OPTIMIZATION AND CHARACTERIZATION OF RHAMNOLIPID AND SOPHOROLIPID PRODUCED FROM *ASPERGILLUS NIGER* IN A SOLID STATE FERMENTATION SYSTEM USING POTATO PEELSAS CARBON SOURCE

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BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,
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DEPARTMENT OF BIOCHEMISTRY
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JULY, 2015

DECLARATION

I hereby declare that the work in this thesis entitled "OPTIMIZATION AND CHARACTERIZATION OF RHAMNOLIPID AND SOPHOROLIPID PRODUCED FROM *ASPERGILLUS NIGER* IN A SOLID STATE FERMENTATION SYSTEM USING POTATO PEELSAS CARBON SOURCE*"* was performed by me in the Department of Biochemistry under the supervision of Dr. A.B Sallau and Dr. M.A Ibrahim. The information here in derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree or diploma at any university to the best of my knowledge.

Abba Daniel _____ _____

Signature Date

CERTIFICATION

This Thesis entitled "Optimization and characterization of rhamnolipid and sophorolipid produced from*Aspergillusniger*in a solid state fermentation system using potato peelsas carbon source*"* by DANIEL ABBA, meets the regulations governing the award of the degree of MASTER of Science Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my late Father, Mr. Abba Duniya

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ABSTRACT

This study was aimed at optimizing and characterizing rhamnolipid and sophorolipid produced by *Aspergillusniger* in a solid state fermentation system using potato peels as carbon source. A two-level Plackett–Burman (PB) design was implemented to screen the medium components that significantly influence the production and response surface methodology was used for the optimization. Following the one factor at a time analysis (OFAT), a central composite design was conducted to optimize the three selected factors for rhamnolipid and sophorolipid productions. Characterization of the biosurfactants involved determination of Emulsification index, surface tension; blood hemolysis and fourier transform infrared analysis. Three vital components influencing rhamnolipid production were identified as magnesium sulphate, sodium chloride and zinc sulphate while the three vital component influencing sophorolipid productions were identified as magnesium sulphate, sodium chloride and potassium dihydrogen phosphate. The validity of the model developed was verified, and the optimum medium containing 0.49% (w/v) magnesium sulphate, 0.2% (w/v) sodium chloride and 0.49% (w/v) zinc sulphate, 0.74%(w/v) magnesium sulphate, 0.1%(w/v) sodium chloride and 0.9%(w/v) of potassium dihydrogen phosphate led to a maximum rhamnolipid and sophorolipid production of 226.60±50.91 mg/gds and 84.41±8.73 mg/gds respectively. The analysis of variance indicated that the established models were significant ($P < 0.05$). Characterization of rhamnolipid and sophorolipid showed that they were able to form clear zone with a hemolyic activity. The biosurfactants were able to emulsify petrol with an emulsification index of 54.87±1.23 and 54.86±3.64 for rhamnolipid and sophorolipid respectively. The produced rhamnolipid and sophorolipid were able to reduce surface tension of distilled water from 71mN/m to 28.09 and 30.30mN/m respectively. The results of this work reveals that rhamnolipid and sophorolipid can

be produced from*Aspergillusniger* using potato peels and the production could be optimize using statistical tools.

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LIST OF ABBREVIATIONS

3D- Three Dimensional

AW- Water activity

BS- Biosurfactants

CMC- Critical Micelle Concentration

CV- Coefficient of Variation

CWI- Cell Wall Intergrity

EC- Effective Concentration

FTIR- Fourier Transform Infrared Spectroscopy

Gds- gram per dry substrate

HIV- Human Immune Virus

LC- Lethal Concentration

MEL- Monnosylerythritollipids

NARICT- National Institute for Research and Chemical Technology

OFAT- One Factor at a Time

PB- Plackett-Burman

PP- Potato Peels

RLs- Rhamnolipids

RSM-Response Surface Methodology

SLF-Submerged Liquid Fermentation

SLs- Sophorolipids

SSF-Solid State Fermentation

USA- United State of America

CHAPTER ONE

1.0 INTRODUCTION

Surfactants are surface active agents that form integral part of our everyday life with a worldwide production. They are amphipathic molecules that accumulate at interfaces, decrease interfacial tensions and form aggregate structures such as micelles (Van Hamme *et al*., 2006). The hydrophobic portion of the molecule is long-chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxyl fatty acids. The hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Thesecompounds find applications in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Desai *and* Banat, 1997 and Banat *et al*., 2000). Currently, almost all the surfactants being produced are chemically derived from petroleum (Suresh *et al*., 2012). However, microbial surfactants, referred to as biosurfactants which are produced by certain bacteria, filamentous fungi and a number of yeast have gained attention in recent years because they offer several advantages over chemical surfactants, such as lower toxicity, higher degree of biodegradability, higher foaming capacity and optimal activity at extreme conditions of temperatures, pH and salinity (Chen*et al*., 2007).

Biosurfactantsare structurally diverse and can have various chemical structures mainly consisting of glycolipids, lipopeptides, phospholipids, neutral lipids, polymeric surfactants and particulate biosurfactants, depending on the producing microorganism, raw material and process conditions (Syldatk and Hausman, 2010; Makkar *et al*., 2011). They can also be high molecular weight biosurfactants which are generally polyanionic heteropolysaccharides containing both polysaccharides and proteins. The low molecular weight biosurfactants are often glycolipids and are the most common types of

biosurfactant. The constituent mono-, di-, tri- and tetrasaccharides include glucose, mannose, galactose, glucuronic acid, rhamnose, and galactose sulphate. The fatty acid component usually has a composition similar to that of the phospholipids of the same microorganism. The glycolipids can be categorized as: rhamnolipids, trehalose lipids and sophorolipids. Amongst glycolipids, rhamnolipids, which are secondary metabolites produced by strains of *Pseudomonas aeruginosa*, have drawn particular attention as they can be produced in high yields from easily available raw materials such as used oils or wastes from the food industry and have several potential industrial, agricultural and environmental applications (medical, bioremediation) (Nitschke *et al*., 2011; Henkel *et al.,* 2012).Rhamnolipids are composed of one (for mono-rhamnolipids) or two (for dirhamnolipids) rhamnose sugar moieties linked to one or two β-hydroxyl fatty acid chains (Perfumo *et al*., 2006; Raza *et al*., 2009). Rhamnolipid induces a remarkably larger reduction in the surface tension of water from 72 to values below 30 mN/m and it reduces the interfacial tension of water/oil systems from 43 to values below 1 mN/m. Rhamnolipid also has an excellent emulsifying power with a variety of hydrocarbons and vegetable oils (Abalos *et al*., 2001).

Sophorolipid are produced mainly by yeast such as *Torulopsis bombicola, Torulopsis petrophilum* and *Torulopsis apicola* consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxylfatty acid by glycosidic linkage. Sophorolipids are the most important biosurfactants used in cosmetics and therapeutics. The amphipathic structure of sophorolipids imparts them with surfactant type properties. These biosurfactants are readily isolated in high yield, nontoxic and biodegradable (Casas and Garcia-Ochoa, 1999)

Numerous microorganisms such as yeast, bacteria, and filamentous fungi have been widely used in the production of biosurfactants (Mata-Sandoval *et al*., 2000),but the type and quantity of microbial surfactants produced depends primarily on factors such as carbon sources, nitrogen sources, microelements, temperature, pH and degree of aeration (Desai and Banat, 1997).*Pseudomonas spp*was shown to be capable of using both water soluble carbon substrates, such as glycerol, mannitol and fructose (Sim *et al*., 1997) as well as water immiscible n-alkanes to produce rhamnolipid-type biosurfactants (Henkel *et al*., 2012).

Fungi are among the best adapted species in the utilization of agricultural residues based on their ability to grow on surfaces of various substrates and penetrate into the interparticle spaces of the solid substrates (Viniegra-Gonzalez and Favela-Torres, 2006). Additionally, tolerance to minimal water condition renders fungi to be more efficient in the bioconversion of several renewable substrates (Diaz *et al*., 2006).

Aspergillus niger, a member of the black *aspergilli,* is widely used in biotechnology for the production of food ingredients, pharmaceuticals and industrial enzymes. In their natural habitat *Aspergillus niger*strains secrete large amounts of a wide variety of enzymes needed to release nutrients from biopolymers. This high secretory capacity is exploited by industry in both solid state and submerged fermentations (Pandey *et al.*, 1999). It alsohas a long tradition of safe use in the production of enzymes and organic acids.*Aspergillusniger* is used as host for the production of heterologous proteins (Archer and Turner, 2006) and as cell factory for the production of citric acid and gluconic acid (Ruitjter *et al*. 2002). Itexhibits a remarkably versatile metabolism,

enabling growth on a wide range of substrates under various environmental conditions (Bashir *et al*., 2011).

Solid State Fermentation (SSF) is the growth and/or cultivation of micro-organisms under controlled conditions in the absence of free water for the production of desired products of interest. Examples of products of solid state fermentation include industrial enzymes, fuels and nutrient enriched animal feeds. The application of modern biotechnical knowledge and process control technologies can extremely lead to significant productivity increases from this process. These processes offer more advantages such as low level of contamination, low capital investment, easy and simple process and above all provide higher yield when compared with submerged liquid fermentation (SLF) (Bashir *et al*., 2011).

Despite the aforementioned advantages of SSF over SLF, SSF is beset with following disadvantages as reported by (Cannel and Moo, 1980; Raimbault, 1998): The substrate in most cases requires pretreatment which include size reduction by grinding, physical or chemical and enzymatic hydrolysis, cooking or vapour treatment. Microorganisms like bacteria which may require high moisture levels can perform poorly in SSF. Difficulties are usually encountered inbiomass determination.

1.1 Statement of Research Problem

Most surfactants in use are chemically produced and therefore constitute an environmental hazard because of their high toxicity, low biodegradability and low ecological acceptability.Agroindustrial residues are continuously being generated in vast quantity especially in developing countries and their disposal is associated with several environmental problems.

1.2 Justification

It is important to produce surfactants of biological origin (biosurfactant) which are usually less toxic, biodegradable and environmentally friendly. Utilization of agro residues like potato peels to produce useful substance such as biosurfactant is very important and could be a source of revenue generation.To date there has been no report on production and characterization of biosurfactants from *Aspergillus niger*usingpotato peels as carbon source.

1.3 Aim

To optimize andcharacterizerhamnolipid, sophorolipid from *Aspergillus niger*in a solid state fermentation system using potato peels as carbon source.

1.4 Specific Objectives

i. To produce rhamnolipid and sophorolipid from *Aspergillus niger* via a solid state fermentation system using dried potato peels.

ii. To optimize culture medium component required for rhamnolipid and sophorolipid production by *Aspergillus niger.*

iii. To characterize rhamnolipid and sophorolipid produced from *Aspergillus niger.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Surfactant

Surfactants are amphiphilic surface active agents possessing both hydrophilic and hydrophobic moieties that reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids like oil and water, signifying that surfactants moreover assist the solubility of non-polar compounds in organic solvents(Saharan*et al*., 2011). Surfactants have been used industrially as flocculating, wetting, foaming agents, adhesives, emulsifiers and lubricants(Mulligan and Gibbs, 1993). The ability to reduce surface tension is a major characteristic of a surfactant. Due to their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces. As a result, surfactantsreduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily (Bodour and Maier, 2002). Also due to the presence of surfactants, less work is required to bring a molecule to the surface, and the surface tension is reduced. It is obvious that their surface and membrane-active properties play an important role in the expression of their activities. Commercially, surfactants are key ingredients used in detergents, shampoos, toothpaste and oil additives. These amphiphiles (hydrophilic and hydrophobic) are classified as nonionic, anionic, cationic, or amphoteric which is dependent on their molecular structures (Surekha *et al*., 2010).

2.2 Biosurfactants

Biosurfactants are amphiphilic compounds produced in livingsurfaces, mostly on microbial cell surfaces or excreted extracellularhydrophobic and hydrophilic moieties that confer the ability toaccumulate between fluid phases, thus reducing surface and interfacialtensions at the surface and interface respectively (Cunha *et al.*, 2004). Their hydrophilic moiety is mainly comprised of an acid, peptide cations, or anions, mono-, di- or polysaccharides while their hydrophobic moiety can be an unsaturated or saturated hydrocarbon chains or fatty acids. They possess thecharacteristic property of reducing the surface and interfacial tensionusing the same mechanisms as chemicals surfactants (Singh *et al*., 2007). Primarily, biosurfactants attracted attention as hydrocarbon dissolution agents in the 1960s, and their applications have been greatly extended in the past five decades as an improved alternative to chemical surfactants (carboxylates, sulphonates and sulphate acid esters), especially in food, pharmaceutical and oil industry (Banat *et al*., 2000).

2.3 Properties of Biosurfactants

Biosurfactants are of increasing interest for commercial use because of the continuallyincreasing spectrum of available substances. There are various advantages of biosurfactantscompared to their chemically produced counterpart. The major distinctive features of biosurfactants and a brief description of each property are given below.

2.3.1Surface and interface activity

A good surfactant can lower surface tension of water from 72 to35 mN/m and theinterfacial tension of water/ hexadecane from 40 to 1 mN/m (Mulligan, 2005). Surfactinproduced from*B. subtilis* are able to reduce the surface tension of water to 25

mN/m andinterfacial tension of water/hexadecane to <1 mN/m (Cooper *et al.,* 1981). Rhamnolipidsfrom *P. aeruginosa* reduce the surface tension of water to 26 mN/m and the interfacialtension of water/hexadecane to <1 mN/m (Hisatsuka *et al.,* 1971). The sophorolipids from *T.bombicola* reduce the surface tension to 33mN/m and the interfacial tension to 5mN/m(Cavalero and Cooper, 2003). In general, biosurfactants are more effective and efficient andtheir critical micelle concentration (CMC) is about 10–40 times lower than that of chemical surfactants, i.e. less amount surfactant is required to get a maximum decrease in surface tension (Desai and Banat, 1997).

2.3.2Biodegradability of biosurfactants

Unlike the synthetics, biological surfactants are easily degraded by microorganism (Mohan *et al.,* 2006). This, according to Mulligan (2005) elucidates its use in environmental application such as bioremediation and dispersion of oil spills.

2.3.3Low toxicity of biosurfactants

Corexit (a synthetic chemical surfactants displays an LC_{50} (lethal concentration to 50% of test species) against *Photobacterium phosphoreum*, ten times lower than rhamnolipids, thus demonstrating the higher toxicity of the chemical-derived surfactants. It was also reported that biosurfactants showed higher EC_{50} (effective concentration to decrease 50% of test population) values than synthetic dispersants. Similarly, when a biosurfactant from *Pseudomonas aeruginosa*, was compared with Marlon A-350 (a synthetic surfactant widely used in industry with respect to its mutagenic and toxicity properties), both assays indicated higher effects for the chemical derived surfactants whereas the biosurfactants was considered slightly non-toxic and non-mutagenic (Desai and Banat, 1997).

2.3.4Availability of raw materials for biosurfactant production

Biosurfactants can be produced from very cheap raw materials which are available in large quantities. The carbon source may come from hydrocarbons, carbohydrates and /or lipids, which may be used separately or in combination with each other (Kosaric, 2001).

2.3.5Biocompatability and digestibility

Biological origin imparts on biosurfactant inherent characteristic of compatibility, which allows their unabated usage in cosmetics, pharmaceuticals and as functional food additives (Kapadia and Yagnik, 2013).

2.4 Classification of Biosurfactants

Unlike chemically synthesized surfactants, which are usually classified according to the nature of their polar groups, biosurfactants are generally categorized mainly by their chemical composition and microbial origin. Rosenberg and Ron (1999) classified biosurfactants into low molecular-mass molecules, which efficiently lower surface and interfacial tension; and high molecular-mass polymers, more effective as emulsion-stabilizing agents. The major classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants.

2.4.1 Low molecular- weight biosurfactants

These are compounds that lower the surface and interfacial tensions 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 hydroxyl fatty acids (Ron and Rosenberg, 2001).

2.4.2 High- molecular weight biosurfactants

These are usually referred to as bioemulsans (Salihu *et al*., 2009). They are more effective in stabilizing oil in water emulsions. 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. When classification is based on their polar groupings most of the biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long chain fatty acids or fatty acid derivatives whereas the hydrophilic portion can be carbohydrates, amino acid, phosphate or cyclic peptide (Nitschke and Coast, 2007). Desai and Banat (1997) stated that in general, the structure of a biosurfactant 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.

2.5 Classification of Biosurfactants Based on Chemical Composition

2.5.1 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 eitheron

ether or an ester group. Among the glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids (Desai and Banat, 1997; Muthusamy *et al.,* 2008).

2.5.1.1*Rhamnolipid*

Rhamnolipids (RLs), the glycolipid biosurfactants produced mainly by *Pseudomonas aeruginosa*, are the most intensively studied biosurfactants. These arises from the facts that they display relatively high surface activities and are produced in relatively high yields after relatively short incubation periods by a well-understood, easy to cultivate microorganism (Abdel-Mawgoud *et al*., 2010).The discovery of RLs dates back to 1946 when Bergstrom *et al.,* (1946b) reported an oily glycolipid produced by *Pseudomonas pyocyanea* (now *P. aeruginosa*) grown on glucose. This substance was named pyolipic acid and its structural units were identified as L-rhamnose and β-hydroxydecanoic acid (Bergstrom *et al*., 1946a, b; Hauser and Karnovsky, 1954).Rhamnolipids are composed of one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid (Fig. 2.1). The hydroxyl group in one of the acids has a glycosidic linkage with the reducing end of the rhamnose disaccharide, while the hydroxyl group of the second acid is involved in ester formation. As one carboxylic group is free, the rhamnolipids are anions above pH 4 (Karanth *et al*., 2005).

The role of these biomolecules for the producing microorganisms as well as their interactions with otherbiological systems, especially the human body was considered with key interest (Van Hamme *et al*., 2006; Abdel-Mawgoud *et a*l., 2010). One of these roles is to promote the uptake of poorly soluble hydrocarbons (Koch *et al*., 1991). Other physiological functions include the control of the bacterial cell surface hydrophobicity for attachment and detachment on different substrates (Yuan *et al*., 2007; Zhong *et al*., 2007;

Sotirova *et al*., 2009; Al-Tahhan *et al*., 2000), and the enhancement and modulation of surface motility (Caiazza *et al*., 2005; Tremblay *et al*., 2007).

Studies about the interactions of rhamnolipids with other biological systems are numerous. The antibacterial (Abalos *et al.*,2001; Onbasli and Aslim, 2008; Shen *et al*., 2009; Sotirova *et al.*, 2008), antifungal (Kim *et al*., 2000; Yoo *et al*., 2005), antiviral (Remichkova *et al*., 2008), antiphytopathogenic (Kim *et al*., 2000; De Jonghe *et al*., 2005; Nielsen *et al*., 2005, 2006), and algicidal (Wang *et al*., 2005a) properties of RLs have been extensively investigated. RLs released by *P. aeruginosa* have long been known as the heat-stable extracellular hemolysin (Fujita *et al*., 1988).

Due to their excellent surface activity, the physicochemical properties of RLs have received considerable interest (Abalos *et al*., 2001;Ochoa-Loza *et al*., 2001 Chen, 2004; Ozdemir and Malayoglu, 2004; Ozdemir *et al*., 2004; Cohen and Exerowa, 2007; Hansen *et al*., 2008;Abdel-Mawgoud *et al*., 2009 and Pornsunthorntawee *et al*., 2009). Due to their hydrocarbonsolubilizingproperties, they also have been used in the fields of bioremediation and biodegradation (Asci *et al*., 2007; Benincasa, 2007; Asci*et al*., 2008; Cameotra and Singh, 2009).

The potential industrial and biotechnological applications ofrhamnolipids are thus quite diverse (Singh *et al.*, 2007). RLs have been used for the synthesis and stabilization of nanoparticles (Xie *et al*., 2006; Palanisamy and Raichur, 2009), the preparation of microemulsion (Xie *et al*., 2007; Nguyen and Sabatini, 2009), as an antiagglomeration agent (York and Firoozabadi, 2008), as dispersing agent (Raichur, 2007; Tripathy and Raichur, 2008), in cleaning soap mixtures and as a source of rhamnose (Linhardt *et al*., 1989).

Clinical testing of RLs as pharmacoactive compounds has been performed. Some successful trials proved the potential applications of RLs for the treatment of ulcers (Pilja, 2008) and of full-thickness wounds (Stipcevic *et al.,* 2006).

These promising properties and potential application of RLs have encouraged researchers to improve the production of RLs, using industrially safe and more affordable processes in order to reduce production costs, which currently restrict the competitiveness of RLs vis-à-vis petroleum-derived surfactants.This goal has been sought through different approaches. First, many attempts have been made to isolate RL producers other than the opportunistic pathogen *P. aeruginosa* (Abouseoud *et al.,* 2008) or to transfer the genes responsible for RL production into more industrially safe heterologous hosts, such as Escherichia coli (Cabrera-Valladares *et al.,* 2006). Second, important efforts have been dedicated to the identification of low-cost and renewable raw material as production substrates, such as agroindustrial wastes (Nitschke *et al.,* 2005). Finally, an even production of RLs through pure chemical synthesis was also reported (Bauer *et al*., 2006).Rhamnolipids can lower the surface tension of water to 25-30 mN m^{-1} and the interfacial tension against *n*-hexadecane to 1 mN m^{-1}; their CMC value range from 10 to 30 mgL^{-1}(Ahmad *et al*., 2011).

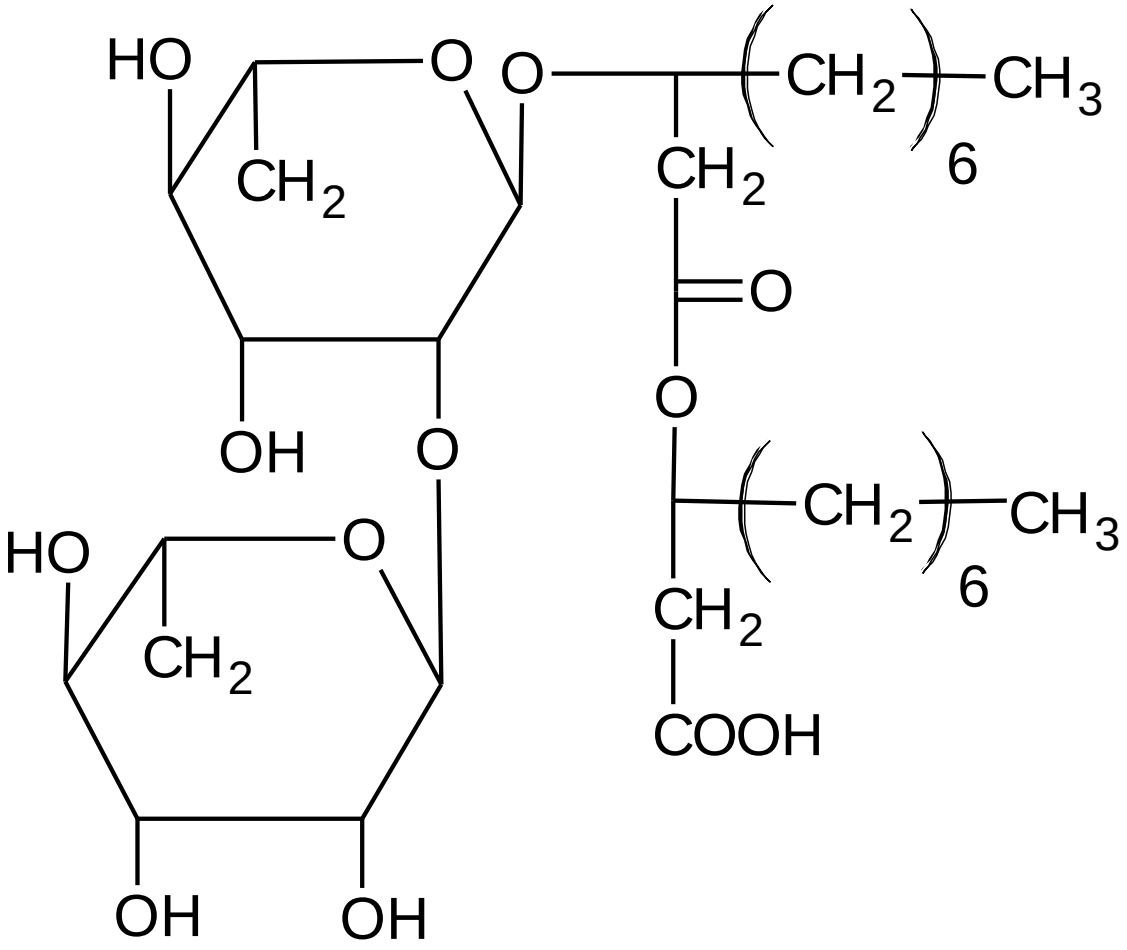


Figure 2.1**:** Structure of rhamnolipid (Kapadia and Yagnik, 2013)

2.5.1.2*Sophorolipid*

Sophorolipids consist of two glucose molecules linked between β-1, 2 (sophorose), with 6- and 6'-hydroxyl groups, generally acetylated, linked to a long-chain hydroxyl fatty acid (Figure 2.2). The terminal carboxyl group can be in the lactonic form or hydrolyzed to give an anionic surfactant. They are produced mainly by yeasts, such as *T. bombicola*, *T. petrophilum*, *T. apicola* and *Candida bogoriensis*. Cutler and Light (1979) showed that *Candida bogoriensis* produces glycolipids in which sophorose is linked to docosanoicacid diacetate. *T. petrophilum* produced sophorolipids on water-insoluble substrates such as alkanes and vegetableoils (Cooper and Paddock, 1983). These sophorolipids, which were chemically identical to those produced by *T. bombicola*, did not emulsify alkanes orvegetable oils. When *T. petrophilum* was grown on a glucoseyeast extract medium, however, sophorolipids were not produced,but an effective protein-containing alkane emulsifying agent was formed (Cooper and Paddock, 1983). These results appear to contradict theconventional belief that microbial emulsifiers and surfactants are produced to facilitate the uptake of water-insoluble substrates.Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents (Cooper and Paddock, 1984).Both lactonic and acidic sophorolipids lowered the interfacial tension between *n*-hexadecane and water from 40 to 5 mN/mand showed remarkable stability toward pH and temperature changes (Cooper and Paddock, 1983).

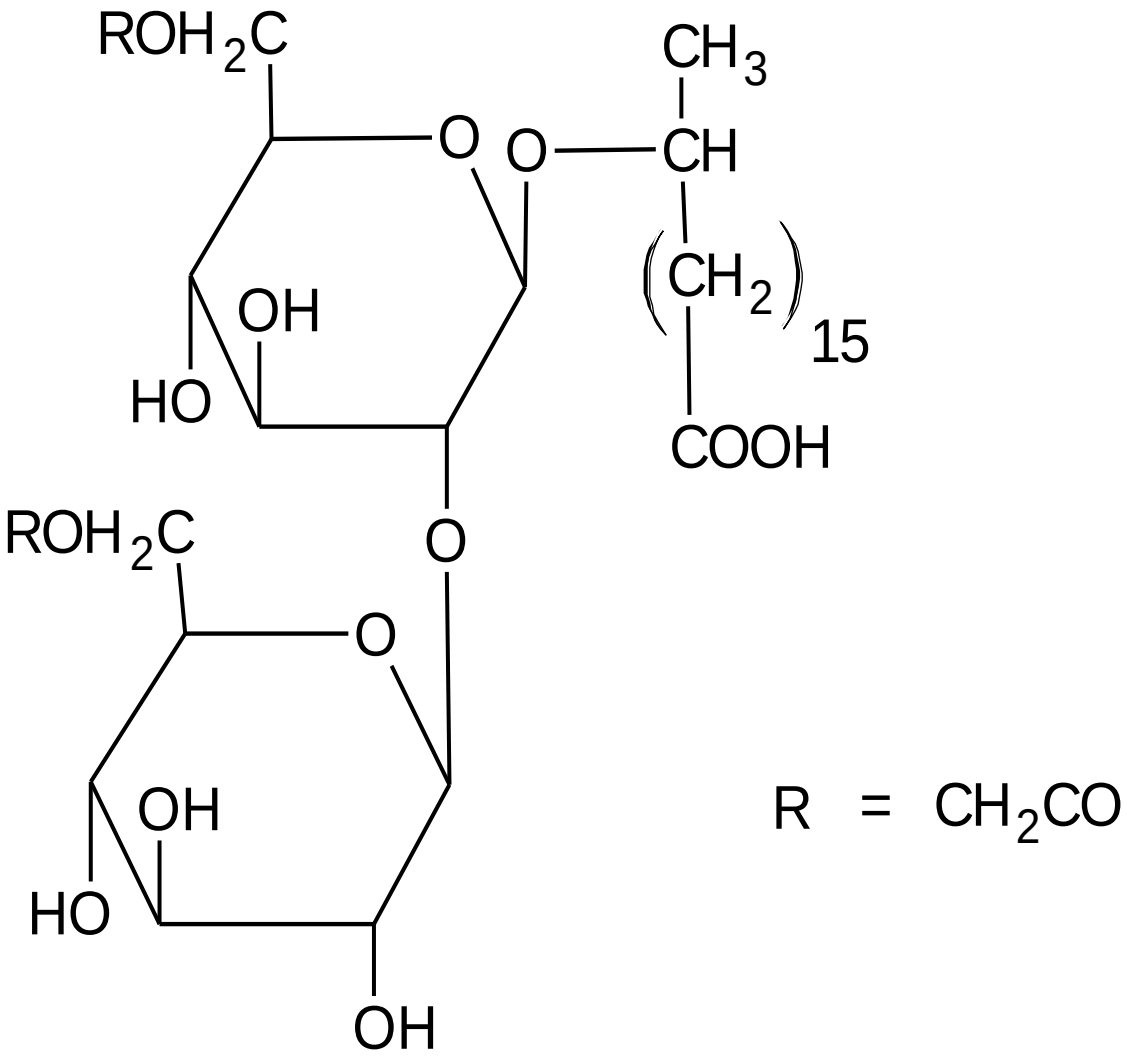


Figure 2.2: Structure of Sophorolipid (Kapadia and Yagnik, 2013)

2.6 Factors Affecting Biosurfactant Production

Biosurfactants are produced by a number of microorganisms, predominantly during their growth on water-immiscible substrates. However, some filamentous fungi may produce biosurfactants in the presence of different types of substrates, such as carbohydrates. 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 (Saharan *et al*., 2011). The composition and characteristics of biosurfactants are influenced by the nature of the nitrogen source as well as the presence of iron, magnesium, manganese, phosphorus and sulphur in the media. Many biosurfactants are not affected by environmental factors such as temperature, pH and ionic strength tolerances. Lichenysin produced by *Bacillus licheniformis* strain was not affected by temperature ranges of up to 50^{o}C, a pH range of 4.5-9.0, and sodium chloride concentration of 50g/l and calcium chloride concentration of 25g/l. (Muthusamy *et al.*, 2008).

2.6.1 Carbon source for biosurfactants production

Till date, biosurfactants are unable to compete inexpensively with chemically synthesized surfactants due to their high production costs and recovery system. These costs may be significantly reduced by the use of alternative sources of nutrients. Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were all used for rhamnolipid production by *Pseudomonas* sp (Desai and Banat, 1997) but biosurfactant production, however, was inferior to that obtained with water-immiscible compounds such as *n*-alkanes and olive oil (Robert *et al.,* 1989). The soy molasses, a by-product used in the production of soybean oil, plus oleic acid were tested as carbon sources for the

production of sophorolipids by the yeast *C. bombicola* (Solaiman *et al*., 2004). The purified SLs were obtained at 21 g l^{-1} and were 97% in lactone form. The surface properties of the SLs obtained from the soy molasses/oleic acid fermentation had minimum surface tension values of 37 mNm^{-1}(pH 6) and 38 mN m^{-1} (pH 9), and critical micelle concentration values of 6 mg l^{-1} (pH 6) and 13 mg l^{-1} (pH 9). Others observed a little biosurfactant production, when cells were growing on a readily available carbon source, only when all the soluble carbon was consumed and when water-immiscible hydrocarbon was available was biosurfactant production triggered (Banat et al., 1991; Banat, 1995). Davila et al., (1992) demonstrated a high yield of sophorolipids by overcoming product inhibition in Candida bombicola CBS6009 through the addition of ethyl esters of rape seed oil fatty acids in D-glucose medium. Using Torulopsis apicola IMET 43747, Stuwer et al., (1987) achieved a high glycolipid yield with a medium containing D-glucose and sunflower oil. Mounting evidence leads to theconclusion that the available carbon source, particularly the carbohydrate used, has a great bearing on the type of biosurfactantproduced (Li *et al.*, 1984).

2.6.2 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 (Zinjarde *et al*.,1997), sodium nitrate (Thanomsub *et al*., 2004), meat extract (Bednarski *et al.,* 2004) and malt extract (Mata-Sandoval *et al*.,2001). Yeast extract is the most widely used nitrogen source for biosurfactant production, but its

required concentration depends on the nature of microorganism and the culture medium to be used. The production of biosurfactants often occurs when the nitrogen source is depleted in the culture medium, during the stationary phase of cell growth. Robert *et al*., (1989); Abu-Ruwalda *et al.,* (1991) observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1 and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth, when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. In *P. aeruginosa*, a simultaneous increase in rhamnolipid production and glutamine synthetase activity was observed when growth slowed as the culture became nitrogen limiting (Mulligan and Gibbs, 1989). Similarly, nitrogen limitation caused increased biosurfactant production in *P. aeruginosa* (Suzuki *et al*., 1974; Ramana *et al*., 1989), *C. tropicalis* IIP-4 (Singh *et al.,* 1990), and *Nocardia* strain SFC-D (Kosaric *et al*., 1990). Syldatk *et al*., (1985) showed that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant produced. Guerra-Santos *et al*. (1984, 1986) showed maximum rhamnolipid production after nitrogen limitation at a C: N ratio of 16:1 to 18:1 and no surfactant production below a C: N ratio of 11:1, where the culture was not nitrogen limited.

2.6.3 Salt and mineral ion concentration requirement in biosurfactant production

Metal ions concentrations play a very important role in the production of some biosurfactants (BS) as they form important cofactors of many enzymes. The overproduction of surfactin biosurfactant occurs in presence of Fe^{2+} in mineral salt medium (Irfan and Asif, 2011).In some cases it was observed that, limitation or supplementation of multivalent cation concentration in the growth media increases the

biosurfactant production (Irfan and Asif, 2011). Guerra-Santos *et al*., (1986) demonstrated that limiting the concentrations of salt of magnesium, calcium, potassium and trace elements resulted in a better yield of rhamnolipid in *P. aeruginosa* DSM 2659. The yield of biosurfactant production by *B. subtilis* MTCC 2423 was increased by adding the metal cations altogether than the individual addition of cations (Makkar and Cameotra, 2002).

2.7 Application of Biosurfactant

2.7.1 Food industry

Apart from their obvious role as agents that decrease surface and interfacial tension, thus promoting the formationand stabilization of emulsions, surfactants can have several other functions in food. For example to control the agglomeration of fat globules, stabilization of aerated systems, improvement of texture and shelf-life of starch-containing products, modification of rheological properties of wheat dough, consistency and texture of fat-based products (Kachholz and Schlingmann, 1987).

In bakery and ice cream formulations biosurfactants act in controlling consistency, retarding staling and solubilizingflavor oils; they are also utilized as fat stabilizer and antispattering agent during cooking of oil and fats (Kosaric, 2001). An improvement of dough stability, texture, volume and conservation of bakery products was obtained by theaddition of rhamnolipid surfactants (Van Haesendonck and Vanzeveren, 2004). The authors also suggested the use ofrhamnolipids to improve properties of butter cream, croissants and frozen confectionery products. Recently, a bioemulsifier isolated from a marine strain of *Enterobacter cloacae* was described as a potential viscosity enhancement agent of interest in food industry especially due to the

good viscosity observed at acidic pH allowing its use in products containing citric or ascorbic acid (Iyer*et al*., 2006).

2.7.2 Application of biosurfactant in cosmetic industry

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

2.7.3 Biosurfactant in bioremediation of petroleum contaminants

The bioavailability of contaminants in the natural environment is one of the key factors limiting the progress of biodegradation processes (Volkering *et al*., 1998). The secretion of rhamnolipids would lead to the solubilization of hydrophobic compounds, which in turn contributed to a stimulation of biodegradation processes (Hisatsuka *et al*., 1971). It was often considered as an integral trait of microorganisms capable of biodegrading petroleum hydrocarbons. Koch *et al*., (1991); Ochsner *et al*., (1994) directly connected the lack of genes responsible for rhamnolipids synthesis with the inability to grow on hydrophobic carbon sources, such as n-alkanes.The ability to produce biosurfactants and change the interfacial tension was accepted as a potential mechanism for enhancing the transfer of hydrophobic substrates (Boulton and Ratledge, 1984; Singer and Finnerty, 1984). Solubilization of compounds from the oil phase and their transport

into the water phase would exclude diffusion as a factor limiting the progress of biodegradation processes (Hommel, 1994). Zhang and Miller, (1994) reported that the addition of rhamnolipids at 300 mg/L stimulated the biodegradation of octadecane. In some other experiments carried out by the same authors, rhamnolipids increased biodegradation of hexadecane for some species and inhibited the biodegradation of octadecane for other (Zhang and Miller, 1995).

2.7.4 Medicine

The use and potential commercial applications of biosurfactants in the medical field have increased in the past decades. Their antibacterial, antifungal and antiviral activities make them relevant molecules for applications in combating many diseases and as therapeutic agents (Banat *et al*., 2000). Their pertinence in these fields is related to their biological properties such as the ability to disrupt membranes leading to cell lysis and metabolite leakage through increased membrane permeability and hence antimicrobial activity. Moreover, similarly to organic-conditioning films, their ability to partition at the interfaces can affect the adhesion properties of cells/microorganisms (Banat *et al*., 2010). Biomedical applications of biosurfactants have been thoroughly described (Cameotra and Makkar, 2004; Rodrigues *et al*., 2006; Seydlová and Svobodová, 2008; Rodrigues and Teixeira, 2010).

2.7.4.1*Antibacterial activity*

Lipopeptides have the most potent antimicrobial activity and have been a subject of several studies on the discovery of new antibiotics. The antibiotics activity is due to the ability of molecules of lipopeptide biosurfactants to self-associate and form a pore-bearing channel or micellular aggregate inside a lipid membrane (Carillo *et al*., 2003;

Deleu *et al.,* 2008).Surfactin in particular, has been associated with several physical and biological actions such as antimicrobial, antiviral, anti-mycoplasm and haemolytic activities (Bonmatin *et al*., 2003). Glycolipids both rhamnolipid (Abalos *et al*., 2001) and sophorolipids (Kim *et al.,* 2002; Van Bogaert *et al.*, 2007) also have shown interesting antimicrobial activities.

2.7.4.2 *Anti-cancer activity*

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

2.7.4.3 *Antiviral activity*

Antibiotic activity and inhibition of growth of human immunodeficiency virus in leucocytes by biosurfactants have been cited in literatures (Desai and Banat, 1997; Muthusamy *et al.,* 2008). 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 - 9 against the human semen (Fakruddin, 2012).

2.8 *Aspergillus niger*and Biosurfactant Production

The filamentous fungus *A. niger*(black mould) has been used for centuries asindustrial versatile microbial cell factory for production of organic acids as well asvarious extracellular enzymes, native or heterologous proteins and antibiotics.*A. niger*shows an amazing nutritional flexibility, metabolic capacity and produceshigh levels of secreted primary and secondary metabolites. Nowadays, it is difficult tothink of filamentous fungus where the metabolic capabilities are of greater interestthan *A. niger* (Andersen *et al*., 2008). As a eukaryotic organism, *A. niger*offersvaluable advantages for enzyme secretion, such as facilitated proteolytic processingand protein folding as well as posttranslational modifications (Nevalainen *et al*., 2005; Lubertozzi andKeasling, 2009). This has made this microorganism apotentially attractive host for the biotechnological industry. Especially the genus*Aspergillus*, frequently applied in enzyme production due to the GRAS status(generally regarded as safe), has received particular attention. Due to enormousdevelopment of genetic engineering and efficient expression systems, *Aspergillus*species have also achieved increased attention as host for industrial production ofhomologues and heterologous proteins (Wang *et al*. 2005b).

The fungal cell wall determines biotechnologically relevant features such as morphology during fermentation and cell integrity. The cell wall of *A. nige*r consists of chitin, 1, 3-β-glucan, 1, 6-β-glucan, 1,3-α-glucan, galactosaminogalactan and galactomannan. Genes required for the biosynthesis of these cell wall components were identified in the genome while in other *aspergilli*, a high degree of redundancy is observed for chitin synthases and chitinases compared to *Saccharomyces cerevisiae* (Bernard and Lagte, 2001).

The cell wall integrity (CWI) signaling pathway is responsible for cell wall remodeling and reinforcement in response to a changing environment (Levin, 2005). Several components of this pathway are present in the genome and indeed expressed during fedbatch culture, ensuring the growth of *A. niger* under harsh industrialprocess conditions.

2.9 Plackett-Burman Experimental design and Response Surface Methodology

The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its propensity and relevance (Salihu *et al*., 2011). Plackett–Burman (PB) design is a valuable method for screening significant medium components that influence biosurfactant production from a lot of variable components, so that insignificant ones can be eliminated to obtain a smaller, reliable and more manageable set of components.

PB designs utilize two levels for each factor, the higher level being denoted "+" and the lower "-" as usual. A further feature of the PB method is that the + and - signs for the individual trial experiments are assigned in a cyclical manner. Such sequences of + and - signs are provided by generating vectors and are widely available in software packages. The effect of each factor is then readily determined from the expression:

$$2[\sum(y+) - \sum(y-)]/N \qquad (2.1)$$

Where N is the total number of experiments and the (y+) terms are the responses when a given factor is at its high level, and the (y-) terms reflect the responses for that factor set to its low level (Plackett and Burman, 1946).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the

objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response (Montgomery, 2005).

Originally, RSM was developed to model experimental responses (Box and Draper, 1987) and then migrated into the modeling of numerical experiments. The difference is in the type of error generated by the response. In physical experiments, inaccuracy can be due, for example, to measurement errors while, in computer experiments, numerical noise is a result of incomplete convergence of iterative processes, round-off errors or the discrete representation of continuous physical phenomena (Giunta *et al*., 1996, Toropov *et al*., 1996). In RSM, the errors are assumed to be random. The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods (e.g. finite element method) and their associated numerical noise. The response can be represented graphically, either in the three-dimensional space or as *contour plots* that help visualize the shape of the response surface. A list of different methodologies is given below:

2.9.1 Full factorial design

To construct an approximation model that can capture interactions betweentotal numbers of experiment design variables, a full factorial approach (Montgomery, 1997) may be necessary to investigate all possible combinations. A factorialexperiment is an experimental strategy in which design variables are varied together, instead of one at a time (Montgomery, 1997).

2.9.2 D-optimal designs

The D-optimality criterion enables a more efficient construction of a quadratic model (Myers and Montgomery, 1995).From a statistical point of view, a D-optimal design leads to response surface models for which the maximum variance of the predicted responses is minimized. This means that the points of the experiment will minimize the error in the estimated coefficients of the response model.

2.9.3 Central composite design

A second-order model can be constructed efficiently with central composite designs (CCD) (Montgomery, 1997). CCD is first-order (2^N) designs augmented by additional centre and axial points to allow estimation of the tuning parameters of a second-order model. The numbers of experiments are reduced and more efficient with CCD when compared to full factorial design and D-optimal design (Salihu *et al*., 2011).

2.1 Solid State Fermentation

Solid State Fermentation (SSF) is the growth and/or cultivation of microorganisms under controlled conditions in the absence of free water for the production of desired products of interest (Bashir *et al*., 2011). Examples of productsof solid state fermentation include industrial enzymes, fuels and nutrient enriched animal feeds (Bashir *et al*., 2011).

Bacteria, yeasts and fungi can grow on solid substrates, and find application in SSF processes. Filamentous fungi are the best adapted for SSF and dominate in research works.Bacteria are mainly involved in composting, ensiling and some food processes (Doelle *et al*, 1992). Yeasts can be used for ethanol and food or feed production (Saucedo-Castaneda *et al*, 1992). But filamentous fungi are the most important group of

microorganisms used in SSF process owing to their physiological,enzymological and biochemical properties. The hyphal mode of fungal growth and their good tolerance to low water activity (Aw) and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates and for theutilization of available nutrients. The basic mode of fungal growth is a combination of apical extension of hyphal tips and the generation of new hyphal tips through branching. An important feature is that, although extension occurs only at the tip at a linear and constant rate, the frequency of branching makes the kinetic growth pattern of biomass exponential, mainly in the first steps of the vegetative stage (Raimbault, 1998). The hyphalmode of growth gives the filamentous fungi the power to penetrate into the solid substrates. The cell wall structure attached to the tip and the branching of the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of SLF, what makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. Penetration increases the accessibility of all available nutrients within particles (Raimbault, 1998).

Substrates from agricultural or industrial wastes (wheat straw or barley, sugar cane bagasse, coffee pulp, grape wastes, copra pasta, among others) or inert materials (as resins of ionic exchange, acreolite or poliurethane foam) can be used for the production of valued added products like enzymes (Caritobal *et al*., 2008).

In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate

that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with some of the agro residues. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes (e.g. ligno-cellulose), thereby making them more easily accessible for microbial growth(Bashir *et al*., 2011).

Among the several factors that are important for microbial growth and biomolecules production using a particular substrate, particle size and moisture level/water activity are the most critical(Pandey, 1992; Pandey, 1994). Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. However, too small a substrate particle may result in substrate aggregate, which may interfere with microbial respiration/ aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration/aeration efficiency (due to increased inter-particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process (Duenas *et al*., 1995; Pandy *et al.*, 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals

Chemicals and reagents used were purchased from Sigma CompanyUSA and were of analytical grade.

3.2 Sample Collection and Preparation

Sweet potato peels were collected from Akenzua hostel canteens, Ahmadu Bello University Zaria and were washed first with tap water followed by distilled water to remove the adhered surface dust particles. Then blanching operation was carried out by immersing the peels in hot water (75–80 ^{o}C) for 20 min. Peels were then oven dried at 45 ^{o}C for 36 h. The dried material was ground in a mixer grinder to form powder and was then stored at 4^{o}C for further use.

3.3 Microorganism Collection and Inoculum preparation

*Aspergillus niger*was obtained from the culture stock from Department of CropProtection, Ahmadu Bello University, Zaria, Nigeria. Seven-day-old potato dextrose agar plate containing *A. niger*was used to prepare the inoculum culture as described by Alam *et al,* (2004). Briefly, each culture plate was washed with 25mL of sterile distilled water using bent glass rod and the suspension was filtered through Whatman No. 1 filter paper to remove the mycelia from spore suspension. The spores were counted using the haemocytometer to maintain an inoculum concentration of 3×10^{8}spores per ml.

3.4 Fermentation Media Preparation Based on Plackett- Burman Design.

Twelve experimental runs were generated using Design-Expert software (version 6.0.8, Stat-Ease Corporation, USA) for 11 media components; these are sodium hydrogen tetraoxophosphate (V) (Na_2HPO_4),magnessium tetraoxoSulphate (VI) heptahydrate ($MgSO_4.7H_2O$), ammonium trioxonitrate (V) (NH_4NO_3),potassium hydrogen tetraoxophosphate (V)(KH_2PO_4), sodium chloride(NaCl), calcium chloride($CaCl_2$), iron (ii) tetraoxosulphate (VI) ($FeSO_4$), zinc tetraoxosulphate (VI)($ZnSO_4$), manganse tetraoxosulphate(VII)($MnSO_4$), Thiamine hydrochloride acid (T-HCl) and sweet potato peels. Each component wastested at two concentration levels, low (−1) and high (+1). The experiments werecarried out in triplicate according to the design matrix in 250ml conical flasks. Table 3.1 shows themediumcomponents under investigation as well as levels of each variable used in the experimental design.

3.5 Biosurfactant Production by Solid State Fermentation

Four grams of potato peels (1mmparticle size) was weighed into a 250ml Erlenmeyer flask, and the medium components of varying concentrations were added into the flask based on the PB design matrix to achieve a desired moisture level of 60%. The contents were thoroughly mixed and autoclaved at 121^{o}C for 20 min.The medium were inoculatedwith 5 % (v/w) *Aspergillusniger*inoculum and incubated at 30^{o}C for 5 days.

Table 3.1: Plackett-Burman Experimental Design for Rhamnolipid and Sophorolipid Production

Run	Na_2HPO_4% (w/v)	KH_2PO_4% (w/v)	NH_4NO% (w/v)	$MgSO_4$% (w/v)	$CaCl_2$ %(w/v)	NaCl %(w/v)	$ZnSO_4$ %(w/v)	$FeSO_4$ % (w/v)	$MnSO_4$% (w/v)	T-HCl %(w/v)	Carbon source %(w/v)
1	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)
2	0.6(+1)	0(-1)	0(-1)	0(-1)	0.0014(+1)	0.1(+1)	0.025(+1)	0(-1)	0.025(+1)	0.1(+1)	0(-1)
3	0(-1)	0.3(+1)	0.1(+1)	0(-1)	0.0014(+1)	0(-1)	0(-1)	0(-1)	0.025(+1)	0.1(+1)	5(+1)
4	0.6(+1)	0(-1)	0.1(+1)	0(-1)	0(-1)	0(-1)	0.025(+1)	0.025(+1)	0.025(+1)	0(-1)	5(+1)
5	0(-1)	0(-1)	0(-1)	0.025(+1)	0.0014(+1)	0.1(+1)	0(-1)	0.025(+1)	0.025(+1)	0(-1)	5(+1)
6	0(-1)	0(-1)	0.1(+1)	0.025(+1)	0.0014(+1)	0(-1)	0.025(+1)	0.025(+1)	0(-1)	0.1(+1)	0(-1)
7	0(-1)	0.3(+1)	0(-1)	0(-1)	0(-1)	0.1(+1)	0.025(+1)	0.025(+1)	0(-1)	0.1(+1)	5(+1)
8	0.6(+1)	0.3(+1)	0.1(+1)	0(-1)	0.0014(+1)	0.1(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	0(-1)
9	0.6(+1)	0.3(+1)	0(-1)	0.025(+1)	0.0014(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	0(-1)	5(+1)
10	0(-1)	0.3(+1)	0.1(+1)	0.025(+1)	0(-1)	0.1(+1)	0.025(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)
11	0.6(+1)	0(-1)	0.1(+1)	0.025(+1)	0(-1)	0.1(+1)	0(-1)	0(-1)	0(-1)	0.1(+1)	5(+1)
12	0.6(+1)	0.3(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	0(-1)	0.025(+1)	0.025(+1)	0.1(+1)	0(-1)

Key: (−1) indicates the low level and (+1) indicates the high level

3.6 Extraction of Rhamnolipid

For isolation of biosurfactant produced in SSF, a known quantity of fermented substrate was mixed with distilled water (1:5, w/v) by stirring on amagnetic stirrer for 30 min at room temperature (□25 ^{o}C), followed by centrifuging the whole content at 10,000 × g for 10 min to removethe insoluble matters. In order to precipitate the lipids and carbohydrates, the concentrated HCl was added with the supernatant to bring final pH of 2.0 and kept for overnight at 4°C (Yin *et al*., 2009). Resultant Grey white precipitation was collected by centrifugation at 10.000 × g for 20 min at 4°C. For further extraction of biosurfactant compounds, 10mL of chloroform methanol (2 : 1v/v) was added to precipitated pellet and incubated in a rotatory shaker at 30°C for 15 minutes with 250 rpm agitation. The content was subjected tocentrifugation at 10.000 × g for 20 min under cooling condition and the supernatant was evaporated by air drying. The remaining residue was dispensed in sodium phosphate buffer (pH 7.0) and stored at 4°C as the rhamnolipid.

3.7 Quantification of Rhamnolipid

Rhamnolipid was quantified in triplicate by colorimetric technique as described by Candrasekaran and Bemiller, (1980). Orcinol reagent containing 0.19% orcinol in 53% H_2SO_4 was prepared. In this test, pentoses are converted to furfural by this reagent, which forms a blue green color with orcinol. To 100µL of the sample, 90µL of orcinol solution was added. The sample was heated for 30mins at 80^0C and then cooled at room temperature. Absorbance at 421nm was measured. The rhamnolipid concentration was calculated from standard curves prepared with L-rhamnose (0-50 mg/l) and was expressed as rhamnose equivalent.

3.8 Extraction of Sophorolipid

For isolation of biosurfactant produced in SSF, a known quantity of fermented matter was mixed with distilled water (1:5, w/v) by stirring on amagnetic stirrer for 30 min at room temperature (□25 °C), followed by centrifuging the whole content at 10,000 - g for 10 min to removethe insoluble matters. In order to precipitate the lipids and carbohydrates, the concentrated HCl was added with the supernatant to bring final pH of 2.0 and kept for overnight at 4°C. (Yin *et al*., 2009). Resultant precipitate was collected by centrifugation at 10.000 rpm for 20 min at 4°C. For further extraction of biosurfactant compounds, the precipitate was extracted three times using ethyl acetate withthe addition of 0.5g of magnesium sulfate to remove any traces of water present and the resultant brown gum extract was the sophorolipid (Nunez *et al.*, 2001).

3.9 Quantification of Sophorolipid

Sophorolipid was quantified in triplicate by the colorimetric method as described by Candrasekaran and Bemiller, (1980). Orcinol reagent containing 0.19% orcinol in 53% H_2SO_4 was prepared. In this test, pentoses are converted to furfural by this reagent, which forms a blue green color with orcinol. To 100µL of the sample, 90µL of orcinol solution was added. The sample was heated for 30mins at 80^0C and then cooled at room temperature. Absorbance at 421nm was measured. The sophorolipid concentration was calculated from standard curves prepared with sophorose (0-50 mg/l) and was expressed as sophorose equivalent.

3.10 Medium Optimization of Rhamnolipid and Sophorolipid Production by Statistical Approach

3.10.1 One-factor-at-a-time analysis

Following PB design, the classical one-factor-at-a-time (OFAT) approach was employed to evaluate the possible optimum levelsof the medium components that showed positive effects. The components investigated include: potassium dihydrogen phosphate, magnesium sulphate, sodium chloride, zinc sulphate, iron sulphate. Thus the appropriate concentrations and relative availability of these components are critical in overall media design. For magnesium sulphate, zinc sulphate and Iron sulphate a concentration of 0.025-0.1% (w/v) was considered, while for KH_2PO_4, it was 0.3-1.2% (w/v), 0.1-0.4% (w/v) for NaCl for rhamnolipid and sophorolipid respectively.

3.10.2 Response surface methodology (RSM)

Response surface methodology was used to optimize the screened variables for enhancedoptimized rhamnolipid and sophorolipid production. This process involved three important steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Face centered central composite design (FCCCD) under the response surface methodology (RSM) developed by the Design Expert software (Version 6.0.8, Stat-Ease Inc., Minneapolis, USA) was used to optimize the concentrations of the three significant factors namely magnessium sulphate, sodium chloride and zinc sulphate for rhamnolipid and magnessium sulphate, sodium chloride and potassium dihydrogen phosphate for sophorolipid, to find a set of 20 experimental runs with six replicated center points. The independent variables were studied at three different levels, low (−1), medium (0) and high (+1). The experimental design used for the study is shown in Table

3.2 and 3.3 for rhamnolipid and sophorolipid respectively. Experiments were conducted in 150 ml Erlenmeyer flasks (pH 6.0) prepared according to the design. The incubation was carried out at 30^{o}C and 150 rpm for five (5) days. All the experiments were carried out in triplicate and the average of rhamnolipid and sophorolipid production obtained was taken as the response (Y). The relationship between dependent and independent variables is explained by the following second-order polynomial equation:

$$Y=\beta_o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}X^2_1+\beta_{22}X^2_2+\beta_{33}X^2_3 \quad (3.1)$$

Where Y is the dependent variable (Rhamnolipid and sophorolipid production); X_1, X_2 are independent variables (Magnessium Sulphate, Sodium Chloride and X_3 for Zinc Sulphate and Potassium dihydrogen Phosphate concentrations); βo is an intercept term; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are the interaction coefficients; and β_{11}, β_{22} and β_{33} are the quadratic coefficients.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher'sF-test (overall model significance), its associated probability values, coefficient of determination R^2 which measures the goodnessof fit of regression model. The fitted polynomial equation was then expressed in the form of contour and surface plots in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

Table 3.2: Central Composite Design Matrix of Three Factors in Their Natural Units for Rhamnolipid Optimization

Run order	$MgSO_4.7H_2O$ %(w/v)	NaCl %(w/v)	$ZnSO_4$ %(w/v)
1	0.49(0)	0.3(+1)	0.49(0)
2	0.49(0)	0.2(0)	0.49(0)
3	0.49(0)	0.2(0)	0.49(0)
4	0.49(0)	0.2(0)	0.74(+1)
5	0.74(+1)	0.1(-1)	0.74(+1)
6	0.74(+1)	0.2(0)	0.49(0)
7	0.49(0)	0.2(0)	0.49(0)
8	0.49(0)	0.2(0)	0.49(0)
9	0.49(0)	0.2(0)	0.25(-1)
10	0.25(-1)	0.1(-1)	0.25(-1)
11	0.49(0)	0.1(-1)	0.25(-1)
12	0.25(-1)	0.1(-1)	0.49(0)
13	0.25(-1)	0.3(+1)	0.74(+1)
14	0.25(-1)	0.2(0)	0.49(0)
15	0.74(+1)	0.3(+1)	0.74(+1)
16	0.25(-1)	0.3(+1)	0.25(-1)
17	0.74(+1)	0.3(+1)	0.25(-1)
18	0.74(+1)	0.1(-1)	0.25(-1)
19	0.49(0)	0.2(0)	0.49(0)
20	0.49(0)	0.2(0)	0.49(0)

Table 3.3: Central Composite Design Matrix of Three Factors in Their Natural Units for Sophorolipid Optimization

Run order	$MgSO_4.7H_20$ %(w/v)	NaCl %(w/v)	KH_2PO_4 %(w/v)
1	0.49(0)	0.3(+1)	0.60(0)
2	0.49(0)	0.2(0)	0.60(0)
3	0.49(0)	0.2(0)	0.60(0)
4	0.49(0)	0.2(0)	0.90(+1)
5	0.74(+1)	0.1(-1)	0.90(+1)
6	0.74(+1)	0.2(0)	0.60(0)
7	0.49(0)	0.2(0)	0.60(0)
8	0.49(0)	0.2(0)	0.60(0)
9	0.49(0)	0.2(0)	0.3(-1)
10	0.25(-1)	0.1(-1)	0.3(-1)
11	0.49(0)	0.1(-1)	0.3(-1)
12	0.25(-1)	0.1(-1)	0.3(-1)
13	0.25(-1)	0.3(+1)	0.90(+1)
14	0.25(-1)	0.2(0)	0.60(0)
15	0.74(+1)	0.3(+1)	0.90(+1)
16	0.25(-1)	0.3(+1)	0.3(-1)
17	0.74(+1)	0.3(+1)	0.3(-1)
18	0.74(+1)	0.1(-1)	0.3(-1)
19	0.49(0)	0.2(0)	0.60(0)
20	0.49(0)	0.2(0)	0.60(0)

3.11 Characterization

3.11.1 Blood haemolysis test

This is based on the ability of biosurfactant to breakdown red blood cells and form clear zones. Exactly 10µl of cell free supernatant wastaken and inoculated into blood agar plates. The plates were incubated for 48-72 h at 37°C. Then the plates were observed for the presence of clear zone around the colonies. The clear zone indicates the presence of rhamnolipid and sophorolipid (Rashedi *et al*., 2005).

3.11.2Emulsification index

The emulsification index was determined according to the method of Cooper and Goldenberg (1987). The emulsion test is a method to determine the presence of lipids using wet chemistry. Equal volume of supernatant (biosurfactant) and crude oil in a ratio of 1:1 was mixed in a glass test tube. Then, the mixture was vortexed for 2 min and left to stand for 24 h. The %EI24is given as percentage yielded by dividing the height of the emulsified layer (m) by the total height of the liquid in the glass test tube (m), then multiplying by 100. A higher emulsification index indicates a higher emulsification activity of the tested surfactant (Bodour *et al*., 2004).

Emulsification index (%EI24) =Height of the emulsion layer ×100/Total height (3.2)

3.11.3 Surface tension determination

This is based on the fact that any interface is characterized by the free energy which is proportional to its surface. Surface tension was measured using capillary tube method according to Martin (1993) and applying the formula:

$T = f \times r \times g \times h$ (3.3)

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 of fluid in the capillary tube in metre.

3.11.4Fourier transforms infrared spectroscopy analysis

Infra-Red absorption spectra were obtained using FTIR-8400S fourier transform infrared Spectrophotometer. Absorption spectra were plotted using a built-in plotter. IR spectra were collected from 400–4000 wave numbers (cm^{-1}) with resolution of 2 wave numbers per wave number. Samples wereprepared prior to the analysisby dispersing the solid uniformly in a matrix of potassium bromide.

CHAPTER FOUR

4.0 RESULTS

4.1Screening of Media Constituents for Rhamnolipid and Sophorolipid Production Using the Plackett–Burman Design

The product of the eleven medium components screened using Plackett–Burman (PB) design through twelve experimental runs with two levels each for rhamnolipid and sophorolipid production is presented in table 4.1.It can be seen from the resultsin Table 4.1, the highest yields were 224.6mg/gds and 88.53mg/gds for rhamnolipid and sophorolipid production respectively while the lowest were 46.60mg/gds and 17.35mg/gds for rhamnolipid and sophorolipid respectively. Themain effects of each medium component are presented in Fig.4.1 and 4.2, for rhamnolipid and sophorolipid respectively. These figures serve as description of individual components' contributions to the production of the biosurfactants. This was estimated based on the difference between the average of measurements made at the high level (+1) and at the low level (-1) of each component. Results from Fig. 4.1 shows that NaCl > $MgSO_4$>KH_2PO_4> $ZnSO_4$> $FeSO_4$> $CaCl_2$ positively affected both rhamnolipid and sophorolipid production while Na_2HPO_4, NH_4NO_3, $MnSO_4$ and thiamine-HCl were the components that highly affected the rhamnolipid and sophorolipid production negatively.

Table4.1: Plackett-Burman Experimental Design Matrix for Screening of Various Nutritional Components for Rhamnolipid and Sophorolipid Production Using Potato Peels as the Main Carbon Source.

Run	Na_2HPO_4 %(w/v)	KH_2PO_4 %(w/v)	NH_4NO_3 % (w/v)	$MgSO_4$ % (w/v)	$CaCl_2$ % (w/v)	NaCl %(w/v)	$ZnSO_4$ % (w/v)	$FeSO_4$ % (w/v)	$MnSO_4$ % (w/v)	T-HCl %(w/v)	Carbon source % (w/v)	rhamnolipid yield(mg/gds)	sophorolipid yield(mg/gds)
1	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0.00	0.00
2	0.6(+1)	0(-1)	0(-1)	0(-1)	0.0014(+1)	0.1(+1)	0.025(+1)	0(-1)	0.025(+1)	0.1(+1)	0(-1)	120.60	35.58
3	0(-1)	0.3(+1)	0.1(+1)	0(-1)	0.0014(+1)	0(-1)	0(-1)	0(-1)	0.025(+1)	0.1(+1)	5(+1)	68.60	22.65
4	0.6(+1)	0(-1)	0.1(+1)	0(-1)	0(-1)	0(-1)	0.025(+1)	0.025(+1)	0.025(+1)	0(-1)	5(+1)	92.60	40.29
5	0(-1)	0(-1)	0(-1)	0.025(+1)	0.0014(+1)	0.1(+1)	0(-1)	0.025(+1)	0.025(+1)	0(-1)	5(+1)	224.60	88.53
6	0(-1)	0(-1)	0.1(+1)	0.025(+1)	0.0014(+1)	0(-1)	0.025(+1)	0.025(+1)	0(-1)	0.1(+1)	0(-1)	104.60	49.12
7	0(-1)	0.3(+1)	0(-1)	0(-1)	0(-1)	0.1(+1)	0.025(+1)	0.025(+1)	0(-1)	0.1(+1)	5(+1)	174.60	76.17
8	0.6(+1)	0.3(+1)	0.1(+1)	0(-1)	0.0014(+1)	0.1(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	0(-1)	72.60	45.59
9	0.6(+1)	0.3(+1)	0(-1)	0.025(+1)	0.0014(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	0(-1)	5(+1)	212.60	69.71
10	0(-1)	0.3(+1)	0.1(+1)	0.025(+1)	0(-1)	0.1(+1)	0.025(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	124.60	49.70
11	0.6(+1)	0(-1)	0.1(+1)	0.025(+1)	0(-1)	0.1(+1)	0(-1)	0(-1)	0(-1)	0.1(+1)	5(+1)	134.60	55.00
12	0.6(+1)	0.3(+1)	0(-1)	0.025(+1)	0(-1)	0(-1)	0(-1)	0.025(+1)	0.025(+1)	0.1(+1)	0(-1)	46.60	17.35

Key: (−1) indicates the low level and (+1) indicates the high level

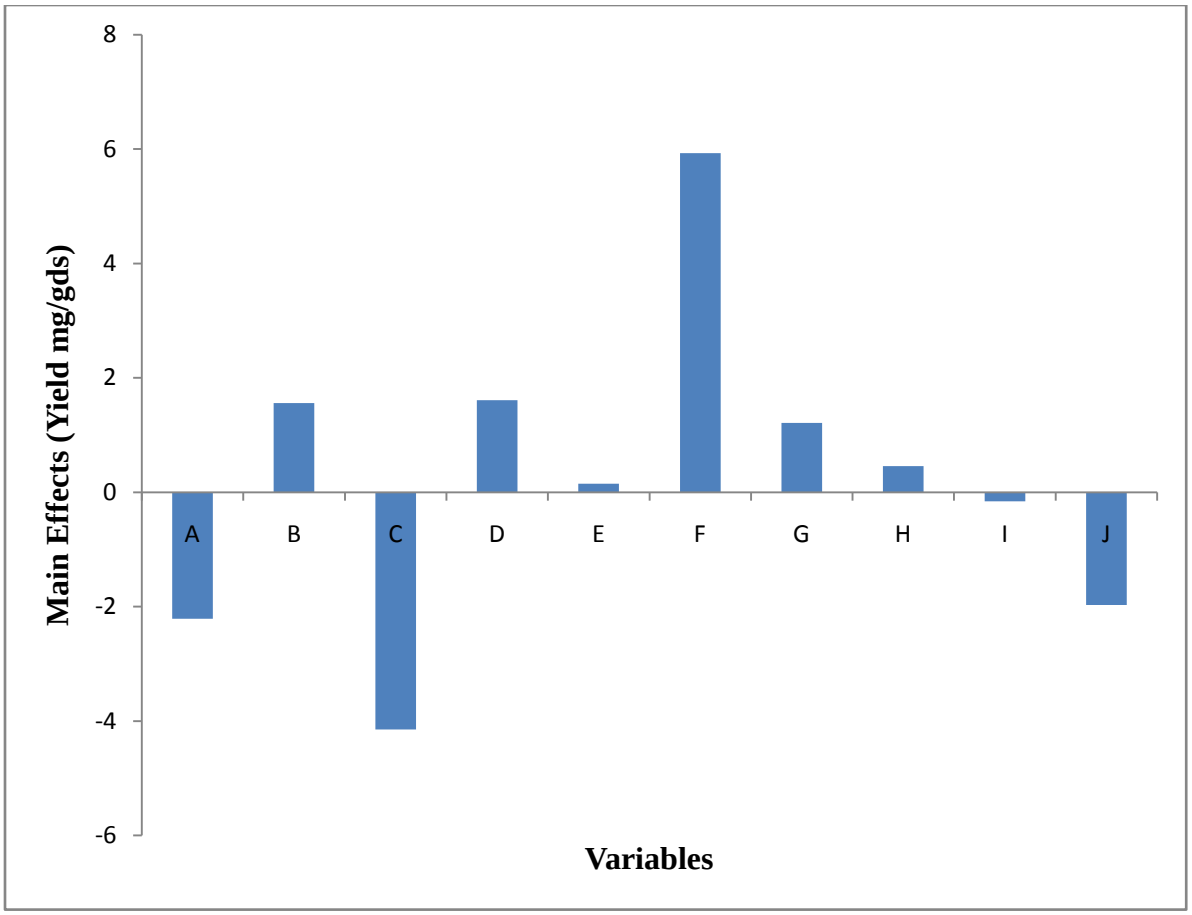


Figure 4.1**:** Main Effects of the Medium Constituents for Rhamnolipid production based on the Plackett–Burman Experimental runs (A, Na_2HPO_4; B, $MgSO_4.7H_2O$; C, NH_4NO_3; D, KH_2PO_4; E, NaCl; F, $CaCl_2$;G, $FeSO_4$; H, $ZnSO_4$; I, $MnSO_4$; J, Thiamine-HCL).

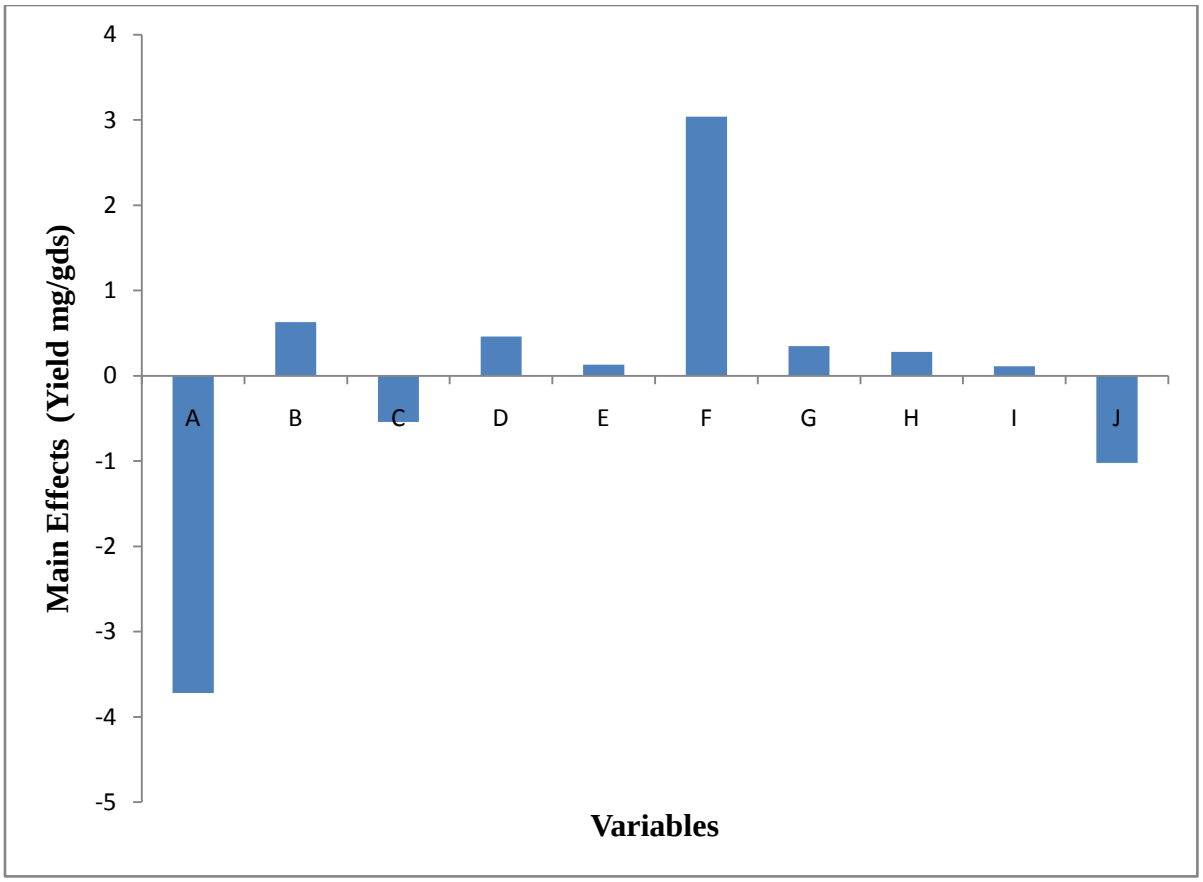


Figure 4.2**:** Main Effects of the Medium Constituents for Sophorolipid production based on the Plackett–Burman Experimental runs (A, Na_2HPO_4; B, $MgSO_4.7H_2O$; C, NH_4NO_3; D, KH_2PO_4; E, NaCl; F, $CaCl_2$;G, $FeSO_4$; H, $ZnSO_4$; I, $MnSO_4$; J, Thiamine-HCL).

4.2 Optimization of Medium Components by Response Surface Methodology

Yields obtained fromPlackett Burman (PB) design matrix and One factor at a time analysis (OFAT) (See appendix III-IX) experiments, face centered central composite design under response surface methodologywas used to determine the optimum conditions of the three significant factors (magnessium sulphate, sodium chloride and zinc sulphate for rhamnolipid and magnessium sulphate, sodium chloride and potassium dihydrogen phosphate for sophorolipid).The experimental and the predicted responses for rhamnolipid and sophorolipid optimization are presented in Table 4.2 and 4.3 respectively. The results demonstrated that the highest amount of rhamnolipid produced by *Aspergillusniger* (226.60 mg/gds) was observed in the runs representing the center points (runs 8) and the lowest amount was observed in run 12 (120.60 mg/gds) in Table 4.2 while the highest amount of sophorolipid produced by *Aspergillusniger* (84.41mg/gds) was observed in the runs representing the center points (runs 5) and the lowest was observed in run 20 (37.35mg/gds) in Table 4.3.

A second order regression equation showed the dependence of rhamnolipid and sophorolipid yield on the medium constituents. An empirical relationshipbetween the response and the screened variables was expressed in terms of second-order polynomial equation:

Y (Rhamnolipid yield, mg/gds) = +209.60+ 4.0A + 15.40B –7.40C −13.0A^2–12.0B^2–18.0C^2−12.25AB–2.75AC +9.25BC.

Y (Sophorlipid yield, mg/gds) = +49.64 – 3.32A – 4.26B + 4.97C – 5.12A^2 + 0.17B^2 + 21.06C^2 – 2.54AB + 3.63AC – 2.10BC.

Where the rhamnolipid and sophorolipid production is the response (Y) and A, B and C are the concentrations of magnesium sulphate, sodium chloride and zinc sulphate, potassium dihydrogen phosphate respectively. The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results are shown in Table 4.3 and 4.4. With model F value of 10.74, 3.10 for rhamnolipid and sophorolipid respectively. The regression equation obtained indicted the R^2value of 0.9063 and 0.7062 as shown in Figure 4.3 and 4. 4. for rhamnolipid and sophorolipid respectively.

Figure4.5a represents the 3D interaction between magnesium sulphate and sodium chloride at fixed level of zinc sulphate. Lower and middle levels of both the magnesium sulphate and sodium chloride did result in higher rhamnolipid yields. The shape of the response surface curves showed a moderate interaction between these tested variables. While figure 4.5b shows the 3D plot corresponding to magnessium sulphate and zinc sulphate concentration, this plot showed somewhat an elliptical contour. In case of sodium chloride and zinc sulphate (figure 4.5c), the response plot was elliptical depicting interaction between the two variables.

While for figure 4.6a the response plot was elliptical while 4.6b, 4.6c the response plot were not elliptical.

Table 4.2: Central Composite Design Matrix of Three Factors in Their Natural Units along with the Responses for Rhamnolipid

Run order	$MgSO_4.7H_20$ %(w/v)	NaCl %(w/v)	$ZnSO_4$ %(w/v)	Rhamnolipid Yield(mg/gds) Predicted	Experimental
1	0.49(0)	0.3(+1)	0.49(0)	213.00	212.60±2.83
2	0.49(0)	0.2(0)	0.49(0)	209.60	200.60±11.31
3	0.49(0)	0.2(0)	0.49(0)	209.60	202.60±2.83
4	0.49(0)	0.2(0)	0.74(+1)	184.20	174.60±2.82
5	0.74(+1)	0.1(-1)	0.74(+1)	148.05	154.60±2.82
6	0.74(+1)	0.2(0)	0.49(0)	200.60	202.60±2.82
7	0.49(0)	0.2(0)	0.49(0)	209.60	200.60±0.00
8	0.49(0)	0.2(0)	0.49(0)	209.60	226.60±50.91
9	0.49(0)	0.2(0)	0.25(-1)	199.00	202.60±14.14
10	0.25(-1)	0.1(-1)	0.25(-1)	148.85	154.60±0.00
11	0.49(0)	0.1(-1)	0.25(-1)	182.20	176.60±8.48
12	0.25(-1)	0.1(-1)	0.49(0)	121.05	120.60±2.82
13	0.25(-1)	0.3(+1)	0.74(+1)	194.85	202.60±2.65
14	0.25(-1)	0.2(0)	0.49(0)	192.60	184.60±11.31
15	0.74(+1)	0.3(+1)	0.74(+1)	172.85	168.60±5.65
16	0.25(-1)	0.3(+1)	0.25(-1)	180.65	180.60±8.48
17	0.74(+1)	0.3(+1)	0.25(-1)	174.65	176.60±5.41
18	0.74(+1)	0.1(-1)	0.25(-1)	186.85	180.60±3.45
19	0.49(0)	0.2(0)	0.49(0)	209.60	212.60±5.65
20	0.49(0)	0.2(0)	0.49(0)	209.60	226.60±2.83

Table 4.3: Central Composite Design Matrix of Three Factors in Their Natural Units along with the Responses for Sophorolipid

Run order	$MgSO_4.7H_2O$ %(w/v)	NaCl %(w/v)	KH_2PO_4 %(w/v)	Predicted	Sophorolipid Yield(mg/gds) Experimental
1	0.49(0)	0.3(+1)	0.60(0)	45.55	60.29±1.66
2	0.49(0)	0.2(0)	0.60(0)	49.64	51.47±1,24
3	0.49(0)	0.2(0)	0.60(0)	49.64	50.88±5.83
4	0.49(0)	0.2(0)	0.90(+1)	75.67	80.29±1.66
5	0.74(+1)	0.1(-1)	0.90(+1)	79.93	84.41±8.73
6	0.74(+1)	0.2(0)	0.60(0)	41.19	52.06±7.07
7	0.49(0)	0.2(0)	0.60(0)	49.64	47.35±1.66
8	0.49(0)	0.2(0)	0.60(0)	49.64	50.29±12.48
9	0.49(0)	0.2(0)	0.3(-1)	65.72	69.71±1.66
10	0.25(-1)	0.1(-1)	0.3(-1)	67.33	75.24±4.99
11	0.49(0)	0.1(-1)	0.3(-1)	54.06	47.94±9.15
12	0.25(-1)	0.1(-1)	0.3(-1)	74.21	72.64±2.07
13	0.25(-1)	0.3(+1)	0.90(+1)	66.58	69.12±1.66
14	0.25(-1)	0.2(0)	0.60(0)	47.83	45.58±1.66
15	0.74(+1)	0.3(+1)	0.90(+1)	62.13	52.06±3.32
16	0.25(-1)	0.3(+1)	0.3(-1)	68.10	61.47±12.89
17	0.74(+1)	0.3(+1)	0.3(-1)	49.11	48.58±2.91
18	0.74(+1)	0.1(-1)	0.3(-1)	58.51	53.83±2.99
19	0.49(0)	0.2(0)	0.60(0)	49.64	43.24±2.49
20	0.49(0)	0.2(0)	0.60(0)	49.64	37.35±0.83

Table 4.4**:** Analysis of Variance of Quadratic Model for Rhamnolipid Production by *Aspergillus niger* using Potato Peels as Carbon Source

Source	**Sum of Squares**	**F-value**	**p-value**
Model	11845.70	10.74	0.0005
$MgSO_4$	160.00	131	0.2798
NaCl	2371.60	19.36	0.0013
$ZnSO_4$	547.60	4.47	0.0606
A^2	464.75	3.79	0.0801
B^2	396.00	3.23	0.1024
C^2	891.00	7.27	0.0224
AB	1200.50	9.80	0.0107
AC	60.50	0.49	0.4983
BC	684.50	5.59	0.0397
Lack of Fit	451.30	0.58	0.7159

R^2= 0.906, adjusted R^2= 0.822, CV = 5.88, adequate precision = 11.748.

Table 4.5**:** Analysis of Variance of Quadratic Model for Sophorolipid Production by *Aspergillus niger*using Potato peels as Carbon Source

Source	Sum of Squares	F-value	p-value
Model	2448.72	3.10	0.0462
$MgSO_4$	110.20	1.25	0.2888
NaCl	181.31	2.07	0.1810
KH_2PO_4	247.51	2.82	0.1239
A^2	72.20	0.82	0.3855
B^2	0.080	0.004	0.9765
C^2	1219.22	13.90	0.0039
AB	51.77	0.59	0.4601
AC	105.63	1.20	0.2981
BC	35.32	0.40	0.5399
Lack of Fit	724.02	4.72	0.0565

R^2= 0.736, adjusted R^2= 0.499, CV = 16.23, adequate precision = 5.85

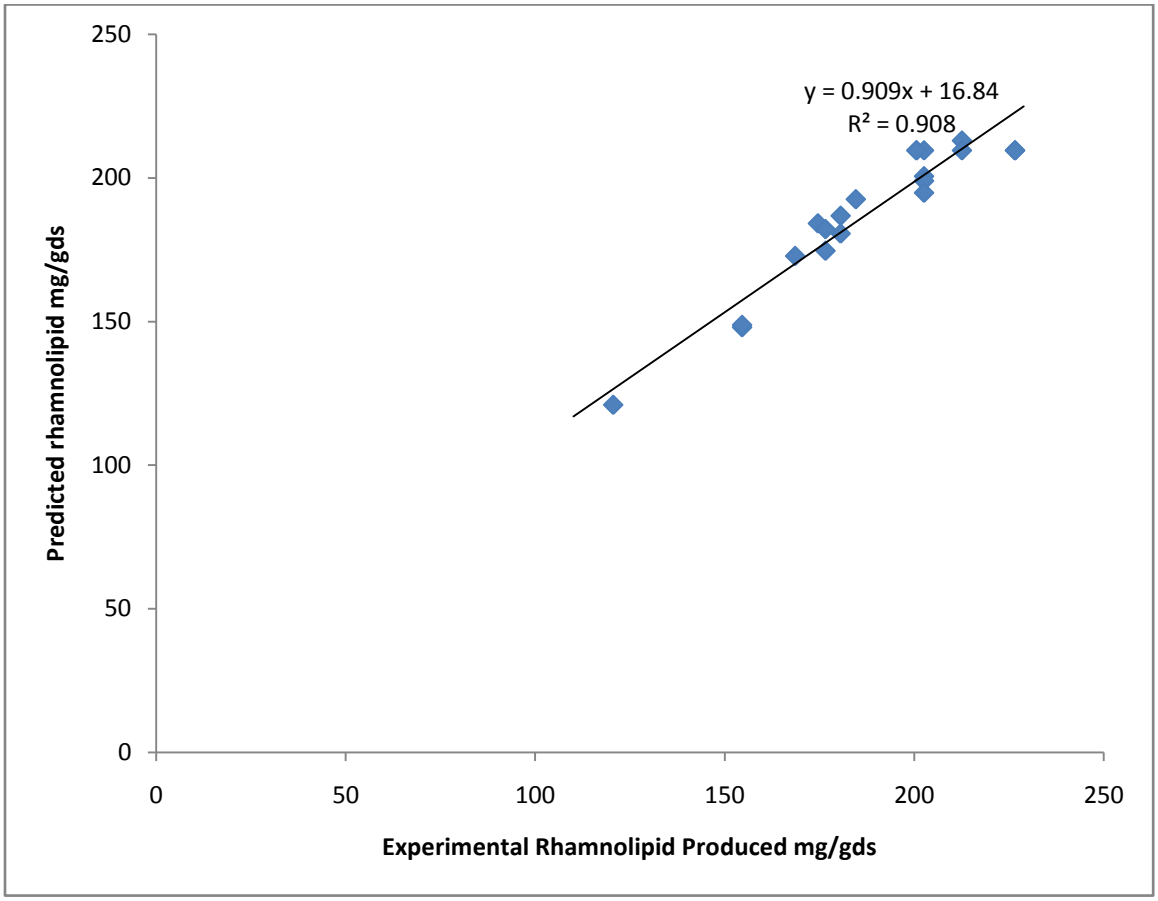


Figure4.3**:** Theoretical Values of Rhamnolipids Predicted by Regression Model Plotted *versus* Experimental Values.

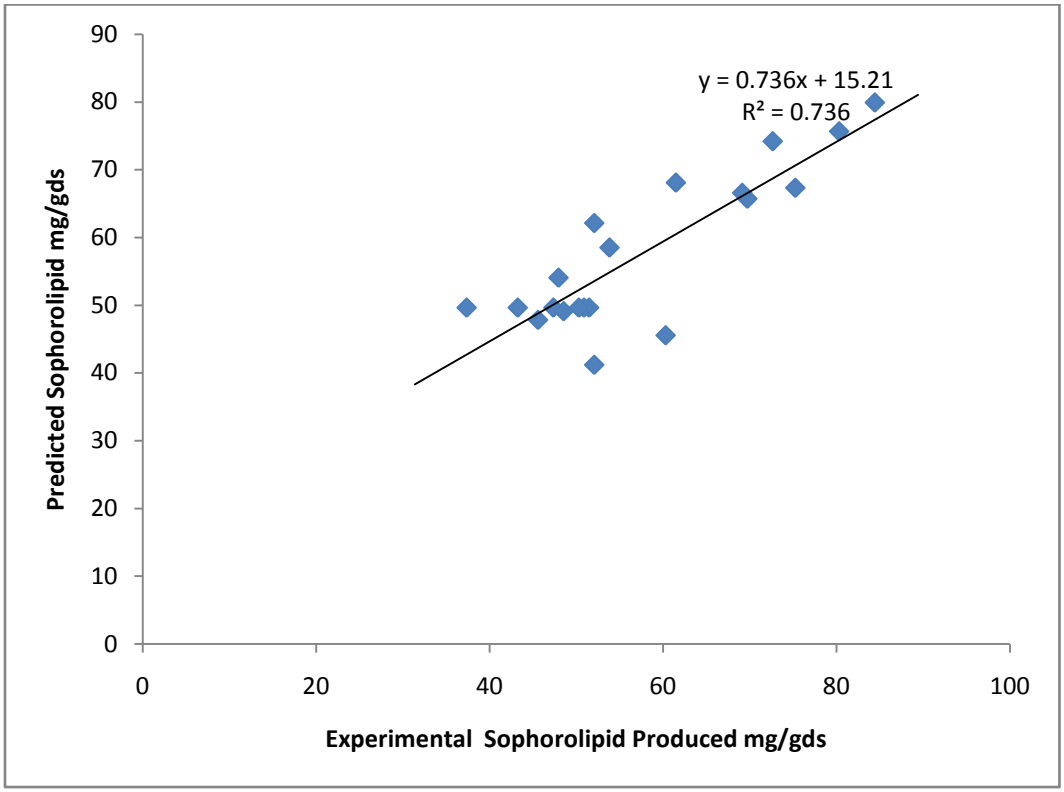
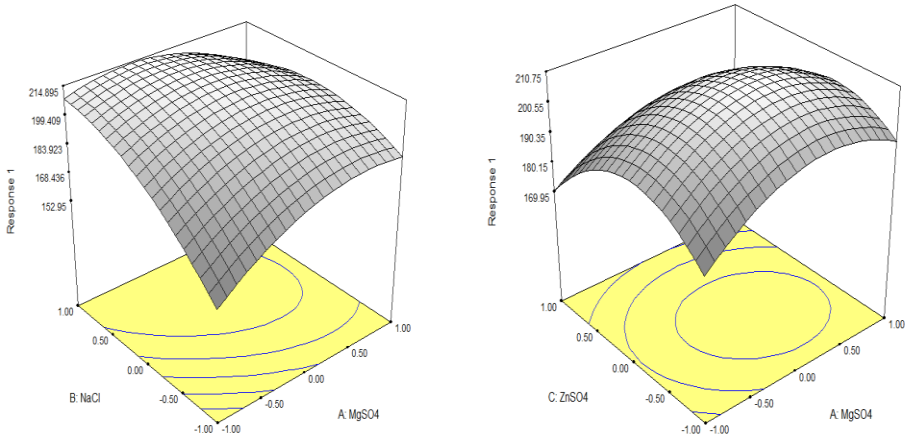


Figure 4.4**:** Theoretical Values of Sophorolipids Predicted by Regression Model Plotted *versus* Experimental Values

(A) (B)



(C)

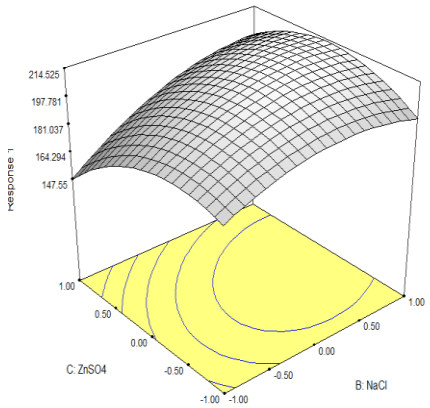
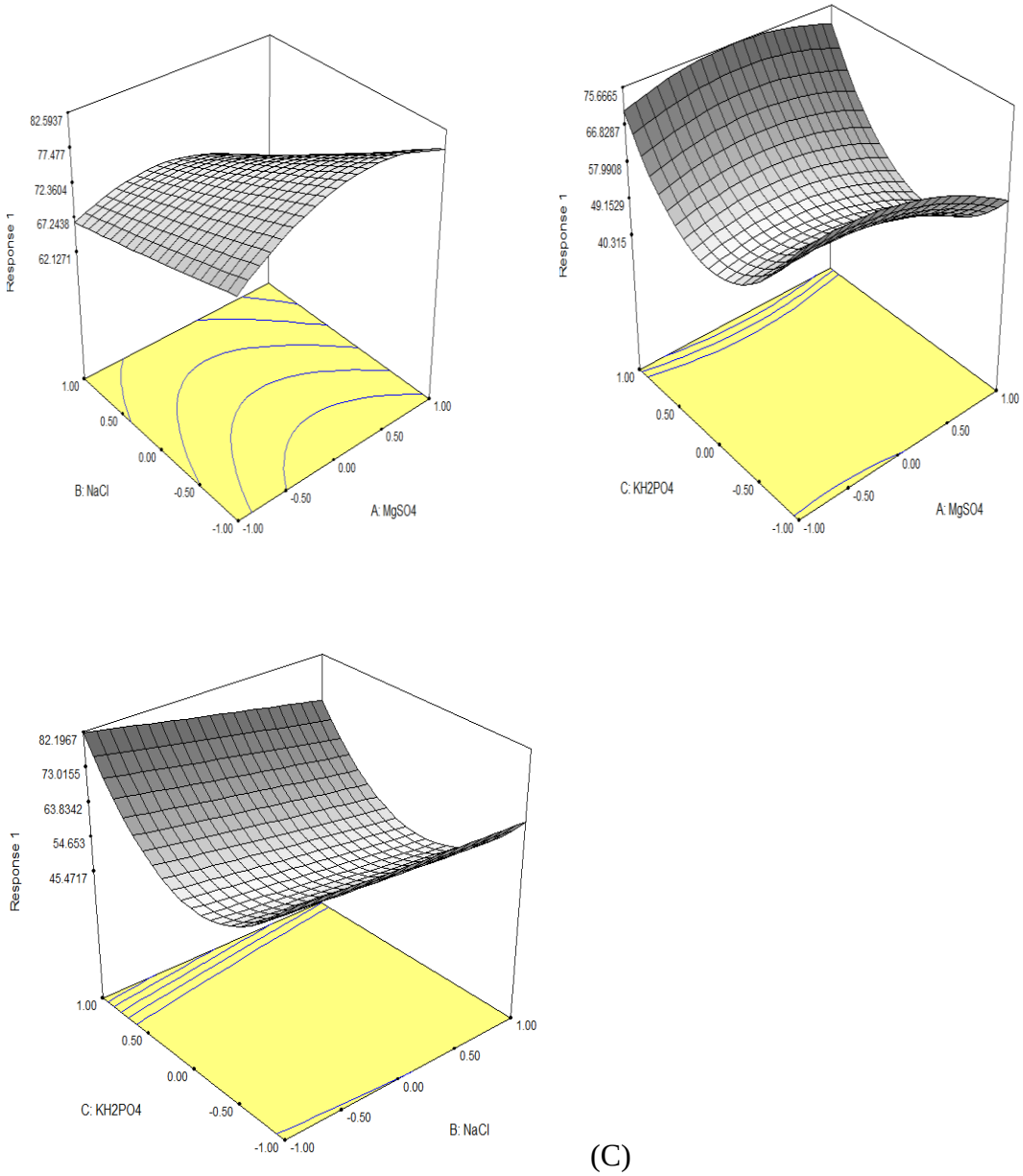


Figure 4.5**:** The 3D response surface curves of the combined effects of magnesium sulphate, sodium chloride and zinc sulphate on rhamnolipid production by *aspergillusniger*: (a) magnesium sulphate and sodium chloride at fixed level of zinc sulphate (b) magnesium sulphate and zinc sulphate at fixed level of sodium chloride, and (c) sodium chloride and zinc sulphate at fixed level of magnesium sulphate

(A) (B)



(C)

Figure 4.6**:** The 3D response surface curves of the combined effects of magnesium sulphate, sodium chloride and potassium dihydrogen posphate on sophorolipid production by *aspergillusniger*: (a) magnesium sulphate and sodium chloride at fixed level of potassium dihydrogen posphate (b) magnesium sulphate and potassium dihydrogen posphate at fixed level of sodium chloride, and (c) sodium chloride and potassium dihydrogen posphate at fixed level of magnesium sulphate

4.3 Blood Hemolysis Test for Rhamnolipid and Sophorolipid

The outcome of blood haemolysis is described in plate 4.2 and 4.2. From the figures below nineteenruns showed formation of a clear zone around the colonies.

4.4 Emulsification Indices for Rhamnolipid and Sophorolipid

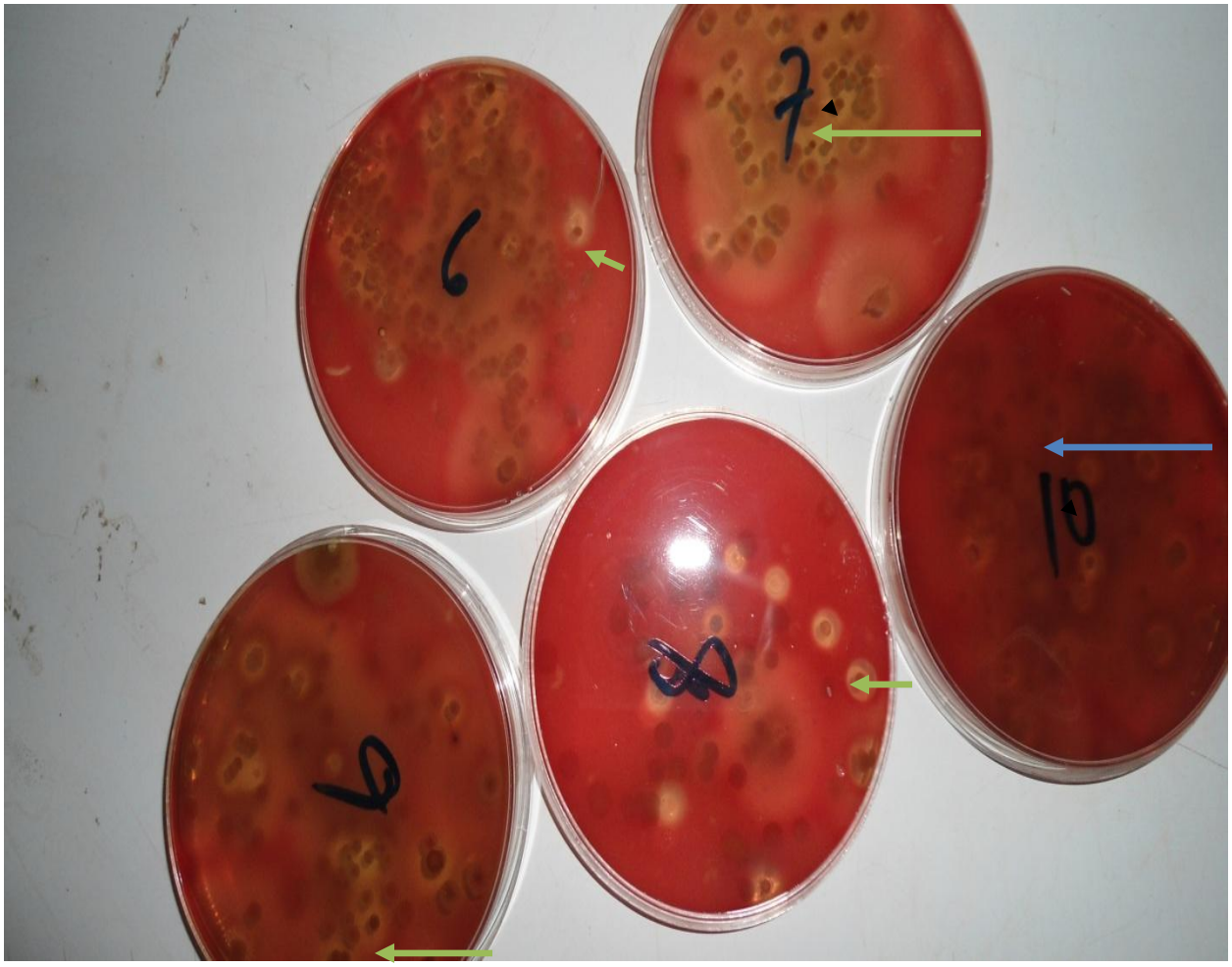
From the emulsification activities results presented in figure.4.7 and 4.8, it was observed that run 11 showed the maximum emulsification values (54.87%) for rhamnolipid while run 5 showed the maximum emulsification activity in sophorolipid (54.86%).

4.5 Surface Tension Indices

The surface tension indices of the rhamnolipid and sophorolipid are presented in figure 4.9 and figure.4.10 and maximum reduction in surface tension of 28.09mN/m and 30.30mN/m was observed for rhamnolipid and sophorolipid respectively.

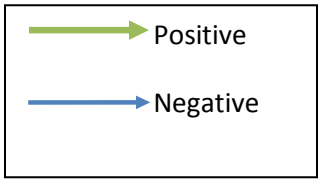
4.6 Spectroscopy of Biosurfactants Produced

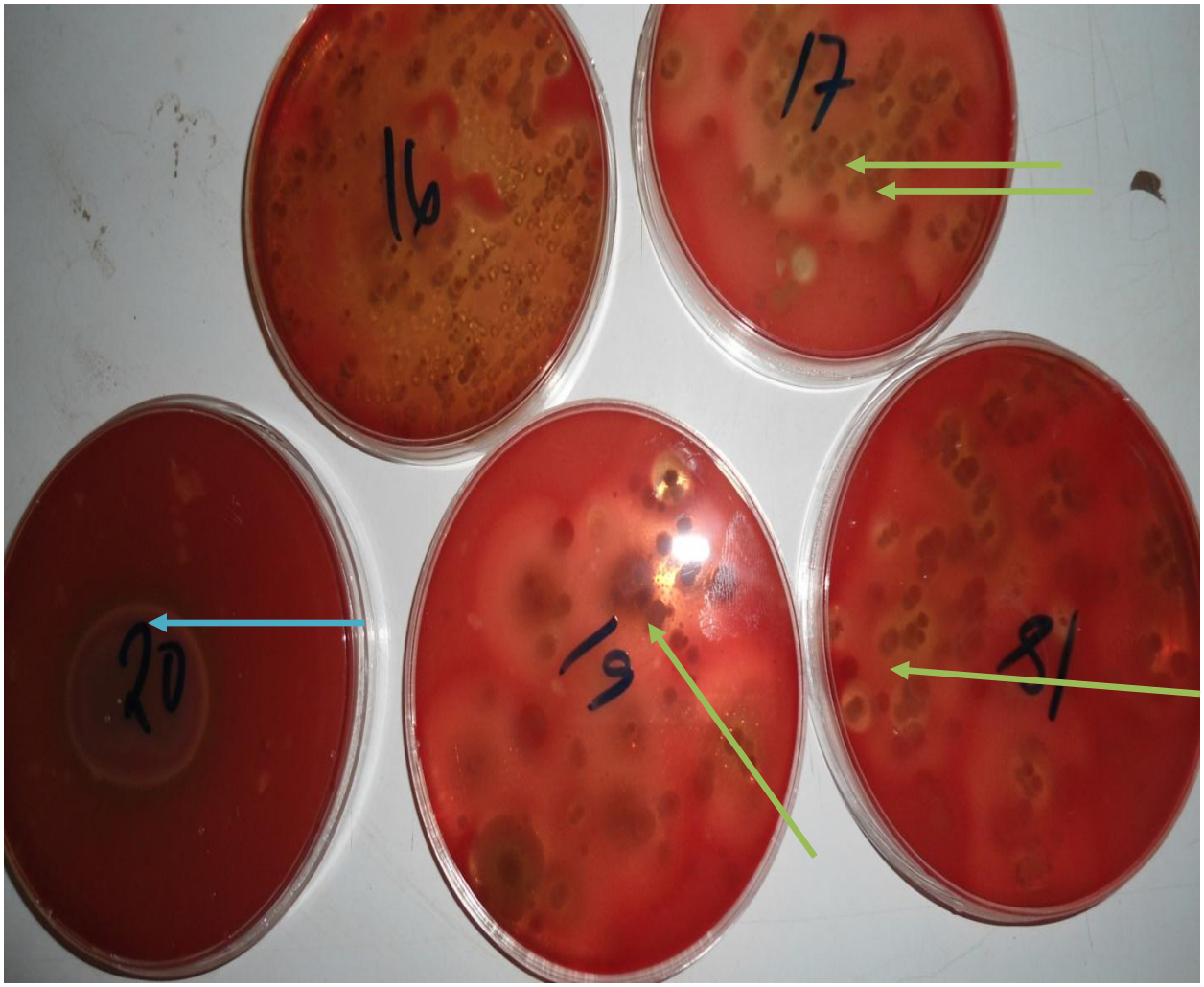
Figure 4.11 shows the FTIR spectrum for the rhamnolipid gotten from *Aspergillus niger* using potato peels as carbon source. It has ten peaks corresponding to seven different functional groups. The peaks are of wave number; of 3834.61cm^{-1}, 3419.90cm^{-1}, 2352.27cm^{-1}, 1641.48cm^{-1}, 1448.59cm^{-1}, 1193.98cm^{-1}, 1032.92cm^{-1}, 863.17cm^{-1}, 596.02cm^{-1} and 390.60cm^{-1} While the analysis for sophorolipid (Figure. 4.12) showed eight peaks corresponding to nine different functional groups of wave numbers; 3459.45cm^{-1}, 2317.55cm^{-1}, 2069.69cm^{-1} 1641.48cm^{-1}, 1212.30cm^{-1}, 1054.13cm^{-1}, 442.68cm^{-1}, 412.78cm^{-1}.



Plates 4.1**:** β-Heamolytic Activity of Rhamnolipid Produced by *A.niger* using Potato Peels

Key:





Plates 4.2**:** β-Heamolytic Activity of Sophorolipid Produced by *A.nger* using Potato Peels

Key

Positive

Negative

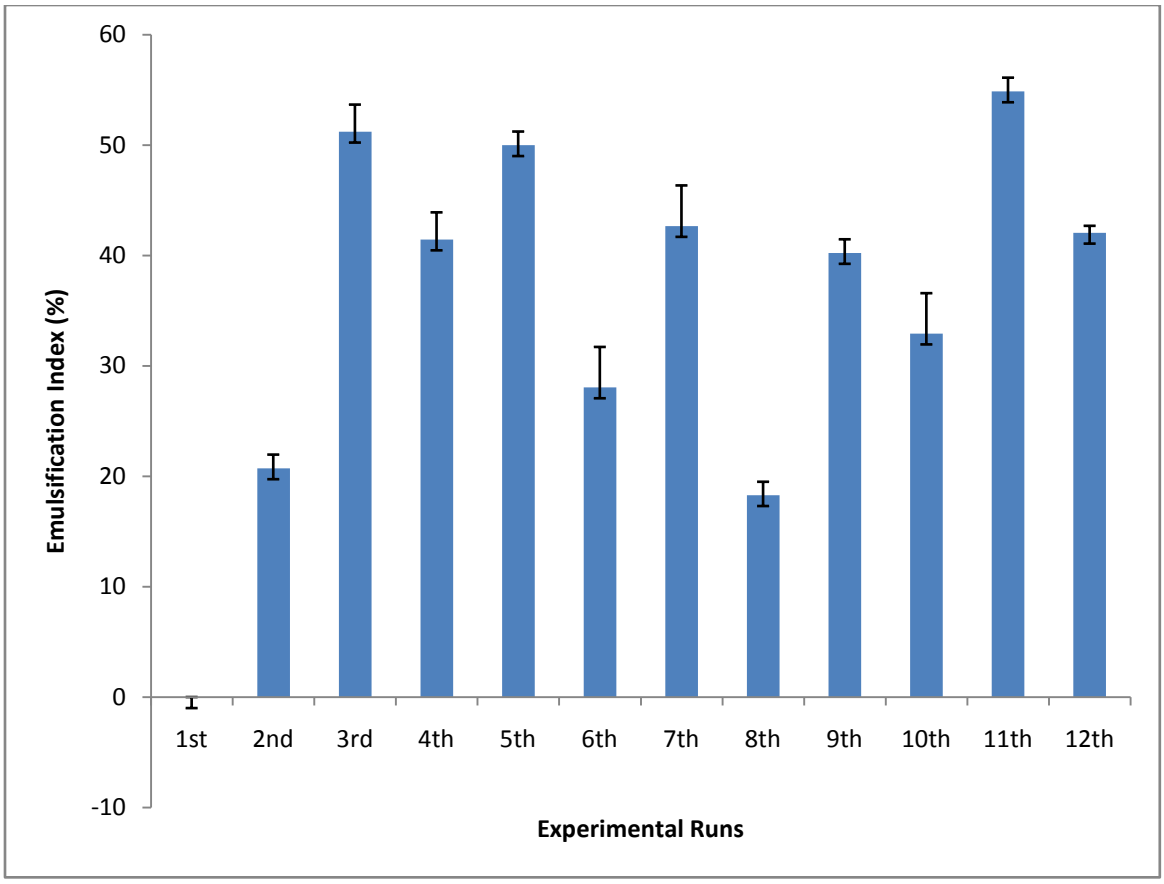


Figure 4.7: Comparison of Emulsification Indices of all the Experimental Runs of Rhamnolipid Produced by *A.niger* using Potato Peels

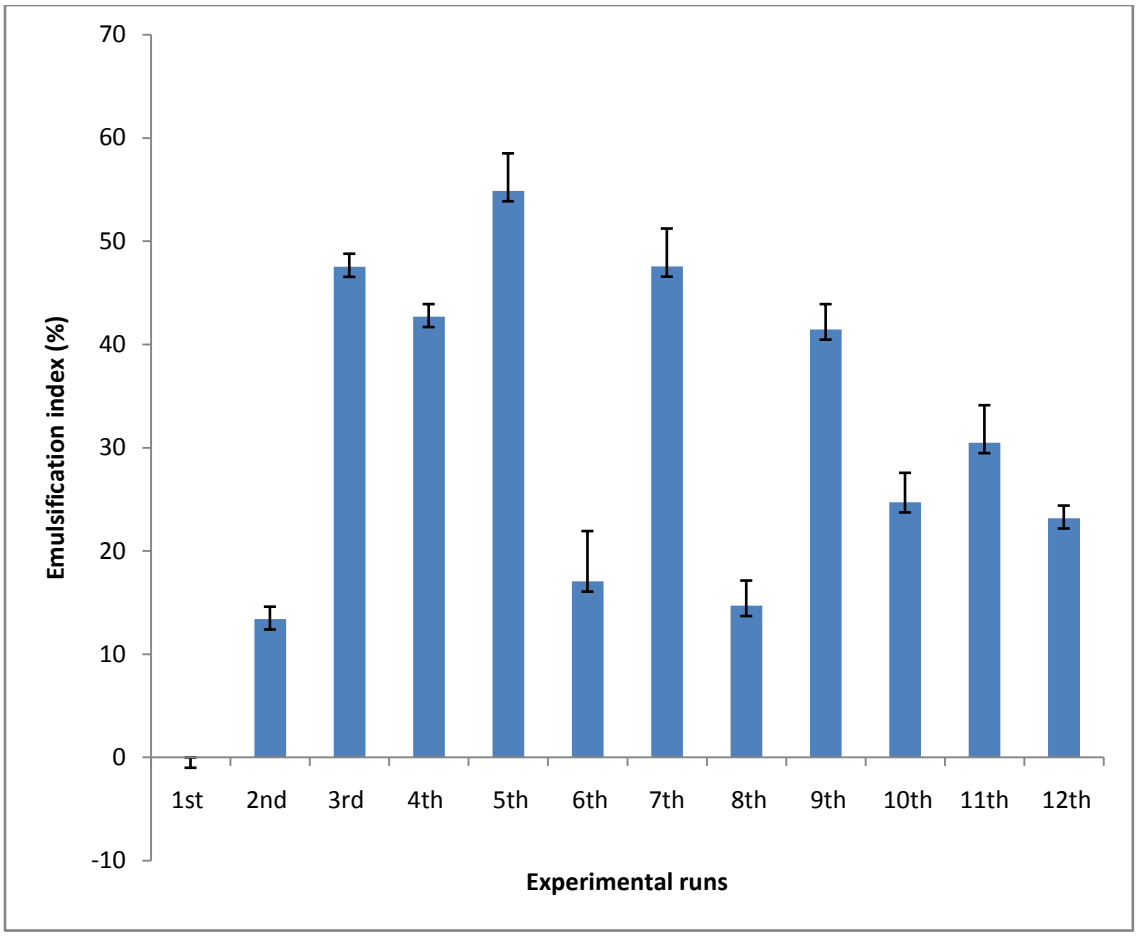


Figure 4.8: Comparison of Emulsification Indices of all the Experimental Runs of Sophorolipid Produced by *A.niger* using Potato Peels

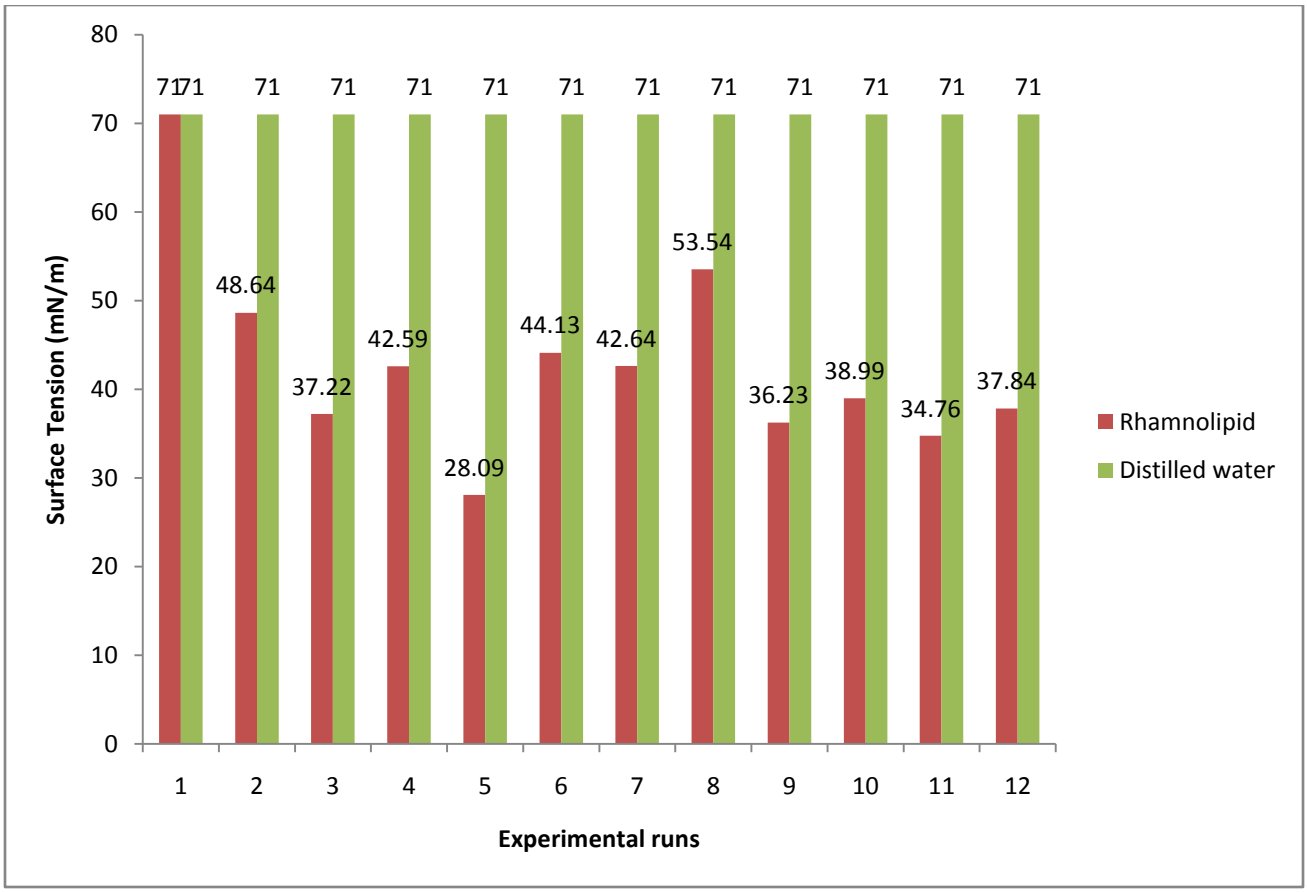


Figure 4.9: Comparison of Surface Tension indices obtained from the Twelve (12) Experimental runs for Rhamnolipid Produced with water

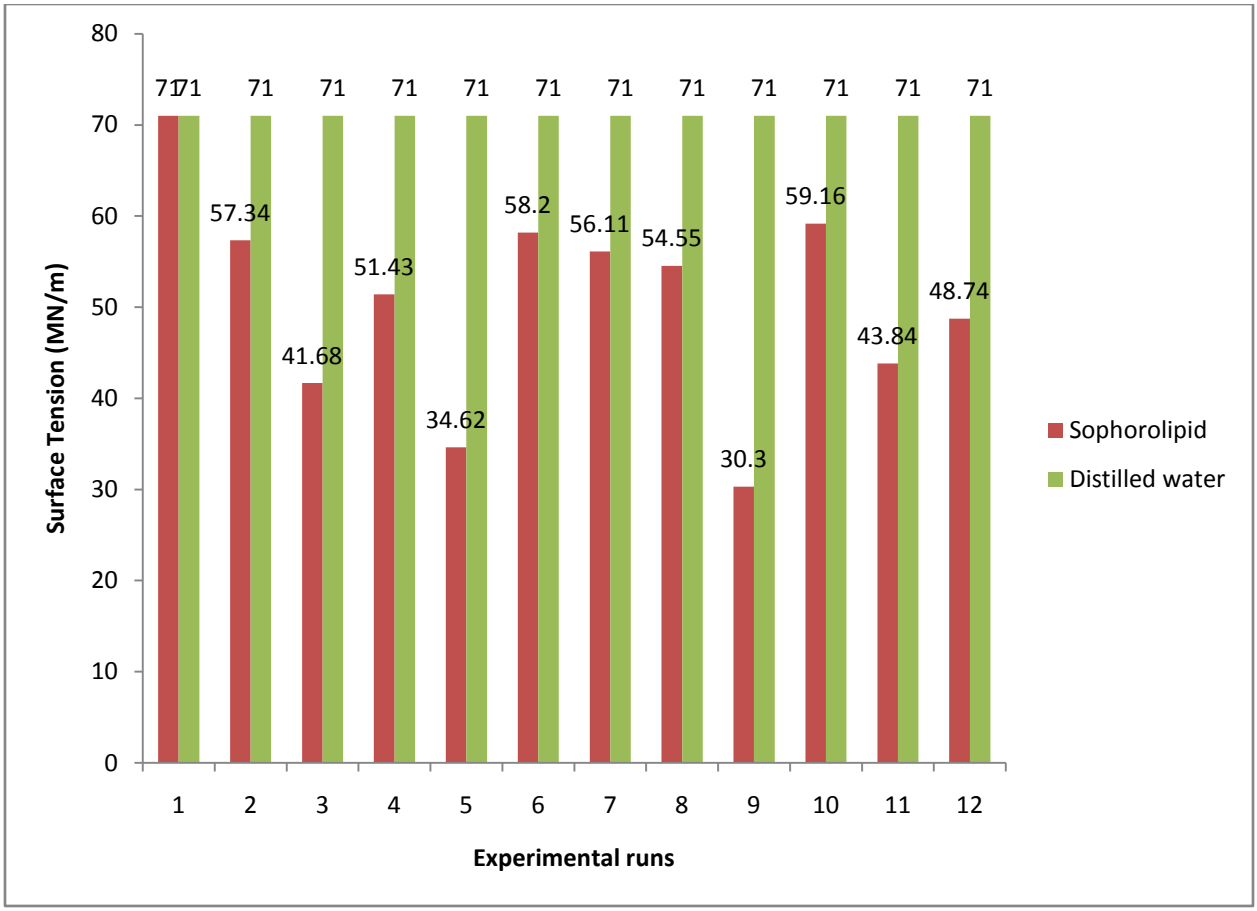


Figure 4.10: Comparison of Surface Tension indices obtained from the Twelve (12) Experimental runs for Sophorolipid Produced with water

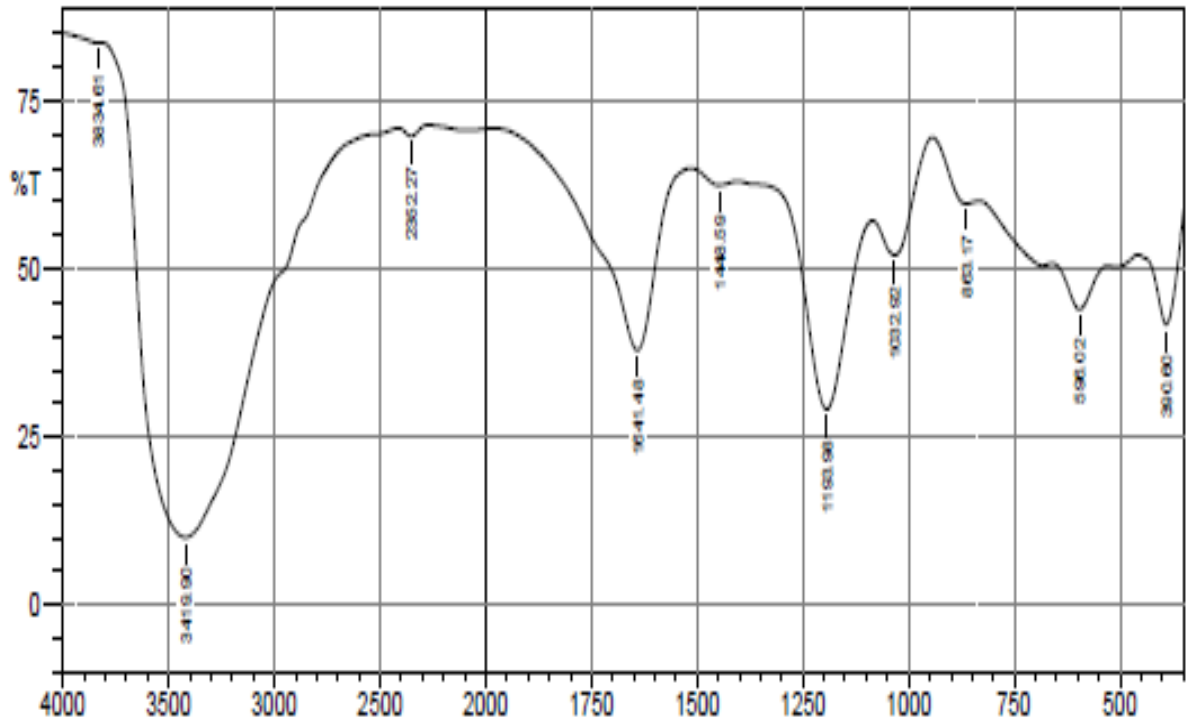


Figure 4.11: Fourier Transform Infrared Spectroscopy Analysis for Rhamnolipid Produced by *A.niger* in a Solid State Fermentation System

Table 4. 6**:** Assisgnment of peaks of Rhamnolipid Produced by *A.niger* using Potato Peels.

Absorption(cm^{-1})	**Intensity**	**Type of Vibration**	**Class of compound**
3419.90	strong	O-H Stretch	Carboxylic acids
1641.48	medium weak	C=C Stretch	alkenes
1193.98	Medium strong	C-O	alcohols
1193.98	Very strong	O=C-O-C	esters
863.17	strong	=C-H bend	alkenes
596.02	strong	C-Br	alkylhalides
390.60	strong	C-I	alkylhalides

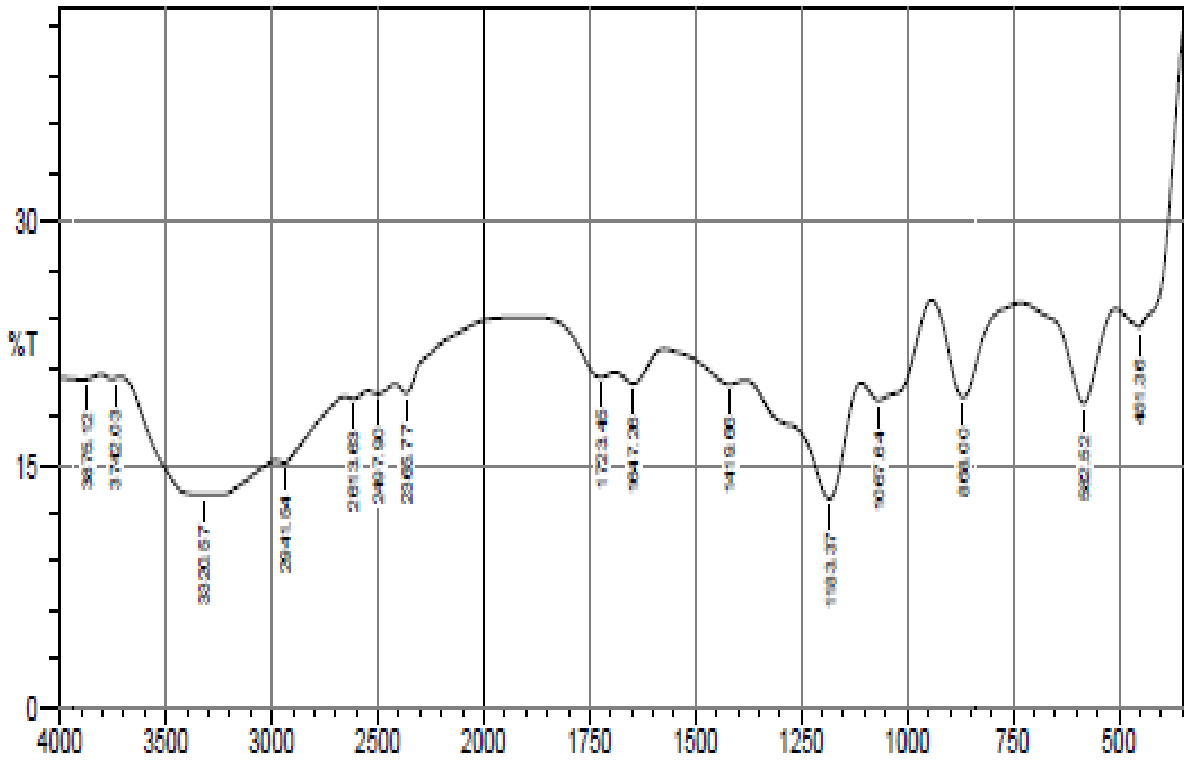


Figure 4.12: Fourier Transform Infrared Spectroscopy Analysis for Sophorolipid Produced by *A.niger* in a Solid State Fermentation System

Table 4.7: Assisgnment of peaks of Sophorolipid produced by *A.niger* using Potato Peels

Absorption (cm^{-1})	**Intensity**	**Type of Vibration**	**Class of compound**
3320.57	strong	=C-H	alkynes
2941.54	strong	C-H	alkanes
2941.54	strong	O-H	carboxylic acids
1723.45	strong	C=O	Aldehydes
1723.45	strong	C=O	esters
1647.26	Very weak	C=C	alkanes
1183.37	Medium strong	C-O	alcohols
1067.64	strong	C-O	alcohols
582.52	strong	C-Br	Alkylhalides
451.36	strong	C-I	alkylhalides

CHAPTER FIVE

5.0 DISCUSSION

The main factor limiting commercialization of biosurfactants is associated with non-economical large scale production. To overcome this obstacle and compete with synthetic surfactants, inexpensive substrate and potent microorganisms must be identified for biosurfactant production. Agro-industrial wastes are considered as the most promising substrate for low-cost biosurfactant production and can alleviate many processing industrial waste management problems. This would lead to the greater possibility for economical biosurfactant production and reduced pollution caused by those wastes (Maneerat, 2005). This work described in writes up aims to achieve all the said goals by identifying *Aspergillus niger*as a microorganism that can use waste (Potato Peels) for bioconversion into rhamnolipids and sophorolipids.In addition, many microorganisms, e.g *Pseudomonas* and *Torulopsis bombicola* have been used to produce rhamnolipid and sophorolipd but the production yield has been low. The use of *Aspergillus niger* and optimization of culture component using statistical models gives another new direction.

In this study, we revealed the possibility of rhamnolipid and sophorolipid production using cheaper carbon source like potato peels which could replaced other traditional carbon sources. Utilization of potato substrates for rhamnolipid and sophorolipid production could alleviate the potato processing industries waste management problem while addressing the economic issues related to rhamnolipid and sophorolipid production costs.

Production economy is the major interest in secondary metabolites production, as in the case with most biotechnological processes. Often, the amount and type of fermentative media components can contribute considerably to the production cost

(Muthusamy *et al*., 2008). Plackett-Burman design analysis shows that Na_2HPO_4, NH_4NO_3, $MnSO_4$ and thiamine-HCl were medium components that highly affected the rhamnolipid and sophorolipid production negatively. It is not surprising that NH_4NO_3appeared non contributory to the production, because reports from Irfan and Asif (2011)indicate that addition of nitrogen source caused inhibition of rhamnolipid biosurfactant in the resting cells of *P. aeruginosa*. A number of investigators have demonstrated the overproduction of biosurfactant by *Pseudomonas aeruginosa* when the culture reaches the stationary phase of growth due to limitation of nitrogen and iron (Syldalk *et al*., 1985). It was reported by Rakeshkumar *et al*., (2013) that unconventional substrate like potato peels contain high level of reducing sugar content which will promote biosurfactant production. Thus, the negative effect of minerals in the PB experimental results suggests that exogenous source of minerals in potato peels based medium is not needed since they are required in smaller quantities, and their high concentration may affect the overall productivity.

At positive levels, the medium components affecting the rhamnolipid and sophorolipid production could be ranked as $NaCl>MgSO_4>KH_2PO_4>ZnSO_4,> FeSO_4> CaCl_2$, The highest yield for rhamnolipid and sophorolipid was found in run 5 and 9, respectively where all the medium components present ($MgSO_4$, $ZnSO_4$, NaCl, $CaCl_2$, KH_2PO_4 and $FeSO_4$).Results from OFAT experiments show that for magnessium sulphate, production of rhamnolipid and sophorolipid where higher between the concentrations of 0.0245-0.074g/L while yield decreases with further increase in concentration. Sodium chloride and potassium dihydrogen phosphate followed the same trend with yield increases from 0.1-0.3g/L, 0.3-0.9g/l which decreases after these

concentrations for both rhamnolipid and sophorolipid respectively. These concentrations were found to be adequate for maximizing the biosurfactant production (Appendix III-IX). As such, further addition of sodium chloride, magnesium sulphate, and zinc sulphate and potassium dihydrogen phosphate into the medium was not considered, since the requirements of the microorganisms were fulfilled by the components of potato peels.

The model F value of 10.74, 3.10 and p-value of <0.0005, <0.0462 (Table 4 and 5) imply that the model is significant, suggesting that there is only 0.05%, 4.62% chance that the model F value could occur due to noise. Model terms with Prob > F (less than 0.05) are considered significant, while those greater than 0.10 are insignificant (Salihu*et al*., 2011). The non significant lack of fit suggested that the obtained experimental responses sufficiently fit with the model. For rhamnolipid yield, the higher values of R^2 (0.9063) and adjusted R^2 (0.8219) also indicated the efficacy of the model suggesting that 90.63% and 82.19% variation could be accounted for by the model equation respectively while the relative lower values of R^2 (0.7363) and adjusted R^2 (0.4990) suggested that 73.63% and 49.90% variation could be accounted for by the model equation respectively. Thus, for a good statistical model, the R^2 value should be in the range of 0–1.0, and the closer the value is to 1.0, the more fit the model is deemed to be (Reddy *et al*., 2008).

Adequate precision measures the signal to noise ratio and a value >4 are considered appropriate for desirable models. The adequate precision value of 11.75, 5.85 for rhamnolipid and sophorolipid production respectively indicate that the model can be used to navigate the design space. Also, the coefficient of variation (CV) indicates the degree of precision with which the treatments are compared, and the low value of CV showed the reliability of experiment. In this study, a relatively lower value of the

coefficient of variation (CV = 2.73) for rhamnolipid suggested a good precision and reliability of the experiment.

The p-values are used as a tool to check the significance of each coefficient, which also indicates the interaction strength between each independent variable; the smaller the p-values, the bigger the significance of the corresponding coefficient. The responses revealed that only one (sodium chloride) had more remarkable effect in rhamnolipid production

The 3D plots shown were based on the function of concentrations of two variables with the other variable being at its optimum level. Significance of the interactions between the corresponding variables is indicated by an elliptical or saddle nature of the contour plots (Salihu *et al*., 2011). From figure 4.5a, 1b and 1C lower and medium values of Magnessium Sulphate, Sodium Chloride and Zinc Sulphate showed higher yield of rhamnolipid while the higher values shows decrease in yield. In figure 4.6a, 2b and 2c the plots where not elliptical depicting lack of much interaction within variables. Thus, it can be seen that the optimized combinations of the selected media components showed strong synergistic effects especially on rhamnolipid production having a maximum yield of 226.60±2.83 with the level of magnesium sulphate, sodium chloride and zinc sulphate at 0.49, 0.2 and 0.49%w/v respectively.

Suresh *et al*., 2012 reported that, biosurfactant produced from *bacillus subtilis* have higher emulsifying properties, where as biosurfactant produced in the present study using potato peels showed moderate or low emulsification activity of 54.87 and 54.86% in rhamnolipid and sophorolipid which followed the same trend with work of Kishore and Ashis, (2007) and Ainon *et al*., (2013) producing a maximum emulsification activity

of 57.0±2.1. Emulsification activity found with biosurfactant produced by *A. niger* in this study inferred that, biosurfactant produced with carbon source like potato peeels might be used to remediate soil pollution.

The surface tension reducing ability of rhamnolipid and sophorolipid biosurfactants produced by *Aspergillus niger* is significantly higher than biosurfactant produced from *Bacillus substilis*, this might be so because the biosurfactant from the former strain is rich in rhamnolipid and sophorolipid isomers (Kishore and Ashis, 2007).

Moran *et al.*, (2002) reported that the biosurfactant produced by *Bacillus* could lyse blood andsince then has been used to quantify rhamnolipids. Johnson and Boese-Marrazzo, 1980 and Carrillo *et al.* (1996) found an association between haemolytic activity and surfactantproduction, and recommended the use of blood agar for observing lysis as a primary method to screen forbiosurfactant activity. Our isolates shows greater area of clear zone when compared with works of Suresh *et al*., (2012).

The deformation vibrations at 3419.90cm^{-1} confirm the presence of a carboxylic acid with an O-H stretch and the presence of alkenes of C=C stretch and C-H bend at 164.48 and 863.17cm^{-1}. The ester carbonyl group was also indicated from the band at 1193.8 cm^{-1}which corresponds to C-O and O=C-O-C deformation vibrations (Fig. 4.11). The presence of alkylhalides could be due to the left over of these compounds during extraction. The Infrared spectroscopy analysis results of rhamnolipid are positively correlated with previous work of Suresh *et al*., (2012) which revealed the presence of carbonyl stretching band and esters bond. The presence of alkynes, alkanes, alcohols, aldehydes, carboxylic acids and esters in Fig. 4.12 could also depict the presence of sophorolipid because closely looking at the structure of sophorolipid shows the presence

of carboxylic (COOH), alkane (CH_3), and alcohol (OH) groups(Kapadia and Yagnik, 2013).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The PB design result shows that $MgSO_4$, $ZnSO_4$, NaCl, $CaCl_2$, KH_2PO_4 and $FeSO_4$ contributed positively for rhamnolipid and sophorolipid production. Production of this biomolecules where high were some of this variables where present in the experimental runs.

Using centered central composite design, highest biosurfactants production were 226.60mg/gds and 84.41mg/gds for rhamnolipid and sophorolipid respectively.

The emulsification indices, surface tension reduction of 54.87, 54.86% and 28.02 and 30.30mN/m for rhamnolipid and sophorolipid, formation of clear zone by blood heamolysis and the FTIR peaks like alkanes, alkynes, esters, carboxylic groups, aldehydes and alcohols shows the presences of these biosurfactants.

6.2 Conclusion

1. Our investigation revealed that *A.niger* can grow on potato peels and produced rhamnolipid and sophorolipid.

2. Production yield were higher when statistical tools like Plackket-Burman and Response Surface Methodology were used in the optimization with optimum medium component containing 0.49% (w/v) magnesium sulphate, 0.2% (w/v) sodium chloride and 0.49% (w/v) zinc sulphate for rhamnolipid and 0.74%(w/v) magnesium sulphate, 0.1%(w/v) sodium chloride and 0.9%(w/v) of potassium dihydrogen phosphate for sophorolipid led to a maximum rhamnolipid and

sophorolipid production of 226.60±2.83 mg/gds and 84.41±8.73 mg/gds respectively.

3. Results from emulsification index, surface tension, blood hemolysis and fourier transform infrared spectroscopy showed the presence of some functional groups that might be associated with biosurfactants.

6.3 Recommendations

These findings should stimulate increased interest in biosurfactant research, which should involve the following:

1. Consideration of potato peels as a cheap substrate for biosurfactants production.
2. Utilization of statisticals tools like Plackett-Burman and Response surface methodology for enhancement in yield.
3. The need to consider novel and efficient multi-step down-stream processing methods to further reduce production cost
4. Further use of recombinant and mutant hyper-producing*Aspergillus* strains can make biosurfactants production economically viable
5. The toxicological aspects of new and current biosurfactants should be emphasized in order to certify the safety of these compounds for food utilization.

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APPENDIX I

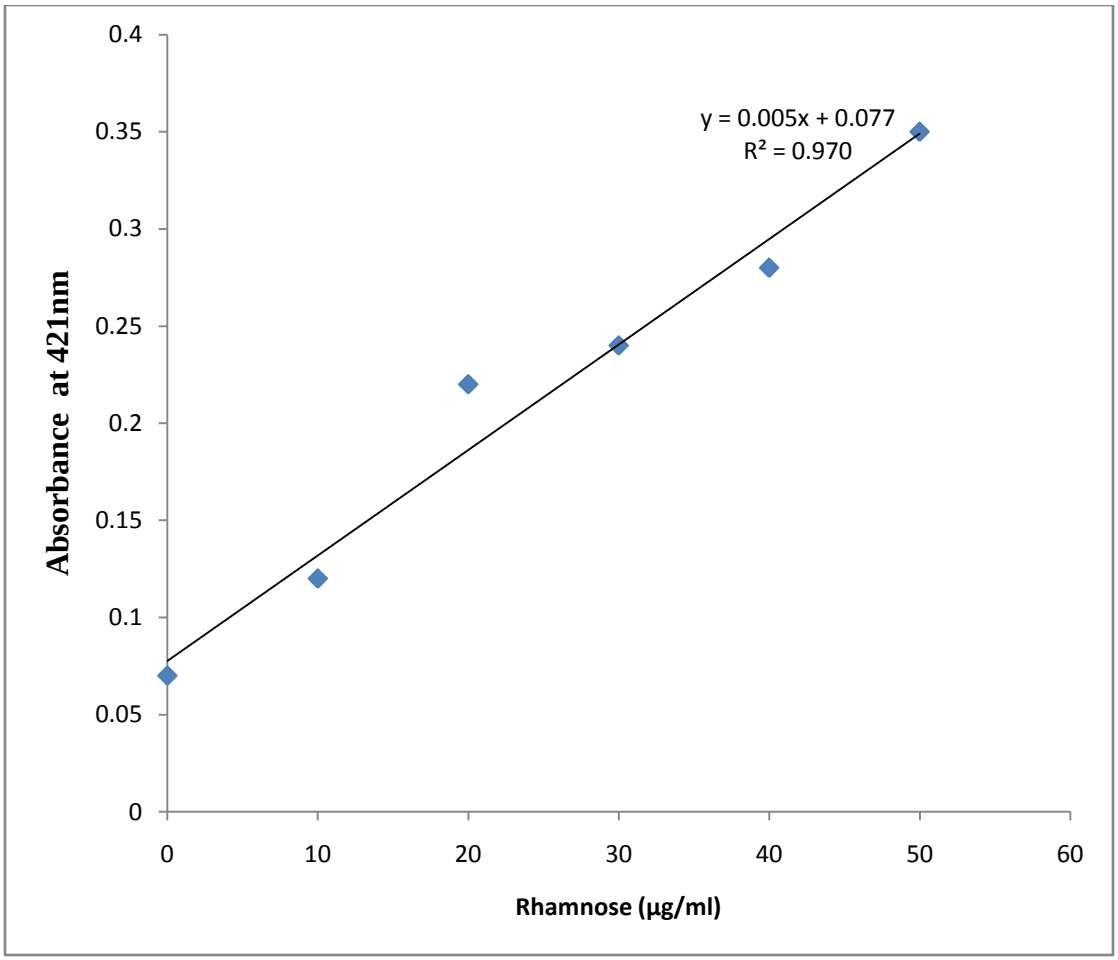


Fig 1: Standard Curve of Rhamnose

APPENDIX II

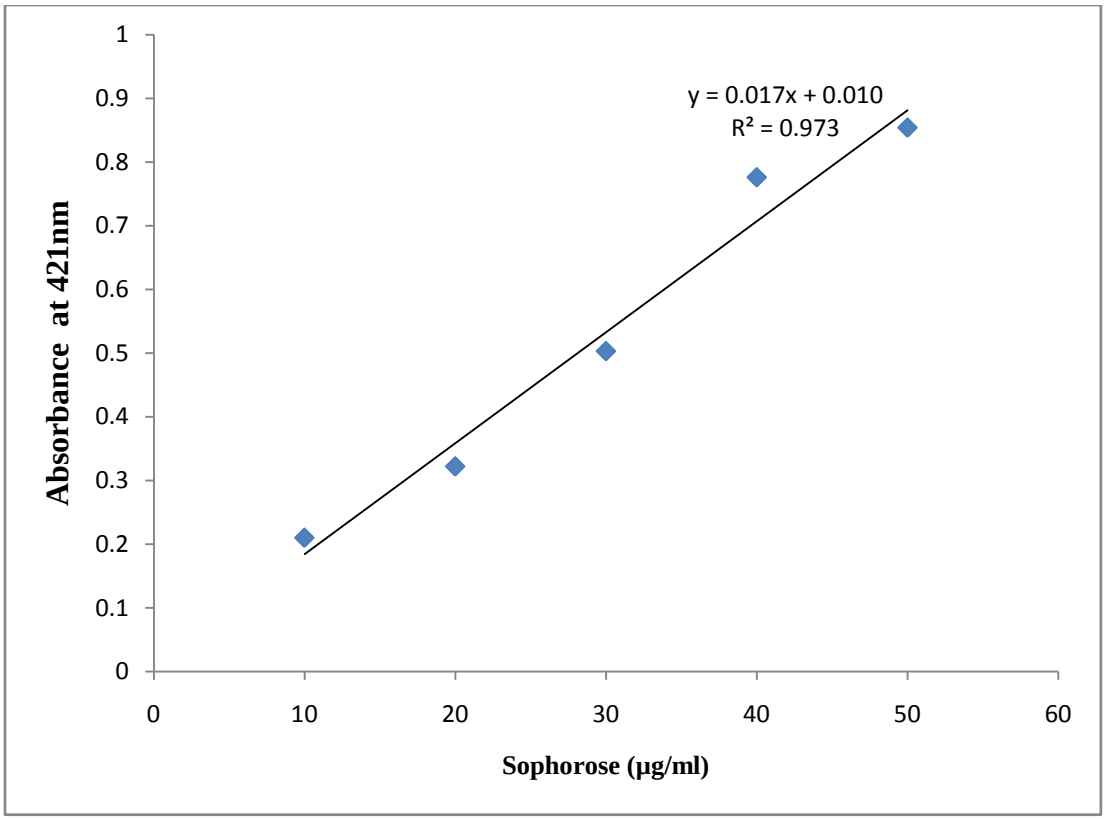


Fig 2: Standard Curve for Sophorose

APPENDIX III

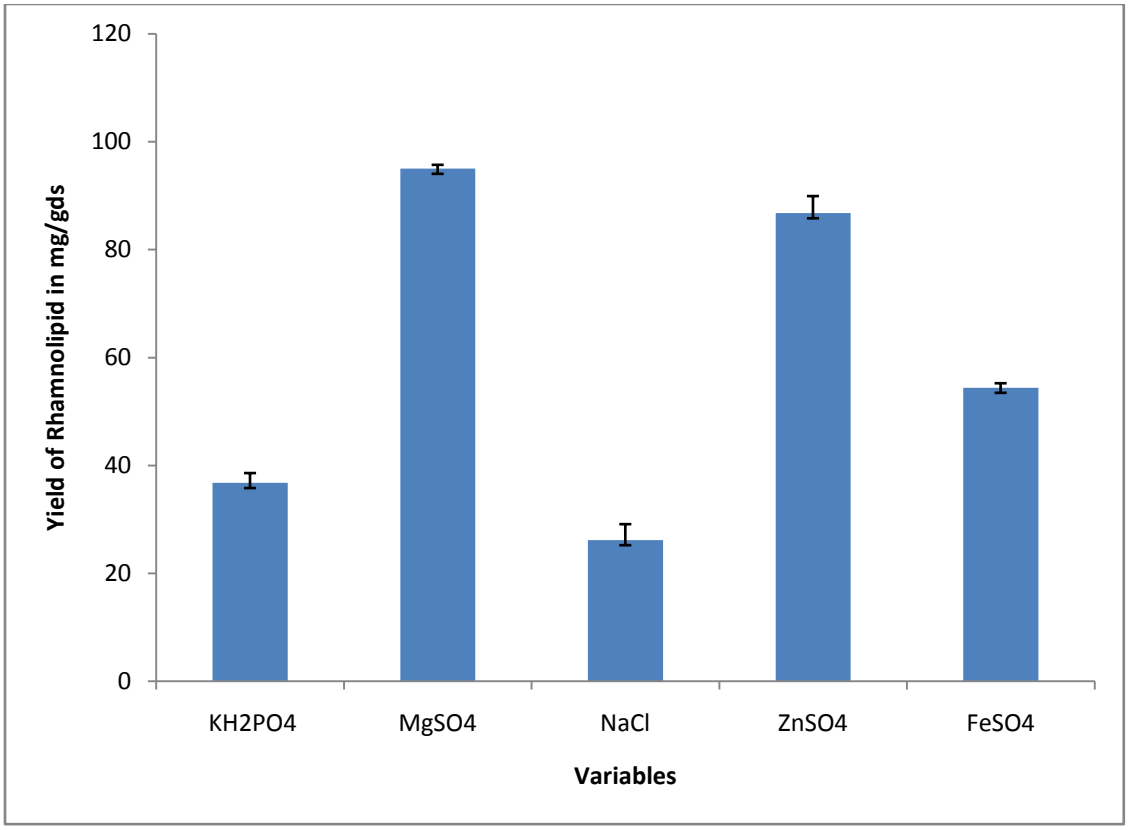


Fig 3: Effects of different medium components (0.25–0.3%, w/v) on rhamnolipid productionby *A .niger*.

APPENDIX IV

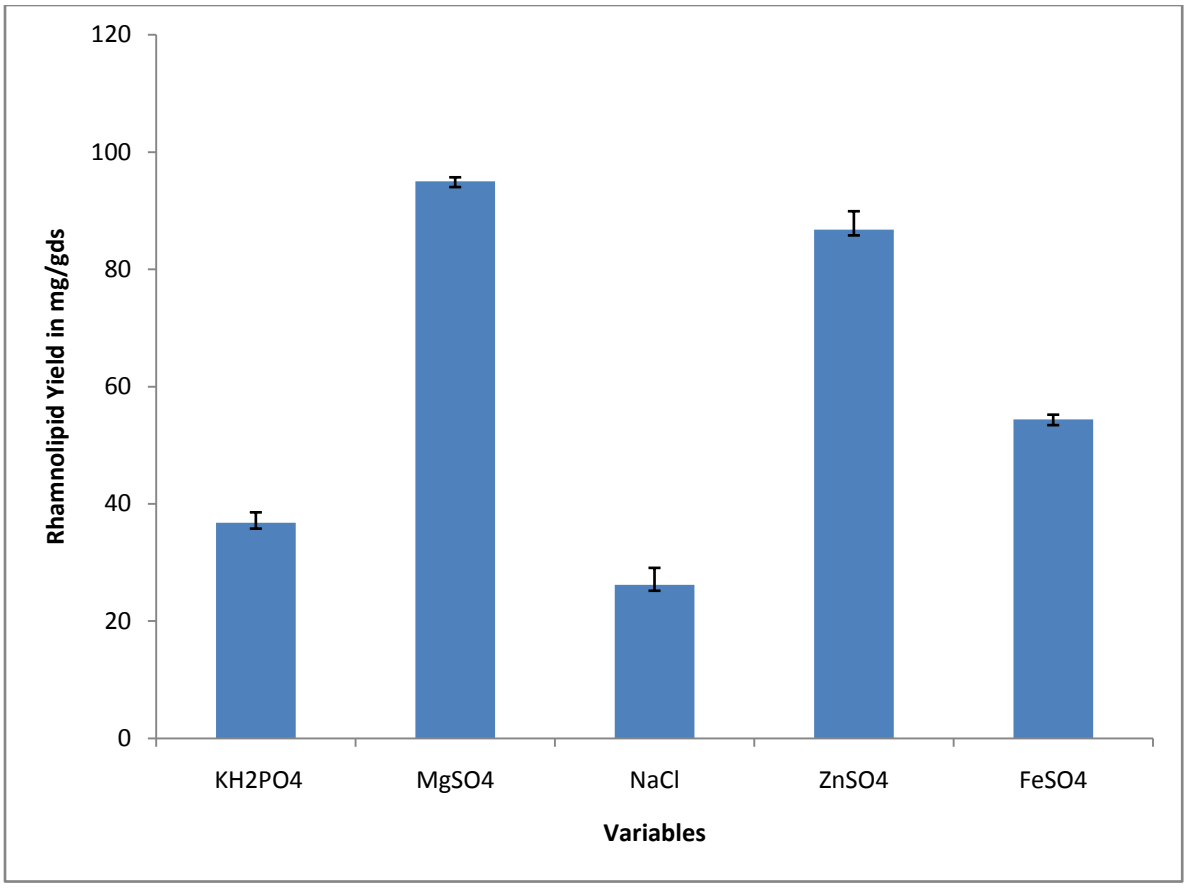


Fig 4: Effects of different medium components (0.049–0.6%, w/v) on rhamnolipid productionby *A .niger*.

APPENDIX V

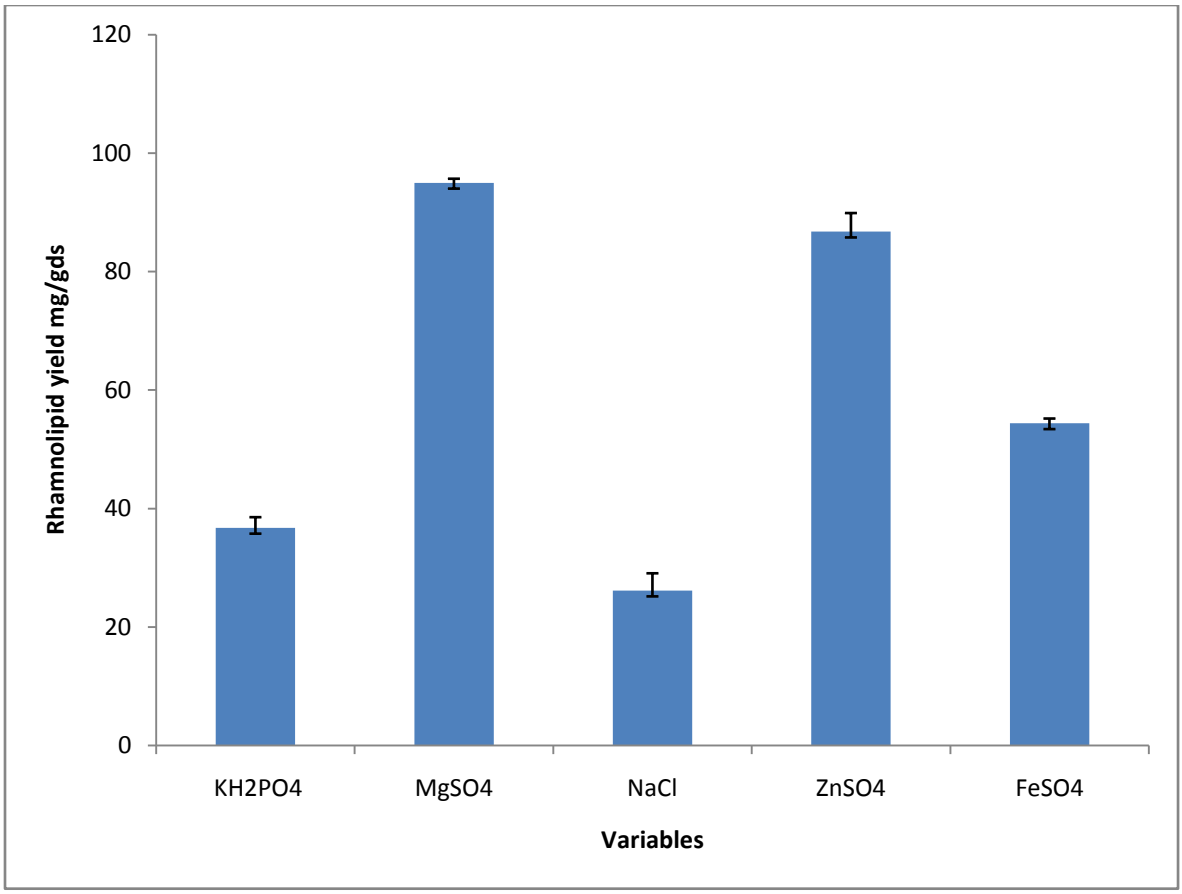


Fig 5: Effects of different medium components (0.075–0.9%, w/v) on rhamnolipid productionby *A .niger*.

APPENDIX VI

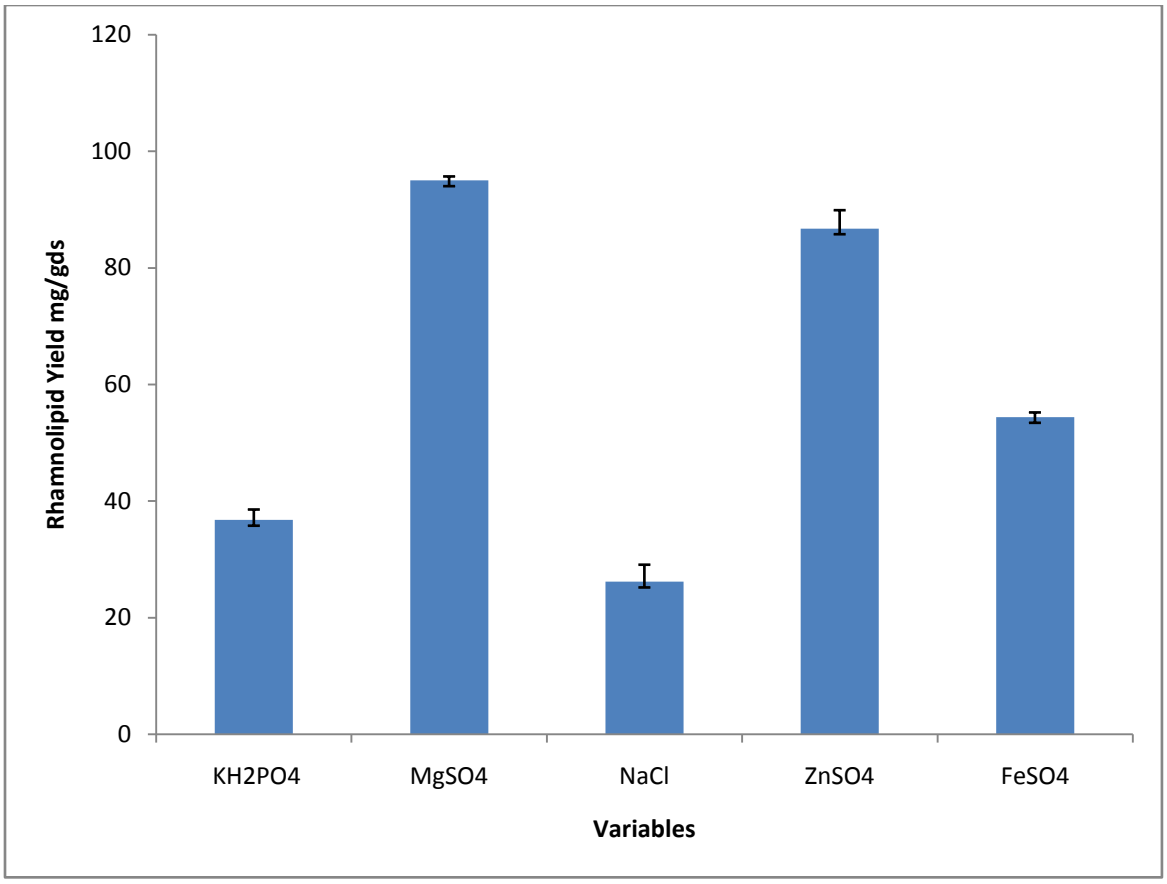


Fig 6: Effects of different medium components (0.1–1.2%, w/v) on rhamnolipid productionby*A .niger*.

APPENDIX VII

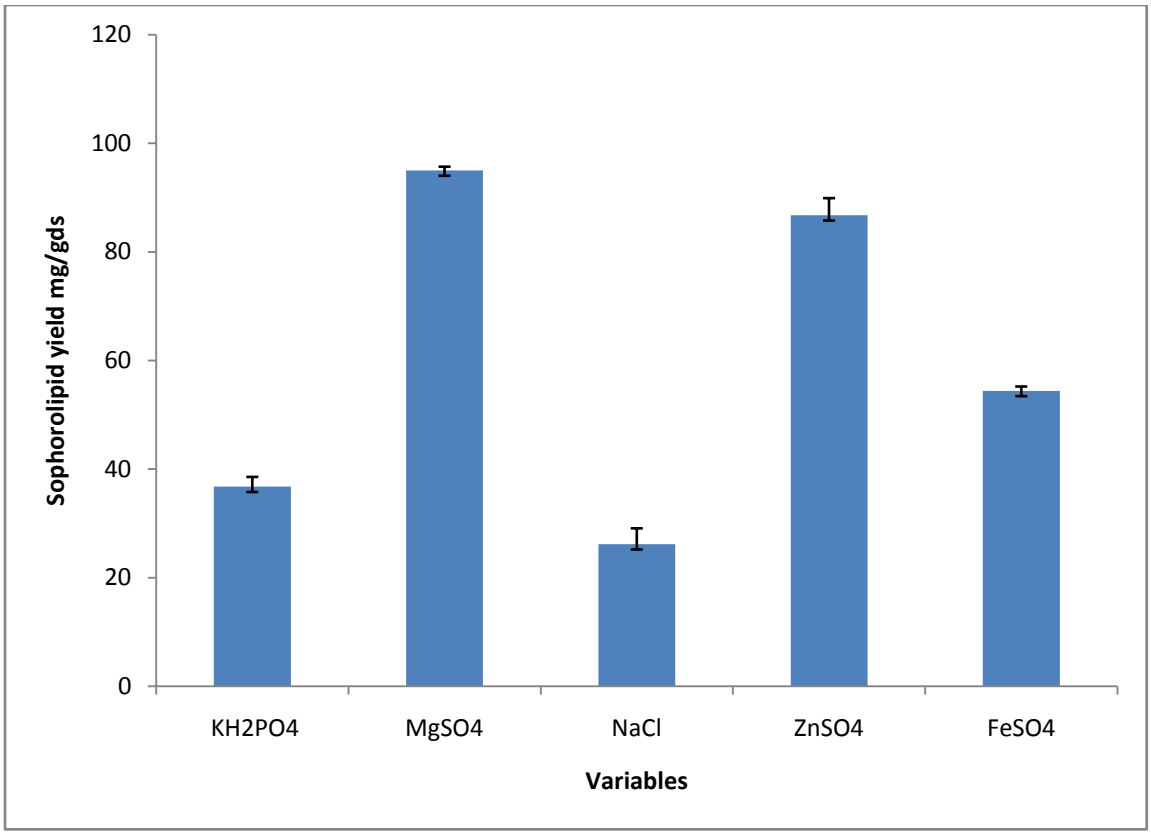


Fig 7: Effects of different medium components (0.25–0.3%, w/v) on sophorolipid productionby *A .niger*.

APPENDIX VIII

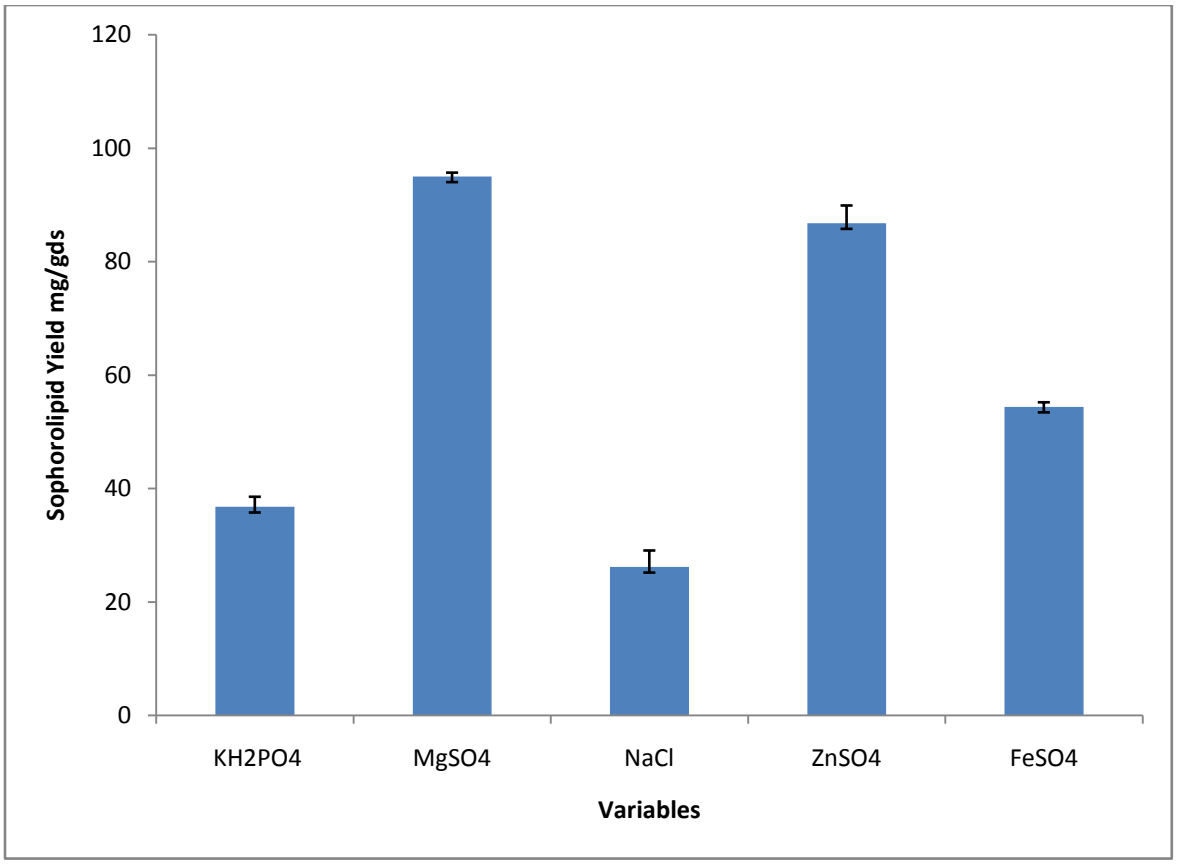


Fig. 8: Effects of different medium components (0.049–0.6%, w/v) on sophoroolipid productionby *A .niger*.

APPENDIX IX

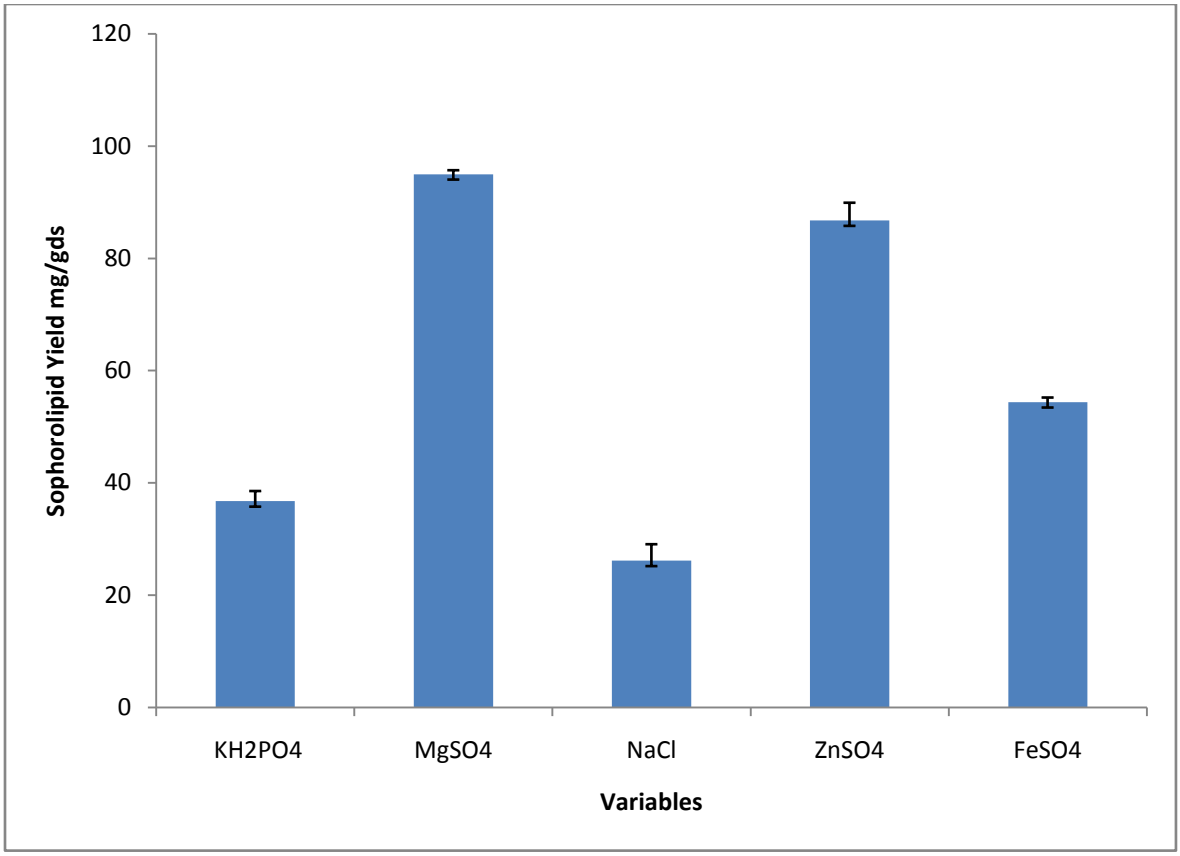


Fig. 9: Effects of different medium components (0.075–0.9%, w/v) on sophorolipid productionby *A .niger*.

APPENDIX X

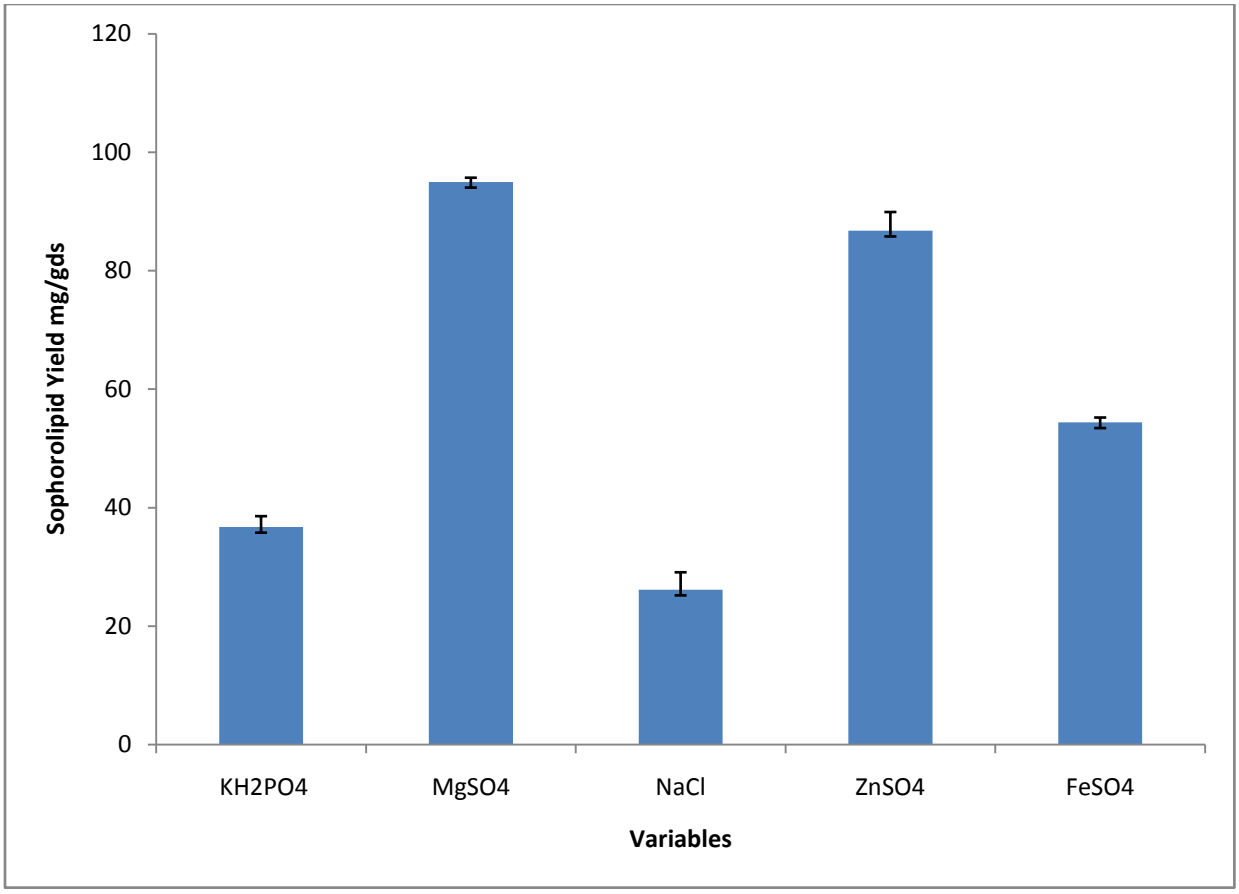


Fig 10: Effects of different medium components (0.1–1.2%, w/v) on sophorolipid productionby *A .niger*.