Study of Microbial Enhanced Oil Recovery using Indigenous Bacteria in Egyptian Oil Reservoirs

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Declaration

I, Hamed Ali Hamed Aboelkhair, declare that this thesis has been composed by myself with the fabulous support of my supervisors. The thesis is submitted for examination in consideration for the award of a degree of Doctor of Philosophy in Chemical, Process and Energy Engineering. I would like to emphasise that it is my effort and that the work has not been submitted for any other degree or professional qualification. Furthermore, I took reasonable care to ensure that the work is original and to the best of my knowledge, does not breach copyright law and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Hamed Aboelkhair

…….. Candidate's Signature

26th September 2022

…….. Date

Dedication

I would like to dedicate my PhD to the godfather of every BUE student and staff member "Mr Mohamed Farid Khamis", who always supported all of us in every step of our life. I always remember and cherish his constant commitment and encouragement to develop Egyptian EG education to serve the community. I was constantly imagining the moment when I would be able to present my "Doctoral Viva" and dedicate all this success to him.

May Allah bless his soul, forgive him, make his grave a garden and grant him the highest levels of paradise. Amen.

Thank you, Mr Mohamed Farid Khamis, for everything

Hamed Aboelkhair

Dedication

I would like to dedicate my PhD thesis to my father "Mr Ali Aboelkhair", who always supports me in every step of my life. I always remember and cherish his constant commitment to my education and encouragement to continue my research work and develop it to serve the community and the world as much as I can. I was constantly imagining the moment when I would be able to present my PhD thesis and dedicate all this success to him.

Thank you, dad, for everything

Your Son “Hamed”

Hamed Aboelkhair

إهداء

أود أن أهدي رسالة الدكتوراه الخاصة بي لوالدي السيد علي أبو الخير الذي يدعمني دائمًا في كل خطوة في حياتي .أتذكر دائمًا وأعتز بالتزامه الدائم بتعليمي وتشجيعي على مواصلة أعمالي البحثية والاستمرار في تطويرها لخدمة المجتمع والعالم بقدر ما أستطيع .كنت أتخيل باستمرار اللحظة التي سأتمكن فيها من عرض رسالة الدكتوراه الخاصة بي وأن أهدي كل هذا النجاح أليه

شكرا والدي الحبيب على كل شيء

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Nomenclature

API	**American Petroleum institute**
BF	Biosurfactant Flooding
BHMS	Bushnell Hass Mineral Salts
BP	British Petroleum
BV	Bulk Volume
^{o}C	Celsius
CFU	Colony Forming Unit
CMC	Critical Micelle Concentration
CNPC	China National Petroleum Corporation
cp	Centi-poise
E24	Emulsification Index
EOR	Enhanced Oil Recovery
EWF	Extended Water Flooding
m	Meter
g/l	Gram per Litre
GW.h	Giga Watt. Hour
h	Hour
IFT	Interfacial Tension
IWF	Initial Water Flooding
JASCO	Joint Assault Signal Company
L	Lower
m	Meter
MBDF	Meters Below Derrick Flow
mD	Milli-Darcy
MEOR	Microbial Enhanced Oil Recovery
mPa.s	Milli-Pascal. Second
MPa	Mega-Pascal
Mw	Average Molecular Weight
N	North

Nm	Nanometre
ppm	Part Per Million
PV	Pore Volume
S	South
ST	Surface Tension
TPEC	Total Primary Energy Consumption
U	Upper
WD	Western Desert
ΔP	Pressure Drop

Abstract

Microorganisms provide a unique opportunity for improving oil recovery economically and environmentally in a technique called “Microbial Enhanced Oil Recovery MEOR”. This study highlights the importance and potential of microbiology in petroleum engineering. Biosurfactant production is one of the most efficient mechanisms in microbial enhanced oil recovery (MEOR) processes. Biosurfactants have recently attained extended attention because they have numerous benefits over chemical synthetic surfactants, including higher biodegradability, lower toxicity, higher foaming, environmental compatibility, and effective properties under harsh conditions.

The present study investigates the production of biosurfactants by indigenous bacteria isolated from Egyptian oil fields, and the use of these biosurfactants in enhancing the oil recovery.

Fifty-nine Egyptian oil reservoirs were screened to investigate the potential for MEOR in Egyptian oil fields. The results showed that 8 reservoirs from the Gulf of Suez and 3 reservoirs from the Western Desert had the potential for MEOR. The bacterial isolation and identification of the collected crude oil samples from the Egyptian oil fields that have the potential for MEOR showed 11 isolated strains, which are *Pseudomonas stutzeri, Clostridium spp, pseudomonas aeruginosa, pseudomonas fluorescens, Brevibacterium spp, Cellulosimicrobium spp, Pseudomonas panipatensis, Enterobacter spp, Bacillus flexus, Bacillus licheniformis, and Bacillus subtilis*. The isolated strains *Bacillus subtilis and Bacillus licheniformis* were selected for further studies in this research because they are reported as good biosurfactants-producing bacteria under facultative or anaerobic conditions, spore forming, and non-pathogenic. The results of surface activity and bacteria growth examination also showed that the selected bacterial strains *Bacillus licheniformis* and *Bacillus subtilis* could produce effective biosurfactants that reached their maximum surface activity and reach maximum after 24 h of incubation. The results of emulsification activity examination showed that produced biosurfactants by *Bacillus licheniformis* and *Bacillus subtilis* could significantly emulsify crude oil with emulsification indices of 50% and 64%, respectively. The contact angle measurement showed that the oil was more detached from the sandstone surface when submerged in an aqueous solution of the produced biosurfactants, where the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* decreased the contact angle of the oil drop from 104.96° and 107.30° to 85.40° and 88.72° after 24 h, respectively. Similarly, the new

proposed medium decreased the contact angle of the oil drop from 112.30° and 110.90° to 63.85° and 69.33° after 24 h, respectively, which could facilitate the recovery of remaining oil. High stability was observed at high temperatures for a long-time period and more than 60% of their surface and emulsification activities were maintained over a wide range of pH and salinity. The core flooding tests showed the potential of the biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* to recover up to 31% and 39% of additional oil over the water flooding residual oil saturation under simulated reservoir conditions, respectively. In addition to the beneficial effects of the selected indigenous bacteria in producing effective biosurfactants, the performed environmental risk assessment showed that *Bacillus licheniformis* and *Bacillus subtilis* are environmentally safe, have no potential for toxicity, and no risk could occur for MEOR.

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Publications

Journal Publications

Aboelkhair, H., Diaz, P., Attia, A., 2022. Environmental comparative study of biosurfactants production and optimization using bacterial strains isolated from Egyptian oil fields. J. Pet. Sci. Eng. 216, 110796. https://doi.org/10.1016/J.PETROL.2022.110796

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CHAPTER 1

INTRODUCTION

Outline of the chapter

This chapter gives a background of the research work, its aims, objectives, and contribution to knowledge and provides an outline of the thesis. The chapter is organised as follows:

1.1. Overview and Background

1.2. Motivation

1.3. Aims and Objectives

1.4. Contribution to Knowledge

1.5. Structure of Thesis

1. INTRODUCTION

1.1 Overview and Background

The world's total primary energy consumption (TPEC) has recorded a steep rise within the last decades reaching 150 TW.h in 2015 (Patel et al., 2015). About 57% growth of this value is expected by 2050 owing to the rapid industrial development and urbanisation that has occurred globally. Presently, fossil fuels including petroleum and natural gas are the main sources of energy (Hajjari et al., 2017). According to the British Petroleum (BP) statistical review of the world's energy, consumption of energy has extensively increased during the last decade as shown in Figure 1-1 2019 (BP Statistical Review of World Energy). The reasons for increases in energy consumption include industrial development, population growth and the invention of new technologies. Industrial developments in different applications require excessive energy for operations using either electricity or heating. Thus, electricity consumption, which until this moment relies mostly on the combustion of fossil fuels, has broadly increased. Moreover, population growth has a direct impact on energy consumption. In addition, new technologies which are created to enhance industrial productivity, preform multitasking requirements, and provide better working environment for humans have excessive impact on energy consumption. These new technologies include special transportation means, heating/cooling equipment and electricity consumptions through electronic devices. All the above-mentioned aspects cause the depletion of fossil fuels reserves (Mardhiah et al., 2017).

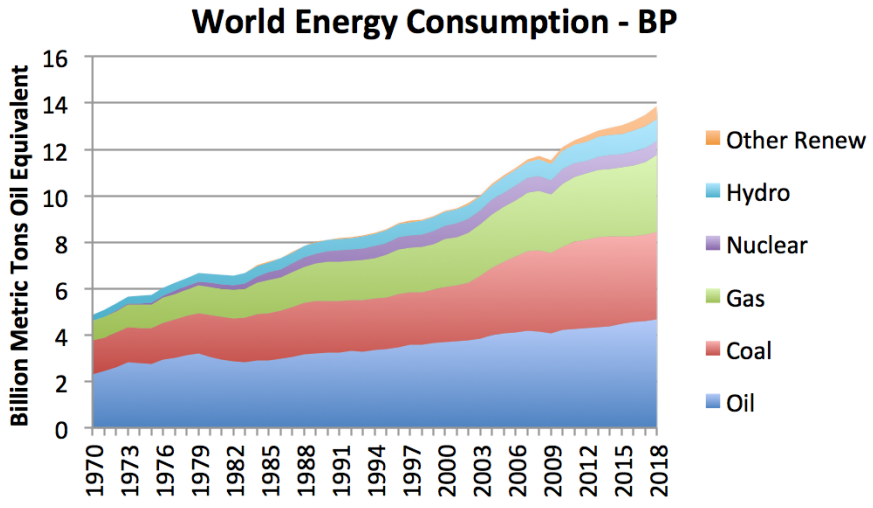

Figure 1-1. World Energy Consumption by fuel through 2018 (based on 2019 BP Statistical Review of World Energy).

The oil industry is one of the most important industries, in term of energy supply in the world. Oil industry represents more than 60% of the global energy supply; therefore, technology must be used to improve sustainable oil recovery. Nevertheless, the high demand for hydrocarbon and the absence of exploring huge new hydrocarbon reservoirs lead to the need of exploiting and optimizing the current mature oil resources to enhance their oil recovery to resolve this critical situation of high energy consumptions. The three main stages of hydrocarbon recovery from oil reservoirs are primary recovery, secondary recovery, and tertiary "enhanced" oil recovery. Primary recovery is the oil recovery process that uses natural production mechanisms without any enhancement process to the natural energy of the reservoir such as fluid injection. The secondary recovery is driven by either gas or water injecting to maintain the reservoir pressure. However, a huge amount of residual oil remains in the reservoir after the stages of primary and secondary oil recovery, which requires proceeding to the tertiary or enhanced recovery stage. Enhanced Oil Recovery methods (EOR) are a range of techniques applied to oil reservoirs to change either reservoir rock or fluid properties (wettability, mobility, etc) to aid production improvement. Primary recovery produces about 20-30% of oil, after applying the secondary recovery the amount of oil produced could reach 40%, but when using the EOR methods the production could be elevated to 60-65% (Al-Sulaimani et al., 2011b; Xiaolin et al., 2012).

Over the last six decades, many EOR methods have been introduced and applied some techniques have shown success while others have failed to fulfil the required demands. EOR methods are classified into four main categories, which are Chemical, Thermal, Miscible, and Microbial. Microbial Enhanced Oil Recovery, MEOR, is a collection of techniques that employ bacteria and their metabolic products to enhance the crude oil recovery from the reservoir rock (Lazar et al., 2007). Microbial Enhanced Oil Recovery (MEOR) is the cheapest EOR method as it can be easily applied after simple adjustments to the existing field facilities, and is also capable of producing up to 50% of the residual oil (Lazar et al., 2007; Sen, 2008). MEOR involves the use of specific microbes to produce useful bioproducts, which enhance oil mobility and recovery. Many bioproducts can be obtained from bacteria such as gas, biosolvents, acids, biopolymers, and biosurfactants. Gas in MEOR has two functions, which are decreasing the oil viscosity and increasing the reservoir pressure (Omoniyi, 2015). Biosolvents are usually used to reduce the viscosity of crude oil (Van Hamme et al., 2003). Acid can dissolve some rock and clean its pores;

hence, it escalates porosity and permeability. Biopolymers can improve the mobility ratio and sweep efficiency because they increase water viscosity which will lead to a decrease in its mobility (AN et al., 2018; Attia M and Musa, 2015). Biosurfactants can improve the mobility of trapped oil because they are surface-acting materials that work on reducing the interfacial tension between crude oil and water, and wettability alteration (Veshareh et al., 2018).

Biosurfactants are structurally diverse surface-active molecular groups, which are mostly produced by microorganisms (Cooper et al., 1980; Desai and Benat, 1997). Glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids are examples of microbial biosurfactants. Many researchers have used different types of bacteria to produce biosurfactants in growth media. The majority of these bacteria are found in contaminated areas that contain petroleum hydrocarbon by-products and/or industrial wastes (Benincasa, 2007; Rahman et al., 2007). Biosurfactants have recently gained popularity because they have numerous benefits over chemical synthetic surfactants, including higher biodegradability, lower toxicity, higher foaming, environmental compatibility, and effective properties under harsh conditions (Bachmann et al., 2014; Banat et al., 2000; Khire, 2010; Mulligan, 2005; Sen et al., 2012).

1.2 Motivation

Three main limitations affect the robustness of the synthetic surfactant flooding in oil reservoirs; these are environmental impacts, synthetic surfactant cost, and oil price. Great concerns have been shown against the ecological effects caused by synthetic surfactants because of their toxicity and degrading difficulty in the environment. Increasing ecological concerns, biotechnology development, and the rise of more rigorous environmental laws have encouraged biosurfactants to be a potent alternative to synthetic surfactants existing on the market. Moreover, synthetic surfactant injection projects are considered the most expensive EOR projects, which could be as high as 50$ per barrel of additional oil. On the other hand, the cost of MEOR processes could be 6$ to 10$ per barrel of additional oil (Badmus et al., 2021; Benincasa, 2007; Dusseault, 2001; Ivanković and Hrenović, 2010; Mulligan, 2005; Sen, 2008; Town et al., 2010; Venhuis and Mehrvar, 2004; Ying, 2006). There are two types of bacteria responsible for MEOR metabolites productions such as biosurfactants; these are indigenous bacteria and exogenous bacteria. MEOR-selected bacteria must have the potential to survive in harsh reservoir conditions and produce the

required bioproducts. Indigenous bacteria are considered the ideal candidates for this MEOR process compared with exogenous bacteria since they are compatible with their reservoir conditions (Lazar et al., 2007; She et al., 2019a). Biosurfactants produced by indigenous bacteria have been proposed as offering an effective mechanism to increase oil recovery from low productive reservoirs (Banat, 1995; Brown, 2010). Consequently, the research has been focused on the capability of producing biosurfactants from indigenous bacteria.

The two main obstacles to the current low acceptance of oil companies to produce biosurfactants from indigenous bacteria for improving oil recovery are (Nikolova and Gutierrez, 2020):

1) This method is based on the assumption that oil reservoirs harbour their indigenous population of microorganisms that can grow or survive under harsh reservoir conditions (high salinity, high temperature) (Nikolova and Gutierrez, 2020). The existence of such indigenous bacteria is still not well described or even fully understood because representative bacteria samples are hard to obtain.
2) Biosurfactants can increase the capillary number by reducing the interfacial tension, but the decrease in interfacial tension must occur by at least one or two orders of magnitude to mobilize the oil. Typically, the interfacial tension between hydrocarbons and water is between 30 to 40 mN/m. For biosurfactants to have any effect in MEOR, they must reduce interfacial tension to 10^{-1} -10^{-2} mN/m (Gray et al., 2008), which are values that have not yet been reported (Nikolova and Gutierrez, 2020).

1.3 Research Aim and Objectives

This work aims to investigate the potential of biosurfactant production by indigenous bacteria isolated from the Egyptian oil fields, the use of these biosurfactants to enhance the oil recovery and the analysis of their environmental aspects for microbial enhanced oil recovery. To achieve these aims, the following objectives have been identified:

1) Collect and statistically analyse the properties of Egyptian oil reservoirs representing the two main Egyptian oil concessions areas (Western Desert and Gulf of Suez) based on MEOR screening parameters to identify the Egyptian oil reservoirs that have potential for MEOR.

2) Isolate indigenous bacteria from collected crude oil samples from the Egyptian oil reservoirs that have potential for MEOR and identify their genus.
3) Select suitable bacterial strains for producing biosurfactants.
4) Investigate the effect of some nutrients on the selected bacterial strains.
5) Investigate the efficiency of the produced biosurfactants.
6) Study the stability of the produced biosurfactants under harsh reservoir conditions (salinity, pH, and temperature), and determine the optimum conditions for the produced biosurfactants to reach maximum surface activity.
7) Investigate the potential of produced biosurfactants in enhancing oil recovery.
8) Study the environmental aspects of the selected biosurfactants producing bacteria for microbial enhanced oil recovery.

1.4 Contributions to Knowledge

This work is concerned with investigating the capability of production of biosurfactants by indigenous bacteria isolated from the Egyptian oil field and proposing a mechanism to optimize these produced biosurfactants. Such a study is an original contribution to the knowledge of microbial enhanced oil recovery. This work contributes to knowledge in terms of statistical analysis, and experimental results to explore several reservoir scenarios to make predictions of their performance after applying MEOR. Firstly, this work statistically analyses Egyptian oil fields based on MEOR screen criteria to create a road map of the Egyptian oil fields that have the potential for MEOR application. Secondly, this work identifies the bacterial strains isolated from the Egyptian oil fields that have the potential for MEOR application. This work proposes a new nutrient medium “H” to optimise the surface activity and emulsification activity of the produced biosurfactants to enhance oil recovery. Further, this work examines the stability of the produced biosurfactants under harsh reservoir conditions (salinity, pH, and temperature), and determines the optimum conditions for the produced biosurfactants to reach maximum surface activity. Finally, this work studies the effect of “in-situ” microbial flooding and “ex-situ” biosurfactant flooding on improving oil recovery on simulated core flooding micromodels with a specific focus on the effect of the interfacial tension reduction and the wettability alteration on reducing the residual oil saturation, hence improving oil recovery.

1.5 Thesis Structure

Brief descriptions of the chapters in the thesis are summarised as follows:

Chapter 1: Introduction

In this chapter, a brief overview and background of enhanced oil recovery processes are given. Challenges and barriers to the application of bacteria in EOR are followed by the highlights of the motivation, aim, and objectives of the research work, and finally, the structure of the thesis has been presented.

Chapter 2: Literature Review

This chapter outlines a critical review of the main areas of research, which are the historical Development of Microbial Enhanced Oil Recovery MEOR, MEOR field trials, methodology for isolation and identification of Bacteria, bio/surfactants, MEOR screening criteria, and finally, the reservoir simulated porous micromodels.

Chapter 3: Research Methodology

This chapter provides a detailed description of the samples, data collection, materials, equipment set-up, and experimental methodology used for this study.

Chapter 4: Results and Discussions

In this chapter, the main results of this study are presented. the analysis and outcome of the laboratory investigations are discussed.

Chapter 5: Environmental Risk Assessment

This chapter assesses the environmental risks of any possible threats of the selected bacteria for producing biosurfactants to the environment.

Chapter 6: Conclusions and Recommendations or Future Work

The overall conclusions of this work are presented in this chapter. In addition, critical suggestions and recommendations are made for future work.

Chapter 6: References

This chapter lists all the references to the literature materials used and cited in this research work.

CHAPTER 2

LITERATURE REVIEW

Outline of the chapter

This chapter gives a detailed review of Microbial Enhanced Oil Recovery MEOR Historical Development. It focuses on the isolation and identification processes of bacteria, biosurfactant production (mechanisms, applications, and approaches), promising bacteria for biosurfactant production, and the effect of nutrients on the growth of bacteria. Finally, the screening criteria of reservoir rock and fluid parameters for the MEOR process are presented. The chapter is organised as follows:

2.1. Introduction

2.2. Historical Development of MEOR

2.3. MEOR Field Trials

2.4. Bacterial Isolation and Identification

2.5. Bio/surfactants

2.6. MEOR Screening Criteria

2.7. Reservoir Simulated Porous Micromodels

2. LITERATURE REVIEW

2.1 Introduction

The continuous search for a low-cost and effective enhanced oil recovery technique is the main driving force behind the development of the Microbial Enhanced Oil Recovery MEOR technique. Microbial Enhanced Oil Recovery MEOR is an eco-friendly and cost-effective process that demonstrates several advantages compared with conventional EOR processes. Generally, the cost of a thermal EOR project could be 25$ per barrel of additional oil, and the cost of carbon dioxide and surfactant injection projects could be as high as 30$ and 50$ per barrel of additional oil, respectively, as shown in Figure 2-1 (Simandoux et al., 1990; Town et al., 2010). However, The MEOR process could cost $6 to $10 per barrel of additional oil (Simandoux et al., 1990; Town et al., 2010), making it the most cost-effective EOR technique because it only requires low-cost nutrient brine solutions and minor modifications to existing secondary recovery facilities. As a result, MEOR offers huge potential as a competitive alternative to conventional EOR chemical techniques. Furthermore, MEOR bioproducts are completely biodegradable and do not accumulate in the environment, which makes them environmentally friendly. The bacterial activity impacts improve with time in the reservoir, whereas the effects of additives in EOR technologies tend to decline with time and distance from the injection well(Marshall, 2008; Maudgalya et al., 2007).

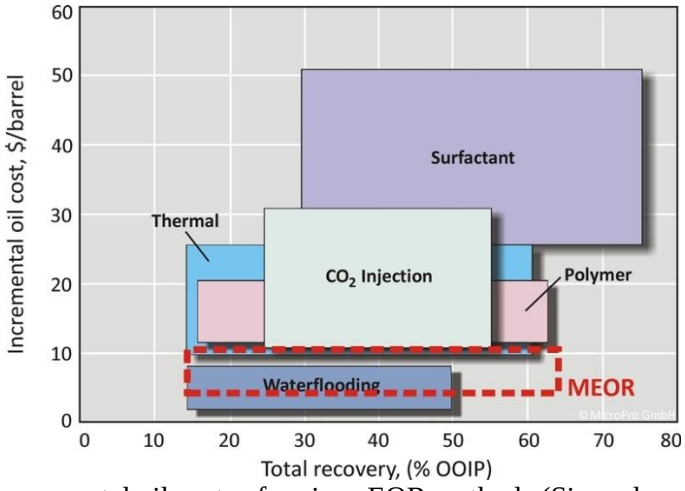

Figure 2-1. Incremental oil costs of various EOR methods (Simandoux et al., 1990)

In 2012, the economical, technological and environmental standpoint of MEOR Technique was examined by Zahner *et al.* (2012) and compared with other EOR techniques (Omoniyi,

2015; Zahner et al., 2012). This study was based on a successful history of field trials. MEOR methods have shown positive results when applied in many countries around the world, not only economical but also environmentally (Bachmann et al., 2014; She et al., 2019b; Zahner et al., 2012). MEOR has many unique advantages such as minimum energy consumption during operation compared with thermal EOR, oil price independence compared with chemical methods because synthetic chemicals is expensive and could be higher than the price of oil barrel based on oil market, and the ability to produce multiple useful by-products from cheap and renewable resources (Safdel et al., 2017; She et al., 2019a).

In this chapter, the definition of microbes (bacteria) and their nutrition system will be highlighted, and then the mechanisms of MEOR will be classified according to the changes in the chemical nature of reservoir rock and fluids. Before proceeding to discuss the different mechanisms of MEOR, a review of the historical development of MEOR investigations is presented.

2.2 Historical Development of MEOR

The concept of using bacteria to improve oil recovery is not a new issue. In 1926, Beckman was the first to suggest that microorganisms could be used to mobilize oil from porous media (Aboelkhair et al., 2020; Lazar et al., 2007). In the 1940s, an actual laboratory experiment was performed by Zobell to confirm Beckman's hypothesis. In 1947, Zobell defined and later patented different processes by which bacterial bioproducts such as gases, acids, solvents, and biosurfactants, liberated oil from sand-pack columns in laboratory tests (Lazar et al., 2007). In 1954, the first field trial of MEOR was performed in Arkansas, the USA by Lisbon field Union County. In 1958, Heinningen suggested the idea of selective plugging recovery, where oil recovery would be achieved from water floods by producing polysaccharide slime *in-situ* from an injected microbial system based on molasses (Sen, 2008). The selective plugging recovery has been recognized as an important additional mechanism of oil release from reservoir rocks (Lazar et al., 2007; Omoniyi, 2015). In the 1960s and 1970s, significant research activity took place in former Czechoslovakia, Hungary, and Poland. The petroleum crisis in the 1970s led to increased research efforts to scientifically evaluate MEOR as a recognized EOR method. Since then, many research projects have been carried out all over the world in countries such as Australia, Bulgaria, Canada, China, Germany, Norway, Poland,

Romania, Russia, the United Kingdom, and the United States (Lazar et al., 2007; Li et al., 2015; Patel et al., 2015; Town et al., 2010; Weidong et al., 2014; You et al., 2016).

2.3 MEOR Field Trials

During field trials, MEOR operations can be achieved by two methods, which are the microbial cyclic method also called "Huff and Puff", and the microbial flooding method. A comparison between microbial "Huff and Puff" and microbial flooding is presented in Table 2-1 (Gao and Zekri, 2011). In microbial cyclic methods, the microbial/bacteria solution is pumped down the well, displaced by a fluid usually brine 2-3% KCl water. Then, the well is shut-in for a period usually ranging from 24 h to 7 days before it is returned to production. This treatment procedure is usually repeated once every 3-6 months period. In microbial flooding methods, bacteria can be added to existing water floods to improve their performance. In this process, MEOR materials are added either periodically or continuously. The biological material is then fed into the reservoir in the injection water or in a form of a slug in front of the water. This method requires little or no modification to the existing water injection systems (Omoniyi, 2015).

Table 2-1. Comparison between microbial huff and puff and microbial flooding (Gao and Zekri, 2011).

Microbial huff and puff	Microbial flooding
▪ Bacteria injected through production tubing	▪ Bacteria injected through injection well
▪ Localized effect near the wellbore	▪ Transport bacteria deep into the reservoir via water flooding
▪ Reservoir shut-in period to allow bacteria to grow	▪ Reservoir shut-in period to allow bacteria to grow
▪ Repeat several times to maximize the gain	▪ Large scale effect
▪ Preferred MEOR option	▪ Involve drilling of injector well unless some are present

More than 400 MEOR field tests have been conducted in the USA. However, the majority were microbial "Huff and Puff". On the other hand, smaller oil and gas companies will consider single-well stimulations if this is an important method by which they produce oil

(Brown, 2010; Maudgalya et al., 2007). MEOR may be attractive to independent oil producers, who mostly produce on average less than half of a tonne of oil per day from "stripper wells," the majority of which are found in the United States (Van Hamme et al., 2003). In Europe, MEOR field projects are mostly confined to Norway (Equinor) (Bødtker et al., 2009), Poland (RAM Biomedicals; Polish and Gas Institute) (Falkowicz et al., 2015), and Germany (BASF; Wintershall) (Alkan et al., 2016, 2014). In China, field trials only started in the last 10–20 years and show some promising, but mixed, results (Li et al., 2015; Weidong et al., 2014).

2.4 Bacterial Isolation and Identification

2.4.1 Sources of Bacterial Isolation

Bacterial species that are biosurfactant production candidates could be isolated from several sources. Depending on the place of extraction, bacteria sources are divided into Indigenous, which exist in the reservoir itself, and exogenous, from an external source. There are four main sources that are suitable for bacterial isolation (Lazar et al., 2007), there are:

1) Formation water.
2) Sediments from the formation of water purification plants (gathering stations).
3) Sludge from biogas operations and effluents from sugar refineries.
4) Oil-contaminated soil could be used as a good source of microbe isolation for MEOR.

2.4.2 Bacterial Identification

Bacteria can be described and classified in three major ways, namely, microscopic examination, morphological characteristics, and biochemical characteristics based on Bergey's manual of systematic bacteriology (Bergey et al., 2012). Morphology means the systematic study of external characteristics of bacteria. The identification of the unknown bacteria could be done by examination of seven characteristics of the unknown bacteria. These seven characteristics are colony morphology, cell morphology, gram stain reaction, presence of endospores in a culture, motility, oxygen intake, and biochemical tests (Bergey et al., 2012).

2.4.2.1 Colony Morphology

The colony morphology method is used to describe the characteristics of an individual colony of bacteria growing on an agar plate. It can be useful in bacterial identification. Colonies are described based on the colony shape (form, elevation, margin, and size of the bacterial colony), and the appearance of the colony surface (consistency/texture, colour, and opacity of the bacterial colony). The form of the bacterial colony refers to the shape of the colony. The most common colony shapes are circular, irregular, filamentous, rhizoid, etc. Elevation of the bacterial colony describes the "side view" of a colony. The most common elevations are flat, raised, umbonate (having a knobby protuberance), crateriform, convex, and pulvinate (cushion-shaped). The margin or edge of a colony may be entire (smooth), irregular, undulate (wavy), lobate, curled, or filiform. The size of the colony can be described by measuring the diameter of a representative colony or by relative terms such as pinpoint, small, medium, and large. Several terms that may be appropriate for describing the texture or consistency of bacterial growth, are dry, moist, viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like) (Bergey et al., 2012).

2.4.2.2 Cell Morphology

The morphology of bacterial cells deals with the study of the shape, size, and arrangement of bacteria cells. The shape of bacteria cells depends on the rigidity of the cell wall. There are three shapes of bacteria as shown in Figure 2-2 (Ribn, 2012):

1) Spherical (cocci) shape
2) Cylindrical (rod) shape
3) Spiral shape

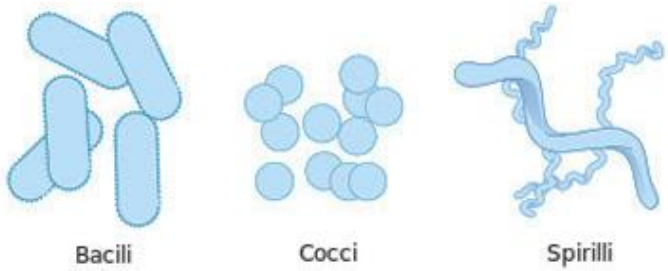

Figure 2-2. Shapes of bacteria cells (Ribn, 2012).

The size of a bacterial cell is less than 3 micrometres. The size of a spherical shape bacteria can be measured in diameter. The size of a cylindrical bacteria is measured by its length and width, in spiral shape bacteria, length is measured. However, this method is not exact due to

spiral form. A variety of arrangements of cells is observed in cocci and rod shape bacteria (Bergey et al., 2012).

2.4.2.3 Gram Staining Reaction

Gram staining is the most important step in the identification of bacteria. It is used to differentiate the bacterial cell into two major groups Gram-positive and Gram-negative which makes it an essential tool for the classification and differentiation of microorganisms. Crystal violet is used as a primary stain and iodine acts as a mordant which increases the affinity of the cells for the stain. Ethyl alcohol 95% is used as a decolourizing agent, which acts as a lipid solvent and also as a protein dehydrating agent, Safranin is used as the secondary stain (Ribn, 2012).

2.4.2.4 Presence of Endospores in a Culture

Bacterial endospores are metabolically inactive, highly resistant structures produced by some bacteria as a defensive strategy against unfavourable environmental conditions. The bacteria can remain in this suspended state until conditions become favourable, and they can germinate and return to their vegetative state. In Schaeffer-Fulton`s method, a primary stain-malachite green is forced into the spore by steaming the bacterial emulsion. Malachite green is water-soluble and has a low affinity for cellular material, so vegetative cells may be decolourized with water (Zhang et al., 2011). Safranin is then applied to counterstain any cells which have been decolourized. At the end of the staining process, vegetative cells will be pink, and endospores will be dark green. Spores may be in the middle of the cell, at the end of the cell, or between the end and middle of the cell. Spore shape may also be of diagnostic use. Spores may be spherical or elliptical (Zhang et al., 2011).

2.4.2.5 Motility

Many bacteria are capable of motility (the ability to move under their power). Most motile bacteria propel themselves by special organelles termed flagella. The bacterial flagellum is a non-contractile, semi-rigid, helical tube composed of protein and anchors to the bacterial cytoplasmic membrane and cell wall using disk-like structures. The motility test medium is a semi-solid motility test medium that may also be used to detect motility. When a non-motile organism is stabbed into a motility test medium, growth occurs only along the stab line and

be very sharp and defined. When motile organisms are stabbed into the soft agar, they swim away from the stab line. Growth occurs throughout the tube rather than being concentrated along the line of inoculation. Growth along the stab line appears much more cloudlike as it moves away from the stab (Bergey et al., 2012).

2.4.2.6 Oxygen Intake

Bacteria are classified based on their oxygen intake into three types, strictly aerobic, strictly anaerobic, and facultative anaerobic bacteria. In the case of aerobic bacteria, oxygen is needed for growth. For strictly anaerobic bacteria, there is no need for oxygen for growth. Facultative anaerobic bacteria can grow either in the presence or absence of oxygen (Ribn, 2012).

2.4.2.7 Biochemical Tests

Many researchers prefer to apply the techniques of biochemical tests in identifying bacteria. Biochemical tests are a conventional and inexpensive means of identifying bacteria; usually performed with phenotypic identification. Frequently less than 15 biochemical tests are required for reliable bacterial identification. Performing more biochemical tests could increase confidence in identification (Bergey et al., 2012).

2.4.2.8 Catalase Test

The catalase test is used to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate between catalase-producing bacteria and non-catalase-producing bacteria. Normally 3% H_2O_2 is used for the routine culture, while 15% H_2O_2 is used for the detection of catalase in anaerobes. The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production (Morobe et al., 2012; Ribn, 2012).

2.4.2.9 IMViC Tests

IMViC tests are used in microbiology lab testing to identify an organism in the coliform group. Coliform is a gram-negative, aerobic, or facultative anaerobic rod, which produces gas from lactose within 48 h. IMViC tests are a set of four reactions that are helpful in distinguishing members of the *Enterobacteriaceae* family. The tests include the Indole test,

Methyl Red test, Voges Proskauer test, and Citrate test. Each of the letters in "IMViC" stands for one of these tests. "I" is for indole; "M" is for methyl red; "V" is for Voges-Proskauer, and "C" is for citrate, lowercase "i" is added for ease of pronunciation. IMViC is an acronym that stands for four different tests (Bergey et al., 2012).

2.4.2.9.1 Indole Production Test

The indole test was used to determine the ability of bacteria to degrade the amino acid tryptophan and produce indole. Indole is detected by combining with Kovac's reagent (50 gm of p-Dimethylaminobenzaldehyde was added to 250 ml of Hydrochloric Acid (37%), and 750 ml of amyl alcohol), which results in a formation of cherry red ring formation on the medium (Bergey et al., 2012).

2.4.2.9.2 Methyl Red Test

Some bacteria can utilize glucose and convert it into a stable acid. These bacteria initially metabolise glucose to pyruvic acid, which is further metabolized through the mixed acid pathway to produce the stable acid. Methyl Red Test was used to detect the production of sufficient acid during the fermentation of glucose. The produced acid decreases the pH to 4.5 or below, which is indicated by a change in the colour of the methyl red indicator that was added at the end of the incubation period from yellow to red (Bergey et al., 2012).

2.4.2.9.3 Voges-Proskauer (VP) Test

The Voges-Proskauer (VP) test is used to determine if bacteria could produce neutral end products such as 2, 3-butanediol or acetoin from glucose fermentation, which could be detected by adding potassium hydroxide KOH to cultures of the studied bacteria. If the studied bacteria can produce a neutral end product, the colour of MR-VP media inoculated by the studied bacteria changes from yellow to red colour (Voges-Proskauer positive reaction) (Bergey et al., 2012).

2.4.2.9.4 Citrate Test

The citrate test was conceived to verify whether a microorganism can use citrate as its sole carbon source and inorganic ammonium salts as the sole nitrogen source. The use of citrate generates alkaline by-products which can be detected by using the inclusion as pH indicator,

bromothymol blue, which is green at a pH of 6.8 and blue at a pH of 7.6 or above (Bergey et al., 2012).

2.4.2.10 Oxidase Test

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When the enzyme is present, the cytochrome c oxidase oxidizes the reagent, which is usually tetramethyl-p- phenylenediamine, to produce (indophenols) a dark blue or purple colour end-product. When the enzyme is not present, the reagent remains reduced and is colourless. All oxidase-positive bacteria are aerobic. However, this does not mean that they are strict aerobic. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative, the oxidase-negative result just means that these bacteria do not have the cytochrome c oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport. Oxidase test is used to assist in the identification of *Pseudomonas, Neisseria, Alcaligens, Aeromonas, Campylobacter, Vibrio, Brucella and Pasteurella* (positive), and the Enterobacteriaceae (all negative) (Bergey et al., 2012).

2.4.2.11 Hydrogen Sulphide (H_2S) Production Test

Some microorganisms can reduce sulphur-containing compounds to hydrogen sulphide during metabolism which is commonly employed as a test measure for their identification in laboratories. An iron compound and a sulphur compound are included in the test medium to test for hydrogen sulphide gas production. Hydrogen sulphide is produced if the sulphur compound is reduced by the bacterial strain. This test is used to determine whether the microbe reduces sulphur-containing compounds to sulphides during the process of metabolism. H_2S is produced by certain bacteria through the reduction of sulphur-containing amino acids like cysteine, and methionine or through the reduction of inorganic sulphur compounds such as thiosulfates, sulphates, or sulphites during protein degradation or when anaerobic respiration shuttles the electrons to sulphur instead of oxygen. In either case, H_2S is produced (hydrogen sulphide gas) which reacts with the iron compound to form the black precipitate of ferric sulphide. The black colour acts as an indicator of the presence of hydrogen sulphide. The detection of hydrogen sulphide (H_2S) gas produced by an organism is used mainly to assist in the identification of that particular bacterium (Bergey et al., 2012).

2.4.2.12 Oxidation-Fermentation (OF) Test

The oxidative-fermentative (OF) test is used to differentiate between oxidative bacteria that produce acid from carbohydrates under aerobic condition only and fermentative bacteria that produce acid both under aerobic and anaerobic conditions. The oxidative-fermentative test determines if certain gram-negative rods metabolize glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in Hugh and Leifson's medium from green to yellow in the presence or absence of oxygen. Certain nonfermenting gram-negative bacteria metabolize glucose using aerobic respiration and therefore only produce a small amount of weak acids during glycolysis and the Krebs cycle. The decreased amount of peptone and increased amount of glucose facilitates the detection of weak acids thus produced. Dipotassium phosphate buffer is added to further promote acid detection. OF Test is used to determine if gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are non-saccharolytic (cannot use the carbohydrate in the media) (Bergey et al., 2012).

2.4.2.13 Gelatin Hydrolysis Test

The gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefies gelatin. Hydrolysis of gelatin indicates the presence of gelatinases. This process takes place in two sequential reactions. In the first reaction, gelatinases degrade gelatin to polypeptides. Then, the polypeptides are further converted into amino acids. The bacterial cells can then take up these amino acids and use them in their metabolic processes. The gelatin hydrolysis test helps identify and differentiate species of *Bacillus, Clostridium, Proteus, Pseudomonas,* and *Serratia*. It distinguishes the gelatinase-positive, pathogenic *Staphylococcus aureus* from the gelatinase-negative, non-pathogenic *S. epidermidis*. Gram-positive, spore-forming, rod-shaped, aerobic, or anaerobic bacteria such as *Bacillus cereus, Bacillus subtilis, Clostridium perfringens and Clostridium tetani,* are also positive for gelatin hydrolysis. It can also be used to differentiate genera of gelatinase-producing bacteria such as *Serratia* and *Proteus* from other members of the family Enterobacteriaceae (Bergey et al., 2012).

2.4.2.14 Urease Test

The urease test is used to determine the ability of bacteria to split urea, through the production of the enzyme urease. Some bacteria can use urea as a carbon source, a by-product generated from the metabolism of proteins by vertebrates. The Catabolism of urea requires the enzyme urease, which hydrolyses urea and consequently generates ammonia, carbon dioxide, and water. The ammonia that is released in the growth medium produces an alkaline reaction, which can be detected by a pH indicator within the medium, phenol red, which turns pink in the presence of alkali products (Ribn, 2012).

2.4.2.15 Nitrate Test

The nitrate test is also called the nitrate reduction test and used for the differentiation of members of *Enterobacteriaceae* based on their ability to produce an enzyme called nitrate reductase that hydrolyses nitrate (NO_3) to nitrite (NO_2), which may then be further degraded to various nitrogen products like nitrogen oxide, nitrous oxide, and ammonia (NH_3) depending on the enzyme system of the organism and the environment in which it is growing. (Bergey et al., 2012).

2.4.2.16 Starch Hydrolysing Test

Starch molecules are too large to enter the bacterial cell, so only bacteria that secrete exoenzymes (α -amylase and oligo-1,6-glucosidase) can hydrolyse starch into subunits (dextrin, maltose, or glucose). These molecules are readily transported into the bacterial cell to be used in metabolism. Starch hydrolysis test is used to differentiate members of various genera which have both amylase-positive and amylase-negative species, including *Bacillus, Clostridium, Corynebacterium, Fusobacterium, Enterococcus, Pseudomonas,* and *Streptococcus* (Ribn, 2012).

2.4.2.17 Mannitol Fermentation Test

The Mannitol fermentation test was used to see if the studied bacteria could ferment the carbohydrate (sugar) mannitol as a carbon source to produce acid-end products. The fermentation broth, presented in Table 3-15, contains mannitol and a pH indicator (phenol red) which is red at a neutral pH 7 and turns yellow at or below pH 6.8 due to the production of organic acids (Bergey et al., 2012).

2.4.2.18 Glucose Fermentation Test

The glucose fermentation test is used to determine the way bacteria metabolize a carbohydrate. The purpose of this test is to see if the studied bacteria can ferment the carbohydrate (sugar) glucose (also known as dextrose) as a carbon source to produce acid end products, which leads to a drop in the pH of the medium. A pH indicator in the medium changes colour to indicate acid production. The phenol red glucose broth is used. The pH indicator phenol red as mentioned above is red at neutral pH but turns yellow at pH<6.8. Furthermore, this medium contains a Durham tube, which is a smaller inverted tube that can serve as a trap for gas bubbles generated during the fermentation of sugar (Bergey et al., 2012).

2.4.2.19 Lactose Fermentation Test

The lactose Fermentation Test is used to examine if the studied bacteria could ferment the carbohydrate (sugar) lactose as a carbon source to produce acid end products, which leads to a drop in the p*H* of the medium. The pH indicator phenol red as mentioned above is red at neutral pH but turns yellow at pH<6.8. It also changes to magenta or hot pink at pH >8.4. Furthermore, this medium contains a Durham tube, which is a smaller inverted tube that can serve as a trap for gas bubbles generated during the fermentation of sugar (Bergey et al., 2012).

2.5 Bio/surfactants

Surfactants are widely employed in a variety of industries, including pharmaceuticals, cosmetics, soap and detergents, textiles, oilfield chemicals, agrochemicals, and food. (Banat et al., 2000). By 2020, the Surfactants Market volumetric demand is expected to be up to 24,037.3 KT (Kilotons) with a money value of $42.12 Billion. During the projected period of 2015 to 2020, it is expected to increase at a rate of 5.5% in terms of value (Mathur et al., 2016).

The amphipathic nature of surfactant compounds enables them to have both polar and non-polar sides, which enables them to interact with two phases of immiscible emulsions. On an industrial scale, surfactants are applied to oil reservoirs for the recovery of residual oil trapped in the reservoir rocks (Perfumo et al., 2010). The use of organic substrates for biological

enhanced oil recovery is considered more favourable than other physical and chemical methods. This has encouraged the necessity of improving oil recovery by utilizing a biologically EOR process, also known as MEOR (Sen, 2008). However, environmental impacts, synthetic surfactant cost, and oil price are the three major limitations that affect the durability of synthetic surfactant flooding in oil reservoirs (Van Hamme et al., 2006).

Accordingly, biosurfactants have become a viable alternative to synthetic surfactants already on the market since they are less toxic, biodegradable, and cost-effective as a result of increasing environmental concerns, more rigorous environmental laws, and the development of biotechnology (Banat et al., 2000; Henkela et al., 2012). Recently, Biosurfactant production is considered one of the most efficient mechanisms in MEOR processes. Biosurfactants are simply surface-active agents produced by different groups of bacteria. Biosurfactants reduce surface tension and interfacial tension and increase the mobilisation of residual oil. Biosurfactants can aggregate at interfaces between fluids having different polarities, such as water and oil, leading to a reduction in interfacial tension. Because of their efficiency in lowering interfacial tension, biosurfactants have been employed for the enhancement of oil production, especially in microbial enhanced oil recovery (Aiad et al., 2015). Biosurfactants can potentially replace virtually any synthetic surfactant and introduce some unique Physico-chemical properties. Currently, the main application is for the enhancement of oil recovery and hydrocarbon bioremediation due to their biodegradability and low critical micelle concentration (CMC) (Banat et al., 2010).

The significance of biosurfactants in MEOR is based on many factors. Biosurfactants can reduce the interfacial tension, they are less adsorbed on the rock surface, and they have low toxicity, biodegradability, and cost-effectiveness. Several bacteria could produce different types of biosurfactants such as glycolipids, phospholipids, fatty acids, amino-acid-containing compounds (i.e., proteins and lipopeptides) and neutral lipids. The production of biosurfactants is affected by the bacterial strain, and the fermentation conditions, e.g., nutrient composition, temperature, pH, presence of metal ions, etc. Many biosurfactant-producing bacteria require oxygen for growth and are unsuitable for in-situ production. Recently, however, microorganisms that produce surfactants under anaerobic conditions have been isolated. Numerous experiments at laboratory scales and field trials have successfully

indicated the effectiveness of biosurfactants in microbial enhanced oil recovery (Khire, 2010). In addition, several studies indicate that the production of relatively large amounts of biosurfactant within the reservoir is feasible, hence the possibility to use biosurfactant instead of synthetic surfactant could be applied to enhance oil recovery and reduce the production cost (S. Mukherjee et al., 2009).

2.5.1 Bio/surfactants Structure

Bio/surfactants are amphipathic structures consisting of a hydrophobic moiety (tail) and a hydrophilic moiety (head) that are produced synthetically or biologically by a variety of microorganisms as by-products of metabolism as shown in Figure 2-3. The hydrophobic moiety (tail) may be a hydroxy fatty acid or a long-chain fatty acid of different lengths. the hydrophilic moiety (head) may be a peptide, phosphate, carboxylic acid, amino acid, carbohydrate, or alcohol (Das et al., 2008). In the past few years, biosurfactants have gained attention because of their biodegradability, low toxicity, and cost-effectiveness. Since biosurfactants can be produced from carbohydrates by the fermentation process, it is possible to produce a huge amount more cheaply than synthetic surfactants, which are also developed for use in the oil industry. The surface properties of a surfactant are controlled by the balance between its hydrophilic and lipophilic characteristics. If the surfactant contains a hydrocarbon chain with less than 12 carbon atoms, it is called water-soluble because the polar head group drags the entire molecule in water. However, when the hydrocarbon chain length is greater than 14 carbon atoms, the compound is called water-insoluble surfactant, because it does not dissolve in water due to the long hydrocarbon chains (Khire, 2010; Obayori et al., 2009).

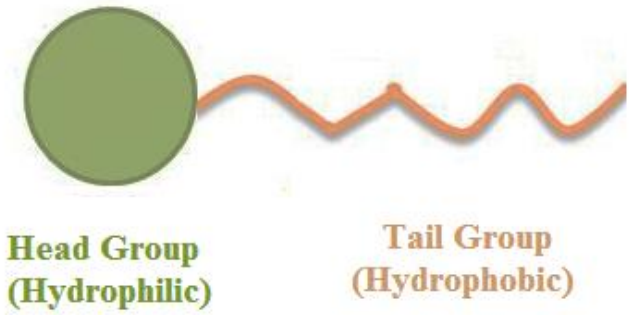

Figure 2-3: Structure of a bio/surfactant molecule

2.5.2 Bio/surfactants Classifications

Surfactants are classified into four general groups, according to the nature of the polar head part of the molecule. These groups are anionic (negative charge), cationic (positive charge), zwitterionic (amphoteric) (both a negative and a positive charge), and non-ionic (wetting agent) surfactants as shown in Figure 2-4 (Roberts, 2005). Among these types, anionic surfactants are widely used in EOR processes due to their lower adsorption on reservoir rocks (sandstones) as compared to other types of surfactants. It is evident to note that, if the biosurfactants produced by bacteria in the reservoir are non-ionic surfactants, they can react with organic acids present in the oil and become ionic. When a surfactant is dissolved in water, its monomers (molecules) form aggregates called micelles. Each monomer is composed of a non-polar moiety (lyophilic) and a polar moiety (hydrophilic) (Lazar et al., 2007).

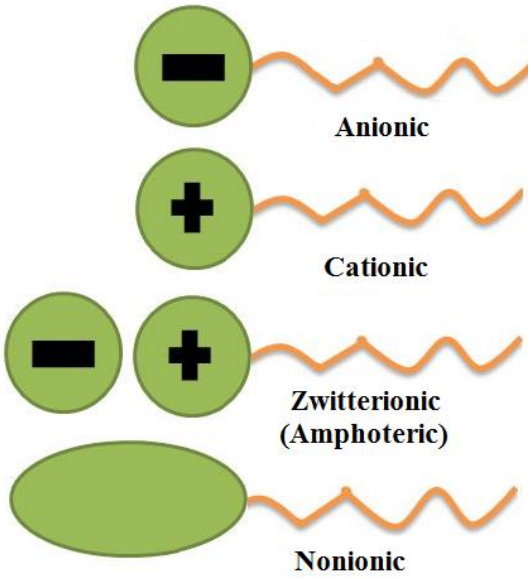

Figure 2-4: Surfactants classification according to the composition of their heads

Biosurfactants are classified based on their microbiological origin and chemical composition, unlike synthetic chemical surfactants which are generally categorized by the nature of their polar grouping. Biosurfactants are classified mainly into two groups based on their molecular mass, these are the “low-molecular-weight biosurfactants” group and the “high-molecular-weight biosurfactants” group. Glycolipids, lipopeptides, fatty acids, neutral lipids, and phospholipids are considered examples of the low-molecular-weight biosurfactants group. Glycolipids and lipopeptide compounds are associated with the potential of reducing surface

and interfacial tensions in liquids. The other low-molecular-weight biosurfactants that have low critical micelle concentration could increase the hydrocarbon's apparent solubility by integrating them into micelles' hydrophobic cavities (Miller and Zhang, 1997). The group of high-molecular-weight biosurfactants like polysaccharides, Liposans, Alasans, Emulsans, and protein complexes are associated with the potential of producing stable emulsions, even if they do not have significant potential in reducing surface tension. However, the production of stable emulsions allows bacteria to strongly adhere to hydrophobic surfaces, hence indicating their high biodegradation potential (Rosenberg and Ron, 1999). Table 2-2 summarizes a list of biosurfactants produced by several bacteria (Al-Sulaimani et al., 2011b; Banat et al., 2000; Mulligan, 2005; Muthusamy et al., 2008; Raaijmakers et al., 2006; Safdel et al., 2017; Youssef et al., 2009).

Table 2-2 Major Biosurfactant classes and their producing microorganisms (Al-Sulaimani et al., 2011b; Muthusamy et al., 2008; Raaijmakers et al., 2006; Safdel et al., 2017; Youssef et al., 2009)

Biosurfactant			Microorganism
Group	Sub-group	Class	
Low Molecular Weight Biosurfactants	Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*
			Pseudomonas sp.
		Trehalose lipids	*Rhodococcus erithropolis*
			Arthobacter sp.
		Sophorolipids	*Candida bombicola*
			Candida apicola
			Candida lipolytica
			Candida bogoriensis
		Mannosylerythritol lipids	*Candida antartica*
		Cellobiolipids	*Ustilago zeae*
			Ustilago maydis
	Lipopeptides	Surfactin/Iturin/Fengycin	*Bacillus subtilis*
		Viscosin/tolaasin/syringomycin	*Pseudomonas fluorescens*
			Pseudomonas sp.
		Putisolvin/Amphisin	*Pseudomonas spp.*
		Lichenysin	*Bacillus licheniformis*
		Serrawettin	*Serratia marcescens*
	Fatty acids, Neutral lipids, and Phospholipids	Fatty acids	*Corynebacterium Lepus*
		Corynomicolic acids	*Corynebacterium insidibasseosum*
		Neutral lipids	*Nocardia erythropolis*
		Phospholipids	*Acinetobacter sp.*
			Corynebacterium Lepus
			Thiobacillus thiooxidans
High Molecular Weight Biosurfactants	Polymeric surfactants	Emulsan	*Acinetobacter calcoaceticus*
		Biodispersan	*Acinetobacter calcoaceticus*
		Alasan	*Acinetobacter radioresistens*
		Liposan	*Candida lipolytica*
		Lipomanan	*Candida tropicalis*
	Particulate biosurfactants	Vesicles and Fimbriae	*Acinetobacter calcoaceticus*
		Whole cells	*Cyanobacteria*

2.5.3 Bio/surfactant Critical micelle Concentration

The presence of surfactants in water causes the hydrophobic tails to aggregate and reduce their exposure to water and form a micelle. However, in an oleic solution, the hydrophilic heads aggregate and form a micelle as shown in Figure 2-5 (Haghighi et al., 2020). To form these micelles, a certain number of molecules must be in solution at a given surfactant concentration. The concentration at which the micelle appears is called critical micelle concentration (CMC) (Joshi and Desai, 2013).

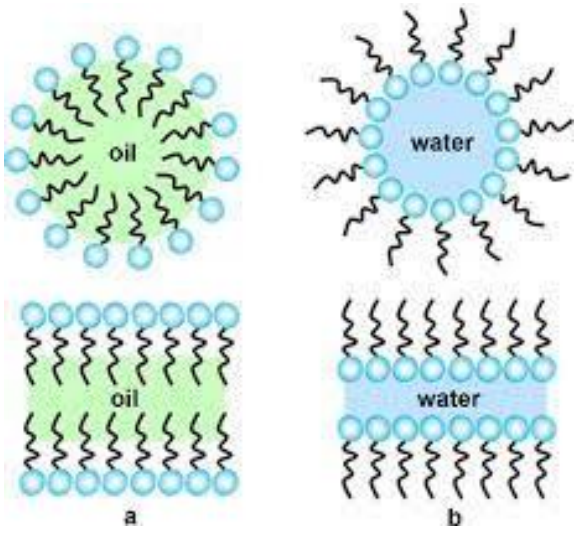

Figure 2-5: Micelle forming in (a) aqueous solution, and (b) oleic solution (Haghighi et al., 2020)

Critical Micelle Concentration CMC is simply the biosurfactant concentration above which micelles aggregate initially start to form. It is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. Figure 2-6 shows the surface tension of a surfactant solution at different surfactant concentrations and the formation of micelles. Surface tension reduces with increasing surfactant concentration until surfactant molecules saturate the surface of the solution at which no more reduction in surface tension is observed. When the formation of micelles is desirable, the CMC is a measure of the efficiency of a surfactant. CMC is an essential characteristic for surfactants because once reaching CMC, there is no more reduction in surface tension even after adding any further amount of biosurfactant. CMC is estimated by plotting a graph between the surface tension versus the concentration of biosurfactant (Haghighi et al., 2020; Joshi et al., 2008a; Joshi and Desai, 2013).

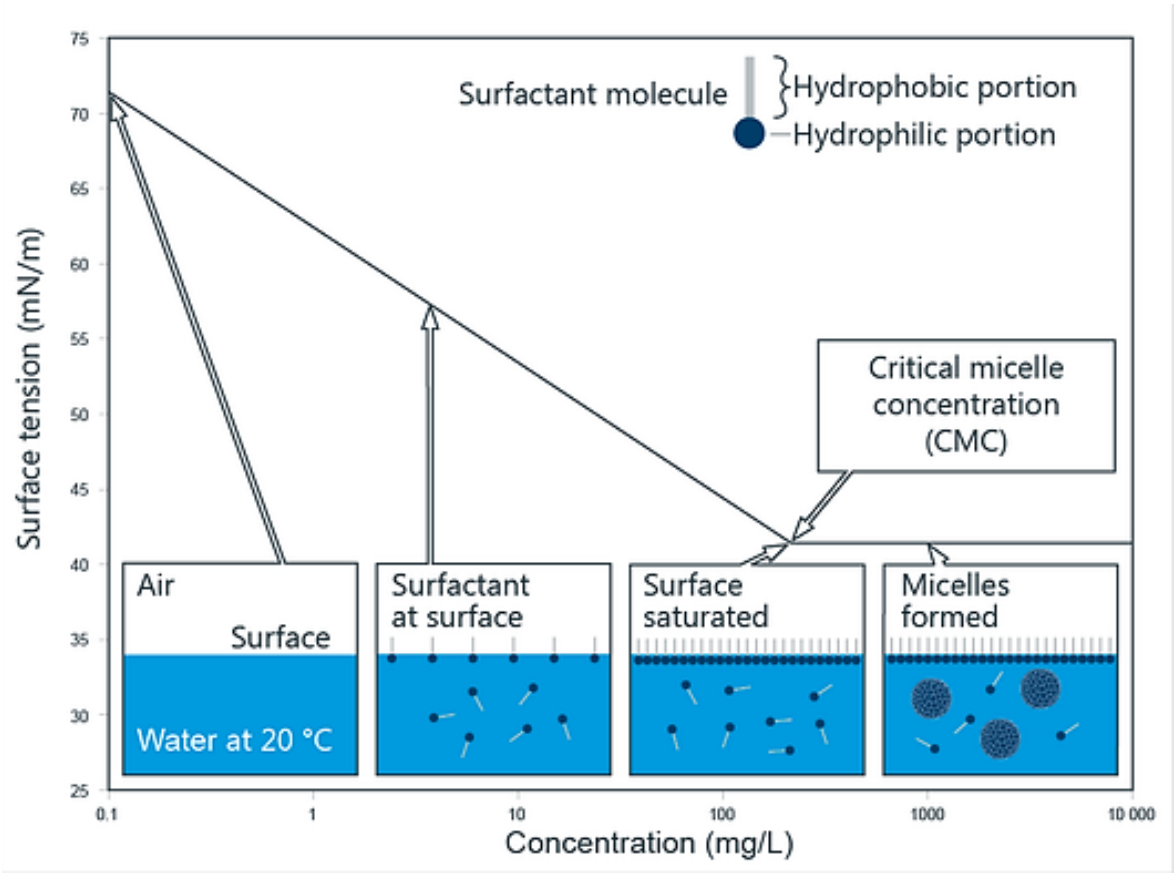

Figure 2-6. Formation of micelles at different concentrations of surfactant (Almgren, 2000)

Biosurfactants have a CMC value about 10-40 times lower than those of chemical surfactants, i.e. less surfactant is necessary to get a maximum decrease in surface tension (Desai and Benat, 1997; Joshi et al., 2013). Above the CMC, biosurfactant molecules aggregate to form supramolecular structures like micelles, bilayers, and vesicles. Between 50 and 100 surfactant molecules usually (aggregation number) form micelles. Micelles arise when the lipophilic part of the surfactant molecule is unable to form hydrogen bonding in an aqueous phase causing an increase in the free energy of the system. One way for the hydrocarbon tail to alleviate this free energy increase is to be isolated from water by adsorption onto surfaces, absorption into an organic matrix or the formation of micelle vesicles where the hydrocarbon moiety of the surfactant becomes towards the centre of the hydrophilic part that in contact with water (Haigh, 1996).

2.5.4 Biosurfactant Purification

Biosurfactant recovery depends mainly on its ionic charge, solubility in water or organic solvents, and location (intracellular, extracellular, or cell-bound). Most of the biosurfactants are secreted into the medium, and they are isolated from either the culture filtrate or the supernatant obtained after the removal of cells. Downstream processes for recovery of important biosurfactants include acid precipitation, solvent extraction, ammonium sulphate precipitation, crystallization, adsorption, foam separation and precipitation, diafiltration and ultrafiltration as represented in Table 2-3, which summarizes all reported biosurfactant recovery methods and their relative advantages (Das et al., 2008; Soumen Mukherjee et al., 2009).

Table 2-3. Biosurfactant recovery methods and their relative advantages (Mukherjee et al. 2006)

Biosurfactant Recovery Methods	Biosurfactant Property Responsible for Separation	Instrument/Apparatus/Setup Required	Advantages
• Acid Precipitation	Biosurfactants become insoluble at low pH values	No set-up required	Low cost, efficient in crude biosurfactants recovery
• Organic Solvent Extraction	Biosurfactants are soluble in organic solvents due to the presence of hydrophobic end	No set-up required	Efficient in crude biosurfactant recovery and partial purification, reusable nature
• Ammonium Sulphate Precipitation	Salting out of the polymeric or protein-rich biosurfactant	No set-up required	Effective in isolation of certain types of polymeric biosurfactants
• Centrifugation	Insoluble biosurfactants get precipitated because of centrifugal force	Centrifuge required	Reusable, effective in crude biosurfactants recovery
• Foam Fractionation	Biosurfactants, due to surface activity, form, and partition into foam	Specially designed bioreactors that facilitate foam recovery during fermentation	Useful in continuous recovery procedures, high purity of the product
• Membrane Ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Ultrafiltration units with porous polymer membrane	Fast, one-step recovery, high level of purity
• Adsorption on Polystyrene Resins	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvents	Polystyrene resin packed in glass columns	Fast, one-step recovery, high level of purity, reusability
• Adsorption on Wood-Activated Carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed using an organic solvent	No setup is required, can be added to culture broth, and can also be packed in glass columns	biosurfactants, cheaper, reusability, recovery from continuous culture
• Ion-Exchange Chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with the proper buffer	Ion-exchange resins packed in columns	High purity, reusability, fast recovery
• Solvent Extraction (Using Methyl Tertiary-Butyl Ether)	Biosurfactants dissolve in organic solvents owing to the hydrophobic ends in the molecule	No set-up required	Less toxic than conventional solvents, reusable, cheap

2.5.5 Bio/surfactants Application

There are three ways through which bio/surfactants could be applied to improve oil recovery, these are the reduction of interfacial tension between oil-rock and oil-brine, wettability alteration of rocks, and emulsification of crude oil (Sen et al., 2012).

2.5.5.1 Reduction of Interfacial Tension

Biosurfactants can reduce the interfacial tension between the flowing aqueous phase and the residual oil saturation to improve oil recovery for a low permeability rock formation, or a high viscosity crude oil as shown in Figure 2-7 (Banat et al., 2000). Biosurfactants can also potentially reduce the capillary forces that prevent oil from moving through rock pores, and hence increasing the capillary number, which is a dimensionless ratio between viscous forces and capillary forces. The capillary number is used to assess the likelihood of mobilizing the residual oil. More residual oil would be mobilized when the capillary number is large. A reduction in the residual oil will result in a higher recovery rate from the reservoir. Biosurfactants can increase the capillary number by reducing the interfacial tension (Gray et al., 2008).

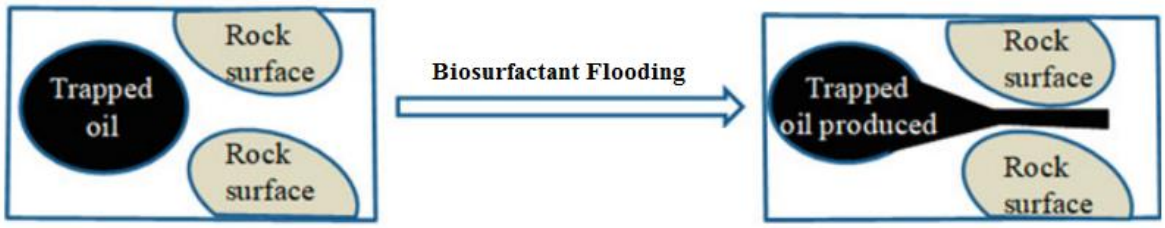

Figure 2-7. The effect of biosurfactant flooding in reducing interfacial tension to produce trapped oil (Kumar and Mandal, 2017).

2.5.5.2 Formulation of Emulsion

Robbins has suggested a molecular theory for lowering the interfacial tension of the oil/water interface leading to emulsification. According to his theory, the hydrophilic heads and lipophilic chains of the interphase are treated as independent interfaces, water interacting with the heads and oil with the chains. Direction and degree of curvature are imposed by a lateral stress gradient in the interface, resulting from differences in interaction on either side of the interphase. This stress gradient is expressed in terms of physically measurable quantities such

as surfactant molecular volume and compressibility. Decreasing head/chain molecular volume and compressibility ratio results in increased oil uptake and decreased water uptake. It is evident to note that, increasing the temperature, the salt concentration and the oil aromaticity have almost the same effect.

2.5.5.3 Wettability Alteration

The oil-wet surfaces lead to poor oil displacement, whereas the water-wet surfaces lead to efficient oil displacement. Biosurfactants could alter the rock wettability from oil-wet to water-wet to create favourable conditions for efficient oil displacement. The wettability can be altered, due to change the wetting state b rock by using surfactant (Krieger et al., 2010). In carbonate reservoirs, changes in the wettability from oil-wet to water-wet are much more significant for oil recovery because, in a water-wet system, the oil tends to be in the large pores of the rock, while water is in small tracks and around the grains (Afrapoli et al., 2011). Consequently, it may seem that water-wet conditions are favourable for increased oil recovery because oil is concentrated in larger pores and hence is easier to access by flooding (Kowalewski et al., 2006). However, in sandstone formations, oil displacement is not fundamentally influenced by the wettability of the rock surface, and hence in this case wettability alterations by microbial activity is not a promising mechanism for improving oil recovery.

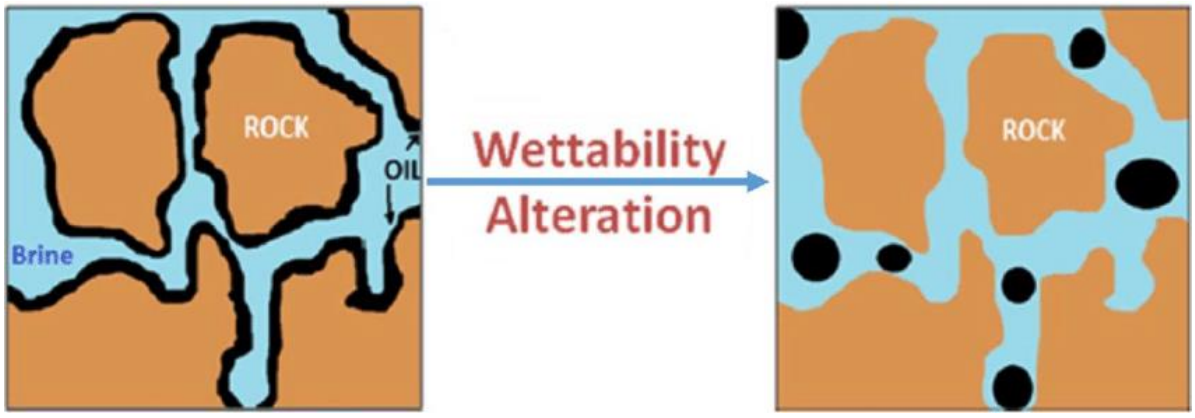

Figure 2-8. The effect of biosurfactant flooding on altering the wettability of the reservoir rock.

2.5.6 Biosurfactants Approaches

A standard practice of biosurfactants does not exist across the energy industry; operators use several different combinations of techniques to extract oil. All applications of biosurfactants can be categorized into one of three main approaches:

(A) Injection of exogenous microbes in the reservoir followed by customized nutrients for cell growth in the subsurface (exogenous approach).

(B) Injection of only nutrients specifically tailored to stimulate the growth of indigenous reservoir microbes (indigenous approach).

(C) Production of biosurfactants on the surface and then injection into the reservoir (biosurfactants injection approach).

Furthermore, implementations of each biosurfactant approach differs within each category based on the presence of carbon in injected nutrients.

2.5.6.1 Exogenous Approach

In a review conducted by Donaldson (1991), many of the world's first biosurfactant field projects utilized an exogenous approach. Simply, the exogenous approach is implemented by injecting exogenous bacteria followed by customized nutrients for cell growth in the subsurface. The advantage of this approach is that the bioproducts that are generated in the subsurface are initially examined and determined in the laboratory. A specific bacterial group presumably produces the same microbial bio-products regardless of their environment (Donaldson, 1991).

In 1979, Johnson implemented a microbial "huff-and-puff" in 150 stripper wells to produce biogas, bioacids, and biosolvents from inoculated *Bacillus* and *Clostridium* bacteria to clean the near-wellbore area. Although performance varied across the study, an average of 20 to 30 % additional oil-in-place was recovered from stimulating the exogenous microbes with molasses and mineral salts. In 1986, Lazar reviewed the results from biosurfactants field tests in seven reservoirs in Romania. After microbial injections, two of the reservoirs experienced a 200% increase in oil production for a few years; however, the remaining five projects had inconclusive results (Donaldson, 1991; Lazar et al., 2007).

Overall, this conventional biosurfactants approach showed promise when implemented in the field; however, there were no explanations for the failed projects. Along with inconsistent outcomes, the exogenous approach requires large expenditures for special facilities and equipment for cultivating microbes. It is unclear whether the mentioned field trials were economical. Moreover, some researchers reported that exogenous bacteria could be incompatible with the reservoir system or were not capable of penetrating the reservoir (Lazar et al., 2007; She et al., 2019a). These disadvantages have encouraged many operators to pursue a different MEOR approach.

2.5.6.2 Indigenous Approach

Some researchers hypothesized that exogenous bacteria are incompatible with the reservoir system or were not capable of penetrating the reservoir (Wang et al., 2008). Reservoirs are the natural habitats to a widely diverse population of indigenous microbes that can vary drastically between reservoirs. These native bacterial populations can compete with injected cultures for nutrients and carbon, resulting in growth hindrance of the foreign microorganisms.

This triggered the development of an alternate approach called “organic oil recovery”, in which in-situ bacterial stimulation shifted to indigenous reservoir bacteria instead of exogenous bacteria. In 1983, Ivanov and his research group recorded the first instance of activating indigenous reservoir microbes. Their successful implementation was based on introducing oxygen and some salts with injection water. Moreover, recent implementations and studies indicated that this MEOR indigenous approach has a higher success rate than the conventional exogenous approach (Zahner et al., 2012).

In 2001, a pilot test was carried out in Dagang Oilfield, PetroChina to evaluate the technical efficiency of biosurfactants in high-temperature petroleum reservoirs. This operation used the microorganisms naturally occurring in the reservoir with injected nutrients into the reservoir. In 2004, after applying biosurfactants in this field, the additional oil production was 35 tons per day compared with the predicted oil production, if applying conventional water flooding (18 tons per day), which means it is more than a 48% increase in oil production. It was noticed that the methanogenic bacteria, which existed in the reservoir formation water could produce

methane after activation by supplied mineral salts, especially in long term, by water flooded reservoirs.

From July 2007 through the end of 2010, more than 100 indigenous approach treatments were conducted in the U.S. and Canada. In a review, 89% of these projects were successful with an average oil production increase of 122% from the pre-nutrient production rate. The need and expenses for large-scale cultivation of microbes are removed, and only a few additional types of equipment are needed to add low-cost nutrients and treat injection brine. Therefore, it was found that the indigenous approach is more economical (Lazar et al., 2007; Zahner et al., 2012).

The five steps to implement this approach are as follows:

1) Initial field screening for suitable conditions
2) Analysis of wellbore fluids for indigenous components and microbes
3) Formulation of nutrient solution to ensure microbial growth
4) Running a pilot test in the injection well
5) Performing field-scale tests

2.5.6.3 Biosurfactants Injection Approach

Biosurfactants applications can be classified into two main classes; in-situ biosurfactants production and ex-situ biosurfactants production. In biosurfactants ex-situ production exogenous or indigenous bacteria are used to produce biosurfactants ex-situ. In this case, mobile plants and industrial fermenters are used to grow bacteria and then inject their produced biosurfactants into the reservoir as an aqueous solution. However, this approach was not recommended for biosurfactants production field applications, because it could be very costly on an industrial scale (Lazar et al., 2007; Omoniyi, 2015; Sen, 2008)

2.5.7 Biosurfactants Producing Bacteria

Selected bacteria for biosurfactant production applications must meet the most important requirement, which is the ability to survive and produce biosurfactants in the reservoir. Ideal candidates that meet these requirements are the indigenous bacteria in the reservoir. Indigenous bacteria have a selective advantage over exogenous bacteria because they are adapted to the reservoir conditions. The main adaptations that bacteria must have are high

tolerance to high temperatures, and salinity, as well as being active under anaerobic conditions (Al-Maghrabi et al., 1999; Lazar et al., 2007; Omoniyi, 2015). Indigenous microbial community structures in oil reservoirs are expected to vary as each reservoir is different in terms of depth, temperature, pressure, salinity, and other characteristic features. Most studies exploring microbial communities use culture-based methods to recover and identify individual microbial isolates and do not provide complete information on how these communities are structured (Lazar et al., 2007). However, these bacteria are likely introduced into the reservoir during drilling and water flooding.

Table 2-4 shows the details of some of the reported biosurfactants along with their producing organisms. The most commonly isolated bacteria for biosurfactants production are *Acinetobacter, Bacillus cereus, Bacillus licheniformis, B. megaterium, B. subtilis, Branhamella catarrhalis, Citrobacter intermedius, Corynebacterium kutscheri, C. xerosis, Enterobacter aerogenes, Escherichia coli, Flavobacterium sp., Klebsiella ozaenae, Lactobacillus casei, L. delbrueckii, Micrococcus, Proteus inconstans, Pseudomonas aeruginosa, Ps. fluorescens, Ps. diminuta, Ps. mallei, Rhodococcus, Staphylococcus aureus,* and *Virgibacillus salaries* (Bodour et al., 2003; Elazzazy et al., 2015; Ganesh and Lin, 2009; Joshi et al., 2013; Liu et al., 2016; Randhawa, 2014; Suthar and Nerurkar, 2016; Thavasi et al., 2011; Viramontes-Ramos et al., 2010).

Table 2-4: Reported biosurfactants (Lazar et al., 2007)

Biosurfactant type	Producing organism
Lipopeptides surfactin	*Bacillus subtilis*
Lychenysin glycolipids	*Bacillus Licheniformis*
Rhamnolipids	*Pseudomonas aeruginosa*
Trehalose lipids	*Pseudomonas sp., r. erythropplis*
Sophorolipids	*Arthobacter sp., mycobacterium sp.*
Phospholipids	*Acinetobacter sp., t. thioosidans*
Polymeric biosurfactant emulsan	*Acinetobacter sp.*

The microorganisms that are most used for biosurfactant production field processes are species of *Bacillus*. These species have a greater potential for survival in petroleum reservoirs because they produce spores. Spores are dormant, resistant forms of cells that can survive

more stressful environmental conditions. *Bacillus subtilis* and *Bacillus licheniformis* are well-known producers of surface-active metabolites. They not only produce good biosurfactants but are also capable of growing under facultative or anaerobic conditions, and have also been reported to be non-pathogenic, which permits their use in food and pharmaceutical industries, apart from environmental applications (Das Neves et al., 2007). Surfactin-cyclic lipopeptide biosurfactant produced by *B. subtilis* is one of the most effective biosurfactants which can lower the ST and IFT of water and water-n-hexadecane system from 72 to 27 mN/m and 43 to 1 mN/m respectively (Nitschke and Pastore, 2006).

2.5.7.1 Thermo-Tolerant Bacteria

In most developed petroleum reservoir conditions, temperatures are expected to vary greatly but can be as high as 70^{o}C, and even 100^{o}C in some cases. To survive such high temperatures, thermophilic bacteria are often spore-forming and possess thermally stable enzymes that allow the normal functioning of cellular processes under such harsh conditions. *P. aeruginosa, B. subtilis* and *B. licheniformis* strains have been repeatedly isolated from many oil reservoirs, as well as from oil-contaminated sites (Al-Sayegh et al., 2015; Al-wahaibi et al., 2014; Cooper et al., 1981; Daryasafar et al., 2016; Gudiña et al., 2015).

Several Thermophilic genera have been isolated from high-temperature reservoirs in China, which are *Bacillus*, *Thermus*, *Thermoanaerobacter*, *Thermococcus*, and *Thermotoga* (90^{o}C) (Lin et al., 2014), California (80–90°C) (Orphan et al., 2000), and North Sea (158°F) (Dahle et al., 2008), and would therefore be ideal candidates for MEOR (Kaster et al., 2009). Three thermophilic species of *Bacillus*, *Geobacillus,* and *Petrobacter* were reported to tolerate 131°F under strictly anaerobic conditions, suggesting their suitability for MEOR (Shibulal et al., 2014).

2.5.7.2 Halo-Tolerant Bacteria

Bacteria that can grow and thrive in relatively high salt concentrations are called "Halo-Tolerant" bacteria. Many species of bacteria are halophilic, some of which with the added ability to also grow under the conditions experienced in oil reservoirs (Head et al., 2014). Strains of *B. subtilis* and *B. licheniformis* can tolerate high salt concentrations and high temperatures (Cooper et al., 1981; Daryasafar et al., 2016). Ollivier *et al.* (1998) reported that

methanogenic *Methanocalculus halotolerant* isolated from an oil well grows at the highest reported salt concentration of 20% NaCl and temperatures up to 45°C (Ollivier et al., 1998).

2.5.7.3 Facultative Anaerobic Bacteria

The isolated bacteria can be categorized based on their oxygen intake into three types, aerobic bacteria (oxygen is needed for growth), strictly anaerobic bacteria (no need for oxygen for growth), and facultative anaerobic bacteria (can grow either in the presence or absence of oxygen). Successful field trials have mostly used the anaerobic and facultatively anaerobic bacteria because they can grow and produce biosurfactants with no need for oxygen supply. Moreover, they have a small cell size, which makes it easier for them to penetrate through the reservoir's porous media. In addition, they can tolerate harsh environments like those in the subsurface reservoirs in terms of pressure, temperature, and pH. Finally, they could produce useful metabolic compounds which will be discussed later (Head et al., 2003; Jones et al., 2008).

2.5.8 Effect of Nutrient Composition

Nutrients are considered the major expense in MEOR projects because they could cost almost 30% of the total cost of the project. A successful MEOR project requires selecting a suitable nutrient in terms of types, concentrations, and nutrient components for optimum bacterial growth and metabolism of the bacteria. Furthermore, it was noticed that there is a relation between nutrient concentrations and bacterial growth rate (Lazar et al., 2007; Omoniyi, 2015). For bacterial growth, nutrients must contain organic carbon sources, nitrogen sources, and salt sources. These nutrients are usually transported in the aqueous phase. Fermentative bacteria usually use molasses, glucose, or sucrose as nutrients (Donaldson, 1991). Generally, molasses have been used as a carbon source in many MEOR field applications, since they are cheap and provide vital vitamins and minerals (Bryant et al., 1994), (Randhir S. Makkar and Cameotra, 1997), (Joshi et al., 2008b). Furthermore, some bacteria use oil as a carbon source, which is excellent for heavy oil production, since it can reduce the carbon chain of heavy oil and increase its quality (Cooper et al., 1980; Lazar et al., 2007; Omoniyi, 2015). Under anaerobic conditions, however, the use of petroleum components as food is thought to be not effective at least within the period required for economic recovery. Even though growth can

occur, the growth can be very slow and hardly detectable for several months (Moses et al., 1993).

2.6 MEOR Screening Criteria

The activity of bacteria employed in MEOR methods depends on the physical and chemical conditions they encounter in the reservoirs, such as temperature, salinity, pressure, pH, pore size, and nutrients. All these factors, which are generally physical and environmental can affect bacterial growth, proliferation, metabolism, and survival, and limit their ability to produce desired quantities of metabolites needed for enhanced oil recovery (Lazar et al., 2007; Omoniyi, 2015). However, it is of the general opinion that with proper planning most of these factors can be overcome. Some of the factors, which are considered limiting to the successful application of MEOR, are explained below.

2.6.1 Temperature

Temperature plays a significant role in bacteria metabolism. With increasing depth, temperature increases. Consequently, bacteria growth and their metabolism will certainly be affected as the increasing temperature can exert negative effects on enzyme function by disrupting important cell activities. The effects of temperature on enzyme function are generally accepted, but it is also to be noted that the temperatures at which these phenomena occur vary widely between organisms. Depending on the temperature ranges for microorganisms' survival, microbes can be classified according to their optimum temperature range as psychrophiles (<77^{o}F), mesophiles (77-113^{o}F), and thermophiles (113-140 ^{o}F) (Marshall, 2008).

2.6.2 Salinity

Sodium chloride makes up about 90% or more of the total dissolved solids found in reservoir brines. Consequently, tolerance of bacteria to salt concentration is considered one of the most important characteristics needed for microorganisms used in MEOR. The effect of salinity on changes in bacterial growth and metabolism depends on the osmotic balance required for such growth because the solute concentration of the surrounding environment can affect cell growth. Grula *et al.* (1983) found that the ability of his isolated Clostridia species to produce

solvents and gases was reduced significantly at high sodium chloride concentrations (5% w/v) (Lazar et al., 2007).

Mostly, the salinity of the oilfield can vary from 100 ppm to over 300,000 ppm (Gran et al., 1992). Most bacteria overcome the osmotic stress by the accumulation of organic compatible solutes within the cytoplasm without the need for a change of intracellular proteins. This method is called the organic osmolyte strategy (Negin et al., 2017; Roberts, 2005).

2.6.3 Pressure

The effect of pressure on bacteria depends not only on the magnitude but also on the duration of pressure applied in combination with temperature, pH, oxygen supply and composition of the culture media (Abe, 2007). The effects of pressure can be very complex and often difficult to interpret. For example, recent results indicate that lactic acid bacteria *Lactobacillus sanfranciscensis* growth at 50 MPa was 30% less than at atmospheric pressure and that increase in temperature did not improve its piezotolerance (Negin et al., 2017; Omoniyi, 2015). In another study, it was shown that treatment of *E. coli* cells at a higher pressure of 75 MPa for 30 min does not readily cause any morphological changes (Kelly and Wood, 2006). The challenges are therefore to establish whether the physiological responses of bacteria cells to high pressure are relevant to their growth and to identify the critical factors in cell viability and lethality under high pressure during microbial enhanced oil recovery.

2.6.4 pH

pH is one of the major environmental factors that affect bacterial growth and is one of the most studied because of its importance in fundamental research. In general, the optimal range pH for bacterial growth is 4.0 to 9.0. However, at very low pH, the metabolic activities of microorganisms can be affected. The detrimental effect of low pH on bacterial growth is well documented, but the mechanisms involved are not well understood. Generally, a near-neutral intracellular pH is maintained in bacteria, but the intracellular pH can decrease considerably when the cell is subjected to an acidic environment (Al-wahaibi et al., 2014; Soumen Mukherjee et al., 2009).

As many enzymes are sensitive to pH, the growth inhibitions that can be seen at low pH could be caused by a direct effect of the H ion on cellular components even though, such direct

effects would not necessarily cause a decrease in the efficiency of growth (Russell and Dombrowski, 1980). pH values normally encountered in oil reservoirs may not pose a problem for the growth of microorganisms, but pH gradients can affect the control of specific metabolic processes required for some MEOR processes (Al-Sulaimani et al., 2011a; Hossein Ghojavand et al., 2008; Jenneman and Clark, 1992).

2.6.5 Pore Size

Pore spaces less than 0.5 nm can place severe restrictions on the ability of most bacteria because most bacteria have dimensions of length approximating 0.5-10.0 µm and width of 0.5-2.0 µm to be able to transport through the rock matrix (Lazar et al., 2007; Negin et al., 2017). In 1983, Updegraff (1983) stated that pores must be at least twice the diameter of cocci or short bacilli for effective transport to occur. Fredrickson *et al.* (1997) also showed that the sizes of pores within the rock, or the pore throat diameter may be an important factor in regulating the observed bacterial activity. Furthermore, Zvyagintsev (1970) stated that placement of bacteria in large capillaries (400 x 150 nm) increased the number of cells 7-10 times, but in small capillaries, not only was an increase of cells observed but the size of the cells was reduced. Generally, 50 mD permeability is thought to be the lower limit for effective microbial transport (Lazar et al., 2007; She et al., 2019a).

In 1993, Sayyouh *et al.* studied the possibility of the application of MEOR to Arabian oil fields. Based on the analysis of data obtained from more than 300 formations, in Egypt, Saudi Arabia, Kuwait, Qatar, Iraq, U.A.E and Syria, they have emphasized that Egyptian, Saudi and Iraqi oil fields have the greatest potential for MEOR because most of their reservoirs characteristics fit the screen criteria of MEOR (Sayyouh et al., 1993).

Screening criteria are usually developed for eliminating poor prospects and for identifying good potential candidates. Table 2-5 lists the screening criteria proposed by China National Petroleum Corporation CNPC (She et al., 2019c). These screening guides cannot be quantified. However, they serve to identify the need for additional evaluations. It could be concluded that microbial growth is favoured when the formation temperature is lower than 80 °C, salinity is below 300,000 ppm, reservoir depth is less than 2,377 m, layer permeability

is greater than 50 mD, API gravity is greater than 15 API, and crude viscosity is less than 500 cp.

Table 2-5. MEOR reservoir screening parameters (She et al., 2019b)

Parameter	Value range	Optimum
Formation Temperature (°C)	≥ 80	30 – 60
Salinity (ppm)	≤ 300,000	≤ 100,000
Depth (m)	≤ 2,377	≤ 2,134
Permeability (mD)	≥ 50	≥ 150
Crude Oil Type (API)	≥ 15°	30° - 40°
Crude Viscosity (mPa.s)	≤ 500	30 -150

2.7 Reservoir Simulated Porous Micromodels

It is apparent that when oilfield parameters are ignored and testing of treatments is conducted under standard lab conditions (Table 2-6), higher recoveries are seen (Banat, 1995; R S Makkar and Cameotra, 1997; Qazi et al., 2013). However, with testing conditions did not mimic those of the reservoir, this, as we know, does not bode well for the accuracy of the microcosm simulations, and will in all likelihood lead to failures in the field as we have seen many times before (Maudgalya et al., 2007). However, in the studies that do mimic reservoir parameters, the most simulated parameters are that of temperature, crude oil density, gravity, and formation water composition, with only a limited number of trials replicating additional parameters such as porosity and well pressure. Up till now the model systems where all oilfield conditions were replicated (temperature, porosity, crude oil formation water, and pressure), produced less oil recovery compared to the less exacting laboratory studies (Dastgheib et al., 2008; Suthar et al., 2009). Most of the conducted laboratory studies have used sand-pack porous micromodels because the construction of a sand-pack column is easy, rapid, and inexpensive. The increased expense in time and money of core-floods experiments, combined with the difficulty of core acquisition, make the use of core floods an impractical process. However, the use of core-flood provides a more accurate evaluation of MEOR potential because it could mimic reservoir parameters (temperature, porosity, crude oil formation water, and pressure), which leads to more accurate microcosm simulations

(Bordoloi and Konwar, 2008; Daryasafar et al., 2016; El-Sheshtawy et al., 2016; Joshi et al., 2008b; Joshi and Desai, 2013; Nazarahari et al., 2021).

Table 2-6: The reported Additional oil recovery studies in different porous micromodel systems and their reservoir simulated conditions.

Microorganism	System Model/ Inoculum	Reservoir Simulated Conditions	Additional Oil Recovery (%)	Oil Type	Reference
• *Bacillus subtilis sp.*	Glass Etched Flow Micromodels/ Bacteria & Nutrient Medium	-	30	Light Oil	(Soudmand-asli et al., 2007)
• *Pseudomonas aeruginosa sp.*	Sand-Pack Column Model/ Crude Biosurfactant	Porosity, Temperature	30	N/A	(Bordoloi and Konwar, 2008)
• *Bacillus licheniformis AC01*	Sand-Pack Column Model/ Bacteria & Nutrient Medium	Porosity, Pressure, Temperature, Crude Oil, Formation Water	22	Light Oil	(Dastgheib et al., 2008)
• *Bacillus licheniformis AC01*	Sand-Pack Column Model/ Bioemulsifier	Porosity, Pressure, Temperature, Crude Oil, Formation Water	< 1	Light Oil	(Dastgheib et al., 2008)
• *Bacillus licheniformis TT42*	Sand-Pack Column Model/ Crude Biosurfactant	Pressure, Formation Water	35	Synthetic	(Suthar et al., 2008)
• *Bacillus licheniformis K125*	Sand-Pack Column Model/ Crude Bioemulsifier	Pressure, Formation Water	43	Synthetic	(Suthar et al., 2008)
• *Bacillus mojavensis JF-2*	Sand-Pack Column Model/ Crude Bioemulsifier	Pressure, Formation Water	29	Synthetic	(Suthar et al., 2008)
• *Bacillus subtilis 20B*	Sand-Pack Column Model/ Crude Biosurfactant	Porosity, Formation Water	25-33	Light Oil	(Joshi et al., 2008a)
• *Bacillus subtilis 20B*	Glass Packed Column Model/ Crude Biosurfactant	Porosity, Crude Oil, Formation Water	30	Light Oil	(Joshi et al., 2008a)

• *Bacillus licheniformis TT33*	Sand-Pack Column Model/ Microbial Biomass in a Nutrient Medium (Selective Plugging)	Pressure, Temperature, Crude Oil, Formation Water	25-32	Heavy Oil	(Suthar et al., 2009)
• *Bacillus sp.*	Glass Etched Micromodels	Temperature, Crude Oil	13		(Gao, 2011)
• *Enterobacter sakazakii/ Bacillus subtilis fusion*	Sand-Pack Column Model/ Engineered Bacteria & Nutrient	Pressure, Temperature, Formation Water	17-25	N/A	(Xu and Lu, 2011)
• *Bacillus subtilis W19*	Berea Sandstone Core Model/ Crude Biosurfactant	Porosity, Crude Oil, Formation Water	23	Light oil	(Al-Sulaimani et al., 2012)
• *Bacillus licheniformis sp.*	Sand-Pack Column Model/ Bacteria &Nutrient	Temperature, Crude Oil	6-25	Light Oil	(Gudiña et al., 2013)
• *Bacillus licheniformis sp.*	Sand-Pack Column Model/ Bacteria &Nutrient	Temperature, Crude Oil	15-17	Heavy Oil	(Gudiña et al., 2013)
• *Bacillus licheniformis R1*	Sand-Pack Column Model/ Crude Biosurfactant	Porosity, Formation Water	32	N/A	(Joshi and Desai, 2013)
• *Fusarium sp. BS-8*	Sand-Pack Column Model/ Crude Biosurfactant	Formation Water	46	Light Oil	(Qazi et al., 2013)
• *Bacillus subtilis W19*	Berea Sandstone Core Model/ Cell-free Biosurfactant	Porosity, Crude Oil, Formation Water	13-28	Light Oil	(Souayeh et al., 2014)
• *Bacillus subtilis B30*	Berea Sandstone Core Model/ Cell-free Biosurfactant	Porosity, Crude Oil, Formation Water	17-26	Light Oil	(Al-wahaibi et al., 2014)
• *Bacillus subtilis B30*	Berea Sandstone Core Model/ Cell-free Biosurfactant	Porosity, Crude Oil, Formation Water	31	Heavy Oil	(Al-wahaibi et al., 2014)

• *Bacillus subtilis R2*	Berea Sandstone Core Model/ Cell-free Biosurfactant	Porosity, Crude Oil, Formation Water	37	Heavy Oil	(Al-wahaibi et al., 2014)
• *Candida albicans IMRU 3669*	Sand-Pack Column Model/ Crude Biosurfactant	Crude Oil, Formation Water	9	Light Oil	(El-Sheshtawy et al., 2016)
• *Bacillus subtilis MTCC 2422*	Sand-Pack Column Model/ Bacteria & Nutrient	-	9	Synthetic	(Kanna et al., 2016)
• *Bacillus licheniformis ATCC 14580*	Sand-Pack Column Model/ Crude Biosurfactant	Crude Oil, Formation Water	17	Light Oil	(El-Sheshtawy et al., 2016)
• *Bacillus licheniformis* W16	Berea Sandstone Core Model/ Cell-free Biosurfactant	Temperature, Crude Oil, Formation Water	24-26	Light Oil	(Joshi et al., 2016)
• *Bacillus licheniformis* L20	Sandstone Core Model/ Cell-free Biosurfactant	Temperature, Crude Oil	14	Heavy Oil	(Liu et al., 2021)

- No petrophysical or geochemical characteristics were simulated in the model system.
N/A Not Applicable

This literature review chapter indicates that indigenous bacteria in the reservoir are the ideal candidates for biosurfactant production applications since they meet the most important requirement, which is the ability to survive and produce biosurfactants in the reservoir. Indigenous bacteria have a selective advantage over exogenous bacteria because they are adapted to the reservoir conditions. The main adaptations bacteria are their high tolerance against high temperatures, and salinity, as well as being active under anaerobic conditions. In addition, the microorganisms that are most used for biosurfactant production field processes are species of *Bacillus*. These species have a greater potential for survival in petroleum reservoirs because they produce spores. Spores are dormant, resistant forms of cells that can survive more stressful environmental conditions. *Bacillus subtilis* and *Bacillus licheniformis* are well-known producers of surface-active metabolites. They not only produce good biosurfactants but are also capable to grow under facultative or anaerobic conditions, and have also been reported to be non-pathogenic, which permits their use in food and pharmaceutical industries, apart from environmental applications. Furthermore, the activity of bacteria employed in MEOR methods depends on the physical and chemical conditions they encounter in the reservoirs, such as temperature, salinity, pressure, pH, pore size, and nutrients. All these factors, which are generally physical and environmental can affect bacterial growth, proliferation, metabolism, and survival, and limit their ability to produce desired quantities of metabolites needed for enhanced oil recovery. Moreover, there are three ways through which biosurfactants could be applied to improve oil recovery, which are the reduction of interfacial tension between oil-rock and oil-brine, wettability alteration of rocks, and emulsification of crude oil. Finally, the use of core-flood provides a more accurate evaluation of MEOR potential because it could mimic reservoir parameters (temperature, porosity, crude oil formation water, and pressure), which leads to more accurate microcosm simulations.

The next chapter discusses the research methodology that helps in achieving the research objectives of this study.

CHAPTER 3

RESEARCH METHODOLOGY

Outline of the chapter

This chapter investigates the Samples and Data Collection, materials, equipment set up and experimental methodology used for this study. The chapter is organised as follows:

3.1. Introduction

3.2. Samples and data collection

3.3. Experimental work

3.3.1. Isolation of bacteria

3.3.2. Identification of bacteria

3.3.3. Biosurfactants production

3.3.4. Examination of Oil Recovery using Core flooding

3. RESEARCH METHODOLOGY

3.1 Introduction

This chapter provides a detailed description of the sampling, data collection, materials, equipment set-up, and experimental methodology used for this study. These are divided into five phases summarized in Figure 3-1, as follows:

Phase I - Sampling and Data Collection: the first phase presents the MEOR screening parameters of 59 Egyptian oil reservoirs that represent the two main Egyptian oil concessions areas (the Gulf of Suez, and the Western Desert). These data are statistically analysed in section (3.2), based on each screening criterion.

Phase II - Microbial Isolation: The second phase presents the process of isolating indigenous bacteria from collected crude oil samples. A description of preparing the isolation media, isolation process , and the pouring of agar on Petri dishes to prevent contamination is detailed in section (3.3.1).

Phase III - Microbial Identification: In the third phase, the process of identifying the isolated bacteria to make a road map representing indigenous bacteria in Egyptian oil fields is presented in section (3.3.2). Proper labelling of agar plates and broths to include name (initials), date, and code are ensured to prevent any mix-up and enable identification of the different species.

Phase IV - Biosurfactants Production: The fourth phase is described in section (3.3.3) and presents the process of selecting the suitable bacterial strains for producing biosurfactants. The methodologies to investigate the effect of some nutrients on the selected bacterial strains, the efficiency of the produced biosurfactants, the stability of the produced biosurfactants under harsh reservoirs conditions (salinity, pH, and temperature), and determine the optimum conditions for the produced biosurfactants to reach maximum surface activity are also presented.

Phase V – Core flooding: A description of the core flooding is presented in section (3.3.4) as well as the process of investigating the effect of produced biosurfactants

on oil recovery. The interaction mechanism between the produced biosurfactants and the oil reservoir under dynamic flow conditions is investigated by core-flooding. The flooding is performed in two stages, water flooding as a secondary recovery stage using the formation water samples of the fields of interest, and the biosurfactants flooding as a tertiary "enhanced" oil recovery stage using biosurfactants produced by the selected indigenous bacterial strains to evaluate their effectiveness in improving oil recovery.

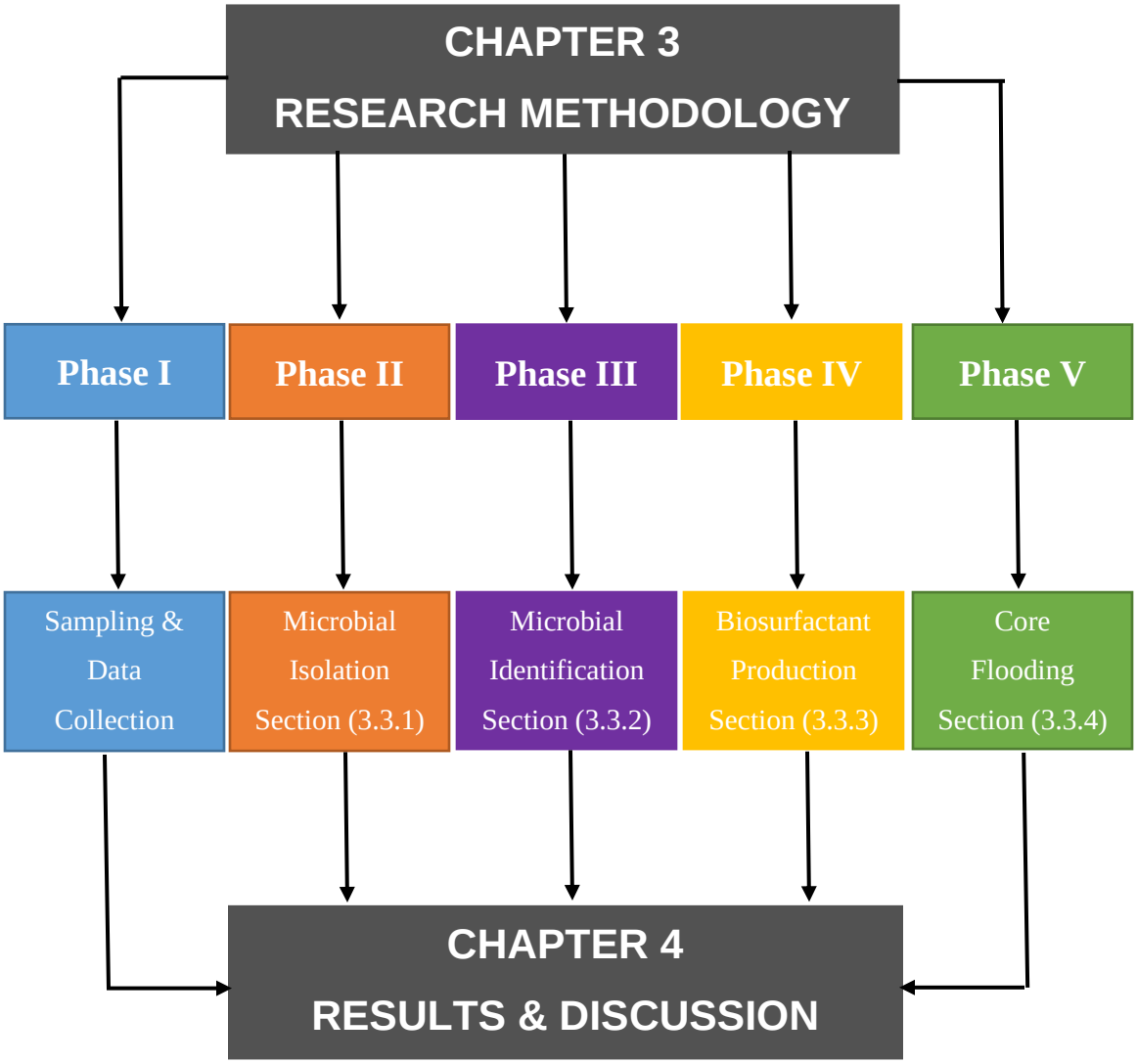

Figure 3-1: The flow chart of the research methodology

3.2 Sampling and Data Collection

MEOR screening parameters of 59 Egyptian oil reservoirs representing the two main Egyptian oil concessions areas (the Gulf of Suez, and the Western Desert), summarized in Table 3-1 and Table 3-2. These data were statistically analysed, based on each screening criterion mentioned in Table 3-3. The crude oil samples were collected from Egyptian oil fields located in the Gulf of Suez and the Western Desert to isolate biosurfactant-producing bacteria that can improve oil recovery. The crude oil samples were preserved in a fridge at 4°C in 50 ml sterilized bottles for further studies.

Table 3-1. MEOR screening parameters for Gulf of Suez Oil Reservoir.

#	Field	Reservoir	Temperature (°C)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
1	EL-MORGAN	N. KAREEM	79	60,000	1,829	190	29.5	0.55
2	EL-MORGAN	S. KAREEM	79	70,000	1,859	300	29.5	1.17
3	EL-MORGAN	N. BELAYIM	71	66,000	1,615	150	26.0	3.40
4	GS-382	NUBIA	138	370,000	3,200	60	33.0	0.49
5	GS-315	KAREEM	79	70,000	1,829	1,400	30.4	0.94
6	GS-315	BALEYIM	72	65,000	1,615	1,000	28.0	1.96
7	JULY	L. RUDIES	116	50,000	2,713	158	29.0	0.62
8	JULY	S. NUBIA	138	250,000	2,926	360	29.9	0.59
9	RAMADAN	NUBIA	146	184,000	3,627	83	31.3	0.72
10	OCTOBER	NUBIA	121	172,000	3,231	450	27.0	1.20
11	OCTOBER	L. SENONIAN	116	144,000	3,048	700	25.2	1.50
12	GS-173	NUKUL	110	39,600	2,957	660	31.0	0.72
13	GS-404	NUBIA	138	170,000	3,139	87	31.6	0.70
14	GS-404	NUKHUL	135	41,300	3,048	300	31.0	0.40
15	GS-336	U. RUDEIS	85	126,000	2,103	300	28.0	1.36
16	GH-376	KAREEM	113	236,000	2,576	187	24.5	0.87
17	RAS FANAR	N. LIMESTONE	46	100,800	642	100	27.7	2.35
28	ZEIT BAY	CARBONATE S.S.	67	220,000	1265	370	38	0.90
19	RAS BADRAN	NUBIAN S.S.	124	143,000	3,277	45	27.3	1.71
20	WEST BAKR	RUDES S.	60	15,000	1,049	3,000	20	30

Table 3-2. MEOR Parameters for Western Desert Oil Reservoir.

#	Field	Reservoir	Temperature (°C)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
1	RAZZAK1	APT. DEL.	82	152,000	2,207	5.3	37.8	1.30
2	RAZZAK2	APT. DEL.	71	84,000	1,698	400	37.5	0.93
3	CYG	UPPER Bah	85	80,000	1,981	132	33	1.50
4	CYG	LOWER Bah	87	143,000	2,286	131	33	1.50
5	BED15	ARC	111	88,000	3,200	307	34	0.65
6	BED3C9	ARG	116	93,000	3,400	40	42.2	0.25
7	BED3-8/3-13	ARC	112	88,000	3,000	303	35	0.25
8	BED3-6/3-11	ARC	107	89,000	2,875	305	38	0.32
9	BED-1	ARC	109	90,000	2,852	304	36.3	0.68
10	BED-1	ARD	113	122,000	2,960	185	33.8	0.80
11	BED-1	KHARITA	128	93,000	3,475	200	40.6	0.50
12	BED-1	Bahariya	121	100,000	3,390	131	39.9	0.22
13	BED-9	Bahariya	121	102,000	3,230	132	36.2	0.36
14	BAHGA	A.R.G	114	93,000	2,812	40	33.2	1.14
15	BAHGA	L. Bah	121	143,000	3,113	131	35.9	0.63
16	AL MAGD	North AR G″	114	97,000	3,048	40	33.2	0.95
17	AL MAGD	South A/R″G″	114	138,000	3,044	40	33.2	0.95
18	AL BARQ	A/R″E″	112	85,000	2,990	239	38.7	0.48
19	AL BARQ	A/R ″G″	112	94,000	3,182	40	31.8	2.10
20	KARAM	ARC	149	90,000	4,220	307	38.7	0.32
21	ASSIL	A/R ″G″	124	92,000	3,870	40	38.6	0.45
22	ASSIL	Bahariya	128	101,000	4,074	132	9	0.45
23	AL FADL	Bahariya	64	122,000	1,200	131	40.4	1.82
24	AL QADR	Bahariya	64	122,000	1,200	133	41.3	1.5
25	NEAG2	Bahariya	96	152,879	2,600	132	42.8	0.87
26	NEAG2 EAST	Bahariya	97	154,720	2,600	132	42.4	0.87
27	NEAG3	Bahariya	92	144,936	2,513	132	41	0.87
28	JG (JD-2BLOCK)	LSA-East	113	133,000	3,235	216	42	0.60
29	JG	LSA- West	114	207,420	3,235	198	37.5	0.77
30	JG (JD- 7BLOCK)	LAC- West	112	185,000	3,235	207	38	0.70
31	JG (JD-10BLOCK)	LSC- East	112	173,00	3,235	195	33	0.50
32	JG	LSB	112	207,420	3260	231	38	0.60
33	SHIBA	Bahariya	96	124,350	2,054	131	36	0.60
34	SITRA-8 BLOCK	A.R.C	109	93,000	2,860	307	29	0.40
35	SITRA-8 BLOCK	A.R.C	110	89,000	2,895	305	39	0.32
36	SITRA-8 BLOCK	ARG Sand1	127	113,000	3,270	100	31	1.29
37	SITRA-8 BLOCK	ARG Sand2	127	107,000	3,270	100	31	1.29
38	SITRA-8 BLOCK	ARE	114	94,000	3,070	163	39	0.50
39	SITRA-8 BLOCK	UPPER Bah	124	91,000	3,410	114	40	0.36

Table 3-3. MEOR reservoir screening parameters (She *et al.*, 2019)

Parameter	Value range	Optimum
Formation Temperature (°C)	≥ 80	30 – 60
Salinity (ppm)	≤ 300,000	≤ 100,000
Depth (m)	≤ 2,377	≤ 2,134
Permeability (mD)	≥ 50	≥ 150
Crude Oil Type (API)	≥ 15°	30° - 40°
Crude Viscosity (mPa.s)	≤ 500	30 -150

3.3 Experimental Work

The experimental work starts with the isolation of bacterial strains collected from Egyptian crude oil samples, identification of their gene, and then the study of the effect of nutrient composition, temperature, salinity, and pH to optimize the biosurfactant production by selected bacteria.

3.3.1 Microbial Isolation

The enrichment culture technique was used for isolating bacteria (Liu et al., 2014). 100ml of BHMS broth medium was prepared in a conical flask for each crude oil sample and autoclaved. 1ml of each crude oil sample was added to the prepared conical flasks, and then the flasks were incubated for 3-10 days on a rotary shaker at 30°C and 180 rpm. the bacteria cultures were streaked out from the conical flasks using a sterile swab or loop and spread on sterile agar (solid medium) plates. The inoculated plates were incubated at 30°C for 24-48 h. A successive streaking was executed by the quadrant pattern method on sterile agar plates to separate single colonies. The whole isolation process described above using the enrichment culture technique and quadrant pattern method is shown in Figure 3-2.

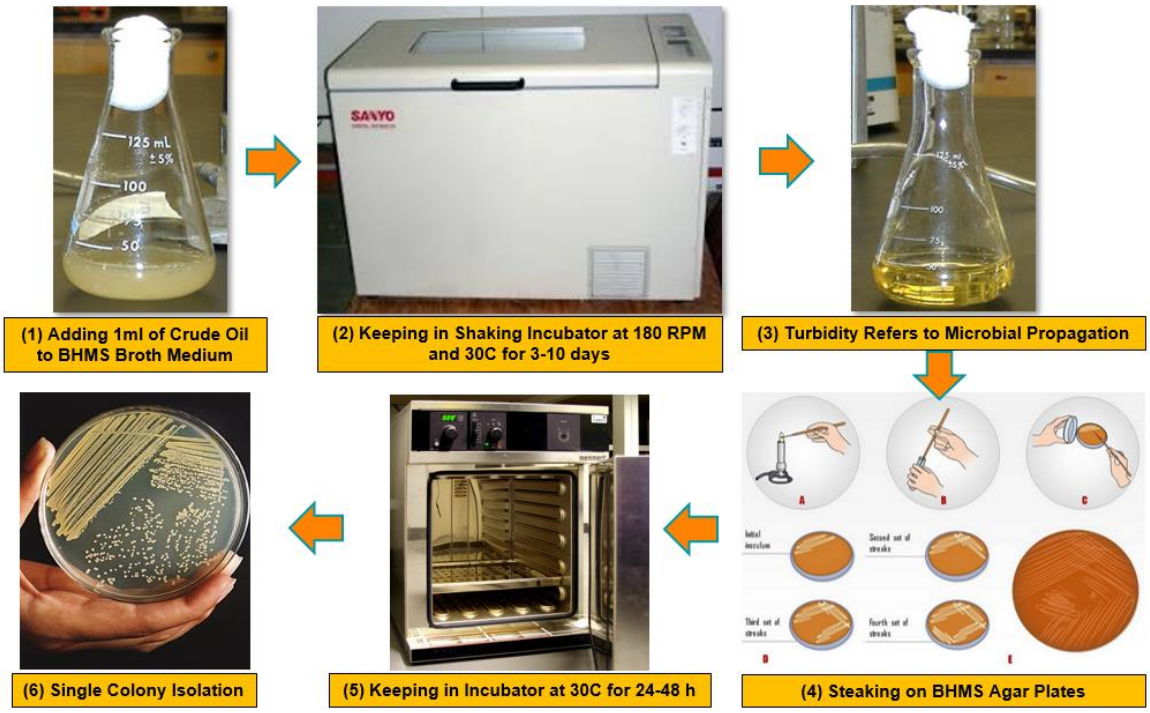

Figure 3-2. Isolation process using enrichment culture technique and quadrant pattern method.

Bushnell Hass Mineral Salts (BHMS) is a recommended medium for studying crude oil bacteria. This medium contains all nutrients except carbon sources, necessary for the growth of bacteria. Bushnell Hass Mineral Salts BHMS media were used to isolate the indigenous bacteria from collected crude oil samples. The composition of Bushnell Hass Mineral Salts BHMS medium is given in Table 3-4, and for the preparation of agar plates (solid medium), 15.0 g/l agar (strength 1300) was added.

Table 3-4. Composition of Bushnell Hass Mineral Salts per litre of distilled water

Composition	Quantity (g/l)
Monopotassium phosphate (KH_2PO_4)	1
Dipotassium phosphate (K_2HPO_4)	1
Ammonium nitrate (NH_4NO_3)	1
Magnesium sulphate ($MgSo_4$)	0.2
Ferric chloride ($FeCl_3$)	0.05
Calcium chloride ($CaCl_2$)	0.02
Final pH (at 25°C)	7.0±0.2

3.3.2 Microbial Identification

The isolated strains were identified based on Bergey's manual of systematic bacteriology by examination of seven characteristics, which are (Bergey et al., 2012):

(1) Colony morphology
(2) Cell morphology
(3) Gram stain reaction
(4) Presence of endospores in a culture
(5) Motility
(6) Oxygen intake
(7) Biochemical tests

3.3.2.1 Colony Morphology

Different types of bacteria produce different-looking colonies, some colonies may be coloured, some colonies are circular, and others are irregular. Figure 3-3 shows the specific terminology used to describe common colony types, which are shape, size, surface, colour, opacity, elevation, and margin.

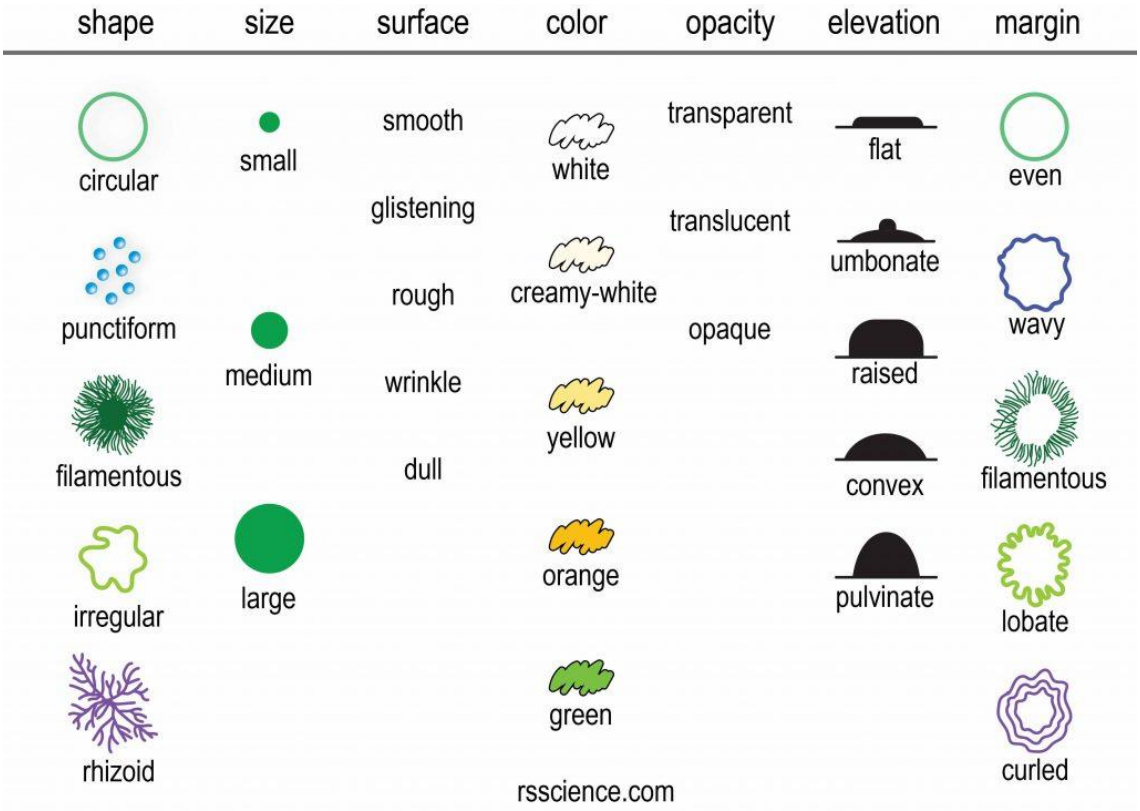

Figure 3-3. Characteristics of bacterial colony morphology (Bergey et al., 2012)

3.3.2.2 Cell Morphology

Morphology of bacterial cells deals with the study of the shape, size, and arrangement of bacteria cells. As mentioned in the literature review, there are three shapes of bacteria spherical (cocci) shape, cylindrical (rod) shape, and spiral shape. As known, all bacteria are microscopic, so their size is also measured under a microscope. The size of bacteria is measured by using a calibrated slide and a calibrated ocular compound microscope. The above method of measuring the size is called micrometry. The size of bacteria can also be measured by electron microscopic micrometry. The units of measurement that are used in bacteriology are micron or micrometre, nanometre (nm) or millimicron, and angstrom (Å).

The arrangements of cocci shape bacteria, as shown in Figure 3-4, are cocci (if a cocci cell appears individually), diplococci (when two cells are attached in pairs), streptococci (If cocci cells are arranged in long-chain and remain attached), and staphylococci (If the cocci cells are arranged in form of a cluster even after dividing them in three) (Bergey et al., 2012).

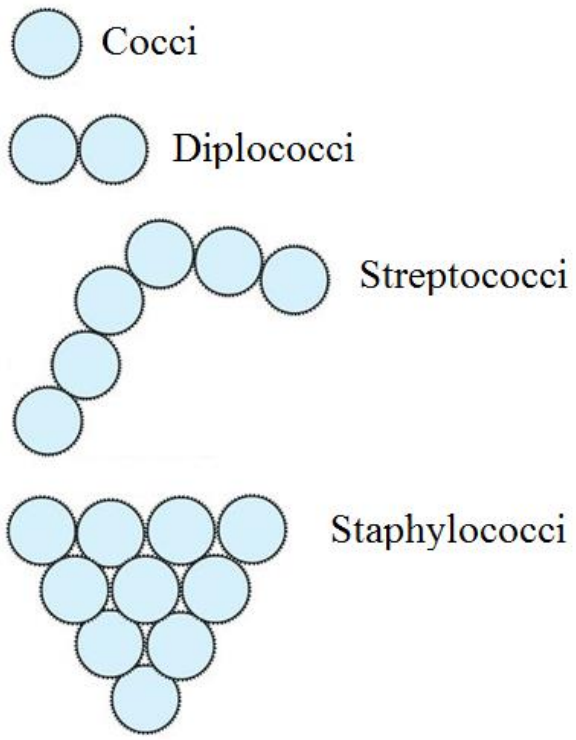

Figure 3-4. Arrangement of cocci shape bacteria.

The arrangements of rod shape bacteria, as shown in Figure 3-5, are bacilli (if a bacilli cell appears individually), diplobacilli (if a bacilli cells are attached in pairs), and streptobacilli (if bacilli cells are arranged in the form of a long-chain).

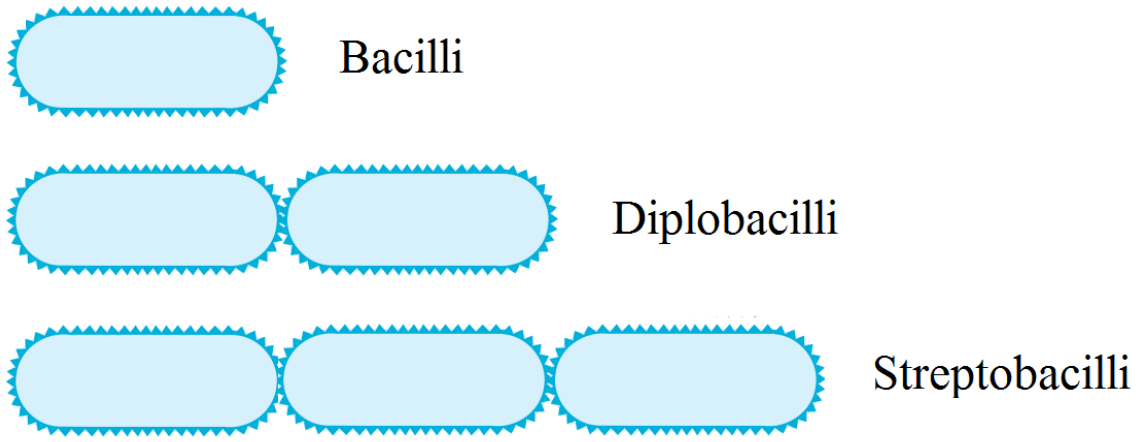

Figure 3-5. Arrangement of rod-shaped bacteria.

3.3.2.3 Gram Staining Reaction

Gram staining as mentioned in the literature review is used to differentiate the bacterial cell into two major groups. Gram-positive and Gram-negative which makes it an essential tool for the classification and differentiation of microorganisms. To find out if the studied bacteria are Gram-positive and Gram-negative, the studied bacteria colonies were picked using a sterile swab or loop and smeared on a clean glass slide. The slide was air-dried, heat-fixed with smear facing up, by running it over the blue flame 3-4 times and left to cool. then, the smear was covered with crystal violet for 60 seconds and washed off using distilled water (the excess water was drained off). After that, the smear was covered with Gram's iodine solution and kept for 60 seconds. The Gram's iodine was poured off and the smear was flooded with ethyl alcohol (95%) for 30 seconds. The slide was washed with distilled water. The counterstain safranin was added to the smear and was kept for 60 seconds. The stain was washed gently for a few seconds. Finally, the slide was air-dried and examined with a light microscope under oil immersion, as shown in Figure 3-6. The AmScope 40X-1000X Compound Microscope was used for the observation of the size and morphology of the bacterial colonies with a 1000× magnification objective (Bergey et al., 2012).

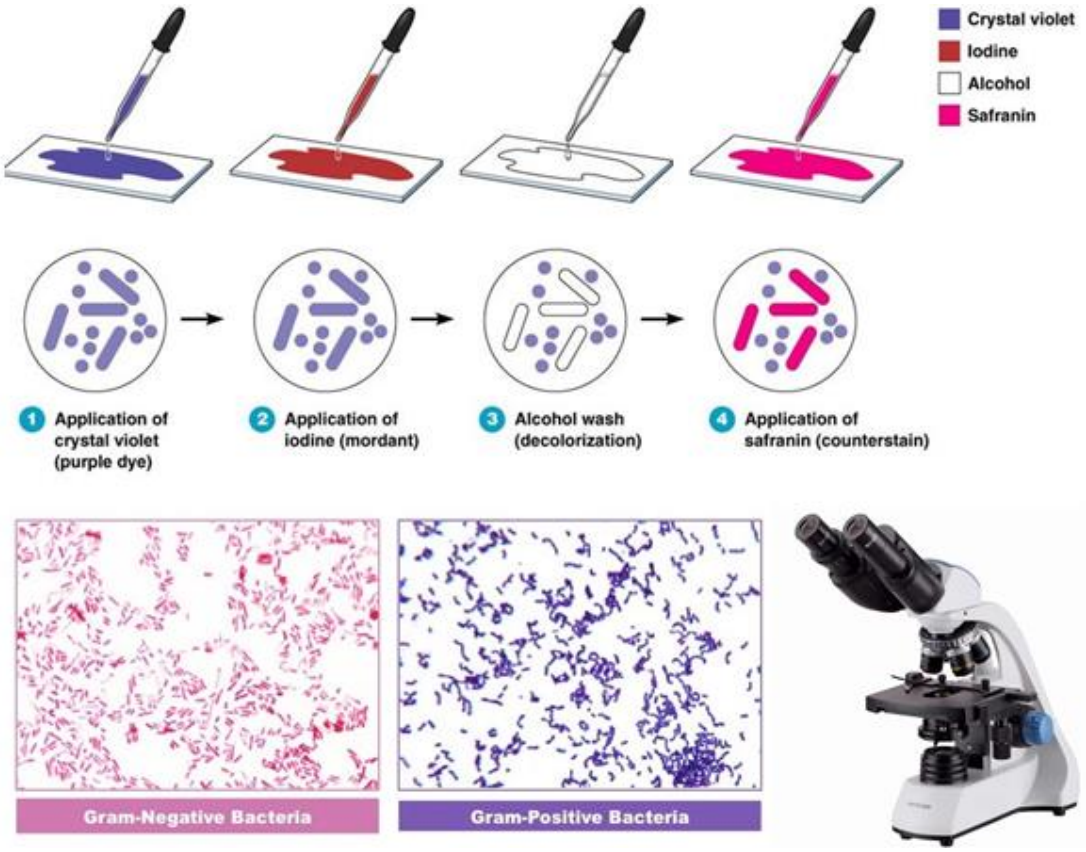

Figure 3-6: Schematic diagram summarising Gram Staining procedure, and microscopic view of gram-positive and gram-negative bacteria using AmScope 40X-1000X (Created by BioRender.com).

3.3.2.4 Endospore Staining

To identify if the studied bacteria are spore-forming or non-spore-forming, the studied bacteria colonies were picked using a sterile swab or loop and smeared on a clean glass slide. The slide was air-dried, heat-fixed with smear facing up, by running it over the blue flame 3-4 times and left to cool. Then, the smear was covered with a piece of absorbent paper, flooded with malachite green for 3-5 minutes and steamed for 3-5 minutes. Then, the stained absorbent paper was removed and the slide was left to cool for 1-2 minutes. After that, the slide was rinsed gently using distilled water by tilting the slide to allow the water to flow over the smeared stain. After that, the smear was covered with safranin (counterstain) for 1 minute, and then the slide was rinsed with water, on both sides to remove the safranin reagent. Finally, the slide was air-dried and, examined with AmScope

40X-1000X Compound Microscope under oil immersion with 1000× magnification objective, as shown in Figure 3-7.

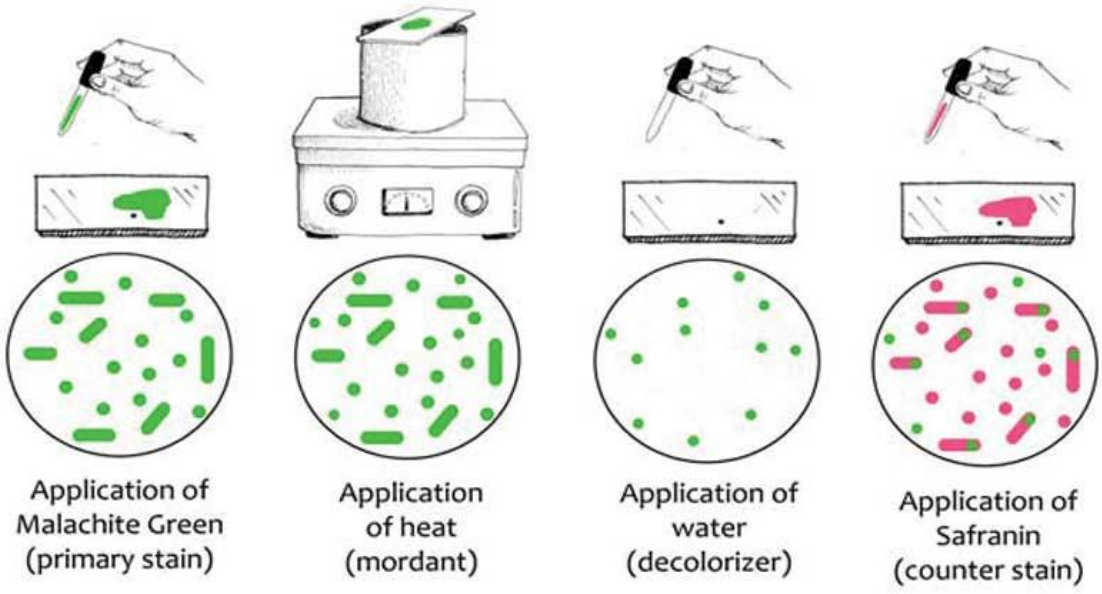

Figure 3-7. The schematic diagram summarizes the Endospores Staining procedure (Microbiology, 2016).

3.3.2.5 Oxygen Intake

Aerobic and anaerobic bacteria can be identified by growing them in tubes of Thioglycolate broth media. Thioglycolate can react with oxygen and consume it. Therefore, in a tube of Thioglycolate broth, the oxygen concentration is decreased with increasing tube depth. In other words, the concentration of oxygen on the surface and bottom of Thioglycolate broth is highest and lowest, respectively. In this test, the position of bacterial colonies determines whether they are aerobic or anaerobic as shown in Figure 3-8. Strictly aerobic bacteria need oxygen because they cannot ferment or respire anaerobically, so they gather at the top of the tube, where oxygen concentration is higher as shown in tube 1. Strictly anaerobic bacteria are poisoned by oxygen, so they gather at the bottom of the tube where the oxygen concentration is low as shown in tube 2. Facultative anaerobes can grow with or without oxygen because they can metabolize energy aerobically or anaerobically, so they gather mostly at the top because aerobic respiration generates more ATP (adenosine triphosphate) than either fermentation or anaerobic respiration as shown in tube 3.

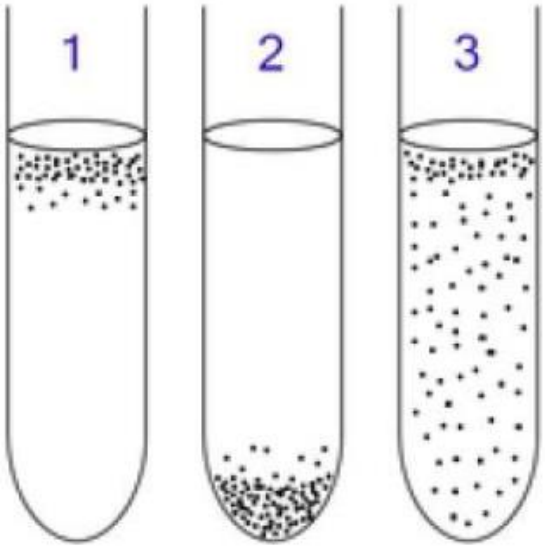

Figure 3-8. Schematic diagram of different forms of bacteria oxygen intakes.

3.3.2.6 Motility Test

To determine if the studied bacteria are motile or not, the motility test was performed. The motility test medium, represented in Table 3-5, was prepared and autoclaved with 2 test tubes. Then, the media were poured into all two test tubes and left for 5 minutes at room temperature for cooling. When the media became semi-solid, inoculate was added to the tube with the help of a sterile toothpick. The second tube was kept as a comparative control. Inoculation should be vertical. Then, the tubes were covered with cotton plugs and wrapped in paraffin and put into an incubator at 37^{o}C and examined daily for up to 7 days. The motile bacteria were spread out into the medium from the site of inoculation, the diffuse zone. However, non-motile bacteria remained at the site of inoculation, as shown in Figure 3-9.

Table 3-5. The composition of Motility test medium.

Components	Quantity (g/l)
Enzymatic digest of gelatin	10
Beef extract	3.0
Sodium chloride	5.0
Agar	4.0
pH at 25^{o}C	7.3 ±0.2

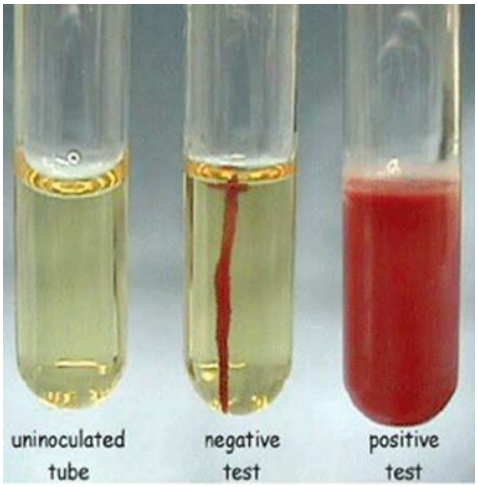

Figure 3-9. Motility Test.

3.3.2.7 Biochemical Tests

Several biochemical tests were implemented namely gram staining, endospore staining, motility test, catalase test, indole test, oxidase test, hydrogen sulphide (H_2S) production test, methyl red test, Voges-Proskauer (VP) test, Oxidative-Fermentative OF test, gelatin hydrolysis test, urease test, nitrate test, citrate test, starch hydrolysing test, mannitol fermentation test, glucose fermentation test, and lactose fermentation test.

3.3.2.7.1 Catalase Test

The catalase test is used to differentiate between catalase-producing bacteria and non-catalase-producing bacteria. 2-3 drops of 3% hydrogen peroxide were taken on a clean glass slide. Then, the studied bacteria were picked using a sterile swab or loop that was just kept over the hydrogen peroxide. After that, the slide was observed for the appearance (catalase-positive) or absence of gas bubbles (catalase-negative), as shown in Figure 3-10.

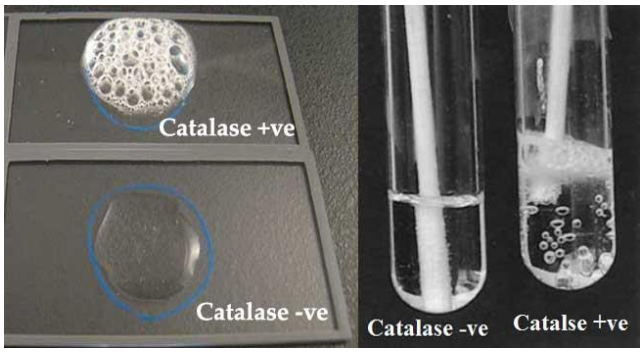

Figure 3-10. Catalase test.

3.3.2.7.2 IMViC Tests

IMViC tests are a set of four reactions that are helpful in identifying and distinguishing among members of the *Enterobacteriaceae* family. These tests include the Indole test, Methyl Red test, Voges Proskauer test, and Citrate test.

3.3.2.7.2.1 Indole test

To test indole production, inoculate the tube of tryptone broth, represented in Table 3-6, with a small amount of pure culture and incubate at 37°C for 24 to 48 h. Then 5 drops of Kovács reagent (50 gm of p-Dimethylaminobenzaldehyde are added to 250 ml of hydrochloric Acid (37%), and 750 ml of amyl alcohol) are added directly to the culture tube. A positive indole test is indicated by the formation of the cherry red ring in the reagent layer on top of the medium within seconds of adding the Kovács reagent. If a culture is indole-negative, the reagent layer will remain yellow or slightly cloudy, as shown in Figure 3-11.

Table 3-6. The composition of Tryptone Broth medium

Components	Quantity (g/l)
Pancreatic digest of casein	10.0
Sodium chloride	5.0
pH at 25°C	7.5 ± 0.2

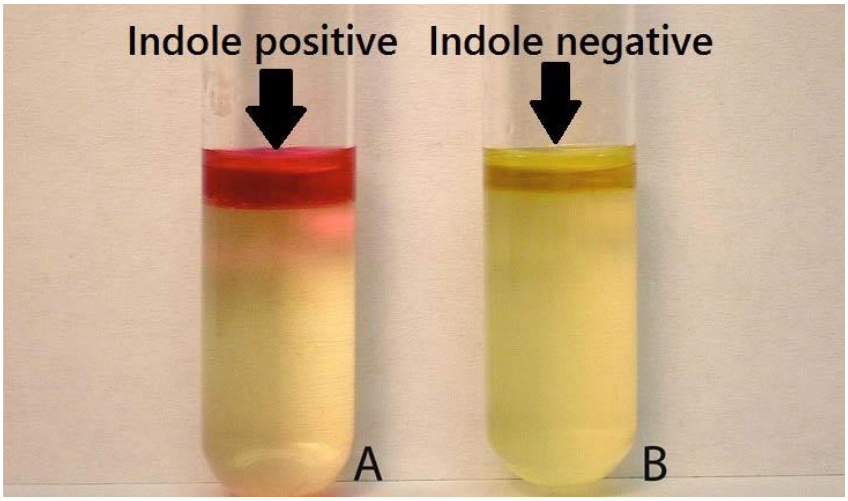

Figure 3-11. Indole test.

3.3.2.7.2.2 Methyl Red Test

Methyl red test is used to detect the production of sufficient acid during the fermentation of glucose. To perform the methyl red test, the culture was inoculated in two test tubes containing MR-VP broth media, represented in Table 3-7, and incubated at 37 ^{o}C for 48 h; one control was also maintained. After incubation was over, 2 to 3 drops of 0.02% methyl red solution (0.1 g of methyl red was added to 300 mL of 95% ethyl alcohol, and 200 ml of distilled) were added. If the red colour was noticed immediately after adding the methyl red indicator, it means that the studied bacteria can utilise glucose to produce a stable acid (Methyl Red Positive). If there was no colour change, it means that the studied bacteria cannot utilise glucose with the production of a stable acid (Methyl Red Negative), as shown in Figure 3-12.

Table 3-7. The composition of MR-VP broth medium.

Components	Quantity(g/l)
Peptone	7.0
Glucose	5.0
Dipotassium phosphate	5.0
pH at 25^{o}C	6.9 ±0.2

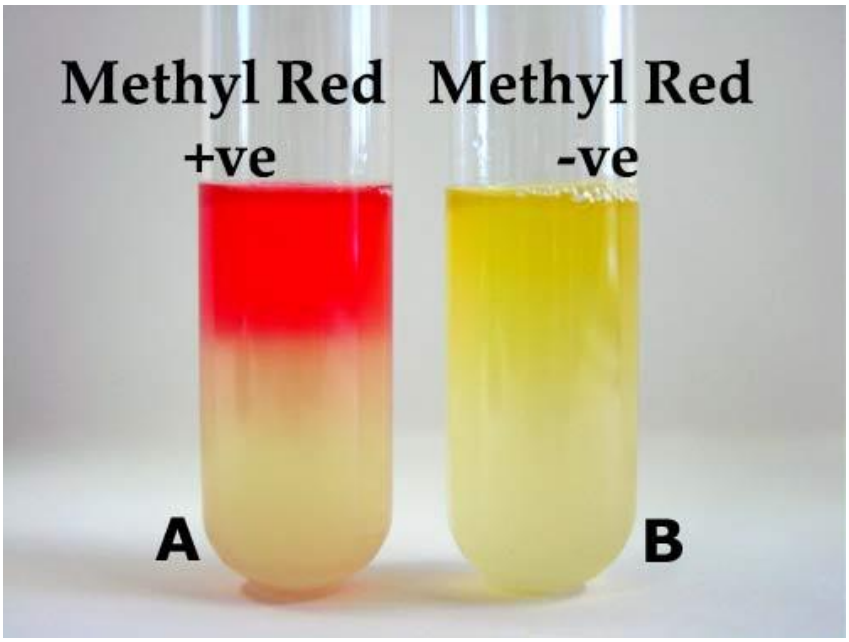

Figure 3-12. Methyl Red Test

3.3.2.7.2.3 Voges-Proskauer (VP) Test

The Voges-Proskauer (VP) test is used to determine if bacteria could produce neutral end products such as 2, 3-butanediol or acetoin from glucose fermentation. To perform this test, the culture was inoculated in two test tubes containing MR-VP broth media, represented in Table 3-7, and incubated at 37^{o}C, for 48 h; a control was also maintained. After the incubation was over, 6 drops of alpha-naphthalin solution (5%) and 2 drops of potassium hydroxide KOH solution (40%) were added. Then, the tube was shaken gently for 30 seconds with the plugs off to expose the media to oxygen. Finally, the tube was observed for colour change. If the colour changed to crimson or yellow colour, it means that the studied bacteria can produce neutral end products (VP positive). If there was no change in colour, it means that the studied bacteria cannot produce neutral end products (VP negative), as shown in Figure 3-13.

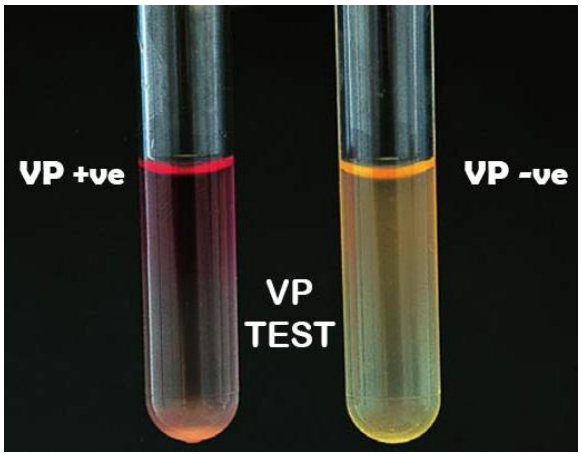

Figure 3-13. Voges-Proskauer (VP) Test

3.3.2.7.2.4 Citrate Test

The citrate test was conceived to verify whether bacteria can use citrate as their sole carbon source and Inorganic ammonium salts as the sole nitrogen source. To perform this test, the culture was inoculated in the tubes containing citrate test agar media, represented in Table 3-8, and incubated at 37^{o}C, for 24 h; one control was also maintained. After the incubation was over, the tubes were observed for the change in colour of the media. A change in colour from green to blue indicates a positive result, while no change in colour indicates a negative result as shown in Figure 3-14.

Table 3-8. The composition of citrate agar media

Components	Quantity (g/l)
Sodium Chloride	5.0
Sodium Citrate	2.0
Ammonium Dihydrogen Phosphate	1.0
Dipotassium Phosphate	1.0
Magnesium Sulphate	0.2
Bromothymol Blue	0.08
Agar	15.0
pH at 25ºC	7.0 ±0.2

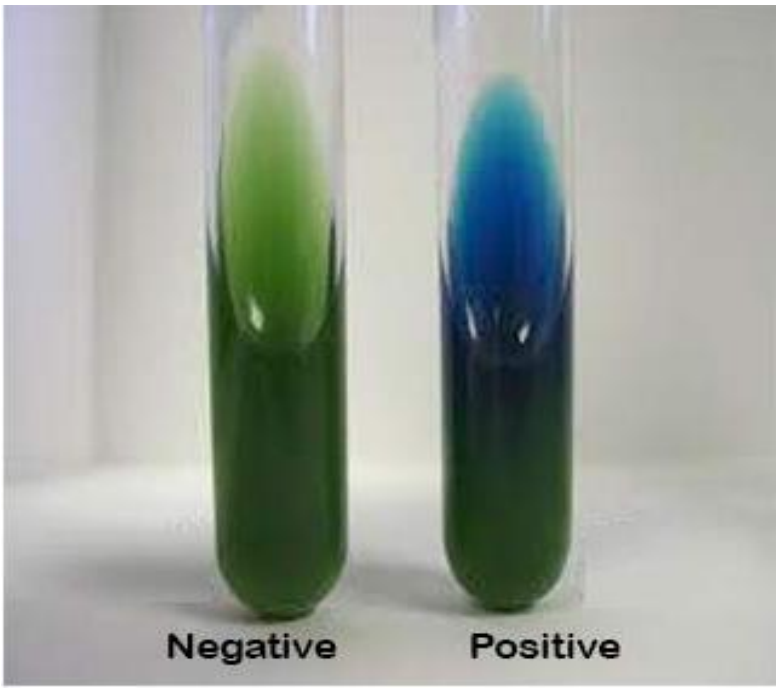

Figure 3-14. Citrate test.

3.3.2.7.3 Oxidase Test

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. To test oxidase production, a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride (1%) was moistened with sterile distilled water. Then, the colony of the studied bacteria was picked by using a

sterile swab or loop and smeared on the filter paper. In the case of oxidase-positive bacteria, dark blue or purple colour (indophenols) could be noticed in the inoculated area within 10 -30 seconds. In the case of oxidase-negative bacteria, no colour change was noticed, as shown in Figure 3-15.

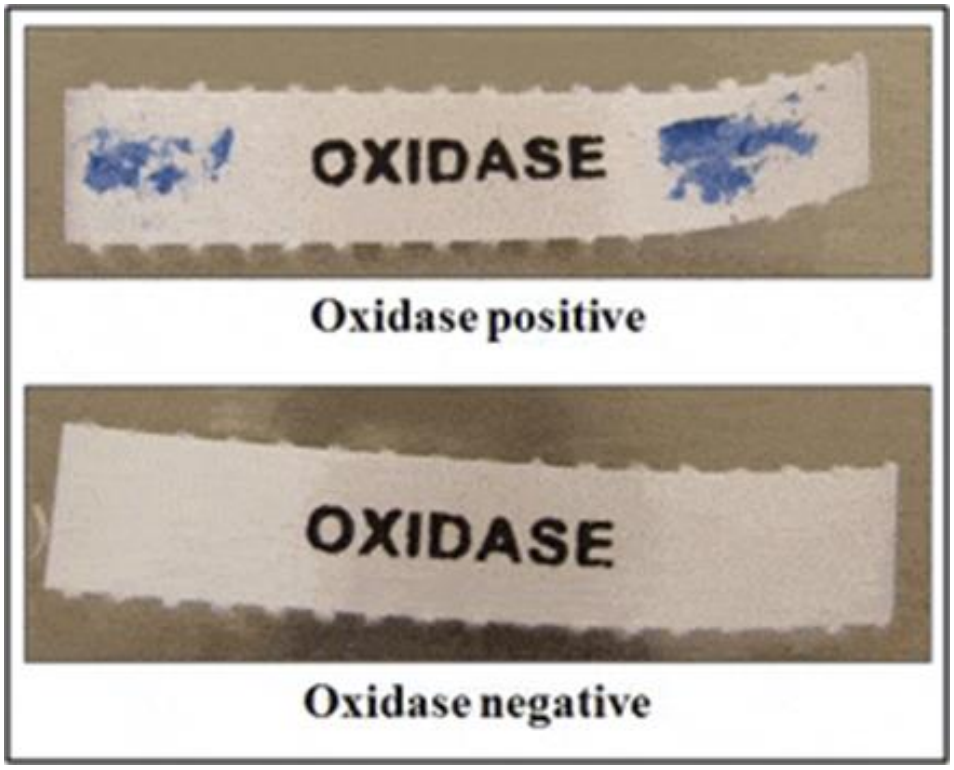

Figure 3-15. Oxidase test.

3.3.2.7.4 Hydrogen Sulphide (H_2S) Production Test

hydrogen sulphide (H_2S) production test is used to determine whether bacteria reduce sulphur-containing compounds to sulphides during the process of metabolism. To test the production of hydrogen sulphide (H_2S), the culture was inoculated in two test tubes containing sulphite indole motility media, represented in Table 3-9, and incubated at 37°C for 24-48 h; one control was also maintained. After the incubation was over, the tubes were observed for the formation of black precipitate on the media. The black colour refers to the presence of hydrogen sulphide, which means that the studied bacteria can produce hydrogen sulphide (H_2S positive). If not, the studied bacteria cannot produce hydrogen sulphide (H_2S negative), as shown in Figure 3-16.

Table 3-9. The composition of sulphite indole motility medium.

Components	Quantity (g/l)
Peptone	30
Beef extract	3.1
ferrous ammonium sulphate	0.2
Sodium thiosulfate	0.025
Agar	3.0
pH at 25ºC	7.3 ±0.2

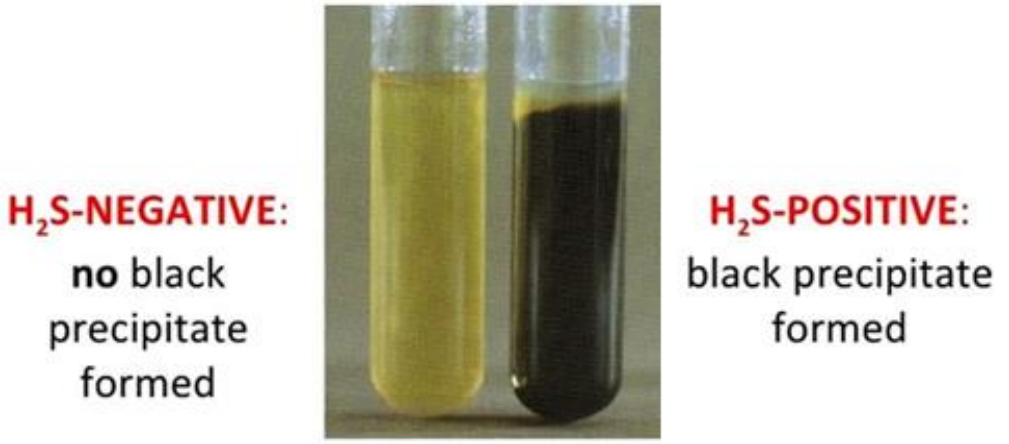

Figure 3-16. Hydrogen Sulphide (H_2S) Production Test

3.3.2.7.5 Oxidation-Fermentation (OF) Test

The oxidative-fermentative (OF) test was used to differentiate between oxidative bacteria that produce acid from carbohydrates under aerobic condition only and fermentative bacteria that produces acid both under aerobic and anaerobic conditions. To perform this test, the culture was inoculated in two test tubes of Hugh and Leifson's medium. One tube was covered with a 1 cm layer of sterile mineral oil or liquid paraffin to create an anaerobic condition in the tube by preventing diffusion of oxygen, and the other tube kept open to the air. Then, both tubes were incubated at 35°C for 48 h (slow-growing bacteria may take 3 to 4 days before results can be observed). Acid production is detected in the medium by the appearance of a yellow colour. In the case of oxidative bacteria, the yellow colour could be noticed in the open tube only. In the case of fermentative bacteria, the yellow colour could be noticed in both open and closed tubes. If there was no colour change in the media

or turned to blue (negative OF result), it means that the studied bacteria were Nonsacchrolytic bacteria (Non-oxidizer/Non-fermenter), as shown in Figure 3-17.

Table 3-10. The composition of Hugh and Leifson's medium.

Components	Quantity (g/l)
Sodium chloride	5.0
Dipotassium phosphate	0.30
Peptone	2.0
Bromothymol blue	0.03
Glucose	10.0
Agar	3.0
pH at 25°C	7.1 ±0.2

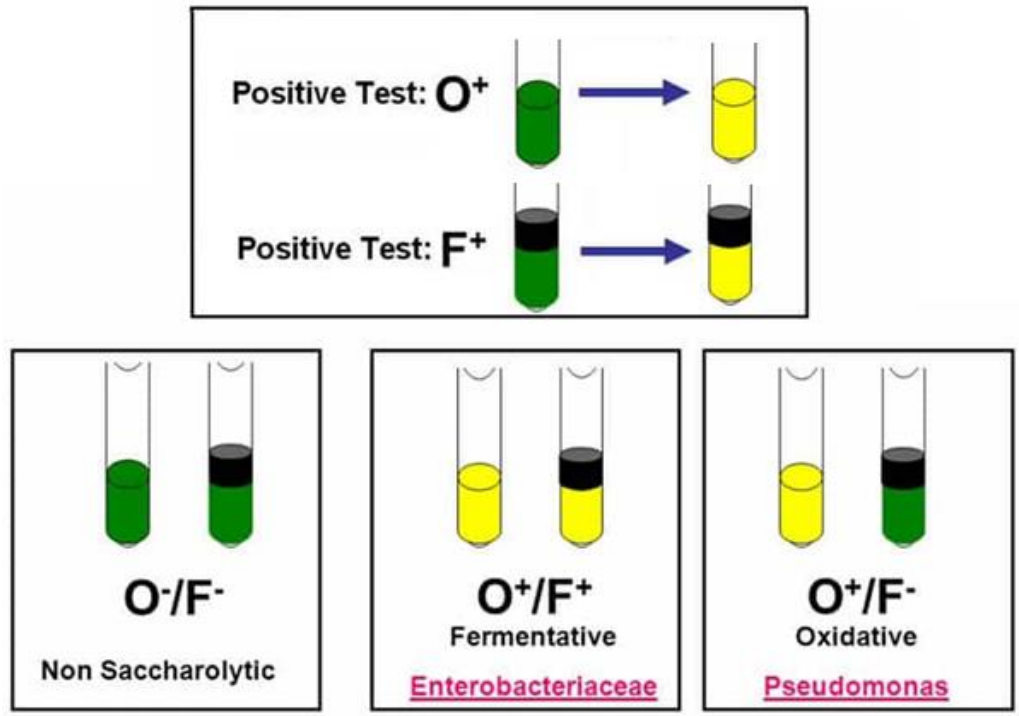

Figure 3-17. Oxidation-Fermentation (OF) Test.

3.3.2.7.6 Gelatin Hydrolysis Test

The gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefies gelatin. To perform this test, the culture was inoculated

in the tube containing nutrient gelatin medium, represented in Table 3-11 and incubated at 35°C for up to 2 weeks. Then, the tubes were removed daily from the incubator and placed in an ice bath or refrigerator at 4°C for 15-30 minutes (until control is gelled) every day to check for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes were immersed in an ice bath or kept in the refrigerator at 4°C. the tube was tilted to observe if the gelatin has been hydrolysed or not as shown in Figure 3-18.

Table 3-11. The composition of Gelatin hydrolysis media

Components	Quantity (g/l)
Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0
Gelatine	120
pH at 25°C	7.0 ±0.2

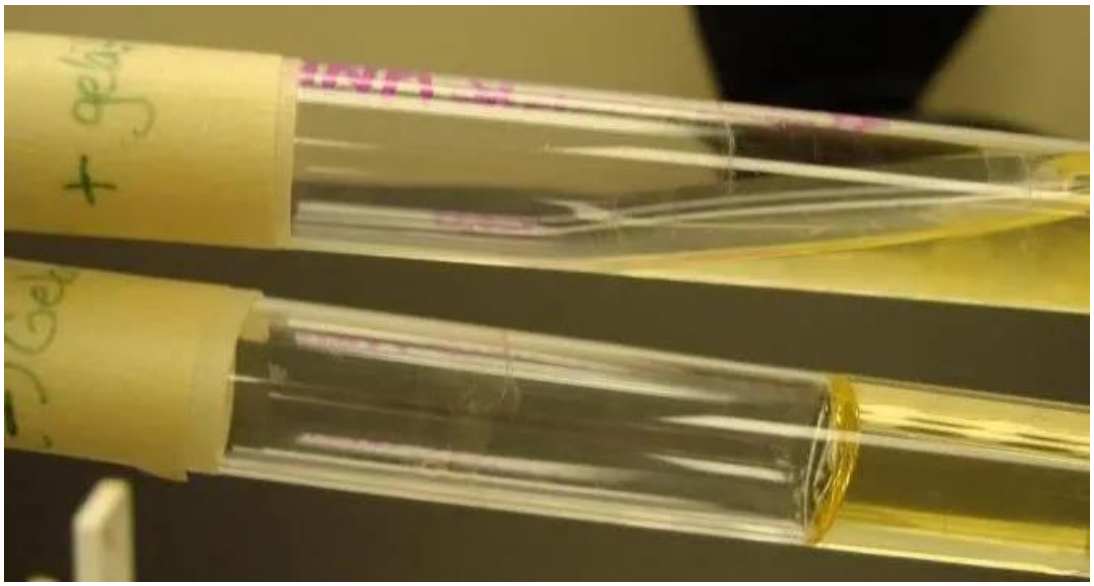

Figure 3-18. Gelatin Hydrolysis Test

3.3.2.7.7 Urease Test

The urease test is used to determine the ability of bacteria to split urea, through the production of the enzyme urease. To perform this test, the culture was inoculated in the

tubes containing urease fermentation broth medium, represented in Table 3-12, and incubated at 37°C, for 24 h; one control was also maintained. After the incubation was over, the tubes were observed for the change in colour of the media. A change in colour from red to yellow indicates a positive result, while no change in colour indicates a negative result, as shown in Figure 3-19.

Table 3-12. The composition of Urease broth medium.

Components	Quantity (g/l)
Urea	20
Sodium Chloride	5.0
Monopotassium Phosphate	2.0
Peptone	1.0
Dextrose	1.0
Phenol Red	0,012
PH at 25°C	6.7 ±0.2

Figure 3-19. Urease test

3.3.2.7.8 Nitrate Test

The nitrate test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO_3). To perform this test, the nitrate broth media, represented

in Table 3-13, were inoculated with the studied unknown bacteria and incubated at 37°C. After incubation, the existence of N_2 gas should be confirmed before adding reagents. 6-8 drops of nitrite reagent A were Added. Then, the same number of drops of nitrite reagent B were added. A reaction should be noticed within a minute or less. If either nitrite or N_2 gas has not been seen, some powdered zinc should be added. The red colour that developed after the addition of zinc could be noticed after at least 3 minutes, as shown in Figure 3-20 (Ribn, 2012).

Table 3-13. The composition of nitrate broth medium.

Components	Quantity (g/l)
Peptone	5.0
Beef extract	3.0
Potassium nitrate	5.0
PH at 25°C	7.2 ±0.2

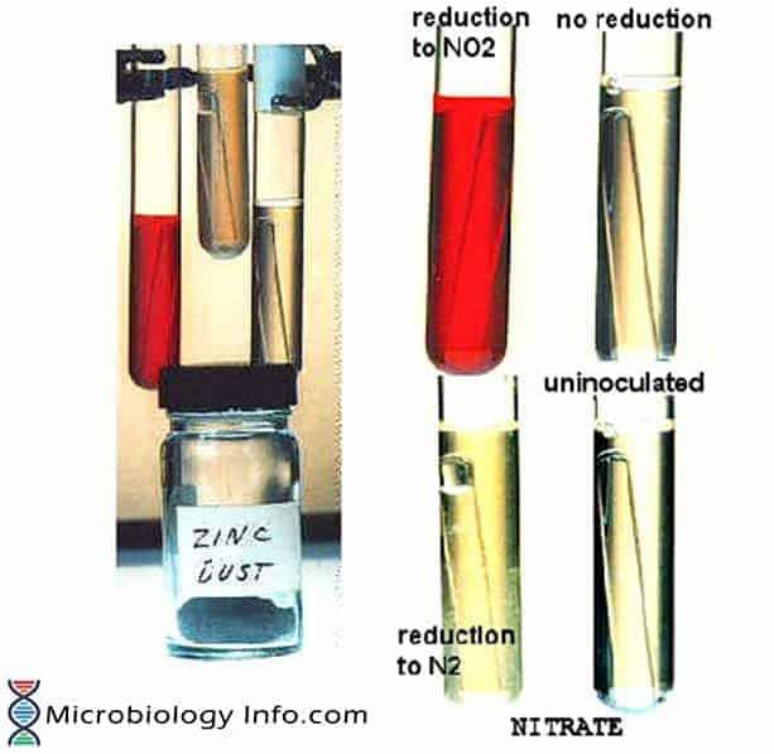

Figure 3-20. Nitrate Test.

3.3.2.7.9 Starch Hydrolysing Test

Starch hydrolysis test is used to differentiate members of various genera which have both amylase-positive and amylase-negative species, including *Bacillus, Clostridium, Corynebacterium, Fusobacterium, Enterococcus, Pseudomonas, and Streptococcus*. To perform this test, a few colonies of the studied bacteria cultures were inoculated onto a starch agar plate, represented in Table 3-14, using a sterile swab or loop and incubated at 37°C for 48 h. Then, 2-3 drops of iodine solution (10%) were added directly onto the edge of the colonies. After 10-15 minutes the results were recorded. the used iodine turned purple-black with a clear halo around the colonies in the presence of starch, which means the studied bacteria can produce α-amylase and oligo-1,6-glucosidase (starch hydrolysis positive). If the purple-black colour appeared in the medium, right up to the edge of the isolated colonies this means that the studied bacteria cannot produce α-amylase and oligo-1,6-glucosidase (starch hydrolysis negative).

Table 3-14. The composition of Starch agar media

Components	Quantity (g/l)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Yeast extract	1.5
Beef extract	1.5
Soluble starch	2.0
Agar	15
pH at 25ºC	7.4 ±0.2

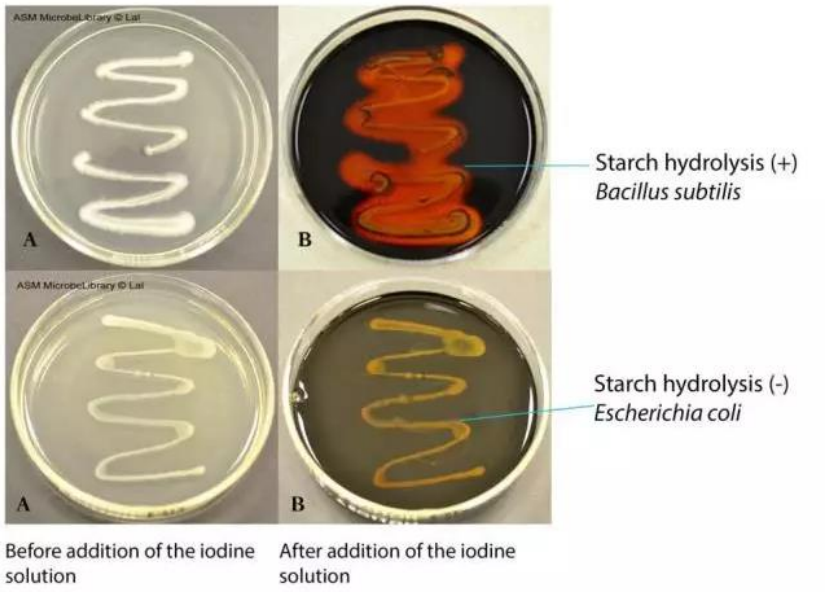

Figure 3-21. Starch Hydrolysing Test.

3.3.2.7.10 Mannitol Fermentation Test

Mannitol fermentation test is used to see if the studied bacteria could ferment the carbohydrate (sugar) mannitol as a carbon source to produce acid end products. To perform this test, the culture was inoculated in the tubes containing mannitol fermentation broth, represented in Table 3-15, and incubated at 37ºC, for 24 h; one control was also maintained. After the incubation was over, the tubes were observed for the change in colour of the media. A change in colour from red to yellow indicates a positive result, while no change in colour indicates a negative result as shown in Figure 3-22.

Table 3-15. The composition of Mannitol fermentation broth medium.

Components	Quantity (g/l)
Agar	15.0
Sodium chloride	7.5
Mannitol	10
Peptone	10
Beef extract	1.0
Phenol red	0.025
pH at 25ºC	7.4 ±0.2

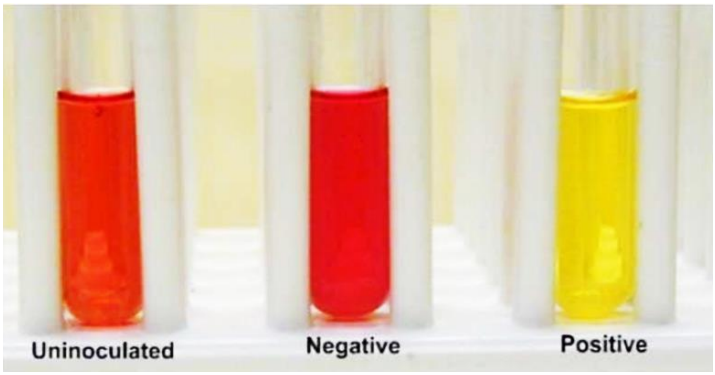

Figure 3-22. Mannitol fermentation Test.

3.3.2.7.11 Glucose Fermentation Test

The glucose fermentation test is used to determine the way bacteria metabolize a carbohydrate. The purpose of this test is to see if the studied bacteria can ferment the carbohydrate (sugar) glucose (also known as dextrose) as a carbon source to produce acid end products, with a drop in the pH of the medium. To perform this test, the studied bacteria culture was inoculated in the tubes containing phenol red glucose broth, represented in Table 3-16; one control was also maintained. Then, Durham tubes were transferred to see whether bacteria were aerobic or anaerobic. After that, the tubes were covered by a cotton plug and placed in an incubator at 37^{o}C for 24 h. Finally, the tubes were observed for the change in the colour of the media. A change in colour from red to yellow indicates a positive result while no change in colour indicates a negative result, as shown in Figure 3-23.

Table 3-16. The composition of phenol red glucose broth medium.

Components	Quantity (g/l)
Agar	15.0
Sodium chloride	7.5
Glucose	10
Peptone	10
Beef extract	1.0
Phenol red	0.025
pH at 25^{o}C	7.4 ±0.2

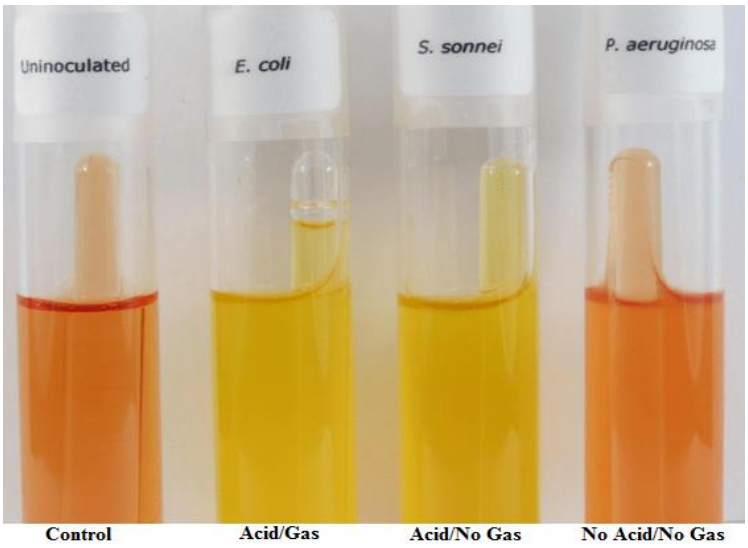

Figure 3-23. Glucose Fermentation Test.

3.3.2.7.12 Lactose Fermentation Test

The lactose Fermentation Test is used to examine if the studied bacteria could ferment the carbohydrate (sugar) lactose as a carbon source to produce acid end products, with a drop in the p*H* of the medium. To perform this test, phenol red lactose broth medium, represented in Table 3-17, were prepared. The medium was a nutrient broth, represented in Table 3-17, to which 0.5-1.0% lactose was added. If lactose is fermented to produce acid-end products, the pH of the medium will drop. A p*H* indicator in the medium changes colour to indicate acid production, as shown in Figure 3-24.

Table 3-17. The composition of phenol red glucose broth medium.

Components	Quantity (g/l)
Agar	15.0
Sodium chloride	7.5
Lactose	10
Peptone	10
Beef extract	1.0
Phenol red	0.025
pH at 25ºC	7.4 ±0.2

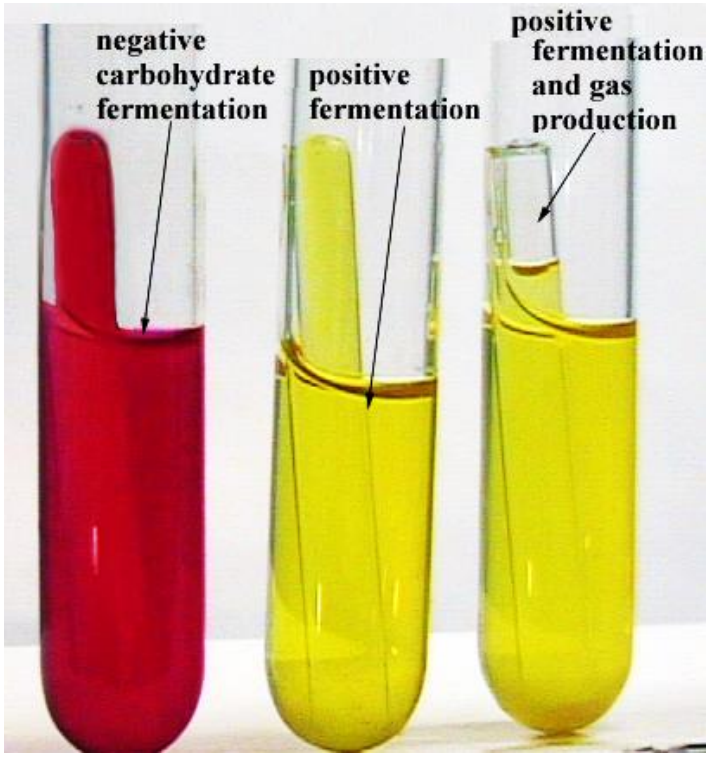

Figure 3-24. Lactose Fermentation Test.

3.3.3 Biosurfactant Production

Selected bacterial strains were examined for their potential to produce biosurfactants using a new proposed nutrient medium nominated "H" and other 10 different reported nutrients media for bacilli species, nominated N1 (Randhir S. Makkar and Cameotra, 1997), N2 (Al-bahry et al., 2013), N3 (Joshi et al., 2008b), N4 (Soumen Mukherjee et al., 2009), N5 (Joshi et al., 2008a), N6 (Landy et al., 1948), N7 (Jenny et al., 1991), N8 (Cooper et al., 1981), N9 (Joshi et al., 2008c), and N10 (Youssef et al., 2007), for making comparative analysis to reach the optimum surface activity of produced biosurfactants. The chemical composition of the nutrient's media is listed in Table 3-18. The reported nutrients media used different carbon sources at different concentrations, which were date molasses, cane molasses, glucose, and sucrose. The carbon source for the new proposed nutrient medium H was glucose (20 g/l). the media were sterilized (120 ^{o}C for 20 min) in an autoclave, then sterilized trace elements were added, and the pH value were adjusted to 7 by adding sterilized 6 N NaOH.

Table 3-18. The compositions of the 10 different reported nutrient media used for Bacilli species and the new proposed medium 'H'.

Composition (g/l)	Nutrient										
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	H
Cane Molasses	80	-	50	-	-	-	-	-	-	-	-
Date Molasses	-	80	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	10	20	-
Glucose	-	-	-	10	11	20	34	40	-	-	20
NH_4NO_3	-	3.3	-	-	-	-	1	4	-	3.3	5
Na- Glutmate	-	-	-	-	-	5	-	-	-	-	4
$NaNO_3$	-	-	-	2.8	4.4	-	-	-	-	-	3
$K2HPO_4$	-	2.2	-	-	-	-	-	-	13.9	2.2	-
KH_2PO_4	1.4	0.14	-	-	-	1	6	4.08	2.7	0.14	4
$Na2HPO_4$	2.2	-	-	-	-	-	2.7	7.12	-	-	6
$MgSO_4.7H_2O$	0.6	0.6	-	0.2	0.8	0.5	0.1	0.2	0.25	0.6	0.3
$FeSO_4.7H_2O$	0.02	0.2	-	0.0003	8	0.15	0.00165	0.0011	0.1	0.2	0.1
$MnSO_4.4H_2O$	-	-	-	0.0002	2.2	0.005	0.001	0.00067	3	-	1.78
KCl	-	-	-	0.5	0.4	-	-	-	-	-	-
$CaCl_2$	0.04	0.04	-	0.03	0.27	-	0.0012	0.00077	0.1	0.04	0.1
Na-EDTA	-	-	-	0.2	30	-	0.000745	0.00148	1	-	-
H_3PO_4 (85.4%)	-	-	-	2ml	1	-	-	-	-	-	-
$CuSO_4$	-	-	-	-	-	0.16	-	-	-	-	-
Yeast Extract	-	-	-	-	-	1	-	-	1	-	1
NaCl	0.1	0.01	-	-	-	-	-	-	50	0.01	-
$(NH_4)_2SO_4$	3	-	-	-	-	-	-	-	1	-	1
KNO_3	-	-	-	-	-	-	-	-	-	-	3
Urea	-	-	-	-	-	-	-	-	-	-	1
Trace elements	-	0.5ml[a]	-	-	10ml[b]	-	-	-	10ml[c]	0.5ml[a]	1ml[d]

[a] $ZnSO_4.7H_2O$, 2.32; $MnSO_4.4H_2O$, 1.78; Na-EDTA, 1.0; $CuSO_4.5H_2O$, 1.0; KI, 0.66; H_3BO_3, 0.56; $CoCl_2.6H_2O$, 0.42; $NiCl_2.6H_2O$, 0.004; and $Na_2MoO_4.2H_2O$, 0.39.

[b] $CoCl_2$, 1.0; $CuSO_4$, 0.6; Na_2MoO_4, 0.5; H_3BO_3, 0.25; and $ZnSO_4$, 6.0.

[c] $ZnSO_4.7H_2O$, 0.1; Na_2MoO_4, 0.1; $CuSO_4.5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; Na_2MoO_4, 0.01; H_3BO_3, 0.01; Na2MoO4, 0.1; Na_2SeO_4, 0.005, and $NiCl_2.6H_2O$, 0.003.

[d] $ZnSO_4.7H_2O$, 2.28; $CuSO_4.5H_2O$, 1.14; H_3BO_3, 0.61; $CoCl_2.6H_2O$, 0.47; and $Na_2MoO_4.2H_2O$, 0.42.

3.3.3.1 Surface Activity

The surface activity of the biosurfactants produced by the selected bacterial strains was studied by measuring their Surface Tension ST and Interfacial Tension IFT, by applying the rod method using the EZ Tensiometer (Model 201, USA), shown in Figure 3-25. All measurements were performed in triplicate at ambient temperature (25±2 ^{o}C) and atmospheric pressure (1 atm), and the average values were reported.

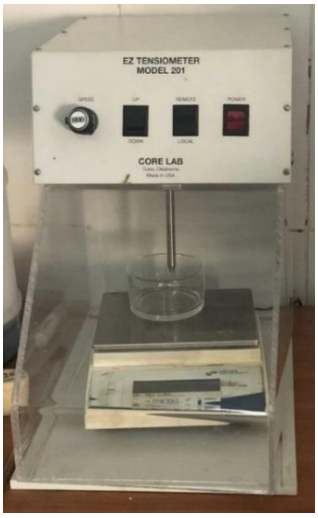

Figure 3-25. EZ Tensiometer (Model 201, USA).

3.3.3.2 Bacterial Growth

The bacterial growth rate was estimated using the optical density method. A JASCO spectrophotometer (V-630, Japan), shown in Figure 3-26, was used to estimate the bacterial growth rate in culture media. The optical density was measured at a wavelength of 600 nm, which is recommended for estimating the bacteria concentration (Joshi et al., 2008a).

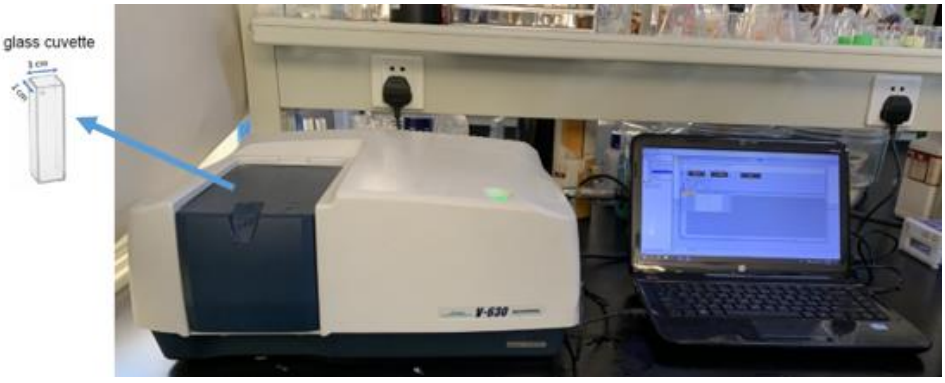

Figure 3-26. JASCO spectrophotometer (V-630, Japan).

3.3.3.3 Biosurfactant Extraction and Purification

There are several methods for biosurfactant extraction and purification. The method used for extracting and purifying the produced biosurfactant from bacteria culture media was based on acid precipitation (Joshi et al., 2008a). 100 ml of culture media was centrifuged using a Sigma centrifuge machine (Model 2-16KL, Germany), shown in Figure 3-27, at 10,000 rpm for 20 minutes in terms of settling the bacterial cells. The pH of the cell-free media was adjusted to 2.0 by adding 6N HCl to precipitate the produced biosurfactant, and the cell-free media were kept at 4^{o}C for 24 h. The precipitates were collected by centrifugation at 12,000 rpm for 30 minutes at 4^{o}C. The collected precipitates were dissolved in distilled water and the pH value was adjusted to 8 by using 6N Na/OH, and finally lyophilized and weighed.

Figure 3-27. Sigma centrifuge machine (Model 2-16KL, Germany).

3.3.3.4 Critical Micelle Concentration CMC

Critical Micelle Concentration CMC is an essential characteristic for surfactants because once reaching a CMC value, there is no more reduction in surface tension by adding more biosurfactant. CMC was estimated by plotting a graph between the surface tension versus the concentration of biosurfactant. Several solutions of extracted biosurfactant with concentrations ranging from 0.01 to 0.1 g/l were prepared in distilled water, and the change of surface tension was observed using the Rod Tensiometer (Joshi et al., 2008a).

3.3.3.5 Emulsification activity

Emulsification activity is the ability of surface-active molecules to form a stable emulsion. The ability of produced biosurfactants to emulsify different hydrocarbons such as hexane, heptane, hexadecane, kerosene, and crude oil was estimated by measuring the emulsification index E_{24}. The emulsification index E_{24} was measured by adding 2 ml of hydrocarbon to 2 ml of biosurfactant aqueous solution at CMC in a sterile test tube, vertexing for 2 minutes, and then keeping at 25°C for 24 h. E_{24} was calculated using Equation (3-1) (Pereira et al., 2013).

$$E_{24}\ (\%) = \frac{Emulsion\ Layer's\ Height\ (mm)}{Total\ Mixture's\ Height\ (mm)} \times 100 \qquad (3\text{-}1)$$

3.3.3.6 Stability Studies

The stability of the produced biosurfactant was examined over a wide range of temperatures, salinity, and pH to examine the effectiveness of the produced biosurfactants at harsh reservoir conditions, and determine the optimum temperature that maximizes the biosurfactant activity (Joshi et al., 2008a).

3.3.3.6.1 Effects of Temperature on Biosurfactant Stability

The thermal stability of biosurfactants is a significant property for their commercial application at extreme temperatures. Therefore, the stability of the produced biosurfactant was examined over a wide range of temperatures (30-90°C) at pH 7, and 0% (w/v) NaCl by measuring ST, IFT, and E_{24} of the biosurfactant aqueous solution at CMC at different temperatures.

In addition, the long-term thermal stability of the produced biosurfactants at high temperatures was also examined by measuring emulsion stability (ES, %) of the produced biosurfactants at the temperature of fields of interest at time intervals (1, 24, 48, 72, 96, 120, 144 and 168 h). The oil-water emulsions were formed in sterile test tubes by adding 2 ml of crude oil of the fields of interest to 2 ml of biosurfactant aqueous solution at CMC and then vertexing for 2 mins. The sterile tubes were then kept in the oven at the temperature of fields of interest for 1 h before measuring the relative emulsion volume (EV, %) and emulsion stability (ES, %) at time intervals (1, 24, 48, 72, 96, 120, 144 and

168 h). The EV (%) and ES (%) were then calculated using Equations (3-2), and (3-3), respectively (Das et al., 1998).

$$EV\ (\%)\ = \frac{Emulsion\ volume\ (ml)}{Total\ liquid\ Volume\ (ml)} \times 100 \quad (3\text{-}2)$$

$$ES\ (\%) = \frac{EV_t}{EV_0} \times 100 \quad (3\text{-}3)$$

Where EV_t is the emulsion volume (%) after the time (t) and EV_0 is the emulsion volume (%) at the time (0).

3.3.3.6.2 Effects of Salinity on Biosurfactant Stability

The salinity effect on surface activity was studied by measuring ST, IFT, and E_{24} of the biosurfactant aqueous solution at CMC at different NaCl concentrations (0-20% w/v) at 25^{o}C, and pH 7 by measuring ST, IFT, and E_{24} of the biosurfactant aqueous solution at CMC at different NaCl concentrations.

3.3.3.6.3 Effects of pH on Biosurfactant Stability

A pH range (2-12) was adjusted, using Jenway pH meter (Model 3505, UK), shown in Figure 3-28, by adding 6N NaOH or 6N HCl to investigate the pH effect on the stability of the biosurfactant by measuring ST, IFT, and E_{24} of the biosurfactant aqueous solution at CMC at 25^{o}C, and 0% (w/v) NaCl concentration.

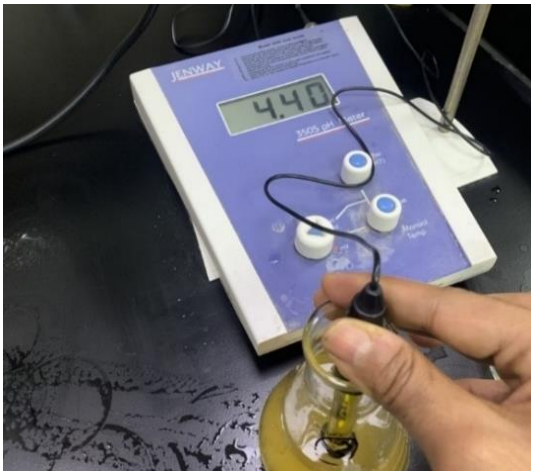

Figure 3-28. Jenway bench pH meter (Model 3505, UK).

3.3.4 Wettability Alteration

Wettability alteration plays a very important role in oil recovery, as altering reservoir rock from oil-wet to water-wet improves water displacement of oil. Recently, wettability alteration has been proposed as one of the mechanisms of MEOR. The contact angle is considered the most recommended method of measuring the natural wettability of a reservoir rock. When a drop of liquid is placed on a surface submerged in another liquid, a contact angle will be formed and ranging from 0^o to 180^o. Contact angle measurements are generally classified into three categories, less than 90^o indicates that the surface is water-wet (hydrophilic) with a higher affinity for water than oil, around 90^o indicates that the surface is intermediate-wet with a neutral affinity for both phases, or greater than 90^o indicates that the surface is oil-wet (hydrophobic) with a higher affinity for oil than water as shown in Figure 3-29.

This study investigated the effect of the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* and also the effect of the new proposed medium "H" alteration on wettability alteration of sandstone surface using the contact angle measurement method shown in Figure 3-29 (Kowalewski et al., 2006; Sayyouh and Al-Blehed, 1995; Zargari et al., 2010; Zekri et al., 2003). The oil drop was equilibrated in contact with the downward of the sandstone rock surface submerged in the aqueous solution of the produced biosurfactants at CMC and in the new proposed nutrient broth medium "H" at different time intervals (6, 12, 24 h) to evaluate the effect of produced biosurfactants and bacterial cells existing already in the oil drop itself in altering the wettability by changing contact angle measurement as shown in Figure 3-30.

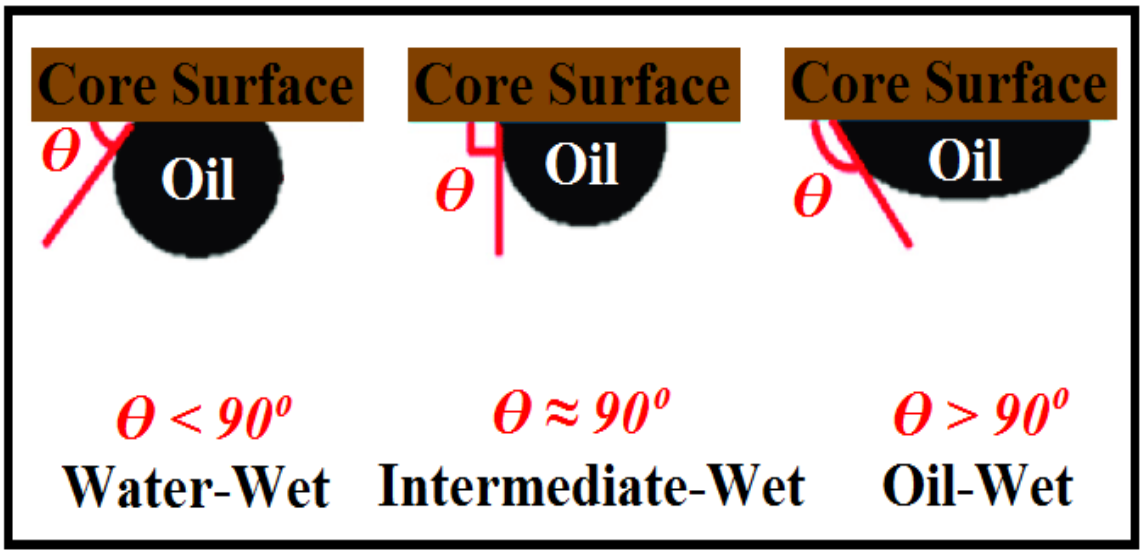

Figure 3-29. Schematic Diagram of wettability classification based on contact angle measurement.

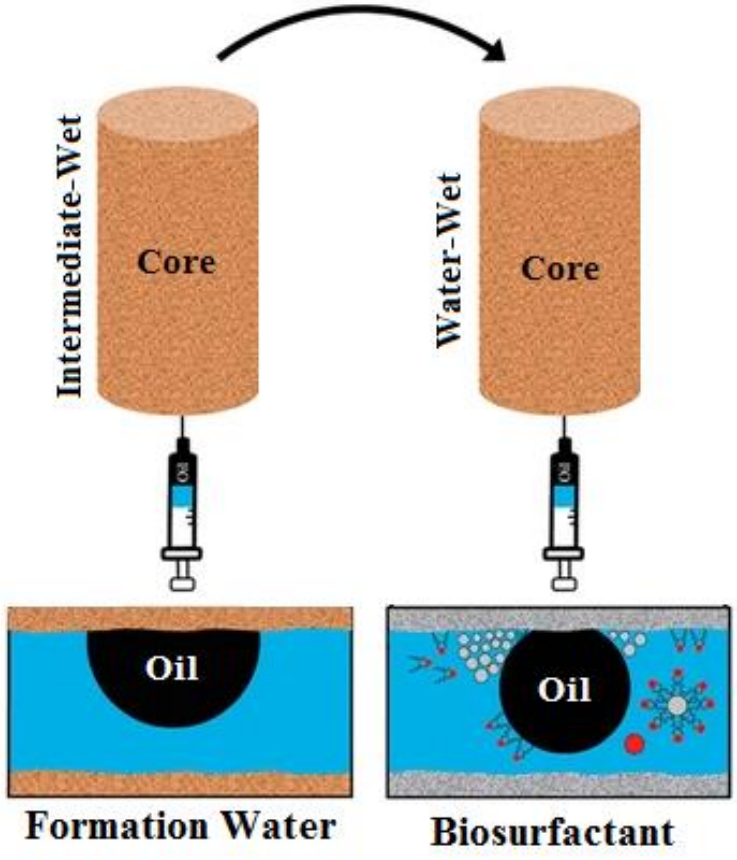

Figure 3-30. Schematic Diagram of the contact angle measurement method.

3.3.4.1 Experimental Apparatus and Materials

The contact angle experiments were conducted using the pendant drop method for the profile of the oil drop in biosurfactant aqueous solution at CMC. In this study, the contact angle of a sessile drop of crude oil samples obtained from the fields of interest was measured on sandstone core samples of the field of interest submerged in the aqueous solution of the biosurfactant produced from the selected bacterial strains isolated from the field of interest at CMC and in the new proposed nutrient broth medium "H" using the goniometer as shown in Figure 3-31. The images of the oil pendant drop were recorded using a goniometer consisting of a Leica Wild stereo microscope (Model M3Z, Switzerland) and a JVC colour video camera (Model TK-C1381, Japan). The shape of an oil drop was analysed by First Ten Angstroms Incorporated Drop Shape Analysis Software Version 2.0 to estimate the contact angle as shown in Figure 3-32.

3.3.4.2 Materials

The materials used for performing the waterflooding /biosurfactant flooding experiments were mentioned below:

(A) Core plugs obtained from the fields of interest
(B) Crude oil samples collected from the fields of interest
(C) Formation water samples collected from fields of interest
(D) Produced biosurfactants at CMC
(E) The new proposed nutrient broth medium "H"

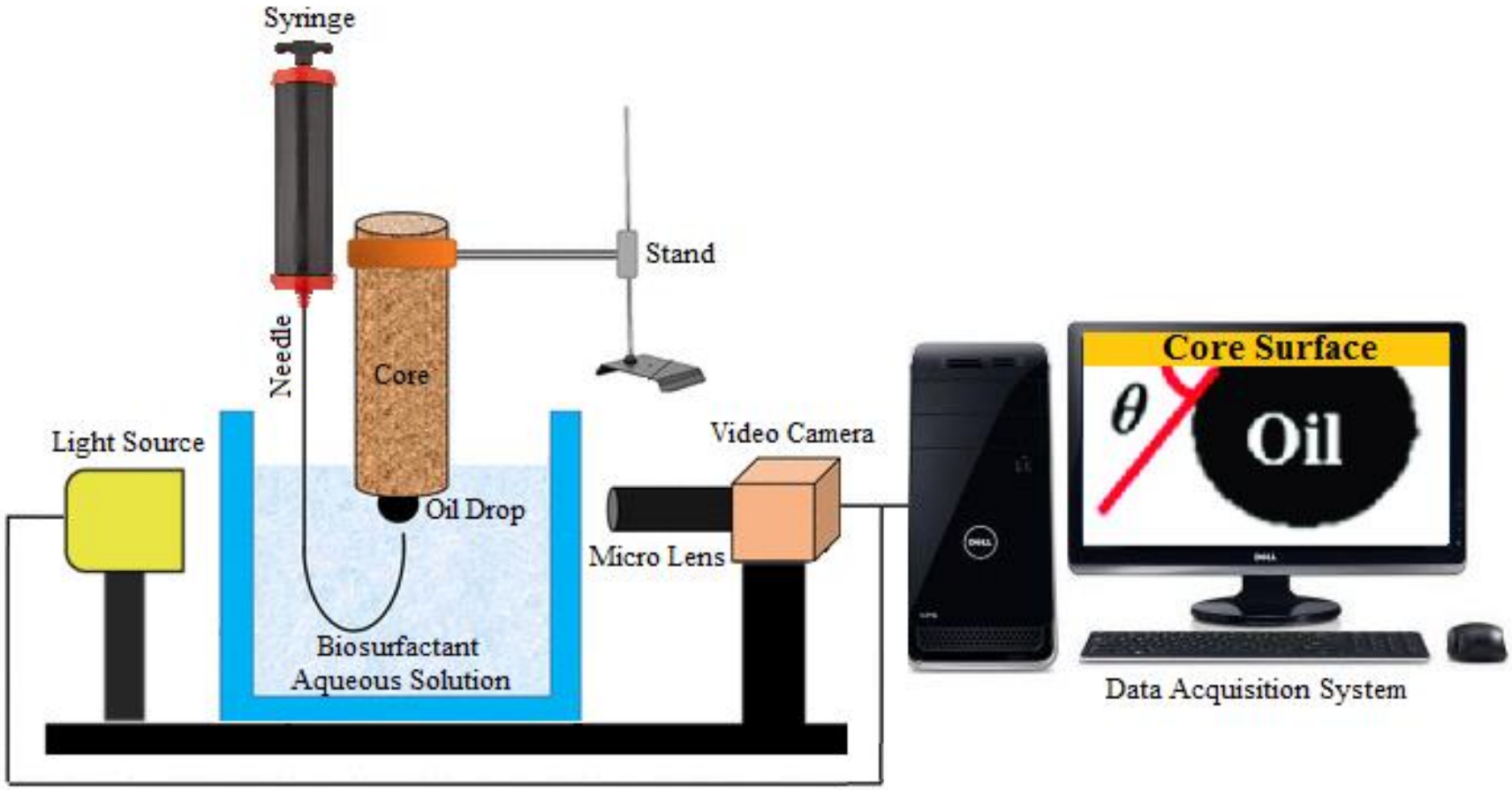

Figure 3-31. Schematic Diagram of the pendant drop visual cell set-up.

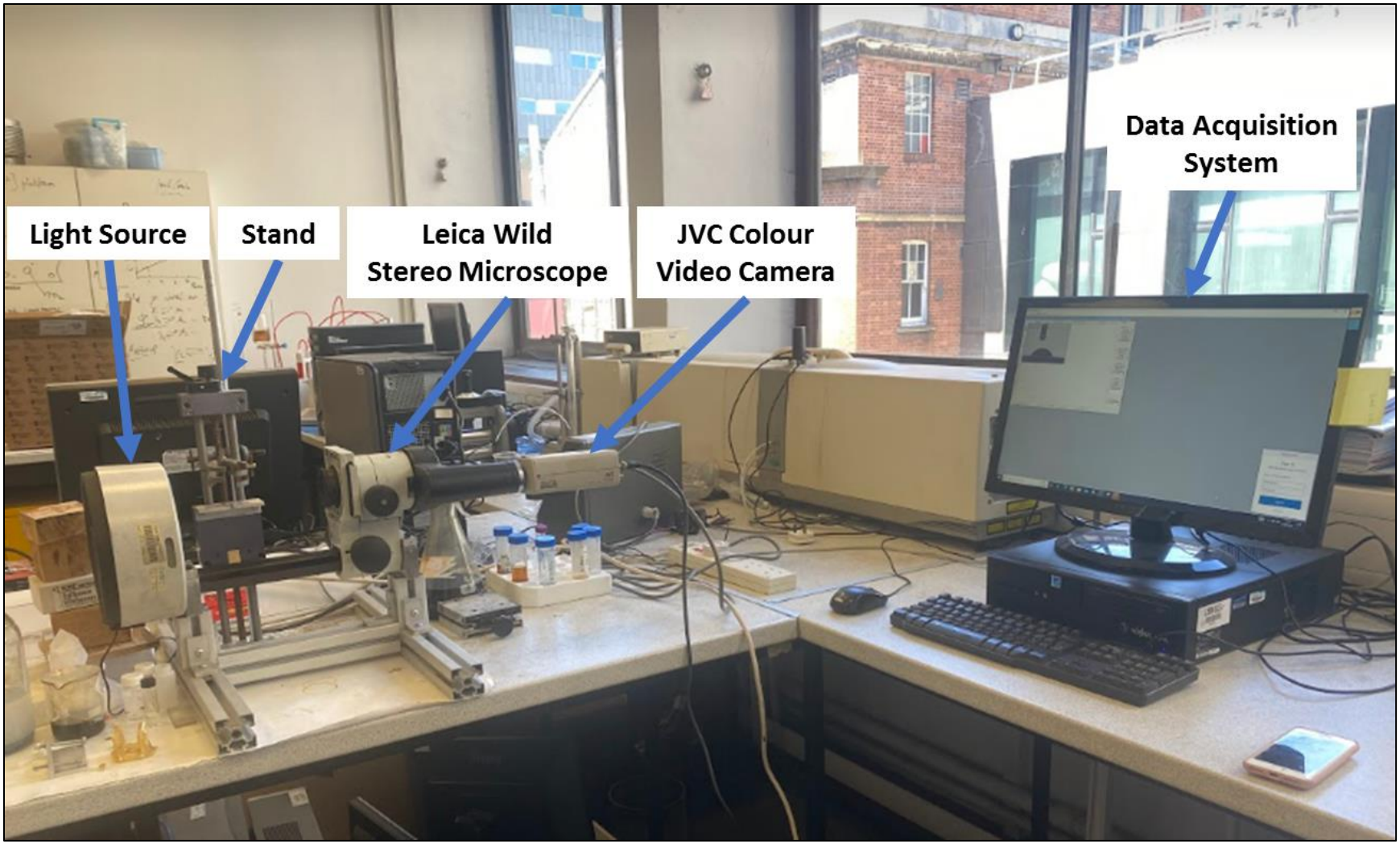

Figure 3-32. The complete set-up of the "pendant drop" visual cell for measuring the contact angle.

3.3.5 Examination of Oil Recovery using Core Flooding

The core flooding tests were carried out to investigate the potential of the produced biosurfactants by the selected indigenous bacterial strains in improving oil recovery. In this section, a series of oil displacement experiments were conducted on sandstone core samples in two stages, water flooding as a secondary recovery stage using the formation water of the fields of interest, and the biosurfactants flooding as a tertiary "enhanced" oil recovery stage using biosurfactants produced by the selected indigenous bacterial strains at simulated reservoir conditions. In addition, the core flooding experimental trials were conducted at different flow rates of 0.25 cm^3/min, 0.50 cm^3/min, and 0.75 cm^3/min to obtain the optimum flow rate that could maximize the oil recovery.

3.3.5.1 Experimental Set-up Apparatus

Core floods are the most representative experiments that can be conducted for reservoir conditions. Water/biosurfactant core floods is influenced by core plugs, pressure, temperature, biosurfactant, formation water, injection strategy, oil saturation, and a few more properties.

In this part of experimental study, a series of core flood tests were carried out for both biosurfactant and the new proposed medium "H" and parameters such as reduction of interfacial tension, wettability alteration, and oil recovery.

A complete high-temperature core flooding apparatus shown in Figure 3-33 and Figure 3-34 was used to conduct the experiments. This core flooding system is located at London South Bank University (LSBU) within the Department of Chemical and Energy Engineering. The flow path of the core flooding experiment was represented in the schematic diagram. The main components of this core flooding apparatus are further explained in detail to highlight their mode of operation and their importance in effectively performing the experiments. It mainly consisted of:

(A) Core Holder
(B) Hydraulic Pump
(C) ISCO Pump
(D) Pressure Transducer

(E) Back Pressure Regulator (BPR)

(F) Floating Piston Accumulators ((FPAs)

(G) Air Bath (Oven)

(H) Data Acquisition System

(I) Collector for Sample Fraction

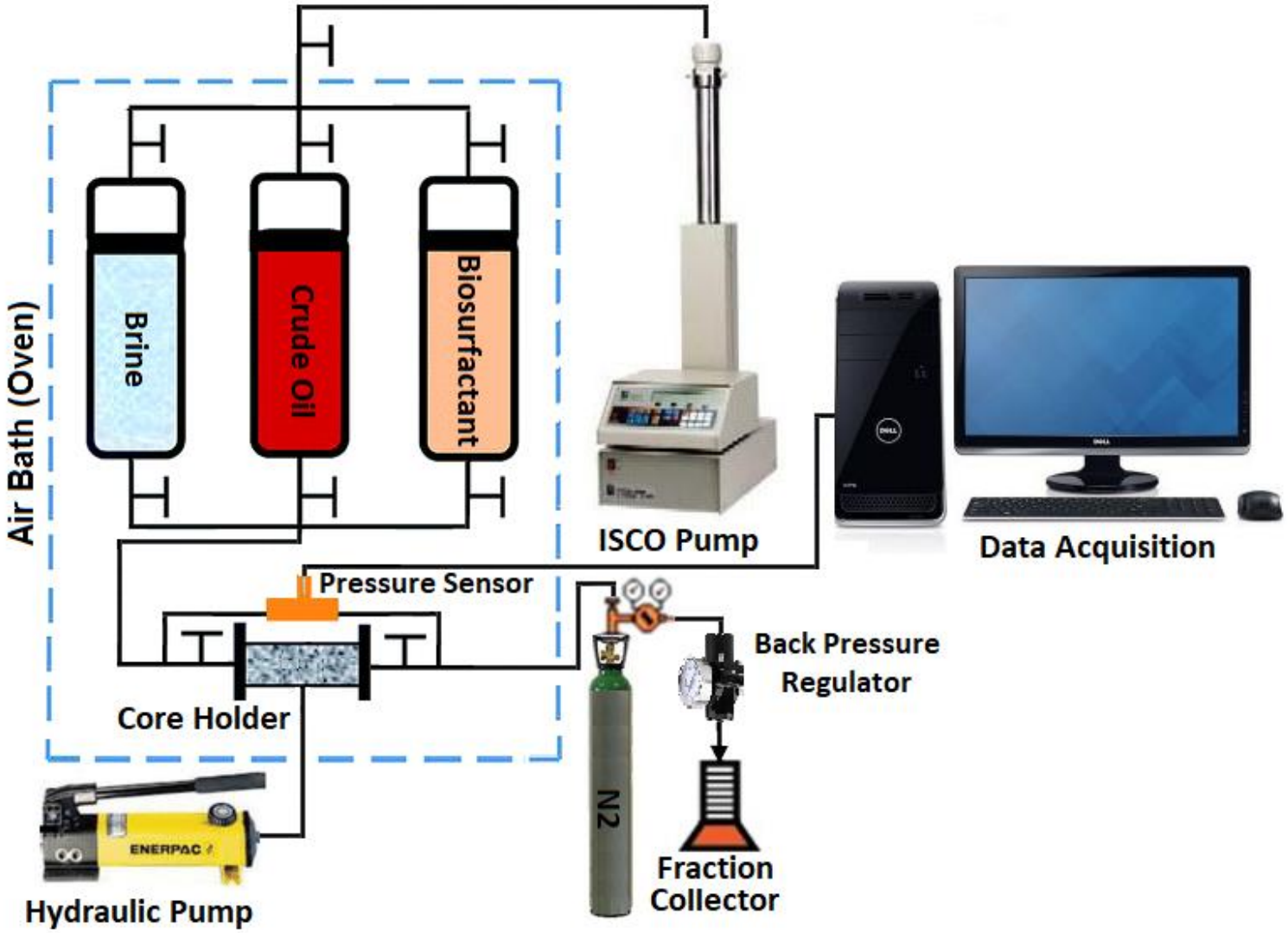

Figure 3-33.Schematic Diagram of Core Flood set-up.

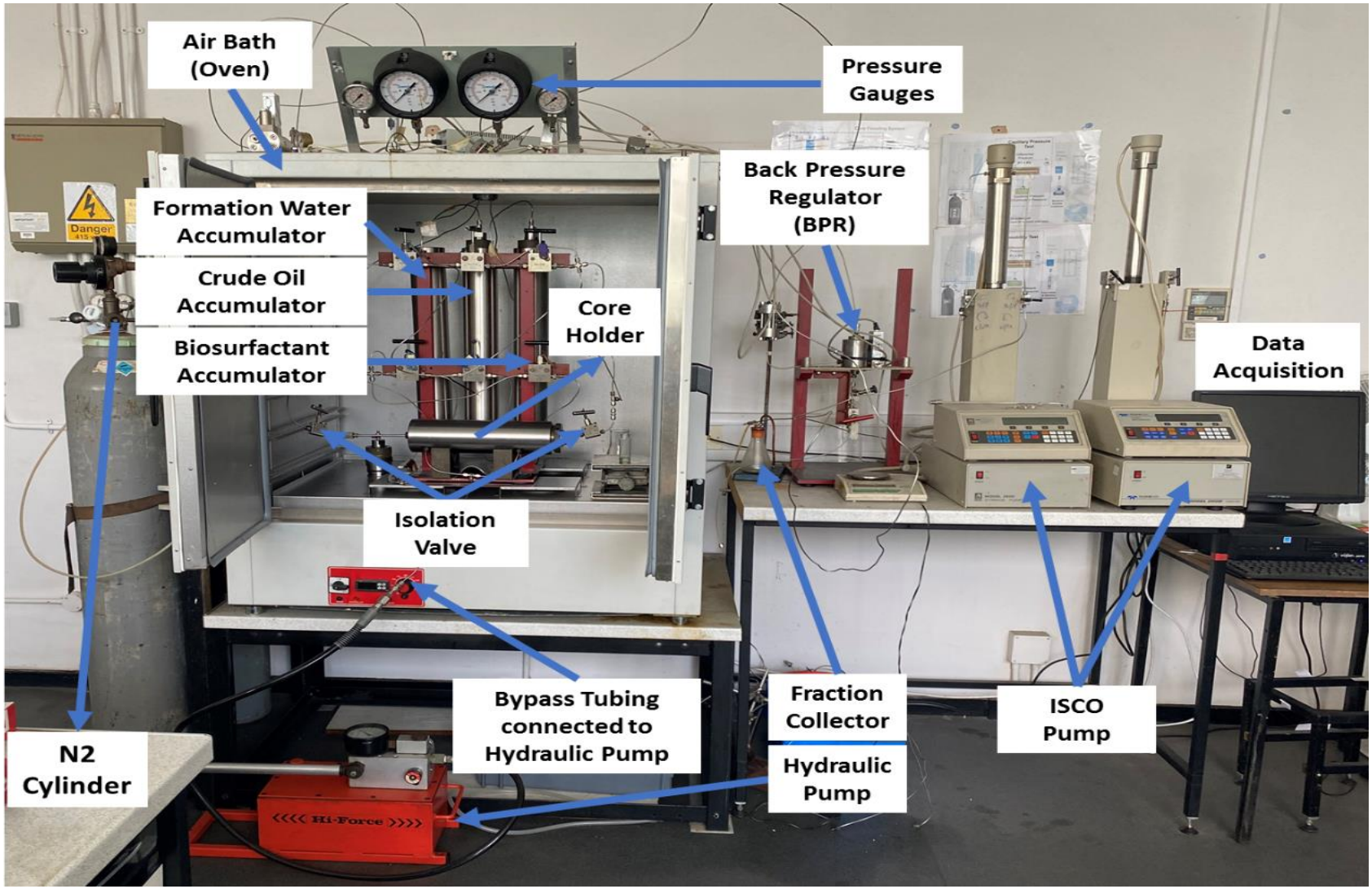

Figure 3-34. Core flood system.

(A) Core and Core Holder

The core holder used in this study has both inlet and outlet mandrels. One mandrel on the left was attached to the cap and the mandrel on the right can slide inside the core holder barrel to accommodate cores of different lengths. The dismounted core holder can be seen on the right in Figure 3-35 and shows the sleeve, a sandstone core and the two inner end pieces.

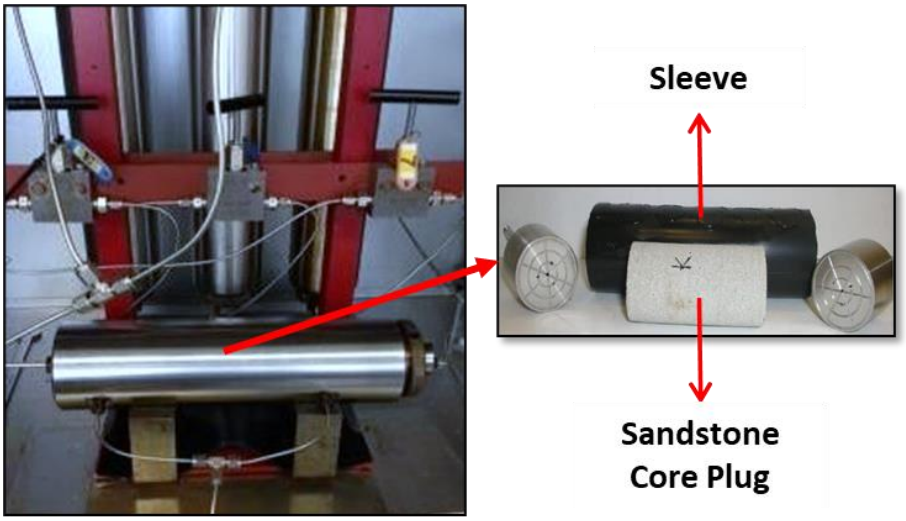

Figure 3-35. Core holder cylinder and dismantled on the right

(B) Hydraulic Pump (Confining Pressure)

After placing the core into the core holder, the sleeve was pressurised to simulate the 3D axis stresses that the core was under in real reservoir conditions. Some of these stresses are caused by the weight of the material above the core which is called “overburden” pressure. In this experiment hydraulic oil was used to provide an overburden pressure of around 13.79 MPa (Figure 3-36).

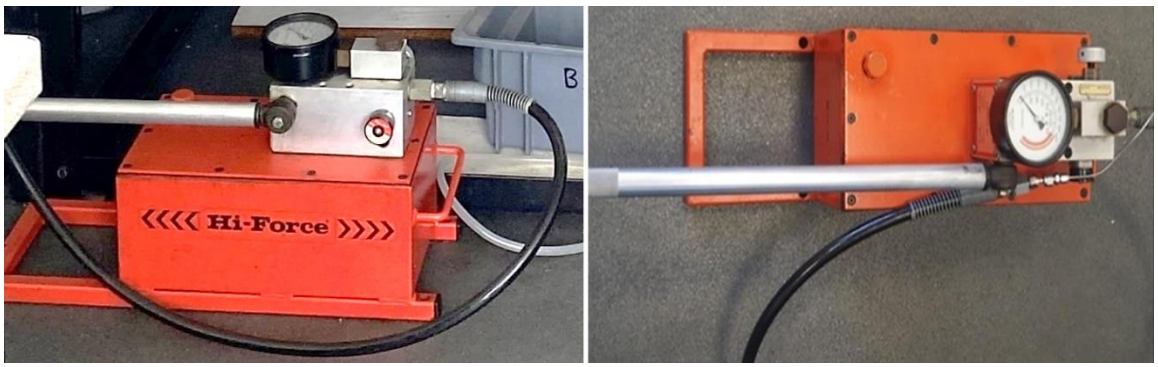

Figure 3-36. Hydraulic Pump.

(C) D-Series ISCO Pump

A Teledyne D-Series ISCO digital syringe pump (Model 500D, USA), shown in Figure 3-37, with a cylinder capacity of 266.05 cm^3 was used to inject fluids. The ISCO injection pump is capable of injecting constant rates over a wide pressure range (up to 68.95 MPa), as well as a constant pressure flow over a wide range of flow rates (0.01 cm^3/min to 100 cm^3/min). The pump injected the driving fluid (distilled water) into the accumulators placed in the oven and then the substance on the other side of the piston (formation water, crude oil, biosurfactant aqueous solution, or toluene/acetone for cleaning the system) was fed into the injection lines.

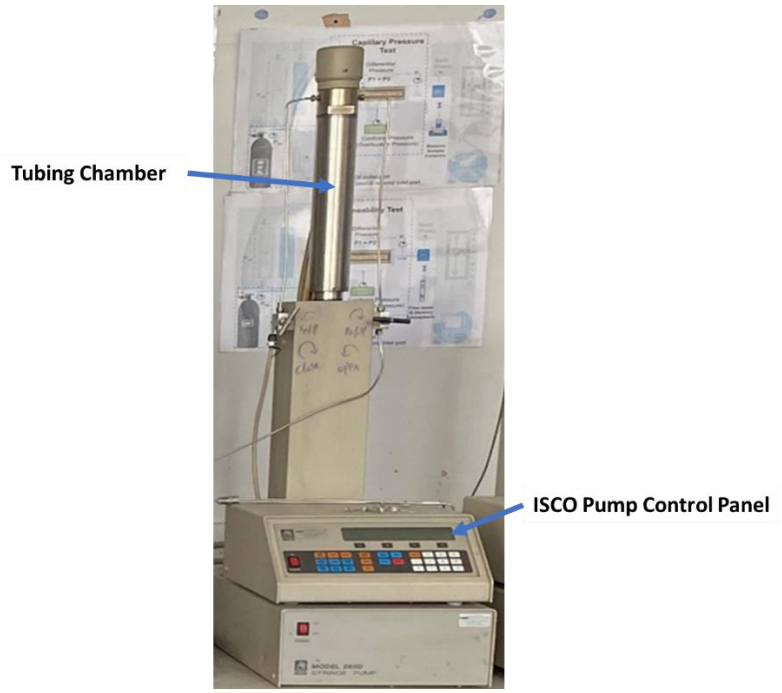

Figure 3-37. Teledyne D-Series ISCO digital syringe Pump.

(D) Pressure Transducers

Pressure transducers were used to measure the pressure of the core inlet, core outlet, pump, back pressure regulator, and overburden. One differential pressure transducer was connected to the core inlet and outlet for the measurement of pressure drop across the core. The pressure transducer and pressure gauges were supplied by Bronkhorst pressure controller Inc with a range of ±0.25% accuracy for the transducer and ±1% accuracy for pressure gauges, respectively. The pressure transducer was connected to a data acquisition system for converting the electrical signals into pressure readings.

(E) Back Pressure Regulator (BPR)

A back-pressure regulator (BPR) was used to control and maintain the pressure inside the system and reduce the pressure drop to a minimum. An accurate BPR was necessary for these experiments, as the pressure needed to remain constant throughout the flooding experiments. The BPR set the outlet pressure (P2) at a level where production did not happen until P2 was increased to that pressure. The back pressure was set manually using a nitrogen cylinder to supply the required pressure for the experiment.

(F) Floating Piston Accumulators (FPAs)

The core flooding system used in this study has three accumulators for injecting fluids and the capacity of each one is 1000 cm^3 as shown in Figure 3-38. The accumulators are cylinders equipped with two end plugs and one floating piston, separating the cylinder into two different chambers; a driving chamber and a test chamber. The driving chamber contained the driving fluid (distilled water) coming from the pump, while the test chamber contained the fluids that were injected into the core holder (formation water, crude oil, and biosurfactant).

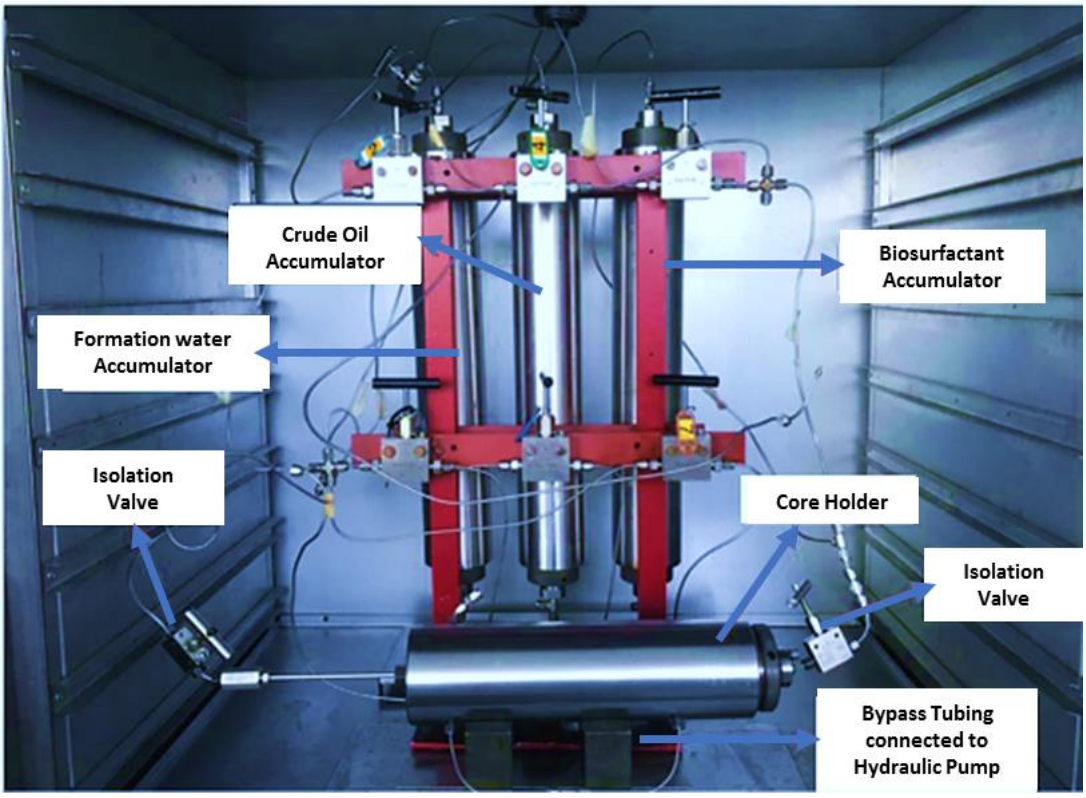

Figure 3-38. The three accumulator cylinders were used for injecting the studied fluids.

(G) Air Bath (Oven)

Measurements were taken at the reservoir temperature of the field of interest using an oven (air bath), which heated the core sample in the core holder and the fluids. The oven maintained a high temperature throughout the different processes of the experiments.

(H) Data Acquisition System

The data acquisition system consists of a personal computer and the entire hardware components and sensors of the system were controlled and monitored with this computer by providing an on-screen display of all measured values (flow rates, pressures, temperature etc.), remote control of flow rates, automatic logging of test data to a data file, alarms, calculations, and graphing.

(I) Collector

The leak-off fluids produced through the core during experimental runs were collected in 10 ml graduated tubes at the outlet of the core. The produced fluids flowed through the back-pressure regulator (BPR) and then into graduated tubes for settling before any measurements.

Materials

The materials used for performing the waterflooding /biosurfactant flooding experiments:

(F) Core plugs obtained from the fields of interest

(G) Crude oil samples collected from the fields of interest

(H) Formation water samples collected from fields of interest

(I) Produced biosurfactants at CMC

3.3.5.2 Experimental Procedure

3.3.5.2.1 Core Preparation

The core plugs were initially cleaned to evacuate any air trapped in void spaces of the core. The experimental procedure was as follows:

- The core plugs were initially cleaned by soaking in methanol for 24h using vacuum desiccators, as shown in Figure 3-39.
- Then, the core plugs were removed from methanol and exposed to dry air for 48 h.
- After cleaning the core plugs, they were submerged in distilled water for 24 h using vacuum desiccators.
- Then, the core plugs were removed and wiped off gently before measuring their wet weight.
- Finally, the core plugs were kept in a vacuum oven at 70°C for 8 h and then the dry weight was measured.
- The bulk volume was obtained by measuring the dimensions using an electronic calliper.
- After these stages, the pore volume (PV, cm^3), bulk volume (BV, cm^3), and effective porosity ($\phi_{eff.}$, %) were calculated using Equations (3-4), (3-5), and (3-6).

$$PV\ (cm^3) = \frac{Wet\ Weight\ (g) - Dry\ Weight\ (g)}{Density\ of\ Distilled\ water\ (g/cm^3)} \quad (3\text{-}4)$$

$$BV\ (cm^3) = \frac{\pi}{4} \times Core\ Diameter^2(cm) \times Core\ Length\ (cm) \quad (3\text{-}5)$$

$$\phi_{eff.}\ (\%) = \frac{PV\ (cm^3)}{BV\ (cm^3)} \times 100 \quad (3\text{-}6)$$

Figure 3-39. The apparatus used in the core saturation process.

3.3.5.2.2 Fluid Preparation

In this step, the three accumulators were filled with the appropriate fluids, as follows:

1) The first accumulator was filled with formation water of the field of interest. The formation water was filtered by a 0.45μm Millipore filter paper before use (Alramahi et al., 2005; Alshibli et al., 2006).
2) The second accumulator was filled with the crude oil of the field of interest.
3) The third accumulator was filled with a biosurfactant produced by the selected bacterial strain isolated from the field of interest.

The cell-free biosurfactant aqueous solution was prepared by filling a sterile beaker with 1000ml of formation water. The purified and extracted biosurfactant was added to the sterile beaker that was filled with the formation water at CMC. Then, a suitable magnet was dropped into the beaker and a magnetic stirrer was used to create a vortex and stirring for one hour. At this point, the biosurfactant aqueous solution was ready to be used in filling the third accumulator.

3.3.5.2.3 Initial Reservoir Condition Set-up

In this experiment, the initial reservoir condition was applied to the core plugs to determine oil recovery. The experimental procedure was as follows:

- After cleaning the core and measuring its porosity, formation water was injected at a low flow rate to estimate the formation water permeability of the core, followed by crude oil injection to establish reservoir conditions for the core.
- After filling the accumulator with the crude oil of the field of interest, all lines were attached and then the crude oil was purged into the line to make sure there is no air in the system before use.
- The confining pressure was applied to the system by closing the hand pump valve first.
- Then, the pumping of hydraulic oil was started until the confining pressure reached 13.79 MPa.
- After that, the back pressure was increased by opening the nitrogen tank valve and the regulator was adjusted to 0.2 MPa.
- All lines were entirely purged and the pressure was monitored during the experiment.
- A low flow rate of a maximum of 0.5 ml/min was applied to a homogeneous propagation of oil in the core plug for better saturation.
- Then, oil injection was carried on until achieving a constant pressure drop across the core.
- At this stage, the original oil in place (OOIP) could be indicated by measuring the volume of displaced water.

- After this, the pump was shut off and the oil valve was closed to stop any further flow of crude oil into the core.
- Initial oil saturation (S_{oi}, %) and initial water saturation (S_{wi}, %) were calculated using Equations (3-7), and (3-8), respectively.

$$S_{oi}\ (\%) = \frac{OOIP}{PV} \times 100 \tag{3-7}$$

$$S_{wi}\ (\%) = \frac{PV - OOIP}{PV} \times 100 \tag{3-8}$$

3.3.5.2.4 Reservoir Simulated Condition Set-up

the core flooding tests were performed by using sandstone cores, crude oil, formation water used in these experiments were extracted from AL FADL (WD2) and AL QADR (WD3) oil fields (fields of interest), which are the same fields from which the selected bacteria strains were harvested at the average reservoir temperature of these fields of interest to make simulated micromodels and mimic the reservoir conditions.

3.3.5.2.5 Water Flooding

The water flooding test was further carried out as a secondary oil recovery stage to determine the volume of recovered oil. The experimental procedure was as follows:

- Initially, the formation water accumulator was filled with the formation water of the field of interest.
- Then, the lines were properly reattached to ensure there is no loss of pressure or fluid during the experiments.
- The pump flow rate was adjusted to the studied flow rate and the pressure data were recorded on the computer.
- The formation water was injected by using an ISCO syringe pump and the effluent was collected in 10 ml graduated tubes.
- The core was injected with several pore volumes of the formation water until no more oil was produced in the collector and the pressure drop across the core plug remained constant.
- The volume of oil recovered; so-called oil recovered after water flooding ($S_{orwf,}$ cm^3) was determined by measuring the volume of displaced crude oil.

- Then, the oil recovered by the water flooding ($OR_{wf,}$ %) and the residual "remaining" oil saturation ($S_{or,}$ %) were calculated using Equations (3-9), and (3-10) respectively.

$$OR_{wf}\ \% = \frac{S_{orwf}}{OOIP} \times 100 \quad (3\text{-}9)$$

$$S_{or}\ \% = \frac{OOIP - S_{orwf}}{PV} \times 100 \quad (3\text{-}10)$$

3.3.5.2.6 Biosurfactant Flooding

The biosurfactant flooding test was finally deployed as a tertiary oil recovery to investigate the effect of biosurfactants on the residual crude oil recovery based on the additional oil that could be recovered. The same sequence of formation water flooding tests was conducted for the biosurfactant flooding test. The experimental procedure was as follows:

- The biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* were used in this experiment and injected into the core plugs that represent the field isolated from its crude oil.
- The core plugs were injected with several pore volumes of the biosurfactant aqueous solution at CMC until no more oil was produced in the collector to achieve the residual oil saturation after biosurfactant flooding (S_{orbf} cm^3) and its volume was determined by measuring the volume of displaced crude oil.
- The additional oil recovered by the biosurfactant flooding (AOR_{bf}, %) was calculated using Equation (3-11).

$$AOR_{bf}\% = \frac{S_{orbf}}{OOIP - S_{orwf}} \times 100 \quad (3\text{-}11)$$

- Biosurfactant flooding continued until the pressure drop across the core plug remained constant.
- After completing the biosurfactant flooding, extended water flooding (EWF) was conducted to investigate if more oil could be recovered.
- Finally, the trial was finished, and the system could be cleaned and reset for a new core.

3.3.5.2.7 Core Cleaning

The core plugs still contained crude oil and formation water that needed to be removed for subsequent experiments. The core plugs were cleaned by using the Soxhlet extraction method, shown in Figure 3-40, using toluene for theoil/water removal and acetone/methanol as an azeotropic mixture in the proportion of (75:25) for the salt removal, and then dried at 65°C for 24 h before reuse (Al-Sulaimani et al., 2011a).

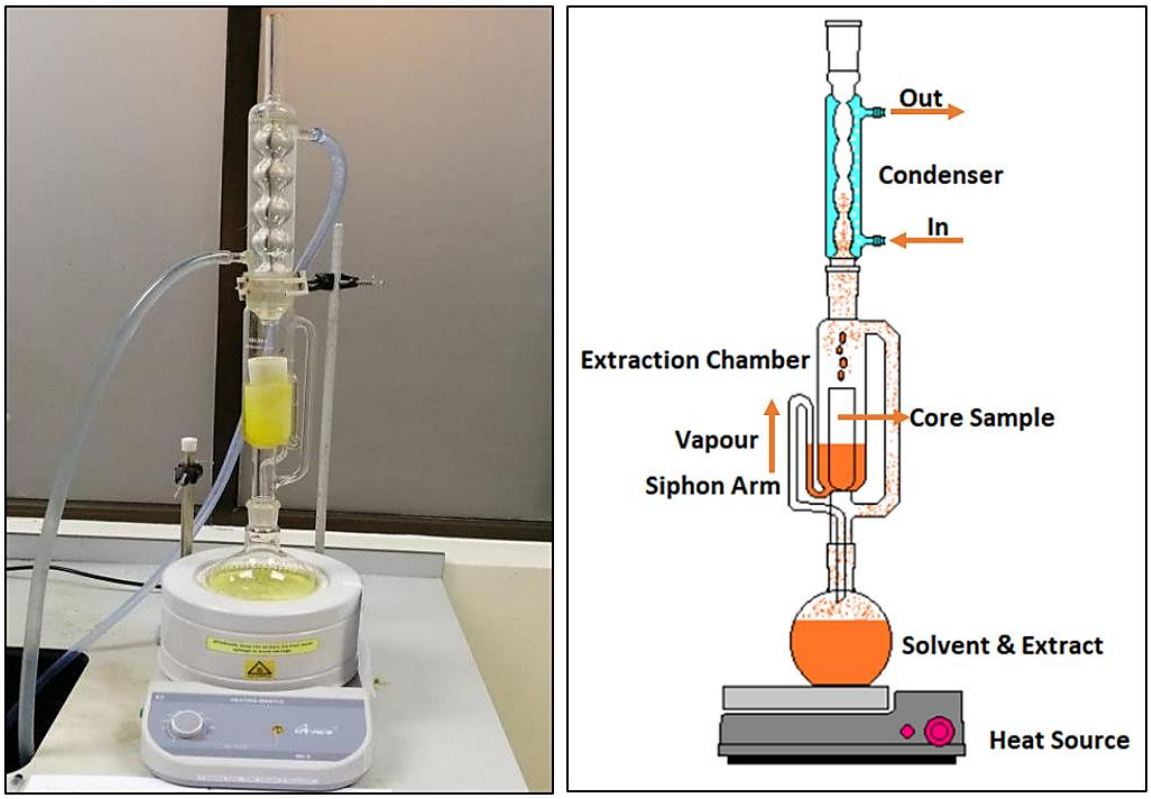

Figure 3-40. Core Cleaning Process using Soxhlet Extractor.

The next chapter describes the statistical analysis of collected data, as well as the experimental analysis and results obtained from this study.

CHAPTER 4

RESULTS AND DISCUSSION

Outline of the chapter

In this chapter, the main results of this study are presented. the analysis and outcome of the laboratory investigations are discussed. The chapter is organised as follows:

4.1. Isolation of bacteria

4.2. Identification of bacteria

4.3. Biosurfactants production

4.4. Examination of Oil Recovery using Core Flooding

4. RESULTS AND DISCUSSION

This chapter introduces the statistical analysis of collected data and the results obtained from the experimental investigation conducted according to the sequence summarised in Figure 3-1, is in line with the original aim of this study in investigating the potential of producing biosurfactants by indigenous bacteria isolated from Egyptian oil fields, and optimizing the production of these biosurfactants to reach the ultimate oil transport and enhanced oil recovery. The results are presented in five phases:

Phase I: the results of the statistical analysis of collected data obtained from Egyptian oil fields are presented in section (4.1). The results of the selection of the Egyptian oil fields with potential for MEOR are presented.

Phase II: the results of indigenous bacteria isolation from collected Egyptian crude oil samples are presented in section (4.2).

Phase III: the results for the identification of isolated bacteria representing indigenous bacteria in Egyptian oil fields are discussed in section (4.3).

Phase IV: the results of the selection of suitable bacterial strains for producing biosurfactants are presented in section (4.4). The findings of the study of the effect of some nutrients on the selected bacterial strains are also presented. Moreover, the results of investigating the efficiency of the produced biosurfactants are also presented. Finally, the results of investigating the stability of the produced biosurfactants under harsh reservoir conditions (salinity, pH, and temperature) are presented.

Phase V: the results of the oil displacement that was performed in two stages, water flooding followed by biosurfactants flooding in simulated core flooding micromodels are presented in section (4.5). Furthermore, the results of studying the effect of flow rate on oil recovery performance are also presented. Finally, the comparison between the obtained results in this study using biosurfactants produced and the results of reported core flooding tests using either *Bacillus subtilis* or *Bacillus licheniformis* are presented.

4.1 Statistical Analysis of Data Collected

The activity of bacteria employed in MEOR applications depends on the physical and chemical conditions they encounter in the reservoirs, such as temperature, salinity, pH, permeability, and nutrients. Although these reservoir conditions vary a great deal from one reservoir to another. All these factors, which are generally physical and environmental can affect bacterial growth, proliferation, metabolism, and survival, and limit their ability to produce desired quantities of metabolites such as biosurfactants that are needed for enhanced oil recovery. Consequently, the data ranges of these physical and chemical conditions of Egyptian reservoirs in the Gulf of Suez and the Western Desert and the number of Egyptian reservoirs that fit each MEOR screening criterion are presented in Table 4-1 and Table 4-2, respectively. Moreover, the frequency diagram of the Gulf of Suez and Western Desert Screening Criteria Analysis are plotted in Figure 4-1 and Figure 4-2, respectively.

Table 4-1. Data Ranges and Number of Egyptian Oil Reservoirs in Gulf of Suez fitting MEOR Screening Criteria

Reservoir Property	Data Ranges for Egyptian Reservoirs in Gulf of Suez	Number of Egyptian Reservoirs in Gulf of Suez Fitting MEOR Screening Criteria
Formation Temperature (^{o}C)	46 - 146	8
Formation Water Salinity (ppm)	15,000 - 370,000	19
Reservoir Depth (m)	642 - 3,627	9
Reservoir Rock Permeability (mD)	45 - 3,000	19
Crude Oil Type (API)	20 - 38	20
Crude Oil Viscosity (mPa.s)	0.4 - 30	20

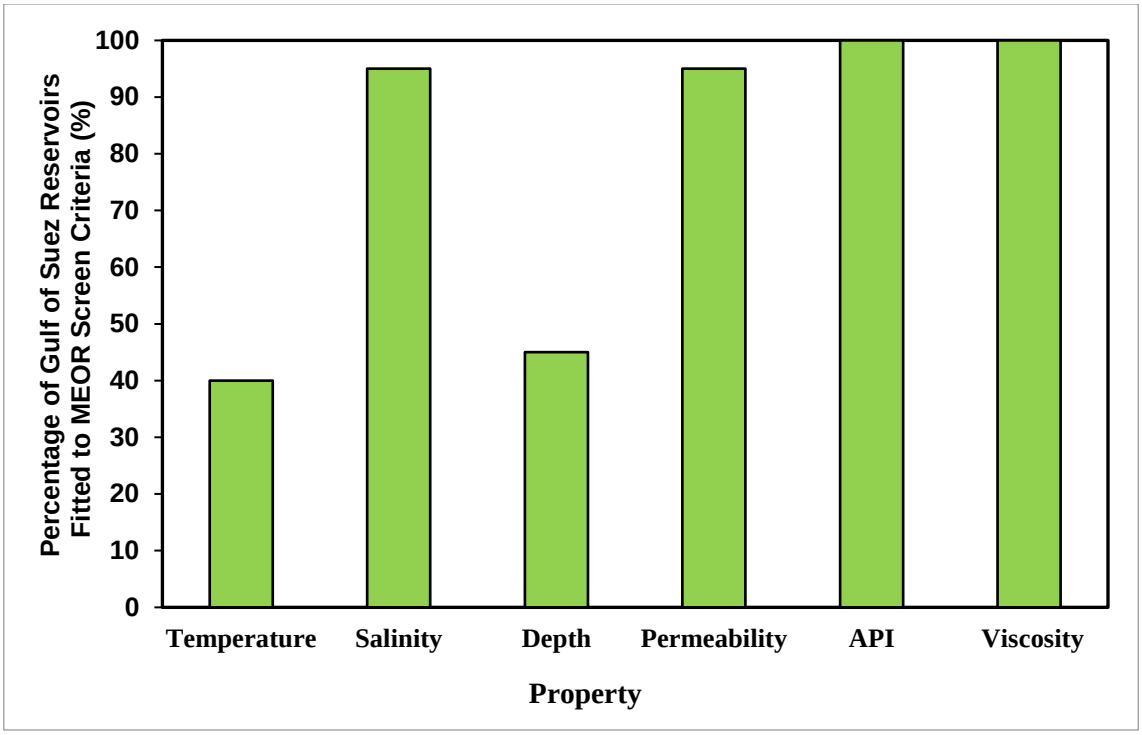

Figure 4-1. Frequency Diagram of Gulf of Suez Screening Criteria Analysis.

Table 4-2. Data Ranges and Number of Egyptian Oil Reservoirs in Western Desert fitting MEOR Screening Criteria

Reservoir Property	Data Ranges for Egyptian Reservoirs in the Western Desert	Number of Egyptian Reservoirs in Western Desert Fitting MEOR Screening Criteria
Formation Temperature (°C)	64 - 149	3
Formation Water Salinity (ppm)	34,000 - 207,420	39
Reservoir Depth (m)	1,200 - 4,220	7
Reservoir Rock Permeability (mD)	5.3 - 400	32
Crude Oil Type (API)	9 - 42.8	38
Crude Oil Viscosity (mPa.s)	0.17 - 2.10	39

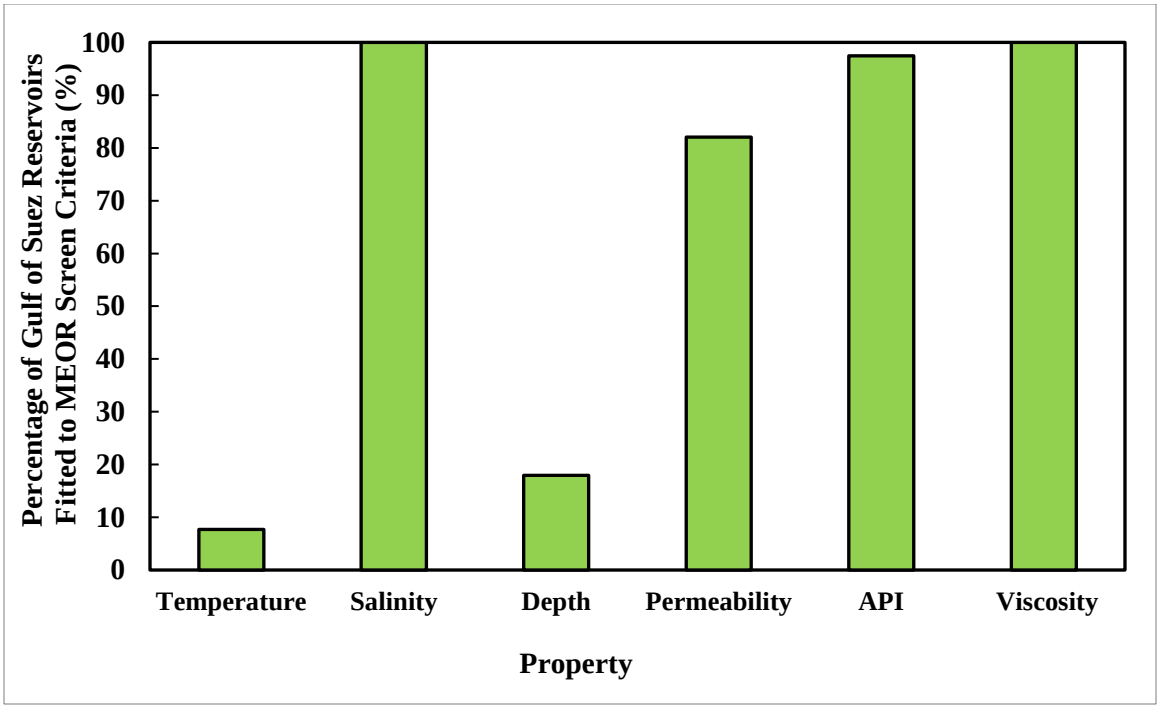

Figure 4-2. Frequency Diagram of Western Desert Screening Criteria Analysis.

It was found that the number of oil reservoirs from the Gulf of Suez and the Western Desert that has the potential to MEOR based on available data is equal to 8 and 3 oil reservoirs, respectively, which are listed in Table 4-3 and Table 4-4. It was also found that the main factors, which are considered limiting for the successful application of MEOR in Egyptian oil fields, are reservoir temperature and depth. Temperature plays a significant role in bacteria metabolism. With increasing depth, the temperature increases. Consequently, bacterial growth and their metabolism will certainly be affected as the increasing temperature can exert negative effects on enzyme function by disrupting important cell activities. However, The effects of temperature on enzyme function are generally accepted, but it is also to be noted that the temperatures at which these phenomena occur vary widely between organisms (Lazar et al., 2007; Marshall, 2008). Marshall *et al.* (2008) reported that bacteria could be classified according to their optimum temperature range as psychrophilic bacteria (<25^{o}C), mesophilic bacteria (25-45^{o}C), and thermophilic bacteria (>45^{o}C), based on the temperature ranges for microorganisms survival (Marshall, 2008). They also reported that in most developed petroleum reservoir conditions, temperatures are expected to vary greatly but can be as high as 70°C, and even 100°C in some cases. To

survive such high temperatures, thermophilic bacteria are often spore-forming and possess thermally stable enzymes that allow the normal functioning of cellular processes under such harsh conditions (Marshall, 2008).

Table 4-3. Gulf of Suez oil reservoirs that have the potential for MEOR.

Field	Reservoir	Temperature (°C)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
EL-MORGAN	N. KAREEM	79	60,000	1,829	190	29.5	0.55
EL-MORGAN	S. KAREEM	79	70,000	1,860	300	29.5	1.17
EL-MORGAN	N. BELAYIM	71	66,000	1,615	150	26.0	3.40
GS-315	KAREEM	79	70,000	1,829	1,400	30.4	0.94
GS-315	BALEYIM	72	65,000	1,615	1,000	28.0	1.96
ZEIT BAY	CARBONATE S.S.	67	220,000	1,264	370	38.0	0.90
RAS FANAR	N. LIMESTONE	46	100,800	642	100	27.7	2.35
WEST BAKR	RUDES S.	60	15,000	1,049	3,000	20	30

Table 4-4. Western Desert oil reservoirs that have the potential for MEOR

Field	Reservoir	Temperature (°C)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
RAZZAK2	APT. DEL.	71	84,000	1,698	400	37.5	0.93
AL FADL	Bahariya	64	122,000	1,200	131	40.4	1.82
AL QADR	Bahariya	64	122,000	1,200	133	41.3	1.50

4.2 Microbial Isolation

11 crude oil samples were collected for the isolation of crude oil indigenous bacteria from the Egyptian oil fields that have the potential for MEOR and were labelled as G1, G2, G3, G4, G5, G6, G7, G8, WD1, WD2, and WD3, as listed in Table 4-5. The collected crude oil samples belonged to the main two different Egyptian oil concessions areas (Gulf, and the Western Desert). They were collected and preserved in the fridge at 4°C in a 50 ml sterilized bottle for further studies.

Table 4-5. Collected samples of Egyptian oil fields that have the potential for MEOR

Sample	Location	Field	Reservoir
G1	Gulf	EL-MORGAN	N. KAREEM
G2	Gulf	EL-MORGAN	S. KAREEM
G3	Gulf	EL-MORGAN	N. BELAYIM
G4	Gulf	GS-315	KAREEM
G5	Gulf	GS-315	BALEYIM
G6	Gulf	ZEIT BAY	CARBONATE S.S.
G7	Gulf	RAS FANAR	N. LIMESTONE
G8	Gulf	WEST BAKR	RUDES S.
WD1	WD	RAZZAK2	APT. DEL.
WD2	WD	AL FADL	Bahariya
WD3	WD	AL QADR	Bahariya

The laboratory studies show the existence of bacterial strains in the collected crude oil samples. The turbidity of broth media after incubation in a rotary shaker for three days at 180 rpm has confirmed the existence of microorganisms in these samples. Single colony was isolated from each species exist in turbid media in agar plates using streak plate method. Figure 4-3 shows the single colony that was isolated from the studied crude oil sample. Initially, 11 pure bacteria cultures were isolated from the collected crude oil samples.

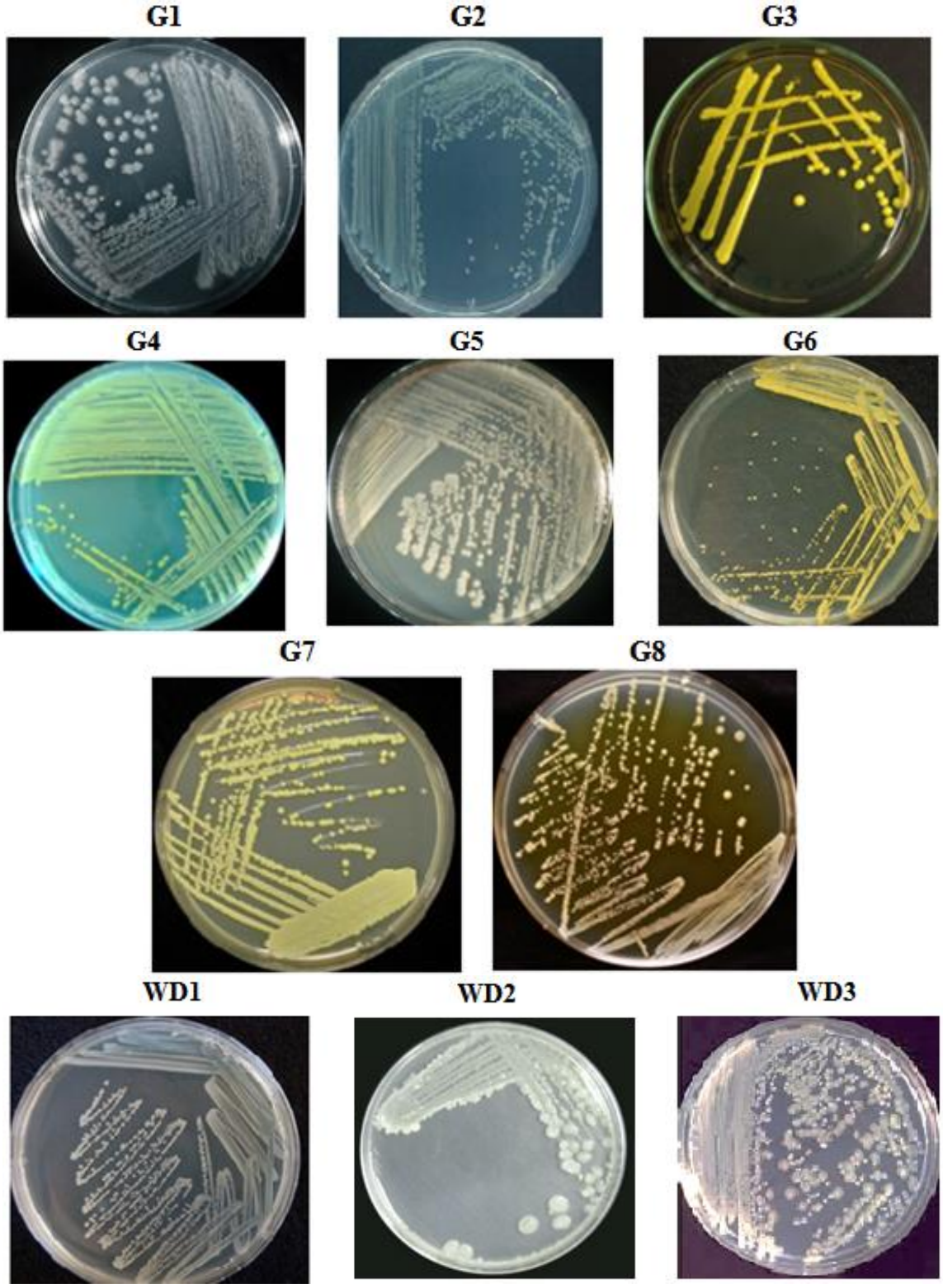

Figure 4-3. Single colonies of isolated bacteria collected from Gulf of Suez (G1-G8), and Western Desert (WD1-WD3) oil fields in agar plates.

4.3 Microbial Identification

Morphological and biochemical analyses were carried out to identify the 11 studied bacteria isolated from the different Egyptian oil fields collected samples.

4.3.1 Colony Morphology

The morphology of isolated colonies has been studied and their visual culture characteristics on an agar plate, shown in Figure 4-3, were listed in Table 4-6. It was found that the colony morphology of the isolated strain G1 is circular shape, small, circular, light-yellow fluorescent, convex, and smooth colonies with irregular edges. The isolated strain G2 is large, circular, light grey, convex, and smooth colonies with irregular edges. The isolated strain G3 is large, circular shape, yellow fluorescent, flat, and smooth colonies with irregular edges. The isolated strain G4 is small, circular, greenish-yellow fluorescent, convex, and smooth colonies with regular edges. The isolated strain G5 is small, irregular, shiny white, convex, and smooth colonies with irregular edges with distinctive cheese odour. The isolated strain G6 is small, circular, shiny yellow, and convex colonies with irregular edges. The isolated strain G7 is small, circular, light-yellow fluorescent, convex, and smooth colonies with irregular edges. The isolated strain G8 is large, circular, slightly yellow, convex, and smooth colonies with irregular edges. The isolated strain WD1 is small, circular, white, raised, and smooth colonies with irregular edges. The isolated strain WD2 is large, circular, white, flat, and finely wrinkled colonies with irregular edges. Isolated strain WD3 is medium, circular, fuzzy white, flat, and mucoid colonies with irregular edges.

Table 4-6. Colony morphology of isolated strains.

Isolates	Colony Size	Colony Shape	Colony Pigmentation Colour	Colony Elevation	Colony Surface	Colony Margin
G1	Small	Circular	Light yellow fluorescent	Convex	Smooth	Irregular
G2	Large	Circular	Light grey	Convex	Smooth	Irregular
G3	Large	Circular	Yellow fluorescent	Flat	Smooth	Irregular
G4	Small	Circular	Greenish-yellow fluorescent	Convex	Smooth, Shiny	Regular
G5	Small	Irregular	White	Convex	Smooth, Shiny	Irregular
G6	Small	Circular	Yellow	Convex	Shiny	Irregular
G7	Small	Circular	Medium yellow fluorescent	Convex	Smooth	Irregular
G8	Large	Circular	light yellow	Convex	Smooth	Irregular
WD1	Small	Circular	White	Raised	Smooth	Irregular
WD2	Large	Circular	White	Flat	Finely wrinkled	Irregular
WD3	Medium	Circular	fuzzy white	Flat	Mucoid	Irregular

4.3.2 Cell Morphology

Figure 4-4 shows the cell morphology of the isolated strains under the microscope after gram staining. It was found that all isolated strains are bacilli (rod-shaped) species. G1, G3, G4, G7, and G8 are gram-negative bacteria. However, G2, G5, G6, WD1, WD2, and WD3 are gram-positive bacteria.

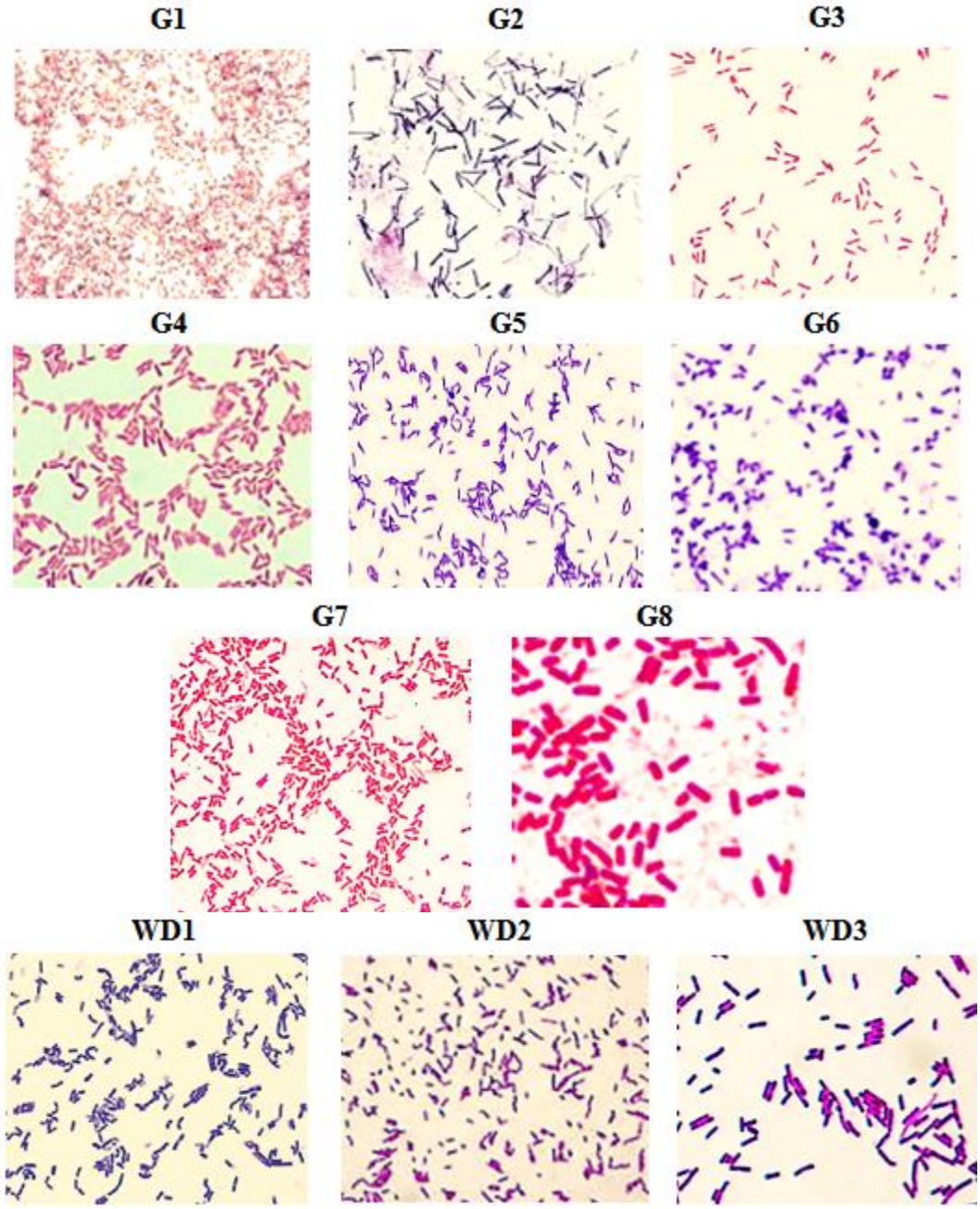

Figure 4-4. Microscopic View of the isolated bacteria collected from Gulf of Suez (G1-G8), and Western Desert (WD1-WD3) oil fields (1000× magnification objective).

4.3.3 Oxygen Intake, Motility, and Endospore Tests

The results of oxygen intake, motility, and endospore tests of all the isolated strains were represented in Table 4-7. Based on the oxygen intake test, it was found that isolated strains G1, G3, G4, G5, G7, and WD1 are strictly aerobic, while G6, G8, WD2, and WD3 are facultatively anaerobic, and G2 is strictly anaerobic. It was also found that all isolated strains are non-spore-forming, except G2, WD1, WD2, and WD3. Based on the motility test, all isolated strains are motile, except G5 and G6.

Table 4-7. Oxygen Intake, Motility, and Endospore Tests of isolated bacteria.

Sample	Oxygen Intake Test	Endospore Staining Test	Motility Test
G1	Strictly aerobic	Non-spore forming	Motile
G2	Strictly anaerobic	Spore forming	Motile
G3	Strictly aerobic	Non-spore forming	Motile
G4	Strictly aerobic	Non-spore forming	Motile
G5	Strictly aerobic	Non-spore forming	Nonmotile
G6	Facultatively anaerobic	Non-spore forming	Nonmotile
G7	Strictly aerobic	Non-spore forming	Motile
G8	Facultatively anaerobic	Non-spore forming	Motile
WD1	Strictly aerobic	Spore forming	Motile
WD2	Facultatively anaerobic	Spore forming	Motile
WD3	Facultatively anaerobic	Spore forming	Motile

4.3.4 Biochemical Tests

Figure 4-5 summarizes the identification process of the studied bacilli bacteria based on their morphological and biochemical characteristics. The results of several biochemical characteristics of all the isolated strains are represented in Table 4-8. The first step in bacteria identification was to determine the Gram staining of the studied bacteria. After Gram testing, the bacterial cell morphology was observed. Bacilli (rod-shaped) bacteria were divided into two categories based on the gram staining reaction results (Figure 4-4) bacilli gram-positive, and bacilli gram-negative. Each of the isolated bacterial strains G2,

G5, G6, WD1, WD2, and WD3 were bacilli gram-positive bacteria, and G1, G3, G4, G7, and G8 were bacilli gram-negative bacteria.

Bacilli gram-positive bacteria (G2, G5, G6, WD1, WD2, and WD3) were tested to distinguish their ability to form spores. If they were spore-forming, they can be *Bacillus spp* or *Clostridium spp*. It was found that each of the isolated bacterial strains G2, WD1, WD2, and WD3 were spore-forming. In this step, the isolated bacterial strains G2, WD1, WD2, and WD3 were tested to distinguish whether they are strictly anaerobic or not. If they were strictly anaerobic, they were *Clostridium spp*. It was found that the isolated strain G2 was strictly anaerobic, which means it was *Clostridium spp*. If they were not strictly anaerobic, they are *Bacillus spp*. It was found that the isolated strains WD1, WD2, and WD3 were not strictly anaerobic, which means they were *Bacillus* spp. In this step, a citrate test was applied to the isolated strains WD1, WD2, and WD3. If they were citrate positive, they can be *B. subtilis, B. licheniformis, B. flexus, or B. coagulans*. It was found that the isolated strains WD1, WD2, and WD3 were citrate positive, which means they can be *B. subtilis, B. licheniformis, B. flexus, or B. coagulans*. In this step, bacteria were tested to distinguish whether they were strictly aerobic, or facultatively anaerobic. If they were strictly aerobic bacteria, they are *B. flexus.* It was found that the isolated strain WD1 was strictly aerobic, which means it is *B. flexus*. If they were facultatively anaerobic bacteria, they can be *B. subtilis, B. licheniformis, or B. coagulans*. It was found that the isolated strains WD2 and WD3 were facultatively anaerobic, which means they can be *B. subtilis, B. licheniformis, or B. coagulans*. In this step, these bacteria can be categorized further by determining their growth ability in a medium containing 6.5% sodium chloride (NaCl). To determine this growth ability, bacteria were added to a sterile test tube containing 6.5% NaCl broth (which is a mixture of nutrient broth and 6.5% NaCl) and incubated for 24 h. A positive test was indicated by the presence of turbidity. If they can grow in a medium containing 6.5% NaCl, they can be *B. subtilis or B. licheniformis*. Otherwise, they are *B. coagulans*. It was found that the isolated strains WD2 and WD3 can grow in a medium containing 6.5% NaCl, which means they can be *B. subtilis* or *B. licheniformis*. In this step, *B. subtilis and B. licheniformis* bacteria can be easily distinguished from each other by testing their ability to grow at 55°C. If the bacteria can grow at 55°C, they were identified

to be *B. licheniformis*. Otherwise, they are *B. subtilis*. It was found that the isolated strain WD2 can grow at 55°C, which means it is *B. licheniformis*. It was also found that the isolated strain WD3 was not able to grow at 55°C, which means it is *B. subtilis*.

If Bacilli gram-positive bacteria were non-spore-forming, they can be *Brevibacterium spp, Cellulosimicrobium spp, Corynebacterium spp, Lactobacillus spp* or *Mycobacterium spp.* It was found that each of the isolated bacterial strains G5 and G6 were non-spore-forming, which means they can be *Brevibacterium spp, Cellulosimicrobium spp, Corynebacterium spp, Lactobacillus spp* or *Mycobacterium spp.* In this step, bacteria were tested to distinguish whether they were strictly aerobic, facultatively anaerobic, or strictly anaerobic. If they were strictly aerobic, they can be *Brevibacterium spp*, *Corynebacterium spp,* or *Mycobacterium spp*.

If they were facultatively anaerobic, they are *Cellulosimicrobium* spp. If they were strictly anaerobic bacteria, they are *Lactobacillus spp.* It was found that the isolated strain G6 was facultatively anaerobic, which means it is *Cellulosimicrobium* spp. It was also found that the isolated strain G5 was strictly aerobic, which means it can be *Brevibacterium spp, Corynebacterium spp,* or *Mycobacterium spp.* In this step, a catalase test was applied to the isolated strain G5. If they were catalase-positive, they can be *Brevibacterium spp* or *Corynebacterium spp*. It was found that the isolated strain G5 was catalase-positive, which means it can be *Brevibacterium spp* or *Corynebacterium spp.* In this step, the isolated strain G5 was tested to distinguish its ability to ferment glucose by applying a glucose fermentation test. If they were glucose fermenters, they are *Corynebacterium spp*. If they were not glucose fermenters, they were *Brevibacterium spp*. It was found that the isolated strain G5 was a glucose fermenter, which means it is *Brevibacterium spp.*

It was found that each of the isolated strains G1, G3, G4, G7, and G8 were Bacilli gram-negative bacteria. In this case, an oxidase test was applied to the isolated strains G1, G3, G4, G7, and G8. If they were oxidase-positive, they can be *Pseudomonas spp, Aeromonas spp,* or *Vibrio spp*. It was found that each of G1, G3, G4, and G7 were oxidase-positive, which means they can be *Pseudomonas spp, Aeromonas spp,* or *Vibrio spp.* In this step, bacteria were tested to distinguish their ability to ferment glucose by applying a glucose

fermentation test. If they were glucose fermentation positive, they can be *Aeromonas spp* or *Vibrio spp*. If they were glucose fermentation negative, they are *Pseudomonas spp*. It was found that each of G1, G3, G4, and G7 were glucose fermentation negative, which means they are *Pseudomonas spp.* In this step, the isolated strains G1, G3, G4, and G7 can be categorized further by observing their colony pigmentation colours in the agar plate. If they were yellow, fluorescent pigments, they can be *P. aeruginosa, P. fluorescens, P. stutzeri, or P. panipatensis*. It was found that the colony pigmentation colours of the isolated strains G1, G3, G4, and G7 were yellow fluorescent, which means they can be *P. aeruginosa, P. fluorescens, P. stutzeri, or P. panipatensis.* In this step, the Voges-Proskauer test was applied to the isolated strains G1, G3, G4, and G7. If they were Voges-Proskauer positive, they are *P. aeruginosa.* it was found that the isolated strain G3 was Voges-Proskauer positive, which means it was *P. aeruginosa.* If they were Voges-Proskauer negative, they can be *P. fluorescens, P. stutzeri, or P. panipatensis*. it was found that the isolated strains G1, G4, and G7 were Voges-Proskauer negative, which means they can be *P. fluorescens, P. stutzeri, or P. panipatensis.* In this step, the methyl red test was applied to the isolated strains G1, G4, and G7. If they were methyl red positive, they are *P. fluorescens.* It was found that the isolated strain G4 was methyl red positive, which means it is *P. fluorescens*. If they were methyl red negative, they can be *P. stutzeri or P. panipatensis*. It was found that the isolated strains G1 and G7 were methyl red negative, which means they can be *P. stutzeri or P. panipatensis*. In this step, a urease test was applied on the isolated strains G1 and G7. If they were urease positive, they are *P. panipatensis.* It was found that the isolated strain G7 was urease positive, which means it is *P. panipatensis*. If they were urease negative, they are *P. stutzeri*. It was found that the isolated strain G1 was urease negative, which means it is *P. stutzeri*.

If Bacilli gram-negative bacteria were oxidase-negative bacteria, they belong to the Enterobacteriaceae family. It was found that the isolated strain G8 was oxidase negative, which means it belongs to the Enterobacteriaceae family. In this step, the isolated strain G8 was tested to distinguish its ability to ferment lactose by applying a lactose fermentation test. If it was lactose fermentation positive, it can be *Enterobacter spp, Citrobacter spp, Escherichia spp, or Klebsiella spp.* It was found that the isolated strain G8 was lactose

fermentation positive, which means it can be *Enterobacter spp, Citrobacter spp, Escherichia spp, or Klebsiella spp*. In this step, the isolated strain G8 was tested to distinguish its ability to motile by applying a motility test. If they were motile, they can be *Enterobacter spp, Citrobacter spp, or Escherichia spp*. If they were nonmotile, they were Klebsiella. It was found that isolated strain G8 was motile, which means it can be *Enterobacter spp, Citrobacter spp, or Escherichia spp*. In this step, the Voges-Proskauer test was applied to the isolated strain G8. If it was Voges-Proskauer positive, it was *Enterobacter spp.* If it was Voges-Proskauer negative. It can be *Citrobacter spp or Escherichia spp*. it was found that the isolated strain G8 was Voges-Proskauer positive, which means it was *Enterobacter spp.*

Based on the above-combined analysis of the isolated bacteria colony morphology results (Table 4-6), cell morphology results (Figure 4-4), Oxygen Intake, and Motility, Endospore characterization results (Table 4-7), and finally biochemical characterization results (Table 4-8), it can be concluded that the 11 types of the isolated indigenous bacterial strains, which were labelled G1, G2, G3, G4, G5, G6, G7, G8, WD1, WD2, and WD3, were *Pseudomonas stutzeri, Clostridium spp, pseudomonas aeruginosa, pseudomonas fluorescens, Brevibacterium spp, Cellulosimicrobium spp, Pseudomonas panipatensis, Enterobacter spp, Bacillus flexus, Bacillus licheniformis*, and *Bacillus subtilis*, respectively.

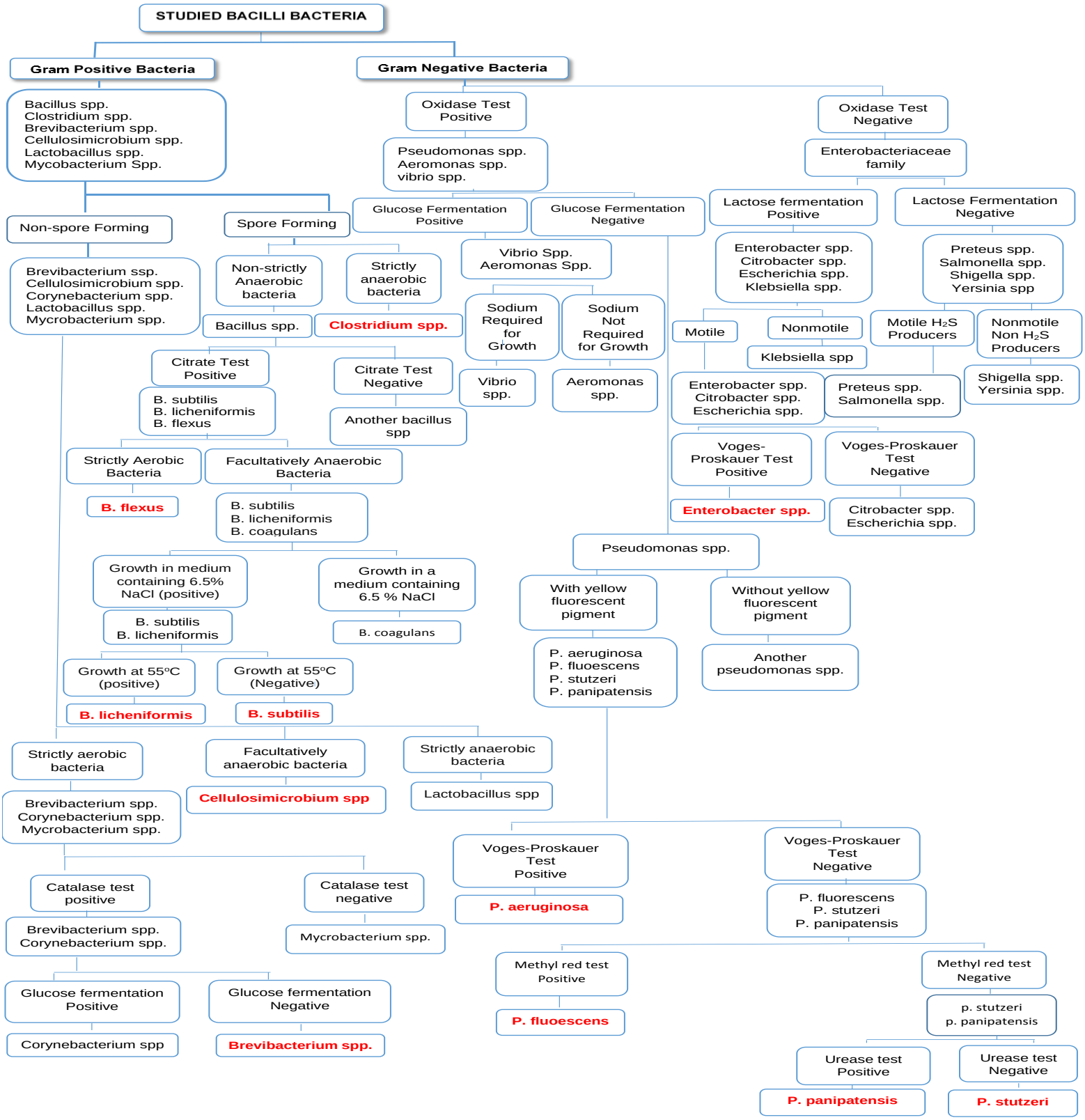

Figure 4-5. A flow diagram summarising the test for classification and identification of studied bacilli bacteria based on their morphological and biochemical characteristics.

Table 4-8. Biochemical characteristics of isolated bacteria.

Sample	Biochemical Tests											
	Catalase	Oxidase	Indole	H_2S	MR	VP	Citrate	Nitrate	Urease	OF	Gelatin	Starch
G1	+	+	-	-	-	-	+	+	-	O	+	+
G2	-	-	-	+	-	-	+	-	-	F	-	-
G3	+	+	+	-	-	+	+	+	-	O	+	-
G4	+	+	-	-	+	-	+	+	-	O	+	-
G5	+	-	-	-	-	-	+	-	+	O	+	+
G6	+	+	+	+	+	-	+	-	+	F	+	+
G7	+	+	-	-	-	-	+	+	+	O	-	+
G8	+	-	-	-	-	+	+	+	-	F	-	-
WD1	+	-	-	+	-	-	+	+	+	O	+	+
WD2	+	-	-	-	-	-	+	+	-	F	-	+
WD3	+	-	-	-	-	+	+	+	-	F	+	+

Sample	Fermentation Tests			
	Mannitol	Glucose	Lactose	Genus
G1	+	-	-	*Pseudomonas stutzeri*
G2	+	+	-	*Clostridium spp*
G3	+	-	-	*pseudomonas aeruginosa*
G4	-	-	-	*pseudomonas fluorescens*
G5	-	-	-	*Brevibacterium spp*
G6	+	+	+	*Cellulosimicrobium spp*
G7	-	-	-	*Pseudomonas panipatensis*
G8	+	+	-	*Enterobacter spp*
WD1	+	+	+	*Bacillus flexus*
WD2	+	+	+	*Bacillus licheniformis*
WD3	+	+	+	*Bacillus subtilis*

+ = Positive, - = Negative, O = Oxidative, F Fermentative

4.4 Biosurfactants Production

As mentioned in the literature review, successful field trials commonly use anaerobic or facultative anaerobic bacteria because they can grow and produce biosurfactants with no need for oxygen supply. consequently, only five indigenous isolates out of all isolated strains have the potential to produce biosurfactants in-situ conditions, these are *Clostridium spp, Cellulosimicrobium spp, Enterobacter spp, Bacillus licheniformis, and Bacillus subtilis*. However, the microorganisms that are most used for biosurfactants production field processes are species of *Bacillus* (Lazar et al., 2007; Omoniyi, 2015). These species have a greater potential for survival in petroleum reservoirs because they produce spores. Spores are dormant, resistant forms of cells that can survive more stressful environmental conditions. *Bacillus subtilis* and *Bacillus licheniformis* are well-known producers of surface-active metabolites. They not only produce good biosurfactants but are also capable of growing under facultative or anaerobic conditions, and have also been reported to be non-pathogenic (Al-Sayegh et al., 2015; Das Neves et al., 2007; Lazar et al., 2007; Liu et al., 2016; Omoniyi, 2015; Suthar and Nerurkar, 2016; Veshareh et al., 2018). Therefore, the isolated strains *Bacillus subtilis and Bacillus licheniformis* were selected for further studies in this research.

4.4.1 Surface Activity

Screening of the selected bacterial isolates (*Bacillus subtilis* and *Bacillus licheniformis*) for biosurfactant production using different media showed a significant increase in surface activity in most of the nutrient media after 72 h. The ability of the produced biosurfactants to increase the surface activity by decreasing ST and IFT against kerosene is shown in Figure 4-6, Figure 4-7, Figure 4-8, and Figure 4-9. It was found that the biosurfactants produced from selected bacterial isolates gave the maximum surface activity within 24 h of incubation, where ST and IFT reduced to lower than 30.0 mN/m and 10.0 mN/m, respectively, when the selected bacterial isolates were grown in nutrients media N7, N8, N10, and the new proposed nutrient medium H. It was also observed that the biosurfactants produced from *Bacillus subtilis* and *Bacillus licheniformis* showed the maximum surface activity when grown in the new proposed nutrient medium H, because ST was reduced from 71.8 mN/m to 25.74 mN/m and 24.13 mN/m, respectively. Similarly, the

biosurfactants produced from *Bacillus subtilis* and *Bacillus licheniformis* by the same nutrient type significantly reduced the IFT from 48.4 mN/m to 0.38 mN/m and 0.27 mN/m, respectively within 72 h of incubation. However, no significant increase in surface activity was shown from nutrient medium N3, which was a raw-material medium supplemented only by 50 g/l cane molasse, and that could be due to the missing of effective nitrogen sources and trace elements required for reaching acceptable surface activity. The comparison between the ability of the biosurfactants produced to increase the surface activity by decreasing the surface tension ST and the interfacial tension IFT against kerosene after the inoculation of their producing bacteria in the new proposed medium H was shown in Figure 4-10. The ability of *Bacillus subtilis and Bacillus licheniformis* to produce biosurfactants has been confirmed by several reports (Randhir S. Makkar and Cameotra, 1997), (Hossein Ghojavand et al., 2008), (Cooper et al., 1981), (H Ghojavand et al., 2008)(Alsharhan, 2003)(R S Makkar and Cameotra, 1997) (Amani et al., 2010). Cooper *et al.* (1981) reported that *Bacillus subtilis* can decrease ST and IFT against hexadecane to 27 mN/m, and 1 mN/m, respectively (Cooper et al., 1981). *B. licheniformis* Strain JF-2 grew and produced lipopeptide anaerobically at salinity up to 8% and temperatures up to 45°C. It was reported to reduce the ST and IFT to 27 mN/m and 0.016 mN/m, respectively (Javaheri et al., 1985). On the other hand, after activating the isolated bacteria by the new proposed medium H, the maximum surface activity was achieved compared with the other media. This important result was due to adding sodium nitrate, potassium nitrate and urea, which are reported to be the best nitrogen sources for *Bacillus species* (Makkar and Cameotra, 2002). Moreover, the added trace elements have important influence on producing biosurfactant. Makkar *et al.* (2002) reported that the most effective trace elements used for biosurfactant production are Zn, Cu, B, Co, and Mo (Makkar and Cameotra, 2002).

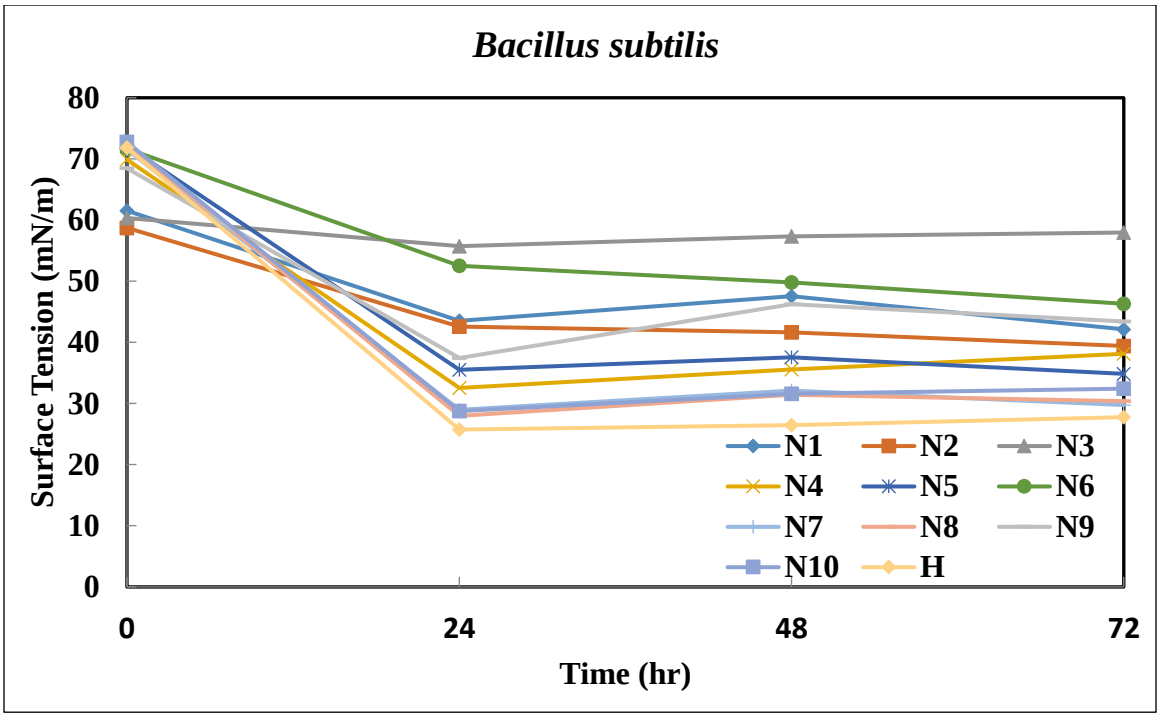

Figure 4-6. The surface tension of different nutrients broth media inoculated with *Bacillus subtilis* isolated from WD3 field.

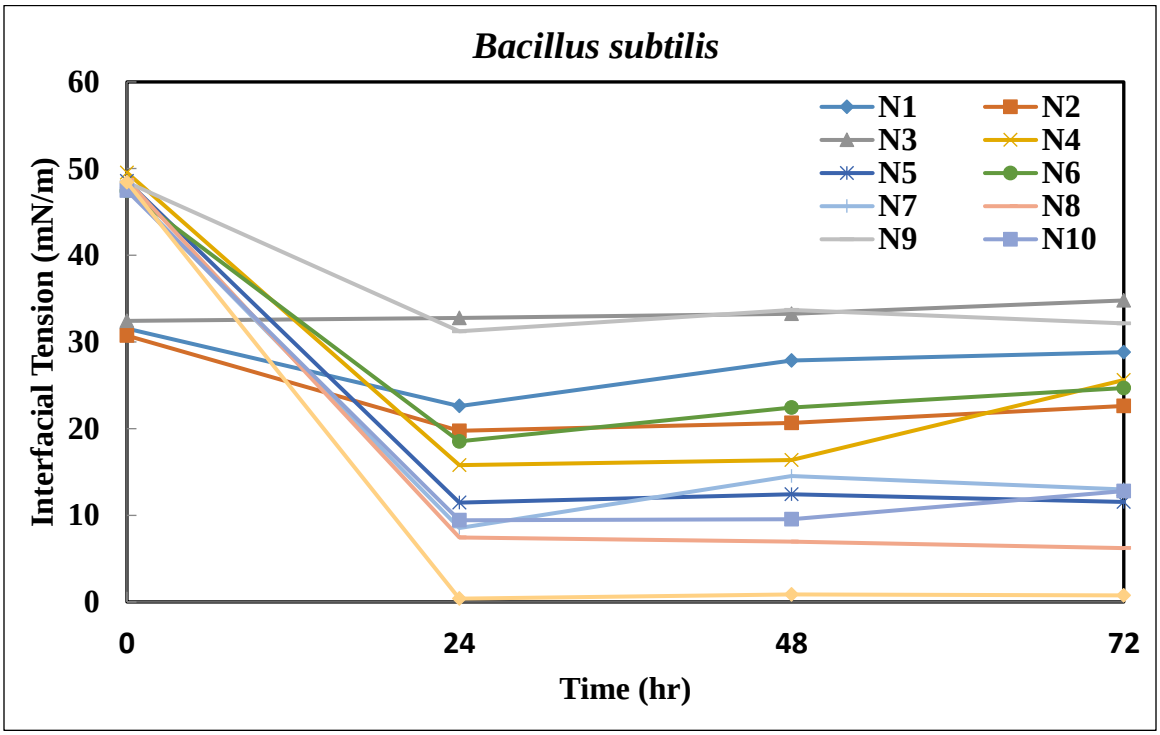

Figure 4-7. The interfacial tension of different nutrients broth media inoculated with *Bacillus subtilis* isolated from WD3 field against kerosene.

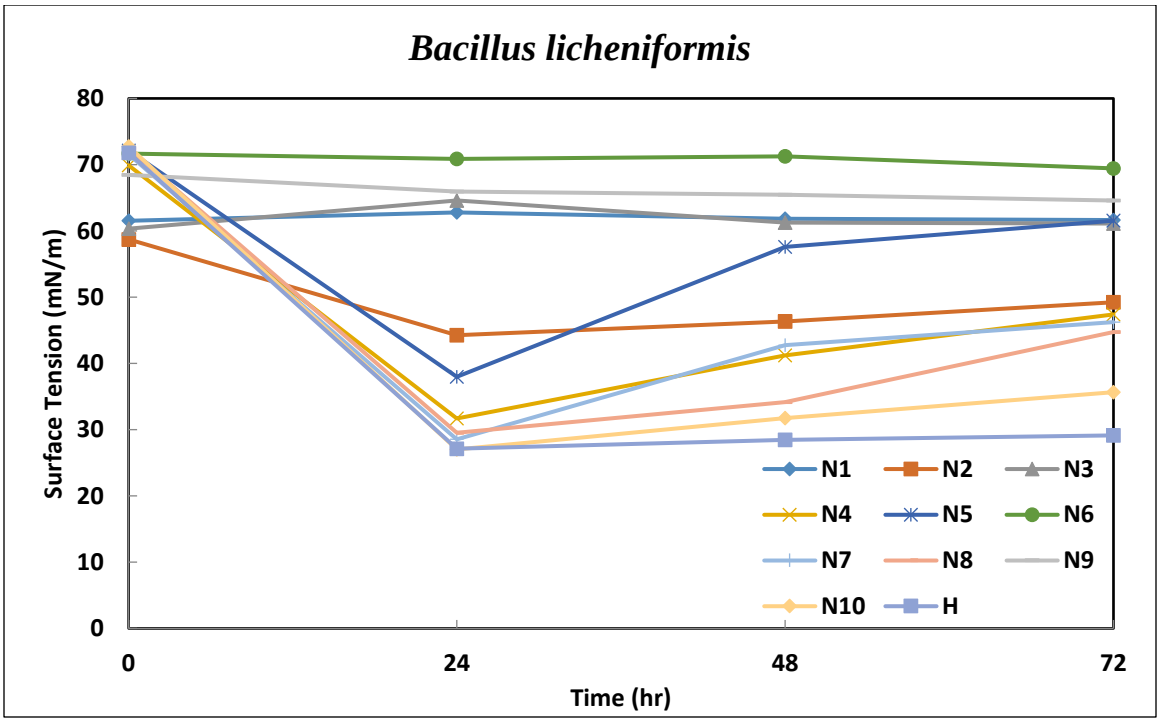

Figure 4-8. The surface tension of different nutrients broth media inoculated with *Bacillus licheniformis* isolated from WD2 field.

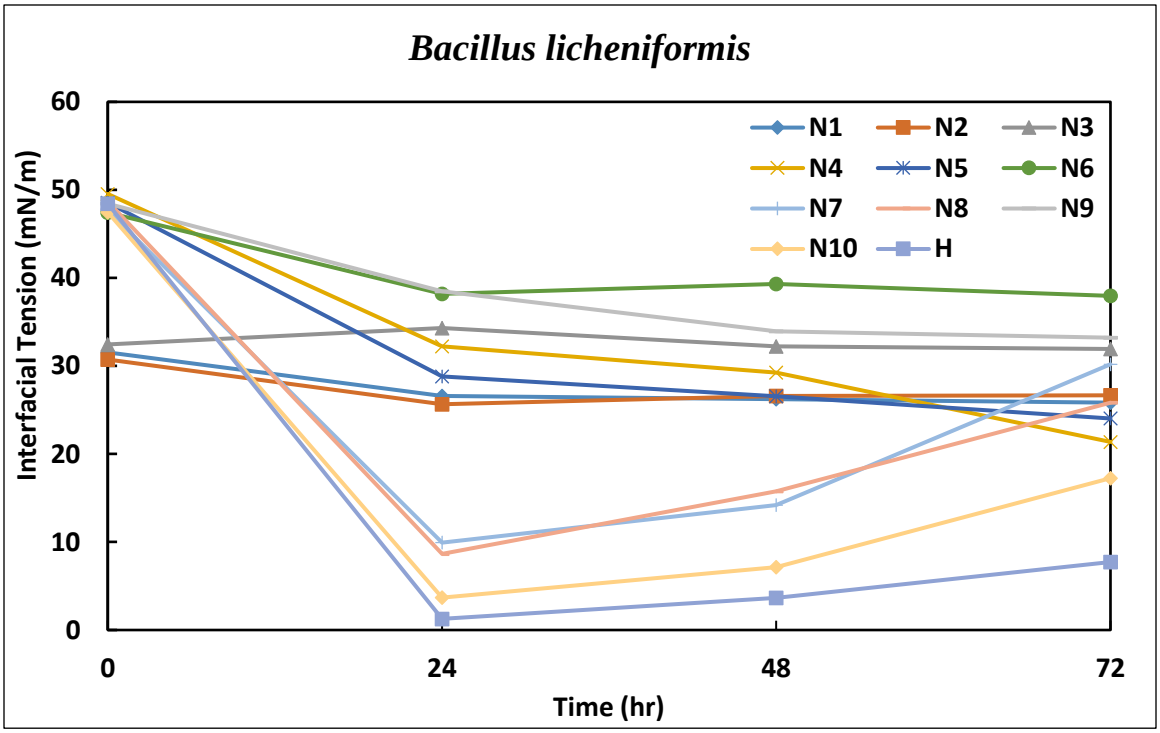

Figure 4-9. The interfacial tension of different nutrients broth media inoculated with *Bacillus licheniformis* isolated from WD2 field against kerosene.

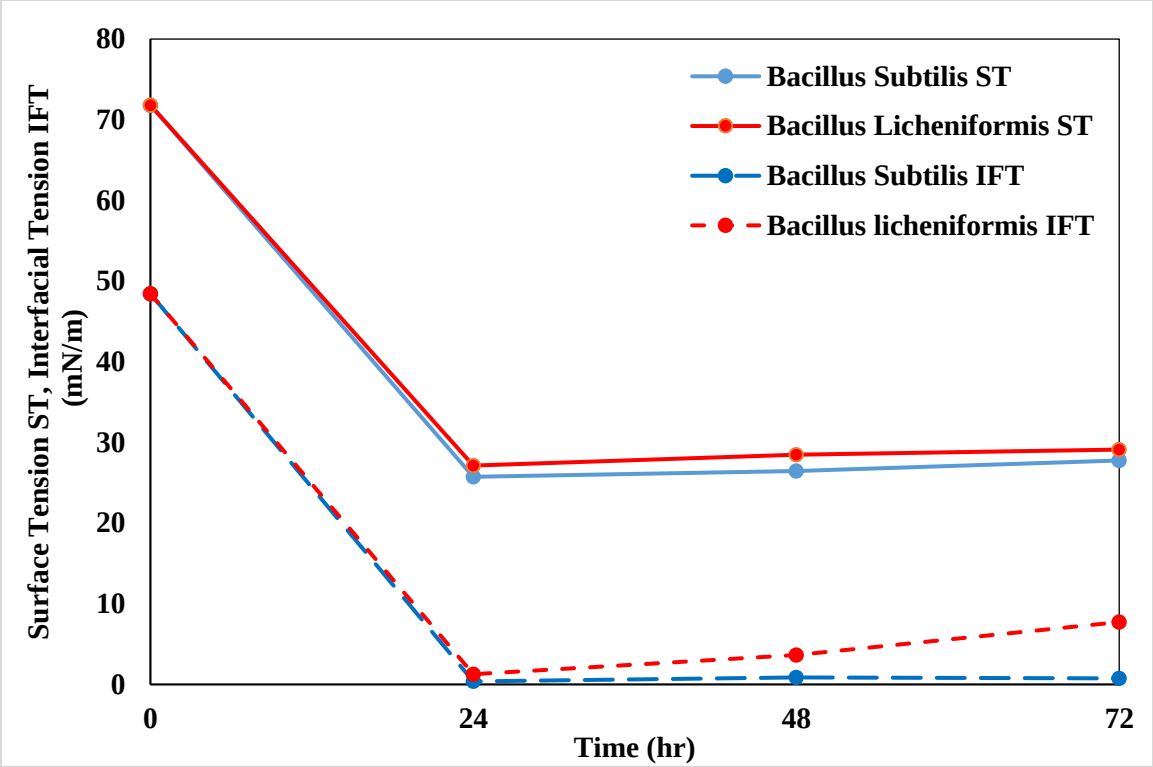

Figure 4-10. The comparison between the surface tension ST and interfacial tension IFT of the produced biosurfactants by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field during 72 h of incubation in the new proposed medium broth H.

4.4.2 Determining the Growth and Decay Rates

Further analysis for the growth and decay rates of *Bacillus subtilis and Bacillus licheniformis* during 72 h of incubation in N7, N8, N10, and the proposed nutrient medium H were implemented as shown in Figure 4-11, and Figure 4-12. A lag phase lasted 3 to 6 h of incubation, after that the exponential growth was observed after 24 h, and then the stationary phase and death phase was observed for 24-72 h. The selected bacterial strains which incubated in the new proposed medium H showed the maximum bacterial concentrations, where the bacterial concentrations of *Bacillus subtilis* and *Bacillus licheniformis* were 2.237 x 10^9 CFU/ml and 2.072 x 10^9 CFU/ml (1 OD_{600} =8 x 10^8 CFU/ml), respectively, before growth decreased and the optical density began to decrease. The comparison between the growth profiles of the selected bacterial strains *Bacillus licheniformis* and *Bacillus subtilis* during 72 h of incubation in the new proposed medium H is shown in Figure 4-13. Since the maximum surface activity and growth rate

results were obtained by the new proposed nutrient medium H, it was used for further studies.

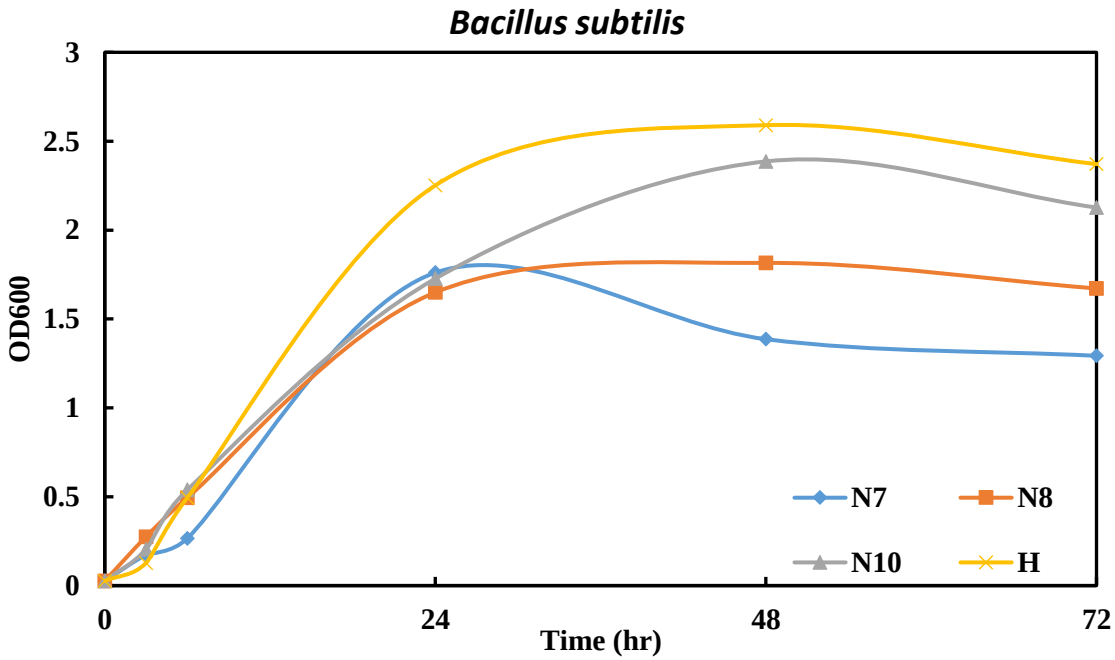

Figure 4-11. The growth curve of biosurfactant-producing bacteria *Bacillus subtilis* isolated from WD3 field in different reported nutrient media and the new proposed medium H.

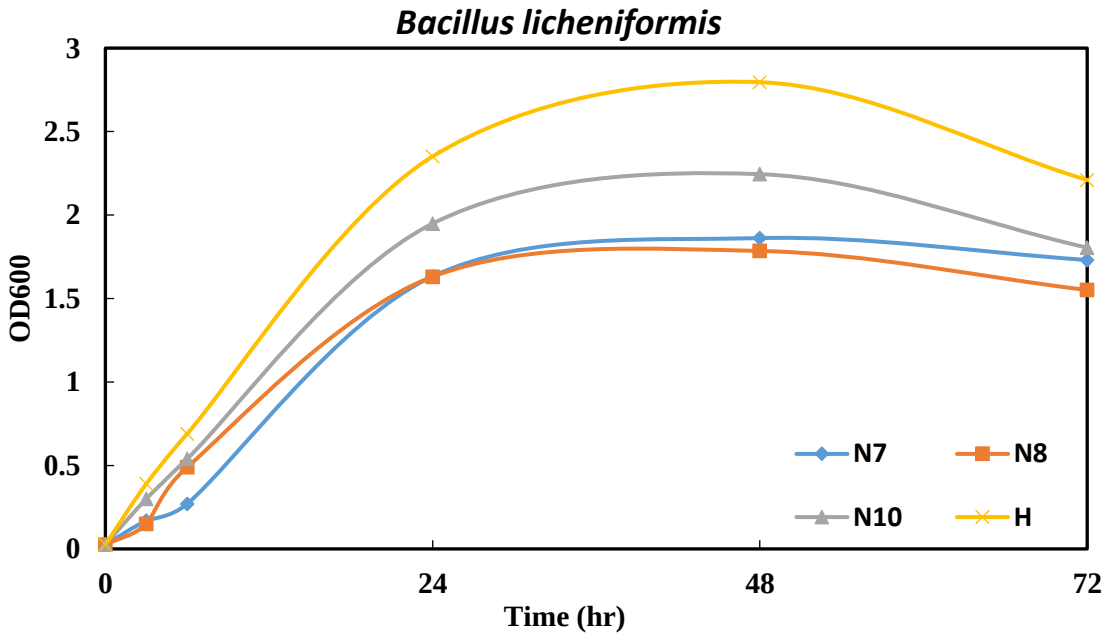

Figure 4-12. The growth curve of biosurfactant-producing bacteria *Bacillus licheniformis* isolated from WD2 field in different reported nutrient media and the new proposed medium H.

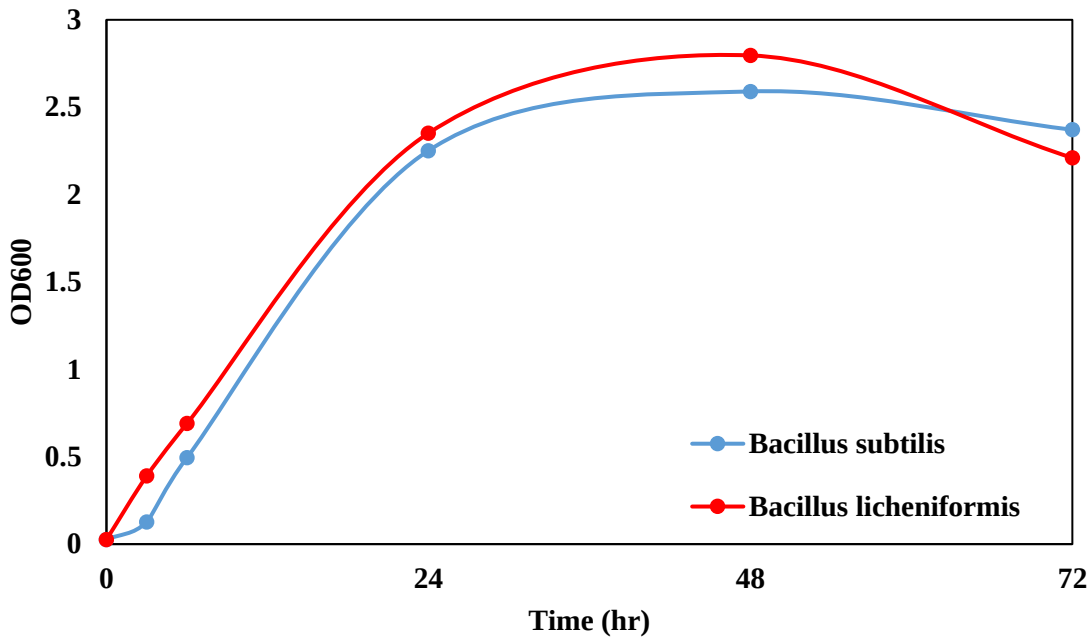

Figure 4-13. The comparison between the growth profiles of the selected bacterial strains *Bacillus licheniformis* isolated from WD2 field and *Bacillus subtilis* isolated from WD3 field during 72 h of incubation in the new proposed medium H.

4.4.3 Determining Biosurfactant Yield and Critical Micelle Concentration

The produced biosurfactants were extracted and purified using the acid precipitation method. The observed biosurfactant yields of the biosurfactants produced from *Bacillus subtilis and Bacillus licheniformis* were 2.85 g/l and 2.47, respectively. Pereira *et al.* (2013) produced 2.56 g/l of surfactin from Bacillus isolate by adding 3 g/l of yeast extract to the culture media (Pereira et al., 2013).

The extracted biosurfactant was dissolved gradually in distilled water to increase concentration, and ST and IFT were measured. Regarding the biosurfactant produced by *Bacillus subtilis*, the maximum reduction in surface tension and interfacial tension were observed at 25.74 mN/m, and 0.38 mN/m, respectively, at a biosurfactant concentration of 0.03 g/l, whereas, the maximum reduction in surface tension and interfacial tension of the biosurfactant produced by *Bacillus licheniformis* were observed at 24.13 mN/m, 1.27 mN/m, respectively, at biosurfactant concentration 0.06 g/l, as shown in Figure 4-14. It was also noticed that no change occurred even after adding more biosurfactants; therefore, these values were considered the CMC of the purified biosurfactants. These CMC values are

slightly higher than the CMC value reported by Cooper *et al.* (1981) and less than the CMC value reported by Makkar *et al.* (1997), which were 0.023 and 0.160 g/l, respectively (Cooper et al., 1981), (Makkar et al., 1997). Santos *et al.* (2016) reported that the value of the critical micelle concentration for biosurfactant applied in MEOR was usually from 0.001 to 2.0 g/l (Santos et al., n.d.). Consequently, this produced biosurfactant was effective and efficient.

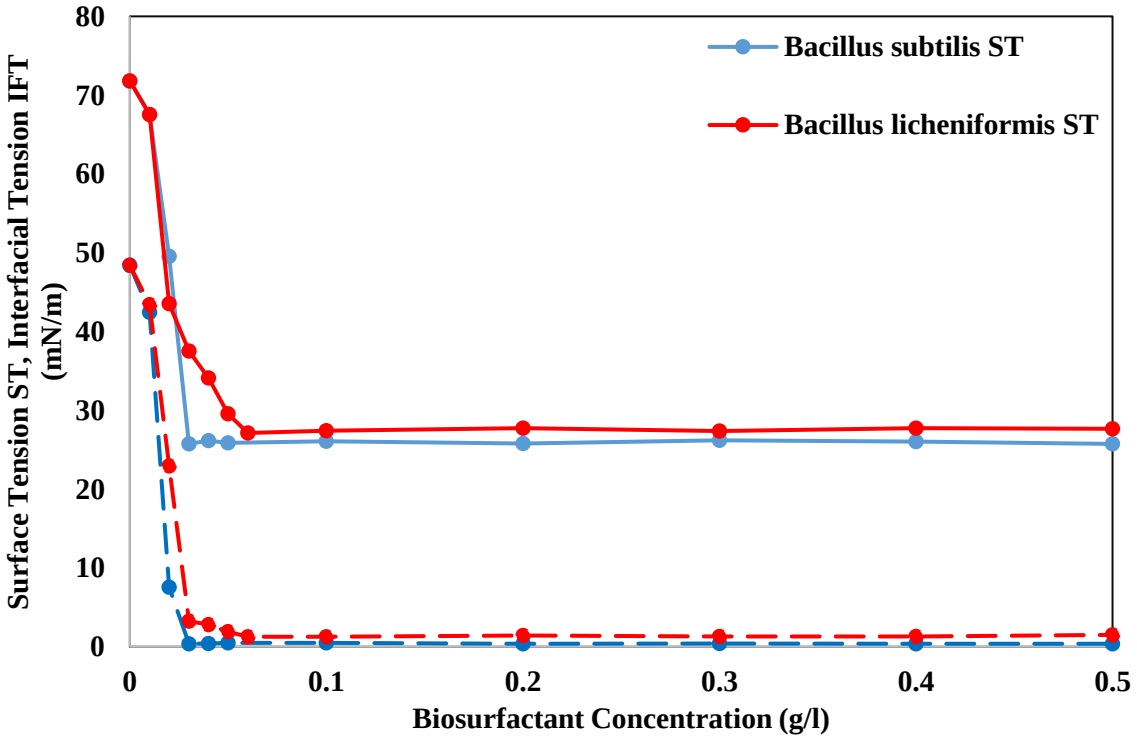

Figure 4-14. The comparison between the critical micelle concentration of the biosurfactants produced by *Bacillus licheniformis* isolated from WD2 field and *Bacillus subtilis* isolated from WD3 field.

4.4.4 Emulsification Activity

The ability of the produced biosurfactants to emulsify different hydrocarbons (hexane, heptane, hexadecane, kerosene, and crude oil) was examined. It was found that all hydrocarbons (hexane, heptane, hexadecane, kerosene, and crude oil) were emulsified with different values as shown in Figure 4-15. The emulsification indices of the biosurfactants produced by *Bacillus subtilis* against the different hydrocarbons were in the range of 64-70%, while the emulsification indices of biosurfactants produced by *Bacillus licheniformis*

for the same different hydrocarbons were in the range of 47-61%. It was also found that the highest emulsification activity was obtained against kerosene, followed by Hexadecane, Heptane, Hexane, and crude oil. The increase of the biosurfactants' emulsification activity against hexane, heptane, and hexadecane, is due to an increase in length of the alkyl chain of hydrocarbons. While the biosurfactants' emulsification activity against kerosene and crude oil could be varied based on the composition of the hydrocarbon. Nitschke and Pastore (2006) reported that the biosurfactants produced by *Bacillus subtilis* LB5a on cassava medium emulsified different hydrocarbons including kerosene, heptane, hexadecane, and crude oil in the range of 67-71% (Nitschke and Pastore, 2006). Ali *et al.* (2019) reported that biosurfactant production by *B. Licheniformis* Ali5 emulsified different hydrocarbons such as kerosene, hexadecane, tridecane, tetradecane, diesel, crude oil, pristane and heptane in the range of 50-64% (Ali et al., 2019). De Faria *et al.* (2011) reported that the biosurfactant produced by *Bacillus species* emulsified hydrocarbons including hexadecane, kerosene, diesel, petrol and benzene in the range of 30-80% (De Faria et al., 2011). In summary, the biosurfactant produced by *Bacillus subtilis* showed higher emulsification activity than the biosurfactant produced by *Bacillus licheniformis*. However, both exhibited a significant increase in emulsification activity against long-chain hydrocarbons such as crude oil, revealing their potential in improving oil recovery.

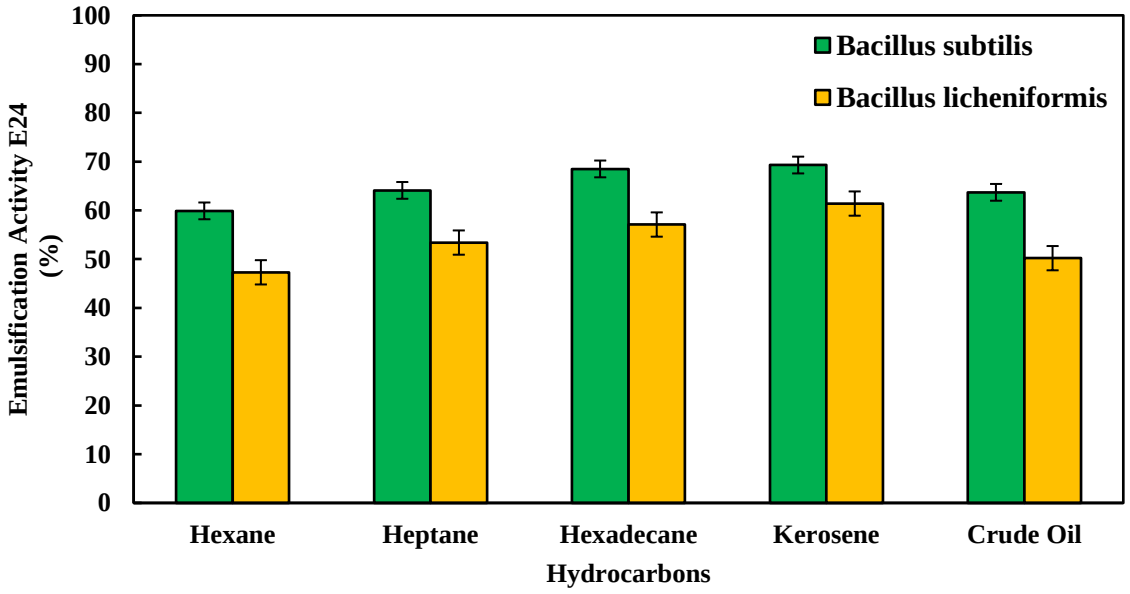

Figure 4-15. Emulsification indices E24 of the biosurfactants produced by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field against different hydrocarbons.

4.5 Wettability Alteration

The measurements of contact angle could give a considerable indication of the wetting tendencies of the surfaces and fluids. The contact angle measurements of the oil drop on the sandstone surface submerged in the aqueous solution of the produced biosurfactants and the new proposed medium "H" at different time intervals (6, 12, 24 h) are shown in Figure 4-16. It was found that the contact angle of the oil drop on the sandstone surface decreased from 104.96° when submerged initially in the aqueous solution of the biosurfactant produced by *Bacillus subtilis* to 85.40° after 24 h. Similarly, the aqueous solution of the biosurfactant produced by *Bacillus licheniformis* also significantly decreased the oil drop contact angle from 107.30° to 88.72° after 24 h. It was also found that the contact angle of the oil drops obtained from AL QADR and AL FADL oil fields submerged in the new proposed medium "H" was decreased from 112.30° and 110.90° to 63.85° and 69.33° after 24 h, respectively. Anderson (1986) reported the generally accepted wetting classification, where 0^o-75^o is a water-wet surface, 75^o-115^o is an intermediate-wet surface, and 115^o-180^o is an oil-wet surface (Anderson, 1986). Therefore, the obtained results in this study reveal that the oil is more detached from the sandstone surface, which means that the wettability of the sandstone surface has been modified toward more water-wet. Al-Sulaimani *et al.* (2012) reported that biosurfactants produced by *B. subtilis* W19 changed the contact angle of distilled water from 70.6° to 25.32° at 0.25% (w/v) biosurfactant (Al-Sulaimani et al., 2012). Al-Wahaibi *et al.* (2014) reported that the biosurfactant produced by *B. subtilis* B30 in glucose or molasses-based minimal media changed the contact angle of a hydrophobic surface from 58.7° to 28.4° and 27.2°, respectively (Al-wahaibi et al., 2014).

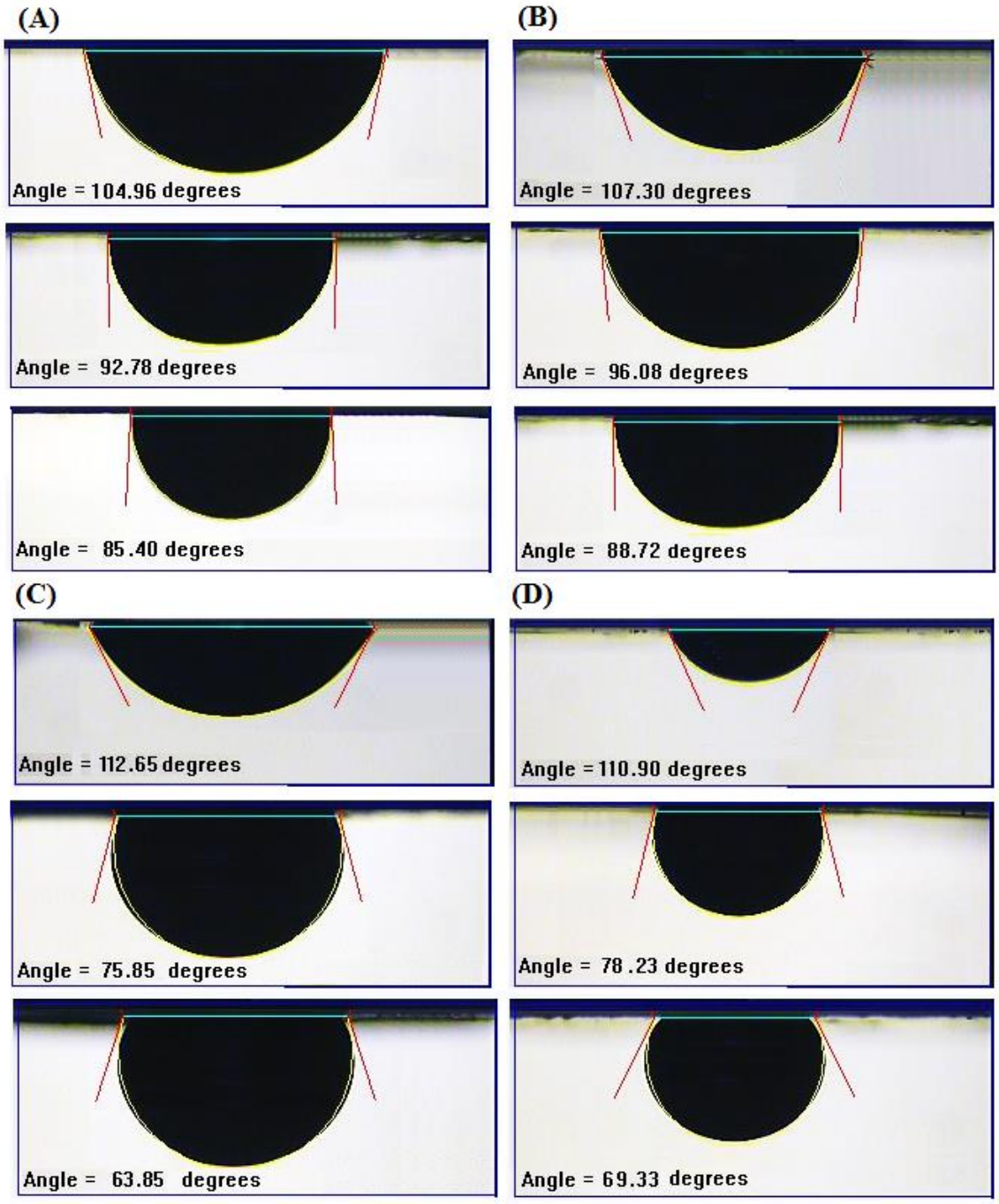

Figure 4-16. Contact angle measurements of oil drop in a contact of sandstone core surface submerged in (A) biosurfactant produced by *Bacillus subtilis* isolated from WD3 field (B) biosurfactant produced by *Bacillus licheniformis* isolated from WD2 field (C) the new proposed medium "H" to grow the *Bacillus subtilis* isolated from WD3 field exists in the oil drop and (D) the new proposed medium "H" to grow the *Bacillus licheniformis* isolated from WD2 field exists in the oil drop at different time intervals (6, 12, 24 h).

It was also found that the contact angle measurements of the oil drop submerged in the new proposed medium "H" showed a greater alteration of wettability compared to the results obtained from the oil drop submerged in the produced biosurfactants as shown in Figure 4-17. Several authors suggested that there were different mechanisms contributing to the alteration of wettability by microbial treatment such as biosurfactant adsorption and bacterial mass attaching to the grain surfaces which lead to biofilm formation (Afrapoli et al., 2009; Al-Sulaimani et al., 2012; Gandler et al., 2006; Karimi et al., 2012; Sarafzadeh et al., 2013; Shabani Afrapoli et al., 2010; Zargari et al., 2010). Consequently, the obtained results reveal that the production of the biosurfactant by growing the bacterial cells that already exist in the oil drop of the fields of interest using the new proposed medium "H" could be responsible for much better results of wettability alteration of the rock surface by biosurfactant adsorption and bacterial mass attaching to the grain surfaces which lead to biofilm formation. In this study, besides all the benefits of the biosurfactants produced by the selected bacterial strains *Bacillus subtilis* and *Bacillus licheniformis* to increase the surface activity by reducing surface tension and interfacial tension and increase the emulsification activity by increasing emulsification index E24, they also have the potential of wettability alteration by changing the wettability of sandstone rock surface toward more water-wet. It could be concluded that the produced biosurfactants could play a significant role in enhancing oil recovery at field-scale applications.

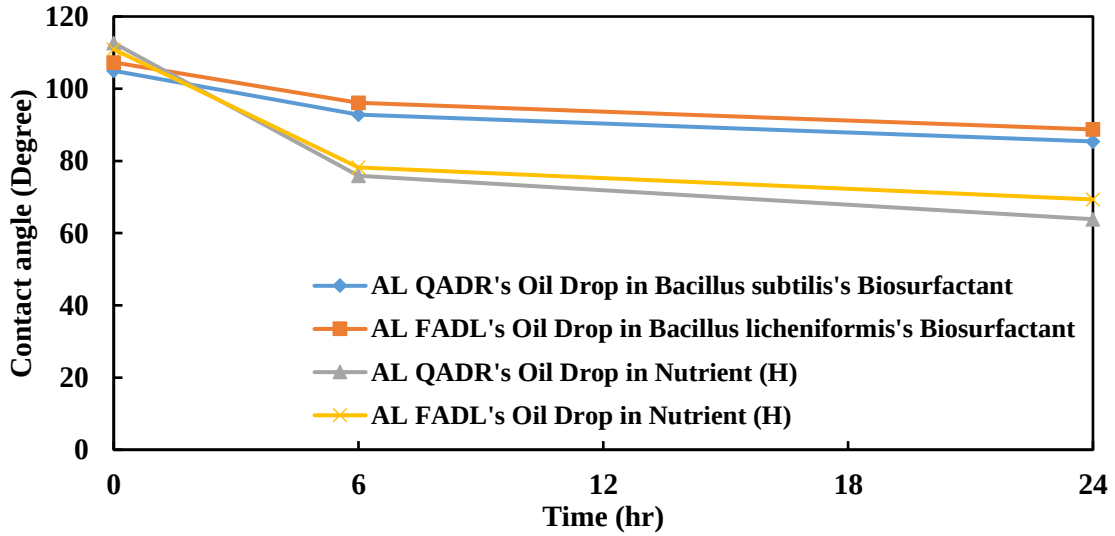

Figure 4-17. The comparison between the contact angle measurements of oil drop submerged in biosurfactants and the new proposed medium "H" at different time intervals (6,12, 24 h).

4.5.1 Stability Studies

Temperature, pH, and salinity are the most important factors of reservoir conditions that can affect the activity of biosurfactants in the MEOR process. Therefore, biosurfactants should have stability in the harsh environment of reservoirs to maintain as much activity as possible.

4.5.1.1 Effects of Temperature on Biosurfactant Stability

The cell-free biosurfactants aqueous solution at CMC were exposed to different conditions, to study the stability of the produced biosurfactants. It was found that no significant change in surface activity over a wide range of temperatures up to 90^{o}C as shown in Figure 4-18. This result is aligned with temperature stability reports (Makkar et al., 1997), (Joshi et al., 2008b), (Randhir S. Makkar and Cameotra, 1997). It was also noticed that the emulsification activity of the produced biosurfactant was slightly affected by temperature, where the emulsification index E24 of the biosurfactant produced by *Bacillus subtilis* decreased from 69% to 63% when the temperature was increased from 30◦C to 90◦C. Similarly, the emulsification index E24 of the biosurfactant produced by *Bacillus licheniformis* decreased from 61% to 53%, when the temperature was increased from 30◦C to 90◦C. Liu *et al.* (2021) reported that the emulsion activity decreased with increasing temperature. When the temperature increased, the increase in thermal motion caused the irregular arrangement of surfactant molecules on the interface, which leads to a decrease in the strength of the interface film and a decrease in the resistance of droplets to coalescence, making it easier to coalesce; on the other hand, increase in temperature reduces the cohesive force between molecules, which leads to the strengthening of the thermal movement of the droplets, and the probability of coalescence increases.

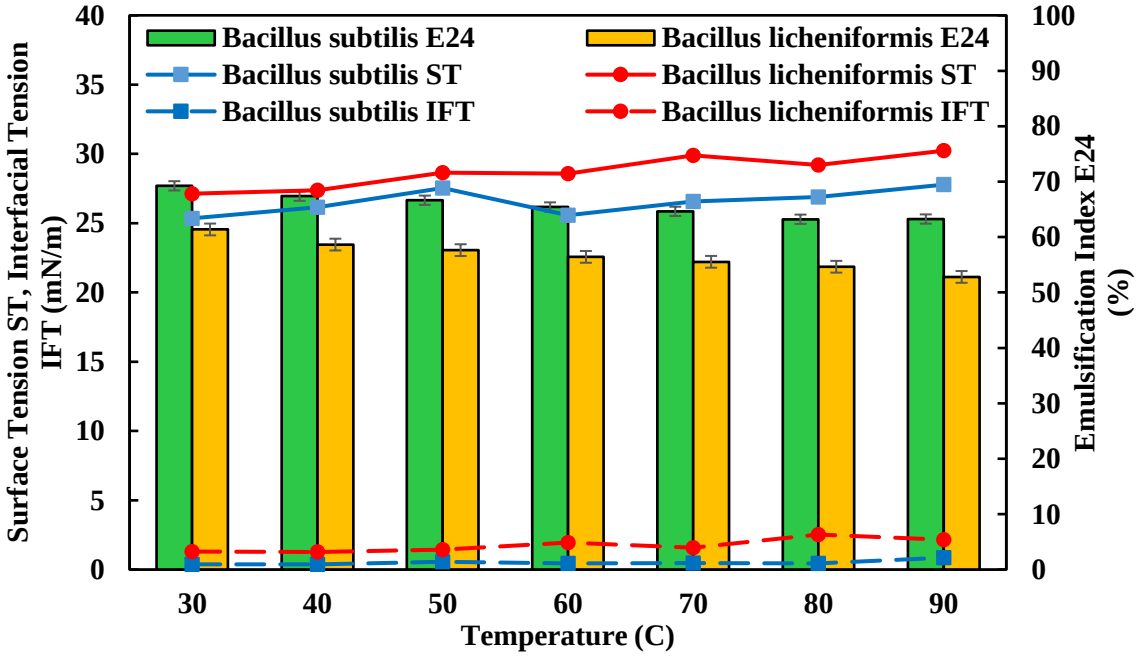

Figure 4-18. The comparison between the stability of biosurfactants produced by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field at different temperatures (30-90°C).

The long-term thermal stability of the produced biosurfactants at high temperatures was also examined at 60°C (temperature of fields of interest) over 168 h (7 days) of formation. The effect of time on the values of emulsion volume and emulsion stability of the aqueous solution containing the produced biosurfactant is shown in Figure 4-19 and Figure 4-20, respectively. It was found that the stability of the oil-water emulsion formed by the biosurfactant was affected by time. The emulsification stability of the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* was 72% and 76%, respectively, after 168 h (7 days) of formation as shown in Figure 4-20. Willumsen and Karlson reported that biosurfactants show a significant emulsification power when they could maintain at least 50% of the original emulsion volume for 24 h after formation (Willumsen and Karlson, 1996). In this study, the oil-water emulsion formed by the produced biosurfactants retained more than 70% of its original emulsion volume after 168 h (7 days) of formation. It was also noticed that the emulsion volume significantly decreased during the first 96 h. However, it almost stabilized after that, which was in agreement with Liu *et al.* (2021) who confirmed that the emulsion formed by the biosurfactant had a certain regularity in the time

distribution under the influence of temperature (Liu et al., 2021). Consequently, the conducted long-term thermal stability study reveals that the produced biosurfactants are thermostable and have high emulsification power for stabilizing crude oil-formation water aqueous solution at high temperatures.

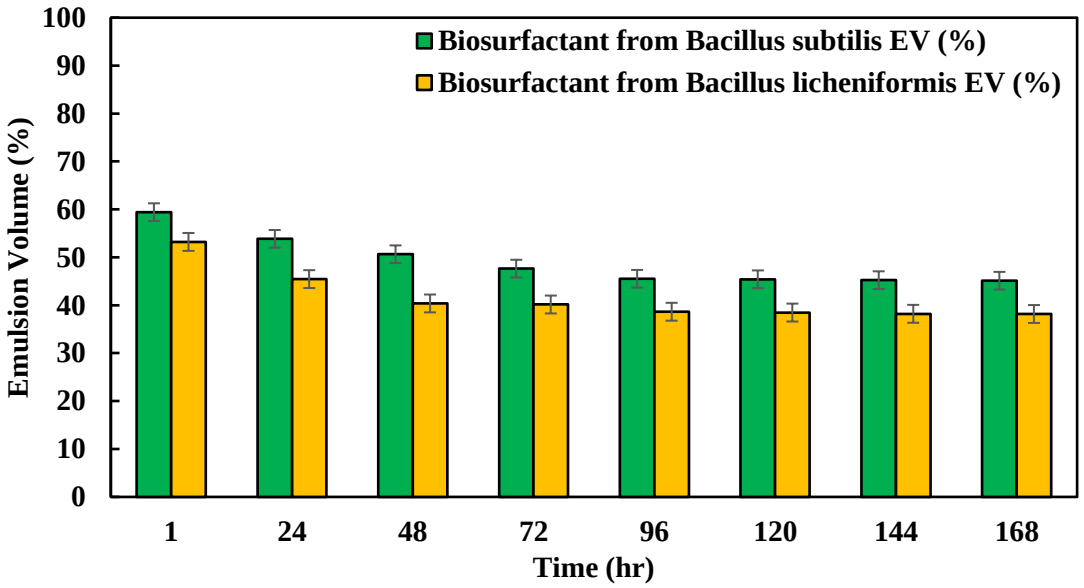

Figure 4-19. The comparison between the emulsion volume of aqueous solutions containing biosurfactants produced by *Bacillus licheniformis* isolated from WD3 field and *Bacillus subtilis* isolated from WD3 field at 60ºC for a long-term time interval.

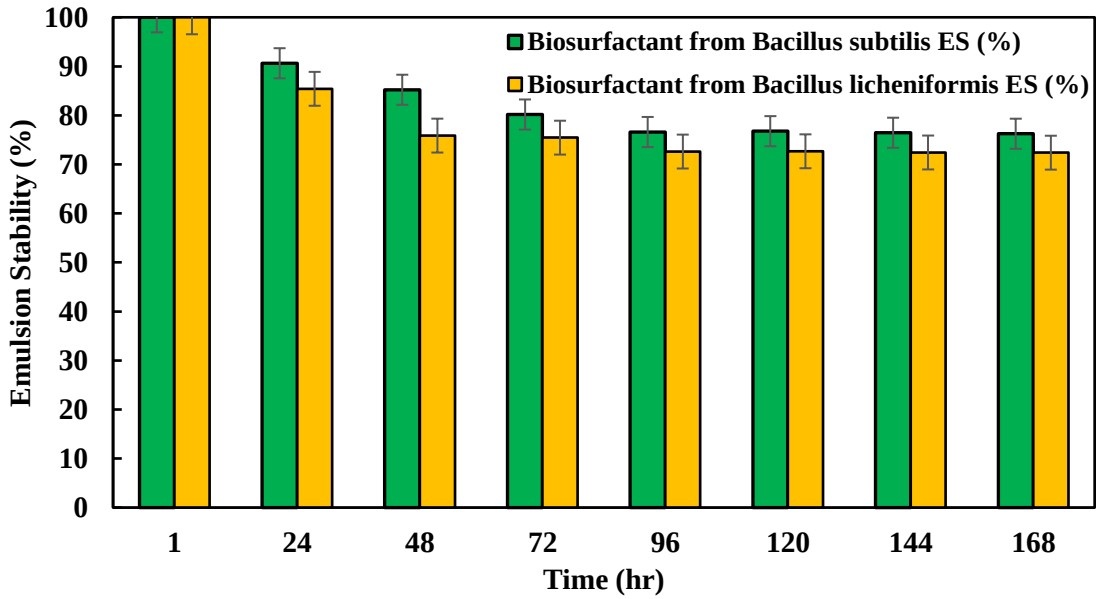

Figure 4-20. The comparison between the long-term thermal emulsion stability of aqueous solutions containing biosurfactants produced by *Bacillus licheniformis* isolated from WD2 field and *Bacillus subtilis* isolated from WD3 field at the temperature of the field of interest.

4.5.1.2 Effects of Salinity on Biosurfactant Stability

The surface activity was marginally affected by increasing NaCl concentration up to 2% (w/v) as shown in Figure 4-21. The effect of salinity was significantly observed at higher NaCl concentrations (4–10%) (w/v), but then the surface activities were stabilized at concentrations of higher salinity (12-20%) (w/v). The emulsification activity was also affected by salinity. It was found the emulsification index E_{24} slightly decreased at low NaCl concentrations up to 2% (w/v) as shown in Figure 4-21. However, the emulsification index E_{24} rapidly decreased, when the NaCl concentration was increased up to 10% (w/v). Then, the downward trend of the emulsification index slowed down at higher NaCl concentrations (12-20%), and the emulsification activity became almost stable. Liu *et al.* (2021) reported that the low concentration of NaCl had little effect on the emulsification activity because a certain concentration of electrolyte ions was conducive to the formation of an electric double layer of this emulsion and helped the occurrence of emulsification. However, when the electrolyte ion concentration was too high, the electric double layer may be compressed and the electric double layer would become thinner, resulting in a decrease in system stability (Liu et al., 2021). However, the produced biosurfactants in this study retained more than 60% of their surface and emulsification activity at the highest salt concentrations.

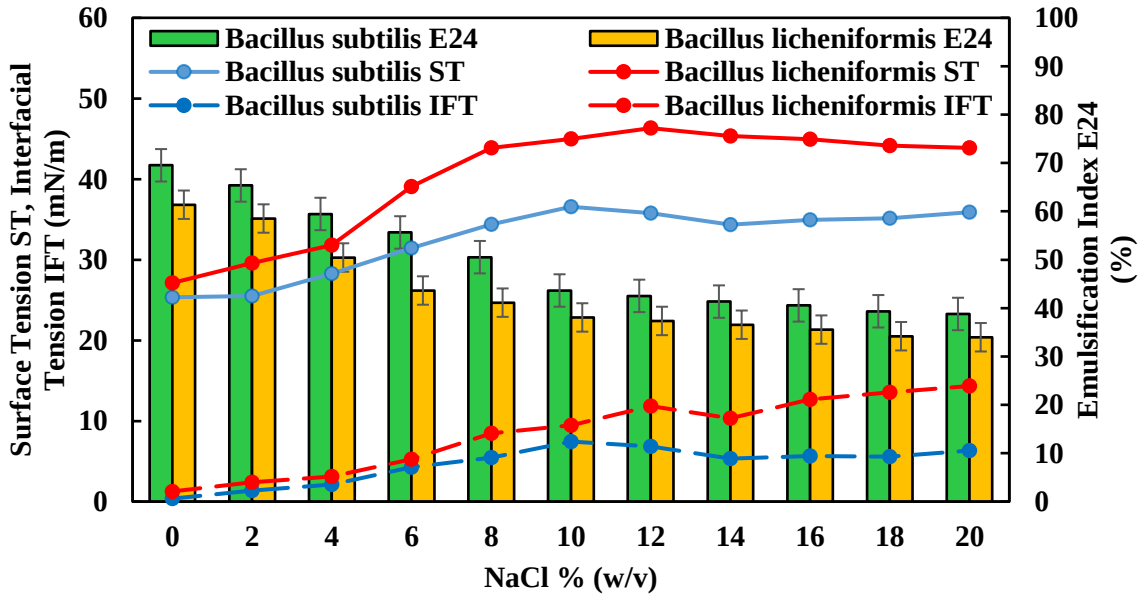

Figure 4-21. The comparison between the stability of biosurfactants produced by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field at different salinities (0-20% (w/v) NaCl concentration).

4.5.1.3 Effects of pH on Biosurfactant Stability

The effect of pH on the surface activity and emulsification activity of the produced biosurfactants is shown in Figure 4-22. It was found that the produced biosurfactants showed their optimum surface activity and emulsification activity at pH 7 (neutral value). There was no significant change in the surface activity of the produced surfactant at pH values range 8-12 (alkaline range), which was in agreement with several reports that confirmed the stability of biosurfactants an alkaline medium (Hossein Ghojavand et al., 2008), (Joshi et al., 2008b), (Batista and Mounteer, 2006), (Gudiña et al., 2010). However, the surface activity and emulsification activity were significantly decreased in the pH range 2-4 because the biosurfactants settle out of the solution as a precipitate under acidic conditions (not soluble). Consequently, the biosurfactants lose their ability to increase the surface activity and emulsification activity due to the precipitation and structural distortion that occurred in acidic conditions. Gudina *et al.* (2010) described the behaviour of biosurfactants in acidic conditions. They reported that the decrease in surface activity could be due to the presence of negatively charged groups at the polar ends of the molecules (Gudiña et al. et al., 2010). furthermore, Dhasayan *et al.* (2014) reported that acidic conditions had a more significant effect on the emulsification activity of biosurfactants due to the coagulation of biosurfactants (Dhasayan et al., 2014). It could be concluded that the produced biosurfactants could retain more than 60% of their surface activity and emulsification activity, which means that they could tolerate the harsh conditions of oil reservoirs and could be promising candidates for MEOR.

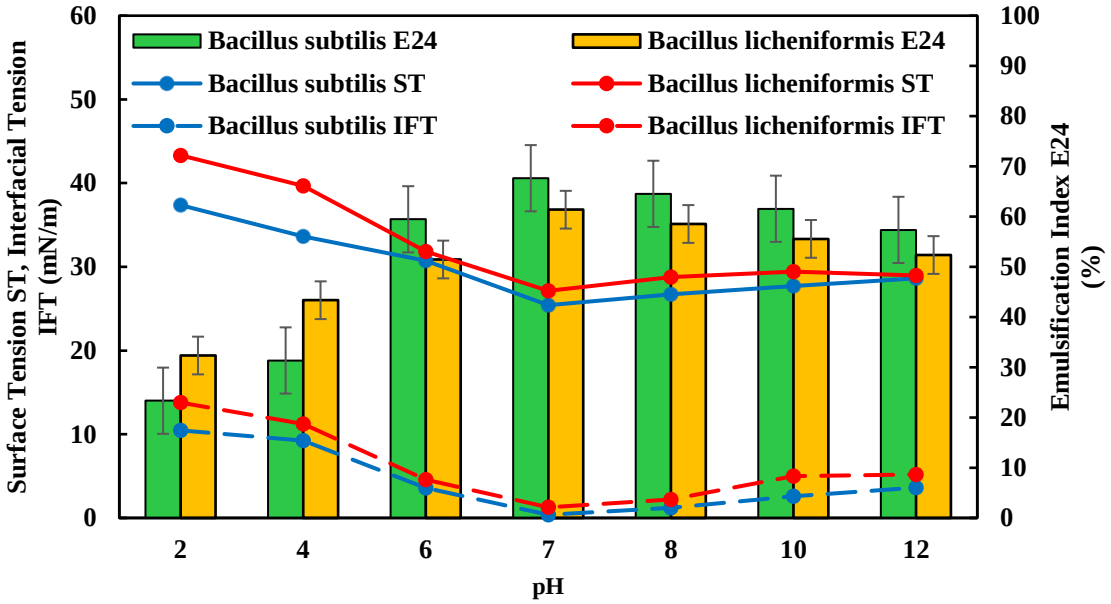

Figure 4-22. The comparison between the stability of biosurfactants produced by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field at different pH values (2-12).

4.6 Examination of Oil Recovery using Core Flooding

The biosurfactant flooding trials were conducted with a specific focus on studying the effect of "in-situ" microbial flooding and "ex-situ" biosurfactant flooding on improving oil recovery by reducing the interfacial tension and altering the wettability of the rock for reducing the residual oil saturation, hence improving oil recovery. All experiments were performed according to the procedures of the core flooding tests that were outlined in Chapter (3).

4.6.1 Core Properties

The selected indigenous bacterial strains *Bacillus licheniformis and Bacillus subtilis* that were used to produce the effective biosurfactants used in this study were originally extracted from the core samples WD2 and WD3, which are collected from AL FADL and AL QADR oil fields, respectively as mentioned in Table 4-5. Therefore, the sandstone core plugs used in this study were extracted from AL FADL and AL QADR oil fields to mimic reservoir conditions. AL FADL and AL QADR oil fields are located, as shown in Figure 4-23, in the eastern part of the Abu El Gharadig Basin, NEAG 1 area, Western Desert, Egypt. These fields belong to Badr El-Din Petroleum Company (BAPETCO) which operates the Northeast Abu El Gharadig Development License (NEAG1) in Western Egypt on behalf of the NEAG stakeholders, Shell Egypt N.V. (SENV), Apache, and Egyptian General Petroleum Corporation (EGPC). The oil production of these fields is mainly from middle and lower Bahariya sand reservoir that has an average reservoir depth of 1300 mbdf (MD), the reservoir temperature of 63 ^{o}C, reservoir lithology mainly consists of shale and sandstone with an average matrix density of 2.69 g/cm^3, virgin pressure around 11 MPa, Pressure gradient of 7.47 KPa/m (clear oil gradient), average porosity of 20%, and average absolute permeability 150 mD. The main properties of the used sandstone core plugs extracted from AL FADL and AL QADR oil fields (fields of interest) including porosity, absolute permeability, and general properties were measured in the laboratory and summarized in Table 4-9.

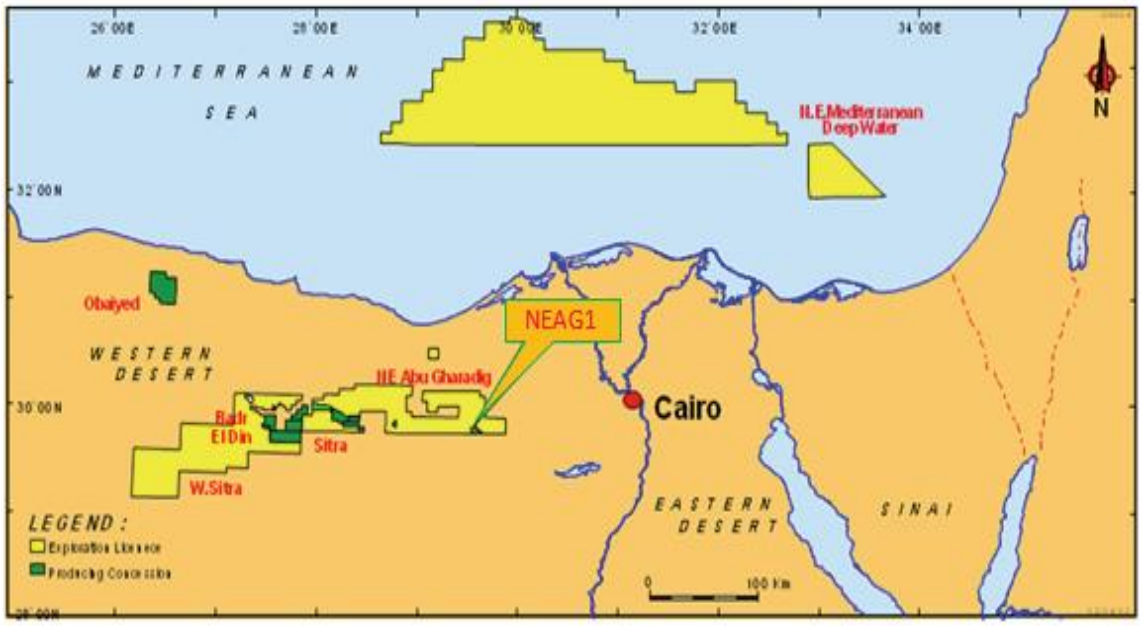

Figure 4-23. The location map of AL QADR (WD3) and AL FADL (WD2) oil fields (Bakr et al., 2010).

Table 4-9. Basic Properties of the sandstone core plugs extracted from AL FADL and AL QADR oil fields and prepared for core flooding tests.

Oil Field						
Core ID/Properties	AL QADR			AL FADL		
	C-F-Q-1	C-F-Q-2	C-F-Q-3	C-F-F-1	C-F-F-2	C-F-F-3
Length (cm)	5.32	5.42	5.43	5.42	5.32	5.52
Diameter (cm)	35.14	35.14	35.14	35.14	35.14	35.14
Bulk Volume (cm3)	51.63	52.6	52.67	52.54	51.63	53.57
Pore Volume (cm3)	10.65	10.6	10.58	10.67	10.79	10.79
Effective Porosity (%)	20.64	20.15	20.09	20.32	20.89	20.14
Absolute Permeability (mD)	205.97	179.68	187.03	133.26	142.07	155.48

4.6.2 Fluid Properties

The crude oil and formation water samples used in this study were also collected from AL FADL and AL QADR oil fields (fields of interest) to mimic reservoir conditions. The API and viscosity of the crude oil sample obtained AL QADR oil field were 41.3°, and 1.5 mPa.s, respectively. Similarly, the API and viscosity of the crude oil sample obtained from

AL FADL oil field were 40.4°, and 1.82 mPa.s, respectively. The compositions of AL QADR and AL FADL crude oil and formation water were also summarized in Table 4-10 and Table 4-11, respectively. The complete PVT analysis reports of AL QADR and AL FADL crude oil are attached in Appendix (8.2) and (8.3), respectively.

Table 4-10. Compositions of Al QADR and AL FADL crude oils by chromatograph up to C_{12}^{+}

Component	AL QADR Oil Field		AL FADL Oil Field		Liquid Density (g/cm^3)	Molecular Weight
	Stock Tank Oil		Stock Tank Oil			
	Mole %	Wt.%	Mole %	Wt.%		
Methane	0	0	0	0	0.30	16.04
Ethane	0.14	0.03	0.12	0.02	0.36	30.07
Propane	1.13	0.30	0.797	0.20	0.51	44.10
I-Butane	1.34	0.47	0.887	0.29	0.56	58.12
n-Butane	2.33	0.81	1.418	0.46	0.58	58.12
I-pentane	2.66	1.15	2.340	0.94	0.62	72.15
n-Pentane	2.59	1.12	2.415	0.97	0.63	72.15
Hexane	6.96	3.60	4.832	2.31	0.66	86.18
Benzene	0.89	0.42	0.884	0.38	0.88	78.11
Heptanes	6.94	4.18	5.355	2.98	0.69	100.20
Toluene	1.34	0.74	1.535	0.78	0.87	92.14
Octane	10.25	7.03	8.476	5.37	0.71	114.23
Ethylbenzene	0.40	0.26	0.233	0.14	0.87	106.16
P, m-xylene	0.91	0.58	0.979	0.58	0.87	106.16
o-xylene	0.32	0.21	0.375	0.22	0.88	106.16
Nonanes C9	7.66	5.90	7.235	5.14	0.72	128.26
Decanes C10	6.33	5.41	6.315	4.98	0.73	142.29
Undecanes C11	5.99	5.28	6.329	5.16	0.79	147.00
dodecanes C12+	41.80	62.51	49.474	69.11	0.90	248.96
Total	100	100	100	100		

Table 4-11. Compositions of Al QADR and AL FADL formation waters.

Ion Composition	AL QADR Oil Field	AL FADL Oil Field
	Concentration (mg/l)	Concentration (mg/l)
Sodium, Na^+	38,906.56	41,359.89
Potassium, K^+	8,310.51	9,225.17
Calcium, Ca^{2+}	1,353.45	1,678.23
Magnesium, $Mg2^+$	373.57	190.78
Bromine, Br^+	2.00	1.0
Chloride, Cl^-	66,222.35	63,022.28
Sulphide, $S2^-$	5,503.71	2,774.83
Bicarbonate, HCO_3^-	1,250.57	2,636.27
Total	121,922.73	120,888.40

4.6.3 The Effect of Flow Rate on Oil Recovery

One of the main objectives of this study was also to produce effective biosurfactants that could ultimately improve oil recovery when compared with conventional water flooding. Therefore, these experiments utilized both the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* that gave the maximum surface activity, emulsification activity, and wettability alteration when grown in the new proposed medium "H" to examine their effect on improving oil recovery after conventional water flooding. Flowrate was the main parameter that required to be established to serve as a control for the initial conditions that could reach the ultimate oil recovery. Consequently, a series of core flooding runs were carried out in triplicate at flow rates of 0.25 cm^3/min, 0.50 cm^3/min and 0.75 cm^3/min to obtain the optimum injection flow rate of the biosurfactants that could reach the maximum oil recovery.

At the early stage of the water flooding, the oil recovery increased dramatically, and the oil recovery rate reached the depletion plateau of the water flooding stage after injecting 3-4 PV of formation water. Then, the water flooding continued until the injected pore volume was 5 PV, and no more oil was produced. Figure 4-24 and Figure 4-25 are the differential pressures across the core sample versus the injected pore volume for the water/biosurfactant flooding runs at different flow rates (0.25, 0.50, and 0.75 cm3/min).

The initial water flooding stage was conducted across the core samples obtained from AL QADR oil field by injecting the formation water obtained from AL QADR oil field for almost 5 PV at different flow rates of 0.25 cm3/min, 0.50 cm3/min and 0.75 cm3/min until it reached a stabilised pressure drop of almost 0.08 MPa, 0.10 MPa, and 0.16 MPa, respectively. Regarding AL FADL oil field, the initial water flooding stage was conducted by injecting the formation water obtained from AL FADL oil field for almost 5 PV at different flow rates of 0.25 cm3/min, 0.50 cm3/min and 0.75 cm3/min until it reached a stabilised pressure drop of almost 0.08 MPa, 0.10 MPa, and 0.21 MPa, respectively. Upon switching from initial water flooding to biosurfactant injection, an immediate response was observed in the oil recovery followed by an increase in the differential pressure across the core samples. The biosurfactant produced by *Bacillus subtilis* that was isolated from the crude oil sample obtained from the same field was injected at different flow rates of 0.25 cm^3/min, 0.50 cm^3/min and 0.75 cm^3/min until it reached a stabilised pressure drop of almost 0.19 MPa, 0.21 MPa, and 0.23 MPa, respectively, after injecting 4-5 PV of biosurfactant. Similarly, the stable pressure drop from biosurfactant injection of the biosurfactant produced by *Bacillus licheniformis* that was isolated from the crude oil sample obtained from AL FADL oil field was reached at 0.19 MPa, 0.22 MPa, and 0.28 MPa for injection flow rates of 0.25 cm^3/min, 0.50 cm^3/min and 0.75 cm^3/min, respectively, after injecting 4-5 PV of biosurfactant. The extended water flood was conducted for an extra 5 PV of formation water of the field of interest, but with no significant increase in pressure drop.

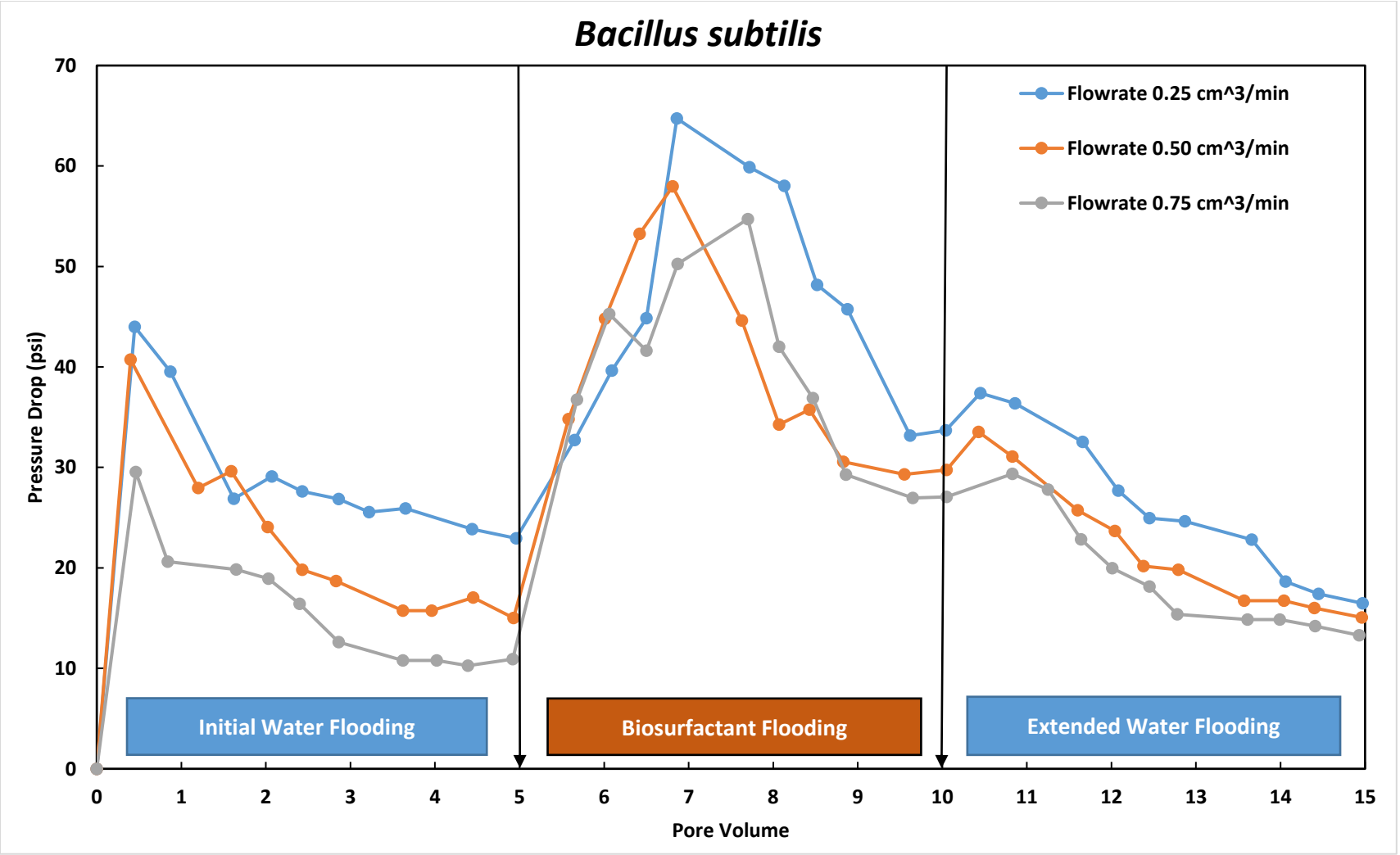

Figure 4-24. Pressure drop during core flooding runs of biosurfactant produced by *Bacillus subtilis* isolated from WD3 field at different flow rates (0.25, 0.50, and 0.75 cm^3/min).

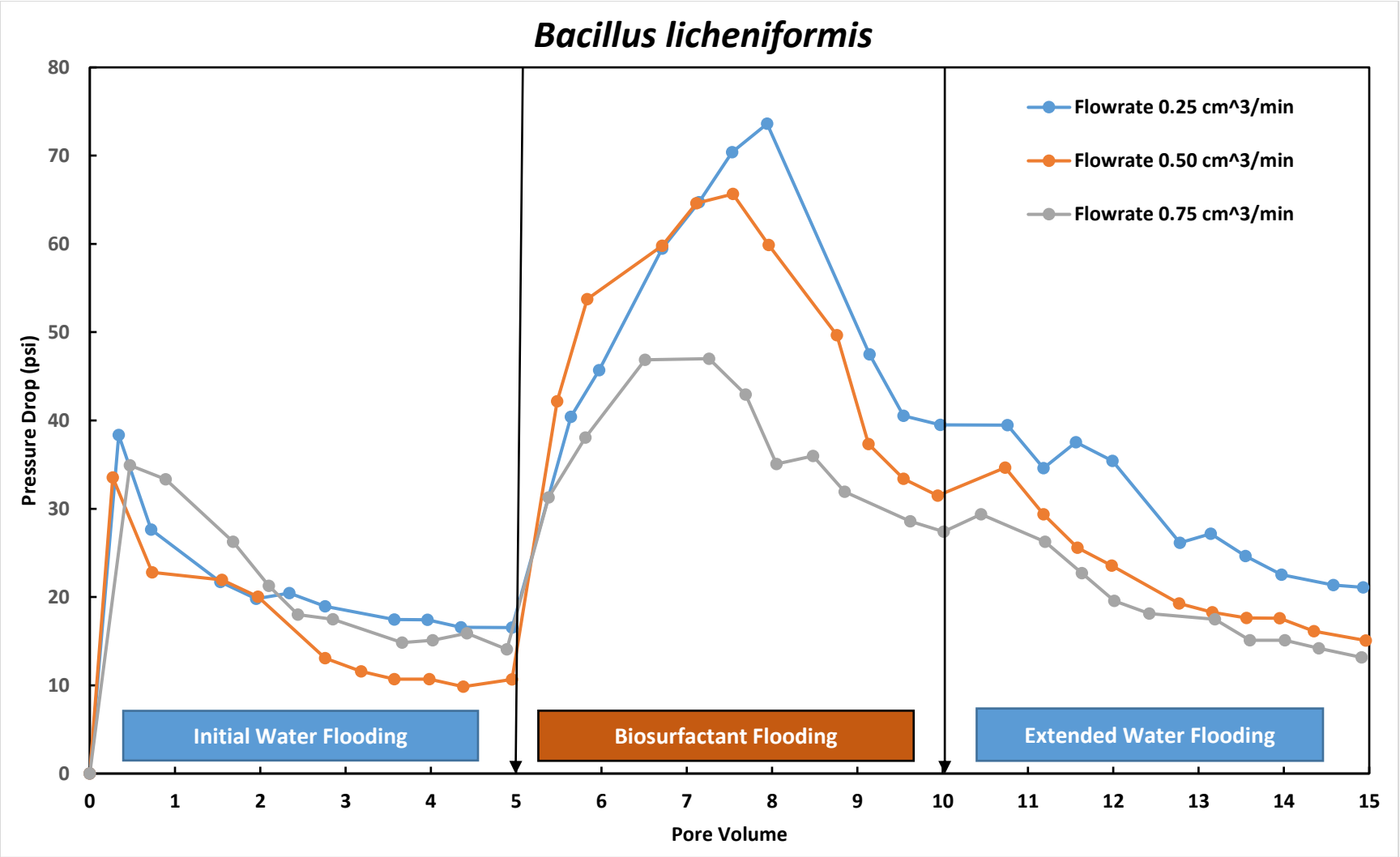

Figure 4-25. Pressure drop during core flooding runs of biosurfactant produced by *Bacillus licheniformis* isolated from WD2 field at different flow rates (0.25, 0.50, and 0.75 cm^3/min).

The effect of the biosurfactants produced by *Bacillus subtilis and Bacillus licheniformis* was examined. Figure 4-26 and Figure 4-27 show water flooding as a secondary stage followed by biosurfactant flooding as a tertiary stage using biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis*. The oil recovery after water flooding reached 40-43% of the original oil-in-place OOIP. It was also found that the maximum additional oil recovery over S_{orwf} was achieved at a flow rate of 0.25 cm^3/min. The maximum additional oil recovery recorded over the water flooding residual oil saturation was 31.41-39% after injecting 2-4 PV of the cell-free biosurfactants produced and the biosurfactant flooding was continued until reaching 5 PV of injected biosurfactant as summarized in Table 4-12. The obtained results indicate that the longer the saturation period is, the more time for the biosurfactant to redistribute in the core, and thus the more crude oil that could be produced. Extended water flooding (EWF) was carried out by injecting 5 PV of formation water after the biosurfactant flooding. However, no significant change was observed because only 1-3% of additional oil was recovered after the EWF. Al-Sulaimani *et al.* (2012) reported that 23% of residual oil was recovered by the biosurfactant produced by *Bacillus subtilis* W19 using core-flood at a flow rate of 0.5 cm^3/min (Al-Sulaimani et al., 2012). Al-Wahaibi et. al (2014) reported that the crude biosurfactant produced by *Bacillus subtilis* B30 enhanced light oil recovery by 26% and heavy oil recovery by 31% in core-flood studies at a flow rate of 0.4 cm^3/min (Al-wahaibi et al., 2014). Souayeh *et al.* (2014) reported that the biosurfactant produced by *Bacillus subtilis* W19 recovered about 13-28% of additional oil recovery over residual water-flood oil saturation from sandstone core in a core flood study under 60˚C at a flow rate of 0.4 cm^3/min (Souayeh et al., 2014). Joshi *et al.* (2016) reported that the biosurfactant production by *Bacillus licheniformis W16* was able to enhance the oil recovery using core-flood by 24–26% over residual oil saturation from sandstone core at a flow rate of 0.4 cm^3/min (Joshi et al., 2016). Liu *et al.* (2021) reported that the biosurfactant produced by *Bacillus licheniformis* L20 recovered about 14% of additional oil from sandstone core in a core-flood study under 80˚C at a flow rate of 0.5 cm^3/min (Liu et al., 2021).

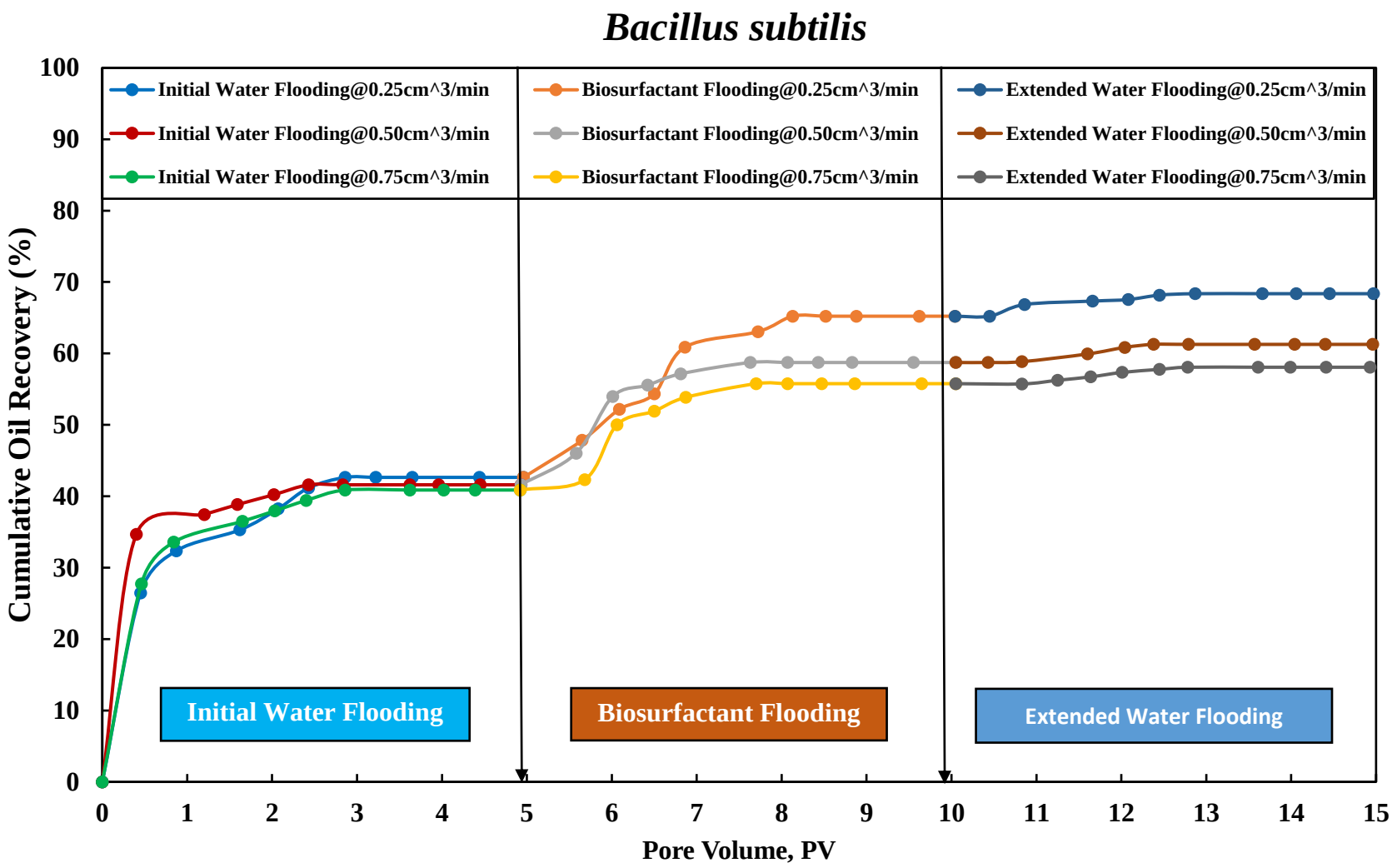

Figure 4-26. Cumulative oil recovery by biosurfactant produced by *Bacillus subtilis* isolated from WD3 field at different flow rates (0.25, 0.50, and 0.75 cm^3/min).

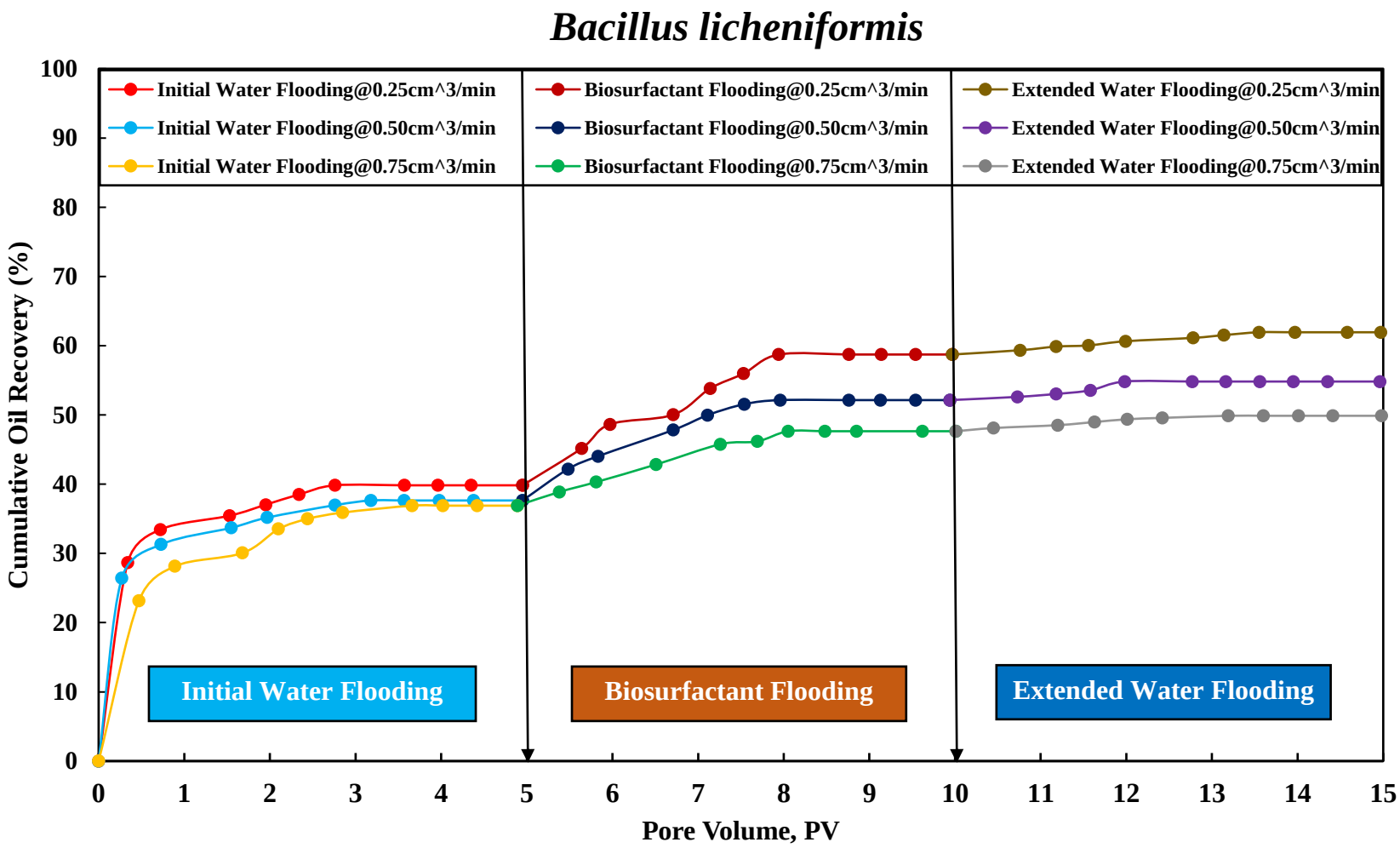

Figure 4-27: Cumulative oil recovery by biosurfactant produced by *Bacillus licheniformis* isolated from WD2 field at different flow rates (0.25, 0.50, and 0.75 cm^3/min).

The comparison between the effect of the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* at the optimum flowrate (0.25 cm^3/min) is shown in Figure 4-28. The reduction of interfacial tension played an essential role in improving oil recovery since it leads to an increase in the capillary number. Increase in capillary number decrease residual oil saturation, and hence additional oil could be recovered (Al-Anssari et al., 2019; Ali et al., 2017, 2015; Haghighi et al., 2020).

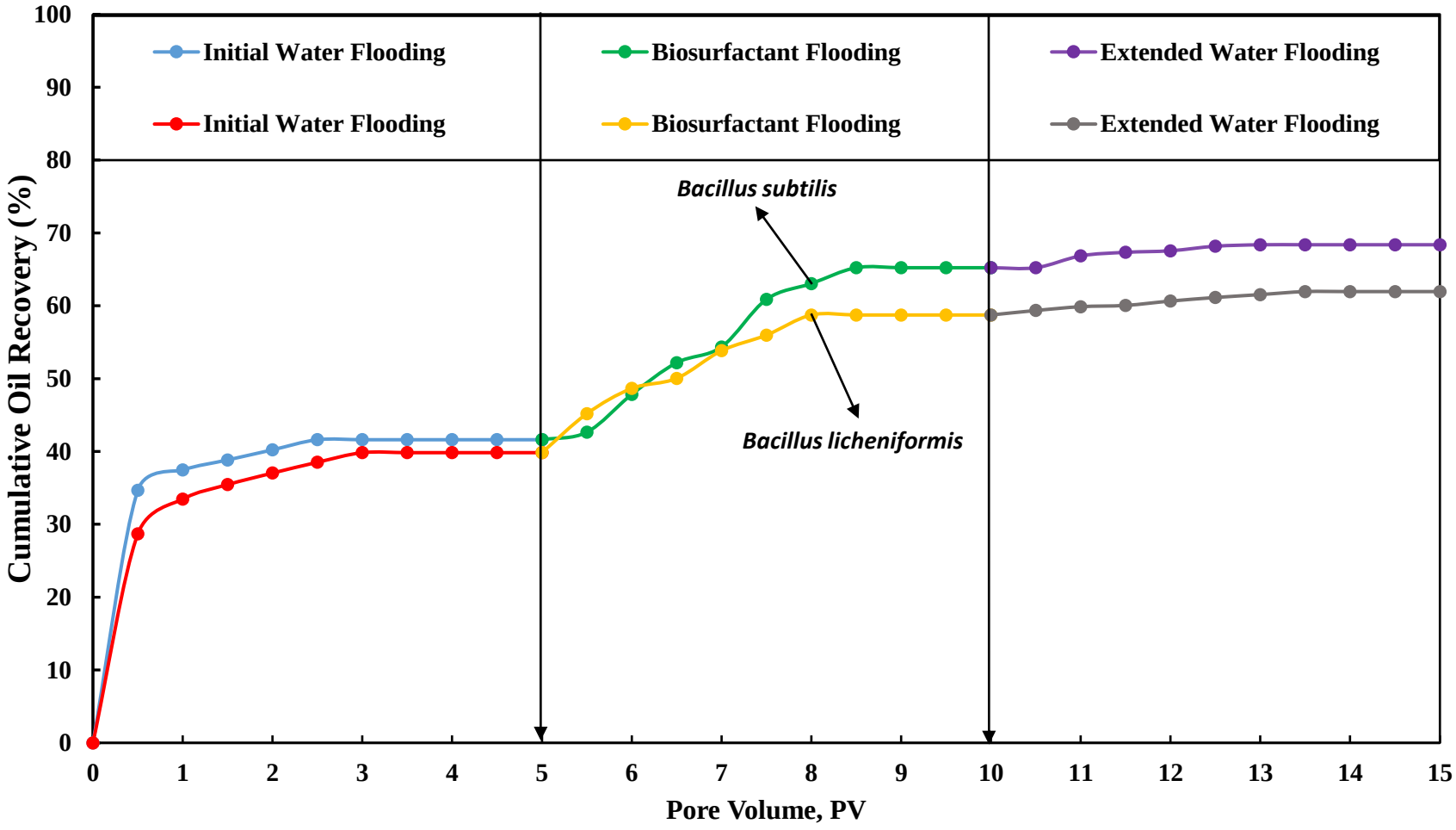

Figure 4-28. The comparison between the effect of the biosurfactants produced by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field at a flow rate of 0.25 cm^3/min.

All core flooding tests reported in the literature review and summarized in Table 2-6 recovered 6–31% of additional oil when injecting biosurfactants produced either by *Bacillus subtilis* or *Bacillus licheniformis* in sandstone cores (Al-Sulaimani et al., 2012; Al-wahaibi et al., 2014; Joshi et al., 2016; Liu et al., 2021; Souayeh et al., 2014). In this study, the additional oil recovery over S_{orwf} reached its maximum with the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* inoculated in the new proposed medium H at a flow rate of 0.25 cm^3/min compared with all reported core flooding tests. The comparison between the results of reported core flooding using biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* and the obtained result in this study are summarized in Figure 4-29. According to the findings, the longer the saturation period, the more time the biosurfactant must redistribute in the core, and hence more crude oil can be extracted. In this study, the produced biosurfactant by *Bacillus licheniformis* and *Bacillus subtilis* showed the most significant improvement in oil recovery (31-39%), which indicates that it could be an effective and promising candidate for MEOR and a promising alternative to synthetic biosurfactant.

Table 4-12. Summary of Core-Flooding Experiments Results

Oil Field	AL QADR			AL FADL		
Core ID	C-F-Q-1	C-F-Q-2	C-F-Q-3	C-F-F-1	C-F-F-2	C-F-F-3
Flow Rate (cm^3/min)	0.25	0.50	0.75	0.25	0.50	0.75
Pore Volume, PV (cm^3)	10.65	10.60	10.33	8.23	8.55	8.38
IOIP (cm^3)	5.32	5.24	5.07	4.74	4.92	4.79
Initial Oil Saturation, S_{oi} (%)	49.95	49.43	49.08	57.57	57.56	57.17
Initial Water Saturation, S_{wi} (%)	50.05	50.57	50.92	42.43	42.44	42.83
Oil Recovered after IWF, S_{orwf} (cm^3)	2.27	2.18	2.07	1.67	1.84	1.79
Residual Oil Saturation after IWF, S_{or} (cm^3)	3.05	3.06	3.00	3.07	3.08	3.00
Residual Oil Saturation after IWF, S_{or} (%)	28.65	28.87	29.02	37.29	36.04	35.81
Oil Recovery Factor after IWF, OR_{wf} (%)	42.65	41.61	40.88	35.23	37.40	37.37
Oil Recovered after BF, S_{orbf} (cm^3)	1.20	0.90	0.76	0.94	0.75	0.53
Residual Oil Saturation after BF, S_{or} (cm^3)	1.85	2.16	2.24	2.13	2.33	2.47
Residual Oil Saturation after BF, S_{or} (%)	38.68	40.97	41.77	46.16	48.79	50.85
Oil Recovery Factor after BF, ORF_{bf} (%)	22.57	17.12	14.89	19.83	15.24	11.06
Additional Oil Recovery after BF, AOR_{bf} (%)	39.35	29.32	25.19	30.62	24.35	17.67
Oil Recovered after EWF, S_{orEWF} (cm^3)	0.06	0.05	0.04	0.04	0.03	0.02
Residual Oil Saturation after EWF, S_{or} (cm^3)	1.79	2.11	2.20	2.09	2.30	2.45
Residual Oil Saturation after EWF, S_{or} (%)	1.13	0.95	0.79	0.84	0.61	0.42
Additional Oil Recovery after EWF, AOR_{EWF} (%)	3.24	2.31	1.78	1.88	1.29	0.81

IWF Initial Water Flooding
BF Biosurfactant Flooding
EWF Extended Water Flooding

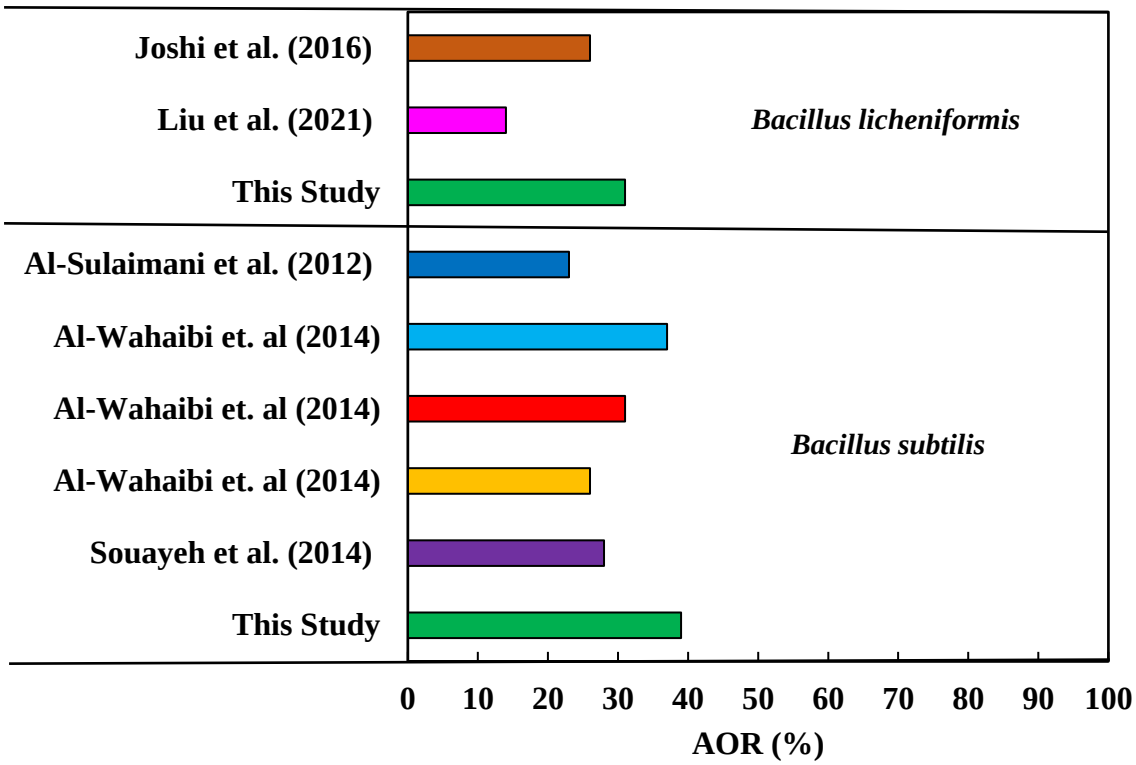

Figure 4-29: Comparison between the results of reported core flooding using biosurfactants produced by *Bacillus subtilis* isolated from WD3 field and *Bacillus licheniformis* isolated from WD2 field and the obtained result in this study.

4.6.4 The Effect of "In-situ" Microbial Flooding and "Ex-situ" biosurfactant Flooding on Oil Recovery after "Shut-in"

The core flooding tests conducted in this section focused on investigating the effect of both wettability alteration and the reduction of interfacial tension mechanisms on improving oil recovery. Based on the results of interfacial tension and contact angles measurements, the time required for the inoculated bacterial strains to reach their maximum reduction values of interfacial tension and ultimate wettability alteration was after 24 h of incubation. Consequently, the effect of microbial treatment in improving oil recovery was studied by injecting 1 pore volume of the new proposed medium "H" in the core plug after the initial water flooding stage and then implementing a shut-in for 24 h to simulate "in-situ" microbial flooding process followed by extended water flooding by injecting 5 PV of formation water at the optimum flow rate obtained in the previous section (0.25 cm^3/min). Similarly, the "ex-situ" biosurfactant flooding process was simulated by injecting 1 pore volume of the produced biosurfactants in the core plug, after the initial water flooding stage, and then implementing a shut-in for 24 h followed by extended water flooding by injecting 5 PV of formation water.

The comparison between the results obtained from the "in-situ" microbial flooding and the "ex-situ" biosurfactant flooding produced by *Bacillus subtilis* and *Bacillus licheniformis* is shown in Figure 4-30 and Figure 4-31, respectively. It was found that the cumulative oil recovery obtained during the "in-situ" microbial flooding was more than the cumulative oil recovery obtained during the "ex-situ" biosurfactant flooding. The reduction of interfacial tension was the dominant mechanism responsible for improving oil recovery efficiency during the "ex-situ" biosurfactant flooding with less effect of wettability alteration due to only biosurfactant adsorption. On other hand, wettability alteration was significantly responsible for the improvement of oil recovery during the "in-situ" microbial flooding due to not only biosurfactant adsorption, but also bacterial mass attached to the grain surfaces leading to biofilm formation. Consequently, wettability alteration and interfacial tension reduction were the dominant mechanisms responsible for the improvement of oil recovery during the in-situ flooding runs. In this study, the obtained results reveal that the presence of bacterial cells in the in-situ runs plays a significant role in altering the wettability,

which means it could be implemented as one of the main mechanisms of wettability alteration that have the potential to improve oil recovery during MEOR processes.

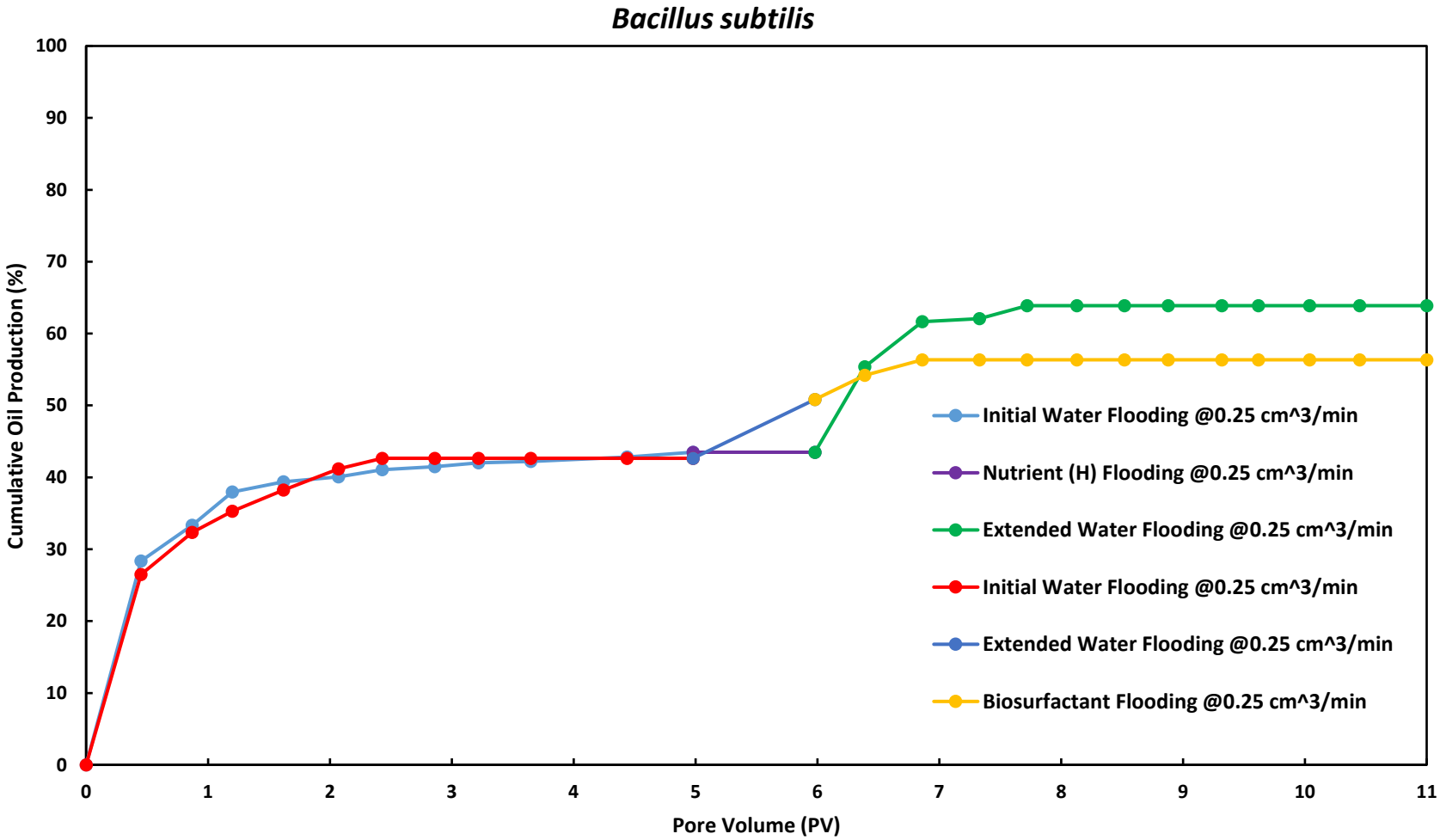

Figure 4-30. Comparison between the effect of "In-situ" Microbial Flooding and "Ex-situ" biosurfactant Flooding using biosurfactant produced by *Bacillus subtilis* isolated from WD3 field on Oil Recovery after a "Shut-in" period of 24 h at a flow rate of 0.25 cm^3/min.

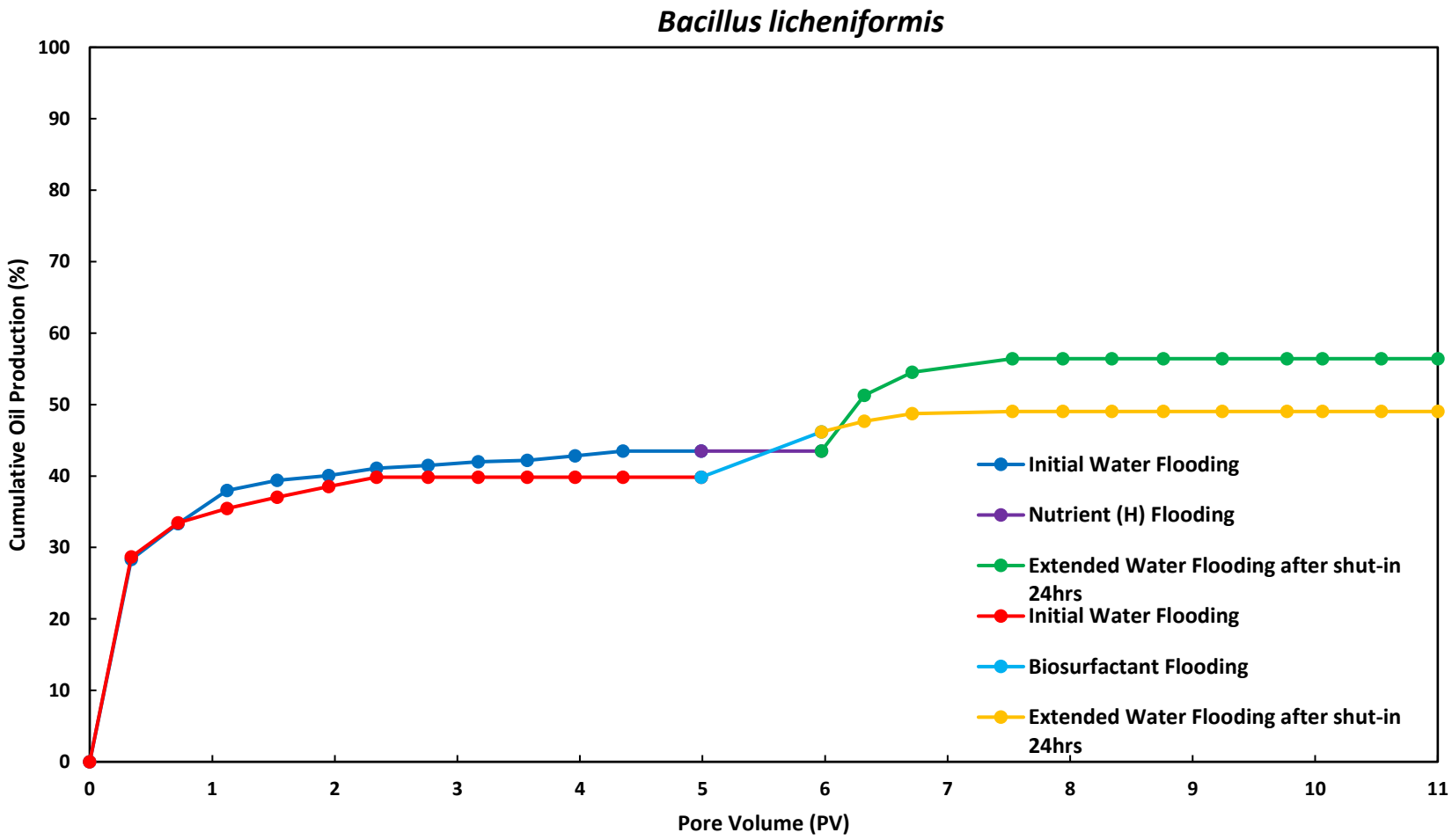

Figure 4-31. Comparison between the effect of "In-situ" Microbial Flooding and "Ex-situ" biosurfactant Flooding using biosurfactant produced by *Bacillus licheniformis* isolated from WD3 field on Oil Recovery after a "Shut-in" period of 24 h at a flow rate of 0.25 cm^3/min.

It can be concluded that the results obtained from the three conducted core flooding scenarios that simulate the continuous biosurfactants flooding without a shut-in period, the "in-situ" microbial flooding with a shut-in period of 24 h, and the "ex-situ" biosurfactants flooding with a shut-in period of 24 h show that the reduction of interfacial tension is dominant mechanism in the scenario of continuous biosurfactants flooding without a shut-in period and the scenario of "ex-situ" biosurfactant flooding with a shut-in period of 24 h. On the other hand, wettability alteration and reduction of interfacial tension mechanisms contribute together to reducing residual oil saturation and improving oil recovery during the shut-in period. However, the effect of wettability alteration on the whole improvement of oil recovery during the "in-situ" shut-in run is greater than the effect of interfacial tension reduction. While during the "ex-situ" run, the contact angle measurements show fewer changes in the wettability and no wettability alteration occurred during the continuous biosurfactants flooding because it is a more time-consuming process. Several studies reported the relationship between interfacial tension reduction and alteration of wetting conditions following microbial treatment (Kowalewski et al., 2006; Zargari et al., 2010; Zekri et al., 2003). Kowalewski *et al.* (2006) reported core flooding studies investigating the effect of interfacial tension reduction and wettability alteration mechanisms on reducing residual oil saturation. These reported studies showed that both gradual reductions in interfacial tension and wettability alteration could be used to reduce residual oil saturation. They also suggested that the reduction in interfacial tension may change the oil/rock contact and the change in wetting properties is dependent on the initial wetting condition where wettability could be altered from oil-wet toward more water-wet and vice versa (Kowalewski et al., 2006). Afrapoli *et al.* (2009) reported that the wettability alteration affects the fluid distribution in the rock and has a strong influence on the spontaneous-imbibition process for oil recovery. They also reported that microbial treatment has shown to change the wetting conditions of core samples leading to a change in the imbibition/drainage behaviour, hence improving oil recovery (Afrapoli et al., 2009).

The next chapter will discuss the environmental risk assessment of the selected bacteria that produced the biosurfactants in this work.

CHAPTER 5

ENVIRONMENTAL RISK ASSESSMENT

Outline of the chapter

This chapter assesses the environmental risk and possible threats of the selected bacteria for producing biosurfactants to the environment. The chapter is organised as follows:

5.1. Introduction

5.2. Oil and gas production wastewater

5.3. Produced water

5.4. Risk matrices

5.5. *Bacillus licheniformis*

5.6. *Bacillus subtilis*

5. ENVIRONMENTAL RISK ASSESSMENT

5.1 Introduction

The environment is a wide concept that defines our biophysical surroundings including air, water, land, and living organisms like plants and wildlife. The environmental risk assessment is a scientific process of identifying and evaluating environmental threats, especially those that relate to living organisms, habitats, and ecosystems. Environmental threats come in many forms; some are natural such as earthquakes and volcanic eruptions, while others such as air pollution, toxic chemicals in food and water, and sanitation, could be the result of human activity (industrialization and urbanisation). Individual companies in the petroleum industry are responsible for conducting risk assessments on the chemicals used during a recovery process, because utilizing these products may pose a risk to the environment (Raz and Hillson, 2005).

In this work, the risk assessment on including ecological and human risk assessments will be examined. This evaluation aims to determine the effects and likelihood of environmental hazards that may arise because of activities, such as bacteria culture in an insufficiently controlled environment or wastewater that has not been treated. There are four steps to these evaluations starting, as shown in Figure 5-1, with hazard identification, being the first and most important step since it establishes the environmental risk assessment scope by defining what needs to be protected from hazards and identifying potential harmful effects. The second step is hazard characterisation, where the potential hazards and the consequences of potential harm are examined. The third step is exposure characterization, which considers the likelihood and level of exposure to the hazards, as well as the likelihood of harm. The fourth step is a risk characterisation, which estimates the level of risk by combining both consequences and likelihood of harm. In some scenarios, risk mitigation strategies are included in the environmental risk assessment. These strategies seek to mitigate risk to an acceptable level. (AIRMIC, 2002). This environmental risk assessment is performed to assure that the biosurfactants produced will not cause unacceptable environmental harm when utilised in an actual field in-situ operation.

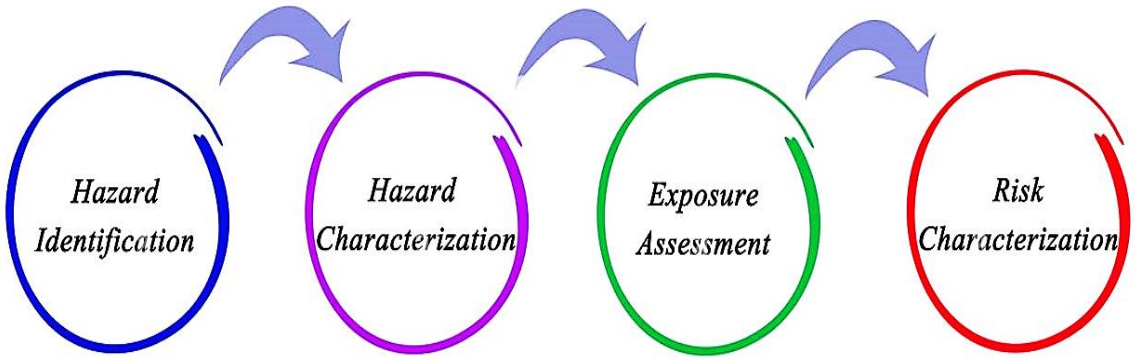

Figure 5-1. Stages of risk assessment

5.2 Oil and Gas Production wastewater

The overall ecology, particularly the wetland ecosystem, has become extremely vulnerable to the damaging impacts of wastewater contamination from the growing of the oil sector. Because produced water is so complex and diverse, there has been less emphasis and attention on water treatment since it is not often a revenue source. Because water quality is such an important aspect of an aquatic ecosystem, wastewater contamination adds another source of stress to aquatic life (Omoregie et al., 1997). Water analysis is the process of determining the state of water. Toxic contaminants in water encompass a wide range of substances that are either leached into groundwater or discharged directly into rivers. Environmental assessment is an essential aspect to consider before beginning any project so that any potential risks may be identified, and mitigation measures can be put in place.

5.3 Produced water

Almost all offshore oil produces large quantities of contaminated water that can have significant environmental effects if not handled appropriately. As oil and gas production proceeds, formation water eventually reaches the production well, and water begins to appear alongside the hydrocarbons. This produced water is a mixture of injected water, formation water, biosurfactants, and hydrocarbons. Tertiary oil recovery processes that use water injection result in the production of even more water with the oil. Most offshore platforms dispose of their produced water directly into the ocean but must meet increasingly stringent regulations on the entrained and dissolved oil and other chemicals

present in the produced water. Some offshore operators are considering reinjection of produced water to avoid meeting these expensive ocean-disposal requirements. The product of the formation and injected water is referred to as produced water.

the main emphasis of this study is the environmental impact of utilising produced biosurfactants for enhancing oil recovery. Consequently, this study will follow the guidelines of the European Federation of Biotechnology, the Canadian Environmental Protection Agency (CEPA), the US Environmental Protection Agency (EPA), and others (Canada, 2015; Edberg, 1997; Frommer et al., 1989). The produced biosurfactants will be reviewed to assess the probability of harm, define the risks of exposure, and propose measures to mitigate such risks.

5.4 Risk Matrices

Risk matrices are possibly one of the most widely utilised risk assessment tools. They are mostly utilised to figure out the size of risk and whether it is well-controlled or not. For this assessment, a bowtie diagram is used to describe the three most common areas of risk matrices, which are low probability impact, medium likelihood impact level, and high or extreme likelihood impact level (Figure 5-2):

- The low likelihood impact level (typically green) implies that an event's risk has been adequately addressed or is not high enough. This generally necessitates no action.
- The medium likelihood impact level (typically yellow) requires efforts to reduce the risk within a set timeframe. Events should be monitored and controlled to the extent that is practically possible, implying that if the risk is maintained at that level, it will be acceptable. Nevertheless, the prevention and control expenses must be considered carefully.
- The high or extreme likelihood, high impact level (typically red) is unacceptable and means that work must not begin or continue until the risk is mitigated the risk. In this case, large resources or additional control actions will be required to reduce the impact or chance (AIRMIC, 2002; Raz and Hillson, 2005).

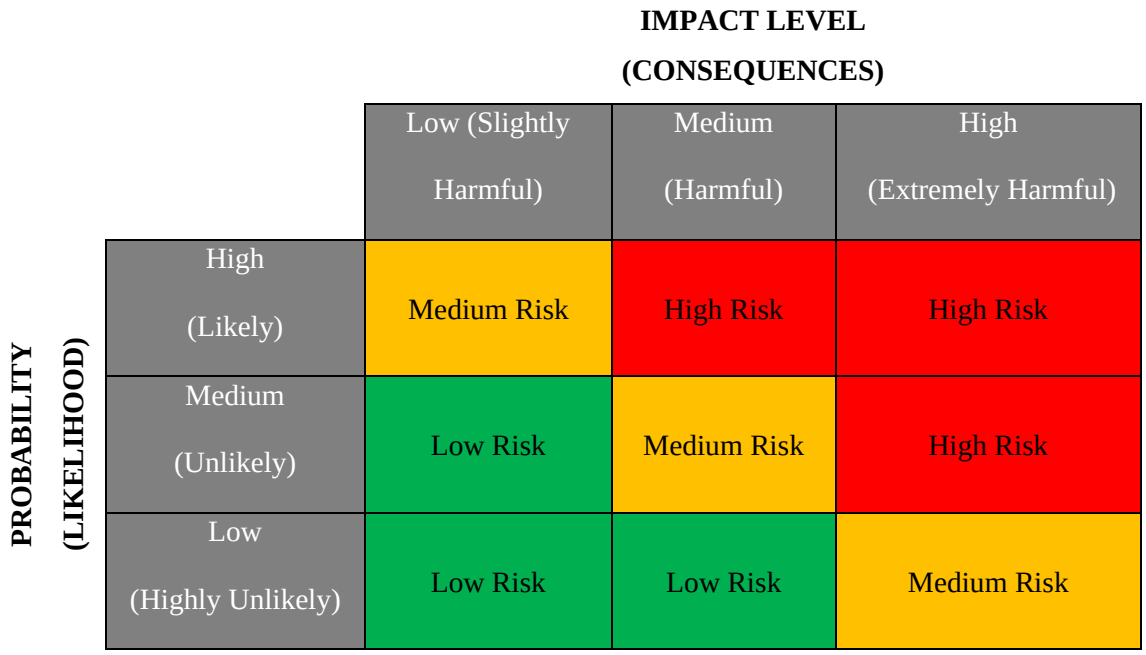

Figure 5-2. (3×3) Probability impact matrix (A. Kassem et al., 2020).

5.5 Bacillus licheniformis

Bacillus licheniformis is a saprophytic bacterium found throughout nature that is believed to aid in a nutrient cycle and has antifungal properties (Claus and Berkeley, 1986). For more than a decade, it was utilised in fermentation to produce antibiotics, proteases, amylases, and speciality chemicals, with no known detrimental effects on the environment or human health. This species can easily be distinguished from other pathogenic genus members in people and animals.

To see if any negative effects from *Bacillus licheniformis* have been reported, a series of literature searches were conducted. *Bacillus licheniformis* does not appear to be pathogenic to plants or estuarine marine organisms. Human infections with *Bacillus licheniformis*, on the other hand, occurred in immunocompromised people or after trauma. There have also been indications of a relationship between livestock abortions and *Bacillus licheniformis*. In most reported cases, few cases contributed to the relationship between *Bacillus licheniformis* and animals' immunosuppression. Since *Bacillus licheniformis* is common in the environment and seems to be an non-pathogenic microorganism, the possible risk connected with using this microorganism in the fermentation industry is in the low range.

5.5.1 Physical/Chemical Properties Assessment

Bacillus licheniformis does not release any extracellular enzymes that may make it susceptible to infection. *Bacillus licheniformis*, unlike numerous other microorganisms in the genus, does not produce toxins. Generally, *Bacillus licheniformis* has a low level of pathogenicity. Although human infection is not impossible, it is unlikely in an industrial environment where very immunocompromised persons are not present. Infection after trauma is a possibility, however in an industrial environment, with basic safety precautions, good laboratory procedures, and proper protective equipment (PPE) including protective clothes, masks, and safety goggles, the risk of employees becoming infected should be in the low range.

5.5.2 Ecological Risk Assessment

Similarly, the risks of *Bacillus licheniformis* use to the environment are in the low range. *Bacillus licheniformis* is suspected to be a cause for livestock abortion. However, these hypotheses have yet to demonstrate that this bacterium was the causative agent. *Bacillus licheniformis* infections in most of these cases occurred in animals that were already immunocompromised due to infection with other pathogens or inadequate nutrition. Immunosuppression is linked to maternal and foetal placentas in pregnant animals, allowing opportunistic bacteria to infect and cause lesions in the foetus. Even though *Bacillus licheniformis* may not be the cause of animal abortion, it has been linked to several cases. Despite this, the number of abortions caused by *Bacillus licheniformis* is insignificant compared with the overall number of livestock abortions caused by all other microbes, including fungi and viruses.

5.5.3 Human Health Risk Assessment

The utilization of *Bacillus licheniformis* for the industrial production of enzymes could not be harmful to the environment because the amount of microorganisms released by the fermentation facility is insignificant. Furthermore, *Bacillus licheniformis* is common in the environment, and the expected releases from fermentation industries under these exemption terms would not considerably expand the population of this microorganism in the environment. Consequently, even if *Bacillus licheniformis* might be linked to livestock abortions, utilization of this bacterium in fermentation industries could not significantly

raise this occurrence possibility. Even if a high-exposure scenario of releasing *Bacillus licheniformis* from the fermentation industry to livestock might be imagined.

It could be concluded *Bacillus licheniformis* is safe to employ in fermentation industries to produce biosurfactants, enzymes, and speciality chemicals. *Bacillus licheniformis*, while not fully harmless, poses a low risk of harming the environment or human health. As a result, the utilization of *Bacillus licheniformis* in the production of biosurfactants is justified without creating any harm. As shown in Table 5-1, the likelihood of risk occurrence is performed to evaluate the risk occurrence probability and its impact on positive events, as well as to reduce the chance of risk occurrence and negative events in a project. Table 5-2 and Figure 5-3 represent the risk matrix rating provided in specific for this study.

Table 5-1. Probability of occurrence of *Bacillus licheniformis.*

Descriptor	Frequency
Low	• The infection could happen to someone who is immunocompromised or after a traumatic event. • Could only happen because of a series of separate system or control failures. • Occurrence is extremely unlikely, and no poisons are produced. • There has never been such an incidence. • An environmental hazard is unlikely to occur because the amount of microorganisms emitted from the fermentation facility is in the low range • Would most likely occur because of a high amount of exposure during the fermentation process. • Infection reports like this have not been reported in the past.

Medium	• Could be caused by predisposing factors in the affected animals' immunosuppression. • It may happen in animals who are already immunocompromised due to infection by other bacteria or inadequate nutrition. • The number of reports concerning animal abortion is quite low.
High	• Occurrence is quite unlikely.

Table 5-2. Risk rating matrix of *Bacillus licheniformis*

Risk Code	Risk Description	Probability (Likelihood)	Consequences (Impact level)
R1	Environment	Low	Low
R2	Human Health	Low	Low
R3	Water	Low	Low
R4	Hazard to animal	Low	Medium
R5	Air	Low	Low
R6	Soil	Low	Low
R7	Worker Exposure	Low	Low
R8	Hazard to plant	Low	Low
R9	Virulence	Low	Low

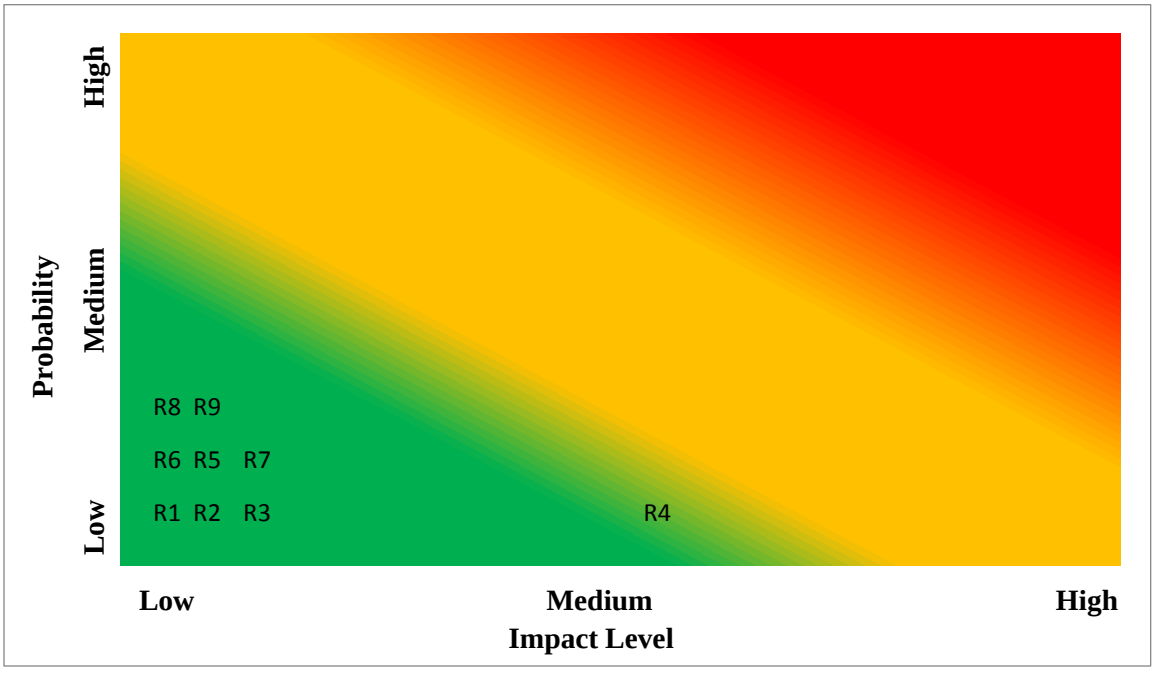

Figure 5-3. Risk assessment chart of *Bacillus licheniformis*

5.6 Bacillus subtilis

Bacillus subtilis is a saprophytic soil microorganism that is considered to have a role in nutrient cycling due to its potential of producing several kinds of enzymes. For more than a decade, this property of the microorganism has been economically exploited, and it has been used to produce antibiotics, proteases, amylases, and speciality chemicals. The US Environmental Protection Agency (EPA) investigated the production of enzymes by genetically modified *Bacillus subtilis* in fermentation facilities and found no unacceptable risks to the environment or human health. It is not pathogenic and does not have disease-causing characteristics (Edberg, 1997).

Historically, the term *Bacillus subtilis* was used to refer to all endospore-forming aerobic bacilli bacteria. Later, *Bacillus subtilis* was taxonomically grouped with two closely similar species, *Bacillus licheniformis* and *Bacillus pumilus*, to form what was called the "*subtilis* group". Recently, *Bacillus subtilis* was distinguished from these other species, due to the development of new methods. Even though it is not a frank human pathogenic microorganism, it is isolated in various cases from human infections (Edberg, 1997; USHHS, 1986).

The infections linked to *Bacillus subtilis* include endocarditis, septicaemia, bacteraemia, pneumonia, and bacteraemia. Nevertheless, these reported infections were detected in immunocompromised patients cases. Before infection with *Bacillus subtilis* could occur, the host must be immunosuppressed and then inoculated in large numbers. Several cases of food poisoning have also been linked to excessive amounts of food contaminated by *Bacillus subtilis*. The amounts of extracellular enzymes or other components produced by *Bacillus subtilis* are not that large to make it susceptible to infection. *Bacillus subtilis* is not toxigenic, unlike the other several species in the genus. *Bacillus subtilis* produces the subtilisin (extracellular enzyme), which was linked to allergy or hypersensitive reactions in people who have been exposed to it frequently (Edberg, 1997).

5.6.1 Human Health Risk Assessment

Workers should have a low risk of infection in an industrial environment if the basic safety precautions, suitable laboratory practices, and proper protective equipment (PPE),

including protective clothes, masks, and safety goggles were used, as described in Table 5-3. The likelihood of allergic reactions from prolonged exposure to subtilisin is the only human health hazard for fermentation industry workers. Generally, *Bacillus subtilis* has a low level of pathogenicity (Edberg, 1997; Ihde and Armstrong, 1973). However, the infection in humans is not impossible, it is unlikely in an industrial environment where bacterial exposure is believed to be in the low range if immunocompromised workers are not present.

Table 5-3. Probability of occurrence of *Bacillus subtilis*

Descriptor	Frequency
Low	• The infection could happen to immunocompromised people or people who have been exposed to the bacteria. It has a low pathogenicity level. • Could only happen because of a series of separate system or control failures. • Occurrence is extremely unlikely, and no toxins are produced. • There has never been such an incidence. • An environmental hazard is unlikely to occur because the amount of released microorganisms from fermentation facilities is in the low range • The risk of allergic responses or hypersensitivity with a high degree of exposure is low, which is a concern for personnel in the fermentation facility. • There have been similar instances of food poisoning linked to many *Bacillus subtilis* contaminated foods.
Medium	• Could be caused by predisposing factors in the affected animals' immunosuppression. • It may happen in animals who are already immunocompromised due to infection with other microorganisms or inadequate nutrition. • There are not many comparable reports concerning livestock abortion.
High	• Occurrence is quite unlikely.

5.6.2 Ecological Risk Assessment

the environmental risks linked to the utilization of *Bacillus subtilis* are in the low range. There have been various reports linking *Bacillus subtilis* to livestock abortions (Fossum et

al., 1985). These few reported cases show that this linking was rare, and in most cases the animals were immunocompromised. *Bacillus subtilis* is also not a causative agent and is not classified as an animal pathogen (Logan, 1988). Similarly, *Bacillus subtilis* is not considered a plant pathogen. Even though some of their produced enzymes like cellulose and polygalacturonase are linked to soft rot in plant tissue, several microorganisms can cause soft rot when inoculated below the layers of the protective epidermal. Unreasonable risks should not be posed to the environment or human health when utilising *Bacillus subtilis* in the industrial environment as evaluated in Table 5-4. Firstly, *Bacillus subtilis* poses a low risk to the environment or human health. Secondly, the amount of microorganisms released by the fermentation facility is insignificant. Furthermore, *Bacillus subtilis* is common in the environment, and the fermentation industry expected released amount will not considerably expand the bacterium's population in the environment.

Table 5-4. Risk rating matrix of *Bacillus subtilis*.

Risk Number	Risk Description	Probability (Likelihood)	Impact level (Consequences)
R1	Environment	Low	Low
R2	Human Health	Low	Low
R3	Soil	Low	Low
R4	Hazard to animal	Low	Low
R5	Food poison	Low	Medium
R6	Worker Exposure	Low	Low
R7	Hazard to plant	Low	Low
R8	Water	Low	Medium
R9	Virulence	Low	Low

Currently, there are no available specific data on the ability of released *Bacillus subtilis* to survive in the atmosphere. Due to point source releases, fermentor off-gas released air could cause nonoccupational inhalation exposures. The soil is considered the natural habitat of *Bacillus subtilis*. Consequently, long-term survival in soil could happen.

Finally, the risk associated with utilizing *Bacillus subtilis* to produce biosurfactants or speciality chemicals in fermentation industries is in the low range. Even though it is not harmless, the industrial utilization of *Bacillus subtilis* poses a low risk of harm to the environment or human health, as shown in Figure 5-4.

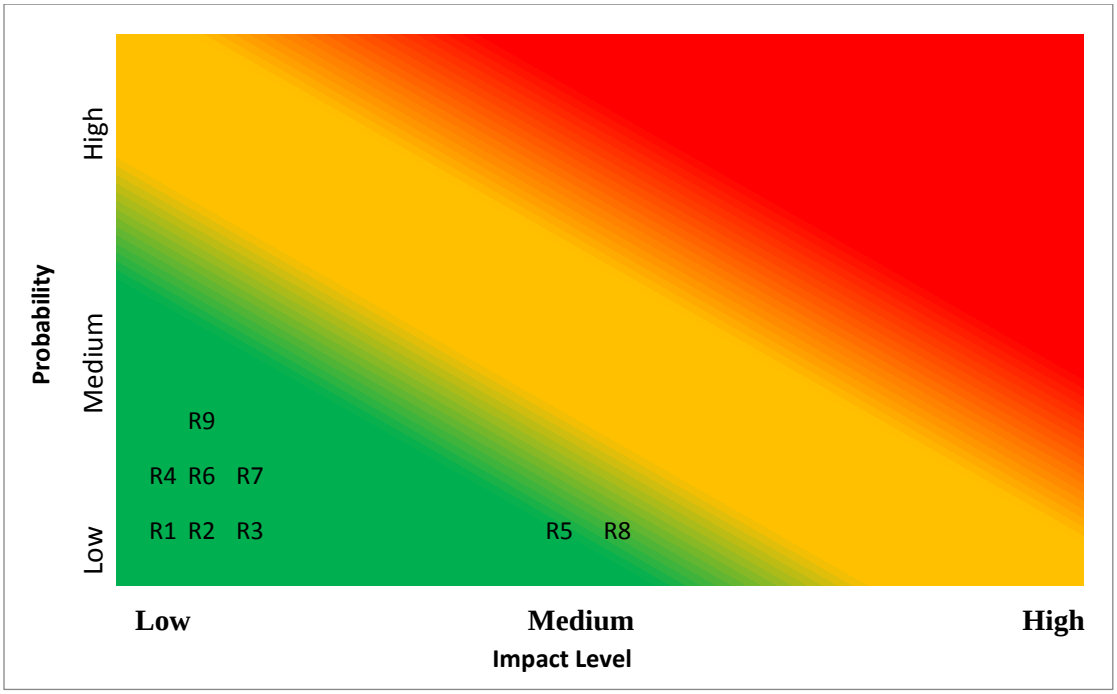

Figure 5-4. Risk assessment chart of *Bacillus subtilis*.

The next chapter will give an overall conclusion of the findings of this thesis, and recommendations for future work that could be further studied.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Outline of the chapter

This chapter provides the overall conclusions of this thesis where the achievement of each research objective has been discussed. Furthermore, research recommendations for future work have been addressed. The chapter is organised as follows:

6.1. Conclusions

6.2. Challenges and recommendations for future work

6. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Conclusions

This study has investigated the potential of producing biosurfactants by indigenous bacteria isolated from Egyptian oil fields and optimised the production of these biosurfactants to reach the ultimate oil transport and enhance oil recovery.

Chapter 2 outlined the detailed review of the main areas of research, which are the significance of MEOR economically and environmentally compared with conventional EOR, the historical development of MEOR, MEOR field trials, isolation and identification of Bacteria, bio/surfactants, and the reservoir simulated porous micromodels. Furthermore, the biosurfactants approaches, mechanisms, classifications, applications, approaches, and significance in improving oil recovery economically and environmentally are also presented. In addition, the most promising bacteria that have the potential for biosurfactant production and the importance of selecting suitable nutrients to reach optimum biosurfactant efficiency are presented. Finally, the screening criteria of reservoir rock and fluid parameters for the MEOR process were presented.

Chapter 3 provided a detailed description of the data collection and sampling, experimental equipment, materials, and procedure used during this experimental work. These procedures followed the conventional and widely accepted standard protocols outlined in the literature, and at the same time were modified to achieve the objectives set for this study. The research methodology performed in this study provided the main impact and outcome.

The results of the statistical analysis of collected data and conducted experiments were interpreted and analysed in Chapter 4. All the obtained results are concluded and listed in five phases, as follows:

Phase I: Statistical Analysis of Collected Data and Sampling

MEOR screening parameters of 59 Egyptian oil reservoirs representing the two main Egyptian oil concessions areas (the Gulf of Suez, and the Western Desert) were summarised and statistically analysed. It was found that 8 oil reservoirs in the Gulf of Suez

and 3 reservoirs in the Western Desert have the potential to MEOR based on available data. Therefore, eleven oil samples were collected from these Egyptian oil reservoirs for further studies.

Phase II: Microbial Isolation

The laboratory studies show the existence of indigenous bacterial strains in collected crude oil samples. Eleven pure bacteria cultures were isolated from collected crude oil samples.

Phase III: Microbial Identification

Combined analysis of morphological, and biochemical characterisation results showed that the 11 types of isolated indigenous bacterial strains, which were coded G1, G2, G3, G4, G5, G6, G7, G8, WD1, WD2, and WD3, are *Pseudomonas stutzeri, Clostridium spp, pseudomonas aeruginosa, pseudomonas fluorescens, Brevibacterium spp, Cellulosimicrobium spp, Pseudomonas panipatensis, Enterobacter spp, Bacillus flexus, Bacillus licheniformis, and Bacillus subtilis,* respectively.

Phase IV: Biosurfactants Production

In this study, *Bacillus subtilis* and *Bacillus licheniformis* isolated from Egyptian oil fields located in the Western Desert were selected for producing metabolic biosurfactants to improve oil recovery. It was found that they can produce a highly active biosurfactant. For reaching the optimal surface activity of this biosurfactant, a comparative analysis by 10 different reported nutrient media for bacilli species, and a new proposed medium nominated H was performed. It was found that the maximum surface activity has been observed after 24 h of incubation in the new proposed nutrient medium H., where the surface tension of biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* was reduced from 71.8 mN/m to 25.74 mN/m and 27.13 mN/m, and the interfacial tension against kerosene was reduced from 48.4 mN/m to 0.38 mN/m and 1.27 mN/m, respectively. The growth profile of selected indigenous bacteria *Bacillus subtilis* and *Bacillus licheniformis* reached their maximum growth rate after 24 h of incubation, which are 2.072 x 10^9 CFU/ml and 2.237 x 10^9 CFU/ml, respectively.

The produced biosurfactants by *Bacillus subtilis* and *Bacillus licheniformis* were extracted and purified from culture media, and their biosurfactant yields were 2.85 g/l and 2.47 g/l,

respectively. the critical micelle concentration CMC of the produced biosurfactant by *Bacillus subtilis* and *Bacillus licheniformis* was also determined, it was 0.04 g/l and 0.06 g/l at minimal surface tension 25.7±1.2 and 27.13 mN/m, respectively. The emulsification activity of the produced biosurfactants was confirmed, and it was noticed that the maximum emulsification power of the biosurfactant produced by *Bacillus subtilis* and *Bacillus licheniformis* against kerosene was 70% and 61%, respectively. Furthermore, the potential of the selected bacterial strains and their produced biosurfactants to alter the wettability of the sandstone rock was examined using the contact angle measurement method which showed that the oil detached from the sandstone surface when submerged in the aqueous solution of the produced biosurfactants, where the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* decreased the contact angle of the oil drop from 104.96° and 107.30° to 85.40° and 88.72° after 24 h, respectively. Similarly, the new proposed medium decreased the contact angle of the oil drop from 112.30° and 110.90° to 63.85° and 69.33° after 24 h, respectively, which means that the wettability of the sandstone surface has been modified toward more water-wet.

The stability of produced biosurfactant in different conditions (temperature, salinity, and pH) was investigated. There was no notable change in surface activity over a wide range of temperatures up to 90^{o}C, which reveals that *Bacillus subtilis* and *Bacillus licheniformis* are thermophilic bacteria and could tolerate the harsh temperature of oil reservoirs. The surface activity of produced biosurfactant exhibited high stability against salt concentration, even at high NaCl concentrations up to 20% (w/v), it retains more than 60% of its surface activity, which reveals that it could tolerate the harsh salinity of oil reservoirs. The optimum salinity of the produced biosurfactant was in the range of 0 to 2% (w/v) NaCl concentration. The optimum pH value of the produced biosurfactant was observed at neutral values.

Phase V: Core Flooding

One of the main objectives of this study was also to produce effective biosurfactants that could ultimately improve oil recovery when compared with conventional water flooding. It was found that the oil recovery after water flooding was 41-43% of original oil in place, whereas 25-39% of additional oil (over the water flooding residual oil saturation) was

recovered after biosurfactant flooding at different flow rates using biosurfactant produced by *Bacillus licheniformis* and *Bacillus subtilis*. The extended water flood was conducted for an extra 5 PV of formation water of the field of interest, but with no significant increase in pressure drop until reaching stable pressure drop.

Flowrate was the main parameter that required to be established to serve as a control for the initial conditions that could reach the ultimate oil recovery. Consequently, a series of core flooding runs were carried out in triplicate at flow rates of 0.25 cm3/min, 0.50 cm3/min and 0.75 cm3/min to obtain the optimum injection flow rate of the biosurfactants that could reach the maximum oil recovery. It was found that the maximum oil recovery was recovered at a flow rate of 0.25 cm^3/min. The maximum additional oil recovery recorded over the water flooding residual oil saturation was 31-39% after biosurfactant flooding using biosurfactant produced by *Bacillus licheniformis* and *Bacillus subtilis*, respectively. The obtained results indicate that the longer the saturation period is, the more time for the biosurfactant to redistribute in the core, and thus the more crude oil that could be produced. Extended water flooding (EWF) was carried out by injecting 5 PV of formation water after the biosurfactant flooding. However, no significant change was observed because only 1-3% of additional oil was recovered after the EWF.

The biosurfactant flooding trials were conducted with a specific focus on studying the effect of “in-situ” microbial flooding and “ex-situ” biosurfactant flooding on improving oil recovery by reducing the interfacial tension and altering the wettability of the rock for reducing the residual oil saturation, hence improving oil recovery. It was found that the cumulative oil recovery obtained during the “in-situ” microbial flooding was more than the cumulative oil recovery obtained during the “ex-situ” biosurfactant flooding. The reduction of interfacial tension was the dominant mechanism responsible for improving oil recovery efficiency during the “ex-situ” biosurfactant flooding with less effect of wettability alteration due to only biosurfactant adsorption. On other hand, wettability alteration was significantly responsible for the improvement of oil recovery during the “in-situ” microbial flooding due to not only biosurfactant adsorption, but also bacterial mass attached to the grain surfaces which lead to biofilm formation. Consequently, wettability alteration and interfacial tension reduction were the dominant mechanisms responsible for the improvement of oil recovery during the in-situ flooding runs. In this study, the obtained

results reveal that the presence of bacterial cells in the in-situ runs plays a significant role in altering the wettability, which means it could be implemented as one of the main mechanisms of wettability alteration that have the potential to improve oil recovery during MEOR processes.

Chapter 5: Environmental Risk Assessment

The main reason for finding an alternative for synthetic surfactants is the increase in environmental awareness. Based on the guidelines used in this study, the probability of risk occurrence of any of the selected indigenous bacteria is low, which means they do not produce toxins.

In summary, the biosurfactants produced by selected indigenous bacteria *Bacillus subtilis* and *Bacillus licheniformis* isolated from Egyptian oil fields have a significant potential to enhance oil recovery, which means that they could be used as a promising alternative to conventional synthetic surfactants due to strong surface and emulsification activities behaviour in harsh conditions(temperature, salinity, and pH). Besides the beneficial effects of the selected indigenous bacteria in producing effective biosurfactants, they do not have the potential for toxicity and couldn’t cause any risk to the health and the environment compared with chemical synthetic biosurfactants.

6.2 Recommendations for Future Work

Further studies could be still carried out to validate and complement this study. The following plans are thereby recommended as proposed for future work:

- A genotypic microbial identification method could be performed to double-check and confirm the results obtained by the phenotypic microbial identification method (Morphological and biochemical tests) applied in this study. Typically, genotypic identification of bacteria involves the use of conserved sequences within phylogenetically informative genetic targets, such as the small subunit (16S) rRNA gene. This process is very expensive and performed by sending the pure culture preserved in a petri dish to Sigma-Aldrich lab which will perform the whole process and send back a report of the genus type and its PCR.
- A rigorous scientific investigation could be performed to support the new proposed nutrient medium "H" by experimental and theoretical evidence.
- The physical characteristics and chemical structure of the biosurfactant produced by the selected indigenous bacteria (*Bacillus subtilis*, *Bacillus licheniformis*) could be determined or identified using Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), and mass spectrometry on each of the samples.
- The biosurfactants producing bacterial strains and the new proposed medium could be examined on a sand pack model as an in-situ production of biosurfactants and compared their effect on oil recovery with the results of injecting biosurfactants directly into the same sand pack model.
- The effect of the produced biosurfactants on oil recovery could be examined on carbonate core samples as well.
- The economical aspect of produced biosurfactants could be studied regarding oil field applications and compare the results with synthetic surfactants extensively used in conventional enhanced oil recovery.

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APPENDICES

8. APPENDICES

8.1 MEOR Worldwide field trials

Table 8-1: Identification of known field trials identified from a comprehensive literature search of any field trials that assessed the alteration of oil recovery after MEOR treatment.

Year	Country	Location	Latitude	Longitude	Site	Oil Type	Recovery	Suggested Method	Microorganism
1954	USA	Nacatoch, Arkansas	32.748916	-93.976776	Onshore	Light Oil	160%	Exogenous plugging	*Clostridia*
1958	Netherlands	Various	52.677081	6.887827	Onshore	Light Oil	0%	Slime forming	*Betacoccus dextrainicus*
1973	Hungary	Demjen	47.846616	20.34801	Onshore	Light Oil	60% (short term)	Indigenous gas and surfactant production	*Mixed, SRB, Pseudomonas, Clostridia*
1988	Canada	Saskatchewan	52.939916	-106.450864	Onshore	Heavy Oil	<5 %	Exogenous	*Leuconostoc*
1990	Australia	Alton, Surat basin	-27.983199	149.315763	Onshore	Light Oil	150%	Exogenous	N/A
1990	Germany	NA	51.162981	10.463035	Onshore	Light Oil	300%	Exogenous	*Clostridia*
1990	Romania	Bragadiru	44.356928	25.994511	Onshore	Light Oil	> 50%	Exogenous	*Bacillus, Clostridium, Arthrobacter, Pseudomonas, Micrococcus*
1990	USA	Delaware-Childers, Oklahoma	36.734439	-95.645795	Onshore	Light Oil	19.20%	Exogenous	*Clostridium*
1991	UK	Lindsey	41.41684	-83.262291	Onshore	Light Oil	0-10%	Exogenous	Acid fracturing
1992	Romania	Caldararu	44.450624	24.971066	Onshore	Light Oil	100-200 % (over 5 months)	Exogenous	*Bacillus, Clostridia*, Gram negatives
1992	Russia	Romashkinskoye, Urals	60.716568	29.798701	Onshore	Light Oil	15-45%	Indigenous	mixed
1992	Trinidad	Numerous	10.149705	-61.100464	Onshore	Light Oil	0%	Gas producers	numerous facultative anaerobes
1992	USA	Jack county, Texas	33.256038	-98.221298	Onshore	Light Oil	5%	Indigenous	N/A

1992	USA	Vassar Vertz, Oklahoma	36.182069	-96.943703	Onshore	Light Oil	300%	Indigenous plugging and gas	Mixed
1993	USA	Vassar Vertz, Oklahoma	36.157125	-96.984901	Onshore	Light Oil	< 5%	Indigenous	Mixed
1993	Venezuela	Maracaibo	10.527982	-71.801147	Onshore	Light Oil	2%	Indigenous	Mixed
1994	Argentina	Diadema	-45.778036	-67.672011	Onshore	Heavy Oil	0-50%	Exogenous plugging	N/A
1994	China	Jilin	43.150884	126.44466	Onshore	Light Oil	30%	Indigenous, Gas and Acid	Mixed, *Eubacterium, Fusobacterium, Bacteroidetes*
1994	Russia	Romashkino, Tatarstan	56.152778	52.489722	Onshore	Light Oil	50%	indigenous	*Clostridia, Pseudomonas*
1995	Russia	Vyangpour, Siberia	57.241722	65.714722	Onshore	Light Oil	3%	Indigenous	Mixed
1995	Russia	Siberia	61.01371	99.196656	Onshore	Light Oil	15-30%	Indigenous plugging	Mixed, *Bacillus, Pseudomonas, Actinomyces*
1996	China	Fuyu	45.013087	126 041336	Onshore	Light Oil	30%	Exogenous	*Enterobacter, Bacillus*
1997	Argentina	Piedras Coloradas	-40.840492	-65.118137	Onshore	Light Oil	66%	Hydrocarbon degradation	various hydrocarbon degraders
1999	China	Qinghai	36.781655	97.198792	Onshore	Light Oil	200%	Exogenous	Mixed hydrocarbon degraders and acid producers
1999	Indonesia	Ledok, Java	-6.885785	111.221904	Onshore	Light Oil	12%	Exogenous/Indigenous	Mixed supplemented with *Bacillus licheniformis*
2000	India	Naharkatiya, Assam	27.286986	95.247552	Onshore	Light Oil	70%	Indigenous	Mixed
2000	Malaysia	Bokor	4.202617	113.5327 15	Offshore	Heavy Oil	2-47%	Indigenous	Mixed
2001	India	Ahmedabad	23.012579	72.501183	Onshore	Light Oil	60%	Indigenous	Multi-bacterial Consortium: *Clostridium Thermoanaerobacterium sp. and Thermococcus sp.*
2004	China	Chaoyanggou	41.547066	120.625763	Onshore	Light Oil	3%	Exogenous	Unnamed
2005	China	Daquing	46.602958	124.570541	Onshore	Light Oil	72%	Hydrocarbon degradation	*Bacillus brevis*
2005	Norway	Nome	65.356532	8.041992	Offshore	Light Oil	NA	N/A	N/A
2006	Africa	Alpha	NA	NA	Onshore	Light Oil	15%	Indigenous	Mixed

2006	Africa	Beta	NA	NA	Onshore	Light Oil	25%	Indigenous plugging	Mixed
2007	China	Dagang, Tianjin	39.043006	117.608991	Onshore	Light Oil	13.8-42%	Exogenous/Indigenous	Mixed consortia, SRB, methanogen, fermentative
2008	Canada	Saskatchewan	52.939916	-106.450864	Onshore	Heavy Oil	200%	Indigenous	Mixed
2010	Brazil	North	-3.713523	-60.952148	Onshore	Light Oil	N/A	Indigenous plugging	N/A
2011	Myanmar	Mann field	20.832502	94.801025	Onshore	Light Oil	10%	EEOR	EEOR
2011	Poland	Pawlowice	50.172439	20.394573	Onshore	Light Oil	70%	Exogenous	Mixed
2012	USA	Viola, Oklahoma	34.111165	-97.472076	Onshore	Light Oil	10%	Exogenous surfactant	*Bacillus licheniformis* and *Bacillus subtilis*
2015	China	Xinjiang	44.069827	84.858398	Onshore	Heavy Oil	307% (short term)	Indigenous surfactant	*Pseudomonas*

8.2 PVT Data of AL QADR Oil Field

Reservoir Fluid Study (PVT)

For

BADR PETROLEUM COMPANY

WELL NAME: Neag C4-1 (Western Desert)

Formation: Bahariya

Field: AL QADR

BOTTOM HOLE SAMPLE

A product of

EPRI-Production Services Centre

File Code: PVT/29/J/467

January 2008

General Well Information

Company	……...	BAPETCO
Well Name	……...	Neag C4-1 (Western Desert)
File Code	……...	PVT/29/J/467
Date Sample Collected	……...	26/10/2007
Sample type	……...	Bottom Hole Sample
Area	……...	W. Desert
Field	……...	NEAG
Oil rate	……...	191 STB/D

Well Description

Formation	……...	Bahariya
Pool (or zone)	……...	*
Producing Interval	……...	(1285-1298.3) MPDF
Depth	……...	*
Choke Size	……...	16/64 "

Pressure Survey Data

Initial Reservoir Pressure	……...	1700 psia (11.72 MPa)
Initial Reservoir Temperature	……...	148 ^{o}F (64.44 ^{o}C)
Well Head pressure	……...	55 psig (0.38 MPa)
Well Head Temperature	……...	88 ^{o}F (31.11 ^{o}C)
Separator pressure	……...	25 psig (0.17 MPa)
Separator Temperature	……...	85 ^{o}F (29.44 ^{o}C)

* Data not forwarded to EPRI PVT Lab.

SUMMARY OF MAIN RESULTS	
G.O.R from flash liberation, SCF/STB	72.2
G.O.R from differential, SCF/STB	72.0
Initial reservoir pressure (psig)	1700 (11.72 MPa)
Bottles Number	814532
Reservoir temperature, ^{o}F	148 (64.44 ^{o}C)
SATURTATION PRESSURE at ^{o}F148 & 305 (psig)	
Viscosity, cp	1.371
Density g/cc	0.76849
F.V.F rb/bbl	1.0860
RESERVOIR PRESSURE at 148 ^{o}F & 1700 (psig)	
Viscosity, cp	1.503
Density g/cc	0.77769
F.V.F rb/bbl	1.0731
Compressibility, $(psi)^{-1}$	7.3776×10^{-6}
STOCK TANK OIL	
Density at 60^{o}F, g/cc	0.81820
Density at 104^{o}F, g/cc	0.80044
Kinematic viscosity at 104^{o}F, Cst	4.81
Dynamic viscosity at 104^{o}F, Cp	3.85
Pour point, ^{o}C	18
Sulphur content, wt.%	0.2
Wax content, wt.%	13.4
Asphaltene content	0.07
Average molecular weight	166.511
^{o}API	41.3
HEPTANE PLUS (C_7^+)	
Density at 60^{o}F, g/cc	0.84076
Average molecular weight	187.111
Wt.% of C_7^+	92.100
^{o}API	36.6
DODECAN PLUS (C_{12}^+)	
Density at 60^{o}F, g/cc	0.90325
Average molecular weight	248.964
Wt.% of C_{12}^+	62.505
^{o}API	25.1

Preliminary Checks of Sample

Cylinder No.	From Sheet		From Lab.	
	psi	ºF (ºC)	Psig (MPa)	ºF (ºC)
814532	1493.5	148 (64.44)	1495 (10.31)	148 (64.44)

Composition of Flashed Gas

(By Capillary Gas Chromatography)

Component	Mole %	Wt.%	Liquid Density (gm/cc)	MW
Nitrogen	0.451	0.436	0.8086	28.013
Carbon Dioxide	0.865	1.315	0.8172	44.010
Methane	62.493	34.633	0.2997	16.043
Ethane	7.472	7.761	0.3558	30.070
Propane	12.134	18.483	0.5065	44.097
I-Butane	5.355	10.751	0.5623	58.123
n-Butane	5.502	11.046	0.5834	58.123
I-pentane	2.261	5.635	0.6238	72.150
n-Pentane	1.552	3.868	0.6305	72.150
Hexane	1.285	3.825	0.6634	86.177
Benzene	0.024	0.065	0.8820	78.110
Heptanes	0.353	1.222	0.6874	100.204
Toluene	0.066	0.210	0.8734	92.140
Octane	0.157	0.619	0.7061	114.231
Ethyl-benzene	0.004	0.015	0.8735	106.160
P, m-xylene	0.008	0.029	0.8671	106.160
o-xylene	0.003	0.013	0.8840	106.160
Nonanes C9	0.015	0.074	0.7212	128.260
Total	**100.00**	**100.00**		

Average Flashed Gas Properties

Average Molecular Weight	…….	28.950
Calculated Gas Gravity (air = 1.000)	…….	0.9996
Equivalent Liquid Density, gm/cc	…….	0.42128
Gross cal value (Btu/Ft^3)	…..…..	1672.132
Net Cal value (Bta/Ft^3)	……..	1527.556

Note: Component properties assigned from literature

[a] Ref.: Gas Producers & Suppliers Association (GPSA) Engineering Data Book

[b] Ref.: "PVT and Phase Behaviour of Petroleum Reservoir Fluids," by Department of Petroleum Engineering Heriot-Watt University, Edinburgh, Scotland-1998 Elsevier Science, New York.

Composition Stock Tank
(By flash/extended chromatography up to C_{38})

Component		Mole %	Wt.%	Liquid Density (gm/cc)	MW
Nitrogen	N2	0.000	0.000	0.8086	28.013
Carbon Dioxide	CO2	0.000	0.000	0.8172	44.010
Methane	C1	0.000	0.000	0.2997	16.043
Ethane	C2	0.143	0.026	0.3558	30.070
Propane	C3	1.129	0.299	0.5065	44.097
I-Butane	i-C4	1.340	0.468	0.5623	58.123
n-Butane	n-C4	2.326	0.812	0.5834	58.123
I-pentane	i-C5	2.661	1.153	0.6238	72.150
n-Pentane	n-C5	2.594	1.124	0.6305	72.150
Hexane	C6	6.955	3.600	0.6634	86.177
Benzene	B	0.891	0.418	0.8820	78.110
Heptanes	C7	6.944	4.179	0.6874	100.204
Toluene	T	1.344	0.744	0.8734	92.140
Octanes	C8	10.251	7.032	0.7061	114.231
Ethyl-benzene	EB	0.403	0.257	0.8735	106.160
m- p -xylene	m, p, x	0.914	0.583	0.8671	106.160
o-xylene	o-x	0.323	0.206	0.8840	106.160
Nonanes	C9	7.663	5.902	0.7212	128.260
Decanes	C10	6.329	5.408	0.7334	142.285
Undecanes	C11	5.986	5.284	0.7890	147.000
dodecanes	C12	5.336	5.158	0.8000	161.000
Tridecanes	C13	4.649	4.886	0.8110	175.000
tetradecanes	C14	4.188	4.779	0.8220	190.000
Pentadecanes	C15	3.552	4.395	0.8320	206.000
Hexadecanes	C16	2.899	3.866	0.8390	222.000
Heptadecanes	C17	2.822	4.017	0.8470	237.000
Octadecanes	C18	2.216	3.342	0.8520	251.000
Nonadecanes	C19	1.958	3.094	0.8570	263.000
Eiconadecanes	C20	1.743	2.880	0.8620	275.000
Eneicosanes	C21	1.620	2.832	0.8670	291.000
Dodeicosanes	C22	1.524	2.790	0.8720	305.000
Tricosanes	C23	1.445	2.761	0.8770	318.000
Tetraicosanes	C24	1.329	2.641	0.8810	331.000
Petaicosanes	C25	1.257	2.605	0.8850	345.000
Hexaucisanes	C26	1.164	2.511	0.8890	359.000
Heptaicosanes	C27	1.014	2.277	0.8930	374.000
Octaicosanes	C28	0.956	2.226	0.8960	388.000
Nonaicosanes	C29	0.831	2.005	0.8990	402.000
Tricontanes	C30	0.428	1.071	0.9020	416.000
Entricontanes	C31	0.315	0.811	0.9060	430.000
Dodetricontanes	C32	0.188	0.500	0.9090	444.000
Tritricontanes	C33	0.147	0.401	0.9120	458.000
Tetratricontanes	C34	0.095	0.269	0.9140	472.000
Pentatricontanes	C35	0.053	0.157	0.9170	486.000
Hexatricontanes	C36	0.033	0.100	0.9200	500.000
Hepatricontanes	C37	0.025	0.078	0.9390	514.000
Octatricontanes	C38	0.017	0.053	0.9410	528.000
Total		**100.00**	**100.00**		

Composition Analysis by Chromatograph up to C_{12}^{+}

Component	Stock tank oil		Liquid Density	MW
	Mole. %	Wt.%	(gm/cc)	
Nitrogen	0.000	0.000	0.8086	28.013
Carbon Dioxide	0.000	0.000	0.8172	44.010
Methane	0.000	0.000	0.2997	16.043
Ethane	0.143	0.026	0.3558	30.070
Propane	1.129	0.299	0.5065	44.097
I-Butane	1.340	0.468	0.5623	58.123
n-Butane	2.326	0.812	0.5834	58.123
I-pentane	2.661	1.153	0.6238	72.150
n-Pentane	2.594	1.124	0.6305	72.150
Hexane	6.955	3.600	0.6634	86.177
Benzene	0.891	0.418	0.8820	78.110
Heptanes	6.944	4.179	0.6874	100.204
Toluene	1.344	0.744	0.8734	92.140
Octane	10.251	7.032	0.7061	114.231
Ethyl-benzene	0.403	0.257	0.8735	106.160
P, m-xylene	0.914	0.583	0.8671	106.160
o-xylene	0.323	0.206	0.8840	106.160
Nonanes C9	7.663	5.902	0.7212	128.260
Decanes C10	6.329	5.408	0.7334	142.285
Undecanes C11	5.986	5.284	0.7890	147.000
dodecanes $C12^{+}$	41.804	62.505	0.9033	248.964
Total	**100.000**	**100.000**		
Gas gravity (air=1.000)				

GOR Flashed SCF/STB

PROPERTIES OF STOCK TANK OIL	
The average molecular weight of stock tank oil	166.511
The density of stock tank oil at 60°F	0.81820
°API	41.3
HEPTANES PLUS (C_7^{+})	
Density at 60°F, (g/cc)	0.84076
Average molecular Weight	187.111
Wt. %	92.100
°API	36.6
DODECANES PLUS (C_{12}^{+})	
Density at 60°F, (g/cc)	0.90325
Average molecular Weight	248.964
Wt. %	62.505
°API	25.1

Composition of Reservoir Fluid

(By flash/extended chromatography)

Flashed Gas/oil Ratio, SCF/STB --------- 72.2

Component	Mole %	Wt.%	Liquid Density (gm/cc)	MW
Nitrogen	0.045	0.008	0.8086	28.013
Carbon Dioxide	0.086	0.025	0.8172	44.010
Methane	6.199	0.651	0.2997	16.043
Ethane	0.870	0.171	0.3558	30.070
Propane	2.221	0.641	0.5065	44.097
I-Butane	1.738	0.661	0.5623	58.123
n-Butane	2.641	1.004	0.5834	58.123
I-pentane	2.621	1.237	0.6238	72.150
n-Pentane	2.491	1.176	0.6305	72.150
Hexane	6.393	3.604	0.6634	86.177
Benzene	0.805	0.411	0.8820	78.110
Heptanes	6.290	4.123	0.6874	100.204
Toluene	1.217	0.734	0.8734	92.140
Octane	9.250	6.912	0.7061	114.231
Ethyl-benzene	0.363	0.252	0.8735	106.160
P, m-xylene	0.824	0.572	0.8671	106.160
o-xylene	0.291	0.202	0.8840	106.160
Nonanes C9	6.904	5.793	0.7212	128.260
Decanes C10	5.701	5.307	0.7334	142.285
Undecanes C11	5.392	5.185	0.7890	147.000
dodecanes $C12^{+}$	37.657	61.331	0.9033	248.964
Total	**100.00**	**100.00**		

*** These values are constants for ideal gas**

Total Well Stream Properties

Molecular weight ---------- 152.864

Properties of Plus Fractions

Component	Mole %	Wt.%	Liquid Density (gm/cc)	MW
Heptane Plus	73.891	90.411	0.8109	187.042
Dodecane Plus	37.657	61.331	0.8536	248.962

Composition Analysis by Chromatograph up to C_{38}

Component		Liquid Density (gm/cc)	MW	Stock Tank		Flashed Gas		Well Stream	
				Mole %	Wt.%	Mole %	Wt.%	Mole %	Wt.%
Nitrogen	N2	0.8086	28.013	0.000	0.000	0.451	0.436	0.045	0.008
Carbon Dioxide	CO2	0.8172	44.010	0.000	0.000	0.865	1.315	0.086	0.025
Methane	C1	0.2997	16.043	0.000	0.000	62.493	34.633	6.199	0.651
Ethane	C2	0.3558	30.070	0.143	0.026	7.472	7.761	0.870	0.171
Propane	C3	0.5065	44.097	1.129	0.299	12.134	18.483	2.221	0.641
I-Butane	i-C4	0.5623	58.123	1.340	0.468	5.355	10.751	1.738	0.661
n-Butane	n-C4	0.5834	58.123	2.326	0.812	5.502	11.046	2.641	1.004
I-pentane	i-C5	0.6238	72.150	2.661	1.153	2.261	5.635	2.621	1.237
n-Pentane	n-C5	0.6305	72.150	2.594	1.124	1.552	3.868	2.491	1.176
Hexane	C6	0.6634	86.177	6.955	3.600	1.285	3.825	6.393	3.604
Benzene	B	0.8820	78.110	0.891	0.418	0.024	0.065	0.805	0.411
Heptanes	C7	0.6874	100.204	6.944	4.179	0.353	1.222	6.290	4.123
Toluene	T	0.8734	92.140	1.344	0.744	0.066	0.210	1.217	0.734
Octanes	C8	0.7061	114.231	10.251	7.032	0.157	0.619	9.250	6.912
Ethyl-benzene	EB	0.8735	106.160	0.403	0.257	0.004	0.015	0.363	0.252
	m, p,			0.914		0.008	0.029		0.572
m- p -xylene	x	0.8671	106.160		0.583			0.824	
o-xylene	o-x	0.8840	106.160	0.323	0.206	0.003	0.013	0.291	0.202
Nonanes	C9	0.7212	128.260	7.663	5.902	0.015	0.074	6.904	5.793
Decanes	C10	0.7334	142.285	6.329	5.408	0.000	0.000	5.701	5.307
Undecanes	C11	0.7890	147.000	5.986	5.284	0.000	0.000	5.392	5.185
dodecanes	C12	0.8000	161.000	5.336	5.158	0.000	0.000	4.807	5.063
Tridecanes	C13	0.8110	175.000	4.649	4.886	0.000	0.000	4.188	4.794
tetradecanes	C14	0.8220	190.000	4.188	4.779	0.000	0.000	3.773	4.689
Pentadecanes	C15	0.8320	206.000	3.552	4.395	0.000	0.000	3.200	4.312
Hexadecanes	C16	0.8390	222.000	2.899	3.866	0.000	0.000	2.611	3.793
Heptadecanes	C17	0.8470	237.000	2.822	4.017	0.000	0.000	2.542	3.941
Octadecanes	C18	0.8520	251.000	2.216	3.342	0.000	0.000	1.996	3.278
Nonadecanes	C19	0.8570	263.000	1.958	3.094	0.000	0.000	1.764	3.035
Eiconadecanes	C20	0.8620	275.000	1.743	2.880	0.000	0.000	1.570	2.825
Eneicosanes	C21	0.8670	291.000	1.620	2.832	0.000	0.000	1.459	2.778
Dodeicosanes	C22	0.8720	305.000	1.524	2.790	0.000	0.000	1.373	2.739
Tricosanes	C23	0.8770	318.000	1.445	2.761	0.000	0.000	1.302	2.708
Tetraicosanes	C24	0.8810	331.000	1.329	2.641	0.000	0.000	1.197	2.592
Petaicosanes	C25	0.8850	345.000	1.257	2.605	0.000	0.000	1.132	2.556
Hexaucisanes	C26	0.8890	359.000	1.164	2.511	0.000	0.000	1.049	2.462
Heptaicosanes	C27	0.8930	374.000	1.014	2.277	0.000	0.000	0.913	2.235
Octaicosanes	C28	0.8960	388.000	0.956	2.226	0.000	0.000	0.861	2.186
Nonaicosanes	C29	0.8990	402.000	0.831	2.005	0.000	0.000	0.748	1.969
Tricontanes	C30	0.9020	416.000	0.428	1.071	0.000	0.000	0.386	1.049
Entricontanes	C31	0.9060	430.000	0.315	0.811	0.000	0.000	0.284	0.798
Dodetricontanes	C32	0.9090	444.000	0.188	0.500	0.000	0.000	0.169	0.492
Tritricontanes	C33	0.9120	458.000	0.147	0.401	0.000	0.000	0.132	0.397
Tetratricontanes	C34	0.9140	472.000	0.095	0.269	0.000	0.000	0.086	0.264
Pentatricontanes	C35	0.9170	486.000	0.053	0.157	0.000	0.000	0.048	0.152
Hexatricontanes	C36	0.9200	500.000	0.033	0.100	0.000	0.000	0.030	0.097
Hepatricontanes	C37	0.9390	514.000	0.025	0.078	0.000	0.000	0.023	0.076
Octatricontanes	C38	0.9410	528.000	0.017	0.053	0.000	0.000	0.015	0.053
Total				**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

Composition Analysis by Chromatograph up to C_{12}^{+}

Component	Stock tank oil		Gas Flashed		Well Stream	
	Mole. %	Wt.%	Mole. %	Wt.%	Mole. %	Wt.%
Nitrogen	0.000	0.000	0.451	0.436	0.045	0.008
Carbon Dioxide	0.000	0.000	0.865	1.315	0.086	0.025
Methane	0.000	0.000	62.493	34.633	6.199	0.651
Ethane	0.143	0.026	7.472	7.761	0.870	0.171
Propane	1.129	0.299	12.134	18.483	2.221	0.641
I-Butane	1.340	0.468	5.355	10.751	1.738	0.661
n-Butane	2.326	0.812	5.502	11.046	2.641	1.004
I-pentane	2.661	1.153	2.261	5.635	2.621	1.237
n-Pentane	2.594	1.124	1.552	3.868	2.491	1.176
Hexane	6.955	3.600	1.285	3.825	6.393	3.604
Benzene	0.891	0.418	0.024	0.065	0.805	0.411
Heptanes	6.944	4.179	0.353	1.222	6.290	4.123
Toluene	1.344	0.744	0.066	0.210	1.217	0.734
Octane	10.251	7.032	0.157	0.619	9.250	6.912
Ethyl-benzene	0.403	0.257	0.004	0.015	0.363	0.252
P, m-xylene	0.914	0.583	0.008	0.029	0.824	0.572
o-xylene	0.323	0.206	0.003	0.013	0.291	0.202
Nonanes C9	7.663	5.902	0.015	0.074	6.904	5.793
Decanes C10	6.329	5.408	0.000	0.000	5.701	5.307
Undecanes C11	5.986	5.284	0.000	0.000	5.392	5.185
dodecanes $C12^{+}$	41.804	62.505	0.000	0.000	37.657	61.331
Total	**100.000**	**100.000**	**100.000**	**100.000**	**100.00**	**100.00**
Gas gravity (air=1.000)			**0.9996**			

Summary of Stock Tank Oil Properties

ANALYSIS	RESULT	METHOD
Density at 60^{o}F, (g/cc)	0.81820	IP 59
Density at 104^{o}F, (g/cc)	0.80044	IP 59
Kinematic viscosity at 104^{o}F, (Cst)	4.81	ASTM D 445
Dynamic viscosity at 104^{o}F, (Cp)	3.85	
Pour point, (^{o}C)	18	ASTM D 97
Sulphur content, (wt. %)	0.2	ASTM D 129
Wax content, (wt.%)	13.4	PPM
Asphaltene content, (wt.%)	0.07	PPM
Average molecular Weight	166.511	
^{o}API	41.3	
Residual sulphide	49.5	PPM
Water content	44	PPM
Salt content	4.24	Ptb
Dissolved H2S	Nil	PPM
Mercaptans	Nil	PPM
Density at 77^{o}F, (g/cc)	0.81125	IP 59
Kinematic viscosity at 77^{o}F, (Cst)	18.84	ASTM D 445
Dynamic viscosity at 77^{o}F, (Cp)	15.28	
HEPTANES PLUS (C_7^+)		
Density at 60^{o}F, (g/cc)	0.84076	
Average Molecular Weight	187.111	
Wt. %	92.100	
^{o}API	36.6	
DODECANES PLUS (C_{12}^+)		
Density at 60^{o}F, (g/cc)	0.90325	
Average Molecular Weight	248.964	
Wt. %	62.505	
^{o}API	25.1	

ASTM-DISTILLATION

Temperature ^{o}C	**Percent volume recovery Vol. %**
90	*Initial boiling point*
115	4
140	9
165	14
190	20
215	26
240	30
265	36
290	44
310	50

Recovery : 51 vol. %
Residue : 47 vol. %
Loss : 2 vol. %

Summary of PVT Data

Reported Reservoir Conditions

Reservoir Pressure	……	1700 psig (11.72 MPa)
Reservoir Pressure	……..	148 ^{O}F (64.44 ^{O}C)

Pressure-Volume Relation

Saturation Pressure	…….	305 psig
Average Single-phase compressibility	…….	6.6306 E-6V/V/PSI (2900 TO1700 PSIG)

Saturation Pressure
(At 305 psig at 148 oF)

Saturation Pressure (Psat.)	……	305 psig (2.10 MPa)
Density at Psat.	……..	0.76849 gm/cc
F.V.F. at Psat	……	1.0860 V at 148 ^{o}F/V at 60^{o}F
Oil Viscosity at Psat.	……..	1.371 CP

Reservoir Pressure
(1700 psig at 148 oF)

Reservoir Pressure	……	1700 psig (11.72 MPa)
Density at Res. pressure	……..	0.77769 gm/c
F.V.F. at Res. pressure	……	1.0731 V at 148^{o}F/V at 60^{o}F
Oil Viscosity at Res. pressure	……..	1.503 cp

Volumetric Data

(At 148 ºF)

Saturation pressure (Psat.)	……..	305 Psig (2.10 MPa)
Reservoir pressure	……	1700 Psig (11.72 MPa)
Reservoir Temperature	……..	148 ^{O}F (64.44 ^{O}C)

Average Single-Phase Compressibility

Pressure Range psig	Single-Phase compressibility v/v/psi
2900 TO 2465	6.0971 E-6
2465 TO 2030	6.7826 E-6
2030 TO 1700	7.0701 E-6
1700 TO 1378	7.8575 E-6
1378 TO 943	8.1223 E-6
943 TO 580	9.1449 E-6
580 TO 305	9.4792 E-6

8.3 PVT Data of AL FADL Oil Field

Reservoir Fluid Study (PVT)

For

BADR PETROLEUM COMPANY

WELL NAME: Neag C3-1 (Western Desert)

Formation: Bahariya

Field: AL FADL

BOTTOM HOLE SAMPLE

A product of

EPRI-Production Services Centre

File Code: PVT/30 /J/468

January 2008

General Well Information

Company	……...	BAPETCO
Well Name	……...	Neag C3-1 (Western Desert)
File Code	……...	PVT/30/J/468
Date Sample Collected	……...	16/11/2007
Sample type	……...	Bottom Hole Sample
Area	……...	W. Desert
Field	……...	NEAG
Oil Rate	……...	264 STB/D

Well Description

Formation	……...	Bahariya
Pool (or zone)	……...	*
Producing Interval	……...	(1266-1269) (1278.5-1281.5) (1309.5-1312.5) MPDF
Depth	……...	1214 m THF (3984F)
Choke Size	……...	16/64 "

Pressure Survey Data

Initial Reservoir Pressure	……...	1700 psia (11.72 MPa)
Initial Reservoir Temperature	……...	148 ^{o}F (64.44 ^{O}C)
Well Head pressure	……...	98 psig (0.68 MPa)
Well Head Temperature	……...	88 ^{o}F (31.11 ^{O}C)
Separator pressure	……...	18 psig (0.12 MPa)
Separator Temperature	……...	62 ^{o}F (16.67 ^{O}C)

* Data not forwarded to EPRI PVT Lab.

SUMMARY OF MAIN RESULTS	
G.O.R from flash liberation, SCF/STB	45.2
G.O.R from differential, SCF/STB	45.0
Initial reservoir pressure (psig)	1700 (11.72 MPa)
Bottles Number	814533
Reservoir temperature, ^{O}F	148 (64.44 ^{O}C)
SATURTATION PRESSURE at 148 ^{o}F & 253 (psig)	
Viscosity, cp	1.650
Density g/cc	0.78312
F.V.F rb/bbl	1.0621
RESERVOIR PRESSURE at 148 ^{o}F & 1700 (psig)	
Viscosity, cp	1.815
Density g/cc	0.79205
F.V.F rb/bbl	1.0501
Compressibility, $(psi)^{-1}$	6.8542×10^{-6}
STOCK TANK OIL	
Density at 60 ^{o}F, g/cc	0.82236
Density at 104^{o}F, g/cc	0.80861
Kinematic viscosity at 104^{o}F, Cst	10.78
Dynamic viscosity at 104^{o}F, Cp	8.72
Pour point, ^{o}C	24
Sulphur content, wt.%	0.08
Wax content, wt.%	15.20
Asphaltene content	0.045
Average molecular weight	180.378
^{o}API	40.4
HEPTANE PLUS (C_7^+)	
Density at 60^{O}F, g/cc	0.83765
Average molecular weight	197.400
Wt.% of C_{7}^{+}	94.450
^{o}API	37.3
DODECAN PLUS (C_{12}^+)	
Density at 60^{O}F, g/cc	0.88149
Average molecular weight	251.951
Wt.% of C_{12}^{+}	69.105
^{o}API	28.9

Preliminary Checks of Sample

Cylinder No.	From Sheet		From Lab.	
	psi (MPa)	ºF ºC	psig (MPa)	ºF ºC
814533	**1493.5 (10.30)**	**148 (64.44)**	**1491 (10.28)**	**148 (64.44)**

Composition of Flashed Gas
(By Capillary Gas Chromatography)

Component	Mole %	Wt.%	Liquid Density (gm/cc)	MW
Nitrogen	0.511	0.578	0.8086	28.013
Carbon Dioxide	1.300	2.310	0.8172	44.010
Methane	74.953	48.542	0.2997	16.043
Ethane	5.748	6.978	0.3558	30.070
Propane	6.744	12.006	0.5065	44.097
I-Butane	2.873	6.741	0.5623	58.123
n-Butane	3.037	7.126	0.5834	58.123
I-pentane	1.574	4.585	0.6238	72.150
n-Pentane	1.239	3.609	0.6305	72.150
Hexane	1.331	4.631	0.6634	86.177
Benzene	0.025	0.079	0.8820	78.110
Heptanes	0.398	1.610	0.6874	100.204
Toluene	0.054	0.201	0.8734	92.140
Octane	0.174	0.802	0.7061	114.231
Ethyl-benzene	0.006	0.026	0.8735	106.160
P, m-xylene	0.008	0.034	0.8671	106.160
o-xylene	0.004	0.021	0.8840	106.160
Nonanes C9	0.021	0.121	0.7212	128.260
Total	**100.00**	**100.00**		

Average Flashed Gas Properties

Average Molecular Weight	…… ….	24.771
Calculated Gas Gravity (air = 1.000)	…… ….	0.855
Equivalent Liquid Density, gm/cc	…… ….	0.3905
Gross cal value (Btu/Ft^3)	…..…. .	1436.409
Net Cal value (Bta/Ft^3)	……..	1307.798

Note: Component properties assigned from literature
[a] Ref.: Gas Producers & Suppliers Association (GPSA) Engineering Data Book
[b] Ref.: "PVT and Phase Behaviour of Petroleum Reservoir Fluids," by Department of Petroleum Engineering Heriot-Watt University, Edinburgh, Scotland-1998 Elsevier Science, New York.

Composition Stock Tank
(By flash/extended chromatography up to C_{38})

Component		Mole %	Wt.%	Liquid Density (gm/cc)	MW
Nitrogen	N2	0.000	0.000	0.8086	28.013
Carbon Dioxide	CO2	0.000	0.000	0.8172	44.010
Methane	C1	0.000	0.000	0.2997	16.043
Ethane	C2	0.121	0.020	0.3558	30.070
Propane	C3	0.797	0.195	0.5065	44.097
I-Butane	i-C4	0.887	0.286	0.5623	58.123
n-Butane	n-C4	1.418	0.457	0.5834	58.123
I-pentane	i-C5	2.340	0.936	0.6238	72.150
n-Pentane	n-C5	2.415	0.966	0.6305	72.150
Hexane	C6	4.832	2.309	0.6634	86.177
Benzene	B	0.884	0.383	0.8820	78.110
Heptanes	C7	5.355	2.975	0.6874	100.204
Toluene	T	1.535	0.784	0.8734	92.140
Octanes	C8	8.476	5.367	0.7061	114.231
Ethyl-benzene	EB	0.233	0.137	0.8735	106.160
P, m-xylene	m, p, x	0.979	0.576	0.8671	106.160
o-xylene	o-x	0.375	0.221	0.8840	106.160
Nonanes	C9	7.235	5.144	0.7212	128.260
Decanes	C10	6.315	4.981	0.7334	142.285
Undecanes	C11	6.329	5.158	0.7890	147.000
dodecanes	C12	5.731	5.115	0.8000	161.000
Tridecanes	C13	5.496	5.332	0.8110	175.000
tetradecanes	C14	4.932	5.194	0.8220	190.000
Pentadecanes	C15	3.972	4.537	0.8320	206.000
Hexadecanes	C16	3.627	4.464	0.8390	222.000
Heptadecanes	C17	3.198	4.203	0.8470	237.000
Octadecanes	C18	2.845	3.958	0.8520	251.000
Nonadecanes	C19	2.471	3.603	0.8570	263.000
Eiconadecanes	C20	2.224	3.39	0.8620	275.000
Eneicosanes	C21	1.984	3.202	0.8670	291.000
Dodeicosanes	C22	1.788	3.023	0.8720	305.000
Tricosanes	C23	1.706	3.007	0.8770	318.000
Tetraicosanes	C24	1.450	2.661	0.8810	331.000
Petaicosanes	C25	1.373	2.626	0.8850	345.000
Hexaucisanes	C26	1.275	2.539	0.8890	359.000
Heptaicosanes	C27	1.118	2.319	0.8930	374.000
Octaicosanes	C28	1.055	2.270	0.8960	388.000
Nonaicosanes	C29	0.925	2.063	0.8990	402.000
Tricontanes	C30	0.785	1.809	0.9020	416.000
Entricontanes	C31	0.621	1.479	0.9060	430.000
Dodetricontanes	C32	0.345	0.850	0.9090	444.000
Tritricontanes	C33	0.220	0.559	0.9120	458.000
Tetratricontanes	C34	0.150	0.394	0.9140	472.000
Pentatricontanes	C35	0.097	0.262	0.9170	486.000
Hexatricontanes	C36	0.041	0.113	0.9200	500.000
Hepatricontanes	C37	0.027	0.078	0.9390	514.000
Octatricontanes	C38	0.018	0.055	0.9410	528.000
Total		**100.00**	**100.00**		

Composition Analysis by Chromatograph up to C_{12}^{+}

Component	Stock tank oil		Liquid Density	MW
	Mole. %	Wt.%	(gm/cc)	
Nitrogen	0.000	0.000	0.8086	28.013
Carbon Dioxide	0.000	0.000	0.8172	44.010
Methane	0.000	0.000	0.2997	16.043
Ethane	0.121	0.020	0.3558	30.070
Propane	0.797	0.195	0.5065	44.097
I-Butane	0.887	0.286	0.5623	58.123
n-Butane	1.418	0.457	0.5834	58.123
I-pentane	2.340	0.936	0.6238	72.150
n-Pentane	2.415	0.966	0.6305	72.150
Hexane	4.832	2.309	0.6634	86.177
Benzene	0.884	0.383	0.8820	78.110
Heptanes	5.355	2.975	0.6874	100.204
Toluene	1.535	0.784	0.8734	92.140
Octane	8.476	5.367	0.7061	114.231
Ethyl-benzene	0.233	0.137	0.8735	106.160
P, m-xylene	0.979	0.576	0.8671	106.160
o-xylene	0.375	0.221	0.8840	106.160
Nonanes C9	7.235	5.144	0.7212	128.260
Decanes C10	6.315	4.981	0.7334	142.285
Undecanes C11	6.329	5.158	0.7890	147.000
dodecanes $C12^{+}$	49.474	69.105	0.8549	251.925
Total	**100.000**	**100.000**		

GOR Flashed SCF/STB	**45.2**
PROPERTIES OF STOCK TANK OIL	
The average molecular weight of stock tank oil	180.378
The density of stock tank oil at 60°F	0.82236
°API	40.4
HEPTANES PLUS (C_7^{+})	
Density at 60°F, (g/cc)	0.83765
Average molecular Weight	197.400
Wt. %	94.450
°API	37.3
DODECANES PLUS (C_{12}^{+})	
Density at 60°F, (g/cc)	0.88149
Average molecular Weight	251.951
Wt. %	69.105
°API	28.9

Composition of Reservoir Fluid

(By flash/extended chromatography)

Flashed Gas/oil Ratio, SCF/STB --------- 45.2

Component	Mole %	Wt.%	Liquid Density (gm/cc)	MW
Nitrogen	0.035	0.006	0.8086	28.013
Carbon Dioxide	0.090	0.023	0.8172	44.010
Methane	5.162	0.488	0.2997	16.043
Ethane	0.509	0.090	0.3558	30.070
Propane	1.207	0.314	0.5065	44.097
I-Butane	1.024	0.351	0.5623	58.123
n-Butane	1.530	0.524	0.5834	58.123
I-pentane	2.287	0.973	0.6238	72.150
n-Pentane	2.334	0.993	0.6305	72.150
Hexane	4.591	2.332	0.6634	86.177
Benzene	0.825	0.380	0.8820	78.110
Heptanes	5.014	2.961	0.6874	100.204
Toluene	1.433	0.778	0.8734	92.140
Octanes	7.904	5.322	0.7061	114.231
Ethyl-benzene	0.217	0.136	0.8735	106.160
P, m-xylene	0.912	0.571	0.8671	106.160
o-xylene	0.349	0.219	0.8840	106.160
Nonanes C9	6.738	5.094	0.7212	128.260
Decanes C10	5.880	4.932	0.7334	142.285
Undecanes C11	5.893	5.106	0.7890	147.000
dodecanes $C12^{+}$	46.066	68.407	0.8549	251.925
Total	**100.00**	**100.00**		

*** These values are constants for ideal gas**

Total Well Stream Properties

Molecular weight ---------- 169.649

Properties of Plus Fractions

Component	Mole %	Wt.%	Liquid Density (gm/cc)	MW
Heptane Plus	80.408	93.526	0.8199	197.330
Dodecane Plus	46.067	68.407	0.8549	251.925

Stock Tank Oil

Average Molecular weight	--------	180.378
Density at 14.73 psia and 60°F	--------	0.82236
°API gravity	--------	40.4

Composition Analysis by Chromatograph up to C_{38}

Component		Liquid Density (gm/cc)	MW	Stock Tank		Flashed Gas		Well Stream	
				Mole %	Wt.%	Mole %	Wt.%	Mole %	Wt.%
Nitrogen	N2	0.8086	28.013	0.000	0.000	0.511	0.578	0.035	0.006
Carbon Dioxide	CO2	0.8172	44.010	0.000	0.000	1.300	2.310	0.090	0.023
Methane	C1	0.2997	16.043	0.000	0.000	74.953	48.542	5.162	0.488
Ethane	C2	0.3558	30.070	0.121	0.020	5.748	6.978	0.509	0.090
Propane	C3	0.5065	44.097	0.797	0.195	6.744	12.006	1.207	0.314
I-Butane	i-C4	0.5623	58.123	0.887	0.286	2.873	6.741	1.024	0.351
n-Butane	n-C4	0.5834	58.123	1.418	0.457	3.037	7.126	1.530	0.524
I-pentane	i-C5	0.6238	72.150	2.340	0.936	1.574	4.585	2.287	0.973
n-Pentane	n-C5	0.6305	72.150	2.415	0.966	1.239	3.609	2.334	0.993
Hexane	C6	0.6634	86.177	4.832	2.309	1.331	4.631	4.591	2.332
Benzene	B	0.8820	78.110	0.884	0.383	0.025	0.079	0.825	0.380
Heptanes	C7	0.6874	100.204	5.355	2.975	0.398	1.610	5.014	2.961
Toluene	T	0.8734	92.140	1.535	0.784	0.054	0.201	1.433	0.778
Octanes	C8	0.7061	114.231	8.476	5.367	0.174	0.802	7.904	5.322
Ethyl-benzene	EB	0.8735	106.160	0.233	0.137	0.006	0.026	0.217	0.136
	m, p,			0.979		0.008	0.034	0.912	0.571
m- p -xylene	x	0.8671	106.160		0.576				
o-xylene	o-x	0.8840	106.160	0.375	0.221	0.004	0.021	0.349	0.219
Nonanes	C9	0.7212	128.260	7.235	5.144	0.021	0.121	6.738	5.094
Decanes	C10	0.7334	142.285	6.315	4.981	0.000	0.000	5.880	4.932
Undecanes	C11	0.7890	147.000	6.329	5.158	0.000	0.000	5.893	5.106
dodecanes	C12	0.8000	161.000	5.731	5.115	0.000	0.000	5.336	5.064
Tridecanes	C13	0.8110	175.000	5.496	5.332	0.000	0.000	5.117	5.279
tetradecanes	C14	0.8220	190.000	4.932	5.194	0.000	0.000	4.592	5.143
Pentadecanes	C15	0.8320	206.000	3.972	4.537	0.000	0.000	3.698	4.491
Hexadecanes	C16	0.8390	222.000	3.627	4.464	0.000	0.000	3.377	4.419
Heptadecanes	C17	0.8470	237.000	3.198	4.203	0.000	0.000	2.978	4.160
Octadecanes	C18	0.8520	251.000	2.845	3.958	0.000	0.000	2.649	3.919
Nonadecanes	C19	0.8570	263.000	2.471	3.603	0.000	0.000	2.301	3.567
Eiconadecanes	C20	0.8620	275.000	2.224	3.39	0.000	0.000	2.071	3.357
Eneicosanes	C21	0.8670	291.000	1.984	3.202	0.000	0.000	1.847	3.169
Dodeicosanes	C22	0.8720	305.000	1.788	3.023	0.000	0.000	1.665	2.993
Tricosanes	C23	0.8770	318.000	1.706	3.007	0.000	0.000	1.589	2.978
Tetraicosanes	C24	0.8810	331.000	1.450	2.661	0.000	0.000	1.350	2.634
Petaicosanes	C25	0.8850	345.000	1.373	2.626	0.000	0.000	1.278	2.600
Hexaucisanes	C26	0.8890	359.000	1.275	2.539	0.000	0.000	1.187	2.512
Heptaicosanes	C27	0.8930	374.000	1.118	2.319	0.000	0.000	1.041	2.295
Octaicosanes	C28	0.8960	388.000	1.055	2.270	0.000	0.000	0.982	2.247
Nonaicosanes	C29	0.8990	402.000	0.925	2.063	0.000	0.000	0.861	2.041
Tricontanes	C30	0.9020	416.000	0.785	1.809	0.000	0.000	0.731	1.792
Entricontanes	C31	0.9060	430.000	0.621	1.479	0.000	0.000	0.578	1.466
Dodetricontanes	C32	0.9090	444.000	0.345	0.850	0.000	0.000	0.321	0.841
Tritricontanes	C33	0.9120	458.000	0.220	0.559	0.000	0.000	0.205	0.553
Tetratricontanes	C34	0.9140	472.000	0.150	0.394	0.000	0.000	0.140	0.389
Pentatricontanes	C35	0.9170	486.000	0.097	0.262	0.000	0.000	0.090	0.259
Hexatricontanes	C36	0.9200	500.000	0.041	0.113	0.000	0.000	0.038	0.113
Hepatricontanes	C37	0.9390	514.000	0.027	0.078	0.000	0.000	0.025	0.076
Octatricontanes	C38	0.9410	528.000	0.018	0.055	0.000	0.000	0.017	0.052
Total				**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

Composition Analysis by Chromatograph up to C_{12}^{+}

Component	Stock tank oil		Gas Flashed		Well Stream	
	Mole. %	Wt.%	Mole. %	Wt.%	Mole. %	Wt.%
Nitrogen	0.000	0.000	0.511	0.578	0.035	0.006
Carbon Dioxide	0.000	0.000	1.300	2.310	0.090	0.023
Methane	0.000	0.000	74.953	48.542	5.162	0.488
Ethane	0.121	0.020	5.748	6.978	0.509	0.090
Propane	0.797	0.195	6.744	12.006	1.207	0.314
I-Butane	0.887	0.286	2.873	6.741	1.024	0.351
n-Butane	1.418	0.457	3.037	7.126	1.530	0.524
I-pentane	2.340	0.936	1.574	4.585	2.287	0.973
n-Pentane	2.415	0.966	1.239	3.609	2.334	0.993
Hexane	4.832	2.309	1.331	4.631	4.591	2.332
Benzene	0.884	0.383	0.025	0.079	0.825	0.380
Heptanes	5.355	2.975	0.398	1.610	5.014	2.961
Toluene	1.535	0.784	0.054	0.201	1.433	0.778
Octane	8.476	5.367	0.174	0.802	7.904	5.322
Ethyl-benzene	0.233	0.137	0.006	0.026	0.217	0.136
P, m-xylene	0.979	0.576	0.008	0.034	0.912	0.571
o-xylene	0.375	0.221	0.004	0.021	0.349	0.219
Nonanes C9	7.235	5.144	0.021	0.121	6.738	5.094
Decanes C10	6.315	4.981	0.000	0.000	5.880	4.932
Undecanes C11	6.329	5.158	0.000	0.000	5.893	5.106
dodecanes $C12^{+}$	49.474	69.105	0.000	0.000	46.066	68.407
Total	**100.000**	**100.000**	**100.000**	**100.000**	**100.00**	**100.00**
Gas gravity (air=1.000)			**0.855**			

Summary of Stock Tank Oil Properties

ANALYSIS	RESULT	METHOD
Density at 60^{o}F, (g/cc)	0.82236	IP 59
Density at 104^{o}F, (g/cc)	0.80861	IP 59
Kinematic viscosity at 104^{o}F, (Cst)	10.78	ASTM D 445
Dynamic viscosity at 104^{o}F, (Cp)	8.72	
Pour point, (^{o}C)	24	ASTM D 97
Sulphur content, (wt. %)	0.08	ASTM D 129
Wax content, (wt.%)	15.20	PPM
Asphaltene content, (wt.%)	0.045	PPM
Average molecular Weight	180.378	
^{o}API	40.4	
Residual sulphide	28.2	PPM
Water content	42.44	PPM
Salt content	92.71	Ptb
Dissolved H2S	Nil	PPM
Mercaptans	Nil	PPM
Density at 86^{o}F, (g/cc)	0.81418	IP 59
Kinematic viscosity at 86^{o}F, (Cst)	16.16	ASTM D 445
Dynamic viscosity at 86^{o}F, (Cp)	13.16	
HEPTANES PLUS (C_7^+)		
Density at 60^{o}F, (g/cc)	0.83765	
Average Molecular Weight	197.400	
Wt. %	94.450	
^{o}API	37.3	
DODECANES PLUS (C_{12}^+)		
Density at 60^{o}F, (g/cc)	0.88149	
Average Molecular Weight	251.951	
Wt. %	69.105	
^{o}API	28.9	

ASTM-DISTILLATION

Temperature ^{o}C	Percent volume recovery Vol. %
85	*Initial boiling point*
110	2
135	7
160	13
185	18
210	22
235	28
260	34
285	42
310	50

Recovery : 51 vol. %
Residue : 47 vol. %
Loss : 2 vol. %

Summary of PVT Data

Reported Reservoir Conditions

Reservoir Pressure	……	1700 psia (11.72 MPa)
Reservoir Temperature	……..	148 ^{O}F (64.44 MPa)

Pressure-Volume Relation

Saturation Pressure	…….	253 psig (1.74 MPa)
Average Single-phase compressibility	…….	6.1131E-6V/V/PSI (2900 TO 1700 PSIG)

Saturation Pressure
(at 253 psig at 148 ^{o}F)

Saturation Pressure (Psat.)	……	253 psig (1.74 MPa)
Density at Psat.	……..	0.78312 gm/cc
F.V.F. at Psat	……	1.0621 V at 148^{o}F/V at 60^{o}F
Oil Viscosity at Psat.	……..	1.650 cp

Reservoir Pressure
(1700 psig at 148 ^{o}F)

Reservoir Pressure	……	1700 psig (11.72 MPa)
Density at Res. pressure	……..	0.79205 gm/c
F.V.F. at Res. pressure	……	1.0501 V at 148^{o}F/V at 60^{o}F
Oil Viscosity at Res. pressure	……..	1.815 cp

Volumetric Data

(At 148 ºF)

Saturation pressure (Psat.)	……..	253 Psig (1.74 MPa)
Reservoir pressure	……	1700 Psig (11.72 MPa)
Reservoir Temperature	……..	148 ^{O}F (64.44 ^{O}C)

Average Single-Phase Compressibility

Pressure Range psig	Single-Phase compressibility v/v/psi
2900 TO 2320	5.7969 E-6
2320 TO 1700	6.3874 E-6
1700 TO 1160	7.3048 E-6
1160 TO 580	7.9902 E-6
580 to 253	8.5867 E-6