PRODUCING BIOSURFACTANTS FROM RENEWABLE HYDROCARBONS (SHEA BUTTER AND PALM-OIL WASTE SLURRIES) USING BACILLUS CEREUS AND PSEUDOMONAS AERUGINOSA

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BY

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MAY, 2016

DECLARATION

I hereby declare that the work presented in this dissertation entitled **"Producing biosurfactants from renewable hydrocarbons (Shea Butter and Palm-oil Waste Slurries) using *Bacillus cereus* and *Pseudomonas aeruginosa*"** was carried out by me in the Department of Microbiology, Ahmadu Bello University Zaria, Kaduna State, under the supervision of **Prof. (Mrs.) H.I Inabo** and **Prof. E.D. Jatau.** The information derived from 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.

Lilian Ouja ADEMU _____ _____

Signature Date

CERTIFICATION

This dissertation entitled: "**Producing biosurfactants from renewable hydrocarbons (Shea Butter and Palm oil waste slurries) using *Bacillus cereus* and *Pseudomonas aeruginosa***" by **ADEMU, Lilian Ouja**, meets the regulations governing the award of the degree of Master of Science (M.Sc.) in Microbiology of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof. (Mrs.) H.I. Inabo _____ (Chairman, Supervisory Committee) Signature Date

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(Member, Supervisory Committee) Signature Date

Prof. I.O Abdullahi _____ _____
(Head of Department) Signature Date

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(Dean, School of Postgraduate Studies) Signature Date

DEDICATION

This project is dedicated to God my Father.

ACKNOWLEDGEMENTS

I owe my utmost gratitude to God Almighty who gives me the strength to pursue my dreams.

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ABSTRACT

A study was carried to produce biosurfactants from Shea butter and palm oil waste slurries using single and consortium of *Pseudomonas aeruginosa* and *Bacillus cereus* and determine the effect of optimizing using pH values, Nitrogen (Sodium nitrate) concentrations and Lipase (NESS Formula 5®) concentrations on the production of biosurfactants. Growth of the organisms was done at room temperature using a Mineral salt medium enriched with the Shea butter and palm oil waste slurries for 120hours on a rotatory shaker. To check for biosurfactant production, Absorbance (mean values), Surface Tension (ST) and Emulsification index (E_{24}) tests were used. Optimum values for pH from single organisms were 0.94 Absorbance, E_{24} of 67% and 22% reduction in surface tension in Shea butter slurry. For Palm oil slurry, 0.75 Absorbance, E_{24} of 45%, and up to 42% reduction in surface tension were observed. The consortium of the bacteria in both Shea butter and Palm oil slurries gave values of up to 1.09 Absorbance, E_{24} of 67% with no observed reduction in Surface tension in Shea butter slurry while 0.64 for Absorbance, E_{24} of 48% and up to 14.2 % reduction in ST was observed for Palm oil slurry. Nitrogen optimization with single organisms gave values of up to 1.16 Absorbance, E_{24} of 79% and up to 33% reduction in surface tension in Shea butter slurry. In Palm oil slurry, 1.52 Absorbance, E_{24} of 0% and up to 42.5% reduction in surface tension was recorded. The consortium of the organisms gave values of 1.32 Absorbance, E_{24} of 58% with 14.2% reduction in Surface tension in Shea butter slurry while 0.61 Absorbance, E_{24} of 55% and up to 30.4% reduction in ST was recorded for Palm oil slurry. Lipase optimization with NESS Formula 5® gave values of up to 0.73 for Absorbance, E_{24} of 65% , with up to 22.2% reduction in surface tension in Shea butter slurry and 0.89 Absorbance, E_{24} of 62% with up to 47.5% reduction in surface tension in palm oil slurry using single cultures of the organisms. 1.19 Absorbance,

$E_{24}$of 55% with up to 14.2% reduction in Surface tension in Shea butter slurry and 0.80 Absorbance, E_{24} of 65% with up to 14.4 % reduction in ST in Palm oil slurry, was recorded for the consortium of the organisms. There were significant differences between the varied environmental parameters (pH, Nitrogen and Lipase) It was concluded that best results for biosurfactant production was from Shea butter slurry while *Pseudomonas aeruginosa* produced more biosurfactant from both slurries compared to *Bacillus cereus*.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Surfactants are compounds that lower the surface tension of a liquid, the interfacial tension between two liquids, or that between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants (Makkar *et al*., 2011).

Biosurfactants are surface active agents of microbial origin and have the unique property of lowering the interfacial tension between two liquids. They act on the interface and are amphipathic molecules with both hydrophilic and hydrophobic moieties present within the same molecule. Biosurfactants and Bio emulsifier are often used interchangeably owing to their unusual emulsifying property that makes them the molecules that enhance the accessibility and bioavailability of hydrophobic chemicals by forming stable emulsions and lowering the surface tension (Mulligan and Gibbs, 2004).

Biosurfactants have been reported by Mulligan (2004) to utilize a number of immiscible substrates and thereby facilitate the biodegradation of panoply of polyaromatic hydrocarbons. They have carved a niche for themselves with their unusual antibacterial, antifungal and antiviral activities (Cameotra and Makkar, 2004; Joshi *et al*., 2008). Some of the uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as therapeutic, probiotic and pharmaceutical agents (Cameotra and Makkar, 2004; Rodrigues *et al*., 2006; Mulligan and Gibbs, 2004;

Eshrat, 2011). In addition biosurfactants have unique characteristics that enables them to degrade a wide range of organic pollutants (Magdalena *et al*., 2011). The prospects of biosurfactants have a great potential because of their applications in the petroleum industry (Mulligan and Gibbs 2004; Amedea *et al*., 2010) and microbially enhanced oil recovery (Okpokwasili and Ibiene, 2006; Youssef *et al*., 2007; Shavandi *et al*., 2011). The *Rhodococcusruber* biosurfactants are found to be 1.4 to 2.3 times more efficient than the synthetic surfactants (Tween 20, Tween 60) in enhanced crude oil desorption and mobilization from soil core, with 65-82% crude oil recovery (Kuyukina *et al*., 2005).

The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection. The uniqueness with unusual structural diversity, the possibility of cost-effective ex-situ production and their biodegrability are some of the properties that make biosurfactants a promising choice for use in environmental applications (Hua *etal*., 2003). Initial focus of industrial interest towards biosurfactants concentrated on the microbial production of surfactants and cosurfactants for the application on microbially-enhanced oil recovery (MEOR) (Hua *etal*., 2003).

The application of biosurfactants industrially is still at the developmental stage because biosurfactant application in industries is dependent on high biosurfactant production yield and the production of highly active biosurfactants with specific properties for specific applications. Hence, majority of surfactants produced today are of petrochemical origin compared to that produced microbiologically from renewable resources.

Hester (2001) estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $US200 million. However, till now, biosurfactants are still unable to compete with the chemically synthesized surfactants in the surfactant market. This is due to their high production costs in relation to inefficient bio-processing methods available, poor strain productivity and the need to use expensive substrates (Cameotra and Makkar, 1998; Deleu and Paquot, 2004).

1.2 Statement of Research Problem

Economy is often the drawback of all biotechnological processes, especially in the case of biosurfactant production. The economics of producing biosurfactants has limited its commercial applications, but the production cost can be reduced by improving yield, rate, recovery and using cheap or waste substrates (Mulligan *et al.*, 2001). Biosurfactants have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties--higher biodegradability, lower toxicity, and effectiveness at extremes of temperature, pH and salinity. However, large-scale production of these molecules has not been realized because of low yields in production processes and high recovery and purification costs (Murkhejee *et al*., 2006).

1.3 Justification

This research aims at using cheap and locally, readily available raw materials to test for bio surfactant production and to test for improved yield and rate of bio surfactant production by modifying some environmental conditions and using a consortium of bacteria.

Most surfactants are petroleum based and are chemically synthesized. However the leading trend towards using environmentally friendly technologies has enhanced the search for biodegradable compounds of natural origin. Biosurfactants are therefore the natural choice for such processes as they possess a host of advantages over synthetic surfactants, such as lower toxicity, biodegradability and effectiveness at a wide range of pH and temperature values (Makkar *et al*., 2011). Most biosurfactants, like synthetic surfactants, exhibit physicochemical properties and characteristics such detergency, emulsification, de-emulsification, foaming and wetting. These molecules have the abilities to reduce superficial and interfacial tension reduction between solids, liquids and gases. Interest in their potential applications by various industries such as Pharmaceuticals, Cosmetics, Food and beverages has significantly increased recently, particularly because of their environmental friendly nature and sustainability (Makkar *et al*., 2011).

In addition, several biosurfactants have been reported to have manifold biological activities covering antibiotics, fungicides, insecticides, antiviral and antitumoral agents, immunomodulators or specific toxins or enzyme inhibitors, which make them interesting for pharmaceutical applications. Of special interest for an industrial use are microbial glycolipids: they occur either as an important part of the microbial cell surface enabling the contact with hydrophobic substrates or they are secreted as emulsifiers in the culture broth when grown on water immiscible or oily substrates. Consisting of carbohydrates moieties bond to fatty acid or hydroxyl fatty acid chains they are the main low molecular weight biosurfactant group possessing many commercially attractive properties and clear advantages compared with their other synthetic counterparts.

Although the production of microbial biosurfactants has been studied extensively in the last decade, their production on a commercial scale has only been realized in very few cases, and up to now they have found few applications due to their distinctly higher prices compared to chemical surfactants. An interesting option for the future not realized yet could be their use for microbial enhanced oil recovery (MEOR), if it is possible to decrease production costs (Dachema,2013). This can perhaps be achieved through this study with the following aims and objectives.

1.4 Aim of the Study

The aim of this study is to screen for production of biosurfactants from renewable waste slurries of Shea butter and Palm oil using *Bacillus cereus* and *Pseudomonas aeruginosa* under different environmental conditions.

1.5 Specific Objectives of the Study

1. To isolate and characterize *Bacillus cereus* and *Pseudomonas aeruginosa* from soil samples.
2. To screen for biosurfactant production activity of *Bacillus cereus* and *Pseudomonas aeruginosa*
3. To screen for production of biosurfactants from Shea butter and Palm oil waste slurries using single cultures and consortium of *Bacillus cereus* and *Pseudomonas aeruginosa*
4. To carry out optimization studies of pH, Nitrogen and lipase on biosurfactant production by the isolates bacterial isolates in Shea butter and Palm oil waste slurries.
5.

1.6 Research Questions

- The possibility of producing biosurfactant from palm-oil and Shea butter wastes using *Bacillus cereus* and *Pseudomonas aeruginosa*?
- The possibility of improved yield when consortium of *Bacillus cereus* and *Pseudomonas aeruginosa* are used?

1.7 Research Hypothesis

Null hypothesis (Ho): Bio surfactants cannot be produced from Shea butter and Palm oil wastes slurries using single and consortium of *Bacillus cereus* and *Pseudomonas aeruginosa.*

Alternative hypothesis (Ha): Bio surfactant can be produced from Shea butter and Palm oil wastes slurries using single and consortium of *Bacillus cereus* and *Pseudomonas aeruginosa.*

CHAPTER TWO

2.0 LITERATURE REVIEW

Biosurfactants are surface-active substances derived from living organisms, especially microorganisms. They are amphiphilic compounds, containing hydrophobic and hydrophilic moieties. The hydrophilic moiety can be carbohydrate, amino acid, phosphate group or some other compounds whereas the hydrophobic moiety usually is a long chain fatty acid (Lang, 2002). The potential applications of biosurfactants in industries include emulsification and foaming for food processing, wetting and phase dispersion for cosmetics and textiles, or solubilization for agrochemicals (Lin, 1996). In addition, biosurfactants can be use in environmental applications such as bioremediation and dispersion of oil spills (Banat, 1995). Biosurfactants can be divided into 4 major groups based on their overall structures. They are glycolipids, phospholipids, lipoproteins or lipopeptides and polymeric compounds (Healy *et al*., 1996).

Biosurfactants are compounds that produce surface-active and emulsifying activities and are themselves produced by microorganisms, such as bacteria, yeast and fungi (Hamzah *et al.,* 2013). The ability to reduce surface tension is a major characteristic of surfactant. Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives, and a number of other consumer and industrial products.

All living cells produce amphipathic molecules. These molecules which consist of both hydrophilic and hydrophobic moieties are called surface-active compounds or surfactants. In many cases, they exhibit surface-active characteristics such as dramatic lowering of surface tension at the air/water interface, lowering interfacial tension at the oil/water

interface, and micelle or pseudomicelle formation (Haddad *et al.,* 2008). Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which make surface-active compound, some of the most versatile process chemicals (Greek, 1991).

Biosurfactants are a group of microbial molecules identified by their unique capabilities to interact with hydrocarbons. Emulsification and de-emulsification, dispersion, foaming, wetting and coating are some of the numerous surface activities that biosurfactants can achieve when applied within systems such as immiscible liquid/liquid (e.g., oil/water), solid/ liquid (e.g., rock/oil and rock/water) and gas/liquid. Therefore, the possibilities of exploiting these bioproducts in oil-related sciences are vast and made petroleum industry their largest possible market at present. The role of bio surfactants in enhancing oil recovery from reservoirs is certainly the best known; however they can be effectively applied in many other fields from transportation of crude oil in pipeline to the clean-up of oil storage tanks and even manufacturing of fine petrochemicals. When properly used, biosurfactants are comparable to traditional chemical analogues in terms of performances and offer advantages with regard to environment protection/conservation (Perfumo *et al*., 2010).

During the last years, several applications of biosurfactants with medical purposes have been reported. Biosurfactants are considered relevant molecules for applications in combating many diseases and as therapeutic agents due to their antibacterial, antifungal and antiviral activities.

Furthermore, their role as anti-adhesive agents against several pathogens illustrate their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction of a large number of hospital infections without the use of synthetic drugs and chemicals (Rodrigues *et al,* 2008).This research would be contributing to the body of knowledge by testing the efficacy of lower cost and increased yield by combining the use of locally available substrates with the use of a consortium of bacteria.

Surfactants are widely used for various purposes in industry, but for many years were mainly chemically synthesized. It has only been in the past few decades that biological surface-active compounds (biosurfactants) have been described and are gaining prominence. They have already taken over for a number of important industrial uses, due to their advantages of biodegradability, production on renewable resources and functionality under extreme conditions; particularly those pertaining during tertiary crude-oil recovery. Conflicting reports exist concerning their efficacy and the economics of both their production and application. At present, their uses are mainly in the oil and petroleum industries, where they are employed primarily for their emulsification capacity in both tertiary recovery and polluted-sites remediation. However, caution is frequently exercised with respect to their use because of possible subsequent microbial contamination of either underground oil reservoirs or products. The limited successes and applications for biosurfactants' production, recovery, use in oil pollution control, oil storage tank clean-up and enhanced oil-recovery are reviewed from the technological point of view (Banat *et al*., 1995).

In comparison to their other chemically synthesized equivalents they have many advantages. They are environmentally friendly, biodegradable, less toxic and non-

hazardous. They have better foaming properties and higher selectivity. They are active at extreme temperatures, pH and salinity as well, and can be produced from industrial wastes and from by-products. This last feature makes cheap production of biosurfactants possible and allows utilizing waste substrates and reducing their polluting effect at the same time.

Because of their potential advantages, biosurfactants are widely used in many industries such as agriculture, food production, chemistry, cosmetics and pharmaceutics. The examples of biosurfactant applications are listed in many review papers. Many properties of microbial surface active compounds such as emulsification/de-emulsification, dispersion, foaming, wetting and coating make them useful in physicochemical and biological remediation technologies of both organic and metal contaminants. Biosurfactants increase the bioavailability of hydrocarbon resulting in enhanced growth and degradation of contaminants by hydrocarbon-degrading bacteria present in polluted soil. In heavy-metal polluted soils biosurfactants form complexes with metals at the soil interface, which is followed by desorption of the metal and removal from the soil surface leading to the increase of metal ions concentration and their bioavailability in the soil solution. The new approach is the use of heavy metal-resistant bacterial strains capable of producing biosurfactants for increasing the metal-removing efficiency by phytoremediation (Pacwa-Plocinizak *et al*, 2011).

Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (CxHy) microorganism facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants. Some bacteria excrete ionic surfactant, which emulsify hydrocarbon substrates in the growth medium. The exact reason why some

microorganisms produce surfactant is unclear (Deziel *et al.,* 1996). Biosurfactants produced by various microorganisms together with their properties are listed in Table 2.1

2.1 Classification of Biosurfactants

Biosurfactants are categorized mainly by their chemical composition and microbial origin (Muthusamy *et al*., 2008; Okoliegbe and Agarry, 2012). However, Rosenberg and Ron (1999) suggested that biosurfactants can be divided into low molecular weight biosurfactants and the high molecular-weight biosurfactants; polar grouping; chemical compositions; phospholipids and polymeric surfactants.

2.1.1 Classification based on low molecular weight

Low molecular- weight biosurfactants are compounds that lower the surface and interfacial tension at the air/water interface (Salihu *et al.,* 2009). The low molecular-weight biosurfactants are generally glycolipids or lipopeptides. The best studied glycolipids are rhamnolipids, trehalolipids and sophorolipids which are disaccharides that are acylated with long- chain fatty acids or hydroxy fatty acids (Ron and Rosenberg, 2001; Okoliegbe and Agarry, 2012).

2.1.2 Classification based on high molecular weight

High- molecular weight biosurfactants are usually referred to as bioemulsans (Salihu *et al.,* 2009). They are more effective in stabilizing oil in water emulsions (Okoliegbe and Agarry, 2012). They are highly efficient emulsifiers that work at low concentrations and exhibit considerable substrate specificity (Dastgheib *et al.,* 2008). Ron and Rosenberg (2001) reported that a large number of bacterial species from different genera produce exo-

cellular polymeric surfactant composed of polysaccharides, proteins, lipopolysaccharides, lipo-proteins or complex mixtures of these biopolymers.

2.1.3 Classification based on polar groupings of biosurfactants

Biosurfactants are either anionic or neutral (Okoliegbe and Agarry, 2012). The hydrophobic moiety is based on long chain fatty acids or fatty acids derivatives whereas the hydrophilic portion can be carbohydrates, amino acid, phosphate or cyclic peptide (Nitschke and Coast, 2007; Okoliegbe and Agarry, 2012).

The structure of a biosurfactants includes a hydrophilic moiety consisting of amino acids or peptides anions or cations, mono-, di- or polysaccharides and a hydrophobic moiety consisting of unsaturated, saturated or fatty acids derivatives (Desai and Banat, 1997).

2.1.4 Classification based on chemical composition

2.1.4.1 Glycolipids

Most known biosurfactants are glycolipids, they are carbohydrates whose constituent mono-, di-, tri- and tetrasaccharides include glucose, mannose, galactose, rhamnose, galactose sulphate and glucoronic acid. These carbohydrates combine with long–chain aliphatic acids or hydroxyaliphatic acids. This linkage is by means of either ether or an ester group. In this taxa, the best known are the rhamnolipids, trehalolipids and sophorolipids (Desai and Banat, 1997, Muthusamy *et al.*, 2008). Other glycolipids produced by microorganisms are cellobiolipids which are produced by *U. zeae*, and *U. maydis* and the Mannosylerythritol lipids that are produced by *Candida antartica* (Desai and Banat, 1997, Muthusamy *et al.*, 2008).

2.1.4.2 Lipopeptides and lipoproteins

Large number of cyclic lipopeptides including decapeptide antibiotics (gramicidin) and Lipopeptide antibiotics (polymxin) produced by *Bacillus brevis* and *Bacillus polymyxa* respectively possess remarkable surface active properties. These consist of a lipid attached to a polypeptide chain. Other examples are orinithine lipids, Iturin with surfactin being the best studied (Desai and Banat, 1997, Muthusamy *et al.,* 2008).

2.1.5 Phospholipids, fatty acids and neutral lipids

Several bacteria and yeast produces large quantities of fatty acids and phospholipids surfactant during growth on n-alkanes. Fatty acids produced from alkanes as a result of microbial oxidations have been considered as surfactant. Apart from these straight chain acids, microorganisms produce complex fatty acid containing OH groups and alkyl branches, example of such complex acids include corynomuolic acids (Rahman and Gakpe, 2008).

2.1.5.1 Phospholipids

Phospholipids are major components of microbial membranes, when certain hydrocarbon degrading bacteria or yeast are grown on alkane substrates the level of the phospholipids increases greatly for example growing *Acinetobacter* sp. on Hexadecane produces the phospholipids which is mainly phosphatidylethanolamine (Muthusamy *et al.,* 2008).

2.1.6 Polymeric microbial surfactants

Most of these biosurfactants are polymeric heterosaccharide containing proteins. The best studied polymeric biosurfactants are emulsan, liposan, mannoprotein and polysaccharide protein complexes (Desai and Banat, 1997).

2.1.7 Particulate biosurfactant

Extracellular membrane vesicles partition hydrocarbons forming micro emulsion which play an essential role in alkane uptake by microbial cells.

2.2 Applications of Biosurfactants

The applications of biosurfactants have been researched and reported in literature proving that the usefulness of this substance to man in most aspects of his existence cannot be over emphasized. Worldwide production of surfactants amount to 17 million metric tonnes in the year 2000 and an expected future growth rates of 3-4% per year globally (Okoliegbe and Agarry, 2012). Applications of surfactants are classified according to how they are applied by the users; detergents and cleaners (54%); auxiliaries for textiles, leather and paper (13%), chemical processes (10%), cosmetics and pharmaceuticals (10%) food industry (3%). Agriculture (2%) and in others (8%) (Rahman and Gakpe 2008; Okoliegbe and Agarry, 2012). These chemically synthesized surfactants are mainly petroleum based and are usually non-biodegradable thus remain toxic to the environment they find themselves (Okoliegbe and Agarry, 2012).

These compounds may also bioaccumulate and their production processes and by-products can be environmentally hazardous, due to this increasing awareness on the need to protect

the ecosystem, environmental scientist have been tightening environment regulations thus necessitating an increased interest in surfactants of microbial origin as possible alternatives to chemically synthesized ones (Benincasa, 2007; Okoliegbe and Agarry, 2012). The various applications of biosurfactants in industries, medicine, agriculture and petroleum are discussed below.

2.2.1 Applications of biosurfactant in industry

Biosurfactants have been used for various food processing industries where they play a role of food formulation ingredient and anti-adhesive agents (Okoliegbe and Agarry, 2012). As food formulation ingredient, biosurfactants promote the formation and stabilization of emulsion due to their ability to decrease the surface and interfacial tension. They are also used to control the agglomeration of fat globules, modify rheological properties of wheat dough and improve consistency, stabilize aerated systems, improve texture and shelf -life of starch containing products, and texture of fat based products (Muthusamy *et al.,* 2008; Okoliegbe and Agarry, 2012).

Furthermore Muthusamy *et al.,* (2008) reported that the use of biosurfactants as anti-adhesive agents given the fact that a biofilm present in food industry surfaces are potential sources of contamination which may lead to food spoilage and disease transmission. They also reported the preconditioning of stainless steel surfaces with a biosurfactant obtained from *Pseudomonas fluorescens* inhibits the adhesion of *Listeria monocytogenes* LO28 strain.

In the cosmetic industry, due to its emulsification, foaming, water binding capacity, spreading and wetting properties effect on viscosity and on product consistency,

biosurfactant have been proposed to replace chemically synthesized surfactants. These surfactants are used as emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, anti-dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleansers etc. (Okoliegbe and Agarry, 2012; Gharaei-Fathabad, 2011).

2.2.2 Applications of biosurfactants in medicine

Wide range of applications of biosurfactants in medicine as reported by Mukherjee *et al.*, (2006) include the following:

2.2.2.1 Anti-cancer activity

Some microbial extracellular glycolipids (MEL) induce cell differentiation instead of cell proliferation in the human promyelocytic (PC) leukemia cell line. Also, exposure of PC cells to MEL enhanced the activity of acetylcholine esterase and interrupted the cell cycle which resulted in the overgrowth of neurites and partial cellular differentiation (Okoliegbe and Agarry, 2012). This suggest that MEL induces neuronal differentiation in PC cells and provides the ground work for the use of microbial extracellular glycolipids as novel reagents for the treatment of cancer cells (Muthusamy *et al.*, 2008; Okoliegbe and Agarry, 2012).

2.2.2.2 Immunological adjuvants

Bacterial lipopeptides constitute potent non-toxic, non-pyrogenic immunological adjuvants when mixed with conventional antigens (Okoliegbe and Agarry, 2012). An improvement

of the humoral humane response was demonstrated with low molecular mass antigens Iturin AL and herbicolin A (Gharaei-Fathabad, 2011).

2.2.2.3 Antimicrobial activity

The diverse structures of biosurfactants gives them the ability to display versatile performance. By its structure, biosurfactants exerts its toxicity on the cell membrane permeability bearing the similitude of a detergent-like effect (Zhao *et al.*, 2010). Several biosurfactants have strong antibacterial, antifungal and antivirus activity (Gharaei-Fathabad, 2011). These surfactants play the role of anti-adhesive agents to pathogens making them useful for treating many diseases as well as its use as therapeutic and probiotic agent (Okoliegbe and Agarry, 2012). A good example is the biosurfactant produced by marine *Bacillus circulans* that had a potent antimicrobial activity against Gram positive and Gram negative pathogens and semi-pathogenic microbial strains including MDR strains (Okoliegbe and Agarry, 2012).

2.2.2.3 Antiviral activity

Antibiotic effects and inhibition of growth of human immunodeficiency virus in leucocytes by biosurfactants have been reported in literature (Desai and Banat, 1997, Muthusamy *et al.,* 2008; Okoliegbe and Agarry, 2012). Furthermore, Muthusamy *et al.* (2008) reported that due to the increased incidence of HIV in women, there arose the need for a female controlled, efficacious and safe vaginal topical microbicide. Sophorolipids surfactants from *C. bombicola* and its structural analogues such as the sophorolipid diacetate ethyl ester is the most potent spermicidal and virucidal agent, it was also reported that this substance has

a virucidal activity similar to nonoxynol against the human semen (Okoliegbe and Agarry, 2012).

2.2.2.4 Anti-adhesive agents

Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites (Okoliegbe and Agarry, 2012). Rodrigues *et al.* (2006) demonstrated that pre-coating vinyl urethral catheter by running the surfactin solution through them before inoculation with media resulted in the decrease in the amount of biofilm formed by *Salmonella typhimurium,Salmonella enterica, Escherichia coli* and *Proteus mirabilis*. Muthusamy *etal*., (2008) also reported that pre-treatment of silicon rubber with *S. thermophilus* surfactant inhibited 85% adhesion of *Candida albicans* and surfactants from *L. fermentum* and *L. acidophilus* adsorbed on glass, reduced by 77% the number of adhering uropathogenic cells of *Enterococcus faecalis*.

2.2.2.5 Gene delivery

Gharaei-Fathabad (2011) stated that the establishment of an efficient and safe method for introducing exogenous nucleotides into mammalian cells is critical for basic sciences and clinical applications Other advantages and applications of bio-surfactant in medicine are the use of surfactants as agents for stimulating stem fibroblast metabolism, immune modulatory action, however it has been reported in literature that the deficiency of pulmonary surfactant, a phospholipids protein complex is responsible for the failure of respiration in prematurely born infants however, isolation of genes for protein molecules of this surfactant and cloning in bacteria have made possible its fermentation production for medical application (Muthusamy *et al.,* 2008).

2.2.3 Application of biosurfactant in petroleum

The petroleum industry have existed for the past 100 years, but the high quality and easily extractable light crude oils are limited, ultimate recoverable resources are estimated at between 2 – 4 trillion barrels which poses two major issues: first, efficiency and maximization of the overall stages of processing and secondly, the challenge of utilizing heavy crude oils, bitumen and tar-sand which may represent the hydrocarbon based energy of the future (Hall *et al.,* 2003; Okoliegbe and Agarry, 2012).

Biosurfactant and bioemulsifier are novel group of molecules and among the most powerful and versatile by-product that modern microbial technology can offer in fields such as bio-corrosion and biofouling degradation of hydrocarbons within oil reservoirs, enzymes and biocatalysts for petroleum up-grading (Perfumo *et al.,* 2010). Furthermore, biosurfactants play a major role in petroleum extraction, transportation, upgrading and refining and petrochemical manufacturing.

2.2.3.1 Microbial enhanced oil recovery

Perfumo *et al.,* (2010) reported that classical oil production technologies involving primary and secondary can only partially recover the oil present in the field with an efficiency estimated at 30 – 40% of the overall amount of oil available. Such efficiencies are expected to decrease with the gradual depletion of light crude resources leaving the viscous crude oil, thus requiring the development of the "tertiary" processes which aim at enhanced oil recovery (Okoliegbe and Agarry, 2012).

Perfumo *et al.,* (2010), also reported that biosurfactant are able to exert oil displacement and movement through oil bearing rocks by three main mechanisms.

1. Modification of the wettability of porous media
2. Emulsification of crude oil.
3. Reduction of interfacial tension between oil–rocks and oil–brine.

In addition, biosurfactant production contributes to the metabolism of viscous oil through microorganism that release lighter hydrocarbon fraction making oil even more fluid. Lichenysin have been used in enhanced oil recovery where it is produced ex-situ by *Bacillus licheniformis* and it is injected into oil resources (Okoliegbe and Agarry, 2012). This surfactant even at low concentrations (10 – 60mg/L) is able to reduce interfacial tension to ultra-low values (less than 10 -2 mN/M) which is required to release the trapped oil. In addition it is not affected by temperature (≤ 140^{o}C), pH (from 6 to 10), salinity (up to 10% w/v NaCl) and calcium concentrations (≤ 340mg/L CaCl2) (Perfumo *et al.,* 2010; Okoliegbe and Agarry, 2012).

Maggot (2005) reported the use of laboratory selected bio-surfactant producing microorganism been injected into oil reservoir with resounding success. These microbes are injected along with their nutrients into oil wells thus their ability to grow and metabolize in these areas. Since most oil extraction processes are carried out at high temperature, most organisms implicated here are extremophiles (Okoliegbe and Agarry, 2012). Thermotolerant *Pseudomonas aeruginosa* strains isolated from injection waters were found to produce molecules which is effective in displacing trapped oil both in laboratory test and within low temperature reservoirs. The rhamnolipids produce at high temperature of 90^{o}C with a critical micelle concentration of 70mg/litre and at lower pH are

only slightly affected by salinity and calcium ions (Maggot, 2005). However, the use of this microbe is limited due to its classification as risk-group 3 organisms with restriction and regulation on its handling and dispersion into the environment. Also, the fact that this organism is typically an aerobic mesophile proves an issue that this organism produces rhamnolipids (Perfumo *et al.,* 2010).

Another strategy is based on the concept that oil reservoirs are inhabited by indigenous microbial Communities. However the knowledge of such microbial ecosystems is limited due to difficulties in collecting representative samples as well as carrying out in situ analysis. Therefore, whether indigenous microorganisms are native or contaminants exogenously introduced through water flooding, drilling or other oil well operations is still to be confirmed as well as their metabolism and activities established (Maggot, 2005, Franzetti *et al.,* 2008; Okoliegbe and Agarry, 2012).

2.2.3.2 Formation of petrochemicals

Petrochemicals are prepared using specific surfactant packages along with a variety of additives (e.g. detergents, lubricity enhancers, anti-foaming agents, ignition improvers, antirust agents and metal deactivators). Petrochemical industry is largely driven by strict environmental conditions rather than product economics. One of such area is the manufacturing of emulsified fuels; emulsified fuels are water–in–diesel emulsion with a typical content of water of 10 – 20% (v/v). Surfactants are expected to stabilize the emulsion and ensure that the finely dispersed water droplets remain in suspension within the diesel fuel. Non-ionic surfactants such as alcohol ethoxylates, fatty acid ethoxylates and sugar ester of fatty acids are currently the most used (Perfume *et al.,* 2010). This is an

interesting technology which is currently applied especially in Europe for public transport fleets, marine engine, locomotive, heat facilities due to the fact that it improves combustion efficiency, the micro exploitation of water particles leads to a reduction of emulsion of hazardous pollutants such as nitrogen oxides (≤ 25%) carbon oxide (≤5%) black smoke (≤ 80%) and particulate matter (≤60%) there is also the reduction of diesel combustion (Perfumo *et al.,* 2010).

2.2.3.3 Crude oil transportation in pipelines

Crude oil presents the need for long distance transportation from extraction fields to the refineries. Oil viscosity is a major factor which affects pipeline as it slows the flow of oil. Traditional methods of heating or dilution with solvents have been applied to reduce oil viscosity but a promising technology consisting of producing stable oil- in- water emulsion that facilitate oil motility have been recently developed and new routes to the application of the bioemulsifier-type of surfactants have been found particularly suitable for this application (Okoliegbe and Agarry, 2012). Emulsan, a surfactant, was applied in field-trial for pipeline transportation of Boscan heavy crude oil with the viscosity of about 200,000 cp, oil ratio of 1:500 producing a 70% w/v oil-in-water stable emulsion. On reaching the refinery, hydrocarbosols can either be de-emulsified and utilized directly without de-watering or treated with specific enzymes emulsanes to depolymerize the bioemulsifiers thus breaking the emulsion before use (Perfume *et al.,* 2010; Okoliegbe and Agarry, 2012).

2.2.3.4 Clean up of oil containers/ storage tanks

Large amounts of crude oil are moved daily and distributed to refineries with oil tankers, barges and trucks thus increasing the problem of clean-up and maintenance of the containers (Okoliegbe and Agarry, 2012).

2.3 Microorganisms Producing Biosurfactants

Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition (Table-2.1) and their nature and the amount depend on the type of microorganism producing a particular biosurfactant. Many microorganisms for industrial utilization for waste products have been isolated from contaminated soils, effluents and waste water sources. Thus, these have an ability to grow on substrates considered potentially noxious for other non-producing microorganisms.

2.4 Physiological Role of Biosurfactant in Microorganisms

Biosurfactants are produced by a variety of microorganisms they are secreted either extracellularly or attached to parts of cells predominantly during growth on water immiscible substrates (Desai and Banat, 1997).

The main physiological role of biosurfactants is to permit microorganisms to grow on water-immiscible substrates reducing the surface tension at the phase boundary, thus making the substrate more readily available for uptake and metabolism. Ron and Rosenberg (2001) suggested that when the surface area becomes limiting, biomass increases arithmetically rather than exponentially, the evidence that emulsification is a natural process brought about by extracellular agents is indirect and there are certain

conceptual difficulties in understanding how emulsification can provide an (evolutionary) advantage for the micro-organism producing the emulsifiers.

Another physiological role of biosurfactants is their antimicrobial activities towards various micro-organisms. As a rule, different surfactant inhibits different taxonomy. In addition biosurfactants have been shown to be involved in cell adherence which imparts greatest stability under hostile environmental conditions and virulence and in cell desorption when organisms need to find new habitats for survival (Desai and Banat, 1997).

The genetics of the producer organisms is an important factor affecting the yield of all biotechnological products because the capacity to produce a metabolite is bestowed by the genes of the organism. The bioindustrial production process is often dependent on the use of hyper-producing microbial strains even though cheap raw materials, optimized medium and culture conditions and efficient recovery processes have been employed. A product economy suggests that a production process cannot be made commercially viable and profitable until the yield of the final product by the producer organisms is naturally high (Muthusamy *et al.,* 2008).

2.5 Factors affecting Biosurfactant Production

The composition and emulsifying activity of the biosurfactant not only depends on the producer strain but also on the culture conditions, thus, the nature of the carbon source, the nitrogen source as well as the C:N ratio, nutritional limitations, chemical and physical parameters such as temperature, aeration, divalent cations and pH influence not only the amount of biosurfactant produced but also the type of polymer produced (Salihu *et al.,* 2009).

2.5.1 Carbon sources

A number of carbon sources have been used in many researches for biosurfactant production. These sources are divided into three categories of carbohydrate, hydrocarbon and vegetable oil which all are used in bacterial culture and biosurfactant production. *Pseudomonas* sp. mostly use glycerol, glucose, mannitol and ethanol as water-soluble carbon sources for production of rhamnolipids. The quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate (Rahman and Gakpe, 2008). Diesel, crude oil, glucose, sucrose, glycerol have been reported to be a good source of carbon substrate for biosurfactant production (Desai and Banat, 1997). It is evident that the importance of carbon substrates play a major role in biosurfactant synthesis. However, its importance is organism dependent as during a production with *Pseudomonas* sp, different carbon sources in the medium affected the composition of the biosurfactant production but substrate with different chain lengths exhibited no effect on the chain length of fatty acid moieties in glycolipids (Desai and Banat, 1997).

The use of different carbon sources alters the structure of the biosurfactant produced and its properties and can be exploited to get products with desired properties for particular applications. Different microbes respond differently to the carbon sources. The soy molasses, a byproduct from the production of soybean oil, plus oleic acid were tested as carbon sources for the production of sophorolipids by the yeast *C. bombicola* (Solaima *et al.,* 2004). The purified SLs were obtained at 21 g l−1 and were 97% in lactone form. Surface tension reduction was better when glucose, sucrose, tri sodium citrate, sodium pyruvate, yeast extract, and beef extract were used as carbon sources.

2.5.2 Nitrogen sources

Nitrogen is important in the biosurfactant production medium because it is essential for microbial growth as protein and enzyme syntheses depend on it. Different nitrogen compounds have been used for the production of biosurfactants such as urea peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extracts. Though yeast extract is the most used nitrogen source for biosurfactant production, its usage with respect to concentration is organism and culture medium dependent. Ammonium salts and urea are preferred nitrogen sources for biosurfactant production by *Arthrobacterparaffineus* whereas nitrate supports maximum surfactant production in *P. aeruginosa* (Adamczak and Bednarski, 2000).

The composition and characteristics of biosurfactants are influenced by the nature of the nitrogen source. Nitrogen is important in the biosurfactant production medium because it is an essential component of the proteins that are essential for the growth of microbes and for production of enzymes for the fermentation process. Several sources of nitrogen have been used for the production of biosurfactants, such as urea, peptone, ammonium sulphate, ammonium nitrate (Thanosub *et al.,* 2004), sodium nitrate (Bednarski *et al.,* 2004), meat extract and malt extract (Matasandoval *et al.,* 2001).

Maneerat (2005) reported that the production of surface active compounds often occurs when the nitrogen source is depleted in the culture medium, during the stationary phase of cell growth. In this nitrogen limiting condition, it causes the decline of the specific activities of NAD and NADP dependent isocitrate dehydrogenase which catalyses the oxidation of isocitrate to 2-oxoglutarate in the citric acid cycle. With the reduction of the

activity of this enzyme, isocitrate accumulates leading to the accumulation of citrate in the mitochondria. Citrate and isocitrate are then transported to the cytosol where the citrate is cleared by citrate synthase forming acetyl-coA which is the processor of fatty acid synthesis and therefore biosurfactant production increases.

2.5.3 Salt concentration

The salt concentration of a particular medium also had a corresponding effect on the biosurfactant production as the cellular activities of microorganisms are affected by salt concentration. Nevertheless, contrary observations were noticed for some biosurfactant products which were not affected by concentrations up to 10% (weight/volume) although slight reductions in the CMC were detected (Desai and Banat 1997).

2.5.4 Metal ion concentration

Metal ions concentrations play a very important role in the production of some biosurfactants as they form important cofactors of many enzymes. The overproduction of surfactin biosurfactant occurs in presence of Fe^{2+} in mineral salt medium. The properties of surfactin are modified in the presence of inorganic cations such as overproduction (Thimon *et al.*, 1992).

2.5.5 Environmental factors

These are extremely important in the yield and characteristics of the biosurfactant produced. To obtain large quantities of biosurfactants, it is always necessary to optimize the bioprocess as the product may be affected by changes in temperature, pH, aeration or agitation speed.

2.5.5.1 Temperature

Most biosurfactant productions are reported to be performed in a temperature range of 25 - 300C, however, in *A. paraffineus* and *Pseudomonas* sp. strain DSM-2874, this temperature range caused alteration in the composition of biosurfactant produced (Desai and Banat, 1997).

2.5.5.2 pH

The effect of pH on biosurfactant produced was studied by Zinjarde and Pant (2002) who reported that the best production occurred when the pH was 8.0 which is the natural pH of sea water which is *Y. lipolytica* natural habitat. With *Pseudomonas* sp, rhamnolipids production was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7.

The effect of pH in the biosurfactant production by several microorganisms have been investigated by many researchers. *P. aeruginosa* has been investigated using phosphate buffer with pH values varying from 4 to 8. All conditions used resulted in a reduction of biosurfactant yield when compared to distilled water (Desai and Banat, 1997). Thimon *et al.* (1992) studied the influence of initial pH in the production of a biosurfactant by *Y. lipolytica*. The best production of biosurfactant occurs when the pH was 8.0, which is the natural pH of sea water. The acidity of the production medium was the parameter studied in the synthesis of glycolipids by *C. antarctica* and *C. apicola*. When pH is maintained at 5.5, the production of glycolipids reaches a maximum. The synthesis of the biosurfactant decreased without the pH control indicating the importance of maintaining it throughout the fermentation process (Bednarski *et al.,* 2004).

2.5.5.3 Aeration and agitation

Aeration and Agitation are important factors that influence the production of biosurfactants as both facilitate the oxygen transfer from the gas phase to the aqueous phase. It may also be linked to the physiological function of microbial emulsifier, it has been suggested that the production of bioemulsifiers can enhance the solubilization of water insoluble substrates and consequently facilitate nutrient transport to microorganisms. While evaluating the influence of aeration in the biosynthesis of a surfactant by *Candida antartica*, Adamczak and Bednarski (2000) observed that the best production value of the surfactant (45.5g/l) was obtained when the air flow rate was 1vvm and the dissolved oxygen concentration was maintained at 50% of saturation. However, changing the air flow rate to 2vvm, there was a high foam formation and the biosurfactant production decreased by 84%, the formation of foam during a bioprocess is undesirable as it causes the removal of biosurfactant, some biomass and lipids from the active medium.

2.5.6 Factors indicating the production of biosurfactant by bacteria

The reduction of surface tension by the test bacteria in Shea butter and Palm oil is indicative of the production of biosurfactant by the microbe. The higher reduction of surface tension indicates a high concentration of the biosurfactant available (Batista *et al.*, 2006; Ade *et al.*, 2012).

Reduction in surface tension of an oil source (such as Shea butter and Palm oil) indicates rapid hydrolysis reaction to produce biosurfactant through the release of fatty acids and glycerol (Ade *et al.,* 2012). In addition, Tulena *et al,* (2009), reported that one of the most important characteristics of the biosurfactant was the ability of a microorganism to reduce

surface tension and interfacial tension between oil and water which can be measured by the method of du-Nouy ring classic. Kaczorek *et al*. (2005) in their research work, reported that the values of surface tension on the characterization of biosurfactant depend on type and concentration of emulsifier, bioemulsifier, quantity and types of strain of bacteria used and the complexity of the oil sources used.

Emulsification properties of the surfactant was measured in terms of emulsification activity (EA) (Ade *et al.*, 2012; Thivaharan and Vytla, 2013). EA is the ability of a surfactant to form emulsions under certain conditions and is directly related to the oil droplet size, the smaller of the size was the greater of the activity (Soniyamby, *et al.*, 2011; Ade *et al.*, 2012).

The ability of organisms to form emulsion on the biosurfactant production process is influenced by many factors, as suggested (Thivaharan and Vytla, 2013). Factors that affect the ability of bacteria in emulsification process consists of: (1). Factors that affect the bacterial cell surface such as: (a) metabolic state of the cell, (b) Species-specific genetic potential for cell wall biochemistry (hydrophobic, hydrophilic), (c) availability of C, N, 0; Substrate form (e.g. sugars and alkanes), (d) Physical organization of cell wall biochemistry (2) Factors of non-biological origin such as organic phase composition, aqueous phase composition (salts and pH), nature of emulsion-stabilizing surfactants, presence of fine particulates (e.g. clays) and temperature (Thivaharan and Vytla, 2013).

2.6 Biosurfactant Production using Substrates of Vegetable Processing Industries

Mercade *et al.* (1993) were the first group to show the production of rhamnolipids by *P. aeruginosa* 47T2 when grown on olive oil mill effluent (OOME) as the sole carbon source

(a major waste problem in Spain). This study was important in demonstrating the possibility of using other lipophillic wastes for wider application. Kitamoto *et al.* (1993) studied the interfacial and antimicrobial properties of two kinds of mannosylerythritol lipids (MEL-A and B) biosurfactants, produced by *Candida antarctica*T-34, when grown on soybean oil as substrate. Since the biosurfactant produced in this study exhibited antimicrobial activity particularly against Gram-positive bacteria, the process could be more economical because of high value application in pharmaceutical industry.

Sim *et al*. (1997) have tested mixture of vegetable oils (canola oil, soy bean and glucose), for rhamnolipid production by *P. aeruginosa* UW-1 and reported 10-12 fold increase in rhamnolipid production on vegetable oils in comparison to glucose. Vollbrecht *et al.* (1999) found the capability of *Tsukamurella* sp. (DSM 44370) growing on sunflower oil rich in oleic acid to produce a mixture of oligosaccharide lipids. They observed approximately 30 g/l glycolipid was produced from 110 g/l sunflower oil. The biosurfactant produced exhibited high surface and interfacial activity and some antimicrobial activities against some bacteria and a fungal strain which can contribute to the economical appeal of the process.

Matasandoval *et al.* (1999) also reported production of a mixture of rhamnolipid from *Pseudomonas aeruginosa* UG2 cultures grown on corn oil as sole carbon. Camargo-de-Morais *et al.* (2004) studied the production of a glycolipid with emulsifier properties during cultivation of *Penicillium citrinum* on mineral medium with 1% olive oil as carbon source. The growth associated emulsifier production reached maximal activity at 60 hours of cultivation with the production yield (Yp/s) of 0.54. Emulsifier which was stable in a wide range of pH and temperature was stimulated by high salt concentration implying a

possible application in industrial waste or marine remediation. (Chang *et al.*, 2005) reported the production of biosurfactant by *Pseudoxanthomonas kaohsiungensis* sp. during cultivation on olive oil as the sole carbon and energy source.

Thaniyavarn *et al.* (2006) examined the biosurfactant production by *Pseudomonas aeruginosa* A41, a strain isolated from seawater in the gulf of Thailand, grown in defined medium containing 2% vegetable oil or fatty acid as a carbon source. Culture medium exhibited excellent surface activity on each carbon source tested and biosurfactant production was observed even after the stationary phase. The type of carbon source affected the biosurfactant yield with maximum yield (6.58 g/l) was obtained with olive oil in comparison to Palm oil (2.91 g/l) and coconut oil (2.93 g/l). Although, less yields was obtained with Palm oil, the produced biosurfactant showed better surface activity and oil displacement values. This study demonstrated the possibility of having a low cost-large scale production of the microbial biosurfactant using Palm oil as a cheap and abundantly available substrate. The biosurfactant produced had good surface activity and stability in wide ranges of pH, temperature and NaCl concentration, properties which allows possible uses and application in bioremediation and under wide range of conditions.

In another study, Thaniyavarn *et al*. (2008) showed the sophorolipid production by *Pichia anomala* PY1, a thermo tolerant strain isolated from fermented food, using 4% soybean oil as carbon source at pH 5.5 at 30°C for 7 days. They observed the surface tension of the medium which decreased to 28 mN/m with crude oil displacement of 69.43 cm^2.

Rufino *et al.(*2007) studied the cultivation of *Candida lipolytica* grown on ground nut oil for production of a new biosurfactant. The preliminary investigation of chemical

composition suggested it was a lipopeptide in nature. The biosurfactant had a yield of 4.5 g/l and exhibited good surface activity, emulsification ability and can withstand high salt concentration but was not thermo stable. They later also applied sequential factorial design to optimize biosurfactant production by *Candida lipolytica* using soybean oil refinery residue as substrate (Rufino *et al.*, 2008). In this study they evaluated the impact of three cultivation factors, amounts of refinery residue, glutamic acid and yeast extract. The biosurfactant product showed high surface activity and emulsifying ability and was very stable at wide range of pH (2-12), temperatures (0-120°C) and salinity (2-10% NaCl). They concluded that combination of an industrial waste and a cheap substrate is a promising approach to reduce production cost. In another study the same group (Sobrinho *et al*. 2008) described a low cost medium for the production of a surfactant by the yeast *Candida sphaerica*. The medium formulation consisted of distilled water containing 5.0% groundnut oil refinery residue plus 2.5% corn steep liquor as substrates. The biosurfactant product had high surface tension reducing activity (26 mN/m), a low CMC value (0.08%) and a yield of 4.5 gl^{-1}. The biosurfactant characterized was an anionic glycolipid (consisting of 75% lipid and 25% carbohydrate) and was stable at wide temperature, pH and salt level. They concluded that it was possible to produce biosurfactants from agricultural materials and use them in potential application in oil recovery from sand. Coimbra *et al.* (2009) also showed the biosurfactant production by six *Candida* strains cultivated in insoluble (n-hexadecane) and soluble substrates (soybean oil, ground-nut oil refinery residue, corn steep liquor and glucose). These biosurfactant were able to remove 90% of the hydrophobic contaminants from sand.

Oliveira *et al*. (2009) used Palm oil, a low-cost agricultural byproduct which is used in as raw material for soap and food industries, for biosurfactant production using *Pseudomonas alcaligenes* (a strain isolated from crude oil contaminated soil). They achieved a biosurfactants concentration of 2.3 g/l and E_{24} more than 70% with the hexane, jet fuel and crude-oil. Abouseoud *et al.* (2008) studied the production of a biosurfactant by *Pseudomonas fluorescens* Migula 1895-DSMZ and reported highest yield of rhamnolipid biosurfactant with olive oil and ammonium nitrate as carbon and nitrogen sources at a C: N ratio of 10. The biosurfactant exhibited good surface activity, emulsifying ability and stability at high temperature, salt concentration and wide range of pH in addition to some antimicrobial activity which gives it additional favorable application related properties.

Pornsunthorntawee *et al.* (2008) used *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4, for biosurfactant production using a nutrient broth with Palm oil as the carbon source. The optimum growth for the organisms was achieved in approximately 48 h and biosurfactant produced exhibited good surface activity. Comparative study of these biosurfactants with three synthetic surfactants showed better oil recovery efficiency by both biosurfactants. Abdel-Mawgoud *et al.* (2009) reported the production of a rhamnolipid by *Pseudomonas aeruginosa* isolate Bs20 on soybean oil amended medium. The rhamnolipid produced was s viscous sticky oily yellowish brown liquid with a fruity odour with a very high surface activity, emulsifying capacity and thermo and halo tolerance properties. These characteristics indicated that rhamnolipids were potential candidate for use in bioremediation of hydrocarbon-contaminated sites or in the petroleum industry a conclusion that was also confirmed by Perfumo *et al.* (2010). Monteiro *et al.* (2009) reported the growth and biosurfactant production using sunflower oil supplemented

mineral medium by the yeast *Trichosporon montevideense*, CLOA 72. The glycolipid produced exhibited good surface and emulsifying activity with vegetable oils, toluene, kerosene, isooctane, cyclohexane, hexane, diesel oil and hexadecane. The biosurfactant was thermo tolerant, halo tolerant and stable in wide range of pH values.

2.7 Biosurfactant Coproduction with Renewable Substrate

An interesting approach for achieving more fruitful results is co-production of biosurfactants and other important metabolites. There are reports of such coproduced metabolites e.g. polyhydroxyalkanotes (PHA), lactic acid and other metabolites.

2.7.1 Biosurfactants and PHA

Pseudomonas aeruginosa produces rhamnolipids and PHA. PHA have been applied for manufacture of bottles, films and fibers as a biodegradable packaging agent (Sudesh *et al*. 2000). Fuchtenbusch *et al*. (2000) attempted the co-production approach for PHA production using the remaining oil from rhamnolipid production. During screening for the bacteria which can use residual oil from biotechnological rhamnose production as a carbon source for growth, they identified *Ralstonia eutropha* H16 and *Pseudomonas oleovorans* as capable of using this waste material as the sole carbon source for growth and production of PHA. The approach adopted, reduced the PHA production cost by using the remaining carbon source for rhamnolipid production.

Hori *et al.* (2002) demonstrated the feasibility of the simultaneous production of PHAs and rhamnolipids, as a novel approach to reduce their production costs, by the cultivation of *Pseudomonas aeruginosa* IFO3924. Large yields of PHAs and rhamnolipids were obtained

using decanoate as a better carbon source than ethanol and glucose for the simultaneous production. Costa *et al.* (2009) evaluated glycerol, cassava wastewater, waste cooking oil and cassava waste with waste frying oils as alternative low-cost carbon substrates for the production of rhamnolipids and PHA by various *Pseudomonas aeruginosa* strains. Cassava waste with frying oil was best substrate for the overall production of rhamnolipids and PHAs. The study demonstrated the feasibility of use of cassava waste with frying oil as an alternate and economical substrate for dual production of biosurfactants and PHAs.

In another study by Marsudi *et al.* (2008) demonstrated Palm oil can be directly utilized for the simultaneous production of polyhydroxyalkanoates (PHAs) and rhamnolipids using *Pseudomonas aeruginosa* IFO3924. Using secreted lipase for Palm oil hydrolysis they showed consumption of fatty acids as carbon sources for PHAs production and glycerol for rhamnolipid production. Both PHA and rhamnolipid production was nitrogen dependent and occurred in nitrogen limited conditions. In their recent work (Hori *et al*. 2011) investigated the effects of temperature and carbon length of fatty acid substrates on the simultaneous production of PHA and rhamnolipids by *P. aeruginosa* IFO3924. Differential temperature optimum obtained for PHA (30°C) and rhamnolipid syntheses (28°C) suggests that the product ratio between these two products can be controlled by changing temperature. This approach for simultaneous production for rhamnolipid and PHAs by using whole or part of the available substrates makes the process economically viable and attractive.

2.7.2 Biosurfactants and proteases

Microbial proteases especially alkaline proteases are an important groups of industrial enzymes that cater to the requirement of nearly 60% of the world enzyme market (Gupta *et al.* 2002). These proteases have numerous industrial applications involving detergents, food, leather, silk, waste management and pharmaceuticals (Gupta *et al.* 2002). However, the single biggest market of their use is in the detergent formulations. Most of these enzyme productions are confined to genus *Bacillus* which is known to be a common biosurfactant producer (Kim *et al.*. 1997; Makkar and Cameotra, 1998; Makkar and Cameotra, 1999). With many complementary properties such as excellent detergency, emulsifying, foaming and dispersing traits to the proteases a concomitant usage of biosurfactant and protease could offer improved efficacy as detergent additives.

Ramnani *et al.* (2005) showed the concomitant production of protease and biosurfactant using cornstarch and soy flour as carbon and nitrogen sources. They achieved an overall 2.3-fold increase for both protease (2954 U/ml) and biosurfactant (41%). They used ultrafiltration (100 kDa cutoff) as a cost-effective purification of both protease and biosurfactant where the surfactant traps the protease on the membrane thereby detaining both in the retenate. These studies signify the ecomomicity of concomitant production of biosurfactant with other metabolite thus easing off the cost factor for the overall biosurfactant production.

2.8 Economics of Biosurfactant Production

Economical large scale production for established and new applications of biosurfactants remains a challenge (Bognolo, 1999). The biosurfactant surfactin (98% purity) available

from Sigma Chemical Company costs approximately $153 for a 10 mg vial. An estimate by Ron and Rosenberg (1997) for the cost of the RAG-1 emulsan containing broth was 50 dollar/kg and would therefore cost much more to extract, concentrate or purify the product. It is important to note however that their higher potency makes them better than commercial surfactant.

Researchers have emphasized the key parameters affecting the efficiency of biosurfactant production in terms of higher yields and lower production costs (Bognolo, 1999; Kosaric, 1992; Mukherjee *et al.,* 2006). According to them the biosurfactant formation and accumulation follows the basic facts of metabolic process and need to be studied comprehensively.

According to (Syldatk and Hausmann, 2010) the reasons for limited use of microbial surfactants in industry are the use of expensive substrates, limited product concentrations, low yields and formation of product mixtures rather than pure compounds. All these factors and other growth and upscale problems like use of antifoaming agents add on to the high costs of the downstream processing.

The main strategy to achieve this are through (i) assessment of the substrate and product output with focus on appropriate organism, nutritional balance and the use of cheap or waste substrates to lower the initial raw material costs involved in the process; (ii) development of efficient bioprocesses, including optimization of the culture conditions and cost-effective separation processes to maximize recovery; and (iii) development and use of overproducing mutant or recombinant strains for enhanced yields (Kosaric, 1992; Bognolo, 1999; Mukherjee *et al.,* 2006).

The use of the alternative substrates such as agro based industrial wastes is one of the attractive strategies for economical biosurfactants production. Kosaric, (1992) suggested the use of industrial and/or municipal waste waters, rich in organic pollutants, to achieve a double benefit of reducing the pollutants while producing useful products.

Another approach for reducing the production costs is developing processes which use renewable low cost raw materials or high-pollutant wastes. A wide variety of alternative raw materials are currently available as nutrients for industrial fermentations, namely various agricultural and industrial byproducts and waste materials (Makkar and Cameotra, 2002; Savarino *et al.*, 2007; Ferreira, 2008; Montoneri *et al.*, 2009a).

Thus the promising future of biosurfactants appears to specifically depend upon the use of abundant and low-cost raw materials and the optimization of the operational cultivation conditions in order to achieve high yields. The development of low-cost processes and raw material can account for 10-30% of the final product cost. Further optimization of culture medium and growth conditions can significantly increase the yield (Cameotra and Makkar, 1998; Mukherjee *et al.,* 2006; Mukherjee *et al.,* 2008; Mutalik *et al.,* 2008). In addition the positive economic outlook can be enhanced by increasing their high throughput values or by harnessing other important properties such as pharmacological, antifungal and antiviral capabilities. Several biosurfactants have recently been used or anticipated to use in cost effective applications in medicine, food and cosmetic industries (Cameotra and Makkar, 2004; Rodrigues *et al.,* 2006a; Singh *et al.,* 2007; Lourith and Kanlayavattanakul, 2009; Mulligan, 2009)

Most biotechnological products processes need high monetary inputs and securing an optimum yield of product at the lowest expense through usage of low cost material (Cazetta *etal*., 2005). However, very low quantities of surface active agents are usually produced by microorganisms and the downstream processing of biotechnological products costs ~60–80% of the total production expenditure. This is why most of the marketable products based on BS and BE are quite expensive. Therefore, it essential to reduce the production costs of BS/BE through the use of inexpensive and renewable substrates (Desai and Banat, 1997; Banat *etal*., 2000; Makkar *etal*., 2011). A diversity of carbon (water soluble and water insoluble) and nitrogen sources have been used for BS production which may consequently vary in structure or production site within the cell (intra or extra cellular, cell associated) depending upon the substrate composition particularly the carbon source (Guerra-Santos *etal*., 1986; Fiechter, 1992). In addition to usual water soluble carbon sources, a variety of unusual carbon sources such as ethanol, blended gasoline, hydrocarbons like heptadecane, hexadecane etc. have been used (Shreve *etal*., 1995; Patricia and Jean-Claude, 1999; Prabhu and Phale, 2003; Cunha *etal*., 2004).

Increased public awareness of issues related to environmental pollution strongly influences the development of technologies that facilitates cleaning hazardous contaminants. This has given imputes for finding suitable cheap BS products that can be used in the treatment of such contaminations. Kapadia and Yagnik (2013) introduced an alternative approach using solid state fermentation to obtain a more economical viable production process worth implementing on a commercial scale. Some of the suggested strategies included the use of more cheaper materials, optimization of environmental conditions and screening for overproducing strain to attain the maximize productivity (Satpute *etal*., 2010). The efforts

taken toward this direction are significant to claim Biosurfactants as the molecules of the future. It is important to note that the results obtained to date show encouraging potentials to drive a beginning for the Biosurfactant production industry.

2.9 Shea Butter

Shea butter a fat extracted from the kernels of *Vitellaria paradoxa*), also known as *Butyrospermum parkii*. The species is found across 19 countries across the African savanna zone from Senegal to Ethiopia. Shea butter contains high levels of UV-B absorbing triterpene esters, including cinnamic acid, tocopherols (vitamin A), and phytosterols. Shea butter does contain a high percentage of unsaponifiables, such as phytosterols (campesterol, stigmasterol, beta-sitosterol, and alpha-spinosterol) and triterpenes (cinnamic acid esters, alpha- and beta-amyrin, parkeol, buytospermol, and lupeol), and hydrocarbons such as karitene (Okullo *et al.,* 2010). Shea butter is not a recent discovery or accomplishment. This all-natural product has been in existence for centuries.

Shea butter tree is an indigenous fruit tree distributed in the Shea parklands of Africa. Olaniyan and Oje (2007) described the Shea fruit as a green epicarp, a fleshy mesocarp (pulp) and a relatively hard shell (endocarp) which encloses the Shea kernel (embryo). The Shea fruit is an important source of food for rural communities especially at time of food shortages, hunger and other disasters in addition to providing enormous health benefit (vitamins A and C, etc.) and income (Okullo *et al.,* 2010). The Shea nuts can contain from 20% up to 50% edible fat. The Shea fruit according to Maranz *et al.* (2004) produces more solid Shea butter as compared to other oil bearing fruits as it contains more stearic acid. The kernel, according to *Axtell et al.* (1993) contains about 60% edible fat (Shea butter)

and the residual product from which the butter is extracted (Shea cake), is an excellent ingredient for livestock feed production. The fat obtained from the Shea kernel is referred to as Shea butter and it is the most valued product from the Shea tree (Hall *et al.,* 1996).

2.9.1 Composition of Shea butter

Native Shea butter consists mainly of triglycerides and a large fraction of unsaponifiable materials. The fatty acid composition of the triglycerides is dominated by oleic, stearic and linoleic acids. The unsaponifiable fraction contains high amounts of cinnamic acid esters of triterpene alcohols but also a smaller fraction of sterols. The most characteristic triterpene alcohol of Shea butter is butyrospermol but other important constituents are lupeol as well as alpha- and beta-amyrin. Shea butter also contains tocopherols (Vitamin E) functioning as antioxidants.

2.9.1.1 Free fatty acids

Shea butter is composed of five principal fatty acids: Palmitic, stearic, oleic, linoleic, and arachidic (Maranz *et al.,* 2004). The fatty acid composition is dominated by stearic and oleic acids, which together account for 85-90% of the fatty acids.

The relative proportions of these two fatty acids produces differences in Shea butter consistency. The high stearic acid content gives the Shea butter its solid consistency, while the percentage of oleic acid influences how soft or hard the Shea butter is.

The proportions of stearic and oleic acids in the Shea kernels and butter differ across the distribution range of the species. Ugandan Shea butter has consistently high oleic acid content, and is liquid at warm ambient temperatures. Ugandan Shea butter fractionizes into

liquid and solid phases, and is the source of liquid Shea oil. The fatty acid proportion of West African Shea butter is much more variable than Ugandan Shea butter; the oleic content ranges from 37 to 55%. Variability can even be high in relatively small local populations; a tree that produces hard butter can be located right next to one that produces soft butter.

2.9.1.2 Phenolics

Phenolic compounds are known to have antioxidant properties. A recent study characterized and quantified the most important phenolic compounds in Shea butter (Maranz *et al.*, 2004). This study identified 10 phenolic compounds in Shea butter, eight of which are catechins, a family of linoleic acids. The unsaponifiable fraction contains high amounts of cinnamic acid esters of triterpene alcohols but also a smaller fraction of sterols. The most characteristic triterpene alcohol of Shea butter is butyrospermol but other important constituents are lupeol as well as alpha- and beta-amyrin. Shea butter also contains tocopherols (Vitamin E) functioning as antioxidants.

2.10 Palm Oil

Cultivation of the oil Palm (*E. guineensis*) hasexpanded tremendously in recent years such that it isnow second only to soybean as a major source of theworld supply of oils and fats. From its home in WestAfrica, the oil Palm (*E. guineensis*) has spreadthroughout the tropics and is now grown in 16 or morecountries (Basri *et al.*, 2003). However, the major centreof production is in South East Asia (SEA) with Malaysiaand Indonesia together accounting for around 83% ofworld Palm oil production in 2001 (Oviasogie *et al.*, 2013). In spite of the huge production, the oil consists of only about 10% of the total biomass produced in

the plantation. The remainder consists of huge amount of oil Palm wastes (biomass) such as oil Palm shells, mesocarp fibers, empty fruit bunch (from the mills), oil Palm fronds and oil Palm trunk (Oviasogie *et al.*, 2013).

Palm oil is one of the two most important vegetable oils in the world's oil and fats market. The extraction and pu rification processes generate different kinds of waste generally known as Palm oil mill effluent (POME). The environmental impact of POME cannot be over emphasized; hence the need for treatment measures to reduce these impacts before discharge (Igwe *et al.*, 2007)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

3.1.1 Sampling area

Soil samples were collected from specific oil contaminated sites around automobile workshops in Zaria and waste effluents channels in Kaduna Refinery Petrochemical Company (KRPC), Kaduna. These sites have been exposed to petrochemical pollution and since the nature of an environment determines the type of organisms that are present in it, we had more chances of isolating hydrocarbon and biosurfactant producing bacteria from these sites.

Samples of Palm oil and Shea butter waste slurries were obtained from their production sites in some *Pampaida* (UNAID assisted villages) in Makarfi LGA, Kaduna state where Palm oil and Shea butter are made in commercial quantities.

3.1.2 Sampling

A total of 12 samples each were collected in sterile containers from both sites and taken to the laboratory for isolation of biosurfactants producing bacteria. The temperature and pH of the soil samples were recorded during sample collection.

A total of 2 samples of 10litres of both the Shea butter and Palm oil waste slurries were also collected in sterile containers and aseptically transported to the laboratory.

3.2 Isolation and Identification of *Bacillus* and *Pseudomonas* Species from Soil Samples

3.2.1 Isolation of *Bacillus* and *Pseudomonas* species from soil samples

An electronic weighing balance was used to weigh 25g of the soil samples and transferred into a 500 Erlenmeyer flask containing 225 ml of sterile distilled water. The flask was shaken vigorously and allowed to stand for 30 minutes. One millilitre of this solution was transferred into 9 mls of sterile distilled water in a test tube using a sterile pipette to obtain a tenfold dilution. Using sterile 1ml pipettes, 0.1ml of prepared dilution from 10^{-3}-10^{-6} was aseptically transferred onto the surface of solidified Mannitol Egg Yolk Polymyxin (MYP) Agar and Cetrimide Agar for the isolation of *Bacillus* and *Pseudomonas* species respectively and spread well using a sterile bent glass rod. Inoculated plates were incubated at 37^{o}C and observed for bacterial growth after 18-48hours incubation (Abouseoud *et al.*, 2007).

Different colonies observed on plates were purified by repeated streaking of each distinct colony on nutrient agar until pure colonies are obtained. Purified bacterial isolates were transferred on sterile nutrient agar slants and stored at 4^{o}C for further identification (Abouseoud *et al.*, 2007).

3.2.2 Characterization and identification of the bacterial isolates

Isolates were identified using various staining and biochemical reactions such as Gram staining (using the primary and secondary dyes crystal violet and fuchsine), motility,

oxidase and catalase tests. Microgen biochemical test-kits were used according to manufacturer's instruction to identify the isolates to species level.

3.3 Preliminary Screening for Biosurfactant Production by the Bacteria Isolates

The purified isolates identified were screened for their ability to produce biosurfactant using the following methods as described by Abouseoud *et al.,* (2007). Oil spreading, Emulsification Index (E_{24}) and Blood Haemolysis were carried out as described in the steps following.

3.3.1 Inoculum preparation and culture

The cultures were grown in nutrient broth for 16–18 h at room temperature. This was then serially diluted from 10^{-1}-10^{-6} and the 10^{-3} dilution was used as inoculum at 2% (v/v) level. For biosurfactant synthesis, a mineral salt medium with the following composition(g/l) was utilized: Na_2HPO_4 (2.2), $KH_2\ PO_4$ (1.4), Mg SO_4 . $7H_2O$ (0.6), Fe SO_4 .$7H_2O$ (0.01), NaCl (0.05), Ca Cl_2 (0.02), yeast extract (0.02) and 0.1 ml of trace element solution containing (g/l) :2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. The pH of the medium was adjusted to 7.0 ± 0.2. Carbon and nitrogen sources were added separately. Cultivations were performed in 250 ml flasks containing 50 ml medium at room temperature, and stirred on a TAITEC NR-20 rotary shaker at 150 rpm for three days and supernatants were used for further analysis (Abouseoud *et al.,* 2007).

3.3.2 Oil spreading technique

This was carried out following the methods described by Hasham *et al.* (2012). Exactly 20 ml of distilled water was dispensed in petriplates to which 1 ml of vegetable oil (Grand pure soya oil manufactured by UAC foods Nigeria and bought from Samaru market, Zaria) was dispensed in the center of the plates containing the distilled water. This was followed by 20 µl of the supernatant of the culture of the bacteria *Pseudomonas aeruginosa* and *Bacillus cereus*. Biosurfactants present in the bacterial supernatant, displaced the oil and spread in the water, after which zone formation (mm) was measured using a centimeter rule. The strains that lacked the ability to produce biosurfactants, showed negative to oil spread by giving no zone of clearance.

3.3.3 Blood haemolysis test

Pure isolates of the organisms were streaked on sterile blood agar plates and the plates were incubated at 37^{o}C for 48-72 hours, after which individual bacterial colonies was observed for the presence of clear zone around them. An area of clear zone around the colonies indicated the presence of biosurfactant producing organisms (Yonebayashi *et al.*, 2000; El-Shahawy, 2014).

3.3.4 Emulsification activity (E_{24}) on various hydrocarbons

Methods described by Cooper and Goldenberg, 1987 was used and 2 ml of various hydrocarbons (Petrol, Diesel, Kerosene and Vegetable oil) were added to the same amount of cell free supernatant and vortexed for 2 minutes, the emulsion stability was determined

after 24 hours and the E_{24} index was calculated as the percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm).

$$E_{24} = \frac{\text{height of emulsified layer (mm)}}{\text{total height of the liquid column (mm)}} \times 100$$

3.4 Preliminary Screening for Biosurfactant Production and Activity on Shea Butter and Palm Oil Wastes Slurries

The bacterial isolates which showed positive test to biosurfactant production were further tested for their activity in Shea butter and Palm oil waste slurries as sources of carbon. The standard tests used were Absorbance (optical density), emulsification stability test, oil spreading and surface tension as described by Hasham *et al.,* (2012). A mineral salt medium as described in 3.3.1 above was used for the culturing of the organisms. The Shea butter wastes and Palm oil waste slurries respectively were added (2ml) to every flask containing the mineral salt medium (96 mls) and autoclaved at 121^{o}C for 15mins. This formed the growth medium to which 2mls of the 10^{-3} dilutions of each of the bacteria inocula (*Pseudomonas* sp. and *Bacillus* sp.*)* were added and placed on a TAITEC NR-20 rotatory shaker at 150rpm at room temperature for 5days.

3.5 Optimization Studies for Physical and Nutrient Parameters Affecting Biosurfactant Production Activities

Optimization of physical (pH) and nutrient (nitrate and lipase) parameters using Shea butter and Palm oil wastes slurries as carbon and energy source was done for The

measurement parameters taken for each optimization were optical density, emulsification stability test and surface tension as described in details below (Hasham *et al.,* 2012).

3.5.1 Bacterial cultures used for optimization studies

Purified *Pseudomonas aeruginosa* and *Bacillus cereus* which showed positive result to the preliminary studies carried out above were selected and used for the subsequent experiments below.

3.5.2 Media used

3.5.2.1 Mineral salt medium (MSM)

The mineral salt medium used for this research had the following composition (g/l): Na_2HPO_4 (2.2), $KH_2\ PO_4$ (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) and 0.1ml of trace element solution containing (g/1): 2.32g $ZnSO_4 \cdot 7H_2O$, 1.78g $MnSO_4 \cdot 4H_2O$, 0.56g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39g $Na_2MoO_4 \cdot 2H_2O$, 0.42g $CoCl_2 \cdot 6H_2O$, 1.0g EDTA, 0.004g $NiCl_2 \cdot 6H_2O$ and 0.66g KI. pH of the medium was adjusted to 7.0 ± 0.2 (Bodour *et al.,* 2003).

3.5.2.2 Nutrient broth

Oxoid supplemented with 0.1% yeast extract (Beal and Betts, 2000). This was used for sub culturing the test bacterial isolates and preparation of bacterial inoculum for this study.

3.5.3 Substrates used for optimization

Raw Shea butter and Palm oil wastes slurries autoclaved at 121^{o}C were used as sources of carbon and energy in biosurfactant production.

3.5.4 Growth medium for optimization

The Shea butter and Palm oil waste slurries were added (2mls) to every flask containing the mineral salt medium (96 mls) and autoclaved at 121^{o}C for 15 minutes. This formed the growth medium that was used for the subsequent steps of this research work (Abouseoud *et al*., 2007).

Dilution 10^{-3} of the inoculum of the single and consortium of *Bacillus cereus* and *Pseudomonas aeruginosa* at 2% (v/v) level were inoculated into the growth medium after sterilization and optimization parameters (pH, nitrogen, Lipase) have been introduced into the growth medium (Bodour *et al.,* 2003).

3.6 Measurement Parameters

3.6.1 Measurement of optical density

Optical density measurement was carried out daily at 590nm from Day 1 through Day 7 using a spectrophotometer to observe the growth of the organism. All measurements were made on culture media obtained aseptically from the batch cultures.

3.6.2 Emulsification stability test (E_{24})

Emulsification activity was measured using the method described by Cooper and Goldenberg, (1987). Two millilitres of kerosene oil was added to the same amount of cell free supernatant and vortexed at for 2 minutes. The emulsion stability was determined after 24 h and the E_{24} index was calculated as the percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm).

$$E_{24} = \frac{\text{height of emulsified layer (mm)}}{\text{total height of the liquid column (mm)}} \times 100$$

3.6.3 Surface tension test

Surface tension was measured using capillary tube method according to Martin (1993) and applying the formula:

$$T = f \times r \times g \times h$$

Where: T = Surface tension, f = Density of the fluid (1000kg/m^3), r = Radius of capillary tube in meter, g = Acceleration gravity (9.81m), h = Height (m) of fluid in the capillary tube. Surface tension of the growth medium was measured daily during all the optimization studies carried out.

3.7 Optimization for Biosurfactant Production with Different pH Values

Four pH values (5, 6, 7 and 8) were used by adjusting the growth medium (MSM containing the Shea butter and palm oil waste slurries) contained in 250ml Erlenmeyer flasks, using acid in the form of 1M HCL or Base in the form of 2M NAOH (Hasham *et al.,* 2012). Triplicates for each flask were made and average reading for each concentration taken. Controls without the bacteria were demonstrated. Sterilization of the growth medium was done after addition of the acid or base in standard medium after which dilution 10^{-3} of the inocula at 2% v/v was added. The measurement parameters (Absorbance, E_{24} and surface Tension) for each pH levels were taken daily for 5 to 7 days on incubation on a TAITEC NR-20 rotatory shaker at 120rpm.

3.8 Optimization for Biosurfactant Production with Nitrogen Source

Four different concentrations of Nitrogen (0.1mg/100ml, 0.2mg/100ml, 0.3mg/100ml and 0.4mg/100ml) in the form of Sodium Nitrate ($NaNO_3$) were added in four different 250ml Erlenmeyer flasks containing 100mls of the growth medium. Triplicates for each flask were made and average reading for each concentration taken. Controls containing only Nitrogen without the bacteria was also demonstrated. Sterilization of the growth medium was done after addition of Sodium Nitrate ($NaNO_3$) after which the 10^{-3} dilution of the inoculum at 2% (v/v) was introduced (Abouseud *et al*., 2007). The measurement parameters (Absorbance, E_{24} and surface Tension) for each concentration were taken daily for 5 to 7 days while incubating on a TAITEC NR-20 rotatory shaker at 120 rpm.

3.9 Optimization for Biosurfactant Production with Lipase

Four different concentrations of Lipase enzyme NESS Formula 5® (0.05mg/100ml, 0.10mg/100ml, 0.15mg/100ml and 0.2mg/100ml) were added to four different 250ml Erlenmeyer flasks containing 100ml of the growth medium. Duplicates for each flask were made and average reading for each concentration taken. Controls containing only Lipase without the bacteria was demonstrated. The Lipase was added after sterilization shortly before addition of the 10^{-3} dilution inoculum at 2% (v/v).The measurement parameters (Absorbance, E_{24} and surface Tension) for each concentration were taken daily for 5 to 7 days on incubation on a TAITEC NR-20 rotatory shaker (120rpm).

3.10 Statistical Analysis

The results obtained from this research were presented in grapH, tables and pictures. Results from the optimization studies and measurements of E_{24}, surface tension and optical density were interpreted using Analysis of Variance ANOVA to compare variables so as to draw conclusions and appropriate inferences.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and Characterization of the Bacterial Isolates

Characterization of the isolates from the environmental samples are presented in Tables 4.1 and 4.2. The isolates were identified as *Bacillus cereus, Bacillus licheniformis, Pseudomonas aeruginosa* and *Pseudomonas fluorescens.*

4.2 Screening for the Biosurfactant Activity of *Pseudomonas spp.* and *Bacillus spp.*

Results for the following tests are presented in Tables 4.3 to 4.5.

4.2.1 Oil Spreading and blood haemolysis screening of *Pseudomonas* spp. and *Bacillus* spp.

The results in Table 4.3 presents the zone formation (mm) of oil spreading (using vegetable oil) and blood haemolysis displayed by the various isolates of *Pseudomonas* and *Bacillus* species. The table revealed that isolates a, b and c of *Pseudomonas aeruginosa* have oil spread zone formation of 29, 30 and 28 millimetres (mm) respectively. Isolates a and b of *P. fluorescens* had 30mm and negative zones of formation respectively. All isolates of *B. licheniformis* showed negative while strains of *B. cereus* showed positive values of 25 mm and 30 mm respectively for zone formation on oil spreading test using vegetable oil.

Nine (9) isolates of the bacteria characterized as *Pseudomonas* and *Bacillus* species were further tested for their haemolytic activity (Table 4.3). Data obtained showed that four (4) of the isolates had the ability to give partial haemolysis (α- haemolysis), one out of the nine

isolates gave complete haemolysis (β- haemolysis) and four out of the nine isolates lacked the ability to give blood haemolysis (γ-haemolysis).

Table 4.4 shows that *P. aeruginosa* showed positive zone formation for all the hydrocarbon sources (kerosene, diesel, Premium Motor Spirit (Petrol), and Vegetable oil (Kings oil brand) *B. cereus* showed positive zone formation for kerosene, diesel and vegetable oil but was completely negative for Petrol. Vegetable oil gave the best and highest values of 30mm for zone formation.

4.2.2 Emulsification stability test (E_{24})

Results for Emulsification stability test are shown in Table 4.5. Five isolates of *Pseudomonas aeruginosa* and *Bacillus cereus* were tested, each of the species could emulsify three of the hydrocarbons used in the study. The best results were however observed in Kerosene where up to 11% E_{24} value was observed.

Table 4.1: Characterization of *Pseudomonas* species isolated from Soil Samples

Test	Isolate A	Isolate B
Growth on CA	green coloured colonies	creamy coloured colony
Gram reaction	-	-
Motility	+	+
Oxidase	+	+
Nitrate Reduction	+	+
Lysine Decarboxylase	-	+
Ornithine Decarboxylase	-	-
H_2S Production	-	-
ONPG	-	-
Indole	-	-
Urease	-	-
VP	-	-
Citrate Utilisation	-	-
TDA	-	-
Gelatin	-	-
Carbohydrate fermentation tests		
Malonate	-	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	-	-
Lactose	-	-
Xylose	+	+
Adonitol	-	-
Raffinose	-	-
Glucose	+	+
Mannitol	-	-
Growth at 25^{o}C	+	ND
Tentative identity	*Pseudomonas fluorescens*	*Pseudomonas aeruginosa*

+ = Positive; − = Negative; ND = Not Determined; ONPG = O-nitrophenyl-β-D-galactopyranoside; VP = Voges Proskauer; TDA = Tyrosine-D-arginine, CA= Cetrimide Agar.

Table 4.2: Characterization of *Bacillus* species isolated from Soil Samples

Test	Isolate C		Isolate D	
	C_1	**C_2**	**D_1**	**D_2**
Growth on MYP agar	Pink coloured colony		Pink coloured colony	
Gram reaction	+		+	
Catalase	+		+	
Motility	+		+	
Carbohydrate fermentation tests				
Arabinose	+	+	–	–
Cellebiose	+	+	–	–
Inositol	+	+	–	–
Mannitol	+	+	–	–
Mannose	+	+	–	–
Raffinose	–	+	–	–
Rhamnose	–	+	–	–
Sorbitol	+	+	–	–
Sucrose	+	+	+	+
Trehalose	+	+	–	–
Xylose	+	+	–	–
Adonitol	–	–	–	–
Gallactose	–	–	–	–
Salicin Test	–	–	–	–
MDM Test	–	–	–	–
MDG Test	+	–	–	–
Inulin Test	–	–	–	–
Melezitose Test	–	–	–	–
Indole test	ND	–	ND	–
ONPG	+	+	–	–
Arginine	+	+	+	+
Citrate Utilisation	+	+	–	–
Voges Proskauer	ND	+	ND	+
Nitrate reduction	ND	+	ND	+
Tentative identity	*Bacillus licheniformis*		*Bacillus cereus*	

+ = Positive; − = Negative; ND = Not Determined; ONPG = O-nitrophenyl-β-D-galactopyranoside; MDG = Methyl-D-Glucoside, MYP Agar = Mannitol Egg Yolk Polymyxin Agar; C_1, C_2, D_1, D_2 = presumptive *Bacillus* isolates

Table 4.3: Oil Spreading and Blood Haemolysis Screening of *Pseudomonas* and *Bacillus* species isolated from soil

Analytical Test	***P. aeruginosa***			***P. fluorescens***		***B. licheniformis***		***B. cereus***	
Isolates	**A**	**B**	**C**	**A**	**B**	**A**	**B**	**A**	**B**
Oil spread test (vegetable oil) mm	29	30	28	30	-	-	-	25	30
Blood haemolysis	+	+	-	-	-	+	-	+	+
	A	β	γ	γ	Γ	α	Γ	A	A

+=positive, -=negative, α=alpha haemolysis, β=beta haemolysis, γ=gamma haemolysis, mm=millimeters

Table 4.4: Oil Spreading Screening for *Pseudomonas aeruginosa* and *Bacillus cereus* isolated from Soil Samples using different hydrocarbons

Sample	Kerosene		Diesel		Petrol (Premium Motor Spirit)		Vegetable Oil	
Organisms	*P. aeruginosa*	*B. cereus*	*P. aeruginosa*	*B. cereus*	*P. aeruginosa*	*B. cereus*	*P. aeruginosa*	*B. cereus*
A	29	30	28	-	29	28	29	25
B	29	25	28	-	29	25	30	30
C	28	-	27	-	29	-	28	-

A, B and C are petridishes containing water, oil sources and test organisms, - = negative, mm = millimeters

Table 4.5: Emulsification Stability E_{24} (%) Screening for biosurfactant production by *Bacillus cereus* and *Pseudomonas aeruginosa*

Strains	Kerosene		Diesel		Petrol		Vegetable Oil	
	BC	**PS**	**BC**	**PS**	**BC**	**PS**	**BC**	**PS**
A	5	10	2.5	7	0	8	5	8
B	9	11	0	0	0	4.5	9	7
C	0	8	0	6	0	7	0	9

BC: *Bacillus cereus* PS: *Pseudomonas aeruginosa*

4.3 Preliminary Testing for Biosurfactant Production and Activity in Shea Butter and Palm Oil Wastes Slurries

Pseudomonas aeruginosa and *Bacillus cereus* which showed positive response for the tests above (biosurfactant production screening) were further tested for their activity in Shea butter and Palm oil wastes for 5 days. These isolates were used in the single and consortium cultures for the following tests; Optical Density, Emulsification Stability test, and Surface Tension tests as described by Hasham *et al.* (2012).

Figures 4.1 and 4.2 shows the daily readings for absorbance for both the single and consortium of bacteria in shea butter and palm oil respectively. Both the single and consortium of the bacteria were able to grow in both Shea butter and Palm oil with absorbance reading of up to 0.87 optical density in Shea butter and Palm oil.

Figures 4.3 and 4.4 shows the daily readings for E_{24} for both the single and consortium of bacteria in shea butter and palm oil respectively. E_{24} of 46% and 42% was recorded by *Pseudomonas aeruginosa* in shea butter and palm oil respectively. Though *Bacillus cereus* also gave up to 46% E_{24} in shea butter, the highest reading it recorded in Palm oil was just 2%. The consortium did much better than the single cultures giving the highest percentage of 50% in Shea butter. However, the highest result of 32% in palm oil.

Figure 4.5 and 4.6 shows the daily surface tension (ST) results. For both shea butter and palm oil, surface tension dropped by at least 1% from Day 1 to Day 5 for all the test samples. Highest results for surface tension reduction was recorded by *Pseudomonas aeruginosa* with 17% surface tension drop in palm oil and 10% drop in shea butter.

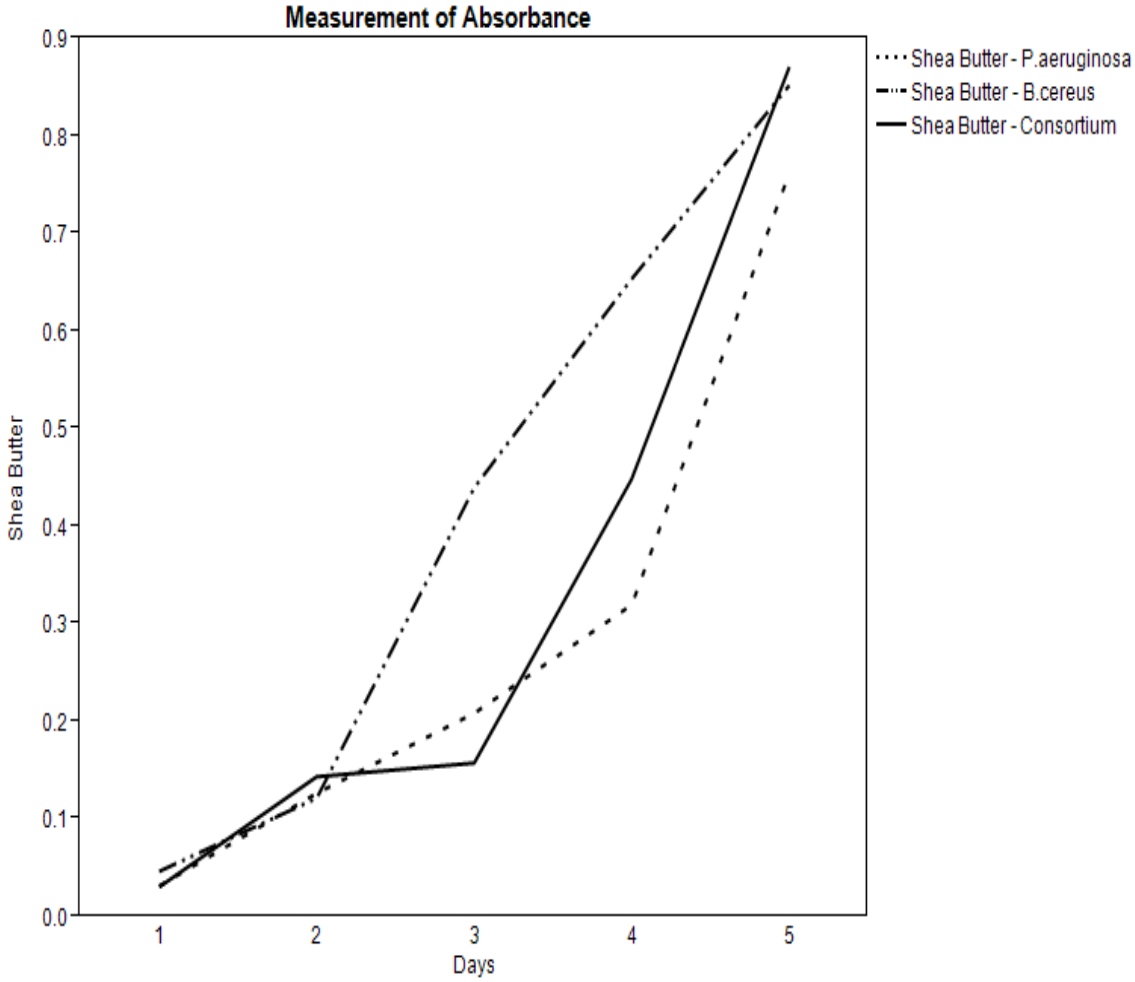


Figure 4.1 **Preliminary Tests for Daily Growth Measurement (Absorbance) of *Pseudomonas aeruginosa* and *Bacillus cereus* Shea Butter Waste Slurries**

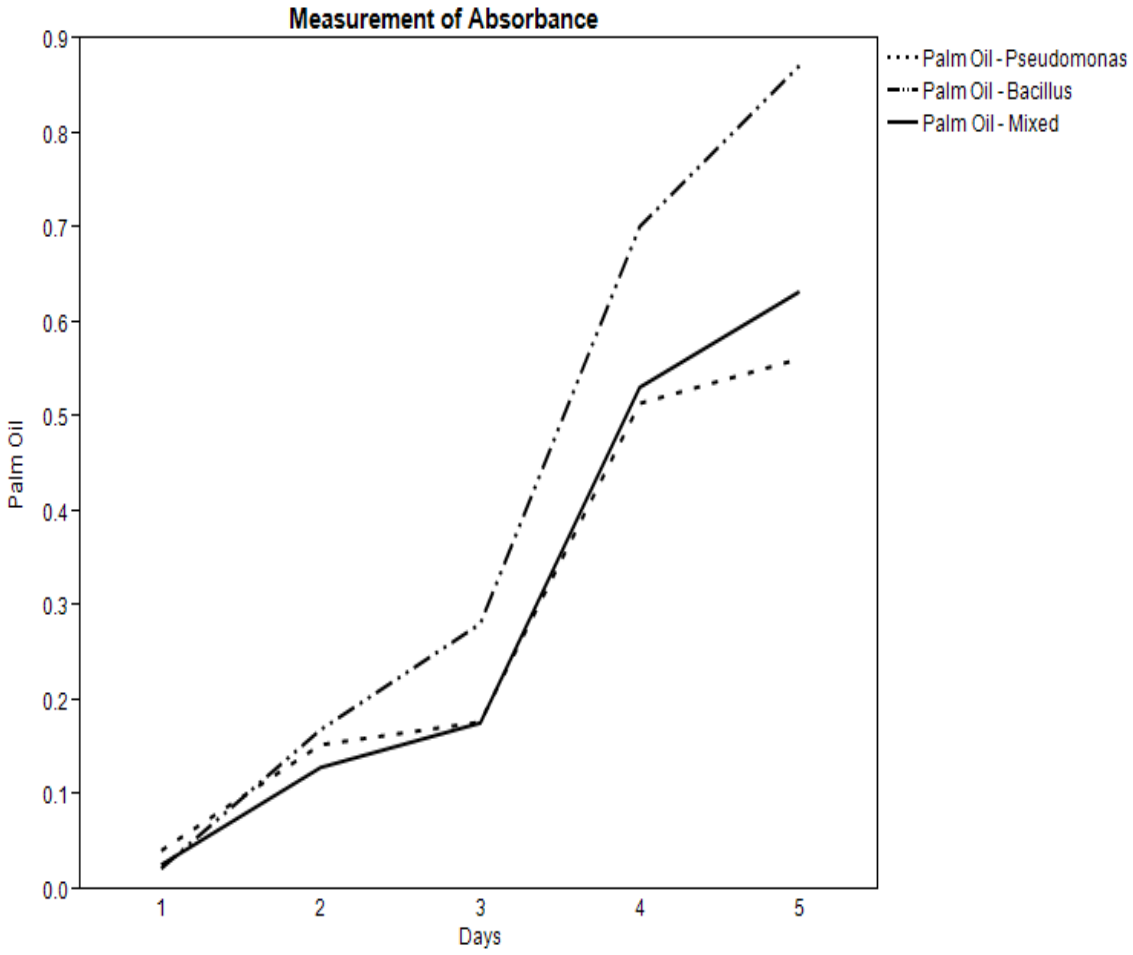


Figure 4.2 **Preliminary Tests for Daily Growth Measurement (Absorbance) of *Pseudomonas aeruginosa* and *Bacillus cereus* Palm Oil Waste Slurries**

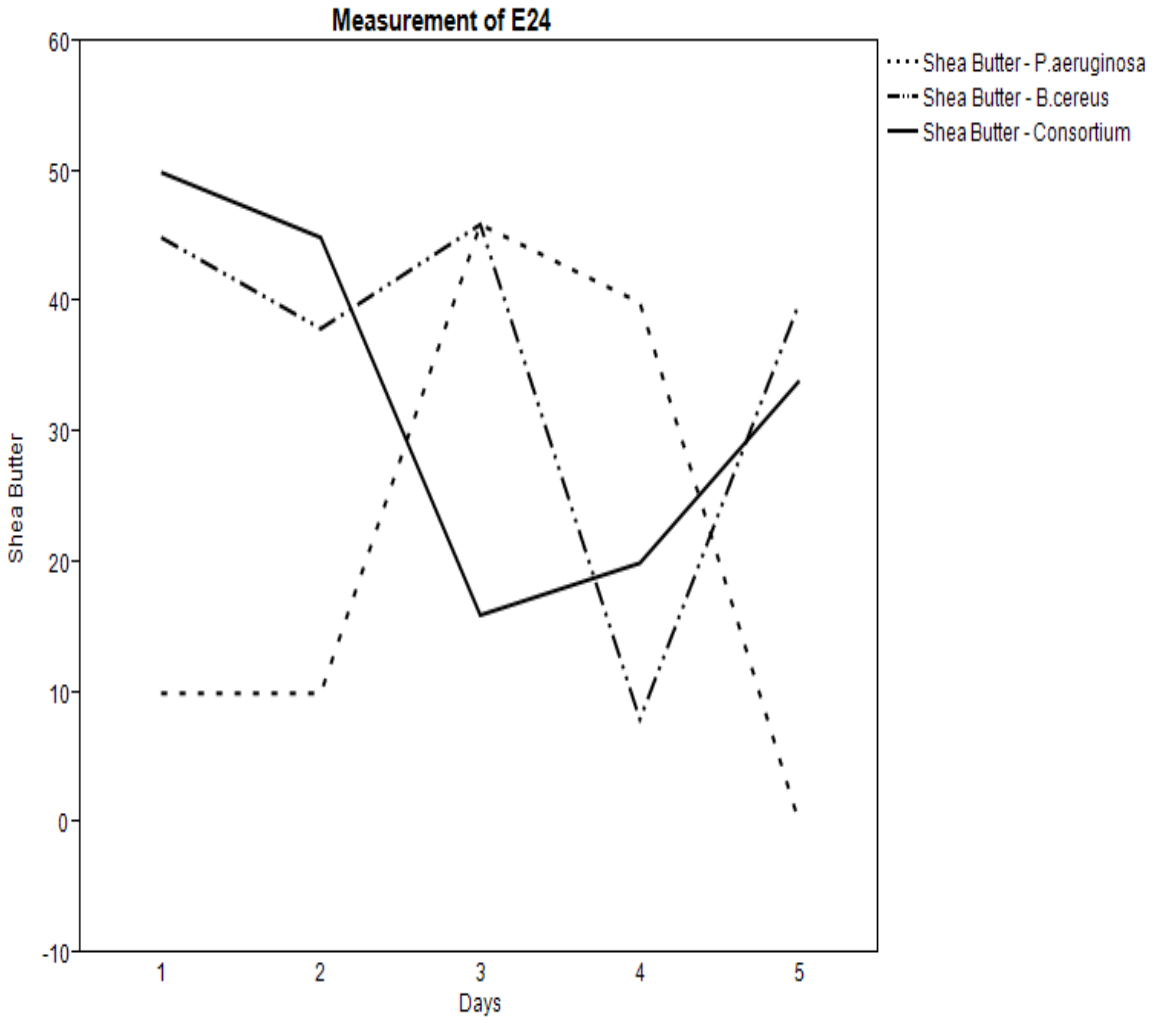


Figure 4.3 **Preliminary Tests for Daily Emulsification Index Measurement (E_{24}) by *Pseudomonas aeruginosa* and *Bacillus cereus* in Shea Butter Waste Slurries**

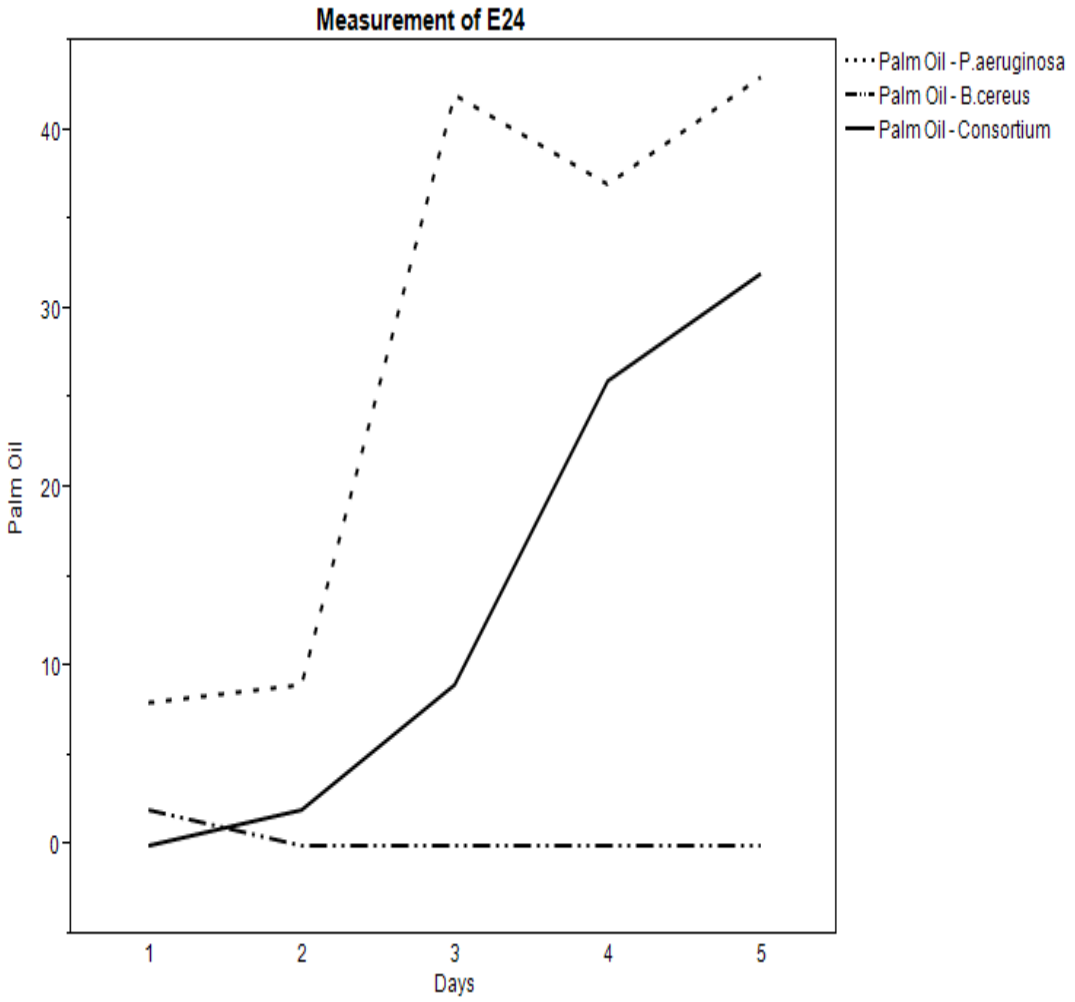


Figure 4.4 Preliminary Tests for Daily Emulsification Index Measurement (E_{24}) by *Pseudomonas aeruginosa* and *Bacillus cereus* in Palm Oil Waste Slurries

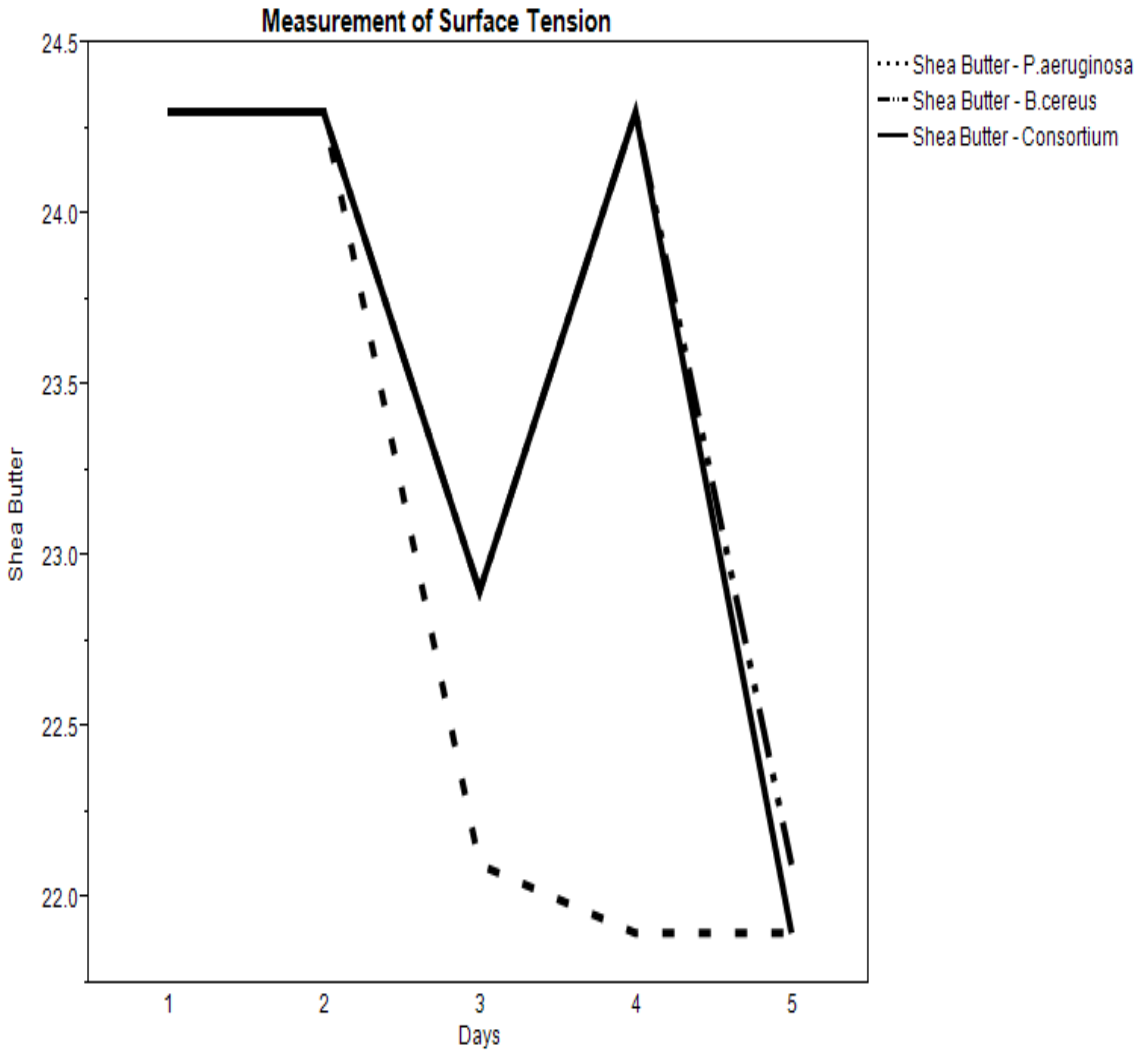


Figure 4.5 Preliminary Tests for Daily Surface Tension Measurement (ST) from*Pseudomonas aeruginosa* and *Bacillus cereus* Shea Butter Waste Slurries

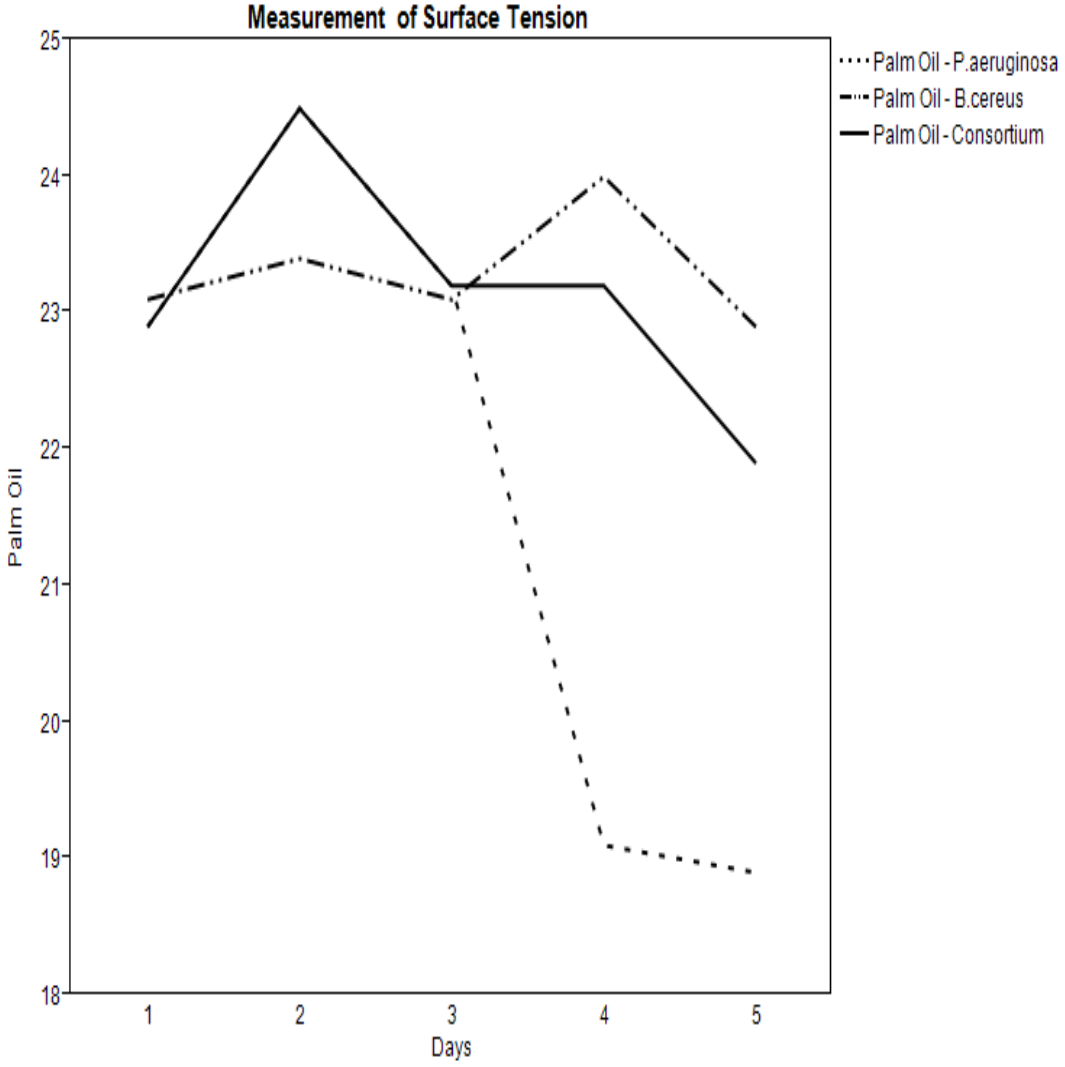


Figure 4.6 Preliminary Tests for Daily Surface Tension Measurement (ST)

from*Pseudomonas aeruginosa* and *Bacillus cereus* Palm Oil Waste Slurries

4.4 Optimization Parameters

pH and nutrient parameters (nitrogen source and lipase) were optimized for growth and biosurfactant production from Shea butter and Palm oil waste slurries by *P. aeruginosa* and *B. cereus* and grown in batch cultures set up on a rotatory shaker at 150rpm for up to 7days. The results of the average of the triplicate daily samples are presented below. Kinetics of the optimization studies are presented in Appendices II to XXVII.

Statistical analysis of all values from the kinetic studies are also presented in Tables 4.6a to 4.8d

4.4.1 pH

Absorbance, E_{24} and Surface Tension results are presented below.

4.4.1.1 Daily growth measurement (absorbance) of the test organisms at varying pH levels

Growth measurement of the test organisms (*P. aeruginosa* and *B. cereus*) in Shea butter and Palm oil at pH 5, 6, 7 and 8, was carried out daily by measuring absorbance at a wavelength of 590nm. (Appendices II to IV)

Daily growth measurement (absorbance) of the single organisms from shea butter waste slurry at varying pH levels:Appendix II shows the daily growth measurement of *P. aeruginosa* and *B. cereus* in Shea butter at varying pH. This resulted in growth (higher absorbance) increases with increase in pH and days. *P. aeruginosa* had the highest absorbance of 0.94 at pH 7 and *B. cereus* had an absorbance of 0.82 at pH 7. It was noted from the results that there was significant growth of both organisms with increase in pH and days. However, at pH 8, a gradual decline in growth was observed for both organisms.

Daily growth measurement (absorbance) of the single organisms in palm oil waste slurry at varying pH levels:The daily growth measurement of the single cultures in Palm oil is presented in Appendix III which depicts a significant growth of the organism with increase in pH and days. The figure shows the highest absorbance of 0.75 at pH 6 by *B. cereus* and 0.70 at pH 7 by *P. aeruginosa* on the 5^{th} day. pH 7 gave the best growth for *B. cereus* from day 1 to Day 3 however, the best growth was recorded at pH 6. On the other hand, *P. aeruginosa* showed no specific pattern in pH and growth for all days but the best growth was recorded at pH 7 on day 5.

Daily growth measurement (absorbance) of the consortium of organisms in shea butter and palm oil waste slurries at varying pH levels:The daily growth measurement of the consortium of the organisms in Shea butter and Palm oil is presented in Appendix IV which depicts a significant growth of the organism with increase in pH and days. The result shows the highest absorbance of 1.09 at pH 7 in Shea butter by the consortium of bacteria. Growth in palm oil by the consortium of bacteria also reached its peak growth (0.64) at pH 7 on Day 5. A general decline in the growth of the consortium of bacteria in both Palm oil and Shea butter was however observed at pH 8.

4.4.1.2 Daily E_{24} measurement of the test organisms at varying pH levels

Emulsification index test (E_{24}) results are presented in Appendices V to VII.

Daily E_{24} measurement of the test organisms at varying pH levels in shea butter waste slurry:Appendix V shows the daily E_{24} readings of *P. aeruginosa* and *B. cereus* in Shea butter. The Appendix shows that E_{24} readings decrease with increase in pH and increased with days for most of the observations. It was noted that at pH 5 on Day 7, *B. cereus* had

the best reading for E_{24} at 74% and *P. aeruginosa* recorded its best at 67% on day 7 at pH 7.

Daily E_{24} measurement of the test organisms at varying pH levels in palm oil waste slurry:The daily E_{24} measurement of the test organisms in Palm oil is presented in Appendix VI which depicts no growth recorded at all for *Bacillus cereus* at all the pH levels. Only *P. aeruginosa* had results at all the pH levels. However, there wasn't any pattern to the reading with respect to the pH levels or days but best reading of 45% was recorded at pH 5 on day 5 for *P. aeruginosa*.

Daily E_{24} measurement of the consortium of organisms at varying pH levels in shea butter and palm oil waste slurries:The daily E_{24} measurement of the consortium of the test organisms in Shea butter and Palm oil is presented in Appendix VII. For Shea butter, excellent results were observed with 67% recorded at pH 6 on day 5. Palm oil on the other hand showed no production of biosurfactant at pH 5, and 6. Production was only observed at pH 7 and pH 8 with readings of 48% and 41% respectively.

4.4.1.3 Daily surface tension measurement for biosurfactant production by the test organisms at different pH evels

Surface Tension results are presented in Appendix V to VIII.

Daily surface tension measurement for biosurfactant production by the single organisms at varying pH levels in shea butter waste slurry:Appendix VIII shows daily Surface Tension results for *P. aeruginosa* and *B. cereus* in Shea butter at different pH. The figure shows that the Surface Tension remained constant for many days before a sharp declined was

recorded on day 7 for *P. aeruginosa* which had a decline of about 14.3% for all levels of pH. *B. cereus* on the other hand showed the highest decline of 22.2% at pH 6. However, the results revealed that there were no significant relationships between pH levels and the surface tension values for both organisms as the pH values for all levels were very similar.

Daily surface tension measurement for biosurfactant production by the single organisms at varying pH levels in palm oil waste slurry: The daily Surface Tension of the test organisms in Palm oil is presented in Appendix IX. The figure shows significant drop in the surface tension for both organisms indicating production of biosurfactants. *P. aeruginosa* gave a 37% reduction in the surface tension readings for all levels of pH from day 1 to 5 while *B. cereus* dropped with up to 42% at pH 6 from day 1 to Day 5. The figure also reveals that there is no significant relationship between the pH and the surface tension readings for both organisms as all pH values for all levels were very similar.

Daily surface tension measurement for biosurfactant production by consortium of the test organisms at varying pH levels in shea butter and palm oil waste slurries: The daily Surface Tension of consortium of the test organisms in Palm oil is presented in Appendix X. Shea butter showed no reduction in the surface tension readings for all levels of pH from day 1 to 5 while Palm oil dropped with up to 14.2% at all the pH values from day 1 to Day 5. The figure also reveals that there is no significant relationship between the pH and the surface tension values for both Shea butter and Palm oil as all pH values for all levels were very similar.

4.4.2 Nitrogen

Absorbance, E_{24} and Surface Tension results are presented below.

4.4.2.1 Daily growth measurement (absorbance) of the test organisms at varying concentrations of nitrogen source (sodium nitrate)

The results are presented and analysed as described in Appendices XI to XIII.

Daily growth measurement (absorbance) of the single organisms in shea butter waste slurry: Appendix XI shows that *P. aeruginosa* gave the highest absorbance of 1.16 at a concentration of 0.3mg/100ml of the sodium nitrate ($NaNO_3$) on the 5^{th} day. *B. cereus* had an absorbance of 0.99 at nitrogen source concentration of 0.4mg/100ml on the 5^{th} day.

Daily growth measurement (absorbance) of the single organisms in palm oil waste slurry: The effect of nitrogen source on growth of the test organisms in Palm oil is presented in Appendix XII which revealed that *B. cereus* had the highest absorbance of 1.52 at 0.4mg/100ml concentration of sodium nitrate on the 3rd day. *P. aeruginosa* had the highest absorbance of 1.30 at 0.3mg/100ml concentration of sodium nitrate also on the 5^{th} day.

Daily growth measurement (absorbance) of the consortium of organisms in shea butter and palm oil waste slurries: The effect of nitrogen source on growth of the consortium of organisms is presented in Appendix XIII which revealed that the best results (1.32) is obtained in Shea butter at the lowest concentration of $NaNO_3$which is 0.1mg/100ml. The highest absorbance recorded on the other hand for Palm oil is also at 0.1mg/ml at 0.61. Growth of the consortium of organisms in both Shea butter and Palm oil increased from

day 1 to 5 and gradually declined with higher concentrations of 0.3mg/100mls and 0.4mg/100mls.

These results indicate that higher concentrations of 0.03mg/100mls to 0.04mg/100mls Sodium Nitrate support growth of the single bacteria but lower concentrations of 0.1mg/100mls supported the consortium. In all, for Nitrogen optimization, Palm oil gave better results with single organisms compared to the consortium or the single cultures in Shea butter.

4.4.2.2 Daily E_{24} measurement of the test organisms at varying concentrations of nitrogen source (sodium nitrate)

Emulsification index test (E_{24}) results are presented in Appendices XIV to XV.

Daily E_{24} measurement for biosurfactant production by the single organisms in shea butter waste slurry: Appendix XIV shows increase in the production of biosurfactant with concentrations of 0.2mg/100ml and 0.3mg/100ml. The production wasn't consistent with days but *P. aeruginosa* gave the best E_{24} reading of 79% at a concentration of 0.3mg/100ml on the 6^{th} day while *B. cereus* gave best production with an E_{24} of 72% at $NaNO_3$ concentration of 0.4mg/100ml on the 7^{th} day.

Daily E_{24} measurement for biosurfactant production by the single organisms in palm oil waste slurry:No biosurfactant production was recorded for all concentrations of the nitrogen source from both organisms.

Daily E_{24} measurement for biosurfactant production by consortium of bacteria in shea butter and palm oil waste slurries: In Appendix XV, biosurfactant production was observed

in both Shea butter and Palm oil. However, for Palm oil, only the concentration of 0.1mg/100ml had E_{24} values of up to 55% all other concentrations of $NaNO_3$ showed no production of biosurfactant at all. For Shea butter all concentrations of the nitrogen source recorded E_{24} readings with values of up to 58% at both 0.2mg/100ml and 0.3mg/100ml.In Palm oil, the consortium gave better results with the Nitrogen than the single cultures.

4.4.2.3 Daily surface tension of the test organisms at varying concentrations of nitrogen source (sodium nitrate)

The results recorded are described below in Appendices XVI to XVIII.

Daily surface tension measurement for biosurfactant production by the single organisms in shea butter waste slurry: Appendix XVI reveals that only *P. aeruginosa* showed a reduction of 14.3% in the surface tension readings for only the concentration of 0.2mg/100ml $NaNO_3$. The readings for *B. cereus* indicated that surface tension reduced for up to 33.3% at 0.1mg/100ml $NaNO_3$ concentration from day 1 to day 5. All other concentrations reduced with up to 25% for *B. cereus*.

Daily surface tension measurement for biosurfactant production by the single organisms in palm oil waste slurry: Appendix XVII reveals that *B. cereus* gave its highest reduction in surface tension of up to 42.5% from day 1 to day 5 at 0.4mg/100ml concentration. *P. aeruginosa* showed its highest reduction of 38.3% also at 0.4mg/100ml concentration from day 1 to day 5. For each organism, the values obtained for all the days were similar for all concentrations.

Daily surface tension measurement for biosurfactant production by the consortium of the organisms in shea butter and palm oil waste slurries: Appendix XVIII reveals that Shea butter showed a 14.2% drop in surface tension from day 1 to day 5 for all concentrations. Palm oil on the other hand,showed the highest reduction of 30.4% at most concentrations from day 1 to day 5.

4.4.3 Lipase

Absorbance, E_{24} and Surface Tension results are presented below.

4.4.3.1 Daily growth measurement (absorbance) of the test organisms at varying concentrations of lipase

The results are presented and analysed as described in Appendices XIX to XXI.

Daily growth measurement (absorbance) of the single organisms in shea butter waste slurry: At varying concentrations of lipase in Shea butter (Appendix XIX), *P. aeruginosa* had the highest absorbance of 5.95 at lipase concentration of 0.2mg/100ml on the 5^{th} day. However, in comparison, the figure clearly revealed that the growth of *B. cereus* at same lipase concentrations for all the days was lower with peak growth observed at 0.73 at 0.15mg/100ml concentration on day 3.

Daily growth measurement (absorbance) of the single organisms in palm oil waste slurry: When tested in Palm oil at varying lipase concentration (Appendix XX), *P. aeruginosa* had its highest absorbance of 0.89 at 0.2mg/100ml of lipase on the 4^{th} day while *B. cereus* gave a high absorbance of 0.66 at lipase concentration of 0.10mg/100ml on day 4.

Daily growth measurement (absorbance) of the consortium of organisms in shea butter and palm oil waste slurries:In Appendix XXI, Shea butter gave the best growth reaching the highest peak at 1.19 at 0.10mg/100ml Lipase concentration. However, growth in Palm oil was not as high with the best growth of 0.80 recorded at 0.10mg/ml Lipase on the 5^{th} day. Growth in both Shea butter and Palm oil increased in days but showed slight decline with higher concentrations of 0.15mg/100ml and 0.2mg/ml.

In all, lower concentrations favoured growth for the consortium while higher concentrations favoured growth for the single cultures used in this study.

4.4.3.2 Daily E_{24} measurement of the test organisms at varying concentrations of lipase

Emulsification index test **(**E_{24}**)** results are presented in Appendices XXII to XXIV.

Daily E_{24} measurement of the single organisms in shea butter waste slurry: At varying concentrations of lipase in Shea butter (Appendix XXII), the values show that with increase in Lipase concentration, the production of Biosurfactants was improved for *B. cereus* with readings for the bacteria peaking at the concentration of 0.15mg/100ml with E_{24} of 65% on Day 4. *P. aeruginosa* on the other hand did not show much significant improvement with the added lipase at all the concentrations. The highest value (47%) for its E_{24} was recorded at the same concentration of 0.15mg/100ml on day 7. For both organisms in Shea butter, the best lipase concentration for production of biosurfactant was 0.15mg/100ml.

Daily E_{24} measurement of the single organisms in palm oil: In Appendix XXIII, biosurfactant production peaked at 0.2mg/ml for both *P. aeruginosa* and *B. cereus*.

However, higher results was recorded for *P. aeruginosa* with little production recorded by *B. cereus*. For both organisms, production increased with lipase concentrations.

Daily E_{24} measurement of the consortium in shea butter and palm oil waste slurries: With the consortium in Shea butter and Palm oil at varying lipase concentrations (Appendix XXIV), production peaked at 0.05mg/ml for Shea butter and at 0.10mg/ml for Palm oil with readings of 55% and 65% respectively. Overall, optimal results were observed in Shea butter compared to palm oil with values at only 0.05mg/ml and 0.10mg/100ml only.

4.4.3.3 Daily surface tension measurement of the test organisms at varying concentrations of lipase

Surface Tension Measurement values are presented below in Appendices XXV to XXVII.

Daily surface tension measurement of the single organisms in shea butter waste slurry: At varying concentrations of lipase in Shea butter (Appendix XXV), *P. aeruginosa* gave a uniform surface reduction (14.2%) for all the concentrations from Day 1 to Day 5. *B. cereus* however showed a better surface tension reduction of 22.2% at 0.1mg/100ml concentration from Day 4 to Day 5. Both organisms show similarities in the pattern of surface tension readings for most concentrations of Lipase.

Daily surface tension measurement of the single organisms in palm oil waste slurry: In Appendix XXVI, *P. aeruginosa* had a high surface tension reduction of up to 47.5% at 0.2mg/100ml of lipase from day 1 to day 5 while *B. cereus* showed up to 28.3% reduction in surface tension at majority of the concentrations of lipase from day 1 to day 5.

Daily surface tension measurement by the consortium in shea butter and palm oil waste slurries: The consortium when tested in Shea butter and Palm oil at varying lipase concentration (Appendix XXVII), Shea butter had a high surface tension reduction of up to 14.2% at all concentrations of lipase from day 1 to day 5 while Palm oil showed up to 14.4% reduction in surface tension at all of the concentrations of lipase from day 1 to day 5.

4.5 Statistical Analysis of Optimization Study

Values of the physical parameters; (pH) and nutrient parameters (nitrogen source and lipase) optimized at varying concentrations in Shea butter and Palm oil waste slurries by *P. aeruginosa,B. cereus* and their consortium were statistically analysed. Measurement parameters (absorbance, emulsification index (E_{24}) and surface tension) carried out during the experimental period is represented in Tables 4.6a to 4.6d, Tables 4.7a to 4.7d and Tables 4.8a to 4.8d.

4.5.1 pH

The results for pH optimization are presented in Tables 4.6a and 4.6b (for Shea butter at varying pH for single and consortium of bacteria) and 4.6c and 4.6d (for Palm oil at varying pH for single and consortium of bacteria).

Table 4.6a revealed that *P. aeruginosa* gave best absorbance of 0.78 at pH 7, emulsification index (E_{24}) of 48.40% at pH 7 and surface tension of 17.54 dyne/cm at pH 7. *B. cereus* gave an absorbance of 0.67 at pH 7, E_{24} of 53.80% at pH 5 and surface tension of 18.22dyne/cm at pH 8. There were significant differences ($P \leq 0.05$) observed between

the pH and the measured parameters for biosurfactant production from Shea butter by the test bacteria.

The consortium of *P. aeruginosa* and *B. cereus* in Shea butter (Table 4.6b) gave best results of absorbance 0.98 at pH 5, E_{24} of 41.00% at pH 8 and equal surface tension of 17.82dyne/cm at all the various pH levels. From the results for single from Shea butter, single cultures produced more biosurfactants than the consortium though more growth was observed in the consortium.

Table 4.6c shows that *P. aeruginosa* gave best results for absorbance 0.35 at pH 7, E_{24} of 20.60% at pH 5 and a surface tension of 17.37 dyne/cm at pH 7. *B. cereus* gave best absorbance 0.47, $\mathbf{E_{24}}$ of 0.00% and surface tension of 17.10 dyne/cm. There were significant differences ($P \leq 0.05$) observed between the pH and the measured parameters.

Results in Table 4.6d reveals that best results for the consortium of the two test organisms in Palm oil at varying pH with absorbance of 0.23 at pH 5, E_{24} of 13.20% at pH 7, and equal reduction in surface tension of 18.16dyne/cm at all the test pH values. Similarly, there were significant differences ($P \leq 0.05$) between pH and the test parameters.

Table 4.6a:pH Optimization of Biosurfactant Production in Shea butter Waste Slurry by *Pseudomonas aeruginosa* and *Bacillus cereus*

Parameters	Organisms	pH (Mean ± SE)			
		pH 5	pH 6	pH 7	pH 8
Absorbance (nm)	*P. aeruginosa*	0.32 ± 0.08^{d}	0.36 ± 0.08^{c}	0.78 ± 0.08^{a}	0.50 ± 0.08^{b}
	B. cereus	0.43±0.12	0.51±0.09	0.67±0.08	0.52±0.11
E_{24} %	*P. aeruginosa*	46.80 ± 5.49^{b}	45.00 ± 2.28^{b}	48.40 ± 2.54^{a}	39.8 ± 2.82^{c}
	B. cereus	53.80±5.99	50.20±3.02	42.60±6.57	29.80±2.18
Surface Tension (dyne/cm)	*P. aeruginosa*	18.36 ± 0.54^{a}	18.36 ± 0.54^{a}	17.54 ± 0.60^{b}	17.54 ± 0.60^{b}
	B. cereus	19.17 ± 0.48^{b}	$19.44 \pm .0.67^{a}$	18.76 ± 0.59^{c}	18.22 ± 2.71^{d}

Means with different superscripted alphabets across the row are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means) E_{24}=Emulsification index, %=percentage, nm=nanometer

Table 4.6b:pH Optimization of Biosurfactant Production in Shea butter Waste Slurry by Consortium of *P. aeruginosa* and *B. cereus*

Parameters	Organisms	pH (Mean ± SE)			
		pH 5	pH 6	pH 7	pH 8
Absorbance (nm)	Consortium	0.98 ±0.18^{a}	0.73±0.16^{c}	0.72±0.17^{c}	0.77±0.18^{b}
E_{24} %	Consortium	23.40±10.55^{c}	35.80±12.10^{b}	40.40±7.72^{a}	41.00±3.24^{a}
Surface Tension (dyne/cm)	Consortium	17.82±0.66^{a}	17.82±0.66^{a}	17.82±0.66^{a}	17.82±0.66^{a}

Means with different superscripted alphabets across the row are significantly (P≤0.05) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer

Table 4.6c:pH Optimization of Biosurfactant Production in Palm Oil Waste Slurry by *Pseudomonas aeruginosa* and *Bacillus cereus*

Parameters	Organisms	pH (Mean±SE)			
		pH 5	pH 6	pH 7	pH 8
Absorbance (nm)	*P. aeruginosa*	0.24 ± 0.05^{c}	0.31 ± 0.06^{b}	0.35 ± 0.10^{a}	0.32 ± 0.09^{b}
	B. cereus	0.35 ± 0.10^{c}	0.34 ± 0.13^{c}	0.47 ± 0.04^{a}	0.44 ± 0.06^{b}
E_{24} %	*P. aeruginosa*	20.60 ± 9.56^{a}	18.4 ± 7.19^{b}	7.20 ± 6.48^{d}	11.2 ± 8.00^{c}
	B. cereus	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Surface Tension (dyne/cm)	*P. aeruginosa*	17.64 ± 1.36^{b}	17.64 ± 1.36^{b}	17.37 ± 1.49^{b}	18.18 ± 1.30^{a}
	B. cereus	17.12 ± 1.05^{b}	18.26 ± 1.69^{a}	17.10 ± 1.06^{b}	17.64 ± 1.36^{b}

Means with different superscripted alphabets across the row are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer.

Table 4.6d:pH Optimization of Biosurfactant Production in Palm Oil by Consortium of *P. aeruginosa* and *B. cereus*

Parameters	Organisms	pH (Mean±SE)			
		pH 5	pH 6	pH 7	pH 8
Absorbance (nm)	Consortium	0.23 ± 0.00^{c}	0.16 ± 0.00^{b}	0.41 ± 0.39^{a}	0.16 ± 0.00^{b}
E_{24} %	Consortium	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	13.2 ± 9.39^{a}	12.00 ± 8.00^{b}
Surface Tension (dyne/cm)	Consortium	18.16 ± 0.54^{a}	18.16 ± 0.54^{a}	18.16 ± 0.54^{a}	18.16 ± 0.54^{a}

Means with different superscripted alphabets across the row are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer

4.5.2 Nitrogen (sodium nitrate)

Sodium Nitrate added to the Shea butter and Palm oil waste slurries was tested at different concentrations of sodium nitrate in milligram per 100ml of the nitrogen source. The results for this section are presented in Tables 4.7a to 4.7b (Shea butter - for single and consortium of bacteria) and 4.7c to 4.7d (Palm oil - for single and consortium of bacteria).

In Table 4.7a, *P. aeruginosa* gave highest values for: absorbance of 0.69 at a concentration of 0.4mg/100ml of sodium nitrate ($NaNO_3$), E_{24} of 51.80% at 0.3mg/ml concentration and surface tension of 18.90dyne/cm at all the varied concentrations of the sodium nitrate.*B. cereus* had highest values for: absorbance 0.62, E_{24} of 36.42% and surface tension of 18.90dyne/cm at 0.3mg/ml concentration of sodium nitrate each.

The consortium in Table 4.7b had maximum absorbance of 0.98 at 0.1mg/100ml concentration, E_{24} of 41% and surface tension of 17.82 both at 0.4mg/100ml sodium nitrate concentrations. There were significant differences observed between the Nitrogen concentrations and the parameters measured.

Nitrogen optimization in Palm oil for biosurfactant production by *P. aeruginosa* and *B .cereus* and their consortium is presented in Tables 4.7c and 4.7d respectively. In Table 4.7c, Absorbance of 0.91 at 0.4mg/100ml; E_{24} of 0.00% at all concentration; and surface tension of 17.10 dyne/cm at 0.2mg/100ml was recorded by *P. aeruginosa* while *B. cereus* had an absorbance of 1.20 at 0.4mg/100ml nitrate concentration, E_{24} of 0.00% at all concentration and surface tension of 17.10dyne/cm at 0.1mg/100ml concentration of

sodium nitrate. There were significant differences ($P \leq 0.05$) observed between the sodium nitrate concentrations and test parameters.

Consortium of the test bacteria in Palm oil (Tables 4.7d), shows that *P. aeruginosa* had highest absorbance of 0.38, E_{24} of 33% at 0.1mg/100ml and constant surface tension of 17.08dyne/cm. Significant differences ($P \leq 0.05$) were observed between the different concentrations of sodium nitrate and the measured parameters.

Table 4.7a: Nitrogen Optimization of Biosurfactant Production in Shea butter Waste Slurry by *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Sodium Nitrate (Mean±SE)			
		0.1 mg/100ml	0.2 mg/100ml	0.3 mg/100ml	0.4 mg/100ml
Absorbance (nm)	*P. aeruginosa*	0.40 ± 0.18^{c}	0.26 ± 0.87^{d}	0.63 ± 0.18^{b}	0.69 ± 0.14^{a}
	B. cereus	0.36 ± 0.10^{d}	0.51 ± 0.13^{b}	0.62 ± 0.12^{a}	0.47 ± 0.19^{c}
E_{24} %	*P. aeruginosa*	31.00 ± 6.54^{d}	42.00 ± 2.28^{c}	51.80 ± 7.70^{a}	47.6 ± 6.05^{b}
	B. cereus	17.42 ± 0.10^{a}	30.43 ± 0.13^{a}	36.42 ± 0.12^{a}	34.28 ± 0.19^{a}
Surface Tension (dyne/cm)	*P. aeruginosa*	18.90 ± 0.00^{a}	18.90 ± 0.00^{a}	18.90 ± 0.00^{a}	18.90 ± 0.00^{a}
	B. cereus	19.44 ± 1.32^{a}	18.90 ± 0.85^{b}	18.90 ± 0.85^{b}	18.90 ± 0.85^{b}

Means with different superscripted alphabets across the rows are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer

Table 4.7b:Nitrogen Optimization of Biosurfactant Production in Shea butter Waste Slurry by Consortium of *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Sodium Nitrate (Mean±SE)			
		0.1 mg/100ml	0.2 mg/100ml	0.3 mg/100ml	0.4mg/100ml
Absorbance(nm)	Consortium	0.98 ±0.18[a]	0.73±0.16[c]	0.72±0.17[c]	0.77±0.18[b]
E_{24}%	Consortium	23.40±10.55[c]	35.00±12.10[b]	40.40±7.72[a]	41.00±3.24[a]
Surface Tension (dyne/cm)	Consortium	17.82±0.66[a]	17.82±0.66[a]	17.82±0.66[a]	17.82±0.66[a]

Means with different superscripted alphabets across the rows are significantly (P≤0.05) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer

Table 4.7c:Nitrogen Optimization of Biosurfactant Production in Palm Oil Waste Slurry by *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Sodium Nitrate (Mean±SE)			
		0.1 mg/100ml	0.2 mg/100ml	0.3 mg/100ml	0.4 mg/100ml
Absorbance (nm)	*P. aeruginosa*	0.80 ± 0.12^{d}	0.83 ± 0.13^{c}	0.89 ± 0.13^{b}	0.91 ± 0.13^{a}
	B. cereus	1.07 ± 0.88^{d}	1.20 ± 0.13^{b}	1.10 ± 0.10^{c}	1.14 ± 0.12^{a}
E_{24} %	*P. aeruginosa*	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	B. cereus	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Surface Tension (dyne/cm)	*P. aeruginosa*	17.64 ± 1.36^{a}	17.10 ± 1.36^{c}	17.64 ± 1.36^{a}	17.60 ± 1.39^{b}
	B. cereus	17.10 ± 1.02^{d}	17.64 ± 1.36^{c}	18.78 ± 1.41^{b}	19.32 ± 1.70^{a}

Means with different superscripted alphabets across the rows are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer

Table 4.7d:Nitrogen Optimization of Biosurfactant Production in Palm Oil Waste Slurries by Consortium of *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Sodium Nitrate (Mean±SE)			
		0.1 mg/100ml	0.2 mg/100ml	0.3 mg/100ml	0.4mg/100ml
Absorbance (nm)	Consortium	0.38 ±0.09[a]	0.19±0.05[c]	0.31±0.08[c]	0.19±0.06[c]
E_{24} %	Consortium	33.00±9.70[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Surface Tension (dyne/cm)	Consortium	17.08±0.66[a]	17.08±0.66[a]	17.08±0.66[a]	17.08±0.66[a]

Means with different superscripted alphabets across the rows are significantly (P≤0.05) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer.

4.5.3 Lipase

The results for lipase optimization in Shea butter and Palm oil waste slurries are presented in Tables 4.8a to 4.8d. Table 4.8a shows that *P. aeruginosa* had a very high absorbance of 3.1 at 0.2mg/100ml lipase concentration, E_{24} of 15.6% at 0.15mg/100ml concentration and surface tension of 19.42dyne/cm at 0.15mg/100ml while *B. cereus* showed highest absorbance of 0.64 at 0.15mg/ml, E_{24} of 25% at 0.15mg/100ml and no significant change in Surface tension at all concentrations. In all, *B. cereus gave a better performance for biosurfactant production than P. aeruginosa* in Shea butter.

The result in Table 4.8b shows that consortium of the test bacteria in Shea butter gave maximum absorbance of 0.92 at 0.1mg/100ml concentration of lipase, E_{24} of 37.07% at 0.05mg/ml and surface tension of 18.90dyne/cm at all the varied lipase concentration.

Lipase optimization in Palm oil (Table 4.8c) revealed that *P. aeruginosa* recorded its highest absorbance of 0.61 at 0.2mg/ml, E_{24} of 43.4% at 0.15mg/ml and had the lowest surface tension of 17.28dyne/cm at 0.2mg/ml of lipase concentration.*For B. Cereus*, growth of up to 0.49 was observed at 0.15mg/100ml concentration of lipase, no evidence of biosurfactant was observed via E_{24} for all the concentrations of lipase. However, there was a significant reduction in the surface tension at 0.2mg/100ml concentration.

In Table 4.8d, the consortium of *P. aeruginosa* and *B. cereus* had highest absorbance of 0.47nm, E_{24} of 23.4% at 0.10mg/100ml each and a constant surface tension of 17.62dyne/cm at all concentration of lipase for Palm oil.

There was significant difference ($P \leq 0.05$) between the lipase concentration and the test parameters in Tables 4.8a to 4.8d.

Table 4.8a:Lipase Optimization of Biosurfactant Production in Shea Butter Waste Slurry by *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Lipase (Mean±SE)			
		0.05 mg/100ml	0.10 mg/100ml	0.15 mg/100ml	0.20 mg/100ml
Absorbance (nm)	*P. aeruginosa*	1.55 ± 0.62^{d}	2.48 ± 1.26^{b}	1.98 ± 0.96^{c}	3.10 ± 1.15^{a}
	B. cereus	0.28 ± 0.07^{d}	0.35 ± 0.08^{c}	0.64 ± 0.08^{a}	0.51 ± 0.09^{b}
E_{24} %	*P. aeruginosa*	15.20 ± 9.31^{a}	6.00 ± 4.65^{c}	15.60 ± 9.88^{a}	9.00 ± 5.88^{b}
	B. cereus	24.80 ± 10.11^{b}	23.60 ± 8.63^{b}	25.60 ± 8.49^{a}	17.60 ± 9.67^{c}
Surface Tension (dyne/cm)	*P. aeruginosa*	17.54 ± 0.60^{a}	17.54 ± 0.60^{a}	17.28 ± 0.66^{b}	17.28 ± 0.66^{b}
	B. cereus	19.98 ± 1.08^{a}	19.44 ± 0.54^{b}	19.42 ± 0.54^{b}	19.44 ± 0.54^{b}

Means with different superscripted alphabets across the rows are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means)
E_{24}=Emulsification index, %=percentage, nm=nanometer

Table 4.8b: Lipase Optimization of Biosurfactant Production in Shea Butter Waste Slurry by Consortium of *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Lipase (Mean±SE)			
		0.05 mg/100ml	**0.10 mg/100ml**	**0.15 mg/100ml**	**0.20mg/100ml**
Absorbance (nm)	Consortium	0.86 ± 0.18^{b}	0.92 ± 0.17^{a}	0.80 ± 0.16^{d}	0.83 ± 0.17^{c}
E_{24} %	Consortium	37.07 ± 9.86^{a}	27.11 ± 11.50^{b}	24.16 ± 10.90^{c}	13.60 ± 7.34^{d}
Surface Tension (dyne/cm)	Consortium	18.90 ± 0.00^{a}	18.90 ± 0.00^{a}	18.90 ± 0.00^{a}	18.90 ± 0.00^{a}

Means with different superscripted alphabets across the rows are significantly ($P\leq0.05$) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer.

Table 4.8c:Lipase Optimization of Biosurfactant Production in Palm Oil Waste Slurry by *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Lipase (Mean±SE)			
		0.05 mg/100ml	0.10 mg/100ml	0.15 mg/100ml	0.20 mg/100ml
Absorbance (nm)	*P. aeruginosa*	0.48 ± 0.14^{c}	0.42 ± 0.12^{d}	0.53 ± 0.13^{b}	0.61 ± 0.15^{a}
	B. cereus	0.49 ± 0.03^{b}	0.58 ± 0.06^{a}	0.49 ± 0.06^{b}	0.42 ± 0.05^{c}
E24 %	*P. aeruginosa*	24.80 ± 11.26^{c}	20.40 ± 7.62^{c}	43.40 ± 10.43^{a}	38.60 ± 11.50^{b}
	B. cereus	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	19.00 ± 11.87^{a}
Surface Tension (dyne/cm)	*P. aeruginosa*	17.54 ± 0.60^{a}	17.54 ± 0.60^{a}	17.28 ± 0.66^{b}	17.28 ± 0.66^{b}
	B. cereus	17.56 ± 1.84^{a}	17.54 ± 1.85^{a}	17.51 ± 1.87^{b}	17.46 ± 1.90^{b}

Means with different superscripted alphabets across the rows are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means) $\mathbf{E_{24}}$=Emulsification index, %=percentage, nm=nanometer.

Table 4.8d:Lipase Optimization of Biosurfactant Production in Palm Oil Waste Slurry by Consortium of *P. aeruginosa* and *B. cereus*

Parameters	Organisms	Lipase (Mean±SE)			
		0.05 mg/100ml	0.10 mg/100ml	0.15 mg/100ml	0.20mg/100ml
Absorbance (nm)	Consortium	0.18 ± 0.05^{b}	0.47 ± 0.12^{a}	0.12 ± 0.02^{c}	0.16 ± 0.04^{b}
E_{24} %	Consortium	0.20 ± 0.20^{b}	23.40 ± 14.48^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Surface Tension (dyne/cm)	Consortium	17.62 ± 0.66^{a}	17.62 ± 0.66^{a}	17.62 ± 0.66^{a}	17.62 ± 0.66^{a}

Means with different superscripted alphabets across the rows are significantly ($P\leq0.05$) different. Values are expressed as means±SE (Standard error of means) E24=Emulsification index, %=percentage, nm=nanometer.

4.6 Comparative Analysis of Biosurfactant Production in Shea Butter and Palm Oil Waste Slurries

Table 4.9 presents a summary of the results on comparative analysis of biosurfactant from Shea butter and Palm oil wastes as carbon source. The Table revealed that Shea butter gave higher absorbance of 0.96, E_{24} of 39.69% and surface tension reduction to 17.62 dyne/cm by the test bacteria than Palm oil which gave an absorbance of 0.68, E_{24} of 23.20% and surface tension to 18.18dyne/cm. However there was no significant difference ($P \leq 0.05$) between the measured parameters for biosurfactant production and the carbon sources (Shea butter and Palm oil).

4.7 Comparative Analysis of Biosurfactant production by *P. aeruginosa* and *B. cereus*

The comparative analysis between *P. aeruginosa* and *B. cereus* in biosurfactant production from Shea butter and Palm oil is presented in Table 4.10. This result clearly shows that *P. aeruginosa* performed better for biosurfactant production in both Shea butter and Palm oil than *B. cereus* from the high absorbance results of 1.52, E_{24} of 38.6% and surface tension reduction to 17.9 dyne/cm in Shea butter and an absorbance of 0.6, E_{24} of 21.64% and surface tension reduction to 17.22dyne/cm in Palm oil. In comparism, absorbance reading results for *B. cereus* was0.75, E_{24} of 19% and surface tension reduction to 18.25 dyne/cm in Palm oil. While in Shea butter, the absorbance was 0.65, E_{24} of 26.5% and surface tension reduction to 18.85 dyne/cm. There were significant differences ($p > 0.05$) observed between the test organisms and the parameters measured for biosurfactant production from Shea butter and Palm oil.

Table 4.9: Comparative Analysis of Biosurfactant Production from Shea Butter and Palm Oil Slurries

Carbon Source	Parameters	Values(means±SE)
Shea butter slurries	Absorbance (nm)	0.96 ± 0.03^{c}
	E_{24} (%)	39.69 ± 0.61^{a}
	Surface Tension (dyne/cm)	17.62 ± 0.07^{b}
Palm Oil slurries	Absorbance (nm)	0.68 ± 0.53^{c}
	E_{24} (%)	23.20 ± 1.87^{a}
	Surface Tension (dyne/cm)	$18.18.\pm0.10^{b}$

Means with different superscripted alphabets along the column are significantly ($P\leq0.05$) different. Values are expressed as means±SE (Standard error of means). E_{24}=Emulsification index, %=percentage, nm=nanometer.

Table 4.10: Mean Comparison of Biosurfactant Production by *P. aeruginosa* and *B. cereus*

Carbon Source	Parameters	Values for each of the Test Organisms (means±SE)	
		P. aeruginosa	***B. cereus***
Shea butter slurries	Absorbance (nm)	1.52 ± 0.41^{a}	0.65 ± 0.12^{b}
	E_{24} (%)	38.6 ± 7.69^{a}	26.48 ± 4.87^{b}
	Surface Tension (dyne/cm)	17.9 ± 0.42^{a}	18.85 ± 1.37^{b}
Palm oil slurries	Absorbance (nm)	0.60 ± 0.12^{a}	0.75 ± 0.07^{b}
	E_{24} (%)	21.64 ± 6.67^{a}	19.0 ± 0.00^{b}
	Surface Tension (dyne/cm)	17.22 ± 1.17^{a}	18.25 ± 1.32^{b}

Means with different superscripted alphabets along the columns are significantly ($P\leq0.05$) different. Values are expressed as means±SE (Standard error of means). E_{24}=Emulsification index, %=percentage, nm=nanometer

CHAPTER FIVE

5.0 DISCUSSION

Microbial surfactants are surface-active metabolites produced by microorganisms belonging to the genera: *Bacillus, Pseudomonas,* and *Acinetobacter* when grown on water miscible or oily substrates (Praveesh *et al.,* 2011). They either remain adherent to microbial cell surfaces or are secreted in the culture broth. They possess the characteristic property of reducing the surface and interfacial tensions using the same mechanisms as chemical surfactants (Praveesh *et al.,* 2011).

For these indications, the aim of this work was pointed out to assay for the production of biosurfactants by *Pseudomonas aeruginosa* and *Bacillus cereus* in renewable hydrocarbons (Shea butter and Palm oil wastes slurries) by attempting to optimize production under conditions of pH, Nitrogen, and Lipase in Shea butter and Palm oil wastes slurries.

5.1 Isolation and Characterization of Biosurfactant Producing Bacteria from Soil Samples

Environmental samples contaminated by hydrocarbon pollutants have been known to be more likely to harbour biosurfactant producing organisms (Jaysree *et al.,* 2011). Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microorganism producing a particular biosurfactant. Many microorganisms for industrial utilization for waste products have been isolated from contaminated soils, effluents and

waste water sources. Thus, these have an ability to grow on substrates considered potentially noxious for other non-producing microorganisms (Saharan, 2012).

Biosurfactant producing organisms such as *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa, Bacillus licheniniformis, Pseudomonas flourescens,Serratia marcescens, Rhodotorula glutinis, Candida bombicola* produce biosurfactants such as rhamnolipis, sophorolipids,Lichenysin, Subtilisin and surfactin.

The soil samples in this study were collected from sites where petrochemical pollution were common place. Using selective media (MYP and Cetrimide) for the isolation of *Pseudomonas*and *Bacillus* spp. based on morphological characteristics, colonies appearing green or cream on Cetrimide and pinkish on MYP were further subjected to gram staining and other tests biochemical test using microgen test kits. A total of 9 isolates identified as *Bacillus cereus, Bacillus licheniformis, Pseudomonas aeruginosa* and *Pseudomonas fluorescens.* All the strains were identified using *Microgen* test kits (Tables 4.1 and 4.2).

These organisms have proven ability to produce biosurfactants and as such have been recommended for wide use in Biotechnological processes (Jaysree, 2011). In order to test for biosurfactant producing abilities of the strains identified in Tables 4.1 and 4.2, the following tests were used.

5.1.1 Haemolytic activity assay

Blood agar haemolysis test have been used by most researchers to check for biosurfactant production (Rodrigues *et al.,* 2006; Thivaharan and Vytla, 2013).

In this present study, in Table 4.3, strains *a* and*b* of *P. aeruginosa* showed haemolytic activity (partial and complete haemolysis) on blood agar plate. This may be due to the presence of rhamnolipids synthesized by some *Pseudomonas* species. Rhamnolipids are surface active glycolipids which possess hemolytic properties of lysing blood cells (Harikshna and Narsimhulu, 2012).

Strains *a* and *b* of *B.cereus* gave partial haemolytic activity on blood agar plate which may be as a result of surfactin, a biosurfactant with haemolytic properties produced by some *Bacillus* species (Thivaharan and Vytla, 2013).

The haemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad (1970), reported that the biosurfactant produced by *Bacillus subtilis*, surfactin lyses red blood cells. Blood agar lyses has been used to identify surfactin and rhamnolipid (Moran *et al.*, 2002; El-Shahawy, 2014). This has been used to screen for biosurfactant production by new isolates (Yonebayashi *et al.*, 2000). They found an association between haemolytic activity and biosurfactant production, and they recommended the use of blood agar lyses as primary method to screen for biosurfactant activity.

Few studies have reported on biosurfactant-producing *B. cereus* strains, for example, Huesh *et al.* (2006) found that, biosurfactant production by *B. cereus* was physiologically related to the formation of a biofilm under nutrient-limiting conditions.

In the same accordance, Vela´zquez-Aradillas (2011)isolated a Gram-positive bacterium produced a biosurfactant from low-quality coffee beans, this isolate was found to be *Bacillus cereus* QD232.

5.1.2 Oil spreading test

In oil spreading test, the quantity of biosurfactant secreted determines the extent of oil spreading activity of a given microorganism (Thivaharam and Vytla, 2013). When the test isolates were subjected to vegetable oil for oil spread test (Table 4.4), all the strains (a, b and c) of *Pseudomonas aeruginosa* displaced the oil significantly with zones formation of 29, 30 and 28 millimeters respectively while strains (a and b) of *Bacillus cereus* moderately displaced the vegetable oil with zones formation of 25 and 30 millimeters respectively. The oil layer remained unchanged with all strains of *P. fluorescens* and *P. licheniformis* without showing zone of formation. Though many studies show that these organisms have proven ability to produce biosurfactants, the strains isolated in this study may lack the gene markers for producing biosurfactants or may have produced them in very minute quantities from the olive oil used as hydrocarbon source in the procedure above. (Thivaharan and Vytla, 2013).

5.1.3 Emulsification stability test (E_{24})

Emulsification activity was measured using the method described by Cooper and Goldenberg, 1987 and Suganya R.S, 2012. The results from the study (Table 4.5) show both the strains of *Bacillus cereus* and *Pseudomonas aeruginosa* isolated are capable of producing biosurfactants that can emulsify at least 3 of the hydrocarbons (Petrol, Diesel, Kerosene and Vegetable Oil) used in the research. The best result was however observed in Kerosene where up to 11% E_{24} value was observed. This is unlike the study conducted by Priya and Ushrani, 2009 where up to E_{24} readings of 52% and 55% were recorded by *Bacillus* sp. and *Pseudomonas* sp. in Kerosene. The amount of biosurfactant produced by

strains with proven capacity to produce biosurfactant is affected by both the substrate and environmental conditions of aeration, agitation, temperature, time and pH (Salihu *et al.,* 2009). This may be the reason for the result of 11% obtained in this research.

5.2 Optimization of Biosurfactant Production in Shea Butter and Palm Oil Waste Slurries

Several studies have aimed to optimize the biosurfactant production process by changing the variables that influence the type and amount of biosurfactant produced by a microorganism. Important variables are carbon and Nitrogen sources (Santos *et al.,* 2002), potential nutrient limitations and other physical and chemical parameters such as Nitrogen, oxygen (Kronemberger*et al.,* 2008), temperature and pH (Mukherjee *et al.,* 2006).

5.2.1 Effect of pH on biosurfactant production

The pH of the medium plays an important role in biosurfactant production through their effects on cellular growth or activity.

Desai and Banat (1997), Salihu *et al.,* 2009elucidated that, environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability affect *Bacillus cereus* biosurfactant production through their effects on cellular growth or activity.

In this present study, the results on the effect of pH on growth (Figures 4.3 to 4.5) and biosurfactant production (Tables 4.6 to 4.8) clearly revealed that maximum production of biosurfactant occurred mostly at pH 7 for the single cultures of bacteria in both Shea butter and palm oil waste slurries.

The consortium of bacteria in both Shea butter and Palm oil wastes slurries also gave good results at same pH 7. This was similar to the research work carried out by Anna and Parthasarathi (2014), who stated that the highest surface tension reduction (29.45 mN/m) and emulsification activity (78.34%) was recorded at a pH of 7.0 for *B. cereus* KBSB1. Any change to both lower or higher pH values caused an appreciable drop in biosurfactant production indicated by surface tension reduction and emulsification index values.

Though less activity occurred at pH 5, 6 and 8. This was in line with the works of Jagtap *et al.* (2010) and Husam and Ahmed (2013), which showed that maximum production occurred at pH 7 and was the optimum pH for biosurfactant production. This neutral pH can save large numbers of acid and alkali used to adjust pH hence providing for high economic gains.

Similar results were obtained for *Pseudomonas aeruginosa* RS29 and *Bacillus cereus* WJ-1 which produced highest biosurfactant at pH range of 7–8 and 6.0–8.0 respectively (Saikia *et al.*, 2012; Xia *et al.*, 2012). It has been demonstrated also that rhamnolipid production in certain *Pseudomonas spp*. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Mata-Sandoval *et al.*, 2001; Al-Araji and Issa, 2004; Rashedi *et al.*, 2005; Kannahi and Sherley, 2012; Swapnil *et al.*, 2014).

In addition, the pH of the culture medium also determines the electrical charge of the cells and the oxidation–reduction potential which can affect nutrient absorption and enzymatic reaction (Salehizadeh and Shojaosadati, 2001; Husam and Ahmed, 2013). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Praveesh *et al.*, 2011).

The important characteristic of most organisms is their strong dependence on pH for their cell growth and production of secondary metabolites and from the results for both single and consortium of the organisms, best growth occurred at pH 7 with the highest recorded by the consortium. This however did not translate to higher production of biosurfactant by the consortium. This may be due to competition for limited nutrients between the organisms and use up of biosurfactants (secondary metabolites) for growth and other cellular activities.

5.2.2 Effect of nitrogen on biosurfactant production

Nitrogen sources play an important role in the production of biosurfactants, because bacteria require Nitrogen to complete their metabolic pathways. Among the different concentrations of the Nitrogen sources (sodium nitrate) used, our results (Tables 4.7a to 4.7d) revealed that 0.3mg/100ml and 0.4mg/ml concentrations of sodium nitrate gave high yields of biosurfactant production (high emulsification activity and surface tension reduction for both the single organisms and their consortium in Shea Butter.

The maximum value of biosurfactant production may be attributed to the presence of sodium nitrate; this salt is inorganic Nitrogen source, very soluble and utilized easily as Nitrogen source for cell metabolism, growth enhancing and plays a significant role in the pathways of biosurfactant or extracellular secretion (Entissar, 2010).

Similar results were obtained by Karkera *et al.* (2012) for *Pseudomonas aeruginosa* R2 and optimum Nitrogen source was found out to be at 0.4% Ammonium nitrate. The nitrate (NH_4NO_3 or $NaNO_3$) form was found to be the best source of Nitrogen for biosurfactant

production by different *Pseudomonas* strains (Santa-Anna *et al.,* 2002, Chi *et al.,* 2008; Tayebe *et al.,* 2009; Kannahi and Sherley, 2012; Xia *et al.,* 2012; Swapnil *et al.,* 2014).

Studies have been carried out to verify the effect of Nitrogen source and the C/N ratio in order to increase the production of rhamnolipid and other biosurfactants (Abouseoud *et al.,* 2008; Wu *et al.,* 2008; Govindammal, 2014). *P. aeruginosa* is able to use Nitrogen sources such as ammonia or nitrate. However, in order to obtain high concentrations of rhamnolipids it is necessary to have restrained conditions of this macronutrient.(Abouseoud *et al.,* 2008; Wu *et al.,* 2008; Govindammal, 2014). This agrees with the results of this research were lower concentrations of Nitrogen favored biosurfactant production by the consortium especially in Palm oil even when no production was recorded by the single cultures in Tables 4.7c and 4.7d. The reason for no evidence of biosurfactant production (E_{24}) by the single organisms in Palm oil may be due to high levels of nitrogen that have been shown to inhibit the production of biosurfactant (Abouseoud *et al.,* 2008). This is because Palm oil wastes effluents have been shown to contain high levels of Nitrogen of up 2.1mg/L as shown in the studies by Parveen *etal*., 2010. Hence addition of Nitrogen in the form of sodium nitrate as carried out in this studies would be detrimental to the production of biosurfactant (Table 4.7c and Table 4.7d).

5.2.3 Effect of lipase on biosurfactant production

Lipases are enzymes belonging to the group of the hydrolases, whose main biological function is to catalyze the hydrolysis of insoluble triacylglycerols to free fatty acids, diacylglycerols and glycerol (Suyambu *et al.,* 2014). In this present study, Lipase was used

to release the glycerol in the Shea butter and Palm oil wastes making them readily available for use by the bacteria.

From the results of the Lipase optimization carried out (Tables 4.8a to 4.8d), the best results for biosurfactant was at concentration 0.1mg/ml and 0.15mg/ml for Shea butter.

5.3 Comparative Study of Biosurfactant Production from Shea butter and Palm oil as Carbon Source

The type, quality and quantity of biosurfactants produced by microorganisms are influenced by the nature of carbon source (Husam and Ahmed, 2013). Media containing Shea butter and Palm oil were used for production of biosurfactant by *P. aeruginosa* and *B. cereus*.

Many studies have been carried out using vegetable oils for the production of biosurfactants. Mercade *et al.*, (1993) were the first group to show the production of rhamnolipids by *P. aeruginosa* 47T2 when grown on olive oil mill effluent (OOME) as the sole carbon source. This study was important in demonstrating the possibility of using other lipophillic wastes for wider application (Kitamoto *et al.*, 1993).

In this present study, the two oil sources were found suitable for production of biosurfactant by *P. aeruginosa* and *B. cereus* (Table 4.9). Shea butter (Table 4.8) gave a higher biosurfactant production activity than Palm oil with absorbance (0.96), E_{24} (39.69%) and surface tension reduction (17.62dyne/cm) by the isolates compared to that of Palm oil having lower absorbance (0.68), E_{24} (23.20%) and surface tension of (18.8dyne/cm).

Shea butter wastes contain a high percentage of unsaponifiables, such as phytosterols (campesterol, stigmasterol, beta-sitosterol, and alpha-spinosterol) and triterpenes (cinnamic acid esters, alpha- and beta-amyrin, parkeol, buytospermol, and lupeol), and hydrocarbons such as karitene (Okullo *et al.,* 2010), Shea butter is also very rich in oleic acid which are short to medium chain fatty acids.

Palm oil like all fats, is composed of fatty acids, esterified with glycerol. Palm oil has an especially high concentration of saturated fat, specifically, of the 16-carbon saturated fatty acid Palmitic acid, to which it gives its name.

Although few research and reports have been made on biosurfactant production from Shea butter, Shea butter performed well in this study than the other substrates (Palm oil) as a carbon and energy source for biosurfactant production for all the measured parameters. The increased activity in Shea butter could be as a result of its higher percentage of shorter chain fatty acids compared to Palm oil. According to Thaniyavarn *etal*. (2008), biosurfactant activities in terms of oil displacement test and rhamnose content were observed to be higher with shorter chain fatty acids than that of the longer chains.

5.4 Comparative Study of Biosurfactant Production by *P.aeruginosa* and *B. cereus*

The comparative study of biosurfactant production from Shea butter and Palm oil by *P.aeruginosa* and *B. cereus* (Table 4.10), shows that *P. aeruginosa* performed better than *B. cereus* by having higher absorbance, E_{24} and surface tension reduction results (1.52, 38.6% and 17.9) when compared to that of *B. cereus,* (0.65, 26.48% and 18.85) in Shea butter.

This result agrees with the work of Sneha *et al.* (2012) whose research confirmed the ability of bacterial isolates to produce biosurfactant produce biosurfactants using different screening methods; including Emulsification test, emulsification index E_{24}, drop collapse method, oil spreading test and blood haemolysis test were assessed. His study suggested that *Pseudomonas* species showed maximum biosurfactant production compared to *Bacillus* spp. *Pseudomonas aeruginosa* was also reported to produce the biosurfactant rhamnolipid (Cameotra and Makkar, 2004) while *Bacillus* sp. is known to produce surfactin (Pornsunthorntawee *et al.*, 2008).

Though Bacillus and Pseudomonas have the ability of emulsifying oils. The research conducted by Priya and Usharani (2009) on biosurfactant production by four isolates of bacillus and pseudomonas from diesel oil, showed that *Pseudomonas* species gave better results compared to the *Bacillus* species. In another study, on biosurfactant production from petrol by *Pseudomonas* and *Bacillus* species, *Pseudomonas* species also recorded higher values for E_{24}, biosurfactants and lower surface tension values compared to *Bacillus sp.* (Suganya, 2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study was carried to isolate and characterize *Bacillus cereus* and *Pseudomonas aeruginosa* from soil samples and to screen for their biosurfactant production activity. These organisms were screened for production of biosurfactants from Shea butter and Palm oil waste slurries using single cultures and consortium of *Bacillus cereus* and *Pseudomonas aeruginosa.* Finally, optimization studies of pH, Nitrogen and lipase on biosurfactant production by the isolates bacterial isolates in Shea butter and Palm oil waste slurries was done. There were significant differences between the varied environmental parameters (pH, Nitrogen and Lipase) It was concluded that best results for biosurfactant production was from Shea butter slurry while *Pseudomonas aeruginosa* produced more biosurfactant from both slurries compared to *Bacillus cereus*.

The results and observations made in this study drew the following conclusions:

Pseudomonas and *Bacillus* isolates from oil polluted soils were identified as *Bacillus cereus, Bacillus licheniformis, Pseudomonas aeruginosa* and *Pseudomonas fluorescens*.

Isolates of *Pseudomonas aeruginosa* and *Bacillus cereus* showed positive results for preliminary screening tests for biosurfactant production via oil spreading, Emulsification index (E_{24}) and blood haemolysis techniques.

The single cultures of the bacteria and their consortium showed positive results for biosurfactant production in Shea butter and Pam oil.

The optimization studies carried out showed that pH, nitrogen sources and lipase levels and concentrations are contributory factors affecting biosurfactant production from Shea butter and palm oil.

This study revealed that at pH of 7, Sodium Nitrate concentration of 0.3mg/100ml to 0.4mg/100mls and Lipase concentrations of 0.1mg/ml to 0.15mg/ml, the production of biosurfactant was at its best. Based on the two substrates (Shea butter and palm oil) used as carbon and energy source for biosurfactant production by the bacterial isolates, Shea butter performed best by giving a high levels of emulsification stability (E_{24}), higher Absorbance, and lower surface tension readings.

Although, both bacterial isolates of *Pseudomonas aeruginosa* and *Bacillus cereus* have the ability to secrete surface active agents, *Pseudomonas aeruginosa* performed better than *Bacillus cereus* in terms of emulsification stability and surface tension reduction. Hence these findings can be applied for industrial and environmental applications.

6.2 Recommendations

1. Bacteria with proven capacity to produce biosurfactants should be applied in industries and environment to replace their chemical counterparts.
2. Large scale production of biosurfactant from microorganisms should be encouraged by building biotechnological systems that provide optimum conditions that encourage high production of biosurfactants at minimal economic costs.

3. Shea butter, Palm oil and other agro-based renewable wastes should be recycled into biosurfactants rather than disposing them into the environment to cause pollution.

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APPENDICES

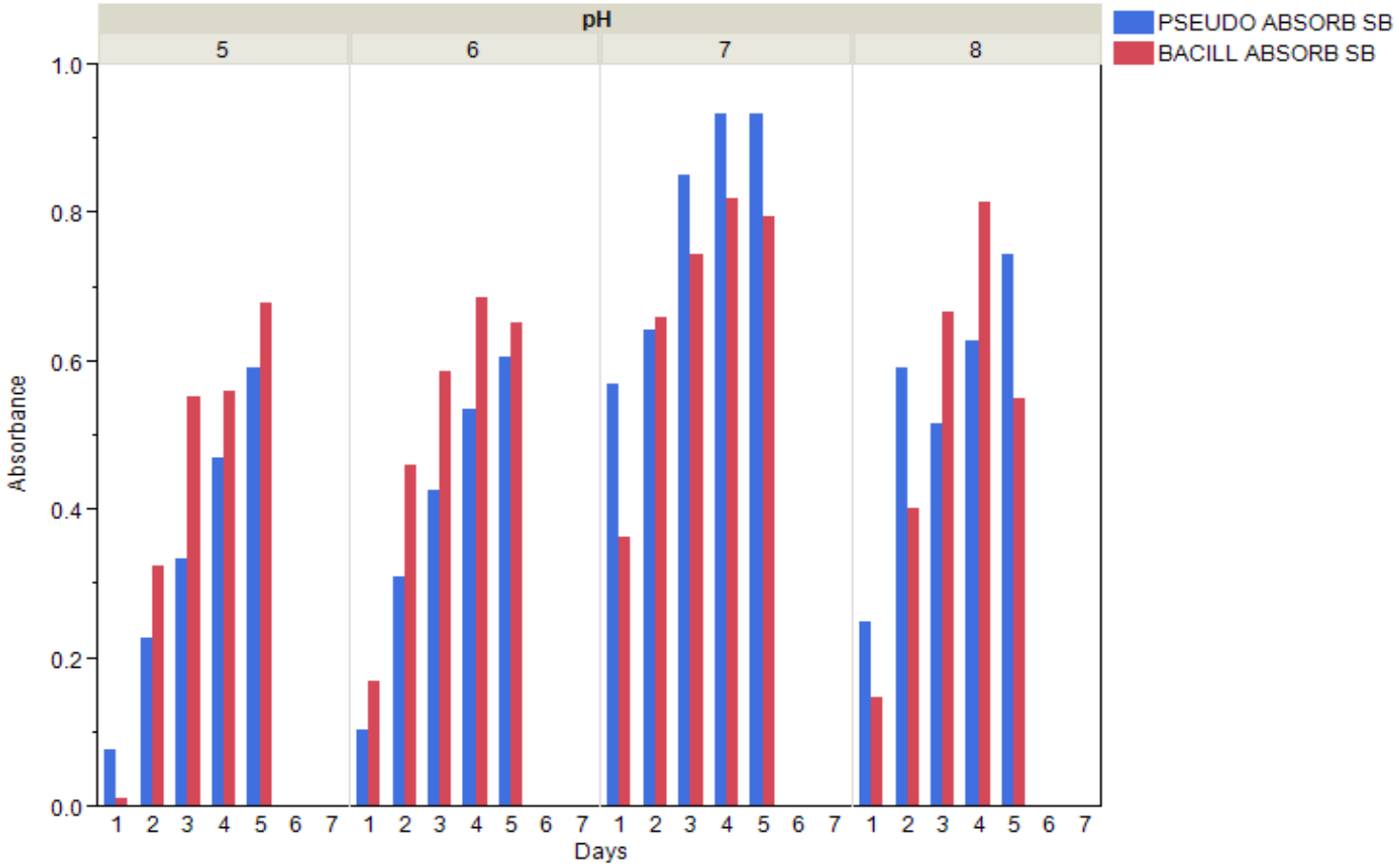
Appendix I: List of some Biosurfactant Producing Microorganisms

Biosurfactants	**Microorganisms**	**Economic importance**	**References**
Rhamnolipids	*Pseudomonas aeruginosa,Serratia Rubidea,*	Antimicrobial, biocontrol properties	Jadhav *et al.,* 2011
Viscosin	*Pseudomonas fluorescens, L. mesenteriods*	lipopeptides	Banat *et al.,* 2010
Sophorolipids	*Torulopsis petrophilum, Candida bombicola,*	Antiviral and Spermicidal	Herman and Maier, 2002
Trehalose lipids	*Rhodococcus erythropolis, Arthrobacter sp., Nocardia erythropolis, Corynebacterium sp.*	Dissolution of hydrocarbons	Muthusamy *et al.,* 2008
Cellobiose lipids	*Ustilago maydis*	Antifungal Compounds	Teichmann *et al.,* (2007)
Ornithine lipids	*Pseudomonas sp., Thiobacillus thiooxidans, Agrobacterium sp. Leuconostoc mesenteriods*	Bio-emulsifiers	Desai and Banat, (1997)
Carbohydrate-lipid	*P.fluorescens, Debaryomyces*	Bio-emulsifiers	Nerurka *et al.,*

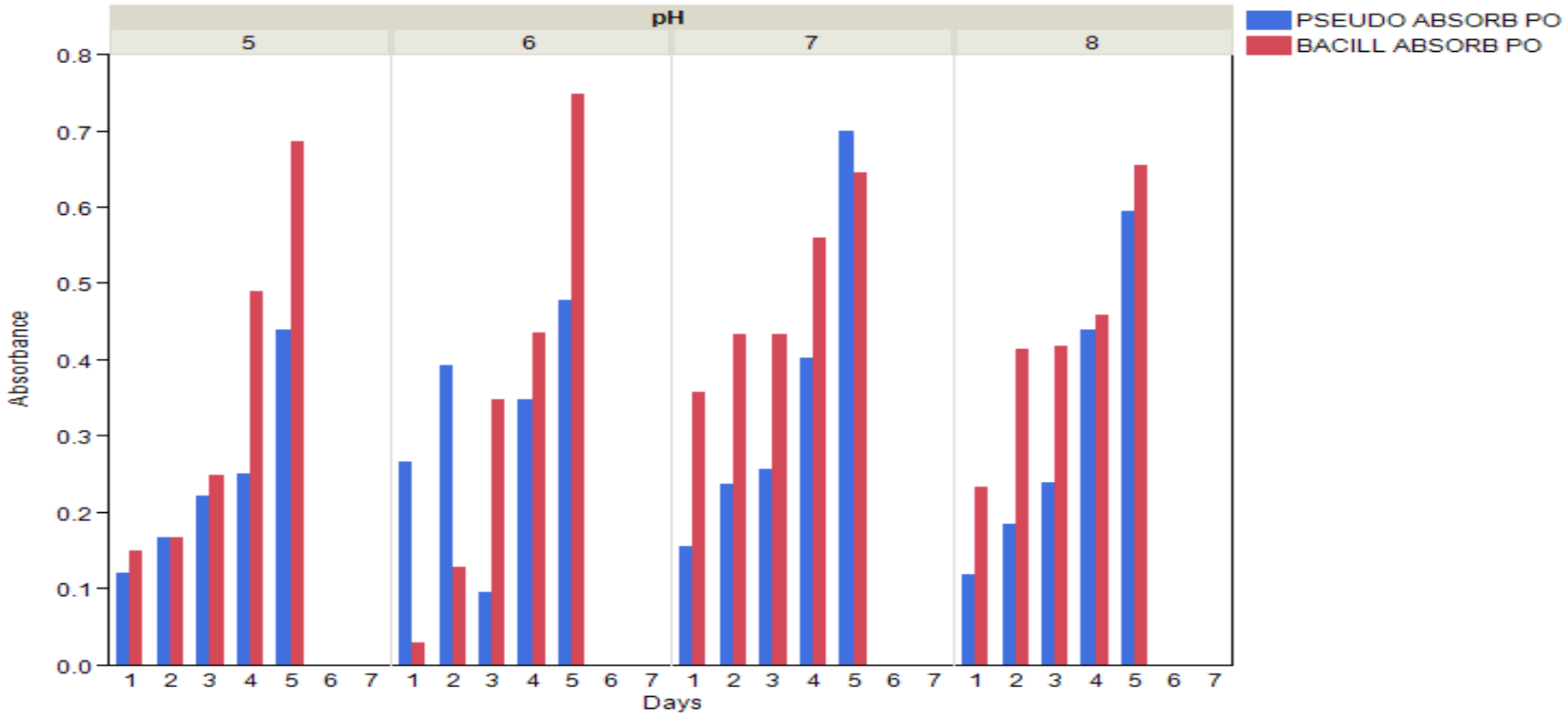
	polmorphus		
Protein PA	*P.aeruginosa*	Bio-emulsifiers	Hisatsuka *et al.*, 1999
Diglycerides	*Lactobacillus fermentum*	Bio-remediation	Mulligan *et al.*, 2001
Whole cell	*Cyanobacteria*	Bio-flocculent	Levy *et al.,* 1990
Fattyacids	*Clavibacter michiganensis*	Bio-emulsifiers	Herman and Maier, 2002
Fattyacids	*Clavibacter michiganensis*	Bio-emulsifiers	Herman and Maier, 2002
Phospholipids	*Acinetobacter sp.*	Bioremediation	Kosaric, 2009
Vesicles, fimbriae	*Acinetobacter calcoaceticus, P.marginilis,*	Bioremediation	Muthusamy *et al.*, 2008
Emulsan	*A. calcoaceticus*	Enhanced oil recovery	Choi *et al.,* 1996
Alasan	*A. radioresistens*	Biodegradation of aromatic compounds	Barkay *et al.,* 1999
Surfactin/Iturin	*B. subtilis, B. amyloliquefaciens*	Antimicrobial properties	Arguella *et al.,* 2009
Subtilisin	*B. subtilis*	Antimicrobial properties	Sutyak *et al.,* 2008
Aminoacids lipids	*Bacillus* sp.	Antimicrobial properties	Cotter *et al.,* 2005
Lichenysin	*Bacillus licheniformis, B. subtilis*	oil recovery	Yakimov *et al.*, 2009
Peptide lipids	*B. licheniformis*	Antimicrobial	Begley *et al.,* 2009

properties

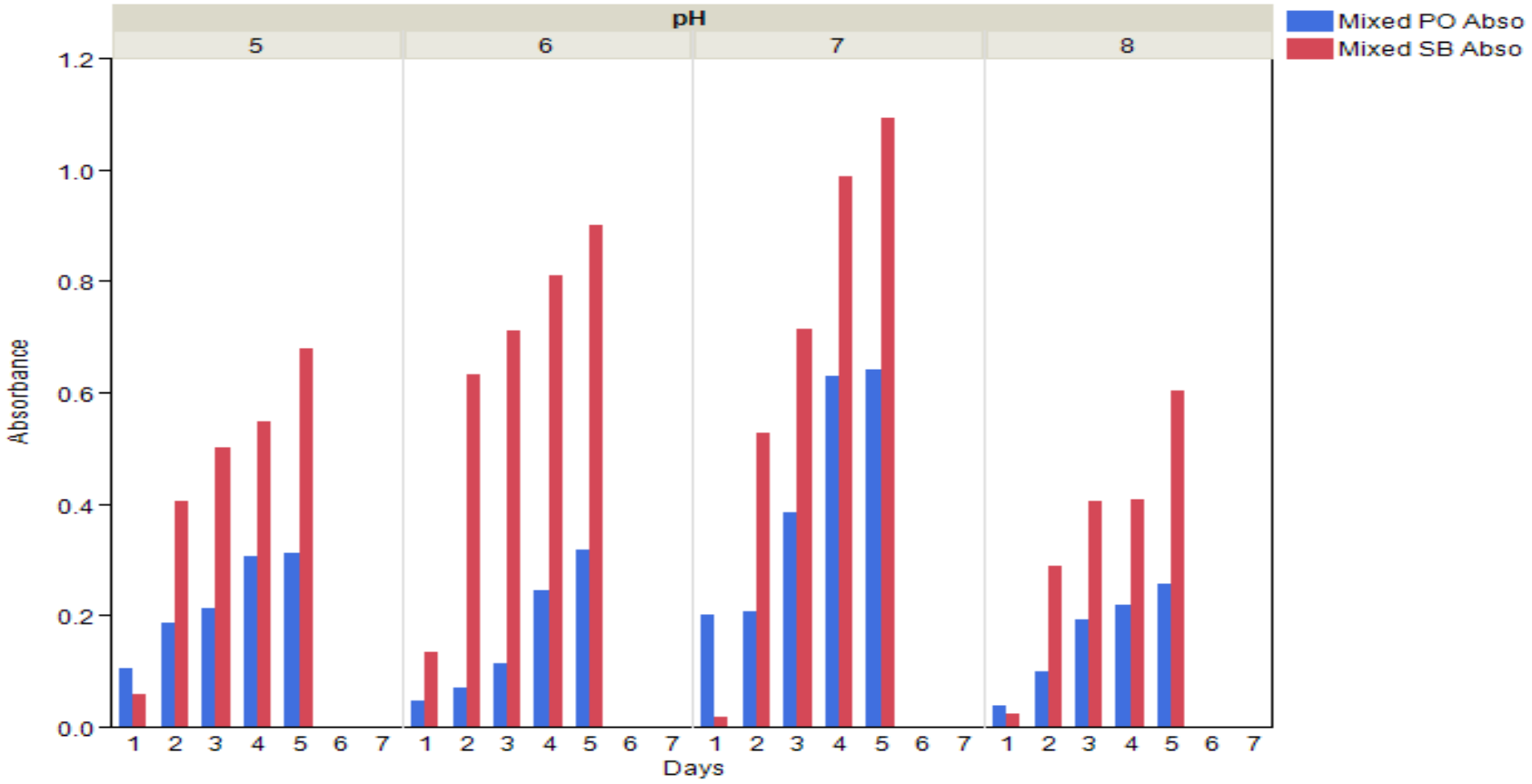
Appendix II: Daily Growth Measurement of *P. aeruginosa* and *B. cereus* in Shea butter Waste Slurry at varying pH Levels



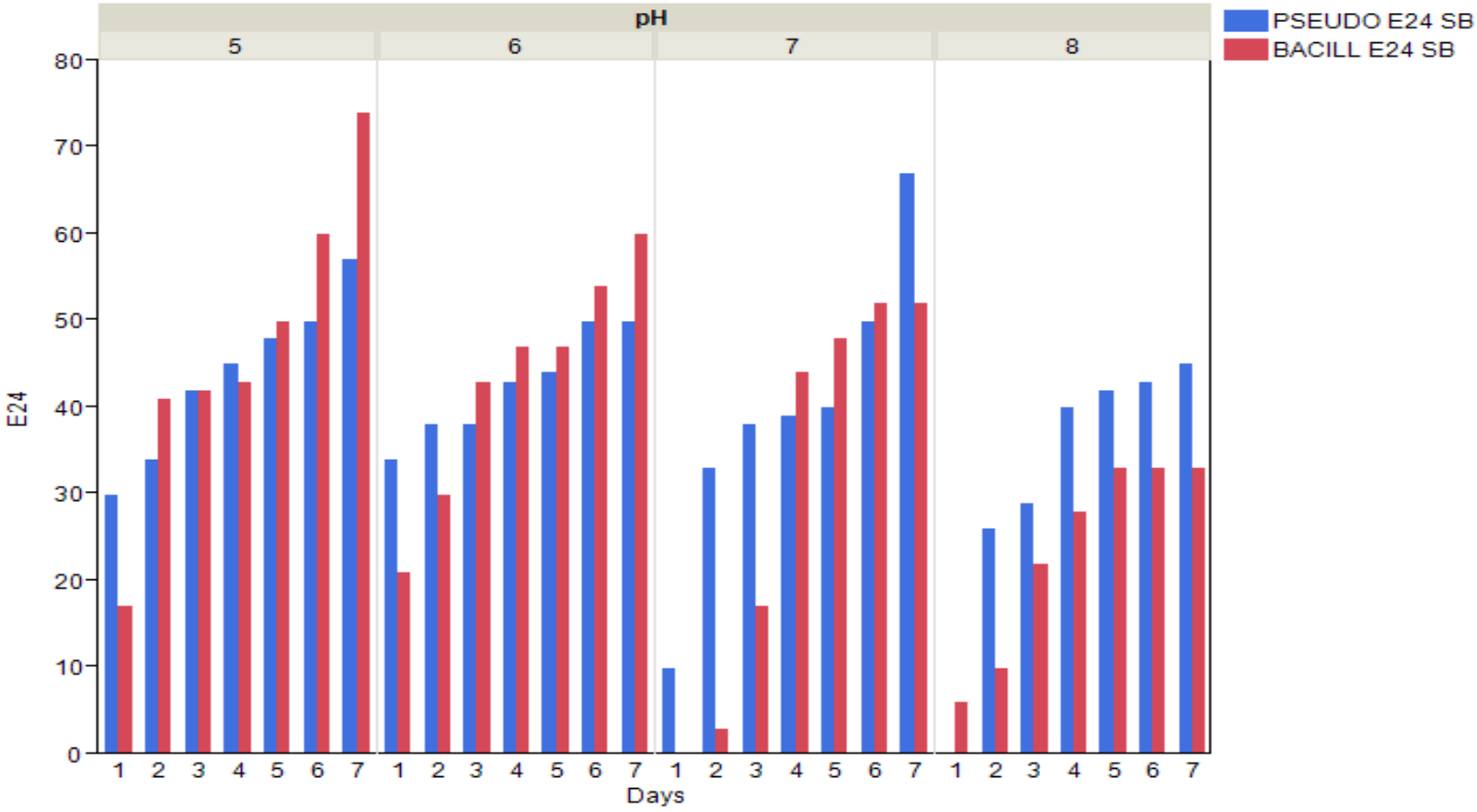
Appendix III: Daily Growth Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at varying pH Levels



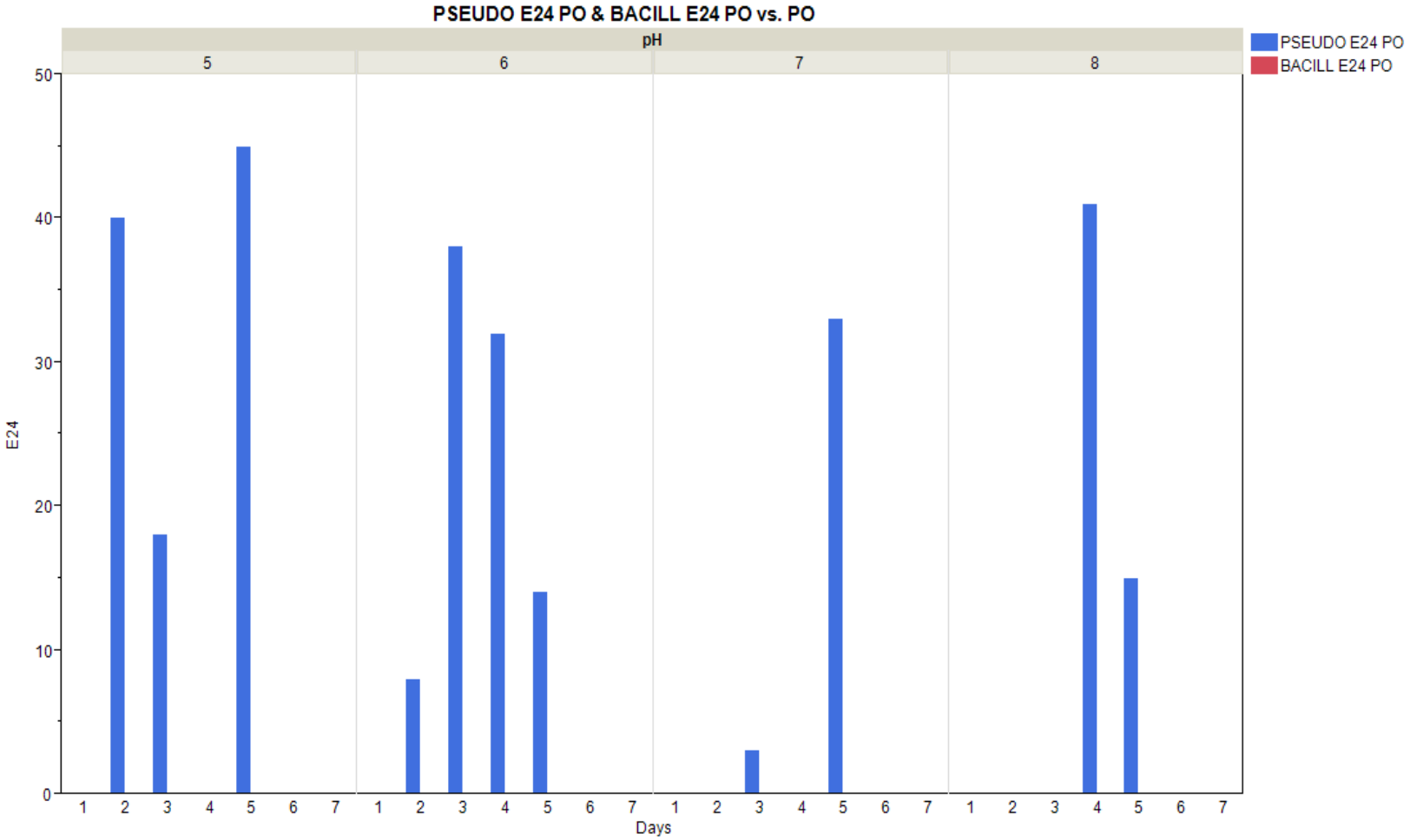
Appendix IV: Daily Growth Measurement of consortium of *P. aeruginosa* and *B. cereus* in Palm Oil and Shea butter Waste Slurries at varying pH Levels



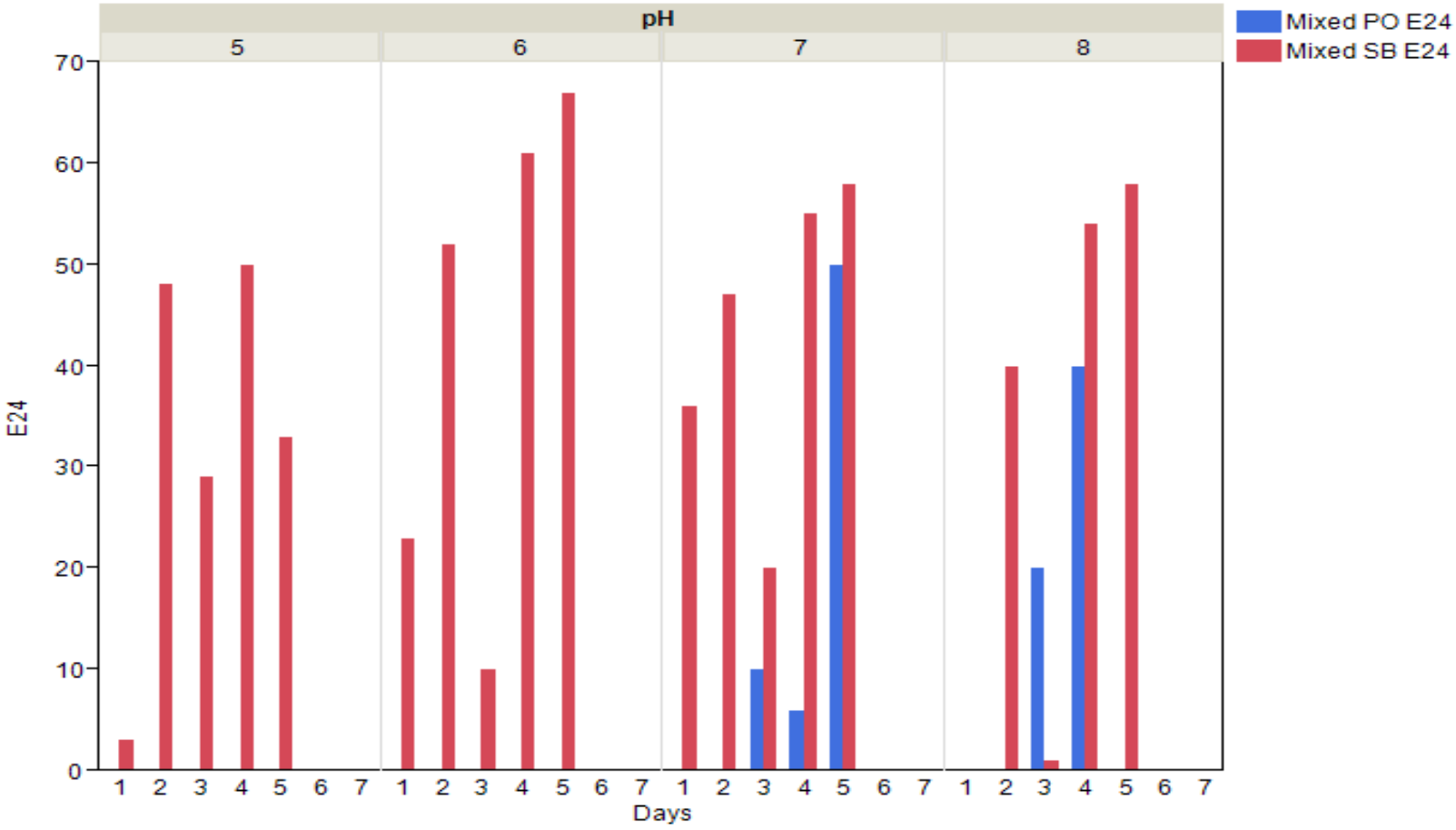
Appendix V: E_{24} Measurement of *P. aeruginosa* and *B. cereus* in Shea butter Waste Slurry at varying pH Levels



Appendix VI: E_{24} Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at varying pH Levels



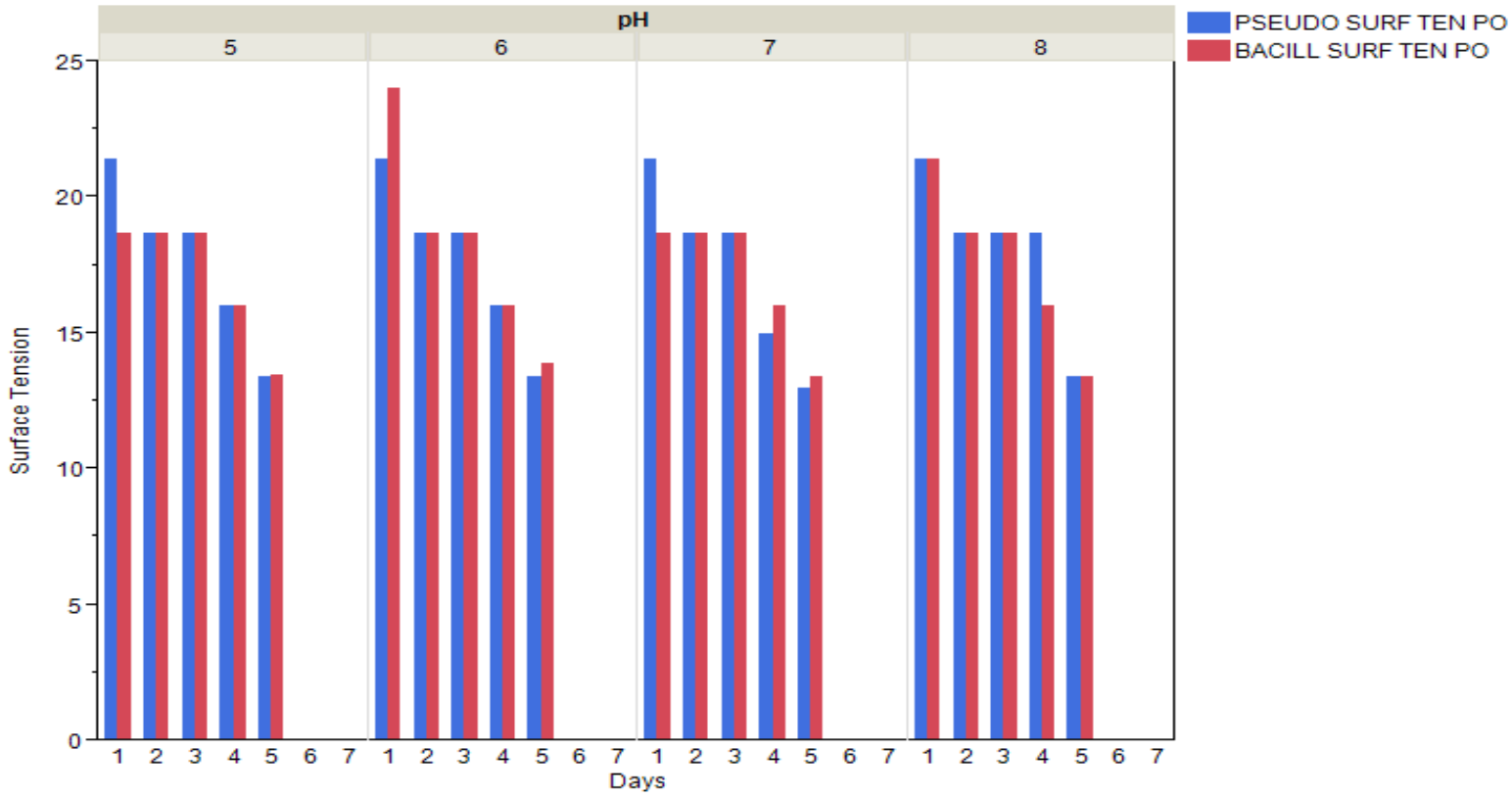
Appendix VII: E_{24} Measurement of consortium of *P. aeruginosa* and *B. cereus* in Shea butter and Palm Oil Waste Slurries at varying pH Levels



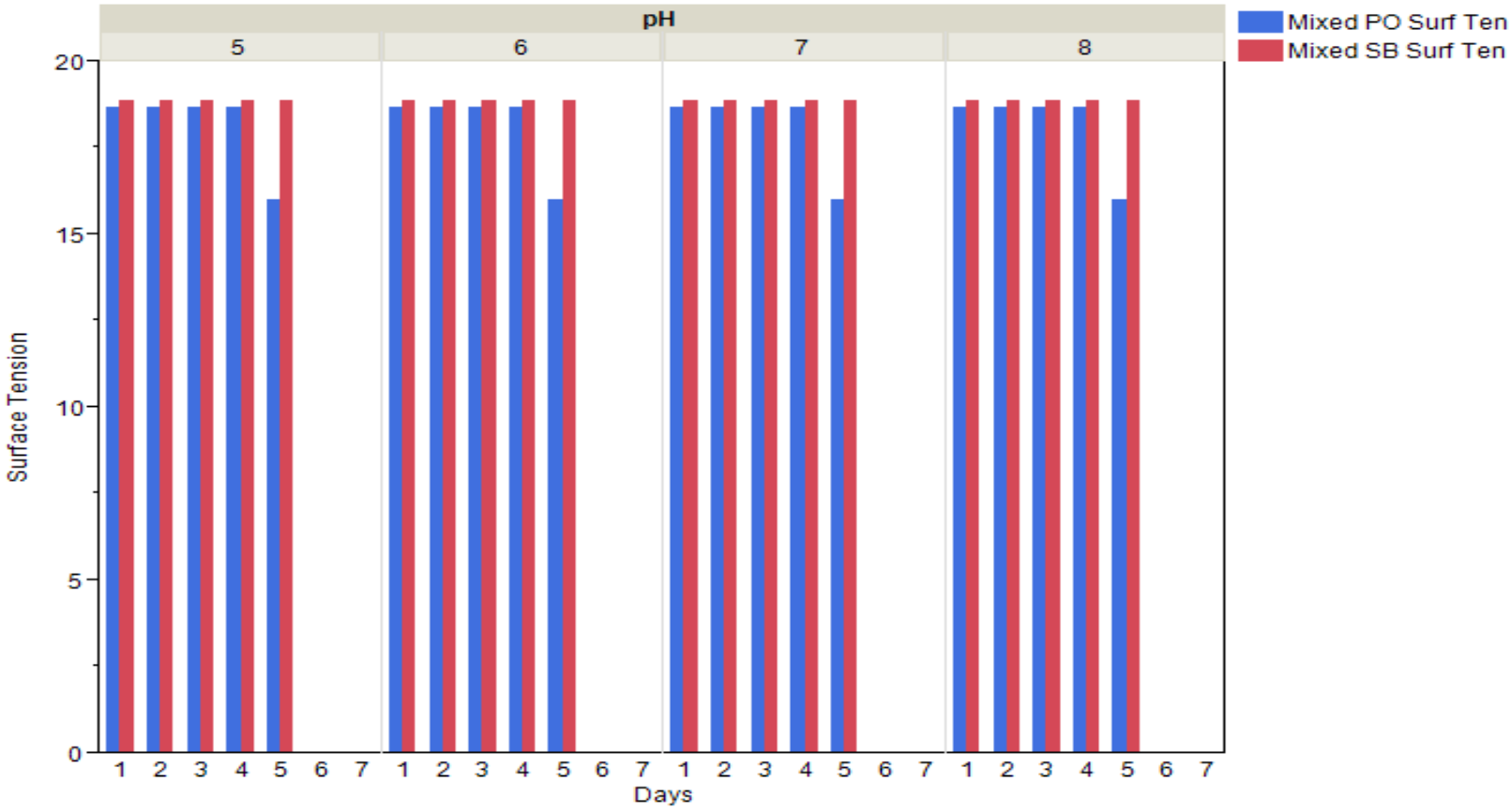
Appendix VIII: **Daily Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Shea Butter Waste Slurry at varying pH Levels**



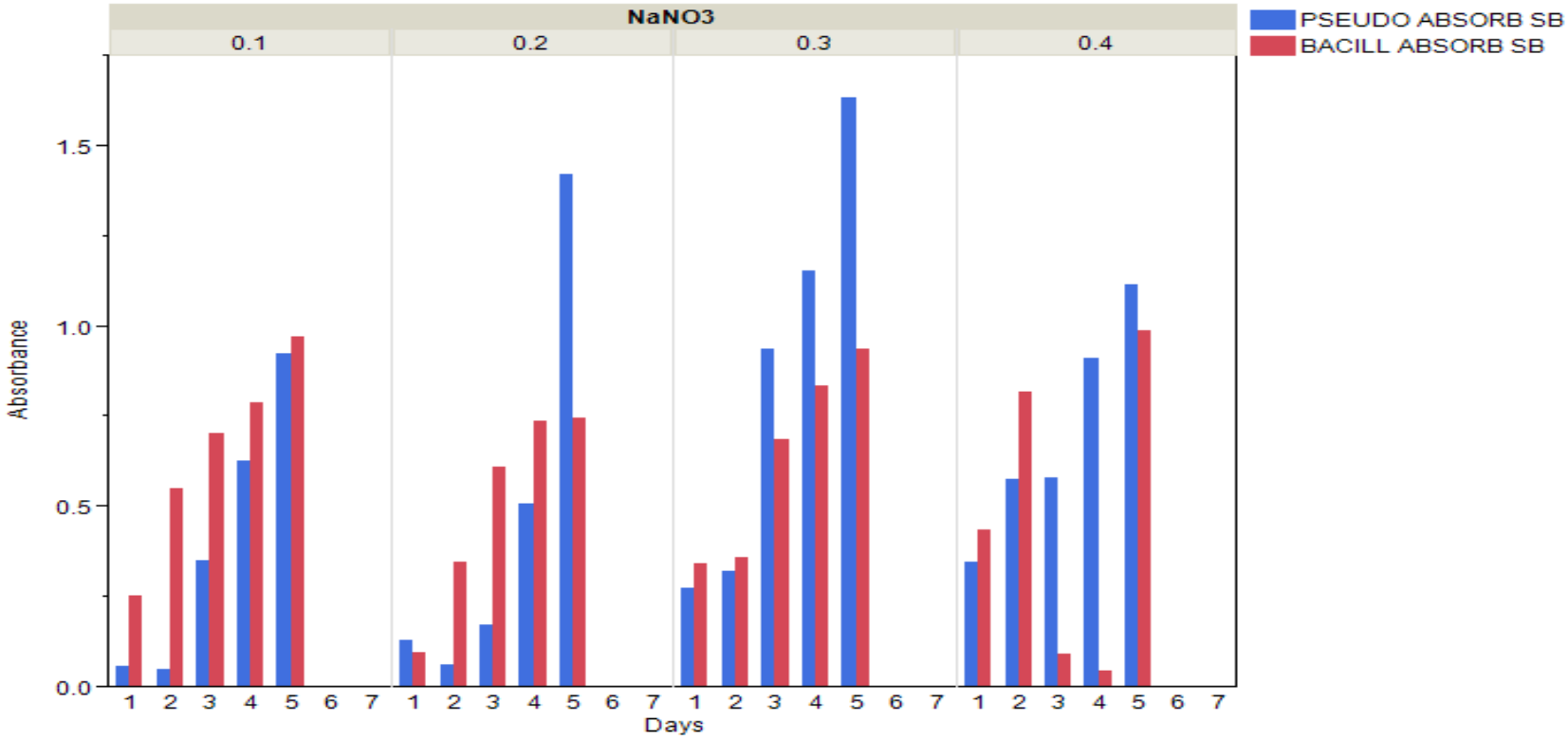
Appendix IX: **Daily Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at varying pH Levels**



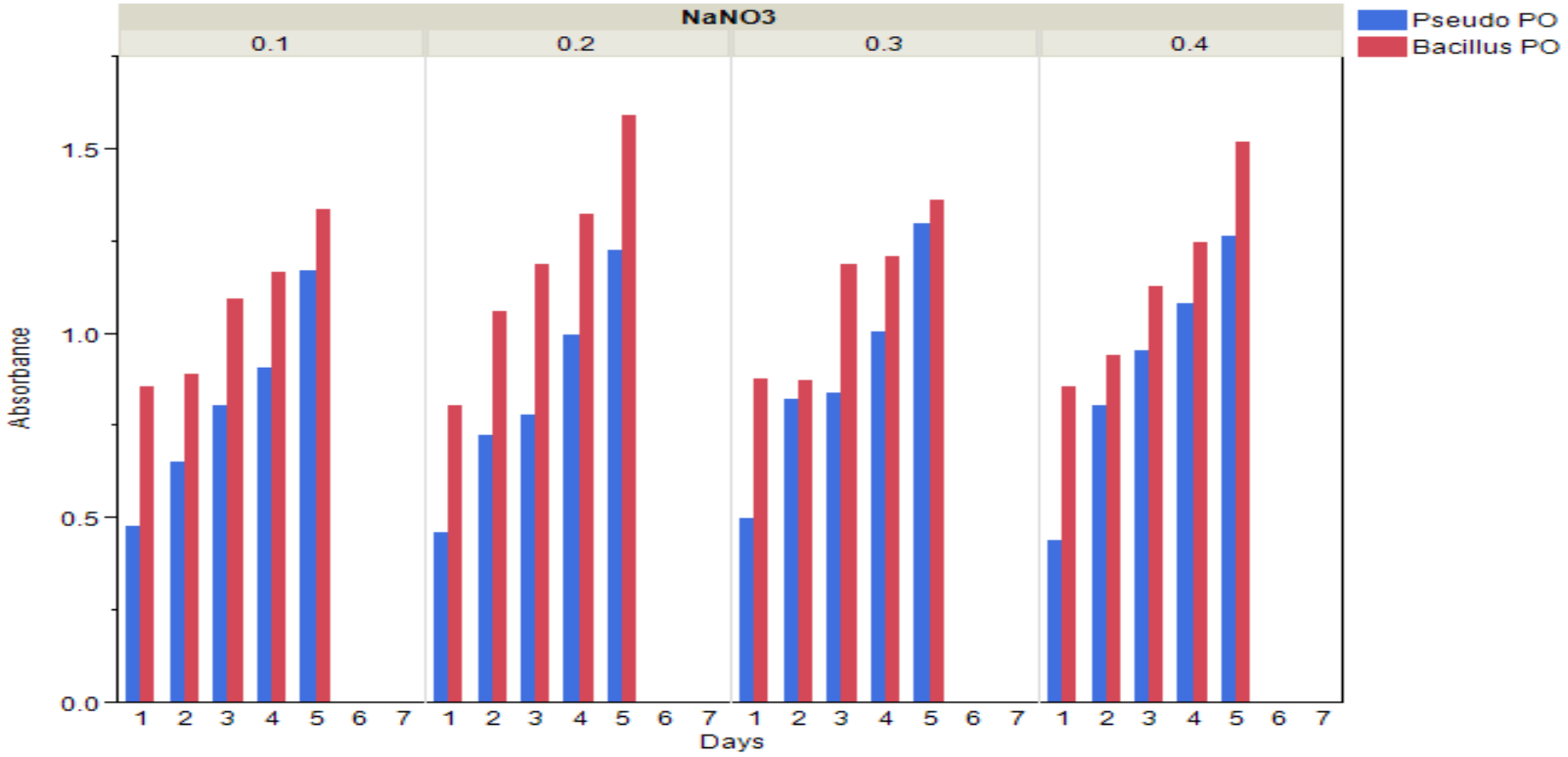
Appendix X: Daily Surface Tension Measurement for consortium of *P. aeruginosa* and *B. cereus* in Shea Butter and Palm Oil Waste Slurries at varying pH Levels



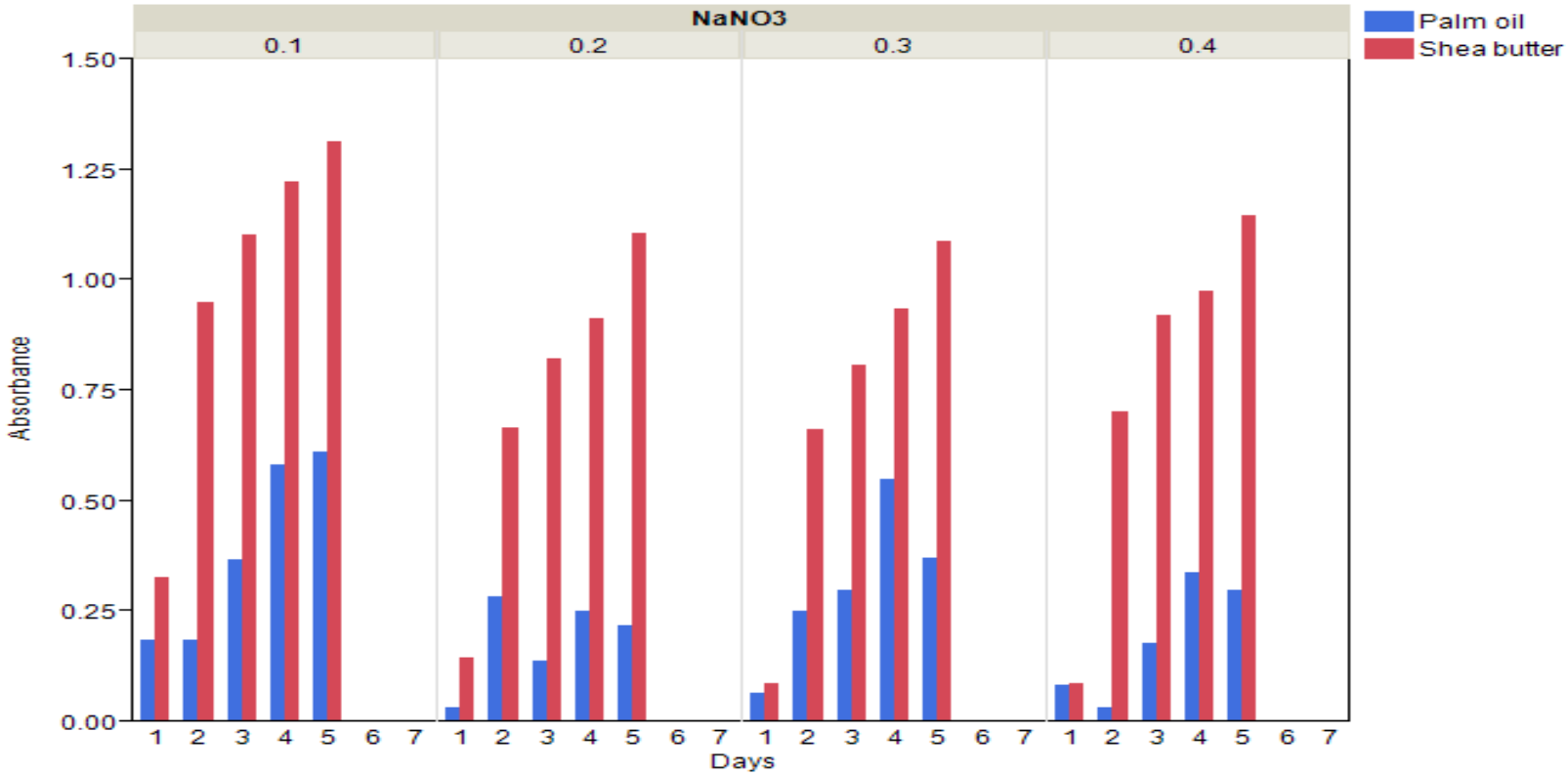
Appendix XI: Daily Growth Measurement of *P. aeruginosa* and *B. cereus* in Shea butter Waste Slurry at varying Concentrations of $NaNO_3$



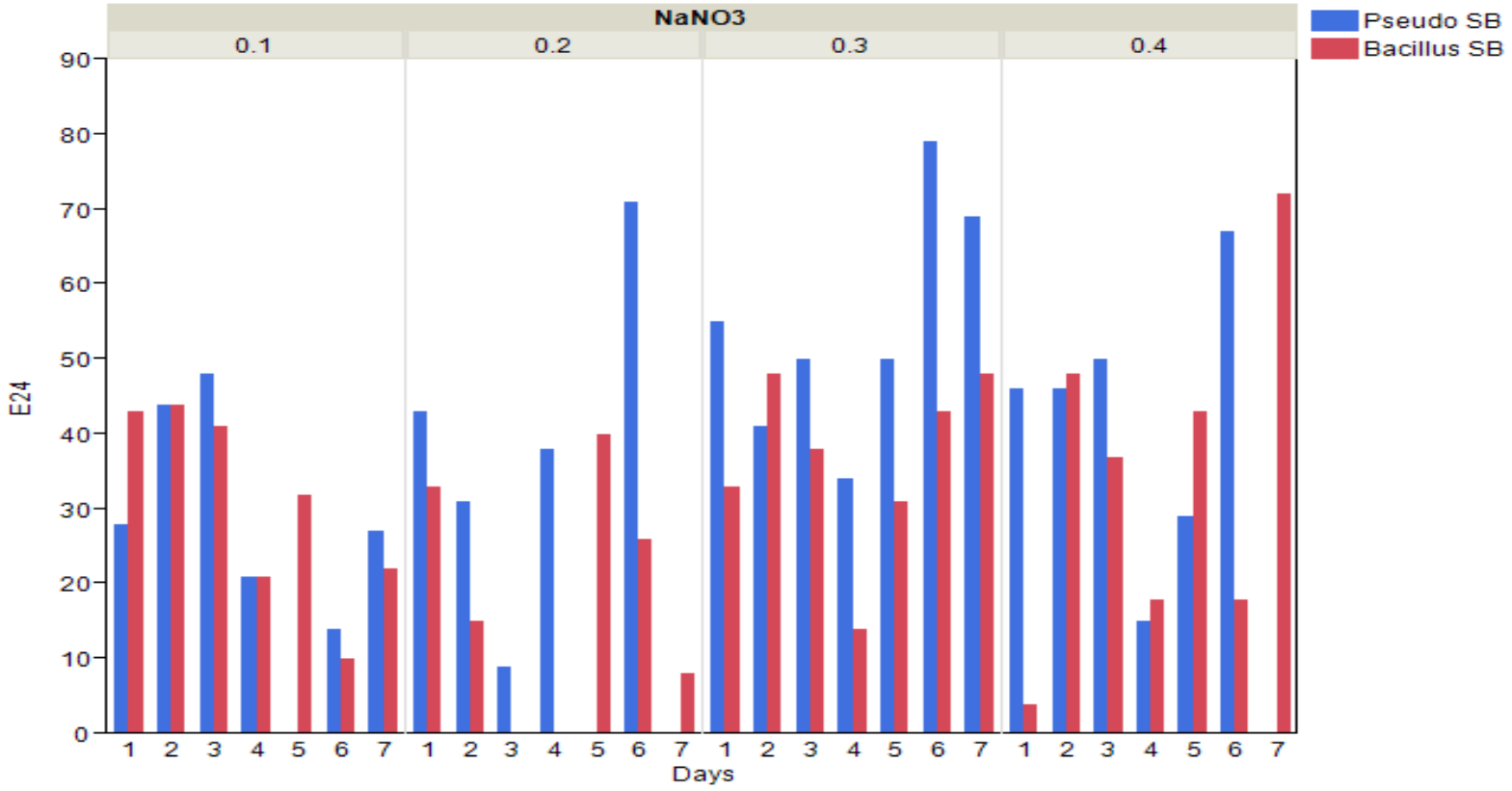
Appendix XII: Daily Growth Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at varying Concentrations of $NaNO_3$



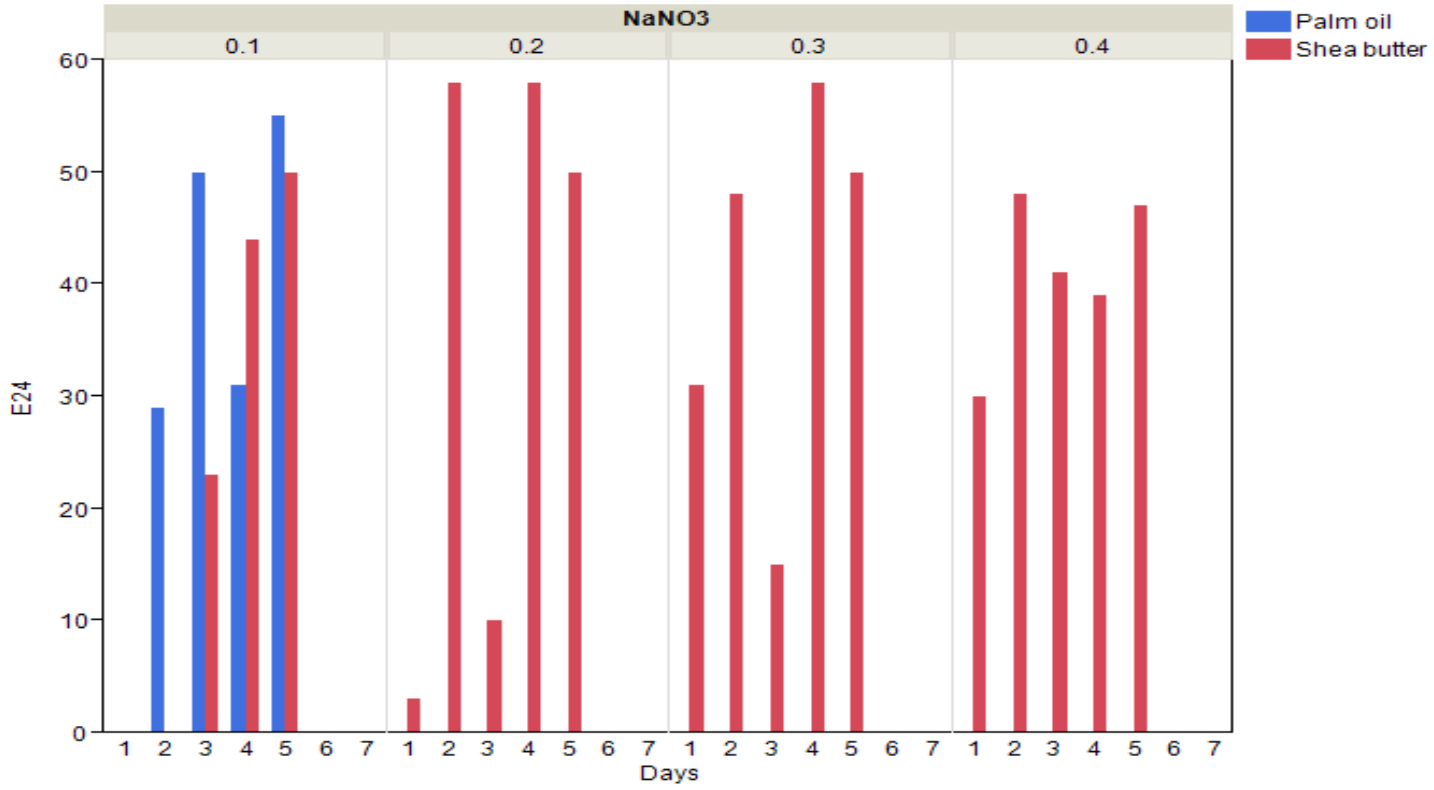
Appendix XIII: Daily Growth Measurement of consortium of *P. aeruginosa* and *B. cereus* in Shea Butter and Palm Oil Waste Slurries at varying Concentrations of $NaNO_3$



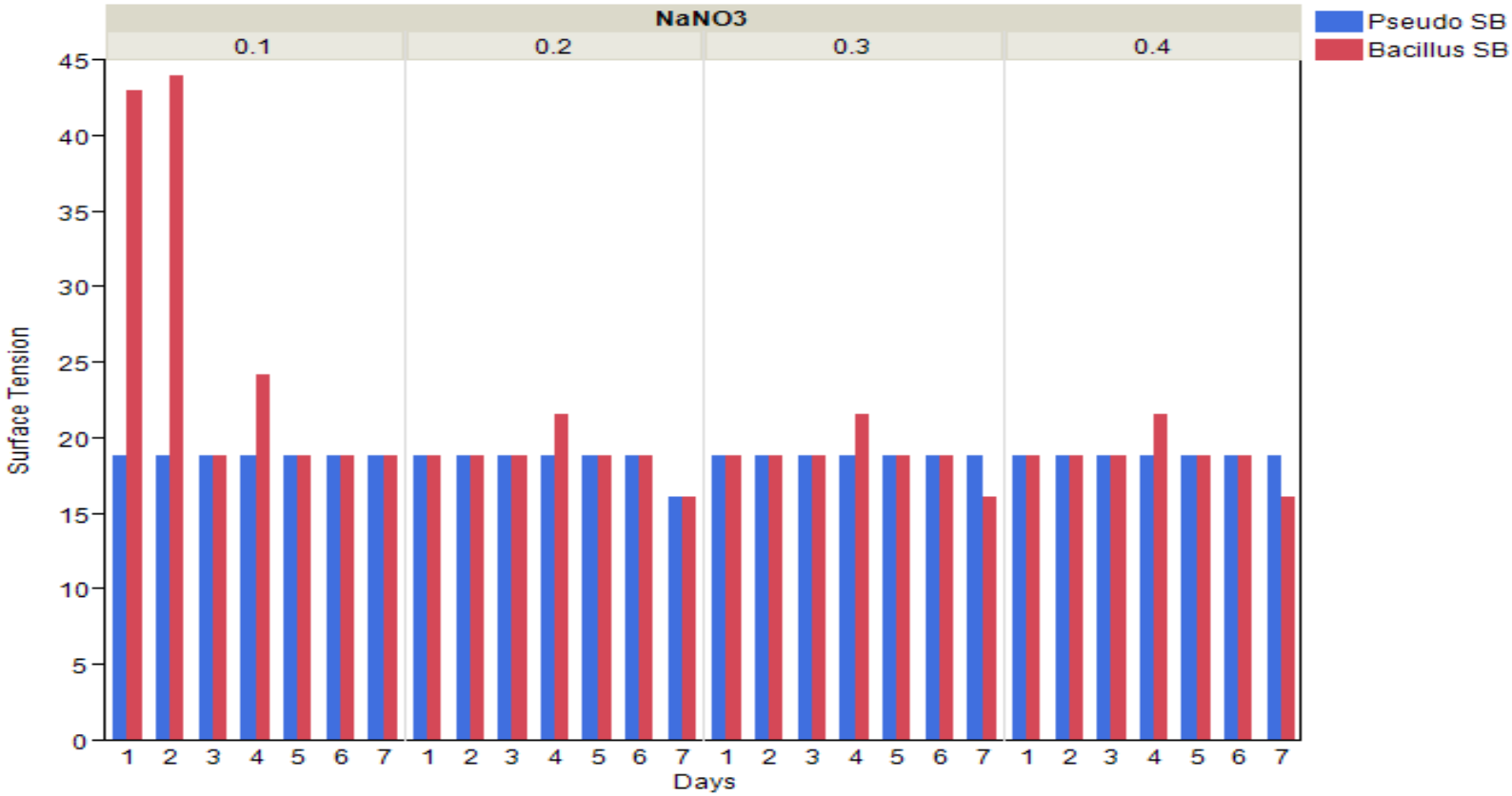
Appendix XIV: E_{24} Measurement of *P. aeruginosa* and *B. cereus* in Shea Butter Waste Slurry at Varying Concentrations of $NaNO_3$



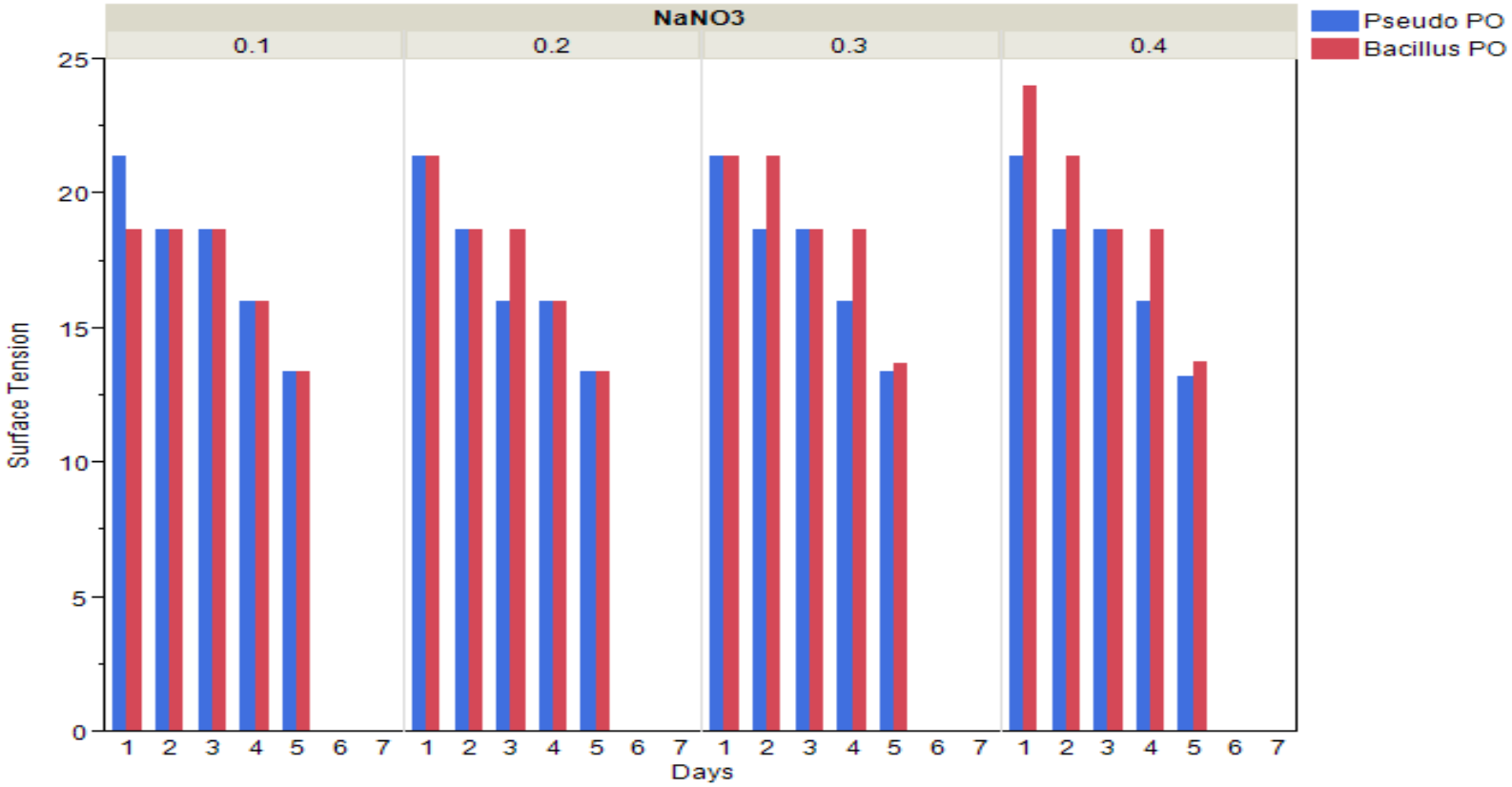
Appendix XV: E_{24} Measurement of consortium of *P. aeruginosa* and *B. cereus* in Shea Butter and Palm Oil Waste Slurries at Varying Concentrations of $NaNO_3$



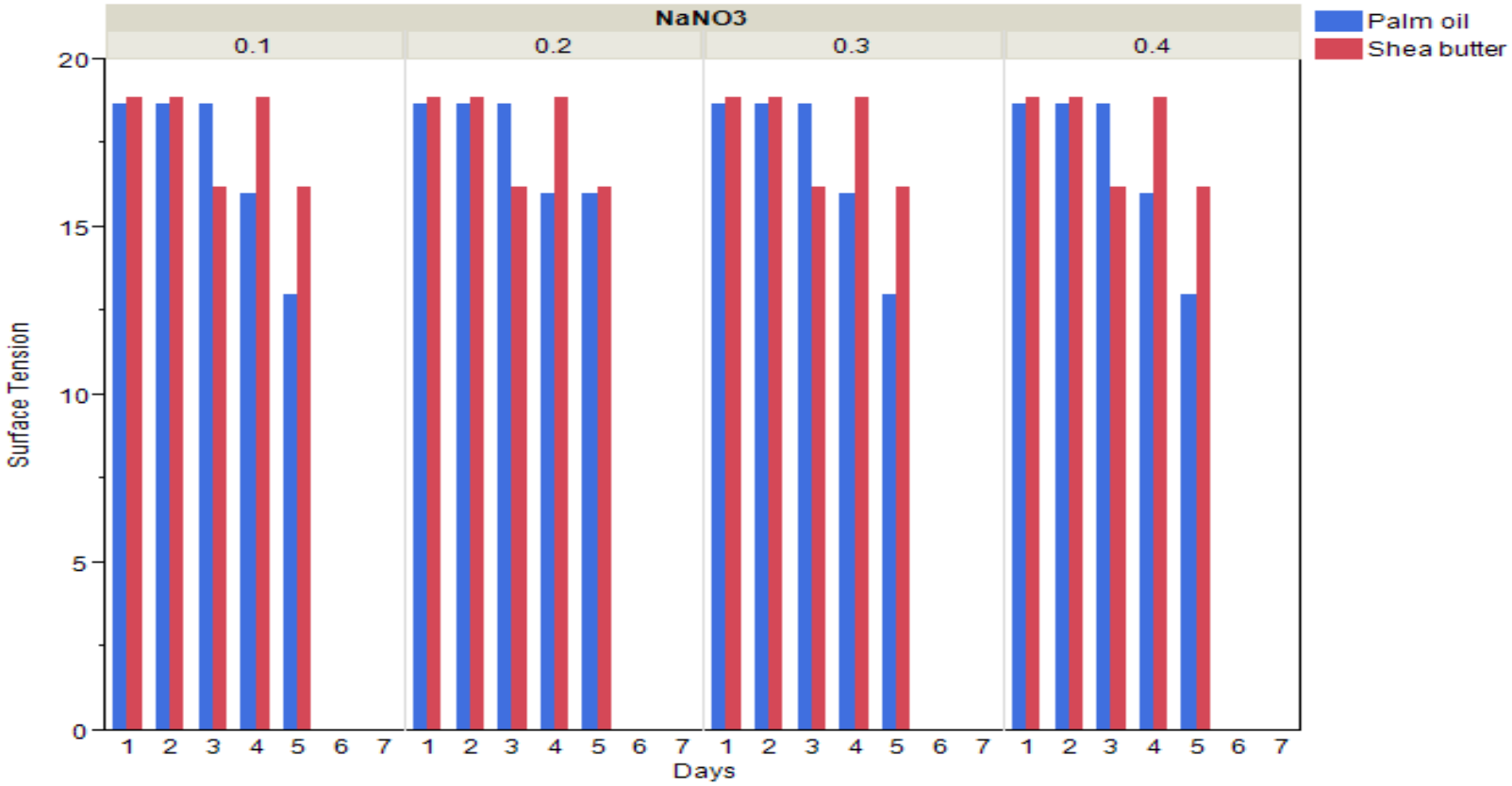
Appendix XVI: **Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Shea Butter Waste Slurry at varying Concentrations of $NaNO_3$**



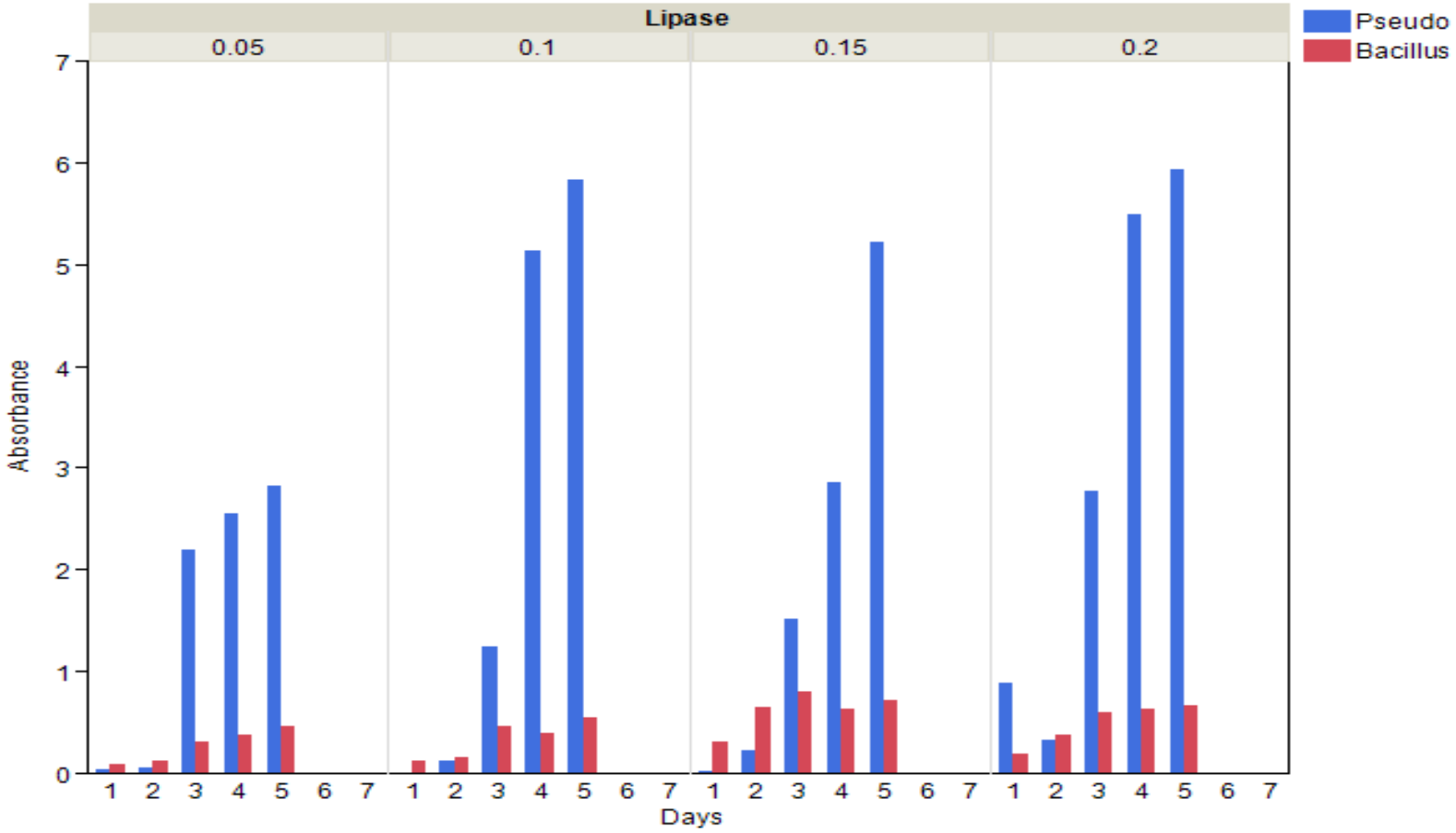
Appendix XVII: **Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at varying Concentrations of $NaNO_3$**



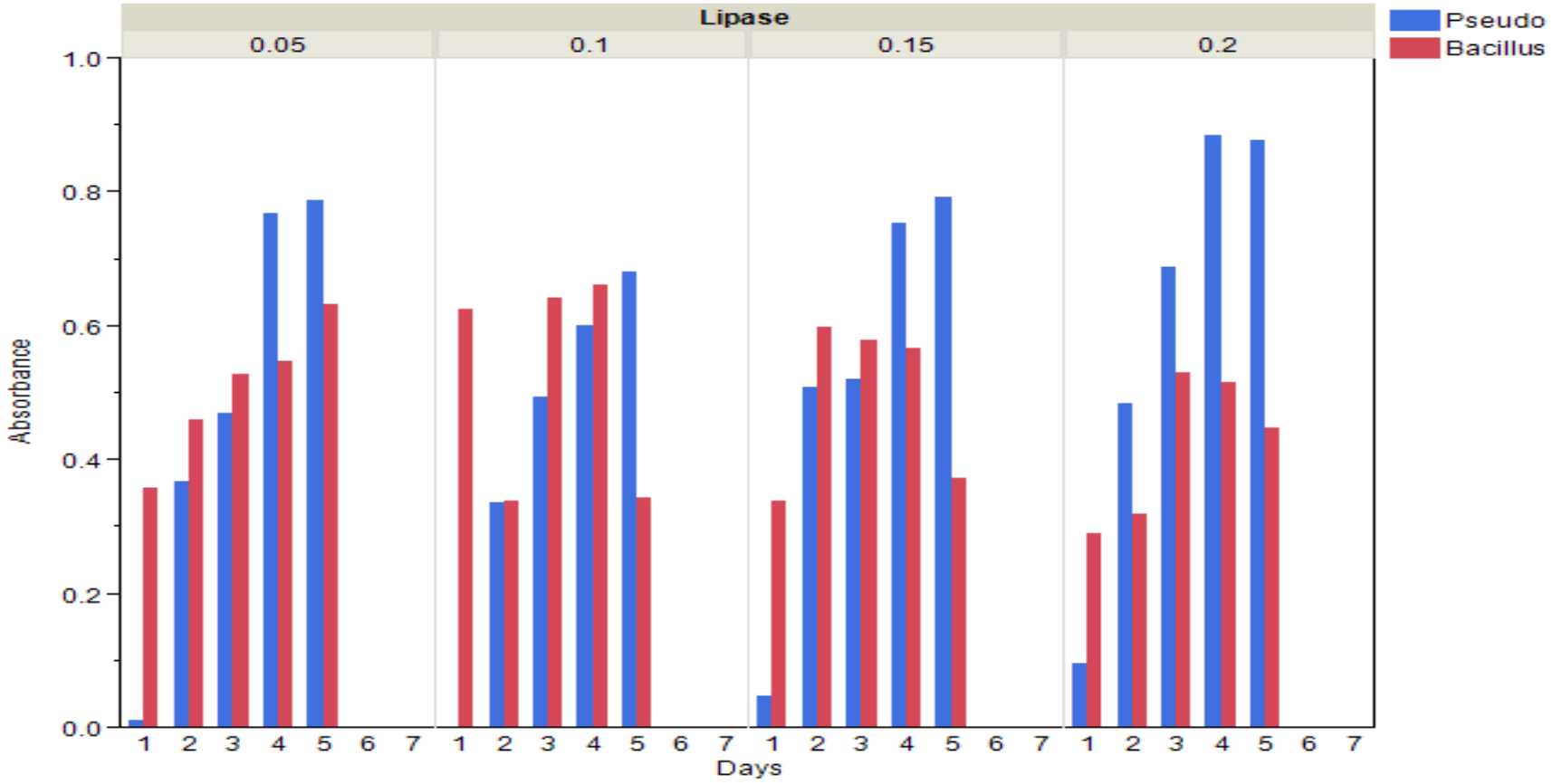
Appendix XVIII: Surface Tension Measurement of consortium *P. aeruginosa* and *B. cereus* in Shea Butter and Palm Oil Waste Slurries at varying Concentrations of $NaNO_3$



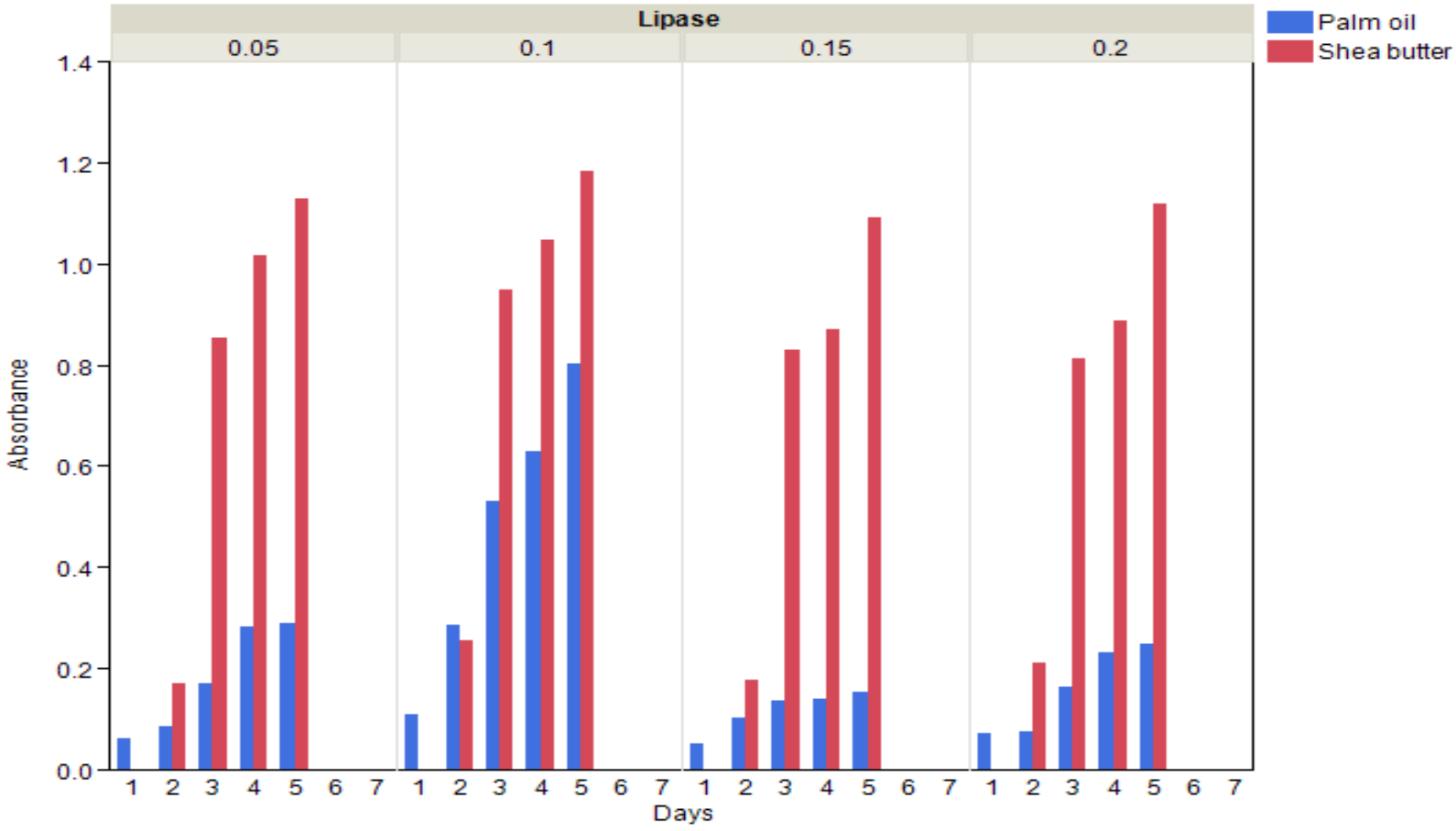
Appendix XIX: **Daily Growth Measurement of *P. aeruginosa* and *B. cereus* in Shea butter Waste Slurry at Varying Lipase Concentrations**



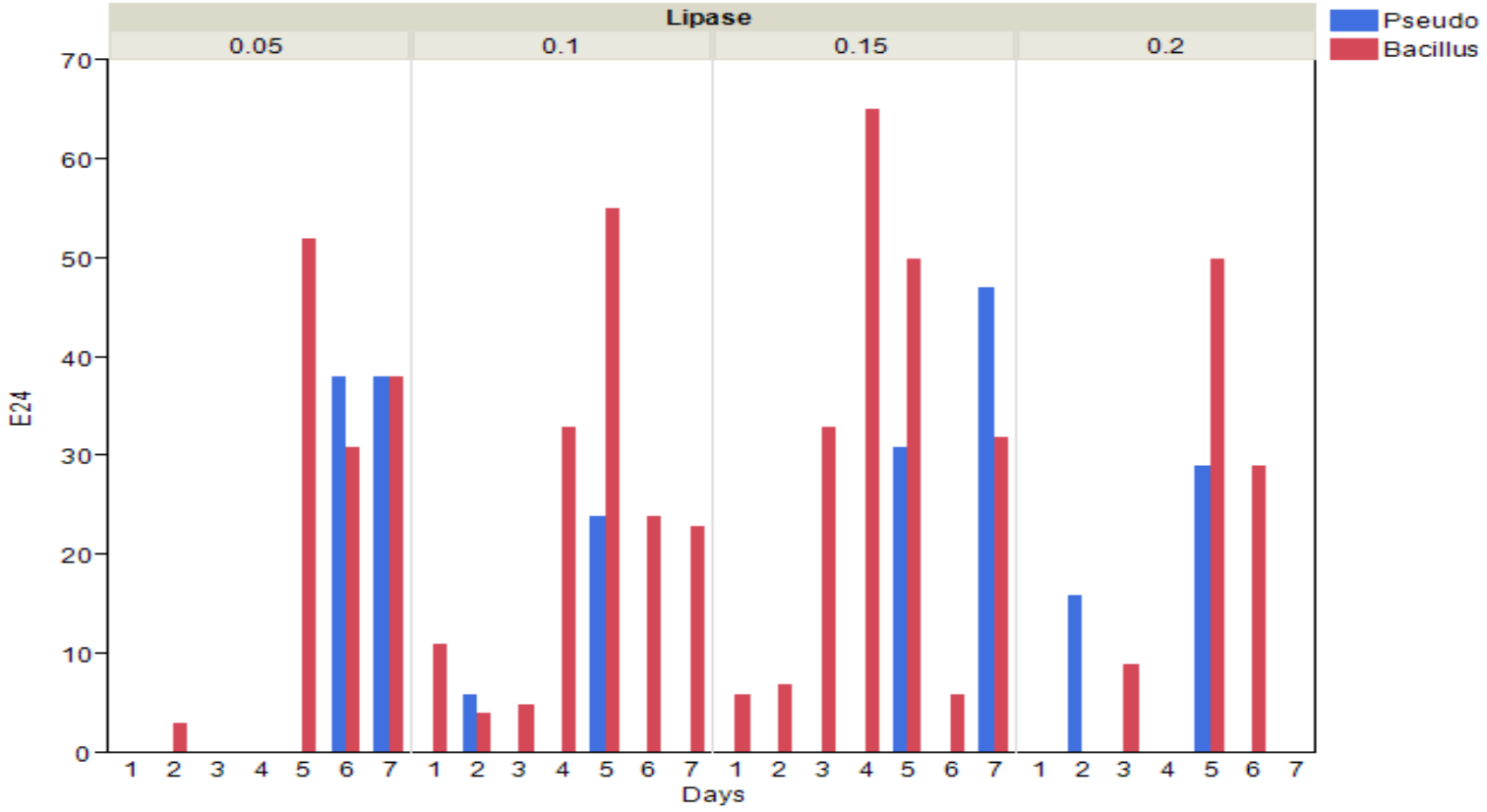
Appendix XX: **Daily Growth Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at Varying Lipase Concentrations**



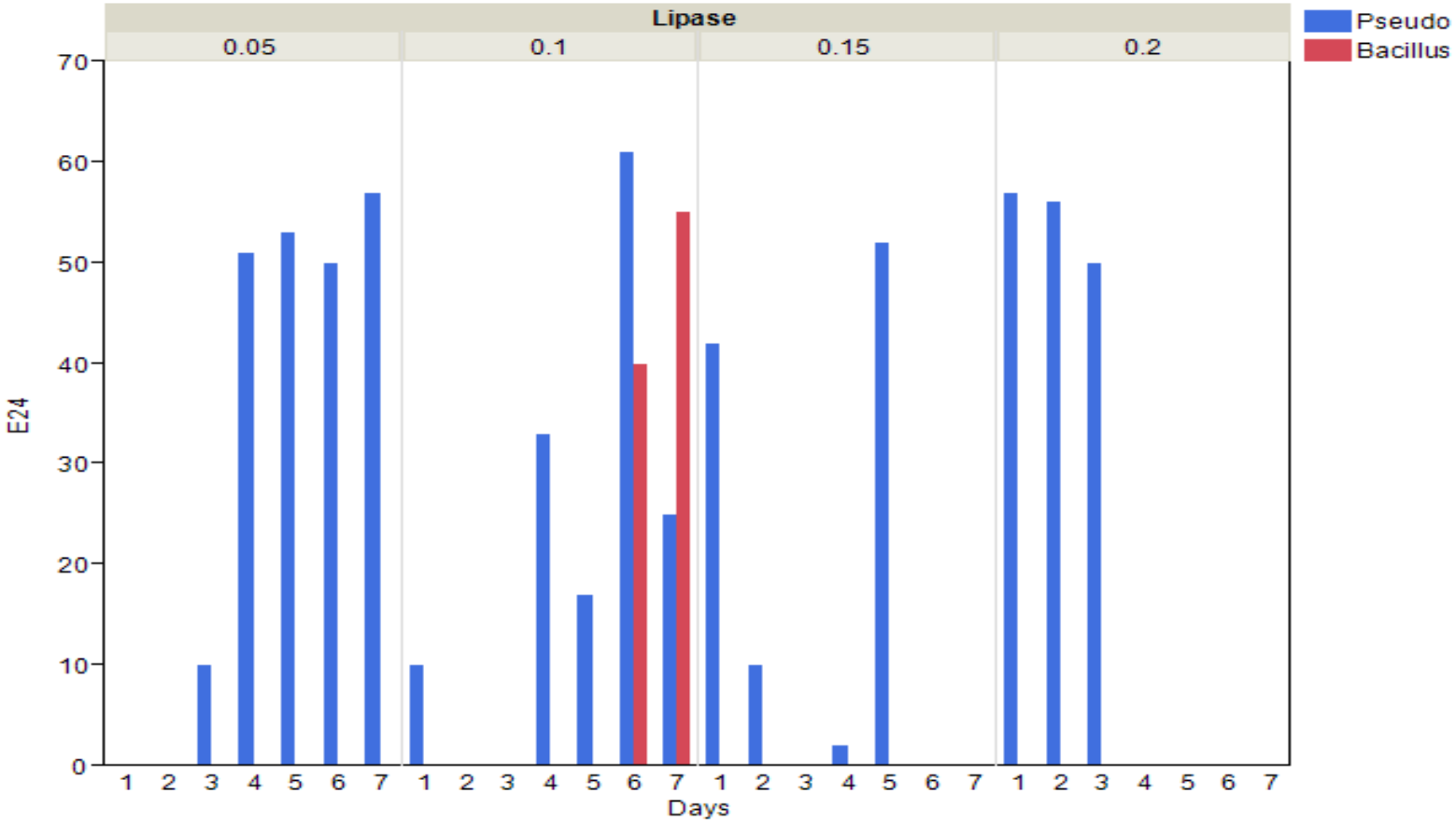
Appendix XXI: **Daily Growth Measurement of consortium of *P. aeruginosa* and *B. cereus* in Palm Oil and Shea Butter Waste Slurries at Varying Lipase Concentrations.**



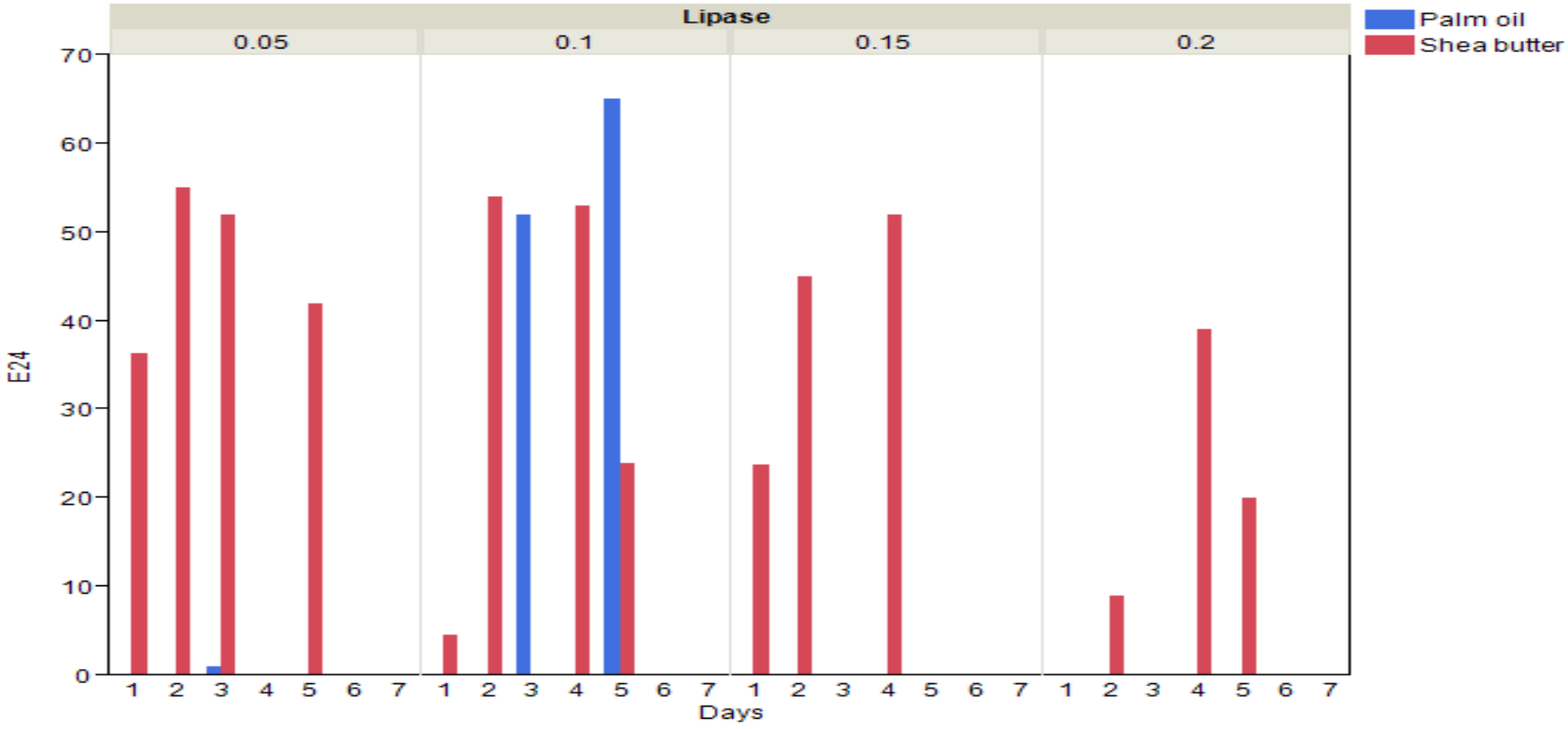
Appendix XXII: Daily E_{24} Measurement of *P. aeruginosa* and *B. cereus* in Shea Butter Waste Slurry at Varying Concentrations of Lipase



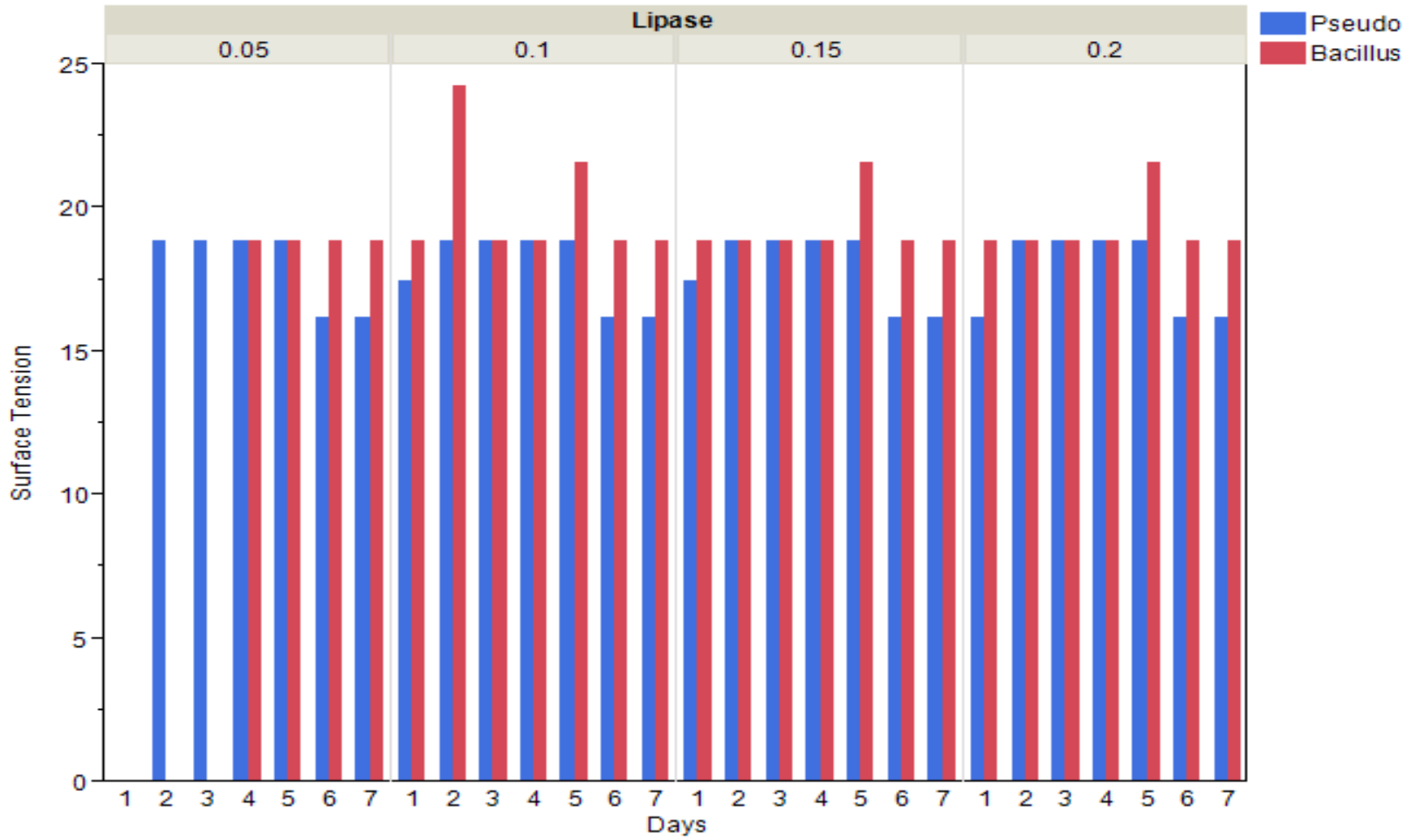
Appendix XXIII: Daily E_{24} Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at Varying Concentrations of Lipase



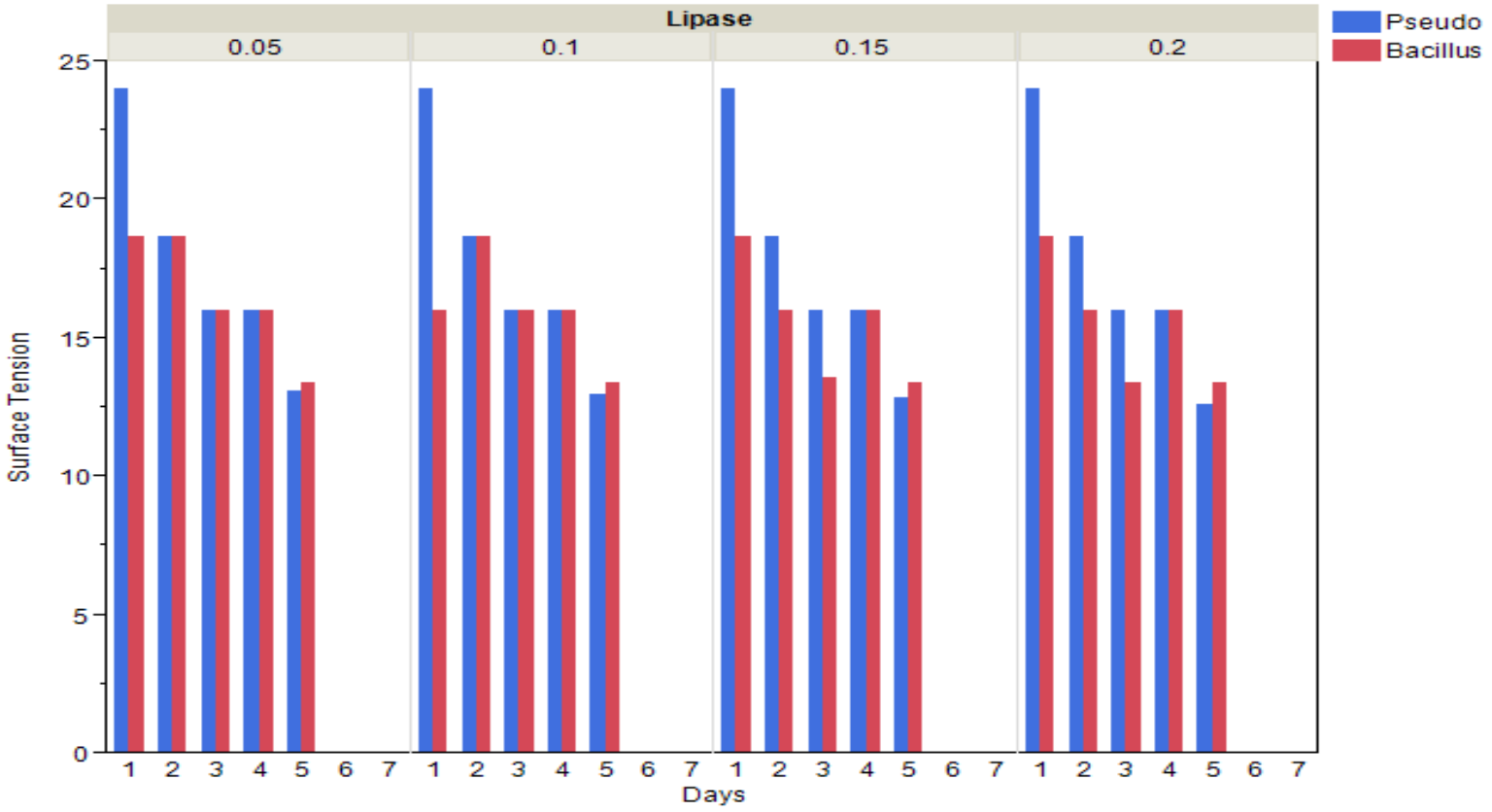
Appendix XXIV: Daily E_{24} Measurement of Consortium of *P. aeruginosa* and *B. cereus* in Shea Butter and Palm Oil Waste Slurries at Varying Concentrations of Lipase



Appendix XXV: Daily Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Shea Butter Waste Slurry at varying Lipase Concentrations



Appendix XXVI: Daily Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Palm Oil Waste Slurry at varying Lipase Concentrations



Appendix XXVII: Daily Surface Tension Measurement of *P. aeruginosa* and *B. cereus* in Shea Butter Waste Slurry at Varying Lipase Concentrations

