DETERMINATION OF PETROLEUM PRODUCT-EMULSIFICATION POTENTIALS OF SOME BACTERIAL ISOLATES FROM BONNY RIVER, RIVERS STSTE

BY

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CERTIFICATION

This is to certify that this work, "Determination of Petroleum Products-Emulsification Potentials of some Bacterial Isolates from Bonny, Rivers State", was carried out by CHARLES OBINNA ANYANWU (20164993458) in partial fulfilment for the award of the degree of M.Sc. in Biotechnology in the Department of Biotechnology of the Federal University of Technology, Owerri.

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DEDICATION

This work is dedicated to my parents Mr and Mrs E.N. Anyanwu for their financial and morale support, to my siblings, I.K, Chigozie and Blessing for their understanding, love and prayers.

Special dedication to my ever sweet, lovely, caring, and compassionate grandmother, Nneoma Dorathy Anyanwu for her elderly love and encouragement.

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ABSTRACT

In this study, the petroleum product-emulsification potentials of some bacteria isolated from Bonny River and its river bank was investigated. A total of ten (10) bacteria isolates, belonging to six genera namely *Bacillus*, *Pseudomonas*, *Klebsiella*, *Escherichia*, and *Salmonella* were isolated. Emulsification assay carried out include drop collapse, oil spread and emulsification index test on the isolates using crude oil, kerosene, diesel and coconut oil as the sole carbon source. Emulsification index assay showed statistical significance ($p<0.05$) in the emulsification potentials of the isolates. *Klebsiella* sp C4 showed the best emulsification activity with 53.3%, 58.2%, 56.0% and 51.9% emulsification index using diesel oil, coconut oil, kerosene and crude oil respectively. It was followed by *Pseudomonas* sp HC4 with 51.0%, 48.0%, 54.0% and 49.0% emulsification activity using diesel oil, coconut oil, kerosene and crude oil respectively, *Bacillus* sp HC6 had emulsification indices of 47.0%, 51.0%, 48.2% and 46.7% for diesel, coconut oil, kerosene and crude oil respectively as sole carbon source. The effects of pH on the growth of the isolates showed that *Pseudomonas* sp HC4 had the highest growth of about 2.25×10^5 cfu/ml at pH 6.5. *Bacilius* sp HC6, *Klebsiella* sp C4 recorded growths of 2.04×10^5 and 1.70×10^5 cfu/ml at pH 7.5 and 7.0 respectively. Statistical analysis showed no significant difference ($p>0.05$) in the growth of the isolates at the various pH ranges tested. These isolates were able to utilize and grow on different nitrogen sources containing ammonium sulphate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$), and ammonium chloride (NH_4Cl). NH_4Cl was the best utilized nitrogen source with growth of 2.37×10^5, 2.17×10^5, and 1.93×10^5 cfu/ml for *Pseudomonas, Bacillus* and *Klebsiella* species respectively. Statistical analysis showed a significant difference ($p<0.05$) between the utilization of nitrogen sources and the growth of the isolates. The effects of pH on the utilization of Bonny light crude oil showed no statistical significance ($p>0.05$) and revealed percentage utilization of about 76%, 68%, and 58% at pH of 6.5, 7.5 and 7.0 respectively for *Pseudomonas, Bacillus and Klebsiella* species. Percentage utilization of about 67%, 63%, and 51.8% were recorded for *Pseudomonas, Bacillus and Klebsiella* species when grown on mineral salt media supplemented with NH_4Cl. The isolates were tested for their ability to utilize bonny light crude oil using the gravimetric method. The percentage utilization of all the isolates ranged between 54.2% and 77.5% after 28 days of incubation for *Pseudomonas, Bacillus* and *Klebsiella* species respectively.

Key words: Emulsification, Emulsification index, Drop collapse, Oil spread.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Biosurfactants are surface-active biomolecules produced by microorganisms with wide-range of applications. In recent years, due to their unique properties like specificity, low toxicity and relative ease of preparation, these surface-active biomolecules have attracted wide interest. Due to their unique functional properties, biosurfactants are used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others. They can be used as emulsifiers as well as demulsifiers, wetting agents, foaming agents, spreading agents, functional food ingredients and detergents. The interfacial surface tension reducing ability of biosurfactants made them to play important role in oil recovery and bioremediation of heavy crude oil (Volkering *et al*., 1998; Vijayakumar and Saravanan, 2015).

Biosurfactants possess both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities such as hydrocarbons and water (Banat, 1995; Karanth *et al*., 1999) hence, decrease interfacial surface tension (Volkering *et al.,* 1998).

When compared to chemical or synthetic surfactants, biosurfactants gained several advantages including their biodegradability, biocompatability and digestibility. Biosurfactants can be used in environmental cleanup by biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil. Their specificity and availability of raw materials also made them most preferred surfactants (Olivera *et al*., 2003).

Surfactants are surface active compound that reduce the interfacial tension between two liquids, or that between a liquid and a solid. Surfactants are organic compound that contain both hydrophobic (head part of the surfactant) and hydrophilic (tail part of the surfactant) moieties. Thus surfactant contains both water insoluble i.e. water repellent group as well as water soluble i.e. water loving group. Biosurfactants are also surface active compound like chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by microbes like bacteria, fungi and yeasts. Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are

usually non-toxic and biodegradable. Recently interest in biosurfactant has increased because of its diversity, flexibility in operation, and more ecofriendly than chemical surfactant (Saharan, 2011)

They are considered as one of the high values of microbial products, which have gained considerable interest in recent years, they have become an important product of biotechnology for industrial and medical applications (Nitschke and Costa, 2007, Makkar *et al.*, 2011). The reasons for their publicity are lower toxicity, specificity of action, simplicity of preparation and extensive applicability. Moreover, they can be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial regions such as: organic chemicals, pharmaceuticals and cosmetics, beverages and foods, metallurgy, mining, petroleum, petrochemicals (Banat *et al*., 2000; Perfumo *et al.*, 2010; Vedaraman and Venkatesh, 2011). Biosurfactants have many advantages over synthetic ones, including bioavailability, structural diversity, specific activity at extreme salinity, temperatures and pH (Datta *et al.*, 2011). In spite of these advantages, good attributions and a variety of potential uses of biosurfactants, efforts to commercial production have failed due to the low yield obtained and high production cost. Increasing biosurfactant yields and decreasing production costs are essential factors affecting the efficiency of biosurfactant production process (Kosaric, 1992; Bognolo, 1999; Moussa *et al*., 2006). Syldatk and Hausmann (2010) found that the use of costly substrates, gave low yields and accumulation of undesirable product mixtures rather than refined biosurfactant compounds, such constraints explain why there is restricted production of biosurfactants in industry.

1.2 Statement of Problem

1 In oil industry about 56% of the subterranean oil are trapped within rocks and as such cannot be pumped out. The oil may also be so viscous that it hinders the normal pumping. These problems are solved by the pumping in of microorganisms of known physiological properties into the wells. These microorganisms produce emulsifiers which reduce the surface tension of the oil and ease its pumping out. (Banat, 1995).

2 In Nigeria, within the coastal regions, there are large surface areas contaminated with petroleum hydrocarbons mainly due to accidental spills or leaks from underground deposits (Chandankere *et al*., 2013). This has caused significant negative impacts and hazards for agro ecosystems and human health (Baldan *et al.*, 2015). Biodegradation of petroleum hydro carbons

is complex and generally requires different microbial species or consortia with specific enzymatic capabilities that accelerate the rate of petroleum degradation. Nevertheless, the efficacy of bioremediation through microbial action is generally limited by the low availability and solubility of petroleum hydrocarbons due to their hydrophobicity and adsorption into solid particles and weathering of the hydrocarbon minerals (Chai-wei *et al*., 2013).

1.3 Aim and Objectives

The aim of this study is to determine the petroleum product-emulsification potentials of bacterial isolates.

The specific objectives are:

- To determine the physicochemical properties of Bonny River and soil
- To isolate and characterize bacteria from Bonny area of Rivers State
- To test for emulsification potential of the bacterial isolates
- To study the effects of various physical and nutrient parameters on bacterial growth and utilization of crude oil.
- To utilize bacteria with considerably high ability in producing emulsifier to enhance crude oil degradation.

1.4 Hypothesis

The following null hypotheses were assessed; (H_0)

- There is no significant difference in the emulsification potentials of bacteria
- There is no significant difference on the effects of nitrogen sources on percentage loss of crude oil
- There is no significant difference on the effects of nitrogen sources on the growth of the isolates.
- There is no significant difference on the effects of pH on the growth of the isolates.
- There is no significant difference on the effects of pH on the percentage loss of crude oil.

1.5 Justification of Study

Biosurfactants have a wide-range of applications due to their unique properties like specificity, low toxicity and relative ease of preparation. These properties hold promise for biosurfactants to increase the degree of microbial utilization of petroleum hydrocarbons, which promotes effective remediation of oil pollution. (Matvyeyeva, *et al*., 2015). They play important role in the environmental cleanup of oil spill both on water and on land. This is because microbial emulsifiers facilitate the growth of bacteria on hydrocarbons; improve the accessibility of the substrate, thereby enhancing the degradation of oil. They also aid in the dispersion of oil. (Matvyeyeva, *et al*., 2015). Biosurfactants enable bacteria to assimilate hydrocarbons as a source of carbon and energy (Pacwa-Plociniczak *et al*., 2011). Microbial hydrocarbons degradation may stop after the emulsifying activity is lost (Plaza *et al.,* 2006). Considering these advantages and benefits of bioemulsifiers, there is therefore need to isolate and characterize microorganisms having the ability to produce emulsifiers.

1.6 Scope of the Study.

This study specifically investigated the physicochemical parameters of Bonny River and the soil at its river bank. Bacterial isolates were characterized and screened for their ability to produce biosurfactants. The effects of physical and nutrient parameters on the utilization of bonny light crude oil was determined and isolates with considerably high emulsification index were used for the utilization of Bonny light crude oil.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biosurfactants

Microorganisms synthesize a wide range of surface-active compounds (SAC), generally called biosurfactants. These compounds are mainly classified according to their molecular weight, physico-chemical properties and mode of action. The low-molecular-weight SACs or biosurfactants reduce the surface tension at the air/water interfaces and the interfacial tension at oil/water interfaces, whereas the high-molecular-weight SACs, also called bioemulsifiers, are more effective in stabilizing oil-in-water emulsions. (Matvyeyeva *et al*., 2015).

Microbial surface-active compounds are a group of structurally diverse molecules produced by different microorganisms and are mainly classified by their chemical structure and their microbial origin. They are made up of a hydrophilic moiety, comprising an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids. These structures confer a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases. These compounds can be roughly divided into two main classes (Neu *et al.*, 1996): low-molecular-weight compounds called biosurfactants, such as lipopeptides, glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteinsthat are collectively called bioemulsons (Rosenberg and Ron, 1997) or bioemulsifiers (Smyth *et al.*, 2010b). The former group includes molecules which can efficiently reduce surface and interfacial tension, while the latter are amphiphilic and polyphilic polymers which are usually more effective in stabilizing emulsions of oil-in-water but do not lower the surface tension as much (Smyth *et al.*, 2010a).

Microbial emulsifiers have many structures, most are lipids which have the typical amphiphilic structures of a surfactant. Amphipathic molecules are molecules having both polar and non-polar portions in their structure. The chemical compounds that feature these molecules are essential to a host of biological and industrial processes. Soaps, detergents and lipoproteins are examples of amphipathic molecules. The non-polar portion of an amphipathic molecule is hydrophobic, meaning that it is repelled from water molecules. This non-polar portion is also lipophilic,

meaning that it is attracted to other organic molecules. The non-polar part is usually a long-chain hydrocarbon. The polar portion of the molecule may be composed of different radical groups including carboxylates, sulfates, phosphates and sulfonates. This polar portion behaves in a way that is almost opposite to the non-polar part, as it is attracted to water and repelled by polar molecules. Amphipathic compounds often have several lipophilic and several hydrophilic parts attached to a central carbon backbone. Phospholipids are a class of amphipathic molecule that is essential to life. These molecules are the main component of biological membranes. The specific amphipathic behavior of phospholipid molecules is determined by how they combine. Other important biosynthesized amphipathic molecules are cholesterol and glycolipids.

2.2 Properties of Microbial Emulsifiers

Several investigators (Cooper and Zajic, 1980; and Cooper, 1986) have outlined certain desirable properties which a good microbial emulsifier must have. These include:

1. A good microbial emulsifier must possess a specific molecular structure with the polar end attracted to the water and non polar end attracted to the oil.
2. It must be non-toxic, safe to handle and relatively inexpensive.
3. It must be able to emulsify the required system upon addition of small concentration.
4. It must influence the viscosity of the emulsion
5. A good microbial emulsifier must be biodegradable
6. It must be substrate specific and has a variety of different applications.

2.3 Classifications of Biosurfactants.

Biosurfactants are categorized by their chemical composition, molecular weight, physico-chemical properties and mode of action and microbial origin. Based on molecular weight they are divided into low-molecular-mass biosurfactants including glycolipids, phospholipids and lipopeptides and into high-molecular-mass biosurfactants/bioemulsifiers containing amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. Low-molecular-mass biosurfactants are efficient in lowering surface and interfacial tensions, whereas high-molecular-mass biosurfactants are more effective at stabilizing oil-in-water emulsions (Rosenberg *et al.,* 1999, Calvo *et al.,* 2009).

Examples of biosurfactants and their producers are depicted in Table 2.1.

Table 2.1 classification of biosurfactants and their uses

BIOSURFACTANT		Microorganism	Applications in Environmental Biotechnology	References
Group	**Class**			
	Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas* sp.	Enhancement of the degradation and dispersion of different classes of hydrocarbons; emulsification of hydrocarbons and vegetable oils; removal of metals from soil	(Sifour, *et al.*, 2007)
Glycolipids	Trehalolipids	*Mycobacterium tuberculosis*, *Rhodococcus erythropolis*, *Arthrobacter* sp., *Nocardia* sp., *Corynebacterium* sp.	Enhancement of the bioavailability of hydrocarbons	(Franzetti, *et al.*, 2010)
	Sophorolipids	*Torulopsis bombicola*, *Torulopsis petrophilum*, *Torulopsis apicola*	Recovery of hydrocarbons from dregs and muds; removal of heavy metals from sediments; enhancement of oil recovery	(Whang, *et al.*, 2008)
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery	(Gerson *et al.*, 1978)
	Spiculisporic acid	*Penicillium spiculisporum*	Removal of metal ions from aqueous solution; dispersion action for	(Ishigami *et* (Ishigami *et al.*, 2000)

BIOSURFACTANT		Microorganism	Applications in Environmental Biotechnology	References
Group	**Class**			
			hydrophilic pigments; preparation of new emulsion-type organogels, superfine microcapsules (vesicles or liposomes), heavy metal sequestrants	
	Phosphati-dylethanolamine	*Acinetobacter* sp., *Rhodococcus erythropolis*	Increasing the tolerance of bacteria to heavy metals	(Appanna *et al*., 1995)
Lipopeptides	Surfactin	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides; removal of heavy metals from a contaminated soil, sediment and water; increasing the effectiveness of phytoextraction	(Awashti *et al*, 1999)
	Lichenysin	*Bacillus licheniformis*	enhancement of oil recovery	(Thomas *et al*., 1993)
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus* RAG-1	Stabilization of the hydrocarbon-inwater emulsions	(Zosim *et al*., 1982)
	Alasan	*Acinetobacter*		(Toren *et al*,

BIOSURFACTANT		Microorganism	Applications in Environmental Biotechnology	References
Group	**Class**			
		radioresistens KA-53		2001)
	Biodispersan	*Acinetobacter calcoaceticus* A2	Dispersion of limestone in water	(Rosenberg, 1988)
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon-in-water emulsions	(Cirigliano, 1984)
	Mannoprotein	*Saccharomyces cerevisiae*		(Cameron, 1988)

Source (Soberón-Chávez, 2011)

Biosurfactants are commonly classified based on their biochemical nature or the microbial producer species. With regard to structure, these compounds are classified into five main groups (Rahman and Gakpe, 2008).

• Glycolipids – the degree of polarity depends on the hydrocarbons used as substrate; examples: rhamnolipids produced by Pseudomonas aeruginosa and sophorolipids produced by species of Candida.

• Lipopolysaccharides – which normally have a high molecular mass and are soluble in water; example: emulsan, an extracellular emulsifier produced from hydrocarbons by the bacteria Acinotobacter calcoaceticus;

• Lipopeptides – example: surfactin produced by Bacillus subtilis (one of the most potent biosurfactants reported in the literature);

• Phospholipids – structures common to many microorganisms; example: biosurfactant from Corynebacterium lepus;

• Fatty acids, neutral lipids (some classified as glycolipids) and hydrophobic proteins.

2.3.1 Glycolipids

Glycolipids, consisting of a carbohydrate moiety linked to fatty acids, are microbial surface active compounds produced by various microorganisms. They are characterized by high structural diversity and have the ability to decrease the surface and interfacial tension at the surface and interface, respectively. Among the glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids. (Ines, 2016).

2.3.1.1 Rhamnolipids

Rhamnolipid, primarily a crystalline acid, is composed of β-hydroxy fatty acid connected by the carboxyl end to a rhamnose sugar molecule. Rhamnolipids are predominantly produced by *Pseudomonas aeruginosa* and classified as: mono and di-rhamnolipids. Other Pseudomonas species that have been reported to produce rhamnolipids *are P. chlororaphis, P. plantarii, P. putida,* and *P. fluorescens*. Some bacteria are known to produce only mono-rhamnolipids while some produce both. The ratio of mono and di-rhamnolipid can also be controlled in the production method. There are enzymes available that can convert mono-rhamnolipids into di-rhamnolipids. The basic Structures of mono and di rhamnolipids is presented in figure 2.1

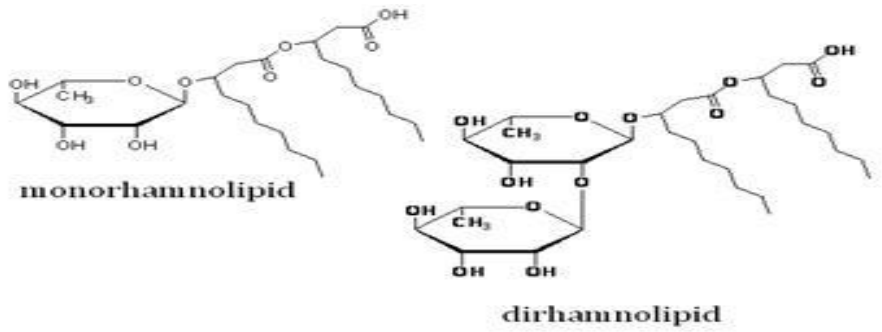

Figure 2.1 Structures of mono and di rhamnolipids.

Source Rahman *et al*., 2003.

2.3.1.1.1 Applications of Rhamnolipids

Over the years rhamnolipids are becoming broadly pertinent in various industries and are posing a serious threat to the synthetic surfactants. A list of five major applications of rhamnolipids that cater to the wide range of industrial demands includes:

1. Bioremediation and enhanced oil recovery (EOR): Rhamnolipids show excellent emulsification properties, efficiently remove crude oil from contaminated soil and facilitate bioremediation of oil spills (Rahman *et al*., 2003; Costa *et al*., 2010).
2. Pharmaceuticals and therapeutics: Rhamnolipids show low toxicity, surface active properties and antimicrobial activities against several microbes (*Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Listeria monocytogenes*) thereby showing promising applications in pharmaceuticals and therapeutics (Magalhaes and Nitschke, 2013).
3. Cosmetics: Rhamnolipid as an active ingredient is found to be effective for several skin treatments i.e., wound healing with reduced fibrosis, cure of burn shock, treatment of wrinkles hence are in demand in the health and beauty industry (Piljac and Piljac, 2007).
4. Detergents and cleaners: Rhamnolipids are natural emulsifiers and surface active agents leading to their wide spread usage in detergent compositions, laundry products, shampoos and soaps (Parry *et al.*, 2013).
5. Agriculture: Rhamnolipids are already used for soil remediation for improving soil quality and are now further getting explored for plant pathogen elimination, for aiding the absorption of fertilizers and nutrients through roots and as biopesticides (Sachdev and Cameotra, 2013).

2.3.1.2 Sophorolipids

Sophorolipids are glycolipid class of microbial biosurfactants which consist of a hydrophobic fatty acid tail of 16 or 18 carbon atoms and a hydrophilic carbohydrate head, sophorose. which is a glucose di-saccharide with an unusual β-1,2 bond and can be acetylated on the 6′- and/or 6″-positions. One terminal or sub terminal hydroxylated fatty acid is β-glycosidically linked to the sophorose molecule. The carboxylic end of this fatty acid is either free (acidic or open form) or internally esterified at the 4″ or in some rare cases at the 6′- or 6″-position (lactonic form). The hydroxy fatty acid itself counts in general 16 or 18 carbon atoms and can have one or more unsaturated bonds (Davila, 1994). The physicochemical and biological properties of sophorolipids are significantly influenced by the distribution of the lactone vs. acidic forms produced in the fermentative broth. In general, lactone sophorolipids are more efficient in reducing surface tension and are better antimicrobial agents, whereas acidic sophorolipids display better foaming properties. Acetyl groups can also lower the hydrophilicity of

sophorolipids and enhance their antiviral and cytokine stimulating effect (Shah, 2005). The basic Structures of lactonic and acid sophorolipids is presented in figure 2.2

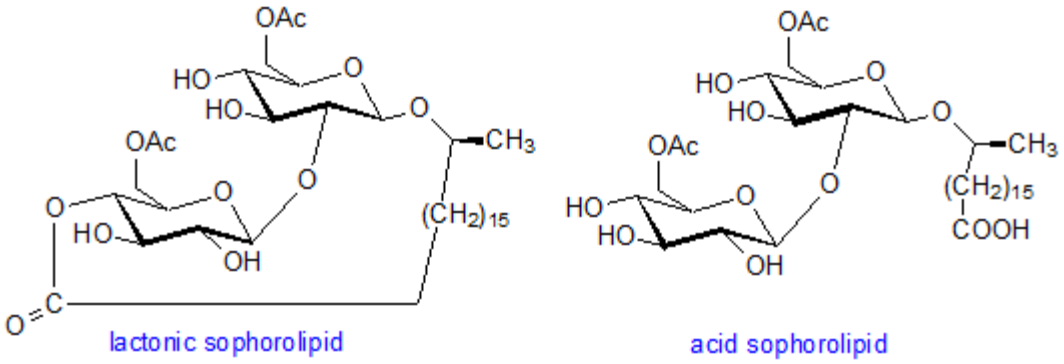

Figure 2.2 Structure of lactonic and acid sophorolipids

Source Shah, 2005

2.3.1.3 Trehalolipids

Trehalolipids or trehalose lipids are glycolipids containing trehalose hydrophilic moiety. Several structural types of microbial trehalolipid biosurfactants have been reported.These trehalose lipids are mainly produced by rhodococci and present interesting physicochemical and biological properties (Lang *et al.*, 1998). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acid is associated with most species *of Mycobacterium*, *Nocardia* and *Corynebacterium*. Mycolic acids are long chain, α-branched-β-hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation (Asselineau and Asselineau 1978). Trehalolipids from *Rhodococus erythropolis and Arthrobacter* sp. were found to lower the surface and interfacial tensions in culture broth from 25-40 and 1-5mN/m, respectively. (Li, 1984)

2.3.2 Lipopolysaccharides

lipopolysaccharides have been studied extensively for over a century. (Luderitx *et al*., 1973) These molecules consists of three distinct regions covalently linked together, a hydrophobic lipid component, a core polysaccharide and the 0-specific lipopolysaccharides.

2.3.2.1 Composition of Lipopolysaccharides

O-antigen

A repetitive glycan polymer contained within an LPS is referred to as the O antigen, O polysaccharide, or O side-chain of the bacteria. The O antigen is attached to the core oligosaccharide, and comprises the outermost domain of the LPS molecule. The composition of the O chain varies from strain to strain. For example, there are over 160 different O antigen structures produced by different *E. coli* strains. (Raetz, 2002). The presence or absence of O chains determines whether the LPS is considered rough or smooth. Full-length O-chains would render the LPS smooth, whereas the absence or reduction of O-chains would make the LPS rough. (Rittig *et al.*, 2003) Bacteria with rough LPS usually have more penetrable cell membranes to hydrophobic antibiotics, since a rough LPS is more hydrophobic (Tsujimoto, 1999). O antigen is exposed on the very outer surface of the bacterial cell, and, as a consequence, is a target for recognition by host antibodies.

Core

The Core domain always contains an oligosaccharide component that attaches directly to lipid A and commonly contains sugars such as heptose and 3-Deoxy-D-manno-oct-2-ulosonic acid (also known as KDO, keto-deoxyoctulosonate) (Hershberger, 1968) The LPS Cores of many bacteria also contain non-carbohydrate components, such as phosphate, amino acids, and ethanolamine substituents.

Lipid A

Lipid A is, in normal circumstances, a phosphorylated glucosamine disaccharide decorated with multiple fatty acids. These hydrophobic fatty acid chains anchor the LPS into the bacterial membrane, and the rest of the LPS projects from the cell surface. The lipid A domain is responsible for much of the toxicity of Gram-negative bacteria. When bacterial cells are lysed by the immune system, fragments of membrane containing lipid A are released into the circulation, causing fever, diarrhea, and possible fatal endotoxic shock (also called septic shock). The Lipid A moiety is a very conserved component of the LPS (Tzeng, 2002). However Lipid A structure varies among bacterial species and Lipid A structure defines an overall host immune activation. (Khan *et al.*, 2018). The basic Structures of lipopolysaccharide is presented in figure 2.3

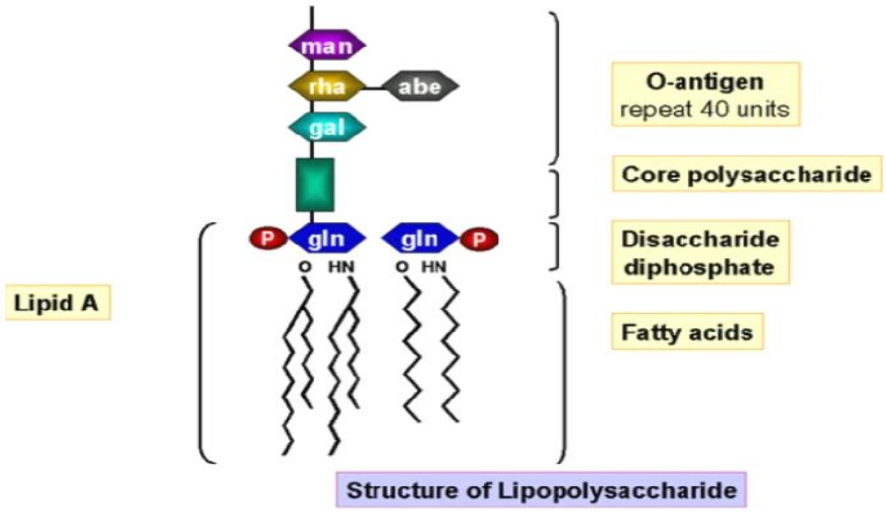

Figure 2.3 Structure of lipopolysaccharide, Source (Khan *et al.*, 2018).

Emulsan, liposan, mannoprotein and polysaccharide-protein complexes are known to be the best-studied polymeric biosurfactants (Desai and Banat, 1997). Using *Acinetobacter calcoaceticus* RAG-1, Rosenberg *et al*. (1979) extracted a potent polyanionic amphipathic heteropolysaccharide bioemulsifier called emulsan. It is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001-0.01%. Additionally, it is noted as one of the most powerful emulsion stabilizers known with the ability to resist inversion even at a water-to-oil ratio of 1:4 (Zosim *et al.*, 1982). Ciriglian and Carman (1984) synthesised liposan, an extracellular water-soluble emulsifier using *Candida lipolytica*. It is composed of 83% carbohydrate and 17% protein with the carbohydrate portion being a heteropolysaccharide consisting of glucose, galactose, galactosamine and galactoronic acid. Cameron *et al*. (1988) demonstrated the production of large amounts of mannoprotein by *Saccharomyces cerevisiae*. When purified, the emulsifier contains 44% mannose and 17% protein. The mannoprotein exhibited excellent emulsifying activity toward several oils, alkanes and organic solvents. Other polymeric biosurfactants such as biodispersan, alasan, food emulsifiers, protein complexes and insectides emulsifiers have also been reported. The basic Structures of Emulsan is presented in figure 2.4

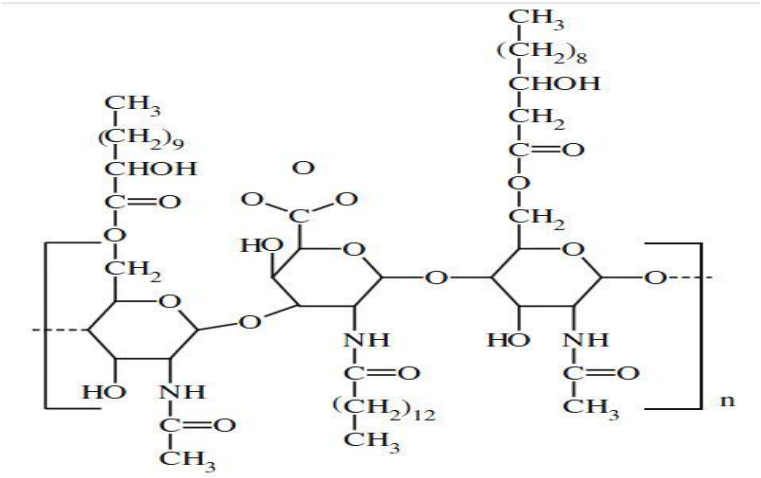

Figure 2.4 Structure of Emulsan. Rosenberg *et al*. (1979)

2.3.3 Lipopeptides and Lipoproteins

These consist of a lipid attached to a polypeptide chain (Rosenberg and Ron, 1999). Several biosurfactants have shown antimicrobial action against various bacteria, algae, fungi and viruses. Besson *et al*. (1976) reported the antifungal property and Singh and Cameotra (2004) reported the antibacterial property of the lipopeptide, iturin which was produced by *Bacillus subtilis.* The two most important lipopeptides are described below.

2.3.3.1 Surfactin

The cyclic lipopeptide surfactin are one of the most powerful biosurfactants composed of a seven amino-acid ring structure coupled to a fatty-acid chain via lactone linkage (Arima *et al*.,1968). Previous study reported that various physic-chemical properties of surfactin from *B. subtilis*.They found that the surfactin are able to reduce the surface tension and interfacial tension of water.

Surfactin, like other surfactants, affects the surface tension of liquids in which it is dissolved. It can lower the water's surface tension from 72 mN/m to 27 mN/m at a concentration as low as 20 µM (Yeh, 2005) Surfactin accomplishes this effect as it occupies the intermolecular space between water molecules, decreasing the attractive forces between adjacent water molecules, mainly hydrogen bonds, creating a more fluid solution that can go into tighter regions of space increasing water's wetting ability (Dufour, 2005) . Overall, this property is significant not only

for surfactin but for surfactants as a whole, as they are primarily used as detergents and soaps. Figure 2.5

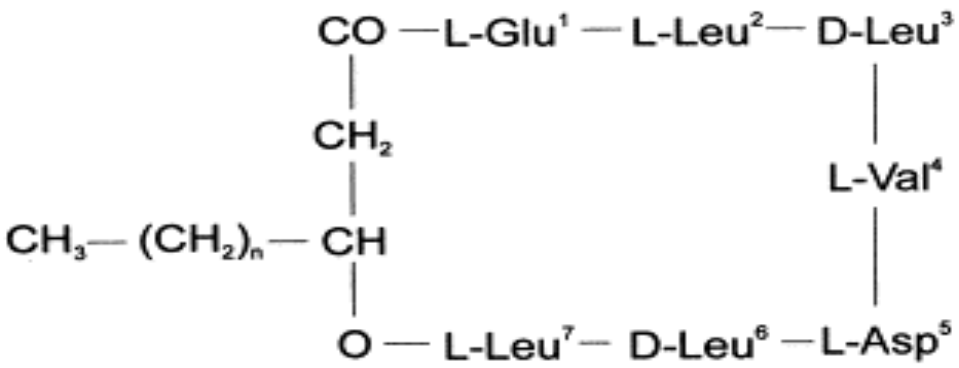

Figure 2.5 shows the structure of surfactin from *Bacillus substilis* (Dufour, 2005)

2.3.3.2 Lichenysin:

Bacillus licheniformis produces several biosurfacants which exhibit excellent stability under extreme temperature, pH and salt conditions which are similar to surfactin. McInerney *et al.* (1990) reported that lichenysin from B. licheniformis are able to reduce the surface tension and interfacial tension of water to 27 and 0.36 mN mG1, respectively.

2.3.4 Phospholipids

Phospholipids are a class of lipids that are a major component of all cell membranes. They can form lipid bilayers because of their amphiphilic characteristic. The structure of the phospholipid molecule generally consists of two hydrophobic fatty acid "tails" and a hydrophilic "head" consisting of a phosphate group. The two components are joined together by a glycerol molecule. The phosphate groups can be modified with simple organic molecules such as choline, ethanolamine or serine. (Mashaghi *et al.*, 2013)

phospholipid head contains a negatively charged phosphate group and glycerol; it is hydrophilic. The phospholipid tails usually consist of 2 long fatty acid chains; they are hydrophobic and avoid interactions with water. When placed in aqueous solutions, phospholipids are driven by hydrophobic interactions that result in the fatty acid tails aggregating to minimize interactions with water molecules. These specific properties allow phospholipids to play an important role in

the phospholipid bilayer. In biological systems, the phospholipids often occur with other molecules (e.g., proteins, glycolipids, sterols) in a bilayer such as a cell membrane. Lipid bilayers occur when hydrophobic tails line up against one another, forming a membrane of hydrophilic heads on both sides facing the water. (Campbell *et al*., 2006). Figure 2.6 shows the basic structure of phospholipids.

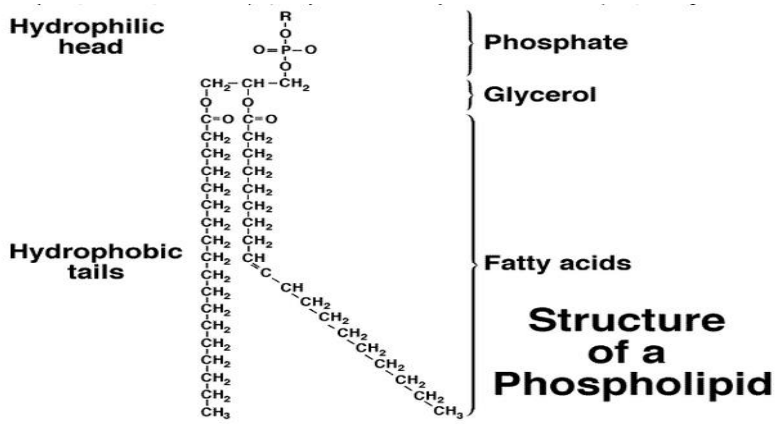

Figure 2.6 structure of Phospholipid (Mashaghi *et al.*, 2013).

2.3.5 Fatty Acids, Neutral Lipids and Hydrophobic Proteins

Fatty acids and neutral lipids are found in all microbial cells and are often observed as extracellular products (Shaw, 1974). Most of these lipids, including carboxylic acids, alcohols, esters, mono-, di-, and triglycerides, have shown to have some degree of surface activity. Most of the examples of the extra cellular production of neutral lipids or fatty acids by microbes have involved organisms growing on hydrocarbons (Zajic and Seffens, 1984).

2.4 Sources of Biosurfactants:

Many of the biosurfactant producing microorganisms are found to be hydrocarbon degraders (Willumsen and Karlson, 1997; Volkering *et al*., 1998). However in the past decades, many studies have showed the effects of microbially produced surfactants not only on bioremediation but also on enhanced oil recovery (Volkering *et al*., 1998; Tabatabaee *et al*., 2005).

2.4.1 Bacterial Biosurfactants:

Microorganisms make use of a wide range of organic compounds as a source of carbon and energy for their growth. When the carbon source is in an insoluble form like a hydrocarbon, microorganisms make possible their diffusion into the cell by producing a variety of substances,

the biosurfactants. Some of the bacteria and yeasts excrete ionic surfactants which emulsify the CxHy substance in the growth medium. A few examples of this group of biosurfactant are rhamnolipids that are produced by different *Pseudomonas* spp. (Burger *et al.*,1963; Guerra-Santos *et al.*, 1986) or sophorolipids that are produced by several *Torulopsis* spp. (Cutler and Light, 1979; Cooper and Paddock, 1984). Some other microorganisms are able to change the structure of their cell wall which are achieved by them by producing nonionic or lipopolysaccharides surfactants in their cell wall. Some examples of this group are: *Rhodococcus erythropolis* and various *Mycobacterium* spp. And *Arthrobacter* spp. which produce nonionic trehalose corynomycolates (Ristau and Wanger, 1983; Kilburn and Takayama, 1981; Kretschmer *et al*., 1982). There are lipopolysaccharides, such as emulsan, produced by *Acinetobacter* spp. (Kretschmer *et al*., 1982) and lipoproteins such as surfactin and subtilisin, that are produced by *Bacillus subtilis* (Cooper *et al.*, 1981).

2.4.2 Fungal Biosurfactants:

Where the field of production of biosurfactants by bacterial species is well explored, relatively fewer fungi are known to produce biosurfactants. Among fungi, *Candida bombicola* (Casas *et al*., 1997), *Candida lipolytica* (Sarubbo *et al*., 2007), *Candida ishiwadae* (Thanomsub *et al*., 2004), *Candida batistae* (Konishi *et al*., 2008), *Aspergillus ustus* (Alejandro *et al*., 2011) and *Trichosporon ashii* (Chandran and Das, 2010) are the explored ones. Many of these are known to produce biosurfactant on low cost raw materials. The major type of biosurfactants produced by these strains is sophorolipids (glycolipids).

Candida lipolytica produces cell wall-bound lipopolysaccharides when it is growing on n-alkanes (Rufino *et al*., 2007). Biosurfactants derived from bacteria and fungi are in table 2.2.

Table 2.2 Biosurfactants derived from bacteria and fungi

Organisms	Biosurfactants	References
Bacteria		
Serratia marcescens	Serrawettin	Lai *et al*. (2009)
Rhodotorula glutinis, R. graminis	Polyol lipids	Amaral *et al*. (2006)
Rhodococcus erythropolis, Arthrobacter spp.,	Trehalose lipids	Muthusamy *et al* (2008)
Nocardia erythropolis, Corynebacterium spp.,		

Mycobacterium spp. *Pseudomonas* spp.,		
Thiobacillus thiooxidans,	Ornithine lipids	Desai and Banat (1997)
Agrobacterium spp.*Pseudomonas fluorescens,*		
Leuconostoc mesenteroides	Viscosin	Banat *et al*. (2010)
Pseudomonas aeruginosa, Pseudomonas chlororaphis,	Rhamnolipids	Jadhav *et al*. (2011)
Serratia rubidea		
P. fluorescens, Debaryomyces polmorphus	Carbohydrate-lipid	Nerurkar *et al*. (2009)
P. aeruginosa	Protein PA	Hisatsuka *et al*. (1971)
Lactobacillus fermentum	Diglycosyl diglycerides	Mulligan *et al*. (2001)
Fungi		
Torulopsis bombicola	Sophorose lipid	Kim *e al*. (1997)
Candida bombicola	Sophorolipids	Casas *et al*. (1997)
Candida lipolytica	Protein-lipidpolysaccharide complex	Sarubbo *et al*. (2007)
Candida lipolytica	Protein-lipidcarbohydrate complex	Rufino *et al*. (2007)
Candida ishiwadae	Glycolipid	Thanomsub *et al*. (2004)
Candida batistae	Sophorolipid	Konishi *et al*. (2008)
Aspergillus ustus	Glycolipoprotein	Alejandro *et al*. (2011)
Trichosporon ashii	Sophorolipid	Chandran and Das.(2010)

Source: Alejandro *et al*. (2011)

2.5 Physiological Role of Biosurfactant.

The physiological role of biosurfactants in microorganisms is to facilitate the contact of bacterial cells with hydrophobic substrates. Surfaces of most bacteria are hydrophilic, this allows them to effectively interact with the water-soluble compounds and ensure the normal operation of membrane-bound enzyme systems. However, it is difficult to contact with hydrophobic substrates such as petroleum products. Nevertheless, the group of microorganisms capable of assimilating oil hydrocarbons as a source of carbon and energy is very extensive. As the hydrocarbon-enzyme systems are localized in the cytoplasm of the bacteria, the ability of a strain to assimilate hydrocarbons depends primarily on the ability to absorb hydrophobic substrate. The absorption process may limit the oxidation process. Thus microbial hydrocarbons degradation may stop after the emulsifying activity loss. (Matvyeyeva *et al*., 2015).

2.6 Advantages of Biosurfactants

When compared to synthetic surfactants, biosurfactants have several advantages including high biodegradability, low toxicity, low irritancy and compatibility with human skin (Banat *et al.,* 2000; Cameotra and Makkar 2004).Therefore they are superior to the synthetic ones. The most significant advantage of a microbial surfactant over chemical surfactant is its ecological acceptance (Desai and Banat 1997; Karsa *et al.*1999; Banat 2000). Some of the advantages of biosurfactants are discussed below.

2.6.1 Biodegradability: Microbial derived compounds can be easily degraded when compared to synthetic surfactants (Mohan *et al*., 2006) and are suitable for environmental applications such as bioremediation/biosorption (Mulligan *et al.,* 2001). The increasing environmental concern forces us to search for alternative products such as biosurfactants (Cameotra and Makkar, 2004). Synthetic chemical surfactants impose environmental problems and hence, biodegradable biosurfactants from marine microorganisms were concerned for the biosorption of poorly soluble polycyclic aromatic hydrocarbon, phenanthrene contaminated in aquatic surfaces (Olivera *et al*., 2003). Lee *et al.* (2008) controlled the blooms of marine algae, Cochlodinium using the biodegradable biosurfactant sophorolipid with the removal efficiency of 90% in 30 min treatment.

2.6.2 Low Toxicity:

Biosurfactants do not cause serious damage/harm of the biotic ecosystem since their toxicity level is low. Many chemical surfactants are toxic to the living beings making them less useful for being used in different industries. Very little data are available in the literature regarding the toxicity of microbial surfactants. They are generally considered as low or non-toxic products and therefore, appropriate for pharmaceutical, cosmetic and food uses.

Although, very few literatures were available regarding the toxicity of biosurfactants. Poremba *et al*. (1991) demonstrated the higher toxicity of the chemical-derived surfactant (Corexit) which displayed a LC_{50} against *Photobacterium phosphoreum* and was found to be 10 times lower than of rhamnolipids. Flasz *et al*. (1998) compared the toxicity and mutagenicity profile of biosurfactant from *Pseudomonas aeruginosa* and chemically derived surfactants and indicated the biosurfactant as non-toxic and non-mutagenic. The low toxicity profile of biosurfactant, sophorolipids from *Candida bombicola* made them useful in food industries (Cavalero and Cooper, 2003).

2.6.3 Specificity: As complex organic molecules with specific functional groups, biosurfactants are specific in their actions, which is of considerable interest regarding the detoxification of specific pollutants as well as in particular applications in the food, cosmetic and pharmaceutical industries.

2.6.4 Surface and Interface Activity: Surfactant helps in reducing surface tension and the interfacial tension. Surfactin produced by *B. subtilis* can reduce surface tension of water to 25 mN mG1 and interfacial tension water/hexadecane to less than 1 mN mG1 (Cooper *et al*., 1981). The rhamnolipids produced by *P. aeruginosa* decreased surface tension of water to 26 mN mG1 and interfacial tension of water/hexadecane to value less than 1 mN mG1 (Syldatk *et al*., 1985). In general, biosurfactants are more effective and efficient and their Critical Micelle Concentration (CMC) is about several times lower than chemical surfactants, i.e., for maximal decrease on surface tension, less surfactant is necessary (Desai and Banat, 1997).

2.6.5 Availability of Raw Materials

Biosurfactants can be produced from cheap raw materials like rapeseed oil, potato process effluents, oil refinery waste, cassava flour wastewater, curd whey and distillery waste, sunflower oil etc. (Muthusamy *et al.*, 2008) which are available in large quantities. The carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used separately or in combination with each other.

2.6.6 Biocompatability and Digestibility

Biosurfactants are biocompatible in nature (Rosenberg *et al.,* 1999) which means they are well tolerated by living organisms. These when interact with living organisms do not change bioactivity of the organisms. This property allows their application in cosmetics, pharmaceuticals and as functional food additives.

2.6.7 Use in Environmental Control

Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil.

2.7 Disadvantages of Biosurfactants

Concerning disadvantages, one of the problems is related to large scale and cheap production of biosurfactants. Large quantities are particularly needed in petroleum and environmental applications, which, due to the bulk use, may be expensive. To overcome this problem, processes should be coupled to utilization of waste substrates combating at the same time their polluting effect, which balances the overall costs. Another problem may be encountered in obtaining pure substances which is of particular importance in pharmaceutical, food and cosmetic applications. Downstream processing is involved with multiple consecutive steps. Therefore, high yields andQ biosurfactant concentrations in bioreactors are essential for their facilitated recovery and purification.

2.8.0 Applications of Biosurfactants

2.8.1 Application of Biosurfactants in Biodegradation Processes

A promising method that can improve bioremediation effectiveness of hydrocarbon contaminated environments is the use of biosurfactants. They can enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan 2004). By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons. In consequence, biosurfactants enhance biodegradation and

removal of hydrocarbons. Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (Nguyen, 2008, Déziel, 1996, Rahman, 2003). Mechanisms of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration is shown in figure 2.7

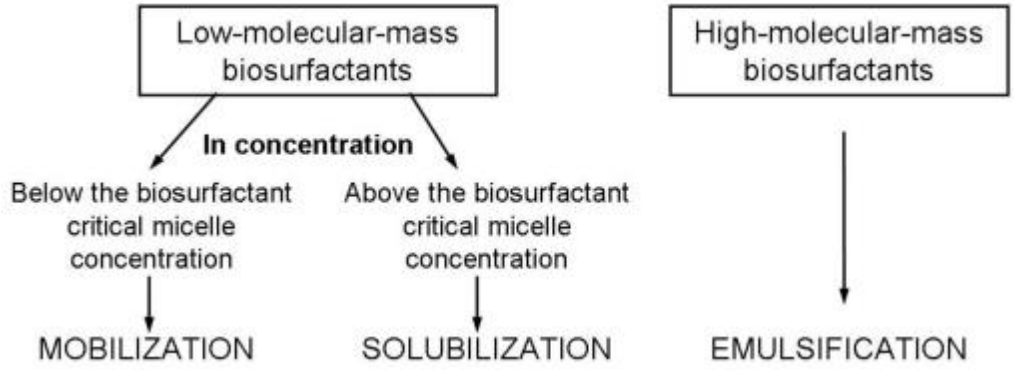

Figure 2.7 Mechanisms of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration (Rosenberg1999).

The mobilization mechanism occurs at concentrations below the biosurfactant CMC. At such concentrations, biosurfactants reduce the surface and interfacial tension between air/water and soil/water systems. Due to the reduction of the interfacial force, contact of biosurfactants with soil/oil system increases the contact angle and reduces the capillary force holding oil and soil together. In turn, above the biosurfactant CMC the solubilization process takes place. At these concentrations biosurfactant molecules associate to form micelles, which dramatically increase the solubility of oil. The hydrophobic ends of biosurfactant molecules connect together inside the micelle while the hydrophilic ends are exposed to the aqueous phase on the exterior. Consequently, the interior of a micelle creates an environment compatible for hydrophobic organic molecules. The process of incorporation of these molecules into a micelle is known as solubilization (Urum, 2004).

Emulsification is a process that forms a liquid, known as an emulsion, containing very small droplets of fat or oil suspended in a fluid, usually water. The high molecular weight biosurfactants are efficient emulsifying agents. They are often applied as an additive to stimulate bioremediation and removal of oil substances from environments.

2.8.2 Bioremediation of Toxic Pollutants:

Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g. nutrients and oxygen), conditions (e.g., pH and moisture content) and prevailing microorganisms. Thus, bioremediation usually consists of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, if necessary, supplying air and often adding bacteria. The addition of emulsifiers is advantageous when bacterial growth is slow (e.g. at cold temperatures or in the presence of high concentrations of pollutants) or when the pollutants consist of compounds that are difficult to degrade, such as PAHs. Bioemulsifiers can be applied as an additive to stimulate the bioremediation process, however with advanced genetic technologies it is expected that the increase in bioemulsifier concentration during bioremediation would be achieved by the addition of bacteria that overproduce bioemulsifiers. This approach has been recently used successfully in the cleaning of oil pipes. Cultures of *A. radioresistens* (Navon-Venezia *et al*., 1995) which produce the bioemulsifier alasan but are unable to use hydrocarbons as a carbon source, were added to a mixture of oil-degrading bacteria to enhance oil bioremediation.

Persistent organic pollutants found in oil containing wastewater and sediments, such as PAHs (phenanthrene, crysene) are also hydrophobic in nature and thus water solubility of PAHs normally decrease with the increasing number of rings in molecular structure. This property induces the low bioavailability of these organic compounds that is a crucial factor in the biodegradation of PAHs. The water solubility of some PAHs can be improved by addition of biosurfactants owing to their amphipathic structure by several folds (Yin *et al*., 2009). In addition, most hydrocarbons exist in strongly adsorbed forms when they are introduced into soils. Thus, their removal efficiency can be limited in low mass transfer phases. However, additions of solubilization agents, such as biosurfactants to the system enhance the bioavailability of low solubility and highly sorptive compounds (Shin *et al*., 2004).

2.8.3 Application of Biosurfactant in Microbial Enhanced Oil Recovery (MEOR)

Biosurfactants can also be involved in microbial enhanced oil recovery (MEOR). MEOR methods are used to recover oil remaining in reservoirs after primary (mechanical) and secondary (physical) recovery procedures (Sen, 2008: Banat, (2000). It is an important tertiary process where microorganisms or their metabolites, including biosurfactants, biopolymers, biomass, acids, solvents, gases and also enzymes, are used to increase recovery of oil from depleted reservoirs. Application of biosurfactants in enhanced oil recovery is one of the most promising advanced methods to recover a significant proportion of the residual oil. The

remaining oil is often located in regions of the reservoir that are difficult to access and the oil is trapped in the pores by capillary pressure (Sen, 2008). Biosurfactants reduce interfacial tension between oil/water and oil/rock. This reduces the capillary forces preventing oil from moving through rock pores. Biosurfactants can also bind tightly to the oil-water interface and form emulsion. This stabilizes the desorbed oil in water and allows removal of oil along with the injection water (Suthar *et al*., 2008). Mechanism of enhanced oil recovery by biosurfactants is represented in figure 2.8.

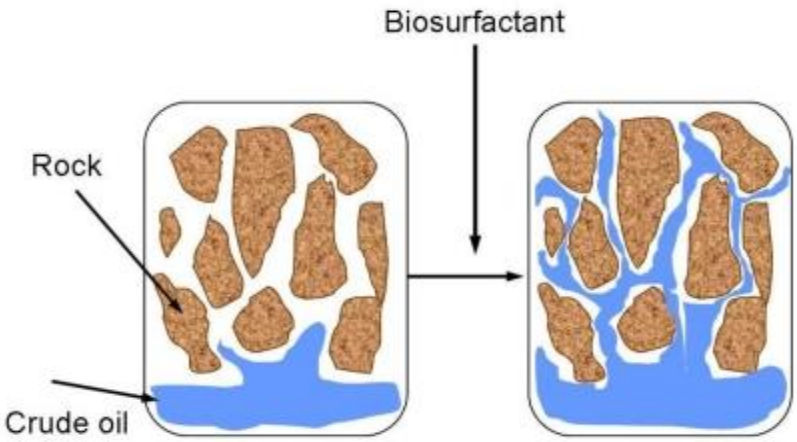

Figure 2.8 Mechanism of enhanced oil recovery by biosurfactants. (Suthar *et al*., 2008).

2.8.4 Removal of Oil and Petroleum Contamination:

Itoh and Suzuki (1972) were the first to show that hydrocarbon culture media stimulated the growth of a rhamnolipid producing strain of *P. aeruginosa*. Recent research findings confirmed the effects of biosurfactant on hydrocarbon biodegradation by increasing microbial accessibility to insoluble substrates and thus enhance their biodegradation (Zhang and Miller, 1992; Hunt *et al*., 1994). Various experiments have been conducted that the effects of biosurfactants on hydrocarbons; enhancing their water solubility and increasing the displacement of oily substances from soil particles. Thus, biosurfactants increase the apparent solubility of these organic compounds at concentrations above the Critical Micelle Concentration (CMC) which enhance their availability for microbial uptake (Chang *et al*., 2008). For these reasons, inclusion of biosurfactants in a bioremediation treatment of a hydrocarbon polluted environment could be really promising, facilitating their assimilation by microorganisms (Calvo *et al*., 2009).

Many of the biosurfactants known today have been studied to examine their possible technical applications (Nayak *et al*., 2009). Most of these applications involve their efficiency in

bioremediation, dispersion of oil spills and enhanced oil recovery. *Alcanivorax* and *Cycloclasticus* genera are highly specialized hydrocarbon degraders in marine environments. *Alcanivorax borkumensis* utilizes aliphatic hydrocarbons as its main carbon source for growth and produces an anionic glucose lipid biosurfactant and thus potentials of *Alcanivorax* strains during bioremediation of hydrocarbon pollution in marine habitats have been studied (Olivera *et al*., 2009); thus, this property needs to be studied extensively in soil to ensure its effectiveness.

Several species of *P. aeruginosa* and *B. subtilis* produce rhamnolipid, a commonly isolated glycolipid biosurfactant and surfactin, a lipoprotein type biosurfactant, respectively; these two biosurfactants have been shown by Whang *et al*. (2008) to increase solubility and bioavailability of a petrochemical mixture and also stimulate indigenous microorganisms for enhanced biodegradation of diesel contaminated soil. *Gordonia* species BS29 grows on aliphatic hydrocarbons.

2.8.5 Bioremediation of Toxic Pollutants: Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g. nutrients and oxygen), conditions (e.g., pH and moisture content) and prevailing

microorganisms. Thus, bioremediation usually consists of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, if necessary, supplying air and often adding bacteria. The addition of emulsifiers is advantageous when bacterial growth is slow (e.g. at cold temperatures or in the presence of high concentrations of pollutants) or when the pollutants consist of compounds that are difficult to degrade, such as PAHs. Bioemulsifiers can be applied as an additive to stimulate the bioremediation process, however with advanced genetic technologies it is expected that the increase in bioemulsifier concentration during bioremediation would be achieved by the addition of bacteria that overproduce bioemulsifiers. This approach has been recently used successfully in the cleaning of oil pipes. Cultures of *A. radioresistens* (Navon-Venezia *et al*., 1995) which produce the bioemulsifier alasan but are unable to use hydrocarbons as a carbon source, were added to a mixture of oil-degrading bacteria to enhance oil bioremediation.

2.8.5.1 Mechanism behind Bioremediation: There are at least two ways in which biosurfactants are involved in bioremediation: increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic compounds.

2.8.5.1.1 Increasing the surface area of hydrophobic water insoluble substrates: For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil (Sekelsky and Shreve, 1999). When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect and there are certain conceptual difficulties in understanding how emulsification can provide an (evolutionary) advantage for the microorganism producing the emulsifier. Stated briefly, emulsification is a cell-density-dependent phenomenon: that is, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water and not be more available to the emulsifier-producing strain than to competing microorganisms.

2.8.5.1.2 Increasing the bioavailability of hydrophobic water-insoluble substrates:

The low water solubility of many hydrocarbons, especially the Polycyclic Aromatic Hydrocarbons (PAHs), is believed to limit their availability to microorganisms which is a potential problem for bioremediation of contaminated sites. It has been assumed that surfactants would enhance the bioavailability of hydrophobic compounds. Several non-biological surfactants have been studied and both negative and positive effects of the surfactants on biodegradation were observed. For example, the addition of the surfactant Tergitol NP-10 increased the dissolution rate of solid-phase phenanthrene and resulted in an overall increase in the growth of a strain of *Pseudomonas stutzeri* (Grimberg *et al*., 1996). A similar effect was obtained by the addition of Tween 80 to two *Sphingomonas* strains, the rate of fluoranthene mineralization was almost doubled. By contrast, the same surfactant inhibited the rate of fluoranthene mineralization by two strains of *Mycobacterium* (Willumsen *et al*., 2001) and no stimulation was observed in other studies using several surfactants (Bruheim and Eimhjellen, 1998).

2.8.6 Application of Biosurfactant in Removal of Metals

Using biosurfactants have unquestionable advantages because bacterial strains able to produce surface active compounds do not need to have survival ability in heavy metal-contaminated soil. However, using biosurfactants alone requires continuous addition of new portions of these compounds.

The usefulness of biosurfactants for bioremediation of heavy metal contaminated soil is mainly based on their ability to form complexes with metals. The anionic biosurfactants create complexes with metals in a nonionic form by ionic bonds. These bonds are stronger than the metal's bonds with the soil and metal-biosurfactant complexes are desorbed from the soil matrix to the soil solution due to the lowering of the interfacial tension. The cationic biosurfactants can replace the same charged metal ions by competition for some but not all negatively charged surfaces (ion exchange). Metal ions can be removed from soil surfaces also by the biosurfactant micelles. The polar head groups of micelles can bind metals which mobilize the metals in water (Mulligan, 2004; Singh, 2004; Juwarkar, 2007) Mechanism of biosurfactant activity in metal-contaminated soil is shown in figure 2.9.

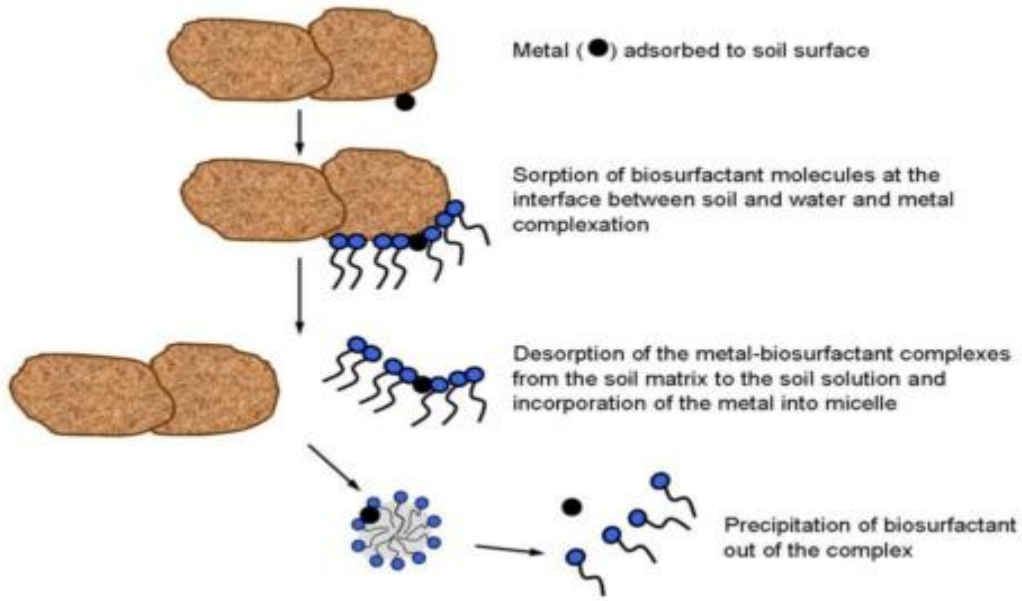

Figure 2.9 Mechanism of biosurfactant activity in metal-contaminated soil (Mulligan, 2005)

2.8.7 Biosurfactants in Food Industries:

The surfactants can have various other functions in food industries, apart from their obvious role as agents that decrease surface and interfacial tension, thus facilitating the formation and stabilization of emulsions. For example, to control the aggregation of fat globules, stabilization of aerated systems, improvement of texture and shelf-life of products containing starch, modification of rheological properties of wheat dough and improvement of constancy and texture of fat-based products (Kachholz and Schlingmann, 1987). In bakery and ice-cream formulations, biosurfactants act by controlling the consistency, slowing staling and solubilizing the flavour

oils; they are agents during cooking of fats and oil. Improvement in the stability of dough, volume, texture and conservation of bakery products is obtained by the addition of rhamnolipid surfactants (Van Haesendonck and Vanzeveren, 2004). The study also suggested the use of rhamnolipids to improve the properties of butter cream and frozen confectionery products. L-Rhamnose has substantial potential as a forerunner for flavouring.

2.8.9 Application of Biosurfactant in Cosmetic Industry

In the cosmetic industry, due to its emulsification, foaming, water binding capacity, spreading and wetting properties effect on viscosity and on product consistency, biosurfactant have been proposed to replace chemically synthesized surfactants. These surfactants are used as emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, anti dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleansers to mention but a few (Gharaei-Fathabad, 2011)

2.8.10 Applications of Biosurfactants in Commercial Laundry Detergents.

Almost all surfactants, an important component used in modern day commercial laundry detergents, are chemically synthesized and exert toxicity to fresh water living organisms. Growing public awareness about the environmental hazards and risks associated with chemical surfactants has stimulated the search for ecofriendly, natural substitutes of chemical surfactants in laundry detergents. Biosurfactants such as Cyclic Lipopeptide (CLP) are stable over a wide pH range (7.0- 12.0) and heating them at high temperature does not result in any loss of their surface-active property (Mukherjee, 2007). They showed good emulsion formation capability with vegetable oils and demonstrated excellent compatibility and stability with commercial laundry detergents favoring their inclusion in laundry detergents formulation (Das and Mukherjee, 2007).

2.8.11 Biosurfactants as Biopesticide

Conventional arthropod control strategy involves applications of broad-spectrum chemicals and pesticides, which often produce undesirable effects. Further, emergence of pesticide resistant insect populations as well as rising prices of new chemical pesticides have stimulated the search for new eco-friendly vector control tools. Lipopeptide biosurfactants produced by several

bacteria exhibit insecticidal activity against fruit fly Drosophila melanogaster and hence are promising to be used as biopesticide (Mulligan, 2005).

2.8.12 Biosurfactant as Antimicrobial Activity: The diverse structures of biosurfactants confer them the ability to display versatile performance. By its structure, biosurfactants exerts its toxicity on the cell membrane permeability bearing the similitude of a detergent like effect (Zhao, 2010).

Gharaei-Fathabad (2011) reported that several biosurfactants have strong antibacterial, antifungal and antivirus activity; these surfactants play the role of anti adhesive agents to pathogens making them useful for treating many diseases as well as its use as therapeutic and probiotic agent. A good example is the biosurfactant produced by marine *Bacillus circulans* that had a potent antimicrobial activity against Gram positive and Gram negative pathogens and Semi pathogenic microbial strains including MDR strain.

2.9 Factors Affecting Biosurfactactant Production

Synthesis of biosurfactants is greatly affected by the type of carbon and nitrogen sources employed as well as environmental conditions like temperature, pH, agitation and aeration.

2.9.1 Carbon Sources

The quality and quantity of biosurfactants produced are affected and influenced by the nature of the carbon substrate. (Rahman and Gakpe, 2008). Two basic types of carbon sources carbohydrates and hydrocarbons are used for the production of biosurfactants. Carbohydrate substrates are used due to relatively less power requirement for dispersion and easy downstreaming operations. Hydrocarbons generate more heat of reaction during cultivation which requires extensive cooling surfaces within the bioreactor system. (Guerra-Santos *et al.,* 1986). Diesel, crude oil, glucose, sucrose, glycerol have been reported to be good sources of carbon substrates for biosurfactant production (Ilori *et al* ., 2005; Desai and Banat, 1997). Hydrophobic substrates like corn oil, lard and long chain alcohols maximized biosurfactant production (100-165 mg/g substrate). Hydrophilic substrates like glucose and succinate delivered poor yields 12-13 mg/g substrate (Mata-Sandoval *et al*., 2000). Water soluble carbon sources such as glycerol, glucose, mannitol and ethanol were all used for rhamnolipid production by Pseudomonas sp. Biosurfactant product however was inferior to that obtained with water immiscible substrates such as n-alkanes and olive oil (Robert *et al.,* 1989).

2.9.2 Nitrogen Sources

Nitrogen plays an important role in the production of biosurfactants, it is essential for microbial growth as protein and enzymes synthesis depends on it. Several nitrogen compounds such as urea, peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extracts have been used for the production of biosurfactants. Ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffines* (Duvnjak *et al* 1982), whereas nitrate supported maximum surfactant production in *Bacillus subtilis* (Guerra-Santos *et al*., 1984, Robert *et al.,* 1989) and *Rhodococcus spp.*

Yeast extract is extensively used as nitrogenous and other vitamin source in the production of sophorolipids from *Candida bombicola.* Casas and Garcia-Ochoa (1999) have shown that 1g/l of yeast extract gives maximum amount of sophorolipids from *Candida bombicola* and categorically stated that at higher yeast extract concentrations, sophorolipids production decreases. Yeast extract is considered to be essential for both cell growth and sophorolipids formation, and its concentration is critical in the culture medium, as sophorolipids synthesis is associated with nitrogen starvation.

2.9.3 Environmental Factors

Environmental factors and growth conditions such as $_{p}$H, temperature, agitation and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity (Desai and Banat, 1997). These are extremely important in the yield and characteristics of biosurfactants produced. To obtain large quantity of biosurfactants, it is always necessary to optimized the bioprocess as the product may be affected by changes in temperature, pH, aeration or agitation speed. Most biosurfactant production are reported to be performed at a temperature range of 25-30^{o}C (Desai and Banat, 1997).

Different microorganisms react differently to changes in pH. Some are insensitive whilst others can have their properties altered by pH adjustment. An optimum pH is therefore needed in the production of microbial emulsifiers. For some biosurfactants, a greater yield may mean a gradual increase in the pH. Zinjarde and Pant (2002), reported that pH 8.0 which is a neutral pH of sea water is the best ph for the production of biosurfactants. The ideal pH for the production of rhamnolipids from Bacillus subtilis as reported by Guerra-Santos *et al.* (1984) is between 6.0 and 6.5 and they have shown that there is drastic decrease in the biosurfactant production beyond the pH 7.0.

2.9.4 Aeration and Agitation

Aeration and agitation are important factors that influence the production of biosurfactants as both facilitate the oxygen transfer from the gas phase to the aqueous phase. It may also be linked to the physiological function of microbial emulsifier. According to Margaritis *et al*. (1980) an increase in the agitation speed results in the reduction of biosurfactant production due to the effect of shear in *Nocardia erythropolis*. While studying the mechanism of biosurfactant production in *Acinetobacter calcoaceticus* RAG – 1 Wang and Wang (1990) revealed that the cell-bound polymer/dry cell ratio decreases as the shear stress increases.

2.9.5 Salt Concentration

Salt concentrations also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products however were not affected by salt concentrations up to 10% (wt/vol), although slight reductions in the CMCs were detected (Abu-Ruwaida *et al* 1991).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling Area

Bonny River, an arm of the Niger River is located in Rivers State, southern Nigeria. At its mouth, 40 km south-southeast of Port Harcourt, is Bonny town, a river pilot station and oil terminal Latitude:4°25' and 4°50' N, Longitude: 7°00' and 7°15'E (Asimiea, 2012).

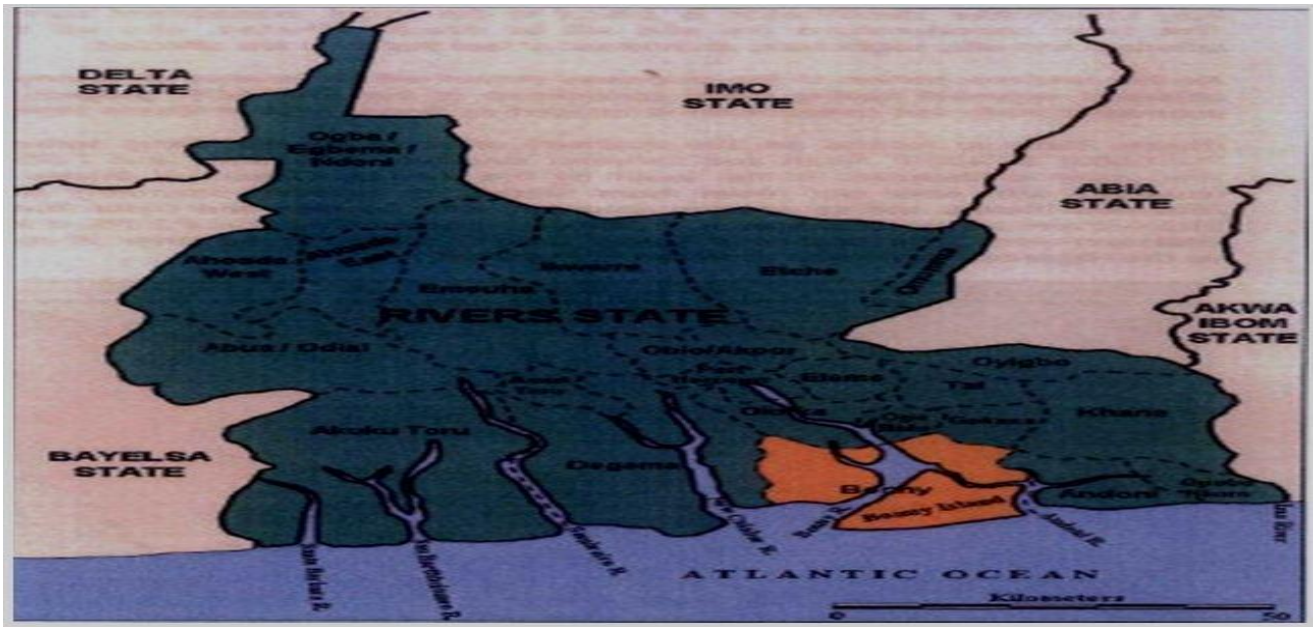

Figure 3.1 Map of Nigeria showing Bonny River (Alaminiokuma, 2018).

3.2 Sample Collection and Preparation

3.2.1 Oil Polluted Soil and Water

Soil samples were collected from Bonny River bank in Bonny local government Area Rivers State. Soil samples were collected at a depth of 0–15 cm using sterile trowel after clearing debris from the soil surface. Samples for chemical analyses were collected in polyethylene bags, while

those for microbiological analysis were collected in sterile screw-capped bottles. Analysis commenced immediately upon arrival in the laboratory. Leftover samples were refrigerated at 4°C. (Lateef, 2011). Unpolluted soil samples which serve as control where collected from some kilometers from the river bank.

Water samples were collected using sterile glass beakers into sterile sampling bottles. The samples were properly labeled and taken to the laboratory in an ice box for analysis. (Onugbolu and Adieze, 2016).

3.2.2 Sample preparation

Soil samples collected from the field were removed of unwanted residues such as dungs, stones and other unwanted materials. The samples were air dried and grinded with a wooden mortar and pestle. After grinding, these samples were sieved and mixed thoroughly to form a bulk sample from where about 1000 g was stored in dry and clean polyethylene bag with proper labeling.

Water sample were serially diluted with distilled water.

3.3.0 Chemical Analysis of Soil Samples:

3.3.1 pH and Conductivity Determination

The pH of the sample were determined using a Jewniary digital pH meter in a sample to water ratio of 1:10 that is 20g of each sample was weighed in a beaker. Then 200 ml of distilled water was added to it. The pH electrode was dipped into the solution and also conductivity was determined using CD303 conductivity meter.

3.3.2 Determination Of Total Organic Carbon

5.0 g of grinded sample was weighed into 500 mL conical flask, 10 mL of 0.5M of potassium dichromate ($K_2Cr_2O_7$) was added and swirled gently. 20 mL of concentrated sulphuric acid H_2SO_4 acid was added into the suspension, the suspensions were gently swirled until the reagents are mixed but becoming vigorous later and for 1 min. The flask was allowed to stand for 30 min.

200 mL of distilled water and 10 mL of concentrated Phosphoric acid (H_3PO_4) were added cautiously to avoid splashing. The suspension was allowed to cool, 3 – 4 drops of ferrion indicator solution was added. Titrate to wine – red colour end point with 0.25M Ferrous Ammonium Sulfate (FAS). (The colour change is usually from blue to deep – green as

the final end point, wine – red, is approached). Note FAS titre reading. Carry out a blank determination as above but without the sample. Note the FAS titre reading.

3.3.3 Determination of Total Hardness

50 ml of filtered soil sample was poured into a 250 ml conical flask and dilute to 100 ml, with deionized water. 4 ml of buffer solution and 6 drops of the mordant black II solution were added. This was titrated with the ethylenediaminetetraacetic acid (EDTA) solution, to a distinct blue endpoint (Vml).

$CaCO_3$ content (mg/Kg) = $\frac{Vx\ E(CaCO_3)\ x\ 100}{50}$

Where; V= ml titration for the sample

E= Calcium carbonate equivalent to 1 ml EDTA titrant.

3.3.4 Determination of Calcium Hardness

50 ml of soil sample was poured into 250 ml conical flask and dilute to 100 ml, with deionized water. Add 2 ml of 2M NaOH solution and 6 drops of the Solochrome dark blue solution. The colour of the solution turn to claret or violet and its pH Value should be at least 12.0

Titrate with the EDTA solution, to a distinct blue endpoint (Vml).

$CaCO_3$ content (mg/Kg) = $\frac{Vx\ E(CaCO_3)\ x\ 100}{50}$

Calcium metal (mg/kg) = $\frac{T\ x\ 400.5\ x\ 1.05}{50}$

T= titre value

3.3.5 Determination of Magnesium Hardness

Magnesium hardness can be calculated from the determined total hardness and Calcium hardness Formula;

Magnesium (mg/l) = (T–C) x 0.243

Where; T= total hardness mg/l (as $CaCO_3$)

C= Calcium hardness mg/l (as $CaCO_3$)

3.3.6 Determination of Total Organic Matter

About 5 g of finely ground, dry soil sample into a porcelain crucible. Char sample on a heater or Bunsen flame inside fume cupboard, to drive off most of the smoke. The sample was transferred into a pre-heated muffle furnace at 550^{o}C. This temperature was maintained for 2 h or until a white or light grey ash results when the residue is black in colour, this were moistened with a small amount of water to dissolve salts, dry in an oven and the ashing process was repeated. Cool in desiccators and reweigh

Calculation:

$$\% \text{ Ash (dry basis)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

$$= \frac{(W_3 - W_1)}{(W_2 - W_1)} \times 100$$

TOM = 100 –%Ash

Where;

W_1 = Initial weight of empty crucible

W_2 = Weight of crucible + sample before ashing.

W_3 = Final weight of crucible + ash.

3.3.7 Heavy Metal Analysis.

Atomic Absorption Spectrophotometer (AAS) model FS 240 Varian atomic absorption spectrophotometer was used in the analysis of heavy metals such as iron, lead, mercury, cadmium, arsenic, and copper.

3.3.7.1 Soil Digestion for Heavy Metal Analysis (Wet Digestion Method)

About 1 g of sample was digested in 250 ml conical flask by adding 30 ml of aqua regia (conc. Nitric acid(HNO_3), Hydrochloric acid (HCl,) Hydrogen fluoride (HF) in ratio 3:2:1), and heated on a hot plate until volume remains about 7-12 ml. The digest was filtered using what-man filter paper and the volume made up to the mark in a 50 ml volumetric flask, and was then stored in a plastic container for AAS analysis.

The sample (digested) was thoroughly mixed by shaking and 50 ml of it was transferred into a glass beaker of 250 ml volume. The sample was aspirated into the oxidizing air-acetylene flame or nitrous oxide acetylene flame of the AAS. When the aqueous sample was aspirated, the sensitivity for 1% absorption was observed. The absorbance of the sample was correlated to obtain the concentration of metal in the sample from the standard calibration curve plotted by the AAS.

3.3.8. Determination of Total Petroleum Hydrocarbon of Soil.

About 5 g of anhydrous sodium sulphate was added to 5 g of soil. 50 ml of n-hexane was added to the mixture. The mixture was transferred to a separating funnel were it was shaken vigorously and allowed to stand for 30 mins. A funnel fitted with sodium anhydrous sulphate was used to collect the layer containing the organic solvent. It was taken for residual oil using the gravimetric method.

3.4.0 Physico-Chemical Analysis of Water

The physicochemical parameters measured include the pH, biochemical oxygen demand (BOD), temperature, total solid (TS), total dissolved solid (TDS), total suspended solid (TSS), electrical conductivity, turbidity, total chloride, physicochemical properties were determined using standard methods for waste water, as described by American Public Health Association, APHA as reported by Abdullahi (2015).

3.4.1 Determination of pH of Water Sample

The pH of the water was determined using ATC pH meter.

3.4.2 Determination of Conductivity/Total Dissolved Solid of Water Sample.

Conductivity and Total dissolved solid was determined using ATC conductivity meter.

3.4.3 Total Solids (TS).

Clean evaporating dish 100 ml was dried at 105^0C in an oven until constant weight was achieved. The dish was cooled to room temperature in a desicator and weigh (w_1). 100 ml of water sample was pipette into the dish and was evaporated to dryness on a steam bath. The outside of the dish was wiped, and the residue was dried in an oven at $105\ ^0$ c for about 1 hour. The dish was quickly transferred to the desicator, cooled and weigh. The dish was returned to the oven to dry further for 10-20 min, cooled in a desicator and re-weighed. The drying was repeated until the weight of the dish plus residue was constant to within 0.05 mg (W_2).

The weight of the dish (W_1) was subtracted from (W_2) to obtain the weight of the total solids.

$$\text{Total solids} = \frac{\text{mg. total solids} \times 1000}{\text{mL of sample}}$$

3.4.4 Determination of Total Suspended Solids (TSS)

The total suspended solid was obtained by calculating from the difference between the total solid and total dissolved solids.

TS = TDS + TSS

3.4.5 Determination of Dissolved Oxygen (DO_2)

Was done using Dissolved oxygen meter (model JPB 607A).

3.4.6 Biological Oxygen Demand (BOD).

Electrometric and Incubation method were used. The water sample was carefully collected in-situ using Winkler's bottle by avoiding bubbling of water. The Winkler's bottle was covered inside the water body without air bubbles. The sample was placed in the cooler and transported to the laboratory. It was incubated at room temperature for 5 days. The DO_2 of the incubated sample was determined by inserting the DO Probe and the reading recorded in mg/l.

The DO_2 of the 5 days was subtracted from the DO_2 of the day 1.

$$BOD_5 = DO_2\,(1) - DO_2\,(5)$$

3.5 MATERIALS USED

3.5.1 Nutrient Agar Medium

Accumix nutrient media was used in purification and maintenance of bacterial isolates. It was prepared by manufacture's direction. Only 28.0 g of commercially formulated nutrient agar was dissolved in 1.0 L of distilled water prior to autoclaving at 121^o C for 15 min. (Sidkey *et al.*, 2016).

3.5.2 Mineral Salt Medium

Bushnell-Haas mineral salt was used in this study comprising of 0.2 g of $MgSO_4$, 0.02 g of $CaCl_2$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NH_4NO_3, 0.05 g of $FeCl_3$. 100 ml of distilled water at pH of 7.0 then sterilization was carried out at 121^{o}C 15 min. (Sidkey *et al.*, 2016).

3.5.3 Petroleum Hydrocarbons

The petroleum hydrocarbons (Bonny light crude oil, diesel and kerosene) was collected from the Shell Petroleum Development Port-Harcourt Refinery in Rivers State, using sterile sampling bottles, this were transported to the laboratory and stored at room temperature

3.6 Enumeration of Total Heterotrophic Bacteria from Soil and Water

The total heterotrophic bacterial (THB) count was determined using the pour plate method on nutrient agar (NA). Soil suspensions were prepared by 6 fold serial dilutions with 10 g of soil in 100 ml of distilled water and 0.1ml of 10^{-2} dilution plated in triplicates. The colony forming units (CFU) of the bacteria were counted after incubation at 28^{o}C for 24-48 h. Colonies from triplicate plates were counted and average count recorded and used for calculation of colony forming units per gram (cfu/g) soil. After incubation, morphologically different colonies observed on the plates were subcultured on a nutrient agar plates to obtain pure culture of the organisms and subsequently transferred into nutrient agar slants. The slants were kept in the refrigerator at 4^{0}C as stock culture. (Onugbolu and Adieze, 2015)

Total heterotrophic count from water was done using the method described by Femi-Ola (2015). Six- fold serial dilution was carried out, pour plate method was used for inoculation, 0.1 ml of 10^{-2} dilution was inoculated on sterile Petri dishes, after which the sterilized media was poured aseptically on the inoculated plates. The plates were incubated at 37^{o}C for 24-48 h. Colonies from triplicate plates were counted and average count recorded and used for calculation of colony forming units per ml (cfu/ml) of water. After incubation, morphologically different colonies observed on the plates were subcultured on a nutrient agar plates to obtain pure culture of the organisms and subsequently transferred into a nutrient agar slants. The slants were kept in the refrigerator at 4^{0}C as stock culture.

3.7 Enumeration and Isolation of Total Hydrocarbon Utilizing Bacteria from Soil and Water

The procedure of (Hamamura, 2006) as reported by Agbor (2012) was used. Crude oil utilizing bacteria in the soil samples were enumerated by the viable count method using the pour plate technique and mineral salts medium. Soil suspensions were prepared by 10 fold serial dilutions with 10 g of soil in 100 ml of distilled water and 0.1 ml of 10^{-2} dilution was poured on the plates in triplicates. After inoculation of the agar plates with the sample, a sterile filter paper (Whatman No.1), saturated with crude oil, was aseptically placed onto the inside of the lid (cover) of the Petri dishes. The filter paper saturated with crude oil served as a sole carbon and energy source

for growth of the organisms on the surface through vapour phase transfer. The plates were then incubated in an inverted position at room temperature for 7 days, after which the average counts from triplicate plates were counted and recorded.

Total hydrocarbon utilizing bacteria from water was done using the pour plate technique, 6 fold serial dilution of water sample was carried out, 10^{-1} and 10^{-2} dilutions were inoculated. After inoculation, a sterile filter paper (Whatman No.1), saturated with crude oil, and was aseptically placed onto the inside of the lid (cover) of the Petri dishes. The filter paper saturated with crude oil served as a sole carbon and energy source for growth of the organisms on the surface through vapour phase transfer. The plates were then incubated in an inverted position at room temperature for 7 days, after which the average counts from triplicate plates were counted and recorded.

3.8 Characterization and Identification of Total Heterotrophic Bacteria and Hydrocarbon Utilizing Bacteria from Soil and Water

Colonies from total heterotrophic bacteria plates and hydrocarbon utilizing bacteria plate on MSA from soil and water were selected based on their colonial characteristics. Bacterial colonies differing in size, shape and colour in different plates were selected. The isolates were purified by streaking on nutrient agar plates. They were then transferred onto nutrient agar slants in Bijou bottles and then stored at 4 ºC in a refrigerator for till further studies. The bacterial isolates were characterized based on colonial and cell morphology, growth on differential/selective media and biochemical tests which include Gram's reaction, indole tests, methyl red, Voges-Proskauer, citrate utilization, Urea test, utilization of different types of sugars, oxidase and catalase test. Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology, Holt *et al*. (1994) as reported by Salam *et al.*, (2011).

3.8.1 Gram Stain

The principle is based on the ability of the bacteria cell to retain the basic dye, methyl violet after decolourization with alcohol. Smears of isolates will be made on clean and greaseless, slides fixed by heat and air dried. The smear was flooded with methyl violet for a minute, and rinsed off using clean water and gram's iodine solution added, and allowed for a minute. The iodine solution was rinsed off while 95% alcohol added and allowed for 30 s. The slides were

counterstained with safranin for 60 seconds, and washed off with water, air dried and examined under oil immersion (100x) objective lens. The organisms that retain the methyl violet solution will appear violet or purple and will be observed as gram positive while those that pick safranin and observed appeared red will be considered gram negative bacteria.

3.8.2 Motility Test

A Semi-solid (half strength) nutrient agar (2.8 g in 200 ml of distilled water) was dispensed 10 ml into test tubes and sterilized. On cooling, the medium was allowed to set in an upright position. The organism was inoculated by stabbing with a sterile straight wire to a depth of approximately 2 cm. the inoculated tubes were the plugged with cotton wool and inoculated at 37^0C for 24 h

After incubation, motility was indicated by diffused brush-like (spreading) growth of the organism from the stab in the medium.

3.8.3 Indole Test

Indole test was employed to determine the ability of certain bacteria to decompose the amino acid tryptophan to indole which accumulates in the medium. The test organism from the inoculums was inoculated into sterile 10 ml peptone water contained in test tubes and incubated at 37^0C for 48 h. Kovacs reagent (0.5 ml) was added in each tube and was shaken gently. Cultures in which red colour developed indicated positive results while absence of red colouration indicated negative result.

3.8.4 Citrate Utilization Test

This test was used to test the ability of some microorganisms to utilize citrate as the sole source of carbon and energy, and ammonium salts as the source of nitrogen. The medium was prepared in slants and the test organism streaked on the surface of the slants before incubation at 37^0C for 48 h. The cultures were observed daily for the development of blue colour and growth along the lines of streak while a negative result showed no change in colour.

3.8.5 Methyl-Red-Voges Proskauer Test (MRVP)

3.8.5.1 Methyl Red Test.

The methyl red test was employed to detect the production of sufficient acid during fermentation of glucose. The test organism was inoculated into glucose-phosphate peptone water medium and incubation at 37^0C for 48 h. At the end of the incubation, 2-3 drops of 0.02% (w/v) methyl red reagent was added to 5 ml of the culture and observed for any colour change.

3.8.5.2 Voges-Proskauer Test

This tests the ability of some bacteria to ferment carbohydrates with the production of acetyl methyl carbinol or its reduction products 2, 3-butylene glycol in the medium. A loopful of the test organism was inoculated into a test tube which contained 10ml of glucose-phosphate peptone water and incubated at 37^0C for 48 h.

At the end of the incubation period, 3 drops of 4% (w/v) potassium hydroxide and 6 drops of 5% (v/v) of α-naphthol in absolute alcohol was added to 5ml of the 48h culture and observed for any colour change.

3.8.6 Catalase Test

The catalase test is used to identify organism which has the ability to produce the enzyme catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.

$$2H_2O_2 \xrightarrow{\text{CATALASE}} 2H_2O + O_2$$

A loopful of 24 h broth culture of each test organism was placed on sterile clean glass slide and mixed with 10% (v/v) hydrogen peroxide solution (about 4 drops) and observed for effervescence. Positive organism produce effervescence while catalase negative ones produce no effervescence.

3.8.7 Triple Sugar Iron Agar Test (TSIA)

This test is used to determine the ability of certain bacteria to utilize a specific sugar incorporated into a basal growth medium with or without the production of gas along with a possible hydrogen sulphide (H_2S) production. TSI agar was prepared in slants; the medium was inoculated by stabbing the butt and streaking the slant with a loopful of the appropriate isolate, incubated at 37^0C for 48 h. the production of gas is marked by cracks in the agar as well as air gap at the bottom of the test tube while (H_2S) production is indicated by the presence of black precipitates which indicates the reduction of sodium thiosulphate to hydrogen sulphide. After incubation period, the following results can be interpreted.

Butt Colour	Slant Colour	Results
Yellow	red	Glucose only fermented
Yellow	yellow	glucose, lactose or sucrose
Red	red	No action on sugars

3.9 Screening of Isolates for Biosurfactant Production

Prior to the screening for biosurfactants, all isolates were inoculated into 20 ml of nutrient broth medium and incubated at 37ºC for 72 h. The culture media were centrifuged at 3000 revolutions per minute (r.p.m.) for 30 min. The supernatant was collected and the cells discarded. The cell free supernatants were used for the various biosurfactant screening tests or assays. Femi-ola (2016)

3.9.1 Drop Collapse Assay

The assay was carried out as described by Jain *et al*. (1991) as reported by Femi-ola (2016) Drops of the supernatant from each of the isolates were placed on a clean glass slide previously coated with hydrocarbon from Diesel. A rapid collapse of the drops show presence of biosurfactant whereas, non-biosurfactant containing drops form balls and does not collapse. Drops of distilled water were used as control.

3.9.2 Oil Spreading Technique

This method was employed to check the efficacy of the culture medium in displacing the oil layer as subscribed by Morikawa *et al.*, (2000), as reported by (Sidkey, 2016). 1 ml of crude oil was added to the surface of 30 ml of distilled water in a Petri-dish to form a thin oil layer, 20 µl of culture supernatant was gently dropped on the centre of the oil layer, after one minute if the sample was +ve (containing biosurfactant), the oil is displaced and a clearing zone was measured.

3.9.3 Emulsification Capacity Test

Emulsification capacity of bacterial isolates towards hydrocarbons (diesel, coconut oil, kerosene, crude oil) was carried out using a mixture of 2 ml oil added to the same amount of cell free supernatant obtained after vortexing sample culture grown on nutrient broth, at high speed for 2 min and left to stand for 24 h. The emulsification index (E_{24}) was calculated as the percentage of

height of the emulsified layer (mm) divided by total height of the liquid (mm) as reported by Okore *et al.,* (2017).

3.10 Preparation of Standard Inoculum

Three different bacteria isolates with highest emulsification index on crude oil were labeled and cultured on nutrient agar and incubated for 24 h at 37°C. Then, a single colony of each bacterium was inoculated into nutrient broth and incubated at 37°C in an orbital shaker for 24 h at 150 rpm. Then, the broths were centrifuged at 4000 for 15 minutes. Supernatants were decanted and pellets containing bacterial cells were centrifuged with distilled water twice to ensure removal of all broth components. The concentration of each inoculum was measured using a spectrophotometer to get 0.1 optical density (Azmy and Hamzah 2007) in 600 nm wavelength. The above standard inoculums were used in the following step. Reported by Kalaivani (2013).

3.11. Determination of the Effects of Physical and Nutrient Parameters on Microbial Growth and Biodegradation of Crude Oil

For all the experiment below, the following standard procedures were used.

Two percent (v/v) of standardized inoculums was inoculated into test tubes containing 24 ml of MSA supplemented with 5% (v/v) of Bonny light crude oil and incubated in an orbital shaker for 7 days. The negative control in these tests was MSM without inoculation. After 7 days of incubation, the cultures were serially diluted 6 fold using normal saline solution and the growth was estimated by plating the bacteria on nutrient agar by the spread plate technique. Residual oil was checked using gravimetric method.

3.11.1 Determination of Effects of pH on Biodegradation of Crude Oil

The pH of each medium was adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0, with 1 m of NaOH or 1 m of HCl. The cultures were incubated at 37°C in an orbital shaker at a speed of 150 rpm.

3.11.2 Determination of the Effects Nitrogen Sources On Biodegradation of Crude Oil

To determine the effect of nitrogen source on the utilization of crude oil, the total amount of nitrogen in MSM which was contained in ammonium nitrate (NH_4NO_3) = 10 g/l was replaced

with different nitrogen sources, namely, ammonium sulphate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl).

3.12 Crude Oil Degradation Studies.

The ability of isolates to degrade crude oil was done using the gravimetric method demonstrated in terms of reduction in the weight of crude oil introduced. The rate of utilization was monitored on the first day (day zero) of the study and subsequently at 4-day interval for 28 days. n-hexane was employed as the extractant. On each day, three samples per single treatment were analyzed for the quantity of residual crude oil using the methods of Udeme and Antai (1988) and described by Nwaogu *et al.,* 2008. Two percent (v/v) of standardized inoculums was inoculated into test tubes containing MSA supplemented with 20% (v/v) of Bonny light crude oil and incubated in an orbital shaker for 28 days. The negative control in these tests was MSM without inoculation.

Extraction of Residual Oil. The residual oil was extracted by using liquid – liquid solvent extraction method. The organic solvent used was n-hexane. This was done by measuring 50ml of n-hexane into the bottles containing OIL-MSM, the contents were later transferred into separating funnel. A funnel fitted with filter paper (Whatman No 1), anhydrous sodium sulphate spread on the filter paper was employed to remove any moisture in the mixture, this was used to collect the layer containing the organic solvent and residual oil in an pre-weighed 50ml pyrex beaker. The organic solvent was allowed to evaporate in an oven, after the evaporation, the amount of residual oil was calculated as thus:

Weight of oil degraded = Original weight of oil minus weight of residual oil obtained after evaporating the extractant.

Percentage degradation = (Weight of crude oil degraded / Original weight of oil introduced) × 100.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS.

4.1.1: Mean Soil Chemical Parameters.

The results obtained the soil analysis as shown in table 4.1 indicates that the soil contains relatively high hydrogen ion concentration (H^+) with pH of 4.24±0.03 as compared with the soil control experiment that recorded a pH value of 7.6±0.05. Other components of the soil showed higher concentration of ions such as potassium, magnesium, chloride, calcium at 11.52±0.5, 11.27±0.34, 1151.614±0.37 and 19.34±0.12 respectively. Analysis of some heavy metals such as iron, cadmium, mercury, arsenic, lead and copper contents of the test soil sample were analyzed.

4.1.2: Mean physicohemical Parameters of Bonny River.

The chemical parameters of Bonny River as shown in table 4.2 showed a relatively low pH of 5.12±0.3 when compared to that of the uncontaminated control at a pH of 7.2±0.02. A temperature of 32.3±0.03 ^oC and 25.1±0.04 ^oC for control and test sample respectively. Dissolved Oxgen (DO) concentrations of the control and test sample were 9.4±0.01 and 5.0±0.03 respectively. Biological Oxgen Demand (BOD) was 0.34±0.01 and 1.8±0.02 for control and test sample respectively. Heavy metals such as mercury and arsenic were below detectable limits in both the control and test samples. Total petroleum hydrocarbon (TPH), total suspended solids (TSS), total dissolved solids (TDS) and total solids (TS) of the test sample were 3.87±0.11 mg/ml, 23036±0.51 mg/ml, 396.5±0.3 mg/ml and 23433±0.4 mg/ml respectively.

4.1.3: Total Heterotrophic and Hydrocarbon Utilizing Bacterial Count.

The results obtained from the microbial isolation and identification as shown in table 4.3 indicates that the soil sample contained relatively high total heterotrophic and hydrocarbon utilizing bacteria with colony forming unit per gram (Cfu/g) with 1.7±0.43 x 10^5 and 6.0±0.36 x 10^4 cfu/g respectively. Total heterotrophic and hydrocarbon utilizing bacteria count from test sample were 2.8±0.32 x 10^5 cfu/ml and 2.3±0.41 x 10^4 cfu/ml respectively. Hydrocarbon utilizers obtained from the soil were 35% and 8.2% respectively.

Table 4.1: Mean Soil chemical Parameters

Physiochemical Parameters	Soil Control Experiment	Test Soil Sample
pH	7.6±0.05	4.24±0.03
Chloride ion (mg/g)	433±0.10	1151.614±0.37
Phosphorus (mg/g)	1.78±0.02	1.23±0.02
Magnesium (mg/g)	6.27±0.1	11.27±0.34
Potassium (mg/g)	7.22±0.2	11.52±0.5
Calcium (mg/g)	18.23±0.1	19.34±0.12
Bicarbonate (mg/g)	1.89±0.03	2.3±0.05
Iron (mg/g)	3.52±0.02	38.7±0.13
Cadmium (mg/g)	BDL	0.015±0.01
Mercury (mg/g)	BDL	BDL
Arsenic (mg/g)	BDL	BDL
Lead (mg/g)	BDL	1.73±0.04
Copper (mg/g)	1.08±0.03	5.49±0.05
Total petroleum hydrocarbon (TPH)(mg/g)	0.34±0.01	5.93±0.13
Total Organic Carbon (TOC)	0.45±0.02	3.64±0.1
Total Organic Matter	0.38±0.01	4.23±0.1

BDL: Below detectable limit.

Table 4.2: Mean Physicochemical Parameters of Bonny River

Parameter	Water Control Experiment	Contaminated Test Water Sample	FEPA Standard
pH	7.2±0.02	5.21±0.03	6-9
Temperature (^{0}C)	32.3±0.03	25.1±0.01	40
Conductance (µS/cm)	433±0.04	610±0.04	200
DO (Mg/l)	9.4±0.01	5.0±0.03	8-10
BOD_5 (Mg/l)	0.34±0.01	1.8±0.02	30
Turbidity (NTU)	12.33±0.04	53.50±0.06	1.0
Chloride ion (mg/ml)	533±0.20	609.82±0.37	600
Phosphorus (mg/ml)	1.8±0.04	1.03±0.02	5
Magnesium (mg/ml)	8.27±0.2	13.44 ±0.42	NS
Potassium (mg/ml)	4.22±0.2	7.68±0.3	NS
Calcium (mg/ml)	20.23±0.1	21.22 ±0.12	0.05
Bicarbonate (mg/ml)	1.89±0.03	4.3±0.07	NS
Iron	12.2±0.13	42.2±0.6	0.05
Cadmium	BDL	0.016±0.01	0.05
Mercury	BDL	BDL	0.05
Arsenic	BDL	BDL	0.1
Lead	BDL	1.9±0.04	0.01
Copper	1.08±0.03	5.2±0.05	0.2
TPH	0.34±0.01	3.87±0.11	NS
TSS	2300±0.5	23036±0.51	10
TDS	392.0 ±0.3	396.5±0.3	200
TS	23431±0.42	23433±0.4	NS

BDL: Below detectable limit. NS: Not specified. FEPA: Federal Environmental Protection Agency.

Table 4.3: Total Heterotrophic and Hydrocarbon Utilizing Bacterial Count.

Sample	Total Heterotrophic Count	Hydrocarbon Utilizing Count	Percentage heterotrophs that are Hydrocarbon Utilizers
Soil	$1.7\pm0.43\times10^{5}$cfu/g	$6.0\pm0.36\times10^{4}$cfu/g	35
Water	$2.8\pm0.32\times10^{5}$cfu/ml	$2.3\pm0.41\times10^{4}$cfu/ml	8.2

4.1.4: Screening of Potential Biosurfactant Production Using Drop Collapse Method.

The ability of the isolates to produce biosurfactants on different hydrocarbon sources such as coconut oil, kerosene, diesel and crude oil were investigated and the results are presented in table 4.4. *Klebsiella* sp C4 and *Bacillus* sp HC6 showed vigorous drop collapse on all the tested hydrocarbon sources. *Escherichia* sp C2, *Pseudomonas* sp HC1 and *Salmonella* sp HC2 were negative to drop collapse using crude oil as sole carbon source.

4.1.5: Screening of Potential Biosurfactant Production Using Oil Spreading Method.

The ability of the isolates to produce biosurfactants was tested using the oil spreading technique on the various hydrocarbon sources and the results are presented in table 4.5. *Pseudomonas* sp C3, *Klebsiella* sp C4, *Pseudomonas* sp HC4 and *Bacillus* sp HC6 recorded clear zone diameter of greater than 6 mm (+++) on coconut oil and kerosene respectively. *Klebsiella* sp C4 showed clear zone diameter greater than 6 mm (+++) on diesel and crude oil respectively. Salmonella sp HC2 was negative showing no clear zone diameter on diesel and crude oil respectively while *Escherichia* sp C2 also showed no clear zone diameter on crude oil as sole carbon source.

4.1.6: Emulsification Index (%E_{24}) Of Bacterial Isolates on Different Hydrocarbons Source

Results obtained from the emulsification Index (%E24) as presented on table 4.6 showed that *Klebsiella* sp C4 showed the best emulsification activity with 53.3±0.6%, 58.2±0.7%, 56±0.56% and 51.9±0.53% emulsification index using diesel, coconut oil, kerosene and crude oil respectively. It was followed by *Pseudomonas* sp HC4 with 51.0±0.40%, 48.0±0.57%, 54.0±0.51% and 49.0±0.52% emulsification index using diesel, coconut oil, kerosene and crude oil respectively. *Bacillus* sp HC6 had emulsification index of 47±0.54%, 51.0±0.32%, 48.2±0.14% and 46.7±0.55% for diesel, coconut oil, kerosene and crude oil respectively. *Salmonella* sp HC2 showed the least emulsification index with 32.8±0.54%, 30.0±0.47%, 34.0±0.4%, and 37.0±0.42 for diesel, coconut oil, kerosene and crude oil respectively.

Table 4.4 Screening of Potential Biosurfactant Production Using Drop Collapse Method.

BACTERIAL ISOLATE	Coconut Oil	Kerosene	Diesel	Crude Oil
Escherichia sp *C2*	+	+	+	-
Pseudomonas sp C3	+	++	++	+
Klebsiella sp. C4	++	++	++	++
Pseudomonas sp. HC1	++	++	+	-
Salmonella sp. HC2	+	-	-	-
Bacillus sp HC3	++	++	+	+
Pseudomonas sp HC4.	++	++	+	+
Bacillus sp. HC5	++	++	+	+
*Bacillus s*p. HC6	++	++	++	++
*Pseudomonas s*p. HC7	+	+	+	+

KEY: Drop-collapse: -, No collapse, + slow drop, ++ vigorous drop.

Cfu value of isolates used.

C1=8.6±0.6×10^5. C2=4.1±0.32×10^5, C3=4.8±0.24×10^5, C4=5.6±0.54×10^5, HC1=4.8±0.46×10^5, HC2=1.28±0.30×10^6, HC3=5.2±0.11×10^5, HC4=8.6±0.6×10^5, HC5=1.15±0.34×10^6, HC6=1.18±0.65×10^6, HC6=1.26±0.07×10^6

TABLE 4.5 Screening of Potential Biosurfactant Production Using Oil Spreading Method.

BACTERIAL ISOLATE	Coconut Oil	Kerosene	Diesel	Crude Oil
Escherichia sp *C2*	++	++	+	-
Pseudomonas sp.C3	+++	+++	++	++
Klebsiella sp. C4	+++	+++	+++	+++
Pseudomonas sp.HC1	++	++	+	+
Salmonella sp.HC2	+	+	-	-
Bacillus sp. HC3	++	++	++	+
Pseudomonas sp HC4.	+++	+++	+++	++
Bacillus sp. HC5	++	++	++	++
Bacillus sp. HC6	+++	+++	++	++
Pseudomonas sp. HC7	++	++	++	+

KEY: Oil spreading technique: - , no clear zone diameter, +, clear zone diameter >1<3 (mm), ++, clear zone diameter >3<6 (mm), +++, clear zone diameter>6 and < 9 (mm).

Table 4.6 Emulsification Index (%E_{24}) Of Bacterial Isolates on Different Hydrocarbons Source

Bacterial Isolate	Emulsification Index (%)			
	Diesel	Coconut Oil	Kerosene	Crude Oil
Escherichia sp C2	37±0.7	36±0.62	41±0.52	39.6±0.45
Pseudomonas sp C3.	45±0.56	43±0.56	44±0.62	43.6±0.52
*Klebsiella.*sp C4	53.3± 0.6	58.2±0.74	56±0.56	51.9±0.53
Pseudomonas sp HC1	40±0.62	42±0.54	40±0.55	46.8±0.51
Salmonella sp. HC2	32.8±0.54	30±0.48	34±0.40	37±0.42
Bacillus sp .HC3	38.0±0.42	40.1±0.34	41.8±0.35	42.6±0.36
Pseudomonas sp.HC4	51.0±0.40	48±0.57	54±0.51	49±0.52
Bacillus sp.HC5	46±0.75	45±0.54	32±0.34	37±0.41
Bacillus sp .HC6	47±0.54	51±0.32	48.2±0.14	46.7±0.51
Pseudomonas sp.HC7	43±0.54	42±0.48	41.5±0.32	41.2±0.55
Control MSM+ Crude oil	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.

4.1.7: Effects of Physical and Nutrient Parameters on the Growth of the Isolates

Considering the effects of various physical and nutrient parameters on the growth of bacterial isolates as presented in figure 4.1, all of the bacterial isolates were able to grow at various pH ranges. *Psuedomonas* sp showed the highest growth at pH 6.5. *Bacillus* sp grew best at pH 7.5 while *Klebsiella* sp grew best at pH 7.0. *Pseudomonas* sp produced the highest growth with 2.25×10^5 cfu/ml while *Bacillus* and *Klebsiella* sp had 2.04×10^5 cfu/ml and 1.70×10^5 cfu/ml respectively. Figure 4.1 shows effects of pH on the growth of the isolates.

The isolates were able to utilize all types of nitrogen sources tested containing ammonium sulphate $(NH_4)_2SO_4$, sodium nitrate ($NaNO_3$) and ammonium chloride (NH_4Cl). Ammonium chloride was the most utilized nitrogen source in this study with *Pseudomonas* sp producing the highest growth of 2.37×10^5 cfu/ml, followed closely by *Bacillus* sp with 2.17×10^5 cfu/ml while *Klebsiella* sp was 1.93×10^5 cfu/ml. Figure 4.2 shows the effects of various nitrogen sources on the growth of the isolates.

4.1.8: Effects of Physical and Nutrient Parameters on the Utilization of Crude Oil

The effects of these isolates on the utilization of crude oil were tested at various pH various ranges and presented in figure 4.3. At pH 6.5, about 76% loss of oil was recorded by *Pseudomonas* sp. *Bacillus* sp recorded a 68% loss at pH of 7.5 while *Klebsiella* sp had a 58% loss at pH of 7.0.

The ability of the isolates to utilize crude oil supplemented with different nitrogen sources were tested and presented in figure 4.4. About 67%, 63% and 51.8% loss of crude oil were recorded for *Pseudomonas, Bacillus* and *Klebsiella* species respectively when supplemented with ammonium chloride. Ammonium chloride was the best utilized nitrogen source. Sodium nitrate recorded a 62.4%, 56.7% and 44.4% loss for *Pseudomonas, Bacillus* and *Klebsiella* species

respectively. Ammonium sulphate was the least utilized with 53.2%, 46.7%, and 36% loss of crude oil for *Pseudomonas, Bacillus* and *Klebsiella* species respectively.

4.1.9 Crude Oil Utilization Studies

I*Pseudomonas, Bacillus*, and *Klebsiella* species were used for the utilization studies and result is presented in figure 4.5. After 28 days of incubation, the highest oil utilization was observed in *Pseudomonas* sp (77.5%), this was followed by *Bacillus* sp (66.8%) and *Klebsiella* sp (54.2%).

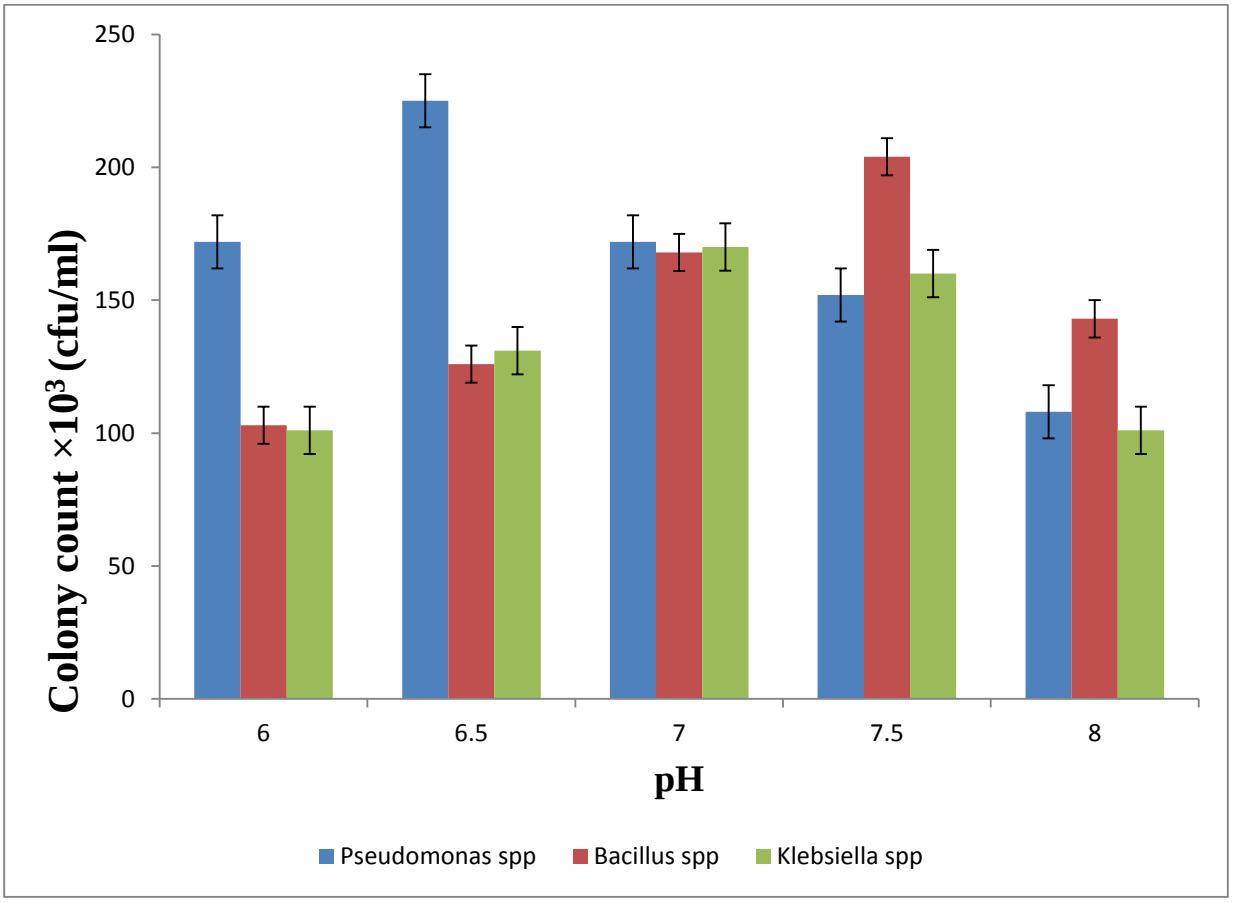

Figure 4.1 Effects of pH on the growth of the isolates.

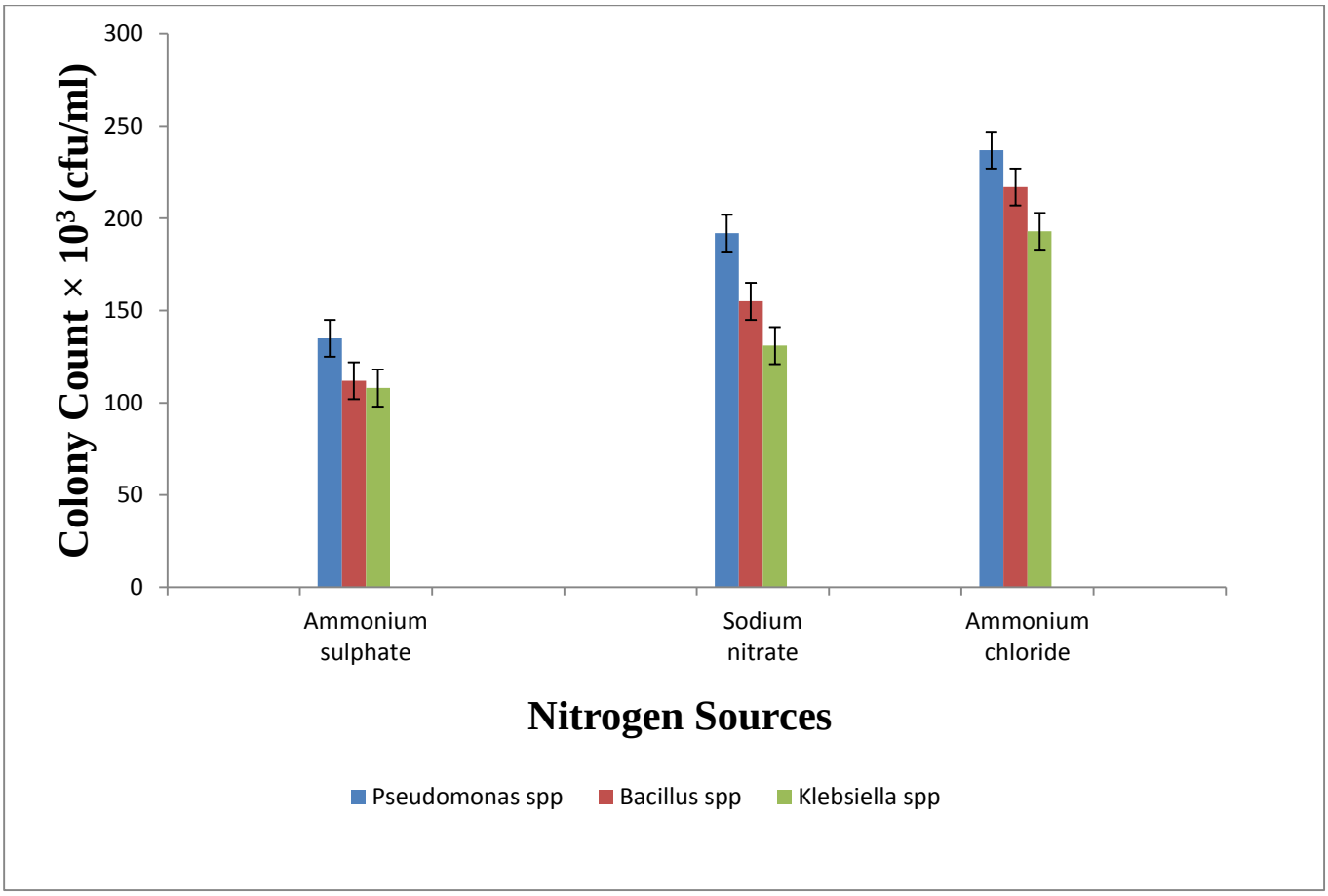

Figure 4.2 Effects of various nitrogen sources on the bacterial growth of the isolates in MSM supplemented with 5% v/v of crude oil.

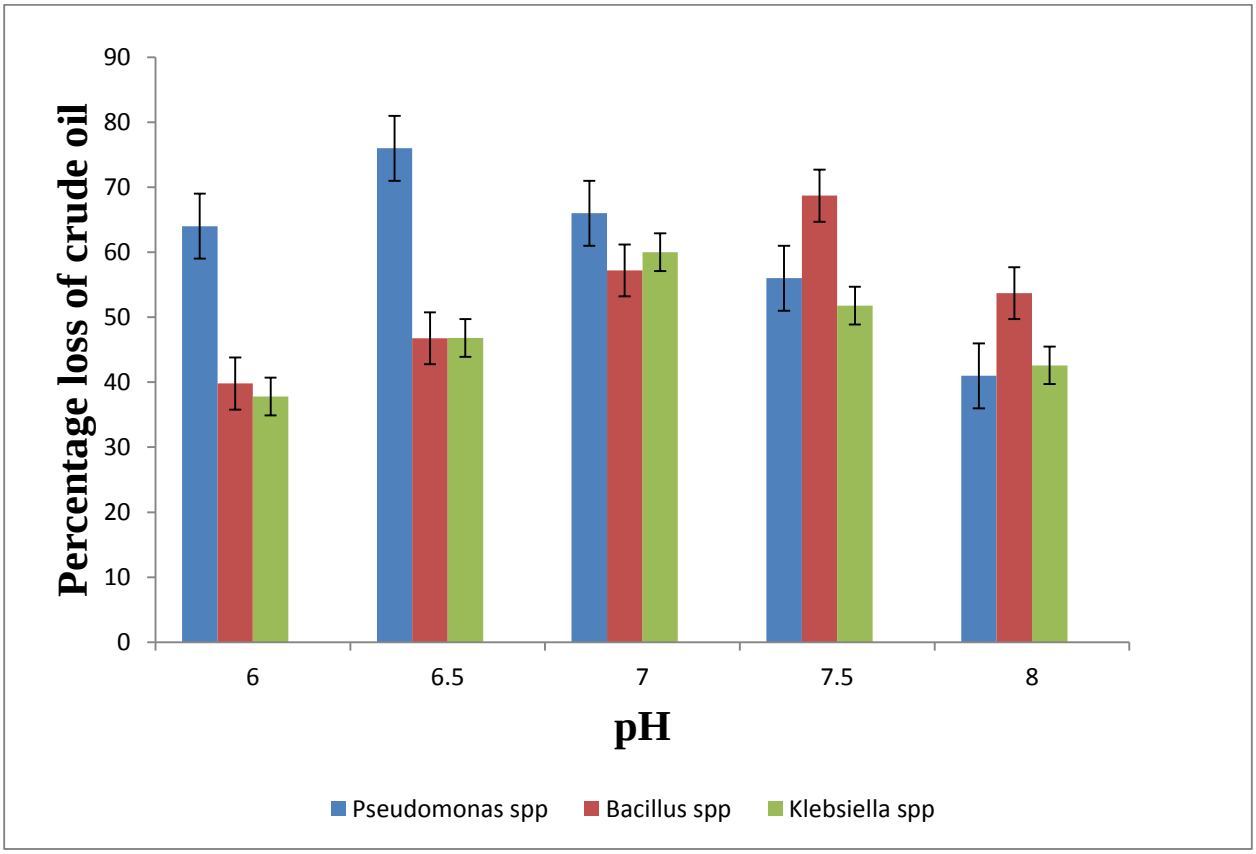

Figure 4.3 Effects of pH on the utilization of crude oil by the isolates.

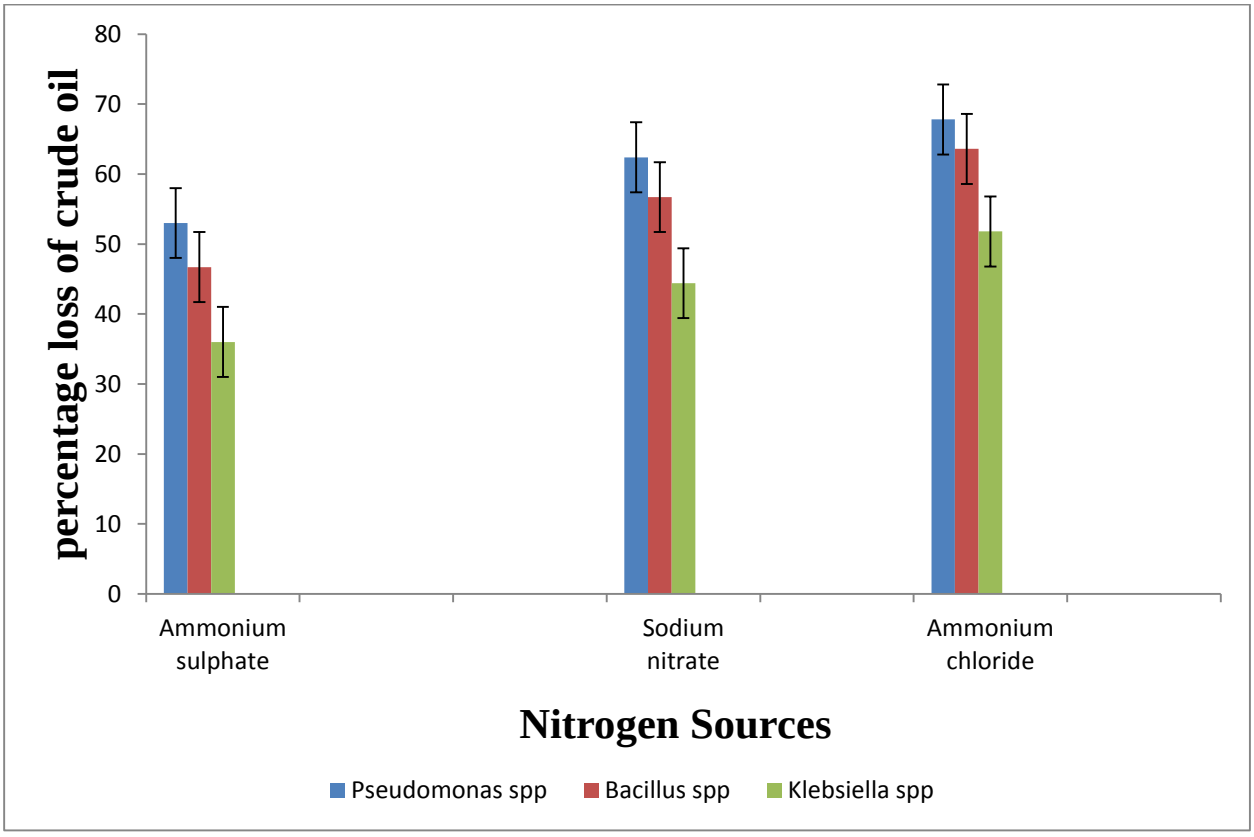

Figure 4.4 Effects of nitrogen sources on the utilization of crude oil

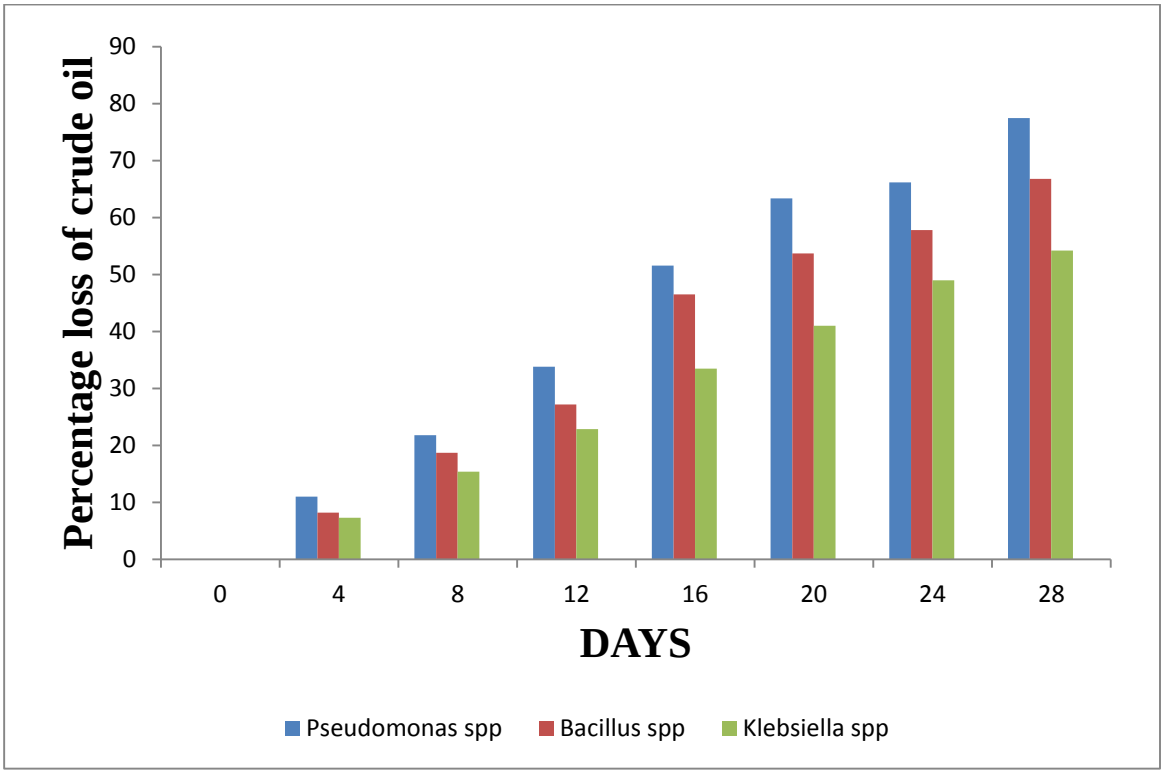

Figure 4.5 The percentage utilization of crude with respect to incubation time (28 days).

4.2 DISCUSSION

4.2.1 Physicochemical Parameters of Soil and Water

The increasing trend of noxious environmental pollutants in our biosystem and possible biological technique of their removal have increased interest in biosurfactants from emulsifying organisms as possible alternative to chemical surfactants owing to their unique qualities. This has resulted in the search for producing microorganisms from diverse environments. From the present study, results obtained from the soil analysis showed that the soil contains relatively high hydrogen ion concentration (H+) with pH of 4.24±0.03 showing an acidic range profiling on pH scale. The same value was observed from the analyzed contaminated water at the same site with pH value of 5.21±0.03. This was significantly different from the uncontaminated soil and water located almost 1.40 Km from the site of sampling site with pH values of 7.6±0.05 and 7.2±0.02 respectively. The low pH range of the contaminated water and soil can be attributed to the nature of the recalcitrant in the soil such as polycyclic aromatic hydrocarbons (PAHS) e.g pyrene, naphthalene etc that contain relatively high acidic contents as stated in the proceedings of the Agency for Toxic Substances and Disease Registry (ATSDR), 2005. These components integrate itself into the soil and surrounding environment such as water and increase the hydrogen ion concentration of the medium. Other components of the soil and water upon analysis showed higher concentration of potassium ions (11.52±0.5 mg/g and 7.68±0.3 mg/ml), phosphorus (1.23±0.02 mg/g and 1.03±0.02 mg/l), Magnesium (11.27±0.34 mg/g and 13.44±0.42 mg/ml), chloride ions (1151.61±0.37 mg/g and 609.82±0.37 mg/ml), calcium ion (19.34±0.12 mg/g and 21.22±0.12 mg/ml) relatively lower bicarbonate ions (2.3±0.05 mg/g and 4.3 mg/ml). The result showed a significant decrease from the unpolluted soil and water sample (control) which upon analysis showed a relative lower concentrations of K, Ca, P, Mg and Cl ion in the following order respectively: 1.78±0.2 mg/g, 18.23±0.1 mg/g, 7.22±0.2 mg/g, 6.27±0.1 mg/g, 433±0.10 mg/g and 1.8±0.04 mg/ml, 20.23±0.1 mg/ml, 4.22±0.2 mg/ml, 8.27±0.2 mg/ml and 533±0.20 mg/ml respectively for both the soil and water. Chikere *et al.,* 2006 in their study at Eleme petrochemical jetting port site reported dissimilar ions concentrations in the contaminated Eleme port soil. They revealed higher concentrations of the mineral ions in the following order 2.28, 1.84, 5.22 and 1789.22 mg/g respectively for K, nitrate, magnesium and chloride ions. Analysis of heavy metal ion (Fe, Pb, Hg, Cd, Cu and As) contents of the crude oil contaminated soil and water using the atomic absorption spectra (AAS), showed a greater proportion of iron (Fe) in both the soil and water with concentration of 38.7±0.13 mg/g and

42.2±0.6 mg/ml respectively, this was followed by copper (Cu) with a concentration of 5.49±0.05 mg/g and 5.2±0.05 mg/ml respectively. Mercury (Hg) and Arsenic were found below detectable limit (BDL) in both tested samples and their respective control experiment. Lead and cadmium are found relatively in lower concentrations of 1.73±0.04 mg/g and 0.015±0.01 mg/ml in the tested soil samples and contain 1.9±0.04 mg/ml and 0.016±0.01 mg/ml in the contaminated water; while they were found below detectable limits in the control samples respectively. This correlate with the findings of Oparaji *et al.*, (2016) on the bioaccumulation of heavy metals in aquatic faunas and sediments at Eleme River Portharcourt. Their results showed a higher proportion of Fe in all the tested species of aquatic fauna in the contaminated Eleme river while they reported Hg to be at below detectable limits (BDL) in all the tested faunas and surrounding sediments. Total organic carbon content (TOC) and total petroleum hydrocarbon (TPH) content of the soil were observed at 3.64±0.1 mg/g and 5.93±0.13 mg/g respectively in the tested sample. This showed a strong significant different from the control experiments in all the tested samples which showed a TOC value of 0.45±0.02 mg/g and TPH value of 0.34±0.1 mg/g respectively. Mbachu *et al*., 2016 reported a dissimilar result in their research on microbial diversities in a spent engine polluted site at Mgbuka, Onitsha Anambra state with Total petroleum hydrocarbon and total organic carbon contents showing the highest in concentrations (4.06 mg/g and 5.21 mg/g respectively). Physiochemical properties of the water such as turbidity showed the presence of high turbid water view with an index of 53.2 NTU. Other physicochemical parameters observed from the contaminated water sample include dissolved oxygen concentration (DO) which was gotten as 5.0±0.03 mg/l from which the biochemical oxygen demand (BOD) needed for oxidation of organic matter in the water was extrapolated in five days. The BOD value of the water was taken after five days of incubation and was 1.8±0.02 mg/l. this observed value significantly varied from the control water sample (unpolluted sample) which showed a BOD of 0.34±0.01 mg/l depicting much available dissolved oxygen for biochemical activities of inhabitants of the water. Total dissolved solid (TDS), suspended solids (TSS) and total solids (TS) of the water were 396.5±0.3 mg/ml, 23036±0.51 mg/ml and 23433±0.4 mg/ml respectively. These showed no significant variation from the control experiment which gave 392.0±0.3 mg/ml, 2300±0.5 mg/ml, 23431±0.42 mg/ml for TDS, TSS and TS respectively.

4.2.2 Enumeration of Total Heterotrohpic and Petroleum Utilizing Bacteria Count from Soil and Water

The results obtained during the microbial isolation and identification showed that soil samples from Bonny have relatively high total heterotrophic and hydrocarbon utilizing bacteria with coliform forming unit per gram CFU/g from the contaminated soil as $1.7\pm0.43 \times 10^5$. Isolates with CFU/g of $6.0\pm0.36 \times 10^4$ were gotten using a differential media specifically for hydrocarbon utilizing organisms formulated by Bushnell and Haas. Microbial isolations from the contaminated water gave the same trend as those isolates from the contaminated surrounding soil. Total heterotrophic count of microbes from the contaminated water was $2.8\pm0.32 \times 10^5$ using the nutrient media. Total hydrocarbon utilizing bacteria recorded a decrease in microbial populations using the Bushnell/Hass formulation media; a count of $2.3\pm0.41 \times 10^4$ was observed at 10^{-2} dilution. This result is an indication that the site may be actively receiving hydrocarbons. According to Adieze *et al*. (2003) and Abu and Ogiji (1996) in their study on hydrocarbon utilizing bacteria showed that bacteria with the ability to degrade a wide range of crude oil components exist ubiquitously in the environment and do appear to respond quite rapidly to the presence of petroleum hydrocarbons. It has also been reported that microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon degrading bacteria and bacterial plasmids encoding hydrocarbon catabolic genes (Leahy and Colwell, 1990).

4.2.3 Morphologiacal and Biochemical Charaterization of Isolates

The characterization and identification of the isolates showed that a total of ten (10) bacteria isolates, belonging to six genera were obtained. These are three spp of the genera *Bacillus*, four of *Pseudomonas*, while a single sp each of *Klebsiella*, *Escherichia*, and *Salmonella* were isolated. Out of the ten (10) isolates from the contaminated soil and water using the nutrient media only seven isolates were competent hydrocarbon degraders owing to their ability to thrive on crude oil spilled on the Bushnell/Hass media as the only carbon source. From the contaminated water, a total of 4 isolates were obtained belonging to 4 genera of bacteria namely: *Escherichia, Salmonella, Pseudomonas,* and *Bacillus* were obtained. 6 isolates were obtained from the contaminated soil, three *Pseudomonas,* two *Bacillus* and one *Klebsiella.* Bacteria are currently optimized active agents in petroleum degradation, and they work as primary degraders of spilled oil in the environment (Rahman *et al.,* 2002; Brooijmans, *et al.,* 2009). This correlates with the finding of Adieze *et al.,* (2003) on microbial diversities during bioremediation of crude

oil polluted site using culture dependent technique. The isolation of high number of certain oil-degrading microorganism from an environment is commonly taken as evidence that those microorganisms are the most active oil degraders of the environment (Atlas and Bartha, 1998).

4.2.4 Emulsification Assays

Upon emulsification assays carried out on the isolated bacteria, only three isolates out of the eleven organisms showed higher emulsification potentials during the study. Three emulsification assay carried out which include drop collapse, oil spread plate and emulsification index test on the isolates using crude oil, kerosene, diesel and coconut oil as the sole carbon source showed the following results: Using drop collapse assay as described by Jain *et al.*, (1991). 90% of the isolates were positive to drop collapse assay using coconut oil as sole carbon source, 18% of the isolates were negative to drop collapse assay using kerosene as sole carbon source, 18% of the isolates were negative to diesel on drop collapse, and 36% of the isolates were negative in drop collapse using crude oil as sole carbon source.

Pseudomonas sp C3, *Klebsiella* sp C4, *Pseudomonas* sp HC4 and *Bacillus* sp HC6, had the highest clear zone diameter of greater than 6 mm. similar trends were observed when using hydrocarbons from kerosene; 36.36% of the isolates had a clear zone diameter of greater than 6mm, while 45% of the isolates had a clear zone diameter greater than 3.0mm but less than 6.0 mm, 9.0% of the isolates had a clear zone diameter of less than 3.0 mm, 9.0% represents isolates with no clear zone diameter.

Almost a similar trend was observed when isolates were testes on diesel as sole carbon source; 18.18% of the isolates had the highest clear zone diameter of greater than 6 mm, these were followed by 45.45% of the isolates that had a clear zone of greater than 3 mm but less than 6.0 mm. 18.18% of the isolates had clear zone diameter of less than 3 mm, 18.18% of the isolates were negative with no clear zone diameter.

Klebsiella sp C4 had the highest clear zone diameter of greater than 6 mm when crude oil was used as the sole carbon source. 36% of the isolates had clear zone diameter of less than 6 mm, 18.18% had clear zone diameter of less than 3mm while 27.27% of the isolates had no clear zone diameter. Oil such as kerosene and coconut oil showed much promising emulsifying feature with the identified organisms than that done by crude oil. Okpokwasili and Amanchukwu (1996) in their impact assessment of biodegradation of crude oil with strains of *Candida* spp stated that crude oil especially that of bonny light are complex mixtures of hydrocarbons comprising of

PAHs, aliphatic hydrocarbons and centrally total petroleum hydrocarbons (TPH) which will take on a hydrocarbonolistic organism(s) to act on and utilize.

Emulsification studies by crude cultures of the isolates showed no statistical significant difference in the emulsification potentials of the isolates at ($p<0.05$). Results from the contaminated soil showed that *Klebsiella* sp C4 showed the best emulsification activity with 53.3%, 58.2%, 56.7% and 51.9% emulsification index respectively using diesel oil, coconut oil, kerosene and crude oil respectively. It was followed by *Pseudomonas* sp HC4 with 51.0%, 48.0%, 54.0% and 49.0% emulsification index. *Bacillus* sp HC6 had emulsification index of 47%, 51%, 48.2% and 46.7% for diesel, coconut oil, kerosene and crude oil as sole carbon source. Upon assessment of isolates from the contaminated water; out of the five isolates three showed a promising emulsification capacity and they include: *Pseudomonas* HC1 sp, *Bacillus* sp HC3 and *Escherichia* sp C2. Their activities in the presence of the oil emulsion showed a significant variation from preparations without culture cell suspensions. *Pseudomonas* showed the highest emulsification activity with 40%, 42%, 40% and 46% index with diesel, coconut oil, kerosene, and crude oil respectively. Ojeh and Onwurah (2016) reported similar results in their findings on emulsifying bacteria consortium isolated from a spent engine mechanic workshop in Nsukka, enugu state. They reported that strains of *Pseudomonas aeroginosa* showed the highest emulsification activity with an index of 49% in the presence of spent engine oil and 53% in the presence of engine oil.

The results from this present study on the biodegradation of the crude oil from the Bonny river port, Rivers state by the strains of bacteria isolated from the contaminated soil using standard microbiology and biochemical technique showed a very promising biodegradation ability of the organisms. As reported in the compendium by Valero (2010) on the significance of organisms during bioremediation of soil contaminated with varying degree of petroleum hydrocarbons (PAHs, TPH and polycyclic aliphatic hydrocarbons) showed that microbial communities present initially in area spilled with the contaminants through various mechanisms as illustrated in the biochemodynamic models breaks down carbon chains of the petroleum hydrocarbons and as well utilize the broken carbon catenation as their sole carbon sources during the process. The compendium also showed various stages of microbial succession during the period of active bioremediation process as insitu organisms present initially during the activity can be augmented or replaced by another seed organism. As reported by Okpokwasili and Amanchukwu, (1996) on cometabolism of fungi (*Candida* sp.) during bioremediation process, their findings revealed a

better of degradation of recalcitrant by the organisms when co metabolized or applied in consortium. Among the organisms isolated, strains of *Pseudomonas*, *Klabsiela* and *Bacillus* showed relatively greater strength in utilization of the bonny light crude during the study using the gravimetry method of biodegradation study (where weight of residual oil was used as a factor of the process) than other organisms isolated from the same contaminated soil. As reported by Valero (2010), bacteria community are active utilizers of petroleum hydrocarbons especially of gram negative rod shaped bacteria domicile within the area of the contamination.

4.2.5 Effects of Physical and Nutrient Parameters on the Growth of the Isolates

Considering the effects of various physical and nutrient parameters on the growth of bacterial isolates, all of the bacterial isolates were able to grow at various pH ranges. *Psuedomonas* sp showed the highest growth at pH 6.5. Hence it could be said that the optimum pH for the growth of *Pseudomonas* sp is 6.5. However, at pH 6.0 and 7.0 the growth of the isolates was the same. This indicates that between pH ranges of 6.0 to 7.0 can be used for the growth of *Pseudomonas* sp. This correlates with the findings of Ainom *et al*., (2010) who reported the optimum pH of *Pseudomonas aeruginosa* at 6.5. Yuan *et al*., (2002) isolated *Pseudomonas auorescens* and *Haemophilus* sp from soil contaminated with petroleum effluent discharge that grew at optimum pH of 7.0. *Bacillus* spp and *Klebsiella* spp were able to grow at various pH ranges, with *Bacillus* sp growing best at pH 7.5 while *Klebsiella* sp grew best at pH 7.0. *Pseudomonas* sp produced the highest growth with 2.25×10^5 cfu/ml while *Bacillus* and *Klebsiella* sp had 2.04×10^5 cfu/ml and 1.70×10^5 cfu/ml respectively. Statistical analysis showed no significant difference ($p<0.05$) in the growth of the isolates at the various pH ranges.

The ability of the isolates to utilize various nitrogen sources was tested. The isolates were able to utilize all types of nitrogen sources tested containing ammonium sulphate $(NH_4)_2SO_4$, sodium nitrate ($NaNO_3$) and ammonium chloride (NH_4Cl). Ammonium chloride was the most utilized nitrogen source in this study with *Pseudomonas* sp producing the highest growth of 2.37×10^5 cfu/ml, followed closely by *Bacillus* sp with 2.17×10^5 cfu/ml while *Klebsiella* sp was 1.93×10^5 cfu/ml. Sodium nitrate was the second best utilized nitrogen source while ammonium sulphate was the least utilized with growth of 1.35×10^5 cfu/ml, 1.12×10^5 cfu/ml, and 1.08×10^5 cfu/ml for *Pseudomonas*, *Bacillus* and *Klebsiella* species respectively, although statistical analysis at ($p<0.05$) showed a significant difference between the tested nitrogen sources and the growth rate. Nitrogen sources play an important role in the production of biosurfactants, because bacteria require nitrogen to complete its metabolic pathways. (Banat, *et al.*, 2000).

4.2.6 Effects of Physical and Nutrient Parameters on the Utilization of Crude Oil

The effects of these isolates on the utilization of crude oil were tested. At the various pH ranges tested, percentage loss of oil was however not statistically significant at ($p<0.05$). At pH 6.5, about 76% loss of oil was recorded by *Pseudomonas* sp. *Bacillus* sp recorded a 68% loss at pH of 7.5 while *Klebsiella* sp had a 58% loss at pH of 7.0.

The ability of the isolates to utilize crude oil supplemented with different nitrogen sources were tested (figure 4.8) and no statistical difference (<0.05) was recorded. 67%, 63% and 51.8% loss of crude oil were recorded for *Pseudomonas, Bacillus* and *Klebsiella* species respectively when supplemented with ammonium chloride. Ammonium chloride was the best utilized nitrogen source. Sodium nitrate recorded a 62.4%, 56.7% and 44.4% loss for *Pseudomonas, Bacillus* and *Klebsiella* species respectively. Ammonium sulphate was the least utilized with 53.2%, 46.7%, and 36% loss of crude oil for *Pseudomonas, Bacillus* and *Klebsiella* species respectively.

4.2.7 Crude Oil Utilization Studies

The use of microorganisms to degrade crude oil is not uncommon since the first publication of bacterial growth on petroleum hydrocarbons (Atlas, 1981, Gerson, 1985, Hidebrandt and Wilson, 1991). Microorganisms of soil and marine habitat are the major sources of bacteria responsible for utilizing petroleum hydrocarbons. (Bossert and Bartha, 1984, Antai and Mgbomo, 1989). In this study, *Pseudomonas*, *Bacillus*, and *Klebsiella* species were used for the utilization studies. After 28 days of incubation, it was observed that all the isolates utilized crude oil at different degrees. The rate of utilization was minimum on day zero and subsequently increased with increase in the days of incubation. The highest oil utilization was observed in *Pseudomonas* sp (77.5%), this was followed by *Bacillus* sp (66.8%) and *Klebsiella* sp (54.2%). Genus *Pseudomonas* has been implicated by many investigators to be a potent hydrocarbon utilizer (Udotong, 1995, Essien *et al*., 1997 and Ezeji *et al*., 2005). The high degrading ability of *Pseudomonas* sp recorded in this study is however not uncommon and is consistent with other reports. Many studies have also reported the efficiency of *Pseudomonas* sp, in degradation of hydrocarbon such as benzenes (Munoz *et al*., 2007), toluene, P-xylene (Yu et al., 2001), biphenyl (Ohta *et al*., 2001).

Bacillus sp capable of degrading hydrocarbons have also been reported (Nwaogu *et al.*, 2008). Linda, (2016) and German, (2016) reported the degradation ability of *Klebsiella* sp. The degrading high ability of *Pseudomonas* sp in this study follows the same trend with the reports of

Das and Mukhereree, (2007), Ezeji *et al*., (2005). However there was a significant difference in the percentage degradation of crude oil at ($p<0.05$).

The degradation of crude oil by microorganisms could be said to be a natural process by which the majority of the pollutant (oil) are been used as organic carbon sources, which results to the breakdown of petroleum compounds, transformation into other organic compounds or as an energy source and or production of other biological product. (Eniola *et al*., 2014). This isolates were able to degrade and utilize crude oil because microorganisms have been reported to be equipped with enzyme systems which enables them to utilize and degrade petroleum hydrocarbons as source of carbon and energy. (Antai and Mgbomo, 1993, Ezeji *el al.*, 2005)

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The results obtained from this present study show that Bonny River and soil contains bacteria with good emulsification and hydrocarbon utilizing ability. Isolates from the soil showed better emulsification and hydrocarbon utilizing potential than those obtained from the water.

5.2 RECOMMENDATIONS

Isolates obtained from this study can be used as good sources of biosurefactant production. However, while screening for biosurfactant production, it is suggested to use multiple test assay methods as only one test is not sufficient to determine the absence or presence of biosurfactants.

Species obtained from in this present study can be used to effect bioremediation

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APPENDIX I

Morphological and Biochemical Characteristics of Bacterial Isolates.

				Tripple sugar ion agar (TSIA)														
ISOLATE CODE	**COLONY MORPH**	**MICROSCOPIC**	**GRAM STAIN**	**SLANT**	**BUTT**	**GLUCOS**	**LACTOS**	**GAS**	**H_2S**	**INDOLE**	**MOTILI**	**OXIDAS**	**CITRAT**	**UREASE**	**CATALASE**	**V.P**	**METYL RED**	**MOST PROBABLE ORGANI**
C2	Shiny creamy colony on Nutrient agar	Rod	-	A	A	+	+	-	+	+	+	-	-	-	+	-	+	*Escherichia* sp
Soil C3	Shiny creamy colony on Nutrient agar	Rod	-	A	A	+	+	+	-	-	-	-	+	+	+	-	+	*Klebsiella a* sp
C4	Mucoidal creamy colony on Nutrient agar	Rod	-	B	B	-	-	-	-	-	+	+	+	-	+	-	-	*Pseudomonas* sp
TPUHC water HC1	Shiny creamy colony on Nutrient agar	Rod	-	B	B	-	-	-	-	-	+	+	+	-	+	-	-	*Pseudomonas* sp
HC2	Creamy colony on nutrient agar	Rod	-	A	A	+	+	+	+	-	+	-	+	-	+	-	+	*Salmonella* sp
HC3	Creamy colony	Rod	+	B	A	-		-	-	-	+	-	+	-	+	+	-	*Bacillus* sp

	on Nutrient agar																	
Soil HC4	Creamy colony on nutrient agar	Rod	-	B	B	-	-	-	-	-	+	-	+	-	+	-	+	*Pseudomonas* sp
HC5	Creamy colony on nutrient agar	Rod	+	B	A	-	-	-	-	-	+	-	+	-	+	+	-	*Bacillus* sp
HC6	Creamy colony on nutrient agar	Rod	+	B	A	-	-	-	-	-	+	-	+	-	+	+	-	*Bacillus* sp
HC7	Creamy colony on nutrient agar	Rod	-	B	B	-	-	-	-	-	+	-	+	-	+	-	+	*Pseudomonas* sp

Key:

A = Acidic condition; **B** = Basic condition; **+** = positive; **-** = negative, V.P; Voges Proskauer

APPENDIX II

Effects of Nitrogen Sources on Percentage Loss of Crude Oil

Pseudomonas sp	Bacillus sp	Klebsiella sp
0.532	0.467	0.36
0.624	0.567	0.444
0.678	0.636	0.518

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*S.Deviation*
Ammonium sulphate	3	1.359	0.453	0.007543	0.0868504
Sodium nitrate	3	1.635	0.545	0.008463	0.0919946
Ammonium chloride	3	1.832	0.610666667	0.00688133	0.0829538
Pseudomonas sp	3	1.834	0.611333333	0.00544933	0.0738196
Bacillus sp	3	1.67	0.556666667	0.00722033	0.0849725
Klebsiella sp	3	1.322	0.440666667	0.00624933	0.0790527

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.037634889	2	0.018817444	370.584245	2.881E-05	6.944272
Columns	0.045571556	2	0.022785778	448.73523	1.969E-05	6.944272
Error	0.000203111	4	5.07778E-05			
Total	0.083409556	8				

If P-values is < the alpha levels selected (0.05), REJECT the NULL hypothesis

If P-values is > the alpha levels selected (0.05), ACCEPT the NULL hypothesis

APPENDIX III

Effects of nitrogen sources on the growth of the isolates. Values represent log Cfu/ml

Pseudomonas sp	Bacillus sp	Klebsiella sp
5.113	5.049	5.033
5.283	5.19	5.117
5.374	5.336	5.285

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*S. Deviation*
Ammonium sulphate	3	15.195	5.065	0.001792	0.04233202
Sodium nitrate	3	15.59	5.196666667	0.006922333	0.08320056
Ammonium chloride	3	15.995	5.331666667	0.001994333	0.04465796
Pseudomonas sp	3	15.77	5.256666667	0.017550333	0.13247767
Bacillus sp	3	15.575	5.191666667	0.020594333	0.14350726
Klebsiella sp	3	15.435	5.145	0.016464	0.12831212

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.106672222	2	0.053336111	83.82519864	0.00054304	6.9442719
Columns	0.018872222	2	0.009436111	14.8301755	0.01412156	6.9442719
Error	0.002545111	4	0.000636278			
Total	0.128089556	8				

If P-values is < the alpha levels selected (0.05), REJECT the NULL hypothesis

If P-values is > the alpha levels selected (0.05), ACCEPT the NULL hypothesis

APPENDIX IV

Effects of pH on the growth of the isolates. Values represents log of cfu/ml

Conditions	Pseudomonas spp	Bacillus sp	Klebsiella sp
pH6	5.235	5.012	5.004
pH6.5	5.352	5.1	5.117
pH7	5.235	5.225	5.23
pH7.5	5.181	5.309	5.204

pH8	5.033	5.155	5.004

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*S.Deviation*
pH6	3	15.251	5.083666667	0.017192333	0.131119538
pH6.5	3	15.569	5.189666667	0.019836333	0.140841518
pH7	3	15.69	5.23	2.5E-05	0.005
pH7.5	3	15.694	5.231333333	0.004656333	0.068237331
pH8	3	15.192	5.064	0.006421	0.080131143
Pseudomonas sp	5	26.036	5.2072	0.0133862	0.115698747
Bacillus sp	5	25.801	5.1602	0.0129887	0.113967978
Klebsiella sp	5	25.559	5.1118	0.0114352	0.106935495

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
pH	0.077732933	4	0.019433233	2.114967006	0.170525956	3.83785335
Organisms	0.022754533	2	0.011377267	1.238216163	0.340021438	4.45897011
Error	0.073507467	8	0.009188433			
Total	0.173994933	14				

If P-values is < the alpha levels selected (0.05), REJECT the NULL hypothesis

If P-values is > the alpha levels selected (0.05), ACCEPT the NULL hypothesis

APPENDIX V

Effects of pH on percentage loss of crude oil. Values represent various percentages.

CONDITIONS	Pesudomonas	Bacillus sp	Klebsiella sp
pH6	0.64	0.398	0.378
pH6.5	0.74	0.4672	0.468
pH7	0.66	0.572	0.581
pH7.5	0.56	0.687	0.5187

pH8	0.41	0.537	0.426

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*	*S. Deviation*
pH6	3	1.416	0.472	0.021268	0.145835524
pH6.5	3	1.6752	0.5584	0.02473408	0.157270722
pH7	3	1.813	0.604333333	0.002344333	0.048418316
pH7.5	3	1.7657	0.588566667	0.007693263	0.08771125
pH8	3	1.373	0.457666667	0.004784333	0.069168875
Pesudomonas sp	5	3.01	0.602	0.01562	0.124979998
Bacillus sp	5	2.6612	0.53224	0.011951188	0.109321489
Klebsiella sp	5	2.3717	0.47434	0.006250628	0.079060913

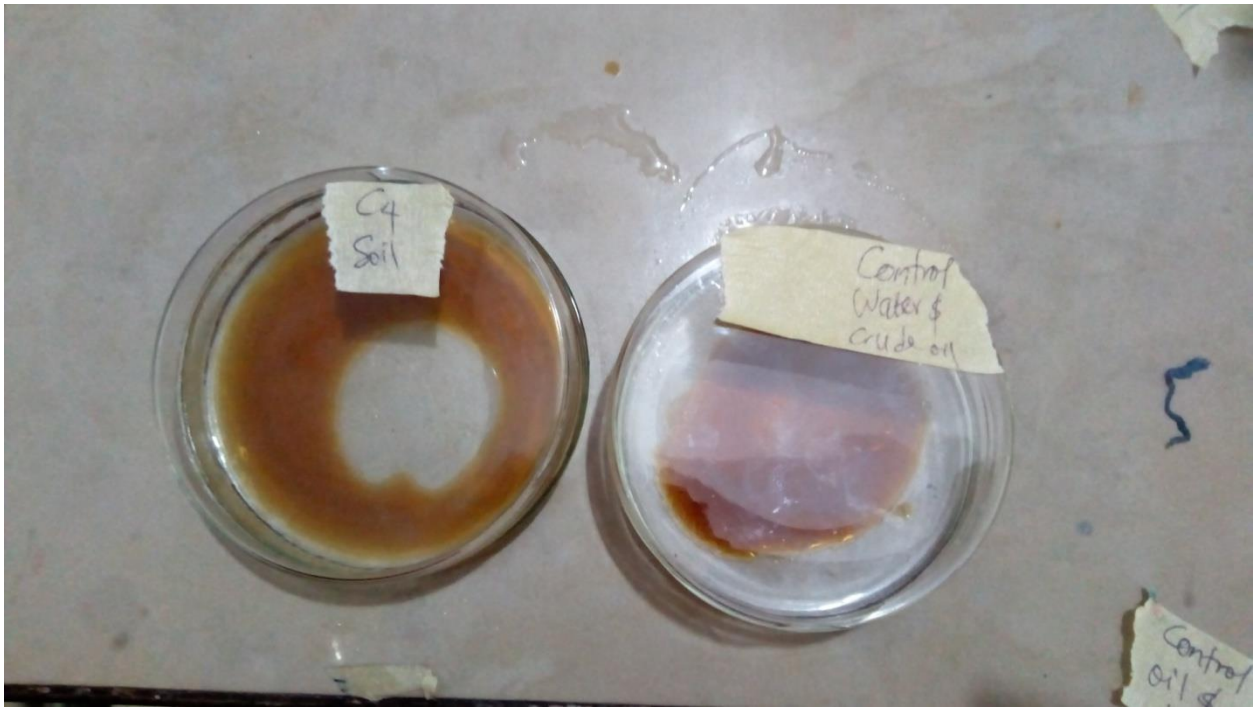
ANOVA

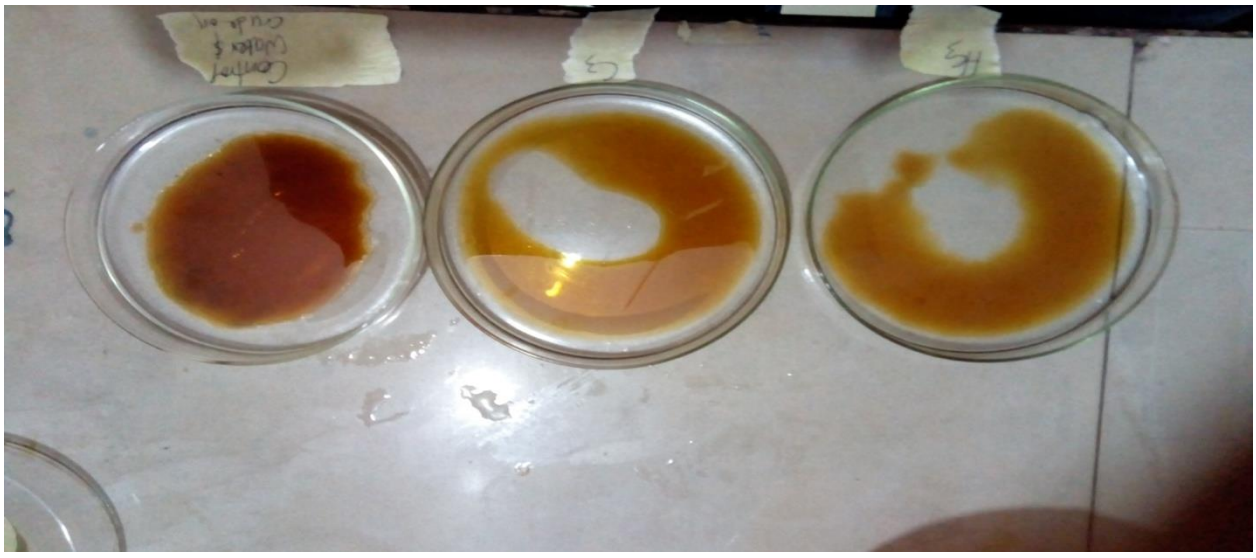
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.054499149	4	0.013624787	1.349187305	0.332069602	3.83785335
Columns	0.040859905	2	0.020429953	2.023065175	0.194522443	4.45897011
Error	0.080788115	8	0.010098514			
Total	0.176147169	14				

If P-values is < the alpha levels selected (0.05), REJECT the NULL hypothesis

If P-values is > the alpha levels selected (0.05), ACCEPT the NULL hypothesis

APPENDIX VI





Pictorial view of some of the isolates on oil spread test.