EVALUATION OF BIOSURFACTANT PRODUCTION BY CRUDE OIL DEGRADING BACTERIA ISOLATED FROM ANAMBRA RIVER SEDIMENT

BY

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NAU/PG/PhD/2008487009P

DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING

FACULTY OF BIOSCIENCES

NNAMDI AZIKIWE UNIVERSITY, AWKA, ANAMBRA STATE.

MAY, 2021.

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING, FACULTY OF BIOSCIENCES NNAMDI AZIKIWE UNIVERSITY, AWKA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (PhD) DEGREE IN ENVIRONMENTAL MICROBIOLOGY

SUPERVISOR: PROF. F.J.C. ODIBO

MAY, 2021

CERTIFICATION

I Nnadi Udoka Patricia with Reg. number NAU/PG/PhD/2008487009P have satisfactorily completed the requirements for course and research work for the award of a Doctor of Philosophy in Environmental Microbiology Department of Applied Microbiology and Brewing. The research work is original and has not been submitted in part or in full for any other diploma or degree of this or any other University.

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NNADI UDOKA PATRICIA Date

APPROVAL

Nnadi Udoka Patricia has satisfactorily completed her research work on the evaluation of Biosurfactant production by crude oil degrading bacteria isolated from Anambra river sediments; in partial fulfillment of the award of Doctor of Philosophy (PhD) in Environmental Microbiology.

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Prof. P.K.Igbokwe Date
Dean School of Post Graduate Studies

DEDICATION

This work is dedicated to God Almighty.

Acknowledgments

With immense gratitude, I humbly acknowledge my indebtedness to God Almighty for His ultimate mercy and for keeping me alive throughout the duration of this programme.

I will remain grateful to my supervisor, Prof. F. J. C. Odibo whose meticulous supervision gave this work the marked difference it now has.

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ABSTRACT

Biosurfactants are valuable microbial amphiphilic molecules with effective surface-active and biological properties applicable to several industrial processes. They are synthesized by microbes, especially during growth on water–immiscible substrates, providing an alternative to chemically prepared conventional surfactants. In this study a rhamnolipid biosurfactant was produced from *Pseudomonas aeruginosa*. Four bacterial isolates recovered from Anambra river sediment using mineral salt agar media were assessed for their ability to produce biosurfactant using some standard parameters such as hemolytic test, drop collapsing test, oil displacement test, emulsification index, cetytrimethyl ammonium bromide (CTAB) agar test and surface tension measurement. One of the isolates identified as *Pseudomonas aeruginosa* using phenotypic characteristics and 16SrRNA gene sequencing analysis was chosen as the best biosurfactant producer as it showed positive results to all the standard parameters used. Biosurfactant synthesis was followed by measuring surface tension (33mN/m) and emulsifying index (96%) after 120h of incubation .The best results were obtained when using crude oil and ammonium nitrate as carbon and nitrogen sources respectively with a C: N ratio of 10(Crude oil 2%;Ammonium nitrate 2.0g). The surface tension and emulsification activity of the biosurfactant remained stable over a wide range of temperature (30^0C-50^0C) and pH (5.0-9.0). The crude

biosurfactant has been extracted from supernatant culture growth by chilled acetone precipitation and the yield of crude biosurfactant was 1.44g/l^{-1}.Structural attributes of biosurfactants were determined by biochemical tests, thin layer chromatography (TLC), gas chromatography which confirmed the biosurfactant was a rhaminolipid in nature. In addition to oil recovery, the *Pseudomonas aeruginosa* degraded crude oil within a 30days of exposure. The result of crude oil degradation also showed that C_{35} fractions of the crude oil were most degraded by the isolate. The ability of the biosurfactant to enhance secondary recovery of crude oil was assessed using a sand packed column. The application of *Pseudomonas aeruginosa* biosurfactant effected recovery of 90% of trapped crude oil over 0% recovery observed for the control (water). This study showed that exposed *Pseudomonas aeruginosa* as a potential of biosurfactant for use in petroleum industries and for bioremediation in crude oil polluted environments.

TABLE OF CONTENTS

List of Tables

List of Figures

List of map

List of slide

List of plates

List of appendices

CHAPTER ONE

1.0 Introduction

Crude oil is a liquid mixture of naturally occurring hydrocarbons, which can be distilled to yield a variety of products. It is usually black or dark brown (although it may be yellowish, reddish, or even greenish) (Zhang and Miller, 1994). Crude oil contains aliphatic, alicyclic and aromatic hydrocarbons. The carbon atoms linked are together in chains of different lengths (Souza *et al*., 2014).

Crude oil can be refined to produce usable products such as gasoline, diesel and various forms of petrochemicals. It is a non renewable resource, also known as fossil fuel, which means that it cannot be replaced naturally at the rate of its composition and is therefore a limited resource.

The world depends on crude oil and the use of crude oil as fuel has contributed to intensive economic development. Although petrochemical plants and oil refineries are beneficial to society, they produce a large amount of hazardous wastes. This hazardous waste is one of the most important pollution sources around the world, due to oil spills. Oil spills during exploration, transportation and refining, have caused serious environmental problems (Franzetti *et al*., 2009; Marchant and Banat, 2012; Souza *et al*., 2014).

Crude oil-degrading bacterial consortia exist in nature and live in oil polluted sites while using petroleum hydrocarbon as a source of carbon and energy

for growth (Heyd *et al*., 2008). In the process of utilizing crude oil as the source of carbon and energy, they secrete a variety of substances known as biosurfactants.

Biosurfactants are a unique class of compounds that have been shown to have a variety of potential application in remediation of organic and metal contaminated sites. They are biological amphipathic compounds consisting of hydrophilic and hydrophobic moieties, where the hydrophobic moiety is either long chain fatty acids, hydroxyl fatty acid, or x-alkyl-β-hydroxyl fatty acid, and the hydrophilic moiety can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid or alcohol (Kosaric, 2001). Their amphipathic nature confers on them the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface, respectively (Konishi *et al*., 2008).

Micro-organisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopetides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Kosaric, 2001). Majority of known biosurfactants are synthesized by micro-organisms grown on water immiscible hydrocarbons, but some are produced on water soluble substrates such as glucose, glycerol and ethanol (Heyd *et al*., 2008).

The various biosurfactants produced at the present time are the rhamnolipids from *Pseudomonas aeruginosa*, surfactin from *Bacillus subtilis,* emulsan from

Acinetobacter calcoaceticus, sophorolipids from *Candida bombicola* and mannosylerythritol lipids (MELs) from Pseudozyma yeasts (Benincasa *et al.*, 2002; Nitschke and Costa, 2007; Muthusamy *et al*.,2008; Banat *et al.*, 2010; Marchant and Banat, 2012).

Marine sediments contain nutrients vital for microbial maintenance and growth, including nitrogen, phosphorus, calcium and magnesium. Carbon in sediments supports diverse microbial populations that are responsible for transforming and retaining nutrients. Nutrients also reach the coastal seas by unwelling. This, together with the large amount of sunlight, makes the continental shelves a productive area. The food for marine sediments consists of fecal pellets, dead organisms and organic debris. This makes the marine sediment a rich community of micro-organisms.

In this study, biosurfactants have a good perspective for clean-up of soil polluted by high concentration of crude oil. In addition, biosurfactants have increased versatility as compared to many synthetic surfactants and are suitable for pollution control through biodegradation (Carrillio *et al*., 1996).

1.1 STATEMENT OF PROBLEM

Crude petroleum oil and its derivatives are considered as one of the most pervasive environmental pollutants because they produce a problem of increasing enormity around the globe (Okoh *et al*., 2001). Accidental and deliberate spillage and instinctive environmental contamination have been a major threat to the ecosystem and biota through the transfer of toxic organic materials including complex mixture of aliphatics, aromatics (including polycyclic aromatic hydrocarbons, i.e., PAHs), nitrogen, sulfur, metals etc. into the food chain (Rufino et al., 2001). Thus, these detrimental hydrocarbon pollutants make the development of a remediation technology essential for cleaning up polluted sites. As compared to other strategies adopted to treat crude petroleum contamination, biosurfactant is recognized as one of the effective, eco-friendly and inexpensive technologies (Rahman et al., 2002).

Generally, tertiary and enhanced oil recovery involves the extraction of residual oil after the primary and secondary phases of production. At this stage, modern and technically advanced are employed to either modify the properties of reservoir fluids or the reservoir rock characteristics, with the aim of gaining recovery efficiencies than those obtained by conventional recovery methods (primary and secondary recovery stages). This can be achieved by the use of biosurfactant.

1.2 Aim and Objectives of Study

The aim of this study is to evaluate the production of biosurfactant from a crude oil degrading bacteria recovered from Anambra river sediment.

The specific objectives are to:

1. isolate the crude oil degrading bacteria from Anambra river sediments.
2. screen the selected bacterium for crude oil degradation
3. screen efficient isolates for biosurfactant production.
4. characterize and identify the isolates.
5. optimize biosurfactant production by the isolates
6. characterize the biosurfactants produced by the bacterial isolates.
7. carryout secondary recovery of crude oil using isolated biosurfactant

1.3 SIGNIFICANCE OF THE STUDY

Evaluation of biosrfactant production by crude oil degrading performance from degrading bacteria is demanded for successful microbial remediation of toxic pollutants. The employment of biosurfactant-producing and hydrocarbon-utilizing microbes enhances the effectiveness of bioremediation as biosurfactant plays a key role by making hydrocarbons bio-available for degradation. This study isolation of a potent biosurfactant producing indigenous bacteria which can be employed for crude oil remediation, along with the characterization of the biosurfactant produced

during crude oil biodegradation. Production of biosurfactant of the bacterial isolates was assayed in terms of drop collapse assay and surface tension reduction of the culture medium.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition and structure of Biosurfactants

According to Banat *et al.* (2010) and Kiran *et al*. (2010), biosurfantants are surface active agents by micro-organisms, which emulsify hydrocarbon in growth medium and reduce surface and interfacial tensions.

Biosurfantants are amphipathic biological compounds produced extracellularly or as a part of the cell membrane by a variety of yeast, bacteria and filamentous fungi (Costa *et al;* 2006). They increase the solubility of hydrophilic molecules thereby reducing both surface and interfacial tensions at the oil water interface (Banat *et al*., 2010). Biosurfantant are biological amphipathic compounds consisting of hydrophilic and hydrophobic moieties, where the hydrophobic moiety is either a long chain fatty acid, hydroxyl fatty acid or α-alkyl-β-hydroxy fatty acid and the hydrophilic moiety can be, a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid or alcohol (Kitamoto *et al*., 2009).

Surfactants reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. They contain a hydrophobic portion with little affinity for the bulk medium and hydrophilic group that is attracted to the bulk medium. Biosurfantants are surface active compounds mainly produced

by micro-organisms and synthesized in cells using diverse substrates, being totally biodegradable in aerobic and anaerobic conditions as well as non-toxic (Varnier *et al.,* 2009; Kosaric, 2001; Banat, 1995).

The molecule of biosurfactant, which in the same molecule balance the hydrophilic and hydrophobic and hydrophobic moiety (Fig. 1)(Banat,1995).

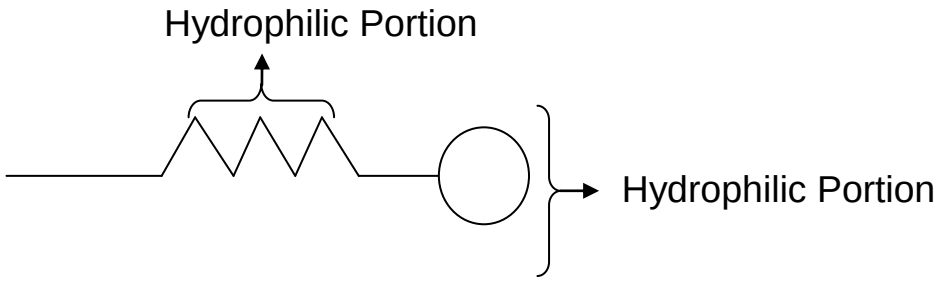


Figure 2.1: The amphiphilic structure of a biosurfantant (Banat, 1995)

The hydrophobic part (moiety) of the molecule is a line chain of fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The water soluble end (hydrophilic) can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxyl acid or alcohol.

Additionally, the hydrophobic moiety is usually a C_8 to C_{22} alkyl chain or alkylaryl that may be linear or branched (Carter, 1984).

2.2. Properties of Biosurfactant

It is necessary to submit a biosurfactant to conservation methods to evaluate its properties (surface tension and dispersion) over a period of 120 days to estimate the commercial validity of the product. Thus, heating methods are used separately

or in combination with potassium sorbate, which is a conservative that inhibits the growth of mould that is widely used in the production and conservation of foods such characteristics are common to the majority of biosurfactants and have advantage over conventional surfactants, as described below (Vijayakumar and Saravanan, 2015).

a). Surface and Interfacial Activity

Effectively and effectiveness are essential characteristics of a good surfactant. Efficiency is measured by the CMC (critical micelle concentration), whereas effectiveness is related to surface and interfacial tensions (Barros *et al.*, 2008). The CMC of biosurfactants ranges from 1 to 2000mg/l, whereas interfacial (oil/water) and surface tensions are respectively approximately 1 and 30mN/m. Good surfactants are able to reduce water surface tension from 72 to 35mN/m and interfacial tension of n-hexadecane from 40 to 1mN/m. The surface tension correlates with the concentration of the surface active compounds until the critical micelle concentration (CMC) is reached (Fig. 7). Efficient surfactants have a low critical micelle concentration (i.e. less surfactant is necessary to decrease the surface tension). The CMC is defined as the minimum concentration necessary to initiate micelle formation (Barros *et al*., 2008). In practice the CMC is also the maximum concentration of surfactant monomers in water aid is influenced by pH, temperature and ionic strength.

Tolerance to temperature, pH and Ionic strength temperatures and pH values ranging from 2 to 12.biosurfactants also tolerate a salt concentration up to 10%, whereas 2% ward is enough to inactive synthetic surfactants.

b. Biodegradability.

Biosurfactants are easily degraded by micro-organisms in water and soil, making these compounds adequate for bioremediation and waste treatment.

c). Low Toxicity

Low degree of toxicity allows the use biosurfactants in foods, cosmetics and pharmaceuticals. Low toxicity is also of fundamental importance to environmental applications. Biosurfactants can be produced from largely available raw materials as well as industrial waste. Glycolipids from *Rhodococcus* sp. 4134 were 50% less toxic than 25% in naphthalene solubilization tests (Kanga *et al*., 1997).

d). Specificity

Biosurfactants being complex organic molecules with specific functional groups are often specific in their action. This is of particular interest in the detoxification of different pollutants and the well as specific food, pharmaceutical and cosmetic applications.

e). Biocompatibility and Digestibility

These properties allow the use of biomolecules in different industries, especially the food, pharmaceutical and cosmetic industries. With their property of

environmental compatibility, biosurfactants have wide environmental application, such as bioremediation and oil recovery (Vijayakumar and Saravanan, 2015).

f). Emulsion forming / Breaking

An emulsion is defined as a "heterogeneous system, consisting of at least one immiscible liquid dispersed in another in the form of droplets, whose diameters, in general, exceed 0.1mm. Such systems posses a minimal stability, which may be accented by such additives as surface active agents, finely defined solids etc (Kanga *et al.,* 1997). Biosurfactants can be either emulsifiers or de-emulsifiers. There are two basic types of emulsion: oil-in-water (o/w) and water-in-oil (w/o). Emulsions have minimal stability, but the addition of biosurfactants can lead to an emulsion that remains stable for months or even years (Velmurgan *et al.,* 1993). Liposan, which is a water soluble emulsifier synthesized by *C. lipolytica*, has been used with edible oils to form stable emulsions. Liposan is commonly used in the cosmetic and food industries for producing stable oil/water emulsions (Campos *et al*., 2013).

g). Availability of Raw Materials

Biosurfactants can be produced from cheap raw that are available in large quantities (Vansileva-Tonckova *et al*., 2001).

h). Acceptable Production Economics

Depending on its application, biosurfactants can be produced from industrial wastes and bye-products and this is of particular interest for their bulk production (Varnier *et al.*, 2009).

i). Use of Environmental Control

Biosurfactants can be effectively used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and bioremediation of contaminated soil (Vanier *et al.,* 2009).

2.3. Biosurfactant producing Micro-organisms

Micro-organisms are capable of producing biosurfantant with different molecular structures and surface activities (Campos *et al.*, 2013). Micro-organisms use a set of carbon sources and energy for growth. The combination of carbon sources with insoluble substrates facilitates the intercellular diffusion and production of different substances (Marchant *et al.*, 2014). In recent decades, there has been an increase in scientific interest regarding the isolation of micro-organisms that produce tension active molecules with good surfactant characteristics, such as a low CMC, low toxicity and high emulsifying activity (Souza *et al*., 2014).

The literature describes bacteria of the genera *Pseudomonas* and *Bacillus* as great biosurfactant producers (Silva *et al*., 2010). However, most biosurfactants of a bacterial origin are inadequate for use in the food industry due to their possible

pathogenic nature (Souza *et al*., 2014). *Candida bombicola* and *candida lipolytica* are among the most commonly studied yeast for the production of biosurfactants. A key advantage of using yeasts, such as *Yarrowia Lipolytica*, *Saccharomyces cerevisiae* and *kluyveromyces lactis*, resides in their generally regarded as safe (GRAS) status. Organisms with GRAS status do not offer the risks of toxicity or pathogencity which allows their use in the food and pharmaceutical industries (Campos *et al.*, 2013).

The first microbiological biosurfactants on the market were sophorolipids. Of all currently known biosurfactants, rhamnolipids have the highest potential for becoming the next generation of biosurfactants introduced in the market (Muller *et al*., 2012).

Micro-organisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Campos *et al*., 2013).

Biosurfactants can be synthesized by a variety of micro-organisms (bacteria, yeasts and filamentous fungi) (Diaz De Rienzo *et al*., 2016) utilizing both water immiscible hydro-carbons i.e., plant/animal derived oil, n-alkanol, polycyclic aromatic hydrocarbons (PAHs) and / or water soluble compounds .i.e., glucose, fructose, sucrose, xylose, galactose, mannitol, glycerol, ethanol (Desai and Banat, 1997; Diaz De Rienzo *et al*., 2016).

Biosurfactants produced by micro-organisms represent a heterogeneous group of secondary metabolites (Campos *et al*., 2013), play major roles in the survival of the producing micro-organisms by increasing the bioavailability of hydrophobic substrate (facilitating nutrient transport), interfering in microbe-host interactions and quorum sensing mechanisms (bacterial pathogenesis), or by acting as antimicrobial, insecticidal, antibiofilm and anti-adhesive agents (Rodrigues *et al*., 2006; Marchant and Banat, 2012; Ines and Dhouha, 2015).

Candida tropicalis, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Brevibacterium casei*, and *Flavobaterium aquatile* were identified as the biosurfactant-producing organisms (Campos *et al*., 2013).

A number of micro-organisms, such as fungi, yeast and bacteria feed on substances that are immiscible in water, producing and using a surface active substance (biosurfactant) (Sobrinho *et al*., 2013; Banat *et al*., 2010). Among bacteria, the genus *Pseudomonas* is known for its capcity to produce extensive quantities of glycolipids. These biosurfactants are classified as rhamnolipids. *Bacillus subtilis* is another micro-organism widely studied for biosurfactant production and is known for its efficiency in producing a lipopeptide with surface activity denominated surfactin or subtilisin (Peypoux *et al*., 1999; Aparna *et al*., 2011; Al-Bahny *et al*., 2013; Souza *et al*., 2014). *Candida bombicola* and *Candida lipolytica* are among the most commonly studied yeasts for the production of

biosurfactants (Campos *et al.,* 2013). Table 1 offers a list of micro-organisms that produce biosurfactants (Pacwa-plociniczak *et al.*, 2011; Sabrinho *et al.,* 2013).

Table 2.1: Main classes of biosurfactants and respective producer microorganisms

Class/Type of Biosurfactant	Microorganisms
Glycolipids:	
Rhamnolipids	*Pseudomononas aeruginosa*
Sophorolipids	*Torulopsis bombicola, T. apicola*
Trehalolipids	*Rhodococcus erythiopolis, Mycobacterium sp*
Lipopeptides and Lipoproteins:	
Peptide-lipid	*Bacillus licheniformis*
Viscosin	*Pseudomonas fluorescens*
Serrawettin	*Serratia marcenscens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidin	*Bacillus brevis*
Polymyxin	*Bacillius Polymyxia*
Fatty acids, neutral lipids and phospholipids:	
Fatty acids	*Corynebacterium lepus*
Neutral lipids	*Nocardia erythropolis*
Phospholipids	*Thiobacillus thiooxidans*

Polymeric Surfactants:	
Emulsan	*Acinetobacter calcoacetius*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carbohydrate-protein	*Pseudomonas fluorescens*
Mannan-lipid-protein	*Candida tropicalis*
Particulate Surfactants:	
Vesicles	*Acinetobacter calcoaceticus*

Source: Sobrinho *et al*. (2013)

2.4. Biosurfactant Classification and their Properties

Unlike chemically synthesized surfactant which are classified according to the nature of their polar grouping; biosurfactants are categorized mainly by their chemical composition and their microbial origin.

There are six major classes of biosurfactants, glycolipids, lipopeptides and lipoproteins, phospholipids, fatty acids polymeric surfactants and particulate surfactants (Persson et al., 1998). Kosaric (2001) classified biosurfactants based on their structure namely, hydroxylated and cross linked fatty acids, polysaccharide-lipid complexes, glycolipids, lipoprotein-lipopeptides, phospholipids and complete cell surfaces.

On the other hand, Biermann *et al. (*1987) grouped biosurfactants as glycolipids, lipopetides, phospholipids, fatty acids, neu tral lipids, polymeric and particulate compounds.

Lastly, Biermann *et al*. (1987) grouped biosufactants into four main categories namely, glycolipids, phospholipids, lipoproteins/lipeptides and polymeric.

Most biosurfactants are either anionic or neutral, whereas those that contain amine groups are cationic. The hydrophobic moiety has long-chain fatty acids and the hydrophilic moiety can be a carbohydrate, cyclic peptide, amino acid,

phosphate carboxyl acid or alcohol. The molar mass of biosurfactants generally ranges from 500 to 1500 Da (Bognolo, 1999). Microbial surfactants can also be divided into two major classes according to their molecular mass. The low molecular mass biosurfactants include glycolipids such as rhamnolipids and sophorolipids, or lipopeptides like surfactin and polymyxin, was a function in lowering the surface and interfacial tensions. The high molecular mass biosurfactants such as lipoproteins, lipopolysaccharides and amphiphatic polysacchardies are more effective at stablizing in water emulsions (Rosenberg and Ron, 1999).

According to Rosenberg and Ron (1999), there three possible properties of biosurfactants in application, increasing the surface area of hydrophobic substance, increasing the bioavailablity of hydrophobic water insoluble substrates, and finally regulating the attachment of micro-organisms to and fro surfaces. The biosurfactant producing microbes are distributed among a wide variety of genera (Rosenberg and Ron, 1999).

Biosurfactants are generally categorized by their microbial origin and chemical composition, as follows (Pattanath *et al.*, 2008; Banat *et al*., 2010; Vijayakumar and Saravanan, 2015):

1. Glycolipids

The most well known biosurfactants are glycolipids, which are composed of carbohydrates linked by means of ether or ester bonds with either long-chain aliphatic acids or hydroxyaliphatic acids. They consist of mono-, di-, tri and tetrasaccharides which include glucose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate. The fatty acid component usually has a composition similar to that of phospholipids of the same micro-organisms (Campos *et al*., 2013).

Also, they are made up of carbohydrates in combination with long chain aliphatic acid and hydroxyaliphatic acids (Kiram *et al.*, 2009*;* Desai and Banat, 1997). These include rhamnolipids, sophorolipids, tretrahalolipids, fructose lipids, glycolipid bioemulsifiers (Rosenberg and Rom, 1999).

Rhamnolipids:

a) Discovery:

The discovery of rhamnolipids (RLS) dates back to 1946, when Benicasa *et al.* (2004) reported an oily glycolipid, which was named piolipic acid, composed of L-rhamnose and β-hydro-oxydicanoic acid (Jarvis and Johnson, 1949), produced by *P. pyocyaneus* (today *P. aeruginosa*), when cultivated on glucose. Further characterization of the structure of Jarvis and Johnson, (1949) showed that the isolated compound was composed of two β-hydroxydecanoic acids (connected via

an ester bond) linked through a glycosidic bond to two rhamnose moieties. Additionally, El-Sheshany *et al. (2015*) using periodate oxidation and methylation, reported that the linkage between the two rhamnose moieties is an α-1, 2-glycosidic linkage. Based on this, they chemically described the rhamnose as 2-O α-rhamnopyranosyl-α-l-rhamnospranosyl-β-hydroxy decanoyl-β hydroxydecanoate (di-Rl-structure). This was the first glycolipid discovered containing a link between a sugar and a hydroxylated fatty acid residue (Sarubbo *et al*., 2006). From 1972 to 2000, various rhammnolipid (RL) structures produced by *P. aeruginosa* strains growing on different carbon sources (n-paraffins, glycerol, n-alkanes, glucose etc), were isolated and reported. The isolated rhamnolipids included all types of RL homologues (RL1, RL2, RL3 and RL4), and their number increased with the progress of analytical methods (Abdel-Mawgoud *et al*., 2010).

Furthermore, some studies reported the complex corporation of bacterially produced RL mixtures, including, for example an RL mixture composed of 67% di-rhamno-di-lipid, 22% mono-rhamno-di-lipid, 9% di-rhamno-di-lipid, and less than 3% of mono-rhamno-mono-lipid (Arino *et al*., 1996). The development of sensitive, high throughput analytical techniques has led to the further discovery of a wide diversity of RL congeners and homologues (about 60) produced in different concentrations by various *Pseudomonas* species and other bacteria (Abdel-Mawgoud *et al*., 2010).

b) *Pseudomonas* Species as Producers of Rhamnolipids

For more than 60 years, much work has been undertaken to stimulate the development and establishment of rhamnolipid production by *P.aeruginosa (*Nitschke *et al.*, 2011). When comparing the production of rhamnolipids by different *Pseudomonas* species, Onbasli *et al*. (1996) found the *P. Luteola* and *P. putida* gave higher rhamnolipid yields than *P. aeruginosa, P. Fluorescens* and *P. Stutzerri*, but the composition and distribution of the rhamnolipid homologues were not described for these strains, while the production of rhamnolipids by *P. Chlororaphis* had lower yield than *P. aeruginosa* Onbasli *et al*.,(1996). Furthermore, reports about rhamnolipids obtained from *Pseudomonas* species other than *aeruginosa* lack information about their surface properties, which is the most important indicator of surfactant quality.

c) Diversity of Rhamnolipids

Interest in rhamnolipids from two contrasting facts. On the one hand, rhamnolipids show relatively high surface activities and are produced in relatively high yields after fermentation process, which are relatively well understood. On the other hand, they are considered as virulence factors involved in the processes of *P. aeruginosa* pathogenesis, resulting in an investigation of rhamnolipid biosynthesis in order to control their production and effect (Abdel-Mawgound *et al*., 2011).

P. aeruginosa produces various extracellular mono-and di-rhamnolipids. Two types of rhamnolipids (Fig. 2&3), termed RL1 and RL3, consisting of one or two L-rhamnose units and two units of β-hydroxy-decanoic acid, are the principal RLs. There are also RL2 and RL4 RLs, which consist of one or two L-rhamnose units and one unit of β-hydroxydecanoic acid (Tahzibi *et al.*, 2004).

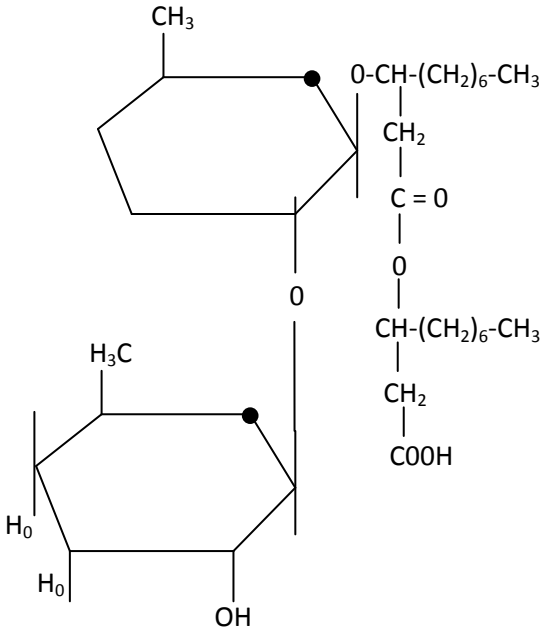


Figure 2.2: Structure of di-rhamnolipid (Tahzibi *et al*., 2004)

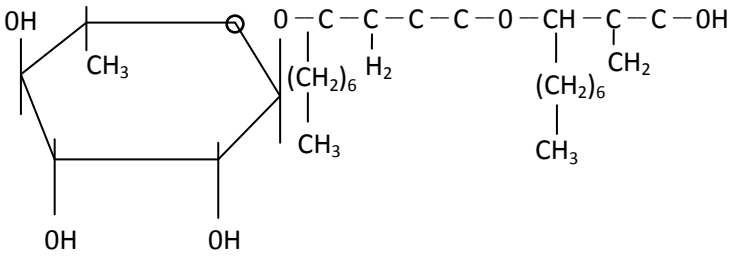


Figure 2.3: Structure of Monorhamnolipid (Tahzibi *et al*., 2004)

d). Composition of Rhamnolipids

Naturally produced rhamnolipids always appear as mixtures of different rhamnolipid congeners, as was observed when produced by various strains of *P. aeruginosa* (Benincasa *et al.*, 2002; Rikalovic *et al*., 2013). The complexity of the composition of rhamnolipid mixtures was found to depend on various factors such as: origin of the bacterial strain, type of carbon substrate (Rahman *et al*., 2002), culture conditions (Costa *et al*., 2006), age of culture (Haba *et al.*, 2003), the *P. aeruginosa* strain itself (Haba *et al*., 2003; Rikalovic *et al.*, 2013), as well as the method or rhamnolipid isolation and punrification (Deziel *et al*., 1999; Nitschke *et al.*, 2010).

Some rhamnolipid congeners are predominant in all rhamnolipid producing *P. aeruginosa* strains and are classified as the major RL structures, while others, produced only sometimes or with low abundance, are the minor rhamnolipid structures. Both the major and the minor rhamnolipid mixtures produced by *P. aeruginosa* strains, it was shown that, aside from their varying composition, these mixtures contain mono-RL and di-RL structures. In the lipid part, chain lengths from C_8 to C_{12} and Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} were the predominant congeners (Haba *et al.*, 2003; Nie *et al.*, 2010; Aparna *et al*., 2011; Rikalovic *et al*., 2013). The ratio of total di-to mono-RL fractions from RL produced by *P.*

aeruginosa is reported to range from 1 to 5 (Dubeau *et al*., 2009; Rikalovic *et al*., 2013).

Despite the diversity of the reported rhamnolipid structures, there are relatively few studies which both quantitatively and qualitatively analyzed rhamnolipids (Deziel *et al*., 1999; Haba *et al.,* 2003; Dubeau *et al*., 2009; Guerra-satos *et al*., 1986; Muller *et al*., 2011; Rikalovic *et al.,* 2013). The composition of rhamnolipid mixtures produced is important, since this defines the physicochemical properties of the products, which further impacts on their potential application.

e). Physicochemcial Properties of Rhamnolipids

Rhamnolipid show physicochemical properties that are different from single rhamnolipid congeners. Hence, the most abundant structure will have the biggest impact in the characteristics and dictate the behavior of the total rhamolipid mixture.

Rhamnolipids can reduce the surface tension of water from 72 to 30mNm^{-1} and interfcicial tension of water/oil to values < 1mNm^{-1} (Benincasa *et al*., 2004). Rhamnolipid are weak acids due to the carboxylic moieties and are known to undergo aggregation in solution (Benincasa *et al.,* 2002). At concentrations above the critical micelle concentration (CMC), rhamnolipids form micelles, vesicles or lamella, depending on the solution properties (Li *et al*., 2002). The CMC values for

Rhamnolipids depend on the chemical composition of the congeners present in each rhamnolipid mixture (the ratio and composition of the homologues, the presence of unsaturated bonds, the branching and length of the alkyl chain, or the size of the hydrophilic head group) and on their chemical environment, and was reported to range from 5 to 200mgL-1 (Haba *et al.*, 2003; Nitschke *et al*., 2003; Dubeau *et al*., 2009 and Rikalovic *et al.*, 2013).

Furthermore, in a comparative study, a statistically significant increase in the CMC values was observed on lowering the ratio of total mono-/di-rhamnolipid and ratio of Rha-C_{10}-C_{10}/Rha-Rha-C_{10}-C_{10} ($P \leq 0.05$), indicating that mono-rhamnolipids start to form micelles at lower concentrations than di-rhamnolipid (Rikalovic *et al*., 2013)

Rhamnolipid were shown to have higher solubilization capacity, which is expressed as the molar solubility ratio (MSR) for non-aqueous phase liquids (NAPL), as well as for solid phase organics, such as polycyclic aromatic hydrocarbons (PAH) and hydrocarbon mixture than some of the commonly used synthetic surfactants (Tween, Triton and alkylbenzyl sulfonate) (Maier and Soberon-Chavez, 2000).

Rhamnolipids are also an example of a readily degraded agent, as determined by the OECD 301 Ready Biodegrability Test (Maslin and Maier, 2000). Furthermore, invertebrate toxicity tests performed in accordance with

OEDC 202 showed rhamnolipid to have very low toxicity (Maier and Soberon-Chavez, 2000). Rhamnolipid also have high affinity for a variety of metals, including cadmium, copper, lanthanum, lead and zinc (Maier and Soberon-Chavez, 2000).

f). Identification and Qualification of Rhamnolipid

The quest for a cost-efficient production of rhamnolipid biosurfantant and alternatives, plus more efficient rhamnolipid producing strains of bacteria is associated not only with economic aspects of their production, but also with new demands in analysis of rhamnolipid mixtures (Heyd *et al*., 2008). A large variety of analytical methods have been employed to identify and quantify the different rhamnolipid species and rhamnolipid production. These methods range from indirect analysis based on the physical properties of rhamnolipids (determination of surface tension and hemolytic test) (Siegmund and Wagner, 1991; Abdel-Mawgoud *et al.*, 2011), colorimetric measurement (cetytrimethyl ammonium bromide agar test, anthrone method and orcinol test) (Hodge and Hofreiter, 1962; Lindhardt *et al*., 1989; Siegmund and Wagner, 1991) to spectroscopic analysis of sample structure by infrared and nuclear magnetic resonance spectroscopy and a sophisticated analysis of composition by mass spectrometry (MS) (Haba *et al*., 2003; Nie *et al.*, 2010).

One current method for rhamnolipid identification and quantification is high performance liquid chromatography (HPLC) analysis coupled with electrospray ionization mass spectrometry (ESI-MS) (Deziel *et al.*, 1999; Benincasa *et al*., 2002; Haba *et al*., 2003; Heyd et al., 2008). Furthermore fragmentation of the pseudomolecular ion using tandem MS analysis of the parent ion is often performed in order to provide additional structural information, such as discrimination of isomeric pairs with subtile structure information variations (Deziel *et al*., 1999). Matrix assisted laser desorption ionization-time of flight MS (MALDI-TOF MS) approaches were recently developed for high throughput screening of naturally occurring mixtures of rhamnolipids from *Pseudomonas* spp. (Peypoux *et al*., 2009).

g). Tension active Properties of Rhamnolipids

Methods based on the tensionactive properties of rhamnolipid biosurfactant are typical for all surface active compounds and involve the calculation of the value of the hydrophilic lipophilic balance (HLB), the drop collapsing test, the oil spreading test, determination of the surface activity and the CMC values (Muller *et al*., 2012 ; Abdel-Mawgoud *et al*., 2011) as well as the determination of the interfacial tension and the wetting properties (Costa *et al*., 2006).

Determination of hydrophilic-lipophilic balance (HLB) value, which varies between O and 20, enables the prediction of the behavior of surface-active

compounds; O-3 antifoaming agents, 4-6 W/O (Water/Oil) emulsifiers, 7-9 wetting agents, 8 -18 O/W (oil/water) emulsifiers, 13-15 typical detergents and 10-18 solubilizers /hydrotropes. The HLB value can only be used as a preliminary guide, and further analysis by more exact and precise analytical techniques is required. According to Griffin (Griffin, 1954), the HLB can be calculated as HLB = 20x (MW_{HP}/MW_{SA}), where MW_{HP} is the molecular height of the hydrophilic part and MWSA is the molecular weight of the whole surface active agent (Muller et al., 2012).

The drop-collapsing test and oil spreading test are methods for rapid screening of rhamnolipid-producing bacterial strains (Abdel-Mawgoud *et al.*, 2011). Both tests are based on a similar approach, where the responses depend on the presence of rhamnolipid in culture supernatant. In the case of oil drop-collapsing test, a sample of supernatant is applied to a polystyrene plate containing shallow wells covered with oil (Jain *et al*., 1991), and spreads over the oil only if the sample of culture contains rhamnolipids. In the oil-spreading test, a drop of bacterial supernatant is added on top of an oil / water interface (Mendes *et al.*, 2015), where the presence of rhamnolipids causes the oil to be repelled, resulting in the formation of a clearing zone, the diameter of which can be correlated with the activity of the tensioactive compounds in the supernatant (Abdel-Mawgoud *et al.*, 2011).

A more precise method for determining the surfactant properties of rhamnolipids in bacterial culture supernatant or of isolated rhamnolipid biosurfactants is the direct measurement of surface activity and the determination of the CMC (specific to each surfactant), which is performed by measurement of the surface tension after sequential dilution of the solution (Abdel-Mawgoud *et al*., 2011). However, the determination of the CMC suffers the drawbacks that it is time consuming and not applicable for high-throughput screening (Abdel-Marwgoud *et al.*, 2011). Additionally, the results of these methods as well as of all the previous indirect tests based on surface tension could be affected by the potential presence of other tension active compounds.

One method to qualify solid-liquid adsorption is to measure the interfacial tension, which can be performed by tensiometry by contact angle goniometry (Costa *et al*., 2006). Surface tensions and contact angle measurements as a function of surfactant concentration are directly related to the difference in the adsorption of surfactants on solid vapor (S-V) and solid liquid (S-L) interfaces. Contact angle measurements also aid the elucidation of the nature of interactions between a surfactant molecule and a solid surface (Ozdemir and Malayoglu, 2004). Ozdemir and Malayoglu (2004) investigated the wetting behavior of a mixture of mono and di- rhamnolipid and SDS (Sodium dodeyl sulfate) molecules on glass, PET (Polyethylene terephthalate) and gold surfaces by measuring the advancing

contact angle, and elucidates the preferences of surfactant molecules adsorbed onto solid liquid (SL) – solid vapour (SV) and (Liquid-vapour) L-V) interfaces.

h). Production of Rhamnolipids

The high cost of production, isolation and purification of rhamnolipids, and the low-yield has determined that, despite 60 years of research in the area of rhamnolipid production, the economic feasibility of these glycolipids is still pending (Muller and Hausanann, 2011). Rhamnolipids from *P. aeruginosa* are officially produced by only a few companies in the USA (Marchant and Banat, 2012). Rhamnolipids are considered to be secondary metabolites and as such, their production coincides with the onset of the bacteria stationary phase (Maier and Soberon-Chavez, 2000).

Reported data indicate that the production of rhamnolipids is possible from simple carbon sources, such as glycerol and glucose, or complex carbon sources (Olive Oil, sunflower oil, crude oil) or wastes (Crude whey, distillery waste, molasses corn steep liquor, sunflower oil mill effluent, olive oil mill effluent (OOME), frying oil and minerals ($NaNO_{3,}$ NH_4Cl) or combined nitrogen source ($NaNO_3$ and yeast extract or NH_4Cl peptone) (Abdel Mawgoud *et al*., 2011; Rikalovic *et al.,* 2013). This indicates that economically viable levels of biosurfactant production could be achieved using renewable resources for the carbon source with special emphasis on the importance of the utilization of

industrial bye products and agricultural wastes as cost effective alternative substrates for microbial growth and biosurfactant production (Mercade *et al*., 1993; Haba *et al.*, 2003; Dubey and Juwarkar, 2001; Rahman *et al*., 2002; Maneerat, 2005; Dagbert *et al*., 2006 and Marchant and Banat., 2012).

The study of Dubey and Juwarkar (2001) is an example of rhamnolipids production from renewable sources, in which *P.aeruginosa* BS_2 was cultivated on whey for 48h, with an rhamnolipid yield of 0.92gl-1. In other studies, Mercade *et al*. (1993) cultivated *Pseudomonas sp* JAMM in a medium with OOME (100gl-1) and $NaNO_3$ (2.5gl-1), which resulted in an rhamnolipid yield of 14gkg-1 OOME after 150h of cultivation. Haba *et al*. (2003) using sunflower and Olive frying oil as carbon sources and $NaNO_3$ as the nitrogen source studied the production of biosurfactant by several *P. aeruginosa*, strains.

Nitrogen compound can significantly influence rhamnolipid production (Mulligan *et al*., 1989). The highest yields are obtained if nitrate is used rather than ammonium as a nitrogen source. The addition of ammonia (less than 5mm) with small concentrations of glutamic acid (170mm) in proteose peptone medium is beneficial for rhamnolipid production. In addition, there seemed to be a correlation between glutamine synthetase activity and rhamnolipid production. Maximum activity occurred as rhamnolipid production was initiated (Mulligan and Gibbs, 1989). Mata-sandoval *et al* (2001a) determined that production of rhamnolipids

increases when ammonium sulphate and trace metals were added throughout the fermentation process as opposed to only at the beginning. Glutamine, however, inhibits production. Matsufuji *et al*. (1997) compared numerous sources of nitrogen and demonstrated that highest yields were obtained at carbon to nitrogen ratios of 3:1 when ethanol was used for carbon and soy flour and yeast extract for nitrogen (3.7g/l). For the substrate, waste frying oil, a ratio of C: N of 8:1 gave the highest production (Haba *et al*., 2003).

Phosphorus sources also influence rhamnolipids yields (Mulligan *et al*., 1989). Limiting phosphate levels (65mm of Phosphate) provided superior results to a phosphate sufficient broth (310mm of Phosphate). Sources of phosphates are sodium phosphate or ammonium phosphates as mono and dibasic. Ammonium phosphates are the least expensive (Mulligan & Gibbs, 1989). Shifts in phosphate metabolism also led to biosurfactant production (Mulligan *et al*., 1989). Limiting calcium, iron, potassium, magnesium, sodium and other trace minerals also has yielded higher amounts of rhamnolipids (Guerra-santos *et al.*, 1986).

Two biosurfactant producing strains of *Pseudomonas aeruginosa* were isolated from a kuwash soil contaminated with oil by the Gulf war (Yateen *et al.*, 2002). Using olive oil as the carbon source, up to 98.4g/l of a rhamnolipid was produced.

Rhamnolipids are produced from both glucose and hydrocarbon substrates. They include alkanes pyruvate, citrate, fructose, glycerol, olive and corn oil, glucose and mannitol. Pilot studies have been performed on their production with final concentrations reaching 2.0g/l (Gurerra santos *et al.*, 1984). More recently Matsufuji *et al*. (1997) showed that ethanol is an excellent substrate that enables production of up to 3.6g/l of rhamnolipid. A comparison of various works performed by Mata-Sandoval *et al*. (2001a) showed that hydrophobic substrates such as corn oil, short and Long chain alcohols provided higher rhamnolipid yield (100-165mg/g substrate) than soluble substrates such as glucose and succinic acid (12 to 36mg/g substrate). Waste frying oils have also shown potential, yielding up to 0.34g of rhamnolipid in 1g of substrate (Haba *et al*., 2003). Most of the rhamnolipids in the biodegradation of hydrocarbons reports showed that rhamnolipids facilitate the uptake of hydrocarbons by *P.aeruginosa* (Beal and Betts, 2000; Noordman and Janssen, 2002; Nie *et al*., 2010).

Although some studies reported positive effects of the biodegradation of petroleum hydrocarbons in the presence of rhamnolipid biosurfactant, a lack of influence or even a negative effect of biosurfactant supplementation was observed just as frequently (Costa *et al*., 2006). Some reports indicated that the potential reason for inhibition of degradation is that rhamnolipids are favored as the carbon source for bacterial metabolism (Mulligan and Gibbs, 1989). Recently, it was

observed that the presence of rhamnolipids, or other surfactant compounds, which in turn corresponded to difference in the degradation patterns (Costa *et al*., 2006). Some earlier reports suggested mechanisms of hydrocarbon biodegradation facilitated by rhamnolipid, and assumed that rhamnolipids, due to their physicochemical properties, increased the hydrocarbon solubility and bioavailability cell, making the cell surface more hydrophilic and easily accessible to hydrophobic substrates (Maslin and Maier, 2000). On the other hand, some recently published studied proposed three mechanisms of interaction between micro organisms and hydrocarbons access to water solubilized hydrocarbons, direct contact of cells with large oil drops and contact with pseudo solubilized or emulsified (Franzetti *et al*., 2010) as well as combinations of these interaction (Chrzanowski *et al*., 2012). However regardless of whether the biodegration process is enhanced or inhibited the effects are bacterial strain specific in the sense of strain characteristic and response to environmental conditions (Zhang and Miller, 1994). Although much work was realized by many groups to explain the role of rhamnolipids and biosurfactants generally, in the degradation of water-immiscible substrate their significance and extract purposed in this process still remain unclear.

i. Flushing Agents for Organic Pollution

Biodegradation of NAPL and soil phase organics, such as PAH is often a slow and non-feasible process (Maier and Soberon-Chavez, 2000). The addition of surfactants to a flushing solution could enhance the flushing efficiency, either by mobilization or by an increase in the solubilization of these compounds (Maier and Soberon-Chavez, 2000). Thus, to be effective, a surfactant must have good solubilization capacity and or be able to reduce interfacial tensions. Rhamnolipids were shown to have an MSR for the model NAPL, hexadecane that was 20 times greater than the MSR for hexadecane alkyl benzyl sulfonate (Maier and Soberon-Chavez, 2000).

In studies examining the use of rhamnolipid for the removal of residual hexadecane from soil, it was shown that rhamnolipid (20% removal) was more effective than other SDS (negligible removal) or Tween 80 (6% removal). Additionally, it was shown the optimal removal of NAPL compounds (60%) could be achieved by altering the pH and ionic strength, thereby maximizing the reduction of the surface tension (Maier and Soberon-Chavez, 2000). Similar results were obtained for rhamnolipid solubilization of solid phase materials. For example, the MSR of rhamnolipid octadecane was ten and five times higher than the MSR for Tritom-X-114-octadecane and for Corexit 0600-octadecane, respectively (Maier and Soberon-Chavez, 2000).

Moreover, the MSR of rhamnolipid-phenanthrene was 1.7 to 2.8 times higher than for 13 different synthetic surfactants that were tested (Maier and Soberon-Chavez, 2000). Furthermore, in a comparison of the removal of a hydrocarbon mixture (Undecane, pentadecane, hexadecane, octadecane, pristane, naphthalene, phenanthrene and pyrene) from soil, rhamnolipids were more effective than Triton x-100 or Tween60 for all hydrocarbon components (Maier and Soberon-Chavez, 2000). Finally, rhamnolipid enhanced removal of phenanthrene, pyrene, and polychlorinated biphenyls and a variety of PAH from soil were reported (Maier and Soberon-Chavez, 2000).

j. Bioremediation of Heavy Metals.

Juwarkar *et al*. (2008) conducted column experiments to evaluate the potential of environmentally compatible rhamnolipids biosurfactants produced by *P*. aeruginosa BS2, to remove Cd and Pb from artificially contaminated soil. Results showed that di- rhamnolipid removed not only the leachable or available fraction of Cd and Pb, but also the bound metals, where as tap water revealed that only ≅ 2.7% of Cd and 9.8% of Pb in contaminated soil were able to be removed, rhamnolipid had removed 92% of Cd and 88% of Pb after 36h of leaching (Juwarkar *et al*., 2008). Wu *et al*. (2018) evaluated the feasibility of using rhamnolipid foam to remove Cd and Ni from a sandy soil. Application of rhamnolipids foam increased the efficiency and enabled the Removal of 73.2% and

68% of Cd and Ni, respectively, whereas the rhamnolipid solution alone flushed 61.7% and 51% of Cd and Ni respectively (Wu *et al*., 2018). Mulligan *et al*. (2001) designed batch washing experiments to evaluate the feasibility of using biosurfactants to remove heavy metals from sediments. Thus, surfactant from *Bacillus subtilis*, rhamnolipid from *P. aeruginosa*, and sophorolipid from *Torulopsis bombicola* were evaluated on sediment containing 110mgKg^{-1} of Cu and 3300mgkg^{-1} of Zn. A single washing with 0.5% rhamnolipid removed 65% of the copper and 18% of Cu and 60% of Zn (Mulligan *et al.,* 2001). Arino *et al.* (1996) studied the chromium (VI) tolerance of *P. aeruginosa* NCAIM (P) BOO1380 and showed that the strain was chromium tolerant and has potential for application in heavy metal bioremediation.

iv. Bioremediation of Co-contaminated Sites

Souza *et al.* (2014) studied the effectiveness of rhamnolipid biosurfactants in the remediation of a Cd and Naphthalene co-contaminated site. They observed that reduced cadmium toxicity as a result of the addition of *P. aeruginosa* rhamnolipid led to enhanced naphthalene biodegradation by *Burkholderia* sp. NCBI U37342 (Souza *et al*., 2014). These authors suggested that reduction of metal toxicity by rhamnolipid might involve a combination of rhamnolipid complexation with cadmium and rhamnolipid interaction with the cell surface to alter Cd uptake, resulting in enhanced rates of bioremediation. In another Co-contaminant study, it

was observed that the inhibition of phenanthrene mineralization in the presence of Cd was reduced by the pulsed addition of rhamnolipid (Maslin and Maier, 2000). Dahrazma and Mulligan (2007) reported higher rates of Cu and Ni removal from sediments by adding 1% NaOH to a solution of rhamnolipid. Efficient removal of Zn and Cu from co-contaminated soil with a 12.6% oil content Cu using rhamnolipid was also demonstrated (Mulligan *et al.*, 1999).

b. Food Industry

Some properties of rhamnolipids, such as emulsion formulation and stabilization, as well as anti-adhesive and antimicrobial activity, make them interesting for the food industry as multipurpose ingredients (Nitschke and Costa, 2007). Apart from their role as surface actives agents, there are reports that rhamnolipids could have several other functions in food industry (Muthusamy *et al*., 2008 and Kosaric, 2001). Some examples are an improvement of dough stability, texture, volume and conservation of bakery products obtained by the addition of rhamnolipids surfactants (Van Haesendonck and Vanzeveren, 2004), while some other authors suggested the use rhamnolipids for improvement of properties of butter cream, croissants and frozen confectionery products (Kozaric, 2001).

Finally, rhamnolipid serve as a source of L-rhamnose, a compound used commercially in the production of high quality flavor compounds. L-Rhamnose is

a methyl pentose natural sugar, classified as one of the rare sugars, and is found in several animal, plant and bacterial polysaccharides, as well as rhamnolipids. This compound was already successfully obtained by hydrolyzing rhamnolipid surfactants produced by *P. aeruginosa* (Linhardt *et al.*, 1989). L-Rhamnose is a sugar that the food and Drug Administration (FDA) has approved as a food additive and hence, it has found its use in the flavor industry as a precursor for the production of 2, 5-dimethyl-4-hydroxy-3(2H) - furanone, the high quality flavor aroma furaneo (trademark of firmenich SA, Geneva) (Muthusamy *et al*. (2008), which resembles strawberry and raspherry. It is also the starting raw materials in the reaction flavors developed during the preparation of various foods, such as bread, grilled meats, etc. thus there is a great deal of interest in obtaining commercial quantities of rhamnolipids to provide a source of L-rhamnose, which already has the above mentioned application in the food industry.

c) Surface Conductioning

Bacterial biofilms present on surfaces in the food industry are potential sources of contamination, which may lead to food spoilage and disease transmission and this, controlling the adherence of micro-organisms to food contact surfaces is an essential step in providing safe and quality products to consumers (Hodge and Hofreiter, 1962). The promising results from studies of the disruption of *Bordetella bronchiseptica* biofilm of rhamnolipid (Irie *et al*., 2005)

and reduction of adhesion of *Streptococcus Salivarius* and *C. tropicalis* by rhamnolipid (Rodrigues *et al.*, 2006) suggested a potential application of rhamnolipids for surface conditioning in the food industry. Moreover, studies by Meylheuc *et al*. (2001, 2006) showed inhibition of the adhesion of the pathogen *Listens monocytogenes* to two types of surfaces typically used in the food industry using biosurfactant obtained from *P. Fluorescens*, while Dagbert *et al.* (2006) showed that the surfactant produced by *P. Fluorescens* also has good potential as a acorrosion inhibitor.

d). Cosmetic and Pharmacy Industries

Cosmetic surfactants perform detergency, wetting, emulsifying, solubilizing, dispersing and foaming effects (Lourith and Kanlayavattanakul, 2009). Biodegradability, low toxicity and ecological acceptability, which at the same time, are the benefits of a natural derived surfactant that promises cosmetic safely, are, therefore in high demand. In particular application of rhamnolipids in the field of cosmetics and pharmaceuticals as emulsifiers, penetrating agents and drug delivery systems is an emerging area of research (Piljac and Piljac, 1995).

Due to their skin compatibility and low irritancy rhamnolipids are used as additives to cosmetics by Iwata Co. of Japan (Maier and Soberon-Chavez, 2000). Ishigami *et al*. (1988) have patents for the use of rhamnolipid in liposomes and emulsions for the cosmetic industry.

Rhamnolipids are used in health care products in several different formulations (Lourith and Kanlayavaltanakul, 2009), for example, in insect repellents antacids, acne pads, anti-dandruff products, contact lens solutions, deodorants, hail care products and toothpastes (Kiran *et al*., 2010; Maier and Soberon-Chavez, 2000). These formulations require surfactants with surface activity and in particular, emulsifying activities (Vasilera-Tonkova *et al*. 2001), which is the essence of the texture consistency of these products (Haba *et al*., 2003). Furthermore, requirements for the biological activities for cosmetics should expand the application of rhamnolipids, and a delivery system has been achieved, not only for emulsion but also for liposomes (Ishigani *et al*. 1988). Patents for cosmetics containing rhamnolipids have been granted for anti-wrinkle and anti aging products (Piljac and Piljac, 1995) which were launched in several dosage forms as commercial skin care cosmetics (Desai and Banat, 1997) because of their skin compatibility and extremely low skin irritation (Haba *et al*.,2003).

e). Biomedicine

Early on, the wide ranging antimicrobial properties of rhamnolipids were noted, interestingly, they were shown to be active against a large variety of bacteria including both Gram-negative and Gram-positive species (Abdel-Mawgoud *et al*., 2010). In several studies, the antimicrobial properties of mixtures of rhamnolipid congeners produced by three different strains of P. aeruginosa were investigated

(Abalos *et al*., 2001; Haba *et al*., 2003; Benincasa *et al*., 2002). The various rhamnolipid combinations displayed antimicrobial ability against nearly all the tested Gram-positive species, including *Staphylococus*, *Mycobacterium* and *Bacillus* and significant activity against a number of Gram-Negative species, with *Serratia marcescens*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* being especially sensitive (Abdel-Mawgoud *et al*., 2011)

Rhamnolipid were also show to affect cellular immuno-suppression (Piljac and Piljac, 1995) and wound healing, treatment and prevention of gum disease and periodontal regeneration (Santa Anna *et al*., 2002) and to display differential effects on human Keratinocytes and fibroblast cultures (Stipceric *et al.*, 2001). Moreover, Piljac *et al*. (2008) reported the successful treatment of decubitus ulcer with an ointment containing 0.1% of a di- rhamnolipid.

Tahzibi *et al*. (2004) tested the cytotoxic activity of a crude rhamnolipid extract, Rha-Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{12}, produced by *P. aeruginosa* B189 isolated from a milk factory, against herpes simplex virus, insect and cancer cell lines. Rha-Rha-C_{10}-C_{10} exhibited significant inhibition of growth of human breast cancer cells line (MCF-7), with minimum inhibitory concentration (MIC) of 6.25μgml^{-1}, Rha-Rha-C_{10}-C_{12} had on MIC of 50μgml-1 against insect cell line C6/36, while the crude rhamnolipid extract shared no cytotoxic activity (Tahzibi *et al*., 2004). The potential mechanism of activity, regarding the structure of the

biosurfactant, is a toxicological effect on the cell membrane permeability. Furthermore, Rha-Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{12} has no inhibition effect on the normal cell line (view cell) at concentrations upto 50μgml^{-1}. This confirmed the specific toxicity of their compounds to the cell lines used. However, the inhibitory mechanisms against these cell lines are as yet unknown and are under investigation (Tahzibi *et al*., 2004).

f). Agronomy

Rhamnolipids also showed the ability to control certain zoosporic plant pathogens, including *Phytophihora cryptogea* and *Pythium spp* (De Jonghe *et al*., 2005; Varnier *et al*., 2009). Purified mono- and di- rhamnolipids, in concentrations ranging from 5 to 30$mgl^{-1,}$ caused cessation of motility and lysis of the entire zoosporic population in less than I minute (Maier and Soberon-Chavez, 2000). This observation led to the development of a rhamnolipid-containing biofungicide formulation, used to prevent crop contamination by pathogenic fungi (Nitschke and Costa, 2011). This product is considered to be non-mutagenic and of low acute toxicity to mammals. It was approved by the FDA for direct use on vegetables, legumes and fruit crops (Nitschke and Costa, 2007). Dorey *et al*. (2007) reported the role of rhamnolipids in triggering defense responses and protection against the fungus *Botrytis cinerea* in grapevines. The authors showed that rhamnolipids inhibited spore germination and mycelium growth, these efficiently protecting

grapevines against the fungus by inducing the plant defense system. A product based on an aqueous rhamnolipid solution (0.01%) was claimed to act as a novel agent for stimulating the natural defense reactions of plants against pathogenic fungi (Dorey *et al*., 2007; Nitschke and Costa, 2011).

Control of the zoospore plant pathogen *Olpidium brassicae* has been accomplished by the addition of rhamnolipids when the pathogen is in the zoospore stage with no cell wall (Tan, 2000).

Rhamnolipids (5 to 3mg/l) have also been able to control the plant pathogens *Pythium aphanadermatum, Phytophythora capsici,* and *Plasmopara lactucaeradicis* but not at sustained levels (Sarubbo *et al*., 2006).

g). Formulation of cleaners and Wetting Agents

One of the major commercial domestic applications of biosurfactants is in the field of cleaning and laundry products. The interfacial chemistry created have absorbed surface-active molecular, of either biological or chemical origin, dominates the end-use properties of materials in many different applications (Ozdemir and Malayoglu , 2004). The properties and efficacy of detergent formulations are crucially dependent on the interfacial activity of various surfactants, which are present in their composition (Ozdemir and Malayoglu, 2004). At present the liquids and powders generally contain alkyl sulfonates, such as linear alkylbenzene sulfonates, but the glycolipid biosurfactants and among

them rhamnolipids, produced by *P. aeruginosa*, are possible candidates to be used for the, at least, partial replacements of these synthetic compounds (Marchant and Banat, 2012).

Batghi and Fazaelipoor (2008) investigated rhamnolipids in the formulation of a washing powder. The results showed that the biosurfactant was effective in removal of oil from the samples. The formulation presented in this study was also compared with some commercial powders for the removal of edible oil, chocolate and albumen stains, the result showed that the rhamnolipid – inclusive formulation was comparable to the commercial powders in terms of stain removal, biodegradability tests performed on pure rhamnolipid and the rhamnolipid-inclusive formulation confirmed the good biodegradability of this biosurfactant.

Ozdemir and Malayoglu (2004) investigated the welting behavior of a mixture of mono-and di- rhamnolipid (in 1:1 ratio of mono: di- rhamnolipid) on glass, PET and gold surfaces by measuring the advancing contest angle, and elucidated the preferences of the surfactant molecules adsorbed onto SL-SV and L-V interfaces, with SDS as the reference surfactant. The study showed that at low concentrations of rhamnolipid and reference surfactant, the contact angle varied in a certain range depending on the character of the surfactant interactions with the surface (Ozdemir and Malayoglu, 2004).

Costa *et al*. (2006) also studied the wetting behavior of rhamnolipids produced by *P.aeruginosa* LBI grown on a waste oil substrate, and the chemical surfactant SDS, on glass, PET, Poly (Vingly Chloride) (PVC), Poly (E-caprolactone) (PCL) and a polymer blend (PVC-PCL) by measuring the contact angle of sessile drops. The comparison of the wetting profiles shared dynamic changes in the contact angle at low SDS and RL concentrations – the contact angle increased and when the concentration of the surfactant increase further, the contact angle decreased (Costa *et al.*, 2006). The results showed that rhamnolipids produced by *P. aeruginosa* LBI exhibited superior wetting abilities compared to SDS. This is the first work that evaluated the wetting properties of rhamnolipids on polymer blends.

h). Bio-and Nano-technology

Biosurfactants have been increasingly attracting attention in the field of nanotechnology as a "green alternative for high performance nano materials (Fracchia *et al.,* 2012). During the last decade, unique properties of biosurfactants, including versatile self assembling and biochemical properties, which do not usually occur among chemically derived surfactants, were studied and analyzed (Kitamoto *et al*., 2005; Kitamoto *et al*., 2009; Nguyen and Sabatini, 2009). Rhamnolipids alone or in combination with other glycolipid biosurfactants, have

potential roles as systems for drug delivery, synthesis of nanoparticles and formulation of microemulsions.

i). Drug Delivery Systems

In 1988, rhamnolipids liposomes were patented as drug delivery systems, useful as microcapsules for drugs, proteins, nucleic acids, dyes and other compounds as microcapsules for drugs, proteins, nucleic acids, dyes and other compounds, as biomimetic models for biological membranes and as sensors for detecting pH variations (Fracchia *et al.*, 2012). These novel liposomes were described as safe and biologically decomposable, with suitable affinity for biologically organisms, stable and with long service and shelf life (Fracchia *et al*., 2012). Recently, in a study of Sharma and Pant (2001), rhamnolipids and sophorolipids were mixed with lecithins to prepare biocompatible microemulsions in which the phase behavior was unaffected by changes in temperature and electrolyte concentration making them desirable for cosmetic and drug delivery applications (Fracchia *et al.,* 2012).

J). Synthesis of Nanoparticles

Another interesting aspect of the applications of rhamnolipid is the synthesis of metal nanoparticles as an alternative (a more ecological technology) to traditional methods of production (Naryanon *et al*., 2010). There are several reports with rhamnolipid applications in this field. Kim *et al*. (1999) synthesized

silver nanoparticles using purified rhamnolipids from *P. aeruginosa* BS-161R, which showed a broad spectrum of antimicrobial activity. Xie *et al.* (2006) successfully synthesized silver rhamnolipids in rhamnolipid reverse micelles. Palanisamy and Raichur (2009) demonstrated a simple and eco-friendly method for synthesis of spherical nickel oxide nanoparticles by a microemulsion technique using rhamnolipids as an alternative surfactant.

In two recent studies, rhamnolipids biosurfactants were used as capping agents for the synthesis of Zns nanoparticles (Narayanon *et al.,* 2010, Hazra *et al*., 2013). Narayanan *et al*. (2010) demonstrated a novel method for the synthesis that rhamnolipid biosurfactant has potential as an effective capping agent for the synthesis of uniform nanoparticles, Hazra *et al*. (2013) reported a facile eco-friendly procedure for biosynthesis of rhamnolipid capped Zns nanparticles, their structural characterization, biocompatibility, cytotoxicity assessment and their applicability as a nanophotocatalyst for the degradation for a textile azo dye. The results obtained explained the importance of environmentally friendly rhamnolipids as an effective and inexpensive capping and stabilizing agent for the development of stable and biocompatible Zns nanoparticles as nanophotocatalysts in the textile industry and for waste water and effluent treatment (Hazra *et al*., 2013).

iii. Microemulsions

Xie *et al*. (2005) showed that rhamnolipids have potential for application in the formulation of microemulsions with medium chain alcohols as co-surfactants. Further, the same authors observed that the phase behavior and micro structure of their micro-emulsions were related to the conformational changes of the rhamnolipids molecules at the surfactant to synthesize spherical nickel oxide nanoparticles by a micro-emulsion technique (Palanisamy & Raichur, 2009).

Nguyen and Sabatini (2009) focused their research on developing alcohol-free biosurfactant based microemulsions rhamnolipids based mixtures were found to have doubled the solubilization parameter as compared to sodium bis (2-ethyl-hexyl) sulfosyccinate/sodium dihexyl sulfosuccinate / sodium mono-and di-methylnaphthalene sulfonate at the same concentration (Palanisamy and Raichur, 2009). Additionally these authors developed a phase diagram for surfactant mixtures containing methyl ester ethoxylate, rhamnolipids and oleyl alcohol with limonene oil, which could be used as a guideline for selecting a surfactant system and surfactant ratio to formulate microemulsions with given oil. The rhamnolipids biosurfactant used by Nguyen and Sabatini (2009) was the least hydrophobic type (fatty acid facts of C_8 chain length since its fall length was the shortest within the typical range, the real length usually varies from C_8 to C_{14}). Nguyen and Sabatini (2009) formulated and evaluated microemulsions of lecithin / RL/sophorolipid biosurfactants with a range of oils.

2.5. Screening of Biosurfactant Producing Bacteria

Recent advances in the field of microbial surfactants are largely attributed to the development of quick, reliable, easy and rapid method to screen biosurfactant producing bacteria with a minimum number of false positive and/or negative. Biosurfactant production is always detected by measuring cell surface hydrophobicity (Pruthi and Cameotra, 1997), hemolytic activity (Yarteen *et al.*, 2002) and the surface activity (Desai and Banat, 1997).

a). Cell Hydrophobicity Test

Hydrophobicity of the cell surface is an important factor in predicting bacterial cell adhesion to surfaces. The hydrophobic nature of the outermost surface of the microbial cells could be used to measure the potential cell affinity to the hydrophobic substrates. Correlations have been found between the adherence of bacteria to hydrocarbons and the attachment of other surfaces including non-wettable solid-surfaces, epithelial cells, teeth (Rosenberg, 1984) and partitioning of bacteria at liquid and liquid air interfaces (Rosenberg and Ron, 1999). Pruthi and Cameotra, 1997) found a direct correlation between cell hydrophobicity and biosurfactant production. Neu and Poralla (1990) used this property to screen for biosurfactant production based on the fact that hydrophobic surfaces are usually associated with molecules that has low surface energy (Youssef *et al*., 2004).

b). Drop Collapsing Technique

Biosurfactants produce surface active agent that contains both hydrophobic and hydrophilic groups. Due to their amphipathic nature, surfactants are not uniformly distributed in the solvent but congregate at solvent surface (Jain *et al.*, 1991). Thus, availability of hydrocarbon and slightly soluble organic compounds can be enhanced by biosurfactants, which can increase dispersion by many orders of magnitude and reduce the surface and interfacial tensions of aqueous medium.

There are two types of intermolecular alterative forces which occurred to molecule lipid (Rosenberg, 1984) cohesive forces are referred to when these forces occur between like molecules. When this cohesive forces at the surface are strong enough, the molecules of water droplet are held together to contribute to surface tension. Both of the attractive forces between molecules in a lipid can be viewed as residual electrostatic forces and this is called Van der Waals forces (Guerra-Santos *et al*., 1984).

A drop collapsing technique has been defined as a qualitative assay to screen biosurfactant producing bacteria. Solutions containing potent biosurfactant will be unable to form stable drops and spread completely over the oily surface, while solutions without surfactant will retain the drop configuration on the oily surface (Jain *et al*., 1991). This method is simple, sensitive, easy to perform, reproducible and requires little specialized equipment (Bodour *et al*., 2003). However, this

technique is not correlated surface tension reduction to confirm its reliability (Youssef *et al.*, 2004).

c). Hemolytic Activity

Hemolysis on blood agar has been widely used to screen biosurfactant producing bacteria and for preliminary identification of many types of clinically important bacterial (Mulligan et al., 1984). Blood agar is purposely used as an enriched medium for growing of fastidious bacteria and as a differential medium. This technique was first discovered by Bohinski (1991).

Hemolytic activity has been used previously to quantify surfactants (Mendes *et al.*, 2015) and rhamnolipids (Juwarkar et al., 2008). Nowadays, many researchers have used this technique to screen for biosurfactant production by new isolated (Yanteen *et al.*, 2002). Hemolytic reactions are generally classified as alpha, beta or gamma according to the appearance of zones around the isolated colonies growing on blood agar (Yanteen *et al*., 2002).

Beta hemolysis indicates a zone of clearing in the blood agar in the area surrounding a bacteria colony. Few or intact of erythrocytes are found. One or more erythrocytes lysing enzymes (hemolysis) caused this type of hemolysis, which completely lyse the red blood cells and with clear zone of colonies. If there is no change in the medium around the colony (no hemolysis) on the blood agar, the reaction is gamma hemolysis.

d). Surface Tension Reduction

Surface tension is a phenomenon involving the cohesive forces between liquid molecules. The molecules at the surface have no neighboring atoms and adhere more strongly to those directly associated with them on the surface (Guerra-Santos *et al*., 1984). This would enhance the intermolecular attractive forces at the surface which makes it more difficult to more liquid molecules when it is completely submerged (Guerra-Santos *et al*., 1984).

The phenomenon of surface tension also can be explained in terms of energy. Surface tension is a measurement of the surface free energy or unit area required to bring a molecule from the bulk phase to the surface (Rosen, 1978).

The larger the surface, the more energy there is. Thus to minimize the energy, most fluids assume the shape with smallest surface area. This is the reason why small drops of water are sphere in shape with minimum surface area for a given volume.

Surface tensions can be defined as;

Surface tension, $Y = \frac{w}{DA}$; where DA is the surface area

It can also be defined as the force, f per unit length, L tending to pull the surface back (Rosen, 1978).

Surface tension, y =f/l; f per length.

Thus, surface tension is measurement of the inter-molecule attractive forces, which is Van der Waals force in a given liquid. The molecules on the surface of the liquid

experience these forces differently to the air than to the liquid. By introducing a substrate into the surface, one with a zero contact angle with the liquid, all of the inter-molecular forces will pull down on the substrate, thus making the surface tension directly proportional to the balance force of a balance connected to the substrate (Tan, 2000).

The association between surfactants and phases of different polarity like water and air-water cause reduction in surface tension. One of the factors that can cause the reduction of surface tension is the presence of microbial surfactants. Biosurfactant is defined as one that can reduce the surface and interfacial tension of aqueous medium. A good biosurfactant producer was defined as one being able to reduce the surface tension of the growth medium of 20mN/m compared with distilled water (Wu *et al*., 2018). The measurement of surface tension has traditionally been used to detect biosurfactant production.

The du Novy Ring Method.

This method measures the force required to pull platinum wire ring through the liquid-air or liquid-liquid interface. It is widely used because of its accuracy, easy to use and it provides a fairly rapid measurement of surface and interfacial tension.

e). Emulsification Measurement

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, and hydrophilic – lipophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer (Rosenberg and Ron, 1999).

The surface tension of distilled water is 72mN/m, and addition of surfactants lowers their value to 30mN/m. when a surfactant is added to air/water or oil/water system at increasing concentrations, a reduction of surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supra molecular structures like micelles, bi-layers and vesicle. This value is known as the critical micelle concentration (CMC) (Mulligan *et al*., 1989).

f). Critical Micelle Concentration (CMC)

Micellization was an important phenomenon in surfactant chemistry, because it affects many interfacial phenomena such as surface and interfacial tension reduction. It is a characteristic property of a biosurfactant. In aqueous solution, biosurfactant tends to form aggregate colloidal sized clusters known as micelles. At very low concentrations, individual molecules are present singly. As the biosurfactant concentration increased, a point called the critical micelle concentration (CMC) is reached (Mulligan *et al*., 2001).

CMC is defined as the lowest concentration of the surfactant required to initiate micelle formation (Mulligan *et al*., 1989). It was proportional to the amount of surfactant present and was generally used to measure the efficiency of a surfactant. These will be no further decrease in surface tension after this point although more surfactant is present in the medium. At the CMC, sudden changes in surface tension, electrical conductivity, detergency, viscosity, density and Osmotic pressure could be observed (Margantis *et al*., 1979; Kim *et al*., 2000).

h). Gas Chromatography

Chromatography is the science of separation as which uses a diverse group of methods to separate closely related components of complex mixtures. During gas chromatographic separation, the sample is transported via an inert gas called the mobile phase. The mobile phase carries the sample through a coiled tubular column where analytes interact with a material called the stationary phase must have an affinity for the analytes in the sample mixture. The mobile phase, in contrast with the stationary phase, is inert and does not interact chemically with the analytes. The only function of the mobile phase is to sweep the analyte mixture through the length of the column.

Gas chromatography is the most widely used chromatographic techniques for environmental analysis, and is used on site in filled investigations and by off-site reference laboratories.

i). Agar Plate Method

Agar plate method helps in the detection of extracellular rhamnolipid (a group of anionic biosurfactant). These biosurfactants form an insoluble ion pair with the cationic tenside cetyltrimethylammonium bromide and the basic dye methylene blue which are included in mineral agar plates (Siegmund and Wagner, 1991).

The method enables to indicate rhamnolipid producing colonies by colour reaction. On the light blue colored plates, colonies producing extracellular anionic biosurfactants are surrounded by dark blue halos.

2.6. Factors Affecting Biosurfactant Production

The production of biosurfactants can be either spontaneous or induced by the presence of lipophilic compounds, variations in pH, temperature, aeration and agitation speed or when cell growth is maintained under conditions of stress, such as a low concentration of nitrogen (Desai and Banat, 1997). The various physicochemical factors are discussed below (Barros *et al.,* 2008);

a) Carbon Sources:

The carbon source plays an important role in the growth and production of biosurfactants by micro-organisms and varies from species to species. A very low yield was found when only either glucose or vegetable oil was used for the production of a biosurfactant by *C. bombicola*, but the yield increased to 70g/l when both carbon sources were provided together (Costa *et al*., 2006). At a

concentration of 80 and 40g/l of glucose and soybean oil, respectively, the maximum yield of sophorose lipids was obtained by *C. bombicola* (Kim *et al*., 2000). Even higher yields of sophorose lipids (120g/l) were produced with *C. bombicola* in eight days when sugar and oil were used as carbon sources (Campos *et al*., 2013).

When canola oil and glucose were used as carbon sources at concentrations of 10% each, maximum yield of sophorolipids (5g/l was obtained from *C. lipolytica* (Sarubbo *et al.,* 2007). Moreover, when industrial waste was used for the production of a biosurfactant by *C. lipolytica*, the yield of the protein-lipid-carbohydrate complex was 4.5g/l, with a reduction in the surface tension of distilled water from 71 to 32mN/m (Rahman *et al*., 2002). A high production of bioemulisifer was obtained with *C. lipolytica* when supplemental with 1.5% glucose (w/v) (Sarubbo *et al*., 2007). *C. antarctica* and *C. apicola* yielded 13.4 and 7.3g/l of sophorolipids, respectively when soapstock was used at a concentration of 5% (v/v) (Bohnisk, 1991). The resting cells of *Pseudozymna* (*C. antractica*) were found to convert C_{12} to C_{18}n –alkanes into mannosylerythritol lipids (MEL), the yield was 140g/l after four weeks and the biosurfactant was able to emulsify soya bean oil (Kitamoto *et al*., 2005). A change in the fatty acid constitution of the final biosurfactant occurred when the fatty acid composition was changed in the fermentation medium containing *C. ingens* (Amezcua-veja *et al*., 2001).

Syldatk and Wagner, (1985) demonstrated that although different carbon sources in the medium affected the composition of biosurfactant production *Pseudomonas spp*. substrates with different chain lengths exhibited no effect on the chain lengths of fatty acid moieties in glycolipids. Mounting evidence leads to the conclusion that the available carbon source, particularly the carbohydrate used, has a great bearing on the type of biosurfactant produced (Syldak and Wagner, 1985).

b). Nitrogen Sources:

This is the second most important supplement for the production of biosurfactants by micro-organisms. In fermentative processes, the C/N ration affects the build-up of metabolites. High C/N ratio (i.e., low nitrogen levels) limit bacteria growth, favoring cell contrast, excessive nitrogen leads to the synthesis of cellular material and limits the build up of products (Kiram et al., 2009). Different organic and inorganic nitrogen sources have been used in the production of biosurfactants. Aparna *et al*. (2011) described the important by *P. aeruginosa* cultivated in a mineral medium containing 3% glycerol. As $NaNO_3$ proved more effective than $(NH_4)_2SO_4$, nutritional limitations clearly guide the cell metabolism to the formation of the product. Mulligan and Gibbs (1989) report that *P. aeruginosa* used nitrates, ammonium and amino acids as nitrogen sources. Nitrates are first reduced to nitrite and ammonium. Ammonium is assimilated either by glutamate dehydrogenase (EC 1.4.1.4) to form glutamate or glutamine synthetase

(EC 6.3.1.2) to form glutamine. Glutamine and α-ketoglutarate are then converted to glutamine by l-glutamine-2-oxoglutarate aminotransferase (EC1.4.1.13).

However, lipid formation rather than sugar is the rate determining factor in the biosynthesis of rhaminolipids and nitrogen limitation can lead to the accumulation of lipids. In comparison to ammonium, the assimilation of nitrate is slower and stimulates nitrogen limitation, which is favourable to the production of rhamnolipids. High yields of sophorose lipids, which are biosurfactants produced by the fungi *T. bombicola* and *C. bombicola*, have been achieved using yeast extract and urea as the nitrogen source (Des hpande and Daniels, 1995). Moreover, high yields of mannosylerythritol lipids by *Candida* sp. Sy16, *C. lipolytica* and *C. glabrata* have been achieved with ammonium nitrate and yeast extract (Kitamoto *et al.,* 2009; Kim *et al.,* 2000; Sarubbo *et al*., 2006; Rufino *et al.,* 2001; Rufino *et al.,* 2008).

The nitrogen source in medium also has a great effect on the production of biosurfactants. They may also contribute to pH control. Organic nitrogen sources include gluten meal, yeast hydrolysates and corn germ, whereas in-organic nitrogen sources include ammonium nitrate, ammonium sulphate, and so on. Among the inorganic salts tested, ammonium salts and urea were preferred for biosurfactant production by *Arthrobacter paraffineus* whereas nitrate supported maximum biosurfactant production by *Arthrobacter paraffineus* whereas nitrate

supported maximum biosurfactant production in *Pseudomonas aeruginosa* (Desai and Banat, 1997). Syldalk and Wagner, (1985) showed that nitrogen limitation not only causes over production of biosurfactant but also changed the composition of the biosurfactant produced.

c). Growth Conditions

Growth conditions (temperature; pH, agitation speed, availability of oxygen, salt concentration) also influence biosurfactant production (Desai and Banat, 1997).

i). pH

The pH plays an important role in sophorolipid production by *T. bombicola* (Gorna *et al*., 2011). Rhamnolipid production in *Pseudomonas spp*. was at its maxinium at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Gorna *et al*., 2011). In contrast, Persson *et al*. (1998) showed that pente- and disaccharide lipid production in *N. corynebacteriariodes* is unaffected in the pH range of 6.5 to 8.

In addition, surface tension and CMCs of a biosurfactant product remained stable over a wide range of pH values, where as emulsification had a narrowed pH range (Abu-Ruwaida *et al*., 1991). Species of the genus *Candida* produce maximum biosurfactant yields in a wide pH range, such as pH 5.7 for *C. glabrata* UCP 1002, pH 7.8 for *Candida* sp. 5416, pH 5.0 for *C. lipoplytica* and pH 6.0 for C. batistae (Kim *et al*., 1999; Cirigliano and Carman, 1984; Sarubbo *et al*., 2007; Konishi *et al*., 2008).

Moreover, *Pichae anamola* and *Aspergillus ustus* produce maximum biosurfactant yield at pH 5.5 and 7.0, respectively (Thaniyavarn *et al*., 2008 and Kiram *et al*., 2009).

J). Temperature

Temperature may cause attraction in the composition of the biosurfactant produced by *Pseudomonas Sp*. DMS-2878 (Syldatk and Wagner, 1985).

A thermophilic *Bacillus* sp grew and produced biosurfactant at temperature above 40^0C (Banat, 1993). However, heat treatment of some biosurfactant properties such as lowering of surface tension and interfacilal tensions and emulsification efficiency, all of which remained stable after autoclaving at 120^0C for 15 minutes (Abu-Ruwada *et al*., 1991). Different microbial processes are affected by even a small change in temperature. The most favourable temperature for the production of biosurfactants by different fungi is 30^0C, as observed for different species of *Candida* viz. *Candida* sp.

Sy16, *C. bombicola*, *C. bastistae* and *T. bombicola* (Deshpande and Daniels, 1995; Kim *et al*., 1999 and Konishi *et al*., 2008). In case of *C. Lipolytica*, 27^0C has been found to be the best temperature. Incubation time also exerts a significant effect on biosurfactant production. Micro-organisms prduce biosurfactants in different time intervals. Maximum biosurfactant production by *Aspergillus ustus* was found after five days of incubation, whereas the incubation periods for *C.*

bombicola were seven, eight and 11 days (Calvo *et al*., 2009; Felse *et al.,* 2007). Maximum biosurfactant production by *C. bombicola* grown in animal fat was found after 68h of incubation (Silva *et al*., 2010).

K). Agitation

An increase in agitation speed due to the sheer effect results by Nocardia erythropolis (Margartis *et al.*, 1979; Mulligan and Gibbs, 1993). While studying the mechanism of biosurfactant production in *A. calcoaceticus* RAG-1, Wu *et al*., (2013) revealed that the cell-bound polymer / dry-cell ratio decreases as the sheer stress increases. On the other hand, in yeast, biosurfactant production increased when the agitation and aeration rates are increased (Sydak and Wagner, 1985). Moreover, an increase in agitation speed favoured the accumulation of a biosurfactant by *P*. *aeruginosa*, UCP 0992 grown in glycerol (Silva *et al*., 2010). Oliveira *et al*. (2009) studied the effect of a change in agitation speed of cultures from 50 to 200rpm on *P. alcaligenes* cultivated in palm oil. The authors found that the increase in rotation velocity favoured a reduction in the surface tension of the cell free broth to 27.6mN/m. In contrast, Carrillo *et al*. (1996) found that agitation had a negative effect regarding a reduction in surface tension using a biosurfactant from *Serratia sp*. SVGG16 grown in a hydrocarbon culture.

L). Availability of Oxygen

Syldak and Wagner, (1985) have recently concluded that oxygen transfer is one of the key parameters for the process optimization and scale up of surfactant production in *B. subtilis*.

M). Salt Concentration

Salt concentrations also affect biosurfactant production depending on its effect on cellular activity. Some biosurfactants were not affected by salt concentrations up to 10% (w/v) although slightly reductions in the CMCs were detected (Abu – Ruwaida *et al*., 1991).

2.7. Recovery of Biosurfactants

The production of low-cost biosurfactant is unlikely due to the complicated recovery process development is conducted in order to obtain biosurfatants that can be recovered easily and in expensively. In biotechnological processes downstream processing accounts for 70%-80% of production costs. For economic reasons most biosurfactant production processes need to involve spent whole cell culture broths or other crude preparations(Helmy *et al*.,2011, Marchant and Banat, 2012).extraction with chloroform- methanol, dichloro methane- methanol butanol, ethyl acetate,pentane, hexane, acetic acid ether, etc, constitutes the most Commonly used method in biosurfactant downstream processing. The most

widely employed products are different ratios of chloroform and methanol which facilitate the adjustment of the extraction agent to the extractable target material. The disadvantages of using organic solvents for biosurfactant recovery include the large amount of solvent required and the increase in production costs due to the price of expensive solvents. Chloroform is a toxic chloro-organic compound that is harmful to human health and the environment. Thus, there is a need for in expensive solvents with low toxicity.

2.8. Industrial application of Biosurfactants

Biosurfactants can be used for oil residue recovery from storage tanks, other oil recovery processes, and the clean-up of oil-spills, bioremediation of both soil and water, removal of hydrophobic organic pollutants, removal of heavy metals, food industry, medicine and nanotechnology (Souza *et al*., 2014).

2. 9. Microbial Enhanced Crude Oil Recovery

Crude oil exists in the small pores and in the narrow fissures and interstics within the body of the reservoir rocks underneath the surface of the earth. The nature pressure of the reservoir causes the oil to flow-up to the surface and provide the so-called primary production, which depends upon the internal energy and characteristics of the reservoir rock and the properties of the hydrocarbon fluids. In some reservoirs, which are the part of a much larger aquifers system, a natural

flow of underground waters may be the drive force (aquifer drive) to push and displace oil. The initial reservoir pressure is usually high enough to lift the oil up to the surface; however as the oil production progresses, the reservoir pressure is continually depleted to a point in which artificial lift or pumping is required to maintain an economical oil production rate, which is termed secondary recovery. The extra energy can be introduced by injecting gas (gas injection) and / or water (water flooding) (Souza *et al*., 2014).

Gas injection is usually only applied to reservoirs which have a gas cap where gas drive would be an efficient displacement mechanism. In water flooding, which nowadays is one of the most common methods of oil recovery, keeps the reservoir pressure around the bubble point, thus preventing the pores to be blocked by dissolved gases. After some years of operation in a field, due to the reservoir heterogeneity, the injected fluids (water or gas) flow preferentially along high permeable layers that cause these fluids to as-pass oil saturated areas in the reservoirs. Therefore, an increasingly large quantity of water (or gas) rises with the oil, and by decreasing the ratio of oil /low water, eventually it becomes uneconomic to continue the process and the field must be abandoned. In this situation, due to the low proportion the oil production in both primary and secondary stages (about 30%), attention will be focused on the third stage of the

oil recovery, so-called tertiary production or enhanced oil recovery(EOR) for recovering more oil from the existing and abandoned oil (Souza *et aly* .,2014).

Generally, tertiary and enhanced oil recovery involves the extraction of residual oil after the primary and secondary phases of production. At this stage, modern and technically advanced are employed too either modify the properties of reservoir fluids or the reservoir rock characteristics, with the aim of gaining recovery efficiencies than those obtained by conventional recovery methods(primary and secondary recovery stages). This can be achieved based on different mechanisms such as reducing the interfacial tension between oil and water, reducing viscosity of the displacing fluid to be more viscous than the oil (Souza *et al*., 2014).

Enhanced oil recovery (EOR) processes can be classified into four main categorie as thermal methods, chemical methods, miscible or solvent injection, and microbial method. Microbial enhanced oil recovery(MEOR), which nowadays is becoming an important and a rapidly developed tertiary production technology, which uses micro-organisms and their metabolites (biosurfactant) to enhances the recovery of residual oil (Banat, 1995).

In this method, nutrients and suitable bacteria, which can grow under the anaerobic reservoir conditions, are injected into the reservoir. The microbial metabolic produces that include biosurfactants, biopolymers, acids, solvent, gases

and also enzymes modify the properties of the oil and the interactions between oil, water and porous media, which increase the mobility of oil and consequently the recovery of oil especially from depleted and marginal reservoirs, thus extending the producing life of the wells (Banat *et al*., 2010).In MEOR process, different kinds of nutrients are injected to reservoirs. In some processes, a fermentable carbohydrate including molasses is utilized as nutrient (Banat *et al*., 2010). Some other reservoirs require inorganic nutrients as substrates for cellular growth or as alternative electron acceptors such as nitrate, are injected into the reservoir, so that anaerobic bacteria can grow by using oil as the main carbon source (Banat *et al*., 2010). The micro-organisms used in MEOR methods are mostly anaerobic extremophiles for their better adaption of the oil reservoir conditions (Banat *et al*., 2010). These bacteria are usually hydrocarbon utilizing, non-pathogenic and are naturally occurring in petroleum reservoir (Aparna *et al*., 2011). *Bacillus substilis* grown on glucose mineral salts medium are one of the most utilized bacteria in MEOR technologies, specifically when oil viscosity reduction is not the primary aim of the operation (Aparna *et al*., 2011).

2.10. Microbial Degradation of Hydrocarbon and Oil Contaminated Soils

Microbial remediation of hydrocarbons and crude oil contaminated soils is an emerging technology involving the application of biosurfactants (Banat, 1993; Banat, 1995). Biodegradation of hydrocarbons by native microbial populations is the primary mechanism by which hydrocarbon contaminats are removed from the environment (Banat, 1995). The effectiveness of enhancing hydrocarbon degradation through addition of microbial inocula prepared from non-indegenous populations (bioaugumentation) has been ambigious (Banat, 1995).

However, the addition of biosurfactant stimulated the indigenous bacterial population those which could be achieved through addition of nutrients alone. Rhamnolipid from *P. aeruginosa* has removed substantial quantities of oil from contaminated Alaskan gravel from the Exxon Valdez oil spill (Healy *et al*., 1996). Recently, on a large scale experiment, the effectiveness of in-situ bioremediation on the Exxon Valdez oil spill has been demonstrated by Bryant and Lickhert, (2000). In another experiment, Sanjeet *et al*. (2004) demonstrated a 25% to 75% and 80% of increase in the recovery of hydrocarbons from contaminated sandy-loam and silt-loam soil, respectively, by rhamnolipid from *P.aeruginosa*.

Similarly, 56% of the aliphatic and 73% of the aromatic hydrocarbons were recovered from hydrocarbon contaminated sandy-loam soil by treatment with *P. aeruginosa* biosurfactant (Sanjeet *et al*., 2004), and also increases hydrocarbon

minerliazation by twofold and shortened the adaptation time of microbial populations to few hours (Muller *et al*.,2012). The efficiency of biosurfactants for the remediation of metal (Muller and Hausmann, 2011), phenanthrene (Peypoux *et al*., 1999) and polychlorinated biphenyl (Varnier *et al*., 2009) contamination in soil has recently been shown.

The ability of biosurfactant to emulsify hydrocarbon-water mixtures has been much documented (Fracchia *et al*., 2012). This property has been demonstrated to increase hydrocarbon degradation significantly and is potentially useful for oil-spill management (Bierman et al., 1987).While screening oil-degrading marine micro-organisms from North Sea, Schumacher (1999) isolated biosurfactant – producing *Alcaligenes* sp. Strain MM-1, *Arthrobacter* sp. Strain EK1, and Arthrobacter sp. Strain. Desanto (2008) observed the development of blue- green mets of oil-degrading organotrophic bacteria attached to mucilage produced by cyanobacteria during the deliberate discharge of oil in the Persian Gulf during the Iraqi war and during the Braer tanker leakage off the Scotland Island. Das and Mukherujee, (2007) recently demonstrated that the use of a mixture of hydrocarbon- degrading microbes for bioaugumentation of soil contaminated wilt slop oil from a petrochemical industry resulted in bioremediation of soil.

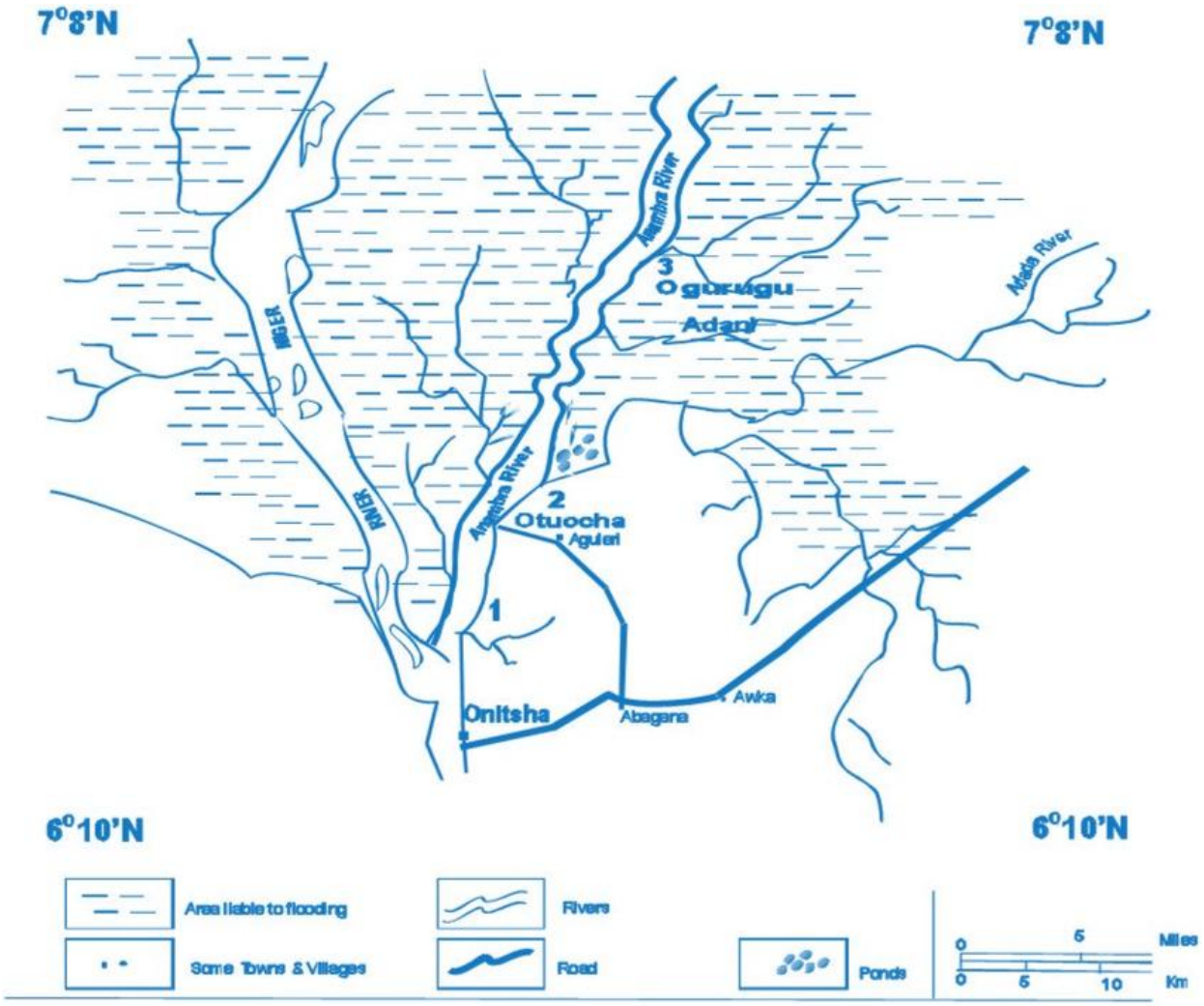
CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Site Description

The study was carried out using a microbial isolate recovered from Anambra river sediment. Anambra river is located in Anyamelum in Anambra West Anambra State of Nigeria. Anambra state lies between latitude 5^0 40^1 N and 6^0 45^1 N and longitude $6^0 35^1$E and $7^0 21^1$E. The climate is tropical with an average annual rainfall of 2000mm and mean temperature of 27^0C.

Anambra river spatially lies between latitude $6^0 00^1$N and $6^0 30^1$N and longitudes $6^0 45^1$E and $7^0 15^1$E. The river is at the south central region of Nigeria, close to the east of the Niger river into which it empties (Map 3.1).



Map 3.1: Anambra river (Shahin, 2019).

Map 3.2: Anambra River, Anambra State Nigeria (Shahin, 2019).

3.2 Collection of River Sediment Samples and Isolation of Crude oil degrading Bacteria

Collection of Sample

Samples were collected randomly at various points within the Anambra river sediment using surface sterilized soil augers by paid divers. Samples from Anambra river sediments were each placed in screw capped plastic containers containing 100ml of distilled water (in order to reduce the viscosity of the sediment samples). Samples were then transported to the laboratory of Applied Microbiology and Brewing Department, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria for processing.

Isolation of Bacteria

Serial dilutions were made from the collected sediment sample by taking 10g of the sample (which is diluted in 100ml of distilled water) and suspending in 9ml of sterile distilled water. One ml of diluted soil sediment was inoculated into a mineral salts medium as described by Sakthipriya *et al*.(2015b) with the following compositions(g/l): 10ml crude oil (by vapor transfer), 0.2g of $MgSO_4$, 0.02g of $CaCl_2$,1.0g of KH_2PO_4, 1.0g of K_2HPO_4, 0.05g of $FeCl_3$, 1g of NH_3NO_3 in agar. The pH of the medium was adjusted with1 N NaOH to 7.0$\pm$0.2 and the plates incubated for 7days at room temperature. The bacterial colonies that

developed on the plates were purified by successive streaking on nutrient agar. The colony characteristics of the purified bacterial strains were observed.

Physiological and biochemical tests performed as described in Bergey' Manual of Systematic Bacteriology (Holt *et a*l., 2000) include:

Gram staining

There are six basic steps:

1. Apply a smear of bacteria on to a slide. Air dry and then heat fix by passing it through a flame a few times. Make sure you air dry the bacteria before heat fixing.
2. Add about 5 drops of Hucker's Crystal Violet to the culture. Let stand for one minute. Bacteria will stain purple. Wash briefly with water and shake off excess.
3. Add about 5 drops of iodine solution to the culture. Let stand for 30 seconds, wash briefly with water and shake off excess.
4. Tilt slide and decolorize with solvent (acetone-alcohol solution) until purple color stops running. Be careful not to over-decolorize. Wash ***immediately (within 5 seconds)*** with water and shake off excess**.**
5. Add about 5 drops of Safranine O. Let stand for one minute, wash briefly with water and shake off excess.
6. Examine under microscope at both 400x and 1,000x oil immersion.

Motility test

Method

1. Touch a straight needle to a colony of a young (18- to 24-hour) culture growing on agar medium.
2. Stab once to a depth of only $^{1/3}$ to ½ inch in the middle of the tube. Be sure to keep the needle in the same line it entered as it is removed from the medium.
3. Incubate at 35°-37°C and examine daily for up to 7 days.
4. Observe for a diffuse zone of growth flaring out from the line of inoculation.

Catalase test

Procedure of Catalase Test

Tube Method

1. Pour 1-2 ml of hydrogen peroxide solution into a test tube.
2. Using a sterile wooden stick or a glass rod, take several colonies of the 18 to 24 hours test organism and immerse in the hydrogen peroxide solution.
3. Observe for immediate bubbling.

Oxidase test

Procedure of Oxidase Test

1. Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride.
2. Moisten the paper with sterile distilled water.
3. Pick the colony to be tested with a wooden or platinum loop and smear in the filter paper.
4. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

Carbohydrate fermentation test

The procedure of Carbohydrate fermentation test

Preparation of Carbohydrate Fermentation Broth

1. Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 ml distilled water and transfer into conical flasks.
2. Add 0.5% to 1% of the desired carbohydrate into all flasks.
3. Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth.

4. Sterilize in an autoclave at 115°C for 15 minutes. (Note: Do not overheat the Phenol red Carbohydrate fermentation broth. The overheating will result in breaking down the molecules and form compounds with a characteristic color and flavor. The process is known as the caramelization of sugar (the browning of sugar).
5. Transfer the sugar into screw-capped tubes or fermentation tubes and label properly.

Inoculation of Bacterial Culture into fermentation medium tube

1. Inoculate each tube with 1 drop of an 18 hour or 24-hour cultural broth in aseptic condition (keep uninoculated tubes as control tubes).
2. Incubate the tubes at 18-24 hours at 37°C
3. Examine the tube for acid and gas production.

Citrate utilization test

Procedure of Citrate Utilization Test

1. Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.
2. Incubate aerobically at 35 to 37 C for up to 4-7 days.
3. Observe a color change from green to blue along the slant.

Gelatin hydrolysis

Procedure of Gelatin Hydrolysis Test

There are several methods for determining gelatin hydrolysis test such as gelatin stab method, plate method, X-ray method, Kohn method, all of which use gelatin as the substrate. The standard and most commonly used method is the nutrient gelatin stab method.

1. Pick up several well-isolated colonies of 24 hour old with a sterile needle.
2. Inoculate the nutrient gelatin medium with a test inoculum by stabbing 4 to 5 times half inch into the medium.
3. Incubate the test and an un-inoculated tube for 48 hour at 37°C. (*Note: incubate the medium at 25°C if the organism grows better at 25°C than at 35°C)*
4. Gently remove the inoculated and uninoculated tubes from the incubator and place in ice bath or refrigerate for at least 30 min. or until the control tube solidifies. (Gelatin is a liquid at 28°C or above). *(NOTE: i. Do not shake or invert the tubes prior to refrigeration. ii. Gently invert to detect liquefaction by the test organism after 30 min of refrigeration.)*
5. Re-incubate a negative test for up to 2 weeks if indicated by the nature of the organism and examine at regular intervals.

Starch hydrolysis test

Test procedure

1. Pick a few colonies of test organism using a sterile swab or loop.
2. Streak a starch plate in the form of a line across the width of the plate. Several cultures can be tested on a single agar plate, each represented by a line or the plate may be divided into four quadrants for this purpose.
3. Incubate plate at 37 °C for 48 hours.
4. Add 2-3 drops of 10% iodine solution directly onto the edge of colonies. Wait 10-15 minutes and record the results.

Urease test

Procedure of Urease Test

1. Streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an overnight brain-heart infusion broth culture.
2. Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 48 hours to 7 days.
3. Examine for the development of a pink color for as long as 7 days.

Hydrogen sulphide production test

Procedure of Hydrogen Sulfide (H2S) Production Test

1. Inoculate the organism into labeled tube by means of stab inoculation in SIM medium.
2. Incubate the inoculated tubes at 37°C for 24-48 hours.
3. Observe for the formation of black precipitate on the medium.

Nitrate reduction test

Procedure of Nitrate Reduction Test

1. Inoculate the nitrate broths with bacterial suspension.
2. Incubate the tubes at the optimal temperature 30°C or 37°C for 24 hours.
3. After incubation look for N2 gas first before adding reagents.
4. Add 6-8 drops of nitrite reagent A and add the 6-8 drops of nitrite reagent B.
5. Observe for the reaction (color development) within a minute or less.
6. If no color develops add zinc powder.
7. Observe for at least 3 minutes for a red color to develop after addition of zinc.

MOLECULAR CHARACTERIZATION

Genomic DNA extractions from the bacterial isolates were carried out using the DNeasy Blood and Tissue Extraction Kit (Qiagen, USA) following the protocol provided by the manufacturer. Overnight bacteria cultures grown in tryptone-soy broth (TSB) were centrifuged for 10 min at 5000 × g, to harvest the cells. The pellet was washed 3 times in TE buffer. Cell pellet already washed in TE buffer was lysed in enzymatic lysis buffer (containing 2 mg/ml lysozyme, 25 Mm Tris HCl pH 8, 10 Mm EDTA, 25% sucrose) and incubated at 37 °C for 30 min in an incubator (Uniscope SM9052, Surgifriend Medicals, England). Proteinase K and extraction buffer were added, mixed by vortexing and incubated at 56 °C in a water-bath (Uniscope SM101 Shaking Water bath, Surgifriend Medicals, England) for 30 min. The DNA was precipitated with ethanol (96 - 100%, v/v) and transferred into the DNeasy Mini spin column for binding of DNA to the column, washed with two different 500 µl washing buffers and eluted with 200 µl elution buffer. The resulting DNA was stored at − 20 °C.

The 16S rRNA gene from resulting DNA was amplified by Polymerase Chain Reaction (PCR) using bacteria universal primers (27 F - AGAGTTTGATCCTGGCTCAG and 1492R - GGTTACCTTGTTACGACTT). The PCR amplification was carried out in a Techne TC-412 Thermal Cycler (Model FTC41H2D, Bibby Scientific Ltd, UK) in a 50 µl reactions containing

25 µl of 2 × PCR Master Mix (Norgen Biotek, Canada), 1.5 µl of template DNA (0.5 Mg), 1 µl of both forward and reverse primers (2.5 µM of each) and 21.5 µl of nuclease free water in a PCR tube added in that order. PCR was carried out at an initial denaturation step at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 2 min, and a final extension step at 72 °C for 5 min. PCR products (amplicons) were separated by electrophoresis on a 1% agarose TAE gel containing ethidium bromide and visualized by UV transillumination (Foto/UV 15, Model 33017, Fotodyne, USA). The gene sequences obtained were compared by aligning the result with the sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) search program at the National Centre for Biotech Information (NCBI). Based on the molecular analysis, the bacterial species were identified and recorded.

3.3 Screening methods for Biosurfactant Production

The biosurfactant producing ability of the isolate was studied using some standard parameters such as hemolysis test as described by Carrillo *et al*. (1996), drop collapse test as described by Morikawa *et al*. (2000), oil displacement test as described by Morikawa *et al*. (2000), emulsification index as described by Dubey and Juwar Kar, (2001), cetyltrimethyl ammonium bromide (CTAB) agar test as described by Morikawa *et al.* (2000) and surface tension measurement as described by Al-Hraji and Issa, (2004). Distilled water was used as control.

3.3.1 Hemolysis Test

The first screening test for identification and isolation of biosurfactant-producing bacterial was the hemolysis test as described by Carrillo et al (1996). Fresh cultures from bacterial isolates were prepared by streaking on nutrient-agar plates and incubating at 37^0C for 24 hours The fresh single colony of culture was then re-streaked on blood agar respectively and incubated at 37^0C for 48-72 hours. Clear zones of hemolysis around the colonies on blood agar were observed.

Results were recorded based on the type of clear zone observed i.e. α-hemolysis indicates a zone of clearing in the area surrounding a bacterial colony as a result of the presence of biosurfactant which completely lyse the red blood cells giving rise to colonies surrounded by clear zone and ¥- hemolysis when there were no change in the medium surrounding the colony.

3.3.2. Drop Collapse Test

Drop collapse test was performed using protocol the method described by Morikawa *et al*. (2000). All isolates were grown for 7 days in nutrient broth. The microbial cells were separated by centrifugation in 500ml falcon tubes for 15mins at 4500rpm. Each of the glass slides used were rinsed with hot water (in order to eliminate contaminants) and then air dried. The slides were then coated with crude oil and equilibrated for 24 hours to ensure a uniform oil coating. A 5µl aliquot of sample fermentation broth was then applied onto the centre of the oil drops visual.

After 1 minute, if the drop remained beaded, the results were scored negative (-ve). If the drop collapsed, the result was scored as positive (+ve).

Distilled water was used as control

3.3.3. Oil Displacement Test

The oil displacement test was done by adding 40ml of distilled water to a petri dish with a diameter of 10cm using the method described by Morikawa *et al*. (2000). Thereafter 15µl of crude oil was dropped to form a thin oil layer on the surface of the water and the 10µ of a test (*Pseudomonas aeruginosa*) solution were dropped on to the surface of oil. The test was conducted at room temperature

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3.3.4. EMULSIFICATION INDEX (E_{24})

T he E_{24} of culture samples was determined by adding 2ml of hydrocarbon (crude oil) to the same amount of culture supernatant, mixing with a vortex for 2 minutes, and leaving to stand for 24 hours at room temperature.

The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm). This was carried out as described by Dubey and Juwar Kar, (2001.

The emulsification index E_{24} was determined by the following equation.

$$\text{Emulsification Index } (E_{24}) = \frac{\text{Height of the emulsified layer}}{\text{Total height of the solution}} \times 100$$

3.3.5. Growth on Cetyltrimethyl Ammonium Bromide (CTAB)

The method of Morikawa *et al*. (2000) was used. Cetyltrimethyl ammonium bromide (CTAB)–methylene blue agar was the selective agar used in the isolation of biosurfactant producing micro organisms. It formed greenish yellow colonies on the surface of the agar.

Biosurfactant producing colonies on CTAB agar plates were identified following the formation of dark halos around the colonies.

3.3.6. SURFACE TENSION MEASUREMENT

The surface tension measurement of cell free supernatant was determined in a K6 tensiometer (KrussGmb H, Hamburg, Germany), using the du Nouy ring method. The values reported are the mean of three measurements. All measurements were made on cell-free broth obtained by centrifuging the cultures at 9000 x g for 30mins.

Surface tension was calculated by the formular given as follows:

Tension reduction $(C/0) = \frac{Y_m - Y_c}{Y_m}$ x 100

Where: Y_m is the surface tension of medium without inoculation

Y_c is the surface tension of the test supernatant.

3.4 Media and Clture Conditions

For biosurfactant synthesis a mineral salt medium with the following composition (g/l) was utilized 0.2g of $MgSO_4$, 0.02g of $CaCl_2$, 0.1g of K_2HPO_4, 1.0g of KH_2PO_4, 0.05g of $FeCl_3$, 1g of NH_3NO_3. pH of the medium was adjusted to 7.0 $\pm$ 0.2. Carbon and nitrogen sources were added separately and aseptically added to flasks containing mineral salt medium. Laboratory scale biosurfactant production was carried out in 250ml Erlenmeyer flasks (containing 50ml of medium inoculated with 3 x 10^{-4}CFU of bacteria isolate) in an orbital shaker (180rev min^{-1}) at 37^0C for 7 days. Isolated bacteria were maintained on nutrient agar.

3.5 Culture Condition for Biosurfactant Production

3.5.1 Estimation of Growth and Biosurfactant Production

Five millilitres of samples of culture broth were collected at 12hour intervals for a period of 168 hour. The dry weight technique was used to quantify microbial growth as bacterial density using a UV-Vis spectrophotometer (Jenway 6305). Optical densities of samples removed from culture were read against a blank of distilled water. Biomass obtained after filtration on a 0.2µ millipore was dried overnight at 105^0C and weighed. Biomass was quoted in terms of mg/ml (dry weight).

The dry weight technique was used to quantify biosurfactant concentration. The culture broth was centrifuged at 4500rpm for 30minutes and kept overnight at 4^0C in the refrigerator. Three volumes of chilled acetone was added and allowed to

stand for 10hours at 4^0C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone and weighed as described by Barros *et al.* (2008);

3.5.2 Effect of initial Carbon Sources on Biosurfactant Production

The experiment was set-up using 250ml Erlenmeyer flasks containing 100ml of mineral salt medium. The medium was inoculated (10%) with 5µl of innoculum strain of the biosurfactant producing bacterium. The pH of growth medium was adjusted to 5.0 (Bezza and Chirwa, 2015) and the cultures were incubated with shaking (200rpm) at 37^0C for 48 hours.

The effects of the following carbon sources were tested for biosurfactant production: crude oil, palm oil, groundnut oil, diesel and tween80.

A basal medium consisting of (g/l): $MgSO_4$,0.2;$CaCl_2$,0.02;K_2HPO_4,1.0; KH_2PO_4,1.0;$FeCl_3$,0.05; NH_4NO_3,1.0;distilled water, 1L(pH 7.0) was used. The carbon source were separately incorporated at 2%v/v and sterilized at 121^0C (15mins). Flasks (250ml) containing the culture medium (100ml) were inoculated with the biosurfactant producing culture($3x10^4$cfu/ml) and incubated on an orbital shaker at 37^0C for 48hours. . The carbon source was added into medium at a concentration of 2% (w/v or v/v). Samples were taken out at regular intervals (24hours) to analyze for growth technique and biosurfactant production (see section 3.6.1).

Effect of carbon sources on the yield of biosurfactant was evaluated by growing the isolate in different carbon sources: crude oil, diesel, palm oil, groundnut oil and tw een 80.

3.5.3 Effect of Nitrogen Sources on Biosurfactant Production

The effects of the following nitrogen sources were tested for biosurfactant production: sodium nitrate, ammonium nitrate, yeast extract, urea and ammonium chloride as described by Barros *et al*. (2008).

A basal medium consisting of (g/l):Most suitable carbon source; $MgSO_4$, 0.2;$CaCl_2$, 0.02;K_2HPO_4, 1.0;KH_2PO_4,1.0;$FeCl_3$,0.05; distilled water, 1L(pH 7.0) was used. The nitrogen source was separately incorporated at 2g and sterilized at 121^0C (15mins).Flasks (250ml) containing culture medium (100ml) were inoculated with the biosurfactant producing culture ($3x10^4$cfu/ml) and incubated on an orbital shaker at 37^0C for 48h. Samples were taken out at regular intervals (24hour) to analyze for growth and biosurfactant production (see 3.6.1). The yield of biosurfactant and biomass were expressed in terms of g/l.

3.5.4 Effect of Initial pH on Biosurfactant Production

Mineral salt medium, supplemented with the optimized 2% initial carbon and nitrogen sources obtained previously was used in this experiment.

The initial pH growth medium was set-up at 5.0, 6.0, 7.0, 8.0 and 9.0 using 1NHcl.The medium was inoculated with shaking (200rpm) at 37^0C for 4 days.

Samples were taken out at 24hours intervals to analyse for growth and biosurfactant production for the 4days fermentation period as decribed by Barros *et al.* (2008).

3.5.5 EFFECT OF TEMPERATURE ON BIOSURFACTANT PRODUCTION

The isolate was inoculated ($10\%^{v}/_{v}$) into mineral salts medium, adjusted to the optimum pH of 7.0 and supplemented with 2%v/v crude oil. The cultures were incubated at 25^0C, 30^0C, 40^0C and 50^0C, respectively for 4days in an orbital shaker as described by Barros *et al.* (2008).

3.5.6 TIME COURSE OF BIOSURFACTANT PRODUCTION

The time course of the production of biosurfactant by the bacterial isolate was carried out in 250ml Erlenmeyer flasks containing 100ml of optimized medium of the following composition (g/l): 2% of crude oil; $MgSO_4$, 0.2; $CaCl_2$, 0.02; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $FeCl_3$, 0.05; NH_3NO_2, 2.0 (nitrogen source) pH of the medium was adjusted to 7.0 with shaking in an orbital shaker (200rpm) at 37^0C.

Samples were analyzed for growth and amount of biosurfactant production at 24hours interval for 7days as described by Barros *et al.* (2008).

3.6 Production of Biosurfactant

For biosurfactant production, a basal medium consisting of (g/l): $MgSO_4$, 0.2; $CaCl_2$, 0.02; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $FeCl_3$, 0.05; NH_3NO_2, 2.0; crude oil,

2%(v/v);distilled water, 1L (pH 7.0) was used. Flasks (250ml) containing the culture medium (100ml) were inoculated with the biosurfactant producing culture($3x10^{-4}$ cfu/ml) and incubated on an orbital shaker at 37^0C for 120hours. Samples were taken out at regular intervals (24hours) to analyse for biosurfactant production by measuring surface tension and emulsification index E_{24} of supernatant samples obtained after cell separation of the crude biosurfactant Rhamnolipids produced by *Pseudomonas aeruginosa* was recovered from the culture supernatant after the removal of cells by centrifugation (4500rpm for 15minutes) to remove the cells and thereafter sterilized with membrane filter. The clear sterile supernatant served as the source of crude biosurfactant.

Rhamnolipids were then precipitated by acidification of the supernatant to pH 2.0 and allowing the precipitate to form at 4^0C overnight. The precipitate thus obtained was pelleted at 4500rpm for 15minutes and dissolved in 0.05M sodium biocarbonate (pH 8.6), reacidified, and recentrifugation at 4500rpm for 15minutes. Following centrifugation, the precipitate was extracted using three volumes of chilled acetone and allowed to stand for 10hours at 4^0C.

The organic solvent was evaporated using a rotary evaporator and a yellowish oily residue was obtained after which it was re-dissolved in 0.05M sodium biocarbonate (pH 8.6).

3.7 Biosurfactant extraction

3.7.1 Determination of Total Carbohydrate for Biosurfactant Characterization

The presence of carbohydrate groups in the biosurfactant was assayed by rhamnose test using the method of Dubeau *et al*. (2009). A volume of 0.5ml of cell supernatant was mixed with 0.5ml of 5% phenol solution and 2.5ml of sulfuric acid. The mixtures were incubated for 15minutes before measuring acid absorbance at 490nm and read against a standard of rhamnose sugar.

3.7.2 Determination of Total Lipids

The lipid content was determined by gas chromatographic test (Dubeau *et al*., 2009). The analysis was carried out in a gas chromatograph equipped with the capillary column HR-SS-IO. The carrier gas was helium at flow rate of 24ml/min. The chromatographic peaks were identified by comparing with the chromatogram of the standard fatty acid methyl mixture.

3.7.3 Determination of Total Protein Content

Biuret reagent (1.0ml) was added to all the three sets of test tubes. The content was mixed thoroughly and incubated for 30minutes at 25^0C as described by Dubeau *et al*. (2009).

Absorbance of the sample (A_{sample}) and the standard ($A_{standard}$) against the reagent blank was read at a wavelength of 530nm.

The total protein concentration was calculated as follows

$$\frac{\text{Abs (sample)}}{\text{Abs (standard)}}$$

Concentration of Total Protein (g/dl) = Conc. of standard x

3.8. Characterization of Biosurfactant using Thin Layer Chromatography (TLC)

The preliminary characterization of biosurfactant was performed by TLC analysis. Biosurfactant was separated on a silica gel 60F254 plate (20cm x 20cm, merk) using chloroform: methanol: glycial acetic acid (65:15:4, $^{v}/_{v}$/v) as mobile phase. The spots were visualized by spraying different colour developing reagents. The developing reagents include ninhydrin (purple), α-naphthol reagent (red) and 4-methoxy-benzaldehyde reagent (green).

The lipid components were detected as green spots after placing the plates in a closed jar saturated with 4-methoxy-benzaldehyde reagent.

Protein spot were visualized by spraying ninhydin reagent followed by heating at 90^{0}C for 5minutes, which generated a purple colour when the compound had an amine function.

Carbohydrate components were detected as red spots on the plates after spraying with an α-naphithol solution followed by concentrated sulphuric acid, heated for 5minutes at 100^{0}C.

Iodine crystals were used to detect lipid fraction of biosurfactant. Three plates were heated at 110^{0}C for 10mins after application of the spraying agents. Rhamnose was used as the standard.

3.10. BIODEGRADATION OF CRUDE OIL WITH BIOSURFACTANT PRODUCING BACTERIA

Perforated aluminum containers (500g capacity) were filled with 100g of soil samples

The experiment was conducted with four different sets (Varnier *et al*., 2009) as follows:

i. Container with Pristine soil (250g)

ii. Container with sterile pristine soil (250g) and crude oil (2v/v)

iii. Container with pristine soil (250g), crude oil (2v/v)

iv. Container with sterile soil (250g), crude oil (2v/v) and biosurfactant producing isolate. Inoculation was done with 24 hours old culture (10^{3} – 10^{-4} cfu/ml).

The set-up was maintained at room temperature for a period of one month. Biodegradation of crude oil was estimated by gas chromatographic technique at 2 weeks intervals.

3.11. Enhanced Oil Recovery using Sand Pack Column.

Sand packed column preparation and oil recovery experiment was carried out with minor modification in the sand packed method described by Souza *et al*. (2014)

A glass column (25 x 500mm) was packed with 100g of acid washed sand (100 mesh size)(Fig.14). In order to wash the sand with acid, 100g of sand was placed

in a 500ml beaker and 25ml of concentrated HCL was added, allowed to stand for 15minutes; the experiment was carried out three times to ensure good result.

Brine (5% NaCl $^{w}/_{v}$) was then passed through the column and pore volume (PV) was determined by measuring of brine required to make the sand matrix wet. To ensure 100% saturation, three PVs of brine were passed through the column under pressure until the column got saturated with oil. Once, oil entered in the column, discharge of brine solution was observed from the matrix of sand. The discharged volume of brine, from sand packed column was collected and measured to calculate initial oil saturation (S_{oi}). The oil saturated column was washed with 4-6 PV of brine until no further oil was discharged in the effluent. The oil retained i.e. residual oil saturation (S_{or}) after brine solution wash was calculated on the basis of oil loaded and oil discharged in the effluent from column. The 0.6Pv of 48 hours old cell free fermentation broth containing biosurfactants was then passed through the oil saturated sand packed column and allowed to stand for 24 hours. The amount of oil recovered upon incubation for 24h was measured by collecting effluent in 100ml quantities. This experiment was repeated thrice to evaluate the reproducibility and efficiency of crude biosurfactant enhanced oil recovery using sand-packed column. The percentage oil recovery was calculated as described by Sandrin *et al*. (2000) as follows:

Pore volume (PV) ml = volume of brine required to saturate the column

Original oil in place (00IP (ml) = Amount of brine solution discharged upon displacement by oil sand pack column.

Sorwf (ml) = oil retained after brine floods

Sorbf (ml) = oil released after the feeding of sand pack column, saturated with residual oil with 0.6PV of biosurfactant preparation.

Initial water saturation (S_{wi} %) = $\frac{001P}{PV} \times 100$

Where x = pore volume = volume of brine displaced after injection of oil in sand packed column

Residual oil saturation (S_{or} %) = $\frac{Xi}{001P} \times 100$

Where Xi = OO1P = volume of oil displaced after water flooding

Oil recovery after water flood (Orecwf)

$$= \frac{Sorwf}{001P} \times 100$$

Additional oil recovery after biosurfactant flooding

$$\frac{\text{Oil recovery using biosurfactant}}{100 \times \text{Oil in column after water flooding}}$$

CHAPTER FOUR

1.0 RESULTS

1.1 Isolation and Identification of Crude oil Degrading Bacteria

The bacterial isolate was examined based on its morphological and biochemical characteristics. The isolated strain produced a diffusible green colored

fluorescent pigment. The bacterial strain was a non-spore forming–gram negative, rod-shaped, motile bacterium. The catalase and oxidase tests were positive, growth on triple sugar iron was neutral bottom and neutral slant, and there was no production of hydrogen sulfide (H_2S) when grown on MacConkey agar (Table 4.2.).

On the basis of partial 16SrRNA gene sequence analysis, isolate 3d was further identified as a member of the genus Pseudomonas revealing 100% identification with *P.aeruginosa* (Fig.4.1).

Comparison with the NCBI sequence after high bor-joining analysis of different *Pseudomonads* indicated that the closest relatives of this strain (3d) were *Pseudomonas aeruginosa*.

Table 4.1: Physiological and Biochemical Characteristics of the Strain 3d

Biochemical Characteristics	Strain 3d
Shape	Rod
Gram Reaction	-
KOH test	+
Acid Test Staining	-

Motility	+
Catalase Tets	+
Oxidase test	+
Nitrate Reduction test	+
Determination of carbohydrate fermentation and hydrogen sulfide production	
Glucose	+
Sucrose	-
Lactose	-
H_2S Production	-
GcS Production	-
Indole test	-
Methyl Red (MR)	-
Voges Proskauer (VP)	-
Citrate test	+
Starch hydrolysis	+
Urea hydrolysis	-
Gelatin hydrolysis	+
Growth on different hydrocarbons (BTex)	+
Decarboxylation of Arginine	+

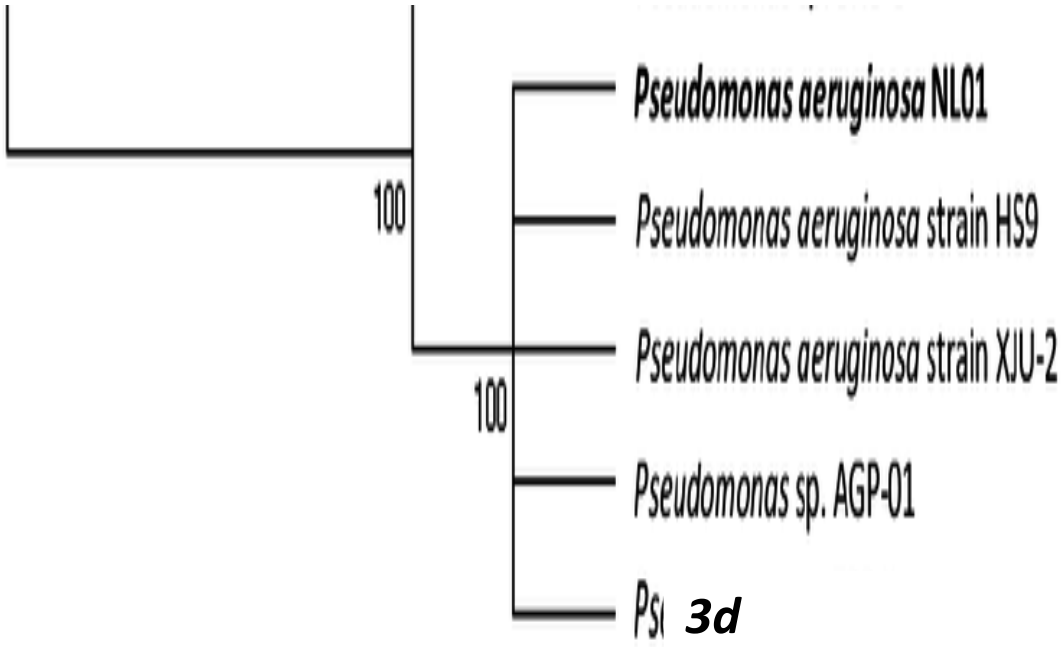

Figure 4.1: Partial 16SrRNA gene sequence analysis revealing 3d as a member of *Pseudomonas aeruginosa*

4.2. Screening of the selected Bacteria for Crude oil Degradation and Production

Four crude oil utilizing bacterial isolates coded 1a, 3d, 6c and 7d, respectively were recovered from the Anambra river sediment using mineral salt agar with crude oil as carbon source. The results of the drop collapse test showed that only isolate 3d gave a positive result by collapsing the liquid droplets. The other isolates and the control did not collapse the liquid droplets. Isolate 3d showed positive reaction for biosurfactant production on CTAB-methylene blue agar medium, as the presence of blue halos around the bacterial colonies were observed after 48 hours of incubation at 37^0C. The results obtained from hemolytic agar test showed that isolates 3d, 6c and 7d hemolysed blood agar, 5cm zone of clearance. The type of zone of clearance is beta- hemolysis (for isolate 3d and 7d) and gamma hemolysis for isolate 6c. On the other hand, the surface tension and emulsification power of the supernatant obtained from the corresponding broth culture of strain from the corresponding broth culture of strain 3d were taken as an indication of the ability of the bacteria to produce biosurfactants strain 3d showed an excellent surface tension reducing capability as it reduced the surface tension of the medium to 33mN/m The bacterial strain (3d) was highly positive for biosurfactant production by given 8cm of oil disablement test. The bacterial isolate 3d also had

the highest emulsification index (96%), followed by isolate 7d (75%) and isolate 1a being the least (0%).

From the results of the biosurfactant-producing abilities of isolates, isolate 3d was chosen for further studies (Table 4.1).

Table 4.1: Screening of bacterial strains for Biosurfactant Production

Isolate	Surface tension (mN/n)	E_{24} (%)	Oil Displacement	Drop collapse	Hemolysis	CTAB
1a	59	0	-	-	-	-
3d	33	96	+	+	+ (α)	+
6c	50	25	-	-	+ (γ)	-
7d	46	75	-	-	+ (α)	-
Control (waste)	60	0	-	-	-	-

CTAB –Cetylrimethyl Ammonium Bromide

E_{24} --Emulsification Index

4.3 Cultural Conditions for Biosurfactant Production by the Choice Isolate

4.3.1 Effect of Carbon Sources on Biosurfactant Production

The effect of different carbon sources on the production of biosurfactant by *Pseudomonas aeruginosa* is shown in Figure 4.2.

In this present study, *Pseudomonas aeruginosa* utilized all the tested carbon sources (crude oil, palm oil, diesel, groundnut oil and tween 80) for growth and biosurfactant production. Crude oil (2%) gave the best biosurfactant yield (1.44g/l) while tween80 gave the least yield (0.05g/l) among the carbon sources evaluated. Hence, crude oil of 2 %(w/v) was used as carbon source for further studies

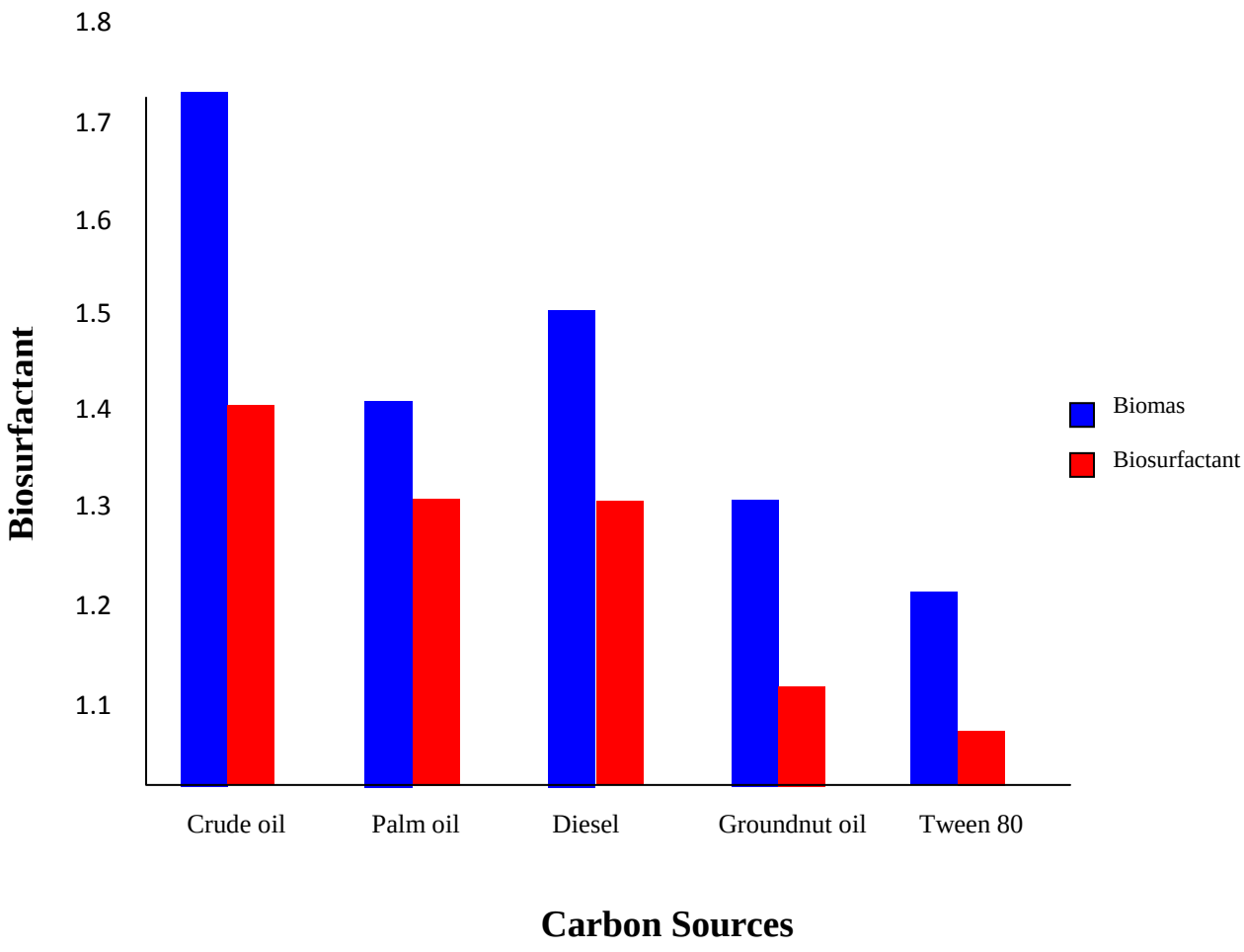

Figure 4.2: Effect of carbon sources on the production of biosurfactant by *Pseudomonas aeruginosa*

4.3.2. Effect of Nitrogen Sources on Biosurfactant Production

The results of the influence of nitrogen sources on biosurfactant production revealed that ammonium nitrate served as the best nitrogen sources for biosurfactant production by *Pseudomonas aeruginosa* (Fig.4.3). Ammonium nitrate gave the best biosurfactant yield (1.4g/l) while Ammonium chloride produced least biosurfactant (.0.05g/l).

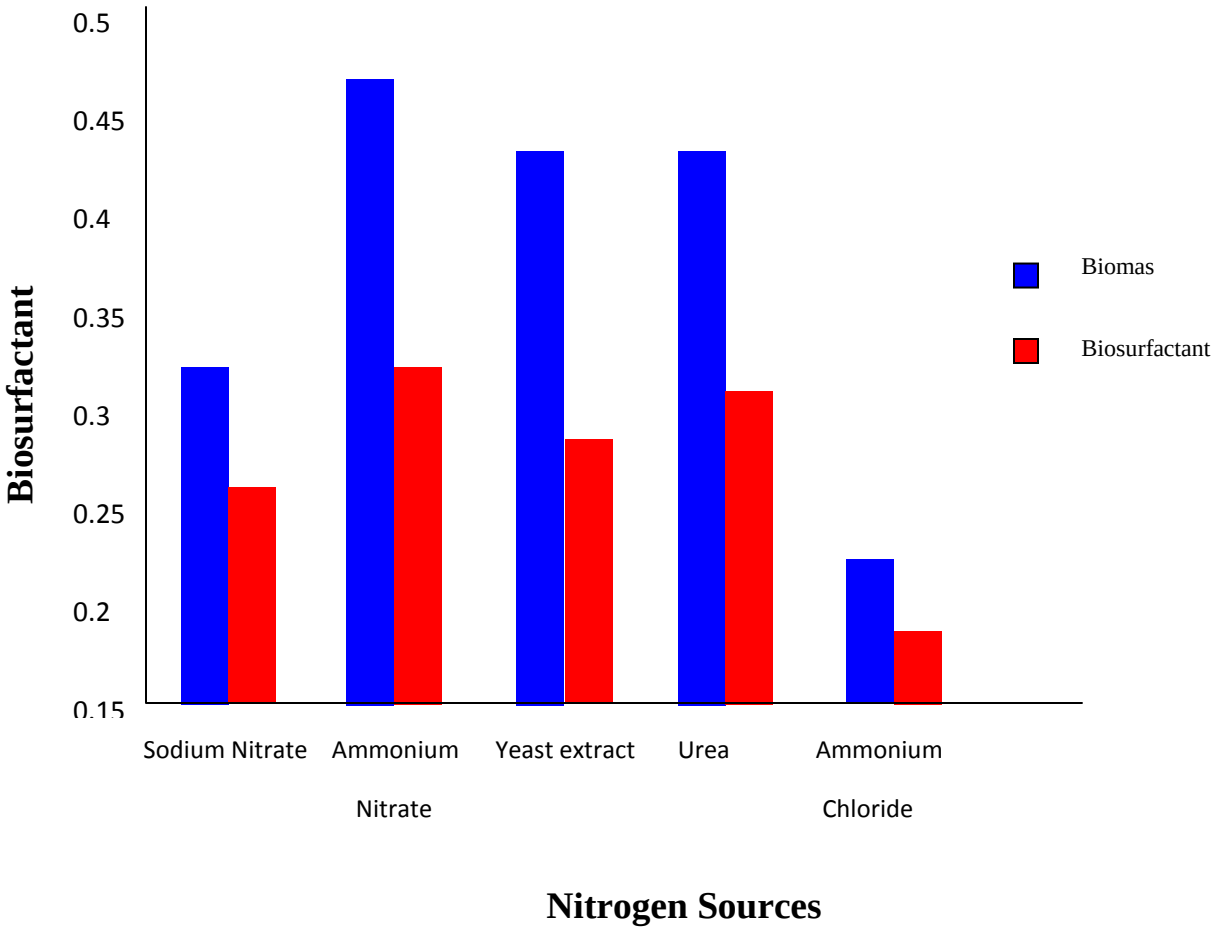

Figure 4.3: Effect of Nitrogen Sources on the production of biosurfactant by *Pseudomonas aeruginosa*

4.3.3 Effect of pH on Biosurfactant Production

The result of the effect of the intial pH on biosurfactant production showed that the pH remained stable at a wide range of pH (5.0-9.0) (Fig.4.4). The highest biosurfactant production (1.44g/l) by *P.aeruginosa* was lower or higher pH values caused an appreciable drop in biosurfactant production indicated by surface tension. This confirmed that maximum biosurfactant production can be obtained at pH 7.0 and therefore pH 7.0 was used for further studies.

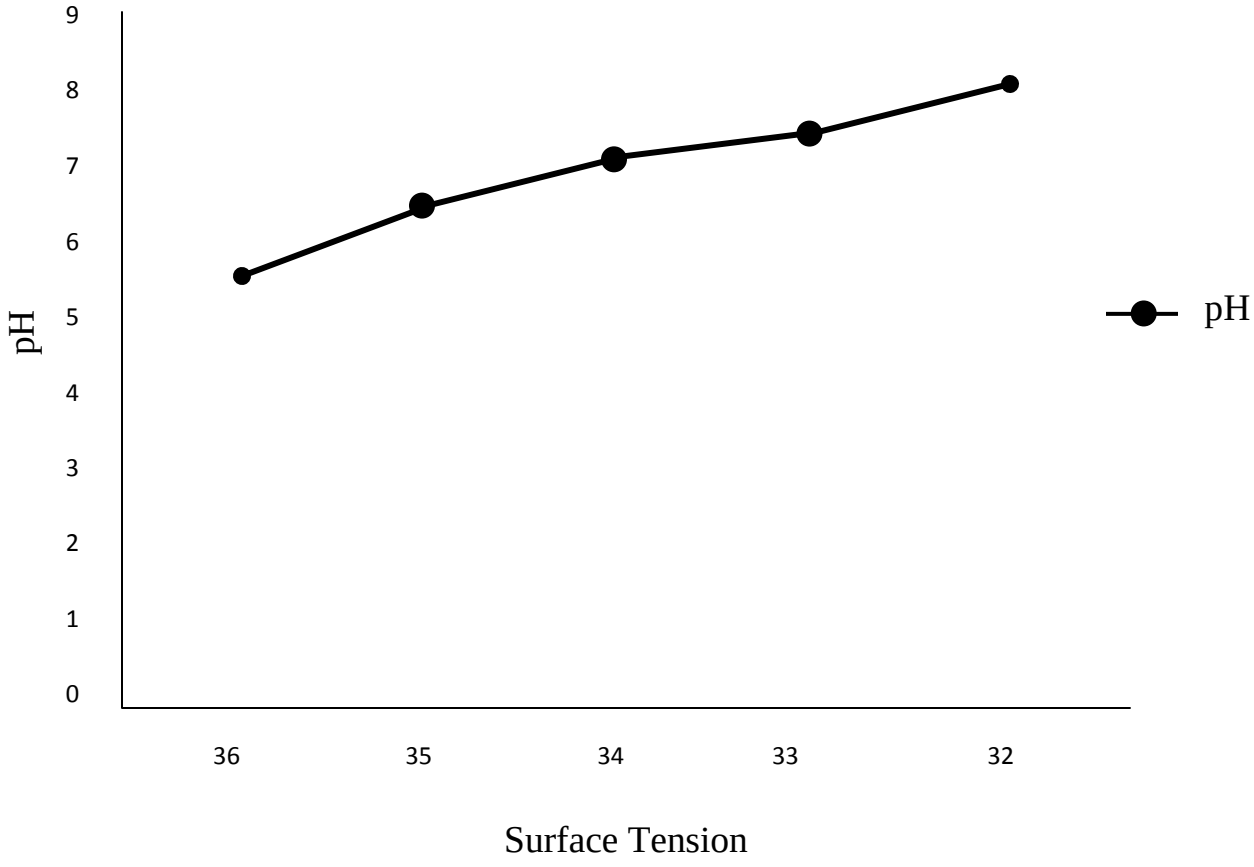

Figure 4.4: Effect of pH on biosurfactant production by *Pseudomonas aeruginosa*

4.3.4. Effect of Temperature

The supernatant of the culture broth was tested over the temperature range of 30^0C – 50^0C.

The results in Fig.4.5 showed that at a temperature of 37^0C, biosurfactant remained stable. The highest biosurfactant production was recorded as 1.4g/l for the *P.aeruginosa*.

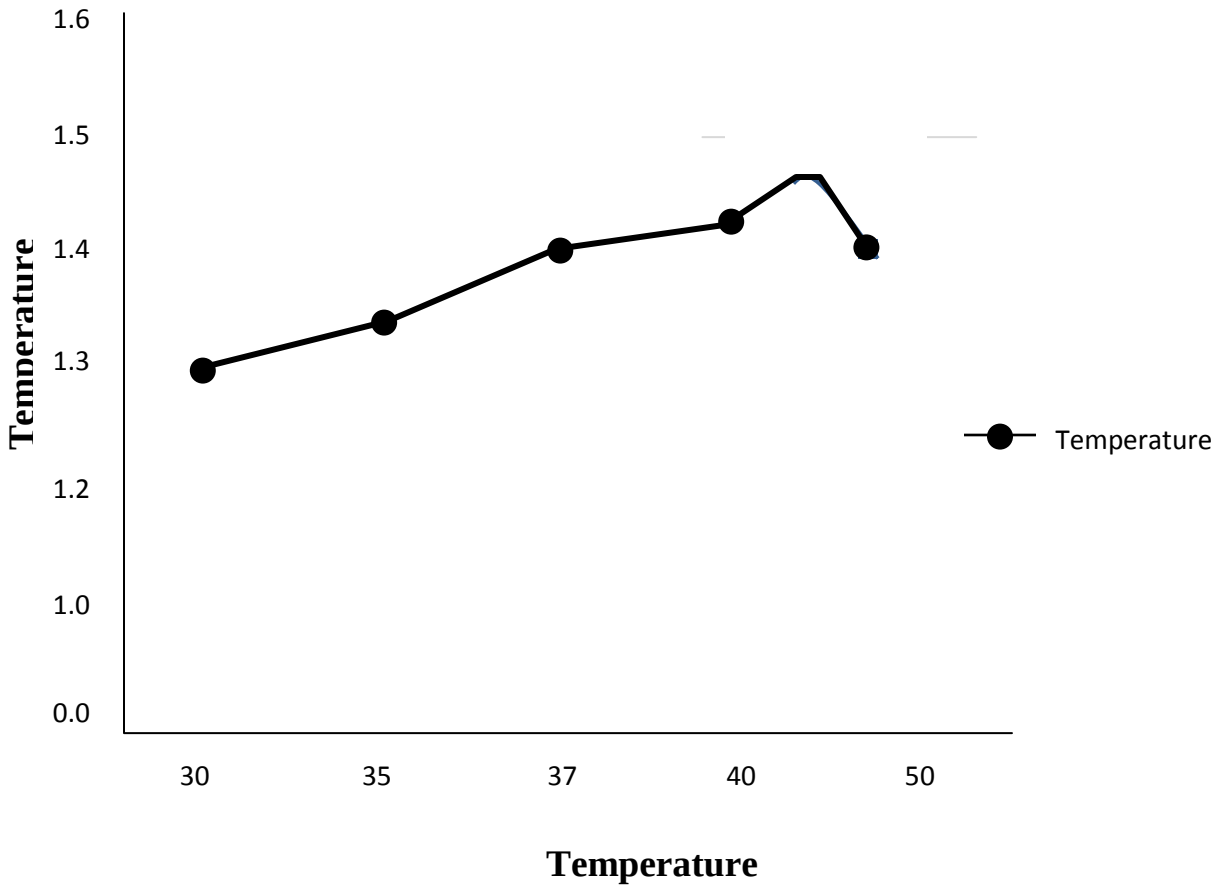

Figure 4.5: Effect of Temperature on Biosurfactant production by *Pseudomonas aeruginosa.*

4.3.5. Time Course of Production of BiosurfactantIOS by *Pseudomonas aeruginosa*

Figure 4.6 shows the time course of biosurfactant production by isolate 3d with crude oil as the sole carbon source and 1g of $NaNO_3$ at pH of 7.0. Maximum biosurfactant concentration of 1.44g/ml occurred at 120h of incubation, when the cells reached their early stationary phase at 37^0C.

Parallel increase in biomass and biosurfactant were found from 24h to 96h.

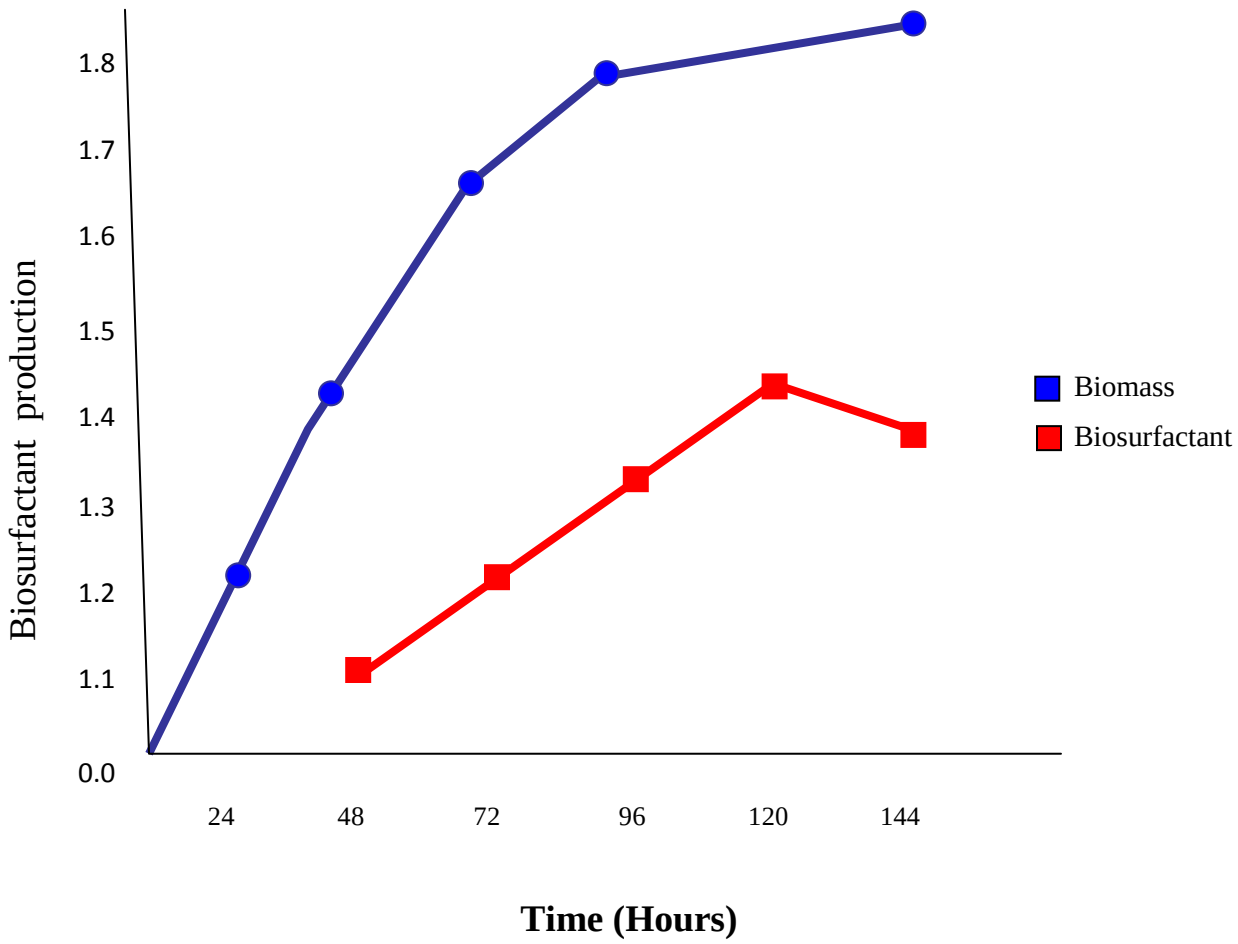

Figure 4.6: Time course on the production of biosurfactant by *Pseudomonas aeruginosa.*

4.4. Biosurfactant Extraction

The biosurfactant was separated by an easy and reliable method with no loss of its activity. A yield approximately 1.44g/ml was obtained (Slide 4.1).

Slide 4.1: Rhamnolipid Biosurfactant produced by *Peudomonas aeruginosa*

4.5. Characterization of Biosurfactant

4.5.1. Determination of Total Carbohydrate

Biochemical analysis revealed that the biosurfactant produced by the strain 3d was mainly composed of carbohydrate. The carbohydrate content was found to be 38mgl (see appendix). The rhamnose test was positive which indicates that the separated biosurfactant could be a glycolipid.

4.5.2 Estimation of lipid

Biochemical analysis using gas chromatographic test revealed that the biosurfactant produced by *Pseudomonas aeruginosa* was composed of 51.45(mg/g) of lipid (see appendix)

4.5.3 Thin Layer Chromatography (TLC)

Thin layer chromatographic test was used to detect the type of biosurfactant produced.

TLC analysis of biosurfactant produced showed a single spot of biosurfactant fraction in replica plates. The biosurfactant fraction showed positive reaction with α-naphitol reagent and with 4-methoxy-benzaldehyde reagent indicating the presence of carbohydrate and lipid moieties (4.8).

Plate 4.1: Thin layer chromatographic result showing a rhamnolipid

The above result of TLC analysis demonstrated the glycolipid nature of biosurfactant.

4.6 Biodegradation of Crude oil with Biosurfactant Producing Bacteria

Figures 4.9 and 4.10 show the results of biodegradation of crude oil by *Pseudomonas aeruginosa*.

The result showed that soil sample containing crude oil and biosurfactant producing isolate has a maximum degradation of crude oil as compared to that of pristine soil and crude oil. Utilization of crude oil by biosurfactant producing bacteria was continuously monitored at 1month of the biodegradation. At the end of the incubation period, the residual crude oil was recovered and used for further characterization to understand the degradation products. Different functional groups present in the residual crude oil were confirmed by Gas chromatographic analysis. Both degrade crude oil spectrum and control spectrum are presented in Tables 6 and 7.

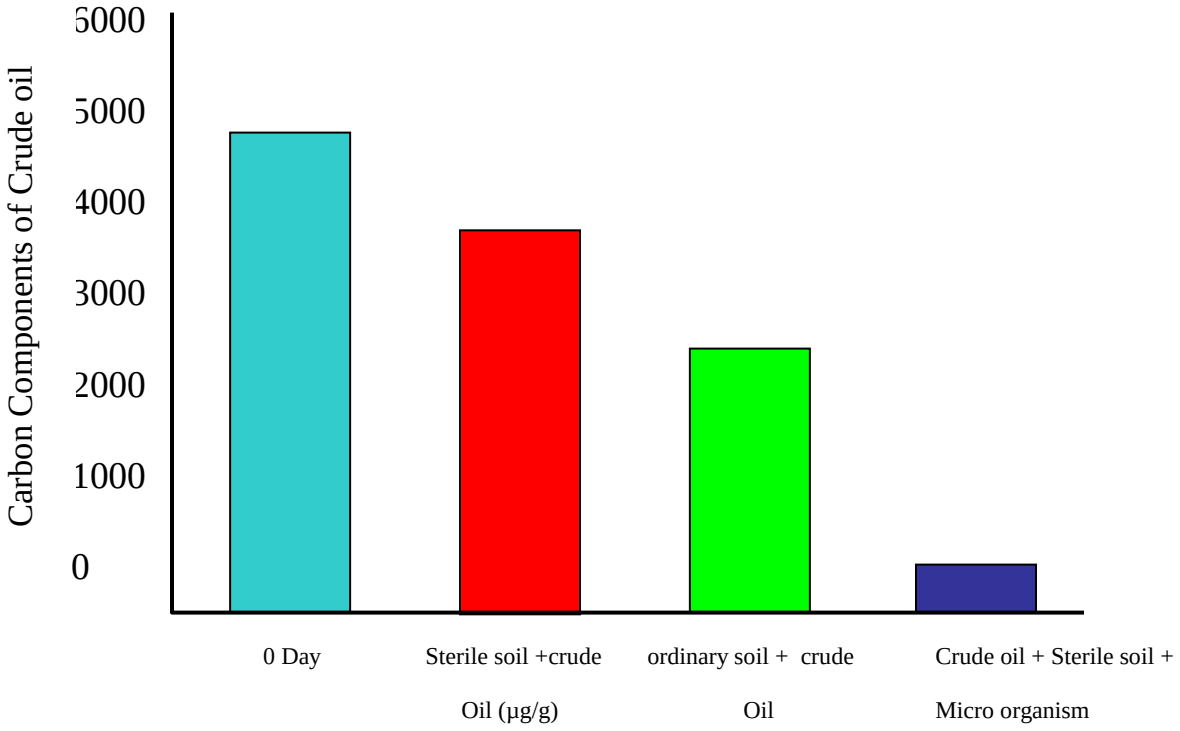

Figure 4.9: Field scale experiment in the biodegradation of crude oil by *Pseudomonas aeruginosa* after 2 weeks of incubation period.

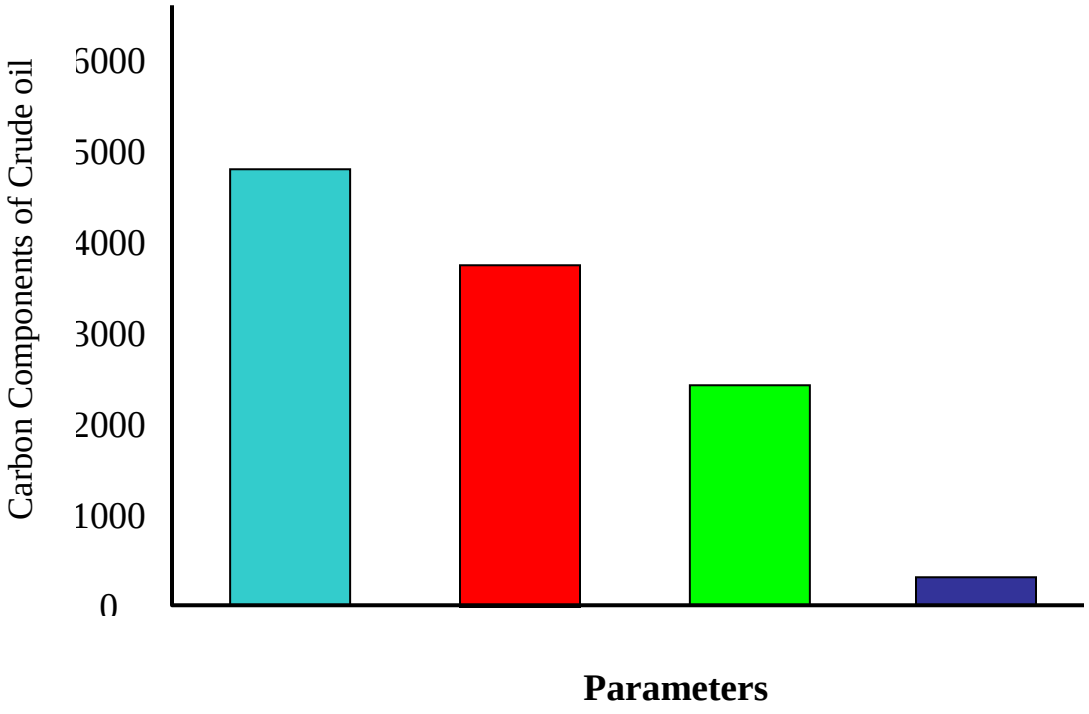

Figure 4.8: Field scale experiment on the biodegradation of crude oil pseudomonas aeruginosa after 1 month interval

4.7. ENHANCED OIL RECOVERY USING SAND PACK COLUMN

Biosurfactant produced by *Pseudomonas aeruginosa* was used to perform the oil recovery technique with crude oil using sand pack column as described by Suthar *et al*., 2008 (Fig.4.10).

Figure 4.10: Sand pack column

The result is presented in Table 4.3; biosurfactant has the ability to enhance oil recovery with sand pack column. The pore-volume (PV) of the column was 43ml while Original oil in Place (OOIP) of the column was 37ml.

After the water flooding process, 32.4% of the oil remained trapped in the column (the soil in the column absorbed most of the oil).

When the biosurfactant of *P. aeruginosa* was introduced into the column and incubated for 24 hours, the amount of oil recovered after biosurfactant flood was 2ml. This means that additional 16.6% of crude oil was recovered due to the action of the biosurfactant from *Pseudomonas aeruginosa*.

Table 4.3: Summary of results obtained in sand pack column for crude oil recovery using *Pseudomonas aeruginosa*.

Parameters	*Pseudomonas aeruginosa*	Control
PV (ml)	43	45
001P (ml)	37	38
S_{oi} (%)	86	84.4
S_{wi} (%)	14	15.6
S_{orwf} (ml)	25	20
001P-Sorwf (ml)	12	18
S_{or} (%)	32.4	47.4
Sorbf (ml)	2	0
AOR (%)	16.6	0

Keynotes:
PV- Pore volume; OOIP-Original oil in place;
S_{oi}- Initial oil saturation;
S_{wi} –Initial water saturation;
S_{orbf} –Oil recovered after biosurfactant flooding;
S_{orwf} - Oil recovered after water flooding;
AOR- Additional oil recovery.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The present study was aimed at the exploration of the indigenous microflora of the Anambra River Sediment and the investigation of their biosurfactant-producing potential. Pollution of the seas, oceans, and coastal zones is a serious issue and contamination of hydrocarbons remains a major threat to the sustainability of the natural environment (Bodour *et al*., 2003). Out of the four bacterial strains, strain coded 3d was selected for further studies. The strain was characterized on the basis of partial 16SrRNA gene sequence analysis, the isolated strain coded 3d was further identified as a member of the genus Peudomonas revealing 100% identity with *Pseudomonas aeruginosa.* Strain 3d showed an excellent positive hemolytic activity (3.05cm) which is generally carried out as a primary method for screening of biosurfactant-producing bacteria (Rodrigues *et al*., 2006). Hemolytic activity appears to be a good screening criterion in the search for biosurfactant-producing bacteria (Carter, 1984).

In this study, strain coded with 3d was found to be positive by oil-spreading method. Other monitoring parameters that estimate surface activity, such as oil-spreading test and the ability to emulsify hydrocarbons are required for verification (Youssef *et al.,* 2004). The oil-spreading method is rapid and easy to carryout,

requires no specialized equipment and only requires a small volume of sample (Plaza *et al*., 2006).Emulsification activity is one of the criteria to determine the potential of biosurfactants. In this present study, *Pseudomonas aeruginosa* gave a good emulsification potential with all the hydrocarbons tested, which included kerosene, diesel, crude oil. Emulsification activity is one of the criteria to determine the potential of biosurfactants. Emulsifying activities (E_{24}) determine the productivity of bio-emulsifier (Bodour *et al*., 2003) and are given as a percentage of the height of the emulsified layer divided by the total height of the liquid column. Present study revealed that *Pseudomonas aeruginosa* was positive on CTAB agar plate method. The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. The assay was developed by Siegmund and Wagner (1991). The drop – collapse test was positive in 3d isolate. The result of the study on the drop collapse assay capability of strain 3d is in-conformity with the works of Jain *et al.* (1991) which states that solutions containing potent biosurfactant will be unable to form stable drops and spread completely over the oily surface, while solutions without surfactant will remain beaded on the oily surface. Jain *et al*. (1991) developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. The drop-collapse method is a sensitive and easy to perform method and has several advantages in requiring a small volume of samples, being rapid and

easy to carry out, and not requiring specialized equipment. Strain 3d showed an excellent surface tension reducing capability as it reduced the surface tension of the medium to 33mN/m. This result is comparable to the recent reports on biosurfactant production by *P. aeruginosa* 112 and PAI (Guerra-Santos *et al*., 1984; Mendes *et al*., 2015). According to the previous report, the biosurfactants produced by bacterial strains for instance *P. aeruginosa* are more effective in decreasing the surface tension of the medium. Crude oil (2%) gave the best biosurfactant yield while tween80 gave the least yield among the carbon sources evaluated. Preference of carbon source for biosurfactants by microorganisms appears to be strain dependent, as different strains produce biosurfactant in different carbon sources which could be either water-miscible or water immiscible substrates(Desai and Banat, 1997; Kumani *et al*., 2012; Sajna *et al*., 2015; DiazDe Rienzo *et al*.,2016; Wu *et al*., 2018). Ammonium nitrate (1g) gave the best biosurfactant yield (1.4g/l). This result can be explained by the fact that nitrate first undergoes dissimilating nitrate reduction to ammonium and then assimilation of nitrate as nitrogen source is so slow that it would stimulate a condition of limiting nitrogen (Barber and Stuckey, 2000). *Pseudomonas aeruginosa* is able to use nitrogen source such as ammonia or nitrate. However, in order to obtain high concentrations of rhamnolipids it is necessary to have restrained conditions of this macronutrients.

This study showed that nitrate is more effective in the production of rhamnolipids than ammonia and urea. This observation is in agreement with other studies reported in the literature (Syldatk and Wagner, 1985; Ochsner *et al*., 1995; Arino *et al*., 1996). According to Maneerat (2005), production of surface active compounds increases when concentration of nitrogen depleted in the medium which is due to reduction in the activity of isocitrate dehydrogenase. This enzyme is NAD and NADP-dependent and catalyzes the oxidation of isocitrate to 2-oxoglutarate in the mitochondria due to decline in the activity of isocitrate dehydrogenase and further transported to the cytosol. In the cytosol, citrate-synthase converts citrate into oxaloacetate and acetylcoA which is the precursor of fatty acid synthesis and hence biosurfactant production increases. Nitrogen limitation has been reported to increase the rhamnolipid production. The rhamnolipid production of the *P. aeruginosa* is dependent not only on carbon source but also on limiting portion of nitrogen sources. Hence, ammonium nitrate (1g) was used as nitrogen source for further studies.

The result of the study also showed that there was no production of biosurfactant when yeast extract was used as a sole source of nitrogen source. The observed disappearance of biosurfactant might be related to the development of competence. There are three possible mechanisms responsible for the decline in the biosurfactant concentration): 1) the biosurfactant was degraded by the enzyme in

the culture, 2) the biosurfactant might be adsorbed on the cell surface, or 3) the biosurfactant was re-internalized and processed intracellulary (Li *et al.*, 2002). Although ammonium sulfate and ammonium chloride supported bacterial growth, production of biosurfactant was very poor. It can be deduced that, at low pH of the culture medium, bacteria could not efficiently synthesize biosurfactant (Li et al., 2002). The temperature is one of the most important parameter that significantly influences the growth of micro-organism and thus the biosurfactant stability. Biosurfactant stability at extreme temperatures was reported by Kiran *et al*. (2009) and Aparna *et al*. (2012) for *Pseudomonas aeruginosa* strain and *Brevibacterium aurem* MSA13, respectively. The results of thermostability of biosurfactant produced by *Pseudomonas aeruginosa* shows the potential application of the biosurfactant in various industries i.e., pharmaceutical, food and cosmetics as well as in microbial enhanced oil recovery (MEOR) where heating step is very important (Abouseud *et al*., 2008; El-sheshawy *et al*., 2015). Additionally, temperature has no significant effect on surface tension reduction properly over a temperature range of 40^0C- 100^0C which is consistent with previous reports where strains showed stability from 40^0C-120^0C (Amani *et al*., 2010; Xie *et al*., 2005). These results confirmed that biosurfactant produced by *P. aeruginosa* exhibits thermal stability. The effect of pH on surface activity has been reported for biosurfactants for different micro-organisms (Kneg and Holt, 1984).The result of

the study showed maximum stability of rhamnolipid at pH of 7.0 using *Pseudomonas aeruginosa*.The result of the study is in agreement with a previous report that maximum biosurfactant stability by *Pseudomonas aeruginosa* 181 was achieved after 120h of incubation at pH 7.0 and 37^0C (Al-Araji and Issa, 2004). For *Bacillus subtilis* the optimal stability of biosurfactant was observed at pH 7.0 (Markar and Cameotra, 2002). It was noticed that pH value induced strong fluctuations in the stability of rhamnolipid, probably due to the kinetic and metabolic behavior of the micro-organism, which produces acids from carbohydrates as it grows thus decreasing the pH (Kneg and Holt, 1984). Parallel increase in biomass and biosurfactant were found from 24h to 96h, but maximum biosurfactant was found at 120h.This result indicate that biosurfactant production from crude oil occurred during the exponential growth phase, suggesting the biosurfactant is produced as primary metabolite accompanying cellular biomass formation (Persson *et al*,. 1988). This property suggests that biosurfactant could be effectively produced under chemostat conditions or by immobilized cells (Kneg and Holt, 1984). The result of this study was comparable to the observation made in a study by Lourith and Kanlayavattarokul (2009). Previous studies reported that rhamnolipid biosurfactant was produced during the logarithmic and stationary phases of bacterial growth and amount of production increased after then (Zhang and Miller, 1994). Onbasli *et al*. (1996) stated that *Pseudomonas luteola* and

Pseudomonas putida produced rhamnolipid at 0.23g/l and 0.24g/l at 48h; 0.38 and 0.36g/l at the 72h, respectively. The increase in extra-cellular biosurfactant concentration might be the result of the cell- bound biosurfactant molecules released into the broth medium (Deziel *et al*., 1999). The results revealed that the production of biosurfactant from crude oil occurred predominantly throughout the exponential phase indicating that the biosurfactant is a primary metabolite and produced accompanying cellular biomass formation (Khopade *et al*., 2012). TLC analysis of purified biosurfactant showed positive reaction with Molish reagent and iodine vapour indicating the presence of rhamnolipid and lipid moieties. The result demonstrated the glycolipid nature of biosurfactant. Similar reports of the production of glycolipids biosurfactant by *Pseudomonas aeruginosa* (RF value of 0.085) and *P. lepacia* (RF value of 0.9) (Silva *et al*., 2010). The RF of the spots (0.30 and 0.82) were near to the RF reported by Syldatk and Wagner (1985) (0.29 and 0.82) using the same solvent system: chloroform-methanol-acetic acid (65:15:2) {(vol/vol)}. Further identification of the sugar moiety after acid hydrolysis confirmed it as rhamnose. This result suggests that *P. aeruginosa* produces rhamnolipid. The result of the biodegradability capacity of *Pseudomonas aeruginosa* showed that *P. aeruginosa* is an efficient crude oil degrader. The result is in conformity of the work of Sakthipriya *et al*. (2015b) which shows that *Pseudomonas aeruginosa* achieved 80% of the degradation efficiency (10 days). In

this work, it was observed that more than 97% of the alkanes of chain lengths C_{13}-C_{14}; C_{31}-C_{34} were utilized by *Pseudomonas aeruginosa*. Degradative enzyme producing capabilities of the bacterial strain make them an efficient strain. Miistra and Singh (2012) have reported that alkane hydroxylase enzyme plays an important role in the degradation of n-hexadecane by bacterial strains *P. aeruginosa* PSA5 and *Rhodococcus* sp.NJ2. These enzymes play an important role in the hydrocarbon degradation and the respective genes that encode those enzymes were identified in earlier studies (Whyte *et al*., 2002; Heyd *et al*., 2008). Several studies have shown that alkanes of chain length between C_{14}-C_{20} were easily utilizable as energy sources by most of the hydrocarbon degrading bacteria (Sanjeet *et al*., 2004). The present study confirms that Gram negative *P. aeruginosa* has the ability to produce biosurfactant of glycolipid nature which exhibits efficient uptake of hydrocarbons in crude oil. Heyd *et al*. (2008) identified many bacterial genera including *Achromobacter* sp., *Bacillus* sp., *Serratia* sp., *Sphingomonas* sp. and *Micrococcus* sp. as crude oil degrading bacteria and biosurfactant producers. The produced biosurfactant was also described as rhamnolipid in nature (Bezza and Chiwa, 2015). Biodegradation of crude oil by micro-organisms appears to be natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecular compounds or transformed into other organic

compounds such as biosurfactant (Chhatre *et al*., 1996). During biodegradation, crude oil is used as an organic carbon source by a microbial process, resulting in the breakdown of crude oil components to low molecular weight compounds (Okoh *et al*., 2001). Treatment of crude oil saturated sand column with biosurfactant containing cell-free supernatant of *P. aeruginosa* resulted in the release of some of the crude oil (32.4%) trapped in the sand column. This was due to the ability of the biosurfactant to reduce surface tension of oil making it more mobile in the column. The result of this work was comparable to the observation made in a study by Bordoloi and Konwar (2008). Banat, (1995) injected biosurfactant produced by *B. subtilis* to oil saturate sand packed columns and reported that 35% release of residual oil as compared to 21% using nutrient solution. Sand pack columns provide a suitable approach to study that ability of injected micro-organisms to increase oil recovery in reserviours, because these models allow the stimulation of oil recovery operations in oil fields. After the water flooding process, residual oil is trapped in the pores of the reserviour rocks. Biosurfactant produced by *P. aeruginosa* reduce the interfacial tensions at the oil-rock interface, thus reducing the capillary forces that prevent oil from moving through rock pore. It was also found that the construction of a sand pack column is easy, rapid and inexpensie and the problems associated with core flood studies like

preservation of live cores. This makes the sand pack column a suitable bench-scale technique for screening microorganisms showing potential for oil recovery.

5.2. CONCLUSION

The result obtained from the present investigations indicated that a promising crude oil degrading and biosurfactant producing strain has been isolated, characterized and identified as *Pseudomonas aeruginosa*. Structural characterization by TLC confirmed that the biosurfactant produced by *Pseudomonas aeruginosa* was glycolipid in nature. The biosurfactant exhibited high emulsification activity and stability in a wide salinity which makes them suitable for various industrial applications such as food, pharmaceutical and cosmetics industries.

Also it enhanced oil recovery in the sand pack column technique. It is found that the isolated biosurfactants have the ability to recover about 16.6% of crude oil entrapped in the sand-pack column and may be useful to recover residual oil from mature oil wells.

5.2.1 CONTRIBUTION TO KNOWLEDGE

The study focused on the environmental application of biosurfactants produced by *Pseudomonas aeruginosa* to treat contaminated soils with crude oil.

In this study also, *Pseudomonas aeruginosa* specie had been applied to generate biosurfactants for oil recovery enhancement. The principle behind this technique is the injection of *Pseudomonas aeruginosa* specie which causes the reduction of oil viscocity and interfacial tension between hydrocarbons and surface of rock matrix, which can facilitate the mobilization of oil and further increment of oil recovery.

5.2.2. RECOMMENDATION

Further studies should be carried out to:

- Investigate the effects of Nitrogen (N), Phosphorus(P) and potassium(K) at different concentrations to get the best Carbon: N: P: K ratios for optimum biodegradation.
- Study the effect of concentration of hydrocarbons on degradation ability to determine if high levels of contaminants may have toxic effects which slow rates of bioremediation.
- Investigate the effect of consortia versus individual bacterial strains on degrading abilities. To use four or more indigenous bacterial strains isolated from crude oil contaminated soil, preferably a consortium that produces a biosurfactant mixture that emulsifies the oil to accelerate biodegradation process.

• Construct a consortium by including pure culture of an alga which will provide oxygen to the aerobic bacteria and consume carbon dioxide.

REFERENCES

Abdel-Mawgound, A., Lepine, F. and Deziel, E. (2010). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Microbiology Biotechnology*. *86*: 1323

Abdel-Mawgound, A. M., Hausmann, R ., Lepine, F., Muller, M.M. and Deziel, E. (2011). *Microbiology Monographys 20-Biosurfactants*: From Genes to Applications, G. Soberon- chavez. A steinbuchel, Eds., Springer, Berlin. P.13

Abalos, A,. Pinaso, A., Infante, M.R., Casals, M., Garcia, F and Manresa, A. (2001). Physico-chemical and antimicrobial properties of new rhamnolipids by *Pseudomonas aeruginosa* At10 from soybean oil refinery wastes. *Langmir*. *17*: 1367-1371.

Abouseound, M., Maachi R., Amrane, A., Boudergua, S. and Nabi, A. (2008). Evaluation of different carbon and nitrogen source in production of biosurfacant by *Pseudomonas fluorescens*. *Desalination*. *223:*143-151.

Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S., Salem, A. and Kadri, M. (1991). Surfactants in consumer products. *Acta Biotechnological*. *11*: 315

Al-Hraji, Y. and Issa, L. (2004). Biosurfactant production by *Pseudomonas aeruginosa* 181 strain. *pHD thesis* University of Putra Malaysia.

Al- Bahmy, S.N., AL- Wahaibi, Y.M., Elshafie, A. E., Al Bemari, A.S., Joshi, S.J., Al-achmani, H.S. and Al–sulamani, H. S .(2013). Biosurfactant production by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. *International Biodegradation. 81:* 141-146:

Amani, H. M., Sarrafzaden, H., Haghighi, M. and Mehrnia, M.R, (2010). Comparactive study of biosurfactant producing bacteria in MEOR applications. *Journal Petroleum Science Engineering*. *75*:209-214.

Aparna, A., Srinikethan, G. and Smitha, H (2012). Production and characterition of biosurfactant produced by a novel *Pseudomonas sp*. 2B. *Colloids surf B. Biointerfaces. 95*:23-29

Aparna, A., Srinikethan, G. and Hedge, S. (2011). *Effect of addition of biosurfactant produced by Pseudomonas sp. On biosurfactant of crude oil.* In International Proceedings of Chemical, Biological & Environmental Engineering, Proceedings of the 2nd International Proceedings of Chemical, Singapore Volume 6. Pp. 71- 75

ASTM(1990).American Standard testing methods for soil analysis. 2217-2280. Arino, S., Marchal, R. and Vandecasteele, J. P. (1996). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Microbiology Biotechnology*. 45: 162.

Bafghi, M. K., Fazaelipour, M. H. and Hassan, M. (2012). Biosurfactants as emulsifying agent for hydrocarbons. *Journal Surfactants Detergent.15*: 679.

Banat, I. M. (1993). The isolation of a thermophilic biosurfactant producing *Bacillus* sp. *Biotechnology Letter*. *15*:591-594

Banat, I.M. (1995). "Biosurfactant production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review". *Bioresource Technology. 51*: 1-12

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Marhnotti, M.G., Fracchia, L., Smyth, J.J. and Marchant, R. (2010). Microbial biosurfactants production applications. *Applied Microbiology Biotechnology*. *87:* 427-444.

Barros, F.F.C., Quadros, C.P. and Pastore, G.M. (2008). Propriedades emulsificantes estabilidade do biossurfactante produzido por *Bacillus subtilis* em manipueira. *Gerics Technology Alignment. 28*: 979-985.

Barber, W. P. and Stickey, D.C. (2000). *Water Resource. 9(34)*:2413-2422.

Benincasa, M., Contiero, J., Manresa, M.A. and Moraes, I. O. (2002). Biosurfactants as emulsifying agent for hydrocarbons. *Journal food Engineering*. *54*:283

Benincasa, M., Abalos, A, Oliveira, I. and Manresa, A. (2004). Biosurfactants as emulsifying agent for hydrocarbons. *Antonie Van Leeuwenhoek. 85:* 1

Bezza, F.A. and Chirwa, E.M.N. (2015).Production and application of lipopeptide biosurfactant for bioremediation and oil recovery by *Bacillus subtilis* CN2. *Biochem. Engineering Journal. 101*:108-178.

Beal, R. and Betts, W. B. (2000). Role of rhamnolipid biosurfactants in the up-take and mineralization of hexadecane in *Pseudomonas aeruginosa*. *Journal Applied Microbiology*. *89*: 158-168.

Biermann, M., Lange, F., Piorr, R., Ploog, U., Rutzen, H., Schindler, J. and Schmidt R. (1987). In Surfactants in Consumer Products Theory, Technology and Application. *journal. Falbe, Springer verlogy, Heidel bergey 2*: 1-3.

Bierman, M. , Lange, F., Piorr, R., Ploog, U., Rutzen, H., Schindler, J. and Schmidt, R. (1987). Surfactants in consumer products, Theory, Technology and Application ed. *J. falbe, Spnngr- Verkg He idelberg*. *6:* 1.

Bryant, S. and Lockhart, T.P. (2000). *Reservoir engineering analysis of microbial enhanced oil recovery*. In proceeding of the SPE Annual Technical Conference and Exhibition, dallis,

Bohnisk, R.C. (1991). *Biozumica*. Editoral Addisionnilsity theroamerikana S.A, 5a Edicion, U.S.A. P.20.

Bordoloi, N. K. and Konwar, B. K. (2008). Microbial surfactant enhance mineral oil recovery under laboratory conditions. *Journal Colloides* and *Surfaces Biosurfactant Biointerfaces*. *63*:73-82.

Bognolo, G. (1999). Biosurfactants as emulsifying agent for hydrocarbons. *Colloids Surfactant. A Phsicochemica. Engineering. 152*:41-52.

Bodour, A. A., Drees, K.P. and Maier, R.M.(2003).Distribution of biosurfactant-producing bacteria in undisturbed and contaminated and South western soils. *Applied Environmental Microbiology. 69*: 3280-3287.

Cameotra, S.S. and Singh, P. (2008). *International. Biodeterioration. Bioengineering. 62*: 274.

Casas, J.A., deLara, S.G. and Garcia-Ochoa, F. (1997). Optimization of a synthetic medium for Candida bombicola growth using factional design of experiments. *Enzyme Microbiology Technology. 21*: 221-229.

Campos, J.M., Stamford T. L. M., Sarubbo, L.A., Luna, J.M., Rufino, R. D. and Banat, I.M. (2013). Microbial biosurfactants as additives for food industries. *Biotechnology Program. 29*:1097-1108.

Calvo, C., Manzanera, M., Silva-castro, G.A,.Vad, I. and Gorizaliz-lopez, J. (2009). Application of bioemulsifiers in soil oil bioremediation processes.Future Prospects. *Science Total Environmental*. 407(*12*):3634-3640.

Carter, C. (1984). Diagnostic Procedures in veterinary Bacteriology mycology, 4th ed. Spring field 11, U.S.A.Charcle C. Thomas.

Carrillo, P. G., Mardaraz, C., Pitta-Alvarez, S. J. and Guihetti, A.M. (1996). Isolation and selection of biosurfactant-producing bacterial. *World journal. Microbiology. Biotechnology*. *12*:82-84.

Chrzanowski, L., Lawnicazak, L. and Czazk, k. (2012). Biosurfactants as emulsifying agent for hydrocarbons. *World Journal microbial Biotechnology*. *28*: 401.

Chandrasekaran, E.V. and Bemiller, J.N. (1980). *Methods in carbohydrate chemistry*. R. L. whistler, Ed., Academic press, New York P.38.

Chhatre, S. A., Purohut, H. J., Shanker, R., Chakrabarti, T. and Khanna, P. (1996). "Bacterial consortia for crude oil spill remediation". *Water* Science *Technology*. *34*:187-193.

Chakraborty, J. and Das, S. (2014). Biosurfactant based bioremediation of toxic metals. In microbial Biodegradation and Bioremediation. 1st ed. Das, S., Ed., Elsevie: Rosurkele Odishe, India. Pp. 167-201.

Cirigliano, M.C. and Carman, G.M. (1984). Isolate of a bioemulsifier from *Candida lipolytica*. *Applied Environmental Microbiology*. *48*: 747-750

Cooper, D.G. and Paddock, D.A. (1984). Production of a biosurfactant from *Torulopsis bombicola*. *Applied Environmental Microbiology*. *47*: 173-176

Costa, S.G.V.A.O., Nitschke, M., Haddad, R. and Eberlin, M.N. (2006), production of *Pseudomonas aeruginosa* LBl rhamnolipids following growth on Brazilian native oils. *Process Biochemical*. *41*: 483-488.

Cvengros, S.Z. and Cvengrosova, Z. (2004). Used fruing oils and fats and their utilization in the production of methyl ester of higher fatty acids. *Biomass Bioenergy*. *27*:173-181.

Dahrazma,B.and Mulligan,C.N. (2007).*Chemosphere.69*:705.

Dagbert, C., Meylheuc, T. and Bellon-Fontaine, M.N. (2006). *Electrochemical Actalogy. 51*: 52.

Dask, K. and Mukherujee, A. K. (2007b). Crude petroleum oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North- East Indis. *Bioresource. Technology*. *98*: 1339-1345.

Desai, J.D. and Banat, I.M. (1997). Biosurfactants as emulsifying agent for hydrocarbons. *Microbial Molecular Biology Review. 61*: 47-64.

Deziel, E., Lepine, F., Dennie, D., Boismenu, D., Mamer, O.A. and Villermur, R. (1999). *Biochemical Biophysics.Actalogy. 1440*: 244—252.

Dejonghe, K., DeDobbeLaere, I., Sarrazyn, R. and Hofte, M. (2005). Biosurfactants as emulsifying agent for hydrocarbons. *Plant Pathology. 54*: 219.

Destipande, M. and Daneils, L. (1995). Evaluation of Sophorolipid biosurfactant produced by *Candida bombicola* using animal fat. *Bioresource*. *Technology. 54*: 143-150.

DiaDeRienzo, M.A., Kamalanathen, I.D. and Martin, P.J. (2016). Comparective study of the production of rhamnolipid biosurfactants by *B. thialandensis* E264 and *P. aeruginosa* ATCC 9027 using foam fractionation. *Process Biochemical. 51*: 820-827.

Dorey, S., Bailheud, F., Clement, C., Varnier, A. and Terganier, M. (2007) (International patent).WO2007/045767.

Dubey, K. and Juwarkar, A. (2001). Biosurfactants as emulsifying agent for hydrocarbons. *World J. Microbio. Biotecnol.17*: 61-69.

Dubeau, D., Deziel, E., Woods, D. and Lepine, F. (2009). Biosurfactants as emulsifying agent for hydrocarbons. *BMC Microbiology. 9*:263

El-sheshtawy H.S., Aiad, I., Osman, M.E., Abo-Elnasr, A.A. and Kobisy, A.S. (2015). Production of biosurfactants by *Bacillus licheniformis* and *Candida albicans* for application in microbials enhanced oil recovery. *Egypt journal Petrol Doii. 7*:18.

Felse, P.A., Shah, V., Chen, J., Rao, K.J. and Gross, R.A. (2007). Sophorolipid biosynthesis by *Candida bombicola* from industrials fatty acid resides. *Enzyme Microbiology Technology 40*: 316-323.

Ferhat, S., Mnuf, S., Badias, A., Eddouaouda, K., Boucherir, A., Mhiri, N., Moulai-Mosrefa, N. and Saydi, S. (2011). Screening and preliminary characterization of biosurfactants produced by *Ochrobacterium* sp. IC and *Brevibacterium* sp. 7G isolated from hydrocarbon- contaminated soils-Int. *Biodeterioration Biodegradation. 65*: 1182-1188.

Franzelti, A., Gandolfi, I., Bestelti, G., Smyth, T. J. and Banat, I.M. (2010). Biosurfactants as emulsifying agent for hydrocarbons. *European Journal Lipid Science Technology. 112*: 617.

Fracchia, L., Cavallo, M., Marhnotti, M.G. and Banat, I. M. (2012). *Biomedical Science, Engineering and Technology*, D.N. Ghista, Ed.,In Tech, Rijeke. P.326.

Franzetti, A., Caredda, P., Rugen, C., laColla, P., Tamburin, E., Papecciring, M. and Bestetti, G. (2009). Potential applications of surface active compounds by Gordonia sp strain BS29 in soil remediation technologies. *Chemosphere. 75:* 810-807.

Guerra-santos, L., Kappeli, O. and Fiechter, A. (1984). Dependence of "*Pseudomonas aeruginosa* biosurfactant production in continous culture with glucose is carbon source". *Applied and Environmental Microbiology 48*(2): 301-305.

Guerra-satos, L., kappeli, O. and Fiechter, A. (1986). "Dependence of *Pseudomonas aeruginosa* continous culture; biosurfactant production on nutritional and environmental factors". *Applied and Environmental Microbiology. 24*: 443- 448.

Haba, E., Abalos, A., Oliveira, I. and Manresa, A. (2003). Biosurfactants as emulsifying agent for hydrocarbons. *Journal. surfactants Detergent. 6*: 155.

Hazra, C., Kundu, D., Chaudhari, A. and Jana, T. (2013). Biosurfactants as emulsifying agent for hydrocarbons. *Journal Chemical. Technoogyl. Biotechnology. 88*: 1039.

Helmy, Q., Kardena, E., Funamizu,N. and Wisjunprapto, W. (2011). Strategies toward commercial scale of biosurfactant production potential substitute for its chemically counterparts. *Journal Biotechnology*. *12:* 66-86.

Heyd, M., Kohnert, A., Tan, T.H., Nusser, M., Kirschhofer, F., Brennor-Weiss, G., Franzerb, M. and Berensmeier. S. (2008). Biosurfactants as emulsifying agent for hydrocarbons. *Annual, Bioannal. Chemmical. 391*: 1579.

Healy, M.G., Devine, C.M. and Murphy, R. (1996). Microbial production biosurfactants. *Resources Conservetion. Recycle. 18*:41-57.

Hodge, J.E. and Hofreiter, B.T. (1962). *Methods in Carbohydrate Chemistry*, R.L. Whistler, M.L. Wolfrom, Eds., Academic Press, New York. P. 380.

Holt, J.G., Krieg, N.R., Sneathm, P.H.A., Staley, J.T. and Williams, S.T. (2000). *Bergey's Manual of Determinative Bacteriology*. Williams and Williams, Baltimore .P. 22.

Ines, M. and Dhouha, G. (2015). Biosurfactant: potential related Biomedical and Biotechnological applications. *Carbohydrate. Resource. 416*:59-69.

Irie, Y., O'Toole, G.A. and Yuk, M.H. (2005). Biosurfactants as emulsifying agent for hydrocarbons. *FEMS Microbial Letter. 250*: 237.

Ishigami, Y., Gama, Y., Nagehors, H., Hongu, T. and Yameguchi, M. (1988) (U.S. patent). 4, 902, 512.

Jain, D.K., Collins Thompson, D.L., Lee, H. and Trevors, J.T. (1991). Biosurfactants as emulsifying agent for hydrocarbons. *Journal. Microbiology Method. 13*: 271.

Jarvis, F.G. and Johnson, M.J. (1949). Biosurfactants as emulsifying agent for hydrocarbons. *Journal. Amount Chemical. Society. 71*:4124.

Juwarkar, A.A., Dubey, K.V., Nair, A. and Singh, S.K. (2008). Biosurfactants as emulsifying agent for hydrocarbons. *Indian Journal Microbiology. 48*:142.

Kanga, S.H., Bonner, J.S., Page, C.A., Mills, M.A. and Autenrieth, R.L. (1997). Solubilization of naphthalene and methyl substituted naphthalene from crude oil using biosurfactant. *Environmental. Science. Technology*. *31*:556-561.

Khopade, A., Biao, R., Lui, X., Mahadik, K., Zang, L. and Kokare, C. (2012). Production and stability studies of the biosurfactant isolated from marine Nocardiopsis sp. B4. Desalination. 3:198-204.

Kiran, S., Thomas, T.A. and Selvin, J. (2010). Production of a new glycolipid biosurfactant from marine *Nocardiopsis lucentensis* MSAO4 in solid state cultivation. *Colloids surfactant Biosurfactant Biointerfaces. 78*:8-16.

Kitamoto, D., Monta, T., Fukuok, T., Konishi, M. and Imura, T. (2009). Biosurfactants as emulsifying agent for hydrocarbons. *Curriculum. Opinon. Colloid Interface Science. 14*:315.

Kitamoto, D., Toma, K. and Hato, M. (2005). *Handbook of nanostructured biomaterials and their applications in nanobiotechnology*, H. Singh Nalwa, Ed., American Sciencepublishers Valencia, CA, USA, Vol. I. P. 239.

Kim, H.S., Yoon, B.D., Choung, D.H., Oh, H.M. and Katsuragi, T. (1999). Characterization of a biosurfactant mannosyleryhritol lipid produced from *Candida* sp. SY 16. *Applied Microbiology. Biotechnology. 52*:713-721.

Kiran, G.S., Herma, T.A., Gandhimah, R., Selvin, J. and Thomas, T.A. (2009). Optimization and production of a biosurfactant from the sponge associated marine fungus *Aspergillus utus* MSF3. *Colloid Surfactant Biosurfactant. Biointerfaces. 73*: 250 – 256.

Kim, S.H., Lim, E.J., Lee, S.O., Lee,J.O. and Lee, T.H. (2000). Purification and characterization of biosurfactants from *Nocardia* sp. L – 417. *Biotechnology Applied Biochemical. 31*: 249 – 253.

Kosaric .N. (2001). Biosurfactants as emulsifying agent for hydrocarbons. *Food Technology. Biotechnology. 39*: 295.

Konishi, M., Fukuoka, T., Morita, T., Imura, T. and Kitamoto, D. (2008).Production of new types of sophorolipids by *Candida batistae*. *Journal. Oleo Science. 57:* 359-369.

Kneg, N. and Holt, J. (1984). *Bergey's Manual of Systematic Bacteriology*, Marcel Dekker, New York Based. 25.

Kumari, B., Singh, S.N. and Singh, D.P. (2012). Characteristics of two biosurfactant producing strains in crude oil degradation. *Process Biochemical. 47*: 2463 – 2471.

Lourith, N. and Kanlayavattanekul, M. (2009). Biosurfactants as emulsifying agent for hydrocarbons. *International Journal.Cosmesic Sci. 31*:255.

Lindhardt, R.J., Bakhit, R., Daniels, R., Mayerl, F. and Pickenhagan, W. (1989). Microbially produced rhamnolipid as a source of rhamnose. *Biotechnology Bioengineering*. 33:365-368.

Li, Q., Kanga, C., Wang, H., Liu, C.H. and Zheng, C.H. (2002). Application of microbial enhanced oil recovery technique to Daquing Oilfield. *Biochemical Engineering Journal*. *II*: 197-199.

Mata-Sandoval, J.C., Karns, J. and Torrents, A. (2001) Effect of nutritional and environmental conditions on the production and composition of rhamnolipids by *Pseudomonas aeruginosa* UGX. *Microbial. Resource*. *155*:249-256.

Makkar, R.S., Cameotra, S.S. and Banat, I.M. (2011). Advances in utilization of renewable substrates for biosurfactant production. *Applied Microbiology Biotechnology. 1*:1-5.

Maneerat, S. (2005). Biosurfactant from marine micro-organisms. Songkianakarin. *Journa Science Technology. 27*: 1263-1272.

Maier, R. M and Soberon-Charez, G. (2000). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Microbiology Biotechnology.54*:625.

Maslin, P. and Maier, R.M. (2000). Biosurfactants as emulsifying agent for hydrocarbons. *Bioremediatio Journal. 4*: 295.

Marchant, R. and Banat, I.M. (2012). Biosurfactants as emulsifying agent for hydrocarbons. *Trends Biotechnology. 30*:558.

Margaritis, A., Zajic, J. and Gerson, D. (1979) "Production and surface active properties of microbial surfactants". *Biotechnological and Bioengineering. 21*:1151-1162.

Makkar, R.S. and Cameotra, S.S. (2002). Effects of various nutritional supplements on biosurfactant production by a strain of *Bacillus subtilis* at 45^0C. *Journal Surfactant Detergent. 5*:11-17.

Matsufuji, M., Nakata, K. and Yoshimoto, A. (1997). High production of rhamnolipids by Pseudomonas aeruginosa growing in ethanol. *Botechnology Letters. 19*:1213-1215.

Marchant, R., Furston, S., Uzoigwe, C., Rahman, P.K. S. M and Banat, I,M. (2014). *Production of biosurfactants from non-pathogenic bacteria*. In Biosurfactants. Taylor & Francis: New York, USA. Pp. 73-81.

Matsufuji, M.,Nakata, K. and Yoshimoto, A. (1997). High production of rhamnolipids by Pseudomonas aeruginosa growing on ethanol. *Biotechnology. Letter*. 19: 1213-1215.

Makar, R. S., Cameotra, S.S. (2002). Effects of various nutritional supplements on biosurfactant production by a strain of *Bacillus subtilis* at 45^0C. *Journal Surfactants Detergents. 5*:11-17.

Mercade, M.E., Manresa, M.A., Robert, M. Espuny, M.J., deAndres, C. and Guinea, J. (1993). Olive oil mill effluent (OOME). New substrate for biosurfactant production. *Bioresource Technology. 43*:1-6.

Meylheuc, T., Heary, J.M. and Bellon-fontaine, M.N. (2001). Biosurfactants as emulsifying agent for hydrocarbons. *Science des Aliments. 21*:591.

Mendes, A.N., Filgueiras, L.A., Pinto, J.C. and Nele, M. (2015). Physico chemical properties of rhamnolipid biosurfactant from *Pseudomonas aeruginosa* PA1 to applications in micro emulsions. *Journal Biomater Nanbiotechnology 6:* 64-79.

Meylheuc, T., VanOss, C.J. and Bellon-Fontaine, M.W. (2006). Biosurfactants as emulsifying agent for hydrocarbons. *International Journal Food Microbiology. 109*:71

Mercade, M.E., Manresa, M.A., Robert, M. Espuny, M.J., de Andres, C. and Guinea, J. (1993). "Ohre oil mill effluent (OOME). New substrate for biosurfactant production". *Bioresource Technology*. *43*:1-6.

Meylheu, T., Van Oss, C.J. and Bellon-Fontaine, M.N. (2001). Adsorption of biosurfactants on solid surfaces and consequences regarding the bioahesion of *Listeria monocytogenes Lo28*. *Journal Applied Microbiology*. *91*: 822-832.

Morikawa, M., Hirata, Y., Imaraka, T.A. (2000). A study on the structure – function relationship of lipopeptide biosurfactant. Biochemcal Biophyics Actalogue. 1488:211-218.

Muller, M.M., Horman, B., Kugel, M., Syldatk, C. and Hausmann, R. (2011). Evaluation rhamnolipid production capacity of *Pseudomonas aeruginosa*, PAOI in comparism to the rhamnolipid over producer strains DSM2874. *Applied Microbiology Biotechnology*. *89*: 585-592.

Muller, M.M., Kuglor, J.H., Henkel, M., Gerlitzi, M., Hormanna, B., Pohnlein, M., Syldate C. and Hausmann, R. (2012). *Journal Biotechnology*. *162*:366.

Muthusamy, K., Gopalakrishan, S., Ravi, T.K. and Sivachidambaram, P. (2008). Biosurfactants as emulsifying agent for hydrocarbons. *Curriclum Science*. *94*:736.

Muller, M.M. and Hausmann, R. (2011). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Microbia*. *Biotechnology*. *91*:251.

Mulligan, C.N., Yong, C.N. and Gibbs, B.F. (1999). Biosurfactants as emulsifying agent for hydrocarbons. *Environmental*. *Microbial*. *66*:4585.

Mulligan, C. N., Yong, R.N. and Gibbs, B.F.L. (2001). Biosurfactants as emulsifying agent for hydrocarbons. *Journal Hazardous Materials*. *85*:111.

Mulligan, C. and Gibbs, B.F. (1989). "Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*". *Applied and Environmental Microbiology*. *55*(11): 3016-3019.

Mulligan, C., Mahmourides, G. and Gibbs, B.F. (1989). "The influence of phosphate metabolism on biosurfactant production by *Pseudomonas aeruginosa*. *Journal of Biotechnology*. *12*:199-210.

Mulligan, C.N., Cooper, D.G. and Neufield, R.J. (1984). *Selection of microbial producing biosurfactants in Biosurfactants, Production*, *Properties, Application*. Pp. 329-371 ed. N Kosanc (New York: Marcel Dekker).

Mulligan, C.N. and Gibbs, B.F. (1993). *Factors influencing the economics of Biosurfactants, in Biosurfactants, Production, Properties*, Application. Pp.329-371 ed. N.Kosaric (New York:Marcel Dekker).

Mistra, S. and Singh, S. N. (2012). Microbial degradation of n-hexadecane in mineral salt medium as mediated by degradative enzymes. *Bioresource Technology*. *111*: 148-154.

Narayanan, J., Ramji, R., Sahu, H. and Gautem, P. (2010). Biosurfactants as emulsifying agent for hydrocarbons. *IET Nanobiotechnology*. *4*:29.

Neu, T.R. and Poralla, K. (1990). Emusifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. *Applied Microbol*ogy *Biotechnology*. *32*:521-525.

Nitschke, M. and Costa, S.G.V.A.O. (2007). *Trends Food Science Tech*nology *18*:252.

Nitschke, M. and Costa, S.G.V.A.O. (2011). Biosurfactants as emulsifying agent for hydrocarbons. *Contiero, process biochemical*. *46*:621.

Nitschke, M., Costa, S.G. and Conhero, J. (2010). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Biochem*ical *Biotechnology*. *160*:2066.

Nie, M., Yin, X., Ren, C., Wang, Y., Xu, E. and Sha, Q. (2010). Biosurfactants as emulsifying agent for hydrocarbons. *Biotechnology Advance. 28:* 635.

Nitschke, M., Costa, S.G.V.A.O. and Contiero, J. (2005). Biosurfactants as emulsifying agent for hydrocarbons. *Biotechnology Programe*. *21*:1593.

Noordman, W.H. and Janssen, D.B. (2002). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Environmental Microbiology*. *68:*4502.

Nguyen, T.T. and Sabatini, D.A., (2009). Biosurfactants as emulsifying agent for hydrocarbons. *Journal Surfactants Detergents. 12*:109.

Ochener, P., Uris, A., Hembach, T. and Fietcher, A. (1995). In:Biotechnology *Engineering Biotechnology*. *53*: 89.

Okoh, A., Ajisebutu, S., Babalola, G. and Treejo-Hermanchia, M.R.(2001). "Potential of *Burkholdina cepacia* RQ1 in the biodegradation of heavy crude oil. *International Microbiology*. *4*:83-87.

Oliveira, F.J.S., Vazquez, L., deCampos, N.P. and deFranca, F.P. (2009). Production of rhamnolipids by *Pseudomonas allaligenes* strain. *Process Biochemical. 44*: 383-389.

Onbasli, D., Marchal, R. and Vandecasteele, J.P. (1996). Biosurfactants as emulsifying agent for hydrocarbons. *Applied. Microbiology Biotechnology. 45*: 162.

Ozdemir, G. and Malayoglu, U. (2004). Biosurfactants as emulsifying agent for hydrocarbons. *Colloids surfaces, Biosurfactant. 39*:1.

Pacwa-Plociniczak, M., Plaza, G.A., Piotrowska-Seget, Z and Cameotra, S.S. (2011). Environmental applications of biosurfactants: recent advances. *International Molecular Science. 12*:633-654.

Palanisamy, P. and Raichur, A.M. (2009). Biosurfactants as emulsifying agent for hydrocarbons. *Material Science Engineering. C. 29*:199.

Pattanath, K.M., Rahman, K.S. and Gakpe, E. (2008). Production, characterization and applications of biosurfactants – Review. *Biotechnology. 7*: 360-370.

Palanisamy, P. (2008). Biosurfactant mediated synthesis of N.O nanorods. *Material Letter. 62*: 743-746.

Persson, A., Osterberg, E. and Dostalek, M. (1998). "Biosurfactant production by *Pseudomonas Fluorescens* 378: Growth and product characteristics". *Applied Microbiology and Biotechnology. 29*:1-4.

Peypoux, F., Bonmatin, J.M and Wallach, J. (1999). Recent trends in the biochemistry of surafctin. *Applied Microbiology Biotechnology. 51*:553-563.

Persson, A., Osterberg, E. and Dostalek, M. (1988). *Applied microbiology and Biotechnology 29*:1.

Plaza, G.A., Zymony, I. and Banat, I.M. (2006) *Petrolum Science Engineering. 50*:71-77.

Pruthi, V. and Cameotra, S.S. (1997). Production, properties of a biosurfactant synthesized by *Arthrobacter protophormiae* an antarctic strain. *World journal Microbiology Biotechnology. 13*:137-139.

Piljac, G. and Piljac, V. (1995). (US Patent). 5, 455, 232.

Piljac, A., Stipceric, T., Piljac-Zagara, J. and Piljac, G. (2008). Biosurfactants as emulsifying agent for hydrocarbons. *Journa Cutanoeus Medical Surgery. 12:*142.

Rahman, K.S., Rahman, I.J., McClean, S., Marchant, R and Banat, I.M. (2002). *Biotechnology Program. 18*.1277-1281.

Rosenberg, E. and Ron, E. (1999). Biosurfactants as emulsifying agent for hydrocarbons. *Applied Microbiology Biotechnology. 52*:154-162.

Rodrgues, L.R., Teixeira, J.A., Vander Mei, H.C. and Oliveira, R. (2006) Biosurfactants as emulsifying agent for hydrocarbons. *Colloids Surfactant Biosurfactant 49*:79-86.

Rosen, M.J. (1978). *Surfactants and Interfacial phenomena* (New York: John Wiley and Sons).P. 20.

Rosenberg, M. (1984). *Bacterial adherence to hydrocarbon*: a useful technique for study cell surface hydrophobicity FEMS.

Rikalovic, M.G., Abdel. Marogoud, A.M., Deziel, E., Nestorovic Z., Vrric, M.M. and Kordzic, I.M. (2013). Recent trends in the biotechnogy. *Journal Surfactants Deterg*ents.*16*: 673.

Rufino, R.D., Luna, J.M., Sarubbo, L.A., Rodngues, L.R.M., Teixeira, J.A.C and Campos-Takaki, G.M (2001). Antimicrobial and anti-adhensive potential of a biosurfactant Refisan produced by Candida Lipolytica UCP 0988. *Coll. Surfactant Biosurfactant Biointerfaces. 84*: 1-5.

Sarubbo, L.A., Luna, J.M. and Campus-Takaki, G.M. (2006). Production and stability studies of the bioemulsifier obtained from a new strain of *Candida glabrataUCP* 1002. *Electrical Journal Biotechnology. 9:*400-406.

Sarubbo, L.A., Fanas, C.B.B. and Campos–Takaki, G.N (2007). Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. *Curriculum Microbiology. 54:*68-73.

Santa-Anna, I.M., Sebashan, G.V., Pereira, N., Jr., Alves, T.L.M., Meneses, E.P. and Freire, D.M.G. (2002). Production of biosurfactant from a new and promising strain of Pseudomonas aeruginosa PAI. *Applied Biochemical Biotechnology*. *91*:459-467.

Sandrin, C., Peypoux, F. and Michel, G. (2000) Co-production of surfactin and Iturin A, Lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. *Biotechnology. Applied Biochemical. 12:* 370-375.

Sajna, K.V., Sukumaran, R.K., Goltumukkala, L.D. and Pandey, A. (2015). Crude oil biodegradation adied by biosurfactants from *Pseudozyma* sp. Nil08165 or its culture broth. *Bioresource. Technology. 191*:133-139.

Sakthipriya, N., Doble, M. and Sangwou, J.S. (2015b).Bioremediation of coastal and marine pollution due to crude oil using a micro-organism *Bacillus subtilis*. *Procedia Engineering*. *116*:213-220.

Sanjeet, M., Pryangshu, M. and Sarma, B. (2004). Crude oil degradation efficiency of a recombinant *Acinetobacter bumunnii* strain and its survival in crude oil contaminated soil microcosom. *FEMS. Microbial Letter.* 235: 323-331.

Schumacher, D. (1999). *Geochemical tools surface geochemical exploration* (w) T. Beaumont, ed.,Exploring for traps and seals:AAPG Treatise of Petroleum Geology Handbook. Pp.1-20.

Sobrinho, H.B.S., Luna, J.M., Rufino, R.D., Porto, A.L.F and Sarubbo, L.A. (2013). Assessment of toxicity of biosurfactant from Candida sphaenca UCP 0995 cultivated with industrial residues in a bioreactor. *Electronical Journal Biotechnol*ogy. *16*.

Souza, E.C., Vessoni-Penna, T.C. and Souza Oliveira, R.P. (2014). Biosurfactant-enhanced hydrocarbon bioremediation: An overview. *International Biodeterioration Biodegradation. 89:*88-94.

Sharma, S.I. and Pant, A. (2001). *Biodegradation. 11*(5): 289-294.

Sharma, S.I. and Pant, A. (2009). Biosurfactants as emulsifying agent for hydrocarbons. *Biodegradation. 11*(5): 289-294.

Shahin, Mamdouh (2002). *Hydrology and water resources of Africa*. Springer. pp. 307–309. ISBN 1-4020-0866-X.

Syldatk, C. and Wagner, F. (1985). *Production of biosurfactant*. In Biosurfactants and Biotechnology; Kosaric, N., Cairns, W.L., Gray, N.C.C; Eds, Marcel Dekke, New York, Ny, USA, Pp. 89-120.

Silva, R.C.F.S., Almeida, D.G., Luns, J.M., Rufino, R.D., Santos, V.A. and Sarubo L.A. (2010). Application of Biosurfactants in the petroleum industry and the remediation of oil spills. *International Journal Molecular Science. 15*:12523-12542.

Silva, S.N.R.L., Fanas, C.B.B., Rufino, R.D., Luna, J.M. and Sarubbo, L.A. (2010). Glycenel as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCPO992. *Cell Surface .B*iosurfactant *Biointerfaces.79*:174-183.

Siegmund, I. and Wagner, F. (1991). Biosurfactants as emulsifying agent for hydrocarbons. *Biotechnology. Technology. 5:*265.

Stipceric, T., Piljac, T., Piyac, J., Dujmic, T. and Piljac, D. Biosurfactants as emulsifying agent for hydrocarbons. (2001)(PCT Patent Application) WO 01/10447 A1.

Siegmund, I. and Wagner, F. (1991). New method for detecting rhamnolipids exerted by pseudomonas species grown on mineral agar. *Biotechnology Technology. 5*:265-268.

Tahzibi, A., Kamal, F. and Assadi, M.M. (2004). Biosurfactant production. *Iran. Biomedical Journal. 8*:25.

Thaniyavarn, J., Chacinguthaui, T., Sangvanich P., Roongsawang, N., Washio, K., Monkawa, M. and Thaniyavein, S. (2008). Production of sophorolipid biosurfactant by picdic anomals. *Bioscicense Biotechnology Biochemical. 72*:2061-2068.

Tan, H.M. (2000). Biosurfactants and their roles in bioremediation. *Cheong Jit Kong. 1*:12.

US Energy Information Administration. Annual Energy Review (2010). Avaliable online: http://large.stanford.edu/courses/2012/ph241/dnizgulsikz/docs/aer/pdf (accessed on 05 September 2015).

Van Haesendinck, I.P.H. and Vanzeveren, E.C.A. (2004). (International application patent PCT), W.O. 2004/040984.

Vansileva –Tonckova, E., Galabova, D., Karpenko, E. and Shulgs, A (2001). Rhamnolipid production. *Letter Applied Microbiol*.ogy *33:*280.

Varnier, A.L., Sanchez, L., Vastsa, P., Boudesocque, L., Grecia-Brugger, A., Rabenoelina F., Sorokin, A., Renault, J.H., Kauffmann, S., Plugin, A., Clement, C., Bacillioul, F. and Dorey, S. (2009). Production of rhamnolipid. *Prodct Plant cell Environmental. 32:*178.

Velmurgan, S., Govindarg, R., Gokutakuma, B. and Ravi, S.(2012). Asia Journal of plant Science and Research. 2(4):473-477.

Vijayakumar, 5. And Saravanan, V. (2015). Biosurfactants-types, sources and applications. *Resource Journal Microbial. 10*: 181-182.

Wu, J.Y., Yeh, K.L., Lu, W.B., Lin, C.L. and Chang, J.S. (2018). Rhamnolipid production with indigenous *Pseudomonas aeruginosa* Emi Isolated from oil-contaminated site. *Bioresource Technology. 99*:1157-1164.

Whyte, L. G., Smites, T. H., Labbe, D., Withol, B., Greer, C. W. and VanBeilen, J.B. (2002). Gene cloning and characterization of multiple alkane hydroxylase syetems in Rhodoccus strains Q15 and NRRL B-16531. *Applied Environmental Microbiology. 68*:5933-5947.

Xie, Y., Li, Y. and Ye, R. (2005). Microbially enhancement oil recovery. Journal Dispersion Science Technology. 26:455.

Xie, Y., Ye, R. and Liu, H. (2006). Microbially enhancement oil recovery. *Colloides Surfaces*. *279*:175.

Youssef, N.H., Duncan, K.E., Nagle, D.P., Savage, K.N., Knapp, R.M. and Mclnerry, M.J. (2004). Comparison of methods to detect biosurfactant production by diverse micro-organisms. *Journal Microbiology Methods. 56*:339-349.

Yakinov, M.M., Timmis, K.N., Wrey, V. and Tredrickon, H.L. (1995). Characterization of a new lipopepide surfactant produced by thermo telorent and halotolerant subsurface *Bacillus lichenformis* BA50. *Applied Environmental Microbial. 61*:1706-1713.

Yateem, A., Balba, M.T., Al.Shayli, Y. and Al. Awadhi, N. (2002). Isolaha and characterization of biosurfactant producing bacterial from oil contaminated soil. *Soil Sediment Contamination . 11:*41-55.

Yoo, I.A., Fashman, M.J.R.(2001). An examination of the continental shelf pump in an open ocean general circulation model. *Global Biogeochemical cycles*. 15(*4)*: 831-844.

Zhang, Y. and Miller, R.M. (1994). "Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobilcity and biodegradation of Octadecane". *Applied and Environmental Microbiology*. 6.(*6*): 2.101-2.106

APPENDIX

4.9. STATISTICAL ANALYSIS

The results of the one way analysis of biosurfactant by Pseudomonas aeruginosa were performed according to design matrix detailed in Table 4.5a. The interactive effect of crude oil (versus palmoil, disel, groundnutoil, tween80); palmoil (versus groundnutoil, tween80); diesel (versus palmoil, groundnutoil, tween80); was negative while the interactive effect of palmoil (versus groundnutoil, tween80); diesel (versus tween80) had positive interactive effects. The ANOVA for the bidegradation of crude oil by *Pseudomonas aeruginosa* after 1month of incubation period was given in Table 11. The stastical significance of biodegradtion of crude oil using biosurfactant produced by *Pseudomonas aeruginosa* was evaluated by F test. The result of F value showed that the biodegradation of crudeoil by pseudomonas aeruginosa was significant (Fig4.8)

The ANOVA for the effects of carbon sources on biosurfactant production was given in Table 4.9. The stastical significance of the carbon sources was evaluated by F test. The result of F value (204.451) showed that the carbon source is highly significant (P: 0.00).

The results of the one way analysis of variance on the effect of nitrogen sources on the production of biosurfactant by *Pseudomonas aeruginosa* were performed

according to design matrix of SPDS 10.0 software detailed in Table 4.6a. The interactive effect of sodium nitrate, ammonium nitrate, urea, ammonium chloride); urea (versus ammonium nitrate); ammonium chloride (versus sodium nitrate,

ammonoium nitrate , urea) was negative while the interactive effect of sodium nitrate (versus yeast extract, ammonium chloride); ammonium nitrate (versus sodium nitrate, yeast extract, urea, ammonium chloride); urea (versus sodium nitrate, yeast extract, ammonium chloride); ammonium chloride (versus yeast extract) had positive interactive effects.

The ANOVA for the effects of nitrogen sources on the production of biosurfactant was given in Table 4.6b. The stastical significance of nitrogen sources was evaluated by the F test. The result of the F value (505.266) showed that the nitrogen sources are highly significant (P: 0.00)(Fig.12).

Table 4.5a: One way on the effects of carbon sources on biosurfactant production

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Crude Oil	3	.4000	.02000	0.1155	.3503	.4497	.38	.42
Palm Oil	3	.2033	.02082	.01202	.1516	.2550	.18	.22
Disel	3	.2067	.01528	.00882	.1687	.2446	.19	.22
Groundnut Oil	3	.1067	.01528	.00882	.0687	.1446	.09	.12
Tween80	3	.0503	.00115	.00607	.0475	.0532	.05	.05
Total	15	.1934	.12405	.03203	.1247	.2621	.05	.42

Table 4.5b: ANOVA on the effect of Carbon sources Biosurfactant Production by Pseudomonas aeruginosa

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.213	4	.053	204.451	.000
Within Groups	.003	10	.000		
Total	.215	14			

Table 4.5c: Multiple comparisons on the different Carbon sources Biosurfactant Production by Pseudomonas aeruginosa

(1) Carbon Sources	(J) Carbon Sources	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude Oil	Palm Oil	.19667	.01317	.000	.1673	.2260
	Disel	.19333	.01317	.000	.1640	.2227
	Groundnut Oil	.29333	.01317	.000	.2640	.3227
	Tween80	.34967	.01317	.000	.3203	.3790
Palm Oil	Crude Oil	-.19667	.01317	.000	-2260	-1673
	Disel	-.00333	.01317	.805	-0327	.0260
	Groundnut Oil	.09667	.01317	.000	.0673	.1260
	Tween80	.15300	.01317	.000	.1237	.1823

Table 4.6a: Oneway on the effect of nitrogen sources on biosurfactant production by Pseudomonas aeruginosa

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	maximum
					Lower Bound	Upper Bound		
Sodium Nitrate	3	.200000	.0200000	.0115470	.150317	.249683	.1800	.2200
Ammonium Nitrate	3	.400000	.0100000	.0057735	.375159	.424841	.3900	.4100
Yeast Extract	3	.000000	.0000000	.0000000	.000000	.000000	.0000	.0000
Urea	3	.213333	.0152753	.0088192	.175388	.251279	.2000	.2300
Ammonium Chloride	3	.050000	.0010000	.0005774	.047516	.052484	.0490	.0510
Total	15	.172667	.1459685	.0376889	.091832	.253501	.0000	.4100

Table 4.6b: Anova on the effect of nitrogen sources on biosurfactant production by Pseudomonas aeruginosa

	Sum of squares	Df	Mean Square	F	Sig.
Between Groups	.297	4	.074	505.266	.000
Within Groups	.001	10	.000		
Total	.298	14			

Table 4.6c: Multiple comparisons on the different sources on biosurfactant production by Pseudomonas aeruginosa

(1) Nitrogen Sources	(J) Nitrogen Sources	Mean Difference	Std. Error	Sign.	95% Confidence Interval	
					Lower Bound	Upper Bound
Sodium Nitrate	Ammonium Nitrate	-2000000	.0098950	.000	-222047	-177953
	Yeast Extract	.2000000*	.0098950	.000	.177953	.222047
	Urea	-.0133333	.0098950	.208	-.035381	.008714
	Ammonium Chloride	.1500000*	.0098950	.000	.127953	.172047
Ammonium Nitrate	Sodium Nitrate	-2000000	.0098950	.000	.177953	.222047
	Yeast Extract	.4000000*	.0098950	.000	.377953	.422047
	Urea	.1866667	.0098950	.000	.164619	.208714
	Ammonium Chloride	.3500000*	.0098950	.000	.327953	.372047
Yeast Extract	Sodium Nitrate	-2000000	.0098950	.000	-222047	-177953
	Yeast Extract	-4000000*	.0098950	.000	-422047	-377953
	Urea	-2133333	.0098950	.000	-235381	-191286
	Ammonium Chloride	-.0500000*	.0098950	.000	-072047	-027953
Urea	Sodium Nitrate	.0133333	.0098950	.208	-008714	.035381
	Ammonium Nitrate	-.1866667	.0098950	.000	-208714	-164619
	Yeast Extract	.21333333	.0098950	.000	.191286	.235381
	Ammonium Chloride	.1633333	.0098950	.000	.141286	.185381
Ammonium Chloride	Sodium Nitrate	-.15000000	.0098950	.000	-172047	-127953
	Ammonium Nitrate	-35000000	.0098950	.000	-372047	-327953
	Yeast Extract	.05000000	.0098950	.000	.027953	.072047
	Urea	-.1633333	.0098950	.000	-185381	-141286

Table 4.7: One way on the biodegradation of crude oil by Pseudomonas aeruginosa after 1 month incubation period

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Biodegradation 2 weekw	0 day	15	343.85758667	433.224611792	111.858113776	103.94579330	583.76938003	7.461400	1756.72460
	Crude oil + sterile soil + Pseudomonas aeruginosa	16	25.50268125	14.775587247	3.693896812	17.52932657	33.27603593	.000000	45.08800
	Ordinary Soil + Crude oil	15	282.0919333	235.569539455	60.82393545	151.64513063	412.55325604	7.460600	777.66090
	Sterile Soil + Crude Oil	16	249.02565625	382.620516733	95.655129183	45.14157462	452.90973788	5.505000	1498.22750
	Total	62	222.26137258	326.700631963	41.491021750	139.29494692	305.22779824	.000000	1756.72460
Biodegradation 1 month	0 day	15	343.8575867	433.22461179	111.85811378	103.9457933	583.7693800	7.46140	1756.7246
	Crude oil + sterile soil + Pseudomonas aeruginosa	16	10.7903375	10.69746203	2.67436551	5.0900624	16.4906126	.00000	27.7069
	Ordinary Soil + Crude oil	12	175.2419417	139.89335008	40.38373166	86.3579476	264.1259358	513840	544.2596
	Sterile Soil + Crude Oil	10	174.0933300	231.64552190	73.25274590	8.3841062	339.8025538	22.91080	823.2994
	Total	53	173.1008642	283.74023048	38.97471807	94.8923706	251.3093577	.00000	1756.7246

Table 4.8: Anova on the biodegradation of crude oil by Pseudomonas aeruginosa

		Sum of Squares	Df	Mean Square	F	Sig.
Biodegradation 2 weekw	Between Groups	907007.801	3	302335.934	3.129	.032
	Within Groups	5603723.678	58	96615.295		
	Total	6510731.478	61			
Biodegradation 1 month	Between Groups	858948.048	3	286316.016	4.216	.010
	Within Groups	332749.090	49	67908.059		
	Total	4186442.958	52			

Table 4.9: Multiple Comparisons on the biodegradation of crude oil by *P.*

Dependent Variables	Materials	Mean Difference (I – J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Biodegradation 2 weeks	0 day	Crude oil + sterile Soil + Pseudomonas aeruginosa	318.454905417	111.711937771	.004	94.83916639	542.07064444
		Ordinary soil+ crude	61.758393333	113.499442275	.588	-165.43552471	288.95221138
		Sterile Soil + crude oil	94.831930417	111.711937771	.399	-128.7838061	318.44766944
	Crude oil + sterile soil + Psedomonas aeruginosa	0 day	-318.454905417	111.71193771	.004	-542.07064444	-94.83916639
		Ordinary Soil + Crude oil	-256.696512083	111.711937771	.025	-480.31225111	-33.08077306
		Sterile Soil + Crude Oil	-223.622975000	109.895362434	.046	-443.60244341	-3.64350659
	Ordinary Soil + Crude oil	0 day	-61.758393333	113.499442275	.588	-288.95221138	165.43542471
		Crude oil+ Sterile Soil + P.aeruginosa	256.696512083	111.711937771	.025	33.08077306	480.32125111
		Sterile Soil + crude oil	33.073537083	111.711937771	.768	-.256.68927611	256.68927611
	Sterile soil + crude oil	0 day	-94.831930417	111.711937771	.399	-318.44766944	126.78380861
		Crude oil+ Sterile Soil + P. aeruginosa	223.622975000	109.895362434	.046	3.64350659	443.60244341
		Ordinary Soil + crude oil	-33.073537083	111.711937771	.768	-256.689276	190.54220194
Biodegradation 1 month	0 day	Crude oil + sterile Soil + P. aeruginosa	333.06724917	93.65606050	.001	144.8583492	521.2761491
		Ordinary Soil + Crude Oil	168.61564500	100.92675019	.101	-34.2042500	371.4355430
		Sterile Soil + crude oil	169.76425667	106.38613582	.117	-44.0266974	383.5552008
	Crude oil + Sterile Soil + P. aeruginosa	0 day	-333.06724917	93.65606050	.001	-521.2761491	-144.8583492
		Ordinary Soil + Crude oil	-164.45160417	99.51511773	.105	-364.4347205	35.5315121
		Sterile Soil + Crude Oil	-163.30299250	105.04789216	.126	-374.4046383	47.798650
	Ordinary Soil + crude Oil	0 day	-168.61561500	100.92675019	.101	-371.4355430	34.2042530
		Crude oil+ Sterile Soil + P. aeruginosa	164.45160417	99.51511773	.105	-35.5315121	364.4347205
		Sterile Soil + crude oil	1.14861167	111.57872057	.992	-223.0772222	225.374455
	Sterile Soil + crude oil	0 day	-169.76425667	106.38613582	.117	-383.5552008	44.0266874
		Crude oil+ Sterile Soil + P. aeruginosa	163.30299250	105.04789216	.126	-47.7986503	374.4046353
		Sterile Soil + crude oil	-1.14861167	111.57872051	.992	-225.3744455	223.0772222

Table 6: Field scale experiment in the biodegradation of crude oil *Pseudomonas aeruginosa* isolates after two weeks of inoculation

Parameters	O day	Sterile soil + crude oil (µg/g)	Ordinary soil +crude oil	Crude oil + soil + micro-organisms
C_{10}	7.4614	5.5050	7.4606	-
C_{11}	316.4808	291.9636	-	45.0887
C_{12}	581.0955	492.1261	577.6609	31.41
C_{13}	581.0955	336.6638	52.7328	-
C_{14}	91.8402	88.1734	86.2177	20.1732
C_{15}	1756.7246	1498.2275	772.3882	38.7234
C_{16}	197.1404	99.4345	196.9879	38.1738
C_{18}	95.1479	88.7450	36.226	22.5606
C_{23}	89.6329	85.0212	59.0784	34.64
C_{21}	139.0285	113.1356	102.4169	30.4161
C_{24}	76.7548	74.74	73.5394	31.5629
C_{29}	335.3675	116.1521	318.4646	37.721
C_{34}	84.2062	62.7240	82.2570	20.2234
C_{32}	89.2776	79.0536	34.1254	-
C_{36}	366.6100	-	-	-
C_{35}	366.6100	297.8320	297.3840	15.860
Total	4800.8638	3729.4974	2696.9388	366.5526

Table 7: Field scale experiment on the biodegradation of crude oil by the *Pseudomonas aeruginosa* after 1 month of incubation period.

Parameters	O day	Sterile soil crude oil (µg/g)	Ordinary soil crude oil	Crude oil + soil + micro-organisms
C_{10}	7.4614	-	5.1384	-
C_{11}	316.4805	95.6877	-	27.101
C_{12}	581.0955	-	44.2596	18.203
C_{13}	581.0955	-	18.8126	-
C_{14}	91.8402	80.4366	10.9586	
C_{15}	1756.7246	823.2994	-	15.3603
C_{16}	190.1404	-	131.1036	11.343
C_{18}	95.1479	13.3930	31.9423	12.029
C_{23}	89.6329	80.2304	487.6856	20.7405
C_{21}	139.0283	111.6822	100.5685	-
C_{24}	76.7548	68.3189	61.1753	19.9361
C_{29}	335.3675	108.5490	-	24.7069
C_{34}	84.2062	40.4252	63.7535	20.2234
C_{32}	89.2776	-	29.7076	-
C_{36}	366.6100	22.9108	-	-
C_{35}	366.6100	22.9108	-	-
Total	4800.8638	1444.9332	985.1046	169.6432