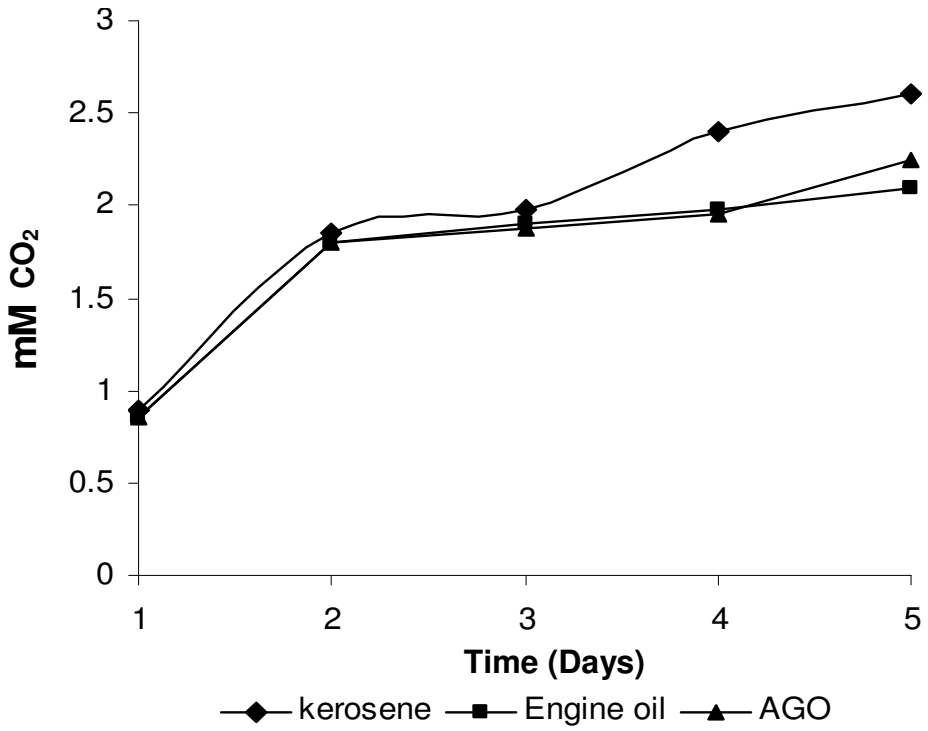

(b)

Figure 5. The growth profile of a bacterial consortium (wild type of *Bacillus* sp., *Pseudomonas* sp., *Klebsiella* sp., *Proteus* sp.) on kerosene, AGO and engine oil.

researchers have described the ability of mixed bacterial consortium to degrade 28 to 51% of saturated and 0 to18% of aromatic compounds present in crude oil (Rahman et al., 2003). The consortium concentration on hydrocarbon degradation obtained in this study is consistent with Bassam and Mohammed (2005) who showed that biodegradation would not occur at a significant rate if population of indigenous microorganism is less than 10^5 CFU/g of soil. According to Atlas (1999) more rapid rates of degradation occur when there is a mixed microbial community than can be accomplished by a single species. Apparently the genetic information in more than one organism is required to produce the enzymes needed for extensive petroleum biodegradation. Results

obtained in this study was under aerobic condition, which is in agreement with other reported studies (Cuhna et al., 2004) where aerobic conditions are generally considered necessary for extensive degrada-tion of hydrocarbons in the environment. This aerobic condition enhanced CO_2 production which have been used to measure mineraliz-ation because it is assumed that 50% of the carbon goes to CO_2 and the cell mass each under aerobic conditions, depicts the growth mean of the pure bacterial cultures *Pseudomonas* sp, *Bacillus* sp. and *Klebsiella* sp. The result revealed that *Pseudomonas* sp was a better utilizer of the hydro-carbons evaluated in this study. The other organisms utilized the hydrocarbon in this decreasing order; *Baciilus* sp. < *Klebsiella* sp. < *Proteus* sp.

Conclusion

The organisms evaulated in this work showed that the three organisms (*Pseudomonas* sp, *Bacillus* sp. and *Klebsiella* sp.) were able to utilize hydrocarbons as the sole carbon source both as a single and mixed culture. Though more degradation was observed when they are used as a mixed culture. Based on the results obtained from the laboratory study, biodegradation could be considered as a key component in the clean-up strategy developed in the future for the treatment of oil-sludge contamination. Some of the pure culture were versatile and persisted throughout the utilization process. Further studies are necessary on more interesting bacterial species and strains. In addition, evaluation of environmental conditions and optimization of biodegradation process based on several factors such as nutrient, biomass type and concentration, oxygen, surface tension, emulsifying activity and gene transfer are areas where further research is necessary. However, it is advantageous and profitable to use native microorganism cultured from areas with historical contamination for degradation of hydrocarbons. This approach is likely to reduce or eliminate the initial lag phase, which can be long and optimize overall process time.

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