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Chemical and Microbial Characterization of North Slope Viscous Oils to Assess Viscosity Reduction and Enhanced Recovery

Submitted by:
Petroleum Development Laboratory
Institute of Northern Engineering
University of Alaska Fairbanks
P.O. Box 755880
Fairbanks, AK 99775-5880

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National Energy Technology Laboratory
3610 Collins Ferry Road
P.O. Box 880
Morgantown, WV 26507-0880
Ph: 304-285-4764 Fax: 304-285-4403

Principal Authors

Shirish Patil

Abhijit Dandekar

Mary Beth Leigh

Submitted by

Petroleum Development Laboratory
Institute of Northern Engineering
University of Alaska Fairbanks
P.O. Box 755880, Fairbanks, Alaska 99775-5880
Telephone: (907) 474 – 7734, FAX: (907) 474 – 5912

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ABSTRACT

A large proportion of Alaska North Slope (ANS) oil exists in the form of viscous deposits, which cannot be produced entirely using conventional methods. Microbially enhanced oil recovery (MEOR) is a promising approach for improving oil recovery for viscous deposits. MEOR can be achieved using either *ex situ* approaches such as flooding with microbial biosurfactants or injection of exogenous surfactant-producing microbes into the reservoir, or by *in situ* approaches such as biostimulation of indigenous surfactant-producing microbes in the oil. Experimental work was performed to analyze the potential application of MEOR to the ANS oil fields through both *ex situ* and *in situ* approaches.

A microbial formulation containing a known biosurfactant-producing strain of *Bacillus licheniformis* was developed in order to simulate MEOR. Coreflooding experiments were performed to simulate MEOR and quantify the incremental oil recovery. Properties like viscosity, density, and chemical composition of oil were monitored to propose a mechanism for oil recovery. The microbial formulation significantly increased incremental oil recovery, and molecular biological analyses indicated that the strain survived during the shut-in period.

The indigenous microflora of ANS heavy oils was investigated to characterize the microbial communities and test for surfactant producers that are potentially useful for biostimulation. Bacteria that reduce the surface tension of aqueous media were isolated from one of the five ANS oils (Milne Point) and from rock oiled by the Exxon Valdez oil spill (EVOS), and may prove valuable for *ex situ* MEOR strategies. The total bacterial community composition of the six different oils was evaluated using molecular genetic tools, which revealed that each oil tested possessed a unique fingerprint indicating a diverse bacterial community and varied assemblages.

Collectively we have demonstrated that there is potential for *in situ* and *ex situ* MEOR of ANS oils. Future work should focus on lab and field-scale testing of *ex situ* MEOR using *Bacillus licheniformis* as well as the biosurfactant-producing strains we have newly isolated from the Milne Point reservoir and the EVOS environment.

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CHAPTER 1: INTRODUCTION

1.1 Overview of the Alaska North Slope

According to the Energy Information Administration Office of Oil and Gas (May 2001), the Alaska North Slope (ANS) is the source of approximately 15% of oil production in the United States. It also contains the largest estimated volume of undiscovered petroleum reserves of any domestic onshore sedimentary basin. Thus, the ANS oil fields offer a measure of independence from foreign oil sources to the nation. To the state of Alaska, North Slope oil offers financial security, as it generates more than 85% of the state's general fund revenue.

The ANS is located north of the Brooks Range, between the foothills and the Chukchi and Beaufort Seas, extending approximately 700 miles westward from the Canadian border to Point Hope. Figure 1.1 shows the geographical location of the ANS. The coastal plain portion is an arctic desert, receiving only 10 inches of precipitation annually. The average temperature ranges from 5°C in the summer to -30°C in the winter. Permafrost extends to a depth of approximately 2000 feet beneath the land surface. The barren, almost flat coastal plain gives way to treeless rolling hills as it extends southward toward the foothills of the Brooks Range.

Figure 1.1: Location of Alaska North Slope (www.columbia.edu/~ari2102/ North%20Slope/ map.jpg, August 2007).

The U.S. Geological Survey (USGS) initiated petroleum exploration on the ANS in the early 1900s. The Prudhoe Bay Field was discovered in 1968 as a result. Several other large fields were also discovered in the area including the Kuparuk River, Milne Point, Endicott-Duck Island, and Point McIntyre fields. Construction began on the Trans Alaska Pipeline System (TAPS) in 1974 to transport ANS oil to the U.S. market. The first oil flowed to the port of Valdez, Alaska, in 1977.

Cumulative ANS oil production through 2000 was 12.941 billion barrels (Bbbl) of oil and condensate and 0.378 Bbbl of natural gas liquids (NGL) for a total liquids production of 13.319 Bbbl. Net production of 13.306 Bbbl resulted from the injection of 0.013 Bbbl of liquid. Cumulative gas production was 42.069 trillion cubic feet (Tcf) with 38.040 Tcf injected, yielding a net production of 4.029 Tcf. Approximately 78% of cumulative oil,

condensate, and NGL, and 88% of cumulative gas production came from the Prudhoe Bay Field. Fifty-seven percent of current oil production is from the Prudhoe Bay Field. Peak ANS production occurred in 1988 when the production rate averaged 2.038 million barrels per day (MMbbl/d). Since then, production has been declining. In 2000, the average production was 1.045 MMbbl/d. The North Slope currently accounts for 16% of U.S. oil production. Gas production averaged 9.132 billion cubic feet per day (Bcf/d) in 2000, but in the absence of a market, 8.430 Bcf/d of that was injected, while the remaining 0.702 Bcf/d was used locally.

1.2 Heavy oils in ANS

According to the Alaska Department of Revenue (2006) *Revenue Sources Book*, the term *heavy oil* refers to high-density oil and low American Petroleum Institute (API) gravity (less than 25°API to 20°API) due to the presence of a high proportion of heavy hydrocarbon fractions. In terms of the ability of the oil to flow underground within the formation, heavy oils are generally those with a viscosity greater than 100 cp—a measurement used to evaluate the ability of a liquid to flow at reservoir conditions. The higher the viscosity number, the slower the flow.

In Alaska, the real issue is the ability of crude oil to flow, or its viscosity as it relates to ability to flow. One of the major reasons that Alaska's oil is viscous is because it is located relatively close to the surface of the earth, where there is thick permafrost. The oil is not found deep in the earth, where the temperatures are warmer, but within 6000 feet of the surface, where temperatures are cooler, thereby reducing the oil's viscosity or ability to flow. For oil production in Alaska, API gravity is not as important as reservoir temperature. Hence, these oil deposits are sometimes referred to as viscous oil deposits, rather than heavy oil. Permafrost affects production, as it cools the oil traveling through the permafrost zone.

There are currently five fields producing viscous oil in Alaska: Orion, Polaris, Schrader Bluff, Tabasco, and West Sak. Four of these fields are shown in Figure 1.2. Not shown is Tabasco, which is a Kuparuk River Unit satellite.

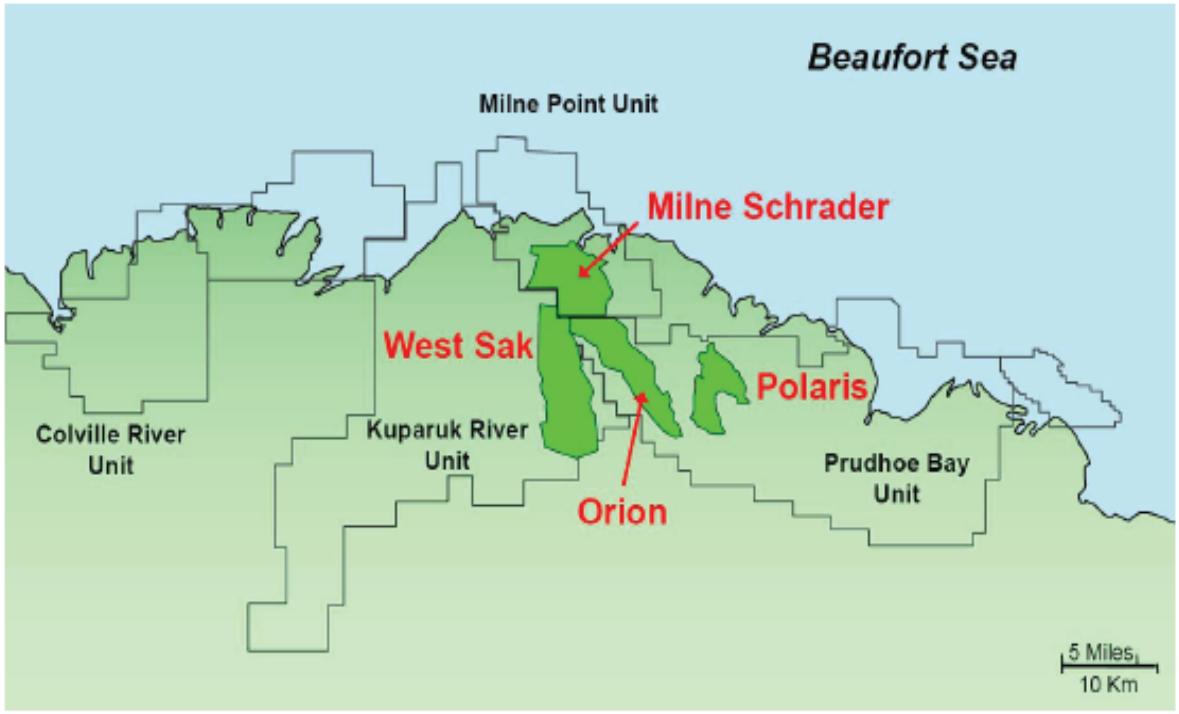


Figure 1.2: Fields producing viscous oil in Alaska (BP Exploration Inc., 2005).

The ANS is underlain by permafrost, which extends to about 1,800 feet in depth. The shallowest oil-bearing formation—Ugnu—is closest to the permafrost. The temperature in this formation is below freezing, its density is 1.014 g/ml, and it has very high oil viscosity. The billions of barrels of reserves in this formation are not presently economical to produce, but work is being done to find economical ways to produce these reserves.

1.3 Developing viscous oil resources

Developing viscous oil is difficult and expensive. Some heavy oils are too viscous to flow at reservoir conditions; they are usually found at relatively shallow depths that are too deep mine. At such depths, temperatures are low, so that viscosity is high. Heavy oils need special production technologies to facilitate their flow from reservoir to wellhead. Traditionally, these technologies have involved "steam flooding" techniques, which involve injecting hot steam to heat the oil *in situ*, thereby reducing its viscosity and allowing it to flow. Also used are techniques involving CO_2 or natural gas injection.

1.4 Microbially enhanced oil recovery (MEOR)

Microbial enhanced oil recovery (MEOR) is the use of microorganisms to retrieve additional oil from existing wells, thereby enhancing the petroleum production of an oil reservoir. In this technique, microbes are harnessed to produce harmless by-products which help to propel oil out of the well. MEOR may be achieved through *in situ* approaches such as biostimulation of indigenous microflora, or *ex situ* approaches in which selected microbes or microbial products are introduced into the well. Because these processes help to mobilize the oil and facilitate oil flow, they allow a greater amount of oil to be recovered from the well.

Microbial enhanced oil recovery is used in the third phase of oil recovery from a well, known as tertiary oil recovery. Oil recovery usually requires two to three stages: Primary Recovery wherein 12% to 15% of the oil in the well is recovered without the need to introduce other substances into the well; Secondary Recovery wherein the oil well is flooded with water or other substances to drive out an additional 15% to 20% of oil from the well; and Tertiary Recovery wherein several different methods may be used, including MEOR, to recover up to 11% more oil from the well. Microbial enhanced oil recovery technology is an attractive candidate for cost-effective solutions to viscosity reduction.

1.5 Objectives

To assess the potential for biosurfactant-mediated MEOR in ANS oil, we conducted research focused specifically on the microbiology of these heavy oil reservoirs. Our goal was to assess the amenability of ANS viscous oils for viscosity reduction processes relying on indigenous or introduced microbial populations. To explore *ex situ* methods, a microbial formulation suitable for application to MEOR was developed using a well-characterized biosurfactant-producing bacterial strain. Several coreflooding experiments were performed to assess the effects of the microbial formulation on incremental oil recovery. Experiments were conducted to monitor the properties of the oil sample used for the experiments in order to study the effect of MEOR on these properties. Properties of oil such as composition, density, and viscosity were monitored. This study was used to propose the mechanism for incremental

oil recovery due to MEOR. Terminal restriction fragment length polymorphism (T-RFLP) of the oil sample was performed to analyze the microbial community present in ANS viscous oil and to monitor survival of the introduced bacterial strain.

We also performed an assessment of the biological potential for MEOR in heavy oils from five important ANS reservoirs by characterizing the total and biosurfactant-producing indigenous microbial community in ANS heavy oils. By applying a combination of state-of-the-art molecular genetic techniques and microbial cultivation methods, we isolated indigenous biosurfactant-producing bacteria native to five different ANS heavy oil reservoirs as well as those from an Alaskan marine oil spill environment (Exxon Valdez oil spill).

EXECUTIVE SUMMARY

Viscous oil reservoirs tend to be low-energy, low-gas/oil-ratio systems with high viscosities, and are difficult to produce, transport, and refine by conventional methods. The large viscous oil deposits in the ANS cannot be produced entirely by conventional methods like pressure displacement or waterflooding. Other methods such as miscible gas injection and water alternating gas (WAG) also have limited success. Microbially enhanced oil recovery (MEOR) is one approach for improving oil recovery for viscous deposits. MEOR can be achieved using either *ex situ* approaches such as flooding with microbial biosurfactants or injection of exogenous surfactant-producing microbes into the reservoir, or by *in situ* approaches such as biostimulation of indigenous surfactant-producing microbes in the oil. MEOR has not been applied yet in any form to the ANS fields. This study includes experimental work to analyze the potential application of MEOR to the ANS oil fields through both *ex situ* and *in situ* approaches.

A microbial formulation using a well-characterized biosurfactant-producing strain of *Bacillus licheniformis* was developed in order to simulate MEOR. Coreflooding experiments were performed to quantify the incremental oil recovery following MEOR simulation. Properties like viscosity, density, and chemical composition of oil were monitored to propose a mechanism of oil recovery. Terminal restriction fragment length polymorphism (T-RFLP) was performed on the oil samples to qualitatively study the effect of the microbial formulation on a molecular scale. The microbial formulation significantly increased incremental oil recovery, and molecular genetic analyses indicated that the strain survived during the shut-in period.

The indigenous microflora of ANS heavy oils was investigated to fully characterize the microbial communities and identify indigenous surfactant-producing bacteria. Enrichment cultures using a variety of media were established using five different ANS oils as inoculum under aerobic conditions. Bacterial growth was obtained from oil from Milne Point and in a culture inoculated with microbes from Exxon Valdez oil spill (EVOS) oiled rocks. Bacteria grown were subjected to a high throughput screening test for their ability to reduce surface tension using an optical distortion assay. Over 90% of organisms screened from both cultures produced positive results. Seven morphologically different surfactant producers from the EVOS culture were isolated into pure culture and surface tension reduction, evaluated using a ring tensiometer. The total bacterial community composition of the five different oils was evaluated using T-RFLP fingerprinting. Each of the five oils tested possessed a unique fingerprint indicating a diverse bacterial community and varied assemblages, even from different production platforms associated with the same reservoir. Peaks corresponding to our surfactant-producing isolates were not detected in the oils, indicating that they likely originated from the EVOS environmental sample rather than the oil in which they were grown. The surfactant producers from EVOS and Milne Point crude oil are worthy of future study, as they may prove valuable for *ex situ* MEOR strategies.

Collectively we have demonstrated that there is potential for *in situ* and *ex situ* MEOR of ANS oils. Future work should focus on lab and field-scale testing of *ex situ* MEOR using

Bacillus licheniformis as well as the biosurfactant-producing strains we have newly isolated from the Milne Point reservoir and the EVOS environment.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The first or primary phase of oil production includes the use of natural stored energy to bring oil to the surface by expansion of volatile components and/or by pumping the individual wells to assist the natural drive. However, using this conventional technology, two-thirds of the oil in place remains unrecovered. The secondary phase of oil production is commonly known as waterflooding. Supplemental energy is added to the reservoir by injecting water. The basic approach in this phase is to maintain the reservoir pressure.

Even with waterflooding a significant amount of oil may remain in place. This remaining oil after waterflooding is typically the target for enhanced oil recovery (EOR) processes. Enhanced oil recovery includes gas injection and chemical and thermal processes to increase oil production. These processes either improve the sweep efficiency in the reservoir or substantially alter the capillary number. The EOR methods that have been tried and tested in the oil fields include steam injection, *in situ* combustion, CO_2 flooding, surfactant, polymer and caustic flooding, or a combination of these processes. Chemical methods such as surfactant, alkaline waterfloods, micellar-polymer, or caustic flooding attempt to reduce the interfacial tension, thus increasing the capillary number. Thermal methods tend to displace the fluids due to a combination of interacting physical changes like reduction in viscosity of the trapped phase and steam distillation. The injection of CO_2 or organic solvents aims at miscibly displacing the oil. At reservoir pressures, CO_2 becomes partially miscible with the oil, increases the effective oil saturation, and allows it to flow.

Conventional EOR processes have several constraints (Teh, 1990). Carbon dioxide flooding is ideal for deep reservoirs and light oil operations. There are certain drawbacks such as early and premature breakthrough, tentative or unknown prediction of miscibility pressure, single contact phase properties and dynamic phase behavior, and precipitation of asphaltene.

Absorption, chemical reactions with various ions and clay minerals in the reservoir, reactions with the crude oil, and temperature instability are some of the most destructive factors related to surfactant flooding.

In polymer flooding, chemical, mechanical, and microbiological instability can lead to ionization in contact with the organic acids in the petroleum. This reaction leads to the formation of macroscopic particles due to cross-linking. These particles can plug the pores, reducing permeability. (Brown et al., 1986)

One of the problems involved in surfactant flooding is adsorption of surfactant on the surface in the reservoir rock. As a result, the concentration of surfactant required for injection in the reservoir to perform the task is increased.

Adverse effects like gravity override and damage to the casing, tubing, pump, and well are encountered for *in situ* combustion. Also, premium petroleum is converted into useless coke. These constraints limit the applicability of these methods economically and technically (Brown et al., 1986).

The necessity to overcome these limitations has encouraged several researchers to explore the technology of enhancing oil production by using microorganisms, commonly referred to as microbial enhanced oil recovery (MEOR) or microbial enhanced hydrocarbon recovery (MEHR). MEOR involves the injection of some selected microorganisms into the reservoir. It also deals with the stimulation and transportation of the growth products of microorganisms, such that these products will further reduce the amount of oil remaining in the reservoir after the secondary phase of recovery.

Beckmann (1926) was the first to suggest the use of microorganisms to increase oil production. But it was Dr. Claude ZoBell who conducted a series of systematic laboratory investigations towards this cause 20 years later (ZoBell, 1946). ZoBell and his research group inoculated sulfate-reducing bacteria (SRB), particularly *Desulfovibrio*

hydrocarbonoclastius and *D. halohydrocarbonoclastius* in a nutrient solution of sodium lactate. The nutrient-covered Athabasca tar sand samples were placed in glass-stoppered bottles. The research team observed that the growth of SRB was accompanied by a gradual separation of oil or tar from the sand. Similar results were also obtained where oil appeared on the surface of the nutrient solution-covered oil-bearing cores. Based on the experiments conducted, ZoBell explained various reasons for the release of oil. His experimental techniques and suggested mechanisms for the release of oil from oil-bearing materials have been widely adopted and are followed even today. He also mentioned potential risks of MEOR if proper precautions were not taken during the application. Many other researchers like Updegraff and Wren (1954), Beck (1947), and others have conducted further research using different species of microorganisms.

Since ZoBell (1947 a/b), significant research efforts have been directed towards MEOR throughout the world. Earlier, due to low oil prices, this work did not receive much attention. With the increase in oil prices, however, the MEOR process has gained importance, as it has become economically viable. Extensive research is conducted in leading universities and laboratories in the United States, the United Kingdom, Canada, China, and Russia. As a result of this research, many successful MEOR projects have already been initiated in many oil fields, some of which are described later in this chapter.

This chapter discusses different aspects related to MEOR such as the fundamentals of the process, the different mechanisms leading to EOR by the use of microorganisms, the transportation of microbes into the porous media (i.e., the reservoirs), design parameters of a successful MEOR field application, the economic analysis of MEOR projects, and successful case studies of MEOR field applications.

2.2 Fundamentals of MEOR

Microbial enhanced oil recovery is used as an alternative to other conventional EOR processes. As with any other process, there are some beneficial and detrimental factors related to MEOR (Davis, 1967).

The beneficial effects related to microbes include the following:

i. Origin of hydrocarbons: Oil contains compounds like porphyrins, steroids, and isoprenoids that directly indicate organic origin. Thus, microbes lead to the development of the reservoirs.

ii. Micropaleontology: In the study and use of fossilized pollen and microbial spores, microbes can be used to identify the geology of particular formations.

iii. Petroleum prospecting: Microbes present on the surface can be used to determine petroleum accumulations at depth.

iv. Microbial enhanced oil recovery: The use of microbes to enhance oil recovery has numerous advantages over conventional EOR processes (Moses, 1983). For waterflooding, MEOR can be easily applied with typical surface equipment; it has low capital costs and is economically attractive for marginally producing fields. The process also has low chemical costs, as the cost of microbial injection fluid is relatively low, and its application is not affected by whether the price of crude oil is high or low. Microbial activity can be easily controlled by controlling the injection of nutrients; it can be applied to heavy as well as light oils. The microbes to be used can be selected and adapted for specific reservoir conditions. As with any other EOR process, MEOR also has some constraints (Moses, 1983): It cannot be applied in reservoirs having high temperatures (greater than 70°C) or high salinities (greater than 10%), and the presence of certain heavy metal ions in the reservoir can be toxic to microbes, leading to failure of the process. The application of MEOR requires laboratory compatibility testing and proper engineering design.

There are three major detrimental effects involved with microbes:

i. Corrosion: Corrosive effects include pitting of the tubulars used in production and injection wells and of the surface production equipment also. Sulfate-reducing bacteria, slime-producing bacteria, and iron-oxidizing bacteria are involved in the corrosion activity. Steel has a natural resistance to corrosion in the form of a

hydrogen layer. However, hydrogen disulfide produced by SRB can cause stress cracking in steel tubulars and souring of the hydrocarbons, and is also a health hazard.

ii. Permeability reduction: Microbial permeability reduction occurs due to microbial cell debris, microbial polysaccharides (gums, slimy secretions, resins), or microbial precipitation of sulfides or calcite. The microbial precipitation of sulfides and calcite occurs due to the presence of SRB. It takes place mainly in the oil-water transition zone and the water zone. Plugging due to microbial cell debris and polysaccharides occurs near the wellbore, especially around the water injection wells. In order to counteract microbial plugging, installation of filtration units and stimulation techniques like acidizing or hydraulic fracturing are used more commonly.

iii. Deterioration of petroleum reserves and drilling fluid additives: Microbes use the petroleum reserve as a carbon source and, in turn, degrade the reserves. The microbes also degrade drilling fluid additives like cornstarch, natural gums, and carboxymethyl cellulose or chrome lignosulphonates, which can lead to formation damage.

Microorganisms can survive in a large variety of conditions: aerobic or anaerobic, acidic or basic (pH 2 to 9), low temperature or high temperature (from 0°C to nearly 100°C), and pressure up to 20,000 psi. Microbes are present in many oilwell environments, and they adapt, grow, and proliferate in the presence of nutrients. These activities lead to the formation of various bioproducts that facilitate oil recovery. Table 2.1 gives a brief summary of some of the common microbial species used in MEOR and their bioproducts.

Table 2.1: Microbial species used in MEOR (Bryant & Birchfield, 1989).

Scientific Name	Aerobic or Anaerobic	Products
Clostridium sp.	Anaerobic	Gases, acids, alcohols and surfactants
Bacillus sp.	Facultative	Acids and surfactants
Pseudomonas sp.	Aerobic	Surfactants and polymers; can degrade hydrocarbons
Xanthomonas sp.	Aerobic	Polymer
Leuconostoc sp.	Facultative	Polymer
Desulfovibrio sp.	Anaerobic	Gases and acids; sulfate reducing
Arthrobacter sp.	Facultative	Surfactants and alcohols
Corynebacterium sp.	Aerobic	Surfactants
Enterobacter sp.	Facultative	Gases, acids

The type of bioproducts produced depends on the type of nutrients consumed by the microbes from the environment. Nutrients required by the microbes basically consist of seven components, namely water, energy source, carbon source, electron acceptor, essential minerals, nitrogen source, and growth factor (Sugihardjo et al., 1999).

The biosurfactants produced act as good emulsifying agents and reduce the interfacial tension between oil and water; they may also result in wetability reversal of the system (Rocha et al., 1992). Some microbes need to use lighter hydrocarbons as their carbon source. These microbes degrade higher hydrocarbons for their use by breaking them into lighter hydrocarbons. This process causes conditional changes such as a decrease in oil viscosity and gas production (Knabe, 1984). The bioacids can change the structure of the carbonate rock by dissolving it, thus connecting the pores in the reservoir and facilitating the movement of oil. The gases formed, such as carbon dioxide, force the oil out of the pores or can dissolve in the oil to reduce viscosity. The biogas formation also helps in maintaining the reservoir pressure (Chisholm et al., 1990). The alcohols produced act as solvents, resulting in reduction of oil viscosity. The production of biopolymers can affect the mobility ratio between the displacing water and the displaced oil, resulting in a piston-like displacement.

The above-mentioned microbial activities can be exploited in two ways: The bioproducts that facilitate the recovery of oil can be produced on the surface by using conventional fermentation techniques. These products can then be injected into the reservoir. Though more capital- and labor-intensive, this *ex situ* approach is helpful in avoiding the injection of microbes into the reservoir, which can help avoid all the detrimental effects related to microbes. Alternatively, the *in situ* process, where the microbes and the nutrients are injected into the reservoir, is less expensive and, hence, more promising for a wider range of conditions. The *in situ* approach is widely used in field application.

A general hydrocarbon reservoir is categorized into five microbial zones: the gas zone, the gas-oil transition zone, the oil zone, the oil-water transition zone, and the aquifer zone, as represented in Figure 2.1. The size of the transition zones depends on the capillary pressure relationship between the two fluids.

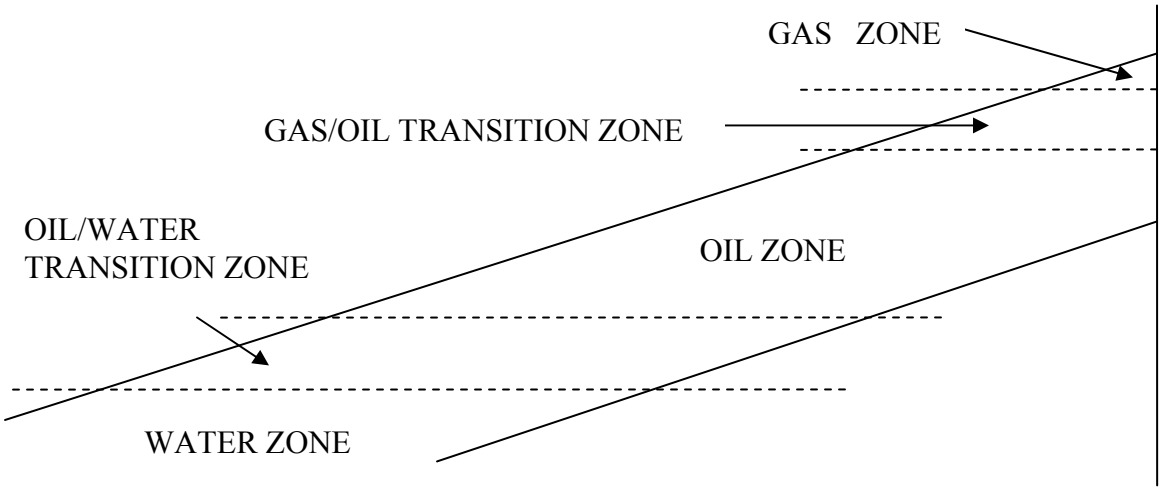


Figure 2.1: A typical hydrocarbon reservoir (modified from Gregory, 1947).

Microbial activity may occur in any of these zones to a variable extent. It is necessary to define certain parameters that will control any microbial inhabitance. Ten parameters related to pore structure and fluid in the rock (Sharpley, 1966) are defined as follows:

i. pH: All microbial species show an optimum pH for growth usually ranging from pH 5 to 8. However, the minimum and maximum pH possible to sustain growth is 2 and 9.5, respectively.

ii. Eh: The electromotive force (Eh) is a quantitative expression of oxidizing and reducing intensity of the system. In an aerobic system, the Eh will be positive, whereas the Eh will be considered negative for an anaerobic system.

iii. Temperature: Microbes can be categorized as a function of temperature: psychrophiles (grow best below 25°C), mesophiles (grow best between 25°C and 40°C), and thermophiles (grow best between 45°C and 60°C). However, microbial population is also observed in the high temperatures of sulfur springs and the low temperatures of the polar ice caps.

iv. Dissolved gases: The most important dissolved gas for the MEOR process is oxygen. Oxygen is usually supplied by using surface water to sustain aerobic microbes.

v. There are four main groups of microbial oxygen usage: aerobic (require oxygen for growth), anaerobic (do not require oxygen for growth), microaerophilic (grow best in the presence of small amounts of oxygen), and facultative (grow either in aerobic or anaerobic conditions).

2.3 Mechanisms of MEOR

Over the years, considerable work has been done to postulate the mechanism for enhanced oil recovery using microorganisms (Peihui et al., 2001; Raiders et al., 1985; Anderson et al., 1986; Sayyouh, 2002). However, the exact mechanism for the facilitation of oil recovery by microorganisms is still uncertain. The reviewed literature indicates that oil production usually can be increased by the following processes:

i. Single well stimulation
ii. Wellbore cleaning
iii. Selective plugging

Different mechanisms that contribute to the recovery of oil were observed by conducting oil displacement experiments in unconsolidated, thin reservoir flow cells (Kianipey and Donaldson, 1985). Three species of microorganisms namely *Bacillus licheniformis*,

Pseudomonas aeruginosa, and *Clostridium acetobutylicum* were used in these experiments. It was concluded that the observed increase in oil production was a direct result of *in situ* growth and metabolism of microorganisms. Microbial products can change the chemical and physical properties of oil, selectively plug high-permeability zones to improve sweep efficiency, and increase wellhead pressures in single-well injections. Oil production can also be increased by removing suspended debris and paraffin from the near-wellbore region.

One of the major mechanisms for EOR due to microbial activity is the production of biosurfactants (McInerney et al., 1999). These biosurfactants include anionic surfactants as carboxylic acids (fatty acids) and certain types of lipids. The biosurfactants produced by microbes reduce the interfacial tension at the oil-water interface. A reduction in interfacial tension results in a reduction of capillary forces. These capillary forces trap residual oil in porous media. The reduction in capillary forces frees the oil droplets, allowing oil to be displaced and thus recovered. The reduction in interfacial tension also reduces the pressure drop across a phase boundary, enhancing the microscopic sweep efficiency. This further increases oil recovery.

The production of biogases such as CO_2 and methane by bacteria as a result of metabolic activity has also been stressed as the mechanism for EOR. It has been proposed that these produced gases increase pressure in the reservoir and thus aid in the release of oil. However, the amount of gas needed to pressurize an entire reservoir is large and could not be easily produced. The produced gases introduce a third flow phase in the reservoir, increasing the mobility of oil by reducing the viscosity and swelling of individually trapped droplets of crude oil caused by solubilization of gas.

Bioacids and other solvents, such as ketone and alcohols, are produced as a result of microbial activities. These cause a removal of sludge and inorganic fine particles from the surface of sand grains in the flow cell. The acids dissolve the carbonate from the porous matrix, increasing permeability and the oil recovery. Alcohol generation can also lower interfacial tension, promote emulsifications, and possibly help to stabilize microemulsions.

Surfactants can also alter the relative permeability of rock to oil by changing the wettability of reservoir rock, thereby increasing oil recovery. Wettability changes toward more water-wet systems have been observed in many cases. This change contributes to the reduction of residual oil saturation, since the oil in water-wet systems is less apt to cling to the porous matrix.

Selective plugging of the pores by microorganisms is also a well-established mechanism for EOR (Stepp et al., 1996). The basic approach is fluid diversion. Producing zones have variable permeability due to stratification, depositional environment, or digenesis. The invading fluid moves rapidly through the more permeable zones, establishing a flow path. A major portion of the injected water flows through this established path. Thus, more oil is swept from the higher permeability zones as compared to the lower permeable portions, significantly affecting oil recovery efficiency. Selective plugging of highly permeable zones by microorganisms can improve sweep efficiency. Plugging can be accomplished by using either non-viable or viable cells. Non-viable or dead cells do not produce slime, precipitates, etc., so they act as particulate agents. Viable cells have the ability to adhere to rock and produce extra-cellular polysaccharides that cover cells and rock surfaces, forming a polymer film (Bryant and Douglas, 1987). These actions result in more effective plugging than particulate plugging by dead cells.

Biosurfactant-mediated MEOR can be achieved by several different strategies. *Ex situ* methods involve cultivation of specific microorganisms, followed by injection of microorganisms and/or their bioproducts into the reservoir. *Ex situ* production and injection of microbes and/or biosurfactants is a complex process requiring carefully controlled cultivation conditions, facilities, and expertise, which are challenging to achieve in the field. Injection of exogenous microbes, also known as bioaugmentation, is additionally problematic due to the challenges of microbial transport in the subsurface, competition with the indigenous microbial community, and induction of surfactant production under prevailing conditions. Alternatively, *in situ* biostimulation is a potentially simpler and less expensive

approach that relies on indigenous surfactant-producing microbes to achieve viscosity reduction. Biostimulation strategies frequently involve injection of nutrients dissolved in readily available production water to promote growth and surfactant-producing activities of indigenous microorganisms.

Assessing the potential success of biostimulation or bioaugmentation approaches requires specific, fundamental information about the microbiology of the targeted oil reservoir. Because microbial communities vary widely, it is first essential to determine whether surfactant-producing microbes are present. If none are detected, then bioaugmentation may be necessary. However, surfactant production is an advantageous trait in petroleum-associated microbial communities, permitting access to hydrophobic carbon sources. Indeed surfactant producers have frequently been isolated from crude oils. They are generally present, however, in low numbers and/or are not expressing surfactant production genes due to unfavorable conditions such as nutrient limitation (Van Hamme et al., 2003). This is in alignment with a fundamental tenet of microbial ecology which states that "Everything is everywhere, but, the environment selects" (Baas Becking, 1934), meaning a diverse array of microbes are ubiquitous but environmental conditions determine which organisms predominate in a particular time and space.

Biostimulation may be achieved if effective surfactant-producing microbes are present in the indigenous reservoir microbial community and if effective methods have been identified for promoting their growth and surfactant production. Strategies for *in situ* biostimulation will vary considerably depending on both the physiology of microbes present and prevailing geochemical conditions. Past MEOR studies have found that providing limiting nutrients to promote microbial growth followed by permitting nutrients to become limiting again effectively induces the production of abundant biosurfactants *in situ* (Sheehy, 1992). Upregulation of surfactant production has been observed in microorganisms under nutrient deficient conditions (Hsueh et al, 2007) and is assumed to be an adaptive strategy to access carbon during starvation. Exactly which nutrients and/or carbon sources are limiting is determined both by the chemistry of the reservoir and the physiology of specific

microorganisms being targeted. Furthermore, redox conditions may be altered to favor surfactant producers. Both aerobic and anaerobic biosurfactant-producing microbes are known (Lin et al, 1994; Youssef et al, 2005). Assessing the efficacy of indigenous aerobic and anaerobic biosurfactant producers, in conjunction with the cost of their biostimulation, can provide valuable information to guide MEOR strategies.

It is suggested that all of the above mechanisms are important for oil recovery, but they are not equally significant. Usually, EOR is a result of a combination of more than one of the proposed mechanisms. It is important to properly understand the mechanism of oil recovery for the selection of appropriate bacterial strains and the design of optimal operational procedures for MEOR. The mechanism of MEOR may be different for various reservoirs due to the complex interactions between microorganisms, the reservoir surface, the aqueous phase, and the oil. However, a better understanding of MEOR can be obtained by simple arguments to evaluate the relative importance of microbial activities.

2.4 Microbial transport in porous media

There are many microorganisms present in a reservoir. The growth of indigenous microbes over the injected microbes can be detrimental to the MEOR project. Also it can affect the permeability of the reservoir. Bactericides are used to inhibit the growth of unwanted bacteria. A proper understanding of the transport of bacteria in reservoir rocks is important for the success of MEOR processes.

The success of a MEOR process depends on the ability of the microbes to move through the reservoir and the production of chemicals. The produced chemicals should come in contact with the oil in order to mobilize it.

Various researchers studied microbial transport in the laboratory (Jenneman et al., 1982; MacLeod et al., 1988; Jang et al., 1984). Chang et al. (1991) compared the simulation and experimental results of the transport of microbes and nutrients in one-dimensional coreflooding experiments. The researchers developed a three-dimensional, three-phase,

multiple-component numerical model to describe microbial transport phenomena in porous media. The mathematical model was developed in two steps: the first step was to develop a mathematical model to predict the propagation and distribution of microorganisms and nutrients in porous media; the second step was to incorporate the transport equations for microorganisms and microbial nutrients into a three-dimensional, three-phase (oil, water, and gas) black oil simulator. To use the simulator, certain parameters such as diffusion coefficient and clogging coefficient of microbes are required. These parameters are determined by performing experiment and simulation matches. The laboratory experiments conducted included microbial coreflood and flask tests. Some field-scale simulations were conducted also.

Studies of the transport of microbes in porous media led to the concept of using microorganisms for selective plugging of reservoir pores (Jack and Stehmeir, 1987; Updegraff, 1983). Microbes that produce polymers, biomass, and slimes have been shown to significantly reduce the permeability of cores.

2.5 Design of MEOR field projects

The selection and design of a MEOR process for application in an oil field involves geological, reservoir, and biological characterization (Robertson et al., 1995). Microbially mediated oil recovery mechanisms are defined by the types of microorganisms used. The engineering and biological character of a given reservoir must be understood to correctly select a microbial system to enhance oil recovery. The steps normally considered in the application of a microbial system to any field are as follows:

i. Site selection: Studies like well log analysis, pressure-transient testing, spinner surveys, and chemical tracer tests are helpful for characterization of the target reservoir. The mineralogy of the rock formation should be characterized by core analysis. The reservoir is chosen in order to meet screening criteria for reservoir evaluation and selection. The screening criteria commonly used are given in Table 2.2.

Table 2.2: Screening criteria for reservoir selection (Bryant, 1990).

Formation Parameter	Recommended
Absolute permeability	> 75 md
Depth	< 7890 ft
Temperature	< 175°F
Oil gravity	> 15°API
Remaining oil saturation	> 25%
Injection-formation water	< 100,000 ppm
Compatibility between indigenous and injection microbial strains	Good

ii. Sampling and analysis of well fluids: Samples of oil, water, and rocks are collected from the sites. Each reservoir has a variation in microbial population. These samples are characterized in the laboratories for the presence of indigenous microbial population.

iii. Selection of microbial formulation: The characterization of microbial population is essential for selection of the microbial formulation to be used. This is useful in comprehending the effects of the indigenous microbes on the injected microbial formulation (Bryant et al., 1986). Samples are inoculated and the indigenous microbes are isolated. In cases where the indigenous microbes are not useful for oil recovery, different microbial formulations are tested for survival in the presence of the reservoir brine, rock, and nutrients. The microbial formulation leading to optimum oil production is selected for injection. Most of the microbial formulations have microorganisms isolated from the reservoir, as these microorganisms are easily adapted to reservoir conditions.

iv. Baseline determination of producing wells: The production history and characteristics of the reservoir should be studied before microbial treatment. Production measurement of the reservoir wells gives an idea of the average production of the reservoir before microbial treatment. This is useful in determining the performance of the MEOR project. Compatibility tests must be performed on core samples at reservoir conditions to get an estimate of oil recovery efficiency.

v. Growth of the microbial formulation and injections of microbes and nutrients: The selected microbial formulation is cultured and grown to the quantities sufficient for injection in the field. The final inoculation is performed at the selected site. Nutrients are injected with the microbial formulation. The microbial formulation is generally a combination of species of *Bacillus* and *Clostridium*. These species have a greater potential for petroleum reservoir survival because they produce spores. These spores are dormant, resistant forms of the cells that can survive more stressful environmental conditions. The nutrients are usually a mixture of numeral salts (a combination of minerals like nitrogen, phosphorous, potassium, etc.) and a carbon source (molasses, etc.). Grula (1986) reported major differences in the composition of molasses, which affects microbial growth and activity. Due to the dependence of microbes on water, the optimum MEOR scheme uses water as an integral part of the nutrients. While injecting the nutrients or water, care should be taken that the indigenous sulfate-reducing bacteria (SRB) are not stimulated or overgrown by the injected microbes.

A proper understanding of the transport of bacteria in the reservoir is very important for designing the injection strategy needed for oil production. If the microbial community is mobile in the reservoir, the injection strategy should be designed such that the microbes receive nutrients adequate to sustain their metabolism. On the other hand, if the microbes are not mobile, then the injection strategy should be designed such that the microbes metabolize the injected nutrients and the produced chemicals propagate through the reservoir. The injection of the microbial formulation and nutrients can be batch injection or continuous injection.

Batch injection tends to affect the reservoir locally, to 25 meters and more from the injection wells. Batch injection can be done in three ways:

a. Injection of a slug of microbes followed by a nutrient slug. The nutrient slug would help displace the microbial slug further into the reservoir.
b. Injection of a nutrient slug followed by a microbial slug. The nutrients absorbed on the rock surface would be used by the microbial slug that follows.

c. Injection of a slug of microbes suspended in a high-concentration nutrient slug chased by water injection.

Continuous injection is considered mainly where the physiochemical nature of the reservoir is more akin to microbial growth. Continuous injection could be done in the following ways:

a. Prolonged spore injection, even until the wells produce them. Selective flooding of germination nutrients could then be carried out.
b. Batch injection of microbes followed by a continuous injection of nutrients.
c. Continuous alteration of microbe slugs and nutrient slugs chased with water.

The size of the slug is decided by consideration of reservoir properties, such as permeability.

vi. Shut-in period: After the injection of the microbes, the wells are shut in for a particular period to allow for growth of the bacteria in the oil-producing formation.

vii. Production follow-up: Production is started after the shut-in period and the increase in oil recovery is observed. It is necessary to monitor and follow up the results for a credible evaluation of the microbial process.

2.6 Economic considerations of MEOR

Research has been carried out on the use of bacteria *in situ* and the use of surface bacteria on-site, where the bioproducts are produced in bioreactors and then applied in EOR processes. An economic analysis of these processes is necessary; as such an analysis may be helpful in identifying problems which could be particularly expensive to overcome. Also, it may provide a useful basis from which to examine the issue of operational investments and further costs, if any.

Conventional EOR methods are undoubtedly becoming more effective as compared with MEOR, as they are better understood, but presently they are expensive to put into operation. For economic assessment of any of these processes, large-scale experience is essential. It is necessary to perform the best calculations possible to determine major cost components and

return on investment (ROI). Also, it is essential to calculate the cost, probable or possible production over time, and initially required selling prices (IRSP) before any EOR process can be field tested.

In many cases MEOR operations have proceeded as part of waterflooding operations, with nutrients and bacteria injected at one point and oil produced at the production well. Although, these processes are less established than conventional EOR methods, they offer several advantages. The economic incentive is that they are much less expensive, because the main raw material used is an inexpensive carbohydrate source such as molasses, with or without inorganic nutrients.

The dedication of capital for MEOR development projects involves consideration of risk factors, including prediction of future events and economic climate and probability of success or failure; rate of return on investment; effect of failure on an organization's economic future; tax ramifications; current investment needs and opportunities; cash generation needs in future years to remain in a sound and dynamic position; and an organization's financial structure. Most of these factors are usually management prerogative, and many of them involve one facet or another of the need for financial continuity from year to year. Stable income must be provided to meet the cost of routine expenses such as fixed direct and indirect overhead, to produce a reasonable ROI for the owners, and to maintain a sufficient operating fund as well as funds to meet any previous financial obligations such as interest on loans. No decision is ever complete without considering what effect the venture's failure might have on the organization's economic structure.

There are usually two risk factors to be taken into consideration: time risk and geologic risk. Time risk is satisfied by payment or similar calculations. Geologic risk forces engineering personnel to express their judgment in quantitative terms. Financial evaluation must consider the technical complexities of the recovery process.

Perry (1981) and Burchfield and Carroll (1987) conducted important work in economic analysis of EOR processes.

2.7 Successful case studies of MEOR application

Alongside the research conducted on MEOR, a number of field tests have been successfully carried out. There are many successfully operating projects worldwide (Sheehy, 1990; Bryant et al., 1994, Matz et al., 1992; Wankui et al., 2006), which basically is an indicator of the feasibility of the MEOR process. Some of the MEOR projects from different parts of the world are discussed below.

Mink Unit Project (Bryant et al., 1990):

A microbial-enhanced waterflood field experiment was initiated during October 1986 by the U.S. DOE, the National Institute for Petroleum and Energy Research (NIPER), Microbial Systems Corp., and Injectech Inc.

Background:

The Mink Unit (Sec. 36, Twp. 27N, Rge. 16E) is located in the Delaware-Childers field in Nowata County, Oklahoma, and is producing from the Bartlesville sandstone formation. The average reservoir properties are listed in Table 2.3.

Table 2.3: Average reservoir properties for Mink Unit project (Bryant, 1990).

Parameters	
Formation	Bartlesville sandstone
Depth	600 ft
Net pay thickness	30 ft
Permeability	60 md
Porosity	20%
Formation temperature	65°F
Number of injection wells	21
Average water injection rate	40 bbl/day per well
Injection pressure	530 psi
Average oil production rate	6.4 bbl/day
Oil gravity	34°API
Oil viscosity	7 cp

The pilot site was four adjacent inverted five-spot patterns within the Mink Unit. The pilot area has four injection and eight production wells.

Treatment:

Chemical tracer studies were initiated in December 1986 to determine the flow patterns of the injected fluids in the Mink Unit. Every injection well was injected with 25 gallons of microbial formulation, NIPER Bac 1 on March 23, 1987. Ten gallons of pure molasses was injected into each well before and after the microbes. The treated injection wells were shut in until April 3, 1987. The four injection wells are being injected with two gallons of molasses every day.

Evaluation:

Field sampling for baseline determination began in November 1986 and continued to March 1987. All the wells produced foam, indicating a large amount of surfactant production and, also, confirming the viability of the microorganism. Oil production was increased after the

microbial injection through May 1988. The predicted and actual average oil production for the Mink Unit project is shown in Figure 2.2.

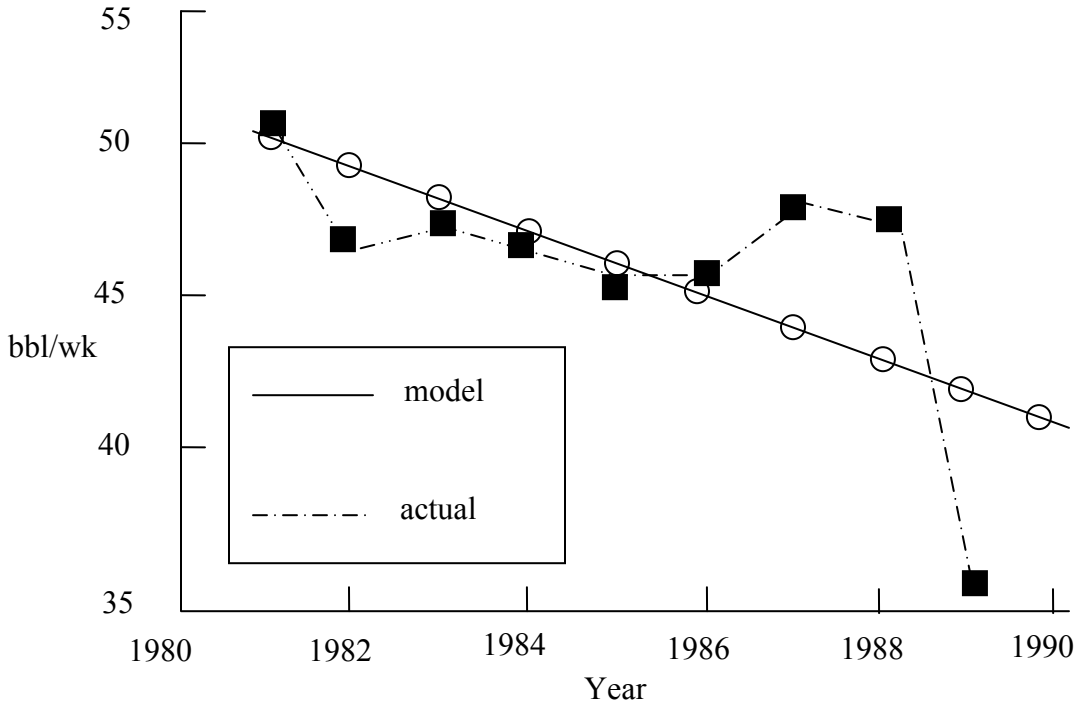


Figure 2.2: Predicted and actual average oil production for Mink unit (Bryant et al., 1990).

Huabei Project (Dietrich et al., 1996):

Background:

The Huabei Project is located at No. 3 Plant, Hebel Province in P. R. China. The average reservoir properties for this field are listed in Table 2.4.

Table 2.4: Average reservoir properties for Huabei Project (Dietrich et al., 1996).

Parameters	
Formation	Sandstone
Depth	6900 ft
Net pay thickness	40 ft
Permeability	240 md
Porosity	20%
Formation temperature	180°F
Number of injection wells	7
Average water injection rate	40 bbl/day per well
Injection pressure	530 psi
Average oil production rate	6.4 bbl/day
Oil gravity	28°API
Oil viscosity	14 cp

This project consists of seven wells that are scattered into different reservoirs. These wells are in the mature stage of waterflooding. Microbial enhanced oil recovery was started in September 1994. The reservoir and fluid parameters are all favorable for microbe growth.

Treatment:

The wells were treated three times. The first two treatments consisted of 150 barrels of microbe-laden fluid, followed by 40 to 150 barrels of displacing water. The third treatment consisted of 50 barrels with displacements ranging from 40 to 125 barrels.

Evaluation:

There was a rapid decline in the project wells before the microbial treatments. The baseline was determined by monitoring daily production for five months before the start of treatment. After a year of microbial treatment, oil production flattened at 150 barrels per day, which is 552% over the baseline value. There was a significant change in the viscosity of the crude. The Huabei Project production response to the treatment is summarized in Figure 2.3.

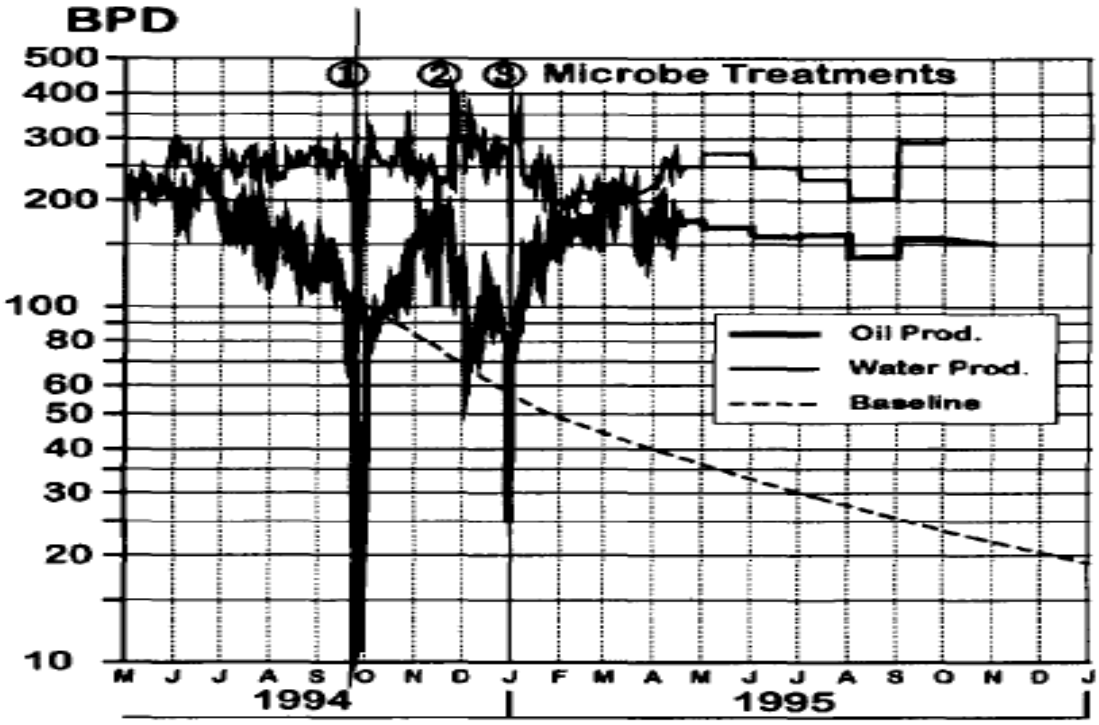


Figure 2.3: Huabei Project oil production response (Dietrich et al., 1996).

Diadema Field (Buciak et al., 1994):

A microbial single-well stimulation field test was conducted at the Diadema Field in Argentina by CAPSA Exploradora S.A. and APAC-Flow Technologies, with the technical support of NIPER, Bartesville, Oklahoma, USA.

Background:

The Diadema oil field is located in the San Jorge Gulf basin in the Province of Chubut in the southern part of Argentina. The formation selected for this pilot test is the Upper Garnet Zone (U.G.Z.) of complex II. The average reservoir properties for this field are listed in Table 2.5.

Table 2.5: Average reservoir properties for Diadema Field (Buciak et al., 1994).

Parameters	
Formation	Upper Garnet Zone
Depth	2953 ft
Net pay thickness	40 ft
Permeability	500 md
Formation temperature	126°F
Number of injection wells	5
Average oil production rate	27.8 bbl/day
Oil gravity	21°API
Oil viscosity	55 cp

Treatment:

The microbial formulation includes a combination of strains NIPER 1A and NIPER 7. This formulation was grown by APAC-Flow Technologies at the laboratory in Buenos Aires. A microbial formulation in the amount of 1 bbl and 176 bbl of nutrient solution were injected in each well. Five wells were treated. The treated wells were shut in for seven days to allow for growth of the bacteria in the oil-producing formation.

Evaluation:

Indications of microbial activity were detected already in the shut-in period, showing a slight increase of wellhead pressure. The oil production behavior of the microbially treated wells was uneven. Two of the five treated wells showed an excellent increase in production after the treatment. Increased production at well D-7 lasted for 70 days, and the amount of additional oil produced was 352 bbl; whereas at well D-47, the increased production lasted 150 days and 862 bbl of additional oil were recovered. The production response of well D-47 is summarized in Figure 2.4. The arrow indicates the initiation of the microbial treatment.

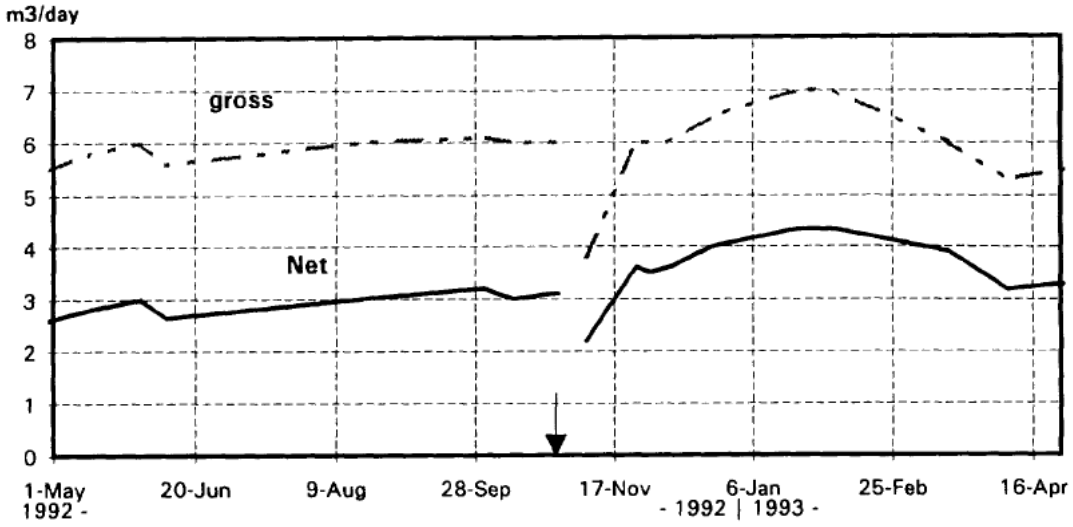


Figure 2.4: Oil production for well D-47 (Buciak et al., 1994).

CHAPTER 3: EXPERIMENTAL WORK

The major objective of this work was to determine the amenability of ANS viscous oils to viscosity reduction by using natural or introduced microbial populations. The ANS viscous oil sample was chemically and microbially characterized to assess the viscosity reduction and enhanced recovery. Careful measurement and analysis of ANS viscous oil were done at molecular and core scales. The following experiments were performed in order to achieve this objective:

1. Design of a biosurfactant-producing microbial formulation.
2. Coreflooding experiments for lab-scale evaluation of the effect of the microbial formulation on oil recovery.
3. Determination of the effect of the microbial formulation on the oil sample composition.
4. Density measurement of the oil sample at stock tank conditions.
5. Viscosity measurement of the oil sample at stock tank conditions.
6. Terminal restriction fragment polymorphism (T-RFLP) of several oil samples.
7. Growth and isolation of indigenous bacteria from ANS oils.
8. Testing of ANS bacteria for surface tension reduction capabilities.

The results obtained from these experiments are used to discuss the application of MEOR to enhance the recovery of oil at the ANS reservoirs.

3.1 Design of the microbial formulation

An ideal microbial formulation for a MEOR application would consist of two major components: the biotic component (i.e., microbes) and the abiotic component (i.e., nutrient medium—usually a combination of carbon source and some chemical, such as nitrogen or potassium, essential for the growth of the microorganisms).

There is a diverse microbial community in the reservoir. This microbial community, called indigenous microbes, can be found in oil samples and connate water samples collected from reservoirs. Due to the diversity of the microbial community, however, it is difficult to identify all the microorganisms present in the oil. Thus, a specific microorganism is injected

in the oil sample and, through metabolic activity, produces bioproducts that facilitate oil recovery. McInerney et al. (1999) isolated the JF-2 strain of the *Bacillus licheniformis* from the connate water of oil fields in Oklahoma, USA. This microorganism produces biosurfactant through metabolic activity when provided with a nutrient medium. The sample was selected because it grows effectively at the temperature commonly observed in ANS reservoirs. A freeze-dried sample of the JF-2 strain of *B. licheniformis* was obtained from the American Type Culture Collection.

A mixture of nutrients commonly known as Bushnell-Haas Broth is recommended for the microbiological examination of fuels by the Society of Industrial Microbiology Committee on Microbiological Deterioration of Fuels (Allred et al., 1963). Difco™ Bushnell-Haas Broth is obtained from Krackeler Scientific, Inc. Bushnell-Haas Broth is prepared according to the formula described by Bushnell and Haas (1941). The formula for the Bushnell-Haas Broth approximated per liter is given in Table 3.1.

Table 3.1: Composition of Bushnell-Haas Broth (Bushnell and Haas, 1941).

Component	**Weight (g)**
Magnesium Sulfate	0.2
Calcium Chloride	0.02
Monopotassium Phosphate	1.0
Diammonium Hydrogen Phosphate	1.0
Potassium Nitrate	1.0
Ferric Chloride	0.05

Magnesium sulfate, calcium chloride, and ferric chloride provide the trace elements necessary for bacterial growth, whereas potassium nitrate acts a nitrogen source and monopotassium phosphate and diammonium hydrogen phosphate provide buffering capability. The Bushnell-Haas Broth lacks the carbon source for growth of microbes, but heavy oil can be utilized by the microbes as a carbon source. For the effective growth of the

microorganisms used, lab grade sucrose (Fisher Scientific Inc.) and lab grade sodium chloride (Fisher Scientific Inc.) are also added to the nutrient medium.

In order to make 1 L of the nutrient medium

i. suspend 3.27 g of the dehydrated Bushnell-Haas in 1 L of deionized water;
ii. add 1% sucrose (10 g) and 5% sodium chloride (50 g) and mix thoroughly;
iii. heat with frequent agitation and boil for 1 min to completely dissolve the powder; and
iv. autoclave at 121ºC for 15 min.

The freeze-dried sample of the bacteria is revived by aseptically adding 0.3 to 0.4 ml of the nutrient medium with a pipette. The entire material is then transferred to a test tube (5 or 6 ml) of the broth medium. Agar gel plates are prepared from agar gel solution made by adding 15 g of agarose powder to 1 L of the nutrient medium. The bacterium is cultured on the agar gel plates to test the purity of the obtained culture and to purify the obtained culture if it is not pure. For this a sterile, cooled loop is used to take a drop of the bacteria-containing broth and streak the agar plate so that it looks like the example shown in Figure 3.1. The agar gel plates are incubated at 30ºC.

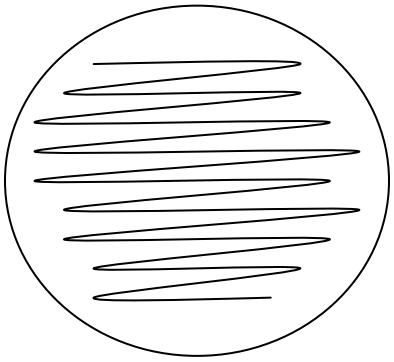


Figure 3.1: Streaking pattern of the broth on agar plate.

The grown culture is transferred into a test tube (about 10 ml) of the nutrient medium. It is allowed to grow overnight and then added to 500 ml of the nutrient medium. The mixture is placed in a shaker for 3 to 4 hours at 30ºC in order to grow the microbial formulation.

The concentration of the microbial formulation can be measured using the Miles and Misra method (1938) described below:

i. Prepare 8 agar gel plates.
ii. Dilute the microbial formulation by 1 to 10, in sterile 1.5 ml tubes with phosphate-buffered saline (PBS), pH 7.4. Continue the dilution until the original microbial formulation is diluted by 1 to 10^8.
iii. Put 1 plate on the turntable and put 100 µl of the diluted microbial formulation on the plate.
iv. Using a "hockey stick," sterilized by dipping in ethanol and then holding over a Bunsen burner, spread the microbial formulation evenly over the plate as shown in Figure 3.2. This step is carried out for each of the dilution.
v. Incubate the agar gel plates at 30ºC for 2 days.
vi. Count the colonies in order to find the concentration of the microbial formulation.

Though still used in laboratories to measure the concentration of microbial formulations, the Miles and Misra method is fairly old. Some recent methods involving spectrophotometer can also be used for this purpose.

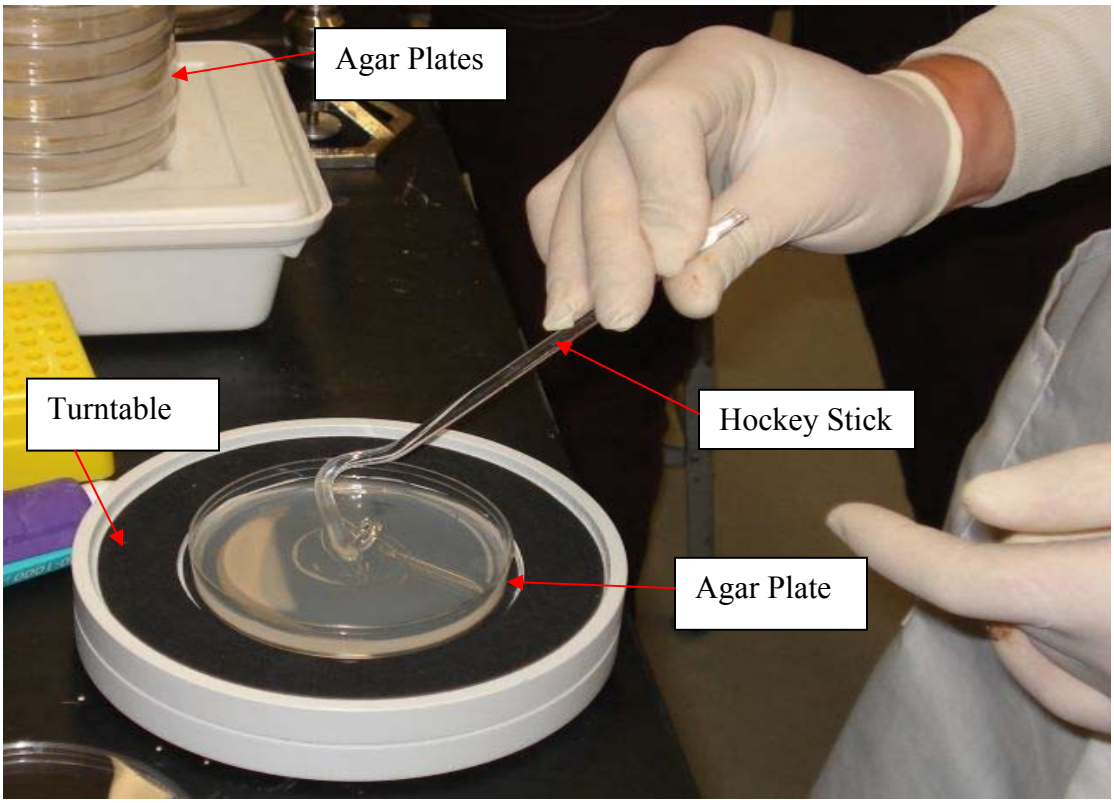


Figure 3.2: Spreading the microbial formulation on the agar plate using a "hockey stick" and turntable.

3.2 Coreflooding experiments for lab-scale evaluation of the effect of microbial formulation on oil recovery

3.2.1 Experimental setup

The coreflooding experiments were designed to examine the effect of microbial formulation on improved oil recovery. Figure 3.3 shows the schematic representation of the coreflooding rig. This rig was designed to conduct experiments at simulated reservoir conditions. The experimental setup of the coreflooding rig is shown in Figure 3.4. The components of the experimental setup for the coreflooding rig, along with their specifications, are listed in Table 3.2.

Table 3.2: Components of the coreflooding rig.

Component	Specifications
Pump	Positive displacement pump Teledyne ISCO D-series pump Model: 260D Maximum pressure: 7500 psi Flow rate: 0 to 90 ml/min Pump capacity: 266.05 ml
Floating piston fluid accumulator	DBR-JEFRI Model: 300-10-P Capacity: 300 ml Maximum pressure: 10,000 psi
Hassler-type core holder	TEMCO RCHR-1.5 Temperature: 100°C Maximum pressure: 3000 psi
Valves	HiP valves Maximum operating pressure: 10,000 psi
Overburden pressure	Nitrogen gas cylinder
Flowline	Swagelok company Tubing diameter: 1/8 inch Fittings: union connector, tee connector Maximum operating pressure: 10,000 psi

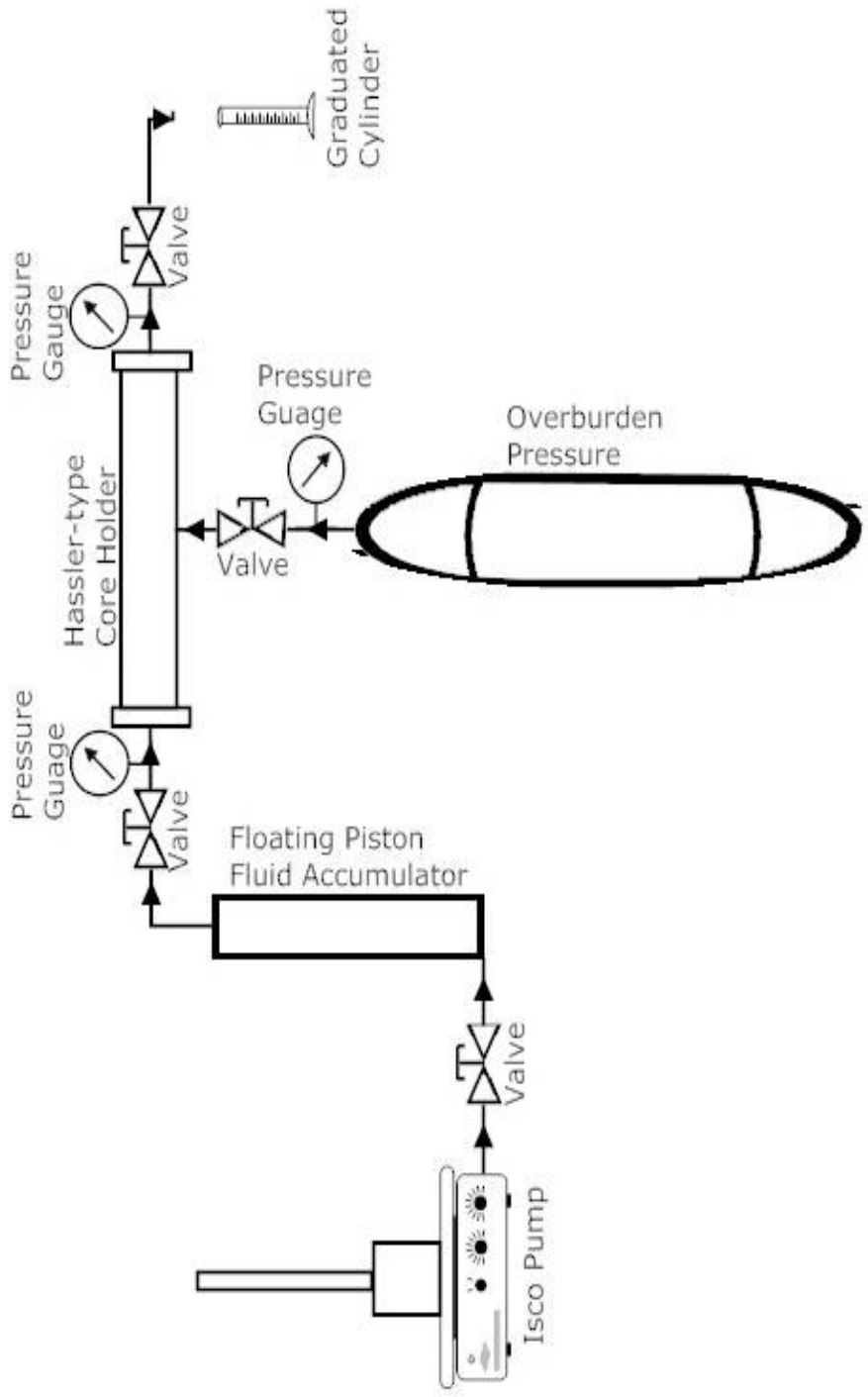


Figure 3.3: Schematic representation of the coreflooding rig.

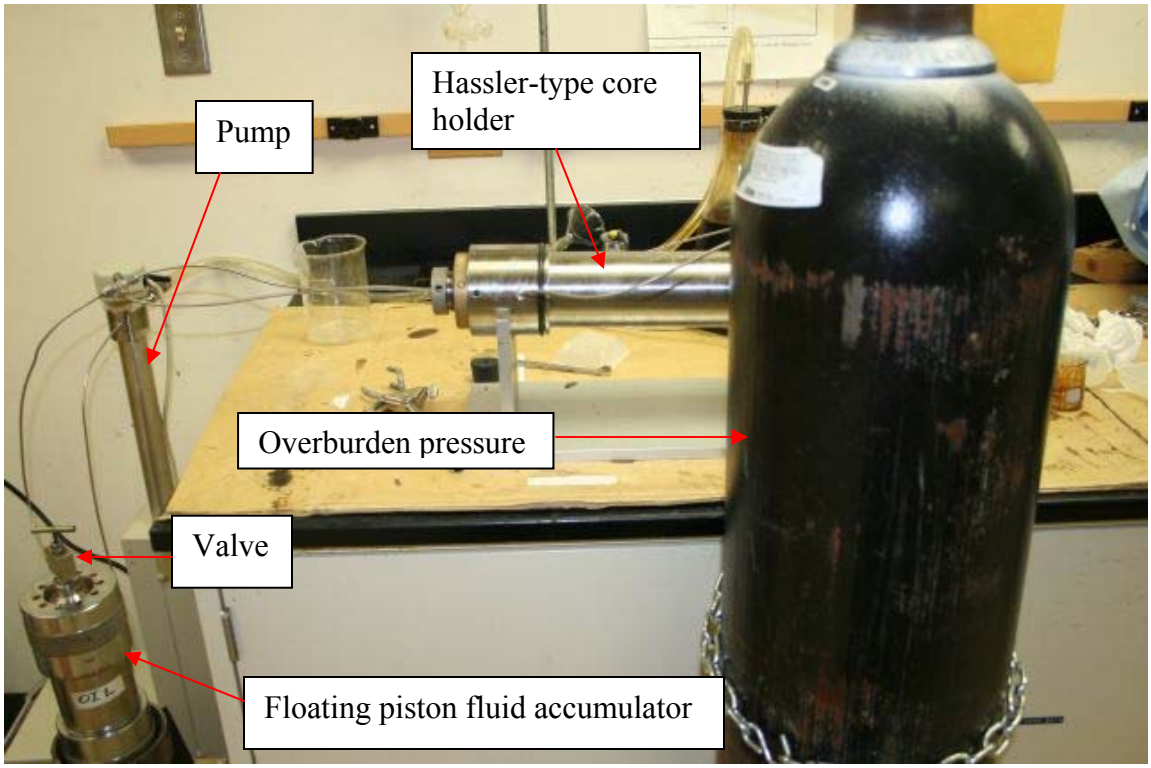


Figure 3.4: Experimental setup of the coreflooding rig.

3.2.2 Materials

Berea sandstone cores were used for coreflooding experiments. Six Berea sandstone cores were obtained from Cleveland Quarries, Ohio, USA. Each core was 1.5 in. in diameter and 6 in. long. The cores were autoclaved at 140ºC for 15 min. This facilitated the transport of microbes when injected inside the cores.

For the experiments, synthetic brine was used as a substitute for connate water. A 2 molar brine solution was prepared by dissolving sodium chloride in deionized water. The mixing ratio of brine and deionized water was measured gravimetrically. If the weight of water is known, then the weight of sodium chloride to be added in order to get a 2 molar brine solution can be obtained using equation 3.1:

$$\frac{W_{NaCl}}{W_{NaCl}+W_{water}}=0.02 \qquad (3.1)$$

where W_{NaCl} is the weight of sodium chloride, and W_{water} is the known weight of water.

The equation can be simplified to equation 3.2:

$$W_{NaCl} = 0.0204 \times W_{water} \quad (3.2)$$

Thus, the brine solution is prepared by adding the calculated amounts of deionized water and sodium chloride. The solution should be thoroughly mixed to completely dissolve the sodium chloride in water. The density of the brine solution can be measured using an Anton-Paar Densitometer, and the viscosity can be measured using a Canon-Fenske Viscometer. However, these values are used as measured by Agbalaka (2006).

Two kinds of fluids were used for the coreflooding experiments. Pure n-decane was used to perform the experiments on the first two core samples (Core #1 and Core #2). An oil sample from ANS oil fields was used to perform corefloods on the remaining core samples (Core #3, Core #4, Core #5, and Core #6).

3.2.3 Method

The procedure for conducting the coreflooding experiments was as follows:

i. The weight of the dry core sample was measured using a weigh scale before it was flooded.
ii. The cores were saturated with the 2 molar brine solution in a flask under vacuum for at least one week. This allowed the brine to achieve ionic equilibrium with the core samples.
iii. The cores were weighed after they were saturated with the brine solution. The pore volume of the core was calculated using equation 3.3.

$$PV = \frac{W_{wet\ core} - W_{dry\ core}}{\rho_{brine}} \quad (3.3)$$

where

PV is the pore volume of the core sample,

$W_{\text{wet core}}$ is the weight of the core sample saturated with brine solution,

$W_{\text{dry core}}$ is the weight of the dry core sample, and

ρ_{brine} is the density of the brine solution.

The bulk volume of the core samples was calculated using equation 3.4:

$$BV = \frac{\pi d^2 l}{4} \tag{3.4}$$

where

BV is the bulk volume of the core sample,

d is the diameter of the core sample, and

l is the length of the core sample.

The porosity (φ) of the core sample is calculated using equation 3.5:

$$\varphi(\%) = \frac{PV}{BV} \times 100 \tag{3.5}$$

iv. The core sample was loaded in the Hassler-type core holder, and an overburden pressure of 500 psi was applied in the radial direction. The brine was injected using high-pressure injection at the flow rate of 10 ml/min from a floating piston fluid accumulator. The brine was not directly injected from the pump, as brine solution can cause pump corrosion. The pump was filled with deionized water to avoid the corrosion. Coreflooding was conducted until a constant differential pressure across the core sample was reached. This ensured achievement of a steady-state condition which is necessary for accurate determination of absolute permeability. The core sample was weighed again after the brine injection.

v. Absolute permeability of the core was determined by application of Darcy's law for linear flow through porous media. The expression for Darcy's law is given in equation 3.6:

$$k = \frac{Q.\mu_{brine}.l}{\Delta P.A} \tag{3.6}$$

where

k is the absolute permeability of the core sample,

Q is the flow rate of the brine injection,

μ_{brine} is the viscosity of the brine solution,

ΔP is the differential pressure across the core sample,

A is the cross-sectional area of the core sample, and

l is the length of the core sample.

The differential pressure is calculated using equation 3.7:

$$\Delta P = P_{inlet} - P_{outlet} \tag{3.7}$$

where P_{inlet} is the inlet pressure noted from the ISCO pump, and P_{outlet} is the outlet pressure, i.e., atmospheric pressure.

vi. The core sample was then flooded with crude oil at a constant pressure of 300 psi to achieve initial water saturation. The flooding resulted in displacement of water from the core. The displaced water was collected in a graduated cylinder. The displacement of water by the oil was continued until no water was produced. The initial water saturation or the connate water saturation was calculated using equation 3.8:

$$S_{wi}(\%) = \frac{(PV - V_{brine})}{PV} \times 100 \tag{3.8}$$

where S_{wi} is the initial water saturation or the interstitial water saturation or the connate water saturation, and V_{brine} is the volume of brine displaced by the oil from the core

vii. The core sample was shut in the core holder for at least 5 days to achieve reservoir conditions.

viii. The core was then injected with a 2 molar brine solution at a constant flow rate of 0.1 ml/min to simulate the waterflooding process. As brine solution was injected in the core, oil was produced from the core. The oil was collected in a graduated cylinder. Brine was injected in the core until no more oil was produced. Oil saturation achieved by waterflooding is called residual oil saturation. Oil saturation was calculated using equation 3.9:

$$S_{or}(\%) = \frac{(V_{brine} - V_{oil})}{PV} \times 100 \tag{3.9}$$

where S_{or} is the residual oil saturation, and V_{oil} is the volume of the oil produced by waterflooding

Waterflooding was carried out on only two cores: Core #1 and Core #6.

ix. Core #2, Core #3, Core #4, and Core #5 were injected with the microbial formulation to produce oil. The microbial formulation was used when the microbes were in the growth phase of their life. The microbial formulation was injected in the cores until no more oil was produced, that is, until residual oil saturation was attained. Core #3 and Core #6 were injected with the microbial formulation after the waterflooding process. Since the cores were already at residual oil saturation, there was no more oil production from these cores.

x. Oil recovery after this process is calculated by equation 3.10:

$$\text{Recovery}(\%) = \frac{V_{oil}}{V_{brine}} \times 100 \tag{3.10}$$

xi. After the microbial formulation injection, the core sample was shut in for a week. This gave the microbes time to perform their metabolic activity and produce biosurfactants. Core #3 was kept in contact with the microbial formulation for 2 months in a conical flask. The conical flask was tightly corked to avoid oxidation of the oil.

xii. A core was produced after a week's shut-in period with the brine solution. The recovery of oil in this process is called as the incremental oil recovery. It was calculated using equation 3.11:

$$\text{Incremental Recovery}(\%)=\frac{V_{\text{increase oil}}}{(V_{\text{brine}}-V_{\text{oil}})}\times 100 \qquad (3.11)$$

where $V_{\text{increse oil}}$ is the increase in the recovery of oil due to injection of microbial formulation.

3.3 Determination of effect of microbial formulation on composition of oil sample

3.3.1 Equipment

The newly acquired Thermo gas chromatograph (Model: Trace GC Ultra) for measuring gas and oil compositions is shown in Figure 3.5. This chromatograph is equipped with a flame ionization detector (FID) and a thermal conductivity detector (TCD).

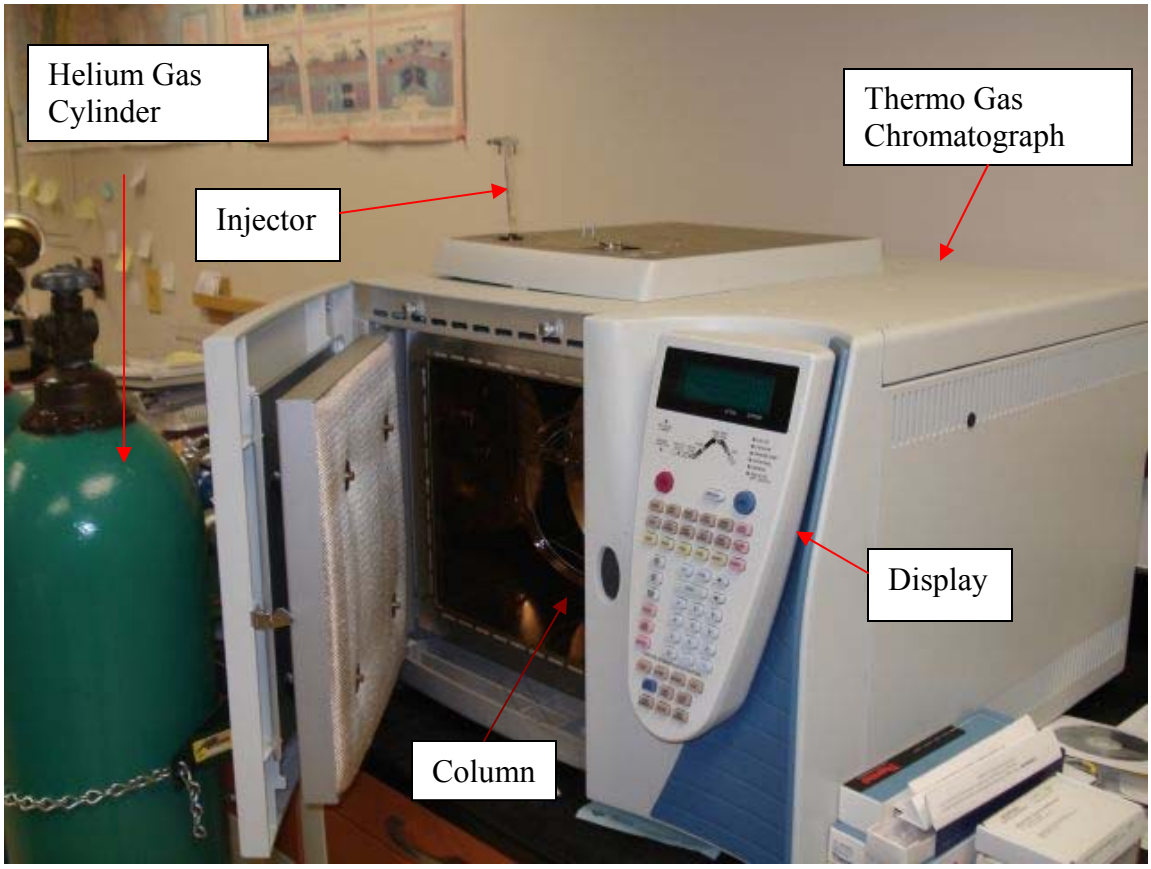


Figure 3.5: Thermo gas chromatograph. (Alurkar, 2007).

Simulated distillation (SimDist) technique was used to analyze crude oil composition. Based on separation using gas chromatography (GC), use of SimDist is widespread in the petroleum industry for evaluation of fossil fuels as well as petroleum feeds and cuts produced by refining and conversion processes. SimDist is based on the assumption that components of crude oil elute in order of their boiling points. Through a calibration curve relating the boiling point of normal paraffins to their elution temperature or retention time, SimDist provides the hydrocarbon distribution of the sample (in weight percent) versus the boiling range of the fraction. The operating conditions used for SimDist are tuned to be in agreement with preparative distillation that gives the true boiling point (TBP) curve. The components of the experimental setup used to determine the composition of the oil samples are listed in Table 3.3.

Table 3.3: Components of gas chromatography.

Component	Specifications
Column	Thermo TR-SimDist capillary column Length: 10 m Diameter: 0.53 mm (ID)
Oven Program	Start temperature: 35°C hold for 1.5 min Ramp: 10°C per minute Final temperature: 350°C hold for 10 min
Carrier Gas	Ultra pure grade helium Flow rate: 15 ml/min
Injector	Split injection Split ratio: 20:1 Sample injection volume: 1 µl Injector temperature: 350°C
Detector	FID Base body temperature: 350°C Hydrogen flow rate: 35 ml/min Air flow rate: 350 ml/min Make up gas (helium) flow rate: 30 ml/min
Reference Standard	ASTM D 2887 calibration mix

3.3.2 Calibration of the gas chromatograph

ASTM D2887 calibration mix was used for calibrating the gas chromatograph. Methylene chloride (chromatographic grade) was used as the solvent to prepare solutions with different concentrations of the calibration mix. This was done in order to calibrate the FID to detect the components over a range of concentrations. The composition of the calibration mix is given in Table 3.4.

Table 3.4: Composition of the calibration mix.

Elution order	Compound	Concentration (% wt/wt)	Purity (%)	Uncertainty (%)
1	n-Heaxane (C6)	6	99	+/- 0.02
2	n-Heptane (C7)	6	99	+/- 0.02
3	n-Octane (C8)	8	99	+/- 0.02
4	n-Nonane (C9)	8	99	+/- 0.02
5	n-Decane (C10)	12	99	+/- 0.02
6	n-Undecane (C11)	12	99	+/- 0.02
7	n-Dodacane (C12)	12	99	+/- 0.02
8	n-Tetradecane (C14)	12	99	+/- 0.02
9	n-Hexadecane (C16)	10	99	+/- 0.02
10	n-Octadecane (C18)	5	99	+/- 0.02
11	n-Eicosane (C20)	2	99	+/- 0.1
12	n-Tetracosane (C24)	2	99	+/- 0.1
13	n-Octacosane (C28)	1	99	+/- 0.1
14	n-Dotriacontane (C32)	1	99	+/- 0.1
15	n-Hexatriacontane (C36)	1	99	+/- 0.1
16	n-Tetracontane (C40)	1	99	+/- 0.1
17	n-Tetratetracontane (C44)	1	99	+/- 0.1
Solvent	Methylene chloride		99.8	

In order to calibrate the equipment, 1 µl solution of the calibration mix in the methylene chloride solvent was injected in the gas chromatographer. A run was made as per the oven program for simulation-distillation method. The components were eluted depending on their boiling points. A component with a lower boiling point was eluted earlier than one with a higher boiling point. Figure 3.6 shows the chromatogram for one of the calibration solutions.

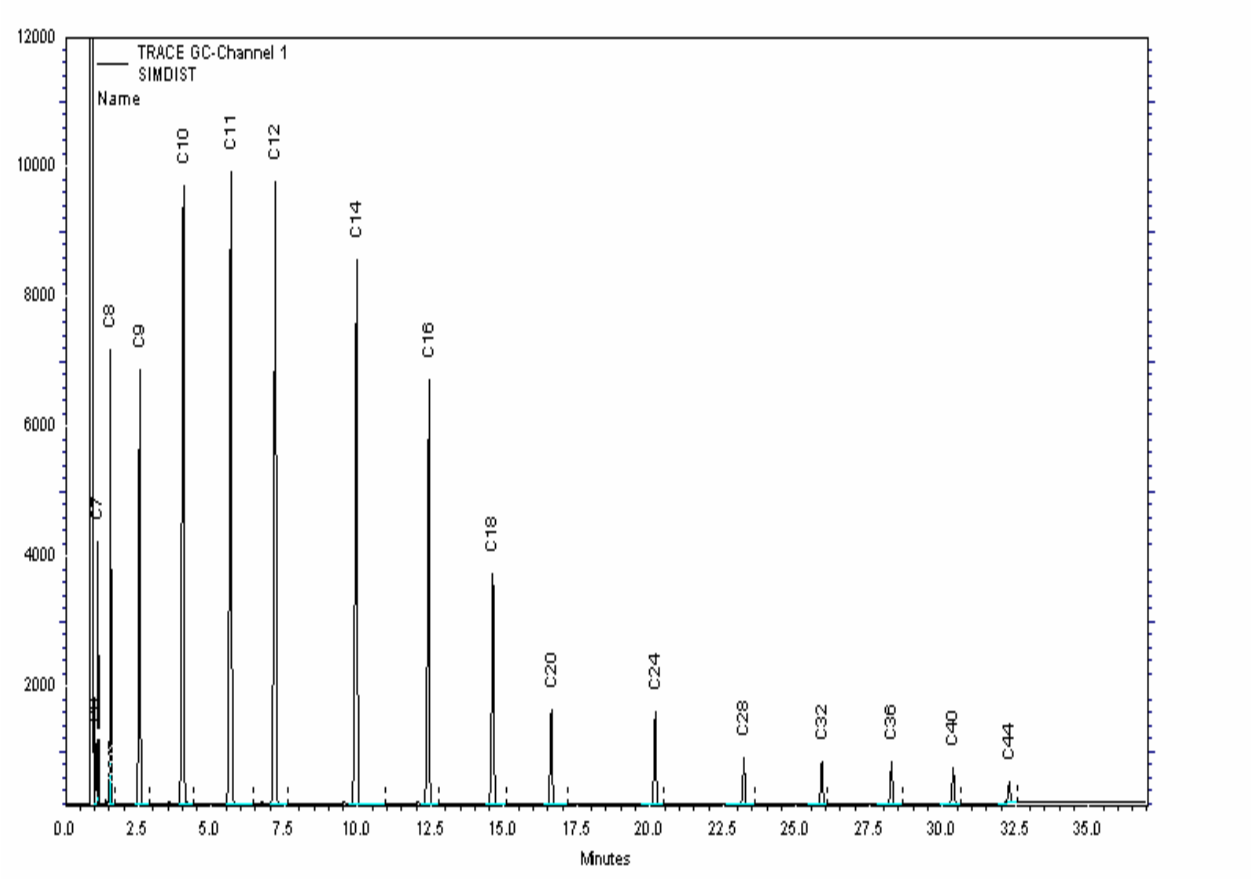


Figure 3.6: Chromatogram of calibration solution for simulated distillation.

The weight of each component in the calibration mix was known. The response factor for the component was calculated with the weight and the area under the peak of that component. Equation 3.12 was used to calculate the response factor:

$$K=\frac{W}{A} \tag{3.12}$$

where

K is the response factor for a component,

W is the weight of the component in the calibration mix, and

A is the area under the peak of the component from the chromatogram.

Response factors of n-paraffins were calculated over a wide range of concentrations, and averaged to get the best fit. The response factors of n-paraffins not present in the standard calibration mix were extrapolated from the plot of carbon number vs. response factor.

Once the chromatographer was calibrated, the sample to be analyzed was injected. The analysis was run as per the oven program to perform a simulated distillation. Equation 3.13 was used to calculate the weight of a component in the unknown samples.

$$W_{unknown} = k \times A_{unknown} \tag{3.13}$$

where $W_{unknown}$ is the weight of the component in the unknown sample, and $A_{unknown}$ is the area under the peak of the component in the unknown sample.

The gas chromatographer was used to determine the composition of the oil sample before and after microbial treatment.

3.4 Density measurement of the oil sample at the stock tank conditions

3.4.1 Equipment

The density of the oil sample was measured to see the effect of microbial treatment on density. Density was measured using the Anton-Paar digital density meter DMA 45. The experimental setup for density measurement consisted of the Anton-Paar digital density meter and a temperature bath (Brookfield TC-500). Figure 3.7 shows the experimental setup for density measurement.

The density meter determines the density of liquids and gases by electronically measuring the period of oscillation. The temperature bath maintains the temperature by circulating an ethylene glycol mixture in water. The calibration of the equipment is necessary before measurement of density.

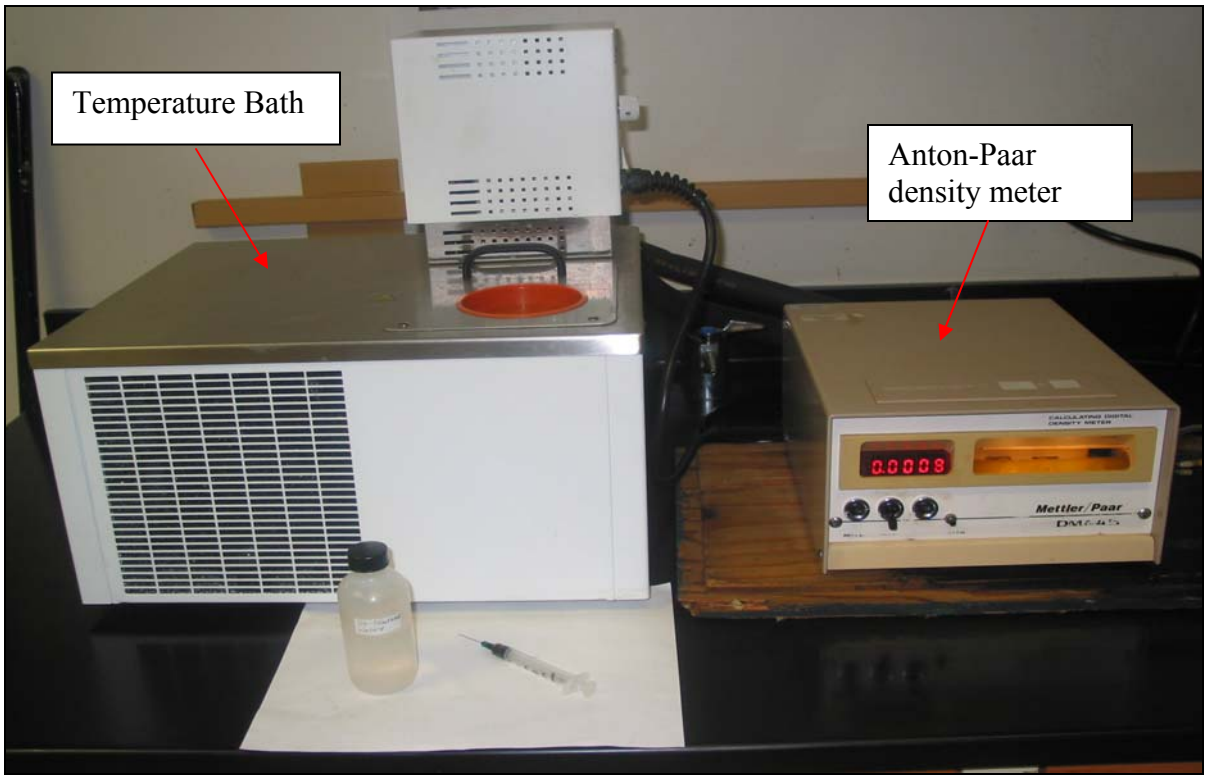


Figure 3.7: Experimental setup for density measurement.

3.4.2 Method of calibration

In order to calibrate the density meter, the two calibration constants A and B were needed. To determine the constants A and B, water and air were injected in the density meter. The following procedure was used to calibrate the density meter:

i. The desired temperature was set and allowed to attain equilibrium.
ii. The dial on top of the density meter was positioned at "T."
iii. Air was injected in the density meter, and the period of oscillation for air was noted. Similarly, the period of oscillation for water was measured also.
iv. The density of air at a particular temperature was calculated using equation 3.14:

$$\rho_a = \left[\frac{0.0012930}{1+(0.00367\times t)}\right]\times\frac{P}{760} \qquad (3.14)$$

where

ρ_a is the density of air at the specific temperature, g/ml,

t is the temperature, °C, and

P is the barometric pressure, torr.

The density of water at various temperatures was obtained from the table of the density of water from Laboratory Manual for standard ASTM D 4052-96 (1996).

v. The period of oscillation and density values for air and water were used to calculate the calibration constants from equation 3.15 and equation 3.16:

$$A=\frac{\left(T_w^2 - T_a^2\right)}{\left(\rho_w - \rho_a\right)} \tag{3.15}$$

$$B=T_a^2 - (A\times\rho_a) \tag{3.16}$$

where

A and *B* are the calibration constants at temperature, *t*,

T_w is the period of oscillation for water,

T_a is the period of oscillation for air, and

P_w is the density of water at the temperature, *t*, g/ml.

The constants A and B for various temperatures are listed in Table 3.5.

Table 3.5: Calibration constants for Anton-Paar density meter.

Temperature (°C)	Density of water (g/ml)	Density of air (g/ml)	T_w	T_a	A	B
0	0.99984	0.0013	8.028	6.1785	26.313	38.14
5	0.999964	0.0013	8.0258	6.176	26.305	38.11
10	0.999699	0.0012	8.0227	6.1738	26.289	38.083
15	0.999099	0.0012	8.0189	6.1715	26.271	38.055
20	0.998203	0.0012	8.0146	6.1694	26.251	38.03
22	0.997769	0.0012	8.0129	6.1688	26.242	38.023
25	0.995645	0.0012	8.012	6.1679	26.295	38.012
30	0.995645	0.0012	8.0086	6.1663	26.259	37.993
35	0.994029	0.0011	8.0049	6.1651	26.257	37.978
40	0.992212	0.0011	8.0005	6.1639	26.248	37.964
45	0.990208	0.0011	7.9943	6.163	26.212	37.953
50	0.98803	0.0011	7.9825	6.1619	26.092	37.941

The constants A and B for any intermediate temperatures were extrapolated from the plot of the temperature against the constants A and B.

3.4.3 Procedure for measuring density

The procedure used to measure density is described below:

i. The temperature bath was set at a constant temperature. Density was measured at stock tank conditions, so the temperature bath was set at 15°C.
ii. The constants A and B were obtained from the table, and the dials at the top of the density meter were adjusted accordingly. The dial was positioned at "ρ."
iii. Once the temperature bath reached equilibrium, the oil sample was injected in the density meter, and the value of density was noted from the digital meter.
iv. Two measurements were made for the same oil sample. In between the two readings, the density meter was cleaned by injecting toluene and acetone.

3.5 Viscosity measurement of oil samples at stock tank conditions

3.5.1 Equipment

The viscosity of oil samples was measured to study the effect that microbial treatment had on them. The experimental setup for viscosity measurement included a viscometer and a temperature bath to control the temperature. A Brookfield Programmable DV II+ Viscometer was used to measure viscosity, and a Julabo FP-50 Refrigerated/Heating Circulator was used for the temperature bath. Figure 3.8 shows the viscometer, and Figure 3.9 shows the temperature bath.

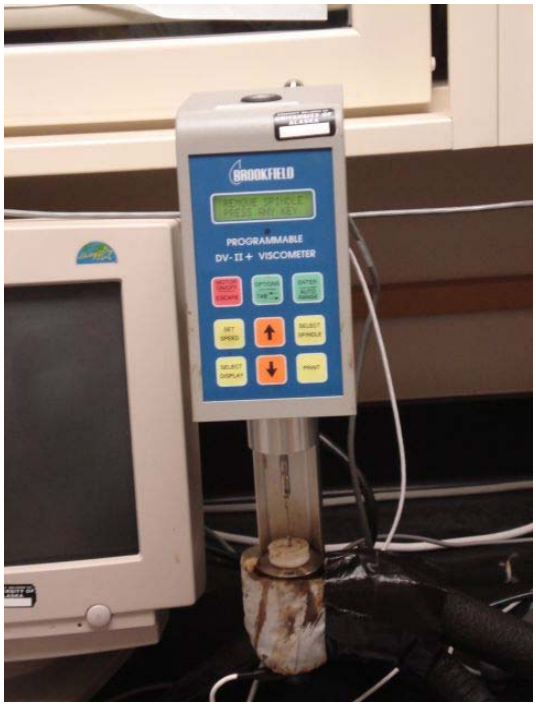


Figure 3.8: Brookfield Viscometer.

Figure 3.9: Julabo FP-50 refrigerated/heating circulator.

3.5.2 Procedure for measuring viscosity

The following is the procedure used to measure the viscosity of oil samples:

i. Turned on the temperature bath. EasyTemp computer software was used to control the temperature bath.
ii. Once the equilibrium was achieved, the viscometer was turned on.
iii. The spindle was removed, and any key was pressed in order to auto-zero the viscometer.
iv. The spindle was replaced. The oil sample was taken in the cylinder, and the spindle was immersed in the oil sample. Any key was pressed.
v. WinGather computer software was executed on a computer. The PC program was turn on in the options menu of the viscometer.
vi. Up to 20 of the custom speeds were selected from the setup menu of the viscometer.
vii. The motor was turned on and the viscosity reading was noted.

viii. The speed of the motor was changed by using the arrow buttons and pressing set speed.

ix. Viscosity readings for all selected speeds were noted and saved in the computer using WinGather.

3.6 Terminal Restriction Fragment Polymorphism (T-RFLP) of the oil samples

Microbial communities can exhibit an enormous range of complexity—from those with a mere handful of populations (Preston et al., 1996) to those with thousands of species derived from all three domains of life (Friedrich et al., 2001; Kudo et al., 1998; Schmitt-Wagner et al., 2003; Torsvik et al., 1990). In order to analyze any microbial community, identifying the diversity of species present is often the first step. This in itself is a very difficult task, as the majority of microbes present in most communities have not yet been cultivated (Amann et al., 1995; Staley and Konopka, 1985). For community analysis, genetic fingerprinting techniques allow the comparative profiling of many environmental samples and thus facilitate the spatial and temporal analysis of microbial communities in ecosystems. In this experiment, the oil sample from the ANS oil field, the developed microbial formulation, the oil sample extracted from the core after one week shut-in period, and the oil sample extracted from the core kept in contact with the microbial formulation for two months were analyzed for the microbial communities. Terminal restriction fragment length polymorphism (T-RFLP) is one of the approaches used for community analysis, commonly referred to as genetic fingerprinting. T-RFLP analysis is based on restriction endonuclease digestion of fluorescently end-labeled PCR products (in this case 16S RNA gene). Digested products are separated by gel electrophoresis and detected on an automated sequencer. The method provides distinct profiles (fingerprints), dependent on the species composition of the communities of the samples. T-RFLP has been used as an effective tool in the dissection of microbial communities (Bruce and Hughes, 2000; Kitts, 2001; Kuske et al., 2002; Marsh, 1999; Marsh et al., 2000). The general protocol for T-RFLP is presented in Figure 3.10.

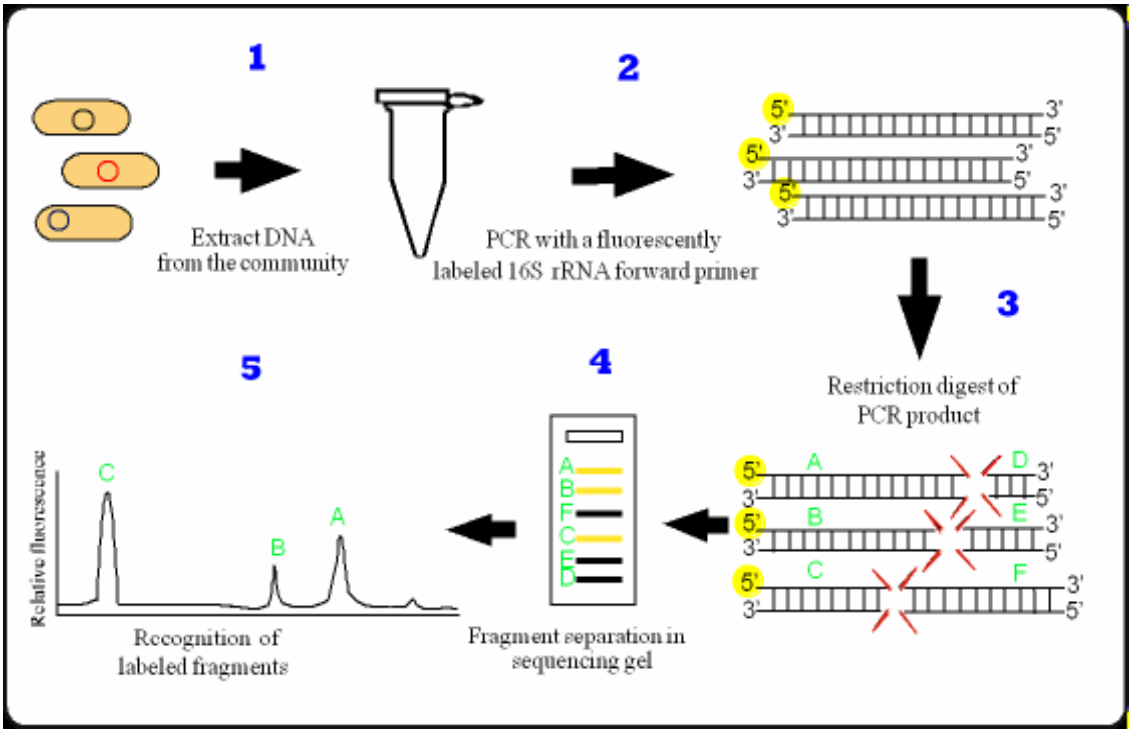


Figure 3.10: General protocol for T-RFLP (Grüntzig et al., 2002).

The T-RFLP method includes DNA extraction followed by PCR amplification. The primer set is usually a fluorescently 5′-labeled forward primer annealing to the 3′ end of the antisense strand and an unlabeled reverse primer annealing to the 3′ end of the sense strand of the template marker gene (here the 16S rRNA gene). The PCR reaction thus results in double stranded DNA fragments all labeled in the 5′ end of the sense strand.

The PCR reaction is followed by restriction enzyme digestion, whereby each DNA fragment renders one labeled terminal fragment and one or more unlabeled fragment. The DNA fragments are then separated (±1 to 2 bases, depending on the total fragment length) by electrophoresis on the ABI sequence analyzer, where the labeled fragments are recognized by the fluorescence detector. Internal standards (labeled fragment length markers) are included in each sample.

The resulting chromatogram reveals the size-fragments present in the sample as well as the relative quantitative distribution among them. By this means, it is possible to compare

samples according to the presence/absence of peaks as well as relative distribution among the peaks.

The DNA of the microbial community from all the samples is extracted by a method using zirconia beads and a stool kit, as mentioned by Tanaka et al. (2002). Usually DNA is isolated because it is more stable than RNA. The DNA is extracted from the cells because PCR cannot be performed on the cells.

The following procedure was followed for DNA extraction:

i. Ordered a QIAamp DNA Stool Mini Kit from Qiagen Inc., Valencia, CA, USA.
ii. Mixed the sample with 2 volumes of zirconia beads (0.1 mm diameter; BioSpec Products, Inc., Bartlesville, OK, USA) and 7 volumes of ASL buffer from the QIAamp DNA Stool Mini Kit. Made 2 replicates for each sample to avoid contamination.
iii. Homogenated the mixture with a Mini-Beadbeater at 560 g for 1 min. The Mini-Beadbeater used is shown in Figure 3.11.

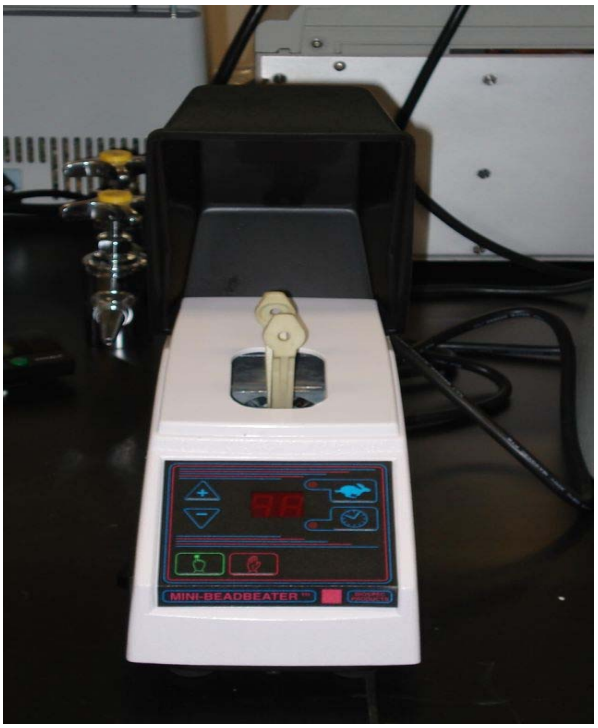


Figure 3.11: Mini-Beadbeater.

iv. Incubated the homogenized sample at 70°C for 5 min in a water bath.

v. Centrifuged the sample using a microcentrifuge. The extracted DNA remained in the aqueous phase, and this DNA was purified with a QIAamp DNA Stool Mini Kit. The microcentrifuge used for centrifugation is shown in Figure 3.12.

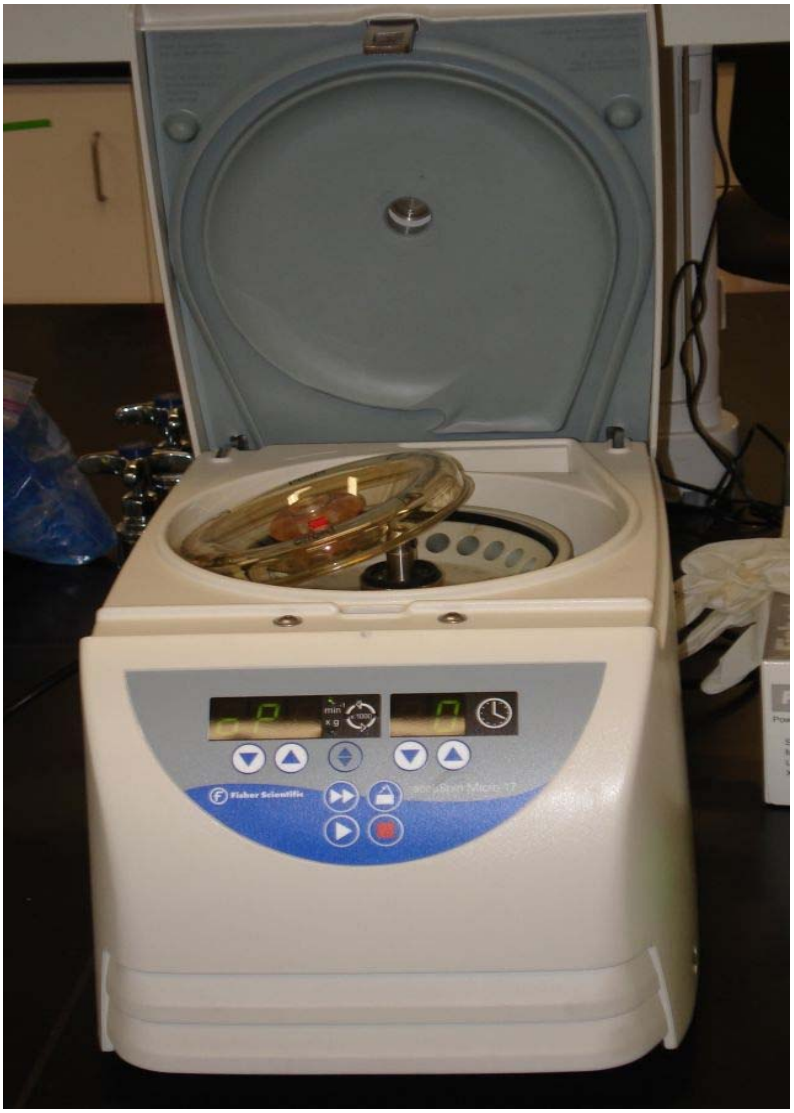


Figure 3.12: Microcentrifuge.

vi. Pipetted 1.2 ml of the supernatant into a new 2 ml microcentrifuge tube, and discarded pellet.

vii. Added 1 InhibitEX tablet to each sample and vortex immediately and continuously for 1 min or until the tablet was completely suspended. Incubate dsuspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

viii. Centrifuged sample at full speed for 3 min to pellet inhibitors bound to InhibitEX.

ix. Pipetted all the supernatant into a new 1.5 ml microcentrifuge tube, and discarded the pellet. Centrifuged the sample at full speed for 3 min.

x. Pipetted 15 µl Proteinase K into a new 1.5 ml microcentrifuge tube.

xi. Pipetted 200 µl supernatant from Step (ix) into the 1.5 ml microcentrifuge tube containing Proteinase K.

xii. Added 200 µl Buffer AL and vortexed for 15 s.

xiii. Incubated at 70°C for 10 min in water bath.

xiv. Added 200 µl of ethanol (96–100%) to the lysate, and mixed by vortexing.

xv. Labeled the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully applied the complete lysate from Step (xiv) to the QIAamp spin column without moistening rim. Closed the cap and centrifuged at full speed for 1 min. Placed the QIAamp spin column in a new 2 ml collection tube and discarded the tube containing the filtrate.

xvi. Carefully opened the QIAamp spin column and added 500 µl Buffer AW1. Centrifuged at full speed for 1 min. Placed the QIAamp spin column in a new 2 ml collection tube and discarded the collection tube containing the filtrate.

xvii. Carefully opened the QIAamp spin column and added 500 µl Buffer AW2. Centrifuged at full speed for 3 min. Discarded the collection tube containing the filtrate.

xviii. Transferred the QIAamp spin column into a new labeled 1.5 ml microcentrifuge tube and pipetted 40 µl Buffer AE to one replicate and 50 µl Buffer AE to the other replicate directly onto the QIAamp membrane. Incubated for 1 min at room temperature, then centrifuged at full speed for 1 min to elute DNA.

After the extraction of DNA, PCR reaction was performed on the samples for amplification of the DNA using the method suggested by Leigh et al. (2007). The PCR reactions were performed on all the replicates: one sample with *E.* coli DNA in it as a pure culture (used as a

positive control) and one negative control with no DNA. The PCR reaction resulted in multiple copies of the 16S rRNA gene—the gene which is responsible for protein synthesis in the cell and present in all bacteria. This gene acts as a marker of the bacteria; it is present in the database and can be used for sequencing after the T-RFLP. The database, however, only gives possible matches and can be objectionable at times. The DNA samples were PCR-amplified using primers 27F and 1392R targeting eubacterial 16S rRNA genes (Johnson, 1994). Primer 27F was labeled with 6-carboxyfluorescien (6-FAM) on the 5′ end. PCR was performed by initially amplifying a 1 ng template with 5 pmol primer in a 25 µl reaction using a thermocycler program of 95°C for 9 min, then 25 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1 min 40 s, and final extension at 72°C for 10 min, followed by a reconditioning step in which 5 µl aliquots of initial PCR product were transferred to new reactions and amplified for 3 cycles under the same PCR conditions, except in 50 µl volumes with 10 pmol primers. Figure 3.13 shows the thermocylcer used for the PCR purification.

Figure 3.13: Thermal cycler.

Agarose gel electrophoresis was employed to check the progression of restriction enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel. The PCR mixture with amplified DNA was put in a well in an agarose gel slab. Under the influence of electric supply, the DNA, which is negatively charged, moved toward the positive electrode. The longer the DNA strand, the slower it will move. When compared with the molecular weight, the sample gives the size of the DNA strand. The equipment for the electrophoresis method included horizontal gel electrophoresis apparatus, a gel casting platform, gel combs, and DC power supply. The procedure for the electrophoresis is described below:

i. The gel was prepared by mixing 1.2 g of electrophoresis-grade agarose into 100 ml of electrophoresis buffer (1x TAE). The mixture was cleaned by melting in the microwave oven.
ii. The mixture was cooled and then poured into a sealed gel-casting platform. The gel comb was inserted in order to make wells or slots.
iii. The seal was removed from the gel casting platform after the gel was hardened. The gel comb was withdrawn.
iv. The gel-casting platform was placed into an electrophoresis tank containing sufficient electrophoresis buffer to cover the gel.
v. DNA samples were prepared by adding 1 µl of loading buffer (dye) to 10 µl of the samples, the negative control and the positive control. Also, a molecular weight marker sample was prepared by adding 1 µl of the dye to 5 µl to the marker. These samples were loaded into the wells with a pipettor.
vi. The electrophoresis was run by attaching the leads at 100 volts for 45 minutes. The leads were attached such that the DNA migrated to the anode or positive lead.
vii. The power supply was turned off when dye from the loading buffer had migrated a distance judged sufficient for separation of the DNA fragments.
viii. The gel was first stained with 0.5 µg/ml ethidium bromide for 10 to 30 min. Ethidium bromide is a potential carcinogen, so gloves must be worn when handling it.
ix. The stained gel was photographed on a UV transilluminator using AlphaImager. Figure 3.14 shows the used UV transilluminator.

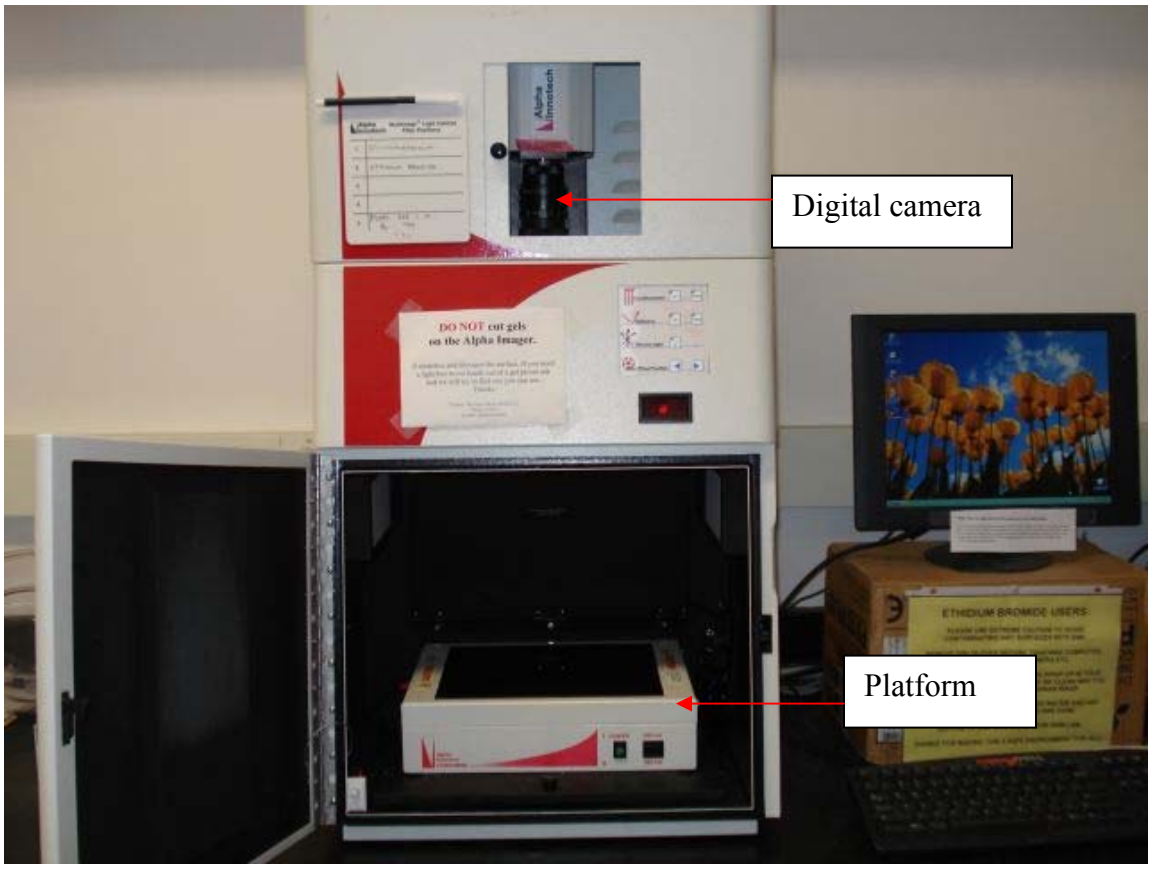


Figure 3.14: UV transilluminator.

The experimental setup for the agarose gel electrophoresis is shown in Figure 3.15.

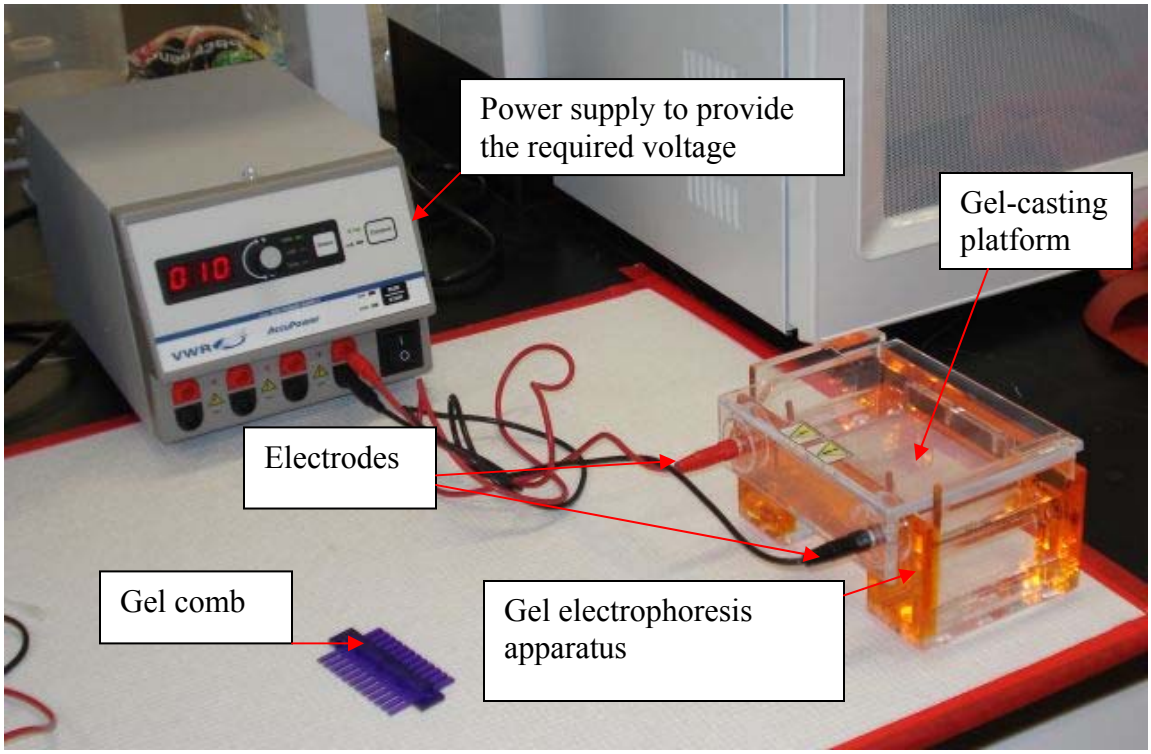


Figure 3.15: Experimental setup for agarose gel electrophoresis.

PCR products of all the samples and positive control were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). The procedure used for the PCR purification is described below:

i. Added 500 µl of Buffer PBI to 100 µl of the PCR sample and mixed by vortexing; it is checked whether the color of the mixture is yellow.
ii. Placed a QIAquick spin column in a provided 2 ml collection tube.
iii. Applied the sample to the QIAquick column to bind DNA and centrifuged for 30 to 60 s.
iv. Discarded the flow-through. Placed the QIAquick column back into the same tube.
v. To wash, added 0.75 ml Buffer PE to the QIAquick column back in the same tube. Centrifuged the column for an additional 1 min.
vi. Placed QIAquick column in a clean 1.5 ml microcentrifuge tube.
vii. Added 30 µl of nuclease-free water to the center of the QIAquick membrane for increased DNA concentration. Let the column stand for 1 min, and then centrifuged.

NanoDrop was used to quantify the purified PCR products for DNA concentration by UV spectrophotometry. Figure 3.16 shows the NanoDrop apparatus used to quantify the PCR products.

Figure 3.16: NanoDrop apparatus.

The purified PCR products were digested with restriction endonucleases. A digestion mixture was prepared for all samples and for positive control. A total of 40 ng of each sample was digested with HhaI endonuclease (New England Biolabs, Beverly, MA, USA) in reaction volumes of 15 µl by incubating at 37°C for 3 h in a water bath. The digest for each sample was precipitated by adding 1.5 µl sodium acetate (0.75 M), 0.3 µl glycogen (molecular biology grade), and 47 µl ethanol (100%). Also, an undigested aliquot (10–20 ng) of positive control was precipitated by adding the above-mentioned components. The mixture was

incubated overnight at room temperature in the dark. The incubated mixture was spun at maximum speed for 35 min at room temperature. The mixture was transferred to a 1.5 ml tube, and 100 µl of cold ethanol (70%) was added to the mixture. The mixture was allowed to stand for 5 min and then spun at maximum speed for 20 min. The supernatant mixture was dried completely in the DNA Speed Vac. Figure 3.17 shows the DNA Speed Vac used for drying the supernatant mixture.

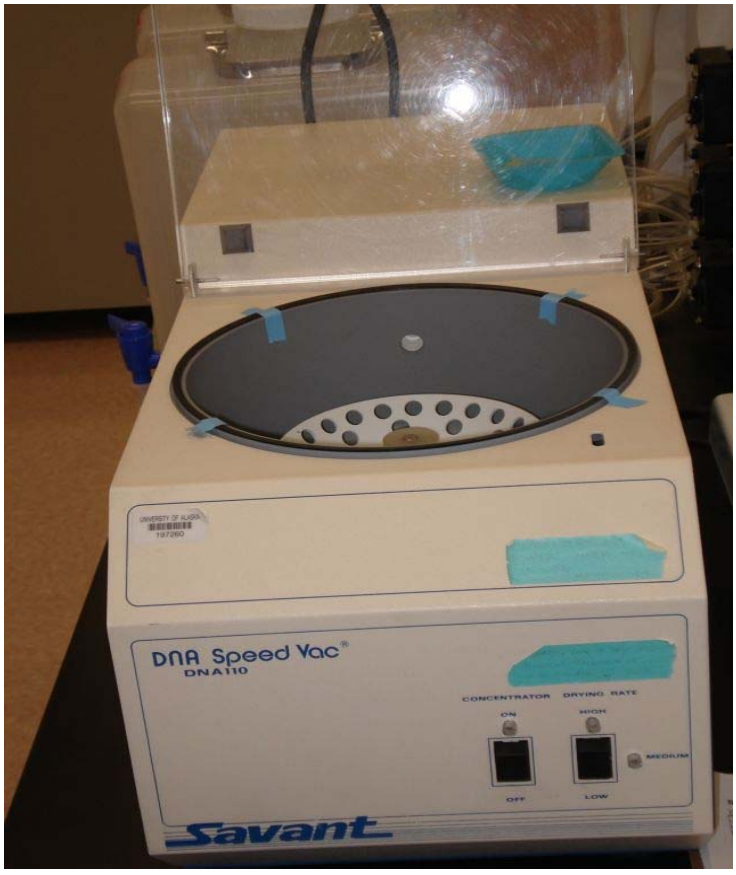


Figure 3.17: DNA Speed Vac.

The dried pellet of each sample was suspended in a mixture of 14.5 µl of HiDi formamide and 0.5 µl of BioVentures ladder. The mixture was mixed by vortexing and set aside for 5 to 10 min. The mixture was transferred to the MicroAmp plate, and then was denatured for 6 min at 97°C using the thermocycler. T-RFLP fingerprinting was carried out on an ABI 3100 genetic analyzer. Figure 3.18 shows the genetic analyzer used for T-RFLP fingerprinting.

Figure 3.18: AB 3100 Genetic Analyzer.

3.7 Isolation and assessment of indigenous surfactant-producing microbes

3.7.1 Enrichment culture conditions

Enrichment culture methods are a valuable tool for obtaining microbes from the environment, particularly microbes in low abundance such as those present in crude oil. The general methodology involves adding an inoculum (crude oil) to a sterile liquid microbial growth medium and incubating on a shaker until substantial growth is observed. This mixed culture is then amenable to further study, such as through the isolation and identification of various strains. In this study, enrichment cultures were established using five different ANS oils in order to first generate a high-biomass mixed culture of indigenous ANS microorganisms. In order to detect a broad range of microbes with a variety of growth requirements, enrichment cultures were established using a variety of media at different temperatures. The media were selected to support growth of populations that utilize a range of different carbon sources, including sugars and proteins or petroleum hydrocarbons. Enrichment cultures were incubated on rotary platform shakers and shaken constantly at 200

rpm. Table 3.6 summarizes the volumes, inocula, media, and incubation temperatures of each of the enrichment cultures established.

Table 3.6: Volumes, inocula media, and incubation temperatures of the enrichment cultures.

Medium	Date Inoculated	Medium volume (ml)	Oil volume (µl)	Inoculum	Incubation temperature (°C)
LB Broth	5/12/2008	10	100	Prudhoe Bay-W	25
	5/16/2008	100	200	Milne Point	25
	5/16/2008	100	200	Kuparuk	25
	5/16/2008	100	200	Prudhoe Bay-P	25
	5/16/2008	50	100	Prudhoe Bay-W	25
	5/16/2008	100	200	West-Sak (D Sand)	25
Bushnell-Haas Broth	4/28/2008	100	100	Milne Point	25
	4/28/2008	100	100	Kuparuk	25
	4/28/2008	100	100	Prudhoe Bay-P	25
	4/28/2008	100	100	Prudhoe Bay-W	25
	4/28/2008	100	100	West-Sak (D Sand)	25
Modified Bushnell-Haas Broth	5/16/2008	100	200	Milne Point	25
	5/16/2008	100	200	Kuparuk	25
	5/16/2008	100	200	Prudhoe Bay-P	25
	5/16/2008	100	200	Prudhoe Bay-W	25
	5/16/2008	50	100	West-Sak (D Sand)	25
Basal Mineral Liquid Medium (BM liquid)	1/23/2008	100	500	Prudhoe Bay + Exxon Valdez Oiled Rock	25
	6/2/2008	100	200	Milne Point	30
	6/2/2008	100	200	Kuparuk	30
	6/2/2008	100	200	Prudhoe Bay-P	30
	6/2/2008	100	200	Prudhoe Bay-W	30
	6/2/2008	100	200	West-Sak (D Sand)	30

Note: Oil samples comprise the inoculum, with the exception of one culture in which Exxon Valdez Oiled Rock was provided as inoculum in addition to Prudhoe Bay crude oil.

Media formulations used for enrichment cultures are presented in Table 3.7.

Table 3.7: Media formulations used for enrichment cultures.

Medium	Ingredient	Mass (g) per liter	Primary carbon source(s) for microbial growth
LB Broth	Bacto tryptone	10	Proteins and yeast extract
	Yeast extract	5	
	NaCl	10	
	* adjust pH to 7.5 with NaOH		
Bushnell-Haas Broth	$MgSO_4$	0.2	Crude oil (inoculum)
	$CaCl_2$	0.02	
	KH_2PO_4	1	
	$(NH_4)_2HPO_4$	1	
	KNO_3	1	
	$FeCl_3$	0.05	
	* Purchased as pre-mixed powder. mix to 1 l water.	Add 3.7 g	
Modified Bushnell-Haas Broth	Bushnell-Haas Broth (above)	1 liter	Glucose
	Glucose	2	
Basal Mineral (BM) Liquid	Na_2HPO_4	2.13	Crude oil (inoculum)
	KH_2PO_4	1.3	
	NH_4Cl	0.5	
	$MgSO_4.7H_2O$	0.2	

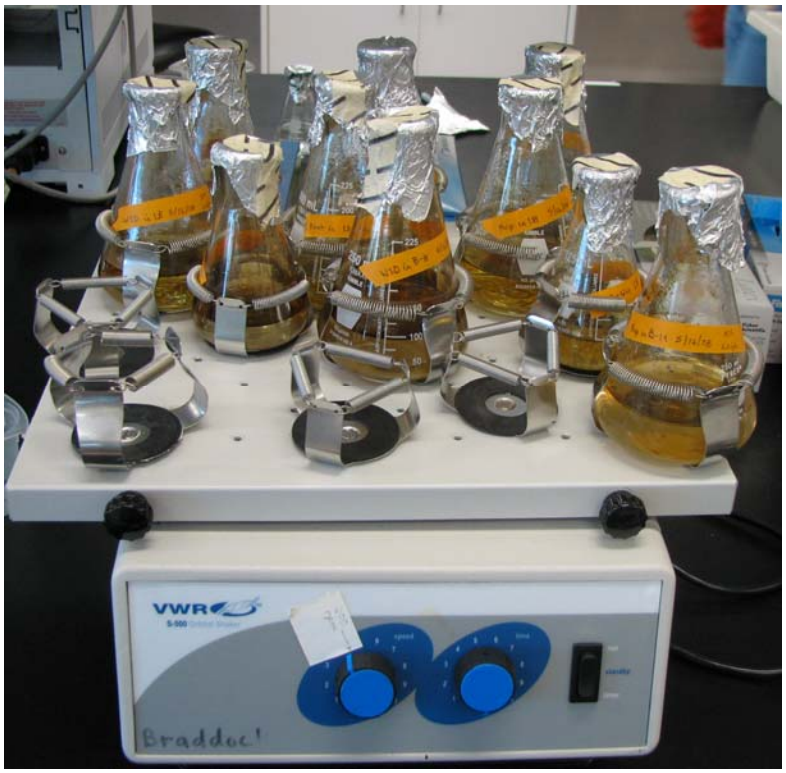


Figure 3.19: Several enrichment cultures on a rotary platform shaker.

3.7.2 High throughput screening for microbial biosurfactant production

Following growth in enrichment culture flasks, a dilution series of the enrichment culture was created using sterile water, and dilutions were then spread onto nutrient agar plates as described previously (Figure 3.2). Agar plates were incubated at 25°C until colonies had grown. Bacterial colonies are the result of a single organism that has grown, thus each colony should represent a different organism from the enrichment culture. Up to 96 colonies per enrichment culture were then individually subjected to high-throughput screening for surfactant production adapted from Chen et al. (2007). To perform the assay, sterile, transparent 96-well microassay plates were used. Each well of the 96-well plate was first filled with 150 µl of sterile M9 medium (refer to Table 3.8 for recipe). Colonies were then individually picked using sterile toothpicks, and inoculated into the wells of the 96-well plates. One well was left uninoculated to serve as a reference. Plates were incubated for 1 week at 25°C. Following incubation and cell growth, an optical distortion assay was used to identify colonies that caused a reduction in the surface tension of the medium in the clear plastic 96-well plate. To visualize the change in surface tension, the clear plate was held over a black and white grid pattern. Optical distortion of the grid in the form of enlargement indicates high surface tension, while reduction in grid size indicates a reduction in surface tension relative to the uninoculated medium. The optical distortion assay is illustrated in Figure 3.20.

Table 3.8: Formulation of M9 medium.

M9 basal medium

Component	Quantity (g per l)
Na_2HPO_4	6.00
KH_2PO_4	3.00
NaCl	0.50
NH_4Cl	1.00

M9 salts

Component	Quantity (g per 100 ml M9 salts)
$MgSO_4*7H_2O$	2.46
$CaCl_2$	0.15

Procedure:

Prepare M9 basal medium
Autoclave to sterilize
Add 10 ml M9 salts per liter M9 medium
Add 10 ml sterile 20% glucose as carbon source

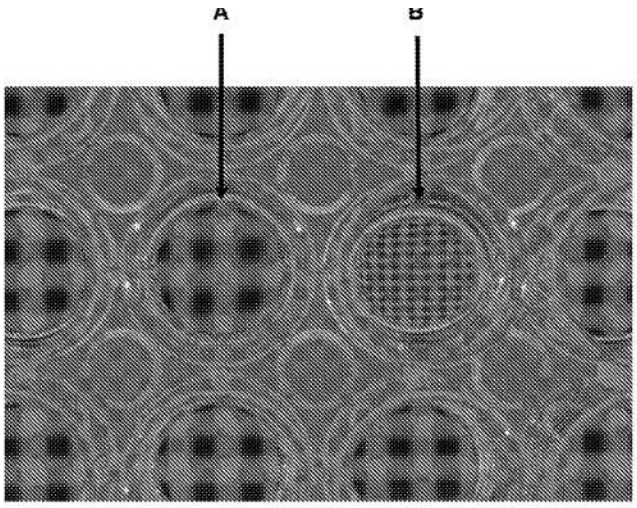


Figure 3.20: Optical distortion assay for detecting surface tension reduction. The well labeled *A* contains water, while the well labeled *B* contains a biosurfactant. (Reprinted from Chen et al., 2007.)

3.7.3. Isolation of biosurfactant-producers

When the optical distortion assay produced a positive response, indicating reduction in surface tension, then bacteria present in the positive wells were selected for isolation.

Standard microbiological streak-plate methods were used to isolate bacteria from the positive wells into pure culture. Organisms were streaked onto a sterile nutrient agar plate and incubated at 25°C until colony growth was observed. Then a single isolated colony was streaked again (three times total) to ensure purity of the culture. Pure cultures were subjected once more to optical distortion assay to verify that the surface-tension reduction properties were still present in the pure cultures.

3.8 Testing surface tension reduction by isolates

The surface-tension reduction capabilities of pure cultures that tested positively with the optical distortion assay were determined more quantitatively using a ring tensiometer. To determine the role of media formulation in surface-tension reduction, isolates were first grown in liquid culture. Strains were inoculated into both LB and M9 liquid media (Table 3.7 and 3.8). Liquid cultures were incubated on rotary platform shakers at 25°C and 30°C until turbidity was observed, indicating that microbial growth had occurred. Cultures were then subjected to centrifugation at 3000 x g for 5 min to remove the cells by pelleting. The cell-free medium was then subjected to surface tension analysis using a ring tensiometer. For reference, the well-characterized biosurfactant-producing MEOR strain *Bacillus licheniformis* RS1 (provided courtesy of Michael McInerney, University of Oklahoma) was analyzed in parallel. A schematic diagram of the ring tensiometer apparatus is shown in Figure 3.21.

Figure 3.21: Ring tensiometer apparatus

3.9 T-RFLP analyses of several ANS oils and surfactant-producing isolates

T-RFLP fingerprinting analyses were performed on five different ANS oils used for enrichment cultures to compare community composition and to determine presence and relative abundance of surfactant-producing isolates. T-RFLPs were prepared from crude oils and isolates as previously described. Peaks corresponding to surfactant-producing isolates were compared to peaks present in oils to determine whether these isolates were present and, if so, at what relative abundance.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Microbial formulation

The culturing of *Bacillus licheniformis* shows that for the nutrient medium designed in Section 3.1 of this work, the bacteria grows very well at a temperature of 30°C, which is the usual reservoir temperature for ANS oil fields. This temperature ensures the proper growth of bacteria in the microbial formulation prepared for injection in the core samples for coreflooding experiments and in the reservoir for any further pilot or field tests. All the experiments involving the microbial formulation can successfully use the designed microbial formulation.

The agar gel plates developed for determining the concentration of microbial formulation showed growth of colonies in the first 4 plates. The colonies in the first 2 plates—that is, the plates for the microbial formulation and the 1 to 100 dilution of the microbial formulation—were difficult to count due to the vast number. The number of colonies in the fourth plate—that is, the plate for the 1 to 10^4 dilution of the microbial formulation—was small (only 2 separate colonies). The third plate—the plate for the 1 to 10^3 dilution of the microbial formulation—was used to calculate the number of colonies. The remaining plates—namely, the plates for the 1 to 10^5 dilution of the microbial formulation to the 1 to 10^8 dilution of the microbial formulation—did not show any colonies. This outcome was expected, as it means that there has been no contamination during the process of dilution.

There were 80 colony-forming units (CFU) per 100 μl counted from the third plate. Thus, for the original microbial formulation used in the experimental work, the concentration was calculated as shown in equation 4.1.

$$\text{Concentration} = \frac{80\ \text{CFU} \times 10^3}{100\ \mu\text{l}} = 80 \times 10^5\ \text{CFU/ml} \tag{4.1}$$

It was assumed that each colony is a progeny of single stem cells. Thus, the number of colonies is equal to the number of cells originally in the solution. The concentration of the microbial formulation was 8 x 10^6 cells/ml. There is always some error in the counting of

colonies because colonies overlap. Taking this into consideration, the error in the concentration of the microbial formulation can safely be assumed to be 1 x 10^7 cells/ml. For every coreflooding experiment, a fresh 500 ml of microbial formulation of approximately equal concentration was made.

4.2 Porosity and absolute permeability of the cores

The brine solution used for coreflooding experiments was made as per the calculations given in Section 3.2 of this work. In order to make 500 ml of brine solution, 10.20 g of sodium chloride is thoroughly dissolved in 500 ml of deionized water. The calculated porosities of the cores ranged from 13% to 16%. Calculated porosities for all of the cores are listed in Table 4.1.

Table 4.1: Calculated porosities of the cores.

Cores	**Weight of dry core (g)**	**Weight of wet core (g)**	**Pore volume (ml)**	**Porosity (%)**
Core #1	349.73	374.74	23.98	13.8
Core #2	342.08	370.34	27.44	15.79
Core #3	343.44	372.18	25.01	14.39
Core #4	341.54	367.91	25.6	14.73
Core #5	345.27	372.18	26.13	15.03
Core #6	344.81	371.56	25.97	15.42

After initial waterflooding to determine absolute permeability, the cores were weighed again. There is always a slight difference in the weight of the cores before and after waterflooding. Though the reason for this variation in the weight is not known, it is attributed to the absence or presence of a few extra droplets of brine solution on the body of the core during the weighing process. The differences did not follow any particular trend. In 4 cores, the weights were higher after waterflooding than they were before waterflooding, whereas in the 2 remaining cores, weights were lower after waterflooding.

Differential pressure across the length of the cores was noted during waterflooding. Absolute permeability was calculated at differential pressure when steady state was attained. The absolute permeability values of the cores ranged between 27 and 68 md. The absolute permeability values of the cores are listed in Table 4.2. The pressure drop profile for determination of absolute permeability for all the cores is shown in Figure 4.1, and the plot for porosity and absolute permeability of the cores is shown in Figure 4.2.

Table 4.2: Absolute permeability of the cores.

Cores	**Differential Pressure (psi)**	**Absolute Permeability (md)**
Core #1	45	27.16
Core #2	25	48.89
Core #3	20	61.11
Core #4	18	67.9
Core #5	19	64.32
Core #6	20	61.11

Core #1 shows high pressure drop across the core. The core was subjected to initial waterflooding three times, but the same differential pressure was obtained every time. Any particular reason for the high differential pressure values is not known.

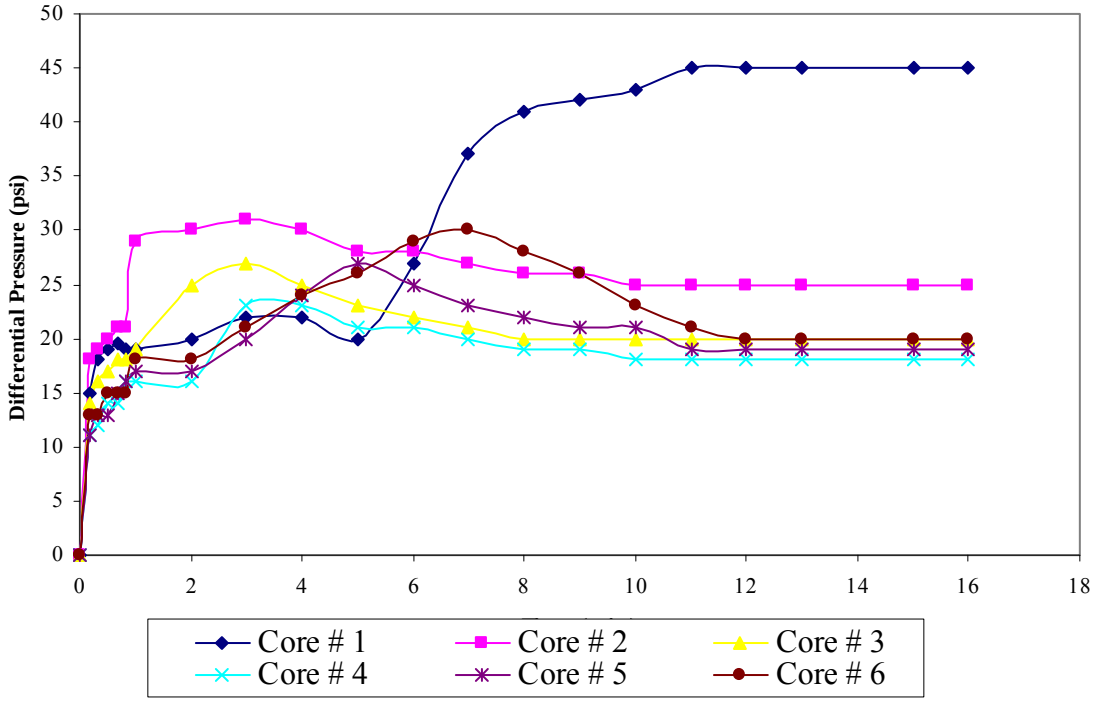


Figure 4.1: Pressure drop profile for tested core samples.

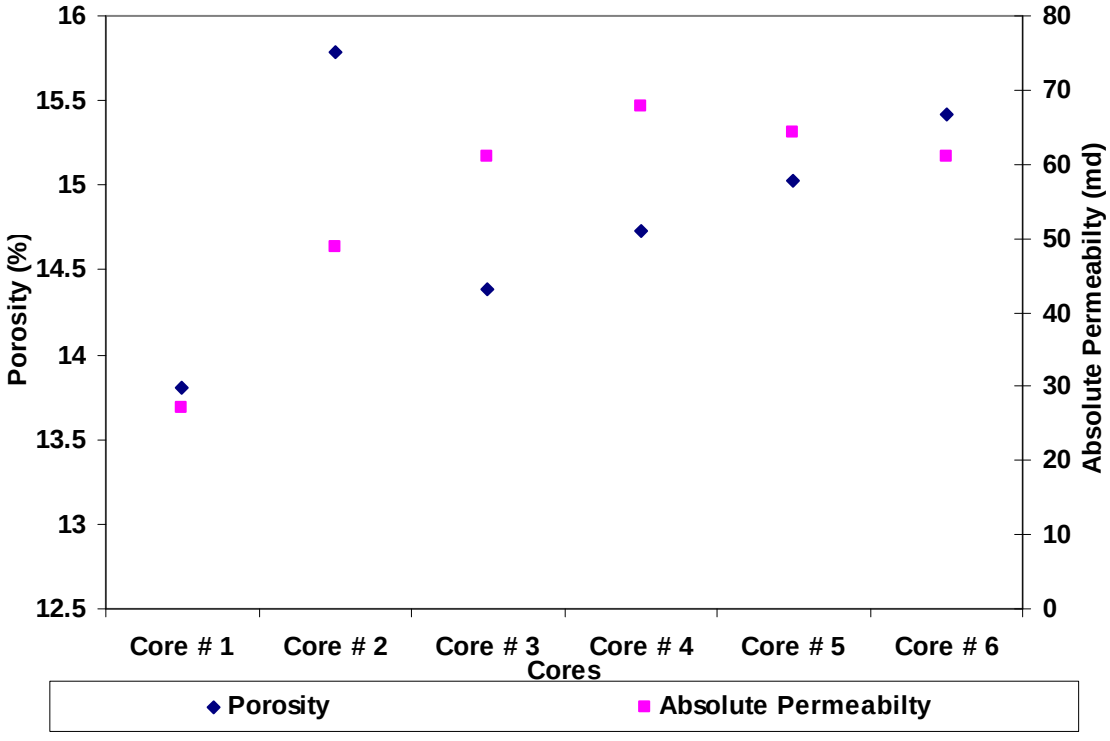


Figure 4.2: Porosity and absolute permeability values of the cores.

4.3 Irreducible water saturation

Irreducible water saturation was achieved by injecting crude oil in the core after initial waterflooding. Crude oil was injected at a constant pressure of 300 psi using the pump. For Core #1 and Core #2, n-decane was injected as a substitute for crude oil, whereas for Core #3, Core #4, Core #5, and Core #6, an ANS crude oil sample was injected. When crude oil is injected, a brine solution is produced from the core. Oil was injected until there was no more brine production. This simulates the process of migration of the hydrocarbons in the reservoir. The core was then shut in for a week so that reservoir conditions were reached.

Irreducible water saturation values for the cores ranged between 32% and 47%. Table 4.3 gives the irreducible water saturation values for the cores, and Figure 4.3 plots the values.

Table 4.3: Irreducible water saturation values for the cores.

Cores	**Volume of brine produced (ml)**	**Irreducible water saturation S_{wi} (%)**	**Initial oil saturation S_{oi} (%)**
Core #1	15.2	36.62	63.38
Core #2	14.7	46.42	53.58
Core #3	14.3	42.71	57.29
Core #4	17.1	33.01	66.99
Core #5	17.6	32.83	67.17
Core #6	17.4	34.96	65.04

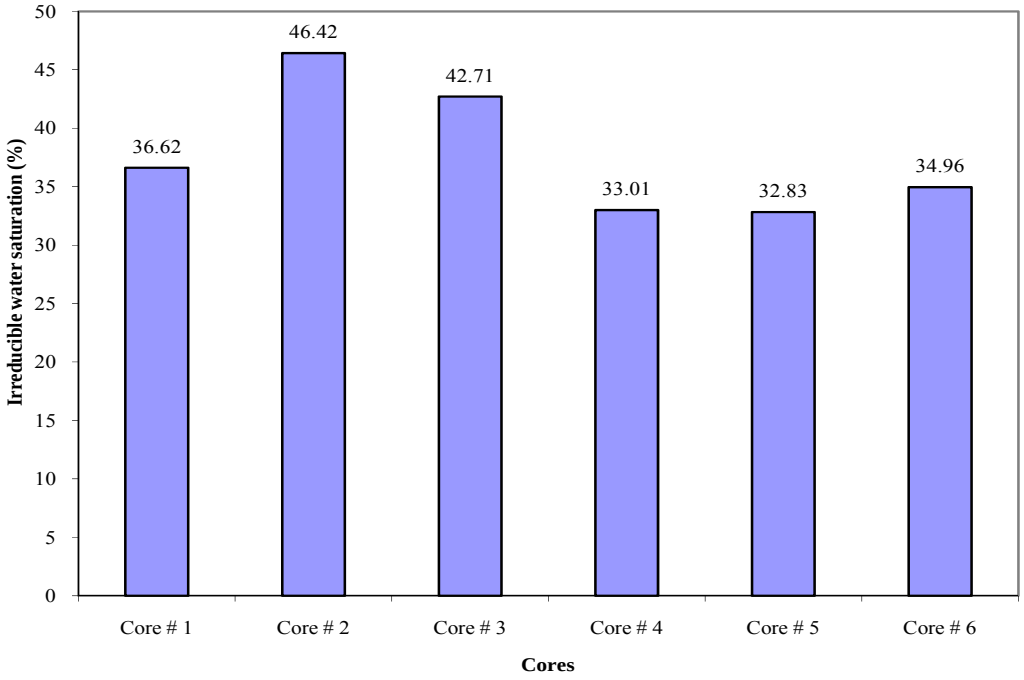


Figure 4.3: Irreducible water saturations for the cores.

4.4 Oil recovery

In this experiment, the conventional waterflooding process was simulated to recover oil from core samples. For Core #1 and Core #6, 2 molar brine solution was injected to produce oil, whereas for the remaining cores—Core #2, Core #3, Core #4, and Core #5—microbial formulation was used to simulate the brine typically used in the waterflooding process.

Oil was produced without any water production until breakthrough was reached. After breakthrough was reached, some brine solution or microbial formulation was also produced along with oil. It was observed that breakthrough was always reached between 0.5 and 1.0 PV injection. Oil remaining in the core after the waterflooding process contributes to the residual oil saturation. It is the aim of any EOR method to minimize residual oil saturation.

For the brine injection, the residual oil-saturation value ranged between 29% and 31%, whereas for the microbial-formulation injection, the residual oil saturation value ranged between 20% and 24%. The oil recovery value for brine injection ranged between 52% and

54%, whereas this value ranged between 62% and 68% for microbial formulation. These results are in conformation with the findings of Crescente et al. (2005) that, with higher concentrations of microbial formulation, there is more oil recovered when the microbial formulation is injected at initial oil saturation, that is, without previous waterflooding. Table 4.4 lists residual oil-saturation values and oil-recovery values for the coreflooding experiments. Figure 4.4 shows the plot of residual oil-saturation and oil-recovery values.

Table 4.4: Residual oil-saturation and oil-recovery values for the coreflooding experiments.

Cores	**Volume of oil produced (ml)**	**Residual oil saturation S_{or} (%)**	**Oil recovery (%)**	**Remarks**
Core #1	8.1	29.61	53.29	Decane, Brine injection
Core #2	9.2	20.04	62.59	Decane, Microbial formulation injection
Core #3	9.3	20.1	64.91	ANS oil, Microbial formulation injection
Core #4	11.6	21.68	67.64	ANS oil, Microbial formulation injection
Core #5	11.5	23.16	65.53	ANS oil, Microbial formulation injection
Core #6	9.4	30.8	52.64	ANS oil, Brine injection

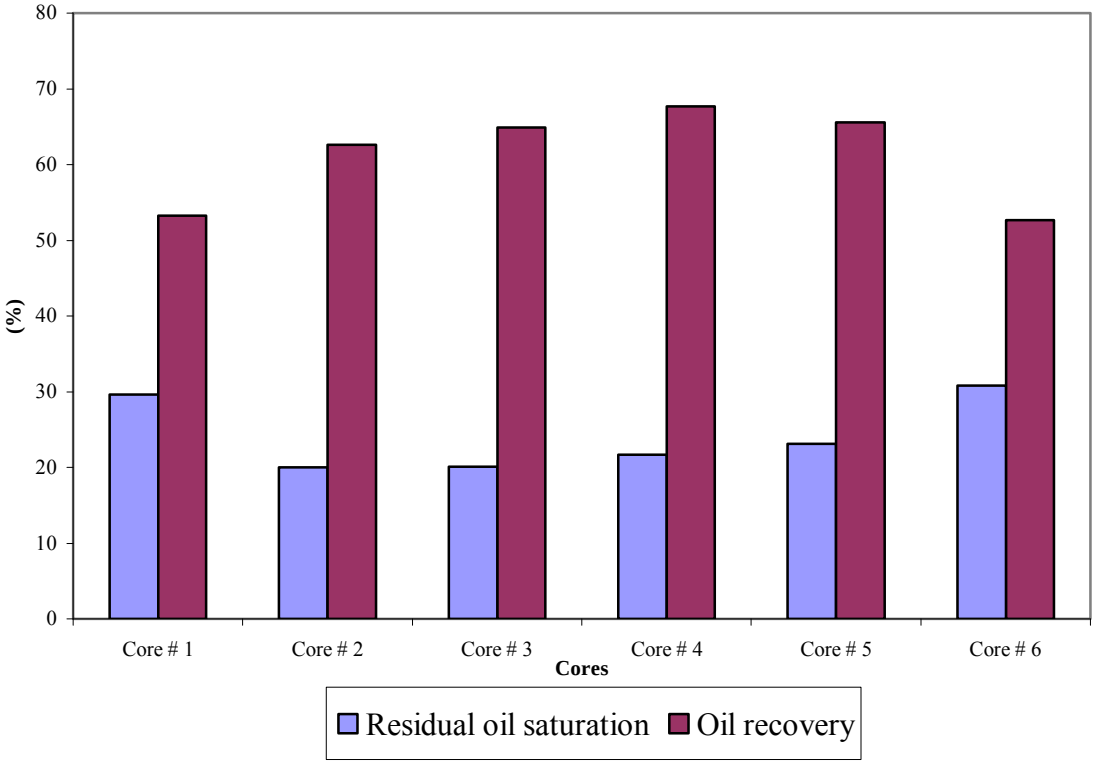


Figure 4.4: Residual oil saturation and oil recovery for the cores.

Figure 4.5 to Figure 4.10 show the oil recovery profile in terms of the percentage of oil recovered from the injection of brine solution and microbial formulation. The tables listing the PV brine injected, volume of oil produced, and oil recovery results for the cores are included in Appendix A.

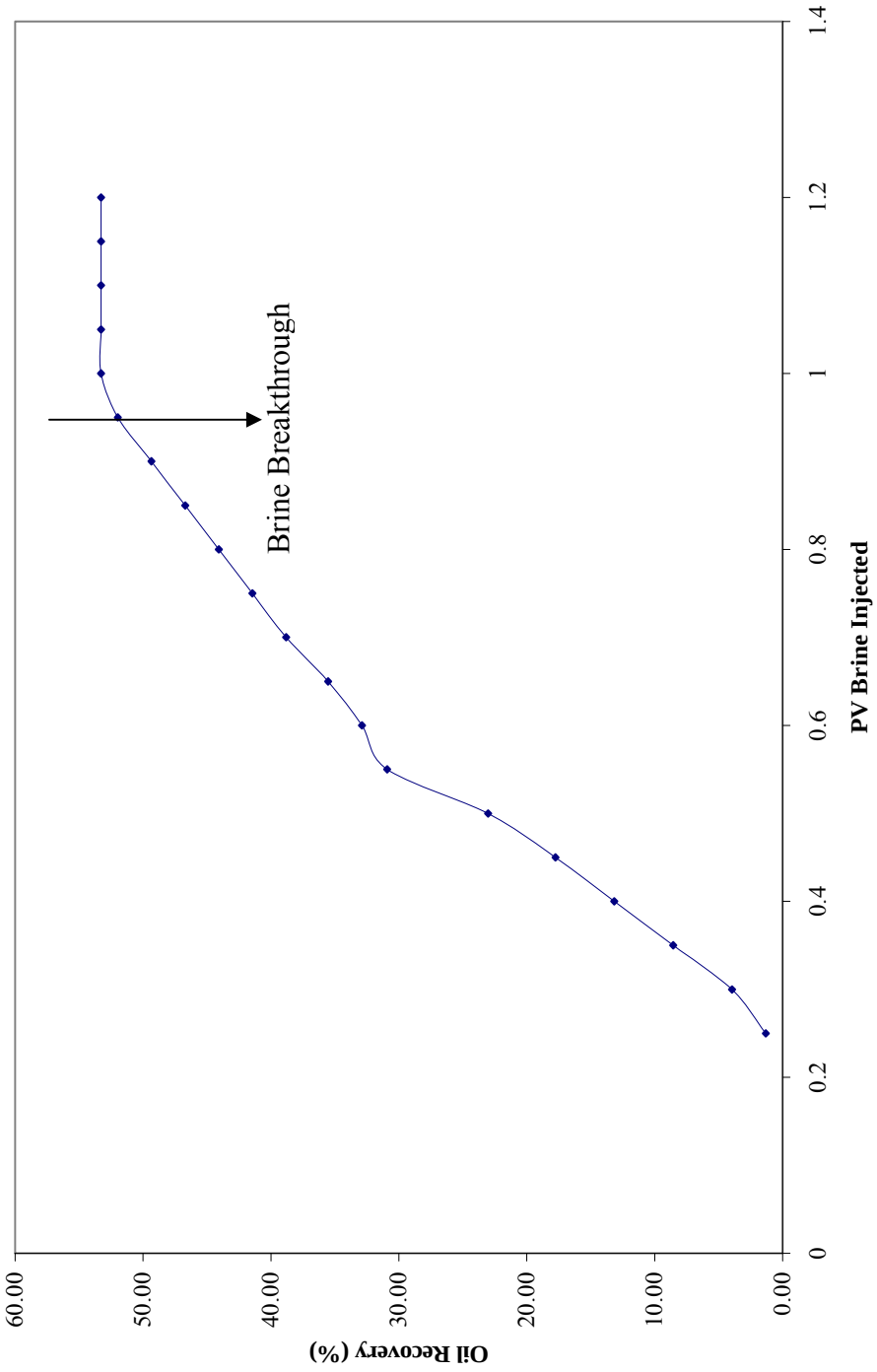


Figure 4.5: Oil recovery profile for Core #1 (φ = 13.8%, k = 27.16 md).

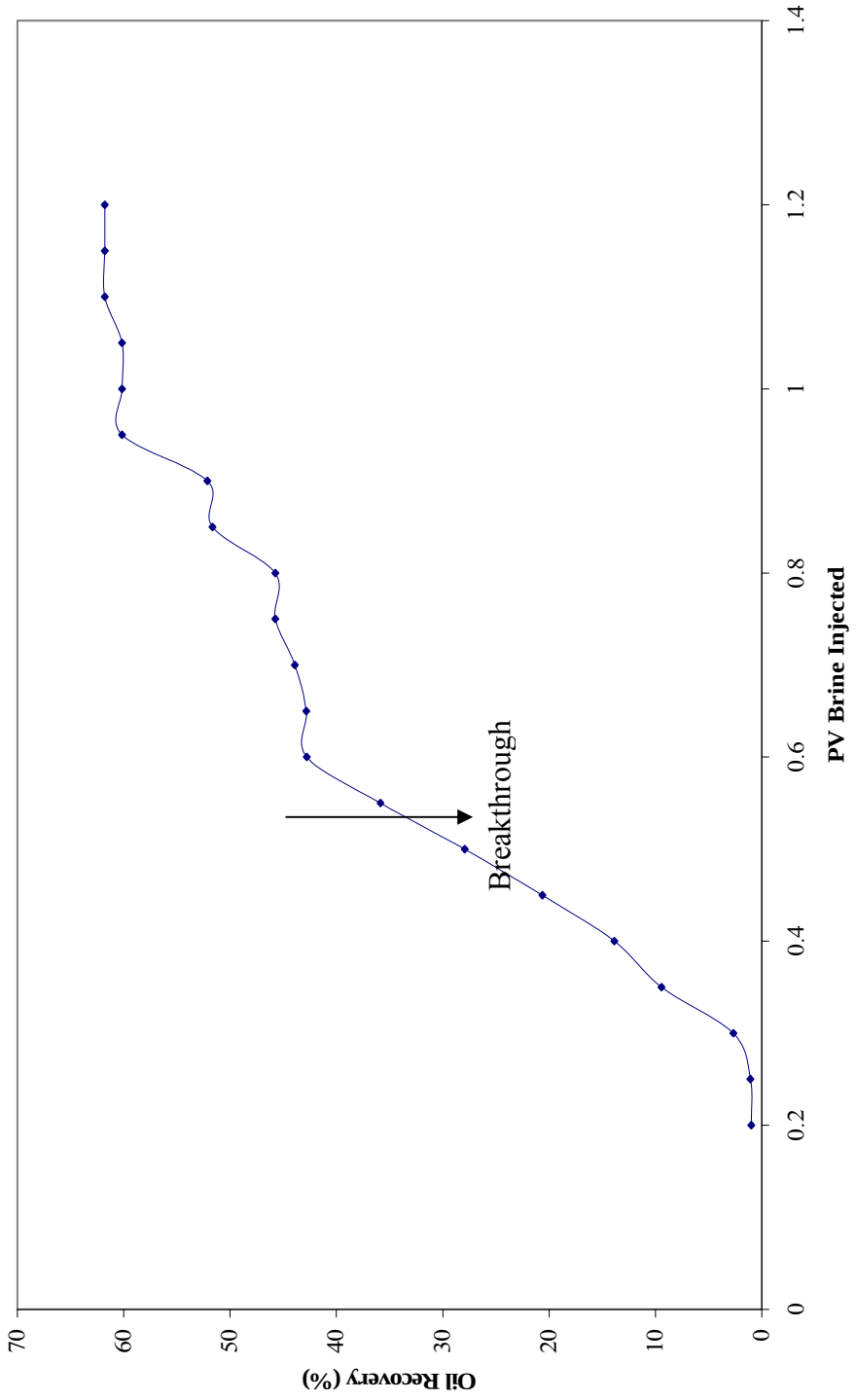


Figure 4.6: Oil recovery profile for Core #2 (φ = 15.79%, k = 48.89 md).

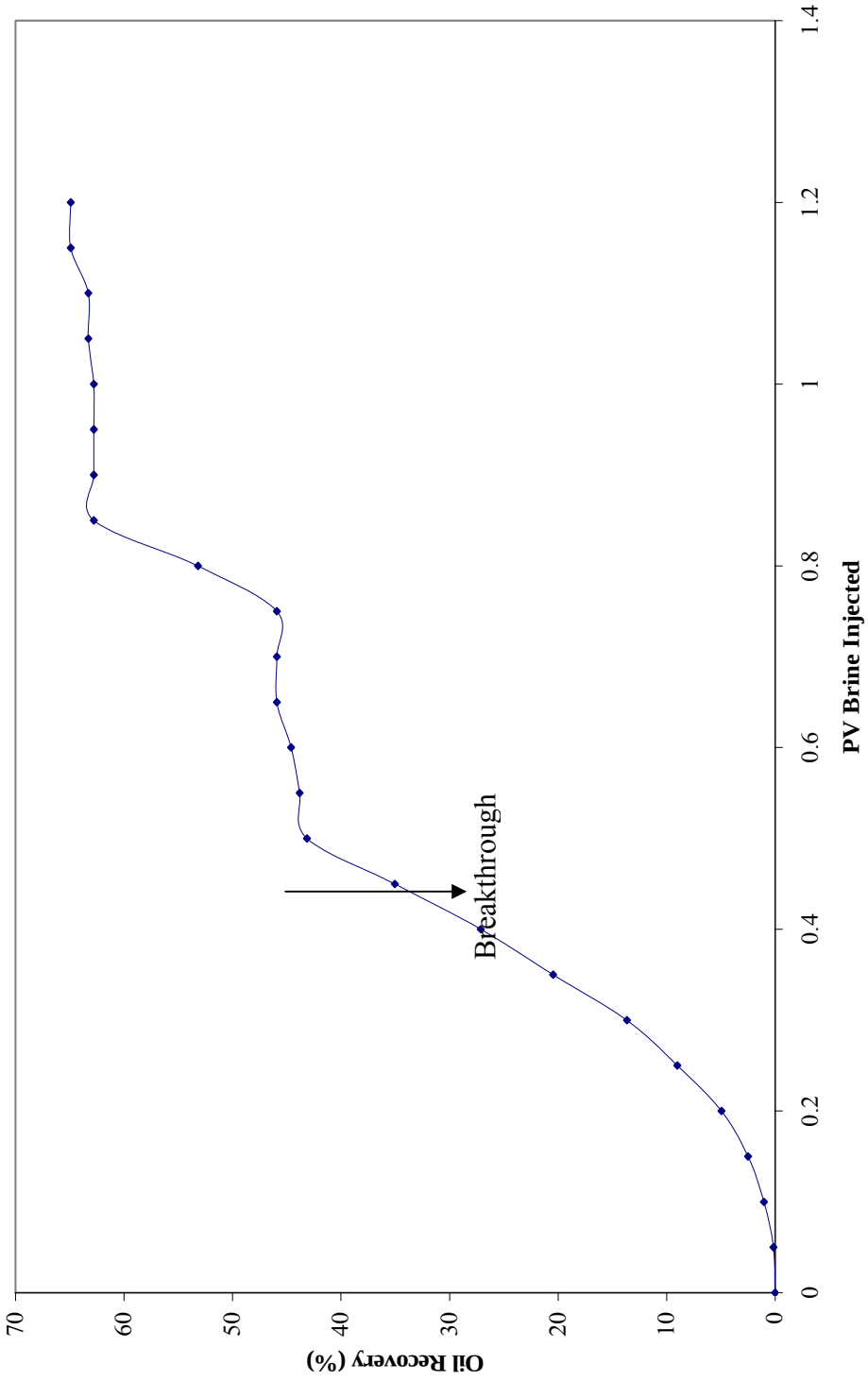


Figure 4.7: Oil recovery profile for Core #3 (φ = 14.39%, k = 61.11 md).

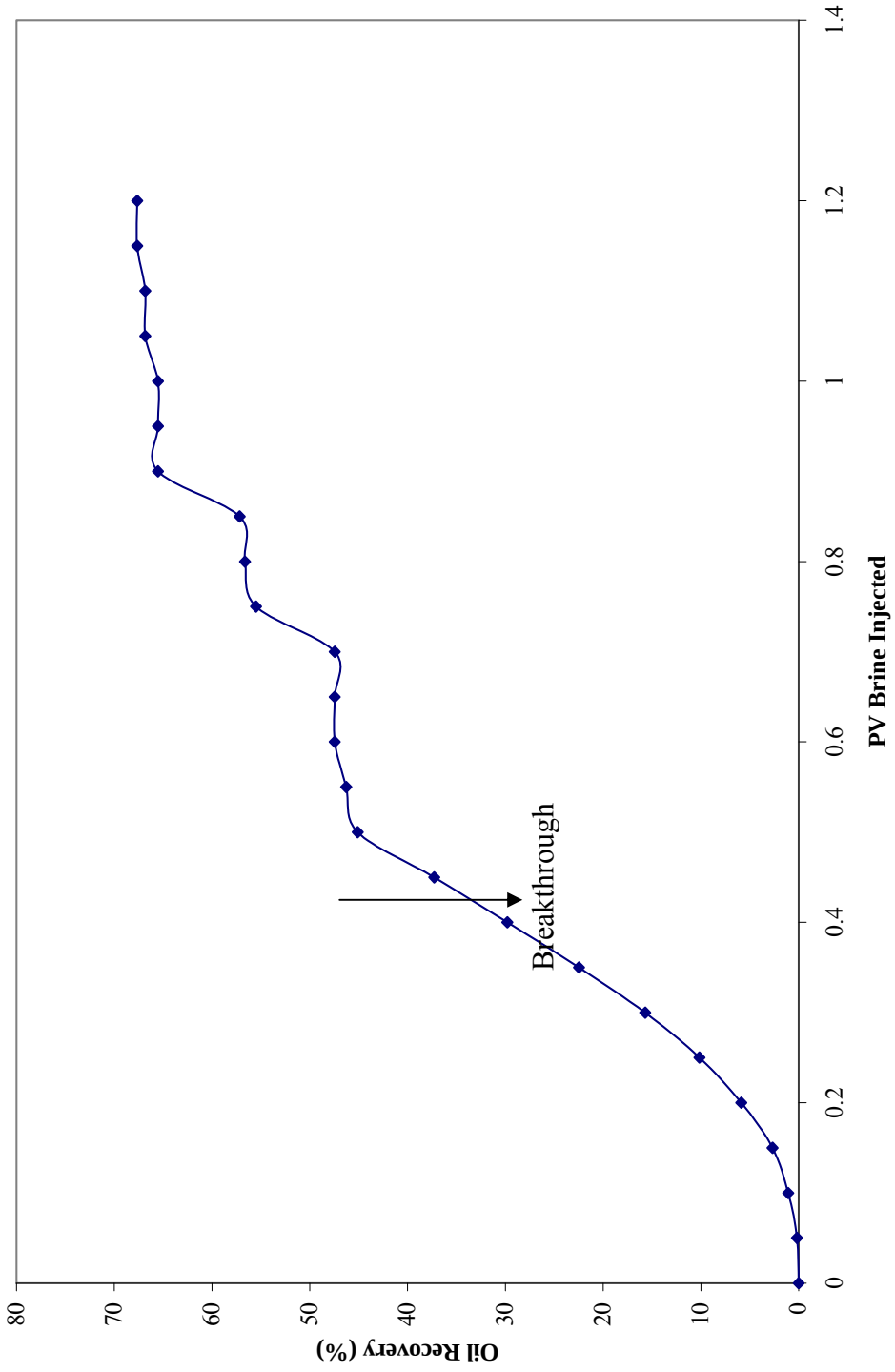


Figure 4.8: Oil recovery profile for Core #4 (φ = 14.73%, k = 67.90 md).

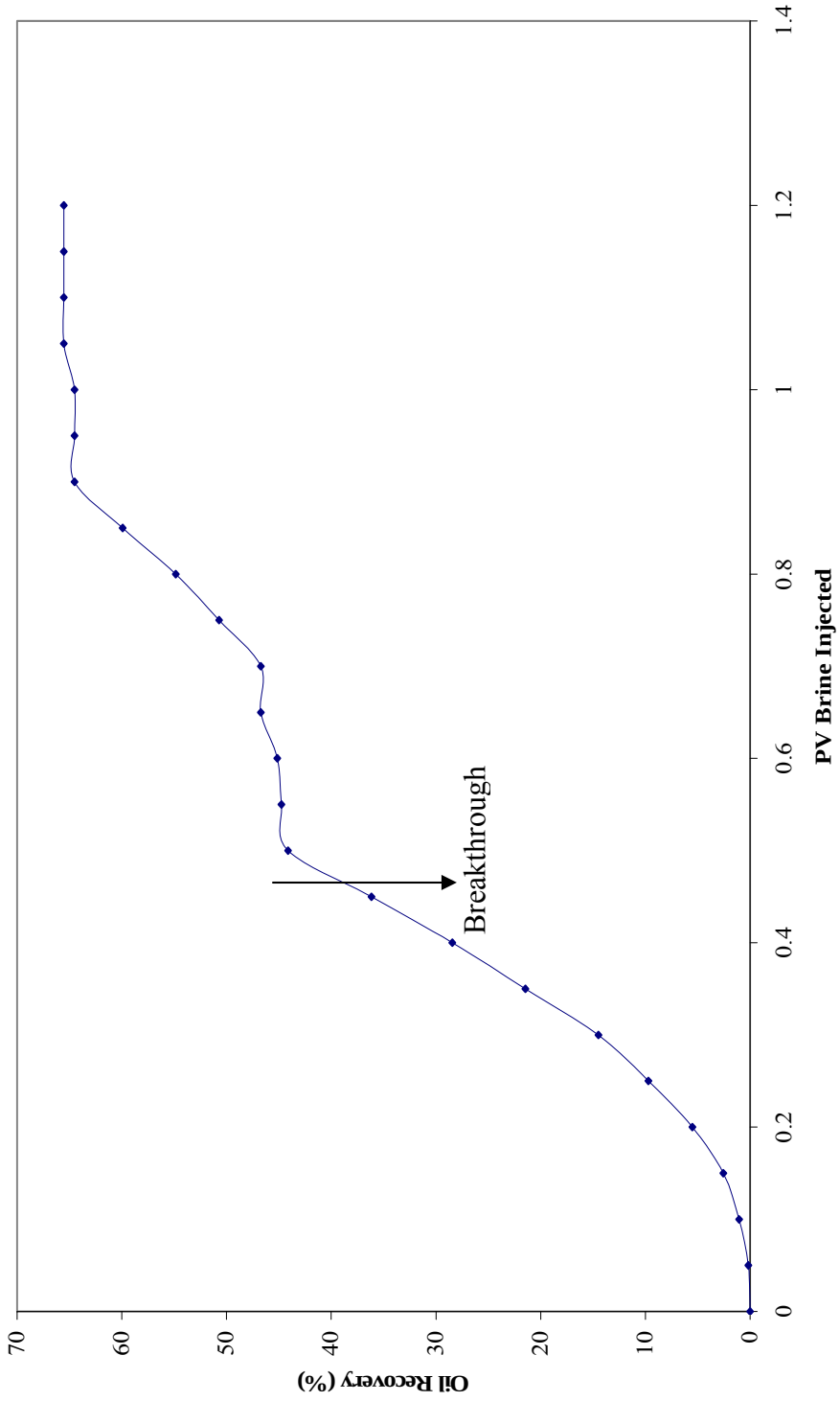


Figure 4.9: Oil recovery profile for Core #5 (φ = 15.03%, k = 64.32 md).

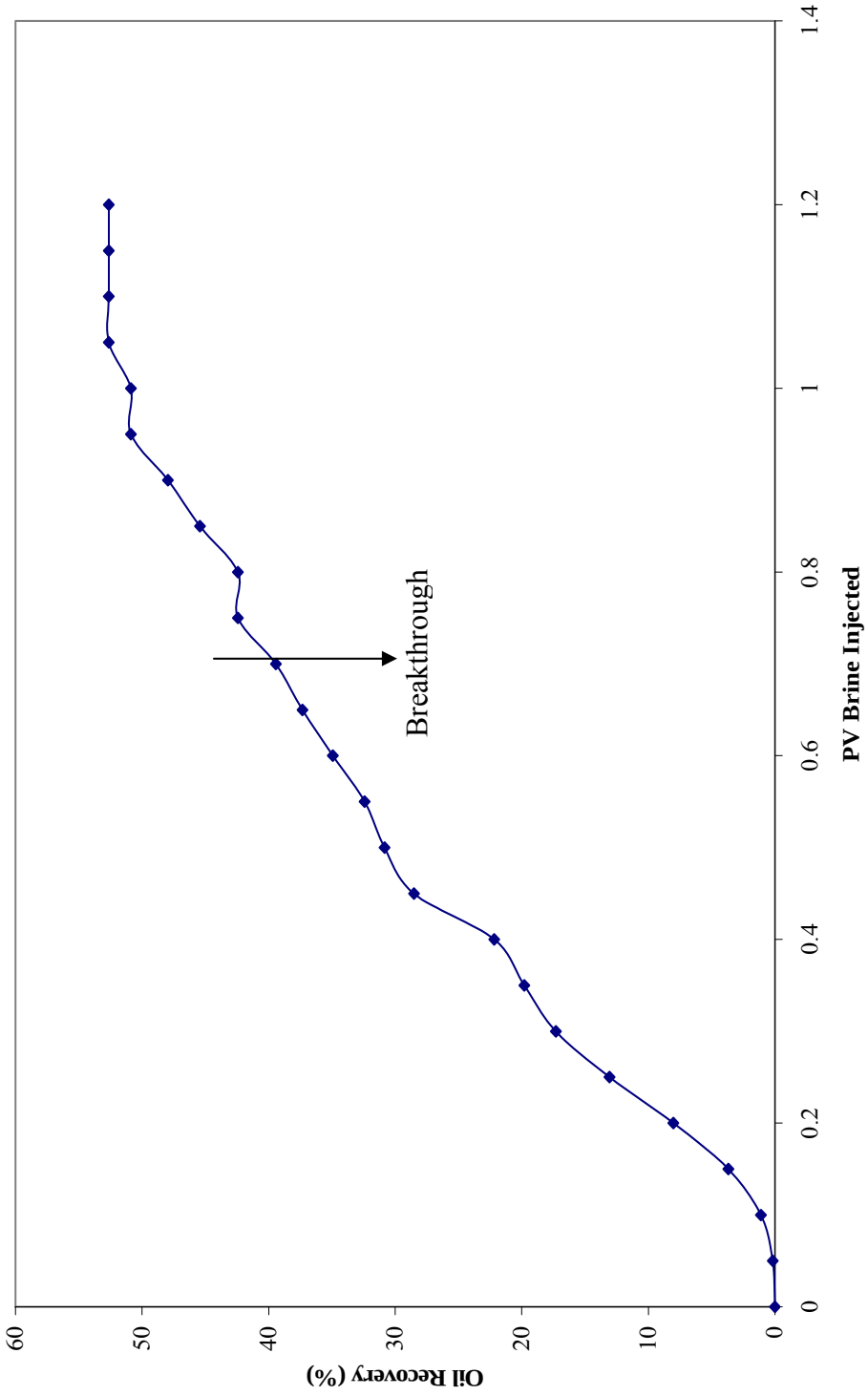


Figure 4.10: Oil recovery profile for Core #6 (φ = 15.42%, k = 61.11 md).

4.5 Incremental oil recovery

Microbial formulation was injected in Core #1 and Core #6 after waterflooding. There was no oil production, as the residual oil saturation was already reached. Core #3 was kept in contact with the microbial formulation in a tightly corked conical flask for two months. This was done to determine whether the variation in shut-in time has any effect on oil recovery. All the cores were shut in for one week after the injection of microbial formulation. During this week, the microbes were expected to reproduce and use the nutrient media to produce bioproducts, including biosurfactants and biogases (mostly carbon dioxide).

The produced biosurfactants reduce interfacial tension between oil and brine in the core. Interfacial tension reduction from 38 mN/m to 0.006 mN/m has been reported in literature (Kowalewski et al., 2005). Interfacial tension reduction helps by releasing droplets of oil captured in the pores. Biosurfactants change the wettability of the system, thus modifying relative permeability. The produced biogases help in oil recovery by increasing pressure. However, at experimental low pressures, this effect is not considerable.

Cores are produced by injecting 2 molar brine solution after the shut-in period. The collected oil is the incremental oil recovery. This recovery is compared with the residual oil saturation. For Core #3, some oil was released in the conical flask, so the mixture of oil and microbial formulation was divided into separated graduated cylinders, and the oil and microbial formulation was allowed to separate under gravity. Core #3 was also produced in the coreflooding rig by injecting brine solution. The produced oil and the released oil, together, contributed to the increase in oil recovery.

The incremental oil recovery due to microbial treatment in Core #1 and Core #6, where the microbial formulation was injected after waterflooding, ranged between 7% and 8%. The incremental oil recovery for the other cores, where the microbial formulation was injected without previous waterflooding, ranged between 11% and 13%. Table 4.5 lists incremental oil recovery for the cores. Figure 4.11 shows the plot of incremental oil recovery for all core samples.

Table 4.5: Incremental oil recovery values for all the cores.

Cores	Volume of oil produced due to microbial activity (ml)	Incremental oil recovery (%)
Core #1	0.5	7.04
Core #2	0.7	12.73
Core #3	0.6	11.94
Core #4	0.7	12.61
Core #5	0.7	11.57
Core #6	0.6	7.27

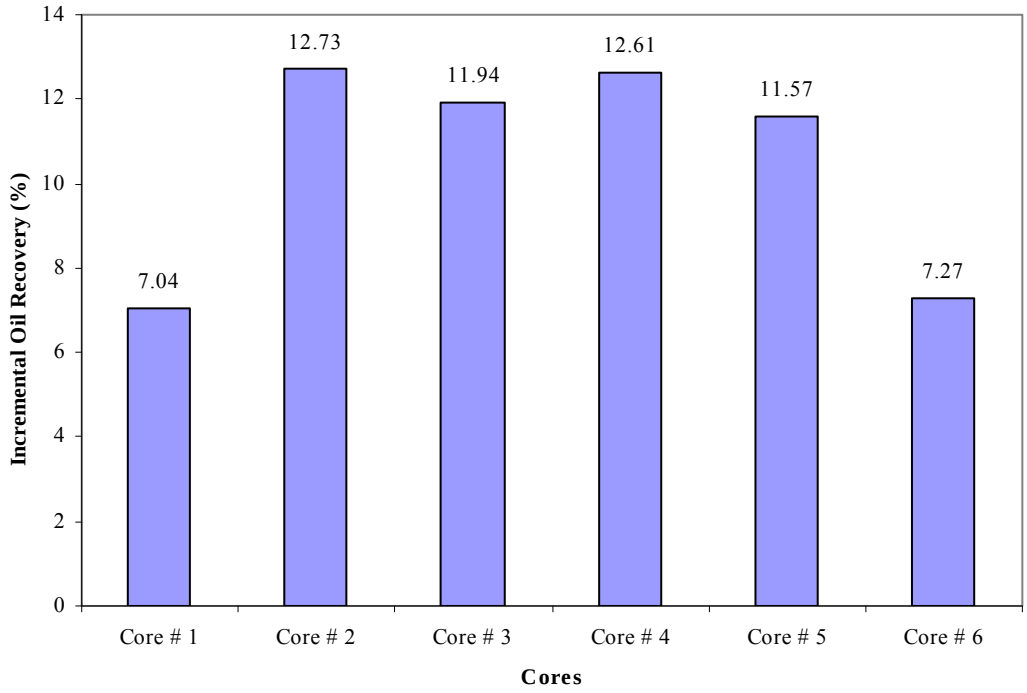


Figure 4.11: Incremental oil recovery for the cores.

Incremental oil recovery after the one-week shut-in period and the two-month shut-in period is between 11% and 13%. It can be concluded that the microbial activity required for incremental oil recovery stops before one week. It is possible that the microbial formulation reaches a stationary phase before one week passes. The reason for reaching the stationary phase could be that all the nutrients are used up by the microorganisms, or there is production

of toxins by the microorganisms which inhibit their further growth. It is possible to determine the life span of the microbial formulation by shaking the mixture of oil sample and microbial formulation in a shaker and observing the concentration of the microorganism in the oil as well as the aqueous phases every day. This can be done by setting up agar gel plates for equal amounts of the oil and the aqueous phase every day and counting the colonies grown over a period of time. This procedure would give a plot of the concentration versus time, showing an exponential increase in concentration in the beginning and then no increase in concentration. The exponential increase represents the growth phase, whereas no increase represents stationary or lag phase. The time needed for the transition from growth phase into stationary phase is the life of the microbial formulation. After this time, there is no further microbial activity in the microbial formulation.

4.6 Total oil recovery

Total oil recovery is the total volume of oil produced from one core sample due to the combined effect of the waterflooding process and incremental oil recovery due to microbial activity. Total oil recovery for Core #1 and Core #6 was 56.58% and 57.47%, respectively, whereas for the other cores it ranged from 67% to 72%. Table 4.6 gives the total oil recovery for all cores. Figure 4.12 shows a plot of total oil recovery of the cores. It can be seen that there was a decrease in residual oil saturation for all cores. The decrease ranged from 2% to 3%. Appendix B includes the coreflooding calculations for Core #1.

Table 4.6: Total oil recovery for all cores.

Cores	Volume of oil produced by waterflooding (ml)	Volume of oil produced due to microbial activity (ml)	Residual oil saturation (S_{or}) (%)	Total oil recovery (%)
Core #1	8.1	0.5	27.52	56.58
Core #2	9.2	0.7	17.49	67.35
Core #3	9.3	0.6	17.71	69.09
Core #4	11.6	0.7	18.95	71.72
Core #5	11.5	0.7	20.47	69.52
Core #6	9.4	0.6	28.49	57.47

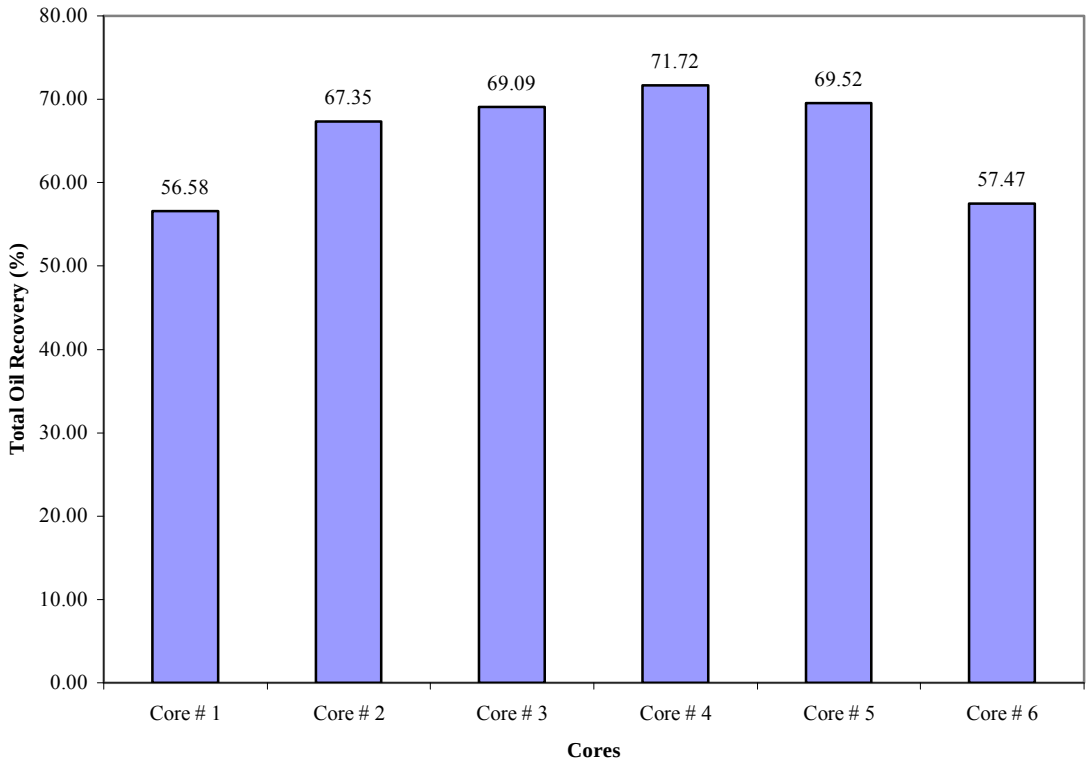


Figure 4.12: Total oil recovery of the cores.

4.7 Compositional analysis

The working and calibrating procedure for the gas chromatograph is described in Section 3.2 of this work. The oil sample from the ANS oil field was run in the gas chromatograph. The oil sample was centrifuged in order to remove any traces of water, as water can interfere with the analysis. Figure 4.13 shows the chromatograph for the oil sample before any microbial treatment. Table 4.7 lists the composition of the oil sample before the microbial treatment.

A similar analysis was run on the oil sample after treatment with microbial formulation. The oil sample extracted from the core sample after one week was analyzed. Table 4.8 gives the composition of the oil sample after microbial treatment. Figure 4.14 shows the chromatograph for the oil sample after microbial treatment.

Table 4.7: Composition of oil sample before microbial treatment.

Component	Concentration (% wt/wt)
C_6	8.1141
C_7	0.4638
C_8	0.9996
C_9	3.1374
C_{10}	3.1276
C_{11}	3.5417
C_{12}	4.5894
C_{13}	6.5752
C_{14}	6.1723
C_{15}	6.9842
C_{16}	4.6520
C_{17}	6.3153
C_{18}	5.7460
C_{19}	4.9002
C_{20}	4.6336
C_{21}	4.6304
C_{22}	2.4470
C_{23}	3.8778
C_{24}	2.1898
$C_{25}+$	16.9025

Table 4.8: Composition of oil sample after microbial treatment.

Component	**Concentration (% wt/wt)**
C_6	4.53538
C_7	0.02819
C_8	0.22068
C_9	0.84800
C_{10}	1.45824
C_{11}	2.74514
C_{12}	5.67851
C_{13}	6.99560
C_{14}	7.71113
C_{15}	9.75397
C_{16}	4.76297
C_{17}	7.15860
C_{18}	6.90621
C_{19}	5.56656
C_{20}	5.36645
C_{21}	4.53684
C_{22}	3.11105
C_{23}	4.06205
C_{24}	2.43360
$C_{25}+$	16.12081

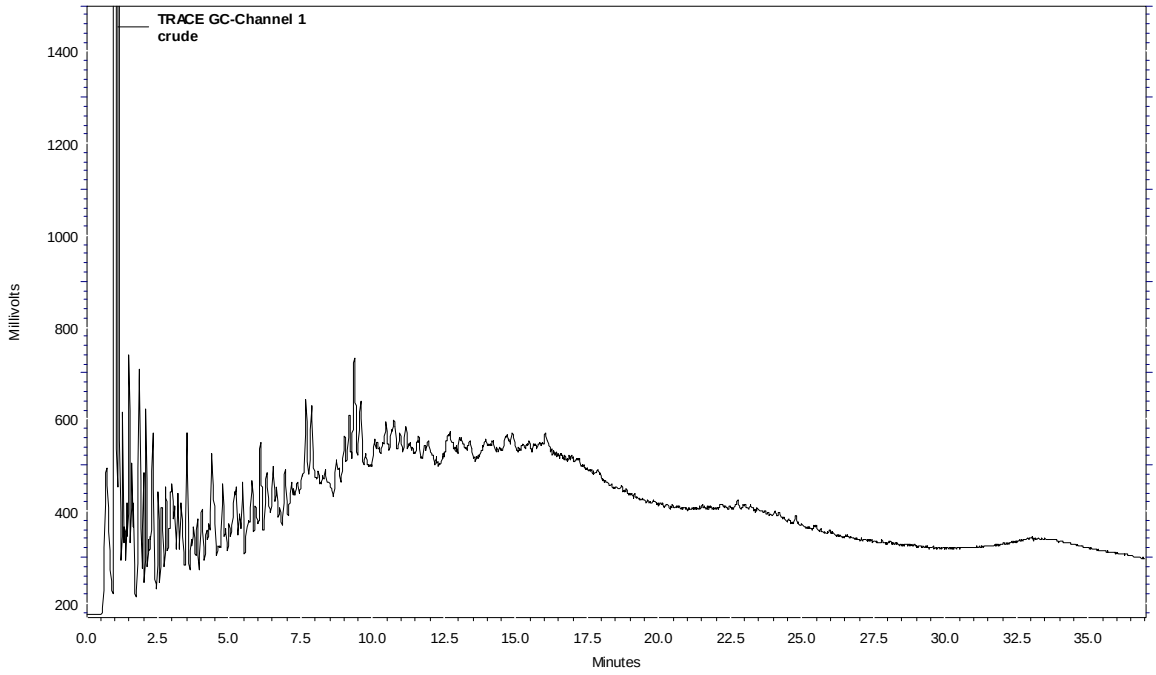


Figure 4.13: Chromatograph of oil sample before microbial treatment.

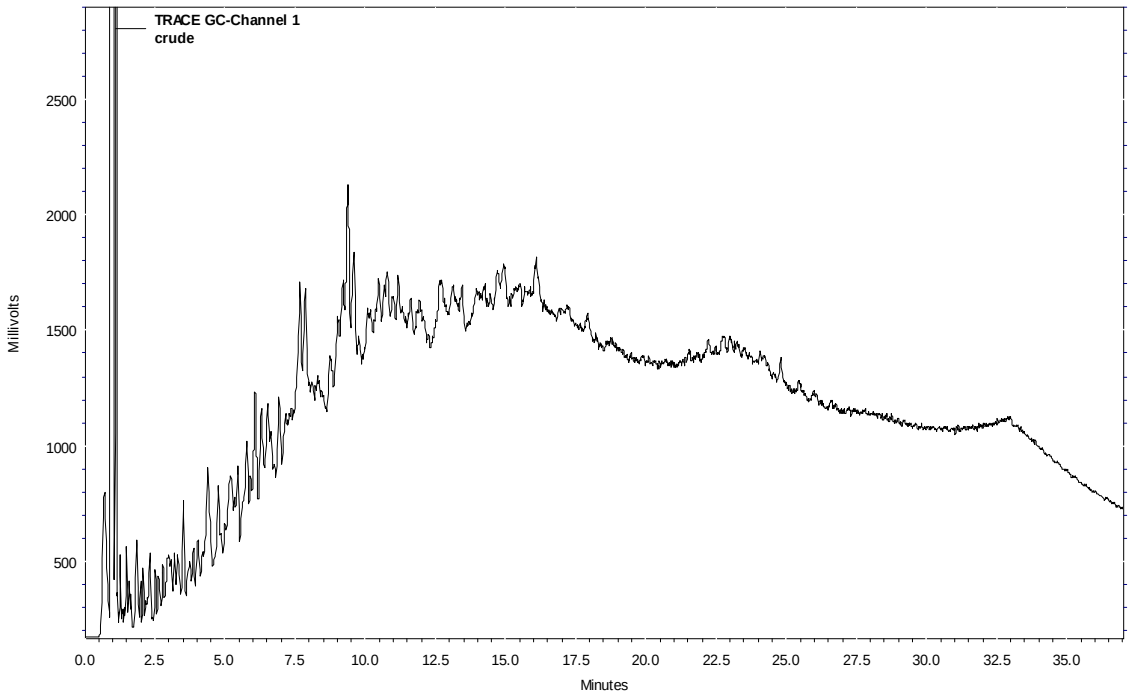


Figure 4.14: Chromatograph of oil sample after microbial treatment.

It can be seen that there is a slight decrease in the concentration of the C_{25}+ group. This slight decrease can be attributed to the microorganisms in the microbial formulation, which degrade long-chained hydrocarbons. There is a decrease in the concentration of light carbon groups, such as the C_6 to C_{11} groups. This decrease mathematically compensates the increase in the concentrations of the middle carbon groups.

The Bushnell-Haas broth is usually used to study the growth of microorganisms in the environment where hydrocarbons can be used as a carbon source. The chemical composition analysis of the oil samples shows that the microbes do degrade the long hydrocarbon chains into lighter hydrocarbons in order to use them as a carbon source. However, the nutrient medium uses sucrose as the carbon source for growth of microorganisms, so the microbes do not use the lighter hydrocarbons formed by degradation of long-chained hydrocarbons. The biodegrading ability of the microbes, therefore, is used as an advantage by degrading heavy oils to lighter oils.

4.8 Density and viscosity measurement

The density measurement equipment setup is discussed in Section 3.3 of this work. The viscosity measurement equipment setup is discussed in Section 3.4. The density and viscosity of the oil sample were measured before and after microbial treatment to determine the effect of microbial treatment on the density and viscosity of the oil, if any. These properties are usually measured at stock tank conditions in the petroleum industry (stock tank conditions represent 15°C and atmospheric pressure). The temperature bath was set, and enough time was given for equilibrium to be achieved before the measurement was started.

The density of the oil sample before microbial treatment was 0.9484 g/cc (i.e., 17.7°API). The density of the oil was also measured after extraction of oil from the core after a one-week shut-in period. The density of the oil sample after microbial treatment was 0.8954 g/cc (i.e., 26.5°API).

The viscosity of the oil sample was obtained at various motor speeds. The value for the lowest motor speed with a torque between 10% and 100% is taken to be the viscosity of the oil sample. The viscosity of the oil sample before microbial treatment was 67.5 cp. The viscosity of the oil sample after microbial treatment was 50.2 cp.

The decrease in density and viscosity of the oil can be attributed to biodegradation of the higher hydrocarbons in the oil to the lighter hydrocarbons. Also, the strain of the bacteria used produced biogases during metabolic activity. These biogases also contributed to the decrease in viscosity and density of the oil sample by dissolving in the oil and by swelling of the oil.

4.9 Terminal restriction fragment polymorphism analyses of coreflooding study

Using the QIAamp DNA Stool Mini Kit, DNA was successfully extracted from the oil sample, the broth, the microbial formulation, the oil sample extracted after a one-week shut-in period, and the oil sample extracted from the core in contact with microbial formulation for two months. The procedure for the extraction is discussed in Section 3.6 of this work. The composition of the PCR and digestion mixture is shown in Appendix C. The extraction can be confirmed from the photograph of the agar gel taken from the UV transilluminator after the agarose gel electrophoresis of the PCR products. Figure 4.15 shows the photograph of the gel.

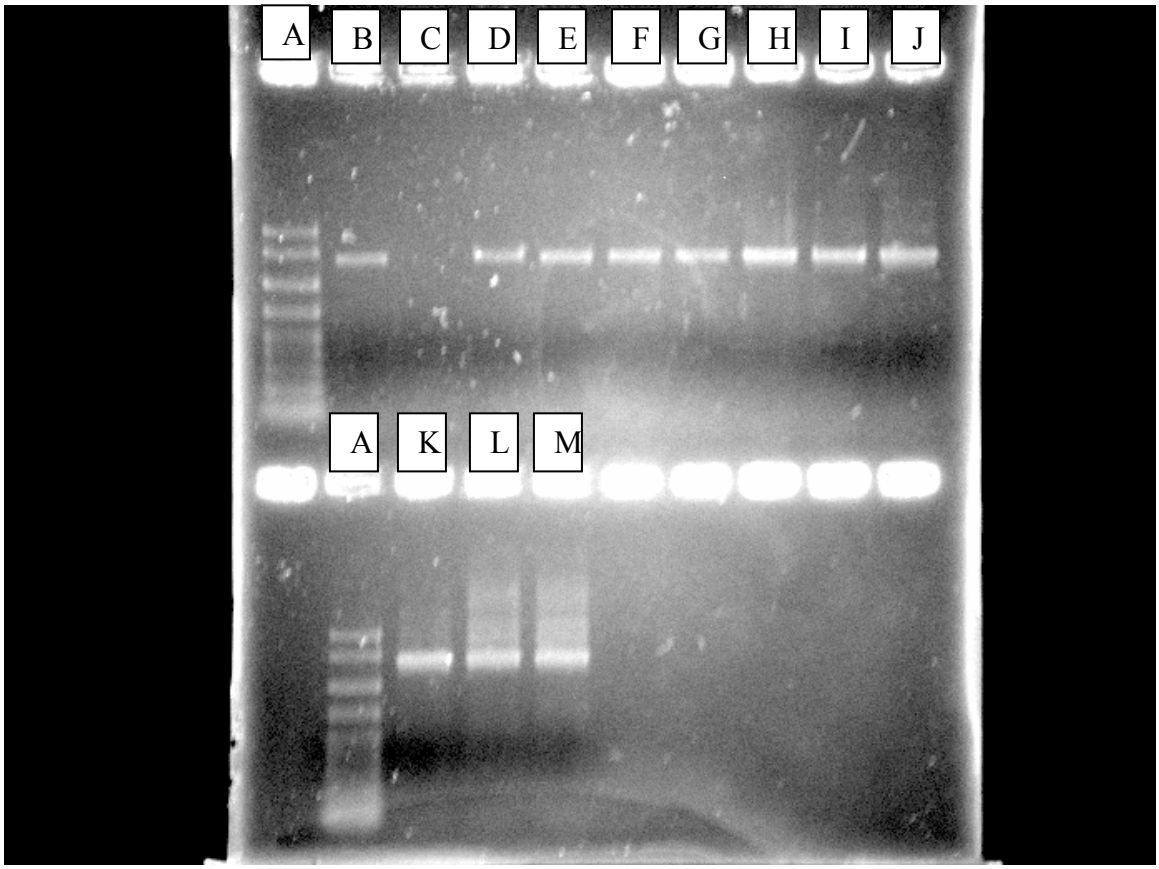


Figure 4.15: Agarose gel of the PCR products. Lanes: *A*, molecular weight marker; *B*, positive control; *C*, negative control; *D* and *E*, oil sample from the ANS oil field; *F* and *G*, the nutrient media for the microbes; *H* and *I*, the microbial formulation; *J* and *K*, the oil sample extracted after a one-week shut-in period; and *L* and *M*, the oil sample extracted after a two-months shut-in period.

The gel shows a band for all the samples except for the negative control, demonstrating that there has not been any contamination until the PCR reaction. However, the nutrient media for the microbes, that is, the broth for the microbial formulation, show a band in the gel. The broth should be sterile, but the gel shows that there has been extraction of DNA from it. This contamination occurred because the broth was transferred in a non-sterile tube before DNA extraction. The broth was kept in sterile condition, however, during preparation of the microbial formulation, so it can be expected that contamination was not carried forward to the microbial formulation. Since the broth used for DNA extraction was contaminated, it was not considered for further analysis. The band for the oil samples in lanes *J*, *K*, *L*, and *M*

shows smudge at the beginning, but the band to be considered is very bright as compared to the smudged bands. Thus, the smudge can be neglected.

The PCR products of all the samples and the positive control were purified using the QIAquick PCR purification kit. The purified products were quantified for the concentration of DNA by using the NanoDrop. Figure 4.16 shows the chromatogram obtained from the NanoDrop for the purified PCR products.

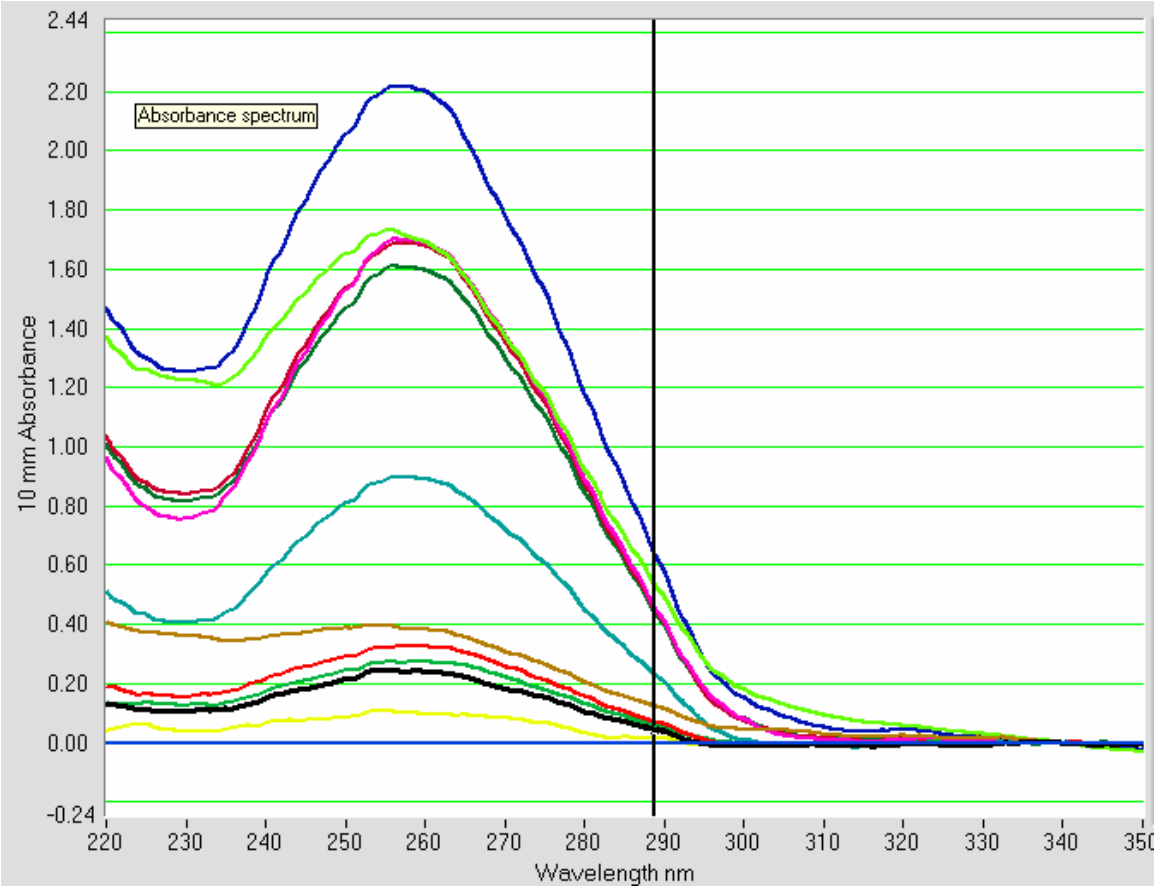


Figure 4.16: NanoDrop chromatogram of the purified PCR products.

The chromatogram shows a peak at the wavelength of 260 nm for all samples, which confirms the finding of the agarose gel electrophoresis that every sample has some DNA in it. As per the concept of the NanoDrop, the DNA concentration of a sample is linear to the absorbance between the ranges of 0.1 and 1.00 mm, meaning that for the samples having a

peak with absorbance more than 1.00 mm, the DNA concentration cannot be estimated precisely. However, what can be said definitely is that these samples have a very high DNA concentration and need to be diluted. The dilution ratio depends on the absorbance value at the wavelength of 260 nm. Dilution should be such that the absorbance is in the desired range. For example, if the chromatogram shows the absorbance of 2.20 mm at the wavelength of 260 nm, then if the sample is diluted with a ratio of 1 to 10 (i.e., adding 1 µl of the sample to 9 µl of nuclease-free water), the absorbance can be expected to be 0.22 mm. Again, the samples are run in the NanoDrop to estimate the DNA concentrations. Table 4.9 gives the DNA concentration for each sample. DNA concentration values are important in deciding the amount of sample added to the digestion mixture.

Table 4.9: DNA concentration values for each sample.

Sample	**Absorbance at 260 nm (10 mm)**	**DNA concentration (ng/µl)**
Oil sample A	0.24	12.12
Oil sample B	0.33	16.23
Microbial formulation A	0.29	23.99
Microbial formulation B	0.89	44.57
Oil extracted after one week A	0.27	51.98
Oil extracted after one week B	0.49	38.21
Oil extracted after two months A	0.84	31.94
Oil extracted after two months B	0.76	36.45
Positive control	0.10	4.84

Terminal restriction fragment length polymorphism (T-RFLP) is also called community profiling or community fingerprinting. The main objective for using T-RFLP was to compare the microbial communities of various samples. The theory and methodology of T-RFLP is described in Section 3.6 of this work. T-RFLP was run on the oil sample, the microbial formulation, the oil sample extracted after a one-week shut-in period, and the oil sample in contact with the microbial formulation for two months. Two replicates of each sample were

run in order to avoid any contamination. The results of the T-RFLP tests are tabulated in Appendix D.

The T-RFLP chromatograms for all the samples were compared using GeneMapper software. All chromatograms showed very high peaks at the size of 204 to 205 base pairs and 236 to 237 base pairs. The peaks at this size were present in all the chromatograms, and can be attributed to some contaminants therefore. The specific reasons for contamination are not known, as utmost care was taken to avoid it. Contamination could have entered the PCR reagents, but this is unlikely, as new reagents were used. Another way that contaminants could enter the solution is through the use of contaminated DNA extraction agents or buffers. However, from the agarose gel photograph (Figure 4.15), it can be seen that there is no band in the lane for the negative control. This confirms that there was no contamination before this point. Contamination can be attributed, therefore, to the buffers used in the PCR purification kit or the buffers used in the digestion mix. Contamination could also have occurred because of incomplete digestion.

All the chromatograms show some peaks for noise. Any peak with a size of less than 50 base pair and/or a height less than 100 is considered a noise. The tables with normalized chromatograms are included in Appendix E.

From the T-RFLP chromatogram for microbial formulation (Figure 4.17), it can be seen that the major peak other than the peaks for contamination is at the size of 225 base pair. On normalization, this peak contributes to 11.66% of the total chromatogram, meaning the microorganism that represents this peak constitutes 11.66% of the total microbial community of the microbial formulation. Ideally, the microbial formulation should be made up of 100% of a single microorganism, but in normalization, the peaks for the contamination are also considered, and thus the concerned peak contributes to a lower percentage. All the peaks other than those for the contamination contribute to less than 1.5% of the total chromatogram. The peaks for contamination contribute to about 36% of the total chromatogram. Thus, the microbial has only one major peak, which can be successfully

attributed to the microorganism, that is, the *B. licheniformis* JF-2 strain. From the literature, it is noted that in a T-RFLP chromatogram for this particular microorganism the peak is observed at the size of 285 base pair. The difference observed in this case can be due to the use of a different buffer in any of the process.

For the oil sample from the ANS oil field, the T-RFLP chromatogram (Figure 4.18) shows a large number of peaks, including those for contamination. All the major peaks, other than those for contamination constitute about 1.0% to 15.0% of the total chromatogram. The peaks for contamination contribute to 18.0% to 42.0% of the total chromatogram. The large number of peaks confirms that the oil sample has a diverse microbial community. The chromatogram also shows a peak at the size of 224.5 base pair. This peak is attributed to the microorganism in the microbial formulation. It could be argued from this observation that the oil sample already had the *B. licheniformis* JF-2 strain, but the peak can be for any other microorganism in the oil sample. The peak could either be a microorganism belonging to the *Bacillus* species or a totally different species. There is no way to know for sure whether the oil sample contained the injected microorganisms.

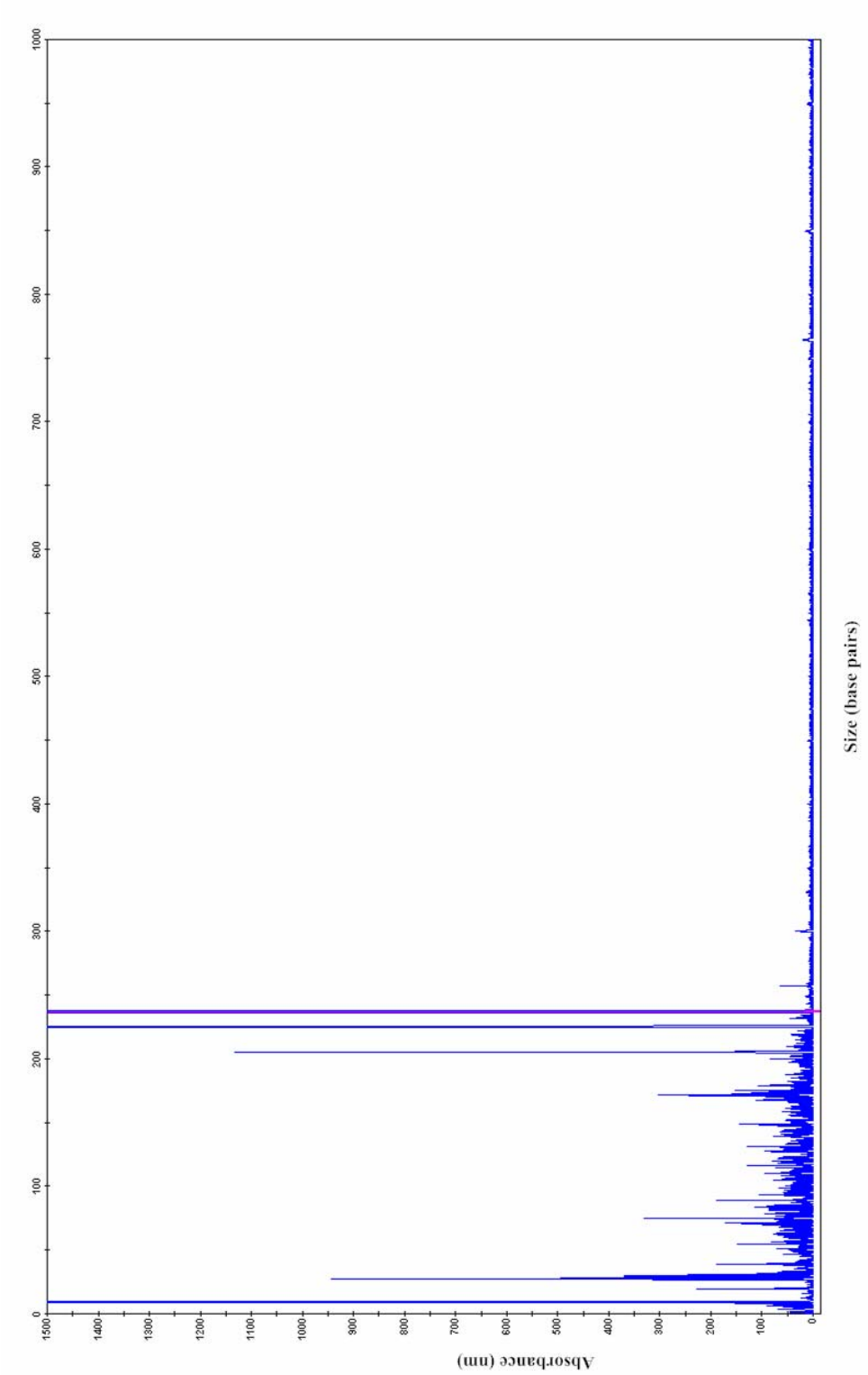


Figure 4.17: T-RFLP chromatogram for microbial formulation.

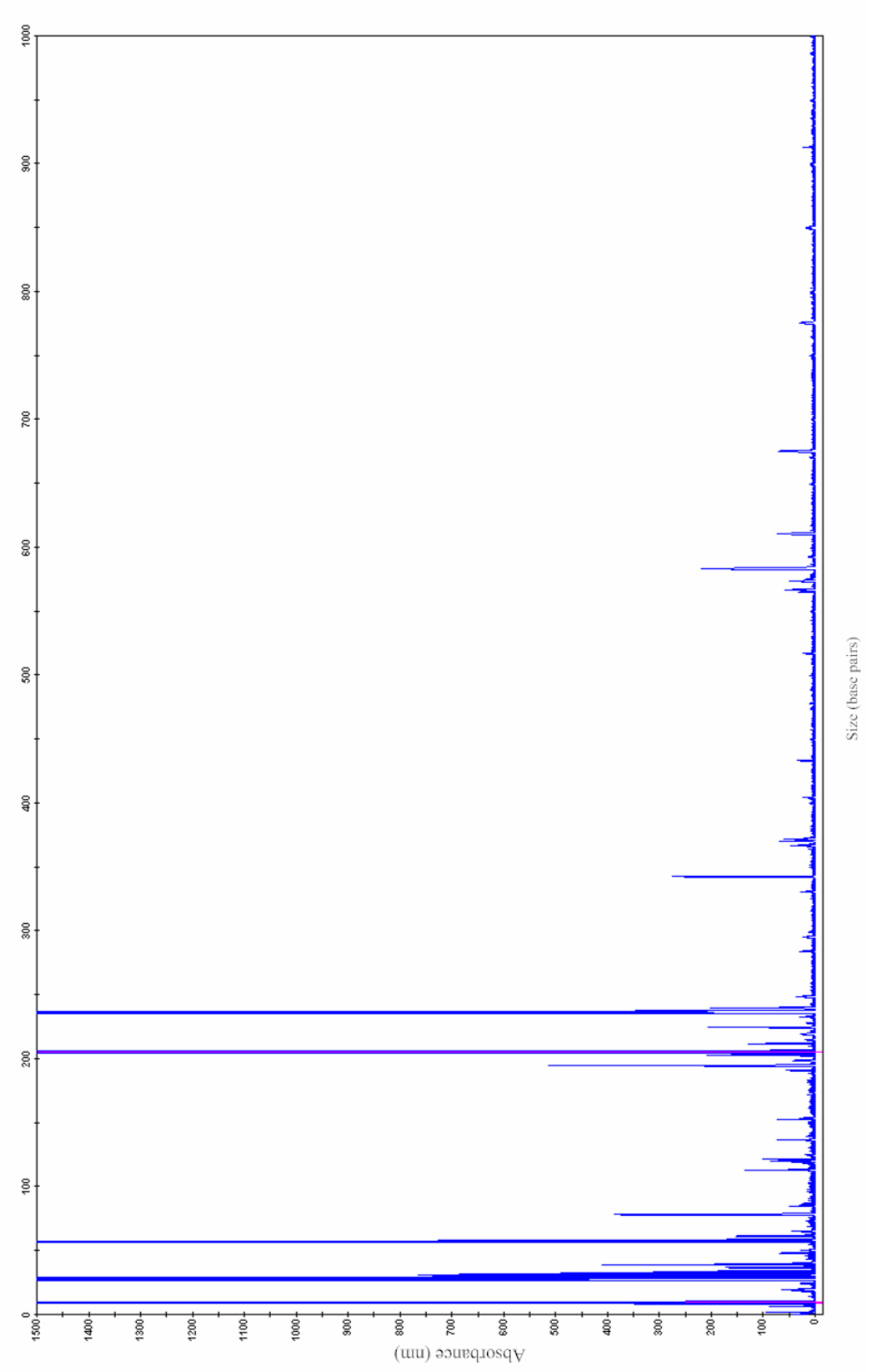


Figure 4.18: T-RFLP chromatogram for the oil sample from ANS oil fields.

The T-RFLP chromatograms for the oil sample extracted from the core with a shut-in period of one week (Figure 4.19 and Figure 4.20) show a large number of major peaks. The emphasis is on the peak for the microorganism, that is, the peak at the size of 225 base pair. The chromatogram for the oil sample where the DNA was eluted in 40 µl Buffer AE (Figure 4.19) on normalization indicates that this peak contributes to 5.43% of the total chromatogram. The chromatogram where the DNA was eluted in 50 µl Buffer AE (Figure 4.20) indicates that the peak contributes to 11.11% of the total chromatogram. Thus, it can be argued that the microorganism contributes to 5% to 12% of the total microbial community of the oil sample. This is definitely more than that in the oil sample from the ANS oil field. Hence, it can be concluded that the microorganism can flourish in the oil sample even if it is not indigenously present.

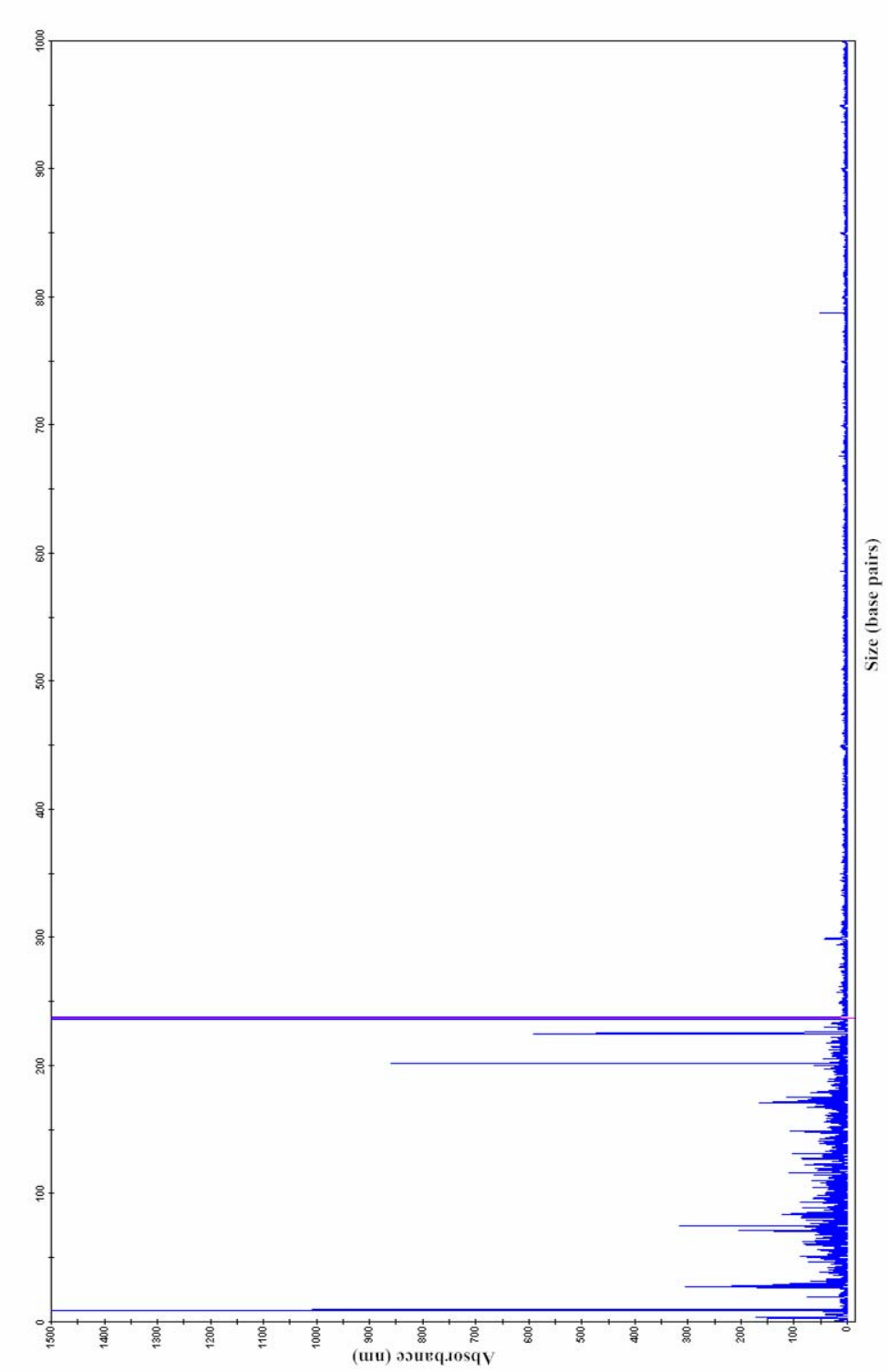


Figure 4.19: T-RFLP chromatogram for oil sample extracted after one week and eluted in 40 µl Buffer AE.

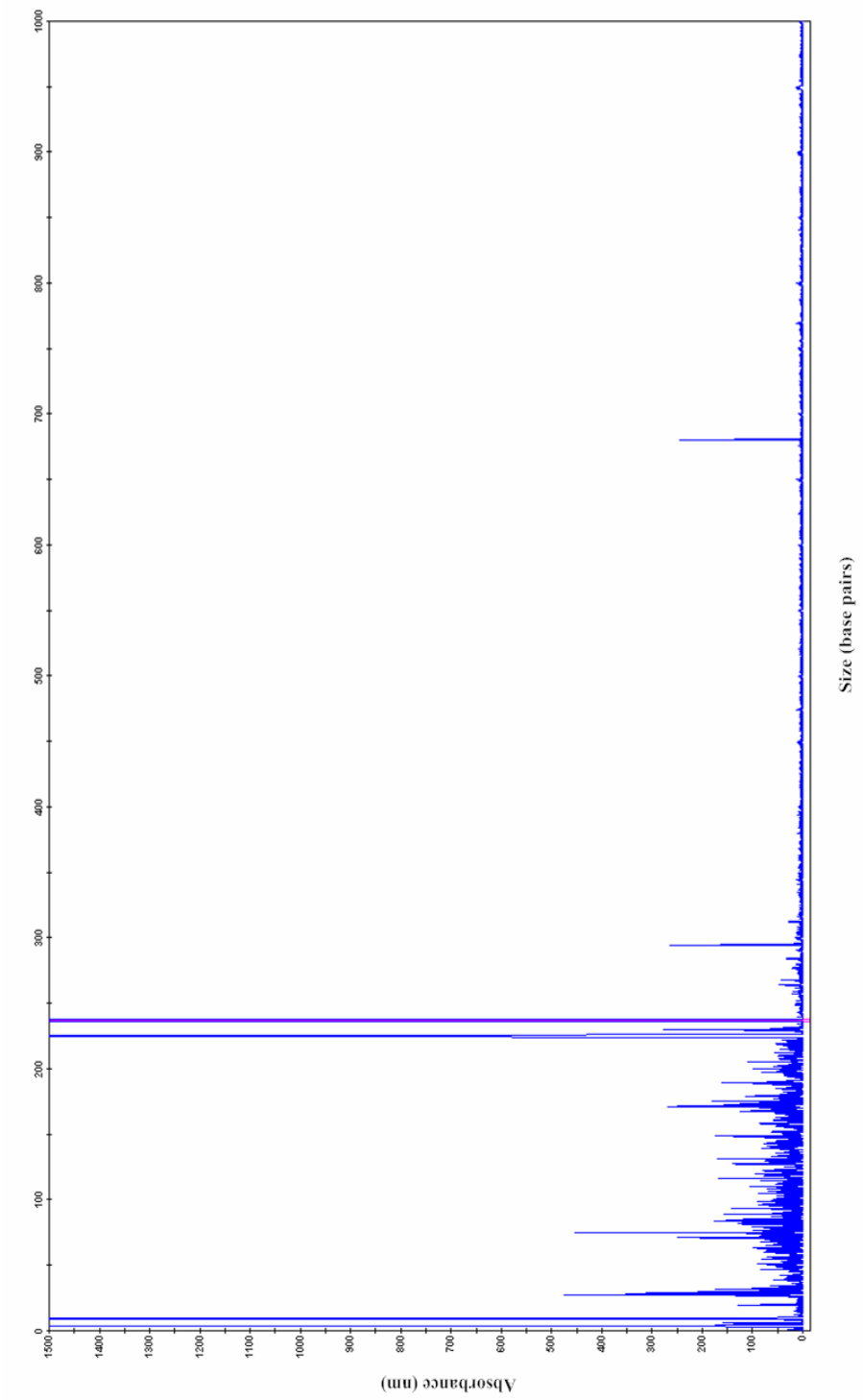


Figure 4.20: T-RFLP chromatogram for oil sample extracted after one week and eluted in 50 µl Buffer AE.

The T-RFLP chromatograms for the oil sample extracted from the core in contact with the microbial formulation for two months (Figure 4.21 and Figure 4.22) also show the peak at the size of 225 base pair. However, this contributes to only 0.4% to 0.7% of the total microbial community of the oil sample. The amount of oil sample produced is not sufficient for T-RFLP analysis, so DNA is extracted from a mixture of the oil and microbial formulation. As the samples are of a different nature, the T-RFLP chromatogram for this sample cannot be compared with any of the previous samples.

It could not be confirmed whether the microorganism in any of the sample was still active, because we were analyzing the DNA extracted from the sample. Even if microorganisms in a sample are dead, DNA can be extracted from the cells. The only way to tell whether or not a microbial community is active is to extract the RNA and analyze it. This is a very complex process and was out of the scope of this work.

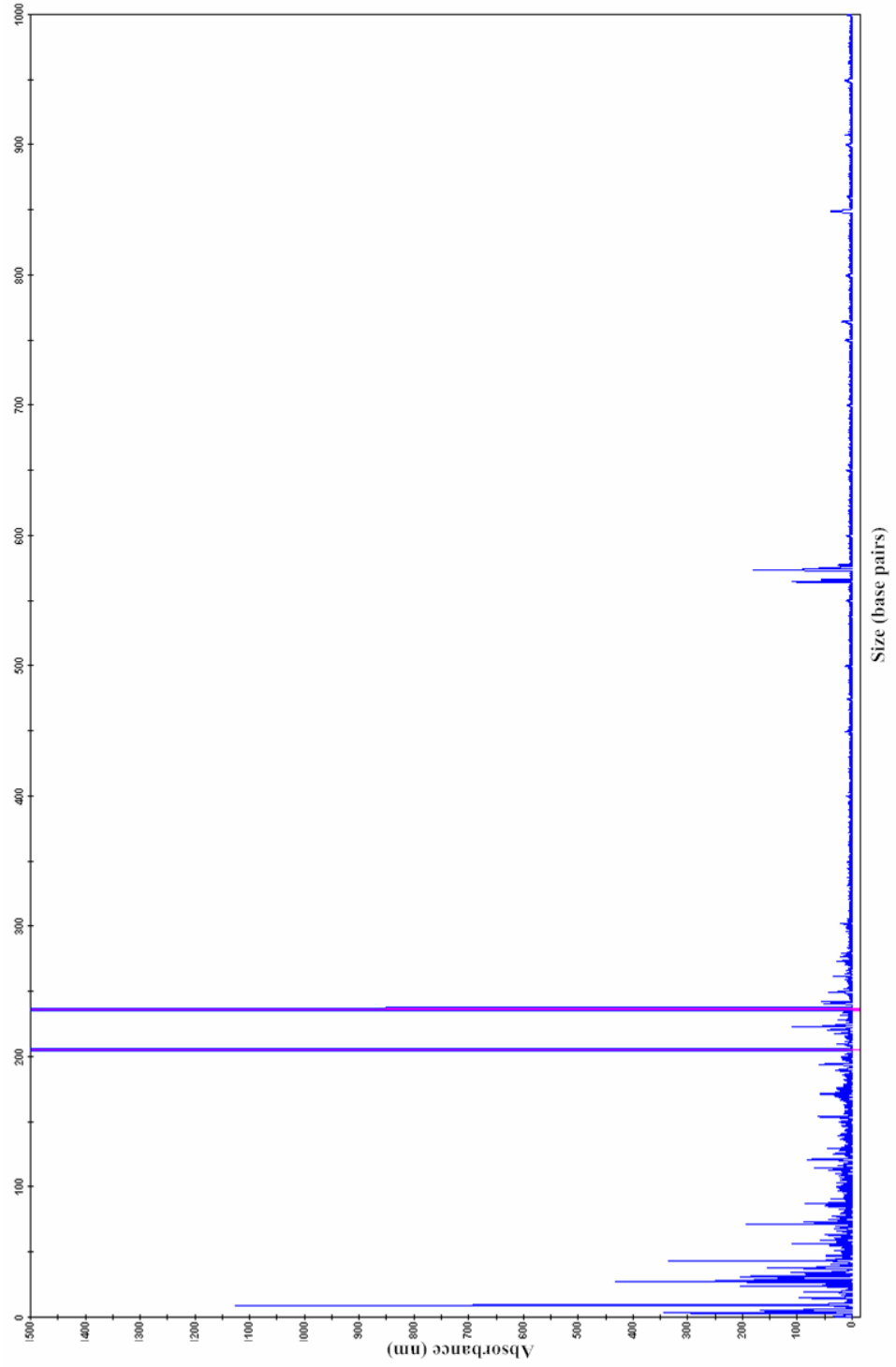


Figure 4.21: T-RFLP chromatogram for oil sample extracted after two months and eluted in 40 µl Buffer AE.

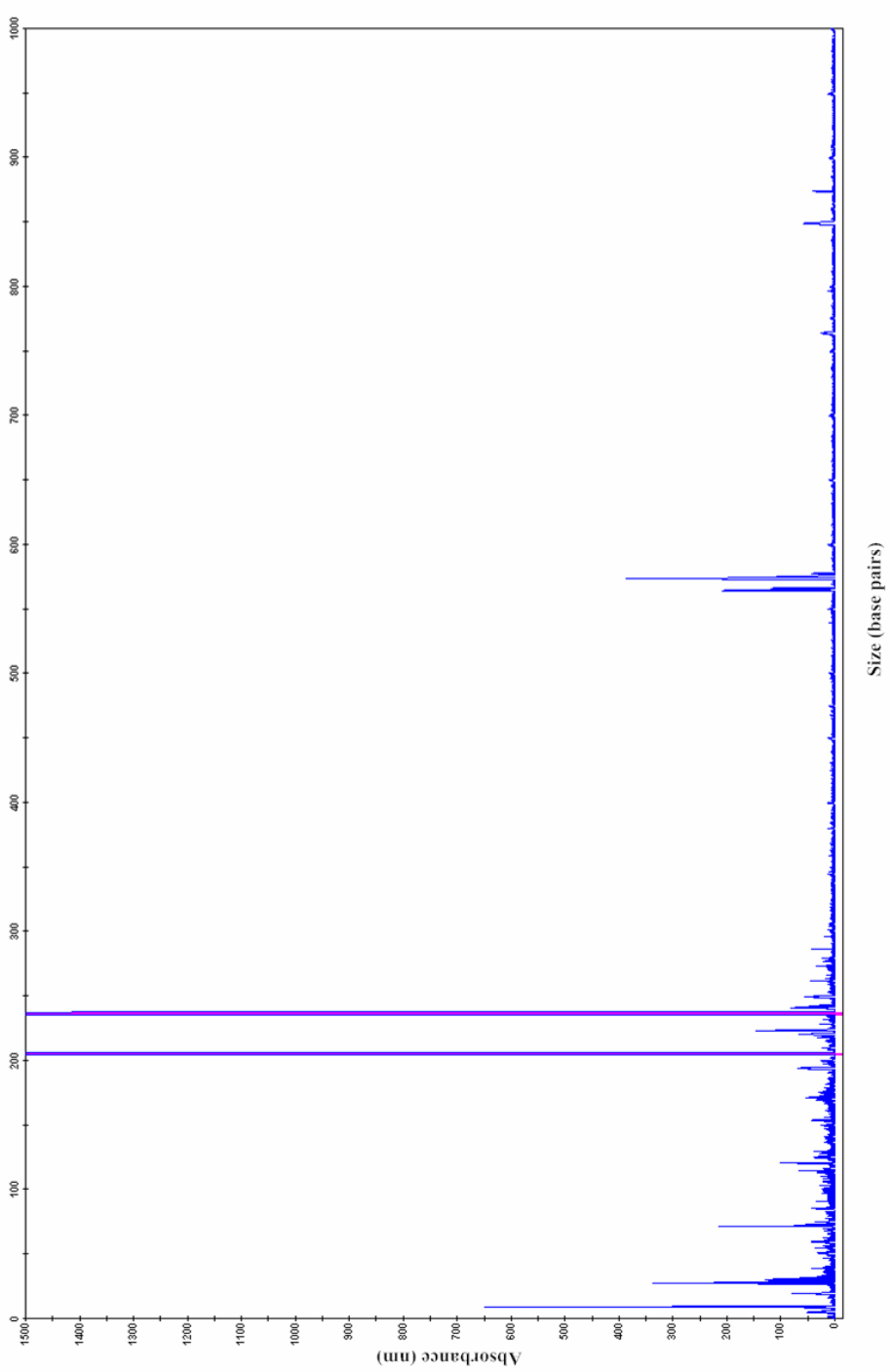


Figure 4.22: T-RFLP chromatogram for oil sample extracted after two months and eluted in 50 µl Buffer AE.

4.10 Isolation of indigenous biosurfactant-producing microbes

4.10.1 Enrichment cultures

Enrichment cultures were established in four different growth media—LB Broth, Bushnell-Haas Broth, Modified Bushnell-Haas Broth, and Basal Mineral Medium—using five different ANS oil samples as inoculums: Prudhoe Bay-W, Prudhoe Bay-P, Point, Kuparuk, and West-Sak-D Sand). An additional enrichment was created using Prudhoe Bay crude in basal mineral medium, which was inoculated with microbes from oiled rock from the Exxon Valdez oil spill (EVOS). Growth was evidenced by turbidity and colony formation when spread onto agar plates. When growth was detected, a dilution series was created in order to obtain isolated colonies for the optical distortion assay.

Growth in the enrichment cultures was obtained from Milne Point, Prudhoe Bay-P, Prudhoe Bay-W, and Kuparuk oils in some but not all media types (Table 4.10). In several cases, growth was detected in enrichments when plated onto agar plates, but when dilution series was plated, there was no growth detected. This lack of growth may be a result of the population in the enrichment having declined during the time when original (concentrated) agar plates were incubating. Growth rates of cells in liquid and on agar media were very slow, requiring weeks to months of incubation time. The slow growth rates may have been because many oil microbes are anaerobic or microaerophilic, and the oxygen in our aerobic culture conditions may have inhibited growth. In future studies, anaerobic cultivation methods would be recommended if the necessary equipment is available.

Table 4.10: Growth in enrichment cultures.

Medium	Date Inoculated	Medium volume (ml)	Oil volume (µl)	Inoculum	Incubation temperature (°C)	Growth in liquid culture	Growth on dilution plates	Optical distortion screening done
LB Broth	5/12/2008	10	100	Prudhoe Bay-W	25	-	-	No
	5/16/2008	100	200	Milne Point	25	+	+	Yes
	5/16/2008	100	200	Kuparuk	25	-	-	No
	5/16/2008	100	200	Prudhoe Bay-P	25	+	+	No (colonies not sufficiently isolated)
	5/16/2008	50	100	Prudhoe Bay-W	25	+	+	No (only fungi present)
	5/16/2008	100	200	West-Sak (D Sand)	25	-	-	No
Bushnell-Haas Broth	4/28/2008	100	100	Milne Point	25	-	-	No
	4/28/2008	100	100	Kuparuk	25	-	-	No
	4/28/2008	100	100	Prudhoe Bay-P	25	-	-	No
	4/28/2008	100	100	Prudhoe Bay-W	25	-	-	No
	4/28/2008	100	100	West-Sak (D Sand)	25	-	-	No
Modified Bushnell-Haas Broth	5/16/2008	100	200	Milne Point	25	-	-	No
	5/16/2008	100	200	Kuparuk	25	+	+	No (only fungi present)
	5/16/2008	100	200	Prudhoe Bay-P	25	+	-	No
	5/16/2008	100	200	Prudhoe Bay-W	25	-	-	No
	5/16/2008	50	100	West-Sak (D Sand)	25	-	-	No
Basal Mineral Liquid Medium (BM liquid)	1/23/2008	100	500	Prudhoe Bay + Exxon Valdez Oiled Rock	25	+	+	Yes
	6/2/2008	100	200	Milne Point	30	-	-	No
	6/2/2008	100	200	Kuparuk	30	-	-	No
	6/2/2008	100	200	Prudhoe Bay-P	30	-	-	No
	6/2/2008	100	200	Prudhoe Bay-W	30	-	-	No
	6/2/2008	100	200	West-Sak (D Sand)	30	-	-	No

Note: Oil samples comprise the inoculum, with the exception of one culture in which Exxon Valdez Oiled Rock was provided as inoculum in addition to Prudhoe Bay crude oil. Growth in liquid culture was evidenced by turbidity and/or growth on agar plates.

4.10.2 High-throughput screening for microbial biosurfactant production

The optical distortion assay was performed on two of the enrichment cultures from which growth on dilution plates was obtained (Table 4.10). Two of the cultures were not tested because dilution plates only grew fungi. Fungi are not desirable MEOR organisms since, although they can produce abundant biosurfactant, they are known to clog wells. Screening was performed on colonies from the EVOS-inoculated enrichment and also from Milne Point oil cultivated in LB Broth. Figure 4.23 shows the results obtained from the assay performed on colonies from the EVOS enrichment culture. A reduction in apparent grid size in many wells clearly indicates a reduction in surface tension has occurred as a result of microbial growth.

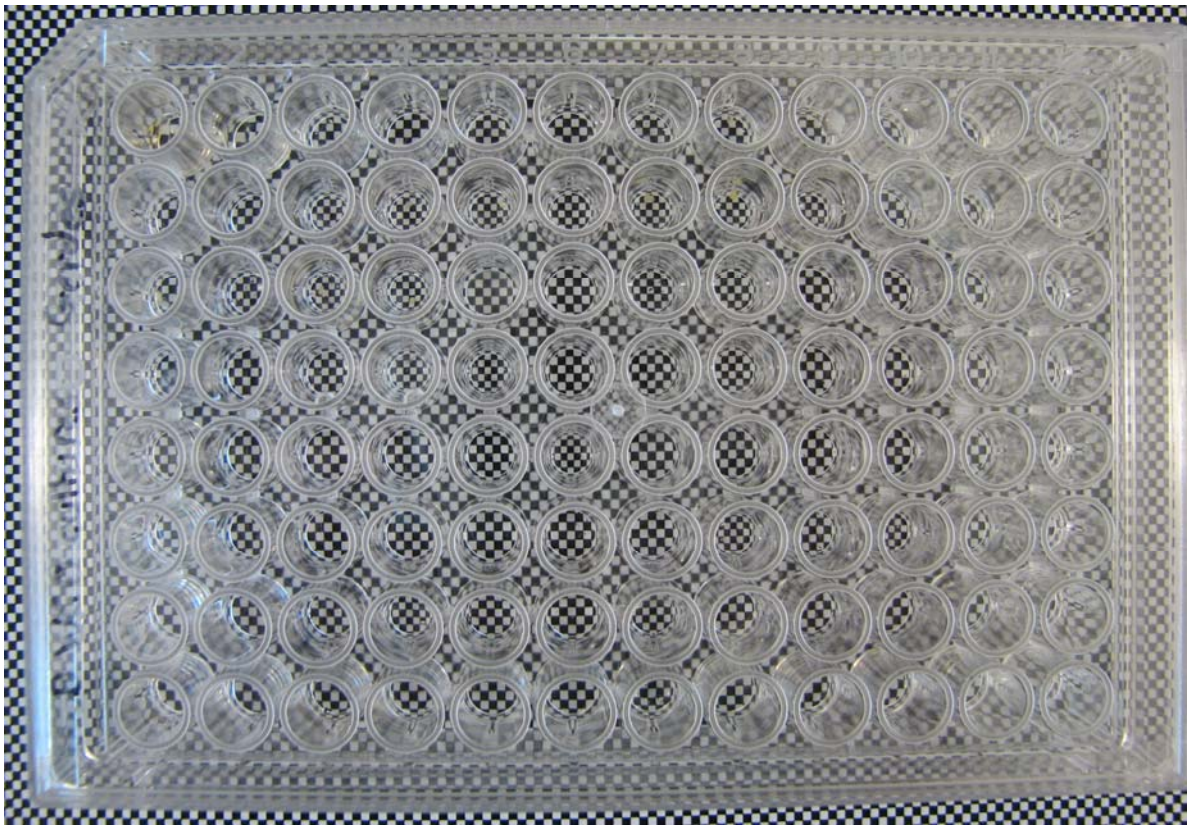


Figure 4.23: Optical distortion assay for surfactant production performed on EVOS enrichment culture. Note the distortion of the grid pattern. A reduction in grid size indicates reduced surface tension.

Table 4.11: Results of optical distortion assay for surfactant production by colonies from EVOS enrichment culture.

Well	**Inoculated**	**Surfactant Production**
A1	Blank	-
A2	Blank	-
A3	Yes	+
A4	Yes	+**
A5	Yes	+*
A6	Yes	+*
A7	Yes	+*
A8	Yes	+
A9	Yes	+
A10	Yes	+
A11	Yes	+*
A12	Yes	+
B1	Yes	+
B2	Yes	+*
B3	Yes	+
B4	Yes	+
B5	Yes	+
B6	Yes	+*
B7	Yes	+
B8	Yes	+
B9	Yes	+
B10	Yes	+*
B11	Yes	+**
B12	Yes	+
C1	Yes	+
C2	Yes	+
C3	Yes	+

C4	Yes	+
C5	Yes	+**
C6	Yes	+**
C7	Yes	+*
C8	Yes	+*
C9	Yes	+**
C10	Yes	+*
C11	Yes	+*
C12	Yes	+
D1	Yes	+*
D2	Yes	+*
D3	Yes	+*
D4	Yes	+
D5	Yes	+*
D6	Yes	+*
D7	Yes	+**
D8	Yes	+*
D9	Yes	+*
D10	Yes	+*
D11	Yes	+**
D12	Yes	+*
E1	Yes	-
E2	Yes	-
E3	Yes	+**
E4	Yes	+*
E5	Yes	+**
E6	Yes	+*
E7	Yes	-
E8	Yes	+*
E9	Yes	+*

E10	Yes	+*
E11	Yes	+*
E12	Yes	+
F1	Yes	+**
F2	Yes	-
F3	Yes	-
F4	Yes	+**
F5	Yes	-
F6	Yes	+*
F7	Yes	+**
F8	Yes	+
F9	Yes	+*
F10	Yes	+*
F11	Yes	+**
F12	Yes	+*
G1	Yes	+**
G2	Yes	-
G3	Yes	+*
G4	Yes	+*
G5	Yes	+*
G6	Yes	+*
G7	Yes	+
G8	Yes	+
G9	Yes	+
G10	Yes	+*
G11	Yes	-
G12	Yes	-
H1	Yes	+**
H2	Yes	+**
H3	Yes	+*

H4	Yes	+
H5	Yes	+*
H6	Yes	+*
H7	Yes	+
H8	Yes	+**
H9	Yes	+
H10	Yes	+**
H11	Yes	+**
H12	Yes	+
TOTALS	-	9
	+	28
	+*	38
	+**	19
-	No surfactant production; same as blank	
+	Lots of surfactant produced	
+*	Some surfactant produced	
+**	Minimal amount of surfactant produced; only slightly different from blank	

Out of the 94 colonies screened from the EVOS culture, 85 colonies reduced the surface tension of the medium visibly.

The enrichment culture established from Milne Point in LB was also tested using the optical distortion assay (Table 4.12). Fewer colonies were tested, reflective of the total number of colonies obtained. In this case, colonies were screened for surfactant production in two different liquid media: M9 (also used for EVOS culture) and a 1% tetrasodium pyrophosphate solution (TPP). Depending on the medium used, 25–32 colonies of the 35 tested produced positive results (Table 4.12).

Table 4.12: Results of optical distortion assay of colonies from Milne Point/LB. Screening was performed in two different media types.

	Medium used for assay	
	M9	TPP
A1	-	+**
A2	-	-
A3	+	+
A4	+	+**
A5	+*	-
A6	+	-
A7	+	+**
A8	+	+**
A9	+*	+**
A10	+	+**
A11	+	+**
A12	Blank	Blank
B1	+	+
B2	+	+**
B3	+	-
B4	+	+
B5	+	-
B6	+	+**
B7	+	-
B8	+	+
B9	+	+**
B10	+	+**
B11	+	+**
B12	+	+**
C1	+*	+**

C2	+*	+**
C3	-	+
C4	+	-
C5	+	-
C6	+*	-
D1	+*	+
D2	+*	+**
D3	+	+**
D4	+	+
D5	+	+**
D6	+	-
TOTALS		
-	3	10
+	25	7
+*	7	0
+**	0	18

The abundance of bacteria in the enrichment cultures that were capable of reducing surface tension indicates that this technique is effective at enriching organisms likely to produce surfactants. This capability should be selected for these conditions, given that crude oil is the sole carbon source provided to cells in enrichment cultures. Because of the lipophilicity of oil components, surfactants are likely needed by the cells in order to facilitate transport into the cell for metabolism. The colony morphologies of cells inoculated into the optical distortion assays were noted. Many colonies were morphologically similar, suggesting that the cultures were dominated by only a few strains that predominated in the liquid enrichment culture.

The presence of microorganisms that can reduce surface tension in the Milne Point enrichment culture is a promising result for MEOR. It indicates that indigenous surfactant producers are present in at least some ANS oils, which could be the target of

biostimulation efforts to produce biosurfactants *in situ*. Slow growth rates precluded further study during this project period, but the organisms will be preserved for future study.

4.10.3 Isolation of biosurfactant producers

Microbes present in positive wells of the optical distortion assay were streaked onto agar plates in quadrants to begin the process of isolation. Once cells had grown, they were grouped based on morphology. Many of the cells shared similar morphology (yellow-orange pigmentation, smooth round colonies). One to three representatives of each morphotype were selected for further isolation and study. Figure 4.24 shows several of the streak plates containing cultures grown after testing positive in the optical distortion assay for surface tension reduction. Figure 4.25 shows selected strains after complete isolation.

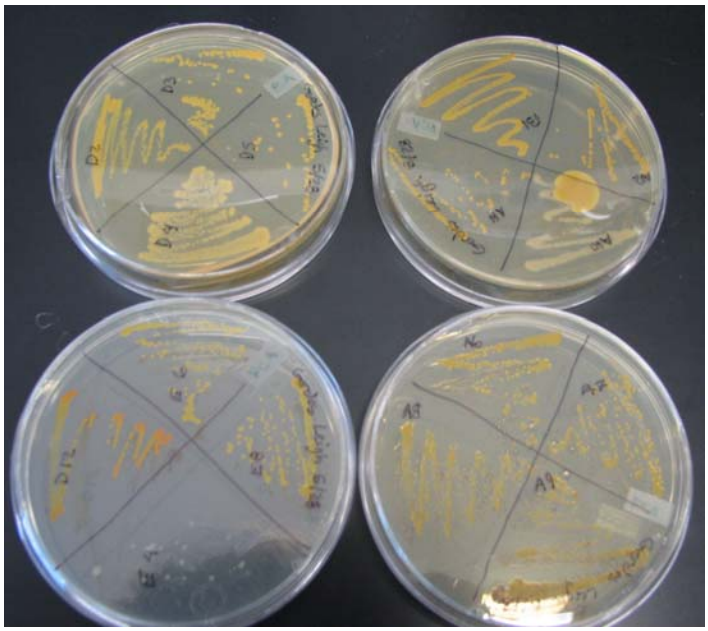


Figure 4.24: Several quadrant streak plates containing bacteria of different morphotypes found to reduce surface tension in the optical distortion assay.

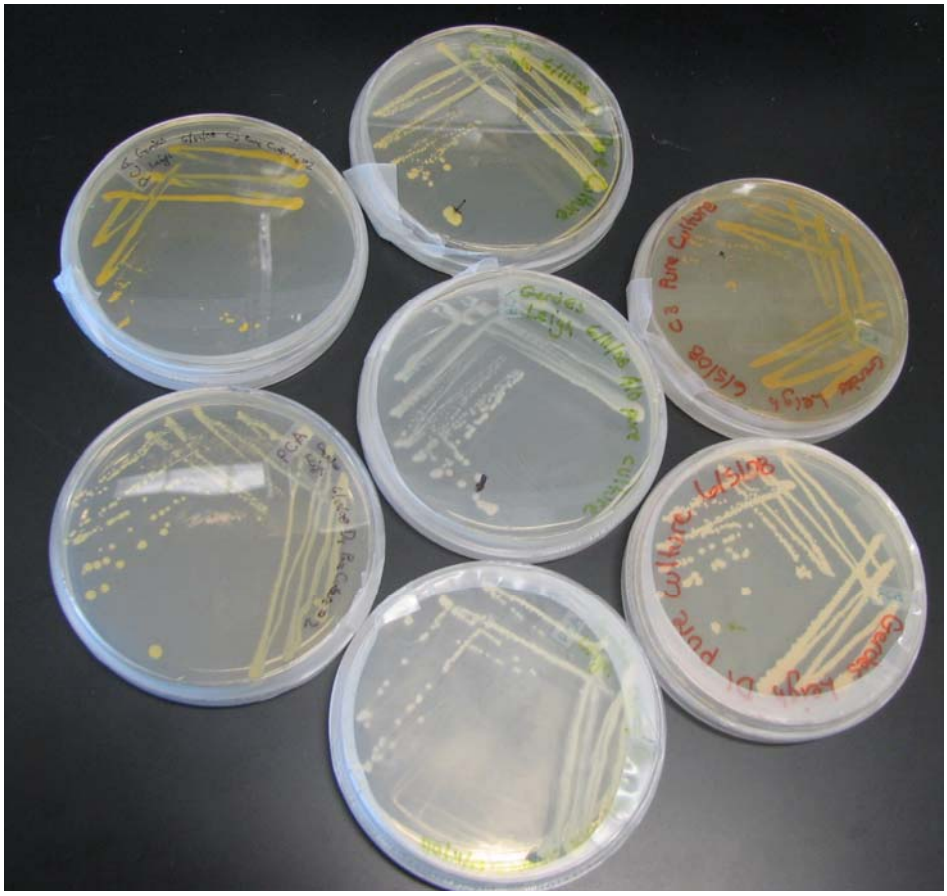


Figure 4.25: Several isolates obtained which had tested positive in the optical distortion assay.

Isolates were only obtained from the EVOS enrichment culture. The Milne Point enrichment grew quite slowly, and did not allow time for complete isolation of organisms during the project period.

4.11 Ring tensiometer analyses to assess surface tension reduction capability of isolates

The surface tension of media following growth was measured more quantitatively using a ring tensiometer. The ability to reduce surface tension in two different media was evaluated for each of the isolates, as well as for *B. licheniformis* RS1, a well-characterized biosurfactant-producing strain used as a positive control. Results are presented in Table 4.13. The two media types had very different surface tensions initially, with the minimal medium (M9) having lower surface tension than the rich medium (LB).

The positive control organism, *B. licheniformis* RS1, successfully reduced the surface tension of LB but not M9. This is explained by the fact that less growth had occurred in the M9 medium. Several strains isolated from the EVOS enrichment culture did appreciably reduce surface tension, specifically D4 when grown in M9 medium, and A10 and D1 when grown in LB. These three organisms are candidates for future study of their biosurfactant chemistry and environmental conditions that favor surfactant production.

Table 4.13: Results of ring tensiometer analyses for surface tension of media following growth with bacterial strains.

Media	Isolate	Measurement (P)	Density (D)	F (correction)	Surface tension (dynes/cm^2)
M9	Sterile control	38	0.9898	0.8441	32.0770
	B. licheniformis				
	RS1	38	0.9847	0.8441	32.0773
	A10	40	0.9872	0.8442	33.7690
	B3	60	0.9826	0.8451	50.7079
	C3	61	0.9896	0.8452	51.5546
	D1	78	0.9796	0.8459	65.9837
	D4	36	0.9812	0.8441	30.3860
LB Broth	Sterile control	82	0.9870	0.8461	69.3798
	B. licheniformis				
	RS1	71	0.9862	0.8456	60.0384
	A10	76	0.9889	0.8458	64.2826
	B3	90	0.9883	0.8464	76.1799
	C3	92	0.9804	0.8466	77.8839
	D1	74	0.9887	0.8457	62.5844
	D4	>90	0.9892	>0.8464	>76.1796

4.12 T-RFLP analyses of several ANS oils and surfactant-producing isolates

In order to characterize the microbial diversity of the five ANS oils from which isolation of surfactant producers was attempted, T-RFLP fingerprinting analysis was performed. As detailed earlier in this report, DNA extraction was performed from the bacterial communities present in the oils. PCR amplification was then performed using primers targeting bacterial 16S rRNA genes. Figure 4.26 shows the results of agarose gel electrophoresis of the PCR products, revealing that the amplification was successful. The appropriately sized bands were produced, and there is no band produced in the negative control, showing that there was no contamination present in the PCR reaction.

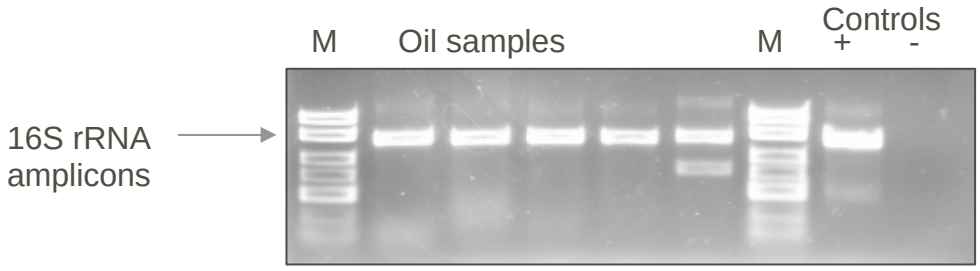
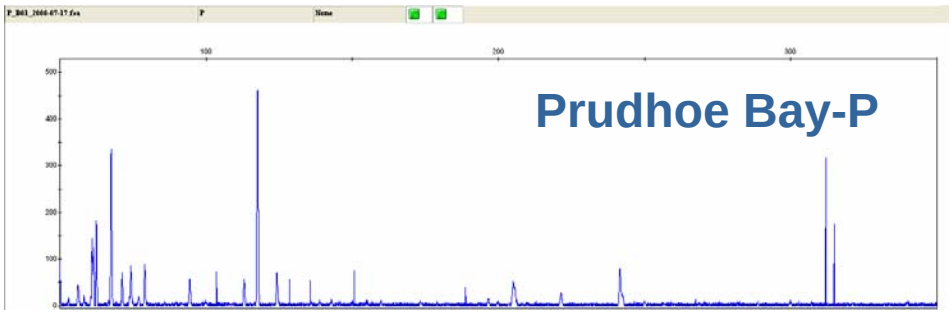
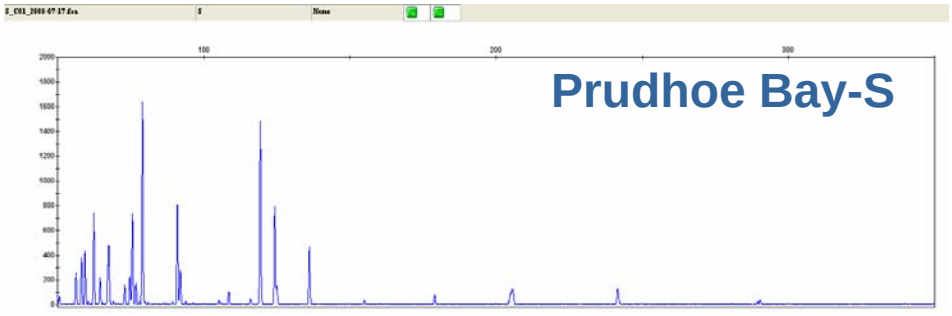
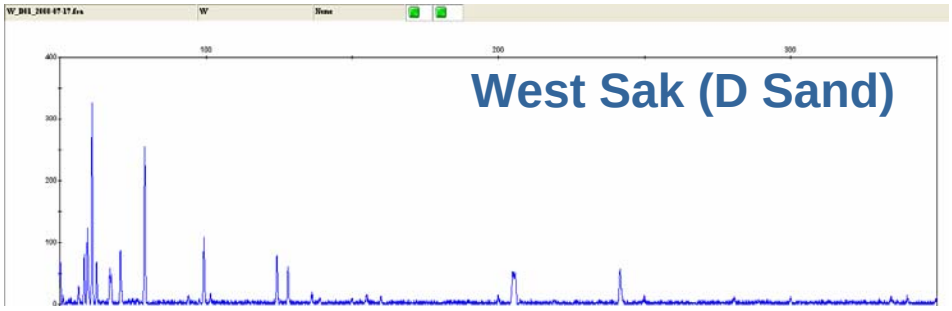


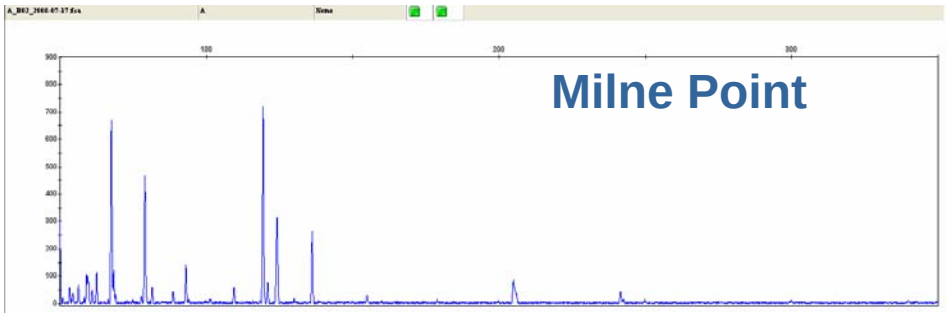
Figure 4.26: Agarose gel electrophoresis image showing successful PCR amplification of 16S rRNA genes from bacteria present in the 5 ANS oil samples. These amplicons were used for T-RFLP analyses.

Next, PCR products were subjected to digestion and T-RFLP analysis as previously described. Figure 4.27 shows the T-RFLP fingerprints generated from each of the five ANS oils examined in this portion of the study. The results show that each of the oils possessed a diverse bacterial community and differed in their community assemblage. Some peaks of the same size were present in different oils, suggesting that members of the same bacterial lineage may be present in multiple reservoirs. Interestingly, the two Prudhoe Bay samples (P and S) possessed different community composition and structure, despite having been recovered from the same reservoir. The two samples were obtained from different production platforms. This suggests that spatial heterogeneity is present across a single reservoir. This observation is important when considering biostimulation approaches for MEOR, since important populations may differ in presence and abundance at different locations within the reservoir.


Prudhoe Bay-P


Prudhoe Bay-S


West Sak (D Sand)


Milne Point

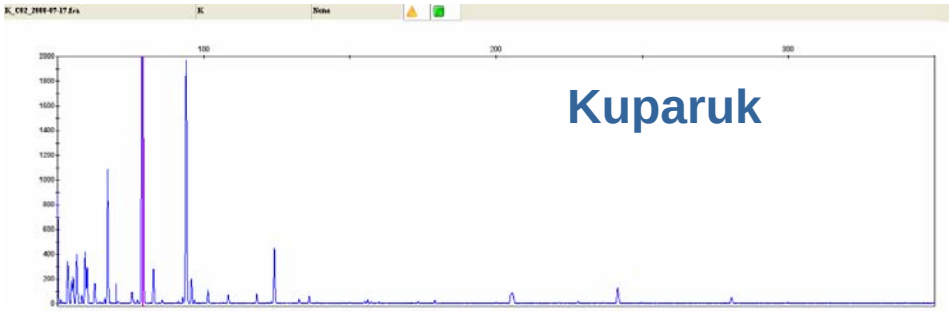


Figure 4.27: T-RFLP profiles generated from each of the 5 ANS oils investigated.

T-RFLP profiles were also obtained from the EVOS enrichment isolates that demonstrated the ability to reduce surface tension in the optical distortion assay. Each isolate produced a single peak, which verifies that the cultures were indeed pure and that digestion was successful. The size of each of these peaks (T-RFs) was compared to peaks present in each of the ANS oils to determine if these organisms or close relatives were present in the native oil communities. None of the peaks were detected in the ANS oils. This supports the notion that these isolates originated from the oiled rock samples from the EVOS, which were used as inoculum for this particular culture. These organisms may prove useful in future MEOR efforts that focus on bioaugmentation with exogenous microorganisms or microbial surfactants.

Figure 4.28: T-RFLP profiles generated by each of the isolates obtained from the EVOS enrichment culture.

CHAPTER 5: CONCLUSIONS

i. The developed microbial formulation using *Bacillus licheniformis* has excellent growth at 30°C. The reservoir temperature of an ANS oil field is about 30°C, so it can be concluded that the microbial formulation could flourish well in the reservoir.

ii. The concentration of microbial formulation used for all the experiments was determined to be approximately 1 x 10^7 cell/ml.

iii. The coreflooding rig was used to estimate the porosity and absolute permeability of the core samples. The porosity values of the cores ranged between 13% and 16%, whereas the absolute permeability values for the cores ranged between 27 and 68 md.

iv. Brine solution or microbial formulation was used to simulate waterflooding with brine injection. Oil recovery due to waterflooding ranged between 52% and 68%. Recovery was 52% to 54% when the brine solution was used for waterflooding, whereas recovery was 62% to 68% when the microbial formulation was used for waterflooding. The brine or microbial formulation breakthrough always occurred in the range of 0.5 to 1.0 pore volume of brine injected.

v. Microbial formulation was injected in the cores, and after a shut-in period of one week, it was observed that there was incremental oil recovery. The incremental oil recovery ranged between 7% and 8% for cases where microbial formulation was injected after previous waterflooding, whereas incremental oil recovery was 11% to 14% when the microbial formulation was injected without waterflooding.

vi. There was a decrease in residual oil saturation in the core after microbial activity. The decrease was in the range of 2% to 3%. The decrease was due to incremental oil recovery.

vii. Incremental oil recovery from the core in contact with the oil for two months was 11.94%, as compared with the incremental oil recovery from cores with a shut-in period of one week, which was 11% to 13%. The incremental oil recovery for both cases was nearly equal. Thus, it is safe to conclude that the major microbial activity resulting in incremental oil recovery ended before the first week. By the

end of the first week, the microbes probably reached a stationary phase of their life, where the bacteria were not growing or producing bioproducts at a high rate.

viii. Total oil recovery from the coreflooding experiments ranged between 56% and 72%.

ix. Compositional analysis using a gas chromatograph shows that there is a miniscule decrease in the concentration of the C_{25}+ group and an increase in the middle carbon number ($C_{12} - C_{23}$) groups. From this analysis, it cannot be quantitatively concluded that the microorganisms are degrading the long-chained carbon compounds into lower carbon number compounds. However, it can be concluded that the combination of biodegradability of higher hydrocarbons into lighter hydrocarbons and biosurfactant and biogas production are the oil recovery mechanisms.

x. There is a decrease in the density of oil after microbial treatment from 0.9484 g/cc to 0.8954 g/cc. Also, there is a decrease in viscosity of the oil from 67.5 cp to 50.2 cp. The compositional analysis of the oil shows that there is degradation of higher hydrocarbons to lighter hydrocarbons in the oil, which results in a decrease in the density and viscosity of the oil.

xi. DNA was extracted from the oil sample, the microbial formulation, the oil sample extracted after one week, and the oil sample extracted from a core in contact for two months. The agarose gel photograph after the PCR reaction shows a band for all the samples, indicating that the oil samples from the ANS oil fields have indigenous microorganisms.

xii. The T-RFLP chromatograph for the oil sample from the ANS oil fields shows that there is a diverse microbial community in the oil sample used for coreflooding studies. The chromatograph shows a peak at the size of 225 base pairs. This peak is also seen in the chromatographs for the microbial formulation, the oil sample extracted after one week, and the oil sample extracted after two months. This indicates that the original oil sample may contain the bacteria of the same species, that is, *Bacillus*.

xiii. Normalizing the peaks from the chromatograms for the samples shows that the bacterium used in the microbial formulation accounts for 1% to 2% of the overall

bacterial community in the original oil sample. The same microorganism contributes to 5% to 12% of the overall microbial community of the oil sample extracted after the one-week shut-in period, whereas in the oil sample extracted after two months, it contributes to 0.4% to 0.7% of the overall bacterial community. However, these two samples cannot be directly compared, because oil after the one-week shut-in period was analyzed, whereas a mixture of oil and microbial formulation was used for analysis after two months due to the small volume of oil. Results indicate that the inoculation increased the relative abundance of this species in the oil microbial community, which was correlated with enhanced oil recovery.

xiv. Efforts to cultivate microorganisms capable of reducing surface tension from five different ANS crude oils yielded positive results from oil collected from Milne Point. The positive results indicate that indigenous surfactant producers are present in at least some ANS oils, which could be the target of biostimulation efforts to produce biosurfactants *in situ*.

xv. A variety of bacterial isolates was obtained from rocks oiled by the Exxon Valdez oil spill, which reduced the surface tension of the liquid media in which they were grown. These organisms are good candidates for future study of their potential for *ex situ* MEOR through bioaugmentation or biosurfactant flooding approaches.

xvi. The optical distortion assay for screening of surface tension reduction capabilities was rapid and simple, but produced several false-positive results. Once organisms were isolated and the surface tension of growth media was tested with a ring tensiometer, several of them did not actually reduce surface tension. Nonetheless, the optical distortion test is a rapid means to narrow down the organisms worthy of the more time-consuming ring tensiometer analysis.

xvii. The microbial community composition of five different ANS oils investigated all differed substantially, as evidenced by T-RFLP profiling.

xviii. Microbial community assemblages from ANS oils of the same reservoir (Prudhoe Bay) but from two different production platforms differed from each other. This difference indicates that significant spatial heterogeneity exists within reservoirs, and has implications for *in situ* MEOR.

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APPENDIX A: Results of Waterflooding the Core Samples

Table A.1: Result of waterflooding on Core #1.

PV brine injected	Volume of oil (ml)	Oil recovery (%)	PV brine injected	Volume of oil (ml)	Oil recovery (%)
0	0	0.00	0.65	5.4	35.53
0.05	0	0.00	0.7	5.9	38.82
0.1	0	0.00	0.75	6.3	41.45
0.15	0	0.00	0.8	6.7	44.08
0.2	0	0.00	0.85	7.1	46.71
0.25	0.2	1.32	0.9	7.5	49.34
0.3	0.6	3.95	0.95	7.9	51.97
0.35	1.3	8.55	1	8.1	53.29
0.4	2	13.16	1.05	8.1	53.29
0.45	2.7	17.76	1.1	8.1	53.29
0.5	3.5	23.03	1.15	8.1	53.29
0.55	4.7	30.92	1.2	8.1	53.29
0.6	5	32.89	1.25	8.1	53.29

Table A.2: Result of waterflooding on Core #2.

PV brine injected	Volume of oil (ml)	Oil recovery (%)	PV brine injected	Volume of oil (ml)	Oil recovery (%)
0	0.0	8.8	0.65	6.3	42.83
0.05	0.0	8.8	0.7	6.5	43.91
0.1	0.0	9.1	0.75	6.7	45.73
0.15	0.0	9.1	0.8	6.7	45.73
0.2	0.1	9.1	0.85	7.6	51.64
0.25	0.2	9.2	0.9	7.7	52.12
0.3	0.4	9.2	0.95	8.8	60.16
0.35	1.4	9.2	1	8.8	60.16
0.4	2.0	9.2	1.05	8.8	60.16
0.45	3.0	9.2	1.1	9.1	61.77
0.5	4.1	9.2	1.15	9.1	61.77
0.55	5.3	9.2	1.2	9.1	61.77
0.6	6.3	9.2	1.25	9.2	62.59

Table A.3: Result of waterflooding on Core #3.

PV brine injected	Volume of oil (ml)	Oil recovery (%)	PV brine injected	Volume of oil (ml)	Oil recovery (%)
0	0.0	0	0.65	6.6	45.91
0.05	0.0	0.16	0.7	6.6	45.91
0.1	0.1	1.03	0.75	6.6	45.91
0.15	0.4	2.5	0.8	7.6	53.18
0.2	0.7	4.95	0.85	9.0	62.79
0.25	1.3	9.01	0.9	9.0	62.79
0.3	2.0	13.64	0.95	9.0	62.79
0.35	2.9	20.45	1	9.0	62.79
0.4	3.9	27.12	1.05	9.1	63.29
0.45	5.0	35.05	1.1	9.1	63.29
0.5	6.2	43.14	1.15	9.3	64.91
0.55	6.3	43.82	1.2	9.3	64.91
0.6	6.4	44.61	1.25	9.3	64.91

Table A. 4: Result of waterflooding on Core #4.

PV brine injected	Volume of oil (ml)	Oil recovery (%)	PV brine injected	Volume of oil (ml)	Oil recovery (%)
0	0.0	0	0.65	8.1	47.45
0.05	0.0	0.18	0.7	8.1	47.45
0.1	0.2	1.07	0.75	9.5	55.51
0.15	0.5	2.67	0.8	9.7	56.62
0.2	1.0	5.88	0.85	9.8	57.18
0.25	1.7	10.16	0.9	11.2	65.52
0.3	2.7	15.69	0.95	11.2	65.52
0.35	3.8	22.47	1	11.2	65.52
0.4	5.1	29.79	1.05	11.4	66.83
0.45	6.4	37.27	1.1	11.4	66.83
0.5	7.7	45.11	1.15	11.6	67.64
0.55	7.9	46.28	1.2	11.6	67.64
0.6	8.1	47.45	1.25	11.6	67.64

Table A.5: Result of waterflooding on Core #5.

PV brine injected	Volume of oil (ml)	Oil recovery (%)	PV brine injected	Volume of oil (ml)	Oil recovery (%)
0	0.0	0	0.65	8.2	46.73
0.05	0.0	0.17	0.7	8.2	46.73
0.1	0.2	1.05	0.75	8.9	50.71
0.15	0.5	2.56	0.8	9.7	54.83
0.2	1.0	5.51	0.85	10.5	59.92
0.25	1.7	9.72	0.9	11.4	64.51
0.3	2.5	14.48	0.95	11.4	64.51
0.35	3.8	21.46	1	11.4	64.51
0.4	5.0	28.43	1.05	11.5	65.53
0.45	6.4	36.16	1.1	11.5	65.53
0.5	7.8	44.12	1.15	11.5	65.53
0.55	7.9	44.75	1.2	11.5	65.53
0.6	7.9	45.16	1.25	11.5	65.53

Table A. 6: Result of waterflooding on Core #6.

PV brine injected	Volume of oil (ml)	Oil recovery (%)	PV brine injected	Volume of oil (ml)	Oil recovery (%)
0	0.0	0	0.65	6.5	37.34
0.05	0.0	0.1754	0.7	6.9	39.44
0.1	0.2	1.11	0.75	7.4	42.44
0.15	0.6	3.68	0.8	7.4	42.44
0.2	1.4	8.03	0.85	7.9	45.44
0.25	2.3	13.07	0.9	8.3	47.96
0.3	3.0	17.3	0.95	8.9	50.89
0.35	3.4	19.8	1	8.9	50.89
0.4	3.9	22.18	1.05	9.2	52.64
0.45	5.0	28.51	1.1	9.2	52.64
0.5	5.4	30.85	1.15	9.2	52.64
0.55	5.6	32.42	1.2	9.2	52.64
0.6	6.1	34.93	1.25	9.2	52.64
0.65	6.5	37.34	1.3	9.2	52.64

APPENDIX B: Coreflooding Calculations for Core #1

1. Core dimensions:

   Diameter = 1.5 inch

   Length = 6 inch

2. Brine Properties:

   Brine viscosity = 1.12 cp

   Brine density = 1.03 g/cc

3. Weight measurement of the core:

   Dry core = 349.73 g

   Wet core = 374.43 g

   Weight of brine = 374.43 -349.73

   = 24.7 cc

4. Pore volume = $\frac{24.7}{1.03}$ = 23.98 ml

5. Bulk volume of the core = $\frac{\pi \times 3.81^2 \times 15.24}{4}$ = 173.77 cc

6. Porosity of the core = $\frac{23.98}{173.77} \times 100$ = 13.80 %

7. Initial waterflood results:

   Flow rate = 3.333 ml/min

   Differential pressure = 45 psi

   Cross-sectional area of core = 11.40 cm^2

8. Absolute permeability of core = $\frac{0.056 \times 1.12 \times 15.24}{3.062 \times 11.40}$ = 27.19 md

9. Volume of brine produced by oil injection = 15.2 ml

10. Irreducible water saturation = $\frac{24.7 - 15.2}{24.7} \times 100$ = 36.62 %

11. Initial oil saturation = 100 – 36.62 = 63.38 %

12. Volume of oil collected after waterflooding = 8.1ml

13. Residual oil saturation = $\frac{15.2 - 8.1}{24.7} \times 100$ = 29.61 %

14. Oil recovery = $\frac{8.1}{15.2} \times 100 = 53.29\ \%$

15. Incremental oil collected = 0.5 ml

16. Incremental oil recovery = $\frac{0.5}{15.2 - 8.1} \times 100 = 7.04\ \%$

17. Total oil produced = 8.1 + 0.5 = 8.6 ml

18. Total oil recovery = $\frac{8.6}{15.2} \times 100 = 56.58\ \%$

APPENDIX C: Composition of the PCR and Digestion Mix

Table C.1: Composition of PCR mix.

Components	**Volume (µl)**
Water	36.65
Buffer (Invitrogen)	5
MgCL2 (Invitrogen)	3
dNTPs (25 mM)	0.4
27F-FAM (100 µM)	0.1
1392R (100 µM)	0.1
Taq (Invitrogen)	0.25
BSA (NEB, 10 mg/ml)	0.5
DNA	4
Total	**50**

Table C.2: Composition of digestion mix.

Components	**Volume (µl)**
10X Buffer 4 (NEB)	1.5
100X BSA (10 mg/ml)	0.15
DNA	40/Conc. of DNA
Water	13 - DNA
HhaI (NEB, 20000 U/ml)	0.5
Total	**15**

APPENDIX D: Results of T-RFLP Run of the Samples

Table D.1: T-RFLP results for the oil sample for the ANS field.

Sample Peak	Size	Height	Sample peak	Size	Height
1	1.65	94	23	119.61	85
2	6.16	88	24	121.27	102
3	8.21	348	25	136.52	73
4	19.11	65	26	152.46	73
5	19.69	57	27	190.84	55
6	27.19	6955	28	194.62	514
7	28.53	2245	29	202.55	208
8	29.73	737	30	203.83	162
9	30.8	765	31	205.28	8630
10	31.96	686	32	206.86	87
11	33.04	320	33	211.73	129
12	34.29	188	34	224.5	207
13	36.61	173	35	235.94	3750
14	38.97	411	36	237.07	1563
15	47.95	68	37	239.71	202
16	56.76	3045	38	342.39	275
17	58.27	727	39	370.29	69
18	59.07	69	40	371.92	60
19	61.17	153	41	566.71	58
20	78	387	42	583.12	219
21	78.6	142	43	610.65	74
22	112.87	136	44	675	71

Table D.2: T-RFLP results for the microbial formulation.

Sample Peak	Size	Height	Sample Peak	Size	Height
1	-	91	32	73.36	66
2	3.32	68	33	73.92	76
3	5.9	90	34	74.91	331
4	7.06	77	35	76.37	73
5	7.93	152	36	78.17	95
6	9.09	6918	37	79.45	74
7	19.51	228	38	80.87	86
8	27.12	944	39	82.34	87
9	28.46	494	40	83.48	114
10	29.66	369	41	84.62	91
11	30.77	244	42	85.62	75
12	31.83	110	43	88.96	190
13	32.98	68	44	92.41	55
14	37.64	57	45	93.56	105
15	38.98	189	46	95.29	57
16	46.54	58	47	96.53	56
17	50.77	72	48	98.89	67
18	54.59	148	49	100.05	53
19	55.28	52	50	105.16	78
20	56.61	81	51	106.7	53
21	60.32	58	52	108.19	62
22	61.61	68	53	110.36	95
23	62.67	77	54	114.44	73
24	63.69	70	55	116.41	129
25	64.98	64	56	117.9	55
26	66.32	56	57	118.48	68
27	67.62	66	58	119.58	79

28	68.46	62	59	121.98	68
29	69.67	99	60	122.89	63
30	71.02	171	61	127.36	94
31	72.1	71	62	128.31	58

Table D-3: T-RFLP results for the oil sample after one-week shut-in period. (Eluted in 40 µl Buffer AE.)

Sample Peak	**Size**	**Height**	**Sample Peak**	**Size**	**Height**
1	-	50	33	84.62	106
2	3.19	173	34	85.56	75
3	9.03	1865	35	88.94	84
4	19.43	76	36	93.61	89
5	27.1	306	37	96.44	64
6	28.42	217	38	98.91	56
7	29.61	108	39	105.14	64
8	31.98	69	40	110.33	67
9	39.01	51	41	114.42	62
10	46.58	73	42	116.32	109
11	48.91	50	43	118.4	56
12	50.76	89	44	119.64	60
13	55.23	50	45	122.82	79
14	56.63	55	46	127.34	87
15	60.36	81	47	131.07	104
16	61.63	57	48	139.5	51
17	62.63	83	49	141.22	54
18	63.68	60	50	143.04	51
19	65.01	58	51	147.59	60
20	67.62	61	52	148.56	107

21	68.5	52	53	167.79	75
22	69.69	55	54	170.8	165
23	71.07	205	55	171.78	139
24	72.09	67	56	172.91	93
25	73.38	59	57	174.08	55
26	73.89	79	58	175.07	114
27	74.86	317	59	179.19	69
28	76.4	68	60	199.81	63
29	79.47	78	61	224.87	592
30	80.92	87	62	236.98	8584
31	82.37	86	63	787.37	51
32	83.5	123	64	1109.81	178

Table D. 3: T-RFLP results for the oil sample after one-week shut-in period. (Eluted in 50 µl Buffer AE).

Sample Peak	**Size**	**Height**	**Sample Peak**	**Size**	**Height**
1	-	75	33	73.85	112
2	3.53	1728	34	74.86	454
3	5.83	158	35	76.29	99
4	9.31	1585	36	78.04	77
5	19.67	130	37	79.43	102
6	27.22	474	38	80.86	121
7	28.57	352	39	82.34	128
8	29.75	209	40	83.46	176
9	30.88	85	41	84.57	153
10	31.87	175	42	85.59	118
11	33	51	43	86.93	62
12	34.45	65	44	88.89	156
13	39.01	58	45	90.05	60
14	46.56	83	46	92.38	63
15	48.92	54	47	93.59	141
16	50.76	90	48	95.18	66
17	54.56	81	49	96.4	88
18	55.28	60	50	97.56	53
19	56.58	71	51	98.88	91
20	58.78	54	52	99.95	62
21	60.31	77	53	101.17	52
22	61.62	72	54	103.24	51
23	62.62	91	55	105.12	88
24	63.61	99	56	106.57	68
25	64.97	73	57	108.17	72
26	66.24	57	58	109.34	51

27	67.56	84	59	110.32	106
28	68.47	65	60	111.73	53
29	69.7	75	61	114.4	83
30	71.02	250	62	116.31	168
31	72.03	86	63	117.86	63
32	73.26	81	64	118.46	77

Table D. 4: T-RFLP results for the oil sample after one-week shut-in period (continued). (Eluted in 50 µl Buffer AE.)

Sample Peak	**Size**	**Height**	**Sample Peak**	**Size**	**Height**
65	119.58	94	94	168.99	62
66	121.92	76	95	169.77	64
67	122.76	80	96	170.78	268
68	127.35	139	97	171.83	249
69	128.22	66	98	173.03	157
70	129.37	75	99	174.13	87
71	130.29	68	100	175.14	181
72	131.02	170	101	177.34	59
73	133.27	63	102	179.13	115
74	135.84	53	103	185.03	54
75	138.77	50	104	187.96	61
76	139.64	81	105	189.15	161
77	140.19	65	106	190.1	72
78	141.24	72	107	197.45	81
79	143.02	78	108	198.5	52
80	144.21	53	109	199.86	99
81	145.44	51	110	202.15	58
82	147.72	105	111	205.32	110
83	148.63	175	112	209.43	50

84	151.94	62	113	212.16	55
85	156	53	114	219.02	54
86	157.16	52	115	224.66	2727
87	158.17	86	116	229.1	116
88	160.38	59	117	230.07	278
89	161.44	59	118	236.76	8262
90	163.1	53	119	237.25	8268
91	165.49	54	120	294.4	265
92	166.5	55	121	680.51	244
93	167.75	124	122	1110.32	254

Table D. 5: T-RFLP results for the oil sample after two-month shut-in period. (Eluted in 40 µl Buffer AE).

Sample Peak	**Size**	**Height**	**Sample Peak**	**Size**	**Height**
1	-	57	23	56.41	109
2	3.91	80	24	58.79	58
3	4.88	167	25	71.45	193
4	5.52	152	26	73.22	88
5	9.02	1127	27	87.41	86
6	9.94	64	28	114.32	69
7	14.78	96	29	121.06	81
8	19.57	89	30	153.76	62
9	23.66	204	31	170.74	59
10	24.91	53	32	171.78	57
11	26.57	133	33	194.16	61
12	27.12	433	34	204.9	8007
13	28.45	250	35	223.05	110
14	29.79	117	36	235.93	7748
15	30.66	204	37	236.98	7069

16	31.81	185	38	240.6	52
17	33.06	87	39	242.01	56
18	34.39	112	40	564.43	109
19	37.11	89	41	566.08	56
20	37.75	155	42	573.55	181
21	38.26	69	43	575.36	60
22	43.19	335	44	1110.42	52

Table D.6: T-RFLP results for the oil sample after two-month shut-in period. (Eluted in 50 µl Buffer AE.)

Sample Peak	**Size**	**Height**
1	4.81	51
2	7.99	55
3	9.01	649
4	19.5	79
5	27.08	338
6	28.42	223
7	29.53	128
8	30.78	113
9	31.75	64
10	71.54	215
11	73.22	54
12	114.37	67
13	120.41	100
14	170.72	54
15	193.46	56
16	194.12	69
17	204.86	7982
18	220.29	66

19	223.11	146
20	235.91	7723
21	237	6836
22	240.84	81
23	249.1	56
24	564.29	209
25	566.03	119
26	573.48	387
27	575.34	108
28	848.7	57
29	1109.79	103

APPENDIX E: Normalization of Peaks of T-RFLP Chromatogram

Table E.1: Normalization of peaks in T-RFLP for oil sample.

Sample peak	Size	Height	Normalized height (Height/Total height)	Normalized height (%)
16	56.76	3045	0.15	14.8168
17	58.27	727	0.04	3.537541
19	61.17	153	0.01	0.744489
20	78	387	0.02	1.88312
21	78.6	142	0.01	0.690964
22	112.87	136	0.01	0.661768
24	121.27	102	0	0.496326
28	194.62	514	0.03	2.501095
29	202.55	208	0.01	1.012116
30	203.83	162	0.01	0.788283
31	205.28	8630	0.42	41.99309
33	211.73	129	0.01	0.627707
34	224.5	207	0.01	1.00725
35	235.94	3750	0.18	18.24729
36	237.07	1563	0.08	7.605469
37	239.71	202	0.01	0.982921
38	342.39	275	0.01	1.338134
42	583.12	219	0.01	1.065642
Total		**20551**		

Table E.2: Normalization of peaks of T-RFLP for microbial formulation.

Sample peak	Size	Height	Normalized height (Height/Total height)	Normalized height (%)
17	50.77	72	0.003083	0.308338
18	54.59	148	0.006338	0.633806
30	71.02	171	0.007323	0.732303
34	74.91	331	0.014175	1.417498
40	83.48	114	0.004882	0.488202
43	88.96	190	0.008137	0.81367
45	93.56	105	0.004497	0.44966
55	116.41	129	0.005524	0.552439
65	131.05	130	0.005567	0.556721
73	148.59	143	0.006124	0.612393
77	167.73	111	0.004754	0.475354
80	170.77	242	0.010364	1.036358
81	171.81	304	0.013019	1.301871
82	172.99	158	0.006766	0.676631
84	175.12	152	0.006509	0.650936
85	179.15	108	0.004625	0.462507
88	205.31	1132	0.048478	4.847758
90	225.04	2648	0.1134	11.34
91	236.89	8481	0.363196	36.31964
92	237.16	8482	0.363239	36.32393
Total		**23351**		

Table E.3: Normalization of peaks in T-RFLP for oil sample extracted after one-week shut-in period. (Eluted in 40 µl Buffer AE.)

Sample peak	**Size**	**Height**	**Normalized height (Height/Total height)**	**Normalized height (%)**
23	71.07	205	0.018818	1.88177
27	74.86	317	0.029099	2.909859
32	83.5	123	0.011291	1.129062
33	84.62	106	0.00973	0.973013
42	116.32	109	0.010006	1.000551
47	131.07	104	0.009547	0.954654
52	148.56	107	0.009822	0.982192
54	170.8	165	0.015146	1.514595
55	171.78	139	0.012759	1.275932
58	175.07	114	0.010464	1.046448
61	224.87	592	0.054342	5.434184
62	236.98	8584	0.787957	78.79567
63	787.37	51	0.004681	0.468148
64	1109.81	178	0.016339	1.633927
Total		**10894**		

Table E.4: Normalization of peaks in T-RFLP for oil sample extracted after one-week shut-in period. (Eluted in 50 µl Buffer AE.)

Sample peak	Size	Height	Normalized height (Height/Total height)	Normalized height (%)
30	71.02	250	0.010182	1.018206
33	73.85	112	0.004562	0.456156
34	74.86	454	0.018491	1.849061
37	79.43	102	0.004154	0.415428
38	80.86	121	0.004928	0.492811
39	82.34	128	0.005213	0.521321
40	83.46	176	0.007168	0.716817
41	84.57	153	0.006231	0.623142
42	85.59	118	0.004806	0.480593
44	88.89	156	0.006354	0.63536
47	93.59	141	0.005743	0.574268
59	110.32	106	0.004317	0.431719
62	116.31	168	0.006842	0.684234
68	127.35	139	0.005661	0.566122
72	131.02	170	0.006924	0.69238
82	147.72	105	0.004276	0.427646
83	148.63	175	0.007127	0.712744
93	167.75	124	0.00505	0.50503
96	170.78	268	0.010915	1.091516
97	171.83	249	0.010141	1.014133
98	173.03	157	0.006394	0.639433
100	175.14	181	0.007372	0.737181
102	179.13	115	0.004684	0.468375
105	189.15	161	0.006557	0.655724

111	205.32	110	0.00448	0.44801
115	224.66	2727	0.111066	11.10659
116	229.1	116	0.004724	0.472447
117	230.07	278	0.011322	1.132245
118	236.76	8262	0.336497	33.64966
119	237.25	8268	0.336741	33.67409
120	294.4	265	0.010793	1.079298
121	680.51	244	0.009938	0.993769
122	1110.32	254	0.010345	1.034497
Total		**24553**		

Table E.5: Normalization of peaks in T-RFLP for oil sample extracted after two months. (Eluted in 40 µl Buffer AE.)

Sample peak	**Size**	**Height**	**Normalized height (Height/Total height)**	**Normalized height (%)**
24	56.41	109	0.004633	0.463317
26	71.45	193	0.008204	0.820369
35	204.9	8007	0.340347	34.03469
36	223.05	110	0.004676	0.467568
37	235.93	7748	0.329338	32.93378
38	236.98	7069	0.300476	30.04761
41	564.43	109	0.004633	0.463317
43	573.55	181	0.007694	0.769362
Total		**23526**		

Table E.6: Normalization of peaks in T-RFLP for oil sample extracted after two months. (Eluted in 50 µl Buffer AE.)

Sample peak	**Size**	**Height**	**Normalized height (Height/Total height)**	**Normalized height (%)**
10	71.54	215	0.009023	0.9023
17	204.86	7982	0.334984	33.49841
19	223.11	146	0.006127	0.612725
20	235.91	7723	0.324114	32.41145
21	237	6836	0.286889	28.68894
24	564.29	209	0.008771	0.877119
25	566.03	119	0.004994	0.499412
26	573.48	387	0.016241	1.62414
27	575.34	108	0.004532	0.453248
29	1109.79	103	0.004323	0.432265
Total		**23828**		

National Energy Technology Laboratory

626 Cochrans Mill Road
P.O. Box 10940
Pittsburgh, PA 15236-0940

3610 Collins Ferry Road
P.O. Box 880
Morgantown, WV 26507-0880

One West Third Street, Suite 1400
Tulsa, OK 74103-3519

1450 Queen Avenue SW
Albany, OR 97321-2198

539 Duckering Bldg./UAF Campus
P.O. Box 750172
Fairbanks, AK 99775-0172

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