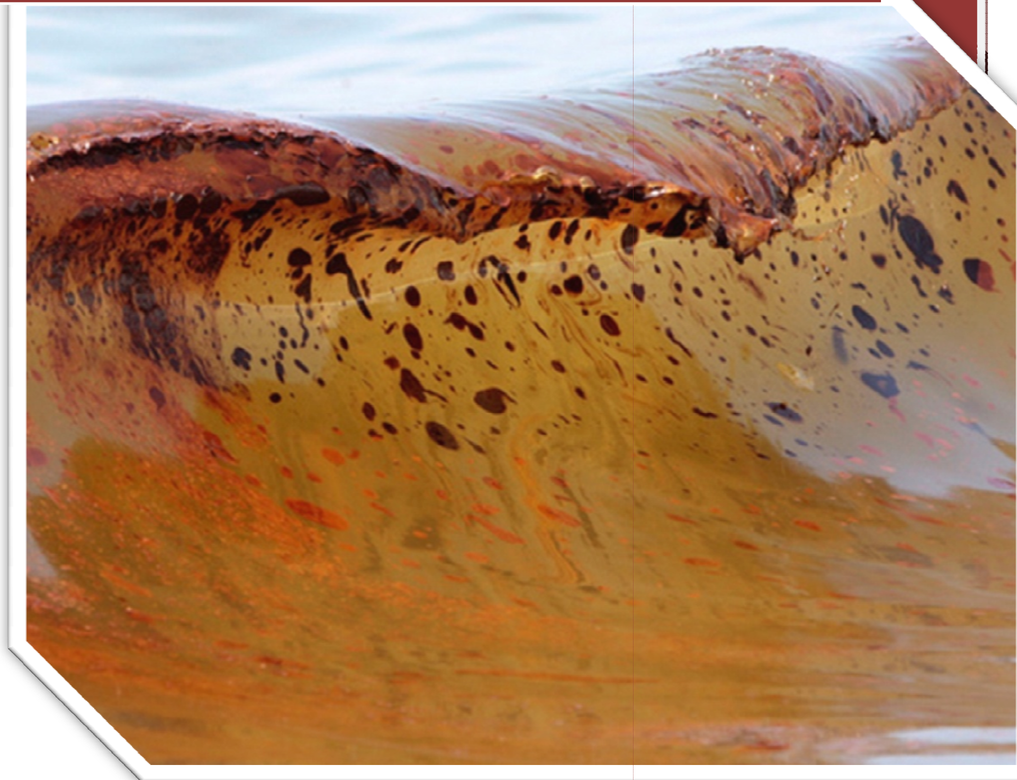
2012

BIODEGRADATION OF PETROLEUM HYDROCARBONS



Thesis submitted in partial fulfillment of the requirement for the degree of

Master of Technology (Research)

In

Chemical Engineering

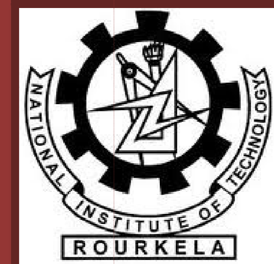
by

Vamsi Krishna Garapati

(Roll No – 609CH309)

Under the guidance of

Dr. Susmita Mishra



NATIONAL INSTITUTE OF TECHNOLOGY ROURKELA, ODISHA

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Department of Chemical Engineering
National Institute of Technology
Rourkela, Odisha – 769008
January 2012

Department of Chemical Engineering
National Institute of Technology, Rourkela
Odisha, India – 769008

CERTIFICATE

This is to certify that the thesis entitled '**Biodegradation of Petroleum Hydrocarbons'** submitted by **Vamsi Krishna Garapati** is a record of an original research work carried out by him under my supervision and guidance in partial fulfillment of the requirements for the award of the degree of **Master of Technology by research** in **Chemical Engineering** during the session January'2010 – January'2012 in the Department of Chemical Engineering, National Institute of Technology, Rourkela. Neither this thesis nor any part of it has been submitted for the degree or academic award elsewhere.

Dr. Susmita Mishra
Department of Chemical Engineering
National Institute of Technology, Rourkela

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Vamsi Krishna Garapatati

Dedicated to

My Parents...

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Nomenclature

% D	Percentage Degradation
Δ_{max}	Global Maximum
Δ_{min}	Global Minimum
γ	Overall Degree Of Standardized Deviation
ξ_i	Gray Relation Coefficient
ξ	Distinguished Coefficient
E_{24}	Emulsification Index at 24 Hours Time
W	Wave length (nm)
V_{max}	Maximum Reaction Rate
K_m	Substrate Concentration

Abbreviations

ANOVA	**AN**alysis **O**f **V**ariance
API	**A**merican **P**etroleum **I**nstitute
BS	**B**io**s**urfactants
CMC	**C**ritical **M**icelle **C**oncentration
CTAB	**C**etyl**t**rimethyl**a**mmonium **B**romide
DoE	**D**esign **o**f **E**xperiments
DO	**D**issolved **O**xygen
FTIR	**F**ourier **T**ransform **I**nfra**r**ed spectroscopy
GC	**G**as **C**hromatography
GRC	**G**rey **R**elational **C**oefficient
GRG	**G**rey **R**elational **G**rade
HC	**H**eavy **C**rude oil

HEPA	**H**igh **Ef**ficiency **P**articulate **A**ir
HPCL	**H**industan **P**etroleum **C**orporation **L**imited
LC	**L**ight **C**rude oil
MSM	**M**inimal **S**alts **M**edia
MTCC	**M**icrobial **T**ype **C**ulture **C**ollection and Gene bank
NAD	**N**icotinamide **A**denine **D**inucleotide
NADP	**N**icotinamide **A**denine **D**inucleotide **P**hosphate
NIT	**N**ational **I**nstitute of **T**echnology
OVHA	**O**rissa **V**oluntary **H**ealth **O**rganization
OD	**O**ptical **D**ensity
ONGC	**O**il and **N**atural **G**as **C**orporation
OTA	**O**ffice of **T**echnology **A**ssessment
OS	**O**il **S**ludge
RL	**R**hamno**l**ipids
RSTPC	**R**ushikulya **S**ea **T**urtle **P**rotection **C**ommittee
SEM	**S**canning **E**lectron **M**icroscope
ST	**S**urface **T**ension
TPH	**T**otal **P**etroleum **H**ydrocarbon

Abstract

Biodegradation of hydrocarbons in the environment is the natural way of cleaning the nature. The potential biodegradation of hydrocarbon contaminants by microorganisms is dependent on the environmental factors and the nutrients available. In this study culture conditions like temperature, p^H, and nitrogen source were optimized by conventional one-factor at a time experimentation and the combination of other nutrients (nitrogen, phosphorus, magnesium, and sulfur) was optimized by using design of experiments (DOE) combined with grey relational analysis (GRA). Total petroleum hydrocarbons of oil sludge, light crude oil and heavy crude oil, degradation was studied for a period of thirty days using microbial strains isolated from the hydrocarbon contaminated sites. They have shown predominant results in the degradation of TPH's under optimized culture conditions and prior addition of biosurfactants in the culture flask has enhanced the degradation process and microbial biomass yield.

Key words: Biodegradation; hydrocarbons; microorganisms; design of experiments; grey relation analysis; oil sludge; light crude oil; heavy crude oil; biosurfactants.

Chapter-1

Introduction

1.1. Introduction:

The Spilled petroleum constitutes (Hydrocarbons) are one of the main environmental pollutants. Their abundance and persistence in several polluted environmental areas have been reported [Mohammed. et al, 2004]. Oil spillage may be caused by natural disasters like earthquakes in the sea surface or due to accidental leaks during exploration, refining, storage and transportation. The causes can be numerous but the consequences are the same. In case of crude oil, the different types such as heavy or light crude oil can affect the clean-up procedures. Crude oil spreads very rapidly on the sea surface and after a short period of time the thickness of the oil film can be at least 1mm. It is therefore necessary to prevent the spreading to reach the shoreline. Once it reaches the shoreline, it contaminates the soil and cause a great damage to the soil ecosystem as well [Web Ref.1].

Some of the largest oil spills that have taken place all over the world are at Iran, Iraq, Persian Gulf, Uzbekistan, Kuwait, United States, Gulf of Mexico, Pakistan and even in India. The most recent oil spill in India is the spill of ONGC Trunk oil pipeline burst, at Uran, Mumbai on 21st January 2011. Around 40 to 45 metric tons of oil was spilled in Mumbai coast that spread around 4 sq km area [Web Ref.2]. Many such disasters reported one seeping oil from a sinking ship [Web Ref.3]. In August 2010, oil spill at Gopalpur beach Orissa in April 2010 causing the death of many Ridley turtles that breed there during that period [Web Ref.4]. It hence becomes a necessity to clean up this oil in order to save the marine life.

Clean-up and recovery of hydrocarbons from an oil spill is difficult and the strategies for cleaning up an oil spill are greatly affected by a variety of factors such as the type of oil spilled, the temperature of the water body, and the types of shorelines and beaches involved [Web

Ref.5]. A number of approaches and technologies have been developed for spreading of oil spills in marine shorelines and freshwater environments. Many mechanical and chemical methods (Table.1.1) can be applied to clean-up the spills [Zhu et al., 2001].

Table.1.1: Methods applied in cleaning of oil spills

Cleaning Methods	Example Technology
1. Physical methods	• Booms. • Skimmers. • Manual removal (Wiping). • Mechanical removal. • Washing. • Sediment relocation or Surface washing. • Tilling. • In-situ burning
2. Chemical methods	• Dispersants. • Demulsifies. • Solidifiers. • Surface film chemicals.

Although various conventional cleaning methods such as physical methods like the first response selection, rarely achieve complete cleanup of oil spills. According to the Office of Technology Assessment [U.S. Congress,1991], current mechanical methods typically recover no more than 10-15 percent of the oil after a major spill. Chemical methods are faster in action but they are toxic to the biota present in the spill environment. Bioremediation has emerged as one of the most promising alternative treatment options for oil removal since its successful application after the 1989 Exxon Valdez spill [Swannell. et al., 1996] [Venosa, 2003]. Bioremediation is a method where microorganisms are used for the clean-up process. Bioremediation has been

defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" [U.S. Congress, 1991].

Bioremediation of oil spills requires the identification of microbes which have the ability to degrade hydrocarbons present in the soil or water, so that in case of a large spill these can be stimulated further in order to clean-up the area. Identification of such strains can ensure better efficiency of remediation as these strains will be well adapted to grow in the respective environment. A number of microorganisms have been found to have the capability to degrade the hydrocarbons from the oil spills. These include strains of bacteria, fungi, yeast, algae etc [Kazuya, 2001].

Bioremediation oil spills involve highly heterogeneous and complex processes. Its application can be limited by the composition of the oil spilled. Bioremediation is a relatively slow process and requires some weeks to months which may not be feasible when immediate cleanup is demanded. The degradation of oil spills by microorganisms mainly depends on the type of microbial species, and its efficiency for the degradation under favourable environmental conditions. Concerns also arise about potential adverse effects associated with the application of bioremediation agents [Prince et al., 1994]. To enhance the bioremediation process addition of nutrients (N and P) were very essential [Wang, 2011]. To identify the adequate amount of nutrients, the optimization study in laboratory has to be carried out. Nutrients are utilised by the microorganisms for the synthesis of organic compounds (Proteins) and for intracellular metabolism.

In biodegradation process, microorganisms consume hydrocarbons as a carbon source. Carbon dioxide and water are released as by products. Microbes which can utilize hydrocarbons, are found naturally in the marine habitats; however, they grow and develop after an oil spill

occurs because additional carbon source (hydrocarbons) are available by following a spill [Harayama et al., 1999]. Biodegradation of petroleum hydrocarbon in the environment is found to be comparatively slow because it is influenced by a number of factors which include the microbial community which degrades the hydrocarbons, temperature and availability of nutrients [Ekpo and Udofia, 2008].

Environmental factors like temperature and pH were considered as important factors as they have a significant effect on the rate of microbial growth and hence on the degradation [Tripathi and Srivastava, 2011]. Temperature is an important factor for biodegradation of petroleum hydrocarbons as it alters the chemical composition and physical nature of the oil, rate of hydrocarbon metabolism by microorganisms, and the population of the microbial community [Atlas, 1981]. The optimum temperature range for biodegradation of hydrocarbon depends on the climatic conditions, sampling sites and type of strains isolated etc. Even though biodegradation of hydrocarbon can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature [Desai and Vyas, 2006].

To enhance the bioremediation process, the microbial population present in the polluted environment is to be stimulated. Bio-stimulation involves the addition of nutrients to increase the rate of biodegradation process. In most shoreline ecosystems that have been highly contaminated with hydrocarbons, nutrients are likely the limiting factors in biodegradation of oil [Evans and Furlong, 2003]. Inadequacy in nutrient concentration affects the biodegradation process. Hence, addition of appropriate nutrients in sufficient quantity is very essential to promote or enhance the microbial growth and thus the biodegradation. For the optimization of nutrients different methods were reported and Taguchi Method is one of the predominant techniques used recently.

Optimization by Taguchi method using orthogonal array (OA) experimental design (DOE) involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels) [Tripathi and Srivastava, 2011]. This approach not only helps in extensive saving in time but also leads to more fully developed process by providing systematic, simple and efficient methodology for the optimization of the near optimum design parameters with only a few well defined experimental sets [Rao et al., 2008].

The selection process of nutrients is based on microbial growth response towards the different nutrient concentration, so the ranking determines the combination of different nutrients which yields the greater biomass. The numeric score for each nutrient will be weighed by a factor, and then summing all scores together. As a result, the highest graded combination would imply the best combination [Tsai et al., 2003]. Hence, the selection of the best combination of nutrients in this traditional method is based on the response of microbial biomass and TPH concentration, which belongs to multiple attributes decision problems. Grey relational analysis in the Grey theory is a simple and accurate method for multiple attributes decision problems [Tsai et al., 2003], especially for those problems with very unique characteristics. Therefore, this study will utilize the Grey relational analysis to establish a complete and accurate evaluation model for selecting appropriate combination of nutrients. This methodology will yield higher microbial biomass with higher TPH degradation efficiency [Tsai et al., 2003].

Here we reported the use of statistical design of the experiments combining with Grey relational analysis (GRA) to reduce the total number of attempts for optimizing the nutrients, and we expected that this statistical tool will derive the best nutrient combination for better growth of fungal biomass with higher TPH degradation efficiency. Statistical design of experiments determines the important effects of factors on a response as well as the interaction effects among

the factors [Rao et al., 2008]. Although statistical experimental design with a combination of GRA has largely been employed in the optimization process parameters in grinding, turning operations and welding works in mechanical industry, it has been rarely applied to bioprocesses [Chung et al., 2008].

The rate of biodegradaction depends on the availability of hydrophobic contaminants i.e, hydrocarbons, to the microorganisms. Microorganisms produces organic compounds such as *Biosurfactants* to emulsify the hydrocarbons. Increase in concentration of biosurfactant in the oil spilled site enhances the emulsification of hydrocarbons which results in improved interaction between the microorganisms and hydrocarbons [Amaral et al., 2010]. This study proposes a degradation of petroleum hydrocarbons using indigenous microorganisms isolated from the hydrocarbon contaminated sites.

1.2. Objectives:

The objective of this study is to develop a new technology to clean up the oil spills in the petrochemical industry effluents and other aqueous environments using microorganisms.

1.2.1. Specific objectives:

- Isolation of microorganisms from hydrocarbon contaminated sites.
- Identification of isolated microorganisms by means of morphological, physiological and biochemical characterization tests.
- Optimization of process parameters to enhance the biodegradation process.
- Performing the total petroleum hydrocarbons (TPH) biodegradation study by using different petroleum oils like oil sludge, light crude oil and heavy crude oil.
- To study the effect of biosurfactants on the biodegradation process.

1.3. Scope of the Study:

This study shall provide a better solution for bioremediation of spilled petroleum hydrocarbons in soil and water ecosystems. It will provide an idea on distribution of microorganisms in the environment which have the ability to degrade the hydrocarbons and investigation of the response of microorganisms towards different petroleum oils. It will also emphasize the effect of biosurfactants on the rate of biodegradation processes.

1.4. Organization of Thesis:

The thesis has been organized in eight chapters. *Chapter-1* is an introductory chapter. *Chapter-2* contains the detailed literature review on various topics related to the present work. *Chapter-3* includes all the materials and methods involved in the work. *Chapter-4* describes the details of isolation of microorganisms from samples collected from various hydrocarbon contaminated sites and characterization of isolated strains. *Chapter-5* deals with the optimization of microbial growth parameters such as temperature, pH and nitrogen source were optimized by following the traditional way of one factor at a time approach and estimation of requisite concentration of nutrient salts used for the preparation of nutrient media in the study of TPH degradation using isolated microorganism was performed using design of experiments (Grey based Taguchi). *Chapter-6* deals with the separation and characterization of biosurfactants produced by the isolated microorganisms. *Chapter*-7 explains the degradation of TPH present in petroleum oil (oil sludge, light crude oil and heavy crude oil by the isolated microorganisms. *Chapter-8* contains conclusions and future perspective.

Chapter-2

Literature Review

2.1. Summary on Hydrocarbons:

Hydrocarbons are naturally occurring, flammable organic compounds in the crude oil found in geologic formations beneath the Earth's surface. Hydrocarbons contained in crude are categorized on the basis of molecular composition as alkanes, naphthenes, aromatics, and alkenes [Scholz et. al., 1999] (Figure 2.1).

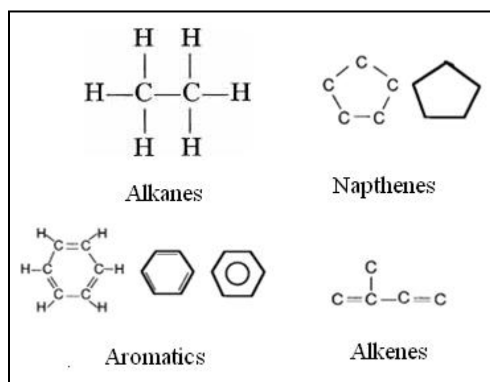

Figure 2.1: Molecular structures of hydrocarbons.

- **Alkanes:**

Alkanes are also called Paraffin's or Saturated hydrocarbons, with a chemcial formula $C_nH_{(2n+2)}$. Alkanes are characterized by branched or unbranched chains of carbon atoms with bonded hydrogen atoms and contain only single carbon-carbon bonds with out any double or triple bonds between carbon atoms [Web Ref. 14].

- **Naphthenes:**

Napthenes are also called Cycloalkanes, with a chemical formula of C_nH_{2n}. Napthenes are similar to alkanes, but are characterized by the presence of one or more rings of carbon atoms in the chemical structure. Napthenes are generally stable and relatively insoluble in water.

- **Aromatics:**

Aromatics are the hydrocarbons that contain alternating double and single bonds between carbon atoms. They are known to be the most acutely toxic component of crude oil, and are also associated with carcinogenic and chronic effects. Many low weight aromatics are water soluble, incerasing the potential for exposure to aquatic resources. The term 'aromatic' was coined before the physical mechanism determining aromaticity and was derived from the fact that many of the compounds have a sweet scent [Web Ref. 6]. They are well known by the number of rings, which ranges from one to six. Aromatics with two or more rings are known as polycyclic aromatic hydrocarbons.

- **Alkenes:**

Alkenes are also called Olefins or Isoparaffins, with a chemical formula of $C_nH_{(2n-2)}$. Alkenes are characterized by branched or unbranched chains of carbons atoms, similar to alkanes excluding for the presence of double-bonded carbon atoms. Aromatic compounds are often drawn as cyclic alkenes, but their structure and properties are different and they are not considered to be alkenes. Alkenes are not generally found in crude oils, but are common in refined products, such as gasoline.

2.2. Hydrocarbons of Crude oil:

Oils are further categorized into three broad groups, according to their molecular weight. General statements can be made for each of the three categories namely light weight, medium weight, and heavy weight components [Scholz et. al., 1999]. Crude oils are composed of various combinations of these three categories with the following general characteristics:

2.2.1. Light Weight Components:

These are carbon atoms range from C_1 to C_{10} which are smaller molecules with few numbers of atoms. They are characterized by high volitility, readily dissolve and evaporate and leave little or no residue because of there short residence time. Many of these components (e.g., benzene, and toluene) are thought to be more bioavailable to animals by primary exposure route (respiratory system). Being highly flammable and readily inhaled, and therefore are of concern for human health and safety.

2.2.2. Medium Weight Components:

These are carbon atoms rangeing from C_{11} to C_{22} which have complex molecules. It has low rate of evaporaton and dissolves very slowly that take several days with some residue remaining. Not as bioavailable as lower weight components, it is less likely to affect aquatic animals. Its primary exposure route is respiratory system and gets readily absorbed through skin.

2.2.3. Heavy Weight Components:

These are components which carbone atoms more than $C_{23.}$ It has the longest residence time with very little loss due to evoparation or dissolition. It can cause chronic effect through smothering as residue in the water column and sediments (tarballs, etc). Its primary exposoure route is direct topical contact. Some heavy weight components contain carcinogens that are absorbed through the skin. Its risk of exposure is increased due to long residence time, probability of contact, and adsorpiton property of the oil components.

2.3. Oil Spills and Their Effects:

- Recently on 21st January 2011, ONGC trunk oil pipeline burst at Uran, Mumbai. About 40 to 45 metric tons of oil was spilled and spread around 4 sq km area [Web Ref. 10]. On 7th August, 2010 a foreign cargo ship, collided with another vessel about 10 km off Mumbai harbor, that spilled the oil in spite of the hectic effect made by Navy and Coast Guard to contain the leak, around 700 tons of fuel oil and 28 tons of diesel oil was spilled [Web Ref. 9]. Thousands of eggs of the endangered *Olive Ridley* Turtles on the beach adjoining river Rushikulya in Ganjam district have failed to hatch this year because of the recent 924 tons of furnace oil spill at a nearby port. Rushikulya Sea turtle protection committee have found that the hatching of the eggs, which ended last week, have gone down by 40 to 50 percent this year as the eggs, numbering thousands, got spoiled after the leaked oil soaked the nesting site on the beach [Web Ref. 11]. A number of incidents have been reported happening every year. Some of the oil spill incidents are listed in the Table 2.1 [Web Ref. 1].
- Oil spills kill wildlife and damage the ecosystem that can last for generations by forcing changes in reproduction and compromising complex food webs. Oil spill damage to the waterways of birds and animals, destroys the animal immune systems, interrupt breeding and fouling of breeding grounds. Besides, they thin bird and turtle egg shells and also damage the fish larvae, causing deformities. They cause damage to sea grass beds and other shelter/feeding areas, tainting of algae, which perform a vital role in waterway ecosystems [Web Ref. 7, 8].
- Due to oil spill on soil the insects and worms living in it are killed due to hydrocarbon toxicity, lack of oxygen supply and it reduce the p^H of the soil. This affects the fertility of soil and its productivity in terms of the growth of plants [Wokocha, 2011]. As a result soil eco

system is getting disturbed and it effects the plant growth. Soil oil also contaminates ground water and it causes illness to the human beings and animals when consumed.

Table 2.1: List of oil spill incidents since 2010 [Web Ref. 1]

S.No	Spill Name	Location	Date	Spilled (tones)
1	Nigeria oil spill	Nigeria, Monga field	21-Dec-11	5,500
2	TK Bremen	France, Brittany, Erdeven	16-Dec-11	220
3	Campos basin oil spill	Brazil, Campos basin, Frade field	07-Nov-11	400
4	North sea oil spill	United Kingdom, North sea	10-Aug-11	216
5	Yellowstone river spill	United states, Billings, Yellowstone river	01-Jul-11	105
6	Bohai bay oil spill	China, Bohai bay	04-Jul-11	204
7	Peace river spill	Canada, Alberta	29-Apr-11	3,800
8	Mumbai-uran pipeline spill	India, Mumbai, Arabian sea	21-Jan-11	40
9	Fiume santo power station	Italy, Sardinia, Porto torres	11-Jan-11	40
10	Mumbai oil spill	India, Mumbai, Arabian sea	07-Aug-10	400
11	Xingang port oil spill	China, Yellow sea	16-Jul-10	90,000
12	Talmadge creek oil spill	United States, Kalamazoo river, Michigan	26-Jul-10	3,250
13	Barataria bay oil spill	United States, Barataria bay, Gulf of Mexico	27-Jul-10	45
14	Jebel al-zayt oil spill	Egypt, red sea	16-Jun-10	Not known
15	Red butte creek oil spill	United states, Salt lake city, Utah	11-Jun-10	107
16	MT bunga kelana 3	Singapore, Singapore Strait	25-May-10	2,500
17	Trans-alaska pipeline spill	United States, Anchorage, Aaska	25-May-10	1,200
18	Exxon mobil oil spill	Nigeria, Niger delta	01-May-10	95,500
19	Deepwater horizon	United States, Gulf of Mexico	20-Apr-10	6,27,000
20	Orissa oil spill	India, Orissa, Rushikulya river	12-Apr-10	24
21	Great barrier reef oil spill	Australia great Keppel island	03-Apr-10	4
22	Port arthur oil spill	United States, Port arthur, Texas	23-Jan-10	1,500
23	Yellow river oil spill	China, Chishui river (Shaanxi)	05-Jan-10	130

2.4. Oil Spill Cleaning Techniques:

Oil is presently a nonrenewable energy source in everyday life. This fossil fuel is used for a variety of purposes including fuel for transportation and factories. Unfortunately, oil spills sometimes occur within the environment due to accidents and unavoidable actions such as

weather and earthquakes or through intentional spills from war and dumping. Several techniques can be used to clean up oil spills and to prevent further destruction by this hazardous constituent.

Cleanup and recovery of spilled oil is difficult and depends upon many factors, including the type of oil spilled, the climatic conditions of spilled site which includes temperature of the water, tidal intensity and the types of shorelines and beaches involved. In general spilled oil can be cleaned in three methods namely physical, chemical and Natural methods.

2.4.1. Physical cleaning methods:

Physical containment and recovery of bulk or free oil is the primary response option of choice in the United States for the cleanup of oil spills in marine and freshwater shoreline environments. Commonly used physical methods include:

2.4.1.1. Booms:

It's an easier way of cleaning-up the oil spill with booms, if it's all in one place. Equipment named containment booms act like a fence to keep the oil from spreading or floating away over the water surface. Booms float on the surface and have three parts: a '*freeboard*' or part that rises above the water surface and contains the oil and prevents it from splashing over the top, a '*skirt*' that rides below the surface and prevents the oil from being pushed under the booms and escaping, and some kind of cable or chain that connects, strengthens, and stabilizes the boom. Connected sections of boom are placed around the oil spill until it is totally surrounded and contained [Vandermeulen and Ross, 1995].

2.4.1.2. Skimmers:

Skimmers are machines that suck the oil from a spilled water bodies. It is a physical method of separating the oil from the water so that it collects the oil over a sea into a tank. Much of the

spilled oil can be recovered with skimmers. The recovered oil has to be stored somewhere, although such storage tanks or barges have to be brought to the spill to hold the collected oil. Skimmers get clogged easily and are limited to large oil spill. When working with heavy crude oils or rough water skimmers suffer from clogging [Zhu et al., 2001] [Schuize, 2010].

2.4.1.3. Sorbents:

These are materials that soak up oils by either *absorption* or *adsorption*. It works on the principle in use of hydrophobic materials to wipe up oil from the contaminated surface. This property helps in removing the oil from the water much easily. Materials like why hay is used to clean oil spill on beaches and whey materials like vermiculite are spread over spilled oil. These materials are often designed as large squares, much like paper towels, or shaped into "*mops*." The squares or mops are used to wipe the shoreline or oily rocks during which time the absorbents are filled with as much oil as they can hold. One problem with using this method is that once the material is coated with oil, it may then be heavier than water. Then the oil-coated material sinks to the bottom where it could harm animals living there. Absorbent materials, very much like paper towels, are used to soak up oil from the water's surface or even from rocks and animal life on shore that becomes coated with oil. Wiping with absorbent materials requires the use of a large quantity of material and several personnel [Chidambaram, 2003].

2.4.1.4. Washing:

Washing can be done to clean the adhered oil along the shorelines to the water's edge. Washing operation can be held with low-pressure cold water flushing to high pressure hot water flushing. This method, using high-pressure or hot water, should be avoided for wetlands or other sensitive habitats. It is time consuming process and requires many persons [Vandermeulen and Ross, 1995].

2.4.1.5. Sediment relocation and tilling:

Oiled sediment are cleaned and relocated by tiling method using *shovels* from one section of the beach to another and the contaminated sediment is mixed to enhance natural cleansing processes by facilitating the dispersion of oil into the water column and promoting the interaction between oil and mineral fines. Tilling may cause oil penetration deep into the shoreline sediments. While selecting this method one should consider the potential environmental impacts from the release of oil and oily sediment into adjacent water bodies [Vandermeulen and Rose, 1995].

2.4.1.6. In-situ burning:

Oil on the shoreline is burned usually when it is on a combustible substrate such as vegetation, logs, and other debris. This method may cause significant air pollution and destruction of plants and animals.

2.4.2. Chemical cleaning methods:

Physical cleaning methods require many additional aids to clean the spills. Clean-up from an oil spill is not considered complete until all waste materials are disposed properly. Another approach of cleaning the oil from the spilled site is by using chemical methods. Chemical methods, particularly dispersants, have been routinely used in many countries as a response option. For some countries, such as the United Kingdom, where rough coastal conditions may make mechanical response problematic, dispersants are the primary choice [Otitoloju et al., 2009].

2.4.2.1. Dispersants:

Dispersants are also be used to dissipate oil slicks. Dispersing agents, which contain surfactants, are used to remove floating oil from the water surface to disperse it into the water column before the oil reaches and contaminates the shoreline. Some types of oil get rapidly dispersed from the sea surface by transferring it into the water column. They will cause the oil slick to break up and form water-soluble micelles that are rapidly diluted [Web Ref. 12]. The oil is then effectively spread throughout a larger volume of water than the surface from where the oil was dispersed. They can also delay the formation of persistent oil-in-water emulsions

Dispersants have two main components, a surfactant and a solvent. Surfactants molecules are made up of two parts, an *oleophilic* part (with an attraction to oil) and a *hydrophilic* part (with an attraction to water). When dispersants are sprayed onto an oil slick, the solvent transport and distribute the surfactants through the oil slick to the oil/water interface where they arrange themselves so that the oleophilic part of the molecule is in the oil and the hydrophilic part is in the water. This creates a reduction in the surface tension at the oil/water interface and small oil droplets will break away from the oil slick with the help of wave energy. These droplets will be of varying sizes. Although the larger ones may rise back to the surface some will remain in suspension and will drift apart and become degraded by naturally occurring bacteria. If dispersion is successful, a characteristic brown plume will spread slowly down from the water surface a few minutes after treatment.

***Limitations**:* Dispersants have little effect on very viscous, floating oils, as they tend to run off the oil into the water before the solvent can penetrate. As a general rule, dispersants are capable of dispersing most liquid oils and emulsions with viscosities less than 2000 cSt, equivalent to a medium fuel oil at 10-20°C. They are unsuitable for viscous emulsions or oils which have a pour

point near to or above that of the ambient temperature. Even those oils which can be dispersed initially become resistant after a period of time as the viscosity increases as a result of evaporation and emulsification. For particular oil, the time available before dispersant stops being effective depends upon such factors as tidal effect and temperature but is unlikely to be longer than a day or two. Dispersants can, however, be more effective with viscous oils on shorelines because the contact time may be prolonged allowing better penetration of the dispersant into the oil.

Types of Dispersants: There are three main types of dispersants:

- Type I dispersants are based on hydrocarbon solvents with between 15% to 25% surfactant. They are sprayed neat onto the oil as pre-dilution with sea water renders them ineffective. Typical dose rates are between 1:1 and 1:3 (dispersant: oil).
- Type II dispersants are dilutable concentrate dispersants which are alcohol or glycol (i.e. oxygenated) solvent based with a higher surfactant concentration. Dilution is normally 1:10 with sea water.
- Type III dispersants are also concentrate dispersants with a similar formulation to type II products. However, they are designed to be used neat and typical dose rates are between 1:5 and 1:30 (neat dispersant: oil).

Type I and II dispersants require thorough mixing with the oil after application to produce satisfactory dispersion. With type III products, the natural movement of the sea is usually sufficient to achieve this.

2.4.3. Natural cleaning methods:

Natural cleaning method of hydrocarbons in the environment is an important route for cleaning of oil spills. After oil is discharged into the environment, oil under goes a wide variety of physical, chemical and biological processes. These chemical and physical processes are collectively called '*weathering*', act to change the chemical composition, route of exposure and toxicity of discharged oil. Weathering process reduces the concentrations of hydrocarbons in sediment, water bodies and alters the chemical composition of oils. The changes in the chemical composition of the oil have profound effects on the oil toxicity and its time of impact on organisms. Hence it adds great difficulties to the identification of the residual spilled oil in the impacted environment. There are seven weathering processes discussed as below:

2.4.3.1. Spreading:

As soon as oil is spilled on water surface, the first weathering process which acts on the oil is spreads. Spilled oil starts to spread over the sea surface immediately following the release and continue for approximately one week to ten days for large slicks, or until it is limited by the shoreline, collection efforts or any other obstruction. Spreading is the movement of the entire oil slick horizontally on the surface of water, and the speed at which this takes place depends on gravity, viscosity and volume of the spilled oil and inertia, friction, surface tension between water surface and oil. Liquid oils initially spread as a coherent slick but quickly begin to break up. Highly viscous oils fragment rather than spreading to thin layers. At temperatures below the pour point, oils rapidly solidify and hardly spread and may remain up to few centimeters thick. Winds, wave action and water turbulence tend to cause oil to form narrow bands or 'windrows' parallel to the wind direction [Badejo et al., 2006]. At this stage the properties of the oil become

less important in determining slick movement. The rate at which oil spreads is also affected by tidal streams and currents. The stronger the combined forces, the faster the spreading process [Scholz. et. al., 1999].

2.4.3.2. Evaporation:

Evaporation is a weathering process which begins after an oil spillage. It is the single most important weathering process in the first several days of an oil spill. The more volatile components of oil will evaporate to the atmosphere with the rate dependent on ambient temperatures and wind speed. Evaporation reduces the volume of the oil, its flammability and toxicity but increases the viscosity and density of the residue [Web Ref. 17]. It starts immediately after a spill and continues up to approximately two weeks. Majority of the lighter fractions evaporation occurs in first 12 hours at a faster rate and disappearing within 48 to 72 hours. The greater the quantity of components with low boiling points, the greater the degree of evaporation. The initial spreading rate of the oil affects evaporation since the larger the surface area, the faster light components will evaporate [Scholz. et. al., 1999].

2.4.3.3. Dispersion:

Dispersion is the weathering process of forming small oil droplets that become incorporated in the water column in the form of a dilute oil-in-water suspension cause all or part of a slick to break up into fragments and droplets of varying sizes. It occurs within 10 hours following the spill but may continue for several weeks; within 100 hours it overtakes spreading as the primary mechanism for transport of oil from the spill site. The speed at which an oil disperses is mainly dependent upon the nature of the oil and the sea environment state, and occurs most quickly if the oil is light and of low viscosity and if the sea is very rough [Scholz. et. al., 1999].

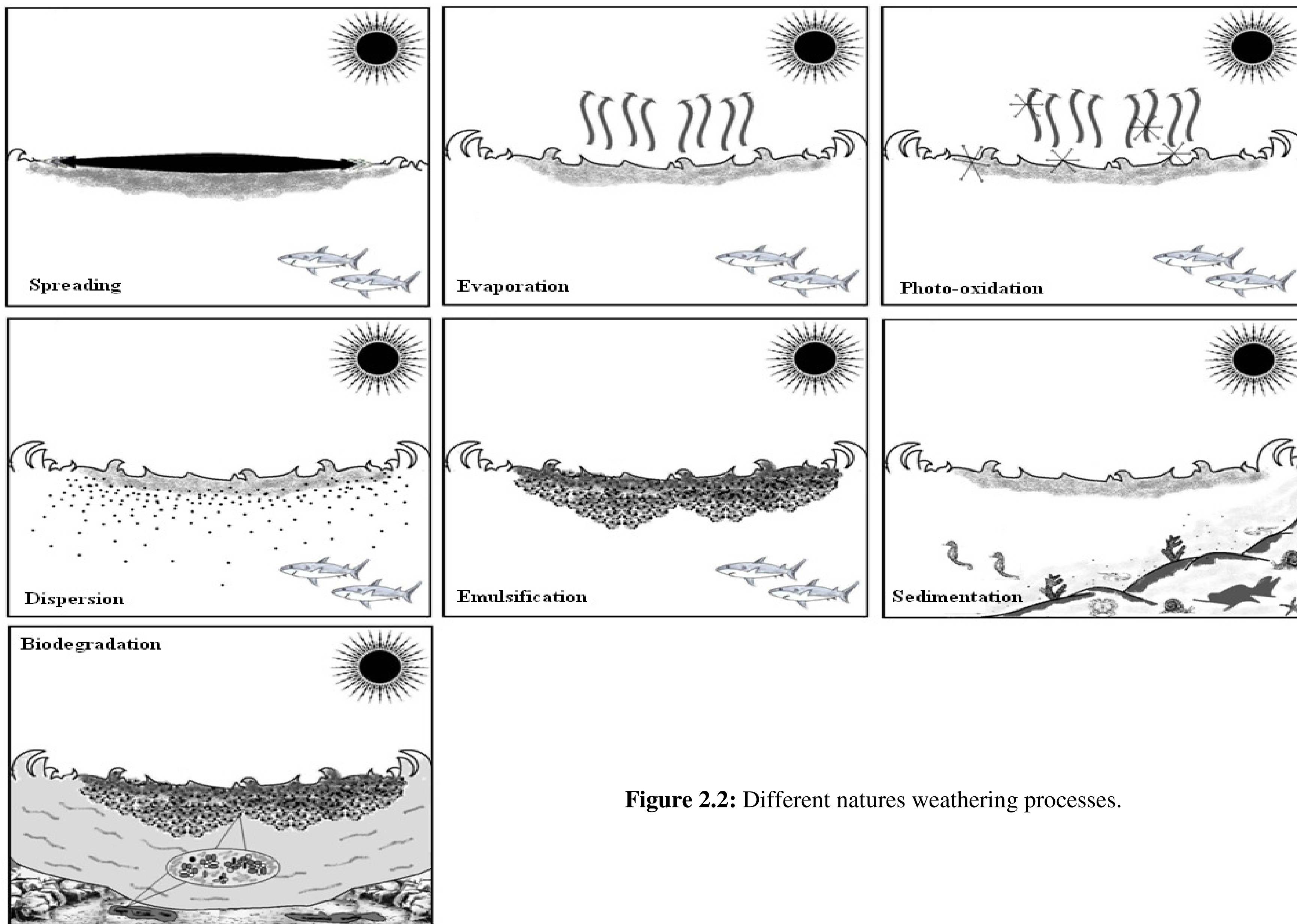

Figure 2.2: Different natures weathering processes.

2.4.3.4. Emulsification:

Emulsification is a process which occurs by the mixing of sea water droplets into oil spilled on water's surface forming water-in-oil emulsions under the mixing promoted by turbulence action of waves on the sea surface. Emulsions form most readily in oils which have a combined Nickel/Vanadium concentration greater than 15 ppm or asphaltene content more than 0.5%. The presence of these compounds and the sea state determine the rate at which emulsions form. The largest volume is typically formed in the first week after the loss of lighter weight components due to evaporation and dissolution. Majority of the weathering processes can be slowed once a stable emulsion is formed; also it greatly increases the volume [Scholz. et. al., 1999].

2.4.3.5. Photo-Oxidation:

Photo-oxidation is when sunlight in the presence of oxygen transforms hydrocarbons by photo oxygenation (increasing the oxygen content of a hydrocarbon) into new by-products. It results in changing in the interfacial properties of the oil, affecting spreading and emulsion formation, and may result in relocation of toxic by-products into the water column due to the by-product's enhanced water solubility. It is relatively insignificant in the overall weathering process as it is interrupted by nights and is narrowed on cloudy days. It does not peak at any particular time as it depends on light intensity, duration, weather conditions and extent of emulsification. It can start within several hours of a spill and can last for several weeks to a month [Scholz. et. al., 1999].

2.4.3.6. Sedimentation:

Sedimentation is a weathering process in which oil is incorporated within both bottom and suspended sediments. Sedimentation can be caused due to a number of reasons; it may be due to adherence of oil to detritus particles or due to undigested oil droplets passing in the fecal matter of marine organisms. Shoreline stranding is the visible accumulation of petroleum along the shoreline following a spill. It is affected by proximity of the spill to the affected shoreline, intensity of current and wave action on the affected shoreline and persistence of the spilled products. Sedimentation can begin immediately after the spill but peaks after several weeks, whereas shoreline stranding is a function of the distance of the shoreline from the spill and chemical nature of spilled oil.

2.4.3.7. Biodegradation:

Biological cleaning method harness natural processes in order to meet today's demands for cleaning, and waste elimination without the use of potentially harmful chemicals [Web Ref. 13]. The biodegradation of these compounds is often a complex series of biochemical reactions and is often different when different microorganisms are involved.

Biological cleaning approach is done by adding microbes which have the potential to consume hydrocarbons to use as a food source, giving out water and carbon dioxide as waste products. It occurs on the water surface, in the water column, in the sediments and at the shoreline. Microbes which can use hydrocarbons occur naturally (Genotypic expression), but multiply further following a spill as additional food sources become available. It is a slow process and is dominant only after the components toxic to the microbes have weathered below a threshold value by other physical processes. It is dominant for light and medium weight components, heavy weight components are too structurally complex for the microbes to degrade

right away, and they may be degraded after several months or even years. Biodegradation process commences immediately following a spill and can continue as long as degradable hydrocarbons are present. It peaks within the first month of the spill but can eventually be limited by the availability of nutrients in the environment for their growth. If nutrients (N, P, and K) are added, the microbes can grow along the shoreline degrading at a faster rate [Shewfelt et al., 2005].

Hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions [Das and Chandran, 2011].

On the basis of mode of application bioremediation is classified in to two types namely In-situ bioremediation and ex-situ bioremediation.

A. In-situ Bioremediation:

The method of In-situ bioremediation requires that the contaminated subsoil must be removed or scraped. Treatment is carried out in subsoil either by biological means such as hydrocarbon degradation by microorganisms, chemical processes, or by physical processes such air sparging or soil venting can be applied to extract contaminants from the soil as well as groundwater. To conduct this treatment vertical and horizontal drilling processes may be required. Also it is more effective on sandy soils than in soils that contain clay. Bioremediation can be applied with the help of spreading units in case the contamination is at the surface. It also accelerated the growth of aerobic bacteria in the contaminated area by oxygen feeding

[Farhadian et al., 2008]. Slurping is a process in which special well completion is required to extract the oil by means of vacuum. It is preferred if the contamination is in the boundary area between groundwater saturated soil and groundwater unsaturated soil. Soil air suction is also a method where suction extraction of contaminated soil air is done by means of vacuum to remove volatile hydrocarbon from contaminated area. In-situ steam injection is another method where steam at high temperature and compressed air are injected into the contaminated soil. The temperature of the injected steam should be greater than the boiling points of volatile components so that the contaminants can be converted to gaseous or volatile phase [Perelo, 2010]

B. Ex-situ Bioremediation:

For these methods soil must be removed to an off site remediation facility. The treatment is carried out by incinerating the soil, chemical extraction or soil washing to remove the hydrocarbon components. The important methods of ex-situ treatment are steam stripping and combustion, extraction methods and biological methods. The chemical extraction method involves the usage of different solvents, where the contaminants are dissociated from the soil, dissolved or suspended in the solvents. It can be applied for different types of crude and soils but is more effective for soil with low clay content. It can be performed in-situ or ex-situ, but in ex-situ the clean-up period is relatively short and the efficiency is better controlled. Surfactant washing too can be applied in-situ or ex-situ. Ex-situ methods can be applied only if the amount of contaminated soil is small or if the contamination has occurred in a residential or industrial area where in-situ treatment cannot be applied. Ex-situ treatment leads to a higher degree of remediation because of controllability of many factors like pH, temperature, salinity, moisture etc [Leila et al., 1994] [Atlas, 1975].

C. Microbial degradation of hydrocarbons:

- The biodegraded petroleum-derived aromatic hydrocarbons in marine sediments by *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* were found to be very effective [Jones et al., 1983]. The extensive biodegradation of alkyl aromatics occurred prior to detectable biodegradation of n-alkane profile of the crude.

- Bacteria strains are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment. Many of the microorganisms have the genotypic ability to utilize the hydrocarbons as a sole carbon source, but they express it only when they are grown in the hydrocarbon contaminated environment. Floodgate isolated 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. A similar compilation by Bartha and Bossert included 22 genera of bacteria and 31 genera of fungi.

- Tropical stream in Lagos, Nigeria which was polluted by petroleum hydrocarbons was cleaned up by bacterial strains isolates from the polluted stream; namely, *Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacterium* sp [Adebusoye et al., 2007].

- Bacterial genera, namely, *Acinetobacter* sp, *Gordonia*, *Brevibacterium*, *Aeromicrobium, Dietzia*, *Burkholderia,* and *Mycobacterium* were isolated from petroleum contaminated soil in North East India. Among them *Acinetobacter* sp. was found to be capable of utilizing n-alkanes of chain length C_{10}–C_{40} as a sole carbon source [Das and Mukherjee, 2007].

- Not only the bacterial stains but fungal genera, namely, *Amorphoteca*, *Neosartorya*, *Talaromyces,* and *Graphium* and yeast genera, namely, *Candida, Yarrowia,* and *Pichia which* were isolated from petroleum contaminated soil had proved to be the potential organisms for

hydrocarbon degradation [Chaillan et. al., 2004]. Singh [2006] also reported a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium,* and *Pencillium* which were also found to be the potential degrader of hydrocarbons of crude oil.

- Not only the soil but from contaminated water also some yeast species, namely, *Candida lipolytica, Rhodotorula mucilaginosa, Geotrichum* sp*, and Trichosporon mucoides* were isolated which have a potential to degrade the hydrcarbons [Bogusławska, 2001]
- Few algal species like *Prototheca zopfi* are capable of utilizing crude oil and a mixed hydrocarbon substrate and exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons [Walker et al., 1975]. Few Green algae, red alga, brown alga, and diatom species are capable of degrading hydrocarbons and they could oxidize naphthalene [Cerniglia et al.1980].

D. Microbial metabolism of long-chain n-alkanes:

Two pathways have been proposed for the oxidation of long chain n-alkanes by many researchers. [Marino, 1998] alkane's metabolic pathway explained as follows.

1) The mono-terminal oxidation pathway yielding an alcohol intermediate which is oxidized further in to an aldehyde and then to an acid.

2) The mono-terminal oxidation yielding an n-alkyl hydroperoxide which is then transformed to a peroxy acid, an aldehyde and finally to an acid. The former is the most popular pathway which is generally accepted. The n-alkane undergoes an oxygen-dependent oxidation to an alcohol catalyzed by a *monooxygenas*e. The alcohol is then oxidized further by an *alcohol dehydrogenase* to an *aldehyde*. Then, an *aldehyde dehydrogenase* transforms the aldehyde to a *fatty acid*. The fatty acid finally undergoes *β-oxidation* during which two carbons are cleaved from the organic acid to give *acetyl-CoA* and a *fatty acid-CoA* (Figure 2.3) [Marino, 1998].

Three different types of induced *aldehyde dehydrogenases* ($NADP^+$ and NAD^+ dependent and nucleotide independent) and 2 different types of constitutive *alcohol dehydrogenases* have been identified ($NADP^+$ and NAD^+ dependent). The *aldehyde dehydrogenases* have been found associated with hydrocarbon vesicles and bound to the cytoplasmic membrane with the active center of the enzyme in the direction of the periplasmic space This suggest that there could be two separate destinations for the products such as *β-oxidation* and wax ester synthesis by *aldehyde reductases* (used as carbon reserves when the cells are under carbon limitation). Work with yeast cells by Ludvik, showed that the cytoplasmic membrane underwent physiological changes when growing on hydrocarbons as carbon source. The membrane became thicker and showed deep invaginations indicating that the membrane could be involved in both transport and metabolism of the substrate. It was also demonstrated that maximum reaction rate (V_{max}) and substrate concentration (K_m) for this enzyme decreased with chain length.

The second pathway not involving alcohols intermediate was proposed by Fimerty [1968]. The *n-alkane* is first oxidized to an *n-alkyl hydroperoxide* by an enzyme *dioxygenase*. The *n-alkyl hydroperoxide* is sequentially converted to a *peroxy acid*, then to an aldehyde and finally to a fatty acid before undergoing *β-oxidation* (Figure 2.4) [Marino, 1998]. The *dioxygenases* isolated thus far were found in the cytoplasm of bacteria and did not need any co-enzymes. When grown in the presence of long chain hydrocarbons (hexadecane and higher fractions), they were more active toward solid than liquid n-alkane.Enzymatic studies have shown that increased lipid synthesis was activated by hydrocarbons. Total cellular lipid in solid n-alkane grown cells increased two and a half time. When the substrate was changed abruptly from glucose to hexadecane in yeast fermentations, the cells were unable to instantaneously utilize the hexadecane. Before the hexadecane could be used, the lipid concentration of the cells was

doubled. Therefore the authors argued that the lipids acted as solvents for the transfer of alkanes from the ce11 surface to the site of enzymatic action.

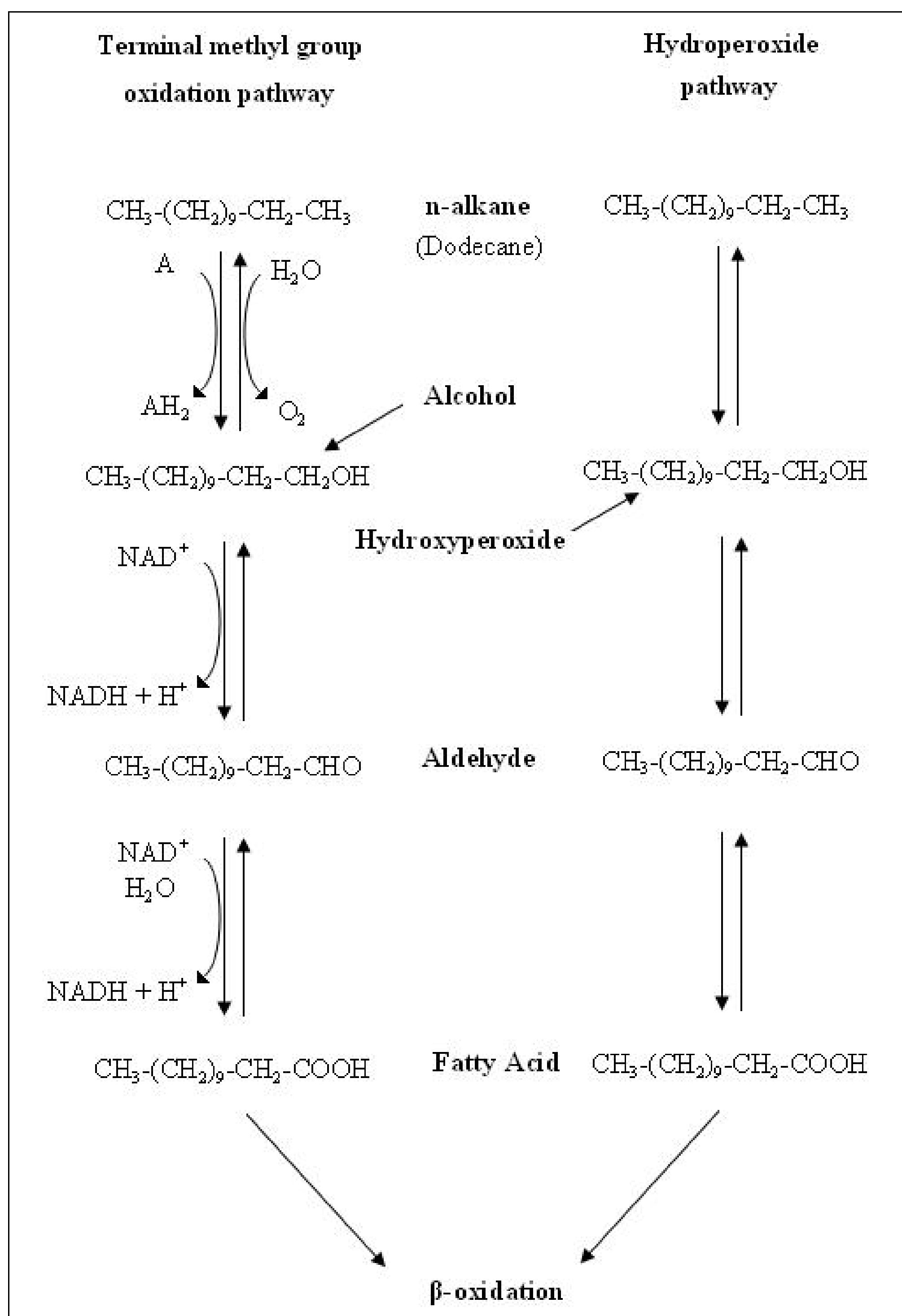

Figure 2.3: Metabolic path way of n-alkanes [Marino, 1998].

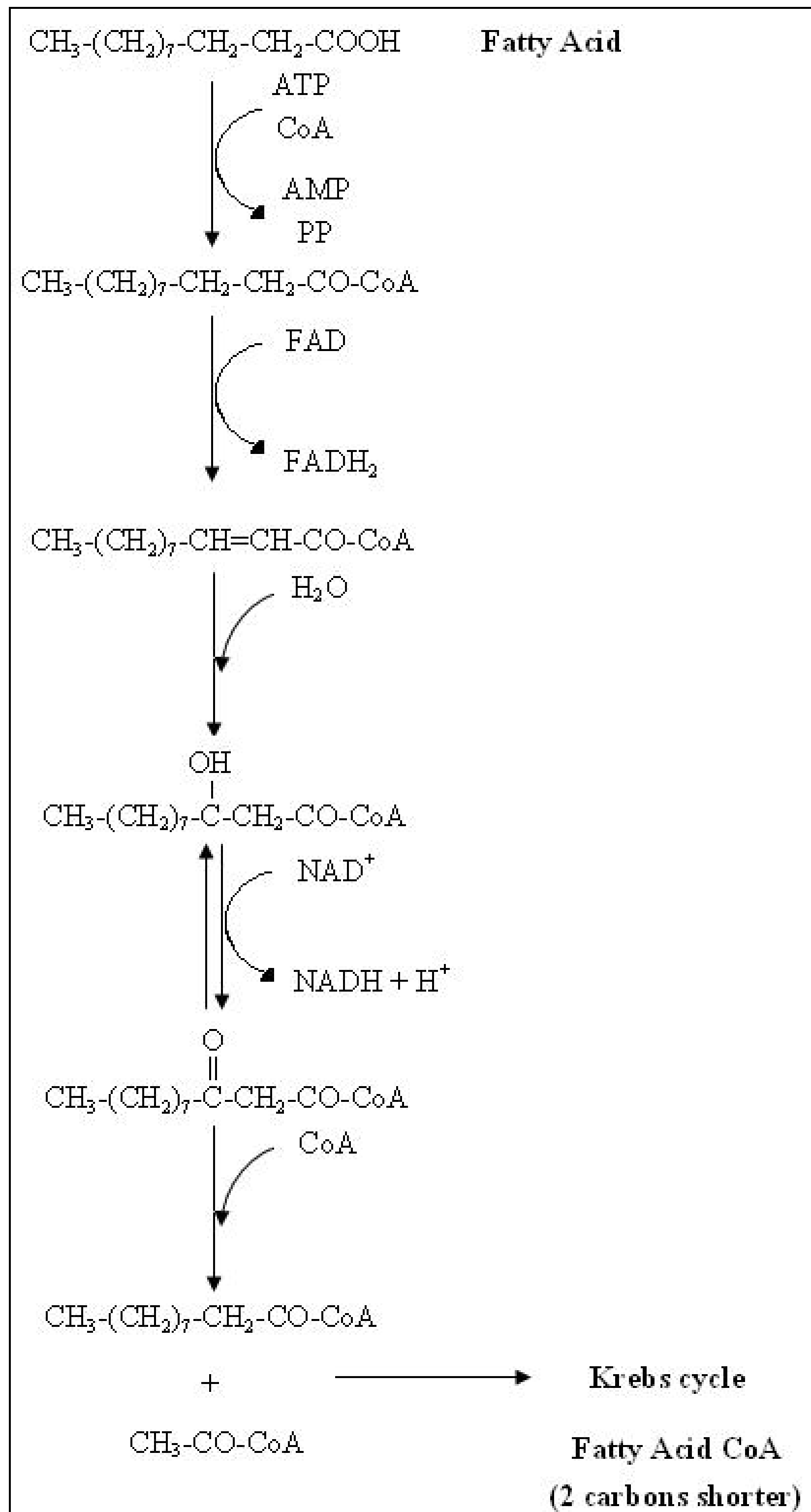

Figure 2.4: β-oxidation metabolic path way [Marino, 1998].

2.4.3.8. Biosurfactants and its Classification:

Application of biosurfactants (BS) is a natural way of cleaning the hydrocarbons in the environment. In general naturally occurring surface-active compounds derived from micro-organisms are called microbial biosurfactants [Mulligan, 2009]. BS are amphiphilic biological compounds produced extracellular or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi [Jennings and Tanner, 2000]. The ability to reduce surface tension is a major characteristic of surfactant.

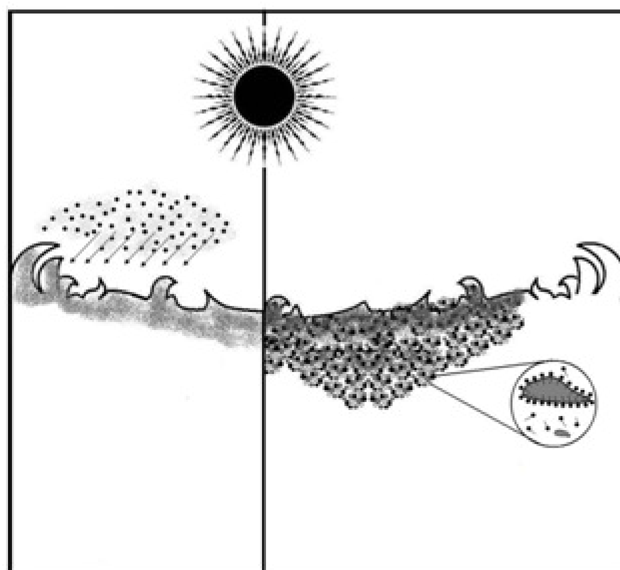

Figure 2.5: Emulsification of oil slick by biosurfactant molecules.

Similar to synthetic surfactants, BS molecules are made up of two parts: an *oleophilic* part (with an attraction to oil) and a *hydrophilic* part (with an attraction to water). When dispersants are sprayed onto an oil slick, the solvent will transport and distribute the surfactants through the oil slick to the oil/water interface where they arrange themselves so that the oleophilic part of the molecule is in the oil and the hydrophilic part is in the water [Makkar and Rockne, 2003]. This creates a reduction in the surface tension at the oil/water interface and small oil droplets will break away from the oil slick with the help of wave energy (Figure 2.5) [Jingliang, 2004]. These

droplets will be of varying sizes and although the larger ones may rise back to the surface some will remain in suspension and will drift apart and become degraded by microorganisms.

Classification of Biosurfactants:

A. Glycolipids:

Glycolipids are the most common types of BS. The constituent mono, di, triand tetrasaccharides include glucose, mannose, galactose, glucuronic acid, rhamnose, and galactose sulphate [Desai and Vyas., 2006]. The fatty acid component usually has a composition similar to that of the phospholipids of the same microorganism [Okoh, 2006] [Jennings and Tanner, 2000]. The glycolipids can be categorized as follows:

a. Rhamnolipids:

Some *Pseudomonas* sp. produces large quantities of a glycolipid consisting of two molecules of rhamnose and two molecules of b–hydroxydecanoic acid. While the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acids is involved in ester formation (Figure 2.6). Since one of the carboxylic acid is free, the rhamnolipids are anions above pH 4.0. Rhamnolipids are reported 46 mN/m to lower surface tension, emulsify C_xH_y, and stimulate growth of *Pseudomonas* on *n*-hexadecane [Christova et al., 2004]. Formation of rhamnolipids by *Pseudomonas* sp, the pure rhamnolipid lowered the interfacial tension against *n*-hexadecane in water to about 1 mN/m and had a critical micellar concentration (CMC) of 10 to 30 mg/l depending on the p^H and salt conditions [Christie, 2012].

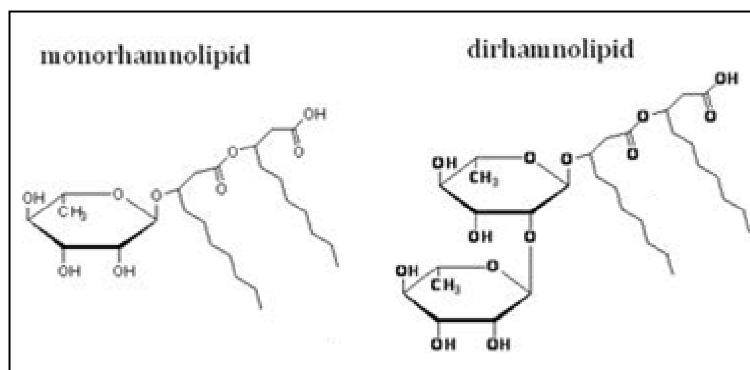

Figure 2.6: Rhamnolipid with mono and di- disaccharides linkage with b-hydroxydecanoic acid [Christie, 2012].

***b. Sophorolipids*:**

Few yeast species, and in particular *Candida bombicola*, secrete extracellular glycolipids known as sophorolipids (or sophorosides), as they contain the sugar sophorose (β-D-Glc-(1→2)-D-Glc). This is linked glycosidically to the hydroxyl group of a 17-hydroxy-C_{18} saturated or monoenoic (*cis*-9) fatty acid. The carboxyl group is usually linked to the 4'-hydroxyl group of the second glucose unit to form a lactone, though it can also remain in free form and then have more powerful detergency properties. One or both of the 6-hydroxyl groups on the glucose units are acetylated. With the organism *C. bogoriensis*, the fatty acid is 13-hydroxydocosanoate, while in *C. batistae* it is 18-hydroxy-stearic acid (and the acidic form of the lipid predominates) (Figure 2.7) [Christie, 2012].

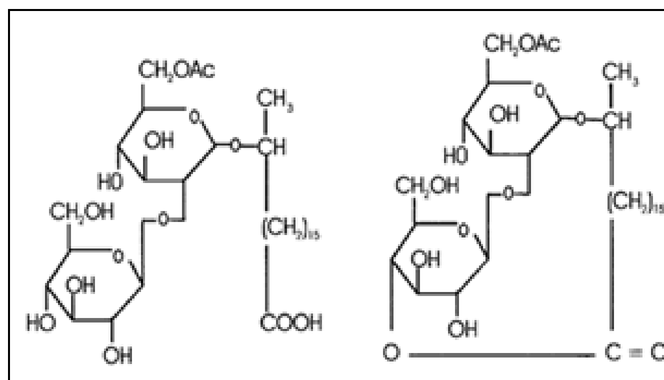

Figure 2.7: Structure of sophorolipids [Christie, 2012].

Biosynthesis involves sequential transfer of activated glucose molecules, UDP-glucose, to a hydroxy acid in processes catalysed by two different *glycosyltransferases* . Finally, the molecule is acetylated by an *acetyltransferase*. The fatty acid constituents can be synthesized *de novo* from acetate or by modifying alkanes in the growth medium.

***c. Trehalose lipids*:**

In many members of the genus *Mycobacteriu,* separate growth is observed du to the presence of trehalose esters on the cell surface Size and structure of the mycolic acid esters in different species of *Mycobacteria*, *Corynebacteria*, *Nocardia,* and *Brevibacteria* varies different cord factors.

B. Phospholipids:

Phospholipids are major components of microbial membranes. When certain hydrocarbon degrading bacteria or yeast were grown on alkane substrates, the level of phospholipids increases greatly. Phospholipids produced on hexadecane media by *Acinetobacter* sp have potent

surfactant properties. *Thiobacillus thiooxidans* produced phospholipids have been reported to be responsible for wetting elemental sulphur, which is necessary for growth.

C. Fatty acids:

Some microbes produce fatty acids which can be used as biosurfactants. They are produced from n-alkanes by oxidation processes. Besides the straight-chain acids, microorganisms produce surfactants with complex fatty acids containing OH groups and alkyl branches. Some of these complex acids were Corynomucolic acids [Karanth et al., 2005].

D. Surface active antibiotics:

Many microorganisms produce antibiotics which have surfactant activity. *Gramicidin Soviet* is one of such antibiotic which is produced by gram positive bacterium *Bacillus brevis***.** *Gramicidin Soviet* is a cyclosymmetric decapeptide antibiotic which is effective against some gram positive and gram negative bacteria as well as some fungi. Spore produced by *Brevibacterium brevis* contain large amounts of gramicidin S bound strongly to the outer surface of the spores. Mutants lacking *gramicidin S* germinate rapidly and do not have a lipophilic surface. The antibacterial activity of gramicidin S is due to its high surface activity (Figure 2.8).

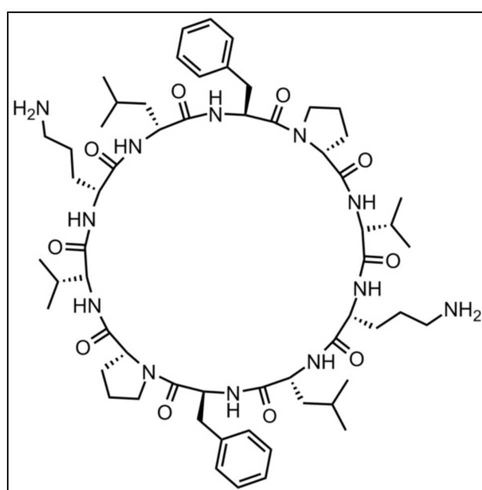

Figure 2.8: Structure of Gramicidin-S [Web Ref. 18].

Chapter-3

Materials and Methods

3.1 Chemicals:

Pure and analytical grade chemicals used in all experiments including media preparation for microbial culture growth and culture maintenance. Nutrients used for microbial culture and the testing kits were supplied by *M/s Hi Media chemicals*, India. Petroleum crude oil was collected from *Oil and Natural Gas Corporation Limited* (ONGC), Rajahmundry, Andhra Pradesh and Petroleum oil sludge collected from the *Hindustan Petroleum Corporation Limited* (HPCL), Vishakhapatnam, Andhra Pradesh. Other chemicals and nutrient salts procured from *Merck Chemical*, India and *Sigma Aldrich.*

3.2 Glassware and Instruments:

All glassware's (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes etc.) purchased from Borosil. The instruments and apparatus used throughout the experiment listed in Table 3.1.

Table 3.1: List of instruments used in the present work

Instrument	Make	Function	Operation conditions or specification
Analytical balance	Sartorius (BS223S)	Weight measurement	1mg - 100g
pH meter	Systronics (361)	Measurement of pH	pH 1 to 12
Vertical autoclave	Test master	Sterilization of nutrient media	121°C temperature, 15 psi pressure, 15 min.
Laminar airflow chamber	Zhichen (ZhJH-1109C)	Aseptic Environment	• Air filter: HEPA • Filter efficiency: FS209E, Class 100. • Air Velocity: 0.3-0.6m/s • Visible Light source: Fluorescent Lamp • UV light source: 8watt, mercury lamp (254nm)

BOD incubator	Vikram scientific instruments	Incubation of cultures	Temperature range: 5 - 70 ºC.
Incubator shaker	Environmental orbital Shaker	Shaking of culture flasks used in degradation study	• Speed range: 50 - 200 rpm. • Temperature: 5 - 70 ºC.
Spectrophotometer (UV/Vis)	Jasco (V-530)	Estimation of biomass and analysis of organic compounds	• Bacterial Biomass: 600 nm • TPH analysis: 228 nm • Anthrone reagent: 620 nm • Biuret reagent: 540 nm
Bioreactor	IIC Industrial corporation	Biosurfactant production	2 liters capacity
Scanning Electron Microscope	JEOL (JSM-6480 LV)	To study the clear morphology about the structure and shape of the microbial cells with dimensions.	• Magnification: up to 500k. • Resolution : 3 nm • Detector: Everhardt Thornley secondary electron detector and Solid state backscattered detector. • X-Ray Analysis: Oxford Instruments ISIS 310 system with "windowless" detector. • Light element analysis: silicon detector with ATW.
Optical microscope	Hund (H-600)	Examining the morphology of microbial cells	100x, 250x, 400x and 1000x magnification.
Surface tensiometer	Data physics (Dcat-11)	Measuring the surface tension of the solution at air/liquid interface	• Method: Wilhelmy Plate. • Plate material: Platinum. • Temperature: Ambient
Fourier Transform Infrared Spectroscopy (FTIR)	Perkin Elmer (3500)	Analyze the organic functional groups present in the Biosurfactant sample	• Measured range: wave number 600- 4000 cm^{-1}. • Resolution: 4 cm^{-1}
Micro Centrifuge	Remi (RM12C)	Separation of biomass from the culture broth	8000 RPM for 10 min
Digital colony counter	EI Products (D12)	Measure the number of microbial colonies	• Dimension: L 274 x B 320 x 167 mm • Magnification :110 mm
Gas Chromatography (GC)	Shimadzu (QP 2010)	Analysis of Hydrocarbon present in oil	• Column : VF-5ms • Length : 30.0m • Diameter : 0.25mm • Film Thickness : 0.25um • Oven Temperature : 70˚C • Injector Temperature: 260˚C

			• Injection Mode : Split • Split Ratio :10 • Flow control mode : Linear velocity • Column Flow : 1.51ml/min • Carrier gas : Helium • Gas Purity: 99.9995% purity

3.3. Methodology:

3.3.1. Sampling for microbial isolation:

Microorganisms used in the degradation study were isolated from sand and soil samples collected from the hydrocarbon contaminated sites namely Rushikulya river beach (Gangam district, Odisha), garden soil (NIT Rourkela, Odisha) and local automobile workshop (Sector-II, Rourkela, Odisha).

3.3.2. Enrichment of microbial community:

Soil samples of garden soil and local automobile workshop were inoculated in a jar containing 500ml Minimal salts media (MSM) (g 1^{-1}): 2.0g of Na_2HPO_4, 0.17g of K_2SO_4, 2.0g of $(NH_4)_2SO_4$, 0.53g of KH_2PO_4 and 0.10g of $MgSO_4$, $7H_2O$. 5 ml of engine oil was used as hydrocarbon carbon source and left alone for 4 months under room conditions [Adekunle and Adebambo, 2007] .

3.3.3. Isolation of pure microbial cultures:

Microbial isolation experiments performed in solid Minimal salts media (MSM) containing 1.5% agar and 1% of the n-Octane as a sole carbon source. The medium was autoclaved before inoculation. Rushikulya beach sand samples and broth samples from microbial enriched jar was

serially diluted and 1ml aliquot of 10^{-4} dilution samples was added to sterile petri plate by spread plate technique. Agar plates were incubated for four days at room temperature (28 - 30ºC) at a pH of 7.4 ± 0.2 [Ilyina et al., 2003].

3.3.4. Hydrocarbon utilization efficiency:

Isolated pure cultures were tested for their ability to grow on solid MSM with 1% of the following pure hydrocarbons: n-Hexadecane and petroleum crude oil. Agar plates were incubated for one to two weeks at room temperature (28 - 30ºC) at a pH of 7.4 ± 0.2.

3.3.5. Sub culturing of pure cultures and inoculum preparation:

Isolated pure cultures were sub-cultured at an interval of every 15 days and the inoculum used in the degradation study was acclimatization using n-Octane.

3.3.6. Characterization of microorganisms:

Isolated microbial strains were characterized to identify the family it belongs. Microbial characterization was done based on morphological, physiological and biochemical tests and the results were compared with *Bergey's manual of determinative bacteriology*. Microbial characterization was done at Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, Punjab, India.

3.3.7. Estimation microbial biomass:

The quantitative determination of bacterial populations is most widely done in two methods namely standard or viable method. Spectrophotometric (turbidimetric) analysis is the standard method and Plate count method is the viable cell count method. Although the two methods are somewhat similar in the results they yield, there are distinct differences. The standard plate count method is an indirect measurement of cell density and reveals information

related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead and alive.

***3.3.7.1. Standard biomass analysis*:**

Cultured broth (1ml) was centrifuged at 8000 gravity for 10 min and the obtained cell pellet was washed with distilled water and centrifuged again. Finally after centrifugation cell pellet was suspend in 1ml distilled water and bacterial biomass concentration was estimated by comparing the optical density (OD) observance at 600nm wavelength with the standard graph plotted with the OD values of different cells concentrations [Mukhopadhyay, 2007].

3.3.7.2. Viable cell count method:

Bacterial solution of known dilution and quantity solution was inoculated on the nutrient agar plate using spread plate technique. The plates were incubated at 32°C for 24 hours in BOD incubator. At the end of the incubation period, the cells on the petri plates were counted to estimate the number of cell per milliliter using digital colony counter. The numbers of colonies were multiplied with the dilution factor and amount of microbial solution was platted over the nutrient agar [Web Ref 15].

3.3.7.3. Fungal biomass analysis:

Fungal biomass was estimated on basis of mycelia dry weight. The fungal culture solution was filtered in Whatman grade no 3 filter paper. The filter paper washed with sterilised distilled water was oven dried and re-weight after cooling it in a desiccator. The difference in the weight of the filter paper measures the weight of the fungal biomass [Web Ref 15].

3.3.8. API gravity estimation: American Petroleum Institute gravity of petroleum oils (oil sludge, light crude oil and heavy crude oil) used in the degradation study was analyzed as per the ASTM standard method D287.

3.3.9. Total petroleum hydrocarbons (TPH) analysis:

TPH were analyzed by two methods and the results were compared. Degradation of TPH was measured gravimetrically and by measuring the OD and comparing the results with the standard plot. The residual oil content in the culture flask was extracted by using a 1:1 proportion of organic solvent (liquid-liquid extraction). The organic solvent was n-hexane. The extracted crude oil was detected spectrophotometrically by measuring the OD at 228 nm using UV/Visible spectrophotometer and followed by evaporating the solvent phase and measuring the weight of the dry extract [Osuji, 2006] [Aparna et al., 2011]. All the experiments were carried out in triplicate and the mean values were considered.

3.3.10. Nutrient salts analysis:

Nutrient salts which are the sources of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), sulfur (S), and Iron (Fe) were analysed by means of colorimetric detection method using Himedia salts testing kits. Details of the kits used in the analysis were given in Table 3.2.

Table 3.2: Nutrient salts detection kits and their reaction color

Element	Detection kit code	Color of reaction
N	WT013 and K054	Pink
P	WT008A and K054	Yellow
K	K054	Muddy yellow
S	WT005A	Violet blue
Mg	WT001C	Blue

3.3.11. Dissolved oxygen estimation:

Dissolved oxygen (DO) content of the broth was measured by a dissolved oxygen meter (Hach-HQ10). DO meter was equipped with a DO sensor which was immersed in the broth to measure the DO content.

3.3.12. Optimization of process parameters:

It is evident from the literature review that the factors affecting the process of biodegradation are temperature, pH and nutrients. Nutrients required for growth are nitrogen, phosphorus, magnesium and sulphate [Jelena et. al., 2009]. These parameters were optimized so to obtain maximum growth of the microorganisms and the growth was analysed in terms of biomass. Process parameters like temperature, pH and nitrogen source were optimized based on one-factor at a time approach.

The temperatures considered for optimization study were 20ºC, 25ºC, 30ºC, 35ºC and 40ºC. The cultures are prepared in five different flasks; each was maintained at different temperature. In this study pH of 7.4 ± 0.2 was maintained, since isolation of microbial strains was conducted at this pH. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with 1% of Petroleum oil sludge used as a carbon source. The flasks were kept in shaker incubator at 120 rpm, for 15 days.

Similarly, the pH optimization study was considered by varying the pH 6.0, 7.0, 8.0 and 9.0 and the flasks were incubated at optimized temperature. Nutrient salts used in the optimization of nitrogen source were Urea, Peptone, Ammonium chloride and Ammonium Sulphate. The culture flasks were incubated at optimized temperature and pH for a time of 15 days.

Nutrients (concentration of nitrogen, phosphorus, magnesium and sulphate) used in degradation were optimized by Gray based Taguchi method using Minitab 14 software. The factors (nutrients) and their levels (concentrations) were given in Table 3.3.

Table 3.3: Factors and their levels used in the nutrient optimization study

Factors	Optimization parameters	Unit	Level 1	Level 2	Level 3	Level 4
A	Sulphates	g/L	0.4	0.6	0.8	1.0
B	Phosphates	g/L	0.4	0.6	0.8	1.0
C	Nitrogen	g/L	0.4	1.5	2.0	2.5
D	Magnesium	g/L	0.1	0.2	0.3	0.4

The grey relational analysis based on the grey system theory was used to solve the complicated interrelationships among the multiple responses effectively. In a grey system data pre-processing is the primary stage since the range and the unit in one data sequence may differ from the other. Data pre-processing is a means of transferring the original sequence to a comparable sequence. Depending on the characteristics of a data sequence, there are various methodologies of data pre-processing available for this analysis [Tosun and Pihtili, 2010]

The optimization of the process was performed in the following steps:

1. Normalizing the experimental results of fungal biomass and residual oil content for all experimental run.
2. Calculating the Grey Relational Coefficient (GRC).
3. Calculating the Grey Relational Grade (GRG) by averaging the GRC's.
4. Performing statistical analysis of variance (ANOVA) for the input parameters with the GRG and identifying the parameters that significantly affected the process.
5. Selecting the optimal levels of process parameters.

The indication of the better performances of microbial strain in TPH degradation study was biomass yield "higher the better" whereas it was "lower the better" for residual oil content. In the analysis of Gary relation for "higher the better" response normalization used Equation 1 and when the response is "lower the better", response normalization used Equation 2.

Response normalization for larger is better condition. $x_i^*(k)$ [Sanjit. et. al., 2010].

$$x_i^*(\mathrm{k}) = \frac{x_i(\mathrm{k}) - \min x_i(\mathrm{k})}{\max x_i(\mathrm{k}) - \min x_i(\mathrm{k})} \tag{1}$$

Response normalization for smaller is better condition [Sathiya and Abdul, 2010]

$$x_i(\mathrm{k}) = \frac{\max x_i(\mathrm{k}) - x_i(\mathrm{k})}{\max x_i(\mathrm{k}) - \min x_i(\mathrm{k})} \tag{2}$$

Where $x_i^*(k)$ and $x_i(k)$ were the normalized data and observed data, respectively, from i^{th} experiment using k^{th} response. The smallest and the largest value of $x_\mathrm{i}(k)$ for the kth response were min $x_\mathrm{i}(k)$ and max $x_\mathrm{i}(k)$, respectively. After pre-processing the data, the Gray relation coefficient $\xi_\mathrm{i}(k)$ for the kth response characteristics in the ith experiment was expressed as follows.

$$\xi_\mathrm{i}(\mathrm{k}) = \frac{\Delta\min + \xi\,\Delta\max}{\Delta_\mathrm{i}(\mathrm{k}) + \xi\,\Delta\max} \tag{3}$$

Where $\Delta i(k)$ was the kth value in Δ_i different data series. Δ_{max} and Δ_{min} were the global maximum and minimum values in the different series, respectively. The distinguished coefficient ξ lies between zero and one, which was to expand or compress the range of GRC. The distinguishing coefficient was selected by decision maker's judgment, and different distinguishing coefficients usually provided different GRG results. The most preferred value of ξ was considered as 0.5. After calculating GRC, for 'n' number of responses, the GRG (γ) was being calculated using Equation 4 [Panda and Mohapatro, 2008].

$$\gamma = \frac{1}{\mathrm{n}} \sum_{\mathrm{i}=1}^{\mathrm{n}} \xi_\mathrm{i}(\mathrm{k}) \tag{4}$$

The magnitude of γ reflects the overall degree of standardized deviation of the ith original data series from the reference data series. In general, a scale item with a high value of γ indicates that the respondents, as a whole, have a higher degree of favorable consensus on the particular item.

3.3.13. Total Petroleum Hydrocarbon Degradation (TPH):

To studying, the degradation of TPH, three different petroleum oils were used namely oil sludge, heavy and light crude oil. In this study 2%, oil was used as carbon source. The optimized nutrients are used and the degradation study was preformed for a period of 30 days at120 RPM at optimized temperature and pH.

Samples analyzed at a regular interval of five days to examine the microbial biomass growth rate and the oil degradation. Microbial growth was measured by applying different techniques discussed earlier. On twentieth day of degradation study residual oil content was extracted from the culture flask using solvent extraction technique and samples were analysed in Gas chromatography (GC) to study the degradation percentage of hydrocarbon fractions. Second set of TPH degradation study was conducted by adding biosurfactants in the culture flask and the results were compared to assess the role of biosurfactants in the degradation efficiency of TPH's by the microorganisms.

3.3.14. Biosurfactant Analysis:

3.3.14.1. Recovery of biosurfactant: The culture broth was centrifuged at 10, 000 rpm for 10 min and biosurfactant was extracted with three folds of chloroform and methanol (2:1 *v/v*). The solvents separated by rotary evaporation and the crude biosurfactant obtained as residue. Dry

weight of the biosurfactant was expressed in terms of grams per liter (g/L) [Heyd et al., 2008] [Chandran and Das, 2010].

3.3.14.2. Emulsifying Index: The emulsifying capacity was evaluated by an emulsification index (E_{24}). The E_{24} of culture samples was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, vortexes at high speed for 2 min and allowed to stand for 24h. The E_{24} index was expressed as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm). The percentage emulsification index was calculated using Equation 5 [Adebusoye et al., 2007]

$$E_{24} = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100 \qquad (5)$$

3.3.14.3. Surface tension measurement: Surface tension of the aqueous solution at different surfactant concentrations were measured by using a Wilhelmy Plate type Surface tensiometer. The surface tension measurement was carried out at ambient temperature after dipping the platinum plate in the solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was considered. The critical micelle concentration (CMC) was then determined from the break point of the surface tension versus its log of bulk concentration curve. Instrument was calibrated by measuring the surface tension of the pure water before each set of experiments.

3.3.14.4. Oil spill test: The oil displacement test is a method used to measure the diameter of the clear zone, which occurs after dropping a biosurfactant containing solution on an oil water interface. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant. The oil displacement test was studied by adding 50 ml of distilled water

to a Petri dish of diameter of 15 cm. about that, 20 µl of crude oil was dropped onto the surface of the water, followed by the addition of 10 µl of an aqueous solution containing a biosurfactant onto the surface of the oil. The diameters of the clear zones of triplicate experiments from the same surfactant sample were evaluated to obtain the average value of the clear zone diameter [Chandran and Das, 2011].

3.3.14.5. Blue agar plate test: This semi quantitative agar plate cultivation test is based on the formation of an insoluble ion pair of anionic biosurfactants with the cationic surfactant CTAB and the basic dye methylene blue [Chandran and Das, 2010]. As the constitution of the agar medium, containing 0.5 mg/ml CTAB and 0.2 mg/ml methylene blue can be altered; this quick and simple test is well suited for detection of biosurfactant producing strains [Chandran and Das, 2011]. Rhamnolipids were detected as dark-blue halos around the colonies, with the spot diameter being dependent on rhamnolipid concentration. Nevertheless, care was taken during quantification, as the spot diameter is influenced by variable cell growths of the bacteria, cultivation times, migrations of the rhamnolipids, and filling levels of the agar plates [Pinzon and Ju, 2009].

***3.3.14.6. Haemolysis test*: ** Normal human venous blood collected in blood donation camp (500 µl) was mixed with 3.5 ml sterile filtered supernatant from batch microbial cultures. It was vortexed for 2 min and left for 10 min and then observation was made to examine the hemolytic property of the biosurfactant [Heyd et al., 2008].

3.3.14.7. Measurement of microbial adhesion to the hydrocarbon: The microbial adhesion to the hydrocarbon (MATH) was used to denote the cell surface hydrophobicity of the microbial species. It was measured according to the protocol suggested by Thavasi et al [2010]. On the 7th

day of cultivation, cells harvested by centrifugation were rinsed with phosphorus buffer (pH 7.0) twice and then diluted to an initial optical density measured at 600nm (initial OD_{600}) of around 0.8–1.0. Four milliliter of the cell suspension was then vortex mixed with 4 ml of kerosene at 1800 rpm for 3 min. The mixture was left undisturbed for 20 min and then the final OD_{600} of the aqueous phase was measured. MATH was calculated as follows:

$$\text{MATH} = \left(\frac{1-\text{OD}_{600}\,(\text{final})}{\text{OD}_{600}\,(\text{inetial})}\right) \times 100\% \tag{6}$$

3.3.14.8. Biochemical test of surfactant: Chemical composition of the biosurfactant was analyzed by following standard methods [Thavasi et al., 2010]. Carbohydrate content of the biosurfactant was determined by the anthrone (9,10-dihydro-9-oxoanthracene) reagent method which forms a green colored complex with carbohydrates present in the biosurfactant sample. D-glucose was used as a standard and OD readings were taken at 620 nm [Chandran and Das, 2011] [Heyd et al., 2008]. Lipid content was estimated adopting the procedure reported elsewhere [Folch *et al*., 1956]. Protein content of the biosurfactant was determined by using biuret method, which forms purple colored complex by reacting with peptide bond present in the proteins. Bovine serum albumin was used as a standard and OD readings were taken at 540 nm [Thavasi et al., 2010].

3.3.14.9. FTIR Analysis: The organic functional groups present in the biosurfactant sample were determined using FTIR analysis. The analysis was carried out using FTIR-3500 spectrophotometer. To measure the absorption spectra, solvent extracted biosurfactant sample was dropped on the Potassium bromide (KBr) crystal at a resolution of 4 cm^{-1} and measurement wave length range from 600 to 4000 cm^{-1} [Saher et al., 2011].

Chapter-4

Isolation and Characterization of Microorganisms

4.1. Sample collection from hydrocarbon contaminated sites:

Most of the microbes species present in the environment genetically have an efficiency to utilize the hydrocarbons as a carbon source. This ability comes to expression only when they are grown in the hydrocarbon environment [Atlas, 1981]. Sample collection from petroleum oil's contaminated sites will facilitate to isolate microbial strains with hydrocarbon degradation efficiency [Li et al., 2000] [Length, 2010]. So, samples have been collected from three sites, namely sand and water samples from oil spilled site of Gokharkuda beach, Ganjam district Odisha. Soil samples from the local automobile work shop, Sector II, Rourkela Odisha; soil samples from the garden soil, NIT, Rourkela, Odisha, India.

4.2. Isolation of Microorganisms:

To augment and isolate the efficient hydrocarbon degrading microorganisms soil sample collected from the garden soil was inoculated (5%) in 500 ml Minimal salts media (MSM) contain 1% engine oil as sole carbon source and left for 2 months at room temperature. At the end of 2 months some mycelia growth was observed over the hydrocarbon contaminated water (Figure 4.1). 500µl of 10^{-4} serial diluted microbial enriched water sample from the garden soil inoculated liquid MSM, sand and water samples collected from oil spilled site of Gokharkuda beach and soil samples from automobile work shop were inoculated on solid MSM of pH 7.4 ± 0.2 containing 1% *n-Octane* as a carbon source. Culture plates were incubated for 7 days at temperature 28 to 30ºC. After seven days of incubation microbial colonies were observed on the eight petri dishes (Table 4.1).

Microbial colonies grown on n-Octane were further tested to examine the growth on higher hydrocarbon fractions. MSM with *n-Hexadecane* and *Petroleum crude oil* was used as a

carbon source and the culture plates were incubated at 28 to 30ºC temperature for 7 days and 15 days respectively. After incubation period growth of microbial colonies were observed on the petri dishes and emulsification efficiency of petroleum oils like Kerosene (Table 4.1).

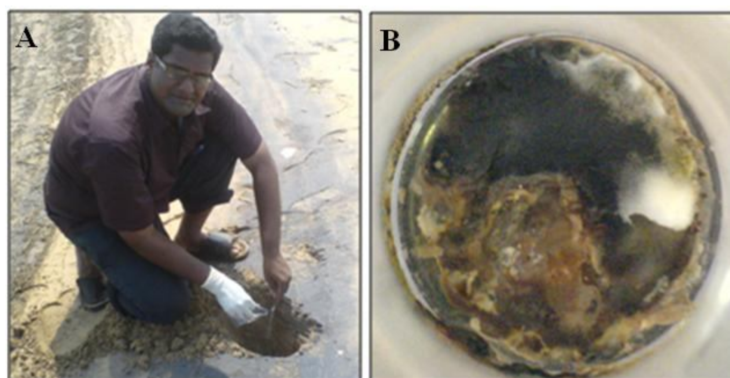

Figure 4.1: (A) Sand sampling from Gokharkuda beach and (B) Fungal growth on MSM inoculated with garden soil

Table 4.1: Microbial colonies isolated from various sampling sites

Sample Name	Sampling site	Microorganism Type	Growth on Hydrocarbon (No of colonies)			Emulsification Activity
			C_8H_{18}	$C_{16}H_{34}$	Crude oil	
WS	Automobile work shop	Bacteria	57	31	11	71
S-1	Garden Soil	Bacteria	44	18	2	16
S-2	Automobile work shop	Bacteria	26	9	2	8
F	Garden soil	Fungus	FC	8	3	NE
G	Gokharkuda beach	Bacteria	61	39	14	83
B-2	Gokharkuda beach	Bacteria	33	20	1	31
B-3	Gokharkuda beach	Bacteria	31	17	1	16
B-4	River water	Bacteria	19	14	0	2

“FC”- Fully covered; “NE”- Non emulsification

4.3. Characterization of Microorganisms:

4.2.1. Bacterial Characterization:

Among the eight strains isolated from three different sampling sites, two bacterial and one fungal strain's have shown good results in utilizing hydrocarbons as carbon source. Preliminary identification procedures were carried out for the three strains. According to the Berge's manual of systematic bacteriology bacterial strains are characterized on the basis of morphological features (Table.4.2), standard physiological tests (Table.4.3) and biochemical tests (Table.4.4) [Bayoumi, 2009] [Oberhofer et al., 1977].

In the morphological characterization study, Grams staring 'WS' bacterial cells retained purple stain and they are gram positive and 'G' bacterial cells retained pink safranin colour and they are gram negative. It is due to the dark purple crystal violet stain retained by the thick layer of peptidoglycan which forms the outer layer of the gram positive cell. In gram-negative bacteria, the thin peptidoglycan layer in the periplasm does not retain the dark stain, and the pink safranin counter stain stains the peptidoglycan layer. 'WS' and 'G' are rode shaped Bacillus cells (Figure.4.1). "WS" bacterial colony off white colour and 'G' bacterial colony produces a green colour water soluble pigment called Pyocyanin. 'WS' bacterial cells do not posses any flagella so it is non motile and 'G' bacterial cells posses' locomotion due to the presence of flagella. 'WS' and 'G' bacterial cells exhibit growth between 25°C to 42°C temperatures. So, they can be considered under class *Mesophiles*. "WS" and 'G' bacterial cells exhibit very good growth rate in pH range from 7.0 to 9.0 and still it exhibited growth up to an higher pH 12.0 so they can be considered under class obligate *Neutrophiles*. 'WS' and 'G' bacterial cells exhibit growth up to

12% NaCl concentration. So, they are considered as *Halopiles* (Moderate halophiles) [Web Ref 16].

Table 4.2: Morphological test results of bacterial strains

Tests	WS	G
Colony configuration	Circular	Circular
Colony margin	Entire	Wavy
Colony elevation	Flat	Flat
Colony surface	Smooth	Smooth
Colony texture	Moist	Slimy
Pigment production	Off white color	Green pigment color
Opacity	Opaque	Opaque
Gram's reaction	+	-
Cell shape	Rod	Rod
Spore(s)	+	-
Motility	Non motile	Motile

'+': Positive; **'-':** Negative

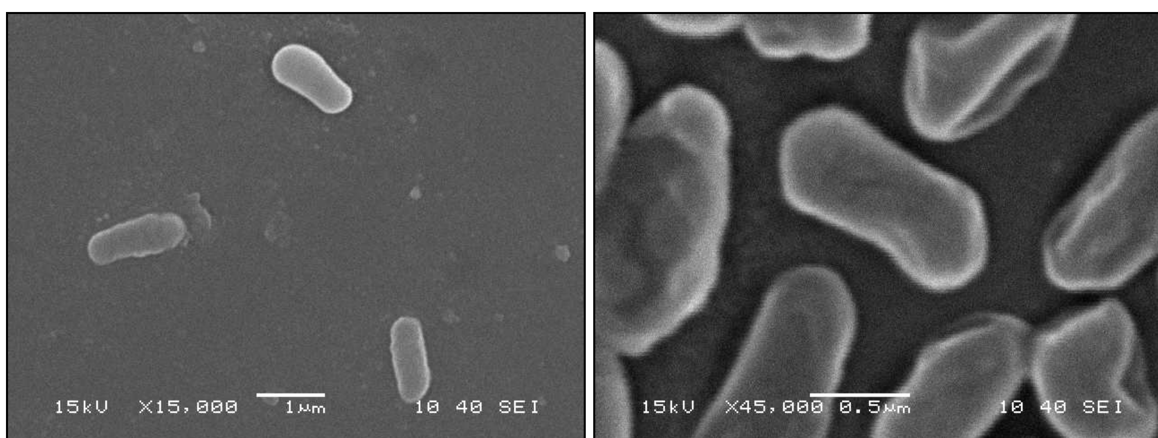

Figure 4.2: SEM pictures of *'WS'* and *'G'* bacterial cells

Table 4.3: Physiological test results of bacterial strains

Growth at Temperatures	**Observation**		**Growth at pH**	**Observation**		**Growth on NaCl (%)**	**Observation**	
	WS	**G**		**WS**	**G**		**WS**	**G**
4°C	-	-	pH 5.0	-	-	2.0	+	+
15°C	-	-	pH 6.0	+	+	4.0	+	+
25°C	+	+	pH 7.0	+	+	6.0	+	+
30°C	+	+	pH 8.0	+	+	8.0	+	+
37°C	+	+	pH 9.0	+	+	10.0	+	+
42°C	+	+	pH 10.0	+	+	11.0	+	+
55°C	-	-	pH 11.0	+	+	12.0	+	+
			pH 12.0	+	+			

‘+’: growth observed; **‘-’:** growth not observed

Biochemical tests were conducted to identify the bacterial strain (Table.4.4). The tests results are discussed below.

- Methyl-red test is performed to exam the mixed acid fermentation by ‘WS’ and ‘G’ bacterial cells. In this test bacterial cells were grown on Methyl red, Voges-Proskauer (MR-VP) broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. After incubation, the pH indicator Methyl Red is added to the broth that shows yellow color which indicate pH above 6.0. So it is considered as negative result.
- Voges-Proskauer test is done to determine whether an organism can produce acetylmethylcarbinol (acetoin) from fermentation of glucose. The cultures are incubated in Clark and Lubb's medium which contain alpha-naphtol (5%) and potassium hydroxide (40%). After incubation it doesn’t produces any pink reddish colour which indicates negative result.

- Casein hydrolysis test is done to examine the production of exoenzyme *Casesase* which hydrolyses the Casein, a complex protein present in milk agar. '*WS*' and '*G*' inoculated plates didn't produce any clear zones which indicate the negative result.
- The citrate test performed to detects the ability of a microorganism to use citrate as the sole source of carbon. '*WS*' and '*G*' Bacteria are inoculated on a medium containing sodium citrate and a p^H indicator bromothymol blue is used. The culture tubes produced blue colour which indicated the production of enzyme *citritase*. Enzyme *citritase* breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH. This positive result indicated by change of the medium's color from green to blue.
- In nitrate reductases test broth of the '*WS*' and '*G*' strains remain colour less which indicates the bacterial cells are able to reduce nitrate (NO_3^-) to nitrite (NO_2^-) using anaerobic respiration and the result was positive.
- The indole test is a performed on bacterial species '*WS*' and '*G*' to determine the ability of the organism to split indole from the amino acid tryptophan. Yellow color in the surface alcohol layer of the '*WS*' and '*G*' strains inoculated broths represents the result as negative. It may be due to lack of production of intracellular enzymes *tryptophanase* that split indole from the amino acid tryptophan.
- Arginine test is performed to distinguish the *Pseudomonas* species from other microbial species. *Pseudomonas* species produces the enzyme, arginine dihydrolase. It releases ammonium from arginine which results in alkalinity pH indicated by pH indicator phenol red, that turns a dark pink color under oil as in contrast to the orange pink of arginine dihydrolase-negative

cultures. Here '*WS*' bacterial strain exhibited negative result and '*G*' strain exhibited positive results which indicated that '*G*' strain belongs to *Pseudomonas* species.

- Gelatin test is done to tests the ability of an organism to produce an exoenzyme, called *gelatinase* that hydrolyzes gelatin present which is present in the media. Both '*WS*' and '*G*' strains inoculated tubes have liquefied the solid gelatin present in the media. It represents positive result which indicated production of gelatinase enzyme to hydrolyse gelatin into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism.
- Starch hydrolysis is performed to tests the ability of '*WS*' and '*G*' strains to produce certain exoenzymes, including α-amylase or oligo-1,6-glucosidase, that hydrolyze starch. A clear zone around the '*WS*' bacterial colony observed which indicates that the organism has hydrolyzed starch by producing α-amylase or oligo-1,6-glucosidase. And the '*G*' bacterial strain has shown negative result.
- Catalase test is performed to check the ability of '*WS*' and '*G*' strains to produce enzyme *catalase* that breaks hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). When the '*WS*' and '*G*' strains were inoculated bubbling was seen which is due to the evolution of O_2 gas. It indicates that both the bacterial strains produce enzyme *catalase* and the test result was positive.
- In oxidase test broth of the '*WS*' and '*G*' strains colour changed to blue which indicates that bacterial cells are producing the enzyme *Cytochrome oxidase*. *Cytochrome oxidase* participates in the electron transport chain by transferring electrons from a donor molecule to oxygen. The colour change is due to the oxidation of a reducing agent, chromogenic present in oxidase reagent. So the test result for both the strains was positive.

- On McConkey agar the '*WS*' and '*G*' strains has grown by utilizing the peptone instead of lactose content of agar. So called non lactose fermentation (NLF) which was indicated by the formation of random circular colonies without change in colour to pink.
- Along with the biochemical tests some fermentation tests are also done to identify the acid production characteristics of the microorganism on various carbon sources which are discussed in Table 4.4.
- On observing the results of morphological tests, physiological tests and biochemical tests bacterial strain '*WS*' was considered as ***Brevibacterium casei*** and '*G*' as ***Pseudomonas aeruginosa.***

Table 4.4: Biochemical test results of bacterial strains

Test	WS	G	Acid Production from	WS	G
Methyl red test	-	-	Dulcitol	-	-
Voges Proskauer test	-	-	Trehalose	+	-
Casein hydrolysis	-	-	Sorbitol	+	-
Citrate	+	+	Raffinose	+	-
Nitrate	+	+	Melibiose	+	+
Indole	-	-	Salicin	+	-
Arganine	-	+	Sucrose	+	+
Gelatin hydrolysis	+	+	Rhamnose	+	-
Starch hydrolysis	+	-	Fructose	+	+
Esculin hydrolysis	+	-	Cellobiose	-	-
Catalase test	+	+	Galactose	+	+
Oxidase test	+	+	Inositol	+	-
Growth on McConkey	NLF	NLF			

'+': growth observed; **'-':** growth not observed; 'NLF': Non lactose fermentation

4.3.2. Fungal Characterization:

Fungal strain was characterized on the morphological basis such as size, shape, and color of spore formation and the texture of fungal growth. After 2-4 days of the growth of the fungi the spore bearing mycelia were then carefully sectioned teased out and stained on a slide and then observed with a light microscope. The fungi identified were confirmed by comparing their morphology and cultural characteristics with reported results [Guarro et al., 1999] and [Klich et al., 2003]. On examining the colonies and mycelia structure of fungal strain '*F*' the following characteristic features were observed. Figure 4.3 shows the conidial structure of fungal strain '*F*'.

Colonies were light yellowish to brown; reverse pink colored. Conidiophores were colorless. Vesicles were elongate with metulae and phialides, covering most of the surface. Conidia were globose to subglobose, smooth to echinulate, 4-5µm in diameter. These characteristics help to conclude that the isolated fungal strain belonged to the genus *Aspergillus*, species was *versicolor* and the strain was ***Aspergillus versicolor***. Culture was deposited in the microbial bank at MTCC, Chandigarh with a reference number **MTCC10950**.

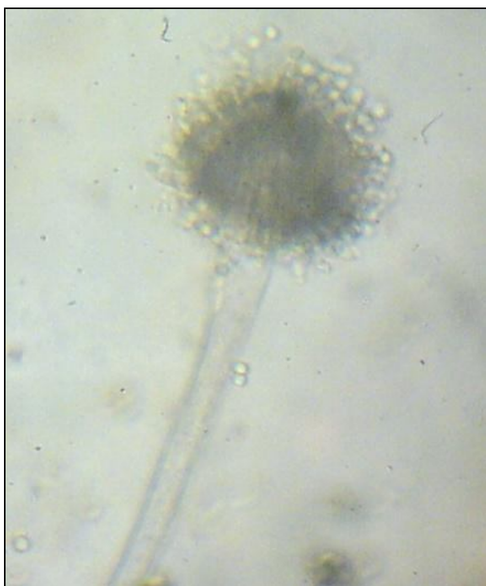

Figure 4.3: Microscopic image of *A. versicolor* conidia

Chapter-5

Optimization of Process Parameters

Biodegradation of petroleum hydrocarbon in the environment is found to be comparatively slow because it is influenced by a number of factors which include the microbial community which degrades the hydrocarbons, temperature and nutrient availability [Ekpo et al., 2008]. In the present study, process parameters like temperature, pH and nutrients effect on the growth of microorganisms was investigated. As, these parameters plays an important role in the growth of microorganisms and in production of organic compounds (Enzymes and Biosurfactants) [Dong. et. al., 2010], which plays an important role in the intracellular and extracellular activities and in uptake of hydrocarbons. The optimization experiments were carried out in conventional one factor at a time approach.

5.1. Temperature Optimization:

Among the environmental factors, temperature is one of the important factors controlling activity and survival of microorganisms as well as the rate of degradation [Leila and Hamidi, 1994]. Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community [Atlas, 1975].

The temperatures considered for optimization study are 20ºC, 25ºC, 30ºC, 35ºC and 40ºC. The cultures are maintained in five separate flasks at varying temperature. In this study a pH of 7.4 ± 0.2 is maintained, as it was maintained earlier at the time of isolation of microbial strains. Experiment is carried out in 100 ml conical flasks containing 50 ml MSM media with 0.5gms of petroleum oil sludge. The flasks were kept in shaker incubator at 120 rpm for 15 days. Biomass of bacterial cell was measured by taking the OD absorbance at 600nm wave length. Fungal strain was measured based on the mycelium dry weight basis. Figure 5.1 shows the *Pseudomonas aeruginosa, Brevibacterium casei* and *Aspergillus versicolor* biomass growth at different

temperature. By the end of the incubation period the *P. aeruginosa* biomass in culture flask incubated at 35ºC temperature was 1.64 g/L which produced highest biomass, *B. casei* and fungus *A. versicolor* incubated at 30ºC temperature were 0.43 mg/ml and 1.43 g/L respectively.

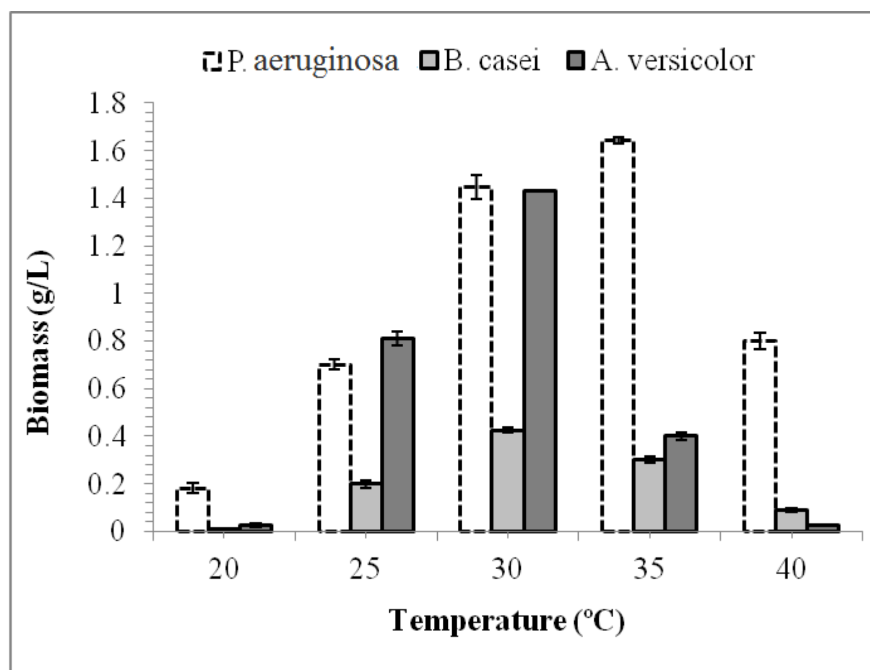

Figure 5.1: Biomass yield at different incubation temperatures

5.2. pH Optimization:

The pH considered for optimization ranges from 6 to 9. The cultures are prepared in 3 different sets containing 4 flasks in each set. Each was maintained at a different pH. The concentration of the broth and the carbon source was the same as in temperature optimization. The p^H was adjusted by adding 0.1N solution of NaOH and HCl. The flasks were sterilized, and kept in shaker incubator chamber at 120 rpm for 15 days and the temperature maintained at 30ºC for *B. casei* and *A. versicolor* and 35ºC for *P. aeruginosa* which is an optimum temperature obtained in the earlier study. At the end of the incubation period the *P. aeruginosa* biomass in

culture flask incubated at pH 8.0 yield the higher biomass i.e. 1.66 g/L, *B. casei* culture flask incubated at pH 7.0 and 8.0 have a similar reading i.e. 0.43 g/L and 0.42 mg/ml which produce highest biomass and 1.67g/L of biomass was produced by *A. versicolor* in the culture flask incubated at pH 7.0. Figure 5.2 shows the growth of microbial biomass at different pH.

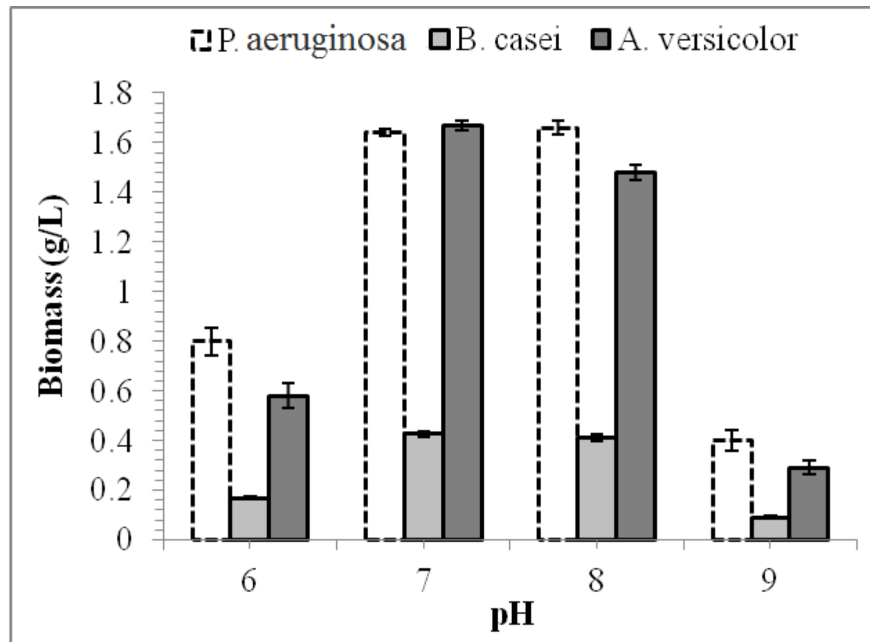

Figure 5.2: Microbial biomass yield at different pH

5.3. Nutrients Optimization:

5.3.1. Nitrogen source optimization:

Nutrients such as nitrogen, phosphorus, magnesium, and sulfur play a much more critical role in limiting the rate of hydrocarbon biodegradation. The ratio of available carbon to nitrogen in the bioremediation system has been identified as the significant parameter affecting degradation rate [Shewfelt et al., 2005].

Recent studies found that the hydrocarbon biodegradation rate depends on the nutrient concentrations in the contaminated site which provides important guidance for nutrient applications [Atlas, 1981]. Minor trace elements are usually present in sufficient amounts in the

environment [Ekpo & Udofia, 2008]. So, optimization of macro nutrients (nitrogen, phosphorus, magnesium, and Sulfur) to promote fungal biomass growth is very essential.

Optimization of nitrogen source (peptone, ammonium sulfate, ammonium chloride, and urea) was conducted used 5g/L concentration. The cultures were prepared in 4 different flasks and maintained at an optimized temperature and pH which are optimized earlier. The concentration of the broth and the carbon source was the same as in temperature and pH optimization. The yield of *P. aeruginosa* biomass using different nitrogen sources such as peptone, ammonium chloride, ammonium sulphate and urea were obtained as 1.52, 1.66, 1.65 and 1.05g/L, for *B. casei* were 0.68, 0.48, 0.43 and 0.48 mg/ml and for *A. versicolor* were 3.24, 0.92, 1.62 and 2.19 g/L respectively (Figure 5.3).Thus biomass yield was maximum with peptone and best with ammonium sulphate for *Aspergillus versicolor*.

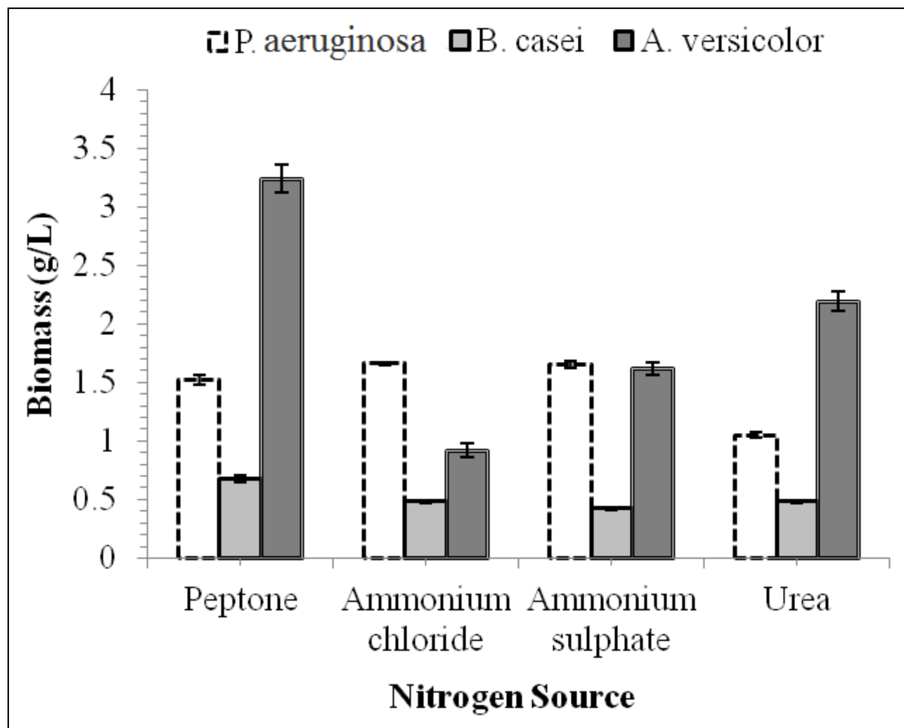

Figure 5.3: Biomass yield at different nitrogen sources

5.3.2. Nutrients dosage concentration optimization:

To optimize the dosage concentration of the nutrients such as nitrogen, phosphorus, magnesium, and sulfur one factor at a time approach was followed. The conditions followed for this study were, temperature 30ºC and 35ºC, pH 8.0, 7.5 and 7.0, nitrogen source ammonium chloride and urea which are optimized earlier and the concentration of the broth and the carbon source is the same as in earlier optimization study. After an incubation period of 15 days unforeseen result was obtained. Growth of microorganisms was not proper and in case of *A. versicolor* this mode of optimization process affected the biomass yield. Figure 5.4 represents the uneven growth of fungal mycelia.

Figure 5.4: Culture flasks of inactive mycelia - *A. versicolor*

5.4. Grey based Taguchi approach in Optimization:

The conventional one factor at a time approach of optimization is not only incapable in its interactions but often time consuming. The method becomes impractical when a large number of nutrients in the culture medium have to be considered since too many combinations have to be considered to optimize the growth medium composition. Taguchi established that statistically planned experiments are important for a successful design of parameter. Statistically designed experiments have been used earlier in industry. However, Taguchi's contribution adds a new aspect to this experimentation by providing a quick and accurate way of determining

optimization [Rao et al., 2008]. Taguchi established that statistically planned experiments are essential for a successful parameter design [Swan and Savage, 1998].

The statistically planned experiments (orthogonal matrix method) can be used to investigate the relationships between variables of nutrient medium components and optimize their concentrations for microbial cells growth and percentage degradation of TPH's. The orthogonal matrix method, as a result of the suitable design of factors, can give effective responses and reduce performance variation [Hoon. et al., 2004] [Wang. et al., 2012].

5.4.1. Design of orthogonal array (L_{16}):

The control factors showed in Table 5.1 which includes four different levels of sulphates, phosphates, nitrogen and magnesium were used as nutrient salt sources in the TPH degradation study.

Table 5.1: Factors and levels in the nutrients optimization study

Factor	Optimization parameters	Unit	Level 1	Level 2	Level 3	Level 4
A	Sulphates	g/L	0.4	0.6	0.8	1.0
B	Phosphates	g/L	0.4	0.6	0.8	1.0
C	Nitrogen	g/L	1.0	1.5	2.0	2.5
D	Magnesium	g/L	0.1	0.2	0.3	0.4

Taguchi method designed an orthogonal array of sixteen experiments (L_{16}) arranging the four input factors and corresponding levels in crisscross way. These sixteen experiments were performed under culture conditions which optimized earlier by one factor at a time approach, was with optimum temperature, pH and nitrogen source. The carbon source as petroleum oil sludge (0.5%) the flasks were kept under shaking at 120 RPM for a time period of 15 days. Microbial biomass and residual oil % were taken for consideration and results of L_{16} experiments and their signal to noise ratio (S/N ratios) are given in Table 5.2.

Table 5.2: S/N ratios of Biomass and residual oil concentration obtained in L_{16} design

Exp. No.	Factors				Biomass concentration (g/L)			Residual oil (%)			S/N ratio of biomass			S/N ration of residual oil		
	A	B	C	D	G	WS	F	G	WS	F	G	WS	F	G	WS	F
1	0.4	0.4	1.0	0.1	0.862	0.630	1.425	25.77	36.00	22.61	-1.28	-4.01	3.08	-28.22	-31.13	-27.09
2	0.4	0.6	1.5	0.2	0.575	0.520	1.250	20.79	29.00	14.45	-4.80	-5.68	1.94	-26.36	-29.25	-23.20
3	0.4	0.8	2.0	0.3	2.258	0.860	2.285	07.94	25.60	14.58	7.07	-1.31	7.18	-18.00	-28.16	-23.28
4	0.4	1.0	2.5	0.4	1.952	1.050	2.390	07.27	28.90	15.52	5.80	0.42	7.57	-17.23	-29.22	-23.82
5	0.6	0.4	1.5	0.3	0.711	0.610	1.700	30.95	52.20	14.09	-2.96	-4.29	4.61	-29.81	-34.35	-22.98
6	0.6	0.6	1.0	0.4	0.812	0.600	2.225	41.63	29.90	13.99	-1.80	-4.44	6.95	-32.39	-29.51	-22.92
7	0.6	0.8	2.5	0.1	0.672	0.580	1.940	26.81	40.30	15.73	-3.45	-4.73	5.76	-28.57	-32.11	-23.93
8	0.6	1.0	2.0	0.2	1.444	0.920	2.545	39.35	26.10	12.71	3.19	-0.72	8.11	-31.90	-28.33	-22.08
9	0.8	0.4	2.0	0.4	0.821	0.550	2.025	13.17	27.30	19.10	-1.71	-5.19	6.13	-22.39	-28.72	-25.62
10	0.8	0.6	2.5	0.3	0.838	0.620	2.275	13.02	47.90	13.75	-1.53	-4.15	7.14	-22.29	-33.61	-22.77
11	0.8	0.8	1.0	0.2	1.298	0.590	2.425	21.93	30.30	12.27	2.26	-4.58	7.69	-26.82	-29.63	-21.78
12	0.8	1.0	1.5	0.1	0.945	0.860	2.300	37.07	27.80	16.51	-0.49	-1.31	7.23	-31.38	-28.88	-24.35
13	1.0	0.4	2.5	0.2	0.799	0.610	2.195	15.21	54.30	18.66	-1.94	-4.29	6.83	-23.64	-34.70	-25.42
14	1.0	0.6	2.0	0.1	1.047	0.520	2.025	29.62	43.00	17.10	0.39	-5.68	6.13	-29.43	-32.67	-24.66
15	1.0	0.8	1.5	0.4	1.202	0.580	1.980	25.06	30.40	13.79	1.59	-4.73	5.93	-27.98	-29.66	-22.79
16	1.0	1.0	1.0	0.3	1.706	0.660	2.335	22.35	35.20	13.14	4.63	-3.61	7.37	-26.99	-30.93	-22.37

5.4.2. Normalization of experimental data:

From the results of L_{16} (4^4) orthogonal arrays (Table 5.2), two procedures were conducted to obtain GRG (γ) as shown in Table 5.4. The response graph is based on the average values of the grey relational coefficient. Accordingly, optimal conditions were selected from the response graph. The raw data of microbial biomass at different levels for specific factor are responsible in Table 5.2 Normalized data and normalized function of GRA (based on longer-the-better) as per *Equation 1* is summarized in Table 5.3. Similarly normalized function (residual oil percentage) of GRA based on smaller-the-better as per *Equation 2* is also illustrated in Table 5.3. Grey relational coefficients ($\xi_i(k)$) and their grey grades can then be obtained through *Equation 3* and *Equation 4* to evaluate the multiple performance characteristics, respectively.

Table 5.3: Response table for Grey relational analysis

Exp. No.	Normalized Values						G R Coefficient					
	Biomass			Residual oil			Σ Biomass			Σ Residual oil		
	G	WS	F	G	WS	F	G	WS	F	G	WS	F
1	0.83	0.79	0.86	0.54	0.36	1.00	0.38	0.39	0.37	0.48	0.58	0.33
2	1.00	1.00	1.00	0.39	0.12	0.21	0.33	0.33	0.33	0.56	0.81	0.70
3	0.00	0.36	0.20	0.02	0.00	0.22	1.00	0.58	0.71	0.96	1.00	0.69
4	0.18	0.00	0.12	0.00	0.11	0.31	0.73	1.00	0.81	1.00	0.81	0.61
5	0.92	0.83	0.65	0.69	0.93	0.18	0.35	0.38	0.43	0.42	0.35	0.74
6	0.86	0.85	0.25	1.00	0.15	0.17	0.37	0.37	0.67	0.33	0.77	0.75
7	0.94	0.89	0.47	0.57	0.51	0.33	0.35	0.36	0.52	0.47	0.49	0.60
8	0.48	0.25	0.00	0.93	0.02	0.04	0.51	0.67	1.00	0.35	0.97	0.92
9	0.85	0.94	0.40	0.17	0.06	0.66	0.37	0.35	0.56	0.74	0.89	0.43
10	0.84	0.81	0.21	0.17	0.78	0.14	0.37	0.38	0.71	0.75	0.39	0.78
11	0.57	0.87	0.09	0.43	0.16	0.00	0.47	0.37	0.84	0.54	0.75	1.00
12	0.78	0.36	0.19	0.87	0.08	0.41	0.39	0.58	0.73	0.37	0.87	0.55
13	0.87	0.83	0.27	0.23	1.00	0.62	0.37	0.38	0.65	0.68	0.33	0.45
14	0.72	1.00	0.40	0.65	0.61	0.47	0.41	0.33	0.56	0.43	0.45	0.52
15	0.63	0.89	0.44	0.52	0.17	0.15	0.44	0.36	0.53	0.49	0.75	0.77
16	0.33	0.74	0.16	0.44	0.33	0.08	0.60	0.40	0.76	0.53	0.60	0.86

5.4.3. Gray Relation Grade Calculation:

GRG is the overall representative of both the responses. The multi response optimization problem was transformed into a single response by using this approach. S/N ratio values obtained from the GRG values were shown in Table 5.4. The experimental trial number 3 of *P. aeruginosa* had a higher grey relational grade, 0.9814, than other experimental trials and the experimental trial number 6 had a lower grey relational grade, 0.3506. The experimental trial number 4 of *B. casei* had a higher grey relational grade, 0.9510 than other experimental trials and the experimental trial number 13 had a lower grey relational grade, 0.4190 and incase of fungal strain experimental trial number 8 of *A. versicolor* had a higher grey relational grade, 0.9609 than other experimental trials and the experimental trial number 1 had a lower grey relational grade, 0.3498. Normally, the larger the grey relational grade, the closer the product quality to the ideal value.

Table 5.4: S/N ration and GRG ranking of GRG values

Experiment No	GRG (γ)			S/N Ratio			Gray ranking		
	G	WS	F	G	WS	F	G	WS	F
1	0.429	0.483	0.350	-7.351	-6.321	-9.119	10	11	16
2	0.446	0.571	0.518	-7.013	-4.867	-5.713	9	6	14
3	0.981	0.791	0.702	-0.167	-2.036	-3.073	1	3	7
4	0.867	0.907	0.710	-1.240	-0.848	-2.975	2	1	5
5	0.386	0.363	0.587	-8.268	-8.802	-4.627	14	15	10
6	0.351	0.570	0.710	-9.094	-4.883	-2.975	16	7	6
7	0.407	0.427	0.558	-7.808	-7.391	-5.067	13	12	11
8	0.429	0.819	0.961	-7.351	-1.734	-0.346	11	2	1
9	0.557	0.620	0.493	-5.083	-4.152	-6.143	5	5	15
10	0.561	0.386	0.741	-5.021	-8.268	-2.604	4	13	4
11	0.503	0.559	0.922	-5.969	-5.052	-0.705	7	8	2
12	0.378	0.725	0.637	-8.450	-2.793	-3.917	15	4	9
13	0.525	0.355	0.548	-5.597	-8.995	-5.224	6	16	12
14	0.422	0.393	0.536	-7.494	-8.112	-5.417	12	14	13
15	0.467	0.555	0.653	-6.614	-5.114	-3.702	8	9	8
16	0.568	0.502	0.805	-4.913	-5.986	-1.884	3	10	3

The optimum nutrients concentration required to yield higher biomass and to attain maximum TPH degradation by bacterial strain *P. aeruginosa* were, sulphates 0.4 g/L (A_1), phosphates 0.8 g/L (B_3), nitrogen 2.0 g/L (C_3) and magnesium 0.3 g/L (D_3) and in case of *B. casei* were, sulphates 0.4 g/L (A_1), phosphates 1.0 g/L (B_4), nitrogen 2.5 g/L (C_4) and magnesium 0.4 g/L (D_4) and sulphates 0.6 g/L (A_2), phosphates 1.0 g/L (B_4), nitrogen 2.0 g/L (C_3) and magnesium 0.2 g/L (D_2) for *A. versicolor*.

5.4.4. ANOVA calculation for *Pseudomonas aeruginosa*:

Using GRG value, ANOVA was designed for identifying the significant factor. The results of ANOVA were presented in Table 5.5. Predominance of sulphates 0.4 g/L on maximum *Pseudomonas aeruginosa* biomass growth and higher percentage of TPH degradation is clearly represented. Subsequently phosphates 0.8 g/L, nitrogen 2.0 g/L and magnesium 0.3 g/L showed their responses in decreasing order. The mean of these response graph based on GRG with four variables and levels illustrated in Figure 5.5. This study using Grey based Taguchi enables to understand the influence of four different nutrients and their concentration on the growth of fungal biomass and TPH degradation efficiency of *A. versicolor*.

Table 5.5: ANOVA table of *P. aeruginosa*

Source	DOF	Seq SS	Adj MS	F	P	% Contribution	Rank
A	3	0.17155	0.05718	8.45	0.057	37.92	1
B	3	0.05664	0.01888	2.79	0.211	12.52	4
C	3	0.09707	0.03235	4.78	0.116	21.45	3
D	3	0.10683	0.03560	5.26	0.103	23.61	2
Residual Error	3	0.02030	0.00676			04.50	
Total	15	0.45238				100	

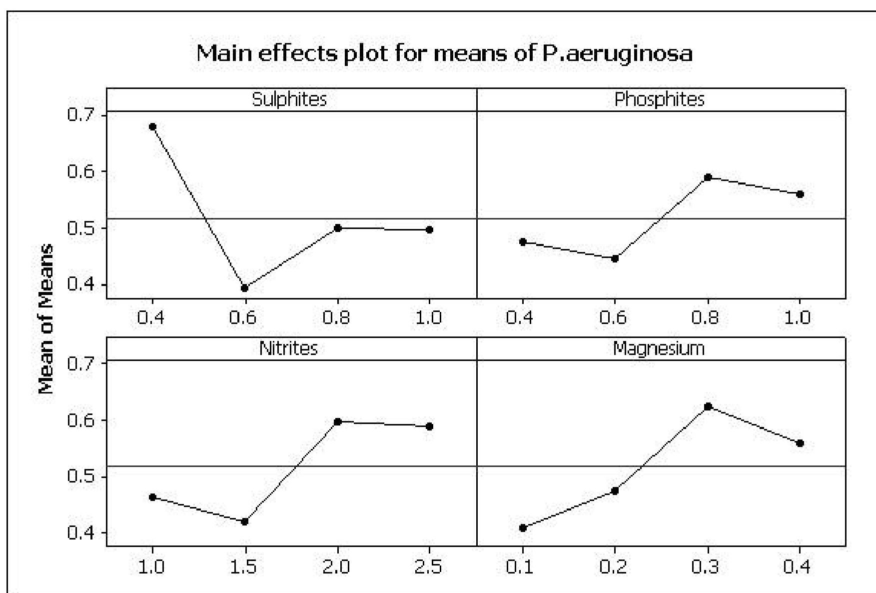

Figure 5.5: Main Effect Plot for means of *P. aeruginosa*

5.4.5. ANOVA calculation for *B. casei*:

Similarly, phosphates 1.0 g/L showed maximum effect on *B. casei* biomass growth and higher percentage of TPH degradation. Subsequently sulphates 0.4 g/L, magnesium 0.4 g/L and nitrogen 2.5 g/L showed their responses in decreasing order. The mean of these response graph based on GRG with four variables and levels illustrated in Figure 5.6. *B. casei's* ANOVA data of means was given in Table 5.6.

Table 5.6: ANOVA table of *B. casei*

Source	DOF	Seq SS	Adj MS	F	P	% Contribution	Rank
A	3	0.11413	0.03804	08.45	0.057	26.08	2
B	3	0.19843	0.06614	14.69	0.027	45.34	1
C	3	0.04734	0.01578	03.50	0.165	10.82	4
D	3	0.06422	0.02140	4.75	0.116	14.67	3
Residual Error	3	0.01351	0.00450			03.09	
Total	15	0.43763				100	

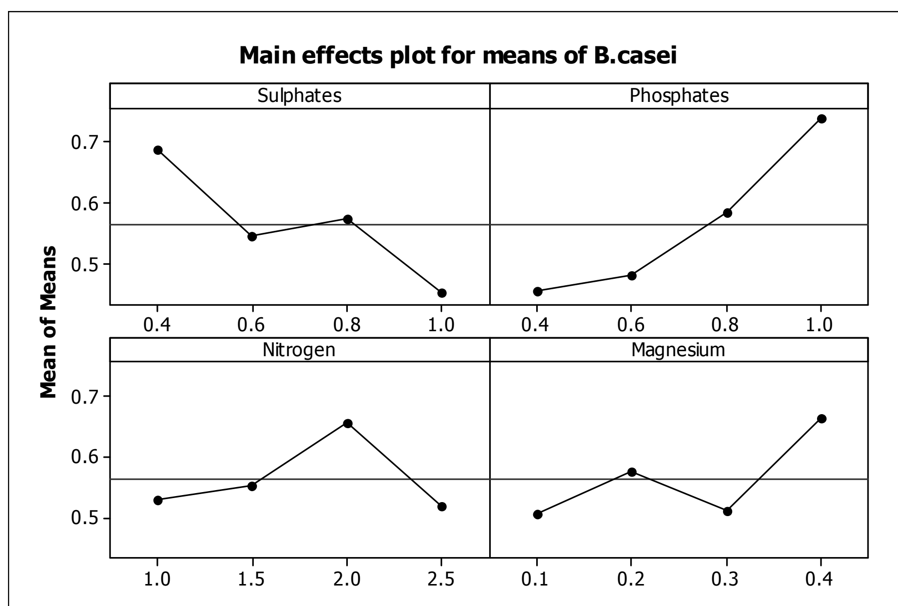

Figure 5.6: Main Effect Plot for means of *B. casei*

5.4.6. ANOVA calculation for *A. versicolor:*

Phosphates 1.0 g/L, magnesium 0.2 g/L, sulphates 0.6 g/L and nitrogen 2.0 g/L showed their subsequent responses in decreasing order on maximum biomass growth and higher percentage of TPH degradation by *Aspergillus versicolor* (Table 5.7). The mean of these response graph based on GRG with four variables and levels illustrated in figure 5.7. It enables to understand the influence of nutrients and their concentration on the growth of microbial biomass. One factor-at-a-time experimental approach showed a negative impact on the growth of *A. versicolor*. But optimization by Grey based Taguchi has shown a good response (Figure 5.8) in formation of higher biomass content with good TPH degradation efficiency.

Table 5.7: ANOVA table of *A. versicolor*

Source	DOF	Seq SS	Adj MS	F	P	% Contribution	Rank
A	3	0.04724	0.01574	2.20	0.266	12.40	3
B	3	0.17893	0.05960	8.35	0.057	46.95	1
C	3	0.02164	0.00721	1.01	0.497	05.68	4
D	3	0.11187	0.03729	5.22	0.104	29.35	2
Residual Error	3	0.02143	0.00714			05.62	
Total	15	0.38111				100	

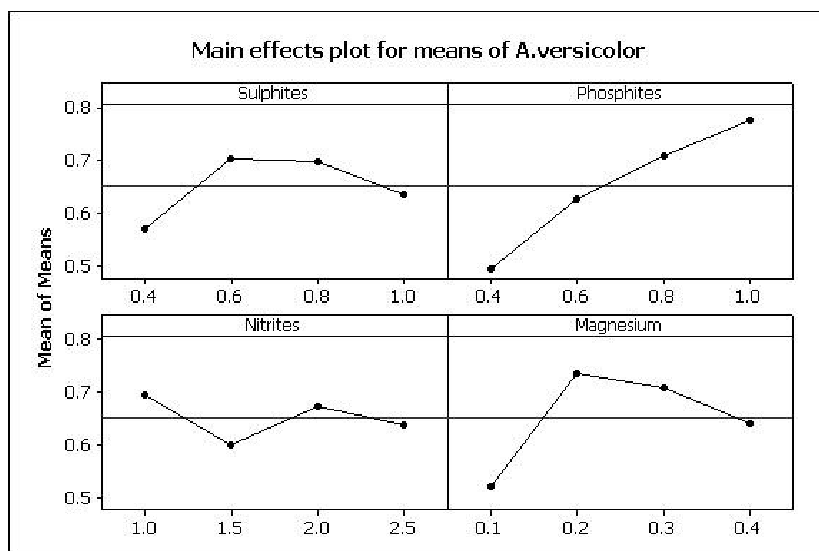

Figure 5.7: Main Effect Plot for means of *A. versicolor*

Figure 5.8: Culture flask with active mycelia of *A. versicolor*

Chapter-6

Biosurfactant Characterization

6. Biosurfactant Characterization:

Alkane droplets pose a problem between two immiscible surfaces for utilization of alkanes by microorganisms that prefer water soluble substrates immediately. Biosurfactants can consequently improve the bioavailability of alkanes to the microbial cells by increasing the area at the aqueous-alkane interface. Many micro-organisms produce biosurfactants during growth on alkane substrates [Swaranjit and Pooja, 2009]. This increases the rate of alkane dissolution, therefore increasing their availability for utilization by the microbial populations [Borjana et. al, 2002] [Gerson et. al, 1993].

6.1. Detection of biosurfactants:

Biosurfactant produced by *P. aeruginosa* and *B. casei* were detected by the Blue agar plate test suggested by Pinzon and Ju et. al [2009]. Figure 6.1 shows the formation of dark blue halos around the colonies formed due to the reaction between insoluble ionic pair of anionic biosurfactants with the cationic surfactant CTAB and the basic dye methylene blue. By this test we can conclude that the biosurfactants produced by *P. aeruginosa* and *B. casei* belongs to the class *Glycolipids.* Biosurfactant was produced in a MSM using glucose as a carbon source. Biosurfactants produced by the *P. aeruginosa* and *B. casei* were separated from the culture broth by centrifugation, acid precipitation and solvent extraction as per the method prescribed by Chandran and Das [2010].

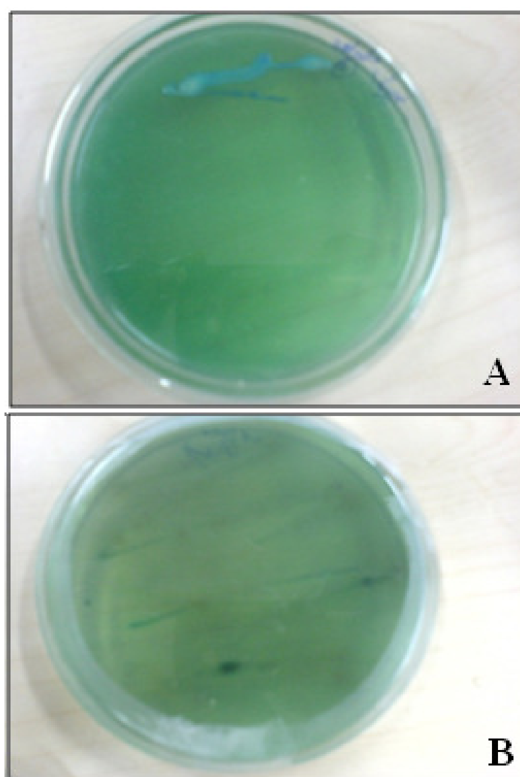

Figure 6.1: Positive result of blue agar plate test inoculated with (A) *Pseudomonas aeruginosa* and (B) *Brevibacterium casei*

6.2. CMC calculation of Biosurfactants:

Different concentrations of the biosurfactant were prepared by dissolving crude biosurfactant in millipore water. CMC of the biosurfactant was calculated by measuring the surface tension readings in surface tensiometer and the results were explained in Figure 6.2 and 6.3. Surface tension readings have shown that till a particular concentration the surface tension of the water was decreasing gradually and after a point it showed a linier straight. It indicates the formation of micelle due to the aggregation of surfactant molecules at the surface of the water. This point is referred as critical micelle concentration (CMC). CMC concentration of biosurfactant produced by *P. aeruginosa* and *B. casei* are 22.4 and 24.9 mg/L.

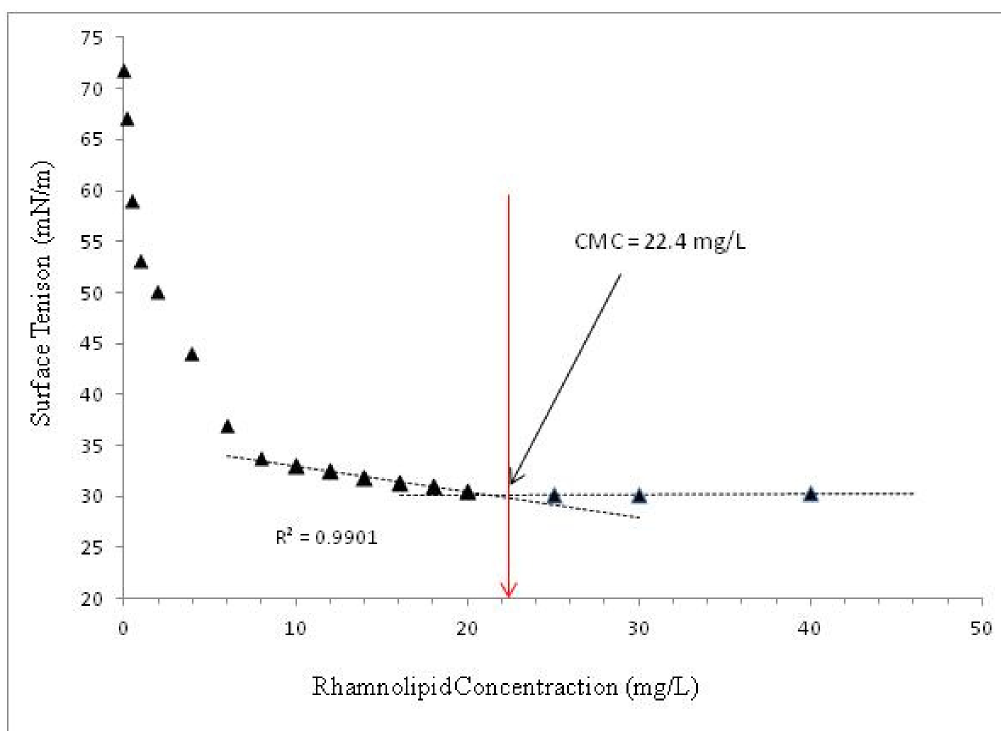

Figure 6.2: CMC value of biosurfactant produced by *P. aeruginosa*

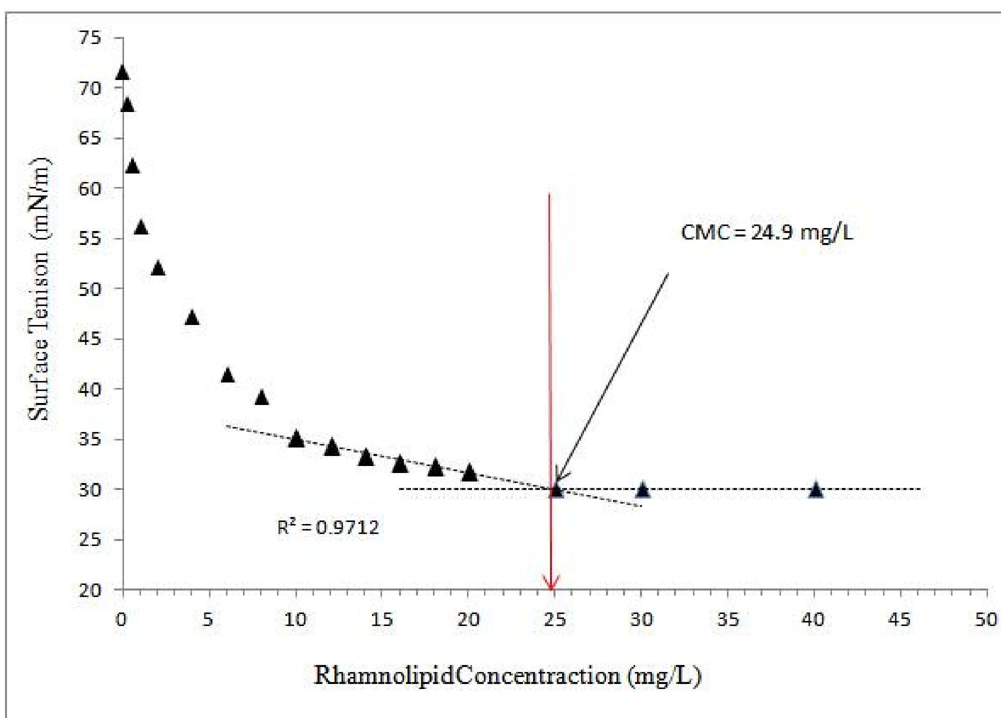

Figure 6.3: CMC value of biosurfactant produced by *B. casei*

6.3. Foam stability test of Biosurfactants:

Foaming is an important property of the biosurfactants. It was observed that the foam that was produced by shaking the cells free supernate of *P. aeruginosa* broth was stable for 115min and of *B. casei* for 102min. It is a clear indicative the biosurfactant is quite stable for a long period (Figure 6.4). Foaming is a function of surface area of the liquid and the interfacial tension. The biosurfactants reduces the surface tension of the liquid and there by reduces the work required for foam formation. This is the reason for sudden appearance of foams even by gentle shaking of the liquid system.

Figure 6.4: Foam produced by *P. aeruginosa* and *B. casei* biosurfactant

6.4. Emulsification activity of Biosurfactants:

The efficiency of the biosurfactants to make the oil available to the bacterial cells was studied by measuring the emulsification activity (E_{24} index) of the biosurfactants. The two concentration of biosurfactants used in this study are 22.4 and 24.9 mg/L (CMC value). Results of emulsification index were measured using *equation 5* and, *equation 6* was used to measure the

microbial adhesion to hydrocarbons and dispersed oil diameter (cm) was measured for oil spill test (Table 6.1). Tests made using crude oil proves that the biosurfactants are responsible for emulsifying and dispersing the oil slick and making them available to the microorganisms.

6.5. Biochemical composition of the biosurfactant:

Biochemical composition of the biosurfactant was analyzed by anthrone reagent method; lipid content was estimated adopting the procedure of Folch *et al*. and protein content of the biosurfactant is determined by using biuret method. Biosurfactant solution doesn't respond to biuret test it indicate that the absence of protein content in the biosurfactants. Presence of carbohydrates and lipids was detected (Table 6.1).

Table 6.1: Properties of Biosurfactants produced by isolated microorganisms.

S.No	Analysis	P. aeruginosa	B. casei	A. versicolor
1	General tests			
	Emulsification assay	83	71	0
	Blue agar plate method	+	+	-
	Oil dispersion test	8cm	7.2cm	-
	Foam stability testing	115 min	102 min	NF
	Hemolysis (%)	+	+	-
2	Interfacial properties			
	Surface tension	29.62 ± 0.71 mN/m*	30.26 ± 0.48 mN/m*	66.21± 0.82 mN/m*
	CMC	22.4 mg/L	24.9 mg/L	NA
3	Biochemical Properties			
	Carbohydrate	+	+	-
	Lipid	+	+	-
	Protein	-	-	-

'+': Present/ positive; **'-'**: Absent/ negative; **'NA'**: Not applicable; **'NF'**: Non foaming;
'*': Surface tension of the culture broth supernate

6.6. FTIR analysis of the biosurfactant:

The organic functional groups present in the biosurfactant sample were determined using FTIR analysis. The results of FTIR were compared with the data given by Saher et. al, [2011] and Olesja et. al, [2010]. In the FTIR analysis (Figure. 6.5 and 6.6) of biosurfactent produced by *P. aeurignosa* and *B. casei* we came to know that the biosurfactant sample contain free OH group in zone A, CH_3, CH_2 and C-H bond stretching in zone B and D, carbonyl bond stretching in zone C, C-O-C bond stretching in zone E and zone F and G represents the pyranyl I and II sorption bonds.

So, by this we can consider that the biosurfactant produced by the *P. aeurignosa* and *B. casei* belongs to *Rhamnolipids* and it suports the results of the earlier blue agar plate test.

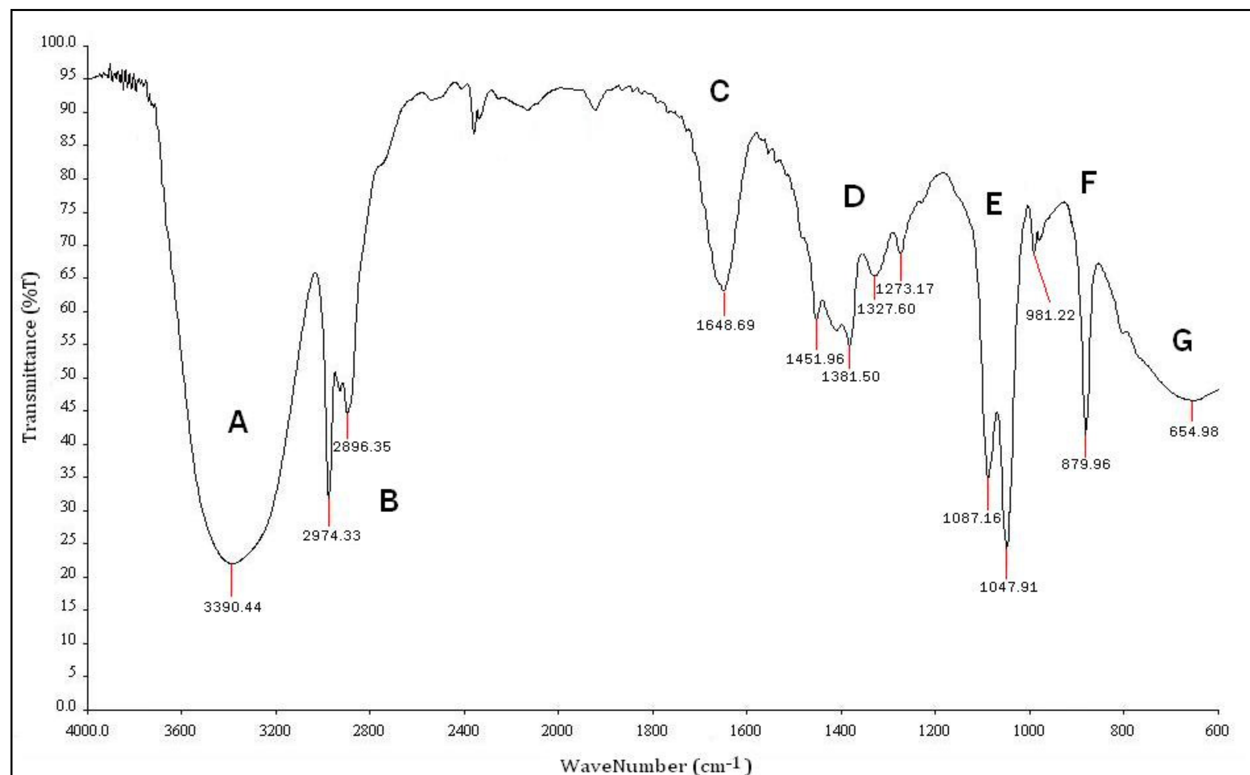

Figure 6.5: Foam produced by *P. aeruginosa* and *B. casei* biosurfactant

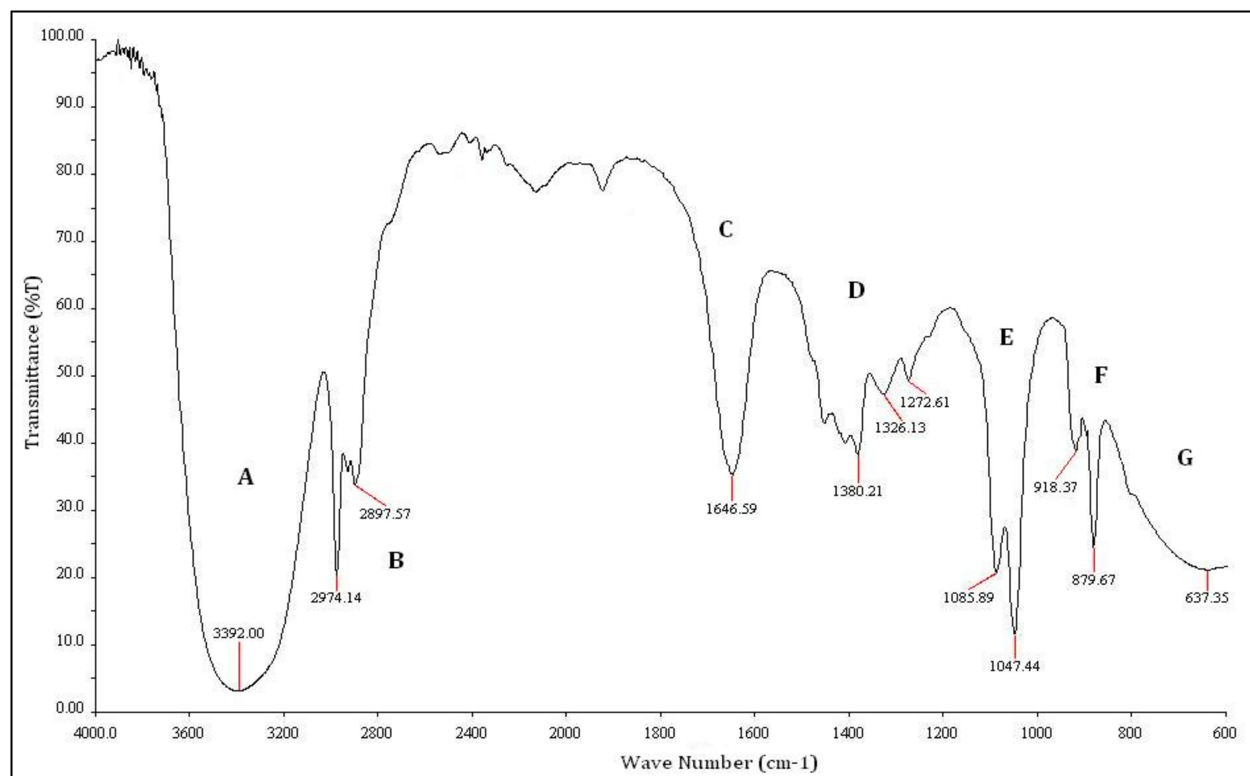

Figure 6.6: FTIR analysis of biosurfactant produced by *B. casei*

Chapter-7

TPH Degradation Study

Degradation of total petroleum hydrocarbon (TPH's) in petroleum oil was studied in a 100 ml nutrient broth under optimum culture conditions for a period of thirty days. The optimized conditions of temperature 35°C, pH 8.0, and ammonium chloride (as nitrogen source) for *P. aeruginosa* was obtained through experimental study. These experiments were conducted in nutrient media containing nitrogen (2 g/L), phosphates (0.8 g/L), sulphates (0.4 g/L) and magnesium (0.3 g/L). For *B. casei* are temperature 30°C, pH 7.5, urea (as nitrogen source) and the concentration of nutrients used were nitrogen 2.5 g/L, phosphates 1.0 g/L, sulphates 0.4 g/L and magnesium 0.4 g/L. similarly *A. versicolor,* through experimental investigation established optimum condition at 30°C temperature, pH 7.0, urea (as nitrogen source) in presence of nutrients such as nitrogen (2 g/L), phosphates (1.0 g/L), sulphates (0.6 g/L) and magnesium (0.2 g/L).

Experiments were conducted using six culture flasks which maintained three different set of conditions. Each flask filled with 100 ml nutrient media (supplement) contained 2gm of oil (as carbon source). Three different oils used in the investigation included oil sludge, light crude oil and heavy crude oil. The culture flasks were maintained at 120 rpm and samples were collected at an individual of 5 days to measure the biomass concentration and % oil degradation (% D).

7.1. TPH Degradation by B. casei:

Degradation of oil sludge (OS), light crude oil (LC) and heavy crude oil (HC) by bacterial strain B. *casei* was studied. By the end of thirtieth day culture flasks containing oil sludge, light crude oil and heavy crude exhibited 41.6%*,* 25.9% and 23.0% of oil degradation respectively. Subsequently the final biomass concentration of 0.48 g/L, 0.39 g/L and 0.29 g/L were measured for oil sludge, light crude oil and heavy crude oil respectively (Figure 7.1). The

rate of TPH degradation and biomass production was higher till day 10, later the biomass production and efficiency of TPH degradation was progressively decreased.

The optimized culture conditions were maintained throughout the incubation period. The nutrients composition of the culture medium was also prepared as per the earlier optimization results. The reasons for the failure were investigated by measuring the final nutrients concentration and dissolved oxygen content. Nutrient salts concentration was measured by using Himedia salts testing kits and DO content was measured by using DO meter and the results were listed in the Table 7.1.

Table 7.1: Nutrient profile of *B. casei* culture broth after biodegradation

Nutrients	Initial concentration	Final concentration	Color of reaction	% Consumed
Nitrogen	2.5	0.9	Pink	64
Phosphates	1.0	0.41	Yellow	59
Sulphates	0.4	0.18	Violet blue	55
Magnesium	0.4	0.17	Blue	57
DO (PPM)	5.21	0.15	--	97.12

The colorimetric and instrumental results of residual nutrient salts and D.O content after the degradation study by *B. casei* has revealed the importance of oxygen. *B. casei* was an aerobic strain and it has utilized 97.12% of dissolved oxygen content of the broth. As the oxygen content decreased the microbial growth decelerated. So, further degradation study with *B. casei* was maintained at airflow rate (0.5 L/min). On the contras, *P. aeruginosa* is facultative anaerobic bacteria could survive in very low oxygen concentrations, requires no additional aeration.

Degradation of hydrocarbon fraction in the petroleum oil (oil sludge, light crude oil and heavy crude oil) were analysed in GC and the results were compared with results of un-inoculated control sample. Figure 7.2, 7.3 and 7.4 are the hydrocarbon fraction of control oil (oil

sludge, light crude oil and heavy crude oil) and figure 7.5, 7.6 and 7.7 are residual fraction after degradation.

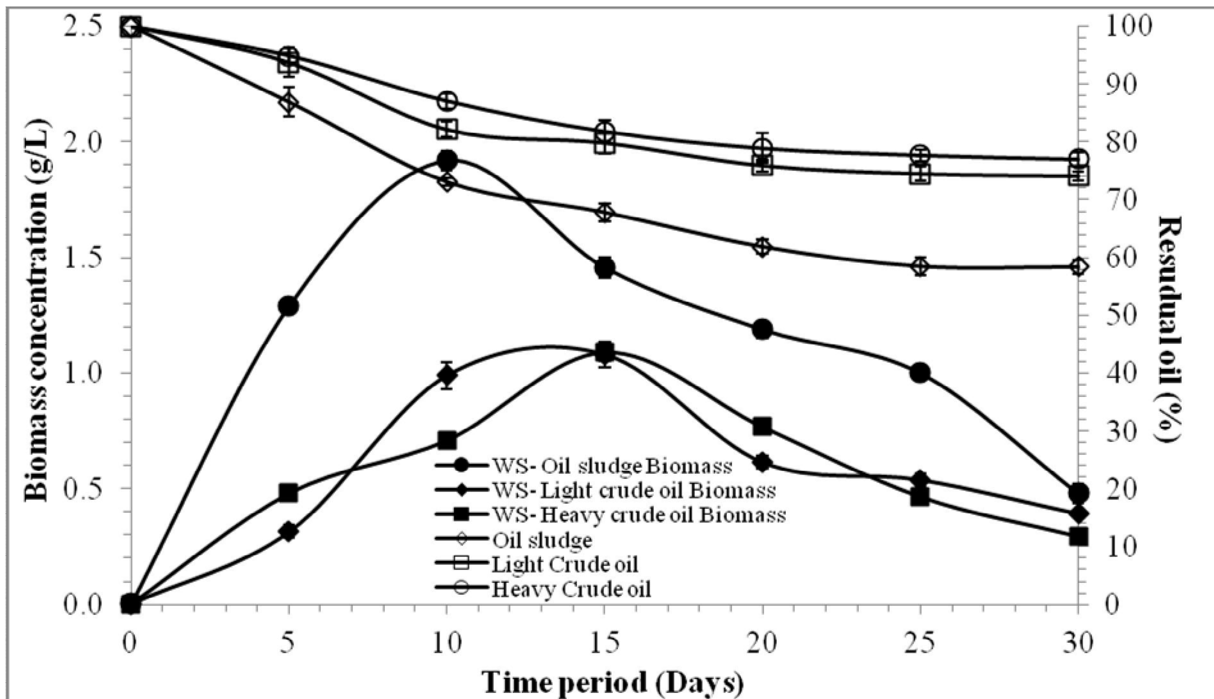

Figure 7.1: TPH degradation by B. casei without aeration

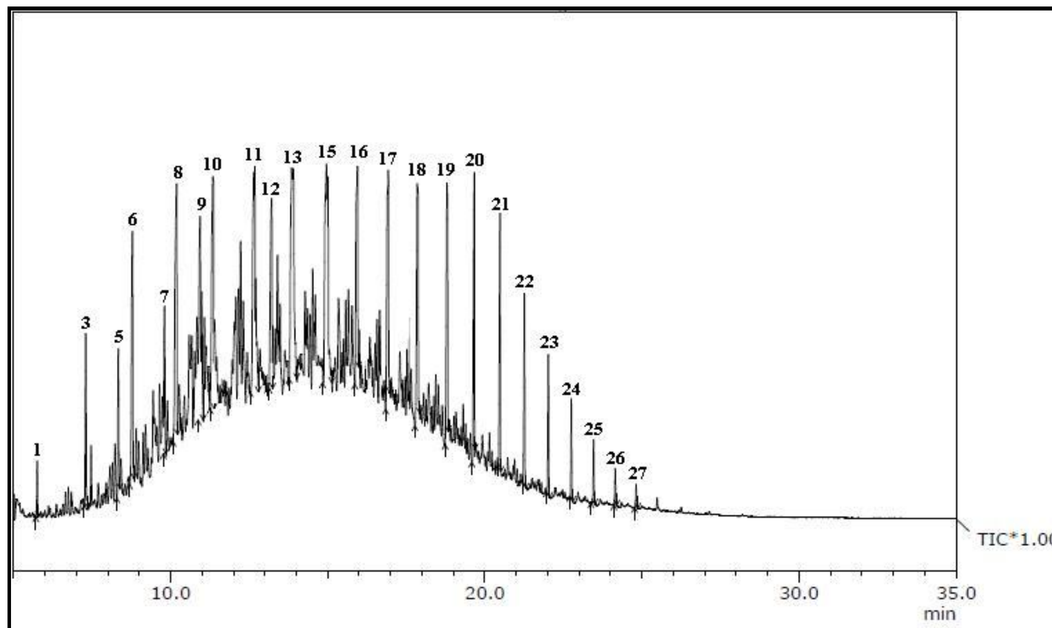

Figure 7.2: Hydrocarbon fraction in oil sludge (control sample)

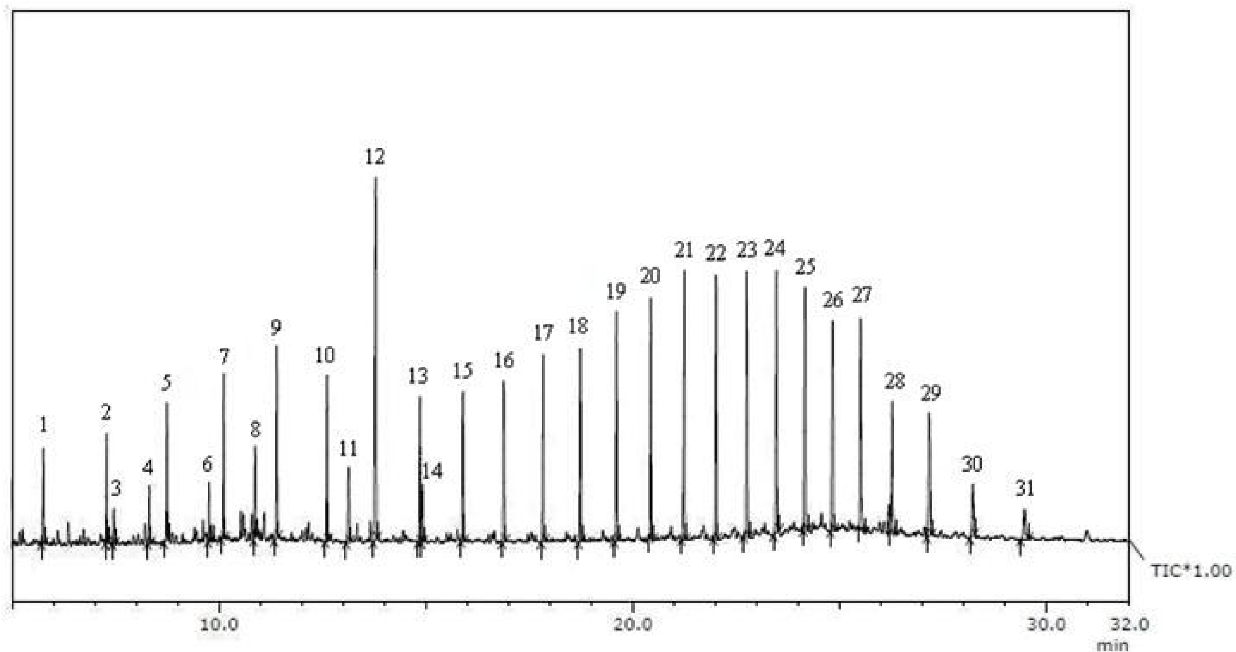

Figure 7.3: Hydrocarbon fraction in light crude oil (control sample)

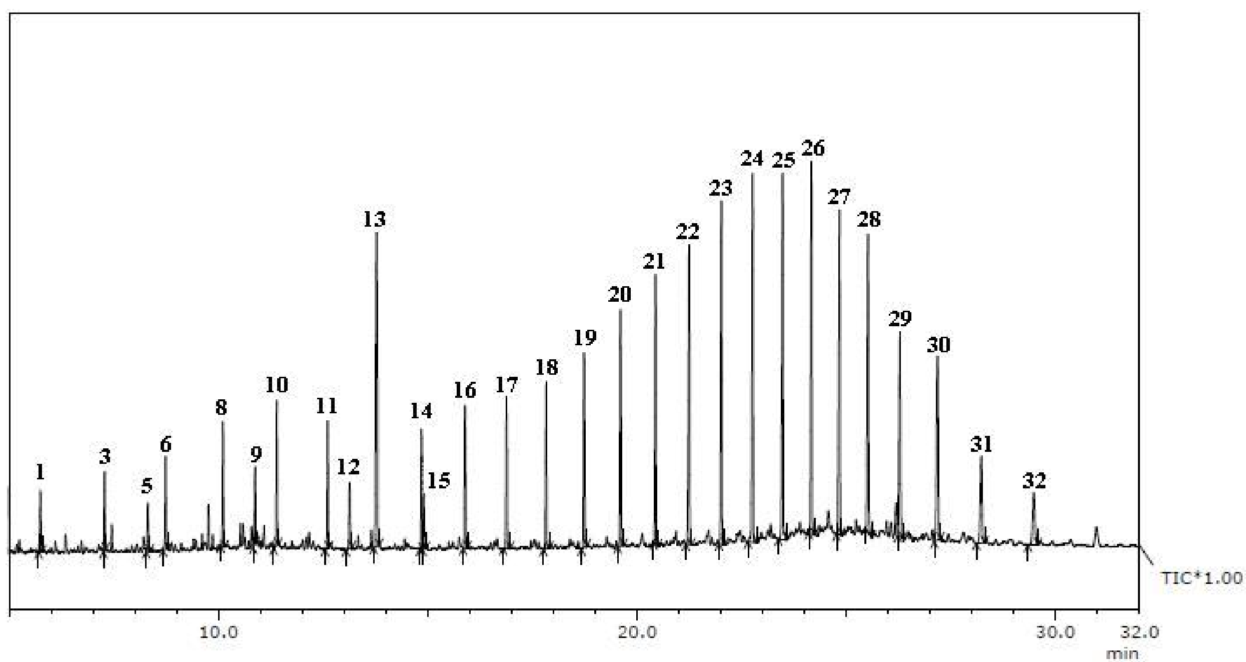

Figure 7.4: Hydrocarbon fraction in heavy crude oil (control sample)

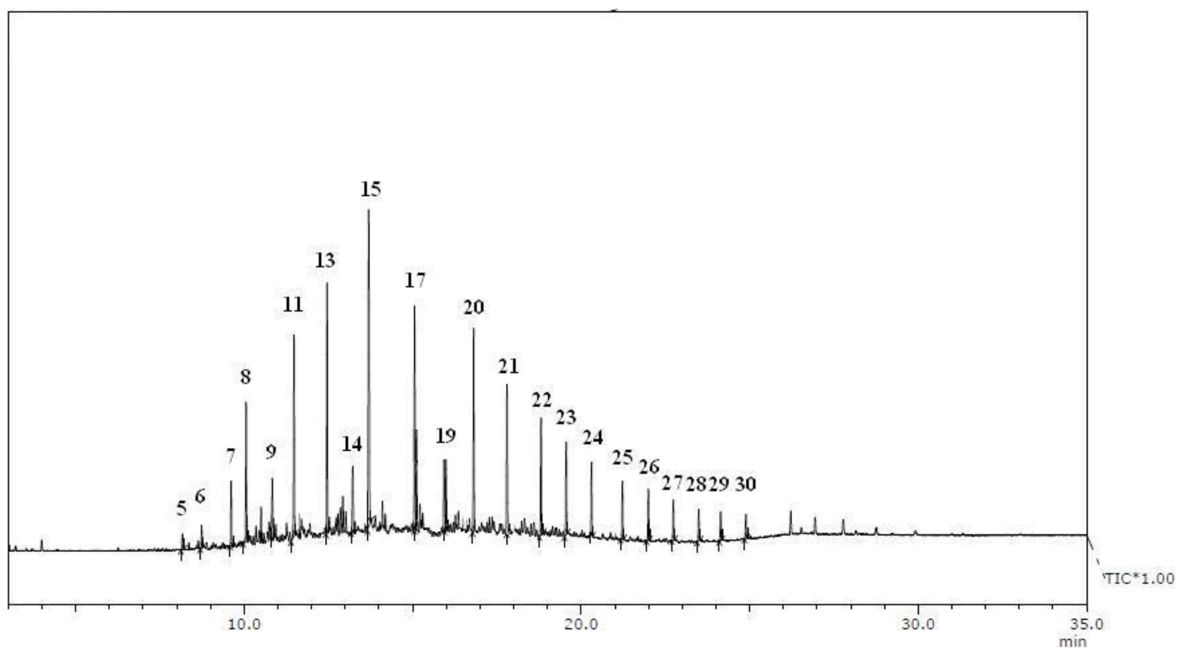

Figure 7.5: Residual hydrocarbon fraction in *B. casei* degraded oil sludge sample without aeration

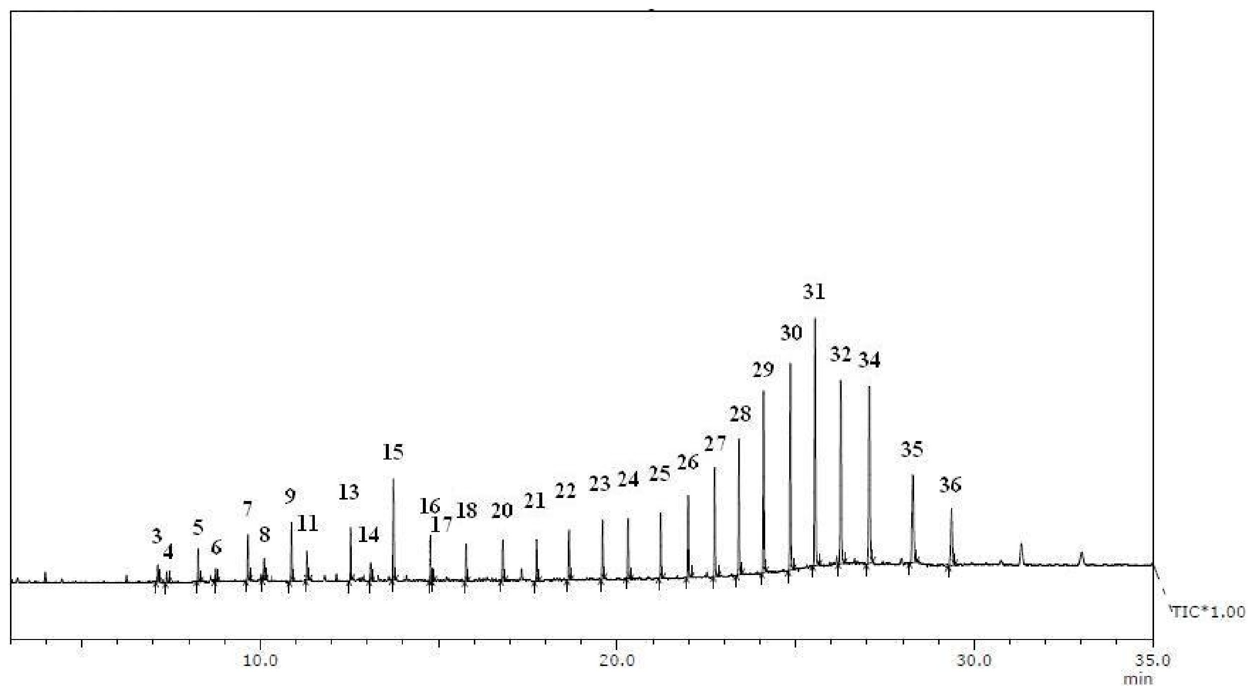

Figure 7.6: Residual hydrocarbon fraction in *B. casei* degraded light crude sample without aeration

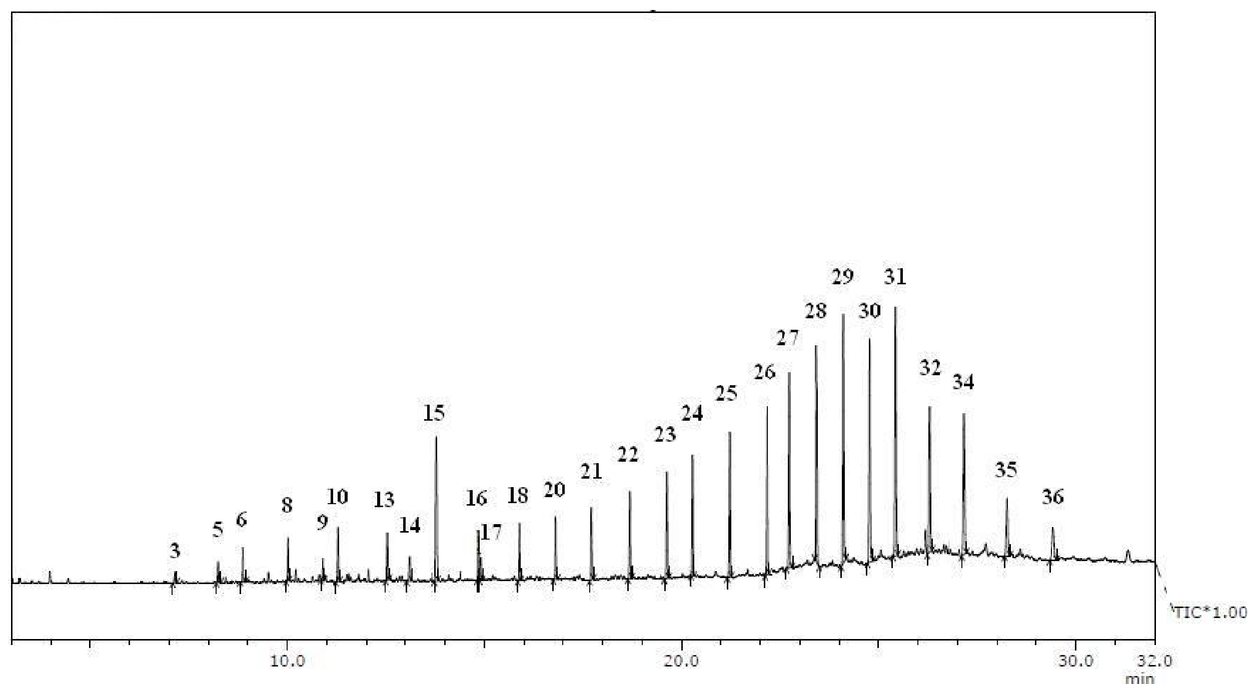

Figure 7.7: Residual hydrocarbon fraction in *B. casei* degraded heavy crude oil sample without aeration

7.2. TPH Degradation without addition of biosurfactant:

7.2.1. TPH Degradation of Oil sludge:

By the end of thirtieth day culture flask containing oil sludge as a carbon source has shown 98% degradation by *P. aeruginosa,* 91.0% by *B. casei* and 87.1%, by *A. versicolor*, with a final biomass concentration of 2.98, 2.69 and 2.71 g/L. significant biomass growth and decrease in oil concentration suggested that oil sludge is easily degraded by the 3 isolates under optimized culture conditions (Figure 7.8). It might be due to the higher API gravity i.e. 32.27 which made the microbes to accept easily. Oil sludge has emulsified in a very short time in bacterial culture flasks and the fungal mycelia contour was spherical and larger.

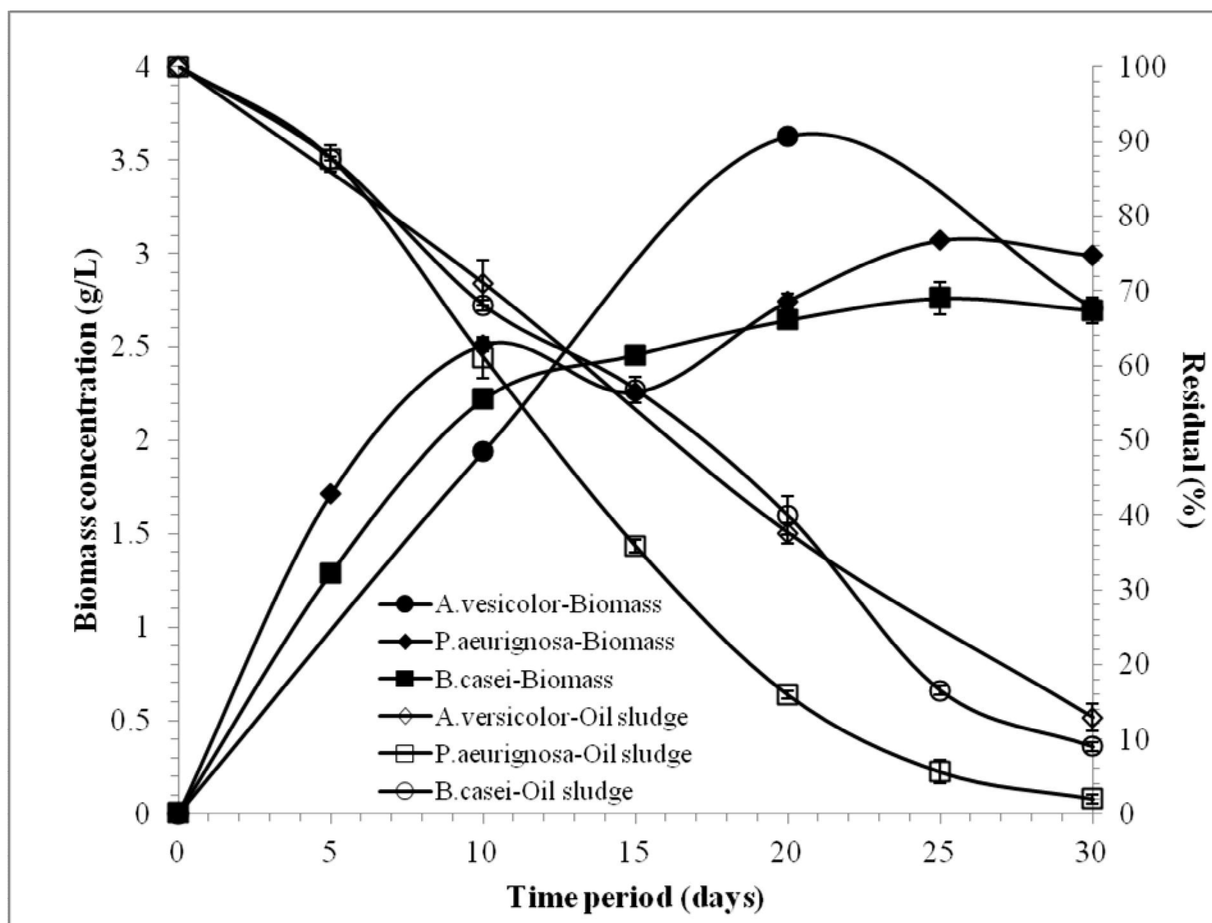

Figure 7.8: Oil sludge degradation profiles of isolated microorganisms for 30 days

Degradation of hydrocarbon fractions of oil sludge was studied by comparing with GC results of control samples. Samples for GC analysis were collected on the twentieth day of the study. Figure 7.2 denotes the GC results of control sample and figure 7.9, 7.10 and 7.11 for oil sludge after degradation. All three microorganisms have degraded almost all the hydrocarbon fractions due to their easy availability to the microorganisms. *B. casei* have degraded the hydrocarbon fractions very effectively when compared to the earlier degradation study without aeration. By this GC results it was conformed that oxygen plays an important role in the hydrocarbon metabolism of *B. casei*. Degradation of individual Hydrocarbon fractions was discussed in Table 7.2.

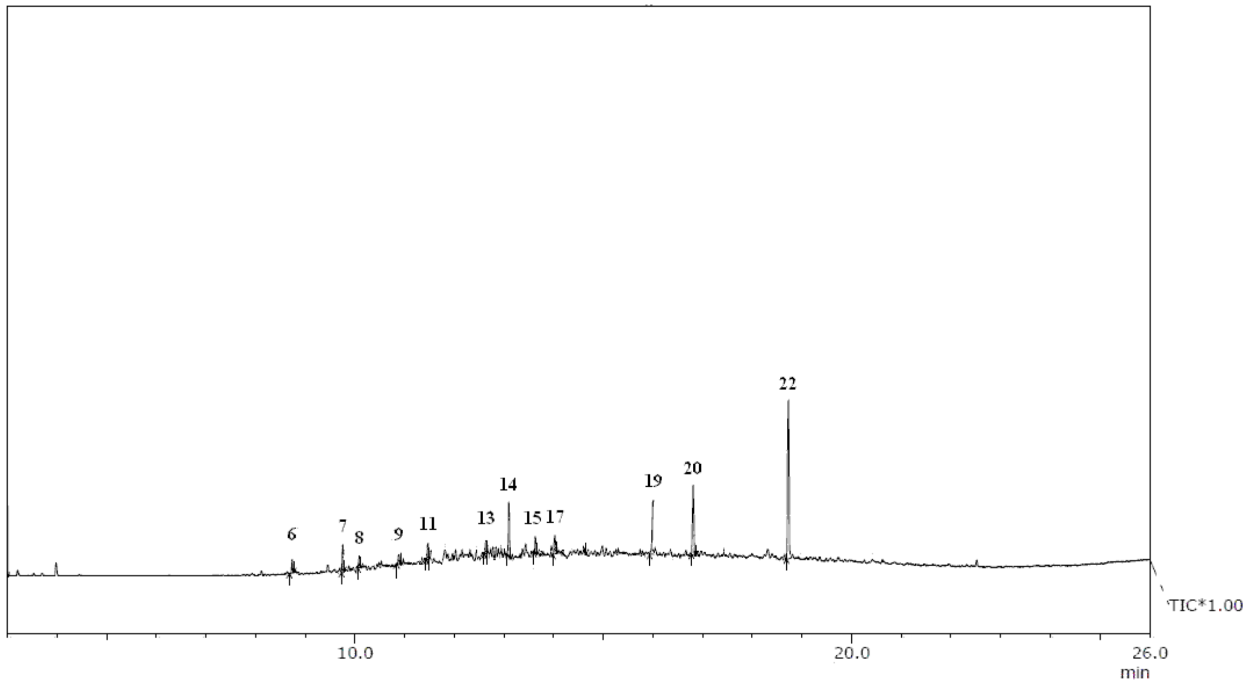

Figure 7.9: Residual hydrocarbon fraction in *P. aeruginosa* degraded oil sludge sample

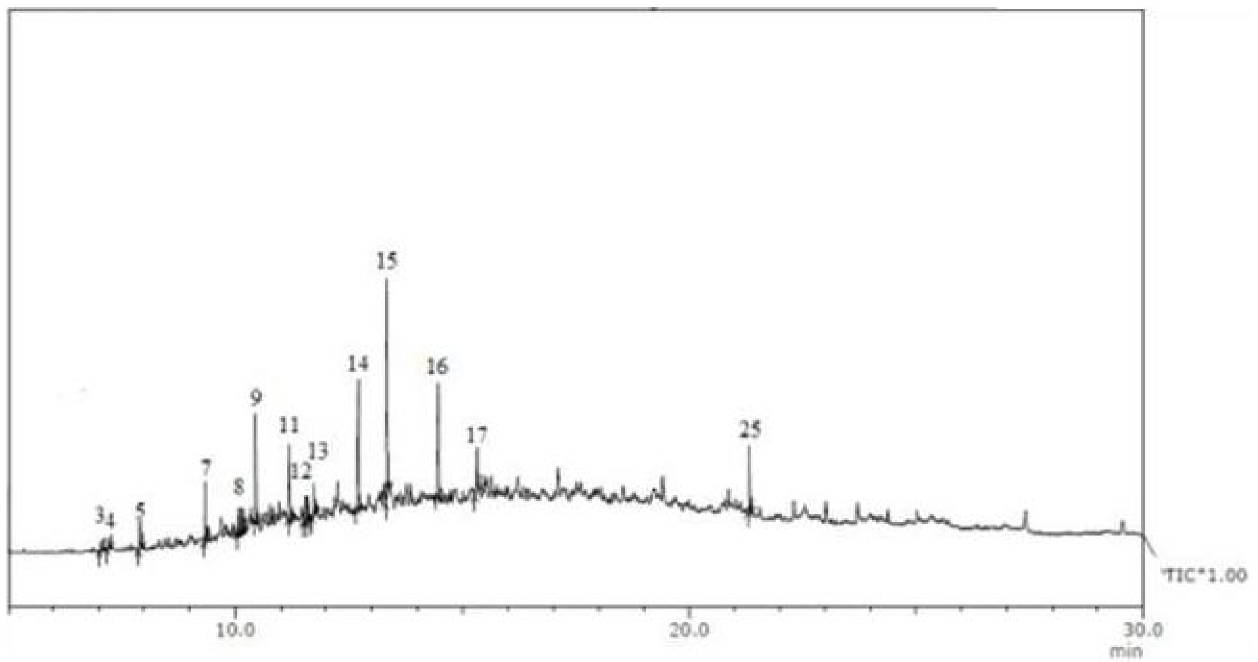

Figure 7.10: Residual hydrocarbon fraction in *B. casei* degraded oil sludge sample

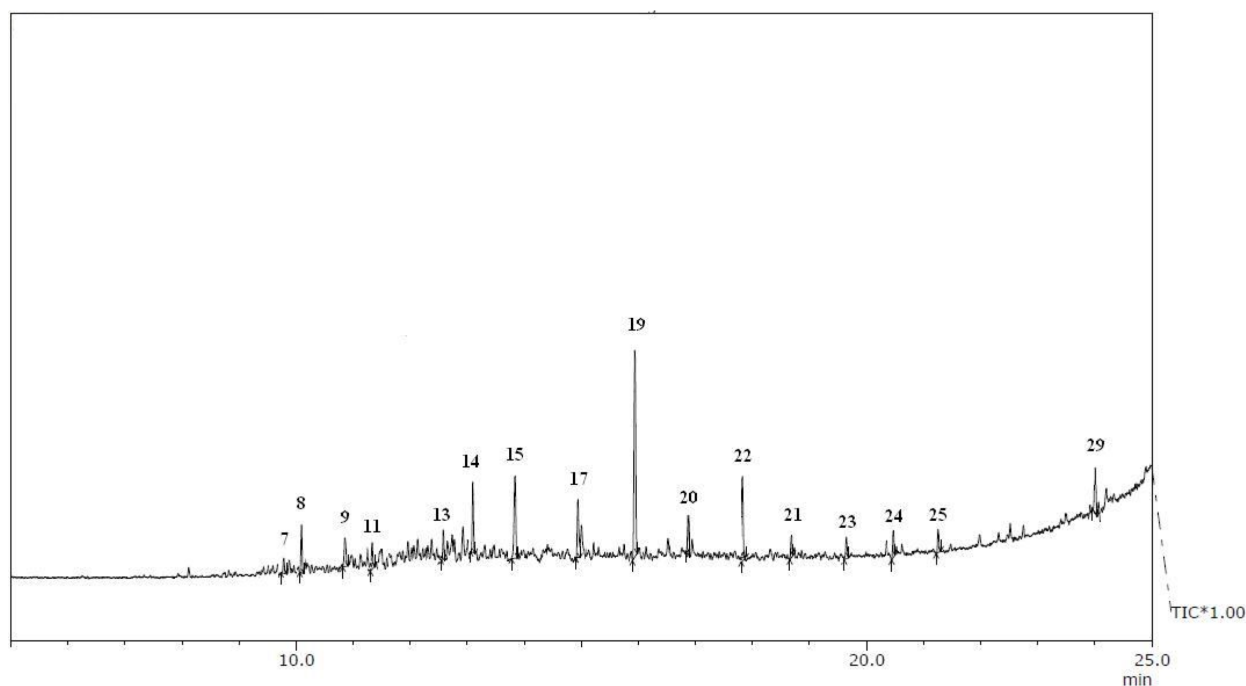

Figure 7.11: Residual hydrocarbon fraction in *A. versicolor* degraded oil sludge sample

7.2.2. TPH Degradation of Light crude Oil:

Degradation of light crude oil by three isolates seems to be similar to the degradation of oil sludge. It might be due to the similar API gravity between the oil sludge and light crude oil. The API gravity of light crude oil was 32.19. There is a constant increase in the biomass concentration till twentieth day and the concentration of oil was decreasing with respective time (Figure 7.12). By the end of thirtieth day culture flask containing light crude oil as a carbon source has shown 90.0% of degradation by *P. aeruginosa,* 83.9% by *B. casei* and 78.0% by *A. versicolor*, with a final biomass concentration of 2.28, 2.19 and 2.42 g/L. By observing the biomass growth and decrease in oil concentration in the culture flask it has been concluded that light crude oil can be degraded in an efficient way using three isolates under optimized culture conditions.

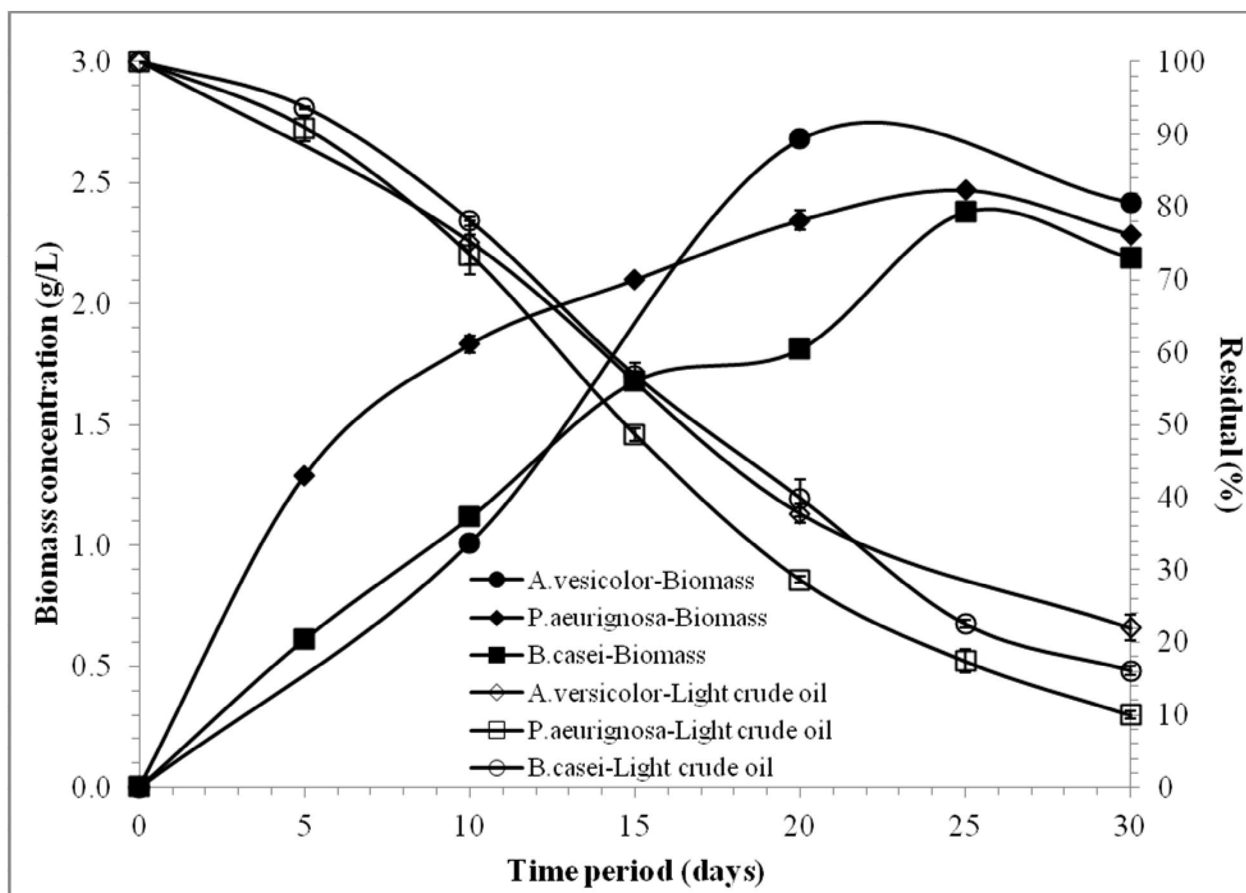

Figure 7.12: Light crude oil degradation profiles by isolated microorganisms for 30 days

Degradation of hydrocarbon fractions of oil sludge was studied by comparing with GC results of control samples. Samples for GC analysis were collected on the twentieth day of the study. From figure 7.13, 7.14 and 7.15, degradation of heavy crude oil fractions was studied. The profile of oil sludge hydrocarbon fractions was discussed in Table 7.3.

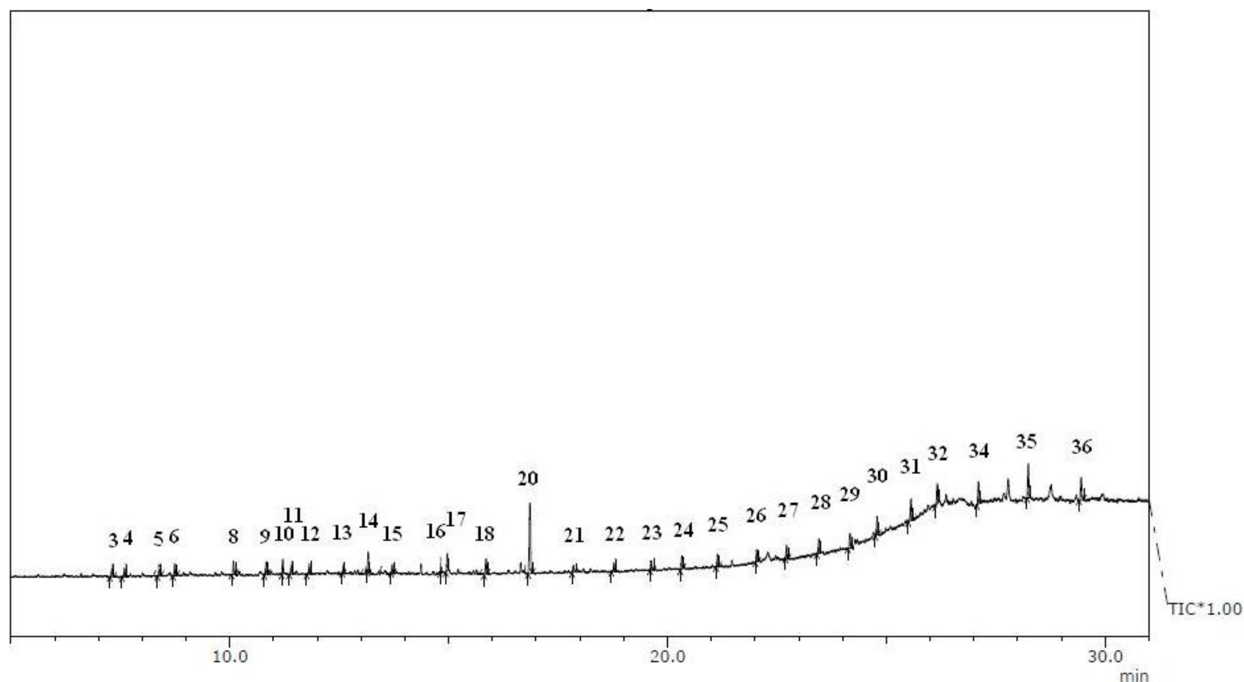

Figure 7.13: Residual hydrocarbon fraction in *P. aeruginosa* degraded light crude oil sample

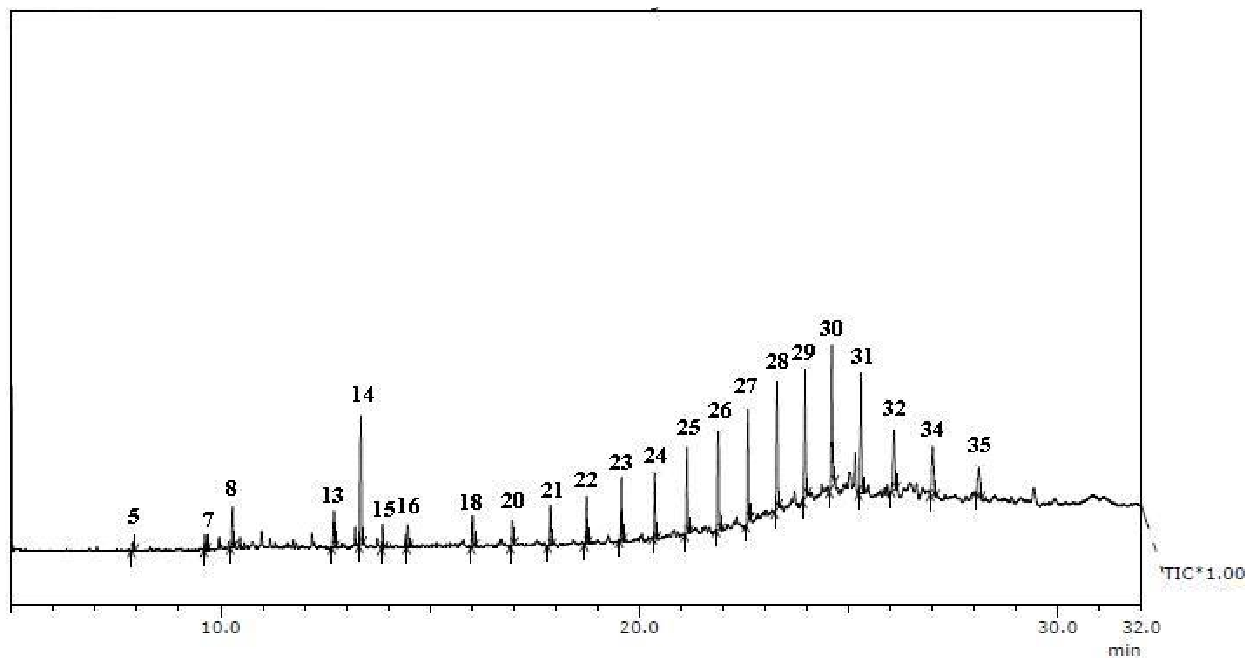

Figure 7.14: Residual hydrocarbon fraction in *B. casei* degraded light crude oil sample

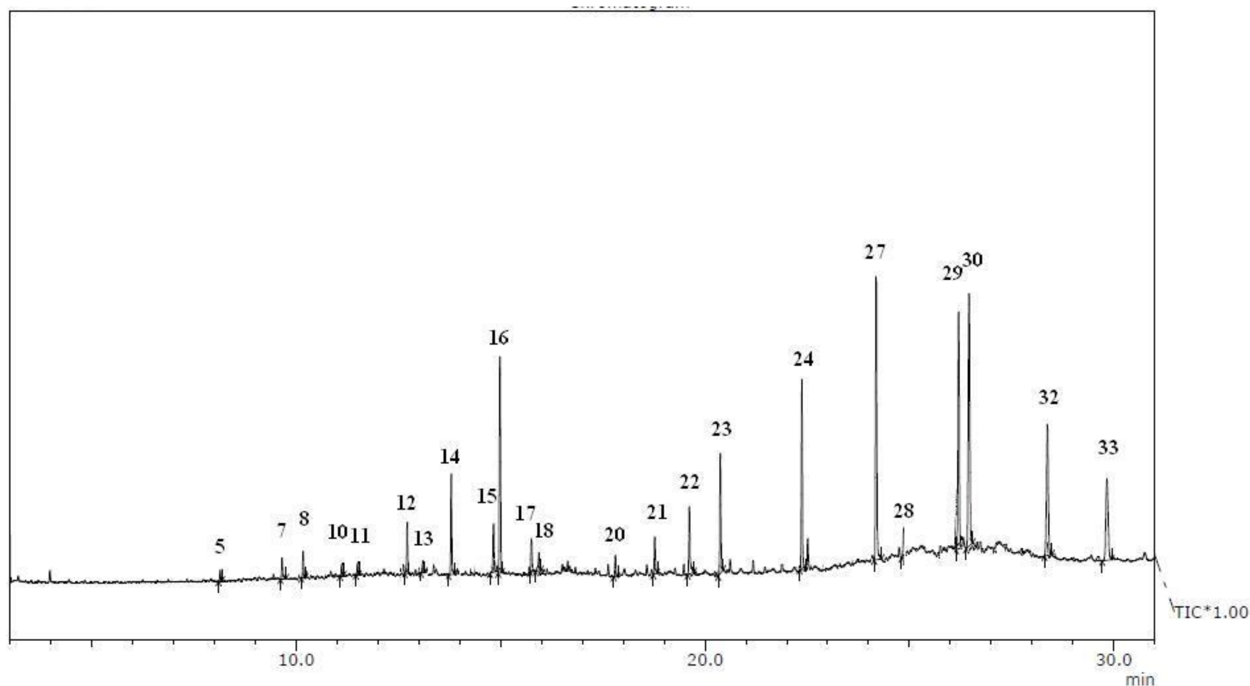

Figure 7.15: Residual hydrocarbon fraction in *A. versicolor* degraded light crude oil sample

7.2.3. TPH Degradation of Heavy crude Oil:

Heavy crude oil degradation is seen to be complicated. The response of microorganisms is slow when compared with oil sludge and light crude oil. Among the three microorganisms *Pseudomonas aeruginosa* have shown a faster response (Figure 7.16). The heavy crude (API gravity 31.82) oil was emulsified and the bacterial strains *B. casei* has shown a stepwise pattern in growth curve. In case of heavy crude oil degradation *A. versicolor* respond time was very slow as compared to bacterial strains. It might be due to complex nature of the crude oil.

By the end of thirtieth day 76.2% of heavy crude oil was degraded by *P. aeruginosa,* 71.5% by *B. casei* and 52.8% by *A. versicolor*, with a final biomass concentration of 2.0, 1.85 and 1.89 g/L respectively. By observing the biomass growth and decrease in oil concentration it

can be postulated that heavy crude oil biodegradation is a complex process as compared to other petroleum oils.

Degradation of hydrocarbon fractions of oil sludge was studied by comparing with GC results of control samples. Samples for GC analysis were collected on the twentieth day of the experimental period. Figure 7.4 denotes the GC results of control sample and figure 7.17, 7.18 and 7.19 for light crude oil after degradation. Degradation of individual Hydrocarbon fractions was discussed in Table 7.4.

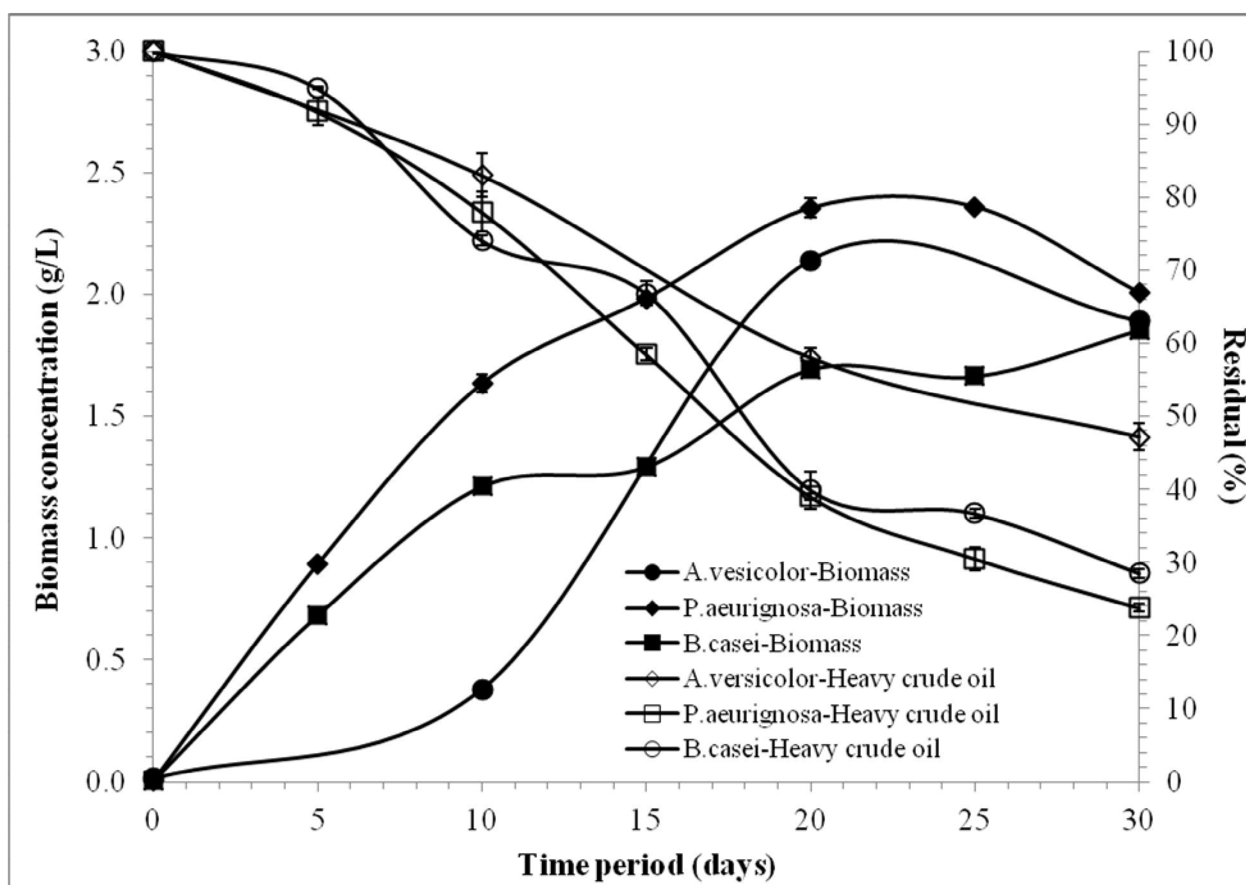

Figure 7.16: Heavy crude oil degradation profiles by isolated microorganisms for 30 days

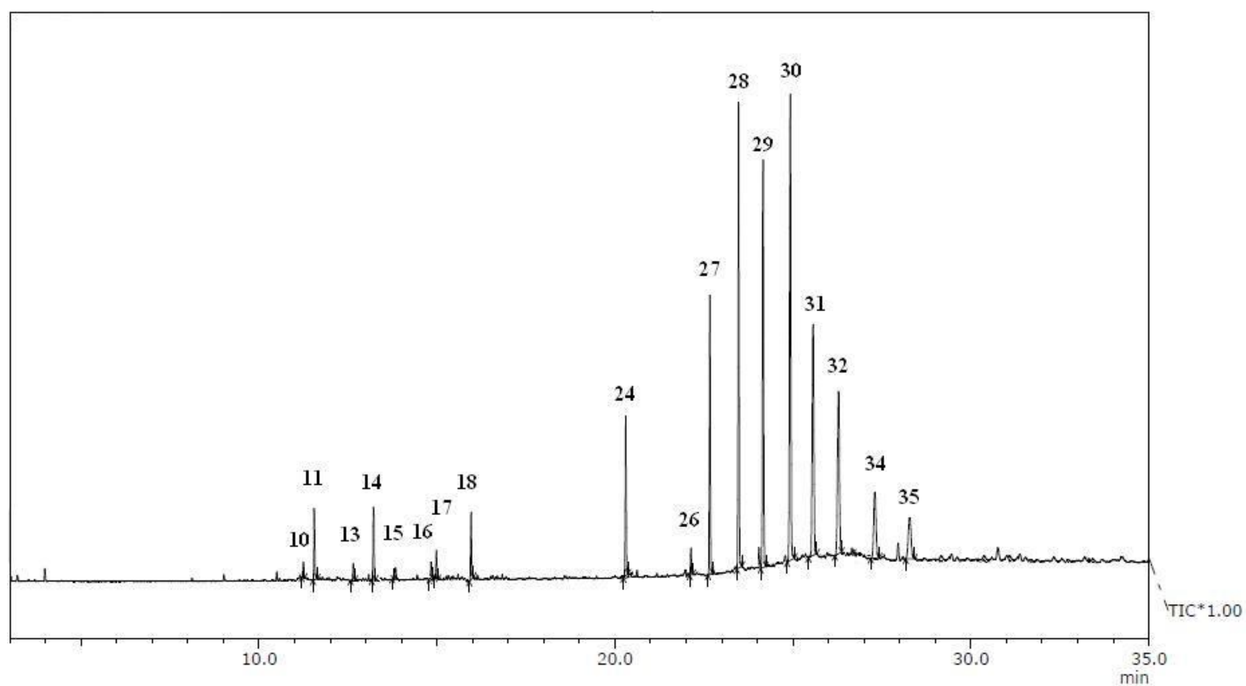

Figure 7.17: Residual hydrocarbon fraction in *P. aeruginosa* degraded heavy crude oil sample

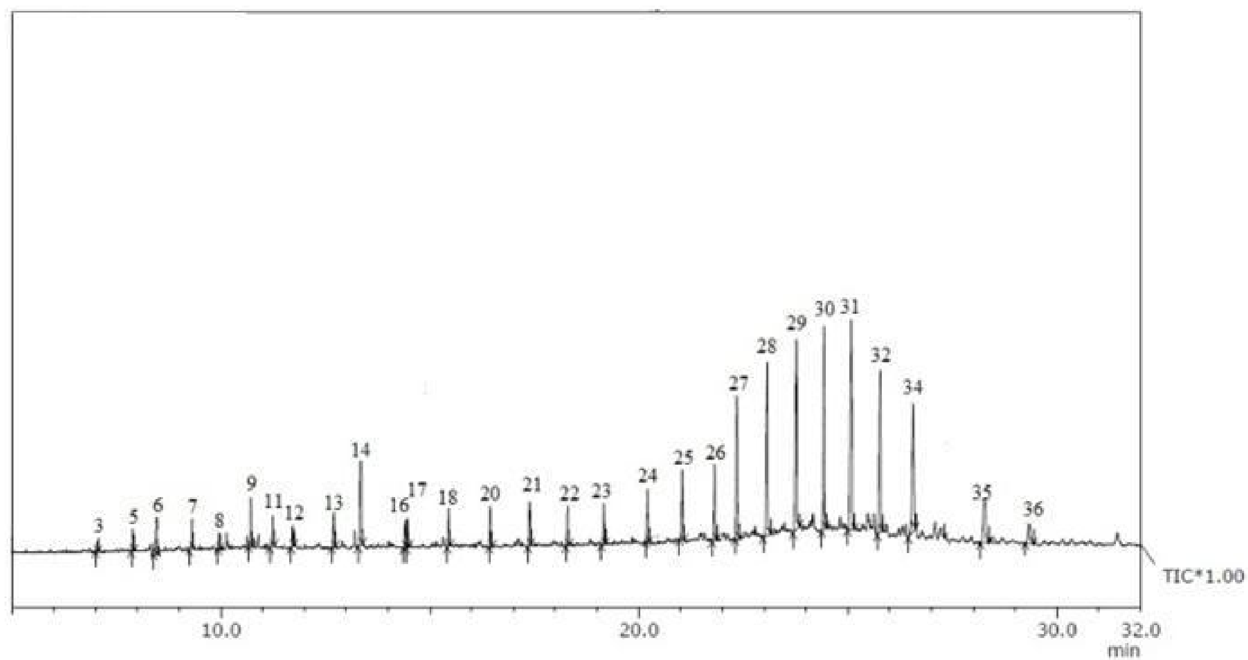

Figure 7.18: Residual hydrocarbon fraction in *B. casei* degraded heavy crude oil sample

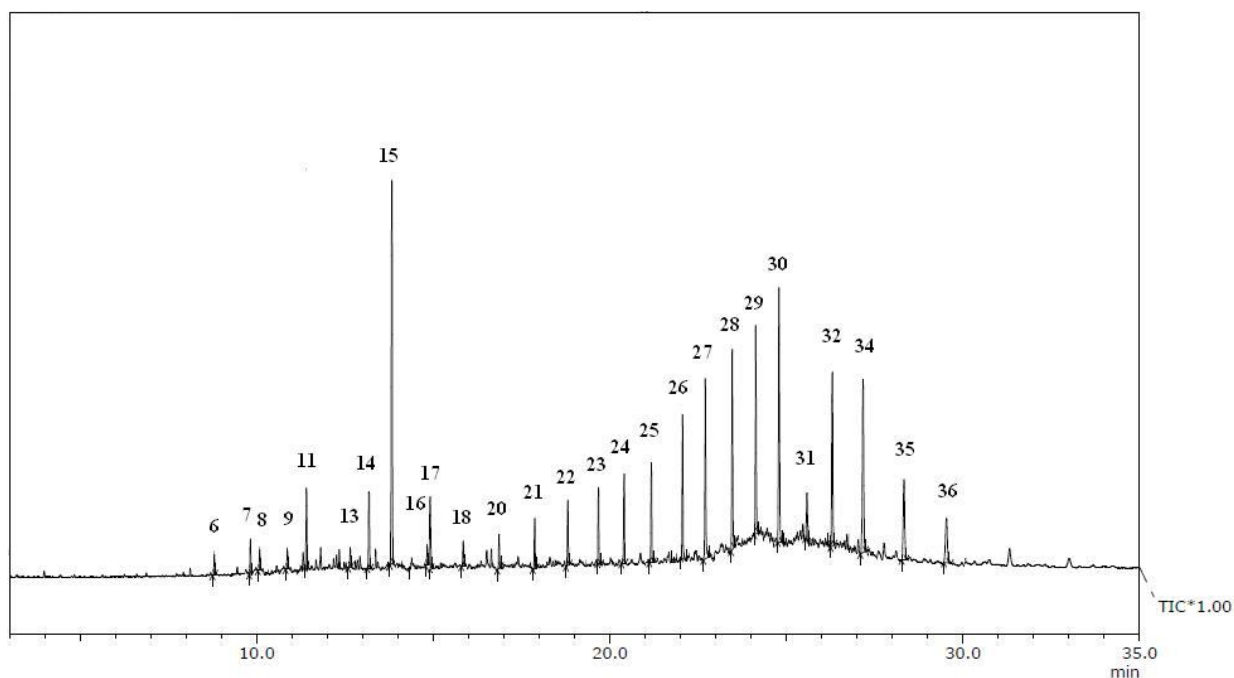

Figure 7.19: Residual hydrocarbon fraction in *A. versicolor* degraded heavy crude oil sample

7.3. TPH Degradation with addition of biosurfactant:

Surfactants can affect hydrocarbon solubilization and mobilization and influence the achievement of bioremediation, since the physical state of a hydrocarbon can determine its rate of biodegradation. Surfactants can increase the bioavailability and improve microbial utilization rates. Solubilization of an organic contaminant by a surfactant depends on a process called micelle formation. As a result of its amphiphilic nature, a surfactant molecule may dissolve in water as an monomer, adsorb at an interface or be incorporated with other surfactant molecules as part of a micelle [Aparna et al., 2011]. When the surfactant concentration is less than a specific concentration, surfactant molecules arranges predominantly in monomer form. Aggregates of micellar surfactant molecules create a hydrophobic less polar core into which contaminant are accommodated. Therefore, the solubilization of contaminants is markedly increased [Pekdemir et al., 2005].

Emulsification of oil was observed in the culture flasks of *P. aeruginosa* and *B. casei*. It might be due to the emulsification action of surfactants. This experimentation was done to study the response time of bacterial strains and rate of hydrocarbon degradation. Detailed studies of the surfactants which are responsible for the emulsification of oil were discussed in chapter 6. In this study microbial cells were inoculated in the nutrient broth which contains biosurfactant with a critical mycelia concentration (CMC) of 22.4 mg/L with *Pseudomonas aeruginosa* culture and 22.7 mg/L with *Brevibacterium casei* culture. Surfactants added in the culture flasks were produced by their respective strains. Culture conditions and composition of nutrient media was same as in earlier TPH degradation study.

7.3.1. TPH Degradation of Oil sludge:

The experiments were conducted with addition of biosurfactants to the inoculum broth in 100 ml nutrient media. By the end of thirtieth day *P. aeruginosa* and *B. casei* degraded oil sludge by 98.78% and 95.80% respectively. Simultaneously the concentration of 2.96 and 2.89 mg/ml were evaluated respectively. Emulsification of oil started very quickly and it has a positive impact on the growth of microbial biomass and on biodegradation of oil sludge. It might be due to the emulsification action of surfactants which made the hydrocarbons available to the bacterial cells. Figure 7.20 shows the decrease in oil sludge concentration with respective growth of biomass. *Brevibacterium casei* has shown a very good result, there is an additional increase of 0.2 g/L concentration of bacterial cells and 4.2% decrease in oil sludge content. It might be due to the increase in availability of hydrocarbons microbe which utilizes them as a carbon source and grown well.

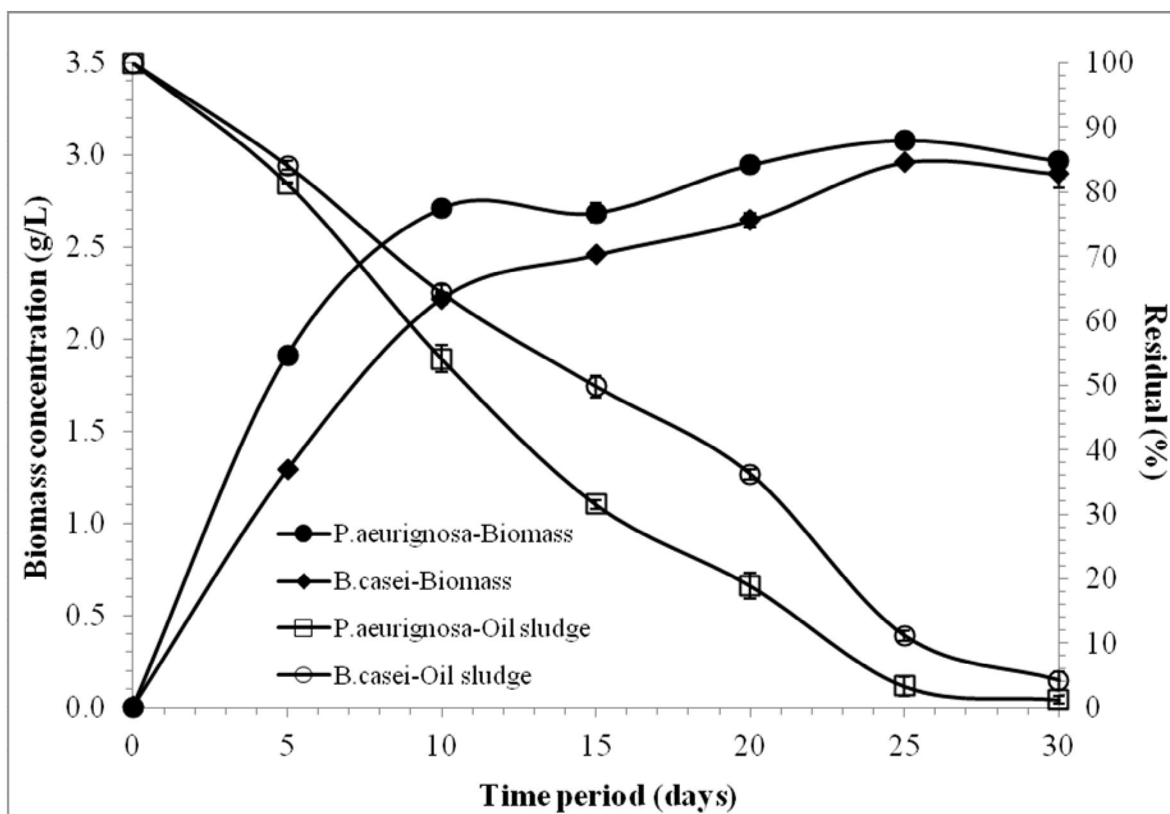

Figure 7.20: Biosurfactant added oil sludge degradation profiles of bacterial strains for 30 days

7.3.2. TPH Degradation of Light crude Oil:

By the end of thirtieth day culture flask containing oil sludge as a carbon source has shown 92.8% of degradation by *P. aeruginosa* and 88.9% by *B. casei*, with a final biomass concentration of 2.48 and 2.22 g/L. There is an increase in biomass growth and decrease in light crude oil weight in the culture flask due to the emulsification action of the biosurfactant. Figure 7.21 shows the decrease in oil percentage with respective growth of biomass. There is an increase in 0.2 mg/ml biomass concentration and 2.8% degradation of light oil content in flask inoculated with *P. aeruginosa, B. casei* has illustrated very good result showing additional

increase of 0.2 g/L concentration of bacterial cells and 5.0% decrease in light crude oil content as compared to flask without adding prior biosurfactant.

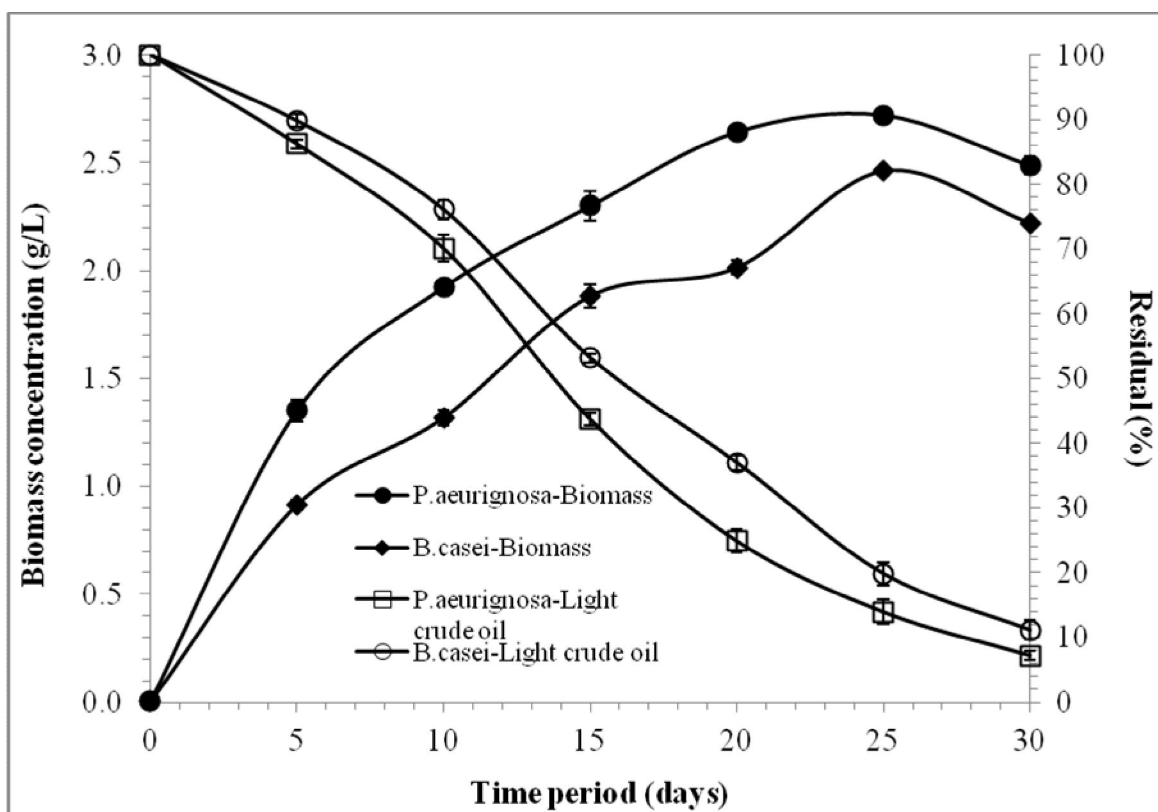

Figure 7.21: Biosurfactant added light crude oil degradation profiles of bacterial strains for 30 days

7.3.3. TPH Degradation of Heavy crude Oil:

The response of biodegradation of heavy crude oil is faster when compared with heavy crude oil degradation without adding prior biosurfactant. By the end of thirtieth day 78.9% of heavy crude oil was degraded by *P. aeruginosa* and 75.2% by *B. casei*, with a final biomass concentration of 2.22 and 2.14 g/L respectively. Figure 7.22 denotes the bacterial biomass growth and decrease in heavy crude oil percentage in the culture flask. There is an additional increase in 0.22 g/L and 0.29mg/ml of biomass concentration about 2.7% and 3.7% degradation

of heavy crude oil content in flask inoculated with *P. aeruginosa* and *B. casei* respectively were observed.

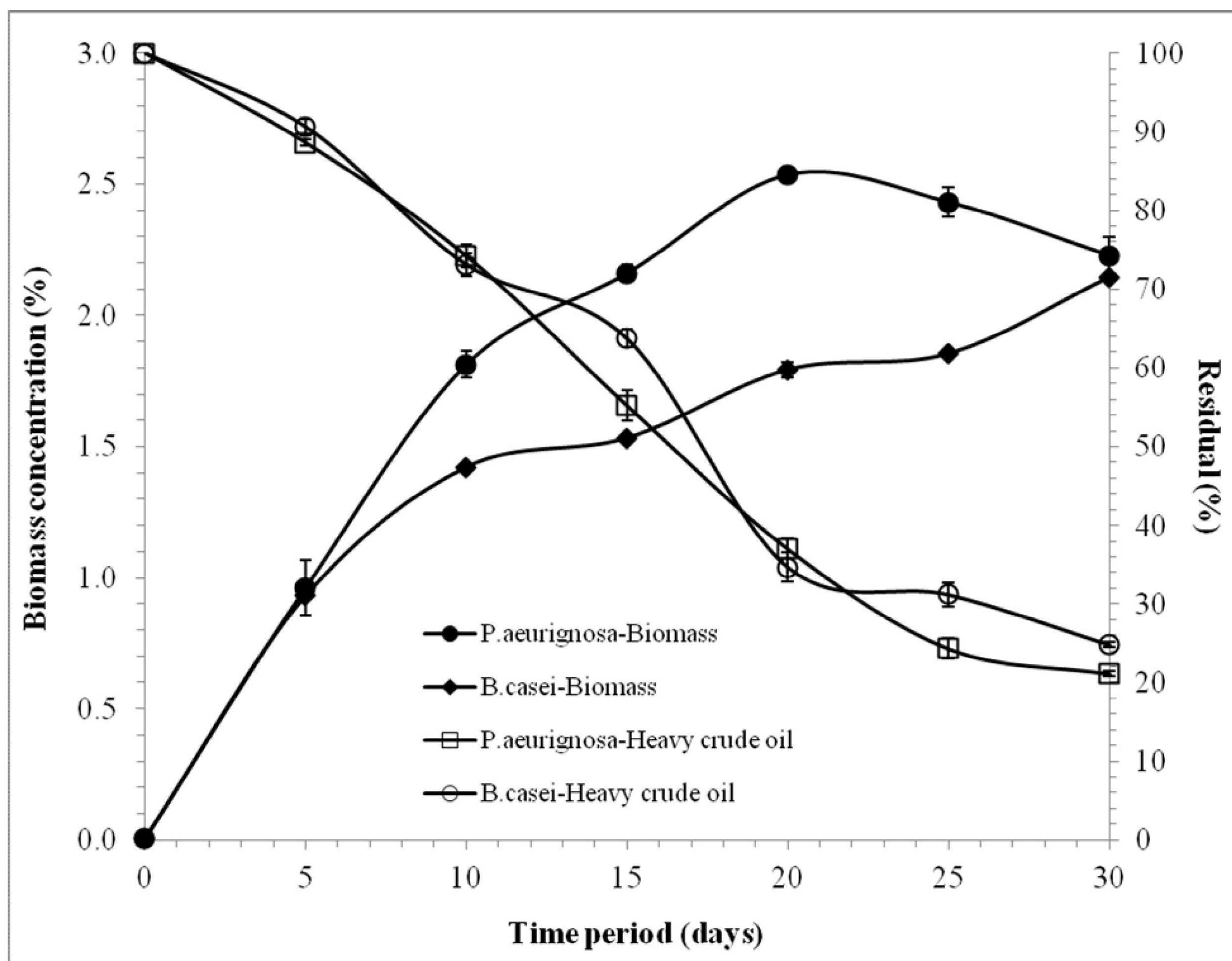

Figure 7.22: Biosurfactant added heavy crude oil degradation profiles of bacterial strains for 30 days

By this study it was proven that biosurfactants can increase the bioavailability of hydrocarbons and improve microbial utilization rates. Emulsification of oil started very quickly and it has a positive impact on the growth of microbial biomass and on biodegradation of oil sludge. Microorganisms follows the β-oxidation metabolic path way to oxidation long chain n-alkanes [Marino, 1998].

Figure 7.23: (A) Control flasks of OS, LC and HC (B) *P. aeruginosa* degradation flasks (OS, LC and HC) (C) *B. casei* degradation flasks (OS, LC and HC) (D) *A. versicolor* degradation flasks (OS, LC and HC)

Table 7.2: Composition of hydrocarbons in oil sludge before and after degradation studies

Peak #	Retention time	Name of the fraction	Chemical formula	Oil sludge Control	*P. aeruginosa*	*B. casei* without aeration	*B. casei* with aeration	*A. versicolor* with aeration
1	5.751	Hendecane	$C_{11}H_{24}$	8285316	0	0	0	0
2	5.758	Undecane	$C_{11}H_{24}$	0	0	0	0	0
3	7.288	n-Dodecane	$C_{12}H_{26}$	31525764	0	0	0	0
4	7.466	2,6-Dimethyl Undecane	$C_{13}H_{28}$	0	0	0	55419910	0
5	8.322	n-Tridecane	$C_{13}H_{28}$	28853609	0	6013009	106399313	0
6	8.743	4,6-Dimethyl dodecane	$C_{14}H_{30}$	70919630	625448	7935770	0	0
7	9.766	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	33842622	1394515	23839941	46554397.5	5198505
8	10.115	n-Tetradecane	$C_{14}H_{30}$	94247844	732737	52964166	66666437.5	3098257.5
9	10.878	2,6,10, 14-Tetramethylpentadecane	$C_{19}H_{40}$	96939704	731120	25168923	47471060	7927400
10	11.401	n-Hexadecane	$C_{16}H_{34}$	106687940	0	0	0	0
11	12.615	n-Octadecane	$C_{18}H_{38}$	131389763	894145	75117395	238248595	7434862.5
12	13.139	2,6,10,14-Tetramethylpentadecane	$C_{19}H_{40}$	73612422	0	0	190386950	0
13	13.791	2,6,10,14-Tetramethylpentadecane	$C_{19}H_{40}$	158281052	762599	83915402	70626790	16085908
14	14.857	n-Heptadecane	$C_{17}H_{36}$	0	2893728	33622585	57188917.5	4948287.5
15	14.908	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	162131851	839898	155986267	455689065	11426610
16	15.896	n-Heneicosane	$C_{21}H_{44}$	81135703	0	0	362922105	0
17	16.886	n-Eicosane	$C_{20}H_{42}$	83581292	1159109	78130872	119508513	12887713
18	17.832	Docosane	$C_{22}H_{46}$	81599907	0	0	0	0
19	18.737	Tricosane	$C_{23}H_{48}$	76526908	4062779	31543216	0	20817973
20	19.607	n-Tetracosane	$C_{24}H_{50}$	64533631	11704058	68934754	0	49673585
21	20.440	Nonacosane	$C_{29}H_{60}$	47468120	0	55031229	0	6257095
22	21.241	n-Pentacosane	$C_{25}H_{52}$	35425028	0	44867685	0	18753605
23	22.011	Hexacosane	$C_{26}H_{54}$	25223111	0	33786989	0	5496952.5
24	22.753	n-Tetratriacontane	$C_{34}H_{70}$	16038000	0	24239239	0	4951765

25	23.470	n-Hexatriacontane	$C_{36}H_{74}$	10775015	0	22304343	182991413	4298190
26	24.162	Tetracontane	$C_{40}H_{82}$	6146407	0	18926543	0	4286262.5
27	24.829	n-Hexatriacontane	$C_{36}H_{74}$	3754537	0	15524702	0	0
28	25.506	Tetracontane	$C_{40}H_{82}$	0	0	13233017	0	0
29	26.274	n-Tetratetracontane	$C_{44}H_{90}$	0	0	11519466	0	0
30	27.168	n-Tetracontane	$C_{40}H_{82}$	0	0	9579746.6	0	0
				1528925176	25800136	892185260	2000073465	183542970

Table 7.3: Composition of hydrocarbons in light crude oil before and after degradation studies

Peak #	Retention time	Name of the fraction	Chemical formula	Light crude oil Control	*P. aeruginosa*	*B. casei* without aeration	*B. casei* with aeration	*A. versicolor* with aeration
1	5.751	Hendecane	$C_{11}H_{24}$	5992415	0	0	0	0
2	5.758	Undecane	$C_{11}H_{24}$	6133398	0	0	0	0
3	7.288	n-Dodecane	$C_{12}H_{26}$	2939648	103283	1416390	0	0
4	7.466	2,6-Dimethyl Undecane	$C_{13}H_{28}$	4358969	98702	963840	0	0
5	8.322	n-Tridecane	$C_{13}H_{28}$	8865810	162050	2708322	193503	318768.4
6	8.743	4,6-Dimethyl dodecane	$C_{14}H_{30}$	4686630	160234	1053747	0	0
7	9.766	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	10440533	0	3855421	330492	337225.7
8	10.115	n-Tetradecane	$C_{14}H_{30}$	5599548	182062	1835421	1378764	1086163
9	10.878	2,6,10, 14-Tetramethylpentadecane	$C_{19}H_{40}$	10894849	133505	4878179	0	0
10	11.401	n-Hexadecane	$C_{16}H_{34}$	10826561	196351	0	0	379362.2
11	12.615	n-Octadecane	$C_{18}H_{38}$	5527500	127857	2619028	0	624925.3
12	13.139	2,6,10,14-Tetramethylpentadecane	$C_{19}H_{40}$	32824675	127645	0	0	2299439
13	13.791	Tetramethylpentadecane	$C_{19}H_{40}$	8779134	121142	4414420	925550	625989.6

Peak #	Retention time	Name of the fraction	Chemical formula	Light crude oil Control	*P. aeruginosa*	*B. casei* without aeration	*B. casei* with aeration	*A. versicolor* with aeration
14	14.857	n-Heptadecane	$C_{17}H_{36}$	3213255	258705	1979944	8086056	3694835
15	14.908	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	8827782	138538	12629892	659011	1489204
16	15.896	n-Heneicosane	$C_{21}H_{44}$	9674561	222390	4171460	773975	6702549
17	16.886	n-Eicosane	$C_{20}H_{42}$	12389497	255195	1232614	0	1091399
18	17.832	Docosane	$C_{22}H_{46}$	12419694	118717	4148116	884860	636842
19	18.737	Tricosane	$C_{23}H_{48}$	14419610	0	0	0	0
20	19.607	n-Tetracosane	$C_{24}H_{50}$	16264080	546197	4421071	1195755	666830.2
21	20.440	Nonacosane	$C_{29}H_{60}$	18654889	92312	4714149	1350185	1120694
22	21.241	n-Pentacosane	$C_{25}H_{52}$	17395943	156246	5238614	1456798	2306427
23	22.011	Hexacosane	$C_{26}H_{54}$	17428919	162852	5742003	2069115	5119705
24	22.753	n-Tetratriacontane	$C_{34}H_{70}$	17093675	168016	5923678	2231052	6533779
25	23.470	n-Hexatriacontane	$C_{36}H_{74}$	16995846	173536	7253802	3285273	0
26	24.162	Tetracontane	$C_{40}H_{82}$	13950521	186727	8375389	6338221	0
27	24.829	n-Hexatriacontane	$C_{36}H_{74}$	14922625	182802	11327536	6523304	10928672
28	25.506	Tetracontane	$C_{40}H_{82}$	8363851	224222	14590955	6532515	1328807
29	26.274	n-Tetratetracontane	$C_{44}H_{90}$	7398851	250482	19478735	8346696	8381175
30	27.168	n-Tetracontane	$C_{40}H_{82}$	4054486	275697	21743848	9519005	9491878
31	28.215	n-Heptatriacontane	$C_{37}H_{76}$	1894683	345024	28922330	8272782	0
32	29.470	n-Pentatriacontane	$C_{35}H_{72}$	0	383259	24399905	4892093	5474853
33	11.254	n-Pentadecane	$C_{15}H_{32}$	0	0	0	0	2613226
34	11.810	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	0	395961	28419948	3085743	0
35	26.850	n-Octatriacontane	$C_{38}H_{78}$	0	1337880	15725516	1625386	0
36	29.321	n-Hexatriacontane	$C_{36}H_{74}$	0	392755	11519119	0	O
				333,232,438	7680344	265703391	79956134	73252747

Table 7.4: Composition of hydrocarbons in heavy crude oil before and after degradation studies

Peak #	Retention time	Name of the fraction	Chemical formula	Heavy crude oil Control	*P. aeruginosa*	*B. casei* without aeration	*B. casei* with aeration	*A. versicolor* with aeration
1	5.751	Hendecane	$C_{11}H_{24}$	2997782	0	0	0	0
2	5.758	Undecane	$C_{11}H_{24}$	0	0	0	0	0
3	7.288	n-Dodecane	$C_{12}H_{26}$	3978610	0	202823	2919664.7	0
4	7.466	2,6-Dimethyl Undecane	$C_{13}H_{28}$	0	0	0	0	0
5	8.322	n-Tridecane	$C_{13}H_{28}$	2563291	0	464006	648092.3	0
6	8.743	4,6-Dimethyl dodecane	$C_{14}H_{30}$	5516677	0	568686	1003716.6	248847
7	9.766	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	0	0	0	1239700.6	456864
8	10.115	n-Tetradecane	$C_{14}H_{30}$	6683732	0	1201504	678325.86	299616
9	10.878	2,6,10, 14-Tetramethylpentadecane	$C_{19}H_{40}$	4516784	0	763151	1576098.8	2764498
10	11.401	n-Hexadecane	$C_{16}H_{34}$	8178014	332984	580693	0	0
11	12.615	n-Octadecane	$C_{18}H_{38}$	7114765	1710283	0	1497228.5	1091344
12	13.139	2,6,10,14-Tetramethylpentadecane	$C_{19}H_{40}$	4467700	0	0	993609.41	0
13	13.791	2,6,10,14-Tetramethylpentadecane	$C_{19}H_{40}$	28657931	451021	1377745	6494033	271669
14	14.857	n-Heptadecane	$C_{17}H_{36}$	6988252	1972492	6497590	1574595.7	1343410
15	14.908	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	3803228	416848	841887	0	329279
16	15.896	n-Heneicosane	$C_{21}H_{44}$	7759334	391700	1156623	908412.22	7172996
17	16.886	n-Eicosane	$C_{20}H_{42}$	8432615	618380	1154198	1784101.1	1456213
18	17.832	Docosane	$C_{22}H_{46}$	10157126	618898	1401036	1995902.1	3550898
19	18.737	Tricosane	$C_{23}H_{48}$	11741959	0	0	0	0
20	19.607	n-Tetracosane	$C_{24}H_{50}$	13859315	0	1528440	2312945.7	450035
21	20.440	Nonacosane	$C_{29}H_{60}$	15770399	0	1791121	2706667.4	654992
22	21.241	n-Pentacosane	$C_{25}H_{52}$	20193137	0	2186590	3236719.1	848241

Peak #	Retention time	Name of the fraction	Chemical formula	Heavy crude oil Control	*P. aeruginosa*	*B. casei* without aeration	*B. casei* with aeration	*A. versicolor* with aeration
23	22.011	Hexacosane	$C_{26}H_{54}$	22165399	0	2499529	3676700.5	1030071
24	22.753	n-Tetratriacontane	$C_{34}H_{70}$	25826733	1648847	3590599	4785534.4	1331297
25	23.470	n-Hexatriacontane	$C_{36}H_{74}$	25437241	0	4769383	5511194.4	1506819
26	24.162	Tetracontane	$C_{40}H_{82}$	25486970	4033716	6776073	6894663.7	2054954
27	24.829	n-Hexatriacontane	$C_{36}H_{74}$	22250996	6840362	8280674	7328161.9	2940986
28	25.506	Tetracontane	$C_{40}H_{82}$	24132872	12151070	9504338	8595001.8	31409688
29	26.274	n-Tetratetracontane	$C_{44}H_{90}$	17080540	12487472	8781338	8172191.7	30273427
30	27.168	n-Tetracontane	$C_{40}H_{82}$	18999795	16862297	10087746	9576156.3	43504207
31	28.215	n-Heptatriacontane	$C_{37}H_{76}$	10681240	10703963	74515340	6593175.1	8501614
32	29.470	n-Pentatriacontane	$C_{35}H_{72}$	7555585	8592710	73725875	7089098.6	32972593
33	11.254	n-Pentadecane	$C_{15}H_{32}$	O	0	0	0	0
34	11.810	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	O	4383175	40885772	3651809	4027920
35	26.850	n-Octatriacontane	$C_{38}H_{78}$	O	3284726	2890728	2549158.4	2095975
36	29.321	n-Hexatriacontane	$C_{36}H_{74}$	0	0	1309896	648092.3	1474115
				366021630	87500946	269333384	106640751	184062568

Chapter-8

Conclusions and Future Perspective

8.1. Conclusion:

- Eight microbial stains were isolated from various sampling sites namely automobile work shop, garden Soil, Gokharkuda beach and Rushikulya River that showed existence of these species on hydrocarbon.

- Among the eight isolates one of the fungal species and two of bacterial species have shown very good growth on hydrocarbons (n-octane, n-hexadecane and crude oil).

- The morphological, physiological and biochemical tests shows that bacterial strain 'G' is *Pseudomonas aeruginosa*, 'WS' is *Brevibacterium casei* and fungal species stain 'F' is *Aspergillus versicolor*.

- Optimization of process parameters like temperature, pH and nitrogen source were conducted by one factor at a time approach and the optimum temperatures were 35ºC, 30ºC and 30ºC and optimum pH were 8.0, 7.5 and 7.0 and the nitrogen source were ammonium chloride, urea and urea for the maximum yield of biomass by *P. aeruginosa, B. casei* and *A. versicolor*.

- Nutrients were optimized by using Grey based Taguchi approach and the best combination of sulphates, phosphates, nitrogen and magnesium for the higher biomass yield and TPH degradation for *P. aeruginosa* were sulphates 0.4 g/L (A_1), phosphates 0.8 g/L (B_3), nitrogen 2.0 g/L (C_3) and magnesium 0.3 g/L (D_3) and in case of *B. casei* were, sulphates 0.4 g/L (A_1), phosphates 1.0 g/L (B_4), nitrogen 2.5 g/L (C_4) and magnesium 0.4 g/L (D_4) and similarly sulphates 0.6 g/L (A_2), phosphates 1.0 g/L (B_4), nitrogen 2.0 g/L (C_3) and magnesium 0.2 g/L (D_2) for *A. versicolor*.

- It can be concluded that impact of aeration on the TPH degradation rate of *B. casei* is significant.

- Biodegradation of TPH (Petroleum oil sludge, light crude oil and heavy crude oil) by *P. aeruginosa* are 98.0%, 90.0% and 76.2% and *B. casei* are 91.0%, 83.7% and 69.0% and *A. versicolor* are 87.1%, 78.0% and 58.2% respectively.

- Addition of surfactant in the initial stage of growth of bacterial cells has observed a predominant increase in the rate of biodegradation and the percentage reduction of TPH's by *P. aeruginosa* and *B. casei* are 98.78% and 95.80% of oil sludge, 92.8% and 88.9% of light crude oil and 78.9% and 75.2% of heavy crude oil.

- The biochemical tests have proven that the biosurfactant extracted from *P. aeruginosa and B. casei* is *Rhamnolipid* which belongs to a group of surfactants called *Glycolipids.*

- It was proven that Rhamnolipid biosurfactants are responsible for the emulsification of oil and making the hydrocarbons available to bacterial cells, by which the degradation process was enhanced.

By observing the above experimental results I conclude that microorganisms having the potential to degrade hydrocarbons exist in the nature. May be they have this ability genetically, but when they come in contact with the hydrocarbons they exhibit the degradation characteristic. So collection of samples from the hydrocarbon contaminated sites will help in the isolation of microorganisms with hydrocarbon degradation ability. Environmental conditions like temperature, pH and dissolved oxygen content, plays a key role in the growth of microorganisms. Microorganisms require proper nutrients to perform the metabolic activates and to grow. Adding nutrients (N, P, S and Mg) will help to maintain proper metabolism and it aids to produce intracellular and extracellular organic compounds (enzymes and biosurfactants) which improves the hydrocarbon degradation rate by increasing uptake of hydrocarbons and in their metabolism to yield biomass, energy, water and CO_2.

Microbes produce biosurfactants to make hydrocarbons available to them. This is done by emulsifying the oil slick by reducing the surface tension and by forming the micelles. Biosurfactants

modifying the surface charge of the microbes and enhance their contact the hydrocarbons. So, addition of biosurfactants will also speed up the biodegradations process.

8.2. Scope for Further Research:

- Impact of other metallic, organic and inorganic contaminants on the biodegradation process is to be studied.
- Metabolic path way followed by fungal strain is yet to be studied.
- Field trials for biodegradation of hydrocarbons in aqueous environment by the microbial isolates are to be done.
- While applying the isolated microorganisms and produced biosurfactants in large quantity to degrade hydrocarbons, biocompatibility study is to be conducted to ensure the safety of other living organisms present in the contaminated environment.
- Degradation studies of petroleum hydrocarbons present in soil by the three isolates are to be done.
- Impact of environmental conditions like temperature, pH and metal ions on action of Rhamnolipid biosurfactants are to be examined.
- Leaching study of oil from rock and sand using biosurfactants is to be explored.

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VITAE

VAMSI KRISHNA GARAPATI

Date of Birth: 05th May 1986.

Mail Id: vamsi_nit@yahoo.com

Education Qualification:

- M.Tech (Research) in the Chemical Engineering from National Institute of Technology, Rourkela (January 2010 - January 2012, Thesis submitted).
- B.Tech in Biotechnology from Godavari Institute of Engineering & Technology, Rajahmundry. Affiliated to JNTU, Hyderabad (2003-2007).

Industrial Experience:

- Worked as Production Engineer (July 2008 – December 2009) in Bio Max Life Sciences Limited, S.P Biotech Park, Shameerpet, Rangareddy district-78, Andhra Pradesh.
- Worked as Trainee Process Engineer (September 2007 – June 2008) in Sentini Bioproducts Pvt Ltd at Gandepalli village, Vijayawada, Andhra Pradesh.

Academic Achievements:

Publications Based on Present work:

Journal Paper:

- Vamsi Krishna Garapati and Susmita Mishra, "Hydrocarbon Degradation using Fungal Isolate: Nutrients Optimized by Combined Grey Relational Analysis", International Journal of Engineering Research and Applications (IJERA), Vol. 2, Issue 2,Mar-Apr 2012, pp.390-399.

Conference Paper:

- Vamsi Krishna. G and Susmita Mishra, "Degradation of petroleum hydrocarbons using microorganisms", International Conference on Recent Advances in Chemical Engineering and Technology (RACET), 10-12 March 2011, Cochin, India.

Other Conference Papers:

- Presented 'Production of Primary Metabolites from Cellulose Waste' and won first prize in Bec'Tagon-06 a national level student's technical symposium conducted by department of Biotechnology at Bapatla Engineering College, Bapatla.
- Presented 'Production of Vanilla flavored Beer and won first prize in Chain de pence-07 a national level student's technical symposium conducted by department of biotechnology at ANITS, Vishakaptanam.
- Presented 'Production Of Primary Metabolites From Industrial Waste' and won second prize in Biomical-07 a national level student's technical symposium conducted by department of biotechnology at GITAM, Vishakaptanam.

Project works:

- I have done a project work on "Production of Vanilla flavored Beer" for the award of B.Tech degree in Biotechnology.
- I have done a project work on "Production of Primary Metabolites by Continues Fermentation Techniques from Industrial Wastes" at Jaypore Sugar Company limited, Chagallu, West Godavari Dist, Andhra Pradesh.

Working Models:

- Exhibited a model entitled 'Bioreactor for Enzymes Production' and won first prize in Medha-06 a national level project exhibition conducted by GIET, Rajahmundry.
- Exhibited a model entitled 'Bioreactor' and won second prize in Medha-05 a national level project exhibition conducted by GIET, Rajahmundry.