COMPARATIVE ANALYSIS OF BIOSURFACTANT PRODUCTION FROM BREWERY EFFLUENT USING PSEUDOMONAS SPECIES (PSEUDOMONAS AERUGINOSA AND PSEUDOMONAS FLUORESCENS)

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FACULTY OF SCIENCES

AHMADU BELLO UNIVERSITY ZARIA

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BY

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Declaration

I declare that the work in this thesis entitled "Comparative analysis of Biosurfactant Production from Brewery effluent using *Pseudomonas* Species (*Pseudomonas aeruginosa* and *Pseudomonas fluorescens)*" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Oladimeji Tayo, Kolawole	_____	_____
(Name of Student)	Signature	Date

Certification

This thesis entitled "COMPARATIVE ANALYSIS OF BIOSURFACTANT PRODUCTION FROM BREWERY EFFLUENT USING PSEUDOMONAS SPECIES (PSEUDOMONAS AERUGINOSA AND PSEUDOMONAS FLUORESCENS)" by OLADIMEJI TAYO, KOLAWOLE meets the regulation governing the award of degree of Master of Science (Microbiology) of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

Prof. Joseph .B. Ameh (Chairman, Supervisory Committee)	Signature	Date
Prof. Saleh .A. Ado (Member, Supervisory committee)	Signature	Date
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Prof. Kabir Bala (Dean, Postgraduate School)	Signature	Date

Dedication

This thesis is dedicated to the Glory of God for strength, guidance and support through the course of study. Also dedicated to my parents, siblings, wife and daughter for the support and encouragement through the years.

Also to my friend late Miss Bukola Akintimehin (R.I.P) who was snatched by the cold hands of death in the course of her studies, you will always be remembered.

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ABSTRACT

The increasing demand for surfactant calls for an alternative to the chemically synthesized surfactant, which poses a lot of threat to the environment due to bio-accumulation of its un-degradable toxic component. Accumulation of industrial and domestic waste also threatens the environment by contributing unfriendly gases and toxic compounds, therefore due to tighten environmental regulations and increasing need to protect the ecosystem, alternatives to chemically synthesized surfactant, which is biosurfactant, is a necessity. Biosurfactants are biodegradable and stable at extreme temperatures and pH. Brewery effluent is an excellent substrate for the production of biosurfactant. *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* isolated from soil produced glycolipid biosurfactant from brewery effluent. The brewery effluent used as substrate had chemical oxygen demand of 15,000mg/l, dissolved oxygen of 2.9mg/l and biological oxygen demand of 1.8mg/l. Screening both isolates for their ability to produce biosurfactant, both were positive for drop collapse assay and hemolytic test. For the oil displacement assay 28mm and 23mm were observed for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. Fermentation process was observed for 168hrs and readings of surface tension and optical density was taken every 24hrs. Three factors were evaluated in the fermentation of the effluent which include; incubation period, pH and temperature. For the incubation period 31.30mN/m and 33.76mN/m was observed for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. For ionic concentration ranging from pH 4.0 to 10.0, pH 7.0 had the least surface tension reading with 31.87mN/m and 33.04mN/m for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. Among the pH considered, difference in surface tension was insignificant, indicating a stable biosurfactant. For the temperature ranging from 25^{o}C to 80^{o}C, temperatures 30^{o}C and 35^{o}C had the least surface tension reading though with minimal difference across the temperatures range.

The growth of the cells used in fermentation was monitored using the colorimeter at 600nm. The peak growth was generally recorded at 72hrs and peak growth highest at 1.07 for incubation factor. The best growth for pH factor was observed at pH 5.0 and 7.0. The control set-up for fermentation had the least surface tension reading as compared to all other factors with 30.87mN/m and 32.87mN/m for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. The application of the biosurfactant produced was tested by checking it ability to emulsify hydrocarbons (diesel, premium motor spirit, kerosene and olive oil). Also applying the various factors (incubation period, pH and temperature), they all showed stable emulsion in 24hrs excluding pH4 with poor or no emulsion formation. Ionic concentration 10.0 had the highest emulsion formation within 24hrs with 87% to 80%. Biosurfactant extracted from *Pseudomonas aeruginosa* induced fermentation was 3.6g/l and 2.8g/l from *Pseudomonas fluorescens*. The spectra analysis (FT-IR) of the extract revealed the probable presence of glycolipid biosurfactant as the surface active compound produced by both isolate. From statistical evaluation of data collected, neither of the isolates performed better than the other but shows that there is a significant association between growth rate and surface tension reduction (biosurfactant production)in both isolates at P=0.005

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List of Abreviations

B.O.D ……..Biochemical Oxygen Demand

C.O.D ……..Chemical Oxygen Demand

D.O ……..Dissolved Oxygen

mN/m ……..Milli-Newton per Meter

^{o}C ……Degree Centigrade

FT-IR ……..Fourier Transformed Infra-Red

Rpm …… Revolution per Minute

CHAPTER ONE

1.0 INTRODUCTION

Biosurfactants are surface active agents. They are amphiphilic biological compounds produced extracellularlly or as part of cell membrane by a variety of yeasts, bacteria and filamentous fungi (Rismani *et al.*, 2006;Mata-Sandoval *et al*., 2002; Adebusoye *et al*., 2008).

Biosurfactant is a structurally diverse group of surface-active molecule synthesized by microorganisms. Their capability for reducing surface and interfacial tension with low toxicity and high specificity and biodegradability, led to an increasing interest in these microbial products as alternatives to chemical surfactants (Banat *et al.,* 2000). From the technical insights, it was estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $US200 million. However, up to now, biosurfactants are still unable to compete with the chemically synthesized surfactants in the surfactant market. This could be due to their high production costs in relation to inefficient bioprocessing methods available, poor strain productivity and the need to use expensive substrates (Cameotra and Makkar, 1998).

The reason why organisms produce surfactants is not always so obvious. (Rismani *et al,* 2006). Although most biosurfactants are considered to be secondary metabolites, some play essential role in the survival of the biosurfactant producing organisms through the facilitation of nutrient transportation or microbe-7host interaction or by acting as biocide agent (Rodrigues *et al.*, 2006a)

Industrial wastes with high content of carbohydrate or lipids meet the requirement for use as substrate for biosurfactant production (Nitschke *et al*; 2004). Brewery waste is a potential substrate for the production of biosurfactant. Organic component in brewery effluent mainly consist of sugars, soluble starch, ethanol and volatile fatty acids among other compounds.

This waste is usually rich in nitrogenous compound and phosphorus containing compound (Dubey and Juwarkar, 2001; Driessens and Veriejken, 2003)

Various microorganisms are known to produce specific kind of biosurfactant depending on the molecular composition of the substrate. *Pseudomonas* species depending on strain produce different types of biosurfactant ranging from rhamnolipids, lipopolysacharisdes, viscosin, particulate biosurfactant to biosur particulate surfactant (Mulligan,2005), which is also affected by the carbon substrate used, therefore affecting the type, quality and quantity of biosurfactant produced (Raza *et al.,* 2007)

It is also evident that the nutrient concentration, pH, and the age of the culture affect the yield of biosurfactant. Microorganisms utilize a variety of compounds and energy for their growth. When the carbon source is an insoluble substrate like hydrocarbon, microorganism facilitates the diffusion into the cell by producing a variety of substances such as biosurfactant. (Karanth *et al*., 1999). *Pseudomonas aeruginosa* and *Pseudomonas flourescens* are known to utilize variety of carbon sources such as eleven and twelve carbon alkane, succinate, pyruvate, citrate, fructose, glycerol, olive oil, glucose and manitol among others (Robert *et al*., 1989, Karanth *et al*., 1999).

Factors such as carbon source, pH, salinity and temperature, calcium and magnesium, nitrogen, phosphorus, iron, manganese, agitation and dilution rate affect biosurfactant production (Ilori *et al*., 2005), though some *Pseudomonas* strains have also been reported not to be affected by temperature, pH, calcium, magnesium, nitrogen, phosphorus, iron and manganese (Karanth *et al*., 1999)

Biosurfactants vary in their chemical properties and molecular size. The low molecular weight surfactants are often glycolipids; high molecular weight surfactant are generally either

polyanionic heteropolysaccharides containing covalently linked hydrophobic side chain or complexes containing both polysaccharides and proteins (Karanth *et al*, 1999).

Biosurfactants have been applied as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredient and detergents in various sectors such as petroleum and petrochemical, organic chemicals, food and beverages, cosmetics and pharmaceuticals. They also have applications in biomedicine and therapeutics as antimicrobial agent, immunoregulators, immunomodulators and their possible role in signaling and cytotoxic activity, mining and metallurgy, agrochemicals and fertilizers, environmental control and management, pulp and paper producing industries and many others (kosaric, 1999; Muthusamy *et al*., 2008).

1.1 Statement of Problem

The ever increasing population has resulted in ever increasing market demand for surfactant which invariably calls for alternatives to chemically synthesized surfactants.

The chemically synthesized surfactants though seem to be meeting the ever growing population has also posed a lot of threat to lives and environment. The environment over the years has become a cause of concern to local and international organizations due to the bio-accumulation of the various toxic compounds which is partly contributed by these chemically synthesized surfactants. These bio-accumulated compounds indirectly or directly affect the inhabitants of the environment (Rahman and Gakpe, 2008).

From environmental findings, some compounds from these chemically synthesized surfactants have been known to accumulate in trees, tissues, fishes, snails among many other living object, which when consumed result in cases such as tumors, cancer, mental ill-health, irritations among many others (Banat *et al*; 2000, Adebusoye *et al.*, 2008*)*

Also the rate of accumulation of domestic and industrial waste has also become a serious threat to the environment. These wastes contribute a lot of unhealthy gases and toxic compounds to the environment which has being reported to bio-accumulate in living objects, posing serious cases of ill-health in humans and animals (Raza *et al.,* 2007).

1.2 Justification

The enormous market demand for surfactant is currently being met by the synthetic form, mainly petroleum-based chemical surfactants. These compound are usually toxic to the environment and may bio-accumulate and their production process and by-product can be environmentally hazardous. Due to tightening environmental regulation and increasing awareness for the need to protect the ecosystem, there has been an increasing interest in biosurfactant as an alternative to chemical surfactant (Rahman *a*nd Gakpe, 2008) Biosurfactants have advantages over their chemical counterpart in that apart from being biodegradable with low toxicity, it is also stable at extreme temperatures and pH values and also their unique structure which provides new properties that classical surfactant may lack (Maneerat, 2005; Rodrigues *et al.,* 2006a; Muthusamy *et al.,* 2008).

Biosurfactant have become recently important product of biotechnology for industrial and medical application. The unique properties of biosurfactant allow their use and possible replacement of chemically synthesized surfactant in a great number of individual operations. Biosurfactants are known to reduce surface tension and interfacial tension in both aqueous solutions and hydrocarbon mixture (Kosaric, 1999)

Biosurfactants are generally biodegradable, non-toxic and eco-friendly substances (Rahman Gakpe, 2008) from various substrates such as sugars, oil and waste of domestic and industrial origin (Rahman *et al.,* 2000a; Raza *et al.,* 2007)

1.3. Aim

This research is aimed at comparing the biosurfactant production and activity by *Pseudomonas* species using brewery effluent as substrate and the effect of some environmental factors on biosurfactant activity.

1.4. Specific Objectives

1. To determine the physico-chemical properties of brewery effluent
2. To isolate and characterize Pseudomonas species mainly *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* from soil.
3. To screen the Pseudomonas species for biosurfactant production.
4. To characterize the biosurfactant produced by the isolated *Pseudomonas* species.
5. To determine the effect of some environmental factors on activity of the biosurfactant produced.
6. To check for the emulsification application of the biosurfactant on some immiscible liquids.

CHAPTER TWO

2.0 LITERATURE REVIEW

Biosurfactants are amphiles consisting of two parts – a polar head (hydrophilic) moiety and non polar (hydrophobic) group. These different polar hydrophilic head and hydrophobic tail moieties decrease surface and interfacial tension by accumulating at the interphase between immiscible fluids such as water and oil or air and liquid (Tahzibi *et* al., 2004; Nitschke *et al*., 2004; Dehghan-Noudeh *et al*., 2009; Almansorry *et al.,* 2014; Zainatul *et al*., 2014; Vandana and Peter, 2014).

These amphilic compounds have functional properties like; surface and interfacial activity, emulsification, wetting, foaming, detergency, phase dispersing, solubilization, and density reduction of heavy hydrophobic compound and also finds wide industrial applications (Dehghan-Noudeh *et al* 2009; Priya and Usharani, 2009).

Biosurfactants are produced by different microorganisms such as bacteria, filamentous fungi and yeast (Priya and Usharami, 2009) from various substrates such as sugar, oil, alkanes and organic waste (Rismani *et al*, 2006). Biosurfactants increase the affinity between a microbial cell and substrate (Padmapriya *et al.*, 2013; Anna-Joice and Parthasarathi, 2014)

Biosurfactants are of six (6) major types; hydroxylated and cross linked fatty acids (mycolic acid), glycolipids, polysaccharide-lipid complexes, lipoprotein-lipopeptide, phospholipids and complete cell surface (Kosaric., 1999; Rodriques *et al*., 2006a; Deka and Das 2009). The activities of these biosurfactants depend on their structural component, e.g, the type of hydrophilic and hydrophobic groups and their spatial orientation (Bonmatin *et al*., 1994).

2.1 Isolation of Pseudomonas species.

In the environment, microorganisms occur in mixed culture of different species. To analyse the properties of any microorganism of interest from a mixed culture, a pure culture is required. Apart from direct isolation of strains by diluting and plating, enrichment cultures with hydrophobic substrates are very promising for isolation of biosurfactant producing microorganism. The principle of enrichment culture is to provide growth conditions that are very favorable for the organism of interest and unfavorable for other competing organism that are not of interest. Hence, the microorganisms of interest are selected and enriched. For the screening of biosurfactant producing microorganisms, enrichment medium utilizing hydrophobic compound as the sole carbon source are applied (Huy *et al*., 1999; Walter *et al*., 2000; Bento *et al*., 2005)

2.2 Classification and Chemical Properties of Biosurfactant.

Classification of biosurfactants is based their microbial source and their chemical composition which is unlike the chemically synthesized surfactants which are classified based on the nature of their polar grouping (Kapadia and Yagnik, 2013). Biosurfactants are broadly categorised as those that decrease surface tension at the air-water interphase; those that decrease interfacial tension between liquids that are generally regarded as immiscible and those that reduce interfacial tension between solid-liquid interphase. Biosurfactants are known to exhibit emulsifying properties but bio-emulsifiers do not necessarily decrease surface tension (Luna *et al*., 2012).

It has also been suggested that biosurfactants can be grouped based on their molecular sizes into low molecular-mass molecules and high molecular-mass molecules. Low molecular mass molecules efficiently lower surface and interfacial tension; they include glycolipids, lipopeptides and phospholipids. The high molecular mass molecules are more effective as emulsion-stabilizing agents they include; polymeric and particulate surfactants. Most

surfactants are either anionic or neutral. The hydrophobic moieties of biosurfactants are made up of long chain fatty acids and their derivatives, while, the hydrophilic moiety can be carbohydrate, amino acid, phosphate or cyclic peptide. Differences in glycolipids are majorly due to the carbohydrate compounds that make up the polar head which also affects their activities (Karanth *et al*, 1999; Muthusamy *et al.,* 2008; Rikalovic *et al.,* 2012; Singh, 2012)

2.2.1 Glycolipids.

Glycolipids are carbohydrates in combination with long chain aliphatic acids or hydroxyl-aliphatic acids linked by either ether or an ester group (Muthusamy *et al*, 2008, Okoliegbe and Agarry, 2012).

Glycolipids include Rhamnolipids, sophorolipids, trehalolipids and fructose lipids.

I. Rhamnolipids

Rhamnolipids are usually classified based on two different structures which are L-rhamnolipids-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-β-hydroxyldecanoyl-β-hydroxyldecanoate (Rashedi *et al*., 2005; Priya and Usharani, 2009).

The hydroxyl group of one of the acids (β-hydroxydecanoic acid) is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide while the hydroxyl group of the second hydroxydecanoic acid is involved in ester formation (Karanth *et al*., 1999).

Javis and Johnson in 1949 were the first to describe the synthesis of rhamnolipid by *Pseudomonas aeruginosa*. Over the past decades, different researchers have reported the use of different substrates as carbon and nitrogen sources ranging from whey milk to molasses to produce rhamnolipid (Patel and Desai, 1997; Onbasli and Aslim, 2009).

Burger *et al*., (1963), proposed the sequential glycosyl transfer biosynthetic pathway for the synthesis of rhamnolipid while Ochner *et al*., (1994), isolated and analyzed the regulation of rhamnolipid synthesis at the genetic level.

II. Sophorolipids

Sophorolipids are capable of lowering interfacial tension, but, they are not effective bio-emulsifiers (Cooper and Paddock, 1984; Kitamoto *et al.*, 2002). *Torulopsis bombicola*, *Torulopsis apicola* and *Torulopsis petrophilium* produces Sophorolipid as extracellular metabolite consisting of dimeric carbohydrate (sophorose) linked to β-1-2-long chain hydroxcarboxylic acids (Fatty acid derivative). The sophorose and the lipid portion of the glycolipid are linked at the reducing end by a glycosidic linkage ((Desai and Banat, 1997; Rosenberg and Ron 1999; Muthusamy *et al*., 2008).

Sophorolipids are composed of mixtures of macrolactones and free acids. The lactone forms of sophorolipids are most preferred for many industrial applications. Sophorolipid is a mixture of six to nine different hydrophobic sophorolipids (Muthusamy *et al*., 2008).

Sophorolipid stimulates the biodegradation of hydrophobic organic contaminant such as fourteen to sixteen carbon chain alkanes, Pristane, phenyldecane, naphthalene and phenanthrene (Makkar and Rockne, 2003).

III. Trehalolipids

Mycobacterium, Nocardia and Corynebacterium are known to produce disaccharide trehalose linked to mycolic acid. Several structural types of trehalolipids have been previously reported. The mycolic acid composition of trehalolipid is made up of long chain α-branched – β-hydroxy fatty acid. The structure, size, number of carbon and degree of saturation of the mycolic acid portion of the trehalolipid depends on the microbial source. Disaccharide trehalose linked at C-6 and C-6^1 to mycolic acids is associated with species of *Mycobacterium, Nocardia,* and *Corynebacterium*. *Rhodococcus erythropolis* synthesised a novel anionic trehalose lipid. Trehalose lipid from *Rhodococcus erythropolis* and *Arthrobacter* species lowered the surface and interfacial tensions of it culture broth to 25-40 and 1-5mN/m respectively (Desai and Banat, 1997, Gautum and Tyagi, 2006)

2.2.2 Lipopeptides and lipoproteins.

Lipopeptides are mixtures of related compounds showing some difference in amino acid composition and lipid proportion. The lipid portion is mostly fatty acid or it derivative. Cyclic lipopeptides consist of eight to seventeen amino acids while the lipid portion is composed of eight to nine methylene groups in a mixture of linear and branched tails (Desai and Banat, 1997).

Surfactin is composed of seven peptide loop of amino acid (L-asparagine, glycine, two L-leucines, L-valine and two D-leucine) in ring structure linked to the fatty acid terminal at the thirteen to fifteen carbon chains via a lactone linkage. *Bacillus subilis* ATCC 21332 produces a cyclic lipopetide known as surfactin which is made up of 3-hydroxyl-13-methyl tetradecanoic acid linked by amination to the N-terminal amine of the heptapeptide. The carboxyl terminal of the peptide is esterified to the hydroxyl terminal of the fatty acid (Cameotra and Makkar, 2004; Muthusamy *et al*., 2008; Priya and Usharani, 2009; Salihu *et al.,* 2009).

Bacillus lincheniformis produces a lipopeptide-lipoprotein biosurfactant known as Lichenysin which is stable at extreme temperatures, pH and salinity. Lichenysin exhibit similar structure and physio-chemical properties to that of surfactin. *Bacillus licheniformis* produces various biosurfactants which lower the surface tension of distilled water from 71mN/m to 27mN/m and interfacial tension between water and hexadecane to 0.36mN/m (Muthusamy *et al*, 2008). Viscosin a peptidolipid biosurfactant produced by *Pseudomonas* species lowers the surface tension of de-ionized water to 27mN/m (Neu and Paralla, 1990).

Streptofactin, an extracellular hydrophobic peptide; a mixture of structurally related peptides of 1003 to 1127Da in size is produced by *Streptomyces tendae* (Ron and Rosenberg, 2001). *Bacillus brevis* produces the cyclo-symmetric deca-peptide antibioticknown as gramicidin S.

Polymyxcin B which is a decapeptide is made up of three to ten amino acids forming a decapeptide with branched chain fatty acid attached at the terminal 2, 4-diaminobutyric chain.

The structure of the biosurfactant (Polymyxcin B) differ in substituents at residues 3 (Diaminobutyric or D-serine), 6 (D-leucine or L-isoleucine) or 7(D- or L-Diaminobutyric) (Suzuki *et al*, 1969). The cationic γ-amino groups of the diaminobutyric residues together with the hydrophobic side chain of the fatty acid give this antibiotic the surface-active properties of a cationic detergent (Ron and Rosenberg, 2001).

2.2.3 Fatty acids, phospholipids and neutral lipids.

Large volumes of fatty acids and phospholipid biosurfactant are produced by several bacteria and yeast when grown on n-alkanes. The hydrophilic and lipophilic balance is directly proportional to the length of the hydrophobic chain in the structure of the biosurfactant (Rosenberg *et al* 1999). Fatty acid, phospholipid and neutral lipid biosurfactants produce an optically clear micro-emulsion of water-alkane mixture. These biosurfactants are produced by *Acinetobacter* species strain H01-N and sulphur producing bacteria. (Desai and Desai, 1993; Desai and Banat, 1997). Phosphatidylethanolamine rich vesicles which form optically clear micro-emulsion of water-alkane mixture are produced by *Acinetobacter* species strain H01-N *Rhodococcus erythropolis* also produces phosphatidylethanolamine when grown on n-alkane causes the lowering of interfacial tension between water and hexadecane to less than 1mN/m and a critical micelle concentration of 30mg/l (Muthusamy *et al.*, 2008).

I. Polymeric biosurfactant.

Emulsan, Liposan, Alasan, Lipomanan and polysaccharide-protein complex are produced by bacteria species of different genera. *Acinetobacter calcoaceticus* RAG-1propduces emulsan, an extra-cellular potent anionic amphipatic hetero-polysaccharide bioemulsifier which effectively emulsify water hydrocarbon mixture (Aparna *et al.*, 2012)

Candida lipolytica produces a water soluble bioemulsifier which is composed of 83% carbonhydrate and 17% protein known as liposan (Rosenberg *et al* 1979; Desai and Banat

1997, Rosenberg and Ron 1999, Muthusamy *et al*, 2008). Emulsan heteropolysaccharide backbone is composed of repeated sequence of trisaccharide of N-acetyl-D-galactosamine, N-acetyl-galactosamine, uronic acid and an unidentified N-acetylamino sugar. Fatty acid composition of liposan is covalently linked to the polysaccharide through an ester linkage (Karanth *et al*, 1999).

Biodispersan, an extracellular non dialyzable dispersing agent produced by *Acinetobacter calcoaceticus* A2, is an anionic heteropolysaccharide with an average molecular weight of 51,400Da. It contain four sugars namely glucosamine, 6-methylaminohexose, galactosamine, uronic acid and an unidentified amino acid (Desai and Desai, 1993).

II. Particulate biosurfactant.

Extracellular membrane vesicles partition hydrocarbon to form a micro-emulsion which plays an important role in alkane uptake by microorganisms. Vessicles produced by *Acinetobacter* species strain HO1-N are composed of protein, phospholipids and lipopolysaccharide (Muthusamy *et al*., 2008).

2.3 Substrates for Biosurfactant Production

In recent decades, biosurfactants have been produced from renewable resources such as the agro-industrial waste. Most agro-industrial waste contain large amount of usable proteins, sugars and some oil residues. The availability of this agro-industrial waste in large quantities, have aided it use as raw material for the production of biosurfactant, therefore reducing cost of production (Fontes *et al.,* 2012; Waghmode *et al.,* 2014). Plaza *et al*., (2011), noted that the use of agro-waste as substrate is largely dependent on the amount of free utilizable sugars and micro-elements available for microbial growth and biosurfactant production.

The effective production of biosurfactants from agro-waste does not require the addition of refined substrates such yeast extract and mineral salt medium and can therefore ensure

sustainability and economic viability of biosurfactant production. These biosurfactants can thus be used in continuous bio-remediation of polluted environments such as those of oil spill sites or contamination from hydrocarbon related substances (Amodu *et al.,* 2014).

It is generally accepted that the use of in-expensive substrates such as industrial waste as substrate for formulation of fermentation media, will lower the cost of production significantly, since large volume (in tons) of raw industrial or agro-wastes are generated annually. Harnessing these wastes as substrate could yield economic benefit. Agro-wastes such as cassava peels, soya bean hull, molasses, potato peel, rice and wheat bran and sugarcane bagasses among other agro-wastes are being use as substrates for biosurfactant production. Whey milk and distillery waste have also found application and can therefore be used as substrate (Maneerat, 2005; Saharan *et al.,* 2011; Zainatul *et al*., 2014). Obtaining biosurfactant from renewable sources reduces production cost and encourages the possibility of commercial production (Zeraik and Nitchke, 2010; Waghmode *et al*., 2014).

Brewery waste can also be a good substrate for biosurfactant production. Brewery waste is composed mainly of liquor pressed from wet grain and wash water from various departments. The biochemical oxygen demand levels are quite high as are the total solids. Typically about half the biochemical oxygen demand and over ninety percent of suspended solids are generated in the brewing operations. There are solid waste, spent grains, hops, sludge that are formed in this process (Noorjahan and Jamuna, 2012).

Another potential renewable source of microbial biosurfactant production is glycerol from biodiesel production in which glycerol has been successfully used as a water soluble carbon source for different microbial production. Fonte *et al., (*2012), observed *Actinetobacter calcoaceticus* was able to produce biosurfactant from cashew juice reducing surface tension of the medium from 72mN/m to 62mN/m.

The use of different substrate as carbon source alters the structure of the biosurfactant produced and it properties can be exploited to get product of desired properties for particular

application. The composition of the biosurfactant is greatly influenced by the nature and source of the nitrogen, iron, phosphorus, sulfur and magnesium in the media (Waghmode *et al.*, 2014)

2.4 Screening of Biosurfactant Producing Microorganisms

Biosurfactant production can be detected in various organisms using the following measures:

1. Measuring of cell surface hydrophobicity test (Pruthi and Cameotra, 1999)
2. Haemolytic activity (Dehghan-Noudeh *et al*, 2009).
3. Surface activity (Desai and Banat, 1997).
4. Drop-collapse test (Yalcin and Aysun, 2009; Amrane *et al*., 2008).

2.4.1 Measurement of cell surface hydrophobicity.

This property of cell surface hydrophobicity was used by Neu and Porralla (1990) to screen microorgainisms for their ability to produce biosurfactant based on findings that hydrophobic surfaces are usually associated with low surface energy molecules.

Direct correlations exist between cell surface hydrophobicity and microbial biosurfactant production (Pruthi and Cameotra, 1997). Hydrophobicity test is expressed as the percentage of cell adherence to water insoluble surfaces, such as hexadecane (Deziel *et al*., 1996).

2.4.2 Hemolytic activity.

Blood agar is an enriched medium for growing fastidious bacteria and also as a differential media used to screen for biosurfactant production in micro-organism (Mulligan *et al*, 1984; Ilori *et al*, 2005)

Hemolytic activity as a technique was first discovered in 1970, establishing the production of surfactin by *Bacillus subtilis* (Moran *et al*., 2002).

Hemolysis can be categorized into three alpha hemolysis, beta hemolysis and gamma hemolysis. These represent area of clearance on blood agar medium upon growth of bacteria, which has the capability of effecting hemolysis.

Identification of biosurfactant producing strain with blood agar plate test was previously reported by Mulligan *et al,* (1984). However, the extent of hemolytic zone formation on blood agar is not the solely dictated by the concentration of biosurfactant and may be affected by divalent ions and other haemolysin produced by the microbe under investigation. (Tahzibi *et al.*, 2004)

2.4.3 Surface activity measurement and critical micelle concentration.

Surface tension is defined as the free surface enthalpy per unit area and is the force acting on the surface of a liquid leading to minimization of the area of the surface (Al-Araji *et al.,* 2007)

Surface tension is the measurement of surface free energy per unit area required to bring a molecule from bulk to the surface. Surface tension reduction is an effective and efficient criterion for surfactant activity. The effectiveness of surfactant is the measurement of the minimum value to which surface tension of the growth medium could be reduced. The effectiveness of the measurement of the surfactant concentration is required to produce a significant reduction in the surface tension of water. Biosurfactant generally can be quantified indirectly by their surface activity. Every biosurfactant mixture thereof has a specific physicochemical activity characterizing it. At certain concentration the biosurfactant reaches a minimum surface tension which does not decrease any further (Mulligan *et al.,* 1989; Sumiardi *et al.,* 2012)

A good surfactant can lower surface tension of water from 72 to 35mN/m and the interfacial tension of water –hexadecane from 40 to 1mN/m. Surfactin from *Bacillus subtilis* can reduce the surface tension of water to 25mN/m and interfacial tension of water-hexadecane to

<1mN/m. Rhamnolipid of *Pseudomonas aeruginosa* decreases surfaces tension of water to 26mN/m and the interfacial tension of water –hexadecane to <1mN/m. Sophorolipid of *Torulopsis bombicola* have been reported to reduce the surface tension to 33mN/m and the interfacial tension to 5mN/m. Biosurfactant are more effective and efficient and their critical micelle concentration is about 10 to 40 times lower than that of chemical surfactant, that is, less surfactant is necessary to get a maximum decrease in surface tension (Desai and Banat, 1997, Muthusamy *et al*, 2008)

Stoimenova *et al*, 2009, described the production of rhamnose by *pseudomonas fluorescens* from different carbon source and biosurfactant production was detected by the measuring surface and interfacial tension of which it was detected that the surface tension of supernatant varied from 28.4mN/m with Phenanthrene to 49.6mN/m with Naphthalene and heptane as carbon source. The interfacial tension was also said to change in a narrow interval between 6.4 and 7.6mN/m.

In-terms of work, W, surface tension can be defined as

$\gamma = w/\Delta A$

Where ΔA is the change in the surface area (Garret, 1972)

It can also be defined as the force F per unit length L tending to pull the surface back Therefore

$\gamma = F/L$

The association between surfactant and the phases of different polarity like oil-water and air – water cause reduction in surface tension. One of the factors that can cause the reduction in surface tension is the presence of microbial surfactant. A good surfactant producer is defined as one (microorganism) being able to reduce the surface tension of growth medium by ≥20mN/m compared with distilled water (Willumsen and Karlson, 1997).

Quantifying biosurfactant indirectly by their surface activity has a few potential problems in that surface tension is very sensitive to specific condition in the medium. The pH of the

medium can greatly influence the surface activity measurement and also how readily available the carbon or the substrate is also determines the rate at which surface active compound and invariable affects the rate of reduction of surface tension readings (Zhang and Miller 1992)

The Du Nouy ring and capillary methods are widely used method for the measurement of surface and interfacial tension.

2.4.4 Drop collapse method.

This method is used to qualitatively determine the presence of surface active compound in a mixture. Jain *et al.,* (1991), developed the drop collapse assay which relies on the destabilization of liquid droplets by surfactants that correlate with the surface and interfacial tension. Droplets of a cell suspension or culture supernatant are dropped on a surface (such as glass slides or micro-wells on plastics) previously coated with mineral oil, if the liquid does not contain surfactant the polar water molecules are repelled from the hydrophobic surface and droplet remains stable. Droplets of broths or supernatants containing surface active compound spread or collapse due to the reduction in interfacial forces between the liquid and the hydrophobic surface. Stability of drops depends on the concentration of surface active compound and the correlation with surface and interfacial tension.

Drops containing surface active compounds will be unable to form stable droplets and would spread easily over an oily surface. Solution without surface active compound will remain beaded on the oily surface. Tugrul and Cansunar, (2005) and Umeji *et al.*,(2010) confirmed the reliability of drop collapse method using polystyrene micro-well plates coated with mineral oil, wells in which droplets collapsed indicated that the culture broths contain surface active compounds. There was no change in the shape of the droplet in the absence of surface active compounds.

2.5 Physiological Role of Biosurfactant

Biosurfactant have being reported to enhance the uptake of hydrophobic substrates. The methods of hydrophobic substrate uptake include; direct interfacial uptake of the hydrophobic substrate by hydrophobic cell membrane, emulsification enhanced interfacial uptake and solubilization of hydrophobic substrates by biosurfactant. Emulsification and solubilization are influenced by biosurfactant (Senthil and Jayalaksmi, 2013).

Biomass increases arithmetically when surface area of the growth medium becomes limiting rather than exponentially giving credence to the evidence that emulsification is a natural process brought about by extracellular surface active compound. There are conceptual difficulties in understanding how emulsification can provide evolutionary advantages for the microorganism producing bio-emulsifier (Ron and Rosenberg, 2001; Okoliegbe and Agarry, 2012)

Bioavailability of a chemical compound is governed by physico-chemical processes such as sorption, desorption, diffusion and dissolution. The bioavailability of hydrophobic nutrients is improved by microorganisms when they produce surface active compounds such as biosurfactant. The success rate of microbial cells in colonising a nutrient restricted environment is often related to their ability to produce polymers with surface activity (Pacwa-Plociniczak *et al.,* 2011).

Biosurfactants also function as antagonistic molecules against other microorganisms (Ron and Rosenberg, 2001). The roles of biosurfactants also include motility, bio-film formation, quorum sensing, cellular differentiation, storage of carbon and energy containing molecules and avoidance of toxic substances such as toxic metals. Microorganism avoid toxic compounds by sorption, desorption or precipitation. Biosurfactant also protect cells against high ionic strength (Soberon-Chavez *et al.,* 2005; Pacwa-Plociniczak *et al.,* 2011; Fakrudin, 2012).

2.6 Biochemistry of Biosurfactant Production

Surface active compounds (biosurfactants) are amphilic compounds. The hydrophilic moieties of these surface active compounds are made up of carbohydrates, amino acids, cyclic peptides or phosphates while the hydrophobic moieties are usually made up of fatty acids and its derivatives. The synthesis of biosurfatants involves two major metabolic pathways; namely the fatty acid synthetic pathway and the gluconeogenesis pathway (Kitamoto *et al.,* 2002; Desai and Banat, 1997).

Rhamnolipd, a biosurfactant produced by *Pseudomonas aeruginosa* is a mixture containing two 3-hydroxy fatty acid of varying length linked to the rhamnose (sugar) moiety. Burger *et al*., (1963), proposed that the synthesis of rhamnolipids proceed by two sequential glycosyl transfer reactions involving different rhamnosyltransferases. The first rhamnosyltransferase catalyses the transfer of thymidine diphosphate-L-rhamnose to 3-hydroxyalkanoyloxy alkanoic acid encoded by the *rhl*AB operon (Ito and Suzuki 1972; Deziel *et al*., 1999; 2003; 2005).

Different microorganisms utilise different pathways depending on the types and nature of substrates available to them and the types of biosurfactant to be produced. *Rhodococcus erythropolis* utilises a different pathway from that of rhamnolipid in the synthesis of nonionic trehalose mono and di-corynomycolates. Often the sugar moieties of biosurfactants are formed by the de-novo biosynthetic pathway or by the gluconeogenesis pathway, while the chain length of the fatty acid portion is dependent on the hydrocarbon source available to the microorganism. For example, the synthesis of glycolipids from carbohydrate is jointly regulated by the lipogenic and the glycolytic metabolic pathways. The lipogenic pathway may be short down if lipophilic compounds are made available in the substrate, therefore conserving the required enzymes and energy (Rapp *et al*., 1979; Boulton and Ratledge 1987; Mulligan and Gibbs, 1990).

Metabolism of lipopeptides such as iturin, lichenysin and arthrofactin are mediated by non-ribosomal peptide synthetase complex (Nagorska *et al*., 2007). Metabolism of hydrocarbons such alkanes to biosurfactant involves the oxidation of alkanes to mono alkanoic via the alkanols. The mono alkanoic make up the fatty acids or the hydrophobic moieties of the biosurfactants. Fatty acids can also be metabolised through β-oxidation to yield co-enzyme A which is further converted in the tricarboxylic acid cycle to sugars and amino acids, part of which is used in the biosynthesis of biosurfactant (Nagorska *et al*., 2007).

2.7. Genetic Regulation of Biosurfactant Production

Rhamnolipid synthesis by *Pseudomonas aeruginosa* proceeds by two sequential glycosyl transfer reactions each is catalyzed by a different rhamnosyl transferase. The first transferase which catalyzed the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkanoic acid is encoded by *rhl*AB operon. *rhl*A and *rhl*B genes co-expressed from the same promoter are essential for rhamnolipid synthesis but where as *rhl*B is known to encode the catalytic subunit of rhamnosyltransfrase, *rhl*A is probably an inner membrane bound protein presumably involved in the synthesis or transport of rhamnosyltransferase precursor substrate or in the stabilization of rhlB. Enviromental factors, especially nutritional conditions influence rhamnolipid production. Furthermore cell-to-cell signaling known as quorum sensing; regulates the expression of the *rhl*AB operon. This quorum sensing system is composed of *rhlI*, *las* the N-butyrylhomoserine lactone autoinducer synthase gene and *rhl*R which encode the transcriptional activator. The second rhamnosyltransferase encoded by *rhl*C has being characterized and its expression shown to be coordinately regulated with *rhl*AB by the same quorum sensing system (Ochner *et al*., 1994, 1996; Desai and Banat., 1997; Deziel *et al*., 2003; Zhu *et al.,* 2010; Mathiyazhagan *et al.,* 2011).

Quorum sensing is a phenomenon where microorganisms communicate and co-ordinate their behavior via the accumulation of signaling molecule, the mechanism relies upon a process

such that when the concentration of these signaling molecules reach a critical threshold, activation or repression of certain target cells occur (Raina *et al.,* 2009).

Quorum sensing, a cell density dependent gene regulation process allowing bacteria cell to express certain specific gene on attaining high cell density, regulates the production of biosurfactant (Das *et al*., 2008)

One vital factor determining the biosynthesis of biosurfactant is the genetic make-up of the producer organism. Studies on molecular genetics and biochemistry of the synthesis of several biosurfactant have revealed operon, the enzymes and the metabolic pathways required for extracellular production. Among all the biosurfactant reported till date, the molecular biosynthetic regulation of rhamnolipid, a glycolipid produced by *Pseudomonas aeruginosa* and a lipopeptide biosurfactant called surfactin produced by *Bacillus substilis* were the first to be deciphered. Other biosurfactant whose molecular genetics have been delineated in recent years include arthrofactin from pseudomonas species, iturin and lichenysin from bacillus species, mannosylerythriol lipid (MEL) from *Candida* and Emulsan from *Acinetobacter* sp. (Das *et al*, 2008)

Surfactin synthesis is catalyzed by surfactin synthetase consisting of three protein sub unit SrfA, comA, SrfC. The peptide synthetase required for amino acid moiety of surfactin is encoded by four operons. The operons are namely SrfAA, SrfAB, SrfAC and SrfAD or SrfA-TE. The operon also contain ComS gene lying within and out of frame with the SrfB while SrfAD is not essential for surfactin biosynthesis (Ramana and Karanth, 1989).

Sfp is another gene encoding phosphopantetheinyl transferase required for activation of surfactin synthetase by post translation modification.

Another gene is that of acyl-transferase which is responsible for the transfer of hydroxyl fatty acid moiety to srfAA but it is yet to be characterized. *Bacillus subtilis* has been found to

regulate surfactin production by a cell density-responsible mechanism not based on homoserine lactose but utilizing a peptide pheromone comX.

Quorum sensing controls srfA expression by comX which when interact with comP and comA, activates the signal transduction system. ComP (histidine protein kinase) donates a phosphate to the response regulator comA which gets activated and stimulates the transcription of the srf operon. Srf transcription is also activated by the pheromone Csf by inhibiting the comA phosphate phosphatase RapC. Since Csf is an extracellular peptide factor, it has to be imported inside the cell (Das *et al*., 2008).

The three genes of arthrofactin operon of pseudomonas are; arfA, arfB and arfC encode ArfA, ArfB and ArfC containing 2, 4, 5 functional modules. Each module has condensation, adenylation and thiolation domains (Roongsawang *et al.,* 2003).

Viscosin is produced by *Pseudomonas fluorescens* pfA7B. Braun *et al.,* (2001), indicates that a 25Kb chromosomal DNA after transcription and translation forms three proteins which forms a synthetase complex and is required for viscosin production (Das *et al*, 2008)

2.8. Kinetics of Fermentative Production of Biosurfactant

Researchers have previously reported the kinetics of cell growth, substrate utilization and biosurfactant production as a vital parameter in commercial production processes. Production of biosurfactants is largely dependent on the substrate, substrate concentration, pH, aeration, temperature, agitation, salinity and carbon dioxide which all interact in a complex way to affect the kinetics of biosurfactant production (Novon-Venazia *et al.,* 1995). Abu-Ruwaida *et al*., (1991). Patel and Desai (1997) observed that the production of biosurfactant is enhanced under nitrogen limiting condition.

Khopade *et al.,* (2012), using marine *Norcardiopsis B4* in a batch fermentation process, studied the kinetics of biosurfactant production. They reported that the biosurfactant from this

halotolerant strain exhibited potential application for bio-remediation of oil spill contaminations. They concluded that surface and interfacial tension, emulsification and cell separation provides information into understanding the production of biosurfactant.

Silva *et al. (*2010) used *P. aeruginosa* UCP0992 to investigate the effect of both carbon and nitrogen (source and concentration) on biosurfactant production at different cultivation conditions such as aeration, temperature, and agitation speed. Growth and biosurfactant production in mineral medium formulated with 3% glycerol and 0.6% $NaNO_3$, at 28^0C during 120hrs incubation at 200rpm was monitored. They reported an almost parallel relationship between biosurfactant production, cell growth, consumption of glycerol, emulsification, surface and inter-facial tension reduction, hexadecane, and other substrate utilization. They concluded that biosurfactant production is associated with growth starting shortly after inoculation with a two phase profile, the first up to 24hrs and remaining constant until 48h, while in the second phase, production increase data slower rate up to 96hrs with yields of 8.0g/L. Biomass concentration was high (4.0g/L) and glycerol consumption profile showed a similar pattern to surface and interfacial tension reduction, while, the hexadecane emulsification followed biosurfactant production. Such observations support the use of surface and interfacial tension and emulsification as indicative measures for the presence of biosurfactant molecules in the medium. Roodriques *et al*., (2006b,c), reported the production of biosurfactant by *Lactobacillus pentosus* CECT-4023 using cheese whey as substrate.

2.9 Factors Affecting Biosurfactant Production

Different parameters affect the fermentative production of biosurfactants, these parameters among others include; microbial strain, carbon source, nitrogen source (organic or in-organic), pH, temperature, oxygen and metallic ions (Desai and Banat, 1997).

2.9.1. Effect of carbon source on biosurfactant production

Stoimenova *et al* 2009 also reported that *Pseudomonas fluorescens* isolated from industrial waste water have the capability to produce glycolipid, from mineral oil, hexadecane, glycerol and vegetable oil. The carbon sources used in biosurfactant production can be divided into three categories: Carbohydrate, hydrocarbon and oils. The use carbohydrate is largely dependent on it amount of free and usable sugar (Plaza *et al.*, 2011)

El-Sersy (2012), observed that *Bacillus subtilis* N10 when grown interchangeably on glucose, fructose, rhamnose, galactose, and arabinose as carbon sources produced different concentration of biosurfactant.

Pseudomonas species have been reported to be able to utilize various carbon sources for the production of rhamnolipid biosurfactant. Carbon sources such as glycerol, mannitol, glucose, hydrocarbon, vegetable oil have been utilized but the composition of the carbon source affects the quality, composition and type of the biosurfactant. Substrates with variation in carbon chain length have also been reported to have no effect on the chain length of fatty acid moiety in the biosurfactant (Shafiei, *et al*., 2014).

Surfactin was produced by *Bacillus subtilis* ATCC 21332 from agricultural waste such as cassava flour waste water, chesse whey and molasses (Nitschke *et al*., 2004).

2.9.2 Effect of nitrogen sources on biosurfactant production

Depending on the microbial strain, nitrogen source greatly affects the production of biosurfactant. Among the in-organic sources of nitrogen that have been investigated by various researchers, ammonium salt and nitrate are the most preferred nitrogen source. *Rhodococcus* and *Pseudomonas* species prefer nitrate as in-organic source while *Athrobacter paraffineus* prefer ammonium as in-organic nitrogen source. Yeast extract and urea are the most prefer organic nitrogen sources for the production of biosurfactant (Gautum and Tyagi, 2006).

Wei *et al*., (2005), observed that high nitrogen concentration in a fermentation medium could limit production of bisourfactant, therefore limitation in nitrogen concentration aid the production of biosurfactant. They also reported that *Pseudomonas aeruginosa* J4 preferred sodium nitrate as it nitrogen source.

Abouseoud *et al*., (2007) using vegetable oil as substrate, reported the impact of nitrogen source on biosurfactant production by *Pseudomonas fluorescens*. They observed that ammonium nitrate and sodium nitrate are the best nitrogen source for biosurfactant production by *Pseudomonas fluorescens* under their conditions of study and also that depletion of nitrogen at the stationary phase of cell growth aided the yield of biosurfactant.

According to studies by Haddad *et al.,* (2009), in which ammonium sulphate was used as nitrogen source resulted in the highest reduction in surface tension while comparing it with sodium nitrate. They also include ammonium nitrate, potassium nitrate and soya bean flour as other sources of nitrogen.

2.9.3 Effect of environmental factors.

An important feature of bacteria lifestyle is that their environment changes constantly. Environmental factors and growth conditions such as pH, temperature, agitation and oxygen concentration affects biosurfactant production through their effects on cellular growth or activity.

The ionic concentration of the medium greatly affects biosurfactant production by *Pseudomonas* species and organism such as *Torulopsis bombicola*. Surface tension and critical micelle concentration of biosurfactant remains stable over a wide range of pH value of which also emulsification has a narrow pH range (Gautum and Tyagi, 2006, Rapp *et al*., 1979 and Abu-Ruwaida *et al*., 1991)

Heat treatment of some biosurfactant caused no appreciable change in biosurfactant properties such as surface and interfacial tension, emulsification and efficiency; all which

remained stable at 121^{o}C for 15mins. The applications of biosurfactant for industrial purposes greatly depend on the thermal stability of the biosurfactant at varying temperature values.

According to Khopade *et al*., 2012, the biosurfactant produced by *Norcardiopsis* B4 strain was shown to be thermal stable at temperature as high as 100^{o}C causing no significant effect on the biosurfactant performance and also emulsification was still stable at high temperature with E_{24} = 66% in comparism to synthetic biosurfactant.

An increase in agitation speed results in the reduction of biosurfactant yield due to effect of shaking in *Norcadia erythropolis.* On the other hand, in yeast, biosurfactant production increases when the agitation and aeration rates are increased. (Gautum and Tyagi, 2006, Khopade *et al*., 2012).

2.10 Application of Biosurfactant

The commercial application of biosurfactant is determined by their cost and properties in relation to competing synthetic compound (Georgiou *et al*., 1999). Because of these superior properties, biosurfactant have a potential use in food, pharmaceuticals and cosmetic industries (Pornsuthorntawee *et al*., 2008). Biosurfactant have carved a niche for themselves with their unusual antibacterial, antifungal and antiviral properties. In biomedical science, some of the uses of biosurfactant include their role as anti-adhesive agents of pathogens, making them useful for treating many diseases and as therapeutic probiotic and pharmaceuticals agent (Sekhon *et al.,* 2011).

Contamination of the environment by heavy metals is of growing concern because of the health risk posed by human and animal exposure. A survey of remedial action sites revealed that heavy metals e.g cadmium, copper, lead, zinc etc., were the most prevalent class of contaminant (Pacwa-Plociniczak *et al.*, 2011)

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Collection, Isolation and Characterization of samples.

3.1.1 Collection and handling of brewery effluent and soil sample.

Five liters of raw brewery effluent was collected from International Beer and Beverage Industries (IBBI) Kudenda industrial estate, Kaduna. The effluent was collected directly from channel through which it passes through before getting into the treatment chambers.

The soil sample used for the isolation of *Pseudomonas aeruginosa* and *Pseudomonas flourescens* was collected from the bank of the stream into which treated effluent is being discharged.

Both samples were collected into separate sterile containers (5L capacity gallon for brewery effluent and a 100g capacity glass ware with lid for soil) which have been previously autoclaved (autoclaving at 121^{o}C for 15min) and covered air tight while being transported to the laboratory.

3.1.2 Isolation of Pseudomonas species from soil sample.

The soil sample (5mg) was serially diluted in 45ml of physiological saline (0.85% normal saline solution). Further dilution in the proportion of 1ml of previous dilutions to 9ml of physiological saline was carried out till a dilution of 10^{-4} was obtained. Aliquots of 0.1ml of the dilution (10^{-4}) were plated on Cetrimide agar for *Pseudomonas aeruginosa*. Also aliquot of 0.1ml of same dilution (10^{-4}) was plated on Pseudomonas selective agar, cetrimide agar and nutrient agar for *Pseudomonas fluorescence* using the spread plate technique (using a sterile bent glass rod to spread aliquot over the surface of the media). This was then be incubated at 37^{0}C for 24hrs (Prescott *et al.*, 1999)

Isolates obtained were streaked on another Petri-dish containing nutrient agar. This process was repeated until distinct pure colonies were obtained (Jayabarath *et al.,* 2009)

Isolates were preserved on trypticase soy agar slant.

3.1.3 Characterization of Pseudomonas isolated from soil sample.

Distinct colonies observed on the petri-dishes were used for characterization of the isolates obtained. In characterizing the *Pseudomonas* species, cultural, microscopic examinations and biochemical test were carried out. This includes; Gram staining, oxidase and catalase test. Microgen^{TM} Test kit GnA + GnB-ID (Microgen Bio-products, UK) was used for further biochemical characterization of isolates.

3.1.3.1 Cultural characterization:

The cultural appearances of the *Pseudomonas* species on pseudomonas selective agar, cetrimide agar and nutrient agar were observed for pigmentation, spread of colony and the ability of *Pseudomonas fluorescens* to grow on Cetrimide agar at 37^0C (Prescott *et al.,* 1999)

3.1.3.2 Microscopy:

In the microscopic examination, the Gram staining technique was used. Smear of organism was prepared on a clean, grease free glass slide and allowed to air dry which was then carefully heat fixed to prevent the cell damage. The heat fixed slides were then flooded with crystal violet solution for 30secs, washed off with slow running tap water and the slides drained. The slides were further stained with Grams iodine solution for one (1) minute then washed off and drained. Slides were then flooded with 95% ethyl alcohol for 10sec and then washed off. Finally safranin (2.5% w/v) was used to counter stain for thirty seconds and then washed off and the blotted slides were examined under the oil immersion objective (Prescott *et al*., 1999)

3.1.3.3 Catalase test:

One milliliter (1ml) of Hydrogen peroxide was transferred into a clean test tube, and then a colony of the test isolate was transferred into the test tube to observe the presence of effervescence if any. Presence of effervescence means positive and otherwise negative (Prescott *et al.*, 1999).

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

3.1.3.4 Oxidase test:

Using a forceps a strip of oxidase paper was rubbed on the colony of interest and colour change was observed out for 60sec. Purple color on the strip meant positive and no colour change indicates negative (Prescott *et al*., 1999)

3.1.3.5 Biochemical characterization with Microgen kit:

The Microgen^{TM} GN-ID system was used to biochemically characterise the isolates from soil. The kit (Microgen^{TM} GN-ID) consists of two separate microwell strips; GNA and GNB; each strip contains 12 standardised dehydrated biochemical substrates in each of the microwells. The GNA and GNB microwell test strip were used together for the identification of the isolates (oxidase test was first carried out as described above). Thereafter a single colony of 24hr culture was emulsified in 5ml of sterile normal saline (0.85%, NaCl). Using a sterile pipette 4 drops of the bacterial suspension was transfer into each of the 24 wells of the titer plates. Wells 1, 2, 3 and 9 on GNA strip and well 24 on GNB strip were over laid with 4 drops of mineral oil. The wells are then carefully sealed with a sealing tape provided along and incubated at 37^0C for *Pseudomonas aeruginosa* and 25^oC *Pseudomonas fluorescens* for 48hr.

After the incubation period, colour changes for reactions were noted and read with the aid of a colour chart provided in the manual. Thereafter 2 drops of Kovasc reagent is added to well eight and read 60sec, a formation of red color indicates a positive result.

A drop of Vogues Prausker reagent one and a drop of Vogues Prausker reagent two were added to well 10 and read after 30mins, formation of a red color indicates a positive result.

A drop of TDA reagent was also added to well 12 and read after 60secs, formation of a cherry red color indicates a positive result.

The nitrate reduction test was carried out after reading the result of ONPG on well seven, one drop of nitrate reagent A and one drop of nitrate reagent B was added to well seven and read in 60sec. the development of a red color indicates Nitrate reduction.

On GNB strip, the gelatin liquefaction result was read in 48hrs for oxidase positive isolates by the presence of a black particle visible throughout the well. Also the arginine well (24) for oxidase positive isolate was also read with a blue color change after 48hr.

Results obtained from the reaction above were inputted into a data form which is then added up and interpreted by software provided by the manufacturer of the kit.

3.2. Determination of Physico-chemical Properties of the Brewery Effluent

3.2.1 pH

The pH of the effluent was determined using an automated pH meter (Jenway pH meter 3150) by dipping the electrode of a pre-standardized pH meter into a beaker containing the brewery effluent. Measurement of pH was carried out in the laboratory and not at the point of collection.

3.2.2 Chemical oxygen demand (COD)

To determine the COD, the reflux digestion and titration approach was added. Fifty milliliters (300ml) of distilled water was transferred into reflux flask as blank and into another flask thirty milliliter (30ml) of effluent was transferred which was then made up to 3000ml with distilled water. Both flasks were placed in ice-bath.

Into each flask one gram (1g) of mecuric sulphate ($HgSO_4$), five milliliters (5ml) of 95% sulphuric acid (H_2SO_4) and beads boiling stones was place and content in both flasks was mixed to dissolution. Still in the ice-bath, five milliliters (25ml) of 0.04167M of standard potassium dichromate ($K_2Cr_2O_7$), one gram (1gm) of silver sulphate Ag_2SO_4 and 70ml of 95% sulphuric acid (H_2SO_4) was slowly added to the flask with blank. The reflux flasks were attached to the condenser and cold water was made to run through. The flask was then heated and refluxed for two hour (2hrs). After refluxing, the flasks were cooled and condenser washed down with distilled water before removing the flasks. Acid solution (30ml) was diluted to three hundred milliliters (300ml) with distilled water and allowed cool at room temperature.

Three drops of phenanthroline ferrous sulphate solution was added to each flask as indicator and the excess dichromate was titrated with 0.25N ferrous ammonium sulfate (FAS) solution until a sharp colour change from bluish-green to reddish brown. If a reddish brown colour is observed, the analysis was repeated with a smaller aliquot.

The COD was obtained using the following equation

$$COD\ (mg/l) = (A-B) \times N \times 8000/S$$

Where

A= titre value of $FeSO_4\ (NH_4)SO_4.6H_2O$ of Blank in ml.

B= titre value of $FeSO_4(NH_4)SO_4.6H_2O$ of effluent in ml

N = normality of $FeSO_4(NH_4)SO_4.6H_2O$

S= ml of effluent used for analysis

(American Public Health Association *et al.*, 1999)

3.2.3 Total solids

To measure total solids, clean evaporating dish (100ml capacity) was heated at 103^{o}C for one hour and cooled in a desicator. The weight of the dish was immediately measured. Thereafter 50ml of the well mixed sample was pippetted into the pre-weighed evaporating dish. The content of the dish was then placed in the oven and evaporated to dryness. The dry evaporated sample is heated at 105^{o}C in an oven. This was removed from oven and cooled in a desicator to a balanced temperature and the weight obtained.

The weight of the total solid was determined as

$$\text{Total solid (mg/l)} = \frac{A - B \times 1000}{\text{Sample volume (ml)}}$$

A = weight of dried residue + dish (mg)

B = weight of dish (mg)

(American Public Health Association *et al.*, 1999)

3.2.4 Total dissolved solids

Thoroughly mixed sample (100ml) was pipetted unto a clean glass-fiber, filtered with applied vacuum, then wash thrice successively with 10ml of reagent grade water and allowed to completely drain between wash and then continued suction for 3min after filtration is complete. The total filtrate was transferred to a pre-weighed evaporating dish and evaporated

to dryness in an oven at 103^{o}C. The evaporated sample was further dried at least for one hour in an oven at 180^{o}C and then allowed to cool in a desicator to a balanced temperature and weighed. The drying cycle and desiccating and weighing were repeated until a constant weight was obtained.

Therefore weight of total dissolved solids (mg/l) = $\frac{(A-B) \times 1000}{\text{Sample volume (ml)}}$

A = weight of dried residue + dish (mg)

B = weight of dish (mg)

(American Public Health Association *et al.,* 1999)

3.2.5 Dissolved oxygen (DO)

300ml biological oxygen demand stoppered bottle was filled with sample water to the brim and then 2ml of managanese sulphate (reagent grade) was carefully added to sample without allowing air bubble formation. Also 2ml of sodium iodide-azide (reagent grade) was added to the sample and bottle stoppered carefully to avoid air bubbles. The content of the bottle was mixed by inverting several times after which a brownish orange cloud of precipitate was observed which was allowed to settle to the bottom of the bottle and then mixed by inverting several time again and then allowed to settle.

To the sample 2ml of 10N sulphuric acid was added via caliberated pipette and carefully stoppered to avoid air bubbles and then thoroughly mixed by invertingseveral times till precipitate dissolve. The bottle and content is then stored at 20^{0}C for 8hrs in a dark room.

In an Erlenmeyer flask, 201ml of sample was titrated with sodium thiosulphate to a pale straw color and slowly mixed on a magnetic stirrer. 2ml of starch solution was added to give

a blue color and titration continueduntil aclear solution was of the sample was obtained and the end point noted.

The concentration of dissolved oxygen in the sample is equivalent of the volume of milliliters of titrant used. Each milliliter of sodium thiosulphate used equal 1mg/l of dissolved oxygen. (American Public Health Association *et al.,* 1999)

3.2.6 Biological oxygen demand.

Two 300ml glass stoppered biological oxygen demand (BOD) bottles (one each for sample and blank), of which into one 300ml of sample was carefully transferred and into the other 300ml of dilution water was also transferred carefully. Both bottles were stoppered immediately and incubatored at 20^0C for five days. After day five, both bottles were removed from the incubator.

Into each bottle the following was added, 2ml of manganese sulphate just below the liquid surface to avoid introduction of air followed by 2ml of alkali-iodide-azide reagent and thoroughly mixed till a brownish-orange cloud of floc appear and the allowed to settle to the bottom of the bottle. The bottles were thoroughly shaken again by turning it upside down 20 times. Then 2ml of 10N sulphuric acid was added via a pipette and bottles stoppered and then inverted several times to till floc dissolve. Into Erlenmeyer flask, 203ml of sample was carefully dispensed and titrated with sodium thiosulphate until the yellow colour of the liberated iodine faded. Using starch solution as indicator, 1ml of the indicator was added into the Erlenmenyer flask content and titration continued until the blue color of the indicator became colourless.

The volume of the sodium thiosulphate solution used was recorded as the final volume dissolved oxygen after five days of incubation (DO_5). Note that the initial DO of the sample was taken in section 3.2.5

The equation for the determination of a BOD_5 value is:

$$BOD_5 \text{ (mg/L)} = \frac{D_1 - D_2}{P}$$

Where

D_1=initial DO of the sample,

D_2=final DO of the sample after 5 days,

P =decimal volumetric fraction of sample used.

(American Public Health Association *et al.,* 1999)

3.3 Screening of isolated*Pseudomonas* Species for Biosurfactant Production.

The following methods were used for screening the ability of the isolates to produce biosurfactant:

3.3.1 Drop-collapse test and preparation of mineral media.

i. The *Pseudomonas aeruginosa* isolate was inoculated into a 150ml capacity Erlenmeyer flask containing 100ml brewery effluent supplemented with mineral salt medium composed of basal salts in g/l (NH_4SO_3 (4.0), Na_2HPO_4 (5.9), KH_2PO_4(4.1), NaCl(0.5), $MgSO_4$(0.096), $CaCl_2$(7.74 x 10-4), Na_2EDTA (1.48 x 10^{-3}) and yeast extract 0.05% w/v. The flask was incubated at room temperature on an orbit shaker (Lab Line Orbit Environ Shaker-18) for 120hrs at 120rpm (Yalcin and Aysun, 2009). McFarland scale 2 representing 600 x 10^6 colony forming unit per milliliter was used for inoculation.

ii. The *Pseudomonas flourenscens*, isolate was inoculated into a 150ml capacity Erlenmeyer flask containing 100ml of brewery effluent supplemented with mineral salts medium in g/l ($NaHPO_4$ (2.2), KH_2PO_4(1.4), $MgSO_4.7H_2O$ (0.6), $FeSO_4.7H_2O$ (0.01), NaCl(0.05), $CaCl_2$(0.02), yeast extract(0.02). 1ml of trace elements solutions containing (g/l)

of 2.3g $ZnSO_4.7H_2O$, 1.78g $MnSO_4.4H_2O$, 0.56g $H3BO_3$, 1.0g $CuSO_4.5H2O$, 0.39g Na_2MoO_4. $2H_2O$, 0.42g $CoCL_2.6H2O$, 1g EDTA, 0.004g$NiCl_2.6H_2O$, 0.66g KI) (Abouseoud *et al.,* 2007). McFarland scale 2 (600 x 10^6 colony forming unit per milliliter) was used for inoculation and incubated at room temperature for 120hr on an orbit shaker at 120rpm. (Tehaoei *et al*., 2003; Medina-Moreno *et al.,* 2011)

For both isolates (*Pseudomonas aeruginosa* and *Pseudomonas fluorescens*) after fermenting for 120hrs, 10ml of culture broth was collected in separate tubes for each isolate and centrifuged at 8000rpm for 25mins. Crude supernatant collected was used for the drop-collapse test, oil displacement test and hemolytic test according to Adebusoye *et al.*, (2008), to confirm the presence of surface active compound.

The drop collapse test was carried out by coating a glass slide with mineral oil and incubated at room temperature for 2hrs to get the oil to be properly affixed to the slide. The slide was then placed on a flat surface and thereafter a drop of the culture supernatant earlier centrifuged at 8000rpm for 25mins and left at room temperature for one minute. The shape of the slide was carefully observed. A beaded drop after 60secs indicates the absence of surface active compound in the supernatant, but a flattened drop that spreads easily over the surface of the slide is confirmed positive for surface active compound (Maneerat and Phetrong, 2007; Umeji *et al.,* 2010)

3.3.2 Hemolytic test:

Pseudomonas aeruginosa and *Pseudomonas fluorecens* was streak on a solidified blood agar medium (Nutrient agar with 5% v/v human blood) to check for the ability of the isolate to lyse blood cells. Plates were incubated at 37^{o}C for 24hrs, thereafter observed for cleared zone around wells created. Zone of clearance indicate positive and if none indicates negative (Dehghan-Noudeh *et al*., 2009).

3.3.3 Oil displacement assay

Fifteen microliter of mineral oil was placed on the surface of 10mls of distilled water in a petri-dish, thereafter 10µl of the culture supernatant was placed gently on the centre of the oil film. The diameter and area of cleared zone was measured after 30secs. The presence of surface active compound (biosurfactant) will cause displacement of oil which spreads in the water (Maneerat and Pheetrong, 2007; Walter *et al.*, 2000 and Anandaraj and Thivakaran, 2010)

3.4. Measurement of Cell Growth and Incubation Period

Incubation was carried out for a time period of 168hrs with interval sample collection of 24hrs. Cell growth was determined by placing two milliters of culture broth in a cuvette and measured using a colorimeter at 600nm.

3.5. Production, Activity and Characterization of Biosurfactant

3.5.1 Fermentation of brewery effluent, measurement of surface tension and optical density.

The initial surface tension of the effluent was measured with the use of the capillary method. Mineral salts was constituted according to Abouseoud *et al.*, (2007) and Yalcin and Aysun (2009) which was then added to two hundred milliliters (200ml) of brewery effluent dispensed into a 250ml Erlenmeyer flasks and then autoclaved at 121^0C for 15mins and thereafter, inoculated with isolates (*Pseudomonas aeruginosa* and *Pseudomonas fluorescens)* in separate flasks (each isolate to a flask). This was agitated at 150rpm at room temperature for 168hrs on an orbit shaker (Lab line orbit environ shaker-18).

Ten milliliters (10mls) from each of the fermentation flask was collected every 24hr for 168hr while agitation continued with the remaining in the flask. The 10ml collected was centrifuged at 8000rpm for 25mins to remove cells and debris. The supernatant collected was used to measure the surface activity of the broth to confirm the presence of surface active

compound (biosurfactant) through the reduction in surface tension. The supernatant was also used for emulsification assay (Abouseoud *et al.,* 2007; Priya and Usharani 2009). Two milliliters (2ml) each of the fermentation broth was also collected every twenty hours for 168hrs to measure the optical density of the broth as a measurement of cell growth.

After 168hrs, 50ml of the broth was collected and centrifuge at 8000rpm for 25mins, the supernatant collected from each flask was filtered using a 0.22µm milipore membrane filter (Milipore S.A, France). The sterile crude supernatant was collected for further extraction of surface active compound (Abouseoud *et al.,* 2007).

3.5.2 Extraction of surface active compound

The bio-surfactant was quantitatively extracted by liquid-liquid extraction. The cell free crude supernatant which was acidified with 6N hydrochloric acid (HCL) and kept in the refrigerator at about 4^0C for 24hr, and then mixed with chloroform and methanol in 2:1 ratio respectively. The organic phase containing the surface active compound was collected and the aqueous phase further extracted three times.

The organic extract was collected in sterile twenty milliliters glass petri-dish and evaporated in a fume cupboard to dryness. Empty dish was weighed before drying and also weighed after drying. Weight of biosurfactant (surface active compound) was calculated as

Dry weight of biosurfactant = Weight of plate after drying – weight of empty plate

The extract was re-dissolved in ten milliters of methanol for futher analysis (Nitschke *et al.,*2004; Rismani *et al.,* 2006; Adebusoye *et al.,* 2008, Parthasarathi and Sivakumaar 2009; Priya *et al.,* 2009; Onbasli and Aslim, 2009; Anandaraj and Thivakaran., *2010;* Bishwambhar *et al,* 2011*)*

Analysis of extract was carried out to verify the class and type of biosurfactant present. The shimazu FT-IR 8400S Fourier transformed intra-red spectroscopy was used (Parthasarathi and savakumaar, 2009; Yalcin and Aysun, 2009). This procedure was carried out at the National Research Institute for Chemical Technology (NARICT), Basawa, Zaria.

3.6. Determination of Effect of Some Environmental Factors on Biosurfactant Production and Activity

3.6.1. Determination of effect of pH on biosurfactant activity.

To determine the effect of pH on biosurfactant production and activity, nine pairs 250ml Erlenmeyer flasks containing 150ml each of brewery effluent with pH adjusted to the following 4, 5, 6, 7, 8, 9, 10 were sterilized at 121^0C for 15mins, allowed to cool and then inoculated with the isolates under aseptic conditions. 1N HCl was used to adjust the pH towards acidity (4-6) while 1N NaOH was used to adjust the pH towards alkalinity (8-10) and agitated at 150rpm on an orbit shaker (Lab line orbit Environ Shaker-18). Temperature was maintained at room temperature. Phosphate buffer was used to maintain the pH and all other parameters remained the same (Abouseoud *et al.,* 2007).

3.6.2. Determination of effect of temperature on biosurfactant activity.

To determine the effect of temperature on the biosurfactant activity, two pairs of 250ml Erlenmeyer flasks containing 150ml of brewery effluent supplemented with mineral salt in g/l (NH_4SO_3 (4.0), Na_2HPO_4 (5.9), KH_2PO_4(4.1), NaCl(0.5), $MgSO_4$(0.096), $CaCl_2$(7.74 x 10-4), Na_2EDTA (1.48 x 10^{-3})) for *Pseudomonas aeruginosa* and for *Pseudomonas fluorescens* in g/l ($NaHPO_4$ (2.2), KH_2PO_4(1.4), $MgSO_4.7H_2O$ (0.6), $FeSO_4.7H_2O$ (0.01), NaCl(0.05), $CaCl_2$(0.02)). 1ml of trace elements solutions containing (g/l) of 2.3g $ZnSO_4.7H_2O$, 1.78g $MnSO_4.4H_2O$, 0.56g $H3BO_3$, 1.0g $CuSO_4.5H2O$, 0.39g $Na_2MoO_4.$ $2H_2O$, 0.42g $CoCL_2.6H2O$, 1g EDTA, 0.004g$NiCl_2.6H_2O$, 0.66g KI (Abouseoud *et al.,* 2007)) was

inoculated with *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* in separate flask and the agitated at 150rpm on an orbit shaker for 168hr after which 100ml of the agitated effluent was collected and centrifuged. The supernatant collected was subjected to heating in a vibrating water bath (SHA-C) at varying temperatures (25^oC, 30^0C, 35^0, 40^0C, 45^0C, 50^0C, 55^0C, 60^0C, 65^0C, 70^0C and 80^0C) for 25min and allowed to cool to room temperature and the surface tension and emulsification activity of the biosurfactant measured (Maneerat and Phetrong, 2007; Haddad *et al.,* 2009; Umeji *et al.,* 2010).

3.6.3 Production of biosurfactant without addition of mineral salt and pH adjustment (control)

To determine the effect of fermentation without the addition of mineral salt to the effluent and no pH adjustment, two (2) flasks of one hundred and fifty milliliter (150ml) each of sterilized raw brewery effluent inoculated with the isolates(*Pseudomonas aeruginosa* and *Pseudomonas fluorescens)* was agitated at 150rpm for 168hrs on Orbit shaker. Ten milliliter of culture broth was collected every twenty four (24) hours of which two (2) milliliters was used to measure cell biomass growth as optical density and the remaining eight milliliters was centrifuge and two milliliters (2ml) was used for surface tension measurement and another for emulsification test.

3.7. Emulsification Activity (E_{24}) With Diesel, Premium Motor Spirit and Olive Oil

The emulsification test was carried out by adding 2ml of oil (Vegetable oil, diesel, kerosene and petrol) to 2ml of cell free supernatant obtained from each of the processes above, which was then mixed by vortexing for 2min using the Votex Mixer XH-C and left to stand for 24hrs. The E_{24} is measured as the percentage height of the emulsified layer (mm) divided by the total height of liquid column (mm). (Priya and Usharani, 2009; Mbawala and Mouafo,

2012). Brand of petroleum products used were sourced at OandO filling station opposite Ahmadu Bello University Samaru Zaria.

$$E24 = \frac{\text{Height of Emulsification}}{\text{Total height of solution}} \times 100$$ (Techaoei et al., 2003)

3.8. Surface Tension Measurement

To measure the surface tension of the substrate and the culture broth, the capillary rise method was used. A narrow capillary tube was dipped into the liquid (broth), held to a clamp on travelling microscope with calibrated lens in millimeter. The liquid rise through the capillary tube until equilibrium is reached. At this point the liquid in the column is supported by surface tension. Capillary rise is measured from the flat surface of the liquid in the beaker to the height of the lowest part of the meniscus in the capillary. The difference in height reached in the tube and the surface of the broth in the beaker is inputted into the following mathematical formular

$\gamma = 1/2\rho ghr_c$

where

ρ = density of the liquid

g = gravity due to acceleration

h = Capillary rise

r_c = radius of the capillary or tube.

Surface tension is measured as dyne/cm

1dyne/cm = 1mN/m.

(Joyban and Fathi-Azarbayani, 2012)

3.9. Statistical Analysis

The Independent Chi square was used for the analysis of data collected.

CHAPTER FOUR

4.0. RESULTS

4.1. Physicochemical Analysis of Brewery Effluent

Table 4.1 shows the data collected from the physico-chemical analysis of the effluent, which showed a result of 300mg/l of phosphate, 1.8mg/l of biochemical oxygen demand, 1500mg/l of chemical oxygen demand, 2.9mg/l of dissolved oxygen, 249mg/l of chloride ion, 3.8mg/l of nitrate and total solid of 3,500mg/l. Ammonium content of 0.64mg/l, calcium hardness of 9,716mg/l.

4.2. Isolation and Characterization of Isolates

Table 4.2 shows the cultural and microscopic characteristic of the isolates obtained from the soil samples collected. Colonies on cetrimide agar (Isolate A) produce a yellowish-green pigmentation which is *Pseudomonas aeruginosa* while isolate on pseudomonas selective agar (Isolate B) also grow on cetrimide with large spreading yellowish colony identified as *Pseudomonas fluorescens.* Both isolates appeared as Gram negative rods when viewed under the oil immersion objective.

4.3 Biochemical Characterization of Isolates.

Further biochemical characterization showed that the isolates were oxidase, catalase. Using the Microgen GN-ID system, the isolates were identified as *Pseudomonas aeruginosa (*isolate A) and *Pseudomonas fluorescens (*isolate B) as is reported in Table 4.3.

4.4 Screening of isolates for biosurfactant activity.

Table 4.4 shows the screening activities carried out on both isolate from table 4.3. Both isolate (*Pseudomonas aeruginosa* and *Pseudomonas fluorescens*) drop collapse assay and haemolysis were observed to be positive indicating the presence of surface active compound.. For haemolysis zones of clearance was observed. In the oil displace test, 28mm diameter was observed for *Pseudomonas aeruginosa* while 23mm diameter was observed for *Pseudomonas fluorescens.*

Table 4.1 Physicochemical properties of the brewery effluent

S/N	PARAMETER	RESULT
1	Color	70
2	C.O.D (mg/l)	15,000
3	D.O (mg/l)	2.9
4	B.OD (mg/l)	1.8
5	Calcium Hardness (mg/l)	9,716
6	Magnesium Hardness (mg/l)	10,485.6
7	Total Hardness (mg/l)	20,201.6
8	Chloride (mg/l)	249.9
9	Total Solids (mg/l)	3,500
11	Phosphate (mg/l)	300
12	Nitrate (mg/l)	3.8
13	Ammonia –N (mg/l)	0.64

Table 4.2: Cultural and Microscopic Characterization of isolates

CHARACTERISTIC	ISOLATE A	ISOLATE B
Grams stain	Gram negative Rods	Gram negative Rods
Pigmentation	Pyocyanin	Nil
Cultural appearance	Large spread colony	Large spread colony
Growth on Cetrimide at 37^{o}C	Positive	Positive
Probable Isolate	*P. aeruginosa*	*Pseudomonas* species

Table 4.3: Biochemical Characterization of isolates

	GNA Strip									GNB strip															
Test	**Oxi**	**Nit**	**Lys**	**Orn**	**H_2S**	**Glu**	**Man**	**Xyl**	**ONPG**	**Ure**	**VP**	**Cit**	**TDA**	**Gel**	**Mal**	**Inn**	**Sor**	**Rha**	**Suc**	**Lac**	**Ara**	**Ado**	**Raf**	**Sal**	**arg**
A	+	+	+	-	-	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	+	-	-	-	+
B	+	+	+	-	-	_	+	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-	-	-	+

This test was carried out using a Microgen^{Tm} test Kit GnA + B-ID system.

Key

A= *Pseudomonas aeruginosa*

B= *Pseudomonas fluorescens*

Oxi= oxidase; Nit= Nitrate; Lys= Lysine; Orn= Ornithine; H_2S= Hydrogen sulphide; Glu= Glucose; Man= Mannitol; Xyl= Xylose

ONPG= Ortho-nitrophenylgalactose; Ure= Urea; VP= Vogues Prausker; Cit= Citrate; TDA= Tryptophan deaminase test

Gel= Gelatin; Mal= Malonate; Inn= Innositol; Sor= Sorbitol; Rha= Rhamnose; Suc= Sucrose; Lac= Lactose; Ara= Arabinose

Ado= Adonitol; Raf= Raffinose; Sal= Salicin; Arg= Arginine

Table 4.4: Screening of isolates for biosurfactant activity

Test	Isolate A	Isolate B
Drop collapse	**+**	**+**
Haemolysis	+	+
Oil Displacement	28mm	23mm
Inference	Biosurfactant producing	Biosurfactant producing

4.5 Fermentation of Brewery Effluent for the Production of Biosurfactant.

4.5.1 Measurement of Surface activity and optical density.

Figure 4.1 shows the surface activity and optical density reading at 600nm for fermentation with surface tension of the medium as 31.30mN/m from 59.54mN/m and optical density reaching a peak of 1.07 before gradually reducing to 0.20 after 168hrs for *Pseudomonas aeruginosa* and fermentation using *Pseudomonas fluorescens* showed that surface tension dropped to 33.76mN/m from 59.54mN/m after 168hrs and an optical density of 1.07 after 72hrs was observed which reduced thereafter.

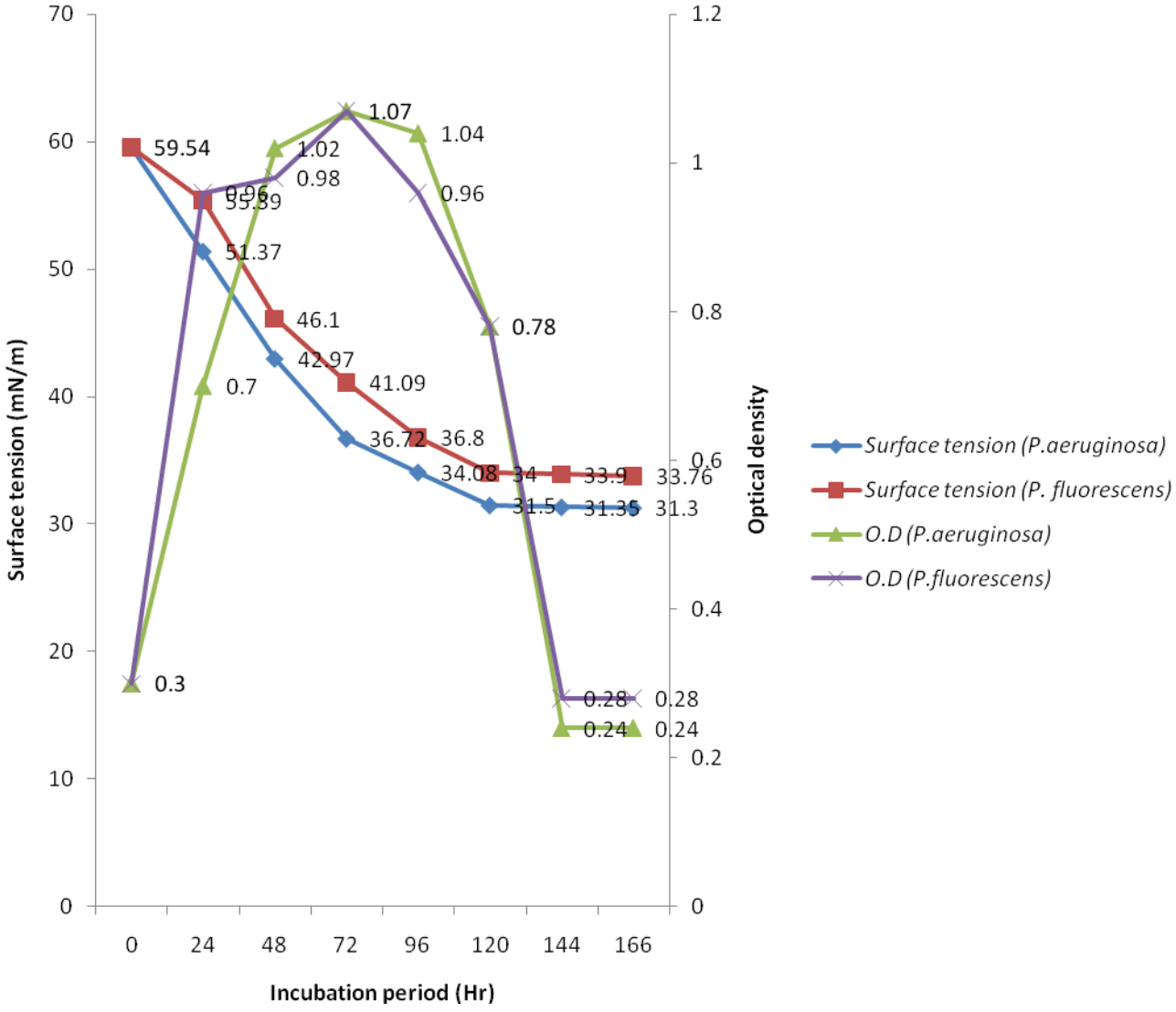
4.5.2. Extraction, purification and quantification of biosurfactant.

Using the chloroform:methanol extraction in ratio 2:1, the organic phase was collected and dried in a fume board and a resultant fine brown colored oil layer was left in the petri-dish representing the surface active compound (biosurfactant). Dried extract from *Pseudomonas aeruginosa* induced fermentation weighed 0.18mg per 50ml or 3.6g/l while dried extract from *Pseudomonas fluorescens also* weighed 2.8mg/l of organic extract or 0.14mg/l of 50ml of extract.

4.5.3. Characterization of biosurfactant

Plate I and II shows the fourier transformation infra-red spectrophotometer (FT-IR) spectrum profile of the biosurfactant present in the fermentation product of the effluent for both isolates (*Pseudomonas aeruginosa* and *Pseudomonas fluorescens).*

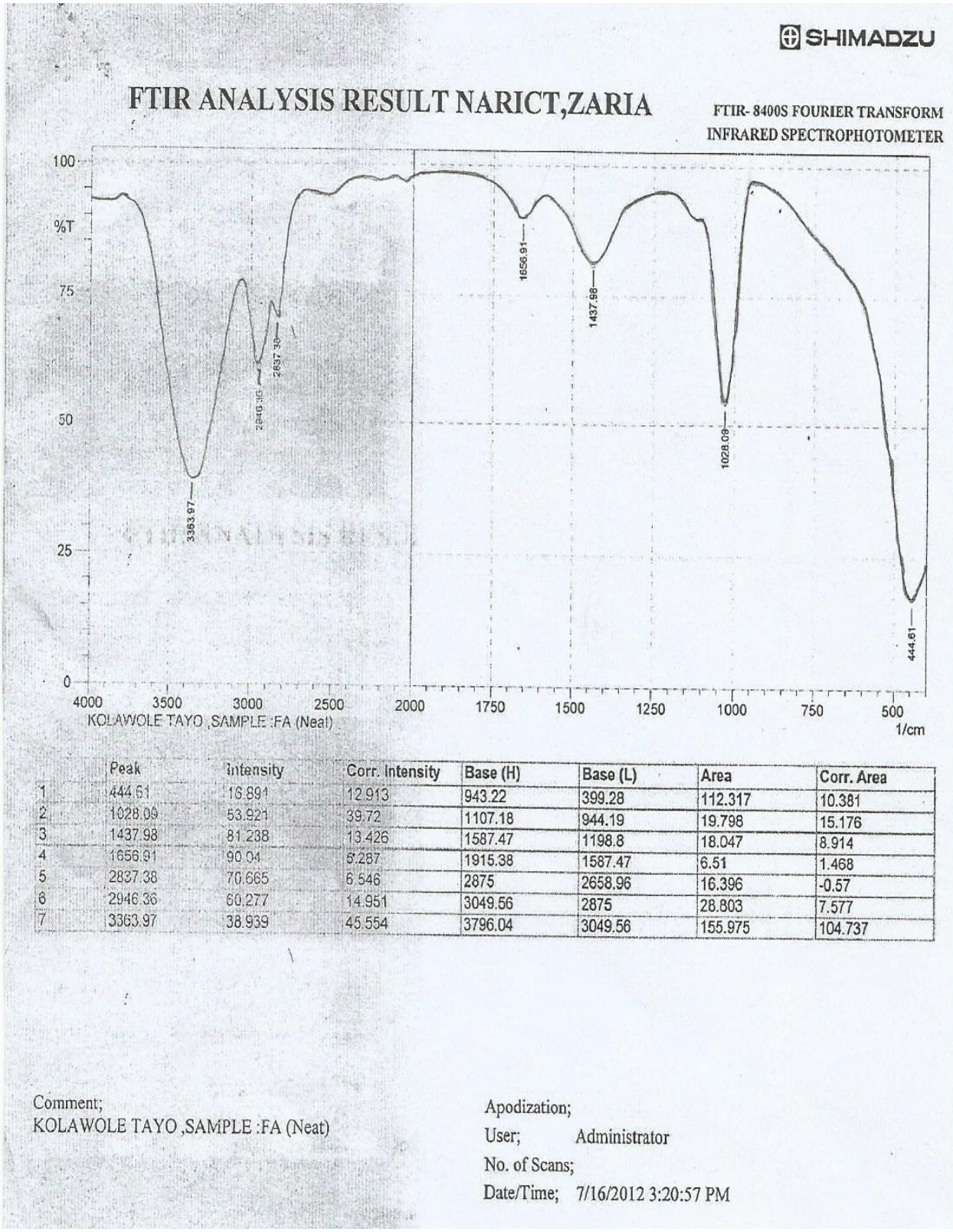
The FT-IR spectra of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* disclosed a spectrum stretched peak of 3363cm^{-1} and 3349cm^{-1} respectively which is characteristic of an overlapping hydroxyl group and CH_2-CH_2 and CH_3 spectrum of an aromatic compound. The absorption spectrum around 2946cm^{-1} (*P. aeruginosa*) and 2944cm^{-1} (*P. fluorescens*) is a characteristic symmetric stretch of an alphatic chain –nCH of CH_2 and CH_3 in both isolates. The corresponding symmetric stretch seen at 2837cm^{-1} *Pseudomonas aeruginosa*) or 2835cm^{-1} (*P.fluorescens*) also indicates the presence of hydroxyl group in the extract.



X^2=56,000 P=0.229[a]

Figure 4.1. Surface tension and Optical density after 168hrs for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induce fermentation with mineral salt added and no pH alteration

a, the level of statistical significant between both isolate in relation to the surface tension values



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	444.61	16.891	12.913	943.22	399.28	112.317	10.381
2	1028.09	53.921	39.72	1107.18	944.19	19.798	15.176
3	1437.98	81.238	13.426	1587.47	1198.8	18.047	8.914
4	1656.91	90.04	5.287	1915.38	1587.47	6.51	1.468
5	2837.38	70.665	6.546	2875	2658.96	16.396	-0.57
6	2946.36	60.277	14.951	3049.56	2875	28.803	7.577
7	3363.97	38.939	45.554	3796.04	3049.56	155.975	104.737

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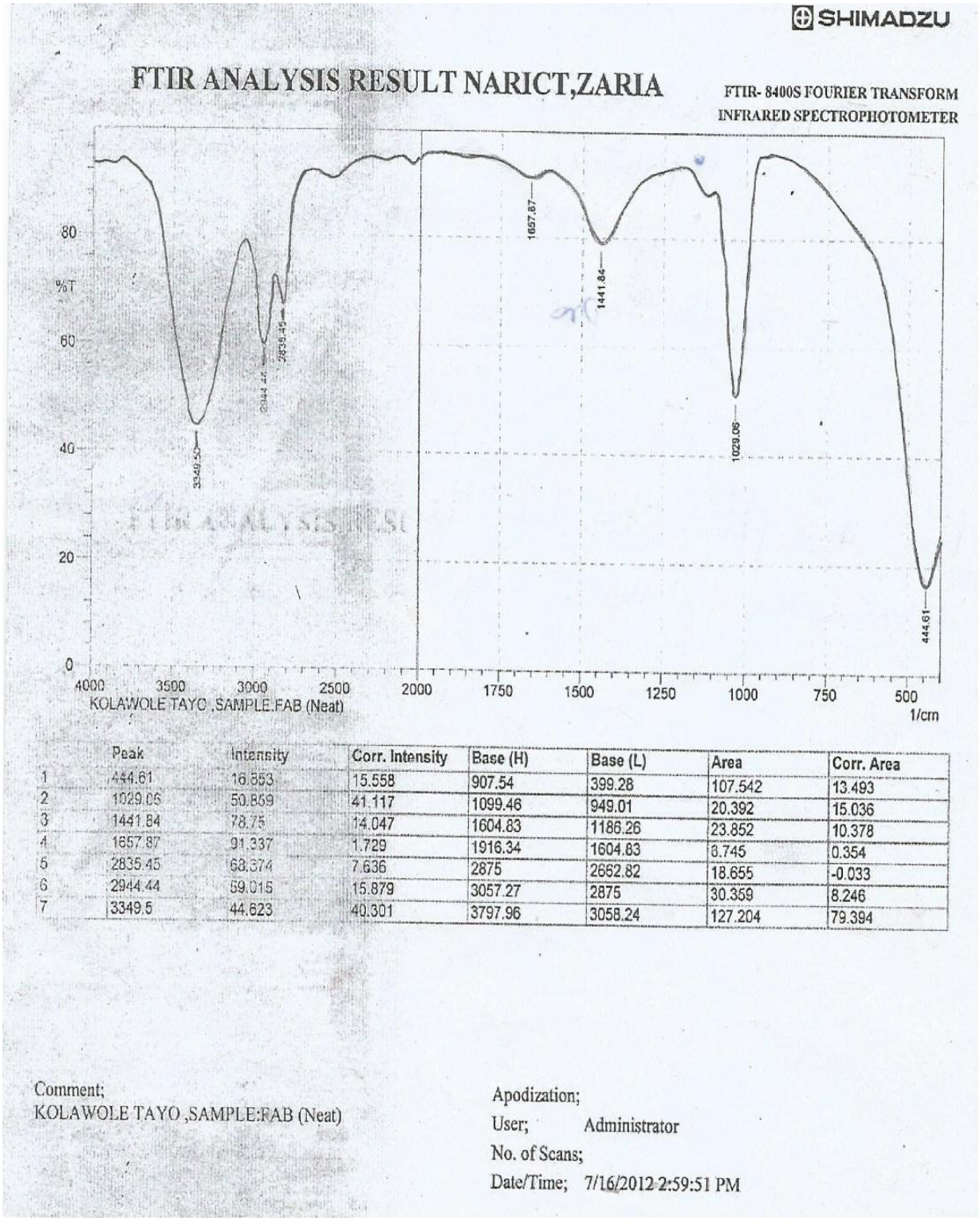
Plate I: The FT-IR spectroscopic analysis of biosurfactant produced by *Pseudomonas aeruginosa*



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	444.61	16.853	15.558	907.54	399.28	107.542	13.493
2	1029.06	50.859	41.117	1099.46	949.01	20.392	15.036
3	1441.84	78.75	14.047	1604.83	1186.26	23.852	10.378
4	1657.87	91.337	1.729	1916.34	1604.83	8.745	0.354
5	2835.45	68.374	7.636	2875	2662.82	18.655	-0.033
6	2944.44	59.015	15.879	3057.27	2875	30.359	8.246
7	3349.5	44.623	40.301	3797.96	3058.24	127.204	79.394

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Plate II: The FTIR spectroscopic analysis of biosurfactant produced by *Pseudomonas fluorescens*

4.6 Effect of Environmental Factors

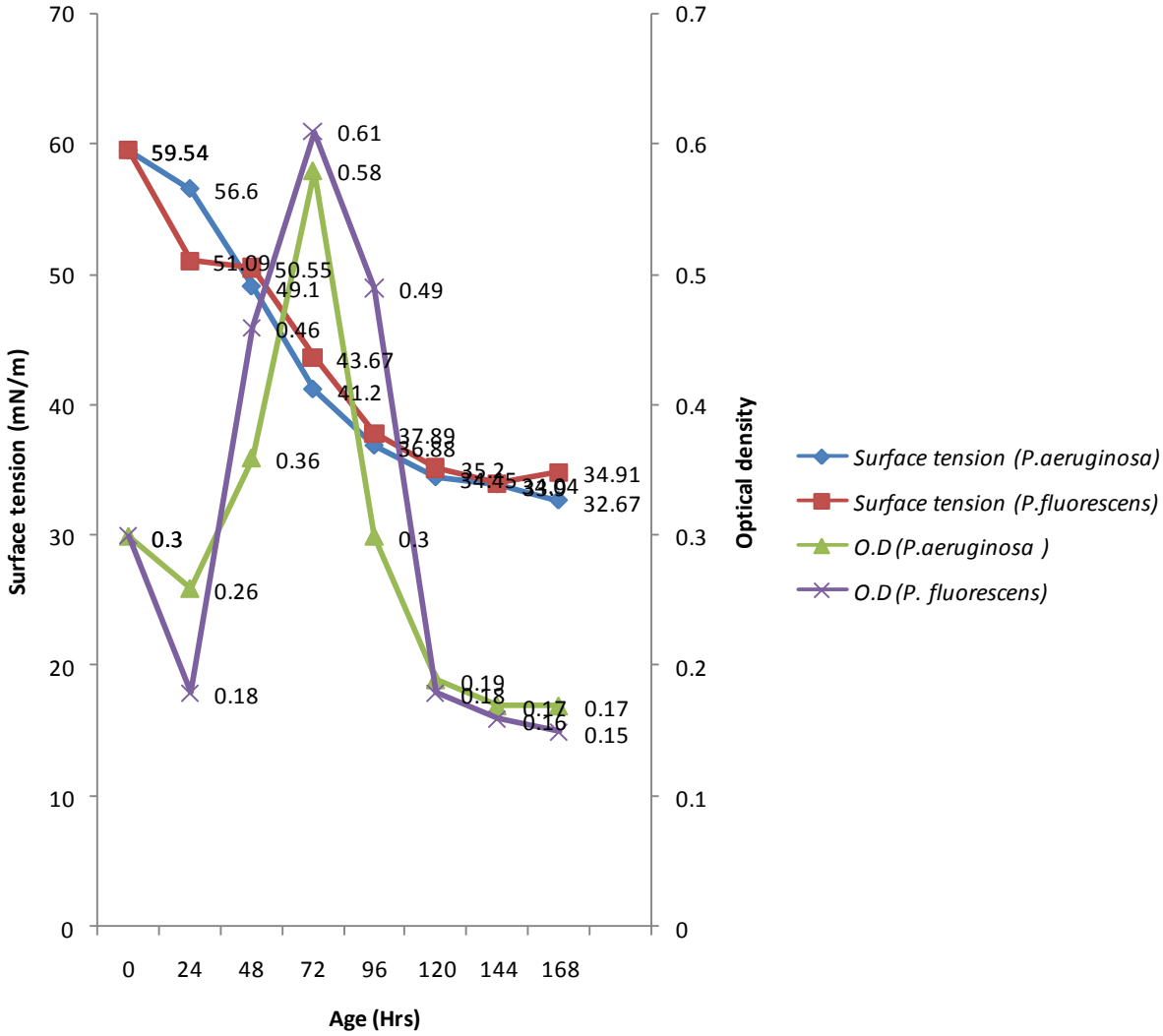
4.6.1 Effect of pH on surface activity and optical density during fermentation.

Figure 4.2 shows the reading of both cell biomass growth (optical density) and surface tension for fermentation at pH4 for 168hr. The optical density peaked at 72hr and a decline thereafter for both isolates. Peak absorbance (optical density) reading of 0.58 was observed for fermentation with *Pseudomonas aeruginosa* and 0.61 was observed for fermentation with *Pseudomonas fluorescens.*

Figure 4.3 shows that surface tension reading of both isolate at pH5. *Pseudomonas aeruginosa* induced fermentation at pH5 reduced from 59.54mN/m to 32.00mN/m at an optical density of 0.32. Peak absorbance of 1.01 was recorded while *Pseudomonas fluorescens* induced fermentation reduced surface tension of the effluent to 34.34mN/m with peak optical density at 0.99 at 600nm.

Figure 4.4 represents the reading at pH6 of surface tension and optical density of the sterilized effluent with mineral salt. *Pseudomonas aeruginosa* induced fermentation reduced the surface tension of the growth medium to 31.90mN/m after 168hrs and peak optical density of 1.02 was reached after 96hrs of fermentation. The reduction in surface tension by *Pseudomonas fluorescens* induced fermentation was 34.19mN/m from 59.54mN/m with peak optical density at 0.99 at 72hrs.

Figure 4.5 shows the data gathered from fermentation of effluent at pH7. Surface active compound produced during the fermentation by *Pseudomonas aeruginosa* reduced the surface tension of the effluent to 31.87mN/m after 168hrs with cell biomass growth peaking at 1.03 on the 96hrs while that *Pseudomonas fluorescens* had surface tension reduced to 33.04mN/m with peak optical density at 0.99 at 72hrs.



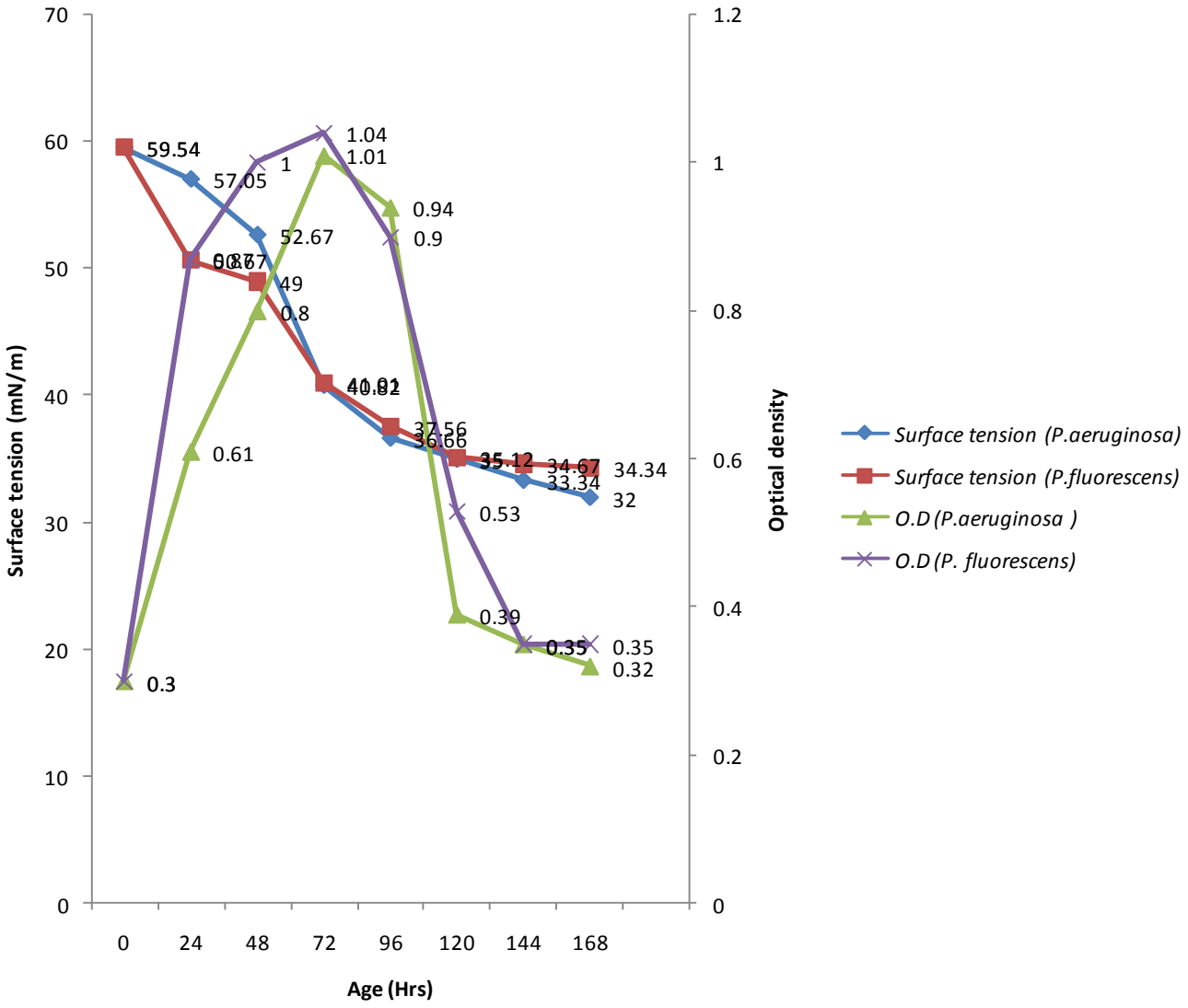
X^2= 56,000 P=0.229[a]

X^2=32,000 P=0.368[b]

Figure 4.2: Surface tension and Optical density at pH4 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induce fermentation

a, the level of statistical significant between both isolate in relation to the surface tension values

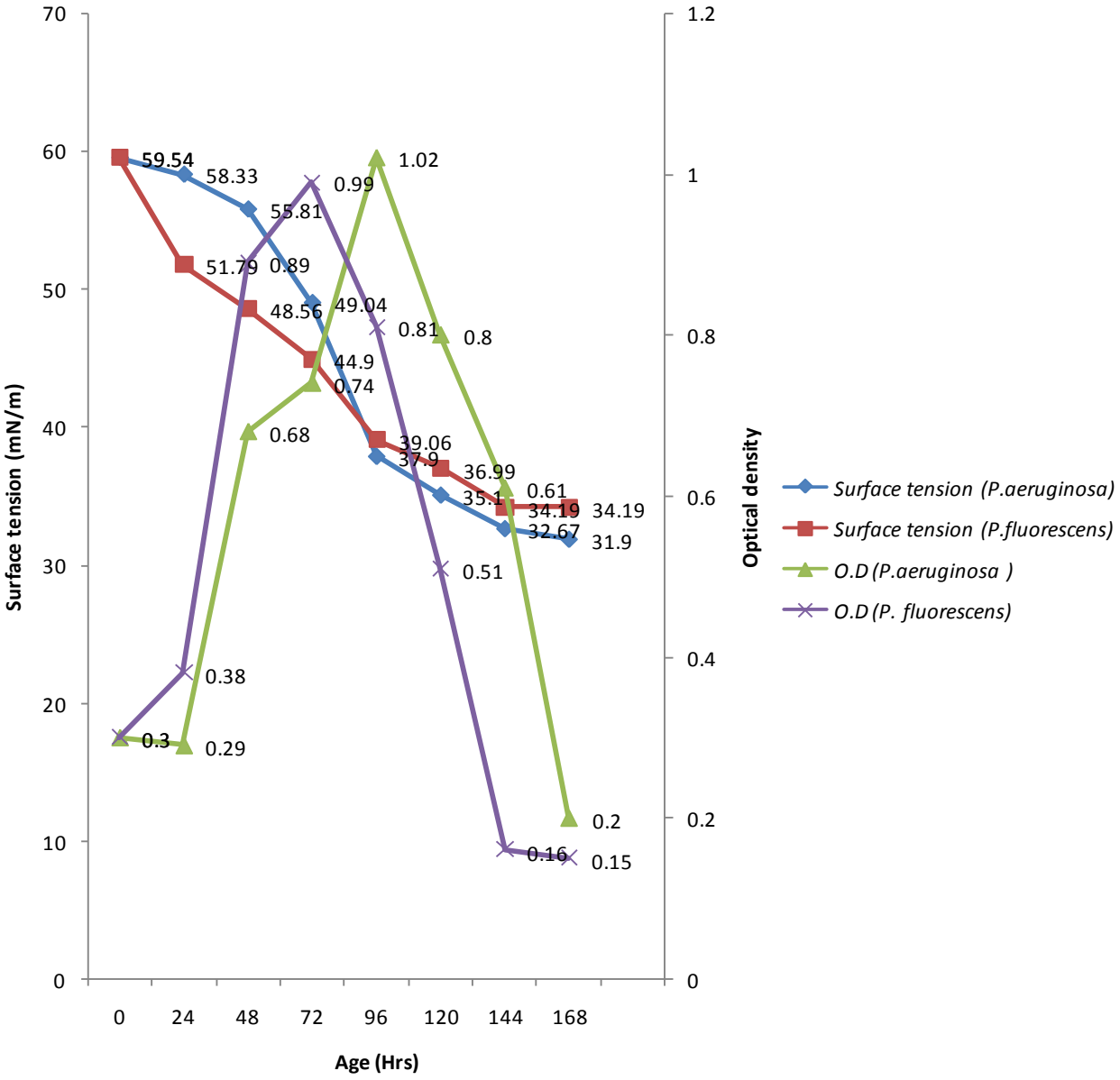
b, the level of statistical significant between both isolate in relation to the optical density values



X^2=56,000 P=0.229

X^2=48,000 P=0.243

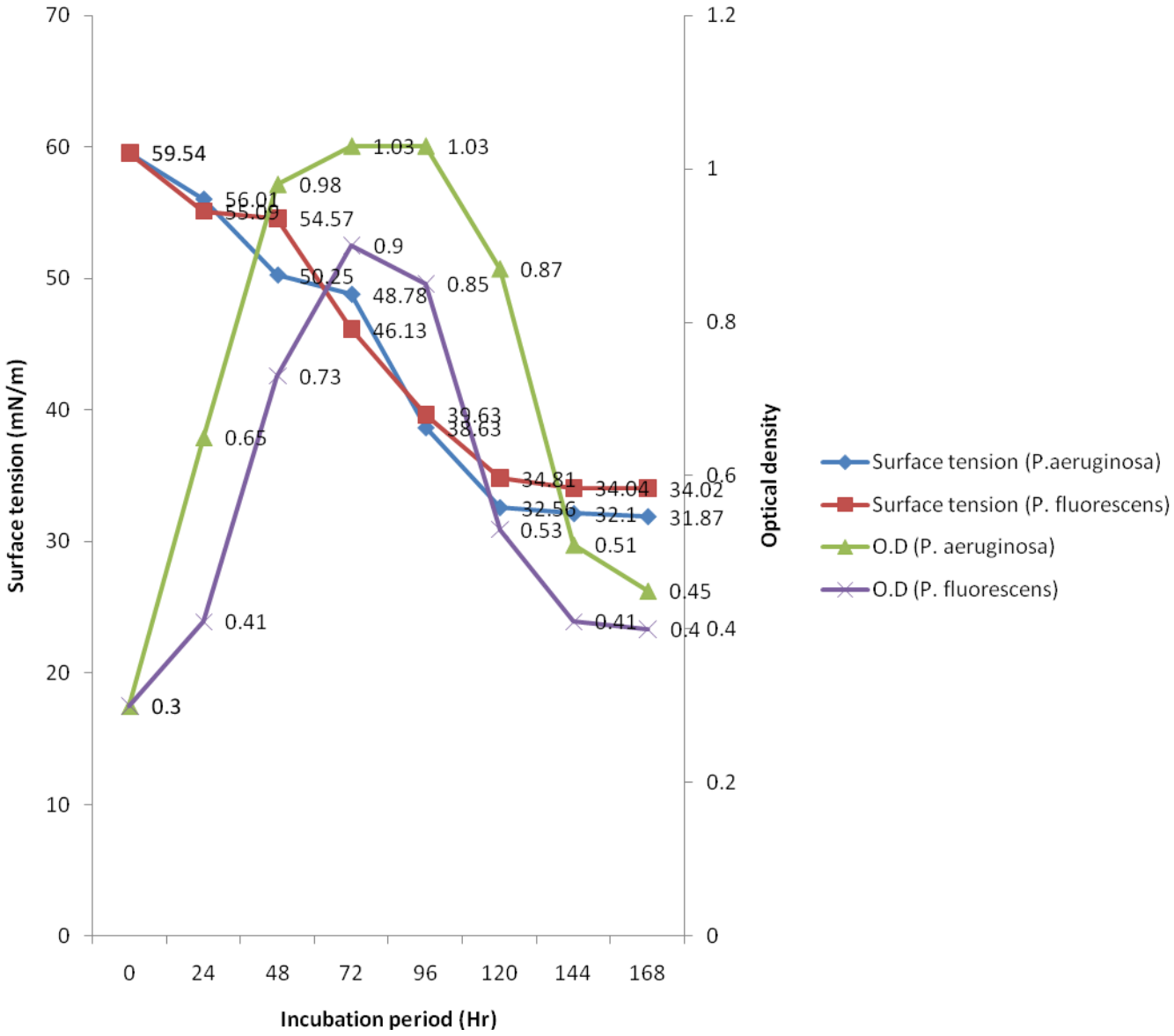
Figure 4.3: Surface tension and Optical density at pH5 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induce fermentation



X^2=48,000 P=0.243

X^2=56,000 P=0.229

Figure 4.4: Surface tension reading and optical density at pH6 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induced fermentation.



X^2=56,000 P=0.229

X^2=40,000 P=0.297

Figure 4.5: Surface tension reading and optical density at pH7 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induced fermentation

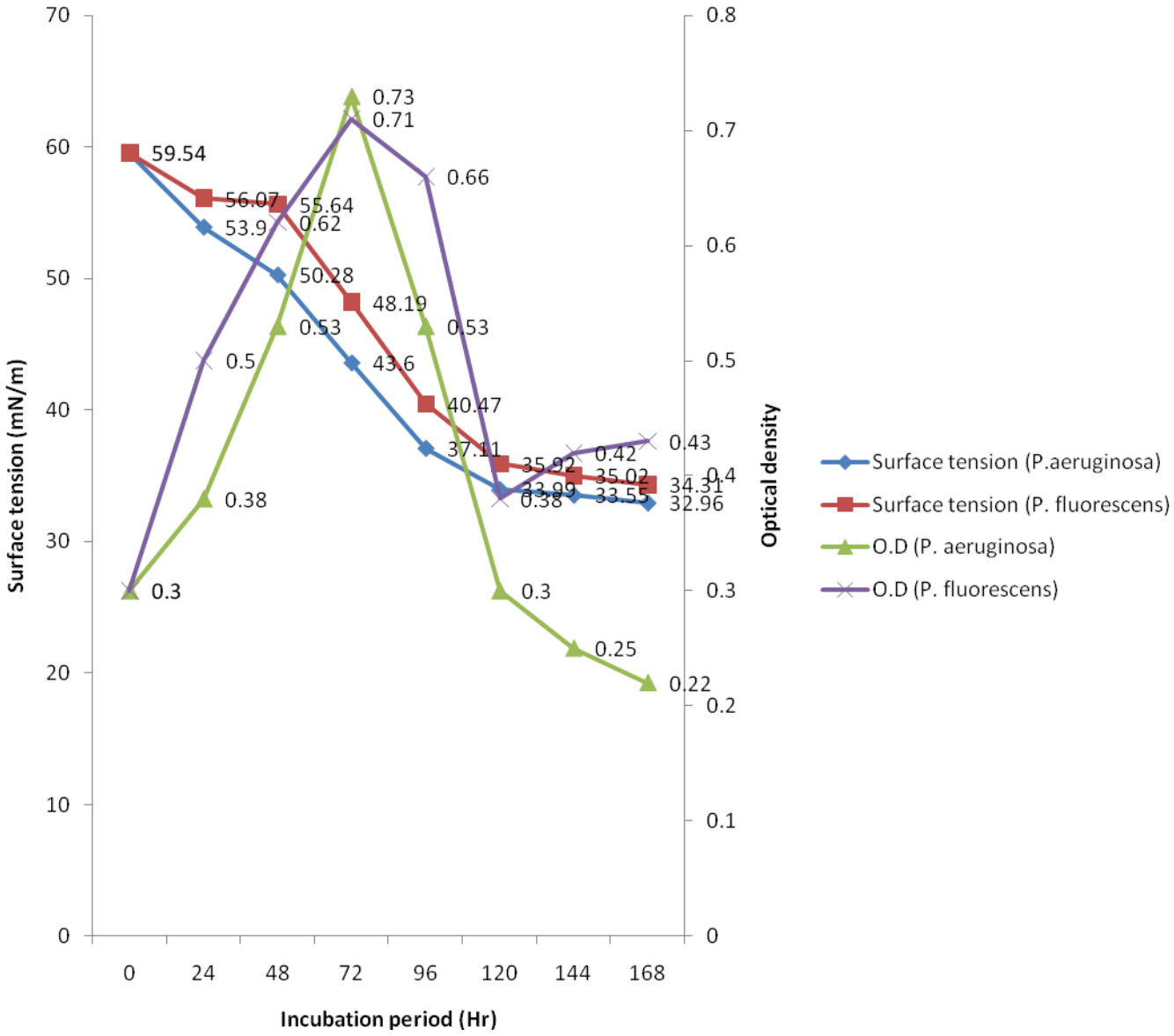
Figure 4.6 shows the reading of both isolate at pH8. *Pseudomonas* aeruginosa induced fermentation showed a reduction in surface tension of effluent to 32.06mN/m and peak optical density of 0.73 after 72hrs of growth while that of *Pseudomonas fluorescens* which had surface activity of 33.31mN/m and peak optical density reading of 0.71.

Figure 4.7 represents data collected during fermentation of effluent at pH9 which shows *Pseudomonas aeruginosa* induced fermentation had surface tension reduced to 31.91mN/m with peak absorbance at 0.70 while *Pseudomonas fluorescens* had peak absorbance of 0.79 at 72hrs extending till the 96hrs with surface tension reduced to 34.44mN/m.

Figure 4.8 illustrates the data obtained during fermentation for 168hrs at pH10 for both isolates. *Pseudomonas aeruginosa* induced fermentation had a peak absorbance at 0.75 and surface tension reduced to 31.98mN/m while *Pseudomonas fluorescens* had a peak absorbance of 0.79 at 72hrs extending till 96hrs.

4.6.2 Effect of fermentation without mineral salt or pH adjustment on surface activity and cell growth (control)

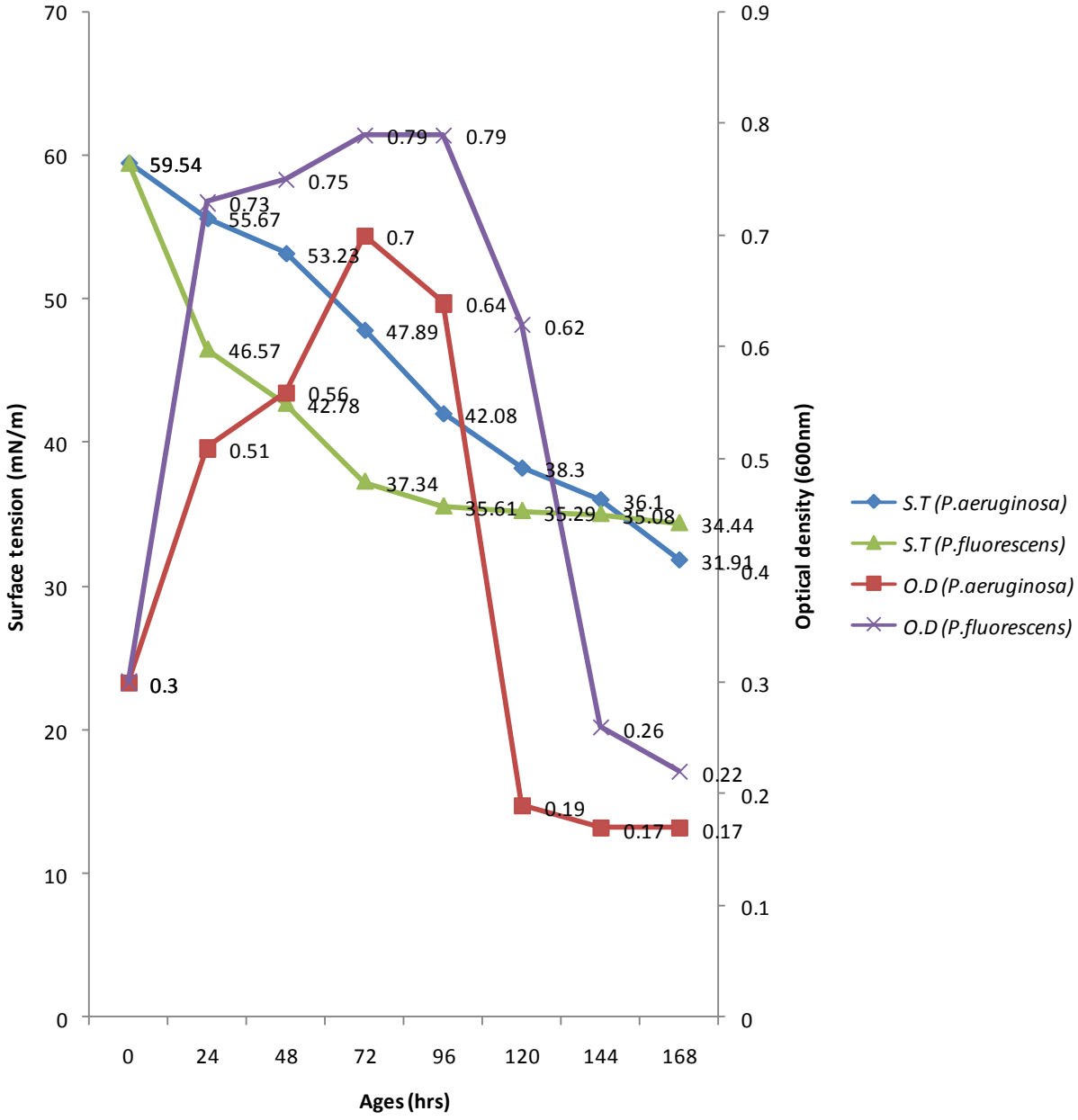
Figure 4.9 shows the relationship between surface tension which reduced from 59.54mN/m to 30.67mN/m at 168hrs of fermentation and a peak absorbance at 0.95 after 72hrs while optical density dropped to 0.17 at 168hrs for *Pseudomonas aeruginosa* Surface tension dropped to 32.78mN/m with *Pseudomonas fluorescens* as the fermenter with peak absorbance of 0.76 at 72hrs. In this process neither the pH was adjusted nor mineral salt added. The pH of the medium was 7.8 at start of fermentation.



X^2=56,000 P=0.229

X^2= 49,000 P=0.258

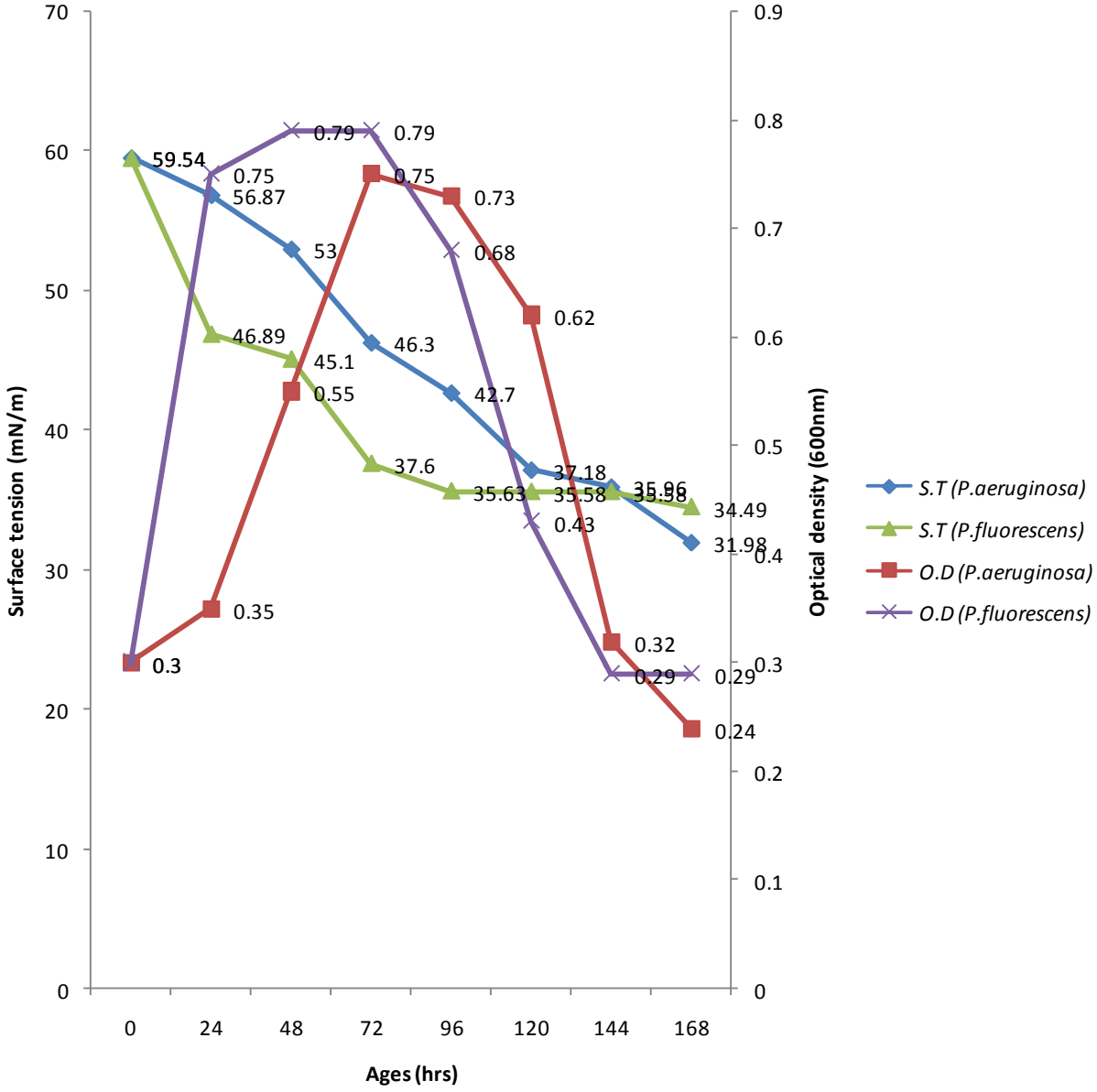
Figure 4.6: Surface tension reading and optical density at pH8 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induced fermentation



X^2=56,000 P=0.229

X^2= 40,000 P=0.243

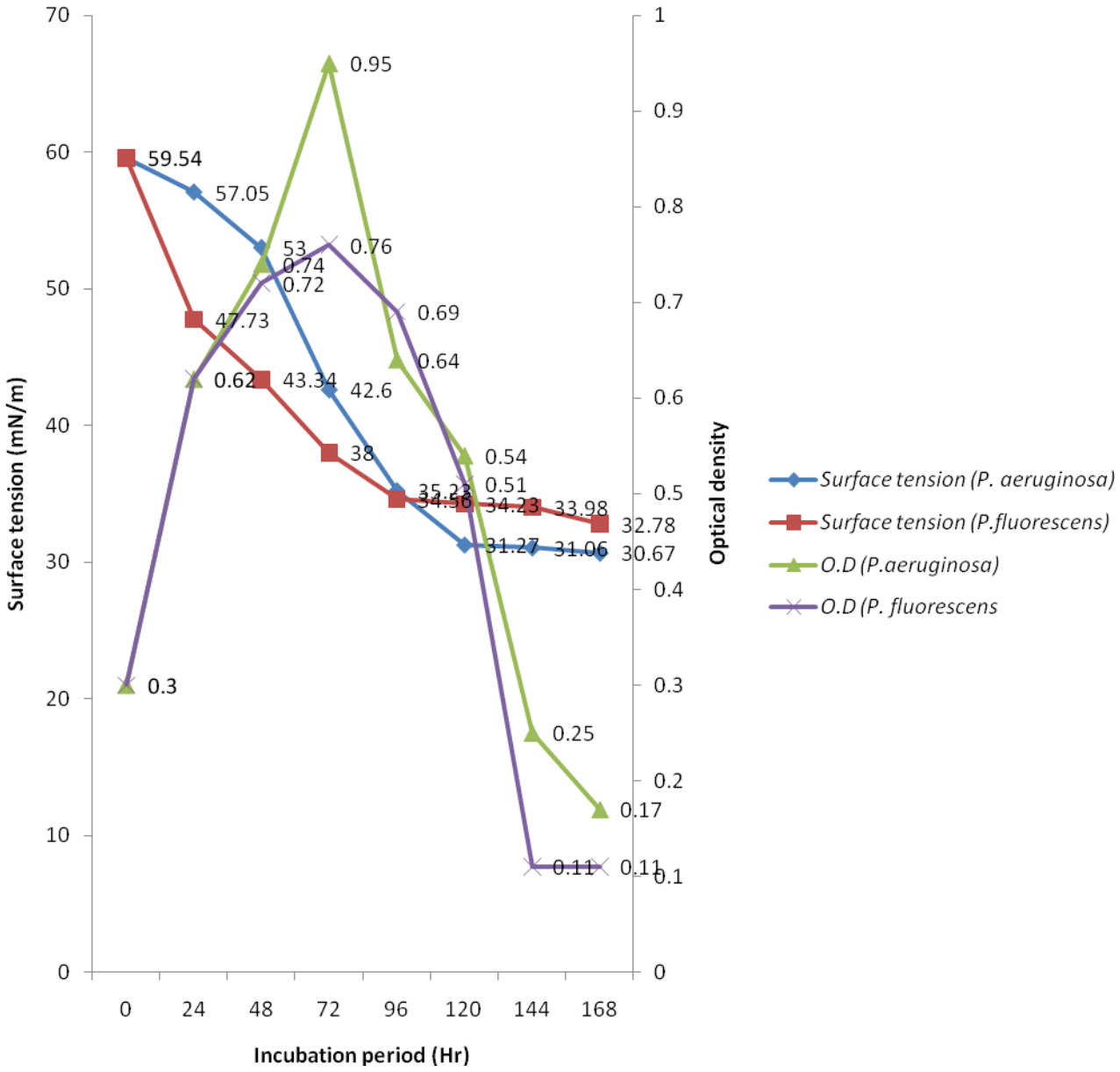
Figure 4.7: Surface tension reading and optical density at pH9 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* induced fermentation.



X^2=56,000 P=0.229

X^2= 40,000 P=0.258

Figure 4.8: Surface tension reading and optical density at pH10 for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens.*



X^2=56,000 P=0.229

Figure 4.9: Surface tension reading and optical density of control for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*

4.6.3 Effect of temperature on biosurfactant activity.

Figure 4.10 shows the effect of temperature on the activity (surface tension) of the crude culture supernatant. For *Pseudomosa aeruginosa*, the change in surface tension upon heating at 30^0C was least at 32.10mN/m with the highest recorded at 60^0C as 32.99mN/m. Also the table shows the effect of temperature on surface tension of the crude culture supernatant fermented with *P. fluorescens* with change in surface tension upon heating at 30^0C showing the lowest deviation recorded as 33.19mN/m and the highest deviation at 70^0C with 33.71mN/m. The initial surface tension of the medium before heating was 32.00mN/m and 33.25mN/m for *Pseudomonas aeruginosa* and *Pseudomonas fluourescens* repectively

4.7. Emulsification Activity.

4.7.1 Emulsification of diesel

Figure 4.11 illustrates the emulsification activity of the supernatant containing biosurfactant on diesel. The emulsion forming ability of the supernatant from fermentation without variation in pH and the addition of mineral salt on diesel showed that the supernatant from *P. aeruginosa* induced fermentation emulsified 68% of the diesel added to it while supernatant from *P. fluorescens* emulsified 58% of the diesel.

The effect of pH on the emulsifying ability of the supernatant on diesel varied among the various pH (4, 5, 6, 7, 8, 9, 10). pH10 had the highest emulsion formation with 83% diesel emulsified with supernatant collected from *P.aeruginosa* and87% of diesel was emulsified by the supernatant collected from *P.fluorecens.* pH4 in both isolates had the emulsion formation with 7.3% for *P.aeruginosa* and 0% for *P.fluorescens.*

The supernatant collected from fermentation process with mineral salt (control) after 168hrs of fermentation showed that *P.aeruginosa* and *P. fluorescens* had 60% and 56% respectively

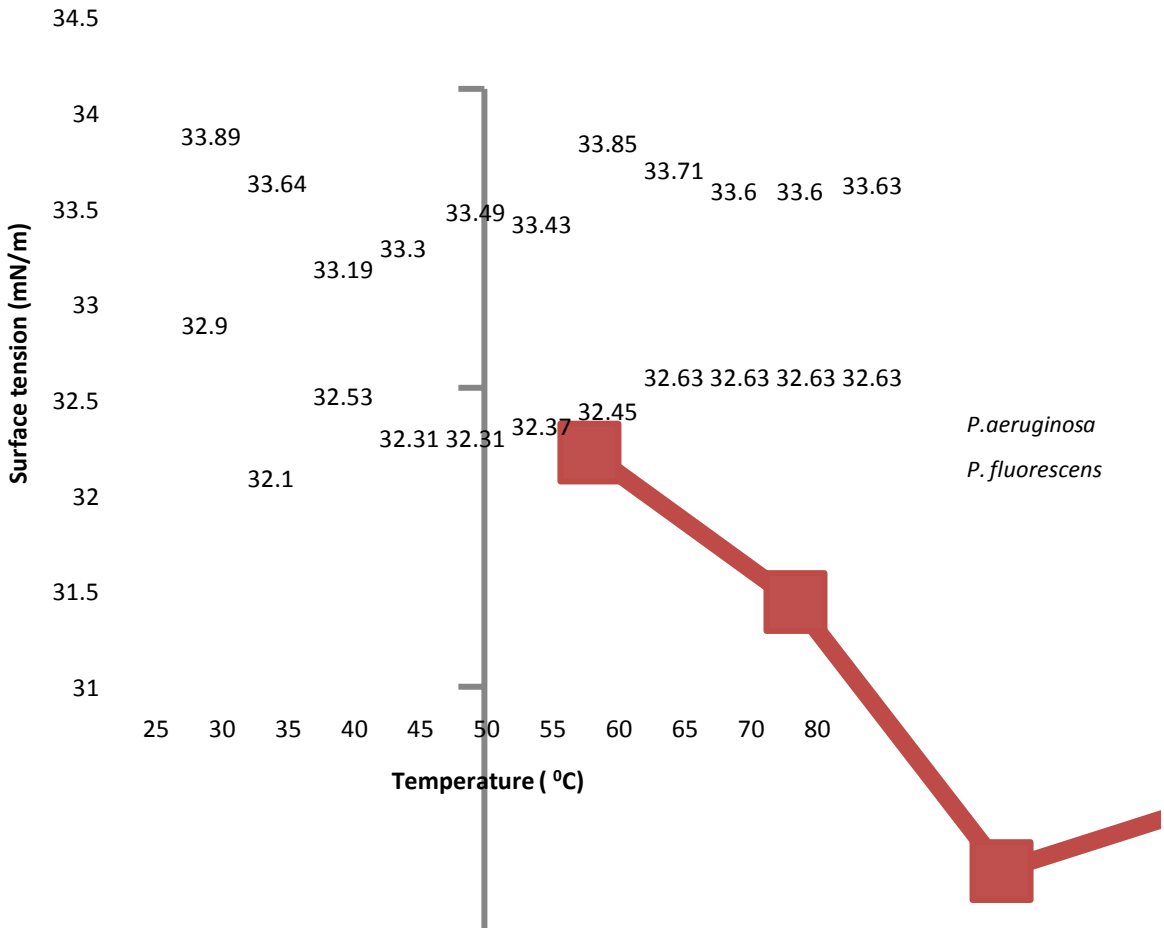


Figure 4.10: stability test on supernatant collected after 168hrs from *P. aeruginosa* and *P. fluorescens*

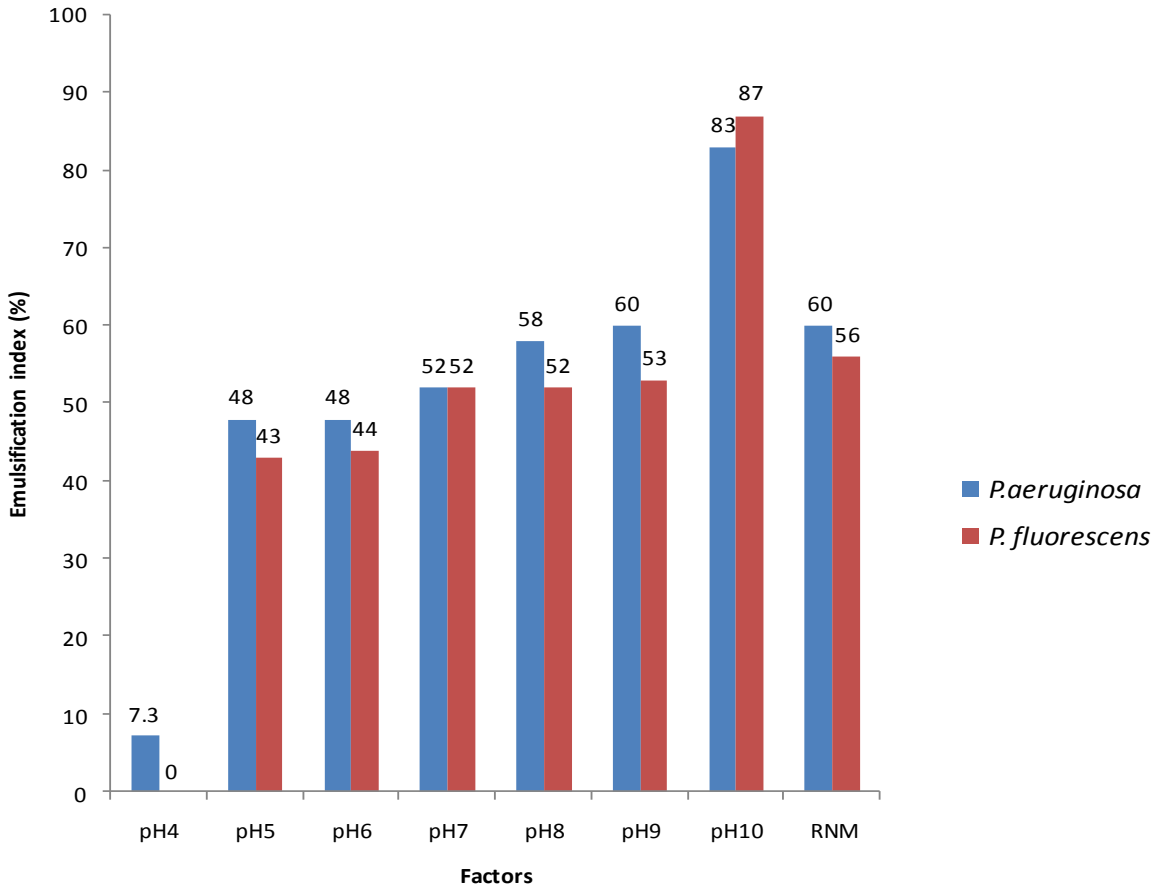


Figure 4.11: Emulsification index of supernatant from *P. aeruginosa* and *P. fluorescens* with diesel in 24hrs.

Key

RNM = Fermentation of brewery effluent without mineral salt and no pH adjustment

4.7.2 Emulsification of premium motor spirit

Figure 4.12 shows the emulsification activity of the supernatant containing biosurfactant on premium motor spirit. The emulsifying ability of crude supernatant collected after 168hrs of fermentation showed that *P. aeruginosa* induced fermentation emulsified 68% of premium motor spirit (petrol) while *P. fluorescens* emulsified 60% of petrol.

For the pH effect on emulsification activity of the supernatant, supernatant from pH10 had the highest emulsion formation stability with 80% and 65% for *P. aeruginosa* and *P. fluorescens* respectively. pH4 had 0% emulsification in both isolates' supernatant.

For the control (that is raw effluent without pH adjustment or mineral salt addition) showed that 67% and 59% of petrol was emulsified by supernatants from *P. aeruginosa* and *P. fluorescens* induced fermentation respectively.

4.7.3 Emulsification of kerosene.

Figure 4.13 illustrates the ability of the supernatant containing biosurfactant to emulsify kerosene. The emulsification test of supernatant collected after 168hrs of fermentation of effluent without variation in pH but with the addition of mineral salt showed that 55% of kerosene was emulsified by *P. aeruginosa* induced fermentation while 51% of kerosene was also emulsified by *P. fluorescens* induced fermentation.

The effect of pH on the emulsifying ability of supernatant collected from fermentation process with varied pH showed supernatant from both isolates induced fermentation emulsified 60% and 63% of the kerosene added by *P. aeruginosa* and *P. fluorescens* respectively. The least emulsification ability was observed at pH4 with 5.4% and 0% in *P .aeruginosa* and *P. fluorescens* induced fermentations respectively.

The supernatant collected from fermentation without mineral salt (control) after 168hrs of fermentation showed that *P.aeruginosa* and *P. fluorescens* had 58% emulsification of the kerosene.

4.7.4 Emulsification of olive oil.

Figure 4.14 indicates the emulsion forming ability of supernatant collected from the fermentation processes under varying condition on olive oil. The emulsification with olive oil was generally low after 24hrs. The emulsification property of the crude biosurfactant after 168hrs of fermentation on the substrate (olive oil) showed that 54% and 47% of the substrate was emulsified by supernatant from *P. aeruginosa* and *P. fluorescens* induced fermentation respectively.

For the effect pH variation on supernatant activity, pH10 had the best emulsion formation for *P. fluorescens* with 43% of the olive oil emulsified while pH10 had the best for *P. aeruginosa* with 48% emulsion formation with substrate.

The supernatant collected from fermentation process with mineral salt (control) after 168hrs of fermentation showed that *P.aeruginosa* and *P. fluorescens* had 43% and 49% emulsification of the kerosene respectively.

4.7.5 Emulsification of diesel, petrol, kerosene and olive oil at varying temperatures.

Figure 4.15 shows the effect of temperature ranging between 25^{o}C and 80^{o}C on various hydrocarbons and oil used. Exposing the supernatant collected after 168hrs of fermentation to heat variation, 30^{0}C and 35^{0}C had the best emulsion formation with 52% of diesel for *P. aeruginosa*, while 35^{0}C for *P. fluorescens* 46% of diesel emulsified.

The effect of heat on emulsification ability of crude supernatant on premium motor spirit showed that the activity was best at 35^{0}C with 51% for *P. aeruginosa* and 35^{0}C with 46% for *P. fluorescens.*

Exposing the supernatant collected after 168hrs of fermentation to heat variation, 30^{0}C and 35^{0}C had the best emulsion formation with 47% of kerosene for *P. aeruginosa*, while 35^{0}C for *P. fluorescens* 45% of kerosene emulsified.

The heat effect on emulsifying property was highest 30^{o}C and 35^{o}C for both *P. aeruginosa* and *P. fluorescens* induced fermentation with 42% and 40% respectively.

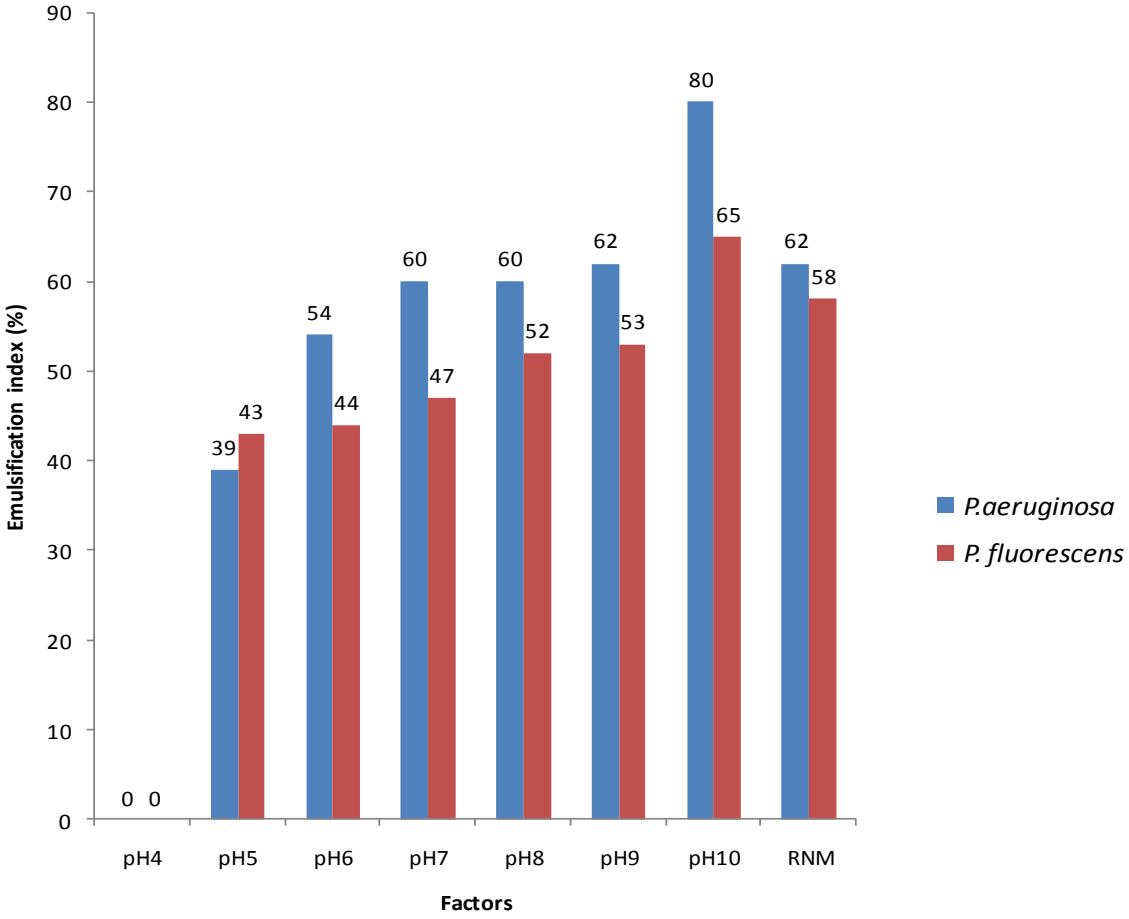


Figure 4.12: Emulsification index of supernatant from *P. aeruginosa* and *P. fluorescens* with premium motor spirit (Kerosene)l in 24hrs.

Key

RNM = Fermentation of brewery effluent without mineral salt and no pH adjustment

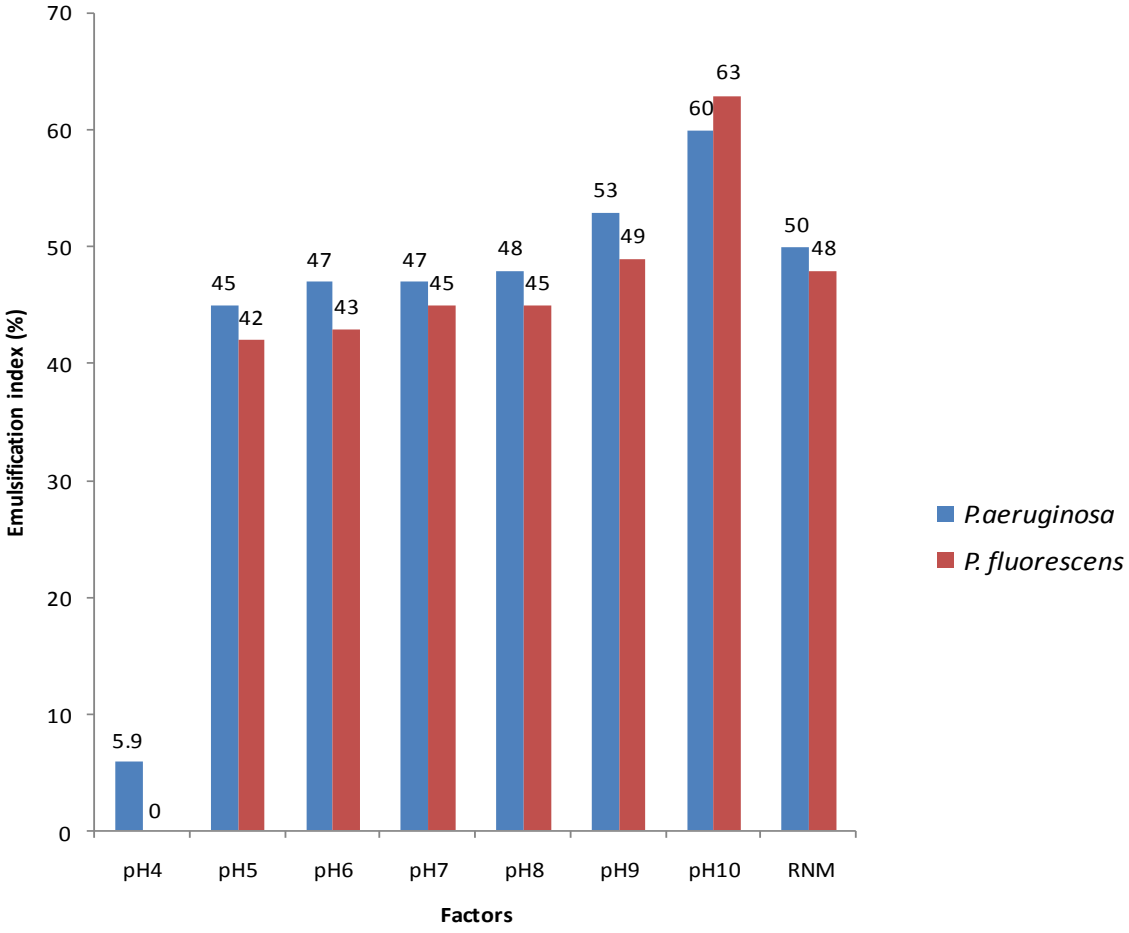


Figure 4.13: Emulsification index of supernatant from *P. aeruginosa* and *P. fluorescens* with Kerosene in 24hrs.

Key

RNM = Fermentation of brewery effluent without mineral salt and no pH adjustment

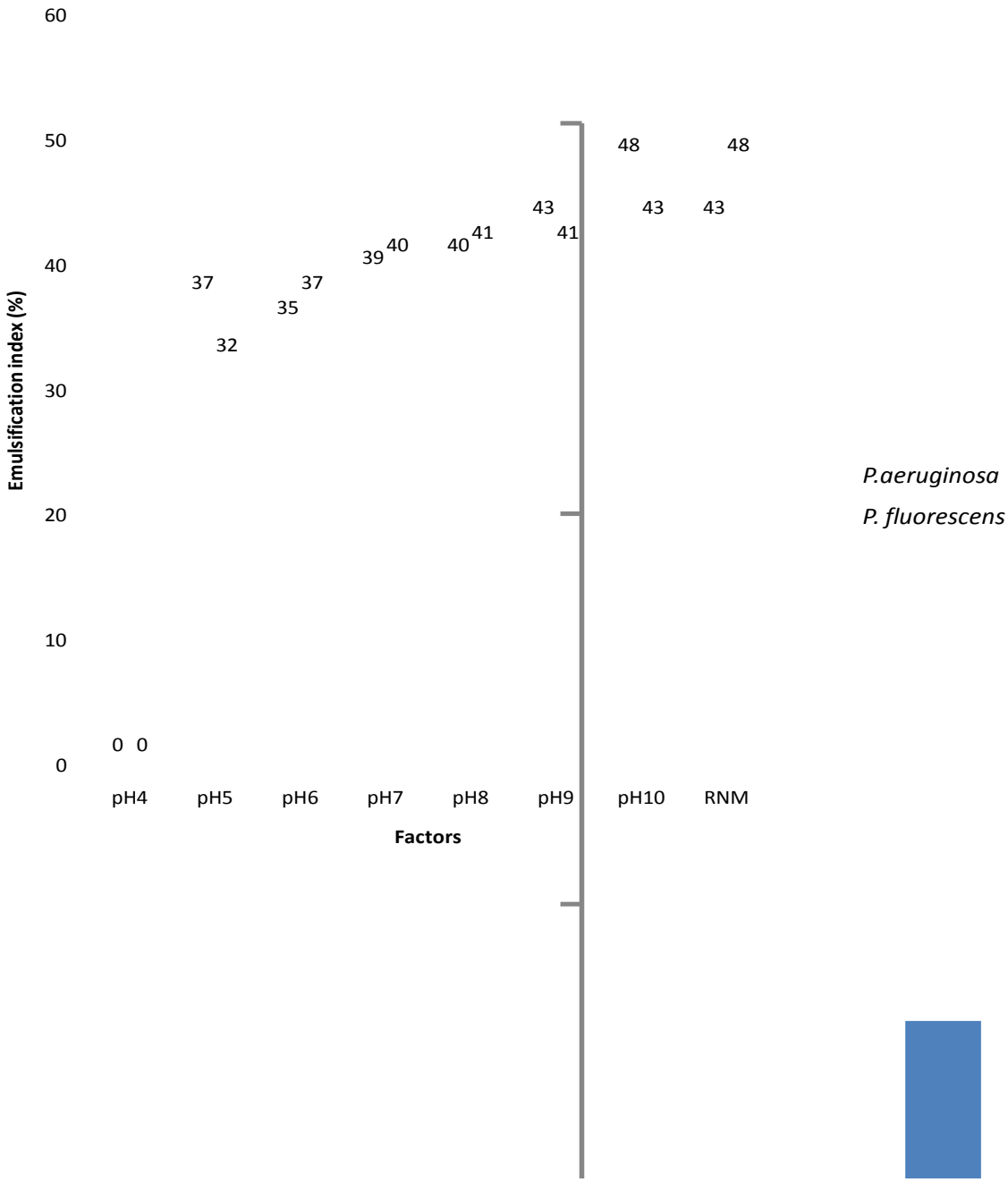


Figure 4.14: Emulsification index of supernatant from *P. aeruginosa* and *P. fluorescens* with olive oil in 24hrs.

Key

RNM = Fermentation of brewery effluent without mineral salt and no pH adjustment

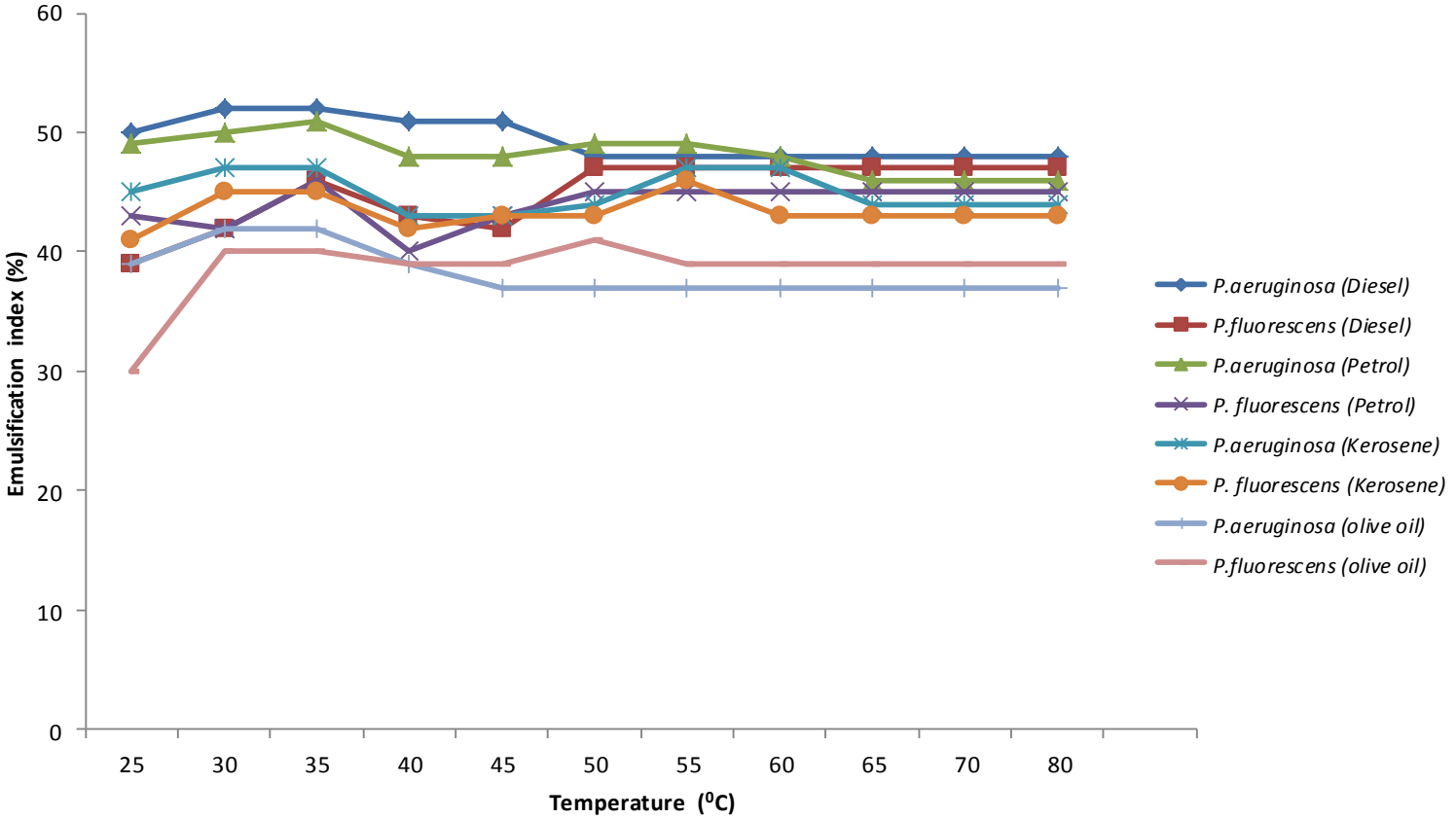


Figure 4.15: Emulsification index by *P. aeruginosa* and *P. fluorescens* at various temperatures

CHAPTER FIVE

5.0 DISCUSSION

Recently biosurfactants have gained numerous industrial and environmental applications which frequently involve exposure to extreme conditions such as temperature, pH, and agitation at high speed (Aparna *et al.,* 2011; Davishi *et al.,* 2011).

The range of substrate available for biosurfactant production could be challenging but substrate with high content of organic composition such as the brewery effluent with content such as sucrose, starchy waste of sorghum, ethanol, nitrogenous and phosphorus compound makes brewery effluent a good substrate for microbial growth and biosurfactant production as was also observed in the course of this work (Dubey and Juwarkar, 2001; Driessen and Veriejken, 2003; Saharan *et al.,* 2014)

The physico-chemical properties of the effluent which serves as the substrate for microbial fermentation, indicates the inorganic and organic composition of the effluent which includes the oxygen requirements for the oxidation of the organic content of the medium by microorganisms. The dissolved oxygen (measured as 2.9mg/l) and the biochemical oxygen demand (1.8mg/l), chemical oxygen demand of 15,000mg/l and total solids of 3,500mg/l was evident during the initial microbial growth multiplication as observed in the optical density readings at 600nm during fermentation. The decline in cell growth and cell death which was observed as drop in optical density after 72hrs may be due to the limits imposed by insufficient oxygen and other nutrients for the growth of the microbial population (Dubey and Juwarkar, 2001).

The assays used in the screening of the isolates showed positivity to the ability of the isolates to produce surface active compound initially determined by drop collapse test which confirms that the biosurfactant is extracellular and not cell-bound since the drop collapsed, in contrast,

the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that caused aggregation of droplets (Mbawala and Mouafo, 2012).

The oil displacement and hemolytic assays were only to further confirm the presence of surface active compound in the supernatant as initially observed with drop collapse assay. Carrillo *et al.,* 1996 reported an association between haemolytic activity and surfactant production. They recommended the use of blood agar lysis as a primary method to screen biosurfactant producing organism. There is a direct relationship between the diameter of the zone of clearance by haemolysis and concentration of the biosurfactant.

The positivity of the haemolysis is not specific and can not be solely used for screening for the presence of surface active compound because other lytic enzymes could lead to zone clearance. Also the diffusion restriction in surfactant can inhibit the formation of a zone of clearance as reported by Mulligan *et al.,* (1984) and Youssef *et al.,* (2005). All assays used to verify the presence of surface active compound have several advantages such as small amount of sample is required, it's rapid and easy to carry out (Carrillo *et al.,* 1996)

Biosurfactant concentration and activity was estimated by indirectly measuring the surface tension of the fermentation medium base on the previous findings that surface activity of any surface active compound is dependent on the presence and concentration of the active compound (Guerra-Santo *et al.,* 1984). Biosurfactant production and surface tension also depends on the ability of the microorganism to grow in and utilize the medium or substrate. This approach was employed to check the growth of the organism in the medium vis-à-vis the reduction in surface tension before changing other parameters such as pH variation and temperature stability check. Tuelva *et al.,* 2002 observed that enough biosurfactant (rhamnolipid) was produced within the initial 24hrs to cause a drop in surface tension from 71to 37mN/m which reached a minimum of 29mN/m after 120hrs of incubation but findings

form this research showed the initial drop in surface tension at 72hrs was from 59.54mN/m to 36.80mN/m which reached the minimum of 31.30mN/m after 168hrs.

When neither pH nor temperature was varied but with addition of mineral salt, it was observed that the rate of reduction in surface tension was accompanied by increase in cell biomass as indicated by the optical density measurement at 600nm also indicating a growth associated kinetic in the fermentation process since rate of surface tension reduction was more at the exponential phase than at the other phases of fermentation process. Reduction in surface tension indicates the production of surface active compound (biosurfactant) by the microorganism which also aids in the metabolism of the substrate and stimulates microbial growth by increasing the surface area leading to bioavailability of the substrate. Faster reduction in surface tension at the exponential phase of growth suggests that the surface active compound (biosurfactant) is a primary metabolite and therefore has aided the availability of the substrate (Khopade *et al*., 2012). This was also observed by Ilori *et al*., (2005) and Adebusoye *et al.,* (2008) but differs from the finds of Tuelva *et al., (*2002) in which biosurfactant activity was best noticed at stationary phase.

Extraction of biosurfactant from crude supernatant revealed that 3.2g/l and 2.4g/l of biosurfactant were produced by *P. aeruginosa* and *P. fluorescens* relatively higher than finding in Mulligan and Gibbs, 2004 in which 0.92g/l of biosurfactant was collected from fermentation of distillery waste of 90,000mg/l of C.O.D. This finding also represents a lesser volume of biosurfactant compared to 5.9mg/l and 112.0g/l for *P.aeruginosa* as also reported by Mulligan and Gibbs 2004.

The effect of pH on biosurfactant activity for pH ranging from four to ten showed a reduction in surface tension also accompanied by cell growth. Both isolates used for the studies (*P. aeruginosa* and *P. fluorescens)* reduced the surface tension by more than 20mN/m at the end

of the fermentation process (168hrs). *P. aeruginosa* reduced the surface tension by more than 2.1mN/m compared to that of *P. fluorescens.* Increase in cell growth was higher at the initial 72hrs after which growth began to decline across the pH ranges used except for pH6 for *P. aeruginosa* and pH9 for *P. fluorescens* where growth was observed till 96hrs and a decline there after. Khopade *et al.,* (2012) observed that as pH increase from five to eight the negative charge of the polar head of the biosurfactant being produced increases, promoting an increase in the aqueous solubility of the substrate enhancing cell growth and reduction in surface tension. According to Kitamoto *et al.,* (2002), the superiority of hydrophilic substrate over hydrophobic substrate will require a less complex metabolic pathways for substate utilization by the microorganism, therefore can also account for the increase in cell biomass in the process of fermentation in the first 48hrs due to availability of the substrate.

Ionic concentration of 7 gave the least surface tension among the pH factors (that is the best surface activity) for both isolates. This is in tanderm with Rapp *et al.,* (1979), Abu-Ruwaida *et al., (*1991) and Gautum and Tyagi (2006) in which they reported that the best pH for biosurfactant production and activity is between pH6 and 7. The important characteristic of most organisms is their strong dependence on pH for their cell growth and production of metabolite. Patil *et al,* 2014, observed that *P. aeruginosa* F23 demonstrated optimum yield of biosurfactant (measured indirectly by surface tension) at pH8. Also the researcher observed that *P. aeruginosa* RS29 and *P. aeuginosa* WJ-1 produced the highest biosurfactant at pH range of 7.0 to 8.0 and 6.0 to 8.0 respectively. The findings of Patil *et al.,*2014 also align with the findings in this studies in which fermentation at pH7.8 (the pH of the raw effluent without adjustment in pH and addition of mineral salt in Figure 1 and 8 respectively) showed the best surface tension reduction which signifies the best biosurfactant producing pH.

Generally among all a parameters (incubation period, pH and control), the drop in surface tension was not significant in the initial 24hrs which had between 10 to 15% drop, this could

probably be due to initial slow growth rate except at pH9 where surface tension drop within 24hrs was above 40% of the overall drop in surface tension. Although a direct relationship exist between incubation period, cell division and cumulative metabolite production, this relationship will only exist up to a specific time in a batch fermentation beyond which it becomes detrimental to the organism as observed by the drop in cell growth after 72 or 96hrs in the study (Biria *et al., 2007)*

The fermentation of the effluent by both isolates without the addition of mineral salt or adjustment in pH was used as control. This process had the best surface activity (reduction in surface tension) compared to other processes suggesting that the raw substrate contained required nutrient for the growth and production of biosurfactant though agitation would certainly have played a role in enhancing cell multiplication. Also due to the hydrophilic nature of the substrate (brewery effluent), this could also have enhanced substrate utilization and also the phosphate and nitrate content of the effluent could also have aided the rate of cell growth. Mulligan *et al*., (1989) and Santa-Anna *et al.*,(2002) reported that the best nitrogen source for biosurfactant production by *Pseudomonas* species is the nitrate form (as measured in the effluent to 3.8mg/l) claiming it enhances biosurfactant production. AbuShady (2005) also confirm that nitrogen as inorganic form (NH_4NO_3 and $NaNO_3$) enhances cell growth and biosurfactant yield.

Statistically, there is an association between the growth rate and reduction in surface tension using the P=0.005 level of significance, therefore it can be said that the reduction in surface tension is dependent on the rate at which the cell multiples while utilizing the medium. Also irrespective of the factors (pH, incubation period and temperature) both isolates performed at par, in-other-words both *P. aeruginosa* and *P. fluorescens* have the same activity across all pH and other factors as there was no significant association between the pH and the reduction in surface tension. This appears to be in constrast to works done by Rapp *et al.,*

(1979) and Patil *et al*, (2014) in which they indicated that pH affect surface tension reduction. Though from the data gather in the course of this work, it could be said that pH is a determining factor though contradicted by statistical calculations at P=0.005.

Considering the effect of adding mineral salt to the medium without varying the pH and also not adding mineral salt; indicated that mineral salt is not a factor determining the activity of both isolates on the medium. Therefore with or without the mineral salt the isolates will effectively reduce the surface tension of the medium (produce biosurfactant), this does not differ from graphical analysis which showed that the control performed better by reducing the surface tension lesser than any other parameter.

The stability of any biosurfactant over a range of temperature is of interest in industrial applications. Stability studies on the effect of temperature variation on the surface activity of the supernatant collected after 168hrs of fermentation show that no appreciable change was observed in the surfactant activity, although surface tension values at different temperatures differ by 0.53mN/m for *P. aeruginosa* and 0.70mN/m for *P. fluorescens*. The surface activity value remained considerably stable after expose to heat at temperatures between 25^0C and 80^0C. The stability of this biosurfactant over the range indicates a stable surface active compound by both isolates studied and its suitability for industrial applications.

The practical application of every surface active compound is its' ability to turn immiscible liquids into stable emulsion for application in various industrial processes that requires formation of stable emulsion. Biosurfactant form emulsion when the surface tension and interfacial tension between the hydrophilic and hydrophobic mixture reduces providing an excellent properties in-terms of reduction in surface tension (Almansorry *et al.,* 2014). The biosurfactant produced by both isolates showed the ability to form stable emulsion within 24hrs with values between 87% and 39% with diesel, kerosene, petrol and olive oil. This

ability to form stable emulsion suggests applicability in cleaning and emulsification in the food, pharmaceutical and cleaning industries.

The best emulsion forming activity across the hydrocarbon emulsified was at pH10 than other pH. Generally the emulsion forming ability increased from acidity to alkalinity. Increased pH has a positive effect on emulsion stability due to the stability of the fatty acid-surfactant micelle in the presence of sodium hydroxide; note that sodium hydroxide was used in pH adjustment (Amrane *et al.*, 2008 and Prieto *et al.*, 2008). Also on the formation of stable emulsion, biosurfactant have optimum aqueous solubility at neutralility to alkalinity which is attributed to their acidic nature. Activity loss at low pH scale of less than pH5 is probably due to the occurrence of precipitate caused by the consequent insolubility of the biosurfactant produced at acidic pH (Luna *et al.*, 2012*;* Almansorry *et al.*, 2014).

The weak or no emulsion formation at pH4 could also be due to the presence of secondary metabolite which interfere with the formation and absorption of the surfactant molecule at the oil-surfactant inter-phase. The emulsification effect could be affected by structural alignment or affinity making the biosurfactant of less effect as it was observed amongst the hydrocarbons used in which emulsion formation was high with diesel, kerosene and petrol but lesser with vegetable oil even at alkaline pH (Bonmatin *et al.*, 1994)

The vibration spectroscopic technique provides rapid and convenient solutions to routine analytical problems. It's increasingly been adopted for identification of molecules and function groups present in the unknown solution. Therefore as vibration technique the Fourier Transformed Infra-Red (FT-IR) is a reliable method. The result of structural analysis (plate 1 and 2) of the purified extract from the supernatant reveals the presence of biosurfactant. The FT-IR spectra obtained showed that the biosurfactant produced would probably be

glycolipids in nature (Coates, 2000; Parthasarathi and Sivakumaar 2009; Rahman *et al.*, 2010).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Pseudomonas aeruginosa and *Pseudomonas fluorescens* isolated from the soil by the bank of the stream into which partially treated brewery effluent was being discharge into were able to utilize brewery effluent as substrate for the production of biosurfactant.

Considering three major factors (incubation period, temperature and pH) for both isolate, the least surface tension reading for incubation period (mineral salt was added but no pH adjustment) of 168hr was 31.30mN/m and 33.76mN/m from 59.54mN/m for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. Also considering the pH range of 4.0 to 10.0, pH7.0 had the least surface tension with 31.87mN/m and 33.04mN/m for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. Cell growth across all the factors was observed to be highest at 72hr and 96hr with values as high as 1.07 for both isolates.

Subjecting the supernatant collected from the cultivation process to temperatures ranging from 25^{o}C to 80^{o}C, 30^{o}C and 35^{o}C had the least surface tension observed with minimal or insignificant differences between the temperatures indicating that a temperature stable biosurfactant was produced by the isolates from brewery effluent.

A portion of the effluent was set up as control in which neither mineral salt nor pH of the media was adjusted, this set up had the least surface tension reduction compared to all other factors considered.

The biosurfactant produced under the aforementioned conditions were all subjected to emulsification assay with various hydrocarbons (diesel, premium motor spirit, kerosene and olive oil), stable emulsion was formed with 24hr emphasizing it applicability.

Pseudomonas aeruginosa produced 3.6g/l of biosurfactant and 2.8g/l of biosurfactant was produced by *Pseudomonas fluorescens*. The FT-IR spectra suggest the presence of a glycolipid.

6.2 Conclusion

In conclusion *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* are good fermentors of brewery effluent resulting in the production of biosurfactant with both isolate performing at par in terms of surface tension reduction and growth in the medium used. Also biosurfactant produced by both isolate are considerably stable at the pH and temperature considered.

The biosurfactant produced (probably a glycolipid) is an effective emulsifier of hydrocarbons and olive oil and effective in reducing surface tension where such effect is important.

6.3 Recommendations

The studies above have shown the potential use of brewery effluent in the production of biosurfactant and also a comparative analysis between the two isolates used have shown to be a good fermenter therefore as suggestion the following could be looked into:

1. Develop a continuous fermentation process that would be effective and economical in the production and extraction of the biosurfactant without harming the fermenting organisms and avoid build-up of toxic substances as this would limit the process.
2. Further molecular manipulation and amplification of the genes of the organisms should be carried out to find a means of boosting the production of the biosurfactant as a substitute to the chemically synthesized surfactant presently available in the market.
3. Also researches should be carried out to check for the medical compatibility and structural rearrangement of the biosurfactant if need be to allow it fit into medical applications.

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Appendix 1: Map showing location of International Beer and Beverage Industries.

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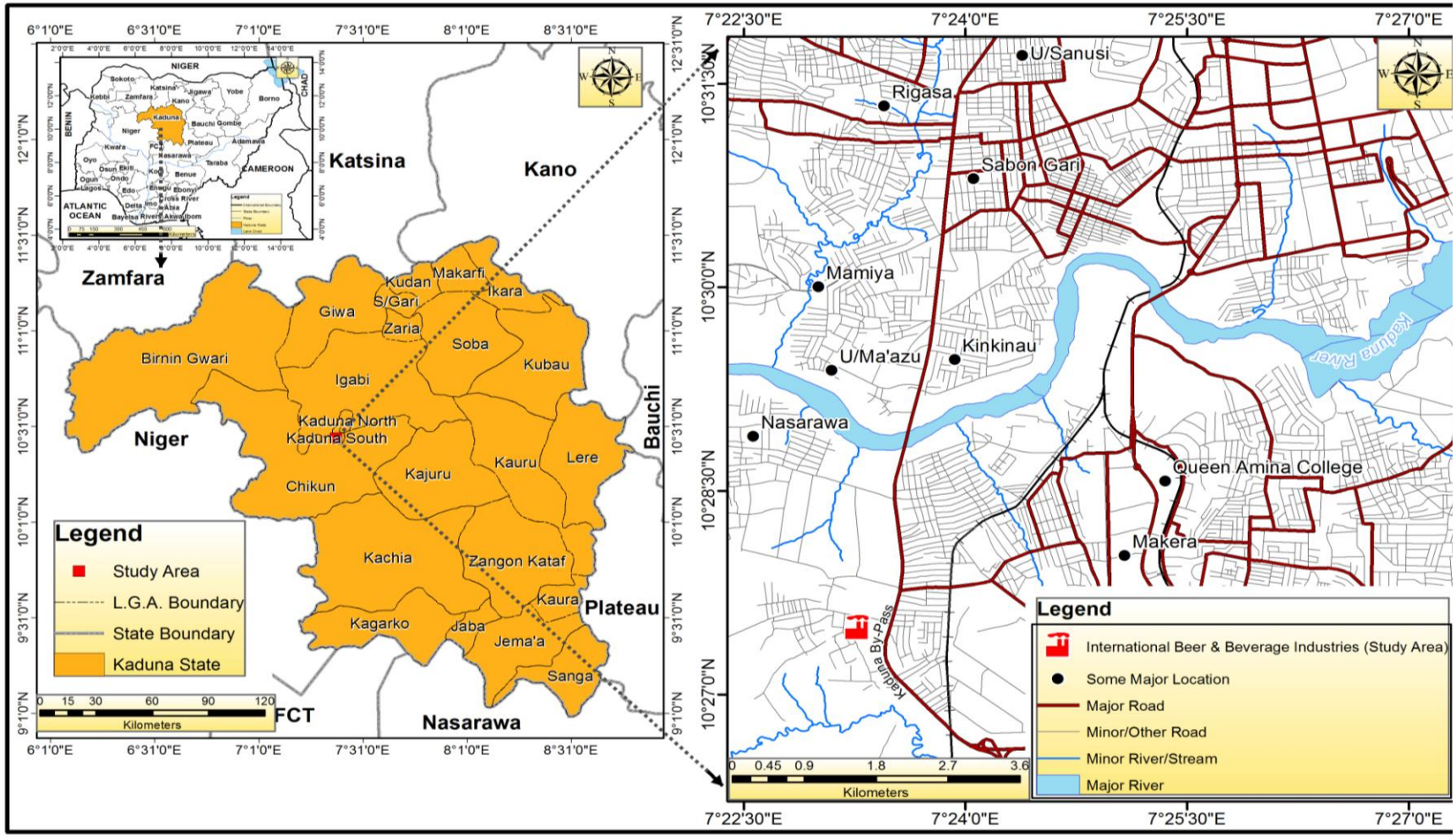


Map of International Beer & Beverage Industries (Study Area) in Kaduna Metropolis
Source: Modified from Kaduna Metropolis Street Map

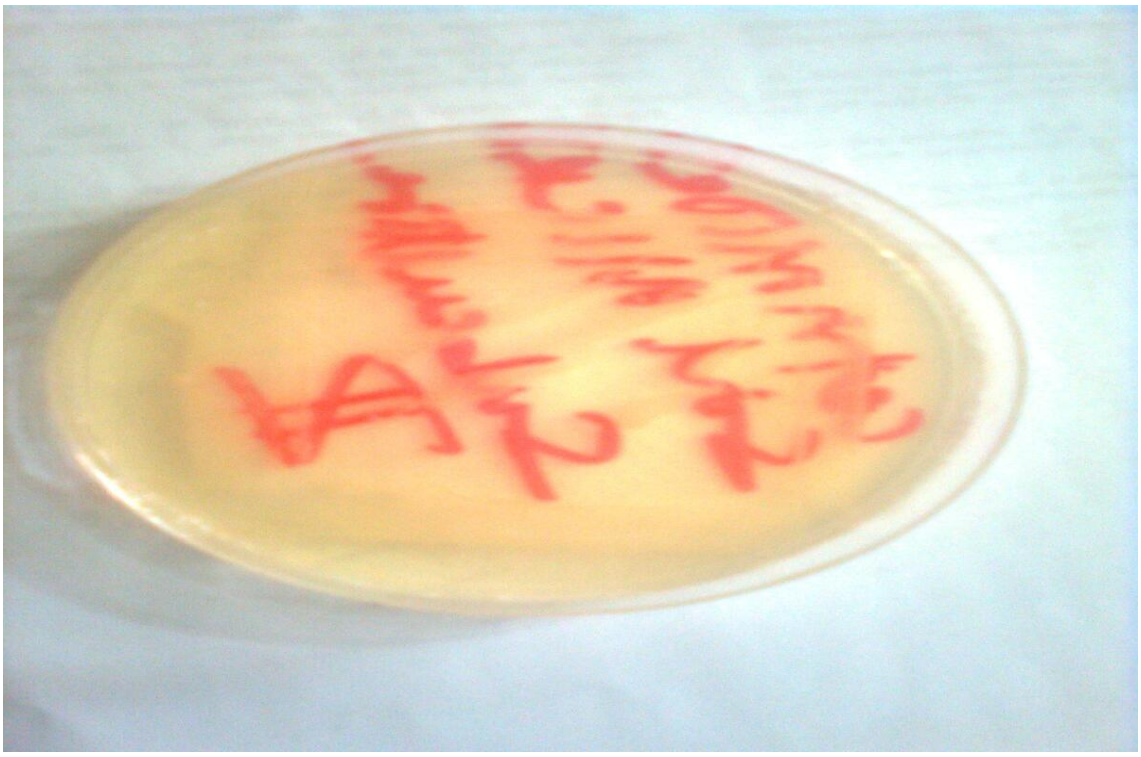
Appendix 2: Biosurfactants produced by various microorganisms

Surfacta nt class	Microorganism
Glycolipids	
4 Rhamnolipids	-*Pseudomonas aeruginosa, P. flourescens*
5 Trehalose lipids	-*Rhodococcus erythropolis, Arthrobacter spp*
6 Sophorolipids	-*Candidda bombicola, Candida apicola,* *Candida antartica, Arthrobacter paraffineus*
Lipopeptides	
1. Surfactin, iturin, fengycin	-*Bacillus subtilis*
2. Viscocin	-*Pseudomonas fluorescens*
3. Lichenysin	-*Bacillus liceniformis*
4. Serrawettin	-*Serratia marcescens*
Phospholipids	*Acinetobacter sp, Corynebacterium spp*
Surface-active antibiotics	
1. Gramicidin	-*Brevibacterium brevis*
2. Polymicin	-*B. polymyxa*
3. Antibiotic TA	-*Myxococcus xanthus*
Fatty acids / neutral lipids	
1. Corynomicolic acids	-*Corynebacterium insidibasseosum*
2. Neutral lipids	-*Norcardia erythropolis, Corynebacterium salvonicum*
Polymeric surfactants	
1. Emulsan	-*Acinetobacter calcoaceticus*
2. Alasan	-*A. radioresisten*
3. Liposan	-*C. lypolytica*
4. Lipomanan	-*C. tropicalis*
Particulate biosurfactant	

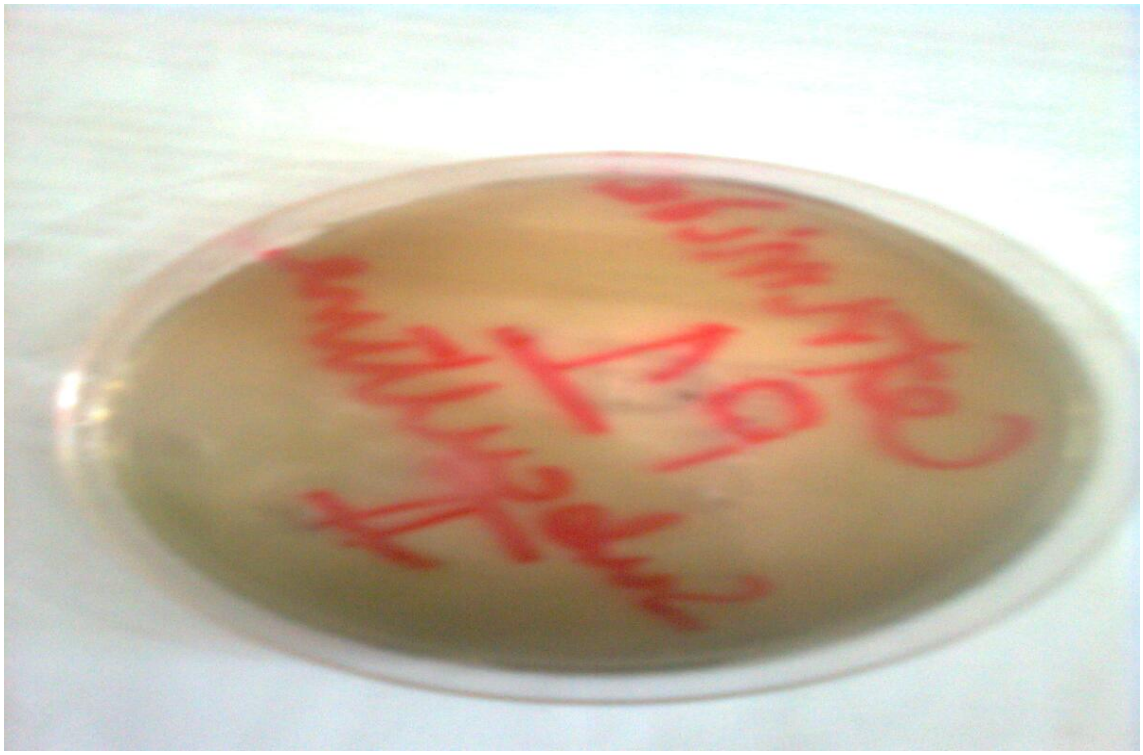
1.	**Vesicles**	*-A. calcoaceticus*
2.	**Whole microbial cells**	*Cyanobacteria spp*

(Desai and Banat, 1997; Georgiou *et al*., 1999; Mathusamy *et al*., 2008,)

Appendix 3: Pictures from laboratory processes



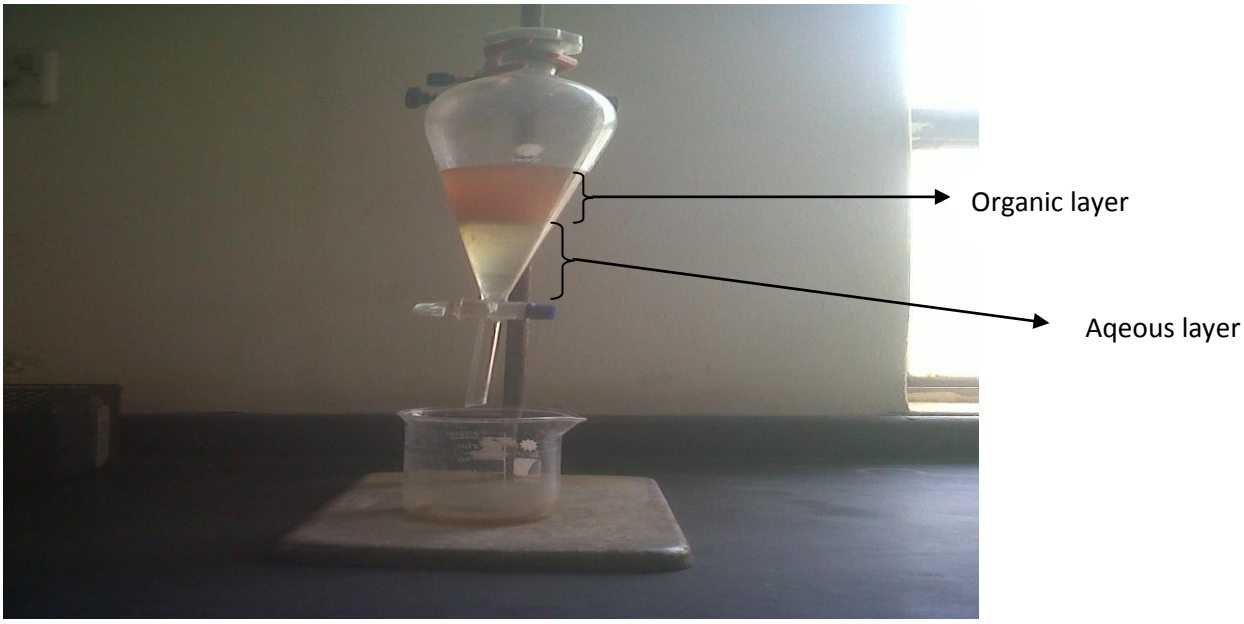
(a)



(b)

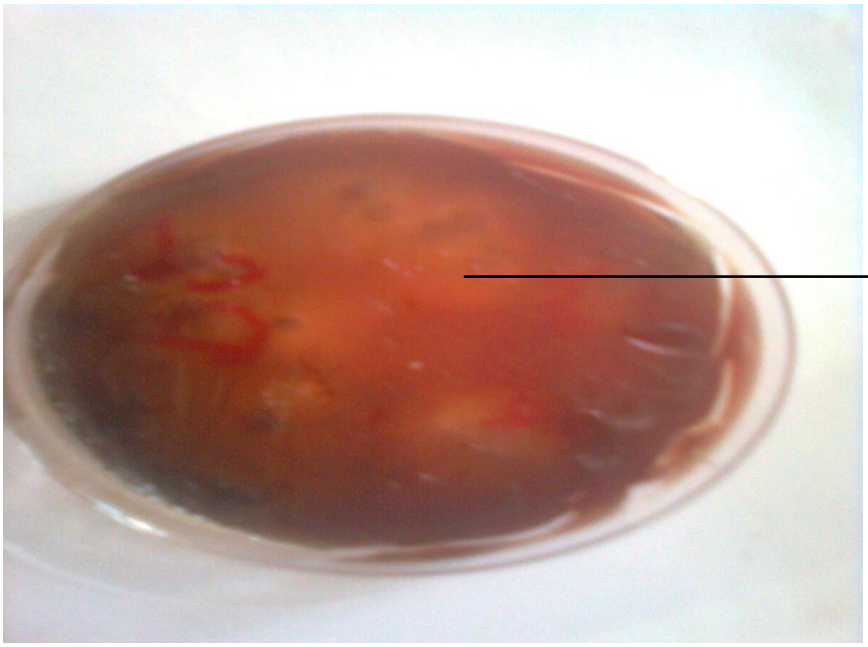
Appendix 3 continued

(c)



(d)

Appendix 3 continued



(e)

a) Culture plate of *P. fluorescens* nutrient agar. (b) Culture of *P. aeruginosa plate with pyocyanin* pigmentation. (c) Emulsification of hydrocarbon (d) Solvent extraction of biosurfactant. (e) plate show haemolysis on blood agar

Appendix 4: Least surface tension and Peak optical density observed with standard error of mean.

	Pseudomonas aeruginosa		***Pseudomonas fluoresens***	
	Optical density	Surface tension(mN/m)	Optical density	Surface tension(mN/m)
AG	1.07±0.08	31.30±0.06	1.07±0.04	33.76±0.14
RNM	0.95±0.110	30.67±2.08	0.76±0.18	32.78±1.63
pH4	0.58±0.101	32.67±0.97	0.61±0.071	34.91±3.29
pH5	1.01±0.007	32.00±4.01	1.04±0.99	34.34±2.79
pH6	1.02±0.042	31.90±1.03	0.99±0.014	34.19±0.56
pH7	1.03±0.042	31.87±3.34	0.9±0.113	34.04±1.13
pH8	0.73±0.014	32.06±5.52	0.71±0.022	34.31±1.0
pH9	0.70±0.028	32.91±1.15	0.79±0.127	34.44±0.87
pH10	0.75±0.7	31.98±0.09	0.079±0.05	34.49±0.51

Appendix 5: Emulsification value with standard error of mean for *P. aeruginosa*

Substrate/ Factor	**Diesel %**	**Petrol %**	**Kerosene %**	**Olive oil %**
AG	68±1.37	68±0.41	55±3.14	45±1.90
RNM	60±3.11	62±0.15	50±0.53	43±0.93
pH4	7±0.12	0±0.00	6±0.62	0±0.00
pH5	48±0.18	39±0.11	45±0.97	37±0.93
pH6	48±2.17	54±0.37	47±0.20	35±1.14
pH7	47±1.12	60±0.73	47±1.28	39±2.16
pH8	58±0.1	60±0.24	48±0.11	40±1.05
pH9	60±1.16	62±0.87	53±0.03	43±1.56

pH10	83±0.92	80±2.10	60±0.13	48±0.06

Appendix 6: Emulsification value with standard error of mean for *P. fluorescens*

Substrate/ Factor	**Diesel %**	**Petrol %**	**Kerosene %**	**Olive oil %**
AG	58± 00.08	60±0.68	51±1.16	43±3.24
RNM	56±1.21	58±0.49	48±0.77	48±0.93
pH4	0±0.00	0±0.00	0±0.00	0±0.00
pH5	43±0.14	43±0.18	42±0.15	32±1.72
pH6	44±1.1	44±0.10	43±0.09	37±1.21
pH7	47±2.09	47±1.86	45±0.81	40±2.33
pH8	52±0.45	52±0.05	45±1.17	41±0.07

pH9	53±1.35	53±0.93	49±0.61	41±0.81
pH10	87±0.43	65±0.06	63±1.13	43±0.03

Appendix 7: Surface tension at different temperature with standard error of mean

	P. aeruginosa	***P. fluorescens***
Temperature (^{o}C)	Surface tension (mN/m)	Surface tension (mN/m)
25	32.9±0.03	33.89±0.77
30	32.10±0.66	33.64±1.56
35	32.53±0.19	33.19±0.15
40	32.13±0.00	33.30±0.34
45	32.31±0.27	33.49±0.08
50	32.37±1.38	3343±0.08
55	32.45±0.87	33.85±2.17

60	32.63±0.06	33.71±0.47
65	32.63±0.17	33.60±0.11
70	32.63±0.70	33.60±0.25
80	32.63±0.59	33.60±0.33