INTERNATIONAL JOURNAL OF ENVIRONMENT
Volume-3, Issue-2, Mar-May 2014 ISSN 2091-2854
Received:27 March *Revised:23 April* *Accepted:13 May*

BIODEGRADATION OF SPENT ENGINE OIL BY BACTERIA ISOLATED FROM THE RHIZOSPHERE OF LEGUMES GROWN IN CONTAMINATED SOIL

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Abstract

Biodegradation of spent engine oil (SEO) by bacteria isolated from the rhizosphere of *Cajan cajan* and *Lablab purpureus* was investigated. It was with a view to determining most efficient bacterial species that could degrade SEO in phytoremediation studies. Hydrocarbon degrading bacteria were isolated and identified by enrichment culture technique using oil agar supplemented with 0.1% v/v SEO. Total heterotrophic and oil utilizing bacterial count showed the occurrence of large number of bacteria predominantly in the rhizosphere soil, ranging between 54×10^8 - 144×10^8 CFU/g and 4×10^8- 96×10^8 CFU/g respectively. Percentage of oil utilizing bacteria ranged between 0% (uncontaminated non rhizosphere soil) to 76% (contaminated rhizosphere). Turbidimetrically, five bacterial species namely *Pseudomonas putrefacience* CR33, *Klebsiella pneumonia* CR23, *Pseudomonas alcaligenes* LR14, *Klebsiella aerogenes* CR21, and *Bacillus coagulans* CR31 were shown to grow maximally and degraded the oil at the rate of 68%, 62%, 59%, 58%and 45% respectively. Chromatographic analysis using GC-MS showed the presence of lower molecular weight hydrocarbons in the residual oil (indicating degradation) after 21 days, whereas the undegraded oil (control) had higher molecular weight hydrocarbons after the same period. The species isolated were shown to have high ability of SEO biodegradation and therefore could be important tools in ameliorating SEO contaminated soil.

Key words: SEO, Biodegradation, Rhizosphere, Soil, Bacteria

Introduction

With an ever increasing human population, there is concomitant increase in demand for energy used for transportation, domestic and industrial consumption. Petroleum-based (fossil) fuels are the major sources of energy since 1950s (American Petroleum Institute, API, 2004). Increased utilization of petroleum and its derived products such as gasoline, diesel and motor oils has led to a marked increase in soil contamination worldwide (Alizera and Asli, 2011), in addition to emission of greenhouse gas leading to climate change. The environmental impact of exploration, production, refining and transportation of petroleum is a major concern in both developed and developing countries (Okieimen and Okieimen, 2005). The oil destroys living organisms, soil and marine environment (API, 2004). The environmental contamination results from oil spills due to several causes such as blowouts, leakage tanks, dumping of waste, tanker accident, sabotage, and carelessness (Alison *et al.*, 1999; Ijah and Abioye, 2003; Agbonlabor *et al*., 2004).

The illegal dumping of used motor oil is an environmental hazard with global ramifications (Blodgett, 2001). Akoachere *et al*. (2008) reported the discharge of used crankcase oil from vehicles as a major cause of oil pollution in Buea, Cameroon. Studies by Thenmozhi *et al*. (2011) and Ugoh and Moneke (2011) have also reported soil pollution due to discharge of used engine oil in Pudukkottai region, India and Gwagwalada area, Nigeria, respectively. Various contaminants such as used engine oil and heavy metals have been found to alter soil biochemistry, including alteration in soil microbial properties, pH, Oxygen and nutrient availability (Odjegba and Sadiq, 2002).

A number of innovative physical and chemical technologies are available to remediate soil contaminated with hydrocarbon pollutants especially in developed countries (Dominguez-Rosado and Pichtel, 2004). These methods however, are expensive, and may only be partly effective.

Bioremediation has emerged as an effective technology for treatment of hydrocarbon contaminants in recent years. A diverse group of micro-organisms is capable of degrading a wide range of hydrocarbon molecules. Recent studies indicate that plant roots provide a beneficial habitat for hydrocarbon-degrading bacteria (Cunningham *et al*., 1996, Ibrahim *et al*., 2009). The diversity and structure of bacterial communities is plant-specific and varies over time; and is affected by the plant age, the soil conditions and genotype of the microorganisms and plants

involved as well as on the environmental conditions (Brimecombe *et al*., 2007, Hrynkiewicz *et al*., 2009). Legumes are known to have an advantage over other plants in phytoremediation because of their ability to fix nitrogen; as they do not have to compete with microorganisms and other plants for limited supplies of available soil nitrogen at oil-contaminated sites (Aprill and Sims, 1990). Ibrahim *et al*. (2009) isolated crude oil (Escravos light – Nigerian oil) degrading bacteria in the rhizosphere of some plants. This study was aimed at determining the bacterial species associated with rhizosphere of two leguminous plants; *Cajanus cajan* (Pigeon pea) and *Lablab purpureus* (Hyacinth bean) and their effectiveness in SEO biodegradation. This is with a view to finding effective bacterial species that could play an important role in phytoremediation of SEO in association with the plant species.

Material and Methods

The experiments were carried out at the Botanical Garden of Usmanu Danfodiyo University, Sokoto. Sokoto is located to the extreme Northwest of Nigeria between longitudes 4º 8'E and 6º54'E and latitudes 12ºN and 13º 58'N.

A garden soil with no previous history of spent engine oil contamination was used in the experiment. The soil was intentionally contaminated for phytoremediation studies a month before our sampling. Two leguminous plants: *Cajanus cajan* and *Lablab purpureus* were grown on the contaminated soil. The samples were collected once in a month for a period of three months (November 2012 to January 2013).

Physicochemical analysis of the soil sample was carried out as described in the methods below. Soil pH was determined using pH meter as described by IITA (1979). Percentage carbon and nitrogen were determined according to the method of Uriyo and Singh (1974), while soil particle size, temperature, moisture and electric conductivity were determined by the method of IITA (1979).

Rhizosphere soils were collected from the root zone of plants grown in SEO contaminated soil. Similar samples were also obtained from uncontaminated control (rhizosphere and non rhizosphere) soils. The samples were either immediately taken to the lab for analysis, or stored at 4^0C for later analysis.

Total heterotrophic bacteria were enumerated by inoculating serially diluted ($\times 10^8$) soil samples in nutrient agar (Oxoid) after 24 hours incubation while spent engine oil degrading bacteria were

enumerated and subsequently isolated by inoculating the same dilution into oil agar as described by Ijah *et al*. (2008) The composition of the oil agar was 1.2g KH_2PO_4, 1.8g K_2HPO_4 4.0g NH_4Cl, 0.2g MgSO4.$7H_2O$, 0.1gNaCl, 0.01g $FeSO_4.7H_2O$ and 20g agar per liter at pH 7.4; supplemented with 0.1% SEO, after 5 days. The isolates were identified based on colonial, morphological and biochemical characteristics as described by Barrow and Feltham, (1993); Prescott and Harley, (2002). The isolates were screened for ability to utilize the SEO by inoculating aliquot (McFarland 8) into broth containing 0.1% oil for seven (7) days. Change in turbidity was used as measure of bacterial utilization of SEO with un-inoculated broth as control. The rate of degradation by most efficient isolates was determined gravimetrically after 21 days. The residual oil was extracted using n-hexane and the solvent was allowed to evaporate properly prior to measurement (Ijah *et al.*, 2008). The residual oil was also subjected to Gas Chromatography-Mass spectroscopic analysis using GCMS-QP2010 PLUS SHIMADZU (JAPAN) machine, equipped with flame ionization detector, to determine the extent of bacterial degradation.

Results and discussion

The experiment was conducted on a sandy soil (characterized by large particle size and low water retention capacity) with an average pH of 6.72, 28.12^0C temperature, 279.03µs/mg electric conductivity, 0.84% carbon, 0.10% nitrogen and 2.75% moisture. The results showed that the soil was poor in carbon and nitrogen contents. This is the common phenomenon in most sandy soils as large amount of the nutrients are lost through leaching. Low nutrient concentrations in sandy soils have been reported by Diab (2008) and Diab (2011). The pH of the soil was weakly acidic to neutral, an ideal pH for most crops. The slight acidity may be attributed to microbial activities and root exudation in the rhizosphere soil. Hamza (2008) recognized microbial activities, root respiration and exudation as important causes of soil acidity. This is also in accordance with the findings of Stephen and Ijah (2011), where acidic soil pH was observed in phytoremediation studies. Other physicochemical parameters (temperature, moisture and EC) might be influenced by prevailing weather conditions during the course of the experiment.

The results showed that the overall range of total heterotrophic count was between 5×10^8 and 144×10^8cfu/g, as shown in Table 1. Contaminated *L. purpureus* rhizosphere had higher (range 96×10^8 - 144×10^8cfu/g) counts as compared to that of *C. cajan* (54×10^8 - 80×10^8cfu/g). This may

be due to species variation between the two plants and possibly exudation of more nutrients from the former, which attracted more bacterial species. Brimecombe *et al*. (2007) observed that the effect of rhizosphere bacteria depends mostly on the genotype of the microorganisms and plants involved as well as on the environmental conditions. Root exudates provide sufficient carbon and energy to support large numbers of microbes in the rhizosphere (Erickson *et al*., 1995). The contaminated rhizosphere of the two plants had higher counts (96×10^8 - 144×10^8cfu/g) compared to the uncontaminated rhizosphere (57×10^8 - 74×10^8cfu/g), as the case was between rhizosphere (54×10^8 - 144×10^8cfu/g) and non rhizosphere (5×10^8 - 37×10^8cfu/g) soil. Higher bacterial population in rhizosphere soil was associated with increased nutrient availability due to primarily hydrocarbon contamination and biostimulation as a result of root exudates. Cunningham *et al.*(1996) reported that plants provide root exudates of carbon, energy, nutrients, enzymes and sometimes oxygen to microbial populations in the rhizosphere. Due to these exudates, microbial populations and activities are 5 to 100 times greater in the rhizosphere than in the bulk soil (Aprill and Sims, 1990).

In the oil utilizing bacteria count, a pattern similar to that of the heterotrophic count was observed (Table 1). Contaminated rhizosphere of *L. purpureus* had the highest count (28 - 96×10^8cfu/g) followed by contaminated (27 - 49×10^8cfu/g) and uncontaminated (4 – 34×10^8cfu/g) *C. cajan* rhizosphere soils. The uncontaminated non rhizosphere however, had the lowest (4 – 20×10^8cfu/g) oil utilizing bacteria count. Hydrocarbon contamination has been a factor that increases the number of degrader communities in a polluted environment. This agreed with previous findings by Stephen and Ijah (2011) and Diab (2008). The degree of deviation above unpolluted reference site appears to quantitatively reflect the degree or extent of exposure of the ecosystem to hydrocarbon contaminants (Atlas and Bartha, 1993).

The contaminated soils generally had higher percentages of oil degraders especially in the first and third months. The higher percentages observed might be attributed to exposure and later adaptation respectively. This agreed with the findings of Atlas and Bartha, (1993) who observed that in unpolluted ecosystem, hydrocarbon utilizers generally constitute less than 0.1% of the microbial community; in oil polluted ecosystem, they can constitute up to 100% of the viable microorganisms. Figure 1 shows the monthly percentage of oil utilizing bacteria from the total heterotrophic count, indicating contaminated *C. cajan* rhizosphere soil having the highest percentage of oil utilizing bacteria.

Five bacterial isolates were shown to grow luxuriantly and with high optical density in oil broth after 7 days incubation. These bacteria were able to degrade SEO at rates ranging from 45% to 68%. The species were *Pseudomonas alcaligenes* LR14, *Klebsiella aerogenes* CR21, *Klebsiella pneumonia* CR23, *Bacillus coagulans* CR31, and *Pseudomonas putrefacience* CR33. These organisms were able to degrade the SEO at the rate of 59%, 62%, 58%, 45% and 68% of the oil respectively after 21 days incubation. Biodegradation of hydrocarbons by members of *Pseudomonas*, *Klebsiella* and *Bacillus* genera have been well known and documented, and thus emergence of these organisms in this study was not unprecedented. Ibrahim *et al.* (2009) reported that *K. pneumonia* LR01 was able to degrade Escravos light (Nigerian) crude oil by 69.78%. Similarly, Ijah and Antai (2003) reported the ability of *Pseudomonas* spp to degrade Transniger pipeline (Nigerian) crude oil by 68.5%.

Table 1: Bacterial Count of Soil Samples

Bacteria	Monthly Counts (cfu/g)																	
	CCR			UCR			CLR			ULR			CNR			UNR		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
TH	68	54	80	70	57	64	96	124	144	64	74	58	29	26	37	28	42	5
OU	49	27	32	34	4	13	58	28	96	31	15	14	12	6	24	20	0	2

CCR contaminated *C. cajan* rhizosphere, UCR uncontaminated *C. cajan* rhizosphere, CLR contaminated *L. purpureus* rhizosphere, ULR uncontaminated *L. purpureus* rhizosphere, CNR contaminated non-rhizosphere, UNR uncontaminated non-rhizosphere TH total heterotrophic, OU oil utilizing.

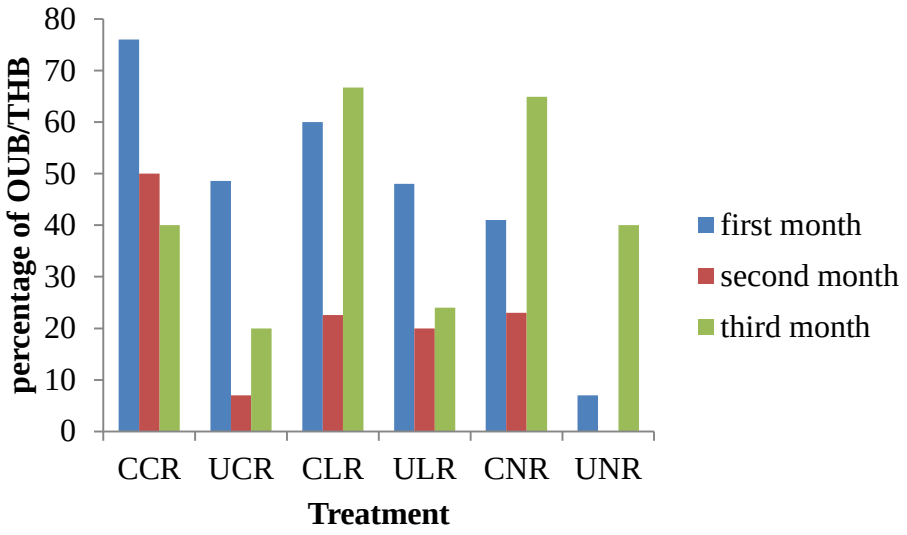

Figure 1. Percentage of SEO utilizing bacteria

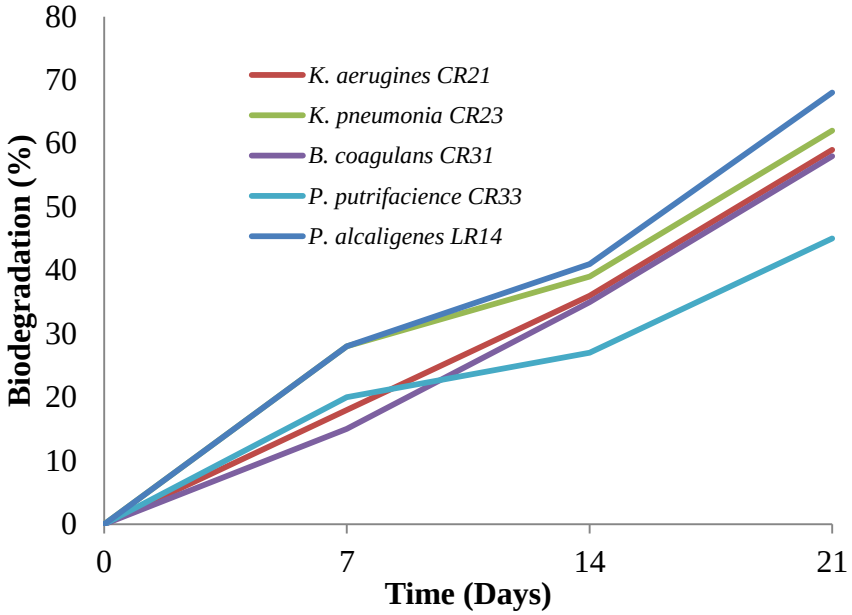

Figure 2.Rate of SEO biodegradation by bacterial species

Moneke and Nwangwu (2011) also observed the ability of *Pseudomonas* species to reduce different types of oil under laboratory conditions. The present finding supports the view that *Pseudomonas* and *Klebsiella* species are among the efficient hydrocarbon degrading bacteria prevalent in Nigerian environment. According to Brimecombe *et al*. (2007), *Pseudomonas* spp. and *Bacillus* spp. belongs to the largest groups of rhizosphere bacteria. The ability of these

organisms to utilize oil may be attributed to enzymatic efficiency and ability to withstand harsh environmental conditions (Ijah *et al*., 2008).

Chromatographic analysis using GC-MS revealed the extent of SEO degradation by the organisms. The results showed the presence of paraffinic compounds with carbon atoms ranging from C_{15} to C_{54} in un-degraded (control) SEO as the major components of the oil. Little of unsaturated compounds were detected, which were primarily C_{18} and C_{19} (mainly octadecenoic acid and their alkylated derivatives) compounds. The result showed that lower carbon ($C_{15} - C_{20}$) containing compounds were aliphatic acids (pentadecanoic, hexadecanoic, etc.) and cycloketonic (cyclopentadecanone) compounds, while the higher carbon compounds were mainly paraffinic (Fig. 3a).

However, compounds ranging from C_7 to C_{13} were generally the most occurring compounds observed in degraded oil. These compounds when compared with the control were absent or otherwise introduced. The chromatograms obtained for *Pseudomonas putrefaciens* CR33 (Fig. 3b) showed the presence of C_7 to C_{20} compounds. No compounds with carbon atoms higher than C_{20} were detected in the oil analyzed. The compounds detected comprised alkane (including alkylated and cycloalkanes), some aromatics (mainly benzen and alkylated derivatives) and few organic acids. Similarly, the same compounds were detected in the profile of *K. aerogenes* CR21 in addition to methyl esters of the organic acids (Fig. 3c).

Majority of the compounds observed (Fig. 3d) for SEO degradation by *P. alcaligenes* LR14 were C_8 and C_9. These include *n*- alkanes, methylated cyclic and substituted aromatic hydrocarbons. Other compounds observed were alkanoic acids (C_{18} and C_{19}) and alcohol (C_{18}). When compared with the control, the profiles of *K. pneumoniae* CR23 (Fig. 3e) and *B. coagulans* CR31 (Fig. 3f) were also reduced. Substituted cyclic compounds (C_8 and C_9) were the major compounds observed in both cases, followed by *n*-alkanes and carboxylic acids. In addition, substituted aromatics (benzen) and some alcohols (C_{12}) were observed.

The extent of SEO biodegradation by the five bacterial species has shown the ability of the isolate to degrade long chain hydrocarbons to smaller chain hydrocarbons. All of the species were able to degrade the oil to smaller levels like benzen and substituted alkanes. These compounds were known to be difficult for most microbes to degrade further as a result of toxicity or extensive alkyl substitution. According to Okoh (2006), methyl branching generally increases the resistance of hydrocarbon to microbial attack. Some researchers showed that

terminal branching inhibit biodegradation of hydrocarbon also (Atlas, 1981). A number of organic acids were observed as important components of the oil. This may be part of the initial constituents of the SEO as many companies add organic acid esters as part of the synthetic base fluids of lubricating oil. Schwindaman (2005) opined that some synthetic lubricants are ester based and reversal of the esters to acids is one of their major weaknesses.

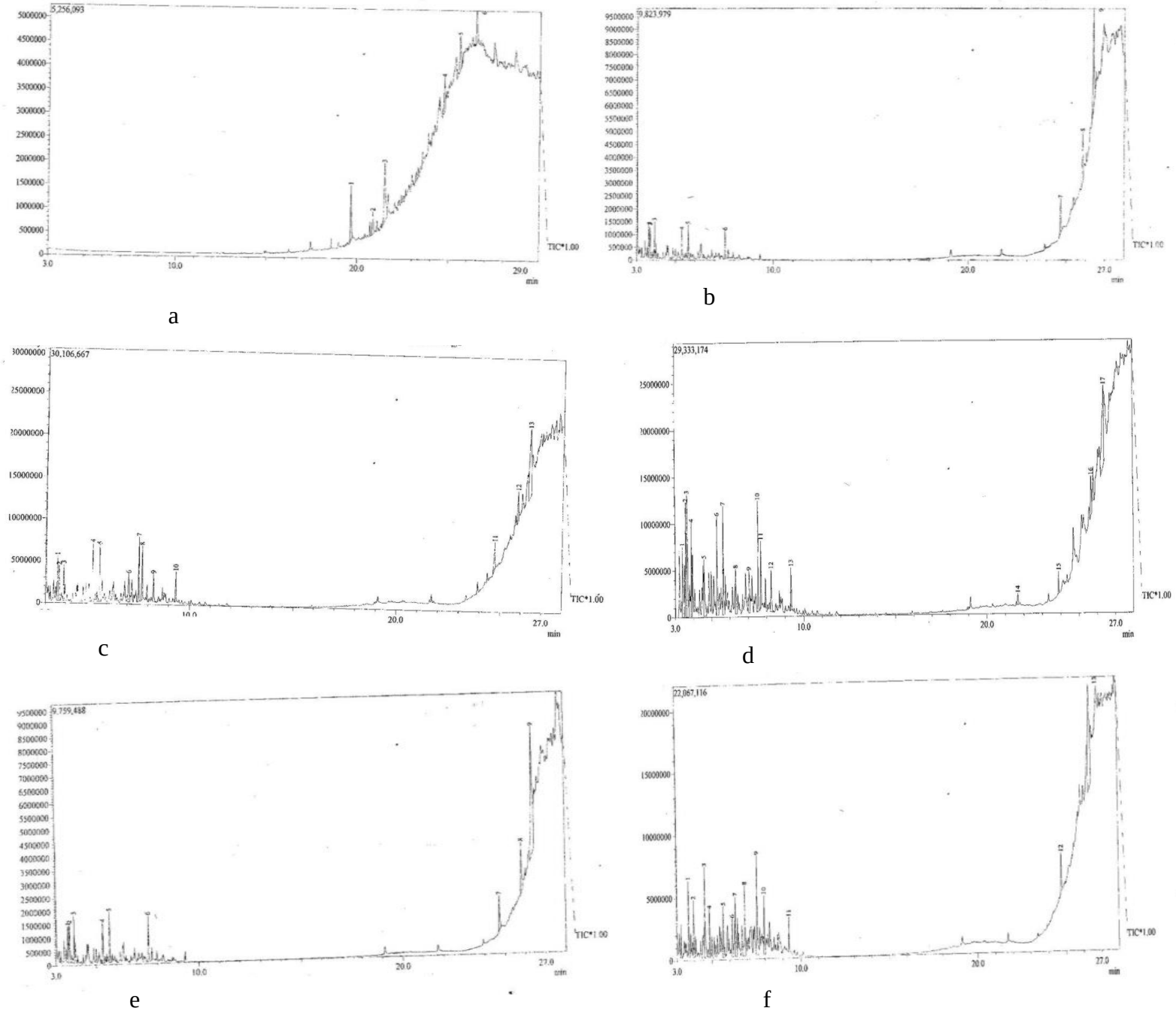

Fig 3. Chromatograms of SEO degraded by: (a) control, (b) *P. putrefaciens* CR33, (c) *K. aerogenes* CR21, (d) *P. alcaligenes* LR14, (e) *K. pneumoniae* CR23 and (f) *B. coagulans* CR31.

Alternatively, the occurrence of the organic acids may be attributed to bacterial metabolism of the hydrocarbons and as such, they are said to be metabolic intermediates. For an effective biodegradation of hydrocarbons, the compounds need to be transformed to carboxylic acids before they can be channeled into ß- oxidation pathway (Van Hamme *et al*., 2003; Okoh, 2006). This may probably be the same for some alcohols observed in the profiles of *P. alcaligenes*LR14 and *B. coagulans* CR31. It could be observed from Fig. 3a through 3e that reduction in area and intensity of chromatographic peaks was more pronounced in *P. putrefaciens* CR33 than the other species as compared to the control. This was followed by *K. aerogenes* CR21, *K. pneumonia* CR23 and *P. alcaligenes* CR14. The least among the studied organism was *B. coagulans*CR31 where the peaks were much more pronounced. This could be associated with enzymatic capability of each organism and possibly, accumulation of intermediate end products that may resist degradation. Van Hamme *et al*. (2003) reported that inhibition of hydrocarbon degradation may occur, presumably due to competition for enzymes involved in oxidation or transport, accumulation of by-products resulting in cytotoxicity, and blockage of enzyme induction. Similarly, this result corresponds to the one obtained in gravimetric analysis were the rate of degradation followed almost similar pattern. Hence, *P. putrefaciens* CR33 is said to be the potent SEO degrader while *B. coagulans* CR31 was least among the five bacterial isolates. This finding corresponds to the study carried out by Mittal and Singh (2009), where *Pseudomonas* SP-I was shown to perform better than *Bacillus* spp when compared to standard strain of *Acineto bacter coaceticus* in petroleum hydrocarbon biodegradation.

Conclusion

This study showed the presence of high number of bacteria in the rhizosphere of *C. cajan* and *L. purpureus*. Five bacterial species were shown to grow and utilize SEO luxuriantly at the rate of 45% to 68% within 21 days. This shows that the organisms were potent SEO degraders and when associated with the plants species, could efficiently cleanup contaminated environment. Hence the bacterial species are still important tools for combating oil pollution.

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