Isolation and Study of the Optimum Growth Conditions of Diesel Degrading Bacteria from Soil

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

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DECLARATION

I, Tasmin Naila, hereby declare that the thesis project titled "Isolation and Study of the Optimum Growth Conditions of Diesel Degrading Bacteria from Soil" submitted by me has been carried out under the supervision of **Dr. M. Mahboob Hossain**, Associate Professor, Microbiology programme, Department of Mathematics and Natural Sciences. 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.

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ABSTRACT

The rise in industrialization and globalization has made oil spills an environmental threat all over the world. Bioremediation can be considered as an effective aid to the clean-up process of spilled oil aside from expensive and invasive physical and chemical methods. The natural degradation of oil by microorganism can help remove residual oil pollutants. Hydrocarbon compounds present in petroleum products can have deteriorating effects on both wild life and humans. Diseases, disorders and even cancer of different organ systems have been linked to the exposure to these hydrocarbons. Microorganisms have metabolic capabilities to utilize the carbon compounds and degrade them to innocuous by-products. These abilities can be targeted for bioremediation purposes. The present study screened for bacteria with capabilities of utilizing diesel as sole source of carbon for growth. Eight isolates were derived from soils of four locations, among which three seemed to have prior exposure to diesel. The bacteria were individually cultured in a mineral salt broth devoid of carbon source except for diesel for 7 days at 35ºC. The growth of bacteria was observed from visible increase of turbidity and was enumerated by CFU/ml on mineral salt agar of same composition after 7 days of culture in the broth. The genera of the eight isolates were identified as *Nocardia*, *Corynebacterium*, *Bacillus*, *Pseudomonas* and *Arthrobacter* by morphological characterization and biochemical test results compared to standard references. Among the eight isolates, *Pseudomonas* sp. exhibited relatively higher ability to grow on diesel while *Bacillus* sp. and *Arthrobacter* sp. showed relatively less growth. *Nocardia* sp. and *Corynebacterium* sp. showed no tangible evidence of utilizing diesel as carbon source. This *Pseudomonas* sp. isolate was further used assess the effect of different concentration of diesel and pH values on growth. The result showed that optimal growth occurred at 3% (v/v) diesel concentration especially with agitation at 120 rpm and pH variation tests showed that near neutral pH values represent better growth conditions.

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LIST OF ABBREVIATIONS

Abbreviations	Descriptions
et al.,	and others
ºC	Degree Celsius
MS	Mineral Salts
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	microliter
mL	milliliter
g/L	grams per liter
v/v	volume by volume
CFU	colony forming unit
TNTC	too numerous to count

CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

Introduction

The use of petroleum based products has increased over the years in the 21st century, particularly for the purpose of transportation. This activity is essential in almost all the industries, especially in manufacturing and agriculture. Even without major oil spills, small amounts of hydrocarbons are being released into the nature during transport, distribution processes or other human activities (Kumar *et al.,*2011). Petroleum hydrocarbons come in different forms such as lighter refined oils such as gasoline, kerosene, diesel as well as heavy crude oils. These oils are complex mixtures of other organic compounds such as aromatic cyclic hydrocarbons, complex branched aliphatic and cyclic alkanes as well as other residual substances. Some of these are termed recalcitrant because they do no degrade easily in nature despite being organic. These man-made substances have become persistent pollutants (Roy *et al.*, 2014). Their presence in soil, water and air has an adverse effect on the wildlife, biogeochemical cycles, soil fertility and also the human population through direct or indirect exposure (Kumar *et al.*, 2011). Oil contaminants in the soil are toxic to the plant community due to their detrimental effects on seed germination and growth. The soil ecosystem is also threatened because oil particles clumping with soil particles reduce the availability of necessary factors such as aeration, water and mineral nutrients (Tang *et al.*, 2011). Over 4 million metric tons of refined petroleum products are being consumed yearly in Bangladesh according to the 2012 Statistical Yearbook of Bangladesh. The leaks and spills occurring on minor scale every day are unaccounted for although major spills are being reported in other parts of the world. The oil spills that occur in fewer amounts (< 7 tons) are difficult to report. During 2013 alone, the oil spilled in the environment added to over 7000 tons and majority of the volume is distributed among three major spills. (ITOPF, 2013) The clean up strategy for oil spills may include physical, chemical or thermal methods and these are invasive as well as expensive strategies. The invasion can damage the ecological balance of the soil or water body (Lundstedt *et al.*, 2003). Researchers are looking towards bioremediation as an environmentally friendly and cost-effective option for restoring contaminated sites. The search for microbial community

efficient in degradation of hydrocarbons had begun as early as 1900s (Bushnell and Haas, 1941) and encouraging results are being produced in the recent years (Nocentini*et al.*, 2000; Odokuma & Dickson, 2003; Lee *et al.*, 2006; Zhuang *et al.*, 2007)

1.1 Diesel

Diesel fuels are middle distillates of crude petroleum separated by fractional distillation. Other middle distillates include kerosene and aviation fuel. This refined oil is used mainly for road vehicles and other transportation modes such as ships, trains and airplanes. Many electricity generators also use diesel as fuel. At room temperature, diesel oil appears as brownish liquid which is flammable, slightly viscous and volatile with a distinct pungent odor similar to kerosene. The boiling point of this oil ranges between 140-385ºC depending on additives. Distributors may add different additives such as organic nitrates, amines, phenols and polymeric substances. The color of the liquid can also vary due these additions. Diesel has low solubility in water (0.5 mg per 100 ml). It has a specific gravity of 0.82 – 0.95 at 15 °C. The components of sample diesel fuels are studied by various fuel and energy companies and researchers using gas chromatography and mass spectrometry. Diesel mainly consists of hydrocarbons ranging from C_8-C_{22} along with varying concentrations of BTEX (benzene, toluene, ethyl-benzene and xylene) hydrocarbons. This includes branched alkanes (paraffins), cycloalkanes (naphthenes), polynuclear aromatic hydrocarbons (naphthalene) and alkenes (olefins) (IARC, 1990)

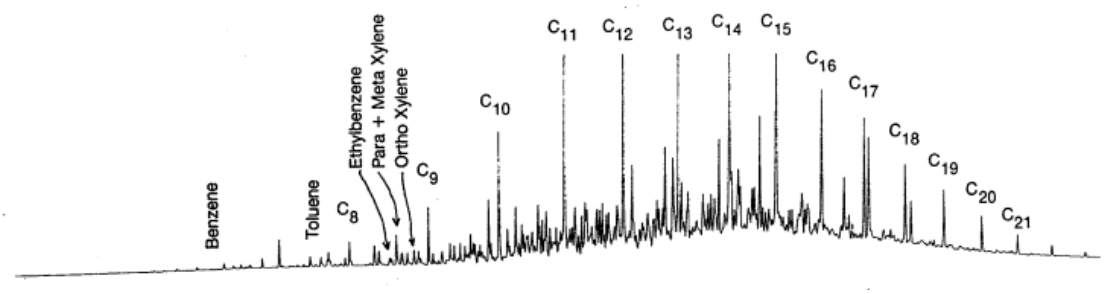

Figure 1.1: Gas Chromatogram of Diesel (Dunlap & Beckmann, 1988)

The hydrocarbons are present in oil in different molecular weights and boiling points, some of them are volatile while others are not. The level of toxicity and effects on biological systems are characterized for only some of the components in petroleum

products. The data on long term effects such as carcinogenic or adverse toxicity is limited. The method and time of exposure plays a sensitive role in affecting the health of human beings and wild life. Diseases, disorders and damage to organ systems such as respiratory, reproductive and circulatory systems have been associated with hydrocarbons from petroleum products (Costello, 1979). The direct exposure to diesel may occur during refueling automobiles, working at petroleum industry or servicing by mechanics and repairmen. Skin contact with diesel may result into dermatitis. Inhalation of diesel vapor can cause dizziness, nausea and headaches. Accidental ingestion of diesel can lead to choking or breathing troubles (UK Health Protection Agency, 2006).

1.2 Bioremediation strategies

The clean up or containment of oil spills by physical means involve skimming oil up from floating surface or absorbent barriers to prevent spreading. On land, the contaminated soil is physically separated. However, the drawbacks of these methods are that there is always residual oil, damage to the ecosystem and disposal of contaminated soil and water. Another method is in situ burning of oil, but it can be done only in low-wind weather and causes air pollution. There have been major improvements in remediation of oil spills since the 1989 Exxon Valdez oil spill in the United States because the clean-up processes used then had caused damage to the ecosystem (Oil Spills, 2011). The current remediation techniques include addition of chemical dispersants, biological agents such as microorganism cultures and nutrients for them. By definition, bioremediation is the use of biological agents such as microorganisms to detoxify or degrade harmful environmental pollutants into less toxic forms or reduce their concentrations below a standard limit established by regulatory authorities (Mueller *et al.,* 1996). It is seen as a cost effective and environmentally friendly alternative to physical and thermal clean up. It degrades the pollutants completely or transforms them into harmless substances using biological activity. As a natural process it is less invasive to the environment and can be engineered for on-site (*ex situ*) remediation as well as off site (Vidali, 2001).

However, as with any technology bioremediation has downsides. There are limitations to the range of contaminants that can be tackled and for implementation of a successful

bioremediation technique, considerable amount of research data and experience must be attained. Also, for the use of microbial culture, the contaminated site must support the growth as well as biological activity of the organisms.

Aside from simply adding microorganisms to a contaminated site, bioremediation strategies have developed into different technologies using biological agents. These can be classified into *in situ* or *ex situ* bioremediation. The different approached to bioremediation are briefly mentioned below (Adapted from Vidali, 2001).

In situ

- Biosparging: Injection of air under pressure to increase oxygen concentration in groundwater and enhance rate of biological degradation processes by microorganisms.
- Bioventing: Stimulating indigenous bacteria by supplying nutrients and air through wells to contaminated soil.
- Bioaugmentation: Addition of microorganisms (indigenous or exogenous) with suitable contaminant degrading abilities to the site.
- Biostimulation: Occur through the addition of fertilizers, oxygen, etc., that help encourage the growth of the pollution-eating microbes within the medium.

Ex situ

- Land farming: Periodic tilling of contaminated soil over a prepared bed to stimulate natural aerobic degradation by microbes.
- Composting: Mixing of polluted soil with manure or agricultural supplements that promote development of rich microbial community.
- Biopiles: Combination of land farming and composting in an aerated engineered cell that promotes controlled physical loss of contaminants by volatilization and leaching and also supports aerobic and anaerobic microorganisms.
- Bioreactors: Engineered containment systems that treat contaminated soil or water in slurry or aqueous phases such that microorganisms can perform degradation of pollutants at controlled and favorable conditions.

The outcome of bioreactor based bioremediation is much more predictable than *in situ*. On the other hand, being an *ex situ* method, the contaminated soil or water require excavation or pretreatment for separation of pollutants to place into the bioreactor.

1.3 Organisms important for petroleum product biodegradation

There have been many successful studies isolating and observing different potentials of microorganisms capable of degrading petroleum hydrocarbons. Due to complex nature of the compounds, a single organism is not capable of degrading all hydrocarbons. Table 1.1 represents a list of microorganisms able to biodegrade different petroleum hydrocarbons.

Table 1.1: Organisms reported for degradation of petroleum hydrocarbon

Hydrocarbon compound	Organism	Reference
Alkane	*Pseudomonas* sp.	Churchill *et al.*, 1995
	Bacillus sp.	Cybulski, *et al.*, 2003
	Micrococcus sp.	Das *et al.*, 1998
	Serratia marcescens	Ijah, 1998
	Alcaligene sodorans	Lal & Khanna, 1996
	Acinetobacter	Harayama *et al.*, 1997
	Brevibacillus sp.	Grishchenkov *et al.*, 2000
	Arthrobacter	Jogdand, 1995
Mono-aromatic hydrocarbon	*Pseudomonas*sp	Churchill *et al.*, 1995
	Alcaligene sodorans	Lal & Khanna, 1996
Poly-aromatic hydrocarbon	*Pseudomonas* sp.	Churchill *et al.*, 1995
	Mycobacterium	Churchill *et al.*, 1995
	*Corynebacterium*sp	Omotayo *et al.*, 2012
	Nocardia	Jogdand, 1995
	Flavobacterium	Jogdand, 1995
	*Rhodococcus*sp	Dean-Ross *et al.*, 2002

Most of these organisms are aerobic bacteria and require oxygen for the biodegradation of pollutants.

1.4 Factors affecting biodegradation

Environmental conditions for the growth of bacteria and rate of degradation is characterized by some physical factors (temperature, pH, nutrients, oxic or anoxic conditions). Concentration, composition and bioavailability of the hydrocarbons are important factors to be considered for choosing a bioremediation approach. The optimization of limiting factors can lead to more efficient clean-up technology (Norris, 1994).

Temperature

The degradation of hydrocarbons has been reported over a variety of temperature, but extreme temperature ranges (psychrotrophic and thermophilic) have been found to be a limiting factor. Elevation in temperatures decreases the viscosity of hydrocarbons, which results into greater rates of diffusion. This allows the petroleum pollutants to be more accessible for the microbes (Atlas, 1981). For soil environments, the optimum rate of biodegradation is found within 30 -40ºC, although the range is lower (15-30 ºC) for aquatic ecosystems (Bartha & Bossert, 1984).

Nutrients

In the natural environment, available concentrations of nitrogen (N) and phosphorus (P) get depleted through continuous biodegradation by microorganisms. To remedy this, addition of fertilizers or organic supplements (such as poultry manure) have reported enhanced conditions for hydrocarbon degradation. Though essential for microbial growth, high concentrations of nitrogen and phosphorus limit the degradation rates (Dibble & Bartha, 1979). In low iron environments, iron supplements could have stimulating effects (Dibble & Bartha, 1976).

Oxygen

Most of the microbial hydrocarbon biodegradation are aerobic pathways involving molecular oxygen and cellular oxygenases. Anaerobic degradations, though reported, are much less explored. The slower rates of anaerobic pathways are undesirable for remediation approaches (Vidali, 2001).

pH

Bacteria can survive at different ranges of pH values but the degradation of hydrocarbons tends to favor near neutral pH of 6.5-8 (Norris, 1994). Although, biodegradation have been reported in acidic forest soils (pH value of 4 to 6), the degradability declines as the pH shifts farther from neutral (Amadi *et al.,* 1996).

1.5 Mechanism of hydrocarbon degradation

The mechanisms that mediate biodegradation of hydrocarbons involve attachment of substrates to the microbial cells and their uptake inside the cell where specific enzyme systems process them. Some microbes produce biosurfactants to enhance bioavailability of the hydrophobic compounds (Fritsche & Hofrichter, 2000).

1.5.1 Degradation pathway for aliphatic compounds

Aliphatic compounds of petroleum products include short and long chains of alkanes (n-alkanes). These substrates are oxidized by substrate-specific terminal or sub-terminal monooxygenases into corresponding alcohol. Subsequent reactions result into formation of aldehydes first and then fatty acids. The carboxylic acids are joined with CoA which forms acetyl CoA or propionyl-CoA by β-oxidation. These metabolites can enter central metabolic pathways like Krebs cycle (Wentzel *et al.*, 2007). The short chained alkane (C_2-C_{10}) degradation can be initiated by non-hememonooxygenases, copper containing monooxygenases and also Cytochrome P450 type heme-iron monooxygenases. The longer alkane chains (C_n>C_{10}) are also activated by flavin-binding monooxygenases aside from the aforementioned enzymes (Sierra-Garcia & Oliveira, 2013).

1.5.2 Degradation pathway for aromatic compounds

Aromatic compounds are much complicated and are processed by different peripheral pathways to produce a few common intermediates. This involves oxygenation reactions by monooxygenases or hydroxylation by dioxygenases that gives intermediates like catechol or protocatechuate. These ringed compounds are cleaved by dioxygenases to give chained metabolites that can eventually form CoA derivatives with the help of CoA ligases. The CoA derivatives can assimilate into the central metabolism of cell

(Harayama&Timmis, 1992). The initiating enzymes involved in aromatic compound degradation include the following (Sierra-Garcia & Oliveira, 2013).

- Rieske non-heme iron oxygenases
- Flavoproteinmonooxygenases
- Soluble diiron multicomponent monooxygenase

Catabolic genes required for mineralization of compounds containing one or more benzene ring have been described within the TOL plasmid of *Pseudomonas putida*. The presence of genetic information for degradation abilities in plasmids can prove to be an efficient system for the spread of such capabilities (Abril *et al*., 1989)

1.6 Recent bioremediation agents

Bioremediation agents can mean microbial cultures (bioaugmentaion), enzyme or nutrient additives (biostimulation). The United States Environmental Protection Agency (EPA) is required to update the latest and most effective bioremediation agents on to the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) product schedule based on field trials and applications. Table 1.2 shows some bioremediation agents that showed high efficiency in regulatory tests by the EPA (Stout & Rubini, 2014).

Table 1.2: Bioremediation agents enlisted in NCP 2014.

Bioremediation Agent	Product type	Company
ACT TERRA FIRMA	Microbial culture	Franssen Enterprises, Inc.
BIOREM-2000 OIL DIGESTER	Microbial culture	Cliff Industries
PRO ACT (OIL CLEAN w/Activator)	Microbial culture w/ nutrient additive	Pro-Act Microbial, Inc
SHAMANTRA GREEN	Nutrient additive	Molecular Mediation LLC
WHITZORB	Microbial culture	LBI Renewable

1.7 Objectives

The **2014 Sundarbans oil spill** was an oil spill that occurred on 9th December, 2014 at the Shela River in Sundarbans, Bangladesh, a UNESCO World Heritage site. The spill resulted from a collision between an oil-tanker named *Southern Star VII*, carrying 350,000 litres of furnace oil, and a cargo vessel. The tanker sank in the river spilling the oil which had spread over a 350 km^2 area by 17th December. The oil spread to a second river and a network of canals in Sundarbans, which blackened the shoreline endangering the trees, plankton, and vast populations of small fish and dolphins. The spill occurred at a protected mangrove area, home to rare wildlife like horseshoe crabs, northern river terrapin turtles and the Irrawaddy and Ganges dolphins. 70,000 litres of oil had been cleaned up by local residents, the Bangladesh Navy, and the government of Bangladesh by January (Kibria, 2015). The importance of research on bioremediation becomes obvious in the light of this type of catastrophe.

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 diesel and their optimum conditions for biodegradation can be a useful approach towards better soil quality and also remediation technology for major spills. The main objectives of this study are as follows.

- To isolate and identify potential microorganisms with the ability to utilize diesel or its components as source of energy from local soil samples.
- To determine optimum concentration of diesel for supporting growth of selected microorganism as well as tolerance levels in diesel.
- To observe the effect of non-agitation condition on the growth of selected microbial isolate.
- To determine the optimum pH for degradation of diesel by the selected microbe.

CHAPTER 2:

MATERIALS AND METHODS

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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 isolated in this study was soil samples of topsoil (0-15 cm depth) collected from an oil contaminated area which is a parking station for vehicles (cranes, bulldozer and lorries) and petroleum filling stations. The locations from where the soils were collected seemed to have prior exposure to diesel oil. These soils were stored in sterile plastic bags.

Table 2.1: Locations of soil sample and designation of isolates.

Soil Sample	Location	Isolate number	Designation of isolates
A	Agricultural field, Barisal,	2	A1, A2
B	Petrol filling station, Bogura	2	B1, B2
C	Construction locomotive storage and servicing site, Moghbazar, Dhaka	2	C1, C2
D	Petrol filling station, Barisal	2	D1, D2

2.2.2 Hydrocarbon (Diesel)

The hydrocarbon used in this study i.e. diesel was collected from a local filling station. The oil was syringe filtered under a biosafety cabinet, stored in sterile test tubes and sealed properly with parafilm strips. Sterility of the oil was determined before each experiment by spread plating an amount of 100 µl diesel on Nutrient agar. The experiment was carried out with the oil only if no growth was observed after 24 hours. No seals of tubes were broken outside of the biosafety cabinet.

2.2.3 Isolate selection from soil sample

Isolates of microorganisms were grown using serial dilution and spread plate technique on Nutrient agar. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} serial dilutions were made from a soil suspension containing 9ml 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 four soil samples, two colonies were selected from each soil sample. Most of the colonies from the spread plate method showed recurring morphological characteristics. The predominant colonies were selected having different 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.

2.3 Degradation studies of Diesel

2.3.1 Inoculum preparation

The suspension of inoculum was prepared by mixing a loopful of pure culture of bacteria in 0.85% NaCl (physiological saline). This solution was mixed by a vortex machine for 5-10 seconds to gain a homogenous mixture. 1 ml of this suspension was used for a 50 ml volume of culture broth. Lesser amount was added in the case of the experiment carried out in test tubes.

2.3.2 Culture medium preparation and screening for diesel 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^oC for 15 minutes. The degradation studies with this broth (mineral salts broth) were carried out by inoculation of an isolate, addition of diesel with its concentrations in the form of v/v % and incubation at 35^oC for 7 days with 120 rpm agitation.

2.3.3 Variations of parameters

i) Inoculum

Eight isolates of bacteria selected from the soil samples were each tested for diesel degradation in a culture containing MS broth and diesel as sole source of carbon. The isolate with highest degradation capabilities was selected for studying optimum conditions in varying parameters.

ii) Concentration

Different concentrations of the diesel oil (v/v %) were used to find the optimum concentration of diesel for the growth of the selected microbe. The concentrations studied were 0.5 %, 1 %, 2 %, 3 %, 4 %, 5 % and 6 %.

iii) Non-agitation and concentration

The importance of agitation was also determined by comparing cultures placed in 120 rpm shaker and cultures incubated without agitation.

iv) pH

The optimum pH for degradation was determined by culturing bacteria in mineral salts broth with initial pH 2, 4, 6, 7, 8 and 10.

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 the mineral salts agar with the same composition as the aforementioned culture broth with 12 g/L agar-agar. Serial dilutions were made from the culture broth with physiological saline for plating on mineral salt agar plates spread with 50µl diesel. These plates were incubated without agitation at 37ºC for approximately 7 days and then colony number was counted.

2.4 Identification of bacterial isolates

The bacteria screened for diesel 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 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 30 seconds 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 Carbol fuchsin 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 Carbol fuchsin. Acid fast negative bacteria would be stained blue.

2.4.2 Biochemical Tests

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.

i) 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 color in the media to pink from orange due to the production of ammonia after incubation.

ii) 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.

iii) 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 is a positive result. Delayed reactions are ignored and concluded as negative.

iv) Citrate 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 color was observed after an incubation period of 24 hours at 37ºC.
- In a positive reaction, the color of the medium turned blue. No change in color indicated negative result for citrate utilization.

v) Starch Hydrolysis Test

- Bacteria from a pure culture was streaked on a starch agar plate along a straight line and kept for incubation for 24 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.

vi) 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 color 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 color change was observed after addition of reagents A and B. The appearance of red color after this step confirmed a negative result for nitrate reduction. Colorless solution after zinc powder addition indicated presence of both nitrate reductase and nitrite reductase.

vii) 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 discoloration or clearing of medium indicated gamma- hemolytic bacteria (negative for hemolysis).

viii) Salt Tolerance test

- Single colony of bacteria were inoculated into 6.5% NaCl broth and incubated at 37ºC for 3 days and checked for change at 24 hours intervals.

- Turbidity in the broth indicated growth of salt tolerant bacteria. No change in broth observed over the incubation period indicated negative result.

ix) 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 color 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 color on the reagent layer signified positive reaction.

x) Fermentation Tests: Lactose, dextrose, sucrose, arabinose, 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 color of broth to yellow indicated acid formation from fermentation with or without gas production.

- **No color 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.

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 6 hours for the bacteria to reach log phase and then 200µl sterile glycerol was added over the inoculated and incubated agar. These vials were stored at room temperature.

CHAPTER 3:

RESULTS

CHAPTER 3: RESULT

Eight microorganisms were isolated from four soil samples to test for diesel degradation capability. The ability to utilize diesel as carbon source was determined by observing growth in mineral salt media with only diesel as carbon source. The growth was visibly qualified by increased turbidity and quantitatively studied by measuring colony forming units on mineral salt media plates. The most successful isolate was selected for studying effects of different parameters such as pH, concentration of oil and presence of agitation. CFU/ml was measuring using the formula

$$\text{CFU/ml} = \frac{number\ \ of\ colonies\ \times dilution\ \ factor}{volu\,me\ of\ culture\ \ plate}$$

For ease of graphical presentation, the value of CFU/ml was converted to log value.

The eight isolates were designated as A1, A2, B1, B2, C1, C2, D1 and D2 throughout this study and later identified as *Nocardia* sp., *Corynebacterium* sp., *Bacillus* sp., *Pseudomonas* sp. and *Arthobacter* sp.

3.1 Diesel degradation ability of different isolates

The eight isolates were individually cultured in 50 ml mineral salt broth (pH 7) for up to 7 days at 35ºC in a shaking incubator at 120 rpm with 1% (v/v) diesel as sole carbon source. The bacteria showed varying degree of growth. The 7 day old cultures were serially diluted and plated on mineral salt agar media. CFU/ml of each isolate was calculated from plates with viable counts.

Table 3.1: Colony counts of different isolates after its culture in mineral salt broth containing only diesel as carbon source.

Isolate	Bacteria	CFU at 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.	32	0	0	0	0	0	3.2×10^3	3.51
B2	*Bacillus* sp.	TNTC	TNTC	326	52	7	0	5.2×10^6	6.72
C1	*Pseudomonas* sp.	TNTC	TNTC	TNTC	TNTC	237	14	2.3×10^8	8.37
C2	*Bacillus* sp.	TNTC	TNTC	130	12	3	0	1.3×10^6	6.11
D1	*Arthrobacter* sp.	TNTC	35	4	0	0	0	3.5×10^4	4.54
D2	*Arthrobacter* sp.	TNTC	41	6	0	0	0	4.1×10^4	4.61

*TNTC = Too numerous to count

From the eight isolates, *Pseudomonas* sp. (C1) showed the highest growth of 8.37 $\log_{10}$ CFU/ml, while (B1) and (C2) belonging to *Bacillus* genera showed the next highest growth in diesel with bacterial count of 6.72 $\log_{10}$ CFU/ml and 6.11 $\log_{10}$ CFU/ml respectively. *Nocardia* sp. (A1) and *Corynebacterium* sp. (A2) did not show any growth in the mineral salt agar plates.

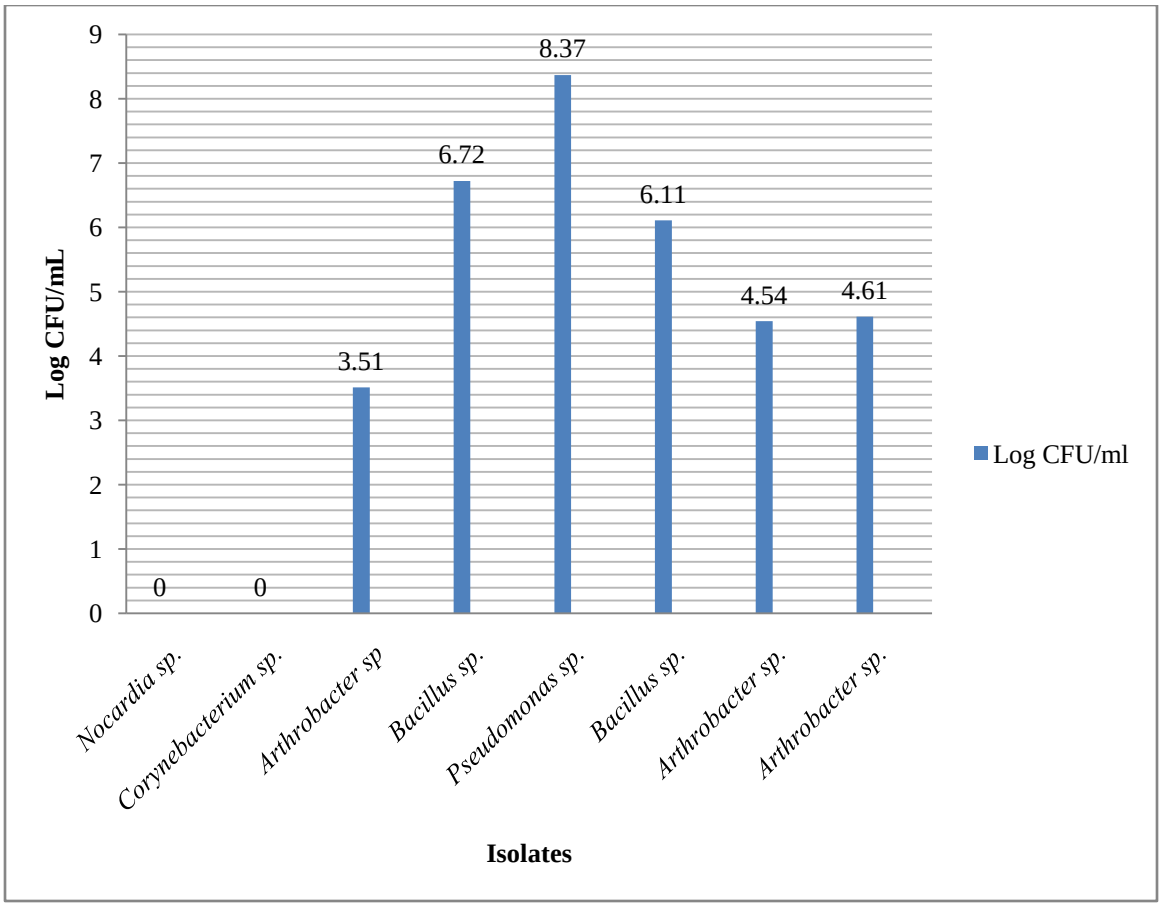

Figure 3.1: Log CFU/ml representing growth of different isolates after its culture in mineral salt broth containing only diesel as carbon source

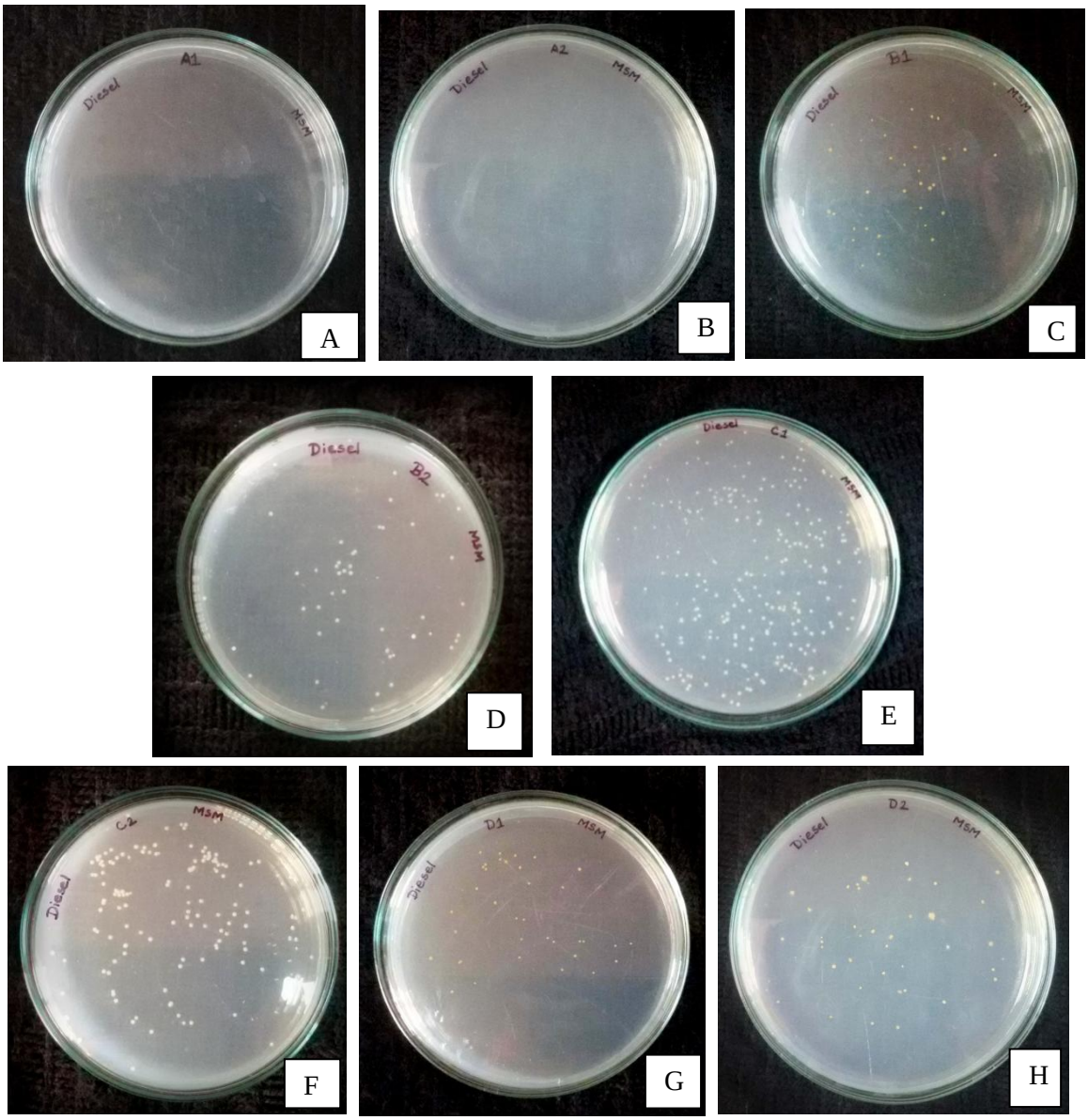

Figure 3.2: Viable counts of different isolates grown in mineral salt agar with diesel as carbon source. Plates **A** and **B** show no growth of bacterial isolates A1 and A2 respectively. Plates **D**, **E** and **F** show high counts of bacteria of isolates B2, C1 and C2 respectively whereas plates **C**, **G** and **H** show lower counts of isolates B1, D1 and D2.

3.2 Variation in concentration of diesel (with and without agitation)

The effect of varying concentrations of diesel was tested to find the optimum concentration for the isolate C1 (*Pseudomonas* sp.), which was selected based on the previous experiment. The bacteria were cultured in six flasks (50 ml) in mineral salt media (pH 7.0) containing different concentrations ranging from 0.5% - 6% (v/v). The flasks were incubated for 7 days at 35ºC in a shaking incubator with agitation of 120 rpm. Another set of this experiment was carried out in an incubator with no agitation. The 7 days old culture were serially diluted and plated on mineral salt agar media. The viable counts (30-300) were used to determine CFU/ml.

Table 3.2: Colony counts of *Pseudomonas* sp. after its culture in mineral salts broth containing varying concentrations of diesel with agitation (120 rpm)

Concentration of diesel (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	382	35	6	0	3.82×10^{6}	6.58
1%	TNTC	TNTC	TNTC	TNTC	171	4	1.71×10^{8}	8.23
2%	TNTC	TNTC	TNTC	TNTC	TNTC	139	1.39×10^{9}	9.14
3%	TNTC	TNTC	TNTC	TNTC	TNTC	276	2.76×10^{9}	9.44
4%	TNTC	TNTC	TNTC	TNTC	TNTC	225	2.25×10^{9}	9.35
5%	TNTC	TNTC	TNTC	TNTC	111	20	1.11×10^{8}	8.05
6%	TNTC	395	38	4	0	0	3.8×10^{5}	5.58

*TNTC = Too numerous to count

The isolate C1 (*Pseudomonas* sp.) showed ability to grow in diesel of varying concentration from as low as 0.5 % to 6 % (v/v). The best growth was observed at 3% and bacterial count was 9.44 $\log_{10}$ CFU/ml. Visual increases in turbidity corroborated with these results. A decline in growth and turbidity was seen from 4% to 6% (v/v).

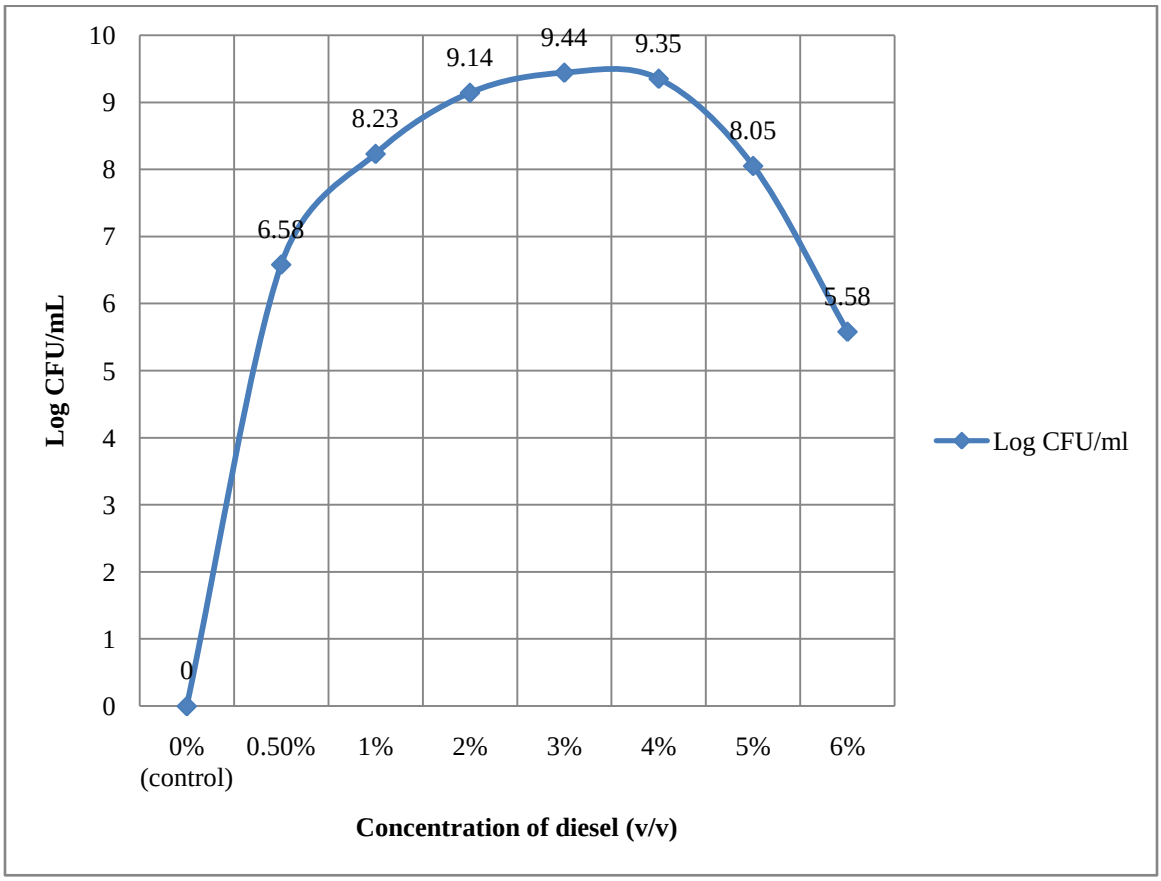

Figure 3.3: Effect of varying concentrations of diesel on the growth of isolate C1 after 7 days incubation at 35°C, with agitation (120 rpm)

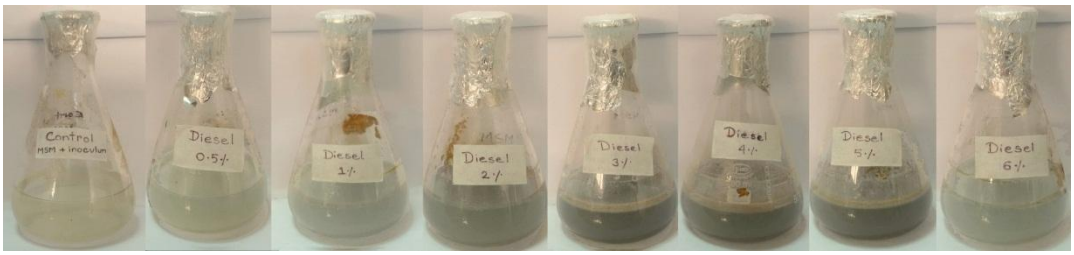

Figure 3.4: Growth of isolate C1 in mineral salt broth with different concentrations of diesel. The change in turbidity observed in mineral salt broth inoculated with isolate C1 after 7 days of culture at 35°C. Compared to the control, visible increase of turbidity was observed.

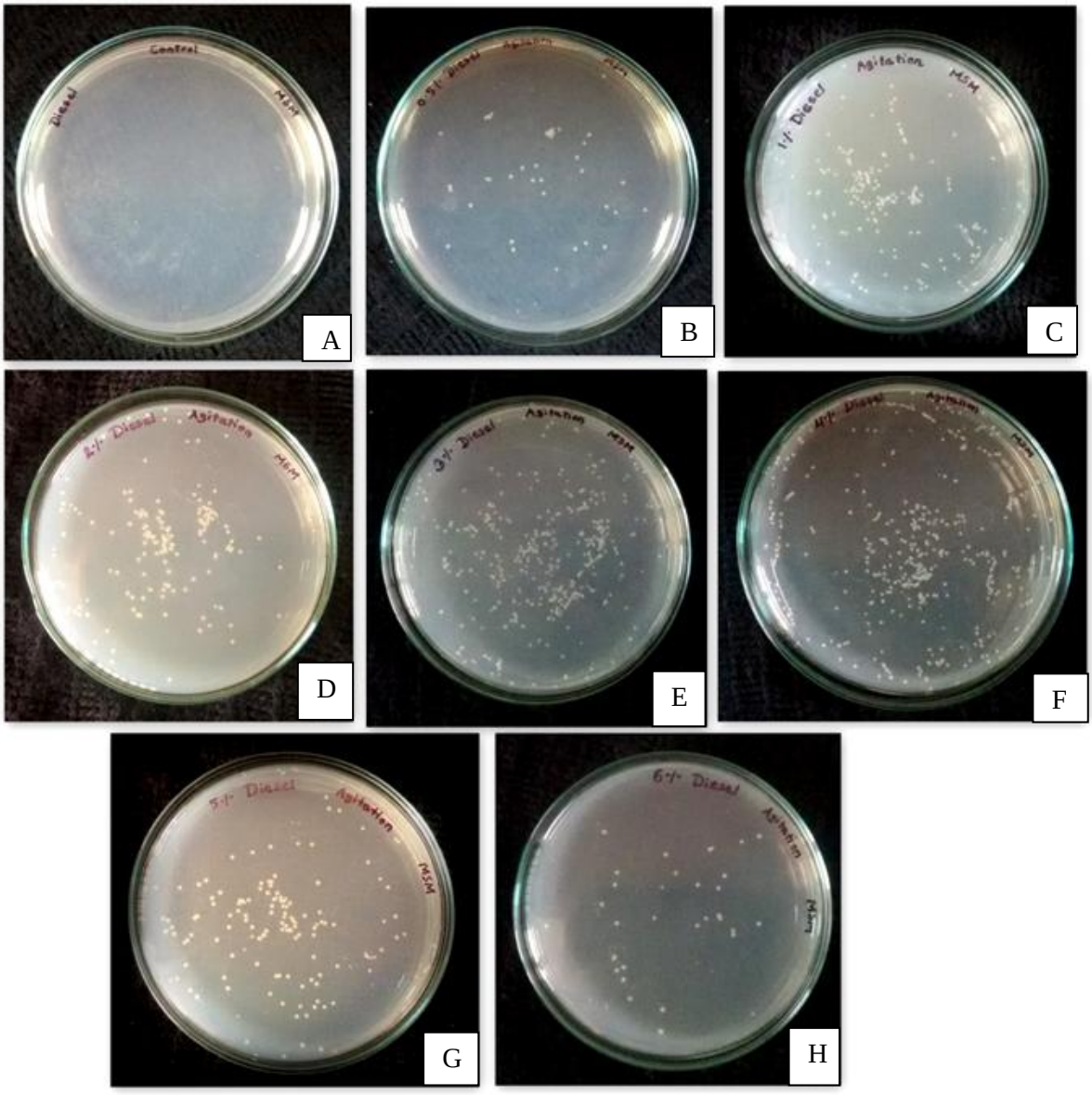

Figure 3.5: Viable counts of isolate C1 on mineral salt agar from various concentrations of diesel (0.5% - 6%) and control plate. **A.** Control culture of mineral salts broth and inoculum plated on mineral salts agar plate. **B-H.** Mineral salts agar plates showing viable counts of bacteria from which CFU/ml were calculated.

Table 3.3: Colony counts of *Pseudomonas* sp. after its culture in mineral salts broth containing varying concentrations of diesel without agitation

Concentration of diesel (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	377	29	3	0	0	2.9×10^5	5.46
1%	TNTC	TNTC	396	38	5	0	3.8×10^6	6.58
2%	TNTC	TNTC	TNTC	368	52	0	5.2×10^7	7.72
3%	TNTC	TNTC	TNTC	TNTC	212	26	2.12×10^8	8.33
4%	TNTC	TNTC	TNTC	TNTC	171	14	1.71×10^8	8.23
5%	TNTC	TNTC	TNTC	110	23	2	1.10×10^7	7.05
6%	387	36	3	0	0	0	3.6×10^4	4.56

*TNTC = Too numerous to count

It can be observed that the lack of agitation had lowered the growth of C1 relative to the observation with agitation. The maximum growth still occurred at 3 % (v/v) diesel with a bacterial count of 8.33 $\log_{10}$ CFU/ ml and declining growth at higher concentration was observed.

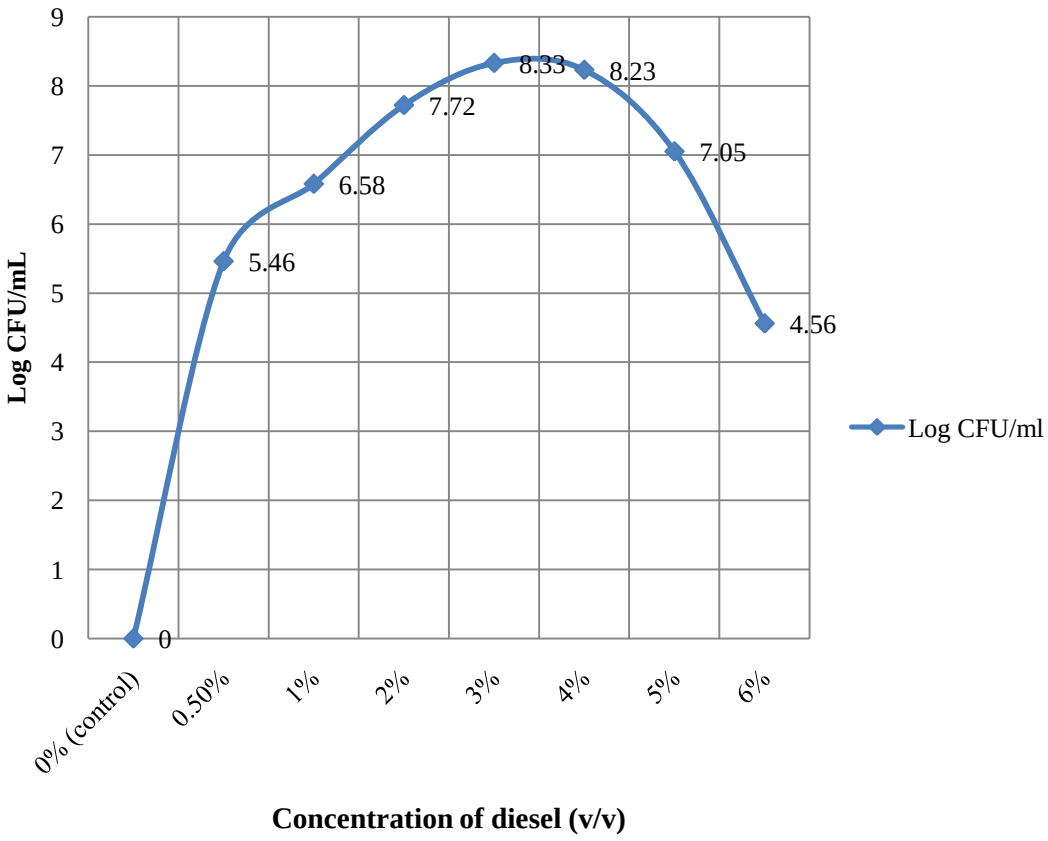

Figure 3.6: Effect of varying concentrations of diesel on the growth of the isolate C1 after 7 days incubation at 35ºC, without agitation.

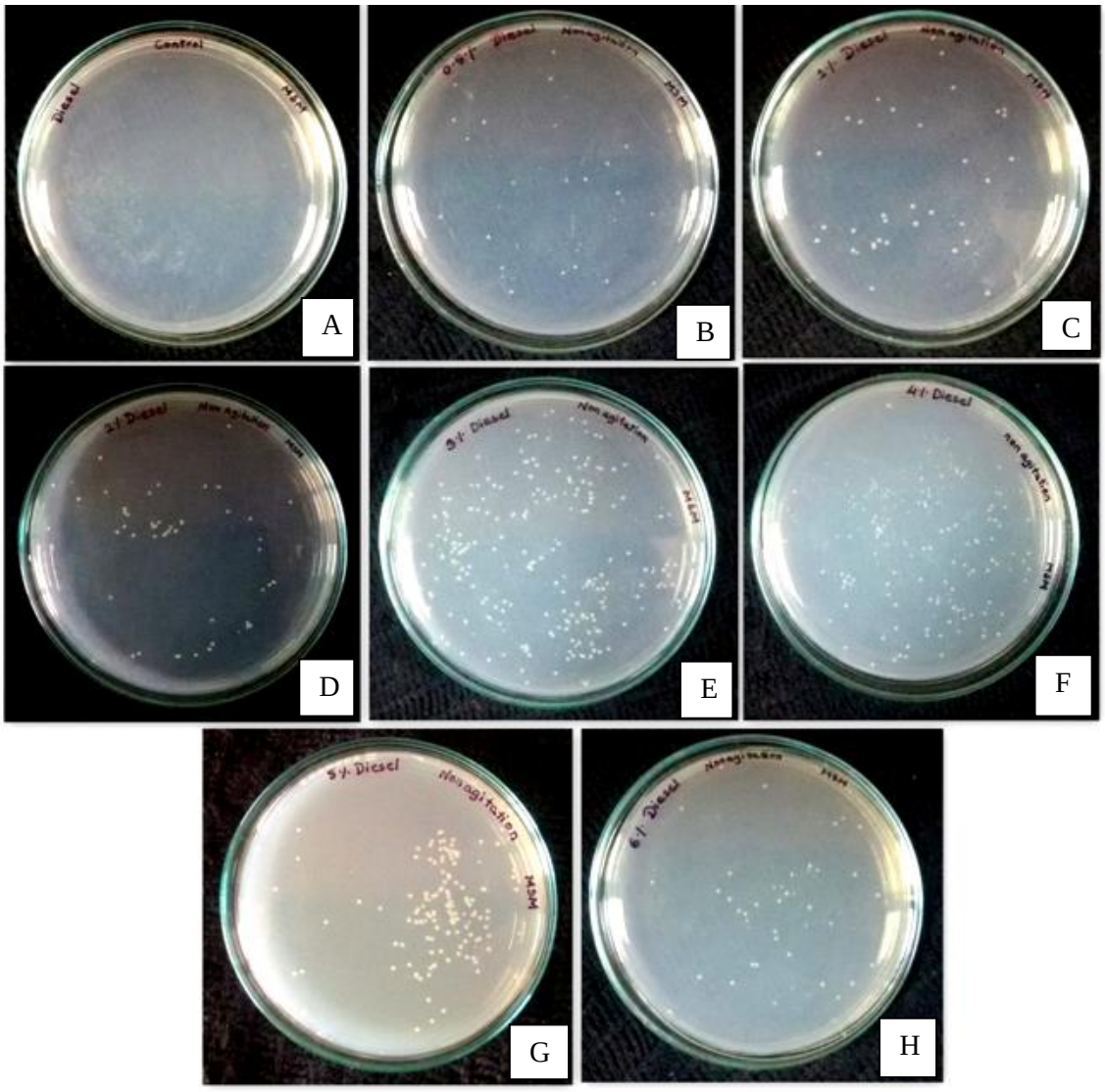

Figure 3.7: Viable counts of isolate C1 on mineral salts agar plate from 7 days culture of mineral salts broth with diesel as sole carbon source in varying concentrations and no agitation. **A.** Control plate. **B-H.** Viable counts of isolate C1 from which CFU/ml was measured.

3.3 Effect of pH

The effect of initial pH on the growth of the isolate C1 was studied in mineral salt broth with 1% (v/v) diesel as carbon source. The flasks were incubated for 7 days in a shaking incubator at 120 rpm and 35ºC. The pH variations used were pH values of 2, 4, 6, 7, 8 and 10. The cultures on day 7 were serially diluted and plated for CFU/ml measurement.

Table 3.4: Colony counts of *Pseudomonas* sp. after its culture in mineral salt broth containing varying pH values

pH	CFU at different dilutions						CFU/ml	Log CFU/ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
pH 2	48	3	0	0	0	0	4800	3.68
pH 4	286	30	2	0	0	0	2.8×10^4	4.45
pH 6	TNTC	178	12	0	0	0	1.78×10^5	5.25
pH 7	TNTC	TNTC	TNTC	TNTC	242	25	2.42×10^8	8.38
pH 8	TNTC	TNTC	TNTC	180	14	0	1.8×10^7	7.26
pH 10	31	3	0	0	0	0	3100	3.49

*TNTC = Too numerous to count

With varying initial pH, different degrees of growth were observed. The best growths were observed at neutral pH 7 with bacterial count of 8.28 $\log_{10}$ CFU/ml and pH 8 with a bacterial count of 7.26 $\log_{10}$ CFU/ml. A sharp decrease in growth was found at pH 10 (3.49 $\log_{10}$ CFU/ml) from pH 8. Growth at pH 2 and pH 4 indicated tolerance of acidic conditions by the isolate C1, *Pseudomonas* sp.

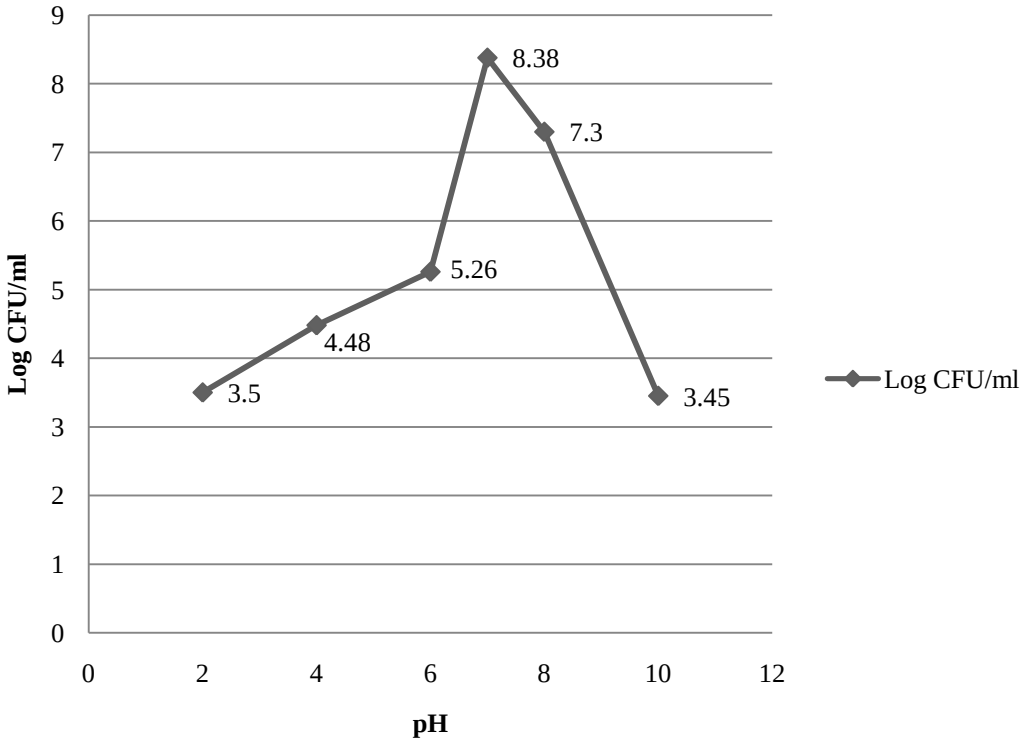

Figure 3.8: Effect of pH on utilization of diesel by isolate C1 after 7 days incubation at 35ºC, 120 rpm

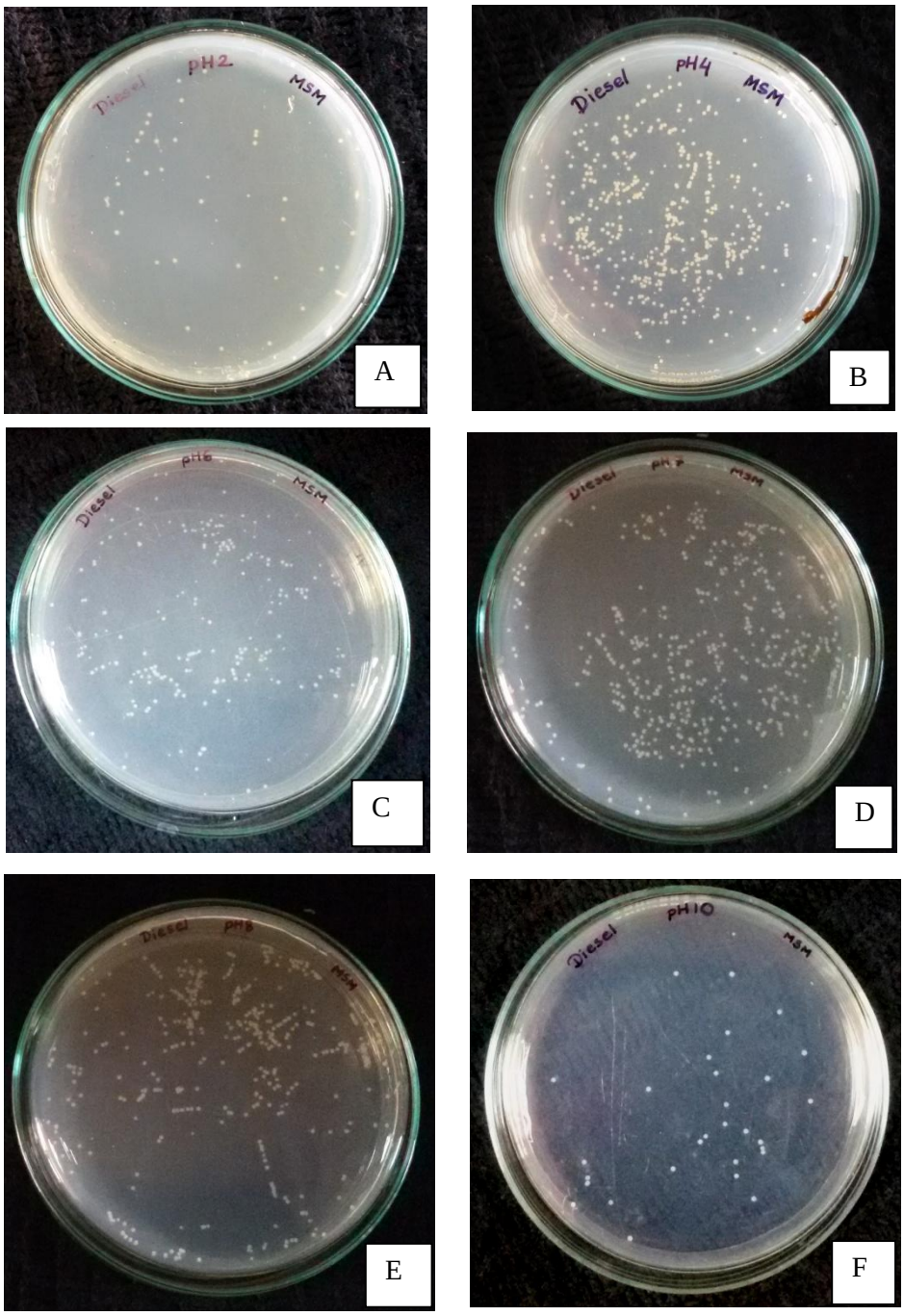

Figure 3.9: Mineral salts agar showing viable counts of isolate C1 from mineral salt broths of varying pH (pH 2, 4, 6, 7, 8 & 10). **A – F.** CFU counted from these plateswere used to measure CFU/ml. **D**. The highest bacterial count was measure from the growth in pH 7.

3.4 Identification 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 Microbiology Laboratory Manual (Cappuccino & Sherman, 2005) and with the help of online laboratory tool for bacterial identification, Advanced Bacterial Identification Software (ABIS)

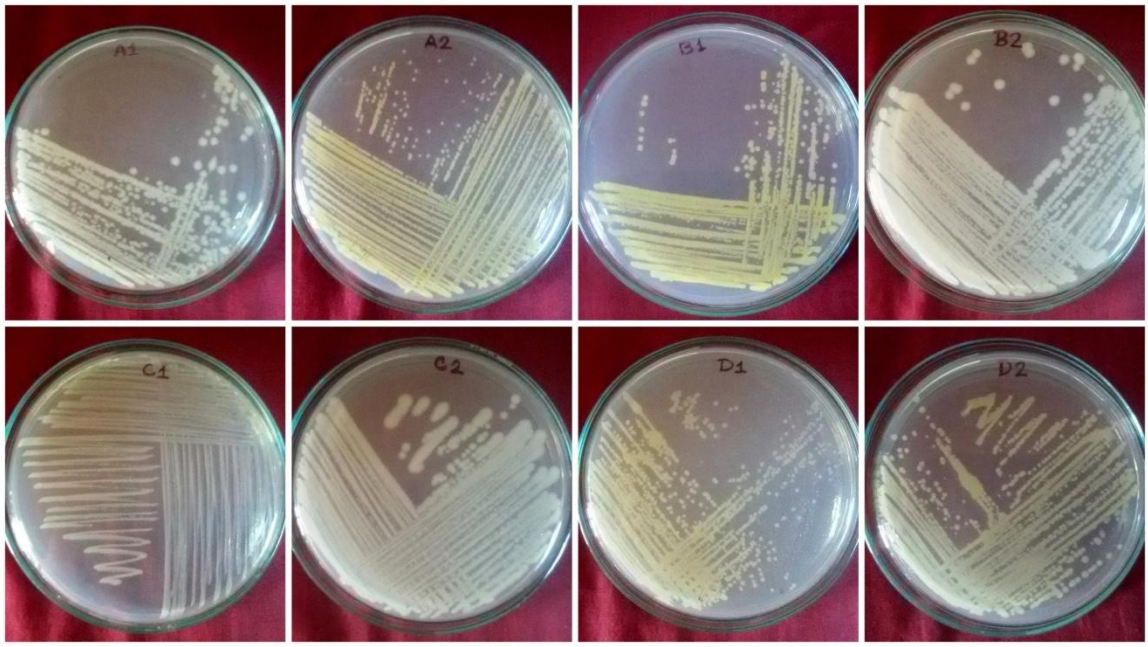

Figure 3.10: Eight isolates streaked four-way on Nutrient Agar.

Table 3.5: Colony morphology of eight isolates in Nutrient agar

Isolate designation	Shape	Margin	Elevation	Size	Texture	Pigmentation	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 3.6: Gram's staining, spore staining and acid fast staining results of eight isolates

Isolate 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 3.7: Biochemical test results of eight isolates and their identification through the use of ABIS online

Isolate designation	Growth at 6.5% NaCl	Catalase	Oxidase	Motility	Indole produc-tion	Urease	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 3.7: Biochemical test results of eight isolates and their identification through the use of ABIS online (continued)

Isolate designation	Methyl-red test (MR)	Voges - Proskauer test (VP)	Fermentation tests					Presumptive organism
			Dextrose	Lactose	Sucrose	Arabinose	Mannitol	
A1	-ve	-ve	+ve	-ve	-ve	-ve	-ve	*Nocardia* sp.
A2	-ve	-ve	+ve	-ve	+ve	+ve, NG	-ve	*Corynebacterium* sp.
B1	-ve	-ve	+ve	-ve	+ve	+ve, NG	-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, NG	-ve	*Bacillus* sp.
D1	-ve	-ve	+ve	-ve	+ve	-ve	+ve	*Arthrobacter* sp.
D2	-ve	-ve	+ve	-ve	+ve	-ve	+ve	*Arthrobacter* sp.

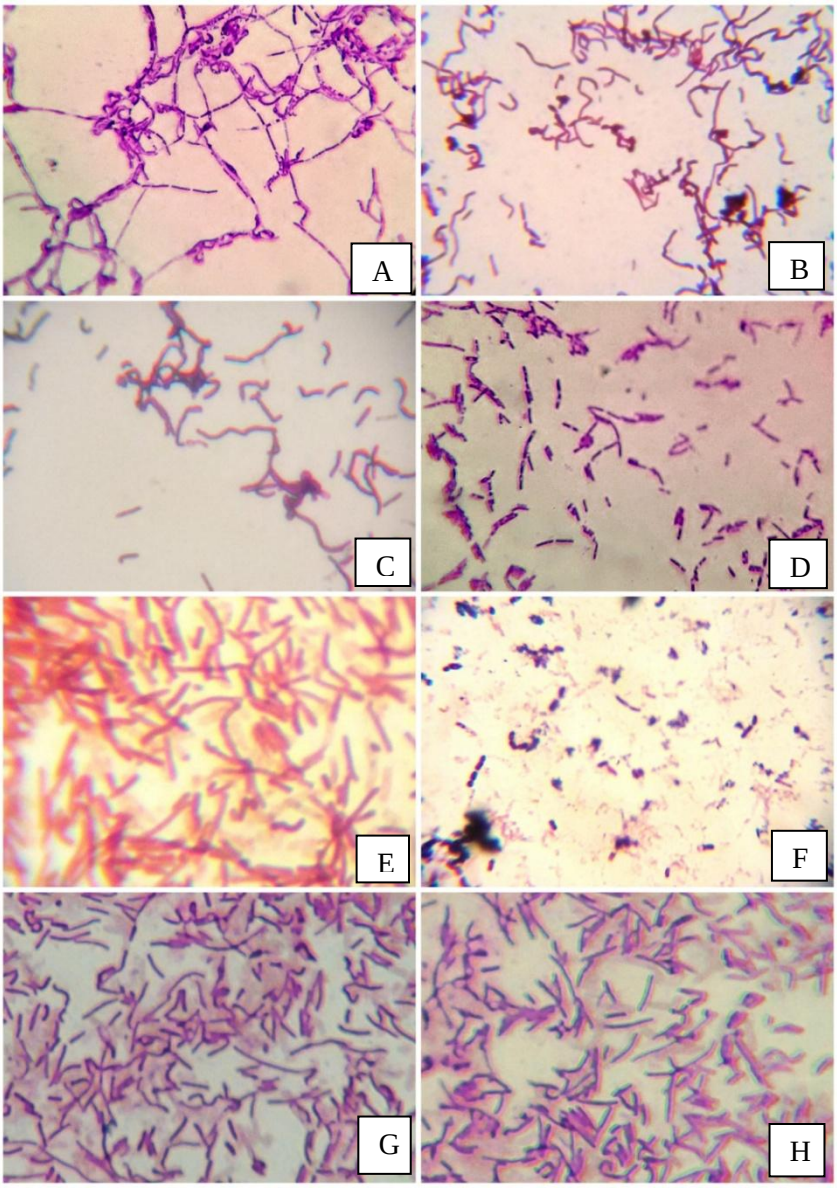

Figure 3.11: Microscopic observations of the isolates after grams staining. **A.** Isolate A1 observed as gram positive bacteria with branching filaments. **B-D & F-H.** Gram positive isolates A2, B1, B2, C2, D1 and D2 respectively. **E.** Isolate C1 stained pink representing gram negative bacteria.

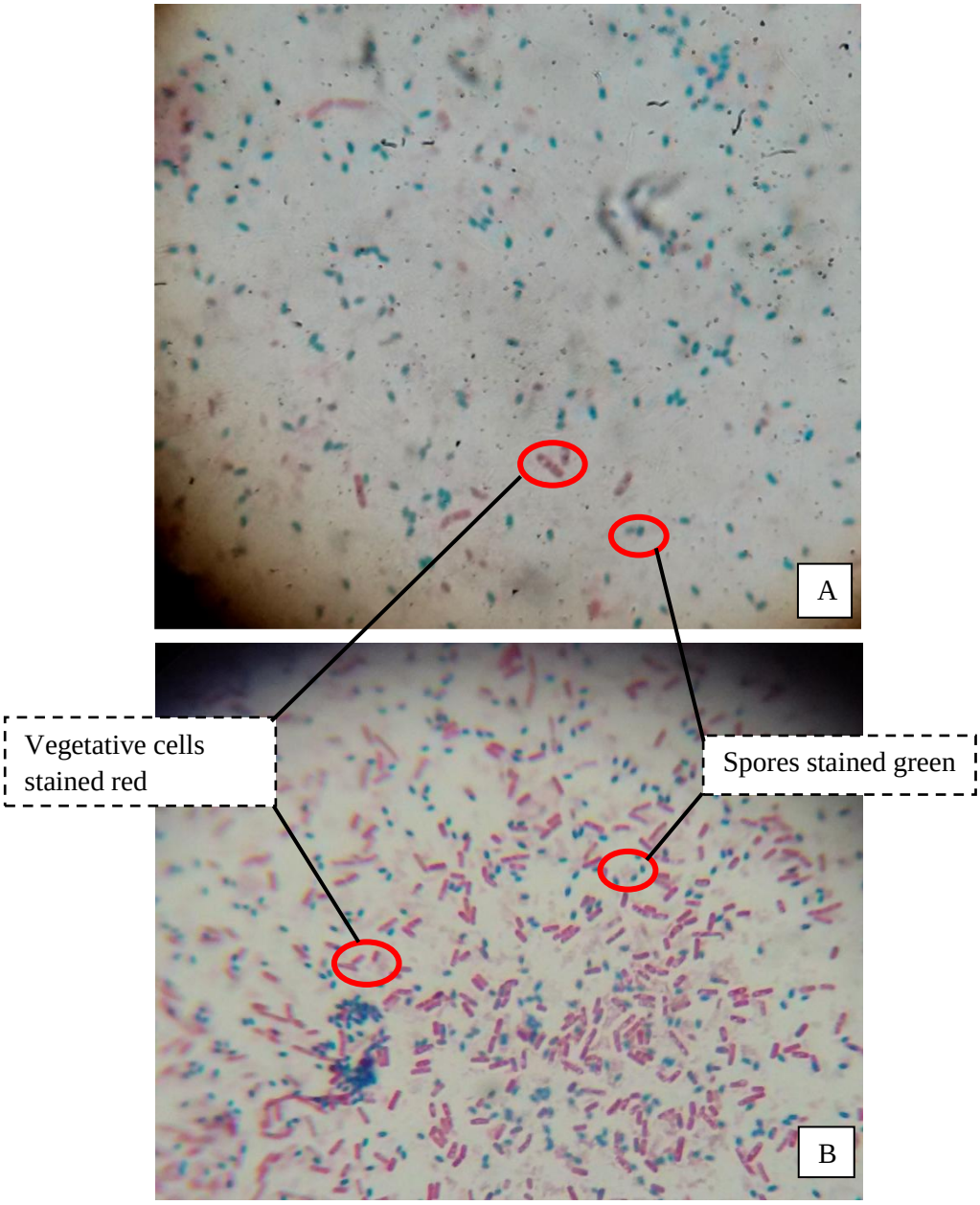

Figure 3.12: Microscopic observations from endospore staining of isolates. **A**. Isolate B2. **B.** Isolate C2

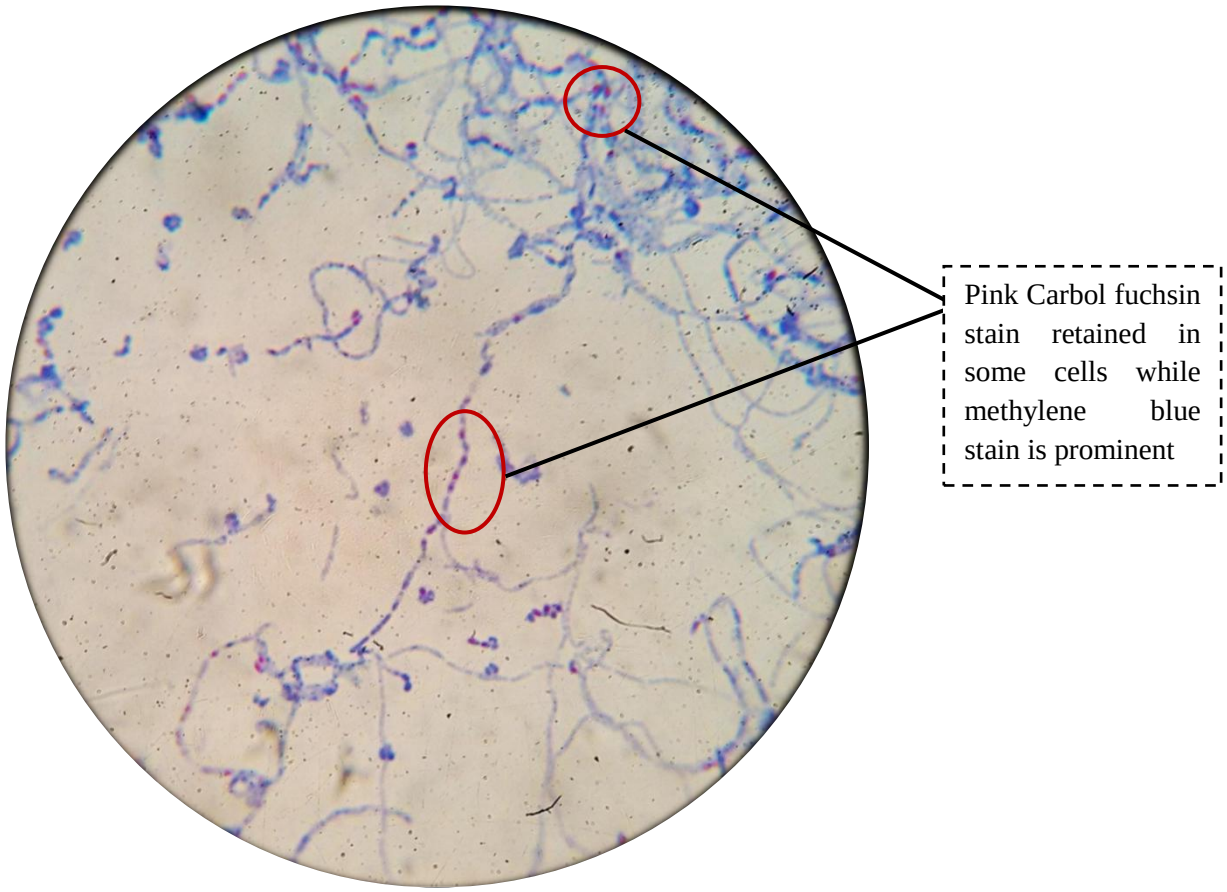

Figure 3.13: Microscopic observation of isolate A1 after acid fast staining showing a partial positive result.

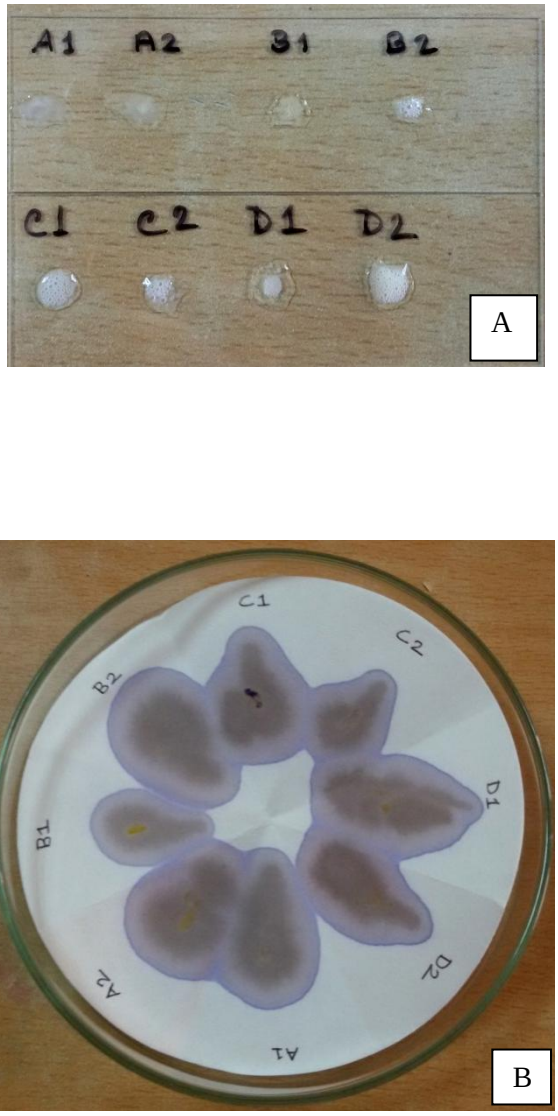

Figure 3.14: Catalase and oxidase test. **A.** Catalase test results of eight isolates, all showing positive indicated by bubble formation. **B**. Oxidase test results of eight isolates among which only C1 showed positive result.

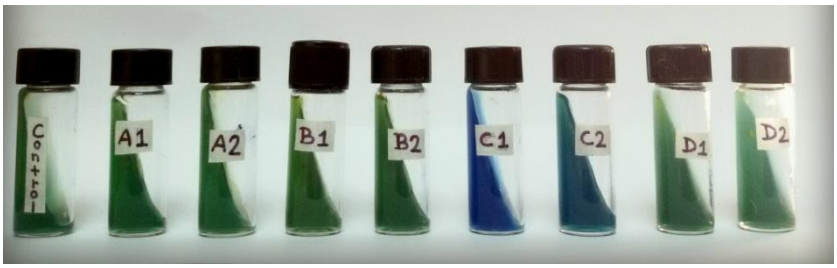

Figure 3.15: Biochemical test result for citrate utilization.

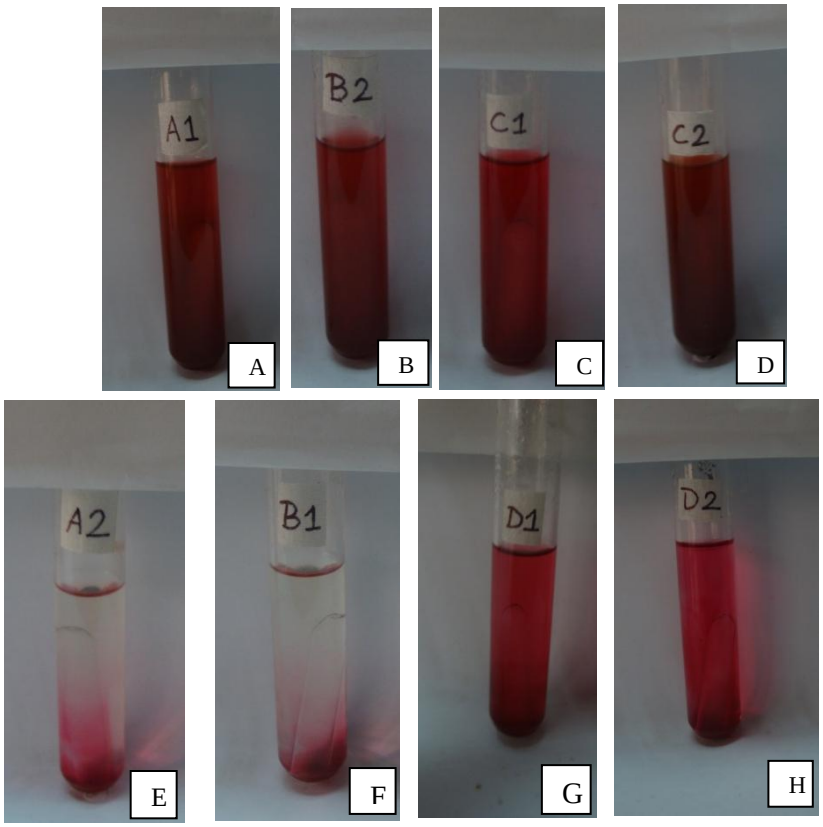

Figure 3.16: Nitrate reduction test result of eight isolates. **A-D.** Positive results for nitrate reduction test of isolate A1, B2, C1 and C2 respectively. **E-H.** Negative results of nitrate reduction test of isolate A2, B1, D1 and D2 respectively.

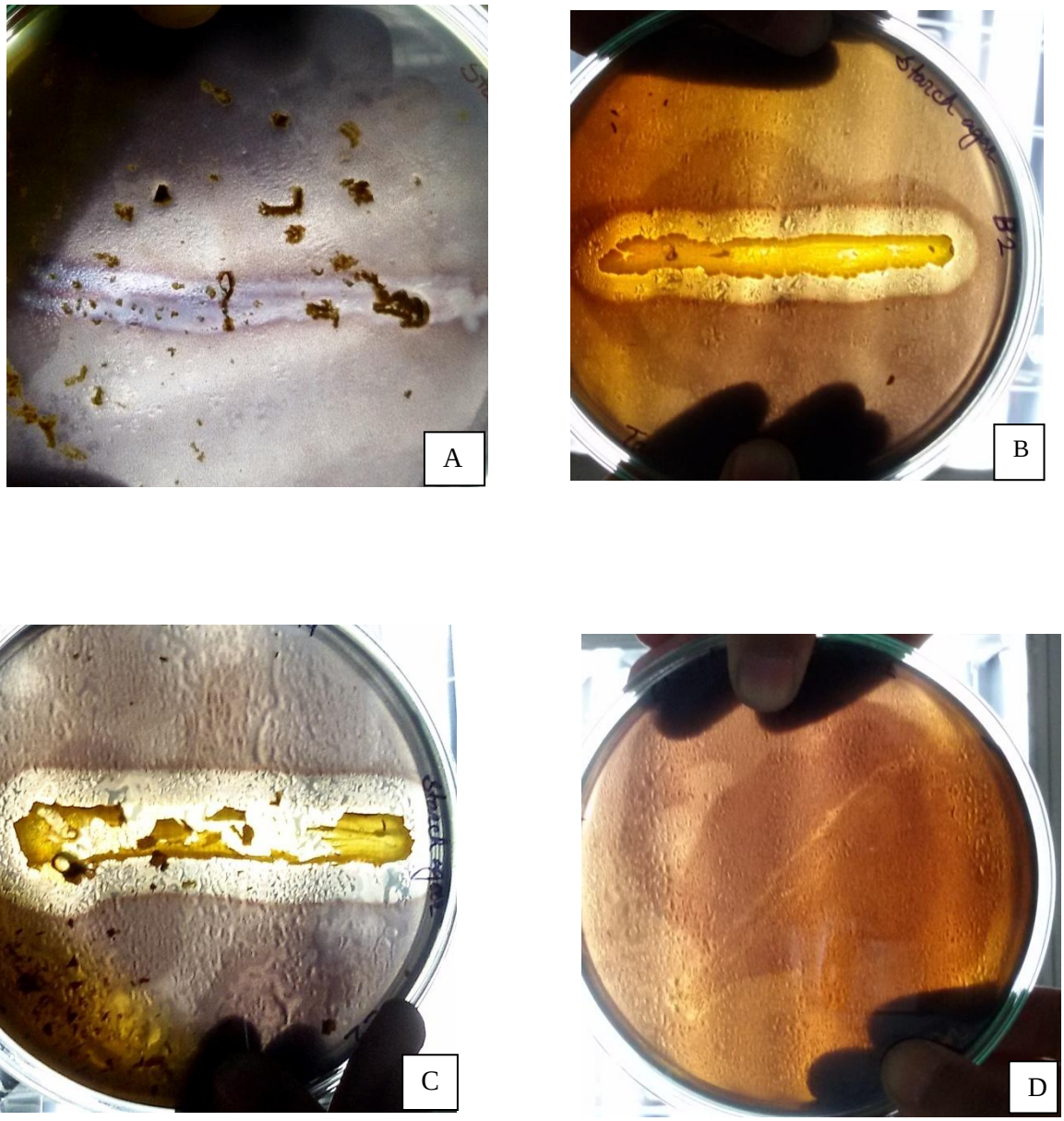

Figure 3.17: Positive Starch Hydrolysis test results. **A-C**. Represent positive results of A1, B2 and C2 respectively for starch hydrolysis observed from the clearing of media where the growth had occurred after addition of reagent. **D.** Negative result where clearing of media did not occur.

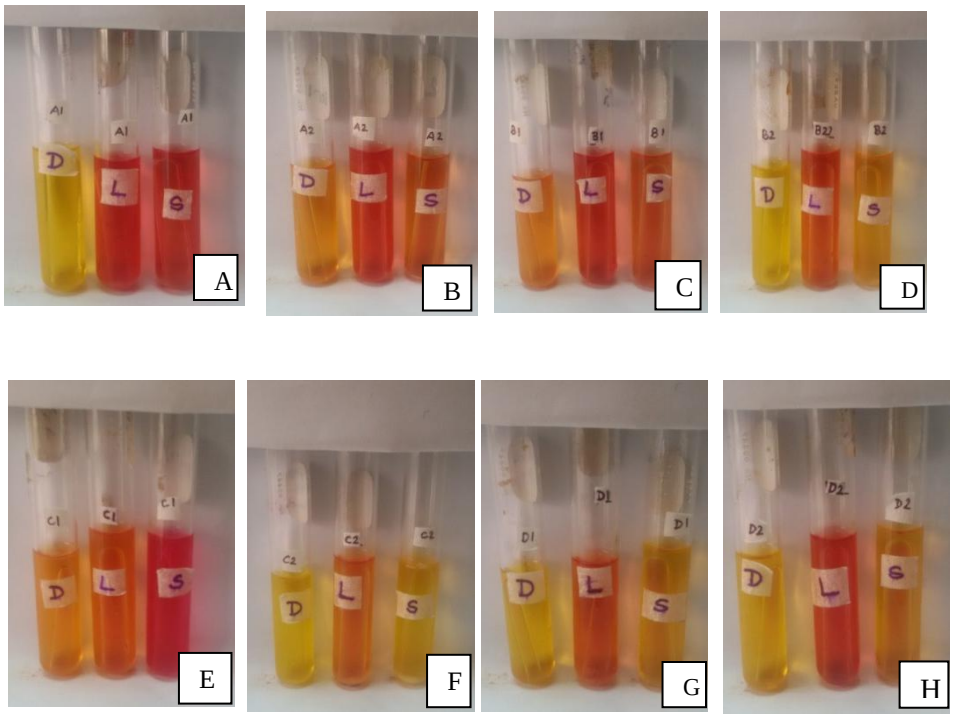

Figure 3.18: Carbohydrate fermentation test results. **A.** Fermentation result of isolate A1 in which, fermentation occurred in the tube labeled D for dextrose. **B, C, D & H**. Represent the fermentation result of isolates A2, B1, D1 and D2 respectively which show the same result; positive for dextrose and sucrose fermentation. **D & F.** Show the fermentation results of B2 and C2 respectively which were positive for dextrose, sucrose and lactose fermentation. **E.** This shows the fermentation results of isolate C1 in which were positive for dextrose and lactose and negative for sucrose.

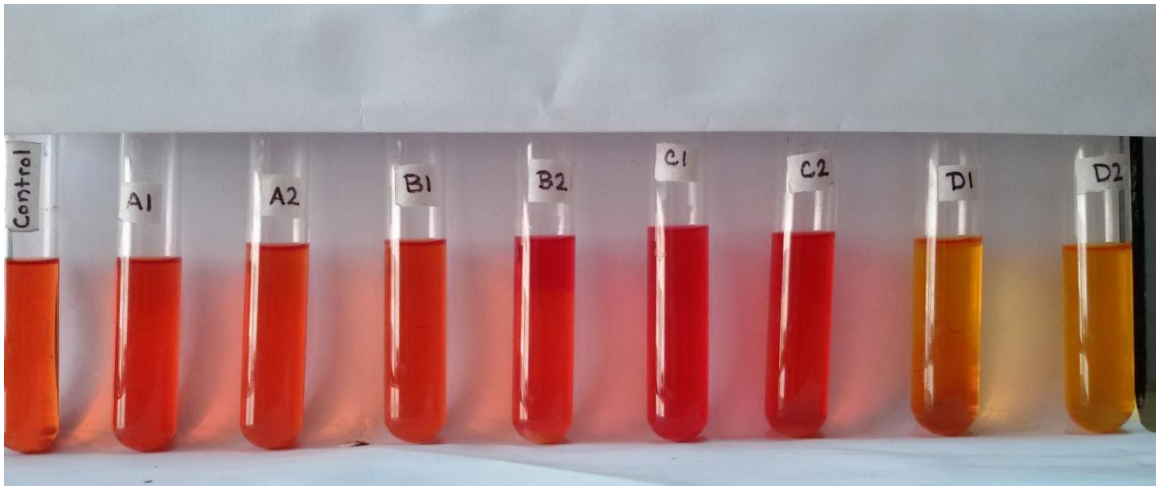

Figure 3.19: Mannitol fermentation result, in which D1 and D2 gave positive result.

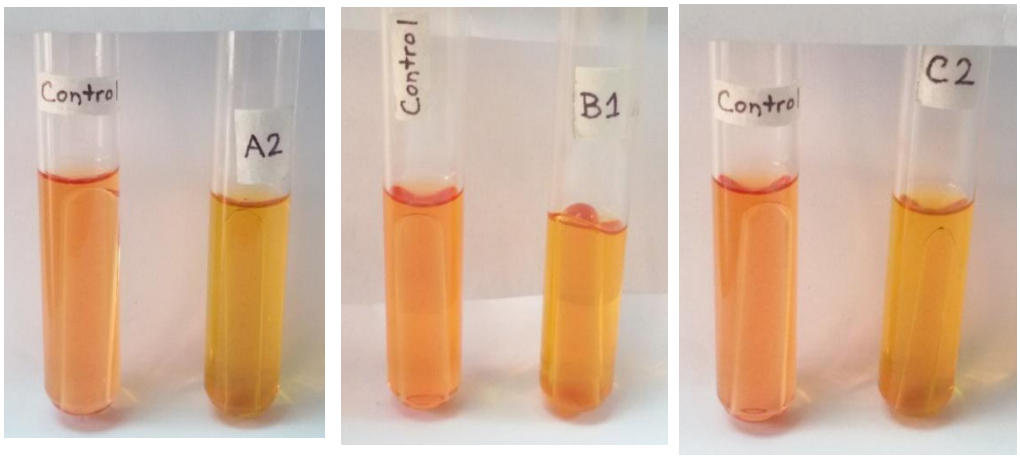

Figure 3.20: Isolates A2, B1 and C2 positive for Arabinose fermentation.

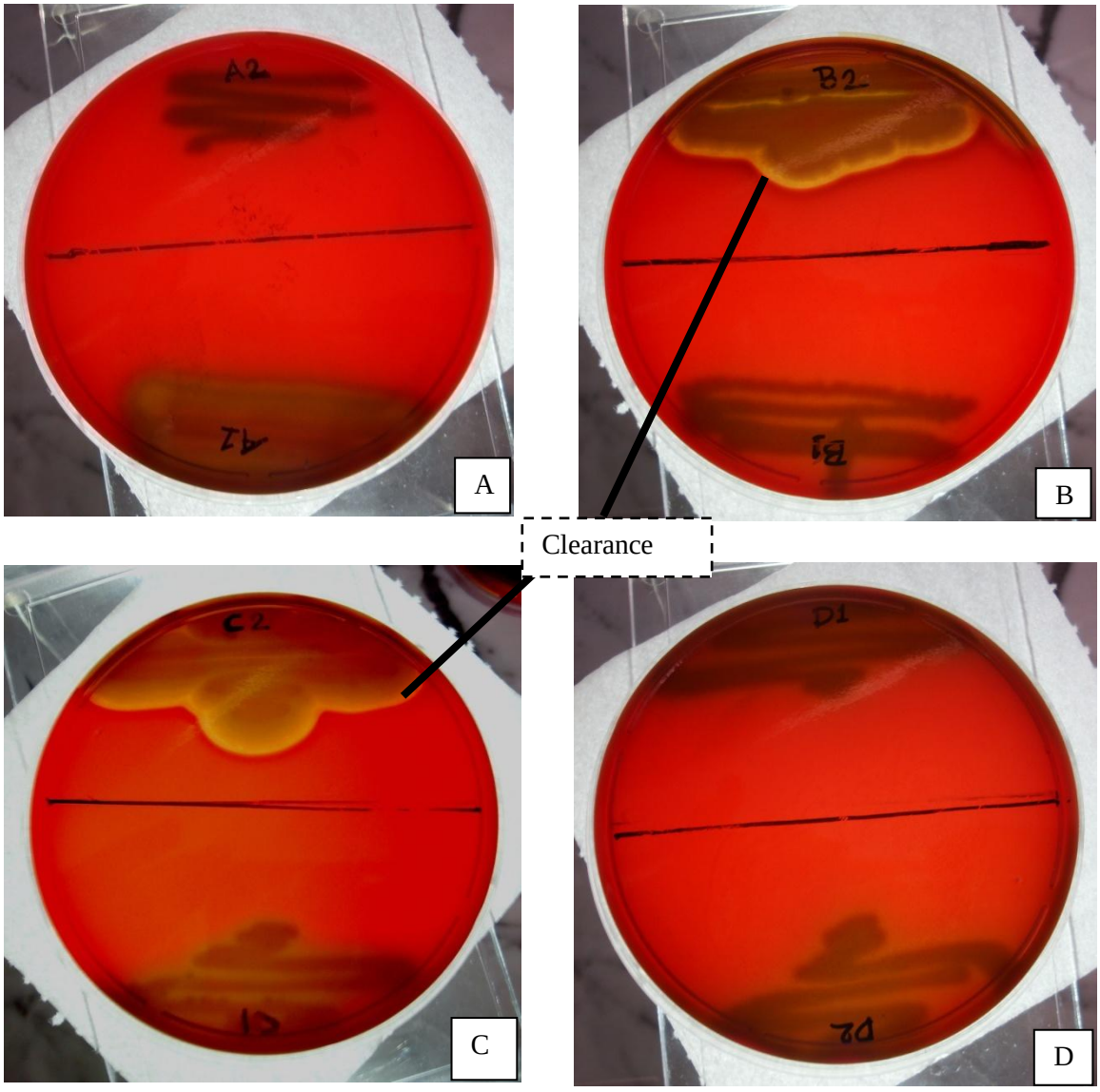

Figure 3.21: Hemolysis test on blood agar (sheep's blood). **A & D.** Results of isolate A1, A2 and D1, D2 respectively were showing no clearance of red blood cells from the media. **B & C.** The distinct clearance of red blood cells in media clearly observed for isolates B2 and C2 indicated β-hemolysis.

CHAPTER 4:
DISCUSSION

CHAPTER 4: DISCUSSION

4.1 Discussion

Chemical and physical treatments for oil spills are costly and invasive to the environment. As an alternative to these invasive methods, biodegradation has proved to be more cost effective. The increasing number of patents in biodegradation products shows a commercial interest and need of bioremediation agents. Bacteria and fungi are being studied for various degrees of biodegradation abilities, their metabolic processes and extreme conditions in which they can function as bioremediation agents. Indigenous bacteria can be screened for their biodegradation abilities and optimized for bioremediation purposes in tropical regions. In the present study, microorganisms were isolated from four different locations. The location of sample A was an agricultural field, soil B was from a fuel filling station, soil C was from a car servicing area and soil D was also from a fuel filling station. The locations of soil B, C and D were more likely to have been exposed to diesel than the location of soil A.

Diesel oil is a complex mixture of hydrocarbons of different lengths ranging from C_8 to C_{22} and includes paraffins, olefins and aromatic compounds (Bawase *et al.,* 2012). With the variation of hydrocarbon compounds in its composition, diesel stands as a great substrate for screening hydrocarbon degradation capababilities in microorganism. The isolates with oil degrading ability from this study may be used to study degradation of other hydrocarbon based substrates. The present study tested 8 isolates from 4 soil samples of different locations. Among the isolates, microorganisms of varied genus were identified; *Nocardia* sp., *Corynebacterium* sp., *Bacillus* sp., *Pseudomonas* sp. and *Arthobacter* sp. The isolate that showed the greatest growth in the diesel oil and mineral salt media belongs to *Pseudomonas* sp. This organism was isolated from soil sample C, collected from a field that had higher exposure to diesel and other fuel oils than the other soils. *Bacillus* sp. showed the next best ability to grow on diesel oil as sole carbon source. It has been reported that the environmental exposure to hydrocarbon has a sensitive role in biodegradation abilities of a microorganism (Rahman *et al.,* 2002). *Pseudomonas* species have been associated with biodegradation of hydrocarbons and specifically petroleum products in many studies and (Churchill *et al.,* 1995; Kothari *et al.,* 2012;

Onwurah, 2003; Rahman *et al.,* 2002). Onwurah (2003) also reported the genera *Bacillus* having capabilities to utilize toxic components of crude oil for growth. Although biodegradation activity of the genera *Nocardia*, *Corynebacterium* and *Arthobacter* on diesel and other hydrocarbon have been documented, in this study, these isolates showed little to no ability to utilize diesel for growth (Jogdand, 1995; Omotayo *et al.,* 2012). This could be due to the difference of species from the reported studies or the lack of hydrocarbon exposure in the locations of the soil sample. Atlas (1981) had related prior hydrocarbon exposure to the development of necessary metabolic pathways that breakdown hydrocarbon components.

In this study, variations of certain parameters were tested using *Pseudomonas* sp. isolate because it exhibited the highest growth in mineral salt broth with diesel as sole carbon source. The parameters mainly tested were concentration and pH. The effect of agitation was observed when compared with the results of growth in non-agitated conditions.

The growth of *Pseudomonas* sp was observed to be highest at 3% (v/v) diesel in mineral salt broth and bacterial count was 9.44 $\log_{10}$ CFU/ml. The concentration 2% (v/v) and 4% (v/v) had sub-optimal growth but the growth decreased at 5% and more at 6% (v/v) diesel. This shows that the optimal growth of the Pseudomonas isolate lays around 3% (v/v) diesel, higher concentration becomes toxic for its growth. This result is comparable to the result observed by Shukor *et al.,* (2009). The declining growth above 5% (v/v) diesel is also observed in the aforementioned study. The high concentration of diesel could be harmful to the bacterial population in enclosed systems due to its solvent effect that may damage the bacterial cell membrane. In the present study, the highest concentration tested was 6% (v/v) diesel which showed bacterial count of 5.56-5.58 $\log_{10}$ CFU/ml. Therefore the tolerance of diesel for the isolate *Pseudomonas* sp. may exceed 6% (v/v). A study showed some growth up to a concentration of 8 % (v/v) diesel. (Ku Ahamad *et al.,* 2013).

The experiment condition for the concentration variation was studied in a non-agitated condition. The results showed that growth of the isolate was slightly lower after incubation period without agitation. The maximum growth was observed at 3% (v/v) and sub-optimally at 2% and 4% (v/v) diesel. However the growth decreased to a bacterial

count of 8.33 $\log_{10}$ CFU/ml in non-agitation condition compared to the result from the experiment at agitation at 120 rpm (9.44 $\log_{10}$ CFU/ml). This suggests that the agitation plays a role in promoting the utilization of diesel oil. The main area of interaction between bacteria and oil occurs at the broth's water-oil interface. Agitation increases the surface area for interaction and thus gives a better growth condition. Also, the aerobic pathways of mineralization of diesel component require oxygen, agitation provides better oxygen supply. Most of the studies on mineral salt broth and oil are carried out with agitation of 100-150 rpm (De Domenico et al. 2004; Ku Ahamad *et al.*, 2013; Shukor *et al.*, 2009).

In the present study, the range of pH tested was pH 2- pH 10. The bacterial isolate Pseudomonas species showed growth in different degrees based on the pH. The maximum growth was obtained in the mineral salt broth at pH 7 and sub-optimally at pH 8. The bacterial count reached 8 $\log_{10}$ CFU/ml and 7.26 $\log_{10}$ CFU/ml respectively. Although there were lower degree of growth at pH 2 and pH4, the declining of growth was sharper from pH 8 to pH 10 (3.41 $\log_{10}$ CFU/ml). The reason for this can be ascribed to the nitrate reducing capability of the isolate C1, Pseudomonas sp. It may have the ability to convert the nitrogen source, NH_4^+ from Ammonium sulfate in the broth to Ammonia. This can result in producing alkaline condition in the broth over the incubation period. A broth with alkaline condition such as pH 10 may hinder the metabolic process of the bacteria and result in low growth. Norris (1994) emphasized the need of near neutrality for the biodegradation activity of bacteria.

4.2 Further research

The present study is a preliminary screening of microorganism capable of utilizing diesel as a carbon source. 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 diesel 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. Only one species of bacteria may not be sufficient to degrade the entire hydrocarbon constituents of oil spills due to lack of enzymatic ability. A combination of microbes can improve degradation range of pollutants and ability through co-metabolism. Sophisticated methods for determining the species of the isolates can be applied. 16S rRNA sequencing 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. The genes of enzyme classes that are involved in degradation pathway must be studied for greater success in bioremediation. 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, 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. Mixed microbial communities can be used to enhance biodegradation processes by providing different enzyme classes and pathways.
5. Environmental manipulation using pH, temperature, oxygen availability may be undertaken to optimize the degradation ability.

In the light of threatened environmental pollution due to spillage of oil, extensive studies should be undertaken to protect our environment.

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APPENDIX – I
Media composition

Blood Agar

Composition	Amount (g/L)
Agar	15
Meat extract	10
Peptone	10
Sodium chloride	5
Sheep's blood	5%

Motility Indole Urea Agar (Oxoid)

Composition	Amount (g/L)
Casein enzymichydrolysate	10.00
Dextrose	1.00
Sodium chloride	5.00
Phenol red	0.01
Agar	2.00

MR-VP broth

Composition	Amount (g/L)
Peptone	7
Dextrose	5
Potassium phosphate	5

Nitrate Broth

Composition	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Potassium nitrate	5.0

Nutrient Agar (Himedia, India)

Composition	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

Peptone Water

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

Phenol red (Lactose, Dextrose, Sucrose, Mannitol, Arabinose) Broth

Composition	Amount (g/L)
Trypticase	0.4
Lactose	0.2
Sucrose	0.2
Dextrose	0.2
Mannitol	0.2
Arabinose	0.2
Sodium chloride	0.2
Phenol red	0.00072
Final pH	7.3

Simmon's citrate agar (Oxoid, England)

Composition	Amount (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
Bactobromothymol blue	0.08

APPENDIX – II
Reagents

Barritt's reagent

Solution A: 5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4oC.

Solution B: 40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminum foil and stored at 4ºC

Catalase reagent

35% Hydrogen peroxide

Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4ºC.

Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4ºC.

Oxidase reagent

100 mg of N,N,N1,N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4ºC.

Nitrate reagent

Solution A, Sulfanilic acid 1gm of sulfanilic acid was dissolved in 125 ml of 5N acetic acid. Solution B, Alpha-napthylamine 0.625 gm of α-napthaylamine dissolved in 120ml of 5N acetic acid.

Appendix – III

Instruments

Instruement	Model	Company
Autoclave,	Model no: HL-42AE	Hirayama corp, Japan
Electric balance	Scout, SC4010	USA
Refrigerator (4^{o}C)		Samsung, Korea
Shaking Incubator	Model: WIS-20R	Daihan Scientific, Korea
Incubator		Japan
Micropipettes		Eppendorf, Germany
Microwave oven,	Model: D90N30 ATP	Butterfly, China
pH meter,	Model no: MP220 :	Toledo, Germany