Isolation of Kerosene Degrading Bacteria from Soil Samples and Determination of Optimum Growth Conditions

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

Submitted by

Mashiat Nawar Chowdhury

Student ID: 11136002

April, 2015

Department of Mathematics and Natural Sciences
Biotechnology Program
BRAC University
Dhaka, Bangladesh

DECLARATION

I hereby certify that this thesis project entitled **"Isolation of kerosene degrading bacteria from soil samples and determination of optimum growth conditions"** is submitted by me, Mashiat Nawar Chowdhury (ID – 11136002), to the Department of Mathematics and Natural Sciences under the supervision of Dr. Mahboob Hossain, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. This dissertation was done as a part of my requirement for the degree of BSc in Biotechnology. I also declare that this work is entirely based on the original results I have found. Materials and knowledge that I have consulted from the published works accomplished by other researchers have been properly cited and acknowledged within the text of my work.

____

Candidate

Mashiat Nawar Chowdhury

ID: 11136002

____

Certified by

Dr. Mahboob Hossain

Supervisor

Associate Professor

Microbiology Program

Department of Mathematics and

Natural Sciences

BRAC University

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ABSTRACT

Oil spills are global catastrophes transpiring annually from minute to substantial amounts, threatening the lives of plants, animals and the health of human. Current physical and chemical management systems are very costly and less efficient. Bioremediation through utilization of oil-degrading bacteria proves to be our auspicious hope for the future. Kerosene is a mixture of hydrocarbons that is deployed in aviation fuels and contributes to extremely noxious pollution during spills. It is a conglomerate of benzene, toluene, ethylbenzene, xylene, along with high percentage of aromatic hydrocarbons that are carcinogenic. This makes kerosene imperative to be removed from the environment before its significant spread over large habitat. In this study, several bacterial isolates from four soil samples were assessed for their ability to degrade kerosene. They were grown on mineral salts broth for 7 days at 35°C in the absence of any carbon source apart from kerosene. Growth of the isolates were observed by visible turbidity and evaluated by CFU/mL on mineral salts agar. The isolates were identified as *Nocardia*, *Corynebacterium*, *Bacillus*, *Pseudomonas* and *Arthrobacter* through morphological characteristics and biochemical tests. The best potential isolate was tested for its optimum concentration of kerosene, optimum pH for growth and optimum concentration of ammonium sulfate (nitrogen source) requirement. *Pseudomonas* sp. exhibited to be the most promising amongst the isolated microbes at utilizing kerosene, followed by *Bacillus* sp., both of which were isolated from soils that were previously contaminated by oil. Isolates such as *Nocardia* and *Corynebacterium* taken from soil sample dearth of prior exposure to oil contamination were unable to grow in the presence of kerosene. *Pseudomonas* sp. tolerated kerosene at concentration as high as 6%, and showed optimum growth at 3% kerosene (v/v). *Pseudomonas* sp. was also found to grow best at near neutral pH and at nitrogen concentrations (ammonium sulfate) of 1 g/L.

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LIST OF ABBREVIATIONS

Abbreviations	Descriptions
MSM	Mineral Salts Media
NA	Nutrient Agar
RBC	red blood cell
NPK	Nitrogen, phosphorus, potassium
MIU	Motility, Indole, Urease test
MR	Methyl Red
VP	Voges-Proskauer
GC	Gas chromatography
rpm	rotations per minute
µL	microliter
mL	milliliter
g/L	grams per liter
v/v	volume by volume
CFU	colony forming unit
TNTC	too numerous to count

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Chapter 1: Introduction

Chapter 1: Introduction

Petroleum hydrocarbons are the world's major sources of fuel because of their potential to produce massive amount of energy. According to their boiling points, petroleum constituents are separated into various fractions, of which alkanes (C_5H_{12} – C_8H_{18}) are refined to petrol, whereas the mid distillates (C_9H_{20} – $C_{16}H_{34}$) are used for fuels such as diesel, kerosene and jet fuel (Speight, 1999). About 85% of the refined crude oil is used for the manufacture of gasoline and kerosene (Pelczer *et al*., 2004).

Figure 1: Oiled pelican from the disaster of Gulf of Mexico (Cole, 2010)

High activity from petrochemical industries worldwide has led to serious environmental problems through accidental spills of the fuels. Such leaks and petroleum spillage ensue during their production, refining, transportation and storage (Das *et al.*, 2010). According to Bartha (1986), about 0.08% to 0.4% of the world's petroleum manufactured wind up as ocean spillage. Same amount of fuels end up polluting the land and can contaminate ground water aquifers through their seepage (Bossart *et al*., 1984). It has been estimated that approximately 600,000 metric tons of oil seepage occurs annually (Kvenvolden & Cooper, 2003). This is of a prior concern since petroleum components are mostly carcinogenic and neurotoxic. Accumulation of these pollutants can not only result in mutation or death of plants and animals, but is a threat to human health through contaminating water supplies and seafood (Das *et al.*, 2010).

Management techniques of an oil spill catastrophe today include physical and chemical methods, both of which are not very effective and can further endanger lives of other organisms (Gulf Oil Spill, 2010). Fortunately, it has been discovered that several indigenous microbes have the capability of removing these pollutants from marine environment and soils. These 'green' microbes are our only hope to salvage lives of animals when accidents befall. Scientists have been working for decades to identify these microbes from oil contaminated sites (Faerber, 2010). However, no two oil spills are exactly alike (Gulf Oil Spill, 2010). A single bacterial species will not be able to optimally metabolize oil at all climatic environments. Therefore, it is of utmost importance to identify several different microbes with the ability to degrade different types of oil at various environments, so that they can be used for the purpose of bioremediation.

1.1 Kerosene: an overview

Kerosene is a refined petroleum constituent having a mixture of 6-16 carbon atoms. It consists of both aliphatic and aromatic hydrocarbons. Gas chromatogram of kerosene shown in Figure 1.1 suggests the presence of benzene, toluene, ethylbenzene, xylene, along with highest percent of naphthalene, 1-methyl naphthalene and 2-methyl naphthalene (C10 and C11 compounds) (Dunlap & Beckmann, 1988).

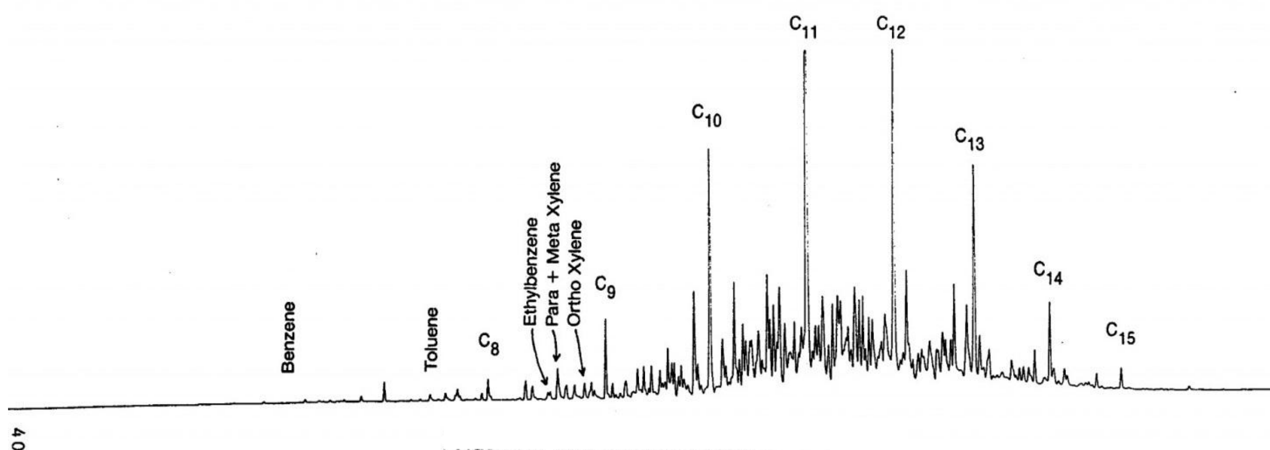


Figure 1.1: Gas chromatogram of kerosene (Dunlap & Beckmann, 1988)

Kerosene is readily available and less expensive, which makes it commonly used for energy both for domestic and industrial purpose. Daily, about 1.2 million barrels of kerosene are used worldwide (Petroleum & Other Liquids, 2014). It is mainly utilized for cooking, particularly in developing countries, as well as for heating and lighting. In industries, kerosene is used as an important solvent as well as a lubricant. It is also an essential constituent of aviation fuels and some rocket fuels. Jet fuels like Jet A, Jet A-1, JP-5 and JP-8 are kerosene type fuels while Jet-B and Jet-4 contains some proportion of kerosene (Rand, 2010). Thus, small to considerable amount of spillage is unavoidably as a consequence of the extensive application of kerosene. A noteworthy aviation spillage that required immediate evacuation of approximately 1,500 residents was reported in 2013 at Slocan Valley, Canada (Maxey, 2013).

1.2 Bacteria involved in biodegradation of petroleum

Microorganisms can utilize unusual organic compounds such as insoluble hydrocarbons as source of carbon and energy. Bossert and Bartha (1984) listed 22 genera of bacteria that have the capability to degrade hydrocarbons. The most notable of the bacteria, as reported in many studies, include *Acinetobacter, Alcaligenes, Arthobacter, Bacillus, Flavobacterium, Nocardia,* and *Pseudomonas* sp. (Table 1.2).

Table 1.2: Soil bacteria known to degrade aliphatic and aromatic hydrocarbons, PAH and chlorinated compounds (Fritsche & Hofrichter, 2008)

Gram negative bacteria	Gram positive bacteria
Pseudomonas sp.	*Nocardia* sp.
Acinetobacter sp.	*Mycobacterium* sp.
Alcaligenes sp.	*Corynebacterium* sp.
Flavobacterium sp.	*Arthrobacter* sp.
Xanthomonas sp.	*Bacillus* sp.

1.3 Mechanism of kerosene degradation

Microorganisms usually degrade most of the organic pollutants under aerobic condition. Aerobic degradation of hydrocarbons occurs in the following steps (as illustrated in Figure 1.3):

1) Contact between pollutants and microbial cell is enhanced through metabolic processes. For example, several microbes produce biosurfactant to emulsify hydrophobic pollutants.
2) Once inside cells, the hydrocarbons are attacked by oxygenases and peroxides.
3) Hydrocarbons enter peripheral pathways in which they are converted into intermediates through a series of steps in the TCA cycle.
4) The pollutants are converted into acetyl-CoA, succinate, pyruvate etc. which can be utilized as cell biomass.

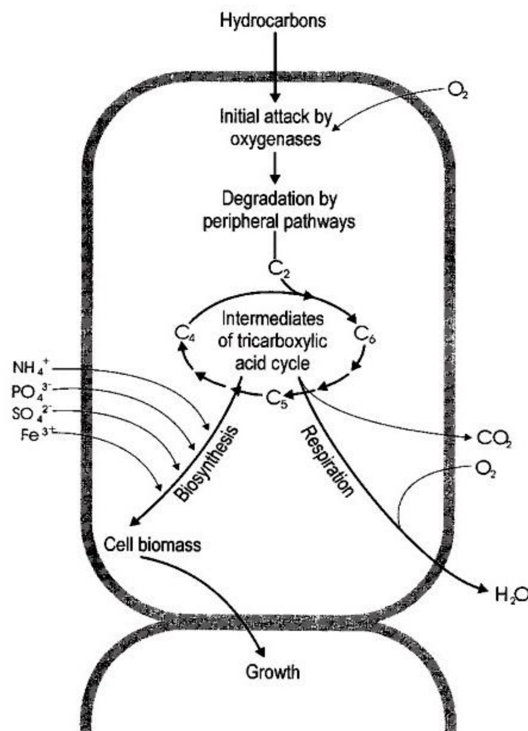


Figure 1.3: Main pathway for degradation of kerosene (Fritsche & Hofrichter, 2008)

1.4 Biosurfactants

Microorganisms produce surface acting substances called biosurfactants in order to facilitate the diffusion of these substrates into their cells through emulsification (Hommel, 1990; Neu, 1996; Desai & Banat, 1997; Bredholt *et al.*, 1998). These biosurfactants increase the bioavailability of the insoluble hydrocarbons, allowing them to be easily broken down and utilized by the enzyme system (Banat *et al*., 2000; Hommel, 1990). Bioremediation can be accomplished through addition of extracted biosurfactants alone (Zhou & Zhu, 2007).

Microbial surfactants can be peptides, fatty acids, phospholipids, glycolipids, antibiotics or lipopeptides, of which glycolipids are the most common. Table 1.4 lists the prominent microorganisms responsible for producing different types of glycolipid biosurfactants (Reis *et al*., 2013).

Table 1.4: Classification of glycolipid biosurfactants and the responsible microorganisms producing them (Reis *et al.*, 2013)

Glycolipid biosurfactant	**Microorganism**
Rhamnolipids	*P. aeruginosa, P. putida, P. chlororaphis, Bacillus subtilis* and *Renibacterium salmoninarum*
Sophorolipids	*Candida bombicola, C. apicola, Torulopsis*
Trehalose lipids	*Rhodococcus* sp., *Corynebacteria, Mycobacteria, Nocardia* and *Brevibacteria*
Mannosylerythritol lipids	*Candida antarctica*

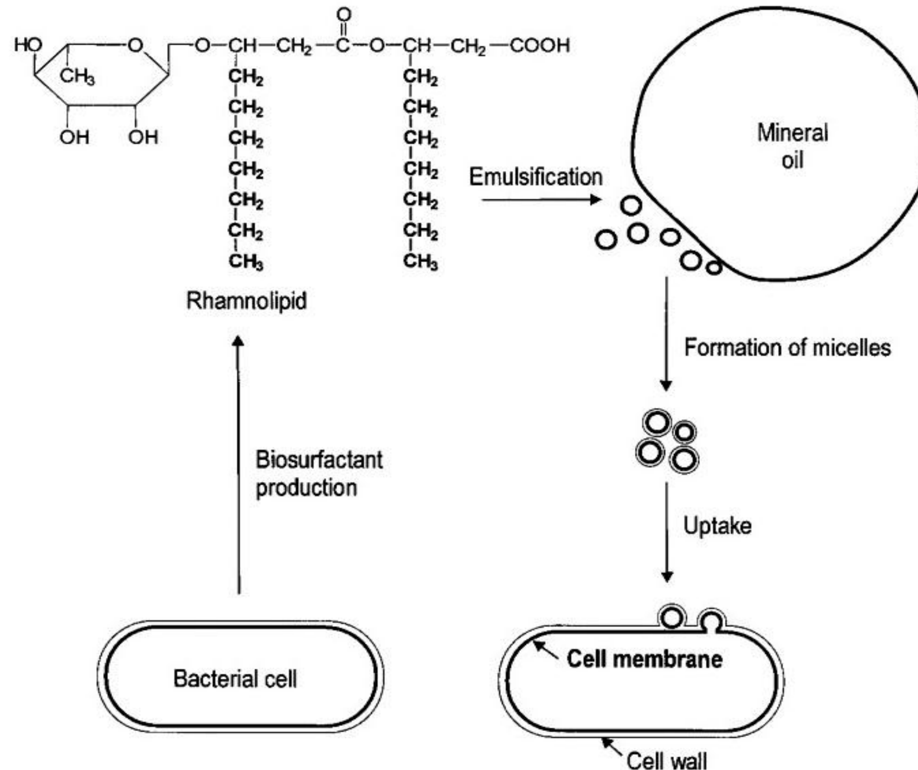


Figure 1.4: Bioavailability of oil through production of rhamnolipid (Reis *et al.*, 2013)

1.5 Aliphatic and aromatic degradation

Kerosene consists of mixture of aliphatic and aromatic hydrocarbons. Both need to be degraded, but they undergo slightly different pathway.

1.5.1 Utilization of aliphatic hydrocarbons

Most short chain aliphatics (<C9) are toxic to microorganisms, however does not pose a threat to environment because of their high volatilization and evaporation from contaminated site. Long chain n-alkanes (C10-C24) usually make up most proportions of fuels and are rapidly degraded by monoterminal or diterminal oxidation process (Figure 1.5.1) (Fritsche & Hofrichter, 2008). Cytochrome P450 is an oxidase enzyme that has an enormous role in the various biodegradation of oil, fuel and chlorinated hydrocarbons (Beilen *et al*., 2007). These enzymes attack the terminal methyl group and convert the alkanes into alcohol. Further oxidization of the alcohol results in the formation of first aldehyde, and then to fatty acids. Fatty acids are later broken down by β-oxidation to

acetyl-CoA (n-alkane with even carbon number) or propionyl-CoA (n-alkane with uneven carbon number) (Fritsche & Hofrichter, 2008).

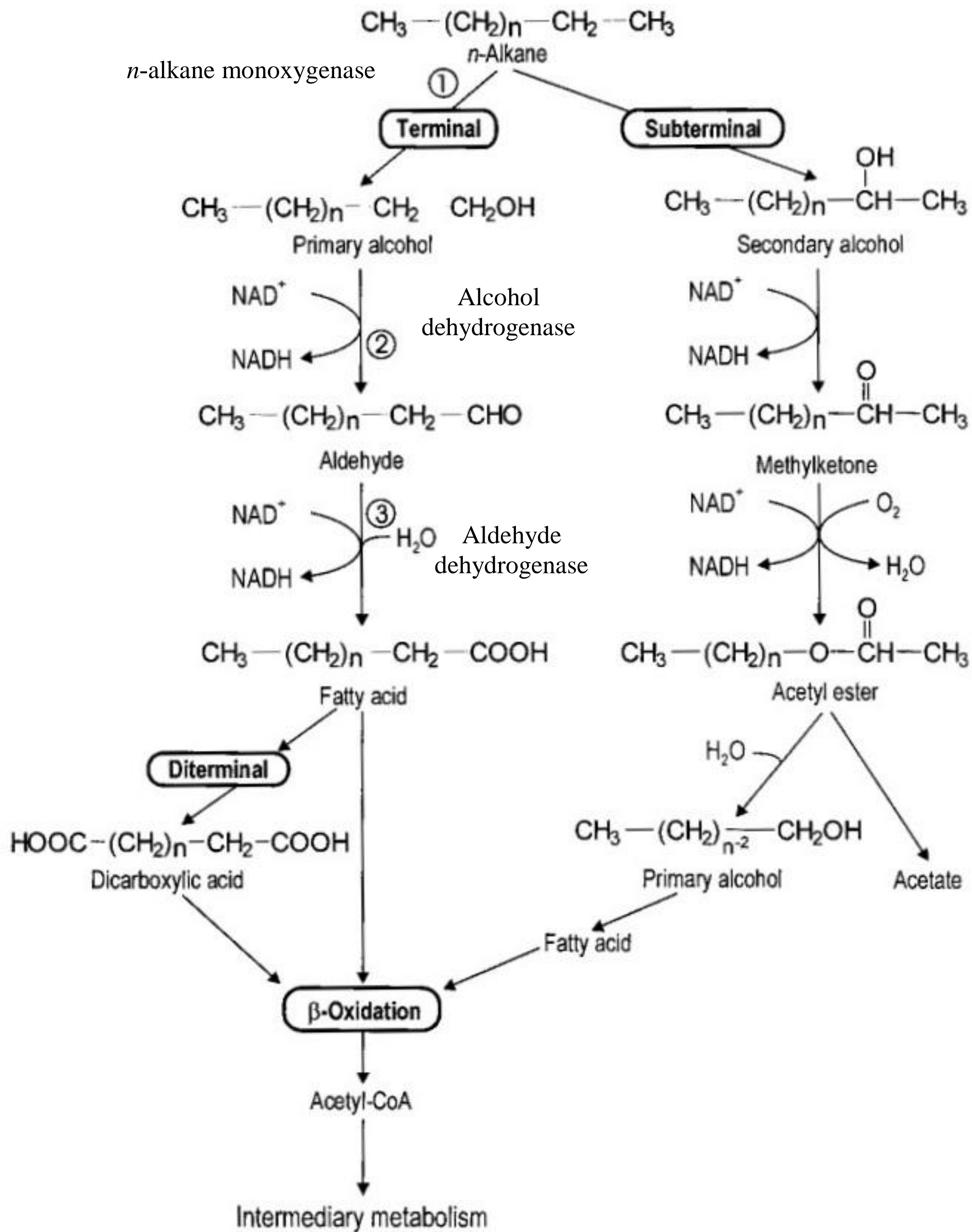


Figure 1.5.1: Degradation of alkanes (Fritsche & Hofrichter, 2008)

1.5.2 Utilization of aromatic hydrocarbons

Degradation of aromatic hydrocarbons occurs less readily than aliphatics. Kerosene and many other fuels have some quantities of BTEX (benzene, toluene, ethylbenzene, and xylene) (Dunlap & Beckmann, 1988). These xenobiotics are converted into intermediates catechol and protocatechuate by several microorganisms. It has been found that most mineralization capability of microbes, mainly *Pseudomonas*, is plasmid borne. TOL plasmid has been found to be related to degradation of toluene, xylene and ethyltoluene (Fritsche & Hofrichter, 2008).

1.6 Abiotic conditions affecting biodegradation

Petroleum mineralizing bacteria and other microbes require environmental conditions with adequate levels of oxygen, source of nutrients, ambient temperature and particular pH (Stegmann *et al.,* 1991). The abiotic conditions are briefly described below:

1.6.1 Oxygen

The very first step of hydrocarbon degradation involves oxidation process by oxygenases. Thus, aerobic conditions are extremely necessary. Rate of oxygen consumption by microbes, type of soil and presence of substrates that might exhaust oxygen determine oxygen levels within soils (Bossert & Bartha, 1984). Indigenous bacteria, deep in the Gulf of Mexico, that were feeding on the oil stopped only after 5 months from the time of disaster due to depletion in the oxygen level (McKay, 2012). However, several studies have confirmed small amount of degradation at anaerobic condition (Bailey *et al.*, 1974; Jamison *et al.*, 1975). Grbic-Galic & Vogel (1987) have reported anaerobic degradation of BTEX, 1, 3- dimethylbenzene, acenaphthene, and naphthalene.

1.6.2 Temperature

Temperature has an effect on biodegradation of fuels as it has an influence on physical and chemical composition of the hydrocarbon, rate of microbial metabolism and on composition of microbial flora (Atlas, 1981). At low temperature, biodegradation is reduced by the increase in viscosity of oil, the decrease in their volatilization and the

increase in solubility in water (Atlas & Bartha, 1972). Reduction of enzymatic activity at low temperature also reduces microbial degradation of hydrocarbons (Atlas & Bartha, 1972; Gibbs, 1975). The optimum temperature usually lies in the range of 30°C to 40°C. Higher temperature increases membrane toxicity for most bacteria, except thermophilic alkane-utilizing bacteria (Bossert & Bartha, 1984).

1.6.3 Nutrients

High nitrogen or phosphorus reduces microbial growth. In soil, however, nitrogen and phosphorus concentrations are low, and thus require the addition of NPK fertilizers to facilitate the degradation of gasoline or crude oil (Dibble & Bartha, 1979; Jamison *et al*., 1975; Jobson *et al.*, 1974). Agarry *et al.* (2010) have reported the benefit of degradation was higher when poultry (73% degradation) or piggery manure (63% degradation) was added instead of NPK fertilizers (39% degradation).

1.6.4 pH

pH of soils can vary considerably from 2.5 (mine soils) to 11.0 (alkaline deserts) (Bossert & Bartha, 1984). Most bacteria survive at near neutral pH and thus soils with extremes of pH are considered to have lower petroleum biodegradation rate. It had been reported that rate of degradation almost doubles when pH of soil is raised from 4.5 to 7.4. However, when pH was farther increased to 8.5, reduced degradation was observed (Verstraete *et al*., 1976).

1.7 Bioremediation

Physical or chemical removal (Figure 1.7) of petroleum spillage can be expensive and less effective. Currently, the chemical removal of oil in marine environment involves the addition of dispersants. It has been estimated that dispersant-oil mixture can be more toxic to deep sea animals than oil alone (Gulf Oil Spill, 2010.). Biological removal or bioremediation is the only cost-effective and eco-friendly method for complete removal of contaminants (Das *et al.*, 2010). Once an isolate have been identified to possess the capability of degradation, it can be further enhanced through gene manipulation to speed up the process. If an organism is already showing optimum mineralization capability, it

can be directly utilized in-situ or ex-situ bioremediation. Removal of petroleum contamination is mainly accomplished in-situ, in order to avoid further delays (Evans & Furlong, 2003).

In-situ bioremediation: In-situ bioremediation can be accomplished through biostimulation or bioaugmentation. Biostimulation involves the supplementation of nutrients to speed up the rate of biodegradation of indigenous polluting degrading bacteria in soil or water. This can be achieved through the addition of water soluble inorganic nutrients or fertilizers (Nikolopoulou & Kalogerakis, 2010). Bioaugmentation involves the addition of oil degrading inoculums to soil or water. Agarry *et al.* (2011) had shown that the rate of degradation of kerosene through the combination of biostimulation and bioaugmentation are far greater (rate constant, k = 0.0597) than when the techniques are individually applied. Currently, bioremediation for oil spill products are manufactured by several companies, most of which are available in liquid or powder form (Table 1.7).

Ex-situ bioremediation: Several ex-situ treatment systems are also being studied upon. Benyahia (2005) had demonstrated the benefits of a laboratory biopile system to which contaminated soils were added along with minerals and bacterial strain for bioaugmentation. Much work is needed in this sector before a fully functional biomechanism can be utilized in practice.

Figure 1.7: Current chemical removal of oil pollutants include the spraying of carcinogenic dispersants (Montara Well Head Platform, 2009)

Table 1.7: List of in-situ bioremediation products that are currently available in market (Bioremediation and Oil Spill Products, 2014; Soil and Water Remediation, 2014; Soil Remediation Products, 2014; A Leader in Bioremediation & Spill Response for Oil, 2014; Oil Spill Cleanup using Bioremediation, 2014).

Manufacturers	Products	Description
BioWorld	BioWorld Hydrocarbon Microbes	Bioaugmentation for crude oil, gasoline, diesel, MTBE and other petrochemicals.
	BioWorld Bioremediation Enhancer	Biostimulation liquid formulation for soil and water petrochemical wastes
EnviroLogic	Remediact	For hydrocarbon wastes in soil
	Liquid Remediact	For hydrocarbon wastes in deep water and soil
	HC-100	Bioremediation of soil and ground water hydrocarbon
NASA	PRP powder; Bio-Boom; Bio-Sok; WellBoom; Oil Busters	Nanotechnology that biostimulates through microencapsulation of nutrients for diesel degrading bacteria in water and soil
Select Synthetics	Wonder Microbes	Composed of 20 microbes with the ability to degrade kerosene, diesel, lubricating oil, gasoline and light petroleum
Soil Bioremediation Inc (Altogen Labs)	Remediation Bio Reagent	Bioaugmentation for oil, benzene, dichloroethylene, petroleum, toluene, pesticides in soils

1.8 Objectives

On 9th December 2014, Bangladesh had met its worst oil spill disaster due to the collision between oil tanker and cargo vessel at the Shela River in Sundarbans. About 350,000 liters of furnace-oil spilled out, endangering the lives of fishes, rare dolphins and vegetations. The disaster had threatened the entire food cycle (Bangladesh oil spill 'threatens rare dolphins', 2014; Disaster in the Sundarbans, 2014). Such tragedies make it indispensible to focus on research in this sector to protect our environment.

Biodegradation of petroleum, particularly kerosene, has not been studied in detail in Bangladesh. Most oil spills, including that of Sundarbans, are not mitigated in time or even mishandled to create further damage due to lack of research. Being an environmentally friendly method, bioremediation needs to be explored in detail. It is of primary importance to identify oil degrading microbes and to find out optimum conditions for degradation in order to utilize the potential bacterial strain for bioremediation. The objectives of this study are as follows:

- To isolate and identify potential microbial isolates capable of degrading kerosene from a range of kerosene utilizers from local soil.
- To determine optimum concentration of kerosene for growth of the potential microbe
- To determine the optimum pH for degradation of kerosene by a potential isolate
- To determine the optimum nitrogen concentration for kerosene degradation by that potential isolate.

Chapter 2: Materials and Methods

Chapter 2: Materials and methods

2.1 Place of research

The study was performed at Microbiology and Biotechnology Research Laboratory under Department of Mathematics and Natural Sciences (BRAC University).

2.2 Soil sample collection

Soil samples were aseptically collected at about 5 cm depth (top soil without contamination from surface) from local fuel contaminated areas and petrol pump station sites. The soils were stored in sterile plastic bags at 4°C for later use (Islam *et al.*, 2013).

Table 2.2: List of soil samples that were collected for this study

Soil sample	Location
A	Agricultural site, Barisal
B	Petrol pump station, Bogura
C	Construction locomotive storage site, Moghbazar, Dhaka
D	Petrol pump station, Barisal

2.3 Kerosene

Kerosene, collected from local petrol pump station was filter sterilized and stored in autoclaved test tubes and sealed with parafilm. For sterility confirmation, 100 µL of the fuel was spread on Nutrient agar (NA) plates and incubated for 48 hours at 37ºC before the start of every experiment. Lack of growth on plates ensured the oil to be uncontaminated.

2.4 Isolation of bacteria from soil samples

One gram from each of the soil samples were individually suspended and serially diluted in 9 mL saline solutions. 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on nutrient agar plates through spread plate technique and incubated for 24 hours at 37°C to obtain soil isolates. The different bacterial colonies based on their morphology were selected in a way to ensure no two colonies displayed the same characteristics. These were four-way streaked on Nutrient agar plates to obtain single colonies of pure isolates. The plates were sealed with parafilm, refrigerated at 4°C and were frequently subcultured.

Table 2.4: Eight isolates based on their different morphology were selected and used throughout the study

Soil samples	Number of isolates	Designation of isolates
A	2	A1, A2
B	2	B1, B2
C	2	C1, C2
D	2	D1, D2

2.5 Evaluating degradation properties of different bacterial strains

2.5.1 Preparation of MS broth (culture media)

Mineral salts media was prepared by first dissolving $(NH_4)_2SO_4$ (1.0 g/L), $CaCl_2$ (0.02 g/L), $MgSO_4.7H_2O$ (0.2g/L), K_2HPO_4 (1.0g/L) and KH_2PO_4 (1.0 g/L) in distilled water. $FeCl_3$ (0.05 g/L) was dissolved separately in distilled water and added to the media. The final volume and pH of the MS broth was adjusted to 7.0 with K_2HPO_4.The mixture was then heat stirred in hotplate and autoclaved (Bushnell & Haas, 1940).

Magnesium sulfate, calcium chloride and ferric chloride provided the required trace elements for bacterial growth. Ammonium sulfate provided nitrogen source, while the potassium phosphates acted as buffering agents. Thus the final media (slightly amber color) contained all important nutrients and buffers except any carbon source.

2.5.2 Culturing kerosene tolerant bacteria

The eight single isolates based on their morphological characteristic were suspended into individual 3 mL saline solutions. The solutions were vortexed and 1 mL inoculum was transferred into the prepared 50 mL MS broth in 100 mL Erlenmeyer flask. 1% kerosene (v/v) was transferred into the flask as the sole carbon source. The flasks were sealed with aluminium foil and parafilm to prevent fungal contamination and kerosene evaporation, and were incubated in shaker at 120 rpm, 35°C for 7 days period.

2.5.3 Evaluation of growth

In order to prevent growth of original inoculum, MS agar plates were used instead of Nutrient agar plates (Bushnell & Haas, 1960). 100 µL of the culture was plated into MS agar plate by spread plate technique. The MS agar media had the following composition: NH_4SO_4 (1.0 g/L), KH_2PO_4 (1.0 g/L), K_2HPO_4 (1.0 g/L), $FeCl_3$ (0.05 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $CaCl_2$ (0.02 g/L) and 10 g/L agar-agar with pH adjusted to 7. Kerosene (50 µL) was next spread once moisture had evaporated from the first spread. The plates were incubated at 35ºC in an upright position to prevent dripping of the oil. After 5 days incubation, colonies were counted and CFU/mL calculated.

2.6 Incorporating variations in growth conditions

2.6.1 Tolerance of different concentrations of kerosene

The experiment was repeated by culturing C1 isolate in eight 50 mL MS media (pH 7.0). Several different concentrations of kerosene were added into the culture media: 0% (control), 0.5%, 1%, 2%, 3%, 4%, 5% and 6%. The culture flasks with varying concentrations were incubated in the shaker at 120 rpm (35ºC) for a period of 7 days.

2.6.2 Effect of pH on degradation of kerosene

C1 isolate was cultured in separate 50 mL MS media whose pH were adjusted to pH 2, pH 4, pH 6, pH 7, pH 8 and pH 10 with K_2HPO_4. The flasks were incubated in shaker at 37°C for 7 days and then plated in mineral salts agar plates. The plates were incubated for 5 days at 35°C to count colonies.

2.6.3 Effect of varying concentration of nitrogen source

The C1 isolate was cultured in six 50 mL MS media in Erlenmeyer flasks with the MS media having different concentrations of ammonium sulfate: 0.25 g/L, 0.5 g/L, 0.75 g/L, 1.0 g/L, 1.25 g/L and 1.5 g/L. After 7 days of incubation at 35°C, 120 rpm, the cultures were plated in mineral salts agar plates and CFU/mL measured once colonies appeared.

2.7 Identification of bacterial isolates

Bacterial isolates used in this study were identified based on:

1. Morphological characteristics of colony
2. Staining and microscopic visualization
3. Biochemical tests

Gram staining, spore staining and biochemical tests were performed on the bacterial isolates according to Microbiology Laboratory Manual (Cappuccino & Sherman, 2005). Presumptive identification was performed through using ABIS Online software.

2.7.1 Staining

1) Gram staining

Procedure: A small amount of a single bacterial colony was transferred onto saline drop over a slide and heat fixed. The slide was next flooded with crystal violet and left to stand for 30 seconds. After a 10 seconds wash with tap water, mordent iodine was used to flood the slide for 30 seconds. The slide, rinsed with tap water, was flooded with 95% acetone as a decolorizing agent. After immediate removal of the acetone with tap water, the slide was counterstained with safranin for 30 seconds and rinsed again. The dried slide was viewed under microscope.

Inference: Gram positive cells would appear violet while Gram negative cells would appear red.

2) Spore staining

Procedure: With the aid of a sterile loop, smears were prepared on a slide and heat fixed. The slide was flooded with malachite green and mounted on top of a beaker containing boiling water for steaming. Malachite green was reapplied every time it started to dry out. After 5 minutes, the slide was rinsed with tap water and counterstain safranin was added for 45 seconds. Once rinsed and dried, the slide was observed under microscope.

Inference: Endospores would appear green while vegetative cells would appear red.

3) Acid fast staining (Ziehl-Neelsen staining)

Procedure: Gram positive rod shaped bacteria that were spore negative were used to prepare smears on slide and heat fixed. The slide was mounted on top of a beaker producing steam, and the smear was flooded with carbol fuchsin stain. After 5 minutes, the stain was rinsed with tap water and acid alcohol. Once the stain turned from deep red to light pink, methylene blue was used to flood the smear for 20 seconds. The counterstain was rinsed with tap water, and the slide was air dried. The slide was visualized under microscope.

Inference: Acid fast bacteria would stain red while non-acid fast bacteria would stained blue. Weakly acid fast bacteria stain mostly blue, with the presence of some carbol fuchsin stained cells due to the presence of mycolic acids in their cell walls.

2.7.2 Motility test

Procedure: Motility of isolates was determined through picking up bacterial colony with sterile needle and stabbing soft agar (MIU agar base) in test tubes. After overnight incubation at 37°C, diffusion of cloudiness was observed.

Inference: Motile bacteria would migrate readily through the soft media, away from the line of stab, and create cloudiness upon incubation. Non motile bacteria would grow, but only along the line of stab inoculation.

2.7.3 Enzyme tests

1) Indole utilization test

Procedure: Indole test was performed to identify isolates capable of degrading tryptophan and produce indole. Bacteria to be tested were inoculated from fresh plates in individual tubes with peptone water containing tryptophan. After overnight incubation at 37ºC, a few drops of Kovac's reagent were added.

Inference: A positive test would produce a red layer on top of the agar, whereas the presence of original yellow or brown layer would confer a negative test result.

2) Urease test

Procedure: Urease test was performed to identify bacteria that are capable of hydrolyzing urea. The test was accomplished by inoculating well-isolated bacterial colony into urea base containing urea and phenol red. Change in color of the agar, incubated at 37°C, was observed after 24 hours and every day for a period of 6 days (for slow hydrolyser).

Inference: Urease positive bacteria would produce ammonia which would turn phenol red pink. The culture medium would remain yellow for bacteria unable to hydrolyze urea.

3) Citrate utilization test

Procedure: Bacterial colonies from fresh agar plate were streaked on the slope of Simmons' citrate agar (Oxoid ltd, England) and incubated at 37ºC for 24 hours.

Inference: Bacteria with the ability to utilize citrate would turn the agar blue through creating alkaline condition. Bacteria unable to utilize citrate would show no change in the agar.

4) Oxidase test

Procedure: Oxidase test identifies bacteria that are able to produce cytochrome c oxidases. A small piece of filter paper was soaked with a few drops of freshly prepared Kovac's reagent (tetra-methyl-p-phenylenediaminedihydrochloride). With the aid of a

toothpick, a single bacterial colony was transferred on to the soaked paper. Change in color of the treated paper was observed within 5-10 seconds.

Inference: Oxidase positive bacteria would turn the soaked paper dark purple by oxidizing Kovac's reagent. Oxidase negative bacteria would display no change.

5) Catalase test

Procedure: This test was used for the detection of the enzyme catalase present in bacterial isolates. 1 drop hydrogen peroxide (3%) was placed on a slide with the aid of a dropper. A small amount of bacterial colony was transferred with a sterile loop onto the applied hydrogen peroxide (H_2O_2).

Inference: Catalase positive bacteria breakdown H_2O_2 into water and oxygen and would give off effervescence of bubbles. Catalase negative bacteria would not produce bubbles.

6) Starch hydrolysis test

Procedure: This test was performed to determine the presence of alpha-amylase activity in bacterial isolates. Bacterial isolate was streaked back and forth across starch agar plate. After overnight incubation at 37°C, the plate was flooded with iodine reagent with the aid of a dropper.

Inference: Iodine reagent changes the color of starch to blue-brown. If alpha-amylase was produced by the bacteria during incubation period, it would digest the starch around its growth, creating halos in the middle of the plate. This would suggest a starch hydrolysis positive result. Absence of clear halo would determine a negative test result and non-starch utilizing bacteria.

7) Nitrate reduction test

Procedure: Single colony of bacteria was inoculated into nitrate broth containing Durham tube, with the aid of sterile loop. Following overnight incubation at 37°C, 5 drops of sulfalinic acid and 5 drops of alpha-naphthylamine reagents were added.

Inference: On addition of the reagents, a bright pink or red color would appear for the strains having the ability to reduce nitrate to nitrite. Bubble formation in Durham tubes

would confer the farther reduction of nitrite to nitrogen gas. A negative test result would give no change when reagents are added. Farther addition of a pinch of zinc to the negative test tubes would provide a pink color.

8) Hemolysis

Procedure: Bacterial isolates from fresh plates were streaked on blood agar plates and incubated overnight at 37°C.

Inference: Clear zone around bacterial growth would indicate complete hemolysis (β hemolysis) by the bacterial isolate. Partial hemolysis (α hemolysis) would provide a greenish color on the agar plate. Bacteria unable to produce hemolysins would simply grow with no clearing or color change (γ hemolysis).

2.7.4 Fermentation tests

1) Carbohydrate fermentation test

Procedure: Single bacterial colony was aseptically suspended into individual glucose, sucrose and lactose broth containing phenol red. The tubes had inverted Durham tubes for observation of gas formation. The tubes were incubated for 48 hours at 37°C for slow fermenters.

Inference: The ability of bacteria to ferment a particular sugar would turn the broth yellow in color through acid formation. Slow fermenters would change it to a yellowish orange color, while non-fermenters would let the broth retain its original red color.

2) Methyl red (MR) test

Procedure: Methyl red test was performed to determine the ability of isolate to carry out mixed acids fermentation when supplied glucose. Bacterial colonies from fresh plates were inoculated into individual potassium phosphate broth (MR-VP broth) containing peptone, dextrose and potassium phosphate. These were incubated overnight at 37ºC. A few drops of methyl red reagent were added to test the pH of the broth.

Inference: If the organism produced sufficient acid to overcome the phosphate buffer, red color would be produced on the addition of MR reagent to indicate a positive test. Prevalence of original yellow color would indicate a negative test.

3) Voges-Proskauer (VP) Test

Procedure: This test was carried out to identify bacteria capable of fermenting glucose via butanediol pathway. Bacteria to be tested were inoculated into MR-VP broth and incubated overnight at 37ºC. Few drops of Barritt's reagent A was added to the broth and slightly shaken to disperse the cloudiness. An equal amount of Barritt's reagent B was next added and the tubes allowed standing for 15 min.

Inference: A positive test result would be indicated by appearance of pinkish-red color, whereas a negative test would show no change in color.

4) Arabinose test

Procedure: Bacterial colony was inoculated in L-arabinose broth with sterile loop. After overnight incubation at 37°C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment arabinose would change the red broth into yellow in color. Non arabinose fermenter would display no change.

5) Mannitol fermentation test

Procedure: Bacteria from fresh NA plates were streaked on mannitol salt broth containing phenol red indicator. The plates were incubated overnight at 37°C.

Inference: Bacteria with the ability to ferment mannitol would turn the solution yellow on growth. Non fermenters would create an alkaline condition and turn the broth bright pink.

2.7.5 Selective and differential media tests

1) MacConkey agar plate

Procedure: To avoid false positives and false negatives during Gram staining, all bacterial isolates were streaked on MacConkey agar plates. The plates were incubated for 24 hours at 37°C.

Inference: Growth observed on plate reconfirmed any isolate to be Gram negative. Lack of growth conferred Gram positive bacteria.

2) Salt tolerant test

Procedure: With the aid of sterile inoculating loop, single bacterial colony from fresh plate was transferred to 6.5% NaCl broth in a test tube. The tube was loosely capped to maintain aerobic condition, and incubated at 37°C. Observation was made at 24 hours and every day for a period of 3 days if no change occurred.

Inference: High salt tolerant bacteria would turn the broth cloudy after incubation, giving a positive result. Bacteria would be unable to grow and make the broth turbid for negative test result.

2.8 Preservation of bacteria

3 mL T_1N_1 agar was inoculated through stabbing each bacterium from nutrient agar plate. The vial was incubated for 5 hours to allow the bacteria to acquire log phase. 200 μL of sterile glycerol was next added and the vial sealed with parafilm and stored at room temperature.

Chapter 3: Results

Chapter 3: Results

In this study, eight isolates from four soil samples (Figure 3) were individually cultured for 7 days in mineral salts broth containing kerosene as the sole carbon source. The ability of each isolates to degrade and thus grow in the presence of kerosene was determined through plating them in mineral salts agar and counting colonies. Higher colony counts of any isolate suggested rapid degradation; lower counts suggested slow degradation; whereas absence of growth determined the inability of the isolate to utilize kerosene. Along with comparisons among different isolates, the best kerosene degrading bacteria was also tested for its usage of optimum concentration of kerosene and its requirement for optimum pH and nitrogen concentrations.

Figure 3: Four soil samples were used to isolate kerosene degrading bacteria

3.1 Kerosene degrading ability of different isolates

Eight isolates (designation A1, A2, B1, B2, C1, C2, D1 and D2) that were isolated from four different soil samples were cultured separately in 50 mL mineral salts media (pH 7.0) for 7 days at 35 ºC and 120 rpm. These isolates were later identified as *Nocardia* sp., *Corynebacterium* sp., *Bacillus* sp., *Pseudomonas* sp. and *Arthrobacter* sp. One milliliter of the 7 day culture was serially diluted and plated on mineral salts agar. Plates with viable counts were used to calculate CFU/mL.

Table 3.1: Colony counts of eight bacterial isolates on mineral salts agar after its culture in mineral salts broth containing 1% kerosene concentration (v/v)

ID	Bacteria	CFU of different dilutions						CFU/ml	Log CFU /mL
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
A1	***Nocardia* sp.**	0	0	0	0	0	0	0	0
A2	***Corynebacterium* sp.**	0	0	0	0	0	0	0	0
B1	***Arthrobacter* sp.**	33	0	0	0	0	0	3,300	3.52
B2	***Bacillus* sp.**	TNTC	TNTC	476	50	3	0	5×10^6	6.70
C1	***Pseudomonas* sp.**	TNTC	TNTC	TNTC	TNTC	240	18	2.4×10^8	8.38
C2	***Bacillus* sp.**	TNTC	TNTC	110	5	0	0	1.1×10^6	6.04
D1	***Arthrobacter* sp.**	320	35	2	0	0	0	3.5×10^4	4.54
D2	***Arthrobacter* sp.**	327	38	3	0	0	0	3.8×10^4	4.58

*TNTC = too numerous to count

When the eight isolates were cultured in mineral salts broth for 7 days and subsequently plated on mineral salts agar, *Pseudomonas* sp. (C1) showed highest growth of 8.38 $\log_{10}$ CFU/mL, followed by *Bacillus* sp. of growth 6.70 $\log_{10}$ CFU/mL (B1) and 6.04 $\log_{10}$ CFU/mL (C2). *Nocardia* sp. (A1) and *Corynebacterium* sp. (A2) did not show any growth.

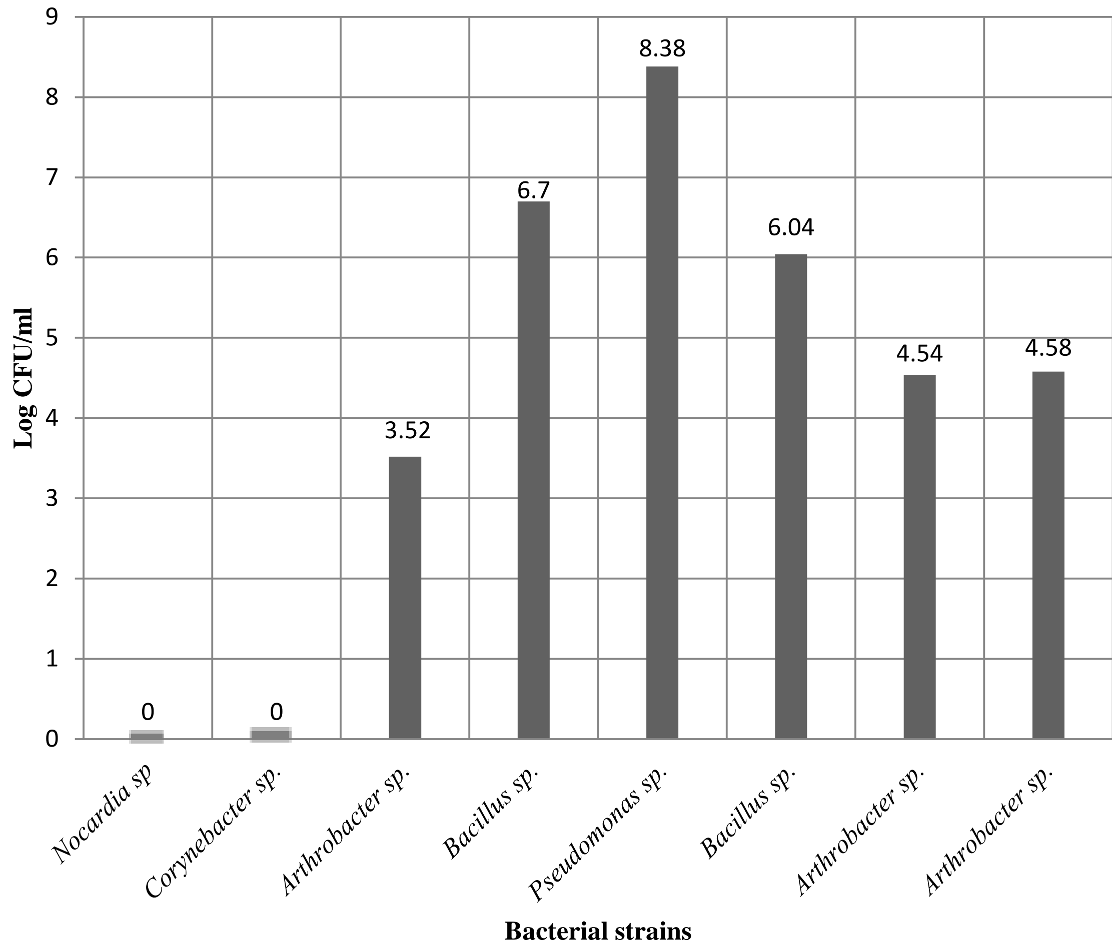


Figure 3.1: Growth of eight bacterial strains through utilization of 1% kerosene (v/v) for 7 days incubation at 35 ºC and 120 rpm

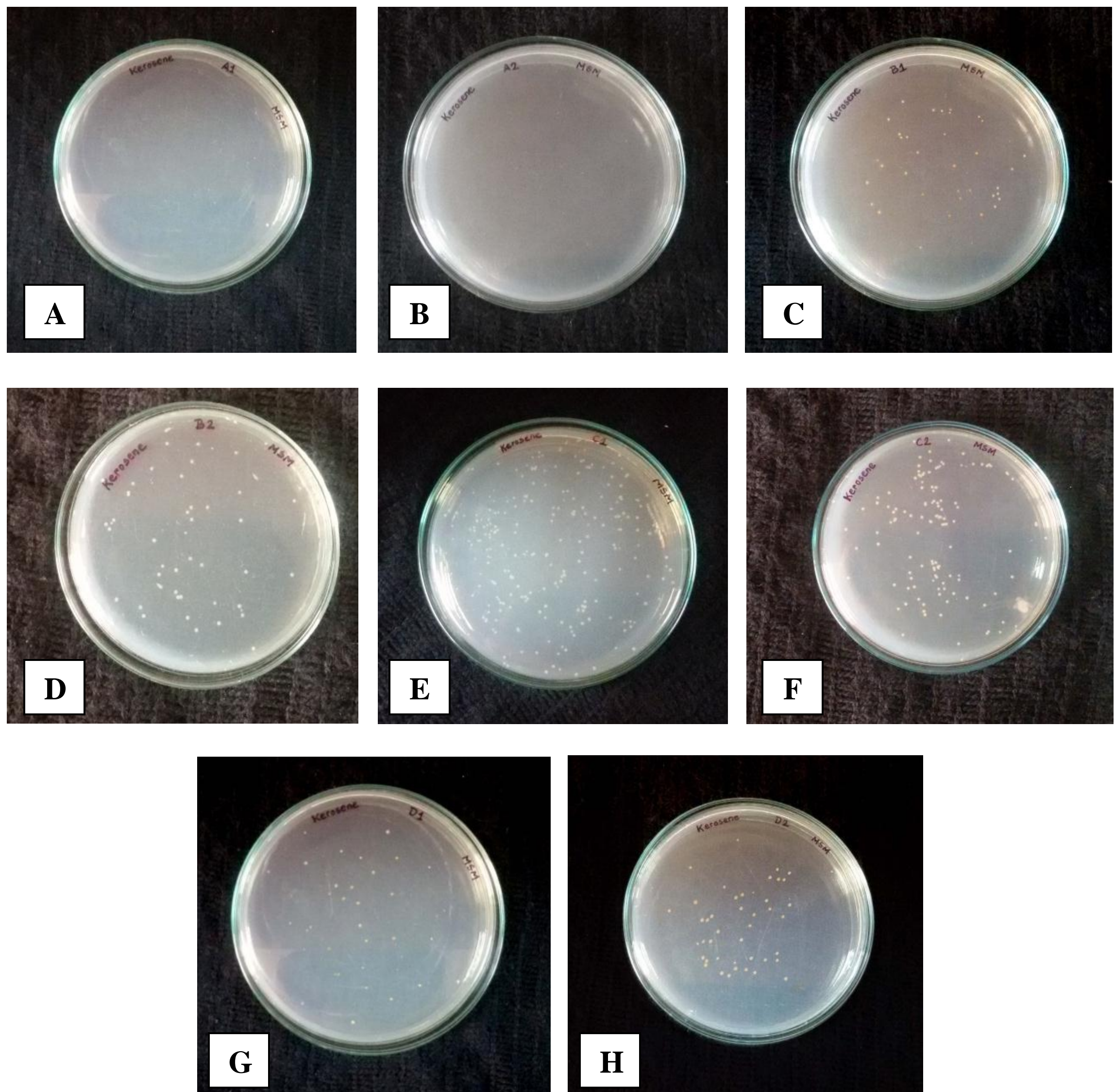


Figure 3.1.1: Mineral salts agar plates showing viable counts of eight isolates. **A-H.** Bacterial cultures of the eight isolates were spread on plates after their 7 days incubation in mineral salts broth containing 1% kerosene (v/v) as the sole carbon source. **E.** C1 isolate showed highest CFU/ml of all the eight isolates. **A-B.** A1 and A2 isolates from agricultural soil samples were unable to grow in the presence of kerosene.

3.2 Effect of different concentration of kerosene on growth of selected isolate

Isolate C1 was cultured in seven 50 mL mineral salts broth (pH 7) containing varying kerosene concentrations. These were incubated for 7 days at 35 ºC and 120 rpm. One milliliter of the culture was serially diluted and plated on mineral salts agar. Plates with viable counts (30-300 colonies) were used to calculate CFU/ml.

Table 3.2: Colony counts of *Pseudomonas* sp. on mineral salts agar after its growth in mineral salts broth containing varying kerosene concentration

% Concentration of kerosene (v/v)	CFU at different dilutions						CFU/ml	Log CFU/ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
0% (control)	0	-	-	-	-	-	-	-
0.5%	TNTC	TNTC	312	35	4	0	3.5×10^{6}	6.54
1%	TNTC	TNTC	TNTC	TNTC	179	13	1.79×10^{8}	8.25
2%	TNTC	TNTC	TNTC	TNTC	TNTC	135	1.35×10^{9}	9.13
3%	TNTC	TNTC	TNTC	TNTC	TNTC	267	2.67×10^{9}	9.43
4%	TNTC	TNTC	TNTC	TNTC	TNTC	158	1.58×10^{9}	9.2
5%	TNTC	TNTC	TNTC	TNTC	106	16	1.06×10^{8}	8.03
6%	TNTC	355	34	2	0	0	3.4×10^{5}	5.53

*TNTC = too numerous to count

The isolate *Pseudomonas* sp. showed maximum growth at 3% kerosene (9.43 $\log_{10}$ CFU/mL) and was able to tolerate as high as 6% kerosene with growth of 5.53 $\log_{10}$ CFU/mL. Gradual decline in growth below 3% kerosene was also observed.

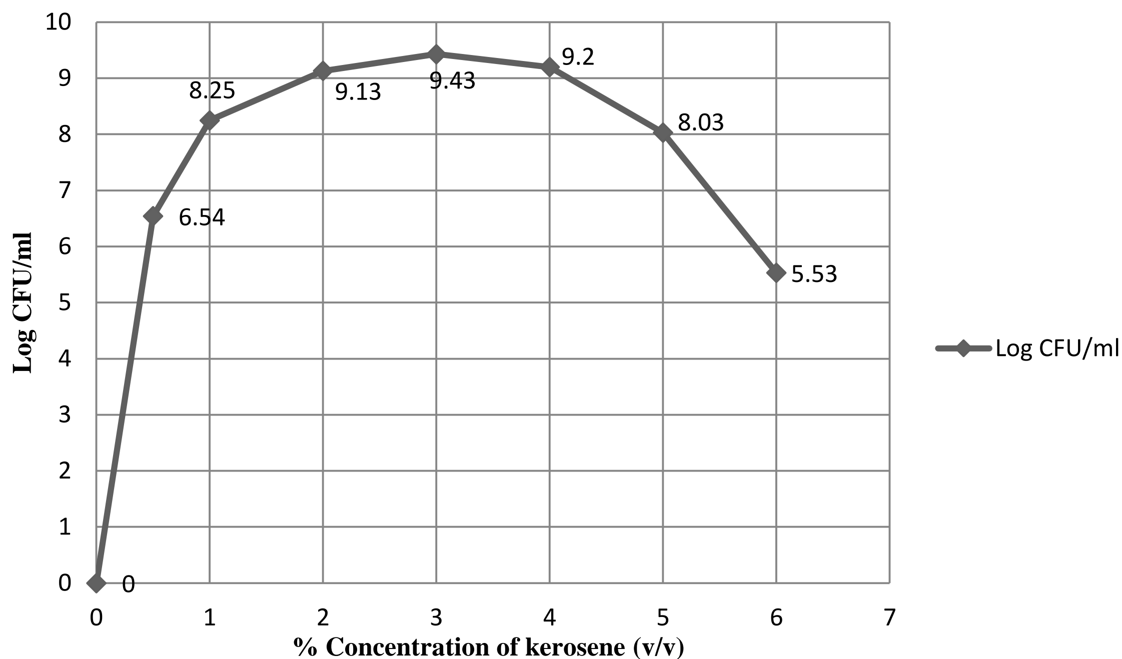


Figure 3.2: Effect of % concentration of kerosene (v/v) on *Pseudomonas* sp. after 7 days incubation at 35 ºC and 120 rpm

Figure 3.2.1: Growth of C1 isolate in mineral salts broth containing varying concentrations of kerosene. After 7 days incubation at 35°C and 120 rpm, the broth turned turbid due to growth of the microbe. Turbidity was observed to gradually increase, showing maximum turbidity at 3% kerosene, and again decline at concentrations higher than 3% kerosene.

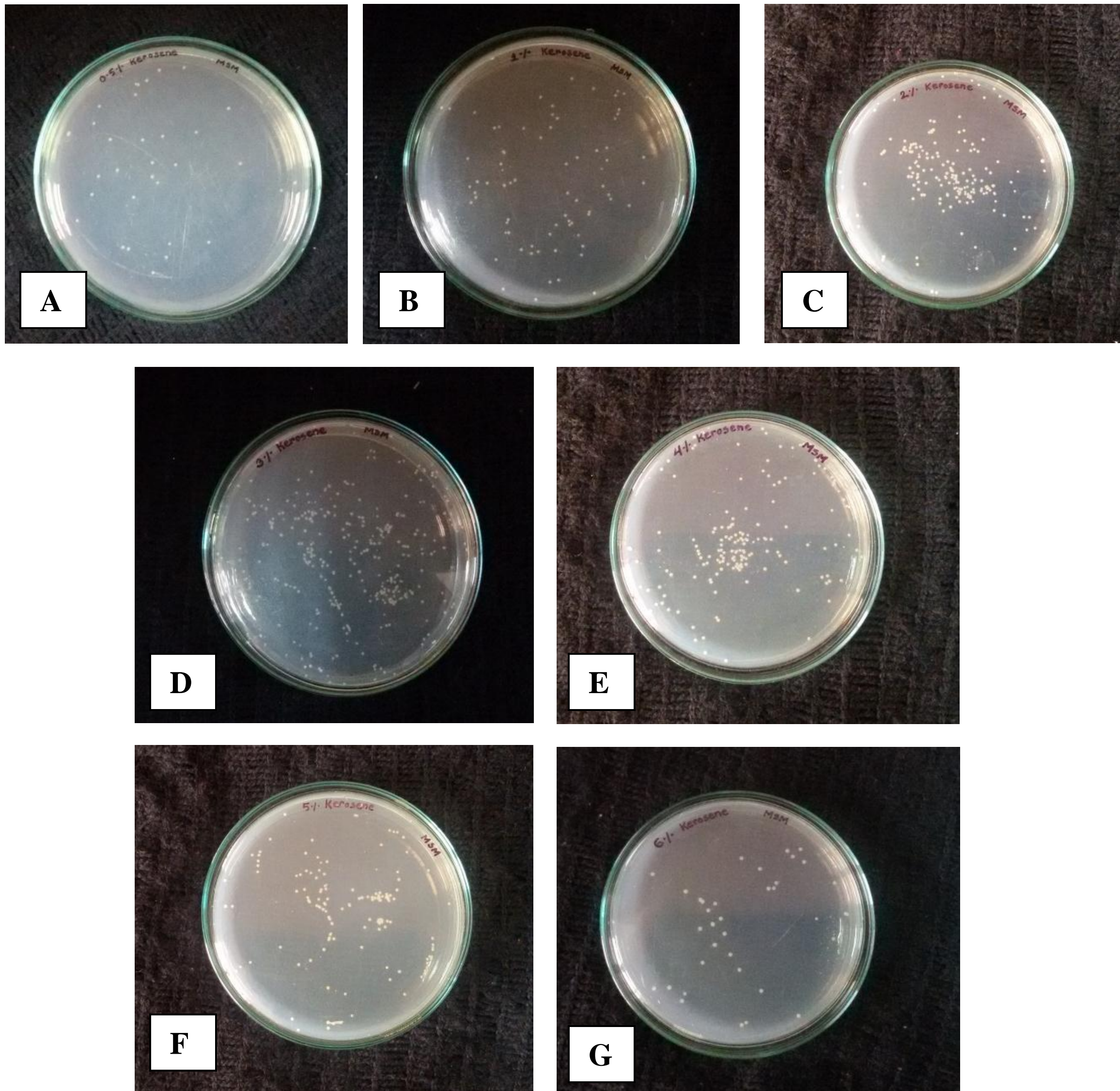


Figure 3.2.2: A-G. Mineral salts agar plates showing viable counts of *Pseudomonas* sp. after its growth in mineral salts broth containing varying kerosene concentration. **D.** Highest count was observed at 3% kerosene (v/v).

3.3 Effect of pH

The C1 isolate was cultured in six 50 mL mineral salts broth containing 1% kerosene (v/v) with pH being varied from pH 6 – pH 10. These were incubated for 7 days at 35 ºC and 120 rpm. One milliliter of each culture was serially diluted and plated on mineral salts agar. Plates with viable counts were used to calculate CFU/mL.

Table 3.3: Colony counts of *Pseudomonas* sp. on mineral salts agar after its growth in mineral salts broth of varying pH

pH	CFU at different dilutions						CFU/ml	Log CFU/ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
pH 2	46	4	0	0	0	0	4,600	3.66
pH 4	313	30	1	0	0	0	3×10^4	4.48
pH 6	TNTC	182	16	0	0	0	1.82×10^5	5.26
pH 7	TNTC	TNTC	TNTC	TNTC	239	23	2.39×10^8	8.38
pH 8	TNTC	TNTC	TNTC	199	17	0	1.99×10^7	7.30
pH 10	28	1	0	0	0	0	2,800	3.45

*TNTC = too numerous to count

The isolate *Pseudomonas* sp. was able to grow within the pH range of pH 2-10 by utilizing kerosene as the sole carbon source. The isolate showed optimum growth at pH 7 (8.38 log_{10} CFU/mL), and high growth at pH 8 (7.30 log_{10} CFU/mL). Further decrease or increases in pH from near neutral retarded its growth.

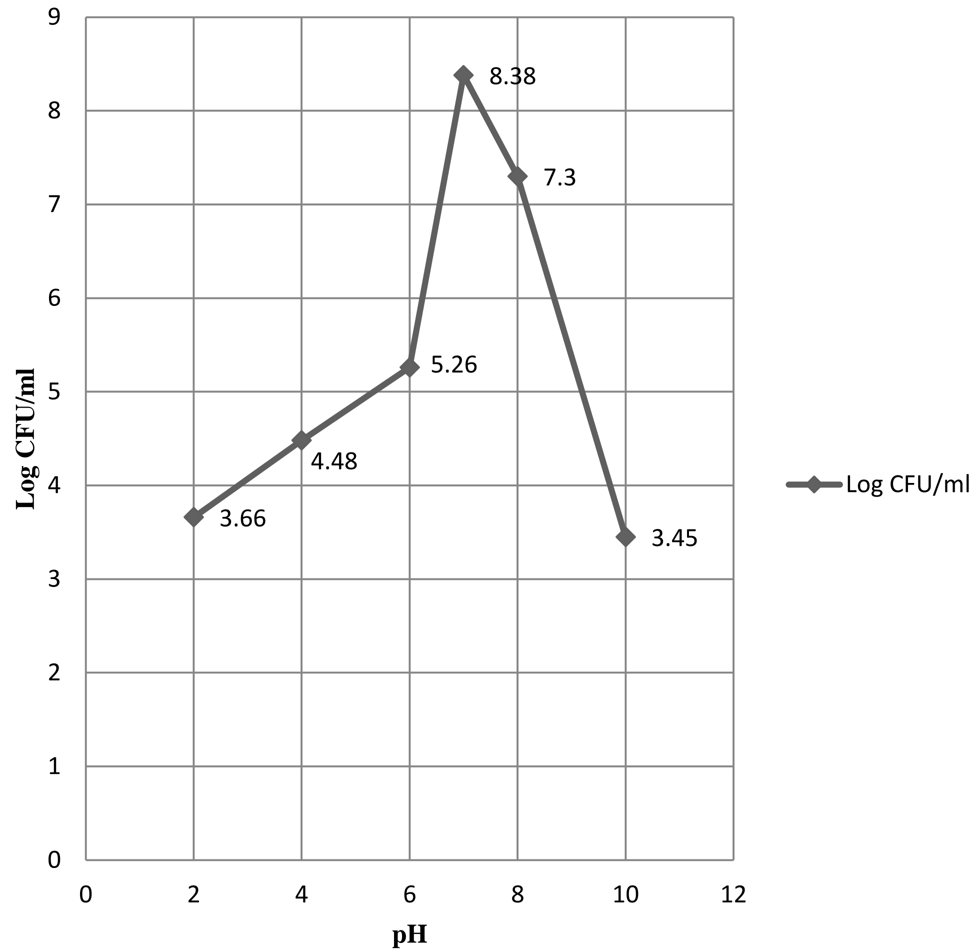


Figure 3.3: Effect of pH on the utilization of kerosene by *Pseudomonas* sp. after 7 days incubation at 35 ºC and 120 rpm

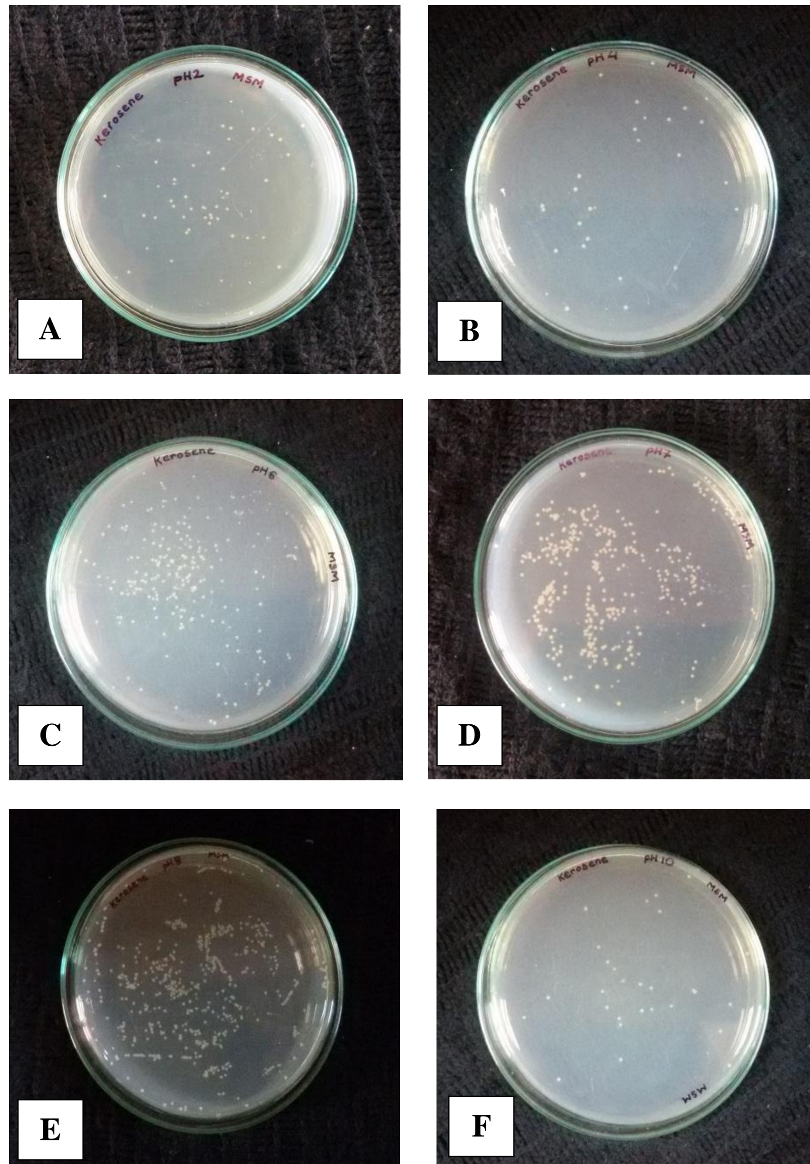


Figure 3.3.2: A-F. Mineral salts agar plates showing viable counts of *Pseudomonas* sp. after its growth in mineral salts broth containing varying pH. **D.** The isolate showed optimum growth at pH 7. **E.** Sub optimum growth was observed at pH 8. **A & F.** Extremes of pH 4 and pH 10 was unfavorable for the growth of the isolate *Pseudomonas* sp. as it produced low CFU/mL.

3.4 Effect of different concentrations of nitrogen source

Strain C1 was cultured in six 50 mL mineral salts broth (pH 7.0) with ammonium sulfate concentration (nitrogen source) being varied from 0.25g/L – 1.5g/L. These were incubated for 7 days at 35 ºC and 120 rpm. One milliliter of each culture was serially diluted and plated on mineral salts agar. Plates with viable counts were used to calculate CFU/mL.

Table 3.4: Colony counts of *Pseudomonas* sp. on mineral salts agar after its growth in mineral salts broth containing varying concentrations of ammonium sulfate

Nitrogen concentration (g/L)	CFU at different dilutions						CFU/ml	Log CFU/ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
0.25 g/L	311	30	2	0	0	0	3×10^5	4.48
0.5 g/L	TNTC	TNTC	TNTC	58	4	0	5.8×10^6	6.76
0.75 g/L	TNTC	TNTC	TNTC	TNTC	186	23	1.86×10^8	8.27
1.0 g/L	TNTC	TNTC	TNTC	TNTC	237	24	2.37×10^8	8.37
1.25 g/L	TNTC	TNTC	TNTC	TNTC	167	11	1.67×10^8	8.22
1.5 g/L	TNTC	TNTC	326	30	1	0	3×10^6	6.48

*TNTC = too numerous to count

The isolate *Pseudomonas* sp. gave optimum growth in the presence of 1.0 g/L ammonium sulfate used as nitrogen source. Growth declined significantly when concentration of ammonium sulfate was reduced below 0.75 g/L or when increased above 1.25 g/L.

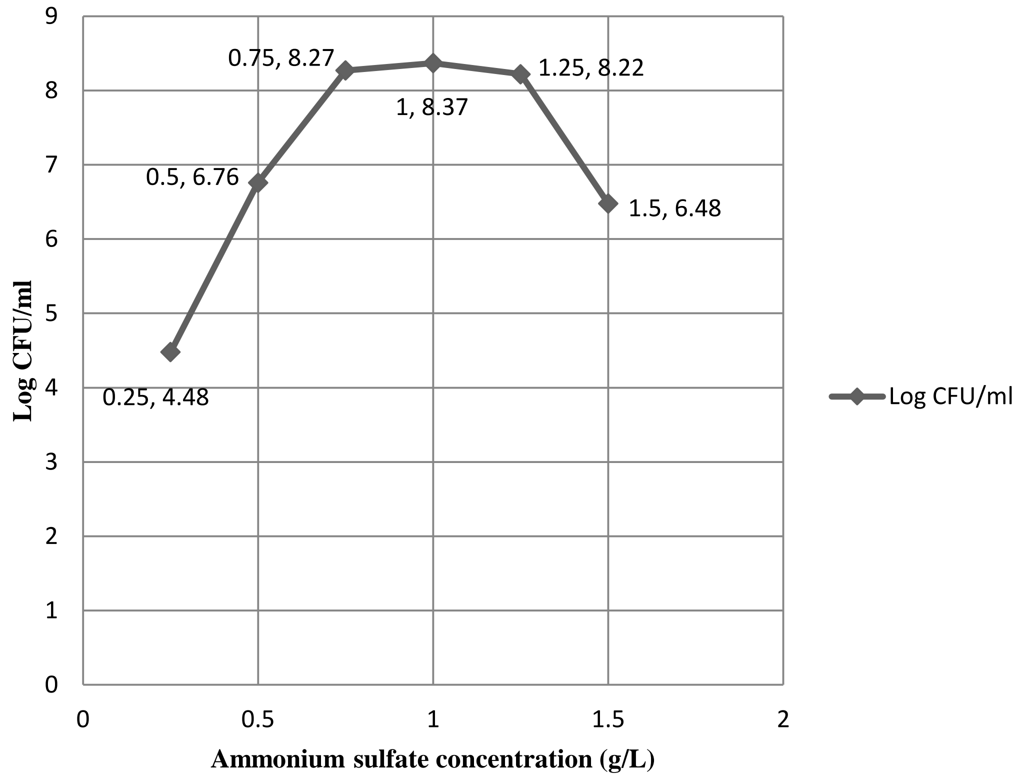


Chart 3.4: Effect of ammonium sulfate concentration on the utilization of kerosene by *Pseudomonas* sp. after 7 days incubation at 35 ℃ and 120 rpm

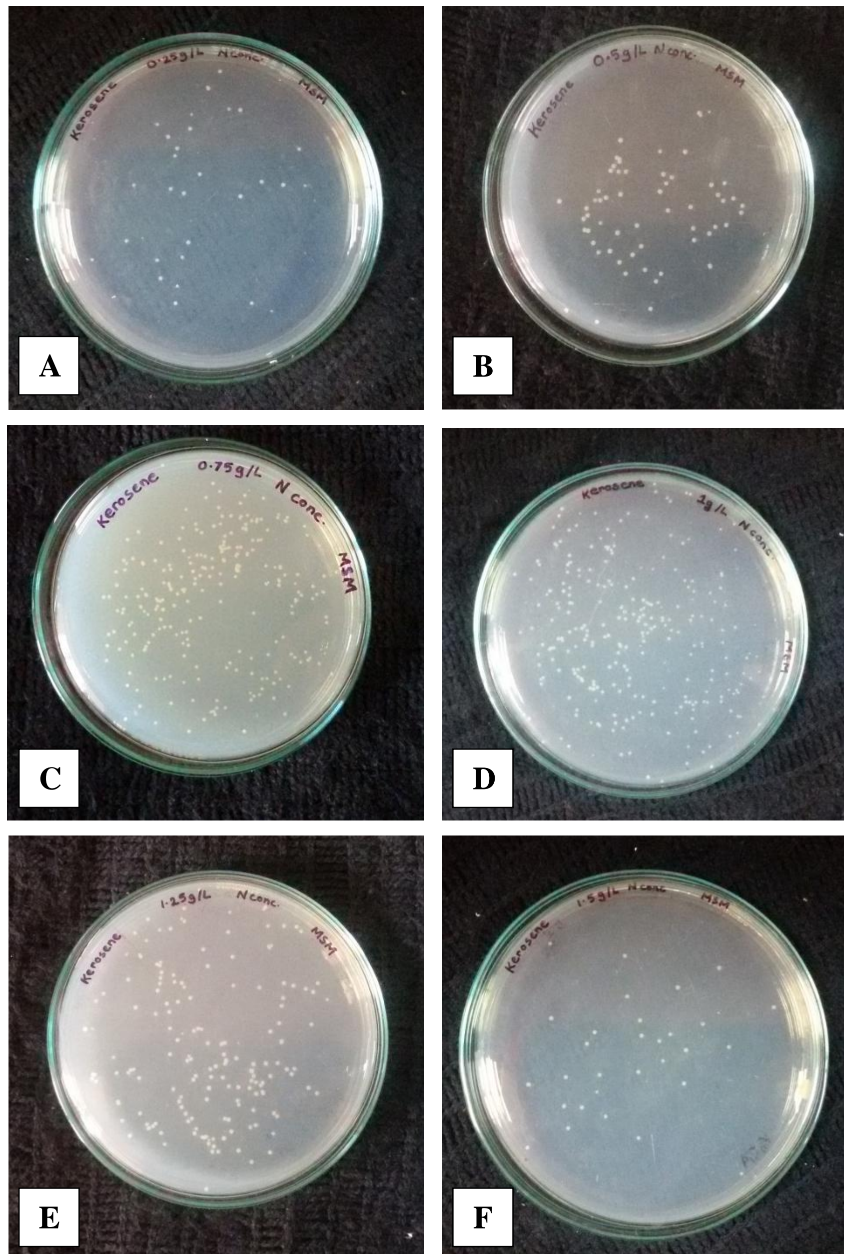


Figure 3.4.2: A-F. Mineral salts agar plates showing viable counts of *Pseudomonas* sp. after its growth in mineral salts broth containing varying concentrations of ammonium sulfate. **D.** Optimum growth of the isolate was observed at 1 g/L of ammonium sulfate. **C.** Sub optimum growth was observed at 0.75 g/L of ammonium sulfate. **E & F.** High concentrations of ammonium sulfate retarded growth of the isolate.

3.5 Identification tests

The eight isolates were streaked on Nutrient agar plates and their distinct colony morphologies were subsequently analyzed. The isolates were viewed under microscope after Gram staining, spore staining and acid fast staining. Biochemical tests were also performed and assumptive identification of the bacterial strains made through the use of ABIS software online. Isolates identified were *Nocardia* sp., *Corynebacterium* sp., *Pseudomonas* sp., two distinct *Bacillus* sp., and three *Arthrobacter* sp.

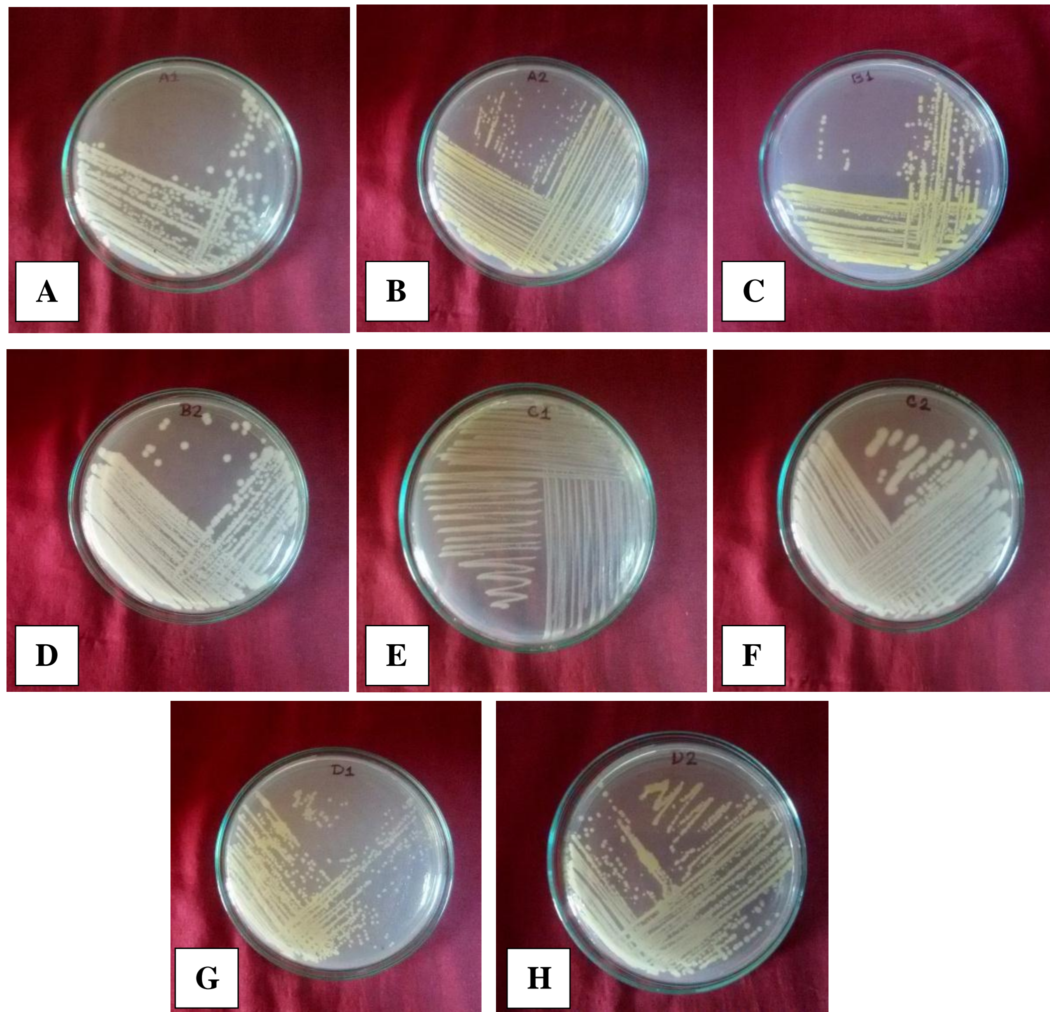


Figure 3.5: **A-H.** Eight bacteria isolated from soil samples were streaked on nutrient agar plates for isolated colonies.

Table 3.5.1: Colony characteristics of eight isolated bacteria in Nutrient agar

Strain designation	Shape	Margin	Elevation	Size	Texture	Color	Appearance
A1	Circular	Undulate	Umbonate	Large	Textured	White	Dull
A2	Circular	Entire	Raised	Small	Smooth	Yellow	Shiny
B1	Circular	Entire	Raised	Small	Smooth	Yellow	Shiny
B2	Circular	Undulate	Umbonate	Large	Textured	White	Dull
C1	Circular	Entire	Convex	Small	Smooth	Transparent white	Shiny
C2	Circular	Irregular	Umbonate	Large	Textured	White	Dull
D1	Circular	Entire	Umbonate	Moderate	Rough	Yellow	Dull
D2	Circular	Irregular	Raised	Moderate	Smooth	Yellow	Shiny

Table 3.5.2: Gram's staining, spore staining and acid fast staining results of eight isolated strains

Strain designation	Gram's staining				Spore staining	Acid fast staining
	Gram's	Morphology	Snapping Division	Rod-coccus cycle		
A1	+ve	Rods arranged as branching filaments	-ve	-ve	-ve	Weakly acid fast
A2	+ve	Irregular rods	+ve	-ve	-ve	- ve
B1	+ve	Irregular rods	+ve	+ve	-ve	-ve
B2	+ve	Irregular rods	-ve	-ve	+ve	--
C1	-ve	Irregular rods	-ve	-ve	-ve	--
C2	+ve	Irregular rods	-ve	-ve	+ve	--
D1	+ve	Irregular rods	+ve	+ve	-ve	-ve
D2	+ve	Irregular rods	+ve	+ve	-ve	-ve

Table 3.5.3: Biochemical test results of eight isolated strains and their identification through the use of ABIS online software

Strain designation	Motility	Growth at 6.5% NaCl	Catalase	Oxidase	Urease	Indole production	Citrate utilization	Nitrates reduction	Hemolysis (Sheep's blood agar)	Starch hydrolysis
A1	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	α hemolysis	+ve
A2	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	γ hemolysis	-ve
B1	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	α hemolysis	-ve
B2	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	β hemolysis	+ve
C1	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	α hemolysis	-ve
C2	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	β hemolysis	+ve
D1	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	γ hemolysis	-ve
D2	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	α hemolysis	-ve

Table 3.5.3: Biochemical test results of eight isolated strains and their identification through the use of ABIS online software (continued)

Strain designation	Methyl-red test (MR)	Voges - Proskauer test (VP)	Dextrose fermentation	Lactose fermentation	Sucrose fermentation	Arabinose fermentation	Mannitol fermentation	Presumptive organism
A1	-ve	-ve	+ve	-ve	-ve	-ve	-ve	*Nocardia* sp.
A2	-ve	-ve	+ve	-ve	+ve	+ve	-ve	*Corynebacterium* sp.
B1	-ve	-ve	+ve	-ve	+ve	+ve	-ve	*Arthrobacter* sp.
B2	-ve	-ve	+ve	+ve (partial)	+ve	-ve	-ve	*Bacillus* sp.
C1	-ve	-ve	+ve	+ve (partial)	-ve	-ve	-ve	*Pseudomonas* sp.
C2	-ve	-ve	+ve	+ve (partial)	+ve (partial)	+ve	-ve	*Bacillus* sp.
D1	-ve	-ve	+ve	-ve	+ve	-ve	+ve	*Arthrobacter* sp.
D2	-ve	-ve	+ve	-ve	+ve	-ve	+ve	*Arthrobacter* sp.

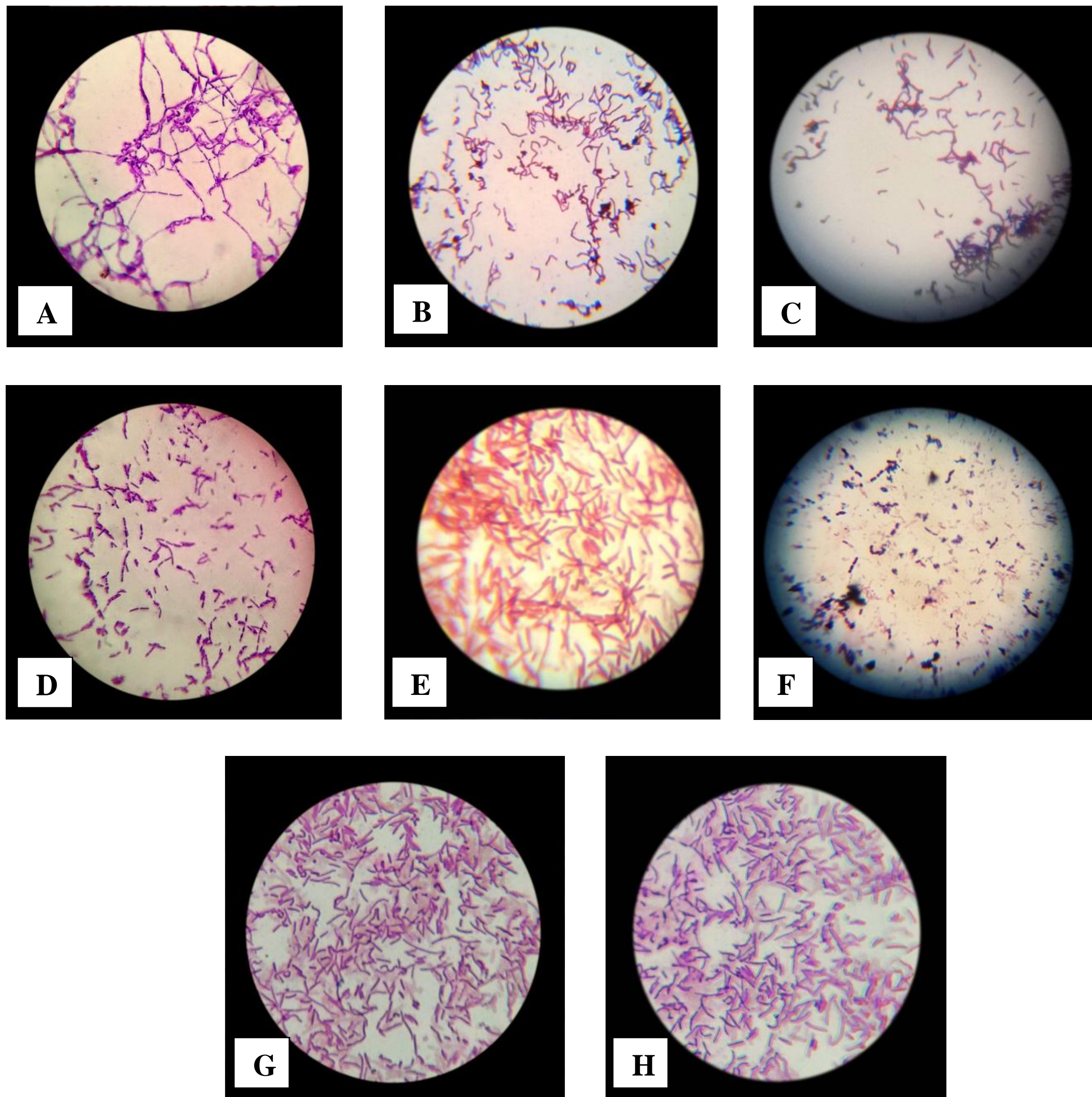
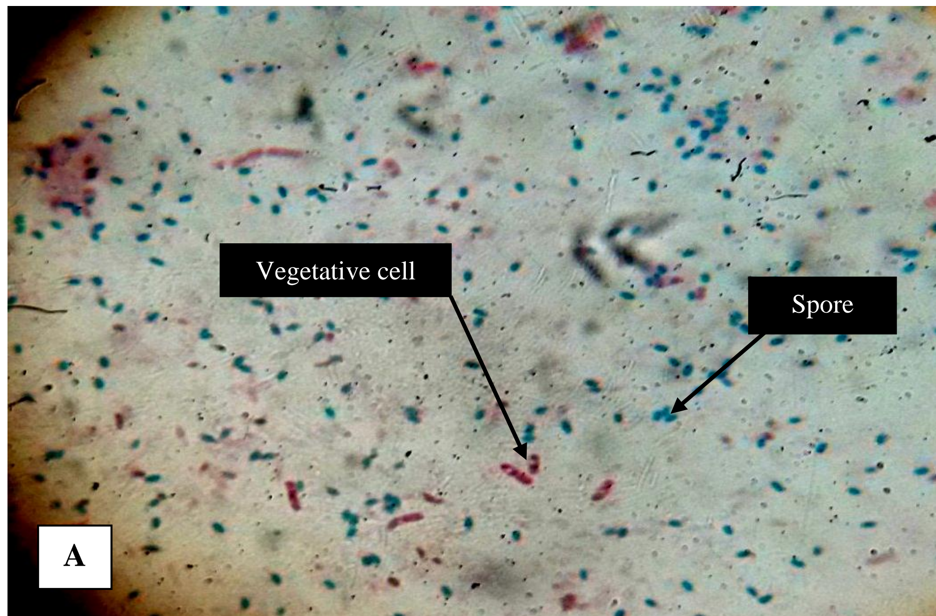


Figure 3.5.1: Gram staining of the eight isolates. **A**. The isolate A1 stained purple or gram positive with cellular morphology of rods arranged in branching filaments. **B**. The isolate A2 stained purple with irregular rods and palisades characteristic of snapping division. **C, G & H**. The isolates B1, D1 and D2 stained purple with irregular rods, few cocci and palisades. **D & F.** The isolates B2 and C2 stained purple with irregular rods. **E**. The isolate C1 stained red or Gram negative with irregular rods.



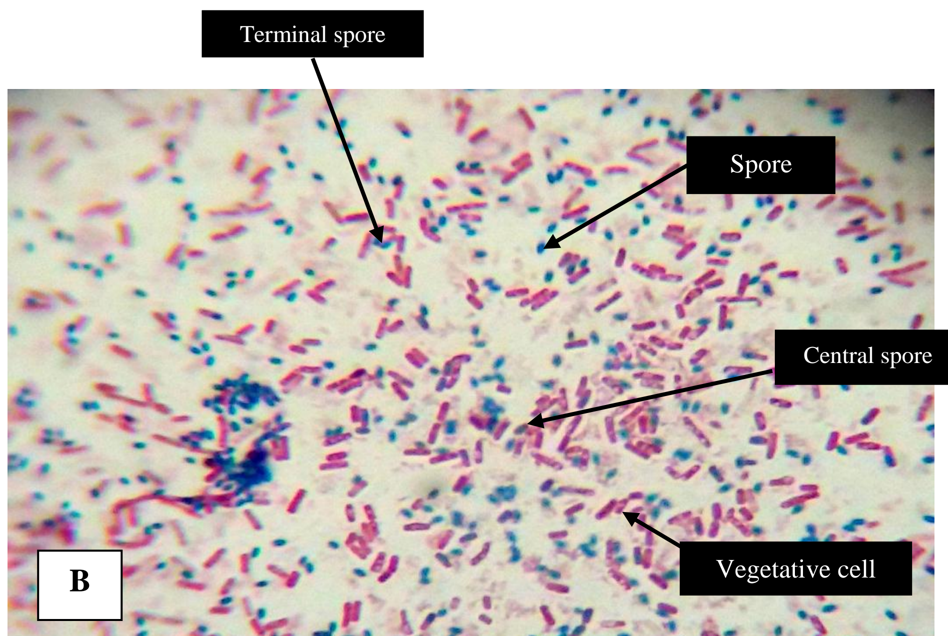


Figure 3.5.2: Spore staining of isolates B2 and C2. **A.** isolate B2 and **B.** isolate C2 stained blue-green with malachite green, characteristics of presence of spore. Cells stained red with the counter stain safranin were vegetative cells.

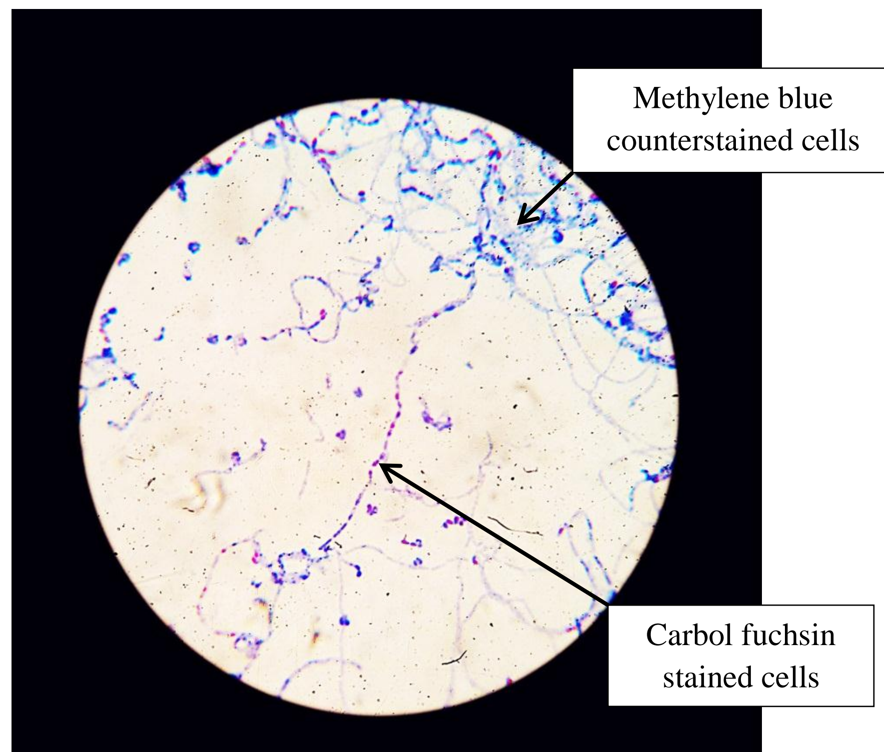


Figure 3.5.3: Acid fast staining of A1 isolate. The isolate A1 was partially positive for acid fast staining. Carbol fuchsin stained the cells red due to the presence of mycolic acids in the bacteria's cell wall. Methylene blue counterstain stained most cells blue since the bacterial isolate was weakly acid fast. All other isolates in this study were non -fast bacteria.

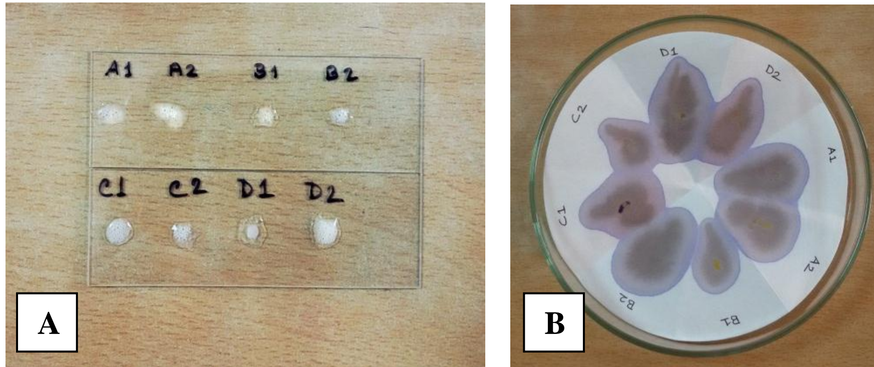


Figure 3.5.4: Catalase and Oxidase test were performed. **A.** All isolates were catalase positive. **B.** The isolate C1 was the only oxidase positive bacterium.

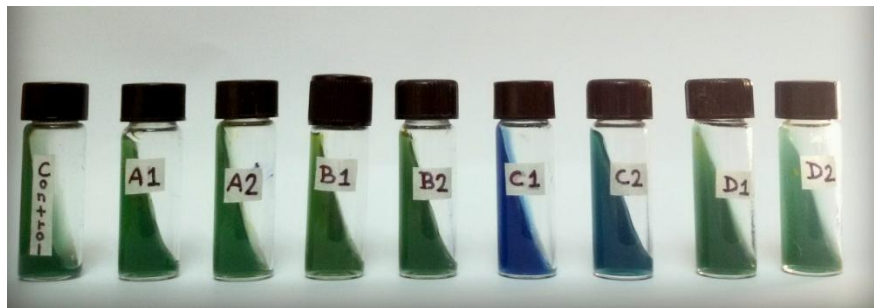


Figure 3.5.5: Simmons' citrate test was performed. The isolates C1 and C2 were citrate positive and turned the agar blue through creating alkaline condition. All other isolates were citrate negative and showed no change in agar.

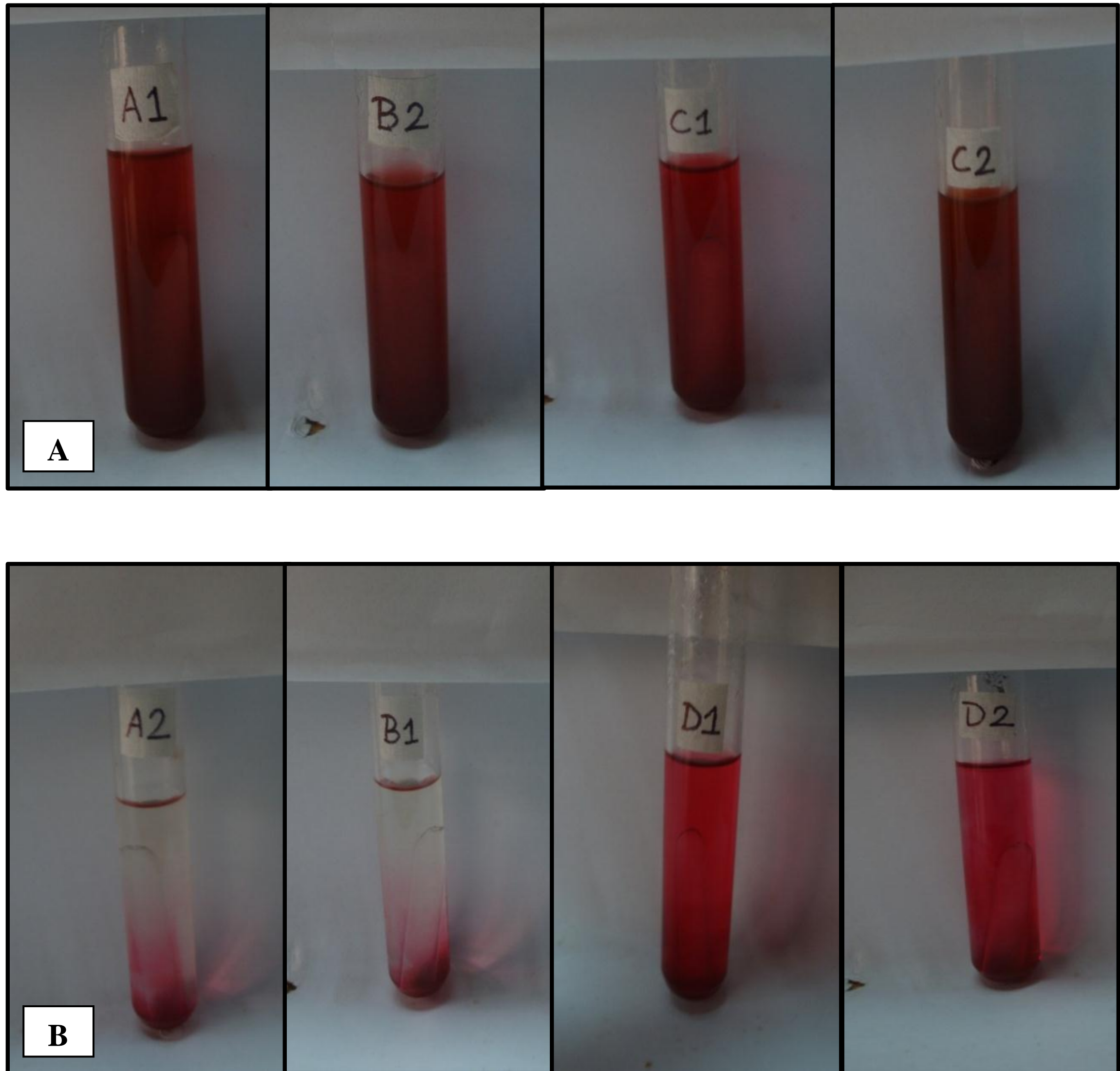


Figure 3.5.5: Nitrate reduction test. **A.** Isolates A1, B2, C1 and C2 reduced nitrate to nitrite without bubble/nitrogen gas formation. The presence of nitrite was observed through the formation of red color when reagents sulfalinic acid and α-naphthylamine were added.

B. Isolates A2, B1, D1 and D2 showed no change on the addition of the reagents. When zinc powder was added, the solutions became colorless first and then turned bright pink, proving unreduced nitrate were still present.

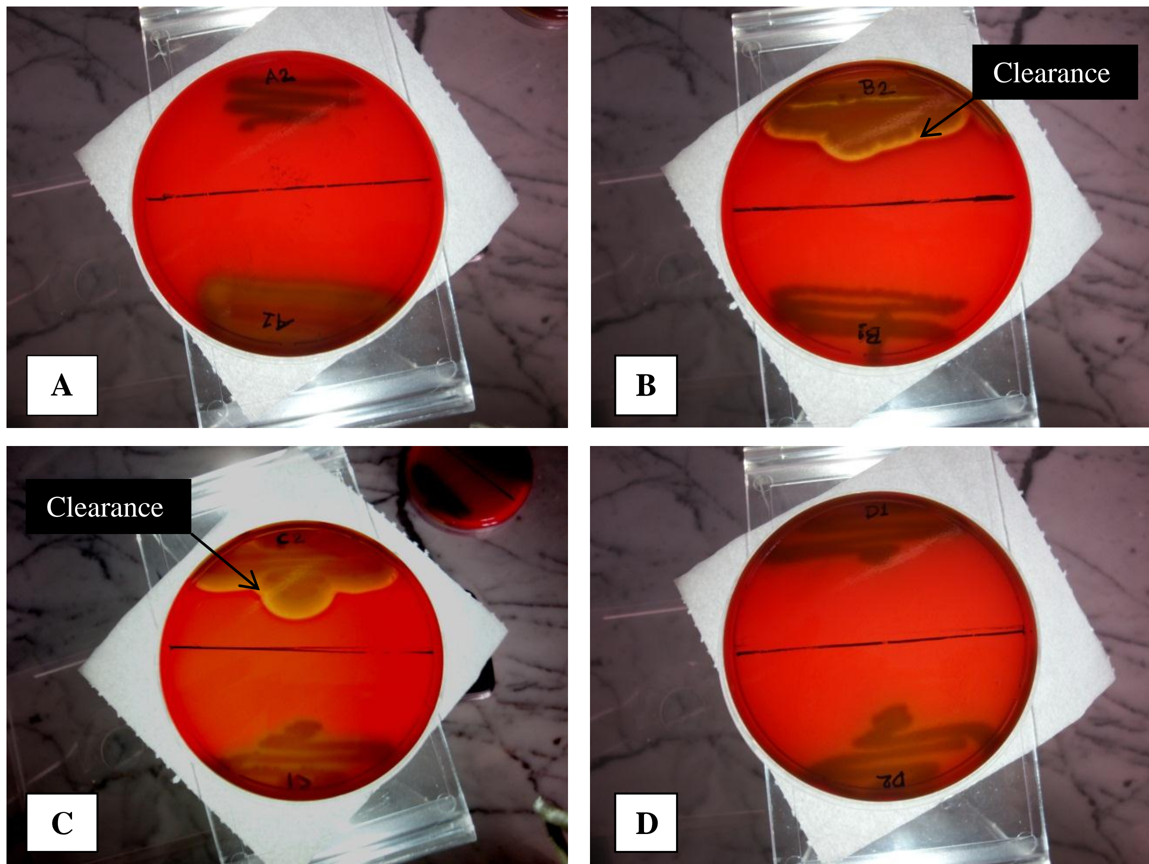


Figure 3.5.7: Ability of the isolates to hemolyse sheep's blood was tested on blood agar plates. **A.** Isolate A1 was unable to break down RBC (α hemolysis). Isolate A2 hemolysed RBC partially, creating a green zone around its growth (β hemolysis). **B.** Isolate B1 showed inability to break down RBC while isolate B2 completely hemolysed blood and produced a zone of clearance around its growth (γ hemolysis). **C.** Isolate C2 was positive for partial hemolysis and isolate C2 demonstrated complete hemolysis. **D.** The isolate D1 showed partial hemolysis while the isolate D2 were unable to hemolyse RBC.

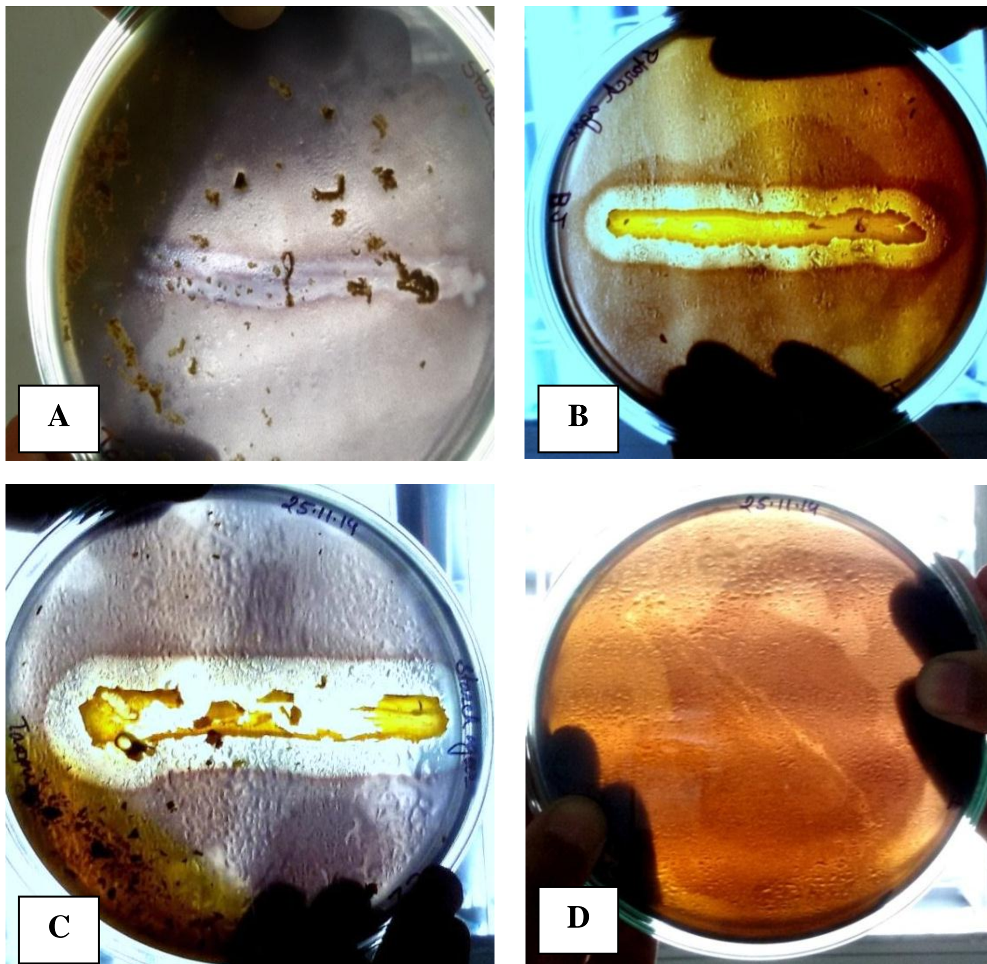


Figure 3.5.8: Starch hydrolysis test. The addition of iodine solution displayed characteristic halo clearing around the growth of **A.** Isolate A1, **B.** Isolate B2, and **C.** Isolate C2 for their ability to hydrolyse starch. **D.** Isolates A2, B1, C1, D1 and D2 did not show any clearing of agar when iodine solution was added.

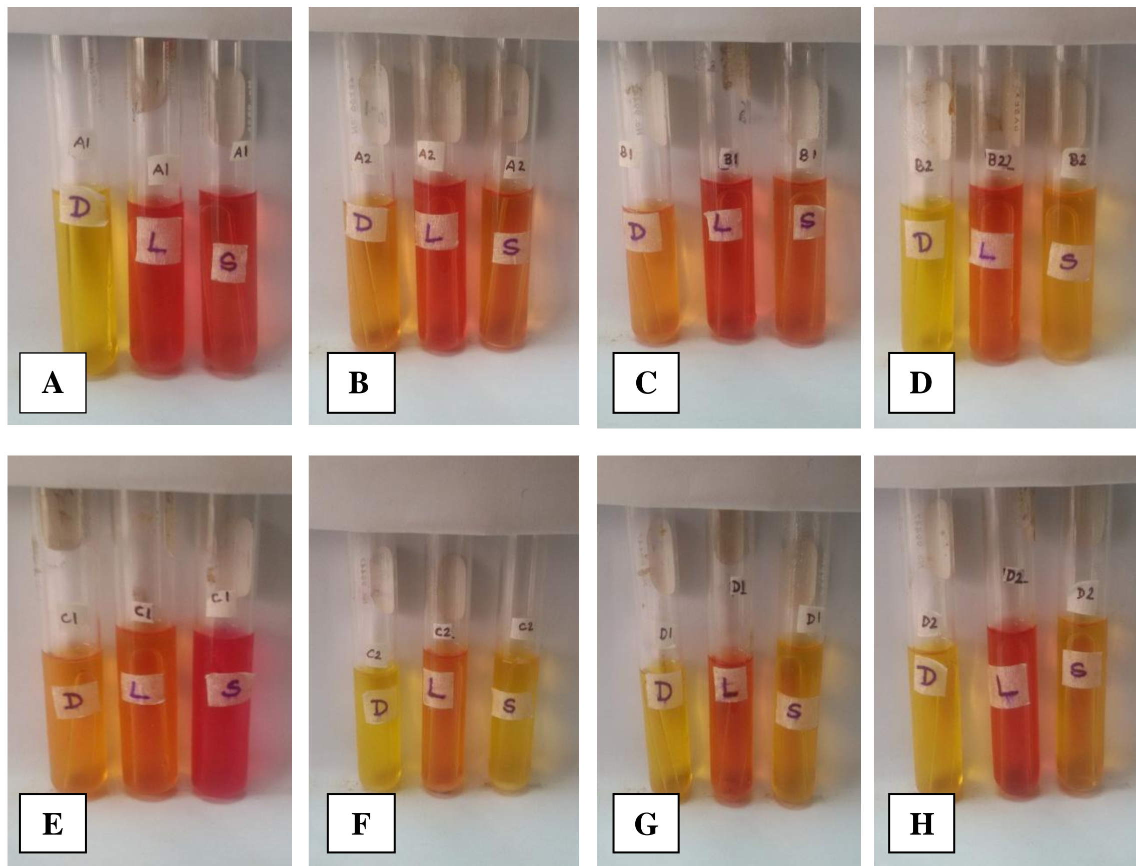


Figure 3.5.9: Carbohydrate fermentation test was performed on the eight isolates to evaluate the fermentative capability of dextrose, sucrose and lactose. **A.** Isolate A1 demonstrated dextrose positive, and lactose and sucrose negative. **B-C.** Isolates A2 and B1 were dextrose and sucrose positive, and lactose negative. **D & F.** Isolates B2 and C2 showed dextrose and lactose positive, while lactose partial positive. **E.** Isolate C1 was able to ferment dextrose, partially fermented lactose, and unable to ferment sucrose. **G-H.** Isolates D1 and D2 fermented dextrose and sucrose and showed inability to ferment lactose.

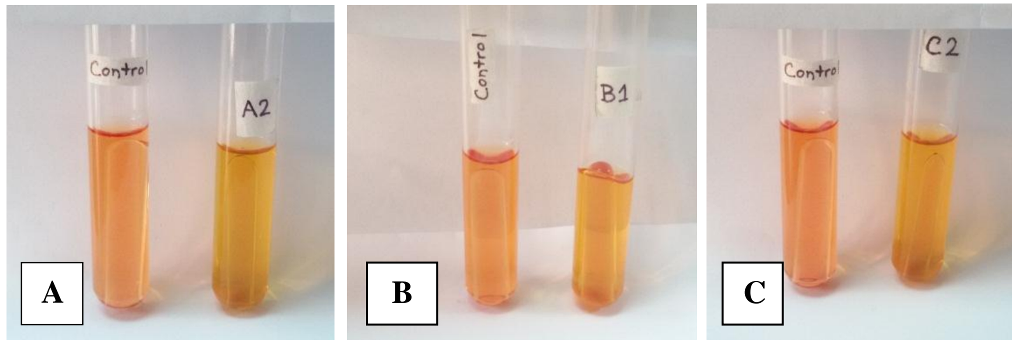


Figure 3.5.10: Arabinose fermentation test. **A-C.** The isolates A2, B1 and C2 were able to ferment arabinose to produce an acidic yellow solution when arabinose test was performed.

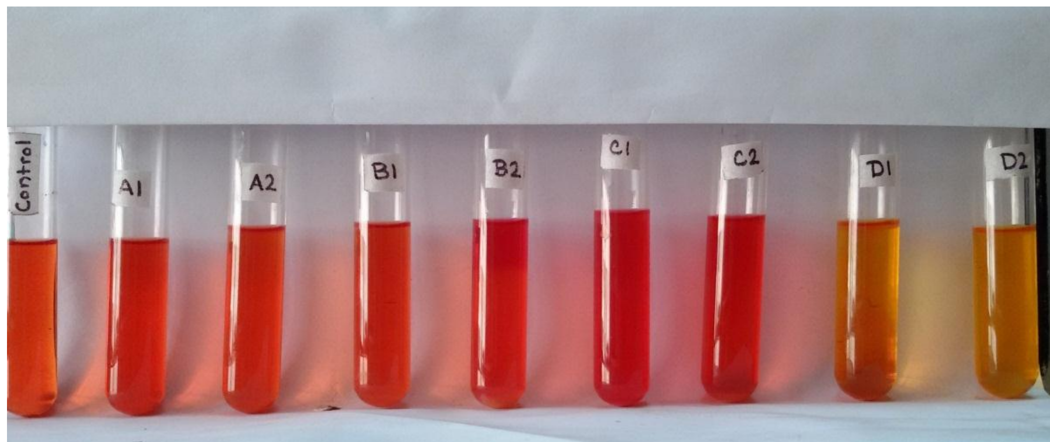


Figure 3.5.11: Mannitol fermentation test. The isolates D1 and D2 demonstrated the ability to ferment mannitol. The color of the solution turned from reddish orange to yellow as acid was produced by the mannitol positive bacteria. The isolates A1-C2 were unable to ferment mannitol and turned the solution slightly deep red from alkaline condition during growth.

Chapter 4: Discussion

Chapter 4: Discussion

Oil pollution is a macabre global problem that obliterates environment, decimates living organisms and inflict potential harm to human. Existing physical and chemical techniques have proven to be less efficient and costly, and thereby requiring the advent of biological techniques to be an imperative solution. This preliminary study have been performed to isolate strains with the disposition to degrade kerosene in particular, but with further research deploying gas chromatography, the specific compound or compounds constituent of the kerosene that is being utilized by the bacteria can be identified. With such result, it will not only be possible to alleviate oil spills of aviation fuel or kerosene, but might also be used to degrade oils containing those specific compounds.

Eight bacteria that had been isolated from soil samples were identified as *Nocardia* sp. (A1), *Corynebacterium* sp. (A2), *Arthrobacter* sp. (B1), *Bacillus* sp. (B2), *Pseudomonas* sp. (C1), *Bacillus* sp. (C2), *Arthrobacter* sp. (D1), *Arthrobacter* sp. (D2), all being different strains.

4.1 Kerosene degrading ability of different isolates

The growth characteristics of eight isolates from soil samples were investigated. When the eight different isolates were cultured in mineral salts broth containing 1% (v/v) kerosene for 7 days at 35ºC and 120 rpm, *Pseudomonas* sp. showed the highest growth as indicated by high turbidity of the culture, followed by *Bacillus* sp. Subsequent plating of diluted 100 µL of each culture in mineral salts agar had also given results consistent with the visualized turbidity, with *Pseudomonas* sp. giving the highest growth of 8.38 log CFU/mL and the two *Bacillus* sp. giving near similar results of 6.70 $\log_{10}$ CFU/mL and 6.04 $\log_{10}$ CFU/mL. The non-fermenter Pseudomonads had been unequivocally identified in innumerable studies to possess the highest degradable capability of organic pollutants (Fritsche & Hofrichter, 2008; Panda *et al*., 2013; Shukor *et al*., 2009, Nilesh & Hardik, 2013). A study presented the isolate *Pseudomonas lundensis* to degrade crude diesel sample with a similar 8 $\log_{10}$ CFU/mL when grown for three days on Bushnell-Hass plates (Rahman & Kelang, 2009).

Apart from the *Pseudomonas* sp. isolate, two *Bacillus* sp. taken from Bogura and Dhaka oil contaminated soil gave near similar results of 6.70 $\log_{10}$ CFU/mL and 6.04 $\log_{10}$ CFU/mL. *Arthrobacter* sp. was identified as three of the isolates taken from different soils and had given a lower growth on kerosene (3.35 $\log_{10}$ CFU/mL, 4.54 $\log_{10}$ CFU/mL and 4.58 $\log_{10}$ CFU/mL). *Pseudomonas*, *Bacillus* and *Arthrobacter* sp. had been the most reported hydrocarbon degrading bacteria, along with many *Nocardia* and *Corynebacterium* sp. (Bossar & Bartha, 1984; Austin *et al.,* 1977). However, *Nocardia sp.* and *Corynebacterium* sp. in this study had not been able to utilize kerosene as the sole carbon source in this study.

Isolates of soil B, C and D taken from oil contaminated sites had all shown the ability to grow in the presence of kerosene. *Nocardia* sp. and *Corynebacterium* sp. isolated from agricultural site (soil A) deficit of oil failed to utilize kerosene and grow in the absence of usual carbon sources. This supports the findings that bacteria must develop their ability to utilize unusual carbon sources through adaptation by prior exposure (Leahy & Colwell, 1990). Prior exposure of microbes to hydrocarbons through accidental oil spills, transportation activities, oil seepage or waste oil disposals determines the rate at which they will be able to degrade external oil inputs (Bartha & Bossert, 1984). Such adaptation to degrade oil develops among exposed microbial community through induction of required enzymes, genetic modifications and selective enrichment of the microbes able to break down the toxic material and had been discussed in a few studies (Spain *et al.*, 1980; Spain & Veld, 1983). The isolates of soil B, C and D being from petrol pump stations and locomotive storage site had been highly exposed to oil for several years. Soil A of agricultural site had not been as exposed to oil components and therefore microbes only adapted to hydrocarbons had been able to break down kerosene into constituents necessary for growth.

4.2 Optimum concentration of kerosene and the level of tolerance for kerosene for the potential isolate

Further research was accomplished utilizing the isolate (*Pseudomonas* sp.) that showed maximum growth in the presence of kerosene. The isolate was found to tolerate as high as 6% kerosene (v/v) and showed optimum growth at 3% kerosene (v/v), with bacterial

count of 5.53 log_{10} CFU/mL and 9.43 log_{10} CFU/mL respectively. High concentrations of hydrocarbon, particularly the volatile ones, can be noxious to bacterial growth. Dibble and Bartha (1979) reported the increase in carbon dioxide emissions in soil when hydrocarbon concentrations were raised from 1.25% to 5%, but no such changes observed on further increase to 10%. Nonvolatile hydrocarbons such as crude oil had also shown similar results as high concentrations prevent adequate oxygen availability to microbes (Fusey & Oudot, 1984). Therefore, optimum degradation was observed only at a certain threshold hydrocarbon concentration, and presence of any higher concentration will require more agitation and dispersion, as reported by Rashid (1974) when they compared hydrocarbon degradation in enclosed stagnant bays to that in sea with greater wave energy.

4.3 Optimum pH for growth of the potential isolate

Factors that can affect the utilization of kerosene and therefore the growth of bacteria were studied in a series of experiments. pH of the media was found to strongly affect bacterial growth since pH changes during bacteria production of wastes and therefore requiring adjustments of environmental conditions. The isolate *Pseudomonas* sp. was able to degrade kerosene and grow within a pH range of 2-10, showing maximum growth at pH 7 and high growth at pH 8. As pH gradually reduced or increased from near neutral, growth declined. Growth nearly doubled when pH of the media was increased from pH 4 (4.48 log_{10} CFU/mL) to pH 7 (8.38 log_{10} CFU/mL), and dropped significantly when further increased to pH 8 (7.30 log_{10} CFU/mL). Growth also declined when pH was increased from pH 7 to pH 10 (3.45 log_{10} CFU/mL). Such extremes of pH was unfavorable for growth of the bacterium as most bacteria lack internal pH homeostasis. The requirement of near neutral pH for optimum growth had been reported in several studies (Espeche *et al.*, 1994; Kwapisz *et al.,* 2008; Shukor *et al.,* 2008). Thus, for bioremediation of oil spills, the isolate *Pseudomonas* sp. can be used in near neutral soil or water body for optimum activity. Extremes of pH as observed in many ecosystems will not be beneficiary to degradation of oil unless the isolate is utilized ex-situ and media pH adjusted accordingly.

4.4 Optimum nitrogen concentration for kerosene degradation of the potential isolate

The *Pseudomonas* sp. isolate showed maximum growth (8.37 log_{10} CFU/mL) through utilizing 1% (v/v) kerosene when 1 g/L ammonium sulfate was provided as the nitrogen source. It gave sub optimum growths at 0.75 g/L and 1.25 g/L of 8.27 log_{10} CFU/mL and 8.22 log_{10} CFU/mL respectively. Further reduction of ammonium sulfate to 0.25 g/L and 0.5 g/L had lowered growth drastically to 4.48 log_{10} CFU/mL and 6.76 log_{10} CFU/mL respectively. Nitrogen is an essential component for bacterial growth in the production of amino acids, and therefore concentrations any lower than 0.75 g/L of ammonium sulfate was limiting to the utilization of carbon source kerosene. This complies with the findings that improper carbon/nitrogen ratio limits bacterial degradation of hydrocarbons through saturation of one and deficiency of the other.

Growth was observed to be reduced when concentrations of ammonium sulfate higher than 1.25 g/L was used. Shukor *et al.* (2008) in a study on degradation of diesel had recorded that growth of isolates lowered from 11.2 log_{10} CFU/mL to 10.5 log_{10} CFU/mL when nitrite concentration was increased from 1.25 g/L to 1.5 g/L. Such decline in hydrocarbon degradation is due to toxic effect of high nitrogen content. Excess ammonium in the media inhibits the production of biosurfactants such as rhamnolipids which are produced by several *Pseudomonas* sp. (Kohler *et al.*, 2000). Rhamnolipid is extremely essential in emulsifying oil for their easier absorption.

Many oil spills can already account to low level of nitrogen supply (Atlas *et al.,* 1977; Cooney *et al.*, 1984). Moreover, inorganic nutrients such as nitrogen or phosphorus avail in many soil sites or water bodies, especially in closed ecosystems (Atlas & Bartha, 1973; Atlas & Busdosh, 1976). However, nutrients tend to wash out in non-stagnant environments (Atlas & Bartha, 1973; Atlas & Busdosh, 1976), resulting in the imperative external addition of nitrogen source to be neither in excess nor too limiting. Bossert and Bartha (1984) had also demonstrated that the variation in soil composition and presence of nitrogen-fixing bacteria can contribute to the amount of inorganic nutrient amendments. Since in this study, the *Pseudomonas* sp. was observed to possess nitrogen reductase in biochemical test, it could tolerate a higher nitrogen concentration and speed

up degradation as opposed to many non-denitrifying bacteria that show no effect on hydrocarbon degradation with increasing nitrogen supply, as observed by Lehtomaki and Niemala (1975).

4.5 Further research

This had only been a preliminary study. Further work could be performed through observing the degradation ability of the isolate reported in this work to other oils, duration of time required for significant degradation, production of harmful metabolites during growth or degradation, and its compatibility to other hydrocarbon degrading microbes. The species of the *Pseudomonas* isolate also needs to be identified through more biochemical tests and 16S rRNA, and its plasmid profiling can be accomplished. The stability of the strain and that of its plasmid are extremely important determining factors at the prudent use of a strain in bioremediation. Further research can be carried out with gas chromatography at determining the exact amount of mass reduction of kerosene or other oils, and the particular components that are being degraded (Wongsa *et al.*, 2004). Only then can the isolate be used for wide range of oil in contaminated sites. Heavier oils would be degraded possibly at a higher rate than kerosene, as they are not as toxic and can be much readily emulsified for absorption. Since this study focused particularly on the ability to utilize kerosene when no other carbon source was available, it had used pure media components devoid of carbon. Additional research could be performed at determining the rate of degradation based on cheaper raw materials that could be easily available for the manufacture of bioremediation product. The study had also been conducted at temperature 35°C, and the utilization of oil by the isolate at other temperatures is impertinent to be determined (ZoBell, 1969; Gunkel, 1967).

No single technique can obliterate oil pollution. Biological remediation must be used hand in hand with physical techniques of containment and chemical techniques of non-toxic dispersion to provide the most benign and efficient combinatorial solution. Thus, a big research challenge is to find out an optimum combination of physical, chemical and biological methods to face the menace of oil pollution of the environment.

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Appendix – I

Media composition

The following media was used during the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise

1. Arabinose broth (pH 7.3)

Ingredients	Amounts (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Arabinose	5.0
Phenol red	0.018

2. Blood agar

Ingredients	Amounts (g/L)
Casein enzyme hydrolysate	14.0
Peptic digest of animal tissue	4.5
Yeast extract	4.5
Sodium chloride	5.0
Agar	12.5
Sheep blood*	5 %

* Sheep blood was aseptically added after autoclaving rest of the media and cooling it to 45-50°C

3. MacConkey agar (Oxoid, England)

Ingredients	Amounts (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

4. Mannitol Salt broth

Ingredients	Amounts (g/L)
Tripticase	10.0
D-Mannitol	5.0
NaCl	5.0
Phenol red	0.018

5. Mineral Salt broth (pH 7.0)

Ingredients	Amounts (g/L)
Ammonium sulfate	1.0
Potassium di-hydrogen phosphate	1.0
Di-potassium hydrogen phosphate	1.0
Calcium chloride	0.02
Magnesium sulfate heptahydrate	0.2
Ferric chloride	0.05

6. Mineral Salt agar (pH 7.0)

Ingredients	Amounts (g/L)
Ammonium sulfate	1.0
Potassium dihydrogen phosphate	1.0
Di-potassium hydrogen phosphate	1.0
Calcium chloride	0.02
Magnesium sulfate heptahydrate	0.2
Ferric chloride	0.05
Agar-agar	20.0

7. MIU agar (HiMedia, India)

Ingredients	Amounts
Casein enzyme hydrolysate	10.0 g/L
Dextrose	1.0 g/L
Sodium chloride	5.0 g/L
Phenol red	0.010 g/L
Agar	2.0 g/L
Urea*	5 mL (40%) per 95 mL media

* Sterile urea solution added to cooled autoclaved rest of the media

8. MR-VP broth

Ingredients	Amounts (g/L)
Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0

9. 6.5% NaCl broth

Ingredients	Amounts (g/L)
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	6.5
Di-basic potassium phosphate	2.5
Glucose	2.5

10. Nitrate broth

Ingredients	Amounts (g/L)
Peptone	5.0
Beef extract	3.0
Potassium nitrate	5.0

11. Nutrient agar (Himedia,India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.5
Sodium chloride	5.0
Yeast extract	1.5
Agar	15.0

12. Phenol red dextrose broth

Ingredients	Amounts (g/L)
Trypticase	10.0
Dextrose	5.0
Sodium chloride	5.0
Phenol red	0.018

13. Phenol red lactose broth

Ingredients	Amounts (g/L)
Trypticase	10.0
Lactose	5.0
Sodium chloride	5.0
Phenol red	0.018

14. Phenol red sucrose broth

Ingredients	Amounts (g/L)
Trypticase	10.0
Sucrose	5.0
Sodium chloride	5.0
Phenol red	0.018

15. Simmon’s citrate agar (Oxoid, England)

Ingredients	Amounts (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bactobromthymol blue	0.08

16. Starch agar

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	1.25
Beef extract	0.75
Starch soluble	2.0
Agar	15.0

17. T_1N_1 soft agar

Ingredients	Amounts (g/L)
Tryptone	0.6
Sodium chloride	0.3
Agar	0.42

Appendix II

Reagents

The following reagents were used throughout the study:

1. Barritt's reagent

Solution A: 5 g alpha-naphthol was dissolved in 95% ethanol. The reagent was covered in aluminum foil and stored at 4°C.

Solution B: 40 g KOH was dissolved in distilled water. Once the mixture cooled, creatine was added. Final volume was adjusted with distilled water and the reagent covered with aluminum foil was stored at 4°C.

2. Carbol Fuchsin Stain (0.3%)

For the preparation of 100 mL solution, 0.3 g of carbol fuchsin (Sigma) was dissolved in 9.5 mL ethanol (100%). To this, 90.5 mL distilled water was added. The solution was left to stand for several days and filtered into sterile reagent bottle.

3. Crystal violet Stain (2%)

2 g of crystal violet was dissolved in 20 mL of 95% ethyl alcohol. 0.8 g ofammonium oxalate monohydrate was next dissolved in 80 mL distilled water. The two solutions were mixed and filtered into sterile reagent bottle.

4. Iodine solution (Gram's)

6.7 g potassium iodide was dissolved in 100 mL of distilled water. To this, 3.3 g of iodine was added, stirred, and the solution made up to 1 liter with distilled water. The reagent bottle was covered in aluminium foil and stored at room temperature.

5. Kovac's reagent

5 g para-dimethylaminobenzaldehyde was dissolved in 75 mL amyl alcohol. To this, hydrochloric acid (1M) was added to make up the final volume of 25 mL. The reagent bottle was covered with aluminium foil and stored at 4°C.

6. Malachite green (0.5%)

0.5 g malachite green was dissolved in 100 mL distilled water. The solution was stored at room temperature by covering the reagent bottle with aluminium foil.

7. Methylene blue solution (1%)

1 g of methylene blue was dissolved in 75 mL of distilled water, and then diluted to make 100 mL. The solution was filtered out and stored in reagent bottle.

8. Methyl red reagent

0.1 g methyl red was dissolved in 300 mL of 95% ethyl alcohol. To this, distilled water was added to make up the final volume of 500 mL. The reagent was covered with aluminum foil and stored at 4°C.

9. Oxidase reagent

100 mg of N, N, N^1, N^1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 mL distilled water. The solution was covered with aluminum foil and stored at 4°C.

10. Safranin

0.1 g of safranin was dissolved in 75 mL of distilled water. The solution was diluted to 100 mL, filtered and stored in clean reagent bottle.

Appendix III

Instruments

Instrument	Company
Autoclave	SAARC
Cellulose filter paper (9.0 cm)	Whatman
Colorimeter, ISO 9001	Labtronics, India
Freeze (-20°C)	Siemens
Incubator	SAARC
Hotplate stirrer	LabTech
Micropipette (10-100 µL)	Eppendorf, Germany
Micropipette (100-1000 µL)	Eppendorf, Germany
Microscope	Optima
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Refrigerator (4°C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Surgical Millipore syringe filter (0.22µm)	Millex-GS
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Weighing balance	ADAM EQUIPMENT™, United Kingdom