Bioremediation of Hydrocarbons by *Lysinibacillus fusiformis* BTTS10

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DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

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Dedicated to Infant Jesus and to the memory of my father N.J.Alexander and my sister Jasmine Paul who left us for their heavenly abode

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DECLARATION

I hereby declare that the research work presented in the thesis entitled "Bioremediation of Hydrocarbons by Lysinibacillus fusiformis BTIS10" is based on the original research work carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance and supervision of Prof.(Dr) M.Chandrasekaran, Department of Biotechnology, Cochin University of Science and Technology and the co-guidance and supervision of Dr.Sarita G.Bhat, Head, Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy and the thesis or no part thereof has been presented for the award of any other degree.

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Roselin Alex

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Abbreviations

%	Percentage
^{o}C	degree Celsius
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cm	centimeter
DNA	Deoxyribo Nucleic Acid
dNTP	deoxy Nucleotide tri phosphate
DW	Distilled water
dH_2O	Distilled water
Fig.	Figure
FT-IR	Fourier Transform Infrared Spectroscopy
gm	gram
g/L	grams per Liter
h	hours
kb	kilobase
LB	Luria Bertani
GC-MS	Gas Chromatography Mass Spectrometry
L	Litre
M	Molar
mg	milli gram
ml	milli litre
µl	micro litre
mM	milli molar
min	minutes
NCBI	National Center for Biotechnology Information
OD	Optical Density

PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
sec.	seconds
SmF	Submerged Fermentation
sp	Species
ASW	Artificial Seawater
Taq	*Thermus aquaticus*
UV	Ultraviolet
V	Volt
v/v	volume per volume
w/v	weight per volume
ZMB	Zobell Marine Broth
Dcpip	2,6 dichlorophenolindo phenol
BH	Bushnell Haas
cfu	colony forming units
DNTP	deoxy nucleotide tri phosphate
E.coli	*Escherichia coli*
GC	Gas Chromatography
OD_{600}	OpticalDensity $_{600}$
CO_2	carbon dioxide
µ g/mL	microgram/ml
BE	biodegradation efficiency
M	molar
N	normality

Chapter 1

INTRODUCTION

Contents

1.1. General Introduction

One of the major environmental problems in recent times is hydrocarbon pollution resulting from the activities related to the petrochemical industry. Accidental releases of petroleum products are of particular concern in the environment. Pollution of the environment with toxic hydrocarbons and heavy metals causes great damage to all living things. Crude oil, refined petroleum products and pyrogenic hydrocarbons are the most frequently occurring pollutants in the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants.

United States Energy Information Administration projects (2006) reveal that world consumption of oil will increase to 98.3 million

barrels per day in 2015 and 118 million barrels per day in 2030 (EIA,2006). Oil spills are inevitable with such a high consumption. Oil spill from Exxon Valdez, which spilled thousands of tonnes of oil is the most notable oil spill in sea (Paine *et al*., 1996; Albaiges *et al*., 2006). Oil spills have taken place all over the world - Iran, Iraq, Persian Gulf, Uzbekistan and even in India. Recent oil spills occurred in India were in the Mumbai coast in August 2010, January 2011 and August2011.The coast guard reported that the oil is leeching at the rate of 2 tonnes per hour and has spread up to seven nautical miles from the sinking ship of August2011. Two years after the Mumbai oil spill of August 2010, it is reported that the mangroves are still contaminated with crude oil. Petroleum is widely used as fuel and chemical compounds worldwide. The uncontrolled release of petroleum hydrocarbons from underground storage tanks, petroleum refineries, bulk storage facilities**,** broken oil pipelines, spills of petroleum products in chemical plants and transportation processes cause adverse effect on both flora and fauna. Service stations, garages, scrap yards, waste treatment plants and saw mills are other sources of petroleum pollution on land (Sherman and Stroo, 1989). The development of petroleum industry into new frontiers, acute accidents during transportation, leaking from old storage facilities and the inevitable spillage that occur during routine operations have called for more studies into oil pollution problem.

1.1.1. Consequences of marine oil spills

Oil spill causes extensive mortality to algae, corals, sea grass and to the largest and most mobile organisms. Oil residues contaminate the

tissues of organisms which then pass into food chain. Soluble components of crude oil are toxic to small organisms which have no protective covering. The most macroscopic impact of oil spill is the coating of large birds and animals with oil. Animals that are surface breathers like dolphins, sea turtles and whales inhale and, or ingest toxic vapours and tar balls and physically block their intestine and respiratory tracts.

The impact of oil spill on the estuaries, shallow waters, coastal estuaries, marshes and in the sea can be devastating. Tar balls form coating over plants and animals and prevents photosynthesis and breathing. The contaminants may remain in the sediments for many years Oil stains decrease the insulation of birds' feathers and freeze the birds to death. Oil diminishes the ability of birds to fly, swim and dive which leads to starvation.

1.1.2. Health effects of petroleum hydrocarbons

Compounds in petroleum hydrocarbons affect the human body in many different ways. The damage caused depends on the type of chemical compounds in petroleum, duration of exposure and concentration of the chemicals in contact. Aromatic compounds like benzene toluene and xylene affect human central nervous system. Death can occur if exposure is high enough. Breathing toluene at concentrations higher than 100 ppm for several hours causes fatigue, nausea, drowsiness and headache. Long term exposure causes permanent damage to nervous system. Hexane at a concentration of 500-2500ppm in air causes a nerve disorder called 'peripheral neuropathy', characterised by numbness in feet and legs and

paralysis in severe cases. Swallowing of petrol and kerosene causes irritation of throat and stomach and difficulty in breathing and pneumonia will develop later. Some components are potent carcinogen and also affect kidney, liver, spleen and developing foetus carcinogenic and toxic compounds cause cell damage, developmental disorders and impair reproduction. Prolonged exposure causes kidney damage and damage to bone marrow (Mishra *et al.*, 2001).

1.1.3. Bioremediation of hydrocarbon contaminated environment

The bioremediation uses living organisms usually bacteria and fungi to remove pollutants from soil, air and water with minimal disturbance to the environment. Bioremediation is not a new technology. Twenty years after the sinking of the super tanker Torrey Canyon in England, scientific community threw attention to the problems of oil pollution and fate of petroleum in various ecosystems. Marine environmentalists have given lot of focus and interest in this, as the world's oceans are the largest and ultimate receptors of pollutants from major oil pollution.

For many decades microorganisms have been used to remove organic materials and toxic chemicals from manufacturing and domestic waste effluent. Several case histories relevant to the role of microbial degradation in assessing the fate of petroleum pollutants from major oil spills have also been brought into focus. In the field of research and development, in academics, government and industry, bioremediation has taken a key role because all the countries have imposed strict laws to abate pollution. Bioremediation, which is potentially more cost-effective

than traditional techniques, requires an understanding of how organisms transform chemicals, how they survive in polluted environments, and how they can be used in the field.

There are five different methods for bioremediation. They are, above ground bioreactors, solid phase treatment, composting, land farming and *in situ* treatment. *In situ* treatment is done by the modification of the environment through nutrient addition and aeration and augmentation by the addition of appropriate microorganisms through seeding. The availability of oxygen is usually limiting in soil sediments and aquifers. In sub surface soil oxygenation can be provided by proper drainage and cultivation. Oxygen availability in ground water and deep soil layers contaminated by hydrocarbons can be achieved by pumping down appropriate concentrations of hydrogen peroxide (Brown e*t al.*,1984). Decomposition of hydrogen peroxide releases oxygen and support aerobic microbial metabolism. Above ground bioreactors are used to treat liquids, solids in a slurry phase and polluted air from factories. Bioremediation becomes successful only if the contaminant is readily biodegradable and the end products of biodegradation are nontoxic. Biodegradation of a pollutant depends on various factors such as the nature and amount of pollutant present, prevalent abiotic factors at the site and the composition of the indigenous microbial community (Atlas 1981; Leahy and Colwell 1990; Hinchee and Olfenbuttel, 1991).

Bioremediation is not an expensive process and the end products of bioremediation are carbon dioxide and water which are readily utilized by environment. Bioremediation can be applied on the site where

conventional technologies need the movement of toxic contaminated soil to incinerators and thus become highly expensive.

In order to prove that bioremediation technology is efficient, it has to be documented under controlled condition. Laboratory conditions that closely model environmental conditions can most likely produce relevant result. The most direct measure of bioremediation efficacy is by monitoring the disappearance rate of pollutants. Field evaluation of bioremediation is done by enumerating the number of pollutant degrading microorganisms and the recovery and analysis of residual pollutants.

Johnson *et al*., (1985) described successful application of defined microbial population for the abatement of pollution. Commercial mixtures of microorganisms are marketed in large scale to treat petroleum contamination in both bioreactors and *in situ* treatment.

Microorganisms are capable of biodegradation of all the pollutants which have similar structure as that of natural organic compound but the synthesis of new compound having no relation to natural compounds will remain as recalcitrant compounds in the environment. The absence of metabolic pathways for the degradation of xenobiotic compounds appears to be the main obstacle for their degradation. But this obstacle is no longer a permanent one. Microorganisms evolve new pathways by exchange of genes in plasmids between microorganisms. New mutants can be developed spontaneously by enrichment technique or can be induced by radiations. Recombinant technology holds promise for developing strains with better capabilities. Many genes coding for

biodegradation and metal accumulation are situated on the transposable chromosomal elements or on plasmids (Eaton and Timmis, 1984). Transfer and recombination of these movable elements play an important role in the evolution of new strains with novel capabilities for biodegradation of new contaminants produced by humans.

1.1.4. Biochemistry of petroleum hydrocarbons

The term hydrocarbon embraces all those organic molecules composed of carbon and hydrogen. There are huge deposits of complex mixture of hydrocarbons present on the surface of earth and below the ground. These are believed to be produced by the combined effect of heat and pressure on the dead remains of the plant and animal material buried during the past, 600 million years ago. Biochemical changes made over these periods in these sedimentary deposits as a result of microbial activities lead to the formation of petroleum.

All petroleum products have their origin from crude oil. It is a complex mixture of thousands of hydrocarbons and some organo metallo constituents. Petroleum products are used as fuels, as solvents and feed stocks in the plastic industries, textile and pharmaceutical industries. It is a complex mixture of hundreds of thousands of aliphatic, branched and aromatic hydrocarbons and heavy metals. Petroleum components can be separated in to four fractions, the saturated, aromatic, resin and asphaltene fraction by absorption chromatography. Large numbers of compounds are present in each of these fractions (Karlsen and Larter, 1991). Saturates are defined as hydrocarbons containing no double bonds.

They are further classified according to their chemical structure into alkanes (paraffins) and cycloalkanes. Highest percentage of crude oil constituents are saturates. Alkanes are either branched or unbranched. Cycloalkanes have one or more carbon rings. Majority of alkanes have alkyl substituent(s). Aromatic hydrocarbons have one or more aromatic rings usually substituted with different alkyl groups. Resins and asphaltenes contain non hydrocarbon polar compounds. Resins and asphaltenes have very complex and mostly unknown carbon structure with trace amounts of nitrogen, sulphur and /or oxygen. These compounds form complexes with heavy metals. Asphaltenes are the most recalcitrant component of the crude oil. The precipitation of these compounds cause problems like blockage of pipelines during extraction, refining and transportation and also pollution of the environment.High molecular compounds in asphaltene make it insoluble in n- heptanes, while resins are n-heptane soluble polar compound. Resins contain heterocyclic compounds acids and sulfoxides (Harayama, 2004.) The composition of a particular petroleum product varies from one reservoir to another in their physical properties and composition.

1.1.5. Microorganisms oxidizing hydrocarbons

The hydrocarbon degrading capacity of microorganism was reviewed by Zobell, (1946).He stated that the microbial utilization of hydrocarbon was dependent on the chemical nature of the compound and the environmental factors prevalent in that area. Hydrocarbon degrading microorganisms are ubiquitous in nature are found at higher densities in petroleum contaminated sites, estuaries, oceans, marine sediments, deep seas, thermal vents, and arctic

environments. Predominant hydrocarbon utilizers isolated from aquatic environment are *Pseudomonas, vibrio, Achromobacter, Arthrobacter, Micrococcus, Corynebacterium Acinetobacter, Nocardia* etc. While *Aureobasidium, Rhodotorula, Candida and Sporobolomyces* were the most common fungi isolated from marine sediments. Attempts to determine microbial diversity in natural environment are limited by the inability to culture all microbes present as indigenous population in the contaminated sites.

There are conflicting reports in the literature pertaining to the ability of individual species to degrade both the aliphatic and aromatic components in crude oil. Bushnell and Haas, (1940) reviewed the literature on this subject and reported that a single species alone could not degrade both aromatic and aliphatic compounds. In many studies microbial consortium were used to degrade crude oil. Similarly, Austin *et al.,* (1977) demonstrated that there was some degree of specificity in the types of hydrocarbons degraded by given bacterial species.

1.1.6. Immobilization of whole cells in bioremediation

Immobilized cells of microorganisms have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization simplifies separation and recovery of cells and makes the application reusable which reduces the overall cost. The use of immobilized cells can overcome adverse environmental conditions that threaten microbial survival. Wilson and Bradely, (1996) used free cell suspension and immobilized cells of *Pseudomonas* sp. to degrade petrol in an aqueous

system. The study indicated that immobilization resulted in a combination of increased contact between cell and hydrocarbon droplets.

1.1.7. Behaviour of petroleum in marine environments

Petroleum undergoes many modifications when it is spilled in to the sea. The composition of petroleum changes with time. This is mainly due to dissolution of water soluble components, evaporation of low molecular weight fractions, mixing of oil droplets with sea water photo chemical oxidation and biodegradation. The components of petroleum with a boiling point below 250^0C are easily evaporated. Therefore n-alkanes shorter than C_{14} are reduced by weathering. Aromatic hydro carbons below this boiling point also get weathered as they are subjected to both evaporation and dissolution. Mixing of oil with sea water takes place in different forms. Emulsification takes place when petroleum contains polar components. Dispersion of water droplets takes place by the action of waves

1.1.8. Degradation pathway of alkanes

The aerobic biodegradation of hydrocarbons is a well studied process. The hydrocarbons are broken down by a series of enzyme mediated reactions. Hydrocarbons in the contaminants will act as an electron donor and oxygen serves as an external electron acceptor. Biodegradation of n-alkanes occur more frequently than all other components of the crude oil. Biodegradation of hydrocarbons up to c_{14} have been demonstrated (van Hamme,2003). NADPH dependent monoxygenase oxidize alkanes to corresponding alcohols which is

subsequently oxidized to aldehyde and then to fatty acid thus formed is assimilated into cellular carbon via β oxidation and TCA cycle. During each cycle of β oxidation one CO_2 is released along with a new fatty acid which is two carbon units shorter than the parent molecule.

Biodegradation pathway of higher alkanes is broadly categorized in to three routes on the basis of initial attack on the alkane molecule. Route 1 involves the terminal oxidation of methyl groups to carboxylic acids via primary alcohol catalyzed by rubredoxin bearing monooxygenase as in the case of *Pseudomonas oleovorans* or by P450 monooxygenase as in the case of *Cornybacterium* sp.The alcohol is subsequently oxidized to aldehyde by two NADP linked dehydrogenase (Fig.1.1). The fatty acid is further metabolized by β oxidation. The second route oxidizes both ends of the molecule to form α, ω-dicarboxylic acids. In the third pathway subterminal oxidation takes place to form secondary alcohols and Ketones.

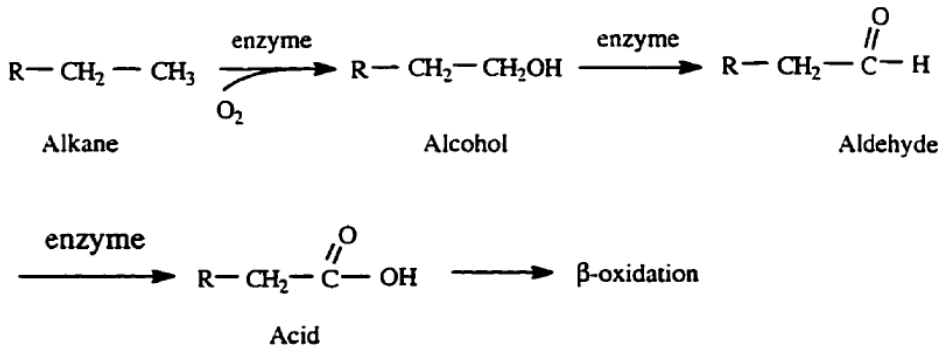


Figure1.1 Degradation of Aiiphatic Hydrocarbon (After Gaudy, and Gaudy. 1980)

1.1.9. Biodegradation of aromatic compounds

Simple aromatic compounds are easily degraded by bacteria. But polyaromatic compounds and aromatic nuclei with side chain substituent particularly with halogens are recalcitrant compounds. Derivitization of aromatic nuclei with various substituents particularly with halogens make them more recalcitrant. While alkyl substituted compounds such as isomeric xylenes, cresols, xylenols etc are amenable to microbial degradation (Atlas and Bartha, 2005).

In aerobic systems the critical step in the metabolism of aromatic compound is catalysed by dioxygenase which cleave the resonance structure by hydroxylation and fission of the benzoid ring. Based on the substrate, aromatic metabolism can be grouped in to three pathways as catechol path way, protocatechuate path way and gentisate pathway. In all these pathways ring activation by the introduction of hydroxyl group is followed by the enzymatic ring cleavage. The products of the ring fission then undergo transformations and enter in to the general metabolic pathways of the organisms (Nair, 2006).

Most of the aromatic pathways converge at catechol. From the substituted and non substituted mono and polyaromatic compounds catechols are formed as intermediates. Most aromatic hydrocarbons such as benzene and its derivatives are converted into dihydro benzene by the incorporation of molecular oxygen on the ring. The enzyme dehydrogenase then convert dihydro benzene to catechol and then it is cleaved between two closed hydroxylated carbon atoms to form muconic

acid by dioxygenase enzyme. The muconic acid is further metabolised in to β, Keto adipic acid and then to succinic acid and finally to acetyl- CoA which is an intermediate of TCA cycle. Oxidation of substituted hydrocarbon takes place by beta- oxidation of side chain followed by ring cleavage. The degradative pathway for highly branched aromatic hydrocarbons such as pristine or phytane may proceed by omega oxidation producing dicarboxylic acid instead of monocarboxylic acid (van Hamme et al., 2003).

Most of the haloaromatics are degraded through the formation of the respective halocatechols, the ring fission of which takes place via ortho-mode. Most of the non halogenated aromatic compounds are degraded through meta pathway. The fission product of metacleavage would be cis,cis- muconic acid or its derivative depending on whether the catechol is substituted or not (Fig.1.2). The meta fission product of catechol would be 2 -hydroxy muconic semialdehyde and the products of both ortho and meta pathways are further metabolised as intermediates of TCA cycle. Ortho pathway is the most productive pathway for the organism as it involves less expenditure of energy. (Nair, 2006)

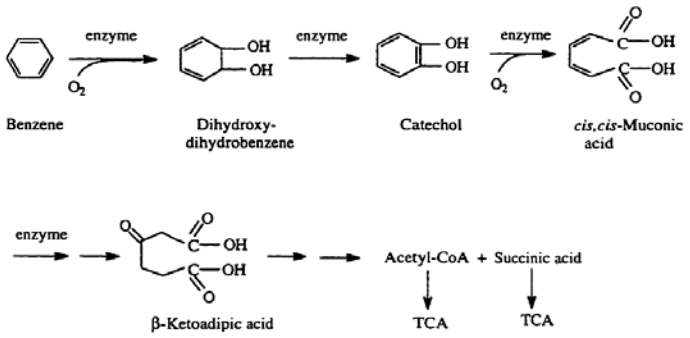


Fig:1.2 Degradation of Typical Aromatic hydrocarbon

The degradation pathway of naphthalene, anthracene and phenanthrene were reported by Schigel, (1993). Unlike benzene, here salicylate is formed instead of catechol. The salicylate is then converted into catechol and then further degradation takes place.

Table1.1: Aromatic hydrocarbon degrading bacteria.

Bacterial species	Strains	Aromatics
Achromobacter sp.	NCW	CBZ
Alcaligenes denitrificans		FLA
Arthrobacter sp.	F101	FLE
Arthrobacter sp.	P1-1	DBT, CBZ, PHE
Arthrobacter sulphureus	RKJ4	PHE
Acidovorax delafieldii	P4-1	PHE
Bacillus cereus	P21	PYR
Brevibacterium sp.	HL4	PHE
Burkholderia sp.	S3702, RP007, 2A-12TNFYE-5, BS3770	PHE
Burkholderia sp.	C3	PHE
Burkholderia cepacia	BU-3	NAP, PHE, PYR
Burkholderia cocovenenans		PHE
Burkholderia xenovorans	LB400	BZ, BP
Chryseobacterium sp.	NCY	CBZ
Cycloclasticus sp.	P1	PYR
Janibacter sp.	YY-1	DBF, FLE, DBT, PHE, ANT, DD
Marinobacter	NCE312	NAP
Mycobacterium sp.		PYR, BaP
Mycobacterium sp.	JS14	FLA
Mycobacterium sp.	6PY1, KR2, AP1	PYR
Mycobacterium sp.	RJGII-135	PYR,BaA, BaP
Mycobacterium sp.	PYR-1, LB501T	FLA, PYR, PHE, ANT
Mycobacterium sp.	CH1, BG1, BB1, KR20	PHE, FLE, FLA, PYR
Mycobacterium flavescens		PYR, FLA
Mycobacterium vanbaalenii	PYR-1	PHE, PYR, dMBaA
Mycobacterium sp.	KMS	PYR
Nocardioides aromaticivorans	IC177	CBZ
Pasteurella sp.	IFA	FLA
Polaromonas naphthalenivorans	CJ2	NAP
Pseudomonas sp.	C18, PP2, DLC-P11	NAP, PHE
Pseudomonas sp.	BT1d	HFBT
Pseudomonas sp.	B4	BP, CBP

Pseudomonas sp.	HH69	DBF
Pseudomonas sp.	CA10	CBZ, CDD
Pseudomonas sp.	NCIB 9816-4	FLE, DBF, DBT
Pseudomonas sp.	F274	FLE
Pseudomonas paucimobilis		PHE
Pseudomonas vesicularis	OUS82	FLE
Pseudomonas putida	P16, BS3701, BS3750, BS590-P, BS202-P1	NAP, PHE
Pseudomonas putida	CSV86	MNAP
Pseudomonas fluorescens	BS3760	PHE, CHR, BaA
Pseudomonas stutzeri	P15	PYR
Pseudomonas saccharophilia		PYR
Pseudomonas aeruginosa		PHE
Ralstonia sp.	SBUG 290 U2	DBF NAP
Rhodanobacter sp.	BPC-1	BaP
Rhodococcus sp.		PYR, FLA
Rhodococcus sp.	WU-K2R	NAT, BT
Rhodococcus erythropolis	I-19	ADBT
Rhodococcus erythropolis	D-1	DBT
Staphylococcus sp.	PN/Y	PHE
Stenotrophomonas maltophilia	VUN 10,010	PYR, FLA, BaP
Stenotrophomonas maltophilia	VUN 10,003	PYR, FLA, BaA, BaP, DBA, COR
Sphingomonas yanoikuyae	R1	PYR
Sphingomonas yanoikuyae	JAR02	BaP
Sphingomonas sp.	P2, LB126	FLE, PHE, FLA, ANT
Sphingomonas sp.		DBF, DBT, CBZ
Sphingomonas paucimobilis	EPA505	FLA, NAP, ANT, PHE
Sphingomonas wittichii	RW1	CDD
Terrabacter sp.	DBF63	DBF, CDBF, CDD, FLE
Xanthamonas sp.		PYR, BaP, CBZ

(PYR, pyrene; BaP, Benzo[*a*]pyrene; PHE, phenanthrene; FLA, fluoranthene; FLE, fluorene; ANT, anthracene; NAP, naphthalene; BaA, benz[*a*]anthracene; dMBaA, dimethylbenz[*a*]anthracene; DBA, dibenz[*a,h*]anthracene; COR, coronene; CHR, chrysene; DBF, dibenzofuran; CDBF, chlorinated dibenzothophene; HFBT, 3-hydroxy-2-formylbenzothiophene; BP, biphenyl; CBP, chlorobiphenyl; NAT, naphthothiophene; BT, benzothiophene; BZ, benzoate; ADBT, alkylated dibenzothiophene; CBZ, carbazole; DD, dibenzo-*p*-dioxin; CDD, chlorinated dibenzo-*p*-dioxin; MNAP, methyl naphthalene).

1.1.10. Bioremediation of heavy metals

Microbe metal interaction plays an important role in the remediation of metal. Microorganisms remove metals and metalloids by reducing them to lower redox states. Many microorganisms use metals and metalloids as terminal electron acceptors in anaerobic respiration. (Lovely *et al.*,1994). Various mechanisms involved in microbe metal interaction are precipitation, intracellular accumulation, extracellular metal complexation, metal sorption on the microbial cell wall surfaces and metal transformation. The use of bacterial biomass in metal sorption is of great interest owing to its great diversity but few attempts have been made to exploit this in practice.

In this context, the present study was aimed at developing a bioprocess for degradation of crude oil hydrocarbon using bacteria capable of rapid biodegradation of the same.

1.2. Research Objectives

In the context of need for recognizing potential bacterium for effective bioremediation of crude oil pollutants in the environment it was desired to isolate a potential bacterium from marine sediment, which often experiences oil pollution and develop a bioprocess for crude oil biodegradation. Efficacy of the selected strain under free cell suspension state as well as under immobilized conditions was also aimed at towards confirming the true potential of the oil degrading bacterium.

The specific objective of the present study included the following:

i. Isolation, identification and characterization of the selected bacteria with potential for degradation of crude oil and its fractions.

ii. Biodegradation of crude oil and its fractions with bacteria under submerged culture condition and optimization of process conditions for maximizing biodegradation of crude oil.

iii. Biodegradation of crude oil and its fractions with immobilized whole cell biomass.

iv. Biodegradation of toluene and asphaltene fraction of crude oil.

v. Isolation of plasmids and confirming the role of plasmids in biodegradation of hydrocarbons.

Chapter 2

REVIEW OF LITERATURE

Contents

Petroleum is a complex mixture of aliphatic, alicyclic, and aromatic hydrocarbons and a smaller number of nonhydrocarbon compounds such as naphthenic acids, phenol, thiol, heterocyclic nitrogen, sulfur compounds and metalloporphyrins (Atlas and Bartha, 1992). Accidental spills of crude oil and its refined products occur on a regular basis during routine operations of extraction, transportation, storage, refining and distribution. Recent estimates reported that between 1.7 and 8.8 million metric tons of oil is released into the world's water every year (Nikolopoulou and Kalogerakis, 2008).

2.1 Bioremediation of petroleum hydrocarbons

The most important principle of bioremediation is that microorganisms can be used to destroy hazardous contaminants or transform them to less harmful form. However, susceptibility of crude oil to biodegradation varies with the type and size of the molecule (Atlas, 1984). Ever since Zobell, (1946) reported the biodegradation of hydrocarbons, the use of microbial catalysis in the biodegradation of organic compound has advanced significantly during the last three decades. Atlas, (1981) studied

the bioremediation of petroleum hydrocarbon in contaminated ground water. He reported that microorganisms in the polluted areas adapt according to the environment as a result of which mutations are caused in the subsequent generations, changing them to become hydrocarbon degraders. In unpolluted environment the numbers of degraders are less than 1% of the population while in polluted areas they are 1% to 10% of the population.

It was reported that petroleum and creosote are most frequently the pollutants of concern, comprising about 60% of the sites where bioremediation is being applied in field demonstrations or for full-scale operations (Hinchee and Olfenbuttel, 1991).

To show that a bioremediation technology is potentially useful, it is important to document improved biodegradation of the pollutant under controlled conditions. This generally cannot be accomplished *in situ* and thus must be done in laboratory experiments. Laboratory experiments demonstrate the potential of a particular treatment in the removal of a xenobiotic from a contaminated site (Bailey *et al.,* 1973) Laboratory experiments that closely model real environmental conditions are most likely to produce significant results (Bertrand *et al.,* 1983).

The parameters typically measured in laboratory tests of bioremediation efficacy include enumeration of microbial populations (Song and Bartha, 1990) measurement of rates of microbial respiration (oxygen consumption or carbon dioxide production), and determination of degradation rates (disappearance of individual or total pollutants) as

compared to untreated controls. The methodologies employed in these measurements are critical.

In the case of soil contaminated by oily sludge or fuel spills, seed germination and plant growth bioassays were documented to study the progress of bioremediation and the decrease in toxicity (Dibble and Bartha, 1979c; Bossert and Bartha, 1985; Wang and Bartha, 1990).

2.2. Degradation of hydrocarbons by microbes.

Microorganism in environment is known to degrade organic compounds including hydrocarbons as their source of carbon and nutrient for their growth and proliferation. These microorganisms are in fact affecting natural bioremediation in various environments as a microcosm. To study hydrocarbon degrading microbial communities, three general experimental protocols are used: physiological, metabolic and genetic.

The oldest technology used is the traditional culture method developed by Koch. Microbes are isolated by culturing and studied individually or collectively (Komukai *et al*., 1996. Fang and Barcelona, 1998*)* proposed whole community fatty acid analysis (PFLA) to get a qualitative view of the community structure.

Metabolic characterization use quantification of specific metabolic activities or biochemical markers. Berthe-Corti and Bruns, (1999) developed Biolog breath print which uses 95 different substrates in 96 well microtitre plates to examine community activity. For many years only those microorganisms which are culturable are isolated and identified as oil degraders. With the recent decline in the cost of DNA sequencing,

metagenomics has made it possible to determine the collective genome of a microbial community. Bioinformatics tools help scientists to design gene sequences which code for enzymes involved in oil biodegradation (MacNaughton, 1999; Theron and Cloete, 2000).

Screening of hydrocarbon degrading microorganisms are done by various methods which includes growing bacteria in a liquid or solid mineral medium supplemented with hydrocarbon as the sole source of carbon, measurement of turbidity in microtitre plates, O_2 consumption, the most probable number technique and sheen screen technique. All these techniques are expensive, time consuming and laborious. A rapid and simple screening technique using redox indicator, 2, 6, dichlorophenol indophenol (2, 6, DCPIP) has been developed to isolate potential hydrocarbon degraders (Hanson *et al.*,1993*)*. This technique is based on the principle that during the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as O_2, sulphates and nitrates. When an electron acceptor such as DCPIP is incorporated in to the culture medium, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the colour change of DCPIP, which is blue in colour (oxidized) to colourless (reduced).

Plasmids play an important role in conferring biodegradation potential to bacteria. Degradation of aromatic compounds is usually mediated by plasmids. Number of plasmids has been found to be increased with exposure of microbial community to hydrocarbon. Frantz and Chakraborty, (1986) reported the role of conjugative plasmids in regulating many bacterial catabolic pathways.

Rahman *et al.*,(2002) isolated 5 species of bacteria capable of degrading crude oil. Among 130 bacteria isolated from oil contaminated soil sample, *Micrococcus sp.GSS 66; Flavobacterium sp DSS 73;Bacillus spDs6-86,Corynebacterium sp GS556 and Pseudomonas sp DS10-129* were found to be efficient in the cleaning of crude oil contaminated sites. Das and Singh, (2006) isolated *Bacillus subtilis and Pseudomonas aeruginosa* strains from petroleum oil contaminated sites from North East India and demonstrated the efficiency of these strains *in situ* bioremediation. They also found that these strains produced biosurfactant. Obuekwe and Al-Zarban, (2009) isolated six crude oil degrading bacteria: *Pseudomonas, Bacillus, Staphylococcus, Acinetobacter, Kocuria and Micrococcus* and showed that bacteria which are predominant in a contaminated area were better crude oil utilizers than less frequently occurring bacterial isolates. Wang *et al.,* (2012) isolated a novel strain of *Dietzia* capable of utilizing wide range of n- alkanes (C_6-C_{40}), aromatic compounds and crude oil as sole source of carbon.

Aromatic hydrocarbon such as benzene, toluene ethyl benzene and xylene causes serious problems in ground water, surface water and in soil. Mazzeo *et al.,* (2010) isolated BTEX degrading microorganism, *Pseudomonas putida* from the effluents of petroleum refinery. They used *Allium cepa* and hepatoma tissue culture (HTC) cells to test the mutagenecity and genotoxic damage to assess the potential of BTEX degrading organisms.

A significant number of hydrocarbon degrading microorganisms are present in the soil and ocean sediment and it was found that their number increases considerably in oil polluted areas (Bragg *et al.,* 1994,

Harayama *et al.,* 2004 and Head *et al.,* 2006). Bacteria which are highly specialized in degrading hydrocarbons are called hydrocarbonoclastic bacteria and they play a key role in the removal of hydrocarbons from the polluted environment (Harayama *et al.,* 2007 and Yakimov *et al.,* 2007). *Alcarnivorax borkumensis* is a marine bacterium which originally found in low concentration in the sea was found to be increased enormously after a spill of crude oil. *Alcanivorax* strains are believed to play an important role in natural bioremediation of oil spills worldwide (Kasai *et al.,* 2002, Harayama *et al.,* 2004 Mckew *et al.,*2007 ab). *Thlassolitus* sp (Yakimov *et al.,* 2004), *Olephilus* (Golyshin *et al.,* 2002), and *Oleispira* (Yakimov *et al*., 2003) were reported as hydrocarbonoclastic genera of bacteria.

Elshafie *et al.,* (2007) isolated fungi capable of degrading n- alkanes and crude oil from the beaches of Oman. They compared the biodegradation potential of three fungi, *Aspergillus niger, Aspergillus terreus and Pencillium chrysogenum* and found that *A terreus* and *P. chrysogenum* can be used as potential organisms for the removal of crude oil from contaminated sea.

2.3. Factors that influence biodegradation of hydrocarbons

The rate of biodegradation is influenced by many factors such as pH, temperature, oxygen, composition, concentration and bioavailability of the contaminants, chemical and physical characteristics of the contaminated environment (Margesin *et al*., 2003).

2.3.1. Temperature

Temperature influences biodegradation by its effect on the physical nature and chemical composition of the pollutant, nature of microbial flora, and the rate of hydrocarbon metabolism (Venosa and Zhu, 2003). Hydrocarbon biodegradation occurs over a wide range of temperatures and biodegradation of hydrocarbons generally decreases with decreasing temperature. Highest degradation in the soil environment was observed to be in the range of 30^0C to 40 ^{0}C, 15 ^{0}C to 20^0C in marine environments and 20^0C to 30^0C in fresh water environment.

2.3.2. Oxygen

Oxygen is an important factor for biodegradation of hydrocarbons since major degradative pathways for both saturates and aromatics take place aerobically involving oxygenases. Bacterial activity proceeds rapidly if oxygen is provided sufficiently. During aerobic biodegradation, petroleum hydrocarbon is oxidized to create energy, carbon dioxide and cell mass, while molecular oxygen is reduced to water. Dineen *et al.,* (1990) reported that the requirement of oxygen to degrade hydrocarbon is 3.1 g of oxygen for 1.0 g of hydrocarbon. In order to enhance the activity of microbial flora, a process called soil venting has been used and three bio venting projects in Southern California were reported (Dineen *et al*., 1990). Role of oxygen as a critical point in the biodegradation of hydrocarbons made Brown and his co workers to use hydrogen peroxide as an oxygen carrier (Brown *et al*., 1984). Treating the soil with dilute hydrogen peroxide at a concentration up to 1000 mg was successfully used.

Conditions of oxygen limitation do not occur in the upper levels of water column and the surface layers of soil, but it may become limiting in the subsurface, deeper layers of water, anoxic sediments and most fine - grained marine shore lines. Oxygen availability is influenced by wave action, physical state of the oil, and the concentration of available substrate .Recent studies proved the role of anaerobic metabolism in the degradation of hydrocarbons (Head and Swannell, 1999). Biodegradation of BTEX compounds occurs under a variety of anaerobic conditions (Leahy and Colwell, 1990; Zhu *et al*., 2004).

2.3.4. Nutrients

In marine waters, nutrients such as phosphorus, nitrogen and iron play much more critical role than oxygen in limiting the rate of biodegradation of hydrocarbons. Several investigators on major oil spills have focused attention on the problem of hydrocarbon contamination in marine and estuarine environments and the potential use of bioremediation through nutrient addition to remove petroleum components. When nutrient deficiency limit the rate of biodegradation of hydrocarbon, addition of nitrogen and phosphorus offers a great promise as a counter measure for combating oil spills. (Pritchard and Costa, 1991; Atlas and Bartha, 1973a). Oleophilic fertilizers that place the nitrogen and phosphorus at the oil water interface, the site of active oil biodegradation, have been developed. In the aftermath of the *Amco Cadiz* oil spill of 1978 and Exxon Valdez spill 1989, Inopol EAP22 was used. Ding Juan *et al,* (2008) *studied* the biodegradation of polycyclic aromatic hydrocarbon biodegradation by *Phanarochaete chrysosporium*. They found that

addition of tween 80 increased the biodegradation of polycyclic hydrocarbon by increasing its solubility.

2.3.5. pH and salinity

pH and salinity are the other important factors affecting biodegradation of petroleum hydrocarbons. pH of the sea water is slightly alkaline and generally stable but the pH of soil and fresh water are highly variable. Degradation of oil increases with increasing pH and slightly alkaline condition is optimum for biodegradation of petroleum hydrocarbon (Zhu *et al*., 2004). The rate of hydrocarbon biodegradation decreases with increase in salinity in the range of 3.3 to 28.4 % (Leahy and Colwell, 1990). The reduction in degradation rate is attributed to reduction in metabolic activities. In estuaries salinity values as well as nutrient levels and oxygen concentration are quite different from those in costal or ocean areas.

2.3.6. Nitrogen and phosphorous

Oil remediation by indigenous microorganisms is limited by the availability of nitrogen and phosphorous. When these salts are supplied in large volumes of water, it will get diluted. Sook *et al*.,(2001) used Slow Release Fertilizers (SRF) by adding urea and phosphate attached to silica and latex as support and reported the enhanced efficiency of crude oil biodegradation. Chainaeu., (2005) studied the effect of application of fertilizers on the degradation hydrocarbon in soil and reported that high inputs of nutrients have adverse effect on the degradation hydrocarbons and the degradation of asphaltenes and aromatics are reduced by the addition nutrients. Nikolopoulou *et al*., (2007) reported the use of hydrophobic nitrogen

and phosphorus, the lecithin and uric acid respectively for stimulating the growth of indigenous bacteria.

2.3.7. Inoculum and inoculation

Ragheb *et al.,* (2011) studied the effect of successive inoculation with hydrocarbon degrading bacteria on the dynamics of petroleum hydrocarbon in soil. The study revealed that stage wise bioaugmentation is more effective in removing TPH than applying the whole inoculum at the beginning of the degradation process. Sang–Jin Kim, (2005) evaluated the bioremediation effectiveness on crude oil. They suggested the importance of high concentration of microorganism for enhancing the mineralization of crude oil. They also found that high concentration of crude oil limit the efficiency of crude oil because of the reduction in the transport of essential factors such as oxygen and nutrients. When bioremediation is applied for cleanup of contaminants in long term weathered contaminated sites, low bioavailability of contaminants and low number of bacteria capable of degrading hydrocarbons reduce the speed of bioremediation. Liang *et al.,*(2009) developed bio carrier for immobilization of indigenous hydrocarbon degrading bacteria using porous material such as activated carbon and zeolite, the microbial biomass reached 10^{10} cells g^{-1} on activated carbon and 10^{6} cells g^{-1} on zeolite.

2.3.8. Biostimulation of native microbial flora

Carillo *et al.,*(2011) evaluated the effect of biostimulation of native microbial flora at different nutrient ratios for biodegradation of sludge.

Microtox assay and Ames test proved that biodegradation of the sludge by native microbes resulted in the conversion of the toxic components in the sludge to nontoxic products.

Gogoi *et al.,* (2003) investigated the bioremediation of petroleum hydrocarbon in contaminated soil at crude oil spill site in the Babahola oil field in Assam, India. The field study conducted by applying nutrient and microbe rich solution showed that crude oil components were reduced by 75% under a life span of 1 year. They suggested that more studies are required to deal with high concentration of contaminants. Computer simulation studies can develop better models to deal with the problem and are more advantageous than lab studies since lab experiments take long periods of the order of 1 year.

Obuekwe *et al.,(*1998) reported that pieces of stones and other solid materials found in oil lakes formed in Kuwait after Iraqi invasion appeared clean. This is an indication of surface enhanced crude oil degradation. SEM studies revealed the presence of active colonies of microbes. The resulting mixed material degraded crude oil effectively in the presence of inert carrier materials than in the absence of inert materials. The inert materials were extensively colonised by microorganisms just as those observed in the stones obtained from the lakes. Vanloosdercht, (1990) suggested that surfaces are important in the degradation of contaminant in nature. Wilson and Bradley, (1996) also reported the increased efficiency of crude oil degradation by surface associated microorganisms. Radwan *et al.,* (1995) reported that plants growing with in the oil lake sites showed clean roots lacking any sign of oil smears indicating enhanced root surface associated crude oil pollution action.

2.4. Biodegradation of aromatic compounds by bacteria.

Aromatic hydrocarbons such as benzene, toluene, ethyl benzene and xylenes (BTEX) are environmental pollutants that cause serious problems in groundwater, in surface water and in soil. Mazzeo, (2010) identified and selected BTEX-biodegrading microorganisms present in effluents from petroleum refinery and evaluated the efficiency of microorganism in the biodegradation process for reducing genotoxic and mutagenic BTEX damage through two test-systems: *Allium cepa* and hepatoma tissue culture (HTC) cells. Heinfling *et al.,* (1997) reported the degradation of azodye- pthalocyaninedye by *Tarmetes versicolor* and *Bjerkandera adjusta*. They compared the specific activities of exoenzyme preparation with the oxidation rates of commercial horse radish peroxidase.

Biodegradation of polychlorinated biphenyl was reported by Gilbert and Crowley, (1998) using *Arthrobacter sps.* Similarly biodegradation of toluene by *Pseudomonas putida* in trickling filters (Peixota and Mota, 1998) and nitrobenzene to aniline *by Commamonas audiovorans* (Peres *et al.,* 2000) were also attempted.

Gouz *et al.,* (2000 a) developed an efficient microbial consortium for the degradation of atrazine. Mason *et al.,* (2000) reported a microbial consortium promising in the biodegradation of benzene, toluene, ethyl benzene and mixed xylenes. The study was conducted in a completely mixed bioreactor in the presence of activated carbon where bacteria were constantly in a flux between absorbent and free phase.

Five distinct pathways have been described for the biodegradation of toluene. First step in all the pathways were oxidation reactions. But five different products were formed. In *P. Putida Fi*, toluene 2, 3 dioxygenase enzyme oxidizes the aromatic ring of toluene, incorporating two oxygen atoms. Then a dehydrogenation step (Finette *et al.,*1984*;* Gibson *et al.,*1990) produces 3 methylcatechol which is further degraded via meta ring fission. *P. putida paw15* converts toluene to benzoate by degrading the methyl group of toluene. (Williams *et al.,*1994) Then benzoate is converted into catechol which is further cleaved by metacleavage pathway.

In *Burkholderza cepacia G4* toluene – monooxygenase attacks toluene at 3 positions of toluene, to form o-cresol and to methyl catechol (Shields *et al.,1989*). In *Ralstonia pickertii*, m- cresol is formed at the 3 position from toluene and then second mono oxygenase attacks at the 2 position of m- cresol, to form 3- methyl catechol. (Yabuuchi *et al.,*1995).

2.5. Biodegradation of alkanes.

Bacteria, filamentous fungi and yeast can degrade alkanes as a source of carbon and energy. (Wentzel *et al*., 2007). Alkanes are insoluble in water. There is a universal relationship between chain length and solubility. Low molecular weight alkanes are directly taken by the bacteria. For medium and long chain length alkanes micro organisms gain access to the hydrocarbons either by adhering to hydrocarbon or by a surfactant facilitated process (Wentsel *et al*.,2007). Lyle *et al*., (1998) investigated the alkane catabolic pathway of *Rhodococcus* strain QI5 a

psychrotroph. They found that Q15 oxidizes alkanes by both terminal oxidation pathway and subterminal oxidation pathway.

2.6. Enzymes involved in alkane degradation

Enzyme involved in the oxidation of methane is methane mono oxygynase. There are two types of methane mono oxygynase, membrane bound particulate methane monooxygenase. (pMMO) and soluble methane mono oxygenase (sMMO). In strains containing both, expression of sMMO occurs under conditions of low copper availability (Lieberman and Rosenzweig. 2004; Hakemian and Rosenzweig 2000). *Pseudomonas butanovora* assimilate C_2-C_4 alkanes by a sequential oxidation of the terminal methyl group of hydrocarbon. (Arp 1999). Butane monooxygenase is a non-haem iron monooxygenase which requires chaperonin like protein for its proper assembly (*Kurth et al.,* 2008). *Gordonia Sp TY-5* oxidize propane at the subterminal position using a narrow – substrate range propane to produce 2 propanol. (Kotani *et al.,* 2007). 2 Propanol is further oxidized to acetone and then transformed into methyl acetate and finally to acetic acid and methanol.

Alkane hydroxylases have wide application in industries for use in biotransformation process. Johnson and Hyman, (2006) reported that Alk B alkane hydroxylase present in *P. putida GP I,* can oxidize propane, n – butane as well as C_5 to C_{13} alkanes. van Beilen *et al.,* (2005 b) elucidated the structure of the hydrophobic pocket of Alk B alkane hydroxylase. More than 60 Alk B homologues are reported to date, both in gram positive and gram negative bacteria (Smits *et al.,* 1999). In *P. putida G Po1 an* unusual rubredoxin has

been reported. It is an iron sulphur protein with two domains $AlkG_1$ and $AlkG_2$ connected by a linker, while rubredoxin from other micro organisms have only one of these domains (van Beilen *et al.*, 2002 a).

Cyctochrome P450 alkane hydroxylase are home proteins. They hydroxylate a large number of compounds. Bacteria which degrade C_5 – C_{10} alkanes contain alkane hydroxylases that belong to the family of soluble cytochrome P450 Monoxygenases. Schmitz *et al.,* (2000) reported that yeast isolated from oil contaminated environments can degrade alkanes. Cytochrome P450 was involved in this oxidation (Zimmer *et al., 1996).*

Alkane hydroxylases that degrade long chain alkanes larger than C_{20} are different from those degrading (C_{10} -C_{20}). Maeng *et al.,* (1996) and Tani *et al.,*(2011) reported *that Acinetobacter Sp* M_1 which can catalyze C_{10}- C_{44} alkanes contain a soluble Cu^{2+} dependent alkane hydroxylase and it has been proposed to be a dioxygenase. *Acinetobacter strain DSM 17874* has been found to contain a flavin binding monoxygenase named AlmA (Throne – Holst *et al*., 2007). Feng *et al*., (2007) indentified a different long chain alkane hydroxylase, name *Lad A* in *Geobacillus thermodendrificans NG80-2*. It generates primary alcohols by oxidizing C_{15}-C_{30} alkanes. It is a flavin dependent oxygenase belonging to the bacterial luciferase family of proteins (Li *et al.,* 2008).

Fatty alcohols generated by the terminal oxidation of alkanes are further oxidized to aldlehydes by an alcohol dehyrogenase (ADH). There are different kinds of ADHs. Some are NAD $(P)^+$ dependent and others are NAD $(P)^+$ independent. NAD $(P)^+$ independent ADHs have pyroloquinoline

quinone as prosthetic group. Some bacteria have several different ADHs with different affinities towards different alcohols (Vangnai *et al.,* 2002).

Heinfling *et al*.,(1997) reported the degradation of azodyes pthalo-cyanamine dyes by *Tarmetes versicolor* and *BjerKandera adusta.* They compared the specific activities of exoenzyme preparation with the oxidative rates of commercial horse radish peroxides.

Biodegradation of polychlorinated biphenyl by *Arthrobacter sp* was reported (Gilbert and Crowley 1998). *S*imilarly biodegradation of toluene by *Pseudomonas putida* in trickling filters was also reported. Peixoto and Mota (1998) studied conversion of nitrobenzene to aniline by *Commamonas acidovorans.* Peres *et al*.,(2000) ; Gouz *et al*., (2000a) developed an efficient microbial consortium for the degradation of atrazine. Manson *et al*., (2000) reported a microbial consortium promising in the biodegradation of benzene toluene ethyl benzene and mixed xylems. The study was conducted in a completely mixed bioreactor in the presence of activated carbon where bacteria were constantly in a flux between absorbent and free phase. van Beilen *et al.,* (1992b, 1994) reported that *Acinetobacter calcoacceteices Hol – N* contains two ADHs of which one showed preference to Dodecanol and other towards tetradecanol. In *P. putida GPol* both the chromosomes and OCT plasmid carries genes coding for alcohol and alelehyde dehydrogenases.

Subterminal oxidation of alkanes produces secondary alcohols. These are further converted into ketones by ADHs. Kotani *et al., (*2007) reported that *Gordinia Sp strain Ty-5* contains three NAD^+ dependent secondary ADH. Secondary ADHs are reported in *Rhodococcus rhodochrous PNKbl*

(Ashraf *et al*., 1990) and *Pseudomonas flurescens NRRL B 124* (Hou *et al*., 1983).

Degradation of branched chain alkane is more difficult than linear n-alkanes. Several bacteria preferentially assimilated n-alkanes over branched alkanes. Hara *et al.,* (2003) reported that *Alkanivorax Sp*. degraded phytane and pristane effectively. *P.putida GPO1 Alk B* produce epoxides from alkanes and other (Van Beilen *et al*., 2001) oxidation in regio or stereo specific regions which opens new vistas in fine chemistry. Optically active epoxides can be used to generate number of useful precursors from which several value added products can be derived (Rojo, 2009).

Dietzia capable of utilizing wide range of n- alkanes (C_6-C_{40}), aromatic compounds and crude oil as sole source of carbon was reported to possess alkB gene (coding for alkane monoxygenase,) CYP153 gene (coding for P450 alkane hydroxylase of the cytochrome CYP153 family) Wang *et al.,* (2012).

Lysinibacillus fusiformis

Lysinibacillus fusiformis was first identified by Ahmed, (2007). He isolated three strains of spore-forming, Gram positive, motile, rod shaped and boron tolerant bacteria from soil. The strains could tolerate 5 %(w/V) NaCl and up to 150 mM boron. Optimum growth was found in Luria-Bertani agar medium without NaCl or boron. The optimum pH was 7-8 and optimum temperature was 37^0C. 16S rRNA gene sequence analysis showed 97.2% similarity to *Bacillus fusiformis* DSM 2898^T and 96.9% similarity to *Bacillus sphaericus* DSM 28^T. DNA–DNA relatedness was greater than 97% among

the isolated strains and 61.1% with *B. fusiformis* DSM 2898^T and 43.2% with *B. sphaericus* I AM 13420^T. The phylogenetic and phenotypic analyses and DNA–DNA relatedness indicated that the three strains belong to the same species, that was characterized by a DNA G+C content of 36.5–37.9 mol%, MK-7 as the predominant menaquinone system and iso-$C_{15:0}$ (32% of the total) as a major cellular fatty acid. But in contrast to the type species of the genus bacillus, the isolates contain peptidoglycan with lysine, aspartic acid, alanine and glutamic acid. Based on the distinctive peptidoglycan composition, phylogenetic analysis and physiology the strains are assigned to a novel species with in a new genus. The type strain is *Lysinibacillus boronotolerans.gen.nov.sp* is proposed. *Bacillus fusiformis* is transferred to the genus *L.fusiformis.comb.nov*. and *B sphericus to Lysinibacillus sphericus*. Raja *et al.,* (2012)isolated a boron resistant and accumulating bacterium *L.fusiformis M1* from mining site at Hokkaido Japan.

Lysinibacillus fusiformis is a potential organism for the commercial production of many useful products by biotransformations. Kim *et al.*,(2012) isolated a gene encoding oleate hydratase from *Lysinibacillus fusiformis*, cloned and expressed in *Escherichia coli*. The enzyme produced 10-hydroxystearic acid from oleic acid and olive oil. Hydroxy fatty acids can then be employed in the production of important industrial materials such as waxes, nylons, plastics resins, lubricants, and cosmetics, as additives in paintings and coatings and as precursors of lactones. Now the enhanced production of hydroxy fatty acids via biotransformation is gaining importance.

Production of enantiopure esomeprazole by biocatalysis has great demand in pharamaceutical industries. Babiak, (2011) reported that *Lysinibacillus* sp.B71 isolated from soil polluted with elemental sulphur catalysed the enantio selective synthesis of esmoprazole from prochiral sulfide without its consequent oxidation into sulfone. Thomas, (2011) reported that the *Lysinibacillus* spores could tolerate 90% ethanol. Generally 70-80% ethanol is recommended as the most effective bactericidal level which allows sufficient contact time with bacterial cells before evaporation unlike higher concentrations.

The xylan is a hemicellulosic polysaccharide in cell walls of land plants, made up of a backbone of xylose residues linked by β-1,4-glycosidic bonds. Xylanolytic enzymes from microorganism have attracted a great deal of attention in the last decade, particularly because of their biotechnological characteristics in various industrial processes, related to food, feed, ethanol, pulp, and paper industries. Alves-Prado, (2010) isolated xylanase from *Lysinibacillus sp. strain P5B1*.

Chapter 3

MATERIALS AND METHODS

Contents

3.1. Screening and isolation of a hydrocarbon degrading bacteria by enrichment culture technique.

3.1.1. Sample collection

Sediment samples were collected from the Munakkal beach (Trichur dist, Kerala) located on the west coast of India immediately after the major event Tsunami in 2004. The site mentioned here experienced impact of Tsunami and hence the sediment deposits after tsunami were subjected to screening of potential bacteria that has ability to degrade hydrocarbons. The collected sediment samples were immediately transported to laboratory under refrigerated conditions and analyzed for bacterial composition and subsequent isolation.

3.1.2. Isolation and screening of hydrocarbon degrading bacteria

Ten gram of sediments was added to 90 ml of sterilized sea water and homogenized by placing the flasks on an orbital shaker at 150 rpm for

1 hour. This homogenate was used as inoculum for plating purposes. 100ml of Zobell Marine Broth (ZMB) prepared in a 250 ml conical flask was inoculated with 1 ml of the prepared sediment homogenate solution and incubated at 37^{o}C for 24h in a shaker at 150 rpm. Enrichment technique using Bushnell - Haas medium (BH) (Appendix 1) supplemented with 1% v/v hydrocarbon substrates was used for the isolation of hydrocarbon degrading bacteria associated with sediment. Bombay High Crude oil Samples obtained as gift by Bharat Petroleum Corporation, Cochin, India were used as substrates for the study. The crude oil used in the enrichment medium contained equivalent mixture of octane, cyclohexane, hexane, benzene, toluene, ethyl benzene, xylene besides other fractions in the crude oil. The culture obtained from Zobell Marine broth was transferred to the enrichment medium and incubated at 37^{o}C for one week in a shaker at 150 rpm. After incubation 1 ml of the culture from primary enrichment medium was transferred to a fresh Bushnell– Hass medium containing the same hydrocarbon mix and incubated for one more week. After second enrichment, 0.1 ml of the culture broth was plated on Zobell Marine agar medium supplemented with same hydrocarbons and incubated at 24 h at 37^{o} C.

Those colonies which were capable of utilizing the given hydrocarbons as sole source of carbon in the medium were isolated, purified and stocked in a phosphate buffer (50Mm KH_2PO_4 /K_2 HPO_4, pH 7.2) containing 20%(v/v) glycerol at –20^{o}C. Permanent stock cultures were made by lyophilization. Working cultures were maintained by sub culturing on mineral salt agar slants containing crude oil at 1%(v/v)

concentration, at intervals of 10 days. The isolate which showed maximal degradation had ability to utilize most of the hydrocarbons tested was selected as the potential strain and used in the study.

3.1. 3. Inoculum preparation and inoculation

A loop full of the culture was transferred from the agar slope into 5ml of ZMB medium taken in a boiling tube and incubated for 16 h at 37^{o}C at 150 rpm in an orbital shaker. Later using this preculture as inoculums 50 ml ZMB medium taken in an Erlenmeyer flask was inoculated (1% (v/v)) and incubated until the Optical Density (OD) reached 1.00. This culture was used as inoculum for further studies unless otherwise mentioned.

3.2. Identification of the selected bacterial strain

The selected bacterial strain was identified based on its morphological and biochemical characteristics as outlined in Bergeys Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974). Molecular ribotyping was also done towards confirmation of the identity of the strain.

3.2.1. Molecular ribotyping

3.2.1.1. Isolation of genomic DNA (Sambrook *et al.*, 2000)

1) Mid-log phase culture of the bacteria (40 ml) were taken in a sterile oakridge tube and centrifuged at 5000 rpm for 10 min at 4°C.
2) The supernatant was discarded and the pellet blot dried.
3) The cell pellet was dissolved in 8.75 ml of TE buffer.

4) To the content, 50 µl of Proteinase K (10 mg/ml) and 10 % SDS (1ml) were added, mixed gently, and incubated at 37°C for 1 hr.

5) To this equal volume of phenol-chloroform mixture (1:1) was added, mixed gently, and kept for 10 min. at 4°C.

6) The contents were centrifuged at 10,000 rpm for 10 min. at 4°C, and the supernatant was transferred to a fresh sterile tube using sterile cut tip.

7) The steps 5 and 6 were repeated three times.

8) The DNA was precipitated by adding 0.1ml of 5 M sodium acetate (pH 5.2) and 20 ml of isopropanol.

9) The precipitated DNA was washed gently with 70 % ethanol.

10) The prepared DNA was dissolved in 1 ml of TE buffer.

3.2.1.2. Agarose gel electrophoresis (Sambrook *et al.*, 2000)

The agarose gel electrophoresis was done in order to check the quality of the DNA prepared.

1) Agarose gel with a concentration of 0.8 % (w/v) was prepared.

2) 10µl of the DNA sample was loaded on to the gel and electrophoresed at 80 V until the migrating dye (Bromophenol blue) had travelled two-thirds distance of the gel. Lambda DNA cut with E.coR1 and Hindlll (Bangalore Genei) was used as markers.

3) The gel was stained in a freshly prepared ethidium bromide solution (0.5 mg/ml) for 20 min.

4) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Biorad).

3.2.1.3. Ribotyping

Ribotyping was performed using universal primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5 kbp) was amplified from the genomic DNA (Reddy *et al.,* 2000; 2002a, 2002b; Shivaji *et al.,* 2000). The sequences of forward (16SF) and reverse (16SR) primers were used for amplifying 16S rDNA were as follows:

Sequence	Reference
16F (5'-AGTTTGATCCTGGCTCA-3')	(Shivaji *et al.,* 2000)
16R (5'- ACGGCTACCTTGTTACGACTT-3').	(Reddy *et al.,* 2002a, 2002b)

(All chemicals were procured from SIGMA -ALDRICH, USA; PCR reactions were carried out in a Biorad thermal cycler under the following conditions standardized in our laboratory).

PCR mix composition

1.5mM $MgCl_2$	200µmol
Deoxy nucleotide mix	200µmol
Primer	0.1µ mol each
Taq polymerase	1unit
Tris-HCl -pH 8.3	100mM

KCl	500 mM
Deionized water	6 µl

PCR conditions

1. Initial denaturation	-	94°C (90 sec.)
2. Denaturation	-	94°C (30 sec.)
3. Annealing	-	56°C (30sec.)
4. Primer extension	-	72°C (2 min.)
5. Repeat step 2, 3 and 4 for 34 times		
6. Final extension	-	72°C (10min.)
7. Hold	-	4°C (5 min.)
8. End		

3.2.1.4. DNA sequencing

Nucelotide sequence of the amplicon was performed after checking the concentration in a micro-volume spectrophotometer (Thermo Scientific Nano Drop™ ND-2000) and purification using Nucleospin purification column (Macherey-Nagel). Sequencing was done in an ABI 3730xl cycle sequencer. Forward and reverse sequences obtained were assembled and contigs were generated after trimming the low quality bases.

3.2.1.5. Phylogenetic tree construction.

The nucleotide sequences of the partial gene sequences of the 16sRNA subjected to multiple sequence alignment and homology search

using multiple sequence alignment software Clustal W and bioinformatics tool BLAST of NCBI. Based on maximum identity score, first few sequences were selected and a dendrogram was constructed**.**

3.3. Characterization of bacteria with potential for degradation of crude oil and its fractions.

3.3.1. Antibiotic sensitivity

Antibiotic sensitivity profile of the bacterial strain was determined using the octadiscs of Himedia in which 8 antibiotic discs were incorporated in one test (Octadisc. Combi-69, Octadisc.T Combi-61, Octadisc. Combi-1 Octadisc-G-V111-plus, Octadiscs-G-V1 minus). The strain was evaluated against 27different antibiotics. Log phase bacterial cultures were inoculated by spread plating on the Zobell Agar and the antibiotic discs were carefully placed over the inoculum and incubated at 37^{o}C for 24 h and checked for the sensitivity.

3.3.2. Enzyme profile of the culture

Enzyme profile of the selected strain was determined as described below.

3.3.2.1. Protease

The proteolytic activity was measured according to Nitkowski *et al.,* (1977). The proteolysis medium consisted of 0.3 % beef extract, 0.5 % peptone, 3% NaCl and 1.5 % agar in distilled water. Casein was provided as substrate for protease in the form of diluted skimmed milk to the medium such that the final concentration of milk was 1.5 %. The medium containing

skimmed milk was swirled gently and poured into petriplates, which were subsequently dried at room temperature. A loop full of bacterial culture was spot inoculated on the solidified skimmed milk agar plate and the plates were incubated at room temperature (28 ± 2°C) for 72 h. An uninoculated media was kept as control. Formation of a clear halo around the bacterial colony was considered as positive for casein hydrolysis.

3.3.2.2. Lipase

Lipase assay was done according to Kim and Hoppe, (1986). The Zobell Marine agar medium was supplemented with 1 % (w/v) Tween 80 (sorbitol monooleate) as lipase substrate, autoclaved and poured into sterile petriplates. A loop full of bacterial culture was spot inoculated on to the top of agar plates and incubated at room temperature (28 ± 2°C) for 72 h. An uninoculated media was kept as control. Appearance of dense opacity around the colony was considered as positive indicating production of extracellular lipase.

3.3.2.3. Alpha amylase

Alpha amylase was assayed according to Kim and Hoppe, (1986). To the Zobell Marine agar medium 1% of soluble starch (w/v) was added, autoclaved and poured to pre sterilized petriplates. Bacterial culture was spot inoculated on the solidified agar plates and incubated at room temperature (28 ± 2°C) for 72hours. After incubation the plate was flooded with Iodine reagent. Iodine reagent reacts with starch and forms a blue coloured complex. A clearing zone around the bacterial colony is an

indication of extracellular alpha amylase production. An uninoculated media was kept as control.

3.3.3. Optimization of growth conditions.

Various process variables namely incubation temperature, pH, sodium chloride concentration, and carbon sources, that influence growth were optimized by growing the bacterium in Zobell Marine broth at different conditions as detailed below. 100ml of Zobell Marine broth prepared in 250 ml conical flasks was inoculated with mid-log phase culture (01 OD @1% v/v) and incubated for a total period of 48 h at 28 $\pm 2^0$C in an orbital shaker at 150 rpm unless otherwise specified. At the end of incubation samples were drawn and assayed for growth in terms of OD at 600nm in a UV–Visible spectrophotometer (Shimadzu-Japan). Uninoculated Zobell Marine broth was used as control. All the experiments were conducted in triplicate and analyzed statistically using Zigma plot.

3.3.3.1. Incubation temperature

Optimum temperature for maximum growth was evaluated by incubating the inoculated media (ZMB) at various temperatures, *viz* 25^0C, 30^0C, $37\ ^0$C, 40^0C, 50^0C and 55^0C, for 24 h at 150 rpm in an environmental shaker.

3.3.3.2. Sodium chloride concentrations

Effect of sodium chloride on growth was studied by incubating the media (ZMB) supplemented with different concentrations of sodium chloride

*viz.*100mM, 200mM, 300m M, 400mM, 500m M, 600m M, 700mM 800m M, 900m M and 1 M.

3.3.3.3. pH

To find the optimum pH for growth, the bacteria were grown in media (ZMB) prepared with different pH ranging from pH 2 to 12.

3.3.3.4. Carbon sources

3.3.3.4.1. Carbohydrates as carbon sources.

Various carbohydrates were tried as source of carbon for growth of bacteria. Mineral salt medium supplemented with starch, sucrose, cellulose, maltose, lactose, dextrose, galactose, sorbitol, manitol and fructose at 100mM concentration was used for the study.

3.3.3.4.2. Organic solvents as source of carbon.

Various organic solvents namely acetone, benzene, toluene, ethyl benzene, chloroform, dichloromethane, octane, pentane, heptanes, hexane, decane, ethanol, methanol, propanol, phenol and cyclohexane were tried as source of carbon by the bacteria towards determining the ability of the bacteria to utilize them. The solvents were added at a concentration of 10% and 50% (v/v) to the mineral salt medium.

3.3.4. Growth curve

Growth curve of the bacteria was studied using Zobell Marine Broth as growth medium. The prepared medium (100 ml) was inoculated with mid-log phase culture (01 OD @1% v/v) in a 250 ml conical flask and incubated for a total period of 48 h at 28 $\pm 2^0$C in an orbital shaker at 150

rpm. At regular intervals samples were drawn and assayed for growth in terms of OD in a UV–visible spectrophotometer (Shimadzu-Japan). Uninoculated Zobell Marine broth was used as control. All the experiments were conducted in triplicate.

3.3.5. 2,6,dichlorophenol indo phenol (DCPIP) as an indicator of biodegradation of hydrocarbons by bacteria

Aerobic biodegradation of hydrocarbons was studied using DCPIP as redox indicator. The principle of using DCPIP is that during the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as O_2, sulphates and nitrates. When an electron acceptor such as DCPIP is incorporated in to the culture medium, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the colour change of DCPIP which is blue in colour (oxidized) to colourless (reduced). This technique has been employed in several works (Hanson *et al.*,1993). BH medium was taken in 100 ml screw capped vial and aliquots of 100µl of different solvents viz. acetone, pentane, heptanes, hexane, cyclohexane, benzene, toluene, xylene, dichloromethane, propanol and methanol were added to the medium and inoculated with bacteria (01 OD @1% v/v)) and kept under shaking for 24 h. Concentration of DCPIP added was 27mg/ml. All the bottles were kept in darkness and the colour change was observed. All the experiments were conducted in triplicate.

3.3.6. Tolerance and accumulation of metals by bacteria.

Tolerance to various metals and their accumulation in cells of bacteria were evaluated using 0.1M of mercuric chloride, cadmium sulphate, copper

sulphate, lead nitrate, zinc sulphate and sodium meta arsenate by addition of the same in mineral salt medium (B.H.) supplemented with sucrose as the source of carbon. The pH of the medium was kept as 9. Inoculated with bacteria (01 OD @1% v/v)) and kept under shaking at 150rpm for 24 h at 37^{o}C. The controls were made without adding any metals. The growth of the cells were measured in a U.V–visible spectrophotometer at OD_{600} nm.(Higham *et al.*,1985).

Accumulation of arsenic, mercury and cadmium by bacteria was studied by growing the bacteria in the corresponding medium containing these metals for 24h. After incubation, the cells were harvested by centrifugation at 1000 rpm in a centrifuge and washed thrice in physiological saline. The harvested cells were dried in a hot air oven at 100^{o}C and their dry weight was weighed. The dried cells were digested by adding 2ml HNO_3, slightly heated, vortexed thoroughly to mix well and then made up to 50 ml with deionized water. The contents were analyzed by Inductively Coupled Plasma -Atomic Emission Spectroscopy (IC-AES).

3.3.7. Accumulation of L-citrulline capped ZnS:Mn nanoparticles by bacteria *Lysinibacillus fusiformis BTTS10 by fluorescence microcopy*

Accumulation of L-citrulline capped ZnS:Mn nanoparticles by the bacteria *Lysinibacillus fusiformis* BTTS10 was analyzed by fluorescence microcopy. Different concentrations of (250µl, 500µl, 1000µl, 2000 µl and 4000 µl) the nanocolloids were added into 10 ml nutrient broth, inoculated with mid-log phase cells of the bacterium and incubated at 37^{o}C in a rotary

shaker with a speed of 150 rpm for 24 h. *Salmonella typhymurium* (no autofluorescence) was taken as control. The growth was measured in a U.V -visible spectrophotometer at OD_{600} nm. The experiment was conducted in triplicate. The cells were harvested by centrifugation at 1000 rpm in a centrifuge, washed in physiological saline and observed under Fluorescence Microscope. Bacterium grown in the medium lacking nano particles was used to find whether the bacteria have the capacity to produce auto fluorescence.

3.3.8. Bio surfactant production

Bio surfactant production by the bacterium was studied by drop collapse assay (Jain *et al.*,1991). This assay relies on the destabilization of liquid droplets by surfactants. 4 drops of oil was placed on a clean glass slide. 100µl of supernatant of the culture grown in hexadecane, benzene, and crude oil independently was added on to a drop of the hydrocarbons and observed the drop. If the liquid contains surfactants, then the drops collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. One drop was kept as control without adding sample supernatant.

3.4. Biodegradation of crude oil and its fractions with bacteria under submerged culture conditions.

3.4.1. Biodegradation of crude oil

Biodegradation was carried out in modified Bushnell and Haas (BH) medium supplemented with 0.2M sodium chloride. The pH was

adjusted to 9.5 with 1N NaOH and Nitrogen and Phosphorus solutions were prepared separately and autoclaved at 121^{o}C for 15 min.

100 ml of B.H medium supplemented with 1% crude oil as sole source of carbon was taken in a 250 ml of conical flask and inoculated with 1 gm wet weight of 24 h old culture of bacteria. The experimental set up was done in triplicate and one control. The inoculated flasks were incubated at 37^{o} C and at 150 rpm in a rotary shaker. Samples were drawn after incubation for 72, 120 and168 h and the extent of biodegradation was determined by estimating the growth of bacteria and total residual hydrocarbons in the flasks gravimetrically and variation in pH besides analysing the intracellular and extracellular products of biodegradation. 1 ml pristane was added as an internal standard.

3.4.2. Saturates, Aromatics, Resins and asphaltene (SARA) separation of crude oil

Crude oil was fractionated by silica gel column chromatography the various components were separated (Mishra *et al.*, 2001). The total petroleum hydrocarbons TPH extracts were dissolved in 10 ml pentane and the insoluble fraction (asphaltene) was removed using Whatman filter paper and weighed. The soluble fraction was loaded on top of silica gel G (60-120 mesh) column (2cm x30 cm) activated at 80^{0}C and eluted with solvents of different polarities. The alkane fraction was eluted with 100 ml of hexane; aromatic fraction was eluted with 100ml benzene, and finally NSO (non saponifiable organic acid) fraction was eluted with methanol and chloroform (100 ml each). The methanol and chloroform

fractions were evaporated and weighed to get the weight of NSO compounds.

3.4.3. Extraction of residual total petroleum hydrocarbons

Extraction of the residual hydrocarbon was conducted according to the method of Mishra *et al.*, (2001).At the end of incubation, residual crude oil was extracted twice from the experimental flask with 100ml of hexane, methylene chloride and chloroform respectively. Each time the solvent was evaporated at 60^0C in a vacuum evaporator. The amount of residual hydrocarbon recovered was determined gravimetrically in an analytical electronic balance (Sartorius Element ETL602).The biodegradation efficiency (BE), based on the decrease in total weight of crude oil was evaluated by the following expression

$$BE = (M_0 - M) - (M_{C0}-M_C)/M_0).X100 \qquad \text{Eq .1}$$

Where,

Mo = the weight of crude oil sample before inoculation,

M = weight of crude oil of sample after inoculation,

M_{C0} = weight of crude oil of control,

M_C = weight of crude oil of control after the same treatment as that of sample without inoculation.

After gravimetric quantification, the extracts were suspended in 5ml hexane and dried by passing through sodium sulphate and analyzed by FTIR and a gas chromatograph (SIMDIS Analyser, VARIAN CP-3800. Gas Chromatogram). The Gas chromatograph fitted with CP-7562

column. The column was 10m x1.2µm. The detection was by flame ionization at a temperature range of 35^0C to 450^0C. Helium was used as carrier gas. The column pressure was 2.5 psi.

3.4.4. GC- MS analysis of biodegradation products.

The biodegradation products after solvent extraction were analyzed using high resolution GCMS -Agilent 7890. The column used was 30m x 25mm x 0.25µm.The temperature of the programme was 40^0C isothermal time, heating up to 250^0C with a heating rate of 40^0C /min. Helium was used as carrier gas with a flow rate of 1.2ml /min and the molecules were scanned from 50-550(m/z). The most relevant peaks were identified on the basis of mass spectra data bases (NIST). Pristane was added as internal standard.

3.4.5. Estimation of pH during biodegradation of crude oil by *L.fusiformis* BTTS10

The pH of the growth medium was measured at intervals during the course of biodegradation to study the change in pH during biodegradation of crude oil as described by Olajide *et al*., (2010).

3.4.6. Dry biomass determination.

After the extraction of residual crude oil, dry biomass was determined by centrifugation of the aqueous phase at 1000rpm for 10 min. The cell pellet was washed with 10ml acetone: hexane 3:1 to remove adherent hydrocarbon. The pellet was then resuspended in 10ml hexane

and centrifuged again and dried in an oven at 80^0C overnight for determination of dry mass (Verma *et al*.,2006).

3.5. Factors affecting biodegradation of hydrocarbons

Various factors affecting biodegradation was studied and analyzed statistically and mean values were used as results.

3.5.1. Effect of agitation on biodegradation

The effect of agitation on biodegradation of crude oil was studied by incubating the inoculated media taken in a conical flask on a rotary shaker at different rpm (100, 110, 120, 130, 140, and 150). Control was maintained by incubating the inoculated media without agitation. The biodegradation was studied by gravimetric analysis of residual crude oil. The medium preparation, inoculation, incubation and gravimetric analysis were performed as described in the section 3.4.1.

3.5.2. Effect of sodium chloride on biodegradation.

The effect of sodium chloride concentration on biodegradation of crude oil was studied by incubating the media with different concentrations of sodium chloride *viz.* 0 .1M, 0.2M, 0.3M, .4M, .5 M, .6 M, .7M .8M, .9M and 1M in the BH medium at 150rpm for 5 days in an environmental shaker. The medium preparation, inoculation, incubation and gravimetric analysis are as described in the section 3.4.1.

3.5.3. Effect of inoculum concentration on biodegradation

Optimum concentration of inoculum that gives maximum degradation of crude oil was studied by adding different concentration of

inoculum (1%, 2%, 3%, 4% and 5% w/v) prepared as mentioned in the section 3.1.3. The medium preparation, inoculation, incubation and gravimetric analysis were done as described in section 3.4.1.

3.5.4. Effect of pH on biodegradation

To find the effect of pH on biodegradation bacteria were grown in media prepared with different pH ranging from pH 6 to 12. Growth was determined by measuring the turbidity at 600nm in UV-visible spectro-photometer (Shimadzu, Japan) after 4 days of incubation in an orbital shaker at 150 rpm. The medium preparation, inoculation, incubation and gravimetric analysis were done as described in section 3.4.1.

3.5.5. Biodegradation of crude oil in a biometric system

Biodegradation of crude oil in a biometric system was studied using biometric flasks. Four, 250 ml biometric flasks were taken; one flask was taken as control. 100 ml Bushnell-Haas(BH) medium added with 1% crude oil as sole source of carbon was taken in a conical flask and inoculated with 1 gm wet weight of mid- log phase cells of bacteria. The side arm of biometric flask was filled with 10 ml of 0.1 M KOH. The experiment was done in triplicate and a control was maintained. All the flasks were incubated at 37^0C and 150 rpm in a rotary shaker. Samples were drawn after incubation for 72, 120 and168 h and carbon dioxide ($CO_{2)}$ was measured by taking the sample out using a pipette from the side arm of the flask. Evolution of CO_2 during the growth of bacteria utilizing crude oil as sole source of crude oil was estimated by colorimetric titration. CO_2 produced during the microbial activity was

captured by 0.2 M KOH solution (10 ml) located by the side of the biometric flasks. To the KOH solution, 1 ml of 0.5 M barium chloride solution and 3 drops of 1% phenolphthalein indicator were added. The residual KOH was titrated with 0.1 M standard HCl solution.

The amount of CO_2produced was computed using the equation CO_2 generated (mg) = (VB - VA) .MCO_2 = 2. M HCl. CF, Eq.2

Where, VB and VA are the volume of HCl (0.1M) used to titrate the blank and the treatment in ml, respectively;

MCO_2 is the molar mass of carbon dioxide in g/mol. M HCl is the molar concentration of HCl standard solution in mol/L; and CF is the correction factor for acid/base molarity (M HCl/M KOH). All the experiments were conducted in triplicate and analyzed statistically .

3.6. Biodegradation of Toluene (aromatic compound)

Biodegradation of toluene was studied using the B.H medium taken in sterilized screw capped brown bottles was added with 0.5% toluene (v/v) and inoculated with 500 mg wet weight of 24 h old culture. The growth of the cells utilizing aromatic hydrocarbon as sole source of hydrocarbon was measured by studying the increase in turbidity just after the inoculation and during the subsequent days of incubation. The rate of biodegradation was determined by analyzing the degraded sample by GC-MS.

3.7. Biodegradation of crude oil and its fractions with immobilized whole cell biomass

Biodegradation of crude oil and its fractions was studied with whole cell biomass immobilized on petiole cuttings of aquatic weed *Eichhornia crassipes* which is quite common on backwaters, and polystyrene beads.

3.7.1. Immobilization of viable bacterial cells on *Eichhornia* petiole

Eichhornia crassipes petiole was used for immobilization of bacteria. After washing in Tween 20 and in distilled water three times, the petioles were cut into small cubes of approximately 1 cm^3 , washed again in distilled water, dried in an oven at 100°C for 1 h, and then sterilized by autoclaving. Log phase cells grown in the mineral salt crude oil medium for 24 hours at 37^0C in a rotary shaker at 120 rpm were harvested by centrifugation at 10,000 rpm. The pellet obtained was washed in Mineral salt medium phosphate buffer (pH. 10) and 1gm (wet weight) cells were added to 50 ml of ZMB medium which was used as immobilization medium. 1gm of sterilized *Eichhornia* petiole was added to the above medium and kept for incubation at 37±2°C for 24 h at 60 rpm in a shaker. The immobilized petioles were pelleted by centrifugation at 1000 rpm and washed in sterile distilled water. Immobilized petioles were used for cell viability testing, SEM analysis, and biodegradation studies.

3.7.1.2. Enumeration of Viable bacterial cells immobilized on *Eichhornia crassipes* petiole

To assess the number of viable bacterial cells attached to petioles, 3 cuttings were washed thrice in 5ml physiological saline and then 1gm of cutting was suspended in physiological saline. The cuttings in suspension were disintegrated with a vortex mixer for 1 min. Successive decimal dilution were made from the supernatant and 0.1ml of the dilutions were spread on nutrient agar medium. The numbers of CFUs were calculated on a regular interval of 45 days for 225 days to study the viability of bacteria immobilised on Eichhornia petiole (Rajeev *et al.*, 2001). All the experiments were conducted in triplicate and analyzed statistically and mean values were taken as result.

3.7.1.3. Estimation of total protein content of viable bacterial cells immobilized on *Eichhornia* petiole

Total protein content in the immobilized petioles were quantified using three petiole cuttings which were recovered, washed thrice in 5ml physiological saline and suspended in 5ml distilled water in a test tube. 5ml of 1M NaOH was added and mixed in a cyclomixer. The contents were boiled by keeping the tubes in a boiling water bath and after which they were rapidly cooled under tap water. The protein released by alkali lysis of the cells were measured by Bradford method (1976) using Bradford kit (Biogene,USA). The samples were diluted to 100ml with 0.15N NaCl. One ml of Bradford reagent was added, vortexed and allowed to stand for 2 minutes and absorbance was read at 595 nm. Quantification of protein was done by comparing with a standard curve plotted with BSA as standard and was expressed as microgram/ml. (Beena, P.S. 2010).

3.7.1.4. Scanning Electron Microscopic (SEM) examination of immobilized viable bacterial cells

Immobilized cells were examined by Scanning Electron Microscopic (SEM) method. For SEM, the cuttings were suspended in 10ml of B.H medium and stirred at 500 rpm for 1 min in order to wash off the non-attached cells. Washed cuttings were taken with sterile forceps and placed in fixative (buffer phosphate, pH 7.2 and 1% v/v glutaraldehyde) in phosphate buffer (pH7.2) at room temperature for 1 h. Washed in the same buffer, post fixed in 1%w/v osmium tetroxide for 1 hr, dehydrated in graded alcohol(50,75,90,100.), and then mounted . The petiole cuttings were finally observed in a scanning electron microscope (JEOL 6390LA).

3.7.2. Crude oil biodegradation by immobilized viable bacterial cells

Biodegradation of crude oil by viable bacterial cells immobilized on petioles of *Eichhornia* was carried out in artificial sea water (ASW) with 1% (w/v) nitrogen and phosphate solution (N &P solution) to stimulate biodegradation (Foght *et al*., 1989). The composition of ASW included per litre of distilled water

NaCl,	23.4g
KCl	0.75g
$MgSO_4 7H_2O$	7g
$CaCl_2.2H_2O$	0.67g
$FeSO_4.7H_2O$.	0.001g

Nitrogen and Phosphorus (N&P) solution contained (per litre):

K_2HPO_4 70 g

KH_2PO_4 30 g

NH_4NO_3. 100 g

ASW and the N&P solutions were prepared separately and autoclaved at 121°C for 15 min. Final pH was adjusted using 1M NaOH.

1% crude oil was added to100ml of ASW medium taken in a 250ml Erlenmeyer flask and inoculated with 10g of petiole cuttings containing immobilized viable bacterial cells. The flask was incubated at 37^0C and 150 rpm in a rotary shaker. The experiments were done in triplicate. Samples were drawn after incubation for 72,120 and 168 h and the rate of biodegradation was monitored by estimating the pH of the medium, total viable bacterial counts, and total residual hydrocarbons by gravimetric analysis.

3.7.3. Immobilization of viable bacterial cells on polystyrene beads

Immobilization of whole cells of viable bacteria was done on polystyrene beads which are commercially available as a packaging material according to the method of Nagendraprabhu and Chandrasekaran, (1995). Polystyrene beads of 4-5mm diameter were autoclaved at 121^0C for 20 min during which time the beads collapsed and reduced in size (Brydson,1982). Pre treated beads of uniform size (0.0084cc) were selected for immobilization. 50 cc (21.786 gm) sterilized beads were used for immobilization. Log phase cells were grown in the mineral salt crude oil medium for 24 h at 37^0C in a rotary shaker at 120 rpm. Cells

were harvested by centrifugation at 10,000 rpm, pellet was washed in Phosphate buffer (pH. 10) and 1gm (wet weight) cells were added to 50 ml immobilization medium. L.B medium was used as immobilization medium. Immobilization was performed by growing the cells along with pretreated polystyrene beads in an immobilization medium. The cells were attached to the support matrix and colonized the surface as a biofilm during their growth. The immobilized polystyrene beads were separated by filtration on sterilized filtration unit, washed in sterile mineral salt medium, and used for cell viability testing, SEM analysis, and biodegradation studies.

3.7.3.1. Enumeration of viable cells immobilized on polystyrene beads

Enumeration of viable cells immobilized on polystyrene beads was done as described in section 3.7.1.2 mentioned above.

3.7.3.2. Estimation of total protein content of cells immobilized on *polystyrene beads*

Estimation of total protein content of viable bacterial cells immobilized on *polystyrene beads* was estimated as described in section 3.7.1.3. mentioned above.

3.7.3.3. Scanning Electron Microscopic (SEM) examination of viable bacterial cells immobilized on polystyrene beads

Scanning Electron Microscopic (SEM) examination of viable bacterial cells immobilized on polystyrene beads was done as described in section 3.7.1.4 mentioned above

3.7.4. Crude oil biodegradation by viable bacterial cells immobilized on polystyrene beads

Biodegradation of crude oil by viable bacterial cells immobilized on polystyrene beads was carried out in artificial sea water (ASW) with nitrogen and phosphate solution to stimulate biodegradation (Foght *et al.*, 1989). The protocol adopted for biodegradation studies is as that described under section 4.2 mentioned above.

3.8. Biodegradation of Asphaltene.

3.8.1. Extraction of asphaltene from crude oil

Asphaltene was precipitated from paper and washed with hot heptanes. The filter paper was placed in the extractor and extracted with n- heptanes till clear. Discarded the extract and the filtrate was then refluxed with 30-60ml of toluene till the asphaltene were dissolved completely. It was then transferred into a glass beaker and dried at 100± 10 for 30 min. (Tavassoli *et al.*,2011).

3.8.2. Asphaltene biodegradation.

Asphaltene biodegradation was studied in a biometric flask.100 ml. of modified BH mineral salt medium was taken in a pre sterilized biometric flask. To this 200 mg of asphaltene and 1% (w/v) of 18h of old culture were added and incubated for 14 days in an orbital shaker at 37^0C

and 150 rpm. The side arm of biometric flask was filled with 10 ml of 0.1 m KOH. All flasks were incubated at 37^0 C and 150 rpm in a rotary shaker. At 72, 120 and168 h of incubation the samples were drawn for measuring CO_2 from the side arm of the flask . Evolution of CO_2 during the growth of bacteria utilizing asphaltene as sole source of carbon was estimated by colorimetric titration. CO_2produced during the microbial activity was captured by 0.20 M KOH solution (10 ml) located by the side arm of the biometric flasks.

To the KOH solution, 1 ml of 0.5 M barium chloride solution and 3 drops of 1% phenolphthalein indicator were added and the residual KOH was titrated with 0.1 M standard HCl solution. The amount of CO_2produced was obtained through the equation

$$CO_2 \text{ generated (mg)} = (VB - VA).MCO_2 = 2.\ M\ HCl.\ CF.$$

Where, VB and VA are the volume of HCl (0.1 M) used to titrate the blank and the treatment in ml, respectively.

MCO_2 is the molar mass of carbon dioxide in g/mol M HCl is the molar concentration of HCl standard solution in mol/L; and CF is the correction factor for acid/base molarity (M HCl/M KOH).

At definite time intervals the growth of the bacteria was studied by measuring the reduction in pH and the number of CFU for 24 days. The biodegraded sample was extracted with hexane and subjected to GC analysis. FTIR analysis of both the control and biodegraded sample was also performed.

3.9. Genetic study of biodegradation

3.9.1. Isolation of Plasmid.

Isolation of plasmids from the bacteria that degraded crude oil was done using alkaline extraction procedure (Birnboim and Doly,1979)

Materials

LB medium
TE buffer (*APPENDIX-2*)
NaOH/SDS solution
Potassium acetate solution
100%ethanol
70% ethanol

1. Inoculated 5ml LB medium with single bacterial colony grown in the presence of crude oil (overnight).
2. Transferred 1.5 ml of the saturated culture to a centrifuge tube and pellet the cells by spinning 20sec.
3. Resuspended the pellet in 100µl TE buffer and let sit 5min at room temperature.
4. Added 200 µl NaOH/SDS solution, mix by tapping tube with finger, and placed on ice for 5min.
5. Added 150µl potassium acetate solution and vortex at high speed for 2 sec to mix. Placed on ice for 5 min.
6. Spinned 1 min in microcentrifuge to pellet cell debris and chromosomal DNA.

7. Transferred supernatant to a fresh tube, mix it with 0.9 ml of ethanol, and kept for 2 min at room temperature to precipitate nucleic acids.
8. Spinned at room temperature to pellet the plasmid DNA.
9. Removed supernatant ,wash pellet with 1 ml of 70% ethanol and dry pellet.
10. Resuspended the pellet 20µl TE buffer, resuspended DNA was used for further studies.

3.9.1.1. Agarose gel electrophoresis (Sambrook *et al.*, 2000)

The agarose gel electrophoresis was done to visualize the plasmids

a) Agarose gel with a concentration of 0.8 % (w/v) was prepared for electrophoresis.
b) 10 µl of the DNA was loaded on to the gel and electrophoresed at 80V until the migrating dye (Bromophenol blue) had travelled two-thirds distance of the gel. Lambda DNA cut with E.coR1 and Hindlll (Bangalore Genei) was used as the marker.
c) The gel was stained in a freshly prepared 0.5 mg/ml ethidium bromide solution for 20 min.
d) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Biorad).

3.9.2. PCR amplification of aromatic dioxygenase gene.

Primer of the subunits of type D iron sulfur multi-component of aromatic dioxygenase gene was purchased from sigma Aldrich.USA. It was then amplified using plasmids isolated from the bacteria in a PCR. The PCR conditions are as follows.

Primer pair	Proteins targeted	Sequences	Amplicon size (bp)
TODC1-F/TODC1-R	Subfamilies D.1.B + D.1.C + D.2.A + D.2.B + D.2.C of α-subunits of Type D iron–sulfur multi-component of aromatic dioxygenases.	16F 5′-CAGTGCCGCCACCGTGGCATG-3′ **16R5′ GCCACTTCCATGCCCACCCCA-3′.**	510 bp

Reference: Hendrickx *et al., (2006).*

PCR mix composition

Plasmid	100ng
1.5mM $MgCl_2$	200 µmol
Deoxy nucleotide mix	200 µmol
Primer	0.1mM each
Taq polymerase	1unit
Tris-HCl pH 8.3	100mM
KCl	500 mM
Deionized water	6 µl

PCR conditions

1. Initial denaturation - 94°C (90 sec.)

2. Denaturation - 94°C (30 sec.)
3. Annealing - 65°C-75°C (30sec.)
4. Primer extension - 72°C (2 min.)
5. Repeat step 2, 3 and 4 for34 times
6. Final extension - 72°C (10min.)
7. Hold - 4°C (5 min.)
8. End

3.9.3. Transformation of *E.coli* DH5α with plasmid isolated from oil degrading bacteria

Plasmid isolated from the oil degrading bacteria BTTS was used to transform *E.coli* DH5α to characterize the properties of the plasmid.

3.9.3.1. Competent cell preparation

E.coli DH5α was used as the host cell for transformation. A single colony of *E.coli* DH5α was inoculated in 5ml Luria Bertani (LB) broth and incubated with a constant shaking of 150 rpm at 37^0C overnight. 1%(v/v) of overnight culture was inoculated into 50ml LB broth and incubated at 150rpm at 37^0C until the OD was 0.4 to 0.6. The cells were harvested by centrifugation at 10000 rpm for 10 min at 4^oC and the pellet was suspended in 10 ml of ice cold 0.1 M $CaCl_2$ and incubated in ice for 30 min. The cells were harvested again by centrifugation at 4^oC for 5 min at 7000 rpm. The pellet was resuspended in 1ml of 0.1 M $CaCl_2$. This was

aliquoted as 80 µl fractions and 20 µl chilled glycerol was added and stored at -80 until use (Sambrook *et al.,* 1989).

3.9.3.2. Transformation of *E.coli* DH5α with Plasmids

Transformation of *E.coli* DH5α with plasmids coding for hydrocarbon degradation was performed adopting the methods of Sheikh *et al.,(*2003); Fujii *et al.*,(1997) and Trevors, (1986).10 µl of plasmid was added to 100µl of competent cells and incubated in ice for 40 min. Then the cells were given heat shock for 40 sec at 42^0C in a water bath then the cells were plunged quickly on ice for 5 min. 250 µl of LB medium was added to the transformed cells and incubated at 37^oC of 1hour. 50 µl of the cells were plated on LB agar plates . The agar plates were incubated in an inverted position by keeping 1 % BTEX in the lid of the plates and the plates were sealed firmly by paraffin film and kept for incubation for 24 hours. The colonies which developed on the agar plates in presence of solvent were selected as the transformed cells .The control was done in the absence of any solvent. The experiments were conducted in triplicate. Transformed colonies were further utilized for plasmid isolation to confirm transformation.

3.9.4. Curing of plasmid from *Lysinibacillus fusiformis* BTTS10

Curing of plasmid DNA was done as suggested by Hardy (1993). The bacterial isolates were grown in100ml Zobell Marine broth medium containing the hydrocarbon of preference. The cells were collected from the late exponential phase and added to media containing different concentrations of ethidium bromide ranging from 100µg/ml to 1mg /ml.

This was incubated at 37^0C for 24 h. The highest concentration of ethidium bromide showing the growth of bacteria was selected. Thereafter the broth was homogenized by vortexing and loop full of the broth was sub cultured on Zobell Marine agar plates(control) and also on BH agar medium (supplemented with hydrocarbon). The colonies that failed to grow on the above medium were identified by replica plating (Sambrook *et al.,* 1989) and were considered as cured.

Chapter 4

RESULTS

Contents

4.1. Screening, selection, and identification of a potential hydrocarbon degrading bacterium

4.1.1. Screening and selection of potential bacteria.

The sediment deposits in the beach of Munakkal, Trichur district, Kerala, collected immediately after the major event Tsunami in 2004 and analyzed by enrichment culture technique was found to harbor several bacteria with potential for degrading hydrocarbons. However, among them isolate no. BTTS 10 showed capabilities for utilizing both alkanes and aromatic hydrocarbons and hence was selected for further studies.

4.1.2. Identification of the bacterium.

The morphological and biochemical characteristics of the isolate BTTS 10 presented in Table 4.1 indicated that the bacterial isolate belongs to the genus *Bacillus* sp. The identity of the isolate was further confirmed by molecular ribotyping based on the results of the partial gene sequence of 16S rRNA gene which was amplified and sequenced. The size of the amplicon was found to be 1500bp (Fig 4.1) and the partial nucleotide sequence of the amplicon of 16S rRNA gene obtained from BTTS10 is presented in Fig. 4.2. The same was submitted to Gene bank (accession number **JN674158**) through Bankit programme, at NCBI site (http:www.ncbi.nlm.nhi.gov/Bankit). The sequence similarity score of the partial 16s rRNA partial gene sequence of BTTS10 with other known sequences are shown in the Fig.4.3. The partial gene sequence of 16S rRNA of BTTS10 showed 82% similarity with the other reported 16S rRNA gene sequence of *Lysinibacillus fusiformis* and hence the identity of isolate BTTS10 is considered as *Lysinibacillus fusiformis.* A phylogram was constructed with the partial gene sequence of *16SrRNA of Lysinibacillus fusiformis* BTTS10 using CLUSTALW N-J programme and is presented in Fig 4.4.

4.2. Growth curve of *Lysinibacillus fusiformis* BTTS10.

From the data presented in Fig 4.5 it was concluded that the bacterium *L. fusiformis* BTTS10 is fast growing and demonstrated exponential phase during 3 to 10 hrs of growth and enter in to stationary phase after 12 hrs of incubation.

4.3. Factors affecting the growth of *L. fusiformis* BTTS10

4.3.1. Temperature

Results obtained for the studies on the effect of incubation temperature on the growth of *L. fusiformis BTTS10* presented in Fig.4.6 indicated that the bacterium prefers 40^0C for achieving maximum growth although it could show considerable growth in terms of OD at a wide range of temperature from 30^oC to 55^oC. The strain may be said to be thermo tolerant since it could also show growth at 60^oC.

4.3.2. Sodium chloride.

Studies on the effect of salinity on the growth of BTTS10 indicated that the optimum salinity for the growth of bacterium was 200mM although significant levels of growth could be recorded at concentrations 100mM to 400mM (Fig.4.7). Further it was also observed that the bacterium could tolerate NaCl above 800mM.

4.3.3. pH

The results presented in the Fig 4.8 showed that pH influence the degradation of hydrocarbon. It was observed that the bacterium could grow well only under alkaline conditions with optimum pH being 10 and could not grow below pH 6. Nevertheless the bacterium was able to tolerate pH of 13.

4.3.4. Carbon source

4.3.4.1. Carbohydrates

Data presented in Fig.4.9 for the effect of different carbon sources on growth of bacterium indicated that it preferred to use simple sugars effectively compared to other carbon sources. Among the various carbon sources tested the bacterium recorded maximal growth with glucose followed by sucrose, starch, mannitol, fructose, sorbitol, lactose and maltose.

4.3.4.2. Organic solvents

Organic solvents, both aliphatic and aromatic, could serve as source of carbon for the bacterium. However, only methanol could support growth at levels similar to that of control (Fig.4.10). Whereas, other solvents caused a decline in the growth. However more than 50% of growth obtained with control could be achieved with benzene, acetone, octane, butanol, ethanol, heptanes, xylene, hexane, ethyl benzene, cyclohexane and toluene. Dichlormethane and propanol resulted in near inhibition of growth.

4.3.5. Nitrogen sources

Result presented in Fig.4.11 clearly evidenced the inhibitory role of organic nitrogen sources such as peptone, tryptone, beef extract and yeast extract except urea which supported slightly enhanced growth of the bacterium when compared to control. Whereas, inorganic nitrogen sources supported growth which was marginally less than the control. Nevertheless,

considerable growth was supported by ammonium nitrate followed by ammonium sulphate and potassium nitrate.

4.4. Responses to antibiotics by *L.fusiformis* BTTS10

The antibiotic sensitivity studies indicated that the bacterium was sensitive to the 27 antibiotics evaluated for sensitive/resistance (Fig:4.12 & Table 4.2). The bacterium could record resistance only towards 3 antibiotics viz; Nalidixic acid, Nitillin and Co-Trioxazole. Results suggested that bacterium is highly sensitive to various antibiotics produced by other microorganisms.

4.5. Use of DCPIP as an indicator of biodegradation of hydrocarbons

The results documented in the Fig:4.13 and Table 4.3. demonstrated the advantage of using DCPIP as an indicator of microbial oxidation of hydrocarbons. Thus after incubation for 24 hrs change of colour was observed to occur from blue to colourless with respect to all the solvents, except in the case of dichloromethane and propanol. Methanol and acetone were found to got decolorised very quickly after 12 hrs. Whereas, the hydrocabons with short carbons got decolorised faster than long chain hydrocarbons. Nevertheless in the case of dichloromethane and propanol slight change in color was observed after 31hrs.

4.6. Heavy metal tolerance by *L.fusiformis* BTTS10

The bacterium was found to have significant level of tolerance to the various metals studied at 0.1M concentration. The results depicted in

Fig.4.14 clearly evidence that none of the metals could support enhanced levels of growth when compared to the control and instead led to a marginal decline in growth. Nevertheless zinc sulphate, copper sulphate, lead nitrate, mercuric chloride and sodium meta arsenate could support considerable levels of growth. Tolerance of bacteria to metallic mercury is shown in the Fig.4.15.

4.7. Accumulation of zinc nanoparticles *by L.fusiformis* BTTS10.

Ability to accumulate L-citrulline capped ZnS:Mn nanoparticles by *L.fusiformis* BTTS10 was confirmed by observation under fluorescent microscope (both bright field and UV light) and the results are shown in Fig.4.16. Bright field image obtained for the pure *L.fusiformis* BTTS10 (control) is shown in Fig.4.16a. The same sample when observed under UV light showed no fluorescence indicating that *L.fusiformis* BTTS10 has no autofluorescence. The image presented in Figure 4.16b for the bacteria attached with L-citrulline capped ZnS:Mn nanoparticles, observed under UV light and fluorescent images, confirm that L-citrulline capped ZnS:Mn nanoparticles were accumulated by *L.fusiformis* BTTS10.

4.8. Accumulation of metals by *L.fusiformis* BTTS10 analysed by ICP –AES

Ability to accumulate metals through biosorption by cells of *L.fusiformis* BTTS10, during growth in the presence of cadmium, mercury and arsenic was confirmed by ICP-AES analysis. The results

given in the Table 4.4 indicated that the bacterium could accumulate maximum levels of cadmium followed by mercury.

4.9. Biodegradation of crude oil and its fractions by *L.fusiformis* BTTS10

4.9.1. Biodegradation of crude oil by *L.fusiformis* BTTS10

The biodegradation of crude oil was studied by growing *L.fusiformis* BTTS10 in mineral salt medium supplemented with crude oil as the sole source of carbon. Bacteria were observed to grow by assimilating the carbon present in the crude oil for their metabolism in the process of biodegradation of crude oil occurred. The biodegradation of crude oil can be visualized from the images presented in the Fig.4.17**.** The results obtained indicated that crude oil began to dissolve into the aqueous medium from the third day onwards and the viscosity of the medium was found to be greatly enhanced on the 5^{th} day. However, as the biodegradation progressed into the 7^{th} day the viscosity was found to get decreased as it may be seen in the Fig.4.17. It was also found that there was a reduction in pH concomitant with the degradation of crude oil (Fig.4.18) and thus the pH of the medium decreased from 10 to 8.4 on the 7^{th} day indicating the presence of acidic intermediates released into the medium.

4.9.2. Biodegradation of crude oil by *L.fusiformis* BTTS10 in a biometric flask

The biodegradation potential was also confirmed by estimating the rate of respiration in a specially designed biometric flask (Fig. 4.19)

since CO_2 is released when respiration occurs**.** The results shown in Fig 4.20 evidenced that maximum production of carbon dioxide took place from 5^{th} day onwards which is in agreement with the other data that indicated biodegradation efficiency of the bacterium.

4.9.3. Effect of various process variables on biodegradation of crude oil by *L.fusiformis* BTTS10.

Impact of process variables namely sodium chloride, pH, inoculum concentration and agitation on biodegradation of crude oil by *L.fusiformis* BTTS10 was evaluated.

4.9.3.1. Sodium chloride

Results documented in Fig 4.21 indicated that the optimum NaCl concentration for effective biodegradation of crude oil was 200 mM and the percentage of biodegradation declined along with increase in the concentration of NaCl.

4.9.3.2. pH

Results documented in Fig 4.22 suggested that the optimum pH for effective biodegradation of crude oil was pH 9.5 although about 80% of degradation could be observed at pH 8 and pH 10. At other pH conditions, the rate of biodegradation was very much less.

4.9.3.3. Inoculum concentration

The variation in the concentration of inoculum was found not to have any profound influence on the rate of biodegradation of crude oil (Fig. 4.23) since the percentage of degradation of crude oil was in the range of 90-96%

for the various concentration of inoculums evaluated (1%-7%) although there was marginal increase in the percent of degradation from 90% at 1% inoculum to 95% at 3% inoculums concentration. Further increase in inoculum concentration of *L.fusiformis* BTTS10 did not show any significant increase in degradation. Nevertheless maximum biodegradation was recorded at 4% (w/v). The biodegradation frequency remained constant after increasing the inoculum concentration higher than 4 % (w/v). Depletion of nutrients in the medium may be the reason for the steady state of biodegradation after increasing inoculum concentration above 4% (w/v).

4.9.3.4. Agitation

Agitation rate was observed to have impact on the rate of biodegradation of crude oil. From the results presented in Fig.4.24 it could be seen that agitation of 110 rpm was required for the efficient biodegradation of crude oil and 120 rpm was found to be optimum for maximal biodegradation by *L.fusiformis* BTTS10. Steady state was obtained in the biodegradation percentage from 120rpm onwards. Agitation of the medium increases the availability of oxygen and enhances the mixing of nutrient and bacteria with hydrocarbons

4.9.4. Separation of SARA components of crude oil fractionated by Column Chromatography

Crude oil is a complex mixture of hydrocarbons consisting of saturates, aromatics, resins and asphaltene. These different components were separated by column chromatography and the result is shown in the Table 4.5. It was found that saturates (52%) formed major component of crude oil followed by aromatics (21%), asphaltene (18% and resins (9%).

4.9.5. FTIR analysis of the biodegraded crude oil during different incubation periods.

The library search report indicated that vibrations, 1654 cm^{-1} -1649 cm^{-1} are representing symmetric C=O stretch of carboxylic acids. The FTIR analysis of samples obtained on the 3rd day (Fig. 4.25.) showed the presence of vibration at 1653cm^{-1} indicating the presence of symmetric C=O stretch of carboxylic acids formed by the biodegradation of hydrocarbons in the crude oil. Vibration 2924 cm^{-1} represents antisymetric CH2 stretch of *n*-Alkanes and 2854 cm^{-1} represents symmetric CH_2 stretch of *n*-Alkanes**.** On the 5th day of incubation a new peak appeared in the FTIR spectrum of the extract of biodegraded crude oil (Fig. 4.26.). The library search results (Correlation Table for Characteristic Raman and Infrared frequencies), point out that the vibration 1740 cm^{-1} -1720 cm^{-1} indicates C=O stretch of aliphatic aldehydes. In the Fig 4.26 vibration 1731 cm^{-1} is present which represents the accumulation of aliphatic aldehyde which might have been formed from the oxidation of carboxylic acids. Fig 4.27 represents the results obtained for the FTIR analysis of samples of the 7th day of biodegradation. Here the intensity of the peaks decreased and many peaks disappeared.

4.9.6. GC analysis of biodegradation of crude oil by *L.fusiformis* BTTS10

Results obtained in terms of gas chromatogram for the biodegradation of crude oil by *L.fusiformis* BTTS10 after GC analysis of samples evidence that *L.fusiformis* BTTS10 is a potential strain with

ability to degrade crude oil. Results of the GC analysis of the control crude oil is presented in Fig.4.28 and Table.4.6. Results obtained from GC analysis of biodegraded crude oil are shown in Fig 4.29, 4.30, and 4.31 respectively for the 3rd, 5th and 7th day of incubation.

Gas chromatographic analysis of crude oil after five days of incubation (Fig 4.30) showed the appearance of 5 large peaks indicating the formation of 5 new hydrocarbons with retention time viz. 5.92 , 6.94, 6.76, 8.12, 8.39, 8.42, 8.817, 9.940. From the star data available it was found that retention time 5.9 corresponds to hydrocarbon with chain length 15 carbons, 6.94 to 18 carbon compound, 8.12 for 21 carbon and 9.94 to 26 carbon containing compound. The chromatogram shows decrease in the area of peaks in the chromatogram as degradation proceeded when compared with control. More degradation has taken place for long chain hydrocarbons as evident from the chromatogram. Gas chromatographic analysis of crude oil on the 7th day of incubation (Fig 4.31) indicated that all new peaks found on the 5th day disappeared and complete degradation of crude oil took place.

4.10. Biodegradation of alkanes in the crude oil.

Results obtained for the GC analysis of the samples alkanes used as control (Fig4.32), and samples obtained from the flasks after biodegradation of alkanes on the 3rd day (Fig4.33) indicated that the bacterium could efficiently degrade tested alkanes.

4.11 GC-MS analysis of biodegraded crude oil and crude oil components.

4.11.1. GC-MS analysis of biodegrdation of crude oil by by *L.fusiformis* BTTS10

Results shown in the Fig.4.34 clearly indicated that there was considerable level of biodegradation of crude oil by *L.fusiformis* BTTS10. Length of the peaks were significantly reduced in the chromatogram of the biodegraded crude oil. New peaks were also visible indicating the production of new intermediates during the biodegradation process.

4.11.2. GC-MS analysis of the intermediates of crude oil biodegradation by *L.fusiformis* BTTS10

Results of the GC-MS analysis of crude oil obtained on the basis of mass spectra data bases (NIST) shown in the Fig. 4.35-4.48 & Table 4.7 indicated that several intermediates are formed during the course of biodegradation of crude oil. Formation of an alcoholic group could be inferred from the Fig 4.35 which is an indication of conversion of pristane which was added as an internal marker into corresponding alcohol. 13-Heptadecyn-1-ol $C_{17}H_{32}O$. In the next step removal of one carbon has taken place and a 16 carbon compound, 2-Cyclopropylcarbonyloxydodecane $C_{16}H_{30}O_2$ was formed. It was found that cyclopropyl ring was formed during this step. In the subsequent step, oxidative cleavage of cyclopropyl ring has taken place and new alkenyl compound 3-(Prop-2-enoyloxy) dodecane $C_{15}H_{28}O_2$ was formed. All the other intermediates represented in Figures 4.38-4.46 are oxidation products formed from the alkanes and

intermediates shown in Figures 4.47 and 4.48 are oxidation products of aromatic compounds. The figures 4.49 to 4.54 represent the components recognized by the library.

4.12. Biodegradation of Toluene by *L. fusiformis* BTTS10

Biodegradation of toluene was analyzed by GCMS. The peak at 11.2 min found in the chromatogram of GC-MS (Fig.4.55 & 4.56) of the extract of biodegraded toluene showed the formation of orcinol (3,5 dihydroxy toluene) as an intermediate. Orcinol was formed by the oxidation of toluene by the dioxygenase enzyme.

4.13. Biodegradation of crude oil with immobilized whole cell biomass of *L. fusiformis* BTTS10

Biodegradation of crude oil was evaluated using viable cells immobilized on two different substrate namely *Eichhornia crassipes* petiole cuttings and polystyrene beads. Biodegradation of the crude oil was monitored by gravimetric analysis and depletion of pH in the biodegradation medium.

4.13.1. Biodegradation of crude oil by *L. fusiformis* BTTS10 immobilized on *E. crassipes* petiole.

Results documented in Fig. 4.57 indicated the progress of cell growth and adherence of cells on to the carrier. The maximum value of protein concentration reached was 273 µg after 4 days of incubation indicating viability and active growth of bacteria under immobilized conditions. Further, from the results documented in Fig 4.58 it was

evident that the bacterial cells remained viable for the test period of 225 days. The number of colony forming units showed a sharp decline up to 50^{th} day and then gradually reduced from 100^{th} day onwards.

4.13.2. Scanning Electron Microscopic (SEM) examination of immobilized viable bacterial cells

Scanning Electron Microscopic (SEM) examination of immobilized viable bacterial cells (Fig 4.59 & Fig 4.60) testified high microbial colonization of rods on the outer surface of the petiole cuttings. The results further showed that large inter cellular spaces were present in the *Eichhornia petiole* providing more area for colonization of bacteria and diffusion of gases and nutrients

4.13.3. Visual observation *of L.fusiformis* BTTS10 immobilized on *E.crassipes* petiole during the biodegradation of crude oil.

Visual observation of the immobilized cells of *L.fusiformis* BTTS10 on *E.crassipes* petiole cuttings **(**Fig.4.61**)** indicates that immobilized cells could degrade crude oil better than free cells. It was found, macroscopically, that dissolution of crude oil into aqueous phase occurred on the 4^{th} day unlike the case with free cells where it occurred only on 5^{th} day. This observation suggests that the bacterial cells have better access to crude oil for utilization under immobilized condition than under freely floating conditions.

4.13.4. Study of biodegradation of crude oil by *L.fusiformis* BTTS10 immobilized on *E. crassipes* petiole

The data obtained from the study of biodegradation of crude oil by gravimetric analysis and observation of reduction in pH presented in Fig 4.62 asserts that immobilized cells were efficient in biodegradation of crude oil. Unlike in the case of free cells where biodegradation started on 5^{th} day herein biodegradation began on the fourth day. It was noted that 70% of the crude oil was degraded on the 5^{th} day and 84 % degradation was achieved on the 7^{th} day. On the seventh day the pH got declined to pH 8.2 from initial pH 10, and no further degradation was noted.

4.14. Biodegradation of crude oil by *L.fusiformis* BTTS10 immobilized on polystyrene beads.

From the visual examination of the flaks containing biodegraded crude oil presented in Fig 4.63 it was inferred that crude oil got quickly dissolved in to the medium because of biodegradation by the immobilized viable bacterial cells. It was also observed that the clumped beads got separated as a result of dissolution of crude oil in to the aqueous medium.

4.14.1. Scanning Electron Microscopic (SEM) examination of viable bacterial cells of L.*fusiformis* BTTS10 immobilized on polystyrene beads

Scanning Electron Microscopic (SEM) examination of viable bacterial cells immobilized on to polystyrene beads presented in Fig 4.64

& Fig 4.65) testified high microbial colonization of rods on the surface of the polystyrene beads.

4.14.2. Study of biodegradation of crude oil by *L.fusiformis* BTTS10 immobilized on polystyrene beads.

Results obtained for the study on the biodegradation of crude oil by viable cells immobilized on polystyrene beads (Fig.4.66.) indicated that polystyrene beads were more efficient as carrier for biodegradation of crude oil compared to the *E.crassipes* petiole cuttings. Gravimetric analysis of biodegradation of crude oil and observations on reduction in pH confirmed that immobilized cells were efficient in biodegradation of crude oil. Unlike free cells where biodegradation started on the 5^{th} day herein biodegradation began on the fourth day. 76% of the crude oil was observed to be degraded on the 5^{th} day and 95% degradation was achieved on the 7^{th} day. The variation in pH of the medium was found to decrease from pH 10 to pH 7.0 on 7^{th} day along with increase in percent of biodegradation of crude oil.

4.15. Biodegradation of asphaltene by *L.fusiformis* BTTS10.

Asphaltenes are petroleum hydrocarbons with extremely complex molecular structure, which are resistant to biodegradation. The results shown in Fig 4.66 indicated a reduction in the pH of the medium during biodegradation which was brought about by the release and dissolution of components of asphaltene degradation by *L.fusiformis* BTTS10. The initial pH of the medium which was pH 10 got declined to pH 7.7 as a result of utilization of asphaltene as sole source of carbon by the bacteria.

4.15.1. Gravimetric analysis of biodegradation of asphaltene.

The residual hydrocarbons formed as result of degradation of asphaltene was evaluated by gravimetric analysis and the results were shown in Fig 4.67. From the results it was inferred that biodegradation gradually began on the 3rd day and 50% degradation of asphaltene was noted on the 25th day.

4.15.2. Respirometric study of Biodegradation of asphaltene

Data obtained from the respirometric study depicted in Fig 4.68 and Fig.69 revealed that carbon dioxide was produced during growth of *L. fusiformis* BTTS10 utilizing asphaltene. This observation testified the potential of this bacteria to utilize asphaltene as sole source of carbon. The evolution of CO_2 increased with incubation period evidencing utilization of asphaltene as sole source of carbon by the bacteria.

4.15.3. Gas chromatographic and FTIR analysis of asphaltene biodegradation

Data obtained by FTIR (Fig 4.70- 4.71) and Gas chromatographic (Fig 4.72-4.73) analysis of products of biodegradation of asphaltene confirmed the potential of *L. Fusiformis* BTTS10 to degrade asphaltene. Formations of new peaks were observed in the FTIR spectra compared to control indicating biodegradation of asphaltene. Gas chromatogram of the extract of the biodegraded asphaltene showed the disappearance of a major peak at 8.71 min (Fig. 4.72 and Fig 4.73) which indicated the biodegradation of components of asphaltene. Many small peaks were also found to get degraded.

4.16. Molecular genetic study of *L.fusiformis* BTTS10

4.16.1. Isolation of plasmid from *L. fusiformis* BTTS10

Agarose gel electrophoresis of the DNA isolated from *L. fusiformis BTTS10* show the plasmid DNA in the gel (Fig 4.74). From the marker ladder it was found that the molecular size of the plasmid DNA is 1.5kbp.

4.16.2. Curing of Plasmid from *Lysinibacillus fusiformis*

Plasmid curing was done using ethidium bromide and the result indicated that no growth of the bacteria occurred in the medium with ethidium bromide concentration above 100µgm/ml. Therefore the sample with maximum concentration of ethidium bromide which showed growth was the 4th sample (Table 4.9). It was selected as cured and used for further study.

4.16.3. Isolation of plasmid from transformed and plasmid cured cells.

Plasmid isolated from transformed *E.coli, L. fusiformis* BTTS10, and plasmid cured *L. fusiformis* BTTS10, were run in an agarose gel by electrophoresis. The results presented as Fig.4.75 & 4.76 confirmed the presence of plasmid in both transformed *E.coli* and *L. fusiformis* BTTS10.

Table 4.1 Morphological and biochemical characteristics of BTTS10.

	variable	Characteristics
Colony and cell morphology	Colony shape	Round
	Colony size	Medium
	Colony edge	Smooth
	Surface	Smooth
	Opacity	Opaque
	Colour	Light brown
	Motility	Motile
	Cell shape	Rod
Biochemical characteristics	Gram staining	positive
	Indole	Positive
	Methyl Red	Positive
	Voges- Proskauer	Positive
	Citrate utilization test	Positive
	Catalase	Positive
	Urease	Positive
	Nitrate reduction	Positive
	Enzyme profile	
	Protease	Negative
	Lipase	Negative
	Alpha amylase	Positive

Table 4.2 Study of biodegradation of organic solvents in presence of DCPIP as redox indicator

Sl.No	Name of solvent	Observation
1	pentane	positive
2	hexane	positive
3	heptane	positive
4	octane	positive
5	cyclohexane	positive
6	benzene	positive
7	toluene	positive
8	xylene	positive
9	naphthalene	positive

Table.4.3 ICP–AES Analysis of the metals accumulated by BTTS10.

Sl/No	Name of metal accumulated	Concentration (ppm)
1	Cadmium	47.09
2	Mercury	17.70
3	Arsenic	3.10

Table 4.4 Antibiotic sensitivity profile of L. *fusiformis* BTTS10

NO	Name of Antibiotic	Result
1	Ciprofloxacin	Sensitive
2	Ofloxacin	Sensitive
3	Saprefloxacin	Sensitive
4	Galifloxacin	Sensitive
5	Aztreonam	Sensitive
6	Azithromycin	Sensitive
7	Vancomycin	Sensitive
8	Doxycyclin Hydrochloride	Sensitive
9	Impepenum	Sensitive
10	Meropenem	Sensitive
11	Tobramycin	Sensitive
12	Moxifloxacin	Sensitive
13	Bacitracin	Sensitive
14	Chloramphenicol	Sensitive
15	Co -Triaxozole	Resistant
16	Pencillin G	Sensitive
17	Erythromycin	Sensitive
18	ceftacnidine	Sensitive
19	cephataxime	Sensitive
20	Nalidixic acid	Resistant
21	Clentamycin	Sensitive
22	Nitillin	Resistant
23	Polymixin	Sensitive
24	Gentamycin	Sensitive
25	Neomycin	Sensitive
26	Tetracyclin	Sensitive
27	Cephalothrin	Sensitive

Table.4.5 Study of surfactant production by drop collapse test

Sl.No	Hydrocarbon	Result(Average)	
		Initial size of drop	Final size of drop
1	Hexadecane	5mm	5mm
2	Benzene	4.8mm	4.8mm
3	Crude oil	4mm	4mmm

Table .4.6 Components of crude oil fractionated by Column Chromatography

TPH	Weight (%)wt/wt
Saturates	52
Aromatics	21
Resins	9
Asphaltene	18

Table.4.7 Gas chromatographic analysis of the components of the BH. crude oil(hexane Fraction)

Name of the compound	Number of carbon	Retention time
Pentane	C_5	0.347
Hexane	C_6	0.556
Heptane	C_7	1.026
Octane	C_8	1.757
Nonane	C_9	2.5
Decane	C_{10}	3.05
Uncecanes	C_{11}	3.6
Dodecanes	C_{12}	4.I
Tridecanes	C_{13}	4.8
Tetradecanes	C_{14}	5.4
Pentadecanes	C_{15}	5.8
Hexadecanes	C_{16}	6.359
Heptadecane	C_{17}	6.790
Octadecane	C_{18}	7.0
Nonadecane	C_{19}	7.7
Eicosanes	C_{20}	8.0
Henecosanes	C_{21}	8.7
Docosanes	C_{22}	9.2
Triacosanes	C_{23}	9.5
Tetracosanes	C_{24}	9.7
Pentacosanes	C_{25}	10.02
Hexacosanes	C_{26}	10.2
Heptacosanes	C_{28}	10.464
Nonacosanes	C_{29}	10.7
Tricontanes plus	30+	< 11

Table 4.8 Intermediates formed during the biodegradation of hydrocarbons analyzed by GCMS

Sl.No	compound	Molecular formula
1	2,Hexanone 3,4, dimethyl	$C_8H_{16}O$
2	Hydroperoxide 1 ethyl butyl	$C_6H_{14}O_2$
3	2Pentene4,4dimethyl	C_7H_{14}
4	Trimethylene glycol monodecyl ether	$C_{18}H_{38}O_4$
5	2cyclopropyl carbonyl oxy dodecane	$C_{16}H_{30}O_2$
6	Pentan 2 ol 4 alloxy 2 methyl	$C_9H_{18}O_2$
7	Heptadyne syne 2,4,dimethyl	$C_{17}H_{32}O$
8	Hydroxyl amine O decyl	$C_{10}H_{23}NO$
9	Hydrazine carboxylic acid	$C_8H_{10}N_2O_2$
10	Benzene dicarboxylic acid	$C_{16}H_{22}O4$
11	2 penten -1-ol-2- methyl	$C_6H_{12}O$
12	3-(Prop-2-enoyloxy) dodecane	$C_{15}H_{28}O_2$

Table 4.9 Curing of plasmid from *L.fusiformis.BTTS10* using ethidium bromide

Sl /No	Concentration of ethidium bromide(µgm/10ml)	Growth of BTTS10 (OD at 600nm)Average of triplicate experiment.
1	20	1.2
2	40	11.
3	60	6.2
4	80	4.1
5	100	3.2
6	120	0

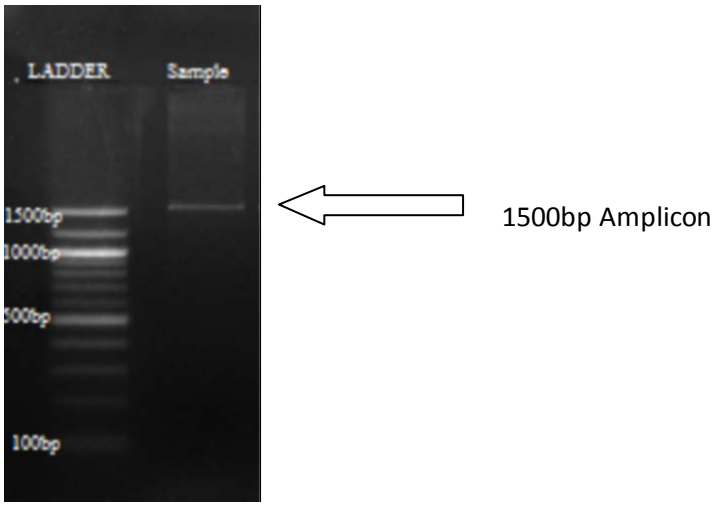
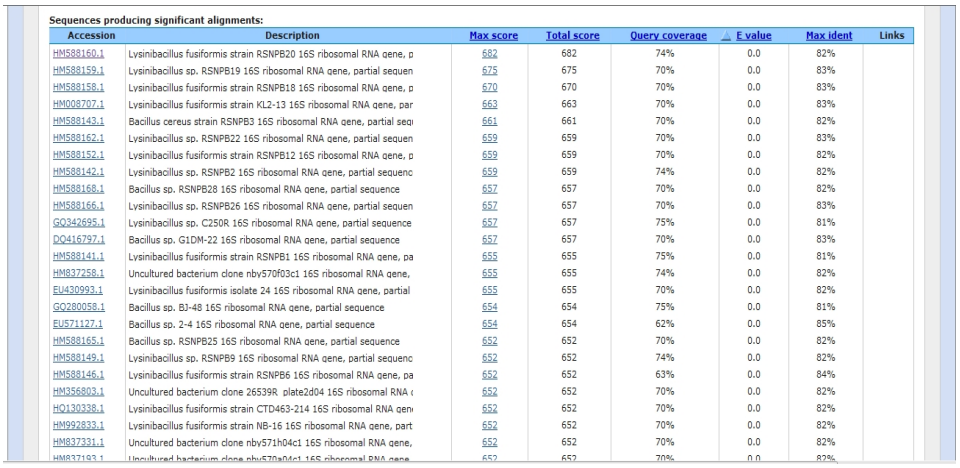


Fig. 4.1 The PCR amplicon of 16S rRNA gene obtained from *L.fusiformis*.BTTS10
Lane 1 -1 Kbp ladder (NEB biolab U.S)
Lane 2 Amplified 16S rRNA gene

TTTCTCTTGTTGGTGCTATCTGCAGTCGTGCGAGGTATCCGTACTTGCTCCT
TTGACGTTAGCGTCGGACGGGTGAGTAACACGTGGGCAACCTACCCTATA
GTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCTCTTTTGC
TTCGTGGCGAAAGACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGC
CCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAACCGACCTGAAAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCATACTCCTACGGGACGCAGGAGTAGGGAATCTTCCACAATGGGCG
AAAGCCTGATGGATCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA
AAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTT
GACGGTACCTTATTATAAAGCCACGGCTAACTACGTGCCATCAGCCGCGG
TAATACGTATGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGCGGTCCTTTATGTCTGATGTGAAAGCCCACGGCTCACCCGTGCAG
GGTCATTGGAAACTGTGGGACTTGAGTGCACAGAGGAAAGTGAAATTCCC
AGTGTATCGTGAAATGCGTAGAGATTTGGAGGACCACCAGTGACGAACGT
GATTTCCTGGTCTGTTAACTGACGCTGATGCCCGAAAGCGTGCGAAGCAA
CCACGATTACATTACGCTGTTAATCCACGCCGTTAACCGATGAAGGCCAA
AGTGATTGGAAGGTTTCCGCCCCTTAATGCCTGCCACTACCGCATTAACCA
CTCTGACCTGCGGAGTATGGACGCAAGACTGAAACTTAAAAGAAATTGAC
GGGGGCCCCGCCAAGCTTGTGCACCATGGTGGTTTATAGTCGAAACATGC
CAAAAAACCTTAACCCAGGCTTTGCTACTCCTGTAAACCCTGTCGAATATA

Fig.4.2 16S rRNA gene sequence of *Lysinibacillus fusiformis* BTTS10



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
HM588160.1	Lysinibacillus fusiformis strain RSNPB20 16S ribosomal RNA gene, p	682	682	74%	0.0	82%	
HM588159.1	Lysinibacillus sp. RSNPB19 16S ribosomal RNA gene, partial sequen	675	675	70%	0.0	83%	
HM588158.1	Lysinibacillus fusiformis strain RSNPB18 16S ribosomal RNA gene, p	670	670	70%	0.0	83%	
HM008707.1	Lysinibacillus fusiformis strain KL2-13 16S ribosomal RNA gene, par	663	663	70%	0.0	83%	
HM588143.1	Bacillus cereus strain RSNPB3 16S ribosomal RNA gene, partial seq	661	661	70%	0.0	82%	
HM588162.1	Lysinibacillus sp. RSNPB22 16S ribosomal RNA gene, partial sequen	659	659	70%	0.0	83%	
HM588152.1	Lysinibacillus fusiformis strain RSNPB12 16S ribosomal RNA gene, p	659	659	70%	0.0	82%	
HM588142.1	Lysinibacillus sp. RSNPB2 16S ribosomal RNA gene, partial sequenc	659	659	74%	0.0	82%	
HM588168.1	Bacillus sp. RSNPB28 16S ribosomal RNA gene, partial sequence	657	657	70%	0.0	82%	
HM588166.1	Lysinibacillus sp. RSNPB26 16S ribosomal RNA gene, partial sequen	657	657	70%	0.0	83%	
GQ342695.1	Lysinibacillus sp. C250R 16S ribosomal RNA gene, partial sequence	657	657	75%	0.0	81%	
DQ416797.1	Bacillus sp. G1DM-22 16S ribosomal RNA gene, partial sequence	657	657	70%	0.0	83%	
HM588141.1	Lysinibacillus fusiformis strain RSNPB1 16S ribosomal RNA gene, pa	655	655	75%	0.0	81%	
HM837258.1	Uncultured bacterium clone nby570f03c1 16S ribosomal RNA gene,	655	655	74%	0.0	82%	
EU430993.1	Lysinibacillus fusiformis isolate 24 16S ribosomal RNA gene, partial	655	655	70%	0.0	82%	
GQ280058.1	Bacillus sp. BJ-48 16S ribosomal RNA gene, partial sequence	654	654	75%	0.0	81%	
EU571127.1	Bacillus sp. 2-4 16S ribosomal RNA gene, partial sequence	654	654	62%	0.0	85%	
HM588165.1	Bacillus sp. RSNPB25 16S ribosomal RNA gene, partial sequence	652	652	70%	0.0	82%	
HM588149.1	Lysinibacillus sp. RSNPB9 16S ribosomal RNA gene, partial sequenc	652	652	74%	0.0	82%	
HM588146.1	Lysinibacillus fusiformis strain RSNPB6 16S ribosomal RNA gene, pa	652	652	63%	0.0	84%	
HM356803.1	Uncultured bacterium clone 26539R_plate2d04 16S ribosomal RNA	652	652	70%	0.0	82%	
HQ130338.1	Lysinibacillus fusiformis strain CTD463-214 16S ribosomal RNA gen	652	652	70%	0.0	82%	
HM992833.1	Lysinibacillus fusiformis strain NB-16 16S ribosomal RNA gene, part	652	652	70%	0.0	82%	
HM837321.1	Uncultured bacterium clone nby571h04c1 16S ribosomal RNA gene,	652	652	70%	0.0	82%	
HM837193.1	Uncultured bacterium clone nby570a04c1 16S ribosomal RNA gene	652	652	70%	0.0	82%	

Fig 4.3 The sequence similarity score of the partial 16s rRNA partial gene sequence of BTTS10 with other known sequences.

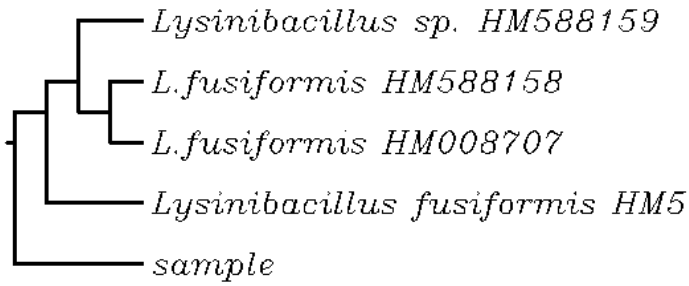


Fig .4.4 Phylogram constructed *with* the partial gene sequence of *16S rRNA of L .fusiformis* BTTS10

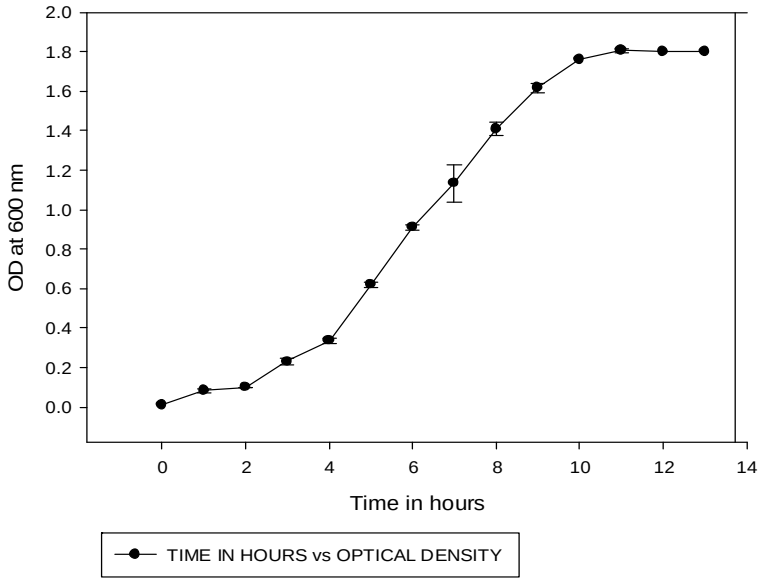


Fig.4.5 Growth curve of *L.fusiformis* BTTS10

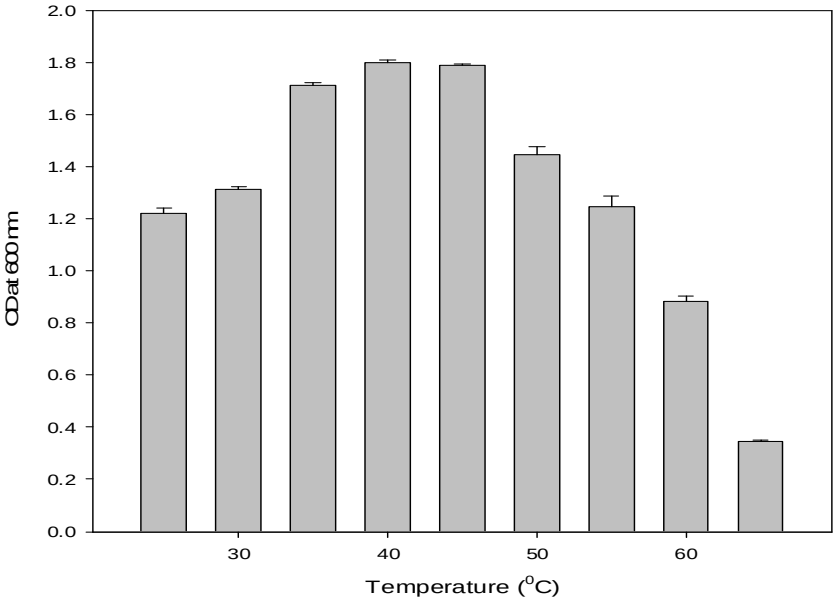


Fig.4.6 Effect of temperature on the growth of *L.fusiformis BTTS10*

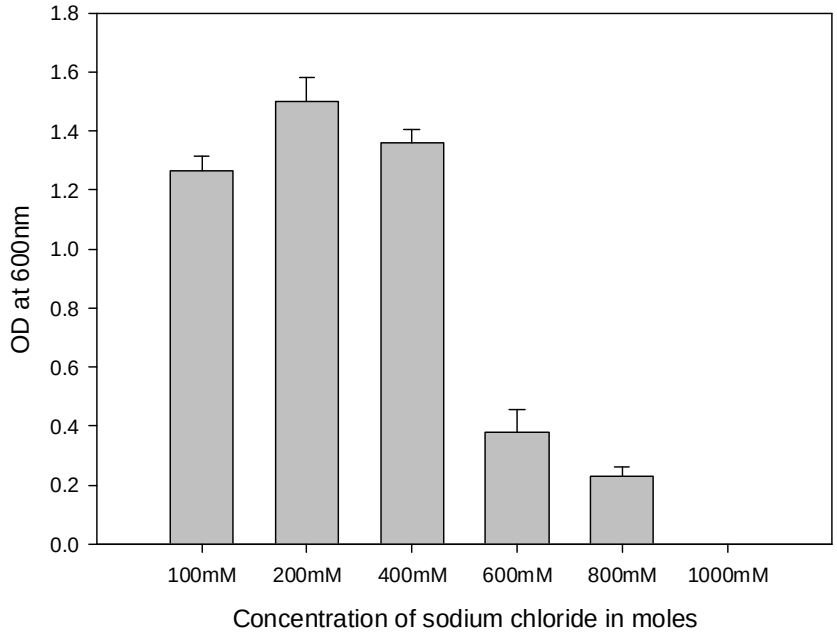


Fig.4.7. Effect of salinity on the growth *L.fusiformis BTTS10.*

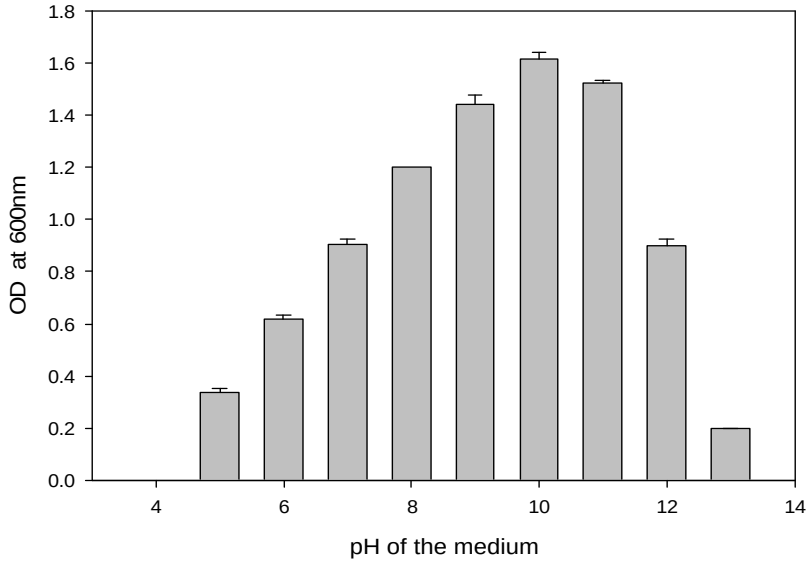


Fig.4.8 Optimum pH for growth *of L.fusiformis BTTS10*

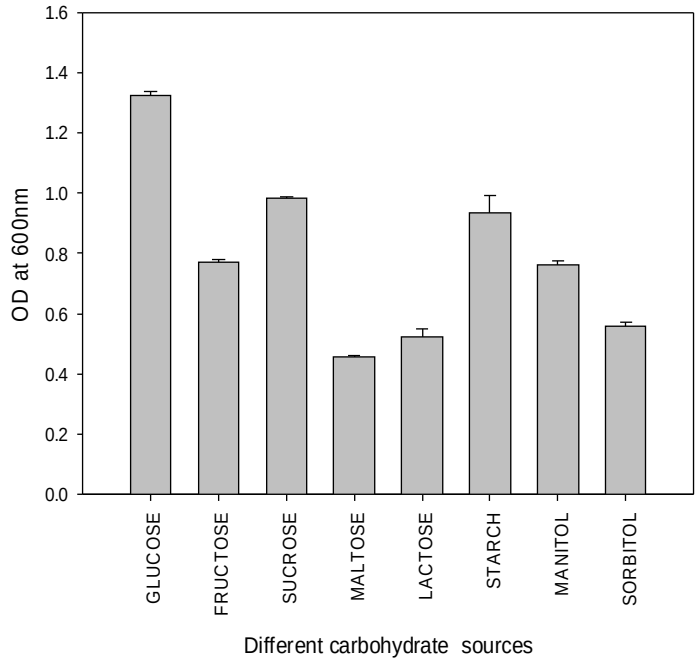


Fig.4.9 Utilization of various carbohydrate sources *by L.fusiformis BTTS10*

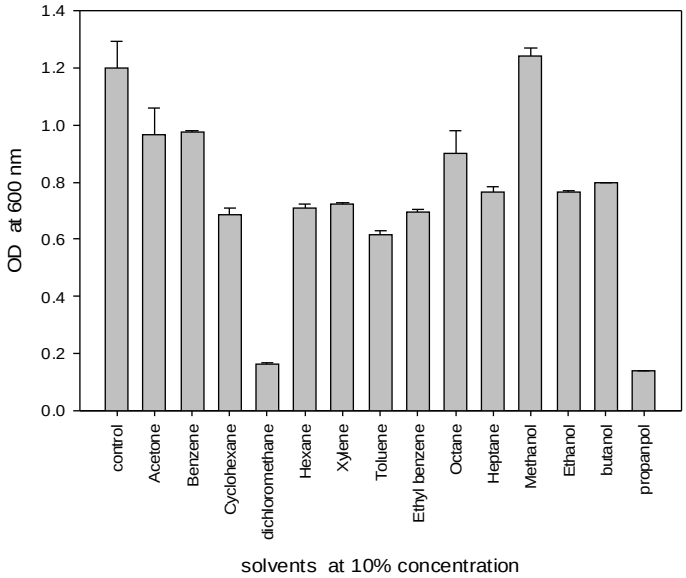


Fig.4.10 Utilization of organic solvents as source of carbon by *L.fusiformis* BTTS10

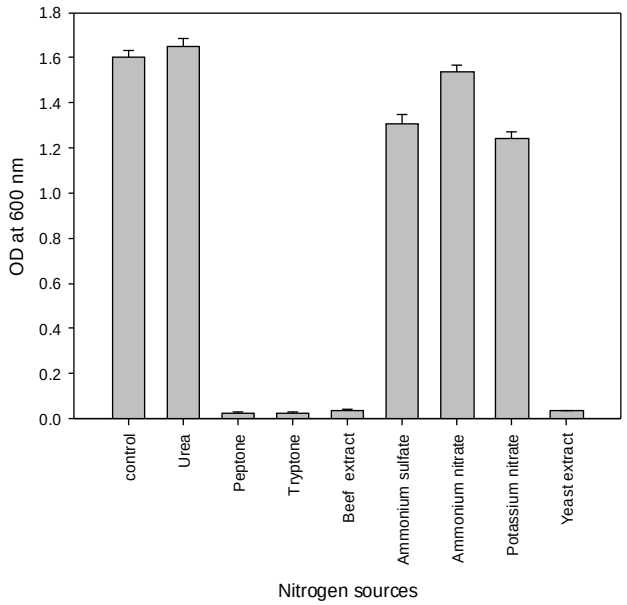


Fig.4.11 Utilization of different nitrogen sources by *L.fusiformis* BTTS10

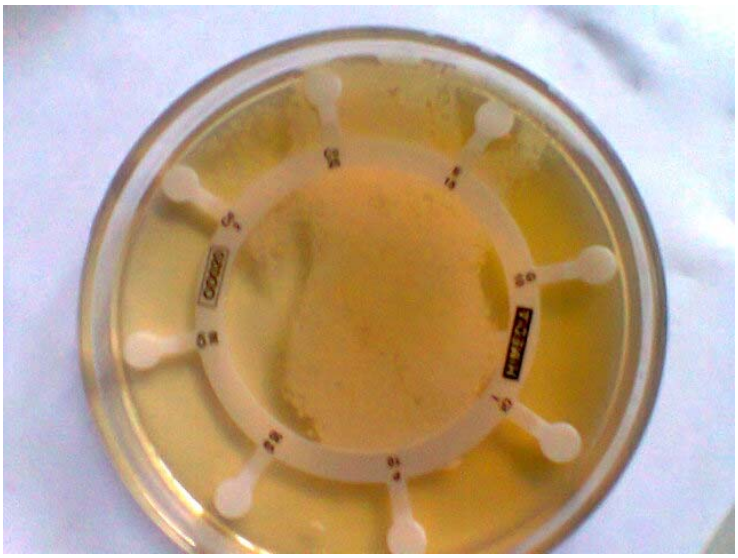


Fig.4.12 Antibiotic sensitivity test showing sensitivity of *L.fusiformis* BTTS10 to 27 antibiotics and resistance to co-trimoxazole.

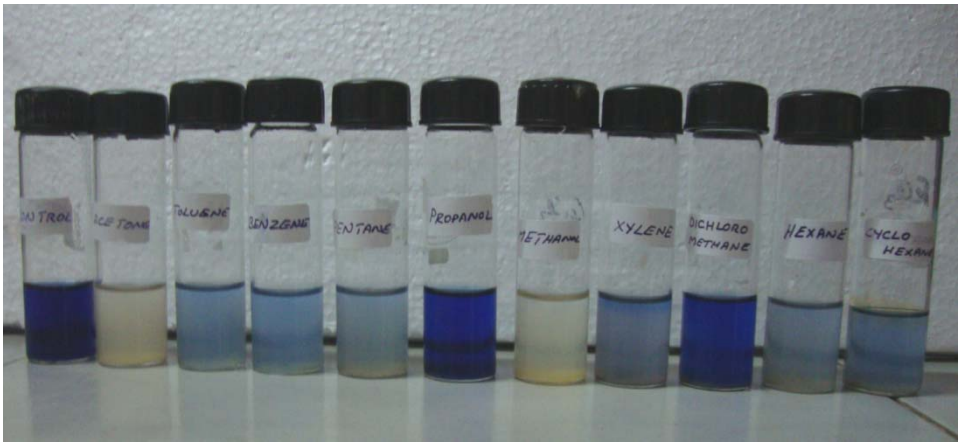


Fig: 4.13.Use of DCPIP as an indicator of biodegradation of hydrocarbons by *L.fusiformis* BTTS10

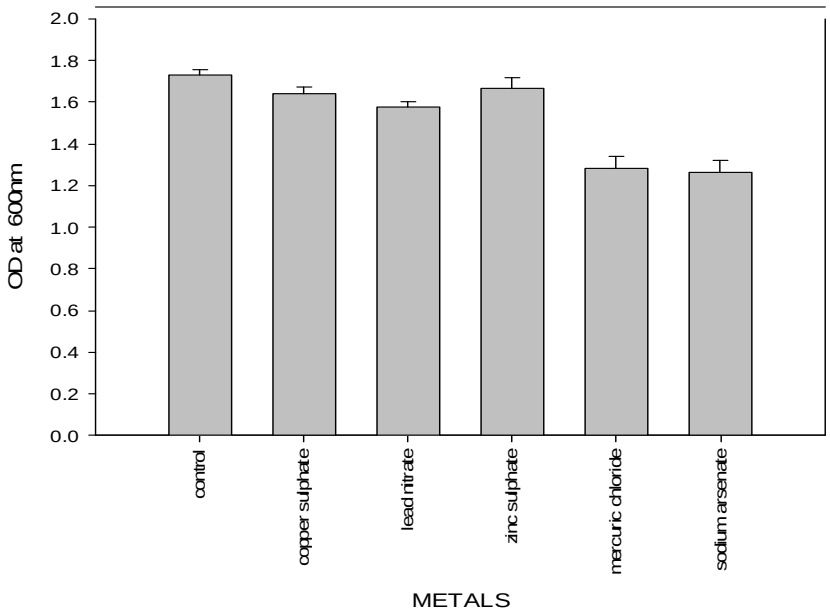


Fig. 4.14 Heavy metal tolerance of *L.fusiformis* BTTS10

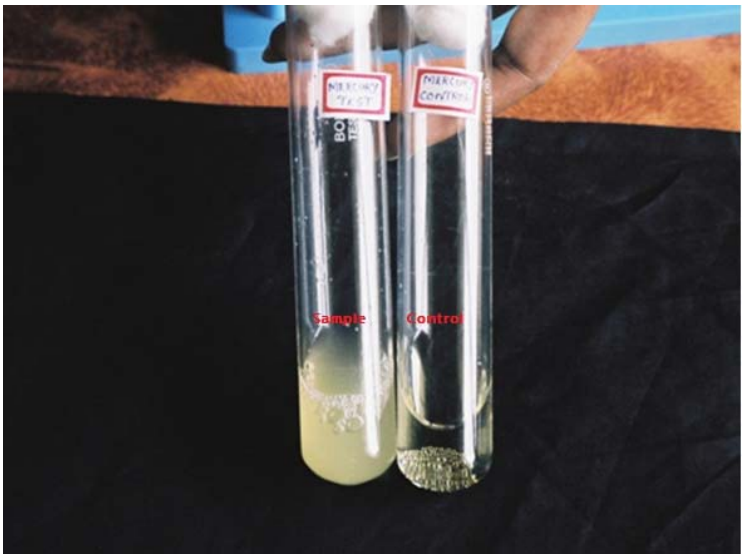
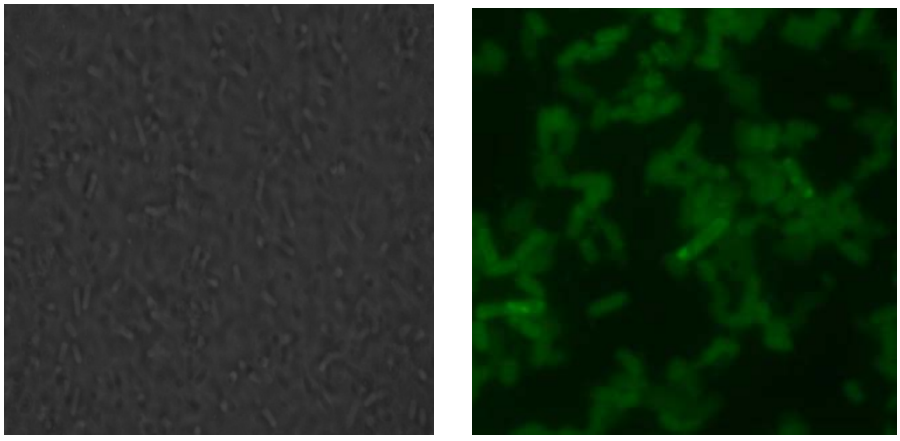


Fig. 4.15 Tolerance of *L.fusiformis* BTTS10 to metallic mercury (control was inoculated with *E.coli* and sample with *Lysinibacillus fusiformis* BTTS10).



(**4.16**a) Control (**4.16**b) Sample

4.16 Fluorescence microscopic observation of *L.fusiformis* BTTS10 treated with ZnS:Mn nanoparticles

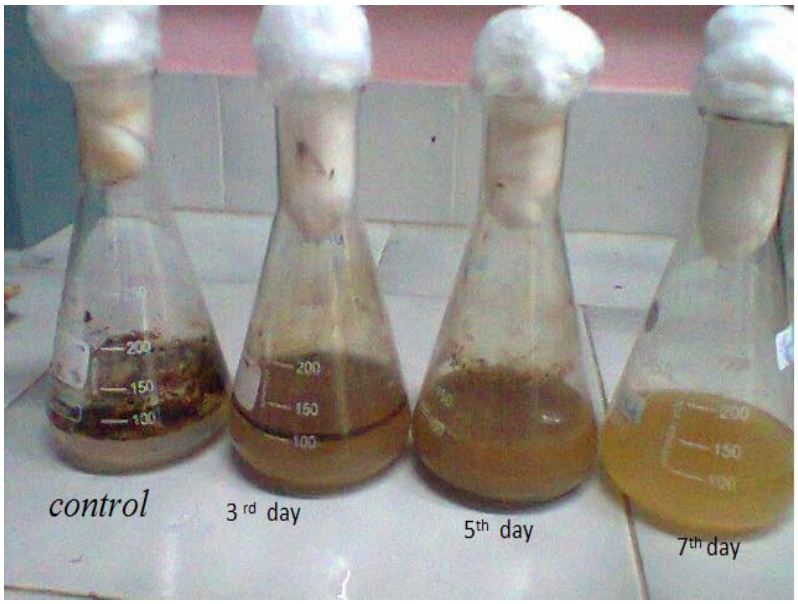


Fig.4.17 Macroscopic view of biodegradation of crude oil by *L.fusiformis* BTTS10

(Control and inoculated Erlenmeyer flask during biodegradation of crude oil on the3rd, 5th and 7th day of incubation at room temperature, shaking at 130 rpm in BH. medium supplemented with 1% crude oil)

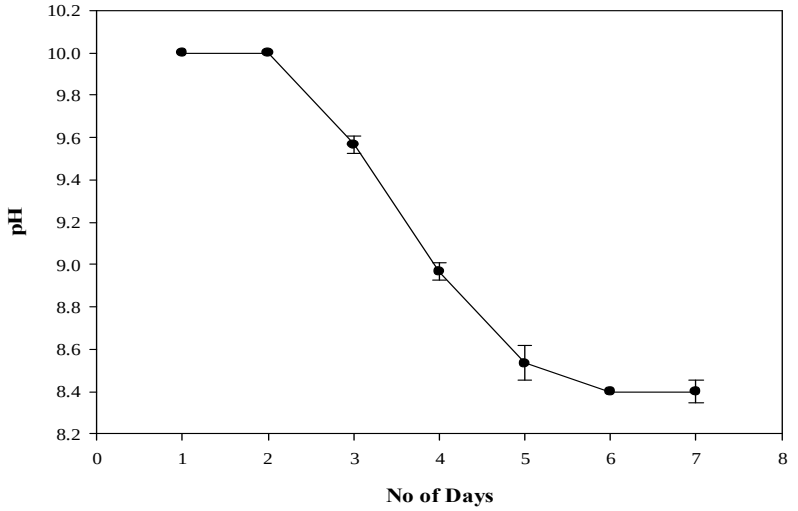


Fig.4.18 Study of pH during biodegradation of crude oil by *L.fusiformis* BTTS10

Fig.4.19 Study of biodegradation of crude oil by *L.fusiformis* BTTS10 in a biometric flask

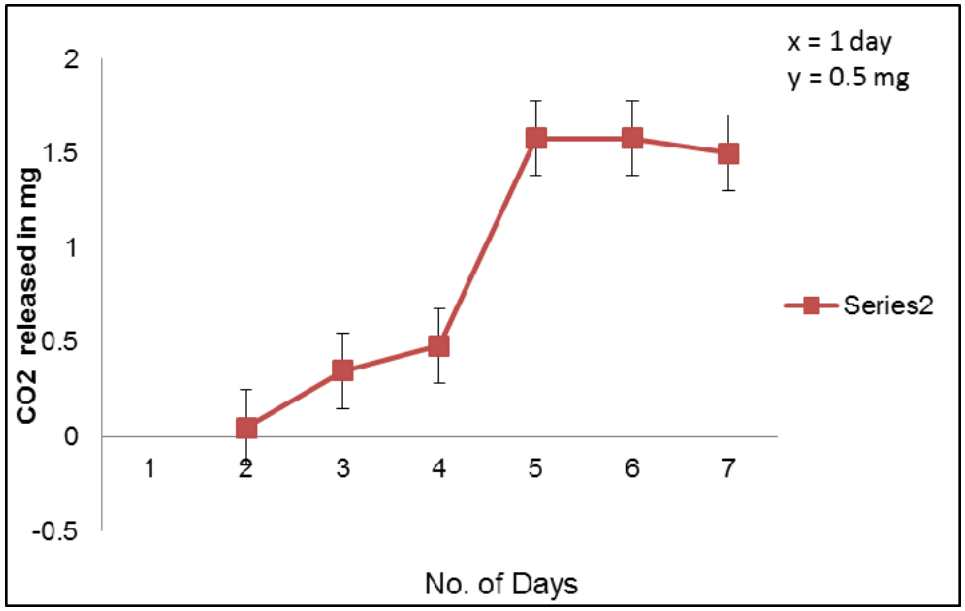


Fig.4.20 Carbondioxide released during biodegradation of crude oil in a biometric flask.

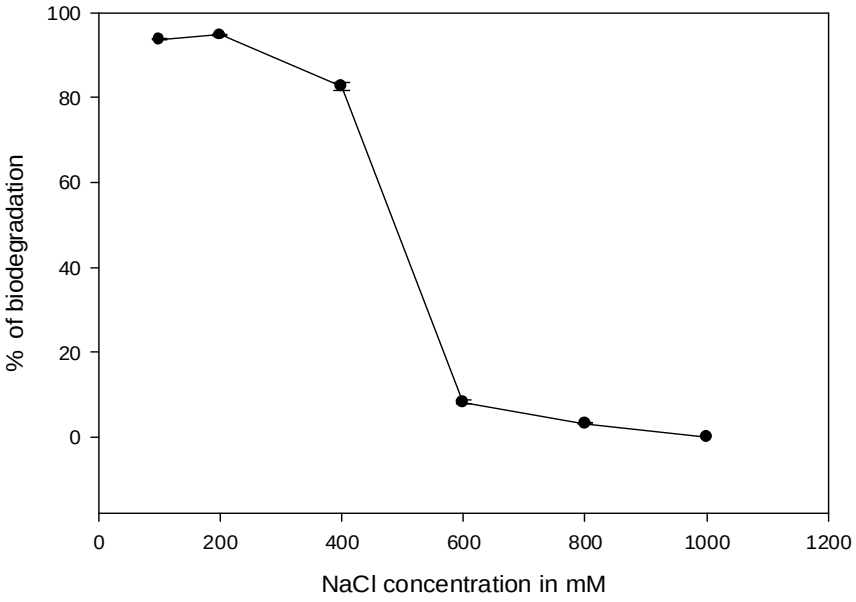


Fig. 4.21 Effect of NaCl on the biodegradation of crude oil by *L.fusiformis* BTTS10 (% of biodegradation in mg).

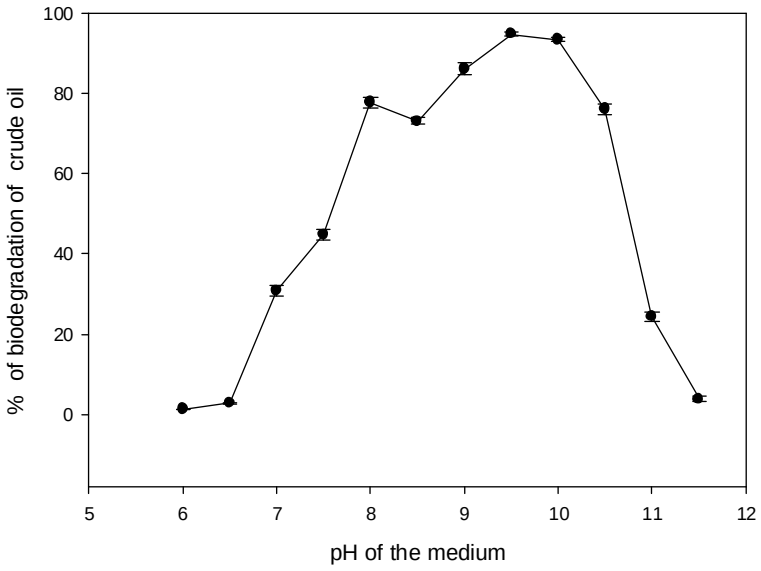


Fig 4.22 Effect of pH on the biodegradation of crude oil by *L.fusiformis* BTTS10

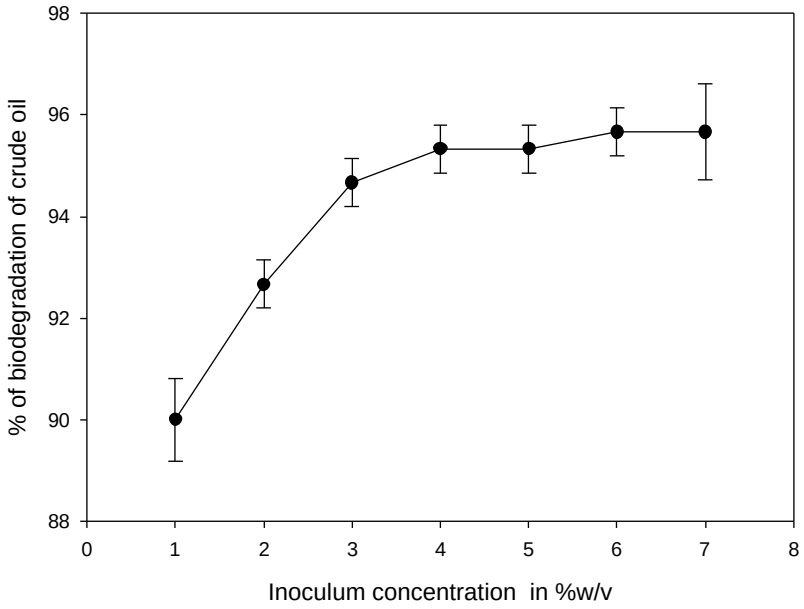


Fig 4.23 Effect of inoculum concentration on the biodegradation of crude oil by *L.fusiformis* BTTS10.

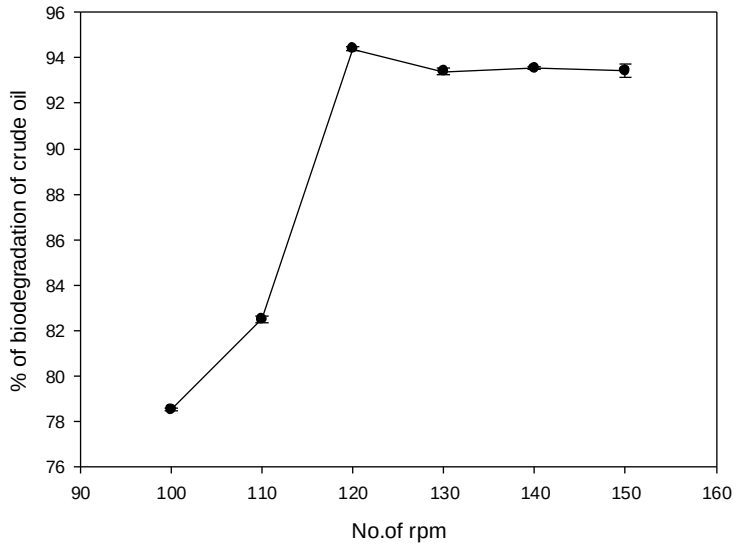


Fig.4.24 Effect of agitation on the biodegradation of crude oil by *L.fusiformis* BTTS10

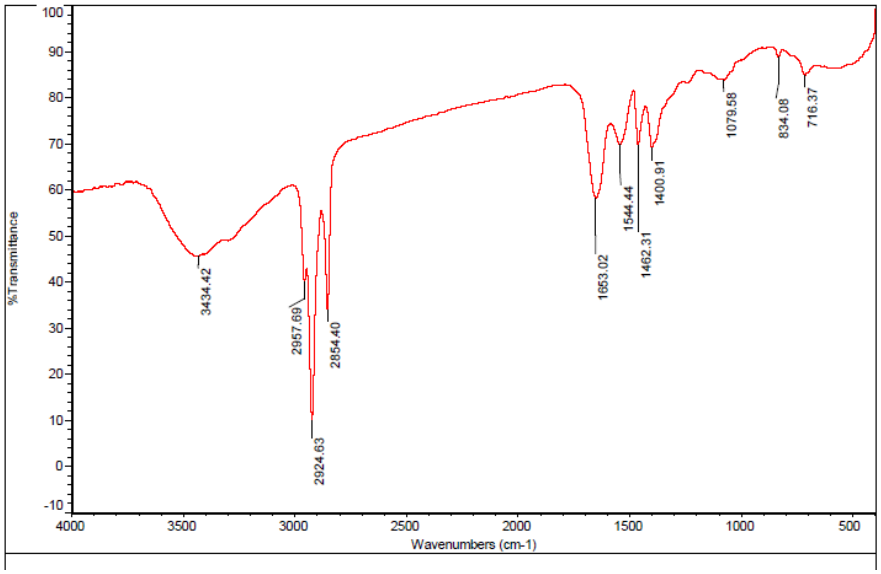


Fig 4.25 FTIR analysis on the 3rd day of biodegradation of crude oil

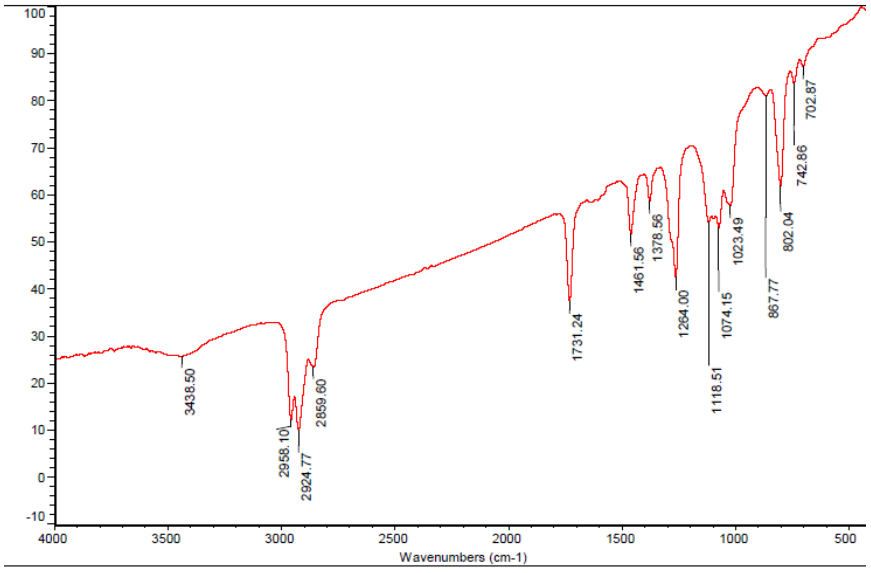


Fig 4.26 FTIR analysis of crude oil on the 5th day of biodegradation

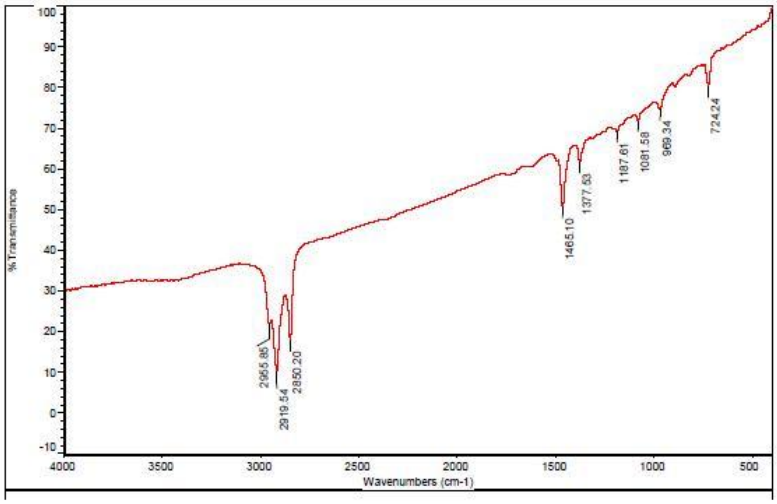


Fig 4.27 FTIR analysis of crude oil on the 7th day of biodegradation

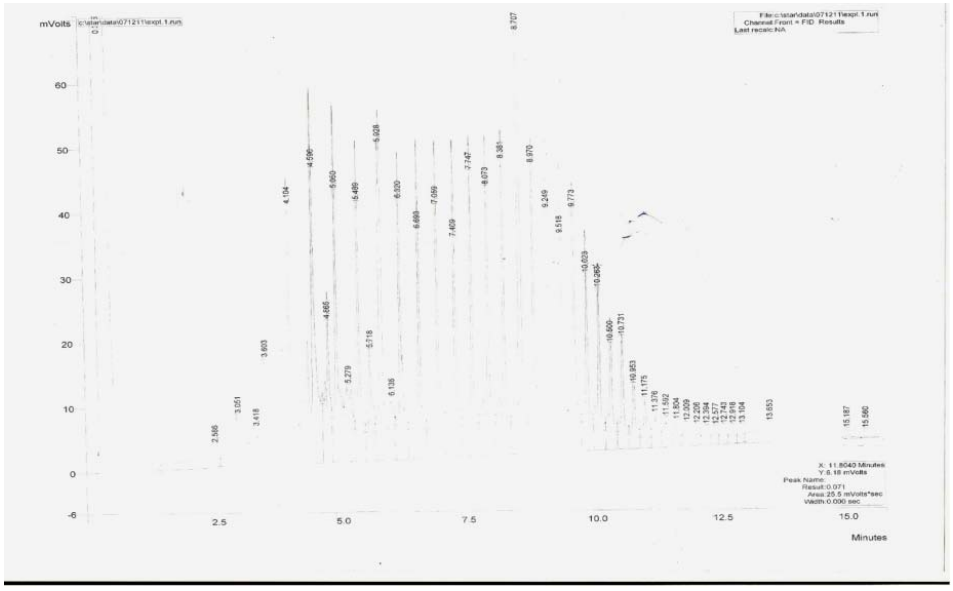


Fig.4.28 GC analysis of hydrocarbons in control crude oil.

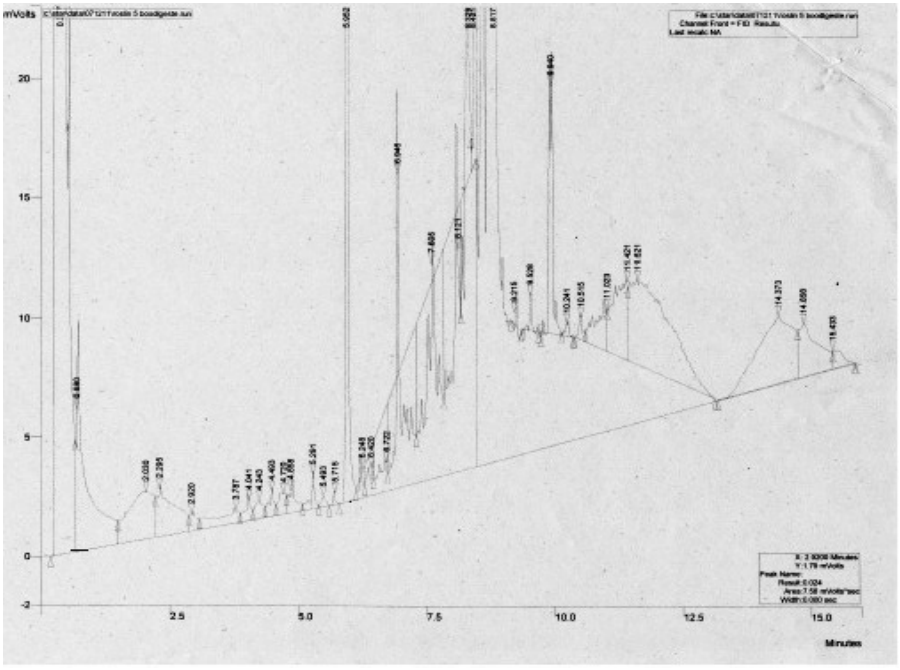


Fig.4.29 GC analysis of the biodegraded crude oil on the 3 rd day of incubation

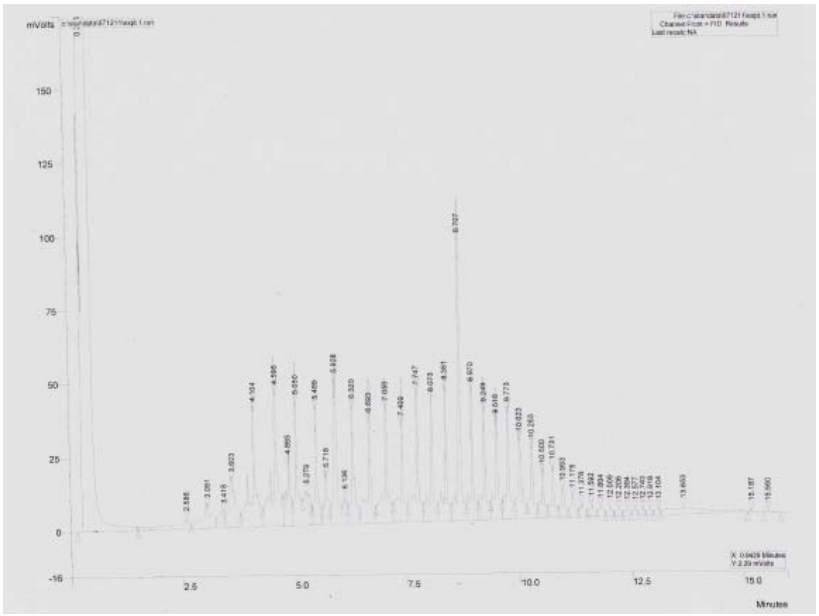


Fig 4.30 GC analysis of the biodegraded crude oil on the 5th day of incubation.

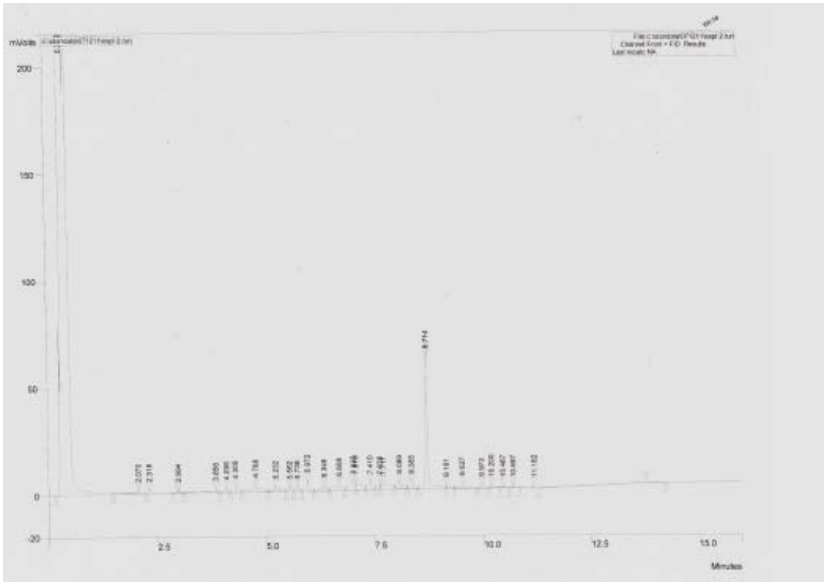


Fig.4.31 GC analysis of biodegraded crude oil on the 7th day of incubation

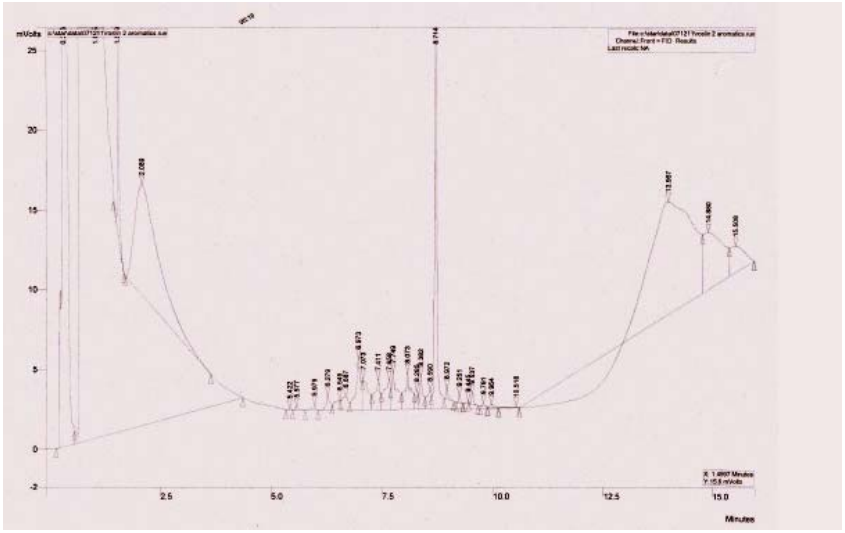


Fig.4.32 GC. Analysis of alkane control

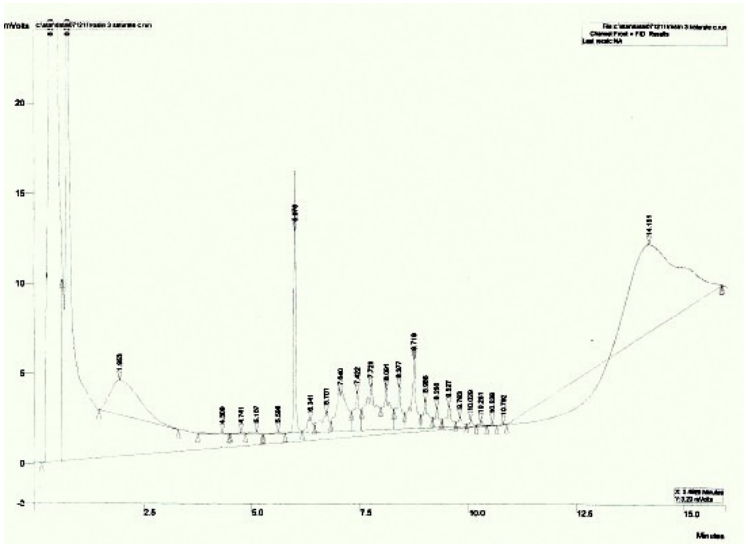


Fig.4.33 GC of biodegraded alkane on 3rd day of incubation.

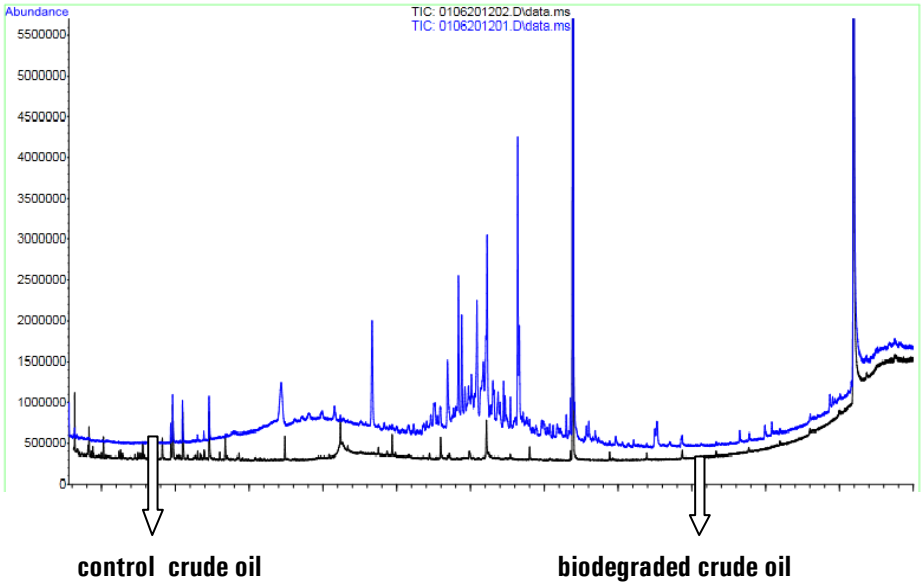


Fig .4.34 GC-MS analysis of control and biodegraded crude oil.

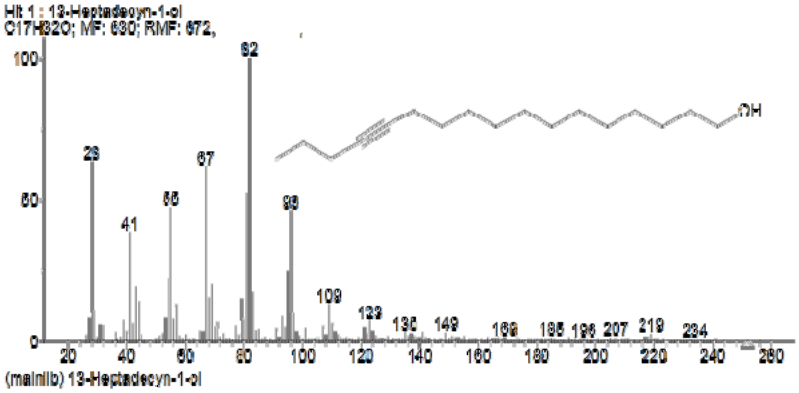


Fig. 4.35 13-Heptadecyn-1-ol (intermediate product of biodegraded crude oil identified by GC-MS).

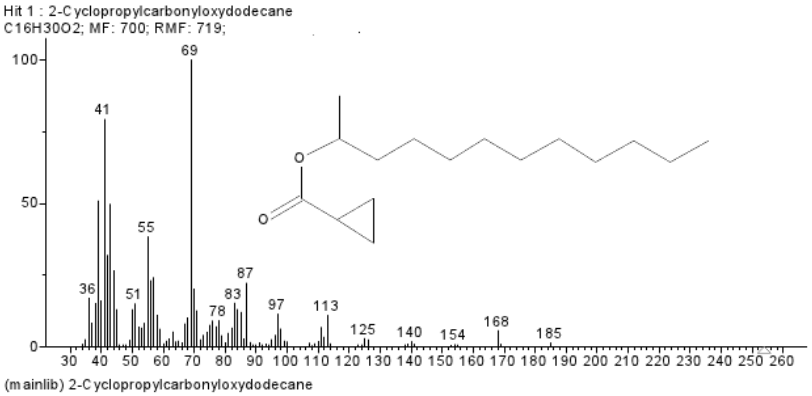


Fig.4.36 2-Cyclopropylcarbonyloxydodecane (intermediate product of biodegraded crude oil identified by GC-MS).

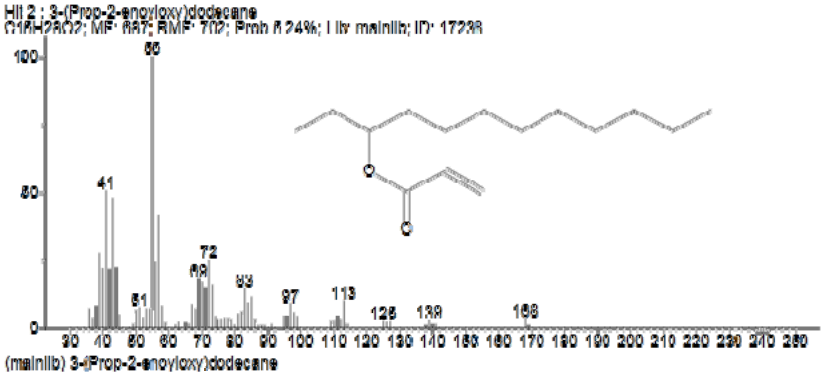


Fig.4.37 3-Prop-2-enoyloxy dodecane (intermediate product of biodegraded crude oil identified by GC-MS).

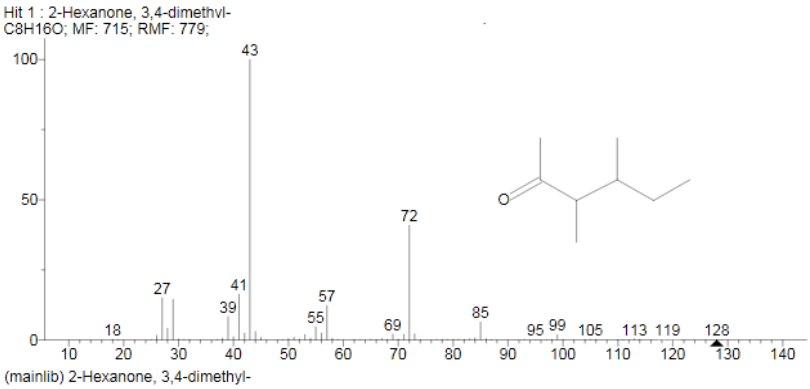


Fig.4.38 2-Hexanone, 3, 4-dimethyl (intermediate product of biodegraded crude oil) identified by GC-MS).

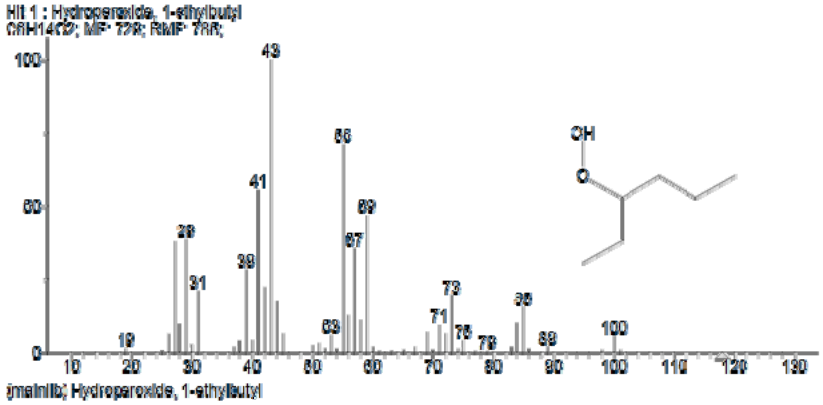


Fig.4.39 Hydroperoxide,1-ethylbutyl (intermediate product of biodegraded crude oil identified by GC-MS)

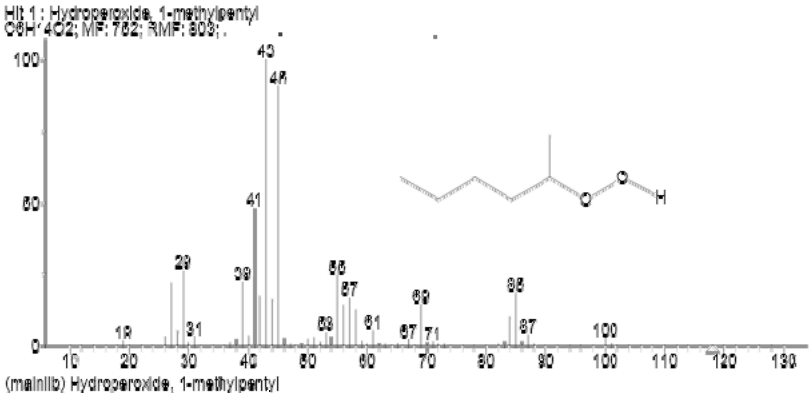


Fig.4.40 Hydroperoxide 1methyl pentyl (intermediate product of biodegraded crude oil identified by GC-MS).

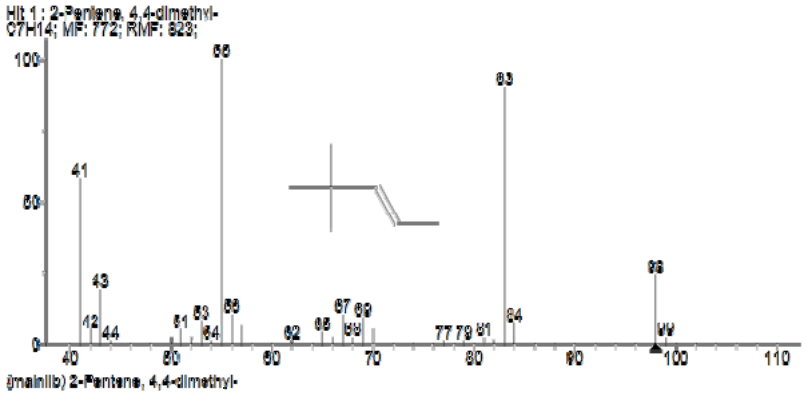


Fig.4.41 2-Pentene 4, 4 dimethyl (intermediate product of biodegraded crude oilidentified by GC-MS).

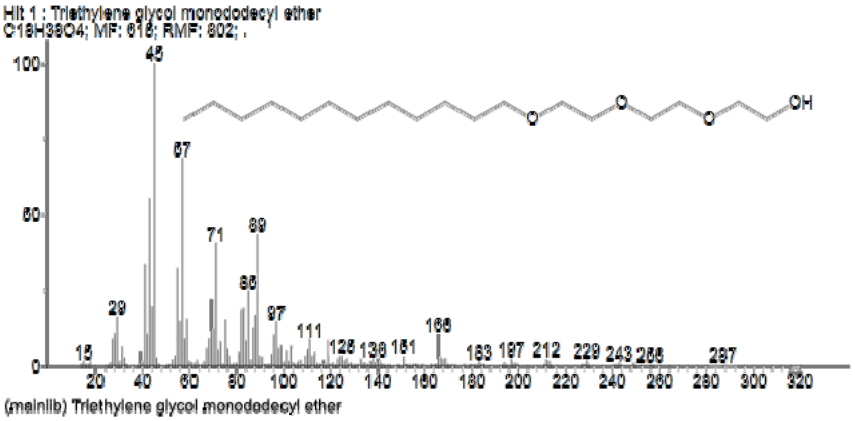


Fig.4.42 Trimethylene glycol monodecyl ether (intermediate product of biodegraded crude oil identified by GC-MS).

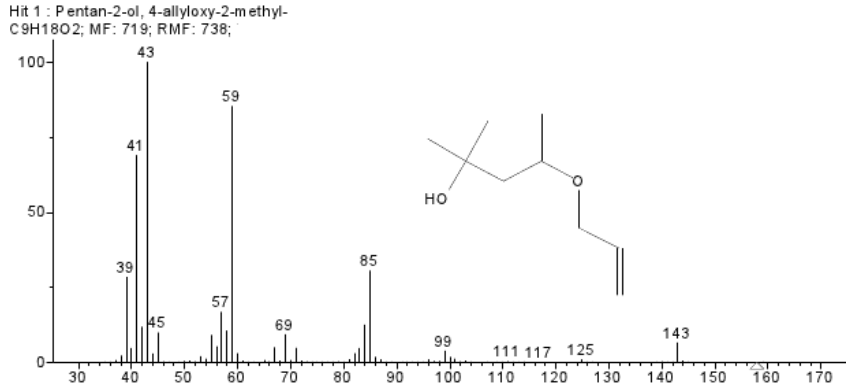


Fig.4.43 Pentan 2 ol 4 alloxy 2 methyl (intermediate product of biodegraded crude oil identified by GC-MS).

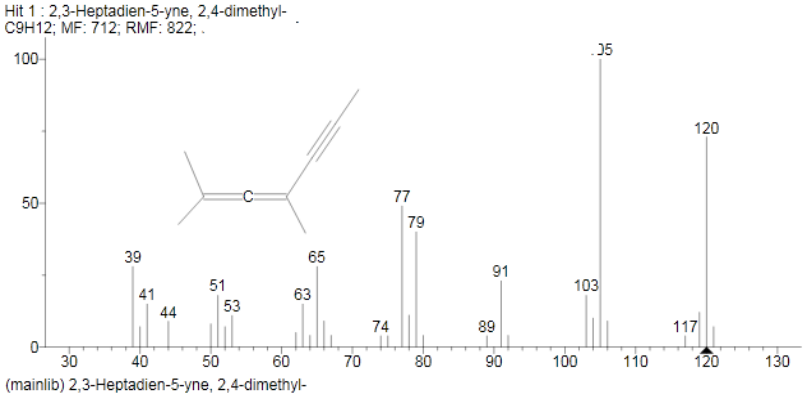


Fig 4.44 2, 3 - Heptadien - 5-yne 2, 4, dimethyl (intermediate product of biodegraded crude oil identified by GC-MS).

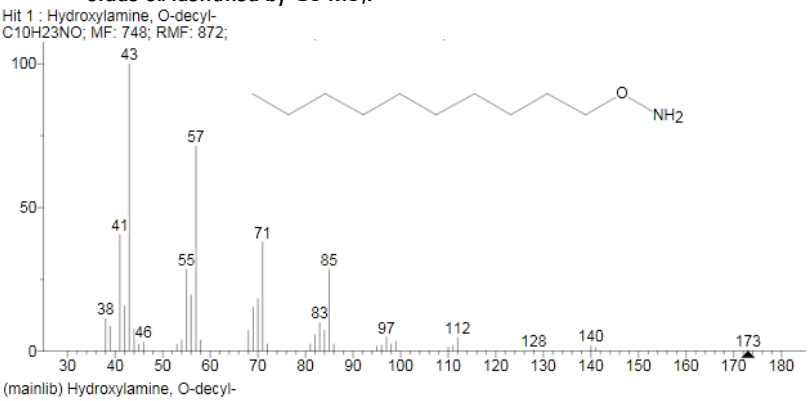


Fig4.45 Hydroxylamine O decyl (intermediate product of biodegraded crude oil identified by GC-MS).

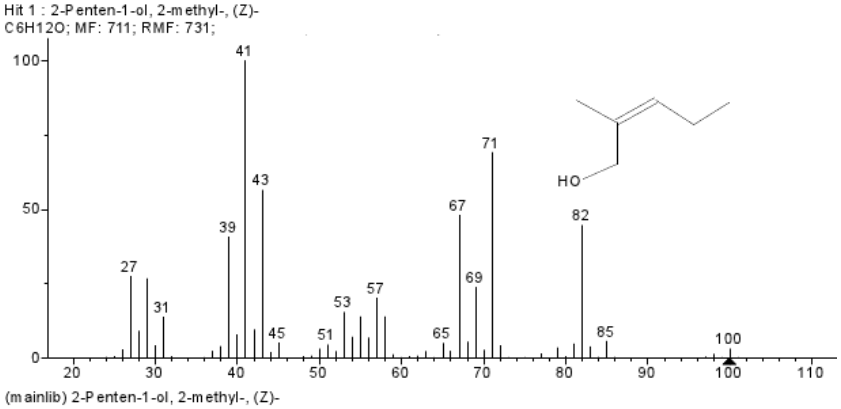


Fig4.46 2 penten -1-ol, 2- methyl (intermediate product of biodegraded crude oil identified by GC-MS).

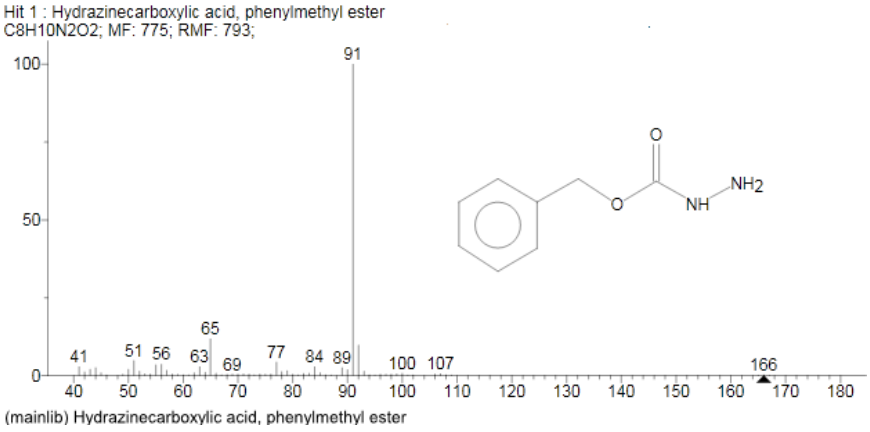


Fig4.47 Hydrazine carboxylic acid, phenylmethyl ester(intermediate product of biodegraded crude oil identified by GC-MS).

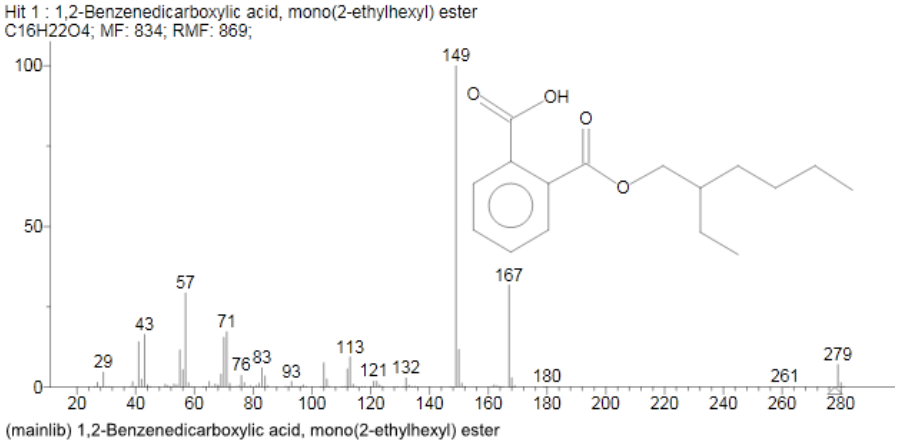


Fig 4.48 Benzene dicarboxylic acid,mono (2-ethylhexyl) ester (intermediate product of biodegraded crude oil identified by GC-MS).

(GC-MS analysis of biodegraded crude oil was done using high resolution GC-MS-Agilent 7890. The fig.4.35-4.48 represents the components of biodegraded crude oil recognized by the library).

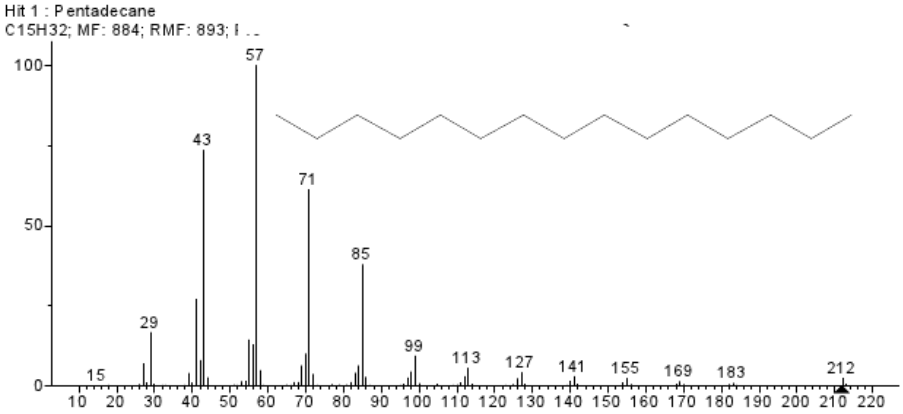


Fig.4.49 Pentadecane (component in crude oil identified by GC-MS).

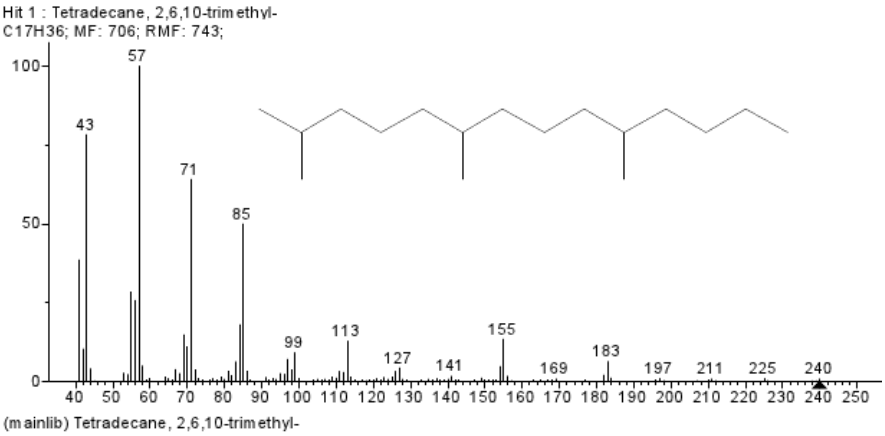


Fig .4.50 Octdecane (component in crude oil idntified by GC-MS).

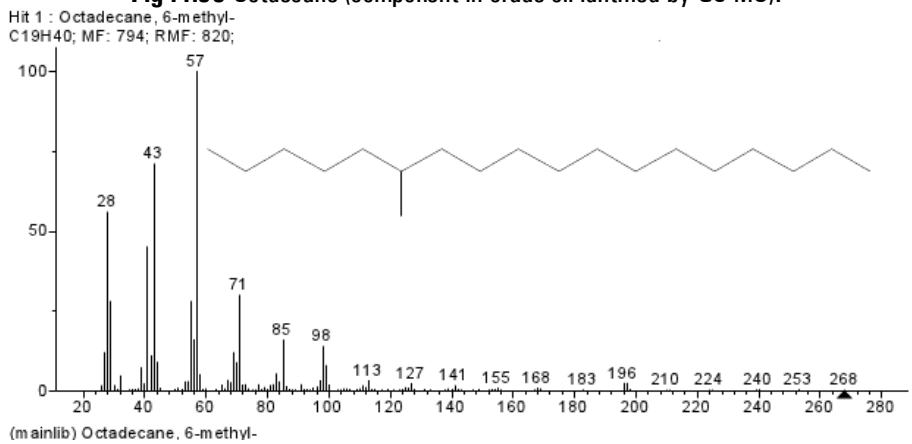


Fig.4.51 Octadecane ,6-methyl (component in crude oil identified by GC-MS).

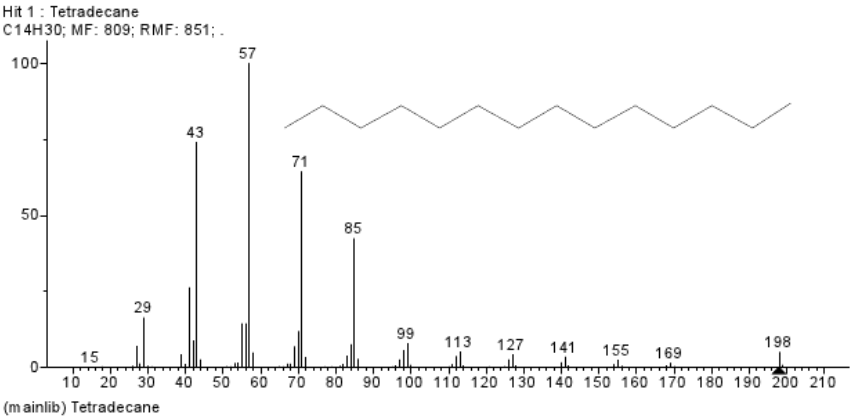


Fig.4.52 Tetradecane (component in crude oil identified by GC-MS).

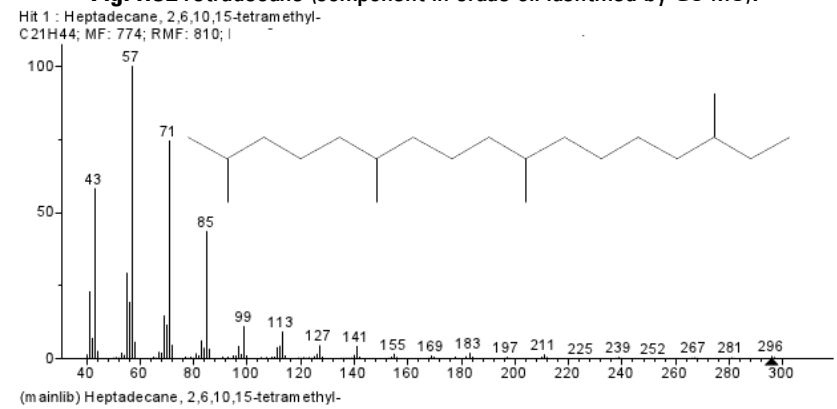


Fig.4.53. Heptadecane, 2,6,10,15 –tetramethyl (component in crude oil identified by GCMS)

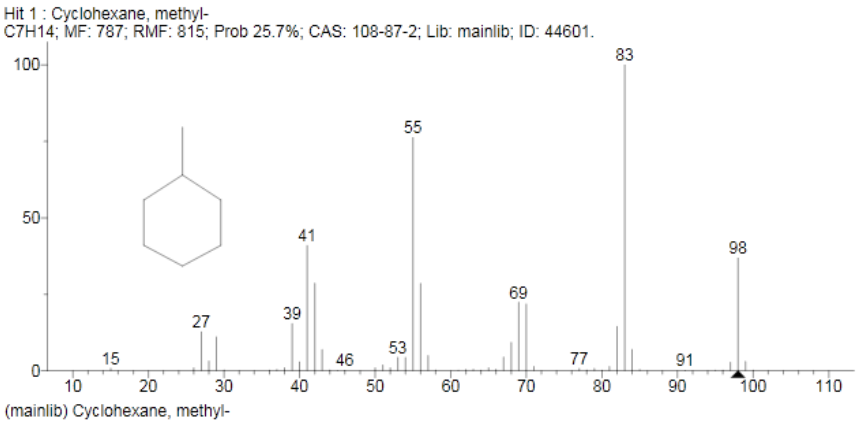


Fig.4.54 Cyclohexane, methyl (component in crude oil identified by GC-MS).

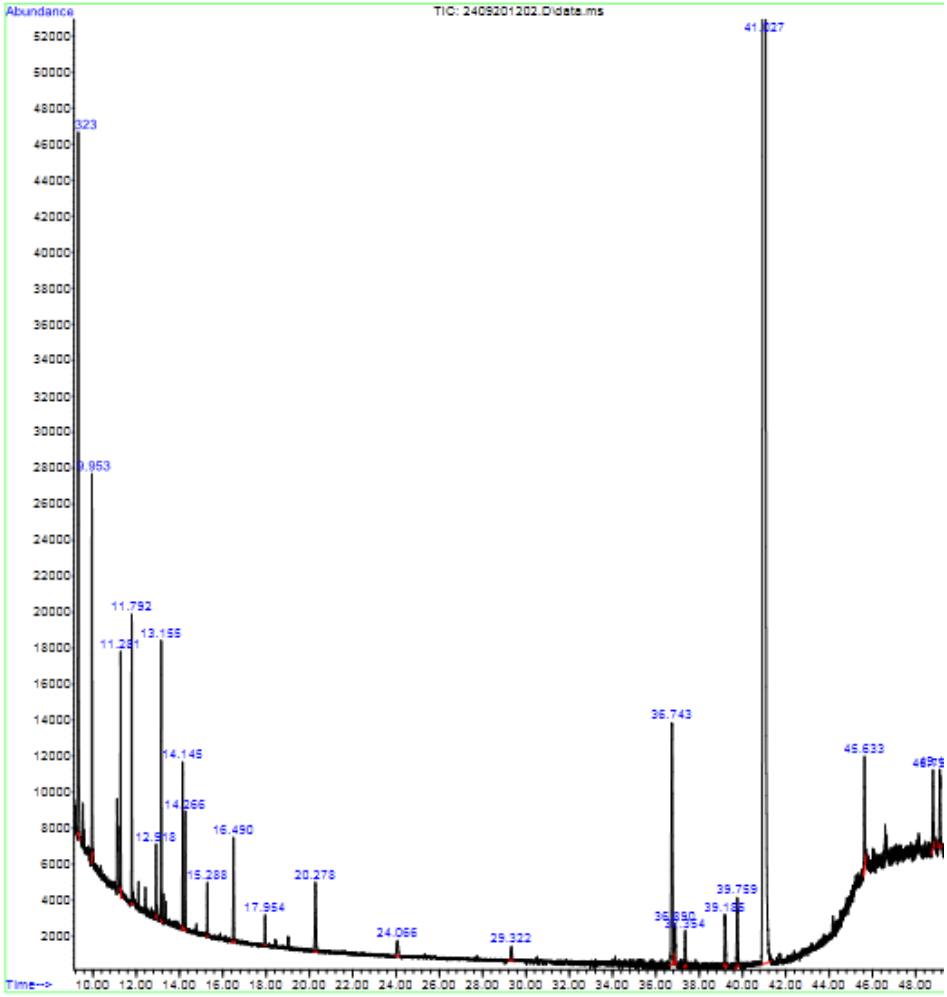


Fig4.55 GC-MS analysis of extract of biodegraded toluene

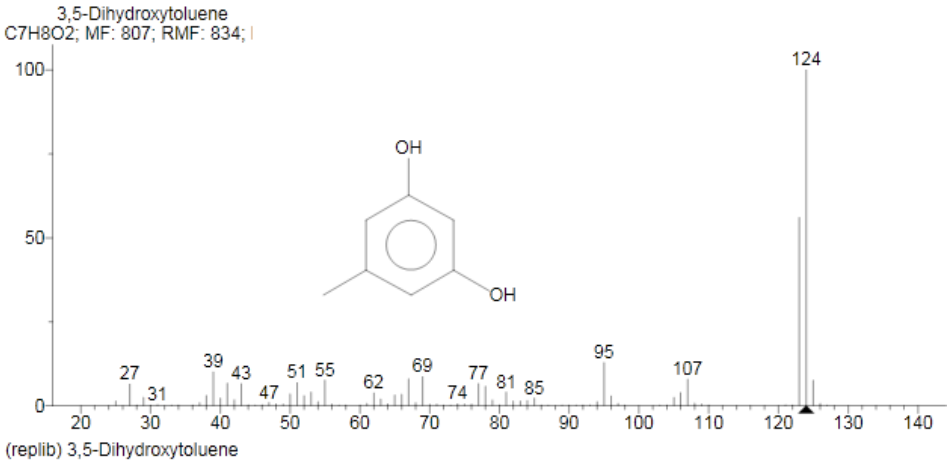


Fig 4.56 3, 5, Dihydroxy toluene (Intermediate product of biodegraded toluene identified by GC-MS)

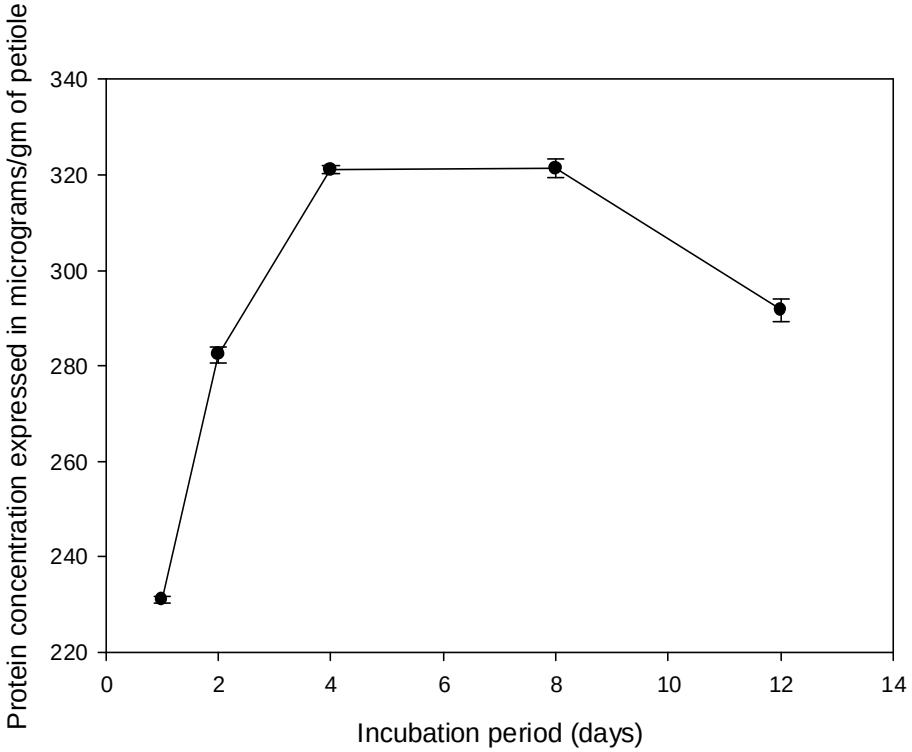


Fig.4.57 Total cell proteins immobilized on the *E. crassipes* petiole

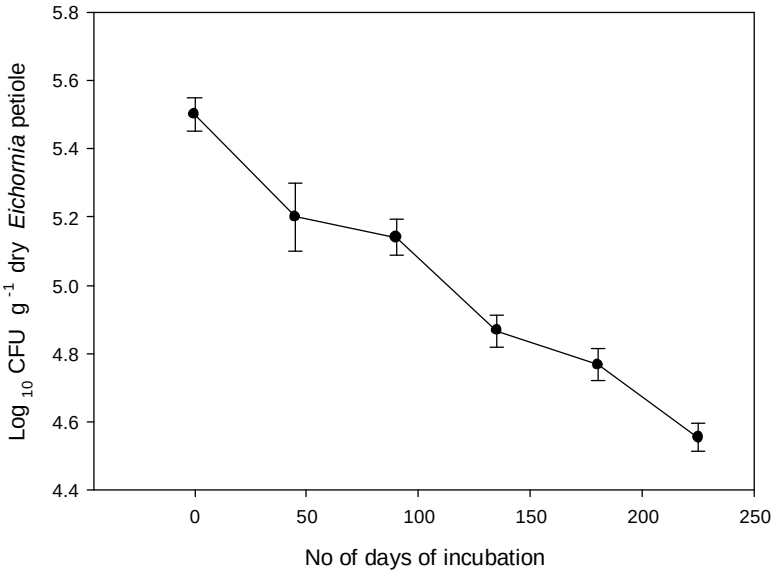


Fig.4.58 Viable counts obtained from *L.fusiformis* BTTS10 immobilized on *Eichhornia* petiole.

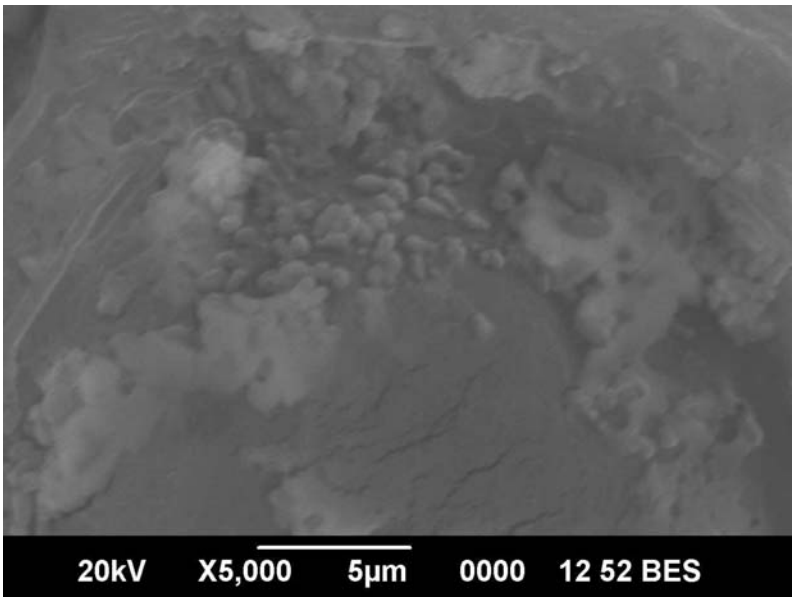


Fig. 4.59 SEM of *L.fusiformis* BTTS10 immobilized on *E. crassipes* petiole (x5000 magnification).

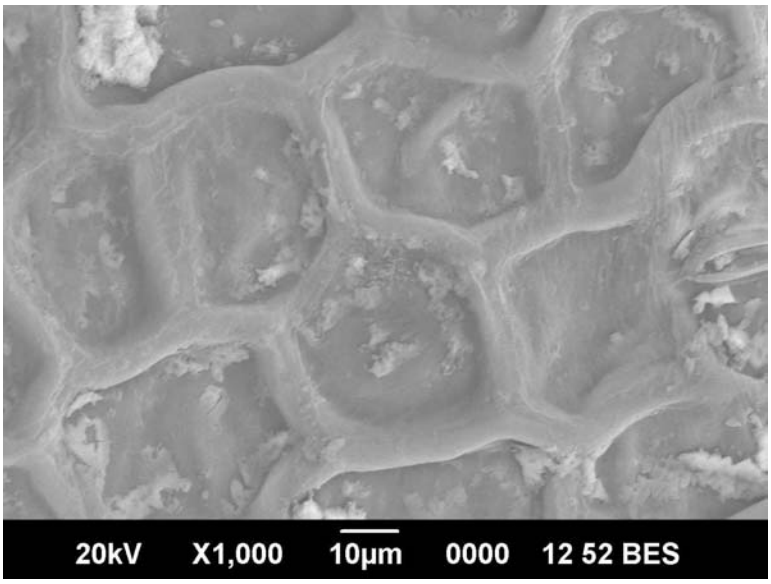


Fig.4.60 SEM of *L.fusiformis* BTTS10 immobilized on *E.crassipes* petiole (x1000 magnified)

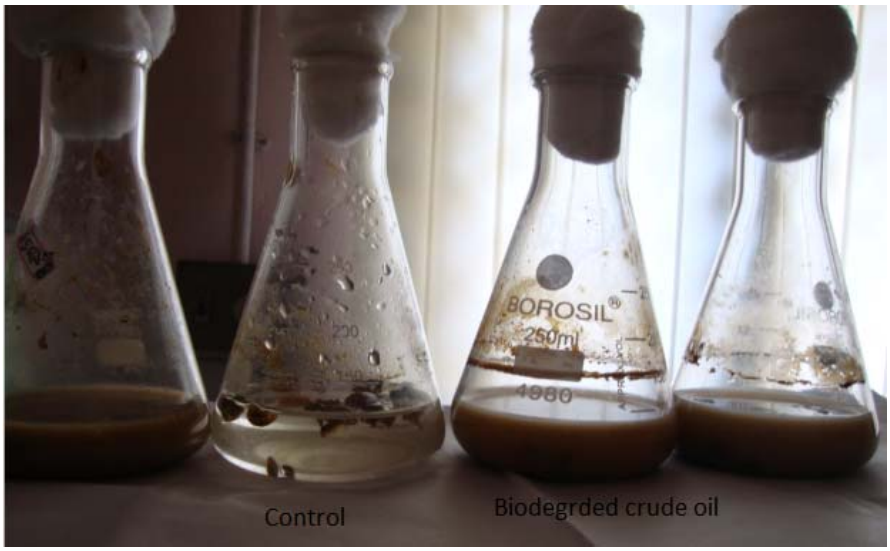


Fig 4.61 Visual observation *of L.fusiformis* BTTS10 immobilized on *Eichhornia crassipes* petiole during the biodegradation of crude oil.

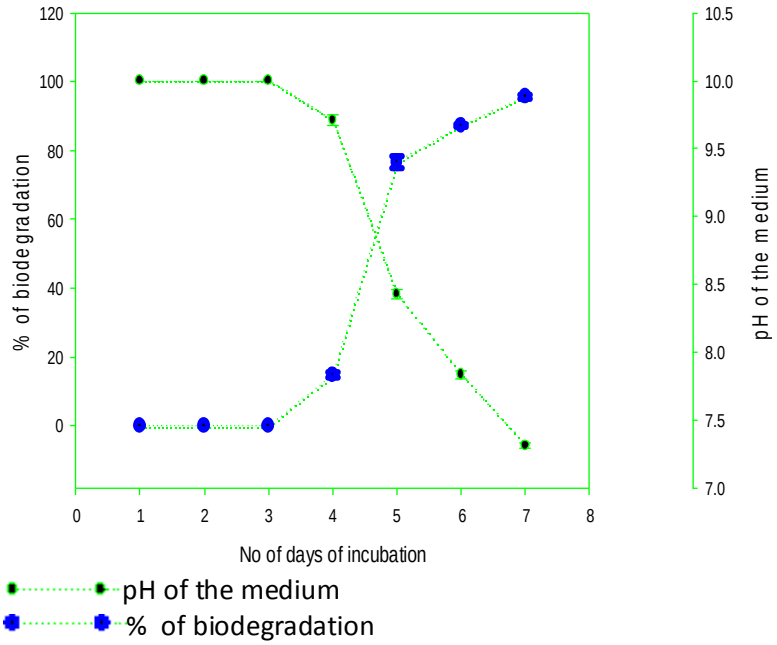


Fig.4.62 Study of biodegradation of crude oil by gravimetric analysis and observation of pH

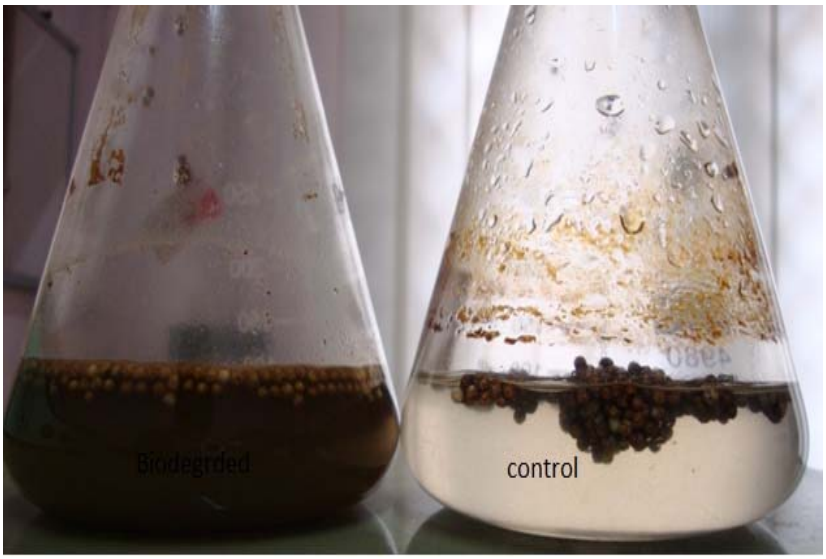


Fig.4.63 Visual observation of *L.fusiformis* BTTS 10 immobilized on polystyrene beads on the fourth day of crude oil biodegradation.

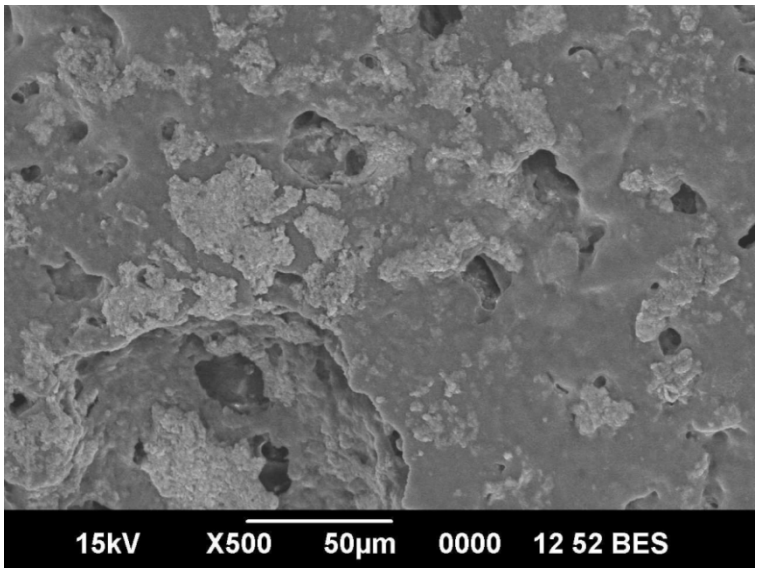


Fig. 4.64 SEM of *L.fusiformis* BTTS10 immobilized on polystyrene beads

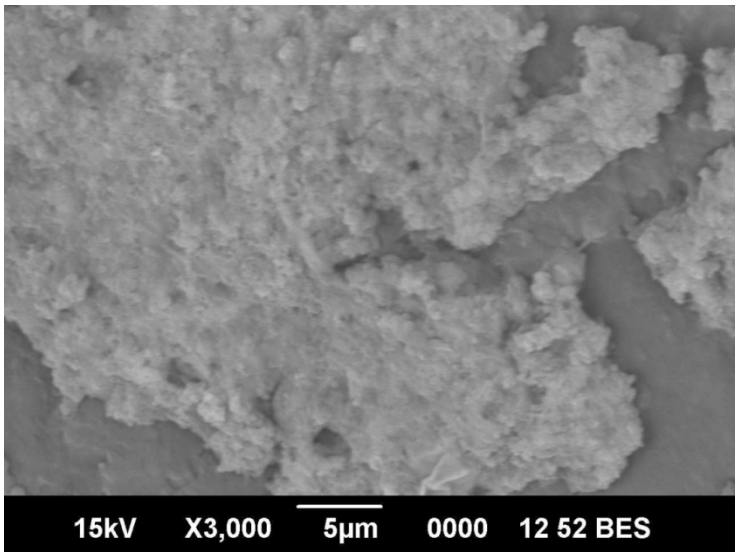


Fig 4.65 SEM of *L.fusiformis* BTTS10 immobilized on polystyrene beads

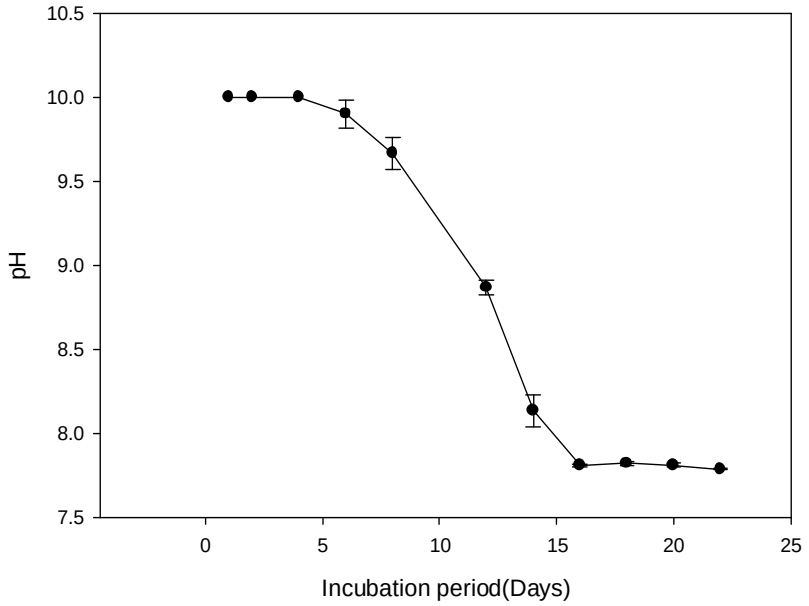


Fig.4.66 Study of biodegradation of asphaltene by pH estimation

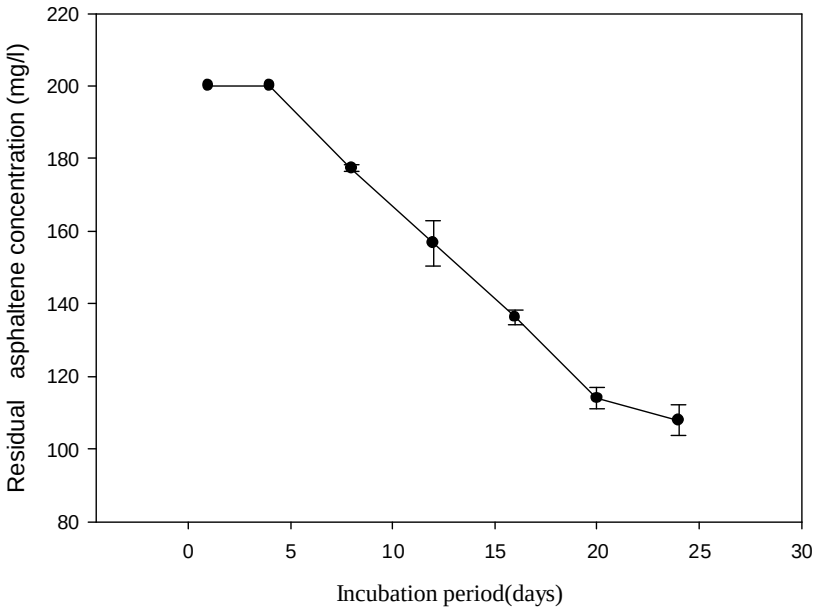


Fig.4.67 Gravimetric analysis of the residual asphaltene after biodegradation by *L.fusiformis* BTTS10

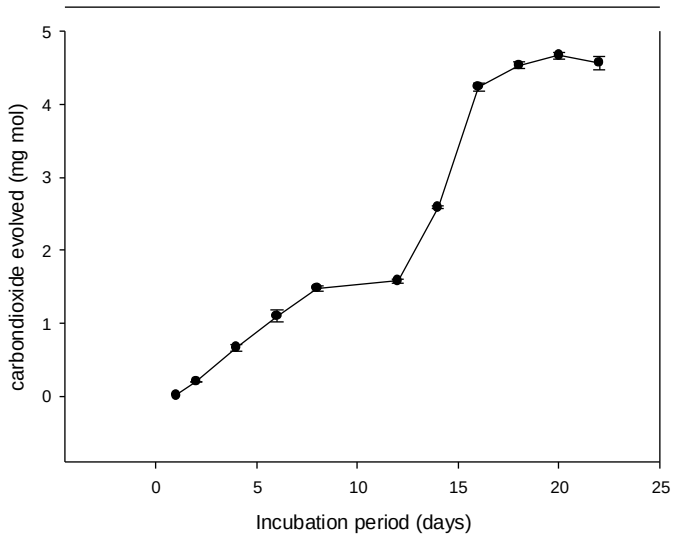


Fig4.68 Respirometric study of biodegradation of asphaltene by *L.fusiformis* BTTS10

Fig.4.69 Biodegradation of asphaltene by *L.fusiformis* BTTS10 in biometric flasks

(Production of carbondioxide during biodegradation of crude oil in a biometric flask containing 100ml Bushnell-Haas medium, inoculated with *L. fusiformis* BTTS10,and 1%(v/v) asphaltene. In the flask labeled biodegraded, dissolution of asphaltene in to the aqueous medium can be visualized).

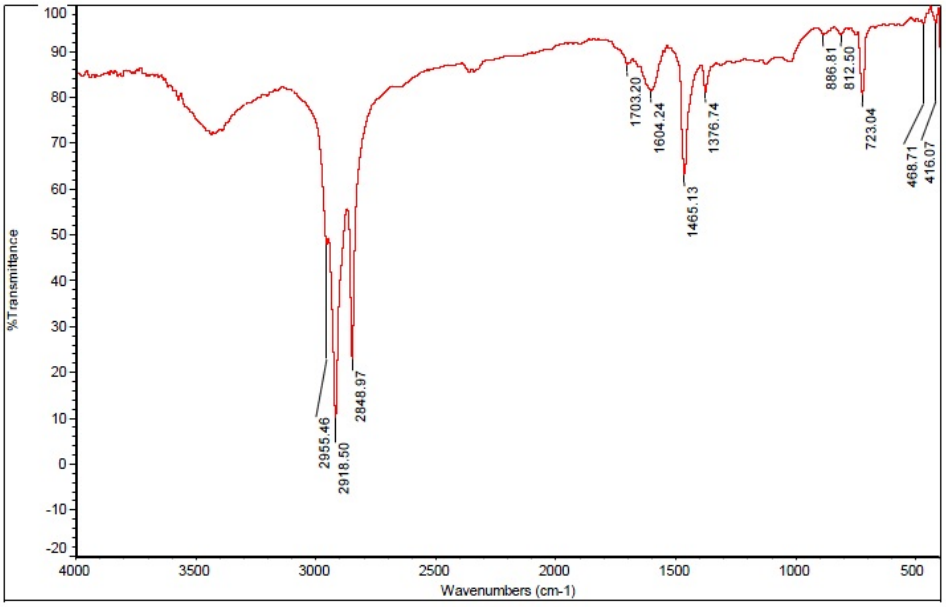


Fig.4.70 FTIR analysis of control asphaltene

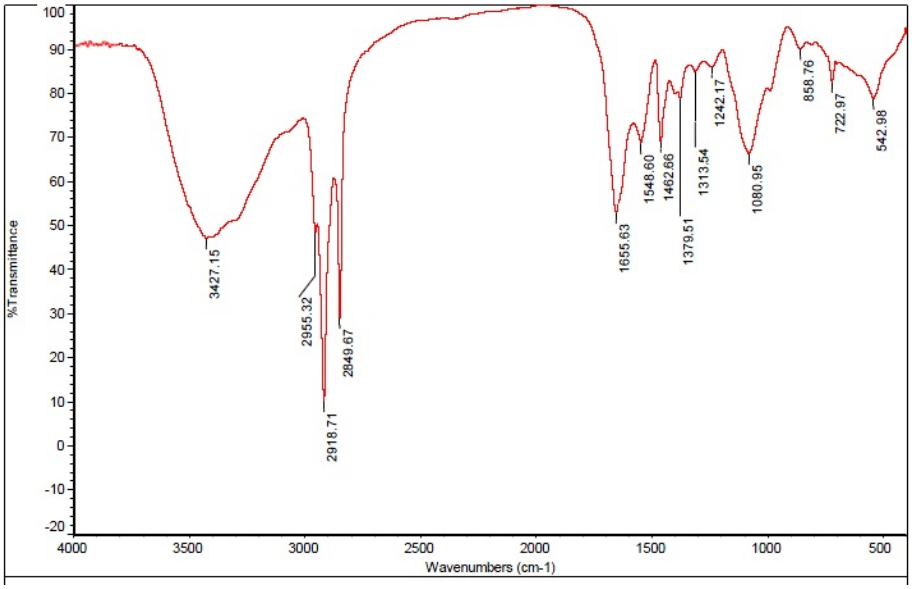


Fig 4.71 FTIR analysis of the biodegraded asphaltene on the 7thday of incubation with *L.fusiformis* BTTS10.

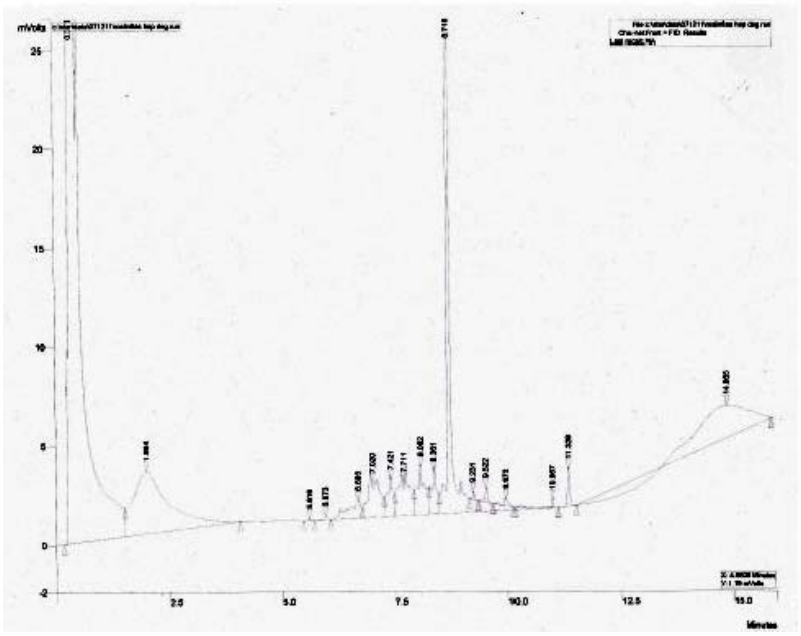


Fig. 4.72 GC analysis of asphaltene control

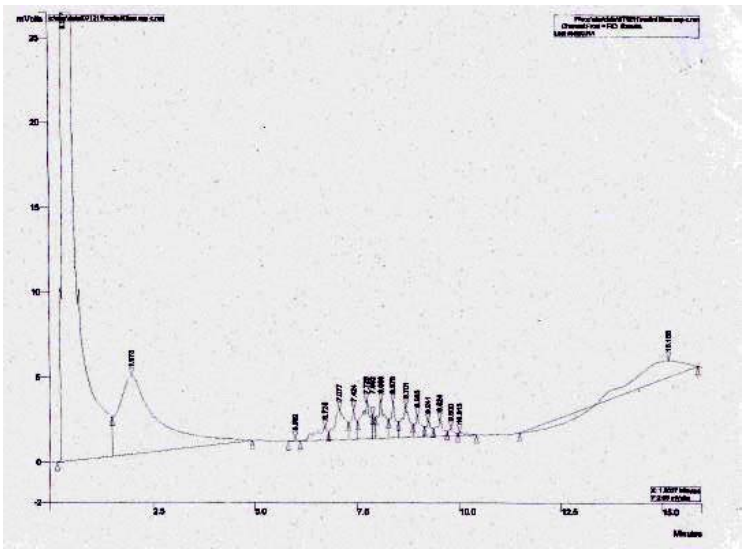


Fig .4.73 GC. analysis of the biodegraded asphaltene on the 14th day of Incubation with *L.fusiformis* BTTS10

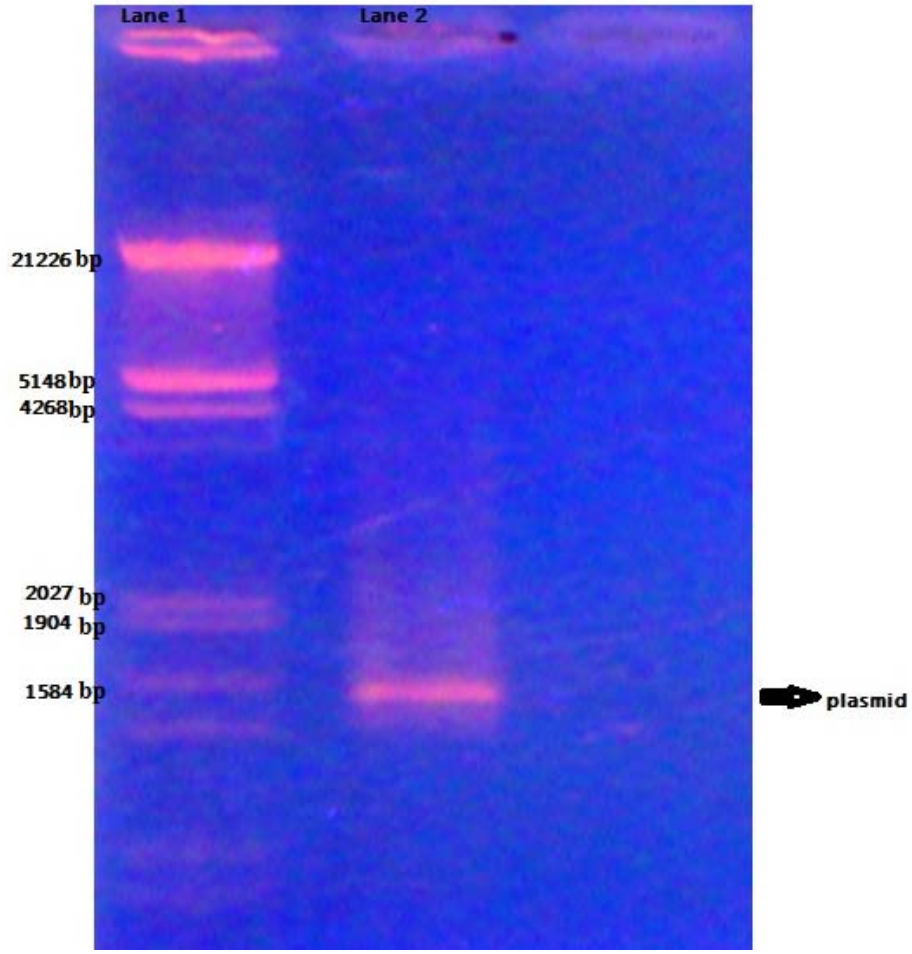


Fig .4.74 Agarose gel showing plasmid isolated from *L.fusiformis* BTTS10.

Lane I, Lamda *DNA E.coRI-Hind* III Double Digest marker.
Lane 2, Plasmid isolated from *L. fusiformis* BTTS10

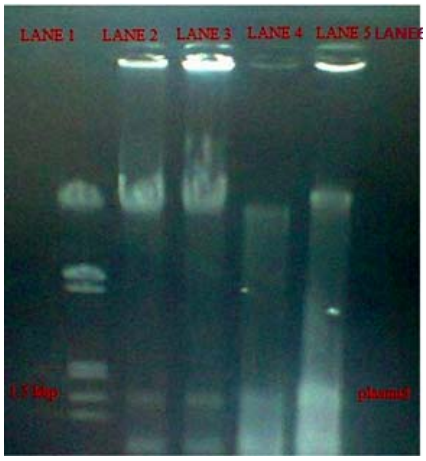


Fig.4.75. Agarose gel electrophoresis of plasmid of *L.fusiformis*, transformed *E.coli* and plasmid cured *L. fusiformis*.

Lane 2. Lamda DNA EcoRI-Hind III Double Digest marker,
Lane 3& 4 plasmid isolated from *L.fusiformis* BTTS10.
Lane4&5 plasmid isolated from transformed plasmid cured *L.fusiformis*

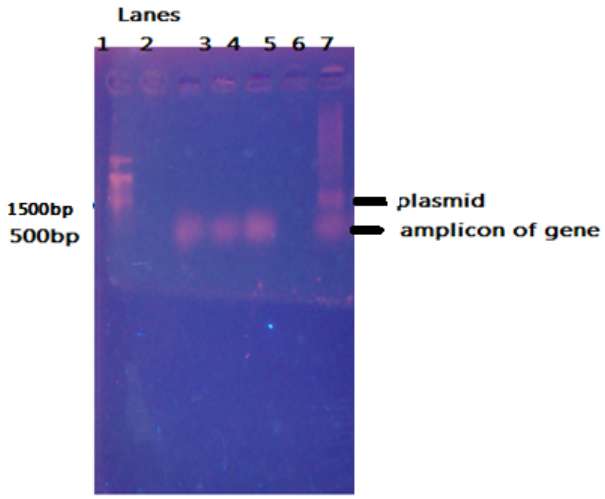


Fig.4.76 Agarose gel electrophoresis of 500bp amplicon of aromatic dioxygenase gene. Lane 1. DNA ladder (EcoR1 digest.), Lane 3, 4& 5. amplicon of aromatic gene, Lane 7. plasmid and amplicon of aromatic gene.

Chapter 5

DISCUSSION

Contents

5.1. Screening, selection, identification and characterization of bacteria with potential for biodegradation of crude oil

Spills and leaks of crude oil hydrocarbons and their fractions from distribution system and storage facilities result in contamination of soil and water ways worldwide. Petroleum is a mixture of thousands of hydrocarbons and includes both biodegradable and recalcitrant compounds. Biodegradation of recalcitrant compound is a significant challenge in effective management of oil pollutants.

Of the various techniques used for remediation, bioremediation based on metabolic activity of microorganism is the most effective and environment friendly technique. Isolation of organic solvent tolerant bacteria is a less explored area in the field of industrial biotechnology. In the presence of organic solvents most bacteria and enzymes are destroyed or inactivated whereas organic solvent tolerant bacteria are extremophilic that can thrive in

the presence of high concentration of organic solvents. These bacteria are being explored for their potential application in environmental and industrial biotechnology for conducting biotransformation.

5.1.1. Screening, selection and identification of a potential bacteria degrading crude oil

Enrichment culture has been used extensively by microbiologists to isolate microorganisms with specific desirable characters in pure culture. This technique has given key information on the metabolic characteristics of thousands of microorganisms. Pure cultures are required to isolate the enzymes involved in degradation of hydrocarbons. In the present study, enrichment culture was used to isolate hydrocarbon degrading bacteria. Three different combinations of hydrocarbons were supplemented in enrichment medium for potential strain isolation viz. equivalent mixture of octane, cyclohexane and hexane, equivalent mixture of benzene, toluene, ethyl benzene and xylene, and crude oil. In the preliminary screening techniques different hydrocarbons, aliphatic and aromatic hydrocarbons were used as sole source of carbon in the mineral salt medium, to find whether the isolate has the potential to degrade wide range of hydrocarbons.

Enrichment of the Bushnell and Hass (BH) medium progressively with hydrocarbon enabled isolation of several hydrocarbon utilizing bacteria and selection of a potential strain capable of degrading hydrocarbons. The isolate was identified as *Lysinibacillus fusiformis* BTTS10. In fact species of *L. fusiformis* is not reported to degrade

hydrocarbons in literature and it is for the first time it is recognized to have ability to degrade crude oil fractions. May be the marine sediments harbor this kind of rare species of bacteria have potential for hydrocarbon degradation.

Results of the present study on characterization of *L. fusiformis* BTTS10 indicated that the bacterium is capable of tolerating and accumulating wide range of heavy metals which included mercuric chloride, cadmium sulphate, copper sulphate, lead nitrate, zinc sulphate and sodium meta arsenate. It was found that the isolate tolerated all the metals and maximum tolerance was observed for zinc sulphate and minimum tolerance for sodium met arsenate. ICP-AES analysis also indicated that the bacteria could accumulate mercury, cadmium, and also arsenic. All these three metals are included in the US Environmental Protection Agency's list of priority pollutants.

A new screening technique was developed using fluorescent Zn-Mn nanoparticles capped with citrulline to screen metal accumulating bacteria. In this method only those bacteria capable of accumulating metal accumulated fluorescent nanoparticles and were visualized in a fluorescent microscope. Interestingly *L.fusiformis* showed positive results and demonstrated rare characteristics for accumulating metals in their cells and in the process exhibited potential for application in bioremediation. Heavy metal removal by bioremediation is an attractive method in comparison with physiochemical methods. (White and Gadd, 1993). There are different mechanisms employed by bacteria for conferring metal resistance. They include precipitation of metals as

phosphates, carbonates and/or sulphides, physical exclusion of electronegative components in membranes, volatilization via methylation or ethylation, energy-dependent metal efflux systems, intracellular and extracellular polymeric substances (EPS) and sequestration with low molecular weight, cysteine-rich proteins (Silver, 1996). Single bacterial strains can be resistant to many metals. Cànovas *et al.,(* 2003) reported that the genome sequence of *P. putida* KT2440 contains 61 open reading frames providing tolerance and resistance to vast variety of metals. *L. fusiformis* BTTS10 is also found to have tolerance to wide variety of metals. The metal accumulation property of BTTS10 was confirmed by utilizing the study of metal accumulation by fluorescent nanoparticles of zinc capped with the amino acid citrulline.

The results of the present study showed that the bacteria could tolerate both aromatic and aliphatic solvents with maximum tolerance for acetone, benzene and octane and minimum tolerance towards dichloromethane among the various organic solvents *viz.* acetone, benzene, toluene, ethylbenzene, dichloromethane, xylene, octane, heptane, hexane and cyclohexane studied at 10% (v/v) concentration. These results strongly indicated the potential of this *L.fusiformis* for application in bioremediation in environment.

The present study also indicated that the bacterium has short generation time capable of demonstrating rapid growth similar to many other species of *Bacillus.* The bacterium was found to be alkaliphilic with preference for pH 10 as optimum for maximal growth and tolerance to pH 13 although it could grow over a pH range of pH6 to pH 12. Similarly the

bacteria was observed to tolerate sodium chloride concentrations up to 800mM although optimum NaCl for maximal growth being 200mM.

The bacteria were observed to have ability to grow well over a wide range of temperatures from 30^{o}C to 55^{o}C. The results suggested that the bacteria are moderately thermophilic in nature. Further the bacterium could utilize all simple sugars as sole source of carbon effectively and preferred inorganic nitrogen sources compared to organic sources except for urea for maximal growth. Probably the bacterium did not prefer complex organic substances such as beef extract, peptone and yeast extract as nitrogen sources due to lack of suitable enzymes for degrading those substances and release nitrogen. Whereas it was observed that the bacterium has high potential for producing amylase that was highly resistant to organic solvents. May be the organism could be explored for possible isolation of solvent tolerant amylase.

5.2. Biodegradation of crude oil hydrocarbon by *L.fusiformis* BTTS10

2, 6-Dichlorophenol indophenol (2, 6-DCPIP) is a redox indicator that detect the oxidation of NADH to NAD+, which is related to hydrocarbon-degradation potential of bacteria. The 2, 6-DCPIP assay has been used in the present study to analyze the range of hydrocarbons utilized by the bacterium. The results were in accordance with the solvent tolerance potential of the bacteria. The least change in blue colour was noticed for dichloromethane. It has been reported that a halogen substituted alkanes are highly recalcitrant (Atlas and Bartha, 2005).

Dehalogenation of the organic compounds takes place only in anaerobic conditions. The microbes will produce dehalogenases and carry out reductive dehalogenation (Suflita *et al.,* 1982; Atlas and Bartha, 2005).

In the present study the biodegradation efficiency was assessed by quantifying the residual hydrocarbons gravimetrically and impact of sodium chloride concentration, inoculum concentration, pH , temperature and agitation. The results showed that there is an absolute requirement for sodium chloride and the optimum concentration of sodium chloride was found to be 0.2M. Further NaCl concentrations above 0.4 M led to a sharp decline in the biodegradation.

A note worthy feature of this bacterium is its alkaliphilic nature. The degradation of crude oil was found at its maximum at pH 10 which got gradually decreased when there was further increase in pH. Below pH 8 there was a sharp decline in the rate of biodegradation. With the increase in inoculum concentration a gradual increase in biodegradation percentage was found and a steady state was reached at 4% inoculum concentration. Liu *et al*.,(2006) reported the enhancement in biodegradation when high concentration of inoculum was added. Increase in agitation also brought about more degradation effect. The enhancement in biodegradation with agitation may be due to the supply of proper aeration and mixing of nutrients since the bacterium is not a producer of surfactant.

The initial pH of the medium was 10 in this experiment. Interestingly, during the progress of incubation to the third day of

incubation the pH of the medium was found to decline sharply and finally reached a pH value of 8.4 on the 6^{th} and 7^{th} day of incubation. The results confirmed the chemical change of the crude oil components brought about by the acidic intermediates formed during biodegradation (Carillo *et al.*, 2011).

Intermediates formed during the course of biodegradation of crude oil were analyzed to infer the mechanism of degradation and nature of the products of biodegradation since knowledge of the intermediates may endorse the process of biodegradation that take place effectively.

Analysis of the biodegradation of crude oil by various analyses including FTIR spectroscopy, gas chromatography mass spectroscopy, gas chromatography, and gravimetry of control and biodegraded crude oil provided sufficient proof for the potential of *L. fusiformis* BTTS10 to effectively degrade both aliphatic and aromatic hydrocarbons. FTIR spectrum of the extract of the biodegraded sample showed bands corresponding to that of alcohols and aliphatic aldehydes. Biodegradation of hydrocarbons always produce carboxylic acid and aldehyde intermediates.

The FTIR spectrum on the 3^{rd}day showed the presence of vibration at1653cm^{-1} representing symmetric C=O stretch of carboxylic acids formed by the biodegradation of hydrocarbons in the crude oil. On the 5^{th} day band at 1731cm^{-1} indicated the presence of aliphatic aldehydes. Oxidations of acids produce aldehydes. Thus presence of vibration corresponding to an aldehyde in the FTIR spectrum is an indication of

hydrocarbon biodegradation. The 7^{th} day spectrum clearly confirmed the biodegradation potential of the bacterium where many peaks got disappeared and the intensity of already existing peaks also got reduced suggesting the degradation of hydrocarbons by the bacterium.

Mass spectroscopic analysis results of biodegraded crude oil also proved the potential of the bacterium to degrade hydrocarbon. In the biodegradation study of crude oil, pristane was added as an internal standard. When the intermediates formed during the degradation of pristane was analyzed using GC MS it was found that there was step by step removal of carbon. The first intermediate formed by oxidation was an alcohol 13-Heptadecyn-1-ol $C_{17}H_{32}O$; then 16 carbon compound, 2-Cyclopropylcarbonyloxydodecane $C_{16}H_{30}O_2$. It is found that a cyclopropyl ring is formed during this step . In the next step, oxidative cleavage of cyclopropyl ring has taken place and new alkenyl compound 3-(Prop-2-enoyloxy) dodecane $C_{15}H_{28}O_2$ was formed with a double bond. These results proved that *L. fusiformis* BTTS10 is capable of degrading long chain hydrocarbons.

Various other oxidation products were also found when crude oil was degraded. They include 1, 2-Benzenedicarboxylic acid mono (2-ethylhexyl) ester $C_{16}H_{22}O_4$, Hydroxylamine, o-decyl, $C_{10}H_{23}NO$, Pentan-2-ol, 4-allyloxy-2-methyl- $C_9H_{18}O_2$ etc. The gas chromatogram of the GC-MS also showed the reduction in the area of the peaks which also confirmed the biodegradation potential of the bacterium.

Gas Chromatographic analysis of the biodegraded and control crude oil also gives additional support to confirm the ability of bacterium to degrade hydrocarbon. The results showed that on the third day of biodegradation new peaks corresponding to long chain hydrocarbons from C_{15} to C_{26} was obtained. These new peaks correspond to the new products formed during the biodegradation process. These results proved the efficiency of *L.fusiformis* BTTS10 to degrade long chain hydrocarbons The results found in the GCMS analysis of degradation of pristane mentioned earlier also confirms the ability of bacterium to degrade complex hydrocarbons. Baker and Herson (1994) reported that degradation of hydrocarbons with long chains from C_{18} to C_{25} proceeds only slowly. In this context isolation of a bacterium capable of degrading complex hydrocarbon is highly useful in the bioremediation of hydrocarbon polluted areas.

5.3 Biodegradation of crude oil hydrocarbon by immobilized cells of *L.fusiformis* BTTS10.

Immobilization of cells is the transfer of cells from a free state to state of localization or confinement in a certain defined region of space with the retention of catalytic activity and viability (Flint, 1987). Marine bacteria are recognized for the ability to colonize and adsorb on solid support (Chandrsekaran,1996). The most widely used immobilization technique is entrapping cells in polymer gels, such as alginate or carrageenan. But there are number of limitations for this technique such as the problem of gel instability, limitations of mass transfer, the

complexity and cost of the process and requirement of sophisticated equipment for the large scale production of gel beads These problems can be overcome by immobilization by passive cell adhesion to economically cheap carriers. (Rajeev *et al*., 2001*)*.

An ideal porous carrier for immobilization of bacteria should have large specific area for the adherence and growth of bacteria, be non toxic and cost effective (Omar *et al.,1990;*Cassidy *et al*.,1995;and Liang *et al.,* 2011). Immobilization by adsorption was adopted for immobilizing viable cells of *L.fusiformis* BTTS10 using *E.crassipes* petiole cuttings and polystyrene beads. High microbial colonization of rods was observed in the electron micrographs of the outer surface of the petiole cuttings as well as on polystyrene beads. The results also showed that the large inter cellular spaces present in the *Eichhornia* petiole provided more area for colonization and diffusion of gases.

The initial pH was 10 in this experiment. The pH measurement showed decrease of pH in the experimental flasks as biodegradation proceeded. This result confirmed the occurrence of chemical change in the crude oil hydrocarbons which was brought about by monoxygenase and dioxygenase enzymes.

Microbial degradation of hydrocarbons produced organic acids, aldehydes and other metabolic products thus causing reduction in pH of the biodegradation medium (Olajide *et al.,* 2010). The results of the study also evidenced the viability of bacterial cells even after 225 days and endorsed the advantages of the technique and application of immobilized

cells. The survival of the bacterial cell is critical to the successful bio augmentation treatments.The amount of residual hydrocarbon was determined by gravimetric analysis and the results showed that biodegradation began only on the third day of inoculation and there was a sharp increase in the biodegradation of hydrocarbons on the fifth day onwards. Maximal degradation up to 96 % was noted on the 7^{th} day as shown by the results of gravimetric analysis.

5.4. Biodegradation of Toluene and Asphaltene.

GC-MS analysis of the biodegraded crude oil adds evidence for the potential of *L. fusiformis* BTTS10 to degrade aromatic hydrocarbon. To further confirm this ability, biodegradation of toluene and analysis of the intermediates was also attempted. Intermediate products of toluene biodegradation studied by GCMS analysis showed the presence orcinol (Dihydroxy toluene) a novel oxidation product at 11.87 min. Two OH groups were added at 1, 3 positions. Dioxygenase enzyme was involved in this reaction. The presence of dioxygenase enzyme was further confirmed by the isolation of plasmid encoding aromatic dioxygenase gene. The orcinol is a reagent used in the estimation of RNA. There is scope for further investigation in this direction of biosynthesis of orcinol in large scale from toluene employing *L. fusiformis* BTTS10.

Precipitation of heavy components of crude oil is one of the major problems involved in oil industries. The precipitation of these compounds causes problems like pollution of ecosystems and the blockage of crude oil extraction and transport. Thermal, mechanical, chemical and magnetic means

have been used to solve these problems .But all the above methods are troublesome and expensive since the complex molecular structured, asphaltenes are resistant to biodegradation causing their accumulation in ecosystems. Only few studies have been conducted for the removal of asphaltene precipitation by biological means (Hoaki *et al.*, 1995.,Tavassoli *et al.*,2011).

In the present study the results indicated that 50% of biodegradation of asphaltene is possible in 25 days if *L. fusiformis* BTTS10 is employed for augmentation. FTIR analysis showed the appearance of new peaks at1655.63 cm^{-1}, which indicated the presence of symmetric C=O stretch which corresponds to carboxylic acid which was formed during biodegradation of the hydrocarbon in the asphaltenes. In the GC analysis disappearance of the existing peaks provide evidence for the biodegradation of the components of asphaltene.

5.5. Molecular studies on *L. fusiformis* BTTS10

Results of the molecular studies conducted with *L. fusiformis* BTTS10 indicated that the bacterium harboured small sized plasmid which may play a critical role in the biodegradation of crude oil fractions. Experiments on curing of plasmids and transformation suggested possible role of plasmids in conferring crude oil biodegradation potential to the bacterium. Growth of the transformants with plasmids isolated from the wild strains in presence of organic solvents confirmed the ability of the bacterium to grow and degrade crude oil and its fractions. These observations strongly

indicate the potentials of this bacterium for possible strain improvement and enhancement of crude oil biodegradation potential.

Chapter 6

SUMMARY AND CONCLUSION

There exists a need for potential microorganism that could facilitate effective bioremediation of crude oil pollutants in the environment. Hence it was desired to isolate a potential bacterium from marine sediment, which often experiences oil pollution and develop a bioprocess for crude oil biodegradation. In the present study the sediment deposits in the beach of Munakkal, Trichur dist, Kerala, collected immediately after the major event Tsunami in 2004 was collected and analyzed by enrichment culture technique towards isolation of potential strains that could degrade crude oil and its fractions. From the results obtained it was found that the sediment deposits harbor several bacteria with potential for degrading hydrocarbons. However, among the strains obtained, isolate no. BTTS 10 showed capabilities for utilizing both alkanes and aromatic hydrocarbons and hence the same was selected for further studies.

Based on the morphological and biochemical characteristics the isolate BTTS 10 was tentatively identified as *Bacillus* sp and the identity was further confirmed by molecular ribotyping based on the results of the partial gene sequence of 16S rRNA gene which was amplified and sequenced. The size of the amplicon was found to be 1500bp. The partial nucleotide sequence of the amplicon of 16S rRNA gene obtained was

submitted to Gene bank (accession number **JN674158**). The partial gene sequence of 16S rRNA of BTTS10 showed 82 % similarity with the other reported 16S rRNA gene sequence of *Lysinibacillus fusiformis* and hence the identity of the isolate BTTS10 is considered as *Lysinibacillus fusiformis.* A phylogram was constructed with the partial gene sequence of 16S rRNA of *Lysinibacillus fusiformis* BTTS10 using CLUSTALW N-J programme.

Growth studies indicated that the bacterium *L.fusiformis* BTTS10 is fast growing and the bacteria pass through exponential phase during 3 to 10 hrs of growth and enter into stationary phase after 12 hrs of incubation. The studies on the effect of incubation temperature on the growth of *L.fusiformis* BTTS10 indicated that the bacterium prefers 40^0C for achieving maximum growth although it could show considerable growth in terms of OD at a wide range of temperature from 30^oC to 55^oC. The strain may be said to be thermo tolerant since it could also show growth at 60^oC.

Studies on the effect of NaCl on the growth of *L.fusiformis* BTTS10 indicated that the optimum NaCl for the growth of bacterium was 200mM. It was observed that the bacterium could grow well only under alkaline conditions with optimum pH 10 and could not grow below pH 6. Nevertheless the bacterium was able to tolerate pH 13.

Growth of the bacterium was observed to be affected by the different carbon sources such that simple sugars could support enhanced growth compared to other carbon sources. Among the various carbon sources tested

the bacterium recorded maximal growth with glucose followed by sucrose, starch, mannitol, fructose, sorbitol, lactose and maltose.

Organic solvents, both aliphatic and aromatic, were observed to serve as source of carbon for the bacterium. However, only methanol could support growth at levels similar to that of control. Whereas, other solvents led to a decline in growth. Nevertheless, more than 50% of growth obtained with control could be achieved with benzene, acetone, octane, butanol, ethanol, heptanes, xylene, hexane, ethyl benzene, cyclohexane and toluene. Moreover Dichlormethane and propanol resulted in near inhibition of growth.

Organic nitrogen sources such as peptone, tryptone, and beef extract and yeast extract, except urea which supported slightly enhanced growth of the bacterium compared to control, were found to play an inhibitory role on growth. Whereas, inorganic nitrogen sources supported growth, which was marginally less than the control. Nevertheless, considerable growth was supported by ammonium nitrate followed by ammonium sulphate and potassium nitrate.

The antibiotic sensitivity studies indicated that the bacterium was sensitive to the 27 antibiotics evaluated for sensitivity/resistance. The bacterium could record resistance only towards 3 antibiotics viz; Nalidixic acid, Nitillin and Co-Trioxazole. Results suggested that bacterium is highly sensitive to various antibiotics produced by other microorganisms.

It was observed that using DCPIP as an indicator of microbial oxidation of hydrocarbons has an advantage since after incubation for 24

hours change of colour was observed to occur from blue to colourless with respect to all the solvents, except in the case of dichloromethane and propanol. Methanol and acetone were found to got decolorised very quickly after 12 hrs. Whereas, the hydrocabons with short carbons got decolorised faster than long chain hydrocarbons. Nevertheless in the case of dichloromethane and propanol slight change in color was observed after 31hrs.

The bacterium was found to have significant level of tolerance to the various metals studied at 1M concentration. The results indicated that zinc sulphate, copper sulphate, lead nitrate, mercuric chloride and sodium meta arsenate could support considerable levels of growth.

Ability to accumulate L-citrulline capped ZnS:Mn nanoparticles by *L.fusiformis* BTTS10 was confirmed by observation under fluorescent microscope. Ability to accumulate metals through biosorption by cells of *L.fusiformis* BTTS10, during growth in the presence of cadmium, mercury and arsenic was confirmed by ICP- AES analysis.

The biodegradation of crude oil was studied by growing *L.fusiformis* BTTS10 in mineral salt medium supplemented with crude oil as the sole source of carbon. Bacteria were observed to grow by assimilating the carbon present in the crude oil for their metabolism and in the process biodegradation of crude oil occurred. The results obtained indicated that crude oil began to dissolve in to the aqueous medium from the third day onwards and the viscosity of the medium was found to be greatly enhanced on the 5^{th} day. However, as the biodegradation progressed into the 7^{th} day the viscosity was found to get decreased

significantly. It was also found that there was a reduction in pH concomitant with the degradation of crude oil.

The biodegradation potential of the isolate was also confirmed by estimating the rate of respiration in a specially designed biometric flask, since CO_2 is released when respiration occurs**.** The maximum production of carbon dioxide took place from 5^{th} day onwards which is in agreement with the other data that indicated biodegradation efficiency of the bacterium.

The optimum NaCl concentration for effective biodegradation of crude oil was 200 mM and the percentage of biodegradation declined along with increase in the concentration of NaCl. The optimum pH for effective biodegradation of crude oil was pH 9.5 although about 80% of degradation could be observed at pH 8 and pH 10. At other pH conditions, the rate of biodegradation was very much less.

The variation in the concentration of inoculum was found not to have any profound influence on the rate of biodegradation of crude oil. Depletion of nutrients in the medium may be the reason for the steady state of biodegradation after increasing inoculum concentration above 4% (w/v). Agitation rate was observed to have impact on the rate of biodegradation of crude oil. Agitation of 110 rpm was required for the efficient biodegradation of crude oil and 120 rpm was found to be optimum for maximal biodegradation by *L.fusiformis* BTTS10. Agitation of the medium increases the availability of oxygen and enhances the mixing of nutrient and bacteria with hydrocarbons

Crude oil is a complex mixture of hydrocarbons consisting of saturates, aromatics, resins and asphaltene (SARA). These different components were separated by column chromatography. It was found that saturates (52%) formed major component of crude oil followed by aromatics (21%), asphaltene (18% and resins (9%). The FTIR analysis of samples obtained on the 3rd day showed the presence of vibration at 1653cm^{-1} indicating the presence of symmetric C=O stretch of carboxylic acids formed by the biodegradation of hydrocarbons in the crude oil. Vibration 2924 cm^{-1} represents antisymetric CH_2 stretch of *n* –Alkanes and 2854 cm^{-1} represents symmetric CH_2 stretch of *n*-Alkanes**.** On the 5th day of vibration 1731 cm^{-1} is present which represents the accumulation of aliphatic aldehyde which might have been formed from the oxidation of carboxylic acids

Results obtained in terms of gas chromatogram for the biodegradation of crude oil by *L.fusiformis* BTTS10 after GC analysis of samples evidence that *L.fusiformis* BTTS10 is a potential strain with ability to degrade crude oil.

Gas chromatographic analysis of crude oil after five days of incubation with *L.fusiformis* BTTS10 showed the appearance of 5 large peaks indicating the formation of 5 new hydrocarbons with retention time viz.5.92, 6.94, 6**.**76, 8.12, 8.39, 8.42, 8.817, 9.940. From the star data available it was found that retention time 5.9 corresponds to hydrocarbon with chain length 15 carbons, 6.94 to 18 carbon compound, 8.12 for 21 carbon and 9.94 to 26 carbon containing compound. The chromatogram shows decrease in the area of peaks in the chromatogram as degradation

proceeded when compared with control. More degradation has taken place for long chain hydrocarbons as evident from the chromatogram. Gas chromatographic analysis of crude oil on the 7^{th} day of incubation indicated that all new peaks found on the 5^{th}day disappeared and complete degradation of crude oil took place

GC-MS analysis of biodegraded crude was done to analyze the intermediates of crude oil degradation. Mass spectra data bases (NIST) indicated that several intermediates are formed during the course of biodegradation of crude oil. Pristane added as an internal marker was converted into corresponding alcohol. 13-Heptadecyn-1-ol $C_{17}H_{32}O$; in the next step removal of one carbon has taken place and a 16 carbon compound, 2-Cyclopropylcarbonyloxydodecane $C_{16}H_{30}O_2$ was formed. It was found that cyclopropyl ring was formed during this step. In the subsequent step, oxidative cleavage of cyclopropyl ring has taken place and new alkenyl compound 3-(Prop-2-enoyloxy) dodecane $C_{15}H_{28}O_2$ was formed. All the other intermediates formed are oxidation products of alkanes and aromatic compounds.

A study on the biodegradation of toluene was done and it was then analyzed by GC-MS. The peak at 11.2 min found in the chromatogram of GC-MS of the extract of biodegraded toluene showed the formation of orcinol (3, 5 dihydroxy toluene) as an intermediate. Orcinol was formed by the oxidation of toluene by the dioxygenase enzyme.

Biodegradation of crude oil was evaluated using viable cells immobilized on two different substrates namely *E.crassipes* petiole cuttings and polystyrene beads. Biodegradation of the crude oil was

monitored by gravimetric analysis and depletion of pH in the biodegradation medium. Scanning Electron Microscopic (SEM) examination of immobilized viable bacterial cells testified high microbial colonization of rods on the outer surface of the petiole cuttings and polystyrene beads.

Asphaltenes are petroleum hydrocarbons with extremely complex molecular structure, which are resistant to biodegradation. Potential of bacterium to degrade asphaltene was also attempted. The results indicated a reduction in the pH of the medium during biodegradation which was brought about by the release and dissolution of components of asphaltene degradation by the bacterium. Gravimetric and respirometric study of biodegradation of asphaltene testified the potential of this bacteria to utilize asphaltene as sole source of carbon. Gas chromatographic and FTIR analysis of biodegraded asphaltene further testified the above inference.

Molecular genetic study of *L.fusiformis* BTTS10 was done by isolating a 1.5Kbp plasmid from the bacterium,then *curing* plasmid from it and transforming the plasmid in to *E.coli* DH5α. Transformants were selected by growing the bacterium in presence of organic solvents. A degenerate primer was used for isolating aromatic dioxygenase gene of ~500bp from the plasmid and confirmed the role of plasmids in biodegradation of hydrocarbon.

The results obtained from the present study strongly indicate that the *L.fusiformis* BTTS10 has the potential to degrade both aromatic and aliphatic hydrocarbons in crude oil. The results showed that this

bacterium has the potential to degrade recalcitrant asphaltene too. It was also found that the bacterium has the potential to tolerate and accumulate heavy metals like mercury, arsenic, cadmium and zinc.

In this context it is strongly suggested that the bacterium *L. fusiformis* BTTS10 can be used for the bioremediation of hydrocarbon and heavy metal polluted environment. Further, the study proved the ability of bacteria to tolerate high concentration of different organic solvents. Isolation of organic solvent tolerant bacteria is a less explored area in the field of industrial biotechnology. In the presence of organic solvents most bacteria and enzymes are destroyed or inactivated whereas organic solvent tolerant bacteria are extremophilic that can thrive in the presence of high concentration of organic solvents.

L.fusiformis BTTS10 can be further explored for its potential application in environmental and industrial biotechnology for conducting biotransformation. It was observed that the bacterium has high potential for producing amylase that was highly resistant to organic solvents. May be the organism could be explored for possible isolation of solvent tolerant amylase. Application of fluorescent Zn-Mn nanoparticles capped with citrulline for screening metal accumulating bacteria is a new technique developed in the present study. This technique opens up new frontiers in the bioremediation of heavy metal contaminated sites

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1. Roselin Alex and Sarita G.Bhat(2012):Isolation and characterization of a solvent tolerant alkaliphilic marine bacteria,International Journal of Scientific and Engineering Research,vol**3,1ssue 8 (1-3)**.
2. Sajimol Augustine,M., Roselin Alex, V.G Sreevalsa, G.D. Deepa, Sarita.G Bhat and S.Jayalekshmi. Highly fluorescent, bio compatible, L-citrulline capped ZnS:Mn nanocrystals for screening metal accumulating *Lysinibacillus fusiformis* BTTS10. (Submitted for final review). Journal of Luminescence. (Elsevier).
3. Roselin Alex, Bindya E.S, Jeena. K, Sarita G.Bhat, M.Chandrasekaran, Biodegradation of crude oil by *Lysinibacillus fusiformis* BTTS10 immobilized on the petiole of aquatic weed *Eichhornia crassipes* (Communicated).

APPENDIX

APPENDIX .1

Bushnell and Haas medium

Magnesium Sulfate .....0.2 g/l

Calcium Chloride .....0.02 g/l

Monopotassium Phosphate..... 1.0 g/l

Diammonium Hydrogen Phosphate .....1.0 g/l

Potassium Nitrate .....1.0 g/l

Ferric Chloride .....0.05 g/l

APPENDIX-2

TE buffer.

10 mM Tris-Cl, pH 7.5

1 mM EDTA