Isolation of Furnace Oil and Crude Oil Degrading Bacteria from Soil

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by
Cinderella Akbar Mayaboti
Student ID: 11236001
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Department of Mathematics and Natural Sciences
Biotechnology Program
BRAC University
Dhaka, Bangladesh

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Cinderella Akbar Mayaboti

August, 2016

DECLARATION

I, Cinderella Akbar Mayaboti, hereby declare that the thesis project titled "Isolation of furnace oil and crude oil degrading bacteria from soil", submitted by me has been carried out under the joint supervision of Dr. Mahboob Hossain, Associate Professor, Microbiology program, Department of Mathematics and Natural Sciences and Abira Khan, former Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University and current lecturer at Dhaka University. The contents of this thesis have not been submitted elsewhere for publication or award of any degree. It is also to be declared that the research work presented here is based on actual and original work carried out by me. Information sources or reference to research works performed by other people or institution have been duly cited and referenced.

Cinderella Akbar Mayaboti
Candidate

Certified:

Dr. Mahboob Hossain

Associate Professor, Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University, Dhaka, Bangladesh.

Abstract

Oil pollution is one of the major problems of the environmental issues. It is important to clean up oil spill as it causes disturbance to the balance of nature, hampering animals, plants and humans life. Many diseases, disorders and cancers are caused due to oil pollution throughout the world. Even exotic marine and terrestrial species are becoming endangered. Chemical and physical methods towards oil spillage are expensive, less effective and has other side effect on the environment. On the other hand biological process i.e. bioremediation is more effective, inexpensive and environment friendly. Microorganisms found in nature are capable of utilizing these hydrocarbons and break them down to innocuous by-products. The purpose of this study was to isolate microorganisms which were capable of using furnace oil and crude oil as sole carbon source. Four isolates were derived from two different locations and from furnace oil itself. The bacteria were individually cultured in a mineral salt broth devoid of carbon source except for furnace oil and crude oil for 7 days at 35°C. The growth of bacteria was observed from visible increase of turbidity and was enumerated by CFU/ml on nutrient agar of same composition after 7 days of culture in the broth. The four microorganisms were identified as *Bacillus megaterium, Bacillus badius, Penibacillus chibensis* and *Aerococcus viridians* through colonial morphology and biochemical tests. Among all four *Bacillus badius* showed higher ability to grow on both furnace oil and crude oil individually. The bacterial count for furnace oil on day 7 was 7.73 logCFU/ml and for crude oil on day 7 it was 7.76 logCFUml. *Aerococcus viridans* showed the next best ability to grow on both furnace oil and crude oil individually. The result on day 7 was 6.7 CFUlog/ml for both furnace oil and crude oil from 6.64 logCFU/ml and 6.69 logCFU/ml respectively on day 1. *Bacillus megaterium* and *Penibacillus chebensis,*had relatively no or little ability to grow on either of the oils.

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LIST OF ABBREVIATIONS

Abbreviations	Descriptions
ºC	Degree Celsius
MSM	Mineral Salts Medium
NA	Nutrient Agar
RBC	red blood cell
MIU	Motility, Indole, Urease test
MR	Methyl Red
VP	Voges-Proskauer
GC	Gas chromatography
Rpm	rotations per minute
µl	Microlitre
Ml	millilitre
g/L	grams per litre
v/v	volume by volume
CFU	colony forming unit
PAH	Polycyclic Aeromatic Hydrocarbon

CHAPTER 1

INTRODUCTION

1.1 Introduction

Oil spillage and oil pollution in soil and water have been a major threat to the ecosystem and human being through the transfer of toxic organic materials into the food chain (A Sei and B.Z. Fathepure *et al.,* 2009). Petroleum based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Kvenvolden& Cooper *et al.,* 2003). Soil contamination with hydrocarbons causes extensive damage of local system since accumulation of pollutants in animals and plant tissue may cause death or mutations. Presence of polycyclic aromatic hydrocarbons (PAHs) in soil and water is major problem as environmental contaminants and most of these PAHs are recalcitrant in nature despite being organic material (Zhao *et al.,* 2008). Oil spill has become a global problem in industrialized and developing countries. Attention has been focused on the marine environment, because of the largest and most dramatic spills (Cooney *et al.,* 1984). Volatilization, chemical oxidation, photooxidation, mechanical, burying, dispersion, washing , and bioaccumulation are physical and chemical methods which are seldom successful process for hydrocarbons (Prince *et al.,* 1997; Alvarez *et al.,* 1984) but are rarely successful in rapid removal and cleaning up hydrocarbons. In addition these methods are neither environment friendly nor cost effective. It is observed that, for the removal of these hazardous products bioremediation is more suitable alternative (Ojo *et al.,* 2006). Bacteria have long been considered as one of the predominant hydrocarbon degrading agents found in the environment, which are free living and ubiquitous (Dasgupta *et al.,* 2013).

Due to its complicated composition, petroleum has the potential to elicit multiple types of toxic effects. It can cause acute lethal toxicity, sub-lethal chronic toxicity, or both depending on the exposure, dosage, and the organism exposed. Some components of petroleum have the potential to bioaccumulate within susceptible aquatic organisms and can be passed by trophic transfer to other levels of the food chain (Ojo *et al.,* 2006).

1.2 Microorganisms

The success of bioremediation technologies applied to hydrocarbon-polluted environments highly depends on the biodegrading capabilities of native microbial populations or exogenous microorganisms used as inoculants. Most important requirement is the presence of proper

metabolic pathway. The continued progress of oil spill bioremediation also depends on one's ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment (Leahy & Colwell *et al.,*1990; Zobell *et al.,* 1946; Atlas *et al.,*1981; Atlas *et al.,*1984; Atlas &Bartha *et al.,* 1992; Foght& Westlake *et al.,* 1987) . The communities which were exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes (Leahy & Colwell *et al.,* 1990; Atlas and Bartha *et al.,* 1998). The adapted microbial communities can respond to the presence of hydrocarbon pollutants within hours (Atlas and Bartha *et al.,* 1998) and exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination (Leahy & Colwell *et al.,* 1990). So, the ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the most active oil degraders of that environment (Atlas and Bartha *et al.,* 1998) and can be used in the bioremediation of petroleum oil polluted sites. Since crude oil is made of a mixture of compounds, and since individual microorganisms metabolize only a limited range of hydrocarbon substrates (Atlas *et al.,* 1984; Atlas &Bartha *et al.,* 1992), biodegradation of crude oil requires mixture of different bacterial groups or consortia functioning to degrade a wider range of hydrocarbons (Britton *et al.,* 1984; Al-Saleh *et al.,* 2009). If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustainedby ensuring that adequate concentrations of nutrients and oxygen are present, and that the pH is between 6 and 9.

Despite the difficulty of degrading certain fractions, some hydrocarbons are among the most easily biodegradable naturally occurring compounds. Many more as-yet-unidentified strains are likely to occur in nature (Emtiazi *et al.,* 2005). All marine and freshwater ecosystems contain some oil-degrading bacteria. No one species of microorganism, however, is capable of degrading all the components of given oil. Hence, many different species are usually required for significant overall degradation. Both the quantity and the diversity of microbes are greater in chronically polluted areas. In waters that have not been polluted by hydrocarbons, hydrocarbon-degrading bacteria typically make up less than 1 percent of the bacterial population, whereas in most chronically polluted systems they constitute 10 percent or more of the total population (Hedlund *et al.,*1999).

1.3Factors Affecting Degradation by microorganisms

1.3.1 Physical nature of the oil:

If the oil is in a single large slick, there is little surface area for the microbes to gain access to the oil, so degradation is slower. Furthermore, if the oil is heavy and viscous, the biodegradable components must first diffuse through that thick matrix to the oil-water interface so that the microbes can access them. Lighter the oil, faster this diffusion, making the biodegradable compounds more available to the microbes (Atlas *et al.,* 2011).

1.3.2 Chemical nature of the oil:

Biodegradation rates vary depending on the particular hydrocarbons that make up the spilled oil. Oil is composed of thousands of different compounds, some may be preferred food sources and be consumed very quickly, others are degraded more slowly or not at all. In a marine environment like the Gulf of Mexico, hydrocarbons, in which carbons are arranged in an unbranched chain, can degrade quickly-in a matter of days or weeks. Hydrocarbons that have a branched structure, or those in which the carbons are arranged in multiple rings, can be far more difficult to biodegrade and therefore persist longer. The most recalcitrant fractions of crude oil including resins and asphaltenes can last for millennia(Atlas *et al.,* 2011)..

1.3.3 Availability of nutrients:

Microbes require nutrients like nitrogen, phosphate and other nutrients. These substances are found in nature but may be present in limiting quantities. When hydrocarbon levels are high, the microbes can only degrade the hydrocarbons as fast as the availability of other nutrients allows. If nitrogen and phosphate levels are very low, biodegradation of oil constituents will take place slowly(Atlas *et al.,* 2011).

1.3.4 Availability of oxygen:

The enzymatic process of breaking down oil is usually most rapid in the presence of oxygen. Theoretically, given enough oil and other nutrients, microbial populations could grow so quickly that they exhaust the oxygen from the water in the vicinity of an oil spill. In practice, oxygen has not proven to be as important a limiting factor as nutrients in restricting oil degradation in the ocean, although degradation rates could be slow if a spill occurred in a location where oxygen levels are low(Atlas *et al.,* 2011).

1.3.5 Water temperature:

Generally, oil is degraded more quickly in warmer waters. The problem is not that microbes cannot live in cold water plenty of oil-degrading microbes are fully adapted to life at cold temperatures but that oil metabolism proceeds more slowly in cold habitats. However, in environments that are always cold, e.g. in the arctic or deep ocean, cold-loving microbes have adapted so that they can degrade oil as quickly as warm water adapted microbes do in their normal habitat. But colder temperatures also have physical effects on the speed of degradation oil evaporates more slowly, so there is more oil left in the water for the microbes to degrade. The oil is also more viscous, so it spreads out and disperses less readily providing less surface area for the microbes to access (Atlas *et al.,* 2011).

1.3.6 Pressure:

The Deepwater Horizon oil spill released oil at a depth of over 1500 meters, where the temperature is low and the pressure high. These are conditions where degradation might have been expected to be quite slow. However, early results show a high number of oil-degrading microbial species adapted to life even in these extreme conditions (Atlas *et al.,* 2011).

1.3.7pH and salinity:

In most of the ocean, pH and salinity do not vary enough to make a big difference in oil degradation rates. Some specific environments, like salt marsh sediments, exhibit not only high salinity, but also rapid fluctuations in salinity, oxygen, and pH—all characteristics that typically slow oil degradation.

1.3.8 Other microbes:

Natural microbial communities are diverse, with many different types of microbes that both compete and cooperate. The complex interactions that characterize healthy, natural microbial communities are only beginning to be understood, but interdependence is the norm. This is one reason why adding microbes to oil spills in the hope of speeding degradation is challenging; outsiders have a hard time breaking into the existing community structure and competing with the local species that have evolved together over the millennia in a particular habitat (Atlas *et al.,* 2011).

1.4 Soil

Soil is abundant with microorganisms of any type. It is the home to a lot of useful microbes. Contaminated soil is non fertile, but also is the provider of the microbes required for bio degradation. If right conditions are given to the contaminated soil, then the degradation of the oil would become faster and easier.

Oil product spills are possible in all stages of oil extraction, transportation or usage. After being emitted into the environment, these products strongly affect the ecosystems: pH and aeration level of soil change most often, biota is exposed to toxicity and its biological activity is weakened, soil degradation probability increases (Shabir *et al.,* 2008). Some of the volatile oil product components evaporate as discussed earlier (Laškova *et al.,*2007; Paulauskienė *et al.,* 2009), others contaminate the soil (Vasarevičius *et al.,* 2005). In order to restore the biological properties of soil, the biological treatment is preferred. Soils contain a very large number of microorganisms, which can include a number of hydrocarbon utilizing bacteria and fungi (Namkoong*et al.,* 2002). In addition, cyanobacteria and algae found in the soil have been found to be able to degrade hydrocarbons. The rate of degradation of organic compounds will be dependent on the structure of the compound. Some compounds can be absorbed by clays and are thus rendered invulnerable to degradation. To overcome this problem surfactants have been added to contaminated soils to improve the availability of hydrocarbons in certain experiments.

The rate of degradation depends on the microbial population, the type and level of contamination (Boldu-Prenafeta*et al.,* 2004). Another way of increasing the biodegradation efficiency can be improving the aeration; the addition of biogenic elements like phosphorus, nitrogen, potassium has also been proposed (Liužinas*et al.,* 2003). Moreover, it is also being offered to introduce biopreparations or other active substances, rich in microorganisms. Usage of complex biocultures artificially made of several microorganism species for hydrocarbon degradation which possess well-known oil oxidation properties, or naturally formed microorganism combinations (Shabir*et al.,* 2008) was known a long while ago. Adapted autochthonous microorganisms are considered as the most prospective and efficient measure.

It is possible to use biowaste like vegetables, fruits, waste from pruning and other gardening operations for composting, during which the soil is cleaned of oil products (Gestel *et al.,* 2003). Meanwhile, other authors (Chander *et al.,* 1997) indicate that the diesel fuel would be biodegraded more intensely if the diesel-contaminated soil were supplemented with

additional organic matter, e.g., sewage sludge, since this organic matter is abundant in substances beneficial for microorganisms. This is probably due to the fact that the additional sludge microflora is introduced, which can break down organic acids, capture atmospheric nitrogen, etc. Comparison of the ability of different bacteria to dispose of oil products suggests that cocci can "soak up" the substrate with the whole surface of the cell while depleting less energy for being more active (Maier *et al.,* 2000). It is estimated that there are about 70 genera of bacteria that are capable of breaking down hydrocarbons (Repečkienė *et al.,* 2002; Prince *et al.,* 2005). Biodegradation activity of micromycetes promotes the decomposition of heavy oil fractions, depending on their systematic affinity, the number of origins, the ability to synthesize various enzymes and release organic acids and other metabolites into environment (Ellis *et al.,* 2001).

1.5 Crude oil

Oils that are found in natural reservoirs are principally derived from ancient algae and plant material. It is basically a natural product generated from organisms that long ago used sunlight as their energy source through the process of photosynthesis. The algae were buried deep in the Earth and heated at great pressure over millions of years. The resulting material is oil, in which is stored the energy generated by that ancient photosynthetic activity. Therefore, the components of crude oil are a great source of energy, not only as fuel to power internal combustion engines, but also as food for microbes. Much of the Earth's crude oil is trapped in underground reservoirs, but some leaks to the surface, and has been doing so for millions of years. It is estimated that about half of the oil entering the world's oceans today comes from these natural seeps and the rest from human activities. Oil enters waterways not only when an offshore rig blows up, a pipeline ruptures, or a tanker runs aground, but also when it is rinsed off roads and parking lots, spilled at marinas and discharged by outboard motors, released during offshore oil operations, or washed out of ships' ballast tanks(Atlas *et al.,* 2011).

Crude oils vary from source to source, containing different proportions of hydrocarbons ranging from methane (natural gas), to light materials similar to gasoline, to heavy materials that resemble asphalt. Refineries convert crude oils to products ranging from gasoline and aviation fuel to the heavy fuel oils used for ship engines and the asphalts used for roofing tiles and roads. Microbes can biodegrade up to 90% of some light crude oil, but the largest and most complex molecules like the ones that make up road asphalt are not significantly biodegradable. When refined petroleum products are spilled, their fate depends

on their composition. Gasoline, kerosene, and diesel fuel are so volatile and easily biodegradable that they rarely persist in marine environments, although they can remain longer if buried in sediment, soils, groundwater, or marshes where oxygen levels are very low(Atlas *et al.,* 2011).

Crude oil components:

Volatile compounds - low molecular weight compounds, like methane (natural gas) or propane, that are normally gaseous or evaporate very quickly at room temperature.

Saturated hydrocarbons - compounds with carbon and hydrogen atoms connected only by single bonds. Saturated hydrocarbons can be arranged in straight or branched chains of up to about 25 carbon atoms. Saturated hydrocarbons are readily biodegraded although degradability decreases with chain length.

Aromatic compounds - compounds that contain rings of carbon atoms held together with double bonds between the carbon atoms. The smallest aromatic compounds in petroleum have six carbons in such a ring structure (e.g. benzene and toluene), but other compounds contain multiple rings. These are known as polycyclic aromatic hydrocarbons, often abbreviated 'PAH'. Most aromatic molecules in petroleum have multiple attached hydrocarbon chains. The smallest aromatic molecules (one- and two-rings) are both volatile and readily biodegraded, even with attached side-chains, but four-ring and larger aromatic compounds are more resistant to biodegradation. They are, however, susceptible to photooxidation. Some larger PAHs are of concern because they are potentially carcinogenic; 16 different PAHs are designated as priority pollutants by the EPA (Atlas *et al.,* 2011).

The percentage of PAHs in crude oil varies, but the 'priority pollutants' are present at low levels in crude oils; they are much more common as a byproduct of burning carbonaceous materials such as fuel, coal, wood, tobacco and other materials. Asphaltenes are examples of high molecular weight PAHs that have additional chemical side chains attached to their aromatic rings. Asphaltenes are not soluble in water and most organic solvents (Atlas *et al.,* 2011).

1.5.1 Physical and Chemical Processes for removal of crude oil:

a) Evaporation

The volatile hydrocarbons evaporate quickly into the atmosphere when they reach the water surface. Under controlled experimental conditions, about 50% of atypical light crude oil evaporates within 20 hours. The characteristics of the spilled oil and environmental conditions make a big difference; light oil on a calm sea will evaporate much faster than heavier oil that has been churned into the water by heavy waves. Evaporation will also be faster at warmer temperatures.

b) Dissolution

Some components of crude oil dissolve in water. These compounds are the most likely to be acutely toxic to sea life, but they are also among the most volatile and readily biodegradable under most conditions. Not all toxic compounds are lost through evaporation some, like heavy polycyclic aromatic hydrocarbons (PAHs), are poorly soluble in water and more likely to adhere to particles and thus remain in the water or sediment. PAHs can be broken down by microbes over time. However, this process is often slow enough that these hydrocarbons can accumulate in such invertebrates as shellfish. Fish and other vertebrates metabolize them rapidly (Atlas *et al.,* 2011).

c) Dispersion

Dispersion is the process by which oil is broken up into small droplets and spread through the water. This is the same physical process that is at work when we whisk oil and vinegar together to make salad dressing. Just as with salad dressing, the stability of the resultant emulsion can vary. Physical dispersion can only happen in the presence of adequate mixing energy (e.g. by wave action or high pressure leaks) under turbulent conditions, dispersion can prevent oil from reaching the surface where it might otherwise evaporate. Dispersion can also drive floating oil into the water column and largely prevent it from forming surface slicks that can threaten birds and mammals. One advantage of dispersion is that oil is broken up into tiny droplets with more surface area, which facilitates microbial degradation. A potential disadvantage is that it might increase exposure of some inhabitants of the ecosystem to the oil. Dispersion can be enhanced by the addition of chemical dispersants, which will be discussed later (Atlas *et al.,* 2011).

d) Photo-Oxidation

Sunlight reacts with some oil constituents, especially the polycyclic aromatic hydrocarbons (PAHs). The process, known as photolysis, is important because by breaking aromatic ring

structures, it enhances the availability of such compounds to microbes and hence microbial degradation. On the other hand, the photooxidized PAHs have been shown to be substantially more toxic to water-dwelling organisms. The physical processes of evaporation, dissolution, dispersion, and photo-oxidation begin as soon as oil is spilled or reaches the surface. Prompt human action can also physically remove some of the oil. When the danger of an oil spill reaching shorelines or other sensitive environments is imminent, physical removal, via skimming or burning, can be a critically important means of minimizing damage. With the exception of burning, which comes with its own set of risks and limitations, these physical processes, however, do not destroy the oil. They do not break it down into harmless carbon dioxide and water. Only living organisms or high temperature combustion can do that (Atlas *et al.,* 2011).

1.6 Furnace oil:

Furnace Oil is one of the cheapest fuels available for industrial use. It is a by-product of petroleum refineries. While processing the Crude Oil, Furnace Oil is one of the products along with other petroleum fuels like Diesel, Petrol etc.

Typically it has a calorific value as 10000 cal/gm. The furnaces which are used mainly for heating or pre-heating a large quantity of metal, are the main users of FO. This can be stored in vertical as well as horizontal tanks, above ground or even underground in some cases

For direct burning, FO is viscous as well as not suitable for complete combustion. It is pre-heated to 55 degrees Celsius onwards, depending upon application. The flow increases with temperature and it improves the combustion also. A water based scrubber is used in the exhaust chimney of furnace, which arrests considerable amount of carbon suite and improves the emissions.

1.7 Objective

There have been very few studies in Bangladesh concerning the biodegradation of hydrocarbons and its importance has been so far understated due to lack of oil spill reports. The data on mineralization abilities of indigenous microorganisms of Bangladesh is very limited. Microbes capable of degrading furnace oil and crude oil and their optimum conditions for biodegradation can be a useful approach towards better soil quality and also remediation technology for major spills. The main objective of this study are:

- Isolationof furnace oil degrading bacteria from soil.
- Isolation of crude oil degrading bacteria from soil.
- Identification of the isolates.

CHAPTER 2

MATERIALS AND METHOD

2.1 Place of Study

The present study was carried out in the Biotechnology and Microbiology Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Collection of soil samples, oil and isolation of organism

2.2.1 Soil sample collection

The source of microorganism tested in this study was top-soil samples collected from oil contaminated area which is a parking lot of trucksand buses in Malibagh, Dhaka and soil from shallow water regions of Buriganga River was also collected. These soils samples were stored in plastic bags.

2.2.2 Hydrocarbon (Furnace oil and Crude oil)

The hydrocarbons used in this study i.e. Furnace oil and crude oils were collected from Rahim Steels situated at Shyampur kadamtoli, DN road, Dhaka and Bangladesh Petroleum Corporation, Easter Refinery Limited respectively, Chittagong. The oils were syringe filtered under a biosafety cabinet prior to each experiment. For confirmation of sterility 100µl of oil was spread on NA plates (incubation at 37°C for 24hrs) to check for any growth.

2.2.3 Isolate selection from soil sample

Isolates of microorganisms were grown using serial dilution and spread plate technique on Nutrient agar. Soil samples were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} times from a soil suspension containing 9 ml of sterile 0.85% NaCl solution and approximately 2 g of soil sample. These dilutions were spread plated on fresh Nutrient agar plates. After an incubation period of 24 hours, the morphological characteristics of colonies grown on each plate were noted. From two soil samples, three colonies were selected. Most of the colonies from the spread plate method showed recurring morphological characteristics. Different colonies were selected based on their colonial characteristics. The selected colonies were streaked again on Nutrient agar plates to obtain pure isolates. Sub-cultures from these pure cultures were regularly made for the oil degradation studies.

2.2.4 Isolate selection from oil sample

Prior to the start of the experiments carried out, both Furnace oil and crude oil was spread on NA plates to observe any kind of growth. Crude oil spread plate did not have any kind of growth but Furnace oil showed distinctive growth of one kind of colony. These colonies were further streaked on NA plates for pure colonies and sub-culture was prepared regularly for the study of oil degradation study.

2.3 Degradation studies of Furnace oil and Crude oil

2.3.1 Inoculum preparation

The suspension of inoculum was prepared by mixing a loopful of pure culture of bacteria in 3 ml of 0.85% NaCl (physiological saline). This solution was vortexed for 5-10 seconds to gain a homogenous mixture. One militre of this suspension was used for a 100 ml volume of culture broth.

2.3.2 Culture medium preparation and screening for furnace oil and crude oil degrading bacteria

The medium used to study the degradation of hydrocarbons like oil is a mineral salt media (MSM) such as Bushnell Haas medium .This MSM lacks a carbon source and is chiefly made up of mineral salts; KH_2PO_4 (1 g/L), K_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $FeCl_3$ (0.05 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $CaCl_2$ (0.02g/L) (Bushnell & Haas, 1941). Salts of higher proportions were dissolved separately in small parts to ensure complete dissolution. The iron salt was also dissolved separately and added to the media. The pH of the salt solution was adjusted to 7 - 7.2 with NaOH before autoclaving at 121°C for 15 minutes. The degradation studies with this broth (mineral salts broth) were carried out by inoculation of an isolate, addition of furnace oil and crude oil with the concentrations in the form of v/v % which was 1ml and incubation at 35°C for 7 days with 150 rpm agitation.

2.3.4 Measurements of bacterial growth

The approach for quantification of bacterial growth was CFU (colony forming units). For measuring bacterial count, the medium plate used was nutrient agar. Serial dilutions were made from the culture broth with physiological saline for plating on nutrient agar plates. Plating was done on day 03 and day 07. These plates were incubated without agitation at 37ºC for 24hrs and then colony number was counted.

To check change in turbidity of the culture broth, on the 7th day OD (optical density) was checked using spectrophotometry set at 600 nm.

2.4 Identification of bacterial isolates

The bacteria screened for Furnace oil and Crude oil degrading abilities were identified based on the following characteristics.

1. Colonial morphology
2. Microscopic observations
3. Biochemical reactions

Colonial observations were made from spread and streaks plates that gave isolated colonies of the bacterial strains.

2.4.1 Microscopic evaluations

The microscope was used to observe the shape, size, arrangement and Gram reaction properties. Endospore staining was performed for suspected endospore producing bacteria. Acid-fast staining was carried out for identification purposes. All of these staining were completed according to the methods mentioned in Microbiology Laboratory Manual(Cappuccino & Sherman, 2005).

i) Grams staining: After heat fixing a smear was prepared using each isolate, crystal violet was applied on the bacterial smear for 30 seconds and then washed off by distilled water for 10 seconds. Gram's iodine (mordant) was then given over the stain and kept for 30 seconds. 95% alcohol (decolorizing agent) was used on the smear and then Safranin (counter stain) was applied on the smear for 1 minute and then washed off with distilled water.

   Observation microscope: under Gram positive bacteria stained purple and gram negative bacteria stained pink from the counter stain.

ii) Endospore staining: A heat fixed smear of an isolate was flooded with malachite green over a heated surface or a beaker with boiling water giving off steam for 5 minutes. After draining the malachite green, counter stain Safranin was applied for 1 minute and washed off with tap water.

   Observation under microscope: Endospores stained green while vegetative bodies appeared red.

iii) Acid fast stain (Ziehl–Neelsen stain): A smear of an isolate was heat fixed on a slide and then placed on a stand or beaker such that it was heated from the bottom. The slide was then flooded with Carbolfuchsin stain for 5 minutes and reapplied to replace evaporated stain. Acid alcohol was used to decolorize the stain and then methylene blue was used to flood the smear for 20 seconds.

   Observation under microscope: The acid fast positive bacteria would retain pink stain from Carbolfuchsin. Acid fast negative bacteria would be stained blue.

2.4.2 Biochemical Tests (following Microbiology Laboratory Manual (Cappuccino & Sherman., 2005)

Several biochemical tests were carried out to identify the bacteria with the aid of Microbiology Laboratory Manual. The bacteria tested were grown on nutrient agar plates at 37ºC in the incubator.

Motility, Urease Activity and Indole Production Test

- A motility indole urea (MIU) semisolid medium was used to determine motility, indole production and urease activity of the bacteria. Fresh bacteria from a pure culture were used to inoculate with a needle. The needle was stabbed 2/3rd way of the medium in the test tube approximately at the center.
- The medium was kept at 37ºC in an incubator for 24 hours.
- The growth and result was interpreted as follows.
    - Motility: The bacteria were considered positive for motility if there was turbid growth spreading from the stab line in the media.
    - Indole production: Kovac's reagent was added at the end of incubation. Appearance of cherry red reagent layer indicated positive reaction for indole production while negative reaction was considered if the layer remains yellow or brown.
    - Urease activity: Positive urease activity was signified by the change of colour in the media to pink from orange due to the production of ammonia after incubation.

Catalase Test

- A bacterial colony was placed on a clean dry glass slide with a clean toothpick.
- Hydrogen per oxide (catalase reagent) was dropped over the bacteria on the slide and mixed.
- Production of bubbles (oxygen) within 5-10 seconds of the addition of reagent indicated positive for catalase activity.

Oxidase test

- A Whatman filter paper (1mm) was soaked with the Oxidase reagent (N, N, N′, N′ - tetramethyl-p-phenylenediamine)
- A loopful of pure culture bacteria was streaked on it.
- Within 1 – 30 seconds, the appearance of purple color over the bacteria indicate a positive result. Delayed reactions are ignored and concluded as negative.

Citate Utilization Test

- Simmon Citrate agar medium was used to test for citrate utilization. The bacteria from a pure culture were streaked on the slant of the agar in a tube.
- The change in colour was observed after an incubation period of 24 hours at 37ºC.
- In a positive reaction, the colour of the medium turned blue. No change in colour indicated negative result for citrate utilization.

Starch Hydrolysis Test

- Bacteria from a pure culture was streaked on a starch agar plate along a straight line and kept for incubation for 48 hours at 37ºC.
- After incubation, a mordant (iodine solution) was dropped over the growth.
- A positive result was indicated by clearing of the media surrounding the bacterial growth.

Nitrate reduction test

- Nitrate broth was inoculated with loopful of bacterial isolate and incubated for 24 hours.
- Nitrate reduction test reagents A and B were added in equal amounts after incubation period.
- The observation of colour change to a deep red within a few moments of addition of reagents A and B indicated presence of nitrate reductase enzyme.
- Zinc powder was added if no colour change was observed after addition of reagents A and B. The appearance of red colour after this step confirmed a negative result for nitrate reduction. Colourless solution after zinc powder addition indicated presence of both nitrate reductase and nitrite reductase.

Hemolysis test

- Blood agar was streaked with bacterial isolate and incubated for 24 hours
- The result was interpreted as follows.
- Alpha-hemolysis: This was indicated by presence of brown-green discoloration under the bacterial growth
- Beta- hemolysis: This was observed from the clearing of red color from the agar around the bacteria due to breakdown of RBC in the agar.

- Gamma-hemolysis: The lack of discolouration or clearing of medium indicated gamma- hemolytic bacteria (negative for hemolysis).

Mannitol salt agar test

- MSA was streaked with single colony.
- The Plates were incubated at 35°C
- It contains 7.5% sodium chloride and mannitol as sole carbon source.
- Growth on MSA plate and colour transformation to yellow from red indicates salt mannitol fermenting capability.
- A nonfermenting bacterium that withstands the high salt concentration would display a red to pink area due to peptone breakdown.

MR-VP test

- Potassium phosphate broth (MR-VP broth) containing dextrose, peptone and potassium phosphate was inoculated by a loopful of pure bacterial isolate and incubated at 37ºC for 24 hours.
- The broth (6 ml) was divided equally among two tubes to perform MR (methyl red) test and VP (Voges-Proskauer) test.

MR test reaction:

- Bacteria may have produced acid through the incubation period to suppress the phosphate buffer and make the broth acidic. Methyl red was added and the test was considered as positive if red colour is formed.

VP test reaction:

- Barritt's reagent A was added to an already incubated potassium phosphate broth and shaken slightly.
- Barritt's reagent B was added in equal amounts to reagent A. The test tube was kept still for 15 minutes.
- Appearance of a red colour on the reagent layer signified positive reaction.

Fermentation Tests: Arabinose, fructose, galactose, lactose, dextrose, sucrose, rhamnose, mannitol

- Labeled test tubes were filled with a carbohydrate broth (dextrose, sucrose, lactose, mannitol and arabinose) and inverted durham tubes were placed in them fully filled with the broth.

- Each tube was aseptically inoculated with pure bacterial culture from nutrient agar plates.
- The tubes were incubated for 24 hours at 37ºC.
- The result was interpreted by observing change of colors accordingly.
  - Change of color to yellow: The change of the colour of broth to yellow indicated acid formation from fermentation with or without gas production.
  - No colour change: The retaining of color (red) meant absence of fermentation or alcohol fermentation depending on gas formation.
  - Gas production: The presence of bubbles in the inverted durham tube indicated gas production accompanied by acid production or alcohol fermentation.

TSI agar

- TSI slant was prepared and using an inoculating needle single colony was taken and the butt was stabbed, withdrawing the needle streaking on the slant surface. The tube was loosely screw capped for access of air.
- Incubation was done at 37°C for 18 to 24 h. Three kinds of data may be obtained from the reactions.

Interpretation of result:

(a) Sugar fermentations

Acid butt, alkaline slant (yellow butt, red slant): glucose has been fermented but not sucrose or lactose.
Acid butt, acid slant (yellow butt, yellow slant): lactose and/or sucrose has been fermented.
Alkaline butt, alkaline slant (red butt, red slant): neither glucose, lactose, nor sucrose has been fermented.

(b) Gasproduction

Indicated by bubbles in the butt. With large amounts of gas, the agar may be broken or pushed upward.

(c) Hydrogensulfideproduction

Hydrogen sulfide production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of H_2S with the ferrous ammonium sulfate to form black ferrous sulfide.

The black precipitate indicates that the bacteria were able to produce hydrogen sulfide (H_2S) from sodium thiosulfate. Because H_2S is colourless, ferric ammonium citrate is used as an indicator resulting in the formation of insoluble ferrous sulfide. Formation of H_2S requires an acidic environment; even though a yellow butt cannot be seen because of the black precipitate, the butt is acidic. The results would be recorded as acid over acid (A/A), H_2S positive.

Lipid hydrolysis

- Tributyrin agar plates were inoculated with organisms in a zig-zag pattern.
- The plates were incubated at 37 ºC for 24-48 hours.
- Results were recorded upon observation of clear areas around the growth of the organisms.
- Clear areas indicate lipolysis, whereas absence of clear areas indicate no lipolysis.

Gelatin hydrolysis

- Medium was prepared by dissolving 5.0g peptone, 3.0g beef extract and 120g liquid gelatin per litre.
- 2 to 3 ml of the medium was dispensed into test tubes, and autoclaved. • The media was inoculated with an inoculating needles before being incubated at 37ºC for 48 hours.
- The cultured were placed in the refridgerator, 4ºC for 30 minutes, and then rapid liquefaction of gelatin was checked.
- The solidified cultured were returned to the incubator for another five days. Afterwards, the cultures were kept at 4ºC for 30 minutes and liquefaction of gelatin was checked again.
- Rapid liquefaction: If media liquefies at 4ºC for within 48 hours, that means the organisms produce gelatinase and demonstrate rapid gelatin hydrolysis • Liquefaction: If media liquefies at 4ºC after five days, that means the organisms are capable of producing gelatinase.
- No liquefaction: If media is solid at 4ºC, then this indicates that organisms do not produce gelatinase.

2.4.3 Identification of bacteria

The colonial and cellular morphologies, physiological and biochemical characteristics were noted from the aforementioned reactions and observations. From those results, identification of the bacteria was done with reference to Microbiology Laboratory Manual (Cappuccino & Sherman., 2005) and online laboratory tool for bacterial identification, Advanced Bacterial Identification Software (ABIS).

2.5 Preservation of Isolates

The preservation of the bacterial cultures was done in 3 ml T_1N_1 agar in small vials. Inoculation was done from isolated pure cultures with a stab of the needle into the agar. The vial was incubated for up to 48 hours for the bacteria to reach log phase and then 400µl sterile glycerol was added over the inoculated and incubated agar. The junction of cap and vial was enclosed with parafilm. These vials were stored at room temperature.

CHAPTER 3

RESULTS

Four microorganisms were isolated from two soil samples and one furnace oil sample to test for furnace oil and crude oil degradation capability. The ability to utilize furnace oil and crude oil as carbon source was determined by observing growth in mineral salt media with only furnace oil and crude oil as carbon source. The growth was visibly qualified by increased turbidity and quantitatively studied by measuring colony forming units on nutrient agar plates.

3.1 Furnace oil and crude oil degradation ability of different isolates

The four isolates were individually cultured in 100 ml mineral salt broth (pH 7) for up to 7 days at 35ºC in a shaking incubator at 150 rpm with 1% (v/v) crude oil and furnace oil as sole carbon source individually. The bacteria showed varying degree of growth.

3.2Plate count

For measuring the growth of the organisms, spread plate technique was used. Fifty micro litresuspension was diluted with 0.85% NaCl and spread on nutrient agar plates on day 3 and day 7.

Table 1: Organism and its growth in 100 ml MSM with 1% Furnace oil

Organism	**Day 0 (Inoculum size)**		**Day 3**		**Day 7**	
	CFU/ml	**logCFU/ml**	**CFU/ml**	**logCFU/ml**	**CFU/ml**	**logCFU/ml**
Bacillus megaterium	3x10	6.48	$8.6x10^3$	3.93	$6.2x10^3$	3.79
Bacillus badius	4.8x10	6.68	$5.48x10^7$	7.74	$5.42x10^7$	7.73
Paenibacillus chebensis	$3.48x10^6$	6.54	$4x10^4$	4.6	$5.36x10^5$	5.73
Aerococcus viridans	$4.4x10^6$	6.64	$4.8x10^6$	6.68	$5x10^6$	6.7

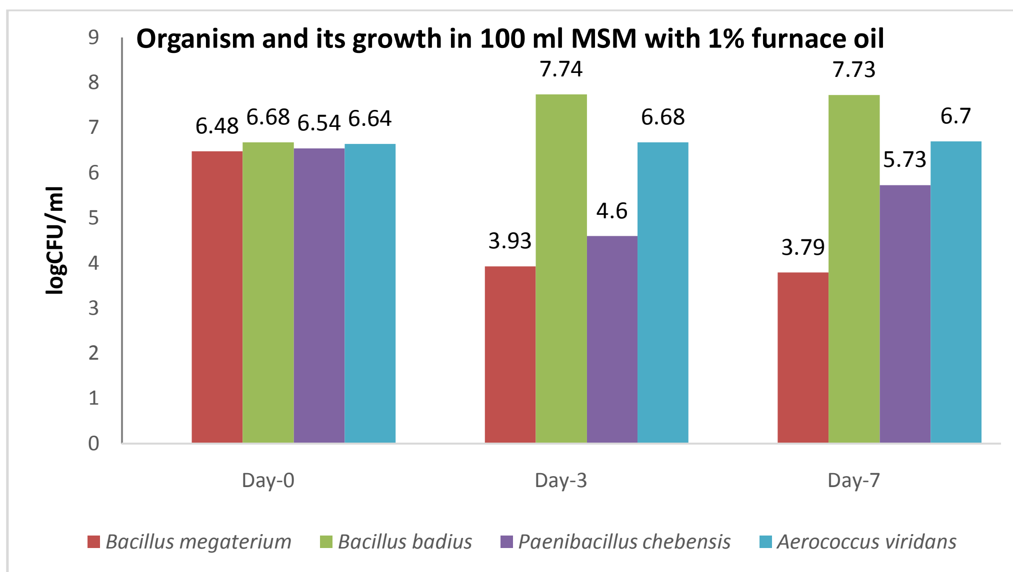

Figure 1: Growth of organisms with time in 100 ml MSM with 1% furnace oil and sample organism.

In the present study, *Bacillus badius* showed the highest growth with increased time. *Aerococcus viridans* showed medium growth. *Paenibacillus chebensis* showed a drop onday 3 and again growth reappears, probably takes time for adaptability. *Bacillus megaterium* with no increase in growth at all but was able to survive.

Table 2: Organism and its growth in 100 ml MSM with 1% Crude oil

Organism	Day 0 (Inoculum size)		Day 3		Day 7	
	CFU/ml	logCFU/ml	CFU/ml	logCFU/ml	CFU/ml	logCFU/ml
Bacillus megaterium	$5x10^6$	6.69	200	2.3	$2.4x10^3$	3.38
Bacillus badius	$2.4x10^6$	6.38	$2.6x10^6$	6.41	$5.7x10^7$	7.76
Paenibacillus chebensis	$3.24x10^6$	6.51	400	2.6	$1x10^3$	3
Aerococcus viridans	$5x10^6$	6.69	$2x10^6$	6.3	$5x10^6$	6.7

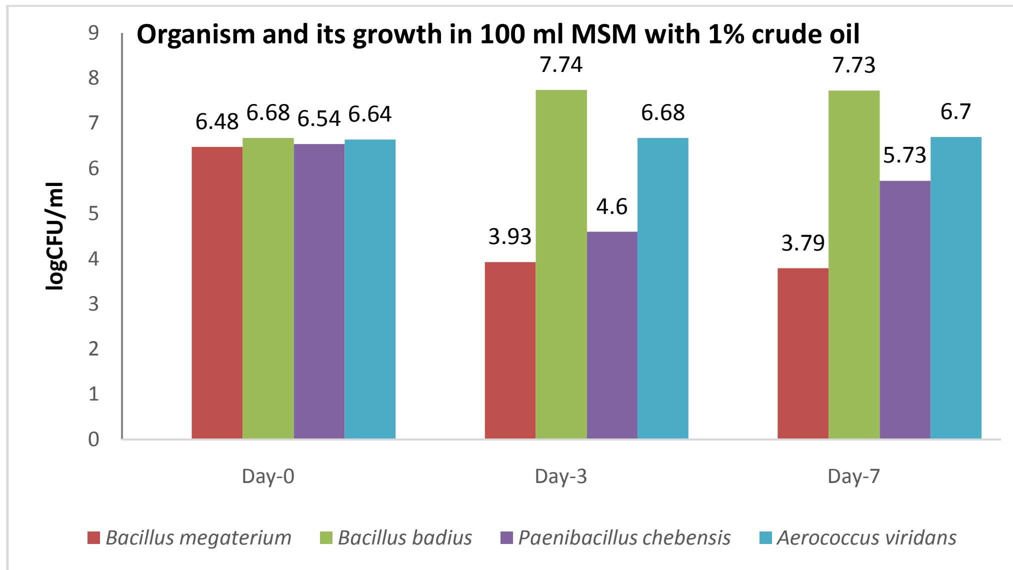

Figure 2: Growth of organisms with time in 100 ml MSM with 1% crude oil and sample organism.

In the present study,*Bacillus badius* showedthe highest growth 7.73 logCFU/ml on day 7. *Aerococcus viridians* showed a decrease in growth then a rise again, which probably means it requires time for the organism to adapt with the environment. Table 2 shows the decrease in growth of*Paenibacilluschebensis*and *Bacillus megaterium* and again an increase of the same with time.

3.3Optical density

Optical Densitywas measured to observe the difference between turbidity of each flask's content with distilled water. The spectrophotometer was set at 600 nm for recording the absorbance. Turbidity is proportional to growth of microorganisms.

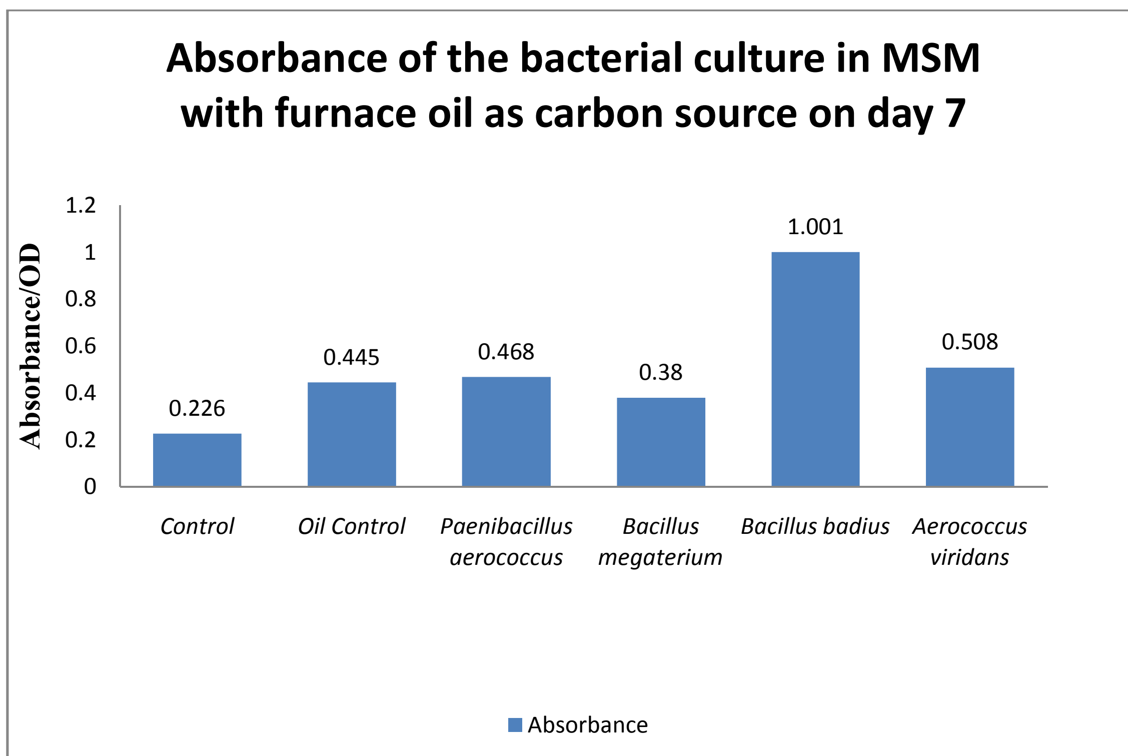

Figure 3: Absorbance of the bacterial culture in MSM with furnace oil as carbon source on day 7.

In the present study, the spectrometer was set at 600 nm. *Bacillus badius* showed the highest absorbance which was 1. *Aerococcus viridans* has an absorbance of 0.508. *Paenibacillus chebensis* and *Bacillus megaterium* with least absorbance of 0.468 and 0.380 respectively, as growth in these flasks were negative. The control (MSM only) and oil control (MSM plus furnace oil) also showed absorbance as shown in the figure 3 when distilled water was used as blank.

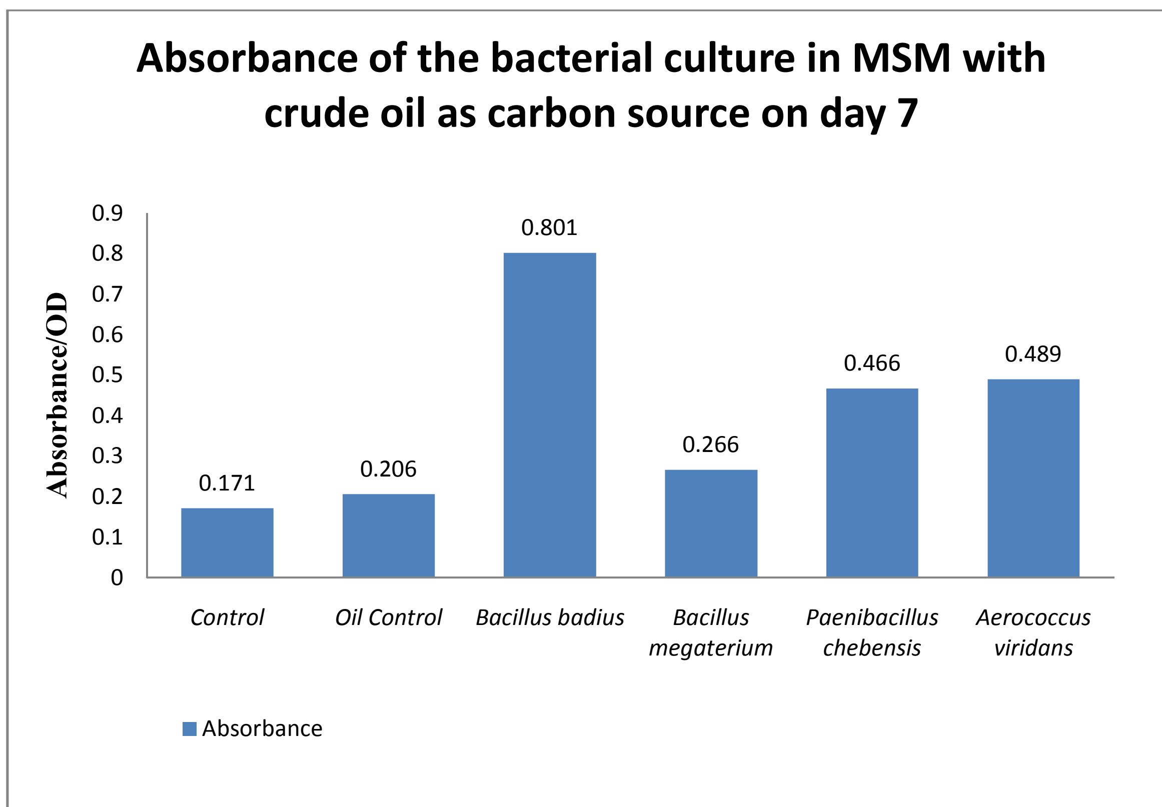

Figure4: Absorbance of the bacterial culture in MSM with crude oil as carbon source on day 7.

In the present study, the spectrometer was set at 600 nm. *Bacillus badius* showed the highest absorbance with 0.801. *Aerococcus viridans* showed absorbance 0.489. *Bacillus megaterium* and *Paenebacillus chebensis* show least absorbance of 0.266 and 0.466 respectively. The control (MSM only) and oil control (MSM plus crude oil) also showed absorbance as shown in the figure 4 when distilled water was used as blank.

3.4Identification of bacterial isolates

The identification of bacteria genera was accomplished through microscopic evaluation, morphological characterization and biochemical tests with comparison to standard references mentioned in Bergey's Manual of Systematic Bateriology, Microbiology Laboratory Manual (Cappuccino & Sherman, 2005) and with the help of online laboratory tool for bacterial identification, Advanced Bacterial Identification Software (ABIS).

Table 3: Colony morphology of four isolates on Nutrient agar

Isolate	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation
Bacillus megaterium	Circular	Smooth	Convex	Small	Smooth	Dull	White
Bacillus badius	Circular	Smooth	Raised	Small	Smooth	Shinny	White
Paenibacillus chebensis	Circular	Irregular	Umbonate	Large	Textured	Dull	Off White
Aerococcus viridans	Circular	Smooth	Raised	Small	Smooth	Shiny	White

Table 4: Biochemical test results of four isolates and their identification through the use of ABIS online and Microbiology Laboratory Manual (Cappuccino & Sherman)

Sample	Gram staining	Shape	Acid fast staining	Spore Staining	Citrate Utilization	Indole Production	Voges-Proskauer	Methyl-red	Sugar fermentation	H_2S production	Gas production	Nitrate Reduction	Motility	Oxidase	Catalase	Gelatin hydrolysis	Hemolysis	Growth at7..5% NaCl	Tributonin lipid hydrolysis	Starch Hydrolysis	Arabinose fermentation	Dextrose fermentation	Galactose fermentation	Fructose fermentation	Maltose fermentation	Sucrose fermentation	Mannitol fermentation	Lactose fermentation	Rhamnose fermentation	Presumptive Organism
SB1	+ve	Rod	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	α	+ve	+ve	+ve	+--	++-	+--	+--	+++	++-	+--	+--	---	*Bacillus megaterium*
SB2	+ve	Rod	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	α	-ve	+ve	-ve	---	---	---	---	---	---	---	---	---	*Bacillus badius*
SM2	+ve	Rod	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	α	+ve	+ve	+ve	++-	++-	---	++-	+--	---	+--	---	---	*Paenibacillus chibensis*
FOI	-ve	Cocci	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	α	-ve	+ve	-ve	++-	+++	+++	+++	+++	---	+++	+++	---	*Aerococcus viridans*

Key:

+++ : Excellent fermentation

++- : Good Fermentation

+- - : Poor fermentation

- - - : No fermentation

α: Alpha hemolysis

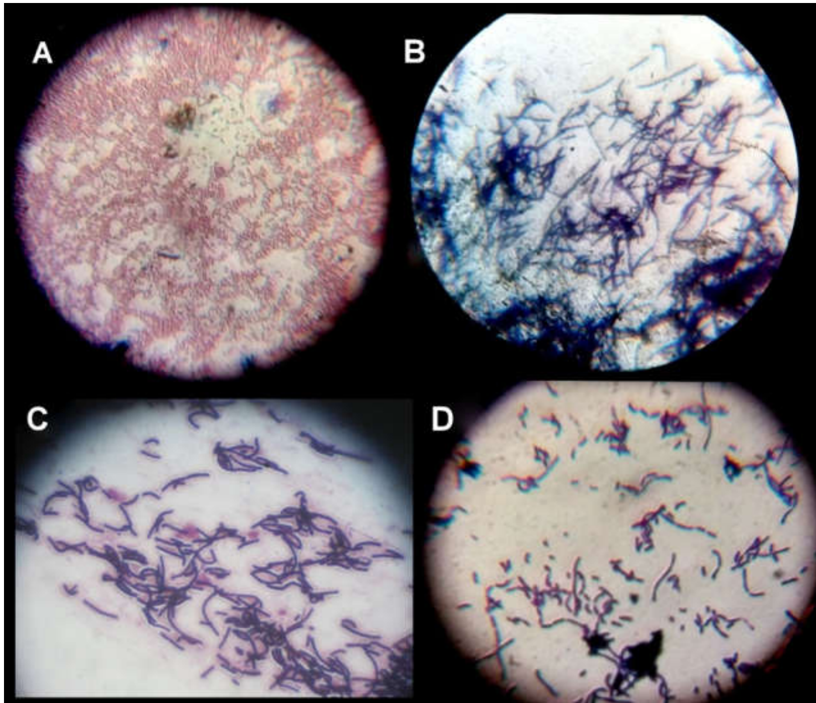

Figure 5: Microscopic observation after Gram Staining (100x oil emulsion).

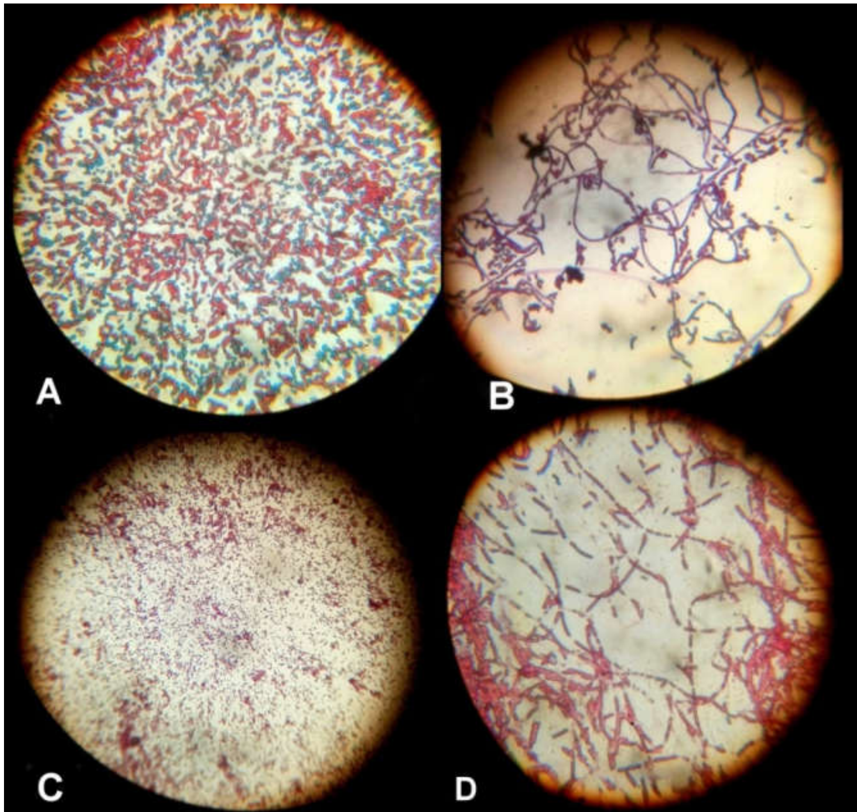

Figure 6: Microscopic observation after Spore Staining (100x oil emulsion).

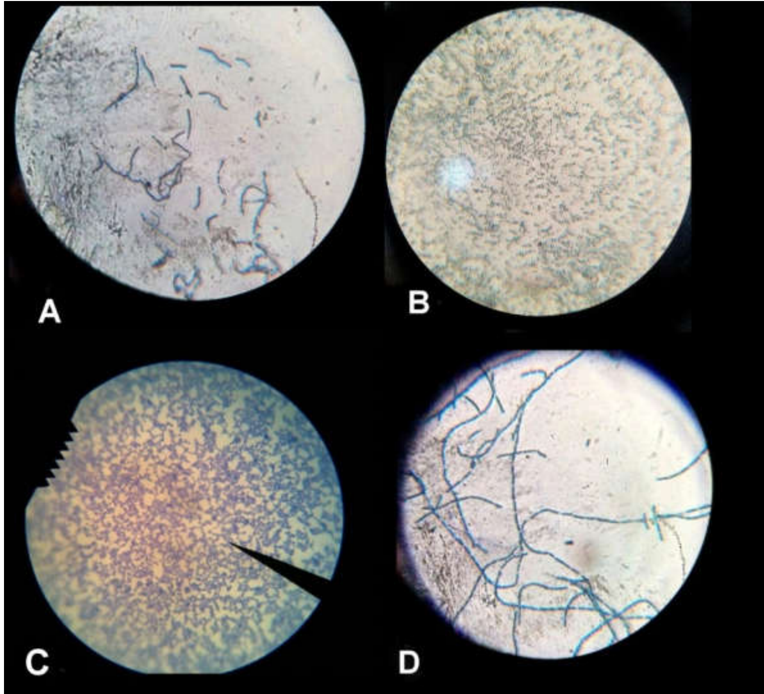

Figure 7: Microscopic observation after Acid Fast Staining (100x oil emulsion).

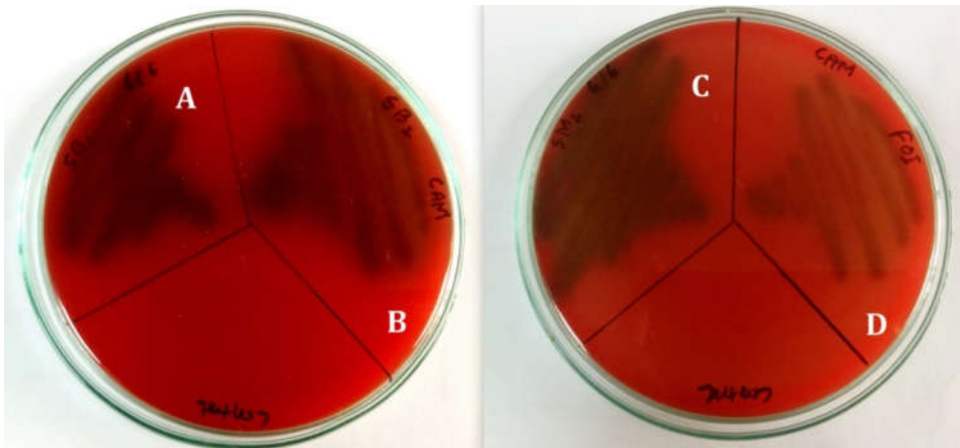

Figure 8: Hemolysis. Alpha hemolysis shown by all samples.

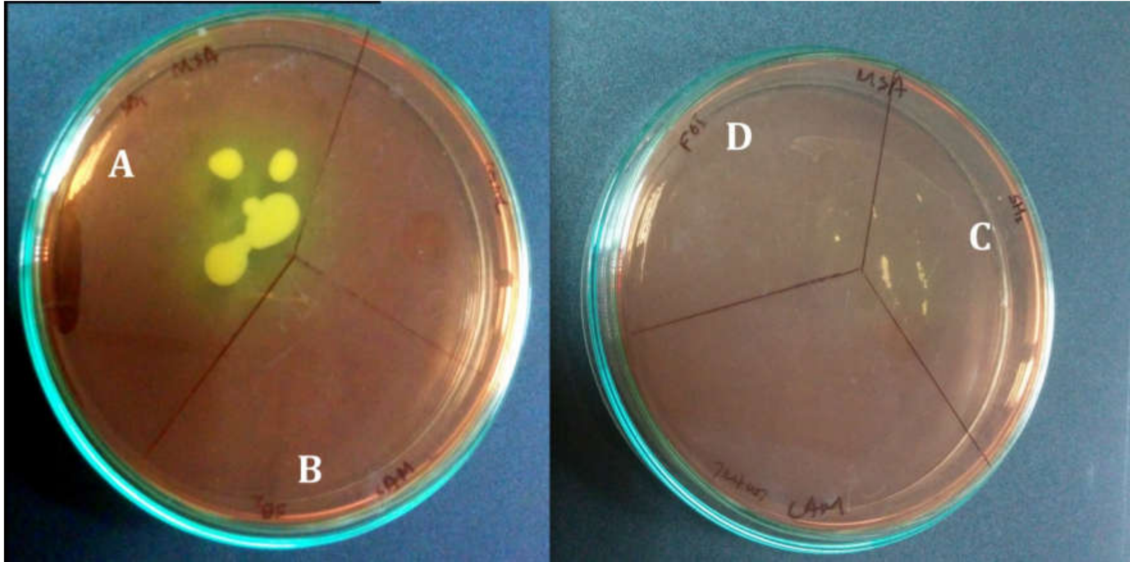

Figure 9: Growth on Mannitol salt agar (7.5% NaCl)

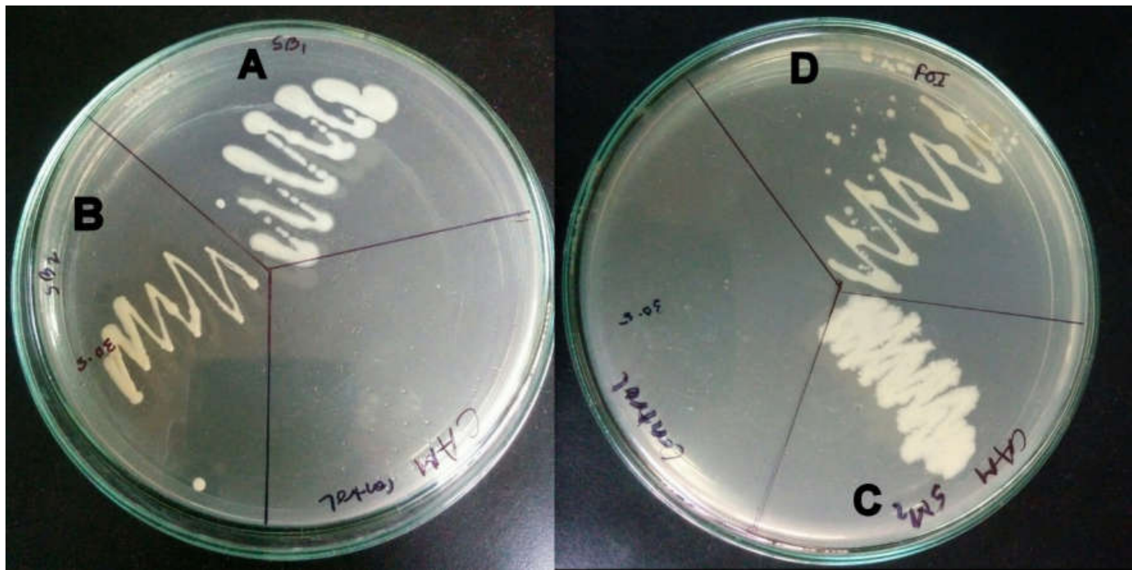

Figure 10: Tributonin lipid Hydrolysis test result, all positive.

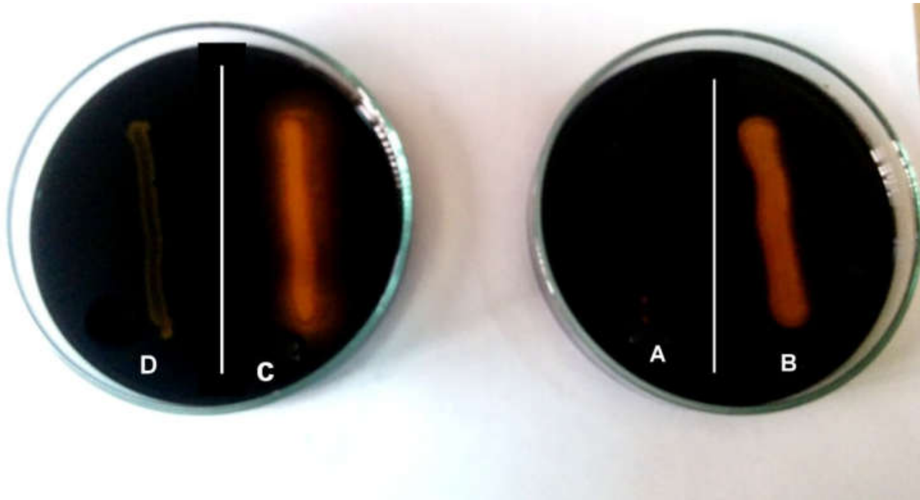

Figure 11: Starch hydrolysis test result.

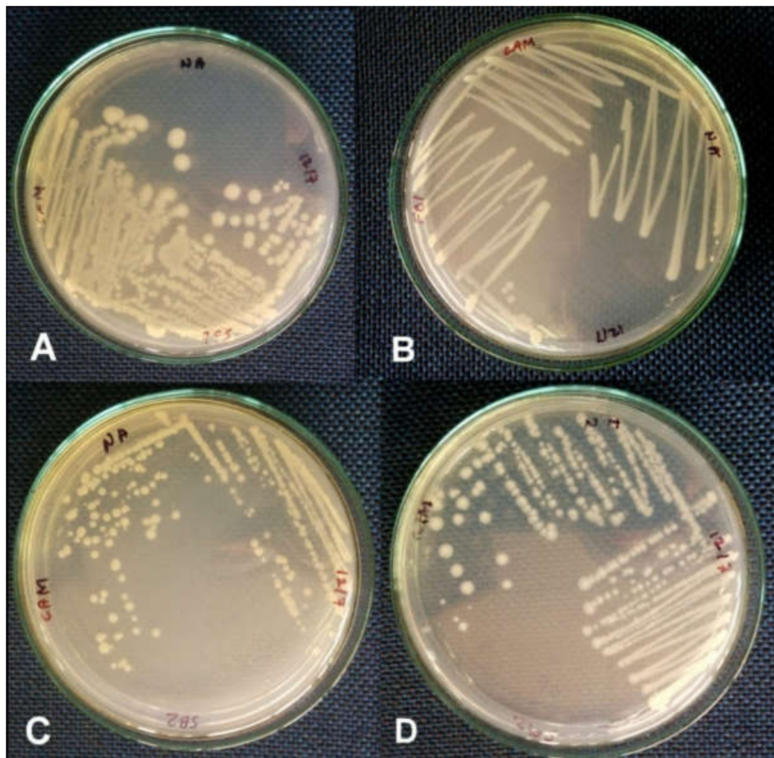

Figure 12: Streak plates of Designated Isolates on NA.

Key: **A-** *Bacillus megaterium* **B-** *Bacillus badius*

C-*Paenibacillus chebensis* **D**- *Aerococcus viridans*

CHAPTER 4
DISCUSSION

4.1 Discussion

Oil spill and pollution is a major problem, which is causing irreversible environmental changes all over the world. It is affecting lives on land and in marine. Different measures are taken in order to clean the spill; physical methods are costly and not as effective. On the other hand chemical methods may cause further toxic problems. Biodegradation has given much effective and safer result. The process is slow, but optimizing conditions and isolating and introducing favorable strains may speed up the process, without causing any affect and further damage to the environment.

In the present study, microorganisms were isolated from two different locations and from one of the two oil samples. The location of sample SB was Shadarghat Buriganga River (underwater soil approx. 2m), two isolates were taken from the sample soil: SB1 and SB2. Location of SM was Malibagh truck stand (top soil), and FOI (Furnace Oil Isolate) was isolated by spreading furnace oil on NA plate, only single try of colonies were observed on the plate after 24hrs of incubation at 37°C.

Among all the isolates three were identified as *Bacillus sp.* and another as *Streptococccus sp*. The isolate which showed the greatest growth on MSM and both crude oil and furnace oil was SB2 identified as *Bacillus badius.* It also showed the highest turbidity of all with 1.001 OD for furnace oil and 0.801 OD for crude oil. The CFU count for furnace oil on day 7 was 7.73 logCFU/ml and for crude oil on day 7 it was 7.76 logCFUml. As mentioned earlier, this organism was isolated from Buriganga River, which is highly polluted with different oils from ships, ferries, launches, oil driven boats etc. It was reported the genera *Bacillus* having capabilities to utilize toxic components of crude oil for growth (Das *et al.,* 2007).

After SB2 isolate FOI identified as *Aerococcus viridians* showed optimum growth in MSM broth with furnace and crude oil as carbon source. The bacterial count on day 7 was 6.7 logCFU/ml for both furnace oil and crude oil from 6.64 logCFU/ml and 6.69 logCFU/ml respectively on day 1.How the organism came in contact with the oil is unknown.

The other two isolates SB1 and SM2 identified as *Bacillus megaterium* and *Paenibacillus chinbensis* respectively showed no or little growth. For furnace oil *B. megaterium* count reduced to 3.79 logCFU/ml on day 7 from 6.48 logCFU/ml at day 1 and *P. chebensis* count reduced to 5.73 logCFUml on day 7 from 6.54 logCFU/ml on day 1. Both showed a reduction in number of CFU and then a leap, which possibly means, they take time to adapt with the

environment. Probably by changing the conditions and adding growth factor may enhance their growth and utilization of both oils.

In addition, it has been studied that other species of *Bacillus* like *B. subtilis* and *B.licheniformis* showed success in degradation of oil, especially when emulsifier was given (Javaheri *et al.,* 1985). *Bacillus*is also found to make biosurfactants e.g.: *B. subtilis* produces Surfactin, B. *polymyxa* produces Polymixins and *B. brevis* produces Gramicidin S. From this study *B. Badius* is thought to produce biosurfactants, as both furnace oil and crude oil was found to lose viscosity after 7 days treatment with *B. Badius* (Banat *et al.,* 1995; Banat & Makkar *et al.,* 2000)

In another study on crude oil degrading bacteria held at Federal University of Technology, Akure, Ondo State, Nigeria showed that *B. cereus*, *B. subtilis* and *Aerococcus viridians* gave 14% to 28% degradation over 7 weeks (Cooney *et al.,* 1984).

4.2 Further Research

The research work in this experiment involved only isolation of furnace oil and crude oil utilizing bacteria. The growth optimization and varying the parameters was not conducted as lab work. Varying time, pH, oil concentration, temperature, oxygen concentration, and presence of carbon dioxide, rotations per minute and additional nutrient can help to determine better growth conditions and degradation of oil by the organisms. Also more biochemical tests would have helped to determine the species better. Most of the studies regarding oil degradation involved varying time, as the both furnace oil and crude oil both has complicated structure, definitely giving more time would have resulted into better degradation.

The degree of degradation can be determined by employing gas chromatographic analysis and the rate of degradation can be analyzed by gravimetric methods. Gas chromatography reveals the individual hydrocarbon components and can be used to compare control and experiment sample of furnace oil and crude oil after degradation by bacteria. A gravimetric method of analysis involves period measurement of the decrease in substrate in soil samples and determines the rates of biodegradation. The experimental condition in a laboratory does not mirror the performance of the bacteria in a polluted environment. That is why pilot scale operations of bioremediation with selected bacteria must be performed and optimized to get effective results.

More sophisticated methods for determining the species of the isolates can be applied. 16S rRNA sequence and phylogenetic tree analysis can be used to identify the species of the screened bacteria. Plasmid profiling can reveal many information related to the genes associated with biodegradation pathway enzymes. PCR methods can also be developed for fast screening of bacteria based on enzyme gene classes. Metagenomics is a promising method that utilized large scale sequencing and comparison against metagenomic library to find enzyme classes. Therefore, this method can be used for screening on a large scale and by-pass cultivation method of screening (Sierra-Garcia & Oliveira *et al.,*2013). Though, the systems and analysis for sophisticated screening requires enriched facilities and expense of resource, it can engender long term benefits for bioremediation.

Recommendation for future work:

1. More extensive screening programme may reveal better hydrocarbon degrading bacteria. Thus more diversified areas may be selected for isolation of suitable degrading organism.
2. Potential isolate may be subjected to genetic manipulation for improvement of pollutant degrading ability.
3. Biostimulation strategy may be adopted to improve the degrading ability of selected isolates.
4. Environmental manipulation using pH, oil concentration, temperature, oxygen concentration, presence of carbon dioxide, rotations per minute and additional nutrient may be undertaken to optimize the degradation ability.
5. PCR for proper identification and determination of the metabolic pathway responsible for oil degradation.

4.3Conclusion

The information obtained in the study implies that bacteriahave the ability to utilize crude oil and furnace oil.Their proliferation in crude oil and furnace oil implies the utilization of the oils as possible source of energy for the bacteria. Thus, the bacteria are able to breakdown the crude oil and furnace oil. However, molecular level analysis is necessary to confirm the issue. In turn, this makes the crude oil less harmful to the activities of plants and animal in the environment. Therefore, the bacteria could be used as agent of bioremediation, useful for the breakdown of the oil pollution in the environment.In order to protect our environment from the spilled oil, extensive research work must be carried out.

References

- AL-Saleh, E., Drobiova, H. and Obuekwe, C. (2009). Predominant culturable crude oil-degrading bacteria in the coast of Kuwait. *International Biodeterioration& Biodegradation, 63*(4), 400-406.
- Alvarez, P. J. J and Vogel, T. M. (1991). Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Applied and Environmental Microbiology, 57*(10), 2981-2985.
- Atlas, R. M. (1982). Microbial degradation of petroleum hydrocarbons: an environmental perspective.*MicrobiologicalReviews*, *45*(1), 180-209.
- Atlas,R. M. and Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation.*Advances in Microbial Ecology*, *12*, 287–338.
- Atlas, R. M., Grimes, D. J. and Hazen, T. C. (2011). Microbes and oil spills.
- Banat, I. M. (1995). Biosurfactant production and possible uses in microbial enhanced oil recovery and oil pollution remediation. *Bioresource Technology, 51*(1), 1-12.
- Banat, I. M., Makkar, R. S. and Cameotra, S. S. (2000). Potential commercial applications of microbial surfactants. *Applied Microbiology* and *Biotechnology*, 53(5), 495-508.
- Boldu-Prenafeta, F. X., Ballerstedt, H., Gerritse, J. and Grotenhuis, J. T. (2004). Bioremediation of BTEX hydrocarbons: effect of soil inoculation with the toluene-growing fungi *Cladophialopora* sp. strain T1. *Biodegradation, 15* (1), 59-65.
- Chander, K., Brookes, P. C. and Harding, S. A. (1995). Microbial biomass dynamics following addition of metal - enriched sewage sludge to a sandy loam. *Soil Biology and Biochemistry, 27*(11), 1409-1421.
- Cooney, J. J. (1984). The fate of petroleum pollutants in fresh water ecosystem, in Petroleum Microbiology, 399.
- Das, K. and Mukherjee, A. K. (2007). Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresource Technology*, *98*(7), 1339-45.
- Dasgupta, D., Ghosh, R. and Sengupta, T. K. (2013). Biofilm-mediated enhanced crude oil degradation by newly isolated *Pseudomonas* species. *International Scholarly Research Notices Biotechnology, 2013*, 1-13.

- Ellis, L. B. M., Hershberger, C. D., Bryan, E. M. andWackett, L. P. (2001). The university of Minnesota biocatalysis/biodegradation database: emphasizing enzymes.*Nucleic Acids Research, 29*(1), 340–34.
- Foght, J. M. and Westlake, D. W. S. (1987). Biodegradation of hydrocarbons in freshwater, in *Oil in Freshwater: Chemistry,Biology, Countermeasure Technology*, J. H.Vandermeulen and S. R. Hrudey, Eds., pp. 217–230, Pergamon Press, New York, NY, USA.
- Gestel, K. V., Mergaert, J., Swings, J., Coosemans, J. and Ryckeboer, J. (2003). Bioremediation of diesel oil-contaminated soil by composting with biowaste. *Environmental Pollution, 125*(3), 361–368.
- Giti, E.,Mehdi,H.and Nasser, G. (2005). Development of a microtiter plate method for determination of phenol utilization, biofilm formation and respiratory activity by environmental bacterial isolates. *International Biodeterioration& Biodegradation*, *56*(4),231-235.
- Hedlund, B. P., Geiselbrecht, A. D., Bair, T. J., Staley, J. T. (1999). Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonasnaphthovorans* gen. nov., sp. nov.*Applied Environmental Microbiology, 65*(1), 251-259.
- Javaheri, M., Jenneman, G. E., McInnerney, M. J. and R. M. Knap. (1985). Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2.*Appllied and Environmental Microbiology, 50*(3), 698–700.
- Kvenvolden, K. A. and Cooper, C. K. (2003). Natural seepage of crude oil into the marine environment.*Geo-Marine Letters*, *23*(3), 140–146.
- L. N. Britton, (1984). Microbial degradation of aliphatic hydrocarbons, in Microbial Degradation of Organic Compounds, D. T. Gibson, Ed., pp. 89–129, Marcel Dekker, New York, NY, USA.
- Laškova T., Zabukas V., Vaitiekūnas P. (2007). Influence of meteorological conditions on volatile organic compound spread in the atmospheric boundary layer. Journal of Environmental Engineering and Landscape Management, 15 (3): 135–143.
- Leahy, J. G. and Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment.*Microbiological Reviews*, *54*(3), 305–315.
- Liužinas R., Jankevičius K. (2003). Microbiological processes inmunicipal sewage. Aplinkostyrimai, inžinerijairvadyba, 3 (25): 21–28.

- Maier, R. M., Soberon-Chavez, G. (2000). *Pseudomonas aeruginosarhamnolipids*: biosynthesis and potential applications. *Applied Microbiology and Biotechnology, 54*(5), 625-633.
- Namkoong, W., Hwang, E. Y., Park, J. S. and Choi, J. Y. (2002). Bioremediation of diesel-contaminated soil with composting. *Environmental Pollution, 119*(1), 23-33.
- Ojo, O. A. (2006). Petroleum-hydrocarbon utilization by native bacterial population from a waste water canal Southwest Nigeria. *African Journal of Biotechnology, 5* (4), 333-337.
- Paulauskienė, T., Zabukas, V. andVaitiekūnas, P. (2009). Investigationof volatile organic compound (VOC) emission in oil terminal storage tank parks. *Journal of Environmental Engineering and Landscape Management, 17*(2), 81-89.
- Prince R. C. (2005). The microbiology of marine oil spill bioremediation. Ollivier B., Magot M. (eds). Microbiology. Washington, USA, 317.
- Prince, R. C. (1997). Bioremediation of marine oil spills. *Trends in Biotechnology, 15*(5), 158-160.
- R. M. Atlas and R. Bartha. (1998). Microbial Ecology: Fundamentals and Applications, Benjamin/Cummings Publishing Company, Inc., An imprint of Addison Wesley Longman, Inc, Menlo Park, Calif, USA, 4th edition.
- R. M. Atlas, Ed. (1984). *Petroleum Microbiology*, Macmillion, New York, NY, USA.
- Repečkienė, J., Januška, V., Paškevičius, A. andLugauskas, A. (2002). Amount and species diversity of microorganisms in fuel oil-contaminated soil. BotanicaLithuanica, 4: 91–101.
- Roy, A. S., Baruah, R., Borah, M., Singh, A. K., Boruah, H. P. D., Saikia, N., Deka, M., Dutta, N. and Bora, T. C. (2014). Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. *International Biodeterioration & Biodegradation*, *94*, 79-89.
- Sei, A. &Fathepure, B. Z. (2009). Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake. *Journal of Applied Microbiology, 107*(6), 2001-8.
- Shabir, G., Afzal, M., Anwar, F., Tahseen, R. and Khalid, M. Z. (2008). Biodegradation of kerosene in soil by a mixed bacterial culture under different nutrient conditions. *International Biodeterioration and Biodegradation, 61* (2), 161-166.

- Sierra-Garcia, I. N. and Oliveira, V. M. (2013). Microbial hydrocarbon degradation: efforts to understand biodegradation in petroleum reservoirs. In *Biodegradation - Engineering and Technology,* 47-72.
- Vasarevičius, S., Greičiūtė, K. andŠiaulytė, E. (2005). Investigationand evaluation of soil pollution with oil products in the most intensively used territories of Gaižiūnai military training ground. *Journal of Environmental Engineering and Landscape Management, 13*(4), 160-167.
- Zhao, H. P., Wang, L., Ren,J. R., Li, Z., Li, M. and Gao, H. W. (2008). Isolation and characterization of phenanthrene-degrading strains *Sphingomonas* sp. ZP1 and Tistrella sp. ZP5. *Journal of Hazardous Materials, 152*(3), 1293-1300.
- Zobell, C. E. (1946). Action of microorganisms on hydrocarbons. *Bacteriological Reviews, 10*(1-2), 1-49.

Appendices

Appendix- I

Media compositions

The composition of all media used in the study is given below.

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline

Component	Amount (g/L)
Sodium Chloride	8.5

Starch Agar

Component	Amount (g/ L)
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniundihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bactobromothymol blue	0.08

Tryptophan Broth

Component	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Methyl red Voges- Proskauer (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Gelatin Broth

Component	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
Final pH	6.8 ± 0.2 at 25°C

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Blood Agar Base

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

Sugar Fermentation Broth

Component	Amount (g/L)
Sugar	5.0
Trypticase	10.0
Sodium chloride	5.0
Phenol red	A very small amount until the broth turns red

Appendix – II

Reagents and Buffers

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room

Folin reagents:

Reagent A: To make 100 ml of reagent A, 50 ml of 2% sodium carbonate was mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)

Reagent B: To make 20 ml of reagent B, 10 ml of 1.56% copper sulphatesolution was mixed with 10 ml of 2.37% sodium potassium tartarate solution.

Reagent C (analytical reagent): To make 100 ml of reagent C, 2 ml of reagent B was mixed with 100 ml of reagent A. This was prepared prior use.

Folin - Ciocalteau reagent solution (1N): To make 4 ml of Folin - Ciocalteau reagent solution 2 ml of commercial reagent (2N) was diluted with an equal volume (2 ml) of distilled water on the day of use.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100 ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temeperature.

Malachite green (100 ml)

To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution was transferred to the

reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was stored at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediaminedihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light. Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Nitrate Reagent A (100 ml)

5Nacetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N,N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N)and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)[a] to form a colourless solution and stored at 4°C.

Appendix – III

Instruments

The instruments used in the study are given below.

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Spectrophotometer, UV mini – 1240	Shimadzu Corporation, Australia
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
pH Meter: pHep Tester	Hanna Instruments, Romania
Microscope	A. Krüssoptronic, Germany
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
Conical Flask	Amber
Petri Plate	Amber
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Syringe	JMI Syringes and Medical Devices ltd.
Filter Unit (Millipore)	MILLEX GS