1.1. INTRODUCTION

Microorganisms play an important role on nutritional chains, that are an important part of this biological balance. Adapting several abilities, microorganisms have become an important influence on the ecological systems, making them necessary for superior organisms and their life in this planet. Ability of microorganisms to transform and degrade many types of pollution in different pollutant matrixes (Soil, Water, Sediments and Air) has been widely recognized during the last decades (Saval, 1998 and Autry and Ellis, 1992). Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche. Contaminates are often potential energy for microorganisms. (Madigan et al., 1998).

Petroleum hydrocarbons are a major source of energy, providing over 50% of the energy used all over the world. In addition, petroleum is the principal source of lubricants, solvents and a variety of large volume of chemical feedstock for synthesis of plastics, fibers, detergents, pharmaceuticals and cosmetics. A large scale of operation necessitated by the above demands renders the petroleum industry a potential source of air, water and soil pollution. Petroleum as crude oil while drilling, refinery effluents, slop oil emulsion solids and as water from ballast tanks may find its way into the adjoining land and surface or ground water through accidental or deliberate disposal. In India petroleum exploration has been in operation for over 100 years. Petroleum product spills during oil exploration, refining and transportation are very common in India. Many farmers have lost their agricultural land due to oil spills in their fields. Many fishermen in India have lost their livelihood due to oil slick in lakes and rivers. Currently, India has 18 oil refineries in the public and private sectors, with a gross refining capacity of over 112 million tones. Petroleum industry by-products have a detrimental effects on the environment which is evident from their classification as new waste categories (Waste category number 10 and 12) under the recent Hazardous Waste act of 1998 (Government of India).

Total petroleum hydrocarbons are composed of compounds varying from simple aliphatic and aromatic compounds to complex, multi-ring structure of high molecular weight hydrocarbons. It also contains a wide range of substances that contain sulfur, nitrogen, oxygen and other elements. On average, crude oil contains about 84%
carbon, 14% hydrogen, 1 - 3% sulfur and less than 1% each of nitrogen, oxygen, metals, and salts. Petroleum hydrocarbons can be broadly divided into four fractions: the aliphatic fraction, the aromatic fraction, the asphaltene fraction and the resins. Among the aromatics, polycyclic aromatic hydrocarbons (PAHs) represent a unique class of petroleum hydrocarbons because of their pyrogenic nature and the complexity of the assemblages in which they occur. Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant pollutants, found to have toxic, mutagenic and carcinogenic properties and are listed among the priority pollutants by the U.S. Environmental Protection Agency (EPA).

Petroleum hydrocarbon-metabolizing microorganisms are widely distributed in nature. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, cyanobacteria and microalgae. However, bacteria play the important in hydrocarbon degradation. Over the last two decades the microbiological investigation of total petroleum hydrocarbon impacted environments have revealed the presence of a physiologically diverse microbial flora. Due to the wide variety of complex individual substrates constituting total petroleum hydrocarbons and different metabolite interactions, there are certain inherent difficulties in characterizing microbial communities impacted by total petroleum hydrocarbons. Despite these problems, there has been extensive research to analyze the microbial abundance in such contaminated sites and to associate the microbial communities with the ecosystem function. For the proper exploitation of the bioremediation potential of indigenous microflora, the study of microbial diversity of site contaminated with total petroleum hydrocarbons with a polyphasic approach becomes imperative. The elucidation of diversity at the genomic level will help to understand the evolutionary and phylogenetic perspectives and will lead to conservation of indigenous microflora, which are of economic and environmental importance. Whereas detection of the functional variability of the bacterial strains from the contaminated sites by investigating the degradative phenotype will allow the selection of specific bacterial strains for designing bioremediation strategies.(Riser-Roberts, 1992 and Bundy et al., 2004).

Petroleum hydrocarbon waste is considered to be a degradation challenge to the microorganisms because of its persistence in the ecosystems. Further the low solubility in water (hydrophobic nature) and low volatility of the petroleum waste poses a
significant threat to the environment. Thus there has been an increased interest in studying the microbial diversity of indigenous microorganisms capable of degrading pollutants such as crude oil, polycyclic aromatic hydrocarbons and polychlorinated biphenyl in different environments. Microorganisms adapt their catabolic activities to make use of toxic organic pollutants as food sources; thereby mineralizing complex organic compounds to simple compounds such as carbon dioxide and water. Identification of key organisms that play a role in degradation of the pollutants is relevant to development of optimal in situ bioremediation strategies. Extensive efforts have been made to characterize bacterial communities and their response to pollutants. Isolation of the potential degraders and identification of their functional genes involved in a particular degradation process have been elaborately studied. Many studies have also demonstrated the microbial diversity of different contaminated environments.

With an ever increasing world’s population, there is a concomitant increase in the demand for petroleum and petroleum products, which apparently constitutes a source of environmental pollution (Raven et al., 1993). Oil pollution is a major environmental concern in many countries, and this has led to a concerted effort in studying the feasibility of using oil-degrading bacteria for bioremediation. The discharge of used engine oil from vehicles is the main source of oil pollution in the environment. Engine oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents (Butler and Mason, 1997) that is used to lubricate the parts of an automobiles engine, in order to keep everything running smoothly (Hagwell et al., 1997). The most important characteristic of the lubricating oil for automotive use is its viscosity. Large amounts of lubricating oils, composing long-chain saturated hydrocarbons (base oil) and additives are used in car engines. The main components of the base oil are cyclic alkanes (c-alkanes). Long-chain hydrocarbons and c-alkanes are known as recalcitrant to microbial degradation. The base oil contains $C_{16}-C_{36}$ hydrocarbons, and more than 75% of the alkanes. The rings number of c-alkanes in the base oil is from 1 to 3 and any ring contains 5 or 6 members. Most of the c-alkanes in the base oil have long alkyl side chains (Koma et al., 2003). It is mainly comprising base stroke of tailored having alkyl benzene having carbon atom mainly $n-C_{21}-n-C_{25}$ in the range of 80–90% by weight, antioxidant in the range of 0.06–0.05% by weight, extreme pressure additive in the range 0.01–0.05% by weight, antifoaming agent in the range of 0.01–1.0% by weight. Chemical analysis of
engine oil demonstrated a high contents of PAH’s (naphthalene, anthracene, phenols and their derivatives) and a small fraction of aliphatic hydrocarbons (n-C_{15}-n-C_{40}).

New motor oil contains a higher percentage of fresh and lighter (often more volatile and water soluble) hydrocarbons that would be more of a concern for acute toxicity to organisms. Used motor oil contains more metals and heavy polycyclic aromatic hydrocarbons (PAHs) that would contribute to chronic hazards including mutagenicity and carcinogenicity (Keith and Telliard, 1979; Hagwell et al., 1992; Boonchan et al., 2000). Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra et al., 2001; Propst et al., 1999; Lloyd and Cackette, 2001). Environmental pollution with petroleum and petrochemical products has attracted much attention in recent times. The illegal dumping of used motor oil is an environmental hazard with global ramifications (Blodgett, 2001). Thousand million gallons of waste engine oil is generated annually from mechanical workshops and discharged carelessly into the environment (Faboya, 1997). According to USEPA (1996), only 1 liter of used engine oil is enough to contaminate one million gallons of freshwater. Used engine oil also renders the environment unsightly and constitutes a potential threat to humans, animals, and vegetation (ATSDR 1997; Edewor et al., 2004 and Adelowo et al., 2006). The presence of various kinds of automobiles and machinery vehicles has caused an increase in the use of motor oil. Spillages of used motor oils such as diesels or jet fuels contaminate our natural environment with hydrocarbon compounds. (Husaini et al., 2008).

Engine oil is the most widely used petroleum product. Research on biodegradation of engine oil is scarce, to which screening of mixed bacteria is more important than of a single strain. Persistent screening and domestication are necessary to obtain a mixture of degrading bacteria through natural selection to produce a symbiosis system. Such a mixed bacterial flora is less vulnerable to environment and is more applicable than traditional treatment process.

With this in view, an attempt has been made to isolate engine oil degrading bacteria from engine oil contaminated sites of Pudukkottai (78.25’ and 79.15’ of the East of Longitude, 9.50’ and 10.40’ of the North of Latitude) Tamilnadu. This study resulted in isolation and characterization of potential used engine oil degrading bacterial isolates, their innate biotechnological potentials namely bioremediation of used engine
oil contamination by consortium cultures and biosurfactant production by a selected bacterial isolates.
1.2. REVIEW OF LITERATURE

Total petroleum hydrocarbons in many natural sources coexist with heterocyclic molecules where one of the carbon atoms in a ring gets substituted by nitrogen, oxygen and sulfur. This leads to a greater diversity of this class of molecules. The total petroleum hydrocarbons occur in the environment as products both of natural biosynthesis and industries based on coal and petroleum. There are several anthropogenic and natural sources leading to ubiquitous distribution of total petroleum hydrocarbons in air (Dagley, 1985 and Georgiadis et al., 2001), soil and sediment (Langworthy et al., 1998; Lim et al., 1999) surface water, ground water, and road runoff (Holman et al., 1999; Ohkouchi et al., 1999).

Petroleum refining is also a major contributor to localized loading of total petroleum hydrocarbons into the environment. Such loading may occur through discharge of industrial effluents, through accidental release of raw and refined products, coal liquefaction and gasification process. Petroleum refineries unavoidably generate considerable volumes of oily sludge. Oily sludge is composed of hydrocarbons, water and minerals. Common sources of oily sludge are storage tank bottoms, oil-water separators, floatation and biological wastewater treatment unit, cleaning of processing equipment’s and soil from occasional minor leakage on refinery grounds (Dibble and Bartha, 1979).

Petroleum and coal provide the largest source of petroleum hydrocarbon compounds. In addition, these aromatic chemicals are commonly found in the environment as a result of biosynthesis by plants (Muller et al., 1996). They are present as natural constituent of fossil fuels and are formed during incomplete combustion of organic material (Lee et al., 1981). There are natural incidences of oil seepage, surface run off, and geological processes leading to release of total petroleum hydrocarbons in the environment. They are also formed due to pyrolysis of organic substances in nature. Clusters of benzene are commonly found in pyrolysates, when the pyrolysis products are rapidly cooled (Mueller et al., 1989). Surveys done in terrestrial and aquatic environment show that some compounds consisting of total petroleum hydrocarbons, like polycyclic aromatic hydrocarbons occur, from the geochemical cycles. Volcanic
eruptions and forest fires are other sources of polycyclic aromatic hydrocarbon contamination to nature (Lee et al., 1981).

Modern day contamination of soil sediment and groundwater by total petroleum hydrocarbon has originated from four primary waste sources: creosote, coal-tar, petroleum and industrial effluents (Cripps and Warkinson, 1990) Atmospheric deposits originate primarily from high temperature, incomplete combustion of fuels, typically from industrial activities and automobile exhaust. Another non-point source introduction of total petroleum hydrocarbon into soils is through land treatment procedures (Wild and Jones, 1993). The use of sewage sludge as fertilizers in agriculture fields has been shown to have increased the total petroleum hydrocarbon concentration considerably. Large-scale spillage and disposal of oily wastes and sludge are also major contributors to total petroleum hydrocarbon pollution. Tanker accidents, seepage from sludge tanks, pipeline blasts are some of the common sources of total petroleum hydrocarbon contaminations. The release of total petroleum hydrocarbon is not only restricted to fossil origins but is also released in tobacco smoke (Georgiadis, et al., 2001).

Petroleum industry by-products have a detrimental effect on the environment, which is evident from its classification as Waste Categories no. 10 and 12 under the recent Hazardous Waste act 1998, Government of India. The compounds that constitute total petroleum hydrocarbons are extremely toxic to human beings as well as animals. They are highly carcinogenic and mutagenic. Moles (1998) studied the sensitivity of ten aquatic species to long-term crude oil exposure using fractions of crude oil and found them to be sensitive, however chronic oil exposure appears to minimize the differences in hydrocarbon sensitivity between species.

Among the total petroleum hydrocarbons, the polycyclic aromatic hydrocarbons (PAHs) are known to have acute toxic effects and also possess mutagenic, teratogenic and/or carcinogenic characteristics (Kanaly and Harayama, 2000). Polycyclic aromatic hydrocarbons and their metabolites may lead to covalent bonding of cellular macromolecules, such as DNA and proteins, leading to cell damage, mutagenesis, teratogenesis and cancer. Benzo(α)pyrene is one of the most potent chemical carcinogens and was first studied for its biological activity (Phillips, 1983). The microbial diversity of a contaminated environment may be influenced by the complexity
of the chemical mixtures present and the length of time the populations have been exposed to these contaminants (Macnaughton et al., 1999). Nyman (1999) observed that environments contaminated with crude oil consists of toxic components that reduces the microbial diversity of the soil.

The culture-dependent methods are based on differential morphological, metabolic and physiological traits. These include isolation and cultivation in different media, most probable number (MPN) liquid assays, and more recently Biolog substrate utilization pattern (Winding and Hendricksen, 1997; Lehman et al., 1995; Garland, 1996). There have been improvements in the designing of media which have accounted for documentation of novel bacterial isolates. Hedlund et al., (1999 ) reported novel bacterial strain, Neptunomonas naphthovorans with modification in the enrichment protocol and media. There have been other reports of improvements on methods and media that have increased the proportion of bacterial strains that can be cultured from the environmental samples (Janssen et al., 2002, Sait et al., 2002, Zengler et al., 2002). The Biolog system is based on the utilisation of a suite of 95 different carbon sources, which are present in the wells of the microtiter plates (Garland and Mills, 1991; Haack et al., 1995). The carbon utilization profile data is analyzed by multivariate statistical analysis (Haack and Griffiths, 1997; Hitzl et al., 1997; Hill et al., 2000). Mafham et al., (2002) evaluated the functional diversity of the bacterial community by studying the utilization pattern of the different carbon sources in the Biolog bacterial identification systems. However, there are certain limitation with Biolog system as the substrates present in the microtiter plate do not reflect the substrates present in the contaminated sites (Konopka et al., 1998). Aside from isolating and identifying bacterial strains in hydrocarbon impacted environment, the culture dependent techniques provided a direct functional characterization of the bacterial strains. But it has been acknowledged that only a small fraction (<1 %) of the bacterial strains in a given sample can be cultured.

With the rapid expansion of the field of molecular biology, culture independent approaches to study bacterial diversity has attracted a lot of investigations. There are numerous publications advocating nucleic acid based approaches that have resulted in exploration of uncultivated microbial community. Morris et al., (2002) reviewed the scientific literature on microbial diversity and reported that the percentage of published articles that have used DNA based characterization techniques rose from 9% in 1988 to
over 50% in 1995. It has been reported in various studies that the use of culture independent approaches removes the inherent bias imposed by isolation dependent techniques (Van Hamme, 2003).

Different molecular approaches have been performed for assessing the diversity and structure of soil bacterial community. According to Woese (1987) rRNA gene sequences are suitable for microbial community analysis because as they are found in all forms of life They are composed of highly conserved regions and also of regions of considerable sequence variation. Of the various DNA based techniques used to estimate microbial community composition and diversity in complex habitats, the most useful is the determination of the sequences of 16S rRNA genes encoded by rDNA in prokaryotes (Zhou et al., 1996). The use of the rRNA gene to study the structures and activities of microbial communities has become of great interest in recent years (Ogram et al., 1995). Numerous studies have applied these techniques to study soil microbial communities and survivability of microorganisms (Lee et al., 1996; Bintrim et al., 1997). It is essential to evaluate the bacterial community of a hydrocarbon-contaminated site since a contaminated soil causes changes in the composition of the microbial community (Kozdroj and Elsas, 2001). Friedrich et al., (2003) elucidated high bacterial diversity of hydrocarbon-degrading microbial community in an industrial bio-filter as shown by 16S rDNA clone library.

A plethora of microorganisms belonging to different genera are capable of utilizing hydrocarbons as sole source of carbon and energy. In the year 1946, Zobell first reported the existence of oil degrading microbes. These microorganisms are widely distributed in nature (Zobell, 1946). Davis and Updegraff (1954) studied the growth of these microorganisms on hydrocarbons as a source of protein and food. While hydrocarbon-degrading microorganisms are ubiquitous, hydrocarbon-degraders normally constitute less than 1% of the total microbial community. Hydrocarbon degrading microorganisms are widely distributed in the nature. Many microorganism including bacteria, fungi, yeast and algae have the capacity to mineralize petroleum hydrocarbons. Biodegradation of petroleum oil by using bacteria belonging to the genera Achromobacter, Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Burkholderia, Brevibacterium, Corynbacterium, Micrococcus, Mycobacterium, Nocardia Pseudomonas, Vibrio, Rhodococcus (Juhasz et al., 2000;
Daane et al., 2002; Samanta et al., 2002; Emtiazi et al., 2005) and fungi like *Aspergillus*, *Candida*, *Cladosporium*, *Penicillium*, *Rhodotorula*, *Sporobolomyces*, *Trichoderma* etc., has been reported (Colombo et al., 1996; Head and Swannnel, 1999). Bacteria are considered to represent the predominant agents of hydrocarbon degradation in the environments because they possess the capability to utilize oil as energy sources (Song et al., 1990), whereas, other species may not possess this ability and are gradually eliminated (Atlas and Bartha, 1992). Using an indigenous bacterial consortium ensures that the organisms have a higher tolerance to the toxicity of hydrocarbon and are resistant to changes in the environment (Dibble and Bartha, 1979; Eriksson et al., 1995). Hence in the last two decades, the field of microbiology has increasingly focused on the use of microorganisms for environment clean up (Abdel-El-Haleem, 2003).

Besides heterotrophic bacteria and fungi, algae and cyanobacteria have also been reported to degrade hydrocarbons. Walker et al., (1975) isolated an algae, *Prototheca zopfi* that exhibited extensive degradation of *n*-alkanes and branched-alkane as well as aromatic hydrocarbons. Studies indicated that algae might enhance the degradation of the oily waste by providing oxygen and possible nutrient from dying algae for the bacterial community. *Chactoceros calcitraus* (diatom) was shown to contribute to the degradation of hydrocarbons in the seawater. Cyanobacteria have also been reported to degrade hydrocarbons (Cerniglia et al., 1984). This assumes greater importance from the fact that cyanobacteria are the usual inhabitants in the marine environment but the extent of their involvement in hydrocarbon biodegradation is largely unknown.

Bossert and Bartha (1984) have listed 22 genera of hydrocarbon degrading bacteria and 31 genera of fungi isolated from soil. The most important and efficient hydrocarbon degrading bacteria in both soil and marine environments are *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Achromobacter*, *Bacillus*, *Flavobacterium* and *Nocardiia* sp. Among the fungi, *Aureobasidium*, *Candida*, *Rhodotorula* and *Sporobolomyces* sp. are the most common marine isolates and *Trichoderma* and *Mortierella* sp. are the most common soil isolates (Leahy and Colwell, 1990). Protozoans have also been identified for utilizing alkanes. Kaska et al., (1991) have successfully isolated a marine *Amoeba*, *Trichosphaerium*, which feeds on macro algae to be capable of utilizing *n*-alkanes, *n*-alkenes and potentially also halogenated alkanes.
Anaerobic mixed cultures have been studied for dechlorination of chlorobenzenes (Holliger et al., 1992). Bacterial co cultures have been reported for the degradation of chlorinated hydrocarbons (Awasthi et al., 1997). Most of the biodegradative bacteria belong to Gram-negative group (MacNaughton, 1999). These latter microorganisms can degrade a wide range of target constituents of TPH present in oily sludge (Eriksson, 1999) making it possible to employ the bacterial flora present at such a site for reclaiming contaminated soil. A large number of bacteria for example Yokenella sp., Stenotrophomonas sp., Alcaligens sp., Roseomonas sp., Alcaligens sp., Roseomonas sp., Flavobacter sp., Corynebacterium sp., Streptococcus sp., Providencia sp., Sphingobacterium sp., Capnocytophaga sp., Moraxella sp., Bacillus sp. (Rusansky et al., 1987; Antai, 1990; Bhattacharya et al., 2002; Herrera et al., 2008), Enterobacter sp., Escherichia sp. and Hafnia sp. (Grant 1967; IJah 1998 and Diaz et al., 2001), Pseudomonas citronellolis KHA (Sadouk et al., 2009) have been reported for hydrocarbon degradation.

Stapleton et al., (2000) evaluated the catabolic and genetic diversity of indigenous degradative bacteria from fuel contaminated aquifers, which were capable of degrading hydrocarbon compounds. They detected the presence of the members of proteobacteria: Pseudomonas,Ralstonia, Burkholderia, Sphingomonas, Flavobacterium, by 16S rRNA gene sequence analysis (Stapleton et al., 2000 and Watanabe et al., 2000).). Bakerman and Madsen (2002) recorded wide microbial diversity in a coal tar contaminated aquifers. The 16S rDNA sequence analysis indicated the presence of 7 genera Pseudomonas, Ralstonia, Burkholderia, Sphingomonas, Flavobacterium, Commonomonas and Bacillus. The bacteria associated with the ε-Proteobacteria have also been associated with the petroleum-contaminated ground water which accumulated at the bottom of the underground crude oil-storage cavities (Kodama and Watanabe, 2003).

Kubo et al., 2003 reported that seventeen types of bacteria were isolated from different soil samples and finally two isolates (Rhodococcus sp and Gordonia sp) which grew in W medium containing the 1% c-alkane fraction from the base oil as a sole carbon and energy source, were further analyzed for the degradation abilities. Acinetobacter and Nocardia species demonstrated higher ability in utilizing hydrocarbon when inoculated directly into minimal salt broth containing used engine oil.
as the sole source of carbon and energy. It may be due to the presence of efficient 
hydrocarbon degradative enzyme systems and the presence of catabolic genes involved 
in hydrocarbon degradation in the bacterial species (Kyung-Hwa et al., 2006; Majid et 
al., 2008). Adelowo (2006) isolated *Pseudomonas fragi* and *Achromobacter aerogenes* 
from used engine oil polluted soils, they were grown in minimal salts medium (MSM) 
supplemented with used engine oil as sole carbon and energy source to evaluate their 
ability to biodegrade used engine oil.

Three bacterial isolates capable of utilizing used engine-oil as a carbon source 
were isolated from contaminated soils using the enrichment technique. Three isolates 
were identified as *Flavobacterium* sp., *Acinetobacterium calcoaceticum* and 
*Pseudomonas aeruginosa* based on biochemical tests and 16S rRNA sequencing 
(Mandri and Lin, 2007). Shojaosadati et al., 2008 reported that five mixed cultures and 
3 single bacteria strains, *Pseudomonas* sp., Arthrobacter sp. and *Mycobacterium* sp. 
were isolated from hydrocarbon-contaminated soils by enrichment technique using 
either crude oil or individual hydrocarbon, as the sole carbon sources. The strains were 
selected based on their ability to grow in medium containing crude oil, used engine oil 
or both. Their ability to degrade hydrocarbon contamination in the environment was 
investigated using soil samples contaminated with used engine oil.

A study was designed to evaluate the bacterial diversity of soil environment 
contaminated with used engine oil. Ten mechanic workshops within were selected and 
six soil samples were collected from each site. These were analyzed using Bushnell 
Haas enrichment medium. Samples were enumerated using ten fold dilutions from 1:10 
to 1:100000 from the soil samples. The result showed the isolation of *Bacillus 
Stearothermophilus* (8.3%) and *Cyanobacteria* (1.7%) from the sites sampled. 
(Udeani et al., 2009). Gupta et al., (2010) reported that five bacterial strains, capable 
to utilizing 2T engine oil as a sole source of carbon and energy, were isolated from 
petroleum contaminated soil of high altitude. Among this *Enterobacter cloaca* which 
was confirmed by partial sequencing of 16S rDNA, showed good growth on 2T engine 
oil and was selected for further studies.
1.3. MATERIALS AND METHODS

Cleaning of glasswares and sterilization

All the glasswares used for the research were cleaned with soap water, and then washed with tap water. The glasswares thus cleaned were rinsed with distilled water and dried in oven before used. In all experiments microbiological techniques were followed for sterilization of the media, glassware like petri plates, flasks etc (Johnson and Case, 1995; Aneja, 1996; Kannan, 2002).

Collection and transportation of samples

The soil samples were collected from the contaminated sites of various automobile workshops in Pudukkottai, Tamil Nadu, India. It was collected at depth of 0 to 5cm with sterile spatulas, transported in coolers to the Microbiology laboratory, and stored at 4°C until they were analyzed.

Analysis of physico-chemical parameters in sediment soil samples

The physico-chemical parameters such as pH, EC, N, P, K were analyzed.

Determination of pH

20 g of air-dried sediment was taken in a clean conical flask and 100 ml distilled water was added for making 1:5 soil suspension. It was shaken for one hour at regular intervals. After shaking, the suspension was filtered through Whatman No: 42 filter paper. The pH of the sample was determined using a digital pH meter MK IV (Systronics, India).

Estimation of sediment Electrical conductivity (Levine, 2001)

1g of soil was mixed with 1ml of water was prepared in the ratio of 1:1 suspension. The suspension was filtered using suction. A round Whatmann no: 42 filter paper was put in the Buchner funnel, and the filter paper was moistened with distilled water and make sure that it is tightly attached to the bottom of the funnel that all holes are covered. The vacumm pump was started. The suction was opened and the suspension
is added to Buchner funnel. The clear filtrate was transferred into 50 ml bottle and the conductivity cell was immersed into the solution and the readings were noted.

**Estimation of total Nitrogen** (Hale *et al.*, 1949)

0.2 ml to 1 ml of working standard solution was pipetted out in five test tubes (S1 to S5) respectively. 0.2 mg of sample was taken in fresh test tube. All the test tubes were made up to 9 ml using sterile distilled water. 1.5 ml of sodium hydroxide and 1 ml Nessler’s reagent were added to all the standard and sample test tubes. The intensity of the color development was read at 540 nm by using green filter. The concentration of nitrogen of the solution can be calculated using standard.

Calculation:

\[
\text{Test OD / test OD} \times \text{conc. of Standard} \times 100 / \text{volume of sample taken.}
\]

**Estimation of total Phosphorous** (Olsen and Sommers, 1982)

0.5 g powdered sediment was taken in a china dish and moistened with distilled water to the consistency of a thin paste. 2 ml of Conc. HNO₃ followed by 2 ml Conc. Perchloric acid were added. The contents were heated slowly on a hot plate until they become nearly dry. The dish was cooled and 1 ml perchloric acid was added and the contents were treated again until they were dry. The dish was cooled and 20 ml diluted H₂SO₄ was added. It was boiled slowly for 10 minutes and allowed to cool. The contents were filtered through Whatmann No. 42 filter paper and the final volume was made up to 250 ml. 50 ml aliquot was taken in a beaker. 2 ml ammonium molybdate and 5 drops of SnCl₂ were added of it. After the development of colour, the absorbance was read in a spectrophotometer at 690 nm.

Calculation:

\[
\% \text{ Total Phosphorus} = \frac{\text{mg/1 of digest} \times \text{OD}}{20}
\]

**Estimation of total potassium** (Richards, 1954)

5 g of air-dried and sieved sediment sample was taken in a beaker. 10 ml of ammonium acetate was added to it and stirred. The supernatant was kept overnight. The
supernatant was filtered through Whatmann No. 42 filter paper. The sample was leached 4-5 minutes with equal amount of ammonium acetate and the final volume was made up to 100 ml and it was used for determination of potassium using flame photometer. Flame photometer was switched on and the flame was lit following the instructions given in the user’s manual. The standard solutions were aspirated into the flame and the instrument was calibrated. The sample was aspirated into the flame and the concentration of potassium present in the sample was determined.

**Hydrocarbon used**

The hydrocarbon used as carbon source was used engine oil, sterilized by filtration through a Millipore membrane 0.22mm, being obtained directly from automobile workshops in Pudukkottai, Tamil Nadu, India.

**Enrichment and isolation of hydrocarbon degrading bacterial strains**

Soil samples (10g) from engine oil contaminated sites was suspended in 100ml of Bushnell-Hass Mineral media (Bushnell and Hass, 1941) which was used as the enrichment media with 10% (v/v) used engine oil added as the sole carbon source to isolate engine oil degrading bacteria. These were incubated at 30°C at 170 rpm kept for one week. After 1 week, 1ml of enriched media was transferred into freshly prepared enrichment media and incubated at the same conditions as mentioned above and it was followed for second enrichment and third enrichment respectively. Serial dilutions (1/10) from each enrichment process were carried out and plated on to BH agar plates, which were covered with 100 µl of used engine oil and incubated at 30°C. The single colonies were streaked onto nutrient agar plates, incubated at 30°C overnight, and stored at 4°C until further use. For long term preservation, the bacterial isolates were stored in 40% glycerol at -70°C.

The initial number of total viable cells in the original sample (before enrichment) was determined by serial dilution-agar plating procedure on Plate count agar medium (PCA) plates and incubated at 26°C for 48 hours.
Composition of Bushnell Hass media

Magnesium sulphate 0.20g  
Calcium chloride 0.02g  
Monopotassium phosphate 1g  
Ammonium nitrate 1g  
Ferric chloride 0.05g  
Di potassium phosphate 1g  
Distilled water 1000ml  
pH 7.2

Composition of PCA media

Peptone 5.0 g  
Yeast extract 2.5 g  
Glucose 1.0 g  
Agar 14.0  
Distilled water 1000ml  
pH 7.2

Screening for biodegradation and biosurfactant potential

Initial screening for oil degradation was carried out as described by Okoh et al., (2001) with some modifications.

Screening on solid media:

Bushnell Hass medium (Bushnell and Hass, 1941) was prepared and sterilized, 10% used engine oil was added to the medium before solidification, when the temperature was between 50°C-60°C, vigorously shaken and then poured in to
petriplates. All the isolated bacterial cultures were streaked on each of the solid plate. The plates were incubated at 37°C for 7 days and examined for colony formation.

**Screening on Liquid media**

Bushnell Hass medium was prepared, sterilized and 10% used engine oil was added to the media. All the isolates were aseptically inoculated into the media. Flask were incubated at 37°C on 170 rpm for 7 days and examined for turbidity development.

**Screening for the biosurfactant activity**

Haemolytic assay was performed using blood agar. The blood agar plates were prepared by the adding 5% of sheep blood to sterilized molten nutrient agar, after solidification the plates were utilized for assay method. The fresh single colony was streaked on the freshly prepared blood agar plate and incubated at 37°C for 48-72 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996).

**Phenotypic and biochemical characterization:**

The test conducted for this study were listed in Table. Gram staining was performed as described by Johnson and case (1995) and endospore staining was performed according to Kannan (2002). Using standard procedures citrate utilization test, catalase and oxidase activity were tested (Johnson and case, 1995). Hydrogen sulphide production, nitrate reduction and gelatin hydrolysis experiments were performed as recommended by Kannan (2002). Indole, methyl red, voges proskauer test, lipase and starch hydrolysis were carried out as described by Aneja (1996).

Based on the observation of the obtained results identification of the isolated bacterial strain was carried out by using Bergey’s Manual of Systemic Bacteriology 9th Edition (Holt et al., 1994)

**Molecular characterization of the potential isolates**

**Isolation of genomic DNA**

The genomic DNA isolation was performed using the reagent PrepMan (PE Applied Biosystems, CA, USA). A loopful of bacterial cells grown on Luria-Bertani
broth was suspended in 245 µl 0.1M TE. (10 mM Tris Cl, 0.1 mM EDTA; pH 7.00) The cell suspension was incubated with 5 µl (50 mg/ml) of lysozyme solution at 56 °C for 45 min. The following reagents were then added: 196.2 µl of 0.1M TE, 5 µl of dithiothreitol (1 M), 20 µl of EDTA (0.25 M), 25 µl of sodium dodecyl sulphate (10 %) and 3.8 µl of Proteinase K (20 mg/ml). The reaction mix was incubated at 37 °C for 1 hr. A 500 µl of PrepMan solution was then added and incubated at 56°C for 30 min. The reaction mix was finally heated at 100 °C for 8 min and centrifuged at 8500 ×g for 2 min. The supernatant obtained was diluted at a ratio of 1:10 with nuclease free sterile de-ionized water.

**Luria-Bertani (LB)-agar**

Peptone 10g  
Yeast extract 5g  
Sodium chloride 5g  
Agar 20g  
Distilled water 1000ml  

pH 7.2

**Agarose gel electrophoresis**

The genomic DNA was resolved on 0.8 % agarose gel. The agarose gel was prepared in 1× TAE buffer (ref appendix 1). The PCR amplified DNA and restricted DNA fragments were separated on 2 % sieving agarose gel prepared in 1× TAE buffer. The electrophoresis was performed in 1× TAE buffer for 5 to 6 hrs at a constant voltage of 140 V at 25°C. The DNA samples were visualized by staining with 0.6 µg/ml of ethidium bromide. The agarose gel DNA profiles were observed and photographed in UVI gel documentation (UVItec, Cambridge, UK). The data analysis was done with UVI photo V.99 and UVI band / map V.99 software (UVItec.). All of the thermocycling reactions were processed in Geneamp 2400 PCR system (Perkin Elmer, USA).
Sequencing of the genes encoding 16S rRNA

The Microseq™ kit is a complete system for identification of the cultured bacterial isolates. The partial gene encoding 16S rRNA were sequenced with Microseq™ 500 bacterial sequencing kit (PE Applied Biosystems, USA).

Partial 16S rDNA sequencing

Amplification of the partial 16S rDNA sequences

The 500 bp sequences of 16S rDNA were amplified with Microseq™ 500 16S rDNA-PCR module (PE Applied Biosystems, USA). A volume of 1 µl of the genomic DNA was diluted in 24 µl of nuclease free sterile de ionized water. The various reagents of PCR; primers 8f (5’AGAGTTTGATCCTGGCTCAG3’) and 1492r (5’GGTTACCTTGGTACGACTT-3’), dNTPs, AmpliTaq Gold DNA polymerase, MgCl₂ and buffer are pre-mixed into a single tube as the “PCR master mix”.

A 50 µl of the reaction mixture was prepared which consisted of 25 µl of the diluted genomic DNA (1 ng/µl) and 25 µl of the PCR master mix.

The cycling conditions for the amplification reaction were as follows:

Initial denaturation 95 °C for 10 min
This was followed by 30 cycles of
Step I  95 °C for 30 sec
Step II  60 °C for 30 sec
Step III  72 °C for 45 sec
A rapid thermal ramp of 1°C/sec was maintained between the steps
Final extension  72 °C for 10 min
Final temperature 4 °C

A 5 µl of the amplified 16S rDNA was confirmed on a 2% agarose gel.

Purification of the amplified 16S rDNA

The PCR products were purified with Microcon 100 PCR centrifugal filter device. The Microcon column was hydrated by adding 500 µl of sterile MilliQ water to the column. The column was spinned at 500× g in a fixed angle microcentrifuge for 6
min. After hydration of column, 400 µl sterile MilliQ water was added to the column and then 45 µl of the PCR product was loaded on to the column. The column was spined at 500 × g in a fixed angle microcentrifuge for 15 min. Collection tube was removed and discarded. The column was now inverted and attached to a new collection tube. 25 µl Sterile MilliQ water was added to the inverted column and spinned the inverted column at 10000 × g for 3 min to collect the purified DNA in the collection vial. The purified DNA was recovered in 25 µl of de-ionised water.

**Cycle sequencing of the amplified 16S rDNA**

The amplified 16S rDNA was subjected to cycle sequencing with Microseq™ 500 16S rDNA sequencing module. The forward and reverse sequencing reactions were assembled in separate reactions.

The 20 µl of the reaction mixture consisted of 3 µl Purified PCR product and 13 µl of sequencing reaction mix. The final volume of 20 µl was made up by 4 µl of de-ionised water.

The cycling conditions were as follows:

25 cycles of

- **Step I**  96 °C for 10 sec
- **Step II**  50 °C for 5 sec
- **Step III**  60 °C for 4 min
- **Final**  4 °C soak

A rapid thermal ramp of 1°C/sec was maintained between the steps.

**Analysis of the DNA sequences**

The cycle sequenced DNA was resolved by ABI PRISM™ 310 genetic analyzer (PE Applied Biosystems). The DNA samples were sequenced with the short capillaries (5-47 cm × 50 µm) and long capillaries (5-61 cm × 50 µm). The electrophoresis was performed with 1× electrophoresis buffer with EDTA and performance optimised polymer (POP6).
The parameters set for the electrophoresis in ABI PRISM™ 310 genetic analyzer are as follows:

Temperature 50 °C
Current 4 μA
Voltage 12 KV
Argon ion Laser power 9.7 MW

**Restriction Fragment Length Polymorphism (RFLP) analysis of 16S rDNA**

The amplified 16S rDNA were purified with Microcon PCR centrifugal filter devices as per the protocol mentioned above.

**Reagent**

1. 100 bp ladder.
2. TBE (5 x) : 54 tris base ,27.5 g boric acid , 20ml .5 EDTA (PH 8.0 ) dissolved in one liter water.
3. Ethidium bromide (0.5 μl).
4. Loading dye (6X) (0.25% Bromophenol blue in 40% sucrose.
5. Restriction endonuclease (*Alu*I, *Msp* I and *Hae* III) along with the buffer (10 X).

All the reagent tubes were assembled on ice bath. The contents of all the reagent tubes were thawed and mixed so as to have a homogenous suspension (except for the restriction enzyme, which was not to be thawed or vortexed mixed). The purified fragments were digested with single restriction enzyme *Alu*I, *Msp* I and *Hae* III. The 10× restriction enzyme buffer was diluted to a working concentration of 1×.

The 20 μl of the reaction mixture was prepared, which consisted of:

Nuclease free sterile MQ water 9 μl
PCR product 10 μl
Restriction enzyme (10U/μl) 1μl
Total volume 20 μl

The reaction content was mixed by pipetting and the reaction mixture was incubated at 37°C for 2 hrs. The reaction was terminated by inactivating the enzyme by
heating the reaction mixture at 65 °C for 10 min. The restricted DNA was checked on a 2 % agarose gel.

Data analysis

The computer assisted data analysis was performed for the RFLP profiles. The similarity matrices were calculated by Jaccard’s coefficient and the cluster analysis of similarity matrices was performed by “unweighted pair group method” (UPGMA) using NTSYS PC-Version 2.0 computer package program (Rohlf,1992) and a dendrogram was constructed.

Identification and phylogenetic analysis

The identification and phylogenetic relatedness of the isolates was assessed based on the partial 16S rRNA gene sequences. To identify unknown bacterial isolates, the 16S rDNA sequences obtained were subjected to basic local alignment search tool (BLAST) search. This search was performed with Microseq identification and analysis software “Microseq™ Analysis software v. 1.40, Microseq™ 16S rDNA Sequence Databases v. 1.01” (PE Applied Biosystems, USA). The sequences were also analyzed with the BLAST (N) search against the non-redundant Genbank+EMBL+DDBJ+PDB databases using NCBI web service: www.ncbi.nlm.nih.gov/blast. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated through bootstrap analysis by MEGA 4.0 package (Tamura et al., 2007)

Biolog substrate utilisation profile

Some of the dominant bacterial isolates which grow either in solid or liquid media were characterized by determining their substrate utilization profiles with Biolog GN and GP plates (Biolog Inc., Hayward, CA, USA).

Experimental protocol

The bacterial isolates were grown overnight in 5 ml of LB broth at 30°C. The cell pellet was harvested by centrifugation at 10,000×g for 10 min. A cell suspension was prepared by re-suspending the pellet in sterile 0.85 % NaCl solution. The cell density was adjusted to a density of ~ 3 ×10⁸ cells per ml by comparison with the turbidity standards supplied by the manufacturer. The Biolog GN and GP microplates
were inoculated with 150 µl of the cell suspension. These inoculated plates were then incubated at 30°C for 24 hrs, leaving out the 4 hrs measurement as recommended by Homes et al., (1994).

The color development in the microplate wells was interpreted visually as positive (+), negative (-) or borderline (/), when it was not possible to demarcate the positive from negative. The readings were also entered in the Biolog microlog GN release 1 4.01B database to provide comparative identification, of the isolates which was acknowledged when the similarity index used by Biolog was 5.0 or more.

**Antimicrobial susceptibility test**

*In vitro* antimicrobial sensitivity test for the isolates PDKT-1, PDKT-2, PDKT-5 were determined by Kirby Bauer disc diffusion method (Kirby et al., 1966). The Muller Hinton agar plates were prepared and sterilized. The 24 h old cultures were inoculated on the medium separately by spreading the inoculums with a sterile cotton swab. The antimicrobial agents used in this study were Amikacin (30 mcg), Amoxycilin (25 mcg), Cephalexin (30 mcg), Cefazolin (30 mcg), Co-Trimoxazole (25 mcg), Gentamycin (10 mcg), Tetracyclin (30 mcg), Streptomycin (10mcg), Amoxyclavin (30mcg), Norfloxacin (10mcg), Cefixime (30 mcg), Roxithromycin (30 mcg). These disc were kept on swabbed medium and the plates were incubated at 37°C for 24-48 h. After incubation zone of inhibition was measured and recorded.
1.4. RESULT

Physiography

The present study site Pudukkottai district covers an area of 4663 Sq. Km. which has a coast line of 39 Kms. The district is located between 78°.25' and 79°.15' of the East of Longitude and between 9°.50' and 10°.40' of the North of Latitude (Fig. 1). The used engine oil (Fig. 2) and its contaminated soil samples were collected from various automobile workshops in Pudukkottai (Fig. 2) showed marked variation in the physicochemical as well as in the biological parameters.

Physicochemical parameters

a) Hydrogen ion concentration (pH)

The pH of the contaminated soil samples showed fluctuations with lowest value of 6.8 to the highest value 7.9. (Table.1)

b) Electron conductivity (EC)

The EC of the contaminated soil samples showed variations with lowest value of 0.22 to the highest value 0.47. (Table.1)

c) Nitrogen content

The nitrogen content of the contaminated soil samples ranged from 0.67 -1.1. The highest level of nitrogen content was observed in soil sample B and lowest level recorded in soil sample A. (Table.1)

d) Phosphorous content

The phosphorous content of the contaminated soil samples ranged from 0.2-0.6. The lowest level of phosphorous content was observed in soil sample D and highest level recorded in soil sample C. (Table.1).
e) Potassium content

The potassium content of the contaminated soil samples ranged from 0.22 - 0.55. The soil sample C having highest level and soil sample B showing least amount of potassium. (Table.1)

**Isolation of the total petroleum hydrocarbon degrading bacteria**

Prior the screening of hydrocarbon degrading microorganisms, the bacterial populations were estimated in each original sample. Appreciable number of bacteria ranges from $10^6$ - $10^8$ colony forming units (CFU) has been found to exist in the investigated soil samples. Indigenous organisms isolated in this study were selected by enrichment culturing technique. The cultivable bacterial strains were isolated from used engine oil contaminated soil samples. After 72 hrs of incubation under aerobic conditions at 37°C, the bacterial colonies of different morphological characteristics grew on the BH agar media plates with steam sterilized used engine oil as the sole carbon source (Fig.3) As the results in Table .2 indicate a significant increase in hydrocarbon-degrading microorganisms observed after the first and the second week of enrichment.

A total of 25 cultivable bacterial strains were isolated by enrichment culture technique from soil samples contaminated used engine oil procured from five different sampling sites (Fig.4-7). The microscopic examination of the bacterial cells showed different morphology (Table. 3). The bacterial strains also had varying pigmentation (red, yellow, white and pale colour). All the bacterial strains were given a unique strain designation. One of the objectives of this study was to isolate as many culturable strains as possible in order to determinate their hydrocarbon biodegradation potential in standardized culture conditions. For this reason, a first screening of strain was done after Gram staining and microscopic examination for bacteria to eliminate apparently similar strains.

The present study was designed to isolate a novel hydrocarbon-degradation microorganism(s). For this purpose all the 25 bacterial strains were primarily tested for their ability to grow on hydrocarbon containing media. Colony development and turbidity were used as indices of biodegradation potential (Nweke and Okpokwasili, 2003). The efficiency of colony development varied among the isolates, only three
isolates (JJ-4, JJ-7, JJ-21) were able to grow on such media indicating their hydrocarbon degradation ability (Fig. 8). The strains JJ-8 and JJ-20 showed moderate growth and remaining isolates were unable to grow on solid media containing used engine oil as the sole source of carbon and energy. Experiment with Bushnell and Haas liquid culture also confirmed the above result. The isolates JJ-4, JJ-7, JJ-21 showed hemolytic activity as indicated by the clear zone formation around the colonies on blood agar medium and the diameter of the haemolytic zone was 0.9 mm, 1.1 mm, 1.0 mm diameter respectively (Fig. 9). Based on the above result the three isolates were selected for further investigation.

By following Bergey’s Manual of Systemic Bacteriology, the isolates (JJ-4, JJ-7, JJ-21) which were able to grow on the medium supplemented with used engine oil as the sole source of carbon and which also had the ability to produce biosurfactant was subjected to biochemical, cultural, and morphological determination and the results were tabulated (Table. 4 & 5). The substrate utilization profile revealed that the isolates which were able to grow in BH agar plates (JJ-9, JJ-11, JJ-12) were identified as Pseudomonas bathycetes, Pseudomonas stutzeri, Acinetobacter calcoaceticus and Vibrio vulnificus, and in BH broth (JJ-13, JJ-5, JJ-1, JJ-3) as Escherichia vulneris, Micrococcus luteus, an unidentified strain respectively (Fig.12-18).

The potential isolates (JJ-4, JJ-7, JJ-21) and the isolates which showed moderate growth (JJ-8, JJ-20) were selected for 16S rRNA sequencing. The alignment and comparison of the 16S rDNA sequences with the Microseq microbial identification and analysis software (PE Applied Biosystems) gave up to 95 % to 100 % sequence similarity. The analysis of the 16S rDNA sequences with the BLAST (N) search against the Genbank + European molecular biology laboratory (EMBL) + DNA databank of Japan (DDBJ) and with ribosomal database project (RDP) also gave identical results of up to 98 % 16S rDNA sequence homology. The BLAST search results depicted that the strains JJ-4, JJ-7, JJ-21, JJ-8, JJ-20 had a closest 16S rDNA sequence homology to Serratia marcescens, Pseudomonas aeruginosa, Bacillus licheniformis, Pseudomonas putita and Bacillus subtilus respectively. All the five strains were deposited in Genbank, USA and the strain JJ-4 (Serratia marcescens PDKT-1) was assigned the accession number HM 998315 and for remaining strains JJ-7 (Pseudomonas aeruginosa PDKT-
2, Bankit No;1376434), JJ-21 (Bacillus licheniformis PDKT-5 Bankit No;1379954), JJ-8 (Pseudomonas putita PDKT-3, Bankit No;1379945,) and JJ-20 (Bacillus subtilus PDKT-4, Bankit No;1379952) the process are in runway (Fig.19-23). The RFLP profiles of the potential degraders Serratia marcescens PDKT-1, Pseudomonas aeruginosa PDKT-2, Bacillus licheniformis PDKT-5) revealed distinct banding pattern (Fig. 25-28). A dendogram was constructed for RFLP profiles was carried out on the basis of the 16S rDNA RFLP profiles, the UPGMA analysis clustered the three strains PDKT-1, PDKT-2, PDKT-5 in to two genotypic groups (Fig. 29)

The antibiotic sensitivity profile of the isolates PDKT-1, PDKT-2, PDKT-5 was tested against some commonly used broad spectrum antibiotics. The antibiotic sensitivity pattern showed significant difference. The isolates PDKT-1, PDKT-2 were sensitive to Streptomycin and Roxithromycin while the isolate PDKT-5 was sensitive to Streptomycin, Roxithromycin and Norfloxacin. The three isolates were resistant to all the remaining tested antibiotics (Fig. 30-32).
1.5. DISCUSSION

In this study five different used engine oil contaminated sites were selected for the present investigation. The age of the used engine oil contamination at the sampling sites also varied. The used engine oil contaminated sites were selected for the present study because the soils at these locations get contaminated with the toxic petroleum hydrocarbons generated in their day to day activities. The different contaminated sites were also selected to elucidate the diversity among bacterial strains surviving in different conditions. The age of the used engine oil contamination could have also influenced the diversity of the bacterial population.

Microbial diversity has also received particular attention in the areas of environmental microbiology, where microbial activity has important implications for the restoration and bioremediation of polluted sites. The resilience in the nutrient preference of the microorganisms and adaptation in extreme environments make these microbes ideal candidates for investigation to mitigate problems related to reclamation of polluted sites. The evaluation of microbial diversity in soil contaminated with total petroleum hydrocarbons is important for being a source of microorganisms that could degrade the recalcitrant hydrocarbon constituents in the contaminated sites. With the gaining importance and awareness of preserving the indigenous microflora, documentation of such biological agents has become an absolute necessity.

The diversity of the cultivable total petroleum hydrocarbon (TPH) degrading bacterial strains was evaluated by the culture dependent approaches and by molecular analysis of the community DNA isolated from hydrocarbon contaminated soil. The cultivable TPH degrading bacterial strains was isolated by “enrichment culture technique”. The culture enrichment technique has been suggested by Watanabe et al., (1998) as the commonly used technique for the isolation of the microorganisms that are capable of degrading a variety of hydrocarbons. Alternative methods such as direct spread plating and enrichment in chemostat have been proposed for the isolation of hydrocarbon degrading bacteria (Watanabe et al., 1998; Fulthorpe et al., 1998).

In motor mechanics workshops there is a constant change in the soil microorganism as a result of deliberate spillage of used engine oil. These alter the biomass and ecology of the soil such that both microbial communities and grasses can no longer
grow on the soil spots. The colour and texture of the soil are affected; this leads to
different microbial flora establishment in an attempt to remedy the petroleum product
spillage (Bartha and Atlas, 1977). The analysis of used engine oil contaminated soil
samples revealed physicochemical variations. These variations greatly influence the
distribution of microorganism. Soil pH was an important factor in biodegradation. In
the present study pH range (Table 1) of the used engine oil contaminated soil samples
indicates that soil buffering capacity was sufficient to maintain the soil pH in the neutral
range, which is favourable for biodegradation, stated by Atlas and Bartha (1992); Norris
(1994). Various factors may limit the rate of petroleum hydrocarbon degradation
including lack of essential nutrients such as nitrogen. Positive effects of nitrogen
amendment on microbial activity and/or petroleum hydrocarbon degradation have been
widely demonstrated (Jørgensen et al., 2000; Margesin et al., 2000, 2007; Brook et al.,
2001; Margesin and Schinner, 2001; Riffaldi et al., 2006). The presence of appreciable
quantities of nitrogen, phosphorus and potassium (Table 1) in the investigated soil
samples which was a necessary nutrient for bacterial biodegradative activities was in
agreement with the previous studies by Nakasaki et al., (1992); Ijah and Antai (2003);

In the present investigation it was observed that there was a significant increase
in hydrocarbon-degrading microorganisms after the first and the second week of
enrichment. The total heterotrophic bacteria count range from 1.2 x 10^6 to 2.2 x 10^8 in
this work. These results suggest that slightest differences were observed in total
bacterial count and total hydrocarbon degrading bacteria because mostly growing
bacteria in petroleum contaminated soil were petroleum hydrocarbon degrader and only
these bacteria were capable to grow in high concentration of NSO (Nitrogen, Sulphur,
and Oxygen) compounds. Similar results were observed by Abioye et al., (2009). Butier
and Mason (1997) indicated that there is an increase in heterotrophic bacterial
population in the presence of dispersant agent. Antai (1990) reported that two major
responses to crude oil in which there is an increase in biomass. An important fact is that
the medium employed for the isolation of petroleum degrading bacteria may have
significant selective effect on bacteria population that was isolated (Mishra et al., 2001).
The type of enrichment substrate significantly affected the microbial population. The
transfer of microorganisms after first enrichment to a fresh medium resulted in an
increase of numbers of hydrocarbon utilizers. These results confirmed the fact that repeated exposure to petroleum products at a site will usually increase the adaptive capabilities of the microorganisms and there is an increase in the rate of degradation with a new exposure to such a compound.

A total of 25 bacterial species isolated by enrichment and spread plating of the used engine oil contaminated soil samples affiliated to seven different genera. Of the total bacterial strains isolated by enrichment culture technique, both Gram negative and Gram positive bacteria were obtained but Gram negative bacterial strains were dominant. This corroborates with the previous report where MacNaughton, (1999) who have stated that hydrocarbon contaminated site is dominated by Gram negative bacteria. The species of these genera have been previously reported to degrade hydrocarbons (Stapleton et al., 2000; Watanabe et al., 2000; Bakeman and Madsen, 2002; Van Hamme et al., 2003). In this study, it was observed that the majority of the cultivable bacterial strains belong to the proteobacteria group. The bacterial strains were predominantly affiliated to the γ-subclass of proteobacteria. This indicated the limited bacterial diversity and selective population on succession at the petroleum hydrocarbon contaminated environment. MacNaughton (1999) stated that microbial communities within contaminated ecosystems tend to be dominated by those organisms capable of utilizing and surviving on the components present in the contaminants. Therefore, these microbial communities are typically less diverse than those in non-stressed conditions.

There are limited reports describing the distribution of hydrocarbon degrading microorganism with suitable biosurfactant production. According to Carillo et al., (1996) the hemolytic activity appears to be a good screening criterion for surfactant-producing strains. Baruah et al., (1997) isolated 40 bacterial strains from soil and sludge samples, which were capable of using hydrocarbon as sole source of carbon and energy. From this 40 only 6 microbial strains were capable of producing biosurfactants. Tuleva et al., (2002) reported that from the 14 isolates screened, only 5 bacterial strains were able to grow with hexadecane as the sole carbon source, and only two of them decreased the culture medium surface tension below 35 mN m\(^{-1}\) and formed kerosene water emulsions. The results of the present study correlated with the observations made by the author earlier. Among 25 isolates, only three isolates JJ-4 (PDKT-1), JJ-7 (PDKT-
2), JJ-21(PDKT-5), were able to degrade the hydrocarbon together with biosurfactant production in this study.

The identification of microorganisms and their affiliation to a particular taxonomic hierarchy is an important prerequisite in the documentation of microbial diversity (Bertilsson et al., 2002). In the present study, the bacterial strains isolated by the culture-dependent methods which were able to grow either in solid or liquid media were identified with Biolog microbial identification. Previously Mafham et al., (2002) evaluated the functional diversity of the bacterial community by studying the utilization pattern of the different carbon sources in the Biolog bacterial identification systems. The bacterial strains grown in both medium revealing their hydrocarbon degrading ability were identified by sequencing of their 16S rRNA genes. During the past decade, Ribosomal RNA based identification and detection methods have become techniques routinely used in all disciplines of microbiology. As molecular chronometers (Woese, 1987) these molecules have preserved their evolutionary history. Highly conserved region carry the information on early evolutionary events and more recent changes are documented within less conserved position or stretches. The degree of divergence of present day rRNA sequences given an estimate of their phylogenetic distance. The 16S rRNA genes have been used for microbial identification and 16S rRNA gene sequences are indicative of identity of the genomes at the strain level (Woese, 1987, Klappenbach et al., 2000 and 2001, Regenhardt et al., 2002). For the identification and taxonomic affiliation of 16S rDNA gene sequences in this study, the common criterion where 16S rRNA sequences that are over 97% similar were placed under same species as described by Kissand and Wilkner 2003 was used. A total of 12 bacterial species belonging to 7 genera; Pseudomonas sp., Bacillus sp., Serratia sp., Micrococcus sp., Escherichia sp., Vibrio sp., Acinetobacter sp. were identified from different sampling sites. These bacterial species had been implicated in hydrocarbon degradation by different researchers (Ijah, 1998; Ahn et al., 1999; Van Hamme et al., 2003; Bento et al., 2005; Das and Mukherjee, 2007, Akoachere et al., 2008). The distinct RFLP pattern of the three strains could be due to increased mutation under stress, facilitating adaptation of the strains to stressful environments and also be due to the different types of oily sludge contamination and varying age of contamination at the sites.
In this study the 50% of the bacterial strains were found to belong to the genus *Pseudomonas*. The discovery of *Pseudomonas* was not surprising based on their frequency in the soil as well as their frequent biodegradability. This observation corroborates with the previous report where Stoffels *et al.*, (1998) suggested that during microbial degradation of complex aromatic hydrocarbons, population of γ-proteobacteria especially *Pseudomonas* are predominant. The dominance of *Pseudomonas* was probably due to their survival ability under stressful conditions imposed by diverse nature of contaminants at the sites. As a result of which only a selective bacterial population could strive on toxic moieties of hydrocarbons (Stoffels *et al.*, 1998). Greene *et al.*, 2000 have suggested the evolution of a few dynamic microbial populations during growth on hydrocarbon irrespective of their geoclimatic location. The presence of *Pseudomonas aeruginosa* in used engine oil contaminated sites was reported by Mandri and Lin (2007) and Shojaosadati *et al.*, 2008. The second most dominant genus identified at the different sampling sites was the *Serratia marcescens*. This result is in agreement with Akoachere *et al*.,(2008) who reported the occurrence of *Serratia marcescens* being dominant at the lubricating oil contaminated sites which coincides the present result. The contaminated sites were also characterized by the presence of *Bacillus licheniformis* at all sites. Previously Ohenhen *et al.*, 2006 and Nwaogu *et al.*, 2008 reported the presence of *Bacillus* sp in crude oil and diesel oil contaminated sites respectively. Udeani *et al.*, 2009 stated that *B. stearothermophilus* was found to reoccur in most sites mechanic workshops’ soil environment contaminated with used engine oil. Recently Gerard Nkwelang *et al.*, (2008) observed that the genera active in oily sludge polluted soil were *Pseudomonas, Bacillus, Serratia* and *Acinetobacter*. *Vibrio* sp., *Acinetobacter* sp., *Micrococcus* sp., and *Escherichia* sp, was in line with the present study results. Stapleton *et al.*, 2000 reported that the presence of plasmid may be one of the reason for the presence of both antibiotics resistance and hydrocarbon degradation capabilities supports the present study results.

The metabolic diversity of microorganisms in the natural environments is an important factor in the biodegradation of hydrocarbons. As the results indicate original samples from each location were characterized with a very high diversity of microorganisms. Extensive degradation of petroleum pollutants is generally accomplished by mixed microbial populations, rather than single microbial species.