BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CERTAIN BACTERIA FOR APPLICATION IN BIOREMEDIATION OF PETROLEUM CONTAMINATED SOIL

A THESIS
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BY
NABA KR. BORDOLOI, M.Sc.
Registration No. 011 of 2006

School of Science and Technology
Department of Molecular Biology and Biotechnology
ONGC – Centre for Petroleum Biotechnology
Tezpur University, Napaam-784028
Tezpur, Dist. Sonitpur, Assam
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DEDICATION

I DEDICATE THIS THESIS

TO

MY EVER LOVING MOTHER AND BROTHER

MRS. GUNADA BORDOLOI

AND

MR. ANJAN KR. BORDOLOI

Abstract

Petroleum comprises of a vast number of varied compounds that are categorized into aliphatics, aromatics and paraffins. These compounds often become pollutants after being released into different ecosystems at various stages of operations relating to the exploration, transportation, refining, downstream processing and product applications. Considering the huge economic and ecological costs involved in the depletion of the environment resulting from such petroleum pollutions, effective strategies have to be evolved to remediate and restore such polluted environments. The advent of Biotechnology has presented opportunities for cleaning up of petroleum contaminated sites through integration of microbial approaches with other conventional strategies. Bioremediation of polluted sites has emerged as a viable option in the context of operational efficiency and economic considerations.

Assam witnessed industrial activities in its petroleum sector for over a hundred years. This obviously brought in its wake the inherent environmental problems necessitating strategies for rectifying such polluted conditions. The present work which was conceived against this perspective has the following objectives:

1. To isolate bacterial strains from crude oil contaminated soil and water and their characterization.
2. To evaluate their efficiency in biodegradation of crude oil.
3. To isolate and characterize metabolites produced by bacteria isolated from crude oil contaminated sites.
4. To test surface – active metabolites produced by the bacteria in enhanced oil recovery.

Screening of environmental soil samples collected from various petroleum hydrocarbon contaminated sites of Assam yielded 58 pure bacterial isolates. The initial *in vitro* experiments enabled selection of 13 isolates for assessing their potential to degrade petroleum hydrocarbons. Ten of the isolates were subjected to growth kinetics study in different

culture media supplemented with crude oil components like hexadecane, octadecane, dodecane (aliphatic compounds), benzene, toluene, xylene (aromatic compounds), pyrene, fluorene, carbazole (polycyclic aromatic) and hydrocarbon derivatives, like phenol, pyridine and other simple carbon sources and 2,4-Dichlorophenoxy acetic acid (2,4-D).

The synergistic and antagonistic effects of these isolates were studied by culturing them in 24 different combinations in media supplemented with the constituents of crude oil. Two promising combinations, Consortium I and II were selected from the study. Laboratory scale experiments confirmed the potential of the bacterial consortia in bioremediation of soil contaminated with 8% and 20% crude oil contamination. A contamination level of 8% was brought down to 1.9-2.1%; whereas, the contamination level of 20% was brought down to 5.3-6.8% after 180 days of inoculation, the Consortium II displaying better efficiency in both the cases.

Experiments were conducted to assess the extent of bioremediation in terms of plant growth and yield of three crop plants: rice, Bengal gram and green gram. The rice plants performed the best in the remediated soil showing higher growth and yield even without the application of manures and fertilizers.

Out of the bacterial isolates assessed, five were found to be promising biosurfactant producer. These isolates could reduce surface tension of culture medium from 68 $mN.m^{-1}$ to 30.2 $mN.m^{-1}$. Biosurfactants produced by the isolates were found to be Rha $C_8 - C_{10}$ and Rha $C_{10} - C_8$ and lipopeptide in nature. Crude oil and polycyclic aromatic hydrocarbons were found to be solubilized with the addition of isolated biosurfactants.

Plasmid DNA of the selected bacterial isolates was isolated and characterized through restriction digestion. Curing experiments were carried out to confirm the presence of the biosurfactant producing gene(s) in the plasmid DNA. Thermophilic bacterial isolates (>70°C) devoid of biosurfactant producing ability were genetically transformed by mobilizing

the plasmid DNA from the biosurfactant producing bacteria. The transformants were selected by culturing in selective medium supplemented with tetracycline. However, the transformed bacteria failed to survive in petroleum hydrocarbon supplemented selective liquid medium at temperature above 70°C.

The efficiency of biosurfactants isolated from bacterial isolates in the mobilization of crude oil at different temperatures was investigated using crude oil- saturated sand packed column. The biosurfactant treatment could release 60±1.2% crude oil from the saturated sand packed column at 90°C. The result indicated the potentiality of biosurfactants in microbial enhanced oil recovery.

Declaration

I hereby declare that the research work presented in the thesis was carried out in the Department of Molecular Biology and Biotechnology under the School of Science and Technology, Tezpur University, Napaam, Tezpur–784028, Dist. Sonitpur, Assam. No part of the thesis was reproduced elsewhere for award of any other degree.

Date: 29.12.2006
Place: Tezpur University, Napaam

(Naba Kr. Bordoloi)
Registration No.011 of 2006

TEZPUR UNIVERSITY (CENTRAL)
Department of Molecular Biology & Biotechnology
Napaam, Tezpur-784 028
Dist. Sonitpur, Assam, India

B.K. Konwar, Ph.D. (London) DIC
Professor & Head
E mail: bkkon@tezu.ernet.in

Ph. (O) 91-03712-267172 -267007/8/9 Ext.5401/02
(R) 91-03712-267316 Mobile: 9435380139
Fax 91-03712-267005/6

CERTIFICATE OF THE SUPERVISOR

This is to certify that the thesis entitled "Biochemical and Molecular characterization of certain bacteria for application in bioremediation of petroleum contaminated soil" submitted to the Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research work carried out by Mr. Naba Kr. Bordoloi under my personal supervision and guidance.

All helps received by him from various sources have been duly acknowledged.

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Date: 29.12.2006
Place: Tezpur University, Napaam

(B. K. Konwar)
Signature of Principal Supervisor
Professor and Head
School of Science and Technology
Dept. of Mol. Biol. and Biotech.

TEZPUR UNIVERSITY

CERTIFICATE OF THE EXTERNAL EXAMINAR AND ODEC

This is to certify that the thesis entitled "Biochemical and Molecular characterization of certain bacteria for application in bioremediation of petroleum contaminated soil" submitted by Mr. Naba Kumar Bordoloi to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us on 17th Jan, 08 and found to be satisfactory.

The Committee recommends for the award of the degree of Doctor of Philosophy.

Signature of:

Principal Supervisor

Date: 17.01.08

External examiner

Date: 17/01/08

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List of Abbreviations

Abs.	Absorbance
A.U.	Absorbance unit
ADSA	Axisymmetric drop shape anaysis
BSA	Bovine serum ablbumin
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
CMC	Critical micelle concentration
CMD	Critical micelle dilution
C:N:P	Carbon, Nitrogen, Phosphorus
CSH	Cell surface hydrophobicity
DNA	Deoxyribonucleic acid
EPA	Environmental protection agency
EOR	Enhanced oil recovery
E.I.	Emulsification index
ft	Feet
g	gram
GC	Gas chromatography
h	hour
HAB	Hydroxylamino benzene
HPLC	High performance liquid chromatography
l	litre
LPH	Light petroleum hydrocarbon recovery
LEL	Lower explosion limit
LPH	Liquid phase hydrocarbon
LPG	Liquid petroleum gas
LNAPLs	Light non aqueous phase liquids
LB	Luria Bertani
MTBE	Methyl tert-butyl ether
MeOH	Methanol
MEK	Methyl ethyl ketone
MEOR	Microbial enhanced oil recovery
MSM	Mineral salt medium
mg	milligram
$mN.m^{-1}$	Milli Newton per meter
min	minutes
n	Normal
NaCl	Sodium chloride
Nm	Nanometer
NAPLs	Non aqueous phase liquids
O.D.	Optical density

PBS	Phosphate buffer saline
PCBs	Polychlorinated biphenyls
PCE	Perchloroethylene
ROI	Radius of influence
rmp	Revolution per minute
SNO	Sulphur, Nitrogen, Oxygen
SVE	Soil vapor extraction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TCA	Trichloroethene
TCE	Trichloroethylene
TPH	Total petroleum hydrocarbon
UST	Underground storage tank
USEPA	United State Environmental Protection Agency
UV/Vis	Ultra violet/Visible
v/v	Volume/volume
viz.	Namely

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Chapter 1
Introduction

Chapter 1

Introduction

The origin of petroleum on earth might have taken place millions of years ago. There are unmistakable evidences that oil was formed deep inside the earth through a very long geo-evolutionary process. The detection of crude oil in the mud volcanoes in several parts of the world is well reported. Seepage of oil from the depths of the earth must have been possible through the fissures, fractures, faults and other such geological conduits (Deshpande, 1992). The oil that is occasionally detected on the oceanic and soil surfaces is obviously the result of such seepages. The significant point to be noted however is that the changes in some of the physical properties of oil and also in its chemical composition had taken place due to the interaction of oil with various external factors like solar radiation, influence of wind etc.

Drake (1857) and Sidorov (1885) had drilled oil in the USA and the USSR respectively from the sedimentary rocks. The occurrence of oil mostly in the sedimentary rock strongly points towards the biogenic origin of oil. The subsequent detection of many constituents of oil that have close similarities with compounds of plant and animal origin gives credence to this theory. The generally high molecular weights of the compounds of organic origin, presence of porphyrins and other nitrogenous compounds in oil, the ability of petroleum to rotate the plane of polarized light resembling similar property of plant sugar and several other evidences strongly point towards the biogenic origin of crude oil.

The discovery of petroleum and its production in Assam in the last century has placed the name of India in a select band of countries that have completed a hundred years in oil industries. Oil exploration in India started as early as the first part of the 19^{th} century that too in Assam. The drilling of oil in Assam started just after 7 years of Drake's work in the USA. In India,

the first well was dug by hand at Nahorpung, near Jaipur, Dibrugargh, Assam in 1889. However, drilling was started at Digboi in 1889 and the well was completed in the next year making the beginning of the oil industry in India. It is noteworthy that the first well in Digboi oil field which was completed as producer in 1890, still producing to commemorate the oil centenary. Assam has one of the largest oil deposits in India. The major oil fields of the country are in Naharkatia, Moran, Lakuwa, Rudrasagar, Galeky, Borhola, Kharaghat and Uriumghat, Bombay High, Godabory basin etc.

1.1. Petroleum hydrocarbons

Crude oil is not a uniform substance: its appearance and characteristics vary widely from oil field to oil field and even from well to well in the same oilfield. It may not be even a homogenous substance, as it can contain, besides dissolved gases, dissolved solids and colloidal suspensions. It can be defined as a naturally occurring mixture, consisting predominantly of hydrocarbons, and or of sulphur, nitrogen and oxygen derivatives of hydrocarbons, which is removed from the earth in liquid state.

The physical characteristics of crude oil; can vary from light, mobile liquids of reddish brown color with a large proportion of easily distillable fractions, to highly viscous, semi – solid black substances with very little distillable material before decomposition occurs. The density can vary from 0.8 to almost 1.0.

In spite of the wide differences in the physical aspect of different crude oils, their ultimate or elemental analysis show a remarkable consistency. The elements present in crude vary in a limited extent.

Table 1.1. Constituents of crude oils (in percent weight)

Elements	% of weight
Carbon	83.9 – 86.8
Hydrogen	11.4 – 14.0
Sulphur	0.06 – 8.00
Nirtogen	0.11 – 1.70
Oxygen	0.5
Metals (Fe, V, Ni etc.)	0.03

1.2. Constituents of crude oils

Most natural materials like crude oil are complicated still, being mixtures of many substances, which are often difficult to separate. Most of the substances, which make up crude oil, have atoms of hydrogen and carbon only in their molecules. For these reasons they are called "hydrocarbons". Hydrocarbons are present in crude oil in a great variety of forms, which differs in the number of carbon and hydrogen atoms in their molecules and in the way in which the atoms are linked with one another.

The hydrocarbons present in crude oils range from methane (one carbon atom in the molecule) up to molecules containing 60 or more carbon atoms i.e., a range of molecular weights from 16 up to 850 or more. They are members of the homogenous series of even hydrogen numbers or lower in hydrocarbon content.

$$C_n H_{2n+2}, C_n H_{2n}, C_n H_{2n-2} \text{ --------}$$

These homogeneous series are grouped into the following classes:

1. Normal or straight - chain paraffins (*n* - alkanes)
2. Branched – chain paraffins (iso – paraffins, iso - allkanes)
3. Napthenes (cycloparaffins, cycloalkanes)
4. Aromatics (arenes)
5. Mixed naptheno – aromatics

Normal or straight - chain paraffins {*n* – alkanes (30 – 70%)}:

Saturated hydrocarbons are formed by open chains. They are important constituents of light crude oils (paraffinic crudes), particularly in the low boiling fractions and their concentration decreases progressively with increasing molecular weight of the fractions. Straight chain alkanes (*n* - alkanes) of well over 30 carbon atoms.

C-C-C-C-C-C-C-C-C-C

Normal Paraffin

Branched – chain paraffins (iso – paraffins, iso - allkanes):

Saturated hydrocarbons, with one or more alkyl side chains. Branched isomers (methyl *n* - alkanes) in the range C_6 –C_8.

Napthenes or cycloalkanes (16 – 64%):

The cycloalkanes or cycloparaffins commonly present in petroleum are mainly, methylcyclopentane, alkylcycolhexane etc.

Aromatics (arenes) Hydrocarbons (8 –15 %):

Low molecular weight mononuclear aromatics (bezene, toluene, xylene, ethyl benzene etc) are present in appreciable amounts in virgin gasoline from light crude oils and with increasing number of alkyl substituents as they appear in kerosene and higher boiling fractions.

Mixed naptheno – aromatics:

Compounds containing napthenic rings, aromatic rings and alkyl side chains in the same molecule. As a class they begin to appear in the kerosene fraction, increasing in concentration in the higher boiling fractions and residues.

Non – hydrocarbon components (S, N, O compounds):

Crude oil contains appreciable amount of organic non- hydrocarbon constituents, mainly sulphur, nitrogen and oxygen containing compounds and in smaller amounts, organo metallic compounds in solution and inorganic salts in colloidal suspension. The sulphur compounds present to the extent of 6 % include marcaptants (R - SH) and sulphides (R –S -R). The nitrogenous compounds found include alcohols, phenols and resins. These constituents appear throughout the entire boiling range of crude oil, but concentrate mainly in the heavier fractions and in the non – volatile residues.

1.3. Soil contamination by crude oil and their remediation

Soil contaminated with petroleum products is a pervasive problem. Petroleum industry has been growing for decades, at the same time, there have been increasing cases of leakage and spill in various petroleum-related processes. These are the major sources of petroleum contamination and have caused significant impacts on the environment (Norris *et al.,* 1994; Riser-Roberts, 1998). The petroleum contaminants may remain on the ground surface, volatilize into the atmosphere, or leach through subsurface, resulting in extensive pollution concerns (Norris *et al.,* 1994). Bioremediation, which harnesses naturally occurring biochemical processes, is a more environmentally acceptable method to remove oil pollution than chemical treatment alone. Bioremediation destroys contaminants, rather than transfer them.

Most of the chemicals, such as benzene, toluene, ethylbenzene and xylenes (BTEX) are listed as toxic constituents (Freeman *et al.,* 1998) and priority water pollutants (Liu *et al.,* 2000). These toxic condensates are frequently stored in underground storage tanks that are subject to corrosion and structural failures with subsequent leaks, which is recognized as a major environmental concern (Craig *et al.,* 1995).

Among various pollution-control technologies, bioremediation has been under use since the last 30 years and receiving more importance (Ryan *et al.,* 1991) in the recent years. It is evaluated as a safe and cost-efficient method for the disposal of underground petroleum contaminants (Burke *et al.,* 2000).

Bioremediation has been defined by Madsen (1991) as a managed or spontaneous process in which biological, especially microbial, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination. Harmful hydrocarbon contaminants may be assimilated by microorganisms and converted into biomass or transformed by cells or cell free enzymes (Babel, 1994). Bacteria capable of biodegrading petroleum hydrocarbons may commonly be found in subsurface soils; however, natural breakdown of the compounds will occur to slowly without intervention to prevent accumulation of the pollutants to unacceptable levels (Lyman *et al.*,1990). Bioreclamation or bioremediation refers to the enhancement of this native capability of the microorganisms. The naturally occurring microbes can be stimulated, or specially developed microorganisms can be added to the site to degrade, transform, or attenuate organic and organometallic compounds to low levels and nontoxic products (Catallo and Portier, 1992).

Environmental contamination by petroleum products is widespread, and development of remediation efforts must accommodate the fact that there are vast number of sites that need to be treated and that there will be individual requirements for each site (McGugan *et al.*, 1995). Appropriate measure must also be available for those areas of the world, which cannot afford expensive remediation practices and may not even have the driving legislation to enforce them to clean up the pollution. Site remediation must be simple and cost-effective for both industrialized and developing countries to be able to confront and resolve this enormous problem.

More than $1 million a day was spent in a partially successful attempt to clean up the oil spill at Price William Sound, Alaska (Atlas, 1991). Neither

government nor private industry can afford the cost of physically cleaning up the known toxic waste sites. Because of the large volumes of unsaturated soils contaminated with metals and organic solvents, *in situ* remediation is often the most economically attractive remediation technique (Lindgren and Brady, 1995). Therefore, bioremediation is now being considered as a viable solution to this problem.

1.3.1. Bioremediation approaches

There are two bioremediation approaches: microbiological approach and microbial ecology (Piotrowski, 1991). The microbiological approach involves supplying microorganisms that have been conditioned to degrade target compounds, along with appropriate nutrients, to the subsurface. These organisms could be prepackaged 'superbugs', which are the strains developed in the laboratory and shipped to a contaminated area, or there could be site-specific superbugs, which have been isolated from the affected area itself and reintroduced at higher concentrations. The second approach, on the other hand, involves altering the environment of the indigenous microorganisms to optimize biodegradation of the contaminants.

Bioremediation can also be expressed as being engineered or intrinsic. Any modification of the bioremediation process is considered engineered bioremediation, and the lack of intervention is intrinsic bioremediation, or natural attenuation (Hart, 1996). Intrinsic remediation results from several natural processes, such as biodegradation, abiotic transformation, mechanical dispersion, sorption, and dilution that reduce contaminant concentrations in the environment (Morin, 1997). For natural attenuation to be a viable approach, the site must have a high natural supply of nutrients and oxygen, and the source of contamination must be small (Hart, 1996). Intrinsic bioremediation, also known as natural or passive bioremediation, is essentially allowing nature to take its own course. But, it does not entail a 'no action' approach. The presence of indigenous bacteria capable of breaking down site-specific contaminants must first be verified.

Engineered *in situ* bioremediation involves the design and installation of systems designed for the purpose of supplying microbe- simulating materials into the subsurface. Engineered systems can, in turn, be broadly categorized as either biostimulation or bioaugmentation systems. Biostimulation refers to the addition of oxygen alone (in aerobic systems) or the addition of both oxygen and nutrients to the subsurface. Bioventing pertains to the process of supplying induced air flow, whereas air sparging or biosparging refers to the process of injecting air into the groundwater under low pressure. Bioaugmentation is the process of adding nonnative bacteria to the subsurface to work together with the indigenous bacteria in breaking down the contaminants. Bioaugmentation typically also includes adding electron acceptors and nutrients. Significant evaluation up front and follow-up monitoring are necessary to ensure removal of contaminants of concern at reasonable rates. There are various approaches for determining whether or not a site would be appropriate for remediation via biological means. Ogunseitan (1996) advocated environmental testing and analysis to characterize the site first as to determination of the chemical nature, concentration, and hydrogeological context of the pollutants; the existence, diversity, and population densities of relevant biodegradative organisms; engineering constraints to implement *in situ* or contained system bioremediation; as well as microcosm bioassays (remediation cost estimations and establishment of conditions supportive to bioremediation).

Sites recently contaminated by oil spills, containing high contaminant concentrations, toxic metals and organic compounds as well as those containing extremely low, but unacceptable contaminant concentrations tend to favor the microbiological approach (Piotrowski, 1991).

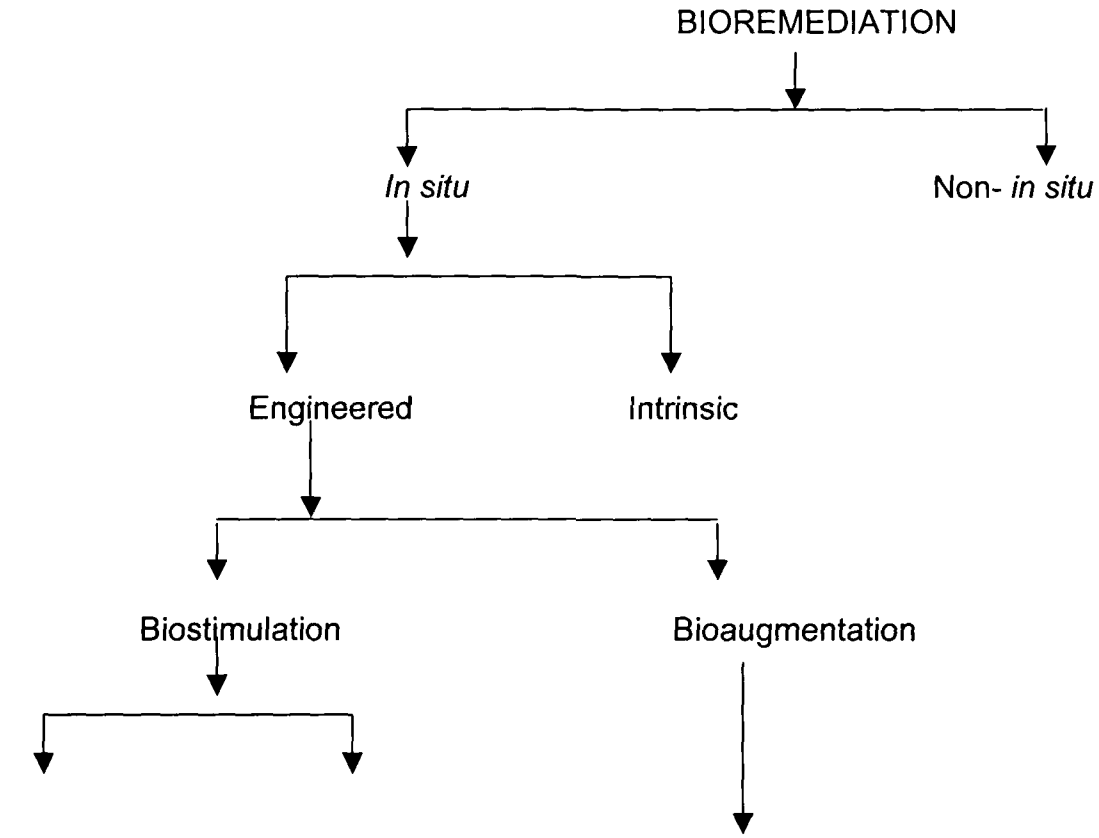


Fig.1.1. Types of bioremediation

Note: For Aerobic Systems

1.3.2. *In situ* bioremediation

In situ treatment of waste and soil at contaminated sites offers an alternative to the traditional approach to site remediation involving excavation and redisposal or on site isolation or treatment (Ghassemi, 1988). They include biodegradation, air/steam stripping, neutralization, solidification/stabilization, and oxidation and can be used in combination as a treatment train.

Bioremediation is sometimes referred to by other names such as biorestoration, biodegradation, or bioventing. Bioventing actually refers to the process of supplying air to the vadose zone to stimulate *in situ* biological activity. Bioremediation treatment systems can be broadly grouped into two categories: *In situ* and non- *in situ*. Basically, in situ systems are those where the contaminated medium (soil groundwater) is not physically moved or transported from its original location. Non-*in situ*, or above-ground systems involve brining the contaminated medium to the surface for treatment.

Non-*in situ* bioremediation usually involves the design and construction of an above ground bioreactor or biofilter for the treatment of contaminated groundwater. Biological treatment of excavated soil is also considered a non-*in situ* method.

Table 1.2. Petroleum hydrocarbon remediation technologies and their applicability

Technology	**Applicability**	**Soil types and saturated zone characteristics**
Light petroleum hydrocarbon recovery LPH withdrawal	All lighter- than-water petrochemicals except for the most viscous fuel and lube oils	Works better with more permeable soils
Vadose zone Soil vapor extraction	LPH less than about 0.5 ft, contaminants with Vp>1mmHg(BTEX, gasoline, MTBE, PCE, TCE, TCA, mineral spirits, MeOH, acetone, MEK, etc.)	Permeable soils, ROI>10 ft, depth-to-water greater than 3 ft
In situ percolation (bioremediation)	Any aerobically biodegradable chemical in the vadose zone	Works better in permeable soils; depth-to-water greater than 3 ft
Excavation	All soils and contaminants	All soil types

Saturated zone Sparging	Contaminants in saturated zone with K_H>0.1 and Vp>1 mmHg; contaminants: BTEX, gasoline, PCE, TCE, TCA, mineral spirits	Hydraulic conductivity>10^{-5} cm/s(silty sand or better); at least 5 ft of saturated thickness
In situ Bioremediation	Any biodegradable chemical in the saturated zone; inhibited by pH extremes, heavy metals, and toxic chemicals	Nutrients are transported better in more permeable soil
Excavation	All soil and contaminants	All soil types
Ground water recovery and treatment Groundwater recovery	Uses: (1) LPH recovery, (2) provides hydraulic control of contaminant plume, (3) pump and treatment technologies	Transmissivity, depth-to-water and saturated zone thickness determine optimal strategy
Liquid phase carbon	Removal of compounds with low solubility/ high adsorptivity	See ground water recovery
Air stripping	Compounds with K_H> 0.1; contaminants with K_H between 0.01 and 0.1 may require an air water ratio >100	See ground water recovery
Advanced oxidation	Most effective on sulfide cyanide, doublebonded organics (PCE, TCE), BTEX, phenols chlorophenols, PCBs, PAHs, some pesticides	See ground water recovery

Table 1. 2. (continued)Technology Applicability

Technology	Applicability	Soil type and saturated zone characteristics
Bioreactors	Any biodegradable compound	See ground water recovery
Off-gas treatment Vapor-phase carbon	Adsorptive capacity generally increases with increasing molecular weigh	NA
Catalytic oxidation	Conventional units can treat all compounds containing carbon, hydrogen,and oxygen; concentrations should not exceed about 20% of the LEL	NA
Thermal oxidation	Compounds containing carbon, hydrogen, and oxygen; usually no amenable to halogen-containing compounds	NA

NA, not applicable; LEL, lower explosion limit; ROI, radius-of- influence; LPH, liquid-phase hydrocarbon; MTBE, methyl *tert-* butyl ether; PCE, perchloroethylene; TCE, trichlorethylene; TCA, trichloroethane; MeOH, methanol; MEK, methyl ethyl ketone; BTEX, benzene, toluene, ethylbenzene, and xylenes; PCBs, polychlorinated biphenyls; PAHs, polycyclic aromatic hydrocarbons.

Source: Eve Riser-Roberts, Remediation of Petroleum contaminated soils, Lewis publishers, 1998.

1.3.3. Natural attenuation and intrinsic bioremediation

The basic principle of attenuation is the mixing of contaminated soil (or waste) with clean soil to reduce the concentrations of hazardous compounds to acceptable levels. Natural decay or attenuation describes the naturally occurring assimilation of underground contaminants through physical, chemical, and/or biological means. Intrinsic bioremediation is a remediation technology that relies on these measures. Intrinsic bioremediation processes do not include any enhancement of on-site conditions, but comprise a series of site assessment and data processing

methods that can identify the capacity of a pristine site to remediate contamination.

Assessment of an intrinsic bioremediation process usually includes (a) determination of the existence of steady-state contaminant plumes, (b) estimation of natural contaminant degradation rates, (c) estimation of the mass of contaminant source, (d) estimation of the source lifetime, (e) prediction of long-term plume behavior with and without source removal, (f) decision making regarding the use of intrinsic bioremediation and desirability of source removal at a given site, and (g) development of a long-term monitoring strategy if intrinsic bioremediation is selected for plume management (Hyman and Dupont, 2001).

However, the indigenous species may act, but often not sufficiently fast to prevent the spreading of a local contamination. Intrinsic bioremediation process is thus not appropriate when a rapid biodegradation rate is required, i.e., the plume is moving out of the controlled area or the rate of contaminant release from the source area is higher than the contaminant degradation rate observed at the site. Such an improper application of intrinsic bioremediation may result in the movement of the contaminants through soil to underlying groundwater and the further transport of pollutants to water bodies used for human, animal or plant consumption; also the uptake by on-site plants of toxicants in contamination plume. The enhancement of the bioremediation processes is then desired (Alexander, 1999).

1.3.4. Enhanced *in-situ* bioremediation

In situ bioremediation is a commonly accepted cost-efficient method that has been widely applied to subsurface contamination problems (Norris and Kerr, 1994; O'Mara, 1996). It has larger treatment zones than *ex situ* options, and reduces the risk to the workers and the community by minimizing disturbances of contamination and produces no solid residues for further cleanup (Ward *et al.*, 1997).

Disadvantages of *in situ* bioremediation are set forth by Hyman and Dupont (2001) as follows: (a) toxicity within the site limits the applicability and performance (b) injection well clogging is a recurring problem, (c) transfer-efficiency limitations of nutrient and electron acceptors caused by disadvantageous soil properties can severely limit the remediation efficacy, and (d) high concentrations of nutrients can impact groundwater quality adversely.

The potential utility of *in situ* bioremediation lies in the capabilities of functional microorganisms. Thus, the process of *in situ* bioremediation can be enhanced by inoculation of specific microorganisms known to degrade on-site contaminants and/or enhancement of overall indigenous microorganisms by adding nutrients or electron acceptor.

Generally, petroleum degraders can be isolated from petroleum contaminated sites and re-injected to the site after an enlarged culturing to increase the microorganism quantity so that underground biodegradation can be amplified (Wainwright, 1999). The problem of this method is that inoculated microorganisms may not survive or would have a hard time competing with the existing species. Moreover, the addition of externally acclimated organisms to a contaminated site may be perceived by the public as the addition of engineered, mutant organisms that may not be publicly acceptable (Hyman and Dupont, 2001).

Nutrients are often present in lower-than-necessary concentrations in both aquifers and vadose zone soils (Eweis *et al.*, 1998). To enrich the nutrient level, groundwater is typically recovered, treated, amended with nutrients, and introduced back into the subsurface either by injection wells for deep aquifers or infiltration galleries for shallower ones (Wong *et al.*, 1997).

Nitrate can be applied as a growth nutrient as well as an electron acceptor. Nitrate, sulfate, ferric iron, carbon dioxide and, in some cases, hydrogen peroxide are injected to the subsurface as electron acceptors to

overcome the limitation imposed by oxygen depletion or its low solubility in water (Lee *et al.*, 1991 and Borden *et al.*, 1995).

In addition, surfactant-enhanced bioremediation has been suggested as an economically and technically feasible approach (Churchill *et al.*, 1995). Synthetic surfactants, such as Triton X-100 and Tween 80, have been used to increase the rate of organic pollutant desorption from soil particles, and thus increase the bioavailability of contaminants (Smith and Burns, 2001). In recent years, biosurfactants have been used in many fields like food processing and chemical industries (Rahman *et al.,* 2002). Biosurfactants are biologically produced surfactants, which are generated by microorganisms during growth on some insoluble organic substrates. They are often complex mixtures of protein, lipid, and carbohydrates. A few are identified as rhamnolipids, trehalose-containing glycolipids, phospholipids, and lipopolysaccharide (Bitton, 1984). Compared with chemical surfactants, biosurfactants are biodegradable, more economical, less toxic, and highly resistant to environmental changes (Banat, 1995).

There are many technologies available for treating sites contaminated with petroleum hydrocarbons; however, the selection of the treatment method depends upon contaminants and site characteristics, regulatory requirements, costs, and time constraints (Ram *et al.*, 1993). The successful treatment of a contaminated site depends on designing and adjusting the system operations based on the properties of the contaminants and soils and the performance of the systems, and by making use of site conditions rather than force-fitting a solution (Norris *et al.*, 1994).

1.3.5. Bioventing (soil) and bioslurping

Bioventing is an *in situ bio*remediation technology that introduces air or oxygen through extraction or injection wells into the unsaturated zone to enhance the activities of the indigenous microorganisms to biodegrade organic constituents adsorbed to soil particles. Nutrients are added when

necessary. Soils in the saturated zone and the capillary fringe are not affected in this process.

Bioventing defers from soil vapor extraction (SVE) due to inclusion of its

biological components. While SVE removes constituents primarily through volatilization, bioventing systems stimulate biodegradation of contaminants and minimize volatilization by lower airflow rates. Also, bioventing is not constrained by volatility of the underground contaminants and therefore is applicable to less volatile chemicals (Hinchee, 1994). Moreover, since treatment of the off-gas is not required, bioventing can be more cost-effective than SVE (Reisinger *et al.*, 1994).

Bioslurping is a relatively new technology that integrates pump and treatment, SVE and bioventing methods. The process is designed to recover free-, gaseous- and aqueous-phase contaminants through the same extraction well, and simultaneously enhance the activities of the indigenous microorganisms in unsaturated zones as bioventing does. The vacuum-enhanced pumping minimizes free-phase contaminant and groundwater draw down near the extraction well and maximizes fluid recovery as free product moves horizontally along high-transmissibility flow paths and into the well. At the same time, air flow due to pressure induced gradient in vadose zone provides oxygen for indigenous microorganisms and thus intensifies the on-site biodegradation (Hyman and Dupont, 2001).

1.3.6. Biosparging

Biosparging has the same mechanism as bioventing does, while it enhances the activities of the indigenous microorganisms to biodegrade organic constituents by air (or oxygen) and nutrient (if needed) injection. These constituents can be dissolved in the saturated zone, adsorbed to soil below the water table, or retained within the capillary fringe. The process is similar to air sparging, except that the gas is injected at lower pressure with

the intent of enhancing biodegradation rather than volatilization of contaminants. (Brown *et al.*, 1994).

1.3.7. Phytoremediation

Phytoremediation, referred to as vegetative remediation, natural plants can help remediate a contaminated site by accumulating contaminants (Johns and Nyer, 1996). It is a cost-effective, technically effective, and environmentally favorable *in situ* bioremediation technology that uses plants to absorb and metabolize inorganic or organic chemicals, and thus realizes the destruction and removal of contaminants from groundwater and soil (Nyer, 1998). Meanwhile, the rhizosphere has been shown to be a source of diverse microbial populations, which can increase the degradation of a series of less soluble organic compounds (Nyer, 1998).

1.3.8. On-site or *ex situ* processes

On site or *ex situ* method is based on the principle of physically removing the contaminants from the contaminated site by excavation. Excavation is easy to perform, and it rapidly removes the contamination from the site in a matter of hours, as opposed to other remediation methods, which may require several months. It is often used when urgent and immediate action is needed.

However, there are problems associated with excavation. It allows uncontrolled release of contaminant vapors to the atmosphere. Nearby buildings, buried utility lines, sewers, and water mains could be in the way, and above ground treatment approaches tend to be more expensive than *in situ* methods. Contaminated soil may be considered a hazardous waste, and disposal is becoming increasingly restricted by regulation. In addition, the excavation site must be filled.

1.3.9. Microorganisms in Bioremediation

Microorganisms are the principal agents responsible for the recycling of carbon in nature. In many ecosystems there is already an adequate indigenous hydrocarbonoclastic microbial community capable of extensive oil biodegradation, provided that environmental conditions are favorable for oil-degrading metabolic activity. The ability to utilize hydrocarbons is widely distributed among diverse microbial populations (Atlas, 1977, 1981). Many species of bacteria, cyanobacteria, filamentous fungi, and yeasts coexist in natural ecosystems and may act independently or in combination to metabolize aromatic hydrocarbons (Gibson, 1982; Cernigila, 1984; Fedorak, Semple, and Westlake, 1984). Bacterial genera capable to degrade hydrocarbons are *Achromobacter, Aerobacillus, Alcaligenes, Arthrobacter, Bacillus, Bacterium, Beijerinckia, Botrytis, Citrobacter, Clostridium, Corynebacterium, Desulfovibrio, Enterobacter, Escherichia, Flavobacterium, Gaffkya, Methanobacterium, Micrococcus, Micromonospora, Mycobacterium, Pseudomonous, Sarcina, Serratia, Spirillum* and *Thiobacillus;* Actinomycetes - *Actinomyces, Endomyces* and *Nocardia;* filamentous fungi -*Aspergillus, Cephalosporium, Cunninghamella, Torulopsis, Trichoderma* and *Saccharomyces* and Yeasts - *Candida, Rhodotorula* and *Torula.*

In general, population levels of hydrocarbon utilizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons (Atlas, 1981). In unpolluted ecosystems, hydrocarbon utilizers generally constitute less than 0.1% of the microbial community; in oil polluted ecosystems, they can constitute up to 100% of the viable microorganisms. This difference seems to quantitatively reflect the degree or extent of exposure of an ecosystem to hydrocarbon contaminations.

Individual organisms are not restricted to one oil or a limited range of oil types. Each organism attacks many different oils with comparable facility. However, this is not the case when individual hydrocarbons or classes of

hydrocarbons are considered (Bausum and Taylor, 1986). The overall effect of an organism on a complex substrate is limited by its capacity to attack only certain substrates or to accumulate intermediates that it cannot further degrade. Extensive degradation of petroleum pollutants generally is accomplished by mixed microbial populations, rather than single microbial species (Atlas, 1978a).

The heterotropic bacteria are the most important organisms in the transformation of organic hazardous compounds, and soil treatment schemes may be directed towards enhancing their activity. Heterotrophs can use the organic contaminants as sources of both carbon and energy. (Knox *et al.*, 1986). Some organic materials are oxidized for energy while the rest is use as building blocks for cellular synthesis. Bacteria are predominantly involved with degradation of those chemicals that have a higher degree of water solubility and are not strongly absorbed (Kaufman and Plimmer, 1972).

1.3.10. Factors influencing biodegradation of petroleum hydrocarbon

There are three key factors - physical, chemical and biological are influencing microbial activities, and therefore either accelerate or restrain the microbial deconstruction of petroleum hydrocarbons. Meanwhile, the characteristics of the hydrocarbons and the underground soil texture and structure also have great impacts on biodegradation processes. A successful bioremediation needs to identify these rate-limiting factors and realize the delivery of those factors to the bacteria.

1.3.11. Factors influencing microbial growth

Microorganisms can live and reproduce in rigid environments. At the same time, suitable conditions normally lead to a better growth and faster metabolism, which enables the biodegradation of hazardous organic compounds surrounding the microbial cells. The most important soil factors that affect degradation are water, soil pH, aeration or oxygen supply,

available nutrients, i.e., nitrogen (N), phosphorus (P), potassium (K), sulfur (S), oxidation/reduction potential, and soil structure and texture.

Soil temperature is one of the most important parameters regulating the activities of microorganisms. It influences the response of microorganisms directly by its effects on growth rate, enzyme activity, cell composition and nutritional requirement. Under unfavorable temperature, metabolism in microbe cells becomes slow or even stops and the cells turn into the dormant state. Soil temperature use to have more pronounced influence on light petroleum contaminant degradation than soil nitrogen and phosphorus levels (Walworth *et al.,* 1995). The optimal temperature for common petroleum-degradable microorganisms is usually about the room temperature 25°C (Moran *et al.,* 1997). Availability of psychrophilic microorganism in subsurface and groundwater is therefore an important factor during the operation of bioremediation.

Nutrient availability is another critical factor. Deficiency of nutrients may severely limit growth of microorganisms and thus biodegradation of petroleum contaminants. In addition to organic compounds including petroleum hydrocarbons that serve as carbon and energy source, a group of other nutrient elements are required, such as nitrogen, phosphorus, potassium, sulfur, magnesium, calcium, iron, sodium, amino acids, B vitamins, fat-soluble vitamins, and other organic molecules. In case of underground bioremediation, nitrogen and phosphorus are frequently in short supply among these nutrients (EPA, 1985). Ratio for C:N:P is of significance as well. Deficiency or plethora of N or P will reduce the efficiency of biodegradation. Typical ratios that have been proposed are 100:15:3 (Zitrides, 1983), 120:10:1 (Alexander, 1977), 250:10:3 or 100:10:2 (Staps, 1990), and 100:10:1 (Miller, 1990). Besides, it has been recognized that a low concentration of N (approximately 300 mg/kg soil) is more realistic due to toxicity considerations (Wibowo, 1996).

Biodegradation of waste chemicals in the soil requires water for microbial growth and for diffusion of nutrients and by – products during the

breakdown process. Extremes of very wet or very dry soil moisture markedly reduce waste biodegradation rates. Biodegradation of simple or complex organic materials in soil is commonly high at 50 to 70% of the soil water holding capacity (Pramer and Bartha, 1972). Inhibition at levels below 30 to 40% is due to inadequate water activity, and high values interfere with soil aeration. Moisture is a critical parameter for degradation of two, three, and four ring polycyclic aromatic hydrocarbons (PAHs), and it has been found that degradation is considerably greater at 80% than at 40% of field capacity (Loehr, 1992). Holman and Tsang (1995) determined that a water content of 50 to 70% of field capacity was optimum for biodegradation of aromatic hydrocarbons to proceed at maximum rate. However, excessive water will displace air from pores in soil, which inhibits gas exchange and results in anaerobic zones and elimination of aerobic processes (Eweis, 1998).

Usually, at moderate conditions, biodegradation tends to be faster. For example, extreme acidity or alkalinity will lead to a decline of microbial activities; moderate soil pH (6 to 7) always results in a higher biodegradation rate (Wainwright, 1999). It is a common practice to add lime to bioremediate acid soils or subsoil materials containing harmful organic compounds (Alexander, 1999). High salinity is harmful to most microorganisms. Microbial processes in such environments are inhibited due to the extraordinarily high osmotic pressure (Alexander, 1999).

Natural inhibitors (e.g. toxins) that affect microbial growth or survival are present in polluted or even unpolluted soils and waters. Bacteria must be resistant to these toxins in order to function during the biodegradation processes. Petroleum contaminants may be cellular poisons inhibiting both microbial growth and activity (Wang *et al.*, 2002; Kropp and Fedorak, 1998; Okpokwasili and Odokuma, 1996). Different microorganisms have varied thresholds to petroleum hydrocarbons, while these hydrocarbons could serve as a stimulant to microbial growth provided that the concentration is not toxic (Ward *et al.*, 1997). Surface vegetation also acts as a factor affecting microbial growth. Bacteria numbers in soil are substantially greater

with vegetation on soil surface as compared to those without plants. The rhizosphere is usually a zone of intense biodegradation activities (Alexander, 1999). However, the presence of plants is not always expediting biodegradation, as in the case of benzene degradation in soils planted with alfalfa (*Medicago sativa*). Evidences of existence and proliferation of specific microbial groups that degrade contaminants must be shown to confirm the rhizosphere effects (Ferro *et al.*, 1997).

In addition, with the presence of hydrocarbon-degrading microorganisms, some other organisms often act as predators, parasites or lysis inducers, such as protozoa, bacteriophages, viruses and organisms that excrete enzymes which destroy cell walls of fungi and bacteria and thereby cause their lysis. These organisms may largely reduce the number of bacteria by grazing. However, they might also facilitate the cycling of limiting inorganic nutrients especially P and N, and excrete essential growth factors. Moreover, microorganisms often require an adaptation period before they can manufacture enzymes necessary to biodegrade contaminants. This period may be especially extended to months in anaerobic metabolism of some organic compounds (Alexander, 1999). It was reported that the microbial degradation rate for organic contaminants was increased when the microorganisms had been pre-exposed to the contaminants. Therefore, the indigenous microorganisms that are acclimated to the contaminants should be more capable of degrading the contaminants than those from a pristine site (Atlas and Bartha, 1973).

1.3.12. Factors influencing bioavailability

Microbial movement in soils may often be restricted by the filtering effect of soil particles. Clay grain size is 1- 2μ, while most of the individual bacterial cells are 0.3 - 50 μ, e.g., cell diameter of cocci is 2μ and bacilli is 10μ in length (Winegardner, 1996). Meanwhile, petroleum hydrocarbons are hydrophobic and tend to sorp to soils; a large proportion is not available in the water phase. Therefore, the overall biodegradation rate of a petroleum

contaminant is often controlled by the bioavailability, i.e. whether the microorganisms can approach the contaminants (Eweis, 1998).

Soil texture and structure are important factors in this aspect. Bacteria cannot move effectively in fine-textured soils (Romero, 1970). On the other hand, composition of soil influences its permeability and infiltration rate, water holding capacity, and adsorption capacity for various contaminants (Hornick, 1983). In addition, fractures in soil textures always provide preferential pathways for water and pollutant migration in consolidated aquifers, which creates higher bioavailability for underground contaminants (Ward *et al.*, 1997).

The existence of surfactants can obviously increase the solubility and mobility of hydrophobic contaminants in water, and therefore facilitate the bioavailability for these contaminants. Thus, *in-situ* surfactant-enhanced aquifer remediation has been suggested as an economically and technically feasible remediation approach (Smith and Burns, 2001).

Except for its effect on microbial metabolisms, temperature also influences the contaminant solubility, ion transport and diffusion, osmotic effects (on cell membranes), surface tension, density and colloidal matter. These also lead to impacts on bioavailability (Khan *et al.*, 2001). In addition, pH level influences bioavailability by its effect on (a) solubility of phosphorus which is maximized at a pH value of 6.5, and (b) metal transport which is minimized while pH value is greater than 6 (Sims *et al.*, 1990).

1.3.13. Availability of electron accepters

The rate and extent of biodegradation are strongly influenced by the type and quantity of electron acceptors present in the contaminated soil. As mentioned above, oxygen is the most efficient electron acceptor in biodegradation. Usually the availability of dissolved oxygen is a dominant limit in bioremediation processes (Barker and Patrick, 1985). The need of oxygen is generally based on a rule-of-thumb that three pounds of oxygen will be consumed to convert one pound of hydrocarbon (Wong *et al.*, 1997).

When oxygen is unavailable, the accessibility of ferric iron, nitrate, sulfate and carbon dioxide become essential. They can be used as terminal and dominant electron acceptors during anaerobic biodegradation. As soon as the available electron acceptors have been consumed, bioremediation process on a contaminated site is limited, and is then controlled by biodegradation at the fringes of the contamination plume where access to electron acceptors is possible (Borden *et al.*, 1995).

1.3.14. Microbial consortia

Many studies on microorganisms capable to grow on oil have dealt with single hydrocarbon degrading single microbial strain (Cooney, 1980). The effect of single microorganism on multiple substrates and, conversely, the effect of multiple microorganisms on a single substrate are poorly understood. However, mixed population studies show that some compounds that are resistant to degradation by a single organism can be degraded by mixed populations (Beam and Perry, 1974). True mutualistic or symbiotic relationships also exist among soil organisms. It is common for degradation of a xenobiotic compound to involve sequential metabolism by two or more microorganisms in a relationship that may benefit only one partner (commensalism) or both (Atlas and Bartha, 1981). In such a commensalistic relationship, microbes cannot oxidize a given hydrocarbon individually, but collectively they are able to do so. Some members of a community might be able to provide important degradative enzymes, whereas others may supply surfactant or growth factors (Wiesel *et al.*, 1993). This form of commensalism may be very widespread in nature with natural mixed populations employing each other's metabolic intermediates as growth substrates.

When a particular chemical is not easily transformed by microorganisms using it as a sole source of carbon, it is sometimes possible to employ commensalism to encourage complete biodegradation (Donoghue *et al.*, 1976). Mutualistic relationships (all members derive some benefit) are

based not only on growth factor interdependence, but may also encompass removal of a product of metabolism produced by one component of the mixed population and used by another (Pfennig and Biebl, 1976), combined metabolic attack or relief of substrate inhibition (Osman *et al.*, 1976). Some microorganisms thrive on metabolic products or products from lysis of other organisms, as a result of a commensalistic relationship (Harder, 1981). A *nocardia sp.* has been identified as a cyclohexane utilizer, but growth occurs only in the presence of an unidentified *pseudomonas* that provides biotin and possibly other growth factors (Sterling *et al.*, 1977).

1.4. Biosurfactant –a microbial byproduct

In recent years, interest in microbial surfactants has been increasing, because of their natural origin and as an alternative or addition to synthetic surfactants. The advantages of biosurfactants as compared to the synthetic products are their biodegradability, low toxicity, and simple production by microbial fermentation processes (Gerson *et al.,* 1979). Biosurfactants show a wide range of applications, such as clean up of oil spills, secondary and tertiary oil recovery. It is also possible to use biosurfactants as additives in cosmetics, foodstuffs, beverages and pharmaceutical products (Kosaric *et al.,* 1987).

Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons can solubilize water or where water can solubilize hydrocarbons. Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity; higher biodegradability (Zajic *et al.,* 1977); better environmental compatibility (Georgiou *et al.,* 1990) and higher foaming (Razafindralambo *et al.*, 1996)

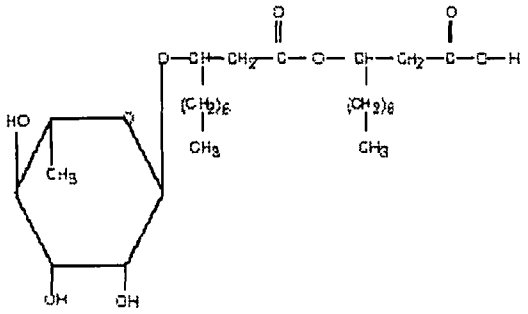
1.4.1. Source, characteristics, and properties of biosurfactants

Many microbes appear to produce a complex mixture of biosurfactants, particularly during their growth on water- immiscible substrates. Among microbes, a majority of biosurfactants are found to be produced by bacteria. Generally, biosurfactants are microbial metabolites with the typical amphiphilic structure of a surfactant, where the hydrophobic moiety is either a long chain fatty acid, hydroxy fatty acid, or α- alkyl-β-hydroxy fatty acid and the hydrophilic moeity can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid alcohol, etc. Physical and chemical properties, surface tension reduction, and stability of the emulsion formed are very important in the search for a potential biosurfactant.

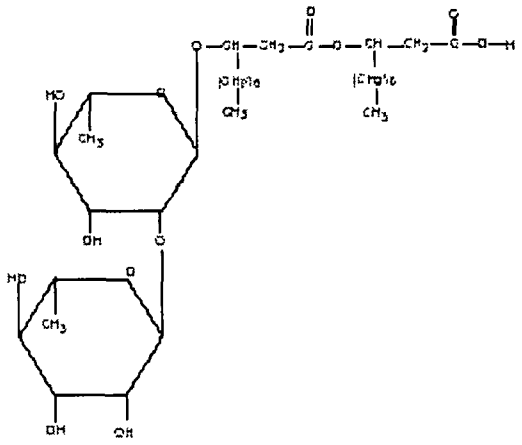
Synthetic surfactants are usually classified according to the nature of their polar group. However, microbial surfactants are commonly differentiated on the basis of their biochemical nature and the microbial species producing them. Major classes of biosurfactants are (i) glycolipids, (ii) phospholipids and fatty acids, (iii) lipopeptide/lipoproteins; (iv) polymeric surfactants, and (v) particulate surfactants.

1.4.2. Glycolipids

Glycolipids, the most commonly isolated and studied biosurfactants, are carbohydrates in combination with long chain aliphatic acids or hydroxy aliphatic acids. Glycosyl diglycerides present in the cell membrane of a wide variety of bacteria are the most common glycolipids. The best examples of glycolipids studied from the point of view of surfactant characterization and properties are (a) trehalose lipids, (b) rhamnolipids, and (c) sophorolipids.



RLL or R1 (α-L-Rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate)



RRLL or R2 (2-O-α-L-Rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate)

Fig.1.2. Chemical structure of rhamnolipids

1.4.3. Phospholipids and Fatty acids

Certain hydrocarbon degrading bacteria and yeast produce appreciable amounts of phospholipids and fatty acids when grown on *n*-alkanes (Asselineau *et al.,* 1972 and Cirigliano *et al.,* 1985). These surfactants are able to produce optically clear microemulsions of alkanes in water. The important candidates are saturated fatty acids in the range of C_{12} to C_{14} and the complex fatty acids containing hydroxyl groups and alkyl branches (Krettschmer *et al.,* 1982 and MacDonald *et al.,* 1981).

1.4.4. Peptides and Amino Acid Containing Lipids

Decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) produced by *Bacillus brevis* (Marahiel *et al.,* 1979) and *B. polymyxa* (Suzuki *et al.*, 1965), respectively possess remarkable surface active properties. Similarly, peptide containing lipids exhibit biosurfactant activity. Surfactin, a cyclic lipopeptide, reported first by Arima *et al.* (1968) in Bacillus subtilis ATCC-21332 is one of the most effective biosurfactants known so far.The ability of surfactin to lyse red blood cells is of limited use,

but this discovery has led to the development of a quick method for the screening of biosurfactant producing microbes (Mulligan *et al.*, 1984). Surfactant BL-86 was reported to be a mixture of lipopeptides with the major components ranging in size from 979 to 1091 daltons with varying increments of 14 daltons. There are seven amino acids per molecule while, lipid portion is composed of 8 to 9 methylene groups and a mixture of linear and branched tails (Horowitz and Griffin, 1991).

1.4.5. Particulate Biosurfactants

Accumulation of extracellular membrane vesicles having 20-50 nm diameter and buoyant density of 1.158 g/cm^3 has been reported in *Acinetobacter sp.* Ho1-N cells (Kappeli and Finnerty, 1979). The vesicles partition hydrocarbons in the form of microemulsion and play an important role in alkane uptake by the cells. The purified vesicles are composed of proteins, phospholipids, and lipopolysaccharides. The vesicles have a phospholipid five times higher and polysaccharide content, 360-fold higher than that observed in the outer membrane of the same organism.

A large variety of microorganisms such as most hydrocarbon degraders, cyanobacteria, and pathogenic bacteria possess surfactant activity (Rosenberg 1986 and Fattom and Shilo, 1985). The surface components that contribute to the surfactant activity include M-protein and lipoteichoic acid on *Streptococci* group-A, protein-A of *Staphylococcus aureus,* layer-A of *Aeromonas salmonicids*, prodigiosin of *Sarratia* spp., gramicidins in *B. brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1.

1.4.6. Biosurfactants and their possible uses in Microbial Enhanced oil recovery

Biosurfactants are a heterogeneous group of surface-active molecules produced by microorganisms. These molecules reduce surface tension, critical micelle concentration (CMC) and interfacial tension in both aqueous solutions and hydrocarbon mixtures. These properties create

micro-emulsions in which micelle formation occurs where hydrocarbons can solubilize in water, or water in hydrocarbons.

Generally, the structure of biosurfactants includes a hydrophilic moiety composed of amino acids or peptides, anions or cations, or mono-, di-, or polysaccharides. The hydrophobic portion is often made up of saturated, unsaturated or hydroxylated fatty acids (Georgiou *et al.,* 1992), or composed of amophophilic or hydrophobic peptides. Worldwide interest in biosurfactants has increased immensely due to their ability to meet most synthetic surfactants' requirements (Morkes, 1993). Biosurfactant(s) spontaneous release and function are often related to hydrocarbon uptake; therefore, they are predominantly synthesized by hydrocarbon degrading microorganisms. Some biosurfactants, however, have been reported to be produced on water-soluble compounds, such as glucose, sucrose, glycerol or ethanol (Hommel and Huse, 1993). In some instances, these compounds have antibiotic properties, which may serve to disrupt membranes of microorganisms competing for food. Examples of these include the lipopeptides of the iturin family produced by *Bacillus subtilis,* which have powerful anti-fungal properties (Thimon *et al.,* 1992), *Candida antarctica,* which have antimicrobial activity (Kitamoto *et al.,* 1993), and *Bacillus licheniformis,* which inhibit bacteria, yeast and filamentous fungi (Fiechter, 1992).

Chemically synthesized surfactants have been used in the oil industry to aid the clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to the environment. Biosurfactants, however, have been shown in many cases to have equivalent emulsification properties and are biodegradable. Thus, there is an increasing interest in the possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, oil-pollution control, cleaning oil sludge from oil storage facilities, soil/sand bioremediation and microbial enhanced oil recovery (MEOR).

MEOR offers major advantages over conventional EOR in that lower capital and chemical/energy costs are required (Sarkar *et al.,* 1989).

1.4.7. Biosurfactants in Microbial Enhanced Oil Recovery (MEOR)

MEOR is an important tertiary recovery technology utilizing microorganisms and/or their metabolic end products for recovery of residual oil. It is generally accepted that approximately 30% of the oil present in a reservoir can be recovered using current EOR technology (Singer and Finnerty, 1984). The main factor of poor oil recovery from existing producing wells is the low permeability of some reservoirs or the high viscosity of the oil, which results in poor mobility. High interfacial tensions between the water and oil may also result in high capillary forces retaining the oil in the reservoir rock (Bubela, 1987). Since, most of the oil remains in the reservoir following primary and secondary recovery techniques, interest has evolved in tertiary recovery techniques (Morkes, 1993). Techniques involving the use of chemical or physical processes such as pressurization, water flooding or steaming, however, is generally unapplicable to most of the oil reservoirs. The use of chemical surfactants for cleaning-up of oil reservoirs is an unfavourable practice that is hazardous, costly and will leave undesirable residues, which are difficult to dispose off without adversely effecting the environment.

1.4.8. Biosurfactants in oil storage tank clean up

Pilot-plant-scale production of the biosurfactant using a 1500 litre up-lift fermenter produced 2 tonnes of culture broth. The biosurfactant-containig growth was used as a substitute for chemical surfactants in a test carried out on an oil storage tank belonging to Kuwait Oil Company, Kuwait. Basal salt medium containing 2% w/v glucose as a readily available carbon source was used and oleic acid, a hydrocarbon source (2% v/v), was added after glucose consumption. Biosurfactant production reached a maximum after 18-19 h, as measured by reductions in the surface and interracial tension in

broth cases (Banat *et al.,* 1991). At the end of the production run, the culture broth was sterilized in the fermenter and stored in 200 liter sterile drums for their use in oil storage tank clean up.

1.4.9. Strategies and factors affecting MEOR

The appropriate remedy for any given oil reservoir will vary and be based on the conditions present. Temperature, pressure, pH, porosity, salinity, geological make-up of the reservoir, available nutrients and the presence of indigenous flora must all be taken into consideration. It is estimated, based on criteria developed by the National Institute for Petroleum and Energy Research, USA that 27% of the oil reservoirs in the major oil-producing states in the USA may be suitable for MEOR (Bryant, 1991). It has also been estimated that 40% of the oil-producing carbonate reservoirs in the USA may also be suitable for MEOR (Tanner *et al.,* 1991). The mechanisms of MEOR's action *in situ* are most probably due to multiple effects of the microorganisms on the environment and oil. These mechanisms include: gas formation and pressure increases; acid production and degradation of limestone matrices; reduction in oil viscosity and interfacial tension by biosurfactant; solvent production; plugging by biomass accumulation or polymer formation; and degradation of large organic molecules in oil, resulting in decreases in viscosity (Khire and Khan, 1994). The presence of different types of microorganisms with varying growth properties and metabolite production will have different effects on the reservoir environment. Thus, it is important to consider all aspects of MEOR while trying to influence oil production by one mechanism, such as the use of biosurfactants. There are several strategies involving the use of biosurfactants in MEOR (Shennan and Levi, 1987): (i) injection of biosurfactant-producing microorganisms into a reservoir through the well, with subsequent propagation *in situ* through the reservoir rock (Bubela, 1985).

(ii) Injection of selected nutrients into a reservoir, thus stimulating the growth of indigenous biosurfactant-producing microorganisms and
(iii) Production of biosurfactants in bioreactors *ex situ* and subsequent injection in to the reservoir

1.5. Statement of the problem in respect of Assam

Assam, the eastern most state of the Indian sub-continent, extends from 22°19' to 28°16' North Latitude and 89°42' to 96°30' East Longitude between the foot hills of the Eastern Himalayas and the Patkai and Naga Ranges. Assam is bordered in the North and East by the Kingdom of Bhutan and Arunachal Pradesh. Along the south lies Nagaland, Manipur and Mizoram. Meghalaya lies to her South-West, Bengal and Bangladesh to her West. Assam is connected with the rest of the Indian Union by a narrow corridor in West Bengal that runs for 56km below the foothills of Bhutan and Sikkim.

Physiographically Assam, which has a total area of 78,438 sq km, is divided into three broad geographic units viz., the lower and central Assam hills (Shillong plateau). The Barail ranges and the low hilly terrains of Mizo hills and the alluvial valley of Brahmaputra, Dhanshree and the Barak rivers.

The alluvial plains of Assam consist of two distinct parts; a) the valley of the Brahmaputra and its tributaries and b) the Barak valley. These are separated from each other by the water shed of the Shillong plateau and the Barail ranges.The Brahmaputra valley separates the sub-Himalayan foothills from the Shillong plateau and the Patkai-Naga hill ranges. The Mizo hills and the Barail ranges die out towards the west and southwest into the plains of Cachar, which is a part of Surma-Kusiyara valley

Assam's soil is classified into the following three types - red loam soil, lateritic soil and alluvial soil. The oil rich regions of the state generally has alluvial soil covers.

The climate of Assam is characterized by its extreme humidity. Its most distinguishing feature is the copious rainfall between March and May at a time when precipitation in upper India is at its minimum. Climatically the year may be divided into the cold season and the rainy. The cold weather lasts from October to February and the rest of the year is rainy. The southwest monsoon begins from middle of June. The neighborhood of Cherapunji and Mawsynram are known to receive the highest rainfall in the world. It is concentrated in four months, June to September. The rainfall in Assam ranks among the highest in the world; its annual rainfall varies from 70 inches in the west to 120 inches per year in the east. The average annual rainfall of 120 inches or more on the great Brahmaputra.

Historically oil was reported for the first time in a well in the Naharpung area in Upper Assam in 1866. Subsequently, the well was set up in March 1867, the first oil well in the Asian continent to be dug using mechanical means was started in the Makum Namdang area also in Upper Assam. They hit oil at 118 feet and over a tone of crude oil was extracted. In 1889 the Assam Railway and Trading Company began massive oil exploration and production in Digboi. The year 1893 saw the formation of Assam Oil Syndicate to handle oil production in Assam and a complex sprung up in north of Digboi. This fructified in 1901 with the establishment of the Assam Oil Company that started producing 500 barrels of crude oil per day and established a refinery to refine this crude in Digboi itself. In 1911, the Burma Oil Company came to Assam with the intention of oil exploration and production and soon they discovered massive oil reserves in Surma Valley. Subsequently, the Oil India Limited was set up to exploit the natural crude oil and natural gas of Assam. The company set up an oil and gas-based fertilizer plant at Namrup, a petrochemical plant as well as a power complex in the same area using gas for the production of electricity. Further in Duliajan a plant was set up to manufacture Liquid Petroleum Gas or LPG and market the same as cooking gas all over India.

The oil rich areas of Assam are very fertile and subjected to traditional agricultural practices since time immemorial. It may be mentioned that the local people of the state are mostly agriculturists whose major crop is rice. Discovery of wild tea (*Camellia assamica* Masters) in upper Assam has given way to the establishment of huge tea plantations, which constitutes a major industry of the state. The typical monsoon climate and the rich alluvial soil have been responsible for the ecological development of rich and unique biodiversity in the state. Exploration activities in the oil fields often cause spillages of crude oil from the oil wells in to the forests and agricultural fields. With the increase in the scale of oil exploration activities the problem of oil spills has also been on the rise. This has affected extensive areas in the upper Assam resulting in the destruction of soil qualities that consequently damages agriculture. The problem of oil pollution has also become a threat to the forests and the rich biodiversity these habitats possess. Oil spills frequently pose a major threat to the environment. Crude oil being lighter than water floats on the water surface, thus, also posing the grave threat of fire hazard. On land, crude oil is transported through pipelines to various locations, where they are processed. There have been instances when these pipelines have been damaged and large quantities of oil discharged in to open fields. On land, oil spills generally pose three types of hazards. These are: fire, ground water pollution and air pollution due to evaporation. Apart from exploration activities, refining and transportation of crude and refined products do contribute towards pollution of soil and water affecting agriculture, aquaculture (fisheries), tea industry and human health. The problem of oil pollution in the Upper Assam areas in particular calls for urgent solutions to restore the polluted environment for protection and sustenance of agriculture, tea industry and the unique biodiversity of the region.

Bioremediation being a less expensive and more effective strategy presents enormous scope for application in the oil contaminated sites in Assam. *In situ* bioremediation is a viable and straightforward method that is

easy to be applied and can eliminate the future liability associated with treatment or disposal of contaminants. Meanwhile, it is also a relatively time-consuming remediation process that could last for months or even years (Wainwright, 1999). Numerous studies have been conducted towards the improvement of on-site physiochemical conditions, such as nutrient availability, bioavailability of contaminants, soil texture, synergism of microorganisms, and availability of oxygen or other electron accepters. However, petroleum hydrocarbon in Assam and adjoining states has peculiarity, e.g. high wax content, high soil surface temperature as compared to cold countries and low microorganism concentrations causing negative effects on *in situ* bioremediation processes. Little work has been done to determine effects of multiple variables and their interactions within the North East India context.

A large fraction of applications of bioremediation to date have been experimental efforts and largely differ from on-site practices (Eweis *et al.,* 1998). Small-scale investigations on the microbial performance do not investigate of on-site geology and hydrology in subsurface has always been a challenging task. Sampling from monitoring wells is the only direct way currently applied to accessing the underground conditions (Liu and Liptak, 2000). The efficiency of site remediation is often restricted with the limited understanding of the subsurface conditions. On-site investigation cannot reflect all possible conditions due to non-availability of data. Given the heterogeneity that exists in the subsurface, it is simply not feasible to completely define subsurface conditions at a given site. Attempting to do so would require a large number of borings, monitoring wells, samples, and analyses. Even after all this work is completed, the results would still be subject to non-unique interpretation (Adams, 1995). Consequently, in a number of practical cases, underground soil structure is simply assumed to be uniform or roughly estimated based on limited data from boreholes (USEPA, 1998). Soil structure in western Canada subsurface is often complex (Environment Canada, 2002) and cannot be simply considered

homogeneous. This leads to difficulties in soil remediation processes. In order to gain better understanding of the contaminated subsurface, a number of scaled studies were conducted. However, there has been no well-controlled pilot-scaled bioremediation research within the country more specifically North East India.

In general, due to the above limitations, most of the previous studies were based on simple experimental works, and were mostly dedicated to one or few existing technologies for the purposes of problem solving and/or consulting (Freeman and Harris, 1995). There has been a lack of integration of various individual components within a remediation-technology. This lack has hindered innovative research on remediation technologies that are suitable and applicable to the country's source, soil and climatic conditions. As a result, most of the existing remediation activities in this field are based on imported technologies. Environmental companies tend to (a) use simple and available technologies even for complicated problems (b) promote the available technologies which they have in hand (with relatively narrow scopes), and (c) over-design remediation systems to make their jobs easier and protect themselves (due mainly to the lack of confidence on the adopted technologies). In fact, natural conditions are different from region to region of the world. Under many situations, none of the existing technologies is directly suitable. Consequently, innovative, advanced, integrated and/or enhanced measures are desired for obtaining improved efficiencies.

1.6. Objectives

The present study has been taken up to analyze the nature and extent of soil contamination by crude oil and to devise possible means to destroy or degrade the contaminant as well as to reclaim such soils for crops cultivation. Furthermore, to ascertain that such remediation technologies are suitable to sources and climatic conditions. The work entails comprehensive investigation on the isolation and regeneration of functional microorganisms and the effects of multiple variables and their

interactions during bioremediation processes as well as systematic research on optimization of microbial activities and improvement of remediation efficiency towards the potential on-site conditions. Accordingly, the following objectives have been taken up under the present investigation:

1. To isolate bacterial strains from crude oil contaminated soil and water and their characterization.

The petroleum hydrocarbon production and exploration sites of North East India are Sibsagar, Dibrugargh, Tinsukia, Jorhat, Golaghat and Silchar districts of Assam, Nagaland, Arunachal Pradesh and Tripura. Crude oil – contaminated soil and water samples from these sites will be collected for the isolation of bacteria using standard culture techniques. Isolated bacteria strains will be characterized on the basis of their colony behaviour and biochemical tests.

2. To evaluate their efficiency in biodegradation of crude oil.

Petroleum hydrocarbon is comprised of more than 200 different constituent chemicals like aliphatic, aromatic as well as nitrogen and sulphur compounds (nitroso). Bacterial species/strains can selectively degrade these compounds. Hence, bacterial isolates will be assessed for their efficiency to degrade the constituents.

3. To isolate and characterize metabolites produced by bacteria isolated from crude oil contaminated sites.

Bacteria are known to produce enzymes as well as biosurfactant to degrade xenobiotics. Bacterial isolates capable of degrading crude oil components will be assessed for their ability to produce biosurfactant on degrading hydrocarbon constituents.

4. To test surface – active metabolites produced by the bacteria in enhanced oil recovery.

Surfactants are surface active chemicals capable to reduce the surface tension of various compounds. The underground crude oil is highly viscous, only 25-30% can be lifted. Biosurfactant obtained from bacteria can be used to reduce the surface tension of crude oil making available for EOR.

Chapter 2
Review of Literature

Chapter 2

Review of literature

2. 1. Bioremediation

Environmental contamination by petroleum hydrocarbons is recognized as a major environmental concern. A variety of bioremediation techniques have been developed to mitigate such a concern. Bioremediation is currently receiving considerable attention as a remediation option for sites contaminated with hazardous organic compounds. Various field and laboratory tests have been conducted to elucidate and improve the technique. This chapter introduces efforts on the understanding of sub-surface contamination as well as the development and application of biological techniques for site remediation. It also explores the possibility of using microbial by-products in other activities like enhanced oil recovery.

In 1985, the U.S. Department of Energy established the sub-surface Science Program to clean up underground contamination by improving the understanding the biology of the sub-surface to make bioremediation a successful approach for environmental remediation. Bartha (1986) reported that over two billion tons of petroleum is produced per year worldwide. Levine *et al.* (1995) observed that the subsurface environment is characterized by a complex natural physiological and biological heterogeneity. Reis (1996) opined that the control and remediation of petroleum contamination either in atmosphere, on ground surface, or in subsurface were becoming more and more critical to public health throughout the world. According to Riser-Roberts (1998) the release of petroleum hydrocarbons in environment had been creating continuous impacts on the environment.

2.1.1. Petroleum hydrocarbons

According to Cooney (1980) crude oil is a highly complex mixture containing thousands of hydrocarbons. Compounds in crude oil can be divided into three general classes consisting of saturated hydrocarbons, aromatic hydrocarbons, and polar organic compounds. Sittig (1985) reported that benzene, toluene, ethylbenzene and xylene, which are frequently referred to as BTEX compounds, are monoaromatics with high water-solubility and relatively low sediment-water coefficient. More importantly, benzene was proved to be a carcinogen, toluene a depressant of human central nervous system, and ethylbenzene a skin irritant; also, long-term exposure to xylene has been correlated with a plastic anemia. Noonan and Curtis (1990) reported that groundwater contamination is one of the most significant environmental impacts from the leaking USTs. A hole, as small as 3 mm, either in the tanks themselves or in the piping, let could cause leakage of 36,000lit. petroleum product in a year. More importantly, one gallon of the leakage could render one million gallons of water unsuitable for drinking. The difficulties of prevention and remediation of UST leaks could result in failure to preserve groundwater quality and quantity. Besides, leaking USTs is also a major component of soil contamination. According to Huesemann and Moore (1993) the saturated hydrocarbons could be separated further in to straight chain and branched alkanes, as well as cyclic alkanes with varying numbers of saturated rings and side chains. Aromatic hydrocarbons contain one or more aromatic rings ranging from simple monoaromatic compounds, such as benzene and toluene to polyaromatic compounds such as pyrene. The polar fraction is made of compounds containing "polar" heteroatoms, such as nitrogen, sulfur and oxygen.

Fan *et al.* (1994) stated that gasoline could contain more than 1,200 different hydrocarbon compounds, of which 923 hydrocarbons in the carbon number range of C_3 to C_{12} were identified. Cookson *et al.* (1995) opined that BTEX compounds could make up over 40 percent of gasoline's composition. Moreover, volatile and injurious chemicals leaked from USTs could

eventually reach the air and finally result in air pollution. All these contributions of leaking USTs to the environment are intensifying the need of effective site remediation. Swaigen (1995) pointed out that a number of these USTs are leaking 10 Leakage of USTs originates from (a) corrosion of old bare steel tanks and the connecting pipes or lines, (b) improper connection and incompatibility between pipes and their fittings, or shifting of the piping after installation, and (c) pump failures. Steffan *et al.* (1997) reported that the major constituents of gasoline are aliphatic, alicyclic and aromatic hydrocarbons as well as other additives. The aromatic hydrocarbons include benzene, toluene, ethylbenzene, m-, o- and p-xylene, trimethylbenzene, and other benzene forms. According to Eweis (1998) the light non-aqueous phase liquids (LNAPLs) in petroleum products are hydrocarbons with low boiling point, e.g., gasoline that consists of a number of light distillates with a boiling-point range of -12 to 200°C. Eweis (1998) stated that BTEXs are often used as indicators of soil and groundwater contamination, especially from leaking underground storage tanks (USTs). Variations in chain length or branching and in ring condensations or interclass combinations as well as the presence of oxygen, nitrogen, or sulfur result in a wide variety of petroleum hydrocarbons. He further stated that aromatic compounds in the petroleum hydrocarbons are among the most recalcitrant substances from petroleum products. According to Clifton (1999) underground storage tanks (USTs) are inevitable facilities during the petroleum production, transportation, and storage processes. Among them, 7,500 to 20,000 were believed to be leaking and contaminating the surrounding environment, causing losses of millions of dollars yearly to petroleum industries. He also reported that the LNAPLs are condensate from natural gas that have a similar composition with gasoline containing hydrocarbons ranging from C_5 to C_{10}. Rooney-Varga *et al.* (1999) reported that BTEX are more stable than other cyclic compounds owing to the sharing of delocalized electrons by the *pi* bonds.

2.1.2. Fate of petroleum contaminants

According to Freeze and Cherry (1979) in aerobic respiration, the substrate molecules, e.g. petroleum hydrocarbons, are broken down by enzyme-mediated (usually oxygenase-mediated) reactions in microbial cells. Most aerobic bacteria decompose organic compounds into carbon dioxide, H_2O and other inorganic compounds with the consumption of oxygen. Zitrides (1983) observed aerobic biodegradation via more efficient and rapid metabolic pathways than anaerobic one. Huling (1991) stated that unsaturated zones, LNAPLs might exist in four phases: (a) gaseous phase – vapor in the pore spaces, (b) adsorbed phase – adsorbed to subsurface solids, (c) aqueous phase dissolved in water, and (d) liquid phase – nonaqueous phase liquids (NAPLs). In saturated zones, they could exist in three phases: (a) adsorbed phase – residual saturation adsorbed to the aquifer solids, (b) aqueous phase - dissolved in water, and (c) liquid phase – NAPLs. The LNAPLs spreading laterally on the surface of the water table could form a mound, which could be compressed into a spreading lens due to upward pressure of the water flow. Fluctuations of the water table due to seasonal variations, pumping, or recharge could result in the movement of bulk LNAPLs further into the subsurface with significant residues present beneath the water table. LNAPLs could also dissolve from the bulk liquid into the water and thus be transported with the migrating ground water. Thomas and Ward (1992) reported that microorganisms are ubiquitous on the earth. They can be found almost everywhere in soil and groundwater even as deep as 600 m in subsurface. In unsaturated soil, microorganisms are attached to surfaces of aggregates, inside pores and crevices, or growing on plant roots. Norris and Kerr (1994) reported release of LNAPLs into the environment could spread readily and lead to a large area of contamination. They might volatilize into air, stick on land surfaces or leach through subsurface and thus be dissolved in groundwater or attach to soil particles. Dissolved LNAPLs could be further transported with groundwater causing extensive agricultural and/or residential contamination. They also

reported observation of LNAPL residues by soil particles, which could be relatively more stable and less exposed to remediation methods. Therefore, LNAPLs in the subsurface, resulting from their seepage entering the vadose zone, would either accumulate on the water table or penetrate deeper into the saturated zone, or adsorbed on solid particles, which could serve as a long-term source for sustained ground water contamination. Eweis *et al.* (1998) opined that in saturated zone, microorganisms had been found floating with the moving water, however the majority are attached to soil surfaces. Bacteria are the most abundant group of organisms present in the soil and functioning in the biodegradation of petroleum hydrocarbons. He also reported that the degradation of petroleum contaminants is usually conducted through a redox reaction during which an electron donor becomes oxidized after releasing electrons while an electron acceptor becomes reduced after receiving electrons. According to him oxygen could be the most efficient electron acceptor in the process of biodegradation. According to Alexander (1999) bacteria produce inducible enzymes in order to acclimatize to the environment in the lag phase. He also suggested that oxygen could be replaced as an electron acceptor by organic or inorganic compounds such as metal ions, nitrate, sulfate and carbon dioxide. Wainwright (1999) reported that the fastest microbial metabolism and utilization of nutrients could occur during the exponential growth phase. Therefore, being carbon and energy sources, hydrocarbon contaminants were most efficiently absorbed and degraded by bacteria in the exponential phase. He also observed that bioremediation of petroleum-contaminated sites was based on the metabolism of petroleum hydrocarbons by microorganisms. This caused destruction of contaminants. A variety of microorganisms were found capable of degrading petroleum hydrocarbons by aerobic, anaerobic or fermentation metabolisms utilizing hydrocarbons as their growth substrates. He also stated that bacteria obtain chemical energy through such a reaction to support their living. Petroleum hydrocarbons, mostly aliphatic or aromatic compounds that contain functional groups like

OH, and Cl, usually function as electron donors; oxygen often acts as terminal electron acceptor or directly reacts with the petroleum hydrocarbon molecules during the microbial metabolism. Energy released from organic compounds is much smaller than that obtained from oxygen, and they cannot react directly to oxidize the contaminants. Rooney-Varga *et al.* (1999) found a series of microbial species capable of degrading petroleum hydrocarbons. Bioremediation technologies rely on such a process which naturally occurs in microbiological colonies and thus eliminate the adverse health and ecological effects. Liu and Liptak (2000) reported that the fate and concentration of organics, e.g. LNAPL-contaminants in the subsurface, are controlled by underground physical, chemical, and biochemical processes. These include (a) hydrolysis in aqueous phase, (b) oxidation-reduction, (c) biodegradation by microorganisms, (d) adsorption by soil particles, and (e) volatilization to the air present in unsaturated zone. During the hydrolysis process, organic chemicals (R-X) reacts with water or hydroxide ion (OH) as follows:

R-X + H2O R-OH + H+ + X-, or R-X + OH- R-OH + X-

Oxidation-reduction of an organic compound frequently involves a gain or loss in oxygen or hydrogen atoms. Oxidation of organic contaminants occurs when electrical potential of the soil becomes greater than that of the contaminants. Biodegradation occurs through microbial metabolism pathways. Microbial cells, carbon dioxide, oxygen and water are generated as ultimate products during the modification or decomposition of the contaminants. Adsorption decreases the mobility and retards the migration of organic contaminants by a temporary adhesion of contaminant molecules to soil particles. Volatilization causes the loss of subsurface organic chemicals from solid to gas phase. It is relatively a small effect on the migration of underground petroleum contaminants. Bachoon *et al.* (2001) observed that the stimulation of the indigenous sediment-microbial:

populations with nutrient amendments could lead to the greatest extent of petroleum hydrocarbons degradation. The enhancement of degradation was most pronounced for alkane hydrocarbon components, which were markedly reduced, compared to controls. The aromatic constituents of petroleum hydrocarbons were reduced by half, at the most, as compared to controls.

Farinazleen *et al.* (2004) reported that biodegradation of complex hydrocarbons requires the cooperation of more than a single species. This is particularly true in pollutants that are made up of many different compounds such as crude oil or petroleum and complete mineralization to $C0_2$ and H_2O is desired. Individual microorganisms could metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further.

Kim *et al.* (2004) reported that a major aspect of engineered *in situ* bioremediation is the supply of nutrients such as nitrogen and phosphorus, electron acceptors such as oxygen, and/ or microorganisms to the contaminated area. Chaineau *et al.* (2005) reported that in strictly aerobic conditions, adding nutrient at an adequate rate could be efficient for the biodegradation of saturated hydrocarbons as compared to natural attenuation. The extent of assimilation of aromatics and hexane-insoluble molecules were not increased with high inputs of nutrient as compared to natural attenuation.

Frederic *et al.* (2005) reported that temperature fluctuations and nutrient availability are among the most important factors for the metabolism of petroleum hydrocarbons present in the contaminated soil.

2.1.3. Fermentation

According to Eweis (1998) in fermentation metabolism, organic compounds are used as both electron donors and electron acceptors. Within the same organic molecule some atoms may become oxidized while the others may be reduced. During this metabolism, organic substrates are not

completely oxidized. Because of low efficiency of energy production, fermentation is seldom utilized in bioremediation.

2.1.4. Aerobic degradation

Zajic (1964) classified oxidation of alkanes as being terminal or diterminal. These terms indicate that the initial breach occurs at one of the terminal atoms. Monoterminal oxidation proceeds with the formation of a free radical and then alcohol, which is readialy oxidized to its respective aldehyde or aliphatic acid. Perry and Cerniglia (1973) reported the relative biodegradability of hydrocarbons in the decreasing order of degradability: linear alkanes (C_{10} to C_{19}), linear alkanes (C_{12} to C_{18}), gases (C_2 to C_4), alkanes (C_5 to C_9), branched alkanes to 12 carbons, alkenes (C_3 to C_{11}) and branched alkanes, aromatics and cycloalkanes. Bartha and Atlas (1977) reported alkanes to be completely saturated hydrocarbons; i.e., they contain only carbon-hydrogen and carbon-carbon single bonds. A large amount of structural diversity is represented by many isomers of alkanes, but only a limited number of this isomer occur in large amounts, and only the *n*-alkanes and the branched alkanes are important environmental contaminants. They also (1977) reported the determination of the ecological succession sequence of hydrocarbon degradation in an oil spill is also likely to be determined by the ecological succession of the degrading microorganism. *n*-alkane degraders with rapid growth rates would outcompete the slow growing decomposers of the more recalcitrant hydrocarbons for the nutritional resources until the *n*-alkanes are depleted. Westlake *et al.* (1978) reported that n-alkanes are the most widely and readily utilized hydrocarbons, with those between C_{10} and C_{25} being most suitable as substrates for microorganisms; on the other hand, they are the most susceptible components in oil to microbial attack. The process is similar to degradation of fatty acids. According to Gibson (1978) microorganisms evolved catabolic enzyme systems for the metabolism of naturally occurring aromatic compounds. In the oxidation of aromatic hydrocarbons; oxygen is

the key to the hydroxylation and fission of the aromatic ring. Bacteria incorporate two atoms of oxygen into the hydrocarbons to form dihydrodiol intermediates. The hydroxyl groups are *cis* –dihydrodiols. Oxidation of the dihydrodiols leads to the formation of catechols, which are substrates for enzymatic cleavage of the aromatic ring. In contrast, certain strains of fungi and higher organisms (eukaryotes) incorporate one atom of molecular oxygen into aromatic hydrocarbons to form arene oxides, which can undergo enzymatic addition of water to yield *trans*-dihydrodiols. Atlas (1978a) reported presence of metabolic pathways for the degradation of a number of simple aliphatic and aromatic structures. The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde, and a fatty acid. The fatty acid then cleaved (decarboxylated), releasing carbon dioxide and formation a new fatty acid, two carbons unites shorter than the parent molecule. This process is known as β-oxidation. The initial enzymatic attack involves the class of enzymes called oxygenases. The general pathway for the degradation of an aromatic hydrocarbon involves cis- hydroxylation of the ring structure formation a diol, e.g. catecol. The ring is then cleaved by oxygenases, forming a dicarboxylic acid, e.g. muconic acid. According to Atlas (1978b) and Cripps and Watkinson (1978) degradation of substituted aromatic compounds generally proceeds by initial β-oxidation of the side chain, followed by cleavage of the ring structure. Simple alkyl substitution of benzene generally increases the rate of degradation, but extensive alkylation inhibits degradation. This also occurs with polyaromatic hydrocarbons. Dietz (1980) reported that some compounds appear to be degraded only under aerobic conditions; others only anaerobic conditions, and some under either condition, while others are not transformed at all. It has been concluded that hydrocarbons are subject to both aerobic and anaerobic oxidation. The first stage of biodegradation of insoluble hydrocarbons is predominantly aerobic, while the organic carbon content is then reduced by anaerobic action. Harvey (1982) reported that diol epoxide is very potent carcinogen, since it binds with DNA. Hornick *et*

al. (1983) critically observed enzymatic oxidation of the terminal methyl group by incorporation. U.S.E.P.A. (1985a) reported rapid and complete aerobic degradation for most of the hydrocarbons. It could be generalized that for the degradation of petroleum hydrocarbons, aromatics, halogenated aromatics, polyaromatic hydrocarbons, phenols, halophenols, biphenyls, organophosphates, and most pesticides and herbicides, aerobic bioreclamation techniques were the most suitable ones. Aerobic degradation with methane gas as the primary substrate appeared promising for some low molecular weight halogenated hydrocarbons. Bausum and Taylor (1986) reported that species of *pseudomonas, Beijerinckia,* and *Nocardia* could degrade aromatic hydrocarbon compounds, usually one phenol ring at a time, although the pathways are not clearly defined. They also reported that the requirement of oxygenase enzymes for the incorporation of oxygen from the environment. The oxygenases required for the initial attack on hydrocarbons were typically inducible enzymes, although induction sometimes was accomplished by molecules other then the substrates being oxidezed. The oxygenases include the following categories:

I. α-Oxygenase - This enzyme is present only in *Arthrobacter simplex* (Retledge, 1978), where pentadecane is readily oxidized to pentadecanoic acid. The organism is also able to degrade some fatty acids by oxidative decarboxylation with the evolution of CO_2.

II. Dioxygenases (Wood, 1982) - Enzymic activation of oxygen from the triplet state to the singlet state is a crucial prerequisite for the biological oxidation to occur (Dagley, 1977; Ratledge, 1978). This is accomplished by dioxygenases, where oxygen is fixed directly into the organic compounds, e.g. oxidation of benzene to catechol, a process commonly found in bacteria, yeast, and Fungi (Giger and Roberts, 1978).

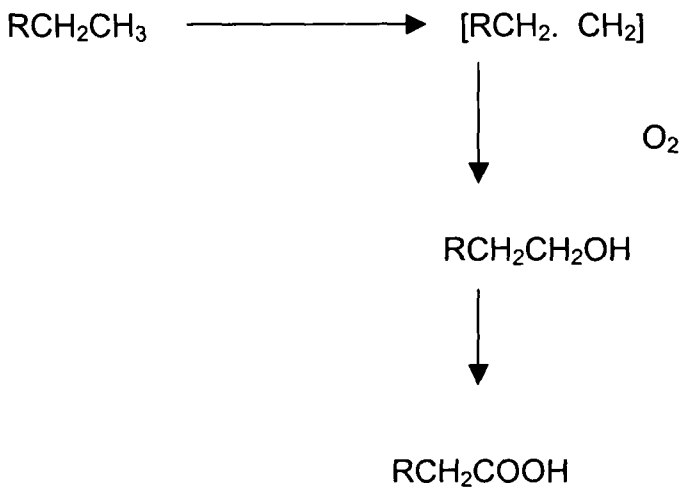
III. Hydroxylases (Giger and Roberts, 1978; Ratledge, 1978; Wood, 1982) - These enzymes accomplish the insertion of an oxygen atom and the transfer of electrons, e.g. in the oxidation of acyclic hydrocarbons with alcohols as

the intermediate product. The enzymes are also active in the degradation of PAHs, as mixed function-oxygenases of microsomal origin.

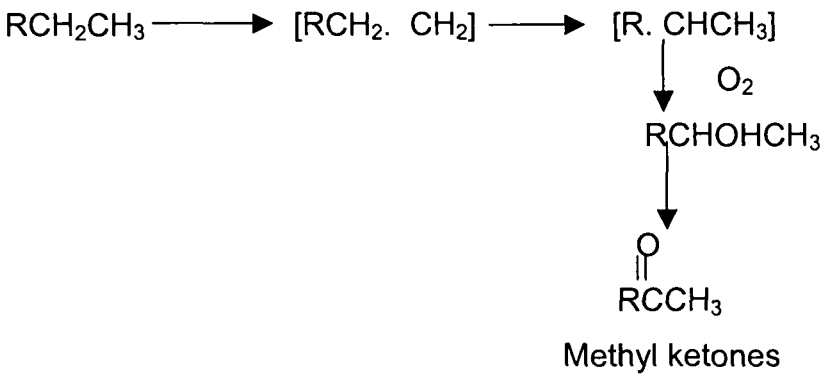
IV. Cytochrome oxidases – These enzymes effect the transfer of electrons in the respiratory chain, usually with the formation of water and an organic acid (Bausum and Taylor, 1986). Among the cytochrome oxidases, a group termed as cytochrome P-450 is found in all types of cells, including mammalian, and also when the cells are stressed by a soluble hydrocarbon molecule or other xenobiotics (Alvares, 1981).

Bausum and Taylor (1986) also reported that if side chains are present, oxidation usually occurs at a point next to the ring, but may occur in more than one molecular region. A mixed function oxygenase is the active enzyme, with formation of an arene oxide. This oxide can from a phenolic compound, or with the addition of water, a diol, which can be further oxidized to a diol epoxide.

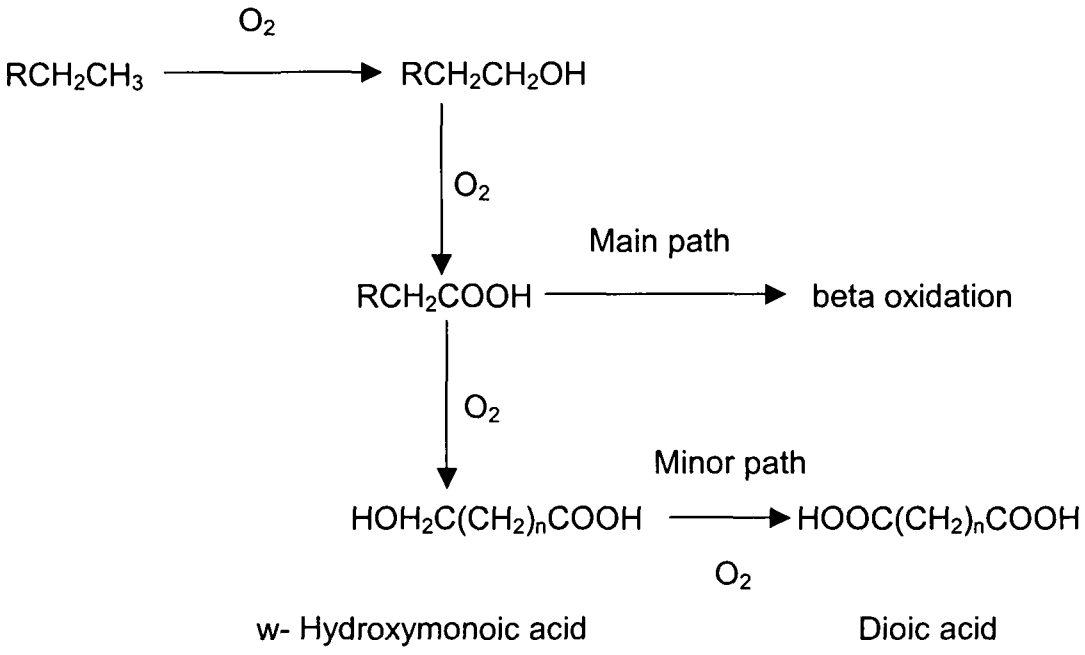
Alexander (1994) reported biodegradation of *n*-alkanes with molecular weights up to C_{44} having solubility as low as 1 ng/ l.



(a)



(b)



(c)

Fig.2.1. Terminal (a), monoterminal (b), (α- oxidation) diterminal and (c) oxidation of alkanes or aliphatics (Zajic, *Devel. Ind. Microbiol.* 6: 16- 27, 1964)

of molecular oxygen by a monooxygenase, producing a primary alcohol with further oxidation to a monocarboxylic acid. β-oxidation of the carboxylic acid

results in the formation of fatty acids and acetyl coenzyme A, with eventual liberation of carbon dioxide. Fatty acids can be toxic and may accumulate during hydrocarbon biodegradation.

2.1.5. Biodegradation of BTEX

Alvarez and Vogel (1991), Yadav and Reddy (1993), Paje *et al.* (1997) and Rooney-Varga *et al.* (1999) observed degradation of aromatic BTEX compounds by many species of bacteria, such as, *Pseudomonas* sp. strain CFS-215, *Arthrobacter* sp. strain HCB, *Phanerochaete chrysosporium* Rhodocuccus sp. strain and *Geobacter* spp. Edwards *et al.* (1992) and Borden *et al.* (1995) reported degradation of toluene as the first among BTEX, followed sequentially by p-xylene or o-xylene. Benzene and ethylbenzene, if degraded at all, tend to be degraded in the last. Wiesel *et al.* (1993) opined transformation of BTEX to medium substances, which might either continue to be degraded by other microorganisms or become dead end-metabolites. Thus, mixed microbial communities are preferential rather than mono-species in bioremediation processes. Alexander (1999) reported degradation of BTEX by mineralization and transformation processes, such as reduction of double bonds, ring cleavage, carboxylation, and therefore could enter the metabolism pathways in microbial cells. According to Weiner *et al.* (1998) mineralization is the complete degradation of BTEX into CH_4, CO_2 and H_2O.

2.1. 6. Bidegradation of PAHs

Park *et al.* (2000) reported mineralization of nitrobenzene (NB) one of the recalcitrant nitroaromatic compounds by microorganisms through oxidative and partial reductive pathways. In the partial reductive pathway NB was first reduced to hydroxylaminobenzene (HAB) by the NB nitoreductase and then HAB was rearranged to 2 – aminophenol (2-AP) by HAB mutase, 2-AP underwent a *meta* ring cleavage to 2-aminomuconic 6-semialdehyde (2-AMS) by 2-AP 1,6- dioygenase. On the other hand, 2-AMS

dehydrogenase converted the resulting 2-AMS to 2-aminomuconate (2-AM), which was, by the action of 2-AM deaminase, finally deaminated to yield 4-oxalocrotonate, a well known intermediate in the catechol *meta* cleavage pathway. Moser and Stahl (2001) reported that alpha subunit genes of intial polyaromatic hydrocarbon (PAH) dioxygenases were used as targets for the PCR detection of PAH-degrading strains of the genera *Pseudomonas, Comamonas and Rhodococcus*. Bonan *et al.* (2001) reported that the efficacy of bioremediation approaches, particularly when used gaseous nutrients (N and P compounds with sufficiently high vapor pressures to allow their conversion to a gas under environmental conditions) supported enhanced PAH bioremediation in soil. Rahman *et al.* (2002) reported presence of biosurfactant producing bacteria in hydrocarbon contaminated sites. Calvo *et al.* (2004) reported surfactant activity of *Bacillus pumilus* strain 28 –11 and the ability of this bacterium to grow and to remove naphthalene from liquid media and from soil microcosms.

2.1. 7. Factors influencing biodegradation of petroleum hydrocarbon

A variety of physical, chemical and biological factors are influencing microbial activities, and therefore either accelerate or restrain the microbial deconstruction of petroleum hydrocarbons. Meanwhile, the characteristics of the hydrocarbons and the underground soil texture and structure also have great impacts on the biodegradation processes. A successful bioremediation needs to identify these rate-limiting factors and realize the delivery of those factors to the bacteria.

2.1.7.1. Microbial growth

According to U.S.E.P.A. (1985) the availability of psychrophilic microorganisms in subsurface and groundwater is an important factor during the operation of bioremediation. Nutrient availability is another critical factor. Deficiency of nutrients may severely limit growth of microorganisms and thus biodegradation of petroleum contaminants. In addition to organic

compounds including petroleum hydrocarbons that serve as carbon and energy source, a group of other nutrient elements are required, such as nitrogen, phosphorus, potassium, sulfur, magnesium, calcium, iron, sodium, amino acids, B vitamins, fat-soluble vitamins, and other organic molecules. In the case of underground bioremediation, nitrogen and phosphorus are frequently in short supply among these nutrients. Ratio for C:N:P is of significance as well. Lee *et al.* (1988) observed requirement of 44 g of N and 22 g of P supplied as aqueous solution for the degradation of gasoline. Deficiency or plethora of N or P would reduce the efficiency of biodegradation. Typical ratios proposed were 100:15:3, 120:10:1, 250:10:3 or 100:10:2 and 100:10:1. McMillen *et al.* (1993) and Wibowo (1996) reported a low concentration of N (~300 mg/kg soil) to be more realistic from toxicity considerations. Walworth *et al.* (1995) found that soil temperature had a more pronounced influence on the light petroleum contaminant degradation than did soil nitrogen and phosphorus levels. Moran *et al.* (1997) reported the optimal temperature requirement to be 25°C for common petroleum-degradable microorganisms to grow.

Ferro *et al.* (1997) reported existence and proliferation of specific microbial groups that degrade contaminants. Ground surface vegetations also act as a factor affecting microbial growth. Bacterial numbers in soil are substantially greater with vegetation on soil surface as compared to those without plants. Eweis (1998) reported moisture level as the limiting factor for microbial growth and activity in unsaturated subsurface. Inadequate supply of water could severely restrict biodegradation in surface soils. However, excessive water would displace air from pores in soil, which would inhibit gas exchange and result in anaerobic zones and elimination of aerobic processes. Alexander (1999) reported harmful effect of high salinity to most of the microorganisms. Microbial processes in such environments were inhibited due to the extraordinarily high osmotic pressure. Natural inhibitors, like toxins affect microbial growth or survival and are present in polluted or even unpolluted soils and waters. Bacteria must be resistant to these toxins

in order to function during the biodegradation process. He also reported the presence of hydrocarbon-degrading microorganisms, some other organisms often act as predators, parasites or lysis inducers, such as protozoa, bacteriophages, viruses and organisms that excrete enzymes which destroy cell wall of fungi and bacteria and thereby cause their lysis. These organisms may largely reduce the number of bacteria by grazing. According to Wang *et al.* (2002) petroleum contaminants might be cellular poisons inhibiting both microbial growth and activity. Different microorganisms had varied thresholds to petroleum hydrocarbons, while these hydrocarbons could serve as a stimulant to microbial growth provided that the concentration was not toxic.

2.1. 7.2. Bioavailability

Hornick (1983) reported influence of composition of soil on its permeability and infiltration rate, water holding capacity, and adsorption capacity for various contaminants. According to Sims *et al.* (1990) pH level influences bioavailability by its effect on (a) solubility of phosphorus, which is maximized at a pH value of 6.5, and (b) metal transport which is minimized while pH value is greater than 6. Winegardner (1996) reported restriction of microbial movement in soils by the filtering effect of soil particles. Clay particle size is 1-2μ, while most of individual bacterial cells are of the size of range of 0.3 - 50μ, e.g., cell diameter of cocci is 2μ and bacilli 10μ in length. Meanwhile, petroleum hydrocarbons are hydrophobic and tend to sorb to soils; a large proportion is not available in the water phase. Ward *et al.* (1997) observed that fractures in soil textures always provide preferential pathways for water and pollutant migration in consolidated aquifers, which creates higher bioavailability for underground contaminants.

Smith and Burns (2001) observed that the existence of surfactants could obviously increase the solubility and mobility of hydrophobic contaminants in water, and therefore facilitate the bioavailability for these contaminants. Thus, *in-situ* surfactant-enhanced aquifer remediation had

been suggested as an economically and technically feasible remediation approach. Except for its effect on microbial metabolism, temperature also influenced the contaminant solubility, ion transport and diffusion, osmotic effects (on cell membranes), surface tension, density and colloidal matter.

2.1. 7.3. Availability of electron acceptors

Barker and Patrick (1985) observed strong influence of the type and quantity of electron acceptors present in the aquifer on the rate and extent of biodegradation. As mentioned above, oxygen is the most efficient electron acceptor in biodegradation. Usually the availability of dissolved oxygen is a dominant limit in bioremediation processes. Borden *et al.* (1995) observed that as soon as the available electron acceptors are consumed, bioremediation process on a contaminated site is limited, and is then controlled by biodegradation at the fringes of the contamination plume where access to electron accepters is possible. Wong *et al.* (1997) reported that the need of oxygen is generally based on a rule-of-thumb that three pounds of oxygen would be consumed to convert one pound of hydrocarbon. When oxygen is unavailable, the accessibility of ferric iron, nitrate, sulfate and carbon dioxide becomes essential. They can be used as terminal and dominant electron acceptors during anaerobic biodegradation.

2.1.7.4. Synergism of microorganisms

According to Alexander (1999) a complete biodegradation process often required more than one microbial species. Synergism of microorganisms might be indispensable either during initial transformation or in later mineralization of petroleum contaminants. Therefore, the variety of on-site microorganisms could be an important concern in bioremediation processes. Mechanisms for synergistic relationships have been described as (a) growth factors which are vital to one species are produced by another species, (b) one species carries out incomplete metabolites which can be growth substrates for another species and be mineralized, (c) one species

co-metabolizes a compound to yield a product that it can no longer be metabolized, and the second species destroys it, and (d) one species converts the substrate to a toxic metabolite that slows down the transformation, while another species destroys the inhibitor.

2.1.8. Bioremediation

Blackburn and Halker (1993) and Mihelcic *et al.* (1993) reported disadvantages of bioremediation, especially for its exceedingly long time required to meet cleanup guidelines. Usually after an initial, quick degradation of the labile fraction of contaminants, further biodegradation of the residual compounds could be very slow as a result of the low bioavailability of residual contaminants to soil microorganisms. King *et al.* (1998) reported decrease of contaminant concentration with the presence of a microbial population that was capable of biodegrading the contaminant in subsurface did not necessarily trigger biodegradation or bioremediation on site. In addition, microbial strains were usually studied in pure culture; the extensive degradation capabilities of numerous microbial species had been demonstrated in this manner. These capabilities might not occur to the same extent or in the same fashion when the species are active in a mixed microbial community found in nature. Thereby, an introduction of petroleum-hydrocarbon degrading microorganisms is far from the success of bioremediation. Favorable environmental and chemical conditions are important for microbial growth; meanwhile, an effective method is needed to trace the contaminants and to monitor the absorption and metabolism of petroleum hydrocarbons by microorganisms. Wainwright (1999) opined that *in situ* bioremediation to be a viable and straightforward method, often offering the potential to remediate contaminated soil and groundwater without excavation, and could be implemented below and around existing buildings, piping and paved surfaces.

According to Burke *et al.* (2000) bioremediation is an emerging technology by which an organic contaminant is converted into simple

inorganic molecules through biological processes. Bioremediation techniques are very diverse and have been commonly applied in the remediation of groundwater, soil, and sludge contaminated with petroleum compounds. It has advantages of low cost, relatively high performance, and being easy for implementation. Different from many other physicochemical technologies, bioremediation can destroy hazardous compounds other than simply transferring them from one phase or location to another. At the same time, it is a natural process that can eliminate the future liability associated with treatment or disposal of contaminants. Hyman and Dupont (2001) identified high specificity of bioremediation as a major disadvantage that cannot degrade all xenobiotic compounds, except for those that can be served as a source of energy and carbon for the microbial cells or suitable for co-metabolic degradation. Concerns have also been raised that in some cases metabolic end products of biodegradation may be more persistent or hazardous than the initial parent compounds. In addition, high concentrations of contaminants may not be amenable to biodegradation because of toxicity and/or inhibition that develops at these high concentration levels.

2.1.8.1. Natural decay/attenuation and intrinsic bioremediation

Alexander (1999) observed that the indigenous species might act, but often not sufficiently fast to prevent the spreading of a local contamination. Intrinsic bioremediation process is thus not appropriate when a rapid biodegradation rate is required, i.e., the plume is moving out of the controlled area or the rate of contaminant release from the source area is higher than the contaminant degradation rate observed at the site. Such an improper application of intrinsic bioremediation may result in the movement of the contaminants through soil to underlying groundwater and the further transport of pollutants to water bodies used for human, animal or plant consumption; also the uptake by on-site plants of toxicants in contamination plume. The enhancement of the bioremediation processes is then desired.

According to Hyman and Dupont (2001) the natural decay or attenuation is describes the naturally occurring assimilation of subsurface contaminants through physical, chemical, and/or biological means. Intrinsic bioremediation is a remediation technology that relies on these measures. Intrinsic bioremediation processes do not include any enhancement of on-site conditions, but comprise a series of site assessment and data processing methods that can identify the capacity of a pristine site to remediate contamination. Assessment of an intrinsic bioremediation process usually includes (a) determination of the existence of steady-state contaminant plume, (b) estimation of natural contaminant degradation rates, (c) estimation of the mass of contaminant sources, (d) estimation of the source lifetime, (e) prediction of long-term plume behavior with and without source removal, (f) decision making regarding the use of intrinsic bioremediation and desirability of source removal at a given site, and (g) development of a long-term monitoring strategy if intrinsic bioremediation is selected for plume management.

2 .1.8.2. Enhanced *in situ* bioremediation

According to Norris and Kurr (1994) and O'Mara (1996) *in situ* bioremediation is a commonly accepted cost-efficient method. It has larger treatment zone than *ex situ* options, and reduces the risk to the workers and the community by minimizing disturbance of contamination and produces no solid residues for further cleanup. Disadvantages of *in situ* bioremediation set forth by Hyman and Dupont (2001) are: (a) toxicity within the site limits the applicability and performance (b) injection well clogging is a recurring problem, (c) transfer-efficiency limitations of nutrient and electron acceptors caused by disadvantageous soil properties can severely limit the remediation efficacy, and (d) high concentrations of nutrients can impact groundwater quality adversely.

2.1.8.3. Bioventing and bioslurping

Crocetti *et al.* (1993), Hinchee (1994) and Reisinger *et al.* (1994) stated that bioventing is an *in situ* remediation technology that introduces air or oxygen through extraction or injection wells into the unsaturated zone to enhance the activities of the indigenous microorganisms to biodegrade organic constituents adsorbed to soil particles. Nutrients are added when necessary. Soils in the saturated zone and the capillary fringe are not affected in this process. Bioventing defers from soil vapor extraction (SVE) due to inclusion of its biological components. While SVE removes constituents primarily through volatilization, bioventing systems stimulate biodegradation of contaminants and minimize volatilization by lower air flow rates. Also, bioventing is not constrained by volatility of the underground contaminants and therefore is applicable to less volatile chemicals. Moreover, since treatment of the off-gas is not required, bioventing can be more cost-effective than SVE.

Hyman and Dupont (2001) reported that bioslurping is a relatively new technology that integrates pump and treat, SVE and bioventing methods. The process is designed to recover free-, gaseous- and aqueous-phase contaminants through the same extraction well, and simultaneously enhance the activities of the indigenous microorganisms in unsaturated zones as bioventing does. The vacuum-enhanced pumping minimizes free-phase contaminant and groundwater draw down near the extraction well and maximizes fluid recovery as free product moves horizontally along high-transmissibility flow paths and into the well. At the same time, airflow due to pressure-induced gradient in vadose zone provides oxygen for indigenous microorganisms and thus intensifies the on-site biodegradation.

2.1.8.4. Biosparging

According to Brown *et al.* (1994) biosparging has the same mechanism as bioventing does, while it enhances the activities of the indigenous microorganisms to biodegrade organic constituents by air and

nutrient injection. These constituents can be dissolved in the saturated zone, adsorbed to soil below the water table, or retained within the capillary fringe. The process is similar to air sparging, except that the gas is injected at lower pressure with the intent of enhancing biodegradation rather than volatilization of contaminants.

2.1.8.5. Phytoremediation

Nyer (1998) reported that phytoremediation, also referred to as vegetative remediation, is a cost-effective, technically effective, and environmentally favorable *in situ* bioremediation technology that uses plants to absorb and metabolize inorganic or organic chemicals, and thus realizes the destruction and removal of contaminants from groundwater and soil. Meanwhile, the rhizosphere (area around the root system) has been shown to be a source of diverse microbial populations, which can increase the degradation of a series of less soluble organic compounds.

2.1.8.6. *Ex situ* bioremediation

According to Hyman and Dupont (2001) *ex situ* bioremediation, contaminant removal or destruction, is conducted during above ground treatment preceded by the excavation of soil and/or extraction of groundwater from the contaminated area. *Ex situ* bioremediation can optimize biological activities through management of aeration, moisture, nutrient, soil texture and toxicity to achieve desired treatment efficiency. It can also prevent contaminants from further migration and pollution. However, as compared to *in situ* treatment, *ex situ* bioremediation is often more costly and cannot cover large volume of contaminated soil. Also, it cannot remove contaminants sorbed to aquifer soil particles.

2.1.8.7. Stimulation of petroleum-hydrocarbon contaminated subsurface

Francois *et al.* (1996), Gonen and Gvirtzman (1997), Pinto *et al.* (1997), USEPA (1998), Walke (1998), Illangasekare *et al.* (2000) and Zhu and Sykes (2000) reported that in order to gain insight of contaminated subsurface and realize effective treatment during on-site remediation, a number of trials were documented in which practical sites and bioremediation processes were physically stimulated. These studies had focused on one- or two-dimensional bench-scale models, on-site demonstrations, and laboratory or field experiments. Garcia *et al.* (2000) designed and tested bench-scale and tank-scale systems to examine transport parameters in heterogeneous aquifers. Illangasekare *et al.* (2000) presented a pilot-scale laboratory evaluation of subsurface restoration technologies for a diesel-contaminated site under different water flow rates.

2. 2. Biosurfactants

Biosurfactants are a heterogeneous group of surface-active molecules produced by microorganisms. Both mesophilic and thermophilic microorganisms are known to produce biosurfactants. Mesophilic microorganisms grow at the temperature range of 28-42°C and thermophiles grow at 42°C and above.

2.2.1. Surfactants and biosurfactants

According to Desai and Desai (1993) surfactant, when present at a low concentration in a system has the property of adsorbing onto the surfaces or interfaces of the system and of altering to a marked degree the surface or interfacial free energies of those surfaces. The term interface indicates a boundary between any two immiscible phases, the term surface denotes as interface where one phase is a gas, usually air. The term biosurfactant has been used very loosely and refers to any usable and

isolate-able compound obtained from microorganisms that has some influence on interface.

Rosen (1988) reported that almost all surfactants currently used are chemically synthesized from petroleum hydrocarbon. Chemically surfactants are classified according to their polar groups. Chemical surfactants are classified into (a) Anionic (b) Cationic (c) Nonionic (d) zwitterionics. Shaw (1994) reported that current worldwide surfactant market is around $ 9.4 billion per annum and their demand is expected to increase at a rate of 35 % towards the end of the century.

2.2.2. Component of biosurfactants

Edward and Hayashi (1965) reported formation of glycolipid, type R-1 containing two rhamnose and two β hydroxydecanoic units by *Pseudomonas aeruginosa*. Wiken and Knox (1970) reported micelle formation by glycosyldiglycerides isolated from *Lactobacillus fermenti*. Hisatsuka *et al.* (1971) reported that rhamnolipid biosurfactant could emulsify alkanes and stimulate the growth of *Pseudomonas aeruginosa* in hexadecane. Hisatsuka *et al.* (1971) measured the surface active properties of the R-1 lipid. Itoh *et al.* (1971) reported a second kind of rhamnolipid (R-2) containing one rhamnose. Shaw (1974) reported that diglycosyl diglycerides are glycolipids and are present in the cell membrane of a wide variety of bacteria. The structure of this class of molecule contains a polar, water-soluble head and two lipophilic alkyl tails. Cooper and Paddock (1983) reported production of sophorolipids by *T petrophilum* on water insoluble substrates such as alkanes and vegetable oil. These surface active compound were chemically identical to those produced by *T. bombicolam*, but could not emulsify alkanes or vegetable oils. These findings contradicted the conventional belief that microbial emulsifier and surfactants are produced to facilitate the uptake of water-insoluble substrates. Although sophorolipids lower the surface and interfacial tension, but they are not effective emulsifying agents. They also reported that a protein containing

alkane emulsifying agent was formed when *T petrophilum* was grown on a glucose- yeast extract medium. Land and Wagner (1987), Syldatk and Wagner (1987) and Cooper *et al.* (1989) reported trehalolipids from different organisms differed in size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation. Geuerra-Santos *et al.* (1986), Lang and Wagner (1987) reported that rhamnolipds from *Pseudomonas sp.* lowered the interfacial tension against n- hexadecane to 1 mN/m and the surface tension to 25-30 mN/m. Hommel *et al.* (1987) reported the production of a mixture of water soluble sophorolipids from yeasts.

2.2.3. Amino acid in surfactants

Marahiel *et al.* (1977) reported the production of decapeptide antibiotiecs (Gramicidins) and lipopeptide antibiotic (Polymyxins) by *Bacillus brevis* and *B. polymyxa* respectively, which possessed remarkable surface-active properties. Surfactin, a cyclic lipopeptide is one of the most effective biosurfactants known so far, which was first reported in *B. subtilis* ATCC-21332. A concentration as low as 0.005 %, of the surfactant could lower the surface tension from 72 to 27.9 mN/m. According to Mulligan *et al.* (1984) the ability of surfactin to lyse red blood cells was of limited use, but this led to the development of a quick method for the screening of biosurfactant producing microbes. Javaheri *et al.* (1985) and McInerney *et al.* (1990) observed that lichenysin is a lipopeptide surfactant produced by *B. licheniformis* JF2, which has similar structure and physiochemical properties like that of surfactin. McInerney *et al.* (1990) observed that *B. licheniformis* could produce several other surface active agents, which could act synergistically and exhibit excellent temperature, pH and salt stability. Horowitz (1990) observed that BL-86, a surfactant produced by *B. licheniformis* 86, lowered surface tension of water to 27 dynes.cm^{-1} and interfacial tension between water and n- hexadecane to 0.36 dynes.cm^{-1}. Horowitz and Currie (1990) reported that the surfactant was stable over a wide range of pH, temperature and NaCl concentrations and could promote

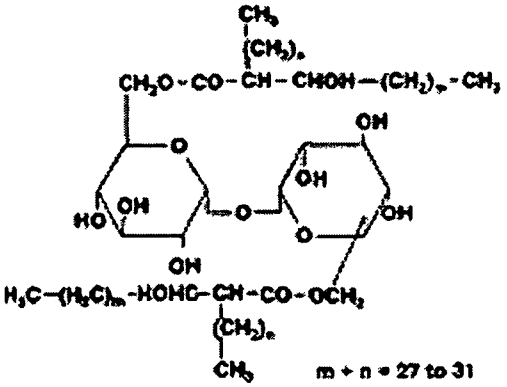
dispersion of colloidal 3- silicon carbide and alumium nitrite slurries much more efficiently then the commercial agents. Horowitz and Griffin (1991) found BL-86 to be a mixture of lipopeptides with major components ranging in size from 979 to 1091 daltons with varying increments of 14 daltons.

2.2.4. Phospholipids and fatty acids

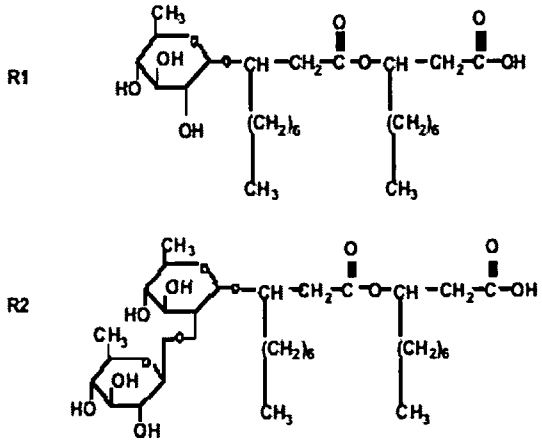
Cirigliano and Carman (1985) reported that certain hydrocarbon degrading bacteria and yeast could produce appreciable amounts of phospholipid when grown on n-alkanes. These biosurfactants could produce optically clear microemulsions of alkane in water. Phospholipids produced by *Thiobacillus thioxidan* could help in wetting of elemental sulfur. Miyazima *et al.* (1985) reported that *Aspergillus* sp. could produce appreciable amounts of phospholipids when grown on hydrocarbons.

Kretschmer *et al.* (1982) observed that certain hydrocarbon degrading microbes could produce extracellular free fatty acids when grown on alkanes and exhibited good surfactant activity. The important candidates were saturated fatty acids in the range of C_{12} to C_{14} and complex fatty acids containing hydroxyl groups and alkyl branches. Wayman *et al.* (1984) and Robert *et al.* (1989) reported that Arthobacter strain AK-19 and *P. aeruginosa* 44Ti could accumulate up to 40-80% (w/w) of such lipids when cultivated on hexadecane and olive oil respectively. They also observed that *R. erythropolis* could produce phosphatidylethanolamine having CMC value of 30mg.l^{-1} and lowered the interfacial tension between water and hexadecane to less than 1 mN.m^{-1}.

2.2.5. Polymeric biosurfactant



(a)



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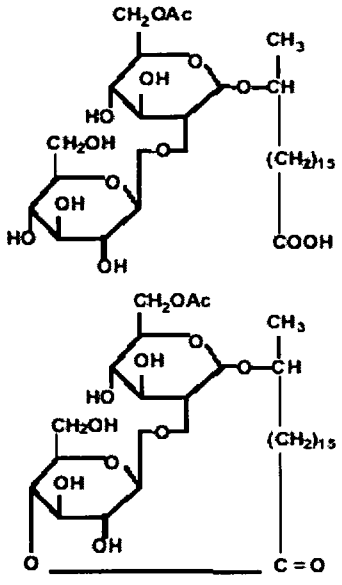

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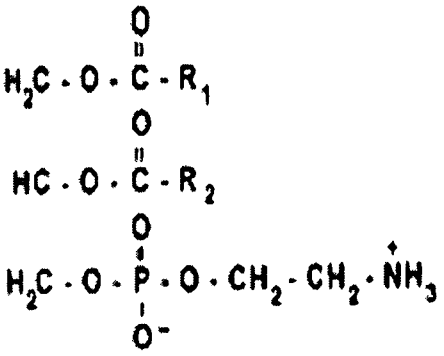
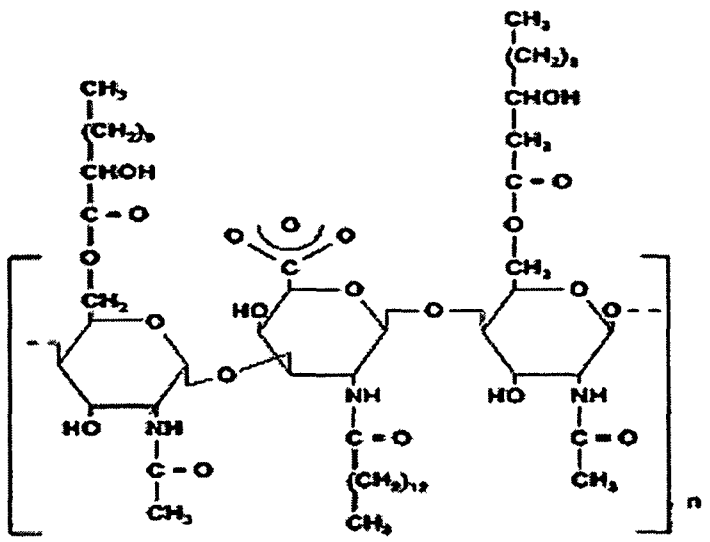


(d)



(e)

Fig. 2.2. Structure of (a) trehalose lipids, (b) R1 and R2 rhamnolipids, (c) sophorolipid, (d) phosphatidylethanolamine, and (e) emulsan

Rosenberg (1979) and Zukerberg *et al.* (1979) reported that *Acinetobacter calcoaceticus* RAG-1 could produce a potent extracellular polymeric bioemulsifier called emulsan. Emulsan was characterized as a polyanionic amphipathic heteropolysaccharide, the heteropolysaccharide backbone consisting of repeating units of trisaccharide of N- acetyl-D-galactosamine, N-acetylgalactosamine uronic acid and an unidentified N-acetylamino sugar. Belsky *et al.* (1979) Gutnik and Shabtai (1987) observed covalent linkage of fatty acids to polysaccharides through O-ester linkages. Zosim *et al.* (1982) reported the composition of the upper cream layer, known as emulsanosol, was approximately 70 - 75% oil. Emulsanosols remain stable for months and have the ablility to withstand enormous shear without any inversion. Shoham *et al.* (1983) has isolated the enzyme responsible for the depolymerization of emulsion, which depolymerize by transelimination. Gutnik and Shabtai (1987) reported that emulsan to be a

very effective emulsifying agent for hydrocarbon in water even at a concentration as low as 0.001% to 0.01%. It is one of the most powerful emulsion stabilizer known to-date and resist inversion even at water-to-oil ration of 1:4. On long standing, emulsion separates into two layers.

Rosenberg *et al.* (1988) reported that *A. calcoaceticus* A-2 could produce an extracellular, nondialyzable dispersing called biodispersan. The surface active component of biodispersan is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and it contains 4 reducing sugars namely glucosamine, 6-methylaminohexose, galactosamine uronic acid, and an unidentified amino sugar.

Navonvenezia *et al.* (1995) first described alasan, is an anionic alanine- containing heteropolysaccharide protein biosurfactant produced by *Acinetobacter radioresistens* KA-53. It was found to be 2.5 to 3 times more active on heating at 100°C under neutral or alkaline condition.

Sar and Rosenberg (1983) demonstrated that polysaccharide had no emulsification activity alone but become a potent emulsifier when combined with some proteins released during growth on ethanol. Cirigliano and Carman (1984) reported that *Candida lipolytica* could produce an extracellular water soluble emulsifier called Liposan, which was composed of 83% carbohydrate and 17% protein. The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid. A potent bioemulsifier with carbohydrate as a major component produced by a gram negative bacterium was reported by Palejwala and Desai (1989).

The production of large amounts of mannoprotein by *Saccharomyces cerevisiae* exhibiting excellent emulsifier activity toward several oils, alkanes, and organic solvents was reported by Cameron *et al.* (1988). The purified emulsifier contained 44% mannose and 17% protein. Kappeli *et al.* (1984) isolated a mannan-fatty acid complex from alkane grown *Candida tropicalis*. This complex stabilized hexadecane-in-water emulsion. Fautz *et al.* (1986) characterized *Schizonella malanogramma* and *Ustilago maydis*

produced biosurfactants as erythritol and mannose-containing lipid. Kitamoto *et al.* (1993) demonstrated the production of two kinds of mannosylerythhritol lipids from *Candida antarctica* T-34.

An emulsifying protein along with rhamnolipid from *P. aeruginosa* was isolated by Hisatsuka *et al.* (1977). It has a molecular weight of 14,300 and it contains 147 amino acids, of which 51 are serine and threonine. Koronelli *et al.* (1983) reported production of peptidoglycolipd by *P. aeruginosa* P-20 which contains 52 amino acids, 11 fatty acids, and a sugar unit. Raddy *et al.* (1983) and Chameotra and Singh (1990) isolated an emulsifying and solubilizing factor containing protein and carbohydrate from *Pseudomonas* sp. grown on hexadecane. Desai *et al.* (1988) reported the production of bioemulsifier, composed of 50% carbohydrate, 19.6 % protein and 10% lipid by *P. fluorescens*.

Kappeli and Finnerty (1978) demonstrated that alkane uptake by microbial cells was also mediated by extracellular membrane vesicles that partition hydrocarbons to form a microemulsion. Accumulation of extracellular membrane vesicles having 20-50 mm dia and a buoyant density of 1.158 g/cm^2 were reported in Acinetobacter sp. HO1-N cells. The purified vesicles were composed of protein, phospholipid and lipopolysaccharide. The vesicles had phospolipid content five times higher and a polysaccharide content 360 fold higher than that of observed in the outer membrane of the same organism.

2.2.6. Detection of potential biosurfactant producing microorganisms

Zajic and Seffen (1984), Rosenberg (1986), Desai (1987) and Lang and Wagner (1987) reported that surface active compounds in most hydrocarbon- degrading and pathogenic bacteria was attributed to several cell surface components which included structures such as M protein and lipoteichoic acid in the case of *Aeromonas salmonicida*, prodigiosin in *Serratia spp.*, gramicidins in *Bacillus brevis* spores, and then fimbria in *A. calcoaceticus* RAG-1. Quick and reliable methods for screening

biosurfactant producing microbes largely attributed for the recent advances in the field of microbial surfactants. Van der Vegt *et al.* (1991) developed the axisymmetric drop shape analysis (ADSA) by profile for the assessment of potential biosurfactant- producing bacteria. In the technique, drops of culture broth are placed on a fluoro-ethylene-propylene surface and the profile of the droplet is determined with a counter monitor. Surface tensions are calculated from the droplet profiles by ADSA. Biosurfactant producing bacterial strains only shows reduction in surface tension. Matsuyama *et al.* (1991) described a direct thin-layer chromatographic technique for rapid characterization of biosurfactant-producing bacterial colonies. Siegmund and Wagner (1991) and Hansen *et al.* (1993) developed the colorimetric method for screening of rhanmolipid producing and hydrocarbon-degrading bacteria respectively. Van Dyke *et al.* (1993) and Makkar and Cameotra (1997,1998) described the methods of emulsification and emulsification index after 24 hours (E-24). E-24 is the emulsification percentage obtained by vigorously shaking culture broth samples with an equal volume of kerosene. This method is most suitable for the emulsifying biosurfactant. Cell surface hydrophobicity is an important aspect in bacterial cell adhesion to surface since hydrophobic surfaces are usually associated with molecules with low surface energy. Neu and Parolla (1990) used this property to screen biosurfactant production. Jain *et al.* (1991) and Bodour and Miller-Maier (1998) reported another simple method of detecting biosurfactant production was the rapid drop - collapsing test. In this method, a drop of a cell suspension is placed on an oil-coated surface, and drops containing biosurfactants collapse whereas non-surfactant containing drops remain stable. Shulga *et al.* (1993) reported that the ability of the anionic surfactants to react with the cationic indicator to form a colored complex forms the basis of calorimetric estimation of biosurfactant. Schenk *et al.* (1995) developed a high performance liquid chromatographic method of detection of rhamnolipid produced by *Pseudomonas aeruginosa*. A similar method of detection of biosurfactant production in the cell-free fermentative broth of *Bacillus subtilis*

ATCC 21332 was proposed by Lin *et al.* (1998). According to Bodour *et al.* (2003) this was an easy method particularly when large numbers of samples were concerned, but it was not been correlated to surface tension reduction to confirm its reliability. Morikawa *et al.* (2000) developed another method of detection of biosurfactant production, the oil spreading technique. This technique measures the diameter of clear zones caused when a drop of biosurfactant-containing solution is placed on an oil-water surface. They used this method to compare the activity of both cyclic and linear forms of surfactin and arthrofactin. However, its ability to detect biosurfactant production in diverse organisms has not been tested. Youssef *et al.* (2004) compared three methods to detect biosurfactant production viz., drop collapse, oil spreading and blood agar for their use and reliability in relation to the ability of the cultures to reduce surface tension and found that this technique is the best.

2.2.7. Uptake of water-insoluble substrates

Lang and Philp (1998) observed accumulation of growth stimulating compounds in the culture medium, when microorganisms were cultured in water- insoluble substrates like *n*-alkanes or vegetable oils. These compounds emulsified the substrate, extending the interfacial area between the microorganisms and the substrate, thus facilitating mass transfer of the substrate to the surface of microorganisms. Lang and Wullbrandt (1999) reported cell surface hydrophobicity in trehalose lipids, which facilitated the attachment and subsequent passive transport of the substrate into the cell. Daniel *et al.* (1998) and Makkar and Cameotra (1998), Maier and Sobron-Chavez (2000) and Rau *et al.* (2001) described that biosurfactants were the prerequisites for the substrate uptake, however, could not explain all physiological observation as there were no reports on the production of biosurfactants from water soluble substrates such as glucose, starch etc.

2.2.8. Use of biosurfactants

Rosenberg *et al.* (1992) demonstrated involvement of two phases in the process of biodegradation. In the first phase, the most water-soluble compounds were degraded and in second phase the most resistant compounds degraded by the microorganisms with high cell hydrophobicity, which allowed them to adhere to high-molecular weight hydrocarbons. Atlas (1992) and Shauver (1993) reported use of physiological potential of microorganisms for bioremediation technologies as documented most readily in laboratory assays, to eliminate or reduce the concentration of environmental pollutants in field sites to level that were acceptable to site owners and regulatory agencies that might be involved. Fry *et al.*, (1993b) successfully remediated machine oil contaminated soil by using microbial inoculation and by biosurfactant treatment. Successful bioremediation of oil contaminated soil and groundwater from a US Arm engineering plant using naturally surfactants producing indigeneous microorganisms was also reported by Fry et al. (1993 a). Bragg *et al.* (1994) reported the effectiveness of bioremediation activities on the Exxon Valdez Alaskan oil spill *in situ*. This was carried out by enriching the indigenous microflora using an oleophilic liquid fertilizer containing N and P. Bioremediation of crude oil contaminated desert soil in Kuwait both *in situ* and *on situ* was reported by Al-Awadhi *et al.* (1994). Hunt *et al.* (1994) reported that permeability of the microbial cell membrane might be adversely affected by the use of synthetic surfactant, which could interfere with the capacity of a microorganism to biodegrade. Chakrabarty (1995) reported that microbial surfactants were generally much less toxic than chemical surfactants, at least as effective, and more readily biodegradable. Wilson and Jones (1993) reported that microorganisms that produce their own biosurfactants could also lower treatment cost. According to Banat (1995) oil-contamination of soil was a common problem and its treatment techniques including excavation, incineration, landfarming and landfilling, could be difficult or economically not feasible. One of the most economically feasible methods included *in situ* bioremediation. According to

Alexander (1994) and Atlas and Cerniglia (1995) biodegradation was the partial simplification or complete destruction of the molecular structure of environmental pollutions by physiological reactions catalyzed by microorganisms. Bioremediation is the intentional use of biodegradation process to eliminate environmental pollutants from sites where they are released either intentionally or inadvertently. Riser-Roberts (1998) observed production of emulsifiers throughout the growth period. The production of emulsifiers during the active growth on hydrocarbons suggested that emulsifiers were involved in the growth on hydrocarbons.

Banat *et al.* (1991) reported the ability of biosurfactants, produced by a bacterial strain (Pet 1006) for cleaning up oil storage tanks and to recover hydrocarbons from emulsified sludge.

2.2.9. In microbial enhanced oil recovery (MEOR)

Singer and Finnerty (1984) reported approximately 30% of the oil present in a reservoir could be recovered using current enhanced oil recovery (EOR) technology. Bubela (1987) reported that poor oil recovery in existing producing wells might be due to the low permeability or the high viscosity of the oil, which results in poor mobility. High interfacial tensions between the water and oil might also result in high capillary forces retaining the oil in the reservoir rock. Shennan and Levis (1987) demonstrated several strategies involving the use of biosurfactant in MEOR. The first involved injection of biosurfactant-producing microorganisms into a reservoir through the well, with subsequent propagation *in situ* through the reservoir rock. The second involved the injection of selected nutrients into a reservoir, to encourage the growth of indigenous biosurfactant-producing microorganisms. The third mechanism involved the production of biosurfactants in bioreactors *ex situ* and subsequent injection into the reservoir. According to Abu-Ruwaida *et al.* (1991a, b) laboratory studies on MEOR had typically utilized core samples and columns containing the desired substrate. The substances were utilized to demonstrate the

usefulness of biosurfactants in oil recovery from sand and limestone. Similarly, core samples were used to model the movement of microorganisms and nutrients through substrates to ascertain their usefulness after injection into oil reservoirs.

According to Morkes (1993) due to failure of primary and secondary recovery techniques to recover oil from reservoirs, interests were evolved in tertiary recovery techniques. Banat (1993) observed that fermentive culture broth containing biosurfactant from *Rhodococcus* ST-5 and the thermophilic *Bacillus* AB-2 could release 80% and 95% oil from sand-pack columns. Biosurfatant containing culture broth Pet 1006 could also release 95% residual oil from sand pack column. Biosurfactant produced by *Bacillus sp* C-14 used at concentration of 0.04 mg/ml could release oil from oily sand.

Banat (1995) reported that the presence of different types of microorganisms with varying growth properties and metabolite production would have different effects on the reservoir environment. Thus, it was necessary to consider all aspects of MEOR when trying to recover oil from reservoir by one mechanism such as the use of biosurfactants.

2.2.10. Biosurfactant production under thermophilic conditions

There are very few reports of biosurfactant production in thermophilic conditions. Thermophiles are heat loving microbes and can survive at a temperature ranging from 42-100°C. Banat (1993) reported a thermotolerant *Bacillus sp.* growing at 50°C on hydrocarbon containing medium. Biosurfactant containing fermentative broth of the bacterium could release 95% oil from the sand pack column suggested a potential application in MEOR and in oil-sludge clean up. Busscher *et al.* (1994) reported a Streptococcus *thermophilus* strains isolated from pasteurization units in a dairy industry to have biosurfactant producing ability. *Bacillus licheniformis* JF2 isolated by Jenneman *et al.* (1983) from oil-field injection water had properties for the potential use in *in situ* MEOR. The organisms could be grown anaerobically and produce biosurfactant using glucose and $NaNO_3$ as

the respective carbon and nitrogen sources. The strain could also be grown in a wide range of pH (4.6-9), temperature (upto 50°C) and salinity (upto10%). McInerney *et al.* (1990) reported that the biosurfactant of *Bacillus licheniformis* JF2 was not affected by typical parameters of many reservoirs like temperature, pH, salinity and calcium concentration. Yakimov *et al.* (1995) reported characterization of a new lipopeptide biosurfactant produced by thermotolerant and halotolerant *Bacillus licheniformis* BAS 50. The organisms could produce biosurfactant when grown both aerobically and anaerobically at a temperature ranging from 35°C and 45°C and salt concentration optimal at 5%. The biosurfactant termed lichenysin A could reduce the surface tension of water from 72 mN/m to 28 mN/m and have a CMC value of 12 mg/litre. The surfactant was purified and chemically characterized. Denger and Schink (1995) isolated two biosurfactant producing new strains of bacteria that produced the surface active compounds at salt concentration of about 8% and temperature 50°C using sugar as the carbon source. Trebbau de Acevedo and McInerney (1996) isolated bioemulsifiers from *Methanobacterium thermoautotrophicum* that form viscous emulsions but failed to reduce the surface tenion of water or interfacial tension between water and hexadecane. The emulsifier was active over a wide range of pH (5-10) and at salt concentrations up to 200g.l^{-1}. Makkar and Cameotra (1997a, b and 1998) reported biosurfactant production by two strains *of B. subtilis* at 45°C. The organisms could produce biosurfactant over a wide range of pH and temperature ranging from 30°C to 45°C. The organism could also grow and produce biosurfactant on molasses like cheaper carbon sources. Das *et al.* (2003) reported biosurfactant production by a *Pseudomonas* DM-01 strain at 45°C. The organisms could grow over a wide range of pH and could release 60 and 80% of crude oil and kerosene from an oil saturated sand pack column indicating its potential in MEOR and oil sludge clean up.

2.2.11. Genetic engineering

Genetic engineering can be used to develop microorganisms with unique metabolic capabilities. Chakrabarty (1974) reported that alkanes and simple aromatic hydrocarbon degrading genes present in the plasmid DNA. He also reported that multiple plasmid transfer has been accomplished between *Pseudomonas* species to construct a strain that can degrade several hydrocarbons. Friello *et al.* (1976) reported that genetically engineered plasmid produce from conjugation of *Pseudomonas* strains with OCT-CAM and plasmid display Oct^+ and Nah^+ degradation phenotype. According to Kamp and Chakrabarty (1979) by manipulating the exchange of hydrocarbon degrading genetic material, it is possible to develop strains with extended degradative capability, i.e., organisms that can degrade more than one xenobiotic substrate or that can completely mineralize highly recalcitrant molecules. Thibault and Elliott (1980) reported that the ability of microorganisms to degrade hydrocarbon has been shown to be encoded on extrachromosomal – DNA. They also reported that the development in recombinant DNA technology permits engineering of genes that code for desired enzymatic capabilities. Kolenc *et al.* (1988) reported that successful transfer of TOL plasmid from a mesophilic *Pseudomonas putida* to a psychotropic *P. putida* by conjugation in which the toluene biodegradation genes were expressed at 0^0C. Haryama and Rekik (1990) reported the isolation and extensive study of TOL plasmid, harbouring genes encoding the enzymes for the oxidation of toluene were done. Ochser *et al.* (1995) reported transform the plasmid Pu 098 from *P. auroginosa* but ramnolipid production was not present there. Cabrera *et al.* (1997) isolated plasmid from *P. auroginosa* strain P3, P6, P18, P27 and P31 their molecular weight being within the range of 280 ± 15 megadelton .Strain P2, P 27 and P 31 were found to poses a plasmid easily isolated using the alkaline bypass method. They also cut the enzyme with different restriction endonuclease. Whyte *et al.* (1997) reported that isolated large catabolic plasmid, similar to known OCT^+ and Nah^+ plasmid from psychotropic *Pseudomonas sp.* Strains

B17, B18 and B19. They digest the isolated plasmid DNA with *Eco* RI and *Bam* H1 and compared with the data of Oct - plasmid. Park and Kim. (2000) isolated two plasmids from *Pseudomonas putida* strain HS121 and one plasmid from *P. putida* strain HS 124. They constructed a physical map of P^{NB1} and P^{NB2} using restriction enzyme *Nhe* 1. Kupier *et al.* (2004) digested the chromosomal DNA and plasmid DNA pmp5459 from a mutant strain PCL 1436 of *Psuedomonas putida* by *Eco* RI to identify the biosurfactant producing gene. Dubey and Juwarkar. (2004) isolated a high molecular weight plasmid 32.08 x 10^6 da from *P. auroginosa* strain BS2 producing biosurfactant. A study on the plasmid profile revealed the presence of chloramphenical, tetracycline, sulphonamide and heavy metal (mercuric chloride) resistant genes in the plasmid. They performed the plasmid curing experiment, but found no effect of biosurfactant production.

Chapter 3
Materials and Methods

Chapter 3

Materials and Methods

Remediation and reclamation of oil-polluted soils require environmentally sound treatment procedures mediated by microorganisms capable to degrade various fractions of hydrocarbon. Formulation of bacterial consortia could be a successful approach towards this end. The effectiveness of bioremediation is often a function of the microbial population. With a single microbial species it is virtually impossible to degrade the hydrocarbons; it needs a mixed population of microbial species/strains having broad substrate specificity.

Hydrocarbon polluted soil and crude oil samples were collected in two phases from different sites for the isolation of microorganisms.

3.1. Materials

3.1.1. Collection of environmental samples

Surface and sub-surface crude oil-contaminated soil samples were collected from three different oil fields of Assam viz., Lakuwa, Geleky and Rudrasagar of Siwasagar district. Collection sites were contaminated with crude oil since the time of drilling and subsequent production of crude oil. Samples were taken from just below the soil surface and sub-surface soil samples were collected from a depth of 8 cm. Soil moisture level was maintained with the addition of 0.85% saline water at regular interval. Samples were stored at normal room temperature for subsequent uses. In addition, water samples were collected from hot springs of Himachal Pradesh for the isolation of thermophilic microbes.

3.1.2. Collection of crude oil samples

Crude oil samples were collected in two phases. In the first phase, from three different oil fields such as, Rudrasagar GGS II, Lakuwa GGS III and Geleky GGS III. Collections were done from the well head - out let in sterile reagent bottles. In the second phase, samples were collected from different oil fields of ONGC, Oil India Limited and Digboi Refinery, such as Rudrasagar - (i) RDS 74 (ii) RDS 79 and (iii) RDS 151; Geleky - (i) G 48 (ii) G 293 and (iii) G 296; Lakuwa - (i) L 64 (ii) L 123 (iii) L 253 and (iv) L 490; Digboi – (i) D1 (ii) D 448 and (iii) D 848; Duliajan – (i) DJN 132 and (ii) DJN 191; and Digboi Refinery – oil and sludge from the tarpit and soil.

3.2. Enrichment culture media

The procedure of isolation, screening and subsequent selection of the potential strains capable of degrading hydrocarbons was based on the following three steps:

Step I: Enrichment culture technique was used to isolate bacteria from crude oil contaminated surface and sub- surface soil samples. Enrichment cultures were initially established by suspending crude oil contaminated soil samples (1 g) in 100 ml of mineral salt medium, to which added 10µl hexadecane.

Table 3.1 *Composition of mineral salt medium*

Sl. no	Chemicals	Compositi on in liter	Sl. no	Chemicals	Composition in liter
1.	Urea	2.0 g	7.	$FeSO_4.7H_2O$	1.0 mg
2.	$(NH_4)_2 SO_4$	2.0 g	8.	$CuSO_4. 7H_2O$	50µg
3.	Na_2HPO_4	3.61g	9.	H_3BO_4	10µg
4.	KH_2PO_4	1.75g	10.	$MnSO_4. 5H_2O$	10µg
5.	$Mg_2SO_4.7H_2O$	0.2g	11.	$ZnSO_4. 7H_2O$	70µg
6.	$CaCl_2 .2H_2O$	50 mg	12.	MoO_3	10µg

pH of the medium was adjusted to 6.8.

The cultures were incubated at 37°C with shaking at 200 rpm for 7 days. After 7 days, 1 ml inoculum was transferred to the fresh medium and incubated for 7 more days under the identical conditions. The process was repeated thrice and finally 1 ml inoculum from the third enrichment culture was serially diluted in sterile distilled water up to 10^{-7}. All chemicals used in mineral salt medium were purchased from Merck, Germany and India (BDH).

Step II: From the dilution of 10^{-7}, 100 µl was plated in the nutrient agar plates. The plates were incubated at 37°C for 12 h. Plate count technique was used with the help of digital colony counter (Lapiz, India) for the determination of bacterial concentration in all samples. The process was repeated thrice. Subcultures were done by inoculating in fresh nutrient agar plates. Isolates exhibiting distinct colony morphology were isolated by repeated sub culturing in nutrient agar plates until purified strains were obtained (as confirmed by assessment of cellular and colony morphology). A total of 58 distinct isolates were obtained from enrichment culture.

Table 3.2 Composition of nutrient agar medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1.	Agar	15.00
2.	NaCl	5.00
3.	Peptone	5.00
4.	Yeast extract	2.00
5.	Beef extract	1.00

pH of the medium was adjusted to 6.8

Chemicals used for the preparation of nutrient agar medium were purchased from Merck, Germany and Himedia, India.

Isolated pure cultures of bacteria were preserved at 4°C in nutrient agar slants. Subcultures were done at an interval of 45 days in fresh nutrient agar plates for preservation and further use. Isolates were also stored in 15% (v/v) glycerol by keeping at -70° C for long time storage.

Step III: Bacterial isolates were screened for their ability to grow on different carbon sources like glucose, fructose and hydrocarbons used as the sole source of carbon and energy. The basic media composition used was Luria-Bertani (LB) due to its suitability and availability in the market. A prick head bacterial colony mass was taken from the plate and resuspended in 5 mM potassium phosphate buffer (pH 7.0) before culturing in the LB medium.

Table 3.3 Composition of Luria-Bertani medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1.	Casein enzymic hydrolysate	10.0 g
2.	Yeast extract	5.0g
3.	Sodium Chloride	5.0g
4.	Agar	1.5 %

pH of the medium was adjusted to 7.0

For the preparation of potassium phosphate buffer, first 1 M stock solutions of K_2HPO_4 and KH_2PO_4 at the concentrations of 174.18 $g.l^{-1}$ and 136.09 $g.l^{-1}$ respectively in distilled water were prepared. By mixing required volumes of 1 M K_2HPO_4 and 1 M KH_2PO_4 the required pH containing 0.1M buffer was prepared.

Table 3.4 Preparation of 0.1M potassium phosphate buffer

pH	Volume of 1M K_2HPO_4 (ml)	Volume of 1M KH_2PO_4 (ml)
6.8	49.7	50.3
7.0	61.5	38.5

An appropriate volume of the bacterial suspension was added to a flask containing 100 ml mineral salt medium (MSM) to yield an initial OD_{600nm} of 0.08 – 0.1. All such cultures were supplemented with 1 % (v/v) hexadecane and non-hydrocarbon sources and incubated at 37°C and 200 rpm for different time intervals. Optical density was measured at 600 nm

from 0 to 96 h of incubation. Isolates exhibiting an increase of turbidity and biomass (on dry weight basis) with the OD value of 1.07± 1.0 and above, possessing biodegradative ability by forming foam, changing color of the broth as well as reducing the surface tension of the culture supernatant were retained. A total 13 isolates were selected based on these criteria.

Isolates exhibiting growth on hydrocarbon-enriched media in pure cultures were characterized and identified up to genus level.

3.3. Biomass determination

Bacterial biomass was determined by centrifuging the culture broths at 12,000 rpm for 15 min, washing the cells twice with phosphate buffer saline (0.9% NaCl, 10 mM K_2HPO_4, and pH 7.2) (PBS, pH 7.0). The biomass was dried overnight at 45°C and weighed. In the case of aliphatic hydrocarbons (hexadecane, dodecane, light paraffin, octane and octadecane), the culture broth was treated with hexane to remove the residual hydrocarbons; and in the case of polycyclic aromatic hydrocarbon (pyrene, flourene and phenathrene), the culture medium was treated with ethyl acetate prior to weighment (Makkar and Cameotra, 1998).

3.4. Taxonomic identification of bacterial strains

3.4.1. Biochemical and morphological tests

The taxonomic identification of the bacterial isolates was conducted following the standard morphological, physiological and biochemical tests as described by Cappuccino and Sherman (1999). The bacterial isolates were taxonomically identified up to genus level with the help of Bergey's Manual of Systematic Bacteriology. The list of biochemical and morphological tests performed are given below:

(a) Morphological tests

1. Size and shape of colony
2. Colony forms
3. Colony margins

4. Colony elevation
5. Growth on agar, broths, different pH levels and temperatures

(b) Bacterial staining

3.4.2. Gram staining

The aim of the test was to differentiate between gram positive and gram negative bacteria, an essential tool for the classification and identification of bacteria. Gram staining employs the principle of differential staining. Bacteria can be differentiated on the basis of their cell wall composition.

Gram staining requires 3 chemical reagents that were applied sequentially to a heat- fixed smear:

(I) Primary stain: Crystal violet and Gram's iodine

(II) Decolorizing agent: Ethyl alcohol 95 %

(III) Counter stain: Safranin

Table 3.5 Biochemical tests and media used

Sl.no	Biochemical test		Media
1	Catalase		Trypticase soy agar slant/ plate
2	Oxidase		
3	Voges-Proskauer test		MR-VP broth
4	Methyl Red test		
5	Carbohydrate fermentation (gas production)	Lactose	Phenol red lactose broth
		Dextrose	Phenol red dextrose broth
		Sucrose	Phenol red sucrose broth
6	Hydrolysis of	Casein	Milk agar
		Gelatin	Nutrient gelatin deep tube
		Starch	Starch agar plate
		Lipid	Tribuyrin agar plate

7	Citrate utilization		Simmons citrate agar slant
8	Nitrate reduction		Triptic nitrate broth
9	H_2S production		SIM medium
10	Indole production		
11	Litmus milk reaction		Litmus milk
12	Urease test		Urea broth
13	Triple sugar iron agar test		Triptic nitrate broth
14	Acid from	D-glucose	Phenol red dextrose broth
		D-Xylose	Phenol red xylose broth
		Mannitol	Phenol red mannitol broth

3.4.3. Trypticase soy agar (TSA) medium

This medium was used to determine the ability of bacterial isolates to degrade hydrogen peroxide by producing catalase enzyme. TSA (Bacto TM, Difco) medium with pH 7.3 was used. Catalase production was determined by adding 3% solution of hydrogen peroxide.

The production of cytochrome oxidase was determined by the addition of the test reagent, *p* - aminodimethylaniline oxalate to colonies grown on a TSA plate.

Table 3.6 Composition of TSA medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Pancreatic digest of casein	17.00
2	Enzymatic digest of soybean meal	3.00
3	Dextrose	2.50
4	NaCl	5.00
5	K_2HPO_4	2.50
6	Agar	20.00

pH of the medium was adjusted to 7.3

3.4.4. Methyl red- Voges – Proskauer (MR-VP) broth

Methyl red (MR) test (Cappuccino and Sherman, 1999) was carried out to test the ability of an organism to produce and maintain stable acid end products from glucose fermentation. In this test, the pH indicator methyl red detects the presence of large concentrations of acid end products. If the tube turns red in colour, it indicates a positive test and if the indicator turns yellow it indicates a negative test.

The Voges – Proskauer (VP) test (Cappuccino and Sherman, 1999) determines the capability of organisms to produce non-acidic or neutral end products, such as acetylmethylcarbinol (acetoin), a natural product formed from pyruvic acid in the course of glucose fermentation. The reagent used in this test, Barritt's reagent (Cappuccino and Sherman, 1999) consists of a mixture of alcoholic α- napthol and 40% potassium hydroxide solution. Development of a deep rose color in the culture after 15 minutes of addition of Barritt's reagent was indicative of the presence of acetoin and this represented a positive result. The absence of rose coloration was a negative result.

Table 3.7 Composition of MR-VP broth

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Buffered peptone	7.00
2	Dextrose	5.00
3	Dipotassium phosphate	5.00

pH of the medium was adjusted to 6.9.

3.4.5. Carbohydrate fermentation medium

This biochemical test was performed to determine the ability of microorganisms to degrade and ferment carbohydrates with the production of acid or acid and gas. For the purpose, different types of carbohydrate-enriched media were used:

(a) Phenol red lactose broth

(b) Phenol red dextrose broth

(c) Phenol red sucrose broth

(d) Phenol red xylose broth

(e) Phenol red mannitol broth

Table 3.8 Composition of phenol red lactose, dextrose and sucrose broths

Sl.No	Chemicals	Composition ($g.l^{-1}$)				
		Phenol red lactose	Phenol red dextrose	Phenol red sucrose	Phenol red xylose	Phenol red mannitol
1	Proteose peptone	10.00	10.00	10.00	10.00	10.00
2	Beef extract	1.00	1.00	1.00	1.00	1.00
3	Sodium chloride	5.00	5.00	5.00	5.00	5.00
4	Phenol red	0.018	0.018	0.018	0.018	0.018

5	Lactose	10.00	-	-	-	-
6	Dextrose	-	1.00	-	-	-
7	Sucrose	-	-	5.00	-	-
8	xylose	-	-	-	5.00	-
9	mannitol	-	-	-	-	5.00
pH		7.4	6.9	6.9	6.8	6.8

Fermented carbohydrates with the production of acidic wastes might cause yellowing of phenol from red colour, thereby indicating a positive reaction. In some cases, acid production is accompanied by the evolution of CO_2 gas which becomes visible as bubbles in the inverted tube. Cultures that are not capable of fermenting carbohydrates are negative ones.

3.4.6. Extracellular enzymatic activities (hydrolysis) test medium

Hydrolysis was carried out in the concerned medium to determine the ability of microorganisms to excrete extracellular hydrolytic enzymes capable of degrading polysaccharides, lipids and proteins (casein and gelatin). The test was used to differentiate microbes based on their ability to hydrolyze starch with the exoenzyme amylase.

In the experiment, tributyrin agar was used to determine the hydrolytic activity of lipase. The medium was composed of nutrient agar supplemented with triglyceride tributyrin as the lipid source. In the experiment, milk agar was used to determine the hydrolytic activity of the exoenzyme. The bacterial isolates produced a clear zone surrounding their growth and loss of opacity, which represented a positive result. In the absence of protease activity, the medium surrounding the growth of the bacteria remained opaque, which was a negative reaction.

Table.3.9 Composition of starch agar, tributyrin agar, nutrient gelatin, milk agar and medium for hydrolysis tests

Chemicals	Concentrations $g.l^{-1}$ for the media			
	starch agar	tributyrin agar	milk agar	nutrient gelatin
Peptone	5.00	5.00	5.00	5.00
Beef extract	3.00	-	-	3.00
Yeast extract	-	3.00	-	-
Skim milk powder			100.00	
Gelatin	-	-	-	120.00
Starch	2.00			
Agar	15.00	15.00	15.00	-
pH	7.0	6.9	6.9	6.8

Gelatin liquefaction test was used to determine the ability of bacteria to produce hydrolytic exoenzymes called gelatinases that digest and liquefy gelatin. The presence of these enzymes, as determined by the liquefaction, was used for identifying certain bacteria.

3.4.7. Citrate utilization test

The citrate utilization test was used to determine the ability of an organism to produce the enzyme citrase for using citrate as its sole source of carbon. The pH indicator, bromthymol blue, turned from green at neutral pH (6.9) to blue when a pH higher than 7.6 was reached (basic or alkaline). If citrate would be utilized, the resulting growth of the bacteria would produce alkaline products (pH > 7.6), changing the color of the medium from green to blue.

Table 3.10 Composition of Simmons citrate agar slant medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Magnesium sulphate	0.20
2	Ammonium dihydrogen phosphate	1.00
3	Potassium phosphate	1.00
4	Sodium citrate	2.00
5	Sodium chloride	5.00
6	Bromothymole blue	0.08
7	Agar	15.00

pH of the medium was adjusted to 6.8

3.4.8. Nitrate reduction test

The test conducted in the tryptic nitrate medium detects the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or some other nitrogenous compounds, such as molecular nitrogen (N_2), using the enzyme nitrate reductase. The nitrate medium contains potassium nitrate as the substrate. If the organism reduces the nitrate to nitrite, the nitrite will react with added reagents namely, sulfanilic acid and α - naphthylamine to produce a red color. If no color is developed, it indicates either of the following two reactions: (i) nitrate is not reduced or (ii) nitrate is reduced even further to compounds other than nitrite (NH_2 or N_2).

Table 3.11 Composition of tryptic nitrate broth medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Casein enzymic hydrolysate	20.00
2	Disodium phosphate	2.00
3	Dextrose	1.00
4	KNO_3	1.00
5	Agar	1.00

pH of the medium was adjusted to 7.6

To distinguish between the negative reaction and the complete reduction, zinc dust was added to the medium. In the case of nitrate being present in the medium, zinc would reduce NO_3 to NO_2, and then a pink color would be observed. This was a negative reaction. No color change after the addition of zinc meant that nitrate was not reduced to NO_2 but to compounds other than NO_2.

3.4.9. Indole production test

The indole test was used to identify bacteria capable of producing indole by using the enzyme tryptophanase. The by-product indole was the metabolite identified by this test. When Kovac's reagent, which contains hydrochloric acid and dimethylaminobezaldehyde and amyl alcohol, was added, a red layer was formed on indole being present. No color development in the layer meant a negative result.

3.4.10. H_2S production test

This test was used to identify those bacteria capable of reducing sulfur. Hydrogen sulfide (H_2S) could be formed by anaerobic respiration. The medium contained cysteine, an amino acid containing sulfur, and sodium thiosulfate with peptonized iron or ferrous sulfate. The H_2S would react with the iron or ferrous sulfate, forming a black precipitate. If the black precipitate would be present, the test would be positive for H_2S production. No precipitate formation would refer to a negative test. The composition of the Sulfide Indole Motility medium (SIM) is given in Table 3.12.

Table 3.12 Composition of Sulfide Indole Motility medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Pancreatic digest of casein	20.00
2	Peptic digest of animal tissue	6.10
3	$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.20
4	$Na_2S_2O_3.5H_2O$	0.20
5	Agar	3.50

pH of the medium was adjusted to 7.3.

3.4.11. Litmus milk reaction test

This test was used to identify those bacteria capable to enzymatically transform different milk substrates into varied metabolic end products. Formation of lactic acid during the reaction would turn the purple litmus to pink colour.

Table 3.13 Composition of litmus milk medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Skim milk powder	100.00
2	Litmus	0.50
3	Sodium sulfate	0.50

pH of the medium was adjusted to 6.8.

3.4.12. Urease test

This test carried out in urea broth could detect the ability of an organism to degrade urea by means of the enzyme urease. The presence of urease is detectable as and when the phenol red turns to a deep pink. This is a positive reaction for the presence of urease. Failure to develop deep pink color is the evidence of a negative reaction. The test was carried out to assess the urease activity of the bacterial isolates. The composition of the urease test medium is presented in Table 3.14.

Table 3.14 Composition of Sulfide Indole Motility medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Urea	20.00
2	Na_2HPO_4	9.50
3	KH_2PO_4	9.10
4	Yeast extract	0.10
5	Phenol red	0.01

pH of the medium was adjusted to 6.8.

3.4.13. Triple sugar iron agar test

This test in tryptic nitrate broth with three sugars and iron was carried out in order to differentiate bacteria due to their ability to ferment glucose, lactose and sucrose or to reduce sulfur to hydrogen sulfide. It was used primarily to distinguish the morphologically similar bacteria of enterobacteriaceae.

Table 3. 15 Composition of tryptic nitrate broth

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Casein enzymic hydrolysate	10.00
2	Peptic digest of animal tissue	10.00
3	Yeast extract	3.00
4	Beef extract	3.00
5	Lactose	10.00
6	Sucrose	10.00
7	Dextrose	1.00
8	Ferric ammonium citrate	0.30
9	Sodium chloride	5.00
10	Sodium thiosulphate	0.30
11	Phenol red	0.024
12	Agar	12.00

pH of the medium was adjusted to 7.4.

Triple sugar iron agar contains peptone, glucose, sucrose, lactose and thiosulphate. Phenol red is the pH indicator (yellow at pH less than 6.8 and red above 6.8).

3.5. Isolation of microbes from other sources

Bacterial strains were isolated from engine oil contaminated soil samples collected from Tezpur, Assam. The samples were serially diluted in the sterile PBS up to 10^{-7} From the dilution, 100 µl was plated in LB agar plates to get pure colonies.

3.5.1. Phosphate buffer saline

The PBS solution was used in screening experiments to suspend crude/engine oil contaminated soil samples in liquid-phase. The PBS was used for serial dilution as because, the soil samples contained high amount of oil. The dilution in PBS would reduce the level of oil content.

3.5.2. Direct isolation of microbes from environmental samples

Water and soil samples collected from hot springs of Himachal Pradesh were serially diluted in sterile distilled water up to 10^{-7}, cultured on LB agar plates followed by incubation at the temperatures of their original habitats (45°C, 70°C and above). Single colonies were picked up by the conventional technique. Isolates were preserved in 15% (v/v) glycerol-supplemented LB broth and kept at -70°C.

3.6. Screening of hydrocarbon degrading bacterial isolates

3.6.1. Bushnell-Hass medium

The pure cultures of bacterial isolates were selected from enrichment broth cultures supplemented with 1% (v/v) hexadecane. Bacterial isolates further grown on Bushnell-Hass medium (Difco) ensured the utilization of hydrocarbons. The medium supplemented with 1% (v/v) n-hexadecane was

used for screening the potential hydrocarbon degrading and biosurfactant producing bacterial isolates (Casellas *et al.*, 1997).

Table 3.16. Composition of Bushnell-Hass medium

Sl.no	Chemicals	Composition ($g.l^{-1}$)
1	Mg SO_4	0.20
2	$CaCl_2$	0.20
3	Monopotassium phosphate	1.00
4	Ammonium phosphate Dibasic	1.00
5	Potassium Nitrate	1.00
6	Ferric chloride	0.05

pH of the medium was adjusted to 7.0.

3.6.2. Hydrocarbon degradation assay

Pure cultures of hexadecane degrading bacterial isolates and putative hydrocarbon degrading bacteria (13 cultures) were tested for their ability to degrade aliphatic (hexadecane, dodecane and octadecane), aromatic (benzene, toluene and xylene) and polycyclic aromatic hydrocarbons (pyrene, fluorine and carbazole). The experiments were conducted using the following procedure:

(a) bacterial isolates were grown in 100 ml mineral salt medium supplemented with 1% (v/v) aliphatic hydrocarbons, 0.2 % (v/v) aromatic hydrocarbons and 0.2% (w/v) polycylic aromatic hydrocarbons in a 250 ml conical flask for 96 h at 37°C and 200 rpm.

(b) at different time intervals, 2 ml sample from each culture was drawn and cells were harvested by centrifugation at 6000 rpm for 10 min, washed twice with cold phosphate buffer saline (pH 7.2), and resuspended in mineral salt medium and determined the OD at 600 nm.

(c) after the preliminary investigation, the bacterial isolates were cultured in enriched mineral salt medium with 1% (v/v) hexadecane, dodecane and octadecane (Merck, Germany).

(d) the isolates, which were able to utilize hexadecane, dodecane and octadecane, were found to be aliphatic hydrocarbon degraders as well.

(e) bacterial isolates were also cultured in media enriched with aromatic and polycyclic aromatic hydrocarbons. Isolates capable to grow in these hydrocarbons were classified as aromatic and PAHs degraders.

(f) the isolates were maintained by sub-culturing on weekly basis.

3.7. Bioremediation of crude oil contaminated soil under laboratory conditions

3.7.1. Formulation of microbial consortia

After five cycles of enrichment with different fractions of hydrocarbons, 100 μl of the culture broth was plated on a mineral salt agar medium with appropriate dilution containing crude oil and solvent evaporated PAHs (0.2%) as the sole source of carbon and energy. Out of 13 bacterial isolates, 10 were selected on the basis of their growth on crude oil supplemented media and for their ability to degrade individual components of crude petroleum in the medium. Two bacterial consortia were formulated by mixing these bacterial isolates.

3.7.2. Determination of bacterial protein content (growth) in culture media

Bacterial growth in hydrocarbon-supplemented medium was assessed in terms of increased protein concentration in the medium, dry biomass production and the residual hydrocarbons remained in the culture medium (Vila *et al.*, 2001). Dry biomass of bacterial cells in the medium was determined after extracting the residual hydrocarbons and then pelleting the cells following the method described by Makkar and Cameotra (1998). The protein concentration was measured using the flask contents of duplicate cultures by modifying the Lowry method (Daniel *et al.*, 1994).

3.7.3. Biodegradation assay

Biodegradation assay was carried out using 100 ml of mineral salt medium in 250 ml Erlenmeyer flasks. Individual hydrocarbon fraction 2 ml (v/v) and crude oil 2 ml (v/v) were separately added to the medium as the sole source of carbon and energy. For each consortium, inoculum was prepared from the enrichment culture maintained in mineral medium. After 7 days of culture, 1 ml sample from each of the enrichment cultures was first washed with PBS (pH 7.0) and then inoculated the mineral medium containing crude oil (of Assam asset basin) as the sole source of carbon. The targeted final concentration of bacterial cells was approximately 1x 10^{6} $cfu.ml^{-1}$.

Culture media supplemented with Assam asset basin crude oil were incubated for 30 days at 37°C in a rotary-shaking incubator at 200 rpm. Non-inoculated flasks were taken as control for assessing abiotic losses.

3.7.4. Laboratory scale bioremediation

Soil samples artificially contaminated with 20% crude oil were subjected to bacterial inoculation for laboratory scale bioremediation. The thickness of the soil substrate was 4 cm with an area of 39 x 30 sq cm. Inoculation density was 10^{6} $cfu.ml^{-1}$ and the treatments were maintained at room temperature. In order to maintain soil moisture and nutrients, the liquid mineral medium was sprayed in each treatment including the control (non inoculated) at an interval of 15 days. After 180 days of bioremediation, the residual crude oil was assayed by extracting with dichloromethane at a monthly interval.

3.7.5. Estimation of total petroleum hydrocarbon (TPH) and its fractionations

The TPH was extracted from the control as well the treated soil at 0, 120 and 180 days of bioremediation. For the purpose, 10 g soil was treated with 100 ml each of hexane, methylene chloride and chloroform. All three

extracts were pooled and dried at room temperature by evaporation of the solvents under a gentle nitrogen stream in a fume hood. After the evaporation of solvents, the amount of residual TPH was determined gravimetrically. Further, fractionation of TPH into aromatic, aliphatic, asphaltene and NSO (nitrogen, sulfur and oxygen- containing) compounds was done on a silica column (30 X 2 cm). TPH extracted were dissolved in *n*-pentane and separated in to soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on a silica gel column and eluted with different solvents. The alkane fraction was eluted with 100 ml of hexane followed by the aromatic fraction (eluted with 100 ml of toluene). The NSO fraction was eluted with methanol and chloroform (100 ml each).

3.7.6. Assay of residual crude oil

The residual crude oil and pure hydrocarbon fractions from cultures and control were extracted with dichloromethane (5 x 15 ml) in duplicate samples. An aliquot of 5 ml culture was extracted with hexane, methylene chloride, and chloroform: methanol (1:1) each separately. All three extracts were mixed together and dried at room temperature over Na_2SO_4 and concentrated using a rotary evaporator to a final volume of 3 ml.

3.7.7. Gravimetric measurement

After solvent evaporation, the residual crude oil was determined by measuring the weight of the dry extract. The rate of biodegradation and abiotic loss were evaluated by comparing their weights against the control samples as well as the fresh weight of 2 ml crude oil.

3.7.8. Fractionation by column chromatography

Three dried extracts were fractionated by column chromatoghraphy according to Aceves *et al.* (1988). Each extract was resuspended in 2 ml hexane and then loaded in a glass column (30 cm long having x 2 cm internal diameter, filled with 20 g of silica of 60-80 mesh (Merck India). The

column was equilibrated overnight with *n*-hexane. Fractions were eluted with 20 ml of *n*-hexane (aliphatic hydrocarbon) using 20 ml of toluene (aromatic hydrocarbon) and followed by 20 ml of chloroform: methanol 1:1 (resins and asphaltene). All these fractions were further analyzed by GC.

3.7.9. GC analysis of the residual hydrocarbon

Analysis of alkane fraction was performed by using Varian 3800 gas chromatography with FID detector. Helium served as the carrier and make up gas. Liquid samples were analyzed by using CPSil 8 low bleed (30 m X 0.25 mm X 0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m X 0.25 mm X 0.25 μm) column. The column temperature was 80-240°C for 30 min with 5°C/min increment and hold at 240°C for 30 min. The injector temperature was 240°C and the injector was in splitless mode. The detector temperature was programmed at 300°C.

3.8. Polycyclic aromatic hydrocarbons (PAHs)

3.8.1. Biodegradation of PAHs

Polycyclic aromatic hydrocarbons were dissolved in N-N-dinitroformamide and used at a level of 200 $mg.l^{-1}$ concentration along with the mineral salt medium with and without phenanthrene (100 $mg.l^{-1}$). PAHs utilization was initially assessed by disappearance of PAH crystals and increase in the optical density of the media containing PAHs/PAHs+ phenanthrene as the sole source of carbon. PAHs were extracted from the culture medium with ethylacetate. The control flask was the same having no inoculation. Residual PAHs were quantified by evaporation of ethylacetate at its boiling temperature (87°C) and then taken the weight.

The residual PAHs were extracted with a mixture of chloroform and methanol (v/v 20:10) as described by Zhang *et al.* (2004). An extracted sample of 1 ml was filtered through a 0.4 μm filter and 20 μl of the filtrate was then analyzed for PAHs content by a HPLC (Waters) with a reverse-

phase C_{18} Nova pak column (3.9 mm X 150 mm) and using isocratic elution with acetonitrile-water (Pickard *et al.,* 1999). Flow rate was adjusted to 1 $ml.min^{-1}$ and elution of PAHs was monitored at 273 nm, 250 nm and 253 nm. The decrease in the amount of pyrene, flourene and phenanthrene in the experimental flasks were estimated by measuring the peak area of UV absorbance at 273 nm, 250 nm and 253 nm respectively and by comparing with the peak area of control flasks. Pyrene, flourene and phenanthrene concentrations were expressed as mean and standard deviation based on the results obtained with triplicated flasks.

3.8.2. Uptake of pyrene, flourene, phenanthrene and crude oil by bacterial isolates

Pyrene, flourene, phenanthrene and crude oil uptake by the bacterial cells were measured using spectrophotometric rate assay as described by Stringfellows and Aitken (1995). Briefly 1.0 X 10^7 bacterial cells (final volume of 3.0 ml in 20 mM phosphate buffer containing 150 mM NaCl, pH 7.0) were placed in a 3.5 ml quartz cuvette of a UV/Vis Spectrophotometer (backman DU-530) along with 60 μg each of pyrene, flourene, phennanthrene and crude oil (in 10 μl acetone). The absorbance was measured in a decreasing wave length of A_{273}, A_{250}, A_{253} and A_{254} from 0 sec to 60 min following the addition of PAHs and crude oil. PAHs and crude oil uptake were also measured in the presence of a suspension of killed cells. From a standard curve of representative PAHs and crude oil, decreases in PAHs and crude oil content were calculated and results are expressed in μg of PAHs and crude oil uptake by bacterial cells (1.0 X 10^7).

To study the uptake of biosurfactant-solubilized PAHs and crude oil by the bacterial isolates, stock solutions of PAHs and crude oil (in acetone) were incubated overnight at 32°C with biosurfactants (0.5 $mg.ml^{-1}$) harvested from the respective bacterial isolates. An aliquot of 60 μg of each of the biosurfactant-solubilized PAHs and crude oil was injected in to the cuvette containing the bacterial cells (final volume of 3 ml). PAHs and crude

oil uptake measurement were done in the same manner as described above.

3.8.3. PAHs and crude oil solubilization assay

PAHs and crude oil solubilization assay were done as described by Barkay *et al.* (1999). An aliquot of 60 μg of PAHs and crude oil (from 6 $mg.ml^{-1}$ stock in hexane) were distributed in to test tubes (10 mm X 170 mm) and kept open in an operating chemical fume hood to remove the solvent. Then 3.0 ml of assay buffer (20 mM Tris-HCL, pH 7.0) and 1.0 ml of biosurfactant solution (0.5 $mg.ml^{-1}$) were added. Tubes were capped and then incubated in a vertical position overnight at 32°C with shaking (200 rpm) in darkness. Samples were filtered through 1.2 μm filter (Whatman); an aliquot of 2.0 ml was removed to a clean test tube, added 2.0 ml hexane prior to extraction by vortexing for 2 min. This emulsion was centrifuged at 10,000 rpm for 10 min to separate the aqueous and hexane phases. PAHs and crude oil in the hexane extracts was measured spectrophotometrically at 273, 250, 253 and 254 nm on the basis of a calibration curve of PAHs and crude oil in hexane. The control experiments were also run in parallel where no biosurfactants were added to PAHs and crude oil before the extraction with hexane.

3.9. Reclamation of bioremediated soil

Soil reclamation experiments were designed to assess whether the bioremediated soil can support normal plant growth. For the purpose, three soil types were used: soil bioremediated with the bacterial Consortium II, normal soil and soil artificially contaminated with 20% crude oil (control). Experimental soil weighing 1.5 kg was taken for the reclamation experiment; five substrates used for growing 3 crops were (1) Normal soil + NPK + organic manure (N1), (2) Bioremediated soil + NPK + organic manure (R1),

(3) Normal soil alone (N3), (4) Bioremediated soil (R2) and (5) Normal soil + 20 % crude oil (C1).

3.9.1. Planting of crops

Pre-germinated seedlings of Bengal gram (*Cicer aretinum*), Green gram (*Phaseolus mungo*) and Rice (*Oryza sativa*) were grown in the soil samples till maturity. Soil moisture in the pots cultivated with the pulses and water level in the pots planted with rice were maintained by adding sufficient water at regular interval.

3.9.2. Observation and record

The following data were recorded on the cultivated crops:

1. Mortality after transplanting in soil samples in pots.
2. Growth rate as the plant height at 30 days interval.
3. Tillering of rice or branching of pulses till flowering.
4. Duration to flowering.
5. Duration to maturity.
6. Yield per plant.

3.10. Screening of biosurfactant producing mesophilic bacteria

3.10.1. Screening of biosurfactant producing bacteria

The pure cultures of bacterial isolates were tested for their ability to produce biosurfactant on the basis of their ability to reduce the surface tension of the growth medium (Maier, 2003 and Mulligan, 2005). Potential biosurfactant producing microbial isolates were screened by a rapid drop-collapsing test (Bodour *et al.*, 1998). In this technique, a drop of culture broth was placed on an oil-coated paper surface and then assessed its collapse. Out of 30 drops of each culture solution, only 4 collapsed indicating the production of biosurfactant.

Along with the production of biosurfactant, each bacterial isolate also exihibited one more character by forming foam during the enrichment culture

in mineral medium supplemented with 2% (v/v) *n*-hexadecane, octadecane, dodecane or glucose (w/v).

3.10.2. Estimation of surface activity of biosurfactants

Surface tension and critical micelle dilution (CMD^{-1} and CMD^{-2}) were determined using a Du-Nouy Tensiometer (Kruss 9KT Tensiometer, Kruss, Germany) at room temperature (25°C) with the ring correction mode of the instrument. All surface activities were tested on cell free culture broths and *aqueous solutions of purified biosurfactants. Cells from the fermentative* broths were pelleted by centrifugation at 12,000 rpm for 15 min in Sorval 5B centrifuge at 4°C.

3.10.3. Critical Micelle Concentration

Critical micelle concentration (CMC) was determined by the sudden break in the curve obtained by plotting surface tension values against the log of concentration of biosurfactants.

3.11. Isolation and purification of biosurfactant

3.11.1. Isolation

Crude biosurfactant was extracted from cell-free culture broth after 96 h of growth following acetone precipitation and acidification of the broth to pH≈2.0. After the removal of acetone, the broth was acidified using 6 N HCl. Overnight precipitation was allowed at 4°C. The product was recovered by centrifugation and drying under the vacuum, then weighed and stored.

3.11.2. Purification of biosurfactants

Biosurfactant was purified by using column chromatography and TLC. Biosurfactants were eluted with a linear gradient of methanol (80%) and 10 mM potassium phosphate buffer, pH 6.0 (20%) at a flow rate of 1 ml. min^{-1}. Detection was monitored at 210 nm and the peak showing the maximum biosurfactant activity was lyophilized and stored. Specific activity of the

purified fraction and that of the crude was defined as the amount of biosurfactant required to reduce the surface tension by 1mN.m^{-1} at 25 °C.

3.12. Biochemical composition of biosurfactants

3.12.1. Quantitation of protein

The protein content in the biosurfactant was estimated by Folin-Lowry method (Lowry *et al.*, 1951) using BSA as the protein standard. The protein content of unknown samples was calculated from the standard curve obtained by plotting optical density vs concentration of BSA (1 mg.ml^{-1}).

3.12.2. Estimation of carbohydrate content

Total carbohydrate was quantitated by Phenol-sulphuric acid method as described by Dubois *et al.* (1956) using D-glucose as the standard. The optical density of the reaction mixture was measured at 490 nm against a reagent blank. The carbohydrate content of the unknown samples was calculated from the standard curve obtained by plotting optical density Vs concentration of D-glucose (0.1 mg/ml).

3.12.3. Estimation of total lipid

The total lipid was estimated gravimetrically as described by Folch *et al.* (1956). Briefly, 10μg of crude biosurfafacant was homogenized with a 2:1 chloroform-methanol mixture. The crude extract was mixed thoroughly with 0.2 its volume of water and the mixture was allowed to separate into two phases. The upper phase was removed by siphoning with micropipette. Finally, the lower phase was taken and dried properly and gravimetrically estimated the total lipid content.

3.12.4. Temperature and pH stability of the isolated biosurfactants

Stability studies were carried out using the cell free broth obtained by centrifuging the cultures at 10,000 rpm for 15 min. Culture broth measuring 20 ml was heated in a boiling water bath for different time intervals(5 to 60

min) and cooled to room temperature. Surface tension and CMD^{-1} and CMD^{-2} values of each sample were measured. To study pH stability of cell free culture broth, the pH of the cell free culture broth was adjusted from 2 to 11 and then surface tension and CMD^{-1} and CMD^{-2} values were determined.

3.12.5. Emulsification and emulsification stability

Emulsification assay of biosurfactant was carried out following a new method developed in the laboratory. Weighed amount of biosurfactant was dissolved in 4 ml PBS buffer pH 7.0. To this, 1 ml of hexadecane was added. The resultant mixture was vortexed at high speed for 1 min and then the optical density measured at 540 nm. Emulsification index is defined as the amount of surfactant in $\mu g.ml^{-1}$ required to increase the optical density at 540 nm by 1.0. For the control same volumes of PBS buffer and hexadecane were used. For determining the stability of the emulsion, the decrease in O.D. value for 1 h was recorded at 540 nm using Beckman DU 530^{R} spectrophotometer. Emulsification stability is defined as the value of decreasing optical density from 0 to 1 h.

3.12.6. Assessment on emulsification index of biosurfactants

Emulsification assay of biosurfactant was also carried out following the method described by Cooper *et al.* (1987). The emulsification activity was measured by adding 6 ml of kerosene to 4 ml of aqueous sample and vortexing the mixture at high speed in SPINIX vortex for 2 min. The resulting mixture was kept at room temperature for 24 h and the emulsification index (E_{24}) was calculated as follows:

E_{24} = Height of emulsion layer (cm) X Total height of the mixture (cm) X100

3.13. Optimization of biosurfactant production

3.13.1. Effect of carbon sources

A total of 16 carbon sources were used to study their effect on biosurfactant production. Dextrose and molasses at the rate of 2% w/v;

glycerol, hexadecane, dodecane, light paraffin (liqued), octane and octadecane at the rate of 2% v/v were used for the purpose. The aromatic hydrocarbons pyrene, fluorene and phenathrene were dissolved separately in N-N-dinitroformamide and then used at the concentration of 200 $mg.l^{-1}$ in the mineral medium. After 48 and 96 h of culture, the surface tension, CMD values, yield of biosurfactant and dry biomass were assessed and recorded.

3.13.2 Effect of concentrations of carbon sources

Different concentrations 0.5, 1, 2 and 3% of glycerol and glucose were used to study the growth behavior of bacterial isolates. After the incubation period the surface tension, CMD values and dry biomass were estimated and recorded.

3.13.3. Effect of agitation on biosurfactant production

Erlenmeyer flask (250 ml) containing 100 ml mineral salt medium was used for the growth of bacterial isolates and biosurfactant production. The flasks were incubated in a shaker with the agitation set at 100, 200 and 300 rpm to evaluate the impact of agitation on biosurfactant production. A flask without any agitation was also set considering it to be 0 rmp. After the required incubation period the surface tension, CMD values and dry biomass were estimated and recorded.

3.13.4. Fermentation studies

For batch fermentation, bacterial isolates were grown in 3.0l working volume of minaral medium in a Bioflow 110 Fermentor (New Brunchwich Scientifc, USA) set at 37°C and pH 7.0 - 8.0 with an agitation of 200 rpm. When the cells reached the late log phase were harvested and biomass determined by the method described in the section 3.3. The cell-free clear supernatant was used for the surface activity assessment.

3.14.1. FT- IR spectra analysis

The IR spectrum of the biosurfactant was recorded using KBr pellet in Nicolas Impact 410 spectrophotometer. The spectra showing the functional group were used to study the composition of the biosurfactant. Absorption spectra were plotted using a built-in plotter. IR spectra were collected from 500-4000 wave numbers (cm^{-1}). Samples were prepared by dispersing the solid uniformly in a matrix of dry nujol (KBr) mull, compressed to form an almost transparent disc.

3.15. *Cell surface hydrophobicity*

The surface hydrophobicity of the cells was measured by the method of Rosenberg *et al.* (1980). Cells were grown either on 2% (v/v) hexadecane or 2% glycerol separately containing basal mineral medium for 20-24 h at 37°C. Then cells were harvested by centrifugation at 7,000x g for 15 min, washed twice with phosphate buffer (50 mM, pH 7.5) and suspended in the same buffer, in such a way that the final absorbance was adjusted to 0.29 – 0.31 at 600 nm. To 3.0 ml of washed cell suspension, different hydrocarbons (hexadecane, hexene and benzene) were added. Each mixture after 5 min of incubation at room temperature was vortexed for 60 sec and then rested at room temperature for 15 min followed by the removal of the aqueous phase with a Pasture pipette. The absorbance was recorded at 600 nm using a Hitachi U-1000 spectrophotometer.

The cell surface hydrophobicity was expressed in terms of percent cell transferred to the hydrocarbon phase, which was calculated as follows:

$$\% \text{ cell transferred to hydrocarbons} = 100 - \frac{\text{Abs. at 600 nm after mixing}}{\text{Abs. at 600 nm before mixing}} \times 100$$

3.16. Oil release from sand pack column

The potential use of biosurfactant in MEOR was evaluated using the sand pack column technique as described by Abu Ruwaida *et al.* (1991). Glass columns (3 x 60 cm^2), packed with 100 g of acid washed sand were saturated separately with 60 ml of kerosene and crude oil. The columns were treated with surfactant solutions and then the release of the crude oil was measured. In the control experiment, the same volume of the medium (devoid of biosurfactant) was applied in to the column.

3.17. Isolation of bacterial DNA

3.17.1. DNA extraction by alkaline lysis

Genomic DNA of bacterial isolates was prepared following the method of Ausubel *et al.* (1995).

(1) The cells were pelleted by centrifuging 15 ml of culture broth at 8,000 rpm for 15 min and the supernatant was decanted.

(2) Pellets were resuspended in 3 ml of solution I (Appendix).

(3) To the resultant mixture, 600 µl lysozyme (10 $mg.ml^{-1}$) was added and incubated at room temperature for 20 min.

(4) Subsequently, 167µl of 10% SDS solution was added and incubated for 10 min at 50°C.

(5) After adding 200 µl of Rnase A (10mg/ml), the solution was incubated at 37°C for 1½ h.

(6) This was followed by addition of 170 µl of Na-EDTA (0.1 M pH 8.0) and incubated at 50°C for 10 min.

(7) To remove the protein, 100 µl of proteinase K (5 $mg.ml^{-1}$) was added and incubated at 50°C for 12 h.

(8) Equal volume of phenol (equilibrated with 0.1 M Tris HCL, pH 8.0) was added and mixed thoroughly.

(9) The mixture was centrifuged at 8,000 rpm for 15 min, the upper (aqueous) phase was aspirated in to sterile tube and the lower phase was discarded.

(10) 5 ml of phenol and chloroform (1:1) – isoamyl alcohol (24:1) was added and mixed thoroughly.

(11) Centrifuging it again at 7000 rpm for 15 min, the upper phase was transferred to a sterile tube.

(12) The DNA was precipited by adding 2 volumes of ice-cold ethanol (100%) and the DNA strand was pooled with a micro - tip and collected in an eppendorf tube.

(13) The DNA strand was again resuspended in 1 ml of 70% ice-cold ethanol and centrifuged at 10,000 rpm for 15 min.

(14) The dry DNA pellet was resuspended in 10 M Tris HCL- 1mM EDTA buffer (pH 8.0) at final concentration of 1 $\mu g.ml^{-1}$ and then added 20 µl of Rnase A and stored at 4 °C.

3.17.2. Isolation of plasmid DNA

Plasmid DNA was isolated from the bacterium by alkaline lysis as described by Sambrook *et al.* (1992).

(1) The cells were pelleted from 15-20 ml of culture by centrifugation at 6000 rpm for 10 min at 4°C.

(2) Pellets were resuspended in 1 ml of ice-cold solution I (Appendix) by vigorous vortexing.

(3) 2 ml of freshly prepared solution II (Appendix) was added and mixed slowly by inverting the tubes.

(4) Then 1.5 ml of ice- cold solution III (Appendix) was added and mixed by gently inverting the tubes followed by centrifugation at 10,000 rpm for 20 min at 4°C.

(5) The supernatant (containing plasmid DNA) was transferred to a fresh tube and equal volume of Tris equilibrated phenol: chloroform- isoamyl alcohol (24:1) was added and mixed thoroughly by vortexing followed by centrifugation at 6000 rpm for 20 min.

(6) The upper phase was transferred to a sterile tube and 0.7 volume of isopropanol was added.

(7) Contents were mixed thoroughly and then centrifuged at 13,000 rpm for 15 min at room temperature.

(8) DNA pellet settled down at the bottom of the tube was washed twice by adding 1.5 ml of 70% ice- cold ethanol.

(9) Plasmid DNA was recovered by centrifugation at 10,000 rpm for 10 min followed by removal of ethanol by drying in air. (10) The dried pellet was redissolved in 40 µl TE (1x) buffer containing 120 µg $.ml^{-1}$ Rnase and resolved in 0.8% agarose gel following staining with etidium bromide.

3.17.3. Curing of plasmid DNA

Plasmid curing was done following the method of Hirota *et al.* (1959).

(1) The test organism was grown in nutrient broth supplemented with acridine orange (final concentration ranging from 100 to 500 $mg.mL^{-1}$) and incubated overnight at 37°C.

(2) The culture tube having the highest concentration of the dye and showing visible growth was selected and diluted ten-fold in fresh nutrient broth.

(3) About 100 µl from each of the dilutions were spread on nutrient agar plates with the help of a sterile spreader.

(4) Mutants lacking the plasmid were isolated by plating in ampicillin $^{+-}$, tetracycline $^{+}$ and chloramphanicol^{+} plates. The mutants which failed to grow in these antibiotics were taken for further studies.

(5) The positive mutants were grown overnight in the nutrient broth medium and modified mineral medium for 48 h followed by isolation of plasmid DNA by alkaline lysis method described in section 3.16.2. 6. The positive mutant along with the wild type were grown in mineral salt medium and the surface activities along with biosurfactant production and growth was recorded after 96 h of incubation at the optimum growth temperature.

(7) After 96 h of incubation, plasmid DNA was again isolated to reinforce the result of the experiment.

3.18. Transfer of bacterial plasmid using calcium chloride method

3.18.1. Preparation of competent cells

Competent cell of bacterial isolates was prepared following the method of Cohen *et al.* (1972).

1. A single colony was picked from a plate freshly grown for 16-20 h at 37°C and transfered in to 100 ml of LB broth medium in a 250 ml flask. The culture was incubated for ~6 h at 37°C with constant shaking at 200 rpm in a rotary shaker.
2. The cells were aseptically transferred in to a sterile pre-cooled polypropylene tube. The cultures were kept at 0°C for 10 minutes.
3. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C in a Sorvall G5B rotor.
4. The medium was decanted from the cell pellets and then tubes were kept inverted for 1 min on a filter paper for draining out.
5. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice.

3.18.2. Plasmid DNA mobilization

Plasmid DNA was mobilized in to competent cells of theromophillic bacteria following the method described by Cohen *et al.* (1972).

1. Using a chilled sterile pipette tip 200 µl of each of competent cell suspensions was transferred to a sterile microcentrifuge tube.
2. An aliquot of 10 µl (not more then 50 ng) of plasmid DNA was added to each tube. Mixed the contents of the tubes by swirling gently. The tubes were stored on ice for 30 min.
3. The tubes were transferred to a shaking water bath having temperature 70°C for 90 sec.

4. The tubes were then immediately removed to the room temperature and allowed the cells to chill for 1-2 min.
5. An aliquot of 800 µl LB medium was added to each tube and then cultures were incubated for 45 min in a water bath at 70°C to allow the bacteria to recover and to express the antibiotic resistance marker gene retained by the plasmid.
6. The transformed competent cell suspension 100 µl was transferred to LB agar plates containing antibiotics such as ampicillin, chloramphanicol and tetracycline. The inoculum of transformed cells was spread over the surface of the agar plates using a sterile glass rod.
7. The plates were left at room temperature until the liquid phase was absorbed.
8. The plates were then inverted and incubated at 70°C.

Chapter 4
Results

Chapter 4

Results

This study highlights the development of innovative *in situ* bioremediation technologies that may be viable in the context of the specific environmental situations of the North East India.

4.1. Bioremediation

4.1.1. Isolation and pure culture of microbes capable of utilizing hydrocarbons

From different environmental crude oil - contaminated soil samples, 58 culturable bacterial isolates were recovered, pure cultured and maintained in stab agar cultures at 4°C and preserved in 15% glycerol at -70°C. The environmental samples included crude oil - contaminated soils from the ONGC oil fields, engine oil contaminated soils from Tezpur and hot spring water samples from Manikaran, Himachal pradesh.

The procedures of isolation, screening and subsequent selection of potential bacterial isolates capable of utilizing hydrocarbons at 37°C was based on two steps (i) isolation of microbes from different environmental samples either by enrichment culture or by direct plating and incubating the cultures at 37°C for 24 h and (ii) culturing of microbes in mineral-salt medium along with a carbon source for enrichment and then incubation for 24 h at 37°C with constant shaking (200 rpm). Sub-culturing was done by re-suspending the microbes in fresh mineral medium. Hexadecane was added as the sole source of carbon and incubated at 37°C for 7 days with constant shaking (200 rpm). The population density was lowered down by serial dilution to 10^{-7} then cultured in nutrient agar (pH 6.8) and Bushnell-Hass media with overnight incubation at 37°C. Subsequent subcultures were made to obtain pure colonies. All these steps helped in obtaining hydrocarbon-degrading microbes.

4.1.2. Density of Bacteria in different samples

The density of bacteria in the environmental samples was determined by the plate counting method and data generated are presented in Table 4. 1. In order to get approximately the same bacterial density of each isolate, the cultures were diluted to 10^{-7} before culturing. Bacterial density was found to be higher in surface samples as compared to sub-surface ones.

Table 4.1. Bacterial density in petroleum contaminated surface and sub-surface soil samples

Sl.No	Oil field	Environmental sample from the well	Bacterial colonies derived from	
			Surface soil	Sub-surface soil
1	Digboi	D#848	35	20
		D#1	0	0
		Mean	35	20
2	Duliajan	DJN#132	52	23
		DJN#191	45	19
		Mean	48.5	21.0
3	Geleky	G#85	40	15
		G#35	45	26
		G#207	52	22
		G# 48	35	08
		G# 296	25	07
		Mean	39.4	15.6
4	Lakuwa	L# 485	41	22
		L# 253	51	14
		L# 490	35	18
		L# 64	53	15
		L# 123	50	26
		L# 453	27	10
		L# 43	48	13
		Mean	43.5	16.8
5	Rudrasagar	R# 79	37	21
		R# 48	25	11
		R# 74	16	08
		R# 38	15	05
		Mean	23.2	11.2
6	Lakuwa	Effluent	13	07
		Mean	13	07
7	Tezpur	TW_1	18	02
		TW_2	25	12
		Fs_1	12	06
		Mean	18.3	6.6

The density of bacteria in the surface and sub-surface soils at different sites were determined. Bacterial colony density was found to be the highest (48.5 colonies) in Duliajan surface soil samples. The corresponding subsurface bacterial colony density at the site was also found to be the highest (21 colonies). The lowest density was recorded at Lakuwa effluent treatment site with respect to both surface and sub–surface samples (13 and 07 colonies respectively). In the remaining sites, bacterial density ranged within the average number of bacterial colonies 43.5 to 18.3 (in the surface) and between 20 to 6.6 (in the sub -surface).

4.1.3. Morphological characterization of bacterial isolates

The bacterial isolates were obtained from different environmental samples and their colony characters studied by growing in nutrient agar medium. Characters recorded are presented in Table 4.2.

Table 4.2. Morphological characters of bacterial isolates obtained from crude oil-contaminated soil samples

S. N	Sample	Size	Pigment	Form	Margin	Elevation	Growth rate
1	R79-I	Large	White	Circular	Entire	Convex	FG
2	R79-II	Small	White	Circular	Serrate	Convex	SG
3	R79-III	Large	White	Irregular	Lobate	Raised	FG
4	R 48-I	Small	White	Circular	Entire	Raised	SG
5	R 48-II	Moderate	White	Circular	Entire	Convex	M
6	R 48-III	Large	White	Circular	Serrate	Raised	FG
7	R 74-I	Moderate	White	Circular	Entire	Flat	FG
8	R 74-II	Small	White	Circular	Entire	Flat	FG
9	DJN 132-I	Large	Yellowish	Circular	Entire	Flat	FG
10	DJN191-I	Large	White	Circular	Undulate	Flat	FG
11	DJN191-II	Small	White	White	Entire	Raised	FG
12	D 848-I	Moderate	White	Circular	Entire	Convex	SG
13	D 848-II	Moderate	Yellowish	Irregular	Lobate	Flat	SG
14	G 48-I	Large	Yellowish	Irregular	Lobate	Raised	FG
15	G 48-II	Moderate	White	Circular	Entire	Raised	M
16	G 48-III	Large	White	Irregular	Lobate	Flat	FG
17	G207 –I	Pin point	White	Circular	Entire	Raised	SG
18	G207-II	Moderate	Yellowish	Circular	Entire	Convex	M
19	G207-III	Large	Yellowish	Irregular	Lobate	Flat	FG
20	G296-I	Large	Yellowish	Irregular	Lobate	Flat	M
21	G296-II	Moderate	Yellowish	Circular	Undulate	Flat	FG

22	G296-III	Pin point	White	Circular	Entire	Convex	SG
23	G85-I	Large	Greenish	Circular	Entire	Raised	FG
24.	G85-II	Small	Greenish	Circular	Entire	Flat	FG
25	L64-I	Large	White	Circular	Entire	Flat	SG
26	L64-II	Pinpoint	White	Circular	Entire	Flat	M
27	L 123-I	Large	White	Circular	Entire	Flat	FG
28	L 123-II	Large	White	Circular	Entire	Flat	FG
29	R 38-I	Pinpoint	White	Circular	Entire	Flat	FG
30	R 38-II	Small	Yellowish	Circular	Entire	Flat	M
31	R 38-III	Large	Yellowish	Circular	Filamentous	Flat	SG
32	L490-I	Large	White	Rhizoid	Entire	Flat	FG
33	L490-II	Moderate	Yellowish green	Circular	Entire	Flat	M
34	L490-III	Small	Yellowish	Irregular	Entire	Flat	SG
35	L 43-I	Large	Greenish	Irregular	Entire	Flat	M
36	L 43-II	Moderate	White	Circular	Entire	Flat	M
37	L 43-III	Pinpoint	Yellowish	Circular	Entire	Flat	M
38	L453-I	Large	White	Circular	Entire	Flat	FG
39	L453-II	Moderate	White	Circular	Entire	Raised	FG
40	Tw_1-I	Large	Green	Circular	Entire	Flat	FG
41	Tw_1-II	Large	White	Irregular	Undulate	Flat	M
42	Tw_1-III	Moderate	Yellowish	Circular	Entire	Flat	SG
43	Tw_2-I	Large	White	Irregular	Entire	Flat	M
44	Tw_2-II	Small	White	Circular	Entire	Flat	SG
45	Tw_2-III	Moderate	Pale Yellowish	Circular	Serrate	Flat	M
46	Fs_{-1}-I	Large	White	Rhizoid	Filamentous	Flat	M
47	Fs_{-1}-II	Large	White	Irregular	Undulate	Flat	M
48	Fs_{-1}-III	Small	White	Circular	Entire	Flat	M
49	L253-I	Moderate	White	Circular	Entire	Flat	M
50	L253-II	Moderate	White	Circular	Serrate	Flat	M
51	ETP-I	Pin point	White	Circular	Entire	Convex	SG
52	ETP-II	Small	White	Circular	Entire	Flat	SG
53	ETP-III	Moderate	White	Circular	Serrate	Convex	M
54	L485-I	Moderate	White	Circular	Entire	Convex	M
55	L485-II	Moderate	White	Circular	Serrate	Flat	M
56	G35-I	Large	White	Rhizoid	Filamentous	Flat	FG
57	G35-II	Large	White	Circular	Entire	Flat	SG
58	G35-III	Small	Yellowish	Circular	Entire	Flat	M

FG = Fast growing, M= Moderate and SG= Slow growing

A total of 58 bacterial isolates were pure cultured and their morphological characters recorded. Morphological studies of bacteria in terms of colony size, pigmentation, form, margin and elevation displayed variable results. The size of colonies was observed to fall in the categories of large, moderate, small and pinhead. While in most of the cases, the colour of the colonies was white. There were yellowish and green colonies

too. The growth rate of the bacterial colonies isolated from different sites was variable; 22 colonies were fast growing, 22 were moderate and 14 slow growing.

4.1.4. Screening of hydrocarbon degrading bacterial isolates

In screening experiments, 58 native bacterial isolates were re-cultured at 37°C in mineral salt medium supplemented with 10µl (v/v) of hexadecane as the sole carbon source after initial screening. Subsequently, the sixth-generation bacterial isolates were cultured on Bushnell-Hass medium containing hexadecane (1% v/v) to ascertain their hydrocarbon degrading ability. The bacterial isolates were allowed to grow for 96 h. Dry biomass yield of the bacterial isolates was determined and are presented in Table 4.3.

Table 4. 3. Isolation and screening of hydrocarbon degrading bacteria at 37°C (Mean ± S.D of 3 experiments)

Isolates no	Dry biomass ($g.l^{-1}$)	Isolates no	Dry biomass ($g.l^{-1}$)
control	0.001±1.0	G207 -I	0.041±1.0
R79 -I	0.002±2.0	G207-II	2.023±5.0
R79 -II	0.008±0.8	G207-II	0.065±2.0
R79 -III	0.001±1.0	G296-I	0.055±4.0
R 48 -I	0.012±3.0	G296-II	0.069±1.0
R 48 -II	0.021±1.0	G296-III	0.035±2.0
R 48 -III	0.002±4.0	G85-I	3.012±1.0
R74 -I	0.005±1.0	G85-II	0.052±0.7
R74 -II	0.002±5.0	G35-I	1.251±1.0
R 38 -I	1.120±1.0	G35-II	0.023±4.0
R38- II	0.001±1.0	G35-III	0.345±2.0
R 38 -III	0.001±6.0	L485-I	0.018±4.0
DJN132-I	0.003±0.8	L485-II	1.425±5.0
DJN191 -I	0.008±4.0	L253-I	0.016±0.6
DJN191-II	0.010±3.0	L253-II	1.150±0.7

D848 -I	-	L 64-I	1.075. ±1.0
D848 -II	-	L64-II	0.001±1.0
G 48 -I	0.028±2.0	L123-I	0.042±1.0
G 48 -II	0.004±5.0	ETP-II	0.018±2.0
G48 -III	0.031±0.9	ETP-III	0.015±3.0
L123-I	2.490±2.0	Tw_1 -I	2.85±1.0
L 490-I	0.002±1.0	Tw_1-II	0.025±2.0
L490-II	1.60±0.6	Tw_1 -III	0.017±0.6
L 490-III	0.005±3.0	Tw_2 -I	0.010±4.0
L43-I	1.250±4.0	Tw_2 -II	0.001±1.0
L 43-II	0.006±1.0	Tw_2 -II	0.025±2.0
L43-III	0.003±0.9	Fs_1 -I	1.955±1.0
L453-I	1.240±1.0	Fs_1 -II	0.042±6.0
L453-II	0.021±4.0	Fs_1 -III	0.003±1.0
ETP-I	0.016±1.0		

As shown in Table 4.3, out of 58 bacterial pure cultures, only 13 such as G85-I, G35-I, G207-I, L64-I, L453-I, L123-I, L253-II, L485-II, L490-II, L43-I, R38-I, Pw_1-I and Fs_1-I possessed better growth in hexadecane supplemented medium on the basis of increased dry biomass yield. These isolates were considered to be promising for the degradation of hydrocarbons. Subsequent studies were carried out with these 13 bacterial isolates.

4.1.5. Growth rate studies

Pure colonies were cultured at a single point in nutrient agar plate and incubated at 37°C for 96 h. The increase in diameter of the colony was measured with a graph paper after 48 h of incubation for the determination of bacterial growth rate. One unit was defined as 1 mm^2 of a graph paper. Results are presented in Table 4.4.

Table 4.4. Growth rate of bacterial isolates (Mean ± S.D of 3 experiments)

Isolate	Units	Growth rate in $mm^2.h^{-1}$
G85-I	460	9.58
G35-I	199	4.14
G207-II	470	9.79
L64-I	247	5.15
L453-I	250	5.20
L123-I	330	6.87
L253-II	292	6.08
L485-II	203	4.79
L490-II	162	3.37
L43-I	162	3.37
R38-I	250	5.20
Pw_1-I	230	4.79
Fs_1-I	221	4.60

Studies on the growth rate of bacterial isolates recorded wide variations. The isolate G207-II was found to possess the highest growth rate of 9.79 $mm^2.h^{-1}$ followed by G85-I, L123-I, L253-II, L453-I, R38-I, L64-I, Pw_1-I and Fs_1-I with 9.58, 6.87, 6.08, 5.20, 5.20, 5.15, 4.70 and 4.60 $mm^2.h^{-1}$, respectively.

4.1.6. Degradation studies on the basis of optical density

On the basis of growth rate in the isolation medium, 10 bacterial isolates were selected for assessing their ability to grow on crude oil and hydrocarbon components: aliphatics (hexadecane) and aromatics (benzene, petroleum benzene) with or without synthetic surfactant. Growth kinetics of the bacterial isolates was determined using the spectrophotometric (UV-Visible, $OD_{600\ nm}$) method. Data thus generated are presented in Table 4.5.

Table 4. 5. Growth behavior of bacterial isolates on crude oil, aliphatic and aromatic hydrocarbon - enriched medium (Mean ± S.D of 3 experiments)

Isolate	Carbon source	Bacterial growth over the days				
		1st day	2nd day	3rd day	4th day	5th day
P aeruginosa (MTCC 8163)	Crude oil (c.o.)	0.26±0.01	0.70±0.01	1.06±0.1	1.14±0.2	1.75±0.1
A. faecalis (MTCC 8164)		0.09±0.01	0.18±0.02	0.69±0.02	0.81±0.01	0.59±0.1
P aeruginosa (MTCC 8165)		0.06±0.01	0.65±0.03	0.95±0.03	1.15±0.1	1.68±0.1
B. circulans (MTCC 8167)		0.06±0.02	0.22±0.03	0.65±0.01	0.88±0.03	0.39±0.02
P. aeruginosa (L43-I)		0.08±0.01	0.18±0.02	0.71±0.03	0.91±0.04	0.87±0.03
P. flourescens (L490-II)		0.06±0.01	0.26±0.02	0.66±0.03	0.89±0.03	0.88±0.01
B. licheniformis (MTCC 8166)		0.08±0.01	0.56±0.03	1.02±0.03	1.25±0.1	1.70±0.1
P.aeruginosa (MTCC 7815)		0.35±0.01	0.67±0.02	1.08±0.2	1.15±0.1	1.76±0.2
Microbacterium (G35-I)		0.08±0.02	0.12±0.02	0.43±0.02	0.75±0.02	0.85±0.02
B. subtilis (R38-I)		0.21±0.02	0.35±0.03	0.56±0.04	0.95±0.03	1.25±0.2
Control	Medium+c.o.	0.05±0.01	0.05±0.01	0.04±0.02	0.06±0.01	0.5±0.01
P aeruginosa (MTCC 8163)	Hexadecane	0.38±0.01	1.45±0.2	1.97±0.2	2.24±0.2	1.75±0.1
A. faecalis (MTCC 8164)		0.11±0.01	0.21±0.02	0.55±0.03	1.09±0.4	2.08±0.1
P aeruginosa (MTCC 8165)		0.08±0.01	0.35±0.02	0.75±0.03	1.25±0.01	2.15±0.1
B. circulans (MTCC 8167)		0.07±0.01	0.16±0.01	0.45±0.02	0.63±0.01	2.17±0.2
P. aeruginosa (L43-I)		0.09±0.02	0.22±0.01	0.58±0.04	0.82±0.02	2.22±0.1
P. flourescens (L490-II)		0.07±0.01	0.23±0.03	0.75±0.04	0.91±0.02	2.02±0.1
B. licheniformis (MTCC 8166)		0.08±0.01	0.26±0.04	1.02±0.02	1.25±0.01	1.90±0.2
P.aeruginosa (MTCC 7815)		0.25±0.01	0.75±0.03	1.18±0.2	2.55±0.2	3.60±0.2
Microbacterium (G35-I)		0.08±0.02	0.22±0.02	0.56±0.01	1.25±0.1	2.25±0.1
B. subtilis (R38-I)		0.21±0.02	0.25±0.03	0.76±0.04	1.95±0.2	2.15±0.3
Control	Medium+ Hexadecane	0.04±0.01	0.05±0.01	0.04±0.03	0.06±0.01	0.05±0.01

Continued table 4.5

P aeruginosa (MTCC 8163)	Benzene	0.05±0.01	0.06±0.02	0.05±0.02	0.04±0.01	0.05±0.03
A. faecalis (MTCC 8164)		0.05±0.02	0.06±0.03	0.06±0.01	0.07±0.02	0.08±0.01
P aeruginosa (MTCC 8165)		0.08±0.01	0.09±0.02	0.15±0.03	0.25±0.01	0.45±0.01
B. circulans (MTCC 8167)		0.05±0.01	0.07±0.03	0.05±0.03	0.04±0.01	0.06±0.04
P. aeruginosa (L43-I)		0.05±0.01	0.05±0.01	0.05±0.01	0.04±0.02	0.04±0.01
P. flourescens (L490-II)		0.06±0.01	0.05±0.01	0.05±0.01	0.04±0.01	0.05±0.01
B. licheniformis (MTCC 8166)		0.08±0.01	0.09±0.02	0.12±0.01	0.25±0.02	0.30±0.01
P.aeruginosa (MTCC 7815)		0.25±0.01	0.35±0.01	0.48±0.02	0.55±0.04	0.80±0.03
Microbacterium (G35-I)		0.08±0.02	0.10±0.01	0.26±0.01	0.35±0.03	0.45±0.01
B. subtilis (R38-I)		0.21±0.02	0.35±0.02	0.38±0.03	0.45±0.01	0.55±0.01
Contro	Medium+Benzene	0.04±0.02	0.05±0.01	0.04±0.01	0.03±0.01	0.05±0.02
P aeruginosa (MTCC 8163)	Petroleum benzene	0 10±0.01	0.11±0.04	0.08±0.01	0.07±0 01	0.07±0.02
A. faecalis (MTCC 8164)		0.07±0.01	0.07±0.04	0.05±0.01	0.05±0.01	0.07±0.03
P aeruginosa (MTCC 8165)		0.05±0.02	0.06±0.03	0.08±0.01	0.10±0.02	0.19±0.01
B. circulans (MTCC 8167)		0.08±0.01	0.12±0.01	0.07±0.01	0.08±0.03	0.08±0.04
P. aeruginosa (L43-I)		0.07±0.01	0.07±0.03	0.07±0.01	0.08±0.01	0.07±0.03
P. flourescens (L490-II)		0.06±0.01	0.05±0.01	0.05±0.01	0.04±0.02	0.04±0.02
B. licheniformis (MTCC 8166)		0.08±0.01	0.09±0.02	0.10±0.04	0.15±0.02	0.20±0.01
P.aeruginosa (MTCC 7815)		0.25±0.01	0.25±0.01	0.31±0.02	0.35±0.04	0.38±0.03
Microbacterium(G35-I)		0.08±0.02	0.10±0.03	0.26±0.01	0.35±0.02	0.45±0.02
B. subtilis (R38-I)		0.21±0.02	0.35±0.01	0.38±0.01	0.45±0.03	0.55±0.04
Control	Medium+ Petro-Benzene	0.04±0.02	0.05±0.01	0.04±0.01	0.06±0.01	0.05±0.03

Growth of the bacterial isolates *P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC 8163), *A. faecalis* (MTCC 8164), *P. aeruginosa* (MTCC 8165), *B. licheniformis* (MTCC 8166), *B. circulans* (MTCC 8167), *P. fluorescens* (L490-II), *P. aeruginosa* (L43-I) and *B. subtilis* (R38-I) were studied in medium supplemented with crude oil, hexadecane, benzene and petroleum benzene. Isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC 8163), *B. licheniformis* (MTCC 8166), *P. aeruginosa* (MTCC 8165) and *B. subtilis* (R38-I) exhibited better growth in crude oil supplemented medium with increased optical density (O.D.) values of 1.76±0.2 to 1.25±0.2 during the culture period. In hexadecane-supplemented medium, isolates *P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *B. circulans* (MTCC 8167), *P. aeruginosa* (L43-I), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC 8165) displayed better growth with O.D. values of 3.60 to 2.15. In the medium supplemented with benzene, the isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I), *Microbacterium* (G35-I) and *P. aeruginosa* (MTCC 8165) showed good growth with O.D. values of 0.80 – 0.45 during the culture period. The growth of the isolates in the medium supplemented separately with benzene and petroleum benzene was much poorer in comparison to the medium supplemented separately with hexadecane and crude oil. Three isolates *B. subtilis* (R38-I), *Microbacterium* (G35-I) and *P. aeruginosa* (MTCC7815) possessed comparatively better growth with O.D. values of 0.55±0.4, 0.45±0.2 and 0.38±0.3 respectively than the other isolates in the medium supplemented with petroleum benzene.

The bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC 8163), *B. licheniformis* (MTCC 8166), *P. aeruginosa* (MTCC 8165) and *B. subtilis* (R38-I) possessed better growth in crude oil supplemented medium. However, all 10 bacterial isolates possessed similar degradation ability in hexadecane-supplemented medium. In benzene-supplemented medium *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC 8165) and *B. licheniformis* (MTCC 8166)

showed similar degradation ability. The isolates *B. subtilis* (R38-I), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC7815), *B. licheniformis* (MTCC 8166) and *P. aeruginosa* (MTCC 8165) possessed better growth in petroleum benzene-supplemented medium.

In a separate experiment, for enhancing the degradation of crude oil and aromatic hydrocarbons, the synthetic surfactant TritonX100 (60μg/ml v/v) was added to the medium. Data generated are presented in Table 4.6. The bacterial isolates *P. aeruginosa* (MTCC 8165), *B. circulans* (MTCC 8167), *P. aeruginosa* (L43-I), *P. fluorescens* (L490-II) and *Microbacterium* (G35-I) exhibited better growth with O.D. values of 3.75 – 2.30 in crude oil supplemented medium as compared to the medium having no addition of TritonX100. The bacterial isolates *B. licheniformis* (MTCC 8166), *P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC 8165) exhibited better growth with O.D. values of 0.45 – 0.17 in petroleum benzene with TritonX100 supplemented medium as compared to the ones without it.

Table 4.6. Growth of bacterial isolates in crude oil and benzene (aromatic hydrocarbon) containing media supplemented with TritonX100 (Mean ± S.D of 3 experiments)

Isolate	Carbon source	Bacterial growth over the days				
		1st day	2nd day	3rd day	4th day	5th day
P. aeruginosa (MTCC 8165)	Crude oil + TritonX100	1.63±0.1	2.34±0.1	2.61±0.1	2.50±0.3	2.30±0.2
B. ciculans (MTCC 8167)		1.64±0.1	3.13±0.4	3.17±0.2	3.13±0.1	3.75±0.4
P.aeruginosa (L43-I)		1.65±0.1	2.15±0.1	2.65±0.1	3.10±0.1	3.73±0.1
P. fluorescens (L490-II)		1.67±0.2	1.85±0.1	2.25±0.3	2.73±0.4	2.95±0.1
Microbacterium (G35-I)		1.75±0.2	1.65±0.4	1.95±0.4	2.35±0.1	2.85±0.2
Control		1.65±0.1	1.65±0.1	1.65±0.3	1.65±0.1	1.65±0.2
P. aeruginosa (MTCC 8163)	Benzene+ TritonX100	0.06±0.1	0.09±0.2	0.15±0.3	0.25±0.3	0.45±0.1
A. faecalis (MTCC 8164)		0.04±0.2	0.06±0.4	0.05±0.4	0.07±0.3	0.08±0.4
B. circulans (MTCC 8167)		0.05±0.1	0.07±0.1	0.09±0.4	0.23±0.5	0.35±0.1

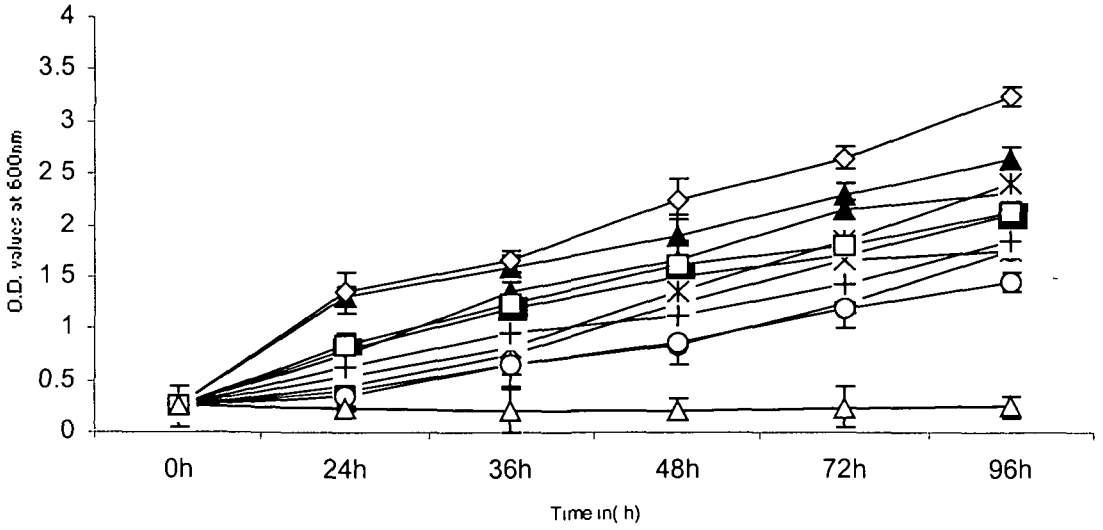
P.aeruginosa (L43-I)		0.05±0.4	0.06±0.2	0.07±0.1	0.15±0.3	0.17±0.2
P. fluorescens (L490-II)		0.08±0.1	0.07±0.1	0.18±0.1	0.21±0.1	0.29±0.1
Control		0.08±0.2	0.06±0.3	0.07±0.3	0.09±0.1	0.08±0.1

4.1.7. Growth of bacterial isolates in different carbon sources

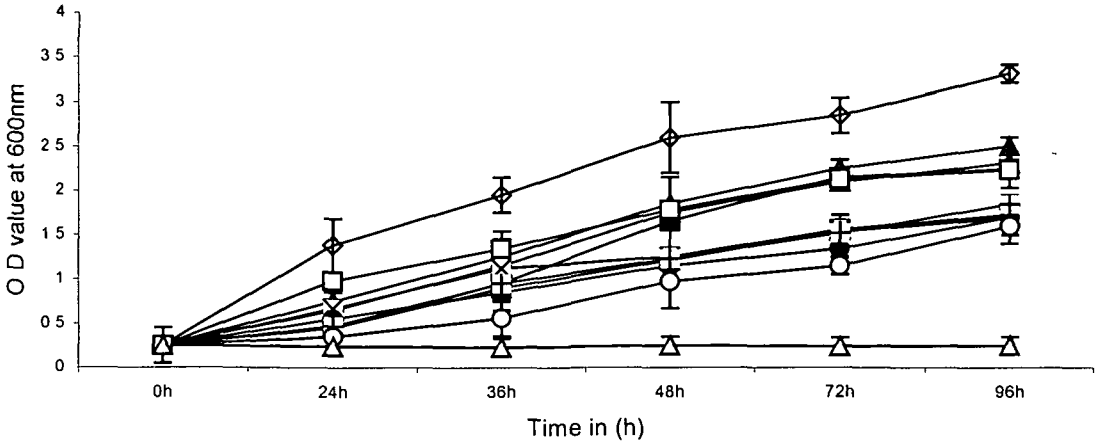
Hydrocarbon degrading bacterial isolates were also inoculated in medium separately supplemented with different petroleum hydrocarbons: hexadecane, octadecane, dodecane, benzene, toluene, xylene, phenanthrene, pyrene, fluorene, carbazole and its derivatives, phenol, pyridine, light paraffin and simple carbon sources like glucose, fructose as well as 2,4-dichlorophenoxy acetic acid (2,4-D). The growth of the bacterial isolates was assessed spectrophotometrically. Data thus obtained are presented graphically in Fig. 4.1. (a – o).

4.1.7.1 Growth of bacteria in glucose and fructose

The experiment was carried out to determine the growth of the bacterial isolates in medium supplemented with simple sugars like glucose and fructose. All the isolates grew in these media and the results obtained are presented graphically in Fig.4. 1. (a - b).



(a)



(b)

[-▲- *P. aeruginosa* (MTCC8163), - ■- *A. faecalis* (MTCC8164), -♦- *P. aeruginosa* (MTCC8165), -x- *B. circulans* (MTCC8167), -Ж- *P.aeruginosa* (L43-I), -●- *P.fluorescens* (L490-II), -I- *B. licheniformis* (MTCC8166), -◊- *P. aeruginosa* (MTCC7815), -o- *Microbacterium* (G35-I), -□- *B. subtilis* (R38-I) and -Δ- Control.]

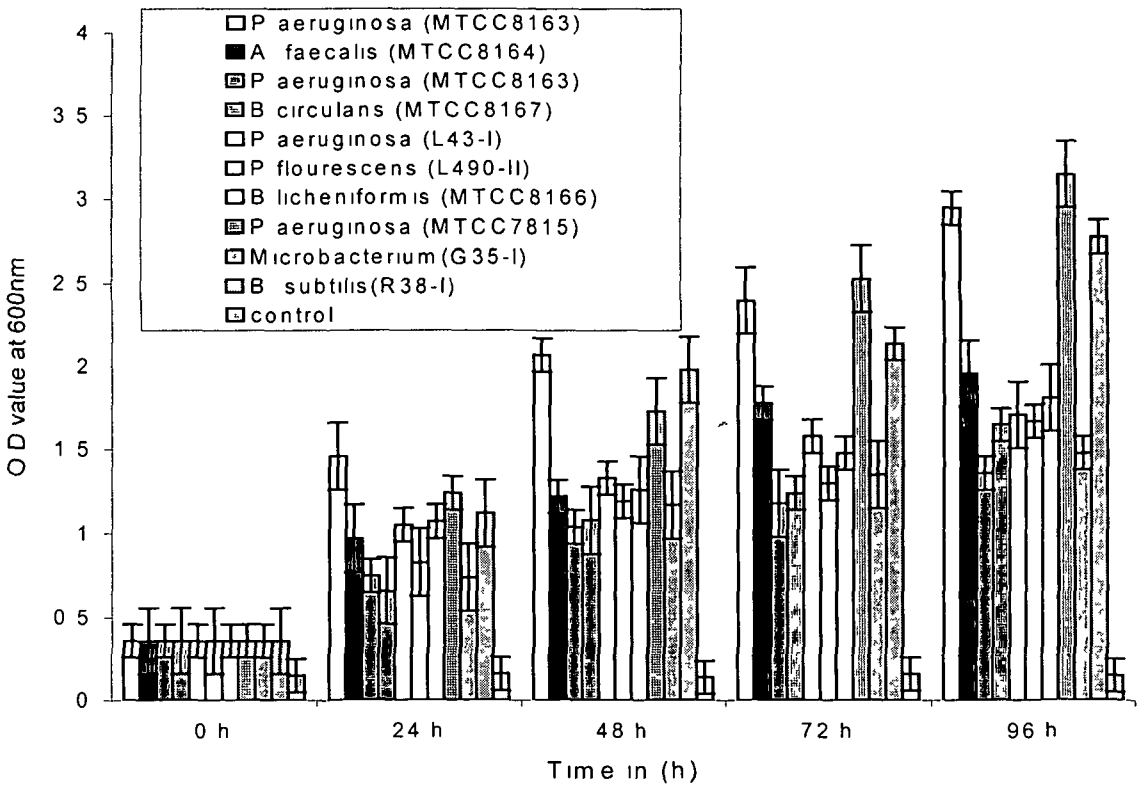
Fig.4.1. Growth of bacterial isolates in different carbon sources (a) glucose and (b) fructose

The rate of utilization of glucose and fructose by the bacterial isolates was higher in the exponential growth phase than in the stationary phase with increased O.D. values. In the glucose-supplemented medium, after the first 24 h of incubation with the isolate *P. aeruginosa* (MTCC7815), the O.D. values dramatically increased from 1.35 - 3.25 over the next 96 h. Similar trend was observed in the case of other isolates, such as *P. aeruginosa* (MTCC8163), *P. aeruginosa* (L43-I), *B. subtilis* (R38-I), *A. faecalis* (MTCC 8164), *B. licheniformis* (MTCC 8166), *P. fluorescens* (L490-II) and *Microbacterium* (G35-I). However, in the case of bacterial isolates *P. aeruginosa* (MTCC8165) and *B. circulans* (MTCC 8167), the O.D. value increased with the increase of incubation time from 24 – 72 h; thereafter the growth rate gradually decreased. In fructose-supplemented medium, the bacterial isolate *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8163) exhibited higher O.D. values of 3.32 and 2.51 respectively at 96 h of incubation. The bacterial isolates *B. subtilis* (R38-I), *P. aeruginosa* (MTCC8165) and *A. faecalis* (MTCC8164) exhibited moderate growth with O.D. values of 2.24 – 2.32. However, the bacterial isolates *Microbacterium* (G35-I), *P. aeruginosa* (L43-I), *P. fluorescens* (L490-II), *B. circulans* (MTCC 8167) and *B. licheniformis* (MTCC 8166) exhibited poor growth with O.D. values of 1.6 – 1.85.

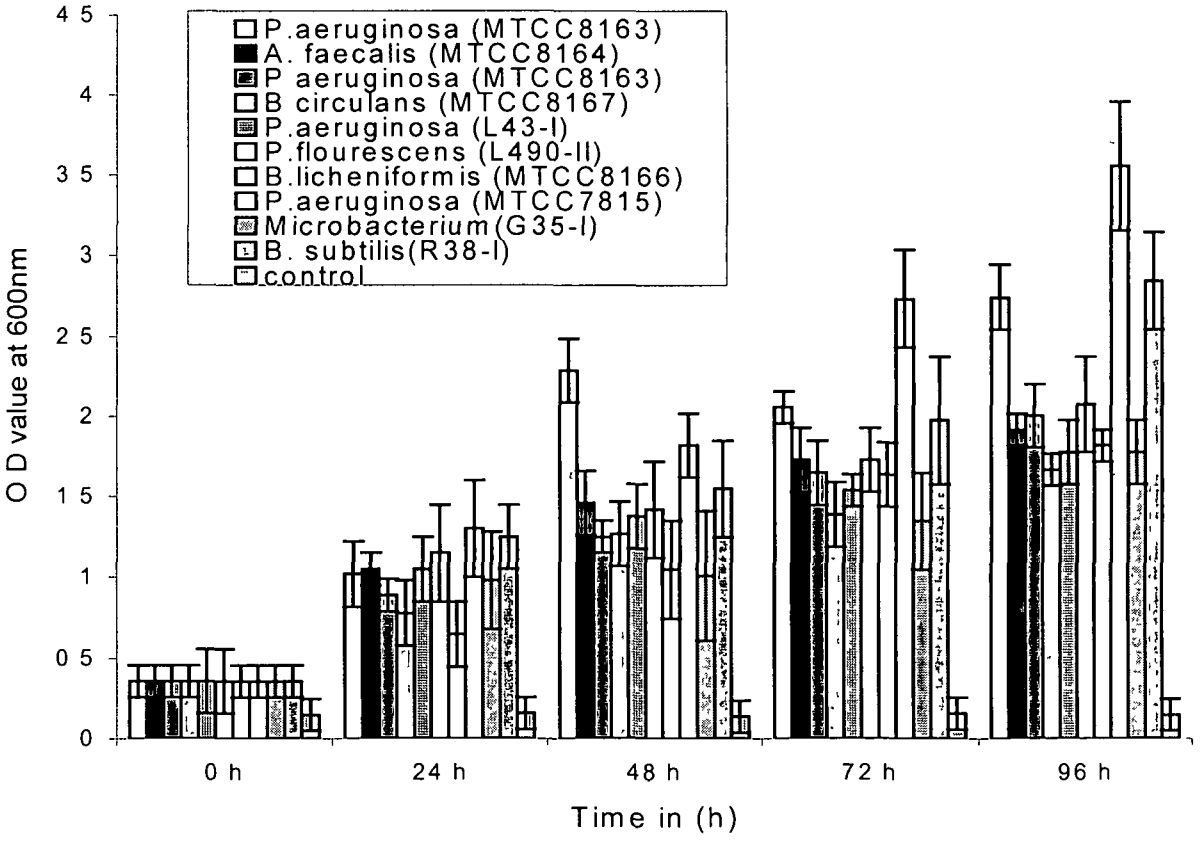
4.1.7.2. Growth of bacterial isolates in aliphatic hydrocarbons

The bacterial isolates having the potentiality to degrade aliphatic hydrocarbons were screened. Data thus obtained are presented in Fig.4.1. (c - e). Among the tested hydrocarbons, the isolate *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) exhibited the highest growth with O.D. values of 2.78 – 3.15 in dodecane-supplemented medium. The isolates *P. aeruginosa* (MTCC8165), *Microbacterium* (G35-I), *B. circulans* (MTCC 8167), *P. fluorescens* (L490-II), *P. aeruginosa* (L43-I), *B. licheniformis* (MTCC 8166) and *A. faecalis*

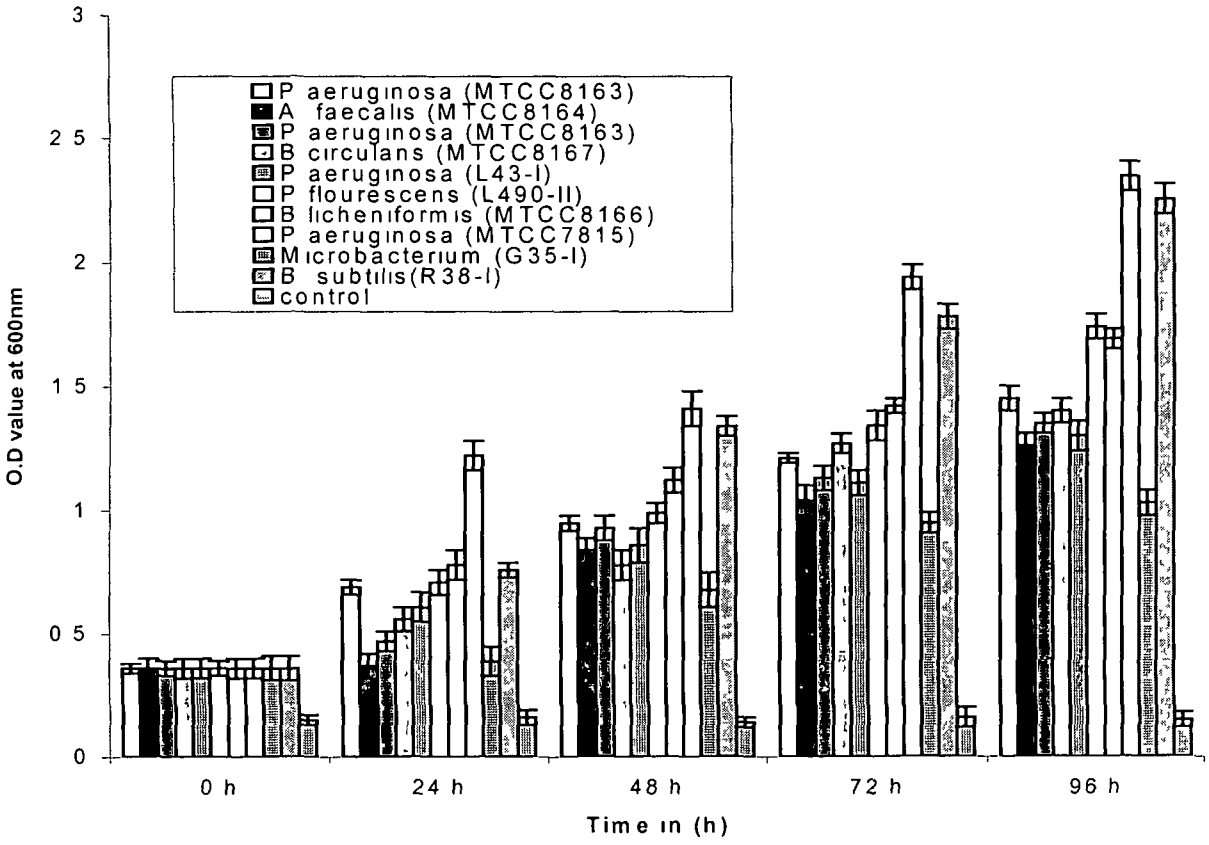
(MTCC8164) possessed comparatively better growth with O.D. values of 1.36, 1.48, 1.65, 1.67, 1.71, 1.81 and 1.96, respectively. In hexadecane-supplemented medium, the isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I), *P. aeruginosa* (MTCC8163), *P. fluorescens* (L490-II) and *P. aeruginosa* (MTCC8165) exhibited better performance with O.D. values of 3.56, 2.85, 2.75, 2.08 and 2.01, respectively. In the case of octadecane-supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7815) and *B. subtilis* (R38-I) showed better growth with O.D. value of 2.35 and 2.26 respectively. The remaining bacterial isolates showed almost the identical growth pattern.



(c)


P.aeruginosa (MTCC8163)
A. faecalis (MTCC8164)
P aeruginosa (MTCC8163)
B circulans (MTCC8167)
P.aeruginosa (L43-I)
P.flourescens (L490-II)
B.licheniformis (MTCC8166)
P.aeruginosa (MTCC7815)
Microbacterium(G35-I)
B. subtilis(R38-I)
control
O D value at 600nm
4 5
4
3 5
3
2 5
2
1 5
1
0 5
0
0 h
24 h
48 h
72 h
96 h
Time in (h)

(d)

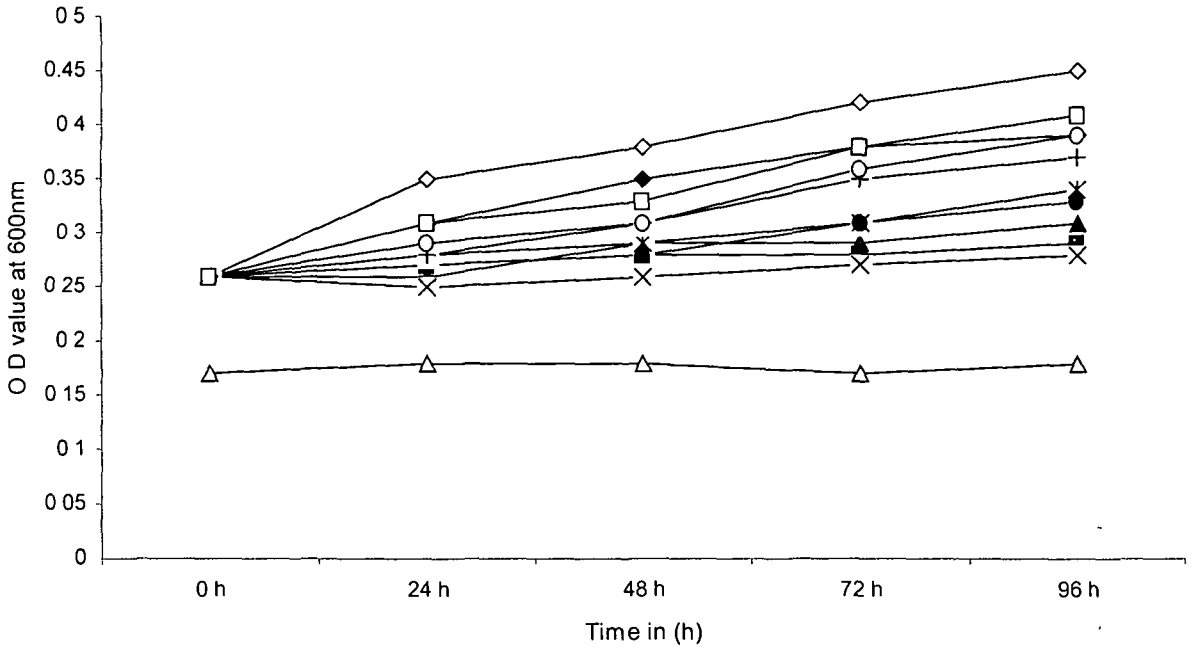


(e)

Fig.4.1. Growth of bacterial isolates in different carbon sources, (c) Dodecane, (d) Hexadecane and (e) Octadecane

4.1.7. 3. Growth of bacterial isolates in aromatic hydrocarbons

Bacterial isolates were grown in aromatic hydrocarbons like benzene, toluene and xylene. Data obtained are presented in Fig. 4.1. (f - h). These components were selected due to their known toxic and mutagenic effect. The potential bacterial isolates were able to grow in aromatic hydrocarbons.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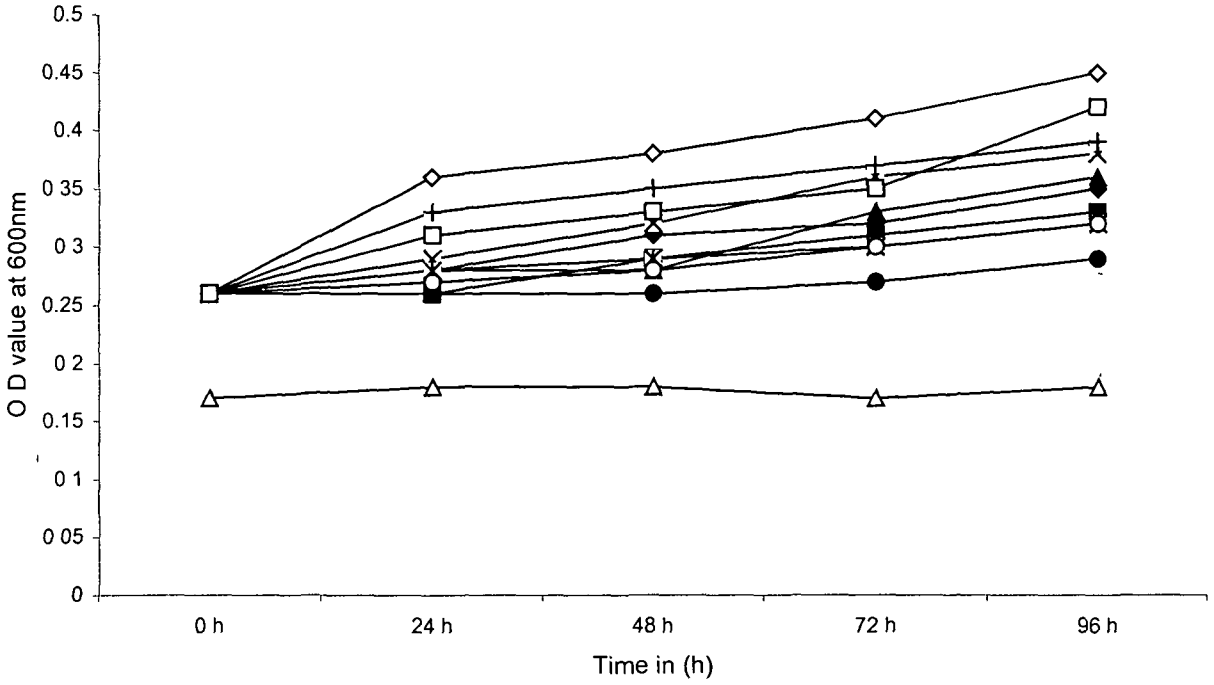
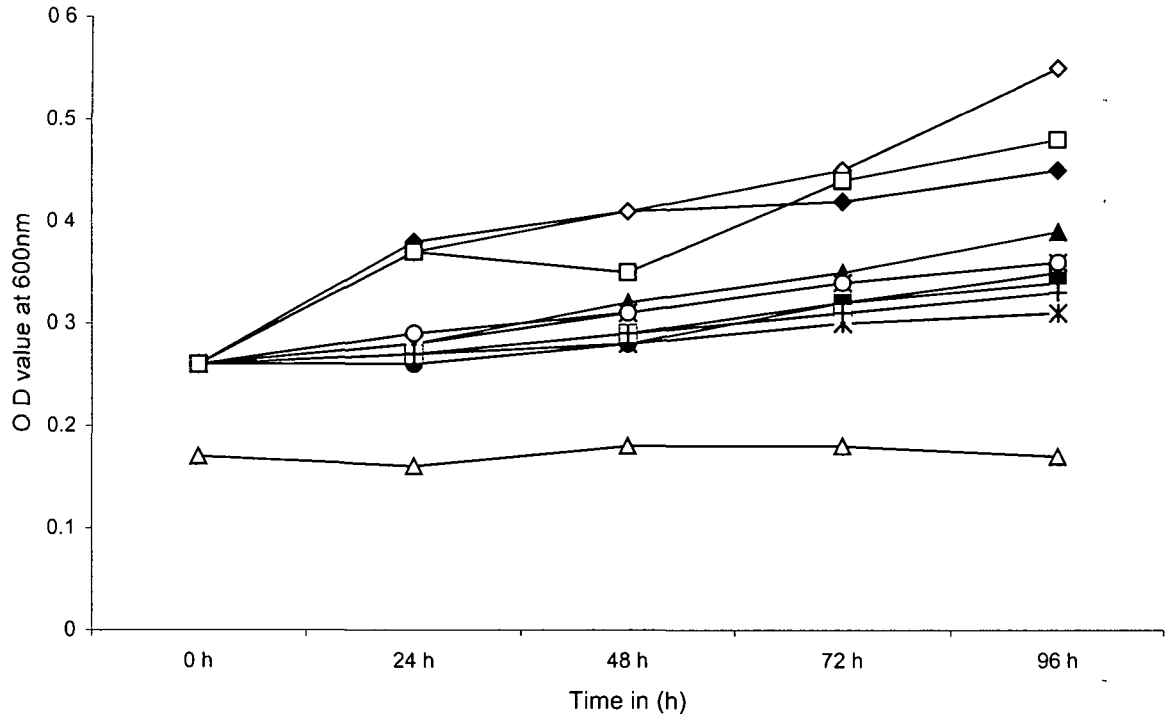
0.45
0 4
0.35
0 3
0 25
0 2
0 15
0 1
0 05
0
O D value at 600nm
0 h
24 h
48 h
72 h
96 h
Time in (h)

(f)


0.5
0.45
0.4
0 35
0 3
0.25
0 2
0.15
0 1
0 05
0
O D value at 600nm
0 h
24 h
48 h
72 h
96 h
Time in (h)

(g)



(h)

[-▲- *P. aeruginosa* (MTCC8163), - ■- *A. faecalis* (MTCC8164), -♦- *P. aeruginosa* (MTCC8165), -x- *B. circulans* (MTCC8167), -Ж- *P.aeruginosa* (L43-I), -●- *P.fluorescens* (L490-II), -I- *B. licheniformis* (MTCC8166), -◊- *P. aeruginosa* (MTCC7815), -o- *Microbacterium* (G35-I), -□- *B. subtilis* (R38-I) and -Δ- Control.]

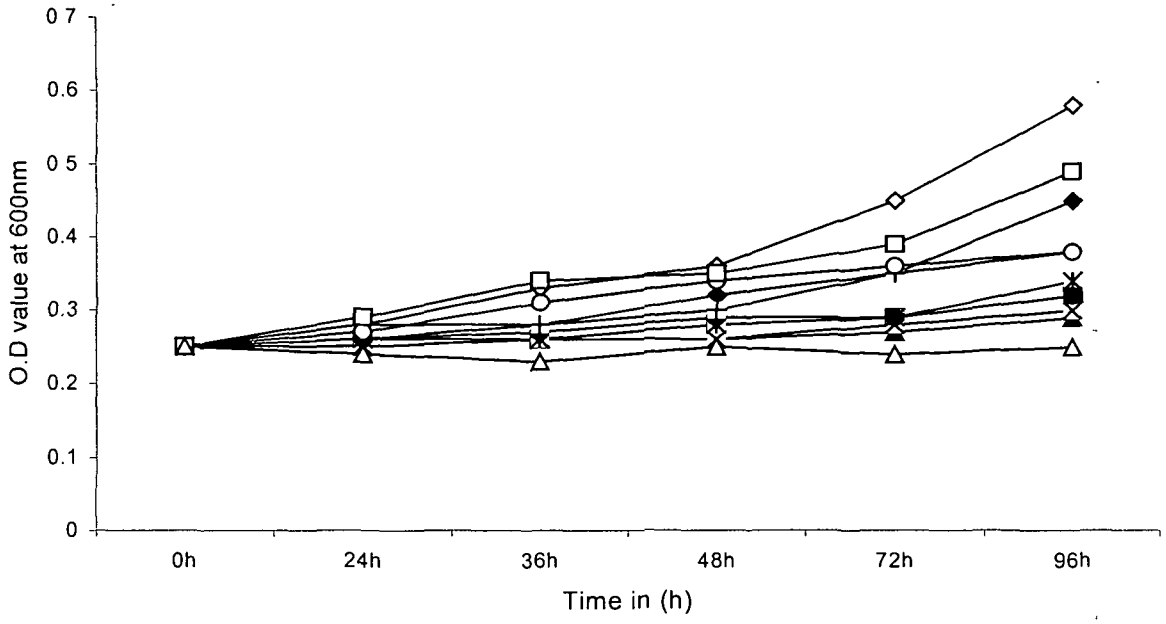
Fig.4.1. Growth of bacterial isolates in different carbon sources: (f) Benzene, (g) Toluene and (h) Xylene

In benzene-supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I) followed by *Microbacterium* (G35-I) and *P. aeruginosa* (MTCC8163) exhibited the highest growth with O.D. values of 0.45, 0.41, 0.39 and 0.39, respectively at 96 h of incubation. The other bacterial isolates *B. circulans* (MTCC 8167), *P. aeruginosa* (L43-I), *P. flourescens* (L490-II) and *P. aeruginosa* (MTCC8165) also possessed higher growth rate with O.D. values of 0.37, 0.35, 0.39, 0.33 and 0.31,

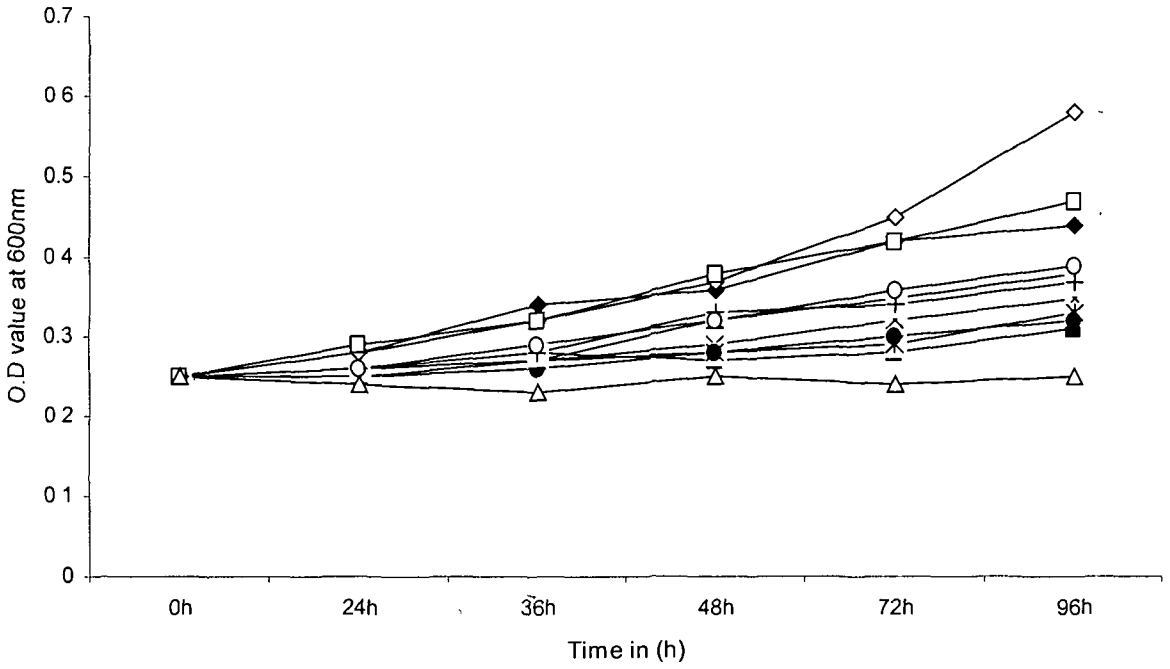
respectively. In toluene-supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I) followed by *B. licheniformis* (MTCC 8166) and *B. circulans* (MTCC 8167) exhibited the highest growth with O.D. values of 0.45, 0.42, 0.39 and 0.38, respectively. High growth rate was also exhibited by isolates *P. aeruginosa* (MTCC8165), *P. aeruginosa* (MTCC8163), *A. faecalis* (MTCC 8164), *Microbacterium* (G35-I) and *P. aeruginosa* (L43-I) with O.D. values of 0.36, 0.35, 0.33, 0.32 and 0.32, respectively. Subsequently, in xylene - supplemented medium the isolates *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *B. circulans* (MTCC 8167) exhibited the highest growth with O.D. values of 0.55, 0.48, and 0.45, respectively. Higher growth rate was also possessed by *P. aeruginosa* (MTCC8165), *B. circulans* (MTCC 8167), *Microbacterium* (G35-I), *A. faecalis* (MTCC 8164), *P. flourescens* (L490-II) and *B. licheniformis* (MTCC 8166) with O.D. values of 0.39, 0.36, 0.36, 0.35, 0.34 and 0.33, respectively.

4.1.7.4. Growth of bacterial isolates in polycyclic aromatic hydrocarbons

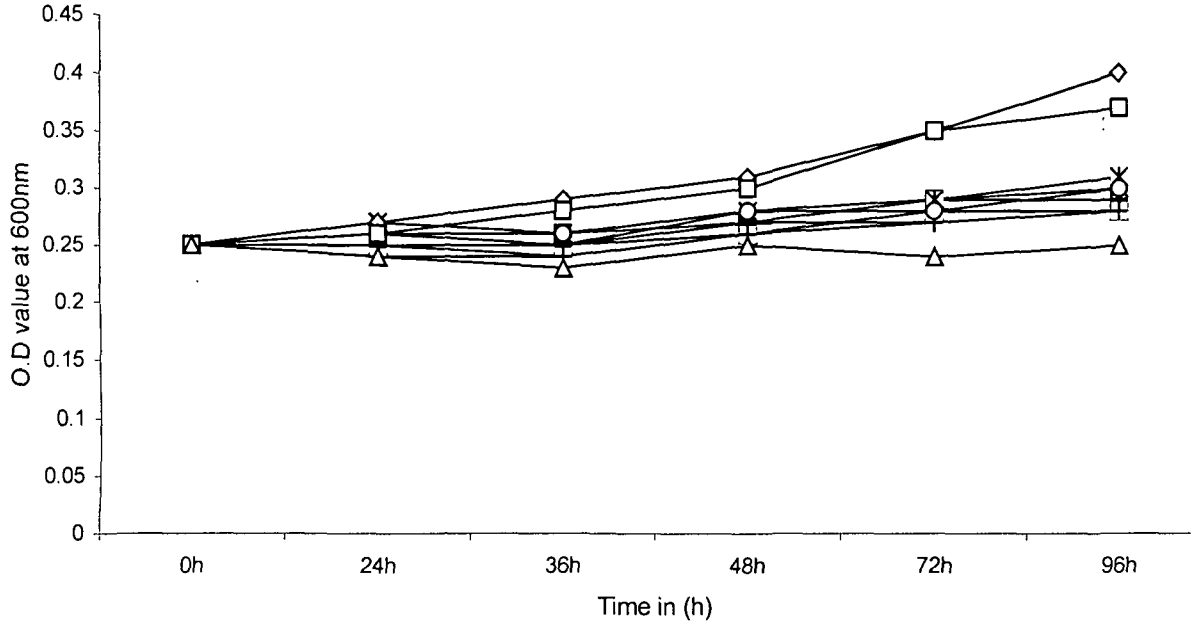
The growth behavior of bacterial isolates on polycyclic aromatic hydrocarbons was assessed and data obtained are graphically presented in Fig. 4.1 (j - l). Among, the tested hydrocarbons, the isolate *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8163) and *B. subtilis* (R38-I) exhibited the highest growth in phenanthrene, pyrene, flourene and carbazole supplemented media. The bacterial isolates showed almost identical growth pattern in all the tested PAHs.


0 7
0.6
0 5
0.4
0.3
0.2
0.1
0
O.D value at 600nm
0h
24h
36h
48h
72h
96h
Time in (h)

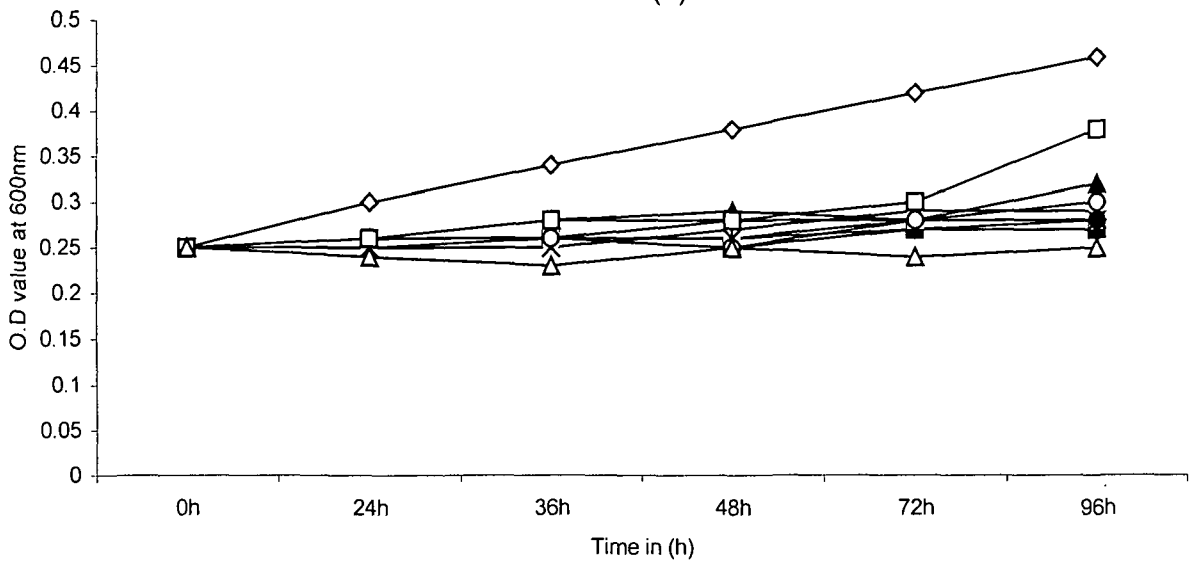
(i)


0.7
0 6
0.5
0 4
0.3
0 2
0 1
0
O.D value at 600nm
0h
24h
36h
48h
72h
96h
Time in (h)

(j)



(k)



(l)

[-▲- *P. aeruginosa* (MTCC8163), - ■- *A. faecalis* (MTCC8164), -♦- *P. aeruginosa* (MTCC8165), -x- *B. circulans* (MTCC8167), -Ж- *P. aeruginosa* (L43-I), -●- *P. fluorescens* (L490-II), -I- *B. licheniformis* (MTCC8166), -◊- *P. aeruginosa* (MTCC7815), -o- *Microbacterium* (G35-I), -□- *B. subtilis* (R38-I) and -Δ- Control.]

Fig.4.1. Growth of bacterial isolates in different carbon sources: (i) phenanthrene (j) pyrene (k) flourene and (l) carbazole

The isolate *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) exhibited the highest growth with O.D. values of 0.58, 0.49, and 0.45, respectively in phenanthrene-supplemented medium. Similar trend was observed in the medium containing pyrene where the isolate *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) exhibited the highest growth with O.D. values of 0.58, 0.47, and 0.44, respectively. The remaining bacterial isolates exhibited almost similar growth pattern. In the case of fluorene-supplemented medium, the isolate *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (L43-I) exhibited the highest growth with O.D. values of 0.40, 0.37, and 0.31, respectively. The remaining bacterial isolates displayed poor growth in fluorine, phenanthrene and pyrene containing media. While in carabazole - supplemented medium, the isolate *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8165) and *B. subtilis* (R38-I) exhibited the highest growth with O.D. values of 0.46, 0.32, and 0.30, respectively.

In a separate experiment, the promising bacterial isolates were tried in minimum salt medium supplemented separately with different concentrations of polycyclic aromatic hydrocarbons. Data obtained are presented in Table 4.7.

Table 4.7. Growth of bacterial isolates in minimum salt (MS) agar solidified PAHs supplemented medium at different concentrations

Isolates	phenanthrene			pyrene			flourene			carbazol		
	MS+ 500 µg	MS+ 300 µg	MS+ 200 µg	MS+ 500 µg	MS+ 300 µg	MS+ 200 µg	MS+ 500µg	MS+ 300µg	MS+ 200 µg	MS+ 500 µg	MS+ 300 µg	MS+ 200 µg
P. aeruginosa (MTCC8163)	-	+	++	-	+	+	+	-	+	-		+
A. faecalis (MTCC8164)	-	-	+	-	-	+	+	-	-	-	-	-
P. aeruginosa (MTCC8163)	-	+	++	-	+	+	+	-	-	-	-	+
B. circulans (MTCC8167)	-	+	+	-	+	+	+	-	-	-	-	+
P. aeruginosa (L43-I)	-	-	+	-	-	+	-	-	-	-	-	-
P. fluorescens(L490-III)	-	+	+	-	-	+	+	-	-	-	-	-
B.licheniformis(MTCC 8166)	-	-	+	-	-	+	+	-	-	-	-	-
P. aeruginosa (MTCC7815)	-	++	+++	-	++	+++	-	-	+	-	+	++
Microbacterium (G35-I)	-	+	++	-	+	++	+	-	+	-	-	-
B. subtilis (R38-I)	-	+	+++	-	+	+++	+	-	+	-	+	++
Control	-	-	-	-	-	-	-	-	-	-	-	-

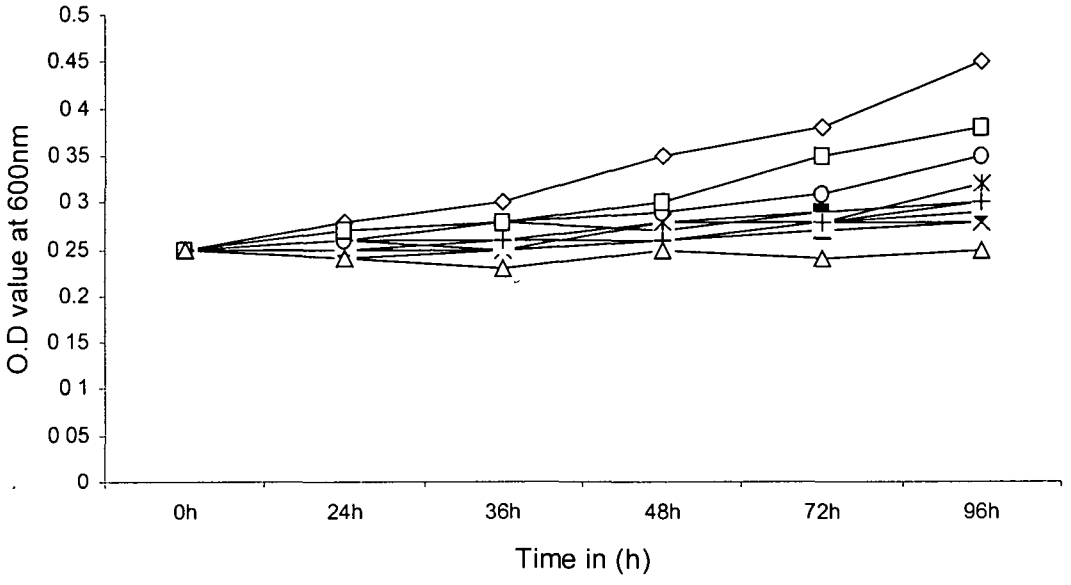
- no growth, +++ high growth, ++ moderate growth, + less growth,

The isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) showed the best growth at the hydrocarbon concentration of 200 $\mu g.l^{-1}$.

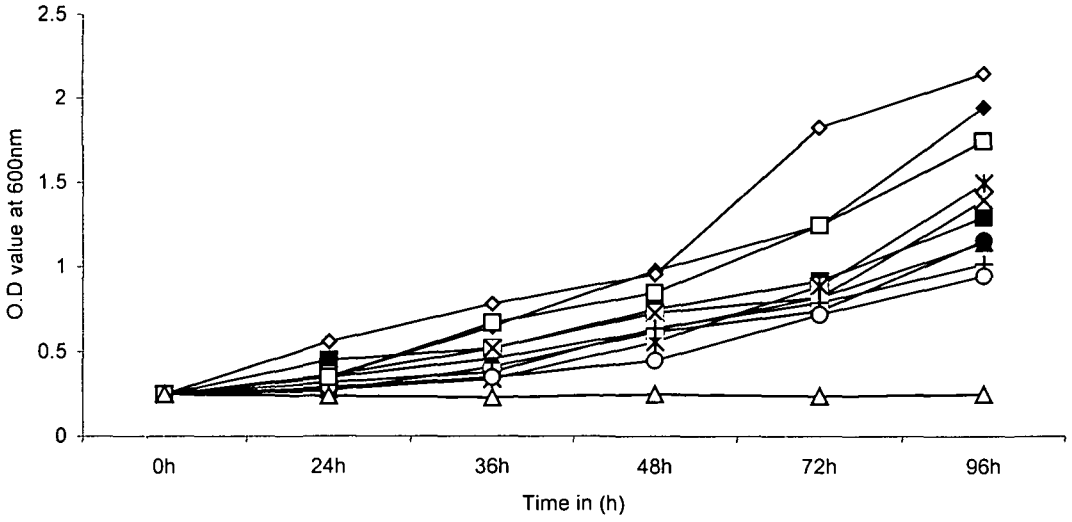
In phenanthrene-supplemented medium at the concentration of 200 $\mu g.l^{-1}$ the best growth was exhibited by *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I) followed by *Microbacterium* (G35-I), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC8163). In the case of pyrene-supplemented medium, the best concentration was 200 $\mu g.\ l^{-1}$ for the bacterial isolates *P. aeruginosa* (MTCC7815) and *B. subtilis* (R38-I). In flourene-supplemented medium the concentration of 500 $\mu g.l^{-1}$ was found to be effective for the isolate *P. aeruginosa* (L43-I). In carbazole containing medium 200 $\mu g.l^{-1}$ concentration was moderately effective for the isolates *P. aeruginosa* (MTCC7815) and *B. subtilis* (R38-I).

4.1.7.5. Growth of bacterial isolates in other hydrocarbons

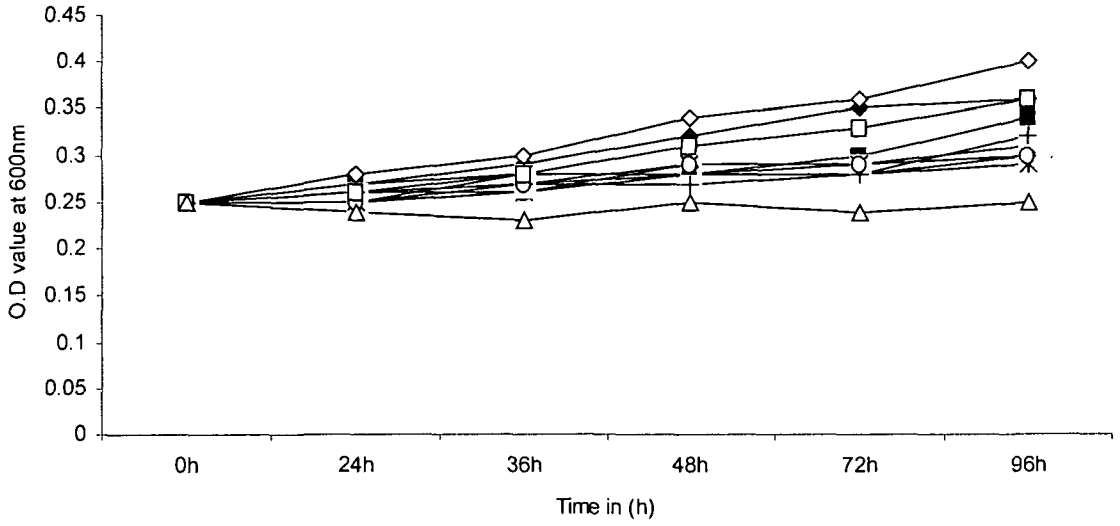
The growth performance of the bacterial isolates was also studied in media containing non-petroleum hydrocarbons like 2,4-D, pyridine and paraffin. The results are presented in Fig.4.1. (m - o).



(m)



(n)



(o)

[-▲- *P. aeruginosa* (MTCC8163), - ■- *A. faecalis* (MTCC8164), -♦- *P. aeruginosa* (MTCC8165), -x- *B.circulans* (MTCC8167), -Ж- *P.aeruginosa* (L43-I), -●- *P.fluorescens* (L490-II), -I- *B. licheniformis* (MTCC8166), -◊- *P. aeruginosa* (MTCC7815), -o- *Microbactrium* (G35-I), -□- *B. subtilis* (R38-I) and -Δ- Control.]

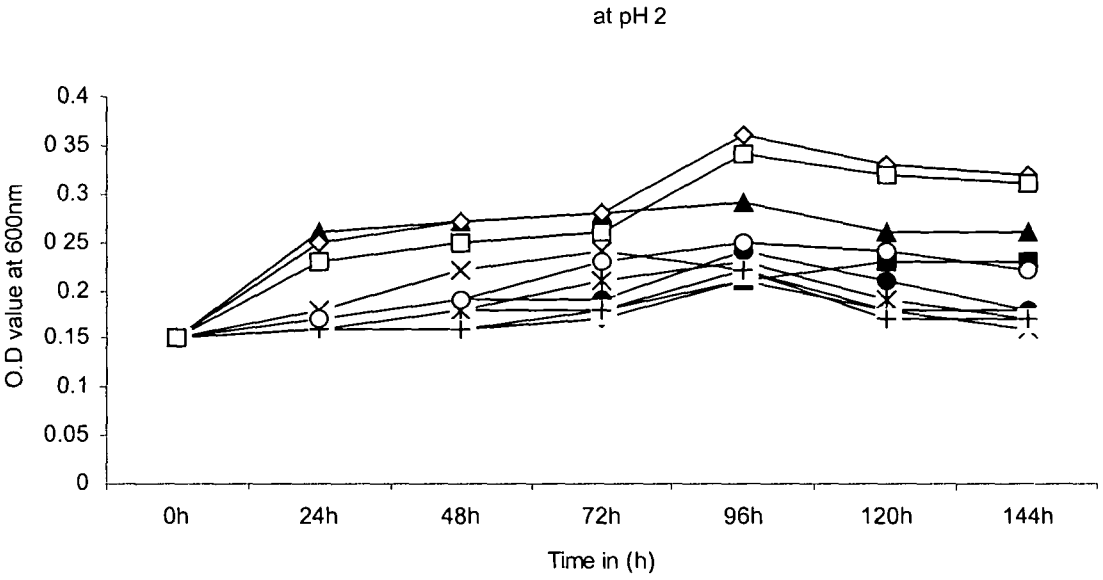
Fig.4.1. Growth of bacterial isolates in different carbon sources: (m) pyridine (n) paraffin and (o) 2-4 D

In pyridine - supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *Microbacterium* (G35-I) exhibited comparatively higher growth with O.D. values of 0.45, 0.38 and 0.35, respectively at 96 h of incubation. The remaining bacterial isolates displayed similar trend of growth with O.D. values of 0.28 –0.32. In the case of paraffin - supplemented medium, the isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8163), *B. subtilis* (R38-I) and *P. aeruginosa* (L43-I) exhibited the highest growth with O.D. values of 2.15, 1.95, 1.75 and 1.50, respectively. The isolates *B. circulans* (MTCC8167), *A. faecalis* (MTCC8164), *P. fluorescens* (L490-II), *P. aeruginosa* (MTCC8163) and *B. licheniformis* (MTCC8166) also showed comparatively higher growth. Subsequently, in 2,4-D - supplemented medium, the isolates *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8163) and *B. subtilis* (R38-I) exhibited better growth with the O.D. values of 0.40, 0.36 and 0.36, respectively. The remaining bacterial isolates exhibited similar trend of growth.

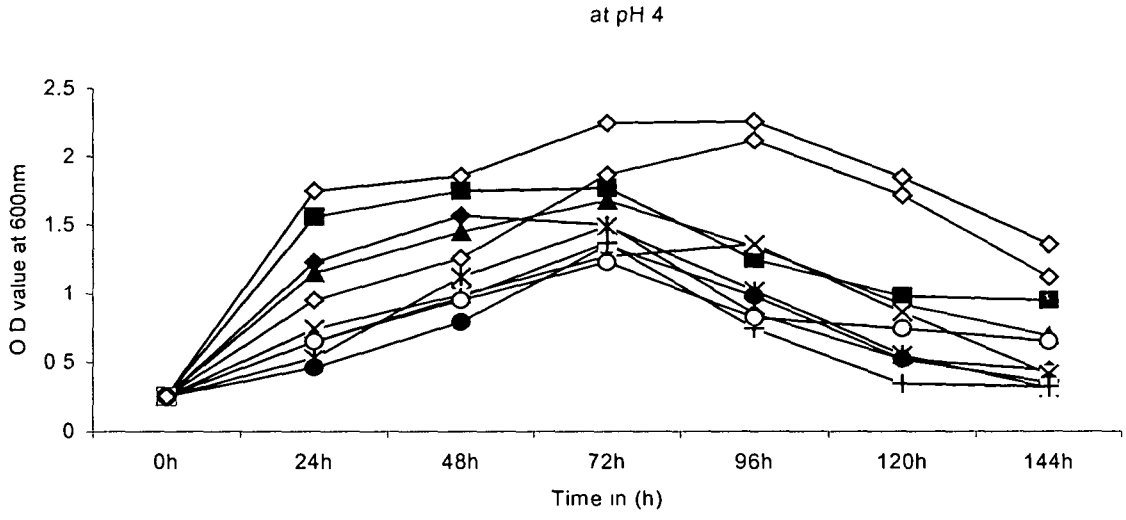
4.1.8. Growth of bacterial isolates at different pH levels

The promising bacterial isolates were also tried in hydrocarbon - supplemented media having acidic to alkaline pH (2 – 10). Data thus obtained are presented graphically in Fig.4.2 (a - e). In highly acidic (pH 2) and alkaline (pH 10) conditions, the growth performance of bacterial isolates was poor with 0.2 – 0.35 $g.l^{-1}$ dry biomass production. However, at pH 2.0, the isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) produced comparatively higher biomass of 0.28 – 0.35 $g.l^{-1}$ having the peak at 96 h. At pH 4.0, the isolates *P. aeruginosa* (MTCC7815) possessed better growth with biomass yield of 1.8 – 2.5 $g.l^{-1}$ for the entire growth period of 144 h. On the other hand, the isolate *P. aeruginosa* (MTCC8165) exhibited better growth with biomass yield of 1.5 – 1.65 $g.l^{-1}$ during the initial culture period of 24 - 72 h, at pH 6.0, the isolate *P. aeruginosa* (MTCC7815) exhibited better growth with the dry biomass yield

of 2.25 – 3.75 $g.l^{-1}$ for the culture period of 24 – 96 h. At pH 8.0, the isolate *P. aeruginosa* (MTCC7815) possessed the better growth with biomass yield of 3 – 3.5 $g.l^{-1}$ and *B. subtilis* (R38-I) with biomass yield of 2.5 – 2.8 $g.l^{-1}$ during the culture period of 72 – 120 h. At the alkaline pH of 10.0, all isolates possessed poor growth; however, the isolate *P. aeruginosa* (MTCC7815) possessed comparatively better growth.

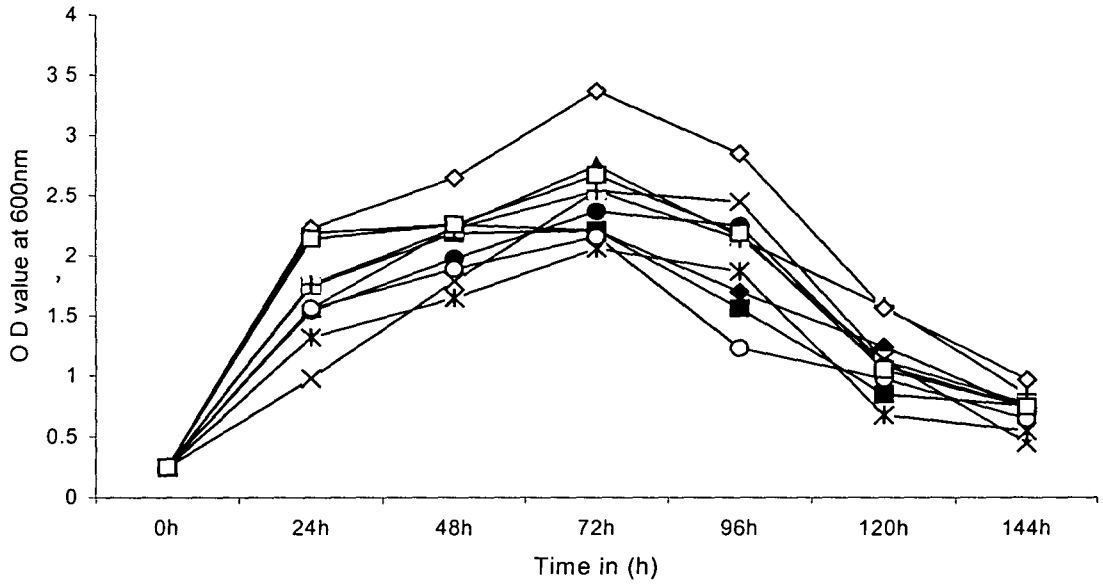


(a)



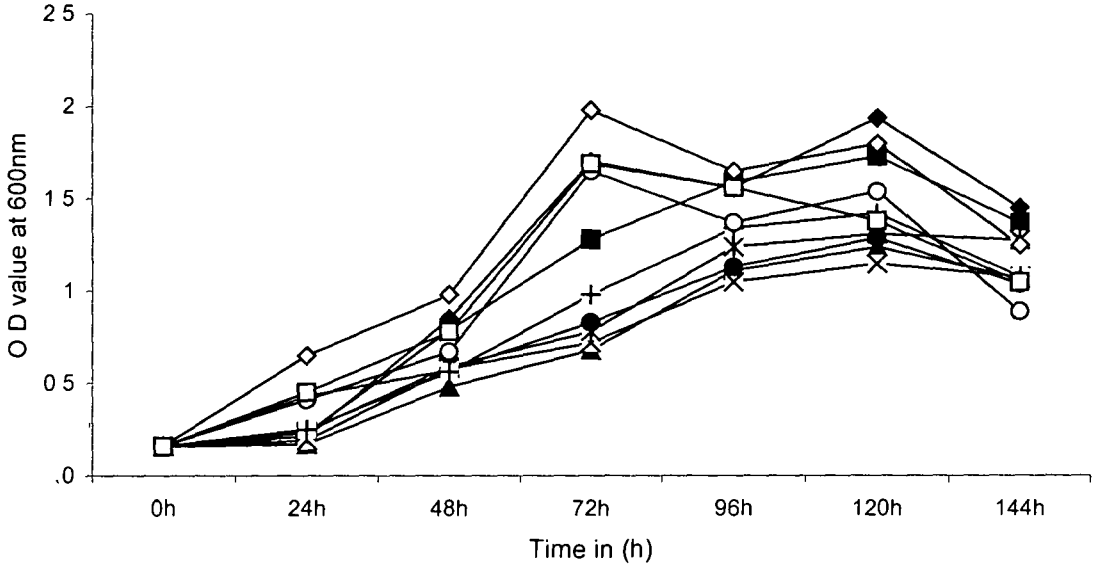
(b)

at pH 6

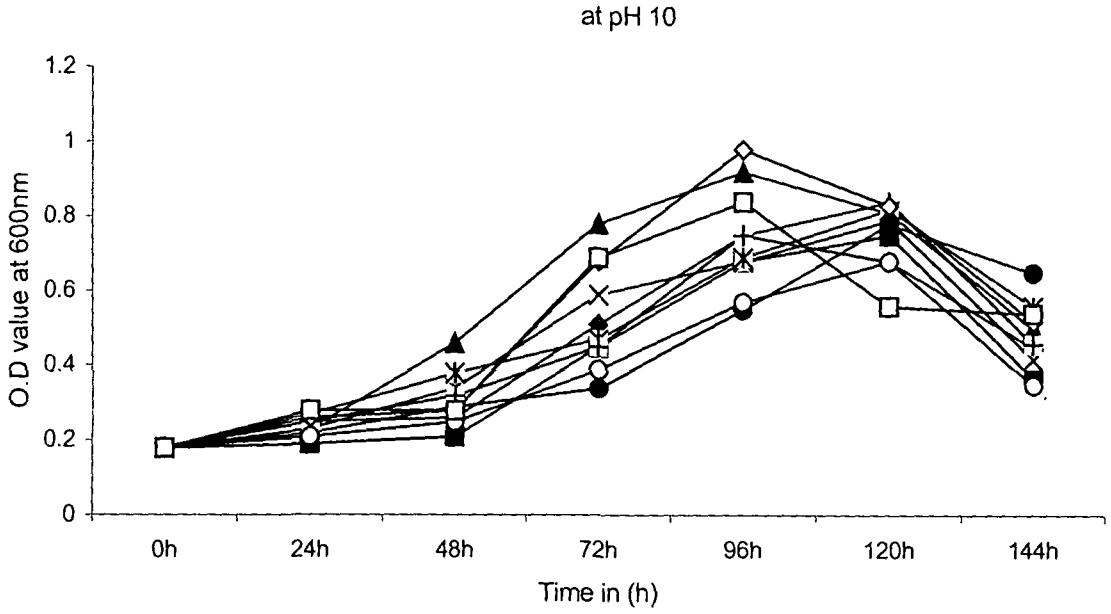


(c)

at pH 8



(d)



(e)

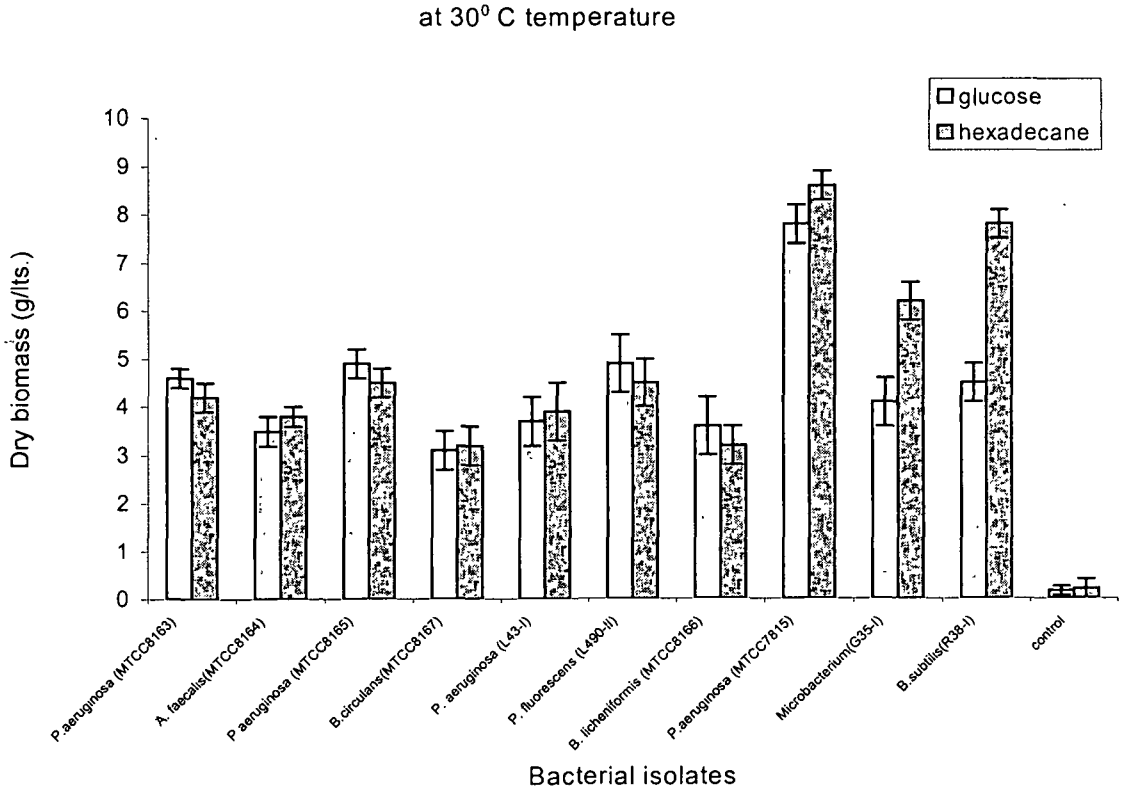
[-▲- *P. aeruginosa* (MTCC8163), - ■- *A. faecalis* (MTCC8164), -♦- *P. aeruginosa* (MTCC8165), -x- *B.circulans* (MTCC8167), -Ж- *P.aeruginosa* (L43-I), -●- *P.fluorescens* (L490-II), -I- *B. licheniformis* (MTCC8166), -◊- *P. aeruginosa* (MTCC7815), -o- *Microbactrium* (G35-I), -□- *B. subtilis* (R38-I) and -Δ- Control.]

Fig.4.2. Growth of bacterial isolates in hydrocarbon - supplemented media having pH 2-10

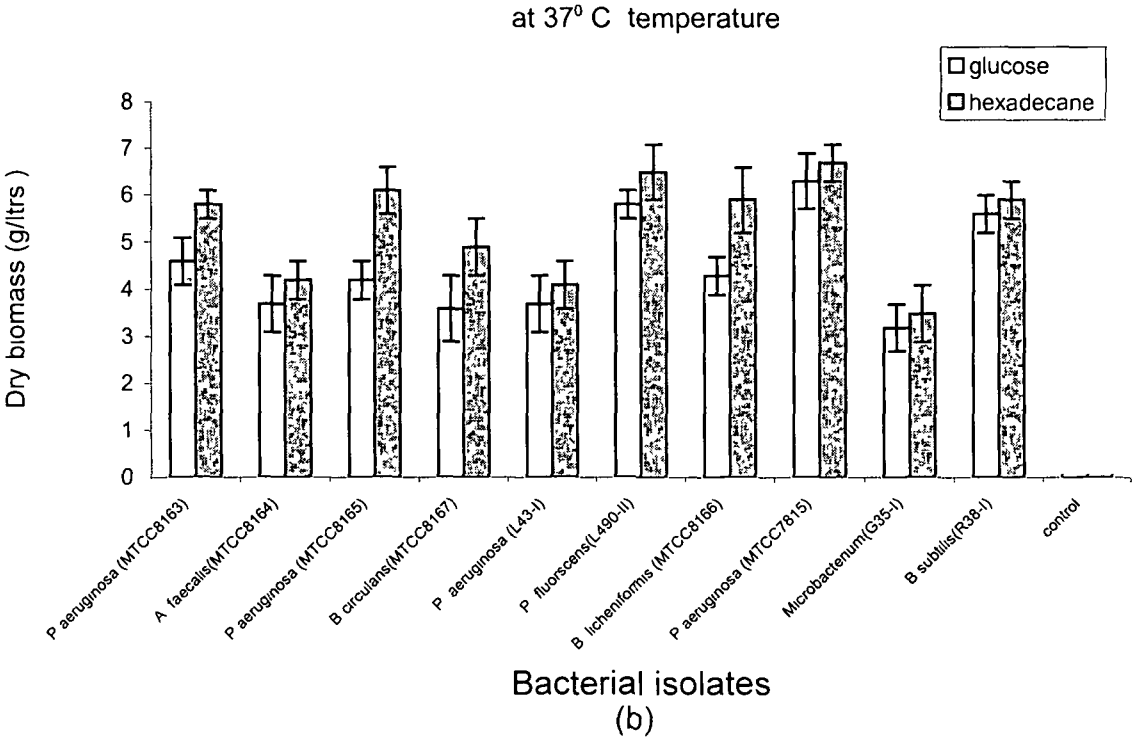
4.1.9. Growth of bacterial isolates at different temperatures

On the basis of better degrading ability, the bacterial isolates were also tried in media supplemented with 1% glucose (w/v) or 1% hexadecane (v/v) as the single source of carbon and at different temperatures ranging from 30 - 45°C. At 30°C, the bacterial isolates *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I), *Microbacterium* (G35-I) exhibited the highest growth with 8.6 – 6.2 $g.l^{-1}$ dry biomass production in hexadecane-supplemented medium. The remaining bacterial isolates exhibited similar trend of growth with 4.5 – 3.2 $g.l^{-1}$ dry biomass production. While in glucose - supplemented medium, at 30°C the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8165), *P. aeruginosa* (MTCC8163) and

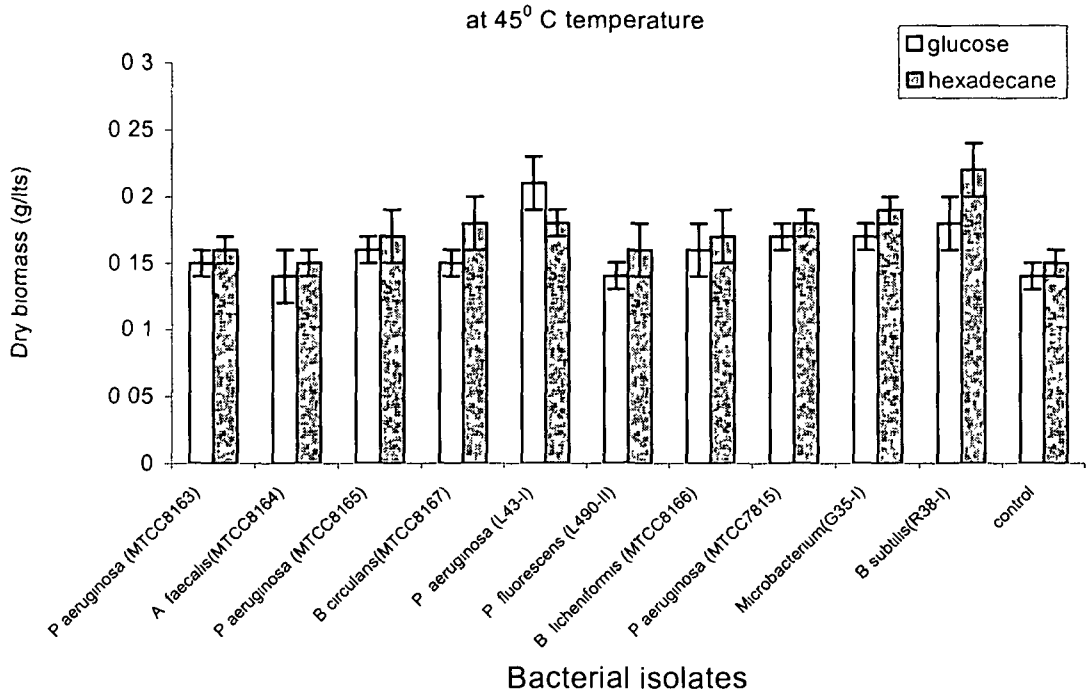
Microbacterium (G35-I) produced the biomass yield of 7.8 – 4.5 $g.l^{-1}$; whereas at 37°C, the isolates *P. aeruginosa* (MTCC7815), followed by *P. fluorescens* (L490-II), *B. subtilis* (R38-I), *P. aeruginosa* (MTCC8163) exhibited biomass production of 6.3 – 4.6 $g.l^{-1}$. Subsequently, in hexadecane - supplemented medium, the bacterial isolates *P. fluorescens* (L490-II), *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I), *B. licheniformis* (MTCC 8166) and *P. aeruginosa* (MTCC8163) produced the dry biomass yield of 6.5 – 5.8 $g.l^{-1}$. The remaining isolates exhibited comparatively poor growth. At 45°C all the bacterial isolates failed to survive in both glucose and hexadecane supplemented media.



(a)



(b)



(c)

Fig.4.3. Growth of bacterial isolates in hydrocarbon supplemented media having different temperatures (a) 30°C (b) 37°C and (c) 45°C

4.1.10. Taxonomic identification of bacterial strains

The taxonomic identification of the bacterial isolates was carried out following the standard morphological, physiological and biochemical tests as described by Cappuccino and Sherman (1999) to identify the genus. Data on growth and morphological characters as well as biochemical tests are presented in Table 4.8 and 4.9.

Table 4. 8. Morphological characters of bacterial isolates. (Mean of 4 experiments)

Bacterial isolates	Morphology	Growth on			
		Agar	Broth	pH	Temperature range
G85-I	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, green pigmentation in the colony and in mineral salt medium broth	Abundant, green	Vigorous growth, with gum like substance	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°C
G35-I	Rod shaped with 1-2 µm in diameter, Gram positive, border regular, surface raised, and no pigmentation in the colony and in mineral salt medium broth.	Abundant,	Moderate growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°C
G207-II	Rod shaped with 1-2 µm in diameter, Gram negative, border regular and surface flat, yellow green pigmentation in the colony and in mineral salt medium broth.	Abundant, pale yellow	Vigorous growth,	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
L64-I	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, greenish pigmentation in the colony and in mineral salt medium broth.	Abundant, greenish	Vigorous growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
L123-I	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, greenish pigmentation in the colony and in mineral salt medium broth.	Abundant, greenish	Vigorous growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
L490-II	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, yellow green pigmentation in the colony and in mineral salt medium broth	Abundant, pale yellow	Vigorous growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C

Table 4. 8. (continued)

Bacterial isolates	Morphology	Growth on			
		Agar	Broth	pH	Temperature range
L453-I	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, green pigmentation in the colony and in mineral salt medium broth	Abundant, light green	Vigorous growth, with gum like substance	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
L485-II	Rod shaped with 1-2 µm in diameter, Gram positive, border regular, surface raised, and no pigmentation in the colony and in mineral salt medium broth.	Abundant, White	Moderate growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
L253-II	Rod shaped with 1-2 µm in diameter, Gram positive, border regular, surface flat, no pigmentation in the colony and in mineral salt medium broth.	Abundant, cream	Vigorous growth,	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
R38-I	Rod shaped with 1-2 µm in diameter, Gram positive, border regular, surface flat, no pigmentation in the colony and in mineral salt medium broth.	Abundant, White	Vigorous growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
Pw_1-I	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, blue pigmentation in the colony and in mineral salt medium broth.	Abundant, blue	Vigorous growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C
Fs_1-I	Rod shaped with 1-2 µm in diameter, Gram negative, border regular, surface flat, yellow green pigmentation in the colony and in mineral salt medium broth	Abundant, pale yellow	Vigorous growth	4.0-10.0, optimum at 6.8± 0.2 –7.5± 0.2	28-37°C, optimum at 30-35°± 0.2C

Table 4.9. Biochemical test of bacterial isolates (Mean of 4 experiments)

Bacterial isolates	Catalase	VP	Methyl Red	Carbohydrate fermentation			Hydrolysis of				Citrate Utilization	Nitrate reduction
				Lactose	Dextrose	Sucrose	Casein	Gelatin	Starch	Lipid		
G85-I	+	-	-	+	+	+	+	+	-	+	+	+
G35-I	+	-	-	-	+	-	-	+	-	+	+	-
G207-II	+	-	-	-	-	-	+	+	-	+	+	+
L64-I	+	-	+	-	+	-	+	+	-	+	+	+
L123-I	+	-	+	-	+	-	+	+	-	+	+	+
L490-II	-	+	-	-	+	+	+	+	-	+	+	+
L43-I	-	-	-	-	+	+	+	+	-	-	+	+
L453-I	+	-	+	-	+	-	-	-	-	-	+	+
L485-II	+	-	-	+	+	-	-	+	-	-	-	-
L253-II	+	+	-	-	+	+	+	+	-	+	+	+
R38-I	-	-	-	+	+	+	-	-	-	+	-	-
Pw_1-I	+	-	-	+	+	+	+	+	-	-	+	+
Fs_1-I	+	-	-	-	+	-	+	+	-	-	+	+

Table 4.9.(continued)

Bacterial isolates	H_2S production	Indole production	Litmus milk reaction	Triple sugar iron	Acid from			Urease test
					D- glucose	D- xylose	D- manitol	
G85-I	-	-	Peptonization	-	+	+	+	-
G35-I	-	-	Peptonization	-	+	-	-	-
G207-II	-	-	Peptonization	-	+	+	+	-
L64-I	-	-	Peptonization	+	+	+	+	-
L123-I	-	-	Peptonization	+	+	+	+	-
L490-II	-	-	Peptonization	+	+	+	+	-
L43-I	-	-	Peptonization	+	+	+	+	-
L453-I	-	-	Peptonization	+	+	-	-	-
L485-II	-	-	Peptonization	+	-	-	-	-
L253-II	-	+	Peptonization	+	-	-	-	-
R38-I	-	-	Peptonization	+	-	-	-	+
Pw_1-I	-	-	Peptonization	-	+	+	-	-
Fs_1-I	-	-	Peptonization	-	+	+-	+	-

The bacterial isolates G85-I, G207-II, L64-I, L123-I, L43-I, Pw_1-I and Fs_1-I were found to be non-spore forming, gram negative, catalase-positive, motile rods, (approx. 1-2 µm in length) strict aerobe growing normally at 30 - 35°C. In contrast, the isolates L43-I was found to be catalase negative. The isolates produced acid from glucose, xylose and manitol, but the isolate Fs_1-I produced acid only from glucose. The isolates reduced nitrate to nitrite. The isolates G85-I and Pw_1-I assimilated lactose, dextrose and sucrose. However, isolates L64-I and L123-I assimilated lactose and dextrose only. Isolates L43-I and Fs_1-I assimilated dextrose only. On the other hand, the isolate G207-II had no effect on carbohydrate. The isolates hydrolysed casein and gelatin. Isolates G85-I, L123-I, L43-I and Fs_1-I had capability to utilize citrate, but other isolates had no activity on citrate utilization. The microbes had no activity for the utilization of starch and urease but isolates L64-I, Pw_1-I and Fs_1-I were found to produce urease. The isolates showed negative results in the production of H_2S and indole. The isolates displayed peptonization of litmus milk. The isolates G85-I, G207-II, L64-I and Pw_1-I possessed mucoid growth on nutrient broth supplemented with glucose and hexadecane while the other isolates were non-mucoid. As per *Bergey's Manual of Systematic Bacteriology* (Krieg *et al.*, 1984), the isolates were grouped in section 4. There were five species belonging to the genus *Pseudomonas- P. aeruginosa, P. fluorescens* (biovar I, II, III, IV and V), *P. chlororaphis, P. aureofaciens and P. putida* (biovar A and B). The morphological and biochemical tests confirmed 80-60% homology of the isolates G85-I, G207-II, L64-I, L123-I, L43-I, Pw_1-I and Fs_1-I with *Pseudomonas aeruginosa*. Accordingly, isolates G85-I, G207-II, L43-I and Pw_1-I were identified as *P. aeruginosa* mucoid, whereas, isolates L64-I, L123-I and Fs_1-I were identified as *P. aeruginosa* non- mucoid. All the isolates characterized have been sent to the Institute of Microbial Technology, Chandigarh for confirmation and detailed identification.

The bacterial isolate L453-I was found to be a gram negative, catalase, MR positive and VP negative, motile rod (approx. 1-2 µm in

length), strict aerobe which grew at 25 -39°C. Biochemical tests showed that the isolate could produce acid from glucose. The isolate L453-I had no ability to hydrolyzed gelatin; it had the capability to peptonize milk protein and utilize citrate. L453-I reduced nitrate to nitrite or molecular nitrogen and assimilated dextrose. The microbe had no activity for the utilization of starch, urease, H_2S and indole production. According to *Bergey's Manual of Systematic Bacteriology* (Krieg *et al.*, 1984), the isolate L453-I was grouped in section 4. There were two species belonging to genus Alcaligenes- *A. faecalis, A. denitrificans (*biovar 2a and 2b). The morphological and biochemical tests confirmed 70 % homology of the isolate L453-I with Alcaligenes. Accordingly, the isolate was identified as *Alcaligenes faecalis.* The isolate was sent to the Institute of Microbial Technology, Chandigarh for confirmation and detailed identification.

The isolate L490-II was found to be a nonspore forming, gram negative, catalase-negative, VP- positive, motile rod (approx. 1-2 µm in length), strict aerobe which grew at 25 - 37°C. Biochemical test confirmed its ability to produce acid from glucose, xylose and manitol. L490-II reduced nitrate to nitrite or molecular nitrogen and assimilated dextrose and sucrose; it also hydrolysed casein and gelatin; it had the capability to utilize citrate, and triple sugar iron and peptonized milk. The microbe had no activity for the utilization of starch, urease, H_2S and indole production. According to *Bergey's Manual of Systematic Bacteriology* (Krieg *et al.*, 1984), the isolate L490-II was grouped in section 4. There were five species belonging to genus Pseudomonas- *P. aeruginosa, P. fluorescens (*biovar I, II, III, IV and V), *P. chlororaphis, P. aureofaciens, P. putida biovar* A and *P. putida biovar* B. The morphological and biochemical tests confirmed about 60% of the isolate L490-II with *P. aeruginosa* and 70% with *P. fluorescens.* Accordingly, the isolate was identified as *Pseudomonas fluorescens.* The isolate characterized have been sent to the Institute of Microbial Technology, Chandigarh for confirmation and detailed identification.

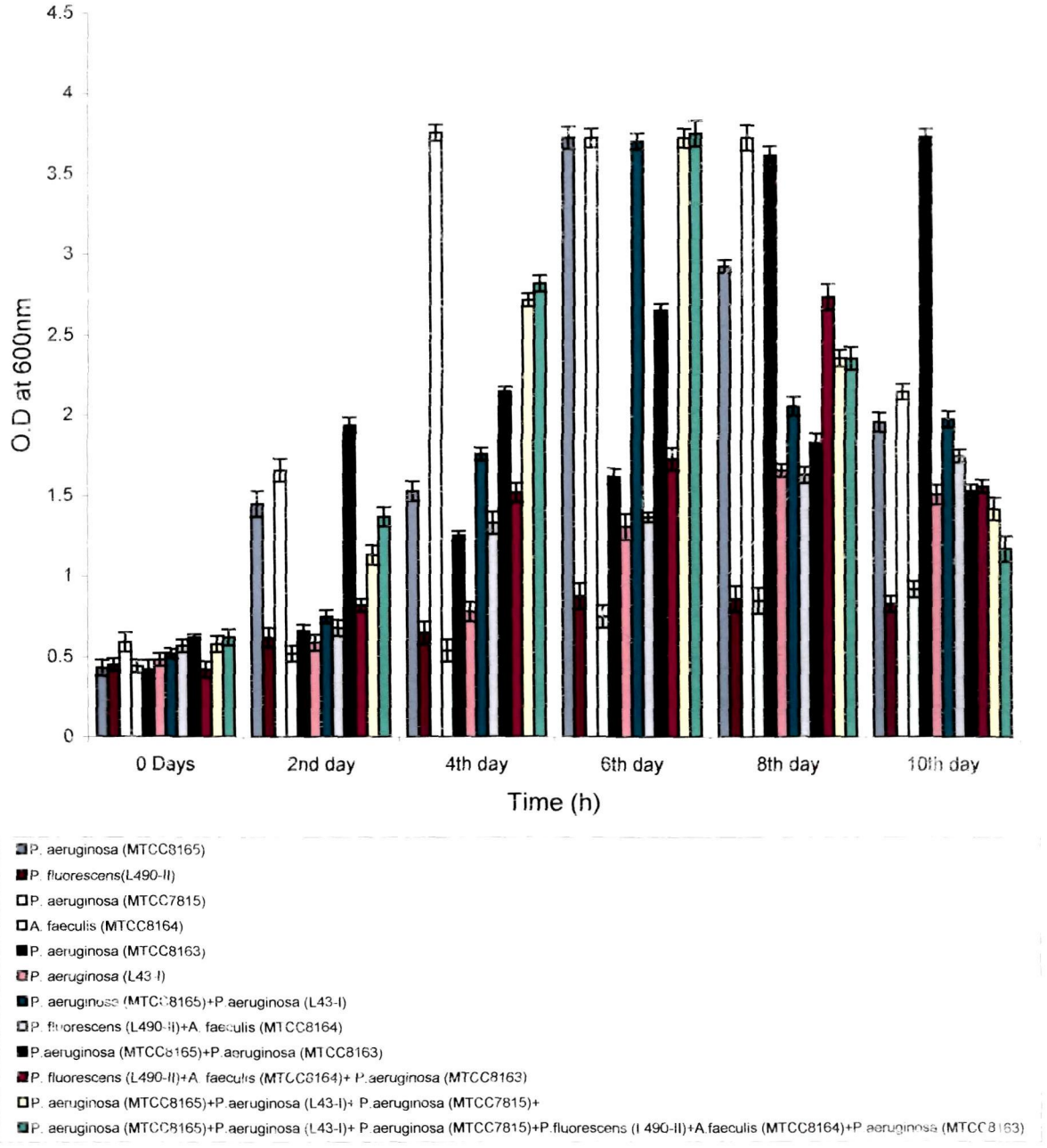
The bacterial isolate G35-I was found to be a gram positive, catalase-positive, motile rod (approx. 1-2 µm in length), strict aerobe which grew at 25 -39°C. Biochemical tests showed that the isolate could produce acid from glucose. The isolate G35-I hydrolyzed gelatin; it had the capability to peptonize milk protein and utilize citrate. The microbe had no activity for the utilization of starch, urease lactose and sucrose. It showed negative results in the production of H_2S, indole and triple sugar iron. According to *Bergey's Manual of Systematic Bacteriology* (Sneath *et al.*, 1986), the isolate was grouped in section 15. There were eight genera in the section including Microbacterium. The morphological and biochemical tests confirmed 70 % homology of the isolate G35-I with *Microbacterium*. Accordingly, the isolate was identified as *Microbacterium sp.*, as the isolate showed motility. The isolate was sent to the Institute of Microbial Technology, Chandigarh for confirmation and detailed identification.

The bacterial isolates L253-II, L485-II and R38-I were gram positive, catalase, VP and MR negative, motile rod (approx. 1-2 µm in length), strict aerobe which grew at 30 - 35°C. The isolates L253-II and R38-I hydrolyzed lipid whereas, isolate L485-II lacked activity on lipid. However, all three isolates had the capability to peptonize milk protein but no acid production from glucose, xylose and manitol was observed. The isolates showed positive results on triple sugar iron. The isolates L253-II and L485-II had no activity on urease production whereas the isolate R38-I produce urease. The microbe had no activity for the utilization of citrate, nitrate, starch and casein. The isolate L485-II displayed gelatin liquefaction activity but other two displayed negative results. It showed negative results in the production of H_2S and indole. According to *Bergey's Manual of Systematic Bacteriology* (Sneath *et al.*, 1986), the isolates were grouped in section 13. There were 34 species belonging to the genera Bacillus. The morphological and biochemical tests confirmed 80% homology of the isolates L253-II, L485-II

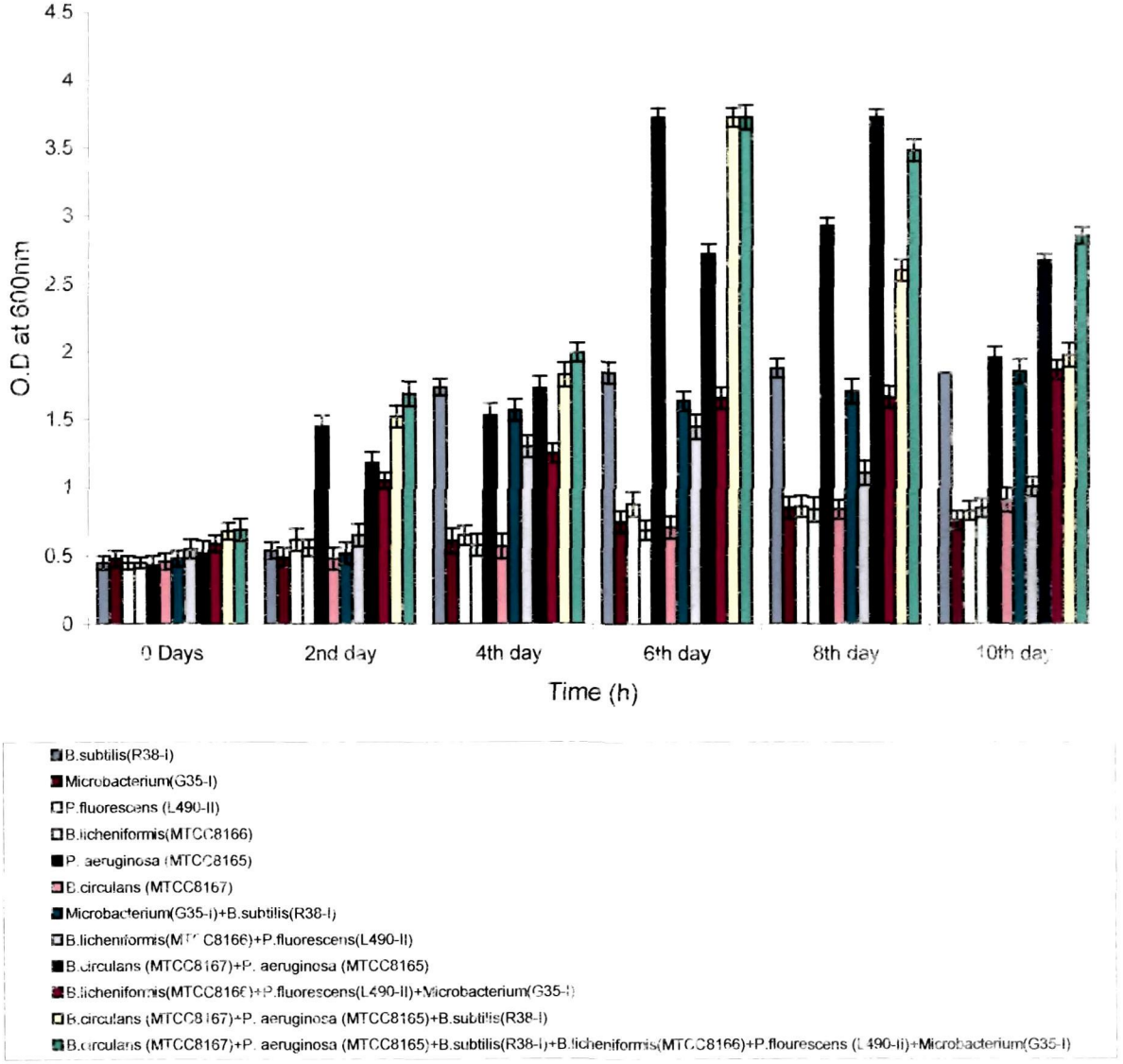
and R38-I with *Bacllus subtilis* ATCC 6051, DSM 10, NCIB 3610 and NCTC 3610. Accordingly the isolates were identified as *Bacillus subtilis*. The isolates were sent to the Institute of Microbial Technology, Chandigarh for confirmation and detailed identification.

4.1.11. Growth of bacterial isolates in consortia

In situ degradation of toxic components of hydrocarbons by microbes could be an effective bioremediation process. However, the strain specificity towards different components of hydrocarbons necessitated developing effective consortia for the purpose. Accordingly, the selected bacterial isolate were grouped in to 24 combinations and their growth with optical density was determined in crude oil supplemented medium. As shown in Fig.4.4 (a) the combination of 12 isolates comprising of *P. aeruginosa* (MTCC8165) + *P. aeruginosa* (L43-I)+ *P. aeruginosa* (MTCC7815) + *A. faecalis* (MTCC 8164) + *P. aeruginosa* (MTCC8163) + *P. fluorescens* (L490-II) exhibited the highest growth rate with O.D. of 3.75 at 10^{th} day of culture. Similar trend was observed (Fig.4.4.b) in the case of the second set of experiments, the combination comprising of *Microbacterium* (G35-I) + *B. subtilis* (R38-I) + *P. fluorescens* (L490-II) + *B. licheniformis* (MTCC 8166) + *P. aeruginosa* (MTCC8165) + *B. circulans* (8167) possessed higher growth rate with O.D. of 3.75 at 10^{th} day of culture.


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
O.D at 600nm
0 Days
2nd day
4th day
6th day
8th day
10th day
Time (h)
P. aeruginosa (MTCC8165)
P. fluorescens(L490-II)
P. aeruginosa (MTCC7815)
A. faeculis (MTCC8164)
P. aeruginosa (MTCC8163)
P. aeruginosa (L43-I)
P. aeruginosa (MTCC8165)+P.aeruginosa (L43-I)
P. fluorescens (L490-II)+A. faeculis (MTCC8164)
P.aeruginosa (MTCC8165)+P.aeruginosa (MTCC8163)
P. fluorescens (L490-II)+A. faeculis (MTCC8164)+ P.aeruginosa (MTCC8163)
P. aeruginosa (MTCC8165)+P.aeruginosa (L43-I)+ P.aeruginosa (MTCC7815)+
P. aeruginosa (MTCC8165)+P.aeruginosa (L43-I)+ P.aeruginosa (MTCC7815)+P.fluorescens (L490-II)+A.faeculis (MTCC8164)+P.aeruginosa (MTCC8163)

(a)

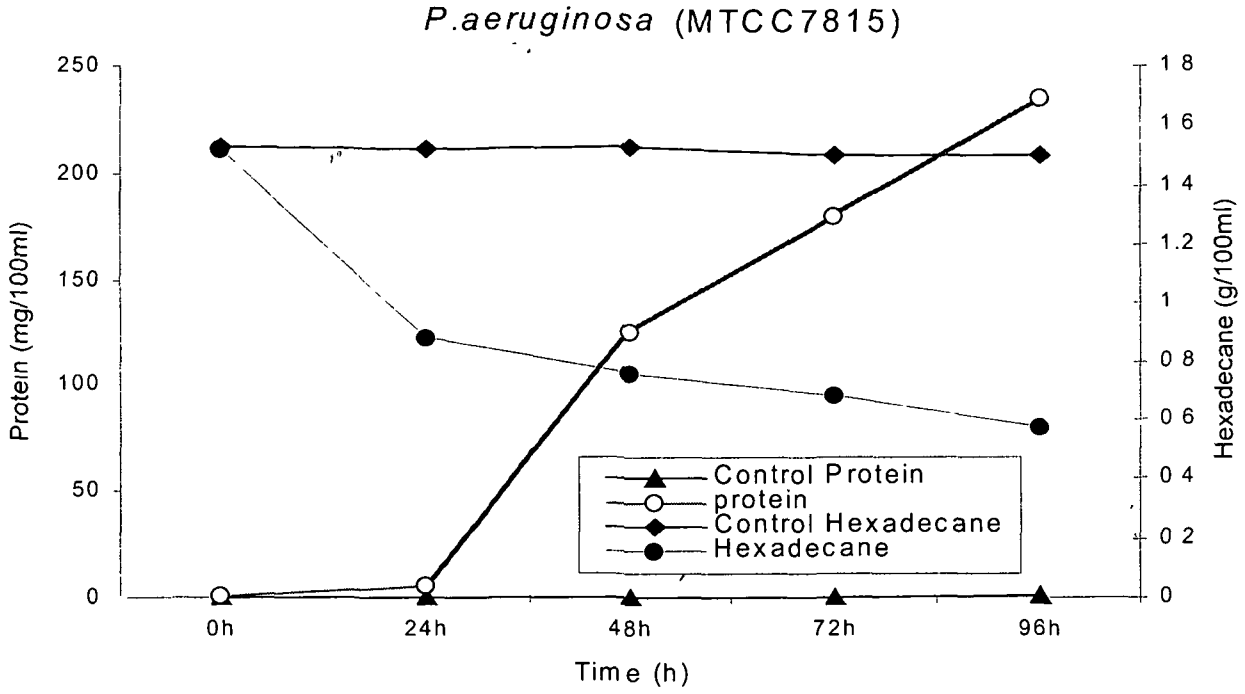


(b)

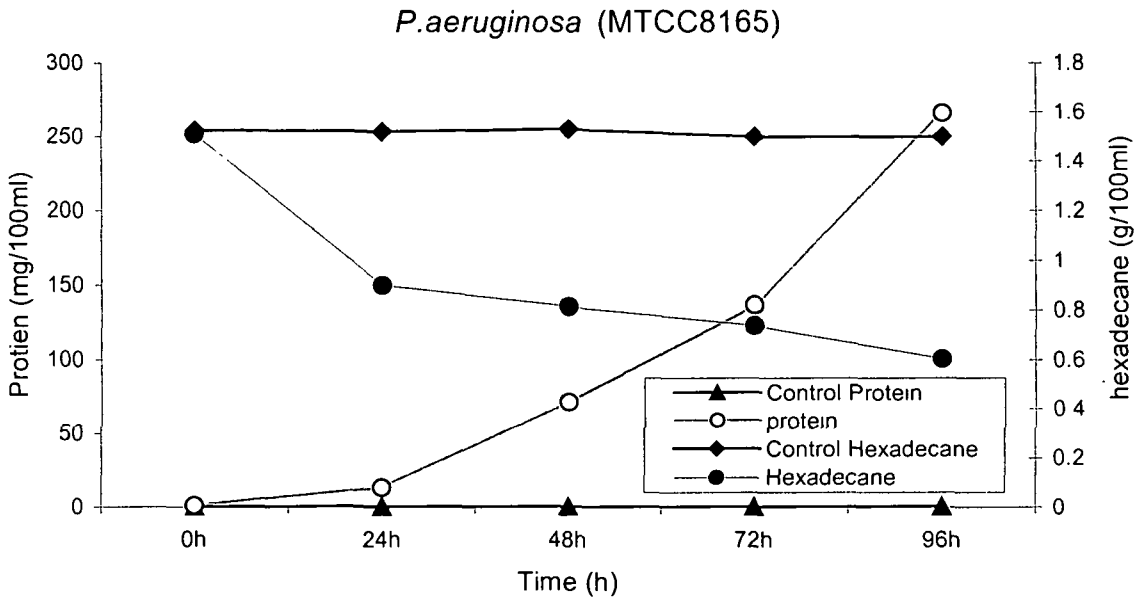
Fig 4.4 Growth of bacterial Isolates in combinations in media supplemented with 100 $\mu l.l^{-1}$ of crude oil (a) first 12 combination and (b) second 12 combinations (Mean ± S.D)

4.1.11.1. Efficacy of bacterial isolates to degrade hydrocarbons

The relative ability of bacterial isolates collected from oil well - sites of Assam asset to degrade hydrocarbons was determined by culturing the bacteria in liquid medium supplemented with an individual hydrocarbon component: hexadecane as the sole source of carbon. A total of 10 isolates were selected on the basis of their better growth as revealed by increased concentration of bacterial protein as against decreased concentration of hexadecane in the culture medium. The increased concentration of bacterial protein in relation to hexadecane concentration in the liquid culture medium over a period of 96 h has been presented in Fig.4.5 (a) to (j)



(a)


P.aeruginosa (MTCC8165)
Protien (mg/100ml)
hexadecane (g/100ml)
Time (h)
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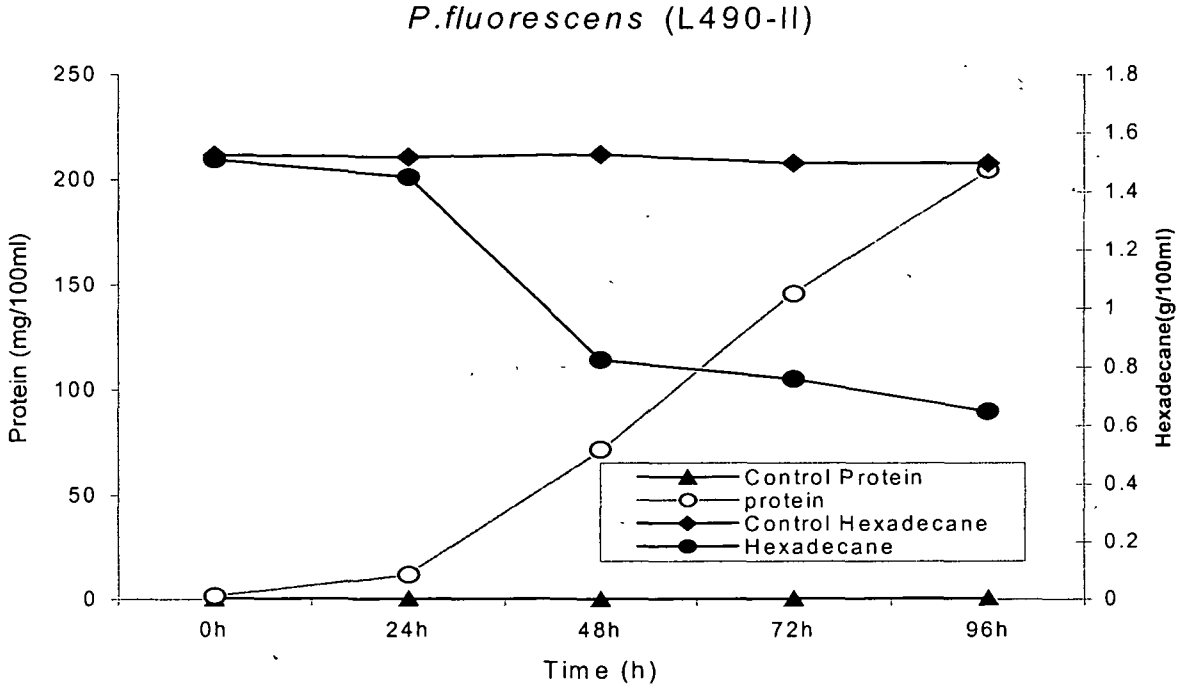
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(b)


P.fluorescens (L490-II)
Protein (mg/100ml)
Hexadecane(g/100ml)
Time (h)
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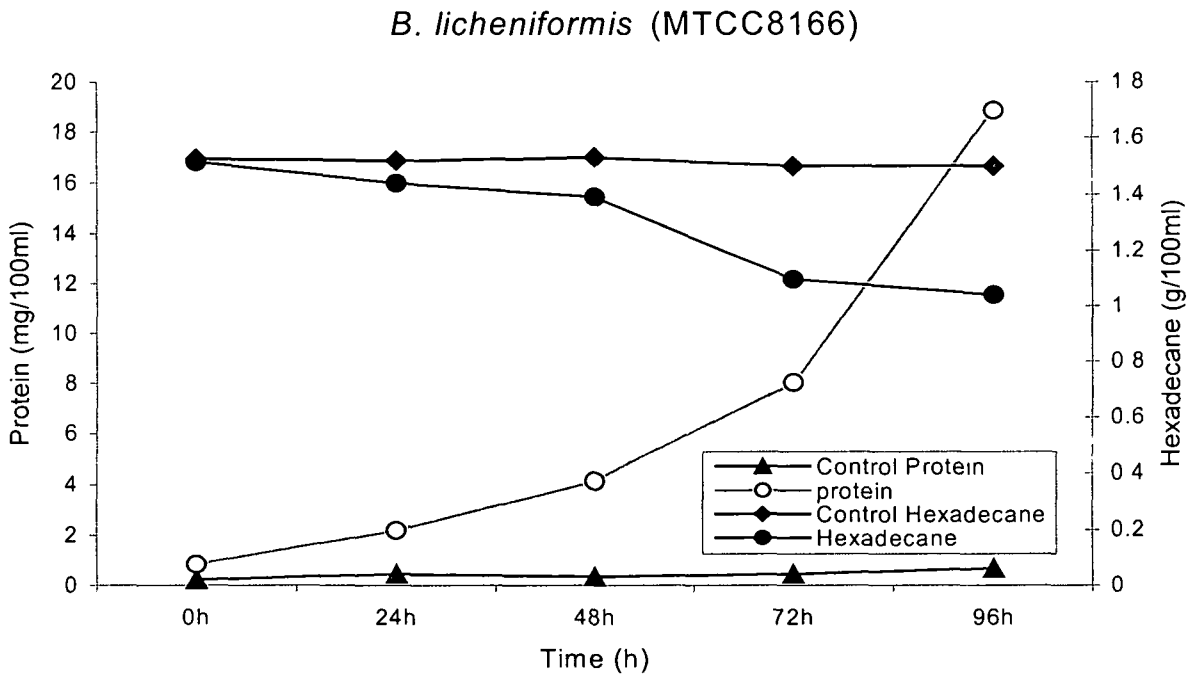
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(c)


B. licheniformis (MTCC8166)
Protein (mg/100ml)
Hexadecane (g/100ml)
Time (h)
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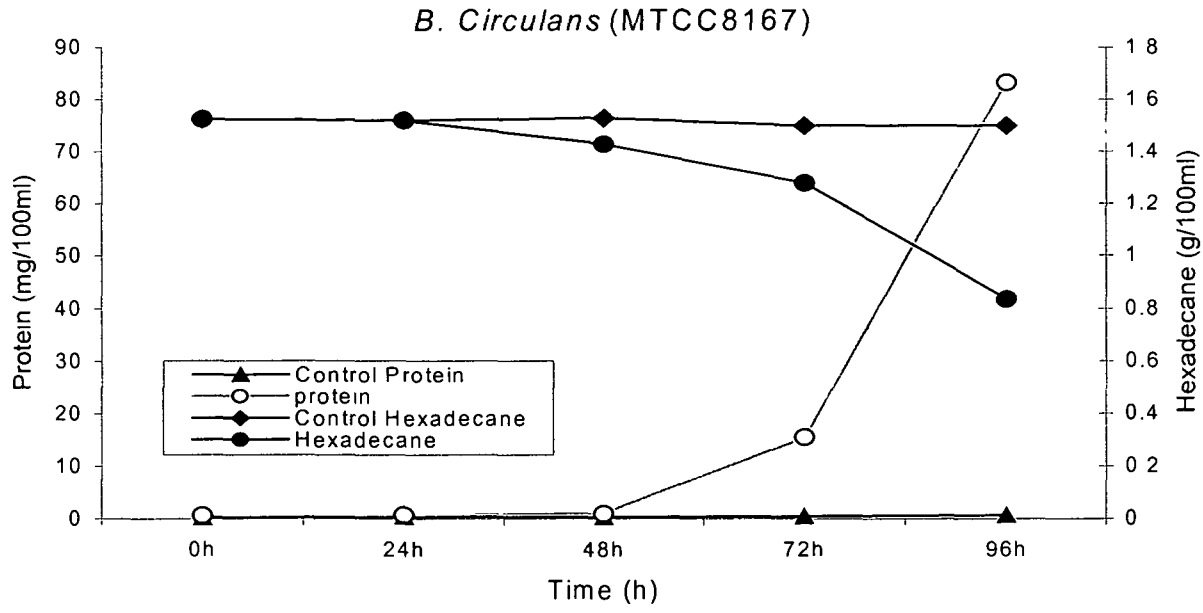
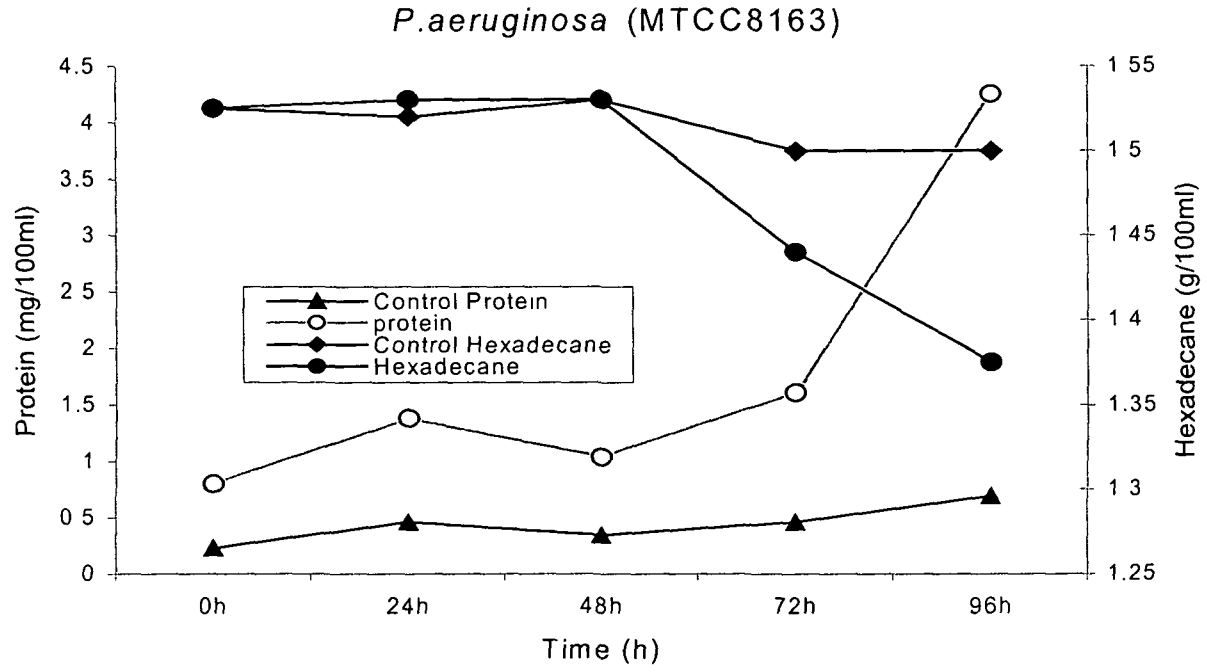
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(d)


B. Circulans (MTCC8167)
Protein (mg/100ml)
Hexadecane (g/100ml)
Time (h)
0h
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(e)


P.aeruginosa (MTCC8163)
Protein (mg/100ml)
Hexadecane (g/100ml)
Time (h)
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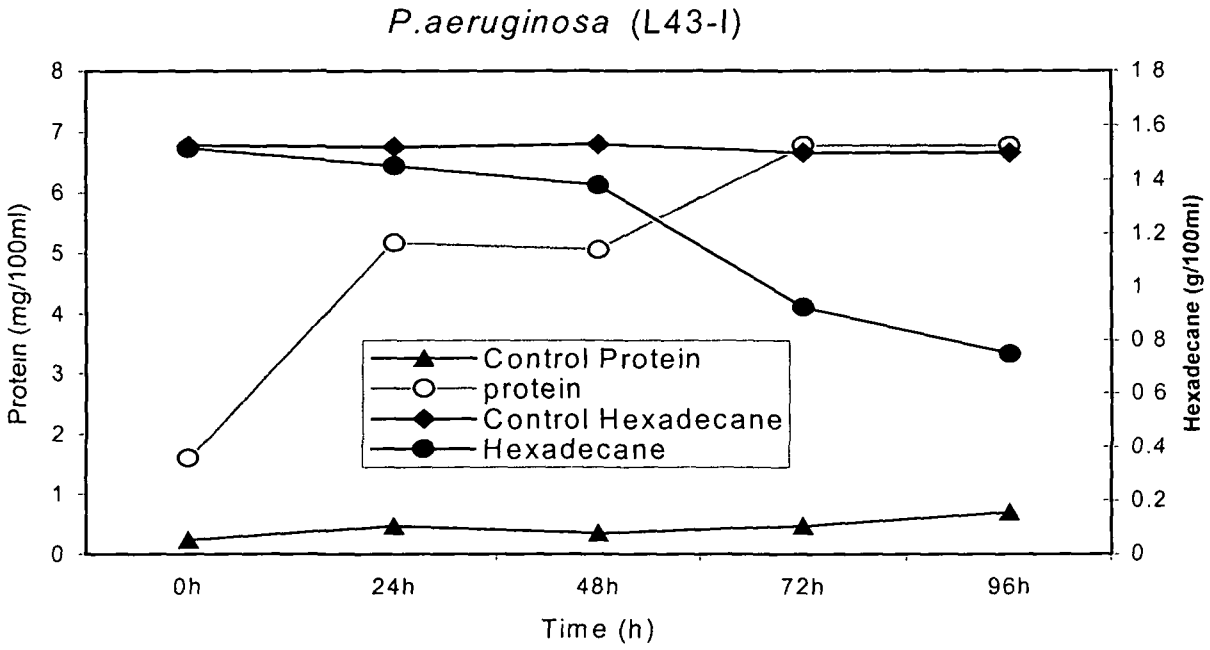
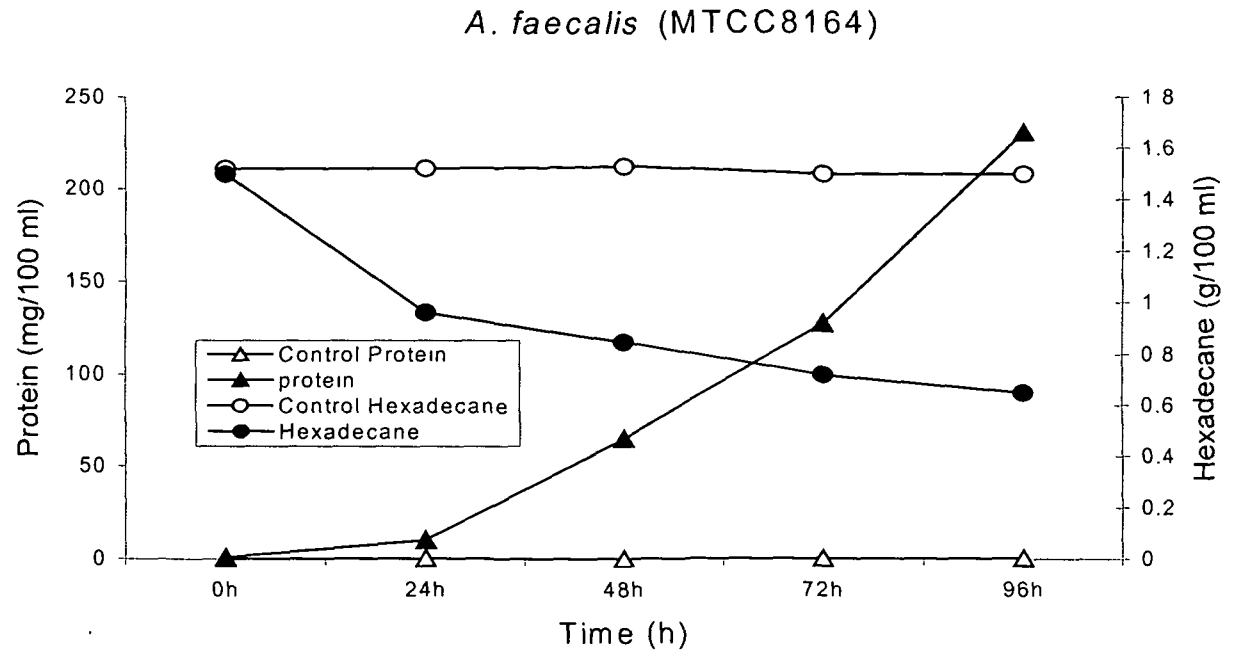
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(f)


P.aeruginosa (L43-I)
Protein (mg/100ml)
Hexadecane (g/100ml)
Time (h)
0h
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(g)


A. faecalis (MTCC8164)
Protein (mg/100 ml)
Hexadecane (g/100 ml)
Time (h)
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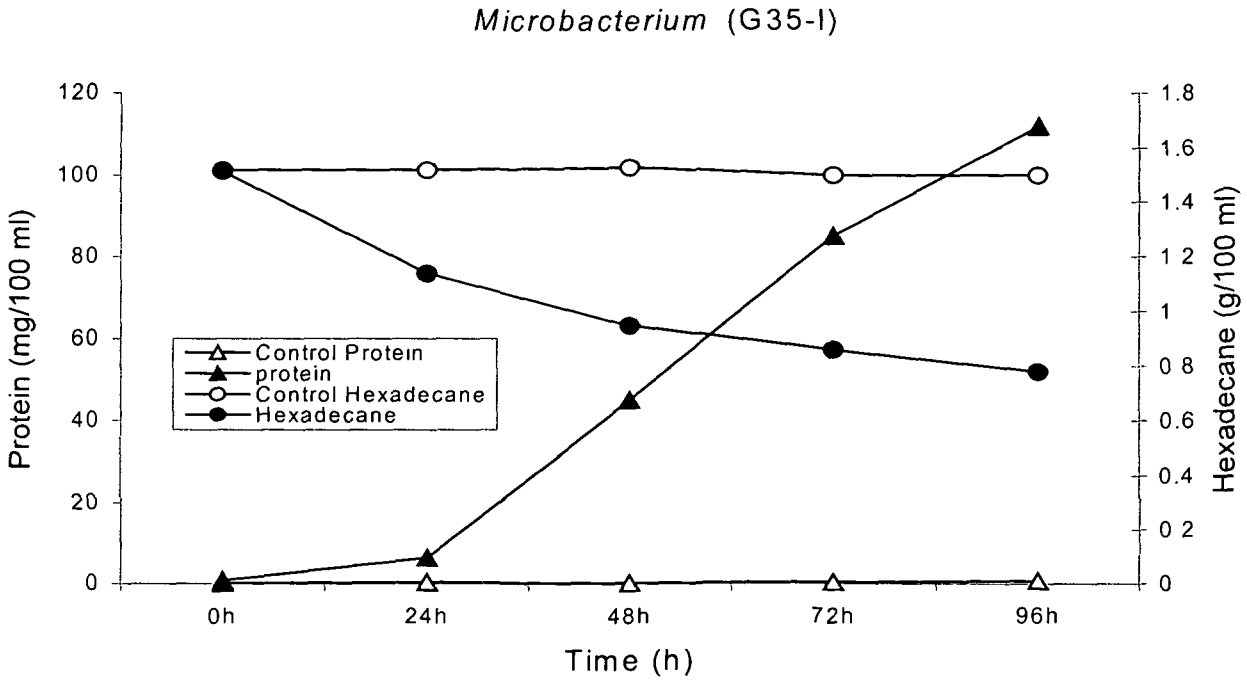
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(h)


Microbacterium (G35-I)
Protein (mg/100 ml)
Hexadecane (g/100 ml)
Time (h)
0h
24h
48h
72h
96h
Control Protein
protein
Control Hexadecane
Hexadecane

(i)

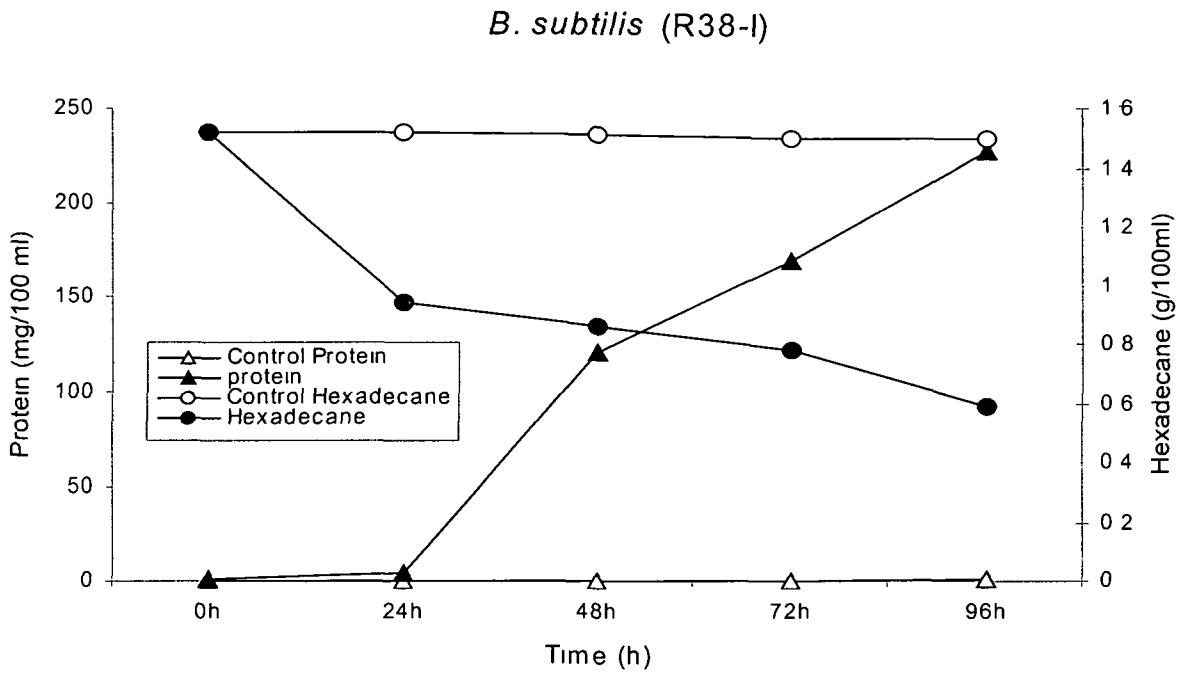


Fig. 4.5. Degradation of hexadecane by the bacterial isolates (a) *P. aeruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC 8165) (c) *P. fluorescens* (L490-II) (d) *B. licheniformis* (MTCC 8166) (e) *B. circulans* (MTCC 8167) (f) *P. aeruginosa* (MTCC8163) (g) *P. aeruginosa* (L43-I) (h) *A. faecalis* (MTCC 8164) (i) *Microbacterium* (G35-I) and (j) *B. subtilis* (R38-I)

The concentration of hexadecane in the culture medium and the total protein content of the bacteria cultured in the medium supplemented with hexadecane were estimated. It is evident from the result that in the case of all 10 isolates grown in hexadecane supplemented medium, there was an inverse relation between the total protein content of the isolates and the residual hexadecane content in the culture medium. The amount of hexadecane in the culture medium decreased as the protein content of the culture medium increased. The isolates *P. aeruginosa* (MTCC8165), *P.*

aeruginosa (MTCC7815) followed by *B. subtilis* (R38-I), *A. faecalis* (MTCC 8164) and *P. fluorescens* (L490-II) used the maximum hexadecane leaving behind residue of 0.57 – 0.65 g in 100 ml culture medium as compared to control ones (1.51g in 100ml). The bacterial isolates *P. aeruginosa* (L43-I), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC8165) and *B. circulans* (MTCC8167) used comparatively less hexadecane as revealed by higher residue of 0.75 –0.83 g in 100ml culture medium. The isolate *B. licheniformis* (MTCC8166) showed poor utilization of hexadecane leaving behind residue of 1.04 g as compared to other bacterial isolates.

Subsequently, these 10 selected bacterial isolates were cultured in the liquid medium supplemented separately with different hydrocarbon components like pyrene, phenanthrene, toluene, benzene, hexadecane as well as glucose as the sole source of carbon. The dry biomass yield of the bacterial isolates was determined and the data thus obtained are presented in Table 4.10.

Table 4.10. Growth of bacterial isolates on different carbon sources over a period of 96 h culture (mean of 3 experiments)

Carbon sources	Dry biomass ($g.l^{-1}$) of bacterial isolates									
	P. aeruginosa (MTCC7815)	*Microbacterium* (G35-I)	*P. aeruginosa* (MTCC8163)	*A. faecalis* (MTCC8164)	*P. aeruginosa* (MTCC816 5)	*B. licheniformis* (MTCC816 6)	*B. circulans* (MTCC8167)	*P. fluoresens* (L490-II)	*P. aeruginosa* (L43-I)	*B. subtilis* (R38-I)
Glucose	3.5±0.4	2.1±0.1	2.5±0.2	3.2±0.2	3.1±0.2	2.1±0.2	1.9±0.1	2.2±0.1	2.5±0.1	1.5±0.1
Hexadecane	3.1±0.3	2.4±0.1	3.1±0.1	3.1±0.1	3.5±0.1	2.3±0.1	2.3±0.1	2.5±0.2	3.1±0.2	1.8±0.1
Crude oil	1.2±0.2	0.9±0.2	1.0±0.1	0.7±0.1	1.1±0.1	0.6±0.2	0.9±0.1	0.9±0.1	0.7±0.1	1.4±0.1
Benzene	0.6±0.2	0.2±0.2	0.7±0.1	0.5±0.2	0.5±0.1	0.4±0.2	0.4±0.1	0.4±0.1	0.5±0.1	0.5±0.1
Toluene	0.8±0.2	0.2±0.1	0.6±0.1	0.6±0.4	0.6±0.2	0.5±0.1	0.5±0.1	0.3±0.2	0.3±0.2	0.7±0.2
Phenanthrene	1.0±0.1	0.3±0.2	0.9±0.4	0.7±0.2	0.8±0.1	0.4±0.1	0.6±0.2	0.5±0.1	0.5±0.1	0.6±0.1
Pyrene	0.7±0.1	0.1±0.1	0.8±0.2	0.7±0.3	0.6±0.2	0.3±0.2	0.7±0.1	0.6±0.1	0.6±0.1	0.8±0.1

In the glucose - supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7815) followed by *A. faecalis* (MTCC 8164) and *P. aeruginosa* (MTCC8165) produced comparatively higher biomass of 3.5 – 3.1 $g.l^{-1}$. The remaining isolates possessed less biomass yield of 2.5 – 1.5 $g.l^{-1}$ for the entire growth period. In the hexadecane-supplemented medium, the isolate *P. aeruginosa* (MTCC8165) followed by *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8163), *A. faecalis* (MTCC 8164) and *P. aeruginosa* (L43-I) exhibited better growth with the biomass yield of 3.5- 3.1 $g.l^{-1}$; in crude oil supplemented medium, the isolates *B. subtilis* (R38-I) followed by *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC8163) showed better growth with dry biomass yield of 1.4 – 1.0 $g.l^{-1}$; in benzene and toluene (aromatic) hydrocarbon - supplemented media, the bacterial isolates produced comparatively low biomass of 0.8 – 0.2 $g.l^{-1}$. Similarly, in phenanthrene and pyrene – supplemented media (polycyclic aromatic hydrocarbons), the isolates possessed comparatively less growth with biomass yield of 1.0 –0.1 $g.l^{-1}$.

The bacterial isolates and their possible 11 combinations were then cultured in liquid medium supplemented with crude oil as the sole source of carbon for a period of 96 h to determine their ability to grow in crude oil. The final dry biomass yield as against the initial yield was determined and the data are presented in Table 4.11.

Table 4. 11. Growth of bacterial isolates and their combinations (dry biomass) in crude oil supplemented medium for a period of 96 h (mean ± SD of 3 experiments)

Bacterial isolates/ consortia	Dry Biomass ($g.l^{-1}$) in crude oil supplemented medium			Bacterial isolates/ consortia	Dry Biomass ($g.l^{-1}$) in crude oil supplemented medium		
	Initial	Final	Difference		Initial	Final	Difference
P. aeruginosa (MTCC8165)	0.29± 0.01	1.1± 0.01	0.81± 0.01	Comb 3	0.45± 0.01	1.30 ± 0.1	0.85± 0.1
P. fluorescens (L490-II)	0.42± 0.01	0.9 ±0.01	0.48± 0.01	Comb 4	0.38± 0.02	1.72 ± 0.1	1.34± 0.2
A. faecalis (MTCC 8164)	0.35± 0.01	0.7 ± 0.01	0.35± 0.01	Comb 5	0.80± 0.01	1.51 ± 0.1	0.71± 0.1
P. aeruginosa (MTCC8163)	0.50± 0.01	1.0 ± 0.01	0.50± 0.01	Comb 6	0.40± 0.01	1.26 ± 0.1	0.86± 0.1
B.licheniformis (MTCC 8166)	0.41± 0.02	0.6 ± 0.02	0.19± 0.02	Comb 7	0.60± 0.01	3.32 ± 0.1	2.72± 0.3
B. circulans (MTCC8167)	0.50± 0.01	0.9 ± 0.01	0.40± 0.01	Comb 8	0.35± 0.01	1.85 ± 0.1	1.50± 0.4
P. aeruginosa (L43-I)	0.35± 0.01	0.7 ± 0.01	0.35± 0.01	Comb 9	0.35± 0.01	3.25 ± 0.2	2.90± 0.1
P. aeruginosa (MTCC7815)	0.25± 0.01	1.2 ± 0.02	0.95± 0.03	Comb 10	0.38± 0.01	3.72 ± 0.1	3.34± 0.1
Microbacterium (G35-I)	0.56± 0.01	0.9 ± 0.02	0.34± 0.01	Comb 11	0.40± 0.01	3.73 ± 0.1	3.33± 0.1
B. subtilis (R38-I)	0.75± 0.01	1.4 ± 0.01	0.65± 0.01				
Comb1	0.65± 0.01	2.10 ± 0.01	1.45± 0.01				
Comb 2	0.50± 0.01	1.85 ± 0.01	1.35± 0.04				

* Comb = Combination, Comb 1: *P. aeruginosa* (MTCC8165)+ *P. fluorescens* (L490-II), Comb 2: *A. faecalis* (MTCC 8164)+ *P. aeruginosa* (MTCC8163), Comb 3: *B. licheniformis* (MTCC 8166)+ *B. circulans* (MTCC8167), Comb 4: *P. aeruginosa* (L43-I) + *P. aeruginosa* (MTCC7815), Comb 5: *Microbacterium* (G35-I)+ *B. subtilis* (R38-I), Comb 6: *P. fluorescens* (L490-II)+ *A. faecalis* (MTCC 8164)+ *P. aeruginosa* (MTCC8163), Comb 7: *P. aeruginosa* (MTCC8165)+ *P. aeruginosa* (L43-I)+ *P. aeruginosa* (MTCC7815), Comb 8: *B. licheniformis* (MTCC 8166)+ *P. fluorescens* (L490-II)+ *Microbacterium* (G35-I), Comb 9: *B. circulans* (MTCC8167)+ *P. aeruginosa* (MTCC8165)+ *B. subtilis* (R38-I), Comb 10: *P. aeruginosa* (MTCC8165)+ *P. aeruginosa* (L43-I)+ *P. aeruginosa* (MTCC7815)+ *A. faecalis* (MTCC 8164)+ *P. aeruginosa* (MTCC8163)+ *P. fluorescens* (L490-II)and Comb 11: *Microbacterium* (G35-I)+ *B. subtilis* (R38-I)+ *P. fluorescens* (L490-II)+ *B. licheniformis* (MTCC 8166)+ *P. aeruginosa* (MTCC8165)+ *B. circulans* (MTCC8167)

The bacterial combination 10th comprised of the isolates *P. aeruginosa* (MTCC8165), *P. aeruginosa* (L43-I), *P. aeruginosa* (MTCC7815), *A. faecalis* (MTCC8164), *P. aeruginosa* (MTCC8163) and *P. fluorescens* (L490-II) followed by the combination 11th, comprised of *Microbacterium* (G35-I), *B. subtilis* (R38-I), *P. fluorescens* (L490-II), *B. licheniformis* (MTCC8166), *P. aeruginosa* (MTCC8165) and *B. circulans* (MTCC8167) produced higher biomass yield of 3.34 – 3.33 $g.l^{-1}$ during the entire growth period of 96 h. The combination 7 followed by 8, 1, 2 and 4 exhibited moderate growth with biomass yield of 2.72 – 1.34 $g.l^{-1}$ as compared to other combinations and single isolates.

4.1.11. 2. Degradation of crude oil by bacterial consortia and isolates

On the basis of the growth performance as depicted by the dry biomass yield, 2 bacterial isolates: *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8165) and 2 combinations, designated as consortium I (combination 10) and consortium II (combination 11) were selected to assess their ability to degrade crude oil in liquid medium. The culture medium was supplemented with 2 ml (1.9 g) of crude oil. As against the initial culture density, the population builds up of the bacterial isolates and consortia in 30 and 180 days of culture was determined. Simultaneously, the residual crude oil in the medium was assayed. Along with the increased population density of the bacterial isolates and consortia the percent crude oil degradation are presented in Table 4. 12.

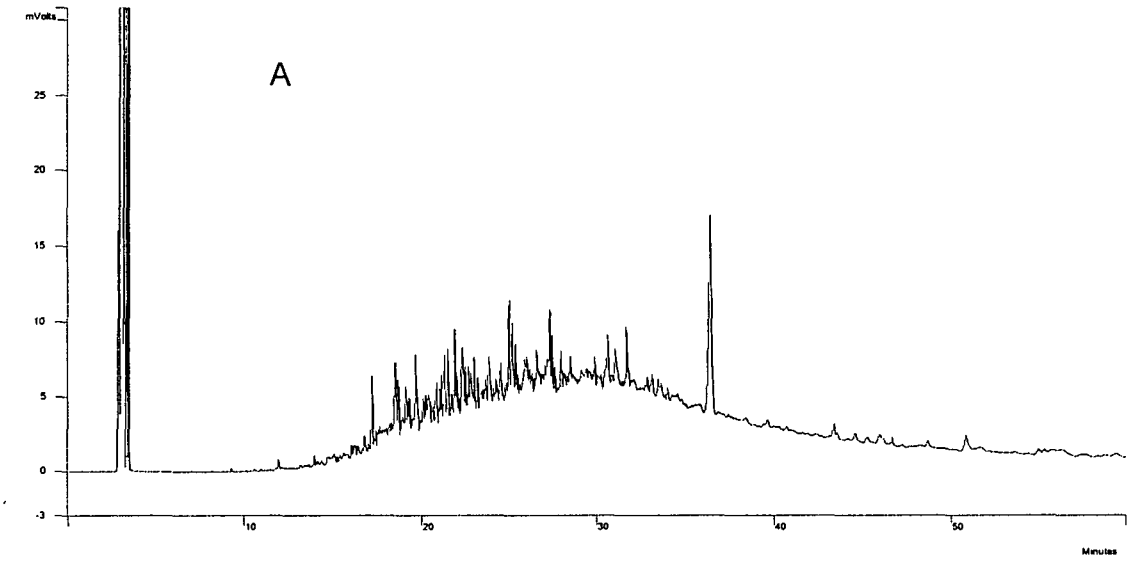
Table 4. 12. Degradation of crude oil in liquid culture medium by bacterial consortia in 180 days (mean ±S.D of 3 experiments)

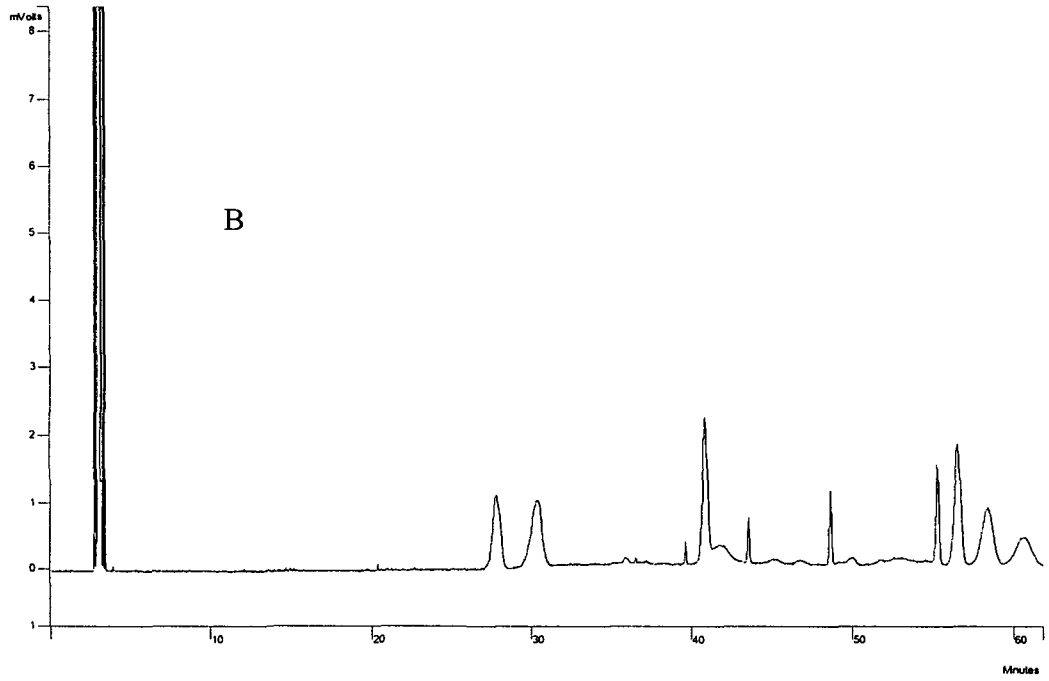
Sample	No of cells/ ml			Level of hydrocarbons 2 ml (1.91 g)	Degradation %	
	0 days	30 days	180 days		30 days	180 days
Consortium I	$1x10^5$	$1.23x10^6$	$1.28x10^6$	2.0	75.71 ± 0.1^a	76.12 ± 0.4^a
Consortium II	$1x10^5$	$1.35x10^6$	$1.40x10^6$	2.0	78.57±0.3	79.34±0.1
P. aeruginosa (MTCC7815)	$1x10^5$	$0.71x10^6$	$0.71x10^6$	2.0	57.14 ± 0.1^a	58.43 ± 0.3^a
P. aeruginosa (MTCC8165)	$1x10^5$	$0.61x10^6$	$0.63x10^6$	2.0	74.28±0.1	75.37±0.2

Significance of difference with respect to consortium 2 $^a p < 0.01$
Significance of difference with respect to *P. aeruginosa* (MTCC 8165) $^a p < 0.01$

As shown in Table 4.12, the consortium II followed by consortium I and single isolate *P. aeruginosa* (MTCC8165) degraded the crude oil by 79.34 – 75.37% during the period of 180 days. On the other hand, the isolate *P. aeruginosa* (MTCC7815) exhibited lesser efficacy with 58.43% crude oil degradation.

The GC-FID profile of the saturated fraction of crude oil in culture medium inoculated with consortium I and consortium II as well as control after 30 days of incubation was determined and the result thus obtained is presented graphically in Fig. 4. 6.

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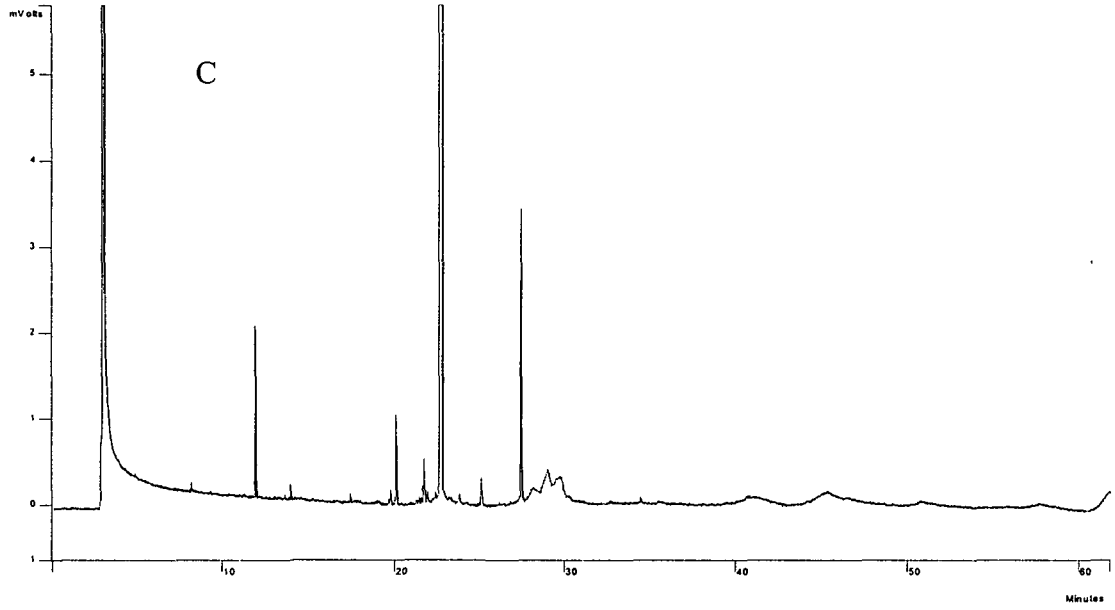
7
6
5
4
3
2
1
0
1
B
10
20
30
40
50
60
Minutes


mVolts
5
4
3
2
1
0
1
C
10
20
30
40
50
60
Minutes

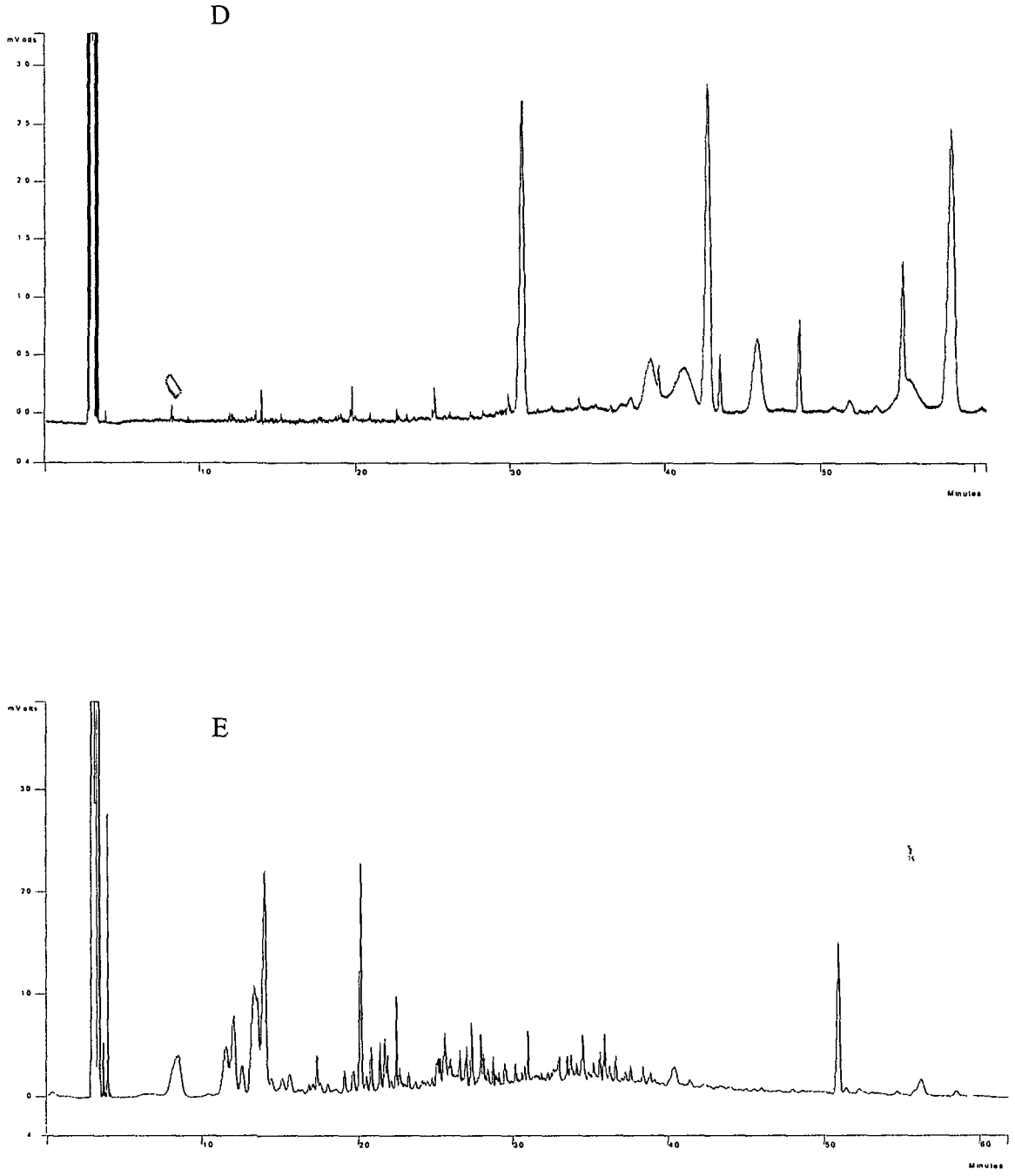


Fig.4.6. Chromatographic profile (GC-FID) of the fraction of crude oil after 30 days of incubation (A) control- not inoculated (B) Inoculated with Consortium II (C) Inoculated with Consortium I (D) Inoculated with isolate *P. aeruginosa* (MTCC7815) and (E) Inoculated with *P. aeruginosa* (MTCC8165)

In a separate experiment, the liquid culture medium supplemented with 1.87 g of crude oil was separately inoculated with the selected 2 bacterial isolates and 2 consortia and cultured for a period of 180 days to determine the degradation of aliphatic, aromatic and NSO compounds. The residual aliphatic and aromatic fractions as well as NSO compounds were estimated and the data on weight basis and percent degradation are presented in Table 4.13.

Table 4.13. Degradation of aliphatic, aromatic and NSO fractions of hydrocarbons by the bacterial consortia in 180 days (mean ±S.D of 3 experiments)

Sample	Media supplemented with crude oil (g)	Aliphatic fraction degraded		Aromatic fraction degraded		NSO compounds degraded	
		Wt in g	%	Wt in g	%	Wt in g	%
Control	1.87	1.06±0.3	56.67±0.4	0.33±0.2	17.92±0.1	0.08±0.1	4.29±0.4
Consortium I	1.87	0.09±0.1	91.38±0.3	0.18±0.1	44.94±0.3	0.06±0.4	18.73±0.4
Consortium II	1.87	0.09±0.1	90.77±0.3	0.21±0.1	36.84±0.2	0.06±0.3	18.48±0.4
P. aeruginosa (MTCC7815)	1.87	0.16±0.2	84.89±0.1	0.32±0.4	2.52±0.2	0.03±0.2	50.00±0.1
P. aeruginosa (MTCC8165)	1.87	0.17±0.1	83.43±0.1	0.14±0.3	56.62±0.1	0.06±0.3	13.77±0.2

*Determination on the basis of crude oil 2.0 ml (1.8748 g)

The degradation percentage of the major fractions of crude oil was determined by the chromatographic assay. In the case of aliphatic hydrocarbons, the consortium I followed by consortium II exhibited the highest degradation with 91.38 and 90.77% in 180 days of culture. On the other hand, the single isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC 8165) possessed 84.89 and 83.43% degradation. In the case of aromatic fraction of crude oil, the consortium I followed by consortium II showed better degradation percentage with 44.94 and 36.84%. The consortium I and II degraded 18.73 and 18.48% of NSO compounds respectively. The isolate *P. aeruginosa* (MTCC7815) exhibited the highest degradation of NSO compounds with 50.00%.

4.3.3. Bioremediation of crude oil in contaminated soil

Crude oil contaminated soil was collected from an oil well site of Assam asset. The crude oil content of the soil was estimated in percentage. The soil was inoculated separately with consortium I, consortium II, isolate *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8165) having the bacterial population density of 10^{6} cfu.ml^{-1}. The residual crude oil content was estimated in 30, 60, 120 and 180 days of bioremediation. Data obtained are presented in Table 4. 14.

Table 4.14. Residual crude oil in contaminated soil collected from an oil well-site of Assam asset after bioremediation for 180 days (mean ±S.D of 3 experiments)

Bacterial consortia/ isolates	Percentage of crude oil in contaminated soils collected from sites				
	0 day	30 days	60 days	120 days	180 days
Control	8.4±0.1	8.01±0.1	7.90±0.3	7.50±0.4	7.3±0.3
Consortium I	8.5±0.1	6.25±0.1	5.30±0.3	2.50±0.4	2.1±0.1
Consortium II	8.6±0.4	4.13±0.3	3.90±0.1	2.00±0.2	1.9 ±0.2
P. aeruginosa (MTCC7815)	8.3±0.5	7.30±0.4	6.40±0.2	4.50±0.1	3.8 ±0.3
P. aeruginosa (MTCC 8165)	8.4±0.2	7.50±0.4	7.20±0.3	4.75±0.2	3.5 ±0.1

At 8% contamination level, the bacterial consortium II followed by the consortium I exhibited the highest degradation with the residual crude oil level of 1.9 and 2.1% after 180 days of bioremediation.

In a separate experiment, the normal soil was artificially contaminated with 20% crude oil and separately inoculated with the above mentioned bacterial consortia and isolates. Residual crude oil content was estimated in 30, 60,120 and 180 days and the data generated are presented in Table 4.15.

Table 4.15. Residual crude oil in artificially contaminated soil after remediation in 180 days (mean ±S.D of 3 experiments)

Bacterial consortia/ isolates	Soil samples contaminated with 20 % crude oil (lab contamination)				
	0 day	30 days	60 days	120 days	180 days
Control	20	19.75±0.1	19.50±0.3	19.05±0.3	18.5±0.3
Consortia I	20	16.40±0.1	11.12±0.3	10.50±0.2	6.8 ±0.2
Consortia II	20	10.20±0.3	7.50±0.1	6.74±0.2	5.3 ±0.1
P. aeruginosa (MTCC7815)	20	15.50±0.2	13.10±0.1	13.00±0.1	9.5 ±0.1
P. aeruginosa (MTCC 8165)	20	18.75±0.3	18.00±0.1	17.12±0.2	11.2 ±0.1

In this experiment, the soil samples artificially contaminated with 20% crude oil, the consortium II followed by consortium I degraded more crude oil with the residue level of 5.3 and 6.8% after 180 days of bioremediation. The isolate *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC 8165) exhibited comparatively poor degradation efficacy with the residual crude oil level of 9.5 and 11.2%, respectively after 180 days.

4.2. Reclamation of bioremediated soil

4.2.1. Mortality of seedlings in bioremediated soil

Pre – germinated seeds of the crops were planted in N1, R1, N2, R2 and C (Soil type description were given in material and methods chapter) types soil. The relative growth rate of each plant species was measured and recorded at every 7 days interval. In the case of 20% crude oil contaminated

soil+ NPK+ organic manure (C), none of the crops survived, all plants wilted one day after the planting. Data thus obtained are presented in Table 4.16.

Table 4.16. Initial growth (30 days) of green gram, bengal gram and rice in bioremediated soil after planting (average of 5 plants)

Crop	Observations	Average growth (cm) of 5 plants in controls and bioremediated soils				
		N1	R1	N2	R2	C
Green gram	1st	3.50±0.5	0.78±0.3	2.72±0.3	0.80±0.2	Died
	2nd	9.36±0.4	3.40±0.4	5.48±0.3	2.68±0.2	
	3rd	14.72±0.3	5.66±0.4	7.83±0.5	4.32±0.3	
	4th	16.28±0.2	7.56±0.3	8.30±0.2	4.70±0.4	
	5th	16.90±0.8	8.40±0.5	9.04±0.4	5.28±0.6	
	6th	17.28±0.7	8.62±0.6	10.24±0.6	5.70±0.5	
Bangal gram	1st	1.70± 0.6	1.40±0.5	1.60±0.8	1.48±0.5	Died
	2nd	9.40±0.4	4.36±0.4	9.00±0.9	3.04±0.3	
	3rd	15.32±0.3	9.72±0.3	18.40±0.3	5.84±0.2	
	4th	15.40±0.4	10.12±0.4	18.42±0.2	9.82±0.1	
	5th	18.20±0.5	12.64±0.2	18.72±0.2	11.98±0.2	
	6th	19.24±0.5	-	19.84±0.1	18.18±0.1	
Rice	1st	19.16±0.7	19.12±0.2	19.14±0.2	19.12±0.3	Died
	2nd	19.88±0.5	20.16±0.1	19.88±0.4	19.64±0.5	
	3rd	21.52±0.4	21.66±0.1	20.5±0.3	20.46±0.6	
	4th	22.42±0.3	22.68±0.2	21.56±0.4	21.42±0.2	
	5th	23.64±0.5	23.70±0.4	23.02±0.5	22.88±0.5	
	6th	24.74±0.6	24.96±0.5	23.94±0.2	23.96±0.2	

Mean data ± S.D. of 3 experiments

The green gram plants in N1 soil, exhibited better growth with 17.21 cm after 30 days of plantation. On the other hand, plants in N2 possessed moderate growth with 10.24 cm. R1 and R2 soils exhibited poor growth with 5.70 cm height after 30 days of plantation.

The growth of bengal gram plants in N1 soil was better with 19.24 cm after 30 days of plantation. Moderate growth was exhibited by R1 soil with 12.64 cm upto 25 days of plantation and thereafter the plants wilted. Plants in N2 soil exhibited higher growth with 19.84 cm in 30 days of plantation as compared to R2 soil.

In N1 soil, rice plants exhibited better growth with 24.74 cm after 30 days of planting as compared to plants in other soils. The growth of plants in other soils was more or less similar.

4.2.2. Growth behavior of crops in bioremediated soil

Each of three crops bengal gram, green gram and rice were grown in 5 soil types till maturity. In the case of bengal gram, death of plants in both N1 and R1 was observed after 30 days of planting in contrast to the survival in the unfertilized soil (N2 and R2). In the case of green gram, plants failed to survive after 30 days of planting in all bioremediated soils. In contrast, the rice plants survived in all types of soils. All three crops failed to survive in C type soil. The result of the experiment is presented in Table 4.17.

Table 4.17. Survival of bengal gram, green gram and rice plants after transplanting

Crop	Soil type	No. of seedlings planted	No of plants survived after 30 days
Bengal gram	N1	5	-
	R1	5	-
	N2	5	5
	R2	5	5
	C	5	-
Green gram	N1	5	5
	R1	5	-
	N2	5	5
	R2	5	-
	C	5	-
Rice	N1	5	5
	R1	5	5
	N2	5	5
	R2	5	5
	C	5	-

4.2.3. Performance of crops in bioremediated soil

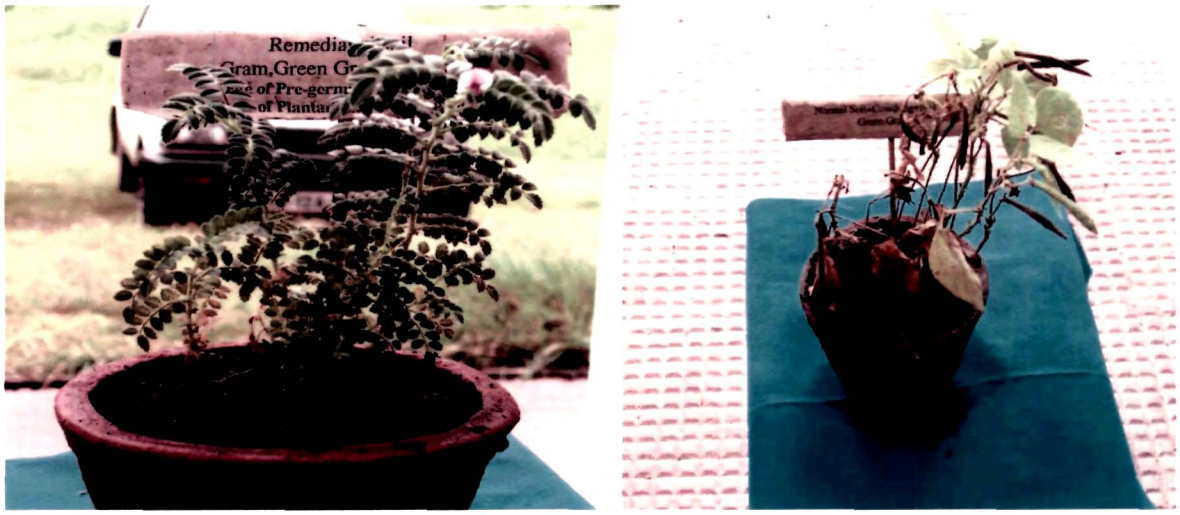
In the case of bengal gram, the plants in R2 soil produced 6.1 tillers as compared to N2 soil. However, the height of the plants was less in the R2 soil with 18.2 cm as compared to N2 soil with 19.24 cm. Higher number of seeds with 11.6 and more weight of seeds with 1.91 g per 100 seeds was observed in the plants grown in R2 soil.

The green gram plants failed to grow in the R1, R2 and C type pots. The plants on an average possessed 7.5 branches per plant in N2 soil and 6.2 in N1 soil. The plants grown in N1 soil were taller (17.28 cm) than those

grown in N2 soil (15.25 cm). The average number of seeds produced per plants in N2 soil (52.2±0.4) was higher than in N1 soil (45.1±0.2). The average weight of seeds produced by a plant grown in N2 soil was 2.81g whereas as in N1 soil it was 1.65 g.

In R1 soil, the rice plants produced 2.4 tillers per plant as against 1.2 tillers per plant in R2 soil. Number of tillers per plant in N1 soil was 1.8 whereas in N2 soil 1.4 tillers per plant.

(a) (b) (c)



(d) (e)

Fig.4.7. Cultivation of rice, bengal gram and green gram in bioremediated soil (a) rice in N1 soil, (b) rice in R2 soil, (c) rice in N2 soil, (d) bengal gram in R2 Soil and (e) Green gram plants in N2 Soil

Table 4.18. Growth performance of crop plants in bioremediated soil

(Mean ± S.D. of 3 experiment)

Crop	Plants in	Tillers / plant	Plant height (cm)		Seeds / Plant	Weight of seeds/plants (g)
			Flowering	Maturity		
Gram	N1		-	-	-	-
	R1		-	-	-	-
	N2	5.3	17.5	19.24	8.2±0.1	1.06±0.1
	R2	6.1	16.2	18.2	11.6±0.2	1.91±0.3
	C1		-	-	-	-
Green Gram	N1	6.2	16.4	17.28	52.2±0.4	2.81±0.1
	R1	-	-	-	-	-
	N2	7.5	13.5	15.25	45.1±0.2	1.65±0.3
	R2	-	-	-	-	-
	C1	-			-	-
Rice	N1	1.8	30.4	36.6	31.6±0.1	0.52±0.1
	R1	2.4	30.2	38.4	38.6±0.3	0.62±0.2
	N2	1.4	28.1	36.6	29.0±0.2	0.40±0.1
	R2	1.2	31.3	46.2	41.0±0.2	0.78±0.1
	C1	-	-	-	-	-

Rice plants in R2 soil exhibited taller plant height of 46.2 cm as against 38.4, 36.6 and 36.6 cm in R1, N1 and N2 soils, respectively. Rice plants in R2 followed by R1 produced 41.0±0.2 and 38.6±0.3 seeds per plant respectively. Plants in N1 soil possessed 31.6±0.1 seeds per plant, whereas, 29.0±0.2seeds per plant in N2 soil. The weight of seeds produced by a plant in R2 was 0.78±0.1g as compared to 0.62±0.2g in R1 soil, whereas, each plant grown in N1 and N2 soil produced 0.52±0.1 and 0.40±0.1g seeds, respectively. The average seed weight per plant was found to be the highest in the case of plants grown in R2 soil.

4.2.4. Properties of soil

Bioremediated and normal soil was subjected to analysis for pH and NPK content prior to the experiment. Data obtained are presented in Table 4.19.

Table 4.19. Chemical analysis of soil samples

Sample No	pH	N ($Kg.ha^{-1}$)	P_2O_5 ($Kg.ha^{-1}$)	K_2O ($Kg.ha^{-1}$)
N2	5.2	242.31	25.00	326.37
R2	5.3	289.65	22.37	461.26

In the case of bioremediated soil N and K level was found to be higher than normal soil. Phosphorous level was found to be higher in normal soil as compared to the bioremediated soil.

4.3. Biosurfactant isolation

4.3.1. Screening of biosurfactant producing bacteria

A screening technique was developed to find out indigenous bacterial strains that readily adapt to moderate-climatic conditions and can efficiently produce biosurfactant. From different oil well- soil samples, 13 hydrocarbon degrading bacterial isolates having the ability to produce biosurfactant, *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC 8163), *A. faecalis* (MTCC 8164), *P. aeruginosa* (MTCC 8165), *P. fluorescens* (L490-II), *P. aeruginosa* (L43-I), *B. licheniformis* (MTCC8166), *B. circulans* (MTCC8167), *B. subtilis* (R38-I), *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC7816) were isolated and pure cultured.

Initially, bacterial cell - free culture broth was assessed for its ability to reduce the surface tension of the medium having 1% (v/v) hexadecane. In the second step, a rapid drop-collapsing test of the culture medium was performed and also formation of foam during enrichment culture recorded. The comparison with the surface tension in the control medium without bacterial inoculation, the rapid drop collapsing test and formation of foam in the culture medium ensured the potential biosurfactant producing isolates. The yield of biomass and biosurfactant of individual bacterial isolates and

surface tension of the hexadecane-supplemented culture medium are presented in Table 4.21.

Table 4.20. Yield of bacterial biomass and biosurfactant of bacterial isolates and surface tension of hexadecane (1%) culture medium supplemented with MSM after 96 h of culture (mean ±S.D of three experiments)

Bacteria Isolates	Mineral salt medium+ 1% hexadecane in 96h		
	D.B* ($g.l^{-1}$)	B.Y**($g.l^{-1}$)	S.T***($mN.m^{-1}$)
P. aeruginosa (MTCC7815)	6.4±1.2	4.6±0.7	29.1±1.0
P. aeruginosa (MTCC7812)	8.6±0.4	4.2±0.8	31.7±2.0
Microbacterium (G35-II)	5.3±0.4	0.8±0.2	37.1±0.4
P. aeruginosa (MTCC8163)	6.2±0.5	2.1±0.3	30.4±1.0
P. aeruginosa (MTCC8165)	6.4±0.6	2.9±0.4	30.4±1.0
P. fluorescens (L490-II)	5.3±0.4	1.8±0.7	35.1±0.5
P. aeruginosa (L43-I)	6.1±0.3	2.0±0.8	34.2±0.2
B. licheniformis (MTCC8166)	4.2±0.3	0.5±0.6	37.4±0.2
B. circulans (MTCC8167)	8.7±0.2	0.3±0.1	37.2±0.2
B. subtilis (R38-I)	8.4±0.1	0.3±0.7	39.6±0.3
P. aeruginosa (MTCC7814)	6.4±1.6	3.5±0.9	31.1±1.0
P. aeruginosa (MTCC7816)	5.6±1.3	2.0±1.0	33.5±0.3
control	0.1±0.1	0.0	68.0±1.0

D.B* = Dry biomass, B.Y**= Biosurfactant yield and S.T***= Surface tension

The bacterial isolate *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC 8165) and *P. aeruginosa* (MTCC8163) exhibited the maximum production of biosurfactant with 4.6, 4.2, 3.5, 2.9 and 2.1, $g.l^{-1}$, respectively at 96 h of incubation. The culture medium inoculated individually with the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8163), *P. aeruginosa* (MTCC8165), *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC7812) exhibited the maximum reduction in surface tension to 29.1±1.0, 30.4±1.0, 30.4±1.0, 31.1±1.0 and 31.7±2.0 $mN.m^{-1}$, respectively as compared to control to 68.0 $mN.m^{-1}$ at 96 h of incubation. The bacterial isolates *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC 8165) and *P. aeruginosa*

(MTCC 8163) were found to produce higher dry biomass yield with 8.6, 6.4, 6.4, 6.4 and 6.2 $g.l^{-1}$, respectively.

4.3.2. Efficacy and production of biosurfactant

Biosurfactants produced by five bacterial isolates were assessed for their ability to lower the surface tension of culture medium supplemented with different carbon sources: glucose, glycerol, hexadecane, octadecane, benzene, toluene, pyrene and flourene. Surface tension of culture medium was determined by drawing samples up to 96 h of culture with an interval of 24h. Data thus obtained are presented in Table 4.21.

Table 4.21. Reduction in surface tension of cell-free culture medium supplemented with different carbon sources over a period of 96 h

Cell free culture media of bacterial isolates	Surface tension (mN/m) of the culture media supplemented with				
Carbon source: Glucose					
Time intervals	0 h	24 h	48 h	72 h	96 h
Control	71.2±0.7	71.1±2.3	71.1±1.8	70.9±0.5	71.0±0.3
P. aeruginosa (MTCC 8165)	70.0±1.3	50.9±1.5[a]	32.4±0.6[a]	34.4±0.7[a]	36.9±0.8[a]
P. aeruginosa (MTCC7815)	70.6±1.6	31.7±0.7[a]	31.5±0.5[a]	31.1±1.7[a]	31.9±0.8[a]
P. aeruginosa (MTCC7812)	70.8±1.2	39.5±0.8[a]	35.7±0.6[a]	36.3±0.5[a]	39.9±0.4[a]
P. aeruginosa (MTCC7814)	70.7±2.8	37.3±0.5[a]	33.6±0.9[a]	34.7±0.6[a]	33.9±1.2[a]
P. aeruginosa (MTCC 8163)	71.1±0.8	48.5±0.6[a]	36.4±1.5[a]	34.4±0.3[a]	33.3±0.5[a]
Carbon source: Glycerol					
P. aeruginosa (MTCC 8165)	70.9±0.4	69.3±1.3[b]	44.1±0.7[a]	31.8±0.9[a]	30.1±1.1[a]
P. aeruginosa (MTCC7815)	70.2±0.5	68.5±0.4[a]	32.5±0.5[a]	31.8±0.5[a]	29.7±1.3[a]
P. aeruginosa (MTCC7812)	70.2±0.7	69.8±0.2[a]	59.0±1.0[a]	59.1±0.4[a]	39.4±0.6[a]

P. aeruginosa (MTCC7814)	68.0±0.9	48.6±0.6[a]	44.1±0.7[a]	34.6±0.7[a]	39.6±0.6[a]
P. aeruginosa (MTCC 8163)	70.1±0.8	69.3±1.3[b]	44.1±0.7[a]	37.8±0.9[a]	34.6±1.0[a]
Carbon source: Hexadecane					
P. aeruginosa (MTCC 8165)	67.6±0.8	35.5±0.7[a]	32.2±0.2[a]	31.5±0.3[a]	30.4±0.6[a]
P. aeruginosa (MTCC7815)	67.0±0.8	34.4±0.3[a]	33.3±0.5[a]	32.6±0.4[a]	29.1±0.6[a]
P. aeruginosa (MTCC7812)	68.5±0.4	34.2±0.4[a]	33.4±0.6[a]	31.7±0.6[a]	38.4±1.2[a]
P. aeruginosa (MTCC7814)	68.4±0.9	64.2±0.6[a]	31.4±0.9[a]	34.1±0.7[a]	31.1±1.3[a]
P. aeruginosa (MTCC 8163)	68.1±0.4	45.2±0.5[a]	37.2±0.7[a]	34.5±0.6[a]	31.5±0.5[a]
Carbon source: Octadecane					
P. aeruginosa (MTCC 8165)	68.3±0.5	35.5±0.5[a]	34.2±0.3[a]	33.5±0.7[a]	31.4±0.3[a]
P. aeruginosa (MTCC7815)	68.4±0.3	35.8±0.5[a]	34.7±0.7[a]	32.6±0.3[a]	31.2±0.8[a]
P. aeruginosa (MTCC7812)	68.1±0.6	36.4±0.3[a]	35.6±0.3[a]	34.5±0.6[a]	33.7±0.5[a]
P. aeruginosa (MTCC7814)	67.9±0.7	42.6±0.5[a]	40.1±0.6[a]	33.6±0.7[a]	31.6±0.9[a]
P. aeruginosa (MTCC 8163)	68.2±0.8	43.2±0.4[a]	36.2±0.5[a]	33.5±0.6[a]	31.7±0.3[a]
Carbon source: Bezene					
P. aeruginosa (MTCC 8165)	68.5±0.5	66.7±0.4[a]	60.8±0.5[a]	58.3±0.6[a]	56.5±0.7[a]
P. aeruginosa (MTCC7815)	68.7±0.6	63.2±0.6[a]	58.4±0.3[a]	55.2±0.7[a]	48.9±0.4[a]
P. aeruginosa (MTCC7812)	68.4±0.7	65.5±0.6[a]	63.5±0.4[a]	58.6±0.5[a]	56.5±0.9[a]
P. aeruginosa (MTCC7814)	68.3±0.6	61.2±0.6[a]	58.7±0.4[a]	50.6±0.7[a]	41.3±0.8[a]
P. aeruginosa (MTCC 8163)	68.5±0.5	64.7±0.6[a]	59.4±0.8[a]	55.3±0.4[a]	51.1±0.5[a]
Carbon source: Toluene					
P. aeruginosa (MTCC 8165)	70.0±1.3	68.5±0.6[a]	63.8±0.3[a]	58.3±0.8[a]	57.3±0.2[a]
P. aeruginosa (MTCC7815)	70.6±1.6	67.2±1.2[a]	59.4±0.7[a]	54.5±0.4[a]	49.5±0.4[a]
P. aeruginosa (MTCC7812)	70.8±1.2	69.7±0.7[a]	63.2±0.6[a]	58.7±0.7[a]	55.5±0.3[a]
P. aeruginosa (MTCC7814)	70.7±2.8	63.2±0.4[a]	57.2±0.5[a]	52.6±0.8[a]	45.6±0.6[a]
P. aeruginosa (MTCC 8163)	71.1±0.8	66.4±0.6[a]	59.7±0.3[a]	53.6±0.7[a]	50.8±1.3[a]

Carbon source: Pyrene					
P. aeruginosa (MTCC 8165)	70.9±0.4	68.9±0.6 [a]	63.8±0.4[a]	57.3±0.6 [a]	54.8±0.7 [a]
P. aeruginosa (MTCC7815)	70.2±0.5	67.8±0.7 [a]	64.1±0.3[a]	57.9±0.2 [a]	55.1±1.4 [a]
P. aeruginosa (MTCC7812)	70.2±0.7	69.8±0.5 [a]	66.3±0.2[a]	61.2±0.4 [a]	59.6±0.8 [a]
P. aeruginosa (MTCC7814)	68.4±0.9	61.5±0.2 [a]	55.8±0.4 [a]	51.6±0.3[a]	52.1±0.6 [a]
P. aeruginosa (MTCC 8163)	70.1±0.8	69.1±1.1 [a]	64.6±0.6[a]	58.2±0.1 [a]	54.3±0.4 [a]
Carbon source: Fluorene					
P. aeruginosa (MTCC 8165)	67.6±0.8	68.2±0.5 [a]	63.5±0.2[a]	56.9±0.5 [a]	55.1±0.2 [a]
P. aeruginosa (MTCC7815)	67.0±0.8	67.5±0.2 [a]	64.3±0.4[a]	58.3±1.2 [a]	56.3±0.9 [a]
P. aeruginosa (MTCC7812)	68.5±0.4	65.5±0.3 [a]	61.7±1.2 [a]	59.8±0.1 [a]	58.7±0.7 [a]
P. aeruginosa (MTCC7814)	68.0±0.9	66.8±0.2 [a]	64.5±0.6[a]	55.4±1.3 [a]	51.8±0.5 [a]
P. aeruginosa (MTCC 8163)	68.1±0.4	65.5±0.6 [a]	59.3±0.5 [a]	52.4±0.7 [a]	53.1±0.8 [a]

Significance at probability levels: [a]$p<0.05$

In glucose-supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812) lowered the surface tension of culture medium from 70.6 to 31.1 $mN.m^{-1}$ and 70.8 to 36.3 $mN.m^{-1}$, respectively at 72 h of incubation. The isolate *P. aeruginosa* (MTCC8165) reduced surface tension of the culture medium from 70.0 to 32.4 $mN.m^{-1}$ at 48 h of incubation. Subsequently, the isolates *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC8163) reduced surface tension from 70.7 to 33.9 $mN.m^{-1}$ and 71.1 to 33.3 $mN.m^{-1}$, respectively at 96 h of incubation. In the case of glycerol - supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC 8165), *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812) and *P. aeruginosa* (MTCC8163) lowered the surface tension of culture medium to 30.1, 29.7, 39.4, and 34.6 $mN.m^{-1}$, respectively after 96 h of incubation.

In the case of aliphatic hydrocarbon hexadecane - supplemented medium, the isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC 8165), *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC 8163) exhibited higher reduction of surface tension of the culture medium to 29.1, 30.4, 31.1 and 31.5 $mN.m^{-1}$, respectively at 96 h of incubation. The isolate *P. aeruginosa* (MTCC7812) reduced the surface tension from 68.5 to 31.7 $mN.m^{-1}$ at 72 h of incubation. All bacterial isolates caused high reduction of surface tension in hexadecane – supplemented medium as compared to octadecane medium. In the aromatic and polycyclic aromatic hydrocarbon - supplemented medium, all isolates except *P. aeruginosa* (MTCC7814) exhibited poor reduction of surface tension.

4.3.3. Effect of concentrations of hexadecane on biosurfactant production

The mineral salt medium was supplemented with four concentrations of hexadecane: 0.5, 1.0, 2.0 and 3.0%. The potential five biosurfactant producing bacterial isolates were incubated in these media till late log phase (post 96 h of culture). Subsequently, bacterial dry biomass and reduction in surface tension of culture medium were estimated and data obtained are presented in Table 4. 22.

Table 4.22. Effect of different concentrations of hexadecane on biosurfactants production (Mean ± S.D of 3 experiments)

Percent of hexadecane (v/v)	Properties	*P. aeruginosa* (MTCC 7815)	*P. aeruginosa* (MTCC 7812)	*P. aeruginosa* (MTCC 8163)	*P. aeruginosa* (MTCC 8165)	*P. aeruginosa* (MTCC 7814)
0.5	Surface tension ($mN.m^{-1}$)	34.2 ± 0.6	35.1 ± 0.5	33.1± 0.2	35.1± 0.4	33.0 ± 0.5
	Dry biomass ($g.l^{-1}$)	1.5 ± 0.5	3.5 ± 0.4	1.8± 0.3	1.7± 0.5	3 .4 ± 0.6
1.0	Surface tension ($mN.m^{-1}$)	29.1 ± 0.6	31.5 ± 0.6	31.5± 0.5	30.2±0.5	31.4 ± 0.5
	Dry biomass ($g.l^{-1}$)	6.4 ± 0.2	7.5 ± 1.5	6.1± 0.5	6.3± 0.6	6.2 ± 0.3
2.0	Surface tension ($mN.m^{-1}$)	32.5 ± 0.4	39.2 ± 0.4	35.3± 0.6	34.4± 0.4	36.3 ± 0.5
	Dry biomass ($g.l^{-1}$)	1.8 ± 0.2	3.8 ± 0.6	2.1± 0.4	2.5± 0.5	1.7 ± 0.3
3.0	Surface tension ($mN.m^{-1}$)	39.6 ± 0.5	42.1 ± 0.8	38.5± 0.2	39.5± 0.7	38.8 ± 0.6
	Dry biomass ($g.l^{-1}$)	1.9 ± 0.5	2.1 ± 0.5	1.6± 0.7	1.3± 0.6	1.8 ± 0.6

The surface tension of the control medium was determined to be 60.5 $mN.m^{-1}$. Surfactants produced by bacterial isolates *P. aeruginosa* (MTCC 7815), *P. aeruginosa* (MTCC8165), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC7812) reduced the surface tension of culture medium to 29.1± 0.6, 30.2± 0.5, 31.4± 0.5, 31.5±0.6 and 31.5±0.5 $mN.m^{-1}$ with the dry biomass yield of 6.4 ± 0.2, 6.3± 0.6, 6.2 ± 0.3, 6.1± 0.5 and 7.5±1.5 $g.l^{-1}$, respectively in 1% hexadecane supplemented medium as compared to other concentrations.

4.3.4. Effect of agitation on biosurfactant production.

Agitation of culture medium in Erlenmeyer flasks was found to have a major role in the growth and biosurfactant production. Agitation helps in the effective mixing of mineral salts of the medium. It also helps inoculum to mix with the media composition, besides also supplying oxygen. The stationary

culture, negative control (0 rpm) possessed poor cell growth and surface activity. The shaking with 200rpm was found to be the optimum for all bacterial isolates in this investigation (Table 4.23).

Table 4.23. Effect of agitation on biosurfactant production (Mean ± S.D of 3 experiments)

Agitation	Properties	Bacterial isolates				
		P. aeruginosa (MTCC7815)	*P. aeruginosa* (MTCC7812)	*P. aeruginosa* (MTCC 8163)	*P. aeruginosa* (MTCC 8165)	*P. aeruginosa* (MTCC 7814)
0 rpm	Surface tension ($mN.m^{-1}$)	49.5 ± 0.6	50.3 ± 0.4	47.8±0.2	48.9 ± 0.5	48.5 ± 0.5
	CMD^{-1} ($mN.m^{-1}$)	58.8 ± 0.8	61.5 ±0.3	62.1±0.4	57.0 ± 0.5	58.0 ± 0.5
100 rpm	Surface tension ($mN.m^{-1}$)	38.4 ± 0.6	34.0 ± 0.3	36.1±0.1	35.0 ± 0.5	32.5 ± 0.5
	CMD^{-1} ($mN.m^{-1}$)	46.5 ± 0.5	39.5 ±0.5	42.8±0.6	40.3 ± 0.6	37.5 ± 0.6
200 rpm	Surface tension ($mN.m^{-1}$)	29.1 ± 0.2	31.05 ±0.5	31.5± 0.4	30.2± 0.2	31.8 ± 0.3
	CMD^{-1} ($mN.m^{-1}$)	34.0 ± 0.8	33.5 ±0.3	35.2±0.5	38.5 ± 0.6	31.0 ± 0.5
300 rpm	Surface tension ($mN.m^{-1}$)	33.2 ± 0.4	33.3 ± 0.6	38.1±0.3	32.5 ± 0.5	29.3 ± 0.3
	CMD^{-1} ($mN.m^{-1}$)	36.8 ± 0.6	36.5 ±0.3	42.2±0.2	40.0 ± 0.8	34.0 ± 0.5

4.3.5. Influence of pH on the efficacy of biosurfactants

The pH of biosurfactant - containing culture medium in normal concentration, CMD^{-1} (10 times dilution) and CMD^{-2} (100 times dilution) was altered from 2 to 11 and the surface tension values of the medium measured. Data thus obtained are presented in Table 4.24.

Table 4.24. Effect of pH on biosurfactants produced by the bacterial isolate in 2% glycerol - supplemented medium and critical micelle dilutions (CMD^{-1} and CMD^{-2} $g.l^{-1}$) cultured for 96 h (Mean ±S.D of 3 experiments)

Bacterial isolates	pH of medium	Surface tension values ($mN.m^{-1}$)		
		Medium (normal concentration)	CMD^{-1}	CMD^{-2}
P. aeruginosa (MTCC 8165)	2	29.1±0.14	29.5±0.78	29.6±0.61
	4	29.4±0.08	29.6±0.66	45.7±0.61
	6.5	30.1±0.66	37.5±0.72	56.7±0.61
	7.5	29.8±0.63	29.9±1.0	46.5±0.90
	11	33.4±0.37	39.4±0.64	64.3±0.78
P. aeruginosa (MTCC 7815)	2	28.7±0.28	28.9±0.56	57.1±0.32
	4	29.5±0.04	34.1±0.35	58.8±0.64
	6.5	29.7±0.88	35.8±0.64	57.6±0.50
	7.5	31.9±0.43	36.2±0.35	58.1±0.53
	11	33.2±0.37	37.8±0.24	48.1±0.49
P. aeruginosa (MTCC 7812)	2	31.8±0.42	40.8±0.62	67.0±0.82
	4	35.1±0.21	50.5±0.71	68.1±0.29
	6.5	39.4±0.50	43.6±0.69	61.2±0.47
	7.5	34.4±0.40	48.1±0.46	62.6±1.43
	11	36.5±1.0	50.7±0.56	68.1±0.21
P. aeruginosa (MTCC 7814)	2	30.9±0.45	37.8±0.96	65.1±0.42
	4	31.5±0.42	36.5±0.78	59.9±0.29
	6.5	31.1±0.45	36.5±0.69	52.1±0.21
	7.5	33.4±0.42	38.4±0.64	59.8±0.25
	11	33.4±0.45	38.5±0.58	68.9±1.23
P. aeruginosa (MTCC 8163)	2	31.5±0.5	35.5±0.85	49.9±0.49
	4	31.9±0.6	37.6±0.86	56.1±0.52
	6.5	32.2±0.4	35.9±0.68	58.5±0.56
	7.5	32.7±0.4	36.7±0.75	58.9±0.39
	11	33.6±0.7	39.5±0.82	65.9±1.20

The biosurfactant of each bacterial isolate caused reduction in surface tension in between 29 – 33 $mN.m^{-1}$ in the normal concentration at all pH levels. At low concentrations CMD^{-1} and CMD^{-2}, the high pH levels showed low efficiency of biosurfactants. At ten times dilution, CMD^{-1}, the biosurfactants of the bacterial isolates *P. aeruginosa* (MTCC 7815) and *P. aeruginosa* (MTCC 8165) possessed almost the same high efficiency as that of the normal concentration at pH 2.0 and 4.

4.3.6. Influence of high temperature on the efficacy of biosurfactant

The biosurfactant of each bacterial isolate was isolated from the culture medium and then dried. The biosurfactant was then exposed to 100°C for 60 min and then its activity to reduce the surface tension of the culture medium was measured. Data thus obtained are presented in Table 4. 25.

Table 4.25. Effect of high temperature (100°C) on biosurfactants produced by the bacterial isolates in 2% glycerol - supplemented medium and at critical micelle dilutions (CMD^{-1} and CMD^{-2} $g.l^{-1}$) cultured for 96h (Mean ±S.D of 3 experiments)

Bacterial isolates	100°C ature for (min)	Surface tension (mN/m) in		
		Culture media	CMD^{-1}	CMD^{-2}
P. aeruginosa (MTCC8165)	5	29.1±0.4	29.5±1.0	46.3±0.6
	10	31.1±0.3	35.0±0.4	50.2±0.6
	20	31.2±0.2	35.0±0.6	51.8±0.8
	30	31.2±0.3	35.8±0.2	63.7±0.2
	60	31.9±1.0	34.6±0.3	59.8±0.2
P. aeruginosa (MTCC7815)	5	31.7±0.2	36.5±0.4	48.0±0.3
	10	31.8±0.2	37.0±0.3	48.2±0.3
	20	31.5±0.4	37.0±0.4	48.1±0.4
	30	32.7±0.6	34.2±0.2	49.5±0.8
	60	32.4±0.3	34.1±0.3	48.5±1.2
P. aeruginosa (MTCC7812)	5	34.4±0.2	41.5±0.4	67.1±0.2
	10	33.3±0.4	41.5±0.3	66.8±0.2
	20	32.1±0.2	40.1±0.3	56.2±0.4
	30	34.3±0.5	41.6±0.5	58.2±0.2
	60	34.3±0.7	41.6±0.3	58.4±0.5
P. aeruginosa (MTCC7814)	5	33.1±0.2	37.9±0.3	61.5±0.4
	10	33.4±0.2	37.8±0.2	59.9±0.4
	20	32.7±0.1	36.5±0.4	58.9±0.9
	30	33.5±0.6	40.6±0.5	60.1±0.2
	60	33.2±0.9	40.9±0.4	59.1±0.1
P. aeruginosa (MTCC8163)	5	31.8±0.7	37.5±0.4	48.7±0.2
	10	31.9±0.6	38.1±0.5	48.9±0.3
	20	32.1±0.2	38.8±0.4	49.1±0.5
	30	32.5±0.5	39.1±0.6	56.2±0.7
	60	32.8±0.3	39.3±0.8	65.9±0.5

Table 4.26. Thermostability of isolated biosurfactants from the bacterial isolates (Mean ±S.D of three experiments)

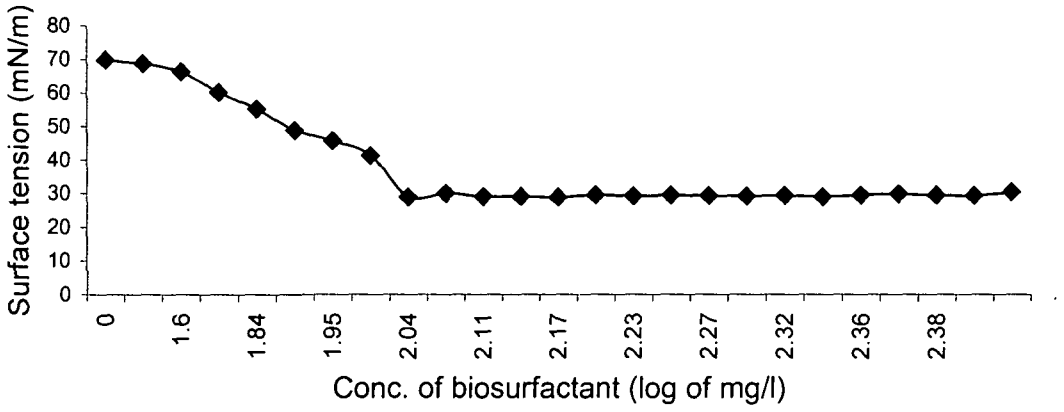
Bacterial isolates	Surface tension of tant		% activity after
	Before mN/m)	Post heating	
P. aeruginosa (MTCC7815)	29.1±1.0	32.4±0.3	88.6
P. aeruginosa (MTCC7812)	31.7±0.6	34.3±0.7	91.7
P. aeruginosa (MTCC8163)	31.5±0.5	32.8±0.3	95.8
P. aeruginosa (MTCC8165)	30.4±0.6	31.9±1.0	95
P. aeruginosa (MTCC7814)	31.1±1.3	33.2±0.9	93.2

The biosurfactants isolated from the bacterial isolates retained their activity even after heating the biosurfactant supplemented aqueous solution at 100°C for 60 min. Biosurfactants from the isolates *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC8165) were more heat stable as compared to the biosurfactants of *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812).

4.3.7. Critical micelle concentration (CMC) of biosurfactants and chemical surfactants

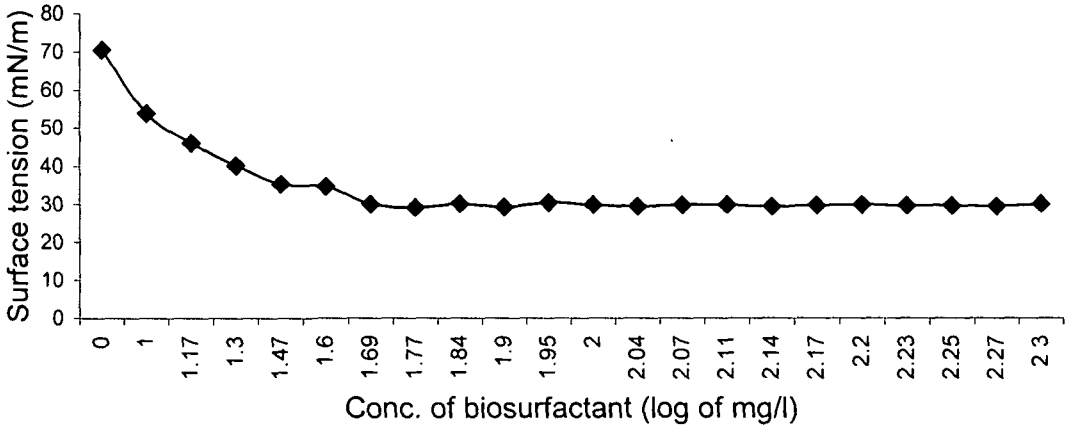
The CMC values of the biosurfactants were determined by separately measuring the surface tension of different concentrations (log of mg l^{-1}) of bacterial biosurfactants and data thus obtained are presented in Fig.4.8. (a - f).

CMC of *P.aeruginosa* (MTCC7815) biosurfactant



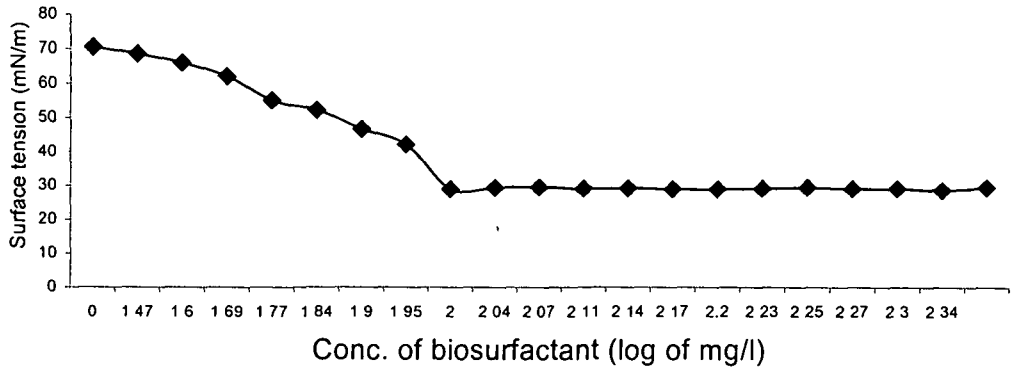
(a)

CMC of *P.aeruginosa* (MTCC7812) biosurfactant



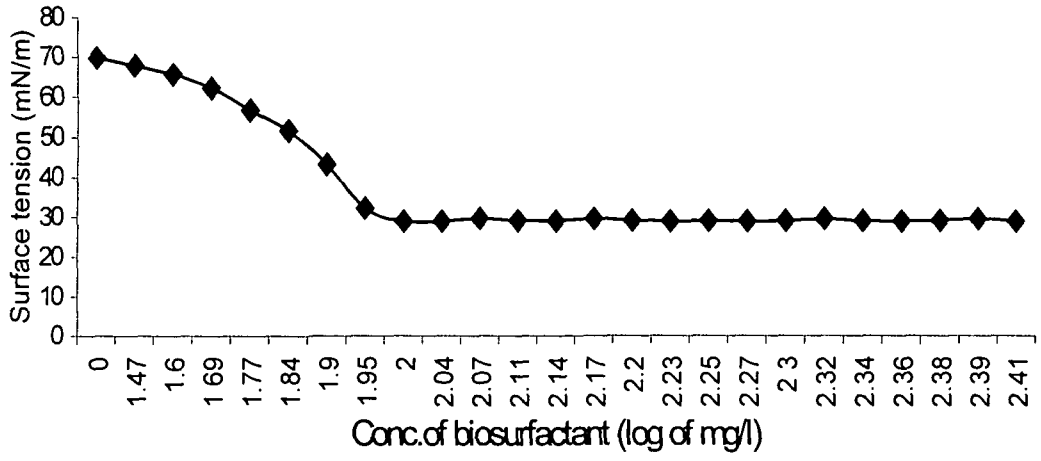
(b)

CMC of *P. aeruginosa* (MTCC8165) biosuractant



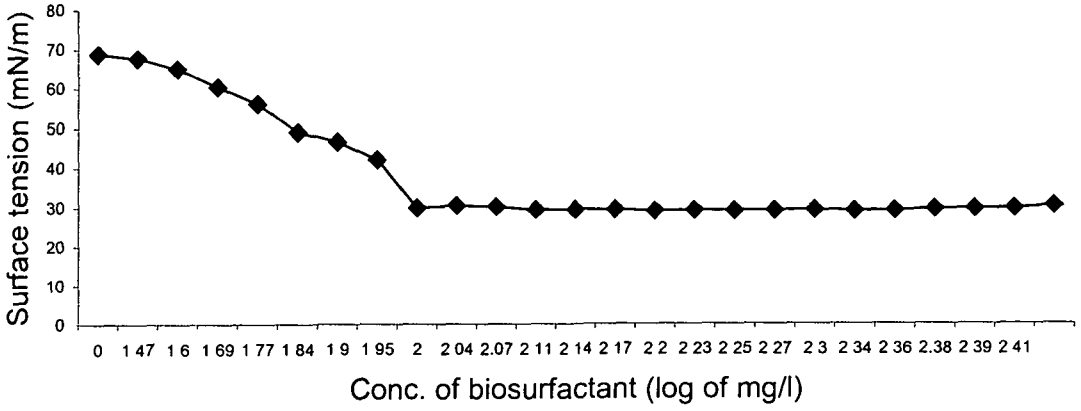
(c)

CMC of *P. aeruginosa* (MTCC7814) biosurfactant

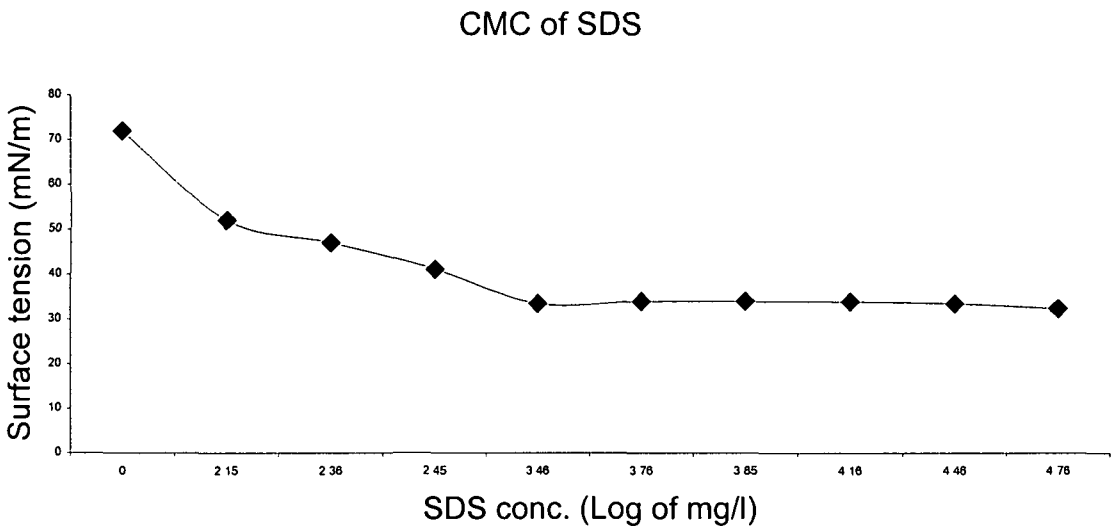


(d)

CMC of *P.aeruginosa* (MTCC8163) bisurfactant



(e)



(f)

Fig. 4.8. CMC of biosurfactant from (a) *P. aeruginosa* (MTCC7815), (b) *P. aeruginosa* (MTCC7812), (c) *P. aeruginosa* (MTCC8165), (d) *P. aeruginosa* (MTCC7814) (e) *P. aeruginosa* (MTCC8163) and (f) SDS (mean ± S.D 4 experiments)

The Mili Q distilled water was found to have the surface tension of 72mN.m^{-1} and the addition of biosurfactant reduced its surface tension to less than 35 mN.m^{-1}. The CMC of biosurfactants from the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC 8163), *P. aeruginosa* (MTCC 8165) and *P. aeruginosa* (MTCC7814) was found to be 100, 110, 110, 100 and 110 mg.l^{-1}, respectively. The CMC value of the chemical surfactant: SDS was found to be 140 mg.l^{-1}.

4.3.8. Emulsification stability

The emulsification index of the biosurfactants in kerosene was determined and data are presented in Table 4. 27. The emulsification index of the crude biosurfactant from bacterial isolates *P. aeruginosa*

(MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC 8163), P. *aeruginosa* (MTCC 8165) and *P. aeruginosa* (MTCC7814) was found to be 65.33, 60.37, 50.51, 70.34 and 63.33 percent, respectively. The emulsion formed by the crude biosufactants isolated from these bacterial isolates were found to be stable as was evident from the result of decay constant (K_d). The biosurfactant emulsion of each isolate was more stable than that of the tested chemical surfactant SDS (Sodium dodecyl sulphate). The biosurfactant from the bacterial isolate *P. aeruginosa* (MTCC 8165) was the most stable with the decay constant of –0.0095 followed by *P. aeruginosa* (MTCC7814) with the value of –0.0115. The biosurfactant from *P. aeruginosa* (MTCC 8163) with K_d value of –0.0216 was comparatively less stable as compared to the biosurfactant of other isolates. The biosurfactants from *P. aeruginosa* (MTCC 8165) and *P. aeruginosa* (MTCC7814) isolates were 8.2 and 6.8 times more stable than that of SDS, which had the K_d value -0.0788.

Table.4.27. Emulsification index and emulsion stability of biosurfactants and chemical surfactant (Mean ±S.D of 4 experiments)

Source of crude biosurfactant/ chemical surfactant	Kerosene	
	Emulsification index (%)	Decay constant (K_d)
SDS	40.67±1.3	-0.0788
P. aeruginosa (MTCC 7815)	65.33±1.2	-0.0158
P. aeruginosa (MTCC 7812)	60.37±1.1	-0.0117
P. aeruginosa (MTCC 8163)	50.51±1.3	-0.0216
P. aeruginosa (MTCC 8165)	70.34±1.4	-0.0095
P. aeruginosa (MTCC 7814)	63.33±1.2	-0.0115

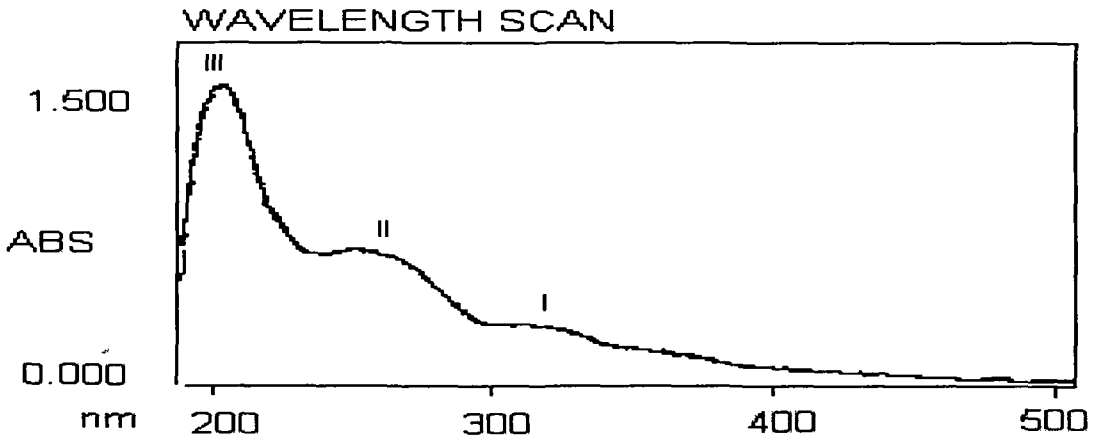
4.3.9. Properties of biosurfactant

4.3.9.1. Light absorption by biosurfactants

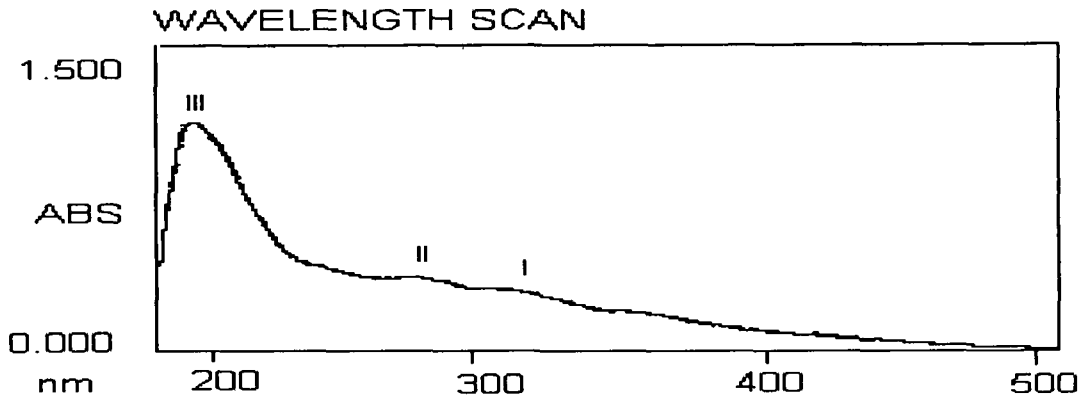
While separating biomass from the culture broth, the surface activity of different fractions of the biosurfactant was analysed. For assaying water - immiscible substrates, the recovered residual hydrocarbons were tested for surface activity, but negative results were confirmed by the tensiometer. The

clumping biomass recovered by centrifugation or from the interface of aqueous and oil layers was washed with PBS buffer and dried. The same was resuspended in distilled water. The biomass did not show significant surface tension reduction. The biomass after centrifugation and then again mixing with medium did not show surface activity. The bacterial cell and oil free aqueous phase, showed surface activity and acidification of which resulted in the precipitation of the biosurfactant.

The crude biosurfactant obtained after acid precipitation was dissolved in 0.05M Na_2HCO_3 and subjected to the wavelength scan to determine the light absorption at 190 - 510 nm to find the maximum absorption (λ_{max}). Data obtained are graphically presented in Fig.4.9. Biosurfactants isolated from *P. aeruginosa* (MTCC7815) was found to have peaks at the wave lengths of 311, 253 and 205 nm; *P. aeruginosa* (MTCC7812) at 309, 279 and 201 nm; *P. aeruginosa* (MTCC 8165) at 321, 278 and 220 nm; *P. aeruginosa* (MTCC 8163) at 313 and 214 nm and that of *P. aeruginosa* (MTCC7814) at 198 nm.



(a)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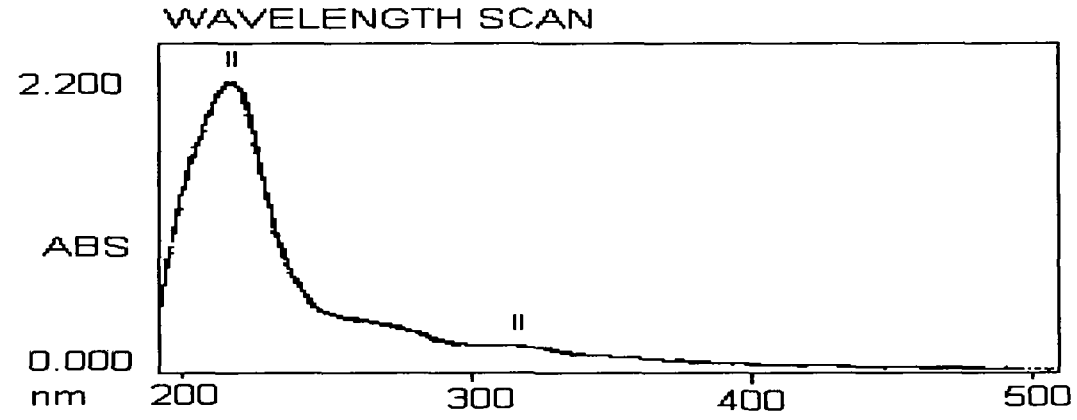
II
I

(b)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nm
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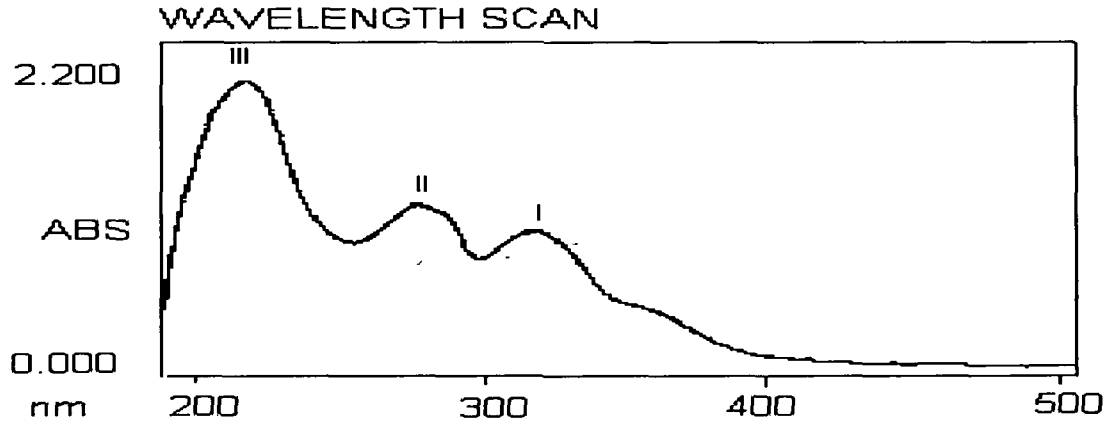
400
500
II
II

(c)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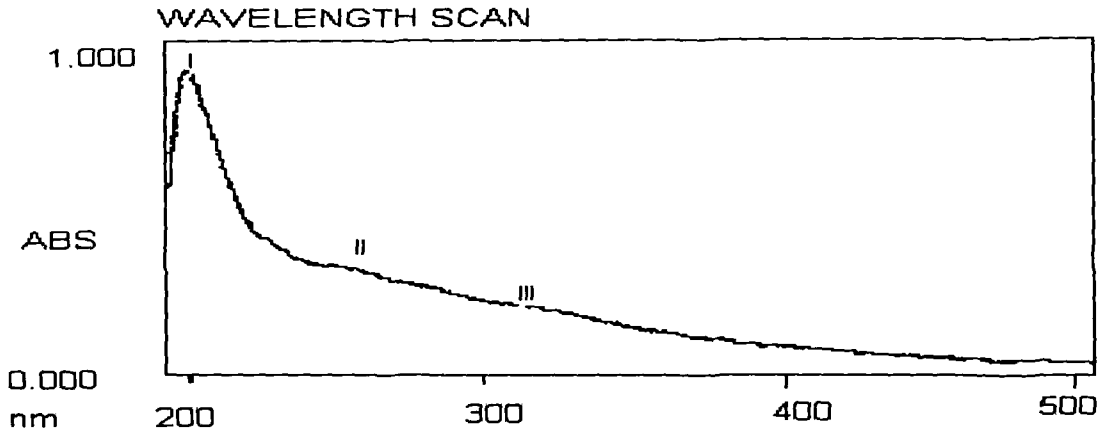
nm
200
300
400
500
III
II
I

(d)



(e)

Fig.4.9. Light absorption characteristics of crude biosurfactant from (a) *P. aeruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC7812) (c) *P. aeruginosa* (MTCC 8163) (d) *P. aeruginosa* (MTCC 8165) and (e) *P. aeruginosa* (MTCC7814)

4.3.9.2. Activity of fractionated biosurfactant by TLC

Fractionation of crude biosurfactants isolated from the bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812) by the TLC resolved each one into three spots. When a spot was tested for surface-active properties, the spot S3 of *P. aeruginosa* (MTCC7815) and S2 of *P. aeruginosa* (MTCC7812) with the R_f values 0.52 and 0.72, respectively exhibited the maximum biosurfactant activity (Table 4.28). Similarly, crude biosurfactants, obtained by acid precipitation of the cell free supernatants of *P. aeruginosa* (MTCC 8163) and *P. aeruginosa* (MTCC 8165) subjected to TLC, they were resolved into two [S1 and S2 of *P. aeruginosa* (MTCC 8163)] and three [S1, S2 and S3 of *P. aeruginosa* (MTCC 8165)] spots, respectively. When individual spots were tested for the surface-active properties, the spot S2 of *P. aeruginosa* (MTCC 8163) and S2 of *P. aeruginosa* (MTCC 8165) with the R_f values of 0.18 and 0.72 respectively

exhibited the maximum biosurfactant activity. These spots were subjected to further purification on the same TLC plate with the solvent system of chloroform: methanol: water (60:30:3 v/v).

Fractionation of crude biosurfactant from the bacterial isolate *P. aeruginosa* (MTCC7814) by the TLC resolved into three spots. When spots were tested for surface-active properties, the spot S3 with the R_f value of 0.53 exhibited the maximum biosurfactant activity.

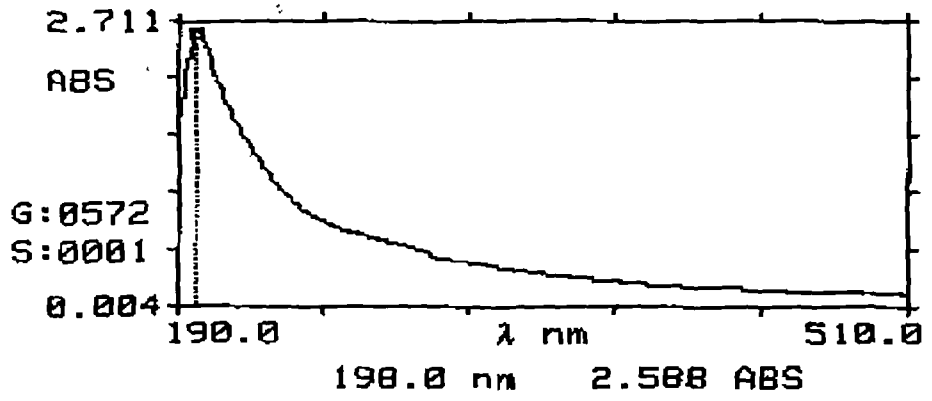
Table 4.28. Summary of isolation of biosurfactants from the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC 8163), *P. aeruginosa* (MTCC 8165) and *P. aeruginosa* (MTCC7814)

Fractions	Surface tension reduction (mN/m)	R_f values of spots
P. aeruginosa (MTCC7815)		
Cell free extract	30.2	
Crude biosurfactant	29.1	
TLC fractions (spots)		
P. aeruginosa (MTCC7815) S1	55.3	0.65
P. aeruginosa (MTCC7815) S2	35.8	0.25
P. aeruginosa (MTCC7815) S3	29.0	0.52
P. aeruginosa (MTCC7812)		
Cell free extract	32.1	
Crude biosurfactant	31.7	
TLC fractions (spots)		
P. aeruginosa (MTCC7812) S1	51.2	0.58
P. aeruginosa (MTCC7812) S2	31.5	0.72
P. aeruginosa (MTCC7812) S3	42.5	0.18
P. aeruginosa (MTCC 8163)		
Cell free extract		
Crude biosurfactant	31.1	
TLC fractions (spots)	30.4	
P. aeruginosa (MTCC 8163) S1	59.0	0.62
P. aeruginosa (MTCC 8163) S2	30.4	0.18

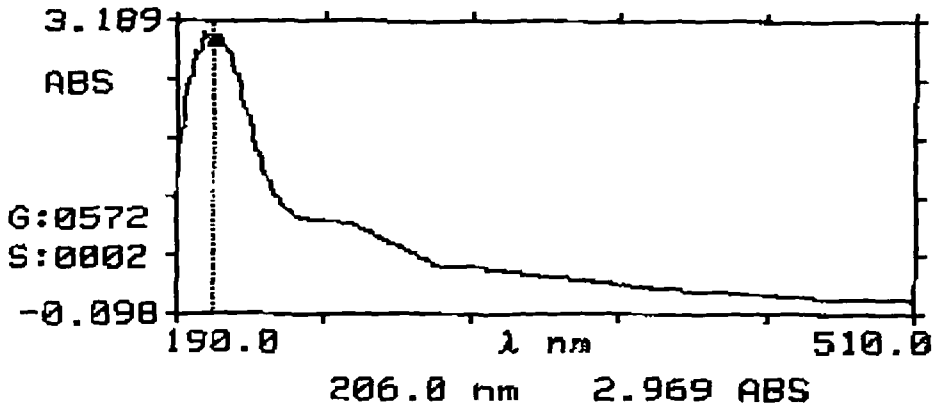
P. aeruginosa (MTCC 8165)		
Cell free extract	29.5	
Crude biosurfactant	30.4	
TLC fractions (spots)		
P. aeruginosa (MTCC 8165) S1	38.5	0.45
P. aeruginosa (MTCC 8165) S2	30.3	0.72
P. aeruginosa (MTCC 8165) S3	52.3	0.24
P. aeruginosa (MTCC7814)		
Cell free extract	32.2	
Crude biosurfactant	31.1	
TLC fractions (spots)		
P. aeruginosa (MTCC7814) S1	54.5	0.32
P. aeruginosa (MTCC7814) S2	38.5	0.25
P. aeruginosa (MTCC7814) S3	31.1	0.53

4.3.9.3. Light absorption by the active fractions from the TLC

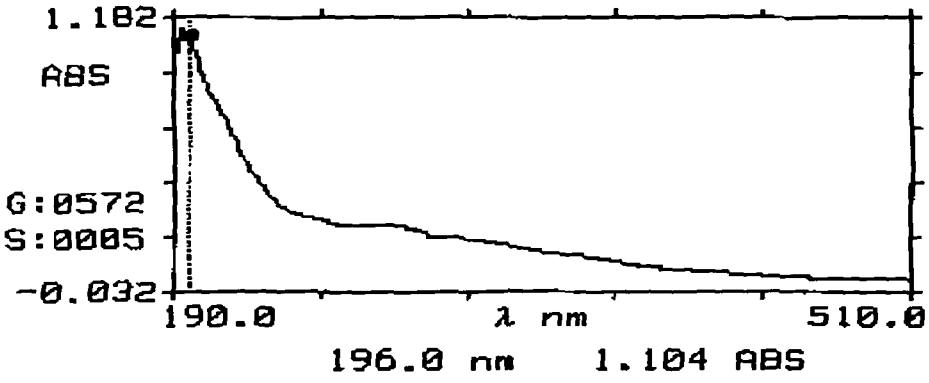
Surface-active fractions of each biosurfactant from the bacterial isolates were obtained from the TCL plates. The fractions were assessed for light absorption (λ_{max}) by wavelength scan. Data thus obtained are presented in Fig. 4.10. The active fraction was again subjected to the wavelength scan to know the purity and λ_{max}. The surface-active property of the spot S3 of *P. aeruginosa* (MTCC7815) and S3 of *P. aeruginosa* (MTCC7815) was observed at 195 and196 nm respectively. In the case of *P. aeruginosa* (MTCC 8163) and *P. aeruginosa* (MTCC 8165), the λ_{max} was observed to be at 206 and 198 nm, respectively. However, the surface-active fractions obtained from the bacterial isolate *P. aeruginosa* (MTCC7814) was observed at 197 nm. The individual peak from each of the active fractions indicated that the TLC fractions might contain single compound.


2.711
ABS
G:0572
S:0001
0.004
190.0
λ nm
510.0
198.0 nm 2.588 ABS

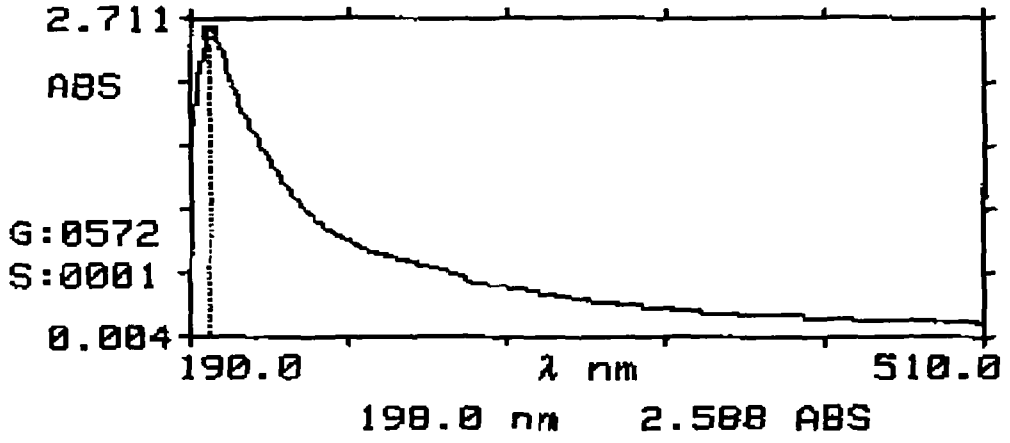
(a)


3.189
ABS
G:0572
S:0002
-0.098
190.0
λ nm
510.0
206.0 nm 2.969 ABS

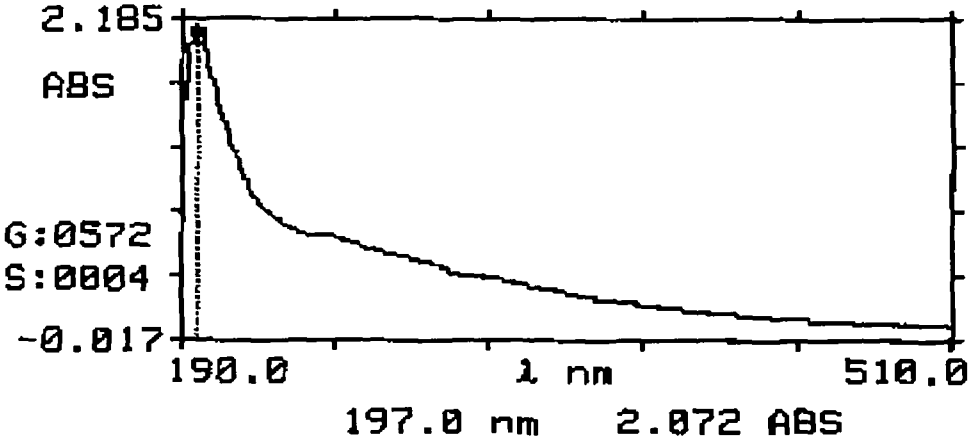
(b)


1.182
ABS
G:0572
S:0005
-0.032
190.0
λ nm
510.0
196.0 nm 1.104 ABS

(c)



(d)



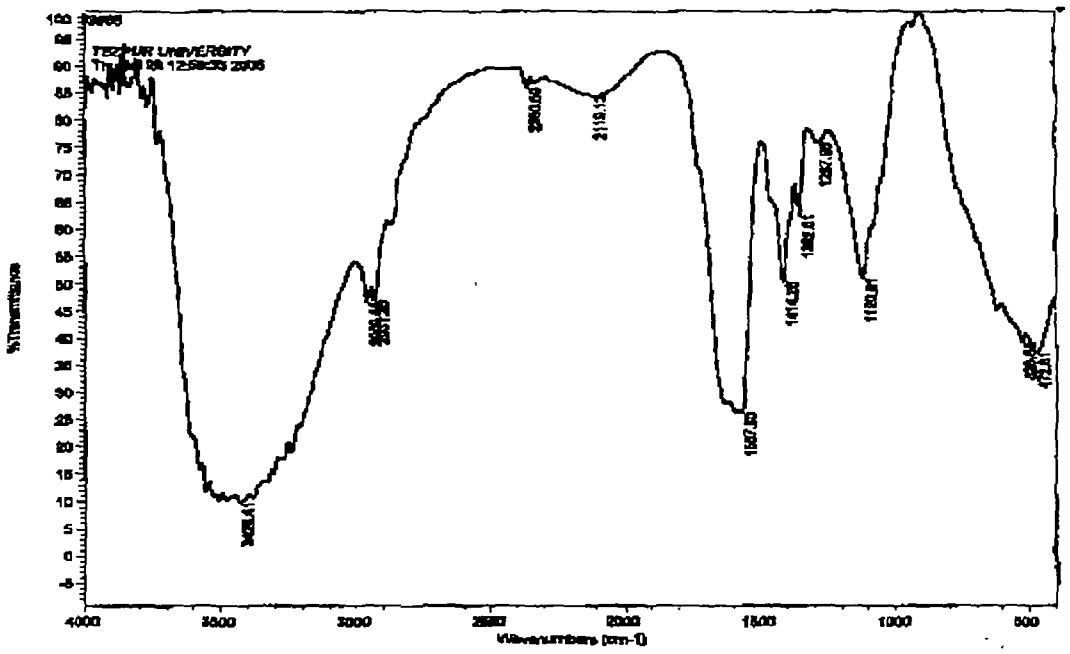
(e)

Fig.4.10. Wavelength scans of TLC fractionated - purified biosurfactant from (a) *P. aeruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC7812) (c) *P. aeruginosa* (MTCC 8163) (d) *P. aeruginosa* (MTCC 8165) and (e) *P. aeruginosa* (MTCC7814)

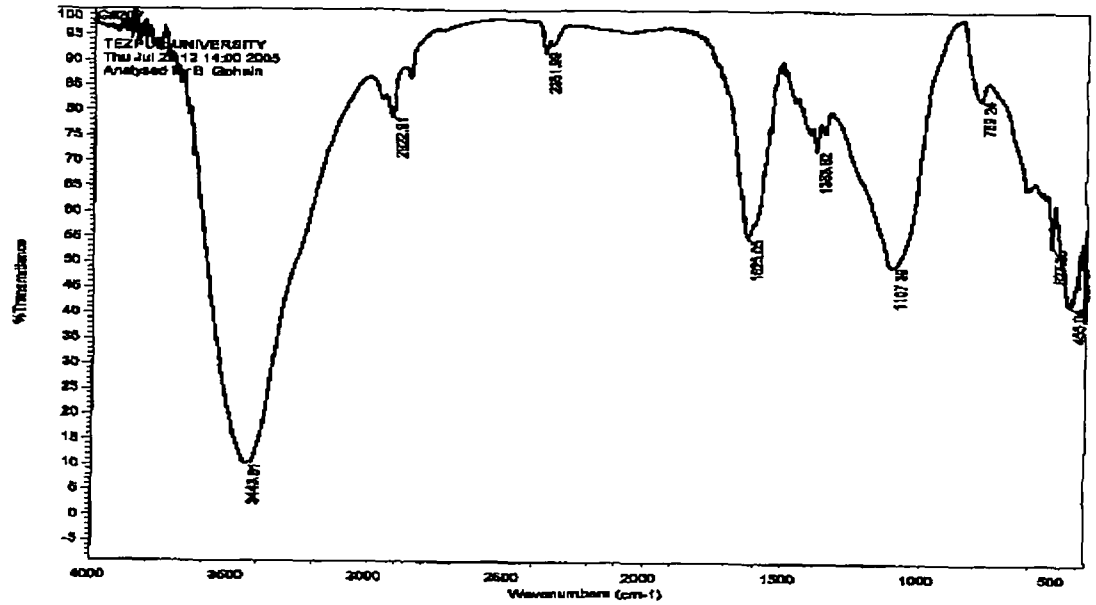
4.3.9.4. FT- IR spectra of the biosurfactant

The IR spectra of the biosurfactant from each of the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa*

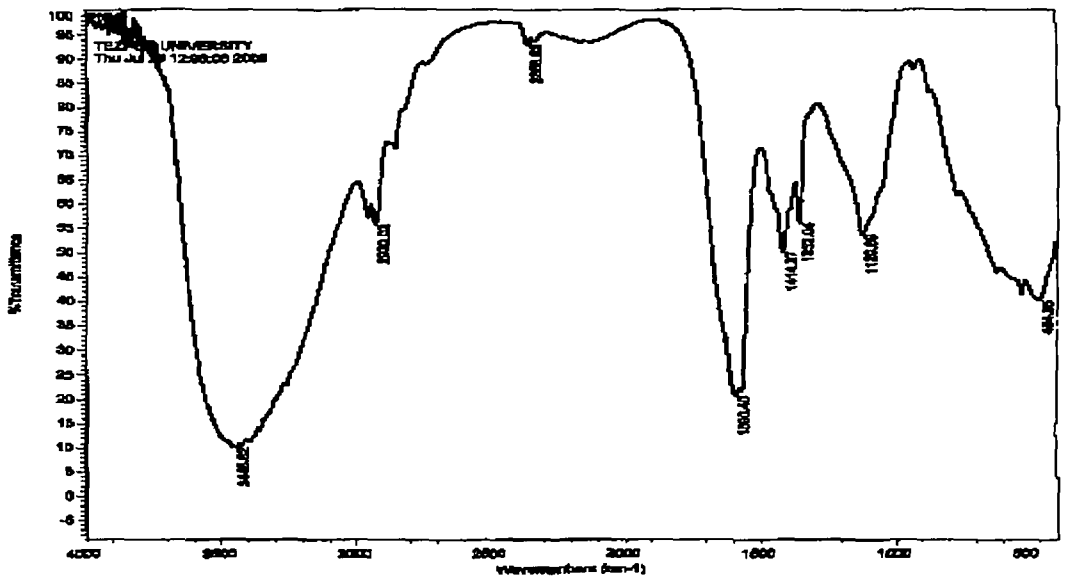
(MTCC 8163), *P. aeruginosa* (MTCC 8165) and *P. aeruginosa* (MTCC7814) in nujol are presented in Fig. 4.11 (a - e). The spectra showed strong absorption bands at 1627 and 1625 cm^{-1} as a result of the stretching mode of CO-N at 1567-1591 cm^{-1} for the deformation mode of the NH bond combined with C-N stretching mode. The presence of an aliphatic chain was indicated by the C-H stretching mode at 2922-2957cm^{-1} and 1351 -1408 cm^{-1}. The C=O stretching band between 1736 - 1567 cm^{-1} was due to a lactone carbonyl absorption. These results indicated the presence of aliphatic and carbonyl moieties in the isolated biosurfactants.



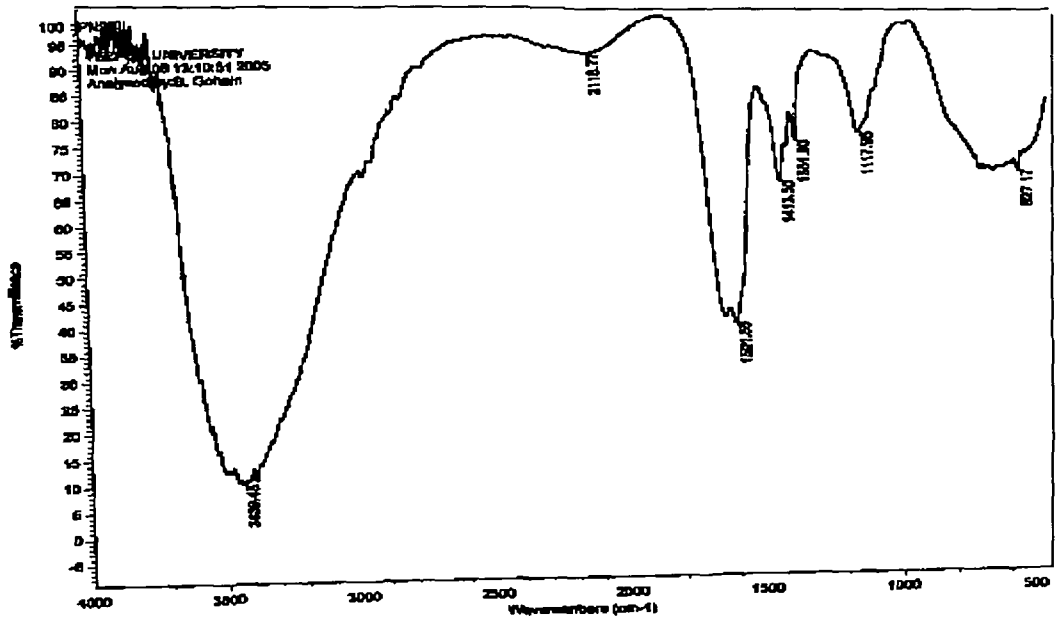
(a)


TEZPUR UNIVERSITY
%Transmittance
Wavenumbers (cm-1)

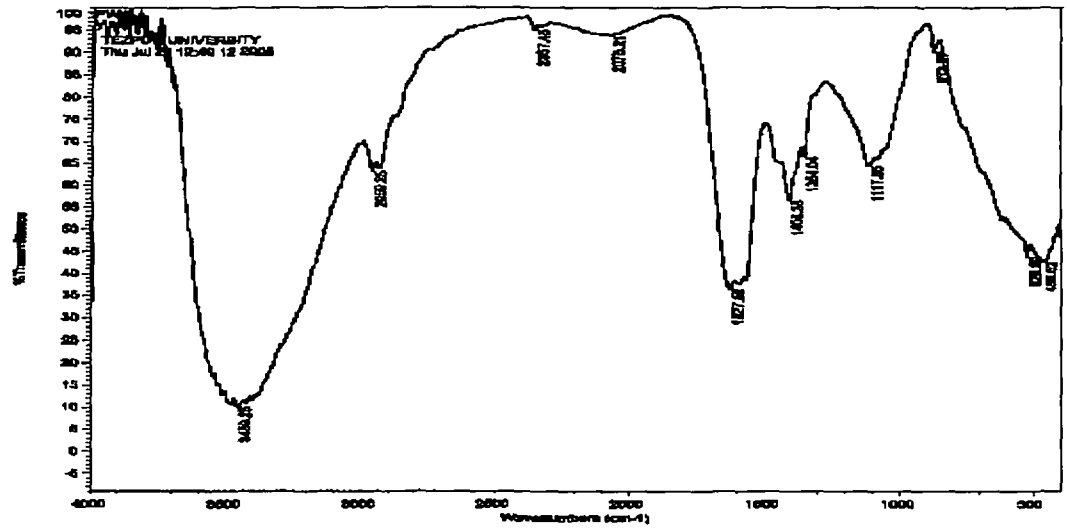
(b)


TEZPUR UNIVERSITY
%Transmittance

(c)



(d)

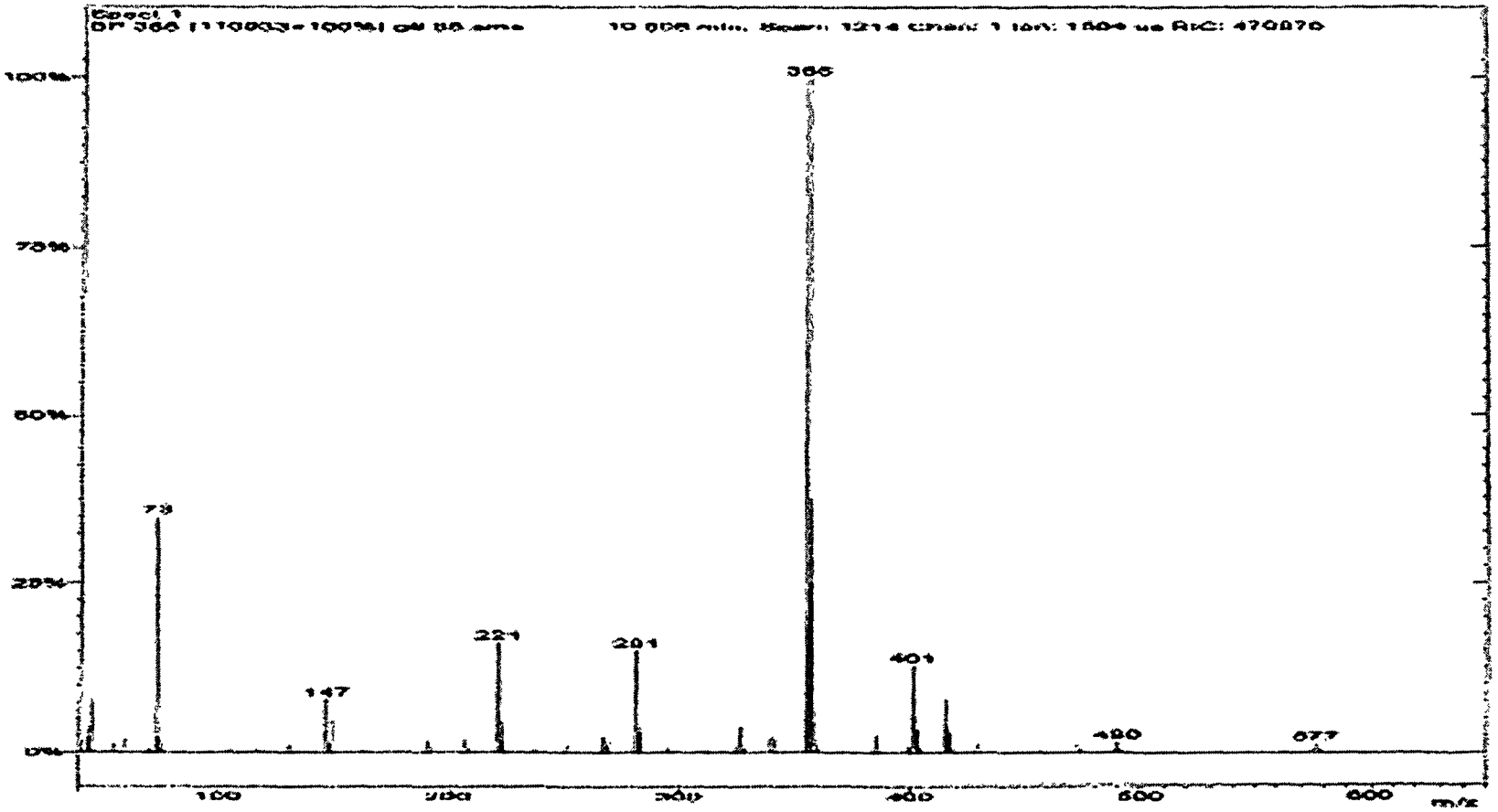


(e)

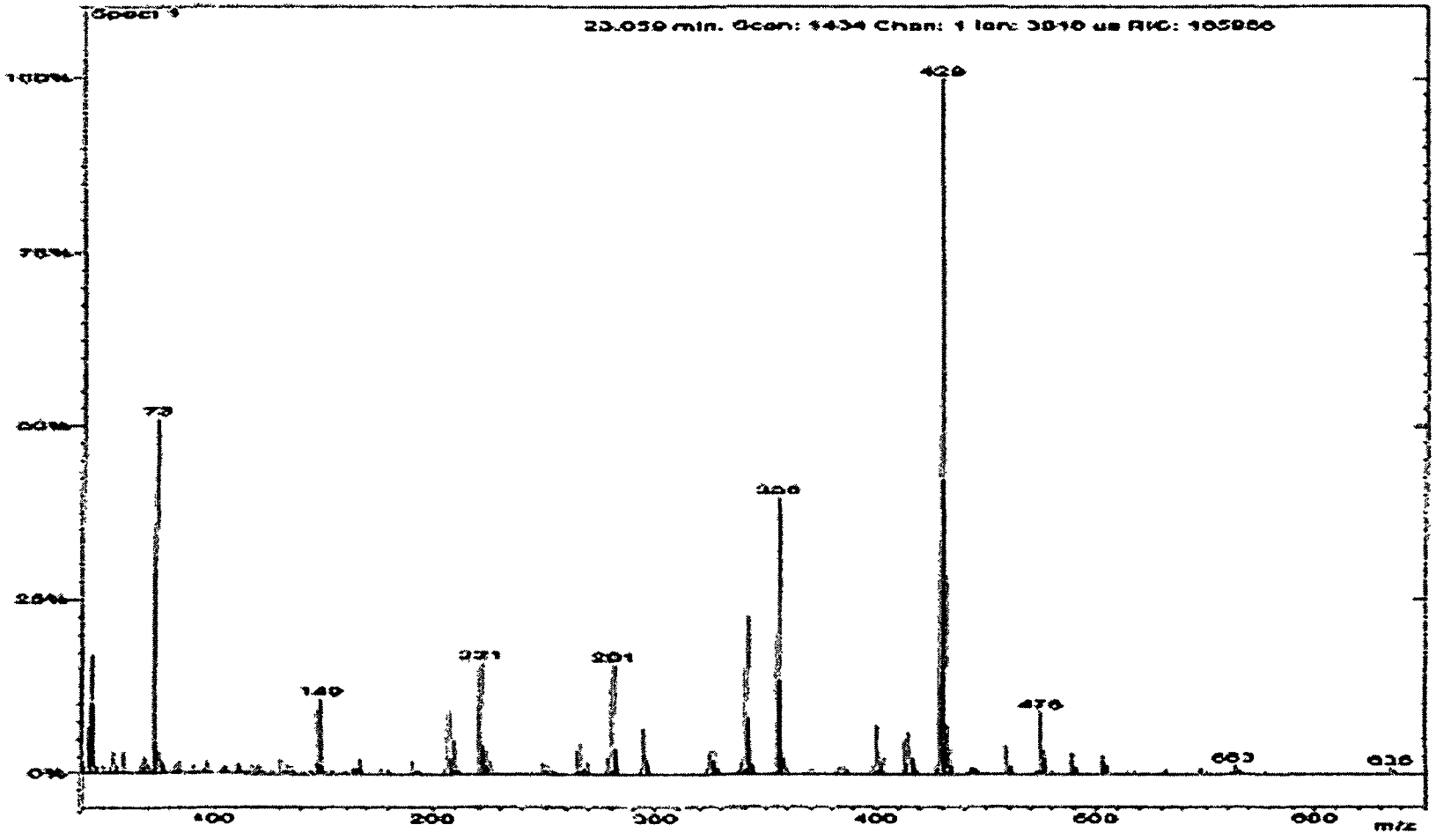
Fig.4.11. FT- IR spectra of purified biosurfactant isolated from (a) *P. aeruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC7812) (c) *P. aeruginosa* (MTCC 8163) (d) *P. aeruginosa* (MTCC 8165) and (e) *P. aeruginosa* (MTCC7814)

4.3.9.5. GC-MS of purified of biosurfactant

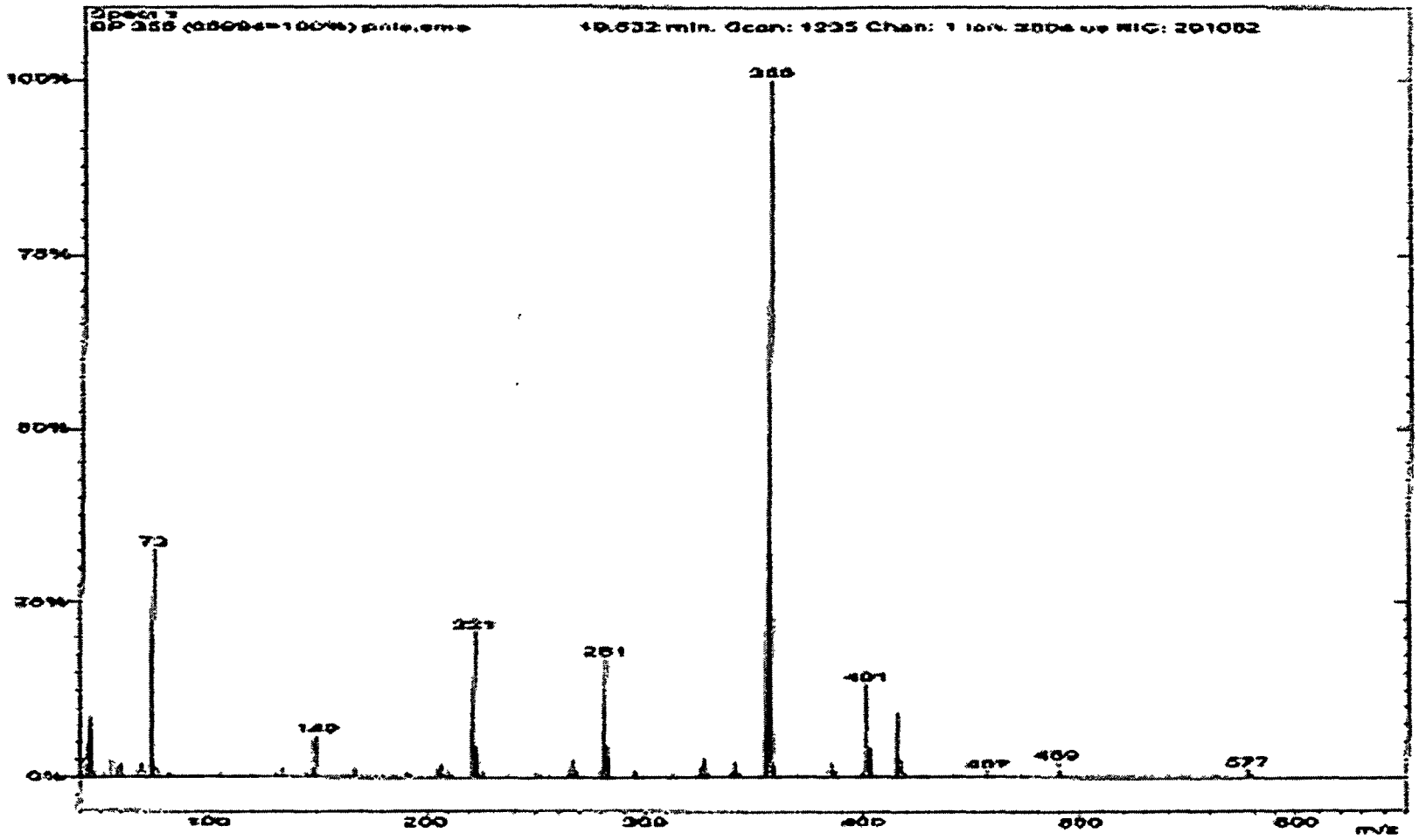
The biosurfactant purified from the culture media of each bacterial isolate was assessed using a Varian GC-MS system and data thus obtained are presented in Fig.4.12 (a - e). In the assay, the biosurfactants of *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC 8163) exhibited peaks at almost the same retention time of 19 min. The pseudomolecular ion at m/z 355 was the base peak of the spectrum, but some fragment ions were seen at m/z 147, 221,281 and 401. However, the purified biosurfactants from *P. aeruginosa* (MTCC7812) and *P. aeruginosa* (MTCC7814) showed peaks at the same retention time of 23 min. The pseudomolecular ion at m/z 429 was the base peak of the spectrum, but some fragments of the ions were seen at m/z 147, 221, 281, 355 and 475. While the purified biosurfactant from *P. aeruginosa* (MTCC 8163) showed the base peak of the spectrum at retention time 26 min with fragmentation ion at m/z 503, but some fragment ions were also seen at m/z 147, 221, 281, 355, 429 and 475.


100%
75%
50%
25%
0%
366
73
147
221
201
401
490
577
100
200
300
400
500
600
m/z

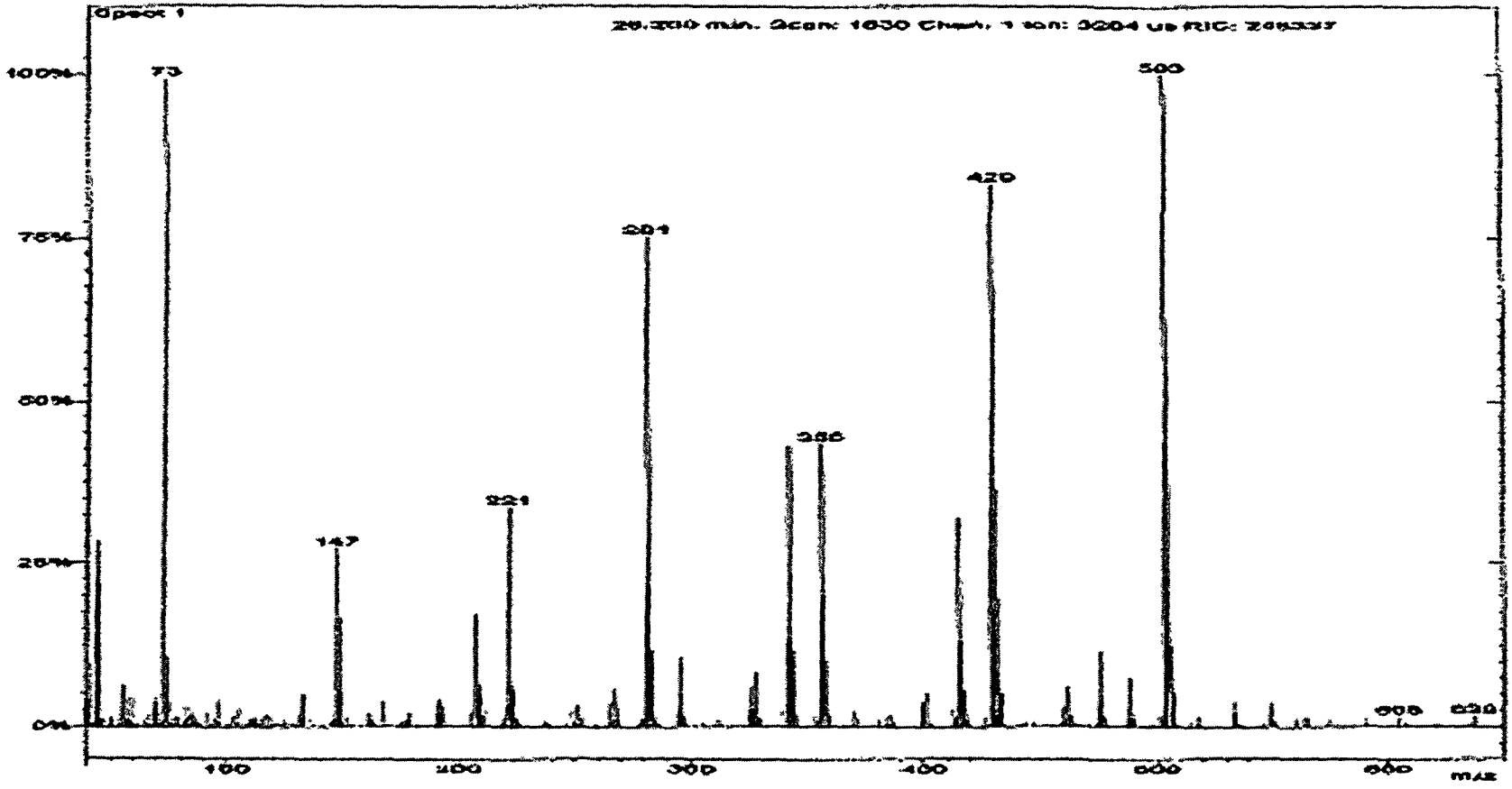
(a)


Spect 1
23.059 min. Scan: 1434 Chan: 1 Ion: 3810 us RIC: 105986
100%
75%
50%
25%
0%
429
326
221
201
140
476
663
836
400
200
300
400
600
800
m/z

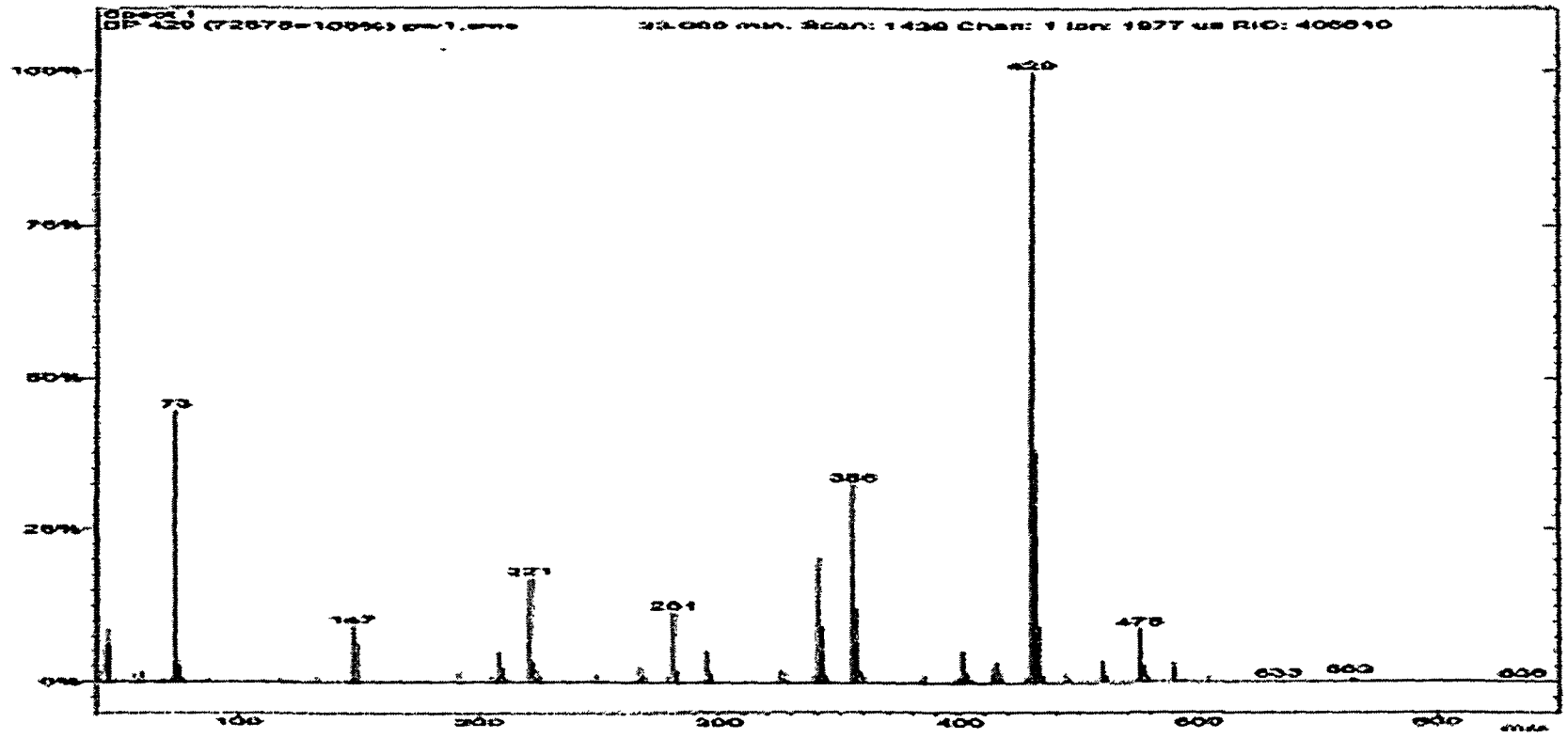
(b)


100%
75%
50%
25%
0%
73
149
221
251
355
401
497
500
577
100
200
300
400
500
600
m/z

(c)


Qpeak 1
100%
75%
50%
25%
0%
73
147
221
294
386
420
500
100
200
300
400
500
600
m/z

(d)



(e)

Fig.4.12. GC-MS spectra of purified biosurfactants isolated from (a) *P. aeruginosa* (MTCC7815), (b) *P. aeruginosa* (MTCC7812), (c) *P. aeruginosa* (MTCC 8163), (d) *P. aeruginosa* (MTCC 8165) and (e) *P. aeruginosa* (MTCC7814)

Table 4.29. GC –MS data of biosurfactants along with other traits

Fraction	Surface* tension reduction to (mN/m)	Amount recovered (mg)	Yield (%)	CMC (mg/l)	m/z ** (Da)	Assignment
P. aeruginosa (MTCC7815)						
CFE	30.2	130.0	100	130	-	
CB	29.1	92.33	71.0	110	-	
TLC fractions						
P. aeruginosa (MTCC7815) S1	55.3	0.115	0.08	140	-	
P. aeruginosa (MTCC7815) S2	35.8	0.210	0.16	130	-	
P. aeruginosa (MTCC7815) S3	29.0	2.70	2.07	110	147, 191, 221, 281, 327, 355, 401	
P. aeruginosa (MTCC7812)						
CFE	32.1	125.00	100	140	-	
CB	31.7	75.00	60.0	110	-	
TLC fractions						
P. aeruginosa (MTCC7812) S1	51.2	0.120	0.09	130	-	
P. aeruginosa (MTCC7812) S2	31.5	1.85	1.48	110	147, 221, 281, 355, 401, 429, 475	Rha-C_8 –C_{10} and C_{10}-C_8
P. aeruginosa (MTCC7812) S3	42.5	0.145	0.11	140	-	
P. aeruginosa (MTCC 8163)						
CFE	31.1	135	100	130	-	
CB	30.4	0.95	0.70	110	-	
TLC fractions						
P. aeruginosa (MTCC81 63) S1	59.0	0.310	0.22	140	-	
P. aeruginosa (MTCC 8163) S2	30.4	0.980	0.72	110	147, 221, 281, 355, 401	

P. aeruginosa (MTCC 8165)						
CFE	29.5	152.0	100	140	-	
CB	30.4	0.85	0.55	100	-	
TLC fractions						
P. aeruginosa (MTCC 8165) S1	38.5	0.315	0.20	130	-	
P. aeruginosa (MTCC 8165) S2	30.3	2.50	1.64	100	147, 221, 281, 355,429, 475, 503	Rha-C_8 –C_{10} and C_{10}-C_8
P. aeruginosa (MTCC 8165) S3	52.3	0.210	0.13	140	-	
P. aeruginosa (MTCC7814)						
CFE	32.2	215.0	100	140	-	
CB	31.1	1.98	0.92	110	-	
TLC fractions						
P. aeruginosa (MTCC7814) S1	54.5	0.145	0.06	140	-	
P. aeruginosa (MTCC7814) S2	38.5	0.310	0.14	130	-	
P. aeruginosa (MTCC7814) S3	31.1	3.5	1.62	110	147, 221, 281, 355, 401, 429, 475	Rha-C_8 –C_{10} and C_{10}-C_8

*CFE – Cell free extract **CB – crude biosurfactant ***Reduction in surface tension ($mN.m^{-1}$) by 100 µg of biosurfactant. Surface tension of control was 72 $mN.m^{-1}$. ****Determined by GC MS mass spectra analysis

4.3.10. Biochemical properties

4.3.10.1. Biochemical composition of purified biosurfactant

The biochemical composition of biosurfactans of the isolates are given in Table 4.30. It was quite evident that biosurfactants from both the *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812) were lipopeptide in nature. However, the bacterial isolates *P. aeruginosa* (MTCC8163), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) produced biosurfactant, which contained proteins, carbohydrates as well as lipids.

Table 4.30. Biochemical composition of biosurfactant from the bacterial Isolates

Biosurfactant from	Protein content (%)	Carbohydrate content (%)	Lipid content (%)
P. aeruginosa (MTCC7815)	26.50	1.2	32.77
P. aeruginosa (MTCC7812)	25.6	1.5	31.8
P. aeruginosa (MTCC8163)	20.60	35.75	6.41
P. aeruginosa (MTCC8165)	41.25	25.6	15.33
P. aeruginosa (MTCC7814)	16.50	40.25	20.28

4. 3.11. Cell surface hydrophobicity (CSH)

The cell surface hydrophobicity was evaluated by studying the ability of the bacterial cells to partition in the organic and aqueous phases. Cells of the bacterial isolates were allowed to grow in the medium supplemented with 2% glucose (w/v). Data obtained are presented in Fig.4.13.

A comparison of CSH of the bacterial isolates among the tested hydrocarbons like hexadecane, hexane and benzene revealed that the cells of *P. aeruginosa* (MTCC8163) followed by *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC7815) exhibited more hydrophobicity with 41.85, 23.71 and 20.4% of cells being transferred in hexadecane as compared to the cells from

P. aeruginosa (MTCC8165) and *P. aeruginosa* (MTCC7812). In unsaturated aliphatic hydrocarbons like hexen, the bacterial isolates *P. aeruginosa* (MTCC8163) followed by *P. aeruginosa* (MTCC7815) showed comparatively more hydrophobicity with 22.77 and 20.16% of cells being transferred. The bacterial isolate *P. aeruginosa* (MTCC7812) exhibited poor transfer of cells in hexen. In the case of aromatic hydrocarbons like benzene, the isolates *P. aeruginosa* (MTCC7814) followed by *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC7815) showed higher hydrophobicity with 25.32, 24.66 and 18.47% cells being transferred.

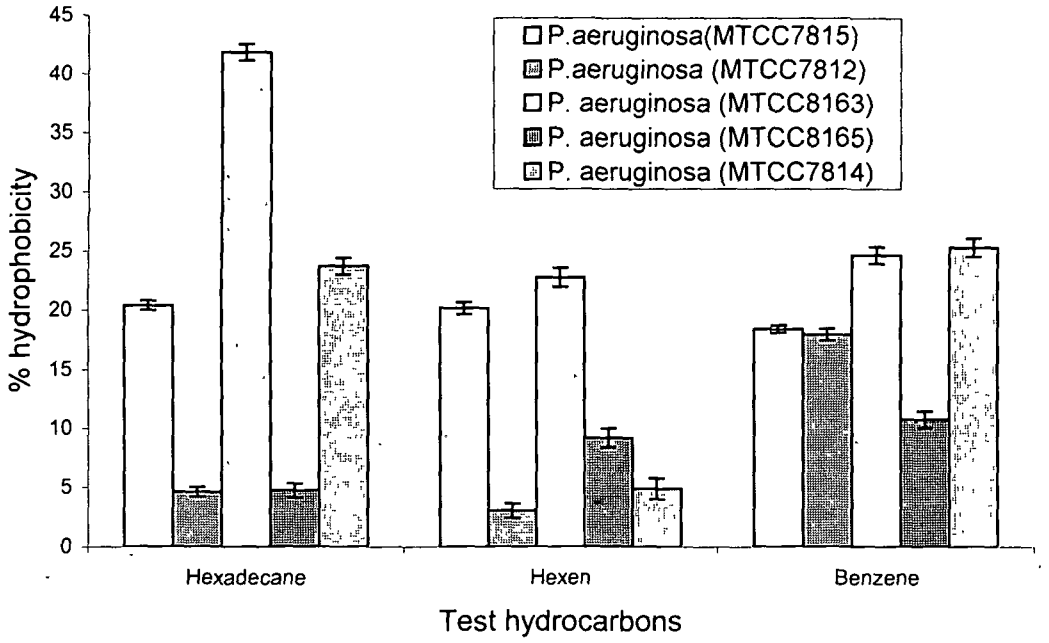


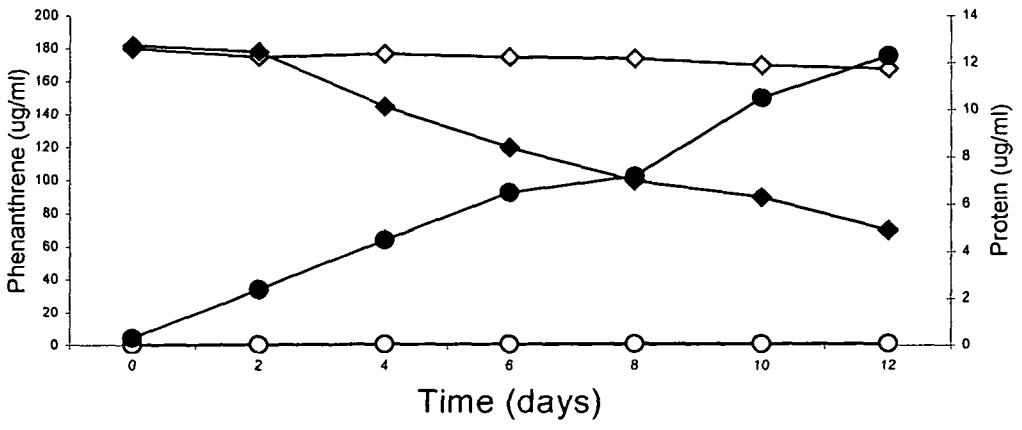
Fig. 4.13. Comparison of cell surface hydrophobicity of bacterial isolates. (mean ± S.D of 4 experiments)

4.4. Polycyclic aromatic hydrocarbon biodegradation

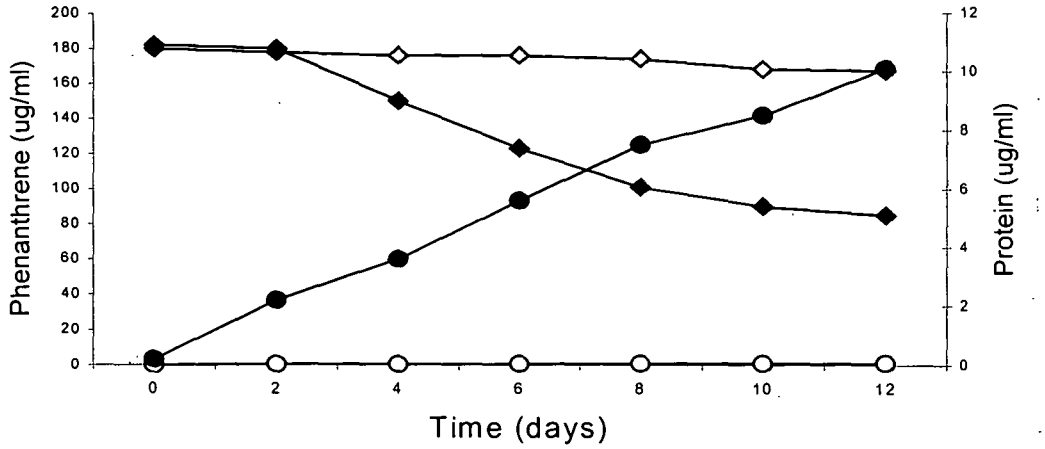
Out of hydrocarbon components, polycyclic aromatics are known to be more dangerous due to their less degradability and air polluting carcinogenic nature. Though 'nitroso' components are the least degraded they are not as harmful as these components. Therefore, a separate experiment was

conducted to assess the degradation of these compounds by the selected bacterial isolates.

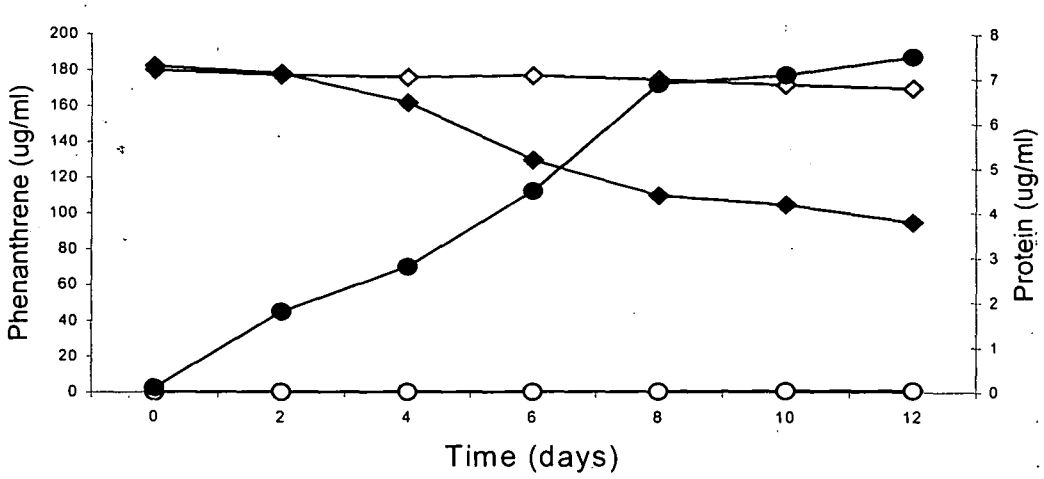
All bacterial isolates used in the present investigation were able to utilize PAHs as the sole source of carbon and energy. This was evident as there was a decrease in the content of PAHs components like phenanthrene, pyrene and fluorene from the medium supplemented with PAHs with a concomitant increase in the bacterial dry biomass and protein content with respect to time. The bacterial isolates were separately cultured in mineral salt medium supplemented with phenanthrene, pyrene and fluorene for 12 days. Increase in protein concentration as the index for bacterial growth and utilization of the hydrocarbon component was estimated at an interval of 2 days. Data thus obtained are presented in Fig. 4.14, 4.15 and 4.16. The utilization of phenanthrene as the sole source of carbon and energy by the bacterial isolates was confirmed by its removal from the medium with a corresponding increase in the bacterial protein. The concentration of phenanthrene decreased dramatically in the culture medium over the next 12 days. The bacterial isolate *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC7812) and *P. aeruginosa* (MTCC7814) exhibited the maximum utilization of phenanthrene to 70, 85 and 87µg from initial application of 180 µg in the medium. The remaining bacterial isolates possessed comparatively less utilization of phenanthrene.



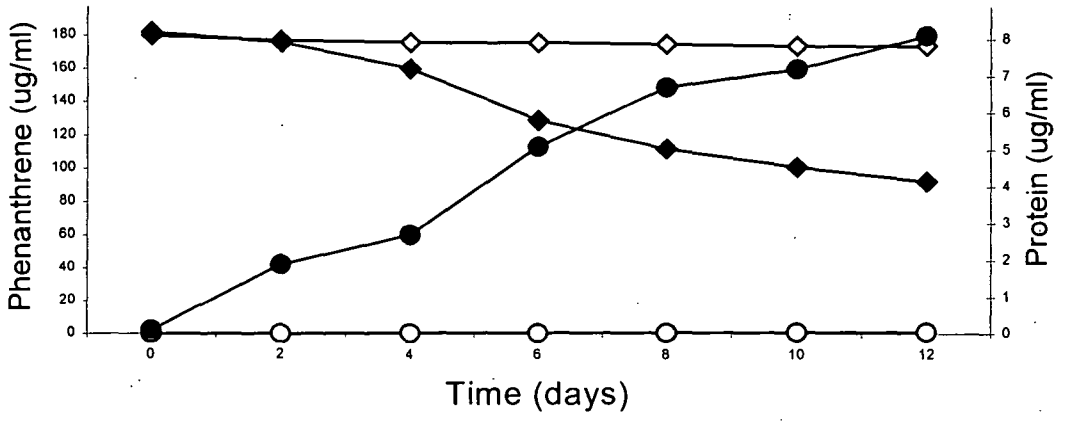
(a)


Phenanthrene (ug/ml)
Protein (ug/ml)
Time (days)

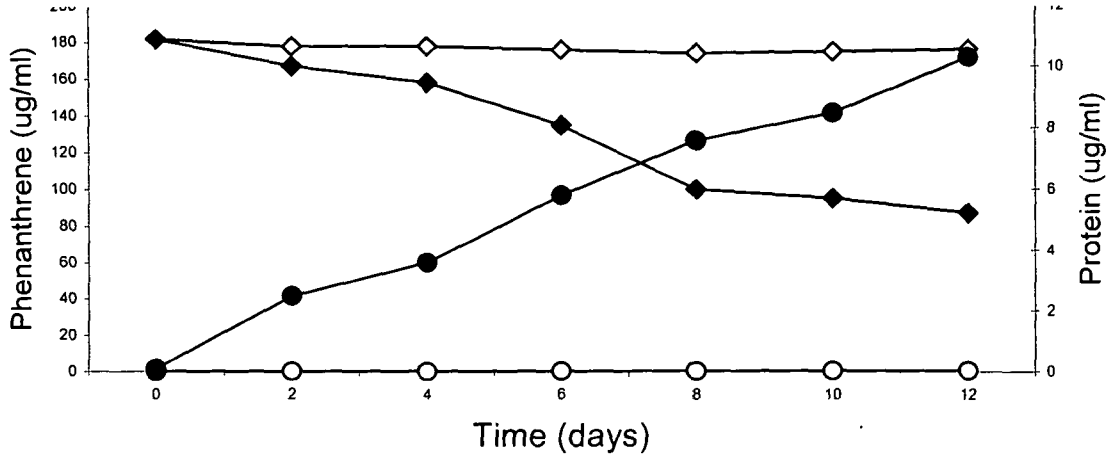
(b)


Phenanthrene (ug/ml)
Protein (ug/ml)
Time (days)

(c)


Phenanthrene (ug/ml)
Protein (ug/ml)
Time (days)

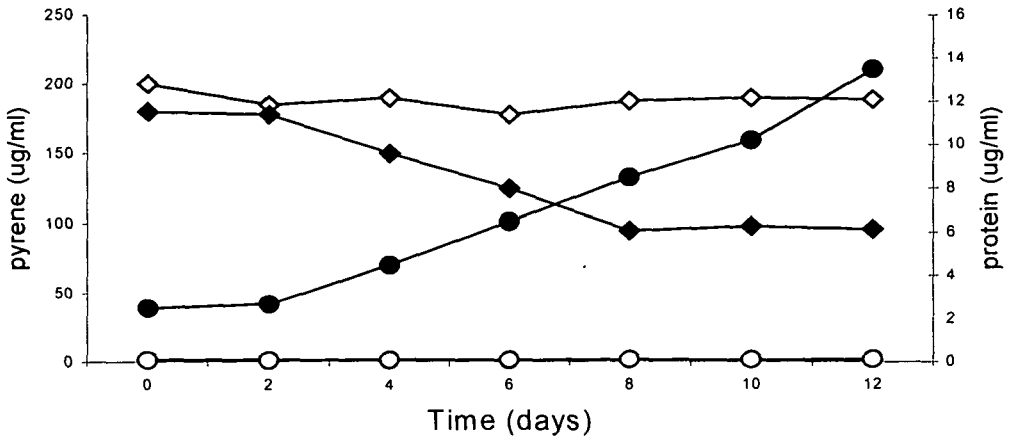
(d)



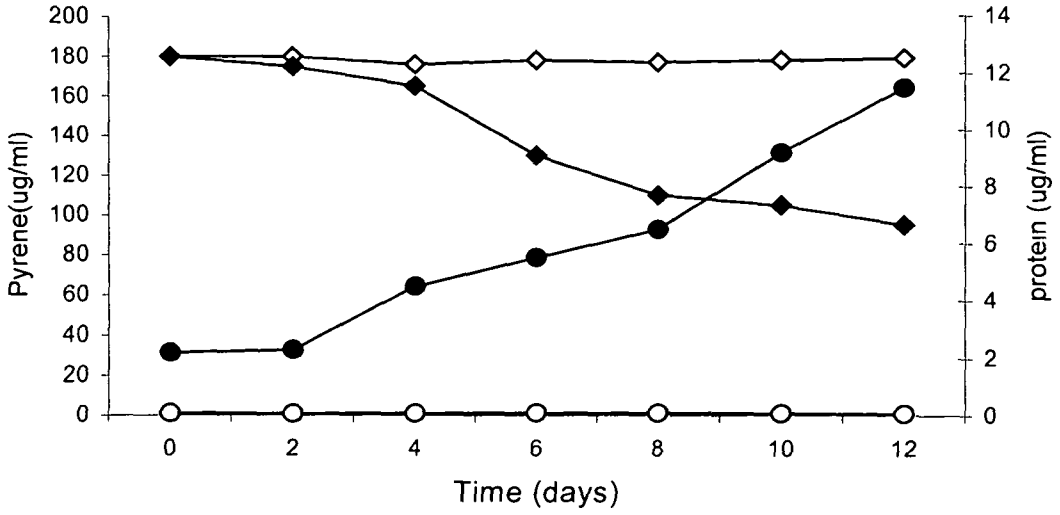
(e)

Fig. 4.14. Utilization of phenanthrene by the bacterial isolates (a) *P. aeruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC7812) (c) *P. aeruginosa* (MTCC8163) (d) *P. aeruginos* (MTCC8165) and (e) *P. aeruginosa* (MTCC7814) in phenanthrene containing medium at 30°C and 200 rpm. [growth as an increase in cell protein in cultures (●) and in controls(○); phenanthrene determination by solvent extraction from cultures (◆) and controls (◇)]

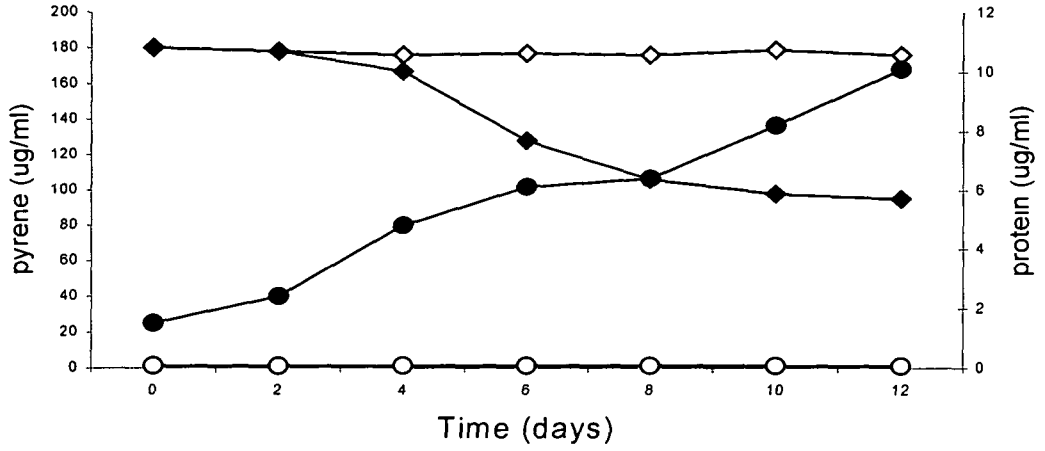
In the case of pyrene supplemented medium no significant growth was observed in the initial 24 h period of culture.



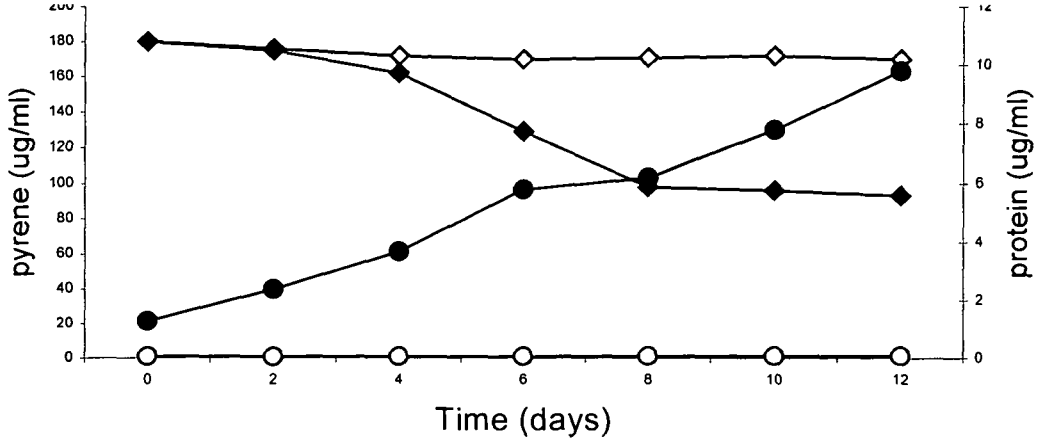
(a)


Pyrene(ug/ml)
protein (ug/ml)
Time (days)

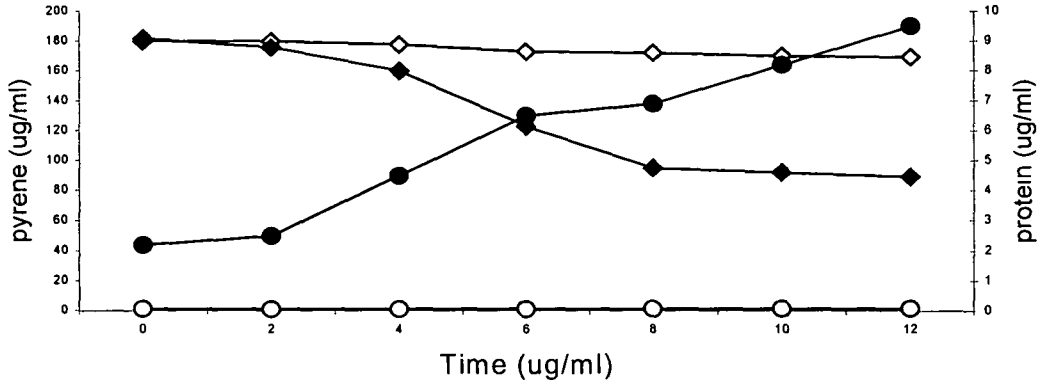
(b)


pyrene (ug/ml)
protein (ug/ml)
Time (days)

(c)


pyrene (ug/ml)
protein (ug/ml)
Time (days)

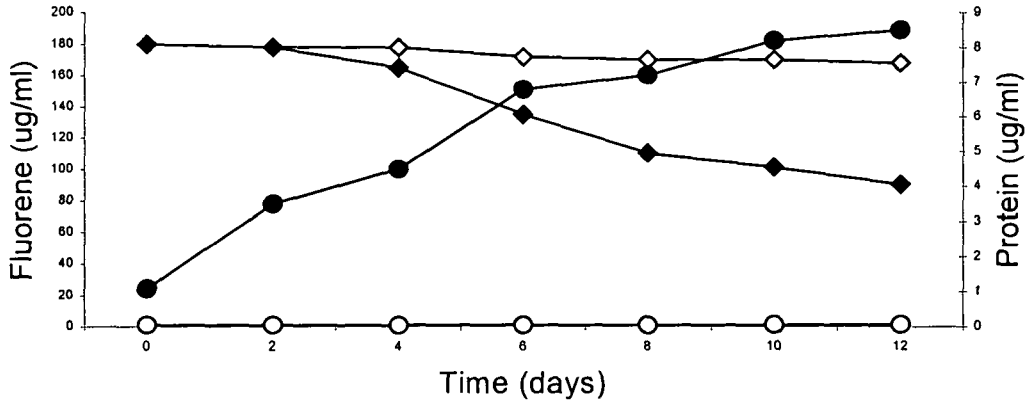
(d)



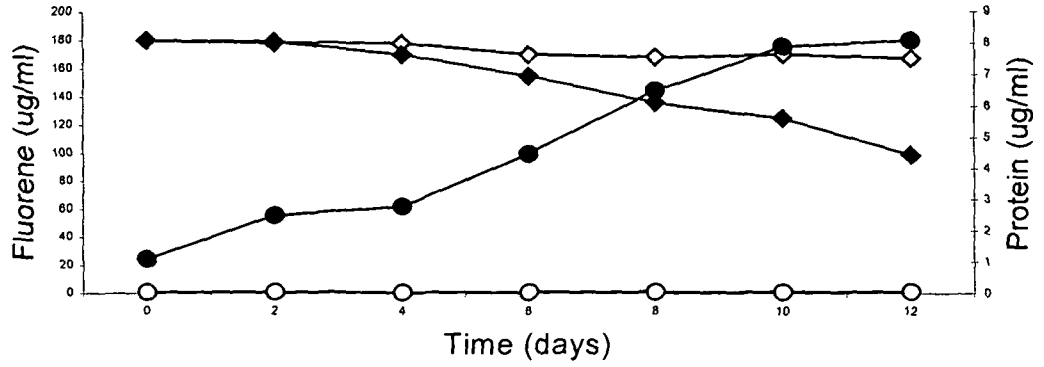
(e)

Fig. 4. 15. Utilization of pyrene by the bacterial isolates (a) *P. aruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC7812) (c) *P. aeruginosa* (MTCC8163) (d) *P. aeruginosa* (MTCC8165) and (e) *P. aeruginosa* (MTCC7814) in phenanthrene containing medium at 30°C and 200 rpm. [growth as an increase in cell protein cultures (●) and in controls (○); phenanthrene determination by solvent extraction from cultures (◆) and controls (◇)]

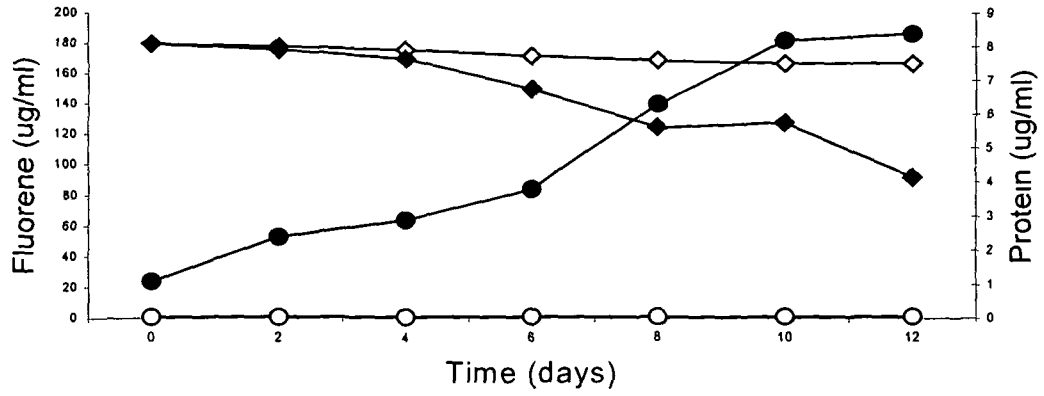
The bacterial isolates *P. aeruginosa* (MTCC7814) followed by *P. aeruginosa* (MTCC8165) exhibited better utilization of pyrene with 89 µg and 93 µg, respectively from the initial application of 180 µ in 12 days of culture. The other bacterial isolates showed less utilization of pyrene.



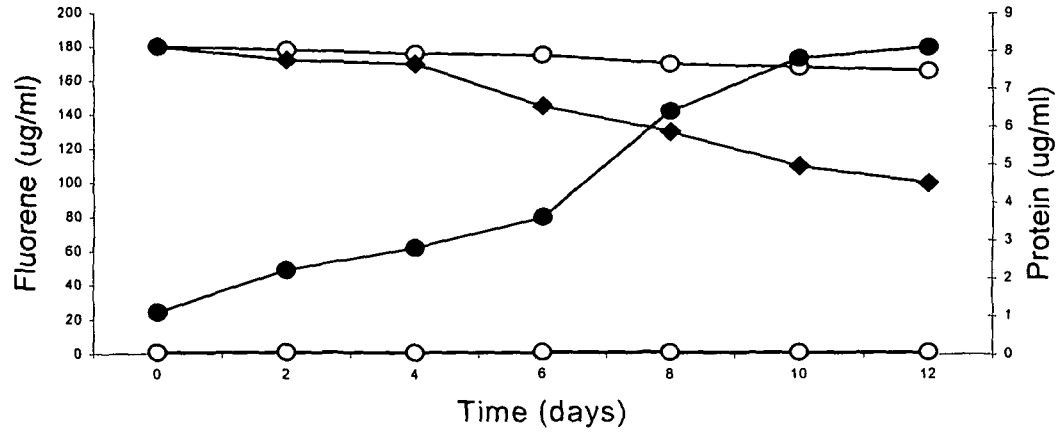
(a)


Fluorene (ug/ml)
Protein (ug/ml)
Time (days)

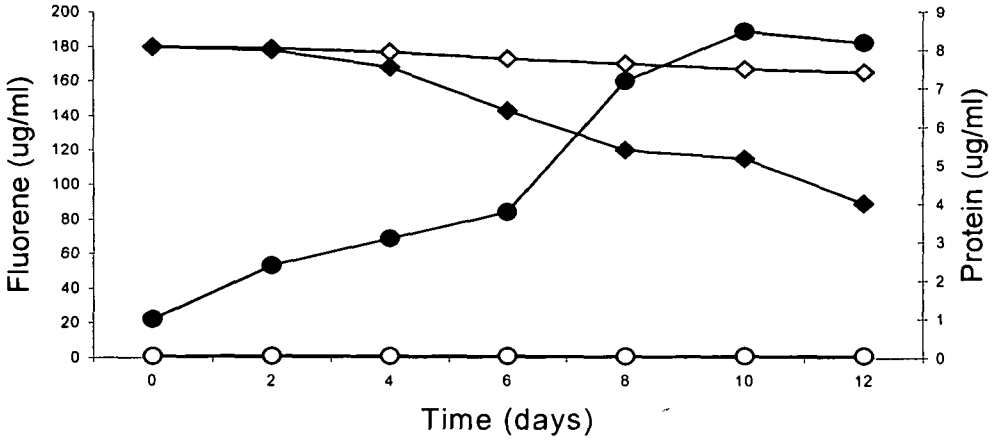
(b)


Fluorene (ug/ml)
Protein (ug/ml)
Time (days)

(c)


Fluorene (ug/ml)
Protein (ug/ml)
Time (days)

(d)



(e)

Fig. 4. 16. Utilization of fluorene by the bacterial isolates (a) *P. aeruginosa* (MTCC7815) (b) *P. aeruginosa* (MTCC7812) (c) *P. aeruginosa* (MTCC8163) (d) *P. aeruginos* (MTCC8165) and (e) *P.aeruginosa* (MTCC7814) in phenanthrene containing medium at 30°C and 200 rpm. [growth as an increase in cell protein in cultures (●) and in controls(○); phenanthrene determination by solvent extraction from cultures (◆) and controls (◇)]

In fluorene-supplemented medium, the bacterial isolate *P. aeruginosa* (MTCC7814) followed by *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) exhibited higher utilization with 89, 90 and 92 µg respectively from the initial application of 180 µg in 12 days of culture. The bacterial isolate *P. aeruginosa* (MTCC8165) exhibited poor utilization of fluorene.

In a separate experiment, growth of bacterial isolates and yield of biosurfactants in pyrene or fluorene - supplemented medium with or without the addition of phenanthrene were assessed and results obtained are presented in Table 4.31 and 32. The yield of biosurfactant in the culture supernatant with acid precipitation increased dramatically after 96 h of culture. The bacterial isolates exhibited better biosurfactant yield of 0.23 – 0.5 $g.l^{-1}$ at 96 h of culture in medium supplemented with pyrene and

phenanthrene as compared to medium without phenanthrene (0.18 – 0.30 $\mu g.l^{-1}$). Concomitantly, the bacterial biomass of 0.7 – 1.20 $g.l^{-1}$ increased to 0.75 – 1.4 $g.l^{-1}$ after 96 h of culture.

Table 4.31. Bacterial growth and yield of biosurfactant in pyrene and pyrene+ phenanthrene containing media in 96 h of culture (Mean ±S.D of 3 experiments)

Bacterial isolates	Carbon source		After 48 h of growth		After 96 h of growth	
	Pyrene	Phenanthrene	Dry biomass ($g.l^{-1}$)	Yield of biosurfactant ($g.l^{-1}$)	Dry biomass ($g.l^{-1}$)	Yield of biosurfactant ($g.l^{-1}$)
P. aeruginosa (MTCC7815)	+	-	0.80±0.01	0.20±0.01	1.00±0.1	0.30±0.01
	+	+	1.00±0.1	0.25±0.01	1.20±0.2	0.50±0.01
P. aeruginosa (MTCC7812)	+	-	0.60±0.02	0.15±0.01	0.80±0.01	0.20±0.01
	+	+	0.70±0.01	0.17±0.01	1.00±0.01	0.25±0.01
P. aeruginosa (MTCC7814)	+	-	0.90±0.02	0.20±0.01	1.00±0.1	0.26±0.01
	+	+	1.10±0.1	0.18±0.01	1.20±0.2	0.35±0.01
P. aeruginosa (MTCC8163)	+	-	0.50±0.01	0.10±0.01	0.80±0.02	0.18±0.01
	+	+	0.72±0.02	0.20±0.01	0.75±0.01	0.23±0.01
P. aeruginosa (MTCC8165)	+	-	0.60±0.01	0.18±0.01	0.90±0.03	0.28±0.01
	+	+	1.20±0.1	0.24±0.01	1.40±0.1	0.45±0.02

Table 4.32. Bacterial biomass and yield of biosurfactant in fluorene and fluorene + phenanthrene supplemented media in 96 h of culture (Mean ±S.D of 3 experiments)

Bacterial isolates	Carbon source		After 48 h of growth		After 96 h of growth	
	Fluorene	Phenanthrene	Dry biomass ($g.l^{-1}$)	Yield of biosurfactant ($g.l^{-1}$)	Dry biomass ($g.l^{-1}$)	Yield of biosurfactant ($g.l^{-1}$)
P. aeruginosa (MTCC7815)	+	-	0.60±0.01	0.20±0.01	0.80±0.01	0.30±0.01
	+	+	1.00±0.04	0.28±0.01	1.30±0.06	0.45±0.03
P. aeruginosa (MTCC7812)	+	-	0.50±0.01	0.18±0.01	0.80±0.01	0.25±0.01
	+	+	0.60±0.01	0.18±0.01	1.00±0.05	0.29±0.01
P. aeruginosa (MTCC7814)	+	-	0.80±0.02	0.23±0.01	0.90±0.01	0.28±0.01
	+	+	1.20±0.05	0.21±0.01	1.50±0.04	0.38±0.02
P. aeruginosa (MTCC8163)	+	-	0.70±0.01	0.15±0.02	0.90±0.03	0.20±0.01
	+	+	0.80±0.03	0.23±0.01	1.10±0.1	0.25±0.01
P. aeruginosa (MTCC8165)	+	-	0.50±0.01	0.21±0.01	1.10±0.2	0.30±0.01
	+	+	0.80±0.02	0.27±0.02	1.50±0.06	0.35±0.03

In the case of medium having the combined addition of flourene and phenanthrene caused better biosurfactant yield of 0.45 and 0.38 $g.l^{-1}$ in the case of bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7814), respectively for the entire growth period of 96 h. The bacterial isolates possessed comparatively poor yield of biosurfactant in fluorene containing medium devoid of phenanthrene. In the same medium, the bacterial biomass increased from 0.6 –1.2 $g.l^{-1}$ at 48 h of inoculation to a maximum of 1.0 – 1.5 $g.l^{-1}$ at 96 h of culture. In fluorene-supplemented medium, the growth of the bacterial isolates was poor.

4.4.1. Solubilization of PAHs and crude oil by biosurfactants

The effect of biosurfactants on the solubility of PAHs (phenanthrene, pyrene and flourene) and crude oil was determined by the test tube solubilization assay (Barkay *et al.*, 1999) in the presence of biosurfactant at the rate of 500 $\mu g.ml^{-1}$. Data obtained are presented in Fig. 4. 17. The solubility of PAHs and crude oil in the mineral salt medium was found to be higher due to the addition of biosurfactant as compared to the one without the addition of biosurfactant. The solubilization of PAHs in biosurfactant-supplemented medium occurred when the concentration of the biosurfactant exceeded the CMC value.

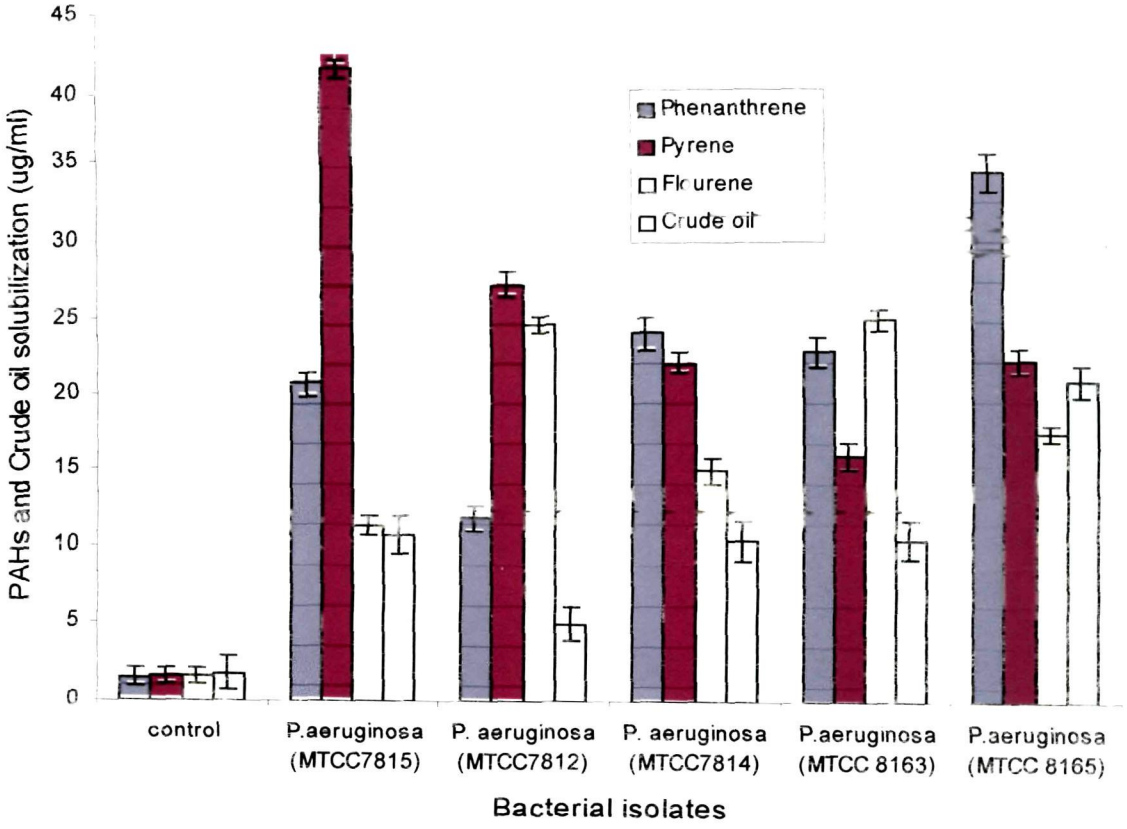


Fig.4.17. Solubilization of PAHs and crude oil by the bacterial biosurfactants (Mean ±S.D of 3 experiments)

Results

The crude biosurfactants isolated from each of five bacterial isolates enhanced the solubility of PAHs at 500 $\mu g.ml^{-1}$ concentrations. However, biosurfactants from the bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812) exhibited 41 and 26 $\mu g.ml^{-1}$, respectively more solubilization of pyrene as compared to the control. The isolate *P. aeruginosa* (MTCC8163) exhibited poor solubilization of pyrene by 15.67 $\mu g.ml^{-1}$ as compared to other bacterial isolates.

In the case of crude oil, the biosurfactant from the bacterial isolate *P. aeruginosa* (MTCC8165) showed 20 $\mu g.ml^{-1}$ more solubilization as compared to the control. The biosurfactant of *P. aeruginosa* (MTCC7812) exhibited low solubility of crude oil as compared to other bacterial isolates. The biosurfactant of *P. aeruginosa* (MTCC8165) displayed three times more solubilization of phenanthrene as compared to the biosurfactant of *P. aeruginosa* (MTCC7812). On the other hand, the crude biosurfactants from the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC8163) displayed almost similar solubilization. The biosurfactant from the bacterial isolate *P. aeruginosa* (MTCC7812) followed by *P. aeruginosa* (MTCC8163) exhibited more solubilization of fluorene with 24.45 – 24.49 $\mu g.ml^{-1}$. But, the biosurfactants from the other bacterial isolates displayed poor solubilization.

4.4.2. Role of biosurfactants in culture media

Following the addition of biosurfactant of the respective bacterial isolates, the uptake of crude oil and its components like pyrene, fluorene and phenanthrene by the cultured bacterial isolates was assayed. Data obtained are presented in Table 4.33.

The uptake of crude oil and its components increased significantly in all bacterial cultures on addition of biosurfactant. The bacterial isolates *P. aeruginosa* (MTCC7814) followed by *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) showed the highest uptake of crude oil with 45.92, 40.31 and 36.28 μg as compared to other isolates during the incubation

period of 60 min. In the case of biosurfactant – supplemented medium, the bacterial isolates *P. aeruginosa* (MTCC7812) and *P. aeruginosa* (MTCC8165) utilized 43.87 and 33.84 μg of phenanthrene, respectively in 60 min of culture. Similarly, the isolate *P. aeruginosa* (MTCC7815) utilized 41.70 μg pyrene from the medium while supplemented with the biosurfactant. The isolate *P. aeruginosa* (MTCC7814) exhibited the highest uptake of flourene with 49.56 μg in 60 min of culture. The isolate *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC7812) exhibited comparatively higher uptake of flourene with 24.49 and 24.45 μg, respectively.

Table 4.33. Reduction of crude oil, phenanthrene, pyrene, and fluorene from the culture medium as acted upon by the bacterial isolates following the addition of biosurfactant (Mean ±SD of 3 experiments)

Carbon sources	Bacterial isolates	Biosurfactant	Uptake (µg)				
			0 min	15 min	30 min	45 min	60 min
Crude oil	*P. aeruginosa* (MTCC7815)	-	0	6.82±0.15	10.00±0.30	10.24±0.02	10.65±0.14
		+	0	36.88±0.08	40.21±0.06	40.31±0.02	40.31±0.02
	P. aeruginosa (MTCC7812)	-	0	1.62±0.02	2.54±0.21	4.40±0.18	4.49±0.07
		+	0	8.81±0.02	11.37±0.31	11.45±0.06	11.53±0.77
	P. aeruginosa (MTCC7814)	-	0	4.52±0.07	7.87±0.47	9.05±0.10	10.2±0.08
		+	0	42.82±0.14	44.79±0.84	45.92±0.10	45.92±0.71
	P. aeruginosa (MTCC8163)	-	0	3.91±0.09	5.21±0.07	8.38±0.58	10.24±0.08
		+	0	34.14±1.04	35.28±0.22	35.28±0.02	35.28±0.26
	P. aeruginosa (MTCC8165)	-	0	4.43±0.40	8.73±0.05	9.00±0.14	9.14±0.11
		+	0	10.75±0.73	18.64±0.28	19.60±0.37	20.31±0.52
Phenanthrene	*P. aeruginosa* (MTCC7815)	-	0	7.90±0.20	10.58±0.16	12.30±0.10	12.57±0.16
		+	0	17.08±0.02	18.32±0.07	19.47±0.52	20.55±0.13
	P. aeruginosa (MTCC7812)	-	0	9.12±0.10	9.69±0.15	11.23±0.05	11.72±0.10
		+	0	21.37±0.31	42.00±0.65	43.12±0.10	43.87±0.79
	P. aeruginosa (MTCC7814)	-	0	16.50±0.43	23.50±0.49	23.71±0.08	23.71±0.06
		+	0	14.26±0.38	25.14±0.05	25.22±0.06	25.05±0.17
	P. aeruginosa (MTCC8163)	-	0	21.12±0.13	22.34±0.09	22.39±0.11	22.44±0.13
		+	0	15.75±0.36	28.12±0.21	28.12±0.18	28.37±0.54
	P. aeruginosa (MTCC8165)	-	0	7.31±0.08	11.30±0.23	10.95±0.23	10.95±0.14
		+	0	42.61±0.55	33.01±0.11	33.26±0.18	33.84±0.20

Pyrene	*P. aeruginosa* (MTCC7815)	-	0	16.01±0.15	15.02±0.13	15.84±0.30	15.68±0.17
		+	0	18.29±0.48	27.80±0.13	35.12±0.09	41.70±0.32
	P. aeruginosa (MTCC7812)	-	0	5.77±0.28	8.07±0.03	8.26±0.08	8.37±0.04
		+	0	5.50±0.43	20.91±0.22	26.42±0.35	26.97±0.09
	P. aeruginosa (MTCC7814)	-	0	9.19±0.17	20.32±0.09	20.80±0.13	21.77±0.14
		+	0	14.13±0.36	21.45±0.21	21.45±0.13	21.54±0.05
	P. aeruginosa (MTCC8163)	-	0	1.04±0.01	3.40±0.14	3.60±0.16	4.15±0.04
		+	0	8.79±0.08	11.83±0.27	15.33±0.08	15.67±0.17
	P. aeruginosa (MTCC8165)	-	0	7.94±0.75	14.35±0.14	14.87±0.09	15.64±0.17
		+	0	20.43±0.17	20.98±0.19	21.35±0.12	21.71±0.09
Fluorene	*P. aeruginosa* (MTCC7815)	-	0	2.45±0.19	4.91±0.46	5.94±0.10	8.27±0.16
		+	0	5.15±0.76	9.12±0.22	10.17±0.52	11.23±0.13
	P. aeruginosa (MTCC7812)	-	0	0.48±0.05	6.91±0.17	15.87±0.05	16.17±0.10
		+	0	11.37±0.81	24.27±0.32	24.36±0.10	24.45±0.79
	P. aeruginosa (MTCC7814)	-	0	13.00±0.23	14.14±0.07	14.52±0.08	14.75±0.06
		+	0	21.91±0.28	48.26±0.26	49.04±0.06	49.56±0.17
	P. aeruginosa (MTCC8163)	-	0	4.34±0.25	10.60±0.21	11.30±0.11	11.65±0.33
		+	0	18.71±0.11	20.36±0.0.19	13.80±0.23	24.49±0.54
	P. aeruginosa (MTCC8165)	-	0	4.12±0.02	6.19±0.26	6.70±0.18	6.70±0.14
		+	0	16.30±0.09	16.06±0.07	16.69±0.30	17.01±0.20

Results

Uptake of crude oil and PAHs like pyrene, fluorene and phenanthrene was also measured in the presence of a suspension of heat killed bacterial cells. Heat killed bacterial cells were cultured separately in the medium supplemented with the addition of biosurfactant from the respective bacterial isolates. Crude oil and PAHs uptake did not occur in the presence of the suspension of heat killed bacterial cells.

Table 4.34. Reduction of crude oil, phenanthrene, pyrene, and fluorene from the culture medium as acted upon by the heat killed bacterial isolates following the addition of biosurfactant

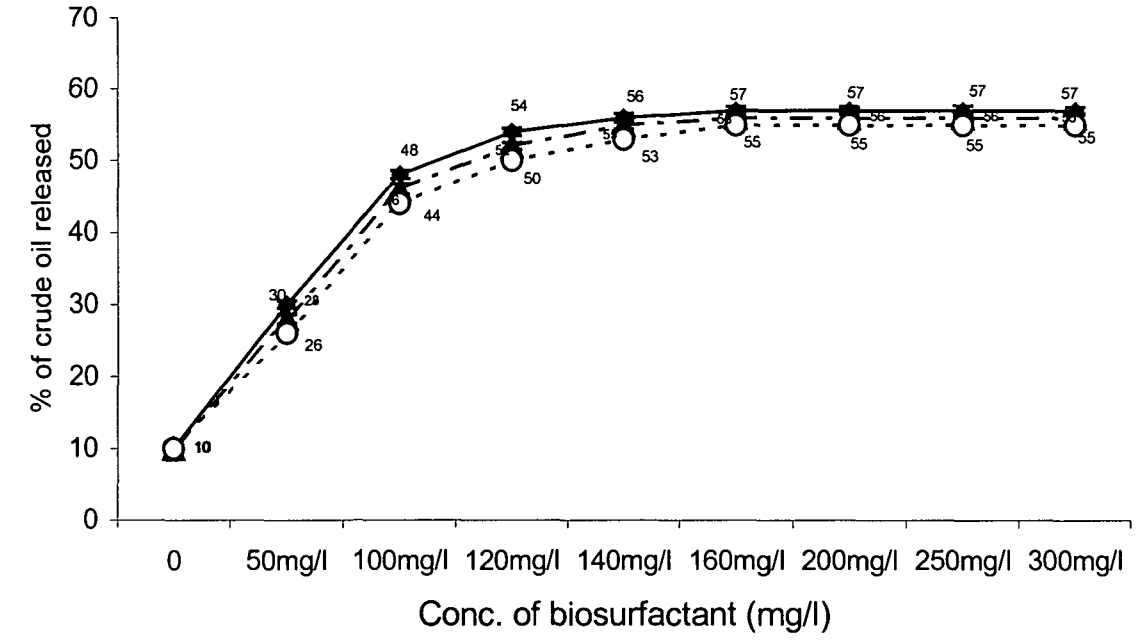
Carbon sources	Heat killed bacterial cells	Biosurf actant	Uptake (µg)				
			0 min	15 min	30 min	45 min	60 min
Crude oil	*P.aeruginosa* (MTCC7815)	+	0	0	0	0	0
Phenanthrene	*P.aeruginosa* (MTCC7812)	+	0	0	0	0	0
Pyrene	*P.aeruginosa* (MTCC8163)	+	0	0	0	0	0
Fluorene	*P.aeruginosa* (MTCC8165)	+	0	0	0	0	0

4.4.3. Application of biosurfactant in microbial enhanced oil recovery

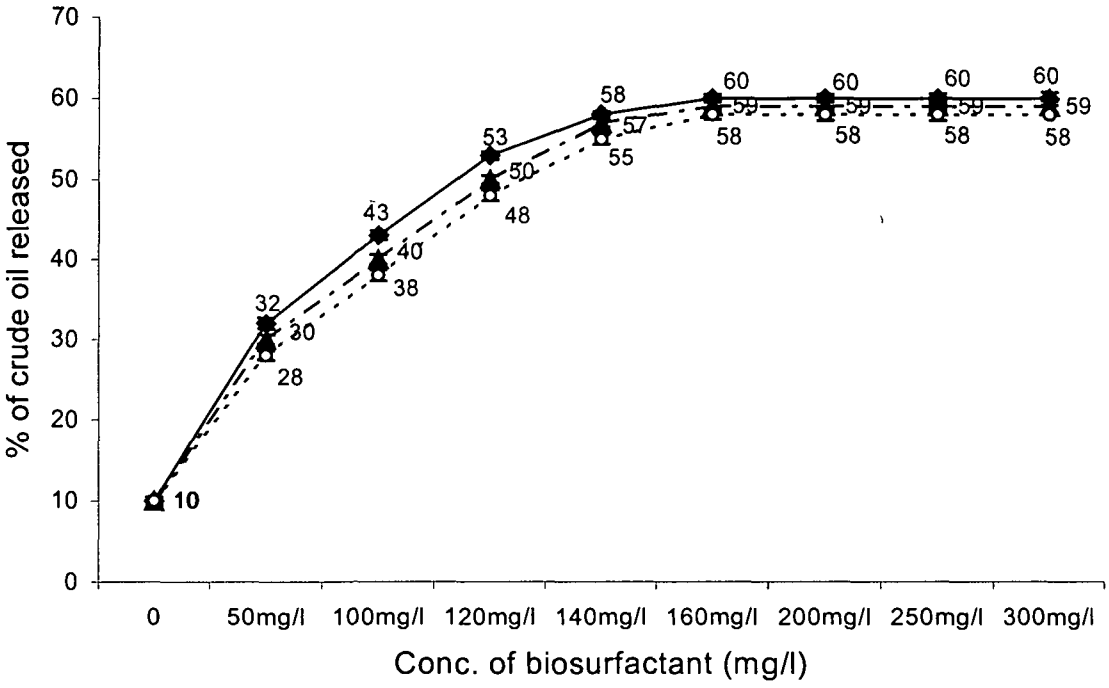
4.4.3.1. Use of biosurfactant(s) in releasing crude oil from saturated sand pack column

The release of crude oil from the sand pack column saturated with crude oil at different temperatures with the treatment of graded amounts of biosurfactants of different bacterial isolates was presented in Fig.4. 25 (a - e). The biosurfactans of *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7812) were found to release 60±1.2% and 60±1.5%, respectively of the crude oil from the saturated sand pack column at 90°C. Subsequently, the crude biosurfactants of *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7814) released 56.7±1.3%, and 51±2.0%, respectively of the crude oil from the saturated sand pack column at 90°C. The maximum percentage of

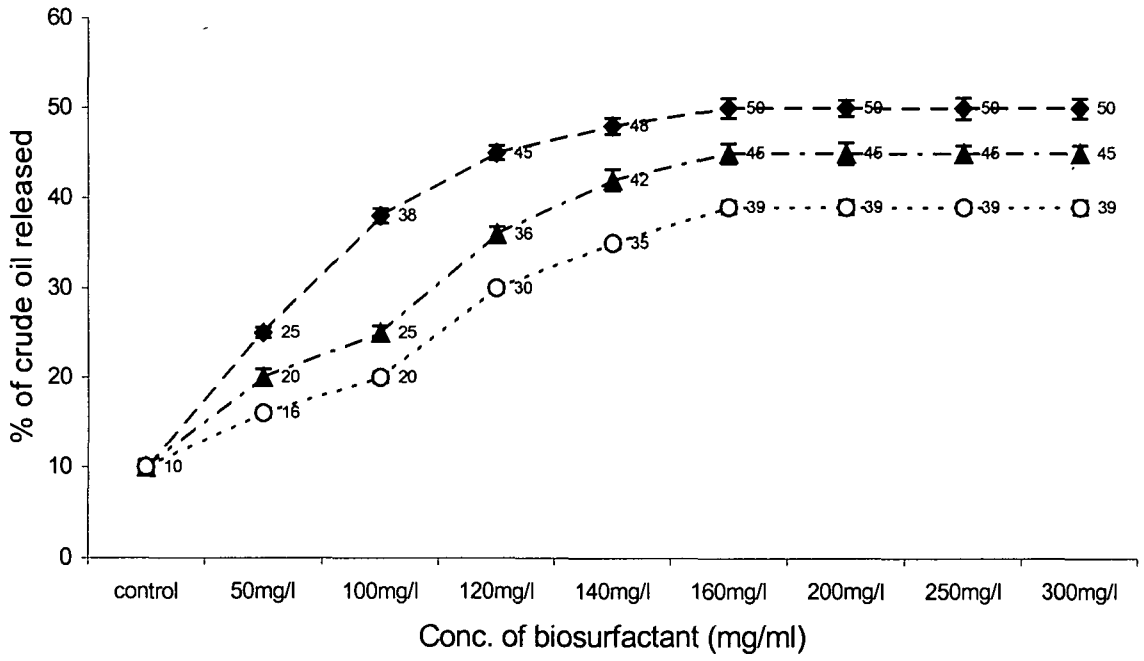
crude oil was released by the crude biosufactant of *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7812) at 90°C.



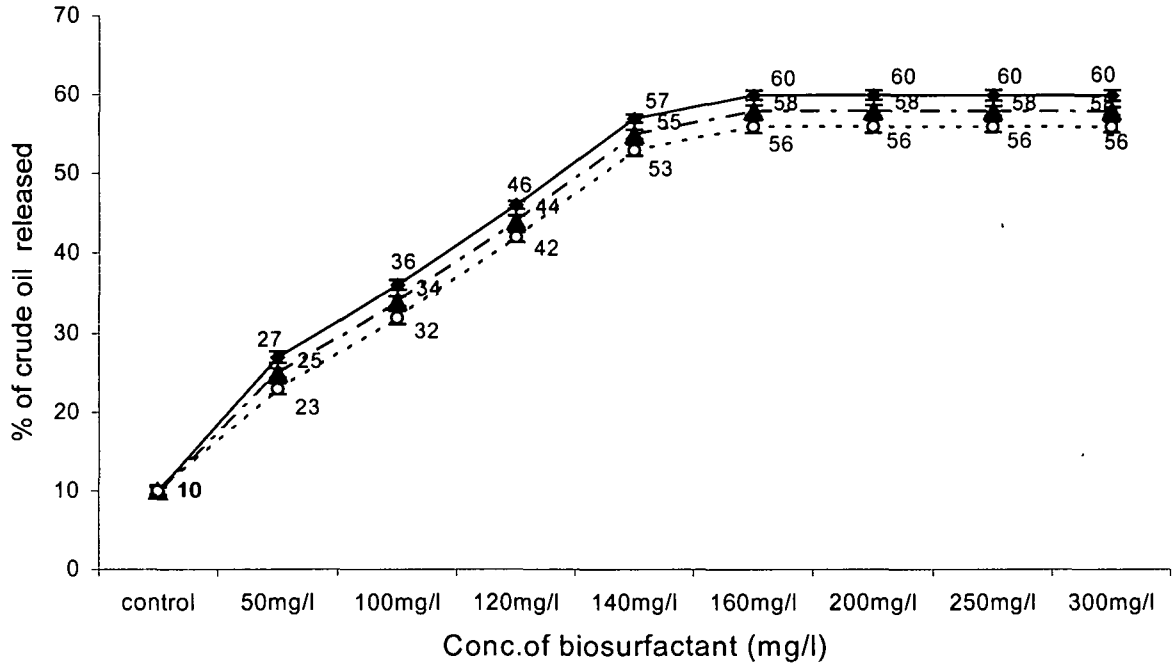
(a)



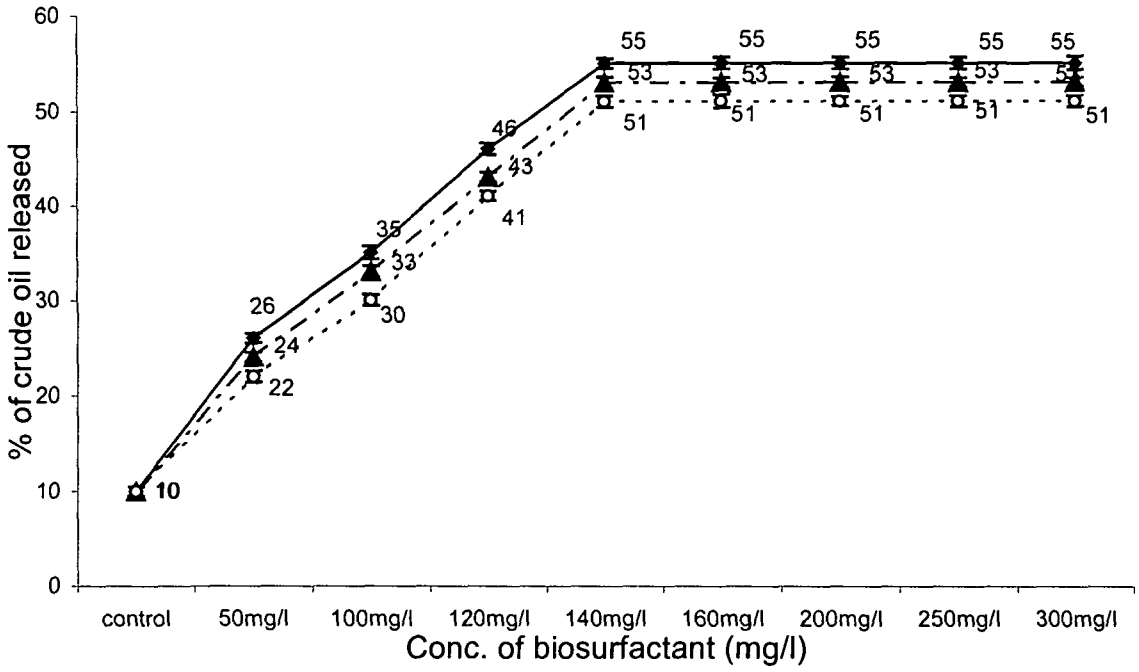
(b)


% of crude oil released
Conc. of biosurfactant (mg/ml)
control
50mg/l
100mg/l
120mg/l
140mg/l
160mg/l
200mg/l
250mg/l
300mg/l

(c)


% of crude oil released
Conc.of biosurfactant (mg/l)
control
50mg/l
100mg/l
120mg/l
140mg/l
160mg/l
200mg/l
250mg/l
300mg/l

(d)



(e)

Fig.4.18. Release of crude oil from saturated sand pack column by different concentrations of biosurfactant isolated from (a) *P. aeruginosa* (MTCC7815), (b) *P. aeruginosa* (MTCC7812), (c) *P. aeruginosa* (MTCC8163), (d) *P. aeruginosa* (MTCC8165) and (e) *P. aeruginosa* (MTCC7814). Percentage oil release at room temperature (--O--), at 70°C temperature (--▲--) and at 90°C temperature (-◆-). (mean ±S.D of 3 experiments)

4. 5. Molecular study on bacterial isolates

4.5.1. Genomic DNA isolation and quantification

Prokaryotes display a considerable diversity in genome organization, with some having a unipartite genome, like *E. coli,* and some others with more complex multipartite genomes. Understanding of the metabolic functions of the bacterial cell at the molecular level involves the study of the bacterial DNA both genomic and plasmid. Studies on the genomic DNA not only reflect the size of the genomes but also allow for reasonable speculation

about the number of genes present. Apart from the necessity in subsequent mapping and sequencing, genomic DNA studies is a basic requirement in the construction of genomic DNA library.

The concentration of bacterial genomic DNA was assessed spectrophotometrically and data obtained are presented in Table 4.35.

Table 4.35. Quantification of genomic DNA

Sl No	Isolates	Vol (µl)	A_{260}	A_{280}	$A_{260/280}$	Conc(µ $g.ml^{-1}$)
1	*P. aeruginosa* (MTCC7815)	10	0.055	0.049	1.11	2.75
2	*Microbacterium* (G35-I)	-do-	0.023	0.028	0.82	2.02
3	*P. aeruginosa* (MTCC8163)	-do-	0.071	0.058	1.21	3.50
4	*A. faeculis* (MTCC8164)	-do-	0.020	0.027	0.74	1.82
5	*P. aeruginosa* (MTCC8165)	-do-	0.069	0.060	1.16	3.45
6	*B. licheniformis* (MTCC8166)	-do-	0.018	0.020	0.90	2.22
7	*B. circulans* (MTCC8167)	-do-	0.029	0.019	1.56	1.45
8	*P. fluorescens* (L490-II)	-do-	0.055	0.044	1.25	1.84
9	*P. aeruginosa* (L43-I)	-do-	0.02	0.022	0.90	2.22
10	*B. subtilis* (R38-I)	-do-	0.042	0.046	0.91	2.10
11	*P. aeruginosa* (MTCC7812)	-do-	0.058	0.059	0.99	2.90
12	*P. aeruginosa* (MTCC7816)	-do-	0.024	0.021	1.14	2.82
13	*P. aeruginosa* (MTCC7814)	-do-	0.102	0.081	1.28	5.01

The bacterial isolates *P. aeruginosa* (MTCC7814), followed by *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC8165) exhibited higher concentration of DNA contents with 5.01, 3.50 and 3.45 $\mu g.ml^{-1}$, respectively. The isolates *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7815) and *B. subtilis* (R38-I) possessed comparatively high amount of DNA with 2.90, 2.75 and 2.10 µg.ml-1, respectively. The isolate *A. faeculis* (MTCC8164) possessed very less amount of DNA with 1.82 $\mu g.ml^{-1}$ as compared to other bacterial isolates.

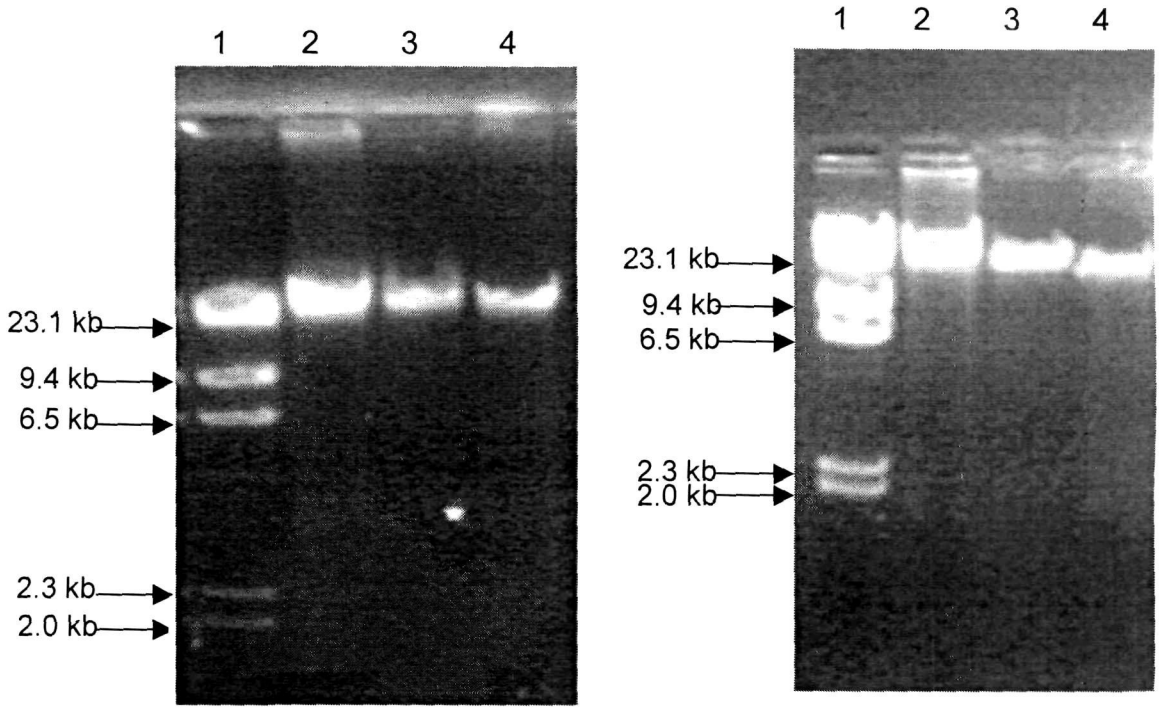


Fig.4.19. (a) Genomic DNA isolated from different bacterial isolates Lane 1: Marker (*Hind III* digested λ DNA), Lane 2 genomic DNA of *P. aeruginosa* (MTCC7815); Lane3: genomic DNA of *P. aeruginosa* (MTCC8163); Lane4: genomic DNA of *P. aeruginosa* (MTCC8163) (b). Lane 1: Marker (*Hind III* digested λ DNA); Lane 2: genomic DNA of *P. aeruginosa* (MTCC7815); Lane3: genomic DNA of *P. aeruginosa* (MTCC7814); Lane4: genomic DNA of *P. aeruginosa* (MTCC8165)

Distinct genomic DNA bands of the bacterial isolates were observed in agarose gel electrophoresis. The size of the genomic DNA of the isolates did not reveal much variation. The average size of the DNA was observed to be more than 23Kb.

4.5.2. Plasmid DNA isolation

The traditional view has been that an entire prokaryotic genome is contained in a single circular DNA molecule. Apart from the single chromosome, prokaryotes may also have additional genes on independent smaller, circular or linear DNA molecules called plasmids. Genes carried by plasmids are useful, coding for properties such as antibiotic resistance or the ability to utilize complex compounds such as hydrocarbons as a carbon source. One of the essential requirements in the development of strategies for the bioremediation of petroleum contaminated soil is the identification of relevant genes involved in the biodegradative pathways or any other gene (s) that may have a role to play in the degradation process in the selected bacteria. It is equally important to find out whether these genes are plasmid-borne or are borne by the genomic DNA.

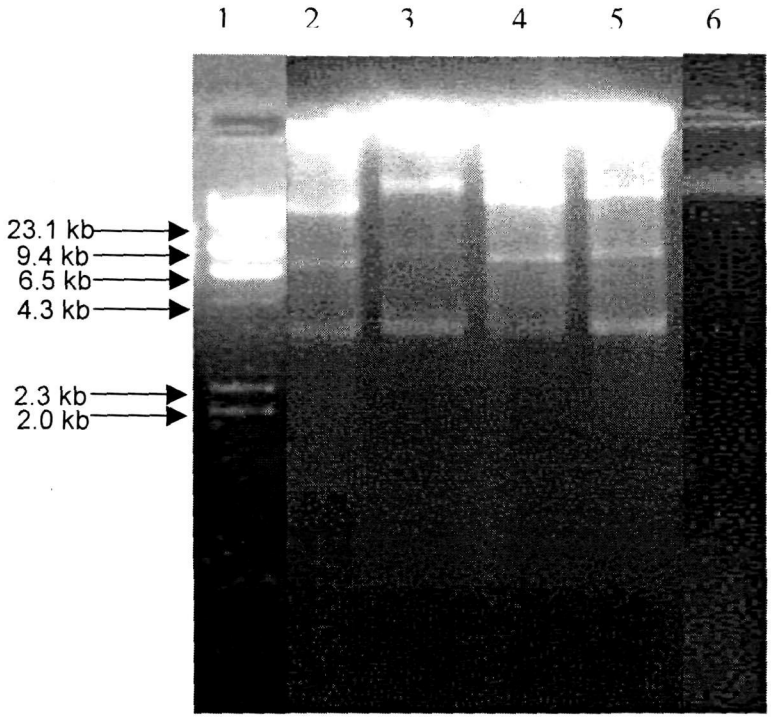


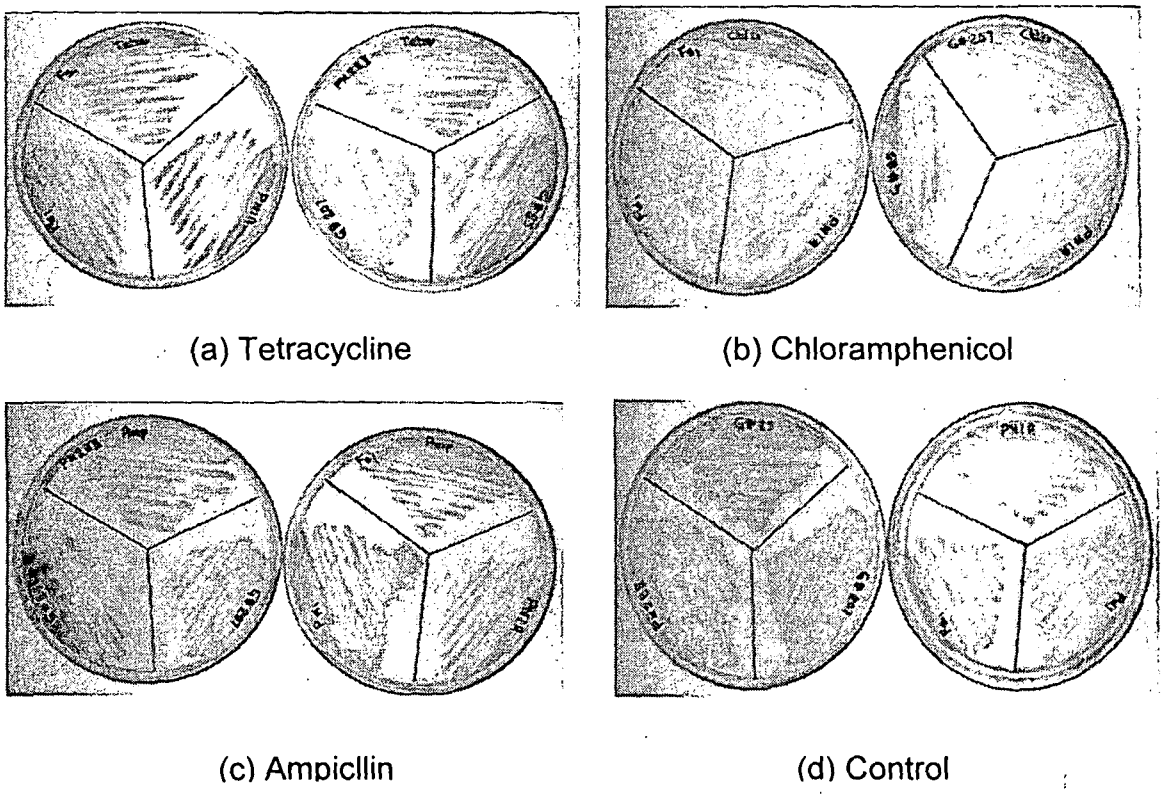
Fig.4.20. Plasmid DNA of bacterial isolates, Lane 1: *Hind III* digested λ DNA, Lane 2: *P. aeruginosa* (MTCC7815), Lane 3: *P. aeruginosa* (MTCC8163), Lane 4: *P. aeruginosa* (MTCC7812), Lane 5: *P. aeruginosa* (MTCC8165) and Lane 6: *P. aeruginosa* (MTCC7814)

Gel document presented in Fig. 4.19. represented the presence of plasmid in all tested bacterial isolates except *P. aeruginosa* (MTCC7814). Three bands exhibited by the bacterial isolates represented the conformations (supercoiled, nicked and linear) of plasmid DNA or there could be more than one plasmid present in an isolate.

4.5.3. Antibiotic sensitivity

Selective bacterial isolates were subjected to *in vitro* antibiotic sensitivity test. Antibiotics ampicillin (10 µg), tetracycline (30 µg), and chloramphenicol (30 µg) containing plates were used. Results are presented in Fig. 4.20. The bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa*

(MTCC7812), *P. aeruginosa* (MTCC8163), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) exhibited tetracycline, chloramphenicol and ampicillin resistance.



(a) Tetracycline (b) Chloramphenicol

(c) Ampicllin (d) Control

Fig.4.21. Resistance of bacterial isolates to antibiotics (a) tetracycline (b) chloramphenicol (c) ampicillin and (d) control (without antibiotics)

4.5.4. Plasmid curing

The elimination of plasmid DNA through the application of chemical agents like acridine orange and selective inhibition of plasmid DNA multiplication is called 'curing'. In the present study acridine orange was used to cure plasmid DNA from the selected bacteria in order to determine the location of the genes involved in the biosynthesis of biosurfactants.

Results

To determine the ideal concentration of acridine orange for plasmid curing, the bacterial isolates were incubated overnight at 32°C in LB broth having different concentrations of acridine orange ranging from 100 - 500 $\mu g.ml^{-1}$ following the method of Fujii *et al.* (1997). Samples of the resulting cultures in triplicate were plated on LB plates to estimate the ideal concentration at which at least 30% reduction of viability was achieved. In comparison to the control by bacterial isolates at 500 $\mu g.ml^{-1}$ displayed a reduction of viability by 50- 55%. Thus, this concentration of acridine orange at 500 µg/ml was used for curing.

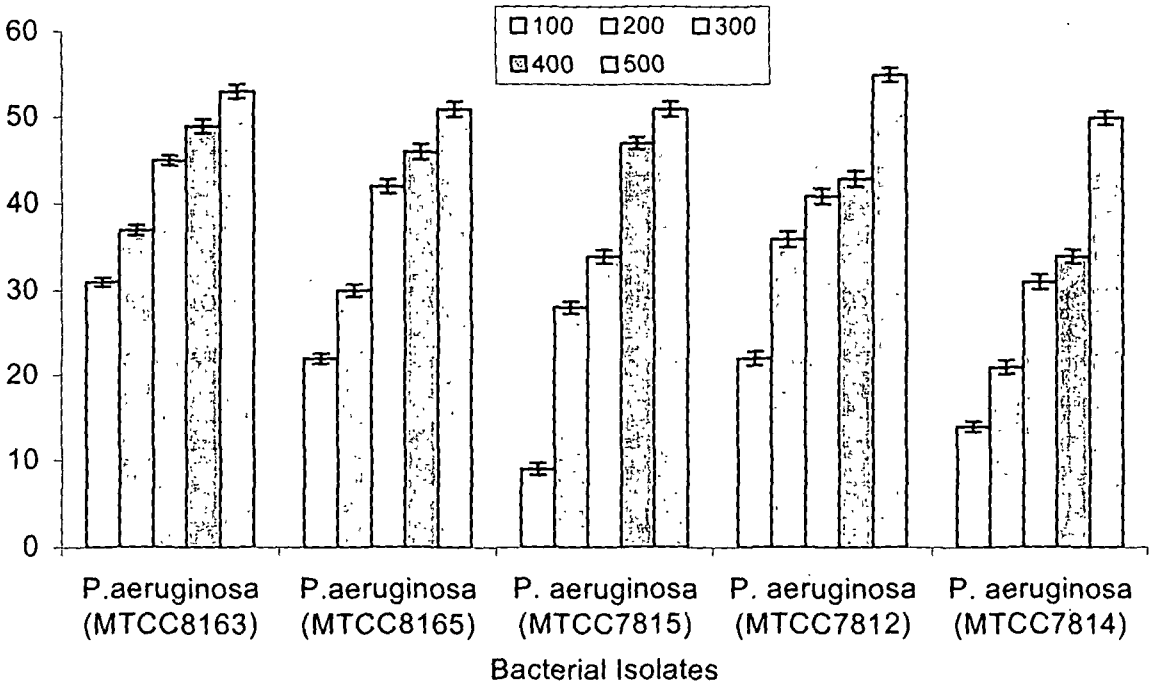


Fig. 4.22. Percent reduction of bacterial cfu on different concentration of arcridine orange

From the replica plates, colonies lacking the plasmid were isolated and recultured in the medium supplemented with 1% (v/v) hexadecane. The colonies failed to grow on this hexadecane-supplemented medium, but found to grow on the normal medium (LB). On the other hand, isolates having

plasmid as usual grew on medium supplemented with hexadecane and reduced the surface tension of the culture medium.

The standard procedure was followed for the isolation of plasmid DNA from normal and cured bacterial isolates. From the cured isolates, no plasmid DNA was obtained. Isolated DNAs were electrophoresed and the photograph of the gel is presented in Fig.4.22. The figure reveals that no plasmid DNA could be obtained from the cured bacterial isolates failing to grow in hexadecane supplemented medium and being antibiotic sensitive. Subsequently, the uncured bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) along with antibiotic resistance possessed the presence of plasmid DNA in the gel. The plasmid curing experiment proved that the gene(s) involved in both hydrocarbon degradation and antibiotic resistance were present in plasmid DNA.

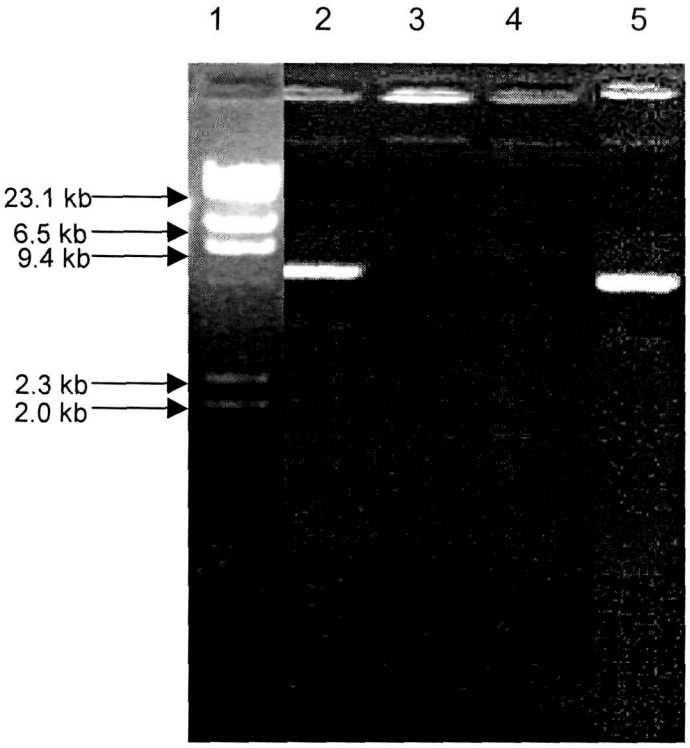


Fig.4.23. Plasmid DNA of bacterial isolates with or without cured plasmid; Lane 1: *Hind* III digested λ DNA Lane 2: *P. aeruginosa* (MTCC7815), Lane 3: isolate *P. aeruginosa* (MTCC7815) cured of plasmid DNA, Lane 4: Isolate *P. aeruginosa* (MTCC8163) cured of plasmid DNA and Lane 5: *P. aeruginosa* (MTCC8163)

4.5.5. Restriction digestion

Plasmid DNA of each isolate was subjected to restriction digestion by *Eco*RI. Digestion patterns of the plasmid DNA from the bacterial isolates are presented in Fig.4. 23. In the case of plasmid DNA of *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7814), two restriction sites with a small size difference were observed. The size of the bands was about 6.5 kb and 5.2 kb (between 6.5 to 4.3 kb). In the case of *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC8165), a single restriction band at about 6.2 was observed. The restriction digestion of the plasmid DNA of the isolates revealed the presence of polymorphism among the bacterial isolates.

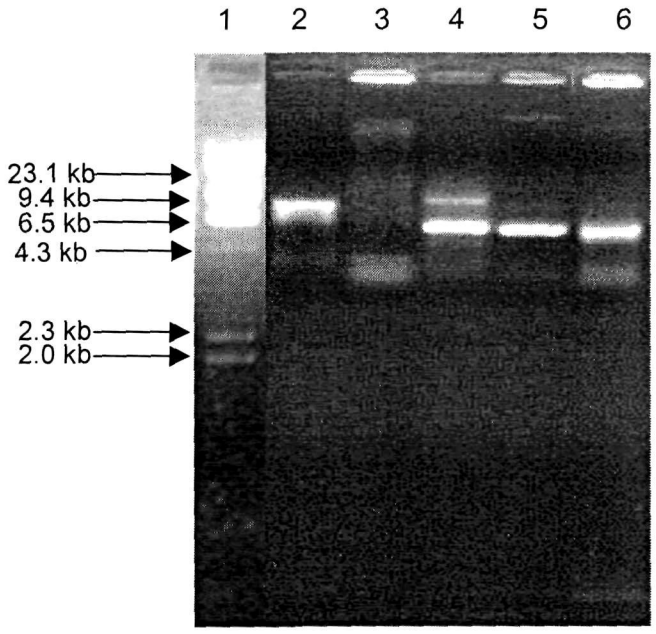


Fig.4.24. *Eco* RI restriction digestion pattern of plasmid DNA of bacterial isolates; Lane 1: *Hind* III digested λ DNA, Lane 2: *P. aeruginosa* (MTCC7815), Lane 3: *P. aeruginosa* (MTCC7812), Lane 4: *P. aeruginosa* (MTCC8163), Lane 5: *P. aeruginosa* (MTCC8165) and Lane 6: *P. aeruginosa* (MTCC7814)

4.5.6. Transformation experiments

Bacterial isolates cured off the plasmid were found to loss the ability to degrade hydrocarbons and also to produce biosurfactant. Transformation experiment were carried out using the bacterial combinations like; *P. aeruginosa* (MTCC7815) x SS2 (thermophilic non-plasmid containing), *P. aeruginosa* (MTCC7815) x MSC1 (thermophilic non-plasmid containing), *P. aeruginosa* (MTCC8163) x SS2 and *P. aeruginosa* (MTCC8163) x MSC1. Prior to transformation, competent cells of SS2 and MSC1 were prepared. The donor isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) were resistant to tetracycline whereas the thermophilic isolates were sensitive. Therefore, using the tetracycline resistance the transformants were selected. Data thus obtained are presented in Fig. 4. 24. The thermophilic bacterial isolates SS2 and MSC1 after the transformation experiment exhibited growth on LB agar plates supplemented with tetracycline at 75°C. However, these transformants failed to grow on liquid medium supplemented with tetracycline. The study therefore requires further investigation.

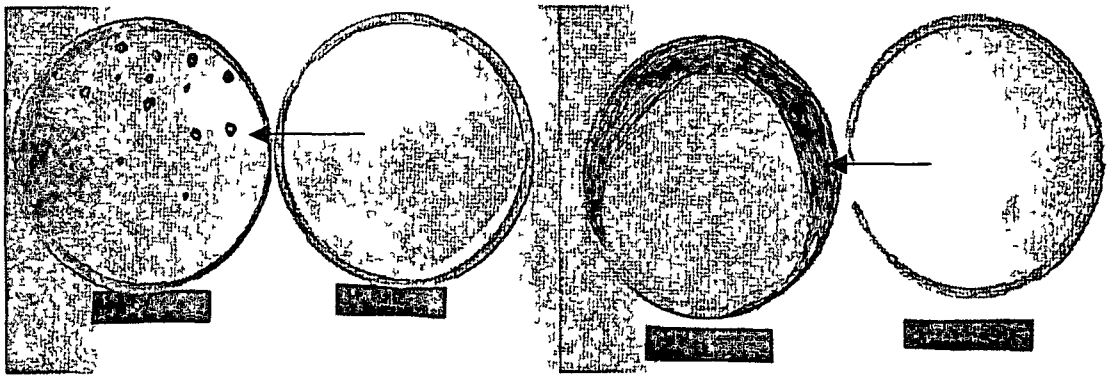


Fig 4 25 Selection of transformed colonies by antibiotic sensitive marker, (a) tetracycline resistance transformed colonies SS2, (b) tetracycline sensitive SS2 strain (control), (c) tetracycline resistance transformed colonies of MSC1 and (d) tetracycline sensitive MSC1 strain (control)

Chapter 5
Discussion

Chapter 5

5. Discussion

Wide diversity exists among hydrocarbon degrading and biosurfactant producing microorganisms, suggesting it to be an important survival tool for the producing microbes and biosurfactant production appears to have evolved in an independent yet parallel fashion (Bodour *et al.*, 2003). Bacteria are known to attack specific alkenes, aromatic, polycyclic aromatic and polar NSO compounds. Crude oil is a complex mixture of such different compounds. Oil spill from various petroleum related processes leads to a significant impact on the environment (Riser-Roberts, 1998; Norris *et al.*, 1994). The petroleum contaminants may remain on ground surface, volatilize into the atmosphere, or leach through subsurface, resulting in extensive pollution concerns. An effective biodegradation of crude oil would require simultaneous action of several metabolically versatile microbes. Atlas (1977) suggested that complex mixture of hydrocarbon degraders would be necessary in order to effectively degrade all the hydrocarbons in a complex petroleum mixture. The role of mixed populations of microorganisms in the degradation of recalcitrant xenobiotics is well recognized. However, in a mixed culture system, the growth of microorganisms cannot be regulated due to the nutrient stress and competition. Many studies of microorganisms capable of growing on oil have been dealt with single microorganism degrading a single hydrocarbon (Cooney, 1980). The effect of single microorganism on multiple substrates and, conversely, the effect of multiple organisms on a single substrate are poorly understood. However, mixed population studies show that some compounds that are resistant to degradation by a single organism can be degraded by a mixed population (Beam and Perry, 1974). Microbial interactions in the soil are very complex and undoubtedly play an important role in the transformation or decomposition of hazardous waste

components. In a commensalistic relationship, microbes cannot oxidize a given hydrocarbon individually, but collectively they can. Some members of a community might be able to provide important degradative enzymes, whereas others may supply surfactants or growth factors (Wiesel *et al.*, 1993).

5.1. Screening and isolation of petroleum hydrocarbon degrading bacteria

5.1.1. Isolation and pure culture of bacteria

In screening experiments, a total of 58 pure bacterial isolates (Table 4.2) were recovered from crude oil contaminated soil, effluent and engine oil- contaminated soil samples on the basis of their morphological variations. Enrichment cultures were conducted for all 58 isolates in media supplemented with hexadecane. The isolates displayed differential abilities with respect to utilization of hexadecane as the sole source of energy and carbon. Mac Elwee *et al.* (1990) and Arino *et al.* (1996) reported that only 27.2%, 25% and 41.6% of the culturable microbes obtained from petroleum sludge, petroleum contaminated soil and petroleum-contaminated effluent samples respectively, were efficient for hydrocarbon degradation and biosurfactant production. *Pseudomonas* strains were isolated from soils contaminated by water-insoluble compounds such as petroleum products indicating their ability to degrade petroleum hydrocarbons.

5.1.2. Density of bacteria in different samples

Plate counting was carried out for determining the bacterial density. The density of bacteria in the samples from the surface soil was found to be higher in comparison to the samples drawn from the subsurface (Table 4.1). There are several reports on the greater microbial diversity on the soil surface environment than in the subsurface (Dunbar *et al.*, 2002; Girvan *et al.*, 2003 and Liles *et al.*, 2003). It is not clear whether the subsurface microbial community is closely tied to the surface soil microbial community

or is an independent ecosystem with a distinct assemblage of microorganisms. Also, subsurface communities are isolated from each other to a greater degree than are surface soil communities. In surface soil, Aeolian transport, flooding, and other mechanisms can move and mix soils over significant distances. There is much less opportunity for this type of transport in the subsurface. Thus, community differences between sites separated even by relatively small distances are potentially greater for subsurface communities (Zhou *et al*, 2003). The relatively higher density of bacteria in the samples from the surface soil in comparison to the same from the subsurface ones in the present study might be viewed in the context of the above findings.

5.1.3. Morphological characteristics of bacteria

Morphological studies of bacteria in terms of colony size, pigmentation, form, margin and elevation displayed wide variations (Table 4.2.). Morphological diversity existed among the microbial communities at the petroleum contaminated surface and sub-surface soil environments. Similar observations were reported by many workers like Borneman and Triplett. (1997), Dunbar *et al.* (2002), Girvan *et al.* (2003) and Liles *et al.* (2003). Zhou *et al.* (2003) hypothesized that spatial isolation and resource heterogeneity were the key mechanisms controlling microbial community diversity and structure. Based on these hypotheses, it could be considered that the levels of morphological diversity of petroleum contaminated soil environments might be reflective of the unequal distribution of carbon content.

5.1.4. Hydrocarbon degrading bacteria

In the present investigation, out of 58 bacterial isolates, only 13, *viz.*, *P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC8163), *A. feacalis* (MTCC8164), *P. aeruginosa* (MTCC8165), *B. licheniformis* (MTCC8166), *B. circulans*

(MTCC8167), *P. fluorescens* (L490-II), *P. aeruginosa* (L43-I), *B. subtilis* (R38-I), *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC7816) showed better growth in hexadecane-supplemented medium in terms of dry biomass yield. These isolates were considered to be promising for the degradation of hydrocarbons (Table 4.3). Using a very limited screening method Bodour *et al.* (2003) reported that 20 of 21 soil samples (including uncontaminated, hydrocarbon-contaminated, metal contaminated and hydrocarbon-metal co-contaminated) contained at least one biosurfactant producing isolate. The association of biosurfactant production with microbial degradation of hydrocarbons is well established. In fact, most of the reports on biosurfactant production have come from the research works on hydrocarbon fermentation (Cooper and Zajic, 1980; Zajic and Steffens, 1984). Therefore, an effort was made to recover the hydrocarbon degrading and biosurfactant producing bacterial isolates by enrichment culture on n-hexadecane-supplemented medium as the carbon source. The enrichment culture technique was extremely useful in the selection and the subsequent isolation of the hydrocarbon degrading bacteria. These bacteria were further assessed for biosurfactant production.

5.1.5. Growth rate studies

Studies on growth parameters of the bacterial isolates revealed considerable variation. This variation was not unexpected as the bacterial community was found to have diversity both at the surface and subsurface soil environments. Bioavailability of nutrients as well as the conditions influencing the growth of bacterial isolates possibly lead to differential growth performance.

5.1.6. Degradation studies

The major objective of the present study was to isolate soil bacteria having the potential to degrade contaminating petroleum hydrocarbons for developing a suitable consortium. Bacterial isolates *P. aeruginosa*

(MTCC7815), *P. aeruginosa* (MTCC8163), *B. licheniformis* (MTCC8166), *P. aeruginosa* (MTCC8165) and *B. subtilis* (R38-I) exhibited better growth in crude oil supplemented medium with increased optical density (O.D.) values of 1.76±0.2 to 1.25±0.2 during the culture period (Table 4.5). In hexadecane supplemented medium, isolates *P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *B. circulans* (MTCC8167), *P. aeruginosa* (L43-I), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8165) and in benzene - supplemented medium the isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I), *Microbacterium* (G35-I) and *P. aeruginosa* (MTCC8165) exhibited better growth as compared to other isolates. The growth of the isolates in benzene and petroleum benzene supplemented media was poor.

The alkane fraction of the crude oil was degraded faster than the aromatic fraction. The degree of degradation of *n*-alkanes was higher, independent of the initial level of contamination. These results are in agreement with the observations of Coulon *et al.* (2005) and Seklemova *et al.* (2001); but in contrast with those of De Jonge *et al.* (1997) who reported that at higher concentrations (1200 - 4000 mg. kg^{-1}), *n*-alkanes were degraded at the same rate regardless of their chain length. The degradation of aromatic hydrocarbons was dependent on the incorporation of molecular oxygen in to the aromatic ring or alkyl constituent (Leahy *et al.*, 2002). The effect of toxicity, enzyme inhibition, and oxygen limitation were found to influence the rate of degradation of high concentration of aromatic hydrocarbon (Leahy *et al.*, 2002). Based on these observations it was concluded that the rate of degradation of aromatic hydrocarbons is inversely proportional to its concentration.

In a separate experiment, for enhancing the degradation of crude oil and aromatic hydrocarbons, the synthetic surfactant Triton X100 (60 μg/ml v/v) was added to the medium. The bacterial isolates *P. aeruginosa* (MTCC8165), *B. circulans* (MTCC8167), *P. aeruginosa* (L43-I), *P. fluorescens* (L490-II) and *Microbacterium* (G35-I) exhibited better growth

(Table 4.6) in crude oil supplemented medium as compared to the control (no Triton X100). The bacterial isolates *B. licheniformis* (MTCC8166), *P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8165) exhibited better growth with O.D. values of 0.45 – 0.17 in petroleum benzene containing medium supplemented with Triton X100 as compared to the ones without it.

The rate of biodegradation of hydrocarbons is dependent upon several physicochemical as well as biological parameters. The ability of hydrophobic compounds to be solubilized and transported into bacterial cells capable of metabolizing them is potentially the rate limiting step in biodegradation (Churchill *et al.*, 1995). Transport limitations have been demonstrated in a study of alkane oxidation (Zhang and Miller, 1992). Churchill *et al.* (1994) reported that addition of purified rhamnolipid, the non-ionic surfactants Triton X-100, Tween 80 and the oleophilic fertilizer Inipol EAP 22 enhanced octadecane dispersion and biodegradation. Thus, the results of the present experiments are therefore in agreement with these findings.

5.1.7. Growth of bacterial isolates in different carbon sources

The rate of utilization of glucose and fructose by the bacterial isolates was higher in the exponential growth phase than in the stationary phase with increased O.D. values. In glucose and fructose -supplemented medium, the isolates *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8163) exhibited higher growth rate after the initial 24h of culture in terms of O.D. values as compared to other bacterial isolates (Fig.4.1 a-b) at 96 h of culture. However, in the case of bacterial isolates *P. aeruginosa* (MTCC8165) and *B. circulans* (MTCC8167), the O.D. value increased with the increase of incubation time from 24 – 72 h; thereafter the growth rate gradually decreased.

5.1.7.1. Growth of bacterial isolates in aliphatic hydrocarbons

The bacterial isolates having the potentiality to degrade aliphatic hydrocarbons were assayed. Among the tested hydrocarbons, the isolates *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) exhibited the highest growth (2.78 – 3.15), (3.56 - 2.85) and (2.35 - 2.26) respectively in dodecane, hexadecane and octadecane-supplemented media (Fig.4.1. c - e). However, the bacterial isolate *P. aeruginosa* (MTCC7815) exhibited better performance with O.D. value of 3.56 in hexadecane-supplemented medium as compared to other bacterial isolates in tested aliphatic hydrocarbons. There are various reports on degradation of short carbon chain (C_8–C_{16}) to very long carbon chain (C_{44}) hydrocarbons (Tsao *et al.*, 1998). Stronguilo *et al.* (1994) reported loss of *n*-alkane from the contaminated soil by both abiotic and biotic processes, the former being more effective for short- chain alkanes and those between C_{10} and C_{25} being most suitable as substrates for microorganisms (Bartha and Atlas, 1977). Degradation of aliphatic hydrocarbon is influenced by the chain length. As the chain length of the alkanes increases, the molecule becomes less soluble in water. However, at chain lengths of C_{11} - C_{12} and above, the liquid *n*-alkane are accommodated in water at a higher concentration than that extrapolated from the solubility of a series of lower alkanes. This might be due to a change in the structure of the water molecules surrounding the alkanes. During the microbial utilization of long- chain liquid alkanes, microbial attachment to droplets of alkane is seen with transport of long-chain alkanes through the cell membrane. Chawala and Lu-Kwang (2000) reported that the hydrophobic hydrocarbons in the environment were often absorbed or trapped in pores by capillary action; thus they were not readily accessible to microbes. Some microorganisms produce extracellular biosurfactants that solubilize and facilitate the penetration of hydrocarbons through the hydrophilic cell wall; these hydrocarbons could be degraded by

enzymes integrated with the cytoplasmic membrane (Syldatk and Wagner, 1987).

The variation in the pattern of degradation of the aliphatic hydrocarbons used in the present investigation might be viewed in the context of bioavailability of the hydrocarbons, which might vary depending on the kind of bacterial isolate and also the differential activities of the enzymes involved in the isolates during the process of degradation.

5.1. 7.2. Growth of bacterial isolates in aromatic hydrocarbons

In the present investigation bacterial isolates were grown in media containing aromatic hydrocarbons like benzene, toluene and xylene. The bacterial isolates *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) exhibited the highest growth rate (0.45, 0.41), (0.45, 0.42) and (0.55, 0.48) at 96 h of culture {Fig. 4.1. (f - h)}. However, the isolate *P. aeruginosa* (MTCC7815) possessed better growth performance (0.55) in xylene-supplemented medium as compared to other aromatic hydrocarbons as well as other bacterial isolates. Degradation of chlorinated aromatics in aerobic condition suggests the need for the mixed – function of oxidases to bring about dehalogenation and ring cleavage of these compounds (Bitton and Gerba, 1985). The present results are in agreement with the observations of Jones and Edington (1968) who reported that only 0.5% of a large group of soil organisms could use benzene as the sole source of carbon. Fewson (1981) reported that toluene could undergo two types of microbial attack: (1) immediate hydroxylation of the benzene nucleus, followed by ring cleavage or (2) Oxidation of the methyl group, followed by hydroxylation and cleavage of ring. The ability to use aromatic compounds can be an induced phenomenon in bacteria (Claus and Walker, 1964). The variation in the genetic make up of the bacterial isolates could exert influence in the process of degradation of the aromatic hydrocarbons.

5.1.7.3. Growth of bacterial isolates in polycyclic aromatic hydrocarbons

PAHs, also called polynuclear aromatics (PNAs), constitute a class of hazardous organic chemicals, made up of two or more fused benzene rings in linear, angular, or cluster arrangements, containing carbon and hydrogen (Cerniglia, 1992; Edwars, 1983). Keith and Telliard (1979) reported that PAHs are in the priority pollutant list of the US Environmental Protection Agency (EPA) due to their carcinogenic and mutagenic nature. Cerniglia (1992) reported that complete mineralization of high molecular weight PAHs could be achieved by only a limited number of microorganisms.

The isolate *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) exhibited the highest growth (0.58, 0.49, and 0.45) and (0.58, 0.47, and 0.44), respectively in phenanthrene and pyrene -supplemented medium (Fig. 4.1. i-j); in fluorene-supplemented medium, *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *P. aeruginosa* (L43-I) (0.40, 0.37, and 0.31) and in carabazole - supplemented medium, *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8165) and *B. subtilis* (R38-I) (0.46, 0.32, and 0.300. Park *et al.* (1988) reported that losses of PAH compounds by abiotic processes might be important for low molecular weight but not in high molecular weight PAHs.

In a separate experiment, the promising bacterial isolates were tried in minimum salt medium-supplemented separately with different concentrations of polycyclic aromatic hydrocarbons. The isolates *P. aeruginosa* (MTCC7815), *B. subtilis* (R38-I) and *P. aeruginosa* (MTCC8163) showed the best growth at the hydrocarbon concentration of 200 $\mu g.l^{-1}$ (Table 4.7). In the ambient conditions, the rate of mineralization of several PAHs was found to be influenced by the concentrations of the compounds. Leahy *et al.* (2002) reported that the effects of toxicity, enzyme inhibition, and oxygen limitation were minimized by using relatively low concentrations

(0.2 mg. L^{-1}) of each hydrocarbon. This observation is in agreement with the results of the present investigation as the bacterial isolates showed the highest efficacy in PAHs utilization with their low concentrations. In some cases, there is a direct correlation between the higher rate of mineralization and the higher concentration of the compounds (Wiesel *et al.*, 1993). Findings contrary to this, indicating an inverse relationship between the rates of mineralization and concentrations of the compounds were also reported (Shiaris *et al.*, 1984).

5.1.7.4. Growth of bacterial isolates in other hydrocarbons

Pyridine occurs in the environment as a by-product of coal gasification (Stuermer *et at.*, 1982) and retorting of oil shale (Leenher *et al.*, 1982). Pyridine causes moderately acute toxicity and is apparently teratogenic (Jori *et al.*, 1983). Biodegradation of pyridine under aerobic conditions has been studied extensively by various workers (Shukla and Kaul, 1975; Watson and Cain, 1975 and Lee *et al.*, 1991). Shukla and Kaul (1975) and Watson and Cain (1975) reported that a number of soil bacteria were capable of growth on pyridine as the sole source of carbon. Some research groups investigated bacterial metabolism of unsubstituted pyridine ring (Korosteleva *et al.*, 1981). Only aliphatic intermediates of pyridine metabolism were identified, because no researchers could produce cell extracts capable of degrading the intact pyridine ring. In the present investigation, the bacterial isolates *P. aeruginosa* (MTCC7815) followed by *B. subtilis* (R38-I) and *Microbacterium* (G35-I) exhibited comparatively higher growth in pyridine - supplemented medium with O.D. values (0.45, 0.38 and 0.35) respectively at 96 h of culture as compared to other bacterial isolates (Fig.4.1.m). The result is in agreement with Desimone *et al.* (2002, 2003) and Krisch and Szajani, (1997); and this could be used to increase microbial pyridine transformation in the presence of other toxic compounds such as phenol. Kim *et al.* (2006) reported that use of immobilized *Pseudomonas sp.* MK1 for the biodegradation of pyridine in industrial waste-

water containing phenol. In the case of paraffin - supplemented medium, the isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8163), *B. subtilis* (R38-I) and *P. aeruginosa* (L43-I) exhibited the highest growth (2.15, 1.95, 1.75 and 1.50), respectively as compared to other bacterial isolates. In 2, 4-D - supplemented medium, the isolates *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC8163) and *B. subtilis* (R38-I) exhibited better growth (0.40, 0.36 and 0.36 respectively) as compared to other bacterial isolates.

5.1.8. Growth of bacterial isolates at different pH levels

Biodegradation of hydrocarbons is affected by pH through the availability of nutrients, toxicants and the tolerance of organisms to pH variations. Some microorganisms can survive within a wide pH range, while others can tolerate only small variations. In the present investigation, promising bacterial isolates were assessed in hydrocarbon - supplemented medium having acidic to alkaline pH (2 – 10). In highly acidic (pH 2) and alkaline (pH 10) conditions, the growth of bacterial isolates was poor with (0.2 – 0.35 $g.l^{-1}$) dry biomass production {Fig.4.2 (a - e)}. In pH conditions 4.0 – 10.0, the bacterial isolate *P. aeruginosa* (MTCC7815) possessed the better growth with higher biomass yield. Thus, the isolate was identified to be the most efficient at a wide pH range of 4.0-10.0. Roberts (1989) reported that the optimum pH range for the rapid degradation of waste and hydrocarbon residues was usually in the range of 6.5 – 8.5. The pH optima for bacteria and actinomycetes were near 7.0. Dibble and Bartha (1979) reported the soil pH of 7.8 to be close to the optimum. Parr *et al.* (1983) reported that pH could influence the solubility or availability of macro- (especially phosphorus) and micronutrients, the mobility of potentially toxic materials, and the reactivity of minerals (e.g., iron and calcium).

5.1.9. Growth of bacterial isolates at different temperatures

On the basis of better degrading ability, the bacterial isolates were also cultured in 1% (w/v) glucose or 1% (v/v) hexadecane - supplemented

medium at temperatures ranging from 30 - 45°C. At 30-37°C, the bacterial isolates *P. aeruginosa* (MTCC7815), *P. fluorescens* (L490-II) and *P. aeruginosa* (MTCC8163) exhibited higher growth with increased dry biomass production irrespective of the carbon source used. At the high temperature of 45°C, all bacterial isolates failed to survive in both glucose and hexadecane supplemented media. Nedwell (1999) reported that the temperature plays a significant role in controlling the nature and extent of microbial metabolism of hydrocarbons. Temperature affects the rate of biodegradation, as well as the physical nature and chemical composition of hydrocarbons (Whyle *et al.*, 1998; Rowland *et al.*, 2000). Although microbial activity is generally reduced at low temperature, many of the components in crude oil and diesel could actually be degraded by psychrophilic and psychrotrophic microorganisms (Leahy and Colwell, 1990; Whyle *et al.*, 1998; Margesin and Schinner, 1999; Delille, 2000; Gibb *et al.*, 2001; Baranieck *et al.*, 2002 and Eckford *et al.*, 2002). Parr *et al.* (1983) reported that most of the soil microorganisms were mesophiles and they exhibited maximum growth in the range of temperature 20 - 35°C. The majority of hydrocarbon utilizers were the most active in this temperature range. Since, many organisms could multiply well at laboratory temperatures of 25- 37 °C but not at lower environmental temperatures (Alexander, 1994). Coulon *et al.* (2005) reported increase of microbial metabolism in each 10°C increase in temperature from 10 to 40 °C.

5.1.10. Morphological and Biochemical tests for taxonomic identification of bacterial isolates

Morphological and biochemical tests are widely used for the identification of bacteria. Biochemical tests are based on the facts that there are a large number of different kinds of cellular metabolites, and the biochemical transformations that occur both outside and inside the cell are governed by biological catalysts called enzymes and that different microorganisms have different types of these enzymes.

Analyses of the data with respect to the tests on Voges-Proskauer, methyl red, hydrolyses of casein, gelatin, starch, nitrate reduction, H_2S and indole production, litmus milk production and acid from D- glucose reveal that all seven isolates identified as *Pseudomonas aeruginosa* mucoid and non- mucoid shared similar properties. Fifty percent of the isolates were found to be positive for sucrose fermentation, lipid hydrolysis, citrate utilization and triple sugar iron. While most of these isolates were positive for catalase, the isolates and L43-I were negative. Similarly the isolates G207-II, L43-I and Fs_1-I were found to be negative with respect to lactose fermentation, the remaining 5 isolates were positive for the test. The isolate G207-II was the only one displaying negative result for dextrose fermentation. The isolate FS_1-I was of interest as it was the lone isolate which could not form acid from xylose and manitol. Only three isolates L64-I, Pw_1-I and Fs_1-I were found to be negative for urease production. The isolates characterized were sent to the Institute of Microbial Technology, Chandigarh for the confirmation of identification.

In this investigation, biochemical characterization of bacterial isolates L485-II, L253-II and R38-I showed negative results with respect to catalase, VP, MR, casein and starch hydrolysis, nitrate reduction, H_2S and indole production, acid from D-glucose, D-xylose and manitol and citrate utilization. All the isolates were found to be positve for activities on lactose, dextrose fermentation, triple sugar iron and peptonization of litmus milk. While the isolates L485-II and L253-II were negative for sucrose fermentation, the isolate R38-I was positive for the same. Similarly the isolates L253-II and R38-I were found to be negative with respect to casein hydrolysis. Similar trend was observed in the case of urease production. The isolate L485-II were found to be an exception with respect to gelatin liquefacation because the other two isolates had no activity on gelatin.

5.1.11. Growth of bacterial isolates in consortia

In situ degradation of toxic components of hydrocarbons by microbes could be an effective bioremediation process. However, strain specificity towards different components of the hydrocarbon necessitated developing effective consortia for the purpose. Accordingly, the selected bacterial isolate were grouped in to 24 combinations and their growth with optical density was determined in crude oil supplemented medium. In this investigation, the combination Nos. 12 and 24 {Fig.4.4 (a) and (Fig.4.5. b)} exhibited the highest growth rate with increased O.D. values (3.75) at 10^{th} day of culture as compared to other combinations. Sorkhoh *et al.* (1995) reported that microbial populations consisting of strains that belong to various genera were detected in petroleum contaminated soil or water. They strongly suggested that each strain or genera had their roles in the hydrocarbon transformation processes. Further evidence for the cooperation of mixed cultures in biodegradation was apparent when Sorkhoh and co-workers (1995) observed a sequential change of the composition of the oil-degrading bacteria over a period of time in sand samples that were contaminated with oil. Venkateswaran and Harayama (1995) reported similar observations in sequential enrichments in medium containing residual crude oil. In an earlier study using pure cultures, it was reported that after exhaustive growth of one strain on crude oil, the residual oil supported the growth of a second and third strain of bacteria (Horowitz *et al.*, 1975). Rambeloarisoa *et al.* (1984) demonstrated a consortium of 8 strains made up of members of 6 genera to be able to effectively degrade crude oil. Mishra *et al.* (2001) reported that a population size of hydrocarbon degrading bacteria at the contaminated site 10^3 cfu.g^{-1} was much negligible, the ideal would be above 10^5 cfu.g^{-1} of soil. He strongly suggested the application of soil bacterial consortium for the effective bioremediation of contaminant crude oil. In contrast to the limited degradation efficiency of pure bacterial isolates in the crude oil contaminated soil, development of

bacterial consortium is useful for the effective bioremediation of crude oil contaminated soil.

5.1. 11.1. Efficacy of bacterial isolates to degrade hydrocarbons

All bacterial isolates tried exhibited better growth as displayed by increased protein concentration in the hexadecane-supplemented medium. It was evident from the results that, against the increased concentration of bacterial protein there was a decreased concentration of hexadecane [Fig.4.5. (a) - (j)]. Increased bacterial biomass production against the decreased concentration of hexadecane exhibited the efficacy of the bacterial isolates to degrade hexadecane. However, the strains *B. licheniformis* (MTCC8166), *A. faeculis* (MTCC8164) and *P. aeruginosa* (MTCC8163) were found to be slow degrader- cum- grower in hexadecane supplemented medium. Out of various hydrocarbon components tried, hexadecane followed by glucose and phenanthrene was more ideal for increased biomass production of the bacterial isolates. The process of biodegradation of contaminating petroleum hydrocarbon is influenced by various factors. Among the environmental factors associated with the biodegradation, the most important one is the availability of nutrients particularly in high altitude regions (Walworth and Reynolds, 1995; Braddock *et al.*, 1997 and Mohn and Stewart, 2000).

5.1.11.2. Development of bacterial consortia

The ability of designed bacterial consortia with wide hydrocarbonoclastic capacity was employed for degradation of various fractions of crude oil. In the present investigation, two bacterial consortia comprising of 10 bacterial isolates [*P. aeruginosa* (MTCC7815), *Microbacterium* (G35-I), *P. aeruginosa* (MTCC8163), *P. aeruginosa* (MTCC8165), *P. fluorescens* (L490-II), *P. aeruginosa* (L43-I), *B. licheniformis* (MTCC8166), *A. faecalis* (MTCC8164), *B. circulans* (MTCC8167) and *B. subtilis* (R38-I)] were developed for the bioremediation

of contaminant-crude oil in soil. The morphologically and genetically different isolates were selected on the basis of their substrate utilization range. In designing the consortia, a key aspect was the solubility and accessibility of the hydrophobic compounds available in the crude oil. Since, only 0.02% of crude oil was water soluble there is a need for emulsification (Suneel et *al.*, 1996) of the crude oil in the medium. Since, the isolates like *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC8163), *P. aeruginosa* (MTCC8165), *P. fluorescens* (L490-II), *B. licheniformis* (MTCC8166) and *A. faecalis* (MTCC8164) were capable of producing extracellular biosurfactants by utilizing short chain aliphatics like hexadecane, dodecane and octadecane; they were included in the consortia as members. Four other isolates were selected due their specialized metabolic potential of utilizing different fractions of crude oil. The ability of bacteria to use each other's metabolic intermediates as growth substance and also to produce biosurfactant is wide spread among the bacterial and aracheal domains. Hydrocarbon degrading and biosurfactant producing microbes were isolated from a wide diversity of environments including soil, seawater, marine sediments and oil fields (Maier, 2003). Ghazali *et al.* (2004) reported that biodegradation of complex hydrocarbons usually required the cooperation of more than a single species. This is particularly true in pollutants that are made up of many different compounds such as crude oil or petroleum and complete mineralization to CO_2 and H_2O is desirable. Individual microorganisms could metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further. Suneel *et al.* (1996) reported that microorganisms were known to attack specific saturates, aromatic and polar compounds. Crude oil is a complex mixture of such different compounds. An effective degradation of crude oil would require simultaneous action of several metabolically versatile microbes. An oil spill in the environment leads to an adaptive process and if metabolically active hydrocarbonoclastic organisms could be added quickly, the lag period

before the indigenous population could respond would be reduced considerably. The necessity for seeding with complementary hydrocarbon degrading bacteria arises from the rationale that indigenous microbial populations may not be capable of degrading a wide range of potential substrates in a complex mixture such as crude oil. Atlas (1977) suggested that complex mixture of hydrocarbon degrader would be necessary in order to degrade effectively hydrocarbons in a complex petroleum mixture. The role of mixed populations of microorganisms in degradation of recalcitrant xenobiotics is recognized. However, in a mixed culture system, the growth of the organisms cannot be regulated due to the nutrient stress and competition. In this investigation, the ability of the designed bacterial consortium with wide hydrocarbonoclastic capacity was employed for degradation of various fractions of crude oil.

Further evidence for the cooperation of mixed cultures in biodegradation became apparent when Sorkhoh *et al.* (1995) observed a sequential change of the composition of the oil – degrading bacteria over a period of time in sand samples that were contaminated with oil. Venkateswaran and Harayama (1995) reported similar observation in sequential enrichment in medium containing residual crude oil. Rambeloarisoa *et al.* (1984) demonstrated a consortium of 8 strains made up of members of 6 genera to be able to effectively degrade crude oil.

5.1.11.3. Degradation of crude oil by bacterial consortia and isolates

On the basis of the growth performance as depicted by the dry biomass yield, out of the 10 bacterial isolates and their 12 combinations tried in crude oil supplemented liquid medium (Table 4.11), the combination 10 and 11, named as consortium I and II, exhibited the highest dry biomass production (3.34 ± 0.1 and 3.33 ± 0.1 $g.l^{-1}$) in 96 h of culture, respectively. The bacterial consortia were found to adapt to the local soil environment as the partner isolates were obtained from the local hydrocarbon contaminated sites. Dibble and Bartha (1979) reported that environmental factors play an

important role in the bioremediation of soil contaminated with petroleum hydrocarbons; and the results obtained in this investigation corroborated the view. Mishra *et al.* (2001) reported an initial population of hydrocarbon degrading bacteria of 10^3 cfu.g^{-1} of soil at the contaminated site. Forsyth *et al.* (1995) reported that the population of hydrocarbon degrading bacteria being less than 10^5 cfu.g^{-1} of soil would be ineffective in bioremediation. He further reported that degradation was higher (55-60%) during the first half (0-55 days) of the study. But, in the present investigation, the degradation of crude petroleum 78.57 and 75.71% upto was achieved during the initial period of 30 days of inoculation with the bacterial consortia II and I, respectively.

The present investigation clearly demonstrates that these two bacterial consortia (II and I) were efficient in degrading about 80% of crude oil in 180 days of culture (Table 4.12). An analysis on the individual fractions of the crude oil revealed that the consortia were efficient in degrading 90-91% of aliphatic, 36-45% of aromatic, and 18-19% of NSO compounds in 180 days of culture (Table 4.13). Ghazali *et al.* (2004) reported the highest reduction by one of their consortia of 57% alkane (C_{14}); whereas, 47.40 - 81.43% aliphatic compounds remained undegraded after 60 days of incubation with the same consortium, and 74.5 - 90.51% aliphatic compounds remained in the soil when inoculated with the consortium.

GC-FID profile of the saturated fraction of crude oil in the liquid medium inoculated with consortium I (Fig.4.6) and consortium II (Fig.4.6 B) exhibited much less noise level as compared to the non - inoculated medium (Fig.4.6 A). This clearly established the fact that both consortia were efficient to degrade crude oil. GC-FID and other analyses demonstrated that *n*-alkanes were preferentially degraded as compared to aromatic and NSO compounds. The outer membrane permeability of bacteria might be one of the factors to determine the biodegradability (Sugiura *et al.*, 1997). Solubility of hydrocarbons could be another factor to influence biodegradability.

5.1.11.4. Bioremediation of crude oil contaminated soil

The present investigation was carried out to assess the efficacy of bacterial isolates and their consortia to degrade crude oil from contaminated soil. Oil pollution is becoming a common phenomenon and it has caused ecological and social catastrophes (Shaw, 1992; Burger, 1993; Burns *et al.*, 1993; Mishra *et al.*, 2001). The ability of biosurfactants to emulsify hydrocarbon- water mixtures has been widely reported. The emulsification properties have also been demonstrated to enhance hydrocarbon degradation in the environment, hence making them potentially useful tools for oil spill pollution-control (Atlas and Bartha, 1992; Atlas, 1993b; Bertrand *et al.*, 1994).

Many of the standard treatment procedures used to decontaminate soil and groundwater have been limited in their application, are prohibitively expensive, or may be only partially effective (Nicholas, 1987). Problems associated with the clean up of leaking disposal sites and spills of toxic substances have demonstrated the need to develop remediation and waste reduction technologies that are effective, economical and rapidly deployable in a wide range of physical setting (Catallo and Partier, 1992).

Traditional methods of treating soil and groundwater contamination have relied upon removal of containments (Brown *et al.*, 1986). Most of these treatments are not completely effective and do not offer permanent solutions for decontamination containment or remediation. Some methods might even create additional uncontrolled hazardous wastes. Therefore, this is the urgent needs of the society to greatly reduce the volume and toxicity of wastes and development of safe, effective and economic alternatives for their disposal (Nicholas, 1987).

An assessment and comparison of the capability of consortium I and consortium II was made to explore biodegradation efficiency of oily sludge, a hazardous hydrocarbon waste generated by the petroleum industry. Survival of the microorganisms after their inoculation is a key-deciding factor in the rate of biodegradation of hydrocarbons (Ramos *et al.*, 1991). Since, all

bacteria in the present study were isolated from a petroleum-contaminated soils, they adapted and survived in the oil-contaminated soil environment very easily. Similar observations were reported by Sugiura *et al.* (1997); Mishra *et al.* (2001) and Rahman *et al.* (2003).

Bacteria present in the oily sludge might be in a dormant or slow-growing state without showing appreciable biodegradation of hydrocarbons even when supplied with glucose or mineral salt media (control). According to Forsyth *et al.* (1995) and Mishra *et al.* (2001) a population of hydrocarbon-degrading microorganisms less than 10^5 cfu/ g in soil would cause very negligible bioremediation. The reports have supported our view that a specific group of bacteria or a bacterial consortium capable of degrading the petroleum hydrocarbons must be inoculated in the soil at a population greater than $1x10^5$ cfu/ g of soil for the effective bioremediation of contaminant crude oil. It was also observed that the supply of phenanthrene as a co-carbon source along with pyrene and fluorene enhanced the rate of degradation of pyrene and fluorene by the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC8165) along with a concomitant increase in bacterial biomass and protein. This observation contradicts the work of Chhatre *et al.* (1996), who described that the addition of nutrients/ co-carbon source in the soil was unlikely to have dramatic effect on the microbial degradation of crude oil.

Soil samples from an oil contaminated habitat having 8.4% crude oil having inoculation with bacterial consortia II and I for a period of 180 days could reduce the crude oil to 1.9±0.2 and 2.1±0.1 % respectively. In the case of non-inoculated control, there was little degradation to 7.3±0.3%. On the other hand, the individual bacterial isolates *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7815) could reduce crude oil contamination to 3.5±0.1 and 3.8±0.3%, respectively (Table 4.14). Artificially contaminated soil with 20% crude oil was inoculated with consortia II and I for 180 days and the treatment could reduce the crude oil to 5.3±0.1 and 6.8 ±0.2 %, respectively

(Table 4.15). From these experiments, the bacterial consortium II comprising of *Microbacterium* (G35-I), *B. subtilis* (R38-I), *P. fluorescens* (L490-II), *B. licheniformis* (MTCC8166), *P. aeruginosa* (MTCC8165) and *B. circulans* (MTCC8167) was found to superior to consortium I comprising of *P. aeruginosa* (MTCC8165), *P. aeruginosa* (L43-I), *P. aeruginosa* (MTCC7815), *A. faecalis* (MTCC8164), *P. aeruginosa* (MTCC8163) and *P. fluorescens* (L490-II) in bioremediation of contaminant crude oil. Although, the biochemical pathways for the biodegradation of various PAHs by microbes have been well established (Gibson *et al.*, 1984), the pathways for PAHs degradation by the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC8165) were yet to be understood. According to Sugiura *et al.* (1997) apart from the biodegradative enzymes, presence of sphingolipids or other specific molecule in the outer membrane structure of bacteria, enabling them to adhere to a specific substrate may be responsible for higher bio-degradation of petroleum hydrocarbons or a specific fraction of petroleum hydrocarbons by a specific group of bacteria. Possibility of the presence of these kinds of molecules in the outer membrane of the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC8163) and *P. aeruginosa* (MTCC8165) needs be investigated in the near future.

5.2. Reclamation of bioremediated soil

Forsyth *et al.* (1995) and Mishra *et al.* (2001) reported that indigenous microorganisms utilize the total petroleum hydrocarbons of crude oil as source of carbon and energy and break them to simpler non-toxic compounds. However, intrinsic bioremediation takes a longer time for the complete reclamation of a contaminated site as the indigenous population of hydrocarbon degrading bacteria is considerably low. The bacterial consortia

with high efficiency for such degradation are thus a proven technique for the efficient bioremediation.

5.2.1. Mortality of seedlings bioremediated soil

The growth performance of three crop plants in bioreclaimed soil alone and fertilized with NPK and organic manure presented different patterns. The green gram seedlings showed rather poor growth within the experimental period of 30 days in both bioremediated soil with NPK and organic manure (R1), without NPK and organic manure (R2) in comparison to the growth in normal soil with NPK and organic manure (N1), and without NPK and organic manure (N2). Addition of NPK and organic manure in the normal soil seemed to have an obvious growth promoting effect. The contrasting poor growth in R1 and R2 indicated that the remediation process failed to restore some of the important conditions critical for the promotion of growth of green gram plants. The growth performance of Bengal gram seedlings in both R1 and R2 were better in comparison to green gram. This indicated variations in the nutrient and soil conditions required for the growth of these two crop plants. The growth of Bengal gram seedlings in R1 and N1 and also in R2 and N2 indicated the restoration of much of the essential elements and conditions following bioremediation for the growth of this crop plant.

The results obtained in the case of rice seedlings appeared to be very interesting as the growth performance of the seedlings in all the soil types such as normal soil with and without NPK and organic manure and bioremediated soil with or without NPK and organic manure were found to be similar. It indicates that the bioremediation of the contaminated soil effectively restored the conditions suitable for the growth and development of rice.

5.2.2. Growth behavior of crops in bioremediated soil

None of the crop plants survived in the crude oil contaminated soil (control). On the other hand, difference in the period of survival of the crop plants was observed. Interestingly, bengal gram seedlings showed larger survival period in the unfertilized soils N2 and R2 than in the fertilized ones. In the first 30 days of plantation (Table 4.16). Bengal gram seedlings showed almost identical growth in all four conditions. The most likely reasons for the lesser length of survival period in the fertilized soil could be due to the imbalance in the soil nutrient regime over longer period after addition of fertilizers.

Green gram seedlings were unable to sustain growth beyond 30 days of plantation in the bioremediated soil with or without fertilizer. This indicated, irreversible changes in the soil conditions disallowing nutrient availability to the plant.

In terms of survival period and growth performance, the rice plants showed better result. Therefore it may be concluded that the bioremediation strategy developed is suitable for the cultivation of rice.

5.2.3. Performance of crops in bioremediated soil

Yield analysis of three crop plants in boremediated soil with or without fertilizer revealed that Bengal gram produced more number of seeds and with higher seed weight when grown in the bioremediated soil without NPK and organic manure (R2). This result was in conformity with the result on growth performance and survival period. There was higher yield and seed weight in the case of green gram plants grown in the unfertilized soil. None of green gram plants survived in contaminated and bioremediated soil with or without NPK and organic manure. The comparative analysis of seed weight and seed number produced by rice plants revealed that the unfertilized remediated soil yielded more with higher seed weight.

The highest growth and yield of rice plants grown in bioremediated soil without any fertilizer strongly indicates that the residual biodegraded compounds left out from the contaminating crude oil are conducive for the growth of the plants. This is very significant, because most of the petroleum hydrocarbon contaminations in Assam occur in fields where rice is cultivated as the major crop. Another significant finding the present investigation is that the soil is rendered unsuitable for supporting any plant growth when the level of contamination by petroleum hydrocarbon is above 6%.

The overall analyses of three crop plants on growth and development parameters revealed that the developed bacterial consortia restored the soil quality of the petroleum-contaminated soil to levels supporting the growth and development of rice plants in comparison to bengal gram and green gram. Addition of fertilizer and organic manure to the bioremediated soil caused lesser growth and development of rice plants.

5.3.1. Screening of biosurfactant producing bacteria

It has been stated that biosurfactant production is an important survival tool for the producing microbes (Ahimou, 2000; Ron and Rosenberg, 2001; Maier, 2003). Production of biosurfactant is related to the utilization of available hydrophobic substrate(s) by the producing microbes from their natural habitat, presumably by increasing the surface area of hydrophobic substrates and increasing their apparent solubility (Ron and Rosenberg, 2001). Bognolo (1998) reported that biosurfactants were produced mainly by aerobic microorganisms in aqueous media from a carbon source feedstock, e.g. carbohydrates, hydrocarbons, oil and fat mixtures. The emulsifiers were secreted into the culture medium during the growth of microorganisms. In the present investigation, bacterial isolates were found to degrade different components of crude oil and could produce biosurfactant in hydrocarbon-supplemented media. Das *et al.* (1998), Syldak and Wagner (1987), Desai *et al.* (1994) and Hommel (1994) reported that surface active properties of biosurfactants depend on the selection of

microorganisms, carbon sources and process parameters. Bodour and Miller (1998) refined the drop-collapse method to use both as qualitative assay to screen for surface producing microbes, and as quantitative assay to determine surfactant concentration. In the investigation, rapid drop-collapsing test and formation of foam during enrichment culture in mineral media -supplemented with different hydrocarbon components like n-hexadecane octadecane, dodecane and glucose established them to be potential for use in MEOR. In environments contaminated by unleaded gasoline, Ridgway *et al.* (1990) identified up to 86% as pseudomonads among 244 bacterial isolates. Exposure to hydrophobic pollutants in contaminated soils appeared to select biosurfactant producers, which could emulsify hydrophobic compounds (Francy *et al.*, 1991; Ron and Rosenberg, 2001).

Biosurfactants produced by *P. aeruginosa* at mesophilic growth conditions (30-37 °C) were characterized as rhamnolipids (Guerra-Santos *et al.*, 1984; Syldatk *et al.*, 1985; Parra *et al.*, 1989) and found to be excellent emulsifiers of hydrocarbons. In the investigation, 58 bacterial isolates were isolated by enrichment culture from hydrocarbon-contaminated soils and some of these selected isolates produced significant amount of biosurfactants at 35°C temperature. The biological activity of a biosurfactant was characterized with an aim to explore their possible industrial application. Mineral salt medium supplemented with 1% (v/v) hexadecane was the best for the growth and biosurfactant production by the bacterial isolates. The yield of biosurfactants with 4.6±0.7 and 4.2±0.8 $g.l^{-1}$ (Table 4.21) from *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812), respectively in the presence of hexadecane as the sole source of carbon was appreciably higher than the reported biosurfactant production by other *Pseudomonas* species. (Pruthi and Cameotra 1995).

Biosurfactant yield of 15.6 $g.l^{-1}$ was reported by Sim *et al.* (1997) by cultivating *P. aeruginosa* ATCC 10145 on corn oil as the carbon source. *P.*

aeruginosa UW-1 produced 24.3 $g.l^{-1}$ biosurfactant in 6% (v/v) canola oil after 9 days of incubation at 30 °C (Sim *et al.*, 1997).

5.3.2. Production and efficacy of biosurfactant

Various key factors such as choice of carbon and nitrogen sources, growth temperature and period, NaCl and mineral-salt concentrations, agitation of culture medium etc influence the bacterial growth along with yield of biosurfactants. Therefore, it is most important to optimize the various culture conditions in an order to improve the biosurfactant yield or to get the best product. Haferberg *et al.* (1986) and Guerra Santos *et al.* (1984) reported that the majority of known biosurfactants were synthesized by microorganisms grown on water-immiscible hydrocarbons, but some had been produced on water- soluble substrates such as glucose, glycerol and ethanol (Cooper *et al.*, 1981; Palejwala and Desai, 1989). In the present study, it was observed that the bacterial isolate *P. aeruginosa* (MTCC8165) preferred glucose and glycerol (1% w/v) for maximum growth and reduction of surface tension of culture medium. Which reflected the difference in the choice of carbon source for the growth and energy production by bacteria at the genus level.

The observation of glucose being the best carbon source for the production biosurfactant by the bacterial isolate *P. aeruginosa* (MTCC7815) (31.1 $mN.m^{-1}$) in the present investigation was found to be in agreement with other workers like Nakano *et al.* (1988), Sandrin *et al.* (1990), Roongsawang *et al.* (2002) and Vater *et al.* (2002). They also reported, saccharose and fructose to be good carbon sources, but glycerol severely decreased surfactin production. According to Cooper *et al.* (1981) and Sandrin *et al.*, (1990) surfactin biosynthesis did not follow stimulation by hexadecane in contrast to other surfactants. Makkar and Cameotra (1997, 1998, and 2001) described the ability of *Bacillus* strains to use starch and sucrose as the preferred carbon source for the maximum growth and biosurfactant production.

The bacterial isolate *P. aeruginosa* (MTCC7815) exhibited the maximum growth and reduction of surface tension (29.7 $mN.m^{-1}$) when the mineral salt medium was supplemented with glycerol (Table 4.22). Glycerol was also used by other investigators like Turkovskaya *et al.* (2001) for the production of biosurfactants. Although growth on glucose had resulted in the maximum decrease in the surface tension of the medium, but the emulsifying activity was only 40%, whereas glycerol was the best source for surfactant synthesis and it exhibited a better emulsifying property of 60% (Turkovskaya *et al.*, 2001). On the basis of reduction of surface tension of culture medium, bacterial isolates were also cultured in aliphatic hydrocarbons (hexadecane and octadecane), aromatic hydrocarbons (benzene and toluene) and polycyclic aromatic hydrocarbons (pyrene and fluorene) supplemented medium. The bacterial isolate *P. aeruginosa* (MTCC7815) exhibited higher reduction in surface tension of culture medium (29.1 $mN.m^{-1}$) irrespective of the carbon source used. (Table 4.22). Glycerol was found to be the second best carbon source for the growth and biosurfactant production by the bacterial isolate *P. aeruginosa* (MTCC7815) which was in agreement with Guerra- Santos *et al.* (1984), Reiling *et al.* (1986), Schenk *et al.*, (1995) and Turkovskaya *et al.* (2001).

As shown by many workers, agitation of the Erlenmeyer flask has a major role in the bacterial growth and biosurfactant production (Syldatk and Wagner, 1987; Turkovskaya *et al*, 2001). Agitation helped in the mixing of mineral salt components of the medium. Intense aeration was crucial for the growth of bacteria and biosurfactant synthesis. Besides supplying oxygen, aeration also helped in mixing the inoculum with the media composition. The agitation at 200 rpm was observed as optimum for all the bacterial isolates in the present investigation study, which was in agreement with the findings of Makkar and Cameotra (1997 and 1998) and Sim *et al.* (1997). However, Turkovskaya *et al.* (2001) reported that agitation at 160 rpm was optimum for the maximum biosurfactant production by *Pseudomonas aeruginosa* 50.3. An increase of agitation speed from 250 to 500 rpm caused a

decrease in biosurfactant production by *Nocardia erythropolis* due to a shear rate effect on the growth kinetics of the microorganism (Syldatk and Wagner, 1987). A similar result was observed in the present investigation.

Chayabutra and Ju (2001) reported that *P. aeruginosa* ATCC 10145 could grow optimally at a hexadecane concentration of 8% (v/v). The requirement of inorganic salt and pH for the optimum growth and biosurfactant production by the bacterial isolates was found to be in agreement with the above finding. Rhamnolipid production is influenced by the nutrients used in the culture media and also on the applied culture parameters. Guerra-Santos *et al.* (1986) reported better yield of rhamnolipids, produce by *P. aeruginosa*, when the concentration of magnesium, calcium potassium, sodium salts and trace elements were minimized. Syldatk and Wagner (1987) reported a similar finding for one Pseudomonas species.

5.3.3. Influence of pH on the efficacy of biosurfactants

The efficacy of biosurfactant produced by each bacterial isolate was found to be independent of pH. There was little reduction of surface tension in acidic and alkaline conditions as compared to the neutral pH range. The dilution of biosurfactants by 10 times (CMD^{-1}) retained the efficacy in reducing surface tension of culture medium similar to that of normal concentration. In 100 times dilution (CMD^{-2}), a similar trend of surface tension reduction in acidic condition only was exhibited by the all bacterial isolates. However, the reduction of surface tension in CMD^{-2} was much less in almost all pH levels. This phenomenon might be related to the presence of higher amount of surfactin isomers in the crude lipopeptide secreted by the isolates due to their preference of higher pH (Morikawa *et al.*, 2000).

5.3.4. Thermostability of biosurfactants

Biosurfactants from the bacterial isolates were found to be thermostable. The activity of the biosurfactants was retained on heating at

100°C for 60 min with a moderate deviation in surface tension at CMD^{-1} and CMD^{-2}. The heating caused a negligible loss in the surface activity. This was in good agreement with the earlier reports demonstrating thermostable nature of biosurfactants from *B. subtilis* strains (Makkar and Cameotra, 1998) and *Pseudomonas* strains (Johnson and Boese-Marrazzo, 1980; Turkovskaya *et al.*, 2001). In the present investigation, the fall of surface activity by 11.5, 8.3, 6.3, 5.0 and 4.3% was observed in the case of biosurfactants from the isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC7814), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC8163), respectively when exposed to 100°C for 60 min. The biosurfactant from *P. aeruginosa* (MTCC 7815) exhibited the maximum (11.4%) loss of surface activity (surface tension and CMC values).

5.3.5. Surface activity of biosurfactants

All five bacterial isolates were found to be efficient biosurfactant producers based on surface tension measurements of culture supernatants obtained from fermentations performed at various temperatures as well as pH. The biosurfactants produced by *P. aeruginosa* (MTCC7815) exhibited better surface tension reducing ability in comparison to other bacterial isolates. This observation supported that the isolate produced higher amount of surface-active compounds as compared to other bacterial isolates. The surface tension reduction values were quite consistent with the reported values for the most efficient biosurfactants, which were isolated and studied so far (Cooper and Zajic, 1980; Cooper *et al.*, 1981, 1989; Desai and Banat, 1997).

The biosurfactants isolated from all the bacterial isolates in the present investigation had lower CMC values as compared to the tested synthetic surfactant sodium dodecyl sulphate (SDS). Biosurfactants possess low critical CMC values as compared to synthetic surfactants and thus have an advantage over chemical surfactants (Lin *et al.*, 1998). Rhamnolipids (Steinbuchel, 1991) and lipopeptides are (Morikawa *et al.*, 2000; Vater *et al.*,

2002) the most efficient biosurfactants known till date. Their CMCs in water can be as low as 10-20 $mg.l^{-1}$, and the corresponding minimal surface tension (MST) ranges from 25 - 30 mN/m. These properties of biosurfactants were compared to those of the synthetic surfactants: SDS which possessed the CMC value at 2023-2890 $mg.l^{-1}$ and MST at 37 $mN.m^{-1}$ and alkylate dodecyl benzene CMC at 590 $mg.l^{-1}$ and MST at 47 $mN.m^{-1}$ (Lenz *et al.*, 1992; Brandl *et al.*, 1990; Margaert *et al.*, 1992). The CMC values and the MST of biosurfactants from the bacterial isolates in the present investigation were in close proximity to those reported by Haba *et al.* (2003), Syldatk *et al.* (1985) and Mata-Sandoval *et al.* (1999). A mutant strain of *P. aeruginosa*, obtained by mutagenesis with N-Methyl-N'-nitro-N-nitrosoguanide designated as *P. aeruginosa* PTCC 1637 produced rhamnolipid biosurfactant having a CMC value of 9 $mg.l^{-1}$ (Tahzibi *et al.*, 2004). Biosurfactants from *Nocardia* was found to have 4.5 times more emulsion stability in comparison to the chemical surfactant SDS (Kim *et al.*, 2000). In the present investigation, it was observed that emulsion stability of biosurfactants from *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) were 8.2 and 6.8 time more stable than that of SDS, as was evident from the calculation of decay constant (K_d).

5.3.6. GC-MS analysis of biosurfactants

Mass spectrometry is an innovative, highly efficient technique to characterize the molecular structure of microbial biosurfactants and their secondary metabolites (Deziel *et al.*, 1999). Schenk *et al.* (1995) used HPLC to charaterise a rhamnolipid mixture produced by *P. aeruginosa* strain DSM2659. The method, in addition to being longer could not provide structural information on various compounds separated. Conversely, mass spectrometry gave useful structural information and allowed the quantitation of chromatographically unresolved molecules.

Arino *et al.* (1996) separated a mixture of rhamnolipids by thin-layer chromatography in several fractions and hydrolysed these fractions into their

respective sugar and 3-hydroxy fatty acids. The sugar was determined to be rhamnose and the fatty acids were analysed by GC/MS. By determining the nature and number of each component in each fraction, they were able to deduce the structure of rhamnolipids. Bonmatin *et al.* (2003) observed that $[M-H]^+$ or $[M-H]^-$ ions generated by this technique were very stable, thus leading to intense signals which were useful for determining rapidly the homogeneity or heterogeneity of the samples by MS analysis including the molecular masses of samples with an accuracy of 0.01 to 0.02%.

In the present investigation, the rhamnolipids with the longest fatty acid side chain tended to have slightly shorter retention time. The rhamnolipids produced by the bacterial isolates *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) differed in both quantities and in structure (Table 4.29). Therefore, GC/MS spectrometry was used as a tool to elucidate the probable structure and determine to the molecular mass. On comparison of mass data obtained for active fractions and the mass numbers of rhamnolipid and lipopeptide complexes isolated from other bacterial strains by Bosch *et al.* (1989), Pajarron *et al.* (1993) and Deziel *et al.* (1999), glycolipids of the bacterial isolates *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) could be identified as Rha- C_8 –C_{10} and Rha -C_{10}-C_8, But it, could not be ascertained whether they were made up one or more fatty acid chains. Syldatk *et al.* (1985a and b) obtained hydrophilic rhamnolipids Rha-C_{10} and Rha- Rha- C_{10} from culture supernatants of resting *Pseudomonas* sp. DSM 2874. Their relative abundance was not reported, but they were apparently present in lower quantity than their two fatty acid-containing analogues, as was also reported by Arino *et al.* (1996). They reported the rhamnolipid profile of *P. aeruginosa* strain GL1 which was isolated from hydrocarbon-contaminated soils. The bacteria were grown with glycerol as carbon source. They observed a variety of mono- and dirhamnolipids containing one or two 3 – hydroxy fatty acids and one or two rhamnoses represented 90% of all rhamnolipids. The fatty acids were

predominantly C_{10} with some C_8, $C_{12:1}$ and C_{12}. Koster *et al.* (1994) observed the same saturated C_8 - C_{12} mono-and dirhamnolipids. Bosch *et al.* (1989) reported the rhamnolipids produced by a *Pseudomonas* species. after column and thin-layer chromatographies. They only observed Rha-C_{10}-C_{10} and Rha- Rha- C_{10}-C_{10}.

The results with hexadecane were similarities with the above mentioned reports. In most of the cases where some quantification results were presented, the predominant rhamnolipids were Rha- C_8-C_{10} and Rha C_{10} - C_8 (Table 4. 29). The most abundant 3- hydroxyl fatty acids were also C_8 and C_{10} observed in rhamnolipids. Deziel *et al.* (1999) reported that the unsaturated fatty acid was always found at the terminal end of rhamnolipids.

In this investigation, the biosurfactants produced by the bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) might be nearly identical demonstrating only minor differences in the isomers. The molecular mass of active biosurfactants from bacterial isolates were detected in the range from m/z 355 to 677 (Jarvis and Johnson, 1949; Chayabutra and Ju, 2001; Haba *et al.*, 2003; Benincasa *et al.*, 2004), but interestingly the m/z of the most active biosurfactant isoforms secreted by *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) were detected in the range from m/z 147 to 401.

5.4.1. Polycyclic aromatic hydrocarbon biodegradation by biosurfactants

Polycyclic aromatic hydrocarbons are ubiquitous pollutants occurring mostly as a result of fossil fuel combustion and also as by-product of industrial activities. Since, many of them are either known or suspected carcinogens and mutagens, exposure to PAHs may result in a significant health risk to human populations (White, 1986), and therefore their fate in nature is of great environmental and medical concern.

In the present investigation, variation was observed in the utilization of phanathrene, pyrene, fluorene and crude oil as sole source of carbon and energy by the bacterial isolates. There was a decrease in the content of phenanthrene, pyrene and fluorene in the media with a concomitant increase in the bacterial dry biomass and protein with respect to time. The bacterial isolates were separately cultured in mineral salt medium supplemented with phenanthrene, pyrene and flourene for 12 days. Increase in protein concentration as the index for bacterial growth and utilization of the hydrocarbon component was estimated at an interval of 2 days (Fig. 4.13, 4.14 and 4.15). The utilization of phenanthrene as the sole source of carbon and energy by the bacterial isolates was confirmed by its removal from the medium, with a corresponding increase in the bacterial protein. The concentration of phenanthrene decreased dramatically in the culture medium over the next 12 days. The bacterial isolate *P. aeruginosa* (MTCC7815) followed by *P. aeruginosa* (MTCC7812) and *P. aeruginosa* (MTCC7814) exhibited the maximum utilization of phenanthrene reducing the content to 70, 85 and 87µg from the initial application of 180 µg in the medium. The remaining bacterial isolates possessed comparatively less utilization of phenanthrene. The role of biosurfactant produced by respective bacterial strains in enhancing the solubility of PAH that leads to a significant increase in PAHs metabolism by bacteria for growth and energy production.

Non-actinomycete bacteria such as *P. aeruginosa*, *P. pudita* and *Flavobacterium* species were reported to utilize pyrene, when supplemented with other forms of organic carbons (Trzesicka-Mlynarz *et al.*, 1995). In the present investigation, the bacterial isolates *P. aeruginosa* (MTCC7814) and *P. aeruginosa* (MTCC8165) exhibited better utilization of pyrene with 89 µg and 93 µg respectively from the initial application of 180 µg as the sole source of carbon in 12 days of culture with increased biomass and protein production, and a concomitant reduction in pyrene content from the culture medium. The growth of the bacterial isolates at the expense of fluorene as the sole source of carbon suggested utilization of 89, 90 and 92 µg of

fluorene respectively from the initial application of 180 µg in 12 days of culture. There were reports on soil *Pseudomonas* species capable of degrading PAHs, but it failed to utilize them as the sole source of carbon and energy (Foght *et al.*, 1988).

Phylogenetic analysis revealed the existence of wide diversity among the biosurfactant producing microbes suggesting biosurfactant production to be an important survival tool for the producing microbes and this has evolved to be an independent but parallel process (Bodour *et al.*, 2003). *Biosurfactant producing bacteria are present in higher concentration in* hydrocarbon-contaminated soils (Bodour and Maier, 2003). In the present investigation, biosurfactant production by all five bacterial isolates increased significantly when the medium was supplied with phenanthrene along with pyrene and fluorene (Table 4.29 and 4. 30). The yield of biosurfactant in the culture supernatant with acid precipitation increased dramatically after 96 h of culture. The bacterial isolates exhibited better biosurfactant yield of 0.23 – 0.5 $g.l^{-1}$ at 96 h of culture in medium supplemented with pyrene and phenanthrene, as compared to medium without phenanthrene (0.18 – 0.30 $\mu g.l^{-1}$). Concomitantly, the bacterial biomass of 0.7 – 1.20 $g.l^{-1}$ increased to 0.75 – 1.4 $g.l^{-1}$ after 96 h of culture. This result might be viewed in the context of increased rate of co-metabolism of pyrene in the presence of phenanthrene (McKenna, 1977, and Cerniglia, 1984). Stringfellow *et al.* (1995) observed that *Pseudomonas saccharophila* p-15 could degrade pyrene on being induced by either phenanthrene or salicylate. In media having the combined addition of flourene and phenanthrene caused better biosurfactant yield of 0.45 and 0.38 $g.l^{-1}$ in the case of bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7814) respectively for the entire growth period of 96 h. In the same medium, the bacterial biomass increased from 0.6 –1.2 $g.l^{-1}$ at 48 h of inoculation to a maximum of 1.0 – 1.5 $g.l^{-1}$ at 96 h of culture. Bouchez *et al.* (1995) reported that addition of fluorene as a cosubstrate could increase utilization of phenanthrene. Bouchez *et al.* (1995) also reported that phenanthrene might be a poor

inducer of its own degradation, but fluorene could enhance phenanthrene biodegradation, possibly by a positive analog effect on enzyme induction.

5.4.2. Solubilization of PAHs and crude oil by biosurfactants

Production of biosurfactant is related to the utilization of available hydrophobic substrates by the producing bacteria from their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility (Ron and Rosenberg, 2001). Therefore, use of biosurfactants has been reported as a mechanism to enhance the bioavailibility of hydrophobic pollutants and PAHs for microbial degradation (Thiem, 1994). Low molecular weight biosurfactants like lipopeptides having low critical micelle concentrations increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities off micelles (Miller and Zhang, 1997). On the other hand, alasan, a high molecular weight bioemulsifier complex produced by *Acinetobacter radioresistens* KA 53 enhanced the aqueous solubility of PAHs by a physical interaction most likely of a hydrophobic nature and increases the biodegradation rate of PAHs (Barkay *et al.*, 1999).

In this investigation, biosurfactant of the bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812) having the concentration of 0.5 mg. ml^{-1} exhibited 41 and 26 $\mu g.ml^{-1}$ solubilization of pyrene. Subsequently, the apparent solubility of pyrene was enhanced by factors 5 - 7 resulting in its higher uptake and metabolism as compared to non-solubilized pyrene. The difference in pyrene solubilization by the biosurfactants from different bacterial strains in this investigation might be related to the chemical nature as well as surface properties of the biosurfactants. The biosurfactants secreted by *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) were lipopeptide in nature containing higher amounts, whereas biosurfactants secreted by *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) were found to be complex mixtures of lipopeptides and glycoproteins. Moreover, the

significantly higher pyrene solubilization effect of biosurfactant of *P. aeruginosa* (MTCC7815) as compared to *P. aeruginosa* (MTCC7812) reinforced the hypothesis that variation in biosurfactant isoforms between these two isolates might result in a large variation of the emulsification property and specificity of biosurfactants. It may be concluded that higher pyrene solubilization effect of biosurfactants from isolate *P. aeruginosa* (MTCC7815) dramatically enhanced the metabolism of pyrene that sustained the growth of this bacterial isolate in pyrene; otherwise it would not be able to grow on pyrene. Similar trends was observed in the case of fluorene solubilization effect of biosurfactant of *P. aeruginosa* (MTCC7812) as compared to *P. aeruginosa* (MTCC8163). Further studies to understand the microbial ecology of PAHs degrading communities and their application for the development of bioremediation strategies for PAHs are necessary.

5.4.3. Biosurfactants in MEOR

The potential use of biosurfactant in MEOR was evaluated using the sand packed column technique. Among the five bacterial isolates under study, biosurfactants from *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7812) were found to release 60±1.2% and 60±1.5 % respectively of crude oil from the saturated sand packed column at 90°C. Biosurfactants of *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7814) could release 56.7±1.3%, and 51±2.0% respectively of crude oil from the column at 90°C. The percent oil release by the bacterial isolates was in agreement with the report by Pruthi and Cameotra (1997). The recovery of oil from the saturated sand pack column by the bacterial biosurfactants in the present investigation was found to be higher than that of the biosurfactants from *Bacillus subtilis* strains as reported by Makkar and Cameotra (1998), but lower than the biosurfactant from a thermophilic *Bacillus* species (Banat, 1993). The ability of the selected five bacterial isolates to grow in high crude oil concentrations and at wide range of temperatures (25-40°C) and pH (4-12), they could be

exploited for such industrial applications, such as for *in situ* oil recovery from oil wells having moderate well temperatures.

5.5.1. Plasmid DNA isolation and restriction digestion

An effort was made in this investigation to find out whether the gene(s) for biosurfactant biosynthesis and hydrocarbon degradation of the bacterial isolates were present in the plasmid DNA. Interestingly all the isolates exhibited antibiotic resistance. Maloy *et al.* (2006) reported that R plasmids render their host cells resistant to certain antibiotics, so in nature a cell containing such a plasmid could survive better in environments in which the antibiotic is present. Esperaza *et al.* (1997) reported that multiple antibiotic resistant gene(s) are linked with the R-plasmid of *Pseudomonas aeruginosa.* Usually the gene(s) conferring antibiotic resistance are located in the plasmid DNA. Plasmid DNA from the bacterial isolates was isolated. Restriction digestion of the plasmid DNA from each of the isolates generated single band, which suggested the presence of specific plasmid in each of the bacterial isolates. On the other hand, restriction-digested DNA fragments from the bacterial isolates exhibited presence of genetic diversity amongst the isolates.

5.5.2. Plasmid curing and antibiotic sensitivity

Plasmid curing experiment was carried out and the antibiotic-sensitive bacterial isolates were recovered assuming the presence of the antibiotic resistant gene in the plasmid DNA. The R plasmid - mediated antibiotic resistance in some bacteria has already been reported which enhanced their survival in environments containing antibiotics (Maloy *et al.*, 2006). Plasmid curing experiments performed in the bacterial isolates *P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC8163), *P. aeruginosa* (MTCC8165) and *P. aeruginosa* (MTCC7814) revealed that 50-55% curing could be achieved with a concentration of 500 $mg.ml^{-1}$ of acridine orange. Toshiki *et al.* (1997) reported that LB medium supplemented with 300$\mu g.ml^{-1}$ of acridine orange cured the plasmid from

aniline-assimilating bacteria. Toshio *et al.* (1982) observed that during the logarithmic phase of *Bacillus subtilis* (*natto*) culture in nutrient broth at pH 7.6 containing acridine organe ($20\mu g.ml^{-1}$) with a cell density of $10^4.ml^{-1}$ cured the plasmid - linked polyglutamate gene. Esperaza *et al.* (1997) reported that multiple antibiotic resistant gene(s) were linked with R-plasmid of *Pseudomonas aeruginosa* and this plasmid was cured with acridine orange in the concentration of 400, 600, 800, 1000, 1200 and 1500 $\mu g.ml^{-1}$. In the present investigation, curing was verified in the isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163) through culture and plasmid isolation. Following plasmid curing, the isolates failed to grow in hydrocarbon -supplemented medium but they could be cultured in LB media devoid of hydrocarbons. These observations confirmed the presence of the gene(s) responsible for the biosynthesis of biosurfactants and biodegradation of hydrocarbons on the plasmid DNA. These two isolates after being cured of their plasmid failed to grow on plates containing ampicilin, tetracycline and chloramphenicol as well confirmed the presence of the genes responsible for the resistance to the aforesaid antibiotics. Subsequent non – recovery of plasmid DNA from the isolates indicated its removal following curing. It might therefore be inferred that the genes for antibiotic resistance are located in the plasmid as was predicted before. Plasmid - based antibiotic resistance is advantageous to identify plasmid-cured bacterial isolates. Study on the behavior of plasmid – cured bacterial isolates revealed their antibiotic sensitivity. The plasmid-cured bacterial isolates not only failed to grow on hydrocarbon-supplemented medium and also failed to produce biosurfactant. Thus, the presence of hydrocarbon degrading gene(s) in the plasmid was confirmed. Thus, an effort was made to transfer the hydrocarbon degrading plasmid from the concerned bacterial isolates into the thermophilic bacteria (above 70°C) devoid of this capability.

5.5.3. Transformation experiment

Having confirmed the presence of the gene(s) conferring antibiotic resistance and biosynthesis of biosurfactant in the plasmid DNA of the bacterial isolates *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC8163), an attempt was made to transform thermophilic bacterial isolates MSC1 and SS2 with the plasmid DNA of the above two bacterial isolates.

Plasmid mobilization experiment, the putative MSC1 and SS2 transforments were cultured on selective plates containing tetracycline. In the solidified selective medium, the thermophilic bacteria MSC1 and SS2 exhibited resistance to tetracycline but failed to grow in liquid medium. They also failed to grow in hydrocarbon-supplemented medium. The observation suggested that these bacteria were neither able to degrade hydrocarbon nor to produce biosurfactant. These might be due to (i) the plasmid might not carry the full complement of the gene(s) required for the degradation pathway and some of the genes might be of chromosomal origin, Maier (2003) reported that major biosurfactant producing genes of *P. aeruginosa* POA1 are located on the chromosome; (ii) the genes might not express due to a lack of an effective induction system in the new host and (iii) the high temperature of above 70°C might inactivate the enzymes produced. The work needs further investigation for finding out a suitable proposition.

Chapter 6
Conclusion

Conclusion 6

The present work was carried out with the primary objective of identifying native soil bacteria with potential for the application in bioremediation of petroleum hydrocarbon - contaminated soil and in microbial enhanced oil recovery. Several soil bacteria were isolated from the petroleum hydrocarbon rich soils of Assam Asset of ONGC and pure cultured. All these isolates were assessed for their degradative ability *in vitro* and *in situ* at petroleum hydrocarbon contaminated sites. The identity of the bacterial isolates was also established at the species level. The synergistic effect of the selected bacterial isolates was studied and bacterial consortia were developed for their application in bioremediation operation. Laboratory and field scale evaluation confirmed the suitability of bacterial consortia in bioremediation of crude oil contaminated soil. The biosurfactant production ability of the selected bacterial isolates was studied. The partially purified biosurfactants were chemically characterized. The plasmid DNA from each of the biosurfactant producing bacteria was isolated and characteristics studied. Plasmid curing experiments indicated presence of some of the biosynthesis of bacterial surfactants as well as antibiotic resistance were plasmid borne. Transformation experiments involving transfer of the plasmid DNA from biosurfactant producing mesophilic bacteria to thermophilic bacteria produced putative transformants, which subsequently failed to grow in the liquid selective medium. Laboratory scale experiments revealed the efficacy of bacterial biosurfactants in enhanced crude oil recovery, however this needs further investigation.

The findings of the work may be summarized in the following points:

1. Thirteen hydrocarbon degrading bacterial isolates were recovered from several oil rich and oil contaminated sites in Assam and identified. Nine of them were confirmed by the IMTECH, Chandigargh to be

Alcaligenes faecalis (MTCC81164), *Bacillus licheniformis* (MTCC 8166), *Bacillus circulans* (MTCC 8167) and *Pseudomonas aeruginosa* (MTCC 8163, 8165, 7812, 7814, 7815 and 7816). Till date, none of these nine bacteria isolated from the soil of Indian subcontinent were previously assessed for bioremediation of crude oil. Hence they could be effective candidates in bioremediation, enhanced oil recovery and storage cleaning in North Eastern region.

2. Bacterial consortium II comprising of isolates *Microbacterium* (G35-I), *B. subtilis* (R38-I), *P. fluorescens* (L490-II), *B. licheniformis* (MTCC 8166), *P. aeruginosa* (MTCC 8165) and *B. circulans* (MTCC 8167) was found to be effective in bioremediation as it could reduce the residual crude oil to 5.3% from 20% in 180 days after inoculation.

3. A bacterial density above 1 x 105 cfu.ml^{-1} was found to be suitable for bioremediation of crude oil contaminated soil.

4. Bioremediated soil was found to contain more nitrogen (313.41kg.ha^{-1}) as compared to normal soil (242.31 kg.ha^{-1}). However, there was a depletion of phosphorus and potash in bioremediated soil as compared to normal.

5. Rice cultivated in bioremediated soil without the application of fertilizers and organic manure yielded more seed as compared to the plants grown in control and bioremediated soils supplemented with inorganic fertilizers and organic manure. On the other hand, leguminous plants green gram and bengal gram failed to grow in bioremediated soil.

6. Five bacterial strains [*P. aeruginosa* (MTCC7815), *P. aeruginosa* (MTCC7812), *P. aeruginosa* (MTCC 8163 and 8165) and *P.*

aeruginosa (MTCC7814)] isolated from crude oil contaminated sites of Assam Asset, ONGC were found to produce biosurfactant.

7. Bacterial biosurfactants were found to be stable at different levels of pH (2-11) and at high temperature (100°C) for 60 min.

8. The requirement of bacterial biosurfactants for the critical micelle concentration was found to be much lesser as compared to commercial surfactants sodium dodecyle sulphate.

9. Biosurfactant producing gene(s) were found to be present in the plasmid of the concerned mesophilic bacteria.

10. Mobilization of the biosurfactant producing plasmid to thermophilic bacteria (70°C and above) - MSC1 and SS2 was successful in solidified selective medium. But, the transformants failed to grow in liquid selective medium.

Future work

Bioremediation of petroleum hydrocarbon contaminated habitats is a major area of focus with greater emphasis being given on the aspect of environment protection in industrial activities. Strain improvement for effective bioremediation may be explored through application of molecular genetic manipulation techniques. Biosurfactants are increasingly recognized as superior to their chemical counterparts in terms of lesser toxicity, greater efficiency and cost effectiveness. The role of biosurfactants is also critical in the success of MEOR. Strain identification with greater genetic ability to produce biosurfactants may be explored. Simultaneously, genetic engineering approaches may be followed to bring about strain improvement in the context of increasing the efficacy of bacterial strain(s) in targeting the specific environmental conditions where these are to be applied.

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List of Publications

In Journals

1. Bordoloi, N. and Konwar, B. K. (2005) Microbial surfactant-enhanced mineral oil recovery under laboratory condition. *International Journal of Biodegradation and Biodeterioration* accepted for publication subject to confirmation of the identification of the relevant bacterial strains by bonafide agency (IMTECH, Chandigarh)

Papers in proceedings of National Conference

1. Bordoloi, N. and Konwar, B. K. (2003) Biosufractant induced enhanced oil recovery. *National Seminar on Hydrocarbon Degrading Microbes 22-23rd December 2003,* Organized by Centre for Petroleum Biotechnology, Department of Molecular Biology and Biotechnology, Tezpur University and sponsored by Oil and Natural Gas Corporation Limited at Tezpur University.
2. Bordoloi, N. and Konwar, B. K. (2004) Bioderadation of contaminant crude oil soil. *National Workshop on Regional Development: Case for North-East India.* 3-6 Feb 2004, Organized by Tezpur University, IIT Guwahati and C-MMACS, Bangalore, India.
3. Bordoloi, N. and Konwar, B. K. (2005) Microbial consortium in bioremediation of contaminant petroleum hydrocarbon. *National Seminar on Value addition to Bioresources of NE-India post harvest technology and cold chain.*19-21 May 2006, Organized by Departmet of Botany Gauhati University.

Seminar/Workshop attended

1. National Symposium on " Microbes in Bioremediation for Eco-Friendly Environment in the New Millennium" 6-7th January 2000, at the Center for Advanced Studies in Botany, University of Madras, Chennai.
2. Patent Awareness Workshop, 9th September 2002, Organized by Tezpur University, Sponsored by Department of Science and Technology, New Delhi.
3. 72nd Annual Session of National Academy of Sciences, India and National Symposium on Biodiversity, NEHU, Shillong Oct 25-27, 2002.
4. National Seminar on " Naturally occurring microbes for the control of Human Diseases" 11-15th October 2004, Defense Research Laboratory Tezpur, Assam.

APPENDIX

Solution I

50 mM glucose
25 mM TrisCl (pH8.0)
10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 15 lb/sq. in. on liquid cycle, and stored at 4°C.

Solution II

0.2 N NaOH (freshly dilute from a 10N stock)
1% SDS

Solution III

5 m potassium acetate	60 ml
Glacial acetic acid	11.5ml
H_20	28.5ml

Resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Annexure

Table 1. Growth of bacterial isolates in glucose as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	1.30	1.60	1.90	2.3	2.65
A. faecalis (MTCC8164)	0.25	0.80	1.20	1.50	1.70	2.10
P.aeruginosa (MTCC8163)	0.25	0.75	1.35	1.65	2.15	2.32
B.circulans (MTCC8167)	0.25	0.45	0.75	1.25	1.65	1.75
P.aeruginosa (L43-I)	0.25	0.54	0.82	1.36	1.85	2.40
P.aeruginosa (L490-II)	0.25	0.40	0.65	0.85	1.25	1.75
B.licheniformis (MTCC8166)	0.25	0.62	0.95	1.12	1.42	1.85
P.aeruginosa (MTCC7815)	0.25	1.35	1.65	2.25	2.65	3.25
Micrococcus (G35-I)	0.25	0.35	0.65	0.86	1.20	1.45
B. subtilis (R38-I)	0.25	0.85	1.24	1.62	1.80	2.12
control	0.25	0.24	0.21	0.22	0.24	0.25

Table 2. Growth of bacterial isolates in fructose as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.75	1.25	1.85	2.25	2.51
A. faecalis (MTCC8164)	0.25	0.45	0.95	1.65	2.15	2.25
P.aeruginosa (MTCC8163)	0.25	0.65	1.15	1.75	2.10	2.32
B.circulans (MTCC8167)	0.25	0.67	1.12	1.25	1.56	1.73
P.aeruginosa (L43-I)	0.25	0.45	0.90	1.21	1.54	1.69
P.aeruginosa (L490-II)	0.25	0.55	0.85	1.15	1.34	1.70
B.licheniformis (MTCC8166)	0.25	0.47	0.95	1.23	1.52	1.85
P.aeruginosa (MTCC7815)	0.25	1.38	1.95	2.60	2.85	3 32
Micrococcus (G35-I)	0.25	0.35	0.56	0.97	1.15	1.60
B. subtilis (R38-I)	0.25	0.98	1.34	1.78	2.13	2.24
control	0.25	0.24	0.23	0.25	0.24	0.25

Table 3. Growth of bacterial isolates in dodecane as determine by optical density

Bacterial isolates	0h	24h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.36	1.46	2.07	2.40	2.95
A. faecalis (MTCC8164)	0.36	0.97	1.22	1.78	1.96
P.aeruginosa (MTCC8163)	0.36	0.75	1.04	1.18	1.36
B.circulans (MTCC8167)	0.36	0.66	1.08	1.24	1.65
P.aeruginosa (L43-I)	0.36	1.05	1.33	1.58	1.71
P.aeruginosa (L490-II)	0.36	0.83	1.19	1.30	1.67
B.licheniformis (MTCC8166)	0.36	1.07	1.26	1.48	1.81
P.aeruginosa (MTCC7815)	0.36	1.24	1.73	2.53	3.15
Micrococcus (G35-I)	0.36	0.74	1.17	1.35	1.48
B. subtilis (R38-I)	0.36	1.12	1.98	2.14	2.78
control	0.15	0.16	0.14	0.16	0.15

Table 4. Growth of bacterial isolates in hexadecane as determine by optical density

Bacterial isolates	0h	24h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.36	1.02	2.29	2.06	2.75
A. faecalis (MTCC8164)	0.36	1.05	1.46	1.73	1.92
P.aeruginosa (MTCC8163)	0.36	0.89	1.25	1.65	2.01
B.circulans (MTCC8167)	0.36	0.78	1.27	1.39	1.67
P.aeruginosa (L43-I)	0.36	1.05	1.38	1.54	1.78
P.aeruginosa (L490-II)	0.36	1.15	1.42	1.73	2.08
B.licheniformis (MTCC8166)	0.36	0.65	1.05	1.64	1.82
P.aeruginosa (MTCC7815)	0.36	1.30	1.82	2.74	3.56
Micrococcus (G35-I)	0.36	0.98	1.01	1.35	1.78
B. subtilis (R38-I)	0.36	1.25	1.55	1.98	2.85
control	0.15	0.16	0.14	0.16	0.15

Table 5. Growth of bacterial isolates in octadecane as determine by optical density

Bacterial isolates	0h	24h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.36	0.69	0.95	1.21	1.45
A. faecalis (MTCC8164)	0.36	0.37	0.84	1.04	1.26
P.aeruginosa (MTCC8163)	0.36	0.47	0.93	1.13	1.35
B.circulans (MTCC8167)	0.36	0.56	0.78	1.27	1.40
P.aeruginosa (L43-I)	0.36	0.61	0.86	1.11	1.30
P.aeruginosa (L490-II)	0.36	0.71	0.99	1.34	1.74
B.licheniformis (MTCC8166)	0.36	0.78	1.12	1.42	1.69
P.aeruginosa (MTCC7815)	0.36	1.22	1.41	1.94	2.35
Micrococcus (G35-I)	0.36	0.39	0.68	0.95	1.03
B. subtilis (R38-I)	0.36	0.76	1.34	1.78	2.26
control	0.15	0.16	0.14	0.16	0.15

Table 6. Growth of bacterial isolates in benzene as determine by optical density

Bacterial isolates	0h	24h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.26	0.31	0.35	0.38	0.39
A. faecalis (MTCC8164)	0.26	0.27	0.28	0.28	0.29
P.aeruginosa (MTCC8163)	0.26	0.26	0.29	0.29	0.31
B.circulans (MTCC8167)	0.26	0.25	0.26	0.27	0.28
P.aeruginosa (L43-I)	0.26	0.28	0.29	0.31	0.34
P.aeruginosa (L490-II)	0.26	0.27	0.28	0.31	0.33
B.licheniformis (MTCC8166)	0.26	0.28	0.31	0.35	0.37
P.aeruginosa (MTCC7815)	0.26	0.35	0.38	0.42	0.45
Micrococcus (G35-I)	0.26	0.29	0.31	0.36	0.39
B. subtilis (R38-I)	0.26	0.31	0.33	0.38	0.41
control	0.17	0.18	0.18	0.17	0.18

Table 7. Growth of bacterial isolates in toluene as determine by optical density

Bacterial isolates	0h	24h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.26	0.28	0.31	0.32	0.35
A. faecalis (MTCC8164)	0.26	0.26	0.29	0.31	0.33
P.aeruginosa (MTCC8163)	0.26	0.28	0.28	0.33	0.36
B.circulans (MTCC8167)	0.26	0.29	0.32	0.36	0.38
P.aeruginosa (L43-I)	0.26	0.28	0.29	0.30	0.32
P.aeruginosa (L490-II)	0.26	0.26	0.26	0.27	0.29
B.licheniformis (MTCC8166)	0.26	0.33	0.35	0.37	0.39
P.aeruginosa (MTCC7815)	0.26	0.36	0.38	0.41	0.45
Micrococcus (G35-I)	0.26	0.27	0.28	0.3	0.32
B. subtilis (R38-I)	0.26	0.31	0.33	0.35	0.42
control	0.17	0.18	0.18	0.17	0.18

Table 8. Growth of bacterial isolates in xylene as determine by optical density

Bacterial isolates	0h	24h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.26	0.38	0.41	0.42	0.45
A. faecalis (MTCC8164)	0.26	0.27	0.29	0.32	0.35
P.aeruginosa (MTCC8163)	0.26	0.28	0.32	0.35	0.39
B.circulans (MTCC8167)	0.26	0.28	0.31	0.34	0.36
P.aeruginosa (L43-I)	0.26	0.27	0.28	0.30	0.31
P.aeruginosa (L490-II)	0.26	0.26	0.28	0.32	0.34
B.licheniformis (MTCC8166)	0.26	0.27	0.29	0.31	0.33
P.aeruginosa (MTCC7815)	0.26	0.37	0.41	0.45	0.55
Micrococcus (G35-I)	0.26	0.29	0.31	0.34	0.36
B. subtilis (R38-I)	0.26	0.37	0.35	0.44	0.48
control	0.17	0.16	0.18	0.18	0.17

Table 9. Growth of bacterial isolates in phenanthrene as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.26	0.28	0.32	0.35	0.45
A. faecalis (MTCC8164)	0.25	0.26	0.26	0.28	0.29	0.32
P.aeruginosa (MTCC8163)	0.25	0.25	0.26	0.26	0.27	0.29
B.circulans (MTCC8167)	0.25	0.25	0.26	0.26	0.28	0.30
P.aeruginosa (L43-I)	0.25	0.26	0.26	0.28	0.29	0.34
P.aeruginosa (L490-II)	0.25	0.26	0.27	0.29	0.29	0.32
B.licheniformis (MTCC8166)	0.25	0.28	0.28	0.3	0.35	0.38
P.aeruginosa (MTCC7815)	0.25	0.28	0.33	0.36	0.45	0.58
Micrococcus (G35-I)	0.25	0.27	0.31	0.34	0.36	0.38
B. subtilis (R38-I)	0.25	0.29	0.34	0.35	0.39	0.49
control	0.25	0.24	0.23	0.25	0.24	0.25

Table10. Growth of bacterial isolates in pyrene as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.28	0.34	0.36	0.42	0.44
A. faecalis (MTCC8164)	0.25	0.26	0.28	0.27	0.28	0.31
P.aeruginosa (MTCC8163)	0.25	0.25	0.27	0.32	0.35	0.38
B.circulans (MTCC8167)	0.25	0.26	0.27	0.29	0.32	0.35
P.aeruginosa (L43-I)	0.25	0.26	0.27	0.28	0.29	0.33
P.aeruginosa (L490-II)	0.25	0.25	0.26	0.28	0.30	0.32
B.licheniformis (MTCC8166)	0.25	0.26	0.28	0.33	0.34	0.37
P.aeruginosa (MTCC7815)	0.25	0.28	0.32	0.37	0.45	0.58
Micrococcus (G35-I)	0.25	0.26	0.29	0.32	0.36	0.39
B. subtilis (R38-I)	0.25	0.29	0.32	0.38	0.42	0.47
control	0.25	0.24	0.23	0.25	0.24	0.25

Table11. Growth of bacterial isolates in flourene as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.25	0.24	0.26	0.28	0.28
A. faecalis (MTCC8164)	0.25	0.26	0.26	0.27	0.29	0.29
P.aeruginosa (MTCC8163)	0.25	0.25	0.25	0.26	0.28	0.28
B.circulans (MTCC8167)	0.25	0.27	0.26	0.28	0.29	0.30
P.aeruginosa (L43-I)	0.25	0.26	0.25	0.28	0.29	0.31
P.aeruginosa (L490-II)	0.25	0.25	0.25	0.27	0.27	0.28
B.licheniformis (MTCC8166)	0.25	0.24	0.24	0.26	0.27	0.28
P.aeruginosa (MTCC7815)	0.25	0.27	0.29	0.31	0.35	0.40
Micrococcus (G35-I)	0.25	0.26	0.26	0.28	0.28	0.30
B. subtilis (R38-I)	0.25	0.26	0.28	0.30	0.35	0.37
control	0.25	0.24	0.23	0.25	0.24	0.25

Table12. Growth of bacterial isolates in carbazole as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.25	0.26	0.26	0.27	0.28
A. faecalis (MTCC8164)	0.25	0.26	0.26	0.25	0.27	0.27
P.aeruginosa (MTCC8163)	0.25	0.26	0.28	0.29	0.28	0.32
B.circulans (MTCC8167)	0.25	0.25	0.25	0.27	0.29	0.29
P.aeruginosa (L43-I)	0.25	0.25	0.26	0.26	0.28	0.28
P.aeruginosa (L490-II)	0.25	0.26	0.26	0.28	0.28	0.28
B.licheniformis (MTCC8166)	0.25	0.26	0.26	0.28	0.28	0.30
P.aeruginosa (MTCC7815)	0.25	0.3	0.34	0.38	0.42	0.46
Micrococcus (G35-I)	0.25	0.26	0.26	0.25	0.28	0.30
B. subtilis (R38-I)	0.25	0.26	0.28	0.28	0.30	0.38
control	0.25	0.24	0.23	0.25	0.24	0.25

Table13. Growth of bacterial isolates in pyridine as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.26	0.25	0.28	0.29	0.30
A. faecalis (MTCC8164)	0.25	0.27	0.28	0.27	0.29	0.30
P.aeruginosa (MTCC8163)	0.25	0.25	0.25	0.26	0.27	0.28
B.circulans (MTCC8167)	0.25	0.24	0.25	0.26	0.28	0.28
P.aeruginosa (L43-I)	0.25	0.26	0.26	0.28	0.28	0.32
P.aeruginosa (L490-II)	0.25	0.25	0.26	0.26	0.28	0.29
B.licheniformis (MTCC8166)	0.25	0.26	0.26	0.26	0.28	0.30
P.aeruginosa (MTCC7815)	0.25	0.28	0.30	0.35	0.38	0.45
Micrococcus (G35-I)	0.25	0.26	0.28	0.29	0.31	0.35
B. subtilis (R38-I)	0.25	0.27	0.28	0.30	0.35	0.38
control	0.25	0.24	0.23	0.25	0.24	0.25

Table14. Growth of bacterial isolates in paraffin as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.35	0.65	0.98	1.25	1.95
A. faecalis (MTCC8164)	0.25	0.45	0.52	0.75	0.92	1.30
P.aeruginosa (MTCC8163)	0.25	0.35	0.46	0.63	0.82	1.15
B.circulans (MTCC8167)	0.25	0.36	0.52	0.73	0.82	1.40
P.aeruginosa (L43-I)	0.25	0.28	0.34	0.56	0.89	1.50
P.aeruginosa (L490-II)	0.25	0.27	0.41	0.62	0.74	1.16
B.licheniformis (MTCC8166)	0.25	0.32	0.38	0.64	0.79	1.02
P.aeruginosa (MTCC7815)	0.25	0.56	0.78	0.96	1.83	2.15
Micrococcus (G35-I)	0.25	0.29	0.35	0.45	0.72	0.95
B. subtilis (R38-I)	0.25	0.35	0.67	0.85	1 25	1.75
control	0.25	0.24	0.23	0.25	0.24	0.25

Table15. Growth of bacterial isolates in 2-4 D as determine by optical density

Bacterial isolates	0h	24h	36h	48h	72h	96h
P.aeruginosa (MTCC8163)	0.25	0.27	0.29	0.32	0.35	0.36
A. faecalis (MTCC8164)	0.25	0.26	0.27	0.28	0.3	0.34
P.aeruginosa (MTCC8163)	0.25	0.27	0.28	0.28	0.29	0.30
B.circulans (MTCC8167)	0.25	0.26	0.26	0.29	0.29	0.31
P.aeruginosa (L43-I)	0.25	0.25	0.28	0.28	0.28	0.29
P.aeruginosa (L490-II)	0.25	0.25	0.26	0.28	0.28	0.30
B.licheniformis (MTCC8166)	0.25	0.26	0.27	0.27	0.28	0.32
P.aeruginosa (MTCC7815)	0.25	0.28	0.30	0.34	0.36	0.40
Micrococcus (G35-I)	0.25	0.25	0.27	0.29	0.29	0.30
B. subtilis (R38-I)	0.25	0.26	0.28	0.31	0.33	0.36
control	0.25	0.24	0.23	0.25	0.24	0.25