Discovery of New Oil-Degrading Bacteria with Biosurfactant Production Ability from Oily Tailings Pond Waste, Refinery-Contaminated Soil, Light and Heavy Crude Oils for Remediation of Crude Oil in Water

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Abstract

Discovery of New Oil-Degrading Bacteria with Biosurfactant Production Ability from Oily Tailings Pond Waste, Refinery-Contaminated Soil, Light and Heavy Crude Oils for Remediation of Crude Oil in Water

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Concordia University, 2019

Contamination of water and soil with crude oil and petroleum compounds frequently occurs. Removal of oil contamination is often not economically feasible by traditional remediation techniques. Therefore, the development of effective, fast, and bio-based remediation methods that can lessen the damaging effects of oil contamination and lower treatment costs are of great importance. Numerous oil-degrading bacteria with biosurfactant production ability have been isolated from oil-related environments. However, less attention has been given to the application of these compounds in the oil-related activities. The objectives of this study were to discover potential oil-degrading bacteria with biosurfactant (BS) production ability with potential application for crude oil bioremediation from oily tailings pond waste, refinery-contaminated soil, light and heavy crude oils by using the enrichment culture technique. The biodegradation tests were conducted in Erlenmeyer flasks containing water (350 mL, pH 7, salinity of 30 ppt) amended with nutrients and crude oil as the only carbon source (2 mL). The contribution of discovered oil-degrading bacteria in the crude oil biodegradation was determined at different periods of biodegradation by analysis of the remaining of total petroleum hydrocarbons using a gas chromatograph (GC-FID). Moreover, the production and properties of produced biosurfactants were assessed through surface tension (ST) and oil-displacement tests. A total of five oil-degrading bacteria species were discovered from oily tailings pond waste (1 species), refinery-contaminated soil (two species), light crude oil (one species) and heavy crude oil (one species). All species showed biosurfactant production ability. The lowest surface tensions of supernatants at the end of the biodegradation period were between 55 mN/m to 40 mN/m. The minimum ST belonged to the BS produced by species discovered from oily tailings pond waste (40 mN/m). Moreover, an average crude oil biodegradation of 70% was obtained with the isolated bacteria from samples following five weeks of incubation. This study confirmed the fast and effective biodegradation of crude oil

by the isolated bacteria with the biosurfactant production as the main mechanism of oil uptake.

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List of Abbreviations

BH	Bushnell Haas Broth
BS	Biosurfactant
CMC	Critical Micelle Concentration
CMD	Critical Micelle Dilution
CTAB	Cetyl Trimethyl Ammonium Bromide
GC-FID	Gas Chromatography- Flame Ionization Detection
H	Heavy Crude Oil
HIC	Hydrophobic Interaction Chromatography
Hx	n-Hexane
L	Light Crude Oil
MSM	Minimal Salt Media
NAPLs	Nonaqueous Phase Liquids
NRC	National Research Council
PAH	Polycyclic Aromatic Hydrocarbons
PPT	Part Per Thousand
ST	Surface Tension
T	Tailings Pond Waste
TPH	Total Petroleum Hydrocarbon

1. INTRODUCTION

1.1. Background

Soil and groundwater contamination by hazardous waste and petroleum hydrocarbons (crude oil and intermediate products) are the most common pollutants of all biological systems in the environment.

The source of this contaminant which is a common groundwater pollutant, are the leakage from underground storage tanks (UST) (Paria 2008), broken oil pipelines, oil-refineries and storage facilities, oil spills in chemical plants and transport processes (Sherman and Stroo 1989). Oil spills often lead to immediate or long-term damage to the environment (Banat *et al.,* 2010). Release may be unintentional or accidental (Banat *et al.,* 2010). One study reported that estimated 6 million tons of petroleum products spill into the soil each year in the United States alone and move into the air or water via the soil (Hutchins *et al.,* 1991).

There is a variety of chemical and biological methods to clean up this type of soil and water contamination such as using dispersants to break down the oil and also adding biological agent to the spill. Remediation technologies can be categorized into ex-situ and in-situ methods. Ex-situ methods involve excavation of polluted soils and subsequent surface treatment as well as removal and surface treatment of contaminated groundwater. In-situ techniques are intended to treat contamination without soil or groundwater depletion. Various technologies for oil-contaminated soil / sediments remediation have been developed. Traditional approaches to remediation consist of soil drilling and disposal of "pump and treat" waste and groundwater. In-situ technologies include but are not limited to: solidification and stabilisation, extraction of soil vapour, permeable reactive barriers, monitored natural attenuation, bioremediation-phytoremediation, chemical oxidation, steam-enhanced extraction and in-situ thermal desorption and have been widely used in the United States. Some of the most popular methods include thermal desorption, a soil remediation technology. During the process, the pollutants (e.g. oil mercury or hydrocarbon) are volatilized by a desorber to isolate them from soil or sludge. After that, the pollutants in an off-gas treatment system can either be removed or destroyed The Surfactant Enhanced Aquifer Remediation (SEAR) is another method commonly used in treatment The surfactant-

enhanced aquifer remediation process involves the injection into the subsurface of hydrocarbon mitigation agents or specialty surfactants to enhance desorption and recovery of non-aqueous phase liquid (NAPL) otherwise recalcitrant. Extraction of soil vapor is an effective soil remediation technology. "Multi-Phase Extraction" (MPE) is also an efficient remediation technique when remediation of soil and groundwater coincidentally occurs. SVE and MPE use various technologies to treat off-gas volatile organic compounds (VOCs) produced from the subsurface after vacuum removal of air and vapours (and VOCs), including granular activated carbon (most widely used historically), thermal and/or catalytic oxidation and vapor condensation. Carbon is commonly used in low (below 500 ppmV) VOC concentration vapor streams, oxidation is used in medium (up to 4,000 ppmV) VOC concentration streams, and vapor condensation is used in high (over 4,000 ppmV) VOC concentration vapor streams. Nanoremediation, another approach used to remediate polluted site as a new technology, is the use of nano-sized reactive agents to degrade or immobilize pollutants. In soil or groundwater nanoremediation, through either in situ injection or a pump-and-treat process, nanoparticles are brought into contact with the contaminant. The nanomaterials then degrade organic contaminants through redox reactions or adsorb metals like lead or arsenic and immobilize them. This technique was primarily used in commercial settings for groundwater remediation, with studies on wastewater treatment. Research is also investigating how nanoparticles can be used for soil and gas cleaning.

In comparison with physio-chemical methods, biological methods have become more acceptable in cleaning up contaminated hydrocarbon sites because they are environmentally friendly, cost-effective and efficient (Zhang *et al.,* 2012; Das and Mukherjee 2007). Bioremediation is one of the biological methods most widely used, but the effectiveness of microbial biodegradation is usually limited by the low bioavailability of hydrocarbons to microorganisms (Van Hamme *et al.,* 2003; Nitschke and Pastore, 2006).

Bento *et al.* (2005) identified bioremediation as the best approach for remediation of contaminated soils and groundwater by petroleum hydrocarbon among several of clean-up technologies. This was also recognized by Franzetti *et al*. (2008) as an economic tool for the management of sites contaminated with organic pollutants. Whang *et al*. (2008) identified bioremediation as an efficient, economic and environmental technology for remediation or clean up petroleum hydrocarbon contaminated sites.

According to Calvo *et al.* (2009), bioremediation involves speeding up the processes of natural biodegradation. It usually involves the use of nitrogen and phosphorus fertilizers, the adjustment of pH and water content and the addition of bacteria (Calvo *et al.,* 2009). The availability of oil to microorganisms is an important limiting factor in bioremediation (Martins *et al.,* 2009). Hydrocarbons like diesel oil or crude oil are hydrophobic compounds with low water solubility and thus low microorganism availability (Calvo *et al.,* 2009). In addition, the bioavailability of diesel oil and crude oil is also limited by its soil particle adsorption (Banat *et al.,* 2010; Bordoloi and Konwar 2009).

Compounds with low solubility and high hydrophobicity, such as diesel or crude oil, tend to adhere strongly to the matrix of soil (Franzetti *et al.,* 2008). These compounds are released slowly into the water phase.

Volkering *et al.* (1997) defined four key factors which contribute to bioavailability. These include the type and physicochemical state of the pollutant, soil type and physicochemical state, micro-organism type and state and external factors (such as temperature and oxygen levels) (Franzetti *et al.,* 2008).

The first two factors determine the shape of contaminants in the ground. The various possible physical forms of organic contaminants are shown in Fig. 1. They can be dissolved in pore water, adsorbed onto soil particles, absorbed into soil particles or present as a separate phase, a liquid or a solid phase (Volkering *et al.,* 1997).

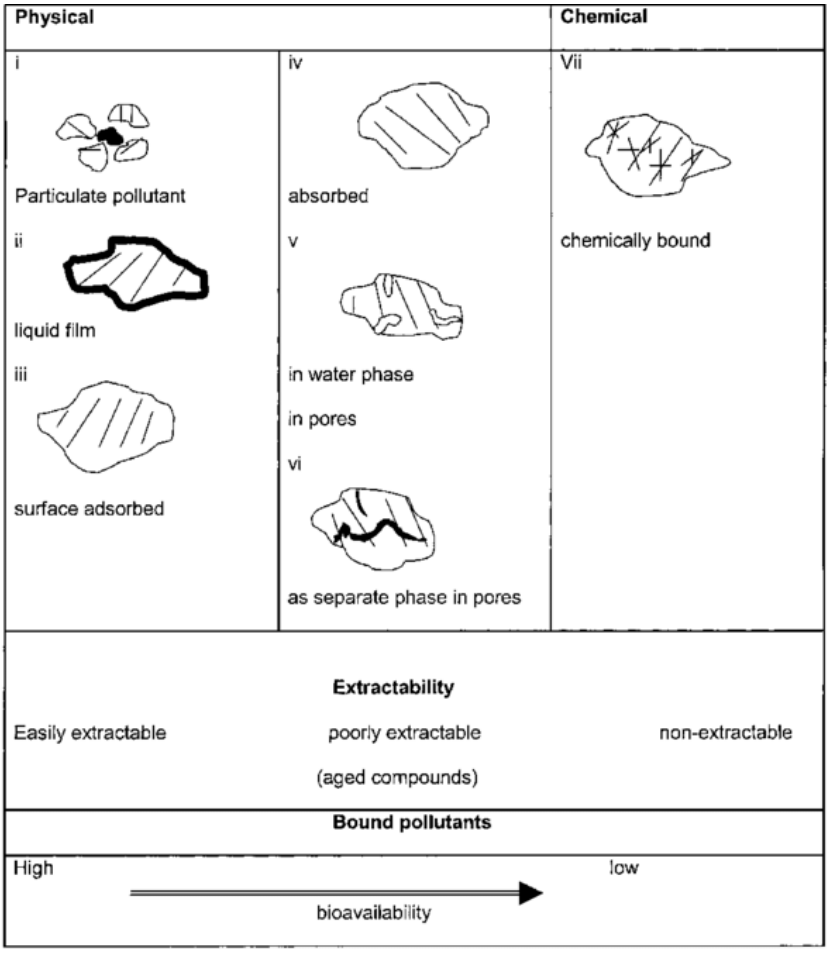


Figure 1.1. Different physical and chemical forms of organic pollutants in soil (taken from Volkering *et al.,* 1997)

Bioremediation has several advantages over conventional approaches to remediation (Alexander, 1999; Romantschuk *et al.,* 2000). First, in-situ bioremediation can be used. This minimizes the cost of excavation and ex situ contaminant treatment (NRC, 1994). Bioremediation also involves less pumping than pumping and processing and while less pumping is involved in bioremediation, the cleanup rate is usually much faster (NRC, 1994). Another major advantage of bioremediation over more conventional approaches is that bioremediation typically only produces carbon dioxide and water as by-products compared to the toxic by-products often associated with certain physical/chemical treatment methods (NRC, 1994).

Biosurfactants are a class of chemicals which are produced by microorganisms. They are low or high molecular weight compounds. Biosurfactants have hydrophilic and hydrophobic moieties mainly due to their amphiphilic nature, allowing biosurfactants to bridge water/air,

oil/air or oil/water interfaces where surface and/or interface tension are reduced (Mulligan *et al.,* 1999).

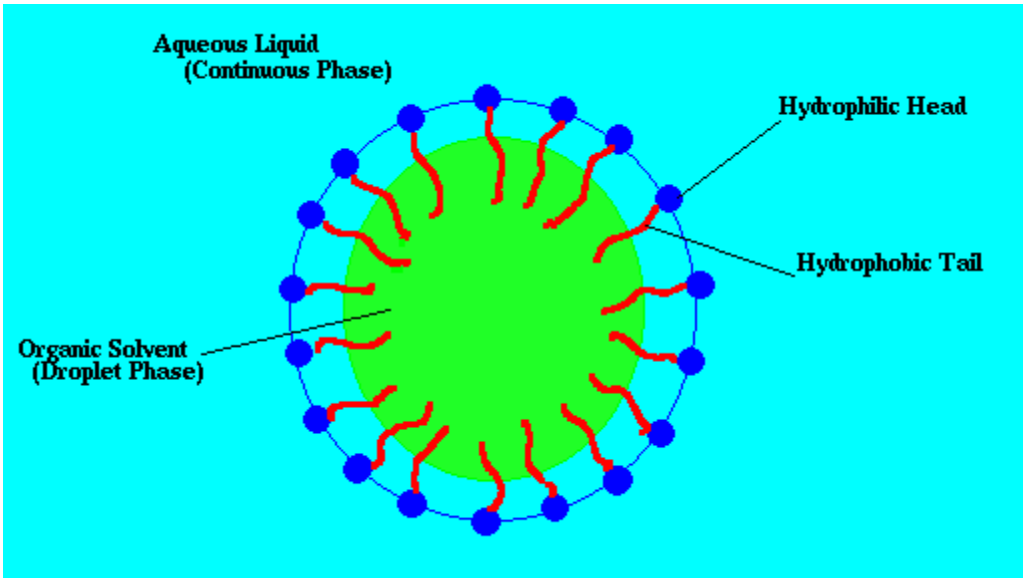


Figure 1.2. Action of surfactants (Source: http://www.physics.emory.edu. "Access date: 12-03-2018")

Chemically synthesized surfactants are used in the oil industry to help clean up oil spills and improve the recovery of oil from oil tanks. These compounds cannot be biodegraded and are toxic to the environment. Biosurfactants, however, have similar emulsification properties and are biodegradable in many cases (Mulligan, 2005). Biosurfactants are naturally produced and biodegradable by microorganisms. Biosurfactants also have low toxicity, high specificity and the potential for extreme environmental use (Mulligan and Gibbs, 2004; Kosaric, 2001).

Biosurfactant production in situ has enormous environmental significance and will eliminate the cost of buying biosurfactants and further reduce the cost of bioremediation (Das and Chandran, 2011). In addition, the biosurfactant produced can more effectively remove organic and metal contaminants by taking advantage of the biosurfactant specificity than currently available commercial biosurfactants (Das and Chandran, 2011).

Singh *et al.* (2006) reported that biosurfactants may replace or potentially increase the activity of surface-active chemically synthesized agents (Desai and Banat, 1997; Georgiou *et al.,* 1992). They have properties that make them promising alternatives to chemically synthesized agents with regards to their biodegradability, better compatibility with the environment, higher foaming activity, lower toxicity and high selectivity at extreme

temperatures, pH and salinity (Desai and Banat, 1997; Georgiou *et al.,* 1992). These microbial products are attractive and environmentally acceptable, particularly for the microbial recovery of oil (MEOR) (Desai and Banat, 1997; Georgiou *et al.,* 1992).

Hydrocarbons such as crude oil and diesel and various carbohydrates such as glucose, sucrose and glycerol have often been used as substrates for biosurfactant production. One important feature of biosurfactants is that they have low critical concentrations of micelle (CMC). The low CMC of biosurfactants means that less biosurfactants are needed. This makes them excellent candidates for green detergents and surfactants. However, more productive strains, better fermentation conditions and cheaper substrates are necessary to reduce costs and expand the application of biosurfactants. Moreover, the chemical and physical characteristics of new biosurfactants are not well investigated. It is therefore particularly important to characterize the biosurfactants produced during the degradation process for hydrocarbons (Kaeppeli and Finnerty 1980).

1.2. Objectives

The aim of this study was to explore the potential of using an oil-degrading bacteria present at contaminated sites for the treatment of petroleum. The use of bacteria as remediation organisms has gained a lot of interest in other pollution areas. Moreover, the use of biosurfactant-producing bacteria as agents of bioremediation in the petroleum industry is fast becoming an area of research that is leading to the development of new processes and technologies. This study confirmed the fast and effective biodegradation of crude oil by the isolated bacteria with the biosurfactant production as the main mechanism of oil uptake. The principal objectives of this study are as follows:

- To explore the possibility of bioremediation of petroleum contaminated soil using biosurfactant-producing bacteria
- To investigate the effect of biosurfactant production on biodegradation of petroleum hydrocarbon contaminated soil

1.3. Thesis Outline

This dissertation consists of five chapters followed by an appendix. The contents are as follows:

Chapter 1 presents the nature and description of the problem and the goals and organization of the thesis. Chapter 2 presents some background information and a literature review of past and current research. Chapter 3 covers the materials and methods used in the study. Chapter 4 presents the results of the study and the implications and significance of the results. Chapter 5 summarizes the conclusions of the findings. Chapter 6 provides suggestions for future work based on the results obtained from this study.

2. LITERATURE REVIEW

2.1. Petroleum Hydrocarbons in the Soil

Today, due to the high worldwide use of petroleum compounds, the pollution caused by these compounds is global (Kardani and Takdastan, 2015). To date, more than 3,600,000 wells have been drilled by oil companies worldwide. In the United States, only about 6,000 wells are produced. There are about 6,000 wells in Western Europe and over 22,000 well-known oil and gas basins in the world.

Oil spills often occur by accident during pumping, transport and refining. Oil is a complex combination of aliphatic and aromatic hydrocarbons that has a negative impact on human health and the environment. The US Environmental Protection Agency (EPA, 1986) considers these hydrocarbons as priorities for pollutants in the environment (Urum *et al.,* 2006).

Many physical and chemical methods are available in the soil to deal with oil pollution. Many physical and chemical methods are available in the soil to deal with oil pollution (Slots and Ting, 1999). Because of high costs and adverse side effects, many of these methods are used less. Biological correction is a refining technology using the biological activity of living organisms to reduce pollutant concentration or detoxification, including oil hydrocarbons (Sarkar *et al.,* 2005).

Soil oil pollution can be caused by petroleum sources such as polycyclic aromatic hydrocarbons (PAHs). This contamination can be caused by oil pipelines, caries, surface or even underground reservoirs and many other incidents often occurring in the production and transport of oil. Low molecular-weight polycyclic aromatic compounds derived from natural sources are mainly found in lower levels of the soil. While high molecular PAHs are often derived from human sources, they can be seen on the surface of the ground. Soil contamination is caused by precipitation of aerosols. The leakage of these compounds from storage tanks in industrial waste dumps also pollutes various soil depths. The rate of PAHs entering the body is influenced by the presence of compounds which are simultaneously exposed to them (Abdel-Shafy and Mansour, 2016).

Today, oil products are one of the chemicals most commonly used in society. With the enormous amount of fuel needed to power automobiles and heat homes and the number of times each gallon of oil is stored, transported or transferred, accidents and leakages are inevitable. Petroleum contamination results from leakage through ground and underground storage tanks, spillage during transport of petroleum products, abandoned petrol sites, other unplanned releases and current industrial processes.

Since oil contains dangerous chemicals such as benzene, toluene, ethylbenzene, xylenes and naphthalene, it can pose a risk to human, animal and plant health (Sarkar *et al.,* 2005). Petroleum contaminated soil is currently being treated by three methods: physical, chemical and biological. The most common methods of physical treatment for contaminated soils such as waste disposal and incineration are expensive. Incineration can cause air pollution (Slots and Ting, 1999).

Chemical treatment involves the direct injection in contaminated soil and groundwater of chemical oxidants. Biological treatment most often involves the breakdown of contamination into non- toxic forms by microbiological processes (Sarkar *et al.,* 2005).

2.1.1. Crude Oil Definition

Petroleum and its equivalent crude oil cover the massive amounts of combined hydrocarbon materials and other compounds that contain variable amounts of sulfur, nitrogen and oxygen that can vary widely in volatility, specific gravity and viscosity. Metal-containing compounds, particularly those containing vanadium and nickel, can usually occur up to several thousand parts per million in viscous oils and can have serious effects on the processing of these foods (Speight, 1997). Because the oil is a mixture of compounds and quantities, it has very diverse physical properties (Speight, 2012).

Oil occurs underground, at different pressures depending on depth. Due to pressure, it contains significant natural gas in solution. The oil underground is much lighter than its surface and is generally under mobile storage conditions because the high temperature (geothermal gradient) in the underground undergoes viscosity reduction. Oil is from aquatic animals and plants that have survived hundreds of years ago (Speight, 2014).

Hydrocarbons, hydrogen and carbon compounds that have various molecular structures are the main components of the oil. A large group of molecules known as paraffins are the simplest hydrocarbons (Speight, 2014). When the oil occurs in a reservoir, the raw material can occur as a light-colored, dark-to-pale color by the pumping operation. It is often referred to as ordinary oil. These materials are much higher in viscosity (and lower in API gravity) than conventional oils and this type of oil usually requires initial recovery by thermal stimulation from the reservoir. Heavy oils out of underground reservoirs are better than light oils. The definition of heavy oils is usually based on the gravity or viscosity of the API, and the definition is completely arbitrary, although attempts have been made to define it based on the concentration, viscosity and density of the API definition (Speight, 2014).

Heavy oils were considered to be crude oils with gravity slightly below 20° API with heavy oils falling within the 10° –15° API gravity range. Cold Lake heavy crude oil, for example, has an API gravity of 12° and extra heavy oils, such as tar sand bitumen, usually have an API gravity of 5° –10° (Athabasca bitumen= 8° API) (Speight, 2014).

Residuals would vary depending on the temperature at which distillation was stopped, but usually vacuum residues are in the 2° –8° API range. In a crude oil with a sulfur content of less than 20° API and usually more than 2 percent by weight, the generic term heavy oil is often used. Furthermore, heavy oils are darker in color and, contrary to conventional crude oils, may be even black. The term heavy oil has also been used arbitrarily to describe both heavy oils requiring reservoir thermal recovery stimulation and bitumen in bituminous sand formations (oil sand, q.v.) from which the heavy bituminous material is recovered through a mining operation (Speight, 2014).

2.1.2. Crude Oil Composition

In fact, the chemical and physical composition (fractional) of crude oil can vary not only with the location and age of the oil field, but also with the depth of the individual well. In fact, two adjacent wells can produce oil with distinctly different properties. Petroleum is a complex blend of hydrocarbons with low quantities of organic compounds including sulfur, oxygen and nitrogen, especially vanadium, nickel, iron and copper. The hydrocarbon content in light paraffin crude oil or 50 percent w/w in heavy crude oil and bitumen can be as high as 97 percent w/w (Speight, 2011).

Petroleum contains an extremely wide range of molecular and organic functionality. The variety is actually so great that it is unlikely that a complete compound-by-compound description would be possible for even a single crude oil. As already noted, the petroleum composition can vary with the location and age of the field in addition to any variations in the depth of the well. Two adjacent wells producing oil with very different characteristics are more than likely (Speight, 2011).

Petroleum, heavy oil, bitumen and residue are in very general terms a complex composition of (1) hydrocarbons, (2) nitrogen compounds, (3) oxygen compounds, (4) sulphur compounds and (5) metal components. This general definition, however, is not sufficient to describe the petroleum composition as it relates to the behavior of these feed stocks. Indeed, the consideration of the atomic ratio of hydrogen to carbon, sulfur content and API gravity is no longer sufficient to determine the refining behavior (Speight, 2011).

The petroleum hydrocarbon content may be as high as 97 percent by weight (e.g. in lighter paraffinic crude oils) or as low as 50 percent by weight or less as illustrated by the heavy crude asphalt oils. However, crude oils with only 50 percent hydrocarbon components are still assumed to retain most of the essential hydrocarbon characteristics. It is nonetheless the non- hydrocarbon (sulfur, oxygen, nitrogen and metal) components that play a major role in determining crude oil 's processing capacity (Speight, 2011).

The isolation of pure petroleum compounds is an extremely difficult task, and the overwhelming complexity of the hydrocarbon components of the higher molecular weight fractions and the presence of sulfur, oxygen and nitrogen compounds are the main causes of the difficulties. Data from synthesized hydrocarbons make it difficult to determine the identity or even similarity of synthetic hydrocarbons to those that make up many of the higher boiling fractions of petroleum. However, it was well established that petroleum hydrocarbon components consist of paraffin, naphthenic and aromatic groups (Speight, 2011).

The hydrocarbon components of petroleum should be divided into the following three classes:

1. Paraffins that are hydrocarbons saturated with straight or branched chains, but without any ring structure

2. Naphthenes that are saturated hydrocarbons with one or more rings, each of which may have one or more paraffinic side chains (more accurately referred to as alicyclic hydrocarbons)
3. Aromatics that are hydrocarbons containing one or more aromatic nuclei, such as benzene, naphthalene and phenanthrene ring systems, which can be connected to naphthene rings and/or paraffinic side chains (substituted) (Speight, 2011).

2.1.3. Environmental Impact of the Petroleum Contaminants

2.1.3.1. Toxicity

The environmental impacts of the petroleum industry have become more and more concerned over the years and are mainly negative. This is due to the oil toxicity which contributes to air pollution, acid rain and different human diseases. Petroleum also fuels climate change in its phases of extraction, refining, transport and consumption due to increased emissions of greenhouse gases.

The toxicity of oils can be understood by the toxic potential or toxicity of each oil component to the water solubility of that component (Di Toro *et al.,* 2007). Many methods can be used to measure the toxicity of crude oil and other petroleum products. Some studies that analyze toxicity levels may use the target lipid model or colorimetric analysis with colored colors to evaluate toxicity and biodegradability (Montagnolli *et al.,* 2015).

Various oil and oil-related products have different toxicity levels. Toxicity levels are influenced by many factors like weathering, solubility and chemical properties like persistence. Increased weathering tends to reduce toxicity levels by removing more soluble and lower molecular weight substances (Di Toro *et al.,* 2007). Highly soluble substances tend to have a higher toxicity level than water-soluble substances.

In general, oils with longer carbon chains and more benzene rings have higher toxicity levels. Benzene is the product associated with petroleum with the highest toxicity. Other substances other than benzene that are highly toxic are toluene, methylbenzene and xylenes (Montagnolli *et al.,* 2015). Crude oil and motor oil are the least toxic substances.

Although the toxicity of various oil variants varies, all petroleum products have adverse effects on human health and the ecosystem. Examples of adverse effects include oil

emulsions in digestive systems in certain mammals which may lead to a reduction in the ability to digest nutrients which may lead to the death of certain mammals. Other symptoms include hair loss and bleeding. Food chains of the ecosystem can be affected by a decrease in the productivity of algae, threatening certain species (Montagnolli *et al.,* 2015).

2.1.3.2. Emissions

Petroleum industry emissions occur in each oil production process chain from extraction to consumption phase. During the extraction phase, not only carbon dioxide but also various other pollutants such as nitrous oxides and aerosols are emitted (Tuccella *et al.,* 2017). Some by-products include carbon monoxide and methanol. When oil or petroleum distillate is combusted, the combustion is usually not complete and the chemical reaction leaves non-water or carbon dioxide by-products.

However, despite the large quantities of pollutants, there is uncertainty about the quantity and concentration of certain pollutants (Tuccella *et al.,* 2017). Petroleum also contributes to large quantities of pollution in urban areas during the refining phases. Due to the toxicity of oil, this increase in pollution has adverse effects on human health. A study in Taiwan investigated the effects of oil-refineries. The study found that premature births occurred more frequently in mothers living in close proximity to oil refineries than in mothers living away from oil refineries (Lin *et al.,* 2001).

2.1.3.3. Climate Change

Petroleum combustion leads to an increase in carbon dioxide emissions and other greenhouse gases. Swedish Nobel chemist Svante Arrhenius studied the first study on the effects of carbon dioxide (Ramanathan and Feng 2009). His mathematical model showed that an increase in carbon dioxide results in an increase in surface temperatures and therefore both factors are correlated. Petroleum combustion for transport, industrial and domestic use is one of the most important forms of air pollution.

The ultimate by-product of oil combustion is carbon dioxide, but other by-products like carbon monoxide and nitrates are present. These by-products react to ozone and other greenhouse gasses with the atmosphere. Increased pollution has global temperature consequences. The atmosphere reflects 30 percent of the incoming long-wave radiation and

keeps 70 percent warm. However, an increased concentration of carbon dioxide in the atmosphere is a "blanket" for increased heat.

Therefore, more longwave radiation is trapped in the atmosphere when there is a higher carbon dioxide concentration and this trapping results in higher surface temperatures. IPCC (2007) states that for carbon dioxide concentration doubling, the climate system will heat up by 3°C (Ramanathan and Feng, 2009). Temperature warming will have a massive impact on rainfall patterns and glacier retreat.

2.1.3.4. Oil Spills

An oil spill caused by human activity is the release into the environment, especially in marine areas, of a liquid petroleum hydrocarbon and is a form of pollution. The term is usually used for marine oil spills, where oil is released into the ocean or coastal waters, but also spills on land. Oil spills may be caused by the discharge of crude oil from tankers, pipelines, railcars, offshore platforms, drilling rigs and wells, as well as by the discharge of refined petroleum products (such as gasoline, diesel) and their by-products, heavier fuels used by large vessels such as bunker fuel, or by the discharge of any oily waste or oil.

Major oil spills include Lakeview Gusher, Gulf War and Deepwater Horizon. Spilt oil penetrates birds ' plumage structure and mammalian fur, reducing their insulating capacity and making them more susceptible to fluctuations in temperature and much less water-buoyant. Cleaning and recovery from oil spills is difficult and depends on many factors, including oil spill type, water temperature (evaporation and biodegradation) and shore and beach types involved (Bower, 2010).

The level of long-term contamination relies on the level of constant input into the environment of petroleum residues and the rate at which the environment is able to clean itself (Nicodem *et al.,* 1997). Spills can take weeks, months or even years to clean up. There are other factors that influence the rate of long-term contamination.

2.1.3.5. Waste Oil

Waste oil contains oils such as hydraulic oil, transmission oil, brake fluids, motor oil, crankcase oil, gearbox oil and synthetic oil. Waste oil create the same problem as with petroleum product. When waste oil from vehicles drips from motors across roads and streets,

the oil enters the water table, bringing with it such toxins as benzene. This poisons soil and potable water. Runoff from storms transports waste oil into rivers and oceans and poisoning the aquatic life (Azbar *et al.,* 2004).

2.1.4. Petroleum Contaminant Fate

Environmental pollution is a major concern for human health and environmental well-being throughout the world. Many chemicals and compounds commonly released into the environment have toxic or carcinogenic properties associated with acute and/or chronic human and other organism exposure. It is extremely important to understand the chemistry that contributes to the fate and transport of a substance in order to develop suitable mitigation and remediation policies. Chemical contaminants can enter the environment by a variety of means, including accidental spills, leaks from storage sites or industrial installations or as industrial activity by-products.

A wide range of physical and chemical remediation techniques are commonly used and are often tailored to the substrates and sites that are cleaned, such as soil, sediments, water bodies, wastewater, leachate, etc., and the contaminants involved (Khan *et al.,* 2004). Common in situ physical and chemical methods are not always fully effective and often require secondary clean-up strategies to destroy or remove the contaminant completely, whereas the excavation of the contaminated substrate is often not feasible or accessible and requires further remediation (Riser-Roberts, 1998).

Biological remediation techniques such as microbial bioremediation and phytoremediation can completely break down and eliminate harmful contaminants in the environment in a non-invasive and effective manner that requires less labor intensive and costly strategies than many physical equipment-based techniques. Bioremediation has the potential to completely eliminate harmful or dangerous compounds by using the natural metabolic capabilities of biota, especially microorganisms.

Petroleum contamination in the environment is ubiquitous. As the primary source of energy in the world and one of the most valuable resources in the world, a necessity for modern culture and the source of numerous economic and political conflicts, it is not surprising that the many forms and substitutes of crude oil can be found almost anywhere (Boehm *et al.,* 2008).

Biodegradation as an organic contaminant consisting of carbon-based compounds is a common process that occurs naturally in a contaminated environment and is actively used for remediation purposes by techniques such as biostimulation, the addition of nutrients to increase the productivity of a microbial community or bioaugmentation, the addition of a microbial strain or community capable of degradation (Boehm *et al.,* 2008).

The persistence of oil residues is a continuous concern in many environments. For example, petroleum residues spilled into the Prince William Sound, Alaska after the oil spill of Exxon Valdez in 1989, were found to persist for many years after the incident. In the French coast, 8 years after the Amoco Cadiz tanker spill in 1979, tar balls and weathered oil were found (Page *et al.,* 1988).

Sediments near West Falmouth, MA were contaminated with oil residues in 1969, 30 years after the barge spill in Florida (Reddy *et al.,* 2002). With such harmful compounds persisting for such long periods of time in a variety of environments, it is extremely important to understand the fate of petroleum hydrocarbons and predict where their transport after release.

Petroleum hydrocarbons released into groundwater can also have complex transportation processes and fates. The problem is complicated by the addition of fuel oxygenators, usually ethanol, which change the properties of the entire mixture and how it reacts in the environment. It is known that adding ethanol to gasoline alters its degradation capacity by providing a labile high-energy carbon source for microbial metabolism (Mackay *et al.,* 2006).

There are a variety of processes when petroleum products of any kind are released into the environment (crude oil, gasoline, etc.). These may be physical abiotic or biological processes through interaction with microorganisms and metabolism. The following are abiotic reactions that contribute to the breakdown or movement of different petroleum compounds and hydrocarbons. Evaporation of light compounds is the quickest physical process. This speed and efficiency can be influenced by environmental factors such as temperature, wind speed, water turbulence or surface properties (Fingas, 1995). Evaporation of light compounds is often a key process in the early stages of oil release and was an important factor in the removal of volatile petroleum components during large-scale spills. Lighter or shorter chain hydrocarbons can lose large proportions of their mass to evaporation soon after initial

petroleum release, up to 99 percent or more for light alkanes such as propane, butane and pentane (Mango, 2001).

Many petroleum hydrocarbons have a high hydrophobic affinity with organic matter. They can adsorb easily onto organic matter and preferably bind to soil and sediments (Yang *et al.,* 2005). These compounds are hard to remove once sorbed and can persist for many years before degradation (Jones and De Voogt, 1999). One of the underlying factors contributing to the persistence of sorbed petroleum hydrocarbons is the limited area of the surface which causes a lack of bioavailability for biodegradation and limited access to physical processes that could otherwise contribute to their breakdown or dilution (Flenner *et al.,* 1991).

If large volumes of petroleum reach shorelines such as large spills, the presence of petroleum hydrocarbons can increase erosion due to soil / sediment matrix interference (Silliman 2012). Once sorbed, these hydrophobic contaminants may be transported by erosion, sediment or mixing of the soil.

2.1.5. Microorganisms Growth

In batch culture, bacteria (or other microorganisms such as protozoa, microalgae or yeasts) can be grown in four different phases: lag phase(A), log phase(B), stationary phase(C) and death phase(D) (Hassan *et al.,* 2017).

During the lag stage, the bacteria adapt to the conditions of growth. It is the period in which the bacteria mature and cannot yet be divided. Synthesis of RNA, enzymes and other molecules occurs during the lag phase of the bacterial growth cycle. During the lag phase, the cells change very little, as they do not reproduce immediately in a new medium. This period of small to no cell division is called the lag phase and can last 1 hour to several days. Cells are not dormant during this phase (Tortora *et al.,* 2004).

The log phase (sometimes referred to as the logarithmic or exponential phase) is a cell duplication period (Steinberg, 2012). The number of new bacteria appearing per unit time is proportional to the current population. If growth is not limited, doubling will continue at a constant rate, so that both the number of cells and the population rate double over each period of time. For this type of exponential growth, the natural logarithm of cell number a straight line against the time. The slope of this line is the organism's specific growth rate,

which is a measure of the number of divisions per unit time cell (Steinberg 2012). The slope of the line in Fig. 2.1. depends on the growth conditions affecting the cell division frequency and the probability of the survival of both daughter cells. Under controlled conditions, cyanobacteria can double their population four times a day and then triple their population (Evans *et al.,* 1998). However, exponential growth cannot continue indefinitely, because the nutrient medium is soon depleted and enriched with waste.

A growth-limiting factor such as the depletion of an essential nutrient and/or the formation of an inhibitor such as organic acid often causes the stationary phase. The stationary phase is the result of a situation where growth and death rates are the same. The growth factor limits the number of new cells produced, and the rate of cell growth corresponds to the rate of cell death. During the stationary stage, the result is a horizontal, "smooth" linear part of the curve. Mutations may occur during the stationary phase.

Bridges *et al.* (2001) demonstrated that many of the mutations resulting from stationary phase genomes or hungry bacteria are responsible for DNA damage. Endogenously produced reactive oxygen species appear to be a major cause of such damage (Bridges *et al.,* 2001). Bacteria die during the death phase (decline phase). This could be caused by a lack of nutrients, environmental temperatures above or below the species tolerance band or other harmful conditions (Bridges *et al.,* 2001).

Batch culture is the most common method of growing bacteria, but it is only one of many. It is ideally unstructured spatially and temporarily structured. The bacterial culture is incubated with a single load of medium in a closed vessel. Certain bacterial cultures are periodically removed in some experimental regimes and added to the fresh sterile medium. This results in the continuous renovation of the nutrients in the extreme case.

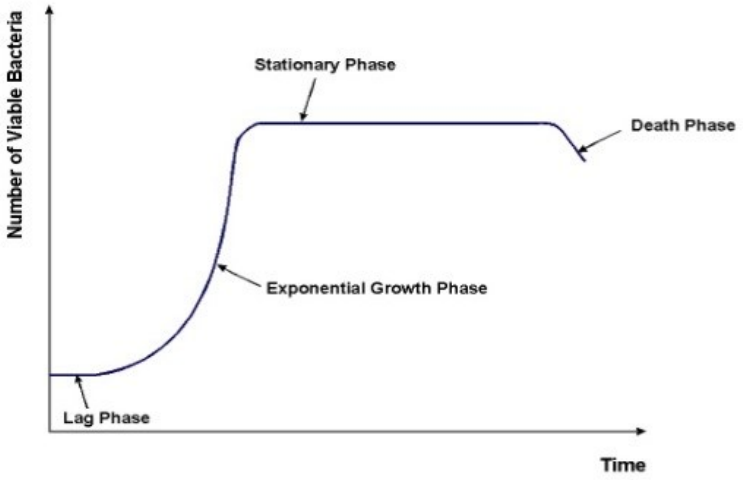


Figure 2.1. A typical growth curve for a bacteria population

2.1.6. Factors that Influence Microbial Growth

2.1.6.1. Nutrients

Concentration of nutrients: If the culture media is rich in a substance that promotes growth, the growth of bacteria occurs faster. The reduction of the concentration of nutrients reduces the growth rate. Various bacteria have different nutritional requirements (Alexander, 1999; Cookson, 1995; Atlas and Unterman, 1999). The relationship between the concentration (nutrition) of the substrate and the growth rate is shown in Fig. 2.2.

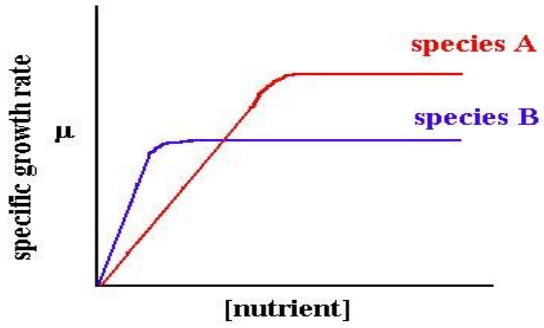


Figure 2.2. Nutrient vs growth rate

As the concentration of nutrients increases, the growth rate of bacteria increases to a certain level, and then the growth rate remains constant regardless of the addition of nutrients.

2.1.6.2. Temperature

Temperature influences bacteria's growth in different ways. The lowest growth temperature is called the minimum temperature, and the highest growth temperature is called the maximum temperature. There is no growth above the maximum temperature and below the minimum. The cell membrane solidifies below the minimum temperature and becomes stiff to transport nutrients into the cell, so that no growth occurs. Cellular proteins and enzymes denature above the maximum temperature, so that the bacterial growth stops. The relation between temperature and rate of growth is shown in Fig. 2.3 (Alexander, 1999; Cookson, 1995; Atlas and Unterman, 1999).

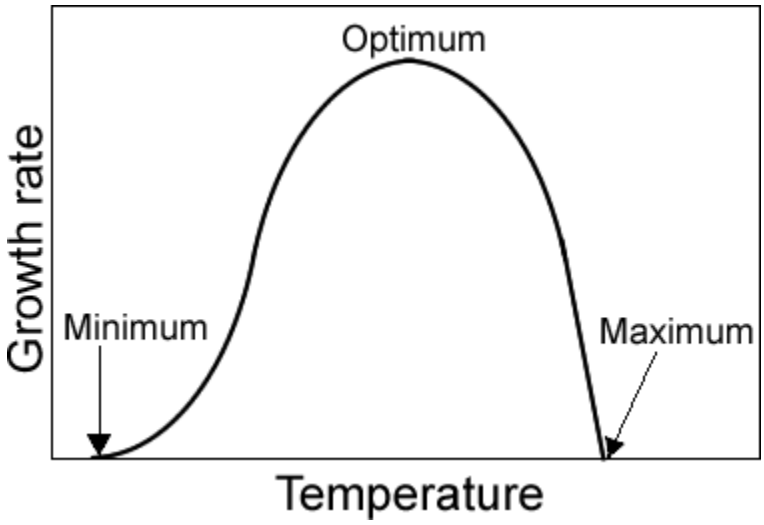


Figure 2.3. Temperature vs growth rate

When the temperature continually increases from its minimum, the growth rate of bacteria increases because the metabolic reaction rate increases with the temperature increase. At some temperature, the maximum growth rate is known as the optimum temperature. When the temperature increases above optimum, the growth rate decreases abruptly and completely with maximum temperature (Alexander, 1999; Cookson, 1995; Atlas and Unterman, 1999).

2.1.6.3. pH

The pH affects the bacterial cell's ionic properties, thus affecting bacterial growth. At neutral pH (6.5- 7.5), most bacteria grow. There are, however, certain bacteria that best grow in

acidic or basic pH. Fig. 2.4 shows the relation between pH and bacterial growth (Alexander, 1999; Cookson, 1995; Atlas and Unterman, 1999).

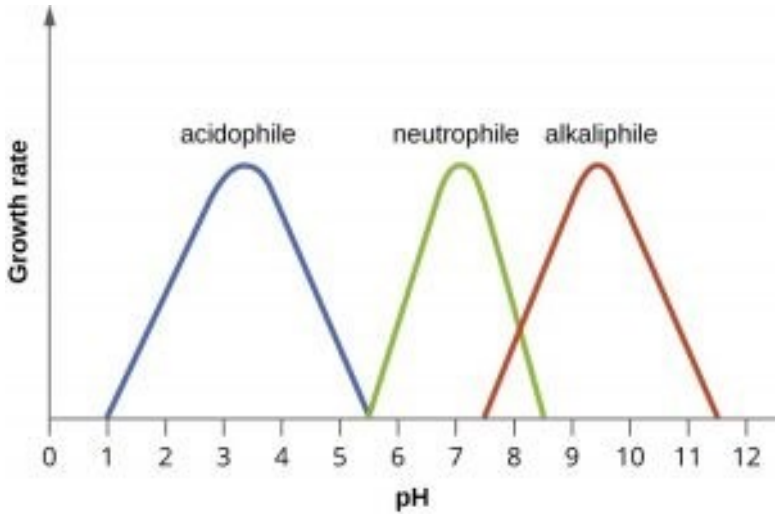


Figure 2.4. pH vs growth rate

2.1.6.4. Ions and Salt

To synthesize enzymes and proteins all bacteria require metal ions such as K+, Ca++, Mg++, Fe++, Zn++, Cu++, Mn++ etc. Most bacteria do not need NaCl in the media, however, they can tolerate very low salt levels (Alexander, 1999; Cookson, 1995; Atlas, 1999). There are some halophilic bacteria such as Archeobacteria that require high concentration of salt in media (Oren, 2008).

2.1.6.5. Gaseous Requirement

Oxygen and carbon dioxide are important gases which influence bacterial growth. Aerobic respiration requires oxygen and mandatory aerobic bacteria need O_2 for growth. Oxygen is harmful or at times lethal for obligatory anaerobic. However, facultative anaerobic bacteria may tolerate low O_2 concentrations (Alexander, 1999; Cookson, 1995; Atlas and Unterman, 1999).

2.1.6.6. Available Water

Water is the primary factor in bacterial growth. The rate of metabolic and physiological activity of bacteria is determined by the available water in the crop media. Sugar, salts and

other substances are dissolved in water and available to bacteria (Alexander, 1999; Cookson, 1995; Atlas and Unterman, 1999).

2.2. Surfactant and Biosurfactant Definition

Biosurfactants are organic compounds produced by microorganisms that have two parts that are hydrophobic and hydrophilic, which can reduce surface and interfacial surface water levels (Mulligan, 2005). Presently, there is only a very limited amount of biosurfactants available on the market, and the most popular biosurfactants are surfactin, sophorolipids and rhamnolipids (Mulligan, 2009).

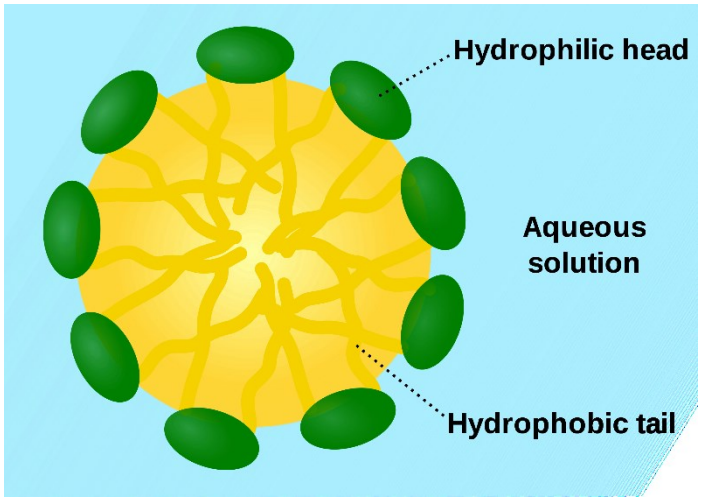


Figure 2.5. Surfactant monomers form a spherical micelle (Pasquali, 2010)

Surfactants are the active components found in soaps and detergents with the ability to concentrate at the air-water interface and mostly used to isolate hydrocarbon components from a particular media because of this fact that they are capable to increase aqueous solubility of Nonaqueous Phase Liquids (NAPLs) by reducing their surface/interfacial tension at air–water and water-oil interfaces (Mulligan *et al.,* 2001).

Therefore, increasing efforts in the discovery of new biosurfactant-producing bacteria are made by using a range of several screening methods. The main goal for screening new biosurfactants is finding new structures with strong interfacial activity, low critical micelle concentration (CMC), high emulsion capacity, and good solubility in wide range of pH (Mulligan and Gibbs, 1990).

For security reasons, biosurfactant-producing bacteria must be nonpathogenic but due to their surface activities biosurfactants may be involved in pathogenic activities. For the screening of biosurfactants, various methods have been developed and successfully used. An effective screening method is the most important part of isolation of new bacteria, because a large number of strains need to be identified (Mulligan *et al.,* 2001).

2.2.1. Assessment of Screening Methods for the Isolation of Biosurfactant

In general, the aim of screening of bacteria is discovery of strains with high efficiency. However, there are three steps for screening of new biosurfactants that consist of:

- sampling
- isolation of strains
- screening methods.

2.2.1.1. Sampling

Biosurfactants can perform different physiological roles and provide various benefits for their producing strains including (Ron and Rosenberg, 2001):

- increasing the surface area of water-insoluble substrates through emulsification,
- increasing the bioavailability of hydrophobic substrates,
- binding heavy metals,
- participating in pathogenesis,
- having antimicrobial activity,
- regulating the attachment/detachment of surface microorganisms.

Therefore, with these roles, biosurfactant-producing bacteria can be found in various environments. Many of them have been isolated from contaminated soil or water with organic hydrophilic compounds (refinery wastes) (Batista *et al.,* 2006; Al-Mallah *et al.,* 1990), some from marine environments (Schulz *et al.,* 1991; Lang and Wanger, 1993; Yakimov *et al.,* 1995), some from intact environments such as natural soils (Bodour *et al.,* 2003), and even from aircraft fuel tanks (kerosene fungus) (Muriel *et al.,* 1996).

2.2.1.2. Isolation

Microbes in natural environments always form a mixed population consisting of many strains and different species. For analysis of the properties of a defined organism by such a mixed population, a pure culture is required. For isolation of biosurfactants, there is direct isolation with diluting and plating and enrichment cultures with hydrophobic substrates. Moreover, other methods such as the replica plate technique and hydrophobic interaction chromatography are also popular methods. The most important role in enrichment culture is providing favorable growth conditions for microbes (Willumsen and Karlson, 1996; Mercade *et al.,* 1996; Rahman *et al.,* 2002).

For the screening of biosurfactants, hydrophobic compounds as the only carbon source are used which is considered as an indirect method because the growth on hydrophobic compounds show the production of biosurfactants. However, it is not always associated with this property. Table 2.1 indicates some samples with their methods of isolation (Willumsen and Karlson, 1996; Mercade *et al.,* 1996).

Table 2.1. Different samples with their methods and their total isolated strains

Origin	Source of contamination	Methods	Strain	Strain producing BS	Reference
Soil	Polyaromatic hydrocarbons (PAHs)	Using PAH-modified liquid minimal medium for culturing then using agar plates covered with different PAHs and agar plates with a PAH-soaked filter in the lid of the petri dish for the selection	57	4	Willumsen and Karlson, 1996
Soil	Petroleum	Using waste lubricating oil as the sole carbon source	44	5	Mercade *et al.,* 1996
Soil	Hydrocarbon-polluted	Using a mineral salts medium containing crude oil as the source of carbon	130	2	Rahman *et al.,* 2002

As a conclusion, sampling of contaminated sites with direct isolation or enrichment culture is a proven strategy to discover new strains producing biosurfactants.

2.2.1.3. Screening Methods

Since most of the screening methods for biosurfactants are based on surface/interfacial activity, the majority of methods are developed for measuring this property.

Measurements of Direct Surface/Interfacial Tension

Direct surface/interfacial tension measurement of the culture supernatant is the most straightforward and initial method for screening of biosurfactant. There are major methods of measuring interfacial/surface tension. Upon reaching the critical micelle concentration (CMC), as concentration of biosurfactant increases the surface tension will decrease (Lin, 1996).

In measuring surface tension, two different cultures may have the same surface tension because an increase of concentration for biosurfactant will not detect if concentration is above the CMC. However, serial dilution is used until the maximum increase in surface tension observed. This dilution correlates to the concentration of the biosurfactant and is called CMD. In the following, various methods are described (Batista *et al.,* 2006; Persson and Molin, 1987).

Du-Nouy Ring Method

This method uses the force for detaching a ring from an interface or surface. The separating force is proportional to the interfacial tension. All contaminants should be removed from the ring by using a flame (Tadros, 2005).

This method has a lot of advantages and disadvantages. The advantage of this method is the accuracy and the ease of use and the disadvantage is that measuring different samples cannot be done at the same time (Bodour and Miller-Maier, 1998).

A culture is good if it reduces the surface tension to 40 mN/m or even less (Cooper *et al.,* 1986). In another definition, a good biosurfactant producer is one that decreases the surface tension to 30 mN/n compared with distilled water (Willumsen and Karlson, 1996).

Stalagmometric Method

Stalagmometry is one of the most frequently used surface tension determination methods. To this end, using the traubestalagmometer, the several drops of the liquid leaked from the glass capillary of the stalagmometer are weighed. The number of drops to determine the surface tension can also be counted if the weight of each drop of the known liquid is counted. The drops formed slowly on top of the capillary of vertical glass. When its weight (volume) reaches the magnitude that balances the surface tension of the liquid, the pendant drop at the

tip begins to decrease. The weight (volume) depends on the properties of the liquid. The surface tension is therefore calculated using Eq. 2.1:

$$\sigma L = \frac{\sigma W \,.\, NW \,.\, \rho L}{NL \,.\, \rho W} \qquad \text{Eq. 2.1}$$

Where σL indicates the surface tension of the supernatant, σW is the surface tension of water, NL is the number of drops of supernatant, NW the number of drops of water, ρL is the density of supernatant and ρW is the density of water.

The problem of this method is that only continuous measurements can be made and, in some cases, different results for the same sample are obtained (Dilmohamud *et al.,* 2005).

Measurements of Indirect Surface/Interfacial Tension

Many screening methods measure surface tension indirectly. In the following, various methods are described.

Drop Collapse Assay

In this assay 25 µl of supernatant were placed on an oil coated solid surface and the shape of drop on the surface was measured after 1 minute and the diameter of droplets was measured. If the supernatant does not have surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops stay stable. If the supernatant has surfactants, the drops spread or even collapse because of the reduction of interfacial tension between the hydrophobic surface and liquid drop. The stability of the drops depends on the surfactant concentration and correlates with surface/interfacial activity (Jain *et al.,* 1991).

Oil Displacement Test

The oil displacement test was done as describe by Morikawa *et al.* (2000). This assay measures the diameter of clear zones from a drop of a biosurfactant containing solution on an oil–water surface. For this assay, 10 µL of crude oil were added to 40 mL of distilled water in a petri dish for making a thin oil layer. Then, 10 µL of supernatant were placed on the center of the oil layer. If the oil was displaced, a clearing zone forms that means the liquid contains a biosurfactant that the magnitude of the clearing zone means more quantity of surfactant. This assay is fast, easy and needs no specialized equipment and just a small

volume of sample. The other advantage of this method is that it is a reliable method for identification of the presence of a biosurfactant (Płaza *et al.,* 2006; Youssef *et al.,* 2004).

Emulsification Capacity Assay

The emulsifying capacity was evaluated by an emulsification index (E24) in this process. The emulsification index of culture sample was determined by adding 2 mL of kerosene and 2 mL of supernatant in a test tube (Techaoei *et al.,* 2007). Then the test tube was vortexed at high speed for 2 minutes and after 24 hours, the E24 index can be measured by Eq. 2.2 that is correlated with the surfactant concentration (Cooper *et al.,* 1987):

$$E24 = \frac{h\ emulsion}{h\ total} \times 100\% \qquad \text{Eq. 2.2}$$

In this assay, the kerosene can be replaced with hydrophobic compounds like hexadecane. The disadvantage of this method is that this gives just indication of the presence of biosurfactant.

Hydrophobic Interaction Chromatography (HIC)

This assay uses hydrophobic interaction chromatography (HIC) that is a chromatographic method based on hydrophobic interaction between the nonpolar groups on a hydrophobic chromatographic resin and the nonpolar zone of a particle (Smyth *et al.,* 1978). A bacterial suspension is added to a gel bed of hydrophobized sepharose. Hydrophobic microbes are held by the gel and the amount of adsorption of the cells to the gel is obtained by the counting of bacteria. For removal of the adherent bacteria, the ionic strength of the buffer is decreased. This method is easy, reliable and used for comparative analysis of the hydrophobic properties of microorganisms (Pruthi and Cameotra, 1997).

Salt Aggregation Assay

In this assay by adding salt, the precipitation of the cell happens. In this assay at low salt concentration first, the most hydrophobic cells precipitate (Lindahl *et al.,* 1981). For doing this test, ammonium sulfate is diluted in sodium phosphate buffer by serial dilution ranging from 4 M to 0.02 M ammonium sulfate. Then the bacterial suspension is mixed with the

same volume of salt solution on glass with depression slides. The suspension was mixed for 2 minutes at 20˚C, and then is read against a black background (Pruthi and Cameotra, 1997).

A positive accumulation reaction indicates a clear liquid and a white aggregate with a diameter of about 0.1 mm. For the positive control the reaction at the highest molarity compared with all readings and for the negative control suspension mixed with 0.002 M sodium phosphate without adding salt. For this assay, no special equipment is required (Pruthi and Comeotra, 1997).

Specialties

The last part deals with two methods that are used specifically for screening biosurfactants named the CTAB agar plate test and the hemolysis test.

CTAB Agar Plate

The CTAB agar plate method is a semi-quantitative measurement for the discovery of anionic surfactants like glycolipids. Blue agar plates including cetyltrimethylammonium bromide (CTAB) (0.2 mg/mL) and methylene blue (5 mg/mL) are used to find extracellular glycolipid production. If the dark blue halos are observed around the colony that means biosurfactant is present. CTAB agar test is easy to carry out, but it is just used for specific biosurfactants.

The advantage of this method is that using different culture conditions can be used on the agar plates, like different substrates or temperature and it can be transferred to liquid culture conditions. The disadvantage is that CTAB is harmful and can prevent the growth of some microbes but the CTAB can be replaced by another cationic surfactant (Siegmund and Wagner 1991).

Hemolysis

Hemolytic activity can be a good assay for screening biosurfactant because biosurfactants are found to be associated with hemolytic activity. For this assay a sample is taken from the culture and put on blood agar plates. The plates are incubated for two days at 25°C. If the supernatant has surfactants, the clear zones around the colony are observed (Mulligan *et al.,* 1984).

The hemolysis assay is often applied for a preliminary screening of biosurfactants on hydrophilic media. Although this media is a rich media for many organisms, there are some disadvantages for this method. This assay is not specific just for biosurfactant because lytic enzymes also cause clear zones and hydrophobic substrates are not the only source of carbon. In addition, the spreading limitation of the biosurfactant can prevent creating clear zones (Schulz *et al.,* 1991).

Although some tests indicated that some biosurfactants do not have any hemolytic activity at all or can give many false negatives and false positive results, some recommend the blood agar method to be applied as a preliminary screening method which should be applied with other techniques based on surface activity measurements (Mulligan *et al.,* 1984).

2.2.2. Economic Factors of Biosurfactant Production

To overcome the cost constraints associated with the production of biosurfactants, two basic strategies are generally adopted worldwide to make them cost-effective:

1. the use of inexpensive and waste substrates for the formulation of fermentation media that reduce the initial raw material costs involved in the process;
2. developing efficient and successfully optimized bioprocesses, including optimizing growth conditions and cost-effective recovery processes for maximum production and recovery of biosurfactants.

Since millions of tons of hazardous and non-hazardous waste are produced worldwide every year, there is a great need for their proper management and utilization. Residues from tropical agricultural crops such as cassava (peels), soybean (hull) (De Lima *et al.,* 2009), sugar beet (Onbasli *et al.,* 2009), sweet potatoes (peel and stalks), potatoes (peel and stalks), sweet sorghum (Makkar and Comeotra 2002), rice and wheat (Krieger *et al.,* 2010) (bran and straw), hull soy, maize and rice, sugarcane and cassava bagasse, coffee processing industry residues such as coffee pulp, coffee husks, spent coffee grounds, reported as substrates for the production of biosurfactants (Pandey *et al.,* 2000).

Other substrates used in the production of biosurfactants include water- mixable waste, molasses, whey milk or distillery waste (Makkar and Comeotra 2002). The advantages of the various substrates previously reported for the production of biosurfactants (Table 2.2) are listed.

Table 2.2. Substrates for microbial surface-active agent production

Source	Substrate	End product	Reference
Cassava	Flour	Biosurfactant	Nitschke and Pastore, 2003
Soybean oil	Seeds	Rhamnolipid	De Lima *et al.,* 2009
Sugar beet	Peels	Biosurfactant	Onbasli *et al.,* 2009
Sweet potato	Peels	Biosurfactant	Makkar and Comeotra, 2002
Sweet sorghum	Peels	Biosurfactant	Makkar and Comeotra,j 2002
Rice and wheat bran	Stem Husk	Biosurfactant	Barrios-Gonzalez *et al., 1988*
Sugarcane bagasse	Stem Husk	Biosurfactant	Krieger *et al.,* 2010
Cashew Apple juice	Pomace	Biosurfactant	Rocha *et al.,* 2007

2.2.3. Environmental Factors Affecting Biosurfactant Production

Biosurfactants are produced by a number of micro-organisms, mainly in water-immiscible substrates during their growth. Some yeasts can, however, produce biosurfactants in the presence of various substrates such as carbohydrates that can alter the structure of the produced biosurfactant and its properties and can be used to obtain products with desired properties for specific applications. There are a number of studies in the production of biosurfactants that optimize their physicochemical properties (Afsora Sarubbo *et al.,* 2006; Sarubbo *et al.,* 2001). The nature of the nitrogen source and the presence of iron, magnesium, manganese, phosphorus and sulphur in the media influence the composition and characteristics of biosurfactants.

2.2.3.1. Carbon Source

Due to the high production and recovery costs, biosurfactants have not yet been able to compete cheaply with chemically synthesized compounds. By using alternative sources of nutrients, these costs can be greatly reduced.

Sarubbo *et al.* (2001) first identified a biosurfactant produced by *Y. lipolytica* IA 1055 using glucose as a carbon source and concluded that the induction of the production of biosurfactants does not depend on the hydrocarbon presence. Biosurfactant production by *B. subtilis* MTCC 2423 has been monitored by measuring cell-free broth surface tension reduction.

It was better to reduce surface tension when glucose, sucrose, trisodium citrate, sodium pyruvate, yeast extract and beef extract were used as sources of carbon. Lactose was also used by *Kluyveromyces marxianus* as a soluble substrate for the production of mannan proteins (Lukondeh *et al.,* 2003). The maximum production of bioemulsifiers was observed when the *C. glabrata* strain, isolated from mangrove sediments, was grown on cotton seed oil (7.5%) and glucose (5.0%), reaching values of 10 g L^{-1} after 144 hours.

The C-sources described, such as glucose, glycerol, acetates and other organic acids and pure n-alkanes, are quite expensive and contribute to the cost of producing biosurfactants. A cost reduction approach is to replace pure reagents with industrial/agricultural waste materials in part or in full.

2.2.3.2. Nitrogen Source

Nitrogen is important in the production medium of biosurfactants because it is an essential component of the proteins necessary for the growth of microbes and the production of enzymes for the fermentation process. Several sources of nitrogen, such as urea, peptone, ammonium sulphate (Zinjarde *et al.,* 1997), ammonium nitrate (Thanomsub *et al.,* 2004), sodium nitrate (Bednarski *et al.,* 2004), meat extract and malt extract (Mata-Sandoval *et al.,* 2001), have been used to produce biosurfactants. Yeast extract is the most commonly used source of nitrogen for the production of biosurfactants, but the required concentration depends on the nature of the microorganism and the culture medium. Biosurfactants are often produced when the source of nitrogen is depleted in the culture medium during the stationary cell growth phase. The use of potassium nitrate in the production of biosurfactant by the yeast *R. glutinis* IIP30 produces better yields compared to other nitrogen sources such as ammonium sulfate or urea (Johnson *et al.,* 1992). Lukondeh *et al.* (2003) investigated the production of *K. arxianus* FII 510700 biosurfactant using yeast extract (2 g L^{-1}) and ammonium sulfate (5 g L^{-1}) as sources of nitrogen.

2.2.3.3. pH

The effect of pH in *C. antarctica 's* biosurfactant production was investigated using phosphate buffers with pH values ranging from 4 to 8. All conditions used led to a reduction

in the yield of biosurfactants compared with distilled water (Kitamoto *et al.,* 2001). Zinjarde and Pant (2002) studied the influence of initial pH on *Y. lipolytica 's* production of a biosurfactant. The best biosurfactant production occurs when the pH was 8.0, the natural pH of marine water.

The acidity of the medium of production was the parameter studied in glycolipid synthesis by *C. antarctica* and *C. apicola.* Glycolipid production reaches a maximum if pH is maintained at 5.5. Without pH control, the biosurfactant synthesis decreased, indicating the importance of maintaining it during the fermentation process (Bednarski *et al.,* 2004).

2.2.3.4. Temperature

Most of the biosurfactant productions reported to date have been performed at temperatures ranging from 25 to 30ºC and Casas and Garcia-Ochoa (1999) observed that the sophorolipid levels obtained in the *C. bombicola* culture medium at temperatures of 25 or 30ºC are similar. However, fermentation at 25ºC shows a lower biomass growth and a higher glucose consumption rate compared to fermentation at 30ºC.

Deshpande and Daniels (1995) observed that *C. bombicola* grows at a maximum temperature of 30ºC while 27ºC is the best temperature for sophorolipid production. Temperature causes variations in biosurfactant production in the *C. antarctic* culture. The highest production of mannosylerythritol lipids was observed at 25ºC for both growth and resting cells (Kitamoto *et al.,* 2001).

2.2.3.5. Metal Ion Concentration

Concentrations of metal ions play an important role in the production of certain biosurfactants because they form important cofactors of many enzymes. Surfactin biosurfactant overproduction occurs in the presence of Fe^{2+} in the mineral salt medium. In the presence of inorganic cations, such as overproduction, the properties of surfactin are modified (Johnson *et al.,* 1992).

2.2.3.6. Aeration and Agitation

Due to increased shear stress, *N. erythropolis* and *A. calcoaceticus* produce less biosurfactant, but on the other hand, biosurfactant production with yeasts usually increases with stirring and aeration rates (Desai and Banat, 1997). Adamczak and Odzimierz Bednarsk

(2000) investigated the effect of aeration on biosurfactant synthesis of *C. antarctica* and observed that optimal production (45.5 g/L) is achieved when the air flow rate is 1 vvm and the dissolved oxygen concentration remains at 50% of the saturation level. The air flow rate, however, is changed to 2 vvm and high foam formation and biosurfactant production is reduced to 84 percent (Guilmanov *et al.,* 2002, Besson and Michel, 1992). There were attempts to reduce the inhibition of the end product in surfactin production by isolating surfactin from the culture using foam separation and aqueous two-phase cultivation (Sandrin *et al.,* 1990).

2.2.4. Product Recovery

Even if optimum production is achieved using optimum media and cultural conditions, the production process is still incomplete without an efficient and cost-effective processing method. The downstream processing costs account for 60% of the total production costs for many microbiological products. A number of methods to improve the recovery of biosurfactants has been developed (Table 2.3.).

Table 2.3. Methods for the recovery of biosurfactants

Number	Method(s)	Mechanism(s)	Reference(s)
1.	Adsorption on wood	Adsorption	Heyd *et al.,* 2008; Dubey *et al.,* 2005
2.	Adsorption on polystyrene	Adsorption	Reiling *et al.,* 1986
3.	Ion exchange chromatography	Charge separation	Reiling *et al.,* 1986
4.	Solvent extraction	Dissolves in organic solvents	Kuyukina *et al.,* 2001
5.	Centrifugation	Centrifugal force	Nitschke and Pastore 2003
6.	Acid precipitation	Insoluble at low pH	Sen and Swaminathan, 2004
7.	Membrane ultra filtration	Micelle formation	Sen and Swaminathan, 2005
8.	Selective crystallization	Redissolution in organic Solvents	Satpute *et al.,* 2010
9.	Ammonium sulphate precipitation	Salting out of protein	Satpute *et al.,* 2010
10.	Organic solvent extraction	Solubility in organic solvents	Dubey *et al.,* 2005
11.	Foam fractionation	Surface activity	Sarachat *et al.,* 2010
12.	Thin layer chromatography	Difference in relative flow against solvent	Priya and Usharani, 2009
13.	Dialysis	Difference in solute concentration	Satpute *et al.,* 2010

14.	Lyophilization	Cryodesiccation	Satpute *et al.,* 2010
15.	Iso-electric focusing	Electric charge difference	Satpute *et al.,* 2010

These procedures take advantage of some of the characteristics of biosurfactants, such as their surface activity or their ability to form micelles and/or vesicles and are especially applicable for the continuous large-scale recovery of extracellular biosurfactants from broth. Examples of such biosurfactant recovery strategies include foam fractionation (Sarachat *et al.,* 2010), ultrafiltration (Goswami *et al.,* 2010), adsorption-desorption on polystyrene resins and chromatography of ion exchange (Reiling *et al.,* 1986), and adsorption-desorption on wood-based activated carbon (WAC) (Dubey *et al.,* 2005).

In recent years, less expensive and toxic solvents such as methyl tertiary butyl ether (MTBE) have been successfully used to recover *Rhodococcus* biosurfactants (Kuyukina *et al.,* 2001). These types of low-cost, less toxic and highly available solvents can be used to significantly reduce recovery costs and reduce environmental risks.

2.2.5. Purification Methods for Biosurfactants

Hydrochloric acid has been used in conventional methods to extract raw biosurfactants from microbial biomass in concentrated form. However, various techniques for isolating and purifying crude biosurfactants, such as membrane-based techniques, foam fractionation, extraction, adsorption, have been developed at this stage. Mulligan *et al.* (2001) initially reported the separation of the membrane for surfactin recovery. The bubbleless membrane bioreactor has now been successfully developed for the production of biosurfactants (Coutte *et al.,* 2013). Microfiltration and ultrafiltration are combined in the bubbleless membrane bioreactor to improve the efficiency of the separation process. Foam fractionation is a method for biosurfactant separation in which acidified hydrochloric acid is added to the biosurfactant precipitate. The precipitates are the extracted with solvent (Cooper and Goldenberg, 1981). Davis *et al.* (2001) indicated that surfactin isolation fractionation is an integrated system. Extraction is now becoming much more attractive to researchers because of its easier operation. For the extraction of biosurfactants, various solvents such as chloroform, methanol, ethyl acetate, di-chloromethane, butanol, pentane, hexane, diethyl ether, isopropanol and acetate are used. In solvent extraction, hydrophobic moieties in some solvents that help in the extraction of the crude product are found to be soluble. The

amberlite XAD 2 or polystyrene resins are used to purify biosurfactants during the adsorption and desorption process. During this process, biosurfactant recovery is governed by various factors such as agitation rate, carbon particle size activated, pH, temperature, initial adsorbent concentration, adsorbent amount and ion strength. Polymer resins are used in newly developed techniques to adsorb biosurfactants and organic solvents are used for desorption. The activated carbon is used as an adsorber for surfactin recovery (Liu *et al.,* 2007). In addition, regenerated active carbon can also be used for biosurfactant recovery (Dubey *et al.,* 2005).

2.2.6. Analytical Methods

Many researchers have used and reported several analytical methods for characterizing biosurfactants as shown in Table 2.4.

Table 2.4. Type of Biosurfactants, Bacteria, Solvent and Analytical Methods Involved

Biosurfactant & bacteria	Analytical method	Chemicals/solvents required	Reference
Rhamnolipids *Pseudomonas aeruginosa*	HPLC	CH_3CN-H_2O	Schenk *et al.,* 1995
	TLC	$CHCl_3/CH_3OH/CH_3COOH$	Arino *et al.,* 1996
	TLC	CH_3OH/H_2O	Rahman *et al.,* 1999
Pseudomonas fluorescens	TLC	CH_3CN/H_2O	Caldini *et al.,* 1995
P. aeruginosa MTCC 2297	HPLC	CH_3CN (Contain 2-bromoacetophenone and triethylamine)	Venkatesh and Vedaraman , 2012
Lipopeptide *Acinetobacter baylyi* ZJ2	FTIR	$CHCl_3/CH_3OH/CH_3COOH$	Zou *et al.,* 2014
Sophorolipid *Candida bombicola*	HPLC with ELSD	CH_3CN/H_2O	Davila *et al.,* 1997
Phospholipid *Acinetobacter sp.*	GC-MS	$CHCl_3/CH_3OH$ (Extraction method)	Koma *et al.,* 2001
Trehalose lipid *Rhodococcus sp. P32C1*	HPLC	CH_3CN	Maghsoudi *et al.,* 2001
Surfactin *Bacillus subtilis ATCC 21332*	HPLC	CH_3CN/TFA	Davis *et al.,* 2001

High-performance liquid chromatography (HPLC) is a method used in analytical chemistry to separate, recognize and quantify each element in a sample. It depends on pumps to pass

through a strong adsorbent material a pressurized liquid solvent comprising the sample combination. Each element in the sample interacts with the adsorbent material slightly differently, causing distinct flow rates for the distinct parts and leading to component separation as they flow out of the column (Gerber *et al.,* 2004).

Typically, the column's active element, the adsorbent, is a granular material made of strong particles (e.g. silica, polymers, etc.), 2–50 μm in size. Because of their distinct degrees of contact with the adsorbent particles, the sample mixture parts are separated from each other.

Typically, the pressurized liquid is a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is called a "mobile phase." In the separation process, its structure and temperature play a significant part by affecting the relationships between sample parts and adsorbent (Gerber *et al.,* 2004).

HPLC is distinguished from traditional liquid chromatography due to significantly higher operating pressures (50–350 bar), whereas ordinary liquid chromatography usually relies on the force of gravity to pass through the column through the mobile phase. Typical column sizes are 2.1–4.6 mm in diameter and 30–250 mm in length due to the limited sample quantity separated in analytical HPLC. Smaller adsorbent particles (2–50 μm in average particle size) also make HPLC columns. This provides HPLC superior solving power when separating mixtures making it a common chromatographic technique (Karger, 1997).

A HPLC instrument's schematics typically include a degasser, sampler, pumps, and detector. The sampler brings in the mobile phase stream the sample combination that carries it into the column. The pumps provide the required mobile phase flow and structure through the column. The detector produces a signal proportional to the quantity of sample element that emerges from the column so that the sample parts can be quantitatively analyzed. The HPLC instrument is controlled by a digital microprocessor and user software and provides data analysis (Karger, 1997).

Thin-layer chromatography (TLC) is a chromatography method used to separate non-volatile mixtures. Thin-layer chromatography is carried out on a sheet of glass, plastic or aluminum foil covered with a thin layer of adsorbent material, generally silica gel, aluminum oxide, or cellulose. This adsorbent layer is referred to as the stationary phase (Moody and Harwood, 1989).

After the sample has been applied to the plate, the plate is drawn up by capillary action by a solvent or liquid combination (known as the mobile phase). Separation is achieved because various analytes reach the TLC plate at different rates. The mobile phase has different characteristics from the stationary phase. For instance, a very polar substance is used with silica gel, non-polar mobile phases such as heptane. The mobile phase can be a combination that allows chemists to fine-tune the mobile phase's bulk characteristics (Moody and Harwood, 1989).

The spots will be visualized after the experiment. This can often be accomplished directly by projecting ultraviolet light onto the sheet; the sheets are handled with phosphorus and dark spots appear on the sheet where compounds absorb light that affects a certain region. For example, anisaldehyde forms colored adducts with many compounds, and sulfuric acid will characterize most organic compounds, leaving a dark spot on the sheet (Vogel *et al.,* 1989).

The distance traveled by the considered substance is divided by the complete range traveled by the mobile phase to quantify the outcomes (the mobile stage cannot reach the end of the stationary phase). This ratio is referred to as the retardation factor (Rf). A substance whose structure is similar to the stationary phase will generally have low Rf, whereas one with a similar structure to the mobile phase will have a high retardation factor. Retardation variables are characteristic but will alter based on the mobile and stationary phase's exact condition. For this reason, before operating the experiment, chemists generally add a sample of a known compound to the sheet. Thin-layer chromatography can be used to monitor a reaction's progress, recognize compounds in a particular mixture, and determine a substance's purity (Vogel *et al.,* 1989).

Fourier-transform infrared spectroscopy (FTIR) is a method used to acquire an infrared spectrum of solid, fluid or gas absorption or emission. At the same time, a FTIR spectrometer collects information of high spectral resolution over a broad spectral range. This gives a important benefit over a dispersive spectrometer, measuring intensity at one time over a limited range of wavelengths (Griffiths and De Haseth, 2007).

The objective of any absorption spectroscopy (FTIR, UV-Vis, etc.) is to assess how much light a sample absorbs at each wavelength. The simplest way to do this, the method of "dispersive spectroscopy," is to shine a monochromatic light beam at a sample, assess how

much light is absorbed, and repeat for each distinct wavelength (Griffiths and De Haseth, 2007).

Spectroscopy of a Fourier-transform is a less intuitive way to acquire the same data. Instead of shining at the sample a monochromatic light beam (a beam consisting of only one wavelength), this method shines a beam comprising many light frequencies at once and measures how much of that beam is absorbed by the sample. The beam is then changed to contain a distinct frequency mix, providing a second data point. This process is repeated several times quickly over a brief period of time. A computer then requires all this information and deduces what the absorption is at each wavelength (Griffiths and De Haseth, 2007).

Starting with a broadband light source, the beam outlined above is generated— one that contains the complete range of wavelengths to be measured. The light shines into an interferometer in Michelson — a certain mirror setup, one of which is shifted by an engine. As this mirror moves, the interferometer blocks, transmits, blocks, transmits, periodically blocks each wavelength of light in the beam owing to wave interference. Different wavelengths are modulated at distinct rates, so the interferometer beam has a distinct spectrum at each time (Griffiths and De Haseth, 2007).

Computer processing is needed, as stated, to convert the raw information (light absorption for each mirror position) into the desired consequence (light absorption for each wavelength). A popular algorithm called the Fourier transform turns out to be the processing needed. The Fourier transforms one domain (in this case the mirror is displaced in cm) into its reverse domain (wavenumbers in cm^{-1}) (Griffiths and De Haseth, 2007).

2.2.7. Application of Biosurfactants

2.2.7.1. Biosurfactants in Metallurgical Industry

Today, due to extensive industrialization, various pollutants are released into the environment. Heavy metals from the metallurgical industries are one of these pollutants. As toxic pollutants, heavy metals contaminate the soil, water and appears to accumulate in the food chain. Heavy metals are inherently persistent and can cause serious environmental problems. Techniques such as excavation have been reported to clean up the heavy metal contaminated soil and to dispose of contaminated soil at landfill sites (Ascı *et al.,* 2010).

Microbes can be used as a whole cell biocatalysts in the bio-reduction of these heavy metals to different states (Bruins *et al.,* 2000). Soil washing and soil flushing is a well-known method of remediation for the treatment of heavy metal soils with biosurfactants. Biosurfactants are injected to the ground with drain pipes and trenches in an in-situ remediation (Singh and Cameotra, 2004). In ex-situ, however, the soil is collected from the site and transported to the washing column and washed with biosurfactant. Biosurfactant could significantly improve the solubility of high concentrations of heavy metals and critical micelle concentrations.

The metal-biosurfactant complex is desorbed from the soil by lowering the surface tension. Generally speaking, the solubilization of metals using biosurfactant is referred to as bioleaching, a process describes the dissolution of metals from mineral sources by certain naturally occurring microorganisms. Biosurfactant converts solid metals into soluble substances. Mechanisms like binding, complexing, desorption and precipitation can occur when heavy metals are removed. The precipitation of heavy metals in water has long been an important treatment method in industrial wastewater.

A combined method of precipitation of biosurfactants with chemical treatment techniques such as ion exchange has been shown to be effective in heavy metal removal. Di-rhamnolipids produced from *Pseudomonas aeruginosa* were used to immobilize metals from soil contaminated with multi-metals (Juwarkar *et al.,* 2008). They are also used to remove various heavy metals from the soil, such as chromium, lead, cadmium and copper. Marine biosurfactants are typical biosurfactants that are isolated from marine bacteria used in polyaromatic hydrocarbon remediation (Das *et al.,* 2009).

The synthesized biosurfactant from the marine organisms is capable of chelating toxic heavy metals. It is therefore used for treating waste water containing heavy metal. Alkali adding improves the removal of heavy metals (Singh and Cameotra, 2004). Foam technology is a further method of advancing remediation based on biosurfactants. Wang and Mulligan (2004) examined the rhamnolipid removal of Cd and Ni from sandy soil. Generally, the foam formed flows into a porous medium and makes it more uniform, making contact with metals more efficient. The rhamnolipid solution was used to remove Cd and Ni is 61.7 per cent efficient and 51 per cent efficient.

But rhamnolipid combined with foam improved the efficiency of Cd and Ni removal by 73.2 percent and 68.1 percent (Wang and Mulligan, 2004). Massara *et al.* (2007) examined the elimination of Cr (III) from chromium-contaminated kaolinite. Factors like pH and NaOH addition could have a positive effect on metal removal. The chelating effect of biosurfactants was greatly improved by pH. The addition of NaOH increases the solubility of biosurfactants and thus promotes better removal of metals (De França *et al.,* 2015). The removal of heavy metals reported by various authors can be found in Table 2.5.

Table 2.5. Removal of Heavy Metals by Biosurfactant Producing Organisms

Number	Metals	Microorganism	Removal (%)	Reference
1.	Cr	*Pseudomonas aeruginosa*	46	Hassen et al., 1998
		Aspergillus niger	21-36	Dursun et al., 2003
2.	Cd	Bacillus strain H9	36	Roane et al., 2001
		Aspergillus terreus	70	Massaccesi et al., 2002
		Pseudomonas aeruginosa	73.2	Wang and Mulligan, 2004
3.	Cu	*Thiobacillus ferrooxidans*	25	Boyer et al., 1998
		Schizosaccharomyces pombe	11-25	Dönmez and Aksu, 1999
4.	Pb	*Pseudomonas aeruginosa PU21*	80	Chang et al., 1997
		Aspergillus niger	13-88	Dursun et al., 2003
5.	Ni	*Pseudomonas spp.*	98	Magyarosy et al., 2002
		Candida spp	29-57	Donmez and Aksu, 2001
		Pseudomonas aeruginosa	68.1	Wang and Mulligan, 2004

2.2.7.2. Biosurfactants in Petroleum Industry

Organisms producing biosurfactants (indigenous or injected) are used to recover oil in oil wells. The microbial enhanced oil recovery process is implemented by direct injection of nutrients with microbes capable of producing desired products for the mobilization of oil, by injecting a specific microorganism or by injecting biosurfactants using this method. This process follows interfacial tension/oil viscosity reduction. Bacteria such as *Pseudomonas aeruginosa, Bacillus licheniformis* and nutrients were injected with biosurfactants to increase the oil recovery by 30-200 percent (Singh *et al.,* 2008).

Microbial improved oil recovery is a way to recover oil from high-viscosity crude oil or low-permeability reservoirs. Oil field emulsions are one of the main challenges for the oil industry. It happens at different stages during the processing of crude oil. The de-emulsification process is one of the best ways to recover oil from these emulsions in order to control the emulsion of the oil field. Centrifugation, heat treatment and chemicals are used to obtain a conventional de-emulsification process.

Biosurfactants are able of replacing the use of a chemical de-emulsifier and can provide an environmentally friendly solution. Some species of bacteria, such as *Acinetobacter* and *Pseudomonas*, are the main de-emulsifiers in mixed cultures (Nadarajah *et al.,* 2002). Microorganisms use the amphiphilic nature of biosurfactants or hydrophobic cell surfaces to disrupt emulsion. Biosurfactant classes such as glycolipids, glycoproteins, phospholipids and polysaccharides are the microbial tools used to displace emulsifiers from the water-oil interface (Mukherjee *et al.,* 2006).

Biosurfactants that may be used to recover oil from bottom sludge petroleum tanks and facilitate heavy raw transport through pipelines. With rhamnolipids (Wei *et al.,* 2005), the oil can be removed from the used oil sorbents. The removal of oil is affected by main factors such as sorbent pore size and wash time. The commercial rhamnolipids were used to remove 95 percent of the oil. In addition to using crude biosurfactants, the fermentation broth could effectively remove crude oil from contaminated sites and motor oil by 85% and 90% respectively (De França *et al.,* 2015).

2.2.7.3. Oil Waste Management and Remediation

Contaminated soil and water with petroleum compounds often occurs at various levels and causes contamination of the environment which is very hard to clean up them by traditional techniques on the other hand they are not economically reasonable. Therefore, it is of excellent significance to develop efficient and possible procedures and techniques that can reduce the negative impacts of oil contamination and treatment expenses.

During petroleum extraction and purification, two sources of waste are produced. It includes water, sand, clay, salts, metals, remaining bitumen, and hydrocarbon diluents and is generally

stored in tailings ponds to promote the gravitational separation of solids from water (Gosselin *et al.,* 2010).

Most of this waste in industrial or municipal waste treatment plants can be recycled or disposed of. In the past, different types of sludge have been generated in refinery operations, requiring handling prior to final disposal using alternative thickening, stabilization, and dewatering. Due to their environmental compatibility, low toxicity, and high efficacy, several biotreatment procedures and techniques have received increased attention (Bognolo, 1999; Das & Mukherjee, 2007).

A recent study on the microbial pre-treatment of contaminated soil with biosurfactant produced by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains during the biodegradation of oil waste has shown that using biosurfactant for treatment of soil has changed the wettability of solids, degrades the asphalt content of bitumen and reduces the bitumen viscosity (Ding *et al.,* 2014).

In this study some methods for isolating biosurfactant-producers were identified and carried out. It was assumed that by using these bacteria that they could be capable of treatment of oil- contaminated soil. Therefore, tests were carried out to evaluate the biodegradation of oil by various strains of isolated bacteria.

3. MATERIALS AND METHODS

3.1. Origin of the Oil

This chapter describes the methods used to carry out this study successfully. This includes isolating and characterizing biosurfactants-producing bacteria and biodegradation studies. Experimental research was conducted at the Environmental Engineering Laboratory (Concordia University). Laboratory tests were conducted using crude-oil degrading bacteria strains, which showed higher growth rates on crude oil. The origin of isolated bacteria included tailings pond waste, light and heavy crude oils and refinery-contaminated soil. The refinery-contaminated soil was obtained from the DaGang oil field (China). The tailings pond waste was obtained by Maria Demeter (Lab Manager / Environmental Engineering Technologist), Civil & Environmental Engineering Department, University of Alberta, CA. Light and heavy crude oils were bought from Petro-Canada (Montreal). According to Roshtkhari (2016) the tailings pond waste consists of bitumen (1-2 wt%), naphtha (<0.1 wt%), clay (30-60 wt%), and water with the pH in the range of 7.3-7.8 (Roshtkhari, 2016). The clay content of 30-60% wt indicates that the samples were taken from mature fine tailing layer from the depth below 10 m of the tailings pond (Foght and Dongshan 2013).

Regarding the refinery-contaminated soil characteristics that have been measured in laboratory, the amount of pH is equal to 7.56 which was done by NADE PHS -2c laboratory pH meter. The pH was measured by direct insertion a glass electrode into slurry of the soil and distilled water. Porosity has been determined as the pores divided by the quantity of the sample based on Eq. 3.1 (Hardie, 2014).

Porosity = (Volume of Voids / Total Volume) x 100%) Eq. 3.1.

The porosity of the soil sample is equal to 0.38, and the bulk density (1.22 kg/L) was calculated based on the Eq. 3.2 (Hardie, 2014):

Bulk density (kg/L) = Dry soil weight (kg) / Soil volume (l) Eq. 3.2.

The buffer capacity (β) for acid and base are 0.892 and 0.5714, respectively. The buffer capacity (β) is determined experimentally by measuring the pH changes when strong base or acid is added to a solution (Yong *et al.,* 1990).

Particle size was measured by HORIBA Laser Scattering Particle Size Distribution Analyzer LA-950 and the results are as follows: mean size: 23.28 (μm); consisting of 46.4% clay, 42% silt and 11.6% sand; particles in the soil samples were found between D_{10} and D_{90} (1.33 and 69.45 μm on average respectively), which are 10% and 90% of the particles finer than those sizes, respectively.

3.2. Enrichment and Isolation of Biosurfactant-Producing Bacteria

A potential biosurfactant producer was isolated by using a minimal salt medium (MSM) enrichment culture technique according to Chandankere *et al.* (2013). $NaNO_3$ (2.0), KCl (0.5), $Na_2HPO_4H_2O$ (1.0), KH_2PO_4 (1.0), $CaCl_2$ (0.025); $MgSO_4$ (0.1) and $FeSO_4.7{\cdot}H_2O$ (0.001) were the composition of the liquid medium (g/L). It also contained a solution of 2 mL/L trace elements (mg/L in distilled water): $FeCl_3{\cdot}6H_2O$ (60), $ZnSO_4{\cdot}7H_2O$ (600), $MnSO_4{\cdot}H_2O$ (200), $CuSO_4{\cdot}5H_2O$ (590), $CoCl_2{\cdot}6H_2O$ (60), H_3BO_3 (150) and $Na_2MoO_4H_2O$ (15). The medium's pH was adjusted to 7.0 and sterilized for 20 min by autoclaving at 121°C. Four clean flasks (250 mL) were filled with 150 mL water medium (MSM) and 5 g of refinery-contaminated soil or 5 g of tailings pond waste or 1 mL light crude oil or 1 mL of heavy crude oil. Then crude oil (2% v/v) was added to each flask as a sole carbon source. The flasks were incubated on the rotary shaker at 200 rpm for 7 days at 28°C.

After the incubation period, 1 mL of culture was transferred to a fresh medium containing crude oil (2% v/v) and re-incubated for another 7 days. Salinity was maintained throughout the enrichment cycles by adding NaCl (30 g/L) to the MSM. After five consecutive enrichment cycles, 1 mL of cultivation was diluted and placed on solidified MSM with agar containing crude oil as the sole source of carbon. Bacterial colonies grown on Luria-Bertani (LB) agar plates containing (g/L): peptone (10.0), NaCl (5.0) and yeast extract (10.0) were further purified.

3.3. Growth Media

Yeast extract (10 g/L), peptone (10 g/L) and sodium chloride (5 g/L) were prepared in a 1 Liter flask and stirred with the help of a magnetic stirrer for 30 minutes. After stirring, agar (15 g/L) was added to produce LB agar. The broth nutrient agar was autoclaved for 45 minutes at 125ºC. The broth was cooled at room temperature and stored at 5ºC for use. Agar was cooled down and then poured into petri dishes and put upside down to prevent condensation before storing. In addition, the plate was poured around a Bunsen burner to prevent contamination by the surrounding air.

3.4. Screening Methods

Biosurfactants are structurally a very diverse group of biomolecules, e.g., glycolipids, lipopeptides, lipoproteins, lipopolysaccharides or phospholipids. Therefore, most methods for of general screening of biosurfactant producing bacteria are based on the physical effects of surfactants.

Alternatively, the ability of strains to interfere with hydrophobic interfaces can be explored. On the other hand, specific screening methods like the colorimetric CTAB agar assay are suitable only to a limited group of biosurfactants. The screening methods can give qualitative and/or quantitative results. For a first screening of isolates, qualitative methods are generally sufficient.

3.4.1. Oil Displacement Test

For this test, 10 µl of crude oil were added to the surface of 40 mL of distilled water in a Petri dish (d, 2.5 cm) and allowed to form a thin oil layer. Then, 10 µl of culture supernatant were gently placed on the center of the oil layer. After 30 seconds the clear zone was observed and the diameter of clear zone was visually measured (Morikawa *et al.,* 2000).

3.4.2. Emulsification Capacity Assay

The Emulsification Capacity Assay of the cell free culture supernatant was measured by using method provided by Cooper and Goldenberg (1987). For this test 6 mL of crude oil were transferred to the test tube containing 4 mL of cell free culture supernatant. The mixture was vortexed for 2 minutes at high speed. The height of the stable emulsion layer was

measured after 24 hours. The E24 emulsion index was calculated as the ratio of emulsion layer height and total liquid height Eq. 3.2:

$$E24 = \frac{h\ emulsion}{h\ total} \times 100\% \qquad \text{Eq. 3.2.}$$

3.4.3. Du-Nouy Ring Method

Fig. 3.1 shows a Du-Nouy Ring tensiometer which is used to measure the surface tension of biosurfactant produce by Bacteria strains isolated in this study. Bacteria strains that showed potential for production of biosurfactant (for example showed oil displacement activity) were tested for surface tension reduction capacity. Strains were cultured in minimal salt media (MSM) using with 2 percent (v/v) crude oil as the source of carbon and then transferred to the incubator for 7 days. For this assay 15 mL of supernatant were transferred to the petri dish and by using the Du-Nouy Ring assay the surface tension reduction was determined each week (Tadros 2006).

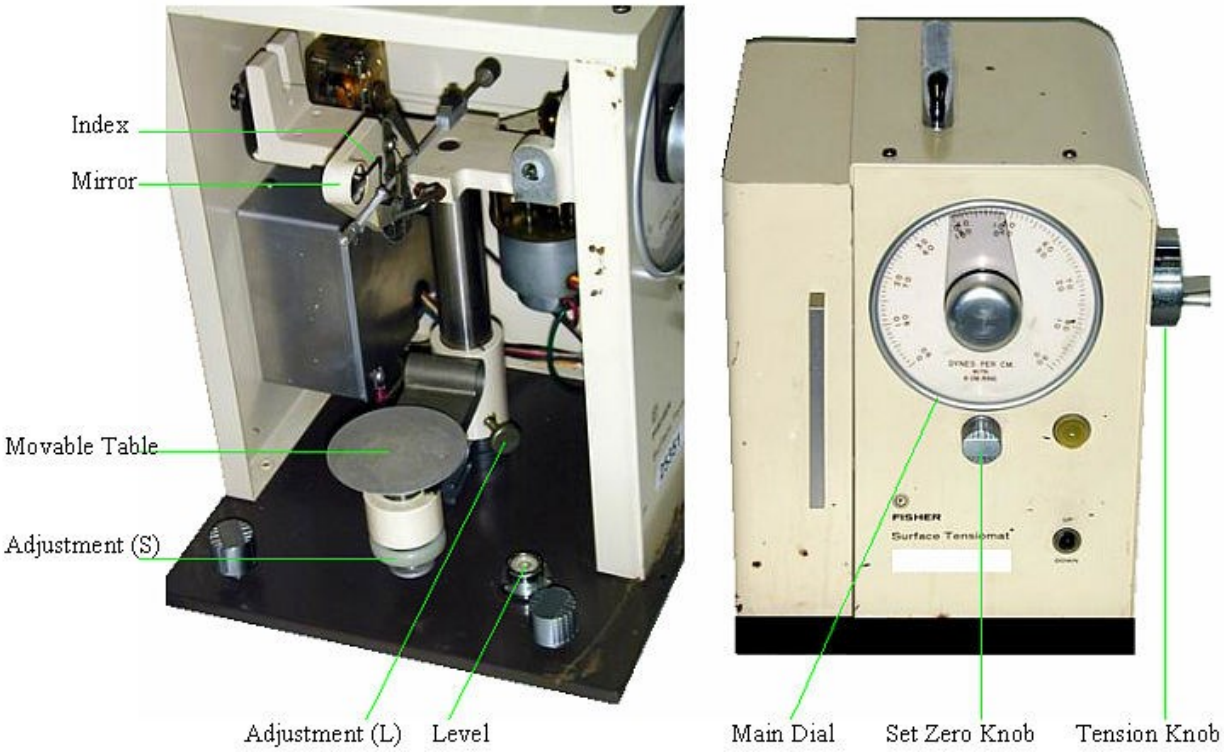


Figure 3.1. Main parts of Du-Nouy-tensiometer, Fisher Scientific, Model 21 (source: Manual of the Machine)

3.4.4. Blood Agar Assay

The growth strains were examined for their potential for producing biosurfactant by using a modified hemolytic assay as reported by Mulligan *et al.* (1984). For this assay, 45 mL of culture broth were transferred to agar plates containing tryptone 10.0 g/L, NaCl 5.0 g/L and agar 15.0 g/L. Then 5 percent (v/v) sterile sheep blood was added to the media. One colony of each isolated strains from tailings pond waste or light crude oil or heavy crude oil or refinery-contaminated soil were streaked on the plate. The plates were incubated for two days at 37°C. Then the clear zone around the colony was observed. Finally, the diameter of the clear zones around the colony was measured.

3.4.5. CTAB Agar Plate

Biosurfactant-producing bacteria that showed hemolytic activity on the blood agar plates were tested using blue agar plate containing Cetyl Trimethyl Ammonium Bromide (CTAB) (0.2 mg/mL). Methylene blue (5 mg/mL) were added to the plates to detect extracellular glycolipid production. One colony of each isolated strains from tailings pond waste or light crude oil or heavy crude oil or refinery-contaminated soil were streaked on the plate. Biosurfactant production was observed by forming the dark blue halos around the colony.

3.5. Biodegradation of Crude Oil

In this study the biodegradation experiment was conducted to determine the role and contribution of the isolated oil-degrading strains in the crude oil biodegrading in water. Two treatments included the control (containing 400 mL MSM media and 2% (v/v) sterilized crude oil) and biodegradation (containing 400 mL MSM media and 2% (v/v) sterilized crude oil and each of the isolated strains). Samples were placed on an orbital shaker (180 rpm) and incubated at 26 ± 1 °C for five weeks. *Bacillus subtilis* (*Bacillus subtilis* ATCC 21332) were used as a control as known-biosurfactant-producing bacteria for comparison. Four mL samples covering days 0, 7, 14, 21, 28 and 35 (for each isolate) were taken to analyze the total petroleum hydrocarbon (TPHs) (Saborimanesh and Mulligan, 2015). In this study light (Alaska North Slope Crude Oil) and heavy (IFO 180) crude oil were evaluated for biodegradation studies.

3.6. Gas Chromatography Analysis

Gas chromatography (GC) is a technique of separation that can separate highly complex mixtures based primarily on differences in boiling point/vapor pressure and polarity. Although chromatography was invented at the beginning of the 20th century, Martin and Synge (1941) saw no reason why the mobile phase was not supposed to be a gas in their GC publication. That year, James and Martin (1941) published their first paper showing GC's use for volatile fatty acid separation (Marriott *et al.,* 2001).

Gas chromatography-also known as gas-liquid chromatography (GLC) is a specific type of chromatography that uses an inert mobile gaseous phase and a stationary liquid phase. Instrumentation continues to improve, but a gas chromatograph's basics—a tool used to perform GC with the same abbreviation—have not changed and remain relatively simple (Jennings *et al.,* 1997).

3.6.1. Analysis Residual Oil in Biodegradation Sample

From each of the biodegradation flasks, four mL samples were taken. The samples were transferred to separatory funnels and 20 mL of n-hexane (95% Sigma-Aldrich) were used for

extraction. The mixture was shaken for 2 minutes and allowed to settle for 3 minutes. The water was then transferred into a 100 mL Erlenmeyer flask through the stopcock and the process repeated three times. An amount of 2 g of sodium sulfate (ACS grade granular anhydrous) was added to the mixture of oil and hexane to remove remaining water and filtered through a Whatman No. 40 filter. The extracts were then transferred to a 20 mL amber vial and stored at 4°C until gas chromatography analysis (GC). A CP-3800 VARIAN gas chromatograph-flame ionization detector (GC/FID) was used to analyze the residual oil. According to the manufacturer, the GC (with a DB-5 fused silica column) data is as follows: 30 m long, 0.25 mm inner diameter, 0.25 μm film thickness with temperature boundaries of -60°C to 325°C. A literature-based technique (Toxics Cleanup Program, 1997) and manufacturer suggestions with modifications were developed. With a steady flow rate of 2 mL/min and a flow rate of 30 mL/min, helium was used as a carrier gas. Hydrogen and airflows were adjusted respectively to 30 mL/min and 300 mL/min. The split-free injection mode was chosen on the suggestions of the manufacturer. Temperatures of the injector and detector remained constant at 250°C. The temperature of the oven was set for 2 minutes at 50°C, increased to 250°C at 8°C/min and kept for 6 minutes at 250°C (complete run time of 33 minutes). From the complete peak area corresponding to the complete petroleum hydrocarbons (TPHs, retention times of 3 to 33 min) the distributed oil concentration was determined. The proportion of residual petroleum was calculated as:

$$(C_{\text{residual oil in sample}} - C_{\text{total oil in sample}}) / C_{\text{total oil in sample}}) \times 100$$

where $C_{\text{total oil in sample}}$ and $C_{\text{residual oil in sample}}$ are the total concentration of oil in the biodegradation sample and the remaining oil after biodegradation treatment respectively (Saborimanesh, 2015).

4. RESULTS AND DISCUSSION

4.1. Introduction

Effective isolation and screening techniques are needed due to the diversity of microorganisms and distinct niches in which they inhabit (Haghighat *et al.,* 2008). For the isolation of biosurfactant-producing bacteria, enrichment cultures with hydrophobic substrates are very promising, apart from direct strain isolation by diluting and plating.

An effective biosurfactant-producing bacteria was effectively isolated using minimal salt medium (MSM) containing crude oil as the only source of carbon and power based on the enrichment and isolation techniques employed in this research.

Cultures of enrichment have been diluted and moved for colony isolation to solid MSM media. The isolated strains have been able to generate a sort of biosurfactant with degradation characteristics as well as surface tension decrease. Lotfabad *et al.* (2009) noted similar results from the biosurfactant-producing bacteria.

In this study, five different crude-oil-degrading bacteria strains that showed growth on crude oil were isolated from different samples including tailings pond waste, light and heavy crude oils, and refinery-contaminated soil. In general, one strain was isolated from each of the B-H media inoculated with the tailings pond waste, light and heavy crude oils and two strains were isolated from the B-H media inoculated with the refinery-contaminated soil. Figs. 4.1 and 4.2 show the isolation and identification of oil-degrading bacteria from the tailings pond waste, light and heavy crude oils, and refinery-contaminated soil. Visual examinations of the strains showed that all the strain cells were circular (cocci) in shape and their size ranged from 0.5 to 3 μm. Visual observation showed that the color of bacteria were different and ranged from white to shiny yellow to pink-reddish. Table 4.1 summarizes morphological characteristics of the isolated oil-degrading strains from different samples.

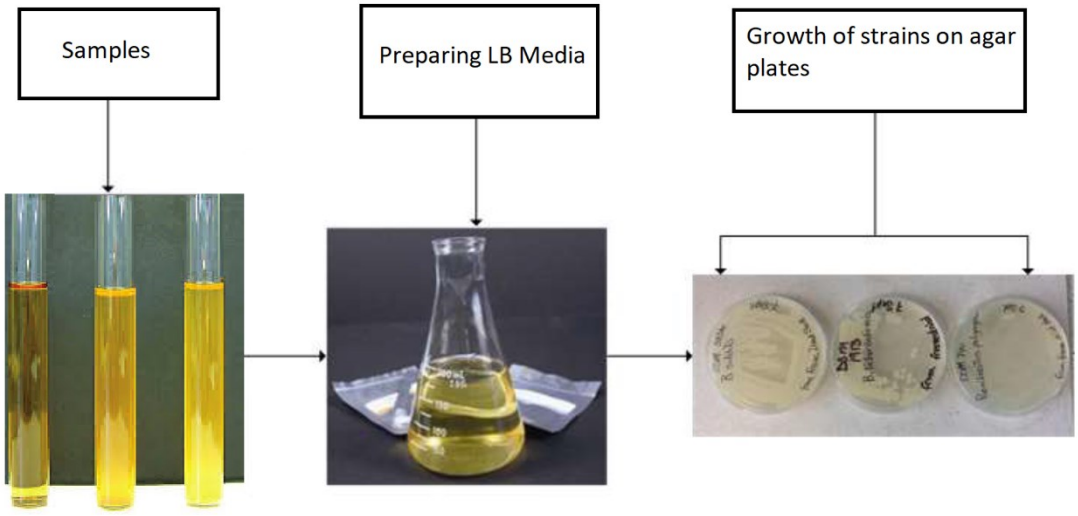


Figure 4.1. Isolation of bacteria and growth of bacteria

Figure 4.2. Oil-degrading bacteria growth on the Bushnell Hass medium after 48 hours of incubation at 37°C

Table 4.1. Morphological characteristics of the isolated oil-degrading strains from different samples

Characteristics	Strain isolated from				
	Tailings pond waste	Light crude oil	Heavy crude oil	Refinery-contaminated soil; strain I (red colony)	Refinery-contaminated soil; strain II (white colony)
Shape (form)	Circular	Circular	Circular	Circular	Circular
Size	Moderate	Moderate	Moderate	Moderate	Moderate
Appearance	Clear	Clear	Clear	Transparent	Transparent
Pigmentation	Yellow	White	Yellow	Pink	Pink

Note: Two strains were isolated from refinery-contaminated soil and identified as strains number I and II

4.2. Biosurfactant Characterization

Following the isolation of the oil-degrading bacteria, several tests were performed to determine if the isolated strains have surface active/emulsification abilities. The tests included (i) oil displacement test, (ii) emulsification activity test, and (iii) surface tension measurement.

4.2.1. Oil Displacement and Emulsification Properties of the Isolated Oil-Degrading Strains

The oil displacement test was conducted in triplicate by adding 20 mL of distilled water to petri dishes followed by the addition of 20 µl of crude oil to the surface of the water. Then 10 µl of cell free culture supernatant was dropped on the crude oil surface. The diameter of the clear zone on oil surface was visualized under visible light and measured after 30 seconds by comparing to 10 µl of distilled water and crude oil without any cell free culture supernatant as the negative control. Biosurfactant obtained from strains isolated from refinery-contaminated soil with red and white colors had the lowest oil displacement so that a 4 mm and 5 mm of oil was respectively displaced as the cell free culture supernatant from these strains were dropped on the liquid medium. The supernatants from the tailings pond waste, heavy and light strains had the oil displacement diameters of 10, 17, and 20 mm, respectively. In addition to oil displacement activity, excellent emulsification is critical for successful biosurfactant use in various environmental and industrial applications. In another study, Ron and Rosenberg (2001) showed that most bacteria use insoluble hydrocarbons by generating biosurfactants that encourage substrate solubilization and emulsification, thereby enabling cells to come into direct contact with the oil phase. In this research, the potential of biosurfactants to emulsify crude oil in cell-free supernatant produced from five strains was explored under specific environmental circumstances. However, the emulsification activity test showed that the produced biosurfactants by the isolated strains didn't show a strong emulsification property. Table 4.2 summarizes the results of the oil displacements and emulsification activities of the biosurfactant produced by the oil-degrading strains.

Table 4.2. Characteristics of biosurfactants produced by the oil-degrading strains in this study

Isolated strains ID	Isolated environments	Oil displacement test, (diameter, mm)	Emulsification activity (E24%)	*Minimum ST (mN/m)	Blood agar lysis
T-1	Tailings pond waste	10	<10	40.3 ± 0.5	++++
L-1	Light crude oil	20	<10	43.1 ± 0.3	++++
S-I (red colony)	Refinery-contaminated soil	5	<10	54.6 ± 0.3	++++
S-II (white colony)	Refinery-contaminated soil	4	<10	51.4 ± 0.5	++++
H-1	Heavy crude oil	17	<10	46.4 ± 0.2	++++
Control-1	*Bacillus subtilis*	ND	ND	51.4 ± 0.3	ND

Note: (-) no hemolysis, (+) incomplete, (++) complete hemolysis with a diameter of lysis between 1 to 2 cm, (+++) hemolysis with a diameter of lysis between 2 to 3 cm, and (++++) complete hemolysis with a diameter of lysis between 3 to 5 cm.
Emulsification activity were done after 10 days of biodegradation.
Bacillus subtilis: Used as known-biosurfactant-producing bacteria for comparison.
Surface tension measurements were conducted at the constant temperature of 28°C.
*Results are expressed as the mean ± standard error mean (SEM) of three independent experiments.
ND: not measured

Compared to the similar study that has been reported by Chandankere *et al.* (2013), the strains showed greater oil-displacement activity by exhibiting a clear halo zone indicating the qualitative evaluation of biosurfactant production. The positive result acquired from the above confirmatory tests verified the strain's output of biosurfactant strongly. Therefore, for further research, the five strains were chosen.

4.2.2. Surface Tension Measurement

As mentioned in the Section 4.1, a total of five oil-degrading strains were isolated from oily tailings pond waste (1 species), refinery-contaminated soil (two species), light crude oil (one species) and heavy crude oil (one species). The surface tension of the supernatants from each isolate was measured to determine the surface activity of the isolated oil-degrading strains at a constant temperature (28°C). For the control, tap water, LB media, and MSM media were used. The tap water, LB media, and MSM media surface tensions were 72.0 ± 2 mN/m, 59 ±

2 mN/m, and 69 ± 0.1 mN/m, respectively. Surface tension measurements showed that all species have surface activity. The results showed that all five strains were able to lower the surface tension of the tap water/LB/MSM. The lowest surface tensions of supernatants obtained were between 55 mN/m to 40 mN/m. The minimum ST belonged to the BS produced by species isolated from the tailings pond waste (40 mN/m). The surface tension values acquired in this research are compatible with previous studies with surface tensions of 34 mN/m (Mulligan *et al.,* 2001), 32.1-34.2 mN/m (Develter and Lauryssen 2010) and 35-36 mN/m (Ashby *et al.,* 2008). Variations in ST values may be due to the impact of materials and circumstances used in biosurfactant production (Ashby *et al.,* 2008; Daverey and Pakshirajan, 2010), biosurfactant purity (Ashby *et al.,* 2008), surfactant dilution solution (e.g., distilled water (Joshi-Navare *et al.,* 2013), buffer solution (Hirata *et al.,* 2009a) or amount of biosurfactant produced. Table 4.3 and Fig. 4.3 summarize the surface activities of the cell-free culture supernatants. It can be assumed that this will help to improve the accessibility and bioavailability of water-immiscible petroleum hydrocarbons to degrading microbes via hydrocarbon solubilization. As can be seen in Fig 4.3 at week one, surface tension of the sample inoculated strain isolated from light crude oil showed the lowest surface tension compared with other strains which was 52.5 mN/m. Heavy crude oil showed the highest surface tension 65.8 mN/m in the same week. The surface tension for the strains isolated from refinery-contaminated soil strains I&II and tailings pond waste were 64.6 mN/m, 57.2 mN/m and 60.7 mN/m, respectively in the same week. During weeks one to three linear changes were observed for all strains. This linear trend in surface tension continued for the strains isolated from light crude oil, refinery-contaminated soil strains I&II. In the strains isolated from tailings pond waste and heavy crude oil a rapid reduction in surface tension occurred during weeks three to five, with changes from 53.8 mN/m to 40.3 mN/m for strains isolated from tailings pond waste and 61.8 mN/m to 46.4 mN/m for strains isolated from heavy crude oil .

Table 4.3. Surface activities of the cell-free culture supernatants

Isolate strains	Isolated environment	*Minimum ST (mN/m) after 5 weeks of biodegradation period
T-1	Tailings pond waste	40.3 ± 0.5
L-1	Light crude oil	43.1 ± 0.3

S-I (red colony)	Refinery-contaminated soil	54.6 ± 0.3
S-II (white colony)	Refinery-contaminated soil	51.4 ± 0.5
H-1	Heavy crude oil	46.4 ± 0.2
Control	*Bacillus subtilis*	**51.4 ± 0.3

Note: *Bacillus subtilis* used as known-biosurfactant-producing bacteria for comparison.
*Results are expressed as the mean ± standard error mean (SEM) of three independent experiments.
**Lower surface tension in control sample inoculated with *Bacillus subtilis* as a known biosurfactant producing bacteria would be possibly due to low biosurfactant production by this strain.

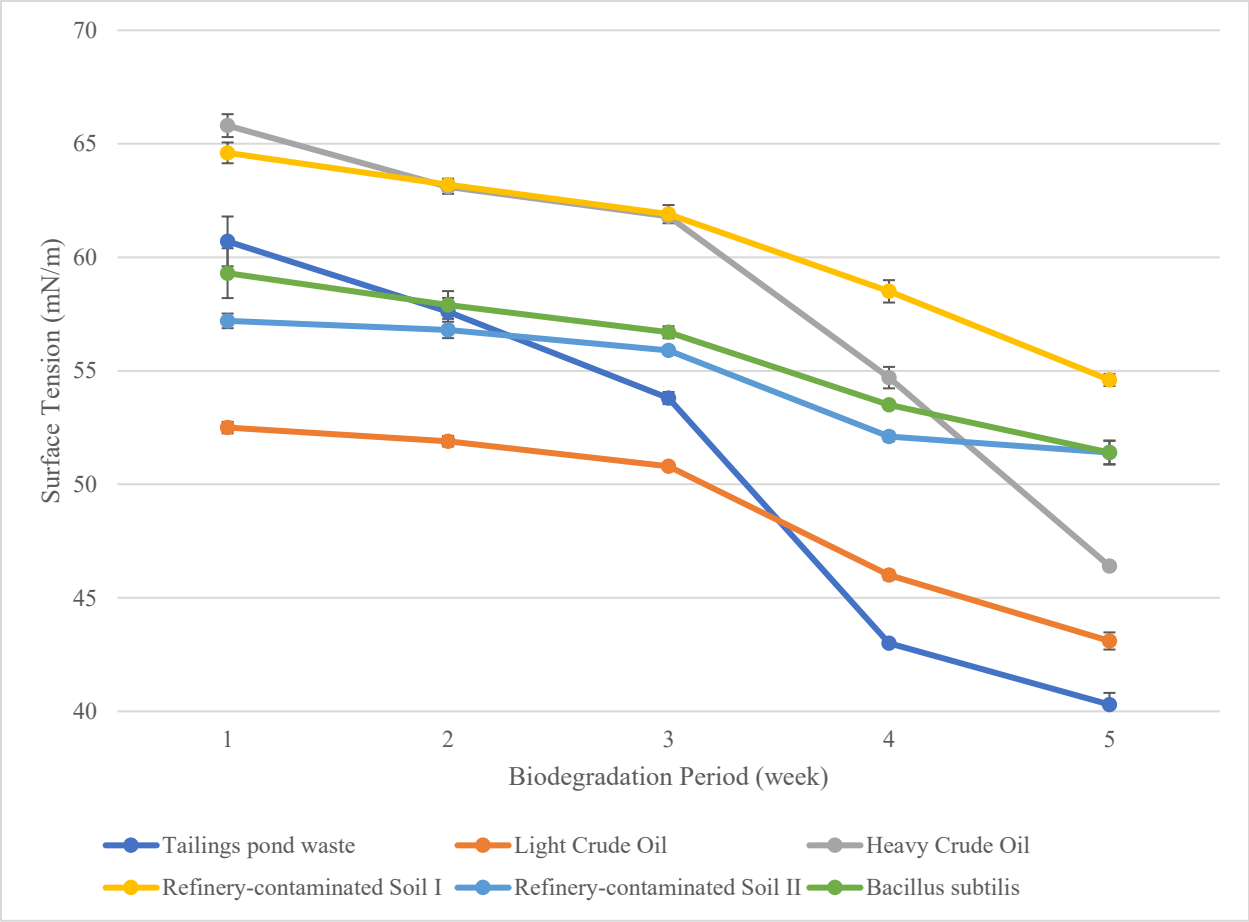


Figure 4.3. Surface tension values of cell-free culture supernatants at a constant temperature of 28°C over time for strains isolated from the L-1, H-1, T-1, S-I (red colony), S-II (white colony), and *Bacillus subtilis* as known-biosurfactant-producing bacteria for comparison. Results are expressed as the mean ± standard error mean (SEM) of three independent experiments

4.2.3. Blood Agar Assay

Kumar *et al.* (2007) established a relation between hemolytic activity and biosurfactant production and suggested the method of blood agar plate as a preliminary screening technique for biosurfactant activity. Chandankere *et al.* (2013) reported in a comparable research that the strain inoculated on the agar plate in the blood, resulted in a substantial clearance area around the colonies of approximately 2 cm confirming biosurfactant production.

In this study bacterial isolates were screened on blood agar containing 5% (v/v) human blood (bought from Fisher). The plates were incubated at 30°C for 48 h. Hemolytic activity was detected as the presence of a clear zone around a colony which is an indication of biosurfactant production. The results of this test showed that all five isolated oil-degrading strains had hemolytic activity. Fig. 4.4 shows the colonies of the enriched bacterial consortium formed on blood plate.

Figure 4.4. Colonies of the enriched bacterial consortium formed on blood plate

Table 4.4. Comparison of the tested methods for detection of strains for biosurfactant production

Methods	Number of positives	Number of negatives
Blood agar lysis	5 out of 5 strains	0
Oil displacement test	5 out of 5 strains	0
Surface activity measurement	5 out of 5 strains	0

Table 4.5. Comparison of the tested methods in predicting biosurfactant production

Isolate	Blood agar lysis	Oil displacement	Surface activity measurement (Du-Nouy Ring method)
L-1	++++	++++	40.3 ± 0.5
H-1	++++	++++	43.1 ± 0.3
T-1	++++	++	54.6 ± 0.3
S-I-(white colony)	++++	+	51.4 ± 0.5
S-II-(red colony)	++++	+	46.4 ± 0.2
a (-), no hemolysis; (+), incomplete, (++) complete hemolysis with a diameter of lysis between 1 to 2 cm; (+++) complete hemolysis with a diameter of lysis between 2 to 3 cm; and '++++,'. Oil displacement: (+) <5 mm, (++) 5-10 mm, (+++) 10-20 mm			

4.2.4. Biodegradation Experiment

In most studies, attention has been paid to the biodegradation experiment (Saborimanesh and Mulligan, 2015). Saborimanesh and Mulligan (2015) concentrated on the role of native bacteria in the oil in the biodegradation of spilled oil. Bacterial degradation of high weathered biodiesel, diesel and light crude oil were verified by chemical evaluation of hydrocarbon (TPH) concentrations over time during the biodegradation experiment.

Other studies have reported the existence of oil-degrading bacteria in oil-contaminated marine settings (Head *et al.,* 2006; Yakimov *et al.,* 2007). Previous studies have demonstrated the existence and role of oil-degrading bacteria in contaminated environments.

The results of this study showed that a maximum crude oil biodegradation of 63 ±4% (the average ± the standard deviation of the biodegradation of crude oil by the five strains) was obtained with the strains after five weeks of the incubation at 28°C. The maximum biodegradation percentage was obtained with the strains isolated from the light crude oil and tailings pond waste with the biodegradation percentages of 12.7% and 18.5%, respectively in the first week of biodegradation. While the biodegradation percentage obtained by the strains isolated from the heavy crude oil was <4.5%. The biodegradation percentage obtained by the

two strains of refinery-contaminated soil (both red and white strains) and *Bacillus subtilis* (as known-biosurfactant-producing bacteria for comparison) were <0.5%, respectively.

However, the biodegradation of crude oil by all five strains increased rapidly to 18.5 ±4% (the average ± the standard deviation of the biodegradation of crude oil by the strains isolated from the heavy and light crude oils, tailings pond waste, refinery-contaminated soil (both strains I & II and *Bacillus subtilis*) in the second week. The maximum biodegradation (43%) in the second week was obtained by the strain isolated from the light crude oil. The biodegradation of crude oil increased further to 48 ±8%, 56 ±5%, and 63 ±4% in the following weeks. Fig. 4.5 shows the level of oil biodegradation at different periods of biodegradation. Another study performed by Saborimanesh and Mulligan (2015) showed that crude oil biodegradation by using biodiesel, diesel and light crude oil were 43 ±1% (biodiesel), 45 ±6% (diesel) and 39 ±5% (light crude oil) in the natural treatment after 35 days.

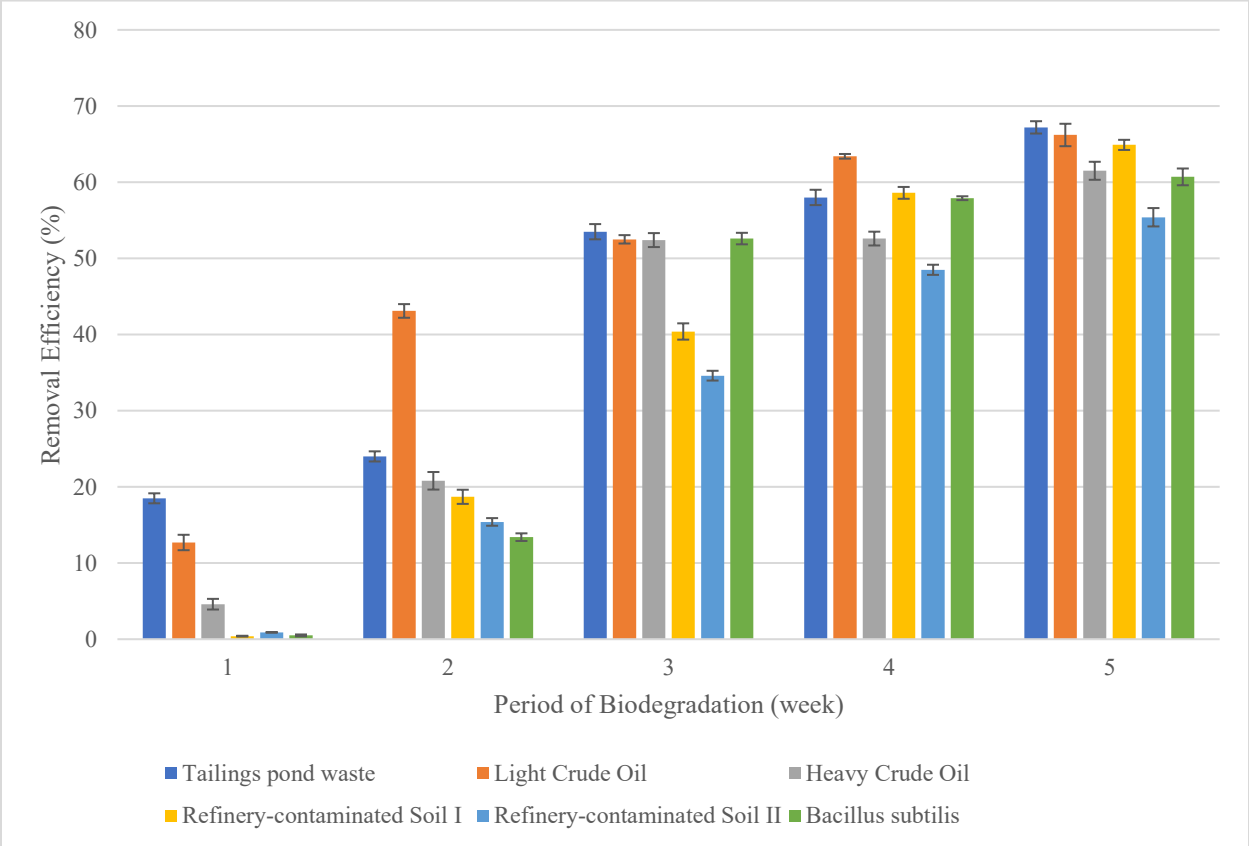


Figure 4.5. Biodegradation of crude oil in MSM (pH 7, salinity of 30 ppt) by five different strains of oil-degrading strain bacteria isolated from the light crude oil, tailings pond waste, heavy crude oil, refinery-contaminated soil and *Bacillus subtilis* as known -biosurfactant-producing bacteria for

comparison. Results are expressed as the mean ± standard error mean (SEM) of three independent experiments.

The comparison of biodegradation results of this study with the previous study (Chandankere *et al.,* 2014) showed that a maximum crude oil biodegradation of 63 ±4% was obtained in this study with the strains after five weeks of the incubation at 28°C and 120 rpm. Moreover, recently Chandankere *et al.* (2014) reported that 82-100 percent of degradation in crude oil by *B. methylotrophicus* strain at the end of biodegradation experiment (14 d) in MSM media incubated at 35°C and 180 rpm. The difference in results may be due to different strains, media conditions and duration of the biodegradation test which affected the amount of biodegradation of crude oil. In both tests, strains were cultured in the same media but with different temperatures and rpm. On the other hand, in both studies the same material has been applied (refinery-contaminated soil) with the same characteristics and also both studies results showed that the isolated strains have biosurfactant production ability. For example, in the study conducted by Chandankere *et al.* (2013) the biosurfactant (cell free supernatant) produced the reduction in surface tension of MSM media from 65 to 28 mN/m, while the bs (cell free supernatant) produced in this study reduced surface tension of MSM media from 69 to a minimum of 40 mN/m. This difference in surface tension reduction could be due to different isolated strain or environmental and experimental conditions.

4.2.5. Crude Oil Biodegradation Rate

A combination of chemical and biological process results in oil biodegradation (Bollag and Liu, 1990 ; Wu and Nofziger, 1999). In this section the biodegradation rate of each sample was determined using regression analysis to a standard first or second order kinetics biodegradation model.

A first-order degradation model is often used to simulate the reduction in residual mass of a chemical compound in environmental system (Dykaar and Kitanidis, 1996; Walker, 1974). If in the degradation process the first-order rate constant or half-life remains unchanged, the residual mass of the degraded chemical decreases exponentially over time. The following describes the first-order degradation kinetics.

Model Description: The first-order degradation kinetics may be expressed as (Dykaar and Kitanidis, 1996; Wu and Nofziger, 1999).

$$\frac{dC}{dt} = -kC$$ Eq. 4.1

Where C is the concentration of the product of interest, k is the first-order rate constant, and t is time. In practice, the first-order rate constant often is replaced by a half-life, H, where

$$H = \ln(2.0)/k$$ Eq. 4.2

The first-order equation can then become

$$\frac{dC}{dt} = -\frac{0.693}{H}C$$ Eq. 4.3

(Rocha and Walker, 1995). If the degradation rate remains constant during the degradation process, the residual concentration, C(t), is given by

$$C(t) = C_0 e^{-kt} = C_0 e^{-0.693t/H} = C_0 (0.5^{t/H})$$ Eq. 4.4

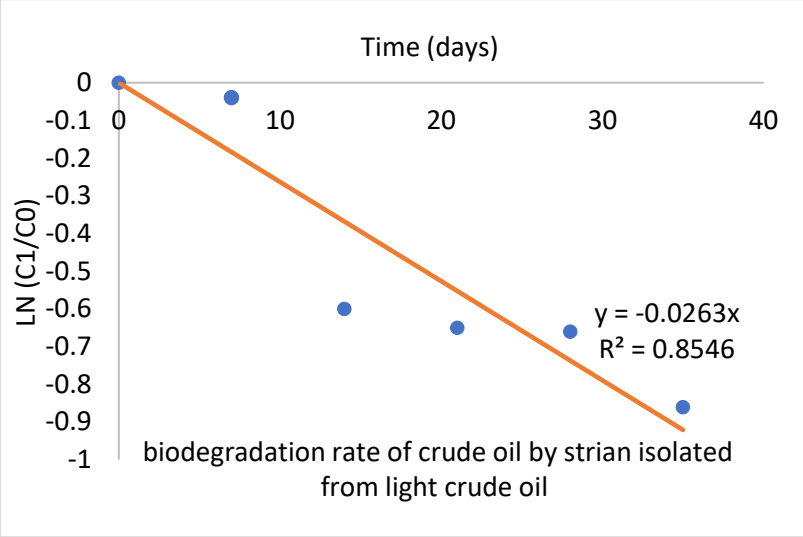
where C_0 is the initial concentration. From equation 4.4, the logarithm of the concentration is a linear function of time.

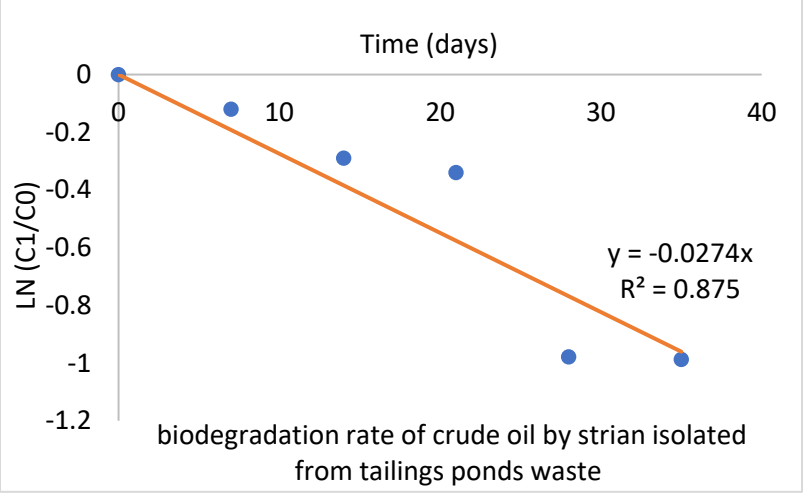
In this study first and second order kinetics were obtained for modeling of the rate of crude oil biodegradation of samples. The rate of coefficient was obtained by fitting the data to (1/C) - ($1/C_0$), over the period of biodegradation from day 1 to day 35. A comparison of first and second order kinetic showed that the crude oil biodegradation rate followed a second-order kinetic model. Fig. 4.6 and Fig. 4.7 showed the first and second order biodegradation rate of crude oil by bacteria isolated from light crude oil, heavy crude oil, tailings pond waste, refinery-contaminated soil I, refinery-contaminated soil II and *Bacillus subtilis*, with the duration of incubation (biodegradation period). By comparing first and second order kinetics the liner regression line has been obtained for second order kinetics which means that the rate of biodegradation followed second-order kinetics during biodegradation period.

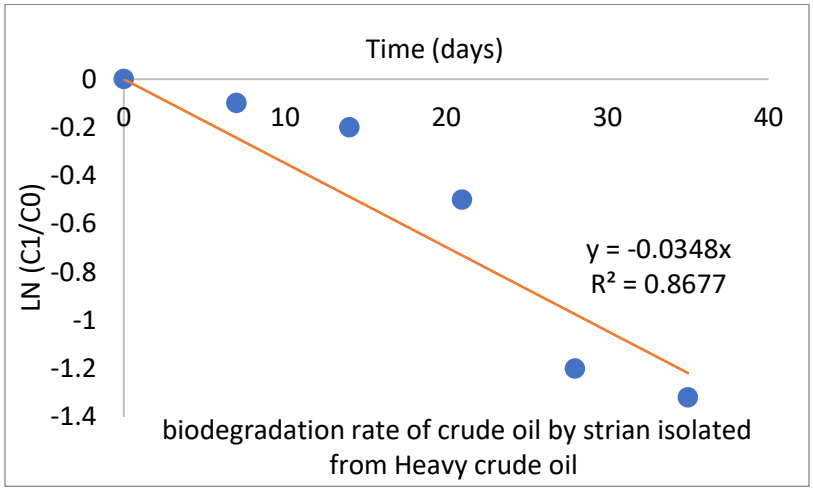
Table 4.6. First-order crude oil biodegradation rates in the samples isolated from light crude oil, heavy crude oil, refinery-contaminated soil I&II, tailings pond waste and *Bacillus subtilis* compared with study conducted by Dagnew (2004).

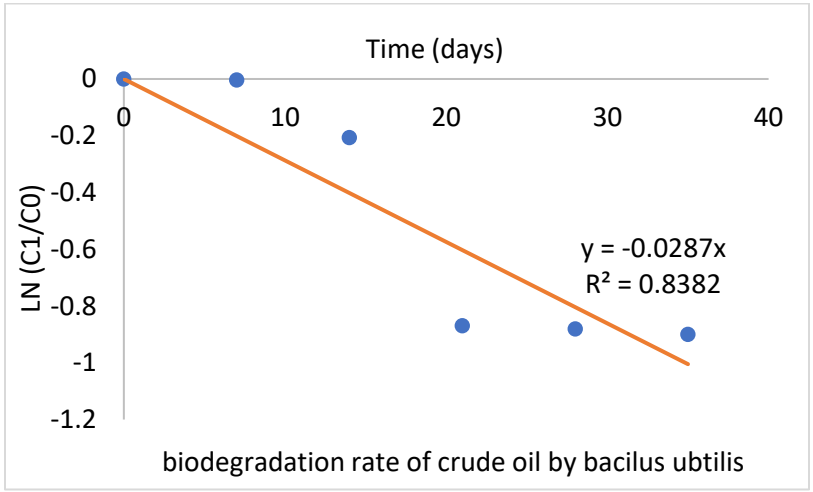
Isolated environment	Oil type	K	R^2	Reaction kinetic	References

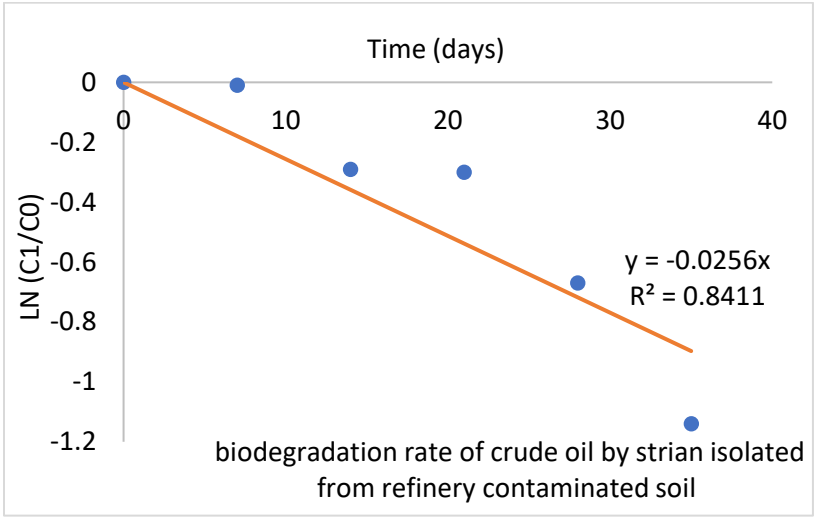
Brent oil	Crude oil	0.038	0.72	First-order	Dagnew, 2004
L-1	Crude oil	0.026	0.85	First-order	This study
H-1	Crude oil	0.027	0.87	First-order	This study
T-1	Crude oil	0.034	0.86	First-order	This study
S-1	Crude oil	0.028	0.83	First-order	This study
S-2	Crude oil	0.025	0.84	First-order	This study
Bacillus subtilis	Crude oil	0.023	0.88	First-order	This study
K: represents the first-order rate coefficient R^2: represents the coefficient of determination					






Time (days)
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
0
10
20
30
40
LN (C1/C0)
y = -0.0348x
R² = 0.8677
biodegradation rate of crude oil by strian isolated from Heavy crude oil


Time (days)
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
10
20
30
40
LN (C1/C0)
y = -0.0287x
R² = 0.8382
biodegradation rate of crude oil by bacilus ubtilis


Time (days)
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
10
20
30
40
LN (C1/C0)
y = -0.0256x
R² = 0.8411
biodegradation rate of crude oil by strian isolated from refinery contaminated soil

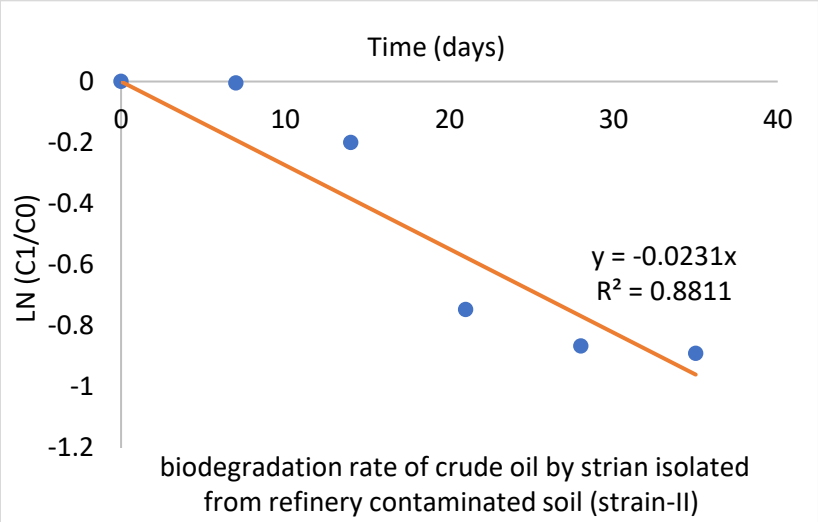
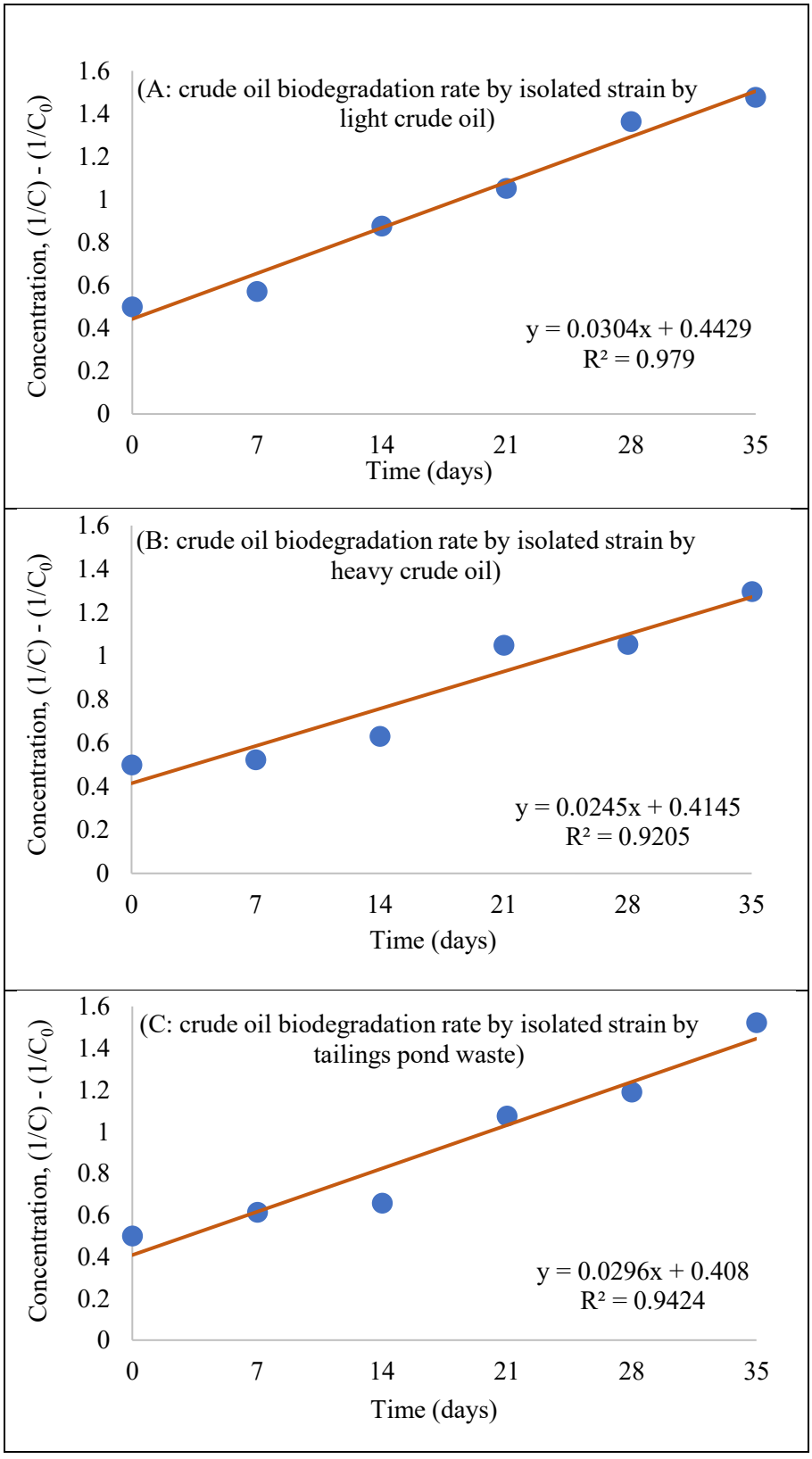


Figure 4.6. Relationship between the first order biodegradation rate of crude oil by bacteria isolated from light crude oil (A), heavy crude oil (B), tailings pond waste (C), refinery-contaminated soil I (D), refinery-contaminated soil II (E), *Bacillus subtilis* (F), with the duration of incubation (biodegradation period).

Table 4.7. Second-order crude oil biodegradation rates in the samples isolated from light crude oil, heavy crude oil, refinery-contaminated soil I&II, tailings pond waste and *Bacillus subtilis* compared with study conducted by Dagnew (2004).

Isolated environment	Oil type	K	R^2	Reaction kinetic	References
Brent oil	Crude oil	0.016	0.91	Second-order	Dagnew, 2004
L-1	Crude oil	0.030	0.97	Second-order	This study
H-1	Crude oil	0.024	0.92	Second-order	This study
T-1	Crude oil	0.029	0.94	Second-order	This study
S-1	Crude oil	0.028	0.91	Second-order	This study
S-2	Crude oil	0.019	0.93	Second-order	This study
Bacillus subtilis	Crude oil	0.026	0.89	Second-order	This study
K: represents the second-order rate coefficient R^2: represents the coefficient of determination					

This study is in agreement with a study conducted by Dagnew (2004) who reported that the biodegradation of BRENT crude oil in the period of 35 days showed second-order kinetics.


(A: crude oil biodegradation rate by isolated strain by light crude oil)
Concentration, (1/C) - (1/C0)
Time (days)
y = 0.0304x + 0.4429
R² = 0.979
(B: crude oil biodegradation rate by isolated strain by heavy crude oil)
Concentration, (1/C) - (1/C0)
Time (days)
y = 0.0245x + 0.4145
R² = 0.9205
(C: crude oil biodegradation rate by isolated strain by tailings pond waste)
Concentration, (1/C) - (1/C0)
Time (days)
y = 0.0296x + 0.408
R² = 0.9424

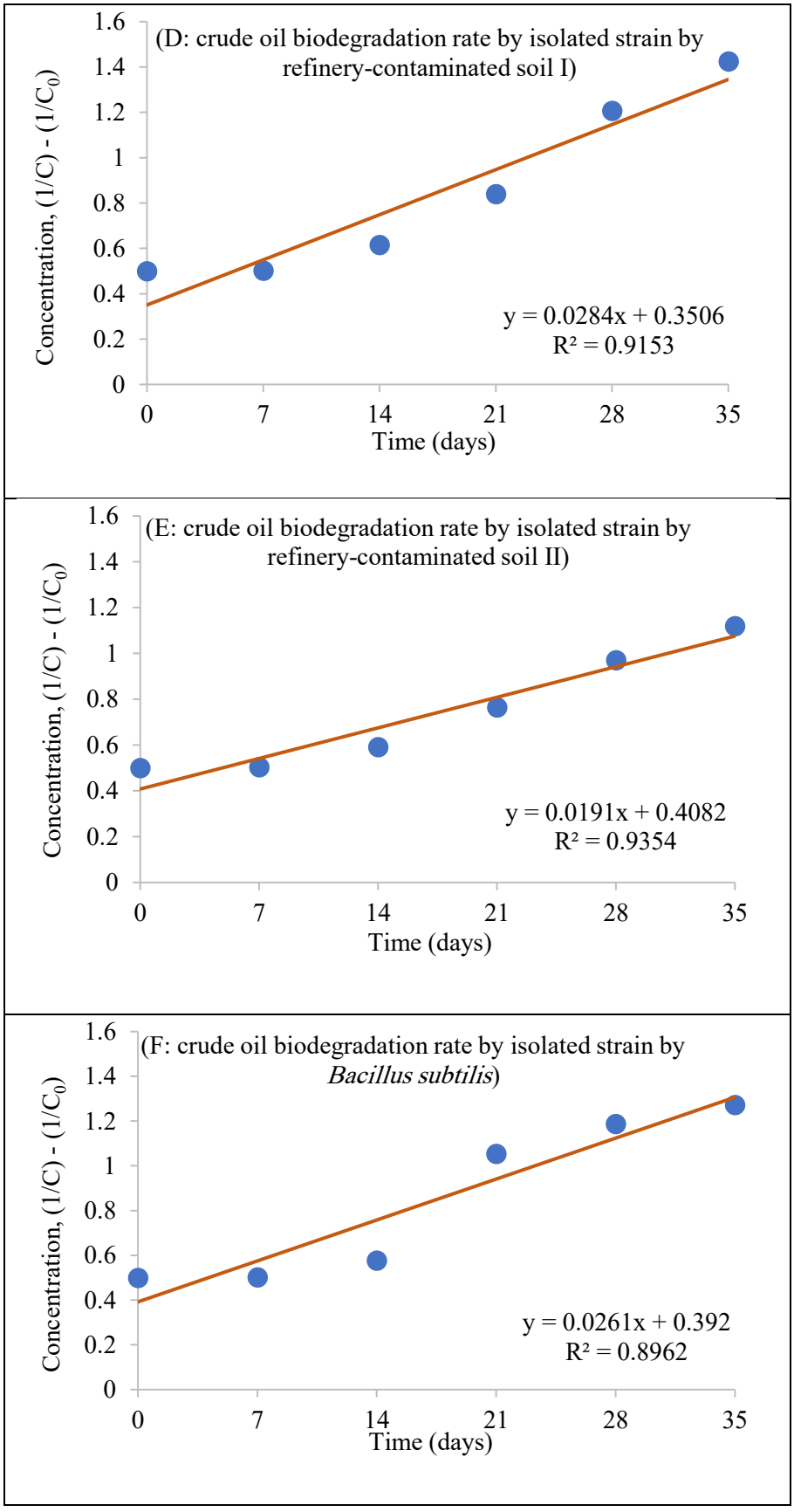

(D: crude oil biodegradation rate by isolated strain by refinery-contaminated soil I)
Concentration, (1/C) - (1/C0)
Time (days)
y = 0.0284x + 0.3506
R² = 0.9153
(E: crude oil biodegradation rate by isolated strain by refinery-contaminated soil II)
Concentration, (1/C) - (1/C0)
Time (days)
y = 0.0191x + 0.4082
R² = 0.9354
(F: crude oil biodegradation rate by isolated strain by Bacillus subtilis)
Concentration, (1/C) - (1/C0)
Time (days)
y = 0.0261x + 0.392
R² = 0.8962

Figure 4.7. Relationship between the second order biodegradation rate of crude oil by bacteria isolated from light crude oil (A), heavy crude oil (B), tailings pond waste (C), refinery-contaminated soil I (D), refinery-contaminated soil II (E), *Bacillus subtilis (F),* with the duration of incubation (biodegradation period).

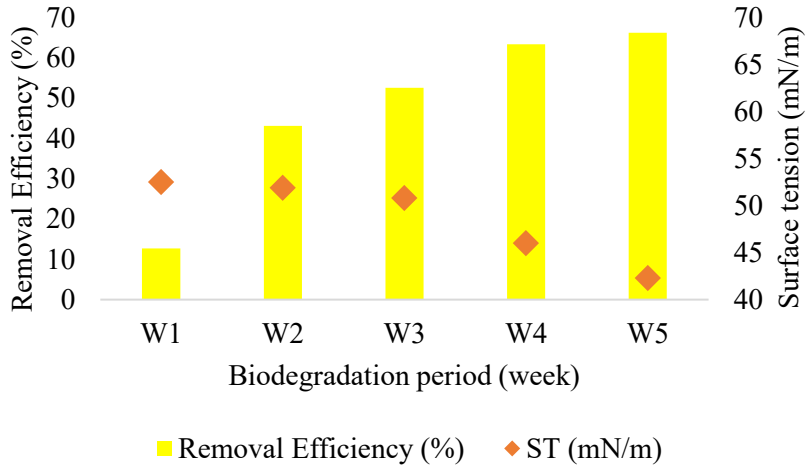
4.2.6. Correlation Between Surface Tension and Crude Oil Biodegradation

The surface tension of the aqueous phase of biodegradation samples at the same sampling days (weeks 1 to 5, and 5) showed that the biosurfactant production seemed to be the main mechanism of crude oil uptake by the isolated strains in this study. This is because a higher biodegradation of crude oil was obtained at a lower surface tension. This implies that biosurfactant production possibly resulted in the crude oil dispersion and increased the availability of crude oil to the oil-degrading bacteria. Fig. 4.8 shows the relationship between the crude oil biodegradation removal by the isolated strains in this study and decreases in the surface tension during the period of biodegradation. As can be seen in Fig 4.7 at week one of biodegradation period the crude oil removal in this week was 18.5% in the biodegradation sample inoculated strain isolated from tailings pond waste which showed the highest removal of crude oil. Light crude oil showed 12.7% removal of the crude oil in the same week. The oil uptake in week one for the strains isolated from refinery-contaminated soil strains I&II and heavy crude oil were between 0.4 to 4.6 %, respectively. The corresponding surface tension at week one of biodegradation period showed that the surface tensions of the samples were between 52 to 66 mN/m. The highest decrease in the surface tension at week one belonged to the strain isolated from light crude oil about 52.5 mN/m and strain isolated from heavy crude oil showed the lowest decrease in the surface tension at week one. Strains isolated from refinery-contaminated soil I&II and tailings pond waste showed reduction in surface tension between 57 to 64 mN/m in the same week. This trend continued for the other weeks and at week 5 nearly 70% removal of crude oil was obtained. Strains isolated from tailings pond waste showed the most removal of crude oil about 67.2% in week 5 and the less removal of crude oil belonged to refinery-contaminated soil strain II about 55.4%. Strains isolated from light crude oil, heavy crude oil and refinery-contaminated soil strain I showed the crude oil removal of about 66.2, 61.5 and 64.9%, respectively.

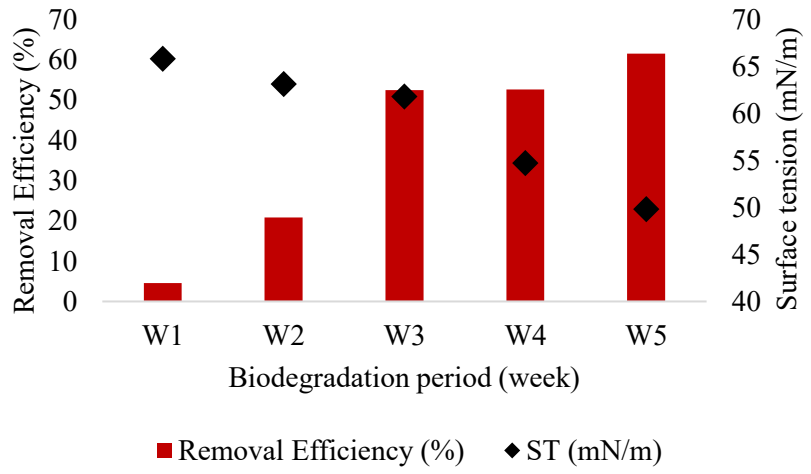
As biodegradation and corresponding surface tension data suggest nearly similar trends in biodegradation was obtained from week one to week five. A comparison of biodegradation and surface tension data from week 1 to 5 show an increasing trend in biodegradation of

crude oil over time. A similar opposite decreasing trend was observed in the surface tension of the sample. This suggests that the increase crude oil biodegradation could be due to the production of biosurfactant.

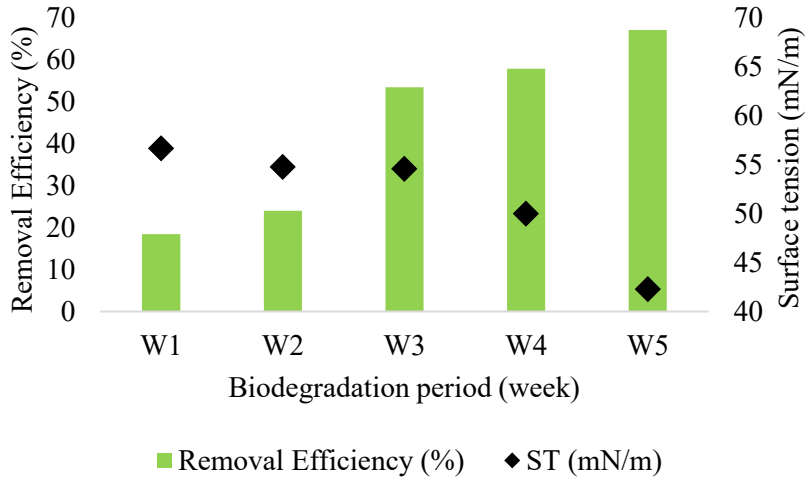
(A: oil-degrading strains isolated from light crude oil)



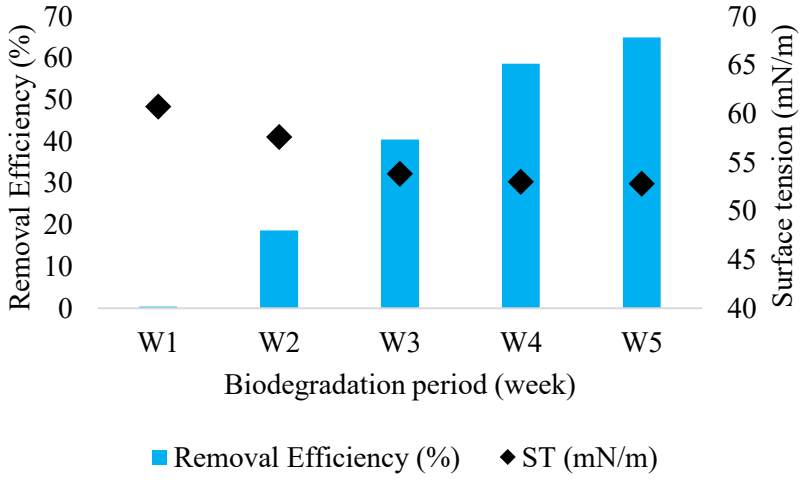
(B: oil-degrading strains isolated from heavy crude oil)



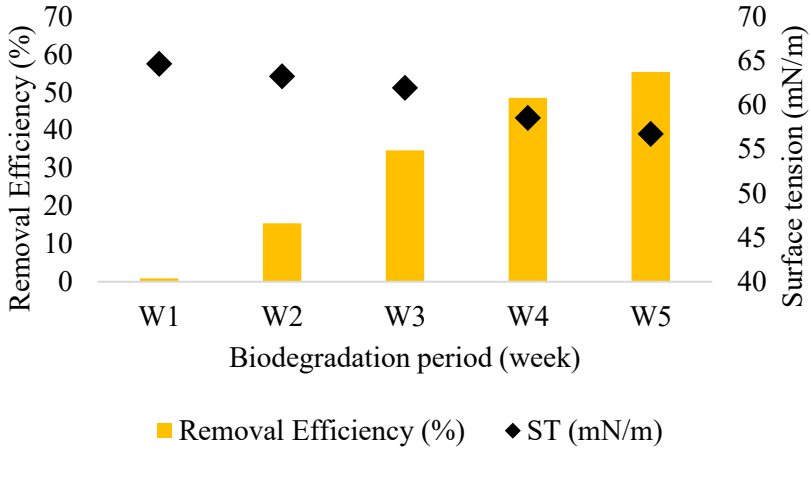
(C: oil-degrading strains isolated from tailings pond waste)



(D: oil-degrading strains isolated from refinery-contaminated soil I)



(E: oil-degrading strains isolated from refinery-contaminated soil II)



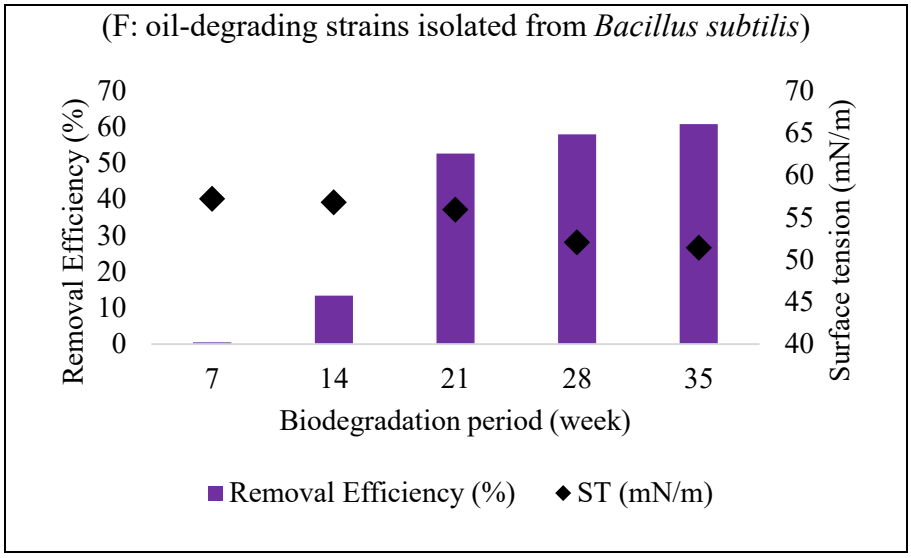


Figure 4.8. Comparison of the biodegradation efficiency of the crude oil by the oil-degrading strains isolated from A) light crude oil, B) heavy crude oil, C) tailings pond waste, D) refinery-contaminated soil I E) refinery-contaminated soil II and F) *Bacillus subtilis* (control) and their surface activity of each strain over time

5. CONCLUSIONS

Worldwide, demand for surfactants is significantly increasing, but most of the surfactants available on the market are chemically based, primarily due to their availability, low price, and expanded areas of application. The market for biosurfactants is in its early stages of development, the use of biosurfactants has been restricted to a few specialized applications due to technical constraints and material costs. Because knowledge on the physiology, genetics, and biochemistry of biosurfactant-producing bacteria needs to be expanded, screening of virulent species and process technology development will help to reduce production costs. Five oil-degrading bacteria with biosurfactant production capacity were discovered using minimal salt medium (MSM) media enrichment techniques from oily tailings pond waste, refinery-contaminated soil and light and heavy crude oils. All strains have high surface activity which could lower the surface tension. The lowest supernatant surface tensions ranged from 55 mN/m to 40 mN/m. The minimum ST belonged to the strain isolated from oily tailings pond waste (40 mN/m). In addition, the present study reported the biodegradation of crude oil under certain conditions and parameters by biosurfactant-producing bacteria nearly 70% biodegradation of crude oil was obtained from samples with the isolated bacteria during the biodegradation period of five weeks. This study confirmed the isolated bacteria's rapid and effective biodegradation of crude oil with the production of biosurfactants as the main mechanism for oil absorption.

6. FUTURE STUDIES

Some future studies could include:

- Investigation of the possibility of biosurfactant production by these microbial strains over a longer period.
- Examination of the pH, substrate addition, mixing, and other factors involved in microbial growth and biosurfactant production to optimize the rate and extent of biosurfactant production and contaminant removal.
- Experiment under different conditions such as different pH, temperature, salinity etc. to obtain better results for degradation.
- Experiment with using different carbon and nitrogen sources. Growth of biosurfactant producing bacteria under optimal conditions can be improved.

- Improving biosurfactant production by culture media optimization and optimizing some other conditions such as temperature.
- Using a combination of biosurfactant producing bacteria strains after isolation may improve the biosurfactant production.

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