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Biodegradation potentials of bacterial isolates from petroleum storage facilities within the Kumasi Metropolitan area

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High on the table of environmental discussions worldwide today is the problems of petroleum hydrocarbon contamination. This research focused on screening for the potentials of five bacterial isolates in utilizing the following hydrocarbon substrates [gasoline, diesel and kerosene (2.0v/v) in Bergs Mineral Salt Medium (BMSM)]. The effects of different nutrients supplementation on biodegradation indices were assessed. The biodegradation indices that were monitored included optical density ($OD_{600\ mn}$), emulsification stability (E_{24}) and gas chromatography (GC) profiles. The results from the GC profiles show that all the isolates had well above 80% reduction in the hydrocarbon substrates. *Enterobacter cloacae* showed the greatest potential with respect to gasoline degradation efficiency under soy supplement (99.66%). For diesel degradation, *Pseudomonas cepacia* and the consortium were at par with respect to the degradative efficiency, that is, 99.80%. For kerosene degradation studies, *E. cloacae* gave the highest degradation efficiency of 95.60%. As compared to the controls, these degradation efficiencies were relatively high. The above results purports that the all the strains especially *Enterobacter cloacae* and consortium are possible candidates for ameliorating the problem of hydrocarbon contamination.

Key words: Bacteria, hydrocarbons, biodegradation, gas chromatography.

INTRODUCTION

Petroleum is a complex mixture of varying molecular weight hydrocarbons and other organic compounds found beneath the earth's surface. It is formed from pyrolysis of hydrocarbon, in a variety of reactions, mostly endothermic at high and/or pressure (Kumar et al., 2011). As a technical term, petroleum encompasses the liquid (crude oil), natural gas and viscous or solid (asphalt and bitumen) forms of hydrocarbons that occur in the Earth, but the meaning is often restricted to the liquid oil form (http://www.britannica.com/EBchecked/topic/454269/petroleumEncyclopediaBritannica).

Industries depend heavily on the use of petroleum and its products. What has increased its usage over the past decades is the development of the automobile which has

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given petroleum the impetus as primary source of energy (Rahman et al., 2002). Today the world is heavily dependent on petroleum not only for motive power but also for lubrication, fuel, dyes, drugs and many synthetics. Chief among the producers of crude oil and natural gas are Iran, Saudi Arabia, the U.S. and Russia, these accounts for more than 60% of world energy consumption; the U.S. is by far the largest consumer. As petroleum production in the United States peaked during the 1960s, however, Saudi Arabia and Russia have surpassed the U.S. (Akiner and Aldis, 2004). According to Lambertson (2008), about 90% of vehicular fuel needs are met by oil; thus making it important or of critical concern to many nations. Petroleum's worth as a portable, dense energy source powering vast majority of vehicles and as the base of many industrial chemicals makes it one of the world's most important traded items. The worldwide consumption is about 30 billion barrels (4.8 km^3) of oil per year, and the top oil consumers largely consist of developed nations. In fact, 24% of the oil consumed in 2004 was by the U.S. alone, though by 2007 this dropped to 21% of world oil consumed (New York Mercantile Exchange (NYMEX) 2006; Mabro, 2006).

Among the numerous contaminants polluting the environment, hydrocarbons play a special role, which is related to their wide-scale distribution and hazardous physicochemical and biological properties (Lisovitskaya and Mozharova, 2008). Their presences in the environment need not only be the result of anthropogenic activities such as exploration, drilling, extraction, refining and combustion, but seepages as well (Kvenvolden and Cooper, 2003). The discharge of petroleum products in large quantities into the environment has impacted negatively on various ecosystems (sea, lands, wetlands and underground water). Their undesirable effects endanger plants and animals lives (Atlas and Philp, 2005). The quality of water is dented in terms of taste and smell even at very low level concentration of these contaminants (Adebusoye et al., 2006; Rahman et al., 2002).

The use of petroleum as a primary source of energy comes in handy; but the detrimental effects it leaves on various ecosystems has far reached consequences, as reiterated by several environmentalists. Media reports indicate that large amount of these toxins are released into both populated areas and ecosystem globally. The most common soil contaminants are petroleum-based. These chemicals tend to spread through soil by diffusion. Hydrocarbons from diesel fuel and gasoline are widespread problems, because of PAHs. Many PAHs are known carcinogens, and others are suspected deleterious chemicals that need to be kept from contaminating drinking water (van Grevenynghe et al., 2005; Samanta et al., 2002). It is believed that shorter carbon molecules facilitate microorganism degradation (Montagnolli et al., 2009).

There are many sinews as to what may constitute toxicity. Many compounds contribute to high toxicity, but the PAHs formed from fuel combustion and lubricant decomposition in high concentration is lethal to soil microorganisms (Henry, 1998). Generally oil products with low boiling points appear to be more toxic than the heavier fuel oils while crude oils are intermediate with respect to toxicity. Small hydrophobic molecules are highly toxic for microorganisms due to their partition into the cytoplasmic membrane (Sikkema et al., 1995). High concentrations of contaminants can be toxic to microbial populations and therefore hinder degradation (Alagappan and Cowan, 2003). They interrupt the protein-lipid and lipid-lipid connections in the membrane and, as a result, cause functional disturbances and increase membrane fluidity and passive diffusion of the hydrophobic compounds into the cell (Sikkema et al., 1995). Toxicity of crude oil on humans includes liver necrosis, congestion of the liver, fat degeneration and dissociation of hepatocytes. Birds and animals in oil-contaminated area are found to have black emulsion in the digestive tract with a petroleum odor. This leads to decrease in the absorption of nutrients and finally leads to death of these birds (Khan and Ryan, 1991). A mixture of wax, oil, sand and water is referred to as slop or sludge, in the petroleum industry.

Rojo (2009) has also reiterated that with the development of many economies and industries (petroleum exploration), contamination of soil with petroleum compounds is of concern worldwide. Hydrocarbon discharges are not only a problem in countries that produce oil but also in countries that purchase, process and use them. Environmental problems caused by oil from cars and trucks, leaky containers, industrial accidents and poorly disposed of wastes are much more common cause for concern. Cleanup may delay while the contaminated soil continues to pollute groundwater resources if on land, and death of aquatic life if on waterways. This problem is most serious in areas which rely on groundwater and river as major source of drinking water (Samanta et al., 2002). Nevertheless, many pilot works carried out indicates that for physical, chemical and biological methods applied for decontamination, microbial biodegradation is the most promising method and the use of efficient microbes has proved to be successful, especially in the treatment of petroleum pollutions.

In Ghana, there is no data on sites that has been contaminated by the storage or operations of hydrocarbons. Wherever the source of contamination may be from, petroleum products may reach groundwater reserves, lakes or water courses providing water for domestic and industrial use. Apart from the obvious altered taste and smell of water when contaminated with minute quantities of petroleum hydrocarbons, Onosode (2001) has intimated that oil spills have destroyed farmlands, polluted surface and groundwater caused drawbacks in fishing and killed many rural Nigerians through fire outbreaks and explosions in the region. Diesel constituents (PAHs) are known to be carcinogenic, mutagenic and a potent immuno-toxicant,

Table 1. Measurement of OD against pH of culture media containing gasoline (2%) supplemented with pito waste over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	$OD_{(600\ nm)}$/ pH							
Bacillus firmus	0.0	0.05	0.28	0.45	0.48	0.45	0.4	0.40
	10.0	8.5	8.3	8.1	7.6	7.4	7.0	6.70
Enterobacter cloacae	0.0	0.18	0.4	0.79	0.8	0.8	0.74	0.70
	10.0	8.75	8.6	8.4	7.8	7.6	7.4	6.9 0
Pseudomonas aeruginosa	0.0	0.12	0.3	0.45	0.5	0.8	0.6	0.54
	10.0	8.77	8.4	8.0	7.7	7.3	7.0	6.90
Proteus mirabilis	0.0	0.48	0.38	0.81	0.90	0.84	0.83	0.70
	10.0	8.74	8.3	8.1	7.8	7.2	7.1	6.50
Consortium	0.00	0.2 0	0.57	0.6	0.75	0.83	0.87	0.86
	10.0	8.84	8.4	7.9	7.3	7.4	7.1	6.85
Control	0.00	0.00	0.00	0.02	0.02	0.04	0.05	0.05
	10.0	10.0	10.0	9.5	9.4	9.4	9.3	9.0

thus posing a serious threat to human, animal health and the ecosystems over a prolonged period of released. This pollutant may also inhibit some microbial communities that are important in some biogeochemical cycles of that ecosystem and this affects the productivity of such ecosystem.

In this research, five bacterial isolates namely *Pseudomonas aeruginosa, Pseudomonas cepacia, Enterobacter cloacae, Proteus mirabilis* and *Bacillus firmus* were investigated for biodegradation efficiencies. The biodegradation potential of the isolates were compared. They biodegradation include emulsifying stability test (E_{24}), optical density ($OD_{600\ nm}$) and the gas chromate-graphy profile of individual species and mix cultures on various petroleum hydrocarbon substrates.

MATERIALS AND METHODS

Preparation of degradative cultures

Nitrogen sources such as soy and malted sorghum (pito) were used in this study. Each of these nitrogen sources was added to 33 ml of Bergs mineral salt medium (BMSM) (Berg et al., 1990) at concentration of 0.5% (w/v) at the pH of 10 ± 0.5. Two percent (2% v/v) of gasoline, diesel and kerosene were each distributed in 100 ml sterilized bottles containing the above MSM under aseptic conditions. The same experiment was repeated but with no nutrient supplement. One heavy loop full of pure bacterial culture isolates namely *P. aeruginosa, E. cloacae*, *P. cepacia, B. firmus* and *P. mirabilis* each aseptically introduced into each of the medium. A mix culture (consortium) was also introduced in another treatment; a control was also set up without any microorganism. The set ups (treatments) were kept at room temperature on a rotary shaker (Lab line No 3590), with shaking at 110 rpm for fourteen days. The indices of growth namely the OD and the E_{24} indices were estimated. The turbidity of the culture media (OD) were measured spectrophotometrically in a fashion similar to what has been describe by Rahman et al. (2002) at an interval of two days to allow appreciable monitoring of bacteria growth under experimental conditions alongside that of sterile control. The pH of various culture media was also monitored as well. Uninoculated control was used to monitor abiotic loss of the petroleum products. Below is the outline of the set up or treatments: MSM + Gasoline + pure culture; MSM + Gasoline + pito waste + pure culture; MSM + Gasoline + soy residue + pure culture; MSM + Gasoline + soy residue + consortium; Control: MSM + Gasoline (no culture added); MSM +Diesel + pure culture; MSM + Diesel + pito waste + pure culture; MSM + Diesel + soy residue + pure culture; MSM + Diesel + soy residue + consortium; Control: MSM + Diesel (no culture added); MSM + Kerosene + culture; MSM + Kerosene + pito waste + culture; MSM + Kerosene + soy residue culture; MSM +Kerosene + soy residue + consortium; Control: MSM + Kerosene (no culture added).

The results for the above treatments measuring OD and pH with in a time course is presented in Tables 1 to 9.

There was a general trend in the pH and OD values across the treatments (Tables 1 to 9) thus a gradual dip in the pH values with concomitant rise in the OD values. An exception to this trend was observed and recorded for the treatments where no microbes were introduced (control); there were marginal increases in the OD values and very little changes in pH values.

Quantitative analysis/recovery of residual hydrocarbon substrates/losses

The hydrocarbon substrates (under graded oil/residual oil) in the MSM were extracted using the liquid-liquid extraction procedure.

Table 2. Measurement of OD against pH of culture media containing diesel (2%) supplemented with pito waste over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD $_{(600\ nm)}$ / pH							
Pseudomonas aeruginosa	0.00	0.75	0.69	0.64	0.90	0.42	0.24	0.27
	9.8	8.7	8.4	7.9	7.7	7.4	7.0	6.8
Enterobacter cloacae	0.00	0.65	0.75	0.69	0.95	0.42	0.29	0.52
	10.0	8.8	8.2	7.6	7.5	7.2	6.8	6.30
Pseudomonas cepacia	0.0	0.85	0.43	0.75	0.45	0.30	0.15	0.60
	0.0	8.5	8.2	7.9	7.4	7.0	6.7	6.40
Bacillus firmus	0.0	0.9	0.75	0.77	0.93	0.45	0.14	0.07
	9.8	8.75	8.3	8.0	7.8	7.2	7.0	6.80
Consortium	0.0	0.85	0.50	0.6	0.60	0.48	0.28	0.28
	10.0	8.5	8.1	7.85	7.5	7.25	6.95	6.70
Control	0.0	0.00	0.00	0.01	0.00	0.02	0.18	0.20
	0.0	0.0	9.8	9.8	9.8	9.4	9.5	9.0

Table 3. Measurement of OD against pH of culture media containing kerosene (2%) supplemented with pito residue over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD $_{(600\ nm)}$ / pH							
Pseudomonas aeruginosa	0.20	0.80	0.55	0.55	0.65	0.41	0.41	0.75
	10.0	8.9	8.6	8.1	7.6	7.2	6.75	6.5
Enterobacter cloacae	0.00	0.37	0.12	0.12	0.38	0.37	0.37	0.40
	10.0	8.6	8.2	7.8	7.4	7.0	6.85	6.45
Pseudomonas cepacia	0.02	0.69	0.66	0.66	0.74	0.06	0.06	0.30
	9.9	8.5	8.1	7.6	7.1	6.9	6.7	6.35
Bacillus firmus	0.0	0.44	0.70	0.70	0.88	0.50	0.56	0.60
	10.0	8.7	8.25	7.85	7.5	6.95	6.6	6.3
Consortium	0.21	0.72	0.45	0.54	0.98	0.80	0.60	0.50
	10.0	8.6	8.40	7.9	7.4	7.1	7.0	6.80
Control	0.00	0.00	0.00	0.02	0.02	0.04	0.02	0.05
	10.2	10.00	10.00	10.0	9.85	9.70	9.50	9.50

Hexane was used in the extraction; double extraction was employed. 30 ml of hexane was used. Initially 15 ml of hexane was added to the culture media in the separating funnel, capped and shaken thoroughly for about 2 min to partition the contaminants into the solvent phase. After settling, the mixture in the funnel separates into two phases, thus the solvent phase and the aqueous phase.

Table 4. Measurement of OD against pH of culture media containing gasoline (2%) over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD $_{(600\ nm)}$ / pH							
Bacillus firmus	0.00 10.0	0.00 8.84	0.00 8.6	0.00 7.8	0.02 7.2	0.06 7.0	0.00 6.5	0.4 6.5
Enterobacter cloacae	0.00 10.0	0.00 8.42	0.00 7.9	0.00 7.5	0.02 7.2	0.05 7.1	0.02 6.8	0.06 6.5
Pseudomonas aeruginosa	0.00 10.0	0.00 8.70	0.00 8.2	0.00 7.9	0.0 7.3	0.00 7.2	0.01 6.8	0.2 6.4
Proteus mirabilis	0.00 10.0	0.00 8.85	0.02 8.4	0.00 8.0	0.01 7.8	0.4 7.3	0.02 6.5	0.05 6.50
Consortium	0.00 10.0	0.18 8.85	0.2 0 8.3	0.20 8.0	0.03 7.7	0.00 7.2	0.04 6.7	0.1 6.40
Control	0.00 10.0	0.00 10.00	0.00 10.00	0.05 10.0	0.05 9.80	0.02 9.80	0.02 9.80	0.03 9.80

Table 5. Measurement of OD against pH of culture media containing diesel (2%) over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD $_{(600\ nm)}$ / pH							
Pseudomonas aeruginosa	0.09 10.0	0.4 8.7	0.6 8.4	0.3 7.9.	0.30 7.0	0.19 6.8	0.04 6.50	0.02 6.3
Enterobacter cloacae	0.05 10.0	0.1 8.8	0.12 8.2	0.14 7.8	0.37 7.1	0.28 7.0	0.20 6.8 0	0.03 6.5
Pseudomonas cepacia	0.00 10.0	0.04 8.7	0.05 8.0	0.29 7.6	0.30 6.9	0.25 6.8	0.10 6.5	0.10 6.2
Bacillus firmus	0.02 10.0	0.24 8.5	0.42 8.1	0.24 7.8	0.27 7.4	0.1 6.8	0.12 6.9	0.09 6.20
Consortium	0.028 10.0	0.42 8.9	0.46 8.20	0.54 7.5	0.6 7.2	0.5 7.0	0.35 6.9	0.21 6.50
Control	0.00 10.0	0.00 10.00	0.00 10.00	0.02 9.8	0.02 9.9	0.00 9.8	0.01 9.8	0.02 9.53

The aqueous phase was drained off into the bottle from which it was initially poured, while the solvent phase was kept in a clean 30 ml bottle capped and stored in a refrigerator (for a month) until analysis. These steps were repeated for all the samples including the controls. The aqueous phase was used for the emulsification (E_{24}) index.

Table 6. Measurement of OD against pH of culture media containing kerosene (2%) over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	$OD_{(600\ nm)}$ / pH							
Pseudomonas aeruginosa	0.00 9.8	0.00 8.95	0.05 8.3	0.02 7.8	0.17 7.4	0.18 6.8	0.21 6.5	0.26 6.2
Enterobacter cloacae	0.00 10.0	0.00 8.72	0.01 8.2	0.00 7.5	0.15 7.0	0.16 7.0	0.18 6.7	0.1 6.4
Pseudomonas cepacia	0.00 10.0	0.00 8.77	0.02 8.2	0.2 7.6	0.05 7.2	0.07 6.8	0.12 6.4	0.2 6.2
Bacillus firmus	0.00 10	0.00 8.80	0.00 8.0	0.08 7.6	0.11 7.0	0.15 6.8	0.18 6.5	0.28 6.1
Consortium	0.00 10.0	0.2 8.5	0.21 7.90	0.16 7.4	0.2 6.7	0.3 7.0	0.36 6.3	0.26 6.30
Control	0.00 10.0	0.00 10.00	0.00 10.00	0.01 10.0	0.01 10.00	0.00 9.8	0.01 9.7	0.00 9.0

Table 7. Measurement of OD against pH of culture media containing gasoline (2%) supplemented with soy residue over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	$OD_{(600\ nm)}$ / pH							
Bacillus firmus	0.3 9.85	0.72 8.54	0.35 8.2	0.35 7.80	0.28 7.2	0.6 6.3	0.52 6.5	0.50 6.3
Enterobacter cloacae	0.28 10.1	0.88 8.5	0.67 8.3	0.65 7.9	0.35 7.2	0.44 6.2	0.5 6.2	0.45 6.2
Pseudomonas aeruginosa	0.02 10.0	0.7 8.35	0.6 8.2	0.54 7.2	0.27 7.0	0.5 6.8	0.57 6.3.	0.54 6.8
Proteus mirabilis	0.25 9.95	1.0 8.6	0.89 8.0	0.5 7.4	0.38 7.2	0.69 6.7	0.48 6.5	0.50 6.5
Consortium	0.2 10.2	0.9 8.7	0.78 8.2	0.7 7.8	0.57 7.3	0.6 6.9	0.4 6.7	0.45 6.5
Control	0.00 10.2	0.00 10.00	0.01 10.00	0.01 9.9	0.05 9.9	0.05 9.8	0.05 9.8	0.02 9.0

Screening for emulsification stability

Emulsification (E_{24}) index

The (E_{24}) indices of the cultured samples were determined in a fashion similar to that described by Desai and Banat (1997). 2% (v/v) of gasoline, diesel and kerosene mixture was added to same volume of the culture medium from the various treatments, respectively in a 15 ml centrifuge tube. The mixture was vortexed for 2 min and left to stand for 24 h. The E_{24} index is expressed as the percentage of the emulsified layer (mm) divided by the total height of the liquid column (mm).

Table 8. Measurement of OD against pH of culture media containing diesel (2%) supplemented with soy residue over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD $_{(600\ nm)}$ / pH							
Pseudomonasaeruginosa	0.0 10.05	0.46 8.9	0.60 8.1	0.6 7.5	0.4 6.9	0.43 6.7	0.45 6.5	0.6 6.5
Enterobacter cloacae	0.02 10.0	0.50 8.5	0.53 8.0	0.53 7.7	0.5 6.8	0.46 7.0	0.16 6.8	0.30 6.6
Pseudomonas cepacia	0.04 9.9	0.75 8.7	0.77 7.3	0.72 7.0	0.43 6.8	0.3 7.1	0.175 7.0	0.5 6.8
Bacillus firmus	0.10 10.0	0.70 8.5	0.72 7.4	0.68 7.1	0.4 7.0	0.16 6.8	0.2 6.5	0.27 6.4
Consortium	0.33 10.0	1.0 8.5	0.8 7.7	0.7 7.2	0.65 7.0	0.35 7.0	0.51 6.8	0.7 6.5
Control	0.00 10.0	0.00 10.0	0.02 10.0	0.00 9.85	0.01 9.8	0.02 9.8	0.02 9.7	0.0 9.5

Table 9. Measurement of OD against pH of culture media containing kerosene (2%) supplemented with soy residue over a period of 14 days.

Species	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD $_{(600\ nm)}$ / pH							
Bacillus firmus	0.05 10.0	0.4 8.6	0.55 8.2	0.6 7.7	0.63 7.3	0.60 7.05	0.54 6.8	0.50 6.7
Pseudomonas aeruginosa	0.02 10.2	0.5 8.55	0.38 8.0	0.4 7.6	0.50 7.0	0.53 7.0	0.40 6.7	0.42 6.70
Enterobacter cloacae	0.05 10.2	0.46 8.50	0.43 8.0	0.46 7.4	0.44 7.1	0.4 6.8	0.38 6.4	0.30 6.30
Pseudomonas cepacia	0.35 10.2	0.52 8.52	0.51 7.9	0.65 7.2	0.64 6.9	0.58 6.7	0.55 6.3	0.44 6.20
Consortium	0.03 9.85	0.20 8.75	0.6 8.0	0.7 7.5	0.64 7.1	0.63 6.9	0.58 6.4	0.54 6.40
Control	0.00 10.2	0.00 10.0	0.01 10.0	0.00 9.5	0.02 9.1	0.02 9.0	0.00 8.9	0.00 8.50

$$E_{24}\ \text{index} = \frac{\text{Height of emulsified layer (mm)}}{\text{Total height of the liquid column (mm)}} \times 100$$

Results for emulsification (E_{24}) index

Emulsification (E_{24}) index

Incorporation of different hydrocarbon (gasoline, diesel and kerosene)

Table 10. Emulsification (E_{24}) index.

Species	Percentage of (E_{24})		
	Pito	**soy**	**Non-supplemented gasoline**
Bacillus firmus	1.5/4 x 100 = 37.5%	1.5/4 x100= 37.5%	N/S (0%)
Enterobacter cloacae	2.5/4 x 100 = 62.5%	3/4 x 100= 75 %	N/S (0%)
Psuedomonas aeruginosa	2/4 x 100 = 50%	1.5/4 x 100= 37.5 %	N/S (0%)
Proteus mirabilis	1.5/4 x 100= 37.5%	1.5/5 x 100=37.5%	N/S (0%)
Control	N/S (0%)	(0%) N/S	N/S(0%)
	Diesel		
Psuedomonas aeruginosa	3.5/4 x 100= 87.5%	2.5/4 x 100 = 62.5%	N/S (0%)
Enterobacter cloacae	1.5/4x100= 37.5%	1.5/4 x 100=37.5%	N/S(0%)
Pseudomonas cepacia	1.75/4x100= 44.0%	1.5/4 x 100=37.5%	1.5/4 x 100=37.5%
Bacillus firmus	N/S (0%)	1.7/4 x 100= 42.5%	1.7/4 x 100= 42.5%
Control	N/S (0%)	1.5/4 x 100 =37.5	N/S (0%)
	Kerosene		
Pseudomonas aeruginosa	2.7/4 x 100=67.5%	0.3/4 x 100=75%	1.7/4 x100=42.5%
Enterobacter cloacae	3/4 x 100=75%	N/S (0%)	2/4 x 100=50%
Pseudomonas cepacia	3.5/4 x 100=87.5%	N/S (0%)	2.5/4x100=62.5%
Bacillus firmus	2/4 x 100= 50%	1.7/4 x 100=42.5%	N/S (0%)
Control	N/S (0%)	N/S (0%)	N/S (0%)

Those less than 0.2/4 were considered as not significant (N/S) given a percentage of zero (0%).

culture extracts showed an appreciable emulsion after 24 h except the uninoculated tubes. Those that were not amended with nutrients and some isolates did not also show any sign of emulsification. Varying percentages of (E_{24}) indices were achieved by various isolates but *E. cloacae* gave the highest E_{24} indices of 75, 625% with addition of pito and soy respectively, for gasoline.

The (E_{24}) indices were 87.5 and 62.5% for *P. aeruginosa* with diesel under soy and pito amendment, respectively. The (E_{24}) indices of the isolates in MSM having kerosene as carbon source were 87.5% for *P. cepacia* (pito amended), 75% for both *E. cloacae* and *P. aeruginosa* 75% under pito and soy amendment, respectively. Emulsification indices or stability of emulsions were high and showed no patterns of relatedness except for the influence of the media from which the isolates grew, that is, whether it was nutrient amended or not. Isolate that were cultured with pito and soy supplements produced bioactive agents and it seem that the nutrients influenced (E_{24}) indices to an appreciable levels thus increased surfactant activities of the isolate as shown in Table 10. This result is similar to that of Monteiro et al. (2006) who recorded an emulsification index of 70% after 30 days of incubation, *P. aeruginosa* did produce emulsions that are stable and could be used in the control of environmental contamination (Monteiro et al., 2006).

Residual extraction of hydrocarbon substrates

The hydrocarbon analysis was performed at the Tema oil Refinery (TOR) quality assurance laboratory. Gas chromatographic device used was Agilent 6890 gas chromatography (GC), equipped with equipped with a flame ionization detector (FID) using an enhanced integrator software. The oven temperature was initially set to 30°C and increased at a rate of 6°C per minute to 300°C. The carrier gas was helium at a constant flow rate of 1.0 ml/min. Three microliter of the extractable substrates was analyzed on a 30 m polydimethylsiloxane capillary column.

From the GC profiles of residual oils left at the end of the 14 day period (and storage period of one month), reductions in area percent reports of all substrates and their components showed a marked effect of the isolates in utilizing the hydrocarbon substrates. Some (G.C) profiles of gasoline, diesel and kerosene are shown in Figures 1 to 4.

The total mixture was assumed to be 100%. The hexane (being lighter and pure) shows the highest peak. The percent area report values of hexane are from total mixture to obtain the percent area report values of the residual hydrocarbons. This is compared with the area percent report values of authentic standards determined initially. The degradation efficiency of the isolates were calculated using the formula stated below.

D% = (sum of area peak values for TPHs of kerosene before (sd)– sum of total area peak values for TPHs of kerosene treated after (trd) / sum of area peak values for TPHs before (sd) x 100

Where sd = standard, trd= treated.

$D\% = \sum \text{TPHs sd} - \sum \text{TPHs trd} / \sum \text{TPHs sd} \times 100$

Gas chromatographic (GC) profiles

From some of the GC profiles, the growth of *Pseudomonas, Enterobacter*, *Bacillus* and *Proteus* as well as the mix culture (consortium) in various substrates resulted in a substantial disappearrance of the fraction within hydrocarbon substrates as shown in Figures 1 to 4. The Gram-negative bacteria (*Pseudomonas, Enterobacter* and *Proteus*) gave a relatively higher petroleum-degradation efficiency when compared with *Bacillus firmus*

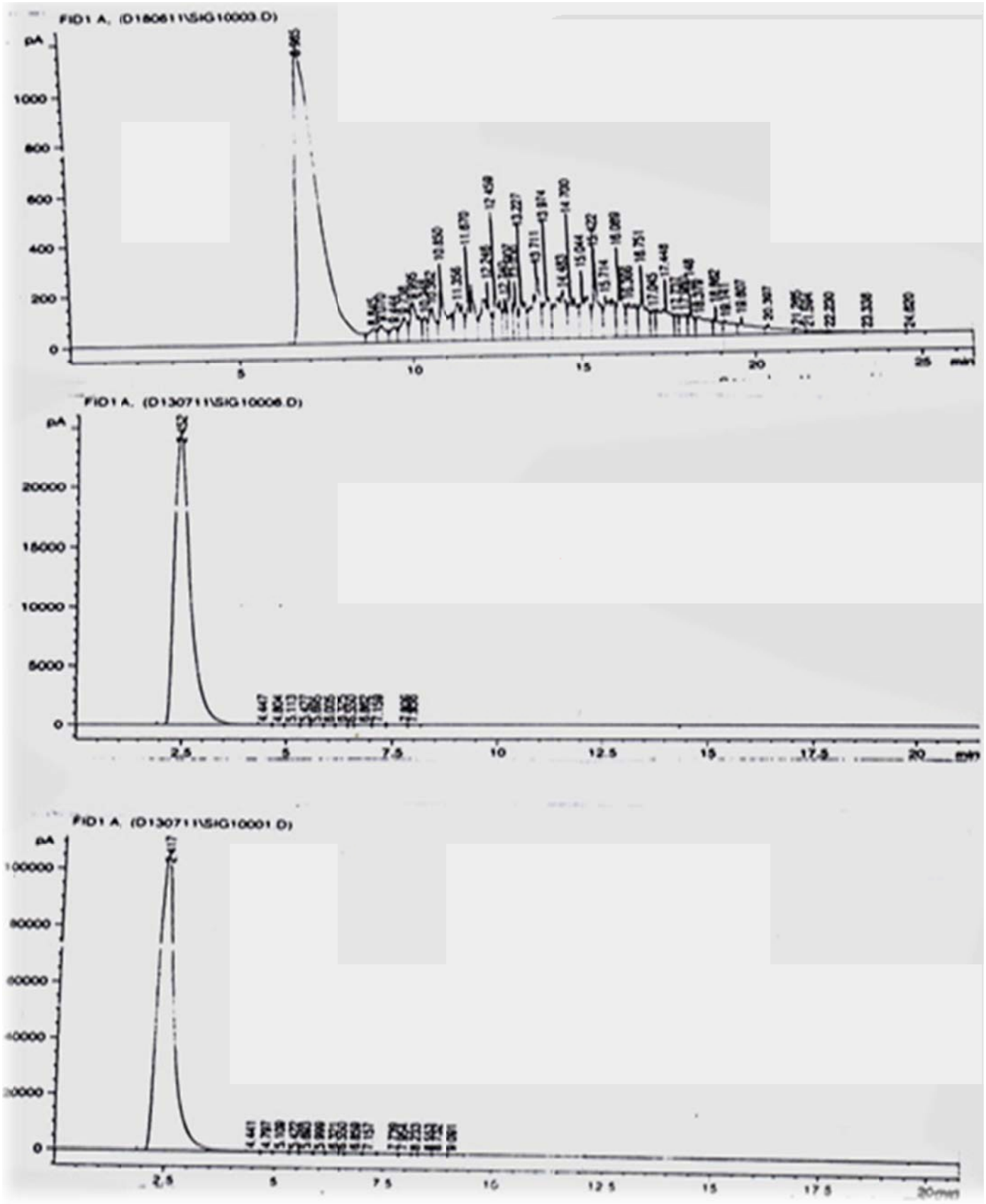

Figure 1. Diesel degradation. GC profile comparing standard (top most), consortium (middle) and *P. cepacia (*middle) with soy added after 14 days period and a storage period of one month.

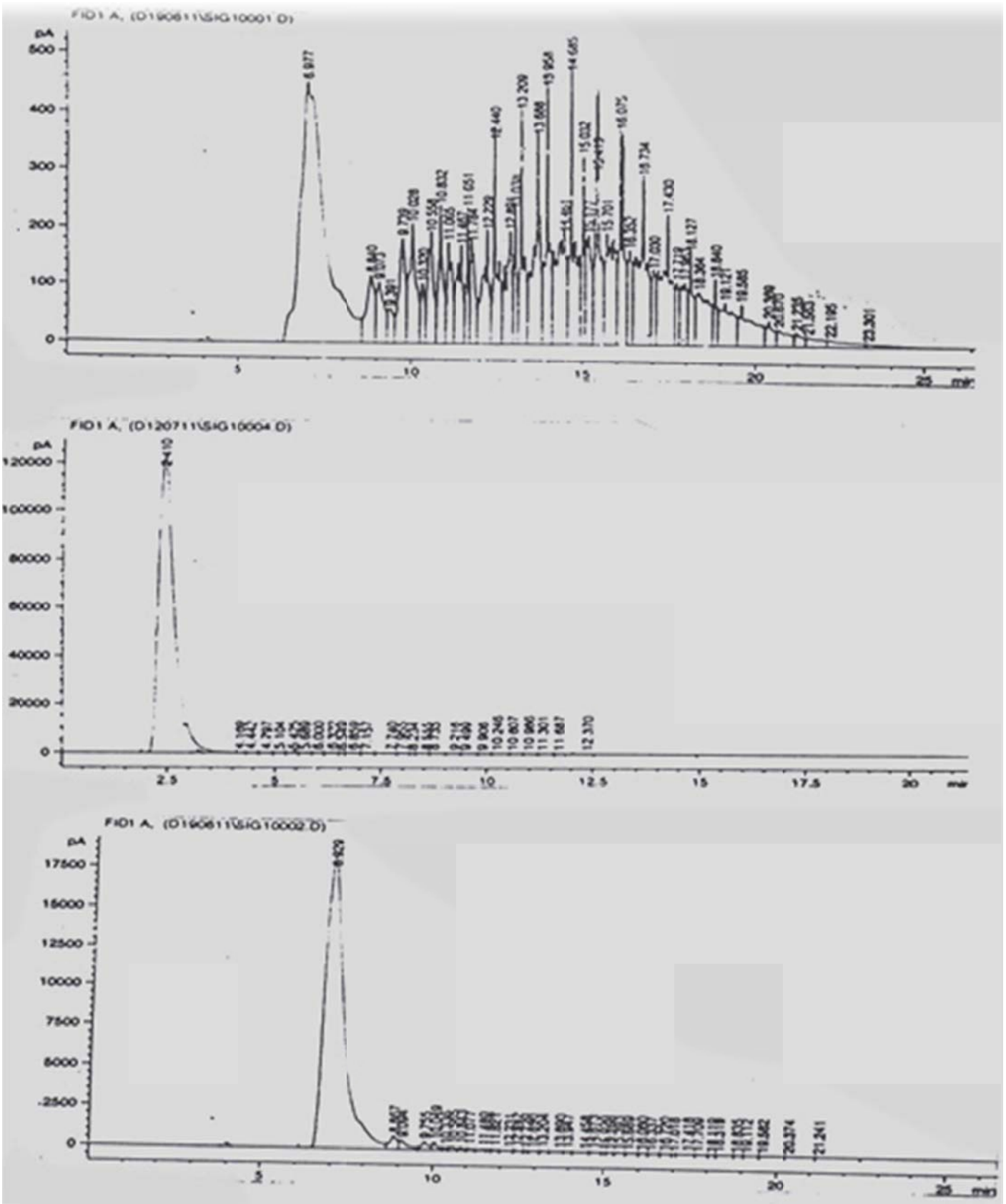

Figure 2. Gasoline degradation. GC profile comparing standard (top most) with *E. cloacae*–soy added (middle) and control (bottom) after 14 days period and a storage period of one month.

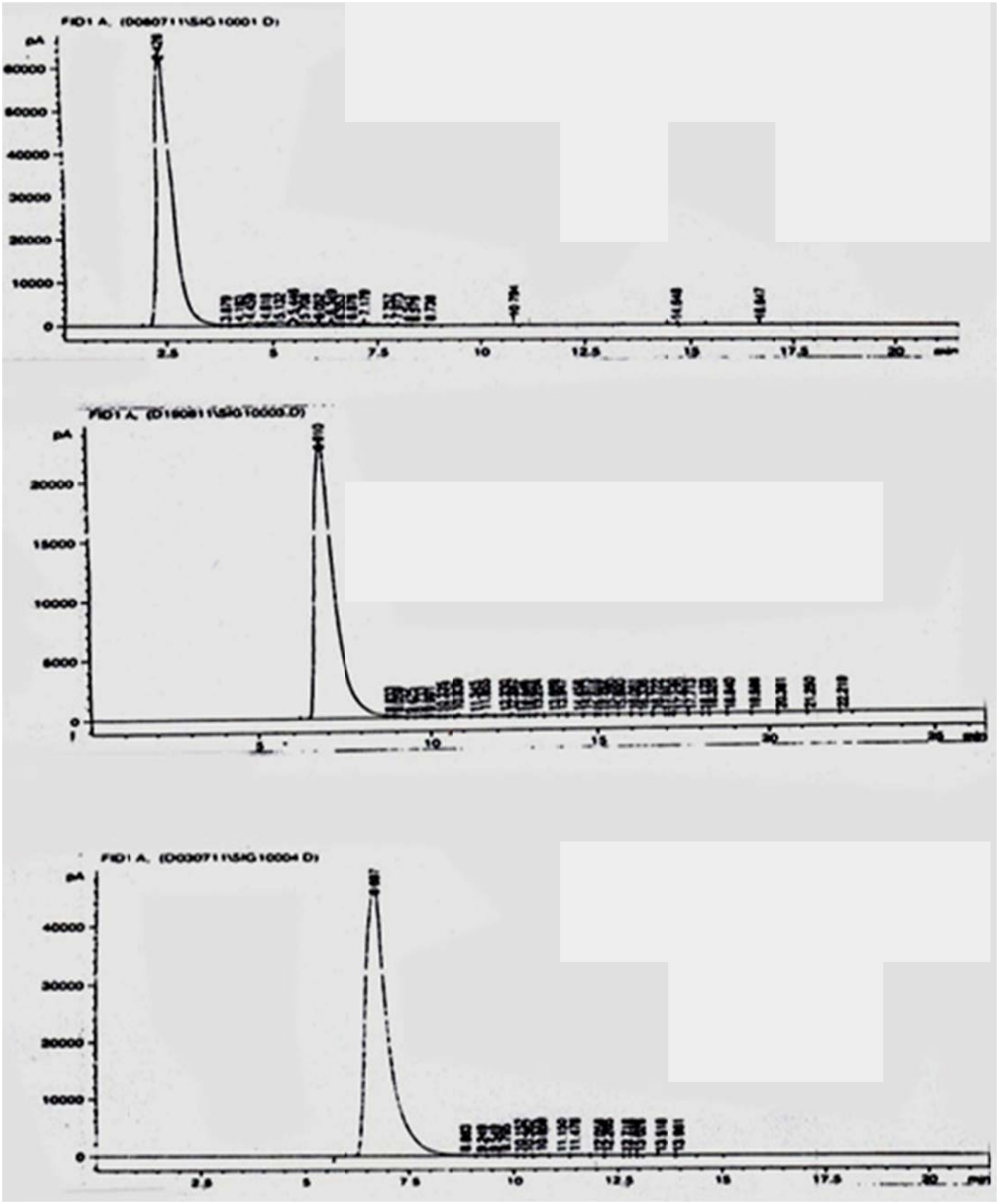

Figure 3. Kerosene degradation: GC profile comparing standard (top most), with control (middle) and *E. cloacae* (bottom) with soy added after 14 days period and a storage period of one month.

Figure 4. liquid-liquid separation technique.

(the only Gram-positive bacteria) and the control. In the degradation of diesel, *P. cepacia* (soy added) and consortium (without nutrient addition) achieved the same level of substrate removal (99.8% efficiency) as seen in Table 13.

Lastly, the gasoline degraded by the expressed in percentages revealed that *E. cloacae* (soy added) and microbial consortium (*B. firmus, E. cloacae, P. aeruginosa, P. mirabilis*) with soy exhibiting the highest degradation potential (99.9 and 99.6%, respectively) followed lastly by *B. firmus* (97.2%).

In the degradation of kerosene, *E. cloacae* exhibited the highest degradation efficiency of 96% (under soy treatment) over a period of 14 days; this was a little higher than microbial consortium degradation efficiency of (95.6%). The differences in the efficiency of the isolates are very close as seen from Table 13. Relatively, the least successful was the attempt to utilize kerosene with *B. firmus* (soy added) (83.3%).

In this study, all the isolate exhibited the ability to degrade hydrocarbon substrates to varying degrees with most of them having an efficiency of over 90%. It is worth noting as the figures suggest that *Pseudomonas* and *Enterobacter* were highest with respect to their efficiency at degrading hydrocarbons. The rate of degradation and or loss of the substrates in the media were relatively fast almost, 100% as revealed in Tables 11-13.

From Tables 11-13, the optimum degradation efficiency was that of gasoline, this was achieved by *E. cloacae* (99.66%), whereas the mix culture and other isolates achieved a rate quite close to the degradation efficiency of *Enterobacter* over the same period of time. All isolates showed high degrees of degradation under nutrient supplementations which were remarkable but the Gram-positive were relatively consistent in their efficiency in all the culture media supplemented with gasoline, diesel and kerosene. These bacteria have a stronger cell envelope and are more tolerant to high levels of hydrocarbons due to their resistant endospores, than Gram-negative bacteria which allow them to thrive in the highly variable hydrocarbon contaminated environment (Zheng and Obbard, 2002).

DISCUSSION

The results from the pH and OD values for the treatments over the 14 days of investigation revealed that the top degrader(s) namely consortium and *Enterobacter* recorded optimum pH and OD values which were within a range of 8.0–5.0 and 0.5–0.9, respectively required for effective bioremediation in accordance with what has been stated by Atlas (1981) and Song et al. (1990). Most heterotrophic bacteria thrived in a neutral pH. Exceptions to this were slight variations in pH and OD values over certain episodes of treatments (control). Therefore, pH and optical density of the microbial cultures were not limiting factors in this study as all the microbes acquitted themselves as being able to grow (increase in OD) thus lowering the pH of the medium in which they grew. The utilization of the hydrocarbon substrates (gasoline, diesel and kerosene) by isolates was evident by the increase in cellular optical density of the culture. The results showed maximal increase in OD with fall in pH values (Tables 1-9) except for the control which experienced marginal decreases. The pH and OD values for the treatments that were supplemented with nutrients (soy and pito) were enhanced due to the nutrients that were provided which enabled them to grow and rapidly utilize the carbon substrates with a concomitant production of organic acids and other metabolites which lowered the pH of the media in line with study of Nwachukwu and Ugoji (1995) and Okposwasilis and James (1995).

Gas chromatography profiles of the inoculated and uninoculated hydrocarbons at the end of the degradation period revealed a reduction in the present area reports of the inoculated hydrocarbons as compared to the uninoculated control refer to as appendices. The incidence of disappearing peaks (percent area report values) may be due to the fact that isolates had an ample supply of their required energy sources and nutrients. The experiments showed hydrocarbon degrading potential of the isolates, buttressing the point that has been made by Bento et al. (2005) that biodegradation of petroleum hydrocarbon depends on the specific microbial

Table 11. A comparison of gasoline degradation efficiencies (%) of microbial isolates.

Treatment	Microorganisms					Abiotic
Gasoline substrates	***Bacillus firmus* (%)**	***Enterobacter Cloacae* (%)**	***Pseudomonas Aeruginosa* (%)**	***Proteus Mirabilis* (%)**	**Consortium *(B. firmus, E. cloacae, P. aeruginosa, P. mirabilis* (%)**	**Control (%)**
Raw	97.2	98.0	97.9	97.7	98.0	
Pito	98.7	97.5	99.2	98.5	99.3	49.0
Soy	98.4	99.6	99.6	99.4	99.8	

Table 12. A comparison of diesel degradation efficiencies (%) of microbial isolates.

Treatment	Microorganisms					Abiotic
Diesel substrates	***Bacillus firmus* (%)**	***Enterobacter Cloacae* (%)**	***Pseudomonas Aeruginosa* (%)**	***Pseudomonas cepacia* (%)**	**Consortium *(B. firmus, E. cloacae, P. aeruginosa, P. mirabilis*) (%)**	**Control (%)**
Raw	98.9	99.2	98.4	97.7	99.8	
Pito	98.8	97.6	93.6	_	99.2	_
Soy	99.1	99.6	99.5	99.8	99.5	

–= not available

Table 13. A comparison of kerosene degradation efficiencies (%) of microbial isolates.

Treatment	Microorganisms					Abiotic
Diesel substrates	***Bacillus firmus* (%)**	***Enterobacter Cloacae* (%)**	***Pseudomonas Aeruginosa* (%)**	***Pseudomonas Cepacia* (%)**	**Consortium *(B. firmus, E. cloacae, P. aeruginosa, P. mirabilis* (%)**	**Control (%)**
Raw	91.0	95.2	95.0	86.0	95.6	
Pito	95.3	92.5	90.0	92.8	86.78	32
Soy	83.3	96.0	95.35	92.1	88.3	

population present. All three hydrocarbons were degraded by microbes at a relatively faster rate, consistent with other report publications; these results indicate that hydrocarbon biodegradation can proceed in the presence of these microbes. The observation that the gasoline and diesel were the most degraded hydrocarbon compounds indicated that these were probably the most preferred substrates by the microbial consortia and the individual microbe(s) that carried out the metabolic process (Figures 1 - 4). With the addition of the nutrients (agro-residues), that is, soy and pito waste, their metabolic ability were enhanced, but the pattern of relatedness was not very clear as some of the treatments indicated that pito addition was not better. There were also few episodes where those treatments that were not supplemented with nutrients did better (Tables 1 to 9). Generally, addition of pito and soy residues as nutrient sources had a significant impact on degradation abilities of the isolates. The combination of soy residue and consortium achieved the highest degradation efficiency of 99.8% for gasoline substrate followed closely by *E. cloacae* (99.6%), *P. mirabilis* (99.4%), *P. aeruginosa* (99.37%) and *B. firmus* (98.4%).

For diesel, microbial consortium achieved the highest degradation efficiency of 99.8% (raw) this value was also achieved by *P. cepacia* with the addition of soy residue (Table 12).

For kerosene degradation profile, a combination of soy and *E. cloacae* gave the highest degradation efficiency of 96.0% followed by consortium (raw), *E. cloacae* (raw), and *P. aeruginosa* (soy added).

The results in Tables 1-9 also revealed that the consortia did not achieve the highest level of degradation for the gasoline, diesel and kerosene in all cases, this was consistent with what has been reported by Ausma et al. (2002) this could be attributed to the assumption that the different microorganism might have acted antagonistically as reported by Okpokwasili and James (1995) and also competition for nutrients or unfavorable change in pH. Relatively, the least successful was the episode

where consortium (soy added) utilized less amount of the kerosene (Table 13).

Recapping and summing up the above outcomes, the soy residue stimulated the hydrocarbon degradation abilities of all isolates better. The use of pure cultures in this study, alongside the mix culture (*B. firmus, Enterobacter, Pseudomonas* sp. and *P. mirabilis*) provides practical advantages by eliminating the ambiguity associated with the process. From our findings, it was evident that *E. cloacae* and Consortium were most effective in utilizing the substances. Many literature states that in a mix culture system; the growth of the organism cannot be regulated because of nutrient stress and competition (Okpokwasilli and James 1995). This could be one of the many possible reasons why in some of the treatments, the single culture tended to be superior to the consortium with respect to their degradative and mineralization profiles. From the research, it seems that the pure culture alone or in a mix culture can make use of most hydrocarbon fuel substances as expressed tentatively by Venkateswaran et al. (1995). Although mix cultures did not give the highest proportion of degradation efficiency in all the treatments, evidence of the cooperation of mixed cultures in dealing hydrocarbons contamination is still relevant as it has been reported elsewhere by Boonchan et al. (2000). Additionally, abiotic losses due to evaporation of low molecular hydrocarbons (aromatic compounds in the substrates) and photo-oxidation may have played a major role in reducing the levels of the oils in the culture media support which has been documented by Mills et al. (2003). Survival of microorganisms in petroleum hydrocarbon media during degradation period was a key factor in the rate of biodegradation of hydrocarbons in substrates (Ramos et al., 1991). Since all the bacteria in the present study were isolated from petroleum sludge, they survived and adapted to the oil substrates easily as report elsewhere by Sugiura et al. (1997). This was evident from the significant increase in OD_{600nm} values viz-a-viz a decline in pH values, floating test and (E_{24}) indices in all cultures as compared to control. The supplementation of soy and pito impacted on the degradation efficiency as compared to the control.

The results from this empirical work affirms the increasing awareness that bioremediation as a means of dealing with oil spill or contamination is real and practicable. The GC percent area report indicates that all five species showed a remarkable effect on removing the contaminants and more so the nutrients that were added enhanced the biodegradation. From our findings, there was no consensus on how best to optimize nutrient addition because in some episodes the raw treatment did better.

Recommendations

Culture collections of these microbes having the potential to metabolize petroleum should be collected by research institutions, universities and government agencies in Ghana since spills are inevitable.

Laboratories in these Universities should be well resourced with modern equipment and logistics to boost and encourage more research work in this area of environment control.

There should be more collaboration between universities, companies, non-governmental organizations (NGOs) and government agencies to ensure that research findings become more meaningful to the development of the country.

Conflict of interest

The author(s) have not declared any conflict of interests

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