2.1.1. INTRODUCTION

The biodegradation of petroleum and other hydrocarbons in the environment is a complex process, whose quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions, and the composition of the autochthonous microbial community (Leahy and Colwell, 1990; Atlas, 1995; van Hamme et al., 2003). Microbial degradation of oil has been shown to occur by attack on aliphatic or light aromatic fractions of the oil, with high-molecular-weight aromatics, resins, and asphaltenes considered to be recalcitrant or exhibiting only very low rates of biodegradation, although some studies have reported their removal at high rates under optimal conditions. In aquatic ecosystems, dispersion and emulsification of oil in oil slicks appear to be prerequisites for rapid biodegradation; large masses of mousse, tar balls, or high concentrations of oil in quiescent environments tend to persist because of low surface areas available for microbial activity. Petroleum spilled on or applied to soil is largely adsorbed to particulate matter, decreasing its toxicity but possibly also contributing to its persistence. Biodegradation rates generally increase with increasing temperature; ecosystems exposed to extremely low temperatures degrade hydrocarbons very slowly. The microbial degradation of petroleum in aquatic environments is limited primarily by nutrients such as nitrogen and phosphorus; salinity and pressure may be important in estuarine and deep-sea regions, respectively. Oxygen, nutrient concentrations, moisture, and pH are predominant factors in determining biodegradation rates in soil.

Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons. Bacteria and fungi are the key agents of degradation, with bacteria assuming the dominant role in marine ecosystems and fungi becoming more important in freshwater and terrestrial environments. Adapted communities, i.e., those which have been previously exposed to hydrocarbons, exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination. The mechanisms of adaptation include both selective enrichment and genetic changes, resulting in a net increase in the number of hydrocarbon
utilizing organisms and in the pool of hydrocarbon-catabolizing genes within the community. The association of such genes with plasmid DNA may also lead to an increased frequency of plasmid-bearing microorganisms. Seeding of petroleum-contaminated water or soils with hydrocarbon utilizing bacteria has met with some success, particularly in situations in which chemostats or fermentors have been used to control conditions and reduce competition from indigenous microflora.

While hydrocarbon-degrading microorganisms are ubiquitous, hydrocarbon-degraders normally constitute less than 1% of the total microbial community. When oil pollutants are present these hydrocarbon-degrading populations increase, typically to 10% of the community (Alexander, 1994). Rates of natural degradation typically have been found to be low and are limited by environmental factors, such as, the contaminant or nutrient (N and P) bioavailability, physical conditions (e.g. temperature, salinity, pH) or microbial competition (Atlas, 1991).

Biodegradation of oil contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as one of the efficient, economic, versatile and environmentally sound treatment (Margesin and Schinner, 1997). Biodegradation of petroleum hydrocarbon pollutants and petrochemicals by bacteria have been extensively investigated (Obire and Nwaubeta, 2001; Ijah and Akpera, 2002; Nweke and Okpowasilli, 2003; Okerentugba and Ezeronye, 2003; Okoh, 2003; Sanni and Ajisebutu, 2003; Oboirien et al., 2005; Ojumu et al., 2005). Other organisms such as fungi are also capable of degrading the hydrocarbons in engine oil to a certain extent. However, they take longer periods of time to grow as compared to their bacterial counterparts (Prenafeta-Boldu et al., 2001).

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds containing carbon and hydrogen, composed of two or more fused aromatic rings in linear, angular and cluster arrangements. They are lipophilic in nature and relatively insoluble in water (Hafez et al., 2008 and Johnsen et al., 2005). PAHs are ubiquitous pollutants and are generated from anthropogenic activities such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants (Ni Chadhain et al., 2006). PAHs have been identified as hazardous chemicals by different State and Central Pollution Control Boards because of their toxic, carcinogenic
and tetrigenic effects on living body (Ruma et al., 2007). PAHs, is a persistent and toxic soil contaminant (Hyotylainen and Oikari, 1999; Lotufo, 1997). Pollution by PAHs is usually found on the sites of gas factories and wood preservation plants.

Bioremediation is an economically and environmentally attractive solution for cleaning those sites (Kastner and Maho, 1996). Bioremediation, a process that exploits the catalytic abilities of living organisms to enhance the rate or extent of pollutant destruction, is an important tool in attempts to mitigate environmental contamination (Autry and Ellis, 1992; Kalyuzhnyi, 2000). Bioremediation achieves contaminant decomposition or immobilization by exploiting the existing metabolic potential in microorganisms with catabolic functions derived through selection, or by the introduction of genes encoding such functions. The effectiveness of bioremediation is often a function of the extent to which a microbial population or consortium can be enriched and maintained in environment. When few or no indigenous degradative microorganisms exist in a contaminated area and practically does not allow time for the natural enrichment of suitable population, inoculation may be a convenient option (Kalyuzhnyi, 2000). Hydrocarbonoclastic microbes play a paramount role in bioremediation. They include bacteria, fungi, yeasts and some algae. These organisms have been isolated from heavily oil-polluted deposits or in a variety of soils and water continuously exposed to hydrocarbon for several years (Ibe and Ibe, 1986). The importance of microorganisms in decomposing natural organic residues in soils, sediments, and aquatic ecosystems had long been recognised. Microbial transformation of organic contaminants normally occurs because the organisms can use the contaminants for their own energy needs, growth and reproduction. The ability of certain microorganisms to degrade petroleum seems to be an adaptive process and is governed by environmental conditions. The presence of petroleum may also affect the microbial community through selection of species.

Biodegradation of complex hydrocarbons; particularly pollutants that are made up of many different compounds, such as crude oil or petroleum; usually requires the cooperation of more than one single species. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so the mixed cultures with overall broad enzymatic capacities are required to increase the rate of petroleum biodegradation.
Several microbial populations have been detected in petroleum-contaminated soils or water. This phenomenon, strongly suggests that each strain or genera has its role in hydrocarbon transformation processes. The degradation capacity of any microbial culture is not necessarily the result of merely adding the capacities of the individual strains (Ghazali et al., 2004).

As the usage of petroleum hydrocarbon products increase, soil contamination with engine oils is becoming one of the major environmental problems. To remediate soils contaminated with oils, bioremediation provides an effective and efficient strategy to speed up the clean-up processes (Mandri and Lin, 2007). Most mechanical methods to reduce hydrocarbon pollution is expensive, time consuming and depends mainly on excavating of these soil, treating in separate area or better treatment facilities. These treatments include incineration and/or burial in secure land fills (USEPA 2001; ITOPF 2006). These are effective treatments but after burning, this soil looses most of its nutritional value and structure. These methods do not remove the contamination but only relocate the problem (Lageman et al., 2005). Bioremediation processes have been shown to be effective methods that stimulate the biodegradation of contaminated soil (Swannell et al., 1996; McLaughlin, 2001) and may restore contaminated soils through the broad biodegradative capabilities evolved by microorganisms towards undesirable organic compounds (Andreoni and Gianfreda, 2007).

The present study was therefore undertaken with a view to elucidate the ability of microbial isolates on the biodegradation of used engine oil and oil contaminated soil by bacterial consortium.
2.1.2. REVIEW OF LITERATURE

Environmental pollution by petroleum hydrocarbons has become a serious problem all around the world. Large-scale incineration plants have been developed, and incineration of hydrocarbon pollutants is carried out to clean up hydrocarbon-contaminated sites. The treatment time is short, but the system requires huge machines and large amounts of heavy oils (Matsumiya and Kubo, 2007). Biological treatments for hydrocarbon-degradation have also been investigated. For the construction of such bioremediation systems, many kinds of hydrocarbon-degrading bacteria have been isolated and analyzed (Pritchard et al., 1992; Komukai-Nakamura et al., 1996; Lal and Khanna, 1996; Korda et al., 1997; Vidali, 2001; Iwamoto and Nasu, 2001; Dua et al., 2002; Aislabie et al., 2006).

In recent years, many microbial ecologists have identified various microbial species that are effective degraders of hydrocarbons in natural environments. Many of these microbial consortia have been isolated from heavily contaminated coastal areas. They were isolated based on their ability to metabolize various carbon sources, such as aliphatic and aromatic compounds and their chlorinated derivates. The microorganisms were obtained originally by enrichment culture procedures, where maximum specific growth rate or maximum final cell concentration was used as the selection criterion. The
driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs. A large number of studies report that low molecular weight alkanes are degraded most rapidly. Mixed cultures carry out more extensive biodegradation of petroleum than pure cultures (Ghazali et al., 2004; Oteyza et al., 2005; Sun et al., 2004; Gerdes et al., 2004; Trindade et al., 2004).

In many ecosystems there is already an adequate indigenous microbial community capable of extensive oil biodegradation, provided that environmental conditions are favorable for oil-degrading metabolic activity (Capelli et al., 2001; Richard and Vogel, 1999; Kim et al., 2004). There are several advantages relying on indigenous microorganisms rather than adding microorganisms to degrade hydrocarbons. First, natural populations have developed through many years. These microorganisms are adapted for survival and proliferation in that environment. Secondly, the ability to utilize hydrocarbons is distributed among a diverse microbial population. This population occurs in natural ecosystems and either independently or in combination metabolizes various hydrocarbons. Many times, when the amount of microorganisms is sufficient in the contaminated environment, microbial seeding is not required.

Microorganisms (bacteria and fungi) have different rates at which they utilize and degrade hydrocarbons in the soil or water. This rate is reflected in the multiplication and colony forming units (cfu) for the isolated organisms. The use of microorganisms to degrade petroleum hydrocarbon resulting from oil spillage has been a subject of extensive research since the first publication of bacterial growth on petroleum hydrocarbons (Atlas, 1981; Gerson, 1985). Several petroleum hydrocarbon degrading microorganisms have been isolated from both soil and marine sources, which are the two major environments affected by petroleum hydrocarbon pollution (Bossert and Bartha, 1984; Antai and Mgborno, 1989). Microorganisms are equipped with metabolic machinery to use petroleum products as a carbon and energy source. The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic (i.e. they utilize oxygen as the primary electron acceptor) or anaerobic (i.e. they utilize an alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic
degradation. One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction.

Bioremediation make use of indigenous oil-consuming microorganism called petrophiles by enhancing and fertilizing then in their natural habitats. Petrophiles are very unique organism that can naturally degrade large hydrocarbons and utilize then as food source (Heider and Rabus, 2008). Microorganisms degrade these compounds by using enzymes in their metabolism and can be useful in cleaning up contaminated sites (Alexander et al., 1997). Pieper and Reineke (2000) published a comprehensive review on engineering bacteria for bioremediation approach. They outlined the understandings of natural diversity and capabilities of microorganisms for degrading aromatic and xenobiotic organic compounds. Briefly noted the bioavailability of xenobiotics, transport and chemotaxis, properties of microorganisms important for biodegradation, molecular tools used to engineer bacteria and tools to characterize organisms present in the environment.

The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde and a fatty acid. The fatty acid is cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation. The initial enzymatic attack involves a group of monooxygenases. The general pathway for aromatic hydrocarbons involves cis-hydroxylation of the ring structure forming a diol (e.g. catechol) using dioxygenase. The ring is oxidatively cleaved by dioxygenases, forming a dicarboxylic acid (e.g. muconic acid). Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the sidechain, followed by cleavage of the ring structure. The degradative pathway for a highly branched compound, such as pristane or phytane, may proceed by omega oxidation forming a dicarboxylic acid, instead of only monocarboxylic acid (Hamme et al., 2003). The degradation of n-alkanes higher than C9 increases with the chain length. The longer-chain aliphatic hydrocarbons are readily degraded by a wide variety of microorganisms under aerobic conditions. For liquid n-alkanes, C12-C16, low solid n-alkanes, C17-C28, and high solid alkanes, above C28, a different degradation pattern can be proposed. Liquid and low solid n-alkanes which are less soluble in water and therefore less toxic to microorganisms, degrade more readily than short chains (<C9) while degradation of n-
alkanes above C28 is a function of the hydrocarbon chain length. (Setti, 1993) About 95% of low molecular weight alkanes (smaller than C30 to C40) are converted to CO2 and water in a few months, alkanes larger than C40 can last for many years. (Weiner, 2000). Some strains of *Bacillus sp* and species of fungi including *Aspergillus spp.* and *Fusarium sp* capable of initiating the degradation of n-alkanes by sub terminal oxidation have been reported (Watkinson and Morgan, 1990).

Research pertaining to biodegradation of aromatic fractions of TPH has documented a vast array of microorganisms capable of utilizing, transforming, or mineralising these toxic compounds. The degradation of aromatic fraction of hydrocarbon has been previously reviewed (Gibson and Subranian, 1984; Kerr and Capone, 1988; Cerniglia, 1992; Sutherland *et al.*, 1995; Kanaly and Harayama, 2000; Juhasz and Naidu, 2000). Light aromatic hydrocarbons are subjected to evaporation and they are available for microbial degradation in a dissolved state. The bacterial degradation of aromatic hydrocarbons normally involves the formation of a diol followed by cleavage of aromatic ring and formation of a diacid such as *cis-cis* muconic acid (Atlas, 1995). Pandey and Jain, (2002) suggested the chemotaxis attraction in certain soil bacteria towards different aromatic hydrocarbons. Kniemeyer *et al.*, (2003) demonstrated the complete anaerobic degradation of ethylbenzene using sulphate as the electron acceptor by marine sulphate-reducing bacteria. In general, indigenous or augmented microorganisms degrade the polycyclic aromatic hydrocarbon compounds in TPH slowly. The ability to degrade polycyclic aromatic hydrocarbons is not limited to individual species but occurs over a wide group of bacterial strains.

Since bacteria initiate PAH degradation by the action of intracellular dioxygenases, the PAHs must be taken up by the cells before degradation can take place. Bacteria most often oxidize PAHs to cis-dihydrodiols by incorporation of both atoms of an oxygen molecule. The cis-dihydrodiols are further oxidized, first to the aromatic dihydroxy compounds(catechols) and then channeled through the ortho- or meta cleavage pathways(Cerniglia, 1984; Smith, 1990). The biological degradation of PAHs can serve three different functions. (i) Assimilative biodegradation that yields carbon and energy for the degrading organism and goes along with the mineralization of the compound or part of it. (ii) Intracellular detoxification processes where the purpose
is to make the PAHs water-soluble as a pre-requisite for excretion of the compounds. Generally it seems that intracellular oxidation and hydroxylation of PAHs in bacteria is an initial step preparing ring fission and carbon assimilation, whereas in fungi it is an initial step in detoxification (Cerniglia, 1984). (iii) Co-metabolism, which is the degradation of PAHs without generation of energy and carbon for the cell metabolism. The low bioavailability of PAH may have prevented the evolution of suitable enzymatic pathways in soil bacteria. The recalcitrant compounds generally do not serve as growth substrates for any single microbial organism, but are thought to be oxidized in a series of steps by consortia of microbes (Perry 1979).

Hanne et al., 2003 published an extensive review on recent advances in petroleum microbiology. They outlined the different way of microbial metabolism (Aerobic alkane, Aerobic PHA and Anaerobic hydrocarbon metabolism), behavioral and physiological responses of microorganisms to various hydrocarbons (Membrane alterations, hydrocarbon uptake and efflux), approaches used for calculating microbial diversity and community, factors affecting the biodegradation rate, ways of treatment contaminated soils and sludge, microbial enhanced oil recovery systems, microbial desulfurization, microbial denitrogenation and applications of biosensors in environmental monitoring. Since the degradation of long chain hydrocarbons, which are solid at temperatures less than 10 °C, is hindered by their limited bioavailability, the waste oil is hardly degraded by microorganisms in nature (Gough and Rowland, 1990; Sorkhoh et al., 1995; Ijaha and Antaib, 2003). In addition, the recalcitrance of hydrocarbons and/or the inhibition of microorganisms by minor ingredients in waste oil, also hinder the degradation of waste oil (Gough and Rowland 1990). Susceptibility of a hydrocarbon to microbial degradation varies with type and size of the hydrocarbon molecule. Alkanes of intermediate chain length (C10–C24) are often degraded rapidly, while very long chain alkanes are increasingly resistant to microbial degradation (Ijaha and Antaib, 2003).

Bioremediation involves the transformation of complex or simple chemical compounds into nonhazardous forms by biological agents resulting in materials of a higher nutritive value or simply reducing the final bulk of the product (Grady, 1985). This then gives rise to a variety of products most of which will be much more water-
soluble than the parent hydrocarbon. Rambeloarisoa et al. 1984 reported a mixed culture, containing 8 strains of 6 genera, which could effectively degrade crude oil. Interestingly, only 5 strains between them could grow as pure cultures, on different hydrocarbons. However, when the other 3 strains were removed from the culture, the effectiveness of the mixed culture was remarkably reduced. These results showed that each member in a microbial community has a significant role and may be dependent on the presence of the other species or strains for surviving. Syoko et al., 1996 reported the sequential degradation of Arabian light crude oil by two different genera. Acinetobacter sp. T4 biodegraded alkanes, then Pseudomonas putida PB4 began to grow on the metabolites and finally degraded aromatic compounds of crude oil. It can be attributed to the effects of synergistic interactions among the members of the association, although the mechanisms may be complex. It is possible that one species can remove the toxic metabolites of the other species, or degrade some compounds better than others (Alexander, 1999). Venosa et al., 1999 reported six of the 22 predominant isolates belonging to the genera Serratia, Alicaligenes, Micrococcus, Aeromonas, Vibrio and Pseudomonas had the capacity to degrade hydrocarbon. Wongsa et al., 2004 reported two isolates, a P.aeruginosa WatG and a Serratia marcescens HOKM, which were able to utilize gasoline, kerosene, diesel, and lubricating oil as the sole carbon and energy sources. Livingston and Islam (1999) reported two isolates, a pseudomonas sp and a Bacillus sp, which were able to utilize kerosene, diesel, and waste oil. They compared the efficiency of bacterial biodegradation at different conditions.

Petroleum hydrocarbon-degrading fungi were isolated from Detarium senegalense seeds. An assessment of the relative ability of each fungus to degrade petroleum crude oil, diesel, unspent and spent engine oils, kerosene and Detarium senegalense oil extract, on minimal salt broth, was done measuring change in optical density read on a spectrophotometer. Five fungi were isolated from diseased namely: Aspergillus flavus, A.niger, Mucor, Rhizopus, and Talaromyces. The fungi isolated were used in the experiment and it was evident that all the fungi were capable of biodegrading the petroleum oil, though at different rates. Aspergillus niger had the highest ability to degrade unspent engine oil and Detarium senegalense oil extract while
Rhizopus had the highest ability to degrade kerosene and diesel, and Talaromyces had the highest ability to degrade spent engine oil (Adekunle and Adebambo, 2007)

Pseudomonas fragi and Achromobacter aerogenes isolated from used engine oil polluted soils 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. The two organisms utilized 73.3 and 80.0% of the oil with a degradation rate of 0.073 and 0.08 ml/day respectively. The utilization rate of the mixed culture did not differ significantly with an 80.0% utilization and 0.08 ml/day degradation rate. However the rate of utilization was reduced significantly after repeated sub culturing of the organisms on nutrient agar for six months with percentage utilization dropping to 33.3, 26.7 and 30.0% respectively for A. aerogenes, P. fragi and the mixed culture. This suggests that the presence of hydrocarbons in the growth medium is necessary for the stability of hydrocarbon utilization potentials of the isolates (Adelowo et al.,2006).

Mancera-López et al.,(2007 ) isolated thirty-seven hydrocarbon-degrading, but only six strains showed a high ability to degrade PAHs, AHs and TPH. These strains were identified as Pseudomonas pseudoalcaligenes, Bacillus firmus, Bacillus alvei, Penicillium funiculosum, Aspergillus sydowii and Rhizopus sp., and they removed 79%, 80%, 68%, 86%, 81% and 67% of TPH, respectively. P. pseudoalcaligenes and P. funiculosum removed 75% of PAHs, while B. firmus and P. funiculosum removed 90% and 92% of AHs, respectively. The highest TPH removal was observed by P. funiculosum, which was isolated from the soil with a high TPH concentration. A. sydowii was also isolated from this soil; no reports were found regarding its capacity to remove PAHs, but it was able to degrade five- and six-ring aromatic compounds.

An unidentified bacterial strain AL-12 from the culture of cyanobacteria was found as the most successful in degrading alkanes from n-C15 to n-C40 present in the fresh motor oil in a quantity of 98%. Small aliquots of motor oil were incubated aerobically for a period of up to 50 days, following the hydrocarbon content by GC/MSD analysis. Within 5 days of incubation up to 70% of n alkanes n-C15-n-C22, up to 45% of n-C22-n-C30 and up to 20% of n-C30-n-C40 were biodegraded. As expected, abiotic losses were smaller with increasing alkane chain length, but increased with incubation time. (Karmen Plohl and Hermina Leskovšek 2002).
Aitken et al., 1998 isolated Agrobacterium, Bacillus, Burkholderia, Pseudomonas, and Sphingomonas from contaminated soil samples by enrichment with phenanthrene as the sole carbon source. All the phenanthrene-degrading strains were examined for their ability to metabolize 12 other polycyclic aromatic hydrocarbons (PAH). Nahar and Quilty (1999) examined the effect of culture conditions such as solid and liquid on toluene degradation using Pseudomonas sp. Strains T0-1, T0-3, T0-5, Na-13 and Aeromonas sp. Isolated from activated sludge. Mycobacterium sp. strain CH1 was isolated from polycyclic aromatic hydrocarbon (PAH)-contaminated freshwater sediments and identified by analysis of 16S rDNA sequences. Strain CH1 was capable of mineralizing three- and four-ring PAHs including phenanthrene, pyrene, and fluoranthene. (Sharin et al., 1999). Widada et al., (2002) isolated and evaluated the diversity of 19 polycyclic aromatic hydrocarbon (PAH) degrading bacteria isolated from geographically diverse sampling sites in Kuwait, Indonesia, Thailand, and Japan.

Adenipekun and Oyinkansola (2008) reported that Pleurotus tuber-regium have the ability to increase nutrient contents in soils polluted with 1 - 40% engine-oil concentration after six months of incubation and reduction in heavy metals after six months of incubation. Hence, the fungus can be employed in decontaminating environment polluted with engine oil. An experiment by Adenipekun and Isikhuemhen (2008) revealed the ability of white rot fungus, L.squarrosulus to improve the nutrient contents of the engine oil contaminated soil and an accumulation of Fe, Zn and Ni to an appreciable extent. This could represent a process that could be exploited in remediation of engine oil contaminated soils.

Various screening and evaluation methods have been developed for isolating hydrocarbon-degrading bacteria. 2, 6-Dichlorophenol indophenol (2,6-DCPIP) is an oxidation–reduction indicator that detects the oxidation of NADH to NAD+, which is related to hydrocarbon-degradation by bacteria. The 2,6-DCPIP assay has been used for rapid and simple isolation of hydrocarbon-degrading bacteria (Hanson et al., 1993). This assay has also been used for estimating hydrocarbon- degrading bacteria in soil (VanHamme et al., 2000). Koma et al., (2001, 2003a, b, 2005) isolated three kinds of long chain hydrocarbon-degrading bacteria (Acinetobacter sp. ODDK71, Rhodococcus sp. NDKK48 and Gordonia sp. NDKY76A) for bioremediation of hydrocarbons. Strain
ODDK71 used long-chain n-alkanes as a sole carbon and energy source, but did not use c-alkanes (Koma et al., 2001). This strain did degrade c-alkanes under limited conditions, such as co-metabolism with n-alkanes (Koma et al., 2003a). On the other hand, strains NDKK48 and NDKY76A used n-alkanes and c-alkanes as sole carbon and energy sources (Koma et al., 2003b), but the metabolic pathways for c-alkanes differed between the two strains (Koma et al., 2003a, 2005).

A mixed bacterial flora was isolated from the soil of two petroleum-contaminated sites, then cultivated and domesticated in an open environment. The bacteria were used to degrade engine oil in wastewater. The optimum biodegradation conditions for all engine oil concentrations of respectively 489 mg L−1, 1 075 mg L−1 and 2 088 mg L−1 are bacterial inoculum concentration of 0.1%, temperature at 30 °C to 35 °C, pH 7.0 to 7.5, and rotation at 190 r min−1 to 240 r min−1. Engine oil concentration barely changes the growth rate of the bacterial consortium. The mixed bacterial flora has a high biodegrading capability for engine oil. (Lei et al., 2007)

Thirty-six bacteria that degraded long chain hydrocarbons were isolated from natural environments using long-chain hydrocarbons (waste car engine oil, base oil or the c-alkane fraction of base oil) as the sole carbon and energy source. A phylogenetic tree of the isolates constructed using their 16S rDNA sequences revealed that the isolates were divided into six genera plus one family (Acinetobacter, Rhodococcus, Gordonia, Pseudomonas, Ralstonia, Bacillus and Alcaligenaceae, respectively). Furthermore, most of the isolates (27 of 36) were classified into the genera Acinetobacter, Rhodococcus or Gordonia. The hydrocarbon-degradation similarity in each strain was confirmed by the 2,6-dichlorophenol indophenol (2,6-DCPIP) assay (Kubo et al., 2008).

Abioye et al., (2010) reported that three organic wastes (banana skin (BS), brewery spent grain (BSG), and spent mushroom compost (SMC)) were used for bioremediation of soil spiked with used engine oil to determine the potential of these organic wastes in enhancing biodegradation of used oil in soil. The rates of biodegradation of the oil were studied for a period of 84 days under laboratory conditions. Hydrocarbon-utilizing bacterial counts were high in all the organic waste-amended soil ranging between $10.2 \times 10^6$ and $80.5 \times 10^6$ CFU/g compared to
unamended control soil throughout the 84 days of study. Oil-contaminated soil amended with BSG showed the highest reduction in total petroleum hydrocarbon with net loss of 26.76% in 84 days compared to other treatments. First-order kinetic model revealed that BSG was the best of the three organic wastes used with biodegradation rate constant of 0.3163 day$^{-1}$ and half-life of 2.19 days. The results obtained demonstrated the potential of organic wastes for oil bioremediation in the order BSG>BS>SMC. The biodegradability of petroleum hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) and n-branched alkanes etc. of 2T engine oil were studied in aqueous media using bacterial strain Enterobacter isolated from petroleum contaminated soil of high altitude. It was capable of degrading 75 ± 3% of n-alkanes, 32 ± 5% PAHs, and the abiotic loss was 24 ± 6% during 10 days incubation period. 85 ± 2% of n-alkanes and 51 ± 3% PAHs were biodegraded in 20 days (Gupta et al., 2010).
2.1.3. MATERIALS AND METHODS

Characterization of the degradation potential

A single colony of the selected isolates (PDKT-1, PDKT-2, PDKT-5) were inoculated into 10ml nutrient broth at 30°C overnight. The overnight culture was centrifuged for 15 minutes at 3500 rpm. The cell pellet was washed twice and was resuspended with Bushnell Hass medium until OD$_{600}$ was equivalent to 1. One ml of bacterial inoculum (1 OD$_{600}$ equivalent) was transferred into 100 ml Bushnell Hass medium with 10ml used engine oil and was incubated at 30°C at 160rpm for four weeks. The loss of hydrocarbon was calculated by gravimetric analysis on 0, 1$^{st}$, 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ week respectively.

Determination of used engine oil degradation

The level of used engine oil degradation was determined using the gravimetric analysis. Conical flasks were pre-weighed in grams prior to gravimetric analysis (Chang, 1998). Gravimetric analysis of used engine oil degradation was performed according to Marquez – Rocha et al. 2001; Mandri and Lin (2007) the aqueous culture phase was dispensed into 500 ml Schott bottles and the organic hydrocarbon phase (engine oil), was extracted with 50 ml of dichloromethane, an organic solvent. The organic phase was then filtered through 10g of sodium sulfate, collected in the pre-weighed flasks and left overnight in the fume cupboard to enable evaporation of the organic solvent. Combined weight of the
pre – weighed flask and oil was recorded and the pre – weight subtracted to ascertain the weight of the oil remaining. This remaining oil was then subtracted from the control and multiplied by 100 to attain the percentage of oil degraded. An average was used for each duplicate.

**Viable count**

One ml of the broth was suspended in 9ml of 0.85% (w/v) saline and was vortexed vigourously for 1 min. Appropriate dilutions were prepared ($10^{-3}$ to $10^{-6}$) and plated in triplicate on nutrient agar medium. The plates were incubated at 37°C for 24-48 h. After incubation, the number of colonies were counted.(Collins and Lynes,1998)

\[
\text{CFU/ml} = \frac{(\text{Average number of colonies} / \text{plate}) \times (\text{Dilution plated}) \times (\text{Volume plated in millilitres})}{10^{3-6}}
\]

**Hydrocarbon Quantification**

The hydrocarbon was extracted from liquid cultures with dichloromethane. The extracts were then measured by direct injection in to a Gas chromatograph equipped with a flame ionization detector (FID) and a 25 m x 0.25 mm (diameter) fused silica capillary column, with an immobilized phase. The hydrogen flow rate was 30ml/min and the sample size was 5µl. The injection and the detector were maintained at 300° C, and the oven temperature was programmed to rise from 80° C to 240° C in 5C/min increments and to hold at 240° C for min . Individual compounds present were determined by matching the retention time with authentic standards (Sigma).The injection were done in triplicate.

**Optimization of the growth patterns of the isolates**

**Effect of pH on growth patterns of the isolates**

In order to determine the optimum pH required for the microbial growth on used engine oil, Bushnell and Hass medium was prepared and sterilized. The pH was adjusted to different level (5, 7 & 9) using 1N NaOH and 1N HCl. Filter sterilized 5%
used engine oil was added aseptically to the medium. The isolated bacterial cultures were inoculated. The flasks were incubated at 37°C for 7 days. After incubation, the growth patterns were obtained by measuring the optical density at 600nm.

**Effect of Temperature on growth patterns of the isolates**

In order to determine the favorable temperature required for microbial growth on used engine oil, Bushnell Hass medium was prepared and sterilized. The pH was adjusted to 7. After sterilization, 5% Filter sterilized used engine oil was added aseptically to the medium. The isolated bacterial cultures were inoculated. The flasks were incubated at different temperature (25°C, 30°C and 35°C) for 7 days. After incubation, the growth patterns were obtained by measuring the optical density at 600nm.

**Effect of Nitrogen sources on growth patterns of the isolates**

In order to determine various nitrogen sources required for microbial growth on used engine oil, Bushnell Hass medium was prepared and sterilized. The pH was adjusted to 7. After sterilization, 5% filter sterilized used engine oil was added aseptically to the medium. The isolated bacterial cultures were inoculated. The flasks were incubated at different nitrogen sources (Sodium Nitrate, Sodium Nitrite, Ammonium Nitrate and Urea) for 7 days. After incubation, the growth patterns were obtained by measuring the optical density at 600nm.

**Plasmid DNA profiling**

The plasmids were isolated from the potential bacterial cultures (PDKT-1, PDKT-2, PDKT-5) by modified alkaline lysis method (Brinboim and Dolly, 1979; Shrivastava, 1995).

The bacterial cells containing were grown over night in 5 ml LB broth with 100 µg/ml of ampicillin at 37 °C. The grown culture was pelleted at 7000-rpm for 5 min and the pellet was re-suspended in 200 µl GTE buffer. Next 300 µl of freshly prepared lysis solution (0.2M NaOH, 1 % SDS) (appendix) was added to the cell suspension and the suspension was mixed by inverting the tube several times and incubated at 4 °C for 5 min. The reaction was further neutralised by adding 300 µl of neutralising buffer (3M
Potassium acetate, pH-4.8) (appendix) and the reaction mix was mixed gently by inverting the tubes several times and incubated at 4 °C for 5 min. The mixture was centrifuged at 12000-rpm for 10 min and the supernatant was transferred to a new eppendorf tube. RNAse (DNAse free) was added to the final concentration of 20 mg/ml and the tube was incubated at 37 °C for 20 min. The sample was extracted twice with 400 µl of chloroform and centrifuged it at 5000-rpm for 1 min and the aqueous phase was then transferred to a new eppendorff tube. The DNA was precipitated with equal volume of 100 % iso-propanol and the tube was centrifuged for 10 min at room temperature. The DNA pellet was washed with 70 % ethanol and the DNA pellet was air-dried. This DNA pellet was then dissolved in 32 µl of de-ionized water and the plasmid DNA was precipitated with 8 µl of 4M NaCl and 40 µl of 13 % PEG 8000. The sample was mixed thoroughly and incubated in ice for 20 min. The DNA was pelleted by centrifugation for 15 min at 4°C in a fixed angle rotor. The supernatant was discarded and the DNA pellet was re-suspended in 450 µl of de-ionized water. The sample was extracted twice with chloroform as per the protocol mentioned above. The final aqueous layer was transferred to a clean tube, 40 µl of 3M sodium acetate (pH 5.5) and 1 ml 95 % ethanol was added. The reaction was mixed thoroughly and the samples were placed on ice for 20 min. The samples were spun at 12000-rpm for 25 min. The pellet was rinsed with cold 500 µl of 70 % ethanol. The plasmid DNA pellet was finally dissolved in 25 µl of nuclease free de ionised water. Plasmid DNA purification was done by using the Norgen Biotek Corporation Plasmid Mini preparation kit. (MW-lambda Hind III digest).

PCR was carried out to check the size of the plasmid using the following reaction. The reaction was assembled as follows:

- Taq polymerase buffer: 1×
- dNTPs: 0.2 mM
- T7 (forward primer): 0.8 µM
- SP6 (reverse primer): 0.8 µM
- Taq polymerase: 1 U

Amplification cycling conditions of the reaction were as follows:

- Initial denaturation: 94 °C for 1 min
This was followed by 35 cycles:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step I</td>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Step II</td>
<td>50°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Step III</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Final</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

The amplified products were resolved on a 2% agarose gel along with 1Kb ladder (lambda *Hind* III digest).

**Dioxygenase enzyme test**

Aromatic ring dioxygenase activity of the potential degraders (PDKT-1, PDKT-2, PDKT-5) was examined using indole because the formation of indigo from indole is presumptive for aromatic ring dioxygenases (Ensley *et al.*, 1983). The potential degraders were pre-grown on YEPG agar plates, and then indole crystals were placed in the in the lids of the petridishes. After one day of incubation at 20°C, colonies producing a blue color were scored positive.

**Yeast extract polypeptone glucose** (Sanseverino *et al.*, 1993)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.25g</td>
</tr>
<tr>
<td>Polypeptone</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.05g</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>0.05g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

**Estimation of hydrocarbon-degrading abilities** (Kubo *et al.*, 2008)

**2, 6-DCPIP** (2, 6-Dichlorophenol indophenol) assay
The selected isolates (PDKT-1, PDKT-2, PDKT-5) were pre-cultured in 5 ml of LB broth at 30°C and 160 rpm until the optical density at 660 nm became 1.0. After centrifugation at 4,000g for 5 min and washing with 0.9% saline, the cell density was adjusted to 1.0 according to the optical density at 660 nm. After sterilization, 750 µl of W medium (Fe-free), 50 µl of FeCl$_3$.6H$_2$O solution (150 µg/ml) and 50 µl of 2,6-DCPIP solution (37.5 µg/ml) were added to a 1.5ml microtube. Subsequently, 80 µl of cell suspension and 5 µl of sterilized hydrocarbons (Phenol, Benzene, Xylene, Aniline, Toluene, Benzyl chloride, Nitro benzene, Phenolpthalene, Anthracene, Napthalene) as substrates were added to the medium, and the cells were cultivated at 30°C and 100 rpm for 48 h. Subsequently, the color of the medium was observed and evaluated as positive for microbial hydrocarbon degrading ability if colorless (degraded) and negative for microbial hydrocarbon-degrading ability if blue (not degraded).

**W medium** (Koma et al., 2003 a)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate</td>
<td>2g</td>
</tr>
<tr>
<td>Disodium hydrogen orthophosphate</td>
<td>14.3204g</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>5.4436g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.2465g</td>
</tr>
<tr>
<td>Ferric sulphate</td>
<td>2.78mg</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>14.7mg</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>2.01mg</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>0.15mg</td>
</tr>
<tr>
<td>Cupric sulphate</td>
<td>0.2mg</td>
</tr>
<tr>
<td>Cobaltous chloride</td>
<td>0.4mg</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>1.49mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>
pH 7.0

Soil microcosms studies

Soil samples

The soil used for this study was obtained from a site free of petroleum hydrocarbon contamination from Pudukkottai (near the study site). The soil sample was collected in pre-cleaned glass jars, air dried (approx.1 week), powdered and sieved through 2mm mesh sieve and then subjected to steam sterilization (1 h at 120°C), repeated three times after a 24 h interval), except one aliquot that was separated to preserve the indigenous soil microflora. The axenic condition of the sterilized soil was tested by incubating the soil particles in nutrient agar (3days at room temperature). None of incubated petri dish showed signs of microbial development.

Soil microcosms (Colombo et al., 1996)

The biodegradation tests were carried out in 500ml vials containing 150g of soil, which was added under laminar air flow chamber and was artificially contaminated with filter, sterilized 10% used engine oil. Several treatment systems were tested as given below.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sterilized soil Control</td>
</tr>
<tr>
<td>2</td>
<td>Sterilized soil Control + consortium</td>
</tr>
<tr>
<td>3</td>
<td>Unsterilized soil control</td>
</tr>
<tr>
<td>4</td>
<td>Unsterilized soil control + consortium</td>
</tr>
</tbody>
</table>

The bacterial consortium was prepared by inoculating the nutrient broth by a loopful of *Serratia marcescens* PDKT-1, *Pseudomonas aeruginosa* PDKT-2 and *Bacillus licheniformis* PDKT-5, having high utilizing potential of used engine oil.
hydrocarbons. The nutrient broth was incubated at 37°C and OD at 660 nm by UV-VIS spectrophotometer was analyzed every day. After obtaining the required absorbancy of 1 at 660 nm, 1 ml from the broth was taken out with a micropipette and centrifuged at 5,000 xg for 5 min in a microfuge (RM10, Remi). The supernatant was discarded and the pellet was used as bacterial consortium inoculums (Anjana and Meenal, 2009). Consortium culture was added to microcosm in the treatment system 1 and 3 (which contained sterile soil and indigenous soil microflora). The initial viable number of cells was measured by the pour plate method both in sterile soil and in unsterilized soil. Viable cell counts were performed at 0, 15 and 30 days by the pour plate method using nutrient agar. The number of colonies was counted after 24h of incubation at 30°C. The cellular quantification was performed in duplicate.

**Hydrocarbon estimation**

The hydrocarbon content in soil microcosm samples was estimated by gravimetric and Gas chromatographic (GC-FID) method. Ten grams of each soil sample were removed in duplicate from the soil microcosms and mixed with an equal mass of anhydrous sodium sulphate. The mixture was placed in a Whatman cellulose extraction thimble. The Soxhlet apparatus was used to extract the remaining engine oil present in the soil samples, using 200 ml of dichloromethane (DCM) for two hours at a rate of four cycles per hour (Helaleh *et al.*, 2001). The DCM fraction was collected in a pre-weighed 250 ml round bottomed flask and rotary evaporated at 40° C. The percentage of degradation was calculated by the following expression:

\[
\frac{[(\text{TPH control} - \text{TPH treatment})/ \text{TPH control}] \times 100.}
\]

Total viable cell count and TPH were performed at 15th and 30th days. TVC was performed by spread plate method using nutrient agar. The number of colonies was counted after 24h of incubation at 30° C. The cellular quantification was performed in duplicate.
2.1.4. RESULTS

The total petroleum hydrocarbon (TPH) degradation efficiency of the isolated potential bacterial strains and consortium culture (PDKT-1, PDKT-2, PDKT-5) was estimated gravimetrically and by gas chromatography. After four weeks of incubation visual observations of biodegradation of used engine oil have been noticed (Fig. 33). The bacterial strains isolated from five different sites could degrade TPH in the range of 60 % to 90 %. Most of the isolates degraded the aliphatic fractions of TPH more than that of aromatic fractions. This could be because the aliphatic fractions are less toxic and are easily degradable fractions of TPH. The least degradation (60%) was obtained by the strain PDKT-5 and highest degradation (81%) was observed by the strain PDKT-2. The strain PDKT-1 showed 72% of TPH degradation. The maximum degradation of 90% TPH in the range of C14 to C 22 was showed by consortium culture. The gas chromatograms depicting the degradation of the different aliphatic and aromatic fractions of TPH by the individual bacterial strains (PDKT-1, PDKT-2, PDKT-5) and their consortium culture areshown in the Figure. 34. The degradation of the different aliphatic and aromatic fractions of TPH by the bacterial strains has been compared to the controls. The controls depict the different fractions of aliphatic and aromatic fractions of TPH.

All the three strains showed maximum growth at pH 7 (Fig. 35) and above pH 7 the growth was declined. Regarding temperature maximum growth of the strain PDKT-1 was observed at 30°C, while the strain PDKT-2 and PDKT-5 preferred 35°C for their growth.(Fig. 36). Different nutrients sources and environmental conditions such as pH and temperature were substituted in the growth pattern assay. The results showed that, *Serratia marcesens* PDKT-1, isolated in this study utilized ammonium nitrate in engine oil degradation metabolic mechanism. The addition of urea or nitrite reduced the cell growth. *P. aeruginosa* PDKT-2 and *Bacillus licheniformis* PDKT-5 on another hand were capable of utilizing different nitrogen sources including nitrite (Fig. 37).
The presence of plasmid DNA was investigated in the isolates PDKT-1, PDKT-2, PDKT-5. The extracted plasmids DNA were resolved 1% agarose gel using TBE buffer. Plasmid DNA profile indicated that the isolates PDKT-1, PDKT-2, PDKT-5 harboured plasmid DNA, which was confirmed by agarose gel electrophoresis. The size of the extracted plasmid from PDKT-1, PDKT-2, PDKT-5 were found to be > 4361 bp (Fig. 38).

All the three isolates (PDKT-1, PDKT-2, and PDKT-5) scored positive results for dioxygenase activity. The indole-conversion to indigo is considered as dioxygenase activity (Fig. 39). Regarding aromatic and polycyclic hydrocarbon-degradation, evaluated by 2,6 DCPIP assay, xylene and nitrobenzene was degraded by all the three strains, where as no isolates degraded benzene, toluene and benzyl chloride. Aniline was degraded by both PDKT-1 and PDKT-2. The strains PDKT-1 and PDKT-5 showed positive results for phenol. Phenolphthalene was degraded only by the strain PDKT-2. For polycyclic hydrocarbons, anthracene was degraded by PDKT-2. Napthalene was not degraded by any of the three isolates (Fig. 40).

Since the consortium culture showed promising result in the preliminary degradation experiments, a study was carried out to assess the capability of the consortium culture to degrade hydrocarbon in soil system and to assess its survivability. For this the pure consortium culture was added to sterile soil taken in conical flasks and made artificial contamination with 10% used engine oil. An uninoculated flask was maintained as control. In another flask un-autoclaved soil with 10% used engine oil was also maintained to check the degradability of the indigenous microflora. After incubation, samples were withdrawn after different days (15, 30) and the residual TPH and TVC was determined. The biodegradation of petroleum oil was also confirmed by GC- FID profile. The result of this study is depicted in Figure.41. It was found that degradation increased with the incubation period and was maximum after 30 days of incubation where nearly 91% of the hydrocarbon was degraded. At the same time the degradation with the indigenous flora was 32% and for indigenous flora and consortium culture the value was 78.5%. The TVC recorded in the different setups is given in Table.7. Maximum number of cells (0.04x10⁶ CFU/ml) (in setup 2: consortium culture alone) was observed in samples collected after 30 days of incubation and there after a
slight decline was observed. The number of cells observed in setup-4 (consortium culture +indigenous flora) at the same period was $0.03 \times 10^6$ CFU/ml.

2.1.5. DISCUSSION

In this study twenty five bacterial strains, capable of utilizing used engine oil as a carbon source were isolated from the contaminated soils. Three isolates with best oil degradation ability were identified as *Serratia marcescens* PDKT-1 (Accession No: HM 998315), *Pseudomonas aeruginosa* PDKT-2 and *Bacillus licheniformis* PDKT-5 using the biochemical tests and confirmed by 16S rRNA sequencing. Figure 7 shows the percentage of used engine oil remaining after degradation by different isolates over a period of four weeks. The result indicates that all three isolates are capable of utilizing engine oil as the nutrient source. The strain *Pseudomonas aeruginosa* PDKT-2 was found to be the best oil degrading isolate in this study with 81% degradation at 30 days of incubation period while 72% and 60% degradation were observed using the strain *Serratia marcescens* PDKT-1 and *Bacillus licheniformis* PDKT-5 respectively, under the standard assay conditions. All three isolates utilized engine oil as a sole carbon source with higher degradation rate. The peak reduction in gas chromatogram of both individual isolates and consortium culture was a strong reason for degradation and
indicates a significant reduction in aliphatic and aromatic compounds under same condition. The most significant reduction is related to the sample seeded by the consortium culture (Fig. 34) which could degrade 90% of aliphatic compounds during 30 days. It degraded long chains of hydrocarbons into smaller chain of hydrocarbon.

Aliphatic compounds are degraded as linear aliphatic compounds > branched aliphatic compounds > cyclic aliphatic compounds, respectively (Sorkhoh et al., 1995). Mancera-López et al., (2007) isolated thirty-seven hydrocarbon-degrading isolates, but only six strains showed a high ability to degrade PAHs, AHs and TPH. These strains were identified as *Pseudomonas pseudoalcaligenes*, *Bacillus firmus*, *Bacillus alvei*, *Penicillium funiculosum*, *Aspergillus sydowii* and *Rhizopus sp.*, and they removed 79%, 80%, 68%, 86%, 81% and 67% of TPH, respectively. In the present work, more reduction of the hydrocarbons was seen with *Pseudomonas aeruginosa* PDKT-2 (Fig. 34) among 3 pure cultures that were investigated. It is obvious that consortium cultures are more effective in the degradative process. Also, the biodegradation of short- and middle-chain aliphatic compounds were more extensive, compared to the long chain hydrocarbons (Fig. 34). Recently the reduction of engine oil hydrocarbons by *Pseudomonas* sp was reported by Mandri and Lin (2007) and Shojaosadati et al., (2008). Nevertheless, isolation and apparently a good growth on hydrocarbon substrates as surprising for *Serratia marcescens*. This bacteria has been not previously considered to be a strong hydrocarbon degrader. It has showed very good crude oil and gasoline degradability (Balows et al., 1992 and Ijah, 1998). Akoachere et al., (2008) reported that *S. marcescens* degraded the highest amount of lubricating oil while *Bacillus mycoides* degraded the least amount and the mixed culture, however, degraded even higher amounts of lubricating oil. There was an increase in cell number of *B. stearothemophilus* during the degradation process demonstrating the ability of utilizing engine oil as energy source for this organism (Udeani et al., 2009). Although *Bacillus* spp. have been frequently isolated from hydrocarbon-polluted sites, as stated above, members of this genus have never been shown unequivocally to directly degrade hydrocarbons. In fact, *Bacillus* spp. are known to act as secondary degraders, i.e., they assimilate the metabolites produced by true primary-hydrocarbon degraders (Challian et al., 2004). The above findings were in accordance with present study results.
In this study the consortia proved (*Serratia marcescens* PDKT-1, *Pseudomonas aeruginosa* PDKT-2, *Bacillus licheniformis* PDKT-5) to be better degrader compared to individual isolate with degradation rates of 90% in 4 weeks. Shojaosadati et al., 2008 reported that during 60 days of experiments, 70% of the engine oil was degraded by the mixed culture. The best degradation was observed by a consortium of three isolates (*Flavobacterium* spp, *A. calcoaceticum*, and *P. aeruginosa*) with a degradation of 90%. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been demonstrated (Alden et al., 2001). A sequential change of the composition of the oil degrading bacteria over a period of time in oil contaminated soil samples (Sorkhoh et al., 1995; Christopher and Christopher, 2004). Komukai-Nakamura (1996) reported the sequential degradation of Arabian light crude oil by *Acinetobacter* sp T4 and *Pseudomonas putida* PB4. *Acinetobacter* sp T4 degraded alkane and other hydrocarbons in the crude oil and produced the accumulation of metabolites that were subsequently degraded by *P. putida* PB4. The use of pure cultures in the study of microbial degradation of fuels provides technical advantages by eliminating the ambiguity associated with constantly fluctuating populations (Reisfeld et al., 1972). However, individual organisms often prefer to metabolize a limited range of hydrocarbon substrates (Marin et al., 1996). Consequently, a mixed population of fungi and bacteria is usually required to provide all the metabolic capabilities for complete degradation of complex mixtures of hydrocarbons (Leahy and Colwell, 1990). Several reports (Obire, 1988; Amund and Nwakaye, 1993; Amund et al., 1993; Facundo et al., 2001; Kulwadee et al., 2001) have confirmed microbial consortia as better degraders than the individual isolates. In a mixed culture, some species utilise intermediates of degradation of the original hydrocarbon produced by other members of the culture leading to a complete degradation of the oil. Thus, a mixed culture is a better inoculum for oil spill clean-up. No single microorganism has been found to be able to completely degrade a petroleum hydrocarbon molecule. However, different species or strains of the same species may be capable of degrading different groups of hydrocarbons, found in oil (Atlas, 1981; Marquez et al., 2001).

The influence of environmental factors rather than genetic capability of a microorganism have been reported to limit the degradation of pollutants (Barther and Atlas, 1977; Jackson and Jackson, 2000). Different nutrients sources and environmental
conditions such as pH and temperature were substituted in the growth pattern assay (Fig. 35 & 36). Various factors may limit the rate of petroleum hydrocarbon degradation including lack of essential nutrients such as nitrogen. Therefore, the addition of inorganic or organic nitrogen-rich nutrients (biostimulation) is an effective approach to enhance the bioremediation process (Hollender et al. 2003; Semple et al. 2006; Walworth et al., 2007). Positive effects of nitrogen amendment on microbial activity and/or petroleum hydrocarbon degradation have been widely demonstrated (Jorgensen et al. 2000; Margesin et al., 2000, 2007; Brook et al., 2001; Margesin and Schinner, 2001; Riffaldi et al., 2006). The results showed that, *Serratia marcesens* isolated in this study utilized NH3NO4 in engine oil degradation metabolic mechanism while the addition of urea or nitrite reduced its cell growth. The strains *P. aeruginosa* and *Bacillus licheniformis* on another hand were capable of utilizing different nitrogen sources including nitrite (Fig. 37).

pH is an important factor which influences the microbiological metabolic activity and growth of microorganism. Different microorganisms can grow over a wide pH range and every organism has its own tolerance level. Generally fat, oil and grease degrading organisms have optimum growth between pH 5.5 and 8.0 with maximization at 7.5 (Lefebvre et al., 1998). From the observed results the isolate actively uptake the hydrocarbons at neutral condition (pH 7), and it was coincide with the above finding.

Climate and season would be expected to select different populations of hydrocarbon-utilizing microorganisms which are adapted to ambient temperatures. Hydrocarbon degradation can occur over a wide range of temperature and psychrotrophic, mesophilic and thermophilic hydrocarbonistics microorganisms have been isolated. Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Atlas, 1981; Margesin and Schinner, 2001). Higher temperatures increase the rates of hydrocarbon metabolism to the maximum, typically in the range of 30 to 40°C, above which the membrane toxicity of hydrocarbons is increased (Bossert and Bartha, 1984). The results obtained by the present study correlates with above finding as the isolate *P. aeruginosa* and *A. calcoaceticum* sp. increased as the temperature increased from 25 to 37°C while
*Flavobacterium* spp, showed a better degradation rate at 30°C. Mandri and Lin (2007) reported that an increase in cell number during the degradation process demonstrating the ability of utilizing engine oil as energy source for this organism supports the present study results.

Plasmids carrying genes influencing degradation of hydrocarbons abound in bacterial populations. Because bacteria are widely distributed in chronically polluted environments, plasmid carriage may provide genetic control for adherence to, or emulsification of, hydrocarbons, ultimately resulting in removal via biodegradative pathway(s). The genes that are responsible for the degradation of hydrocarbons are frequently located on plasmid DNA (Leahy and Colwell, 1999). Plasmid mediation of key steps in the degradation of hydrocarbon molecules is well established (Chakrabarty, 1980). In general, degradation requires an interaction between chromosomal and plasmid genes (Fennewald *et al.*, 1978). It has been suggested that phenanthrene degradation may be under plasmid control in *Alcaligenes faecalis* (Kiyohara *et al.*, 1982). Hada and Sizemore (1981) found that plasmid incidence was greater in *Vibrio* spp. isolated from an oil field in the north western Gulf of Mexico than the isolates from pristine control sites. Catabolic pathways which encode numerous aromatic hydrocarbon degradation pathways are frequently located on plasmids, although the pathways for some xenobiotic compounds such as chlorinated aromatic hydrocarbons can be located on either chromosome or plasmid. In the case of PAHs, it is also supposed that the genes that are responsible for the degradation of PAHs can be located on the plasmid (Cho and Kim, 2001). From the above findings, it is suggested that the presence of plasmid in all the three potential isolates (PDKT-1, PDKT-2, PDKT-5) could be responsible for the degradation of total petroleum hydrocarbon.

The microorganisms capable of surviving in such a polluted environment are those that develop specific enzymatic and physiological responses that allow them to use the hydrocarbon compounds as substrates (Atlas *et al.*, 1991). This response of microorganisms to organic contaminants has been studied for many years. It has been found that individual microorganisms can mineralize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with broad enzymatic capacities are required to increase the rate and extent of petroleum biodegradation. Microbial
degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods (Ijah and Okang, 1993). This is possible because microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy (Ijah and Antai, 1988; Ezeji et al., 2005; Antai and Mgbomo, 1993). For example, *Pseudomonas putida* was found by Britton (1988) to produce enzyme mono-oxygenase linked to electron carrier rubredoxin during hydroxylation of n-alkane to produce n-alkane-1-ol. A species of *Pseudomonas fluorescens* capable of producing dehydrogenase enzyme that attacks aromatic naphthalene to produce catechol had been reported by Mark (1990). Weissenfels et al., (1990) isolated pure cultures of *Pseudomonas paucimobilis* and *Pseudomonas vesicularis* capable of degrading polyaromatic hydrocarbons.

Ahn et al.,(1999) reported that the dioxygenase test suggested a strong relationship between PAH-degrading bacteria and their dioxygenase activity: 98% (87/89) of the screened strains showed dioxygenase activity. The majority of bacteria may initiate degradation via dioxygenases. Although toluene -4- monooxygenase was also reported to produce indigo from indole (Yen et al., 1991), indole-conversion to indigo is considered representative of dioxygenase activity (Ensley et al., 1983). The indole conversion was shown by aromatic ring dioxygenases from not only PAH degraders (Ensley et al. 1983; Goyal & Zylstra 1996) but also monocyclic aromatic hydrocarbon degraders (Ensley et al. 1983; Eaton & Timmis, 1986; Wubbolts et al. 1994). In this study the dioxygenase enzyme activity observed in the isolated potential hydrocarbon degraders (PDKT-1, PDKT-2, PDKT-5) correlates the above findings.

The 2, 6-DCPIP assay has been applied to the enumeration of hydrocarbon-degrading microorganisms in mixed cultures (Van Hamme et al., 2000). This assay has also been applied to the selection of autochthonous microorganisms that can degrade hydrocarbon contaminants in contaminated soils, since these microorganisms would contribute to the cleaning process for hydrocarbon-contaminated soils. Exhaustive analysis of hydrocarbon-degradation can be evaluated by measuring the growth of each strain using many types of hydrocarbons, however growth rate and cell size etc. influence the optical density. The 2,6-DCPIP assay can sensitively detect the primary oxidation of hydrocarbon, therefore the assay is suitable for exhaustive investigation of
hydrocarbon degradation of each isolate. In present study hydrocarbon-degradation by the isolated bacteria was analyzed by the 2, 6- DCPIP assay. The isolated strains showed variation in the degradation of selected aromatic and polycyclic hydrocarbons. In similar studies Kubota et al., (2008) reported that the polycyclic hydrocarbons such as decalin, naphthalene and anthracene were degraded by several strains belonging to 5 genera but tetralin was difficult to degrade. Strains of the genus Rhodococcus degraded more kinds of polycyclic hydrocarbons compared to the other genera, since decalin, naphthalene and anthracene were degraded by various strains of the genus Rhodococcus, respectively. Regarding aromatic hydrocarbon degradation, dodecylbenzene (length of alkyl side-chain: 12) was degraded by many strains throughout all the investigated genera, whereas no strains in the genera degraded benzene, almost supporting the present study.

At present, various microbial genera have been detected in petroleum-contaminated soil or water, which strongly suggests that each has a role in the hydrocarbon transformation process. (Farinazleen et al.,2004). It has been reported that adapted communities previously exposed to hydrocarbons exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination (Leahy and cohwell, 1990). Organic compounds of low molecular weight and simple molecular structure are preferred by many microorganisms (Atlas and Cerniglia, 1995). Compounds with more complex structures, such as polycyclic aromatic hydrocarbons (PAHs), with more than five benzene rings, are more resistant to microbial breakdown (Cerniglia et al.,1985 and Bossert et al.,1986). The extent of hydrocarbon biodegradation in contaminated soils is critically dependent upon four factors, namely the creation of optimal environmental conditions to stimulate biodegradative activity, the predominant petroleum hydrocarbon types in the contaminated matrix and the bioavailavility of the contaminants to microorganisms. The petroleum hydrocarbon degradation is also affected by the molecular composition of the hydrocarbons, a character which is directly related with the bioavailability of such compounds, and as a consequence, the biodegradation rate may be altered (Huesemann, 1995). In the present investigation, the low degradation of the indigenous microflora indicate that these un-adapted microorganisms were unable to grow in the hydrocarbon contaminated site, where as the consortium culture which were isolated from a chronic hydrocarbon polluted site was better adapted and was able to
degrade the used engine oil. It was interesting to note that when consortium culture was added along with the indigenous flora, its degradation ability was not very much compensated. The reduction in degradation ability in this setup might be due to the result of competition with the indigenous flora. Colombo et al., (1996) reported that the biodegradation of aliphatic and aromatic hydrocarbons by natural soil micro flora and pure cultures of some isolated fungal species was highest with the pure cultures than with the indigenous flora. They also observed a reduction in degradation ability when the isolates were used along with the indigenous flora. The result of the present study also confirms the above observation.

Bioremediation is being used or proposed as a treatment option at many hydrocarbon- contaminated sites (Braddock et al., 1997). The effectiveness of bioremediation is often a function of the microbial population or consortium and how it can be enriched and maintained in an environment. The successful use of microbial inocula in soils requires that the microorganisms contact the contaminant (Margesin and Schinner, 1997). When few or no indigenous degradative microorganisms exist in a contaminated area or when there is no time for the natural enrichment of a suitable population, inoculation (bioaugmentation) can be a realistic option. Inoculation of bacteria with hydrocarbon biodegradation capabilities shorten the time of the treatment. The same ecological principles that influence biodegradation with the native microorganism population, in general, will also govern the effectiveness of the inoculation, regardless of whether they are natural isolates or genetically engineered microorganisms (Liu and Suflita, 1993).

The parameters typically measured in laboratory tests of bioremediation efficacy include enumeration of microbial populations (Kastner et al., 1994, Rice et al., 1997 and Peressutti et al.,2003) and determination or fate of hydrocarbon degradation (disappearance of individual hydrocarbons and/or total hydrocarbons) (Okoro, 2008). The most direct measure of bioremediation efficacy is the monitoring of hydrocarbon disappearing rates (Song et al., 1990). In the present microcosm studies, the reduction of TVC in set up 4 (consortium culture +indigenous flora) might have occurred due to the competition that occurred between the indigenous flora and consortium culture. This might also explain the reduction in degradation in dual inoculation (consortium culture
+ indigenous flora). The GC- FID chromatogram obtained from the soil sample supplemented with used engine oil showed varied peaks. All were significantly reduced, when compared with the control. Smaller peaks of hydrocarbon compound indicated that the bacterial consortium had more effectively degraded the used engine oil in soil system. Similar studies have been performed by Shojaosadati et al., (2003); Anjana and Meenal (2009) and Satheeskumar et al., (2009). Previously Harder (2004) estimated that bioremediation accounts for 5 to 10 percent of all pollution treatment and has been used successfully in cleaning up the illegal dumping of used engine oil. These results showed that each member in a microbial community has a significant role and may be dependent on the presence of the other species or strains for surviving. Furthermore, this study has demonstrated a very good biodegradation capability of engine oil hydrocarbons by bacterial consortium.

2.2.1. INTRODUCTION
Hydrophobic pollutants present in petroleum hydrocarbons, and soil and water environment which require solubilization before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Surfactants are amphiphilic compounds containing both hydrophobic (non-polar) and hydrophilic (polar) moieties that confer ability to accumulate between fluid phases such as oil/water or air/water, reducing the surface and interfacial tensions and forming emulsions (Desai and Banat, 1997). Surfactants can increase the surface area of hydrophobic materials, such as hydrocarbons in soil and water environment, thereby increasing their water solubility. Hence, the presence of surfactants may increase microbial degradation of pollutants. Biosurfactant is a structurally diverse group of surface-active molecule synthesized by microorganisms. Their capability of reducing surface and interfacial tension with low toxicity and high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants. (Cameotra and Makkar, 1998; Deleu and Paquot, 2004). They occur in nature as a diverse group of molecules comprising of glycolipids, lipoprotein and lipopeptides, fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants (Desai and Banat, 1997). Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long chain fatty acids or fatty acid derivatives whereas the hydrophilic portion can be a carbohydrate, aminoacid, phosphate or cyclic peptide (Rosenberg and Ron, 1999). They are mainly produced by hydrocarbon utilizing microorganisms exhibiting surface activity. These molecules reduce surface tension and interfacial tension in both aqueous solution and hydrocarbon mixtures. These properties create micro emulsion leading to micelle formation in which hydrocarbon can be solubilized in water or hydrocarbon in water.

The surface active properties make surfactants one of the most important and versatile class of chemical products, used on a variety of applications in household, industry and agriculture (Deleu and Paquot, 2004). Microbial surfactants are categorized by their chemical composition and microbial origin. It was suggested that biosurfactants can be divided into low molecular mass molecules, which efficiently lower surface and interfacial tension, and high molecular mass polymers, which are more effective as emulsion stabilizing agents (Banat, 1995; Rosenberg and Ron, 1999; Karanth et al., 1999; Youssef et al., 2005). The major classes of low mass surfactants include
glycolipids, lipopeptides and phospholipids, whereas high mass includes polymeric and particulate surfactants.

At present, biosurfactant plays an important application in petroleum-related industries which is use in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker clean up, viscosity control, oil emulsification and removal of crude oil from sludge’s. This is due to the ability of biosurfactant producing microorganisms to use petroleum or its’ products as substrates as well as the properties of the biosurfactant which required less rigorous testing than chemical surfactant. To date, there are numbers of reports on the synthesis of various types of biosurfactant by microorganisms using water-soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates. Petroleum-related industry was found to be one of the industries that have a great potential in producing a microorganism that may produced biosurfactant. Hence, there could probably be a potential chance of producing biosurfactant using locally isolated bacteria originated from petrochemical wastes or other wastewater available in this country. It has been focused here that improving the method of biosurfactant production and characterizing the major properties of the biosurfactant are highly important in the commercial application of biosurfactant (Muthusamy et al., 2008).

Initial focus of industrial interest towards biosurfactant concentrates on the microbial production of surfactants, co surfactants and so on for the application on microbial-enhanced oil recovery (MEOR). The applications of biosurfactants however, remain at the developmental stage in industrial level. The development of biosurfactant application in industries has focused mainly on high biosurfactant production yield and the production of highly active biosurfactant with specific properties for specific applications. The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as environmental protection. The uniqueness with unusual structural diversity, the possibility of cost-effective \textit{ex-situ} production and their bio degradability are some of the properties that make biosurfactant a promising choice for use in environmental application (Hua et al., 2003). In recent years there has been a growing interest in the isolation and identification of new microbial surfactants that might have application in enhanced oil recovery processes. The possibility of
discovering a unique bio-emulsifier like emulsan that possesses novel properties allowing its use as a gelling agent, emulsifier, stabilizer, flocculants, lubricant or dispersing agent has encouraged this interest. Biosurfactants are powerful natural emulsifiers capable of reducing the surface tension of water from roughly 76 m N/m to 25-30 m N/m. Biosurfactants are of interest because of their broad range of potential industrial applications, including emulsification, phase separation, wetting, foaming, emulsion stabilization, viscosity reduction of heavy crude oils.

Research in the area of biosurfactants has expanded quite a lot in recent years due to its potential use in different areas, such as the food industry, agriculture, pharmaceutics, oil industry, petrochemical, paper and pulp industries. The development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment. Therefore, the most significant advantage of a microbial surfactant over chemical surfactants is its ecological acceptance because it is biodegradable and nontoxic to natural environments (Karsa et al., 1999; Banat, 2000; Meylheuc et al., 2001). They are potential alternatives of chemically synthesized surfactant in a variety of application because of their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Desai and Banat, 1997; Mukherjee et al., 2006). In recent years, the biosurfactants have been placed on the environmental impacts of chemical surfactants and new surfactants for use in any field. Some of their superior qualities, such as absence of toxicity, biodegrading ability, and their specificity, make these microbial products both attractive for specific industries and environmentally acceptable. Most of the emphasis to date has been on the application of biosurfactants in petroleum-related activities and industries. They offer attractive products for use in enhanced oil recovery, in cleaning oil spills, in oil emulsification, and in breaking industrially derived oil-in-oil emulsions. Their \textit{in situ} and \textit{ex situ} utilization in enhanced oil recovery represent attractive alternatives.

The interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection (Banat et al., 2000 and Rahman et al., 2002).
Biosurfactants enhance the emulsification of hydrocarbons, have the potential to solubilise hydrocarbon contaminants and increase their availability for microbial degradation. The use of chemicals for the treatment of a hydrocarbon polluted site may contaminate the environment with their by-products, whereas biological treatment may efficiently destroy pollutants, while being biodegradable themselves. Hence, biosurfactant producing microorganisms may play an important role in the accelerated bioremediation of hydrocarbon contaminated sites (Rosenberg and Ron, 1999; Del Arco and De Franca, 2001 and Rahman et al., 2002). These compounds can also be used in enhanced oil recovery and may be considered for other potential applications in environmental protection Rahman et al., 2002a and Shulga, 1999. Other applications include herbicides and pesticides formulations, detergents, health care and cosmetics, pulp and paper, coal, textiles, ceramic processing and food industries, uranium ore-processing and mechanical dewatering of peat (Banat et al., 2000; Rahman et al., 2002b and Ron and Rosenberg, 2001).

Successful commercialization of every biotechnological product depends largely on its bioprocess economics. At present, the prices of microbial surfactants are not competitive with those of the chemical surfactants due to their high production costs and low yields. Hence, they have not been commercialized extensively. For the production of commercially viable biosurfactants, process optimization at the biological and engineering level needs to be improved. Improvement in the production technology of biosurfactants has already enabled a 10–20-fold increase in productivity, although further significant improvements are required. However, the use of cheaper substrates and optimal growth and production conditions coupled with novel and efficient multi-step downstream processing methods and the use of recombinant and mutant hyper producing microbial strains can make biosurfactant production economically feasible.

Novel recombinant varieties of these microorganisms, which can grow on a wide range of cheap substrates and produce biosurfactants at high yields, can potentially bring the required breakthrough in the biosurfactant production process. Although a large number of biosurfactant producers have been reported in the literature, biosurfactant re-search, particularly related to production enhancement and economics has been confined mostly to a few genera of microorganisms such as Bacillus,
*Pseudomonas* and *Candida*. As documented biosurfactants are not only useful as antibacterial, antifungal and antiviral agents, they also have the potential for use as major immune modulator molecules, adhesive agents and even in vaccines and gene therapy. A judicial and effective combination of these strategies might, in the future, lead the way towards large-scale profitable production of biosurfactants. This will make biosurfactants highly sought after bio molecules for present and future applications as fine speciality chemicals, biological control agents, and new generation molecules for pharmaceutical, cosmetic and health care industries.

The present study focused on the production of biosurfactant by selected bacterial strains isolated from used engine oil contaminated soil samples. It also includes the optimization of biosurfactant production in terms of productivity and the yield of biosurfactants and further characterization to determine the nature of the biosurfactant produced.
2.2.2. REVIEW OF LITERATURE

Production of rhamnose containing glycolipids was first described in *Pseudomonas aeruginosa* by (Jarvis and Johnson, 1949). They are powerful natural emulsifiers capable of reducing the surface tension of water from roughly 76 mN/m to 25 to 30 mN/m. (Guerra et al., 1984). The rhamnolipids excreted by *Pseudomonas sp.* DSM 2874 consists of one or two L (+) rhamnose units linked to one or two β-hydroxy-decanoic acid compounds (Syldatk et al., 1985). Their composition and yield are dependent on culture condition. These extracellular substances are useful biosurfactant.

Lipopeptides and lipoproteins are a large number of cyclic lipopeptides, including decapeptide antibiotics (gramicidin) and lipopeptide antibiotics (polymyxins). These consist of a lipid attached to a polypeptide chain. Lipopeptides called surfactin are produced by *Bacillus subtilis* ATCC 21332 is one of the most powerful biosurfactant. It is composed of seven amino-acid ring structure coupled to a fatty acids chain via lactone linkage. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.005%, making surfactin one of the most powerful biosurfactants (Kakinuma et al., 1969). Trehalolipids are the group of glycolipids, serpentine group are seen in many members of the genus *mycobacterium* due to the presence of trehalose esters on the cell surface (Asselineau and Asselineau, 1978).

Phospholipids are known to form major components of microbial membrane. When certain hydrocarbon-degrading bacteria or yeast are grown on alkane substrates, the level of phospholipid increases greatly (e.g.) hexadecane-grown *Acinetobacter sp.* (Kaeppeli and Finnerty, 1979). Phospholipids have been quantitatively produced from *Thiobacillus thiooxidans* that are responsible for wetting elemental sulphur necessary for growth. Polymeric biosurfactant are emulsion, liposan, alasan, lipomanan and other polysaccharide-protein complexes. *Acinetobacter calcoaceticus*RAG-1 produces an
extracellular potent poly anionic amphipathic hetero polysaccharide bio emulsifier (Rosenberg et al., 1979)

Lichenysins are produced by *Bacillus licheniformis*, which act synergistically and exhibit excellent temperature, pH and salt stability. These are also similar in structural and physio-chemical properties with other surfactins. The surfactants produced by *B. licheniformis* are capable of lowering the surface tension of water to 27m N/m and the interfacial tension between water and *n* hexadecane to 0.36m N/m. Fatty acids produced from alkanes as a result of microbial oxidation have been considered as surfactant. In addition to the straight-chain acids, microorganisms produce complex fatty acids containing OH group and alkyl branches (Rehn and Reiff, 1981).

Sophorolipids from *T. bombicola* have been shown to reduce surface and interfacial tension, but are not good emulsifiers (Cooper and Paddock, 1984). Sophorolipids are produced mainly by yeast such as *Torulopsis bombicola*, *T. petrophilum* and *T. apicola* consist of a dimeric carbohydrate sophorose linked to a long chain hydroxyl fatty acid by glycosidic linkage. Generally, sophorolipids occur as a mixture of macro lactones and free acid form. It has been shown that the lactone form of the sophorolipid is necessary, or at least preferable, for many applications. These biosurfactant are a mixture of at least six to nine different hydrophobic sophorolipids (Hommel et al., 1987).

**TABLE - 8.**

**MAJOR BIOSURFACTANT CLASSES AND MICROORGANISMS INVOLVED**

<table>
<thead>
<tr>
<th>Biosurfactant</th>
<th>Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.GLYCOLIPIDS</strong></td>
<td></td>
</tr>
<tr>
<td>a. Rhamnolipids</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas sp.</em></td>
</tr>
<tr>
<td>b. Trehalolipids</td>
<td><em>R. erythropolis</em></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>\textit{N.erythropolis}</td>
</tr>
<tr>
<td></td>
<td>\textit{Mycobacterium sp.}</td>
</tr>
<tr>
<td>c. Sophorolipids</td>
<td>\textit{T.bombicola}</td>
</tr>
<tr>
<td></td>
<td>\textit{T.apicola}</td>
</tr>
<tr>
<td></td>
<td>\textit{T.petrophilum}</td>
</tr>
<tr>
<td>d. Cellobiolipids</td>
<td>\textit{Ustilago maydis}</td>
</tr>
<tr>
<td></td>
<td>\textit{U.zeae}</td>
</tr>
<tr>
<td>2. LIPOPEPTIDES &amp; LIPOPROTEINS</td>
<td></td>
</tr>
<tr>
<td>a. Peptide-lipid</td>
<td>\textit{B.licheniformis}</td>
</tr>
<tr>
<td></td>
<td>\textit{P.syringae}</td>
</tr>
<tr>
<td></td>
<td>\textit{P.fluorescens}</td>
</tr>
<tr>
<td></td>
<td>\textit{B.pumilis}</td>
</tr>
<tr>
<td>b. Iturin</td>
<td>\textit{B.subtilis}</td>
</tr>
<tr>
<td></td>
<td>\textit{B.amyloliquefaciens}</td>
</tr>
<tr>
<td>c. Serrawettin</td>
<td>\textit{Serretia marcescens}</td>
</tr>
<tr>
<td>d. Viscosin</td>
<td>\textit{P.fluorescens}</td>
</tr>
<tr>
<td>e. Surfactin</td>
<td>\textit{B.subtilis}</td>
</tr>
<tr>
<td></td>
<td>\textit{B.pumilus}</td>
</tr>
<tr>
<td>f. Subtilisin</td>
<td>\textit{B.subtilis}</td>
</tr>
<tr>
<td>g. Gramicidins</td>
<td>\textit{B.brevis}</td>
</tr>
<tr>
<td>h. Polymyxins</td>
<td>\textit{B.polymyxa}</td>
</tr>
</tbody>
</table>
### 3. FATTY ACIDS, NEUTRAL LIPIDS & PHOSPHOLIPIDS

**a. Fatty acids**
- *Corynebacterium lepus*
- *Penicillium spiculisporum*
- *Nocardia erythropolis*
- *Arthrobacter parafineus*
- *Talaromyces trachyspermus*

**b. Neutral lipids**
- *N. erythropolis*

**c. Phospholipids**
- *Thiobacillus thiooxidans*
- *Acinetobacter*

**d. Flavolipid**
- *Flavobacterium sp.*

### 4. POLYMERIC SURFACTANTS

**a. Emulsan**
- *A. calcoaceticus*

**b. Biodispersan**
- *A. calcoaceticus*

**c. Alasan**
- *A. radioresistens*

**d. Liposan**
- *Sandida lipolytica*

**e. Food emulsifier**
- *C. utilis*

**f. Insecticide emulsifier**
- *P. tralucida*

**g. Mannan-lipid-protein**
- *C. tropicalis*

**h. Carbohydrate-protein-lipid**
- *P. fluorescens*
- *D. polymorphis*
Multi-biotech (a subsidiary of Geodyne Technology) has commercialized biosurfactants for enhanced oil recovery applications. *Bacillus licheniformis* JF-2, an isolate from oil field injection water which, in addition to producing the most effective biosurfactants, has other characteristics like being anaerobic, halo tolerant, and thermo-tolerant produces biosurfactants that are potentially useful for insitu microbial enhanced oil recovery. As such, the viscosity of heavy crude oil can be greatly reduced when it is treated with this biosurfactant. In addition, the use of a biosurfactant for de sludging of a crude oil storage tank for Kuwait Oil Co. has been reported (Banat *et al.*, 1991).

Several biosurfactants have shown antimicrobial action against bacteria, fungi, algae and viruses. The lipopeptide turn from *B. subtilis* showed potent antifungal activity. Inactivation of enveloped virus such as herpes and retrovirus was observed with 80mM of surfactin. Rhamnolipids inhibited the growth of harmful bloom algae species, *Heterosigma akashiwo* and *Protocentrum dentatum* concentrations ranging from 0.4 to 10.0 mg/l. A rhamnolipids mixture obtained from *P. aeruginosa* AT 10 showed inhibitory activity against the bacteria *Escherichia coli*, *Micrococcus luteus*, *Alcaligenes faecalis* (32 mg/ml), *Serratia marcescens*, *Mycobacterium phlei* (16 mg/ml) and *Staphylococcus epidermidis* (8 mg/ml) and excellent antifungal properties against *Aspergillus niger* (16 mg/ml), *Chaetomium globosum*, *Penicillium chrysogenum*, *Aureobasidium pullulans* (32 mg/ml) and the phyto pathogenic *Botrytis cinerea* and *Rhizoctonia solani* (18 mg/ml) (Klich *et al.*, 1994).

Low toxicity, very little data are available in the literature regarding the toxicity of microbial surfactants. (Poremba *et al.*, 1991) reported that a synthetic anionic surfactant (Corexit) displayed an LC50 (concentration lethal to 50% of test species)
against *Photobacterium phosphoreum* ten times lower than rhamnolipids, demonstrating the higher toxicity of the chemical-derived surfactant. It was also reported that biosurfactants showed higher EC50 (effective concentration to decrease 50% of test population) values than synthetic dispersants. They are generally considered as low or non-toxic products, appropriate for pharmaceutical, cosmetic and food uses.

Matsuyama *et al.*, (1995) reported that 3 – hydroxyl fatty acids in serrawettins, the surface active exolipids produced by *Serratia marcescens* was elucidated by chiral column High – performance liquid chromatography. Cameotar *et al.*, (1995 ) have shown that the production of biosurfactant when grown on n- hexadecane as carbon source using *Serratia marcescens* isolated from Gujarat oil field. Matsuyama *et al.*, (1996) stated that *Serratia marcescens* secrete large amounts of wetting agents, are mainly serrawettins, a type of biosurfactant.

Surfactin from *B. subtilis* can reduce the surface tension of water to 25m N/m and interfacial tension of water/hexadecane to <1m N/m (Copper *et al.*,1981).Rhamnolipids from *P. aeruginosa* decrease the surface tension of water to 26mN/m and the interfacial tension of water/hexadecane to <1m N/m (Syldatk *et al.*,1985). Sophorolipids from *T. bombicola* have been reported to reduce the surface tension to 33m N/m and the interfacial tension to 5m N/m .In general, biosurfactants are more effective and efficient and their CMC is about 10–40 times lower than that of chemical surfactants, i.e. less surfactant is necessary to get a maximum decrease in surface tension (Desai and Banat, 1997).

Makka and Cameotra (1997) worked with a strain of *Bacillus subtilis* which was able to grow and produce a biosurfactant on 2% sucrose at 45°C. As a result of biosurfactant synthesis the surface tension of the medium was reduced from 68 dynes cm$^{-1}$ to 28 dynes cm$^{-1}$. The strain had the capacity to produce the biosurfactant at high NaCl concentrations (4%) and a wide range of pH (4.5– 10.5). The biosurfactant retained its surface-active properties after heating at 100°C for 2 hours and at different pH values (4.5–10.5). A maximum amount of biosurfactant was produced when urea or nitrate ions were supplied as nitrogen source. The use of the biosurfactant at high temperatures, acidic, alkaline and saline environments is discussed. As a result of its
action, 62% of oil in a sand pack column could be recovered, indicating its potential application in microbiologically enhanced oil recovery.

Biosurfactant production was studied by *Bacillus licheniformis* K51, *B. subtilis* 20B, *B. subtilis* R1 and *Bacillus* strain HS3 using molasses or cheese whey as a sole source of nutrition at 45°C. The isolates were able to grow and produce biosurfactant under shaking as well as static conditions. Maximum biosurfactant production was achieved with molasses at 5.0–7.0% (w/v). The biosurfactant retained its surface-active properties after incubation at 80 °C at a wide range of pH values and salt concentrations for nine days. Oil displacement experiments in sand pack columns with crude oil showed 25–33% recovery of residual oil (Joshi et al., 2008).

Biosurfactant activity of rhamnolipids makes them excellent candidates for assisting in the breakdown and removal of oil spills. Rhamnolipids also demonstrate antibacterial and antifungal activities, suggesting possible role in the medical and agricultural fields (Desai, 1997). A biosurfactant from *P. aeruginosa* was compared with a synthetic surfactant (Marlon A-350) widely used in the industry, in terms of toxicity and mutagenic properties. Both assays indicated higher toxicity and mutagenic effect of the chemically derived surfactant, whereas the biosurfactant was considered slightly non-toxic and non-mutagenic (Flasz et al., 1998). A *Pseudomonas aeruginosa* strain producing an extracellular surfactant (biosurfactant) was isolated and studied by Turkovskaya et al., (2001). The growth of this strain, referred to as 50.3, on a mineral glycerol-containing medium produces an emulsifying activity (60%) and decreases the surface tension of the culture liquid by a factor of 2.8 (to 25 m N/m). The optimum conditions for its growth and production of biosurfactants are intense aeration, pH 7.0–8.0, and the presence of Mg$^{2+}$. The optimum biosurfactant properties were achieved when glucose was used as the only source of carbon and energy and NH$_4$Cl was used as a source of nitrogen. A new bacterial strain, was isolated from petroleum contaminated soil, identified and named *Pseudomonas aeruginosa* strain LBI. The new strain produced surface-active rhamnolipids by batch cultivation in a mineral salts medium with soap stock as the sole carbon source. Biosurfactant production increased after nitrogen depletion. The maximum rhamnolipid concentration, 15.9 g/l, was reached
when it was incubated in a bioreactor with a constant KLa of 169.9 h\(^{-1}\) (Benincasa et al., 2002).

Ewa Huszcza and Bogdan Burczyk (2003) isolated a new biosurfactant producer, *Bacillus coagulans*, from soil. Its 24 hours old culture broth had a low surface tension (27–29 mN/m). Optimization of cell growth of this bacterium led to maximal biosurfactant production with glucose or starch as the organic carbon source, a pH in the range 4.0–7.5, and incubation temperatures from 20 to 45°C. The crude biosurfactants obtained after neutralization and lyophilization of the acid precipitate yielded a minimal aqueous solution surface tension value of 29 mN/m and an interfacial tension value of 4.5 mN/m against hexadecane. The critical micelle concentration of the crude biosurfactants was 17 mg/L. Addition of NaCl to the aqueous solution of the crude product caused lowering of surface tension at both the aqueous solution–air and aqueous solution–n-hexadecane interfaces. These results indicate that the biosurfactants obtained have potential environmental and industrial applications and may have uses in microbial enhanced oil recovery.

Nutritional requirements for maximal production of biosurfactant by an oil field bacterium *Pseudomonas putida* were determined by Pruthi and Cameotra (2003). The optimal concentrations of nitrogen, phosphate, sulfur, magnesium, iron, potassium, sodium, calcium, and trace elements for maximal production of biosurfactants were ascertained, and a new “Pruthi and Cameotra” salt medium was formulated. Data show that maximal biomass (2.4 g L\(^{-1}\)) and biosurfactant production (6.28 g L\(^{-1}\)) takes place after 72 hours of growth on 2% hexadecane. The biosurfactant was produced optimally over pH and temperature ranges of 6.4–7.2 and 30–40°C, respectively. That the highest biosurfactant yield was obtained during late log phase of growth indicates that the biosurfactant is a secondary metabolite.

Wongsa et al., (2004) reported two isolates, *P. aeruginosa Wat G* and *S. marcescens HOKM*, which were able to utilize gasoline, kerosene, diesel, and lubricating oil as the sole carbon and energy source. Rajkumar Bidlan et al., 2005 observed that production of biosurfactant by *Serratia marcescens* DT-1P, grown in glycerol as carbon source, surfactant was identified as arabinolipid. Thaniyavarn et al., (2006) studied biosurfactant production by *Pseudomonas aeruginosa* A41, a strain
isolated from seawater in the gulf of Thailand. The strain was examined when grown in defined medium containing 2% vegetable oil or fatty acid as a carbon source in the presence of vitamins, trace elements and 0.4% NH₄NO₃, at pH 7 and 30°C with 200 rpm shaking for 7 days. The yield of biosurfactant steadily increased even after a stationary phase. Under such conditions the surface tension of the medium was lowered from 55–70 m N/m to 27.8–30 m N/m with every carbon source tested. However, types of carbon sources were found to affect biosurfactant yield. The yields of rhamnolipid biosurfactant were 6.58 g/L, 2.91 g/L and 2.93 g/L determined as rhamnose content when olive oil, palm oil and coconut oil, respectively, were used as a carbon source. The oil displacement activity was stable to temperatures up to 100°C for 15 h. Surface tension reduction activity was relatively stable at pH 2–12 and 0–5% of NaCl. Emulsification activity tested with various types of hydrocarbons and vegetable oils showed similarity of up to 60% stability. The partially purified biosurfactant via TLC and silica gel column chromatography gave three main peaks on HPLC with mass spectra of 527, 272, and 661 m/z respectively, corresponding to sodium-monorhamnodecanoate, hydroxyhexadecanoic acid and an unknown compound, respectively.

Isolation and identification of key components of the crude biosurfactant produced by *Lactococcus lactis* 53 was studied by Rodrigues et al., (2006). Fractionation was achieved by hydrophobic interaction chromatography which allowed the isolation of a fraction rich in glycol proteins. Molecular (by Fourier transform infrared spectroscopy) and elemental compositions (by X-ray photoelectron spectroscopy) were determined. Critical micelle concentration achieved for the isolated fraction was 14 g/l, allowing for a surface tension value of 36m J/m². Moreover, the isolated fraction, stable to pH changes between 5 and 9, was found to be an anti-adhesive and antimicrobial agent against several bacterial and yeast strains isolated from explanted voice prostheses, even at low concentrations.

Benjamas et al., 2007 in their work chemical structures and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste was investigated. The culture produced two biosurfactants, a and b which showed strong activity and were identified as L-rhamnopyranosyl- L- rhamnopyranosyl
hydroxydecan-hydroxydecanoate or Rha–Rha–C10–C10 and L-rhamnopyranosyl-L-rhamnopyranosyl--hydroxydecanoyl--hydroxydodecanoate or Rha–Rha–C10–C12, respectively. Both compounds exhibited higher surfactant activities tested by the drop collapse test than several artificial surfactants such as SDS and Tween 80. Rhamnolipid a showed significant anti proliferative activity against human breast cancer cell line (MCF-7) at minimum inhibitory concentration (MIC) at 6.25µg/ml while rhamnolipid b showed MIC against insect cell line C 6/36 at 50µg/ml.

A Bacillus subtilis strain isolated from contaminated soil from a refinery has been screened for biosurfactant production in crystal sugar (sucrose) with different nitrogen sources (NaNO3, (NH4)2SO4, urea, and residual brewery yeast). The highest reduction in surface tension was achieved with a 48 hours fermentation of crystal sugar and ammonium nitrate. Optimization of carbon/nitrogen ratio (3, 9, and 15) and agitation rate (50, 150, and 250 rpm) for biosurfactant production was carried out using complete factorial design and response surface analysis. The condition of C/N 3 and 250 rpm allowed the maximum increase in surface activity of biosurfactant. A suitable model has been developed, having presented great accordance experimental data. Preliminary characterization of the bio product suggested it to be a lipopeptide with some isomers differing from those of a commercial surfactin (Fonseca et al., 2007).

Rhamnolipids are biosurfactants produced mainly by Pseudomonas aeruginosa. They, reduce surface and interfacial tension and offer excellent detergent, emulsifying/solubilizing, foaming and dispersing properties. Being biodegradable, they are particularly suitable for bioremediation (Benincasa, 2007; Zhu and Zhang, 2008) and enhanced oil recovery (Harvey et al., 1990; Wang et al., 2007). Rhamnolipid is one of the most effective and commonly used biosurfactant with wide industrial applications. Systematic strategies were applied to improve rhamnolipid (RL) production with a newly isolated indigenous strain Pseudomonas aeruginosa EM1 originating from an oil-contaminated site located in southern Taiwan. Seven carbon substrates and four nitrogen sources were examined for their effects on RL production. In addition, the effect of carbon to nitrogen (C/N) ratio on RL production was also studied. Single-factor experiments show that the most favorable carbon sources for RL production were glucose and glycerol (both at 40 g/L), giving a RL yield of 7.5 and 4.9
g/L, respectively. Meanwhile, sodium nitrate appeared to be the preferable nitrogen source, resulting in a RL production of 8.6 g/L. Using NaNO3 as the nitrogen source, an optimal C/N ratio of 26 and 52 was obtained for glucose- and glycerol-based culture, respectively. To further optimize the composition of fermentation medium, twenty experiments were designed by response surface methodology (RSM) to explore the favorable concentration of three critical components in the medium (i.e., glucose, glycerol, and NaNO3). The RSM analysis gave an optimal concentration of 30.5, 18.1, and 4.9 g/L for glucose, glycerol, and NaNO3, respectively, predicting a maximum RL yield of 12.6 g/L, which is 47% higher than the best yield (8.6 g/L) obtained from preliminary selection tests and single factor experiments (glucose and NaNO3 as the carbon and nitrogen source). The NMR and mass spectrometry analysis show that the purified RL product contained L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (RL1) and L-rhamnosyl L-rhamnosyl-bhydroxydecanoyl- b-hydroxydecanoate (RL2). Meanwhile, HPLC analysis indicates that the molar ratio of RL1 and RL2 in the purified rhamnolipid product was ca. 1:1 (Wu et al., 2008).

Sang et al., (2008) isolated some bacteria from waste soybean oil. Isolated bacterial strain had a marked tributyrin (C4:0) degrading activity as developed clear zone around the colony after incubation for 24 h at 37°C. It was identified as Klebsiella sp. Y6-1 by analysis of 16S rRNA gene. Crude biosurfactant was extracted from the culture supernatant of Klebsiella sp. Y6-1 by organic solvent (methanol: chloroform:1-butanol) after vacuum freeze drying and the extracted biosurfactant was purified by silica gel column chromatography. When the purified biosurfactant dropped, it formed degrading zone on crude oil plate. When a constituent element of the purified biosurfactant was analyzed by TLC and SDS–PAGE, it was composed of peptides and lipid. The emulsification activity and stability of biosurfactant was measured by using hydrocarbons and crude oil. The emulsification activity and stability of the biosurfactant showed better than the chemically synthesized surfactant. It reduced the surface tension of water from 72 to 32mN/m at a concentration of 40 mg/l.

Production of biosurfactant by free and alginate-entrapped cells of Pseudomonas Xuorescens Migula 1895- DSMZ was investigated by Mahmoud Abouseoud et al., (2008) using olive oil as the sole carbon and energy source. Biosurfactant synthesis was
followed by measuring surface tension and emulsifying index E24 over 5 days at ambient temperature and at neutral pH. Divisional limitations in alginate beads Vected the kinetics of biosurfactant production when compared to that obtained with free cells culture. Nevertheless, the emulsion stability was improved and fewer by-products interfered with the biosurfactant activity. A decrease in pH down to 5 in the case of immobilized cells was observed during the last 3 days, after which it returned to its initial value. The minimum values of surface tension were 30 and 35 dynes cm\(^{-1}\) achieved after 40 and 72 h with free and immobilized cells, respectively, while the corresponding maximum E24 values were 67 and 62\%, respectively. After separation by acetone precipitation, the biosurfactant showed a rhamnolipid type in nature, and had a good foaming and emulsifying activities. The critical micelle concentration was found to be 290 mg. The biosurfactant also showed good stability during exposure to high temperatures (up to 120 °C for 15 min), to high salinity (10% NaCl) and to a wide range of pH (4–9).

Manoj Kumar et al., (2008) isolated a hydrocarbon degrading and biosurfactant producing strain DHT2, from oil-contaminated soil. The organisms grow and produced biosurfactant, when cultured in variety of substrates at salinities up to 6 g l-1 and temperatures up to 45°C. It was capable of utilizing crude oil, fuels, alkane and PAHs as carbon source across the wide range of temperature (30–45°C) and salinity (0–6\%). Over the range evaluated, the salinity and temperature did not influence the degradation of hydrocarbon and biosurfactant productions. Isolate DHT2 was identified as *Pseudomonas aeruginosa* by analysis of 16S rRNA sequences (100% homology) and biochemical analysis. PCR and DNA hybridization studies revealed that enzymes involved in PAH metabolism were related to the naphthalene dioxygenase pathway. Observation of both tensio-active and emulsifying activities indicated that biosurfactants were produced by DHT2 during growth on both, water miscible and immiscible substrates, including PAH. The biosurfactants lowered the surface tension of medium from 54.9 to 30.2 dN/cm and formed a stable emulsion. The biosurfactant produced by the organism emulsified a range of hydrocarbons with hexadecane as best substrate and toluene was the poorest.
Pornsumthorntawee et al. (2008) isolated *Pseudomonas aeruginosa* strain SP4 from petroleum-contaminated soil in Thailand, and were used to produce a biosurfactant from a nutrient broth with palm oil as the carbon source. The key components of the crude biosurfactant were fractionated by using HPLC-ELSD technique. With the use of ATR-FTIR spectroscopy, in combination with 1H NMR and MS analyses, chemical structures of the fractionated components of the crude biosurfactant were identified as rhamnolipid species. When compared to synthetic surfactants, including Pluronic F-68, which is a triblock nonionic surfactant containing poly (ethylene oxide) and poly (propylene oxide), and sodium dodecyl sulfate, the crude biosurfactant showed comparable physicochemical properties, in terms of the surface activities. The crude biosurfactant reduced the surface tension of pure water to 29.0 m N/m with a critical micelle concentration of approximately 200 mg/l, and it exhibited good thermal and pH stability. The crude biosurfactant also formed stable water-in-oil micro emulsions with crude oil and various types of vegetable oils, but not with short-chain hydrocarbons.

A locally-isolated *Pseudomonas aeruginosa* USM AR2 possessing the ability to produce glycolipids type biosurfactant (rhamnolipid) was used in this research to explore fermentation technology for rhamnolipid production. Rhamnolipid concentration in 2.5 L fed-batch fermentation was improved from 0.173 to 8.06 g/L by manipulating the feeding strategy and cultivation protocol. The culture was fed with petroleum diesel and complex medium. The highest rhamnolipid concentration was achieved when the culture was initially fed with both petroleum diesel and complex medium, followed by feeding of petroleum diesel only at the end of the stationary phase. Severe foaming problem was resolved by modifying and integrating a foam recycler to the bioreactor. This successfully extended the cultivation period and increased the yield of final rhamnolipid. No antifoam agent was added as this modified bioreactor allowed cultivation to proceed even under foam generation. The viscosity measurement, surface tension activity test, and drop-collapse test were performed as an indirect measure of rhamnolipid presence (Salwa et al., 2009).

*Gordonia sp.* BS29 is a hydrocarbon-degrading bacterium isolated from a site chronically contaminated by diesel. The strain produces extracellular bio emulsifiers, able to produce stable emulsions, and cell-bound glycolipids biosurfactants, able to
reduce surface tension. The aims of this work were to investigate the cultural factors affecting the production of the cell-bound biosurfactants by *Gordonia sp.* BS29 and to find the optimal composition of growth medium for the production. The cultural factors which have a significant influence on surfactant biosynthesis, identified by a two level 2(8-2) Fractional Factorial Design, were the type and concentration of the carbon source, the concentrations of phosphates and sodium chloride, and the interactions among these factors. On these factors, a flask-scale optimization of cultural conditions was carried out. Then, a steepest ascent procedure and a Central Composite Design were applied to obtain a second order polynomial function fitting the experimental data near the optimum. In the optimized cultural condition we obtained a 5-fold increase in the biosurfactant concentration compared to the un-optimized medium (26.00), reaching a Critical Micelle Dilution value (129.43) among the highest in literature. The optimization procedure did not change the number and type of the glycolipid biosurfactants produced by *Gordonia sp.* BS29 (Franzetti *et al*., 2009).

Tayebe *et al*., (2009) isolated bacterial strain and cultured from the oil excavation areas in tropical zone in southern Iran. It was affiliated with *Pseudomonas*. The biochemical characteristics and partial sequenced 16S rRNA gene of isolate, MR01, was identical to those of cultured representatives of the species *Pseudomonas aeruginosa*. This bacterium was able to produce a type of biosurfactant with excessive foam-forming properties. Compositional analysis revealed that the extracted biosurfactant was composed of high percentages lipid (∼65%, w/w) and carbohydrate (∼30%, w/w) in addition to a minor fraction of protein (∼4%, w/w). The best production of 2.1 g/l was obtained when the cells were grown on minimal salt medium containing 1.2% (w/v) glucose and 0.1% (w/v) ammonium sulfate supplemented with 0.1% (w/v) isoleucine at 37 °C and 180 rpm after 2 days. The optimum biosurfactant production pH value was found to be 8.0. The MR01 could reduce surface tension to 28mN/m and emulsified hexadecane up to E24≈70. The results obtained from time course study indicated that the surface tension reduction and emulsification potential was increased in the same way to cell growth. However, maximum biosurfactant production occurred and established in the stationary growth phase (after 84 hours). Fourier Transform Infrared spectrum of extracted biosurfactant indicates the presence of carboxyl, amine, hydroxyl and methoxyl functional groups. Thermo gram of
biosurfactant demonstrated three sharp endothermic peaks placing between 200 and 280 °C. The core holder flooding experiments demonstrated that the oil recovery efficiencies varied from 23.7% to 27.1% of residual oil.

A biosurfactant-producing strain S6 was isolated from oil-containing wastewater and identified as *Pseudomonas aeruginosa* based on physiological and biochemical tests together with 16S rDNA sequence analysis. Thin layer chromatography (TLC) and high-performance liquid chromatography electrospray ionization mass spectra (HPLC-ESI-MS) worked together to reveal that the strain S6 produced rhamnolipid biosurfactant. Mass spectrometry confirmed the presence of some major components in the rhamnolipid surfactant showing m/z of 675.8, 529.6, 503.3 and 475.4, which corresponded to Rha RhaC10C12:1, RhaC12:1C10, RhaC10C10 and RhaC8C10, respectively. The biosurfactant produced by strain S6 had the ability to decrease the surface tension of water from 72 to 33.9 mN m⁻¹, with the critical micelle concentration (CMC) of 50 mg L⁻¹. Emulsification experiment indicated that this biosurfactant effectively emulsified the crude petroleum and the measurements of surface tension demonstrated that the biosurfactant possessed stable surface activity at variable ranges of pH and salinity. The biosurfactant also exhibited good performance of phenanthrene solubilization with about 23 times higher solubility of phenanthrene in water than the control. Thus, this biosurfactant may have a potential for application in bioremediation of crude oil contamination (Hua Yin et al., 2009).

Lima et al., (2009) isolated *Pseudomonas aeruginosa* PACL strain from oil-contaminated soil from a lagoon, for the investigation of the efficiency and magnitude of biosurfactant production, using different waste frying soybean oils, by submerged fermentation in stirred tank reactors of 6 and 10 l capacities. A complete factorial experimental design was used, with the goal of optimizing the aeration rate (0.5, 1.0, and 1.5vvm) and agitation speed (300, 550, and 800 rpm). Aeration was identified as the primary variable affecting the process, with a maximum rhamnose concentration occurring at an aeration rate of 0.5ppm. At optimum levels, a maximum rhamnose concentration of 3.3 g/l, an emulsification index of 100%, and a minimum surface tension of 26.0 dynes/cm were achieved. Under these conditions, the biosurfactant production derived from using a mixture of waste frying soybean oil (WFSO) as a
carbon source was compared to production when non-used soybean oil (NUSO), or waste soybean oils used to fry specific foods, were used. NUSO produced the highest level of rhamnolipids, although the waste soybean oils also resulted in biosurfactant production of 75–90% of the maximum value.

Gesheva et al., (2010) isolated A-3 from Antarctic soil in Casey Station, Wilkes Land, and characterized for the growth on hydrocarbons. Use of glucose or kerosene as a sole carbon source in the culture medium favored biosynthesis of surfactant which, by thin-layer chromatography, indicated the formation of a rhamnose-containing glycolipids. This compound lowered the surface tension at the air or water interface to 27 mN/m as well as inhibited the growth of *B. subtilis* ATCC 6633 and exhibited hemolytic activity. A highly hydrophobic surface of the cells suggests that uptake occurs via a direct cell–hydrocarbon substrate contact. Phylogenetic analysis based on comparative analysis of 16S rRNA gene sequences revealed that strain A-3 is closely related to *Rhodococcus fascians* with which it shares 100% sequence similarity. This is the first report on rhamnose-containing biosurfactant production by *Rhodococcus fascians* isolated from Antarctic soil.

Anyanwu et al., (2011) investigated the production and some properties of a biosurfactant, synthesized by *Serratia marcescens* NSK-1 strain, using glycerol as substrate were investigated. The organism was able to grow and produce surfactant which reduced the surface tension of distilled water to 38.0 dynes/cm and gave a surfactant concentration of 2.2 gl-1 after 48 hr. The surface-active compound retained its properties during exposure to elevated temperatures (up to 100°C), relatively high salinity (12% NaCl) and a wide range of pH values. The surfactant was capable of forming stable emulsions with various vegetable and hydrocarbon oils. Preliminary chemical characterization of the biosurfactant by the use of thin layer chromatography and infrared spectroscopy revealed that the compound is lipopeptide in nature and with a CMC value of 29 mgl-1.

Da Rosa et al., (2011) investigated the effect of the culture medium composition on rhamnolipid production by *Pseudomonas aeruginosa* LBM10, isolated from an estuarine environment in Southern Brazil. Experimental design and surface
response methodology were used in order to improve biosurfactant production using glycerol, a renewable carbon source. The assays were carried out in a rotary shaker at 30°C and 180 rpm for 120 h and the parameters studied were glycerol concentration, C/N (carbon/nitrogen) and C/P (carbon/phosphorus) ratios. Low glycerol concentration and a phosphorus-limiting condition were favorable for rhamnolipid production. Contour plots constructed by predictive polynomial equations led to a glycerol concentration of 13.2 g/l, a C/N ratio of 12.8 and a C/P ratio of 40 in order to maximize rhamnolipid concentration (4.15 g/l) associated with a high emulsification index (61%).

A rhamnolipid producing bacterium, *Pseudomonas aeruginosa* was isolated from contaminated soil with oily wastes. The *Pseudomonas aeruginosa* grown with glucose and corn oil as a carbon source produced biosurfactant. This biosurfactant was purified by procedures that included chloroform-ethanol extraction and 0.05M bicarbonate treatments. The active compound was identified as rhamnolipid by using thin layer chromatography. The emulsification activity of biosurfactant, the coconut oil responded better than the olive oil, groundnut oil and sunflower oil and gave maximum level of 1cm (Sarita Kumari *et al.*, 2010).


2.2.3. MATERIALS AND METHODS

The five bacterial isolates (PDKT-1, PDKT-2, PDKT-5) which showed positive result for haemolysis test coupled with hydrocarbon degrading ability (as described in Section. 2.1.4) were selected for the following biosurfactant activity assays.

Biosurfactant activity assays.

Drop Collapsing Test:

Two microliters of mineral oil was added to each well of a 96-well microtiter plate lid. The lid was equilibrated for 1 hr at room temperature, and then 5 µl of the cultural supernatant of the isolates (PDKT-1, PDKT-2, PDKT-5) were added to the surface of used engine oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive ('+'). Those cultures that gave rounded drops were scored as negative ('-'), indicative of the lack of biosurfactant production (Youseff et al., 2004).

Emulsification Measurement:

Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) a slight modification. To 4 ml of culture supernatant of the isolates (PDKT-1, PDKT-2, PDKT-5), 4 ml of n-hexadecane was added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage.

Oil Displacement Test:

Fifteen µl of used engine oil were placed on the surface of distilled water (40 µl) in a petridish (150 mm in diameter). Then, 10 µl of the culture supernatant of the isolates (PDKT-1, PDKT-2, PDKT-5) were gently put on the center of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 second as described by Morikawa et al., 1993.
Biosurfactant production

Preparation of Inoculum:

The primary inoculum of selected bacterial isolates PDKT-1, PDKT-2, PDKT-5 having biosurfactant activity was prepared in 50ml sterile Luria Bertani medium. The fresh over night culture was used as an inoculum for production of biosurfactant. To the 100 ml of production medium with 2% sterilized used engine oil, 1% inoculum was transferred aseptically and incubated at 37°C for 48 hours. The medium was agitated at 100 rpm for better aeration and growth of the organism.

Production medium (Tahzibi et al., 2004)

Mineral Salts Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>15g</td>
</tr>
<tr>
<td>KCl</td>
<td>1.1g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.1g</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>0.00028g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.4g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>4.4g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in distilled water and sterilized at 121°C at 15lbs for 15 minutes. To the sterilized mineral salt medium, 5 ml of trace elements solution was added (filter sterilized) separately before inoculation.

Trace elements solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.29g</td>
</tr>
</tbody>
</table>
CaCl$_2$·4H$_2$O 0.24g  
CuSO$_4$·5H$_2$O 0.25g  
MnSO$_4$·H$_2$O 0.17g  
Distilled water 1000 ml

The trace element solution was filter-sterilized through a 0.22-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

**Biosurfactant Recovery** (Pruthi and Cameotra, 2003)

The culture broth of the bacterial isolates PDKT-1, PDKT-2, PDKT-5 were centrifuged at 10000 rpm for 15 minutes, to remove the cells and then after sterilized with Millipore membrane filter. The clear sterile supernatant served as the source of the crude biosurfactant. The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation. Three volumes of chilled acetone was added and allowed to stand for 10 h at 4°C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone after which it was re-dissolved in sterile water.

**BIOSURFACTANT CHARACTERIZATION:**

**Chemical Analysis of Biosurfactants** (Sawhnety and Sing, 2000)

**Analysis of Amino Acids**

**Ninhydrin Test**

To a small amount the extracted biosurfactant 2-5 drops of ninhydrin solution was added. The tubes were mixed well and keep for 5 min in boiling water bath and observed the color formation.

**Analysis of Carbohydrate**

**Anthrone Test**

A tiny amount of the isolated biosurfactant was added to the 3ml of anthrone reagent and it was thoroughly mixed. Colour changes were observed.

**Iodine test**
4-5 drops of iodine solution was added to the little amount of the isolated biosurfactant and it was mixed gently. The colour formation was observed.

**Barfoed Test**

2ml of Barfoed’s reagent was added to the little amount of the biosurfactant. The tubes were kept in a boiling water bath. The formation of colour and also the time taken for its appearance was noted.

**Bial’s Test**

2ml Bial’s reagent was added to 1-2 drops of biosurfactant. The tubes were heat in a boiling water bath. The color formation was observed.

**Analysis of Lipids**

**Solubility Test**

Small amount of isolated biosurfactant was taken in three test tubes. To each tube water, alcohol, chloroform was added and their solubility was tested.

**Saponification Test**

2ml of 2% NaOH solution was added to the small amount of biosurfactant and shaken well. The formation of soap was observed.

**Acrolein Test for Glycerol**

1.5 g Potassium hydrogen sulphate was taken in a test tube and a little amount of isolated biosurfactant was added. The added biosurfactant was covered completely by adding more of solid potassium hydrogen sulphate on top of it. The test tube was slowly heated and noted the odour of the fumes evolved from the tube.

**Fourier Transform Infrared Spectroscopy [FTIR] (Thaniyavarn, 2003)**

The biosurfactant produced by the bacterial isolates PDKT-1, PDKT-2, PDKT-5 were extracted from the 2 ml of supernatant fluid with 2ml chloroform, dried with Na2SO4 and evaporated on a rotary evaporator. The IR spectra were recorded by thin film technique using a Spectrum RXI, FT-IR Spectrometer, in the 4000-400cm-1 spectral region at a resolution 2 cm-1, 100 scans for each spectrum, using a 0.23 mm KBr liquid cell.

**Determination of biosurfactant** (Tuelva et al., 2002).
The strain *Pseudomonas aeruginosa* PDKT-2 which showed maximum biosurfactant activity was selected for optimization studies of biosurfactant production. The production work was carried out as described earlier. The spectrometric chemical assay for the determination of biosurfactant from the sample (PDKT-2) was estimated using Orcinol assay method, which was used for the direct assessment of the amount of glycolipids in the sample. Extracellular glycolipids concentrations were evaluated in triplicate by measuring the concentration of rhamnose. To 100µl of the sample 900µl of a solution containing 0.19% Orcinol (in 53% H$_2$SO$_4$) was added. After heating for 30 min at 80°C the samples were cooled at room temperature and the OD at 421nm was measured. Control was prepared with distilled water. The rhamnolipid concentrations were calculated from a standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg ml$^{-1}$)

**Parameter optimisation studies for the production of biosurfactant:**

In order to determine optimum temperature, pH, carbon source and nitrogen source required for biosurfactant production, 100 ml of sterile production medium with 2 % filter sterilized engine oil was prepared in different conical flasks and they were inoculated with 1% inoculum. For temperature optimization studies each flask was incubated at different temperatures such as 25°C, 30°C and 35°C for 48 hours. In order to determine the favourable pH, flasks containing production media was adjusted to different pH such as 6.7, and 8 using 0.1N NaOH and 0.1N HCl respectively. Different carbon sources (1 %) such as fructose, glucose, sucrose, used engine oil and glucose + used engine oil were amended in each flask to determine the optimim carbon source for biosurfactant production. The favourable nitrogen source for biosurfactant production was determined by adding each flask with different nitrogen sources (0.5%) such as ammonium nitrate, sodium nitrate, sodium nitrite and urea. All the flasks were incubated at 37°C for 48 hours. The biosurfactant production was determined using orcinol assay method as described earlier after incubation. All determinations were carried out in triplicates.
2.2.4. RESULT

Biosurfactant producing ability of the selected isolates was screened. All the three bacterial strains (PDKT-1, PDKT-2, PDKT-5) showed positive result for drop collapsing test by giving flat drops. The emulsification activity of the bacterial isolates (PDKT-1, PDKT-2, PDKT-5) ranged from 45-65%. The strain PDKT-2 showed maximum activity of 65% while lowest activity was observed in the strain PDKT-5 with 45%. (Fig. 42). The range of oil displacement activity by the bacterial isolates (PDKT-1, PDKT-2, PDKT-5) was 1.3 cm - 2.5 cm (Fig. 43). The strain PDKT-5 exhibit lowest activity of 1.1cm and the highest activity of 2.5 cm were scored by the strain PDKT-2 (Table-9). Since all the strains (PDKT-1, PDKT-2, PDKT-5) showed biosurfactant activity, they were selected for characterization studies.

All the three strains PDKT-1, PDKT-2, PDKT-5 produced biosurfactant (white precipitate) by using used engine oil as substrate. The isolated biosurfactant was analyzed chemically for the presence of amino acids, carbohydrate and lipids in the extracted biosurfactant (Table-10). The strains PDKT-1, PDKT-5 showed positive result for ninhydrin test, there was violet-blue complex formation which indicated the presence of amino acids. Absence of colour formation was observed in PDKT-2. In anthrone test for carbohydrates, there was a colour change to bluish green which indicates the presence of carbohydrates was observed only in strain PDKT-2. Also, there was the absence of blue or reddish brown complex in iodine test and the formation of red precipitate in Barfoeds tests within 2-5 min, was observed that it indicates the absence of polysaccharides and the presence of monosaccharides respectively. No colour formation in the strains PDKT-1, PDKT-5 revealed the absence of carbohydrates. Bial’s test for pentose sugars, the formation of blue-green coloured complex was observed. It confirms the presence of pentose sugar in the isolated biosurfactant of PDKT-2. In the solubility test for lipids, the tested biosurfactants from three strains were insoluble in water, but soluble in alcohol and chloroform. In saponification test for lipids, NaOH saponifies the lipid, which is present in the
biosurfactant that indicates the presence of lipid in the isolated biosurfactant of three strains (PDKT-1, PDKT-2, PDKT-5). In achrolin test for glycerol the tested biosurfactant, does not produce pungent smell. It indicated the absence of glycerol in PDKT-1, PDKT-2, PDKT-5. The above results revealed that the isolated biosurfactant of PDKT-1, PDKT-5 and PDKT-2 belong to lipopeptide and rhamnolipid type respectively.

Following chemical screening, the molecular structure of the extracted biosurfactants from the strains (PDKT-1, PDKT-2, PDKT-5) were confirmed by FTIR spectroscopy. Infrared spectrum of the extracted biosurfactant from PDKT-1 (Fig. 44) showed strong bands at 3430 cm\(^{-1}\), indicating the presence of a peptide component resulting from the N-H stretching mode and at 1655 cm\(^{-1}\) resulting from the stretching mode of CO-N bond. The bands at 2960 cm\(^{-1}\) to 2860 cm\(^{-1}\) and 1470 cm\(^{-1}\) resulting from the C-H stretching mode suggest the presence of aliphatic chain. These results revealed that the isolated biosurfactant from PDKT-1 was a lipopeptide.

Analysis of the extracted biosurfactant from PDKT-2 showed positive test for sugar and rhamnose. FT-IR Spectrometry strongly suggested a broad absorption valley at 3400 cm\(^{-1}\) indicating the presence of OH groups in the molecules. Strong absorption valleys observed in the range from 2725 to 2813 cm\(^{-1}\) demonstrated typical CH stretching variation in the alkaline chain. Absorption valleys at 1607 cm\(^{-1}\) indicated stretching vibration of C-O and C = O bonds in carboxyl esters. Scissoring vibration of a CH\(_2\) group adjoining a carboxyl ester was also observed at 1351 cm\(^{-1}\). The peak in the region of 1104 cm\(^{-1}\) indicates C–O–C stretching in the rhamnose (Fig. 45). These results confirm that the extracted biosurfactant from PDKT-2 belonged to rhamnolipid type.

Strong bands at 3430 cm\(^{-1}\), indicating the presence of a peptide component resulting from the N-H stretching mode and at 1655 cm\(^{-1}\) resulting from the stretching mode of CO-N bond observed in the biosurfactant extracted from PDKT-5 (Fig. 46). The bands from 3000 cm\(^{-1}\) to 2000 cm\(^{-1}\) resulting from the aliphatic bonds CH3, CH2 and C–H stretching with C-H stretching indicates the presence of aliphatic chain. Scissoring vibration of a CH\(_2\) group adjoining a carboxyl ester was also observed at 1351 cm\(^{-1}\). Infrared spectrum strongly suggest that the extracted biosurfactant from PDKT-5 belongs to lipopeptide type.
In the present study, the strain PDKT-2 showed maximum biosurfactant activity, hence it was selected for biosurfactant optimization studies. The effect of pH on rhamnolipid production by the strain *Pseudomonas aeruginosa* PDKT-2 was tested using mineral salt medium with different pH. The bacterial strain *Pseudomonas aeruginosa* PDKT-2 showed the ability to produce rhamnolipid at a pH range from 6 to 8. However, there was a significant difference in the rhamnolipid production at different pH. Maximum rhamnolipid production was observed at pH 8 than 7 and decreased at pH 6 (Fig. 47). The impact of cultivation temperature on biosurfactant production by the strain *Pseudomonas aeruginosa* PDKT-2 was studied. Cultivation temperature was found to have a significant influence in rhamnolipid production. Maximum rhamnolipid production observed at 35° C than at 30° C and it was decreased at 25° C (Fig. 48). The biosurfactant production by the strain *Pseudomonas aeruginosa* PDKT-2 when grown on different carbon substrates was investigated. The production of biosurfactant occurred in all substrates tested. The carbon sources in terms of induction of biosurfactant production can be graded as follows: glucose + oil < glucose < used engine oil< sucrose < Fructose. The biosurfactant production with glucose and engine oil was high, whereas it was least with Fructose (Fig. 49). The effect of different nitrogen sources on rhamnolipid production by the strain *Pseudomonas aeruginosa* PDKT-2 was evaluated. When the strain *Pseudomonas aeruginosa* PDKT-was grown in medium with sodium nitrite least production was observed, but when grown in sodium nitrate there was a significant production of biosurfactant. The biosurfactant production was maximum in ammonium nitrate ammended medium. Moderate production was observed for urea (Fig. 50).
2.2.5. DISCUSSION

Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (C\textsubscript{x}H\textsubscript{y}), microorganisms facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants. Some bacteria and yeasts excrete ionic surfactants which emulsify the C\textsubscript{x}H\textsubscript{y} substrate in the growth medium. Release of biosurfactants is one of the strategies used by microorganisms to influence the uptake of PAHs and hydrophobic compounds in general (Johnsen \textit{et al}., 2005 and Marin \textit{et al}., 1996). Many hydrocarbon utilising bacteria possess emulsifying activities, as whole cell or by excreting extracellular surface active compounds (Ilori and Amund, 2001). Those biosurfactants are capable of increasing the bioavailability of poorly soluble polycyclic aromatic hydrocarbons (Gilewicz \textit{et al}., 1997; Olivera \textit{et al}., 2003) and resins (Venkateswaran \textit{et al}., 1995). Therefore, the use of biosurfactants should be a promising means to emulsify the polluted oils prior to biodegradation.

Biosurfactant-producing microorganisms can be screened using different assays. Hemolysis of red blood cell was used for screening biosurfactant producing microorganisms (Carrillo \textit{et al}., 1996; Fiebig \textit{et al}., 1997). Colony surrounded by an emulsified halo on L-agar plate coated with oil was determined as biosurfactant producer as described by Morikawa \textit{et al}., (1992). Emulsification power was measured by thorough mixing of an equal volume of the culture supernatant with kerosene for 1 min. The percentage of volume occupied by the emulsion after 24 h was recorded according to the method of Haba \textit{et al}., (2000). Jain \textit{et al}., (1991) stated that the sensitive rapid method, a drop-collapsing test was advised for screening bacterial colonies that produce surfactants. In this study the screening methodologies used for biosurfactant production by bacterial strains was in agreement with above findings. Similar studies on biosurfactant activity assays was done by Maneerat and Pheetrong (2007).

Among all 25 isolated strains from engine oil contaminated soils, only 3 strains (PDKT-1, PDKT-2, PDKT-5) exhibited the hydrocarbon degradation coupled with biosurfactant activity. The strains exhibiting haemolytic activity showed the positive result with drop collapsing test, emulsification activity test and oil displacement test. \textit{Pseudomonas aeruginosa} PDKT-2 showed the highest activity for oil displacement test.
and emulsification test (Table-9). Haemolytic activity therefore appears to be a good screening criterion for surfactant-producing strains (Carrillo et al., 1996). Maneerat and Pheetrong (2007) reported that among 200 bacterial strains only 8 strains exhibited haemolytic activity and emulsification on weathered crude oil and this coincides with the present study results. Emulsification activity was one of the criteria to support the selection of potential biosurfactant producers. Emulsifying activities (E_{24}) determine productivity of bioemulsifier (Bonilla et al., 2005). The present investigation correlates the findings of Ellaiah et al., (2002) who screened 68 bacterial isolates from soil and found only 6% of isolates with good emulsification activity of up to 61%. Measurement of emulsification units help to choose the carbon and energy source for biosurfactant/bioemulsifier production. Patil and Chopade (2001) introduced emulsification assay based on emulsification units of the tested oils. They selected Acinetobacter junii SC14 for bioemulsifier production. By examining emulsification units, it is possible to select a potent biosurfactant/bioemulsifier producer.

When the 16S r RNA gene sequence of the isolates PDKT-1, PDKT-2, and PDKT-5 were compared to previously published sequences on the NCBI database, the high homology was found with Serratia marcescens (99% homology); Pseudomonas aeruginosa (100% homology) and a Bacillus licheniformis (95% homology) strain, respectively. This was similar to previous findings of Rosenberg and Ron, 1997; Chistofi and Ivshina, 2002 who stated that a diverse range of microbes produce biosurfacants and the commonest genera include Corynebacterium, Rhodococcus, Pseudomonas, Serratia and Bacillus. Recently Anyanwu et al., (2011) isolated biosurfactant, producing Serratia marcescens NSK-1 strain from petroleum contaminated site. Previously biosurfactant production by Serratia marcescens was also reported by Matsuyama et al., 1981 and 1989. The present investigation was in accordance with Obayori et al., (2008) who isolated strain Pseudomonas sp. LP1 having biosurfactant activity from contaminated Nigerian soil on the basis of its ability to grow on pyrene, it also has degradative potential for crude oil, diesel and engine oil correlates the present study. Jenneman et al., (1983) reported that Bacillus licheniformis JF-2, isolated from oil-field injection brine has been able to grow and produce a very effective biosurfactant under both aerobic and anaerobic conditions. The biosurfactant activity in Bacillus sp was also documented by Prommachan, 2002; Tabatabaee et al., 2005.
and Mukherjee, 2007; Sing, 2008 and Suwansuko, 2008 which correlates the present study.

Although the identification and characterization of microbial surfactants produced by various microorganisms have been extensively studied, only few reports available on biosurfactant producing microorganisms on used engine oil. In this study, isolated biosurfactant from PDKT-2 was analyzed chemically; the presence of carbohydrates, lipids was confirmed. That particular carbohydrate was found to be a pentose sugar and the glycerol was absent in the lipid, hence this indicates that the isolated biosurfactant was a glycolipid. Glycolipids containing sugar and lipid component and do not containing glycerol (Table-10). The sugar constitutes most prevalently glucose, galactose, mannose and glycolloyamine have all been identified (Sawhney and Singh, 2000). Chemical analysis confirmed the presence of lipid and amino acid component in PDKT-1 and PDKT-5. Similar result on chemical analysis of biosurfactant was reported by Mahesh et al., 2006.

In the present investigation, infrared spectrum of biosurfactant from *Serratia marcescens* PDKT-1 belonged to lipopeptide type. This result correlates with findings of Matsuyama et al., (1995) who reported that serrawettins, surface – active exolipids, nonionic biosurfactants was produced by *Serratia marcescens*. *Serratia rubidaea* produces rubiwettin R1, linked D-3-hydroxy fatty acids and RG1, β-glucopyranosyl linked D-3-hydroxy fatty acids. (Matsuyama et al.,1990) . *Serratia* produces surface active cyclodepsipeptides known as serrawettin W1, W2 andW3 (Matsuyama et al., 1986 and 1989). Different strains of *Serratia marcescens* produce different serrawettins. Serrawettin W1 was produced by strains 274 and ATCC 13880 or NS 38, W2 was produced by strain NS 25 and W3 was produced by strain NS 45. Besides this, *Serratia liquefaciens* produces serrawettin W2 (Matsuyama et al., 1990). Recently studies on Lipopeptide biosurfactant production by *Serratia marcescens* NSK-1 strain isolated from petroleum-contaminated soil by Anyanwu (2011) which coincides the present study.

Rhamnolipids produced by *Pseudomonas aeruginosa* were the most studied biosurfactants due to their potential applications in a wide variety of industries and the high levels of their production (Toribio et al., 2010). Rhamnolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxy-decanoic acid,
are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* (Jarvis and Johnson, 1990). L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa* (Edward and Hayashi, 1965; Hisatsuka *et al.*, 1971; Itoh and Suzuki, 1972; Itoh *et al.*, 1971). The formation of rhamnolipid types 3 and 4 containing one β-hydroxydecanoic acid with one and two rhamnose units, respectively (Sylatk *et al.*, 1985), methyl ester derivatives of rhamnolipids 1 and 2 (Hirayama and Kato, 1982), and rhamnolipids with alternative fatty acid chains (Lang and wagner 1987; Parra *et al.*, 1989; Rendell *et al.*, 1990) has also been reported. In the present investigation the biosurfactant produced by the strain *Pseudomonas aeruginosa* PDKT-2 was characterized as rhamnolipid by FTIR technique. According to the results of the IR spectra, the rhamnolipids produced by belong to *Pseudomonas aeruginosa* PDKT-2 the glycolipid group, which is made up of aliphatic acid and ester. The adsorption bands obtained were consistent with the report of Guo *et al.*, 2009. This result was similar to recent findings. For example Rahman *et al.*, 2010 characterized the biosurfactant produced by the strain *Pseudomonas aeruginosa* DS10-129 by using FTIR technique and reported that it belonged to rhamnolipid type. Recently Da Rosa *et al.*, (2010) reported that the rhamnolipid type biosurfactant was produced by *Pseudomonas aeruginosa* LBM10 which correlates the present study.

Biosurfactants are easily biodegradable and thus they are particularly suited for environmental applications such as bioremediation and the dispersion of oil spills. Among the many classes of biosurfactants, lipopeptides are particularly interesting because of their high surface activities for example, surfactin, a well-studied lipopeptide produced by *Bacillus subtilis*, was a very effective biosurfactant (Cooper *et al.*, 1981). The present study revealed that the biosurfactant from *B. licheniformis* PDKT-5 was a lipopeptide. Previously two other *Bacillus* isolates, *B. licheniformis* 86 by Horowitz and Griffin (1991) and another *B. licheniformis* strain isolated by Jenny *et al.*, (1991) have been shown to produce lipopeptides with peptide moieties containing C-terminal amino acid residues different from those of surfactin. Lin *et al.*, (1994) reported that the surface-active compound from *B. licheniformis* JF-2 was a lipopeptide. The lipopeptide
type biosurfactant producing strain, *B. licheniformis* F2.2 and *Bacillus licheniformis* PTCC 1595 which were isolated by Thaniyavarn *et al.* (2003) and Noudeh *et al.* (2010) respectively correlates the present study.

This study was designed to investigate the effect of various nitrogen sources on rhamnolipid production by the strain *Pseudomonas aeruginosa* PDKT-2. The nitrogen source can be an important key to the regulation of biosurfactant production (Rismani *et al.*, 2006). In the present study biosurfactant production by *Pseudomonas aeruginosa* PDKT-2 was found to be maximum in ammonium nitrate amended medium. Biosurfactant production differs with the nitrogen source available and this varies from one organism to other. For instance, Duvnjak *et al.* (1983) reported that among the inorganic salt tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthobacter paraffineus*. Guerra-santos *et al.*, 1984; Robert *et al.*, 1989; MacElwee *et al.*, 1990 and Rashedi *et al.*, 2005 reported that nitrate supported maximum surfactant production in *P. aeruginosa*. These findings were in accordance with the present study.

Biosurfactant production also depends both on the type and amounts of carbon source present in the medium (Adamczak and Bednarsk, 2000; Davis *et al.*, 1999). Secretion of surface active compounds by *Pseudomonas aeruginosa* PDKT-2 when grown on different carbon substrates was evaluated. In the present work, the carbon sources in terms of induction of biosurfactant production can be graded as follows: glucose + used engine oil > glucose > sucrose > fructose > used engine oil. This result suggest that the soluble sugar are immediate stimulants of biosurfactant activity in *Pseudomonas aeruginosa* PDKT-2 and when engine oil + sugar was added to a culture, maximum increase in biosurfactant activity occurred (Fig. 49). Several authors reported the production of biosurfactants using different carbon sources by using *Pseudomonas* sp. For example, production of biosurfactant by free and alginate-entrapped cells of *Pseudomonas* sp1895-DSMZ was investigated by Mahmoud Abouseoud *et al.*, (2008) using olive oil as the sole carbon and energy source. Lima *et al.*, (2009) documented biosurfactant production by *Pseudomonas aeruginosa* PACL strain, using a mixture of waste frying soybean oil (WFSO) as a carbon source was compared to production when non-used soybean oil (NUSO), or waste soybean oils used to fry specific foods. NUSO produced the highest level of rhamnolipids, although the waste soybean oils also
resulted in biosurfactant production of 75–90% of the maximum value. A locally-isolated *Pseudomonas aeruginosa* USM AR2 possessing the ability to produce glycolipids type biosurfactant (rhamnolipid) when the culture was fed with petroleum diesel and complex medium. The highest rhamnolipid concentration was achieved when the culture was initially fed with both petroleum diesel and complex medium, followed by feeding of petroleum diesel only at the end of the stationary phase (Salwa *et al*., 2009). *Pseudomonas aeruginosa* was known to produce rhamnolipids on glucose (Tayebe *et al*., 2009) and hydrocarbons (Manoj *kumar et al*., 2008). Recently Obayori *et al*., (2008) reported that the strain *Pseudomonas sp*. LP1 had the ability of biosurfactant production using engine oil as the carbon source. Hence the above study revealed that the isolate *Pseudomonas aerugenosa* PDKT-2 was able to produce biosurfactant from both soluble and insoluble substrates.

In some cases, biosurfactant synthesis is regulated by pH and temperature. For example in rhamnolipid production by *Pseudomonas* sp. (Wagner *et al*., 1984 and Ristau and Wagner,1983) pH played an important role, and in the case of *Arthrobacter paraffineus* ATCC 19558 (Duvnjak *et al*.,1982), *Pseudomonas* sp. DSM 2874 (Wagner *et al*.,1983) temperature was seemed to be important. In all these cases however the yield of biosurfactant production was temperature dependent. The isolate *Pseudomonas aerugenosa* PDKT-2 showed the ability to produced rhamnolipid at a pH range from 6 to 8. More rhamnolipid production was observed at pH 8 than 7 and decreased at pH 6 (Fig. 47). Similar result was reported by Tayebe *et al*., (2009) at pH 8 optimum biosurfactant production by *Pseudomonas aeruginosa* MR01 which was isolated from the oil excavation areas in tropical zone in southern Iran was observed. Cultivation temperature was found to have a significant influence on both rhamnolipid production and growth. More rhamnolipid production was observed at 35° C than at 30° C and decreased at 25° C (Fig. 48). Santos *et al*., 1984 observed maximum rhamnolipid production at a temperature of 32- 34° C. Pruthi and Cameorta, 2003 reported that the biosurfactant was produced optimally at temperature ranges from 30–40° C by *Pseudomonas putida*. These findings supports the present investigation.

Rhamnolipids provide good physicochemical properties in terms of surface activities, stabilities and emulsification activities. Moreover, these surface-active compounds
exhibit antimicrobial activities against both phytopathogenic fungi and bacteria. Due to an increase in concerns about environmental protection and the distinguishing properties of the rhamnolipids, it seems that rhamnolipids meet the criteria for several industrial and environmental applications, such as environmental remediation and biological control. Rhamnolipids have already been commercially produced; making them more economically competitive with synthetic surfactants. In the near future, rhamnolipids may be commercially available successful biosurfactants (Pornsunthorntawee et al., 2010).

In the present study three biosurfactant producing organism has been isolated from used engine oil contaminated sites. Biosurfactants have been proven as the promising agents for bioremediation of hydrocarbons, particularly oil polluted environment. Therefore, novel microorganisms should be intensively screened for bioremediation and these organism are the potential resource for surface-active molecules of industrial importance.

SUMMARY

In the present study biodegradation of multi-component used engine oil was studied by both pure and consortium culture. For this, used engine oil contaminated soil samples were collected from various automobile workshops in Pudukkottai. The physiochemical parameters of the soil samples were analysed. Totally 25 bacterial isolates capable of utilizing used engine oil as carbon source was isolated from the engine oil contaminated soil samples by enrichment method. Three isolates with having hydrocarbon degrading ability coupled with biosurfactant activity were identified by 16S rRNA sequencing and designated as Serratia marcescens PDKT-1 (Accession No:HM998315), Pseudomonas aeruginosa PDKT-2 and Bacillus licheniformis PDKT-5. The dendogram by RFLP mapping revealed their genetic variability. Some of the other dominant isolates, which were not used in this study, were identified by16S rRNA
sequences as *Pseudomonas putida* and *Bacillus subtilis* and by BIOLOG as *Pseudomonas bathycetes*, *Pseudomonas stutzeri*, *Escherichia vulneris*, *Acinetobacter calcoaceticus*, *Vibrio vulnificus* and an unidentified strain. The potential degraders showed significant difference in their antibiotic susceptibility pattern.

The gravimetric results indicate that all three isolates are capable of utilizing engine oil as the nutrient and carbon source. *Pseudomonas aeruginosa* PDKT-2 was found to be the best oil degrading isolate in this study with 81% degradation after 30 days incubation period while 72% and 60% degradation were observed using *Serratia marcescens* PDKT-1 and *Bacillus licheniformis* PDKT-5 respectively, under the standard assay conditions. The consortia proved to be a best degrader compared to individual isolate with degradation rates of 90% in 30 days, and it was confirmed by Gas chromatography. Different nutrients sources and environmental conditions such as pH and temperature were substituted in the growth pattern assay. The results showed that, *Serratia marcescens* PDKT-1 isolated in this study utilized NH$_3$NO$_4$ and Urea in engine oil degradation process. The addition of sodium nitrite reduced the cell growth. *P. aeruginosa* PDKT-2 and *Bacillus licheniformis* PDKT-5 on another hand were capable of utilizing different nitrogen sources including nitrite. Plasmid DNA profile indicated that the isolate PDKT-1, PDKT-2, PDKT-5 harboured plasmid DNA. The size of the extracted PDKT-1, PDKT-2, PDKT-5 plasmid were found to be > 4361bp.

The biosurfactant produced by PDKT-1 and PDKT-5 was characterized as lipopeptide and PDKT-2 resembles rhamnolipid type by using biochemical and FT-IR spectral analysis. The strain PDKT-2 which showed maximum biosurfactant activity was selected for biosurfactant production and the condition for optimum biosurfactant production by PDKT-2 was standardized. The synthesis was further induced when engine oil was added along with glucose. Biosurfactant production was optimum when NH$_3$NO$_4$ and NaNO$_4$ were added. Biosurfactant production was high at 35$^\circ$C and at pH 8.

All the three isolates (PDKT-1, PDKT-2, and PDKT-5) showed positive results for dioxygenase activity. The indole-conversion to indigo was considered as dioxygenase activity. Regarding aromatic hydrocarbon degradation, evaluated by 2,6 DCPIP assay, xylene and nitrobenzene was degraded by all the three strains, where as
no isolate degraded benzene, toluene and benzyl chloride. Aniline was degraded by both PDKT-1 and PDKT-2. The strain PDKT-1 and PDKT-5 showed positive results for phenol. Phenolphthaleine was degraded only by the isolate PDKT-2. For polycyclic hydrocarbons, anthracene was degraded by PDKT-2, while naphthalene was not degraded by any isolate.

In soil microcosms studies, it was found that degradation increased with the incubation period and was maximum at 30 days of incubation where nearly 91% of the hydrocarbon was degraded. At the same time the degradation with the indigenous flora was 32% and for indigenous and bacterial consortium the value was 78%. The GC-FID profile showed reduction in the major peak compounds indicating the ability of the consortium to degrade the components of the engine oil. Thus it is clear from this research that the isolates PDKT-1, PDKT-2, PDKT-5 and bacterial consortium of all three isolates was able to degrade used engine oil coupled with suitable biosurfactant production. Hence they have a potential application in the bioremediation of hydrocarbon polluted sites and can also be used in bio augmentation approaches.