CHAPTER ONE

1.0 INTRODUCTION

1.1. Background of the Study

Surfactants (surface active agents) are molecules that contain both hydrophilic and hydrophobic (generally hydrocarbon) moieties, and are thus referred to as amphipathic molecules (Zhang, 2014).Theypartition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. Surfactants are capable of reducing surface and interfacial tension. They also cause hydrocarbons to solubilize in water with the formation of microemulsion. Such features endow excellent detergency, emulsifying, foaming, and dispersing traits, and therefore, make surfactants one of the most multifaceted process chemicals (Muthuprasanna *et al*., 2009).

These chemically synthesized surfactants are mainly petroleum based and are usually non -biodegradable and thus remain toxic to the environment they find themselves. These compounds may bio-accumulate and their production processes and by-products can be environmentally hazardous. Due to the increasing awareness on the need to protect the ecosystem, environmental scientists have been tightening environment regulations thus necessitating an increased interest in surfactants of microbial origin as possible alternatives to chemically synthesized ones (Benincasa, 2007).

Biosurfactants are surfactants of microbial origin. The industrial need for biosurfactants over synthetic surfactant is constantly growing. In fact, the global biosurfactant market was expected to reach 2.2 billion US dollars IN 2018, based on a growth rate of 3.5% per annum (Bertrand *et al*., 2018). These molecules have the abilities to reduce superficial and interfacial tension between solids, liquids and gases. They have various advantages over the chemical surfactants, however, production costs of biosurfactants prevent them from

competing with the chemical counterparts (Kaskatepe and Yildiz, 2016). Therefore, in the light of the economic constraints associated with biosurfactant production, three basic strategies have been adopted worldwide to make the process cost-effective: (i) the use of cheap and waste substrates as carbon source to lower the initial raw material costs involved in the process;(ii) development of efficient bioprocesss, including optimization of culture conditions and cost-effective separation processes for maximum biosurfactant production and recovery (Mulligan *et al*., 2014); (iii) development and use of high-producing mutant and recombinant strains for improved biosurfactant yields(Mukherjee *et al.*,2006).

Currently, the main application of biosurfactant is for enhancement of oil recovery, heavy metal removal and hydrocarbon bioremediation due to their biodegradability and low critical micelle concentration (Banat *et al.,* 2010). They are also used as biostimulants during bioremediation. The use of biosurfactants has also been presented for various industrial applications, such as in food additives (Denice and Fredrico, 2009), cosmetics, detergent formulations and in combinations with enzymes for wastewater treatment (Damasceno *et al.*, 2012). They have also found application in medicine and in pharmaceutical industries (Banat *et al*., 2010). Moreso, a wide variety of roles have been described for biosurfactants, from biofilm formation to inhibitory activity against pathogenic organisms (Van Hamme *et al*., 2006).

Contamination of the environment by hydrocarbons such as crude oil and heavy metals has created vast problems in the environment. These include loss of viable agricultural lands which has resulted in food insecurity and economic loss (Makkar *et al*., 2011), loss of mangrove forests, loss of aquatic animals such as fishes and increasing health issues such as cancer, infertility and even death. Some hydrocarbons, such as the aromatic hydrocarbons have been incriminated as carcinogens and thus the cause of increasing rate of cancer in the society (Bostrom *et al*., 2002).

Bioremediation remains the best method of reclaiming crude oil or/and heavy metals-contaminated soil. This is because of the advantages it has over the conventional physico-chemical treatments such as less or no toxicity and ecofriendliness of its methods (Zrafi-Nouira *et al*., 2012). Bioremediation techniques include natural attenuation, biostimulation, bioventing, composting, landfarming, phytoremediation and bioaugmentation. Natural attenuation involves study of the remediation potential of the indigenous microbial community in the contaminated environment over time. Biostimulation involves introduction of nutrients and oxygen into the soil to stimulate the indigenous microorganisms, while bioaugmentation is the addition of enriched microbial consortium into the soil (Barathi and Vasudevan, 2001).

Assessment of microbial diversity in polluted environment is paramount to understanding the potentials of bioremediation by the autochthonous organisms. The choice of bioremediation technique to be employed requires understanding/knowledge of the autochthonous population present in the polluted environment as they hold the key to abating challenges connected with bioremediation (Verma and Jaiswal, 2016). Investigations of microbial species that are present in petroleum polluted environments have been traditionally conducted using soil samples to grow bacteria cultures in the laboratory. However, laboratory growth medium does not reproduce the actual diversity of the polluted environment (Tyson and Banfield, 2005). Molecular methods can be used to overcome the difficulties associated with the laboratory cultivation of petroleum degrading-bacteria. The use of genetic techniques to detect, identify and quantify bacteria has largely replaced microbial growth tests. These modern biotechnology methods have been recently employed in petroleum polluted environments (Green and Keller, 2006). The emergence of Next Generation Sequencers (NGS) has made studies on microbial diversity robust and fascinating (Di-Bella *et al*., 2013; Ercolini, 2013). NGS analyzes microbial communities more efficiently and with

less analytical variation. Metagenomic sequencing has many advantages over traditional methods of identifying organism, such as culture, polymerase chain reaction and enzyme-linked immunosorbent assay. These advantages include relative speed, the ability to detect non-culturable organisms, and perhaps most importantly, the fact that metagenomic sequencing requires little or no priorknowledge of the organism(s).

1.2Statement of the Problem

Okarki in Ahoada Local Government Area of River state, Nigeria, suffer from recurring pollution of their farmlands resulting from pipeline bunkering and vandalism by indigenes of the locality. The farmlands and streams have been reported to be heavily polluted with hydrocarbon.

Crude oil and heavy metal pollution of the environment pose serious public health challenges.One of the main factors that affect the rate of bioremediation by indigenous organisms is the non-bioavailability of the hydrocarbon to the utilizing organisms, hence, the resultant accumulation of the pollutant in the environment. Selection of the appropriate bioremediation technique to employ in any bioremediation exercise is a serious challenge.

The use of culture-based approach in determining microbial population in polluted soil is rather limited as it selects for culturable microorganisms while ignoring non-culturable ones, leading to incomplete or biased community diversity assessment.

“One factor at a time” traditional method of optimizing process condition is rather cumbersome, time consuming and cannot estimate interaction among process variables.

1.3Aim of the Study

The aim of this study was to optimizethe production of biosurfactants by *Pseudomonas aeruginosa*and its application in bioremediation of crude oil/heavy metal-polluted soil.

1.4. Specific Objectives

The specific objectives of the study are to:

- isolate bacterial organisms from spent lubricating oil-polluted soil, screen the isolates for biosurfactant production and identify the active producers.
- optimize fermentation conditions using response surface methodology.
- determine the surface tension reduction ability and emulsification activity of the produced biosurfactants.
- determine the functional components of the produced biosurfactants.
- determine the physicochemical properties of the crude oil-polluted and control soil.
- determine the microbial diversity in the crude oil-polluted and control soil using culture-based laboratory techniques and metagenomic technique.
- determine crude oil degradation potential of the isolated indigenous microorganisms on shake flask.
- comparatively bioremediate the polluted soil employing natural attenuation, bioaugmentation and biostimulation.
- measure the rate of crude oil and heavy metal removal from the polluted soil.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1 Classification of Biosurfactants and their Microbial Origin

Biosurfactants are complex organic molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids and lipopeptides. Some surfactants referred to as polymeric microbial surfactants (PMS), which are composed of many chemical types are also produced by microorganisms (Zhang, 2014).

Usually, microorganisms (bacteria in most cases) synthesize biosurfactants during their growth on water immiscible substrates. These biosurfactants have a definite structure, with a lipophilic portion which is usually the hydrocarbon (alkyl) tail of one or more fatty acids that can be saturated, unsaturated, hydroxylated or branched and is linked to the hydrophilic group by a glycosidic, ester or amide bond. Most biosurfactants are either neutral or negatively charged and the list includes both ionic and non-ionic surfactants, which range from a short fatty acid to large polymers (Cameotra*et al.*, 2010).

Biosurfactants are mainly classified based on their chemical structure and their microbial origin (Fakruddin, 2012). There are basically five major classes of biosurfactants namely:

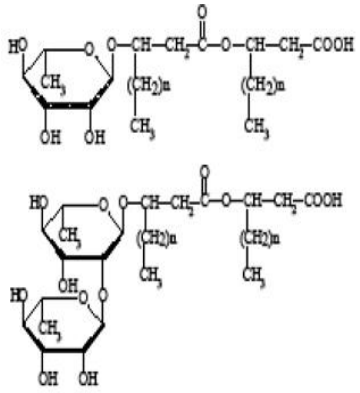
(i) glycolipids (ii) phospholipids and fatty acids (iii) lipopeptides/lipoproteins (iv) polymeric surfactants and (v) particulate surfactants (Table 1).

Table 1: Classification of biosurfactants and their microbial origin

Class of biosurfactant	Microbial origin
1. Glycolipids	
Rhamnolipid	*Pseudomonas aeruginosa*
Sophorolipids	*Torulopsis bombicola, Torulopsis apicola*
Trehalolipids	*Rhodococcus erythropolis, Mycobacterium* sp.
2. Lipopeptides and lipoproteins	
Peptide-lipid	*Bacillus licheniformis*
Viscosin	*Pseudomonas fluorescens*
Serrawettin	*Serratiamarcescens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidin	*Bacillus brevis*
Polymyxin	*Bacillus polymyxia*
3. Fatty acids, neutral lipids and phospholipids	
Fatty acids	*Corynebacterium lepus*
Neutral lipids	*Norcadiaerythropolis*
Phospholipids	*Thiobaciluus thiooxidans*
4. Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carbohydrate-lipid-protein	*Pseudomonas fluorescens*
Mannan-lipid-protein	*Candida tropicalis*
5. Particulate surfactant	
Vesicles	*Acinetobactercalcoaceticus*

Cells	Various bacteria

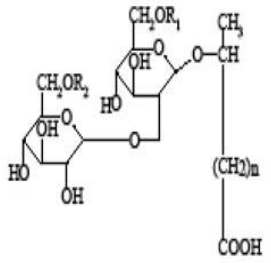
Source: Pacwa-Pociniczak *et al*. (2011).



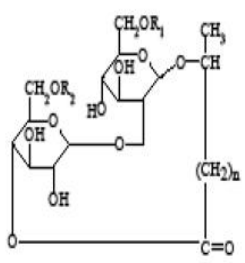
Monorhamnolipids

Dirhamnolipid

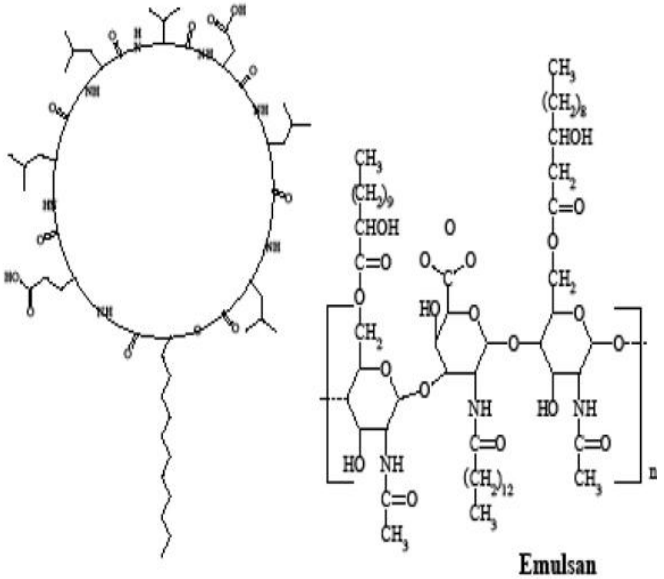
Mannosylerythritol lipids



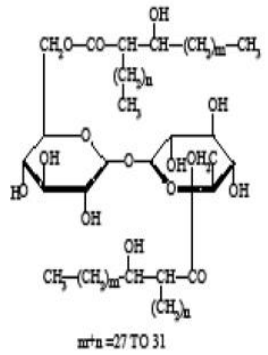
Acidic Sophorolipid



Lactonic Sophorolipid

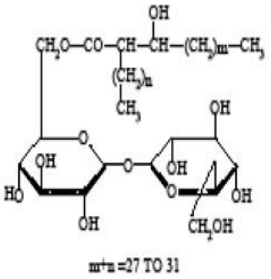


Emulsan



m+n =27 TO 31

Trehalose dimycolates



m+n =27 TO 31

Trehalose monomycolates

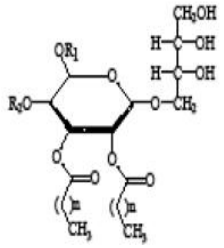


Figure 1:Chemical structures of some common biosurfactants. Source: Makkar *et al*. (2011)

2.1.1 Low Molecular Weight Biosurfactants

Microbial surface-active compounds can be roughly divided into low molecular weight molecules that efficiently reduce surface and interfacial tension (biosurfactants) and high molecular weight polymers that stabilize emulsions but do not lower the surface tension as much (bioemulsans or bioemulsifiers) (Smyth *et al*., 2010a)**.**The most studied low-molecular-weight biosurfactant compounds are glycolipids and lipopeptides.

2.1.1.1 Glycolipids

Glycolipids are commonly mono or disaccharides compounds acylated with long chain fatty acids or hydroxyl fatty acids. Among them, rhamnolipids, mannosylerythritol lipids (MELs), sophorolipids and trehalolipids are the best-studied structural subclasses.

*Rhamnolipids***:** Rhamnolipids are glycosides, produced mainly by *Pseudomonas aeruginosa* and by the genus *Burkholderia*, that are composed of one (for mono-rhamnolipids) or two (for dirhamnolipids) rhamnose sugar moieties linked to one or two β-hydroxyfatty acid chains (Figure 1) (Raza *et al.*, 2009). Most studies involving rhamnolipids focus mainly on assessing the biodegradation efficiency of petroleum hydrocarbons (Szulc *et al*., 2014). These molecules possess many potential applications in the biomedical field due to their reported antibacterial, antifungal, antiviral, antiadhesive properties (Remichkova *et al*., 2008; Sotirova *et al*., 2008). They have also been used in the preparation of nanoparticles (Palanisamy and Raichur, 2009) and microemulsions (Nguyen and Sabatini, 2009). However, researchers have observed a low or no petroleum contaminant biodegradation effect with rhamnolipid (Ławniczak *et al*., 2013).

The mannosylerythritol (MELs): MELs are a mixture of partially acylated derivative of 4-*O*-*β*-D-mannopyranosyl-D-erythritol, containing C2:0, C12:0, C14:0, C14:1, C16:0, C16:1, C18:0 and C18:1 fatty acids as the hydrophobic groups. They are grouped into MEL-A, -B, -C and –D based on the degree of acetylation at C4 and C6 position, and their order of appearance on the thin layer chromatography (Arutchelvi and Doble, 2010). MEL-A representing the diacetylated compound, while MEL-B and MEL-C are monoacetylated at C6 and C4 respectively. The completely de-acetylated structure is known as MEL-D (Rau*et al.*, 2005). MELs have recently gained attention due to their environmental compatibility, mild production conditions, structural diversity, self-assembling properties and vast biochemical functions. In particular, interesting applications have been described in the biomedical field as antimicrobial, antitumor and immunomodulating molecules, in the biotechnological field for gene and drug delivery, and in cosmetic applications as skin moisturizers. Arutchelvi and Doble (2010), have reported production of MELs glycolipid by yeast strains belonging to the genus *Pseudozyma* and *Ustilago* from soybean oil or *n*-alkane.

Sophorolipids: Sophorolipids are another extracellular glycolipids synthesized by some yeast species including *Candida bombicola*, *Candida apicola*, *Rhodotorula bogoriensis, Wickerhaminelladomercqiae* and *Candida batistae* (Van Bogaert and Soetaert, 2010). They consist of two glucose units linked by β-1, 2 – glycosidic bond. The 6- and 6'-hydroxyl groups are generally acetylated. The terminal carboxyl group of the fatty acid can be in the lactonic form or hydrolyzed to generate an anionic surfactant (Rosenberg and Ron, 1999). Sophorolipids have been reported to be suitable for a number of application in the biomedical field including use as antimicrobial, antiviral and anticancer agent. They also have been used in the synthesis of metal-bound nanoparticles in cosmetic and pharmacodermatological products (Van Bogaert and Soetaert, 2010).

Trehaolipids: They are also glycolipids containing trehalose as the sugar moiety, which is a non-reducing disaccharide in which the two glucose units are linked in an β-1,1-glycosidic linkage (Figure 1). It is the basic component of the cell wall of *Mycobacteria* and *Corynebacteria* (Franzetti *et al*., 2010). The most reported trehalose lipid is trehalose- 6, 6'-dimycolate, which is a β -branched chain mycolic acid esterified to the C-6 position of each glucose. Trehalolipids are produced by species of *Mycobacterium*, *Arthrobacter*, *Nocardia*, *Corynebacterium*, *Rhodococcus* and *Gordonia*. Trehalolipids from *Arthrobacter* spp. and *Rhodococcus erythropolis* are able to lower surface and interfacial tensions in culture broth to 25–40 and 1–5 mN/m, respectively (Vijayakumar and Saravanan, 2015). Beside their known industrial applications, trehalose lipids recently attracted attention to their functions in cell membrane interaction and their potential as antitumor therapeutic agents (Shao, 2010). These glycolipids vary in the number and overall chain length (C20–C90) of the esterified fatty acids.

2.1.1.2 Lipopeptides

Bacillus species are well known for the production of lipopeptides. Several variants and families constitute the lipopeptide biosurfactant. The families and variants are differentiated by their fatty acid chain and peptide moiety (Thavasi *et al*., 2011a; Jacques, 2010).

Surfactin: Surfactin is a very active cyclic lipopeptide produced by *Bacillus subtilis* (Ron and Rosenberg, 2001). Surfactin was discovered from the culture broth of *Bacillus subtilis* and it was named thus due to its exceptional surfactant activity. Natural surfactins are a mixture of isoforms A, B, C and D, which are classified according to the differences in their amino acid sequences and possess various physiological properties (Shaligram and Singhal, 2010). Surfactin is composed of seven amino-acid ring structure coupled to a fatty-acid chain

via a lactone linkage. Surfactin-A has L-leucine, surfactin-B has L-valine and surfactin-C has L-isoleucine at the amino acid position involved in the lactone ring formation with the C14–C15 hydroxy fatty acid. The amino-acid residues may vary and the presence of these variants can be related to alterations in the culture conditions such as providing substrate containing some specific amino-acid residues in the culture media (Jacques, 2010).

Lichenysin: This is a lipopeptide biosurfactant produced by *Bacillus licheniformis* culture. It is a surfactin-related compound, having chemical structure and physiochemical properties similar to surfactin (Vijayakumar and Saravanan, 2015). Lichenysinare capable of lowering the surface tension of water to 27mN/m and the interfacial tension between water and *n*-hexadecane to 0.36 mN/m. Other surfactin-like compounds are pumilacidin A, B, C, D, E, F and G, a complex of acylpeptide antibiotics isolated from *Bacillus pumilus* culture supernatants with interesting antiviral properties(Jacques, 2010)**.**

Among the lipopeptides belonging to the iturin family, iturin A is the most studied compound. It is a heptapeptide interlinked with amino-acid fatty acid with carbon chain length from C14 to C17 (Jacques, 2010) produced by *Bacillus subtilis* strains and reported to have antifungal activities. Other members of the iturin family are iturin C, bacillomycin D, F, Lc and mycosubtilin (Bonmatin *et al*., 2003). Other interesting lipopeptides are serrawettins, nonionic cyclodepsipeptide biosurfactants produced by *Serratia marcescens* (Matsuyama *et al*., 2010) and implicated with anti-tumor and anti-nematode activities.

2.1.1.3. Fatty acids, phospholipids and neutral lipids

Several bacteria and yeast produce large quantities of fatty acids and phospholipid surfactants during growth on n-alkanes. In *Acinetobacter* spp. 1-N, phosphatidyl ethanolamine-rich vesicles are produced which form optically clear micro-emulsions of alkanes in water (Vijayakumar and Saravanan, 2015). These biosurfactants are essential for

medical applications. Gautam and Tyagi (2006), reported that the deficiency of phospholipid protein complex is the major cause of respiration failure in the premature babies.

2.1.2. High Molecular Weight (HMW) Biosurfactants

High molecular weight (HMW) biosurfactants are generally classified as polymeric biosurfactants. They are produced by a number of different bacteria and are made up of lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes containing several of these structural types (Ron and Rosenberg, 2001). These high molecular weight biosurfactants generally possess effective emulsifying activity and are referred to as bioemulsifiers. Several bioemulsifiers are effective at high temperature, including the protein complex from *Methanobacterium thermoautotrophium* and the protein-polysaccharide-lipid complex of *Bacillus stearothermophilus* ATCC 12980 (Rosenberg and Ron, 1999). Yeasts produce a number of emulsifiers, which are particularly interesting because of the food-grade status of several yeasts which allows its use in food related industries.

2.1.2.1. Emulsan

Emulsan is the most studied biopolymer. It is a lipopolysaccharide isolated from *Acinetobactercalcoacetius* RAG-1 ATCC 31012 with a molecular weight of around 1,000 kDa. Emulsan is an effective emulsifying agent for hydrocarbons in water, even at a concentration as low as 0.001-0.01% (Hatha *et al*., 2007). The fatty acid moiety which is responsible for its surface activity is attached to the polysaccharide backbone via O-ester and N-acyl linkages.

2.1.2.2. Alasan

Alasan is another HMW biosurfactant. It is a complex of an anionic polysaccharide and a protein with a molecular weight of > 1,000 kDa produced by *Acinetobacterradioresistens* (Smyth *et al*., 2010a)**.**

2.1.2.3. Liposan

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica.* It is composed of 83% carbohydrate and 17% protein (Chakrabarti, 2012). The applications of liposan as emulsifier in food and cosmetic industries were reported by Chakrabarti (2012).

2.1.2.4. Particulate Biosurfactants

Vesicles*:*It is an extracellular film vesicles that can form micro-emulsion with hydrocarbons. They play important role in uptake of alkenes by microbial cells. Vesicles of *Acinetobacter* sp. comprises of protein, phospholipids and lipopolysaccharide (Krishnaswamy *et al*., 2008; Silva *et al*., 2014).

2.2.Properties of Biosurfactants

The physical and chemical properties of biosurfactants are very vital in the assessment of the performance and selection of microorganisms with the ability to produce these agents (Deleu and Paquot, 2004). Despite the diversity in the chemical composition and properties of biosurfactants, a number of characteristics are common to the majority of biosurfactants, many of which offer advantages over conventional chemical surfactants (Nitschke *et al*., 2007).

2.2.1.Surface and interfacial activity: Ability to lower surface or/and interfacial tension is the primary characteristic of biosurfactants. An effective biosurfactant is the one that reduces the surface tension of water (Karlapudi *et al*. 2018). Biosurfactants produce lower surface tension at low concentrations, demonstrating greater effectiveness and efficiency in comparison to conventional chemical surfactants The CMC of biosurfactants (measure of

efficiency) ranges from 1 to 2000 mg/L, wheareas interfacial tension (oil/water) and surface tension are around 1 and 30 mN/m, respectively (Anjum *et al*., 2016).

2.2.2. Tolerance to temperature, pH and ionic strength: Functions and parameters such as temperature and pH of most of the biosurfactants are usually not altered by the environmental conditions (Karlapudi *et al*., 2018). Many biosurfactants can be used under extreme conditions. For instance, the lipopeptide from *Bacillus licheniformis* JF-2 is stable at temperatures around 75°C for up to 140 hours and within a pH range of 5 to 12. At high temperatures beyond autoclavable temperature (121°C) and at low temperatures below -15°C (Karlapudi *et al*., 2018). Lipopeptides produced by *Bacillus subtilis* was recorded to be stable when stored for 180 days. Biosurfactants also tolerate salt concentrations up to 10%, whereas 2% NaCl is sufficient to inactivate conventional surfactants (Cheng *et al*., 2016).

2.2.3.Biodegradability: Biosurfactants are easily degraded by bacteria and other microorganisms in water or soil, which makes them adequate for bioremediation applications and waste treatment (Karlapudi *et al*., 2018). The increasing environmental concern in use of chemical surfactants is that they become recalcitrant and obnoxious in the environment after use (Cameotra and Makkar, 2004), hence, biodegradable biosurfactants are advocated for. Lee *et al*. (2008b), controlled the blooms of marine algae *Cochlodinium* using the biodegradable sophorolipid with the removal efficiency of 90% in 30 min treatment.

2.2.4. Low toxicity: Biosurfactants have received greater attention due to the increasing concern on the part of the population regarding the allergic effects of artificial chemical products. Moreover, the low degree of toxicity of these compounds allows their use in food, cosmetic and pharmaceutical products (Sarrubo *et al*., 2015).

2.2.5.Specificity: Biosurfactants are specific in their actions having complex organic molecules with specific functional groups. This is of considerable interest as it enabled

specificity in its application in detoxification of specific pollutants, in the food, cosmetic and pharmaceutical industries (Rahman and Gakpe, 2008).

2.2.6.Biocompatibility and digestibility: Biosurfactants are biocompatible with living tissues, and are easily digested. Theydo not produce any toxic or immune response when exposed to the body or body fluid. These features allow the application of biosurfactants in the food, cosmetic and pharmaceutical industries (Santos *et al*., 2016).

2.3. Factors Affecting Biosurfactant Production

The composition and biosurfactant activity of biosurfactant not only depend on the producing microorganism, but also on the fermentation conditions such as the nature of the carbon source, the nitrogen source as well as the C:N ratio. Other nutritional factors and environmental factors such as temperature, agitation, medium volume and pH influence not only affect the quantity of biosurfactants produced, but also the type produced (Santos *et al*., 2016).

2.3.1. Carbon sources: The nature of carbon substrate used influences the quality and quantity of biosurfactant production. Different yields of biosurfactant have been reported when different carbon sources are used for production. Diesel, crude oil, glucose, sucrose, glycerol have been reported to be a good source of carbon substrate for biosurfactant production(Raza *et al*., 2007).

Industrial wastes had aroused the interest of researchers as a low-cost substrate for biosurfactant production (Makkar and Cameotra, 2002). The selection of waste products should ensure the proper balance of nutrients to allow microbial growth and consequent biosurfactant production. Industrial waste with a high content of carbohydrates or lipids is ideal for use as substrate. According to Barros *et al*. (2007), the use of agro-industrial waste is one of the steps toward a cost-effective biosurfactant production on an industrial scale.

Quite a number of waste products have been employed in biosurfactant production, such as vegetable oils, oily effluents (Batista *et al*., 2010; Sarubbo *et al*., 2007), starchy effluents (Fox and Bala, 2000; Thompson *et al*., 2000), animal fat (Maneerat, 2005), vegetable fat (Gusmão *et al*., 2010), vegetable cooking oil waste (Maneerat, 2005; Cvengros and Cvengrosova, 2004), soapstock (Maneerat, 2005; Benincasa *et al*., 2002), molasses (Kalogiannis *et al*., 2003; Lazaridou *et al*., 2002), dairy industry waste (whey) (Sudhakar-Babu *et al*., 1996), corn steep liquor (Luna *et al*., 2013; Sobrinho *et al*., 2008; Rufino *etal*., 2007), cassava flour wastewater (Nitshke *etal*., 2004), oil distillery waste (Luna *et al*., 2012; Rufino *et al*., 2007) and glycerine (Silva *et al*., 2010). Use of aqueous extracts of the agro-wastes such as banana peels, orange peels, potato peels and bagasse for biosurfactant production were described by Kulkarni *et al*. (2015).

2.3.2. Nitrogen sources: Nitrogen is important in the biosurfactant production medium because it is essential for microbial growth because protein and enzyme syntheses depend on it. Choice of nitrogen source varies among microorganisms. Different nitrogen compounds such as urea peptone, yeast extract, ammonium sulphate, ammonium nitrate, potassium nitrate, sodium nitrate, meat extract and malt extracts have been used as nitrogen sources for biosurfactant production (Joshi and Shekhawat, 2014; Jorge *et al*., 2013; Onwosi and Odibo, 2012).

Though yeast extract is the most used nitrogen source for biosurfactant production, its usage with respect to concentration is organism and culture medium-dependent (Jorge *et al*., 2013). Ammonium salts and urea are preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* whereas nitrate supports maximum surfactant production in *P. aeruginosa* (Adamczak and Bednarski, 2000). Sodium nitrate was the best nitrogen source for biosurfactant production by *Pseudomonas monteilli* (Anaukwu *et al*., 2015). According to

Joshi and Shekhawat (2014), ammonium nitrate was observed as best nitrogen source for biosurfactant production.

2.3.3. Environmental factors: Environmental factors greatly influence the yield and characteristics of the biosurfactant produced. To obtain large quantities of biosurfactants, it is always necessary to optimize the bioprocess, as the product may be affected by changes in temperature, pH, aeration or agitation speed.

2.3.3.1. Temperature: Most biosurfactant productions are performed in a temperature range of 25-30˚C (Krishnaswamy *et al*., 2008). However certain microorganisms such as *Acinetobacter baylyi ZJ2* which could withstand higher temperature (40–45°C) was identified through the investigation carried out by Changjun *et al*. (2014).

2.3.3.2. pH: pH is another important environmental factor which affects the biosurfactant production. Different microorganisms produce biosurfactant at varying pH levels. Joice and Parthasarathi (2014), studied the biosurfactant production by altering the pH from 5.0 to 8.5 and observed surface tension reduction of water at pH 6.5 and emulsification activity of 75.12% at pH 7.0; they therefore, concluded that biosurfactant production by *Pseudomonas aeruginosa* PBSC1 was maximum at pH 7.0. Zinjarde and Pant (2002), reported that the best pH for bioemulsifier production by marine *Yarrowia lipolytica* NCIM 3589 was 8.0 which is the natural pH of sea water.

2.3.3.3. 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. Adamczak and Bednarski (2000), observed that the best production value of the surfactant was obtained when the air flow rate was 1vvm (that is one liter of air passed through one liter of medium in one minute), and the dissolved oxygen concentration was maintained at 50% of saturation.

2.3.3.4. Salt concentration: Salt concentration of a particular medium do have a corresponding effect on the biosurfactant production, as the cellular activities of microorganisms are affected by salt concentration. Nevertheless, contrary observations were noticed for some biosurfactant products which were not affected by concentrations up to 10% (w/v) although slight reductions in the CMC were detected (Shao, 2010).

2.4. Low-Cost Waste Materials Used For Biosurfactant Production

The use of cheaper, renewable substrates from various industries such as agricultural (sugars, molasses, plant oils, oil wastes, starchy substances, lactic whey), distillery wastes, animal fat, oil industries have been reported (Makkar *et al*., 2011). Various cheap substrates such as soybean oil not only act as nutrients for the microbial growth but also act as an important source for isolation of potential biosurfactant-producing microorganisms (Lee *et al*., 2008a). Rhamnolipids, one of the common biosurfactants are usually produced on soybean oil, soapstock, spent soybean oil, or chicken fat as carbon source (Nitschke *et al*., 2010). Large scale production for most microbial surface active agents has not reached a satisfactory economical level due to their low yields. Such obstacles may be overcome by isolating potential biosurfactant producers that can use the renewable waste substrates to raise the quality as well as quantity of biosurfactant. Several alternative strategies for production at commercial scale have been reviewed by Helmy *et al*. (2011). Different relatively cheap and abundant substrates are currently available for use as carbon sources from various industrial sectors (Table 2)

Table 2: Low –cost materials used for biosurfactant production

Source Industry	Waste/residues as cheaper renewable substrate
Agro-industry waste, crop residues	Bran, beet molasses, bagasse of sugarcane, straw of

	wheat, cassava, cassava flour waste water, straw of rice, hull of soy, sugar cane molasses
Animal fat	Waste
Coffee processing residues	Coffee pulp, coffee husks, spent of free groundnut
Crops	Cassava, potato, sweet potato, soybean, sweet sugar beet, sorghum
Dairy industry	Curd whey, cheese whey, whey waste
Distillery industry	Industry effluents
Food processing industry	Frying edible oils and fats, olive oil, potato peels, rape seed oil, sunflower, vegetable oils
Fruit processing industry	Banana waste, pomace of apple and grape, carrot industrial, pine apple
Oil processing mills	Coconut cake, canola meal, olive oil mill waste water, palm oil mill, peanut cake effluent, soybean cake, soapstock, waste from lubricating oil

Source: Banat *et al*. (2014)

Products such as bran, wheat straw, rice straw, hull of soy, corn, rice, sugar cane molasses, beet molasses, bagasse of sugarcane, cassava flour and its wastewater are representative candidates of agro-industrial waste (Thavasi *et al*., 2014; Benincasa, 2007; Rashedi *et al*., 2005;Nitschke *et al*., 2004). Some waste materials like rice water (by-product from domestic cooking and rice processing industry), corn steep liquor (corn processing industry) and cereals, pulses-processed waste water are rich in starch content. Agro-industrial waste contains high amount of carbohydrates, lipids and hence, can be used as a rich carbon source for microbial growth. Among the agro-industrial waste products, molasses had attracted considerable attention to many researchers (Banat *et al*., 2014).

2.4.1. **Agro-Industrial Wastes**

2.4.1.1. Molasses

Molasses are concentrated syrups by-products of sugar cane and beet processing industries. This cheap substrate contains 75% dry matter, 9–12% non-sugar organic matter, 2.5% protein, 1.5–5.0% potassium and approximately 1% calcium, magnesium, and phosphorus. Other components like biotin, pantothenic acid, inositol, and thiamine at 1–3%

are also present, giving it a thick, dark brown colored appearance (Banat *et al*., 2014). The high sugar content ranging approximately 48-56%, represents a good substrate for growth as well as production of microbial bioactive compounds for various microorganisms. Some of the research laboratories are particularly involved in the use of molasses for production of various microbial metabolites (Banat *et al*., 2014)**.** Raza *et al*. (2007), produced rhamnolipid biosurfactant from *P. aeruginosa* mutant strains using blackstrap molasses with or without supplementary nitrogen source and reported a yield of 1.45 g/L rhamnolipid after 96 h incubation.

Other than above mentioned sources of molasses (sugar cane and beet), soy molasses are the most commonly used wastes from industrial sectors for the production of sophorolipid (SL) type of biosurfactant (Solaiman *et al*., 2007). Molasses produced during the production process of soybean oil have been reported as a good carbon sources for sophorolipid production from *Candida bombicola* (Solaiman *et al*., 2004).

2.4.2.Animal Fat and Oil

Meat processing industries such as food and leather-producing industries produce significant quantities of animal fat, tallow and lard. Demand for animal fats is considerably less than vegetable oils and much of it becomes a problem for utilization as well as for their disposal. In comparison with other renewable substrates, animal fat and oil have not been much explored. Animal fat has been reported to act as a stimulator for the production of sophorolipid biosurfactant from *Candida bombicola* yeast (Banat *et al*., 2014). Mixture of glucose (10% w/v) and fat (10% v/v), enhanced the growth of the yeast and the production of sophorolipid (120 g/L). Santos *et al*. (2013), reported maximum glycolipid biosurfactant

production using animal fat combined with corn steep liquor as compared to other carbon sources using yeast *Candida lipolytica* UCP0988. They also reported the use of the product in bioremediation, oil mobilization, and recovery.

Production of biosurfactants by fermentation of fats, oils, and their co-products has also been reported (Solaiman *et al*., 2003). Nitschke *et al*. (2010), carried out biosurfactant production by using soybean oil waste, along with molasses, whey and cassava flour, as substrates. These cheaper substrates were compared with conventional medium for biosurfactant production. Among eleven isolates tested, eight cultures reduced the surface tension of water to levels below 30 mN/m using soybean oil waste as substrate. They reported improved growth on soybean oil agar for several isolates suggesting a high growth capacity, and concluded that soybean oil represents a potential alternative culture medium for biosurfactant production. Industrial wastes, corn steep liquor and ground-nut oil refinery residue were also reported as low cost nutrients for the production of glycolipid type biosurfactant from *Candida sphaerica* UCP0995(Luna *et al*., 2015).

2.4.3.Dairy and Distillery Industries By-Products

Dairy industries produce large quantities of whey that includes, curd whey, whey waste, cheese whey, lactic whey, all of which are easily available as raw substrate for microbial production of metabolites (Rodrigues and Teixeira, 2008; Dubey *et al*., 2005, Dubey and Juwarkar, 2004). High amount (about 75%) of lactose is present in the lactic whey. Other components like protein and organic acids, vitamins provide good sources for microbial growth and biosurfactant production (Maneerat, 2005). An exciting report was published by Dubey *et al*. (2012) on distillery waste in combinations with curd whey waste,

fruit processing waste and sugar industry effluent for growth and production of biosurfactant from microorganisms. They observed a positive impact of such combinations for biosurfactant production from *Kocuria turfanesis* strain BS-J and *P. aeruginosa* strain BS-P. Similarly Rodrigues *et al*. (2006), reported that *Lactobacillus pentosus* grown on whey at 31^{o}C effectively lowered the surface tension of medium from 54mN/m to 45mN/m.

2.4.4. Oil Processing Industries

Wastes from oil processing industries represent one of the alternative and easily available renewable substrates for production of microbial surface active molecules. Spent oils are usually abundantly available oils that are quite difficult to dispose due to environmental concerns including persistence and resistance to biodegradation (Rodrigues and Teixeira, 2008). They include waste vegetable oil, used motor oil, lubricating oils, jet fuels all of which can act as cheaper source for microbial processes such as biosurfactant production. Usage of such kind of substrates is usually encouraged as a pollution control strategy. Food processing industries use huge quantity of frying oils, where the composition vary depending on the number of times it has been used, modification in its composition, and finally need for pretreatment(Rodrigues and Teixeira, 2008).

Vegetable oil, olive oil and sunflower oil have been proved as potential carbon and energy sources for production of microbial surfactants. The oils that contain low chain length (<C10) fatty acids undergoes modification for incorporation into surface active products. Haba *et al*. (2000), investigated the use of olive and sunflower oils in submerged culture condition by 36 microbial strains. They reported that several *Pseudomonas* strains grew well on 2% waste olive or sunflower oil, reducing surface tension of production medium to <40 mN/m. Abalos *et al*. (2001), also used a soybean oil refinery waste for production of rhamnolipid using *P. aeruginosa* AT10 strain. In addition, Abouseoud *et al*. (2007), achieved

biosurfactant production from *P. fluorescens* DSMZ using olive oil as a carbon source with ammonium nitrate as a nitrogen sources. Canola oil refinery waste supplemented with sodium nitrate was reported best for microbial growth and rhamnolipid production with a yield of 8.50 g/L. Co-utilization of canola oil and glucose has also been carried out successfully for production of biosurfactant from *C. lipolytica* (Sarubbo *et al*., 2007).

Palm oil mill effluent is also a promising substrate for biosurfactant production. Palm oil has also been used for biosurfactant production by *P. aeruginosa* SP4 (Pansiripata *et al*., 2010). Saimmai *et al*. (2012), also documented biosurfactant producing microorganisms from palm oil contaminated industrial sites in palm oil refinery factory. Along with palm oil, they also included other sources like palm oil decanter cake and palm oil mill effluent. Comparative study carried out by Govindammal and Parthasarathi (2013), on glucose, petroleum based substrates, waste fried vegetable oil, and coconut oil cake for biosurfactant production by *Pseudomonas fluorescence* MFS03 isolated from mangrove forest soil, proved that vegetable oil and coconut oil are reliable substrates for biosurfactant production. These oils contain high percentage of oleic acid.

Oil cakes or soapstocks, in spite of being a complex substrate has been successfully shown to produce high yield of rhamnolipid, along with different oily substrates, viz a viz, sunflower oil, olive oil and soy bean oil. Yield up to 15.9 g/L was reported by Benincasa *et al*. (2002), in a medium containing soapstock inoculated with *P. aeruginosa* strain LBI. Soapstock has also been used efficiently for production of extracellular capsular polysaccharides.

2.4.5.Food Processing By-Products

Wastes from soybean, potato, sweet potato and sweet sorghum contain high amount of starch that acts as base material in fermentation process. Waste products like canola meal, coconut cake, peanut cake, soybean cake, also represent suitable candidates for cheaper substrates (Banat *et al*., 2014). Processed olive oil, sunflower oil, ground nut oil, rape seed oil; potato peels are useful as raw material for microbial products. A peat, composed of decomposed vegetable matter contains high amount of carbohydrates and amino acids, which provide excellent nutrient source for the growth of microbes. Other by-products from vegetable oil refining processes are increasingly becoming targeted substrates for microbial biosurfactant production process (Banat *et al*., 2014).

In addition to the above mentioned relatively cheap substrates a number of abundantly available starch base substrates provide another alternative renewable carbon sources. One of the representative examples is the potato processing industry that produces significant quantities of starch-rich waste substrates suitable for biosurfactant production. In addition to approximately 80% water contents, potato waste also has carbohydrates (17%), protein (2%), fat (0.1%), vitamins, inorganic minerals, and trace elements. Thus, potato wastes are a rich source of various components which can support the growth of microorganisms for production of various commercially important products. A commercially prepared potato starch in mineral salts medium was investigated by Fox and Bala (2000). They reported biosurfactant production by *B. subtilis* ATCC 21332 and a significant reduction in surface tension from 71.3 to 28.3 mN/m with a CMC value of 0.10 g/L. Noah *et al*. (2005), carried out studies on surfactin production from *Bacillus* sp. by using cassava waste water. They established that cassava wastewater produced from the cassava flour preparation, a renewable inexpensive and easily available carbon source can be used for surfactin production by *B. subtilis* and other biotechnological processes. Different unconventional carbon sources such as potato peel powder, corn powder, sugarcane bagasse and *Madhuca indica* were also used

by Jain *et al*. (2013). They reported increased viscosity in cultures yet achieved maximum surface tension reduction when compared to other substrates. They reported an unidentified biosurfactant production at a yield of 15.40 ± 0.21 g/L on corn powder base production medium from *Klebsiella* sp. strain RJ-03 and concluded that the use of such cheap substrates have a significant potential for commercialization for applications in bioremediation processes.

2.5. Strategies for Improvement of Biosurfactant Production

Optimization of production processes is needful for an efficient production of biosurfactant at an industrial scale (Abalos *et al*., 2002). Thus, the most significant factors that affect biosurfactant production and their biochemical properties should therefore be screened, and optimum conditions selected and enhanced. Typically, the type and concentration of the carbon and nitrogen sources used are the first factors evaluated, followed by choosing an adequate microorganism. Other nutritional factors such as trace elements, and physicochemical parameters such as pH, temperature, and agitation speed show great effect on biosurfactant production and are therefore optimized (Bertrand *et al*., 2018).

2.5.1. Optimization of medium composition

The culture media and culture conditions are extremely important for feasible biosurfactant production. The use of adequate nutrients (carbon and nitrogen sources) directly influence the microbial metabolism, and thus, production (Ismail *et al*., 2015; Abalos *et al*., 2002). Apart from directly participating in cell growth and reproduction, the carbon and nitrogen sources are used as building blocks for biosurfactant biosynthesis. Different nutrient sources may regulate biosurfactant synthesis by induction or catabolic repression. Immiscible substrates such as different oils and hydrocarbons have been used as biosurfactant production inducers (Pal *et al*., 2009). Catabolic repression has been observed in hydrocarbon degrading

bacterial strains that do not produce surface-active molecules in the presence of carbon sources like glucose and organic acids. This can be explained by the fact that hydrocarbon uptake is promoted by biosurfactant production. Sugars such as glucose stimulate high biomass growth and biosurfactant production in the stationary phase by most organisms (Bertrand *et al*., 2018) such as*Bacillus*sp. and *P. aeruginosa* (Reis *et al*., 2013). However,biosurfactant production has also been detected in the exponential phase by bacteria such as*Acinetobacter* sp. such that when the bacteria reached the stationary phase, biosurfactant production would have reachedits maximum. This was also been reported for *Rhodococcus* sp. and *Aspergillus* sp. strains (Chen *et al*., 2012).

The criteria for choosing adequate carbon and nitrogen sources depends on the producing strain. In the event of the possibility of using numerous carbon and nitrogen sources, traditional "one factor at a time" screening method or statistical design are implemented. Not only are the significant factors determined, but the levels (concentrations) and relevant interactions are inferred (Bertrand *et al*., 2018).

2.5.1.1. Traditional Method of Optimization (One factor at a time or monothetic analysis)

This approach of optimization depends on changing only one factor at time and keeping other factors constant. This procedure is repeated in turn for all factors to be studied. This method cannot study the interactions between factors, it misses optimal settings of factors and also consumes time and materials (Nor *et al*., 2010).

2.5.1.2 Statistical Design for Optimization of Biosurfactant Production

In this method several process factors are varied simultaneously. It saves time and materials, gives precise estimates and estimates interactions between factors (Czitrom, 1999). The use of statistical designs have been demonstrated to be a very efficient tool for enhancing biosurfactant production and properties. The most popular designs employed for optimizing

biosurfactant production are factorial designs and response surface methodologies (RSM). Factorial designs are multifactor linear models, also denominated “the fully crossed design”. In these designs, the factors evaluated and their levels occur in combination with every level of the other factors. They allow for the measurements of different sorts of factor effect; the main effect (the effect of the independent factors) and the interaction between the factors (how much one factor depends on the level of one or more factors) (Quinn and Keough, 2002). The factorial designs reported for biosurfactant production optimization include two-level factorial design, the Plackett–Burman Design (PBD), and the Taguchi design.

On the other hand, the RSM is defined as a combination of mathematical and statistical techniques for empirical model building (Cornell, 1990). Response surface methodology (RSM) consists of a group of empirical techniques devoted to the evaluation of relationship existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria (Khuri and Cornell, 1987, Onwosi and Odibo, 2013).

By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables).

An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. The application of the RSM design for optimization is aimed at reducing the cost of expensive analysis methods (e.g., the finite element method) and their associated numerical noise (Myers *et al*., 2016). The most common RSM implemented for enhancing biosurfactant production and characteristics are the Central Composite Design (CCD) and the Box–Behnken Design (BBD). Because of the limitation of individual statistical designs, a number of researchers reckon the need for the use of more than one statistical design for best optimization results (Betrand *et al*., 2018).

2.6.Downstream Processing

Although cost effective biosurfactant production is the major priority for feasible industrial application, downstream processing should be taken into account. Once the secondary metabolite has been produced, the need for high purity of the tenso-active agent will depend on the application intended (Najmi *et al*., 2018). Recovery and purification has been shown to greatly increase biosurfactant production costs (Varjani and Upasani, 2017). Traditional strategies for biosurfactant recovery and purification include precipitation with acids, salts, or organic solvents which are toxic and cause air pollution. Other more sophisticated methods include foam fractionation, ultrafiltration, adsorption-desorption on polystyrene resins, ion exchange chromatography, and High Pressure Liquid Chromatography (HPLC) (Díaz De Rienzo *et al*., 2016; Reis *et al*., 2013). However, the latter present very low yields, as only small amounts of the crude extract can be treated. A new approach which has recently gained popularity for downstream processing in other biomolecules is Aqueous Two-Phase Systems (ATPS). This strategy has been successful for the recovery and partial purification of enzymes and other micro and macro molecules, but has been hardly used for the treatment of crude biosurfactant extracts (Bertrand *et al*., 2016). Up to date, the application of ATPS for biosurfactant downstream processing is not fully explored in the biotechnological field.

2.7.Applications of Biosurfactant

Currently, the global biosurfactant market has attained growth rate of 3.5% per annum, and is being expected to reach 2.2 billion US dollars (Bertrand *et al*., 2018). Almost all surfactants currently in use are chemically synthesized. Nevertheless, in recent years, much attention has been directed toward biosurfactants due to their broad functional properties and the diverse capabilities of microbes. The structural analysis of biosurfactant

has opened possibilities for their chemical synthesis. Biosurfactants have several applications in agriculture, medicine, petroleum and industry.

2.7.1. Application of Biosurfactants in Agriculture

One way to enhance the solubility of bio-hazardous chemical compounds such as polyaromatic hydrocarbon (PAH), is to apply surfactants as mobilizing agents. This increases the apparent solubility of Hydrophobic Organic Contaminants (HOC). Also surfactants are said to help microbes adsorb to soil particles occupied by pollutants, thus decreasing the diffusion path length between the site of absorption and site of bio uptake by the microorganisms (Makkar and Rockne, 2003).

Also in agriculture, surfactants are used for hydrophilization of heavy soils to obtain good wettability and to achieve even distribution of fertilizer in the soil. They also prevent the caking of certain fertilizer during storage and promote spreading and penetration of the toxicants in pesticides (Makkar and Rockne, 2003). Fengycins are reported to possess antifungal activity, and therefore may be employed in biocontrol of plant diseases. Lipopeptide biosurfactants exhibit insecticidal activity against fruit fly *Drosophila melanogaster* and hence can be used as biopesticide (Mulligan, 2005). The emergence of pesticide resistant insect populations as well as rising prices of new chemical pesticides has stimulated the use of biosurfactant as a new eco-friendly vector control tools (Vandana and Singh,2018).

2.7.2. Application of Biosurfactants in Medicine

Mukherjee *et al.* (2006), elucidated on the wide range of applications of biosurfactants in medicine and they include:

2.7.2.1. 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 *et al*., 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. It has been shown that these biosurfactants are very efficient bacteriostatic agent against *Listeria monocytogenes*, an important food related pathogen, and showed synergistic effect when combined with nisin, a broad-spectrum bacteriocin (Magalhães and Nitschke, 2013).

2.7.2.2. Anti-cancer activity: Some microbial extracellular glycolipids induce cell differentiation instead of cell proliferation in the human promyelocytic leukemia cell line. Also, exposure of PC 12 cells to mannosylerythritol (MEL) enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase with resulting overgrowth of neurites and partial cellular differentiation. This suggests that MEL induces neuronal differentiation in PC 12 cells and provides the ground work for the use of microbial extracellular glycolipids as novel reagents for the treatment of cancer cells (Krishnaswamy *et al*., 2008).

2.7.2.3. Anti-adhesive agents: Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites. Rodrigues *et al.* (2006), demonstrated that pre-coating vinyl urethral catheter by running the surfactin solution through them before inoculation with media resulted in the decrease in the amount of biofilm formed by *Salmonella typhimurium, Salmonella enterica, E. coli and Proteus mirabilis.* Krishnaswamy *et al*. (2008), reported that pretreatment of silicone rubber with *S. thermophilus* surfactant inhibited 85% adhesion of *C. albicans*, and also surfactants from

Lactobacillus fermentum and *L. acidophilus* adsorbed on glass, reduced by 77%, the number of adhering uropathogenic cells of *Enterococcus faecalis.*

2.7.2.4. Immunological adjuvants: Bacterial lipopeptides constitute potent non-toxic, nonpyrogenic immunological adjuvants when mixed with conventional antigens. An improvement of the humoral humane response was demonstrated with low molecular mass antigens Iturin AL and herbicolin A (Gharaei-Fathabad, 2011).

2.7.2.5. Antiviral activity: Antibiotic effects and inhibition of growth of human immunodeficiency virus in leucocytes by biosurfactants have been cited in literature (Desai and Banat, 1997).Furthermore, Krishnaswamy *et al*.(2008), reported that due to the increased incidence of HIV in women, there arose the need for a female controlled, efficacious and safe vaginal topical microbicide. Sophorolipids surfactants from *C. bombicola* and its structural analogues such as the sophorolipid diacetate ethyl ester is the most potent spermicidal and virucidal agent; it was also reported that this substance has a virucidal activity similar to nonoxynol – 9 against the human semen.

Other advantages and applications of bio-surfactant in medicine are the use of surfactants as agents for stimulating stem fibroblast metabolism, immunomodulatory action, however, it has been reported in literature that the deficiency of pulmonary surfactant, a phospholipids protein complex, is responsible for the failure of respiration in prematurely born infants. Isolation of genes for protein molecules of this surfactant and cloning in bacteria have made possible its production for medical application (Krishnaswamy *et al*., 2008). Rhamnolipids significantly reduced the rate of deposition and adhesion of several bacterial and yeast strains isolated from explanted voice prostheses (Rodrigues *et al*., 2006).

2.7.3. Application of Biosurfactants in Commercial Laundry Detergents

Almost all surfactants, an important component used in modern day commercial laundry detergents, are chemically synthesized. These chemicals exert toxic effect 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*et al.*, 2006). 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).

2.7.4. Application of Biosurfactants in Food Processing Industry

Biosurfactants have been used for various food processing application but they usually play a role as food formulation ingredient and anti-adhesive agents. As food formulation ingredient, they promote the formation and stabilization of emulsion. It is also used to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf -life of starch-containing products, modify rheological properties of dough and improve consistency and texture of fat-based products (Krishnaswamy *et al*., 2008).

2.7.5. Application of Biosurfactants 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, biosurfactants 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, insect repellents, antacids, bath products, acne pads, anti-dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste and dentine cleansers (Gharaei-Fathabad, 2011).

2.7.6. Application of Biosurfactants in Microbial Enhanced Oil Recovery

Microbial enhanced oil recovery includes use of microorganisms and the exploitation of their metabolic processes to increase production of oil from marginally producing reservoirs. Microbial surfactants are widely used in oil recovery in recent times. Microorganisms in reservoir are stimulated to produce polymers and surfactants which aid MEOR by lowering interfacial tension at the oil–rock interface (Kumar and Mandal, 2017). *In situ,* microorganisms in the reservoir are usually provided with low-cost substrates, such as molasses and inorganic nutrients, to promote growth and surfactant production. As regards use of microorganisms for MEOR *in situ,*they must be able to grow under extreme conditions encountered in oil reservoirs such as high temperature, pressure, salinity, and low oxygen level. Several aerobic and anaerobic thermophiles tolerant of pressure and moderate salinity have been isolated which are able to mobilize crude oil in the laboratory. The Rhamnolipid is one of the most investigated biosurfactants employed in enhanced oil recovery (Camara *et al*., 2019; Zhao *et al*., 2016), due to its promising characteristics as surface agent and its emulsifying properties. It is the most effective biosurfactant regarding the ability to reduce the water surface tension, as well as the oil–water interfacial tension (Amani and Mehrnia, 2010), besides emulsifying the oil and, therefore, increase the trapped oil mobility (Zhao *et al*., 2016).

2.7.7. Application of Biosurfactants in Hydrocarbon Degradation

Hydrocarbon-utilizing microorganisms excrete varieties of biosurfactants. Biosurfactant, by being a natural product, is biodegradable and ecofriendly (Camara *et al*., 2019).The release of petroleum hydrocarbons in the environment due to oil spill and chronic pollution is a serious major concern. Chemical dispersants are effectively utilized worldwide to minimize oil spill damage. Dispersants are mixtures of one or more surfactants and

solvents that enhance dispersion of oil into droplets leading to increased mobility and bioavailability of hydrocarbons. The dispersed oils are solubilized and degraded by microorganisms.

Crude oils have very low water solubility, high adsorption onto soil matrix and present limited rate of mass transfer for biodegradation (Banat *et al*., 2000). Oil-contaminated soil is especially arduous for bioremediation since oil forms droplets or films on soil particles, which is a powerful barrier against microbial degradation (Urum *et al*., 2004). Bioavailability of contaminants in soil to the metabolizing organisms is influenced by factors such as desorption, diffusion and dissolution. Chemical dispersants are toxic to living organisms in the environment and replacing them with biological non-toxic alternatives would be very advantageous and highly sought after (Tripathi *et al*., 2018).

Biosurfactants are produced to decrease the tension at the hydrocarbon-water interface aiming to pseudo-solubilize the hydrocarbons, thus increasing mobility, bioavailability and consequent biodegradation (McGenity e*t al*., 2012; Perfumo *et al*., 2010). Several biosurfactants are produced by a diversity of microorganisms in order to survive in an oil-rich environment, and this adaptation process selected for surfactants with highly adaptable physico-chemical properties. Biosurfactants are, therefore, very suitable for applications in the oil industry and this is reflected in the market, where the large majority of biosurfactants produced are in petroleum-related applications. The applications are, in general, in oil recovery, oil spill management and microbial enhanced oil recovery (Kanna *et al*., 2014).

Purified rhamnolipid biosurfactants were applied in the removal of oil from contaminated sandy soil (Santa-Anna *et al*., 2007). The authors optimized the biosurfactant concentrations and oil removal by applying statistical experimental design tool that generated a surface response. Sandy soil contained predominantly aromatic and paraffinic hydrocarbons

(5-10% w/w) mixed with reduced concentration of rhamnolipid (6.3-7.9 g/L) resulted in removal of oil by up to 91 and 78%, respectively. Rahman *et al*. (2003), reported that addition of rhamnolipids above the CMC, enhanced the apparent aqueous solubility of hexadecane, the biodegradation of hexadecane, n-paraffins, octadecane, creosotes in soil and promoted biodegradation of petroleum sludges. Rhamnolipids produced by *Nocardioides* sp. A-8 allows the bacterium to grow on aromatic hydrocarbons or n-paraffin as sole carbon source by lowering the surface tension and emulsifying the aromatic hydrocarbons (Vasileva-tonkova and Gesheva, 2005). The authors found similar results for the strain *Pantoea* sp. A-13, which also produced rhamnolipid to grow on n-paraffin or kerosene. Both A-8 and A-13 strains were isolated together with other 15 aerobic microbial isolates from oil-contaminated sites in Antarctica and appear to be very promising source for application in accelerated environmental bioremediation at low temperatures.

Urum and Pekdemir (2004), applied different biosurfactants (rhamnolipid, aescin, saponin, lecithin and tannin) in washing oil-contaminated soil and observed significant removal of crude oil with different concentrations of biosurfactant solution. Oil mobilization was the main cause for its removal, which was triggered by the reduction of surface and interfacial tensions, rather than oil solubilization or emulsification.

The combination of oil-degrading bacteria and biosurfactant or biosurfactant-producing bacteria has also been tested by research groups. *P. putida*, an oil degrading bacterium, was co-cultured with a biosurfactant-producing bacterium, and an improved degradation was observed in both aqueous and soil matrix in comparison with the individual bacterium cultures (Kumar *et al*., 2006). This treatment resulted in increased oil emulsification and also adhesion of hydrocarbon to the bacteria cell surface.

2.8.Crude Oil Pollution in the Environment

The shift in economic base of coal to crude oil and petroleum products, more especially after the World War II, greatly increased the volume of these commodities being transported across the high seas. The above, coupled with their storage underground, involve high environmental risks. For example the wreck of the "Torrey Canyon " off the coast of England in 1967 produced worldwide concern about the consequences of massive oil spills in the marine environment (Onwurah *et al*., 2007). Diverse components of crude oil and petroleum such as polycyclic aromatic hydrocarbons (PAHs) have been found in waterways as a result of pollution from industrial effluents and petrochemical products.

Petroleum hydrocarbon pollution of the environment may arise from oil well drilling production operations, transportation and storage in the upstream industry, and refining, transportation, marketing in the downstream industry, and intentional bunkering of pipeline. Sources of petroleum and its products in the environment also include accidental spills and from ruptured oil pipelines. Today the international oil and gas-pipelines span several million kilometers and this is growing yearly. Just like any other technical appliance, pipelines are subject to ''tear and wear'', thus can fail with time. Spilled petroleum hydrocarbons in the environment are usually drawn into the soil due to gravity until an impervious horizon is met, for example bedrock, watertight clay or an aquifer. Poor miscibility of crude oil accounts for accumulation of free oil on the surface of ground water and this may migrate laterally over a wide distance to pollute other zones very far away from the point of pollution (Wang *et al*., 2013).

Oil contamination can affect soil physical and chemical properties. Oil usually causes anaerobic environment in soil by smothering soil particles and blocking air diffusion in the soil pores, and affects soil microbial communities (Sutton *et al*., 2013). Heavy crude oil

pollution can cause complete mortality of marsh vegetation (Lin and Mendelssohn, 2012). In addition, crude oil-contaminated soils are hydrophobic compared with pristine sites (Aislabie *et al*., 2004). Hydrocarbon contamination can also increase soil total organic carbon (Ekundayo and Obuekwe, 2000), and change soil pH values (Wang, 2010) and other soil chemical properties (Kisic *et al*., 2009). Crude oil affects germination and growth of some plants. It also affects soil fertility but the scale of impact depends on the quantity and type of oil spilled. Pollution of environment affects humans exposed to it. Some diseases have been diagnosed to be the consequences of crude oil pollution. The health problems associated with oil spill may be through any or combinations of the following routes: contaminated food and / or water, emission and / or vapors. Toxic components in oil may exert their effects on man through inhibition of protein synthesis, nerve synapse function, and disruption in membrane transport system and damage to plasma membrane (Onwurah *et al*., 2007). Volatile components of crude oil after a spill have been implicated in the aggravation of asthma, bronchitis and accelerating aging of the lungs. They also affect the liver, kidney and spleen (Anozie and Onwurah, 2001). Epidemiological evidence from the work of Bruederle and Hodler (2019), suggests that oil spills affect neonates, contributes to infant mortality, and also increase the risk of abortion and stillbirth.

2.8.1. Crude Oil Pollution in Nigeria

Nigeria is Africa's largest crude oil producer. The country's crude oil production, estimated at over 300 million liters per day, makes up 70 percent of the Nigerian government's revenue (Adebayo, 2019).Oil from the Niger Delta region accounts for more than 90% of Nigeria’s exports and about 80% of the government’s revenue, from as far back as December 1981. In the present times, the overall contribution of the oil sector to the national economy grew from 84% in 2000 and 95% in 2002 to about 96.7% in 2003 (Twumasi and Merem, 2006).

However, ever since oil was discovered in the country in 1956, it has been a source of strife. It is estimated that between 9 million to 13 million barrels have been spilt since oil drilling started in the country in 1958.

The first oil spill in Nigeria was at Araromi in the present Ondo state in 1908. In July 1979, the Forcados tank Terminal in Delta state incidence spilled 570,000 barrels of oil into the Forcados estuary polluting the aquatic environment and surrounding swamp forest. The Oyakama oil spillage of 1980 with a spill of approximately 30,000 barrels. In 1983, Oshika village in Rivers state witnessed a spill of 5,000 barrels of oil from Ebocha-Brass pipeline which flooded the lake and swamp forest (Ayuba, 2012).

Oil spills are a common occurrence in Niger Delta region of Nigeria where over 40 million liters of crude oil is spilled annually, resulting in human deaths and damage to the local ecosystem. A 2018 study by the Journal of Health and Pollution, found that more than 12,000 oil spill incidents have occurred in the oil-rich region between 1976 and 2014 (Adebayo, 2019).

Half of all spills occur due to pipeline and tanker accidents, other causes include sabotage and oil production operations, with 1% of the spills being accounted for by inadequate or non-functional production equipment (Baird, 2010).

Findings have shown that the major causes of the spills in Ogoni land are worn-out oil facilities like the pipelines as well as oil bunkering and sabotage by youth in the village (Bankole, 2018). Shell Petroleum recorded oil spill due to crude oil theft and sabotage of facilities, as well as illegal refining, as the leading cause of environmental damage from oil and gas operations in the Niger Delta. They recorded that hundred percent of the spill experienced so far in 2019 is due to sabotage of pipelines (SPDC, 2019).

2.9. Heavy Metals Contamination of the Environment

Heavy metals are non-biodegradable and toxic to living things. They accumulate in plants and animals and cause long term effect on humans (Vijaya *et al*., 2010). Although heavy metals are naturally present in the soil, their concentration increases owing toatmospheric depositions (Rai *et al*., 2019) and anthropogenic activities such as combustion, extraction processes, agricultural runoff, transportation of dissolved metals, irrigation of agricultural soil with wastewater, industrial and mining activities (Zwolak *et al*., 2019; Akhilesh *et al*., 2009). Musa *et al*. (2017), reviewed the common heavy metals found in agricultural soils – lead, chromium, arsenic, cadmium, copper, mercury, nickel.

Different studies have reviewed the acute and chronic effects of heavy metals such as iron (Fe), lead (Pb), mercury (Hg) on soil fertility and agricultural output. Under certain conditions, these metals may accumulate to a toxic concentration level which can lead to ecological damages. Heavy metals affect soil enzymatic activities indirectly by shifting the microbial community which synthesizes enzymes and affects microbial activities. Heavy metals such as As, Cd, Hg, Pb or Se are not essential for plants growth as they do not perform any known physiological function in plants. Their presence in the soil inhibits plant growth and accumulates in plants. The rate of accumulation of heavy metals in leafy vegetables and root vegetables is quite high, thus, such vegetables should not be grown on contaminated soil (Zwolak *et al*., 2019). The consumption of vegetables grown on heavy metal-polluted soil by humans and animals is associated with serious public health issues. Singh and Kalamdhad (2011), reviewed the various public health issues caused by ingestion of heavy metal polluted food crops. They include lung damage, kidney damage, proteinuria, allergy, amalgam disease, pneumonitis, inhibition of sex hormones (progesterone

and estradiol), endocrine disruption, encephalopathy, nausea and vomiting, adverse impact on central nervous system, circulatory, and cardiovascular systems, difficulty in learning and concentration in children, hepatomegaly, melanosis,“rice-water”diarrhea, severe neuropathy, peripheral vascular disease, respiratory problems (Rai *et al*., 2019).

2.10.Bioremediation of Polluted Environment

Increasing exploration and production activities coupled with improper waste disposal practices have led to widespread contamination of both the aquatic and terrestrial ecological systems (Odokuma and Ikpe, 2003). Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon and as energy for metabolic activities, and these microorganisms are widely distributed in nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinant (Adeline *et al*., 2009).

Bioremediation recently has evolved as an emerging green technology of environmental conservation by removing, transforming and breaking down various contaminants especially petroleum hydrocarbons, by applying living organisms. Bioremediation is defined as the process by which microorganisms are stimulated to rapidly degrade hazardous organic pollutants to environmentally safe levels in soils, sediments, substances, materials and ground water (Mahmoud, 2016). Degradation can occur aerobically or anaerobically, however, greater percentage of hydrocarbon degradation occur under aerobic condition. Biochemical pathway for aerobic and anaerobic bacterial degradation of hydrocarbon is shown in Appendix I.

This process uses microbial metabolism in the presence of optimum environmental conditions and sufficient nutrients to breakdown contaminants notably petroleum hydrocarbons (Adams *et al*., 2015).The energy and carbon are obtained through the metabolism of organic compounds by the microbes involved in bioremediation processes

(Fulekar *et al*., 2009). Bioremediation process transforms contaminants to non–hazardous or less hazardous forms. Often, the microorganisms metabolize the chemicals to produce carbon dioxide or methane, water and biomass. Bioremediation techniques have advantages of causing little disturbance to soil and water, low cost, simple and convenient operation, and less secondary pollution (Xia *et al*., 2019).

Based upon the process of removal and transportation of wastes, bioremediation technology can be carried out *in situ* and *ex situ*. *In situ* bioremediation deals with pollutants treatments at the same site, while *ex situ* involves the removal of contaminated material completely from one site and its transfer to another site (Xia *et al*., 2019).

Researchers have developed and modelled different bioremediation techniques; however, due to nature and/or type of pollutant, there is no single bioremediation technique that serves as a '*silver bullet'* to restore polluted environments. Autochthonous (indigenous) microorganisms present in polluted environments hold the key to solving most of the challenges associated with biodegradation and bioremediation of polluting substances (Verma and Jaiswal, 2016) provided that environmental conditions are suitable for their growth and metabolism.

2.11. Some Bioremediation Techniques

2.11.1. Natural Attenuation: This technique focuses on the verification and monitoring of passive natural remediation processes (Khan *et al*., 2004). Natural attenuation is an *in situ* treatment method that uses natural indigenous organisms to limit the spread of contamination and to reduce the concentration and the amount of pollutants at contaminated stations (Boparai *et al*., 2008; Khan *et al*., 2004). This indicates that the environmental contaminants are undisturbed during natural attenuation (Gelman and Binstock, 2008). These contaminants include fuels, nonhalogenated volatile organic compounds**,** semivolatile organic compounds, pesticides, and herbicides. The process could deal with halogenated organics, but it requires

longer treatment times. Also, the technology is manipulated to treat especially hydrophobic contaminants such as high molecular weight petrochemicals that tend to adsorb strongly to soil particles and have very low rates of imbibition. Often, communities of adapted degraders will mineralize these contaminants quickly after its absorption to soil particles (Mahmoud, 2016).

2.11.2. Biostimulation: Biostimulation is the process of adding nutrient, electron acceptor, oxygen and other amendment to stimulate existing bacteria involved in bioremediation. This can be done by addition of various forms of limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon, which are otherwise available in quantities low enough to constrain microbial activity (Rhykerd *et al.,* 1999). Perfumo *et al.*(2007), described biostimulation as the addition of nutrients, oxygen or other electron donors and acceptors to the coordinated site in order to increase the population or activity of naturally occurring microorganisms available for bioremediation. Bioremediation requires the evaluation of both the intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the *in situ* process (Margesin and Shinner, 2001).

Hydrocarbon biodegradation in soil can be limited by many factors, including nutrients, pH, temperature, moisture, oxygen, soil properties and contaminant presence (Al-Sulaimani, 2010; Atagana, 2008). Indigenous microorganisms are present in the contaminated site, but they require adequate stimulation for growth and effective remediation (Thapa *et al*., 2012). Additives are usually added to the subsurface through injection wells. Subsurface characteristics such as groundwater velocity, hydraulic conductivity of the subsurface, and lithology of the subsurface are also important in developing a biostimulation system (Vidali, 2001).

The primary advantage of biostimulation is that bioremediation are undertaken by already present native microorganisms that are well-suited to the subsurface environment,

and are well distributed spatially within the subsurface. The primary challenge is that the delivery of additives in a manner that allows the additives to be readily available to subsurface microorganisms is based on the local geology of the subsurface. Tight, impermeable subsurface lithology (tight clays or other fine-grained material) make it difficult to spread additives throughout the affected area. Fractures in the subsurface create preferential pathways in the subsurface which additives preferentially follow, preventing even distribution of additives. Addition of nutrients might also promote the growth of heterotrophic microorganisms which are not innate degraders of petroleum hydrocarbon, thereby creating competition between the resident microflora (Adams *et al*., 2014).

2.11.3. Bioaugmentation: Bioaugmentation is the addition of oil-degrading microorganisms to supplement the indigenous populations. Bioaugmentation involves the introduction of microorganisms isolated from the contaminated site, from a historical site or carefully selected and genetically modified to support the remediation of petroleum-hydrocarbon contaminated sites based on the assumption and/or confirmation that indigenous organisms within the impacted site cannot biodegrade petroleum hydrocarbon (Adams *et al*., 2015). It has been proposed as an alternate strategy for the bioremediation of oil contaminated environments. The rationale for this approach is that the indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum or that they may be in a stressed state as a result of the recent exposure to the spill. Other conditions under which bioaugmentation may be considered are when the indigenous hydrocarbon-degrading population is low, when the speed of decontamination is the primary factor, and also when introduction of allochthonous organisms may reduce the lag period for startup of the bioremediation process (Forsyth *et al.,* 1995). For this approach to be successful in the field, the seed microorganisms must be able to degrade most petroleum components, maintain genetic stability and viability during

storage, survive in foreign and hostile environments, effectively compete with indigenous microorganisms, and move through the pores of the sediment to the contaminants (Adams *et al*., 2015).

Different microbial species have different enzymatic abilities and preferences for the degradation of oil compounds. Some microorganisms degrade linear, branched, or cyclic alkanes. Others prefer mono- or polynuclear aromatics, and others jointly degrade both alkanes and aromatics. The study of microbes in bioremediation systems makes possible the selection of microorganisms with potential for the degradation and production of compounds with biotechnological applications in the oil and petrochemical industry. Successful bioaugmentation treatments depend on the use of inocula consisting of microbial strains or microbial consortia that have been well adapted to the site to be decontaminated. Foreign microorganisms (allochthonous organisms) have been applied successfully but their efficiency depends on ability to compete with indigenous microorganisms, predators and various abiotic factors. Factors affecting proliferation of microorganisms used for bioaugmentation including the chemical structure and concentration of pollutants, the availability of the contaminant to the microorganisms, the size and nature of the microbial population and the physical environment should be taken into consideration when screening for microorganisms to be applied (Eun-Hee *et al*., 2011).

2.11.4. Land Farming: Land farming is amongst the simplest bioremediation techniques owing to its low cost and less equipment requirement for operation. It can be carried out *insitu* and *exsitu*. In land farming, polluted soils are usually excavated and/or tilled, but the site of treatment apparently determines the type of bioremediation. It has been reported that when a pollutant lies <1m below ground surface, bioremediation might proceed without excavation, while pollutant lying >1.7m needs to be transported to ground surface for bioremediation to be effectively enhanced (Nikolopoulou *et al*., 2013). Generally, excavated

polluted soils are carefully applied on a fixed layer support above the ground surface to allow aerobic biodegradation of pollutant by autochthonous microorganisms (Silva-Castro *et al*., 2015; Volpe *et al*., 2012). The construction of a suitable land farming design with an impermeable liner minimizes leaching of pollutant into neighboring areas during bioremediation operation (da Silva *et al*., 2012). Tillage, which brings about aeration, addition of nutrients (nitrogen, phosphorus and potassium) and irrigation are the major operations, which stimulate activities of autochthonous microorganisms to enhance bioremediation during land farming. Nevertheless, it was reported that tillage and irrigation without nutrient addition in a soil with appropriate biological activity increased heterotrophic and diesel-degrading bacterial counts thus enhancing the rate of bioremediation. Dehydrogenase activity can be used as a biological parameter in land farming technology (Silva-Castro *et al*., 2015).

In a field trial, Paudyn *et al*. (2008), reported 80% diesel removal by aeration using roto- tilling approach at remote Canadian Arctic location over a 3-year study period. This further demonstrates that in land farming technique, aeration plays crucial role in pollutant removal especially at cold regions. Land farming is usually used for remediation of hydrocarbon-polluted sites including polyaromatic hydrocarbons (Cerqueira *et al*., 2014); as a result, biodegradation and volatilization (weathering) are the two remediation mechanisms involved in the pollutant removal. Land farming system complies with government regulations, and can be used in any climate and location (Besaltatpour *et al*., 2011). Over all, land farming bioremediation technique is very simple to design and implement, requires low capital input and can be used to treat large volume of polluted soil with minimal environmental impact and energy requirement (Maila and Colete, 2004).

2.11.5. Composting: Composting is a process of piling contaminated soil organic substances such as manure or agricultural wastes. Composting technology combines contaminated soil

with nonhazardous organic matter such as manure or biological wastes. The added organic material supports the development of a rich microbial population and elevates temperature of the pile. Stimulation of microbial growth by added nutrients results in effective biodegradation in a relatively short period of time (Mahmoud, 2016).

2.11.6. Phytoremediation: This technique relies on the use of plant interactions (physical, biochemical, biological, chemical and microbiological) in polluted sites to mitigate the toxic effects of pollutants. Depending on pollutant type (elemental or organic), there are several mechanisms (accumulation or extraction, degradation, filtration, stabilization and volatilization) involved in phytoremediation (Azubuike *et al*., 2016).

Elemental pollutants (toxic heavy metals and radionuclides) are mostly removed by extraction, transformation and sequesteration. On the other hand, organic pollutants (hydrocarbons and chlorinated compounds) are predominantly removed by degradation, rhizoremediation, stabilization and volatilization, with mineralization being possible when some plants such as willow and alfalfa are used (Kuiper *et al*., 2004).

Some important factors to consider when choosing a plant as a phytoremediator include: root system, which may be fibrous or tap depending on the depth of pollutant, surface biomass, which should not be available for animal consumption, toxicity of pollutant to plant, plant survival and its adaptability to prevailing environmental conditions, plant growth rate, site monitoring and above all, time required to achieve the desired level of cleanliness. In addition, the plant should be resistant to diseases and pests (Lee, 2013).

It has been reported that in some contaminated environments, the process of contaminant removal by plant involves: uptake, which is largely by passive process, translocation from roots to shoots, which is carried out by xylem flow, and accumulation in shoot (Miguel *et al*., 2013). Further, translocation and accumulation depend on transpiration, and partitioning between xylem sap and adjacent tissues, respectively. Nonetheless, the

process is likely to differ, depending on other factors such as nature of contaminant and plant type. It is plausible that most plants growing in any polluted site are good phytoremediators. Therefore, the success of any phytoremediation approach primarily depends on optimizing the remediation potentials of native plants growing in polluted sites either by bioaugmentation with endogenous or exogenous plant rhizobacteria, or by biostimulation. It was reported that the use of plant growth-promoting rhizobacteria (PGPR) might play an important role in phytoremediation, as PGPR tends to enhance biomass production and tolerance of plants to heavy metals and other unfavourable soil (edaphic) conditions (de-Bashan *et al*., 2012; Yancheshmeh *et al*., 2011).

Grobelak *et al*. (2015), reported increased plant length, root and stem growth, when *Brassica napus* L. subsp. *napus* and *Festuca ovinia* L. were inoculated with exogenous PGPR during seed germination, and 2 weeks after plant growth. This protected the seeds and plants from growth inhibition on heavy metal-polluted soil. Similarly, during phytoremediation of metal-contaminated estuaries with *Spartina maritima*, bioaugmentation with endogenous rhizobacteria resulted in increased plant subsurface biomass, metal accumulation and enhanced metal removal (Mesa *et al*., 2015). Addition of biosurfactant produced by *Serratia marcescens* to gasoline-contaminated soil to which *Ludwigia octovalvis* were planted, resulted in 93.5 % total petroleum hydrocarbon (TPH) removal and corresponding increase in microbial count. This was attributed to desorption and solubilization effects of biosurfactant, which in turn increased gasoline bioavailability to microbial consortia within *L. octovalvis* rhizosphere (Almansoory *et al*., 2015).

Other bioremediation techniques include bioventing, biosparging, bioslurping and biopiling.

2.12. Analysis of Polluted Environment – Culture-Dependent and Non Culture-Dependent Techniques

Characterization of microbial communities living in oil-contaminated soils and evaluation of their oil-degradation capacities potentially serve as guide for selection of bioremediation technique to be employed in the polluted environments (Peng *et al*., 2015). Culture-independent and dependent methods for microbial community analysis have both been used frequently to describe microorganisms from hydrocarbon-contaminated environments. Culture-dependent approach involves isolation of the microorganisms in the polluted environment using nutrient medium and then identifying the microorganisms following laboratory techniques.

Microbial culture is limited to the isolation and identification of limited numbers of microorganisms, therefore, is not enough for the investigation of the whole microbial diversity and the genomes of all microorganisms present in a particular polluted environment (Abbasian *et al*., 2015). Molecular tools and sequencing have been widely used in determining the bacterial community structure and function and also the catabolic capacity which may be related with the attenuation rate (Beller *et al*., 2002).

Recently, metagenomic approaches enable researchers to analyze the whole microbial diversity, and the genetic capacity for the active metabolic pathways present in a given environment. This technique is especially efficient for the study of the biodiversity and genome analysis of complex environmental samples where most of the microorganisms cannot be cultured under normal laboratory conditions. Moreover, this system enables operators to establish a correlation between microbial diversity and the level of hydrocarbon(s) in a contaminated site (Gong *et al*., 2013).

Up to now, several studies have been performed on the microbial communities and metagenomic capacities of soils contaminated with crude oil and its derivatives (An *et al*., 2013). These studies have revealed large variations in the biodiversity and abundances of microorganisms in different geographical locations. Furthermore, big differences were

observed in the genetic capacities of microbial communities identified at each location. Therefore, metagenomic analysis gives excellent opportunities for finding new microbial strains and the genes involved in both bioremediation of hydrocarbon contaminants and improving crude oil quality through refining biological process (An *et al*., 2013).

Although culture-dependent methods generally recover a small portion of the diversity from soil environments, they are still a critical component of bioremediation development and research (Malaviya and Rathore, 2007). In addition to the potential of *in situ* and *ex situ* applications of cultured isolates, microbial isolation allows for *in vitro* assessments of isolate physiology and hydrocarbon degradation pathways and performance, providing a basis for annotating extensive metagenomic datasets, and helping to identify genes and/or organisms that could be useful in land reclamation (Stefani *et al*., 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Description of Okarki Sampling Site

Okarki community in Ahoada Local Government Area of River State, is a border town between Bayelsa and River state. It is located on latitude: 4˚58’56”N and longitude 6˚25’44”N. The sampling site is one of the sites of illegal refineries of crude oil opertated by the youths of the community. The crude oils refined are obtained through pipeline vandalization. Okarki community is composed majorly of farmers who depend on their agricultural produce as means of livelihood.

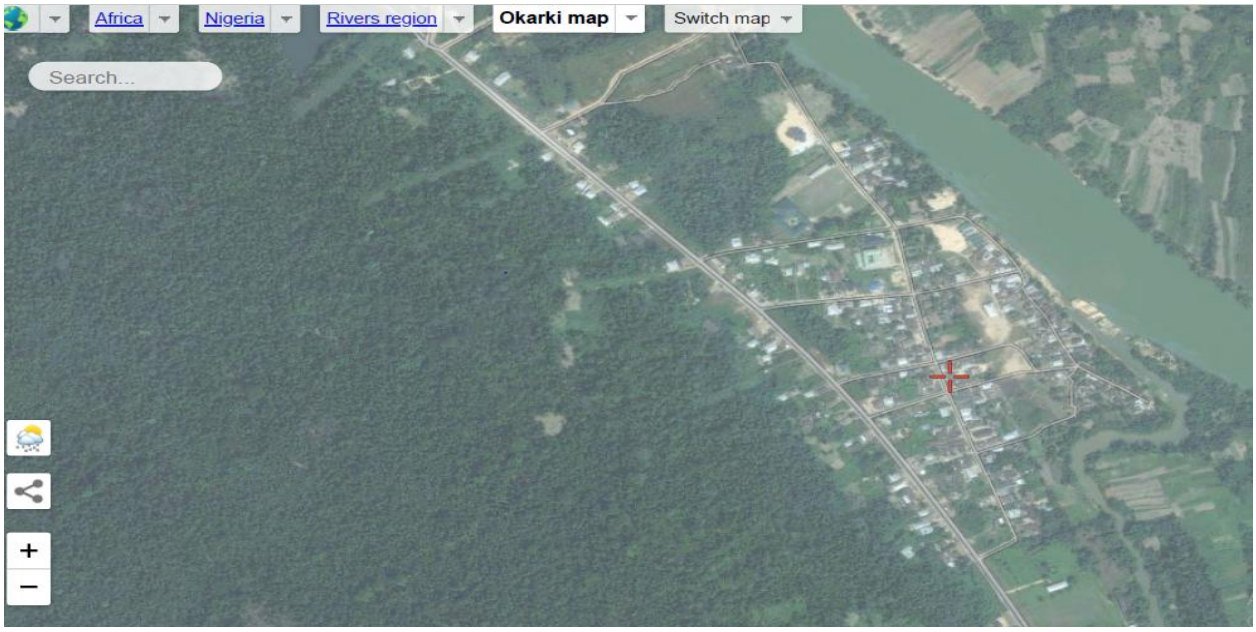


Figure 2: Okarki sampling site using google map

3.2. Sample Collection:

Random soil samples were collected from spent lubricating oil-contaminated sites in five different mechanic workshops in Awka for the isolation of biosurfactant-producing bacteria.

Soil samples contaminated with crude oil were also collected from an illegal oil refinery site located in Okarki, Ahoada Local Government Area, River state, Nigeria, during the rainy season (April 22nd, 2018 precisely). Polluted soil samples were collectedin a sterile sampling bag by random sampling technique at 6cm depth and analyzed in the laboratory. The samples were collected in triplicates. Unpolluted soil samples which served as the control were also collected randomly from a site 100m away from the polluted site.

3.3. Isolation and screening of bacterial organisms for biosurfactant production

Cetrimide agar, a selective medium, was used for the isolation of bacterial species. The m-Cetrimide medium had the following composition: Pancreatic digest of Gelatine, 40.0g; Magnesium Chloride, 2.8g; Potassium sulphate, 20.0g; Cetrimide, 0.6g; Glycerol, 10.0

g; Agar, 15.0 g; Water, 1L; pH, 7.2 ± 0.2 (Atlas, 1995). One gramme of soil sample was suspended in 10ml of sterile distilled water and a ten-fold dilution was carried out. A 0.1ml of 10^{-2} dilution was inoculated onto the selective medium (m-cetrimide medium) and the plates incubated at 30^0C for 24h. Pure cultures were isolated and stored on nutrient agar slants at 4˚C.

3.3.1. Inoculum preparation

A 24h old culture of the isolate was inoculated into 10ml of sterile distilled water in a test tube and standardized using 0.5 Mcfarland standard as described by Patel *et al*. (2015). This served as the seed inoculum. Mcfarland standard was prepared by mixing 0.5ml of 1% BaCl and 9.95ml of 1% H_2SO_4.

3.3.2. Fermentationprocess for biosurfactant production

A fermentation process was carried out according to the method described by Anaukwu *et al.*(2015). A mineral salts medium containing the following components was used: Basal medium (KCl, 1.1 g/L; NaCl, 1.1 g/L; $FeSO_4.7H_2O$, 0.00028 g/L; KH_2PO_4, 3.4 g/L; K_2HPO_4, 4.4 g/L; $MgSO_4.7H_2O$, 0.5 g/L; Yeast extract, 0.5g/L; 2ml of Trace element solution [$ZnSO_4.7H_2O$, 0.29 g/L; $CaCl_2.4H_2O$, 0.24 g/L; $CuSO_4.5H_2O$, 0.25 g/L; $MnSO_4$. $7H_2O$, 0.17 g/L]; $NaNO_3$, 1.5g/L and Glucose, 2% w/v served as nitrogen and carbon sources respectively. The pH of the medium was adjusted to 7.2 with 1N NaOH. Fifty millilitresof the medium in 100ml Erlenmeyer flask were sterilized in an autoclave at 121^0C for 15min, cooled and inoculated with 1ml of the standardized seed inoculum (2.26 × 10^8 cell/ml). The flask was incubated for 72h in an orbital shaker at 150rpm and 30ºC. Uninoculated flask served as control.

3.3.3. Screening for potential biosurfactant-producing organisms

Mineral salts medium was used as the fermentation medium for the screening of biosurfactant-producing bacteria as described by El-Amine (2012).

Fifty millilitre of the fermentation medium in 100ml Erlenmeyer flask were sterilized in an autoclave at 121^0C for 15min, cooled and inoculated with 1ml of the standardized seed inoculum(2.26×10^8 cell/ml). The flask was incubated for 72h in an orbital shaker at 150rpm and 30ºC. The culture broth was centrifuged twice at 4000rev /min for 10min to remove bacterial cells. The cell-free supernatant obtained was used for drop collapse test, oil displacement test and emulsification index measurement.

3.3.3.1. Drop collapse test

The method of Tugrul and Cansunar (2005) was used for the drop collapse test. A drop of the cell-free supernatant was placed on an oil-coated surface in a polystyrene microwell plate. Each well with a diameter of 8mm and 0.03mm depth was coated with 7µl mineral oil and left for 24h at room temperature. A 20µl supernatant was then added to each well using a sterile syringe at an angle of 45^0C. Sterile distilled water was used as control. After one minute, the drops were examined visually for positive or negative result. Drops containing biosurfactant collapsed whereas non-surfactant-containing drops remained stable.

3.3.3.2. Oil displacement test

The oil spreading test measures the diameter of clear zones observed when a drop of a biosurfactant-containing solution is placed on an oil water surface. The method described by Morikawa *et al.*(1993) was adopted.Forty millilitres of distilled water were placed in a large Petri dish, followed by the addition of 15µl of crude oil to the surface of the water. Ten microlitre of the supernatant was slightly placed on the surface of oil film and allowed to stand for 60sec. The diameter of the clear zone on the oil surface was measured using a meter rule.

3.3.3.3. Emulsification index

The method of Ellaiah *et al*. (2002) was used. A mixture of 2ml of the supernatant and 2ml of kerosene was vortically stirred for 2min, and the height of emulsion layer was measured after 24h using a meter rule, to determine the emulsification index. Emulsification index was calculated by measurement of the height of the emulsion layer (a), divided by the total height (b), and then multiplied by 100 (EI = a /b × 100).

3.4. Identification of Selected Biosurfactant Active Producers

The three active biosurfactant producers were subjected to molecular assessment for identification. The isolates were identified by 16S rRNA sequence analysis at Research Resource Center, University of Illinois, Chicago, USA.

Bacterial cells stabilized using Norgen stabilization solution (Norgen Biotek Corp.) were extracted using a Maxwell® 16 Tissue DNA Purification Kit (Promega) with a slight modification. Samples were first incubated with a lytic enzyme mixture (Metapolyzyme) diluted in 1× phosphate buffered saline (PBS) and incubated at 35°C for 2hr, prior to initiation of the extraction protocol implemented on a Maxwell16 device. Genomic DNA extracted from bacterial isolates was PCR amplified using the primer 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT), amplifying nearly the entire bacterial small subunit ribosomal RNA gene (Lane, 1985). PCR amplification was performed using DreamTaq Green PCR Master Mix (2X; ThermoFisher Scientific) using standard PCR conditions. Amplicons were purified and sequenced in two directions using 27F and 1492R primers on an Applied Biosystems 3730XL 96- capillary DNA sequencer. Raw sequence data (.ab1 format) were processed within the software package CLC genomics workbench (v11.0.1) to merge the forward and reverse reads. Merged and quality trimmed reads were analyzed using BLAST analysis as described by Altschul *et al*. (1990) and the software package MEGA7 sas described by Kumar *et al*. (2016).

3.5. Optimization of Fermentation Conditions for Biosurfactant Production

3.5.1. Assessment of renewable waste materials as sole carbon source using one factor at a time method

Six different waste materials (sugar cane molasses, spent lubricating motor oil, pulverized banana peel, pulverized potato peel, pulverized orange peel, and spent lubricating generator oil) were screened for use as sole carbon source by the three isolatesfor the production of biosurfactants. The pulverized banana, potato and orange peel were home-made (Appendix II),the sugar cane molasses was obtained from Dangote Sugar Refinery PLC, Apapa, Lagos, while the spent lubricating motor oil and generator oil were obtained from a mechanic and a generator repair workshops respectively.

Fermentation was carried out in a medium consisting of: Basal medium, $NaNO_3$, 1%(w/v); carbon source, 2% (w/v), pH, 7.2. A 100ml Erlenmeyer flask containing 50ml of the fermentation medium was inoculated with 1ml of the standardized seed inoculum and the flask incubated in an orbital shaker (150rpm) at 30°C. Triplicate flasks were used and uninoculated flask served as control. After 72hr fermentation, biosurfactant activity was determined by measuring the emulsification index. The carbon source that gave the best result was used for further fermentation studies.

3.5.2. Screening for best nitrogen source

Different nitrogen sources (KNO_3, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$) were used in the fermentation medium for the evaluation of most appropriate nitrogen source for the production of biosurfactants by the three isolates. Fermentation was carried out in a medium consisting of: Basal medium, nitrogen source, 1% (w/v); Sugar cane molasses, 2% (w/v); pH, 7.2. A 100ml Erlenmeyer flask containing 50ml of the fermentation medium was inoculated with 1ml of the standardized seed inoculum and the flask incubated in an orbital shaker

(150rpm) at 30°C. Triplicate flasks were used and uninoculated flask served as control. After 72h fermentation, biosurfactant activity was determined based on emulsification index. The nitrogen source that gave the best result was used for other fermentation activities.

3.5.3. Statistical Optimization of Critical Media Components

3.5.3.1. Response surface methodology for enhanced biosurfactant production

Response surface methodology was used for the optimization of fermentation variables as described by Onwosi and Odibo (2013).

Experimental design for nutritional and environmental factors

A 2^4 full factorial Central composite design for four test variables, each at five levels with eight star points and seven replicates at the center points was employed to fit a second order polynomial model. Thirty one experiments were carried out. The maximum biosurfactant activity based on emulsification index was taken as the response of the design experiments. In developing the regression equation, the test variables were coded according to the equation:

$$x_i = \frac{X_i - X_i^*}{\Delta X_i}$$

Where x_i is the coded value of the independent variables, X_i is the uncoded value of the ith independent variable, X_i^* is the uncoded value of the ith independent variable at the centre point and ΔX_i is the step change value. The actual values obtained for the independent variables are shown in Table 3, while the central composite design showing the thirty one experimental runs is shown in Table 4.Minitab software version 17 was used for regression and graphical analysis of the data obtained.

The results obtained were further subjected to Analysis of Variance (ANOVA) to assess the significance of each variable on the biosurfactant production. The extent of

variance that could be explained by the model was determined by the multiple regression coefficient (R^2 value). The experiments were further ran using the optimum conditions given by the response optimizer in order to validate the predicted maximum response.

Table 3: Actual factor values corresponding to the coded factors for fermentation variables

		Actual values				
Variable	Code	-2	-1	0	1	2
Concentration of molasses (g/L)	X1	5	10	15	20	25
Concentration of sodium nitrate(g/L)	X2	5	10	15	20	25
Inoculum size (ml)	X3	1	1.5	2	2.5	3
Medium volume (ml) in 250ml flask	X4	20	30	40	50	60

Table 4: Central composite design runs showing factors and their levels for fermentation variables

Run	Coded value				Actual values			
	X1	X2	X3	X4	CM	CN	IS	MV
1	0	0	0	2	15	15	2.0	60
2	-1	1	-1	1	10	20	1.5	50
3	0	0	0	0	15	15	2.0	40
4	-1	1	-1	-1	10	20	1.5	30
5	1	-1	1	1	20	10	2.5	30
6	1	1	-1	-1	20	20	1.5	30
7	0	-2	0	0	15	5	2.0	40
8	-1	1	-1	-1	10	20	1.5	30
9	0	0	0	0	15	15	2.0	40

10	1	-1	-1	1	20	10	1.5	50
11	0	0	0	0	15	15	2.0	40
12	0	2	0	0	15	25	2.0	40
13	0	0	0	0	15	15	2.0	40
14	0	0	-2	0	15	15	1.0	40
15	-1	-1	1	1	10	10	2.5	50
16	1	1	1	1	20	20	2.5	50
17	0	0	0	0	15	15	2.0	40
18	-1	-1	1	-1	10	10	2.5	30
19	-1	-1	1	1	10	10	2.5	50
20	2	0	0	0	25	15	2.0	40
21	-1	1	1	1	10	20	2.5	50
22	0	0	0	0	15	15	2.0	40
23	1	1	1	-1	20	20	2.5	30
24	1	-1	1	-1	20	10	2.5	30
25	0	0	2	0	15	15	3.0	40
26	-1	-1	-1	-1	10	10	1.5	30
27	1	1	-1	1	20	20	1.5	50
28	0	0	0	0	15	15	2.0	40
29	-2	0	0	0	5	15	2.0	40
30	0	0	0	-2	15	15	2.0	20
31	1	-1	-1	-1	20	10	1.5	20

Key: CM – Concentration of Molasses, CN – Concentration of sodium nitrate, IS – Inoculum size, MV – medium volume.

3.6. Assessment of the Surface Tension Reduction Ability of the Biosurfactant

3.6.1. Surface tension measurement

The surface tension of the cell free culture broth was determined by capillary rise method as described by Adamson (1997). The cell free culture broth was added to 1L of sterile distilled water in increasing concentration (10-80 mg). A capillary tube (0.01cm diameter) was placed inside the solution. Surface tension was measured from height of the water in the capillary tube using the equation: surface tension (γ) = (ρgah)/2. Sodium dodecyl sulphate (SDS) was used as control surfactant.

The concentration at which micelles began to form was represented as the Critical Micelle Concentration (CMC). The CMC value was determined by plotting the surface tension as a function of the biosurfactant concentration.

3.7. Assessment of Emulsification Activityof the Biosurfactant

Bioemulsification activity was assessed by measuring the emulsification index (E24) of cell-free broth with various hydrophobic substrates (kerosene, diesel, crude oil, groundnut oil, palm oil). Emulsification index of the cell-free culture broth was measured as earlier described.

3.8. Detection of Glycolipid-type of Biosurfactant

Anionic biosurfactant, specifically rhamnolipid is detected by this technique as described by Satpute *et al*. (2008). Methylene blue agar which is composed of mineral salts supplemented with glucose, 2%; cetyltrimethylammonium bromide (CTAB), 0.2g; methlyene blue, 0.005g and agar, 20g in 1L, pH 7.2 was used. Wells of 2cm diameter were made on the methylene blue agar plates using sterile cork-borer and0.1ml of the cell-free culture broth was added. The plates were incubated at 25°C for 72 h. A dark blue halo zone around the culture was considered positive for anionic glycolipid biosurfactant production. The diameter of the halo was measured using a meter rule and recorded.

3.9. Determination of Functional Components of the Biosurfactant Using Gas Chromatography-Mass Spectroscopy(GC-MS)

A 1 µl of biosurfactant solution was injected into the Gas Chromatography-Mass Spectroscopy (GC-MS) machine - Agilent Technology 5890 gas chromatograph, with a split detector and Mass Spectrometer Detector (MSD). The GC is usually coupled to a Mass Spectrometer (detector), 5973 Mass Selective Detector (MSD), which records the mass spectrum of the chemical compounds as they elute from the GC and after fragmentation processes by a stream of electrons in the mass spectrum. Helium was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 1 µl, injector temperature 250°C and ion-source temperature 280°C. Total GC running time was 90.67 min. and the total length of

time for running the analysis determined and programmed by the GC-MS analyst. Peaks in the chromatograms produced by these analyses were identified by a combination of references to their mass spectra and the NIST08 mass spectral database as described by ISU (2017).

3.10. Physicochemical Analysis of the Crude Oil-Polluted and Control Soil Samples

Conductivity, total phosphate, total nitrate, total organic carbon, pH, lead, cadmium, chromium, arsenic, mercury andtotal petroleum hydrocarbon content of the polluted and control soil samples were determined by the method of AOAC (1990).

3.10.1. pH

The soil pH was determined using pH meter (Mettler Toled pH meter). Ten grammes of the soil sample was introduced into a 20ml beaker containing 10ml of distilled water. The solution was allowed to stand for 30 min with occasional stirring with a glass rod. The pH meter was first calibrated at pH 7.0 and the electrode inserted into the partly settled suspension and the pH measured.

3.10.2. Conductivity

Ten grammes of the soil sample was suspended in 100ml of distilled water in a beaker. The electrode of the conductivity meter was placed into the solution, and after stabilization, the reading was recorded.

3.10.3. Total nitrate

Ten grammes of the soil sample was suspended in 100ml distilled water in a beaker and allowed to stand for 30min with occasional stirring. The suspension was filtered and 0.5ml of the filtrate mixed with 0.8ml of salicylic acid to form a sample solution. A blank was prepared by adding 0.8ml of salicylic acid to 0.5ml of distilledwater, and allowed to

stand for 20 min. Nineteen millilitres of sodium hydroxide was added to the sample solution and the blank, and their absorbance read in a spectrophotometer (PD303 UV spectrophotometer) at 410nm. Concentration of nitrate in the sample solution was calculated following the equation:

$$\frac{\text{Absorbance of sample} \times \text{concentration of standard}}{\text{Absorbance of standard}}$$

* Concentration of standard = 10mg/ml

3.10.4. Total phosphate

Two grammes of the soil sample in a 250ml beaker was addedto 80ml mixture of HCl and H_2SO_4 (3:1). The beaker was covered with a watch glass and heated for 30min to oxidize the organic matters. The digest was allowed to cool and diluted with 25ml warm distilled water. Whatman No.1 filter paper was used to filter to obtain the filtrate. Four millilitres of ammonium molybdate and 3ml of hydrazine sulphate were added to 20ml of the digest.The blank contained all the reagents without the sample. The mixture was allowed for 10mins and then read on the spectrophotometer (PD303 UV spectrophotometer) at 690nm. Concentration of phosphate was calculated following the equation:

$$\frac{\text{Absorbance of sample} \times \text{concentration of standard}}{\text{Absorbance of standard}}$$

* Concentration of standard = 10mg/ml

3.10.5. Total organic carbon (TOC)

Walkley-Black chromic acid wet oxidation methodwas used to determine total organic carbon. One gramme of soil sample was introduced into 250ml conical flask. Ten millilitres of 1N potassium dichromate was added, and the flask was rocked gently to

disperse the soil sample in the suspension. Twenty millilitres of 98% sulphuric acid was added and the flask rocked to mix for even mixture of the soil and the solution over a hot plate until a temperature of 135°C was reached. The blank was also prepared in the same way as the sample (all the reagents without the sample). Afterwards, the mixture was cooled and diluted to 200ml with distilled water. The mixture was further titrated with 0.4N ferrous sulphate using 3 drops of ferroin as indicator. The solution turned greenish and then reddish-grey. TOC was calculated following the equation: TOC (%) = 3(1-T/S)/w

Where;

T = Volume of FeSO4 used in sample titration (ml)

S = Volume of FeSO4 used in blank titration (ml)

W = Oven-dry sample weight (g)

3.10.6. Heavy metal analysis

Heavy metal analysis was conducted using FS240AA agilent Atomic Absorption Spectrophotometer. Two grammes of the soil sample was heated in a furnace at 550°C for 2h and diluted with 20ml of 20% H_2SO_4. Whatman filter paper was used to filter the suspension. The filterate was placed into the atomic absorption spectroscopy for measurement of cadmium, lead, chromium, arsenic and mercury content. Standard metal solutions of the metals of interest in the optimum concentration range were also prepared. A calibration blank was prepared using all the reagents except for the sample. Calibration curve for each metal was prepared by plotting the absorbance of standards versus their concentrations.

3.10.7. Total petroleum hydrocarbon content

Gas chromatography was carried out to determine the total petroleum hydrocarbon content of the soil. Ten grammes of the homogenized soil sample was mixed with 60g of

anhydrous sodium sulphate in an agate mortar to absorb moisture. The homogenate was placed into a 500ml beaker containing 300ml n-hexane and left for 24h. Crude extract obtained was evaporated to dryness in a rotary vacuum evaporator. Residue obtained was transferred onto a 5ml florisil column for cleanup. Eluate collected after florisil cleanup was evaporated to dryness and dissolved in 1ml n-hexane for chromatographic analysis.Buck 530 gas chromatograph equipped with an on-column, automatic injector, mass spectroscopy(HP 88 capillary column CA, USA) was used. All the parameters (gas flow to the columns, the inlets, the detectors, the injector temperature - 22°C, detector temperatures - 250°C and the split ration) were set to the correct values before the analysis was ran.

3.10.8. Dehydrogenase enzyme activity

Dehydrogenase enzyme activity was monitored following the method of Nwankwegu *et al*. (2016). Dehydrogenase activity (Dhase) which was used as direct determination of microbial activity in the soil, was estimated by the rate of formation of triphenyl formazan (TPF) from triphenyl tetrazolium chloride (TTC). One gramme of the soil sample collected from the treatments was added to a test tube containing 2 ml TTC-glucose solution and 2.5 ml Tris buffer, and then incubated at 30˚C for 96 hr under diffused light. The control contained 2.5 ml of Tris buffer without TTC. After 96 hr, TPF was extracted by addition of acetone (1:1 v/v) and further incubated for 2 hr in the dark. The optical density of the soil filtrate was measured at 485nm, and the result expressed as mgg^{-1} dry soil/96 hr. Concentration of extracted TPF = (Concentration of standard × Absorbance of TPF)/ Absorbance of standard

* Concentration of standard used (Triphenyl formazan) = 2.5 mg/g

3.11. Microbiological Analysis of the Crude oil-polluted and ControlSoil Samples

3.11.1. Enumeration and isolation of indigenous microorganisms

Indigenous microorganisms in the polluted and unpolluted soil samples were enumerated and isolated using standard pour plate method. Ten-fold serial dilution of 1g of the composite soil sample suspension was used to determine the heterotrophic bacterial and fungal count. Nutrient agar and Sabouraud Dextrose agar were used as the culture media for bacterial count and fungal count respectively. One millilitre of 10^{-2} and 10^{-4} dilution (for polluted and unpolluted soil samples respectively) were inoculated onto the nutrient agar plate and sabouraud dextrose agar (SDA) plate, incubated at 30^{0}C for 24h and 72h forbacteria and fungi respectively. Developed colonies on nutrient agar plate and SDA plate were counted and recorded as heterotrophic bacterial count and fungal count respectively. Pure cultures obtained upon subculture of the isolates were stored on nutrient agar slant for bacterial isolates and SDA slant for fungal isolates.

3.11.2. Biochemical tests for identification of the isolates from the crude oil polluted and control soil samples

Several biochemical tests were carried out to identify the bacterial and fungal isolates from the polluted and unpolluted soil samples. They include gram reaction, catalase test, oxidase test, coagulase test, motility test, indole test, methyl red test,Voges-Proskauer test, citrate utilization test, sugar fermentation, fungi wet mount and microscopic examination.

3.11.2.1. Gram Staining

The method described by Cheesbrough (2000) was used. A smear of the isolate on a clean slide was air-dried and heat fixed. The smear was flooded with crystal violet solution and rinsed off after 60 sec with water. Subsequently Lugol's iodine solution and acetone-alcohol were added to the smear and rinsed off after 60 sec and 5 sec respectively. The

smearwas then counterstained with Safranin and rinsed off after 30 sec. The air-dried slide was examined under the microscope using x100oil immersion objective lens. Purple colour of the isolate indicates Gram positive while red colour indicates Gram negative.

3.11.2.2. MotilityTest

The method described by Cheesbrough (2000) was used. Sterile and solidified single strength Nutirent agar medium in a test tube was inoculated with 24hr old culture of the test organisms by stabbing straight into the medium to about half the depth of the medium. The inoculated mediumwas incubated at 37°C for 18-24 hours and thereafter examined. Non-motile organisms will grow only along the line of stab while motile organisms will spread away from the line of stab.

3.11.2.3. Citrate utilization Test

The method described by Cheesbrough (2000) was used. A 24h old culture of the isolate was inoculated into 10 ml Simmon's citrate agar prepared in a test tube and solidified in slanted position, incubated at room temperature for 24 h. Positive results were shown by a change in colour from green to blue.

3.11.2.4. IndoleTest

The method described by Cheesbrough (2000) was used. This test is based on the ability of some organisms to breakdown the amino acid, tryptophan with the enzyme tryptophanase into indole. A loopful of 24 h old culture of the isolate was inoculated into 5ml sterile tryptophan medium and then incubated for 2 days at 37°C. A 0.5ml of Kovac's reagent (para-methylaminobenzylehyde) was added and allowed to stand for 20 min. Formation of red colour ring at the reagent layer indicated indole positive reaction.

3.11.2.5. Coagulase Test

The method described by Cheesbrough (2000) was used. Coagulase test depends on the ability of some bacteria to produce an extra-cellular enzyme called coagulase which coagulate blood plasma. A thick saline suspension was prepared on a clean slide with a 24h old culture of the isolate. A loopful of human plasma was mixed with the suspension, coagulation of cells within five (5) seconds showed positive result.

3.11.2.6. Catalase Test

The method described by Cheesbrough (2000) was used. A drop of water was placed on a clean slide, a loopful of the test organism was picked and dissolved with the water. Drops of hydrogen peroxide were added to the solution and observed for effervescence (production of gas). Presence of effervescence indicated positive result.

3.11.2.7. Oxidase Test

The method described by Cheesbrough (2000) was used. A freshly prepared 1% N-Tetramethy-p-phenylenediaminehydrochloride (oxidase reagent) was used to soak a sterile filter paper. A 24 hours old culture was streaked on the sterile filter paper. A color change to deep purple or blue indicated a positive reaction.

3.11.2.8. Methyl Red (MR) Test

The method described by Cheesbrough (2000) was used. A loopful of 24h old culture of the isolate was inoculated into 10ml sterile peptone water and incubated for 5 days at 37^{o}C. After incubation, five drops of methyl red solution was added to the culture, shaken and allowed to stand for few minutes. Development of re colour indicates a positive Methyl Red reaction while yellow colour development shows a negative reaction.

3.11.2.9. Voges-Proskauer (VP) Test

The method described by Cheesbrough (2000) was used. A loopful of 24h old culture of the isolate was inoculated into 10ml sterile peptone water and incubated for 5 days at 37^{o}C. After incubation, I ml of napthol in absolute ethanol and 1ml of 40% KOH were added to the culture and allowed to stand for 15 min. Red colouration indicatespositive Vogues Proskauer result.

3.11.2.10. Sugar Fermentation Test

The method described by Cheesbrough (2000) was used. The different sugars used were glucose, maltose, sucrose, lactose and rhamnose. A loopful of 24h old culture was inoculated into 10ml sterile peptone water supplemented with the sugars in test tubes containing inverted Durham tubes, and incubated at 37^{o}C for 3 days. Un-inoculated tubes served as control. The tubes were examined after incubation for gas and acid production. A colour change from red to yellow indicates acid production while gas was detected by the displacement on the Durham tubes.

3.11.2.11. Fungi Wet Mount

The method described by Atlas (1995) was used.This was done by placing a drop of lactophenol cotton blue stain on a clean slide. A small piece of mycelium free of medium was picked with the aid of a sterile inoculating needle and placed into the stainand gently teased to avoid distorting of the mycelium with the inoculating needle. A cover slip was placed with care to avoid air bubble on the preparation and then viewed under the microscope at x 40 objective lens.

3.11.3. Metagenomic Analysis of the Crude oil-polluted and Control Soil Samples

Metagenomic analysis was carried out on the crude oil-polluted and control soil samples at Research Resource Center, University of Illinois, Chicago, United States.

3.11.3.1. Genomic DNA extraction

Genomic DNA was extracted from the soil samples with QIAamp PowerSoil DNA kit (Qiagen) and processed on a QIAcube automated nucleic acid extraction system (Qiagen), according to the manufacturer's instructions. Samples were incubated for 10 min at 65°C before being homogenized with FastPrep-24 5G bead-beating device (MP Biomedicals) at 6 m/s for 40 sec.

3.11.3.2. Microbial community characterization using deep sequencing of 16S rRNA gene amplicons

Genomic DNA extracted from soil was PCR amplified with primers CS1-515Fb and CS2-806Rb (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal RNA genes. Amplicons were generated using a two-stage "targeted amplicon sequencing (TAS)" protocol as described by Naqib *et al*. (2018) and Bybee *et al*. (2011). The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) (Green *et al*., 2015; Moonsamy *et al*., 2013). First stage PCR amplifications were performed in 10 microlitre reactions in 96-well plates, using the MyTaq HS 2X mastermix. PCR conditions were 95°C for 5 min, followed by 28 cycles of 95°C for 30min, 55°C for 45min and 72°C for 60min.

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-well plates. A mastermix for the entire plate was made using the MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). These Access Array primers contained the CS1 and CS2 linkers at the 3' ends of the oligonucleotides. Cycling conditions were as follows: 95°C for 5

min, followed by 8 cycles of 95°C for 30min, 60°C for 30min and 72°C for 30min. A final, 7 min elongation step was performed at 72°C. Samples were pooled in equal volume using an EpMotion 5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq mid-output flow cell (2×153 paired-end reads). Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Sequencing Core (UICSQC).

3.11.3.3. Bioinformatic analysis of amplicon sequence data

Forward and reverse reads were merged using the software package PEAR as described by Zhang *et al*. (2014). Merged reads were trimmed to remove ambiguous nucleotides, primer sequences, and trimmed based on quality scores. Chimeric sequences were identified and removed using the USEARCH algorithm with a comparison to GreenGenes 13- 8 reference sequence database (McDonald *et al*., 2012; Edgar, 2010). The standard QIIME pipeline was modified to generate taxonomic summaries using sub-Operational Taxonomic Unit (OTU) resolution of the sequence dataset (Tikhonov *et al*., 2015; Caporaso *et al*., 2010). Briefly, the resulting sequence files were then merged with sample information. All sequences were then de-replicated to produce a list of unique sequences. All sequences that had an abundance of at least 10 counts were designated seed sequences. USEARCH was then used to find the nearest seed sequence for any non-seed sequence with a minimum identity threshold of 98%. For any non-seed sequence that matched a seed sequence, its counts were merged with the seed sequence counts. For any

non-seed sequence that did not match a seed sequence it would remain an independent sequence.

Taxonomic annotations for seed and unmatched non-seed sequences were assigned using the USEARCH and GreenGenes 13-8 reference with a minimum similarity threshold of 90% (Edgar, 2010). In order to improve depth of annotation, the standard QIIME assignment algorithm was modified to only consider hits at each taxonomic level that had an assigned name. Taxonomic annotations and sequence abundance data were then merged into a single sequence chart to give summary of absoluteabundances of taxa for all phyla, classes, orders, families and genera.

3.12. Determination of Crude oil Degradation Potential of the Indigenous Isolates on Shake Flask

The method of Latha and Kalaivani (2012), was used to assess the crude oil degrading potential of the isolated indigenous microorganisms. One millilitre of seed inoculum of the indigenous isolates was inoculated into 50ml of Bushnell-Haas broth supplemented with 5% w/v crude oil in 250ml Erlenmeyer flask. The flask was incubated at 30°C for 7 days in a rotary shaker at 150rpm. An uninoculated medium served as control. After incubation, residual crude oil was extracted and crude oil degradation rate was estimated gravimetrically.

To the culture flask,5ml of n-hexane were added and the contents were transferred to a separating funnel to extract the residual crude oil. Extraction was carried out twice to ensure complete recovery of oil. The extract was treated with 0.4g of anhydrous sodium sulphate to remove the moisture and decanted into a pre-weighed beaker leaving behind sodium sulphate. This was evaporated to dryness by heating in a water bath.

The weight of extracted oil was deducted from the previously weighed beaker.

The % degradation of the crude oil was calculated as:

Weight of residual crude oil= Weight of beaker containing extracted crude oil – Weight of empty beaker.

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil

% degradation = (Amount of crude oil degraded / Amount of crude oil added in the media) x 100

3.13. Comparative Bioremediation of the Crude Oil-polluted Soil Sample

Bioremediation by natural attenuation, biostimulation and bioaugmentation techniques was carried out over a 12week period following the method described by Bento *et al*. (2005). During this period, microbial load, pH and dehydrogenase assay were monitored at 2 weeks interval, while total petroleum hydrocarbon was measured at 4 weeks interval. The crude oil-polluted soil (400 g)sterilized in an autoclave at 121˚C for 15 min served as the positive control soil for the bioremediation experiment.

Four hundred grammes of the crude oil-contaminated soil was placed into eight (8) different plastic pans (Appendix xxii) and kept at 25˚C. Ona weekly interval, the soils were moistened by the addition of 20ml sterile water and evenly mixed to provide sufficient air and oxygen until the end of the experiment.

3.13.1. Natural attenuation*:* The soil's natural ability to degrade the crude oil was studied. The crude oil-polluted soil sample was left for a period of 12weeks and the microbial load, pH and dehydrogenase enzyme activity were determined at 2 weeks interval. TPH was measured at 4 weeks interval.

3.13.2. Bioaugmentation*:* The biosurfactant-producing bacterial isolates served as the allochthonous organisms. A 5% v/v developed inoculaand 5% nutrient(2.5% nitrate: sodium

nitrate and 2.5% phosphate: ammonium phosphate**)** were added to the soil samples. The inocula were standardized using 0.5M Mcfarland standard.

Four set ups were prepared (Table 5), a total of 10% additives were introduced into each set-up.

Set-up A – C were each treated with 20ml of the three active biosurfactant producers plus nutrient(10g/L sodium nitrate and 10g/Lammonium phosphate**).**

Set-up D was treated with the bacterial consortium (6.7ml each of the three biosurfactant producers) plus nutrient(10g/L sodium nitrate and 10g/L ammonium phosphate**)**.

3.13.3. Biostimulation*:*Nutrients were added to improve the natural degradation rate. Three set-ups were prepared (A – C) [Table 5], a total of 10% additives were introduced into each set-up.

In set-up A, the soil sample was treated with 10% v/v (40ml) of nutrient only.

In set-up B, the soil sample was treated with 5% v/v (20ml) of the produced crude biosurfactant and 5% nutrient

In set-up C, the soil sample was treated with 10 % of the produced biosurfactant only.

* Microbial load was monitored by determining the heterotrophic bacterial count and fungal count as described earlier.

* pH of the soil was monitored using a pH meter.

* Dehydrogenase enzyme activity was monitored as earlier described.

* Residual TPH and heavy metals were determined using gas chromatography and atomic absorption spectrometry respectively.

3.14. Measurement of Rate of Crude Oil and Heavy Metals Removal from the Polluted Soil

Crude oil degradation rate and heavy metal removal rate were calculated by subtracting the weight of residual crude oil/heavy metal from weight of the initial crude

oil/heavy metalcontent before remediation, divided by the weight of the initial crude oil/heavy metal and then multiplied by 100 as described by Yakubu (2007).

Table 5: Bioremediation study design

No.	Experiment	Description
1	Control (Co)	Sterile polluted soil with no form of amendment
2	Natural attenuation (Nat. att.)	No form of amendment
3.	Bioaugmentation A (Bioaug. A)	5% Nutrient + 5% *P.aeruginosa* strain CCUG
4.	Bioaugmentation B (Bioaug. B)	5% Nutrient + 5% *P.aeruginosa* strain I3
5.	Bioaugmentation C (Bioaug. C)	5% Nutrient + 5% *P.aeruginosa* strain ST11
6.	Bioaugmentation D (Bioaug.D)	5% Nutrient + 5% bacterial consortium
7.	Biostimulation A (Biostim. A)	10% Nutrient only
8.	Biostimulation B (Biostim. B)	5% Produced biosurfactant + 5 % nutrient
9.	Biostimulation C (Biostim.C)	10% Produced biosurfactant only

3.15 Statistical analysis: Data obtained was subjected to Pearson correlation analysis and one way Analysis of Variance by Student-Newman-Keul (SNK) test at 95% confidence level. IBM SPSS statistics version 20 was used for the correlation and ANOVA.

CHAPTER 4

4.0.RESULTS

4.1. Isolation and Screening results of Bacterial Organisms for Biosurfactant Production

The total number of bacterial isolates from five different mechanic workshops in Awka used for biosurfactant production is shown in Table 6. Bacterial isolates from different sites (A – E) were screened for biosurfactant productionand varying levels of biosurfactants were produced (Table 7).The Emulsification index obtained ranged from 0 – 93.3%, oil displacement ranged from 0 – 2.1 cm, 82.4% were positive for drop collapse test while 17.6% were negative.As shown in Table 7, isolates 1a, 7c and 10c were the best producers of biosurfactants.

4.2. Selected Biosurfactant Active Producers

Three best biosurfactant producers (Isolates 1a, 7c and 10c) were selected and identified based on I6S rRNA sequencing as *Pseudomonasaeruginosa* strain CCUG, *Pseudomonasaeruginosa* strain I3, *Pseudomonasaeruginosa* strain ST11. The phylogenetic tree showing the evolutionary relatedness of the isolates is shown in Figure 3.

Table 6: Number of bacteria isolated from different mechanic workshops in Awka

Sample site	No. of bacteria isolated
A	3
B	1
C	7
D	2
E	4
Total	17

Table 7: Biosurfactant-producing potentials of the isolates

Isolate	E24(%)	Oil displacement (cm)	Drop collapse
1a	93.30	1.80	++
2a	0.00	0.70	++
3a	20.00	0.00	-
4b	16.67	0.30	++
5c	23.30	1.50	+
6c	53.30	1.40	++
7c	77.10	1.70	++
8c	0.00	2.00	++
9c	28.60	0.00	-
10c	77.10	2.10	+++
11c	66.67	0.80	+
12d	0.00	0.60	+
13d	6.67	0.50	++
14e	43.33	2.00	+++
15e	26.67	0.40	+
16e	0.00	0.30	-
17e	63.33	0.00	++

Key: + = positive, - = negative, E24 = Emulsification index at 24hr

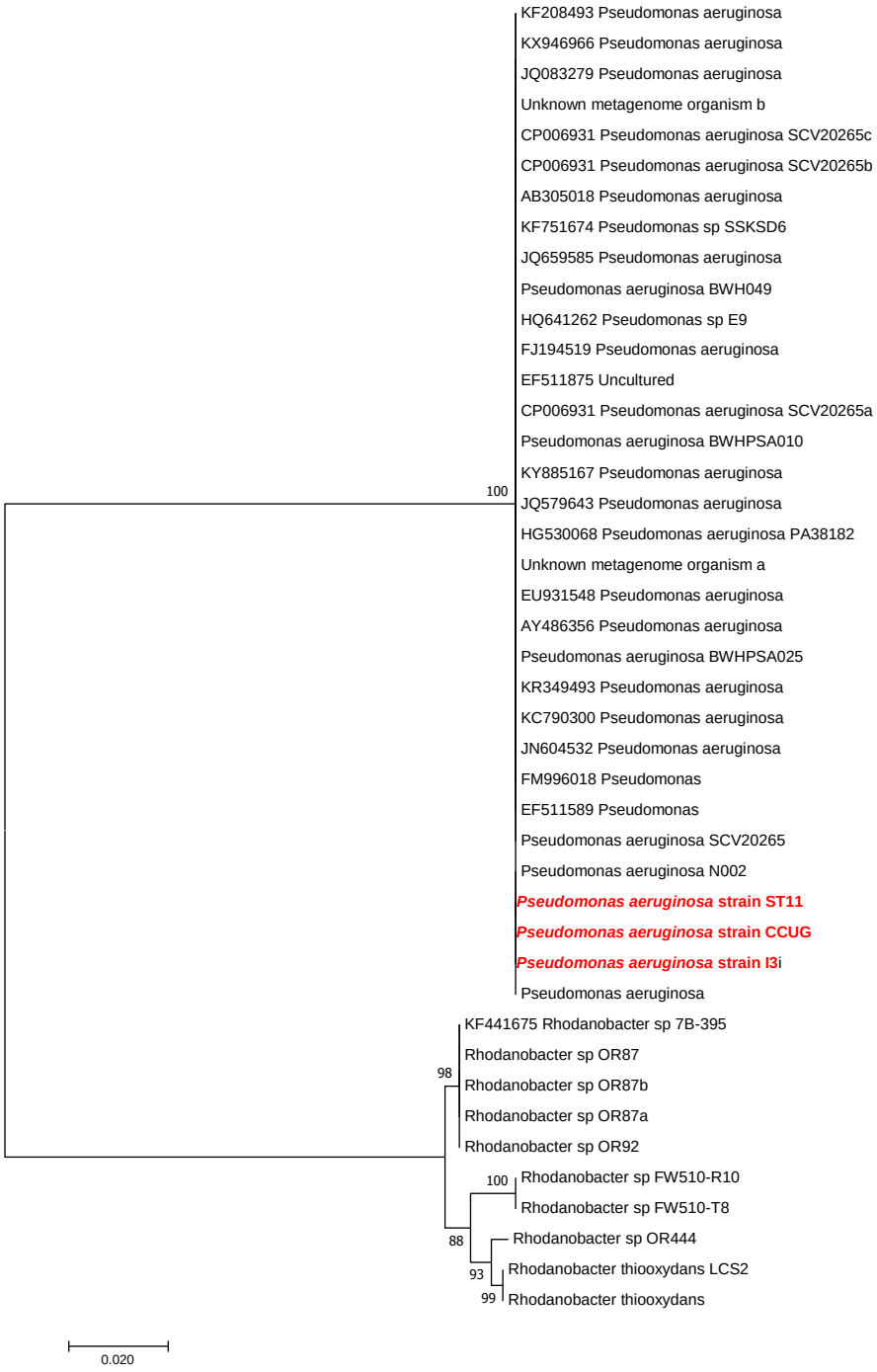


Figure 3: Evolutionary relationship of the isolated *Pseudomonasaeruginosa* strains using the Neighbour-joining method.

4.3. Optimization of Fermentation Conditions for Biosurfactant Production

4.3.1. Results of the screening of renewable waste materials as sole carbon source using "one factor at a time" method

The result of the screening for best renewable waste material to be used as carbon source for biosurfactant production is presented in Figure 4. The result shows that sugar cane molasses gave the best result with *Pseudomonas aeruginosa* strain CCUG, strain I3 and strain ST11, having biosurfactant activity (E24) of 77.6%, 75.3% and 89.4% respectively. Order of performance of the renewable wastes for the isolates are: *Pseudomonas aeruginosa*strain CCUG – sugar cane molasses > waste lubricating oil (motor) > banana peel > potato peel > orange peel > waste lubricating oil (generator), *Pseudomonas aeruginosa*strain I3 – sugar cane molasses > waste lubricating oil (motor) > banana peel > potato peel > waste lubricating oil(generator), *Pseudomonas aeruginosa*strain ST11 – sugar cane molasses > banana peel > waste lubricating oil(generator) > waste lubricating oil (motor) > potato peel. There were significant differences in the performances of the various wastes with the *Pseudomomas aeruginosa* strains (p-values < 0.05) [Appendices iii - v].

4.3.2. Screening results for best nitrogen source

The result of the screening for best nitrogen source for biosurfactant production is presented in Figure 5. The result shows that sodium nitrate was the best nitrogen source of choice by the three *Pseudomonas aeruginosa* strains for biosurfactant production. The nitrogen sources had the same order of performance with *Pseudomonas aeruginosa* strain CCUG and strain ST11 – sodium nitrate > potassium nitrate > urea > ammonium sulphate, while the order of performance with *Pseudomonas aeruginosa* strain I3 is sodium nitrate >

potassium nitrate > ammonium sulphate > urea. The differences in the performances of the nitrogen sources were significant (p-value < 0.05) [Appendices vi – viii].

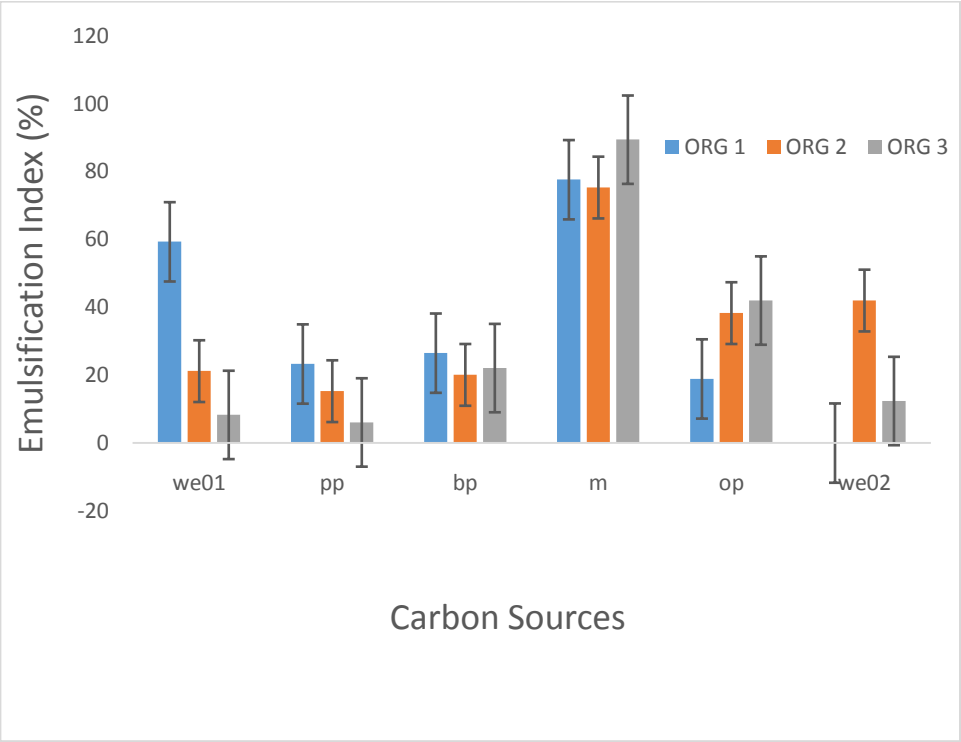


Figure 4: Screening of carbon sources for biosurfactant production

Key: weo1 = waste-lubricating oil (motor)

pp = potato peel

bp = banana peel

m = sugarcane molasses

op = orange peel

weo2 = waste-lubricating oil (generator)

Org 1 = *Pseudomonas aeruginosa* strain CCUG

Org 2 = *Pseudomonas aeruginosa* strain I3

Org 3 = *Pseudomonas aeruginosa* strain ST11

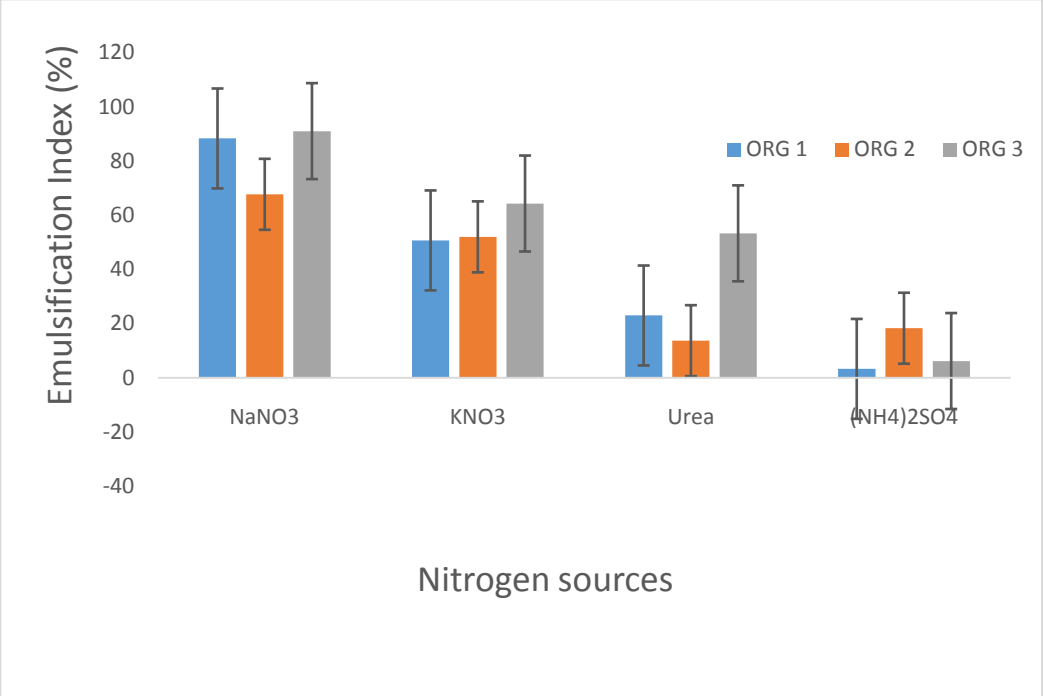


Figure 5: Screening of nitrogen sources for biosurfactant production

Key: Org 1 = *Pseudomonas aeruginosa* strain CCUG

Org 2 = *Pseudomonas aeruginosa* strain I3

Org 3 = *Pseudomonas aeruginosa* strain ST11

4.3.3. Experimental design matrices and actual responses obtained

Table 8 shows the experimental design matrix for optimization of biosurfactant production by *Pseudomonas aeruginosa* strains according to central composite design (CCD) and the actual responses obtained. The response was based on emulsification index after 24hr (E24). Out of the thirty one experiments carried out, the highest responses obtained by *Pseudomonas aeruginosa* strain CCUG, I3 and ST11 were 95%, 93.8% and 93.8% respectively, while the least responses observed were 51%, 13.3% and 13.7% respectively.

Table 9shows the estimates of the parameters in the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain CCUG. Out of the four variables considered, X_1 and X_4 had significant effect on the biosurfactant production by the isolate (p-value < 0.05), while X_2 and X_3 showed no significant effect. Only the quadratic term involving X_3 and the interactions between X1 and X4, and X1 and X_2 showed significant effect on biosurfactant production.

The ANOVA (Table 10), shows that the P-value for regression model, linear, quadratic term and interaction were all less than 0.05. However,the lack-of-fit test was not significant (p-value > 0.05).Table 11 shows the estimates of the parameters in the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain I3. From the result, X_2 and X_4 did not show any significant effect on the biosurfactant production by the isolate (P-value > 0.05), while X_1 and X_3 significantly affected biosurfactant production. All interactions with X_1 and the interaction between X_2 and X_4 showed significant effect on biosurfactant production by the isolate (P-value < 0.05).

Table 8: Experimental design matrix for optimization of biosurfactant production by the *Pseudomonas aeruginosa* strains according to central composite design (CCD) and the actual responses obtained.

Run	Coded values				Responses (E24 in %)		
	X1	X2	X3	X4	Y1	Y2	Y3
1	0	0	0	2	79.4	47.3	60.0
2	-1	1	-1	1	58.0	22.1	50.0
3	0	0	0	0	73.2	46.2	69.1
4	-1	1	-1	-1	81.8	55.0	50.0
5	1	-1	1	1	85.6	52.1	50.0
6	1	1	-1	-1	69.3	75.6	57.1
7	0	-2	0	0	80.0	13.3	33.3
8	-1	1	-1	-1	73.0	41.6	33.0
9	0	0	0	0	86.7	63.3	70.0
10	1	-1	-1	1	88.3	54.7	51.7
11	0	0	0	0	77.0	45.0	70.0
12	0	2	0	0	78.8	35.5	33.3
13	0	0	0	0	71.0	45.0	70.0
14	0	0	-2	0	51.0	60.0	45.0
15	-1	-1	1	1	55.3	50.3	13.7
16	1	1	1	1	78.4	44.3	45.0
17	0	0	0	0	73.2	45.3	76.8
18	-1	-1	1	-1	55.0	47.1	37.8
19	-1	-1	1	1	70.0	40.0	20.0
20	2	0	0	0	93.8	43.3	50.0
21	-1	1	1	1	52.8	20.5	50.0
22	0	0	0	0	72.0	45.2	93.8
23	1	1	1	-1	60.0	33.3	40.0
24	1	-1	1	-1	60.0	35.0	60.0
25	0	0	2	0	60.0	47.3	46.2
26	-1	-1	-1	-1	53.0	50.0	20.0
27	1	1	-1	1	95.0	93.8	60.0
28	0	0	0	0	71.0	45.1	93.1
29	-2	0	0	0	55.0	25.0	30.0
30	0	0	0	-2	65.2	50.2	41.8
31	1	-1	-1	-1	59.3	39.0	50.0

Key: X_1 = Concentration of Sugarcane molasses (g/L), X_2 = Concentration of $NaNO_3$ (g/L), X_3 = Inoculum size (ml), X_4 = Medium volume (ml), Y_1 = Response for *P.aeruginosa* strain CCUG, Y_2 = Response for *P.aeruginosa* strain I3, Y_3 = Response for *P.aeruginosa* strain ST11.

Table 9: Parameter estimates of the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain CCUG

Model Term	Parameter Effect	Coefficient	SD	T-value	P-value
Constant		74.87	2.60	28.77	0.000
X_1	7.275	3.638	0.703	5.18	0.000
X_2	1.642	0.821	0.703	1.17	0.260
X_3	-1.475	-0.737	0.703	-1.05	0.310
X_4	4.183	2.092	0.703	2.98	0.009
X_1*X_1	-0.230	-0.115	0.322	-0.36	0.726
X_2*X_2	0.395	0.198	0.322	0.61	0.548
X_3*X_3	-2.555	-1.277	0.322	-3.97	0.001
X_4*X_4	-0.492	-0.246	0.322	-0.76	0.456
X_1*X_2	-0.712	-0.356	0.430	-0.83	0.420
X_1*X_3	-0.037	-0.019	0.430	-0.04	0.966
X_1*X_4	3.919	1.959	0.430	4.55	0.000
X_2*X_3	-0.787	-0.394	0.430	-0.92	0.374
X_2*X_4	-2.244	-1.122	0.430	-2.61	0.019
X_3*X_4	-0.744	-0.372	0.430	-0.86	0.400

Regression Equation : Y1 (E24) = $74.87 + 7.28\ X_1 + 1.64\ X_2 - 1.47\ X_3 + 4.18\ X_4 - 0.46\ X_1^2 + 0.79\ X_2^2 - 5.11\ X_3^2 - 0.98\ X_4^2 - 1.42\ X_1X_2 - 0.07\ X_1X_3 + 7.84\ X_1X_4 - 1.57\ X_2X_3 - 4.49\ X_2X_4 - 1.49\ X_3X_4$

Table 10: Analysis of variance(ANOVA) for the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain CCUG

Source	DF	Adj SS	Adj MS	F	P
Regression	14	4027.31	287.66	6.07	0.000
Linear	4	1807.12	451.78	9.53	0.000
Square	4	807.49	201.87	4.26	0.016
Interaction	6	1412.70	235.45	4.97	0.005
Error	16	758.46	47.40		
Lack of fit	10	570.21	57.02	1.82	0.240
Pure error	6	188.25	31.38		
Total	30	4785.77			

Key: DF = Degree of freedom, SS = Sum of squares, MS = Mean square, P-value ≤ 0.05 is significant at 95% confidence level

Model Summary: S = 6.885005, R^2 = 84.15%, R^2(adj) = 70.28%, R^2(pred)= 26.02%

Table 11: Parameter estimates of the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain I3

Model Term	Parameter Effect		Coefficient	SD	T-value	P-value
Constant			47.87	3.21	14.91	0.000
x_1	11.48	5.74	1.73	3.31	0.004	
x_2	5.20	2.60	1.73	1.50	0.153	
x_3	-10.95	-5.48	1.73	-3.16	0.006	
x_4	-0.38	-0.19	1.73	-0.11	0.913	
x_1*x_1	-4.56	-2.28	1.59	-1.43	0.171	
x_2*x_2	-9.43	-4.72	1.59	-2.97	0.009	
x_3*x_3	5.19	2.60	1.59	1.63	0.122	
x_4*x_4	2.74	1.37	1.59	0.86	0.401	
x_1*x_2	14.30	7.15	2.12	3.37	0.004	
x_1*x_3	-11.35	-5.67	2.12	-2.67	0.017	
x_1*x_4	15.35	7.68	2.12	3.61	0.002	
x_2*x_3	-13.45	-6.73	2.12	-3.17	0.006	
x_2*x_4	-6.35	-3.18	2.12	-1.49	0.154	
x_3*x_4	2.40	1.20	2.12	0.56	0.580	

Regression Equation : $Y2(E24) = 47.87 + 5.74\ X_1 + 2.60\ X_2 - 5.48\ X_3 - 0.19\ X_4 - 2.28\ X_1^2 - 4.72\ X_2^2 + 2.60\ X_3^2 + 1.37\ X_4^2 + 7.15\ X_1X_2 - 5.67\ X_1X_3 + 7.68\ X_1X_4 - 6.73\ X_2X_3 - 3.18\ X_2X_4 + 1.20\ X_3X_4$

The analysis of variance (Table 12), shows that the regression model, linear term, quadratic term and interaction were all significant at P-value < 0.05, while the lack of fit test was not significant.

The estimates of the parameters in the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain ST11 are presented in Table 13. Only X1 out of the four variables showed significant effect on biosurfactant production by the isolate. All the quadratic term of the variables had significant effect, while all the interactions did not show significant effect on the production. Table 14 shows that only the interaction and lack of fit test were not significant.

Figures 6, 7 and 8 show the probability plot of the actual response obtained from the experiments and the predicted values at 95% confidence level for *Pseudomonasaeruginosa* strain CCUG, I3 and ST11 respectively.

The optimum conditions of the process variables for production of biosurfactants by the *Pseudomonasaeruginosa* strains obtained by the regression model are shown in Table 15 (coded values shown in Appendix ix). The actual responses obtained upon using the optimum conditions are also shown (Table 15).

4.4. Surface Tension Reduction Ability of the Biosurfactants

The results of the surface tension measurement of the biosurfactants produced by *Pseudomonas aeruginosa* strains are presented in Figure 9. The biosurfactants produced by the *P.aeruginosa* strains were able to reduce the surface tension of distilled water significantly (P < 0.05) from 72 mN/m to 45 mN/m, 55 mN/m and 42.3 mN/mfor *P. aeruginosa* strain CCUG, *P. aeruginosa* strain I3 and *P. aeruginosa* strain ST11 respectively. *P. aeruginosa* strain CCUG and *Pseudomonas aeruginosa* strain ST11 attained micelle formation concentration at 60 mg/ml, while*P. aeruginosa* strain I3 reached micelle formation concentration at 50 mg/L. The control (sodium dodecyl sulphate) reduced surface tension to 33.3 mN/m.

Table 12: Analysis of variance(ANOVA) for the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain I3

Source	DF	Adj SS	Adj MS	F	P
Regression	14	5987.51	427.679	5.92	0.001
Linear	4	1673.74	418.435	5.79	0.004
Square	4	1130.09	282.523	3.91	0.021
Interaction	6	3183.68	530.613	7.35	0.001
Error	16	1155.33	72.208		
Lack of fit	10	876.58	87.658	1.89	0.226
Pure error	6	287.75	46.459		
Total	30	7142.84			

Key: DF = Degree of freedom, SS = Sum of squares, MS = Mean square, P-value ≤ 0.05 is significant at 95% confidence level

Model Summary: S = 8.49754, R^2 = 83.83%, R^2(adj) = 69.67%, R^2(pred)= 24.00%

Table 13: Parameter estimates of the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain ST11

Model Term	Parameter Effect		Coefficient	SD	T-value	P-value
Constant			77.54	3.54	21.89	0.000
X_1	14.94	7.47	1.91	3.91	0.001	
X_2	6.82	3.41	1.91	1.78	0.093	
X_3	-2.24	-1.12	1.91	-0.59	0.566	
X_4	2.41	1.20	1.91	0.63	0.538	
X_1*X_1	-18.58	-9.29	1.75	-5.30	0.000	
X_2*X_2	-21.93	-10.97	1.75	-6.26	0.000	
X_3*X_3	-15.78	-7.89	1.75	-4.50	0.000	
X_4*X_4	-13.13	-6.57	1.75	-3.75	0.002	
X_1*X_2	-12.64	-6.32	2.34	-2.70	0.016	
X_1*X_3	-2.29	-1.14	2.34	-0.49	0.632	
X_1*X_4	0.84	0.42	2.34	0.18	0.860	
X_2*X_3	-8.61	-4.31	2.34	-1.84	0.085	
X_2*X_4	7.16	3.58	2.34	1.53	0.146	
X_3*X_4	-2.09	-1.04	2.34	-0.45	0.662	

Regression Equation: $Y3(E24) = 77.54 + 7.47\ X_1 + 3.41\ X_2 - 1.12\ X_3 + 1.20\ X_4 - 9.29\ X_1^2 - 10.97\ X_2^2 - 7.89\ X_3^2 - 6.57\ X_4^2 - 6.32\ X_1X_2 - 1.14\ X_1X_3 + 0.42\ X_1X_4 - 4.31\ X_2X_3 + 3.58\ X_2X_4 - 1.04\ X_3X_4$

Table 14:Analysis of variance(ANOVA) for the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain ST11

Source	DF	Adj SS	Adj MS	F ratio	P-value
Regression	14	9738.5	695.61	7.92	0.000
Linear	4	1684.0	420.99	4.79	0.010
Square	4	6872.6	1718.16	19.57	0.000
Interaction	6	1181.9	196.98	2.24	0.092
Error	16	1405.1	87.82		
Lack of fit	10	656.2	65.62	0.53	0.824
Pure error	6	748	124.81		
Total	30	11143.6			

Key: DF = Degree of freedom, SS = Sum of squares, MS = Mean square, P-value ≤ 0.05 is significant at 95% confidence level

Model Summary: S = 9.37108, R^2 = 87.39%, R^2 (adj) = 76.36%, R^2(pred)= 56.93%

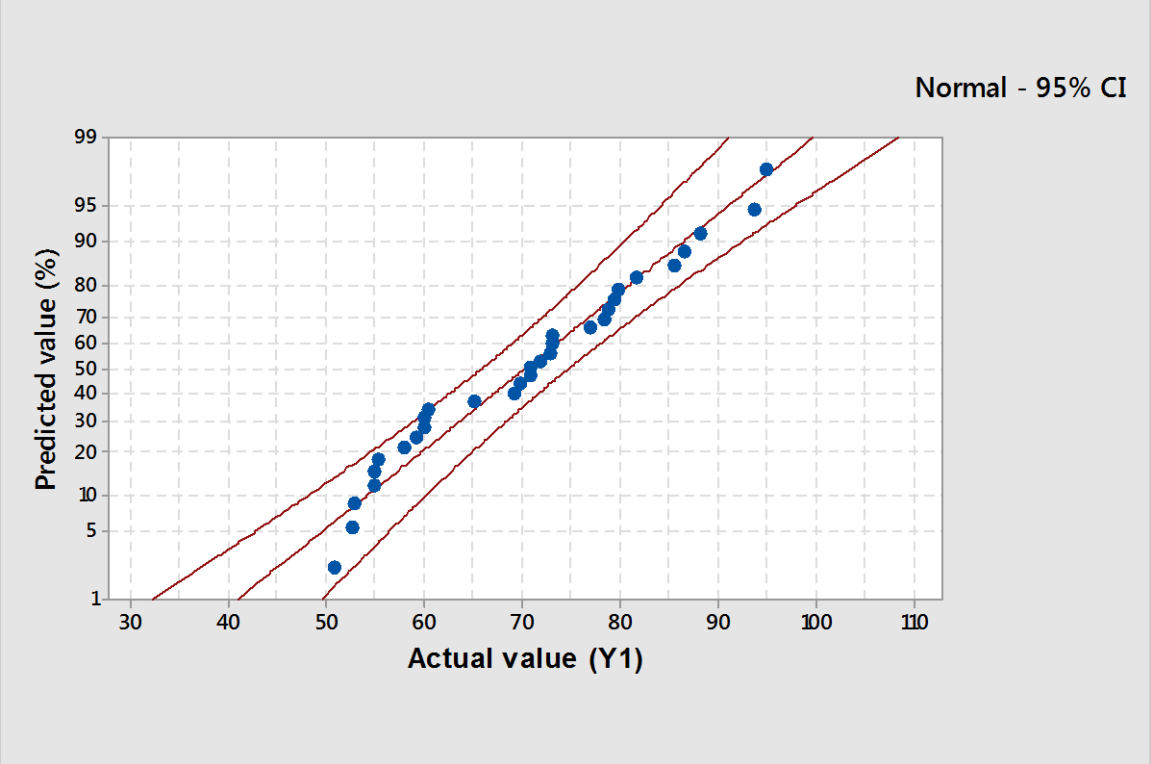


Figure 6: Probability plot of Actual response (Y1)

Key: Y1 - Response (E24) by *P.aeruginosa* strain CCUG

CI – Confidence interval

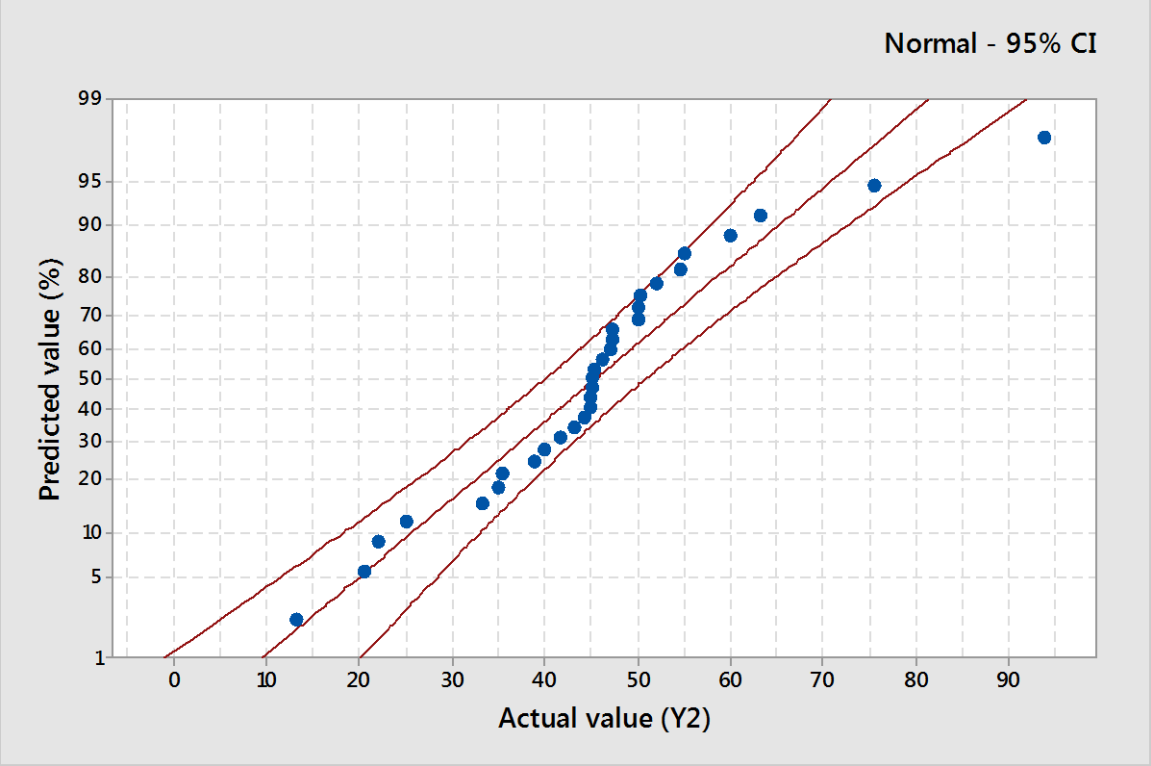


Figure 7: Probability plot of actual response (Y2)

Key: Y2 - Response (E24) by *P.aeruginosa* strain I3

CI – Confidence interval

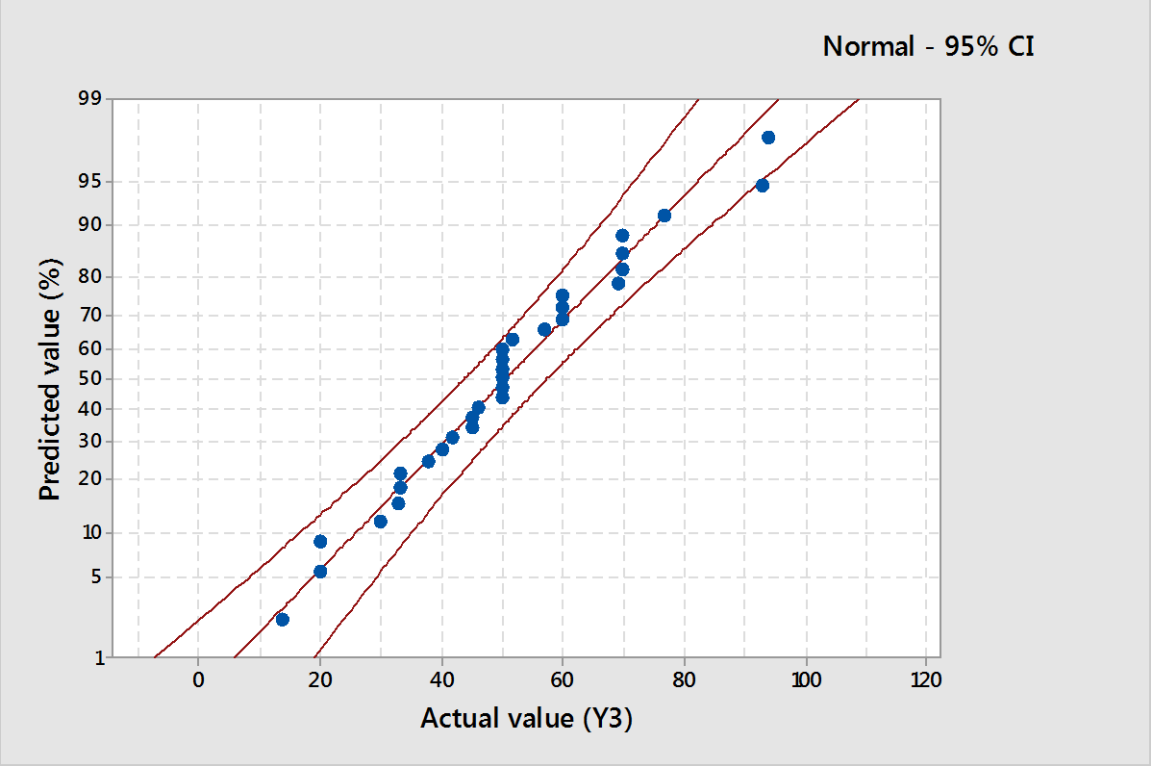


Figure 8: Probability plot of actual response (Y3)

Key: Y3 - Response (E24) by *P.aeruginosa* strain ST11

CI – Confidence interval

Table 15: Actual values of optimum conditions for maximum biosurfactant yield and the actual yield obtained

P. aeruginosa	**X_1(g/L)**	**X_2(g/L)**	**X_3(ml)**	**X_4(ml)**	**Y(E24)**	**Y(pred)**
strain CCUG	25	5	1.93	60	96.3%	147%
strain I3	25	25	1	60	92.3%	154.4%
strain ST11	16.92	15.51	1.93	41.41	80.66%	79.3%

Key:X_1 = Concentration of molasses, X_2= Concentration of sodium nitrate, X_3 = inoculum size, X_4 = medium volume, Y(E24) = Actual response obtained, Y(pred) = Predicted response

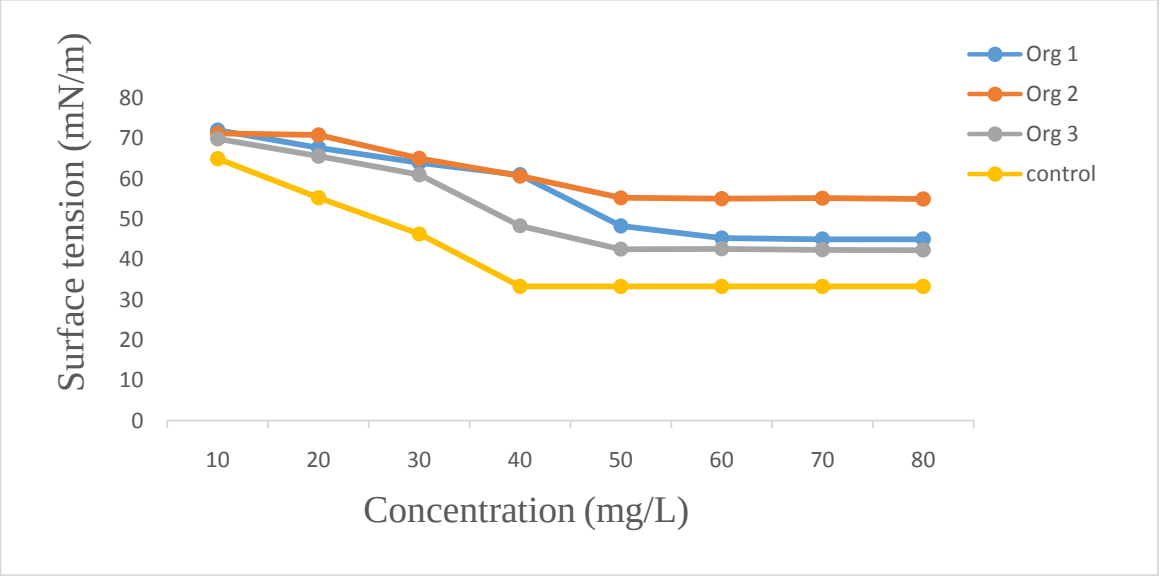


Figure 9: Surface tension measurement of the biosurfactants produced by *Pseudomonas aeruginosa* strains

Key: Org 1 = *Pseudomonas aeruginosa* strain CCUG

Org 2 = *Pseudomonas aeruginosa* strain I3

Org 3 = *Pseudomonas aeruginosa* strain ST11

Control = Sodium dodecyl sulphate (a chemical anionic surfactant)

4.5. Emulsification Activity of the Biosurfactants

The results of the emulsification activities of the biosurfactants produced by *Pseudomonas aeruginosa* strains against five hydrophobic substrates are presented in Figure 10. The biosurfactants produced by *Pseudomonas aeruginosa* strains showed the highest emulsification activity with crude oil, with emulsification index (E24) of 78.6%, 85.5% and 92.3% for *P.aeruginosa* strain CCUG, I3 and ST11 respectively (Figure 10). The order of emulsification activities of the biosurfactants produced by *Pseudomonas aeruginosa* strain CCUG and I3 with the hydrophobic substrates were crude oil > groundnut oil > kerosene > diesel > palm oil, while the biosurfactant produced by *Pseudomonas aeruginosa* strain ST11 had the order – crude oil > kerosene > palm oil > diesel > groundnut oil. One-way ANOVA (Appendices xiii – xv) shows that there was significant differences in the emulsification activities of the biosurfactants produced by the organisms with the hydrophobic substrates.

4.6. Glycolipid-type of Biosurfactanttest result

The production of dark blue halo on methylene blue agar plate by the *Pseudomonas aeruginosa* strains is shown in Plate 1. Biosurfactants produced by *Pseudomonas aeruginosa* strain I3 and ST11 showed dark blue halo of 1.4cm and 1.7cm on methylene blue agar plate respectively, while *Pseudomonas aeruginosa* strain CCUG did not show any dark blue halo.

4.7. The Functional Components of the Biosurfactant

The result from GC-MS analysis of the biosurfactants revealed the presence of several organic acids and esters with their relative abundance (Table 16). Five main fatty acid components were discovered in the biosurfactant produced by *Pseudomonas aeruginosa* strain CCUG, while 4 components and 3 components were found in the biosurfactants produced by *Pseudomonas aeruginosa* strain I3 and ST11 respectively. Mass spectrum from the GC-MS analysis of the biosurfactants from the *Pseudomonas aeruginosa* strains is shown in Appendices xvi – xviii

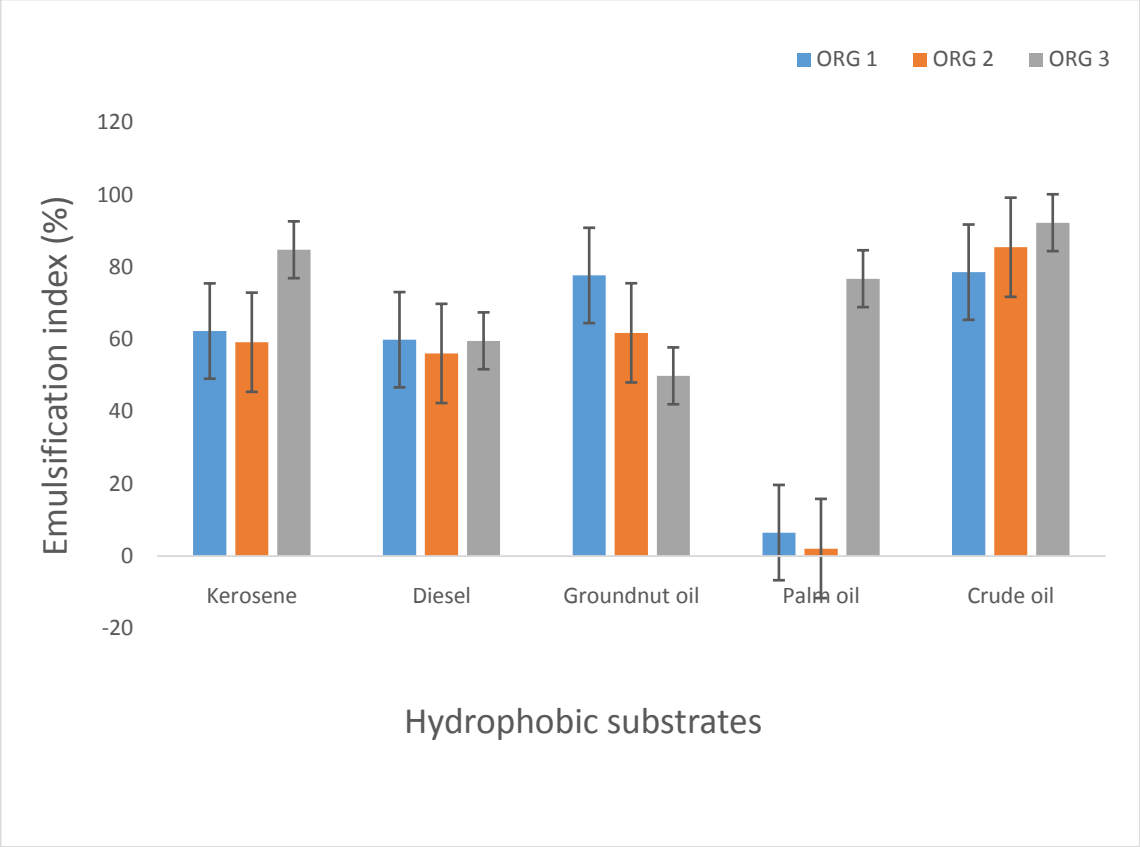


Figure 10: Emulsification activity of the biosurfactants produced by *Pseudomonas aeruginosa* strains

Key: Org 1 = *Pseudomonas aeruginosa* strain CCUG

Org 2 = *Pseudomonas aeruginosa* strain I3

Org 3 = *Pseudomonas aeruginosa* strain ST11

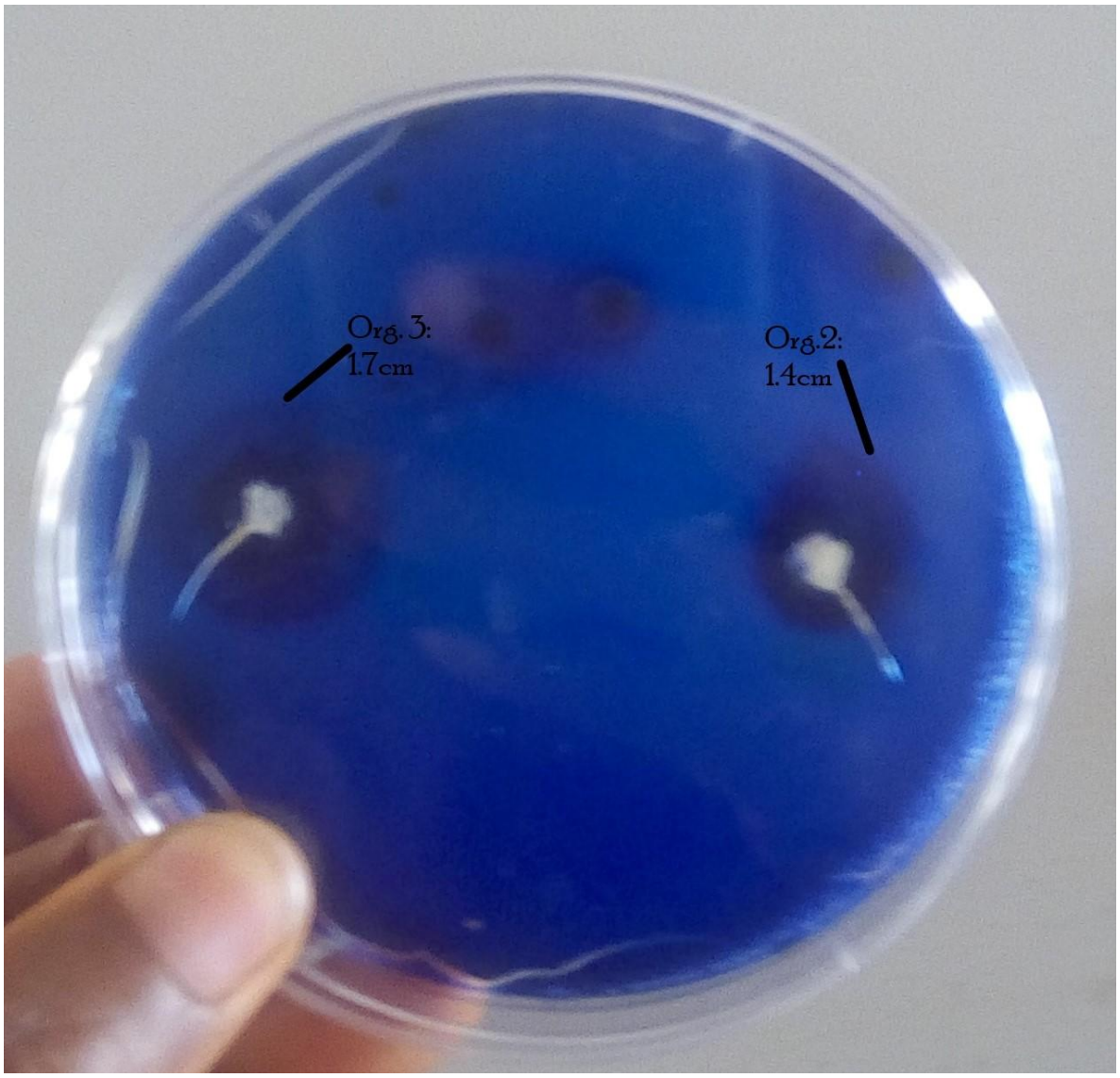


Plate 1:Production of dark blue halo on methylene blue agar plate

Key: Org 2 = *Pseudomonas aeruginosa* strains I3

Org 3 = *Pseudomonas aeruginosa* strains ST11

Table 16: Functional components of the biosurfactants

Biosurfactant source	Functional component	Relative abundance (%)	Formula
P. aeruginosa strain CCUG	Octadecanoic acid	25.90	$C_{18}H_{36}O_2$
	Methyl stearate	7.79	$C_{19}H_{38}O_2$
	Cyclododecanol	18.28	$C_{16}H_{32}O_2$
	Tert-Butyl isopropyl disulphide	0.70	$C_7H_{16}S_2$
	Cyclotetrasiloxane	0.73	$C_8H_{24}O_4Si_4$
P. aeruginosa strain I3	9-Octadecenoic acid	80.80	$C_{18}H_{34}O_2$
	n-Hexadecanoic acid	4.50	$C_{16}H_{32}O_2$
	Cyclotetrasiloxane	0.47	$C_8H_{24}O_4Si_4$
	Trimyristin	3.94	$C_{45}H_{86}O_6$
P. aeruginosa strain ST11	Octadecanoic acid	34.11	$C_{18}H_{36}O_2$
	Methyl stearate	5.67	$C_{19}H_{38}O_2$
	Sulfuric acid	1.89	H_2SO_4

4.8. Physicochemical Properties of the Crude Oil-polluted and Control Soil Samples

As presented in Table 17, the result of the physicochemical characteristics of the soil samples shows that the crude oil polluted soil was a fine sandy loam soil while thecontrol soil was a coarse sandy soil. The polluted soil was acidic (pH 5.8± 0.01) with soil conductivity of 93.3± 0.3 μS/cm, while the pH of the control soil was slightly acidic (pH 6.5± 0.03) with low conductivity of 87.5± 0.3μS/cm. The analysis of the soil samples show that the total nitrate and phosphate values were higher in the polluted soil when compared with the control soil, but had low TOC value than the control.

The result (Table 17) also shows the presence of heavy metals – arsenic, lead, mercury, cadmium and chromium in both the polluted and the control soil, however, the concentrations of the heavy metals were higher in the polluted soil. The total petroleum hydrocarbon concentration in the polluted soil and the control soil were 1066.23±0.69μg/ml and 467.09±0.52μg/ml respectively. Gas chromatogram of the crude oil-polluted and control soil is shown in Appendices xix and xx respectively. Dehydrogenase enzyme activity was relatively low in the polluted soil (Table 17).

4.9. Microbiological Profile of the Crude Oil-Polluted and Control Soil Samples

4.9.1.Indigenous Microorganisms of Crude Oil-Polluted and Control Soil

Table 18 shows the heterotrophic bacterial and fungal count in the polluted and control soil samples. The bacterial and fungal count were relatively higher in the control soil. A total of six bacteria belonging to the genera *Staphylococcus*, *Citrobacter*, *Micrococcus*, *Pseudomonas*, *Bacillus*, *Corynebacterium*, and two fungi of the genera *Aspergillus* and *Penicillium* were isolated from the crude oil-polluted soil. The bacterial and fungal isolates in the control soil were *Staphyloccus aureus*, *Micrococcus* sp., *Bacillus subtilis*, *Streptococcus* sp., *Lactobacillus* sp., *Serratia marcescens*, *Pseudomonas aeruginosa*, *Fusarium solani*, *Aspergillus fumigatus*, *Aspergillus niger* and *Penicillium* sp.

Table 17: Physicochemical properties of the crude oil-polluted and Control soil samples (Mean ±SD)

Parameter	Crude oil-Polluted soil	Control soil
Soil type	Sandy loam soil	Sandy soil
Soil texture	Fine	Slightly coarse
pH	5.80± 0.01	6.50± 0.03
Conductivity (µS/cm)	93.30± 0.30	87.50± 0.30
Total Nitrate (mg/g)	0.09±0.00	0.53±0.06
Total Phosphate (mg/g)	0.70±0.00	0.55±0.00
TOC (%)	1.40±0.17	3.07±0.03
Heavy metals (mg/kg)		
Arsenic	0.126±0.00	0.062±0.04
Lead	0.337±0.00	0.325±0.00
Mercury	1.560±0.04	0.360±0.00
Cadmium	0.184±0.00	0.092±0.00
Chromium	0.572±0.00	0.495±0.00
TPH (µg/ml)	1066.23±0.69	467.09±0.52
DHase enzyme activity (mg/g dry soil/96h)	0.84±0.01	3.60±0.00

Table 18: Heterotrophic microbial count in the crude oil-polluted and control soil

	Polluted Soil (Log Cfu/g)	Control Soil (Log Cfu/g)
Bacterial count	2.57±0.01	5.65±0.03
Fungal count	2.38±0.07	5.26±0.04

The results of biochemical characteristics of the bacterial isolates from the polluted and control soil are shown in Table 19 and 20 respectively, while the percentage occurrences of the bacterial isolates from the control soil are presented in Table 21. The result (Table 21) shows that *Bacillus subtilis* had the highest occurrence of 27.3%.

The morphological characteristics of the fungal organisms isolated from the polluted and control soil are presented in Table 22.

4.9.2. Metagenomic profile of the Crude Oil-polluted and Control Soil Samples

The metagenomic analysis revealed the presence of large communities of microorganisms in both the crude oil-polluted soil and the control soil. The illumina Miseq sequencing showed that there were 47,616 and 53,069 operational taxanomic units (OTU) in the polluted and control soil samples respectively.

The percentage distribution of bacteria and archaea in the soil samples is presented in Figure 11. In the polluted soil sample, the percentage abundance of archaea and bacteria were 3.126% and 96.874% respectively, while out of the organisms recovered from the control soil, 1.083% belonged to the domain archaea.

Figure 12 shows the two archaeal phyla recovered and their relative abundances. Out of 3.126% archea present in the polluted soil, 2.83% were Euryarchaeota while 0.295% were Crenarchaeota. In the control soil, the 1.08% archea were made up of 0.85% Crenarchaeota and 0.23% Euryachaeota.

Table 19: Results of biochemical test for identification of the indigenous bacterial isolates from the polluted soil

Biochemical test	Isolates 1B	2B	3B	4B	5B	6B
Gram reaction	+ cocci	+ cocci	- rod	- rod	+ rod	+ rod
Motility	-	-	+	+	-	+
Citrate	+	+	+	+	-	+
Indole	-	+	-	-	-	-
Coagulase	+	-	-	-	-	+
Catalase	+	+	+	+	+	+
Oxidase	-	+	+	-	-	-
Methyl Red	+	-	-	+	+	-
Voges Proskauer	-	+	-	-	-	+
Sugar fermentation						
Sucrose	+	+	-	+	-	+
Maltose	-	+	-	+	+	+
Glucose	-	+	-	+	+	+
Lactose	-	-	-	+	-	+
Rhamnose	-	+	+	+	+	-

Key : - = negative, + = positive

Isolate 1B = *Staphylococcus aureus*

Isolate 2B = *Micrococcus* sp.

Isolate 3B = *Pseudomonas aeruginosa*

Isolate 4B = *Citrobacter* sp

Isolate 5B = *Bacillus subtilis*

Isolate 6B = *Corynebacterium*sp

Table 20: Results of biochemical test for identification of bacterial isolates from the control soil

Biochemical test	Isolates 1C	2C	3C	4C	5C	6C	7C
Gram reaction	+	+	+	-	+	-	+
Shape	cocci	rod	cocci	rod	rod	rod	cocci
Motility	-	-	-	+	-	+	-
Citrate	+	-	+	+	-	+	+
Indole	-	-	+	-	-	-	-
Coagulase	+	-	-	-	-	-	-
Catalase	+	-	+	+	+	+	-
Oxidase	-	-	+	+	-	-	-
M. R	+	-	-	-	+	-	-
V.P	-	-	+	-	-	+	-
Sucrose	+	+	+	-	-	+	+
Maltose	-	+	+	-	+	+	+
Glucose	-	+	+	-	+	+	+
Lactose	-	+	-	-	-	-	+
Rhamnose	-	+	+	+	+	-	+

Key: - = negative, + = positive, M.R. = Methyl red, V.P. = Voges Proskauer

Isolate 1C = *Staphylococcus aureus*

Isolate 2C = *Lactobacillus* sp

Isolate 3C = *Micrococcus* sp.

Isolate 4C = *Pseudomonas aeruginosa*

Isolate 5C = *Bacillus subtilis*

Isolate 6C = *Serratia marcescens*

Isolate 7C = *Streptococcus* sp.

Table 21: Percentage occurrence of bacterial isolates from control soil

Bacterialisolate	No. of occurrence	% occurrence
Staphylococcus aureus	2	18.2
Micrococcus sp.	1	9.1
Bacillus sp.	3	27.3
Lactobacillus sp.	1	9.1
Serratiamarcescens	1	9.1
Pseudomonas aeruginosa	2	18.2
Streptococcus sp.	1	9.1
Total	11	100

Table 22: Morphological characteristics of fungal isolates from polluted and control soil

Isolate	Macroscopic features	Microscopic features
Fusarium solani[C]	Round, flat, cottony colonies. Colour of colony from the front is white and reddish brown on the reverse	Hyaline septate hyphae, branching and narrow conidiophores, cylindrical thin phialides with aerial oval shaped microconidia
Aspergillus fumigatus[C]	Round woolly dark blue colonies on the front with white edges, and dirty white on the reverse.	Hyphae is septate, conidia head is columnar and uniseriate, conidiophore stipes are smooth and short with clublike phialides
Penicillium sp.[CP]	Greenish rough colonies with white edges on the front and milky colour on the reverse	Septate hyphae, branching conidiophore stipes with clublike philaides and conidia arranged in chains
Aspergillus niger[CP]	Round brownish dark cottony colony with white edges on the front and milky on the reverse	Septate hyhae, variable length and smooth conidiophore, vesicles end in metulae which gives rise to the phialides with globular conidia
Aspergillusflavus[P]	Yellowish green cottony colonies on the surface and brown on the reverse	Septate hyphae, smooth conidiophore with round head. Conidia are clustered on the phialide head

Key: Fungi with superscript "cp" were isolated from both the control and polluted soil, fungus with superscript "p" was isolated from only the polluted soil, and fungi with superscript "c" were isolated from the control soil only

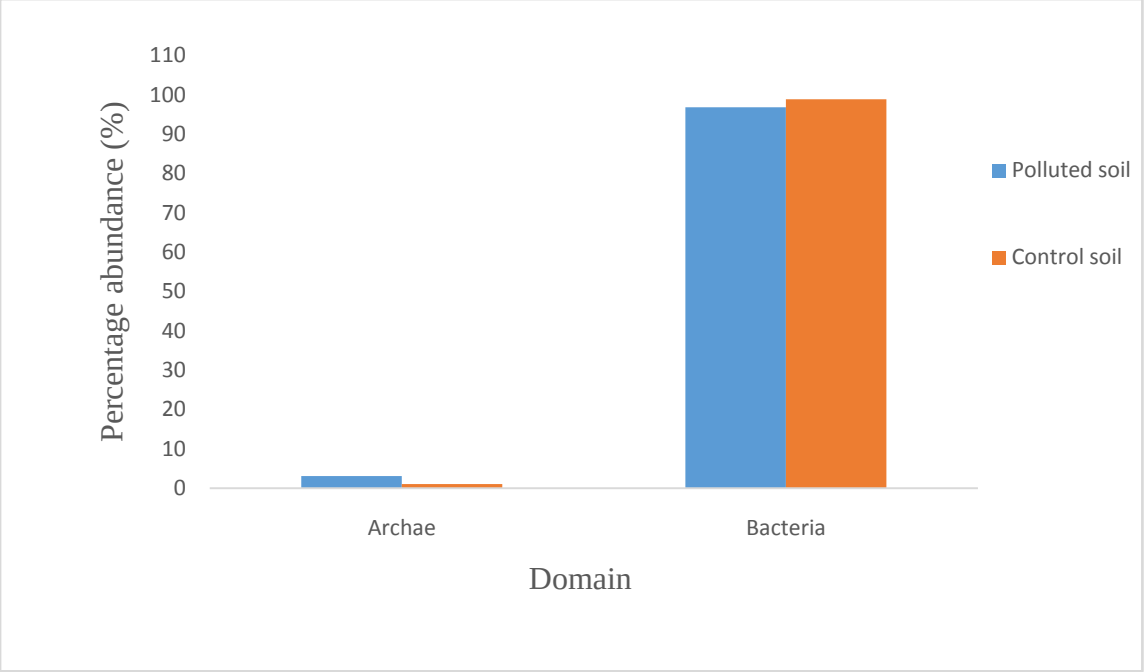


Figure 11: Percentage abundance of the archaea and bacteria in the polluted and control soil samples

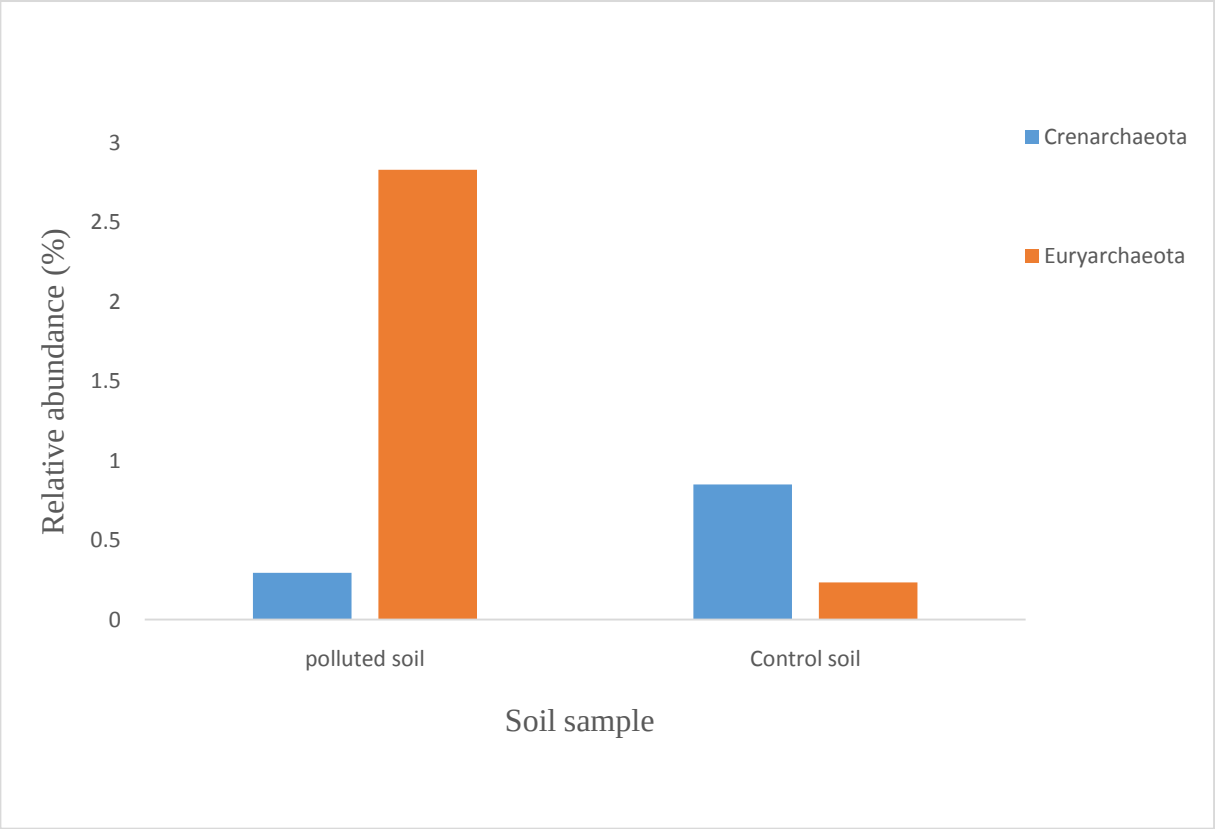


Figure12: Relative abundance of archaeal phyla in the polluted and control soilsamples

At phylum level, 54 taxonomic groups were obtained. The dominant phyla with relative abundance >1% is shown in figure 13.*Proteobacteria* was the most abundant phylum in both soil samples, comprising of 47.4% of all phyla in the polluted soil and 53.5% in control soil. This was followed by the *Firmicutes* (11.2% in polluted soil, 16.1% in control soil) and *Acidobacteria* (8.8% and 9.4% in polluted and control soil respectively). *Actinobacteria, Chloroflexi* and *Planctomycetes* occurred more in the polluted soil than in the control.

At the class level, a total of 133 taxa were recovered. The relative abundance of dominant classes in the soil samples with proportion > 1% is shown in Figure 14.*Alphaproteobacteria*predominated in the polluted soil with relative abundance15.1%, while*Betaproteobacteria* predominated in the control soil with relative abundance 28.7%.

At the genus level, a total of 300 taxa were recovered with varying relative abundances. The dominant genera with relative abundance >0.5% is shown in Figure 15. In the polluted soil, *Candidatus koribacter* occurred most with relative abundance 3.71% while *Comamonas* occurred most in the control soil with relative abundance 14.1%.

4.10. Crude Oil Degradation Potential of the Indigenous Isolates in Shake Flask

The result of the gravimetric assessment of crude oil degradation by indigenous organisms is presented in Figure 16. It shows that crude oil degradation rate ranged from 6.7± 2.3% to 98.8 ± 0.7% with *Bacillus subtilis* achieving the highest degradation of crude oil, while degradation by *Micrococcus* sp. was the least. There was no significant difference in the degradation rates of *S.aureus, P.aeruginosa, B. subtilis* and bacteria consortium (P-value > 0.05), indicating that they had the same level of performance (Appendix xxi).*Bacillus subtilis* was the best crude oil-degrading bacterium, while *Penicillium* sp was the best crude oil-degrading fungus.

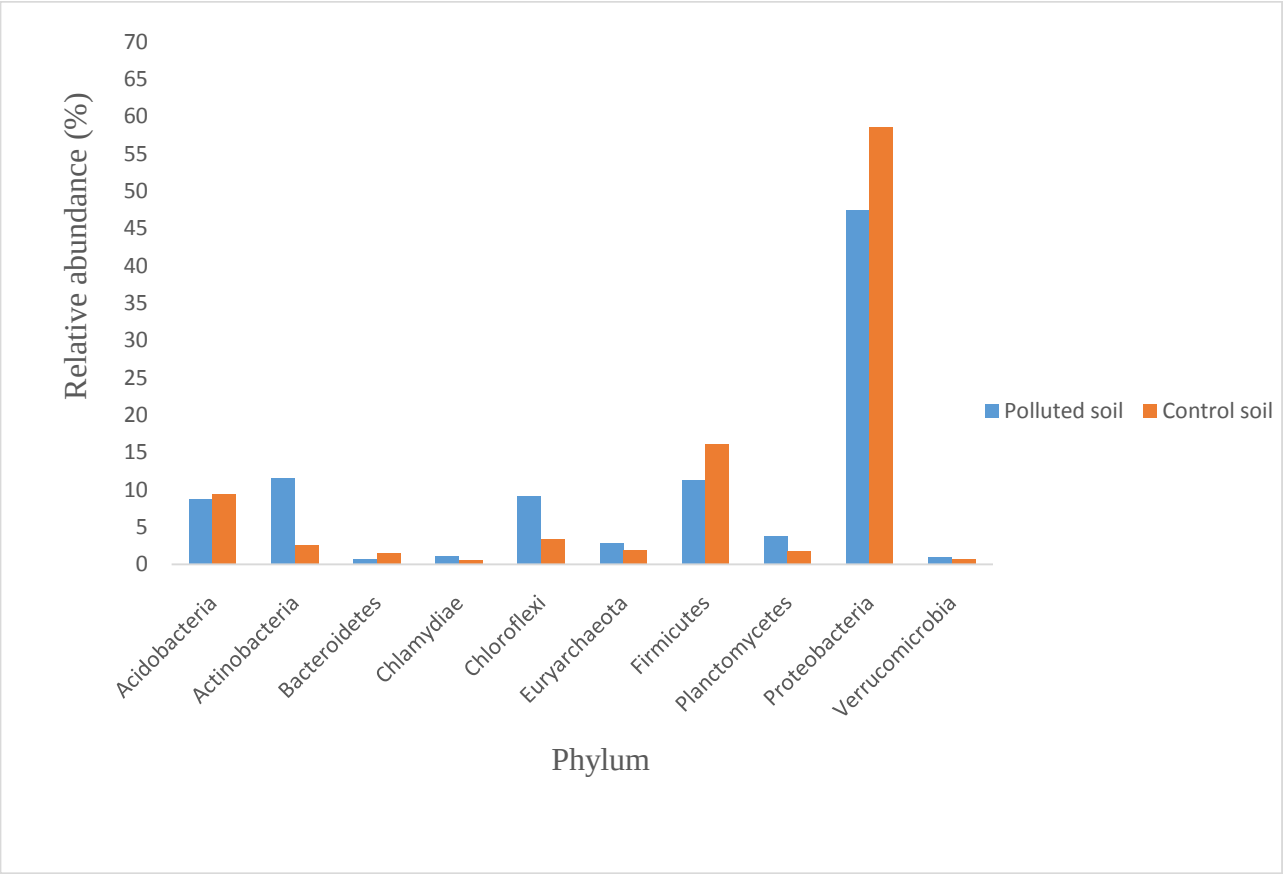


Figure 13: Relative abundance of dominant phyla of bacteria in polluted and control soil with proportion > 1%

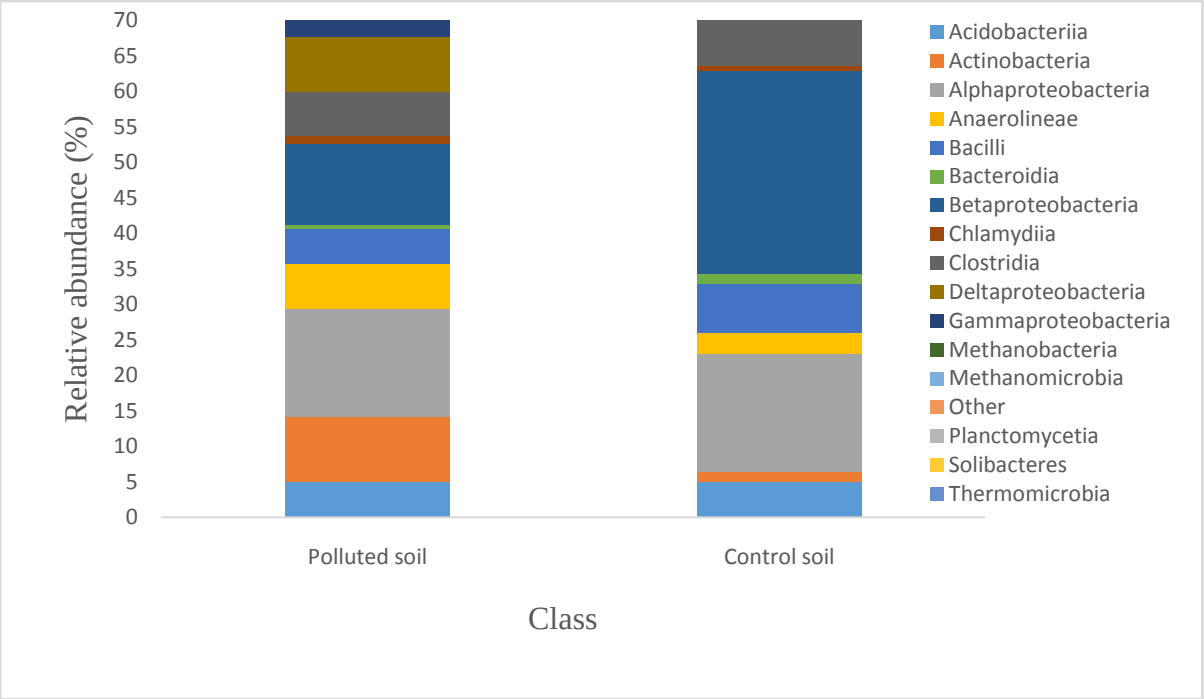


Figure 14: The relative abundance of dominant class of bacteria in the polluted and control soil with proportion > 1%

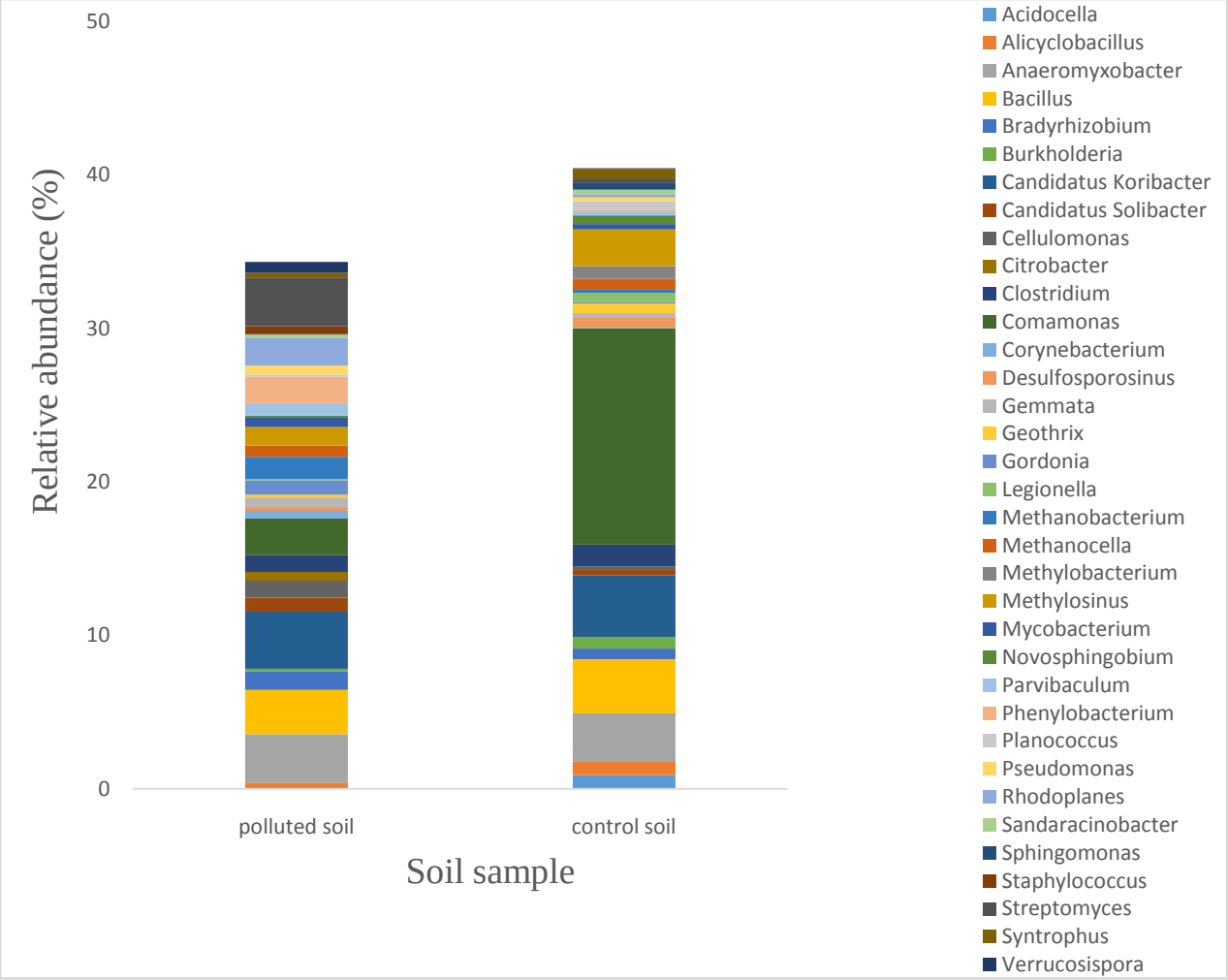


Figure 15: Relative abundance of genera in the polluted and control soil samples with proportion > 0.5%

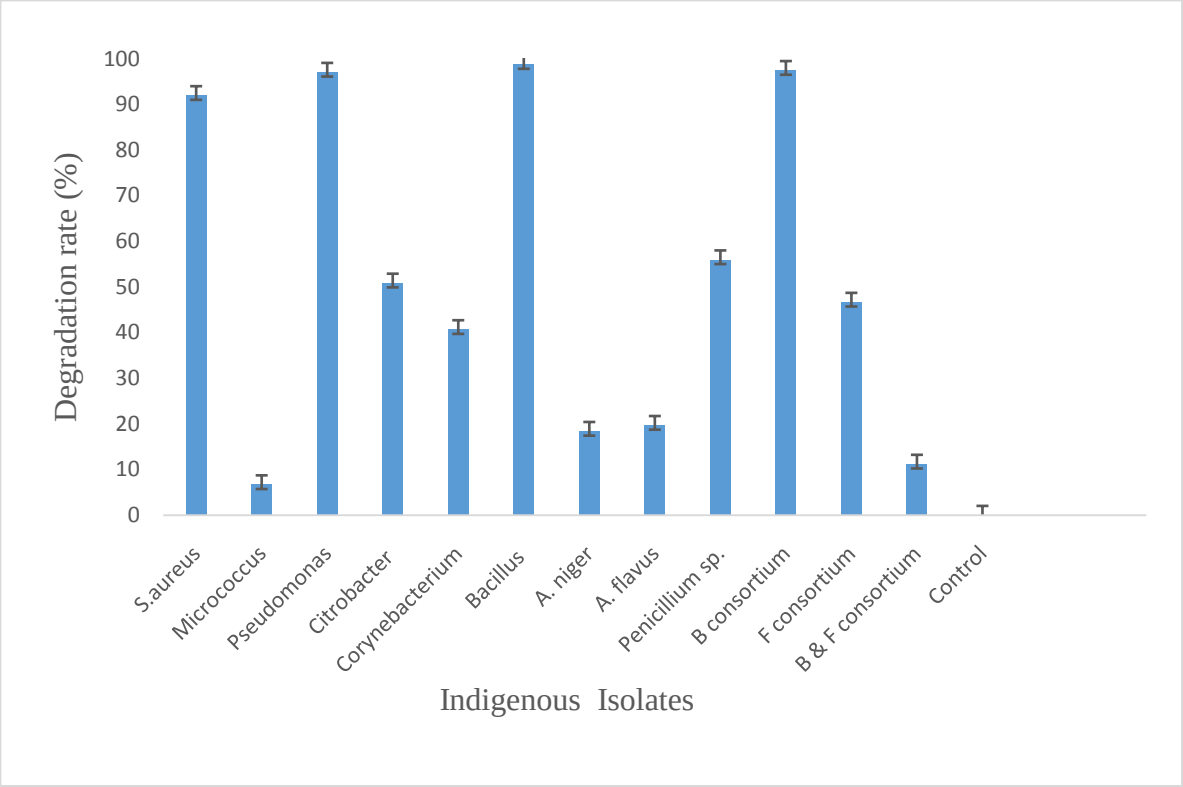


Figure 16: Crude oil degradation potential by indigenous isolates

Key: B = bacteria

F = fungi

4.11. Comparative Bioremediation of the Crude Oil-polluted Soil Sample

Figure 17 shows the heterotrophic bacterial count during bioremediation, while Figure 19 shows the fungal count. There was progressive increase in bacterial and fungal counts in all the treatments throughout the bioremediation period. At the end of the experiment, the bacterial count obtained in all the treatment ranged from 2.77±0.04 – 3.98±0.00 log cfu/g (Figure 17) while the fungal count ranged from 2.3±0.04 – 2.99±0.01 log cfu/g (Figure 19). Percentage increase in bacteria population in the treatments ranged from 34.6% to 54.9% (Figure 18), while the percentage increase in the population of fungi at the end of the experiment ranged from 16.4% to 25.6% (Figure 20). The result of the one-way analysis of variance indicated that there was significant difference in bacterial and fungal counts observed in all the treatments (p-value < 0.05)[Appendix xxiii and xxiv]. While the highest percentage increase in bacterial count (54.9%) was observed in bioaugmentation D (Figure 18), the highest percentage increase in fungal count of 25.6% was recorded in bioaugmentation C (Figure 20).

Figure 21 shows the pH changes during the bioremediation period. The pH of the control soil was relatively steady while the pH of natural attenuation started increasing after wk 8. The pH of the other treatments were changed during the bioremediation period. At the end of the experiment, the highest pH of 7.21 was observed in biostimulation A.

The result of the dehydrogenase enzyme assay is presented in Figure 22. There was significant difference (P-value < 0.05) in dehydrogenase enzyme activity observed in all the treatments [Appendix xxv]. The highest enzyme activity (16.4± 0.40 mg/g) was observed in bioaugmentation D set-up while the least enzyme activity was seen in the control (1.8±0.03 mg/g). Dehydrogenase enzyme activity was observed more in the bioaugmentation set-ups

than in the biostimulation set-ups. There was strong positive correlation between the enzyme activity and bacterial count observed (r = 0.733, P-value < 0.05)[Appendix xxxvii].

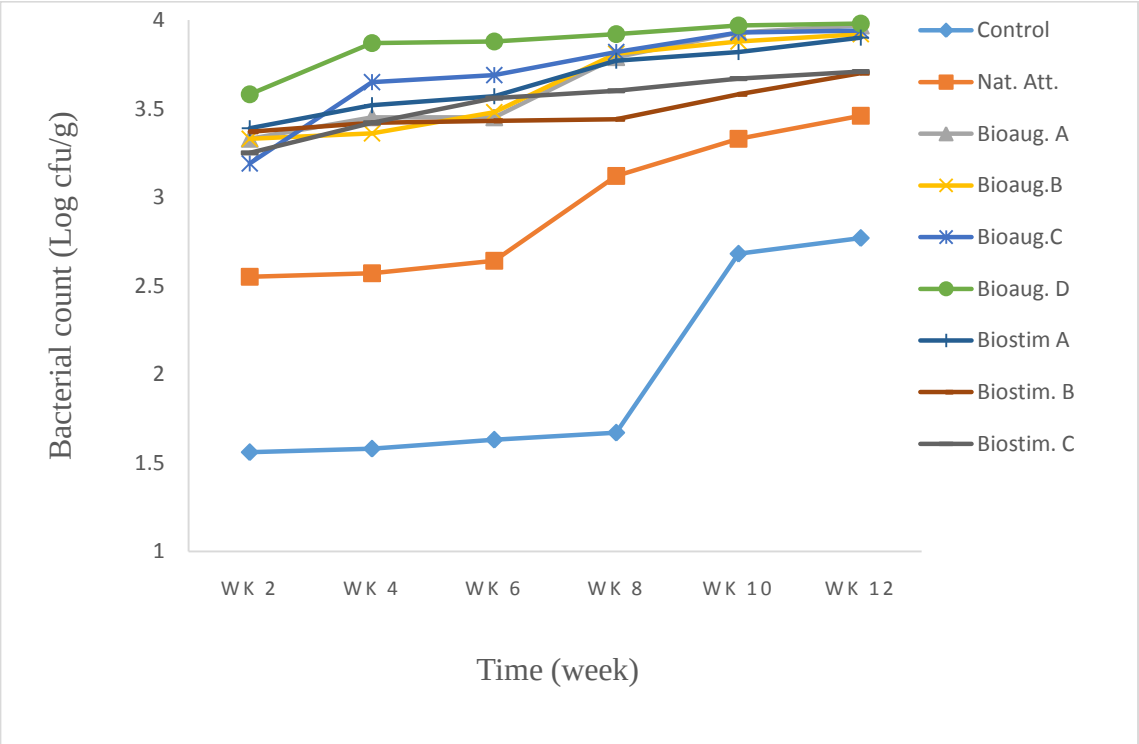


Figure 17: Heterotrophic bacterial count during bioremediation

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

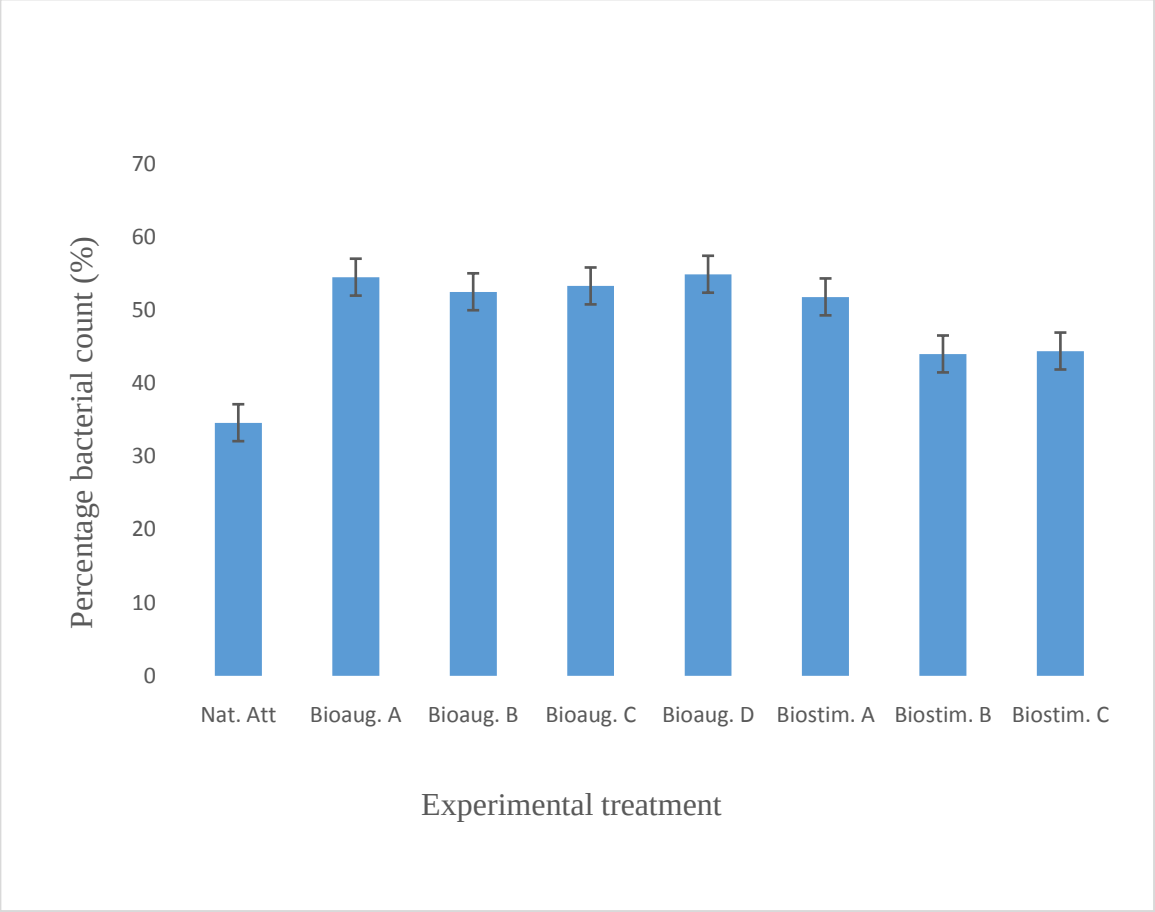


Figure 18: Percentage increase in bacterial count after bioremediation

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

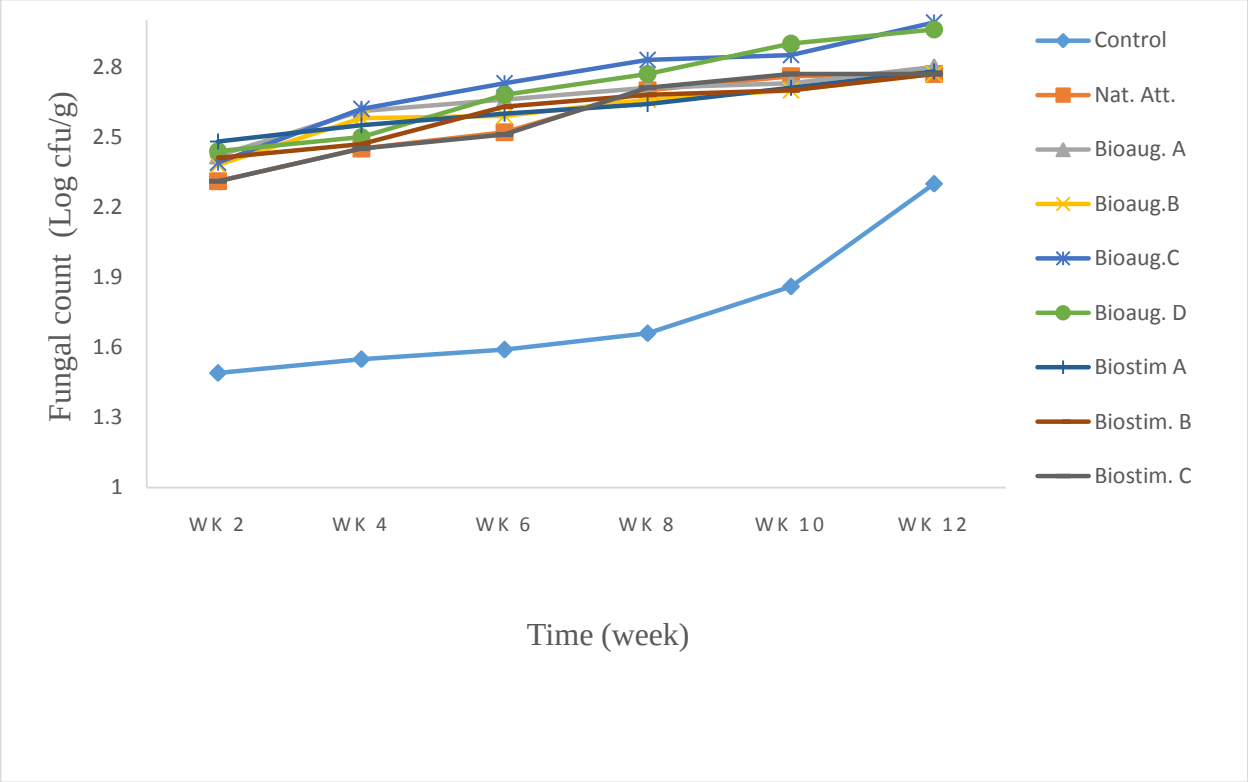


Figure 19: Fungal count during bioremediation.

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

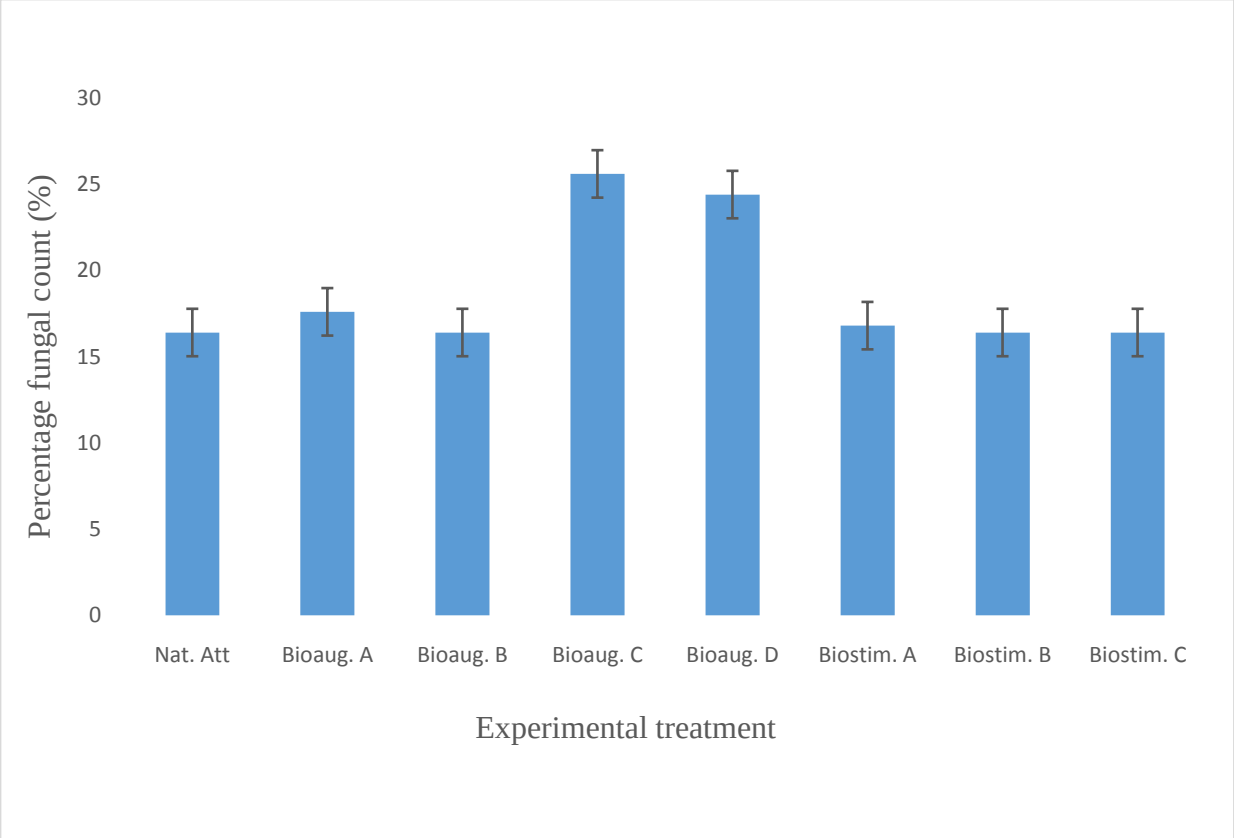


Figure 20: Percentage increase in fungal count after bioremediation

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

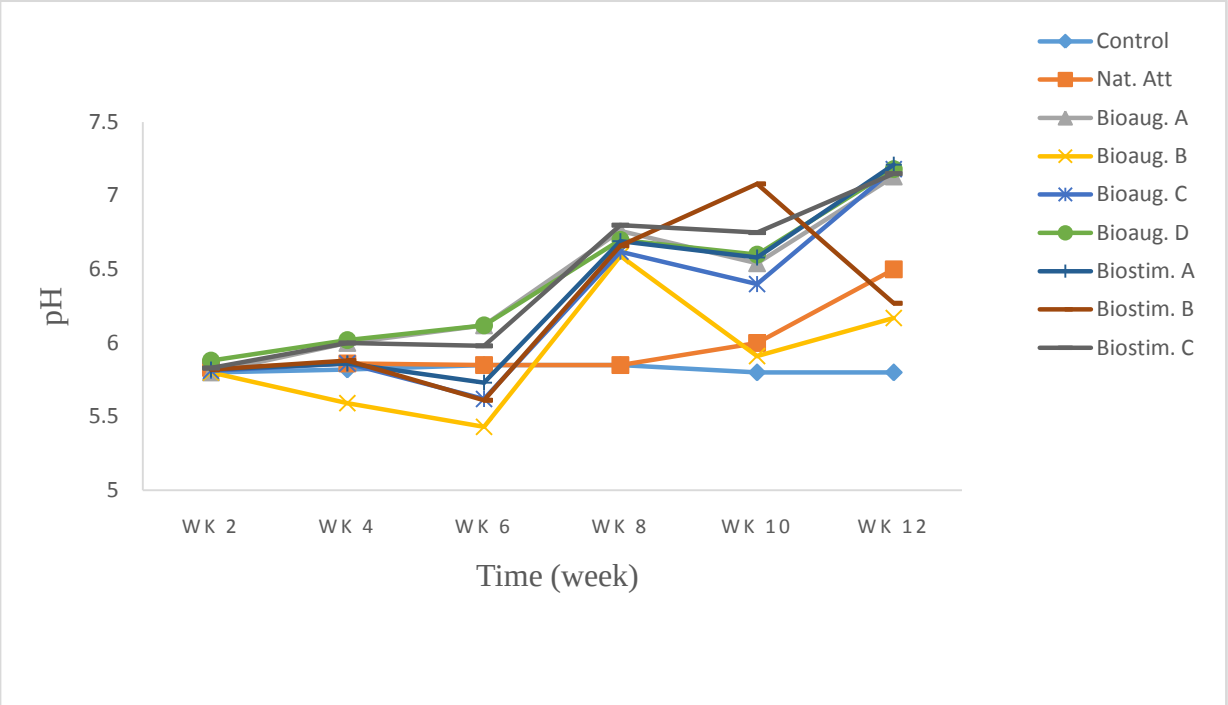


Figure 21: pH changes during the bioremediation period

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

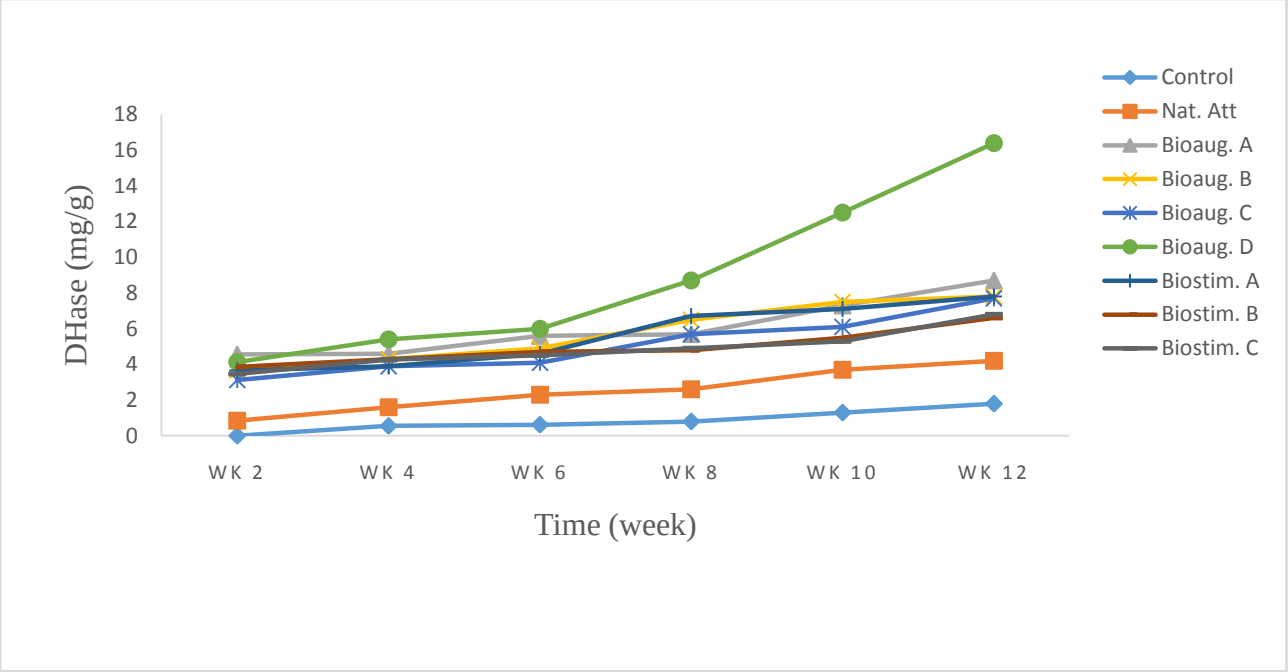


Figure 22: Dehydrogenase enzyme activity during bioremediation

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

4.12. Rate of Crude Oil and Heavy Metals Removal from the Polluted Soil

The result of the residual total petroleum hydrocarbon (TPH) pattern during the bioremediation period is presented in Figure 23, while Figure 24 shows the percentage TPH degradation at the end of the experiment. The results show that there was rapid TPH degradation within the first 4 weeks of treatment and continued throughout the experimental period (Figure 23). The highest residual TPH among the treatments was observed in the natural attenuation set-up (532.53 mg/kg), while the least residual TPH was seen in the biostimulation B set-up (49.15 mg/kg).

TPH degradation was progressive throughout the bioremediation period in all the treatments with biostimulation B attaining the highest TPH degradation (95.4±0.40%), while natural attenuation achieved 49.8±0.15% TPH degradation (Figure 24). The degradation rates observed in all the treatments were significantly different (p-value < 0.05) [Appendix xxvi].TPH degradation showed a significant positive relationship with dehydrogenase enzyme activity result with r-value of 0.651 and p-value < 0.005 (Appendix xxxviii). The degradation rate also correlated significantly with heavy metal removal at 99% confidence level ($r^a = 0.819$, $r^l = 0.948$, $r^m = 0.891$, $r^{cd} = 0.949$, $r^{cr} = 0.699$) [Appendices xxxii – xxxvi].

Figure 25 shows the percentage heavy metal removal in all the treatments. Arsenic was completely removed only in bioaugmentation D. While lead was completely removed in all the treatments except in natural attenuation, 100% mercury removal was observed only in bioaugmentation B. Complete cadmium removal was observed in bioaugmentation C, D and biostimulation A, while chromium was completely removed only in bioaugmentation C. Moreso, the figure shows that 3 out of the 5 heavy metals present in the soil – lead, cadmium and chromium were completely removed in bioaugmentation C, while arsenic, lead and cadmium were completely removed in bioaugmentation D. Statistical analysis are shown in Appendices xxvii – xxxi.

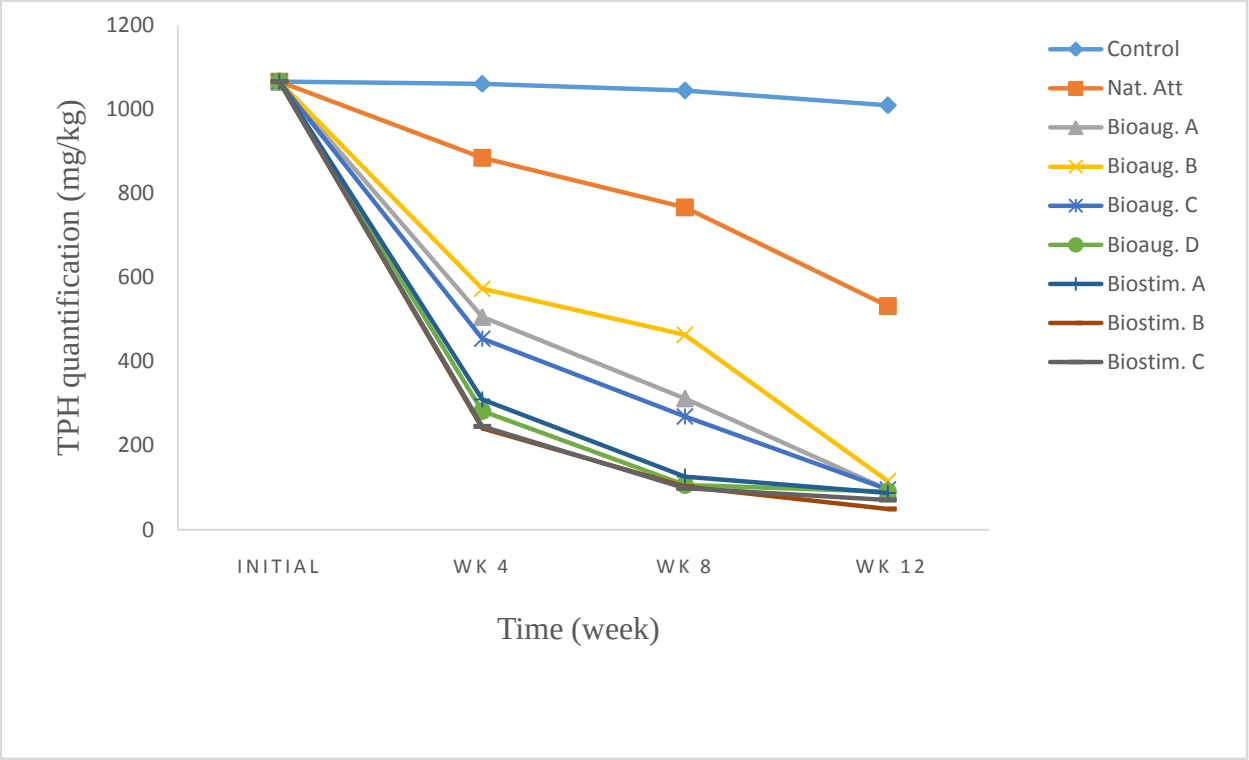


Figure 23: Residual TPH pattern during the bioremediation period

Key: TPH = Total petroleum hydrocarbon, Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation

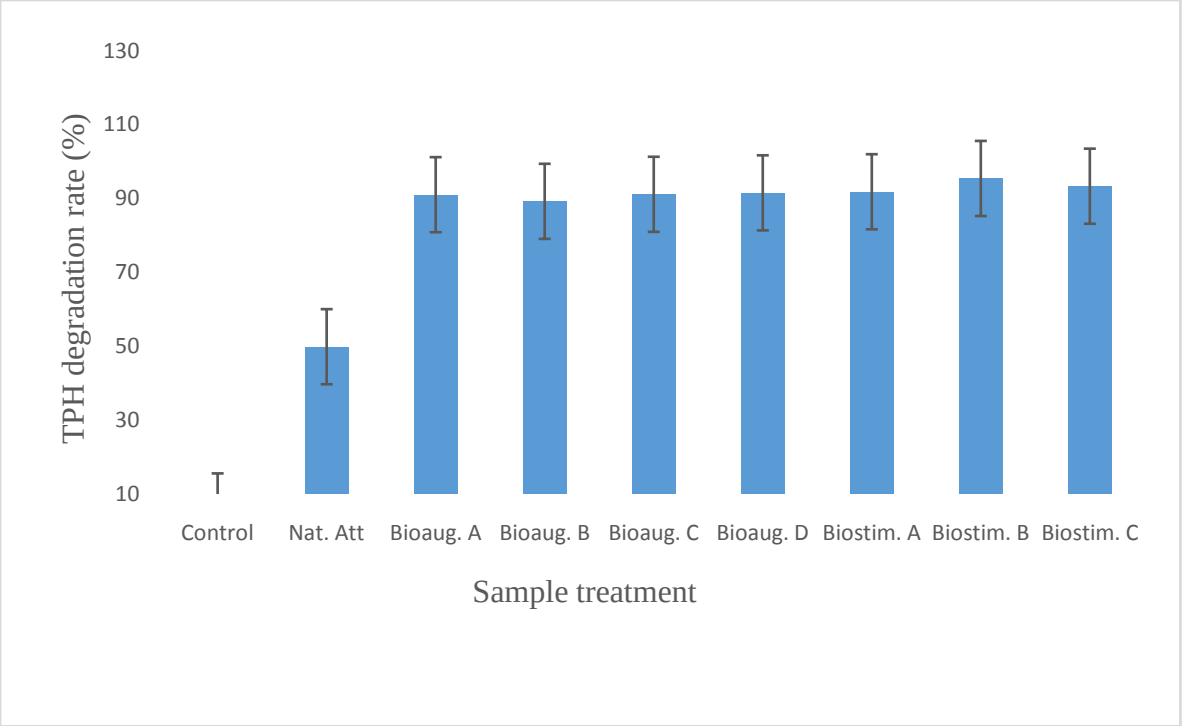


Figure 24: Percentage TPH degradation after 12 weeks bioremediation period

Key: TPH = Total petroleum hydrocarbon, Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

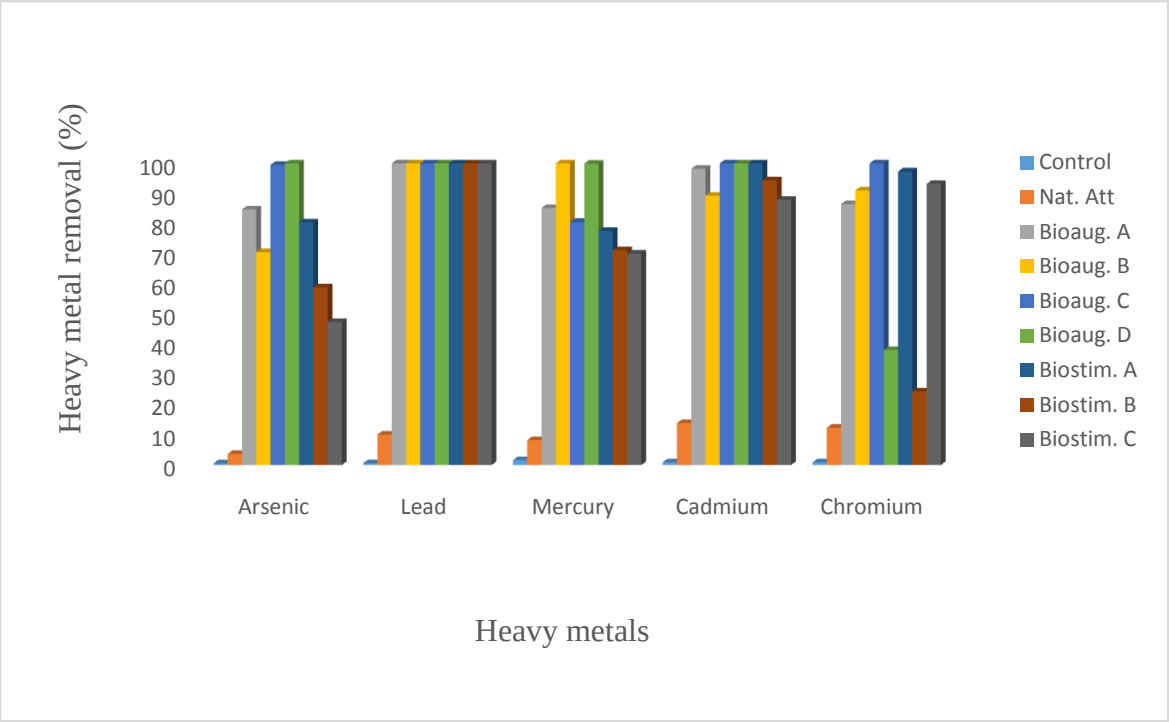


Fig. 25: Percentage heavy metal removal after bioremediation

Key: Nat. Att = Natural attenuation, Bioaug A = Bioaugmentation A, Bioaug B = Bioaugmentation B, Bioaug C = Bioaugmentation C, Bioaug D = Bioaugmentation D, Biostim A = Biostimulation A, Biostim B = Biostimulation B, Biostim C = Biostimulation C

CHAPTER FIVE

5.0 DISCUSSION

Cetrimide agar is a medium for isolation of bacteria of the genus *Pseudomonas* because it contains cetrimide, which is the selective agent against other microbial flora. The isolation of different strains of *Pseudomonas aeruginosa*(strain CCUG, I3 and ST11) with cetrimide agar in this study, is in line with the works of various researchers (Elshafiee *et al*., 2019; Peekate*et al*., 2018; Onwosi and Odibo, 2012; Mokate and More, 2013), who isolated various species of *Pseudomonas* with cetrimide agar. Therefore, Cetrimide agar medium is recommended for the isolation and enumeration of *P. aeruginosa* in environmental samples.

The isolation of different strains of *Pseudomonas aeruginosa* from spent-lubricating oil-polluted soil as seen in this study,showedthat *Pseudomonas* species are hydrocarbon utilizers. They can metabolize and proliferate in the presence of hydrocarbon pollutants.

Table 7 shows the screening results of the isolates on mineral salts medium. The screening procedures used were consistent with previous works (Peekate and Boreh, 2019; Nurul *et al*., 2019; Onwosi and Odibo, 2012 and Satpute *et al*.,2008).

In drop collapse test, drops of cell suspension containing biosurfactants collapsed, whereas non-surfactant containing drops remain stable (Tugrul and Cansunar, 2005). Distilled water which served as control, did not collapse on the oily surface of the well but appeared as a bead. This is because, the oily surface was hydrophobic and, therefore, water molecules tend to aggregate forming droplets (Jain *et al*., 1991). The drop collapse result showed 82.4% positive result and 17.6% negative result. This result agreed with the work of Thavasi *et al.*(2011a), who out of the 105 bacterial strains screened for biosurfactant production, 78.1% were positive for drop-collapse activity. Nurul *et al*. (2019), observed

different kinds of collapse ranging from partial collapse to flattened collapse in their work on comparative screening methods for the detection of biosurfactant-producing capability of antarctic hydrocarbon-degrading *Pseudomonas* sp. Therefore, it is recommended that drop-collapse assays are reliable methods to screen large numbers of samples for biosurfactant production.

The oil displacement test is an indirect measurement of the surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution (Rodrigues *et al*., 2006). The isolates screened produced oil displacement diameter ranging from 0 – 2.1cm (Table 7). This is quite contrary to the works of Hesham *et al*.(2012), and Jaysree *et al*. (2013), who obtained larger oil displacement diameters. While Hesham *et al*.(2012) obtained oil displacement ranging from 2.8cm to 4.1cm in the screening of *Candida* species for biosurfactant production, Jaysree *et al*. (2013), recorded displacement diameter ranging from 3.0cm to 4.2cm in their work on biosurfactant production by halophilic bacteria. Nature of microorganism isolated may have contributed to the small displacement diameters obtained in this study.

Emulsification activity is one of the criteria used in selection of potential biosurfactant producers (Satpute *et al.,* 2008). Most researchers determine surface active properties of biosurfactants from microorganisms based on emulsification activity and surface tension (Varjani and Upasani, 2019; Bueno *et al*., 2019; Nwaguma *et al*., 2019). It has been reported that if a cell-free culture broth used in an assay contains biosurfactant, it will emulsify the hydrocarbon present in the test solution (Thavasi *et al.*,2011a). In this study, kerosene was used as the hydrophobic substrate, and the results (Table 7) revealed that out of the seventeen isolates cultured for biosurfactant production, only two isolates showed zero emulsification activity. The positive ones produced different levels of emulsification, ranging from 6.67 – 93.3%. Contrary to the observation in this study, Ellaiah *et al*.(2002), screened 68 bacterial

isolates grown on mineral salt medium and found only 6% of the isolates with emulsification activity up to 61%. However, Bodour and Maier (2000), suggested that maximum of two or three screening methods should be used for the selection of biosurfactant producers.

P.aeruginosa strains recovered in this study were excellent biosurfactant producers. This agreed with the work of other researchers who had reported that*Pseudomonas aeruginosa*are capable of biosurfactant production (Aransiola*et al*., 2019; Nurul *et al*., 2019, Peekate and Abu, 2017, Abdurrahim *et al*., 2009; Subasioglu and Cansunar, 2008).

Konsoula and Liakopoulou-Kyriakides (2007), suggested that selection of appropriate carbon and nitrogen sources or other nutrients is one of the most critical stages in the development of an efficient and economic biosurfactant production process. Pattanathu *et al.*(2010), opined that biosurfactant production strongly depends on the composition of the medium, which affects the efficiency of production. Although most microorganisms produce biosurfactants in the presence of water-soluble substrates such as glucose, sucrose, glycerol, maltose and other carbohydrates, the use of low-cost raw material as carbon source is being emphasized on, to reduce production cost (Rashedi *et al*., 2005). In line with one of the waste management strategies (reuse) and for a cost effective production of biosurfactant, several waste materials were screened for use as carbon source for biosurfactant production by the isolates in this study (Figure 7).

The biosurfactant yields with sugar cane molasses were significantly high (P-value < 0.05) [Appendix iii - v]when compared to the other waste materials screened. Sugar cane molasses produced biosurfactant with emulsification index (E24) 77.6± 2.6% for *P.aeruginosa* strain CCUG, 75.3±0.6% for *P.aeruginosa* strain I3 and 89.4±0.8% for *P.aeruginosa* strain ST11 (Figure 7). Although *P.aeruginosa* strain CCUG only produced zero percent emulsification index with spent generator-lubricating oil, the three isolates

produced significant amount of biosurfactants with the other waste materials tested, having E24 ranging from 6.08±0.6 – 59.3±2.1%. The high E24 observed with the use of sugar cane molasses in biosurfactant production may be attributed to sugar compositions (sucrose, fructose, glucose and other carbohydrates) of the sugar cane molasses. The result obtained is supported by the work of Raza *et al*. (2016) and Mouafo *et al*.(2018). Raza *et al*.(2016),noted a high biosurfactant yield (1.46g/L) with blackstrap molasses as the carbon source in their work on response surface optimization in biosurfactant production using a renewable growth substrate, while Mouafo *et al*.(2018), obtained 81± 1.14% emulsification index with sugar cane molasses as carbon source for biosurfactant production by *Lactobacillus* strains. On the contrary, Thavasi *et al*.(2007), observed a high lipopeptide biosurfactant production by *Corynebacterium kutscheri*using waste motor lubricating oil as carbon source.

From the results obtained in Figure 5, on the screening of different nitrogen sources for biosurfactant production by the *Pseudomonas aeruginosa* strains, sodium nitrate was observed to produce very high biosurfactants from the isolates. This observation is in agreement with the work of Subasioglu and Cansunar (2008), who observed that out of six nitrogen sources studied, sodium nitrate was found to give the highest production of rhamnolipid. There were significant differences observed in the biosurfactant production by the isolates in the presence of the various nitrogen sources (P-value < 0.05) [Appendices vi - viii].

Multiple regression analysis using response surface methodology was carried out to fit the regression model to the experimental data and investigate the effect of the four variables selected (Abalos *et al*., 2002). Table 8shows the central composite experimental design matrix for optimization of biosurfactant production by the *Pseudomonas aeruginosa* strainsand the actual responses obtained. A total of 31 experiments were performed and emulsification indexes after 24 hr were recorded as the response. ANOVA table was used to

test the significance and acceptability of the regression model developed, while the regression model, a quadratic polynomial equation, gives the best interactions of the variables for an optimum response. Tables 10, 12and 14 (ANOVA tables) show that the regression, linear and their quadratic terms (squares) were statistically significant ($P< 0.05$), with concentration of sugar cane molasses showing significant effect in production by all the *Pseudomonas aeruginosa* strains (Tables 9, 11 and 13).The interactions of the variables showed significant effect on biosurfactant production by *Pseudomonasaeruginosa* strain CCUG and strain I3, but did not show any significant effect on biosurfactant production by *Pseudomonasaeruginosa* strain ST11 (P-value > 0.05). The non-significance of the lack of fit test indicated adequacy of the model for optimum biosurfactant production by the *Pseudomonasaeruginosa* strains (P-value > 0.05) (Tables 10, 12 and 14).

The normal probability plot of the actual value against the predicted values (Figures 6 – 8) demonstrated that the actual values (Y1, Y2 and Y3) were distributed near the straight line and within the confidence limit, indicating that such values were very close to the predicted values, with regression coefficients greater than 80% (R_1^2 = 84.15%, R_2^2 = 83.83% and R_3^2 = 87.39%). Therefore, the model proved to be suitable for the prediction of biosurfactant production by the *Pseudomonasaeruginosa* strains under the experimental conditions.

The optimization of the four variables for the best response (E24)in this study was carried out using the response optimizer of the Minitab software version 17. The optimum process conditions for production of biosurfactant by *P.aeruginosa* strain CCUG obtained by the regression model were found to be 20g/L of molasses, 5g/L of sodium nitrate, 1.93ml inoculum size and 60ml medium volume in 250ml conical flask (Table 15), while the optimum process condition for production by *P.aeruginosa* strain I3 were found to be 25g/L of molasses, 25g/L of $NaNO_3$, 1ml inoculum and 60ml medium volume in 250ml conical

flask. The optimum conditions for production by *P.aeruginosa* strain ST11 are 16.92g/L molasses, 15.51g/L $NaNO_3$, 1.93ml inoculum and 41.4ml medium volume in 250ml conical flask. In order to validate the experimental model, the optimum conditions were employed for each *Pseudomonas aeruginosa* strain, and markedly high biosurfactant yield based on emulsification index were obtained (Table 20). The predicted emulsification index and the actual responseobtainedwith the optimum conditions with *Pseudomonas aeruginosa* strain CCUG and I3 were not close, while that by *Pseudomonas aeruginosa* strain ST11 was quite close, thus validating the model. Khademolhosseini*et al.* (2019), reported that salinity out of three variables (carbon concentration, salinity and inoculation) had greatest effect on biosurfactant production by*Pseudomonas aeruginosa* HAK01 during their study onphysicochemical characterization and optimization of glycolipid biosurfactant production by a native strain of *Pseudomonas aeruginosa* HAK01 and its performance evaluation for the MEOR process. Moshtagh *et al*. (2019),optimized biosurfactant production by *Bacillus Subtilis* N3-1P using response surface methodology with brewery waste as the carbon source, and reported high biosurfactant yield during experimental validation of predicted responses under optimum condition.

A low critical micelle concentration (CMC) and the ability to lower surface tension of aqueous solutions are considered important properties of a potent surface-active agent (Silva *et al*., 2014). The capillary rise method used in this study was based on the fact that the height of the biosurfactant-containing liquid in a capillary tube is directly proportional to the surface tension of the liquid (Adamson, 1997). The biosurfactant, thus reduces the surface tension (cohesive force between water molecules) and suppresses the height of the liquid in the tube. Surface tension measurements of the cell-free culture broths obtained in this study showed that *P. aeruginosa* strain CCUG, strain I3 and strain ST11were able to reduce surface tension of water from 72.1±0.0mN/m to 45.0±0.0mN/m, 55.0±0.1mN/m and 42.3±0.2mN/m

respectively(Figure 12). A much lower surface tension reduction of 28.8mN/m was attained in distilled water by biosurfactant produced by *Rhizopus arrhizus* UCP1607 in low-cost culture medium (Pele *et al*., 2019). Biosurfactant from *Pseudomonas putida* MTCC 2467 culture reduced medium surface tension from 74mN/m to 35 mN/m (Kanna *et al*., 2014).Biosurfactant from *P.aeruginosa* strain CCUG and ST11 attained critical micelle concentrations (CMC) at 60mg/L, while that of *P. aeruginosa* I3 was reached at 50mg/L. At increasing concentration of the biosurfactant above the CMC, no significant reduction in the surface tension was observed (p-value > 0.05) [Appendix x, xi and xii]. These results are indicative of the tensoactive property of the biosurfactants. This is in line with the report of Chittepu (2019), who reported that the lipopeptide produced by *Bacillus pseudomycoides* OR1 attained critical micelle concentration at 60 mg/L and reduced the surface tension of water from 71.6 to 31.6 mN/m.

Emulsification index is an indirect measure of the amount of biosurfactant produced in the culture broth after the fermentation period. Biosurfactants in a solution aid in formation and stabilization of emulsion. The emulsification activity results (Figure 10) show that biosurfactants produced by the isolates formed stable emulsions with the hydrophobic substrates tested except with palm oil. They all gave varying emulsification activity with all the hydrophobic substrates (P -value < 0.05)[Appendices xiii - xv], with E24 ranging from 2.1±3.6 – 92.3±3.6%. The *Pseudomonas aeruginosa* strains performed maximally with crude oil substrate, thus showing their potential for use in crude oil biodegradation. Although *P.aeruginosa* strain CCUG and strain I3 showed poor performance with palm oil, *P.aeruginosa* strain ST11 produced significant amount of biosurfactant activity with palm oil.The high emulsification activity of the biosurfactants produced by the*Pseudomonas aeruginosa* strainsshow that the biosurfactants have very strong affinity for long-chain hydrocarbons (Figure 10). Emulsification index as high as 60, 65 and 100% were recorded by

Santos *et al*. (2019), for biosurfactants produced by *Streptomyces* sp. DPUA1566 with soybean oil, *Caryocar brasiliense* oil (pequi fruit oil) and waste motor oil respectively. Similarly, Thavasi *et al.*(2011b), observed that the biosurfactant produced by *Pseudomonas aeruginosa* was able to emulsify several hydrophobic substrates (waste motor lubricating oil, crude oil, peanut oil, kerosene, diesel, xylene, naphthalene and anthracene) better than the synthetic surfactant tested.

Methylene blue agar plate test is a preliminary screening test for glycolipid biosurfactant production, and the genus *Pseudomonas* is well known for production of rhamnolipid group of glycolipids (El-Sheshtawy and Doheim, 2014). Other microorganisms have also been identified as glycolipid producers(Ekprasert *et al*., 2019; Astuti*et al*., 2019; Chittepu, 2019). As shown inplate 1, biosurfactants from *Pseudomonas aeruginosa* I3 and ST11 produced dark blue halo with diameters of 1.4cm and 1.7cm respectively, while *P.aeruginosa* CCUG did not produce any halo after 72hr incubation. This indicated that the biosurfactants from *Pseudomonas aeruginosa* I3 and ST11 may likely belong to the glycolipid family. This result is similar to that obtained by Bhat *et al*. (2015), who reported that out of 75 *Pseudomonas* spp screened for glycolipid biosurfactant production on methylene blue agar plate, only 10 showed positive results. *Enterobacter cloacae* B14 isolated from petroleum-contaminated soil also gave a positive result for glycolipid production on methylene blue agar plate (Ekprasert *et al*., 2019).

The GC-MS analysis revealed the fatty acid components of the biosurfactants produced by the organisms (Table 16).Cyclotetrasiloxane was common in the biosurfactants from *P.aeruginosa* strain CCUG and strain I3, Octadecanoic acid and methyl stearate were found in both biosurfactants from *P.aeruginosa* strain CCUG and strain ST11. Recovery of these components as the functional components of the biosurfactants produced by the *Pseudomonas aeruginosa* strains are in line with the reports of other researchers (Parthipan *et*

al., 2017; Deepansh *et al*., 2014; Lobna and Ahmed, 2013). These components are likely the active agents in the biosurfactants produced by the *Pseudomonas aeruginosa* strains, which are responsible for enhanced crude oil degradation and heavy metal removal from polluted soil.Octadecanoic acidcommonly called stearic acid, is a surface active agent derived from natural fatty acids,which has excellent surfactant properties and is easily biodegraded (Klein *et al*., 2013). Cyclotetrasiloxane is used as hair conditioner, skin conditioner and in other cosmetics as foaming agent (Johnson *et al*., 2011). 9-octadecenoic acid, commonly called oleic acid, is a good emulsifying agent in soap, a moisturizer in creams (De Villiers, 2009), and a solubilizer in aerosol products (Smolinske, 1992), whilen-hexadecanoic acid, also known as palmitic acid, has excellent surface reducing property (Asadov *et al*., 2012).

The results of the physicochemical analysis of the crude oil-polluted and control soil samples are presented in Table 17.From the result, the electrical conductivity (EC) of the polluted and control soil were 93.3±0.3 μS/cm and 87.5±0.3μS/cm respectively. This result is contrary to the work of Eli and Agusomu (2015), who reported that the electrical conductivity of Otuoke soils in Bayelsa state ranged from 0.004 – 0.009dS/m.

Soil pH expresses the activity of hydrogen ions in the soil solution, and most agricultural crops grow best with mineral soil of pH range 5.5 – 7.5 (Oshunsanya, 2018). Results obtained on pH measurement (Table 17) show that polluted soil sample exhibited pH value of 5.8±0.01, while the unpolluted (control) soil had pH 6.5±0.03. Though the soils were weakly acidic, the pH values were within permissible range for agricultural soil. Ezekiel *et al*.(2017), reported that the pH of surface soils of Bayelsa palm Limited, Elebele – Yenagoa, Bayelsa state ranged from 4.7 – 6.4. Eli and Agusomu (2015), reported quite low pH values in Otuoke soils and opined that such low pH values suggested that the soils may contain pyritic materials (FeS_2).

Total nitrate and total phosphate content of the polluted and control soil as presented in Table 17were0.085±0.00mg/kg and 0.7±0.00mg/kg in polluted soil and 0.53±0.06mg/kg and 0.55 ±0.00mg/kg in control soil. There was observable difference in the nitrate content of the polluted and control soil, while no significant difference was observed in the phosphate content of the soils. Reduced nitrification process as a result of reduced microbial population could have contributed to the low nitrate content observed in this study. This result is contrary to the report of Tanee and Albert (2015), who recorded high nitrate and phosphate content (132±48.60mg/kg and 75.62±6.87mg/kg respectively) in crude oil polluted soil of Ogoni community, Khana Local government Area of River state, Nigeria.

The presence of organic carbon in any soil is indicative of presence of plant and animal residues, root exudates, and dead and living microorganisms (NRCS, 2011). The total organic carbon (TOC) of the polluted and control soils were 1.40 ±0.17% and 3.07 ±0.03% respectively, indicating presence of organic matters in the soils (Table 17). The low TOC value of the polluted soil when compared to the control soil may be as a result of reduced microbial activities and low plant residues in the polluted soil. However, contrary to the observation in this study, Nwankwoala and Omemu (2019), and Osakwe and Okolie (2015), reported quite lower TOC values. While Nwankwoala and Omemu (2019), recorded TOC values ranging from 0.098 – 1.131% during the evaluation of the physicochemical properties of the soil in Elebele Community in Ogbia Local Government Area of Bayelsa State, Osakwe and Okolie (2015), recorded TOC range of 0.09±0.80 - 1.20 + 0.13%. during their work on physicochemical characteristics and heavy metals contents in soils and cassava plants from farmlands along a major highway in Delta State, Nigeria.

The presence of heavy metals were observed in both the crude oil polluted and control soil at varying concentrations (Table 17).As observed, higher concentrations of the heavy metals were found in the polluted soil than in the control. The presence and higher

concentrations of the heavy metals observed in the polluted soil compared to the control soil is in agreement with the report that Nigerian crude oil is rich in heavy metals (Koliander, 2000). Notwithstanding, the heavy metal concentrations in the crude oil-polluted and control soils fell within the permissible limit stipulated by the Department of Petroleum Resources (DPR) for agricultural soil (DPR, 2002).

The total petroleum hydrocarbon content was relatively higher in the polluted soil than the control soil (Table 17). The control soil which was collected based on visual examination 100 metresaway from the polluted site contained relatively small amount of TPH. The dehydrogenase enzyme activity was higher in the control soil than in the polluted soil.Dehydrogenase enzyme assay is indicative of oxidative activities of living organisms in the soil.

Several researchers have reported different populations of bacteria and fungi in crude oil-polluted and unpolluted soil. **(**Borowik*et al*., 2019; Adekunle *et al*., 2015; Udosen and Okon, 2014).Table 18,shows the heterotrophic microbial count in the polluted and control soil before bioremediation. There was significantly higher populations of bacteria and fungi in the control soil than in the polluted soil (P-value < 0.05), and this may likely be as a result of availability of more nutrients and low hydrocarbon pollutant in the control soil. Contrary to the microbial count obtained in polluted soil in this study, Olukunle (2013), observed high bacterial and fungal count (9×10^4 - 31.67×10^4 cfu/g and 4×10^6 - 16×10^6 cfu/g respectively) in his work on characterization of indigenous microorganisms associated with crude oil-polluted soils and water using traditional techniques.

The six indigenous bacteria isolated from the crude oil-polluted soil based on biochemical characteristics belonged to the genus *Staphylococcus, Citrobacter, Micrococcus, Pseudomonas, Bacillus* and *Corynebacterium,* while the fungal isolates were *Aspergillus*

niger, Aspergillus flavus Penicillium sp.The isolates in this study make the list of the commonly isolated microorganisms in hydrocarbon-polluted environment, and this observation is supported by the work of Onifade and Abubakar(2007). This finding is also in agreement with the work of Ataikiru *et al*.(2017), who isolated nine different genera of bacteria including *Staphylococus, Micrococcus, Bacillus, Pseudomonas,Acinetobacter, Enterobacter, Escherichia, Klebsiella* and *Proteus* from hydrocarbon-polluted soil in Effurun, Delta State.Similarly, Umeaku *et al*. (2019), isolated *Bacillus* spp., *Pseudomonas* spp., *Serratia* spp., *Micrococcus* spp., *Arthrobacter* spp., *Proteus* spp. and *Shigella* spp from three different crude oil-polluted sites in Anambra state.Mansi *et al*.(2018), isolated *Penicillium* spp. and *Aspergillus niger* alongside other six fungal organisms from a crude oil-polluted soil in Bayelsa state, Nigeria, and this corroboratesthe findings in this study in which *A. flavus,A. niger* and *Penicillim* sp were isolated from crude oil polluted soil.

Metagenomic analysis carried out on the polluted and control soil was to determine the abundance and diversity of microorganisms present in the soil samples. The analysis revealed that large population and diversity of bacteria were present in the soil samples (Figure 11). A total of 47,616 and 53,069 sequence reads which gave rise to operational taxonomic units were obtained, out of which 3.126% and 1.083% in the polluted and control soil respectively were archaea (Figure 11). Two archaeal phyla were recovered from the soil samples (Figure 12) – Crenarchaeota and Euryarchaeota at 0.295% and 2.83% in the polluted soil, and 0.853% and 0.233% in the control soil. As observed in Figure12, Euryarchaeota occurred more in the polluted soil, while Crenarchaeotawas more in the control soil.

Archaea are mostly extremophiles and can survive extreme polluted environment. Traditionally, the study of microbial diversity in crude oil-polluted soil had focused on eubacteria but recent studies have revealed that archaea exist in large populations in crude oil-polluted soil and works synergistically with bacteria during degradation (Krzmarzick*et*

al., 2018). The long-term pollution of the soil used in this study allowed for the adaptation of archaeal population in the soil.

The phylum Crenarchaeotahave been reported as the archaeal phylum commonly found in natural soils while Euryarchaeota such as the methanogens occur in polluted environment (Bates *et al*., 2011). This report supports the relative high occurrence of Euryarchaeota in the polluted soil in this study. They are well adapted to acidic environments and are highly tolerant to heavy metals. The low proportion of archaea compared to bacteria obtained in this study (Figure 11) is in line with the work of Siles and Margesin (2018), who reported archaeal community abundance of 0.05 – 3.2% in a hydrocarbon-polluted soil.

The operational taxonomic units (OTU) obtained from the NGS amplicon sequencing were assigned to 300 genera, 133 classes and 54 phyla. Figures 13 and 14 show the dominant phyla and classes with above 1% relative abundance, while Figure 15shows the dominant genera with above 0.5% relative abundance. Proteobacteria predominated in the soil samples with relative abundance of 47.4% and 58.5% in the polluted and control soil respectively. While the Actinobacteria, Chlamydiae, Chloroflexi, Euryarchaeota, Plantomycetes and Verrucomicrobia occurred more in the polluted soil, Acidobacteria, Bacteroidetes, Firmicutes and Proteobacteria occurred most in the control soil (Figure 13). These phyla are reportedly involved in aerobic degradation of hydrocarbon (Yergeau *et al*., 2012). While the class Betaproteobacteria predominated in the control soil, Alphaproteobacteria and Betabacteria predominated in the polluted soil (Figure 14). The most occurring genus in the polluted and control soil samples werethe*Candidatus koribacter* and*Comamonas* respectively. The organisms identified based on the metagenomic analysis as the indigenous organisms agreed with the organisms isolated based on culture-dependent technique, and these organisms are known to be associated with hydrocarbon contaminated environment(Gan *et al*., 2018). Although the recovered sequence reads showed that there was little variation in diversity of

bacteria found in the polluted and control soil, the predominant bacteria were found in both soils. Several researchers have reported the identification of these communities of organisms in crude oil-polluted soil (Gan *et al*., 2018; Kumar *et al*., 2018; Galazka *et al*., 2018).

Result of crude oil degradation by the indigenous microorganisms isolated from the crude oil-polluted soil presented in Figure16,shows that all the bacterial and fungal organisms had the ability to degrade crude oil. These bacteriahave the ability to synthesize biosurfactants in the presence of hydrophobic substrate, which makes the hydrocarbon bioavailable to them. This is in line with the works of Nurul *et al*. (2019), Cheng *et al*. (2017) and Eddouaouda *et al*. (2011). Christova *et al*. (2019), also reported similar high crude oil degradation rate (93%) with *Bacillus cereus* during their work on biodegradation of crude oil hydrocarbons by a newly isolated biosurfactant producing strain.

The optimal degradation of the crude oil achieved by the bacteria consortium (Figure 16) could be as a result of synergistic interaction among the bacterial organisms. This observation is in line with the report of Tian *et al*. (2018), who recorded 80.4% crude oil degradation rate with mixed bacteria culture. The fungi consortium also achieved high degradation rate (46.7±6.1) when compared to single fungal isolates. Ramoutar *et al*.(2019), recorded quite high degradation of petroleum hydrocarbon (92%) with fungi consortium comprising of *Aspergillus terreus*-SRF-15,*Fusarium proliferatum*-SRF-50, *Fusarium* sp-SRF-58 and *Aspergillus* sp-SRF-67. The high degradation of crude oil observed with *Penicillium* sp in this study is similar to that reported by Al-Hawash *et al*. (2018).They noted a 57% and 55% crude oil degradation by *Penicillium* sp. RMA1 and *Penicillium* sp. RMA2 respectively isolated from Rumaila oil field. The poor degradation of crude oil (Figure 16) observed with bacteria/fungi consortium and *Aspergillus* spp.in this study is contrary to the report of Ra *et al*. (2018). They recorded a 42.24% degradation of crude oil by bacteria/fungi consortium and 47.72% by *Aspergillus fumigatus*in their comparative study of petroleum

crude oil degradation potential of microbes from petroleum-contaminated soil and non-contaminated soil. Burghal *et al*. (2016), also noted a 94% crude oil degradation by *Aspergillus niger* isolated from petroleum contaminated soil.

The results of the parameters studied during bioremediation are presented in Figures 17 – 25.The count of heterotrophic bacteria present in the contaminated soil upon bioremediation treatments and the percentage increase in bacteria population are shown in Figures 17 and 18.There was significant increase (P-value < 0.05) in bacteria population in the soil treatments throughout the remediation period. The control soil, which was free of microbial growth from the start of the experiment showed significant increase in bacteria at the end of the bioremediation period, having log bacteria count of 2.77±0.04 (Figure 17). The bacteria population present in the control soil are contamination from the surrounding environment. A significant increase in bacterial count of the natural attenuation treatment (34.6%) was observed at the end of the experiment (Figure 18). This could be related to the periodic moistening and aeration of the contaminated soil, which could have provided favorable environment for the proliferation of the indigenous organisms. This result is contrary to the report of Chikere *et al*.(2017) and Ebuehi *et al.* (2005). Chikere *et al*.(2017), recorded a slight decrease in culturable hydrocarbon utilizing bacteria population (from 7.7×10^4 to 6.1×10^4 cfu/g) during their study on remediation by enhanced natural attenuation (RENA) of a crude oil-impacted soil in Ikarama community of Bayelsa, Nigeria, while Ebuehi *et al*. (2005), recorded drastic reduction in heterotrophic bacteria count (from 1.22×10^8 to 5.98×10^5 cfu/g) during remediation of crude oil contaminated soil by enhanced natural attenuation technique.The bioaugmentation treatments (Figure 18) showed maximal increase in bacteria population at the end of the experiment.The allochthonous biosurfactant-producing bacteria used to augment the indigenous organisms in this study adapted to the contaminated soil and maintained synergistic interaction with the indigenous organisms,

hence, the increase in heterotrophic bacterial count observed in the bioaugmentation set-ups.Burghal *et al*. (2015), reported 28.1% increase in heterotrophic bacteria count in the experimental set-up augmented with actinomycetes consortia.Increase in bacterial count was also observed in biostimulation treatments. Stimulation of polluted soil with nutrients enhances proliferation of heterotrophic bacteria (Adams *et al*., 2015), hence, the increase in heterotrophic bacterial count observed in the biostimulation set-ups in this study. Nwogu *et al*. (2015), reported about 8.5% increase in culturable bacterial count during a 14-day biostimulation of petroleum contaminated soil with goat manure.

The total fungal count present in the contaminated soils upon bioremediation treatments and the percentage increase in fungal population are shown in Figures 19 and 20.A significant increase in fungal population was observed in the soil treatments throughout the remediation period(P-value < 0.05) [Appendix xxiv]**.** The percentage increase in all the treatments ranged from 16.4 – 25.6%. There was no significant difference in the percentage increase in fungal count in the treatments amended with nutrient + biosurfactant and biosurfactant only (BiostimulationB and C respectively) having P-value0.34. The introduction of *Pseudomonas aeruginosa* strains in the crude oil-polluted soil (Bioaugmentataion A – D) promoted the growth of the fungal organisms present in the soil. This indicated that a positive symbiotic interaction mayoccurred between the bacteria and fungi population, which resulted in enhancement of their growth (Figure19). The observation is in line with the work of Ataikiru *et al*. (2018), who reported an increase in culturable fungal population (10^2 to 10^3cfu/g) in the first fourteen days of bioaugmenting a crude oil-polluted soil with *Candid*a spp. They noted that the allochthonous microorganisms adjusted rapidly to the crude oil contamination. Ibiene *et al*. (2011), observed that the fungal population in crude-oil polluted soils in Niger Delta changed during their 90 days study of the

sites, and following the natural attenuation protocol, the fungal population increased from 4.3 $\times 10^4$ to 0.74 $\times 10^5$ cfu/g.

The pH values of the contaminated soil under the various treatments are shown in Figure 21**.**The pH values observed in all the treatments ranged from 5.80 – 7.21. There was no steady increase nor decrease in pH observed in the treatment, rather they fluctuated during the bioremediation period. Although the initial pH (5.80) of the soil was within the acceptable pH value for agricultural soil (Oshunsanya, 2018) and microbial activities, the treatments influenced the pH value of the soil relative to time. However, at the end of the experiment, the pH in all the treatments increased compared to the initial pH of the polluted soil. The residual components of the crude oil and increase in the exchangeable bases and metabolites in the polluted soil during these periods may have contributed to the fluctuations and increased pH observed. There was no difference in pH among soils augmented with *P. aeruginosa* strain CCUG (Bioaug A), *P.aeruginosa* ST11 (Bioaug.C), bacteria consortium (Bioaug.D) and the soil stimulated with biosurfactant only (Biostim.C), while the pH of the control soil remained nearly the same during the whole experiment (5.80 – 5.85) [Figure 21]. Similar change in pH was reported by Polyak *et al*. (2018) during their 9-year field study of a site polluted with crude oil. They noted that pH is one of the main factors that affect the success of bioremediation and therefore, should be monitored and controlled to ensure that optimum conditions are maintained for bioremediation.

Dehydrogenase enzyme assay is indicative of microbial oxidative activities in the soil, and was carried out during the bioremediation period to determine the oxidative activities of microorganisms in utilizing the crude oil contaminant in the soil as carbon source (Figure 22). It was also used as an indirect measure of the efficiency of the treatments towards enhancement of aerobic crude oil degradation by the microorganisms in the soil. Figure22shows that significant microbial activities were observed in the soil treatments.

There was progressive increase in dehydrogenase enzyme activity (DHase) observed in all the treatments. Among the bioremediation treatments, the highest microbial activity at the end of the experiment occurred in bioaugmentation, with DHase value of 16.4 ± 0.40 mg/g observed in the treatment containing the bacteria consortium (Bioaugmentation D). The allochthonous organisms used in this study were biosurfactant producers, thus were able to make the hydrophobic crude oil contaminant bioavailable to the microorganisms in the soil, hence the increased microbial activities observed in this study. Significant microbial activity was observed upon natural attenuation relative to the control soil. There was a strong positive correlation between bacterial count and dehydrogenase assay ($r = 0.733$, $p < 0.05$)[Appendix xxxvii], indicating that the increasing DHase activities corresponded with the increasing microbial population in the soil. This is in agreement with the report of Polyak *et al*. (2018), who recorded 77% increase in dehydrogenase activity under bioaugmentation of crude oil-polluted soil after two years of treatment. In contrast, Ogbolosingha *et al*. (2015), reported a decrease in dehydrogenase activity under natural attenuation, bioaugmentation and biostimulation during a 90-day bioremediation of an artificially polluted soil.

Figures 23 and 24 show the residual total petroleum hydrocarbon (TPH) pattern in the bioremediation treatments studied and the percentageTPH degradation after 12 weeks bioremediation respectively. A significant reduction in the quantity of TPH was observed in all the treatments ($p < 0.05$) [Appendix xxvi].Greater than 50% reduction from the 4^{th} week was observed in all the treatments except in natural attenuation and bioaugmentation B. The crude oil degradation correlated significantly with microbial activity ($r = 0.651$, p-value < 0.05)[Appendix xxxviii], which shows that the treatments of the crude oil-polluted soil were efficient in enhancing the biodegradation activities of the indigenous organisms (Figure 23). As the microbial count increased, the microbial activities improved resulting in increased crude oil degradation. At the end of the 12 weeks experiment, the bioaugmentation and

biostimulation treatments achieved above 85% degradation (Figure 24). There was significant difference among the TPH degradation achieved by the three biostimulation treatments used in this study ($P< 0.05$)[Appendix xxvi]. The highest percentagecrude oil degradation (95.4 ±0.40 %) was observed in the soil treated with biosurfactant and nutrient (Biostimulation B), followed by the soil biostimulated with only biosurfactant (Biostimulation C). Biostimulation with only nutrient (Biostimulation A) also performed maximally, achieving 91.8 ±0.26% TPH degradation. Nutrient availability is one of the factors that affect hydrocarbon degradation by microorganisms and from this result (Figure 24), it can be deduced that the presence of nitrogen and phosphorus stimulated the utilization of the crude oil as carbon source by the indigenous organisms. The biosurfactant thus increased the availability of the crude oil contaminant to the organisms, hence, the increased degradation of the crude oil observed in the biostimulation set-ups (Figure 24). Several researchers have reported improved biodegradation of hydrocarbon by addition of nitrogen and phosphorus (Wu *et al*., 2019; Ogbeh *et al*., 2019; Semboung *et al*., 2016). Although the bioaugmentation with bacteria consortium (bioaugmentation D) performed best among the bioaugmentation set-ups, there was no significant difference in the TPH degradation among the bioaugmentation set-ups (A – D) having p-value > 0.05 (Appendix xxvi).The biosurfactant-producing *Pseudomonas aeruginosa* strains were able to augment the degradation potential of the indigenous organisms, exhibiting synergistic effect on the indigenous organisms. This observation is similar to the work of Yanan *et al*. (2019), who recorded improved degradation of diesel by diesel-degrading organisms, in the presence of lipopeptide-producing bacteria. Bidja *et al*. (2019), compared the biodegradation efficiencies of natural attenuation and bioaugmentation for the degradation of highly contaminated soils in China and Kuwait and recorded that bioaugmentation treatment showed better efficiencies than the natural attenuation. Monitoring the autochthonous organisms and maintaining their environmental

factors such as aeration in any contaminated site is the simplest and cheapest approach for remediating the site (Azubuike *et al*., 2016). The natural attenuation technique employed in this study (Figure 24) showed slightly improved crude oil degradation performance when compared with other treatments. This observation is contrary to the report of Piñón-Castillo *et al*. (2017), who reported a crude oil degradation performance order: biostimulation > natural attenuation > bioaugmentation, in their one year study on laboratory-scale biodegradation of Fuel Oil No.6 in contaminated soils by autochthonous bacteria. Also Bento *et al*. (2005), reported that in Hong Kong soil, natural attenuation resulted in highest degradation of light fraction of TPH when compared to bioaugmentation, and opined that such efficiency could be attributed to the more physiological compatibility of the autochthonous organisms with their habitat than the foreign allochthonous organisms.

Figure 25shows the percentage heavy metal removal in the experimental set-ups after the 12 weeks bioremediation. The initial physicochemical examination of the crude oil-polluted soil shows the presence of arsenic, lead, mercury, cadmium and chromium. At the end of the experiment, various percentage heavy metal removal were observed in the treatments, ranging from 0.53±0.46 % to 100%. There was significant difference in the heavy metal removal efficiency of the experimental treatments used in this study ($P< 0.05$)[Appendices xxvii - xxxi]. It can be observed in figure 25, thatthe removal efficiency of natural attenuation set-up was relatively low compared to the bioaugmentation and biostimulation set-ups. Complete removal of arsenic (100%) was achieved in the soil treated with consortium of *Pseudomonas aeruginosa* strains (Bioaugmentation D). The arsenic removal efficiency and crude oil degradation in the bioaugmentation set-ups agreed strongly with correlation coefficient of 0.819 and p-value < 0.01 (Appendix xxvii). The *Pseudomonas aeruginosa* strains used for bioaugmentation in this study may have boosted the activities of the indigenous flora resulting in increased arsenic removal.The genus *Pseudomonas* has been

reported as arsenic oxidizers (Chattopadhyay *et al*., 2017), and are able to volatilize arsenic (Pandey *et al*., 2018; Chen *et al*., 2017). The stimulation of the polluted soil with nutrients and biosurfactant (biostimulation A – C) (Figure 25), showed an increase in arsenic removal, however, biostimulation A gave a better result.

At the end of the experiment, lead was completely removed in all the bioaugmentation and biostimulation set-ups (Figure 25), and percentage lead removal agrees positively with crude oil degradation (r = 0.948) [Appendix xxxviii]. This indicated that the *Pseudomonas aeruginosa* strains used are likely to be efficient at either complexation or biosorption of lead and that the biosurfactants werealso good agents for removal of lead from polluted soil. *Pseudomonas aeruginosa* has been identified as one of the highly efficient bacteria at bioremediation of lead polluted soil (Akhtar *et al*., 2013). Juwarkar *et al*. (2007), reported 88% removal of lead from polluted soil using 0.1% di-rhamnolipid. Xiaoyu *et al*. (2018) recorded 44.8% lead removal from an artificially polluted soil using 8% crude sophorolipid.

There was significant difference in the percentagemercury removal in the bioaugmentation and biostimulation set-ups (Appendix xxix). However, 100% mercury removal was observed only in the soil treated with *P.aeruginosa* I3 (bioaugmentation B). The treatment with bacteria consortium (bioaugmentation D) gave the second best result (99.9 ± 0.07%) in mercury removal. The percentage mercury removal correlated very strongly with crude oil degradation (r value = 0.891) [Appendix xxxiv]. This result is supported by the work of Kotwal *et al*. (2018), who reported that out of the mercury resistant bacteria isolated from metal contaminated site, *P. aeruginosa* showed highest bioremediating capacity for mercury under laboratory conditions.

High percentage removal of cadmium was observed in all the bioaugmentation and biostimulation treatments, with percentage cadmium removal ranging from 87.9±0.31% to

100% (Figure 25). The complete removal of cadmium was seen in bioaugmentation C, bioaugmentation D and biostimulation A. Pearson correlation shows that the rate of cadmium removal correlated significantly at 0.01 level (r = 0.949) [Appendix xxxv]. *Pseudomonas aeruginosa* has been reported as an efficient biosorbent agent for the removal of cadmium from contaminated soil and water (Chellaiah, 2018). The high percentage cadmium removal recorded in the bioaugmentation set-ups supports the work of Bojorquez *et al*. (2016), who carried out a biosorption studies and reported that adapted cells of *P*. *aeruginosa* strain JCM 5962 and genetically engineered (GE) *P*. *aeruginosa* were able to remove cadmium efficiently. The high cadmium removal achieved in the soil stimulated with biosurfactant is supported by the work of Mulligan (2005),who reported that biosurfactants are able to complex and remediate heavy metals such as cadmium, lead and zinc. The biosurfactants thus complexed the cadmium metal and enhanced their removal.

Chromium was completely removed only in bioaugmentation C (Figure 25). High percentage chromium removal was observed in the soils augmented with *Pseudomonas aeruginosa* strains. In comparison with percentage removal of other heavy metals, the bacteria consortium performed poorly (bioaugmentation D). The interaction of the organisms in the consortium may have produced a negative effect in the presence of chromium, hence the poor performance recorded. The combination of nutrient and biosurfactant (biostimulation B) gave low chromium removal, while the introduction of nutrient and biosurfactant individually (biostiimulation A and C) enhanced chromium removal by 97.3±0.10% and 93.18±0.18% respectively. Contrary to the findings in this study, Ashruta *et al*. (2014), reported 75 – 85% removal of chromium by bacteria consortia in less than two hours of contact duration.

5.2.CONCLUSION AND RECOMMENDATION

In this study, *Pseudomonas aeruginosa* strains capable of biosurfactant production were isolated. The screening of waste materials and nitrogen sources for use as carbon and nitrogen sources revealed that sugar cane molasses and sodium nitrate were the carbon and nitrogen sources of choice for biosurfactant production. Response surface methodology was effective at optimizing the process variables. The metagenomic analysis recovered several organisms that were limited on culture plate. The indigenous organisms in the polluted soil showed good ability to remove crude oil and heavy metal, with *Bacillus subtilis* and *Penicillium* sp as the best degrading bacteria and fungi respectively. Highest crude oil degradation was observed with biostimulation treatment, however, augmentation with crude oil degraders recorded above 89% crude oil removal. Therefore, bioremediation is the best alternative for reclaiming crude oil and heavy metal polluted soil, with biosurfactant as an excellent stimulant.

Further study and manipulation of the genome of *Pseudomonas aeruginosa*strainsfor improved biosurfactant yield are recommended. The combination of biosurfactants and nutrient gave the best result at crude oil and heavy metal removal from the soil in this study, therefore, commercial production of biosurfactant incorporated with nutrients such as nitrate and phosphate should be encouraged. Metagenomic analysis should replace the use of culture-dependent laboratory techniques for comprehensive information on community abundances and diversity in polluted environments.

5.3. CONTRIBUTION TO KNOWLEDGE

This study supports the reports by various researchers that bioremediation is highly effective at reclaiming crude oil and heavy metal polluted soil. Biosurfactant production can be optimized by statistical methods which are reliable, easy and saves time. Metagenominc analysis gives comprehensive data on population of microorganisms in polluted soil. Bioaugmentation and biostimulation are the best bioremediation approaches. The combination of biosurfactant and nutrient was excellent at reclaiming crude oil and heavy metal-polluted soil**.**

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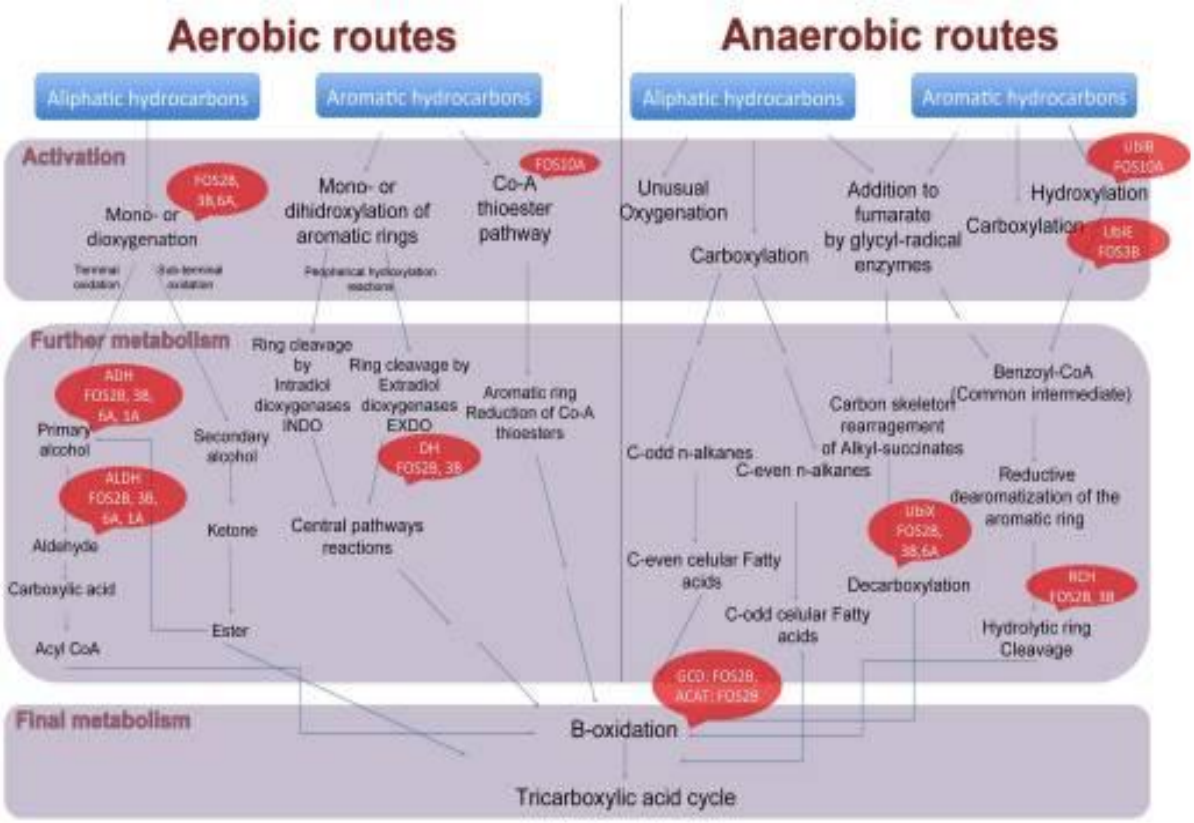
APPENDICES



Appendix i: Biochemical pathways for aerobic and anaerobic bacterial degradation of hydrocarbon compounds (Sierra-Garcia *et al*., 2014).

Appendix ii: Preparation of the substrates used as carbon sources

* Preparation of grounded banana peel

The peels of freshly peeled bananas were oven-dried and grounded to obtain a finely grounded powder which was used for screening

* Preparation of grounded potato peel

The peels of freshly peeled oranges were oven-dried and grounded to obtain a finely grounded powder which was used for screening

Appendix iii: Assessment of renewable waste materials as sole carbon source for biosurfactant production by*Pseudomonas aeruginosa* strain CCUG

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Screening for carbon source**for *P. aeruginosa* strain CCUG**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12017.372	5	2403.474	327.226	.000
Within Groups	88.140	12	7.345		
Total	12105.512	17			

Post Hoc Tests

Homogeneous Subsets

Screening for carbon source for *P. aeruginosa* strain CCUG

Student-Newman-Keuls

CARBON SOURCES	N	Subset for alpha = 0.05			
		1	2	3	4
Waste lubrication oil (generator)	3	.0000			
Orange peel	3		22.2667		
Potato peel	3		23.3333		
Banana peel	3		26.4567		
Waste lubricating oil (motor)	3			59.3333	
Sugar cane molasses	3				77.6333
Sig.		1.000	.183	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix iv: Assessment of renewable waste materials as sole carbon source for biosurfactant production by*Pseudomonas aeruginosa* strain I3

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Screening for carbon source for ***P. aeruginosa* strain I3**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9253.419	5	1850.684	1965.455	.000
Within Groups	11.299	12	.942		
Total	9264.718	17			

Post Hoc Tests

Homogeneous Subsets

Screening for carbon source for *P. aeruginosa* strain I3

Student-Newman-Keuls

CARBON SOURCES	N	Subset for alpha = 0.05				
		1	2	3	4	5
Waste lubrication oil (generator)	3	6.1000				
Potato peel	3		15.2667			
Banana peel	3			20.1233		
Waste lubricating oil (motor)	3			21.2333		
Orange peel	3				38.3000	
Sugar cane molasses	3					75.3333
Sig.		1.000	1.000	.187	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix v: Assessment of renewable waste materials as sole carbon source for biosurfactant production by*Pseudomonas aeruginosa* strain ST11

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Screening for carbon source for ***P. aeruginosa*** **strain ST11**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15245.740	5	3049.148	1408.675	.000
Within Groups	25.975	12	2.165		
Total	15271.715	17			

Post Hoc Tests

Homogeneous Subsets

Screening for carbon source for *P. aeruginosa* strain ST11

Student-Newman-Keuls

CARBON SOURCES	N	Subset for alpha = 0.05				
		1	2	3	4	5
Potato peel	3	6.0800				
Waste lubricating oil (motor)	3	8.3000				
Waste lubrication oil (generator)	3		12.4333			
Banana peel	3			22.1333		
Orange peel	3				42.0000	
Sugar cane molasses	3					89.3667
Sig.		.089	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix vi: Screening of nitrogen sourcesfor biosurfactant production by*Pseudomonas aeruginosa* strain CCUG

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Screening for nitrogen source for***P. aeruginosa*** **strain CCUG**

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	12228.667	3	4076.222	858.152	.000
Within Groups	38.000	8	4.750		
Total	12266.667	11			

Post Hoc Tests
Homogeneous Subsets

Screening for nitrogen source for *P. aeruginosa* strain CCUG

Student-Newman-Keuls

NITROGEN SOURCES	N	Subset for alpha = 0.05			
		1	2	3	4
Ammonium sulphate	3	3.3333			
Urea	3		23.0000		
Potassium nitrate	3			50.6667	
Sodium nitrate	3				88.3333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix vii: Screening of nitrogen sourcesfor biosurfactant production by*Pseudomonas aeruginosa* strain I3

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Screening for nitrogen source for***P. aeruginosa*** **strain I3**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6172.760	3	2057.587	264.812	.000
Within Groups	62.160	8	7.770		
Total	6234.920	11			

Post Hoc Tests
Homogeneous Subsets

Screening for nitrogen source for *P. aeruginosa* strain I3

Student-Newman-Keuls

Screening for nitrogen source for P.aeruginosa strain I3	N	Subset for alpha = 0.05		
		1	2	3
Urea	3	13.6667		
ammonium sulphate	3	18.2667		
potassium nitrate	3		52.0000	
sodium nitrate	3			67.6667
Sig.		.078	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix viii: Screening of nitrogen sourcesfor biosurfactant production by*Pseudomonas aeruginosa* strain ST11

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Screening for nitrogen source for **for *P. aeruginosa* strain ST11**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11291.729	3	3763.910	2243.761	.000
Within Groups	13.420	8	1.678		
Total	11305.149	11			

Post Hoc Tests
Homogeneous Subsets

Screening for nitrogen source for *P. aeruginosa* strain ST11

Student-Newman-Keuls

NITROGEN SOURCES	N	Subset for alpha = 0.05			
		1	2	3	4
Ammonium sulphate	3	6.1667			
Urea	3		53.3333		
Potassium nitrate	3			64.3333	
Sodium nitrate	3				91.0000
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix ix: Coded values of optimum conditions for maximum biosurfactant yield obtained using the regression model

P. aeruginosa	X_1(g/L)	X_2(g/L)	X_3(ml)	X_4(ml)
strain CCUG	2	- 2	- 0.1414	2
strain I3	2	2	- 2	2
strain ST11	0.3838	0.1010	- 0.1414	0.1414

Appendix x: Surface tension measurement for *Pseudomonas aeruginosa* strain CCUG

```
ONEWAY Tension BY Concentration
 /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Surface tension for ***P. aeruginosa* strain CCUG** (mN/m)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2705.310	7	386.473	249.069	.000
Within Groups	24.827	16	1.552		
Total	2730.136	23			

Post Hoc Tests
Homogeneous Subsets

Surface tension for *P. aeruginosa* strain CCUG (mN/m)

Student-Newman-Keuls

Biosurfactant concentration	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
70mg/L	3	45.0000					
80mg/L	3	45.0000					
60mg/L	3	45.2667					
50mg/L	3		48.3333				
40mg/L	3			61.1000			
30mg/L	3				64.0000		
20mg/L	3					67.7333	
10mg/L	3						72.0667
Sig.		.963	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xi: Surface tension measurement for *Pseudomonas aeruginosa* strain I3

```
ONEWAY Tension BY Concentration
 /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Surface tension (mN/m) for ***Pseudomonas aeruginosa* strain I3**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1068.573	7	152.653	488.491	.000
Within Groups	5.000	16	.313		
Total	1073.573	23			

Post Hoc Tests
Homogeneous Subsets

Surface tension (mN/m) for *Pseudomonas aeruginosa* strain I3

Student-Newman-Keuls

Biosurfactant concentration	N	Subset for alpha = 0.05			
		1	2	3	4
80mg/L	3	55.0333			
60mg/L	3	55.1000			
70mg/L	3	55.1667			
50mg/L	3	55.3333			
40mg/L	3		60.6667		
30mg/L	3			65.1000	
20mg/L	3				70.8667
10mg/L	3				71.2667
Sig.		.911	1.000	1.000	.394

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xii: Surface tension measurement for *Pseudomonas aeruginosa* strain ST11

```
ONEWAY Tension BY Concentration
 /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Surface tension (mN/m)for ***Pseudomonas aeruginosa* strain ST11**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2893.218	7	413.317	2517.666	.000
Within Groups	2.627	16	.164		
Total	2895.845	23			

Post Hoc Tests
Homogeneous Subsets

Surface tension (mN/m) for *Pseudomonas aeruginosa* strain ST11

Student-Newman-Keuls

Biosurfactant concentration	N	Subset for alpha = 0.05				
		1	2	3	4	5
80mg/L	3	42.3000				
70mg/L	3	42.3667				
50mg/L	3	42.5000				
60mg/L	3	42.6333				
40mg/L	3		48.3000			
30mg/L	3			61.0000		
20mg/L	3				65.6333	
10mg/L	3					69.8667
Sig.		.747	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xiii: Emulsification activity by biosurfactant produced by*Pseudomonas aeruginosa* strain CCUG

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Emulsification activity for ***P. aeruginosa*** **strain CCUG**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10447.817	4	2611.954	1919.614	.000
Within Groups	13.607	10	1.361		
Total	10461.424	14			

Post Hoc Tests
Homogeneous Subsets

Emulsification activity for *P. aeruginosa* strain CCUG

Student-Newman-Keuls

Hydrophobic substrate	N	Subset for alpha = 0.05			
		1	2	3	4
Palm oil	3	6.5000			
Diesel	3		59.9667		
Kerosene	3			62.3333	
Groundnut oil	3				77.6667
Crude oil	3				78.6333
Sig.		1.000	1.000	1.000	.334

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xiv: Emulsification activity by biosurfactant produced by*P. aeruginosa* strain I3

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Emulsification activity for ***P. aeruginosa* strain I3**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11310.573	4	2827.643	459.929	.000
Within Groups	61.480	10	6.148		
Total	11372.053	14			

Post Hoc Tests
Homogeneous Subsets

Emulsification activity for *P. aeruginosa* strain I3

Student-Newman-Keuls

Hydrophobic substrate	N	Subset for alpha = 0.05			
		1	2	3	4
Palm oil	3	2.1000			
Diesel	3		56.1000		
Kerosene	3		59.2333	59.2333	
Groundnut oil	3			61.7667	
Crude oil	3				85.4667
Sig.		1.000	.153	.239	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xv: Emulsification activity by biosurfactant produced by*P. aeruginosa* strain ST11

```
ONEWAY E24 BY SUBSTRATE
 /MISSING ANALYSIS
 /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Emulsification activity for ***Pseudomonas aeruginosa*** **strain ST11**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3712.617	4	928.154	31.278	.000
Within Groups	296.747	10	29.675		
Total	4009.364	14			

Post Hoc Tests
Homogeneous Subsets

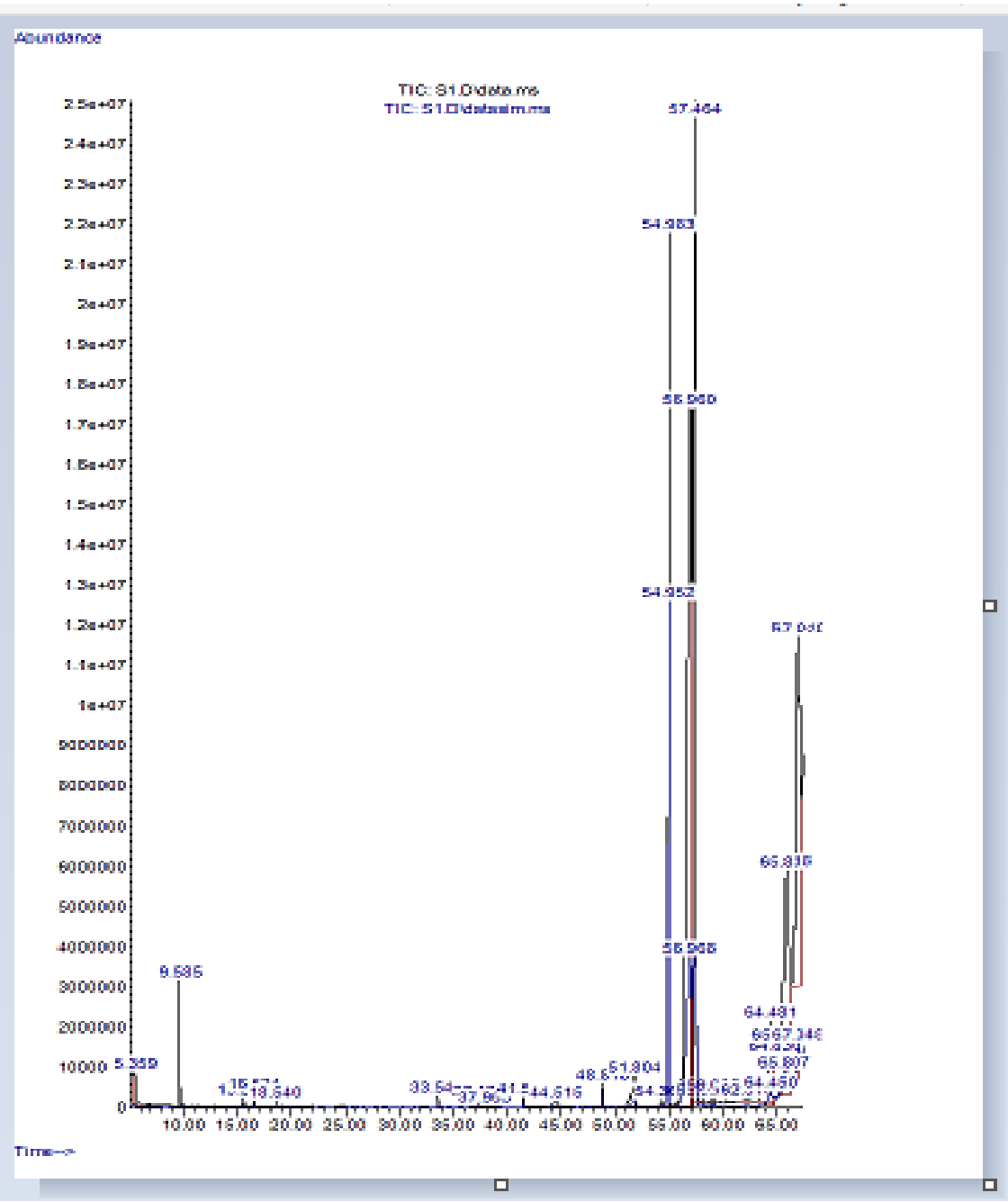
Emulsification activity for *Pseudomonas aeruginosa* strain ST11

Student-Newman-Keuls

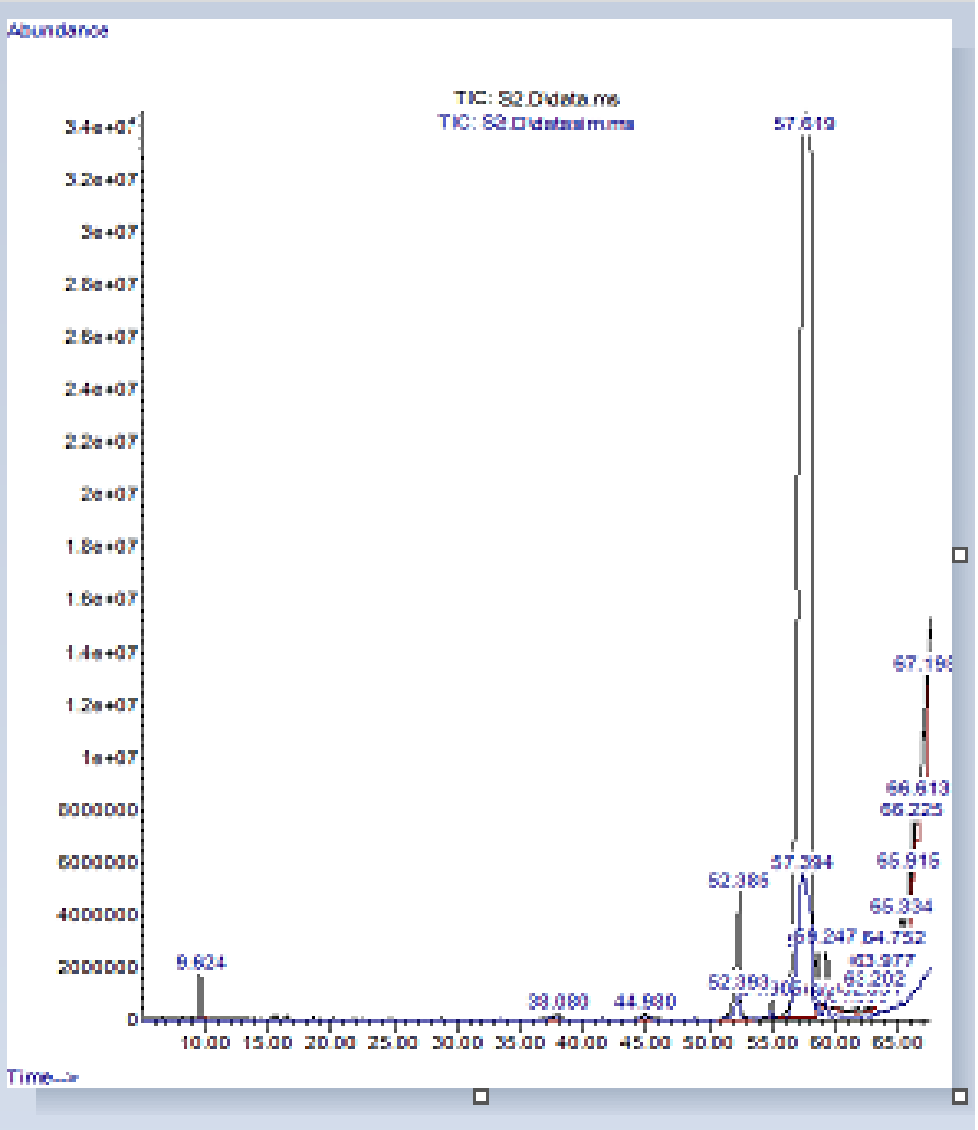
Hydrophobic substrate	N	Subset for alpha = 0.05		
		1	2	3
Groundnut oil	3	49.9333		
Diesel	3	59.6000		
Palm oil	3		76.7667	
Kerosene	3		84.7667	84.7667
Crude oil	3			92.3333
Sig.		.055	.102	.120

Means for groups in homogeneous subsets are displayed.

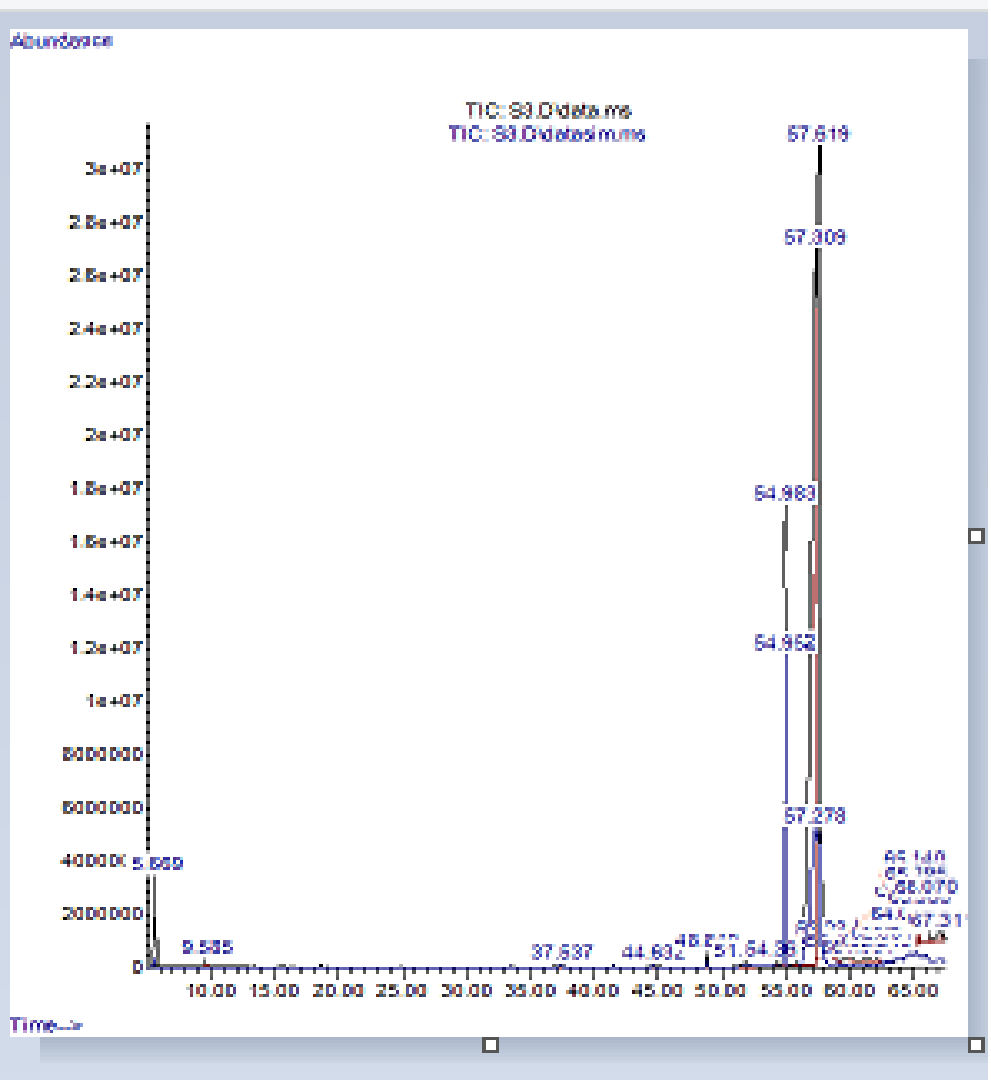
a. Uses Harmonic Mean Sample Size = 3.000.



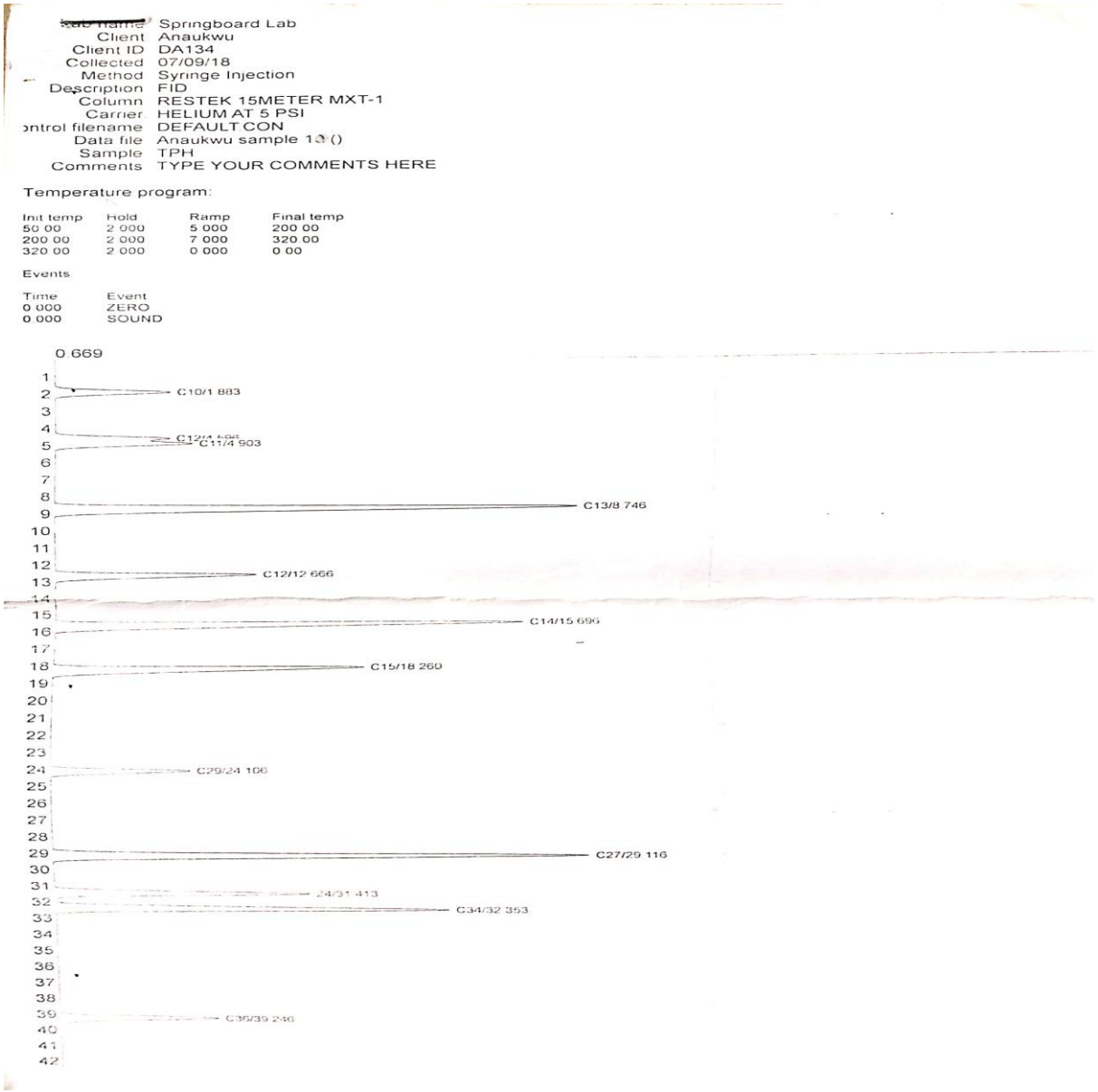
Appendix xvi: Mass spectrum from GC-MS analysis of the biosurfactant from *P.aeruginosa* CCUG



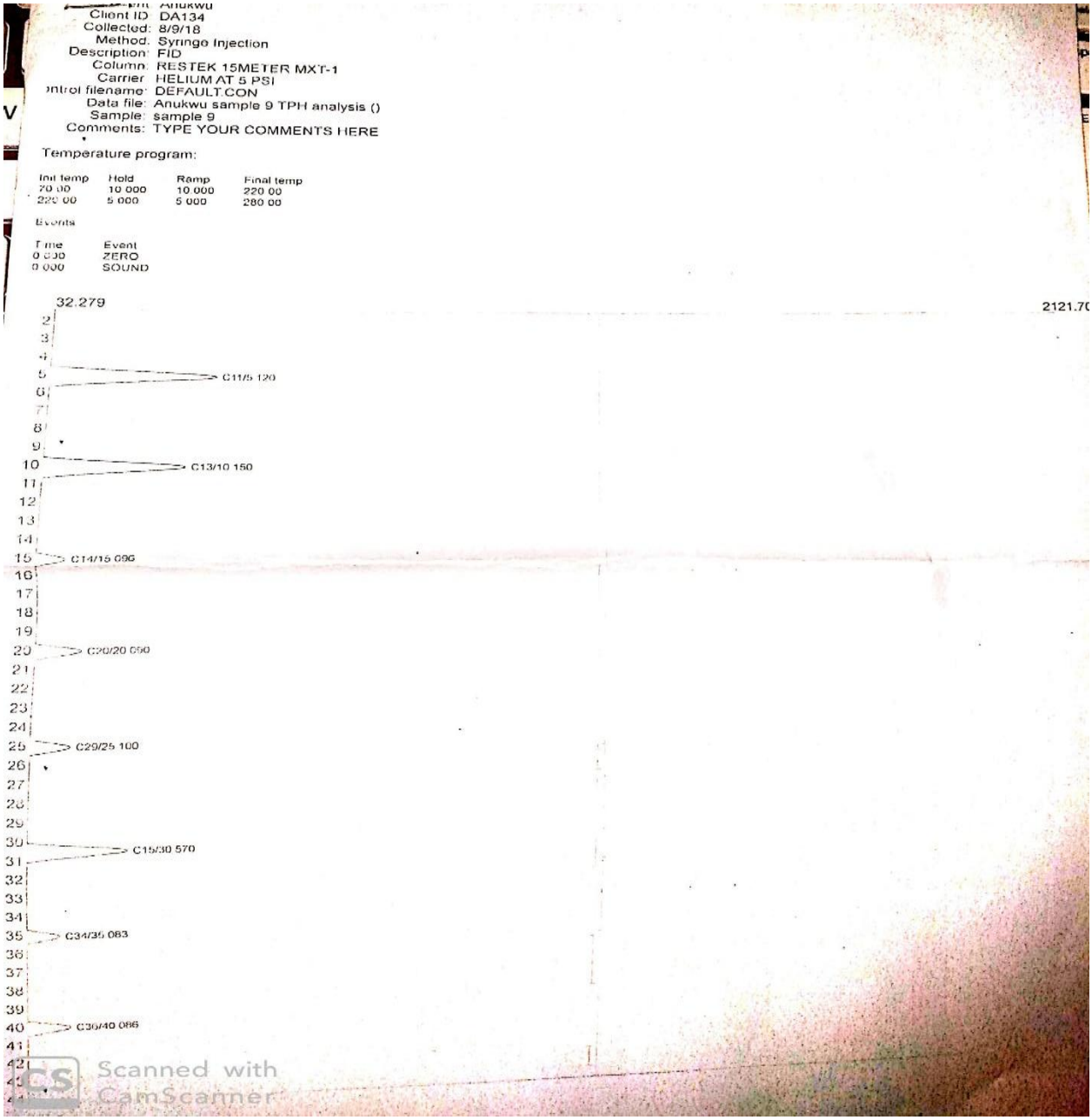
Appendix xvii: Mass spectrum from GC-MS analysis of the biosurfactant from *P.aeruginosa* I3



Appendix xiii: Mass spectrum from GC-MS analysis of the biosurfactant from *P.aeruginosa* ST11



Appendix xix: Chromatogram from gas chromatographic analysis of the polluted soil



Appendix xx: Chromatogram from gas chromatographic analysis of the control soil

Appendix xxi: Determination of crude oil degradation potential of indigenous isolates on shake flask

```
ONEWAY degradation BY Isolates
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Gravimetric percentage degradation

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	44167.166	13	3397.474	143.152	.000
Within Groups	664.533	28	23.733		
Total	44831.699	41			

Post Hoc Tests
Homogeneous Subsets

Gravimetric Crude oil degradation

Student-Newman-Keuls

Isolates	N	Subset for alpha = 0.05								
		1	2	3	4	5	6	7	8	9
Micrococcus sp.	3	6.6667								
Bacteria & Fungi consortium	3		11.2000							
Aspergillus niger	3			18.4000						
Aspergillus flavus	3			19.7333						
Corynebacterium sp.	3				40.6667					
Fungi consortium	3					46.6667				
Citrobacter freundii	3						50.9333			
Penicillium sp.	3							56.0000		
Staphylococcus aureus	3								92.0000	
Pseudomonas aeruginosa	3									97.0667
Bacteria consortium	3									97.4667
Bacillus subtilis	3									98.8000
Sig.		1.000	1.000	.448	1.000	1.000	1.000	1.000	1.000	.582

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxii: The bioremediation experimental setup

Appendix xxiii: Percentage increase in bacterial count in the treatments

```
ONEWAY bacteriacount BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

percentage increase in bacteria count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1070.880	7	152.983	33378.078	.000
Within Groups	.073	16	.005		
Total	1070.953	23			

Post Hoc Tests
Homogeneous Subsets

percentage increase in bacteria count

Student-Newman-Keuls

Bioremediation treatments	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
Natural Attenuation	3	34.6000							
Biostimulation B	3		44.0667						
Biostimulation C	3			44.4667					
Biostimulation A	3				51.7667				
Bioaugmentation B	3					52.5000			
Bioaugmentation C	3						53.4000		
Bioaugmentation A	3							54.5333	
Bioaugmentation D	3								54.9333
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxiv: Percentage increase in fungal count in the treatments

```
ONEWAY fungicount BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05)
```

ANOVA

percentage increase in fungi count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	318.886	7	45.555	9111.036	.000
Within Groups	.080	16	.005		
Total	318.966	23			

Post Hoc Tests
Homogeneous Subsets

percentage increase in fungi count

Student-Newman-Keuls

Bioremediation treatments	N	Subset for alpha = 0.05				
		1	2	3	4	5
Biostimulation C	3	16.4000				
Natural Attenuation	3	16.4667				
Biostimulation B	3	16.4667				
Bioaugmentation B	3	16.5000				
Biostimulation A	3		16.8333			
Bioaugmentation A	3			17.5333		
Bioaugmentation D	3				24.4333	
Bioaugmentation C	3					25.6667
Sig.		.340	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxv: Dehydrogenase enzyme activity

```
ONEWAY DehydrogenaseAssay BY Treatment
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Dehydrogenase enzyme assay

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	375.318	8	46.915	884.629	.000
Within Groups	.955	18	.053		
Total	376.273	26			

Post Hoc Tests
Homogeneous Subsets

Dehydrogenase enzyme assay

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Control	3	1.8033					
Nat.Att	3		4.2333				
Biostimulation B	3			6.6333			
Biostimulation C	3			6.8333			
Bioaugmentation C	3				7.7333		
Bioaugmentation B	3				7.8000		
Biostimulation A	3				7.8000		
Bioaugmentation A	3					8.6667	
Bioaugmentation D	3						16.4000
Sig.		1.000	1.000	.302	.933	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxvi: Rate of crude oil degradationin the treatments

```
ONEWAY Degradationrate BY Treatment
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Crude oil degradation rate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22361.539	8	2795.192	5717.439	.000
Within Groups	8.800	18	.489		
Total	22370.339	26			

Post Hoc Tests
Homogeneous Subsets

Crude oil degradation rate

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Control	3	5.3000					
Nat.Att	3		49.8333				
Bioaugmentation B	3			89.1667			
Bioaugmentation C	3				91.0667		
Bioaugmentation A	3				91.1000		
Bioaugmentation D	3				91.5000		
Biostimulation A	3				91.8000		
Biostimulation C	3					93.3000	
Biostimulation B	3						95.4000
Sig.		1.000	1.000	1.000	.584	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxvii: Percentage arsenic removal in the treatments

```
ONEWAY Arsenicremoval BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Arsenic removal rate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	33478.567	8	4184.821	5279.878	.000
Within Groups	14.267	18	.793		
Total	33492.834	26			

Post Hoc Tests
Homogeneous Subsets

Arsenic removal rate

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
Control	3	.52767							
Nat.Att	3		3.70300						
Biostimulation C	3			47.35433					
Biostimulation B	3				58.99933				
Bioaugmentation B	3					70.63433			
Biostimulation A	3						80.42267		
Bioaugmentation A	3							84.65533	
Bioaugmentation C	3								99.47067
Bioaugmentation D	3								100.00000
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	.476

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxviii: Percentage lead removal in the treatments

```
ONEWAY Leadremoval BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Lead removal rate

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	41950.098	8	5243.762	33509.426	.000
Within Groups	2.817	18	.156		
Total	41952.915	26			

Post Hoc Tests
Homogeneous Subsets

Lead removal rate

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05		
		1	2	3
Control	3	.59333		
Nat.Att	3		10.08867	
Bioaugmentation A	3			100.00000
Bioaugmentation B	3			100.00000
Bioaugmentation C	3			100.00000
Bioaugmentation D	3			100.00000
Biostimulation A	3			100.00000
Biostimulation B	3			100.00000
Biostimulation C	3			100.00000
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxix: Percentage mercury removal in the treatments

```
ONEWAY Mercuryremoval BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Mercury removal rate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	31636.057	8	3954.507	13602.720	.000
Within Groups	5.233	18	.291		
Total	31641.290	26			

Post Hoc Tests
Homogeneous Subsets

Mercury removal rate

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
Control	3	1.61600							
Nat.Att	3		8.25300						
Biostimulation C	3			70.13167					
Biostimulation B	3				71.16667				
Biostimulation A	3					77.61267			
Bioaugmentation C	3						80.62067		
Bioaugmentation A	3							85.15133	
Bioaugmentation D	3								99.91467
Bioaugmentation B	3								100.0000
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	.848

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxx: Percentage cadmium removal in the treatments

```
ONEWAY Cadmiumremoval BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Cadmium removal rate

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	37093.686	8	4636.711	3437.822	.000
Within Groups	24.277	18	1.349		
Total	37117.963	26			

Post Hoc Tests
Homogeneous Subsets

Cadmium removal rate

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Control	3	.90533				
Nat.Att	3		13.94867			
Biostimulation C	3			87.86200		
Bioaugmentation B	3			89.26767		
Biostimulation B	3				94.38367	
Bioaugmentation A	3					98.18833
Bioaugmentation C	3					100.00000
Bioaugmentation D	3					100.00000
Biostimulation A	3					100.00000
Sig.		1.000	1.000	.156	1.000	.259

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxxi: Percentage chromium removal in the treatments

```
ONEWAY Chromiumremoval BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=SNK ALPHA(0.05).
```

ANOVA

Chromium removal rate

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	39817.541	8	4977.193	579.724	.000
Within Groups	154.538	18	8.585		
Total	39972.079	26			

Post Hoc Tests
Homogeneous Subset

Chromium removal rate

Student-Newman-Keuls

Experimental treatment	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
Control	3	.87333							
Nat.Att	3		12.35367						
Biostimulation B	3			24.35833					
Bioaugmentation D	3				38.14800				
Bioaugmentation A	3					86.53367			
Bioaugmentation B	3					91.02533	91.02533		
Biostimulation C	3						93.17900	93.17900	
Biostimulation A	3							97.31867	97.31867
Bioaugmentation C	3								100.00000
Sig.		1.000	1.000	1.000	1.000	.077	.380	.101	.277

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix xxxii: Correlation of crude oil degradation and arsenic removal in the treatments

```
CORRELATIONS
  /VARIABLES=arsenicremoval degradation
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlations- crude oil degradation and arsenic removal

		Arsenic removal	crude oil degradation
Arsenic removal	Pearson Correlation	1	.819**
	Sig. (2-tailed)		.000
	N	27	27
crude oil degradation	Pearson Correlation	.819**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix xxxiii: Correlation of crude oil degradation and lead removal in the treatments

```
CORRELATIONS
  /VARIABLES=leadremoval degradation
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlations- crude oil degradation and lead removal

		lead removal	crude oil degradation
lead removal	Pearson Correlation	1	.948**
	Sig. (2-tailed)		.000
	N	27	27
crude oil degradation	Pearson Correlation	.948**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix xxxiv: Correlation of crude oil degradation and mercury removal in the treatments

```
CORRELATIONS
  /VARIABLES=mercuryremoval degradation
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlations- crude oil degradation and mercury removal

		mercury removal	crude oil degradation
mercury removal	Pearson Correlation	1	.891**
	Sig. (2-tailed)		.000
	N	27	27
crude oil degradation	Pearson Correlation	.891**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix xxxv: Correlation of crude oil degradation and cadmium removal in the treatments

```
CORRELATIONS
  /VARIABLES=cadmiumremoval degradation
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlations- crude oil degradation and cadmium removal

		cadmium removal	crude oil degradation
cadmium removal	Pearson Correlation	1	.949**
	Sig. (2-tailed)		.000
	N	27	27
crude oil degradation	Pearson Correlation	.949**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix xxxvi: Correlation of crude oil degradation and chromium removal in the treatments

```
CORRELATIONS
  /VARIABLES=chromiumremoval degradation
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlations- crude oil degradation and chromium removal

		chromium removal	crude oil degradation
chromium removal	Pearson Correlation	1	.699**
	Sig. (2-tailed)		.000
	N	27	27
crude oil degradation	Pearson Correlation	.699**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix xxxvii: Correlation of dehydrogenase activity and bacterial count

```
CORRELATIONS
  /VARIABLES=Dehydrogenase activity Microbialcount
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlation of dehydrogenase and microbial count

		Dehydrogenase assay	Bacterial count
Dehydrogenase assay	Pearson Correlation	1	.733**
	Sig. (2-tailed)		.000
	N	27	27
Bacterial count	Pearson Correlation	.733**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

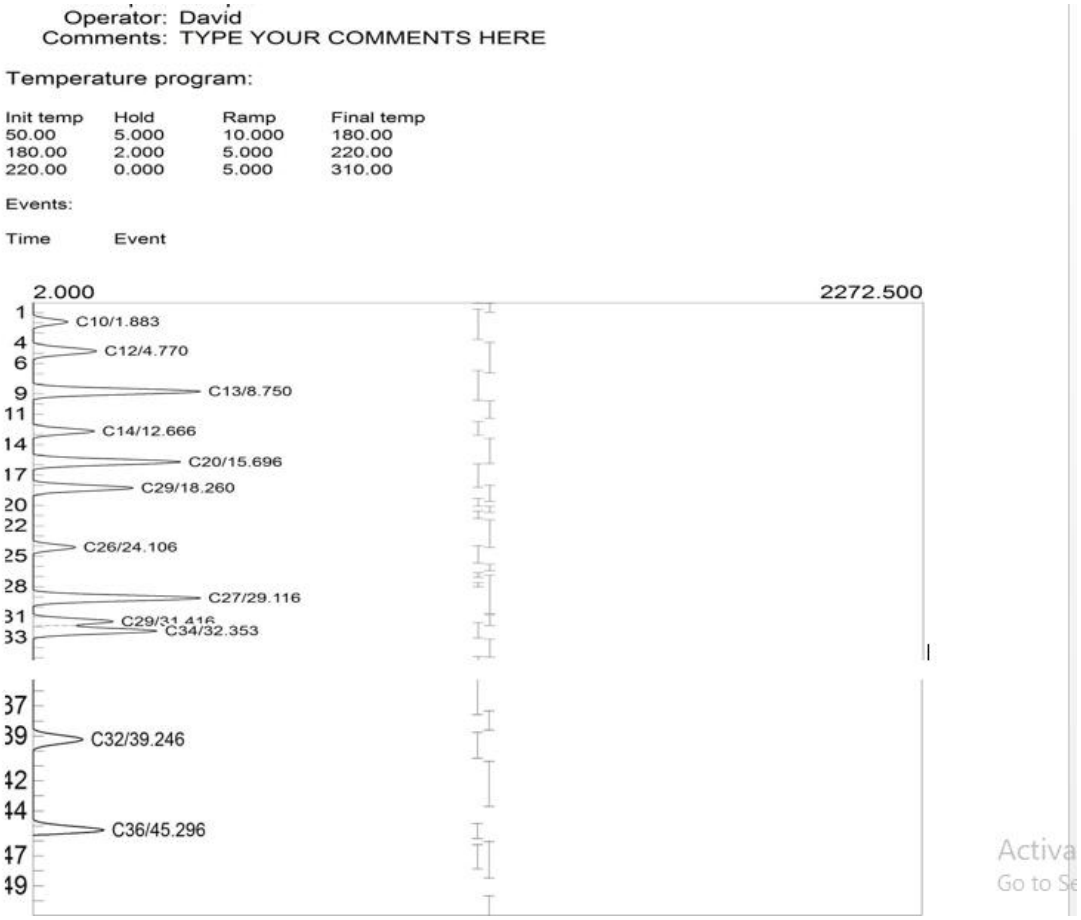
Appendix xxxviii: Correlation of dehydrogenase and crude oil degradation

```
CORRELATIONS
  /VARIABLES=Dehydrogenase activity degradation
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

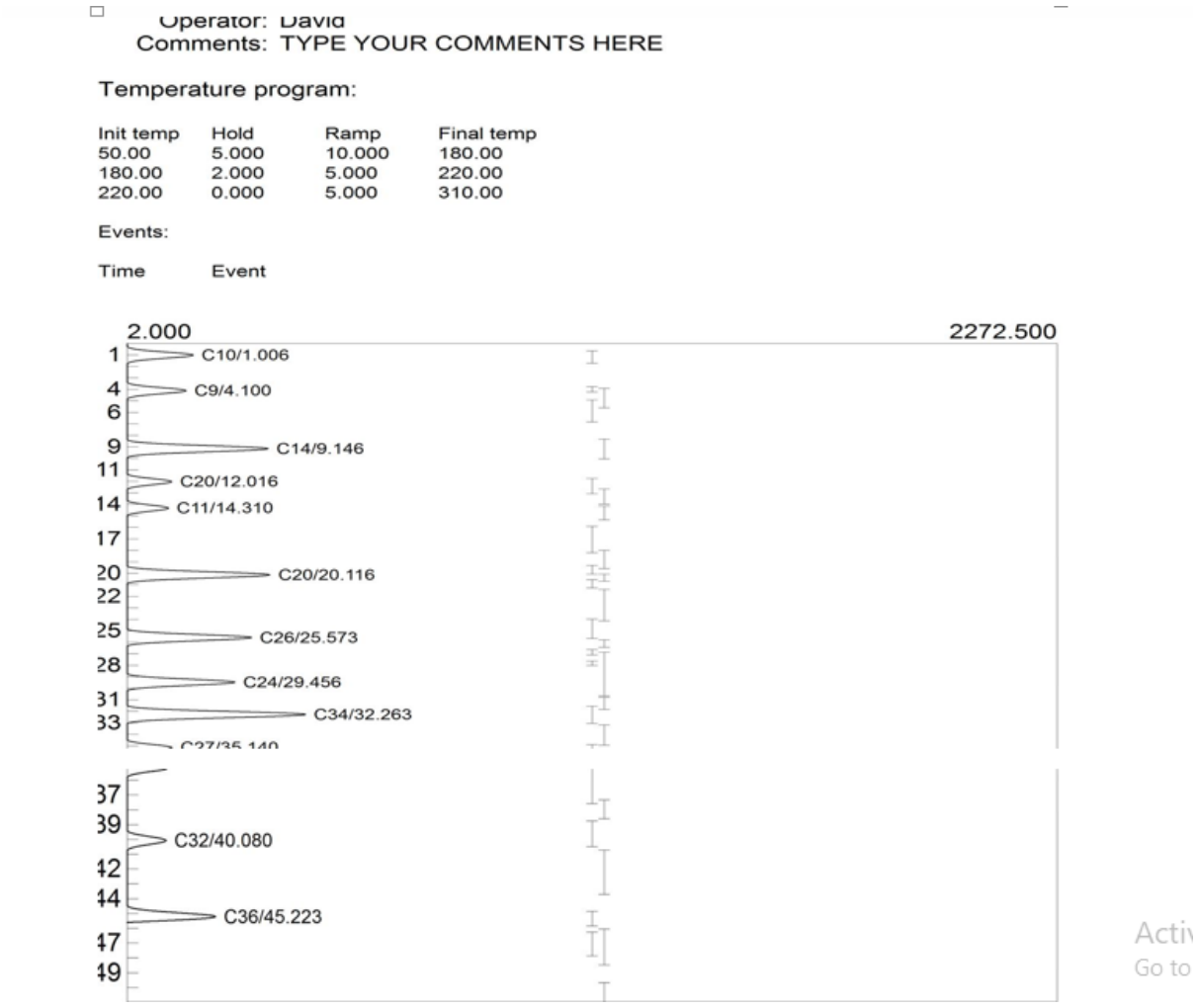
Correlations

		Dehydrogenase assay	crude oil degradation
Dehydrogenase assay	Pearson Correlation	1	.651**
	Sig. (2-tailed)		.000
	N	27	27
crude oil degradation	Pearson Correlation	.651**	1
	Sig. (2-tailed)	.000	
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).



Appendix xxxix: Chromatogram from the chromatographic analysis of the control set-up after bioremediation



Appendix xl: Chromatogram from the chromatographic analysis of the natural attenuation set-up after bioremediation

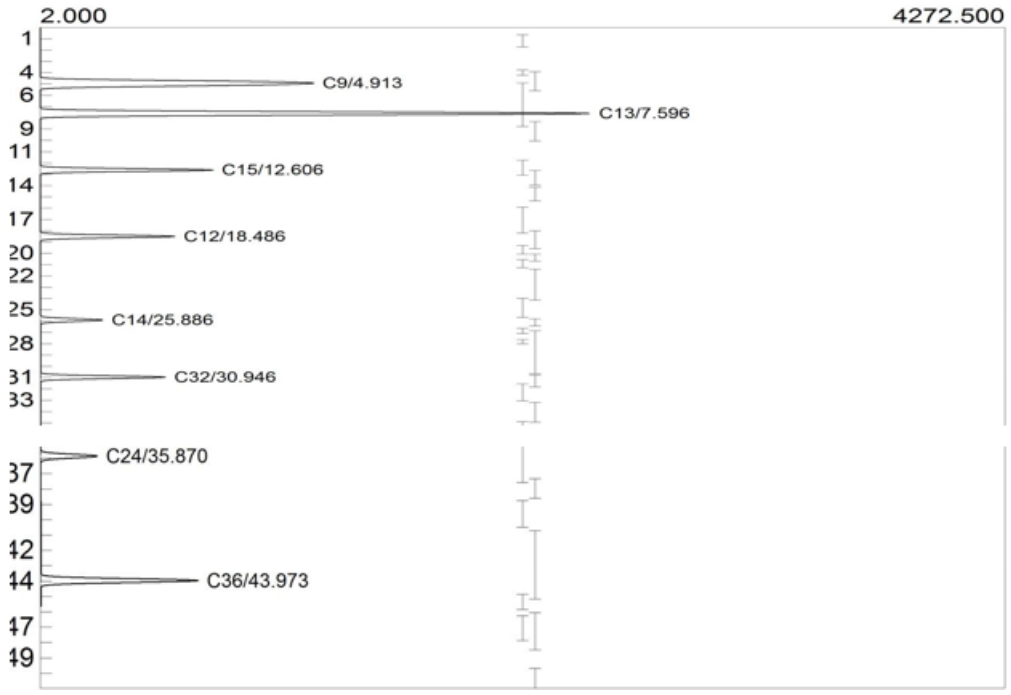
Sample: sample 5
Operator: David
Comments: TYPE YOUR COMMENTS HERE

Temperature program:

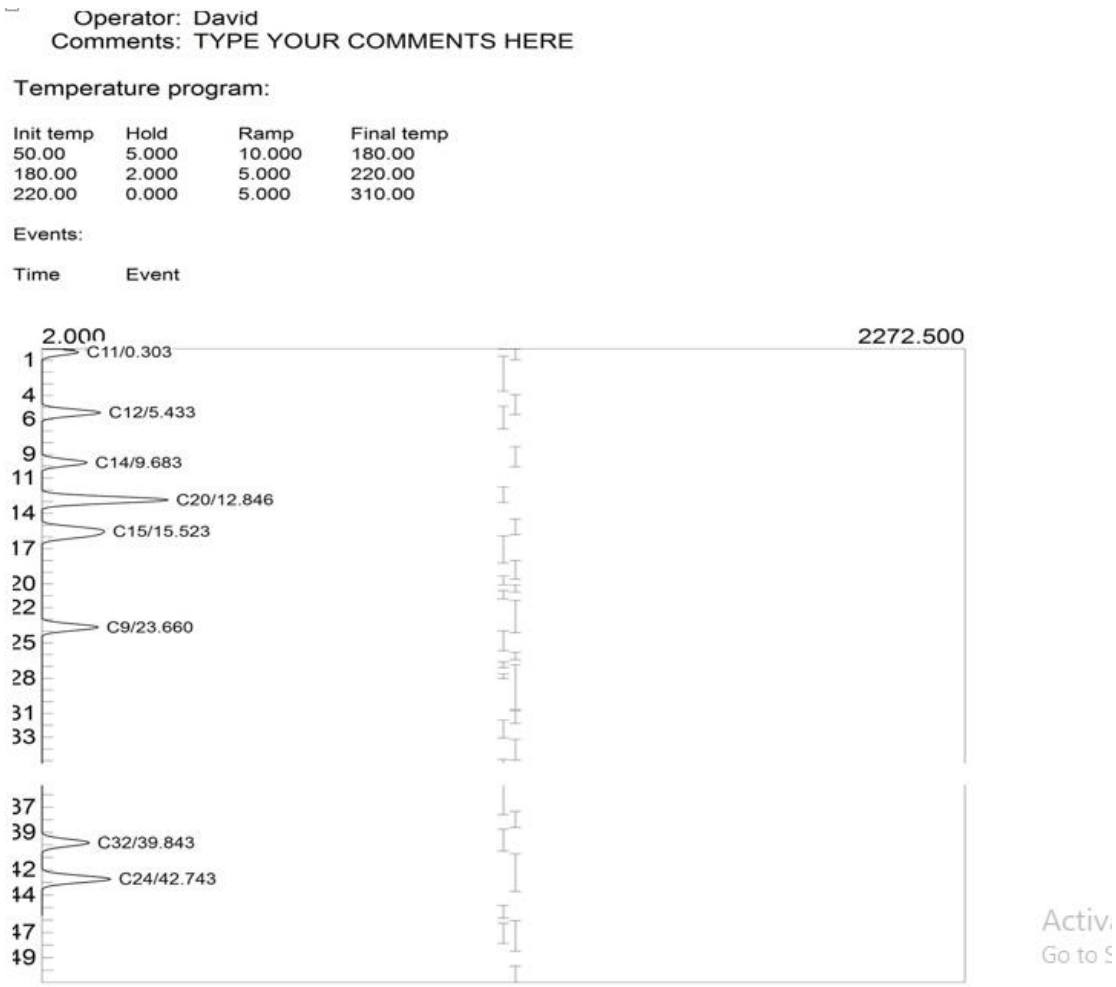
Init temp	Hold	Ramp	Final temp
50.00	5.000	10.000	180.00
180.00	2.000	5.000	220.00
220.00	0.000	5.000	310.00

Events:

Time	Event



Appendix xli: Chromatogram from the chromatographic analysis of the Bioaugmentation A set-up after bioremediation



Appendix xlii: Chromatogram from the chromatographic analysis of the Bioaugmentation B set-up after bioremediation

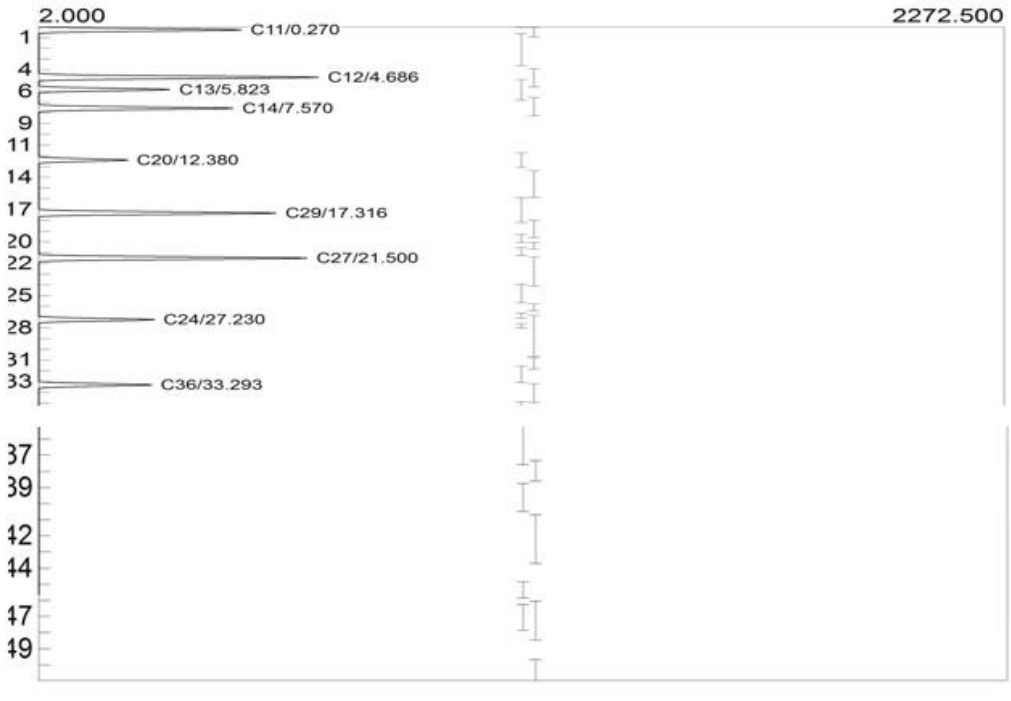
Sample: sample 2
Operator: David
Comments: TYPE YOUR COMMENTS HERE

Temperature program:

Init temp	Hold	Ramp	Final temp
50.00	5.000	10.000	180.00
180.00	2.000	5.000	220.00
220.00	0.000	5.000	310.00

Events:

Time	Event



Appendix xliii: Chromatogram from the chromatographic analysis of the Bioaugmentation C set-up after bioremediation

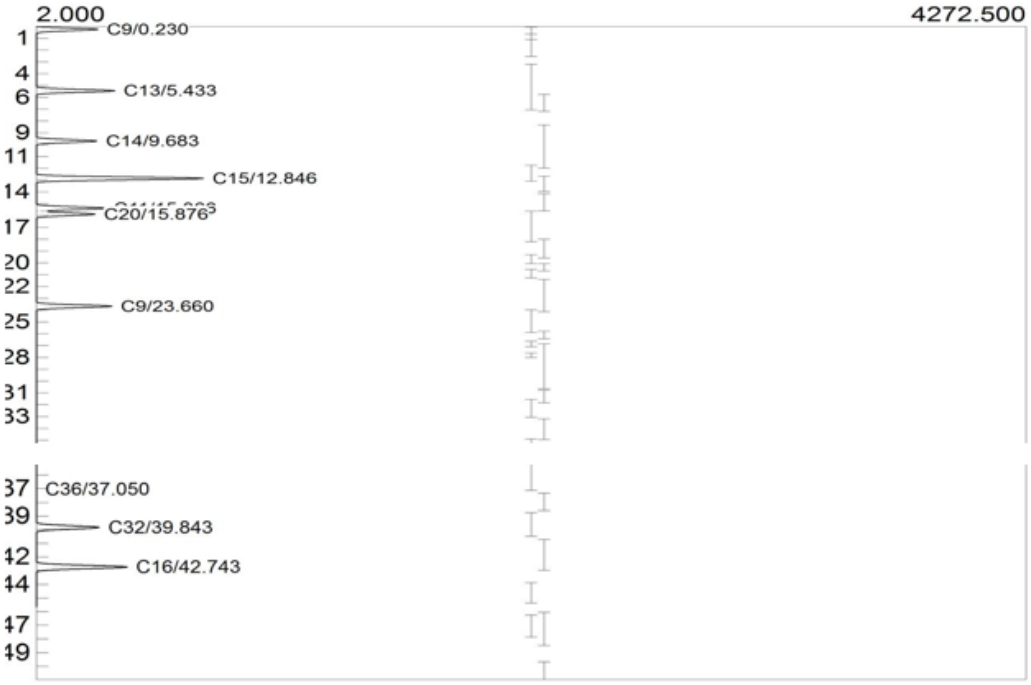
Sample: SAMPLE 9
Operator: David
Comments: TYPE YOUR COMMENTS HERE

Temperature program:

Init temp	Hold	Ramp	Final temp
50.00	5.000	10.000	180.00
180.00	2.000	5.000	220.00
220.00	0.000	5.000	310.00

Events:

Time	Event



Appendix xliv: Chromatogram from the chromatographic analysis of the Bioaugmentation D set-up after bioremediation

Sample: sample 7
Operator: David
Comments: TYPE YOUR COMMENTS HERE

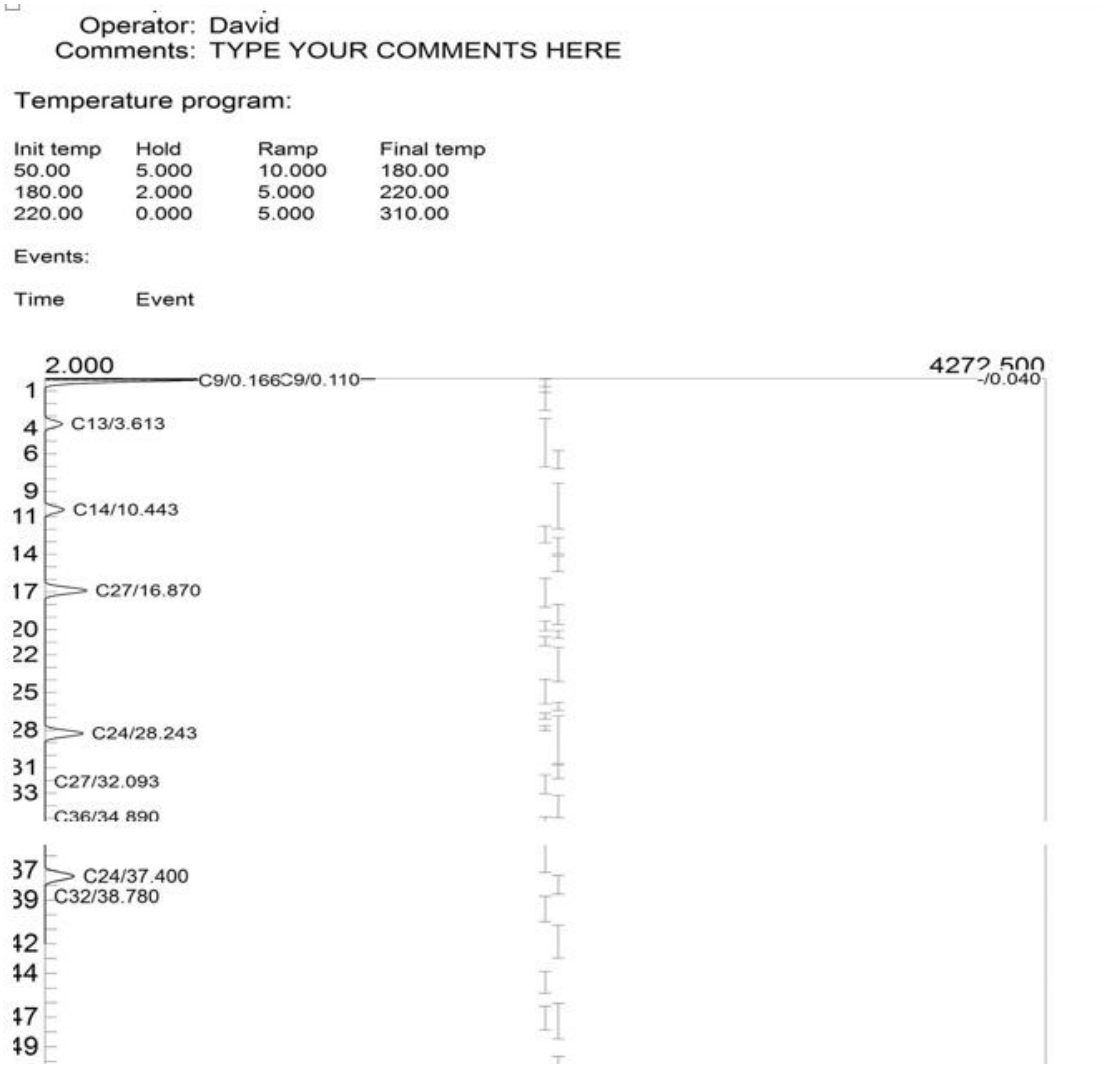
Temperature program:

Init temp	Hold	Ramp	Final temp
50.00	5.000	10.000	180.00
180.00	2.000	5.000	220.00
220.00	0.000	5.000	310.00

Events:

Time	Event

Appendix xlv: Chromatogram from the chromatographic analysis of the Biostimulation A set-up after bioremediation



Appendix xlvi: Chromatogram from the chromatographic analysis of the Biostimulation B set-up after bioremediation

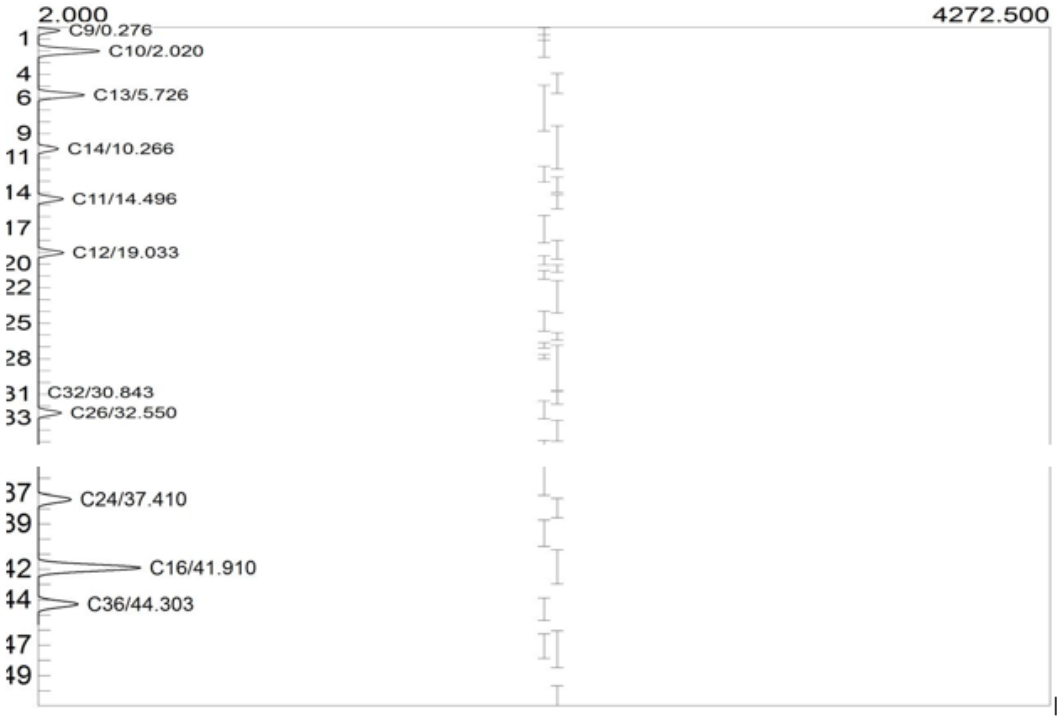
Sample: TPh
Operator: David
Comments: TYPE YOUR COMMENTS HERE

Temperature program:

Init temp	Hold	Ramp	Final temp
50.00	5.000	10.000	180.00
180.00	2.000	5.000	220.00
220.00	0.000	5.000	310.00

Events:

Time	Event



Appendix xlvii: Chromatogram from the chromatographic analysis of the Biostimulation C set-up after bioremediation