# Production of lipopeptide biosurfactant by *Paenibacillus* sp. D9: Basic and Applied Aspects

**by**

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As the candidate's supervisor, I have approved this thesis for submission.

Signed: _____ Name: Prof. Johnson Lin Date: 31/10/2019

# ABSTRACT

The research aimed at analyzing the efficiency of biosurfactant-producing *Paenibacillus* sp. D9, characterization, stability studies, optimization of process conditions, biodegradative enzymes induction, biotechnological applications, and molecular studies. *Paenibacillus* sp. D9 displayed higher hydrophobicity to the long chain hydrocarbons mixtures tested such as 71.50% diesel fuel, 70.0% engine oil and 76.0% *n*-paraffin. The *Paenibacillus* sp. D9 strain could tolerate a high diesel concentration and a wide range of utilization on different hydrocarbons substrates such as *n*-hexadecane, *n*-dodecane, *n*-tetradecane, 1-nonene, *n*-tetracosane, and *n*-toluene respectively. *Paenibacillus* sp. D9 produced a low molecular weight lipopeptide biosurfactant with critical micelle concentration of 200 mg/L, and high surface tension activity. The optimum condition for biosurfactant synthesis was obtained in a medium containing 10% (v/v) diesel fuel with a production yield of 4.7 g/L. The resultant biosurfactant reduced surface tension from 71.4 mN/m to 30.1 mN/m against carbon source utilized. *Paenibacillus* sp. D9 lipopeptide biosurfactant was capable to withstand and survive in toxic hydrophobic compounds. The obtained lipopeptide can proficiently emulsify different hydrophobic compounds inclusive of engine oil, diesel fuel, motor oil, and *n*-paraffin, hydrocarbon substrates such as *n*-hexadecane, *n*-dodecane, *n*-tetradecane, *n*-hexane, chloroform, *m*-xylene, 1-nonene, *n*-tetracosane, and toluene respectively.

The enzyme activities of alkane hydroxylase (82 U), alcohol dehydrogenase (23 U), and esterase (0.220 U) were assessed, with enhanced biosurfactant activity during the biodegradation of diesel fuel and *n*-hexadecane. The production of enzymes and biosurfactant by *Paenibacillus* sp. D9 were shown to be involved in the biodegradative mechanism and pathways. Thus, 98.4% *n*-hexadecane (C16) and 80.2% diesel fuel (C9-C25) were utilized as source of carbon and energy by *Paenibacillus* sp. D9. Hence, *Paenibacillus* sp. D9 was more vigorous in degradation, and pseudo-solubilization of *n*-hexadecane.

The lipopeptide biosurfactant retained surface-active properties under extreme conditions: temperature (≥ 50$^{o}$C), acidic (pH 2-6), alkaline (pH ≥ 8.0), and salt concentration (0-20 %). *Paenibacillus* sp. D9 was evaluated for optimal conditions and improved production yield. The maximum yield of 4.11 g/L occurred at a C/N ratio of 3:1, at pH 7.0, 30°C, 4.0 mM $MgSO_4$ and 1.5% inoculum size. The potential of *Paenibacillus* sp. D9, to utilize different cheap waste frying oils for maximum biosurfactant production resulted in improvement of surface tension reduction and yield of 31.2 mN/m and 5.31 g/L, respectively using response surface methodology. The *Paenibacillus* sp. D9 biosurfactant was effective in the solubilization and removing 49.1%

to 65.1% diesel fuel including *n*-hexadecane, and *n*-dodecane. The application of biosurfactant further resulted in the bioremediation of motor oil and diesel fuel in both shaking and static conditions from contaminated sands and aqueous environment respectively.

Further biotechnological application revealed the removal of 85.90%, 98.68%, 99.97%, 63.28%, 99.93%, and 94.22% for $Ca^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Ni^{2+}$, and $Zn^{2+}$ respectively from contaminated acid mine effluents. The biosurfactant produced a better performance in heavy metals removal from vegetables, as well as improved oil dispersing activity when compared to acid precipitated supernatant, Triton X-100, and sodium deocyl sulfate. There was high removal of heavy metal from both synthetic wastewater, and contaminated sands. A comparative study of different formulations for the removal of tomato sauce and coffee stains proved that the biosurfactant was more effective (>60%) in terms of the stain removal than chemical surfactants (<50%). *Paenibacillus* sp. D9 lipopeptide biosurfactant synergistically enhanced the removal of tomato sauce and coffee stain from 64.0% to 76.7% and 60.5% to 71.5% respectively.

The *sfp* gene encoding a phosphopantetheinyl transferase was cloned and over-expressed in *Escherichia coli* BL21 (DE3) pLysS and purified to homogeneity using cobalt affinity chromatography. The enzyme was recovered efficiently and had specific activity of 87.14 U/mg against 4-nitrophenyl acetate, at an optimal pH of 8.0 and temperature of 30$^{o}$C. The enzyme exhibited stability under a wide range of pH and temperature. Kinetic parameters were obtained having values of 4.52 mg/mL, 35.33 U/mg, 3.64 $s^{-1}$, and 0.104 $mM^{-1}$ $s^{-1}$ for $K_m$, $V_{max,}$ $k_{cat}$, and $k_{cat}/K_m$ respectively. Biosurfactant produced by the recombinant *E. coli* strain was found to be surface active, reducing the surface tension to 35.7 mN/m and enhancement in biosurfactant yield (1.11 g/L) as compared to 0.52 g/L from *Paenibacillus* sp. D9 utilizing 2 g/L glucose substrate. Therefore, these results demonstrated that lipopeptide biosurfactant are green biomolecules to replace synthetic surfactants and detergents, thus reducing hazards, and contaminations caused to the environment. The non-toxic effect of *Paenibacillus* sp. D9 BioS suggests its usefulness in different applications relatable to soil and aquatic environments, as the biomolecule was confirmed to be ecological safe and environmentally-friendly. For future use, this biosurfactant is highly promising in environmental biotechnology.

## Keywords

Bioremediation; Biosurfactant; Biotechnology; Lipopeptide; *Paenibacillus* sp. D9; Phosphopantetheinyl transferase

# DEDICATION

The thesis is dedicated to Almighty God, Alhaji Babatunde Jimoh, and Alhaja Maryam Adekilekun.

# PREFACE

The research contained in this thesis was accomplished by the candidate while situated in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa under the supervision of Professor Johnson Lin from July 2016 to March 2019. The contents of this work have not been submitted in any form to another university and, aside where the work of others is acknowledged in the text, the outcomes detailed are due to investigations by the candidate.

_____

Signed: Professor Johnson Lin

Date: 31/10/219

# DECLARATION 1: PLAGIARISM

I, Abdullahi Adekilekun Jimoh, declare that:

(i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work;

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# DECLARATION 2: PUBLICATIONS

Details of contribution to publications that form part and/or include research presented in this thesis (include publications in preparation, submitted, and published and give details of the contributions of each author to the experimental work and writing of each publication).

**Publication List**

Publication 1

Title: Advances in production, characterization, and application of biosurfactants

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**Authors:** Abdullahi Adekilekun Jimoh, and Johnson Lin

A.A Jimoh; conceptualization, drafting, and editing of the manuscript

J. Lin; conceptualization, supervision, and editing of the manuscript

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**Authors:** Abdullahi Adekilekun Jimoh, and Johnson Lin

A.A Jimoh; conceptualization, experimental design, laboratory work, drafting and editing of the manuscript

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Title: Diesel fuel and *n*-hexadecane biotransformation by *Paenibacillus* sp. D9: Degradative enzymes and biosurfactant mediation

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**Authors:** Abdullahi Adekilekun Jimoh, and Johnson Lin

A.A Jimoh; conceptualization, experimental design, laboratory work, drafting and editing of the manuscript

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**Authors:** Abdullahi Adekilekun Jimoh, and Johnson Lin

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Publication 5

Title: Bioremediation of diesel and motor oil contaminated sands through the optimization of biosurfactant produced by *Paenibacillus* sp. D9 on waste canola oil

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## Conference Presentations

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4. **A.A. Jimoh,** N.P. Ntshingila, J. Lin. " **Response surface methodology for optimizing the production of biosurfcatant by *Paenibacillus* sp. D9 on low cost substrates and its application in the bioremediation of contaminated sands"**. *School of Life Sciences Postgraduate Research Day, School of Life Sciences Building, University of Kwazulu-Natal, Pietermaritzburg, South Africa.* 22nd May 2018. **Oral Presentation**
5. N.P. Ntshingila, **A.A. Jimoh**, J. Lin. "**Optimization, purification, characterization and application of biosurfactant produced by *Acinetobacter* sp. V2 isolated from an engine oil contaminated site".** *School of Life Sciences Postgraduate Research Day, School of Life Sciences Building, University of Kwazulu-Natal, Pietermaritzburg, South Africa.* 22nd May 2018. **Poster Presentation**
6. **A.A. Jimoh,** J. Lin. **"Biosurfactant and degradative enzymes mediated diesel fuel and *n*-hexadecane degradation by bacterium *Paenibacillus* sp. D9".** *College of Agriculture, Engineering and Science Postgraduate Research and Innovation Day, University of KwaZulu-Natal, Durban, South Africa.* 26th October 2018. **Oral Presentation**

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| | |
|---|---|
| $(NH_4)_2SO_4$ | Ammonium sulphate |
| °C | degrees Celsius |
| µ | Micro |
| µm | micrometer |
| BH | Bushnell-Haas |
| BioS | Biosurfactant |
| C | Carbon |
| $CaCl_2$ | Calcium chloride |
| cm | centimetre |
| CMC | Critical Micelle Concentration |
| CSH | Cell Surface Hydrophobicity |
| E24 | Emulsification index after 24 hours |
| $FeCl_3$ | Iron (III) chloride |
| FT-IR | Fourier Transform Infrared Spectroscopy |
| g | Grams |
| *g* | Gravitational rotation |
| GC-FID | Gas Chromatography-Flame Ionization Detector |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| h | Hours |
| HC | Hydrocarbon |
| HCl | Hydrochloric acid |
| $H_2SO_4$ | Sulfuric acid |
| HP-LC | High Performance-Liquid Chromatography |
| HP-TLC | High Performance-Thin Layer Chromatography |
| $K_2HPO_4$ | Potassium hydrogen phosphate |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| $KNO_3$ | Potassium nitrate |
| L | Litres |
| LB | Luria Bertani |
| LC-MS | Liquid Chromatography-Mass spectrometry |
| MALDI-TOF | Matrix-Assisted Laser Desorption/Ionization-Time of Flight |

| | |
|---|---|
| mg | milligram |
| $MgSO_4$ | Magnesium sulfate |
| min | Minutes |
| mL | Millilitre |
| m | Metre |
| mm | Millimetre |
| mol | mole |
| m/v | mass/volume |
| m/z | mass per charge number |
| N | Nitrogen |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide |
| $NaNO_3$ | Sodium nitrate |
| NaOH | Sodium hydroxide |
| NB | Nutrient Broth |
| $NH_4NO_3$ | Ammonium nitrate |
| nm | Nanometre |
| NMR | Nuclear Magnetic Resonance |
| OD | Optical Density |
| OVAT | One Variable at a Time |
| PBS | Phosphate Buffer Saline |
| PAH | Polycyclic Aromatic Hydrocarbon |
| pH | Hydrogen potential |
| Rpm | Revolution per minute |
| RSM | Response surface methodology |
| ST | Surface Tension |
| TLC | Thin Layer Chromatography |
| Tris–HCl | Tris hydrochloride |
| UV-V | Ultraviolet-Visible |
| v/v | volume/volume |
| V | Volume |
| W | Weight |
| w/v | weight/volume |
| × | times |

# Chapter 1

## Introduction

### 1.1 Background

In the present economy, advancement in science and biotechnology have progressively aided mankind in the probe and exploration of natural resources. Different activities such as excavation of fossil fuels, crude oil exploration, crude-oil related products (kerosene, diesel, petrol) usage, arrival of agricultural chemicals and pharmaceutical products have eased the lifestyle of people worldwide. Unfortunately, a few of these interventions have a downside as the chemicals, solvents, and materials required for those developments may instigate health effects on the environment, humans, including the aquatic habitat (Valentín et al., 2013). Numerous human activities have led to deliberate or accidental discharge of pollutants into the Earth's ecological system as these contaminants pose a vast risk to human life, wellbeing and normal biological system (Chen et al., 2015). Any unwanted substances released into the environment are termed pollutants or contaminants. Pollutants are in existence for a while now, and life on Earth has always progressed amongst them (Korjus, 2014). The Earth is incessantly a polluted planet with pollutant similarities from, global warming, comets, space dust, organic dust, volcanic activities, smoke, comets, space dust, and acid rain (Korjus, 2014).

Poisonous chemicals contained in different materials in the Earth's atmosphere can be absorbed on human skin, accumulate in the dust we inhale, or subsequently end up in the surrounding natural environment. Substances that leach into the soil and water environment can indirectly affect humans, by absorption on different vegetables, fruits, fish, and other food products that end up on our eating table. The exhaustive utilization of substances, for example, oil hydrocarbons (such as saturated, unsaturated, polyaromatic, polycyclic aromatic hydrocarbons, and cycloalkanes), heavy metals (such as thallium, copper, zinc mercury, arsenic, iron, titanium, cadmium, nickel), pesticides, herbicides, air contaminants (carbon monoxide, ozone, acid rain, particulate matter), volatile organics (such as benzene, toluene, chloroform, ethylbenzene, xylenes), nitroaromatic compounds, organophosphorus compounds, trichloroethylene, perchloroethylene, solvents, and chlorinated hydrocarbons can be intensely noxious thus inflicting extensive damage such as corrosive injuries, toxicity, and overall illness (Chen et al., 2015; Korjus, 2014; Megharaj et al., 2011). In some cases, some

compounds are synthesized purposely to suit environmental requirements whereas in most cases, the burning of some chemicals such as polyvinylchloride, plastic, radioactive substances, inorganic substances, and organic compounds generate undesired toxic by-products. The destination of the above-listed pollutants is often the soil, lakes, river, and sea leading to bioaccumulation, and biomagnification effects over time. This, however, leads to deleterious and hazardous effect to both aquatic and terrestrial life (Liu et al., 2017; Valentín et al., 2013).

The discharge of crude oil, petroleum, hydrocarbons, heavy metals, and other various pollutants into the environment is a proportional range of approximately 2.0–8.8×$10^6$ metric tonnes annually (Hassanshahian and Cappello, 2013). Many pollutants introduced into the soil environment are degraded biologically while other pollutants have been toxic and not degradable to a number of the soil microbial community. Due to the detrimental consequences of hydrophobic pollutants, it is imperative to propose procedures to annihilate these environmental problems. Several conventional techniques such as alteration, volatilization, photo-oxidation, chemical oxidation, adsorption, landfilling, burning, and chemical treatments are extensively used in polluted sites clean-up, but these methods highlighted above are exceedingly costly, toxic, non-biodegradable and pose additional risks to the environment (Guntupalli et al., 2016; Patowary et al., 2018).

Anionic, non-ionic, cationic and mixed surfactants are different types of surfactants are able to remove concentrated hydrophobic compounds from liquid medium, groundwater, surface water and contaminated soil (Chaprão et al., 2015; Lai et al., 2009; Urum et al., 2006). Surfactants are standout amongst the profitable synthetic products and huge amounts are expended throughout the world for various purposes (Chakraborty et al., 2015). Be that as it may, the remaining surfactants in soil surroundings and receiving water bodies constitute probable danger to environmental conditions and human wellbeing (Chaprão et al., 2015).

One of the promising techniques to restore contaminated environments is the utilization of bioremediation innovations, which is an eco-accommodating, financially productive, and supportable method. Bioremediation is an ecologically-sound technique encompassing the usage of natural biological processes to cleanse or remove pollutants through biochemical solubilization or mineralization. It is the most interesting strategy by which microbes, microalgae, green plants and/or their enzymes use for hydrocarbon biodegradation and bioremediation (Korjus, 2014; Mani and Kumar, 2014; Mnif et al., 2015). They have been recognized as extensive substitutes for regular strategies in settling natural ecological issues (Mnif et al., 2015). Likewise, biodegradation by natural microbial population characterizes one of the significant mechanisms by which hydrophobic contaminants can be expelled or diminished from nature. Biodegradation is a natural process that

involves the *transformation* and breakdown of organic contaminants principally by microbial organisms into simpler substances. The microbial organisms transform the contaminants through their metabolic or enzymatic processes (Katyal and Morrison, 2007; Thomson, 2012). Some physicochemical, just as biological parameters determine the extent of hydrocarbons biodegradation. Due to the exceedingly hydrophobic nature of hydrophobic pollutants, different factors such as low water solubility, strong soil particles attachment, and low biological availability of oil pollutants have constrained the mass transfer proportion to biodegradation and bioremediation. Also, oil and petroleum contaminants are often adsorbed and absorbed onto soil particles, might be available as a liquid or a solid phase (Bezza and Nkhalambayausi Chirwa, 2015; Chaprão et al., 2015; Paria, 2008).

Both eco-friendly techniques highlighted above involves the production of surface-active molecules of microbial origin termed *"biosurfactant"* which aid in the solubilization and remediation of hydrophobic pollutants, petroleum hydrocarbons, oil-related products, and not limited to heavy metals. Biosurfactants (BioSs) are amphiphilic compounds, produced by specific microorganisms (Parthipan et al., 2017; Sharma et al., 2015). BioS synthesis by microorganisms is either secreted intracellularly by being partly attached to the cell membrane or as an extracellular release to the medium. The former mechanism arises commonly when the microorganism is grown in substrates that are insoluble in water. BioSs produced intracellularly assist in nutrient uptake, neutralization of toxic elements, and further ease carbon molecule storage (Ndlovu, 2017). Furthermore, BioSs enable microorganisms to have a surface activity which assists in lowering the surface tension between multiple interphases (liquid-liquid, liquid-air, liquid-gas, and liquid-solid), thus rendering the substrate and aid the mobility of microorganisms in unfriendly environments (Van Hamme et al., 2006).

The BioS molecules which contain the hydrophobic group (water repelling such as unsaturated or saturated hydrocarbon chains or fatty acids) and hydrophilic ends (water-loving, such as, acid, cations, or anions, peptide, mono-, di- or polysaccharides) mediates the surface interactions at the interface (Sharma et al., 2015). The dual nature of BioSs permits the dissolution of both polar and non-polar solvents (Smyth et al., 2010a; Smyth et al., 2010b). Depending on the chemical structure and microorganisms that produce these compounds, BioS are biological-chemical complexes that consist of an extensive kind of biomolecules such as fatty acids, dicarboxylic acids, fatty acid amides, lactones, alkyl glycosides, and sugar molecules (Ndlovu, 2017; Youssef et al., 2005). The main classes include polymeric compounds, lipopeptides, phospholipids, particulate surfactants, and glycolipids. BioSs have several advantages when compared to chemically produced counterparts. Such advantages involve non-toxicity, extensive foaming activities, biodegradability, ecological

acceptability, high selectivity, environmentally friendliness, and effectiveness at extreme environments (Bezza and Nkhalambayausi Chirwa, 2015; Chrzanowski et al., 2012; Hirata et al., 2009). As such, BioSs are well-thought-out as a preeminent option to synthetic surfactants in augmenting solubilization, bioavailability, biodegradation, and bioremediation of hydrophobic pollutants (Mnif et al., 2015). These favorable properties make BioSs suitable in scope of applications, for example, food, agriculture, oil industries, cosmetics, environmental, pharmaceutics, and biotechnological processes (Bezza and Nkhalambayausi Chirwa, 2015; Mnif et al., 2014; Pacwa-Płociniczak et al., 2011).

## 1.2 Statement of problem

Major environmental problems and human health effects have ensued from long-standing contamination arising from different activities such as extensive usage, inappropriate dumping, accidental leakages of aliphatic and aromatic hydrocarbons, organic solvents, heavy metals, and crude oil and other related products such as paraffin, motor oil, diesel fuel, motor oil, and other hydrophobic pollutants (Chen et al., 2015). Due to the recalcitrant nature of these hydrophobic pollutants in contaminated soils or sediments, these contaminants present one of the most demanding problems that require an urgent solution about their solubilization and bio-treatment. BioS-enhanced biodegradation and bioremediation has presented a cost-effective option to conventional techniques, though, bioremediation of petroleum hydrocarbons is limited by the pollutants' low bioavailability, insolubility, hydrophobicity, and strong adsorption to soil particle in the environment (Bezza and Chirwa, 2015; Bezza and Nkhalambayausi Chirwa, 2015; Malik et al., 2011).

Despite several advantages and good properties over chemical surfactants, the drawback still lies in the high production cost, low production yield, expensive downstream and recovery process. In addition, difficulties in synthesizing huge quantities of BioSs for environmental applications is the main limitation to date. Novel discoveries, improvement of fermentation conditions and new recovery processes may allow BioSs usage in a wide range of biotechnological applications. At present, commercial production of BioSs in bulk quantities is restricted (except for rhamnolipids obtainable at http://www.rhamnolipid.com) as the wholesale total production cost is still very high (Al-Wahaibi et al., 2014). Syldatk and Hausmann (2010) discovered that the utilization of expensive substrates gave low production yields, build-up of detrimental mixtures instead of advanced BioS products, and such constrictions clarify why there exists limited production industrially. To address this problem, large-scale production of BioS is dependent on numerous strategies, which involves the

advancement of in-expensive scaled-up methods, isolation of novel BioS group, microbial strain improvement, media components optimization, improved statistical techniques, the use of cheap raw materials, and development of hyper-producing genetic microorganisms (Al-Bahry et al., 2013).

## 1.3 Scope of present study

In the current research, different sequences of BioS enhanced solubilization, biodegradation, optimization, and bioremediation research were analyzed. This study is made from five main parts

- Production, physiochemical, structural, and molecular characterization of lipopeptide BioS by diesel fuel and hydrocarbon-degrading *Paenibacillus* sp. D9.
- Explore BioS synthetic-degradative enzymes, and mechanism as mediated by biodegradation and solubilization of hydrocarbons.
- Optimization of different growth conditions, factors, and parameters for improved BioS production yield.
- Stimulation of BioS production for enhanced solubilization, biodegradation, and bioremediation of hydrocarbons and hydrophobic compounds.
- Development of a genetically hyper-producing recombinant strain for improved synthesis of BioS.

## 1.4 Hypothesis

- It was hypothesized that *Paenibacillus* sp. D9 will produce a novel, low molecular weight lipopeptide BioS with diverse biotechnological applications.
- It was hypothesized that improved production parameters will significantly increase BioS yield thus resolving the associated high production cost associated and its wide usage in environmental, and biotechnological applications via physico-chemical and molecular approaches.

## 1.5 Aims and objectives

- To determine the functions, and structural characterization of novel lipopeptide BioS produced by *Paenibacillus* sp. D9, thus contributing valuable information on the genus *Paenibacillus* with new properties and attributes.

- To produce, characterize, and determine the structural composition of the newly isolated lipopeptide BioS.
- To evaluate the utilization, and degradative ability of *Paenibacillus* sp. D9 on different hydrocarbons, hydrophobic mixtures and survive high toxic concentration of diesel.
- To recover, purify, and determine the physicochemical classification, and stability studies of the BioS synthesized.

- To determine biodegradative mechanism, and activities of BioS-mediated degradative enzymes and role played in improving the biodegradative ability of *Paenibacillus* sp. D9.
  - To explore the correlation between BioS production and biodegradative-mediated enzymes in hydrophobic pollutants.
  - To determine the role of important degradative and metabolic enzymes during degradation of diesel fuel and *n*-hexadecane.
  - To accomplish *Paenibacillus* sp. D9 improved pseudo-solubilization and desorption on hydrocarbon substrates.
- To explore different conditions for maximum production yield and assess *Paenibacillus* sp. D9 BioS capability in improving the bioavailability, solubilization, and biodegradation of hydrophobic pollutants.
  - To determine the optimum conditions required for improved lipopeptide BioS yield when grown on diesel fuel through the utilization of one variable at a time (OVAT) classical design.
  - To assess the stability of the *Paenibacillus* sp. D9 BioS in extreme environmental conditions.
  - To design a medium incorporating a novel cheap carbon substrate with the potentiality of using low cost substrates.
  - To present new, improved production process parameters and conditions of lipopeptide BioS produced by *Paenibacillus* sp. D9 using a combination of Box Behnken Design and response surface methodology.
  - To explore the impact of exogenously included synthetic surfactant, BioS in single or/and combination with *Paenibacillus* sp. D9 on the degradation abilities of high toxic hydrophobic compounds.

- To build up a powerful bioremediation technique through exogenous BioS introduction synthesized from a competent hydrocarbon degrader for advanced biodegradation of hydrophobic polluted sands and liquefied media.
    - To perform experimental design tests by examining the utilization of BioS produced from *Paenibacillus* sp. D9 in enhancing bioremediation of hydrophobic pollutants from contaminated sand and water media using different conditions.
- To present novel biotechnological applications inclusive of oil dispersion, heavy metal removal from contaminated environments, and formulation of detergents on the produced lipopeptide BioS.
- To develop a hyper-producing recombinant strain for improved esterase and BioS production through cloning, and expression, thus making it a commercially practicable bio-process.
    - To clone, and determine the heterologous expression of BioS biosynthetic gene, and its molecular characterization in terms of amino acid-make up and homology.
    - To purify and characterize cloned BioS product and thereby contrast BioS activity of the hyperproducing strain to the parent strain

## 1.6 Thesis organization

This thesis is a production of nine different chapters. Chapter one, and chapter two is made up of the introduction and literature review respectively. The manuscripts (Chapters Four, Six, Seven, and Eight) have been submitted to peer-review international journals. The manuscripts (Chapter Three and Chapter Five) have been published in peer-reviewed international journal. Chapter Nine comprise of the conclusions made from this research as well as future perspectives.

**Chapter 1.** This chapter contains thesis introduction, foundation, problem statement, the scope of research, aims and objectives as well as the thesis layout or organization.

**Chapter 2. Advances in production, characterization, and application of biosurfactants.**

The second chapter examines past reports and ongoing advancements in the area of BioS, different types, properties, advantages, structural composition, the physiology, pathways, and kinetics of BioSs production, factors and strategies for improvement of BioS production, purification and recovery process, characterization, recombinant DNA advancements, as well as the recent environmental, industrial, and biotechnological applications. Part of this chapter has been published in Ecotoxicology and Environmental Safety.

**Chapter 3**. **Production and characterization of lipopeptide biosurfactant-producing *Paenibacillus* sp. D9 and its biodegradation of diesel fuel.**

This chapter focuses on the functions, structures, and characterization of novel lipopeptide BioS produced by hydrocarbon-degrading bacterium *Paenibacillus* sp. D9 capable of withstanding high toxic hydrophobic compounds. The synthesized lipopeptide in the current study can emulsify a number of hydrophobic compounds inclusive of engine oil, motor oil, *n*-parrafin, and diesel fuel, other hydrocarbon substrates such as *n*-hexadecane, *n*-dodecane, *n*-tetradecane, 1-nonene, tetracosane, toluene and intermediary metabolites of polycyclic aromatic hydrocarbon degradation such as benzoic acid, salicylic acid, and phthalic acid. This chapter also contributes valuable information on this species of *Paenibacillus* with novel BioS properties and attributes. This chapter has been published in International Journal of Environmental Science and Technology (2019).

**Chapter 4. Diesel fuel and *n*-hexadecane biotransformation by *Paenibacillus* sp. D9: Degradative enzymes and biosurfactant mediation.**

This chapter exhaustively discusses the role of key metabolic biosynthetic degradative enzymes (such as alkane hydroxylase, alcohol dehydrogenase, and esterase) in the degradation of diesel fuel and *n*-hexadecane. Also, pseudo-solubilization and accumulation of intracellular *n*-hexadecane hydrocarbon were also presented in relation to biodegradation. The chapter examined significant relationship between surface tension, emulsifying activity, and cell surface hydrophobicity as regards lipopeptide BioS synthesis and activity. This chapter has been submitted to Biocatalysis and Biotransformation.

**Chapter 5. Enhancement of *Paenibacillus* sp. D9 lipopeptide biosurfactant production through the optimization of medium composition and its application for biodegradation of hydrophobic pollutants.**

This chapter features the optimal culture parameters advancement and their impact on lipopeptide BioS synthesis, high stability, enhanced desorption and solubilization of hydrophobic contaminants (namely diesel fuel, *n*-hexadecane, and *n*-dodecane). The chapter further highlighted some interesting findings as discussed therein. The study showed significant positive effects on lipopeptide production on the following proportional parameters; diesel fuel (carbon source), ammonium sulfate (nitrogen source) and magnesium sulfate (metal supplementation) with the optimal pH, temperature, and inoculum size found to be 7.0, 30$^{o}$C, and 1.5 mL respectively. The synthesized *Paenibacillus* sp. D9 BioS revealed reliable stabilities in a wide range of pH, temperature and salt concentrations. In addition, the lipopeptide BioS was more efficient in the solubilization

of hydrophobic pollutants than the commercially available chemical surfactant. This chapter is published in Applied Biochemistry and Biotechnology (2018): 1-20.

**Chapter 6. Bioremediation of diesel and motor oil through the optimization of biosurfactant produced by *Paenibacillus* sp. D9 on waste canola oil.**

This chapter reports a new, improved production process parameters and conditions of lipopeptide BioS produced by *Paenibacillus* sp. D9 using a combination of Box Behnken Design and response surface methodology on low-cost substrates. The significant lipopeptide BioS ability and feasibility in the bioremediation from contaminated sands using both the shaking and static conditions were also evaluated and reported. This chapter has been submitted to Bioremediation Journal.

**Chapter 7. Biotechnological applications of *Paenibacillus* sp. D9 lipopeptide biosurfactant produced in low-cost substrates**

The chapter reports the effects of low-cost substrate-produced BioS and its novel environmental, and biotechnological applications such as oil dispersion, heavy metal removal from contaminated effluents, sands, wastewater, vegetables as well as washing performance and detergent formulations. The non-toxic effect of *Paenibacillus* sp. D9 BioS proffers its usefulness in different applications relatable to soil and aquatic environments, as the biomolecule was confirmed to be ecologically safe and environmentally-friendly. This chapter has been submitted to Applied Biochemistry and Biotechnology.

**Chapter 8. Heterologous expression of S*fp*-type phosphopantetheinyl transferase is indispensable in the biosynthesis of lipopeptide biosurfactant.**

This chapter identifies and characterizes the *sfp* gene encoding phosphopantetheinyl transferase from *Paenibacillus* sp. D9 at the molecular level, which provides more insight into the structure and function of *sfp* gene involved in the BioS biosynthesis. This chapter presents, exhaustively the characterization, improved production yield, structural prediction, biochemical characterization, and enzyme kinetics of phosphopantetheinyl transferase enzyme. The recombinant strain (BioSp) was also implicated in the synthesis of BioS as reported in this thesis. This chapter has been submitted to Journal of Biotechnology.

**Chapter 9. Conclusions and Future Perspectives**. This chapter integrates and provides conclusions and documentation of the contributions of this research. Future research possibilities and recommendations are included.

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# Chapter 2

## Advances in production, characterization, and application of biosurfactants

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## Advances in production, characterization, and application of biosurfactants

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## Abstract

Petroleum hydrocarbons, oil, and heavy metals pollution is becoming an additional severe problem due to the growing call for crude oil and crude oil related products in several fields of application. Such pollution leads to ecological damages in both marine, aquatic and terrestrial ecosystems. Thus, different techniques including chemical surfactants and complex technologies have been proposed for their clean up from the environment, which in turn has potential detrimental effects on the environment. As of late, biosurfactant compounds have added much deliberation since they are considered as a reasonable option and eco-accommodating materials for remediation technology. Biosurfactants hold the special property of minimizing and reducing the interfacial tension of liquids. Such features sustain biosurfactants to play a major part in emulsification, de-emulsification, biodegradability, foam formation, washing performance, surface activity, and detergent formulation in diverse industrial set-ups. Conversations on cost-effective technologies, renewable materials, novel synthesis, downstream, upstream, emerging characterization techniques, molecular, and genetical engineering are substantial to produce biosurfactant of quality and quantity. Be that as it may, the extravagant cost drew in with biosurfactants biotechnological synthesis and recovery can hamper their application in those areas. Notwithstanding these costs, biosurfactants can be used as these parts shows outstandingly high benefits that can at present beat the expenses incurred in the initial purification and downstream processes. The production of biosurfactant by microorganisms is relatively considered one of the crucial know-how for improvement, growth, advancement, and environmental sustainability of the 21$^{st}$ century. This review emphasizes exhaustively the key and recent areas to be considered during biosurfactants production by microorganisms.

**Keywords** Applications; Bioremediation; Biosurfactants; Characterisation; Environmental pollution; Microorganisms; Production.

## 2.1 Introduction

Different activities such as oil spillage, leakage and indiscriminate disposal/exposure of petroleum hydrocarbon and petroleum products (fuel oils, hydraulic oil, automotive oil, and lubricating oils) occurs either due to human activities or accidental discharge. This poses a severe threat to ecosystems, thereby leading to severe ecological and environmental pollution effects (Bayoumi et al., 2010; Lima et al., 2011). Also, rudimentary activities encompassing cosmetic products, toothpaste formulations, personal hygiene, detergent formulations, oil paints, bio-commercial detergents, and other pharmaceutical by-products are mostly dependent on the use of surfactants either of chemical, biological origin or biological-chemical complexes. Surfactants are amphiphilic surface-active agents that have both polar and non-polar group that decrease surface tension at the interface between two liquids incapable of forming a homogeneous substance, similar to water and oil. They are either of synthetic or of biological origin which are termed biosurfactant (BioS). BioSs synthesized by microorganisms is garnering a noticeable interest owing to their prospective benefits such as environmental compatibility, biodegradability, non-toxicity, effectiveness at extreme environmental conditions, and higher foaming capacity over their synthetic counterparts (Gudina et al., 2015a; Pereira et al., 2013).

These properties assume BioSs significance in different areas like pharmaceutics, health, cosmetics, oil recovery, bioremediation, biodegradation, food industry, and numerous uses in various modern industrial sectors (Banat et al., 2010; Fracchia et al., 2014). Microorganisms are proficient in producing different kinds of BioSs which range from the low molecular weight BioS to the high molecular weight. They belong to genera such as *Pseudomonas, Acinetobacter, Bacillus, Brevibacterium, Clostridium, Arthrobacter, Gordonia, Rhodococcus, Halomonas, Serratia, Aeromonas, Thiobacillus, Leuconostoc, Citrobacter, Candida, Corynebacterium, Penicillium, Yarrowia, Ustilago, Aspergillus, Torulopsis, Ochrobactrum, Pseudozyma, Saccharomyces, Enterobacter, and Lactobacillus* (Li et al., 2016; Shekhar et al., 2015). The world markets are engrossed with several entrepreneurs who have shown interest in exploiting the BioSs industry since there is increasing responsiveness among consumers for environmentally-friendly compounds. BioSs can substitute man-made surfactants such as Polysorbate 80, Dowfax 8390, Triton X-100, Tween 80, sodium dodecyl sulfate, sorbitan trioleate, Tergitol NP-10, Tergiitol NPX in numerous environmental and industrial applications (Gudina et al., 2015a). ZONIX, a biofungicide, technical grade active ingredient made from rhamnolipid BioSs is currently being sold by a company named Jeneil BioSs, USA. Paradigm Biomedical Inc (USA) is dedicated to the research of pharmaceutical products derived from rhamnolipids BioSs. R95, an anionic amphiphilic

molecule (hydrophilic and lipophilic) which is a high performance-liquid chromatography grade rhamnolipid has been introduced recently by AGAE technologies Ltd, USA. R95 consists of mono- or di-rhamnose sugar heads and ß-hydroxyl alkanoic acid tails of variable lengths (Sekhon et al., 2012). Other prominent and major BioS manufacturers include Ecover Belgium (Belgium), Cognis (Germany and USA), Groupe Soliance (France) and MG Intobio (South Korea). Fraunhofer IGB (Germany) is involved in the distribution glycolipid BioS, mannosylerythritol lipids, and cellobiose lipids. Additionally, Saraya (Japan), have been implicated in the large-scale sale of sophorolipid BioS products. Cognis (China) announced the sale of a green BioS termed APG®, produced from starch or vegetable oil (Sekhon et al., 2012; Sekhon Randhawa and Rahman, 2014).

While different researchers are ardent in substituting synthetic surfactants, the exorbitant cost of synthesizing BioSs is of major concern (Al-Wahaibi et al., 2014). Subsequently, further research needs to be done in increasing production yields together with the exploration for novel kinds of BioSs and application roles in hydrophobic bioremediation processes, antimicrobial agents, microbial enhanced oil recovery, biotechnological, environmental, and industrial applications (Dalili et al., 2015; de França et al., 2015; Ferreira et al., 2017). Likewise, exploring the structural, functional composition, novelty, and applications of BioSs is appealing to several scientists all over the world. Reduction in the cost of BioSs production making them economically attractive mainly hinge on the improvement of inexpensive procedures, utilization of inexpensive raw resources and improved production yields through genetically engineered bacteria and superlative mutants. In this way, investigations on human and environmental sustainability to improve the proficiency of feasible innovations on environmental safety and greener technology are also being explored. The review demonstrates the recent development in BioSs production by microorganisms, with a description of their properties, strategies for the improvement in BioS production, and BioS production economics as well as the new characterization, recombinant DNA technology for enhanced BioS production, application for its usage in the most diverse environmental, industrial and biotechnological industries with major focus on greener technology and environmental sustainability.

## 2.2 Biosurfactant classification

BioSs are produced by an extensive diversity of microorganisms and possess structures of different chemical and surface properties (Martins and Martins, 2018). Microorganisms can make different types of BioSs, which includes glycolipids (mannosylerythritol, rhamnolipids, sophorolipids, xylolipid, cellobiose lipids trehalose

lipids), lipopeptides (subtilisin, vixcosin, serrawetin, surfactin, polymyxin, iturin), polysaccharide-protein complexes, flavolipid, phospholipids, fatty acids, polymeric surfactants (liposan, alasan, emulsan) and lipids (de França et al., 2015; Martins and Martins, 2018). The most frequently produced low molecular weight surface active compounds are glycolipids and lipopeptides. The other group which have often been used substitutively with BioSs to represent biomolecules that are surface active are referred to as bioemulsifiers (Uzoigwe et al., 2015). Bioemulsifiers are surface-active but do not essentially decrease surface tension, however, provide steady emulsions between water mixtures and hydrocarbons (liquids). BioSs in most cases are commonly referred to as bioemulsifiers (Franzetti et al., 2012; Smyth et al., 2010b). Bioemulsifiers are high-molecular-weight as they are combinations of protein, heteropolysaccharides, lipopolysaccharides, and lipoproteins (Bezza and Nkhalambayausi Chirwa, 2015; Perfumo et al., 2010; Smyth et al., 2010b). They are also referred to as high molecular weight biopolymers or exopolysaccharides. The different types of BioSs, and the structural compositions are discussed and provided below.

### *2.2.1 Glycolipids*

Numerous glycolipids, encompassing simple fatty acids esterified to a carbohydrate moiety have been defined varying from different microorganisms (de Jesus Cortes-Sanchez et al., 2013). Their structural composition differs from simple sugars with fatty acyl substituents to complex carbohydrates, that can successively be connected to aromatic compounds, nucleosides, or terpenoids, in addition to having different connection points to “un”, “mono”, “poly”, unsaturated fatty acids by means of glycosidic or ester linkages. Glycolipid BioS structures include rhamnolipids, sophorolipids, mannosylerythritol, trehalose lipids, and xylolipids (Santos et al., 2016; Shekhar et al., 2015).

#### 2.2.1.1 Rhamnolipids

Rhamnolipids are recognized as glycolipid BioS, synthesized by *Pseudomonas aeruginosa* as metabolites during secondary metabolism (Bodour et al., 2003). An oily glycolipid BioS synthesized formerly by *Pseudomonas pyocyanea* was first discovered in 1946. Edwards and Hayashi (1965) additionally explained the rhamnolipid chemical structure as glycosides comprising of one (mono-rhamnolipids) (Figure 2.1a) or two (di-rhamnolipids) (Figure 2.1b) rhamnose sugars connected by an O-glycosidic bond to lipid moieties. The hydrophobic component consists basically of one or two but in uncommon cases, three β-hydroxy unsaturated fatty chains that might be single, double or triple bonded and possess different lengths of C8 to C16. The

hydrophilic component of rhamnolipid comprises of single or twofold rhamnose sugars connected to each other by an α-1, 2-glycosidic bond (Abdel-Mawgoud et al., 2011). Abdel-Mawgoud et al. (2011) indicated the structural composition of more than 60 rhamnolipid congeners have been designated.

#### 2.2.1.2 Sophorolipid

Sophorolipid is a glycolipid complex that are synthesized by a couple of non-pathogenic yeast species. Sophorolipid comprises of hydrophilic sugar head called sophorose and a hydrophobic unsaturated fat of 16 or 18 carbon chain length. Sophorose encloses glucose of disaccharide group connected by an irregular β-1, 2 bonds acetylated on the 6′-as well as 6′′-positions (Van Bogaert et al., 2007). The carboxylic end of sophorolipid could either be lactonized (Figure 2.1c) or an acidic form of sophorolipid (Figure 2.1d).

#### 2.2.1.3 Mannosylerythritol and cellobiose lipids

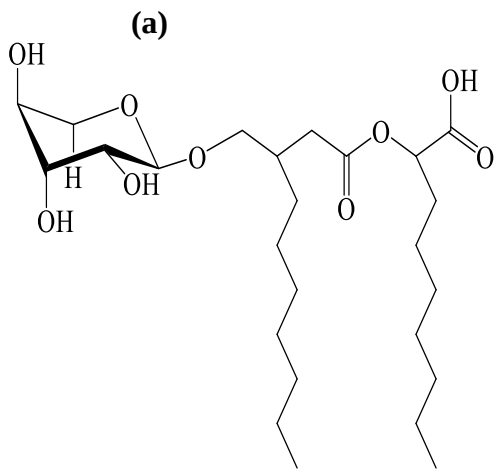
Mannosylerythritol lipids are functional glycolipids also synthesized abundantly by yeast strains. They comprise of fatty acids joined to 4-O-β-D-manno-pyranosyl erythritol or 1-O-β-D-manno-pyranosyl erythritol as the hydrophilic head group (Figure 2.1e) (Morita et al., 2006). Cellobiose lipids is another glycolipid BioSs with the major product recognized as *16-O-(2",3",4",6'-tetra-O-acetyl-β-cellobiosyl)-2-hydroxyhexadecanoic acid* (Figure 2.1f). Yeasts and mycelia organisms are shown to produce a few extracellular glycolipids, including cellobiose, and mannosylerythritol lipids (Morita et al., 2011).

#### 2.2.1.4 Trehalolipids

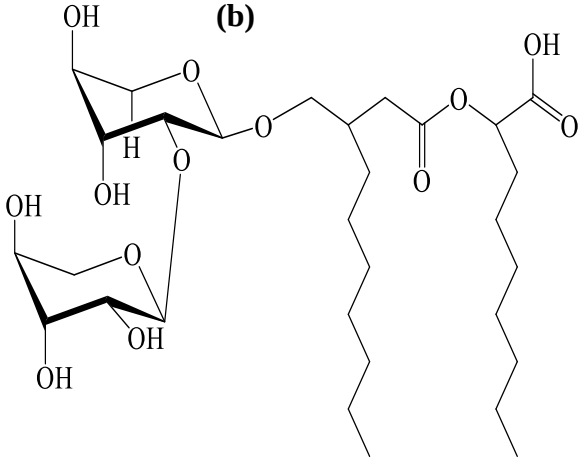
Trehalolipids are made from unsaturated fatty acids group length (hydrophobic components) in a blend with carbohydrate group (hydrophilic component) (Figure 2.1g). The hydrophobic components of trehalolipids are vastly different, comprising of hydroxylated branched fatty acids of varying chain lengths and aliphatic acids. The amounts of the hydrophobic chain in every molecule of trehalose lipids are normally mono-, di-, and tetra-esters, separately connected to long-chain unsaturated fats by an ester bond (de Jesus Cortes-Sanchez et al., 2013).

#### 2.2.1.5 Xylolipids

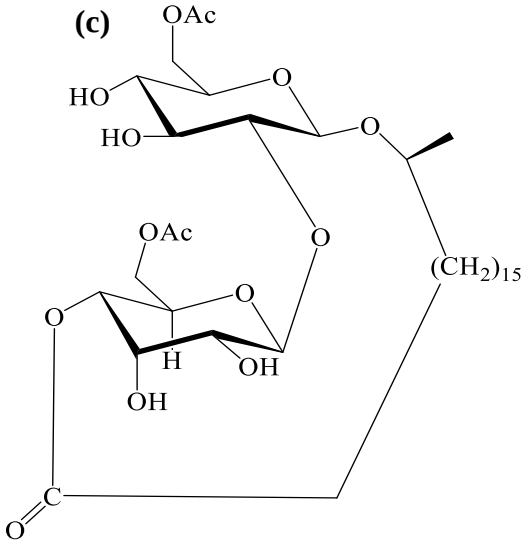
Xylolipid is another class of BioS discovered recently with molecular composition of methyl-2-*O*-methyl--d-xylopyranoside, which is a hydrophilic component connected to hydrophobic parts of the octadecanoic acid (Figure 2.1h) (Saravanakumari and Mani, 2010).



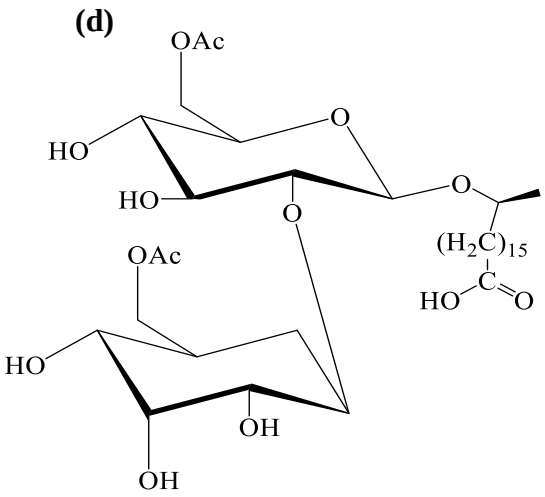

mono-rhamnolipid




di-rhamnolipid



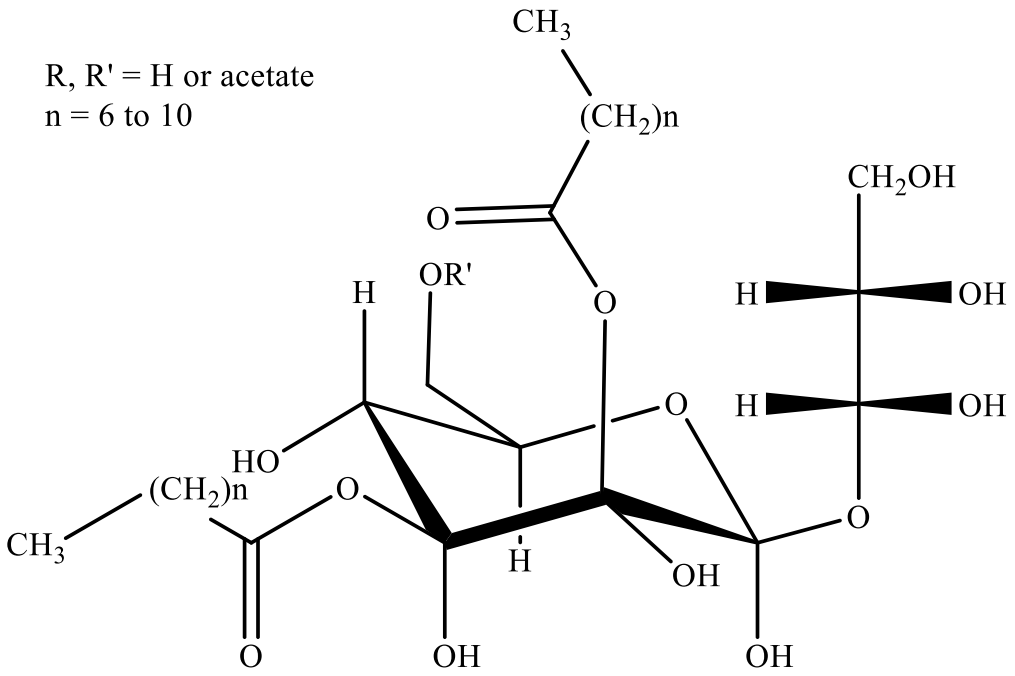

lactonic sophorolipid




acid sophorolipid

**(e)**

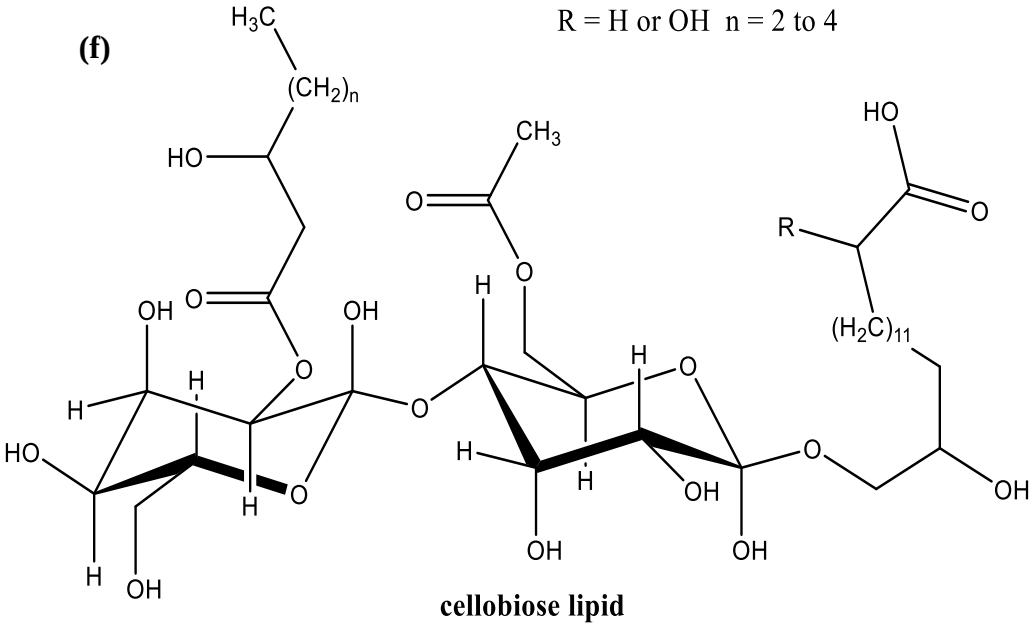
R, R' = H or acetate
n = 6 to 10




**mannosylerythritollipid**

**(f)**

R = H or OH n = 2 to 4




**cellobiose lipid**

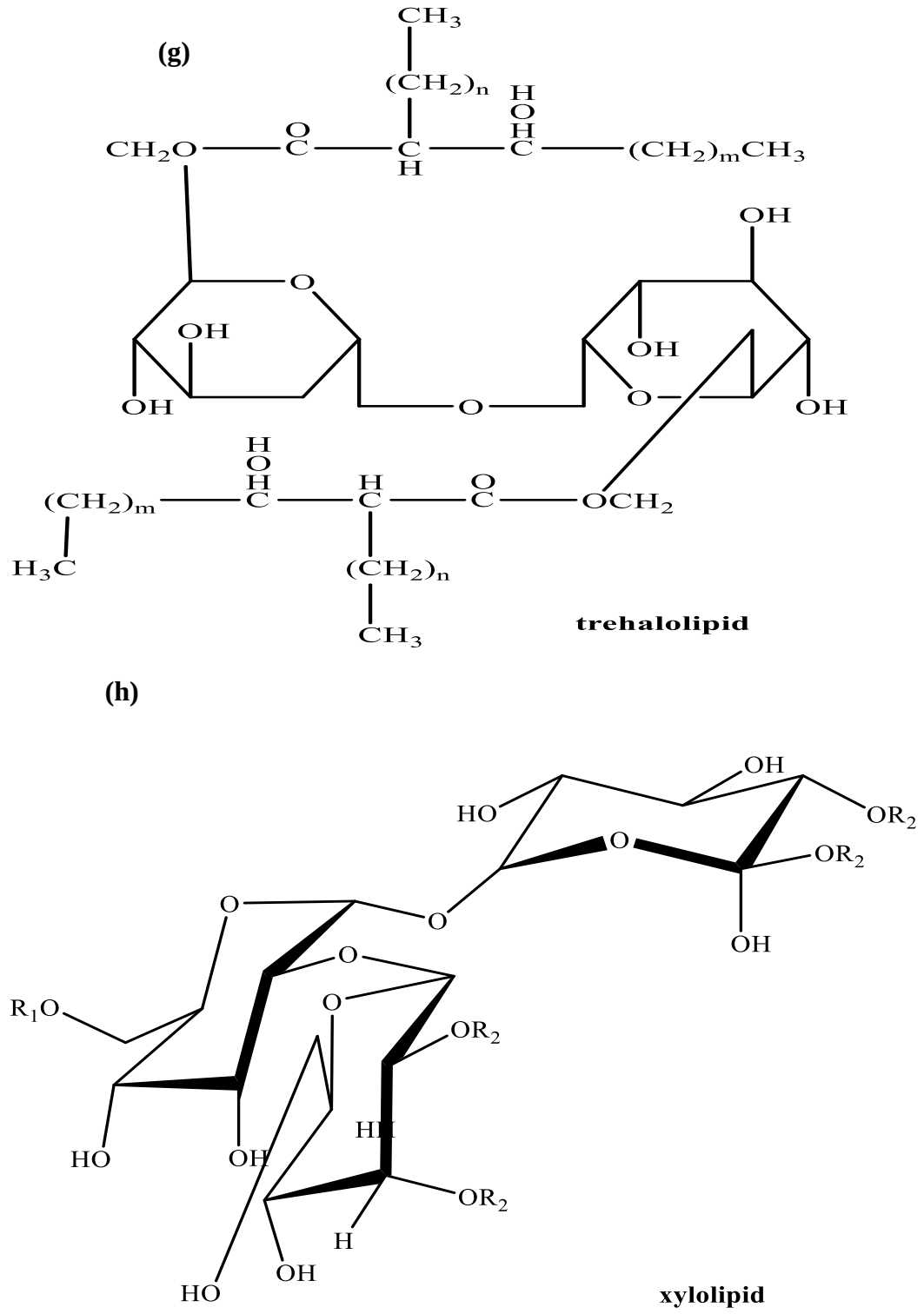



**Figure 2.1** Main glycolipid biosurfactants produced by microorganisms namely (a) monorhamnolipid (b) dirhamnolipid (c) lactonic sophorolipid (d) acid sophorolipid (e) mannosylerythritol lipid (f) cellobiose lipid (g) trehalolipid and (h) xylolpid (Bodour et al., 2004; de Jesus Cortes-Sanchez et al., 2013; Morita et al., 2013; Santos et al., 2016).

### *2.2.2 Lipopeptides*

Lipopeptides are biomolecules comprising of a lipid connected to a peptide, that are small chains of amino acid monomers joined by peptide (amide) bonds. Lipopeptides are synthesized by various bacterial genera such as *Bacillus, Streptomyces, Pseudomonas* and including fungi such as *Aspergillus* (Adetunji, 2012). Lipopeptides have received substantial consideration for their antimicrobial and surfactant properties. *Bacillus subtilis* produced acyclic lipopeptide surfactin which is one of the most recognized BioS (Adetunji, 2012). The major lipopeptide group of BioS is further discussed below.

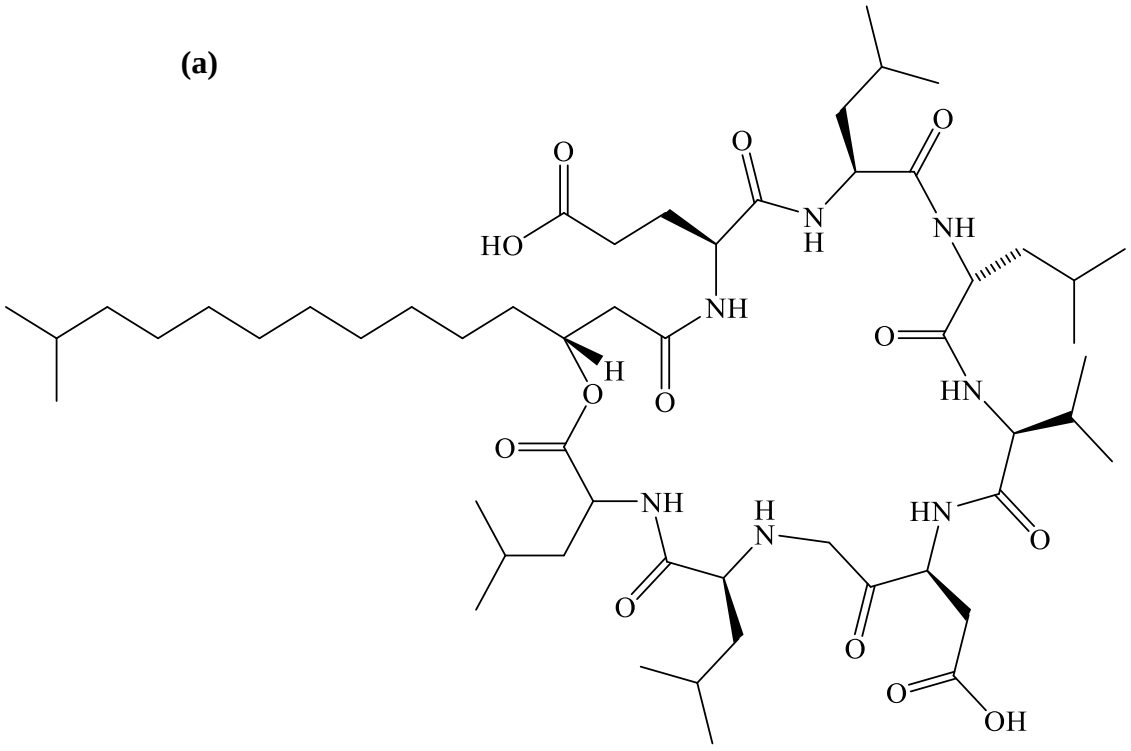
#### 2.2.2.1 Surfactin

Surfactin group is the most prominent lipopeptide (Figure 2.2a) which is made up of a peptide loop of seven different amino acids (L-valine, two L-leucine, L-aspartic acid, glutamic acid, and two D-leucines), and a hydrophobic fatty acid chain, of 13 to 15 carbons length. Surfactin have shown potent antibacterial, antitumoral, antibiofilm, and antiviral activities as well as bioremediation process and environmental applications in recent studies (Ndlovu et al., 2017; Ohadi et al., 2018).

#### 2.2.2.2 Iturin

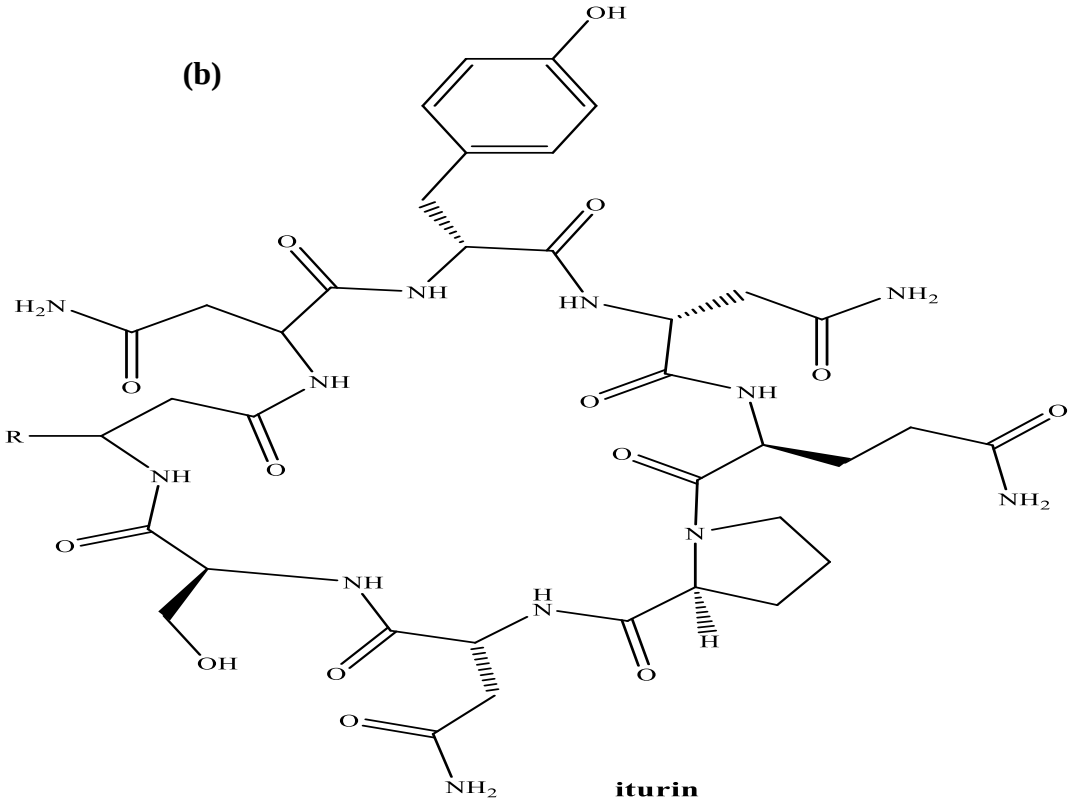
Another lipopeptide group with a hydrophobic fatty acid joined by an amide bond to a peptide moiety (constituent amino acid residual constituent) is *iturin* (Mnif and Ghribi, 2015). They possess a typical arrangement and show variability at four different positions (Figure 2.2b) (Jacques, 2011). The different groups associated with iturin includes bacillopeptin, and mycosubtilin, iturin A, C, D, and E, bacillomycin D, F, and L, respectively. Iturin as a lipopeptide group has also been implicated to be useful in antimicrobial, pharmaceutical and biotechnological applications (Jacques, 2011).

**(a)**



**surfactin**

**(b)**



**iturin**

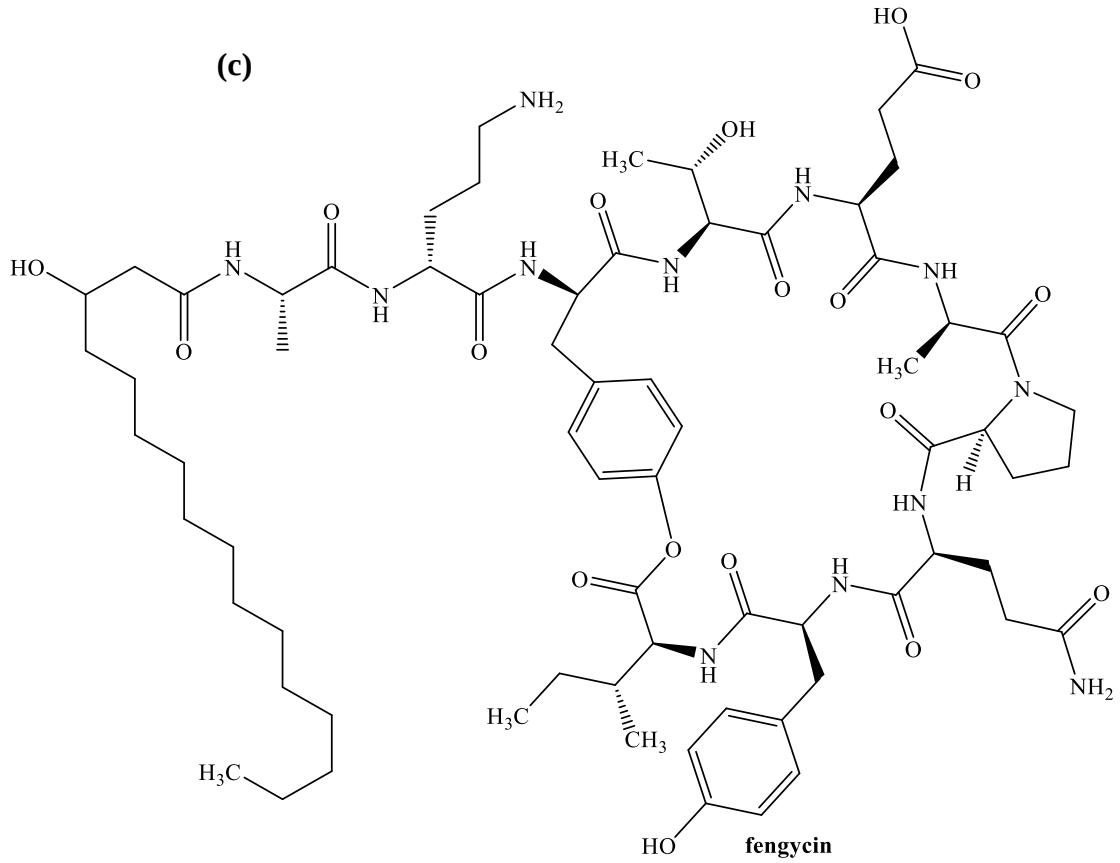



**Figure 2.2** The chemical structures of (a) surfactin, (b) iturin, and (c) fengycin biosurfactant. The cyclic lipopeptide contain fatty acid chain linked with amino acids. The compound subordinate in each group originates from a various amino acid constituent (Mnif and Ghribi, 2015).

### 2.2.2.3 Fengycin

Fengycin is another set of lipopeptide group that possesses a lipidic fraction and 10 different amino acids connected to biomolecule *N*-terminal end. Iturin and surfactin contrast from this group due to the incidence of uncommon amino acids such as allo-threonine and ornithine (Mnif and Ghribi, 2015). Like iturin group, fengycin possess solid antifungal action, inhibit the development of an extensive variety of plant pathogens and application in improved diesel biodegradation. The diverse variety of the peptide component (variations with trademark Alanine-Valine di morphy positioned at 6 in the peptide ring) also authorizes the characterization of a new fengycin B into fengycin family (Figure 2.2c) (Mnif and Ghribi, 2015).

### *2.2.3 Fatty acids and phospholipid*

They are immersed unsaturated BioS of $C_{12}$ to $C_{14}$ lengths and complex unsaturated fatty acids comprising of hydroxyl groups and alkyl branches. Different bacteria produce huge numbers of fatty acids and phospholipid surfactants as the fatty acids are suitable as BioSs due to their surface activity (Adetunji, 2012).

### *2.2.4 Polymeric biosurfactants*

Polymeric BioSs are generally high atomic weight biopolymers, with characteristics, for example, rigidity, high thickness, and shear resistance. Emulsan and liposan, synthesized by *Acinetobacter calcoaceticus* and *Candida lipolytica* respectively are the best studied polymeric BioS (Adetunji, 2012). Different cases of particulate BioS are extracellular vesicles of microbial cells, which aid hydrocarbon emulsification (Shekhar et al., 2015). Emulsan holds a backbone comprising a 2-amino-2-deoxy-hexuronic acid, amino sugars, glucose, fatty acids, and galactosamine (2-amino-2-deoxy-galactose) connected to the main chain by means of amide and ester bonds (Figure 2.3) (Park et al., 2017).

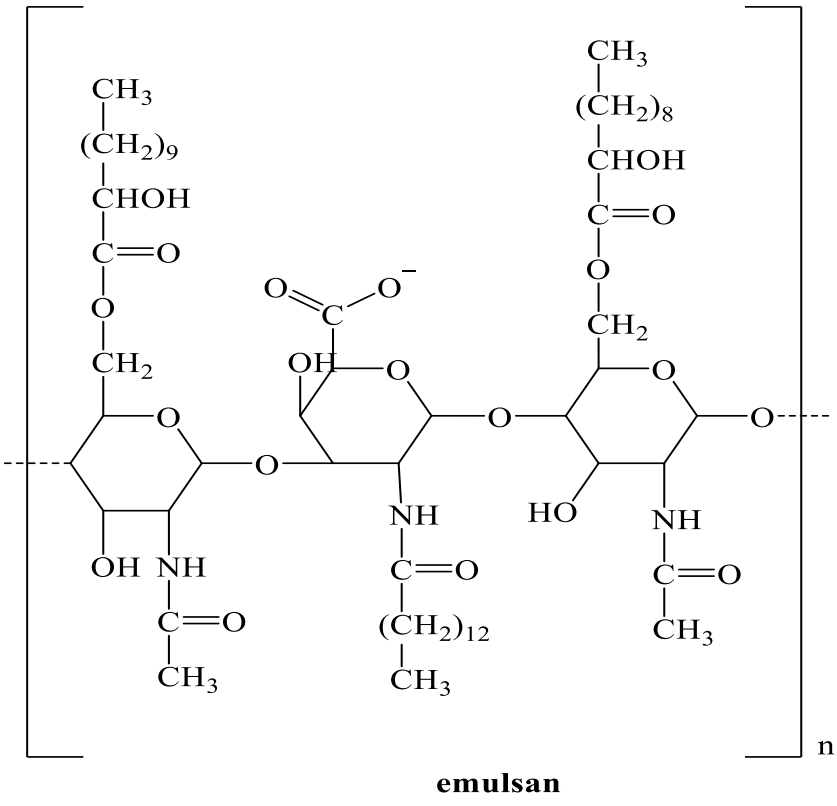
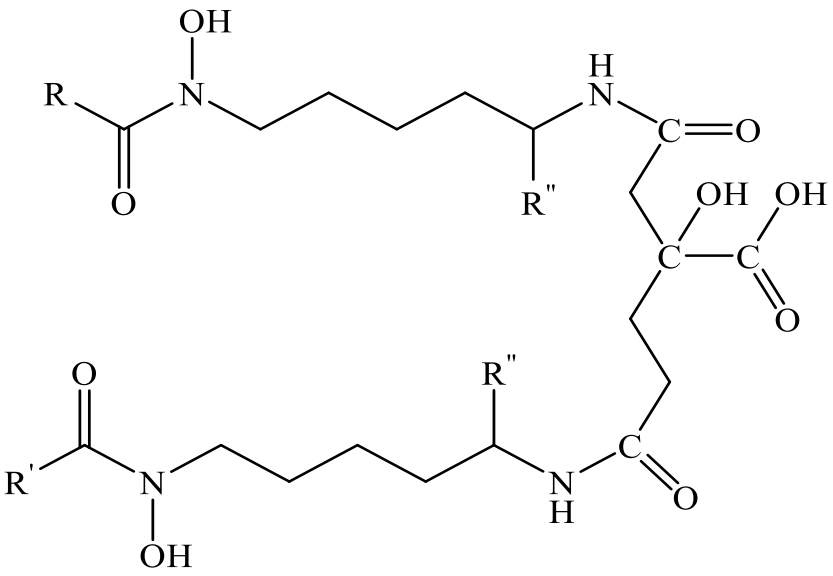



**Figure 2.3** The structural composition of emulsan, a major microbial surface-active compound with high molecular weight (Park et al., 2017; Shekhar et al., 2015).

### *2.2.5 Flavolipid*

A new class of BioS with solid interfacial activity and emulsifying capacity is represented as flavolipids. The polar end of this group (Figure 2.4) possess two cadaverine molecules and citric acid, which is rather dissimilar to the polar groups in other reported BioSs. Flavolipid BioS is of interest for their potentiality in environmental, biotechnology, industrial applications (Bodour et al., 2004).




| | R and R' | | R" |
|---|---|---|---|
| **Flavolipids** | $(CH_3)_2CH(CH_2)_nCH{=}CH$ | and/or | H |
| | $(CH_3)_2CH(CH_2)_nCH_2CH_2$ | n = 0 to 4 | |

**Figure 2.4** Structures of flavolipid biosurfactant isolated from *Flavobacterium* sp. strain MTN11 (Bodour et al., 2004).

## 2.3 General properties of biosurfactant

BioSs possess some properties, which are peculiar to them and therefore have advantages over conventional surfactants. Microbial surfactants are distinct, they are tolerable to extreme pH and temperature, biodegradable, and have high ionic strength (de França et al., 2015). They have low toxicity as compared to chemical surfactants, high emulsifying and demulsifying ability, and act as antimicrobial, and surface activity (Chakraborty et al., 2015). The remarkable properties of BioSs in comparison with their synthetically produced counterparts make them appropriate for large-scale commercial applications (Ferreira et al., 2017; Martins and Martins, 2018).

The vital present movement for industrial, and environmental sustainability has led to increased interest in BioSs. Aside from being produced from renewable raw-materials, BioSs also possess features that are termed 'environmental and ecologically-friendly. Similarly, they are considerably less detrimental to the environment as related to more recalcitrant compounds termed chemical surfactants (Dalili et al., 2015). Also, their ability to endure extreme environmental conditions makes them striking components for many industrial products formulation (Martins and Martins, 2018). The special properties of BioSs are further illustrated below.

### *2.3.1 Temperature, pH and salt tolerance*

The use of BioS in a comprehensive series of applications requires its stability in a range of high temperatures (around 50 –100$^{o}$C), high salt concentrations, and extensive range of pH (2-12) (Al-Wahaibi et al., 2014). This ability is massively vital implicating the importance of BioS in beauty care products, food, pharmaceutical and where heating to accomplish sterility is of significance (Khopade et al., 2012). Since, industrial, environmental and biotechnological processes encompass exposure to temperature, pH and pressure extremity, hence the need to identify innovative microbial products that can work under these conditions (Darvishi et al., 2011). Researchers recently have supported the production of stable BioSs under an extensive array of conditions to support valuable properties highlighted above (Al-Wahaibi et al., 2014; Elazzazy et al., 2015; Jha et al., 2016). In that capacity, the exploitation of such steady and consistent BioS is also profitable in petroleum oil industry oil-sludge cleaning, microbial enhanced oil recovery, storage tanks, and oil immobilization (Jha et al., 2016).

### *2.3.2 Biodegradability and low toxicity*

The increasing environmental concern has necessitated the relative exploration of alternative products as chemical surfactants have been shown to be toxic to the environment (Lima et al., 2011). Therefore, an important advantage to consider is the biodegradable nature and non-toxicity of the BioS as this is needed for any product formulation. BioSs are largely low or non-toxic products making them suitable in pharmaceutics, food uses, detergent formulation, cosmetic, ornamentals, and other applications of human, and health concern. Studies had confirmed that anionic and cationic BioSs have significantly lower toxicity than that of the synthetic surfactants (Ferreira et al., 2017; Freitas et al., 2016; Lima et al., 2011). Therefore, the biodegradability property makes BioSs appropriate for environmental applications such as bioremediation, biosorption, and bio-solubilization.

### *2.3.3 Emulsifying and demulsifying property*

BioSs may act as either emulsifiers or de-emulsifiers (Santos et al., 2016). An emulsion can be characterized as a heterogeneous system (with diameter, greater than 0.1 mm), which comprises of an immiscible liquid distributed in droplet forms. Two types of emulsions namely oil-in-water (o/w) or water-in-oil (w/o) emulsions have been largely defined as reported quite recently (Santos et al., 2016). BioSs, and additionally bioemulsifiers, when contrasted with synthetic surfactants show better or comparative emulsifying activities, and in view of their microbial origin, thus suggesting a more prominent ecological compatibility (Gudina et al., 2015b).

### *2.3.4 Antiadhesive agents*

One of another important advantage of BioS is the ability to act as an anti-adhesive and anti-biofilm agent. Adhesion of microorganisms to different pollutants is enhanced by modification of the surface hydrophobicity. Microbial-surface interactions are intrinsically influenced by the development of surface-active BioS (Sharma and Saharan, 2016). During adherence to the solid surface, the microbial biofilm is formed as a specific group of microorganisms (Sharma and Saharan, 2016). *Streptococcus thermophilus* BioS reduced the colonization of other thermophilic *Streptococcus* strains which is liable for fouling during steel processing (Chakrabarti, 2012). BioS produced by various microorganisms have been accounted for their powerful anti-biofilm properties against different pathogens (Peele Karlapudi et al., 2018; Sharma and Saharan, 2016).

### *2.3.5 Surface and interface activity*

Another advantage of BioSs is the capability to decrease interfacial and surface tension at different interphase. For maximal decline in surface activity, less measure of BioS is required. This is possible as BioS possess effective critical micelle concentration (CMC) which is 10 to 40-fold lower than synthetic surfactants (Al-Wahaibi et al., 2014; Uzoigwe et al., 2015). BioSs have outstanding qualities such as foaming, emulsifying, washing, and dispersing agents due to their surface and interface activity. In addition, BioSs owing to their amphiphilic nature intensify the surface area of water-insoluble hydrophobic pollutants and thus improves the bioavailability and solubilization of contaminated substances (Bezza and Nkhalambayausi Chirwa, 2015; Gudina et al., 2015b).

### *2.3.6 Availability of cheap raw materials*

Unlike chemical surfactants for which the substrates for production are relatively expensive, BioS molecules can be synthesized from different fermentation processes using cheap, renewable substrates, waste products, and waste materials. An extensive variety of cheap raw materials such as oil refinery wastes, potato process effluents, cassava waste water, waste frying oils, curd whey, distillery wastes, sludge palm oil, plant-derived oils, and corn steep liquor has been implicated in the synthesis of BioS (Mukherjee et al., 2006).

## 2.4 Biosurfactant production

During growth and metabolism, different microbes such as bacteria, yeasts, and fungi can synthesize surface-active biomolecules. BioS synthesis often occurs by resting microbial cell systems, in addition to growth dependent production. BioSs are produced biologically from various substrates ranging from hydrocarbons, hydrophobic mixtures, chemicals, solvents, hydrophobic mixtures, vegetable oils, waste products oil wastes, dairy products, and so on. The commercial production of these BioSs is quite limited and costly, as the recovery processes account for 60% of the total cost of production. So, the use of low-cost raw materials is highlighted to overcome expensive costs made from BioS production. It is imperative that the proper management and utilization of harmful and non-harmful waste materials generated in the world entirely is needed desperately (Jimoh and Lin, 2019a). Discussed below are some of the substrates that have been implicated in the production of BioS.

### *2.4.1 Industrial wastes*

Several industrial wastes such as starch waste, molasses, corn steep liquor, soap stock, animal fat are low-cost materials generated from industrial set-ups concerned with the production of BioS. A lot of the waste products are released into the environment sometimes as co-product thus their utilization by the natural population of microorganisms. For the large-scale commercial production of BioS compounds, industrial waste products are available for use as cheap raw materials or substrates making them an alternate option. There was the utilization of soybean oil waste, as well as, cassava flour, molasses, and whey for producing rhamnolipid BioS by *Pseudomonas aeruginosa* LBI strain (Nitschke et al., 2010).

### *2.4.2 Oil processing industries*

Of the readily accessible, inexpensive renewable sources for the synthesis of surface-active biomolecules are the wastes generated from oil processing industries. Also olive oil, sunflower, canola, coconut oil made from oil industries has been discovered as promising carbon and energy substrates for BioS synthesis. Olive oil, a product of oil processing industries was utilized as a carbon source for BioS synthesis coupled with a nitrogen source (ammonium nitrate) by *Pseudomonas fluorescens* 1895-DSMZ (Abouseoud et al., 2008). The utilization of canola oil refinery waste augmented with sodium nitrate gave maximum rhamnolipid BioS production yield of 8.50 g/L at the end of 10 days incubation by *Pseudomonas aeruginosa* mutant (Raza et al., 2007b). Palm oil, another product of the oil industry has additionally been utilized effectively to produce BioS using *Pseudomonas aeruginosa* SP4 (Pansiripat et al., 2010). There was also a report on the synthesis of 1.0 g/L BioS from palm oil mill effluent, as a novel substrate, by *Nevskia ramosa* NA3, a new BioS-producing strain (Chooklin et al., 2013). *Lactobacillus delbrueckii*, a probiotic bacterial system utilized peanut oil for the synthesis of BioS (Thavasi et al., 2011). Soybean oil was also reported to have been used in the production of BioS. In another research conducted, BioS was produced by *Bacillus pseudomycoides* BS6 through the utilization of soybean oil waste (Li et al., 2016).

Furthermore, different vegetable oils have been implicated as consistent substrates for the production of BioS (Khopade et al., 2012; Saravanan and Subramaniyan, 2014). Also, olive oil, as well as phenylalanine as a nitrogen source, was used for the BioS production by marine strain *Nocardiopsis* sp. B4 (Khopade et al., 2012). Saravanan and Subramaniyan (2014) confirmed BioS production on several cheap renewable and vegetable oil substrates as a replacement for routine carbon sources by *Pseudomonas aeruginosa* PB3A. The summary of renewable and inexpensive substrates used to produce BioSs are provided in Table 2.

**Table 2.1** Different renewable substrates used to produce biosurfactants (Sekhon Randhawa and Rahman, 2014; Shekhar et al., 2015).

| **Biosurfactant** | **Producing Microorganisms** | **Raw Materials** |
|---|---|---|
| Glycolipids | *Candida ntarctica, Candida apicola* | Oil refinery wastes |
| Lipopeptides | *Bacillus subtilis*, *B. subtilis* ATCC 21332, *B subtilis* LB5a, *B. subtilis* SK320, | Potato process effluents; cassava flour wastewater; olive oil; sunflower oil |
| Phospholipid | *Klebsiella pneumoniae* WMF02 | Sludge Palm oil |
| Rhamnolipid | *Pseudomonas aeruginosa* 47T2 | Waste frying oils (olive and |
| | 40044 | sunflower oil) |
| | *Pseudomonas* sp. DSM 2874 | Soybean soap stock waste |
| | *Pseudomonas aeruginosa* DS10- | Rapeseed oil |
| | 129 | |
| | *Pseudomonas aeruginosa* BS2 | Sunflower and soybean oil |
| | | Curd whey and distillery wastes |
| Sophorolipid | *Candida lipolytica* IA 1055 | Babassu oil |
| | *Candida bombicola* ATCC 22214 | Turkish corn oil |

### *2.4.3 Dairy products*

Large amounts of raw substrates such as cheese whey, curd whey, whey, whey waste, lactic whey are readily generated from dairy industries, which are accessible for microbial production of BioS (Dubey et al., 2005; González-Siso et al., 2008). In a report, there was a method to utilize valued by-products (soybean oil and sugarcane molasses) for sophorolipids production using *Candida bombicola*. In the experimental medium containing dairy wastewaters, 50 g/L each of soybean oil and sugarcane, a BioS production yield of 38.76 g/L was achieved (Daverey and Pakshirajan, 2009). *Trichosporon mycotoxinivorans* CLA2, a bio-emulsifier producing yeast strain isolated from dairy industrial effluents, grew on a mineral salt medium comprising refinery waste (de Souza Monteiro et al., 2012).

### *2.4.4 Hydrocarbons and hydrophobic mixtures*

Microorganisms utilize an extensive variety of hydrocarbons, organic compounds, hydrophobic mixtures, and chemicals as carbon and energy source. Thus, BioSs increase the solubilization of these compounds at a concentration which enhances their bioavailability, microbial uptake, and utilization (Patowary et al., 2017). *Pseudomonas* sp. strain LP1 was analyzed for its BioS production prospect while utilizing pyrene, diesel fuel, crude oil, and engine oil (Obayori et al., 2009). Recently, *Paenibacillus dendritiformis* CN5 strain could produce lipopeptide BioS by enhancing polycyclic aromatic hydrocarbon (PAH), motor oil and pyrene biodegradation (Bezza and Chirwa, 2017; Bezza and Nkhalambayausi Chirwa, 2015). Crude oil is another hydrophobic pollutant that has been utilized by different microorganisms through the production of BioS (Gudina et al., 2015b; Ibrahim et al., 2013; Patowary et al., 2017).

### *2.4.5 Agro-industrial waste and residues*

Different agricultural products, for example, straw of sugar cane, molasses, wheat, straw of rice, bran, beet molasses, rice, bagasse of sugarcane, hull of soy, corn, cassava flour, and its wastewater have been discovered by researchers as candidates of agro-industrial waste which are very good substrates for BioS production. Many carbohydrates and lipids can be obtained from agro-industrial waste residues and hence, necessary for the growth of microbial life and BioS production. Some waste material like corn steep liquor and processed cereals wastewater, and rice water (gotten from rice processing industry, domestic, and restaurant cooking), have also been implicated in the production of BioS (Joshi et al., 2008; Makkar et al., 2011; Nitschke et al., 2010; Onbasli and Aslim, 2009; Rashedi et al., 2005; Raza et al., 2007a).

## 2.5 Physiology, pathways, and kinetics of biosurfactant production

### *2.5.1 Biosurfactant physiology and metabolic pathways*

BioSs are synthesized through intracellular or extracellular adhesion to microbial cells when cultured on insoluble substrates. Microbial cell function associated with BioS is not understood fully, as speculations have been made about their implication in the emulsifying of insoluble pollutants (Santos et al., 2016). The foremost physiological role of BioSs is enhanced microbial toleration and capacity to synthesize insoluble substrates. This is enabled via reducing the surface tension between the interphase, thus, increasing the availability of substrate for metabolism and uptake (Bezza and Nkhalambayausi Chirwa, 2015). Cell surface hydrophobicity

is conferred on microbes that synthesize BioSs in growth associated manner. This property is needed for successive uptake of hydrophobic substrates by microorganisms (Perfumo et al., 2010; Satpute et al., 2010a; Uzoigwe et al., 2015).

The different pathways for the biosynthesis of BioSs are discussed below ranging from glycolipids (rhamnolipids, sophorolipids, phospholipids, mannosylerythritol, trehalose lipids), lipopeptide (surfactin) including polymeric BioSs (emulsan). BioSs are amphiphilic in nature comprising both hydrophilic polar and hydrophobic non-polar joined ends. Microorganisms exploit the hydrophilic polar moieties for cell metabolism whereas the utilization of hydrocarbon portion is entirely dependent on the hydrophobic moieties (Joshi-Navare, 2013; Santos et al., 2016). The synthesis of precursors for BioS production involves different metabolic pathways which are dependent on carbon substrates utilized in the production culture medium. In the synthesis of glycolipids, the flow of the major carbon source (carbohydrates) is regulated by the lipogenic pathways, while glycolytic pathway, on the other hand, enabled the formation of the hydrophilic moiety (Figure 2.5) (Santos et al., 2016). A major precursor of carbohydrates (glucose 6-phosphate) present in the hydrophilic component of glycolipid BioS is made from the degradation of carbohydrates substrates such as glucose or glycerol. Subsequently, acetyl-CoA is produced from pyruvate, which in turns gives malonyl-CoA in addition with oxaloacetate. This process is thus followed by conversion into an important precursor for the synthesis of lipids namely fatty acids (Joshi-Navare, 2013).

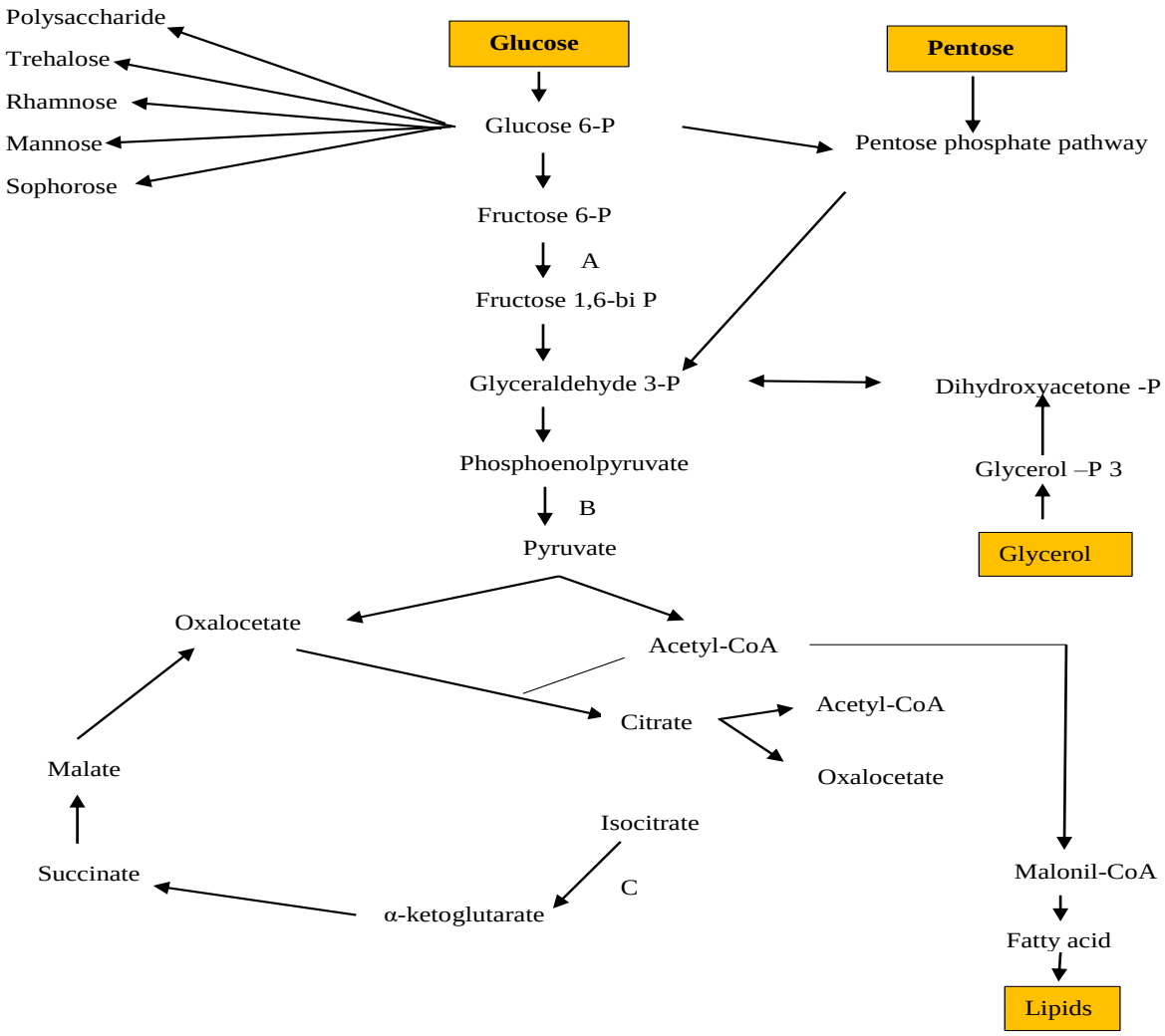



**Figure 2.5** The intermediate metabolism identified with the synthesis of glycolipid biosurfactant precursors with different carbohydrate substrates. The enzymes (**a**) phosphofructokinase; (**b**) pyruvate kinase; and (**c**) isocitrate dehydrogenase are responsible for the flow of carbon (Santos et al., 2016)

In a situation where hydrocarbon is utilized as the substrate source, the mode of action is principally engaged to both the gluconeogenesis and lipolytic pathways thereby allowing its usage to produce sugars or fatty acids (Figure 2.6). The gluconeogenesis pathway is activated to produce sugars which involve fatty acids oxidation to acetyl-CoA or propionyl-CoA. The steps required the production of complex sugars precursors such as glucose 6-phosphate involves the initiation of acetyl-CoA formation, which is the reverse of those steps associated with glycolysis. Conversely, some reactions exclusive to gluconeogenesis processes are irreversible and are catalyzed by pyruvate kinase and phosphofructokinase-1 (Santos et al., 2016; Tokumoto et al., 2009).

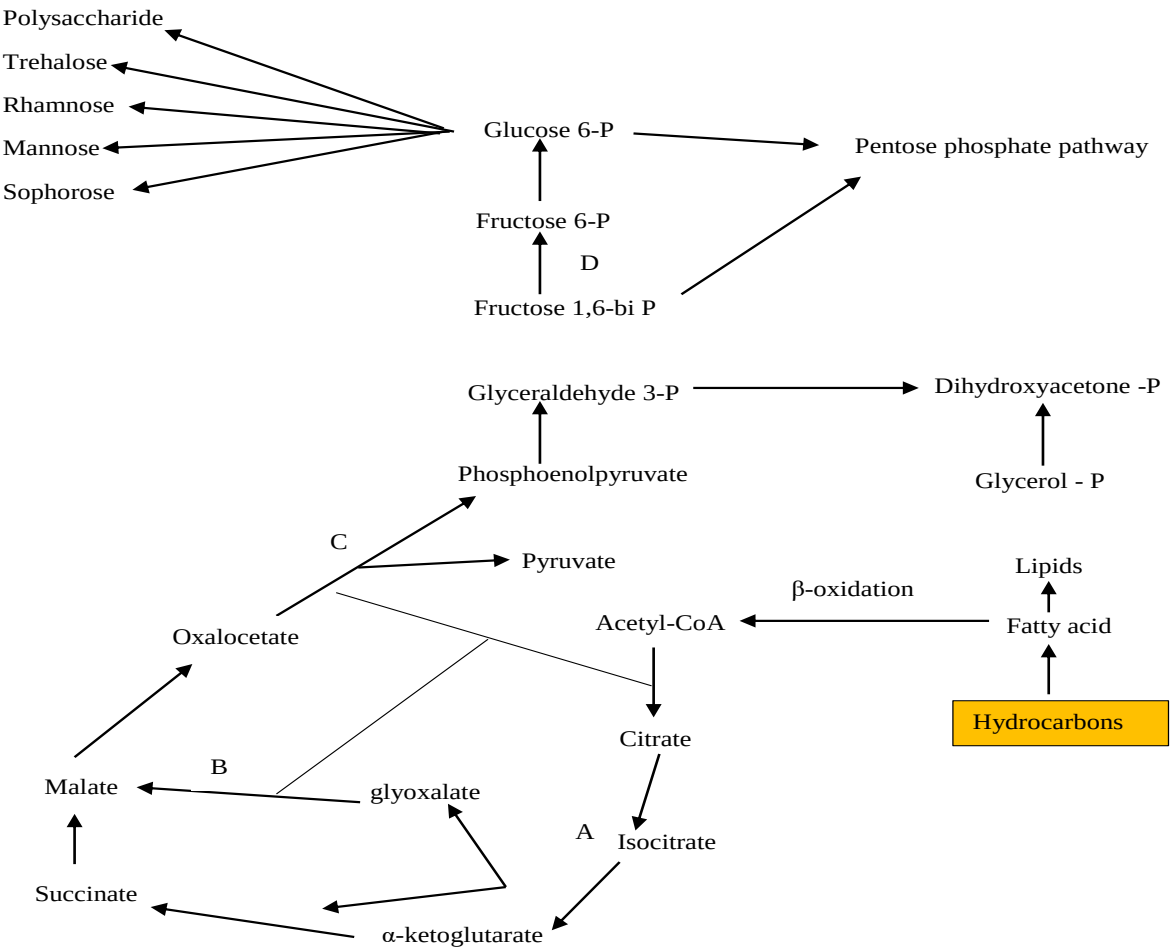



**Figure 2.6** The intermediate metabolism identified with the synthesis of glycolipid biosurfactant precursors utilizing hydrocarbons substrates. The enzymes (**a**) isocitrate lyase; (**b**) malate synthase; and (**c**) phosphoenolpyruvate carboxykinase; (**d**) fructose-1-phosphatase are responsible for the flow of carbon (Santos et al., 2016).

The formation of several polysaccharide species highlights the cross-linkage of the rhamnolipid BioS biosynthesis pathway (Figure 2.7) (Ma et al., 2009). In rhamnolipid biosynthesis, the β-oxidation mechanism is suggested as the key provider of lipid precursors (Abdel-Mawgoud et al., 2014). The β-oxidation confirmed a significant role in rhamnolipids production which is a constituent and should not only occur when the fatty acid is provided as carbon and energy sources. The biosynthesis of rhamnolipid is involved by three main enzymatic reactions (Abdel-Mawgoud et al., 2014). The substrate needed for both mono- and di-rhamnolipids is utilized and activated by rhamnose moiety, which is dependent on the RmlBCAD pathway. This process is further encoded by the catalytic activity of the enzyme AlgC, and RmlBCAD operon. In the synthesis of the rhamnose sugar precursor, the normal D-glucose molecule is converted to D-glucose-1-phosphate which is then catalyzed by the enzyme AlgC. The process then follows the synthesis of dTDP-D-glucose by enzyme,

RmIA. The RmIB further converts the dTDP-D-glucose to dTDP-4-oxo-6-deoxyl-D-glucose by RmIB and subsequent conversion to dTDP-6-deoxyl-L-deoxyl-4-hexulose by enzyme RmIC. The RmID enzymes to end with convert dTDP-6-deoxy-L-lyso-4-hexulose to dTDP-L-rhamnose. The dTDP-D-glucose and dTDP-6deoxyl-L-deoxyl-4-hexulose are rhamnosyl-transferases RhIB and RhIC substrates, needed for the mono- and di-rhamnolipids biosynthesis. Hypothetically, RhIG enzyme functions by relaying intermediates of fatty acid synthesis into the rhamnolipid pathway (Abdel-Mawgoud et al., 2014; Ndlovu, 2017).

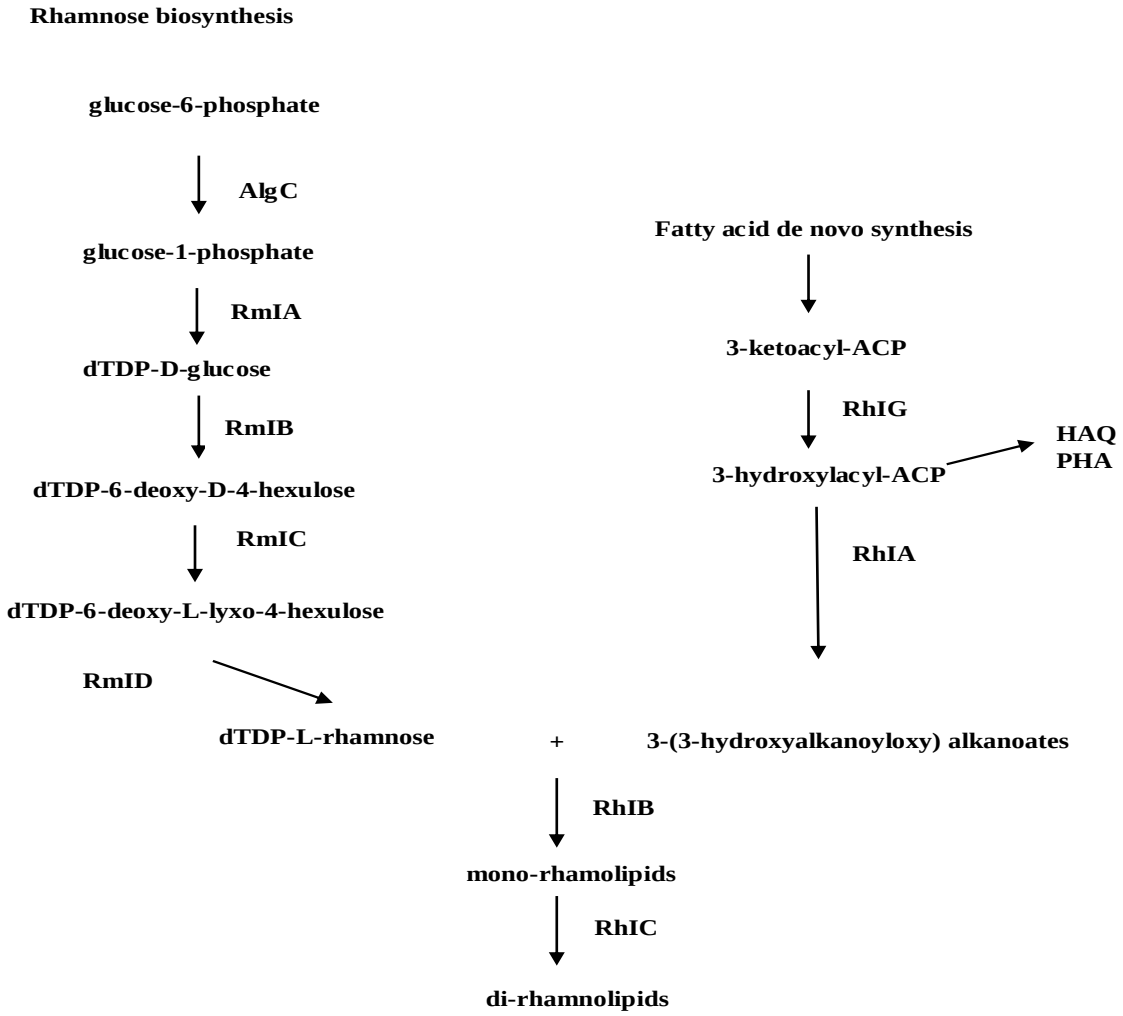



**Figure 2.7** Biosynthesis pathway of mono-rhamnolipid and di-rhamnolipid biosurfactant (Abdel-Mawgoud et al., 2014; Ndlovu, 2017).

So far, the biosynthesis of sophorolipids BioS includes the successive transfer of activated glucose molecules (UDP-glucose) to a hydroxyl acid in reactions catalyzed by two separate glycosyltransferases. The acetyltransferase further acetylates the glucose molecule and the fatty acid constituents can be produced by modifying hydrocarbons or *de novo* from acetate in the growth medium (Van Bogaert, 2008).

In the case of mannosylerythritol biosynthesis, the genes required were formerly described on smut fungi named *Ustilago maydis*, which yields mannosylerythritol inclusive of cellobiose lipids (Morita et al., 2010). Mannosylerythritol BioS is synthesized through different enzymatic reactions. The enzyme mannosyltransferase required in the synthesis of mannosylerythritol is encoded by *emt1*, while *mat1* translates an acetyltransferase catalyzing the mannosylerythritol acetylation at both the C-4′ and C-6′ hydroxyl groups of mannoses. Also, an acyltransferase is required for the acylation of mannosylerythritol is encoded by *mac1* (Hewald et al., 2006; Morita et al., 2010).

Trehalose (trehalolipids) biosynthesis, on the other hand, encompasses glucose transfer from UDP-glucose to glucose-6-phosphate to synthesize trehalose-6-P-UDP. This is synthesized through the catalytic capability of trehalose-6-phosphate synthase. Subsequently, a free disaccharide catalyzed by trehalose-6-phosphate phosphatase is generated by de-phosphorylation (Rao et al., 2006).

The synthesis of phospholipid occurs in the cytosol corresponding to the membrane that is coupled with proteins that act in allocation (flippase and floppase) and synthesis (acyl transferases, phosphatase, and choline phosphotransferase). Ultimately, the phospholipids containing vesicle destined for the cytoplasmic cellular sprout out on its exterior. Also, on the other hand, the exoplasmic cellular membrane generates the release of phospholipids BioS on its inner leaflet (Lodish, 2008).

Biosynthesis of surfactin, which is one of the prominent lipopeptide BioS ensues through a non-ribosomal peptide synthetase mechanism. The step includes multi-enzyme peptide synthase complex which comprises four enzymatic subunits SrfA, SrfB, SrfC, and SrfD. These enzymes are called surfactin synthetases needed for surfactin biosynthesis and are coded by *srf* operon (Vater et al., 2009). The joining of seven amino acids into the peptide component of surfactin is catalyzed by surfactin synthase through a thiotemplate mechanism. This includes amino acids activation by ATP and assemblage of amino acids into a peptides chain. Using an acyltransferase enzyme, lipopeptide is then formed by linking the hydroxyl fatty acid to a peptide group (Eyéghé-Bickong, 2011).

### *2.5.2 Kinetics of biosurfactant production*

The BioS production kinetics has substantial variance amongst diverse systems. The different kinetic parameters to be considered are assembled below,

(a) growth-dependent;

(b) growth-limiting;

(c) synthesis by immobilized or resting cells; and

(d) synthesis with precursor supplements (Desai and Banat, 1997).

In production related to growth, there exists a parallel correlation between cellular growth, substrate usage, and increased BioS production. A heightened increase in BioSs concentration due to the restraint of one or more medium constituents characterize the synthesis under growth-limiting conditions. The synthesis by immobilized or resting cells is a type where the cells use carbon substrates continuously for BioS synthesis, with relatively no cell multiplication. The last kinetic parameter as listed above involves the addition of BioS precursors to the production medium. As revealed by researchers, precursor addition often results in qualitative and quantitative variations in BioS product yield (Santos et al., 2016).

## 2.6 Factors and strategies for improvement of biosurfactant production

BioSs are produced by a diversity of microorganisms, which are either attached intracellularly or extracellularly predominantly during growth (Desai and Banat, 1997). The BioS synthesis by microorganisms occurs during the exponential or stationary growth phase when the nutrient restricting conditions predominate the production medium. Production of BioS might be attributed to certain biosynthetic genes that are stimulated by the incidence of hydrophobic mixtures, hydrocarbons and other different carbon substrates (Sekhon et al., 2012).
The current BioSs industry is aiming at the important factors affecting the production of microbial surfactants relative to production costs and products yield. The significant process to attain these goals include obtaining via screening of the novel microorganisms, usage of inexpensive substrates, media optimization, improved production yield, cost-effective downstream processes, and purification of the end products (Sekhon et al., 2012). The synthesis of BioSs can either be natural or induced by the presence of different compounds, varying pH, temperature, inoculum size, aeration, stress, and also agitation speed (Desai and Banat, 1997). In general, the yield of BioS has been reported to be affected by elements such as carbon, nitrogen, iron, sulfur,

phosphorus, and manganese. (Darvishi et al., 2011; Gudina et al., 2015a). Different elements ratio such as carbon: nitrogen, carbon: phosphorus, carbon: iron or carbon: magnesium and optimization of these rates should be achieved to increase the BioS production yield (Darvishi et al., 2011; Gudina et al., 2015a). The various factors and strategies needed for improved BioS production are further discussed below

### *2.6.1 Carbon Sources*

Carbon sources of different types are hugely important and have been described to largely influence the BioS production yield both in quality and quantity. Such sources range from cheap substrates such as starchy substrates, animal fat, oils, petroleum effluents, plant-derived oils, lactic whey, olive oil mill effluent, distillery wastes, soapstock, molasses, vegetable oils and oil wastes, hydrocarbons (*n*-hexadecane, *n*-dodecane, *n*-tetradecane, pyrene), hydrophobic mixtures (such as motor oil, diesel, crude oil, paraffin, kerosene) with great potential in enhancing and improving BioSs production (Abouseoud et al., 2008; Bezza and Chirwa, 2017; Bezza and Nkhalambayausi Chirwa, 2015; Patowary et al., 2017).

The probable usage of low substrates for improved BioS yield is of great significance to the BioS world market to defeat the high cost of production. There have been reports on rhamnolipid BioS utilizing waste cooking oil with major emphasis on *Pseudomonas aeruginosa* (Lan et al., 2015; Xia et al., 2012) and optimization of production conditions (Lan et al., 2015; Xia et al., 2012). As well, a maximum rhamnolipid BioSs yield of 13.93 g/L by a non-pathogenic microorganism *Pseudomonas* sp. SWP-4 utilizing waste cooking oil as a sole source of carbon (Lan et al., 2015). There was an increase in production of lipopeptide BioS by *Bacillus subtilis* SK320 utilizing olive oil as an unconventional carbon source (Sekhon et al., 2011) and BioS produced by marine *Nocardiopsis* B4 (Khopade et al., 2012). In another study, there was improved production yield of 2.13, 2.20 and 2.45 g/L by bioengineered recombinant strains *BioSa*, *BioSb*, and *BioSc* when cultured with olive oil as a sole source of carbon and energy. The parent strain *Bacillus subtilis* SK320, on the other hand, produced a lower yield of 1.2 g/L, which was considerably lower than to the recombinant strains. However, an enriched BioS production was not only presented by the recombinant strains but also increase in the esterase activity (Sekhon et al., 2011). Correspondingly, *Pseudomonas aeruginosa* and *Corynebacterium kutscheri* on oil from peanut cake and vehicle lubricant oil exhibited lipopeptide BioS production yield of 8.6 mg/mL, and 6.4 mg/mL respectively (Thavasi et al., 2011).

Hydrophobic carbon sources such as insoluble substrates, hydrocarbons (aliphatic and aromatic), oils, and so on have also been implicated in the improvement of BioS by different research report discussed below. The

use of hydrophobic carbon sources was accompanied by high BioS productivity (Abdel-Mawgoud et al., 2011). In an observation similar to the above stated, parameter optimization on different hydrocarbon substrates yielded a 3.6-fold increase in BioS production by *Sphingobacterium detergens* (Burgos-Diaz et al., 2013). There was also high induction of BioS on all the hydrocarbons tested on *Pseudomonas aeruginosa* PBSC1 with the best yield of 4.99 g/L utilized on motor oil produced on par statistically with the *n*-hexadecane (4.76 g/L) (Joice and Parthasarathi, 2014). Obayori et al. (2009) also reported the utilization of *Pseudomonas* sp. strain LP1 on engine oil with poor performance on diesel fuel and crude oil.

The use of low molecular weight carbohydrates has been used to further enhance the production of BioS. Glycerol is a C3 compound and precursor of fatty acid with high solubility in BioS production medium. *Pseudomonas aeruginosa* UKMP14T produced optimum levels of BioS when developed in a mineral salt medium comprising 1% (v/v) glycerol (Hamzah et al., 2013). In fact, the greatest quantity of lipid-based BioS synthesis was achieved in a medium containing glycerol (Chakraborty et al., 2015). Different raw materials utilized in surfactin (lipopeptide) BioS synthesis are pure cottonseed oil and paraffin oil (Joshi et al., 2008). In the synthesis of BioS by different *Bacillus* isolates, the production was improved by utilizing the best carbon source, glucose coupled with molasses, and palm oil (Al-Wahaibi et al., 2014; Pornsunthorntawee et al., 2008). In addition, there was report on the utilization of glycerol and sucrose as the sole carbon sources with an increase in BioS synthesis by different *Bacillus* strains (Pereira et al., 2013; Sousa et al., 2012). In a different study, 2.11 g/L lipopeptide yield after 96 h was observed in a medium encompassing 1% (w/v) glucose as carbon source by *Bacillus clausii* 5B (Aparna et al., 2012), while *Pseudomonas aeruginosa* BS-P and *Kocuria turfanesis* BS-J when cultured on distillery waste in combination with industrial wastes displayed production yields 1.976 g/L, and 0.967 g/L, respectively (Dubey et al., 2012).

### *2.6.2 Nitrogen sources*

Nitrogen is the second most significant nutrient needed to produce BioS by microorganisms. Composite nitrogenous sources are essential for microbial growth, cell constituents' development, and synthesis of bioactive metabolites. Different inorganic and organic nitrogen sources have been described to influence BioS synthesis (Jimoh and Lin, 2019 a; b).

In a past report, there was utilization of nitrates, ammonium, and amino acids by *Pseudomonas aeruginosa* as nitrogen sources. For use, there was a reduction of nitrates to nitrite and thereafter ammonium. Subsequently, ammonium is integrated either by glutamine synthetase to form glutamine or glutamate dehydrogenase to form

glutamate. $KNO_3$ (0.3%) was the optimum preferred nitrogen source to produce 2.8 g/L rhamnolipid BioS by *Pseudomonas aeruginosa* (Patil et al., 2014). Similar outcomes were achieved by *Pseudomonas aeruginosa* R2 with ammonium nitrate (0.4%) as the optimum nitrogen source (Komal et al., 2012). Also, the nitrate form ($NH_4NO_3$ or $NaNO_3$) was observed to be the best source of nitrogen for various methods of BioSs production by different *Pseudomonas* strains (Joice and Parthasarathi, 2014; Kannahi and Sherley, 2012; Patil et al., 2014; Xia et al., 2012). Influence of 0.6% (w/v) sodium nitrate was observed on rhamnolipid BioS activity by *Pseudomonas aeruginosa* UCP0092 in a production medium enclosing 3% (v/v) glycerol (Silva et al., 2010). There was also improved BioS activity in a different report with ammonium nitrate and sodium nitrate as nitrogen sources respectively (Meyer, 2010).

Some elevated BioS production was observed utilizing urea, $NaNO_3$, $NH_4NO_3$ or $KNO_3$ except ammonium sulfate by some *Bacillus* isolates. The variant in choosing inorganic over organic nitrogen source or the inverse may be medium composition or microbial strain dependent (Elazzazy et al., 2015; Ghribi and Ellouze-Chaabouni, 2011). Thus, organic sources of nitrogen possess a strong influence on the increase in BioS activity. The usage, however, becomes significant due to their availability and low cost. Elazzazy et al. (2015) reported the greatest emulsifying activity (82%), and least surface activity (29.5 mN/m) while utilizing urea as an organic nitrogen source by *Virgibacillus salarius* KSA-T. *Vibrio* sp. strain 3B-2 used organic nitrogen sources for microbial growth and improved BioS yield with lower synthesis developed from inorganic nitrogen sources. Also, yeast extract as a nitrogen source was involved in the maximum production of BioS from strain 3B-2 (Hu et al., 2015). The greatest BioS activity was realized when utilizing yeast extract as an organic nitrogen source by *Azotobacter chrococcum* while $(NH_4)_2SO_4$ was the best inorganic nitrogen source (Auhim and Mohamed, 2013). There was a significant drop of surface tension to 29.7 mN/m, with yeast extract as an appropriate nitrogen source for anaerobic lipopeptide synthesis by *Bacillus mojavensis* JF-2 (Liang et al., 2017). Abbasi et al. (2012) during the synthesis of BioS by *Pseudomonas aeruginosa*, they revealed a synergistic association between sodium nitrate and yeast extract positively affected BioS production. In another report, a mixture of yeast extract-$NaNO_3$ showed great influence on bacterial BioS biosynthesis and activity better than the single addition of yeast extract or sodium nitrate (Martínez-Trujillo et al., 2015).

### *2.6.3 Carbon/nitrogen ratio*

In fermentative processes, carbon to nitrogen ratio is one of the utmost significant features affecting the microbial influence and the build-up of metabolites in BioS production (Xia et al., 2012). Lower nitrogen concentrations (i.e., high C/N ratios) inhibits bacterial growth thus supporting cellular metabolism towards the synthesis of these biomolecules. The comparative quantities of multiple elements to carbon, such as C/N affects BioS productivity in the medium compositions (Xia et al., 2012).

Different research has shown the high production of BioS when nitrogen is in limiting condition. Illustratively, optimized carbon: nitrogen at ratio 7:1 was the optimal condition for improved BioS yield by *Pseudomonas aeruginosa* strain F23. *Pseudomonas aeruginosa* also produces rhamnolipid BioS when the amount of nitrogen sources is limiting (Patil et al., 2014; Xia et al., 2012). Similarly, *Pseudomonas aeruginosa* produced the highest activity at the carbon to nitrogen ratio of 10:1 (Abouseoud et al., 2007). In contrast, at the C/N ratio of 22:1 with glucose and sodium nitrate respectively, there was maximum BioS production by *Pseudomonas nitroreducens* (Onwosi and Odibo, 2013). Elazzazy et al. (2015) revealed that the lowest surface tension (29 mN/m), and most extreme emulsifying activity (85%), were accomplished at a C/N ratio of 30:1, while any abundance in proportion has no noticeable impact on BioS synthesis of *Virgibacillus salaries KSA-T*. Additionally, it was discovered that the perfect C/N ratio (waste cooking oil and $NaNO_3$) was 10:1, which gave a most elevated decrease in surface tension to 27.5 mN/m and 5 g/L BioS yield (Lan et al., 2015). From the reports illustrated above, nitrogen is very important to BioS production but needs to be in minimal supply to the medium.

### *2.6.4 Growth conditions*

Different growth conditions such as temperature, pH, incubation time, agitation speed and aeration also affect the BioS production output. It was discovered that the production of BioS was directly influenced by pH (Seghal Kiran et al., 2010). The strong dependence of numerous organisms on pH for cell growth and secondary metabolites production is an important characteristic. Some researches discussed the improved production within the alkaline condition range particularly pH ≥ 8.0. Patil et al. (2014) demonstrated optimum yield of glycolipid BioS at pH 8 by *Pseudomonas aeruginosa* strain F23. Comparative outcomes were obtained from *Pseudomonas aeruginosa* RS29 and WJ-1 which delivered high BioSs at pH range of 7– 8 and 6.0– 8.0 individually (Saikia et al., 2012; Xia et al., 2012). A maximum of 2.5 g/L of lipopeptide BioS was produced

at pH 8.0 by *Pseudomonas putida* MTCC (Kanna et al., 2014). In another research conducted, pH of the medium was at maximum by pH 9.0. Thus, the BioS synthesis increased from pH range 6.0 to 9.0, and pH 10 after which the BioS production started to decrease (Hamzah et al., 2013).

Relatively neutral (pH 7.0) and acidic pH conditions (pH ≤ 6.0) have been tentatively discussed to improve BioS production to sustain its competitiveness on a large scale. For instance, production of rhamnolipid was at its greatest at a pH range of 6 to 6.5 and with a sharp reduction above pH 7 by *Pseudomonas* sp (Kannahi and Sherley, 2012). *Paenibacillus alvei* was able to produce lipopeptide BioS within a pH range of 6–8 and the greatest BioS yield was attained at pH 6.89 (Najafi et al., 2011).

There have also been some studies on the greatest yield of BioS achieved at a neutral pH 7 (Abouseoud et al., 2008; Chakraborty et al., 2015; Kiran et al., 2009). However, the BioSs synthesis from strain *Actinomycetes nocardiopsis* A17 as observed to be the greatest at pH 6.8 though BioSs activity was still maintained at higher and lower pH (Chakraborty et al., 2015).

A slight variation in temperature is another critical factor that affects different BioS production processes. Optimum yield of rhamnolipid BioSs in *Pseudomonas aeruginosa* strain F23 was demonstrated at 30ºC (Patil et al., 2014). In another research conducted by Chakraborty et al. (2015) on *Actinomycetes nocardiopsis* A17, the optimum temperature 28ºC was highly effective for BioS production. However, most of the strains like *Bacillus subtilis* MTCC441 (Chander et al., 2012), *Pseudomonas aeruginosa* RS29 (Saikia et al., 2012), *Pseudomonas aeruginosa* WJ-1 (Xia et al., 2012), and *Pseudomonas putida* (Kannahi and Sherley, 2012), synthesized maximum BioS at 37°C. Another study carried out by Soniyamby et al. (2011) showed that the bacterium *Pseudomonas aeruginosa* at 35°C produced the maximum rhamnolipid BioS and the growth of bacteria and production of BioS was inhibited at 40°C. In addition, BioS from *Brevibacillus brevis* was thermally stable in a range of 30–80ºC. Similar behavior was observed with other *Bacillus* strains (Gudiña et al., 2012; Saimmai et al., 2012).

Agitation speed and aeration are also major factors that affect the production of BioSs by different microorganisms. Production of BioS is influenced by these two factors as both encourage the exchange of oxygen from gaseous to the aqueous phase. Fontes et al. (2010) investigated the influence of aeration and agitation speed on BioS production by *Yarrowia*. The results obtained from the batch fermentation indicated BS activity was influenced as the agitation speed increases from 160 rpm to a speed of 250 rpm. It was pointed out that the changes in agitation speed aid the build-up of BioS by *Pseudomonas aeruginosa* UCP 0992 synthesized in glycerol containing medium (Silva et al., 2010). It was also established that varying agitation

speed from 50 to 200 rpm has a significant effect on the BioS produced by *Pseudomonas alcaligenes* cultured in oil palm (Oliveira et al., 2009). The authors discovered that an increase in the velocity of rotation favor surface tension reduction to 27.6 mN/m in the cell-free broth. The maximum concentration of the rhamnolipid BioS by *Pseudomonas aeruginosa* was detected when the agitation speed remained at the limit of 140-160 rpm with different revolutions of 100-200 rpm (Jamal et al., 2014).

de Kronemberger et al. (2007) have also shown that rhamnolipid production by *Pseudomonas aeruginosa* depends on specific oxygen uptake rate. The mass transfer efficiency of the medium components and oxygen molecules affected by the agitation speed. Similarly, after 21 h in a fed-batch culture with an agitation rate of 150 rpm and 1 vvm aeration rate, the greatest surfactin concentration of 4.7 g/L was achieved (Yao et al., 2015).

The size of inoculum is also another critical factor that has significant consequence on BioS synthesis (Waqas et al., 2013). The size of an inoculum also plays a significant part in the biomass yield and product development since many physiological processes are dependent on cell density. The direct relationship between biomass growth rate and product formation is observed in the case of many bacterial products. In a research carried out on *Bacillus subtilis* SPB1, it was observed that density and the age of inoculum can influence to a large extent the yield and the overall cost of the production process. After optimizing the conditions of the inoculum, there was enhanced lipopeptide BioS yield of 3.4 g/L by *Bacillus subtilis* SPB1 (Mnif et al., 2013). *P*seudomonas *aeruginosa* RS29 gave a rhamnolipid BioS after an incubation period of 48 h at 37$^{o}$C and pH 7-8 and high yield was achieved after optimization of the few environmental factors in comparison to other conditions used in the research conducted (Saikia et al., 2012). At 2.4 mL inoculum of strain SNAU02, the maximum BioS production was obtained, with the size of the inoculum showing a substantial influence on BioS synthesis (Nalini and Parthasarathi, 2017).

In another instance, increase in the size of the inoculum upsurges microbial growth and to a certain extent, any further increase will lead to a reduction in microbial activity due to nutrient limitation (Nalini and Parthasarathi, 2017). The limitation of nutrient constituents and oxygen could arise due to high inoculum ratio while lower inoculum size could give rise to a noteworthy decline in the cell number in the production medium. The lower size of inoculum calls for an extensive period to attain optimum growth and utilize substrates to yield the needed product. For instance using 1 mL inoculum size, a reduced BioS yield of 2.74 g/L BioS was synthesized (Waqas et al., 2013). The above-stated reports show a different variation in the BioS yield under different conditions of bacterial growth and inoculum densities.

### *2.6.5 Response surface methodology*

The improved BioS production yield has been further explored through the introduction of a more innovative statistical approach that takes in cognizance the interaction between different factors responsible in the bioprocess. Response surface methodology (RSM) comprises of a group of statistical methods for experimental designs, models building, simultaneously examining the special effects on the factors and to establish optimum conditions. It is vital for examining the impacts of a few independent factors on the system reaction without the requirement for a foreordained connection between the variables and the objective function (Najafi et al., 2011). RSM utilized various regression investigation by utilizing quantitative information obtained from appropriately composed examinations to understand multivalent conditions concurrently (Kiran et al., 2010; Najafi et al., 2011). RSM has been effectively employed to increase BioS yield by reducing the cost of production of BioSs through selecting a balanced proportions of culture medium compositions and culture conditions optimization (de Cássia FS da Silva et al., 2013; Kumar et al., 2015; Najafi et al., 2011).

## 2.7 Recovery and purification of biosurfactant

There is high budget associated with recovery, purification, and downstream processing as it accounts for about 60% - 80% of the total manufacturing costs for several biotechnological products (Sarachat et al., 2010). The production of low-cost BioS is comparatively improbable because of the intricate recovery process. The procedure improvement is in this way coordinated to achieve BioS that can be integrated economically and effortlessly (Satpute et al., 2010a). Different factors such as location (intracellular, extracellular, cell bound), ionic charge (chromatography), and solubility (water/organic solvents) significantly influence the purification procedure and techniques for the desired BioS extraction. The commonly used and cheap methods for the recovery of BioS from fermentation broths are solvent extraction (Salleh et al., 2011), acid precipitation, foam precipitation (Sarachat et al., 2010).

Crude BioSs such as phospholipids, flavolipid and glycolipids have been recovered using the acid precipitation method which is promptly accessible, readily stress-free, and inexpensive. This method has also been used to purify lipopeptide and rhamnolipid BioS from microbial process cultivated in simple or complex growth conditions. This technique ensues the acid hydrolysis of the culture supernatant to pH 2.0 by HCl or $H_2SO_4$, to allow precipitation of proteins, lipid-containing BioS. This process is thus followed by centrifugation and solvent extraction, after standing overnight at refrigerated temperature 4°C (Gudiña et al., 2012). Conversely,

diverse solvents like chloroform, dichloromethane, diethyl ether, methanol, *n*-hexane, and ethyl acetate are also largely used in centrifugation, pellet extraction and purification of BioSs (Satpute et al., 2010a). Filtration is processed for removal of residues from extracted materials which is thus evaporated to dryness using a rotary evaporator (Satpute et al., 2010a). BioS purification by acid precipitation was also reported by a few publications (Bezza and Nkhalambayausi Chirwa, 2015; Gudina et al., 2015a; Gudiña et al., 2012; Najafi et al., 2011).

Also, extraction process with other solvents, for example, butanol, chloroform-methanol, dichloromethane-methanol, hexane-ethyl acetate, pentane, acetic acid, chloroform, isopropanol, methanol, and ether establishes the technique most frequently used in BioS downstream recovery which are used either singly or in combination. BioS hydrophobic ends are dissolvable in a few solvents which help in removal and separation of crude product. Solvent extraction methods have used to successfully purify different types of BioS namely sophorolipids, cellobiolipids, rhamnolipid, liposan, and trehalose lipids synthesized by different microorganisms (Desai and Banat, 1997; Satpute et al., 2010a; Smyth et al., 2010a; Smyth et al., 2010b). Organic solvents are not widely used for BioS recovery because of the huge amount of solvent required and the exclusive price of the solvent which increase the cost of production. In any case, chloroform as an organic dissolvable solvent, is a destructive chloro-organic compound that is damaging to human wellbeing and the ecosystem at large. It is, therefore, necessary to obtain solvents that are cheap and possess low toxicity for extracting BioS that can serve industrial purposes. Additionally, other cheap techniques such as ammonium sulfate precipitation, centrifugation, and adsorption have been reported for their usage (Helmy et al., 2011; Satpute et al., 2010a). Extraction of BioS with conventional procedures such as crystallization, centrifugation, solvent extraction, precipitation, foam fractionation imparts color to BioS when wastewater from the distillery is used as a medium for synthesis (Satpute et al., 2010a).

Often, a multi-step recovery strategy that employs a series of absorption and purification steps for product recovery is more active than a single downstream processing technique to obtain any degree of purified product (Alcantara et al., 2014). During the recovery process, there should be alternate care as contamination risk with undesired product always occur. Though, a great deal of financial input is essential for the recovery and purification processes, as such, cheap techniques as described above is highly proposed (Satpute et al., 2010a).

## 2.8 Characterization of biosurfactant compounds

New emerging techniques for identifying and characterizing of BioS molecules have been introduced in recent decades due to advancement in science and technology. So, a range of techniques such as thin layer chromatography (TLC), high performance-liquid chromatography (HP-LC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), matrix-assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-TOF), and nuclear magnetic resonance (NMR) spectrometry are introduced to categorise and depict the different BioS compounds produced by a diverse microorganisms (Bezza and Nkhalambayausi Chirwa, 2015; Jimoh and Lin, 2019a).

In context, TLC is the most widely utilized, basic, inexpensive strategy for the detection of groups present in an unknown BioS sample. This technique is carried out on a sheet of aluminum foil, glass, or plastic covered with a *thin layer* of adsorbent materials, normally aluminum oxide (alumina), silica gel, or cellulose. This technique gives the first clue on the presence or absence of either protein, lipids, carbohydrate group in either a crude or purified sample (Jimoh and Lin, 2019a; c). High-performance thin layer chromatography (HPTLC) on other hand is the sophisticated or a more precise quantitative version with a similar approach. BioS synthesized from *Bacillus subtilis* B30 was isolated and examined utilizing a totally automated HPTLC framework (Al-Wahaibi et al., 2014). Different researchers have used this technique including HPTLC for the separation, purification of a crude extract as through a well-developed system of different solvents used, relatively dependent or polar or the non-polar group of a BioS compound (Al-Wahaibi et al., 2014; Bezza and Chirwa, 2017; Bezza and Nkhalambayausi Chirwa, 2015; Chakraborty et al., 2015; Ibrahim et al., 2013; Jha et al., 2016).

In a bid to improve the performance, separation, and purification of crude BioS samples, *HPLC* technique has been further used by different researchers to achieve this purpose. HPLC; called *high-pressure liquid chromatography*, is a method in analytical chemistry required for the separation, identification, and quantification of each component present in a mixture. HPLC allows separation in large volume and inexplicably saves time in the downstream process. HPLC technique was initially used for purification during the structural classification of novel cyclic lipopeptide BioS from *Corynebacterium xerosis* NS5 termed as "*coryxin"* (Dalili et al., 2015). Also, other studies have reported the use of HPLC in a similar capacity (Ibrahim et al., 2013; Janek et al., 2010; Patowary et al., 2017). However, in some cases, HPLC and TLC are both used interchangeably to determine the purity of the separated components. For BioS synthesized in harsh

environmental conditions by an effective combination of microorganisms, *ERCPPI-2*, TLC was used to confirm the fractions that displayed high surface activity as separated by HPLC technique (Darvishi et al., 2011).

Mass spectrometry which is coupled with either gas or liquid chromatography, on the other hand, recognizes the chemical bonds and structures of BioS compounds. The process also gives an account of the qualitative and quantitative analysis of the BioS compound which differentiates its usage to HPLC as it gives the molecular mass determination in Daltons (Sharma et al., 2014). Three significant parameters namely ion source, a molecular mass analyzer, and a detector make up the mass spectrometers. So, samples that are volatile are directly introduced into the apparatus, while, on the other hand, non-volatile samples must be dissolved in volatile solvents. Then, the ionization of the sample takes place as it passes through the electromagnetic field. The ionized particles separation occurs based on the charge and mass before finally getting to the detector. The automated signal is then amplified and relayed to a computer system where it is reported as different chromatograms peaks. As a result, assessment of the overall quantity and quality of the compounds and each ion is given (Sharma et al., 2014). In most cases, a hydrophobic portion (water repelling) of the BioS compound is revealed by GC-MS while LC-MS identifies hydrophilic moiety structural composition (water-loving). There were revelation of free fatty acid methyl esters of various chain length with the GC-MS chromatogram generated from purified BioS by crude oil degrading bacteria (Ibrahim et al., 2013).

Electrospray ionization has also been employed for the ionization of several BioS-based compounds before the molecular mass analysis (Monteiro et al., 2007). Reports have shown the usage of tandem mass spectrometry as a great tool in analyzing complex BioS compounds. This allows efficient differentiation among different homologs and isoforms existing within a mixture of compounds. Liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is an exceedingly delicate method for the identification of biomolecule with low concentrations, secondary metabolites, and a crude extract of natural origin. These procedures are presently utilized by different research centers for identifying BioS biomolecules and has been accounted to be valuable in lessening the possibility of inaccurate characterization. The technique is cost-effective, reduces time, and energy required for identifying innovative BioS compounds. There have been previous reports on the utilization of this technique in distinguishing between dissimilar homologs synthesized by *Paenibacillus* strains. A novel lipopeptide of molecular weight (1240Da) synthesized by *Paenibacillus dendritiformis* CN5 was characterized using LC-MS-MS (Bezza and Nkhalambayausi Chirwa, 2015). Similarly, Qian et al. (2011) stated the synthesis of lipopeptide BioS with 8 amino acid constituents

from *Paenibacillus tianmuensis*, using electrospray ionization recorded in the positive-ion mode on a mass spectrometer. In a different context, high-performance liquid chromatography-electrospray ionization mass spectra revealed the chemical constituents a rhamnolipid BioS produced by *Pseudomonas aeruginosa* S6 as $RhaRhaC_{10}C_{12:1}$, $RhaC_{12:1}C_{10}$, $RhaC_{10}C_{10}$ and $RhaC_8C_{10}$ (Yin et al., 2009).

MALDI-TOF joined with mass spectrometry as well allows the documentation of integral BioS compounds due to its ability for soft ionization (Smyth et al., 2010a). Despite the fact that the MALDI-TOF examinations are costly, previous reports demonstrated that it is quick and delicate, giving high-resolution data for the basic characterization of BioS compounds (Sharma et al., 2015; Singh et al., 2014). A cyclic lipopeptide was isolated from the genus *Paenibacillus*, consisting of a cyclic lipopeptide with 13 amino acid residues using a MALDI-TOF analysis (Guo et al., 2012).

Furthermore, scientists have also used NMR to further clarify, identify the molecular structure of the BioS compounds that contain hydrogen and proton component. This technique is equally efficient and requires a more sophisticated approach, which further reveals the chemical identity of an unknown BioS compound. It also detects the composition and purity of the sample as well as its structural composition. This is a spectroscopic technique in quality control and research to observe local magnetic fields around atomic biomolecule (Chakraborty and Das, 2017). The different chemical composition of BioS dissolved initially in most commonly used deuterated solvents have been reported quite recently using the NMR spectroscopic technique (Dalili et al., 2015; Li et al., 2016).

Lastly, Fourier transform infra-red (FT-IR) spectroscopy is a quick and cheap strategy for describing the chemical composition of BioS and to distinguish the various useful groups present. This procedure gives a basic elucidation of the compound of interest and resolves the functional groups of gases, fluids, and solids. During the FT-IR analysis of BioS, the analysis of spectra and peak tasks are of vital steps. The infra-red spectrophotometer is utilized in the range between around 4000 and 400 $cm^{-1}$, in spite of a few contrasts in FT-IR spectra among BioS or experimental conditions (Kong and Yu, 2007). FTIR techniques are comparatively quick, simple to utilize, inexpensive, and IR spectroscopy is non-destructive, i.e. the structural composition of BioS stays exactly as the same. In identifying the different types of BioS, FT-IR has been utilized in studies reported by different researchers and had closely displayed their structural composition using the interpretation of the absorption spectra (Al-Wahaibi et al., 2014; Bezza and Nkhalambayausi Chirwa, 2015; Ibrahim et al., 2013; Patowary et al., 2018).

## 2.9 Applications of biosurfactants

BioS possesses the exceptional quality of decreasing the surface tension between two miscible or immiscible liquids, block hydrogen bonding and increasing hydrophilic/hydrophobic interactions owing to cell surface hydrophobicity (Anyanwu et al., 2011; Darvishi et al., 2011). Attributable to their potential focal points, BioSs are broadly utilized in a few industrial set ups, for example, horticulture, agriculture, chemistry, cosmetics, food production, food processing industries, and pharmaceutics. By virtue of biodegradation properties, substrate specificity, diversity in chemistry and function, and rapid/ controlled inactivation; these biological products are gaining importance in various industries have potential uses in hydrocarbon bioremediation, microbial enhanced oil recovery, nanotechnology (mediated biosynthesis of metallic nanoparticles), pharmaceutical, medicine, commercial laundry detergent, food, textiles, petrochemicals, paper and paint industries, pollution control, nanotechnology (Janek et al., 2010; Lima et al., 2011). A list of wide range of BioSs types and applications is presented in Table 2.2.

**Table 2.2** Biosurfactant types and applications

| Biosurfactants | Applications | References |
|---|---|---|
| Glycolipids | | |
| Rhamnolipids | Pharmaceuticals and cosmetic formulations, environmental remediation of hydrocarbons, heavy metals, pesticides in soil and water, antibacterial, antifungal, biosynthesis of silver nanoparticles, organic nanoparticles synthesis. | (Gustafsson et al., 2009; Vatsa et al., 2010; Xie et al., 2006) |
| Sophorolipid | Agriculture, phytopathogens control, antifungal properties, antimicrobial/germicidal agent, anti-microbial, antitumor, antiviral and immune-modulator, anticancer activity, cosmetic production, hygienic and pharmacological–dermatological products, contact lens and antimicrobial wound dressings. | (Banat et al., 2010; de Oliveira et al., 2015) |
| Mannosylerythritol and cellobiose lipids | Moisturization activity, repair of damaged air, activation of fibroblasts and papilla cells leading to hair growth, antioxidative effects. | (Morita et al., 2013; Morita et al., 2010) |
| Trehalolipids | Remediation, solubilization, and biodegradation of different hydrophobic organic compounds, oil recovery, de-emulsifying | (Franzetti et al., 2010) |

| | | |
|---|---|---|
| | property, antitumor activity, production of cytokines and enhancement of angiogenic activity, antiviral and antimicrobial properties | |
| **Lipopeptide** | | |
| Surfactin | Biocontrol agent, enhancement of biodegradation, Antimicrobial, antiviral, antiadhesive, and insecticidal, heavy oil washing efficiency | (Hwang et al., 2008; Snook et al., 2009) |
| Iturin | Antifungal activity, heavy oil washing efficiency | (Pathak and Keharia, 2014; Xia et al., 2014) |
| Lichenysin | Surface activity, oil displacement and enhancement of oil recovery | (Anuradha S, 2010) |
| Fengycin | Antifungal activity, removal of petroleum hydrocarbons | (Singh and Cameotra, 2013; Zhao et al., 2014) |
| **Fatty acids and Phospholipid** | Efficiency of drug delivery system, good emulsifying property, surface-active wetting agents, pharmaceutical excipients. | (Li et al., 2015; van Hoogevest and Wendel, 2014) |

## 2.10 Recombinant DNA technology for enhanced production of biosurfactant

In recent years, there has been call for genetic enhancement of BioS production. This is due to relatively low production yields which subsequently limits biotechnological applications of these BioSs (Willenbacher et al., 2016). It is imperative to note that, microorganisms are capable of degrading and utilizing different substrates while producing BioSs. However, fairly little is known about the molecular characteristics, cloning and functional characterization of their degradative, and BioS systems (Aliakbari et al., 2014; Piccolo et al., 2011). In the presence of hydrocarbons and other carbon sources, production of BioSs by some microorganisms might be ascribed to the induction of certain genes (Sekhon et al., 2012). For industrial and biotechnological significance, new or enhanced BioS capabilities by modifying the genetic material of microorganisms through recombinant DNA technology is proposed. One of the general aims to decrease cost and promote BioS activity is enabled by the construction of hyper-producing microorganisms (De Almeida et al., 2016). It is important to develop recombinant or mutant microbial strains with enhanced BioS production yields to further reduce production cost or with the capability to produce effective congeners which are a combination of closely associated bio-products (Bachmann et al., 2014). In different applications such as petroleum and biotechnological industry, BioS producers need to be engineered for resistance to extreme process conditions. An option is to discover new genetic modifications from extreme conditions, for example, high salt concentration, high temperatures, and pH (De Almeida et al., 2016). However, modern and biotechnological utilizations of cloned hyper-producing strains have not been appropriately tried, though hyper-BioS producers have been described (Satpute et al., 2010b). The genetic composition of microorganisms is an essential factor influencing the yield of all biotechnological products (Calvo et al., 2009). What's more, the area of cloning, expression, utilization of novel BioS genes/enzymes and improved BioS synthesis is still in its earliest stages. For any bioproduct, for example, BioS, when new monetarily focused and ecologically-friendly procedures are requested, viable up-to-date engineering strategies must be utilized to fulfill this demand (Kuhn et al., 2010). Hence, it is of extraordinary importance to examine the enhanced roles played by these BioSs biosynthetic genes and enzymes. Khanna et al. (2009) proposed recombinant *Escherichia coli* pSKA clones containing BioS gene *srfA* demonstrated higher esterase and BioS activity with olive oil when contrasted to parent *Bacillus* sp. SK320 strain. In another study, there was overexpression of BioS genes in **BioSa**, **BioSb** and **BioSc** displaying a twofold increase in BioS activity than the parent strain. Also, enhanced esterase production was

conferred on the recombinant cells as compared to *Bacillus substilis* SK320 (Sekhon et al., 2011). In another case, a mutant defective *Acinetobacter calcoaceticus* A2 produced a higher level of biodispersan. The reduction of secreted proteins reduced difficulties related to purification, recovery, and application of biodispersan. Moreover, recombinant strains gave rise to improved product characteristics (Calvo et al., 2009). Additionally, Anburajan et al. (2015) described the heterologous expression of surfactin synthetase genes from *Bacillus licheniformis* NIOT-06. The engineered strain has potential application since it synthesizes BioS at high rates and can maintain a strategic distance from the complex recovery process related to the regular bioprocess (Anburajan et al., 2015). In another report, phosphopantetheinyl transferases are responsible for the activation of polyketide synthases, non-ribosomal peptide synthases, and fatty acid synthases involved in the synthesis of fatty acids, and antibiotics (Bunet et al., 2014). Jung et al. (2012) reported the surfactin synthesis was effectively expanded both separately, and consolidated expression of ComX and PhrC. The overexpression of both extracellular peptides, ComX, and PhrC, is a vital factor for the enhancement of lipopeptide synthesis. The potential utilization and improved application of these hyper-producing strains notwithstanding novel inexpensive bioprocesses minimize difficulties and offer tremendous opportunities for making enhanced BioS production a success story.

## 2.11 Conclusion

Nature has provided tremendous possibilities for the isolation of novel bioemulsifiers or BioSs-producing microbial communities and products that are useful in a various industrial application such as petroleum industry, detergents, pharmaceutical companies, agriculture, and personal health care products. Since the discovery of BioSs, the production cost and selecting suitable inexpensive raw material are the important factors and concern for manufacturing industries. However, with the recent discoveries in this field and increased global awareness among consumers for these biologically synthesized products, it appears inevitable that in years to come chemical emulsifiers will be replaced completely by high-quality BioSs produced by microorganisms in many industrial applications. Owing to their environmentally-friendly nature and increased global awareness among the populace for products obtained from nature, the market today now has a niche for BioSs. Manufacturing industries are staking money on BioSs due to their potential and prospective characteristics and properties. With the use of mutants and hyper-active microorganisms with high producing

capacities and inexpensive renewable substrates as raw material, BioSs production has been ameliorated at the industrial level.

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## Conflict of interest

The authors wish to declare that there is no conflict of interest.

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# Chapter 3

## Production and characterization of lipopeptide biosurfactant producing *Paenibacillus* sp. D9 and its biodegradation of diesel fuel

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ORIGINAL PAPER

### Production and characterization of lipopeptide biosurfactant producing *Paenibacillus* sp. D9 and its biodegradation of diesel fuel

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**Abstract**
The current research aimed at analyzing the biodegradation efficiency of a potent biosurfactant producing *Paenibacillus* sp. D9, along with the characterization of the surface-active compound produced during diesel fuel biodegradation. The biosurfactant production by *Paenibacillus* sp. D9 was evaluated using diesel fuel as culture medium, subsequently analyzed for its structural characteristics using different methods and thus determining the biodegradation utilization. This strain showed wide cell surface hydrophobicity against varieties of hydrocarbon substrate tested. *Paenibacillus* sp. D9 displayed higher hydrophobicity to the long-chain hydrocarbons mixtures tested such as 71.5% diesel fuel, 70.0% engine oil and 76.0% *n*-paraffin. The optimum condition for biosurfactant synthesis was obtained in a medium containing 10% (v/v) diesel fuel with a production yield of 4.7 g/L. The resultant biosurfactant reduced surface tension from 71.4 to 30.1 mN/m against carbon source utilized. The critical micelle concentration value of the biosurfactant was 200 mg/L with emulsification efficiencies against a wide range of hydrophobic pollutants. With different physiochemical and analytical methods, the study demonstrated that the genus *Paenibacillus* produced a low molecular weight lipopeptide biosurfactant. Its emulsifying ability further supports its potential use in environmental and biotechnological applications.

**Keywords** Biotechnological application · Characterization · Emulsification · Hydrocarbon · Surface tension

### Introduction

Different forms of ecological and environmental pollution due to the improper or inherent usage of hydrocarbons (such as aliphatic and aromatic, including polycyclic aromatic hydrocarbons), heavy metals, sulfur, hydrophobic pollutants, solvents, and chemicals affect fauna and flora significantly and continuously (Ismail et al. 2013; Al Disi et al. 2017). Exposure to the afore-mentioned pollutants contribute indirectly to significant economic losses in developing countries and pose a threat to the environment and living organisms including human beings, plants, and other organisms (Ismail et al. 2013; Durval et al. 2018). Conventional remediation such as mechanical, physical, and chemical treatments has been used to contain these contaminations and polluted extreme environments (Ahmad et al. 2014; Al Disi et al. 2017). However, bioremediation has attracted increasing interest in recent years because it is cost-efficient, effective, and environmentally friendly in contrast to physicochemical treatments (Al Disi et al. 2017).

Toxic compounds, inclusive of xenobiotics and pollutants such as crude oil, diesel fuel, engine oil, motor oil, aromatic hydrocarbons, and other hydrocarbon mixtures are degraded to a certain extent through natural processes initiated by indigenous microorganisms (Bezza and Chirwa 2017; Durval et al. 2018). Petroleum-derived diesel is the environmental and ecological pollutant composed of about 25% aromatic hydrocarbons (alkylbenzenes and naphthalenes) with 75% saturated hydrocarbons (primarily paraffin including *n*-, iso-, and cycloparaffins) (Ahmad et al. 2014; Dahalan et al. 2014). Advances in biotechnology have allowed the use of pollutant-degrading microbes effectively to remove

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# Production and characterization of lipopeptide biosurfactant producing *Paenibacillus* sp. D9 and its biodegradation of diesel fuel

**Running Title: Lipopeptide Biosurfactant producing *Paenibacillus* sp. D9 and its Biodegradation of Diesel**

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## Abstract

The current research aimed at analyzing the biodegradation efficiency of a potent biosurfactant producing *Paenibacillus* sp. D9, along with the characterization of the surface-active compound produced during diesel fuel biodegradation. The biosurfactant production by *Paenibacillus* sp. D9 was evaluated using diesel fuel as a culture medium, subsequently analyzed for its structural characteristics using different methods and determining the biodegradation utilization. This strain showed wide cell surface hydrophobicity against varieties of hydrocarbon substrate tested. *Paenibacillus* sp. D9 displayed higher hydrophobicity to the long chain hydrocarbons mixtures tested such as 71.50% diesel fuel, 70.0% engine oil and 76.0% *n*-paraffin. The optimum condition for biosurfactant synthesis was obtained in a medium containing 10% (v/v) diesel fuel with a production yield of 4.7 g/L. The resultant biosurfactant reduced surface tension from 71.4 mN/m to 30.1 mN/m against carbon source utilized. The critical micelle concentration value of the biosurfactant was 200 mg/L with emulsification efficiencies against a wide range of hydrophobic pollutants. With different physiochemical and analytical methods, the study demonstrated that the genus *Paenibacillus* produced a low molecular weight lipopeptide biosurfactant. Its emulsifying ability further supports its potential use in environmental and biotechnological applications.

**Keywords**

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## 3.1 Introduction

Different forms of ecological and environmental pollution, due to the improper or inherent usage of hydrocarbons (such as aliphatic and aromatic, including polycyclic aromatic hydrocarbons), heavy metals, sulfur, hydrophobic pollutants, solvents, and chemicals affect fauna and flora significantly and continuously (Al Disi et al., 2017; Ismail et al., 2013). Exposure to the afore-mentioned pollutants contribute indirectly to significant economic losses in developing countries, pose a threat to the environment and living organisms including human beings, plants, and other organisms (Durval et al., 2018; Ismail et al., 2013). Conventional remediation such as mechanical, physical, and chemical treatments have been used to contain these contaminations and polluted extreme environments (Ahmad et al., 2014; Al Disi et al., 2017). However, bioremediation has attracted increasing interest in recent years because it is cost-efficient, effective, and environmentally-friendly in contrast to physicochemical treatments (Al Disi et al., 2017).

Toxic compounds, inclusive of xenobiotics and pollutants such as crude oil, diesel fuel, engine oil, motor oil, aromatic hydrocarbons, and other hydrocarbon mixtures are degraded to a certain extent through natural processes initiated by indigenous microorganisms (Bezza and Chirwa, 2017; Durval et al., 2018). Petroleum-derived diesel, one of the main environmental and ecological pollutants is composed of about 25% aromatic hydrocarbons (alkylbenzenes and naphthalene) with 75% saturated hydrocarbons (primarily paraffin including *n*-, iso-, and cycloparaffins) (Ahmad et al., 2014; Dahalan et al., 2014). Advances in biotechnology have allowed the use of pollutant-degrading microbes effectively to remove the identified contaminants and pollutants under measured conditions to yield biomolecules or bio-products such as biosurfactants (BioSs) (Datta et al., 2018). As such, the growing environmental awareness, sustainability, and concern, surfactants obtained from biological-based resources are of increased demand in the green ecological market.

BioSs are compounds of surface active origin produced by specific groups of microorganisms that utilize diverse substrates like oils, hydrocarbons, hydrophobic mixtures, carbohydrates, fats, dairy products, vegetable frying oil, and heavy metals from polluted environments (Parthipan et al., 2017; Reddy et al., 2018). BioSs are classified into two different groups: which are low molecular-weight compounds, which effectively reduce interfacial and surface tension (ST); and high molecular-weight compounds frequently mentioned as bioemulsifiers effective as emulsifying agents. The first group which is of interest display an extensive variation of chemical structures, including phospholipids, glycolipids (rhamnolipids, sophorolipids, trehalose

lipids, mannosylerythritol, cellobiose lipids), lipopeptides, fatty acids, or neutral lipids lipopeptides, polysaccharide-complexes, flavolipid, lipids, and polymeric surfactants (Datta et al., 2018).

The features of BioSs over chemically produced counterparts which includes, functional diversity, reliability at extreme ecological conditions, low toxicity, better surface activity, biodegradability, bioremediation, substrate specificity, environmental compatibility, and biocompatibility have expanded its potential use in different applications (Al-Wahaibi et al., 2014; Bezza and Chirwa, 2017; Reddy et al., 2018; Wang et al., 2014). These applications include hydrocarbon bioremediation, microbially enhanced oil recovery, nanotechnology (mediated biosynthesis of metallic nanoparticles), pharmaceutical, medicine, commercial laundry detergent, food, textiles, petrochemicals, and pollution control (Deng et al., 2016; Guo et al., 2012; Wang et al., 2014). The degradation and bio-utilization of hydrocarbons by microorganisms is enhanced by the production of BioS. In fact, hydrocarbon degraders have the potential to produce BioSs *in situ* thus stimulating their growth and survival in environments dominated with hydrophobic compounds (Mesbaiah et al., 2016).

BioSs of different kinds have been synthesized from microorganisms belonging to diverse genera, such as *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, and *Achromobacter* (Deng et al., 2016; Durval et al., 2018; Parthipan et al., 2017; Patowary et al., 2017; Pirog et al., 2015). However, few researchers have identified genus *Paenibacillus* as having the potential of producing BioS, for its wider usage in biodegradation purposes and bioremediation (Bezza and Chirwa, 2017; Govarthanan et al., 2016; Gudina et al., 2015; Mesbaiah et al., 2016; Najafi et al., 2011; Reddy et al., 2018). The discovery of new environmentally-friendly BioS and finding the optimum conditions for improved production yield is pivotal especially for biotechnological applications and effective bioremediation of soils and groundwater polluted with hydrocarbons.

In this research paper, the functions, structures, and characterization of novel lipopeptide BioS produced by hydrocarbon-degrading bacterium *Paenibacillus* sp. D9 was reported. This study will contribute to valuable information on this species of *Paenibacillus* with novel BioS properties and attributes.

## 3.2 Materials and methods

### *3.2.1 Chemicals and reagents*

All reagents, hydrocarbons, chemicals, and media used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA). Hydrocarbon substrates were at least 99% pure, and of analytical grade.

#### 3.2.1.1 Strain growth, media, and culture conditions

*Paenibacillus* sp. D9 GC368737 was identified in a previous study (Ganesh and Lin, 2009) and the whole genome sequence has been deposited at DDBJ/EMBL/GenBank under the accession number JZEJ00000000. The microorganism culture for future use was stored at -80°C in nutrient broth (NB) medium supplemented with 40% (v/v) of glycerol. The composition of the NB medium was (g/L): sodium chloride 5.0; yeast extract 2.0; beef extract 1.0; peptone 5.0, respectively. The culture stocks were streaked on nutrient agar plates, incubated at 30°C for 24 h, and subsequently stored at 4°C for no longer than 3 weeks. For the degradation and BioS production medium, a Bushnell Haas medium (BH); pH 7.4 with the following composition in g/L ($NH_4$) $NO_3$ 1.00, $FeCl_3$ 0.05, $K_2HPO_4$ 1.00, $KH_2PO_4$ 1.00, $MgSO_4$ 0.20, $CaCl_2$ 0.02 were used. The pH was adjusted when required to 7.0 using 1M HCl or NaOH. The media were sterilized at 121°C for 15 min using an autoclave (HL-340 Vertical Type Steam Sterilizer). The *Paenibacillus* sp. D9 strain was grown at 30°C in NB medium for 24 h, followed by centrifugation (13,500 × *g*, for 20 min at 4ºC). The inoculum obtained was washed twice in phosphate buffer saline (PBS) (1×) and further suspended in BH medium until $OD_{600}$ was equivalent to 1.00 (Ganesh and Lin, 2009).

### *3.2.2 Microbial adhesion to different hydrocarbons*

The bacterium cell surface hydrophobicity (CSH) was evaluated by microbial adhesion to hydrocarbon assay. *Paenibacillus* sp. D9 cells were harvested from 24 h grown culture by centrifugation at 13,500 × *g* at 4ºC for 10 mins and washed twice with phosphate urea magnesium sulfate buffer composition (g/L, pH 7.0) containing $K_2HPO_4$ 19.7, $KH_2PO_4$ 7.26, $MgSO_4$. $7H_2O$ 0.2, and Urea 1.8). The cells were suspended in the buffer to an optical density of 1.0 (A0) at 600 nm wavelength. Five mL inoculum was added to different hydrocarbons (500 µL) and the different test-tubes subsequently vortexed for 2-5 min. The final growth of aqueous phase was measured (A1) inclusive of initial optical density (A0) after 1 h (Dahalan et al., 2014). The spectrophotometer was blanked with the medium containing the different substrates during measurement of $OD_{600}$ value.

The CSH was calculated using the formula [1-(A0-A1)/A0 × 100%]

### *3.2.3 Hydrocarbon utilization by strain Paenibacillus* sp. D9

The capability of *Paenibacillus* sp. D9 to grow on various aliphatic hydrocarbons, aromatic hydrocarbons, hydrocarbon mixtures, and intermediary metabolites of polycyclic aromatic hydrocarbon (PAH) degradation was assessed. The different substrates were added to the BH medium at final concentration of 2% (v/v) or 2% (m/v). Uninoculated control flasks with the same quantity of different substrates were incubated in parallel to monitor abiotic losses (Mesbaiah et al., 2016). The production medium allowed for clear separation of hydrophobic layer containing the substrates and hydrophilic layer containing the bacterial cells. Bacterial cell growth was subsequently monitored by measuring the growth density ($OD_{600}$) at two-day intervals using an ultra-violet (UV) spectrophotometer for 14-day period. The spectrophotometer was blanked with the medium containing the different substrates during the measurement of $OD_{600}$ value.

### *3.2.4 Diesel fuel degradation and gas chromatography-flame ionization detector analysis*

One hundred millilitres of BH medium containing different concentrations of diesel fuel, viz 1%, 2%, 5%, and 10% (v/v), in 250 mL Erlenmeyer flask (pH 7.0), were inoculated with 1 mL of inoculum ($OD_{600}$ = 1.00). The flasks were incubated aerobically at 30°C on an orbital shaker (MRC polychem supplies, China) operated at 30°C, 150 rpm for 21 days to determine the biodegradation efficiency of *Paenibacillus* sp. D9. The spectrophotometer was blanked with the medium containing the diesel fuel during the measurement of $OD_{600}$ value. Uninoculated control flasks with the same amount of diesel fuel were included and incubated in parallel to monitor abiotic losses. The samples were measured at three-day intervals for 21 days to monitor the bacterial growth at $OD_{600}$. The remaining diesel fuel was extracted at the end of the experiment by shaking vigorously with 20 mL of *n*-hexane solvent. The diesel fuel degraded was determined using gas chromatography (GC) equipped with a flame ionization detector (FID). In brief, hexane extracts (1.0 µL) were analyzed with the Shimadzu AOC-201 gas chromatograph (GC-2010) equipped with 30 m HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 µm). The injector and detector temperatures were maintained at 250°C and 350°C, respectively. The column was automated to hold for 2 min at an initial temperature of 70°C; then ramped at 10°C $min^{-1}$ to 320°C and subsequently held for 10 min. Nitrogen was used as the carrier gas. Diesel fuel biodegradation was expressed as the percentage of diesel fuel degraded relative to the residual diesel fuel fractions in the control samples (external standard method). Thus, the biodegradation efficiency (BE), centered on the reduction in the total hydrocarbon concentration, was determined as described,

BE (%) = 100 - (As x 100/Abc)

Where Abc = total peak area identified in the abiotic control,

As = total peak area in each appropriate sample.

### *3.2.5 Biosurfactant production, recovery, and purification*

To study the BioS production and activity, the bacterium *Paenibacillus* sp. D9 cultivation was performed as per the diesel fuel degradation experiments above, for an incubation period of 15 days. Uninoculated BH medium was supplemented with different concentrations of diesel fuel and utilized as control samples. The culture broth was checked at three-day intervals for the analysis of bacterial growth, surface tension (ST), and BioS production yield. The growth of *Paenibacillus* sp. D9 bacterium in BH media was determined by measuring the increase or decrease in OD at 600 nm. The production medium was allowed for clear separation of hydrophobic layer containing the substrates and hydrophilic layer containing the bacterial cells. The spectrophotometer was blanked with the medium containing the different substrates during the measurement of $OD_{600}$ value. The samples were centrifuged (13,500 × *g*, for 20 min at 4°C), for BioS production yield and ST determination as defined below. The residual oil was extracted through double extraction using an approximate 40 mL volume of *n*-hexane At the end of the production, the crude BioS was obtained by centrifugation and acid precipitation (Al-Bahry et al., 2013). Acidification of the cell-free supernatant to pH 2 was carried out with 6 M HCl, consequently incubated at 4°C overnight to stimulate the precipitation of BioS. Thereafter, the precipitated crude BioS were collected by centrifugation (10,000 × g, 20 min, and 4°C). The BioS produced was liquefied in a marginal quantity of demineralized water with the pH adjusted to 7 using 1 M NaOH. The BioS solutions were lyophilized, and the products were weighed and stored at –20°C until further usage. To determine and confirm the surface activity of the BioS as either extracellular (cell-free supernatant) or intracellular (cell bound), cell-bound BioS was harvested by centrifugation (10,000 *x g*, 10 min, 4°C), washed twice and resuspended in 100 mL of PBS. The cell-free suspension was incubated at room temperature overnight for the release of cell-bound BioS and filtered through a 0.22 μm pore size sterile filter (GVS, USA). Dialysis of filtered cell-bound BioS was carried out against distilled water at 4°C in a dialysis membrane (6,000-8,000 Dalton) Sigma-Aldrich (St Louis, MO, USA) and freeze-dried for subsequent analysis. The product collected through the above-listed methods was reflected as partially purified BioS. In this case, the partially purified BioS was subsequently confirmed for surface activity before its further usage.

### *3.2.6 Surface active properties of biosurfactant*

#### 3.2.6.1 Surface tension

ST determination of supernatant and cell-bound supernatants were achieved at 25°C according to the Ring method (Burgos-Diaz et al., 2011). Approximately 40 mL cell-free supernatant and cell-bound supernatants was placed in the measuring cylinder and ST readings were actualized. An average of triplicate samples was determined to improve the precision of the ST measurements. Sterile BH medium added with diesel fuel at a final concentration of 1%, 2%, 5%, 10% (v/v) were used as a control to ascertain the effectiveness of the BioS synthesized.

#### 3.2.6.2 Emulsifying activity determination

The emulsification activity index (E24) was determined according to Burgos-Diaz et al. (2011). Cell-free supernatants (2 mL) were added to the same volume of diesel fuel in test tubes. The tubes were vortexed with a vortex machine (V-220, Germany) for 2-5 min and left standing at 25°C for 24 h.

The E24 index was estimated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). The E24 indexes were performed in triplicate. To detect the capability of the BioS molecules to form stable emulsions, diesel fuel was substituted with different hydrocarbons and hydrophobic mixtures.

#### 3.2.6.3 Drop collapse assay

Drop collapse assay was carried out to detect the surface-active compound. Diesel fuel (10 µL) was added to 96 well microtiter plates. The plates were equilibrated for 1 h at 30ºC and 5-10 µL of BioS containing solution was added. The solution that made the oil drop collapse after 1 min was taken as a positive result and the solution that remained as flat was scored as negative when compared with sterile demineralized water (control) (Thavasi et al., 2013).

#### 3.2.6.4 Oil displacement assay

This assay measures the diameter of the clear zone that occurs after dipping a BioS-induced solution on oil-water interphase. The oil displacement test was analyzed by adding 50 mL of distilled water to a Petri dish of diameter 15 cm. Diesel fuel (20 µL) was dropped onto the surface of the water, followed by the addition of 10 µL of BioS containing solution. The diameter of the clear halo zone visualized was measured after 1 min. The diameter allows validation on ST reduction effectiveness of the BioS produced (Thavasi et al., 2013).

#### 3.2.6.5 Critical micelle concentration

Critical micelle concentration (CMC) was analyzed by measuring the ST sequences of a series of dilutions of BioS concentrations using Tris-HCl buffer solution, pH 8 (Sharma et al., 2015). CMC is explained as the lowest BioS concentration to achieve maximum ST and all additional BioS added to the biological system goes into micelles formation. A stock solution of the crude BioS (1 g/L) was prepared and various dilutions made to obtain a range of the concentrations from 10 to 1000 mg/L. The CMC was investigated by plotting the ST as a function against the BioS concentration logarithmic scale. The common experimental procedure is to determine the intersection point of two straight lines that best through the CMC (pre- and post-) data and BS concentration. ST of the different concentrations was measured in triplicate against water as a blank.

### *3.2.7 Structural characterization*

#### 3.2.7.1 Purification of biosurfactant

The crude BioS was partially purified initially according to the procedures defined above. The sample was then liquefied in methanol, mixed with silica gel (230 – 400 mesh) and subsequently oven-dried at 50°C. The silica gel was further mixed with chloroform and then loaded onto a chromatography column (50 cm × 2.8 cm). A mixture of ethyl acetate/chloroform in different proportions (100% to 0% with 10% interval), was used in the sequential washing of the loaded column at a flow rate of 0.5 mL/min. A UV spectrophotometer with a range of 200–800 nm was used to monitor the absorption wavelength of the mixtures. The eluents (20 mL) were collected and the fractions showing oil-displacement activity were thoroughly mixed, followed by evaporation at 80$^{o}$C to acquire purified sample (Deng et al., 2016). The purified BioS was confirmed subsequently for surface activity and properties before its further usage.

#### 3.2.7.2 Compound analysis of purified biosurfactant

The protein content of the purified BioS was estimated by using the total protein kit (Micro-Lowry, Onishi & Barr Modification) from Sigma-Aldrich (St Louis, MO, USA). The phenol-sulphuric acid method was used to determine the total carbohydrate content. Gravimetric method was used to estimate the total lipid content. The purified BioS (1 g) was extracted with chloroform: methanol in different proportions (1:1 and 1:2, v/v), and the lipid content estimated by gravimetric analysis (Ismail et al., 2013). The fatty acids content of the BioS hydrophobic portion was determined by converting and derivatizing it to methyl esters. The methyl esters of fatty acids were suspended in *n*-hexane and analyzed by gas chromatography-mass spectroscopy. The peaks

and spectra generated were analyzed for its molecular weight characterization from obtainable library data (NIST MS library search).

#### 3.2.7.3 Thin layer chromatography analysis

BioS obtained was dissolved in chloroform: methanol (1:1 v/v), and a 50 µL sample was introduced on a thin layer chromatography (TLC) silica plate (Sil 60 F254, 0.2 mm, Macherey-Nagel, GmbH & Co, Germany). A solvent system containing methanol: chloroform: acetic acid (15:65:2 v/v/v) was used in developing the samples. BioS constituents were then spotted after spewing the TLC plate with different visualizing stains including anthrone, ninhydrin, and rhodamine spray reagents. The anthrone reagent (1 g in 5 mL of $H_2SO_4$ mixed with 95% ethanol) was used to detect the incidence of yellow spots which are indicative of carbohydrate. Amino acids present in the BioS solution were detected using ninhydrin reagent 0.05 % w/v (methanol and water 1:1 v/v) heated at 100°C for 10 min, while the presence of lipids was detected using rhodamine reagent (0.25 w/v in 70% ethanol). The bands conforming to BioS constituents were visualized in ultraviolet light.

#### 3.2.7.4 Liquid chromatography and mass spectroscopy analysis

The purified BioS sample was dissolved in methanol-water to obtain 1 mg/mL solution. An ultra-performance liquid chromatography system equipped with a photo diode detector, gradient pump, and auto sampler was utilized in the current study. A C18 column (2.1 µm× 1.7 µm× 100 mm) was used for separation at an oven temperature of 40°C. A multistep linear gradient composed of eluent A (water + 0.1% trifluoracetic acid), and eluent B (acetonitrile + 0.1% trifluoracetic acid) were applied with the temperature, continued at 10°C. The sample solution (10 µL) was injected, and a linear gradient (from 0–13 min) from the mixture A: B (70:30, v/v) to A: B (0: 100 v/v) was applied. A plateau of 100% eluent B from 13 min to 15 min was initialized, then completed with 70% eluent A for 15 min to 16 min. The mobile phase flow rate was 0.3 mL/min. The liquid chromatography system was joined with a Water's mass-spectrometer and an electroscopic interface. The electrospray ionization source was set in positive and negative ionization mode. Helium was used as a collision gas while nitrogen gas was used as a nebulizer.

#### 3.2.7.5 Gas chromatography and mass spectroscopy analysis

The BioS was hydrolyzed with 6 M HCl at 100°C for 24 h. The fatty acids were extracted from the hydrolysate using diethyl ether, followed by vacuum evaporation. The fatty acids derivative was prepared by mixing 5% HCl-methanol (1 mL) with the BioS (10 mg). The reaction was quenched with water (1 mL), thereafter the

methyl ester derivatives were extracted with *n*-hexane. One µL was injected into a Shimadzu gas chromatograph model QP2010 SE equipped with a capillary GC column Rtx-5MS (0.25 µm × 30.0 m × 0.25mm) and a Shimadzu Mass Selective Detector model set to scan from 45 m/z to 600 m/z at a scan rate of 1.2 scans per second. The capillary column used was a column Rtx-5MS (0.25 µm × 30.0 m × 0.25 mm) (Bellefonte, PA 16823, USA). The oven temperature was set from 130ºC to 230ºC at 2ºC/min. The temperature of the injector port was 230ºC and the detector transfer line temperature was 240ºC. The carrier gas was helium at a flow rate of 1 mL/min and a split ratio of 50:1.

#### 3.2.7.6 Fourier transform infrared spectroscopy

For identifying types of chemical bonds (functional groups), Fourier transform infrared spectroscopy (FTIR) was used to elucidate the components of the mixture. One mg of purified lyophilized BioS was grounded with 100 mg potassium bromide salt and pressed for 1 min to obtain translucent pellets. The pellets were analyzed in an FTIR (PerkinElmer Spectrum 100 Series, PerkinElmer, Shelton, CT, USA), in the range of 450 – 4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$. All data were corrected for the background spectra.

### *3.2.8 Statistical analysis*

All the experimental data analyzed were expressed in terms of arithmetic averages obtained from at least three independent replicates, with standard deviation **(±)**.

## 3.3 Results and discussion

### *3.3.1 Cell surface hydrophobicity*

CSH of *Paenibacillus* sp. D9 against varieties of hydrocarbon substrate and mixtures is shown in Table 3.1. There was an increasing CSH as the carbon length of liquid aliphatic hydrocarbons increased (63.20%, 64.90% and 65.50% of *n*-dodecane, *n*-tetradecane, and *n*-hexadecane respectively) (Table 3.1). *Paenibacillus* sp. D9 further displayed higher hydrophobicity to the long chain hydrocarbons mixtures tested (motor oil - 62.70%, diesel fuel - 71.50% and engine oil - 70.00%) with the highest CSH toward *n*-paraffin (76.00%). 1-Nonene, a cyclic unsaturated aliphatic hydrocarbon produced an average hydrophobicity of 50.70%. A relatively fair hydrophobicity to *n*-hexane, a volatile aliphatic hydrocarbon of 49.10% was also observed. In contrast, there was a weak hydrophobicity toward liquid aromatic hydrocarbons such as toluene and phenol with the

hydrophobicity of 42.20% and 41.60%, respectively. Positive CSH is proportional to a solid indication for the determination and production of BioS (Thavasi et al., 2013). The dual cell-surface hydrophilicity, ranging from hydrophilic (remained in the aqueous phase with no interaction with the organic solvent, despite multiple contacts) to very hydrophobic phase (fractionated with the initial addition of solvent) was established for *Paenibacillus* sp. D9.

**Table 3.1** The cell surface hydrophobicity of *Paenibacillus* sp. D9 grown on various hydrocarbon substrates

| **Hydrocarbons** | **Hydrophobicity** | **Hydrocarbons** | **Hydrophobicity** |
|---|---|---|---|
| *n*-Paraffin | 76.00% ± 0.08 | 1-Nonene | 50.70% ± 0.02 |
| *n*-Dodecane | 63.20% ± 0.01 | Phenol | 41.60% ± 0.01 |
| *n*-Hexane | 49.10% ± 0.24 | Motor oil | 62.70% ± 0.01 |
| *n*-Hexadecane | 65.50% ± 0.01 | Engine oil | 70.00% ± 0.01 |
| *n*-Tetradecane | 64.90% ± 0.01 | Toluene | 42.20% ± 0.00 |
| Diesel fuel | 71.50% ± 0.07 | | |

All data points are means ± standard deviation (S.D.) of three independent experiments

Thus, *Paenibacillus* sp. D9 showed heterogeneity in the hydrophobic surface characteristics that affect the ability of cells to use various hydrocarbon substrates. The result above were supported by BioS-producing strain *Dietzia maris* As-13-3 with high CSH towards *n*-hexadecane, *n*-tetradecane, and *n*-paraffin and hydrophilicity to *n*-dodecane and pristine (Wang et al., 2014). In another report, *Acinetobacter* sp. DRY12 showed high CSH towards *n*-hexadecane (Dahalan et al., 2014). Cell adherence with hydrophobic compounds like diesel fuel, *n*-hexadecane, *n*-dodecane, *n*-tetradecane, engine oil, and several hydrocarbon mixtures are a well-thought-out method to screen bacteria for its production of BioS. Microorganisms produce surface active compounds by cell adherence to the oil droplets thus enabling effective solubilization and degradation (Thavasi et al., 2013).

The feat of biological remediation is reliant on the intrinsic biodegradability of the contaminants, availability to pollutant-degrading microbes and microbiological optimization. Considering this, the ability of *Paenibacillus* sp. D9 to utilize several pollutants and contaminants such as hydrocarbons (short and long chain), hydrocarbon mixture, chemicals, and PAH intermediates was investigated. The aim of the experiment was to

ascertain the carbon source preference of *Paenibacillus* sp. D9 to several hydrocarbon constituents and relatively determine the correlation between CSH and substrate specificity. Table S1 showed the ability of *Paenibacillus* sp. D9 to utilize some hydrocarbons as source carbon and energy source inclusive of short and middle chain aliphatic hydrocarbons (from C8 to C20). *Paenibacillus* sp. D9 used *n*-dodecane ($OD_{600}$ max = 1.08), *n*-hexadecane ($OD_{600}$ max = 1.56), *n*-tetradecane ($OD_{600}$ max = 0.65), 1-nonene ($OD_{600}$ max = 1.01), tetracosane ($OD_{600}$ max = 0.99) as carbon and energy sources at the end of the incubation. In addition, the organism grew on hydrocarbon mixtures such as diesel fuel ($OD_{600}$ max = 1.86), *n*-paraffin ($OD_{600}$ max = 0.51), motor oil ($OD_{600}$ max = 0.62), and engine oil ($OD_{600}$ max = 1.20). However, *Paenibacillus* sp. D9 was unable to utilize *n*-hexane ($OD_{600}$ max = 0.003) and cyclohexane ($OD_{600}$ max = 0.10) which are representative of carbon chain C6. Aromatic compounds are difficult to degrade in oil-polluted environments. Taking this into consideration, there was the utilization to certain degree of toluene ($OD_{600}$ max = 0.32) despite its weak hydrophobicity and *Paenibacillus* sp. D9 grew poorly in phenol ($OD_{600}$ max = 0.03). Owing to its vast potential and diversity, *Paenibacillus* sp. D9 showed growth and/or utilization on PAH biodegradation metabolites (benzoic acid, salicylic acid, phthalic acid) as a sole source of carbon and energy. This further explains its suitability and capability in biodegradation, bioremediation, and environmental application.

Previous report showed *Paenibacillus dendritiformis* CN5 could utilize several hydrophobic mixtures as well as polycyclic aromatic hydrocarbons as carbon and energy source (Bezza and Nkhalambayausi Chirwa, 2015). *Paenibacillus* sp. #510, and *Paenibacillus* sp. 1C were stated to have the capability to use-up straight chain hydrocarbons and hydrocarbon mixtures (Gudina et al., 2015; Mesbaiah et al., 2016).

### *3.3.2 Diesel degradation analysis*

The optimum diesel fuel concentration for *Paenibacillus* sp. D9 was assessed in this research. Figures 3.1 and 3.2 indicated that the optimum concentration for growth was 2% (v/v) with an increase in optical density and cell growth in the containing medium. There was a significant change in color and turbidity of the medium after 3 days of incubation which was due to the diesel dispersion in the basal medium coupled with the disintegration of hydrocarbons present. However, the increase in viscosity of the medium in proportion with the density also indicates biodegradation of diesel fuel. At a much higher concentration of 5% (v/v) and 10% (v/v), a reduction in the growth of the *Paenibacillus* sp. D9 was observed evident of an increasing diesel concentration. Although the bacterial growth started to decline, *Paenibacillus* sp. D9 bacterium was still able to grow on 5%, and 10% (v/v) diesel fuel despite a decrease in the optimal growth. The bacterial growth, in

this case, dropped abruptly due to an increase in the concentration of hydrophobic mixtures to the basal medium. Diesel fuel as a carbon source is fundamental; however, it can inflict pressure on microorganisms at high concentrations. Solvent effect destroys bacterial cells by altering the nutrient transfer and literally damages the biological membrane leading to an outflow of macromolecules, and later cell death (Shukor et al., 2009).

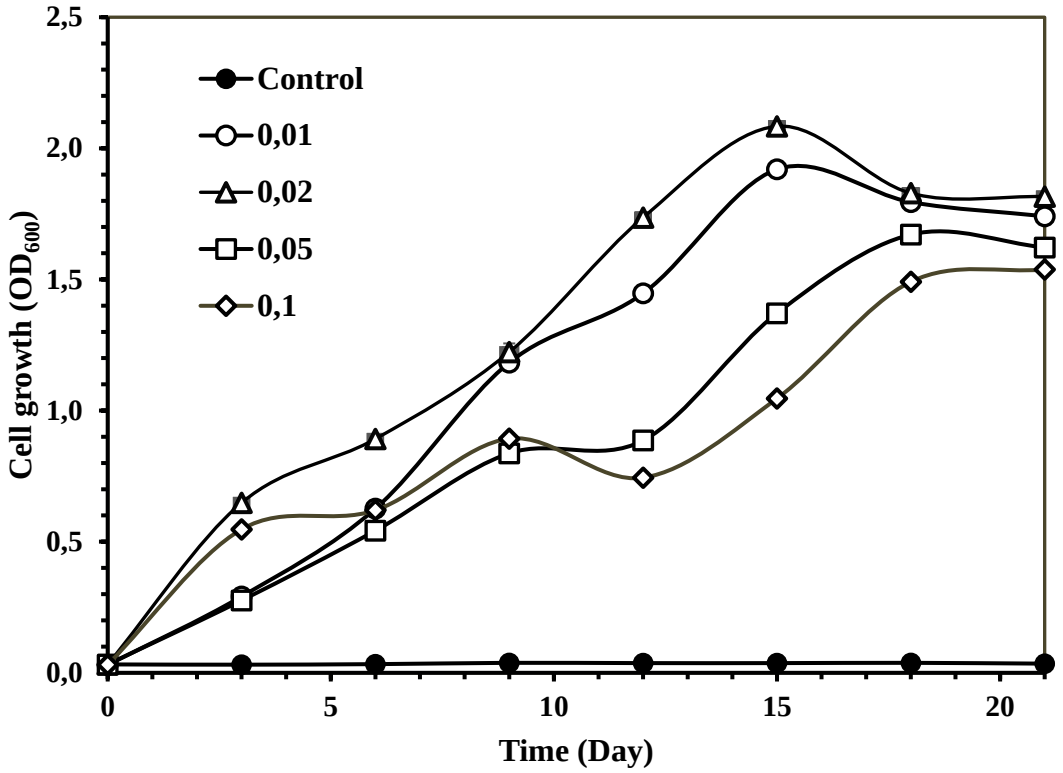


**Figure 3.1** Growth profile of *Paenibacillus* sp. D9 at various concentrations of diesel fuel grown at 30°C, pH 7 in an orbital shaker (150 rpm).

The biodegradation of diesel fuel is usually analyzed using lower diesel fuel concentrations (Ahmad et al., 2014; Shukor et al., 2009). This highlights the significance of this research, where high diesel concentrations were utilized. The optimum carbon source (diesel fuel) concentration for the growth of *Burkholderia* sp. strain DRY27 was 3% (v/v) and it showed tolerance to 4% (v/v) and 5% (v/v) of diesel fuel with a declining bacterial growth (Ahmad et al., 2014). It has been found that biodegradation is usually slow at concentrations higher than 2% (v/v). A diesel fuel-degrading bacterium *Acinetobacter* sp. strain DRY12 was also able to tolerate 10% (v/v) diesel concentration as sole carbon and energy source (Dahalan et al., 2014). Another author recently

reported the degradation and utilization of diesel fuel at a much higher concentration (> 2%) (Al Disi et al., 2017). The fact that *Paenibacillus* sp. D9 could tolerate higher diesel concentrations together with its potential toxicity (5% v/v, 10% v/v), and ability to withstand the hydrocarbon stress and selective pressure makes it a good bioremediation agent (Figure 3.2). The high tolerance level is not unanticipated since the soil pollution with diesel fuel had happened for some years with the acclimatization process favoring higher tolerant microbial strains. Soil microbiologists had proposed that the abundance of carbon and energy materials in soil is relative to the microbes' capability of utilizing such constituents (Dharni et al., 2012). However, diesel concentration affects biodegradation itself as microorganisms can only grow well when their growth rate is slower than the dissolution rate of hydrocarbons present. Excessive diesel fuel concentration may reduce the biodegradation rate due to its toxic effect and selective pressure. On the other hand, low diesel concentrations may also reduce overall degradation rates because contact between microorganism and diesel is limited (Shukor et al., 2009). The activities of wide varieties of microorganisms in a polluted environment enable the biodegradation of hydrophobic mixtures with individual organisms only able to degrade a limited range of aliphatic and aromatic hydrocarbons. However, *Paenibacillus* sp. D9 utilized and grew well on different straight chain hydrocarbons and hydrophobic substrates (Table S1) in all cases with significant disappearance of diesel fuel and biomass accumulation.

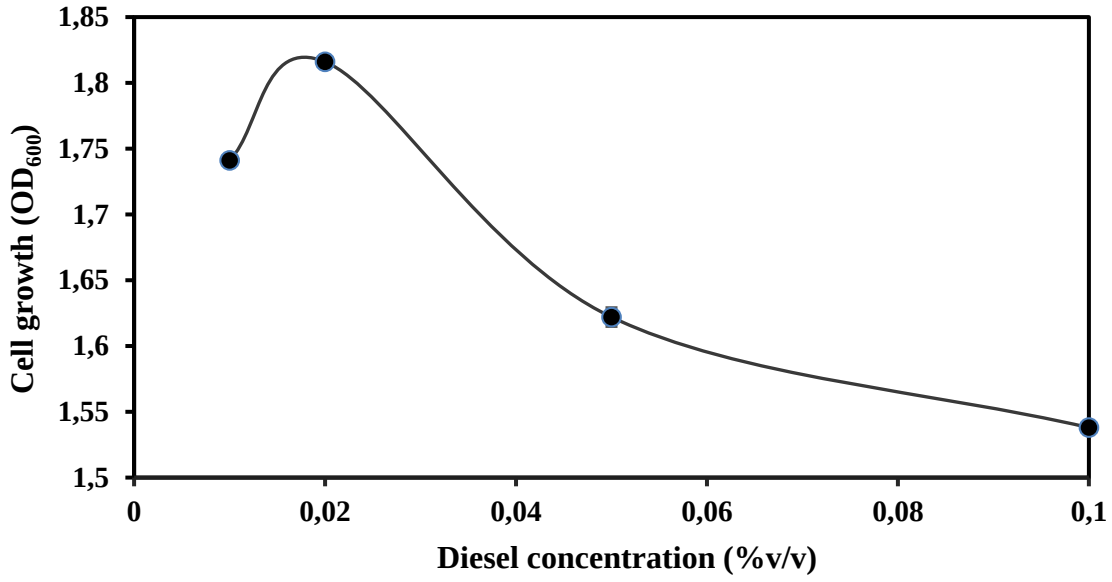


**Figure 3.2** The effect of diesel concentrations on the growth of *Paenibacillus* sp. D9 after 21days of incubation.

The hydrocarbon component changes in optimum concentration of diesel were detected by GC-FID (Figure 3.3a, and 3.3b). The control sample when analyzed comprised of *n*-alkanes (C3-C26), naphthalene derivatives, and branched alkanes. The chromatogram results showed a decrease in the concentration of the hydrocarbon peaks of the sample with *Paenibacillus* sp. D9 when compared to chromatograms of the control diesel fuel (Figure 3.3a). High and sharp chromatographic peaks represent the major components of the control sample (Figure 3.3b). The abundance of each *n*-alkane in the inoculated flask was remarkably lower as compared with that of the control as many peaks decreased significantly showing the capability of degrading a wide range of alkane hydrocarbons.

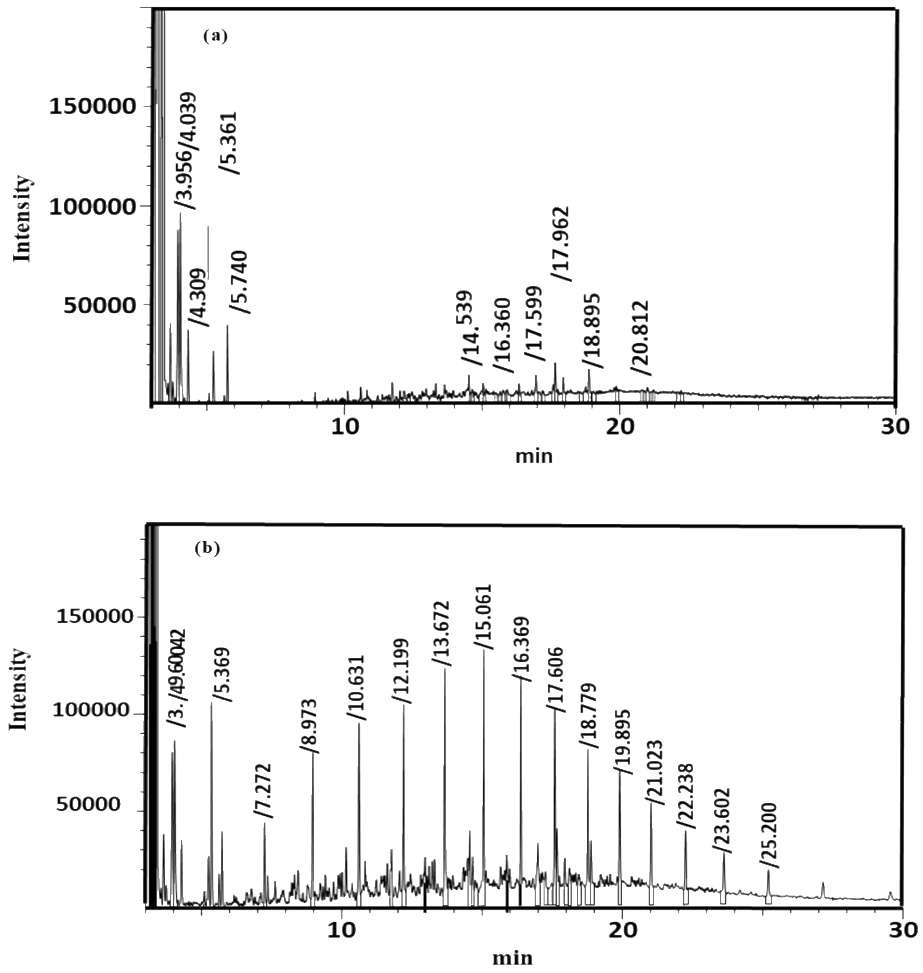


**Figure 3.3** GC-FID profiles of diesel fuel extracted from the aqueous phase of the medium after 21 days of incubation with 2 % (v/v) diesel (a) inoculated with *Paenibacillus* sp. D9 (b), abiotic control (uninoculated).

*Paenibacillus* sp. D9 could utilize and degrade *n*-alkanes ranging from C7 to C30 and important aromatics were removed from the medium as revealed by the gas chromatograph fingerprint. The degradation ability of *Paenibacillus* sp. D9 was estimated as 85-95% efficiency rate and near total removal of the different component mixtures present in diesel fuel. The GC-FID profile outlined showed a total disappearance of different hydrocarbons peak intensity depicting the optimum condition (Figure 3.3a). Other concentrations profile (data not shown) expressed good disappearance and degradation of the hydrocarbons present including the low molecular and high molecular weight compounds. It further demonstrated the ability and availability of *Paenibacillus* sp. D9 for petroleum-derived diesel fuel degradation with the enormous potential of the strain in biodegradation and oil pollution remediation.

### *3.3.3 Biosurfactant production of Paenibacillus* sp. D9

To determine the optimum condition for BioS production by *Paenibacillus* sp. D9, culture media were prepared at different diesel concentrations (Figure 3.4a and 3.4b). The results obtained show the ability to utilize various diesel concentrations for BioS production due to a sharp reduction in ST in the first 3 days of incubation (Table 3.2). Ten percent (v/v) diesel was discovered to be the best inducer of BioS production with ST activity (ST decreased from 71.4 mN/m to 30.1 mN/m). The STs achieved in this research showed high influence of the BioS synthesized as the control sample containing the diesel fuel reduced from 71.4 mN/m to 67.1 mN/m). As shown above, higher concentrations of diesel were toxic to *Paenibacillus* sp. D9, but the optimal BioS production allowed the reduction in toxicity effect for its survival, utilization, and growth. The culture media using lower concentrations of diesel [1, 2, 5 % (v/v)] showed increasing high reduction in ST activity (ST decreased from 71.4 mN/m to 32.0 mN/m, 32.1 mN/m, 31.8 mN/m) with increasing incubation time giving a clue that the increase in production of BioSs is directly proportional to the volume of substrate added. The ST values showed some level of consistency after the third day of incubation until the extinction of the experimental procedures for both intracellular and extracellular production. The consistency in ST results explained the effectiveness and stability of BioS produced. The ST values obtained at the end of the extracellular production experiments include 43.3, 30.3, 31.4 and 30.0 (mN/m). The high value of 43.3 mN/m obtained for 1% diesel fuel concentration was due to complete disappearance and utilization of diesel present.

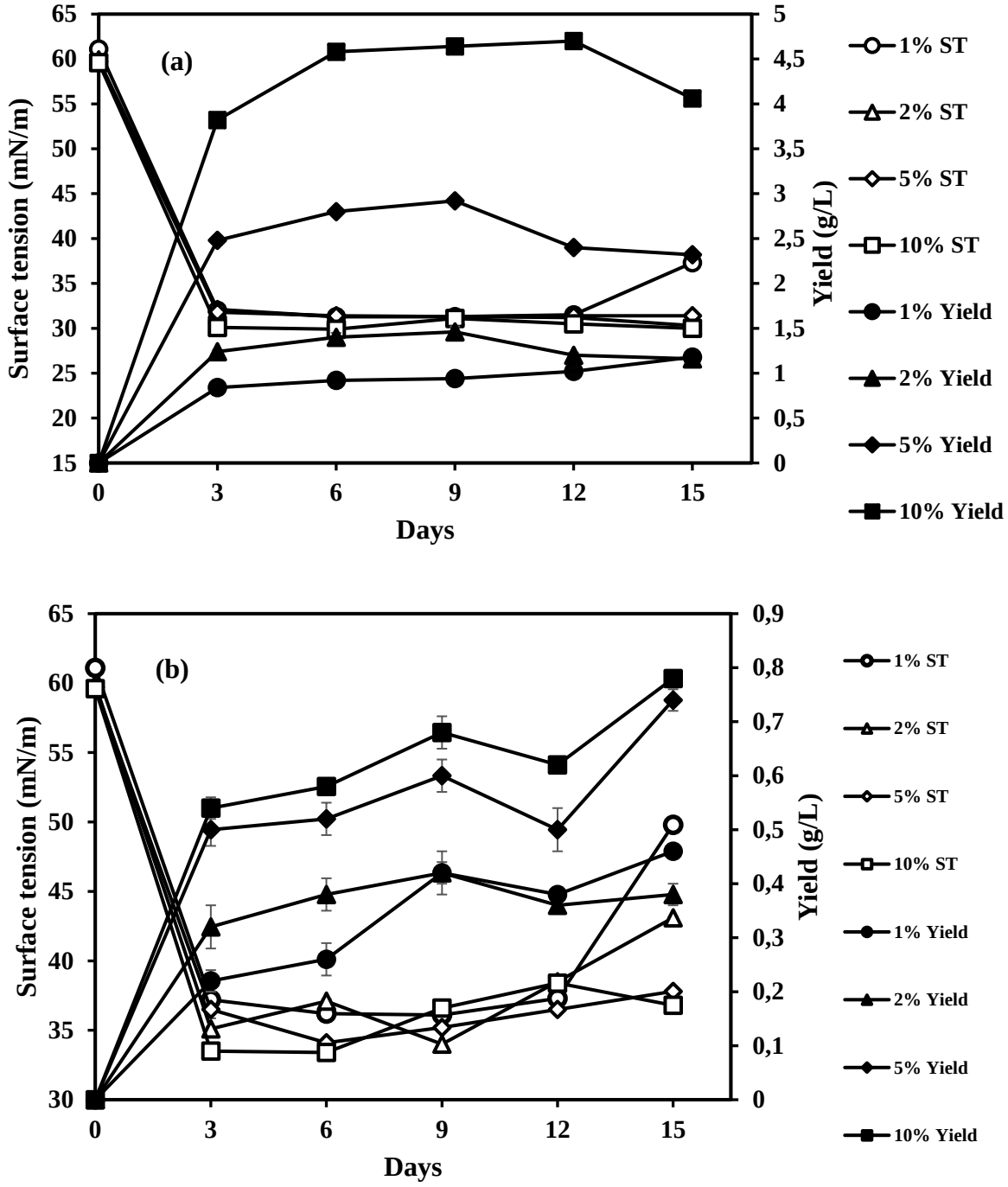


**Figure 3.4** Biosurfactant production profile of *Paenibacillus* sp. D9 with reference to production yield and surface tension reduction (a) extracellular (b) intracellular.

BioSs are generally a combination of complex molecules like polysaccharide, peptides, and fatty acids. BioSs possess the ability to reduce ST through solubilization, leading to exploitation of hydrocarbons (Patowary et al., 2017). According to the data obtained from this study, there is a direct relationship between bacterial cell growth and BioS

production. This indicates that the BioS production was consistent with cell growth which occurred at the exponential growth phase so that a decisive decrease in the ST occurred (Figure 3.1). A study revealed that the growth-associated lipopeptide BioS production by *Bacillus mojavensis* was maximum at the exponential growth phase (Ghojavand et al., 2011). A reduction in the ST of genus *Paenibacillus* due to BioS production was observed by different authors. *Paenibacillus alvei* ARN63 isolated from an oil field reduced ST to 35mN/m, after 17 h of incubation (Najafi et al., 2011). *Paenibacillus dendritiformis* on the other-hand produced BioS activity with ST reduction from 71 mN/m to 34 mN/m, in the first 3 days of incubation (Bezza and Nkhalambayausi Chirwa, 2015).

Figure 3.4a showed changes in the ST readings of *Paenibacillus* sp. D9 supernatant with the minimal value achieved after about 3 days of growth (43.3 mN/m, 32.1 mN/m, 31.8 mN/m, 31.1 mN/m). A slight reduction in ST was observed on the sixth day by the end of the exponential phase. This may be due to the formation of micelle ascribed to the CMC obtained, in which the ST remained stable (30 mN/m). The increase or decrease in extra-cellular BioS concentration results from the interchangeable release of cell-bound BioS molecules into the culture medium (Elazzazy et al., 2015).

This research was also intended to determine if the production of BioS by *Paenibacillus* sp. D9 is extracellular (cell-free supernatant) or intracellular (cell-bound) as shown in Figure 3.4a-b. The low ST readings of the cell-free supernatants and high BioS yields (g/L) showed production by *Paenibacillus* sp. D9 was extracellular as compared to low yield products and high ST readings associated with the cell-bound BioSs. Elazzazy et al. (2015) argued that the increase or decrease in extracellular BioS molecules could result in an alternate release of intracellular BioS molecules into the culture broth medium. The BioS yield of 3.82 g/L was obtained with 10% (v/v) diesel concentration with significant low ST readings (30.1 ± 0.1 mN/m) after the third day of incubation. BioS production reached its maximum (4.70 g/L) at the end of the exponential growth phase with 10% diesel fuel with BioS yield decreasing slightly afterward. The other concentrations of diesel fuel (1%, 2%, 5% v/v) also produced different BioS yields and ST readings of 0.42, 0.62, 1.60 g/L after the third day of incubation. However, optimum yields of 0.51, 1.20, 2.80 g/L were observed at the end of the exponential phase (Figure 3.4a). The outcomes showed significance in relative to control samples with no production of BioS yield. This however, rules out any possibilities of the substrates co-precipitating with the isolated BioS. Al-Bahry et al. (2013) reported an extracellular BioS production yield of 2.29 g/L by *Bacillus subtilis* B20 strain while Al-Wahaibi et al. (2014) reported a low BioS production yield of 0.50 g/L by *Bacillus subtilis* B30. BioS produced by the strain *Paenibacillus* sp. D9 was measured as 4.70 g/L found to be maximum in comparison to another literature report (Parthipan et al., 2017). The high yield of BioS produced at a

higher diesel fuel concentration further confirms the direct correlation between the production of BioS and cellular growth in this study.

The cell-bound production at different concentration of diesel fuel showed a minimal BioS yield at different phases of incubation and production (Figure 3.4b). The yield of *Paenibacillus* sp. D9 cell bound BioS at the end of the experiment were 0.46, 0.38, 0.74, and 0.78 (g/L) for 1%, 2%, 5%, and 10% diesel fuel concentration respectively. Sharma et al. (2015) achieved the production of BioS intracellularly (cell bound) using *Enterococcus faecium*. The data from this research proved that biomolecules produced by *Paenibacillus* sp. D9 were dual in nature (extracellular and intracellular). The biomolecule comes out of the fermentation medium along with the cellular component interchangeably. However, it is imperative to note that the intracellular production of BioSs achieved in this research was relatively low in yield as compared to extracellular production. Future research aims to improve production yield by optimizing low-cost substrates, thus increasing the importance of this BioS in biotechnological and environmental application.

The results in Tables 3.2 and 3.3 showed important ST reduction and E24 activity (32.0 mN/m and 74.60%) by *Paenibacillus* sp. D9 BioS after three days of incubation. The partially purified extracellular BioS thereafter appeared as a crystalline white powder. The physiochemical characterization of the *Paenibacillus* sp. D9 BioS revealed the drop collapsing and oil spreading activities which were both positive, indicating the presence of a surface-active agent (Table 3.2). The analysis confirms the presence of the surface-active agent in the experiments as shown with the cell-bound extract showing negativity in the physiochemical assays. In this study, there was a direct correlation between drop collapse, oil spreading, E24 and ST reduction assays confirming the production of BioS. The physiochemical parameters tested above always give a strong positivity and indication of BioS production (Thavasi et al., 2013).

**Table 3.2** Physiochemical characterization of the biosurfactant produced by *Paenibacillus* sp. D9 after 3 days

| Materials | Surface tension (mN/m) | Emulsification Index (E24) % | Drop collapse | Oil spreading (cm) |
|---|---|---|---|---|
| $DH_2O$ | 71.80 ± 0.05 | 0.00 ± 0.00 | - | - |
| BioSs supernatant | 30.10 ± 0.07 | 72.50 ± 0.10 | + | 8.60 |
| Cell Bound BioSs | 35.50 ± 0.71 | 44.50 ± 0.10 | - | - |
| Partially purified BioSs | 32.00 ± 0.08 | 74.60± 0.10 | ++ | 10.50 |
| Tween 80 | 35.00 ± 0.49 | 61.70 ± 0.10 | + | 8.10 |

All data points are means ± standard deviation (S.D.) of three independent experiments

$DH_2O$ distilled water (- negative control) 10% SDS Sodium dodecyl sulphate (+ positive control)

+ + indicate highly positive drop collapse test + indicate moderate positive drop collapse test

**Table 3.3** Percentage emulsification indexes obtained against different hydrocarbon substrates by biosurfactant produced from *Paenibacillus* sp. D9

| Hydrocarbon substrates | % Emulsification index ($E_{24}$) |
|---|---|
| Diesel fuel | 70.80 ± 0.14 |
| Engine oil | 76.70 ± 0.06 |
| Motor oil | 61.72 ± 0.08 |
| *n*-Hexadecane | 59.24 ± 0.12 |
| *n*-Dodecane | 56.75 ± 0.14 |
| *n*-Paraffin | 60.82 ± 0.10 |
| *n*-Tetradecane | 61.74 ± 0.18 |
| *n*-Hexane | 26.72 ± 0.10 |
| *n*-Toluene | 62.50 ± 0.06 |
| Chloroform | 56.70 ± 0.10 |
| *m*-Xylene | 66.72 ± 0.12 |
| 1-Nonene | 35.06 ± 0.04 |

All data points are means ± standard deviation (S.D.) of three independent experiments

#### 3.3.3.1 Critical micelle concentration

The CMC of the BioS produced as determined by ST reduction continued until micelle was formed in the system (Figure S1). The crude BioS reduced the ST of buffer solution from 65.9 ± 0.5 to 32.0 ± 0.3 mN/m with increasing BioS concentration. The BioS produced in this research has a CMC of 200 mg/L conforming to the minimum ST of 32.0 ± 1.0 mN/m. There was no further decrease in ST with further increase in the concentration of the BioS beyond this point indicating that CMC had been reached due to the formation of micelle. The ability to reduce ST is a major property that enhances the capability of the surface-active compound (BioS). A CMC range of over 100 mg/L has also been reported for *Paenibacillus dendritiformis* CN5 (185 mg/L), and *Bacillus* sp. l-15 (200 mg/L) (Bezza and Nkhalambayausi Chirwa, 2015; Ismail et al., 2013).

#### 3.3.3.2 Emulsifying activity using different hydrophobic substrates

E24 of the diesel substrate by *Paenibacillus* sp. D9 in this study indicates its BioS synthesis and production ability. The BioS obtained could stabilize emulsions and effectively emulsify with diesel fuel as well as other types of hydrocarbon substrates. The E24 index obtained included 63.30% ± 0.15, 67.50% ± 0.10, 69.20% ± 0.15, 73.30% ± 0.12 with the increasing order of concentrations of carbon source tested with the result indicative of high BioS production (Table S2). An emulsification activity in the range of 50% with low BioS production by *Bacillus subtilis* B30 was reported (Elazzazy et al., 2015). Based on these assessments, strain *Paenibacillus* sp. D9 was established as an effective BioS producer with higher emulsification activity.

The ability to also emulsify different hydrocarbon substrates was also investigated (Table 3.3). The capability to form and stabilize emulsions is also a significant factor to determine the quality of a surface-active agent. The emulsification ability of *Paenibacillus* sp. D9 BioS was found to be maximum against diesel fuel-engine oil (76.70%). The BioS produced also formed stable emulsions with long-chain aliphatic (*n*-hexadecane, chloroform, *n*-dodecane, *n*-hexadecane, *n*-tetradecane) and aromatic hydrocarbons (toluene, xylene), along with hydrocarbon mixtures (motor oil, paraffin). The *Paenibacillus* sp. D9 BioS was not efficiently emulsified with short chain aliphatics such as *n*-hexane, and 1-nonene. Thus, a good emulsifying activity potential is essential for the use of BioS in environmental and industrial applications, such as oil tank clean-ups, emulsion-based oil and/or fuel transport, biodegradation of polycyclic hydrocarbons, and bioremediation including heavy metals (Gudina et al., 2015). Thus, *Paenibacillus* sp. D9 BioS possesses the ability to emulsify different varieties of toxic hydrocarbon and hydrophobic compounds.

### *3.3.4 Structural characterization of the biosurfactant*

#### 3.3.4.1 Compound analysis of purified biosurfactant

The BioS produced was analyzed for its lipid, protein, and carbohydrate contents. There were high percentages of lipid (approximately 63%, w/w), protein content (approximately 40%, w/w) in addition to a minute fraction of carbohydrates (approximately 4%, w/w) as revealed by the biochemical analysis of the isolated BioS. The minor fraction of carbohydrates (4% w/w) found in the sample arises from remaining cellular fragments being co-precipitated during the extraction process as suggested by the structural analyses results below. However, the larger fractions of both protein (hydrophilic) and lipids (hydrophobic) give a strategic indication of the amphiphilic nature of the BioS.

#### 3.3.4.2 Thin layer chromatography analysis

The results from the conventional analysis of the isolated BioS were further confirmed qualitatively using the TLC technique to detect the BioS constituents. TLC is the utmost broadly studied method for initial characterization of BioSs followed by post chromatographic detection. The purity and $R_f$ value of BioS product was determined by the TLC data obtainable in this research. TLC analysis of the methanol-$H_2O$ extract displayed a $R_f$ spot of 0.64 on a plate developed on a solvent system (methanol: chloroform: acetic acid (15:65: 2 v\v\v) (Figure S2). Results similar to other lipopeptide type BioS have also been reported (Burgos-Diaz et al., 2011). Rhodamine reagent when sprayed on a TLC silica plate, produced no dark red spot indicative of the absence of sugar molecule. Additional, replica silica plate was spewed with anthrone reagent produced a dark blue spot signifying the existence of lipid fraction. Amino acid fraction, on the other hand, was identified on the same spot when stained with ninhydrin reagent. The above results by TLC representation established the presence of the lipopeptide BioS**.**

#### 3.3.4.3 Fourier transform infrared spectroscopy

For the identification of functional groups (chemical bonds) present in the bioactive part of an isolated BioS, FTIR was used as an utmost spectroscopic tool for the data analysis as shown in Figure 3.5. The extending vibration observed at 3200–3500 $cm^{-1}$ is a representative of O–H stretching vibrations, indicative of a strong hydrogen bonding. The strong absorbance peak observed at 3000 – 2900 $cm^{-1}$, was characteristic of aliphatic chains (–CH3, –CH2) stretching vibrations. The C, N stretch observed is a representation of the advent of a weak absorbance signal at 2300 – 2400 $cm^{-1}$. Also, the absorbance bands observed at 1650 $cm^{-1}$ showed a significant linkage between the amides I and II. The peak absorbance in this area was important in confirming

the presence of the peptide group in the biomolecule. A high-intensity peak at 1372 $cm^{-1}$ was a characteristic of C-O (carbon-oxygen bond), an amine compound that contains a basic nitrogen atom with a lone pair. The high-intensity peak observed in the region of 1000–1100 $cm^{-1}$ was allotted to O–C–O extend vibrations of aldehydes, ketones, carboxylic acids, and carboxylate ester. It is also noteworthy that the oxidation of the hydroxyl groups of hydrolysates originated from the peptide's component in the medium. The absorbance vibrations observed at 800 – 500 $cm^{-1}$ may be suggestive of methylene scissoring vibrations from the peptide component in the lipopeptide BS group. According to FTIR spectrum obtained, the BioS obtained is composed of hydrophobic aliphatic chain (lipid) and hydrophilic group mainly composed of protein. There was a similarity in the FT-IR profile of the *Paenibacillus* sp. D9 lipopeptide BS to surfactin, and other lipopeptide BioS like arthrofactin and lichenysin confirming its presence in the lipopeptide group (Elazzazy et al., 2015).

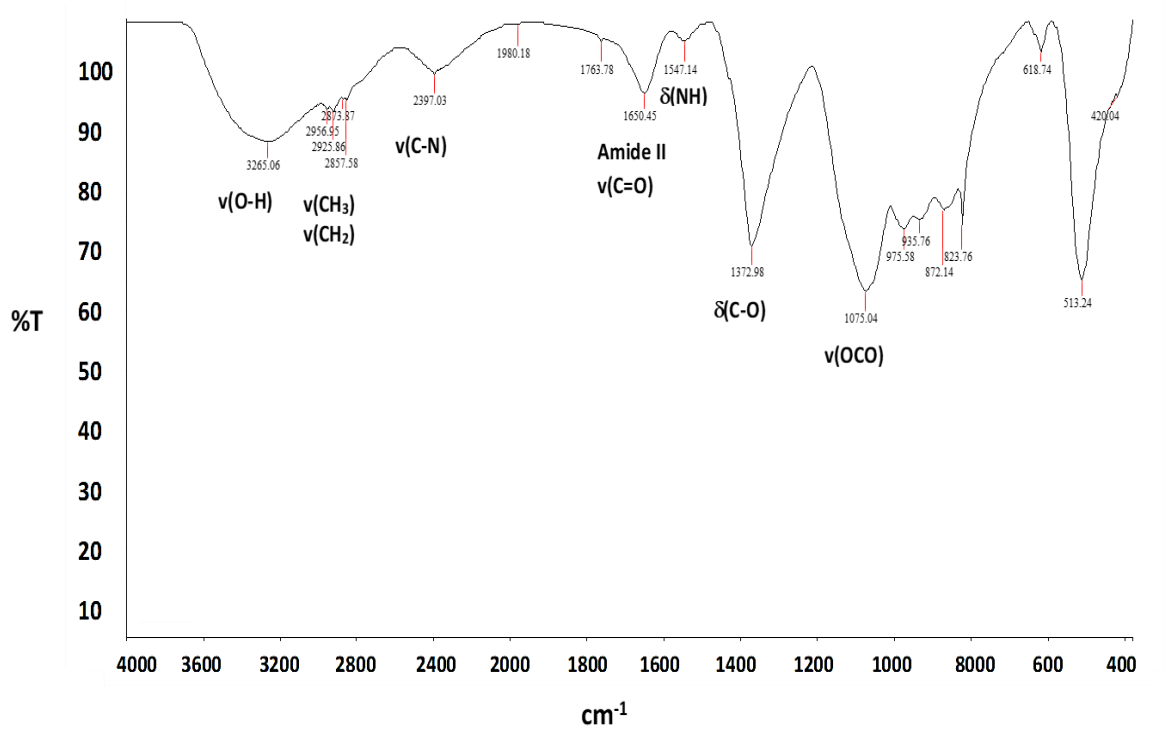


**Figure 3.5** Fourier transform-infra red spectrum of purified biosurfactant isolated from *Paenibacillus* sp. D9.

### 3.3.4.4 Gas chromatography-mass spectroscopy

To obtain a more accurate determination of lipopeptide BioS, fatty acid methyl esters were analyzed by gas chromatography-mass spectroscopy. The spectra were investigated from obtainable NIST Mass Spectral Library and various derivatives were recovered with their different retention times. By and large, lipopeptide BioS mainly comprised of aliphatic hydrophobic chains from C9 to C20 connected to a peptide group. From

this research, *Paenibacillus* sp. D9 BioS chiefly comprised of long chain fatty acids, of carbon length C-17. The fatty acid identified was hexadecanoic acid (Figure 3.6a) at retention times (RT): 15.34, 15.87, 16.52 & 17.46, chemical formula (CF): $C_{17}H_{34}O_2$), MW: 270. Hexadecanoic acid was found as the main fatty acid chain in studies of lipopeptide purified BioS previously (Parthipan et al., 2017).

#### 3.3.4.5 Liquid chromatography-mass spectroscopy analysis

The data obtained from liquid chromatography (LC)-mass spectroscopy (MS) analysis further confirmed the lipopeptide type of BioS. A positive full scan LC-MS/MS chromatogram of the lipopeptide extract (eluted at 1.09) was interpreted by means of MS technique to determine the structural composition. The amino acid sequence of the peptide fraction was inferred by interpreting the MS/MS spectra derived from the mass spectra of ions m/z 1366.7 as the base peak.

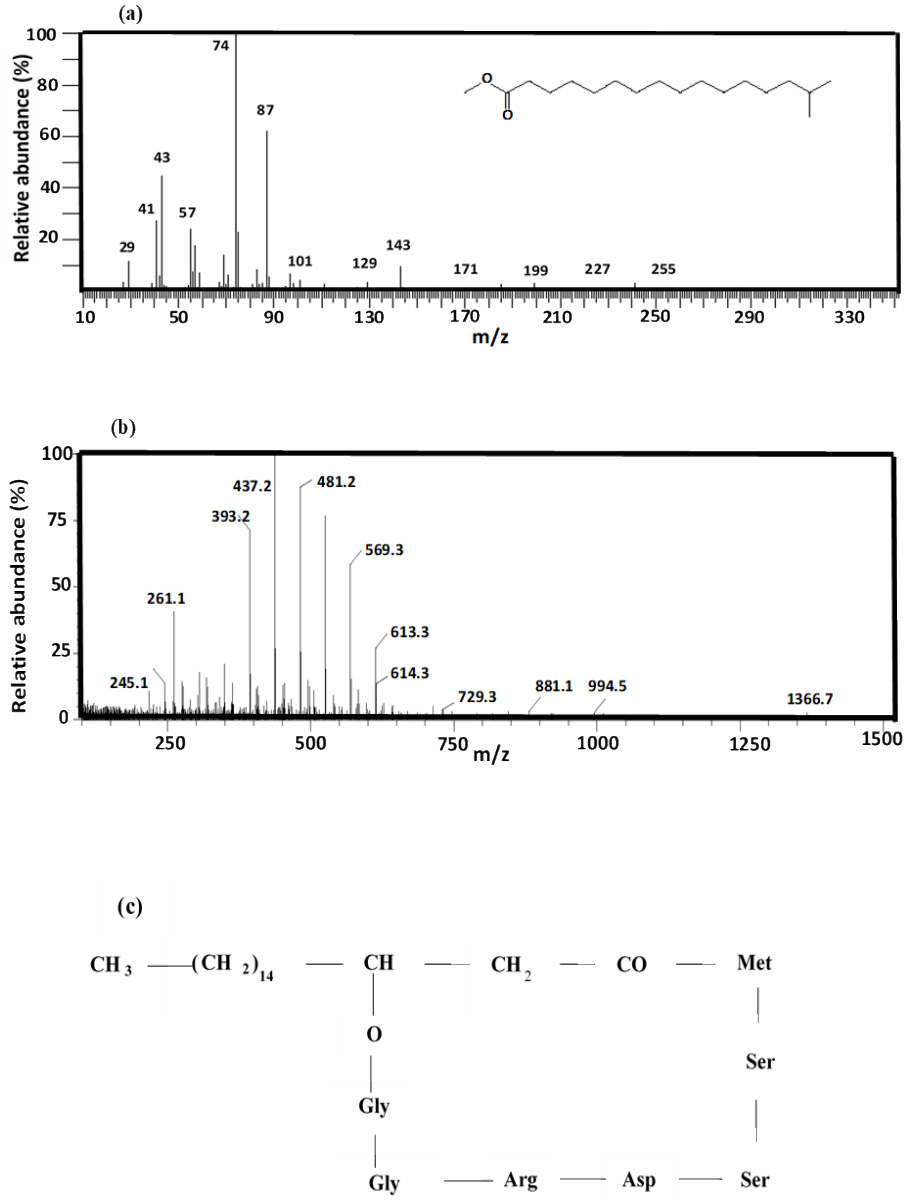


**Figure 3.6** (a) Gas chromatography-mass spectrum of the biosurfactant isolated from *Paenibacillus* sp. D9 (b) TOF MS ES+ spectrum of the purified lipopeptide biosurfactant from *Paenibacillus* sp. D9 (c) The inferred structures of lipopeptide based on GC-MS and LC-MS analysis.

The molecular spectra obtained for different fragmentation in the MS/MS data at m/z 994.5, 881.1, 729.3, 613.3, 481.2, 393.2 and 261.1 display predicted lipopeptide sequence of Met-Ser-Ser-Asp-Arg-Gly-Gly (Figure 3.6b). The C-terminal amino acid in the peptide group is linked to an aliphatic chain of $C_{17}H_{34}O_2$ with a total molecular mass of 1085.85 Da. Ultimately, the molecular structure of the lipopeptide was inferred as $CH_3$-$(CH_2)_{14}$-CHO-$CH_2$-CO-Met-Ser-Ser-Asp-Arg-Gly-Gly as illustrated in Figure 3.6c. The current study reports the production of a new lipopeptide BioS by *Paenibacillus* sp. D9. Based on the chemical structure and

molecular weight (1085.85 Da), it is different from already reported lipopeptides, viz. glumamycin (1290.4 Da), arylomycin A6 (867.4 Da), daptomycin (1620.6 Da), aspartocin (1317 Da), and tsushimycin (1304.7 Da) (Sharma et al., 2015). In addition, lipopeptide structure profile of different molecular mass and forms have been informed with minimal data on genus *Paenibacillus*. This research also presents the identification of lipopeptide of 7 amino acid fragments by *Paenibacillus* sp. D9 which is different from previously reported lipopeptides. According to published reports, lipopeptide BioS usually contain aliphatic hydrophobic chain from C9 to C20, and hydrophilic peptide chains composed of 4-10 amino acids (Deng et al., 2016). In comparison to this research, a lipopeptide BioS named battacin was produced from *Paenibacillus tianmuensis* with 8 amino acid residues (Qian et al., 2011). *Paenibacillus dendritiformis*, on the other hand, produced a lipopeptide of 7 amino acid residues with a long chain fatty acid (Bezza and Nkhalambayausi Chirwa, 2015). Also, a cyclic lipopeptide was also isolated and identified from the *Paenibacillus* genus consisting of 13 amino acid residues and 15 fatty acid chains (Guo et al., 2012). The variation in the length composition, substitution of amino acids, and branching of the fatty acid chains show extraordinary diversities in the lipopeptide group. In addition to possessing a longer hydrophobic fatty acid chain ($C_{17}$), the newly purified lipopeptide contains Met, which is a sulfur-containing amino acid in its structure (Figure S3). This is, however, different to previously discussed cyclic lipopeptide above. Results of TLC, FTIR, LCMS and GCMS spectra suggested that the *Paenibacillus* sp. D9 BioS consists of long-chain aliphatic compounds such as hexadecanoic acid as the main lipids and a peptide component as a hydrophilic part. The surface activities are facilitated by the dual nature of the macromolecules which comprises of hydrophobic regions (unsaturated or saturated hydrocarbon chains or fatty acids) and hydrophilic part (mono-, di- or polysaccharides, acid, peptide cations, or anions) allowing them to act as surfactants (Sharma et al., 2015). The significance and comparison of lipopeptide BioS produced by *Paenibacillus* sp. D9 to other BioS producing strains evaluating the surface-active properties and class of the BioSs are summarized in Table 3.4.

The *Paenibacillus* sp. D9 strain could tolerate a high diesel concentration and a wide range of utilization on different hydrocarbons substrates. The increase in diesel concentration was accompanied by the production of complex biomolecules called lipopeptide BioS. A greater production yield was achieved extracellularly with *Paenibacillus* sp. D9. *Paenibacillus* sp. D9 thus produced a high amount of lipopeptide BioS and was capable to withstand and survive in high toxic hydrophobic compound. The obtained lipopeptide in the present report can proficiently emulsify different hydrophobic compounds inclusive of engine oil, diesel fuel, and other

hydrocarbon substrates. It is proposed that this lipopeptide BioS would be appropriate for highly toxic hydrocarbon solubility and also bioremediation of contaminated soil.

**Table 3.4** Comparison of lipopeptide biosurfactant produced by *Paenibacillus* sp. D9 to other biosurfactant-producing strains evaluating the surface-active properties and class of the biosurfactant produced. ST; surface tension, E24; Emulsification Index, CMC; critical micelle concentration

| Microorganism | Substrate utilized | Surface properties | ST (mN/m) | E24 Index (%) | CMC (mg/L) | Biosurfactant produced | References |
|---|---|---|---|---|---|---|---|
| *Paenibacillus* sp. D9 | Diesel fuel | + | 30.1 | 76.7 | 200 | Lipopeptide | Present study |
| *Paenibacillus* sp. 1C | Olive oil | + | 32.6 | 76.4 | 500 | Lipopeptide | (Mesbaiah et al., 2016) |
| *Paenibacillus dendritiformis* | Oil and anthracene | + | 30.0 | - | 185 | Lipopeptide | (Bezza and Chirwa, 2017) |
| *Paenibacillus alvei* | Iranian oil | + | 35.0 | - | - | Lipopeptide | (Najafi et al., 2011) |
| *Paenibacillus* sp. strain | Crude oil | + | 50.0 | 75.1 | - | Bioemulsifier (oligosaccharide-lipid complex) | (Gudina et al., 2015) |
| *Paenibacillus* sp. PRNK-6, *Pseudoxanthomonas* sp. PNK-04 | Fluorene | + | - | - | 280 | Rhamnolipid | (Reddy et al., 2018) |
| *Bacillus cereus* | Frying oil | + | 27.0 | 98.0 | 500 | Biosurfactant | (Durval et al., 2018) |
| *Pseudomonas aeruginosa* PG1 | Crude oil | + | 29.6 | 100 | 56 | Rhamnolipid | (Patowary et al., 2017) |
| *Bacillus sp. I-15* | Crude oil | + | 42 | - | 200 | Lipopeptide | (Ismail et al., 2013) |
| *Bacillus subtilis* MG495086 | Light-paraffin oil | + | 29.85 | 72.5 | 40 | Lipopeptide | (Datta et al., 2018) |

## 3.4 Conclusion

This study confirmed that *Paenibacillus* sp. D9 produced high amounts of new lipopeptide BioS with significant properties and attributes. The utilization of diesel fuel, as well as other hydrocarbon substrates, contributed to its dual BioS synthesis abilities thereby enabling its efficiency in biodegradation and bioremediation. The lipopeptide produced was found to have a good performance of emulsifying and ST activity. These BioS abilities enhance its potential for further application in the bioremediation of contaminated environments including, environmental, and biotechnological applications. Future research aims at improving the yield of lipopeptide BioS, development of hyperproducing strains and the use of the products in a variety of applications.

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# Chapter 4

## Diesel fuel and *n*-hexadecane biotransformation by *Paenibacillus* sp. D9: Degradative enzymes and biosurfactant mediation

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**Diesel fuel and *n*-hexadecane biotransformation by *Paenibacillus* sp. D9: Degradative enzymes and biosurfactant mediation**

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## Abstract

Elimination of long-chain hydrocarbon and hydrophobic pollutants from contaminated environments is of importance to diminish severe damages to the ecosystem. Degradation of 2% of *n*-hexadecane ($C_{16}H_{34}$), a high molecular weight *n*-alkane, and diesel fuel were studied for *Paenibacillus* sp. D9. During 14 days of incubation, *n*-hexadecane and diesel fuel were degraded to 98.4% and 80.2% as a source of carbon and energy, respectively, by *Paenibacillus* sp. D9. The induction of degradative enzymes such as alkane hydroxylase, alcohol dehydrogenase, and esterase was determined during the biodegradation process. Higher activities of alkane hydroxylase (82 U), alcohol dehydrogenase (23 U), and esterase (0.220 U) were differentially produced, with enhanced biosurfactant activity, indicating their involvement in hydrocarbons degradation. The presence of specific genes responsible for biodegradation and biosurfactant synthesis detected using specific polymerase chain reaction primers, further reinforced these results. The data revealed that the efficiency of the biosurfactant in accelerating the degradation and solubilization of these hydrocarbons was correlated with high cell surface hydrophobicity tendencies, emulsifying abilities, and reduced surface tension. Owing to enzyme production, *Paenibacillus* sp. D9 strain was more effective than other reported biosurfactant-producing bacteria in biodegradation efficiency of both aliphatic hydrocarbon and diesel fuel.

**Keywords: Biosurfactant; biodegradation; hydrocarbon; lipopeptide; *Paenibacillus* sp. D9**

## 4.1 Introduction

A wide cluster of studies has succeeded in the biodegradation and bioremediation of oil hydrocarbons (HCs), thus, explaining the complexities of degradation and knowledge of microbial, physiological, and biochemical reactions (Ismail et al., 2013). In this light, the isolation of probable biodegraders, identification of microbial systems, reactiveness to pollutants, also, unique enzymes and functional genes associated with biodegradation mechanisms have been liable to broad investigation (Lin and Sharma, 2013). Biodegradation is the degradation of pollutants such as HCs, compounds, and substances into a less or nontoxic forms mediated by different metabolic activities of microorganisms (Kim et al., 2017).

The biodegradation of HCs is enhanced by the key roles played by biodegradative enzymes. These biodegradative enzymes include alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase, monooxygenase, and esterase. Esterase is known as a lipolytic enzyme which catalyzes the cleavage of ester bonds, and plays key roles in the hydrolysis of various ester organic contaminants (Chen et al., 2019). Organic pollutants, such as the insecticides, pyrethroids, propoxur, and carbofuran have been initially transformed using genes encoding esterase (Chen et al., 2019; Kim et al., 2017). Though, esterase established much attention for their extensive variety of applications, to date not very many esterases has been described for the role played in the biodegradation of HCs. Several alkane hydroxylase genes are found in HC-degrading bacteria which are equipped at degrading a varied array of HCs. The oxygenation of the terminal methyl group, which is a vital mechanism is carried out by degradative enzymes in the removal of alkanes, and other aliphatic constituents during HC degradation process (Jauhari et al., 2014). *Rhodococcus*, *Pseudomonas*, and *Acinetobacter* have been reported to facilitate aliphatic HC uptake, by enhancing the cell surface hydrophobicity (CSH), enzyme production and changing membrane structure (Jauhari et al., 2014).

The biodegradation of HC is initialized by alkane hydroxylases (*alkB*, *alkM*), transforming alkane to alkanols (Paisse et al., 2011; Singh et al., 2012). About the initial oxidation of *n*-alkanes, four different pathways have been identified, and elucidated. Terminal oxidation pathway, found in a few bacteria, for example, *Geobacillus thermodenitrificans* NG80-2 is the first and the most common. The second pathway is bi-terminal oxidation, in which *n*-alkane end undergo oxidation to the respective unsaturated fatty acids without breakage of the carbon chain that is additionally changed over to a dicarboxylic acid, and in this way enters β-oxidation. In *Pseudomonas aeruginosa*, there exists another major pathway named subterminal oxidation pathway (Ji et al., 2013). Alkane hydroxylases or alkane oxygenases are enzymes with significant roles in the first step of each

of the three pathways featured above. The last pathway is peculiar to *Acinetobacter* sp. strain HO1-N which oxidizes *n*-alkanes to *n*-alkyl hydroperoxides and finally fatty acids (Ji et al., 2013).

Up until this point, the degradative mechanisms of few Gram-negative microbes have been described; for example, *Pseudomonas*, *Acinetobacter*, and *Alkanivorax*. The *alk* gene cluster found in *Pseudomonas putida* GPo1, which degrades *n*-alkanes ranging from *n*-pentane to *n*-dodecane is the most broadly described alkane hydroxylase system (Zampolli et al., 2014). The alkane hydroxylase system featured above involves alkane monooxygenase (*alkB*) and two dissolvable proteins, rubredoxin reductase (*alkT*), and rubredoxin (*alkG*) (Alonso and Roujeinikova, 2012; Zampolli et al., 2014). Alcohol and aldehyde dehydrogenase also plays a significant role in the solubilization of HCs which is the final step though β-oxidation in the degradation pathways. These two enzymes are responsible for solubilizing alcohols formed in the degradation process (Mishra and Singh, 2012).

The bacterial strains such as *Pseudomonas aeruginosa* PSA5, *Rhodococcus* sp. NJ2, *Bacillus subtilis* A1 (Mishra and Singh, 2012; Parthipan et al., 2017), *Ochrobactrum intermedium* (Mishra and Singh, 2012), *Dietzia maris* As-13-3 (Wang et al., 2014), *Pseudomonas* sp. BP10, *Stenotrophomonas nitritireducens* (Jauhari et al., 2014), were described to synthesize degradative enzymes during the biodegradation of HCs. To solve these problems associated with the release of these pollutants, microorganisms produced several types of biosurfactants (BioSs) with diverse structural composition, physicochemical properties, and vast applications (Bezza and Nkhalambayausi Chirwa, 2015; Ferhat et al., 2011). BioSs are amphiphilic compounds comprising both hydrophobic and hydrophilic end (Bezza and Nkhalambayausi Chirwa, 2015). BioS enables emulsification of HCs in solution by increasing cells adhesion to the substrate (Tebyanian et al., 2014). The major importance of BioSs is the ability to increase the surface area of the substrate hydrophobicity, thus decreasing the surface tension (ST), and surface activity, which leads to improved bioavailability and successive HCs biodegradation (Bezza and Nkhalambayausi Chirwa, 2015; Tebyanian et al., 2014). These BioSs possess several advantages such as stability at extreme temperatures, pH, and salinities, biodegradability, low toxicity, environmental compatibility, and huge biodegradative enzymes production (Wang et al., 2014). Therefore, it is imperative to understand the relationship associated with the synthesis of BioS, and degradative enzymes.

With respect to Gram-positive bacteria, much less is known about the degradative systems. For instance, *alkB1* and *alkB2* genes of *Rhodococcus opacus* B-4 expressed in *E. coli* recombinants could utilize *n*-hexadecane and *n*-pentane to their respective alcohols. Additionally, *alkB* gene of Gordonia sp. strain SoCg oxidized *n*-

hexadecane to 1-hexadecanol through heterologous expression in *E. coli* and *S. coelicolor* (Zampolli et al., 2014). A few bacteria have different alkane hydroxylases; for instance, the concurrence of various alkane hydroxylases in *Amycolicicoccus subflavus* DQS3-9A1T 13 while *CYP153* and *alkB* genes were found in *Dietzia* sp. DQ12-45-1b (Nie et al., 2014).

*Paenibacillus* sp. D9 is a Gram-positive bacterium isolated from a diesel fuel contaminated site (Jimoh and Lin, 2019b). *Paenibacillus* sp. D9 utilized in this examination has been shown to possess the elevated capacity to solubilize diesel fuel and hydrophobic pollutants such as *n*-dodecane, *n*-paraffin, engine oil, *n*-tetradecane, and polycyclic aromatic hydrocarbon intermediates by producing BioS as surface-active agent (Jimoh and Lin, 2019b). However, the metabolism and degradative pathways of the strain are not characterized. In this research, the degradation of diesel fuel and *n*-alkane (C16) was achieved, highlighting the metabolic mechanism and pathway of *Paenibacillus* sp. D9. All things considered, the correlation between BioS productions and biodegradative mediated enzymes has not been explored so far in the literature. These outcomes bring new knowledge regarding the physiology and molecular genetics of the genus *Paenibacillu*s. The significance of key metabolic, biosynthetic degradative enzymes in the degradation of diesel fuel and *n*-hexadecane was studied. Also, the role played by the BioS produced in pseudo-solubilization and build-up of intracellular *n*-hexadecane HC was investigated in relation to biodegradation.

## 4.2 Materials and methods

### *4.2.1 Chemicals, media, and culture conditions*

All reagents, chemicals, hydrocarbons utilized were of analytical grade, purchased from Sigma-Aldrich Co. LLC. Diesel fuel was obtained from a garage store in Durban, South Africa. Bushnell Haas (BH), with the following composition (g/L); $(NH_4)\ NO_3$ (1.00), $FeCl_3$ (0.05), $KH_2PO_4$ (1.00), $K_2HPO_4$ (1.00), $MgSO_4$ (0.20), $CaCl_2$ (0.02) was used for production and degradation medium (Bushnell and Haas, 1941). The pH was subsequently adjusted to 7.0 using 1 N NaOH or HCl. The media were sterilized at 121°C for 15 min using an autoclave (HL-340 Vertical Type Steam Sterilizer). The *Paenibacillus* sp. D9 strain was grown at 30°C in liquid broth medium for 24 h, followed by centrifugation (13,500 × *g*, for 20 min at 4°C). The inoculum obtained was washed twice in phosphate buffer saline (1X) and further suspended in BH medium until $OD_{600}$ was equivalent to 1.00. The washed culture was kept at 4°C until further usage. The experimental data were

expressed in terms of arithmetic means obtained from at least three independent replicates, with standard deviation.

### *4.2.2 Cell surface hydrophobicity*

The CSH of *Paenibacillus* sp. D9 strain to *n*-hexadecane and diesel fuel was determined by measuring the bacterial adhesion to HCs according to the method of Jauhari et al. (2014). Cells were developed in nutrient broth, centrifuged, washed and suspended with PUM buffer (g/L) with compositions (g/L): $KH_2PO_4$ 7.26, $K_2HPO_4$ 22.1, urea 1.8 and $MgSO_4$ 0.2; pH 7.1. The initial absorbance was adjusted to 1.0 utilizing a UV–Vis spectrophotometer at 600 nm. The experimental tubes included 0.4 mL sterile *n*-hexadecane and/or diesel fuel and 1.0 mL of culture solution, incubated in an orbital shaker (MRC supplies, China) at 30ºC, 150 rpm at different time intervals namely 30 min, 1 h, 12 h, and 24 h. The tubes were subsequently vortexed for 5 min and kept for 30 min for the separation of aqueous and hydrophobic phases. The lower aqueous phase was prudently removed, and optical density measured at 600 nm. The spectrophotometer was blanked with the medium containing the different substrates during measurement of $OD_{600}$ value.

CSH (%) was calculated using the equation below,

% CSH = 1 - ($OD_{600}$ of the cell suspension - $OD_{600}$ of the aqueous phase/$OD_{600}$ of the cell suspension) × 100

### *4.2.3 The growth of Paenibacillus sp.* D9 *in diesel fuel and n-hexadecane*

Five hundred (mL) Erlenmeyer flasks comprising 98 mL BH augmented with 2% v/v (diesel fuel, *n*-hexadecane) and 1 mL bacterial inoculum (1 $OD_{600}$) were prepared for determination of bacterial growth. The flasks were inoculated with *Paenibacillus* sp. D9 inoculum and incubated in an orbital shaker (MRC supplies, China) at 30ºC and 150 rpm for 14 days. Also, control flasks were inoculated with *Paenibacillus* sp. D9 inoculum, but without the introduction of diesel fuel or *n*-hexadecane. The consecutive $OD_{600}$ was taken at the interval of two days up to 14 days. At each interval, the growth was determined at $OD_{600}$ with an UV-spectrophotometer (Shimadzu UV Spec). The production flasks allowed for clear separation of hydrophobic layer containing the substrates and hydrophilic layer containing the bacterial cells (Jimoh and Lin, 2019b). The spectrophotometer was blanked with the medium containing the different substrates during the measurement of $OD_{600}$ value.

The growth rate of *Paenibacillus* D9 strain in both mediums was determined by the formula below;

Specific growth rate $\mu = (y_t/y_0) / t$

where, $y_t$ = cell mass produced; $y_0$ = initial cell mass; t = incubation time.

### *4.2.4 Bacterial degradation of diesel fuel and n-hexadecane*

For *n*-hexadecane and diesel fuel degradation study, 98 mL of BH each with 2% diesel fuel or *n*-hexadecane was added in 500 mL Erlenmeyer flasks and sterilized at 121ºC for 15 min. Then after, 1 mL *Paenibacillus* D9 (1 $OD_{600}$) was inoculated separately in flasks and control samples were also prepared, but without a bacterial culture to monitor abiotic losses. The flasks were subsequently incubated on an orbital shaker (MRC supplies, China) at 30ºC, 150 rpm for 14 days. The cells were harvested at two-day intervals by centrifugation (Avanti J-26 XPI, Beckman Coulter, USA) at 13,500 × *g* at 4ºC for 20 min to determine alkane hydrogenase and alcohol dehydrogenase activity. The residual substrates were extracted twice with 20 mL *n*-hexane. The degradation of diesel fuel and *n*-hexadecane were measured using a Shimadzu AOC-201 gas chromatograph (GC-2010) set with flame ionization detector and HP-5 column (30 m long) (internal diameter, 0.25 mm; film thickness, 0.25 µm). Both injector and detector were maintained at 280ºC. A temperature of 80ºC for 2 min was set for the initial oven temperature, subsequently increased to 300ºC with a 10ºC increase per min. The chromatogram peaks area of the HCs residues of the different samples were obtained after experimentation and computation. The biodegradation rate was determined as follows (Jia et al., 2018).

Degradation rate = (AH control - AH experimental × AT control/AT experimental)/ AH control

AH is the peak area of the HC remains, and AT is the peak area of *n*-hexane.

### *4.2.5 Medium pH*

The increase or decrease in pH of both media containing *Paenibacillus* sp. D9 bacterial cell during HCs degradation was measured with the aid of 3510 pH meter (Lasec, Jenway).

### *4.2.6 Assessment of enzyme activity*

#### 4.2.6.1 Protein determination

For protein determination, *Paenibacillus* sp. D9 strain grown in both media were extracted by centrifugation at 13,500 × *g* at 4ºC for 20 min and washed twice in 20 mM Tris–HCl (pH = 7.4). The pelleted cells were suspended in the same buffer, sonicated (Omni International Sonic Ruptor 400 Ultrasonic homogenizer) with an operating frequency of 50 kHz for 5-10 min at 4ºC. The samples were subjected to 30s on /30 s off pulses for 10 min at 50% amplitude, and followed by centrifugation at 10,000 rpm at 4ºC for 20 min. The pooled

supernatant preserved at 4ºC and utilized for protein determination and enzyme assays. The protein content was determined using UV–Vis spectrophotometer at 600 nm, using bovine serum albumin as standard.

#### 4.2.6.2 Alkane hydroxylase activity

The activity of alkane hydroxylase during the biodegradation study was determined as described in Parthipan et al. (2017). The cell-free supernatant prepared above was utilized by measuring the decrease of absorbance with UV–Vis spectrophotometer (JASCO V-630) at 340 nm due to the conversion of NADH to $NAD^+$. The testing solution contained 20 mM Tris–HCl and 0.15% CHAPS buffer (pH 7.4), 0.1 mM of NADH, 10 µL of *n*-hexadecane mixture (1% *n*-hexadecane diluted with 80% DMSO) and 50 µL of crude extract in final 1 mL quantity. The alkane hydroxylase activity was expressed as 1 mmole of NADH oxidized per minute. Control samples were used without the introduction of 2% *n*-hexadecane.

#### 4.2.6.3 Alcohol dehydrogenase activity

The activity of alcohol dehydrogenase activity during the biodegradation study was determined as mentioned in Jauhari et al. (2014). In brief, cell-free supernatant was used in the experiment and increase in absorbance was measured with UV–Vis spectrophotometer at wavelength 340 nm due to the conversion of $NAD^+$ to NADH. The reaction solution contained 1 M Tris–HCl buffer (pH 8.8), 4 mM of $NAD^+$, 100 µL of hexadecan-1-ol (99% pure) and 50 µL of crude extract in final 1 mL quantity. The activity of alcohol dehydrogenase was determined as 1 mmole of $NAD^+$ formed per minute.

#### 4.2.6.4 Esterase activity

Esterase activity was carried out using a 75 mM phosphate buffer containing 10 mM $MgSO_4$ (pH 7.0) and 100 mM para-nitrophenyl (pNP) acetate as substrate. The esterase enzyme activity was determined spectrophotometrically by measuring the increase in optical density at 405 nm after 30 min of incubation at 37°C. One esterase unit was defined as the quantity of enzyme required to release 1 µmol of *p*-nitrophenol per minute with the specific esterase activity expressed as µmol/mg protein/min.

#### 4.2.6.5 Determination of emulsifying activity

The change of optical density at 600 nm was used to determine emulsifying activity (Colla et al., 2010). Cell-free supernatant was introduced into 10 mL glass tubes containing TM buffer (20 mM Tris-HCl buffer, pH 7.0; 10 mM $MgSO_4$) and 0.05 mL of a 1:1 (vol/vol) of diesel fuel and 2-methylnaphthalene added to a final volume of 1.5 mL. The tubes were vortexed at room temperature for 60 min with distilled water and BH media

containing diesel fuel and 2-methylnaphthalene used as blanks. The oil in water (O/W) emulsifying activity was obtained by Equation 1. One unit of emulsifying activity is defined as the amount of BioS that yielded an optical density $A_{600}$ of 0.1 using a Shimadzu spectrophotometer. After 24 h, emulsion height and the total height were performed, being the W/O emulsifying activity as seen in equation 2

$$EA_{O/W} = (\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}) / 0.1 \quad (1)$$

$$EA_{W/O} = (E_{\text{height}} / E_{\text{total}}) \times 100 \quad (2)$$

where EA is emulsifying activity; $E_{\text{height}}$ is a percentage of the height of emulsified layer (mm); $E_{\text{total}}$ is the total height of the liquid column (mm); O/W, oil in water; W/O; water in oil

### *4.2.7 Detection of genes involved in degradative and biosurfactant synthesis*

DNA was isolated from the *Paenibacillus* sp. D9 using standard protocols (Zymo Research). The DNA concentration was measured using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fischer Scientific). Primers sequences used for detecting genes involved in BioS and degradative-mediated enzymes are described in Table 4.1. The genes utilized in this study were designed utilizing Snap Gene software (GSL Biotech LLC) according to the whole genome sequence of *Paenibacillus* sp. D9. PCR amplifications were carried out in 10-µL reaction mixtures containing 1 U Phusion DNA polymerase, 25 mM TAPS-HCl (pH 9.3), 2 mM $MgCl_2$ (Qiagen Inc.), 1 mM β- mercaptoethanol, 0.5 µM of each forward and reverse primer, and 2 µL of template DNA (approximately 100 ng of bacterial genomic DNA). The amplifications were performed using a thermocycler with the specified cycle conditions (Table 4.1). Amplified products were separated on 1.2% (w/v) agarose dissolved in 1 × Tris-acetate-EDTA buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.0) stained with 0.5 µg/mL ethidium bromide through electrophoresis. The desired products were eluted from gels using the gel extraction kit (Thermo Fisher Scientific)

**Table 4.1** The primers used in this study and PCR profile

| Primer | Sequence | Mechanism | Product size (bp) | PCR profile | Reference |
|---|---|---|---|---|---|
| Alkb-F | 5'- CCTGCTCCCGATCCTCGA-3' | Biodegradative | 660 | 94°C – 5 min. 30 cycles (94°C for 45 secs, 60°C for 45 secs, 72°C for 45 secs) and 72°C for 7 min. | (Baek et al., 2007) |
| Alkb-R | 5'- TCGTACCGCCCGCTGTCCAC -3' | | | | |
| Alkm-F | 5'-GGATCCGAATGCGATCTTGGAACAGCCTTG-3' | Biodegradative | 1062 | 98°C – 30 secs. 30 cycles (98°C for 10 secs, 61°C for 30 secs, 72°C for 30 sec) and 72°C for 5 min. | This study |
| Alkm-R | 5'-CTCGAGTCAGGCAAGCCTGCCGA-3' | | | | |
| Adh-F | 5'-GGATCCGGTGATTATGAAGGCTGTAAC-3' | Biodegradative | 1146 | 98°C – 30 secs. 30 cycles (98°C for 10 secs, 56°C for 30 secs, 72°C for 30 secs) and 72°C for 5 min. | This study |
| Adh-R | 5'-CTCGAGTTAAGGCTTCAGGACAAATT-3' | | | | |
| Aldh-F | 5'-ATGGACATGGACAGGTTTAAAAAATTCTCCAT-3' | Biodegradative | 1632 | 98°C – 30 secs. 30 cycles (98°C for 10 secs, 58°C for 30 secs, 72°C for 30 secs) and 72°C for 5 min. | This study |
| Aldh-R | 5'-GCCCCCAGGGTGATGGAGTAA-3' | | | | |
| Sfp-F | 5'- ATGAAGATTTACGGAATTTA -3' | Biosurfactant | 675 | 94°C – 25 secs. 30 cycles (98°C for 10 secs, 46°C for 30 secs, 72°C for 1.5 min) and 72°C for 10 min. | (Porob et al., 2013) |
| Sfp-R | 5'- TTATAAAAGCTCTTCGTACG -3' | | | | |
| Nrps-F | 5'-CATATGATGAACGCCACACGGATG-3' | Biosurfactant | 2772 | 98°C – 30 secs. 30 cycles (98°C for 10 secs, 56°C for 30 secs, 72°C for 30 secs) and 72°C for 5 min. | This study |
| Nrps-R | 5'-CTCGAGCTAGACGAGTATTTTTTTGGA-3' | | | | |

### *4.2.8 Surface activity and surface tension*

The *Paenibacillus* sp. D9 containing medium was tested for surface activity. ST was determined using precipitated supernatant obtained during the production process, at room temperature (25ºC) as described in Gudiña et al. (2012). K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. For proper result analysis, ST of BH medium supplemented with diesel fuel and *n*-hexadecane were initially measured as controls. The surface activity was determined as below.

**Surface activity** = ST of uninoculated medium – ST of supernatant (Jimoh and Lin, 2019b).

### *4.2.9 Pseudo-solubilization of n-hexadecane hydrocarbon*

In the pseudo-solubilization experiment, *Paenibacillus* sp. D9 bacterial strain was grown in 50 mL BH in 250 mL conical flask with 2% (v/v) *n*-hexadecane, incubated at 30ºC and 150 rpm for 10 days in an orbital shaker. The sample was taken at 2-day intervals and centrifuged at 13,500 × *g*, at 4ºC for 10 min to determine the effect of BioS released on *n*-hexadecane solubilization. Uninoculated BH medium were also incubated as control in parallel utilizing the same conditions provided above. The cell-free broth was filtered through 0.45 and 0.22 µm Millipore membrane filters. Pseudo-solubilized *n*-hexadecane was extracted using *n*-hexane as a solvent and analyzed by GC under the same conditions as mentioned for *n*-hexadecane biodegradation determination. The solvent was maintained 80ºC for 2 min and subsequently increased to 300ºC with a 10ºC increase per min (Mishra and Singh, 2012).

### *4.2.10 Statistical analysis*

One-Way ANOVA was used to analyze the data in this research, followed by Pearson's correlation coefficient test utilizing GraphPad Prism program. Data were conducted in three independent experiments and presented as mean ± SD.

## 4.3 Results

### *4.3.1 Cell surface hydrophobicity and growth of Paenibacillus* sp. D9 *in diesel fuel and n-hexadecane*

One of the important parameters to define the adhesion of HC to bacteria cell is CSH. In this current research, *Paenibacillus* sp. D9 presented a very high level of CSH to diesel fuel and *n*-hexadecane (Table 4.2). CSH values were documented to be 79.00% with diesel as a substrate and 71.00% with *n*-hexadecane contained medium after 30 min. The cell surface hydrophobicity to the HCs were also stable at the different time intervals (1 h, 12 h, and 24 h, respectively) showing a good hydrophobicity of the bacterium over an extended period (Table 4.2).

**Table 4.2** The cell surface hydrophobicity of *Paenibacillus* sp. D9 against 2% (v/v) diesel fuel and 2% (v/v) *n*-hexadecane

| **Hydrocarbons** | **Cell surface hydrophobicity** | | | |
|---|---|---|---|---|
| **Time** | **30 min** | **1 h** | **12 h** | **24 h** |
| Diesel fuel | 79.00% ± 0.01 | 78.60% ± 0.04 | 78.70% ± 0.07 | 78.80% ± 0.06 |
| *n*-Hexadecane | 71.00% ± 0.02 | 70.00% ± 0.05 | 69.20% ± 0.08 | 71.20% ± 0.1 |

Values are the means of three replications (n=3).

The bacterial growth (*Paenibacillus* sp. D9) in BH medium with 2% *n*-hexadecane and 2% diesel fuel was examined (Figure 4.1). There was a continued increase in bacterial growth through the 14-day incubation period with subsequent decrease in *n*-hexadecane-containing medium due to complete utilization of the *n*-alkane. However, *Paenibacillus* sp. D9 increased faster in the *n*-hexadecane medium than diesel fuel induced medium during the incubation. In *n*-hexadecane-induced medium, exponential growth was observed between day 0 and day 2 with bacterium initializing immediately at the initialization of the experiment. The exponential growth phase was further attained on day 4, with the *Paenibacillus* sp. D9 bacterial growth almost stabilized for an upward to the termination of the experiment. For diesel fuel-induced medium, there was a late growth phase as multiplication of cells occurred between day 2 to day 6. Bacterial growth in BH supplemented with *n*-hexadecane (2%) and diesel fuel (2%) showed the utilization of both substrates for cell growth and

multiplication. There was a stationary phase observed between day 6 and day 10 for the diesel fuel-induced medium followed by a successive increase in growth until day 14 (termination of the experiment).

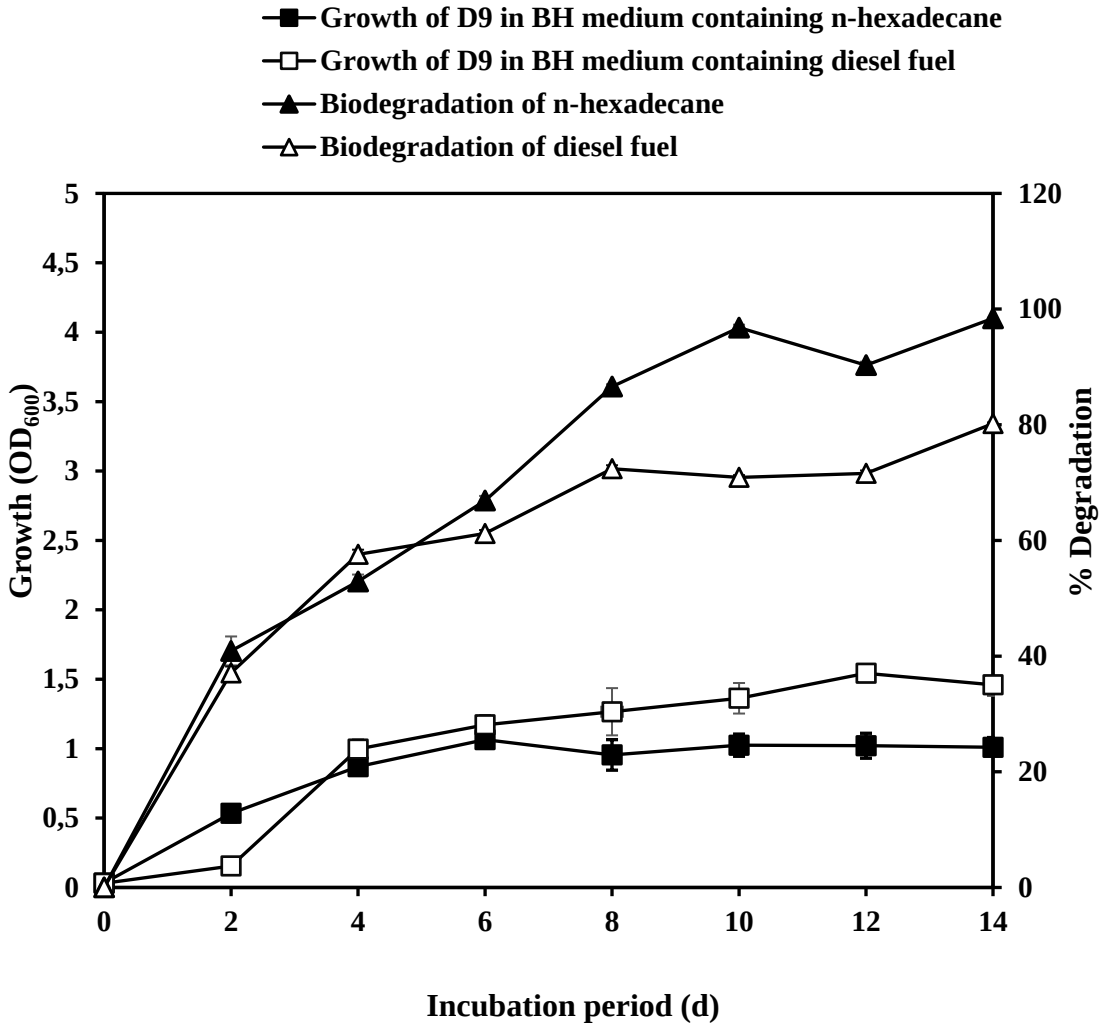


**Figure 4.1** Transformation of diesel fuel and *n*-hexadecane in a Bushnell Haas medium at 30ºC (a) Growth pattern of *Paenibacillus* sp. D9 bacterial strain on 2% (v/v) diesel fuel and 2% (v/v) *n*-hexadecane induced medium (b) Percent degradation of 2% (v/v) diesel fuel and 2% (v/v) *n*-hexadecane. The consumption of diesel fuel and *n*-hexadecane was quantified by GC-FID. In the growth assay, stoichiometric conversion of 2% diesel fuel (v/v) and 2% *n*-hexadecane (v/v) were achieved by *Paenibacillus* sp. D9. The molecular mass of diesel fuel should be 198-202 g/mol while that of *n*-hexadecane was 226.41 g/mol. Values are of mean ± SD; n = 3.

### *4.3.2 Biodegradation of n-hexadecane and diesel fuel*

The degradation of *n*-hexadecane (aliphatic long-chain HC) and diesel fuel (consists of saturated and aromatic HCs) by *Paenibacillus* sp. D9 was analyzed for 14 days by GC-FID. Experimental GC/FID result revealed that *Paenibacillus* sp. D9 can degrade aliphatic long-chain HC fractions, with detectable HCs peaks completely utilized within the 14-day incubation period. The bacterial strain *Paenibacillus* sp. D9 was a proficient HC degrader because, in 2 days of incubation, it degraded *n*-hexadecane by 40.9%. Immediately after, there was an upsurge in degradation level corresponding to an increase in the period of incubation (Figure 4.1).

The biodegradation rate was read based on the GC chromatograms of remaining diesel fuel. The identity of the peaks was established by the corresponding retention time of the chromatograms. There was 57.6% degradation of diesel fuel in 4 days of incubation by *Paenibacillus* sp. D9. The resolved peaks in the diesel fuel chromatograms were observed to be of carbon lengths ranging from C9–C26. There was further intensification in the biodegradation of diesel fuel proportional to an increase with the incubation period up to 6 days with subsequent stabilization. However, diesel fuel degradation continued to increase and was stable throughout the entire experimental period. All the high peaks detected from the control chromatogram disappeared in the test samples inoculated as the incubation period progresses. At the end of the experiment, *Paenibacillus* sp. D9 degraded *n*-hexadecane (98.4%), and diesel fuel (80.2%), as the increase in growth was simultaneous with a reduction in the concentration of *n*-hexadecane, and diesel fuel.

### *4.3.3 pH of incubation medium*

The pH was 7.0 at the beginning of the experiment in both *n*-hexadecane and diesel fuel-induced media. There was a slight decrease in pH of both media observed on the control after the completion of 14 days incubation. The decrease in pH tends towards the acidic range indicating the development of acidic intermediates during biodegradation of diesel fuel and *n*-hexadecane. For the control 1 (diesel fuel) and control 2 (*n*-hexadecane) experiment, no significant drop in pH was observed (Table 4.3). Results obtained from this research showed similarity to the reduction in pH of both HC-induced media.

**Table 4.3** Medium pH changes during 14 days of incubation of *Paenibacillus* sp. D9 in a BH medium containing 2% (v/v) diesel fuel and 2% (v/v) *n*-hexadecane

| Day | Control 1 | Diesel fuel | Control 2 | *n*-Hexadecane |
|---|---|---|---|---|
| Day 0 | 7.00 ± 0.01 | 7.00 ± 0.03 | 7.00 ± 0.01 | 7.00 ± 0.02 |
| Day 2 | 6.98 ± 0.00 | 6.10 ± 0.02 | 6.97 ± 0.02 | 4.40 ± 0.01 |
| Day 4 | 6.92 ± 0.01 | 5.60 ± 0.03 | 6.96 ± 0.02 | 4.30 ± 0.04 |
| Day 6 | 6.92 ± 0.02 | 5.10 ± 0.01 | 6.95 ± 0.00 | 3.70 ± 0.01 |
| Day 8 | 6.91 ± 0.04 | 4.90 ± 0.01 | 6.94 ± 0.02 | 3.50 ± 0.02 |
| Day 10 | 6.89 ± 0.02 | 3.90 ± 0.02 | 6.89 ± 0.01 | 3.40 ± 0.01 |
| Day 12 | 6.87 ± 0.00 | 4.00 ± 0.02 | 6.86 ± 0.00 | 3.40 ± 0.00 |
| Day 14 | 6.87 ± 0.02 | 3.70 ± 0.01 | 6.86 ± 0.00 | 3.40 ± 0.01 |

Values are of mean ± SD; n = 3.

### *4.3.4 Bacterial protein*

The total concentration of the bacterial protein increased continually with the duplication of cells in both the diesel fuel and *n*-hexadecane-induced medium. The initial protein concentration varied between 0.05 and 0.04 mg/mL with a subsequent increase to 0.16 and 0.33 mg/mL at day 2 in both the diesel fuel and *n*-hexadecane medium respectively (Figure 4.2). The bacterial development in BH throughout the incubation period was successively related to improved protein content (Figure 4.2). This pattern reflects that the bacterial strain could have utilized the hydrophobic substance as a source of carbon and energy. However; at the end of the experiment (day 14), the final maximum value attained in diesel fuel induced medium was 0.69 mg/mL protein whereas, a maximum value (0.72 mg/mL) was detected in *n*-hexadecane induced medium (day 6). In the diesel fuel contained medium, protein concentration was enhanced by 14-fold throughout the bioproduction process while an 18-fold increase was observed in the *n*-hexadecane-induced medium.

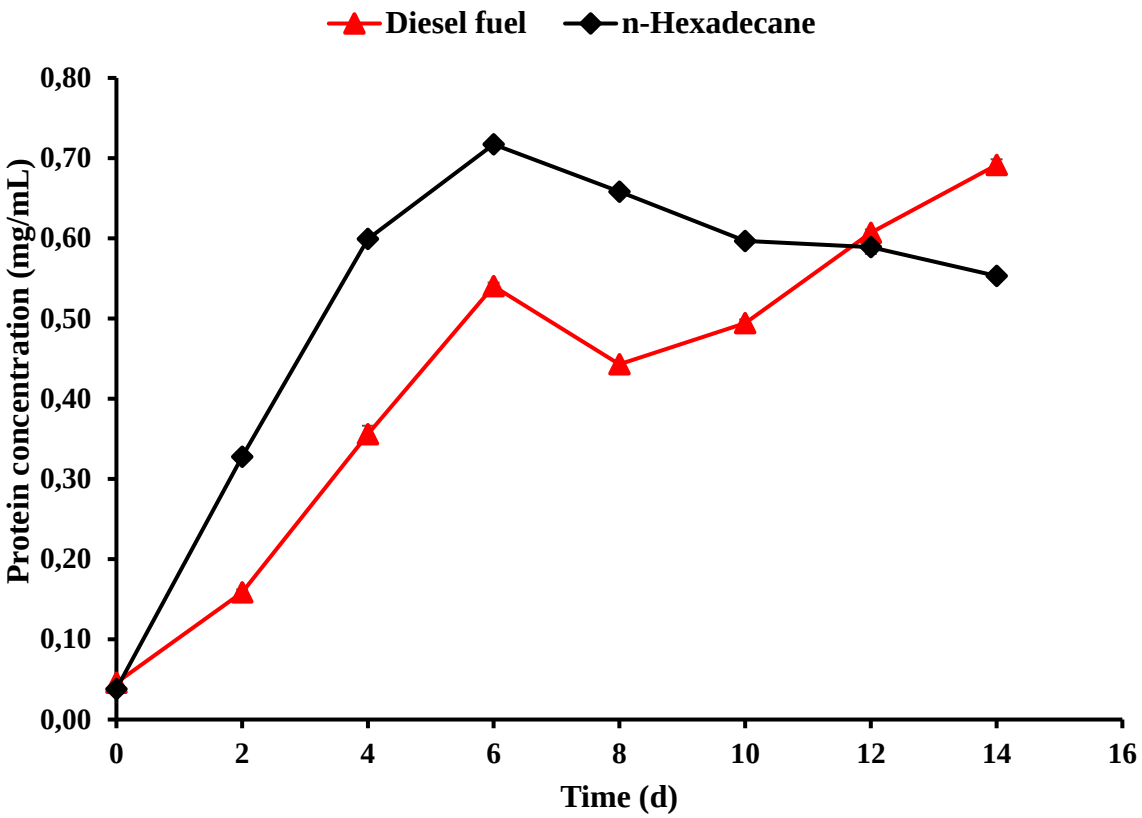


**Figure 4.2** Bacterial protein during the growth of *Paenibacillus* sp. D9 on 2 % (v/v) diesel fuel and 2% (v/v) *n*-hexadecane induced medium. Values are of mean ± SD; n = 3

### *4.3.5 Degradative enzymes and biosurfactant activity*

Alkane hydroxylase, alcohol dehydrogenase, and esterase are general degradative enzymes involved in HC biodegradation process ( Chen et al., 2019; Jauhari et al., 2014; Singh et al., 2012). The induction of these three enzymes through diesel fuel and *n*-hexadecane degradation were studied to evaluate their significance in the biodegradation process.

#### 4.3.5.1 Alkane hydroxylase activity

There was induction of alkane hydroxylase both in presence of *n*-hexadecane and diesel fuel degradation. In this research, more than 98.4% of *n*-hexadecane (C16) and 80.2% of diesel fuel were utilized by *Paenibacillus* sp. D9, due to excessive synthesis of hydroxylase enzyme during the biodegradation process. Initially, the induction of alkane hydroxylase was slow in diesel fuel as compared to the *n*-hexadecane induced medium but increased significantly from day 6 (50.90 µmol/mg protein) to day 8 (76.50 µmol/mg protein) of the incubation period. Similarly, enhanced induction and activity of alkane hydroxylase was observed in the *n*-hexadecane-

containing medium. The alkane hydroxylase activity was high, augmented with increased incubation period simultaneous with high protein content. In the two media, i.e., diesel fuel and *n*-hexadecane, the maximum activity of alkane hydroxylase was recorded as 76.50 µmol/mg protein after 8 days of incubation, while the highest was 82.33 µmol/mg protein in *n*-hexadecane-induced medium, following 6 days of incubation (Figure 4.3). However, there was a preference for alkane hydroxylase enzyme in C16 HC rather than diesel fuel which is a representation of aliphatic and aromatic group of HCs. The enzyme subsequently decreased in both induced media (*n*-hexadecane and diesel fuel) towards the end of the incubation period.

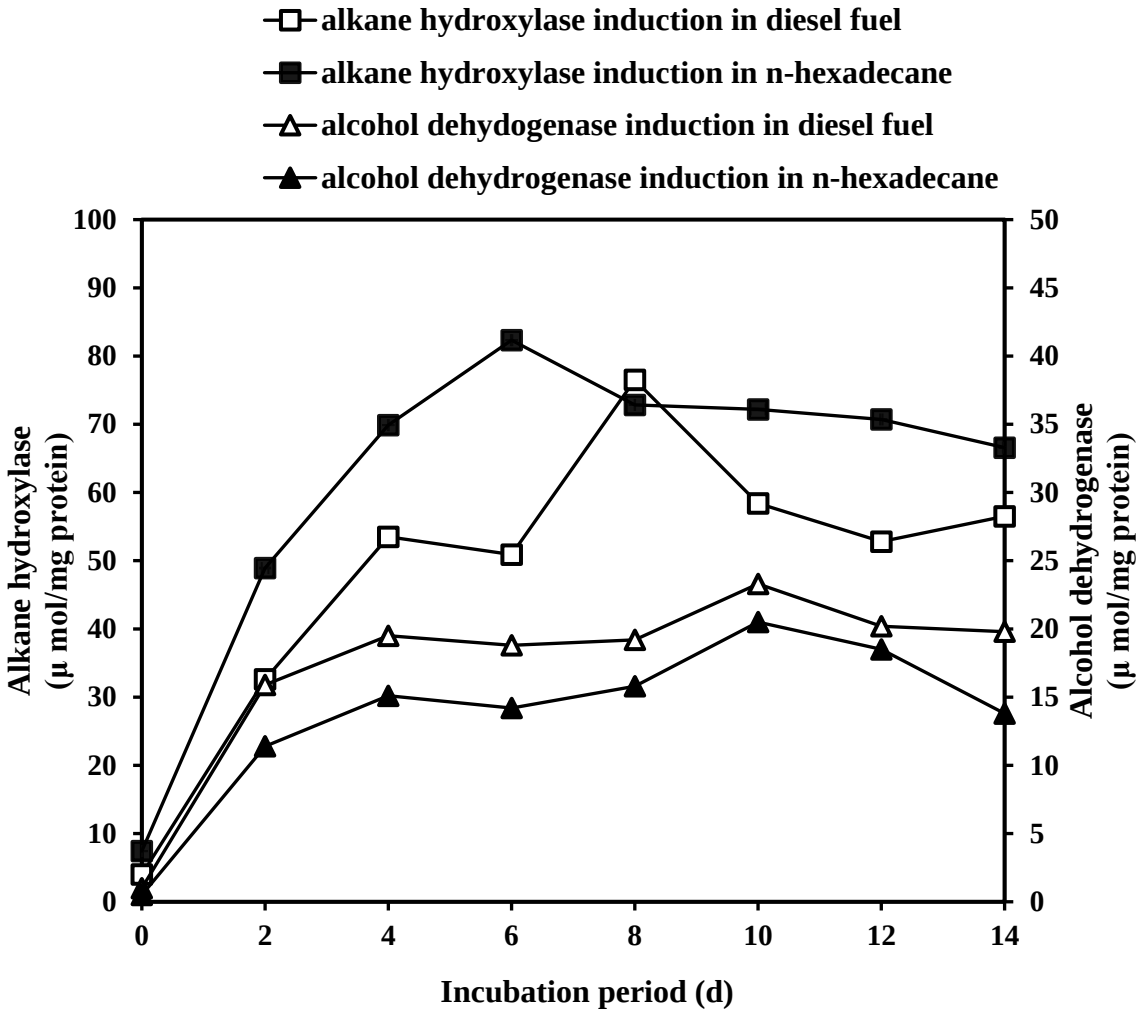


**Figure 4.3** Induction of alkane hydroxylase and alcohol dehydrogenase enzyme by *Paenibacillus* sp. D9 during 2% (v/v) diesel fuel and 2% (v/v) *n*-hexadecane degradation in a BH medium at 30°C. During the degradative assays, a stoichiometric conversion of diesel fuel (2% v/v) and *n*-hexadecane (2% v/v) were achieved by *Paenibacillus* sp. D9. The molecular mass of diesel fuel should be 198-202 g/mol while that of *n*-hexadecane is 226,41 g/mol. Values are of mean ± SD; n = 3

#### 4.3.5.2 Alcohol dehydrogenase activity

The aerobic degradation begins by the oxidation of a terminal methyl group to primary alcohol, by means of alcohol hydroxylase utilizing NADH rubredoxin and rubredoxin reductase. Alcohol dehydrogenase further dehydrogenate the aldehyde to alcohol. The increase in absorbance at 340 nm confirmed $NAD^+$ formation and parallel to oxidation of the aliphatic alcohols was used to determine the alcohol dehydrogenase activity. The activity of alcohol dehydrogenase was lower when compared to alkane hydroxylase activity in both media. The level of alcohol dehydrogenase induction was in parallel to the degradation of *n*-hexadecane and diesel fuel, thus highlighting the significance of this enzyme in the biodegradation process. The alcohol dehydrogenase activities ranged maximum between day 8 and day 12 in both *n*-hexadecane and diesel fuel induced media (Figure 4.3). The maximum activity of alcohol dehydrogenase was observed as 23.00 µmol/mg$^-$protein, on day 10. Likewise, in the same period, the alcohol dehydrogenase enzyme in the presence of C16 had a maximum activity of 20.50 µmol/mg protein (Figure 4.3). Furthermore, there was a subsequent reduction in the alcohol dehydrogenase towards the termination of the experiment.

### 3.5.3 Esterase activity

All enzymes tested in both media, in triplicate, displayed detectable specific esterase activity on the synthetic substrate (4-nitrophenyl acetate). There was a positive correlation (diesel fuel; r = 0.98, *n*-hexadecane; r = 0.96) between esterase activity and BioS activity in *Paenibacillus* sp. D9 as esterase production improved gradually like emulsifying activity. This is due to excessive production of emulsion in the diesel fuel-containing medium indicating its proportionality to the release of esterase and *vice versa* (Table 4.4). The esterase activity was higher in the presence of the diesel fuel-containing medium regardless of the incubation time measured, as compared to *n*-hexadecane-containing medium. In the diesel fuel-induced medium, there was an increase in the esterase activity of (0.008 to 0.220) µmol/mg protein/min from the initial day of incubation till day 10 (Table 4.4). The esterase activity was at a maximum at day 10 of the incubation (0.220 µmol/mg protein/min), thereafter a swift drop was observed until the climax of the experiment. There was a similar observation associated with *n*-hexadecane induced medium. In this case, the esterase activity was at its maximum at day 8 (0.183 µmol/mg protein/min) of the incubation with slight reduction observed from day 10 towards the termination of the experiment (0.174 ─ 0.162 µmol/mg protein/min). There was a slow decline in esterase enzyme production as it reached maximum akin to decrease in bacterial growth explained in Figure 4.1.

#### 4.3.5.4 Emulsifying activity

The emulsifying activities of the BioS supernatant produced by *Paenibacillus* sp. D9 against 1:1 (vol/vol) mixture of diesel fuel and 2-methylnaphthalene were summarized in Table 4.4. Regarding the O/W emulsifying activity, the BioS produced by *Paenibacillus* sp. D9 in diesel fuel induced medium had an emulsification rate ranging from 0.10 to 28.10 UE using diesel fuel and 2-methylnaphthalene as substrate respectively. The best emulsifying activity of 29.20 and 28.40 UE were observed at day 12 and day 14 (Table 4.4). Also, in the *n*-hexadecane induced medium, the BioS produced emulsification rates ranging from 0.10 to 17.30 UE. With respect to water in oil emulsifying activity, the BioS produced by the bacterium *Paenibacillus* sp. D9 induced in diesel fuel had an emulsification index rate of 65.70% as the lipid phase while the same organism produced a lower emulsification rate of 58.70% in a medium induced with *n*-hexadecane.

**Table 4.4** Esterase activity (EA), water in oil (W/O), oil in water (O/W) emulsifying activities obtained using *Paenibacillus* sp. D9

| Time (days) | $A_{600}$ | EAO/W (UE) | EAW/O (%) | EA |
|---|---|---|---|---|
| **Diesel fuel induced medium** | | | | |
| **0** | 0.01 ± 0.01 | 0.10 ± 0.01 | 0.03 ± 0.05 | 0.008`± 0.006 |
| 2 | 0.31 ± 0.23 | 3.10 ± 0.23 | 27.78 ± 0.08 | 0.110 ± 0.005 |
| 4 | 1.20 ± 0.01 | 12.00 ± 0.01 | 40.65 ± 0.12 | 0.143 ± 0.009 |
| 6 | 1.74 ± 0.07 | 17.40 ± 0.07 | 49.61 ± 0.09 | 0.174 ± 0.009 |
| 8 | 2.20 ± 0.02 | 22.00 ± 0.02 | 59.55 ± 0.07 | 0.214 ± 0.023 |
| 10 | 2.71 ± 0.01 | 27.10 ± 0.01 | 60.70 ± 0.10 | 0.220 ± 0.011 |
| 12 | 2.92 ± 0.01 | 29.20 ± 0.01 | 65.70 ± 0.11 | 0.202 ± 0.091 |
| 14 | 2.84 ± 0.02 | 28.40 ± 0.02 | 61.24 ± 0.08 | 0.173 ± 0.007 |
| ***n*-Hexadecane induced medium** | | | | |
| 0 | 0.01 ± 0.03 | 0.10 ± 0.03 | 0.05 ± 0.07 | 0.112 ± 0.002 |
| 2 | 0.68 ± 0.01 | 6.80 ± 0.01 | 29.42 ± 0.09 | 0.120 ± 0.008 |
| 4 | 1.19 ± 0.01 | 11.90 ± 0.01 | 39.10 ± 0.1 | 0.158 ± 0.031 |
| 6 | 1.12 ± 0.01 | 11.20 ± 0.01 | 48.20 ± 0.12 | 0.150 ± 0.018 |
| 8 | 1.73 ± 0.05 | 17.30 ± 0.05 | 58.70 ± 0.06 | 0.183 ± 0.003 |
| 10 | 1.58 ± 0.01 | 15.80 ± 0.01 | 54.30 ± 0.13 | 0.174 ± 0.026 |
| 12 | 1.28 ± 0.02 | 12.80 ± 0.02 | 51.35 ± 0.10 | 0.169 ± 0.007 |
| 14 | 1.25 ± 0.01 | 12.50 ± 0.01 | 52.65 ± 0.08 | 0.162 ± 0.009 |

EA: Esterase activity, UE: Unit emulsification EAW/O: water in oil emulsifying activity, and EAO/W: oil in water emulsifying activity

Values are of mean ± SD; n = 3.

### *4.3.6 Amplification of biosurfactant degradative genes*

Six pairs of primer were used for amplifying genes involved in HC biodegradation and BioS-mediated biosynthesis from *Paenibacillus* sp. D9. The primers *sfp*-F and *sfp*-R amplified a 675 bp region of the *sfp* gene (Figure 4.4) encoding 4'-phosphopantetheinyl transferase involved in lipopeptide BioS biosynthesis Also,

amplified using primers *nrps*-F and *nrps*-R is a 2772 bp of *nrps* gene which is a large multifunctional non-ribosomal peptide synthetase. The two genes highlighted are both responsible for BioS production by the *Paenibacillus* sp. D9 strain in medium induced with hydrophobic mixtures. In this study *alkB* (660 bp), *alkM* (1062 bp), *adH* (1146 bp), and *aldH* (1632 bp) genes were also successfully amplified using their respective primers and conditions (Figure 4.4).

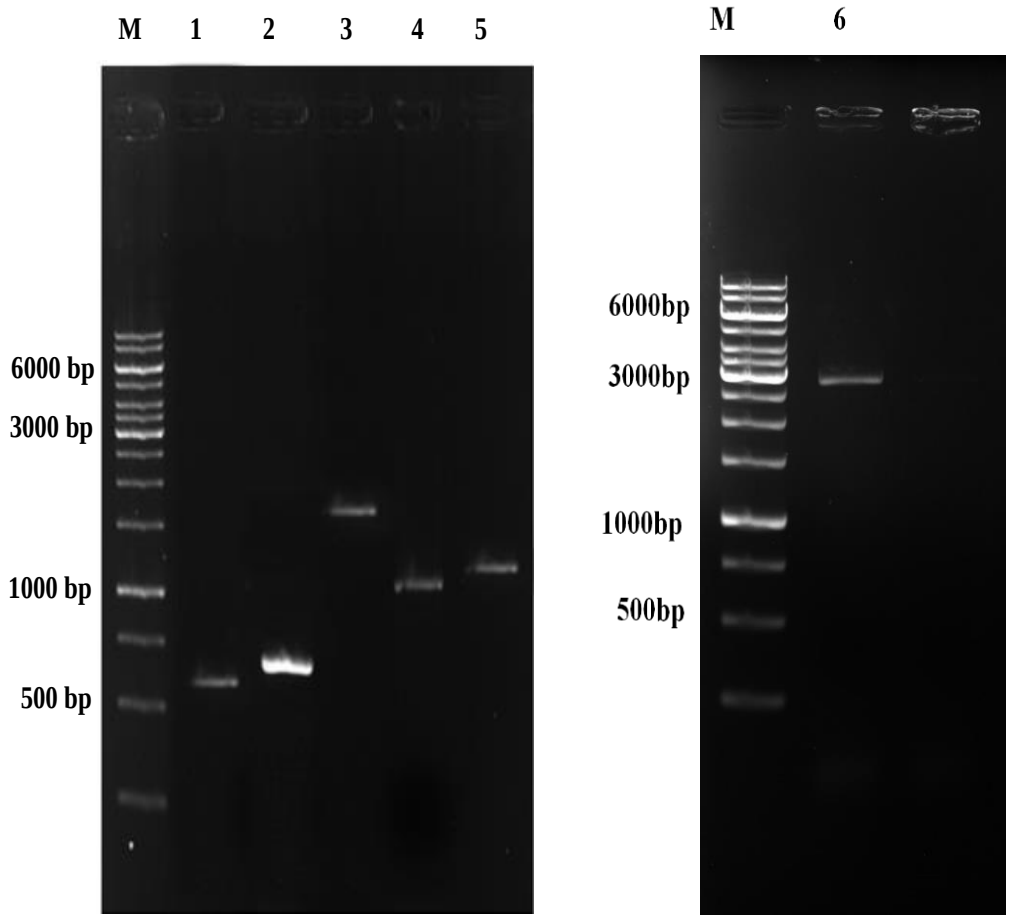


**Figure 4.4** Amplification of degradative and biosurfactant biosynthetic genes blank, M: DNA ladder (Thermo Fisher Scientific), Lane 1: *alkB* (660 bp), Lane 2: *sfp* (675 bp), Lane 3: *aldH* (1632 bp), Lane 4: *alkM* (1062 bp), Lane 5: *adh* (1146 bp), and Lane 6: nrps (2772 bp)

### *4.3.7 Surface tension and surface activity*

The ST was reduced in both induced media (diesel fuel and *n*-hexadecane). Initially, the ST of the two media were 63.4 mN/m and 64.1 mN/m at 0 days, and reduced to 31.6 and 32.6 mN/m, respectively after day 2 of

incubation (Table 4.5). High ST activities were continued in both media from day 2 of incubation till the termination of the experiment. ST reduction of 32.1mN/m (diesel fuel), and 32.8mN/m (*n*-hexadecane) was maintained at the end of the experiment (day 14). The ST activities were maintained in both media, thus showing the significant impact of BioS activity. The STs achieved in this research showed high influence of the BioS synthesized as the control sample containing the diesel fuel, and *n*-hexadecane reduced from 71.4 mN/m to 66.9 mN/m, and 67.8 mN/m) respectively. Because of ST decrease, the surface activity extended maximally prompting quick solubilization of *n*-hexadecane and diesel fuel. This showed the BioS was produced in aqueous medium during the degradation process.

**Table 4.5** Change in surface tension and surface activity with 2% (v/v) diesel fuel and 2% (v/v) *n*-hexadecane incubated with *Paenibacillus* sp. D9 bacterial strain

| **Surface tension (mN/m)** | | | **Surface activity (mN/m)** | | |
|---|---|---|---|---|---|
| **Day** | Diesel fuel | *n*-Hexadecane | **Day** | Diesel fuel | *n*-Hexadecane |
| Day 0 | 63.4 ± 0.2 | 64.1 ± 0.4 | Day 0 | 3.7 ± 0.2 | 2.5 ± 0.3 |
| Day 2 | 28.6 ± 0,3 | 32.6 ± 0.5 | Day 2 | 34.5 ± 0.3 | 30 ± 0.1 |
| Day 4 | 28.9 ± 0.1 | 30.0 ± 0.1 | Day 4 | 34.2 ± 0.1 | 32.6 ± 0.4 |
| Day 6 | 32.9 ± 0.2 | 29.8 ± 0.3 | Day 6 | 30.2 ± 0.2 | 32.8 ± 0.5 |
| Day 8 | 32.2 ± 0.4 | 30.1 ± 0.1 | Day 8 | 30.9 ± 0.4 | 32.5 ± 0.2 |
| Day 10 | 32.0 ± 0.5 | 30.3 ± 0.4 | Day 10 | 31.1 ± 0.5 | 32.3 ± 0.3 |
| Day 12 | 31.6 ± 0.3 | 32.2 ± 0.3 | Day 12 | 31.5 ± 0.3 | 30.4 ± 0.4 |
| Day 14 | 32.1 ± 0.1 | 32.8 ± 0.2 | Day 14 | 31.0 ± 0.1 | 29.8 ± 0.1 |
| Control | 63.1 ± 0.3 | 62.6 ± 0.1 | Control | 0.0 | 0.0 |

Values are of mean ± SD; n = 3.

### *4.3.8 Pseudo-solubilization of n-hexadecane*

The quantity of pseudo-solubilized *n*-alkane in the water phase was analyzed as shown in Figure 4.5. For the ST and surface activity of *Paenibacillus* sp. D9, concentrations of pseudo-solubilized *n*-hexadecane in the water phase increased as time went on during the 10-day incubation time. The concentration of both *n*-hexadecane and BioS decreased markedly at the very beginning indicating the adsorption of *n*-hexadecane/BioS to the cells (Figure 4.5). The BioS significantly enhanced the removal of 84.40% pseudo-solubilized *n*-hexadecane at the end of the experiment. The increase in growth of *Paenibacillus* sp. D9 was proportional to pseudo-solubilization thus yielding maximum activity. Owing to amphiphilic property;

*Paenibacillus* sp. D9 BioS emulsified *n*-hexadecane hydrocarbon accordingly, improving the water solvency, diminishing surface activity, and intensifying the displacement of the pollutant over the cell layer. Due to the water insolubility of the *n*-hexadecane, the hydrophobic nature of *Paenibacillus* sp. D9 cell surface played a major part in the pseudo-solubilization process. The BioS stimulated the substrate uptake by the bacterial cell. The rate of *n*-hexadecane pseudo-solubilization was sufficiently high (84.40%) and uptake happened principally from the pseudo-solubilized substrate. Thus, leading to confirmation that mediated hydrocarbon transport was responsible for relatively fast utilization of *n*-hexadecane by *Paenibacillus* sp. D9. In relation to the control experiment (Figure 4.5), there was no observation of pseudo-solubilization due to no release of the bio-molecule to enable the process.

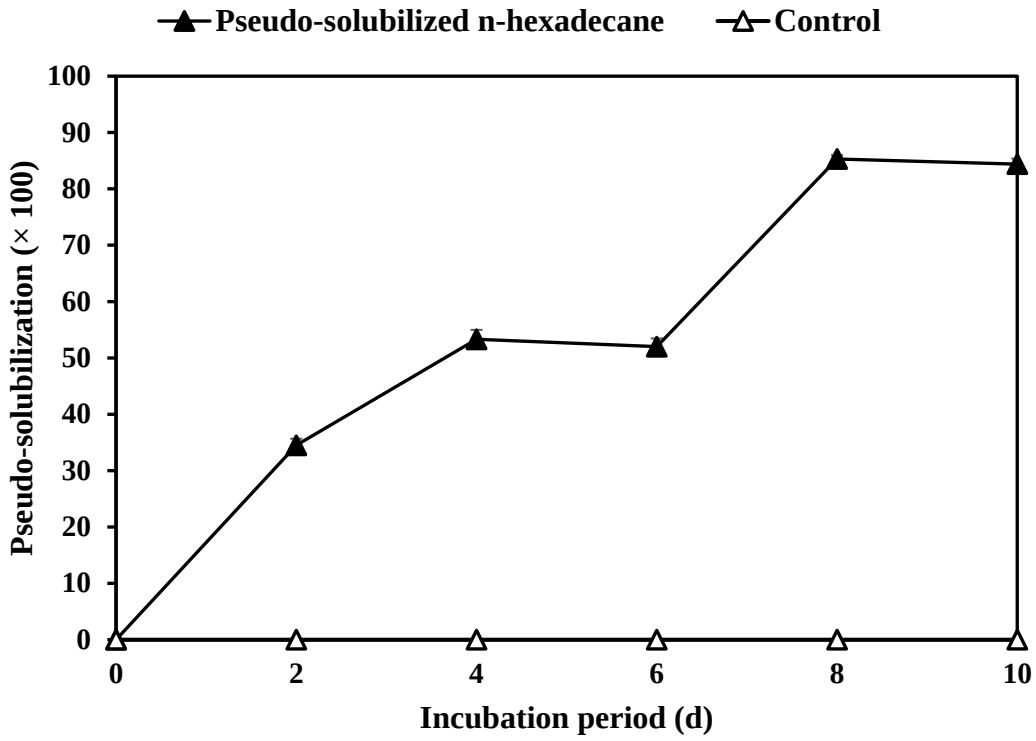


**Figure 4.5** Pseudo-solubilization of (2%) *n*-hexadecane induced by *Paenibacillus* sp. D9 biosurfactant. Control sample was made up of BH medium with no *n*-hexadecane. Values are of mean ± SD; n = 3.

## 4.4 Discussion

The cell surface hydrophobicity (CSH) may be regarded as the most relevant parameter to assay microbes' potential for biodegradation (Das, 2014). *Paenibacillus* sp. D9 possess high cell adherence to both *n*-hexadecane and diesel fuel enabling effective solubilization and production of surface-active compounds, termed *BioS.* There was no significant difference ($p < 0.05$) in the CSH values of both *n*-hexadecane and diesel fuel over an extended time interval. There was a higher CSH showing better acclimatization of *Paenibacillus* sp. D9 for diesel fuel compared to *n*-hexadecane which could be due to long chain (C9-C26) HCs present. Mishra and Singh (2012) reported a higher CSH 99.86% for *Pseudomonas aeruginosa* sp. PSA5, 96.4% for *Ochrobactrum* sp. P2, and *Rhodococcus* sp. NJ2, in minimal salt medium-enriched with *n*-hexadecane. *Trichosporon asahii* showed high CSH to diesel fuel (Chandran and Das, 2010) and *Acinetobacter* sp. DRY12 showed 75% hydrophobicity towards *n*-hexadecane (Dahalan et al., 2014). Tebyanian et al. (2013) also reported low CSH of *S. maltophilia* Q1, *S.maltophilia* M2, and *T. tyrosinosolvens* Q3, (24%, 6%, and 29% respectively) in *n*-hexadecane-containing medium.

This study revealed effective biodegradation of 80.2% for diesel fuel (2% v/v), and 98.4% for *n*-hexadecane (2% v/v) after 14 days of incubation (Figure 4.1). There was difference in mechanism for hydrocarbon uptake and utilization by *Paenibacillus* sp. D9 as the degradation rate in *n*-hexadecane was higher than that of diesel fuel. This would account for the improved CSH of *Paenibacillus* sp. D9 throughout the growth phase. Mohanty and Mukherji (2008) reported similarities in the mechanism of *B. cepacia* for C16 and diesel fuel uptake and a different uptake mechanism observed for *Exiguobacterium aurantiacum.* The discoveries propose the ability to degrade *n*-hexadecane is greater than diesel fuel as aromatic HC components of diesel fuel are difficult to degrade. Diesel fuel is one of the major environmental and ecological pollutants composed of about 25% aromatic HCs (alkylbenzenes and naphthalene) with 75% saturated HCs (primarily paraffin including *n*-, iso-, and cycloparaffins) (Perera, 2015). The components present in the diesel fuel are more recalcitrant than *n*-hexadecane which is an aliphatic long-chain HC. The observed increase in the growth values as provided by this data was agreeable with the other studies on HC degradation (Nwinyi, 2011; Nwinyi et al., 2014). The specific growth rate of *Paenibacillus* sp. D9 when supplemented with diesel fuel and *n*-hexadecane was found to be 0.29 d and 0.38 d in comparison to Meng et al. (2017) that the specific growth rate of *Pseudomonas synxantha* LSH-7 on 1% *n*-hexadecane was found to be 0.7 d. However, in both controls, the specific growth rate was relatively low and documented to be 0.005 d and 0.01 d. In comparison to other research, the specific

growth rate of the bacterial strains E9, BP10 for the aerobic degradation of hexacosane were 0.56 d and 0.48 d respectively (Jauhari et al., 2014). The specific growth rate of diesel fuel- containing medium value in this research is higher than other reported values at 0.0154 and 0.0125 ($h^{-1}$), for alkane-degrading *Pseudomonas frederiksbergensis* and *Rhodococcus erythropolis* (Abdel Megeed and Mueller, 2009), and lower than *Burkholderia* sp. strain DRY27**,** *Rhodococcus rubber*, and *Rhodococcus erythropolis* grown on diesel fuel with maximum growth rates of 0.305, 0.086 and 0.123 h, respectively (Ahmad et al., 2014; Zhukov et al., 2007).

During the growth of *Paenibacillus* sp. D9 on *n*-hexadecane and diesel fuel, pH of the bacterial culture reduced from 7.0 to 3.4, and 3.7, respectively, at the end of the experiment. This change in medium pH may have been induced by acidic intermediates generated from the solubilization of *n*-hexadecane and diesel fuel. In aerobic metabolic pathways, intermediates such as aldehydes, ketones, and ester, are usually synthesized for the duration of biodegradation. These intermediates are further degraded to simple carboxylic acids, acyl COA, which are readily utilized for cell exponentiation and energy requirement before entering tricarboxylic acid cycle through β-oxidation. There was pH reduction of the medium during hexacosane degradation by different bacterial cells and their consortium (Jauhari et al., 2014). In another research, pH of the bacterial culture reduced from 6.8 to 4.8 and 4.3 during the growth of B1 and B2 on *n*-hexadecane (Liu et al., 2012). Likewise, there was a drop in medium pH during fluoranthene degradation confirming the production of acidic intermediates (Kumari et al., 2012; Mishra et al., 2014).

In general, the increase in biodegradation was in correlation (diesel fuel; r = 0.98, *n*-hexadecane; r = 0.96) and accompanied by high emulsification, resulting in a greater oil-water interface. Additional evidence specifies that the larger the oil-water interface, the quicker the decomposition rate by microorganisms (Liu et al., 2012). The ability of this bacterial strain to produce degradative enzymes make them as an effective strain among others. This was comparable to a higher alkane hydroxylase activity by *Rhodococcus erythropolis* EK-1 during degradation of *n*-hexadecane (Pirog et al., 2010). Bacteria possess few genes for alkane hydroxylases and alcohol dehydrogenase which can degrade an extensive range of HCs. However, alkane hydroxylases and alcohol dehydrogenases play a significant part in HC degradation and the individual genes that encode these enzymes were also documented in previous reports (Hassanshahian et al., 2012; Wang et al., 2014). High activities of alkane hydroxylase (82 U), alcohol dehydrogenase (23 U), and esterase (0.220 U) were differentially prooduced, with enhanced BioS activity, indicating their involvement in HC degradation. As observed in this research, there exists a difference in activities, as the alkane hydroxylase activity was higher in *n*-hexadecane-induced medium, while the alcohol dehydrogenase activity was higher in diesel fuel-induced

medium. The overproduction of alkane hydroxylase is possibly due to substrate specificity for *n*-hexadecane which is a straight chain aliphatic HC. In contrast, diesel fuel contains a few numbers of aromatic HCs by which "OH" end is released during the degradation process, hence, the intensification of alcohol dehydrogenase. Alkane hydroxylase enzymes played a vital role in the degradation of crude oil and *n*-hexadecane by *Bacillus subtilis* A1, *Pseudomonas aeruginosa* PSA5, and *Rhodococcus* sp. NJ2 (Mishra and Singh, 2012; Parthipan et al., 2017). Similarly, peak activities of alkane hydroxylase enzyme were recorded as 527 nmol/mg protein in BP10 and 63 nmol/mg protein in E9 during degradation of hexacosane (Jauhari et al., 2014). There was also a higher alkane hydroxylase of 188 µmol/mg during degradation of crude oil by *Bacillus subtilis* A1 (Parthipan et al., 2017).

Therefore, *Alkb* (alkane hydroxylase), AlkM (alkane monooxygenase), Adh (alcohol dehydrogenase), and AldH (aldehyde dehydrogenase) are multiple proteins for hydroxylases accountable for the oxidation and biodegradation of low, medium, and high molecular weight chain alkanes (Lin and Sharma, 2013). Thus, *alkB* genes and the biodegradative genes (*alkM and adH*) have been used to monitor the bioremediation capabilities of petroleum-contaminated environments (Paisse et al., 2011). For the synthesis of BioS, *sfp* gene is a vital member of the *srfA* operon that codes for a non-ribosomal peptide synthetase complex. As such, *sfp* gene translates the phosphopantetheinyl transferase enzyme, required for the non-ribosomal peptide biosynthesis of BioS. In comparison to this research, *sfp* gene found in *Bacillus* species was found to be responsible for BioS synthesis particularly antibiotic production (Porob et al., 2013). Bunet et al. (2014) on the other hand, reported a single *sfp*-type that play a major role in the biosynthesis of NRPS derived metabolites in *Streptomyces ambofaciens* ATCC23877.

From this report, the two HC substrates (*n*-hexadecane and diesel fuel) served as the sole carbon source in the production medium, indicating lipopeptide synthesis is derived from either of the carbon source utilized. The whole genome sequence of *Paenibacillus* sp. D9 (JZEJ00000000) explained a proposed pathway for the biodegradation of hydrocarbons by sub-terminal oxidation associated with the synthesis of lipopeptide BioS (Figure 4.6). The pathway genes identified in the genome included alkane hydroxylase genes (*alkB*), alkane monooxygenase gene (*alkM*), alcohol dehydrogenase (*adH*), aldehyde dehydrogenase (*aldH*), as well as BioS synthesis-related genes, such as phosphothaenithyl transferase (*sfp*), and non-ribosomal peptide synthetase (*nrps*).

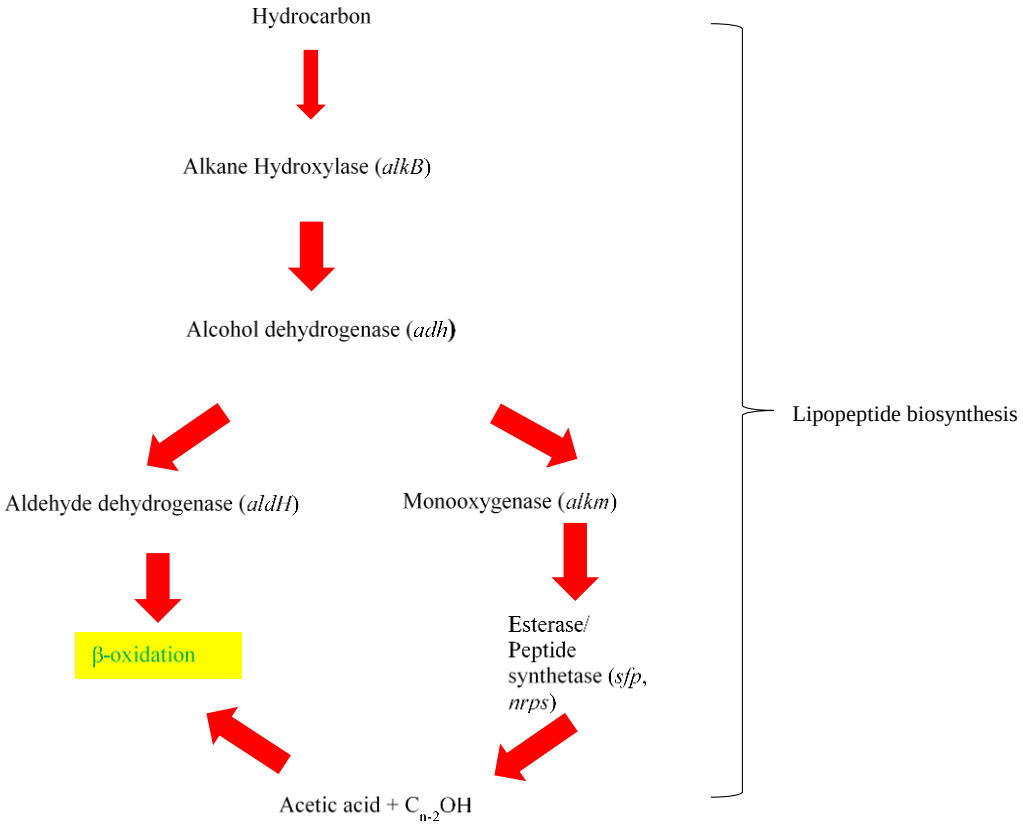


**Figure 4.6** The proposed schematic overview of the hydrocarbon degradation and the lipopeptide biosynthesis pathways by *Paenibacillus* sp. D9

In this investigation, the production of enzymes and BioS by *Paenibacillus* sp. D9 strain led to an increase in the effectiveness of biodegradation. Thus, 98.4% of *n*-hexadecane (C16) and 80.2% of diesel fuel (C9 ─ C25) were used as a source of carbon and energy by *Paenibacillus* sp. D9, which is because of alkane hydroxylase and alcohol dehydrogenase enzyme synthesis throughout the degradation process. Recently Parthipan et al. (2017) confirms that *Bacillus subtilis* A1 can produce BioS which displays effective uptake of HCs in crude oil. Similar to other research, *n*-hexadecane was biodegraded to 95% by *Rhodococcus* sp., 99% by *Pseudomonas aeruginosa* PSA5, NJ2 and 92% by *Ochrobactrum intermedium* P2 during 10 days of incubation (Mishra and Singh, 2012). Similarly, strain BP10 and E9 degraded 82% of hexacosane (50 ppm) after 7 days of incubation (Jauhari et al., 2014).

One of the major enzyme groups responsible for BioS production is the esterase. Much of the esterase produced by *Paenibacillus* sp. D9 in both media were extracellular dissimilar to sonicated cells which gave very low esterase activity. A similar mechanism in production was reported to *Bacillus subtilis* SK320 on different

substrates produced esterase extracellularly (Sekhon et al., 2011). The translational modification of the different gene products involved in BioS synthesis resulted in the multiplication of the esterase enzyme. Increase in the multiplication of the esterase was reported in the expression of BioS gene from endosulfan-degrading *Bacillus* sp. produced on olive oil (Khanna et al., 2009).

BioS properties over chemical surfactant mainly hinge on its capacity to reduce ST, and the formation of a steady emulsion (Rufino et al., 2014). From this research, the BioS produced in diesel fuel-induced medium showed better emulsifying activity than the *n*-hexadecane owing to the high CSH, impurities, and constituents. The emulsions formed were stable in both diesel fuel and *n*-hexadecane, respectively, after 24 h, with greater emulsifying activity of 65.7% and 58.7% being observed. Therefore, it can be resolved that good water in oil emulsifying activities were obtained for *Paenibacillus* sp. D9 BioS. *Stenotrophomonas maltophilia* M2, *S. maltophilia* Q2, and *Tsukamurella tyrosinosolvens*b Q3 degraded hexadecane faster owing to high emulsifying activity (Tebyanian et al., 2013). The W/O emulsifying activities achieved in other studies were comparable to those obtained in this study. Pinto et al. (2009) on the other hand obtained lower emulsifying activities (UE) of 17.9, 20.5, 23.5 and 24.8, using *Corynebacterium aquaticum* (experiment 1), *Corynebacterium aquaticum* and *Bacillus* sp. (experiment 2), *Corynebacterium* sp., *Bacillus cereus* and *Bacillus mycoides* (experiment 3) and *Bacillus subtilis* (experiment 4), respectively. Also, emulsification rates of 45 – 55 UE were obtained using sunflower oil and different HCs (diesel fuel, kerosene, heptane) as a lipidic oily layer with *Pseudomonas fluorescens* BioS (Abouseoud et al., 2008). Similarly, BioS produced by the fungus *Aspergillus* sp. while utilizing soybean oil in a submerged bio-process had an emulsification rate of 42.7% (Colla et al., 2010). *Paenibacillus* sp. D9 showed ST reduction ability indicating the production of BioS when tested on diesel fuel and *n*-hexadecane. *Paenibacillus* sp. D9 BioS showed a better ST value than that of another BioS from *Paenibacillus dendritiformis* (34 mN/m) (Bezza and Nkhalambayausi Chirwa, 2015), that of the BioS from *Paenibacillus* sp. 1C (32.6mN/m) (Mesbaiah et al., 2016), lipopeptide BioS from *Paenibacillus* alvei (35 mN/m) (Najafi et al., 2011), and BioS from *Paenibacillus macerans* TKU029 (35.34 mN/m) (Liang et al., 2014). In contrast, better ST values of 25.8 mN/m were demonstrated by *Staphylococcus* sp. strain 1E (Eddouaouda et al., 2012) and 25.42mN/m by *Bacillus licheniformis* TKU004 (Chen et al., 2012).

BioS synthesis is one of the techniques used to improve and complement the solubilization of the hydrophobic pollutants (Mishra and Singh, 2012). The degradability of hydrophobic organic compounds can be improved through pseudo-solubilization to enhance bioavailability of the HC (Hmidet et al., 2017). The release of BioS enhanced pseudo-solubilization of *n*-hexadecane signifying high surface-biomolecule activity. The outcome

conveyed here was different from other bacteria and surfactant systems (Zhong et al., 2014). Previous research also proposed the enhancement in the solubilization of 10% diesel fuel by BioS produced by *Paenibacillus* sp. D9 which followed a similar mechanism with the one stated in this research (Jimoh and Lin, 2019a). The present study confirms that *Paenibacillus* sp. D9 can produce BioS of lipopeptide nature and synthesize degradative enzymes which exhibit efficient uptake of HCs in diesel fuel and *n*-hexadecane.

## 4.5 Conclusion

*Paenibacillus* sp. D9 synthesizes BioS and degradative enzymes in the presence of diesel fuel and *n*-hexadecane. These enzymes which were produced along with enhanced *n*-hexadecane and diesel fuel biodegradation. Biodegradation efficiency of 98.4% and 80.2% associated with the high synthesis of BioS, alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase, and esterase enzymes, and BioS. Hence, *Paenibacillus* sp. D9 was more vigorous in degradation, solubilization, and mineralization of hydrophobic pollutants, and could be used to successfully decontaminate aliphatic HC compounds or diesel fuel spill at environmental polluted sites.

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## Conflict of Interest

The authors wish to declare no conflict of interest

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## Chapter 5

**Enhancement of *Paenibacillus* sp. D9 lipopeptide biosurfactant production through the optimization of medium composition and its application for biodegradation of hydrophobic pollutants**

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# Enhancement of *Paenibacillus* sp. D9 Lipopeptide Biosurfactant Production Through the Optimization of Medium Composition and Its Application for Biodegradation of Hydrophobic Pollutants

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**Abstract**

Interests in biosurfactant in industrial and environmental applications have increased considerably in recent years, owing to their potential benefits over synthetic counterparts. The present study aimed at analyzing the stability and oil removal efficiency of a new lipopeptide biosurfactant produced by *Paenibacillus* sp. D9 and its feasibility of its use in biotechnological applications. *Paenibacillus* sp. D9 was evaluated for optimal growth conditions and improved production yield of lipopeptide biosurfactant with variations in different substrate parameters such as carbon (C), nitrogen (N), C:N: ratio, metal supplements, pH, and temperature. Enhanced biosurfactant production was observed when using diesel fuel and ammonium sulfate as carbon and nitrogen source respectively. The maximum biosurfactant yield of 4.11 g/L by *Paenibacillus* sp. D9 occurred at a C/N ratio of 3:1, at pH 7.0, 30 °C, 4.0 mM $MgSO_4$, and 1.5% inoculum size. The D9 biosurfactant was found to retain surface-active properties under the extreme conditions such as high thermal, acidic, alkaline, and salt concentration. The ability to emulsify further emphasizes its potential usage in biotechnological application. Additionally, the lipopeptide biosurfactant exhibited good performance in the

**Enhancement of *Paenibacillus* sp. D9 lipopeptide biosurfactant production through the optimization of medium composition and its application for biodegradation of hydrophobic pollutants**

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## Abstract

Interests in biosurfactant in industrial and environmental applications have increased considerably in recent years, owing to their potential benefits over synthetic counterparts. The present study aimed at analyzing the stability, and oil removal efficiency of a new lipopeptide biosurfactant produced by *Paenibacillus* sp. D9 and the feasibility of its use in biotechnological applications. *Paenibacillus* sp. D9 was evaluated for optimal growth conditions and improved production yield of lipopeptide biosurfactant with variations in different substrate parameters such as carbon (C), nitrogen (N), C: N: ratio, metal supplements, pH, and temperature. Enhanced biosurfactant production was observed when using diesel fuel and ammonium sulfate as a carbon and nitrogen source, respectively. The maximum biosurfactant yield of 4.11 g/L by *Paenibacillus* sp. D9 occurred at a C/N ratio of 3:1, at pH 7.0, 30°C, 4.0 mM $MgSO_4$ and 1.5% inoculum size. The *Paenibacillus* sp. D9 biosurfactant was found to retain surface-active properties under extreme conditions such as high thermal, acidic, alkaline, and salt concentration. The ability to emulsify further emphasizes its potential usage in biotechnological application. Additionally, the lipopeptide biosurfactant exhibited good performance in the degradation of highly toxic substances when compared with a chemical surfactant, which proposes its probable application in biodegradation, microbial-enhanced oil recovery or bioremediation. Furthermore, the biosurfactants were effective in a test to stimulate the solubilization of hydrophobic pollutants in both liquid environments removing 49.1% to 65.1% diesel fuel including hydrophobic pollutants. The study highlights the usefulness of optimization of culture parameters and their effects on biosurfactant production, high stability, improved desorption and solubilization of hydrophobic pollutants.

**Keywords**

Application; Biosurfactant; Hydrophobic pollutants; Lipopeptide; Optimisation; Yield

## 5.1 Introduction

Developments in science and technology in relation to green environment, such as biotechnological processes, production of peptides, bioflavours, enzymes, biosurfactants (BioSs) and the like are, of increasing importance worldwide (Burgos-Diaz et al., 2013). Attention to BioSs has been generated because of their probable applications in environmental protection, food, industrial products and processes, as well as in biomedical field (Burgos-Diaz et al., 2013; Ghribi and Ellouze-Chaabouni, 2011; Rufino et al., 2014). For oil spill clean-up and enhanced oil recovery from oil reservoirs and the environment, chemically synthesized surfactants such as Triton X-100, sodium dodecyl sulfate (SDS) and others, have been used, however these compounds can be toxic to the environment and non-biodegradable (Goel, 2014; Silva et al., 2014). Several BioSs manufacturers have made their way into the industrial market seeking to replace some or all chemical surfactants (Marchant and Banat, 2012; Sekhon et al., 2012). Consequently, the global market for BioS has been rising and it is expected to increase from US$ 1.7 billion in 2011 to US$ 2.2 billion by 2018 (Sekhon et al., 2012).

BioSs are amphiphilic group of biologically surface-active molecules produced by specific group of microorganisms such as fungi, bacteria, and yeasts (Chakraborty et al., 2015). The chemical structures of BioSs include both a hydrophilic moiety, consisting of an amino acid or peptide, anions, or cations, mono-, di-, or poly saccharides, or a hydrophobic moiety of unsaturated, saturated, or hydrocarbon fatty acids (Zhang et al., 2016). BioSs occurs in nature as chemical entities such as fatty acids, neutral lipids, phospholipids, glycolipids (rhamnolipids, mannosylerythritol sophorolipids, cellobiose lipids, trehalolipids, and xylolipids), lipopeptides and lipoproteins (serrawettin, surfactin, peptide, viscosin, fengycin, subtilisin, and polymyxin), polymeric surfactants (liposan, carbohydrate–lipid–protein, emulsan, biodispersan), and particulate surfactants (Anjum et al., 2016).

Despite their advantages, BioSs are not yet competitive with their synthetic counterparts (Burgos-Diaz et al., 2013). The main limitation is the availability of the BioSs in bulk as compared to the chemical surfactants (Al-Wahaibi et al., 2014). Therefore, 70 – 75% of all commercial surfactants are synthesized from the petrochemical industry (Campos et al., 2014). The practical application of BioSs depends on the inherent economical production at large-scale (Gudina et al., 2015). To achieve sufficiently great production at reduced costs of such biologically surface-active compound, monitoring the key fermentation parameters (such as biomass, substrate, aeration, pH, and temperature) is necessary. Industrial products have generated substantial

attention from researchers as low-cost substrates for this purpose, as it generally accounts for up to 50% of the final production cost (Das et al., 2009; Rufino et al., 2014).

The production medium optimization, improvement of recovery methods, and the development of BioS-producing microbes, can lead the way to large scale economical and efficient production process (Mukherjee et al., 2006). The growth and environmental factors such as pH, growth medium composition, inoculum size, speed, temperature, and oxygen availability also affect BioS production through their effects on cellular activity. Besides, it has been reported that the addition of various metal and sulfur supplements (such as copper, iron, magnesium, and manganese) to the culture broth medium contribute to increased production, since those elements are enzyme co-factors involved in the synthesis of lipopeptide BioS (Gudina et al., 2015). The established technique for medium optimization includes changing one variable at a time (OVAT) and keeping other factors as fixed levels. Thus, it is difficult to determine the major factors and to optimize them for biotechnological processes as several parameters have multiple effects on different categories of microorganism.

Hence, the utmost important aspect of this study, as enhancement of lipopeptide BioS produced from genus *Paenibacillus*, requires further exploration for greater production yield thus enabling usage of this surfactant in environmental application. In the present study, BioS production by *Paenibacillus* sp. D9 strain was optimized by monitoring the different physicochemical parameters for the greatest lipopeptide production yield. The stability and application of *Paenibacillus* sp. D9 lipopeptide against a chemical surfactant to enhance and solubilize toxic diesel fuel and hydrophobic pollutants biodegradation was also reported.

## 5.2 Materials and methods

### *5.2.1 Chemicals and reagents*

All chemicals, reagents and hydrocarbons were of analytical grade. The purity of all hydrocarbons used in this study was a minimum of 99% as evaluated by instrumental analysis. All chemicals, reagents were purchased from Sigma-Aldrich USA.

### *5.2.2 Strain and growth*

The BioS-producing strain *Paenibacillus* sp. D9 was isolated and identified in a previous study (Ganesh and Lin, 2009). The culture of this microorganism was stored at -80$^{o}$C in Luria-Bertani (LB) medium (g/L: NaCl

10.0; tryptone 10.0; yeast extract 5.0) supplemented with 40% (v/v) of glycerol. When required, frozen stocks were streaked on nutrient agar plates (Merck) and incubated at 30°C for 24 h. The nutrient agar plates were stored at 4°C for less than 3 weeks. The strain used in the study was grown at 30°C in LB medium for 24 h. The cells were recovered by centrifugation at 13,500 × *g*. The cell pellets were washed twice in phosphate buffered saline and resuspended in Bushnell Haas (BH) medium [$MgSO_4$, 0.2; $CaCl_2$, 0.02; $KH_2PO_4$, 1.0; $K_2HPO_4$, 1.0; $NH_4NO_3$, 1.0; $FeCl_3$, 0.05; pH 7.0 (g/L)] until $OD_{600}$ was equivalent to 1.0 (Bushnell and Haas, 1941).

### *5.2.3 Optimization of cultivation medium*

#### 5.2.3.1 Effect of carbon on biosurfactant production

Carbon substrate plays a significant role in the production of BioS. Ten carbon sources were selected for optimization production purposes (Table 5.1). *Paenibacillus* sp. D9 ($OD_{600}$ = 1.0) was inoculated and incubated in 100 mL BH medium containing 2% of each carbon source at 30°C,150 rpm for 7 days. Samples were collected daily for the determination of biosurfactant production (BP), bacterial cell growth ($OD_{600}$), dry cell weight (DCW), and surface tension (ST) (Abouseoud et al., 2008; Lotfabad et al., 2009). At optimum conditions, the time course kinetic profile of growth and BioS of *Paenibacillus* sp. D9 was followed in a modified BH medium. The production medium was allowed for clear separation of hydrophobic layer containing the substrates and hydrophilic layer containing the bacterial cells. Bacterial cell growth was subsequently monitored by measuring the growth density ($OD_{600}$) for the time course experiment. The spectrophotometer was blanked with the medium containing the diesel fuel during the measurement of $OD_{600}$ value.

**Table 5.1** Carbon and nitrogen sources examined for their influence on biosurfactant production by *Paenibacillus* sp. D9

| A. Carbon Sources | B. Nitrogen Sources |
|---|---|
| *n*-paraffin | Ammonium sulfate |
| *n*-dodecane | Sodium nitrate |
| *n*-hexadecane | Yeast extract |
| Sunflower oil | Peptone |
| Canola oil | Urea |
| Sucrose | Potassium nitrate |
| Glycerol | Beef extract |
| Diesel fuel | |
| *n*-tetradecane | |
| Engine oil | |

#### 5.2.3.2 Effect of nitrogen on biosurfactant production

Nitrogen is essential for microbial growth, seven nitrogen (2% N equivalent w/v) sources were selected for the optimization study (Table 5.1) in BH medium containing 2% of diesel fuel as a carbon source as the conditions stated above. BH medium containing ammonium nitrate was utilized as a control. The ammonium nitrate in the subsequent BH medium compositions was substituted with the different nitrogen sources listed above. The different nitrogen substrates were standardized to ensure uniform amount of N was added. At the end of the experiment, DCW, BioS yield, and ST were determined.

#### 5.2.3.3 Effect of the carbon and nitrogen ratio on biosurfactant production

Both optimized carbon and nitrogen sources were added separately in the BH at different carbon: nitrogen ratios from 0.14 to 7.0. The experiment was performed as described above. At the end of the experiment, DCW, BioS yield, and ST were determined.

#### 5.2.3.4 Effect of sulfur sources and metal medium supplementation on biosurfactant production

Once the carbon and nitrogen concentration that led to the greatest BioS production by *Paenibacillus* sp. D9 was selected, the effect of different metals and sulfur sources ($FeSO_4$, $MnSO_4$, $MgSO_4$) on *Paenibacillus* sp.

D9 BioS production were evaluated. The concentrations of compounds used were selected according to previous studies (Gudina et al., 2015; Sousa et al., 2012; Yeh et al., 2005). Control assays were performed using the culture medium without the addition of metal and sulfur supplements. The cultures were performed as described above, and at the end of the experiment, DCW, BioS yield, and ST were determined.

#### 5.2.3.5 Effect of pH and temperature on biosurfactant production

To optimize the temperature and pH on BioS production, a range of pH (4 - 10) was selected with the optimal medium compositions obtained above. Once the pH was optimal, varying temperature conditions (25 ─ 60$^{o}$C) were set for the optimization study. The experiments were accomplished at the same conditions and evaluated as described above.

#### 5.2.3.6 Effect of inoculum size on biosurfactant production

To determine the effect of inoculum size on BioS production, a range of initial bacterial densities ($OD_{600}$ 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5, respectively) was inoculated into 100 mL of BH optimized medium. At the end of the experiment, DCW, BioS yield, and ST were determined.

### *5.2.4 Biosurfactant properties*

#### 5.2.4.1 Determination of dry cell weight

Bacterial cells were removed from BioS-containing medium using a refrigerated centrifuge (Avanti J-26 XPI, Beckman Coulter, USA) at the end of each optimization studies. Bacterial dry cell weight was obtained as described by Darvishi et al. (2011). DCW was determined by centrifugation of 20 mL of culture broth for 20 min at 13,500 × g. The cell pellets were washed twice with distilled water and dried by heating at 105$^{o}$C until constant weight was achieved.

#### 5.2.4.2 Biosurfactant purification and characterization

Cell-free supernatant collected from the optimization study was used for quantifying BioS for ST and emulsifying activities which are described below. The residual oil remaining was extracted with an appropriate volume of *n*-hexane (Obayori et al., 2009). Crude BioS was obtained as described in Gudina et al. (2015). In brief, the centrifuged supernatant was acidified to pH 2.0 using 6 N HCl. The solution, which contained BioS, was precipitated at 4$^{o}$C overnight. The precipitated BioS was further collected by centrifugation at 13,500 × *g* for 20 min. Succeeding centrifugation, the precipitated BioS was dissolved in distilled water and adjusted to

pH 7.0 using 1 N NaOH. The solution was lyophilized, weighed and stored at –20ºC until further usage. For the purification of the crude BioS, the samples were partially purified primarily according to the measures defined above. The sample was then liquefied in methanol, mixed with silica gel (230 – 400 mesh) and subsequently oven-dried at 50°C. The silica gel was further mixed with methanol and then loaded onto a chromatography column (50 cm × 2.8 cm). A mixture of ethyl acetate/chloroform in different proportions (100% to 0% with 10% interval), was used in the sequential washing of the loaded column at a flow rate of 0.5 mL/min. A UV spectrophotometer with a range of 200 – 800 nm was used to monitor the absorption wavelength of the mixtures to confirm surface activity. The eluents (20 mL) were collected and the fractions showing oil-displacement activity were thoroughly mixed, followed by evaporation at 80ºC to acquire purified sample. The purified BioS was confirmed subsequently for surface activity and properties before its further usage.

#### 5.2.4.3 Surface tension

Surface tension was achieved in 40 mL cell-free supernatant obtained by centrifuging the cultures at 13,500 × *g* for 20 min. ST was determined with a KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) using 1.9 cm De Noüy platinum ring at room temperature. For calibration, the ST of distilled water was first measured. The ST of BH medium supplemented with different carbon substrates were used as controls. All readings were produced in triplicate and an average ST value was used.

#### 5.2.4.4 Emulsifying activity determination

The emulsification index (E24) was determined according to Burgos-Diaz et al. (2011). Emulsifying activity was determined through the addition of cell-free supernatants (2 mL) to the same volume of *n*-hexadecane in test tubes. The tube contents were mixed with a vortex mixer (V-220, Germany) at high speed for 2 – 5 min and then left incubated at 25ºC for 24 h. The E24 was calculated as the percentage of the emulsified layer (mm) divided by the total height of the liquid column (mm).

### *5.2.5 Biosurfactant stability studies*

Stability was determined at a range of temperatures (50 – 121ºC), pH (2 – 11) and different salinities (0 – 20% w/v) for a period of 30 min and then cooled to room temperature. All control tests were conducted at pH 7.0, 0% salt concentration, and room temperature. For stability studies, cell-free samples were filled in 50 mL serum bottles, sealed with butyl rubber stoppers to avoid any loss from evaporation, and subsequently incubated at the respective temperatures, pH and salinity. The BioS broth was subjected to autoclave conditions (121ºC, 15

psi for 30 min) to examine the influence of such environment on the surface activity. The samples were also adjusted to analyze different pH stability conditions as highlighted above using 1 N HCl or 1 N NaOH. To determine salt stability effect, different concentrations of NaCl were added to cell-free broth, liquefied completely and incubated at room temperature. The BioS stability under various treatments was determined by measuring the ST and E24 at room temperature as described above.

### *5.2.6 Application in improved biodegradation test*

The degradation experiments were performed in an Erlenmeyer flask containing 100 mL of BH medium. The experimental samples were incubated at 30°C and shaken at 150 rpm for 7 days. A mixture of aliphatic hydrocarbon (*n*-dodecane and *n*-hexadecane, 2:2 v/v) and (10% diesel fuel) were used for the biodegradation test at different experiment sets and conditions. The total hydrocarbon concentration in the experiment was 10% (v/v). SDS as a chemical surfactant was used to compare its effect on hydrocarbon degradation by *Paenibacillus* sp. D9 with that of lipopeptide BioS. The hydrocarbon biodegradation was determined using the standard method for gravimetric determination of hydrocarbon as proposed by Ganesh and Lin (2009).

The rate of biodegradation (%) was calculated as $(Y_0 - Y_1)/Y_0 \times 100$, where $Y_0$ is the initial amount of hydrocarbon, and $Y_1$ is the amount of hydrocarbon after biodegradation. The residual aqueous phase was subjected to the double extractions with dichloromethane. The results were determined with the respect to blank control samples (hydrocarbon with medium, without microorganisms).

### *5.2.7 Statistical analysis*

All the experimental data were expressed an arithmetic mean plus/minus standard deviation of at least three independent replicates. Significance was ascribed using ANOVA at the 95% confidence level.

## 5.3 Results and discussion

### *5.3.1 Effect of carbon on biosurfactant production*

*Paenibacillus* sp. D9 was identified and characterized in a previous study as a promising lipopeptide BioS producer (Ganesh and Lin, 2009). The medium optimization was consequently carried-out in a series of experiments of changing one variable at a time (OVAT), with other parameters at fixed set of conditions. The optimal conditions for BioS production were determined using two factors namely ST reduction and increase

in production yield. To optimize physicochemical conditions, ten carbon sources were selected for BioS production (Figure 5.1).

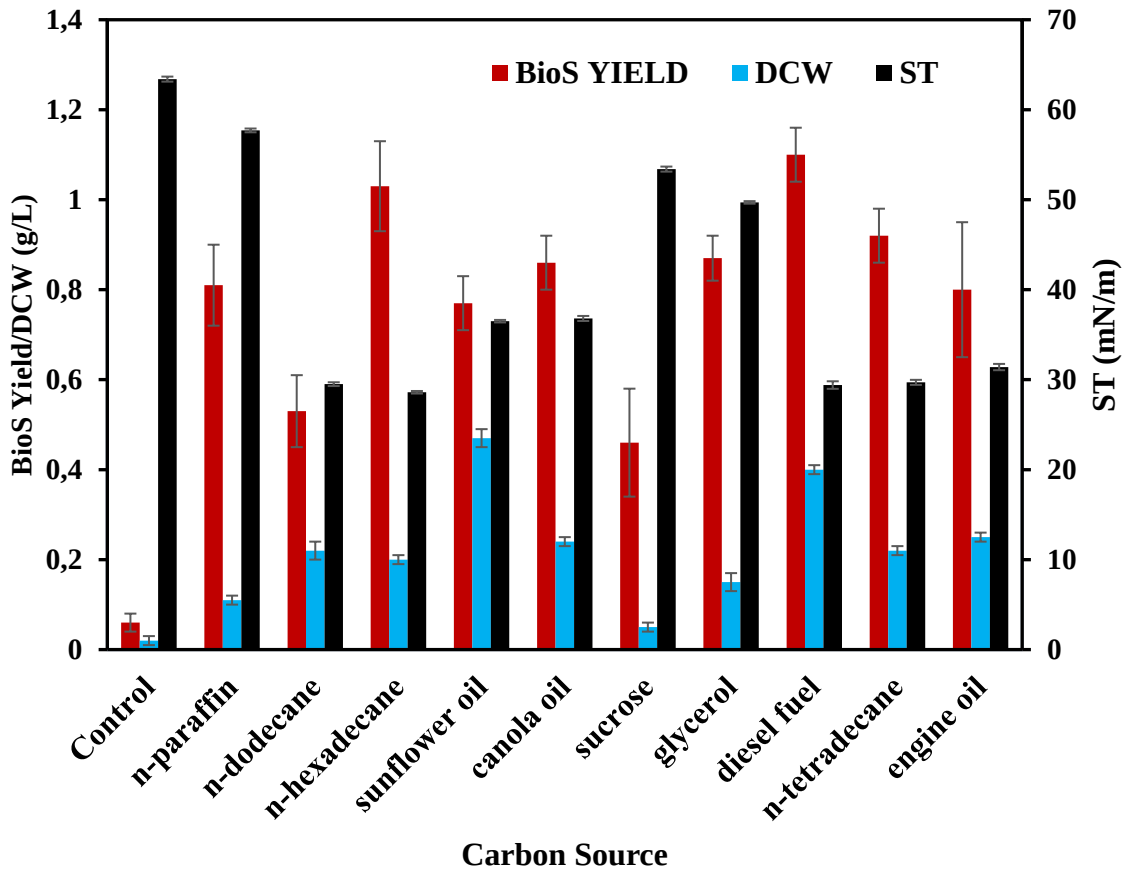



**Figure 5.1** Effect of carbon sources on cell growth, biosurfactant production, and the surface tension values of *Paenibacillus* sp. D9 supernatant grown with different carbon sources in a BH medium. Surface tension values were determined at room temperature (25ºC), with the cell growth calculated as dry weight (105ºC, 48 h). Biosurfactant yield obtained by weighing freeze-dried products. All data points are means ± S.D. (standard deviation) of three independent experiments conducted in triplicate. BioS (Biosurfactant) yield, DCW (Dry cell weight), ST (Surface tension).

*Paenibacillus* sp. D9 was able to produce BioS utilizing each of the carbon nutrients sources tested. Paraffin and sucrose were less effective carbon sources for *Paenibacillus* sp. D9 and the use of diesel fuel as carbon source to produce BioSs obtained the greatest yield and was highly active in reducing ST (Figure 5.1; 1.15 g/L; $p < 0.05$) followed by *n*-hexadecane and *n*-tetradecane. Zhang et al. (2016) reported a similar finding with *Bacillus atrophaeus* 5-2a, which was able to produce BioSs utilizing the some of the carbon sources tested in the present study, except paraffin. The greatest DCW were obtained using sunflower oil and diesel fuel as the

carbon source (0.49 and 0.39 g/L), respectively. Sunflower oil, which is a low-cost substrate, produced the greatest DCW, but cell growth was not accompanied by either maximum BioS production or greater surface ST reduction. The least ST of the culture supernatant was obtained with longer hydrocarbon chain and hydrocarbon mixtures as the sole carbon source [diesel, 29.4; *n*-hexadecane, 28.6; *n*-dodecane, 29.5; *n*-tetradecane, 29.7 (mN/m)]. The results obtained are in good agreement with previous reports, highlighting the use of hydrophobic compounds such as, diesel fuel, *n*-hexadecane, crude oil, engine oil, and kerosene as effective substrates for microbial growth and BioS production (Bharali and Konwar, 2011; Ghribi and Ellouze-Chaabouni, 2011; Khopade et al., 2012; Kiran et al., 2010; Obayori et al., 2009; Pereira et al., 2013; Wang et al., 2014). The outcomes showed significance in relative to control samples with no notable production of BioS yield revealed. This however, ruled out any possibilities of the substrates utilized in this experiment co-precipitating with the isolated BioS. The use of *n*-paraffin as the only carbon source complemented a BioS-like production, but with a limited ST reduction as reported by Pereira et al. (2013). The greater crude BioS yield (1.1 g/L) and a greater ST reduction (29.4 mN/m) when diesel fuel was used as the carbon source confirmed its selection for further experimental study below.

### *5.3.2 Time course profile on biosurfactant production*

Growth kinetics and production of BioSs by *Paenibacillus* sp. D9 strain were determined using the new optimized parameters (BH medium with 2% diesel fuel at 30$^{o}$C, 150 rpm, and pH 7.0) (Figure 5.2). The production of BioS started as early as 24 h, but there was sharp increase in production observed at about 72 – 96 h. However, BioS production continued up to 120 h (day 5) and after that it declined. A reduction in ST of 32.4 mN/m was observed at the third day of incubation indicative of the survival, acclimatization, and adaptational contact time between the hydrocarbon substrate and the bacterium (Obayori et al., 2009). Although BioS production began as early as 24 h in the medium as seen from the ST reduction of 39.4 mN/m, a significant activity of BioS was achieved only after about 48 – 96 h of production which continued to 120 h. The greatest BioS yield of 1.48 g/L was achieved after 120 h of incubation using diesel fuel as the carbon source. The increase of the ST and the reduction in BioS yield after 120 h of culture (Figure 5.2) characterize the termination of BioS biosynthesis. This most likely results from the production of intermediate secondary metabolites, and adsorption of BioS molecules at the water-oil interface which could impede BioS formation. The present study revealed a similar trend for BioS production by *Paenibacillus* sp. D9 as that for a marine bacterium *Nocardiopsis* sp. B4 which began in early log phase with a drastic increase at late growth and early stationary

phase (Khopade et al., 2012). Ghojavand et al. (2008) reported the maximum production of BioS by *B. subtilis* (PTCC 1696) during the growth phase which remained constant even after the exponential phase.

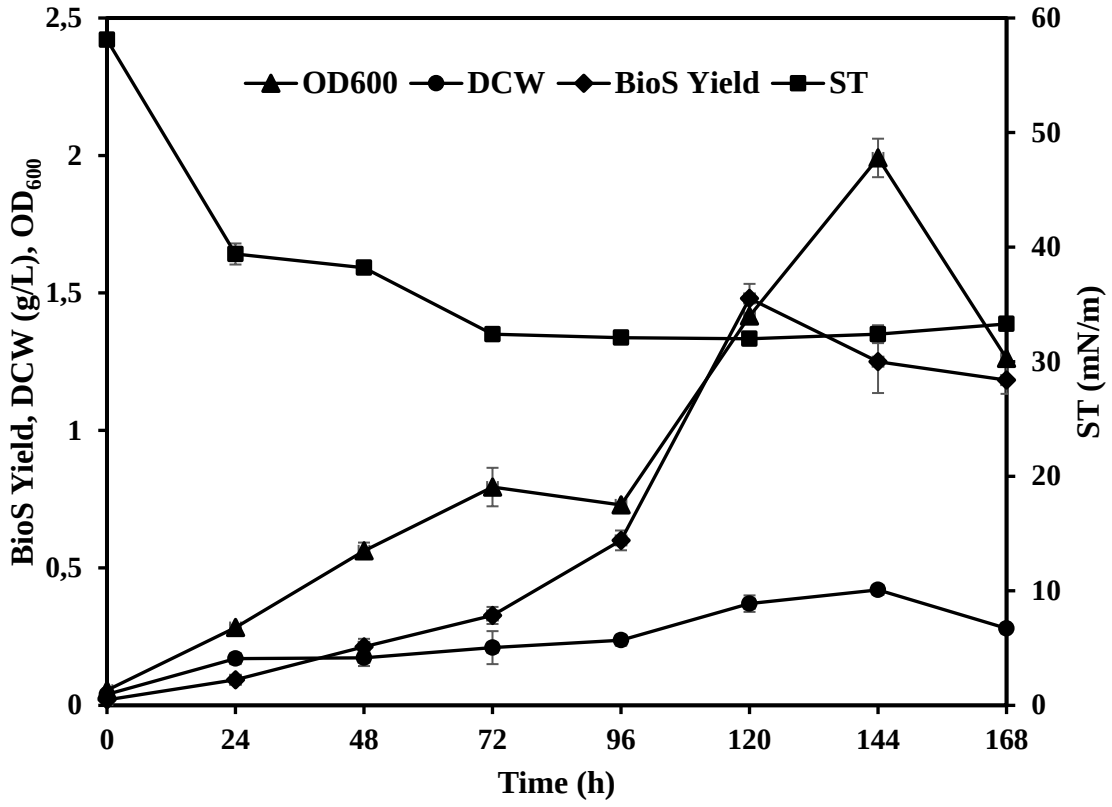



**Figure 5.2** Time-course profile of bacterial growth, biosurfactant production by *Paenibacillus* sp. D9, and the surface tension values of its supernatant grown in a BH medium supplemented with 2% diesel fuel. Samples were retrieved every 24 h and growth was monitored by spectrophotometric measurement of culture turbidity at 600 nm (OD600). Surface tension values were determined at room temperature (25ºC), with the cell growth calculated as dry weight (105ºC, 48 h). Biosurfactant yield obtained by weighing freeze-dried products. All data points are means ± S.D. (standard deviation) of three independent experiments conducted in triplicate. BioS (Biosurfactant) yield, DCW (Dry cell weight), ST (Surface tension).

These results showed that the BioS production from diesel fuel ensued predominantly during the exponential growth phase owing to optical density and dry cell weight data, suggesting primarily produced BioS biomolecule, associated with cellular biomass (growth-associated kinetics). Therefore, the BioS was recovered at day 5 for the subsequent studies. Joshi and Desai (2013) also reported the BioS production by five bacterial strains for up to 72 h with ST values in the range of 28 – 30 mN/m.

### *5.3.3 Effect of nitrogen sources on Paenibacillus sp.* D9 *biosurfactant production*

Complex nitrogenous sources as macro nutrient is essential for growth, formation of cell components, and production of surface-active molecules by a variety of microorganisms (Chakraborty et al., 2015). Observation of these biological functions allows the introduction of different nitrogen sources which tends to affect the quantity of BioS produced (Figure 5.3). Organic nitrogen sources including yeast extract and beef extract had similar effects on the BioS production. Among the organic nitrogen sources, peptone showed the greatest effect on BioS production. The greatest cell weight (0.5 g/L) was achieved using yeast extract as the nitrogen source followed by ammonium sulfate. *Paenibacillus* sp. D9 was able to use the seven nitrogen sources including ammonium nitrate for both bacterial growth and BioS production with (yield ranging from 0.73 – 3.32 g/L) as well as ST reduction of 31.9 to 48.9 mN/m. In the present study, ammonium sulfate as the nitrogen source to produce BioS, gave a maximum yield of (3.32 g/L) and ST reduction of 32.7 mN/m (Figure 5.3). Influence of ammonium sulfate as a nitrogen source was noticeable and significant with the BioS yield increase of 1.48 g/L to 3.32 g/L.

Thus, there was a major effect of nitrogen source on BioS production. Conversely, Bharali and Konwar, (2011) reported urea along with $(NH_4)_2SO_4$ as the best nitrogen sources for BioS production by *Pseudomonas aeruginosa* strain OBP1. Sousa et al. (2012) established that a low BioS yield (0.44 g/L) was produced by *B. subtilis* LAMI005 utilizing a medium containing glycerol and $(NH_4)_2SO_4$. In a previous study, some *Bacillus* strains could not use $(NH_4)_2SO_4$ or $KNO_3$ for growth or BioS production, but they could use $NaNO_3$, $NH_4NO_3$ (Zhang et al., 2016). That *Paenibacillus* sp. D9 could grow and produce BioS utilizing all the nitrogen sources tested indicates its competitiveness for environmental and industrial applications. Inorganic compounds previously reported to increase and improve BioS production include sodium nitrate (Abbasi et al., 2012; Onwosi and Odibo, 2012) ammonium nitrate (Abouseoud et al., 2008), and ammonium sulfate (Bharali and Konwar, 2011; Mata-Sandoval et al., 2001).

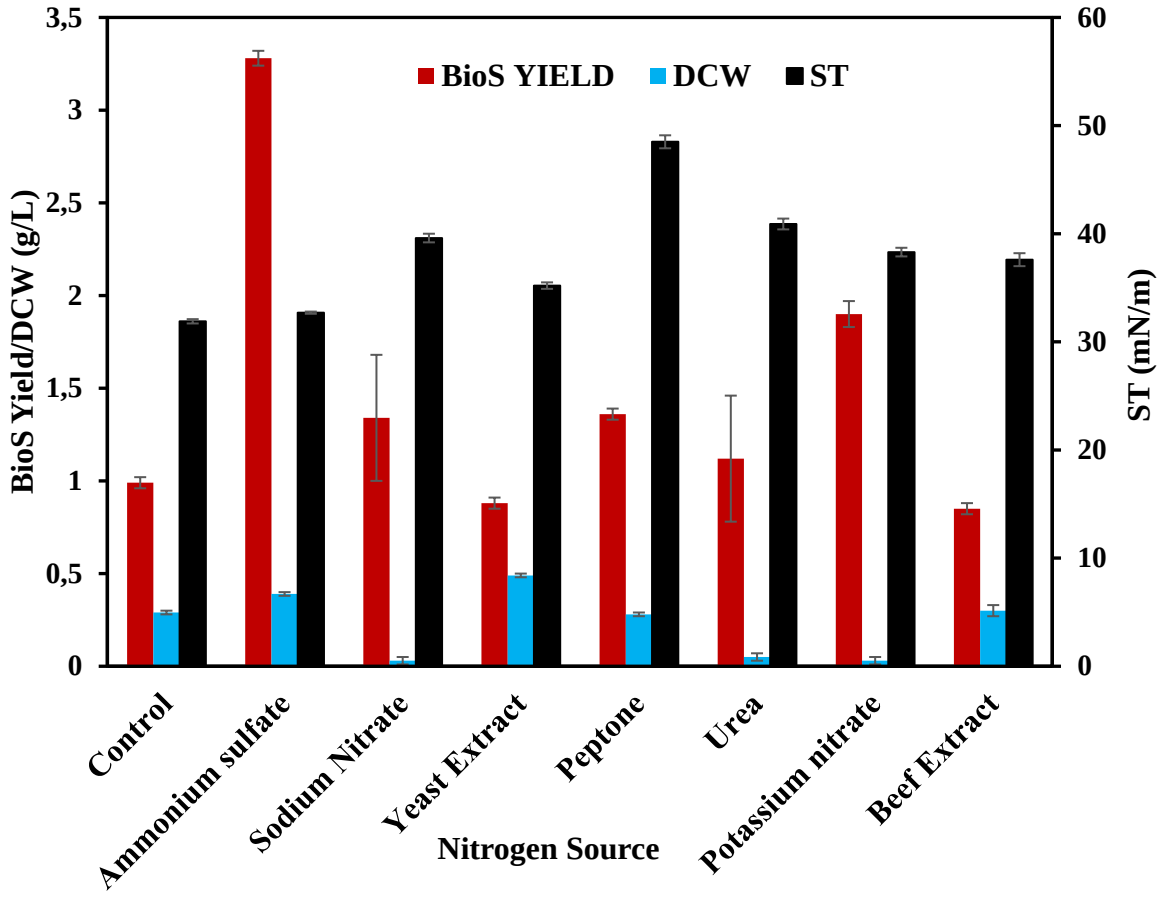



**Figure 5.3** Effect of nitrogen source on cell growth and biosurfactant production of *Paenibacillus* sp. D9 and the surface tension value of its supernatant grown in a BH medium supplemented with 2% diesel fuel as the sole carbon source and energy. Surface tension values were determined at room temperature (25ºC), with the cell growth calculated as dry weight (105ºC, 48 h). Biosurfactant yield obtained by weighing freeze-dried products. All data points are means ± S.D. (standard deviation) of three independent experiments conducted in triplicate. BioS (Biosurfactant) yield, DCW (Dry cell weight), ST (Surface tension).

### *5.3.4 C/N ratio*

C/N ratio is another critical factor required for the improvement of BioS productivity (Onwosi and Odibo, 2012). The best results (ST = 32.7 mN/m, BioS yield = 3.24 g/L, DCW = 0.39 g/L) as obtained above, were attained using diesel fuel (2% v/v) and ammonium sulfate (2% m/v) as carbon and nitrogen source, respectively. The effects of various C/N ratios (0.14 – 7) on cell growth and BioS production were observed (Figure 5.4). The medium system with the lesser amount of ammonium sulfate (greater C/N ratio) produced a

greater BioS yield as compared to the medium with increasing amount of this nitrogen source (lesser C/N ratio).

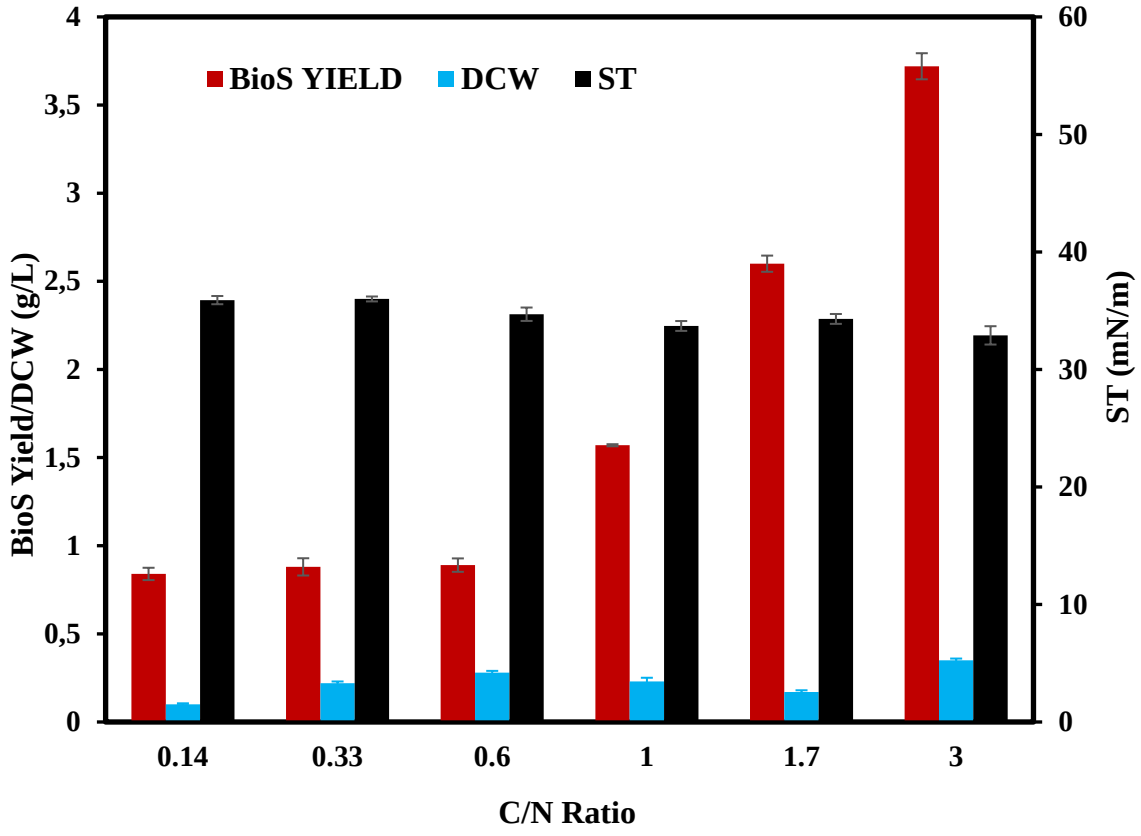



**Figure 5.4** Effect of C/N ratio on cell growth and biosurfactant production of *Paenibacillus* sp. D9 and the surface tension value of its supernatant. Surface tension values were determined at room temperature (25ºC), with the cell growth calculated as dry weight (105ºC, 48 h). Biosurfactant yield obtained by weighing freeze-dried products. All data points are means ± S.D. (standard deviation) of three independent experiments conducted in triplicate. BioS (Biosurfactant) yield, DCW (Dry cell weight), ST (Surface tension).

The presence of diesel fuel: $(NH_4)_2SO_4$, ratio of 3:1 resulted in the greatest *Paenibacillus* sp. D9 BioS production (3.79 g/L), ST reduction (32.0 mN/m) and greatest dry cell weight (0.35 g/L). This revealed that, greater C/N ratios (i.e., reduced levels of nitrogen) limit bacterial growth, and favours cellular metabolism toward production of metabolites. The results were supported by a previous report that increased BioS production occurs under the limitation of nitrogen rather than carbon source (Saimmai et al., 2013). On the other hand, excess of nitrogen directs substrate to the production of cellular material, thus restraining relatively the build-up of surface-active products (Kokare et al., 2007). A C/N ratio of 0.5 was required for the maximum production of BioS by *Nocardiopsis lucentensis* MSA04 in solid-state cultivation (Kiran et al., 2010).

### *5.3.5 Sulfur and metal supplementation*

Iron, manganese, copper and magnesium are enzymes co-factors involved in the synthesis of lipopeptide BioS (Gudina et al., 2015). The medium containing 3% (v/v) diesel fuel and 1% (m/v) ammonium sulfate as identified by C/N ratio was the best medium for BioS production by *Paenibacillus* sp. D9. This optimal condition was supplemented further with $FeSO_4$, $MnSO_4$, or $MgSO_4$ individually at different concentrations to study their effects on BioS production (Table 5.2). The results obtained from metal supplementation tests specified that the presence of metal influenced the maximum BioS produced by *Paenibacillus* sp. D9. Supplementing $MgSO_4$ produced the greatest production yield of 3.76 g/L and the greatest DCW (0.35 g/L). Addition of manganese as a metal supplement produced the greatest ST reduction of 30.3 mN/m and a yield of 3.12 g/L showing a positive interaction on BioS production as compared to the introduction of iron and copper. The amount of BioS produced by the inclusion of iron to the production medium was significantly inhibited, with the greatest ST reduction (39.5mN/m), the least cell growth (0.19 g/L), and the least BioS yield (0.74 g/L). The concentration of different metal elements plays a significant role in the BioS production (Gudina et al., 2015; Wei et al., 2007). There was increased BioS yield for all the metals tested up to 4.1, 4.4, and 3.5 g/L for iron, manganese, and magnesium, respectively from another report (Gudina et al., 2015).

**Table 5.2** The effect of iron, manganese, magnesium, and copper on cell growth, biosurfactant production by *Paenibacillus* sp. D9 and surface tension values of its supernatant.

| **Metal Supplementation** | **DCW (g/L)** | **BioS Yield (g/L)** | **ST(mN/m)** |
|---|---|---|---|
| Control | 0.34 ± 0.03 | 3.60 ± 0.02 | 33.9 ± 1.2 |
| $FeSO_4$ (2.0 mM) | 0.19 ± 0.01 | 0.74 ± 0.03 | 39.5 ± 0.9 |
| $MnSO_4$ (0.2 mM) | 0.26 ± 0.02 | 3.12 ± 0.05 | 30.3 ± 0.4 |
| $MgSO_4$ (4.0 mM) | 0.35 ± 0.01 | 3.76 ± 0.07 | 33.5 ± 0.4 |
| $CuSO_4$ (2.0 mM) | 0.19 ± 0.01 | 1.76 ± 0.05 | 36.5 ± 0.2 |

Control: Medium without supplements, DCW: Dry cell weight, BioS: Biosurfactant yield, ST: Surface tension. The results presented correspond to the optimum production for each medium. Surface tension values were determined at room temperature (25ºC), with the cell growth calculated as dry weight (105ºC, 48 h). Biosurfactant yield obtained by weighing freeze-dried products. All data points are means ± S.D. (standard

deviation) of three different experiments performed on different days (each experiment was conducted in triplicate).

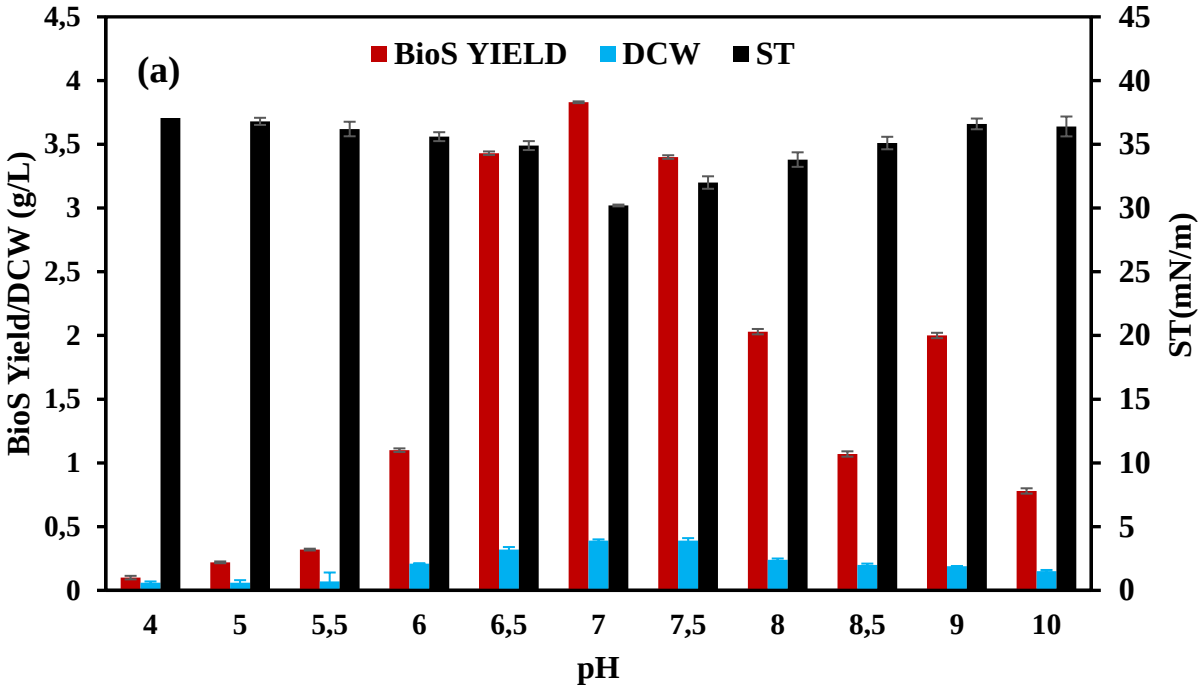
### *5.3.6 pH, temperature, and inoculum size*

Numerous physiochemical factors, for example, pH, temperature, growth conditions, and aeration have been shown to impact microbial development and metabolism (Khopade et al., 2012). The vital attributes of most microorganisms is their dependence on the optimal pH for cell growth and production of secondary biomolecules (Najafi et al., 2011). *Paenibacillus* sp. D9 utilized and produced BioS over a wide pH range from 5.5 to 10.0 under the experimental conditions (Figure 5.5a). The pH 7.0 was found to be optimal for *Paenibacillus* sp. D9 BioS production as compared with media at different pH values. The BioS production yield of 3.83 g/L together with the greatest ST reduction at 30.2 mN/m were obtained at pH 7.0. The production drastically declined under more alkaline ($pH \geq 8.0$) and more acidic ($pH \leq 6.0$) conditions. The pH results in the present study corresponds with previous report. *Paenibacillus alvei* was able to produce BioS in a pH range of 6-8 with maximum yield obtained at pH 6.89 (Najafi et al., 2011). Other studies have reported that the maximum yield of the BioS was achieved at pH 7 (Abouseoud et al., 2008; Chakraborty et al., 2015; Kiran et al., 2009). In another report, BioS production by *Nocardiopsis lucentensis* MSA04 was consistent at pH 7.0 and drastically declined at more acidic pH when compared to the alkaline pH 9.0 (Kiran et al., 2010).

Temperature is another critical factor that is considerably controlled in biological process (Yang, 2011). *Paenibacillus* sp. D9 grown in BH medium at pH 7.0 produced optimum BioS yield and the least ST at 30.9 mN/m when incubated at temperature 30°C, significantly different ($p < 0.05$) from cultures grown at 37°C and greater (Figure 5.5b). While the incubation temperature reached 50°C, BioS production and growth was totally inhibited. The optimal temperature at 30°C was anticipated since the isolated bacterium is a mesophilic organism (Ganesh et al., 2014). *Paenibacillus alvei* BioS production was optimal at 34.76ºC with a ST of 35 mN/m (Najafi et al., 2011).

Inoculum size is another critical factor responsible with significant effect on BioS production (Waqas et al., 2013). It was evident that at inoculum size $OD_{600}$ of 1.5 mL, 3.99 g/L of *Paenibacillus* sp. D9 BioS with ST of 32.4 mN/m was obtained after 5 days (Figure 5.5c). A lesser surfactant yield of 2.74 g/L BioS production was observed with 1 mL $OD_{600}$ inoculum size (Waqas et al., 2013). Increase in inoculum provided no further increase in the D9 BS lipopeptide production and activity. Roy (2017) on the other hand reported an enhanced and significant effect on BioS production at 2 mL inoculum size.

In brief, BioS production by *Paenibacillus* D9 increased to 4.11 g/L under these optimized conditions from 1.15 g/L in BH medium containing 3.0% (v/v) diesel fuel and 1.0% (v/v) ammonium sulfate, 4.0 mM $MgSO_4$, pH 7.0, temperature 30°C and 1.5 mL inoculum size. The result showed that the new optimized conditions favored increased BioS production which was shown to be a growth-associated metabolite. BioSs are valuable products and determining the optimal conditions for improved yield is highly significant from an economic standpoint.



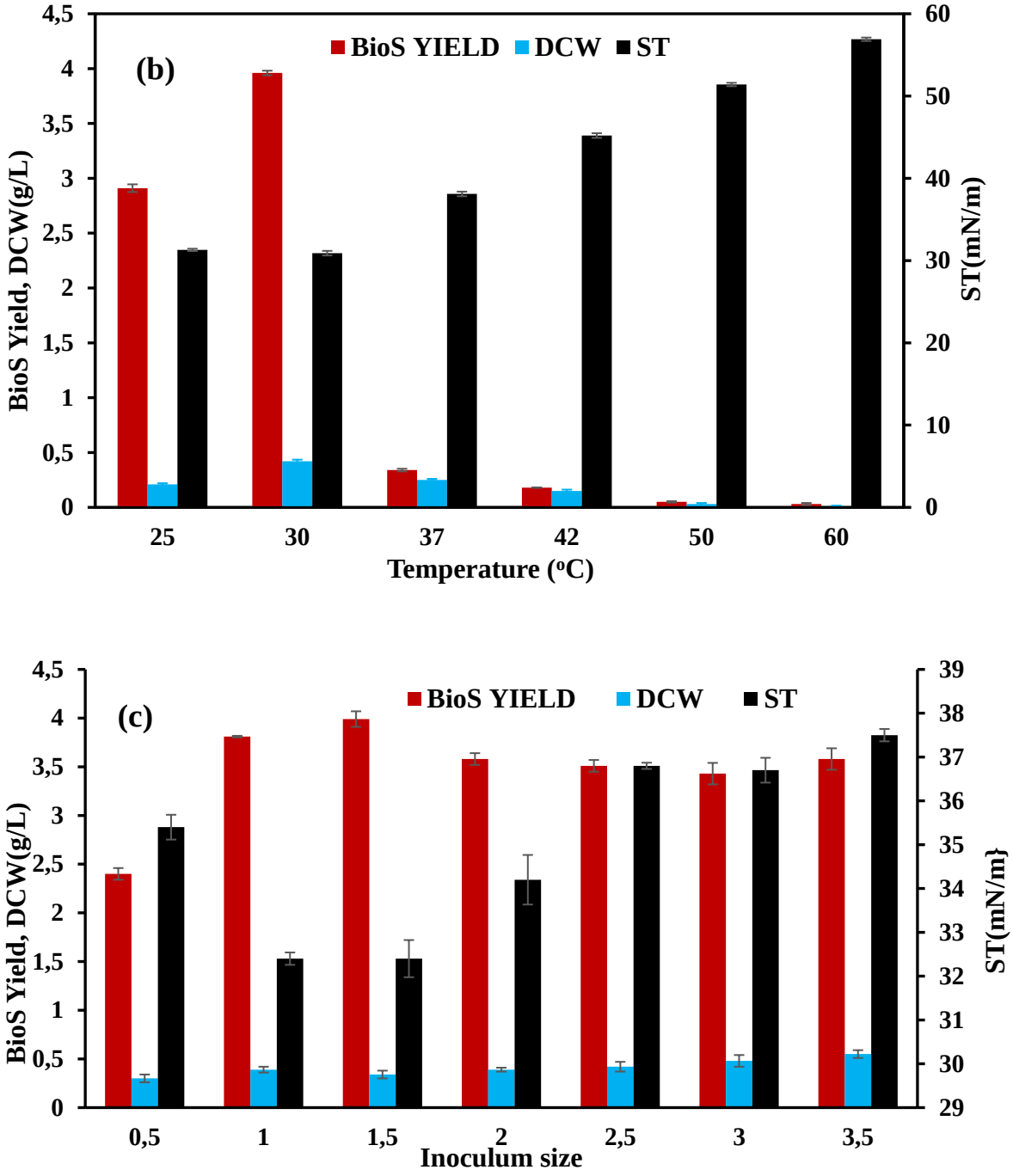
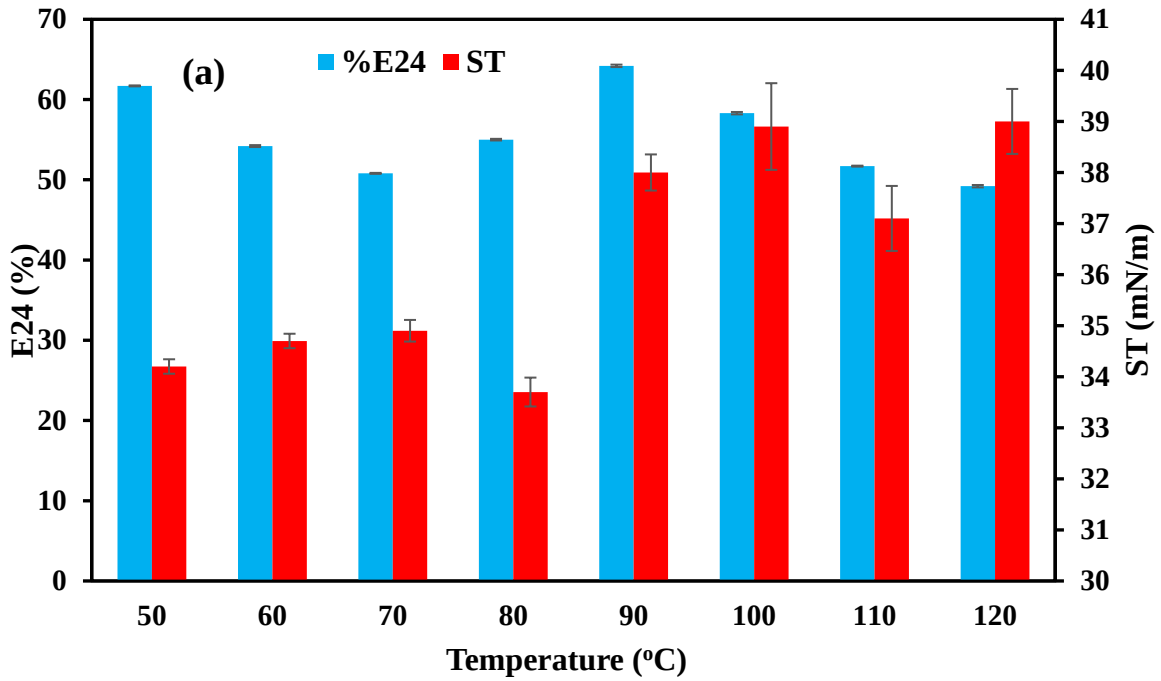
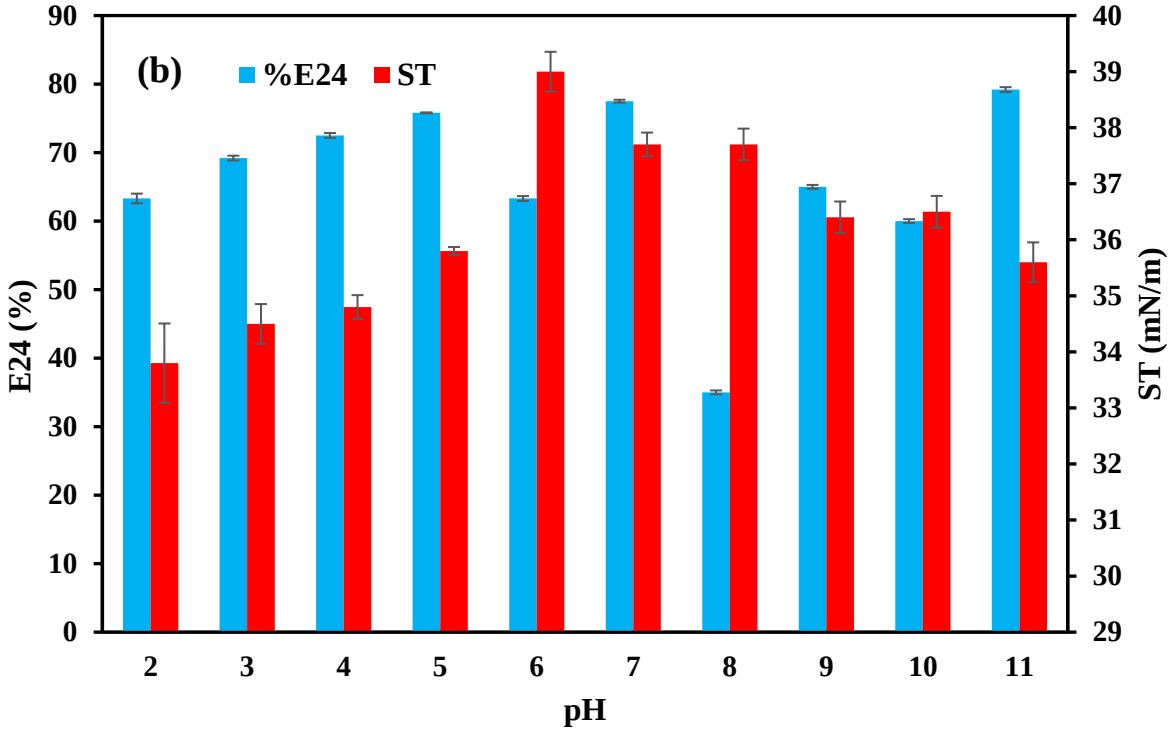



**Figure 5.5** Effect of (a) pH and (b) temperature (c) inoculum size (OD $_{600nm}$) on cell growth and biosurfactant production of *Paenibacillus* sp. D9 and the surface tension value of its supernatant. Surface tension values were determined at room temperature (25ºC), with the cell growth calculated as dry weight (105ºC, 48 h). Biosurfactant yield obtained by weighing freeze-dried products. All data points are means ± S.D. (standard deviation) of three independent experiments conducted in triplicate. BioS (Biosurfactant) yield, DCW (Dry cell weight), ST (Surface tension).

### *5.3.7 Biosurfactant stability studies*

For its potential usage in environmental application, the biological agents must be stable with a wide range of pH, greater temperatures (≥ 50ºC), and salinities (up to 20%) (Al-Wahaibi et al., 2014). Thus, the BioS produced by *Paenibacillus* sp. D9 was considered for its stability at extensive range of extreme environmental conditions. The BioS produced by *Paenibacillus* sp. D9 was found to be thermostable (Figure 5.6a). Exposing the *Paenibacillus* sp. D9 BioS to 120ºC caused no significant effect on the emulsification index (E24) performance. At a range of temperatures from 50 to 80ºC, the BioS was quite stable in the ST reduction ability (Figure 5.6a). As temperature increased; ST decreases, and *vice versa* as cohesive forces decrease with increase in molecular activity. The impact on the surrounding environment is the result of the adhesive action that liquid molecules have at the interface. Thus, movement of molecules disrupts the imbalanced forces on the water surface and weakens the tightly bound molecules, subsequently lowering the ST (Vega and De Miguel, 2007). Khopade et al. (2012) also reported the stability of BioSs under extreme conditions of temperature (50ºC to 100ºC). Similarly, Joshi et al. (2008) reported BioS produced by four *Bacillus* strains to be stable at 80ºC.




(b)
%E24
ST
E24 (%)
ST (mN/m)
pH
0
10
20
30
40
50
60
70
80
90
29
30
31
32
33
34
35
36
37
38
39
40
2
3
4
5
6
7
8
9
10
11

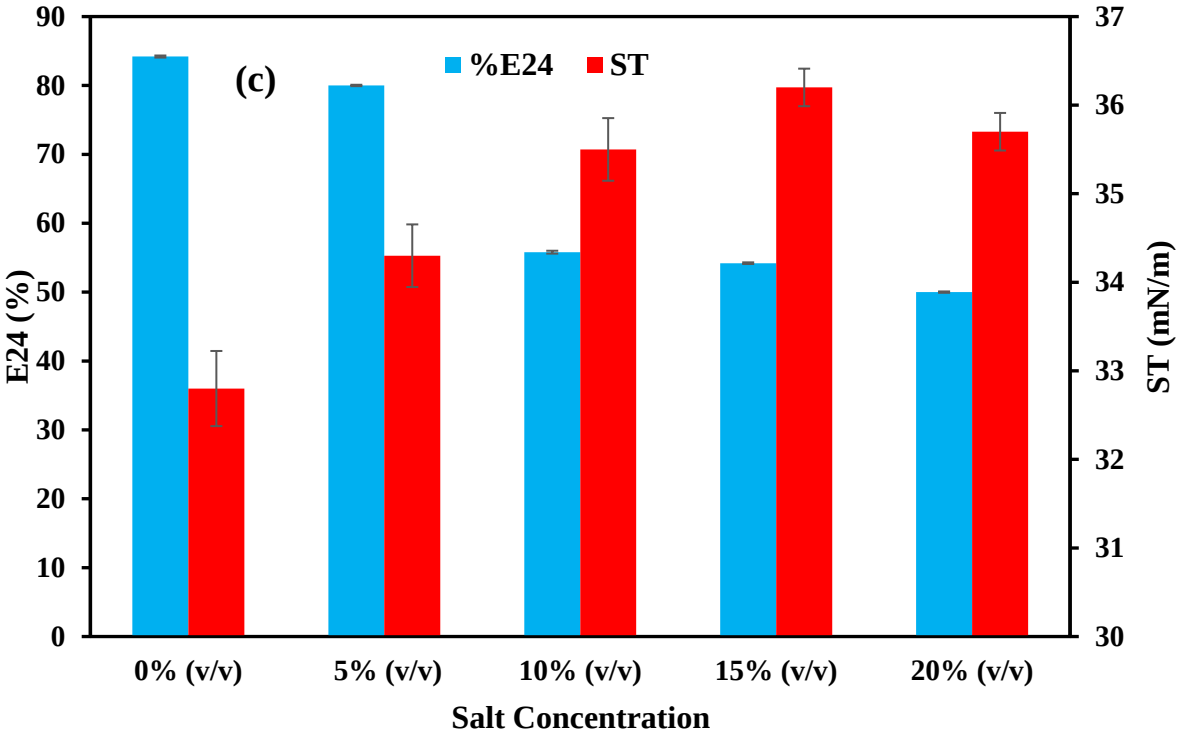

(c)
%E24
ST
E24 (%)
ST (mN/m)
Salt Concentration
0
10
20
30
40
50
60
70
80
90
30
31
32
33
34
35
36
37
0% (v/v)
5% (v/v)
10% (v/v)
15% (v/v)
20% (v/v)

**Figure 5.6** Effects of (a) pH and (b) Temperature (c) Salt concentration on biosurfactant stability. ST (surface tension values, mN/m), E24 (emulsifying indexes, %) and biosurfactant concentrations obtained in cultures performed with *Paenibacilllus* sp. D9 grown in BH medium supplemented with diesel fuel (3%) and ammonium sulfate (1%) at 150 rpm, 30ºC, and pH 7 for 5 days. Surface tension values and emulsification indexes were determined at room temperature (25ºC). Surface tension values (mN/m) obtained with culture broth supernatants with the emulsification indexes performed using *n*-hexadecane. The surface tension and the emulsifying activity of BH medium were 69.3 ± 0.3 mN/m and 0.0%, respectively. All data points are means ± S.D. (standard deviation) of three independent experiments conducted in triplicate.

The *Paenibacillus* sp. D9 BioS was also stable in pH 2 ─ 11 according to the ST activity and the E24 activity data (except in pH 8.0). As the pH decreased from 6.0, there was a significant increase in ST reduction due to the BioS precipitation. At pH ≤ 6, the ST decreases significantly and increased E24 index was observed, indicating adsorption of HCl from the solutions (Figure 5.6b). Interestingly, at pH 8.0 the BioS showed no E24 capacity with the emulsification index of 35.0%. The pI value was greater at pH 8.0 from the introduction of extra positive charge at the basic side chain. However, at pH ≥ 8.0, the ST decreases, indicating a greater surface concentration of hydroxide ion than in the bulk solution. Gudiña et al. (2012) showed that BioS activity was retained through a pH range of 5.0 ─ 11.0 with minimum deviation in ST. The BioS solution also showed stability under a broad salt concentration up to 20% NaCl (Figure 5.6c). From the results in Figure 5.6c, there was a noticeable trend with increase in salt concentration, the ST reduction and E24 index values decrease. This behavior is not far-fetched, since elevated salt concentrations can substantially reduce micelle shape and size, then affecting the BioS chemical and functional properties. Also, the ionic strength in assay solutions affects the interactions between the BioS and substrate molecules (de França et al., 2015). The BioS has stability at high salinity and alkaline pH; such a BioS may be useful for bioremediation of oil spills, and contaminated soil. Several researchers also reported the stability of BioSs at elevated temperatures, at differing pH and salinity (Al-Wahaibi et al., 2014; Gudina et al., 2010; Jha et al., 2016; Joshi et al., 2016; Khopade et al., 2012; Saimmai et al., 2013; Vaz et al., 2012).

The results highlight the application of the lipopeptide BioS produced by *Paenibacillus* sp. D9 at extreme conditions of temperature, salinity, and pH. The bioproduct exhibited great thermal stability, a positive effect at acid and alkaline pH, and great tolerance to varying ionic strength, which illustrates clear standpoints for its possible use in extreme environmental conditions such as bioremediation, improved biodegradation of

hydrophobic pollutants, microbial enhanced oil recovery and other industrial fields. Due to the ability to withstand extreme conditions, BioSs have gained attention in environmental and industrial applications (Darvishi et al., 2011).

### *5.3.8 Application of Paenibacillus* sp. D9 *lipopeptide biosurfactant in biodegradation studies*

Extremely toxic hydrocarbon substrates such as diesel fuel, *n*-dodecane and *n*-tetradecane were used to determine influence of BioS and chemical surfactant on biodegradation. The introduction of *Paenibacillus* sp. D9 only on system 1 (10% diesel fuel) and system 2 (6% diesel fuel + C12, C16) showed little biodegradation of 24.5% and 31.1% respectively (Table 5.3). *Paenibacillus* sp. D9 degraded 76% of diesel fuel in a medium with 1% diesel fuel after a 20 day incubation (Ganesh and Lin, 2009) and only 24.5% with 10% diesel fuel (the present study) confirming the effect of extreme hydrophobic mixtures on *Paenibacillus* sp. D9 cell growth and bio-utilisation potential of this bacterium. Higher concentrations of diesel and other hydrocarbon mixtures are often difficult to remove from the environments due to the hydrophobic end which is water-repelling. In the case of chemical surfactant, diesel fuel biodegradation of 42.4% was achieved using 100 mg/L of SDS. However, lesser diesel fuel biodegradation from 42.4% to 19.2% was observed with the introduction of high concentrations of SDS (Table 5.3). The addition of natural surfactants had a great influence on diesel fuel and hydrocarbon substrates biodegradation (Kaczorek and Olszanowski, 2011). The mechanism proposed for improving degradability of hydrophobic organic compounds involves the ability of BioSs to promote the hydrocarbon bioavailability towards the aqueous phase by pseudo-solubilization (Hmidet et al., 2017).

**Table 5.3** Application of *Paenibacillus* sp. D9 lipopeptide biosurfactant in hydrocarbon biodegradation studies

| System | Biodegradation (%)<br>*Paenibacillus* sp. D9 |
|---|---|
| [1] Diesel fuel | 24.5 ± 0.3 |
| [2] Diesel fuel + C12 + C16 | 31.1 ± 1.1 |
| [3] Diesel fuel + D9 BS (100 mg/L) | 49.1 ± 0.7 |
| [4] Diesel fuel + D9 BS (200 mg/L) | 52.0 ±1.3 |
| [5] Diesel fuel + D9 BS (300 mg/L) | 54.7 ± 1.1 |
| [6] Diesel fuel + D9 BS (400 mg/L) | 60.0 ± 0.5 |
| [7] Diesel fuel + D9 BS (500 mg/L) | 65.1 ± 0.6 |
| [8] Diesel fuel + SDS (100 mg/L) | 42.4 ± 0.6 |
| [9] Diesel fuel + SDS (200 mg/L) | 38.0 ± 0.3 |
| [10] Diesel fuel + SDS (300 mg/L) | 36.5 ± 0.7 |
| [11] Diesel fuel + SDS (400 mg/L) | 27.6 ± 0.9 |
| [12] Diesel fuel + SDS (500 mg/L) | 19.2 ± 1.2 |
| [13] Diesel fuel + C12 + C16 + LPP (500 mg/L) | 55.3 ± 0.9 |
| [14] Diesel fuel + C12 + C16 + SDS (100 mg/L) | 27.4 ± 0.7 |

Time of biodegradation; 7 days, SDS; Sodium dodecyl sulfate, C12; *n*- Dodecane, C16; *n*-Hexadecane. All data points are means ± S.D. (standard deviation) of three different experiments performed on different days (each experiment was conducted in triplicate).

These surfactants cause micelle formation and the uptake of pseudo-solubilized hydrocarbon droplets by microorganisms. Results from this research indicated that the addition of natural surfactants could significantly increase the effectiveness of biodegradation of hydrocarbons and diesel fuel as compared with synthetic SDS. With the presence of *Paenibacillus* sp. D9 BioS in the system, the diesel fuel biodegradation by *Paenibacillus* sp. D9 became a positive BioS-dependent (Table 5.3). The application of *Paenibacillus* sp. D9 BioS augments the removal and solubility of these hydrophobic compounds. BioS causes these hydrophobic mixtures to be more susceptible to degradation. An increase in diesel fuel biodegradation rate up to 65.1% was observed under

the same conditions with the addition of 500 mg/L *Paenibacillus* sp. D9 BioS (Table 5.3; system 7). Compared with the lipopeptide-free inoculated culture, there was a substantial increase in the degradation efficiency of diesel fuel by the *Paenibacillus* sp. D9 strain demonstrating the potential of BioS for bioremediation. Hmidet et al. (2017) reported an increase of diesel fuel degradation capability of 20, 27, and 40% with a maximum of 58% in the presence of 0.5, 1, and 2 g/L, respectively by *Bacillus mojavensis* A21 lipopeptide BioS. The method of improvement of diesel fuel biodegradation by exogenously added BioS was described previously (Ayed et al., 2015; Hmidet et al., 2017; Mnif et al., 2015). These natural surfactants are readily degraded and therefore friendlier to the natural environment. The results support that the use of *Paenibacillus* sp. D9 lipopeptide BioS can provide a better biodegradation and bioremediation approach to environmental protection than chemical surfactants. *Paenibacillus* D9 BioS possesses a better diesel fuel degradation efficiency with a lesser concentration needed than the above-mentioned. Further supplementation of hydrocarbons (C12 and C16) in 10% diesel fuel medium inhibited the diesel biodegradation efficiency (systems 13 and 14). Despite a lesser degradation rate, significant improvement ($p < 0.05$) on diesel fuel degradation by D9 (55.3 %) can be achieved by supplemented *Paenibacillus* sp. D9 BioSs (Table 5.3 system 13). The strain develops as a new class of lipopeptide BioS producer with probable environmental applications, particularly in solubilization, and degradation of hydrocarbons (Jimoh and Lin 2019).

## 5.4 Conclusion

The nature of culture conditions and media composition for optimal production of *Paenibacillus* sp. D9 lipopeptide BioS was developed in the present study. Diesel fuel (carbon source), ammonium sulfate (nitrogen source) and magnesium sulfate (metal supplementation) showed significant positive effects on lipopeptide production with the optimal pH and temperature found to be 7.0 and 30ºC. The produced *Paenibacillus* sp. D9 BioS also exhibited dependable stabilities in an extensive range of pH, temperature and salt concentrations. The addition of lipopeptide BioS at different concentrations increased diesel degradation by *Paenibacillus* sp. D9 strain. The lipopeptide BioS was more effective than the commercially accessible synthetic surfactant. Besides, *Paenibacillus* sp. D9 BioS has the potential to be used in bioremediation, since it is capable of efficiently removing highly toxic hydrophobic compounds.

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## Conflict of interest

There is no conflict of interest.

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# Chapter 6

## Bioremediation of contaminated diesel and motor oil through the optimization of biosurfactant produced by *Paenibacillus* sp. D9 on waste canola oil

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**Bioremediation of contaminated diesel and motor oil through the optimization of biosurfactant produced by *Paenibacillus* sp. D9 on waste canola oil**

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## Abstract

Oil products pollution has serious consequences, and consideration is being given to the advancement of alternative innovations for the eradication of these pollutants. The potential of biosurfactant-producing organism, *Paenibacillus* sp. D9, to grow on cheap carbon sources; waste frying oils (canola, sunflower, castor, and coconut) were investigated as alternate substrates for synthesis of biosurfactant as this may prompt a decrease in the bioprocess cost. This work aimed to study optimum parameters, functional properties, and applicability for bioremediation. Initial lipopeptide biosurfactant yield was 2.11 g/L and a surface tension 33.7 mN/m, which was representative of low biosurfactant production. The two quadratic models developed through response surface methodology were adequate with regards to biosurfactant yield ($R^2$ = 0.9937) and surface tension ($R^2$ = 0.9862). There was an improvement on maximum reduction in surface tension and biosurfactant yield of 31.2 mN/m and 5.31 g/L, respectively. The proficiency of biosurfactant, when contrasted to a frequently utilized surfactant, sodium dodecyl sulfate, brought about the degradation of 73.2% for motor oil, 71.8% for diesel (shaking condition), 63.3% for motor oil, and 59.3% for diesel (static condition) in a solid environment. In contrast, using an aqueous environment, there was bio-removal of 77.6% of motor oil, 74.3% of diesel (shaking condition), 62.2% of motor oil, and 57.4% of diesel (static condition) respectively. Box Behnken design as a response surface methodology tool was suitable in identifying the optimum conditions of low-cost substrates and the biosurfactant is capable for bioremediation of diesel fuel and motor oil from contaminated environment.

**Keywords**

Biosurfactant; Bioremediation; Lipopeptide; Low cost substrate; Optimization; Response Surface Methodology

## 6.1 Introduction

The commercial importance and markets for surfactants are constantly growing as evidenced by their production and the number of new environmental, biotechnological, and industrial applications. Surfactants are amphiphilic as they possess hydrophilic and hydrophobic structural moieties, with the ability to lower surface tension (ST) (Banat et al., 2014). Diesel and motor oil contamination of soil and groundwater is a frequently stated ecological issue. These contaminants have a negative effect on soil quality due to a few factors such as high resistance to degradation, chemical stability, and danger to living biota. There is limitation of microorganisms in degrading these pollutants due to low water solubility and easy bound to soil particles (Vossen, 2014). There has also been a general need to replace synthesized compounds due to their inability to absorb chemical and toxic petroleum products in the environment, and non-biodegradability (Banat et al., 2014; Marchant and Banat, 2012a; Marchant and Banat, 2012b). In resolving these problems, biosurfactant (BioS), due to their amphiphilic property display the propensity to bond at the oil/water interface. Thus, hydrophobic contaminants are enabled into the aqueous phase which occurs through definite contact ensuing in solubilization, emulsification, and bioavailability as facilitated by BioSs. BioS as a biomolecule has been explored due to several benefits and properties it possesses over chemical surfactants (Costa et al., 2010).

The initial output of BioSs in the 2013 world market was 344,068.40 tons and is anticipated to touch 461,991.67 tons by 2020, with an annual compound growth rate of 4.3% (Sidkey et al., 2016). BioSs are naturally promising due to several benefits over chemical surfactants; such as high emulsification, non-toxicity, and their environmental compatibility, biodegradability, and acceptability. BioSs are surface-active agents produced by microbes which are dual in nature (encompassing hydrophobic and hydrophilic group) (Saisa-Ard et al., 2013). Additionally, in contrast to chemical surfactants, BioSs possess improved interfacial and surface activity, bind heavy metals, have high selectivity, biological activity, and can be produced from low-cost raw materials that can be reused over time. BioS presence also enhances oil solubility, potentially increasing their bioavailability for utilization as a source of carbon and energy (Nerurkar et al., 2009).

Currently, low productivities and the high cost of raw materials hinder probable economical BioS production on the large industrial scale (Henkel et al., 2012). The overall production cost, however, can be reduced or eradicated through the selection of efficient BioS-producing microorganisms, optimizing the medium components and the use of inexpensive substitute substrates. The high-quality option of low or free cost raw materials is of huge importance to the entire economic process with the total final production cost resulting to

30 ─ 50% (Ruggeri et al., 2009; Saisa-Ard et al., 2013). One of the waste products associated with the oil processing industries is waste frying oil, which is a prominent low-cost fermentative waste, and readily available resource for BioS production (Maddikeri et al., 2015). The utilization of alternate raw resources, for example, waste cooking oils is one of the appealing methods for cost-effective biosynthesis. Waste cooking oils are vegetative carbon source, lipidic in nature (16-18 carbon atom chains) comprised majorly of saturated or unsaturated fatty acids. The high rich nutritional content available in these waste make them interestingly inexpensive raw materials for different industrial and environmental set-ups involved in resourceful secondary metabolite production (Makkar et al., 2011). In Africa and the world at large, 100 million liters of used frying oil is used up per day; while 700,000-1 million tons are estimated in European countries respectively (Owolabi et al., 2011) have been implicated in the synthesis of BioS.

The improvement in strain selection, process technology, use of low-cost, and renewable substrates play a significant part in improving BioS production process (Banat et al., 2014; Marchant and Banat, 2012a; Marchant and Banat, 2012b). The synthesis of microbial surface-active agents on a huge scale is yet to reach a satisfactory level economically due to low yields output generated which is relatively short for environmental and industrial sustainability. Furthermore, the cost of recovery and purification of BioS is still relatively high (Banat et al., 2014). Thus, the utilization of low-cost substrates by BioS producers can overcome such hindrances to improve the quality and quantity of BioS. Similarly, the stage by stage cost required for BioS production should be kept at a minimum as much as possible. It is imperative to discover oil-rich by-products that could be processed into BioS in large production amounts and substantial yields (Partovi et al., 2013). Residues, such as glycerol (Silva et al., 2010), soybean oil refinery residue (Luna et al., 2011), corn steep liquor (de Cássia FS da Silva et al., 2013), and clarified cashew apple juice (Oliveira and Garcia-Cruz, 2013).

The utilization of vegetable oil and their by-products as a source of BioS is probable but requires a joint multidisciplinary energies and research for its full accomplishments. Reports on the utilization of the enormous capability of these frying oils for BioS production are limited to few (Makkar et al., 2011). This study further emphasizes the prospect of the application of waste cooking substrates and other relatable parameters for increased BioS production. The use of response surface methodology (RSM) as a technique for regression analysis using data attained from experimental design to proffer solution to quadratic equations developed in the bioproduction process (Kiran et al., 2010b; Najafi et al., 2011). RSM technique, in reducing cost of production through the selection of production medium constituents and optimization of culture conditions, has been employed and reported (de Cássia FS da Silva et al., 2013; Najafi et al., 2011). Yet, the use of this

technique is not fully explored on improving BioS production yield from the genus *Paenibacillus* particularly in the aspect waste frying oil utilization. Thus, a high product yield is needed to present its compactivity and compatibility in the world BioS market. The current research aimed at presenting significant BioS ability and feasibility in the bioremediation of diesel and motor oil from solid environment (contaminated sands) and liquid environments using both shaking and static conditions. The study revealed new and improved production process conditions of BioS produced by *Paenibacillus* sp. D9 using a combination of Box Behnken Design and RSM on low-cost substrates.

## 6.2 Materials and methods

### *6.2.1 Chemicals and materials*

Growth media, chemicals and salts were procured from Sigma-Aldrich (USA). Waste frying oils (canola, sunflower, and coconut) were acquired from different restaurants in the city of Durban, Kwazulu-Natal, Republic of South Africa. Waste frying oil of plant, animal, or synthetic fat were previously used in frying, baking, and other types of cooking. On the other hand, samples of used castor oil were obtained from a retailer in local neighbourhood in South Africa. They were stored in the laboratory until further usage. The waste oils are vegetative carbon source, lipidic in nature (16 ─ 20 carbon atom chains) comprised majorly of saturated or unsaturated fatty acids. South African standard sand, 100/50 mesh was used in the experimental set-up. The sand was saturated with motor oil or diesel, left standing for 24 ─ 48 h at 25$^{o}$C until further usage.

### *6.2.2 Microorganism, inoculum preparation, and production medium*

*Paenibacillus* sp. D9 is a significant BioS producer (Jimoh and Lin, 2019) isolated from diesel contaminated site, Durban, South Africa. The microorganism was preserved in nutrient broth supplemented with 40% v/v glycerol at –80°C. Culture transfers were made to fresh agar slants every 2 ─ 3 weeks to maintain its viability. From the nutrient agar slant, *Paenibacillus* sp. D9 single colony was inoculated into 10 mL nutrient broth (Sigma-Aldrich), incubated overnight in an orbital shaker (150 rpm) (MRC, Polychem Supplies) at 30$^{o}$C. Subsequently, 1 mL of overnight culture was inoculated into 100 mL nutrient broth enclosed in a 250 mL Erlenmeyer flask and incubated at 30$^{o}$C for 24 h. The culture was then followed by centrifugation at 10,000 rpm for 10 min. After this period, the pellets were washed twice in phosphate buffered saline (pH 7.4) and further suspended in Bushnell Hass (BH) medium composition (g/L): $K_2HPO_4$ 1.00, $KH_2PO_4$ 1.00,

$MgSO_4 \cdot 7H_2O$ 0.20, $FeCl_3$ 0.05, $CaCl_2$ 0.05, $CaCl_2$ 0.02, $NH_4NO_3$ 1.00, pH of 7.0 ± 0.2 until the $OD_{600}$ was equivalent to 1 (Ganesh and Lin, 2009). One mL of *Paenibacillus* sp. D9 inoculum (1 $OD_{600}$ nm equivalent) was introduced to 500 mL Erlenmeyer flasks encompassing 98 mL liquid BH medium supplemented with 2% of each waste frying oil. Uninoculated control flasks with no addition of bacterium were also incubated to monitor abiotic losses. Cultivations were performed at 30ºC and stimulated on an orbital shaker (Polychem supplies, MRC, China) at 150 rpm for 5 days (Zhang et al., 2016).

#### 6.2.3.1 Biomass determination

Ten mL samples were mixed with cooled distilled water in weighed falcon tubes and centrifuged at 10,000 × *g* for 20 min. The cell pellet was dried in an oven at 105ºC after two washing cycles for 24 h. The samples were eventually weighed to determine the cell biomass. All the assays were carried out in a three set of independent experiments.

#### 6.2.3.2 Surface tension

ST was resolved with a KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) utilizing 1.9 cm De Noüy platinum ring at room temperature. This was carried out by utilizing 40 mL cell-free supernatant acquired by centrifuging culture broth at 13,500 × *g* for 20 min (Gudiña et al., 2012). All readings were produced in triplicate and average ST values of each sample was used. For proper calibration and to avoid error in instrumentation, the ST of distilled water were initially measured. The ST of BH medium supplemented with the different waste frying oils were determined as controls (Zhang et al., 2016).

#### 6.2.3.3 Extraction, isolation, and purification of biosurfactant

The centrifugation (13,500 × *g* at 4ºC for 10 min) of the culture broth was achieved to allow the removal of the bacterial cell. The centrifuged supernatant was acidified to pH 2.0 using 6 N HCl to allow precipitation of the BioS. The precipitated BioS was successively evaporated at 4ºC overnight and subsequently collected by centrifugation at 13,500 × *g* for 20 min (Gudiña et al., 2012). The pooled BioS was liquefied in distilled water with pH adjustment to neutrality (pH 7.0) using 1 N NaOH. The solution was freeze-dried, weighed and stored at –20ºC until additional usage (Gudiña et al., 2012; Zhang et al., 2016). For the purification of the crude BioS, the samples were partially purified primarily according to the measures defined above. The sample was then liquefied in methanol, mixed with silica gel (230 – 400 mesh) and subsequently oven-dried at 50°C. The silica gel was further mixed with methanol and then loaded onto a chromatography column (50 cm × 2.8 cm). A

mixture of ethyl acetate/chloroform in different proportions (100% to 0% with 10% interval), was used in the sequential washing of the loaded column at a flow rate of 0.5 mL/min. A UV spectrophotometer with a range of 200 – 800 nm was used to monitor the absorption wavelength of the mixtures to confirm surface activity. The eluents (20 mL) were collected and the fractions showing oil displacement activity were thoroughly mixed, followed by evaporation at 80ºC to acquire purified sample. The purified BioS was subsequently confirmed for surface properties before its further usage (Deng et al., 2016).

### *6.2.4 One variable at a time optimization for biosurfactant production*

The improvement of BioS synthesis was directed in a progression of one variable at a time (OVAT) experimental conditions while keeping other variables constant. In this manner, proper test models were created to decide the level of associations between the diverse components set up. Different experimental components such as substrate sources (C), nitrogen sources (N), C/N ratio, and metal supplements affecting BioS production were resolved as defined below.

#### 6.2.4.1 Effect of carbon, nitrogen and C/N ratio on biosurfactant production

Carbon sources (C), nitrogen sources (N) and C/N ratio were chosen to aim at higher BioS productivity. The carbon sources tested included the used waste product of canola oil (2% v/v), sunflower oil (2% v/v), coconut oil (2% v/v), and castor oil (2% v/v). The BH medium without the addition of carbon sources were used as control. For the evaluation of the aptest nitrogen source for improved BioS yield, ammonium sulfate $(NH_4)_2SO_4$, potassium nitrate ($KNO_3$), soybean meal, tryptone, casein, and yeast extract was employed at a concentration of 2 g/L. BH medium containing ammonium nitrate was utilized as a control. The ammonium nitrate in the subsequent BH medium compositions were substituted with the different nitrogen sources listed above. The different nitrogen substrates were standardized to ensure uniform amount of N was added in the experiments. Carbon (waste canola oil) and nitrogen substrate ($KNO_3$) which were successfully utilized were employed further employed in C: N ratio optimization for the detection maximum production yield. Both carbon and nitrogen source concentration were introduced respectively in the BH production medium as: 0.25, 0.5, 1, 2, 4, 8, and 16 [% (v/v)/g/L]. Dry cell weight (DCW), biosurfactant production (BP), and ST were examined at the end of the experiment.

#### 6.2.4.2 Effect of metal supplementation

The introduction of various metals (iron, manganese, and magnesium) independently or in combination were assessed to increase BioS synthesis by *Paenibacillus* sp. D9 strain. The experiment was carried out once the appropriate C: N ratio that resulted in the highest BioS yield was determined. The distinctive metal concentrations (mM) were chosen according to previous research reports (de Sousa and Bhosle, 2012; Wei et al., 2007). The production culture medium was kept up until the point that the maximum BioS synthesis was accomplished and at the end of the experiment, DCW, BP, and ST were determined. Control tests were additionally achieved utilizing the production medium without the option of metal enhancements.

#### 6.2.4.3 Effect of optimum conditions on biosurfactant production

The impact of optimal production conditions on BioS synthesis was assessed. The synthesis of BioS was done in 500 mL Erlenmeyer production flasks comprising of optimum C: N proportion, metal supplements and 98 mL BH medium. The medium was adjusted to pH 7 with utilization of 1 N NaOH or 1 N HCl followed autoclaving at 120°C for 15 min. To confirm the effectiveness the OVAT technique, BH medium containing only carbon substrate was considered as control. One mL ($OD_{600}$) inoculum of *Paenibacillus* sp. D9 was introduced into the medium and kept at temperature 30ºC, 150 rpm in an orbital shaker. The flasks were incubated for 5 days while monitoring the DCW, BP, and ST on a daily interval to the end of the experiment following the methods previously described. All experiments were performed in triplicates of independent samples and the standard deviation (±) was indicated.

### *6.2.5 Optimization of biosurfactant production using response surface methodology*

The statistical program Design Expert 11.0 (Stat-Ease, Inc., Taylor Francis Productivity Press, NY, USA) was utilized for the regression analysis of the experimental information, and to plot the response surface graphs. A fractional factorial design termed Box-Behnken design (BBD) was designed by combining two-level factorial designs with block designs. The response was fitted by a second order model to correlate independent variables. The measurable importance of the model quadratic condition and the terms were assessed with Fisher's test. In the present investigation, the basic control factors that impacted the BP as decided in previous OVAT studies (waste frying oil, $KNO_3$ and metal supplementation) were introduced into an RSM model. The autonomous variable was examined as low (−1), center (0) and high (+1) level (Table 6.1). Also included were runs of center points (control) and followed by analysis of the experimental results. Results were validated utilizing

the analysis of variance (ANOVA) method to elucidate the interactions that were most effective for ST reduction and improved BioS yield. At the end of the experiment, DCW, BP, and ST were examined as described above.

**Table 6.1** The scope of the parameters utilized for displaying the biosurfactant synthesis and the predetermined codes for individual parameter

| | Coded values and the relative estimations of factors | | |
|---|---|---|---|
| | **-1** | **0** | **+1** |
| **A: Waste frying oil (v/v)** | **1** | **3** | **5** |
| **B: $KNO_3$ (g/L)** | **1** | **2** | **3** |
| **C: Metal Supplementation (mM)** | **1** | **2** | **3** |

### *6.2.6 Bioremediation of motor oil and diesel fuel from contaminated sand under shaking conditions*

The possibility of BioS in removing diesel and motor oil from polluted sand was assessed. Tests of 50 g sand (100/50 mesh) were polluted with 10% of diesel or motor oil, subsequently transferred to 500 mL conical flasks. About 40 mL of the BioS (500 mg/L; ST; 30.9 mN/m), sodium dodecyl sulfate (500 mg/L; ST; 30.9 mN/m), cell-free fermented broth (ST; 30.2 mN/m) or 40 mL of distilled water (control) were introduced separately to the experimental set-up. The flasks were put in an orbital shaker (Polychem supplies, MRC, China) at 150 rpm and 30$^{o}$C for 48 h. The test samples were subsequently centrifuged at 10,000 × g for 20 min for the sand separation. For bioremediation analysis, there was thorough double extraction with 40 mL dichloromethane and solvent vaporization, with the remaining hydrocarbon estimated gravimetrically. For consideration, a synthetic surfactant SDS (500 mg/L) was additionally tried at similar conditions illustrated previously. The experimental analysis was carried out in triplicate and the outcomes are given as average values ± standard deviation.

Diesel or Motor oil removed (%) = Hi - Hr/Hi × 100

Hi, was the initial diesel or motor oil in the soil (g) before washing while Hr was the diesel or motor oil left over in the soil (g) after washing (Chaprão et al., 2015).

### *6.2.7 Bioremediation of motor oil and diesel fuel from sand-packed column under static conditions.*

Glass columns measuring 200 cm in height × 10 cm in diameter were saturated with 50 g sand mixture containing 10% of diesel fuel or motor oil. About 40 mL of the BioS (500 mg/mL; ST; 30.9 mN/m), sodium dodecyl sulfate (500 mg/mL; ST; 30.9 mN/m), cell-free fermented broth (ST; 30.2 mN/m) or 40 mL of distilled water (control) were introduced respectively to the experimental set-up. The flasks were left to stand alone in an incubator for 48 h at 30$^{o}$C. The saturation of each solution was observed for 48 h until no further percolation. The soil samples were then washed twice with 40 mL dichloromethane for the removal of the residual oil. The oil removed was determined gravimetrically as described above immediately after solvent evaporation.

### *6.2.8 Bioremediation of motor oil and diesel fuel from an aqueous environment*

The prospect of the BioS in eliminating motor oil and diesel fuel from an aqueous environment was evaluated. In the experiment, 50 mL of deionized water was contaminated with 10% of diesel fuel or motor oil and subsequently transferred to 500 mL Erlenmeyer flasks. Approximately 40 mL of the BioS (500 mg/L; ST; 30.9 mN/m), sodium dodecyl sulfate (500 mg/L; ST; 30.9 mN/m), cell-free fermented broth (ST; 30.2 mN/m) or 40 mL of distilled water (control) were added separately to the experimental set-up. The flasks were put in an orbital shaker (Polychem supplies, MRC, China) at 150 rpm and 30$^{o}$C for 48 h. Subsequently, the liquid samples were then washed with 40 mL dichloromethane twice for the extraction of the residual oil. The oil removed was determined gravimetrically as described above immediately after solvent evaporation.

### *6.2.9 Bioremediation of motor oil and diesel fuel from an aqueous environment under static condition*

The prospect of the *Paenibacillus* sp. D9 BioS in eliminating motor oil and diesel fuel from an aqueous environment was evaluated. Glass columns measuring 200 cm in height × 10 cm in diameter were saturated with the pollutants. In the experiment, 50 mL of deionized water was contaminated with 10% of diesel fuel or motor oil and subsequently transferred to 500 mL Erlenmeyer flasks. Approximately 40 mL of the BioS (500 mg/L; ST; 30.9 mN/m), sodium dodecyl sulfate (500 mg/L; ST; 30.9 mN/m), cell-free fermented broth (ST; 30.2 mN/m) or 40 mL of distilled water (control) were added separately to the experimental set-up. The flasks were left to stand alone in an incubator for 48 h at 30$^{o}$C. The saturation of each solution was observed for 24 h

until no further clarification. The liquid samples were then washed twice with 40 mL dichloromethane for the removal of the residual oil. The oil removed was determined gravimetrically as described above immediately after solvent evaporation.

### *6.2.10 Statistical analysis*

Investigation of difference (ANOVA) was utilized to assess the measurable parameters with a probability estimation of <0.05 as the rule for statistical significance. All data points are means ± standard deviation (S.D.) of three independent experiments.

## 6.3 Results and discussion

### *6.3.1 Effect of carbon sources on biosurfactant production*

The effect of the waste substrates, nitrogen source, carbon to nitrogen ratio, and metal supplementation was sequentially estimated in a single-factor experimentation, to determine the greatest conditions for BioS production. On the carbon substrate, all four-different waste cooking oil tested (sunflower oil, canola oil, castor oil, and coconut oil) influenced BioS concentration. Waste canola oil produced the best optimum yield of 1.32 g/L in this research followed by coconut oil of 0.76 g/L. Castor oil and sunflower oil show improved BioS yield of 0.65 g/L and 0.73 g/L, respectively, as compared with the control experiment (Figure 6.1). Thus, canola oil as well as other substrates tested produced high ST values obtained ranging from 34.3 mN/m (canola oil) to 36.8mN/m (castor oil). The STs achieved in this research showed high influence of the BioS synthesized as the control sample containing the different waste frying oils reduced from 71.3 mN/m to 67.1 mN/m ─ 69.1 mN/m. dos Santos et al. (2010) produced BioS with the reduced ST of 49.5 mN/m by *P. fluorescens* utilizing soybean oil as a carbon source. The result obtained in this study proffer a better optimum condition as compared to the above-stated report. The outcomes showed significance ($p < 0.05$) relative to control samples with no production of BioS yield discovered. This however, rule out any possibilities of the substrates co-precipitating with the isolated BioS. With respect to utilization of low-cost substrates, it is worthy to note that all the waste frying oils showed relatively high production yield and supported the growth of *Paenibacillus* sp. D9 with DCW ranging from 0.55 g/L to 0.67 g/L.

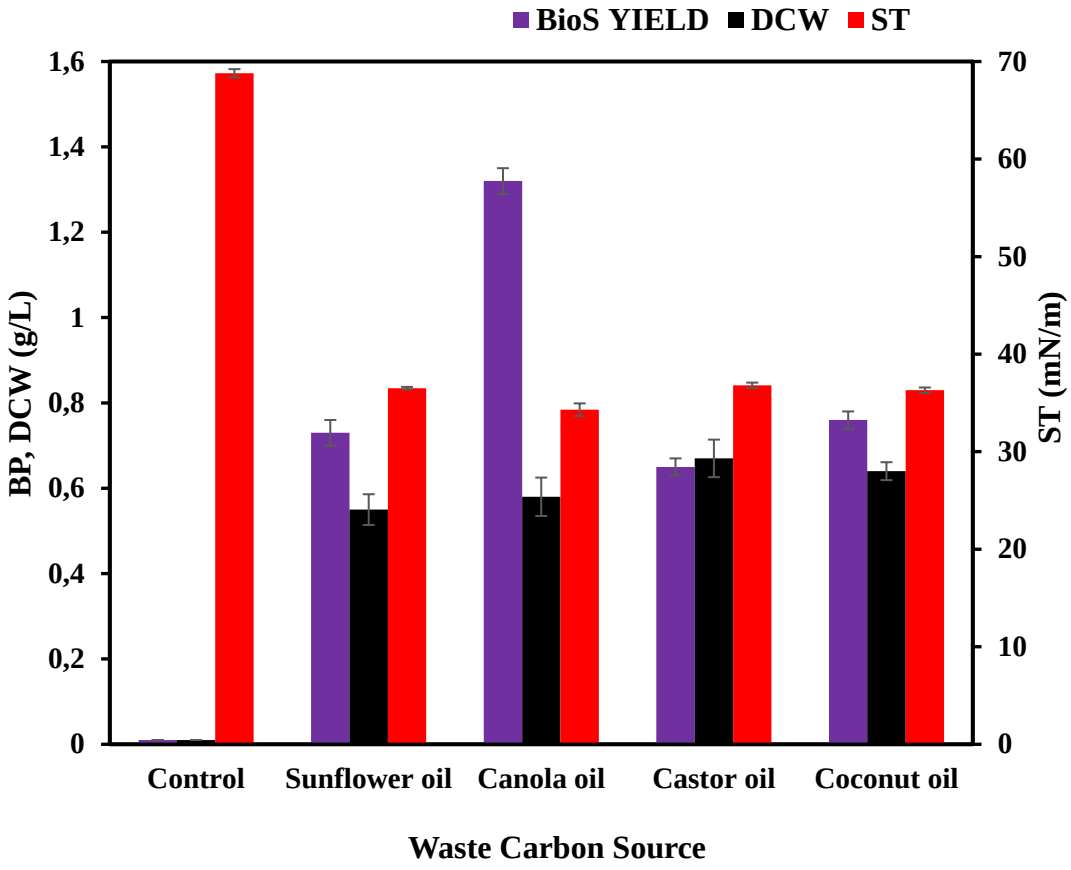



**Figure 6.1** Effect of carbon sources (waste frying oils) on the production of *Paenibacillus* sp. D9. At room temperature (25ºC), the surface tension values were determined, with the cell growth calculated as dry weight (105ºC, 24 h). Biosurfactant production yield achieved by weighing lyophilized products. All data points are means ± standard deviation (S.D.) of three independent experiments.

Be that as it may, this study demonstrates immense capability of the usage of these wasted oils for BioS synthesis. Additionally, in contrast, Gudina et al. (2015) obtained 1.3 g/L BioS yield by *Bacillus subtilis* 573 utilizing the corn steep liquor as a substituent inexpensive culture medium. Similarly, Saravanan and Subramaniyan, (2014) discovered an increase in BioS production on various low-cost substrates with corn oil and cassava flour found to be most efficient. *Pseudomonas aeruginosa* MTCC 2297 utilized coconut oil with a BioS yield of 2.26 g/L (George and Jayachandran, 2013). Sharafi et al. (2014) also reported BioS yield of 5 g/L after 120 h using sunflower oil at 25% by *Aneurinibacillus thermoaerophilus* MK01. In addition, Noudeh et al. (2010) found that the greatest production yield was achieved with olive oil as a sole carbon source by *Bacillus licheniformis* PTCC. Abouseoud et al. (2007) on the other hand, achieved maximum BioS synthesis from *Pseudomonas fluorescens* 1895-DSMZ utilizing olive oil with ammonium nitrate as a nitrogen source.

This current study showed the enormous prospect of these wasted frying oils for enhanced BioS production yield, thus reducing the high cost of production, and hence the significance of this research to literature.

### *6.3.2 Effect of nitrogen sources on biosurfactant production*

The addition of nitrogen is significant in the synthesis of surface-active molecules by a variety of microbes (Saharan et al., 2011). The most commonly used nitrogen sources (organic and inorganic) reported in the literature were assessed for BioS production by *Paenibacillus* sp. D9. It is noteworthy that the addition of nitrogen sources substantially increased BioS production. It was demonstrated that $KNO_3$ and $(NH_4)_2SO_4$ were the most resourceful nitrogen sources that produced the highest BioS concentrations (1.66 g/L and 1.55 g/L, respectively) as seen in Figure 6.2.

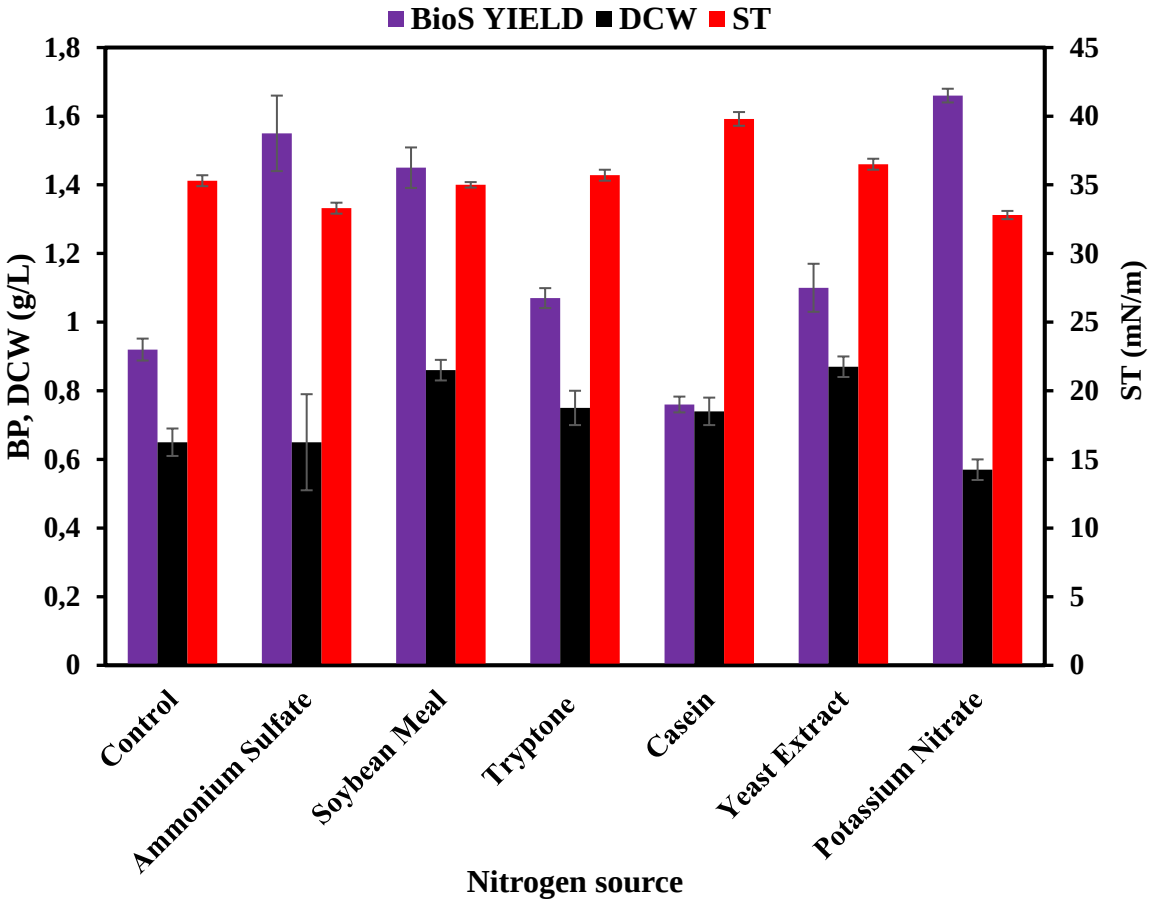



**Figure 6.2** Effect of nitrogen sources on the production of *Paenibacillus* sp. D9. At room temperature (25ºC), the surface tension values were determined, with the cell growth calculated as dry weight (105ºC, 24 h). Biosurfactant production yield achieved by weighing lyophilized products. All data points are means ± standard deviation (S.D.) of three independent experiments.

Although there was no significant difference ($p < 0.05$) between $KNO_3$ and $(NH_4)_2SO_4$ on BioS production, thus $KNO_3$ was chosen for further tests, considering its accessibility as well as higher ST reduction value (32.8 mN/m). Other nitrogen substrates produced considerable BioS yield but $KNO_3$ selection for further optimization analysis was highlighted above. Yeast extract, casein, tryptone, and soybean meal which are representative of organic nitrogen sources showed influenced on cellular growth and BioS production. The use of $KNO_3$ gave better production yields in comparison to other nitrogen sources such as $(NH_4)_2SO_4$ or urea in the BioS produced by *R. glutinis* IIP30 (Johnson et al., 1992). Saharan et al. (2011) also described the use of $KNO_3$ as a nitrogen source for BioS synthesis. The low DCW using of $KNO_3$ (0.57 g/L) and $(NH_4)_2SO_4$ (0.65 g/L) had a significant influence on the high BioS yield and low ST values obtained (32.8 and 33.3 mN/m) respectively. From literature, some *Bacillus* isolates were able to utilize ammonium nitrate, sodium nitrate, or potassium nitrate but not $(NH_4)_2SO_4$ for BioS synthesis (Abouseoud et al., 2008; Makkar and Cameotra, 1997). However, this research also proposed the utilization, with a better ST value and BioS yield of ammonium sulfate (ST; 33.3mN/m, BP; 1.55 g/L), and potassium nitrate (ST; 32.8 mN/m, BP; 1.66g/L) as a nitrogen source for BioS production. Other studies have shown the effects and utilization of potassium nitrate and ammonium sulfate on BioS production (Elazzazy et al., 2015; Zhang et al., 2016). Also, different nitrates as nitrogen nutrients sources were shown to have an impact on BioS synthesis as reported by past researchers include sodium nitrate (Abouseoud et al., 2008; Elazzazy et al., 2015; Pacheco et al., 2010).

### *6.3.3 Effect of the carbon and nitrogen concentration*

C/N proportion is another complex factor, that influences metabolites accumulation in numerous fermentative and production processes (Silva et al., 2010). By using the best carbon source (canola oil) and the nitrogen source (potassium nitrate) kept at constant, the effect of carbon to nitrogen ratios on BioS synthesis was evaluated. These data were obtained using waste canola oil and $KNO_3$ (C/N ratio of 2 = 4; 2) (BP = 1.77 g/L, DCW = 0.75 g/L) as carbon and nitrogen source (Figure 6.3). The best C/N ratio tested was 2 which gave the highest BioS yield of 1.77 g/L (ST = 32.3 mN/m) while the least yield was 0.28 g/L (ST = 37.8 mN/m) which was recorded for C/N ratio of 0.25 (Figure 6.3). The greatest BioS production yield by MSA04 was achieved at a lower C/N ratio of 0.5 (Kiran et al., 2010a). In this research, low level of nitrogen (high C/N ratio), favored the cellular uptake towards the production of BioS. Thus, low C/N ratios (0.25 and 0.5) exhibited inhibition and low production yield due to excess of nitrogen. There was limitation relative to the accumulation of BioS products arising from excess of nitrogen source which direct the substrate to the synthesis of cellular material

only (Silva et al., 2010). There was also an increase in BioS yield by *Rhodococcus erythropolis* ATCC 4277 utilizing higher concentrations of $NaNO_3$, glycerol, and yeast extract respectively (Pacheco et al., 2010).

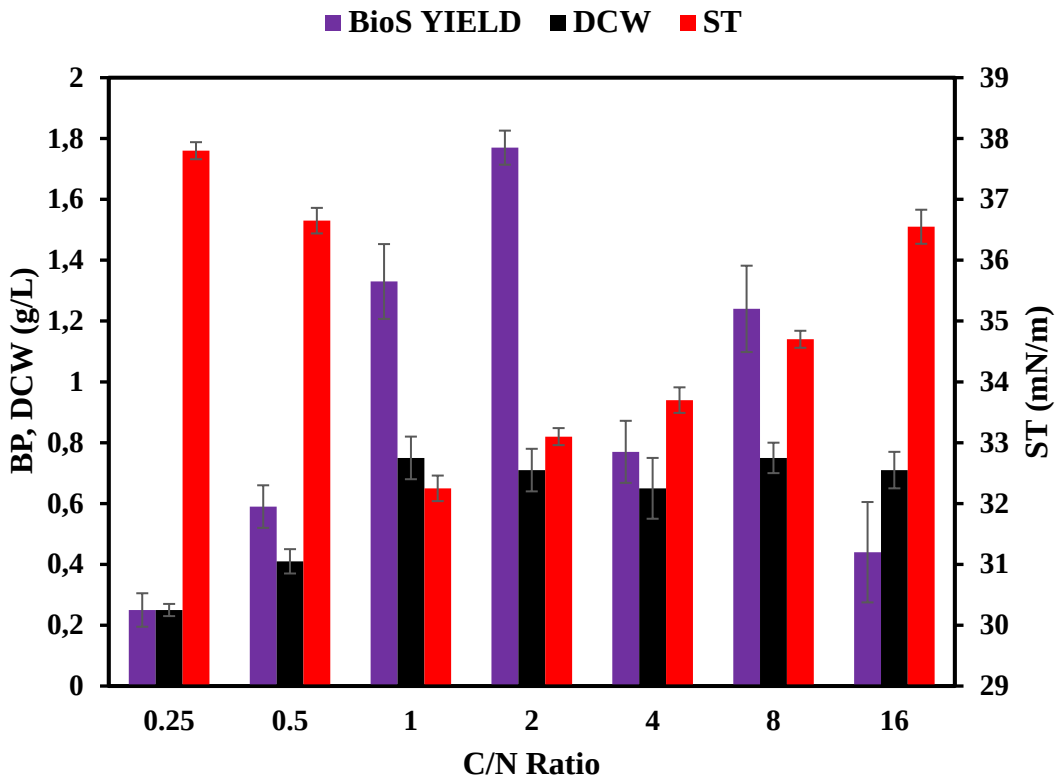



**Figure 6.3** Effect of carbon to nitrogen sources on the production of *Paenibacillus* sp. D9. At room temperature (25ºC), the surface tension values were determined, with the cell growth calculated as dry weight (105ºC, 24 h). Biosurfactant production yield achieved by weighing lyophilized products. All data points are means ± standard deviation (S.D.) of three independent experiments.

### *6.3.4 Effect of metal supplementation*

ST values, BioS yields and DCW in cultures performed with different combinations of metals are shown in Table 6.2. The combinations magnesium-manganese (medium E) produced a better production yield (1.81 g/L) when compared to magnesium-iron (medium D) and manganese-iron (medium F). Medium E produce ST value of (33.8 mN/m), the second-best result in this research regarding interfacial and ST activity. Medium D and F also produced promising ST reduction values of 34.6 mN/m and 36.3 mN/m respectively. The addition of the metals individually (B and C), produced a higher BioS yield compared to its combination, medium F (manganese-iron). Medium F (combination), thus produced 1.45 g/L BioS indicating a negative interaction

between medium B and C. About the combination manganese-iron (medium D), the amount of BioS produced (1.48 g/L) was lower when paralleled to the value obtained with iron (medium C), but better than the one with magnesium (medium A). The combination of magnesium-manganese (medium E) resulted in higher BioS yield (1.81 g/L) when compared with the metals individually (medium A and B) thus indicating a positive interaction on BioS production. Similar to this research, optimization of the trace metals ($Mg^{2+}$, $K^{+}$, $Mn^{2+}$, $Fe^{2+}$, and $Ca^{2+}$) increased BioS yield from (1.74 to 3.34) g/L from *Bacillus subtilis* ATCC 21332 (Wei et al., 2007). Finally, medium G (combination of the three metals) produced the best output with BioS yield (2.11 g/L) and ST reduction (33.7 mN/m). The results were also higher than when the metals were added independently (A-C).

**Table 6.2** Effect of individual metal ion or in combinations on biosurfactant production yield, surface tension and dry cell weight achieved in mediums implemented with *Paenibacillus* sp. D9

| Medium | BioS Yield (g/L) | DCW (g/L) | ST (mN/m) |
|---|---|---|---|
| Control | 1.69 ± 0.12 | 0.36 ± 0.03 | 36.5 ± 0.28 |
| A | 1.36 ± 0.59 | 0.26 ± 0.06 | 36.6 ± 0.28 |
| B | 1.50 ± 0.07 | 0.46 ±0.09 | 34.8 ± 0.21 |
| C | 1.73 ± 0.23 | 0.50 ± 0.04 | 34.1 ± 0.28 |
| D | 1.48 ± 0.03 | 0.41 ± 0.04 | 34.3 ± 0.14 |
| E | 1.81 ± 0.05 | 0.26 ± 0.05 | 33.8 ± 0.28 |
| F | 1.45 ± 0.05 | 0.26 ± 0.07 | 34.6 ± 0.28 |
| G | 2.11 ± 0.22 | 0.39 ± 0.05 | 32.7 ± 0.85 |

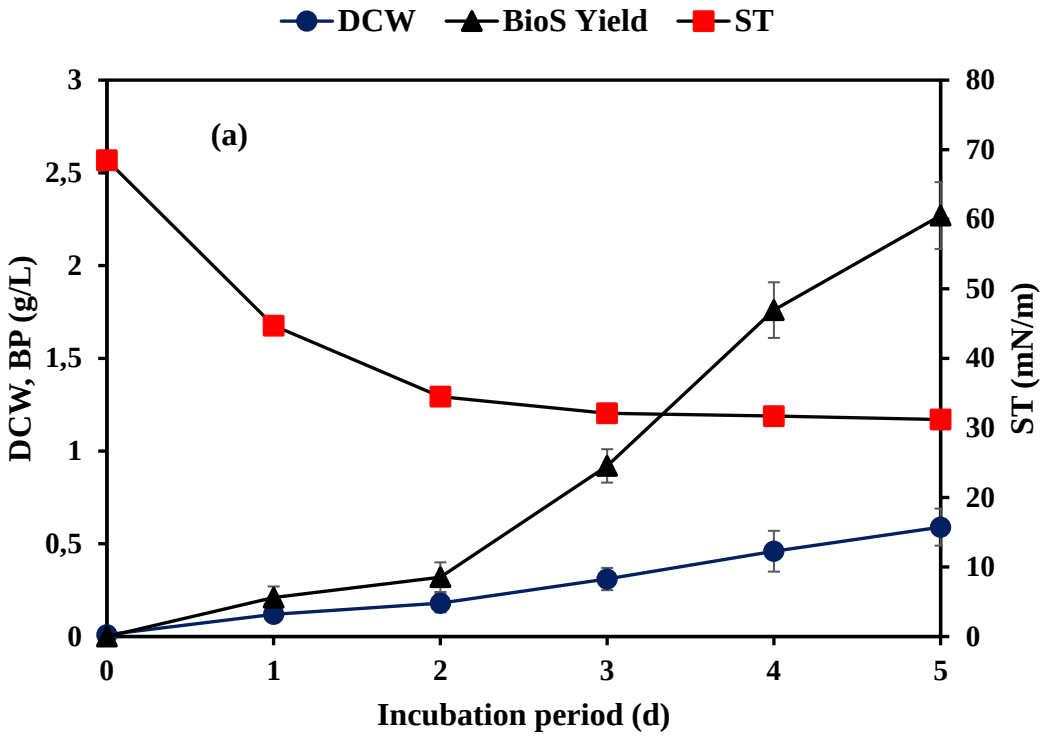
Medium A; 2 mM $MgSO_4$, Medium B; 0.2 mM $MnSO_4$, Medium C; 0.3 mM $FeSO_4$, Medium D; ($MgSO_4$, $FeSO_4$), Medium E; ($MgSO_4$, $MnSO_4$), Medium F; ($FeSO_4$, $MnSO_4$), Medium G; ($MgSO_4$, $MnSO_4$, $FeSO_4$).

Gudina et al. (2015) also reported a positive interaction between iron, manganese, and magnesium on BioS activity. In the experiments conducted with the different media A ─ G, low ST values were obtained ranging from 32.7 to 36.6 mN/m as observed and reported in this research (Table 6.2). However, the result led to higher production yield due to the interaction between manganese, magnesium, and iron. Enzyme co-factors which are mostly metal ions such as iron, manganese, and magnesium are directly involved in lipopeptide BioS

synthesis (Gudina et al., 2015). Therefore, the concentration of these elements which can be considered as helper molecules plays is of significant role in BioS synthesis (Gudina et al., 2015; Wei et al., 2007).

### *6.3.5 Effect of optimum conditions using OVAT studies technique*

From Figure 6.4, the bacterial cell weight in production medium under optimum conditions was high in contrast with the control containing only waste canola oil as carbon source. The cell biomass profile increases and enter the exponential stage under optimum conditions. The trend recommends that *Paenibacillus* sp. D9 indicates better accomplishment when the culturing conditions comprise of waste canola oil and potassium nitrate as supplements at a proportion of 2/1, with a metal enhancement combination ($MgSO_4$, $MnSO_4$, and FeSO4). A combination of optimized culturing conditions enhanced production yield as well as reduction in ST. When waste canola oil and potassium nitrate was selected as the best carbon source and nitrogen source at a ratio of 2/1, *Paenibacillus* sp. D9 synthesized a final improved BioS production yield of 2.27g/L and ST was reduced to 31.2 mN/m at the end of the experiment. From the result, the outputs highlighted in Figure 6.4a produced favourable optimal conditions in comparison to control (Figure 6.4b) where only carbon substrate was utilized.



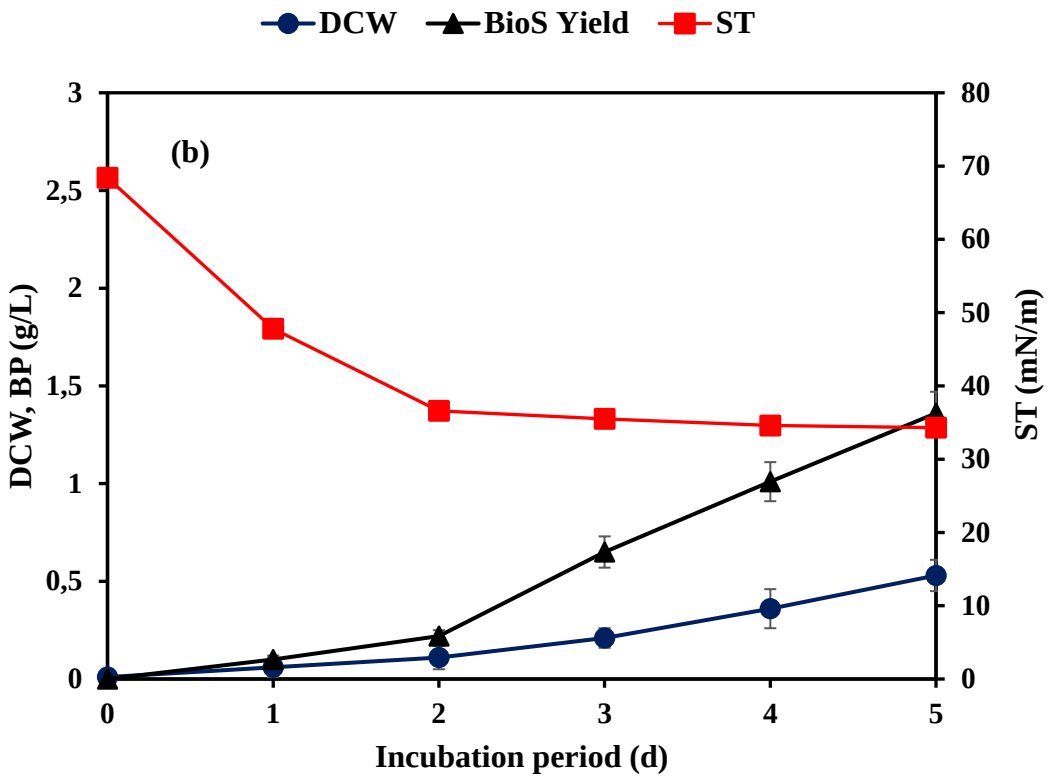



**Figure 6.4** Effect of (a) optimal culturing conditions and (b) control on the cell biomass, surface tension measurement, and biosurfactant yield during biosurfactant synthesis by *Paenibacillus* sp. D9.

These outcomes demonstrate advancement of major parameters that influence BioS synthesis by the OVAT procedure which was successful to decrease production costs. However, this research was also implicated in further enhanced synthesis of BioS to improve their competitiveness in world market. As of now, just a minute quantity of BioSs are economically accessible. This is as a result of few issues such as low yields and production costs (Vossen, 2014). Expanding customer cognizance towards use of environmental products for individual consideration, and other industrial usage may drive BioSs market request. Strong and adaptable guidelines relating to business scale synthesis of BioSs will profit industry members in short and long term. This facilitated the usage of advance statistical technique towards increasing *Paenibacillus* sp. D9 BioS synthesis as described in section 6.3.5.

### *6.3.5 Response surface methodology*

From this study, RSM was utilized to increase the synthesis of BioS by enhancing operational variables. As opposed to basic strategies, the interaction between factors can be predicted by statistical analysis. The simultaneous influence of the three variables was performed using regression analysis suitable for the response function. From the outcome; 17 experimental designs were achieved, and additional runs were done to approximate for controls and error in reproducibility. (Table 6.3). Run 1 provided the best condition for improved BioS production for both response variables, as the highest BioS yield agreed with lowest ST value.

**Table 6.3** Experimental Box–Behnken design runs in Design-Expert 11.0 and corresponding results (the response).

| Run | Factor 1 | Factor 2 | Factor 3 | Response 1 | Response 2 |
|---|---|---|---|---|---|
| | A: Waste frying oil (Canola) | B: Potassium nitrate | C: Metal Supplementation | Surface Tension (mN/m) | BP (g/L) |
| 1 | 5 | 2 | 1 | 31.2 | 5.31 |
| 2 | 3 | 3 | 1 | 34.8 | 2.13 |
| 3 | 5 | 2 | 3 | 32.7 | 4.78 |
| 4 | 3 | 2 | 2 | 33.6 | 3.21 |
| 5 | 1 | 1 | 2 | 36.3 | 1.79 |
| 6 | 3 | 3 | 3 | 34.1 | 2.81 |
| 7 | 3 | 1 | 1 | 33.1 | 3.71 |
| 8 | 3 | 2 | 2 | 33.6 | 3.23 |
| 9 | 3 | 2 | 2 | 33.4 | 3.32 |
| 10 | 1 | 2 | 1 | 36.4 | 1.76 |
| 11 | 1 | 2 | 3 | 36.9 | 1.37 |
| 12 | 1 | 3 | 2 | 37.1 | 1.33 |
| 13 | 3 | 1 | 3 | 34.0 | 2.82 |
| 14 | 5 | 3 | 2 | 32.8 | 4.75 |
| 15 | 3 | 2 | 2 | 34.0 | 3.16 |
| 16 | 3 | 2 | 2 | 33.9 | 3.17 |
| 17 | 5 | 1 | 2 | 31.5 | 5.10 |

The F-value of 55.33 implies the significance of the model and, in this instance, A, B, BC, and $A^2$ were clear significant terms. From the ANOVA, the regression equation specified “Pred R-Squared” of 0.8507 was in practical conformity with the “Adj R-Squared” of 0.9684 (Table 6.4). There exists reproducibility of the experimental data, owing to outstanding low pure error (0.2400).

**Table 6.4** ANOVA results for the produced surface tension quadratic model

| Source | Sum of Squares | df | Mean Square | F-value | p-value |
|---|---|---|---|---|---|
| Model | 47.66 | 9 | 5.30 | 55.53 | < 0.0001 |
| A-Waste frying oil* | 42.78 | 1 | 42.78 | 448.64 | < 0.0001 |
| B-Potassium nitrate* | 1.90 | 1 | 1.90 | 19.94 | 0.0029 |
| C-Metal Supplementation* | 0.6050 | 1 | 0.6050 | 6.34 | 0.0399 |
| AB | 0.0625 | 1 | 0.0625 | 0.6554 | 0.4448 |
| AC | 0.2500 | 1 | 0.2500 | 2.62 | 0.1494 |
| BC* | 0.6400 | 1 | 0.6400 | 6.71 | 0.0359 |
| $A^2$* | 1.11 | 1 | 1.11 | 11.60 | 0.0114 |
| $B^2$ | 0.1901 | 1 | 0.1901 | 1.99 | 0.2008 |
| $C^2$ | 0.0322 | 1 | 0.0322 | 0.3381 | 0.5792 |
| **Residual** | 0.6675 | 7 | 0.0954 | | |
| Lack of Fit | 0.4275 | 3 | 0.1425 | 2.38 | 0.2110 |
| Pure Error | 0.2400 | 4 | 0.0600 | | |
| **Cor Total** | 48.32 | 16 | | | |

The predicted versus experimental plot for ST demonstrated actual values were appropriately close to the straight line (Figure 6.5a), which showed that actual values were near the predicted ones ($R^2$ =0.9862). Along with this line, it was a suitable model to determine BioS synthesis effectiveness utilizing the previously mentioned test parameters. The use of RSM for the valuation of optimal conditions created an experiential association between ST and the process factors. The quadratic polynomial condition featured below best fit the data:

$X_1 = 33.70 - 2.31A + 0.4875B + 0.2750C + 0.15250AB + 0.2500AC - 0.4000BC + 0.5125A^2 + 0.2125B^2 + 0.0875C^2$ in which $X_1$ is ST (m/Nm) and A, B, and C are coded values for waste frying oil, potassium nitrate, and metal supplements respectively.

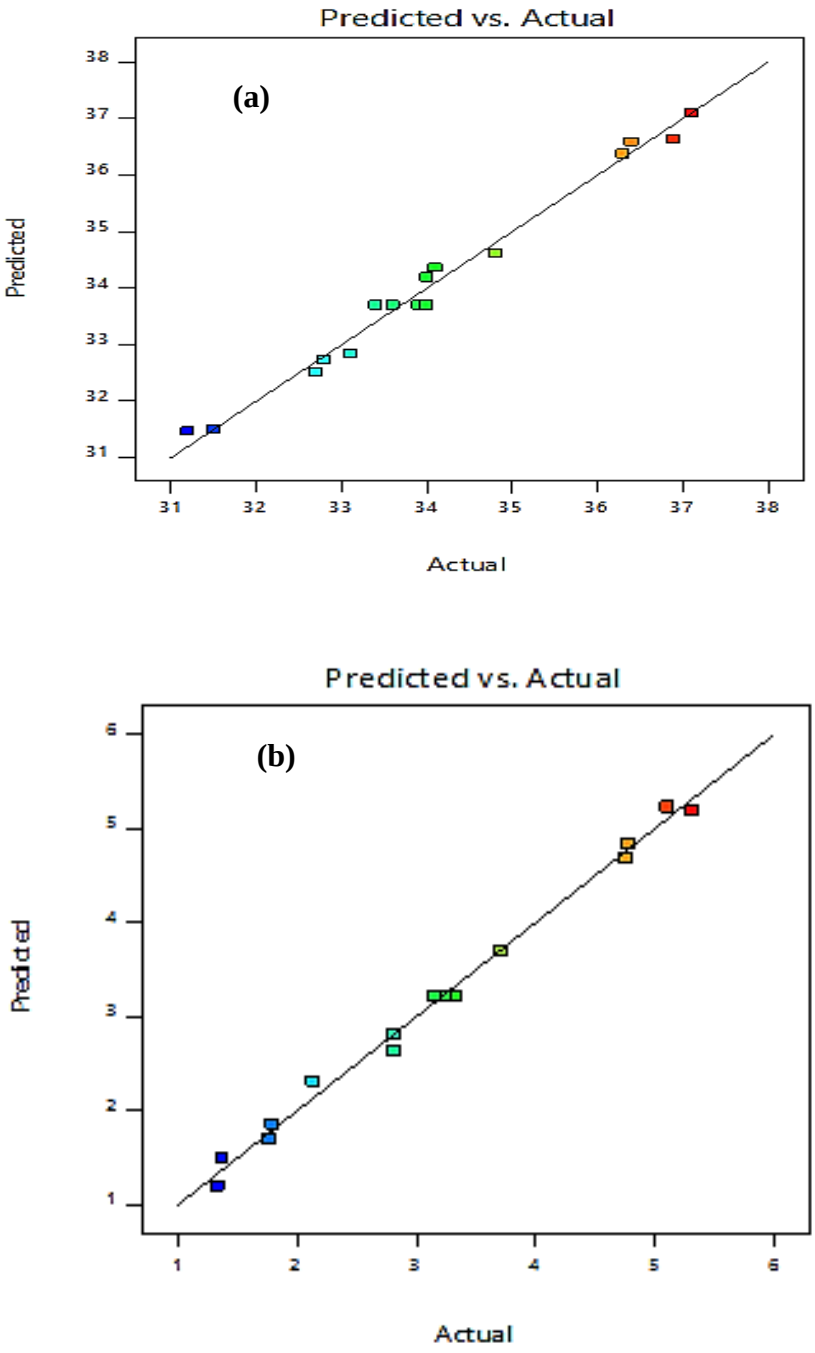



**Figure 6.5** (a) Plot of actual vs predicted surface tension accomplished utilizing biosurfactant synthesized by *Paenibacillus* sp. D9 (b) Plot of actual vs predicted biosurfactant production yield accomplished utilizing biosurfactant synthesized by *Paenibacillus* sp. D9.

Figure 6.6 shows the 3D plots for ST values (BioS production) to determine the interactions of the independent variables tested. Figure 6.6a indicates that potassium nitrate at the middle level with increasing waste canola oil produced the lowest value in ST (31.2 mN/m). Figure 6.6b shows that a decreasing ST (32.7mN/m) was obtained with both factors (metal supplement and waste canola oil) at a maximum level. Figure 6.6c showed that the combination of metal supplement and potassium nitrate at the middle level led to a decrease in ST reduction (33.4 mN/m). The oval nature of the 3D graphs obtainable in Figure 6.6a–b indicated a high degree of positive interaction amongst the factors in each response plot evaluated. Then again, the level curves in Figure 6.6c demonstrated a weak interaction due to parallelism amongst the factors experimented.

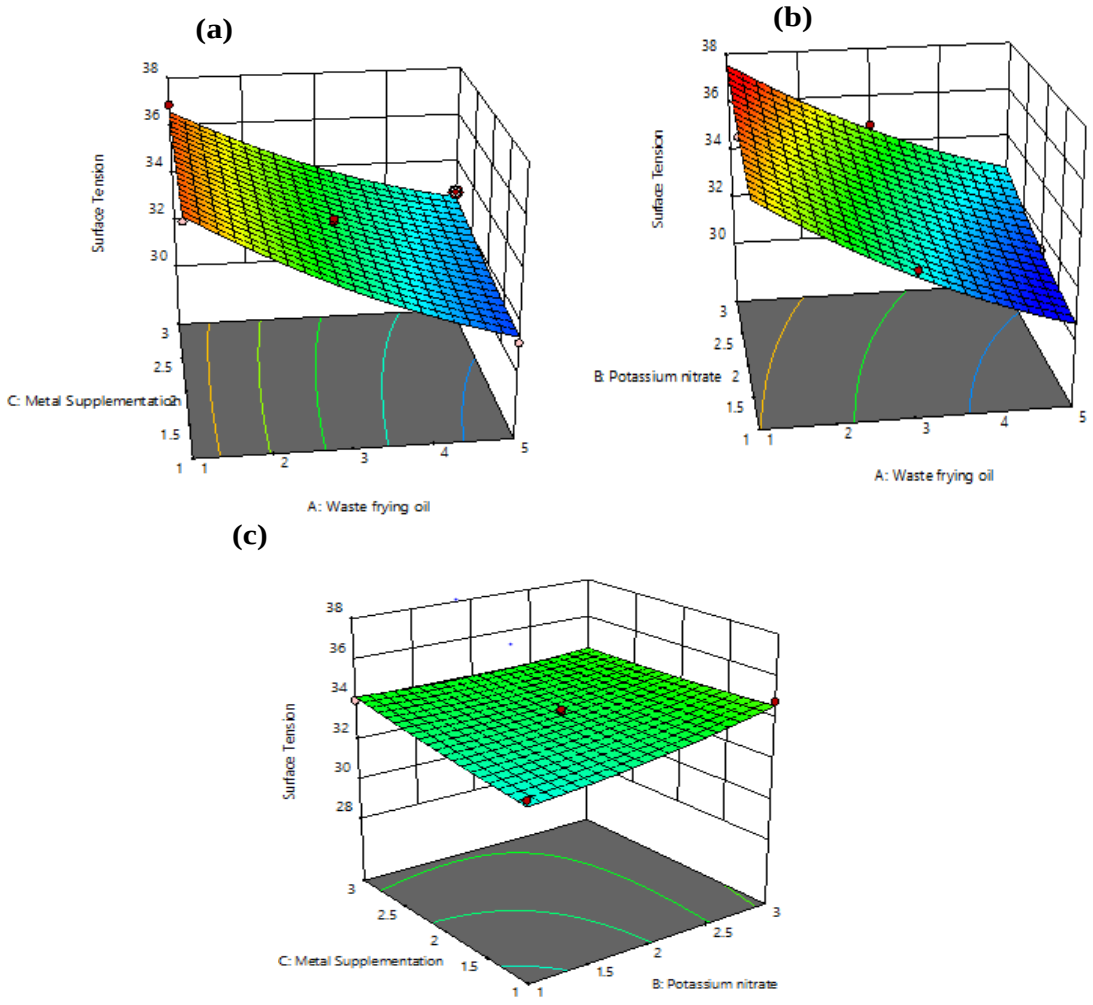



**Figure 6.6** Response surface plots for minimum ST (mN/m) generated using data in Table 4. Reduction in ST as function of (a) potassium nitrate (g/L) and waste frying oil (%); (b) metal supplement (mM) and waste frying oil (%); (c) potassium nitrate (g/L) and metal supplement (mM).

The F-value of 121.99 implies the significance of the model and, in this case, A, B, C, BC, $A^2$, $B^2$ were significant terms. The regression equation obtained after ANOVA (Table 6.5) indicated that the "Pred R-Squared" of 0.9078 is in practical agreement with the "Adj R-Squared" of 0.9855. The predicted versus experimental plot for BP yield showed actual values were distributed near the straight line (Figure 6.5b), which

presented that actual values were near the predicted ones ($R^2$ =0.9937). There also exist excellent reproducibility of the experimental data, owing to the outstanding low pure error (0.163).

**Table 6.5** ANOVA results for the produced biosurfactant production (BP) quadratic model

| **Source** | **Sum of Squares** | **df** | **Mean Square** | **F-value** | **p-value** |
|---|---|---|---|---|---|
| Model* | 25.40 | 9 | 2.82 | 121.99 | < 0.0001 |
| A-Waste frying oil* | 23.43 | 1 | 23.43 | 1012.56 | < 0.0001 |
| B-Potassium nitrate* | 0.7200 | 1 | 0.7200 | 31.12 | 0.0008 |
| C-Metal Supplementation* | 0.1596 | 1 | 0.1596 | 6.90 | 0.0341 |
| AB | 0.0030 | 1 | 0.0030 | 0.1307 | 0.7283 |
| AC | 0.0049 | 1 | 0.0049 | 0.2118 | 0.6593 |
| BC* | 0.6162 | 1 | 0.6162 | 26.63 | 0.0013 |
| $A^2$* | 0.2247 | 1 | 0.2247 | 9.71 | 0.0169 |
| $B^2$* | 0.1795 | 1 | 0.1795 | 7.76 | 0.0271 |
| $C^2$ | 0.0873 | 1 | 0.0873 | 3.77 | 0.0932 |
| **Residual** | 0.1620 | 7 | 0.0231 | | |
| Lack of Fit* | 0.1457 | 3 | 0.0486 | 11.93 | 0.0183 |
| Pure Error | 0.0163 | 4 | 0.0041 | | |
| **Cor Total** | 25.56 | 16 | | | |

The quadratic polynomial equation highlighted below best fit the BP data. The utilization of RSM for the valuation of the optimal conditions created an experimental relationship between the process factors and BioS yield.

$Y_1 = 3.22 + 1.71A – 0.3000B – 0.1412C + 0.0275AB – 0.0350AC + 0.3925BC + 0.2310A^2 – 0.2065B^2 – 0.1440C^2$ in which $Y_1$ is BS production yield (g/L) and A, B, and C are coded values for waste frying oil, potassium nitrate, and metal supplements respectively.

Results revealed in Figure 6.6a–c are analogous to those found in Figure 6.7a–c for ST. The interaction between the three substrates (waste canola oil, $KNO_3$, and metal supplement) should be measured successively as demonstrated by RSM, rather than individually. BioS yield was at a maximum (5.31 g/L) when waste canola oil increased at the maximum level and $KNO_3$ remained at the middle level (Figure 6.7a). The conditions achieved in Run 1 proved to be the most suitable with respect to the process effectiveness and production costs. The oval nature of the 3D plots demonstrates a high level of interaction between the parameters. There was substantial parallelism with the 3D plot curve in Figure 6.7b demonstrating the weak interaction between the two factors (metal supplements and $KNO_3$). However, the combination of the minimum concentration of metal ion supplement and maximum waste frying oil resulted in increased BioS yield with an oval 3D plot curve (Figure 6.7c).

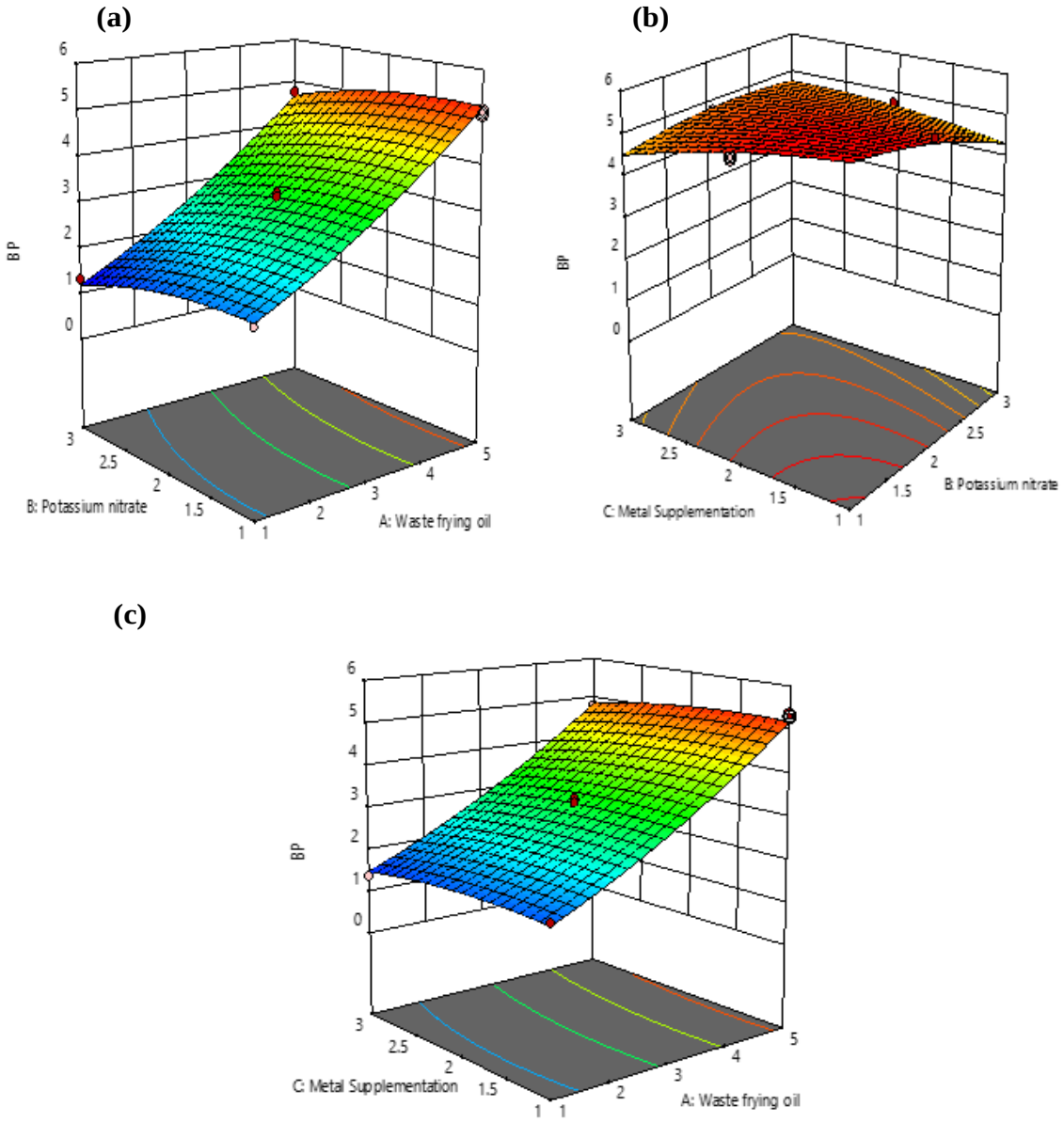



**Figure 6.7** Response surface plots for minimum BP (g/L) obtained with data in Table 4. Biosurfactant production as function of (a) potassium nitrate (g/L) and waste frying oil (%); (b) metal supplement (mM) and potassium nitrate (g/L); (c) waste frying oil (%) and metal supplement (mM).

The result from this study produced a lower ST of 32.7 mN/m thus proposing a better BioS activity. Thus, a better production yield of 5.31 g/L obtainable provides a considerable process parameter in the BioS market enabling its availability as a low-cost alternative for future use. Batista et al. (2010) reported an ST reduction

of 33.66 mN/m and maximum BioS yield of 3.61 g/L utilizing waste frying oil as substrate by *Candida tropicalis.* Oliveira and Garcia-Cruz, (2013) used 5% waste frying oil as carbon substrates, obtained maximum BioS yield of 5.7 g/L and ST of 45 mN/m by *Bacillus pumilus*. The diverse features affecting the BioS synthesis have been considered in the most recent years, however few of these, used appropriate statistical tools for experimental design and modification (Franzetti et al., 2009). The orthodox method of medium optimization includes the OVAT studies approach. This strategy is tedious and leads to negligence in the interaction between factors, with no guarantee in definite determination of the best optimal conditions. In comparison to literature, different microorganisms have been additionally considered utilizing waste frying oil as the carbon source. de Cássia FS da Silva et al. (2013) described the use of RSM in the utilization of low-cost medium formulated with 2% waste frying oil, 3% corn steep liquor, and 0.2% $NaNO_3$. The bacterium, *Pseudomonas cepacia* CCT6659 achieved maximal ST reduction of 26 mN/m and BioS yield of 8.0 g/L. Mnif et al. (2012) employed RSM to increase lipopeptide BioS yield (4.5 g/L) produced by *Bacillus subtilis* SPB1 with a medium made up of sesame peel flour (33 g/L) and diluted tuna fish cooking residue (40%). Economical processes discussed above employ low-cost materials and relatable process parameter. These are important towards successful BioS production and eventually solve the major setback regarding high production cost (e Silva et al., 2014; Rufino et al., 2014).

### *6.3.6 Bioremediation of diesel and motor oil from contaminated sand under shaking and static conditions*

BioS can increase the transport of hydrophobic contaminants toward aqueous phase through some interactions, bringing about emulsification, micellization, pollutants removal and biodegradation (Jimoh and Lin, 2019; Lawniczak et al., 2013). Table 6.6 shows the number of hydrophobic compounds (%) removed from the contaminated sand after treatment with cell-free broth, BioS, a chemical surfactant or distilled water (used as a control). It is imperative that the cell-free supernatant and the isolated BioS removed a comparative measure of oil. The high biodegradation efficiency observed in the cell-free broth over the isolated BioS (76.3% motor oil and 74.4% diesel) might be due to “compounds other than the purified BioS” or during purification, some BioS activity has been destroyed. The use of isolated BioS produced biodegradation efficiency rate of 73.2% for motor oil and 71.2% for diesel from the contaminated sands. Then again, the synthetic surfactant (SDS) demonstrated potential for eliminating diesel and motor oil, yet these qualities were lower than obtained results

in sand treated with BioS. Though the synthetic surfactants present comparable productivity for this application, they are dangerous to the environment because of their toxicity and low biodegradability. The use of BioS for the bioremediation of both diesel and engine oil from polluted sand is ideal over manufactured synthetic surfactant. This was in concurrence with a past report (Marchant and Banat, 2012a). The environmental and ecological friendly BioS had a higher removal efficiency as compared to the chemical surfactant. This gives an inference that BioS has preferable biodegradation proficiency over its chemical counterpart, i.e., SDS in the bioremediation of oil-polluted sand (Table 6.6). As reported by other researchers, BioSs produced are more efficient at removing hydrocarbon mixtures than chemical surfactant (de França et al., 2015; Jimoh and Lin, 2019b). The BioS produced by *Paenibacillus* sp. D9 has feasible application in microbial enhanced microbial oil recovery (MEOR) and solubilization of contaminated sands. This report is also akin to two previous reports. *Bacillus subtilis* CN2 BioS recovered 84.6% of motor oil from contaminated sand and 78–81% of motor oil removal from oil-contaminated sand was achieved by *Paenibacillus dendritiformis* BioS (Bezza and Chirwa, 2015; Bezza and Nkhalambayausi Chirwa, 2015).

**Table 6.6** Removal of diesel and motor oil from contaminated sand by *Paenibacillus* sp. D9 biosurfactant, cell-free fermentation broth, and chemical surfactant

| | **Shaking Assay** | |
|---|---|---|
| | **Motor oil removal (%)** | **Diesel oil removal (%)** |
| Biosurfactant | 73.2 ± 0.3 | 71.8 ± 0.2 |
| Fermentation broth (Cell Free) | 76.3 ± 0.3 | 74.4 ± 0.3 |
| Chemical surfactant (SDS) | 58.8 ± 0.5 | 57.1 ± 0.5 |
| Distilled water | 11.5 ± 0.5 | 11.4 ± 0.6 |
| | **Static Assay** | |
| Biosurfactant | 63.3 ± 0.2 | 59.3 ± 0.1 |
| Fermentation broth (Cell Free) | 67.3 ± 0.3 | 66.4 ± 0.2 |
| Chemical surfactant | 50.3 ± 0.5 | 53.1 ± 0.5 |
| Distilled water | 7.5 ± 0.4 | 7.6 ± 0.6 |

The values are represented as mean, '±' indicates the SD (standard deviation)

From this research, the effect of BioS and chemical surfactant on solubilization of oil-contaminated sands was also determined on static condition (packed column). Studies on microbially enhanced oil recovery provide a bench-scale approach for several reasons such as simultaneous set-up of the column and inexpensive model provision (Chaprão et al., 2015; Suthar et al., 2008). The crude and the isolated BioS synthesized by *Paenibacillus* sp. D9 could eliminate high percentages of motor oil and diesel from packed columns (Table 6.6). In view of its surface activity, *Paenibacillus* sp. D9 BioS was effective in the removal of diesel and motor oil. It is also interesting to observe that kinetic conditions proffered higher removal percentages to static condition. Thus, orbital agitation increased the interaction between the BioS and the hydrophobic pollutants. *Serratia marcescens* BioS produced MEOR efficiency of 76% for crude oil from sand column compared to control experiment (Ibrahim et al., 2013). Water performance in both the removal of motor oil and diesel was negligible as it contributed to 7.5% and 7.6% efficiency of the hydrocarbons, respectively, as shown in Table 6.6. Similarly, Khalladi et al. (2009) proposed water performance in diesel fuel removal was negligible, as it contributed to 24.7% hydrocarbon (*n*-alkanes) elimination. *Rhodococcus ruber* BioS removed oil from the soil core in 1.4–2.3 times better than a chemical surfactant. From this study, *Paenibacillus* sp. D9 BioS removed hydrophobic pollutants better than chemical surfactant tested under static experimental conditions. However, results differ on several factors such as surfactant type, its concentration, kind of soil and sand components (Chaprão et al., 2015; Suthar et al., 2008). BioS adsorbed to soil components was lower compared to a chemical surfactant, due to total penetration through the soil column with 65% – 82% of crude oil removal (Kuyukina et al., 2005).

### *6.3.7 Bioremediation of motor oil and diesel fuel in an aqueous environment*

The widespread use, improper disposal, accidental spills of diesel, motor oil, engine oil, crude oil, and crude oil related products into different ground and surface water sources such as streams, lakes, oceans, rivers, and so on is jeopardizing our human health and wellbeing. Diesel fuel and motor oil pollution of ground water is often a natural or accidental issue (Vossen, 2014). The bioavailability of diesel fuel and motor oil can be increased by the addition of BioSs which will increase diesel oil mobility and solubility, enabling subsequent solubilization of pollutants. Table 6.7 shows the number of hydrophobic compounds (%) removed from an aqueous environment after treatment with cell-free broth, BioS, a chemical surfactant or distilled water (used as a control). This experiment tends to analyze the probable environment that is favourable to the biomolecule in removing the contaminant and to understand factors affecting the performance of the different variables.

The isolated BioS produced biodegradation efficiency rate of 77.6% for motor oil and 74.3% for diesel from the aqueous environment under shaking conditions. It is imperative to note that the cell-free supernatant and the isolated BioS eliminated a comparable amount of oil, emphasizing the efficacy of the biomolecule synthesized by *Paenibacillus* sp. D9. Thus, under shaking conditions, the lipopeptide BioS removed both diesel and motor oil contaminants better than the values observed above in solid environment, however, different results were observed under static assay.

**Table 6.7** Removal of diesel and motor oil from aqueous environment *Paenibacillus* sp. D9 biosurfactant, cell-free fermentation broth, and chemical surfactant

| | **Shaking Assay** | |
|---|---|---|
| | **Motor oil removal (%)** | **Diesel oil removal (%)** |
| Biosurfactant | 77.6 ± 0.5 | 74.3 ± 0.3 |
| Fermentation broth (Cell Free) | 79.2 ± 0.6 | 77.6 ± 0.6 |
| Chemical surfactant (SDS) | 59.2 ± 0.4 | 57.7 ± 0.3 |
| Distilled water | 2.7 ± 0.3 | 2.1 ± 0.3 |
| | **Static Assay** | |
| Biosurfactant | 62.2 ± 0.4 | 57.4 ± 0.4 |
| Fermentation broth (Cell Free) | 64.1 ± 0.2 | 60.3 ± 0.2 |
| Chemical surfactant | 40.8 ± 0.4 | 44.0 ± 0.7 |
| Distilled water | 2.1 ± 0.2 | 1.9 ± 0.4 |

The values are represented as mean, '±' indicates the SD (standard deviation)

The higher removal notable was due to the amphiphilic nature of the BioS, increased solubility, and mobility in the liquid medium as compared to solid environment. Due to the amphiphilic nature of BioS, there was reduction in ST and bioavailability of hydrophobic or insoluble organic compounds (Bezza and Nkhalambayausi Chirwa, 2015). Though, the results indicated some low removal percentages on the contaminants under static conditions when compared to the same experimental conditions in a solid environment. The variance in the output might be due to more time needed for the acclimatization of this amphiphilic BioS to the different contaminants. This explains that agitation increases the interaction between the BioS from *Paenibacillus* sp. D9 and the contaminants (diesel and motor oil). Also, in liquid environment,

both the isolated BioS and cell-free broth produced a better bioremediation removal capability in comparison to chemical surfactant. Surfactants of microbial origin can possibly advance the mobilization and solubilization on hydrocarbons. They do this by emulsification, and pseudo-solubilization which allows expansion of the surface area between oil and water (Vossen, 2014). Pacwa-Płociniczak et al. (2011) likewise express that introduction of BioSs can improve emulsification, solubilization, and subsequent degradation of hydrocarbons. The produced BioS produced from *Paenibacillus* sp. D9 are highly efficient for a sustainable environment devoid of pollutants. The efficacy of the BioS produced in bio-remediating different environmental conditions has also not been relatively tested, hence the impact of this study. As such, the BioS produced on low-cost substrate (waste canola oil) propose a great contribution to literature and overall importance to the world BioS market as the problem associated with high cost of production would become a thing of the past.

## 6.4 Conclusion

This research highlighted the success of BBD and RSM to detect the optimal conditions for increased BioS production yield from *Paenibacillus* sp. D9. The result produced a lower ST of 32.7 mN/m and improved BioS yield of 5.31 g/L. Furthermore, *Paenibacillus* sp. D9 BioS increased the solubilization of hydrophobic motor oil and diesel from contaminated sand and aqueous environment, thereby increasing the pollutants bioavailability and subsequent degradation. As observed, BioS was more effective than the manufactured synthetic surfactant. Thus, the BioS can be utilized as environmentally-friendly and inexpensive approach for bioremediation of oil components.

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## Conflict of interest

There is no conflict of interest.

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# Chapter 7

## Biotechnological applications of *Paenibacillus* sp. D9 lipopeptide biosurfactant produced in low-cost substrates

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# Biotechnological applications of *Paenibacillus* sp. D9 lipopeptide biosurfactant produced in low-cost substrates

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## Abstract

This study assesses *Paenibacillus* sp. D9 biosurfactant synthesis in cheap substrates, its functional properties, and applicability for varying biotechnological processes. Different experimental set-ups were made for oil dispersion, heavy metal removals from contaminated environments, detergent formulations, and washing performances. The detection of concentrated metals from the samples was performed using a multi-element, inductively coupled plasma-optical emission spectrometer (Perkin Elmer) with sample atomization in acetylene flame and compressed air. The study revealed surface tension activities of 31.7 – 32.7 mN/m, and a maximum biosurfactant yield of more than 8 g/L, regardless of the inoculum size used. Removal of 85.90%, 98.68%, 99.97%, 63.28%, 99.93%, and 94.22% was obtained for Ca, Cu, Fe, Mg, Ni, and Zn, respectively from contaminated acid mine effluents. The biosurfactant produced a better performance in removing different metal components from contaminated acid mine effluents and vegetables including improved oil dispersing activity in comparison to chemical surfactants. There was high removal of heavy metals from synthetic wastewater and contaminated sands. A comparative study of different formulations showed that the biosurfactant was more efficient (> 60%) for the removal of tomato sauce and coffee stains than chemical surfactants (< 50%). Besides, *Paenibacillus* sp. D9 biosurfactant synergistically enhanced the removal of tomato sauce and coffee stain from 64.0% to 76.7% and 60.5% to 71.5%, respectively. Therefore, the biosurfactants are probable green biomolecules to substitute chemical surfactants and detergents, thus reducing hazards, and contamination caused to the environment. The future use of this biosurfactant is highly promising in biotechnology.

**Keywords**

Biosurfactant; Chemical surfactants; Detergents; Heavy metals; Low-cost; *Paenibacillus* sp. D9

## 7.1 Introduction

At the present time, there has been huge emphasis positioned at the devastating effects and severity of the usage of synthetic surfactants on the environment (De Almeida et al., 2016). This is based on their highly toxic, persistent nature, and non-biodegradable properties (Sarubbo et al., 2015b). Environmental dangers associated with oil spills in affiliation with heavy metals have limited bio-technological advancements (De Almeida et al., 2016). This ultimately creates a market for alternative "greener" technology. A more sustainable development involving biological molecules called biosurfactants (BioSs) have been given increased recognition over the years (Santos et al., 2016). The desirable functional properties of BioSs have driven their use over the past few decades. They are highly specific, emulsify, have good biocompatibility, are suitable for wetting, foaming, and are overall biodegradable (Santos et al., 2016). In comparison to chemical surfactants, they offer a better and more eco-friendly approach due to their low toxicity as recommended by the World Health Organization (Anyanwu et al., 2011). The stability of a BioS under extraordinary environmental conditions due to surface-active properties depicted its possibility for usage in oil recovery, heavy-metals bioremediation, as well as, food industry (Syahriansyah and Hamzah, 2016; Zhao et al., 2017).

Oil contaminants and petroleum hydrocarbon exists as different forms in the environment such as desorption in water, soil particles adsorption, absorption in soil particles, or presentation at separate phase, which could be a solid or liquid phase (Bezza and Nkhalambayausi Chirwa, 2015). The utilization of surfactants to improve solubilization is one of the viable approaches required to increase the mobilization of hydrophobic contaminants. Notwithstanding, exorbitant cost and toxicity of the synthetic tensioactive agents forestall far-reaching utilization of these surfactants (Santos et al., 2016). Besides hydrocarbons, persistent soil contaminants are heavy metals, posing threats on the ecosystem and human wellbeing indirectly through regular lifestyle or by direct contact to the pollutant (Santos et al., 2016). Since heavy metals are not biodegradable, their removal from soil is particularly challenging, and the conventional remediation usually involves the excavation and transport of the soil for landfill, which is a highly costly process that poses many disadvantages (Usman et al., 2016). Another issue associated with heavy metal contamination is the ability to contaminate plants and undergo subsequent biomagnifications throughout the food chain (Anjum et al., 2016). The continuous ingestion of foods contaminated with heavy metals may lead to detrimental and severe health risks to both humans and animals, as this can lead to the successive accumulation (Singh and Kalamdhad, 2011). In another context; chemical surfactants, household and laundry cleaning products necessitate the innovation of eco-friendly formulations, due to their toxicity on the environment. This need is heightened

further because chemical surfactants comprise almost 15─50% of detergent products composition, with the rest being additives such as fabric softener, enzymes, and bleaching agents (Bouassida et al., 2018).

Despite the plethora of microorganisms producing various BioSs, showing good potential in many applications, there are still a few challenges to overcome in terms of cost and production yield. These problems need to be addressed before BioSs can be considered as commercially viable (Patowary et al., 2017). Exploitation of various low-cost substrates is a means of overcoming these challenges associated with combating the cost implication of the bioprocess (Yañez-Ocampo et al., 2017). Most microorganisms can grow and sustain themselves using the nutrients present in many cheap substrates and waste products, thus minimizing the cost involved (Santos et al., 2016). Cost-effective and renewable carbon sources like molasses, soybean oil, waste frying oil, palm oil, and agricultural residues are now being used for BioS production due to the excessive cost in producing these compounds when using glucose, glycerol, hydrocarbons and other substrates (Santos et al., 2016).

This finding together with the current movement towards sustainability ultimately creates a demand for new formulations and improvements in the environmental and biotechnological industry. Jimoh and Lin, (2019) demonstrated that the lipopeptide BioS produced by *Paenibacillus* sp. D9 exhibited good performance in the degradation of highly toxic substances. Having considered the different prospects of BioS in improving environmental, and biotechnological sustainability, this research was involved in evaluating the effects of low-cost substrates on *Paenibacillus* sp. D9 BioS synthesis and its potential use in oil dispersal, detergent/washing formulations, heavy metals removal from vegetables and contaminated environments. Furthermore, the toxicity and efficiency of the BioS was evaluated in survival trials with *Brassica oleracea*, *Lactuca sativa*, and brine shrimps.

## 7.2 Materials and methods

### *7.2.1 Materials, chemicals, and reagents*

All chemicals were purchased from Sigma Aldrich, Co, USA. Sodium tripolyphosphate and sodium sulfate utilized as builder and filler were of analytical grade. Waste frying oils (sunflower, and coconut) were acquired from different restaurants in the city of Durban, Kwazulu-Natal, Republic of South Africa. Waste frying oil of plant, animal, or synthetic fat were previously used in frying, baking, and other types of cooking. They were stored in the laboratory until further usage. The waste oils are vegetative carbon source, lipidic in nature (16-

20 carbon atom chains) comprised majorly of saturated or unsaturated fatty acids. Two available commercially detergent was purchased from the Durban market, South Africa. Chemical surfactants; sodium dodecyl sulfate (SDS), and Triton X-100 were purchased from Sigma Aldrich, USA for comparative study. The contaminated samples (primary effluent) used in the experiments were obtained from acid mine drainage, northern KZN, South Africa.

### *7.2.2 Growth, and maintenance of Paenibacillus* sp. D9

A culture of *Paenibacillus* sp. D9 was obtained from the Microbiology Department, School of Life Sciences, University of Kwa-Zulu Natal, Westville Campus. A single colony of the bacterial culture was placed in a 5-mL tube for growth overnight at 30°C. The extract was then centrifuged at 10, 000 rpm for 10 min and the pellet washed twice with phosphate-buffered saline of the composition: (g/L) 0.24 $KH_2PO_4$, 1.42 $Na_2HPO_4$, 8.0 NaCl, 0.2 KCl with pH adjusted to 7.6 ± 0.2. The remaining pellet in the Bushnell Haas (BH) medium was then suspended and the optical density (OD) value adjusted to 1.0 at 600 nm. The *Paenibacillus* inoculum was kept at 4°C until further use.

### *7.2.3 Biosurfactant production, extraction, and recovery*

BioS production was carried out in BH medium composition (g/L): $K_2HPO_4$ 1.00, $KH_2PO_4$ 1.00, $MgSO_4 \cdot 7H_2O$ 0.20, $FeCl_3$ 0.05, $CaCl_2$ 0.05, $CaCl_2$ 0.02, $NH_4NO_3$ 1.00, pH of 7.0 ± 0.2. Waste coconut (5.0%), and waste sunflower oil (5.0%) were utilized as low-cost substrates in a 500 mL flask, with the variation of inoculum sizes ranging from 1 to 3 mL. The flasks were incubated at 30°C for 7 days, the solutions were centrifuged, and the culture filtrates were used in the experiments. The increase or decrease in OD was determined using a spectrophotometer (Schimadzu, Japan) at 600nm wavelength. The production medium allowed for clear separation of hydrophobic layer containing the substrates and hydrophilic layer containing the bacterial cells. The spectrophotometer was blanked with the medium containing the waste substrate mixtures during the measurement of $OD_{600}$ value.

At the end of the production period, the crude BioS was obtained by centrifugation (13 500 × *g*, 20 min) and acid precipitation. The pH of 40 mL "cell-free supernatants (CFS) retrieved immediately after centrifugation" was adjusted to 2.0 by the addition of 6 N HCl and maintained at 4°C overnight. The extracts were then washed, lyophilized, weighed, and stored. The crude BioS was purified according to the procedures defined below (Deng et al., 2016). The sample was then liquefied in methanol, mixed with silica gel (230–400 mesh) and

subsequently oven-dried at 50°C. The silica gel was further mixed with chloroform and then loaded onto a chromatography column (50 cm × 2.8 cm). A mixture of ethyl acetate/chloroform in different proportions (100% to 0% with 10% interval), was used in the sequential washing of the loaded column at a flow rate of 0.5 mL/min. A UV spectrophotometer (Cary 60, Agilent Technologies Australia) with a range of 200–800 nm was used to monitor the absorption wavelength of the mixtures to determine the fractions containing the BioS. The eluents (20 mL) were collected and the fractions showing oil-displacement activity were thoroughly mixed. This was followed by evaporation at 80$^{o}$C to acquire purified sample (Deng et al., 2016). The purified BioS was confirmed for surface properties before its further usage. The CFS and the purified BioS described above were utilized for different application set-ups.

#### 7.2.3.1 Surface tension

Surface tension (ST) was determined with a K6 Tensiometer (KRÜSS GmbH, Germany) using 1.9 cm De Noüy platinum ring at room temperature. The culture medium was centrifuged at 13,500 × $g$ for 20 min to obtain a 40 mL CFS (Gudiña et al., 2012). For calibration, the ST of distilled water was first measured. The ST of BH medium supplemented with the waste frying oils (sunflower, and coconut) were analyzed and determined as controls. All readings were produced as three independent experiments with a mean ST value used.

#### 7.2.3.2 Critical micelle concentration

Critical micelle concentration (CMC) was analyzed by measuring the ST sequences of a series of dilutions of BioS concentrations using Tris-HCl buffer solution, pH 8 (Sharma et al., 2015). A stock solution of the crude BioS (1 g/L) was prepared and various dilutions made to obtain a range of the concentrations from 10 to 1000 mg/L. The common experimental procedure is to determine the intersection point of two straight lines that best through the CMC (pre- and post-) data and BioS concentration.

### *7.2.4 Heavy metal removal from contaminated acid mine drainage effluents*

Removal of heavy metals from acid mine drainage samples were evaluated according to the methodology proposed by Dahrazma and Mulligan, (2007). Ten mL of each contaminated sample was transferred to different falcon tubes and, approximately 10 mL of the BioS (500 mg/mL; ST; 30.9 mN/m), sodium dodecyl sulfate (SDS) (500 mg/mL; ST; 30.9 mN/m), CFS (ST; 30.9 mN/m) added separately to the experimental set-up. The samples were incubated at room temperature for 24 h, and subsequently, all samples were filtered through 0.42

µm membrane filter. The detection of the concentrated metals was performed using a multi-element, Inductively Coupled Plasma-Optical Emission Spectrometer (Perkin Elmer) with sample atomization in acetylene flame and compressed air. The initial heavy metal effluent composition included Ca 177.88 ppm, Cu 157.67 ppm, Fe 273.6 ppm, Mg 119.83 ppm, Ni 114.03 ppm, and Zn 315.1 ppm, respectively. The control experiment was achieved without the introduction of the bio-molecule treatments. The percentage of heavy metals removed was determined based on the metals content (control) in the aqueous solution using the following equation;

$\beta = (HMC - HMF)/ HMC \times 100$

Where; HMC; concentration of heavy metals (control, i.e. without treatment), HMF; final concentration of heavy metals (after treatment by lipopeptide BioS).

#### 7.2.4.1 Determination of physicochemical properties of contaminated effluent

Physicochemical analysis of the contaminated acid mine drainage effluents was performed to analyze the effect of some factors, which plays a vital role in the heavy metal removal process. The parameters such as pH, electrical conductivity, salinity, and total dissolved solids were carried out according to standard procedures. Electrical conductivity, salinity, and total dissolved solids were measured as per the instruction manual supplied with the instrument Hatch HQd Portable Meter. The sample pH was analyzed with the aid of 3510 pH meter (Lasec, Jenway). Phosphate and sulfate concentration was determined according to the American Public Health Association standard. Following treatment with *Paenibacillus* sp. D9 BioS, cell-free broth, and SDS, the parameters were measured as stated earlier. All readings were performed in triplicate and deionized water was used as control.

### *7.2.5 Heavy metal removal from the different vegetables*

The different biomass (potato, cucumber, tomato, onion) were washed extensively with running tap water for 30–40 min to remove the particulate matter and dirt. The external parts were pulverized into little pieces, and subsequently immersed in 1:1 HCl solution for 10 min. The different biomass were further washed with twofold deionized water (Anjum et al., 2016). A stock solution of cadmium chloride (1000 mg/L) was prepared in Milli Q for the detection of cadmium (Cd). Upon the introduction of diphenyl carbazide, a violet color was developed, and was measured at an absorbance of 540 nm. Vegetables were exposed to cadmium chloride (0.40, 0.60, and 0.80 mg/mL) for 30 min. The diphenyl carbazide was added to develop violet color and change

in concentration due to absorption was determined by the absorbance at 540 nm. The vegetables from the same stock were treated with BioS, to allow the absorption of cadmium chloride with the biomolecule. The cadmium ion removal percentage due to adsorption was determined as

% Cd removal = (Ci – Cf)/Ci × 100, where

Ci = initial concentration of cadmium (mg/mL), Cf = final concentration of cadmium (mg/mL) (Anjum et al., 2016).

### *7.2.6 Preparation of standard sand with heavy metals*

A metal solution [Pb $(NO_3)_2$ + $ZnSO_4$. $7H_2O$ + $CuSO_4$. $7H_2O$)] was used to contaminate artificial standard sand. The final concentration of 1000 mg/L was achieved through the addition of separate salts dissolved initially in deionized water without pH adjustment. The sand with the salt solutions containing heavy metal was left in contact for proper mixing in an orbital shaker (200 rpm, 25°C) for 2 days. The non-adsorbed metals present in the solution was removed by centrifugation for 10 min at 5000 rpm. The contaminated sands were further dried in an oven at 55°C for 24 h while the supernatant obtained was discarded. The initial and the final weight of the sand was considered to confirm the adsorption of the heavy metals on the contaminated sand.

#### 7.2.6.1 Treatment of contaminated sand with biosurfactant

The sequential treatment of contaminated sand was performed utilizing the purified BioS at full CMC, as well as, crude BioS and CFS. Chemical surfactant (SDS), and distilled water were both used as controls. The experiment was also tested with a 1% HCl solution which was considered both individually and in combination with BioS and CFS. Fifty mL of the solution was introduced at different CMC concentrations and 10 g of sand were subsequently transferred to make a final experimental set-up in a 500 mL Erlenmeyer flask. The samples were incubated in an orbital shaker for 48 h at 25°C, followed by centrifugation at 5000 × $g$ for 10 min. The supernatants collected were analyzed for the residual heavy metal concentration using multi-element, inductively coupled plasma-optical emission Spectrometer (Perkin Elmer). The percentage of heavy metals removed was determined based on the metals content (control) in the aqueous solution of the contaminated sand as described above.

### *7.2.7 Biosurfactant treatment of synthetic wastewater contaminated with heavy metals*

The BioS produced was tested for its capability in removing heavy metals from Phoenix wastewater effluent. The synthetic wastewater after analysis contained a substantial amount of Pb and Zn. The concentrated wastewater was treated separately through the addition of BioS at ½ CMC, full CMC, 2× CMC, and crude BioS to test the ability of the biomolecule to bind to heavy metals present in the aqueous solution (Santos et al., 2017). The conductivity of the resulting solution was measured using the instrument Hatch HQd Portable Meter after removing the metal-BioS precipitate. The Hatch HQd Portable Meter was calibrated with de-ionized water, prior to the measurement of each sample. The percentage of heavy metals removed was also determined based on the metals content (control) in the aqueous solution (synthetic wastewater) as described in the equation above.

### *7.2.8 Oil dispersion assay*

The BioS extracted from *Paenibacillus* sp. D9 was used for its oil dispersing ability according to the methodology described by Andrade Silva et al. (2014). A thin layer of oil on the water surface was formed by the addition of 250 µL of engine oil to the center of 40 mL of distilled water in a petri dish (10 cm). The formation of a clear zone was a positive result for the presence of the BioSs oil dispersing properties. SDS and Triton X-100 were also tested since they are well-known chemical surfactants capable of dispersing oil. The supernatant from the culture was also tested for this property. The oil displacement rate (expressed in %) was attained by measuring the displacement diameter after 30 seconds, relative to the diameter of the petri dish. The rate of oil displacement was calculated as $\frac{\text{Initial Diameter (cm)}}{\text{Petri dish diameter (cm)}}$ x 100. Results were conducted in triplicate and compared relative to negative control of distilled water.

### *7.2.9 Evaluation of wash performance and detergent formulation*

White dry cotton cut into 5 $cm^2$ pieces were stained with 1.25 mL of sunflower oil-tomato sauce and coffee subsequently dried at 40$^oC$ overnight. Two sets of formulations were used with the composition of each set as shown in Table 7.1. To test the wash performance in the first set of experiments, white cloth was either was dipped in any one of the following flasks as stated in Table 7.1 containing (i) 50 mL of tap water (control), (ii) 40 mL tap water and 10 mL of 1.0% (v/v) of each detergent solution, (iii) 40 mL tap water and 10 mL of 1.0%

(v/v) bio-commercial detergent solution, (iv) 40 mL tap water and 10 mL of 1.0% (v/v) BioS solution, or (v) 40 mL tap water and 5 mL each of 1.0% (v/v) detergent and BioS solution.

**Table 7.1** The composition of the experimental set of formulations

| Surfactant/Formulation | BioS | DA | DB | BCD | SDS | X |
|---|---|---|---|---|---|---|
| F1 (Tap only) | - | - | - | - | - | - |
| F2 | 10% w/v | - | - | - | - | - |
| F3 | - | 10% v/v | - | - | - | - |
| F4 | - | - | 10% v/v | - | - | - |
| F5 | - | - | - | 10% v/v | - | - |
| F6 | 5% w/v | 5% v/v | - | - | - | - |
| F7 | 5% w/v | - | 5% v/v | - | - | - |
| F8 | 5% w/v | - | - | 5% v/v | - | - |
| F9 | - | - | - | - | 10% w/v | - |
| F10 | - | - | - | - | - | 10% v/v |

BioS; Biosurfactant, BCD; Bio-commercial detergent, DA; Detergent A, DB; Detergent B, SDS; Sodium dodecyl sulfate; F; Formulation, X; Triton X-100

Flasks were rotated at 200 revs/min for 40 min at room temperature (25°C), followed by the removal of cloth pieces from the flasks, and the left-over wash solution was decanted carefully to avoid soap bubbles. This post-wash water was used to determine the removal of stain from the white fabric cloths. The percentage of stain removal from the white cotton was calculated with the following equations,

Increase in the removal of stain = B – A/B – C (1)

D = B – A; and E = B – C (2)

Where, A = initial weight of the flask before washing, B = weight of the flask + addition of stained white cotton, and C= Final weight of the flask after washing.

% stain removal = (D – E/D) × 100 (3)

### *7.2.10 Toxicity of formulated biosurfactant against Brassica oleracea, and Lactuca sativa*

The phytotoxicity of the produced BioS was assessed by a static test including the seed germination and root development of cabbage (*Brassica oleracea*) and lettuce (*Lactuca sativa*) (Santos et al., 2018). Distilled water was used to prepare isolated BioS in different concentrations of 1 mg/L to 200 mg/L (CMC). The toxicity experiment was determined in sterilized Petri dishes (1 cm × 10 cm) containing filter paper. Twenty-five seeds were inoculated in each Petri-dish containing 5 mL of the test solutions. The seed germination, root elongation (≥ 5 mm) and germination index (GI) were determined below after seven days of incubation (20°C).

Relative seed germination (%) = $(n_s/n_c) \times 100$ (1)

Where $n_s$ it the number of seeds germinated in the sample and $n_c$ that in the control,

Relative root length (%) = $(L_s/L_c) \times 100$ (2)

Where Ls is the sample root length (mean), and Lc that in the control,

GI (%) = [(% seed germination) / (% root length)] x 100 (3)

### *7.2.11 Biosurfactant toxicity to brine shrimp*

Brine shrimp (*Artemia salina*) was used as a toxicity indicator on different concentrations of isolated BioS. Different concentrations of BioS solutions such as, 0, 1, 10, 100 and 200 mg/L (CMC) were tested in this experiment. The assays were carried out using 10 brine shrimp larvae contained in 5 mL aqueous solution (33.3 g/L marine salt solution) in a total of 10 mL glass tubes. Subsequently, 10 mL of each BioS solution at concentrations listed above was introduced in each tube containing the brine shrimp larvae. The tubes were observed for 24 h to determine the rate of mortality. The 50% lethal concentration ($LC_{50}$) to kill brine shrimp within 24 h is defined as the toxicity threshold concentration.

### *7.2.12 Statistical analysis*

All the experimental data were expressed in terms of arithmetic averages of at least three independent replicates, with standard deviation (±). Significance was ascribed using ANOVA at the 95% confidence level.

## 7.3 Results and discussion

### *7.3.1 Biosurfactant production in combination of low-cost substrates*

The capability of *Paenibacillus* sp. D9 to utilize a combination of low-cost substrates for maximum production yield is presented in Table 7.2. However, due to high substrates used (10%), inoculum conditions were varied to ascertain the ability of *Paenibacillus* sp. D9 to withstand selective pressure and concentration. At the end of the experiment, there was increase in OD of the medium indicating a growth-associated BioS production. Results provided in Table 7.2 reveal ST activities of around 31.7–32.7 mN/m, and maximum *Paenibacillus* sp. D9 BioS yield of more than 8 g/L, regardless of the inoculum sizes used. The outcomes showed significance in relative to control samples with no production of BioS yield. This however, ruled out any possibilities of the substrates co-precipitating with the isolated BioS. High reduction in ST from the low-cost production media indicate high production of BioS, thus the great yield obtained. There was no significant difference between the BioS activity output (ST, and BioS yield), thus the differences in inoculum size are inversely proportional to the high concentration of the substrates used. The ST achieved in this research showed high influence of the BioS synthesized as the control sample containing the low cost-substrates only reduced from 71.4 mN/m to 67.8 mN/m). Conversely, a greater rhamnolipid BioS yield of 13.93 g/L was achieved by a non-pathogenic microorganism *Pseudomonas* sp. SWP-4 utilizing waste cooking oil (Lan et al., 2015). Also, the utilization of soybean oil refinery wastes which is another low-cost substrate by *Pseudomonas aeruginosa* MR01 led to maximum production yield of 9.64 g/L (Partovi et al., 2013). Improvement in production procedures and use of inexpensive substrates lowers the initial costs and doubles the benefit of reducing the pollutants while producing useful products. The probable usage of low-cost substrates for improved BioS yield is of great significance to counter the high cost of production. The present work assesses a few residuals from food restaurants to deliver BioS by *Paenibacillus* sp. D9. The waste frying oils utilized was obtained at a relative no expense as an alternate medium. This will significantly diminish the costs associated with large scale production of BioS. This investigation sheds light on the elective usage of waste cooking oil as a high-vitality source for the synthesis of high-value products as lipopeptide BioS.

**Table 7.2** Cell growth, biosurfactant yield, and the surface tension value of its supernatant grown in a BH medium supplemented with 10% low-cost substrates (waste coconut, and sunflower oil). The cell growth was measured by using UV spectrophotometer ($OD_{600}$). Biosurfactant yield attained by weighing lyophilized products while surface tension values were examined at room temperature (25°C).

| ***Inoculum size*** | ***$OD_{600}$*** | ***Biosurfactant yield*** | ***Surface Tension*** |
|---|---|---|---|
| 1% | 1.97 ± 0.04 | 9.05 ± 0.31 | 32.1 ± 0.4 |
| 2% | 2.03 ± 0.06 | 9.56 ± 0.39 | 31.7 ± 0.5 |
| 3% | 2.05 ± 0.07 | 8.14 ± 0.62 | 32.7 ± 0.3 |

Data points are means ± S.D. (standard deviation) of three independent experiments

### *7.3.2 Physicochemical analysis of contaminated acid mine drainage samples*

Determination of the physicochemical properties in contaminated samples is significant because these properties may impact the function of biomolecules, and thus specify their ability for use in applications like heavy metal removal (Velioglu and Urek, 2016).

**Table 7.3** The physicochemical properties of contaminated samples from acid mine drainage before and after treatment with a chemical surfactant, biosurfactant, and cell-free supernatant

| | **Initial** | **Final** | | | |
|---|---|---|---|---|---|
| **Parameters** | | **Control** | **SDS** | **BioS** | **CFS** |
| **EC (ms/cm)** | 6.52 | 6.47 ± 0.57 | 6.23 ± 0.22 | 23.83 ± 0.84 | 3.43 ± 0.06 |
| **TDS (g/L)** | 21.6 | 21.0 ± 0.26 | 18.16 ± 0.27 | 13.94 ± 0.55 | 1.77 ± 0.31 |
| **Salinity (%)** | 3.5 | 3.40 ± 0.07 | 3.05 ± 0.02 | 14.34 ± 0.58 | 1.81 ± 0.02 |
| **pH** | 1.17 | 1.15 ± 0.04 | 1.76 ± 0.05 | 6.03 ± 0.14 | 2.26 ± 0.13 |
| **Sulfate (ppm)** | 1287.68 | 1265.33 ± 7.07 | 821.50 ± 6.62 | 617.12 ± 4.08 | 622.04 ± 13.32 |
| **Phosphate (ppm)** | 5.75 | 5.74 ± 0.03 | 5.50 ± 0.16 | 2.27 ± 0.25 | 3.72 ± 0.09 |

EC; Electrical conductivity, TDS; Total dissolved solids, SDS; Sodium dodecyl sulfate, BioS; Biosurfactant, CFS; Cell-free supernatant. Data points are means ± S.D. (standard deviation) of three independent experiments.

Ultimately, in recording the physicochemical parameters before and after treatment with the BioS, one may, therefore, determine whether treatment affected the use of these molecules as well as whether any alterations in the parameters that occurred (Table 7.3). The initial readings of the physiochemical parameters for the heavy metal effluent displayed that it was highly acidic, with a pH of 1.17 and the salinity of the effluent was rather low at 3%. After treatment with BioS, the pH increased significantly to 6.03 due to BioS structural composition. There was a further surge in salinity to 14.34%, and the electrical conductivity increased from 6.52 to 23.83 µs/cm. In comparison to the BioS, the SDS showed a remarkable reduction in the TDS which is owed in part to its detergent properties. The TDS represents a measure of inorganic salts, organic matter, and various other dissolved material, and in lowering the TDS, the BioS was also able to clear the effluent of these particles (Kim and Vipulanandan, 2006). From this result (Table 7.3), the addition of deionized water which served as a control did not change the physiochemical properties of the contaminated wastewater. However, results following treatment of the effluent with CFS showed a reduction in the electrical conductivity, salinity, and was able to reduce TDS more effectively as compared to BioS and SDS. The pH increase ultimately depicts the ability of the BioS for the treatment of the heavy metal effluent, in raising the pH of the effluent, the conditions of the effluent have become milder and thus more suitable for the environment as opposed to the low pH impact it previously had.

Still, of great concern to the environment is the high incidence of phosphate and sulfate resulting from the contaminated effluents. The BioS including the CFS were both efficient in the removal of both phosphate and sulfate rather to the chemical surfactant (Table 7.3). There was a reduction from initial high concentrations of sulfate and phosphate to 617.12 and 2.27 ppm (purified BioS), 622.04 and 3.72 ppm (CFS) respectively. It was noticed that not much difference come about on phosphate and sulfate removal by CFS and purified BioS. High incidence of these pollutants (sulfate and phosphate) subsequently leading to its release in the water bodies thus creates a long-term effect, in case of algal bloom termed “eutrophication”. Inorganic sources, for example, nitrates, phosphates, and sulfates are some essential contaminants, so it is important to diminish the output levels before releasing. The produced BioS gave great potential since there is a huge market, in removing these pollutants highlighting the usefulness of this biomolecule in environmental sustainability. As such, the toxic, and harmful contaminants present in the effluents was converted into a “less or no” toxic state owing to the complete removal of the different heavy metals. This approach also proffers an ecological safer and cost-effective alternative to the conventional methods as the CFS produced similar efficiency as the purified BioS.

### *7.3.3 Removal of heavy metals from the acid mine drainage effluent*

The extraction of heavy metals by BioS is facilitated through different mechanisms, which includes dissolution, ion exchange, precipitation, and association. The capability in reducing heavy metals, for example, calcium, copper, iron, magnesium, nickel, and zinc, was additionally explored and the outcomes displayed in Figure 7.1. It can be detected that the most astounding potential for eliminating heavy metals occurred when BioS was introduced to the polluted samples. As compared to the chemically synthesized surfactant (SDS), a critical decrease in the grouping of metal was seen after adding the lipopeptide biosurfactant. Removals of 85.90%, 98.68%, 99.97%, 63.28%, 99.93%, and 94.22% were obtained for Ca, Cu, Fe, Mg, Ni, and Zn, respectively, when the purified BioS was used. The results were comparable to the cell-free BioS-containing solution removing (81.18% Ca, 97.9% Cu, 99.65% Fe, 99.79% Ni, 52.15% Mg and 94.22% Zn) from acid mine drainage effluents respectively. These heavy metals become toxic in their ionic species making them difficult to dissociate from the environment (Sarubbo et al., 2015a). The high percentages observed indicated that the removal occurred due to the electrostatic interaction between the molecules of the BioS and the metals. The BioS-metal complex was absorbed from the solution due to the reduction in surface and interfacial tension. The BioS allowed a larger percentage reduction over a period, making the heavy metals present in the non-toxic form. Similar to this research, there was a removal order of Cd = Cr > Pb = Cu > Ni from a multi-element contaminated soil by a di-rhamnolipid produced by *Pseudomonas aeruginosa* BS2 (Juwarkar et al., 2008).

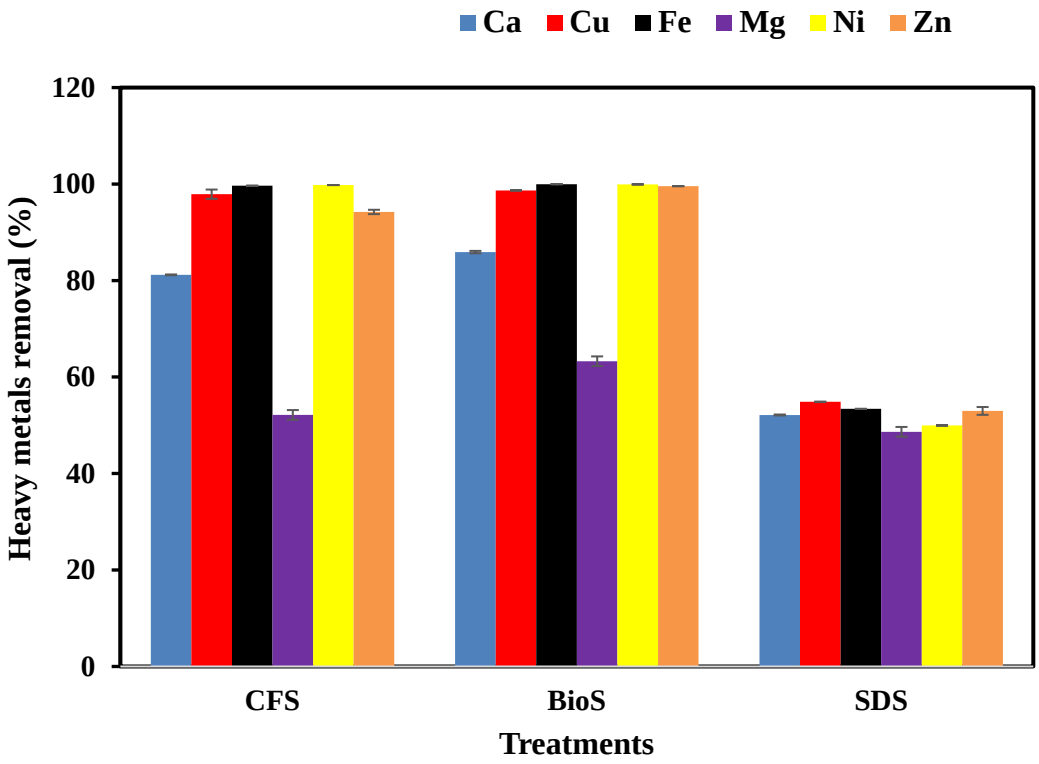


**Figure 7.1** Removal of heavy metals (Ca, Cu, Fe, Mg, Ni, Zn) on contaminated effluent by cell-free supernatant, purified biosurfactant synthesized by *Paenibacillus* sp. D9, and chemical surfactant (SDS)

The addition of BioS and CFS promoted heavy metals desorption from these acid mine drainage effluents through complexation. Still, heavy metals are cations, and this enables their attraction to the negatively charge functional groups present in the biomolecule (CSF and BioS), as this explains their similarities in activity. Thus, the usage of CFS allows for a reduction in production costs, which needs not undergo extraction, recovery, and purification processes which account for 30−50% of the total production cost.

Few reports have mentioned the efficient role of BioS in removing heavy metals from polluted effluents (Elouzi et al., 2012; Hidayati and Surtiningsih, 2014; Sarubbo et al., 2015a). This is the first report to show the effective advantage of BioS in removing heavy metal from acid mine drainage contaminated effluents, and the different physiochemical parameters such as pH, phosphate, sulfates, and so on. This report is therefore of significance to maximize health and environmental benefits associated with BioS treatment. The BioS can be tested further in future environmental applications that involve wastewater from different sources, as the foremost synthetic

contaminants being heavy metals, nitrogen, phosphorus, pesticides, detergents, and hydrocarbons (Akpor, 2011).

Additionally in a polluted soil with Cd and Zn, rhamnolipids BioS increased metal phytoextraction without the conceivable increment of metal mobility in the long term (Wen et al., 2009). However, heavy metals are cations, and this regulates their sorption to negatively charged functional groups present in biomolecule, residual hydroxides ($OH^-$), humic acid, and anionic salts, such as $PO^{4-}$, $SO^{4-}$ (Sarubbo et al., 2015a). The result is novel and significant; hence, the controlled stimulation of the surface-active agent supports in the removal of toxic heavy metals and acid mine drainage pollutants, enabling us to have a safer and cleaner environment.

### *7.3.4 Heavy metal removal from vegetables*

A few health risks in humans have come about due to the overwhelming usage of metal-polluted vegetables (Jimoh and Lin, 2019a). In this setting, different concentrations of cadmium were selectively removed from the varied vegetables like potato, tomato, cucumber, and onion by *Paenibacillus* sp. D9 BioS (Table 7.4). The BioS eliminated a substantial amount of heavy metal from polluted food samples; thus, BioS synthesized could be utilized economically, enabling its usefulness for human health. The concentrations of heavy metal (cadmium) introduced to vegetables in proportion do not have an influence on the % removal capacities of the BioS (Table 7.4). From this study, the BioS selectively removed cadmium from contaminated vegetables in the order of onion = tomato > cucumber > potato. The higher Cd removal ability observed on tomato (71.38%, 73.46%, 74.28%), and onion (65.12%, 66.01%, 67.08%) could be due to absorption to this heavy metal. BioS reduced high absorbed concentrated cadmium from the two vegetables. A comparable result was obtained as the BioS synthesized from *Bacillus* sp. MTCC 5877 removed 61.03% Cd from contaminated onion (Anjum et al., 2016).

**Table 7.4** The cadmium initial, final concentrations, and its percentage removal after treatment with biosurfactant

| S. no | Vegetables | Initial Cd concentration (mg/mL) | Final Cd concentration (mg/mL) | Cd removal (%) |
|---|---|---|---|---|
| 1 | Potato | 0.400 | 0.21 | 47.67 ± 0.01 |
| 2 | Potato | 0.600 | 0.31 | 48.12 ± 0.01 |
| 3 | Potato | 0.800 | 0.41 | 48.79 ± 0.03 |
| 4 | Tomato | 0.400 | 0.11 | 71.38 ± 0.01 |
| 5 | Tomato | 0.600 | 0.16 | 73.46 ± 0.01 |
| 6 | Tomato | 0.800 | 0.21 | 74.28 ± 0.01 |
| 7 | Onion | 0.400 | 0.14 | 65.12 ± 0.02 |
| 8 | Onion | 0.600 | 0.20 | 66.01 ± 0.01 |
| 9 | Onion | 0.800 | 0.26 | 67.80 ± 0.02 |
| 10 | Cucumber | 0.400 | 0.18 | 55.72 ± 0.01 |
| 11 | Cucumber | 0.600 | 0.28 | 52.59 ± 0.02 |
| 12 | Cucumber | 0.800 | 0.38 | 52.15 ± 0.02 |

Data points are means ± S.D. (standard deviation) of three independent experiments

The final reaction between different cadmium concentrations (0.4, 0.6, 0.8 mg/mL) and 1,5-diphenylcarbazide ($C_{13}H_{14}N_4O$) gives a violet colour in the different vegetables set-up (Figure 7.2a). Therein, the use of a chemical surfactant (SDS) showed no effect on the color absorption of the heavy metals of the different vegetables tested (Figure 7.2b). The adherence of biomolecule to the food contact surface needs to be controlled and it is critical to providing healthy and safe food products to the consumers at large. Thus, the utilization of this BioS could be an imperative apparatus for the food industry as the excessive intake of heavy metals through food is highly dangerous to human health. Successively, the introduction of BioS to the experimental set-up was effective as there was a substantial color change from violet to colorless, confirming the BioS ability to remove the metal absorption from the different vegetables (Figure 7.2c). Compared to the control, a significant reduction of the heavy metals was observed with the introduction of the BioS. In this regard, the cautious and measured usage

of this surface-active compound will most likely support improved washing of the compounds from surfaces of vegetable and food crops present in the soil environment. Similar to this research, Anjum et al. (2016) reported the removal of 47%, 61.0%, 62.5%, and 73% Cd, respectively from different vegetables by a BioS produced from *Bacillus* sp. MTCC 5877. Also, the rhamnolipid BioS produced from *Pseudomonas putida* might play a great part in the removal of these toxic heavy metals, as 50% zinc and iron were both removed from the contaminated medium (Meenakshisundaram and Pramila, 2017). The *Bacillus licheniformis* VS16 BioS likewise reduced cadmium (Cd) from contaminated vegetables namely ginger, carrot, radish, and potato with the highest removal being 60.98% (Giri et al., 2017). This *Paenibacillus* sp. D9 BioS thus demonstrate its capacity as a washing agent in heavy metal removal from both contaminated acid mine effluents and vegetables when compared to a synthetic surfactant. This enabled its usefulness in the world market as a bioremediation agent and important tool in biotechnological and environmental sustainability.

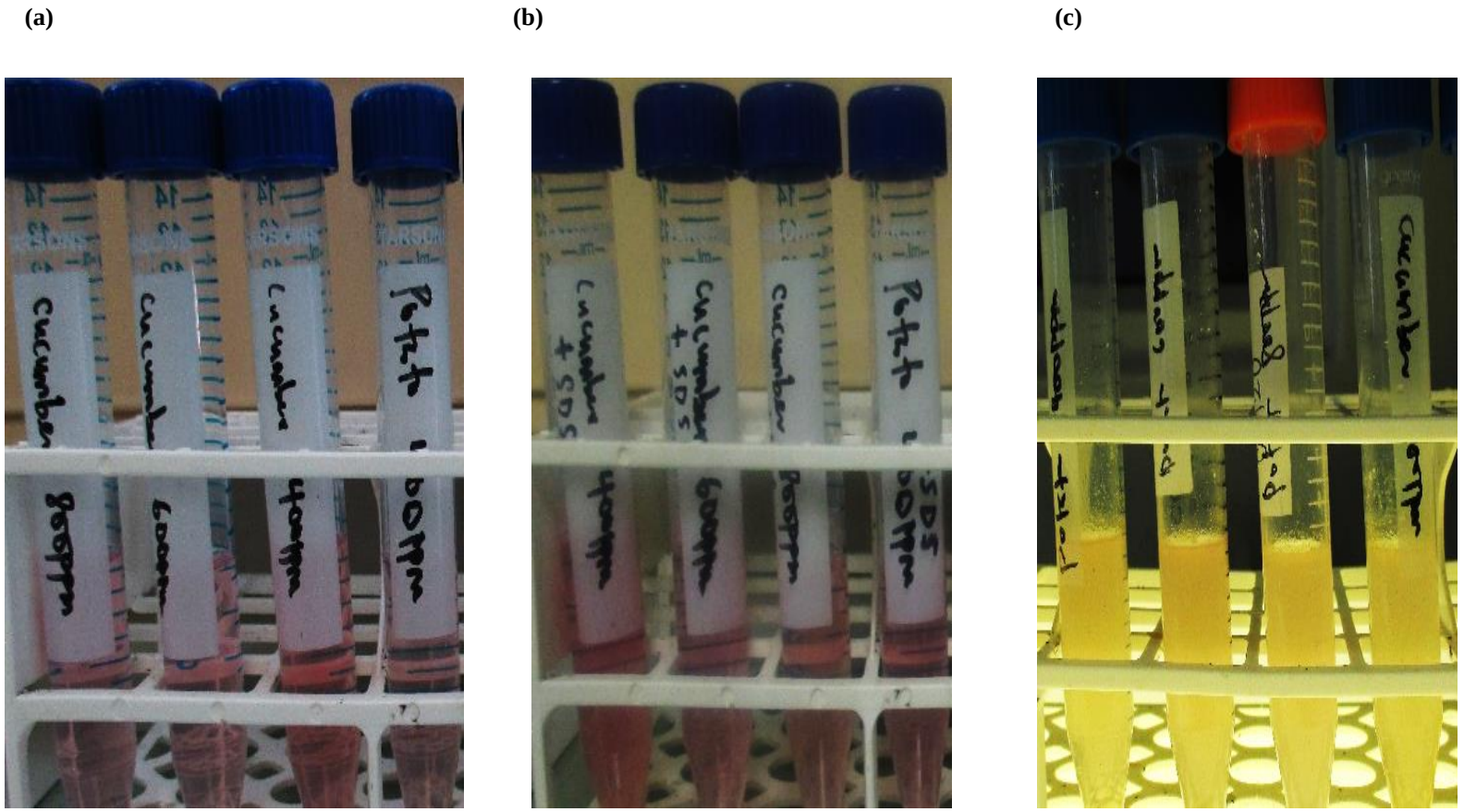


**Figure 7.2** A representative of the heavy metal removal (Cd) from vegetable stocks after the introduction of (a) control (b) chemical surfactant (c) biosurfactant.

### *7.3.5 Removal of heavy metals from contaminated sand*

There is increased interest in the discovery of novel washing procedures and bio-products, such as amphiphilic BioSs equipped for attaching metals and do not present dangers to nature (Santos et al., 2017). Solutions of purified BioS at various concentrations [1/2 × CMC (0.1%), CMC (0.2%), and 2 × CMC (0.4%)] were assessed for the removal of metals with and without micelle formation. From the results obtained, *Paenibacillus* sp. D9 BioS was highly effective in removing copper and lead, while lower percentages were observed for zinc (Table 7.5). As observed from this result, increase in BioS concentration was not proportional to the percentage removal of heavy metals namely copper, lead, and zinc. The BioS-produced possess very low affinity to zinc, giving a low removal efficiency of ≤ 60% at all the concentrations as compared to higher removal percentages (≥ 60%) of copper and lead respectively. The high removal observed for both copper and zinc is frequently identified with BioSs' binding to the constituents of the soil particles. The 1% HCl solution removed 50% – 55% of the metals adsorbed to the sand and the removal rate was enhanced altogether when acid solutions were combined with purified BioS and CFS. However, the introduction of the HCl solution showed no influence on the removal capabilities of the BioS with ≤ 60% zinc removal from contaminated sands achieved.

Diverse BioS have fluctuating attractions to various metals and are constantly influenced by concentrations of acids or alkalines, biomolecules, charge of heavy metal, and soil properties (Ochoa-Loza et al., 2007). The crude BioS removed a higher percentage of copper, and lead from the sand, demonstrating its utilization, as well as, BioS in the decontamination of heavy metal polluted soils. The downstream procedure to purify BioS could account for 60% of the total cost, as such, crude BioS would be highly efficient in achieving a cost-effective bioprocess. Santos et al. (2017) also reported ∼30% of the heavy metal removal from contaminated sand, with a further ≥80% removal was achieved when different additives were introduced. This is like this study by which increasing BioS concentrations was not proportional to the heavy metal removal capacities. *Candida sphaerica* BioS demonstrated 95%, 90%, and 79% removal rates for Fe, Zn, and Pb, respectively, from samples, gathered from a car battery industry. The introduction of HCl solutions increased removal rate when utilized with BioS at concentrations of 0.1% and 0.25% (Luna et al., 2016).

**Table 7.5** Heavy metals removal from contaminated sands using different washing solutions (data expressed as mean ± standard deviation)

| Treatment | Heavy metal removal (%) | | |
|---|---|---|---|
| | Cu | Pb | Zn |
| Distilled water (control) | 22.5 ± 0.3 | 4.1 ± 0.2 | 10.9 ± 0.3 |
| 1% HCl solution | 51.6 ± 0.9 | 54.7 ± 1.2 | 50.8 ± 0.6 |
| Sodium dodecyl sulfate | 48.5 ± 0.8 | 28.6 ± 0.1 | 15.5 ± 0.2 |
| Cell-free supernatant | 82.5 ± 0.3 | 96.4 ± 0.4 | 53.2 ± 2.1 |
| Cell-free supernatant + 1% HCl solution | 88.6 ± 0.5 | 98.1 ± 0.4 | 56.5 ± 0.8 |
| 0.1% biosurfactant solution (1/2 CMC) | 60.1 ± 0.2 | 94.0 ± 0.1 | 53.3 ± 1.2 |
| 0.1% biosurfactant solution (1/2 CMC) + 1% HCl solution | 78.8 ± 0.5 | 96.8 ± 0.3 | 57.8 ± 0.8 |
| 0.2% biosurfactant solution (CMC) | 63.8 ± 0.4 | 96.6 ± 0.3 | 43.8 ± 2.3 |
| 0.2% biosurfactant solution (CMC) + 1% HCl solution | 81.4 ± 1.1 | 98.7 ± 0.5 | 51.5 ± 0.8 |
| 0.4% biosurfactant solution (2 × CMC) | 84.4 ± 0.2 | 96.4 ± 0.1 | 57.9 ± 1.9 |
| 0.4% biosurfactant solution (2 × CMC) + 1% HCl solution | 86.7 ± 0.6 | 98.6 ± 0.4 | 59.1 ± 0.4 |

### *7.3.6 Biosurfactant ability to bind heavy metals in aqueous solution*

The ability of the BioS to bind heavy metals (Pb, and Zn) present in the synthetic wastewater was determined by measuring the conductivities and heavy metal removal capabilities. The initial conductivity of the metal solutions containing concentrations viz, 1/2 CMC, CMC, and 2× CMC was 80 µS/cm, 92 µS/cm, and 76 µS/cm, respectively. Regardless, the conductivity of the solutions comprising zinc (Zn) and lead (Pb) experienced a highlighted decrease when BioS was introduced. The BioS was able to precipitate the positively charged metals from the solution, as such, leading to metal ion reduction and subsequently diminishing its conductivity (Table 7.6). The removal capabilities were observed with CMC (58.1% Pb, 53.3% Zn) and 2× CMC (77.5% Pb, 57.7%

Zn), respectively. There was a very low performance observed with half CMC in terms of conductivity and removal capabilities in both heavy metals. It is notable that high concentration of this BioS eliminated metals in a large proportion. This outcome displayed more micelles incited less free particles and conductivity was accordingly less than in the solutions with no or little BioS. Generally, the different concentrations achieved a greater removal capability of Pb, and Zn in comparison to chemically synthesized surfactant (SDS), and distilled water (negative control). In contrast to this research, no variation in the effect of different BioS concentrations was exhibited on the conductivity of the metal in synthetic wastewater (Santos et al., 2017).

**Table 7.6** The conductivity as well as heavy metal removal of the metal solutions when washing with solutions of *Paenibacillus* sp. D9 biosurfactant (data expressed as mean ± standard deviation)

| Treatment | Conductivity (µS/cm) | | Heavy metal removal (%) | |
|---|---|---|---|---|
| | Pb | Zn | Pb | Zn |
| Initial metal conc. (ppm) | 177.2 ± 0.6 | 194.5 ± 2.5 | | |
| 1/2 CMC | 136.7 ± 0.5 | 158.3 ± 2.4 | 32.2 ± 0.5 | 31.6 ± 1.0 |
| CMC | 96.7 ± 0.6 | 115.0 ± 1.6 | 58.1 ± 1.1 | 53.3 ± 1.3 |
| 2 × CMC | 93.4 ± 0.8 | 100.7 ± 2.0 | 77.5 ± 0.3 | 57.7 ± 0.7 |
| Crude BioS | 163.9 ± 0.2 | 176.0 ± 2.0 | 40.8 ± 0.4 | 37.5 ± 1.4 |
| Distilled water | 175.0 ± 1.7 | 191.4 ± 1.5 | 7.6 ± 1.6 | 16.9 ± 1.1 |
| SDS | 161.6 ± 2.5 | 171.2 ± 1.7 | 13.9 ± 1.8 | 19.3 ± 1.8 |

### *7.3.7 Oil dispersion assay*

Oil dispersion is another technique that depicts the capability of the BioS to remove oil from surfaces using its surface and interfacial tension reducing properties, therefore, providing application in oil clean-up and control of oil spillages. The *Paenibacillus* sp. D9 BioS achieved a significant dispersal rate of 60% whereas the SDS, Triton X-100 had 25 and 20%, respectively. The dispersion rate of the CFS was 30% and the negative control had the smallest initial diameter of 1.5 cm, obtaining a dispersal rate of only 15% (Table 7.7). The oil dispersal technique represents both a means of confirming the presence and screening for BioS production by the microorganism as well as a measure of the surface-active properties. This is because the detection of a zone of

clearing indicates that oil has been displaced due to the presence of the BioS (Ibrahim et al., 2013). The diameter of this zone of clearing positively correlates with the concentration of BioS and depicts oil spreading activity (Chandran and Das, 2010). This shows the efficiency of BioS, and the larger the diameter of the dispersal, the greater the activity of the surfactant (Chandran and Das, 2010). The oil dispersion limit of a BioS is of extraordinary significance when the goal is to treat situations polluted with hydrocarbons (Freitas et al., 2016).

**Table 7.7** The dispersion rate of engine oil after treatment with *Paenibacillus* sp. D9 biosurfactant, SDS, supernatant, Triton X-100, and distilled water

| Sample | Initial diameter (cm) | Final diameter (cm) | Displacement rate (%) |
|---|---|---|---|
| **Biosurfactant** | 6.0 ± 0.4 | 10 | 60 ± 0.4 |
| **SDS** | 2.5 ± 0.3 | 10 | 25 ± 0.3 |
| **Cell-Free Supernatant** | 3.0 ± 0.6 | 10 | 30 ± 0.6 |
| **Triton X-100** | 2.0 ± 0.2 | 10 | 20 ± 0.2 |
| **Control (Distilled $H_2O$)** | 1.5 ± 0.1 | 10 | 15 ± 0.1 |

When comparing the dispersal rate of the BioS to that of the positive controls SDS, Triton-X, it was evident that *Paenibacillus* sp. D9 BioS displays the greatest dispersing ability (Figure S1). Although SDS and Triton-X are good chemical surfactants having been used in many applications around the world, the impact of their use on the environment displays a major drawback (Jimoh and Lim, 2019a). Since the results obtained in Table 7.7 involved the use of minute volumes of BioS, it demonstrates the potential of this biomolecule to withstand much higher concentrations such as oil spills control, and detoxification applications. Likewise, the BioS acquired from *Bacillus licheniformis* culture had the most minimal oil spreading activity (23 mm) in the crude oil-liquid medium while, *Bacillus firmus*, *Bacillus lentus*, *Pseudomonas paucimobilis*, *Serratia marcescens* and *Micrococcus kristinae* had 45 mm, 30 mm, 27 mm, 38 mm and 51mm, respectively (Ibrahim et al., 2013).

### *7.3.8 Fabric wash performance, and formulations*

Figure 7.3 shows the illustrative detergency test representing the relative washing performances of *Paenibacillus* sp. D9 BioS, SDS, Triton X, commercial detergent, and bio-commercial detergent combination

against sunflower oil-tomato sauce and coffee stains. The washing efficiency of *Paenibacillus* sp. D9 BioS formulation was compared to two chemical surfactants, namely anionic surfactant (SDS) and non-ionic surfactant (Triton X-100). This latter, being anionic act as a detergent, and an emulsifier. In the present study, the synthetic surfactant displayed less washing efficacy in comparison to *Paenibacillus* sp. D9 BioS formulation. The *Paenibacillus* sp. D9 BioS influenced washing could eliminate more than 64.3% of tomato sauce and 60.4% of coffee stains while the chemical surfactants removed only 52.2% of tomato sauce, 47.1% of coffee (SDS), and 46.7% tomato sauce, 42.2% of coffee (Triton X-100) from the white cotton fabric respectively. These outcomes are as per with *Pseudomonas aeruginosa* BioS formulation which was effective in removing whiteboard marker stains as compared to chemical surfactants (Turbekar et al., 2014). Also, the formulation *Bacillus subtilis* SPB1 BioS exhibited better cleaning efficiency on oil and tea stains removal as compared to the conventional chemical surfactant (Bouassida et al., 2018).

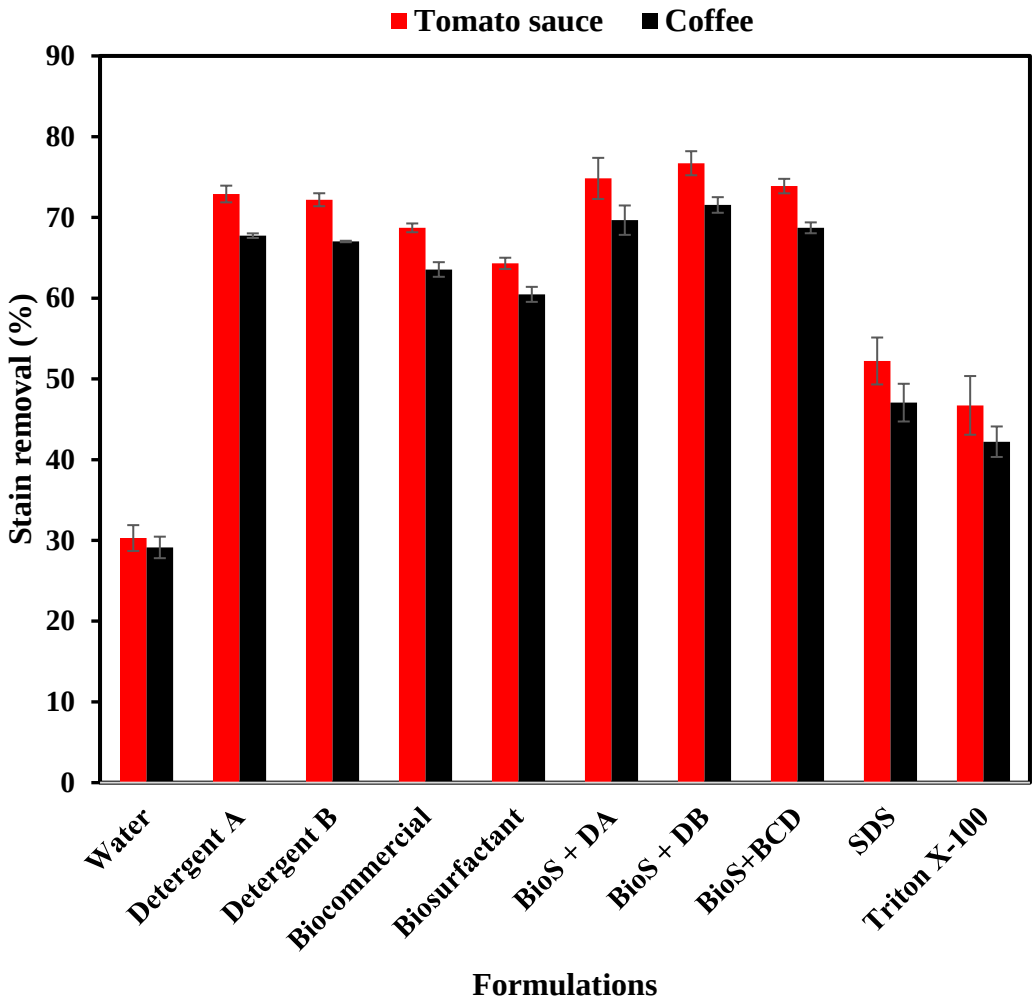


**Figure 7.3** Comparison of the effect of different formulations for stain (coffee, and tomato sauce) removal from fabric cotton. BioS; *Paenibacillus* sp. D9 biosurfactant, DA; Detergent A, DB; Detergent B, BCD; Bio-commercial detergent, SDS; sodium dodecyl sulfate.

The differences in stains (such as a yellow solid containing phenolic, an acrylic group in coffee stain, caffeic acid, and curcuminoids) makes them notorious and difficult to wash (Joshi-Navare et al., 2013). In this regard, there is an indication of BioS ability to remove most of these stains efficiently as well as detergent. For this, it will be imperative to compare to *Paenibacillus* sp. D9 BioS formulation efficiency with two commercial detergents obtainable in Durban, South Africa (Figure 7.3). In this study, the two commercial detergents produced a better washing and removal capability of different stains to when *Paenibacillus* sp. D9 BioS was used alone. The results obtained are not far-fetched as the two-commercial detergents have been processed

industrially with additional chemicals, and additives while the BioS was in its isolated pure form, which could have lost some of its cleaning properties during extraction, isolation, and purification processes. The stain removal by *Paenibacillus* sp. D9 BioS alone was less capable, but also comparable, and effective. As observed in Figure 7.3, 64.3% and 60.4% of removal of tomato sauce and coffee stains, was obtained when using *Paenibacillus* sp. D9 BioS and commercial detergent A removed 72.9% of tomato sauce and 67.7% of coffee stains, while 72.2% of tomato sauce, and 67% of coffee stain were removed by commercial detergent B, respectively.

The bio-commercial detergent removed a percentage like the above listed for commercial detergent A and commercial detergent B (Figure 7.3). Quite similar to this study, Khaje Bafghi and Fazaelipoor, (2012) described that, in as much as commercial formulation was more effective than the natural products in stain removal from white cotton material, the difference was not high. Moreover, the ability of sophorolipids, a glycolipid BioS synthesized by *Candida bombicola* (ATCC22214) was about equivalent to detergent in removing four types of stains (espresso, turmeric, oil, and poster) from cotton and polyester fabrics (Joshi-Navare et al., 2013). Sajna et al. (2013) revealed that stain removal by glycolipid BioS, synthesized by *Pseudozma* sp NII 08165, alone was effective and practically identical to that of the commercial cleanser.

Finally, in this study, *Paenibacillus* sp. D9 BioS was supplemented with each of the two commercial detergents with the ratio of 1:1 (v: v) respectively. The 1:1 (v/v) BioS-commercial detergents formulation gave an increase in wash performance as observed in Figure 7.3. This demonstrated the *Paenibacillus* sp. D9 BioS had positive outcomes on the performance of the commercial formulated detergents rather than using commercial detergents alone. Similarly, Jatropha oil derived sophorolipids BioS and detergent combination lead to an improved coffee stain elimination from cotton fabric rather than the detergent alone (Joshi-Navare et al., 2013). There was also substantial synergy on wash performance between *Paenibacillus* sp. D9 BioS and commercial surfactants in a proportional ratio of 1:1 (w/w) in the role of compost humic acid-like matter in the detergent formulation (Savarino et al., 2010). The detergent-like properties like BioSs hold many applications with respect to laundry and detergent industries. Although lacking the additives present in commercial detergents, BioSs have shown promising results in their ability to reduce stains when compared to commercial detergents (Bouassida et al., 2018).

The formulation displayed in this investigation offers a favourable position in the expulsion of hydrophilic stains in contrast with other formulations presented. This lipopeptide BioS can be a fruitful surfactant and

cleanser formulations. The stain elimination potential of BioS-containing detergent is equivalent to manufactured ones particularly for the removal of hydrophilic and hydrophobic dangerous stains. This research proffers an incredible noteworthiness since microbial BioS are well-thoroughly considered as a substitute for chemical surfactants because of their low or non-toxicity and higher biodegradability.

### *7.3.9 Paenibacillus* sp. D9 *biosurfactant ecotoxicity*

The germination index which joins proportions of the overall seeds' germination and relative development of roots, was utilized to assess the toxic effect of *Paenibacillus* sp. D9 BioS to cabbage and lettuce seeds. The proportion of ≤ 80% GI is considered as a positive indicator, thus indicating the non-existence of phytotoxicity (da Rocha Junior et al., 2018). The outcomes demonstrate that the different *Paenibacillus* sp. D9 BioS solutions tested had no inhibition on germination of the seeds as well as root development (Table 7.8). From this result obtained, the germination index of *Brassica oleracea* (cabbage) was relatively higher than *Lactuca sativa* (lettuce) across all the concentrations of BioS tested. The germination index values of 103.4, 102.9, 104.9, 117.1% were observed for the former while values of 92.6, 87.8, 89.8, and 94.7% for the latter at a BioS concentration of 1, 10, 100, 200 mg/L, respectively. The development of auxiliary roots and the rise of leaves were additionally noticed for the different experimental conditions tested on *Brassica oleracea*, and *Lactuca sativa*. Like this research, higher GI values of 201, 128, 113 and 113% were observed for cabbage, and values of 189, 110, 105, 96%, respectively for lettuce against different concentrations of *Streptomyces* sp. DPUA1566 BioS (Santos et al., 2018).

**Table 7.8** Phytotoxicity of *Paenibacillus* sp. D9 biosurfactant against *Brassica oleracea*, and *Lactuca sativa*

| Biosurfactant concentration (mg/L) | | Phytotoxicity (%) | | |
|---|---|---|---|---|
| | Seeds | Seed germination | Root elongation | GI |
| 1 | *Brassica oleracea* | 90.9 ± 2.6 | 87.9 ± 1.5 | 103.4 |
| | *Lactuca sativa* | 116. 3 ± 2.6 | 125.6 ± 3.6 | 92.6 |
| 10 | *Brassica oleracea* | 109.1 ± 1.0 | 106.1 ± 1.5 | 102.9 |
| | *Lactuca sativa* | 98.0 ± 2.0 | 111.6 ± 2.0 | 87.8 |
| 100 | *Brassica oleracea* | 97.0 ± 1.5 | 92.4 ± 1.5 | 104.9 |
| | *Lactuca sativa* | 87.8 ± 2.1 | 97.7 ± 1.7 | 89.8 |
| 200 | *Brassica oleracea* | 72.7 ± 3.6 | 62.1 ± 3.1 | 117.1 |
| | *Lactuca sativa* | 83.7 ± 1.5 | 88.4 ± 1.5 | 94.7 |

Experiments were processed in triplicate and the results are the mean ± standard deviation of three independent experiments

The *Paenibacillus* sp. D9 BioS solutions (in various concentrations: 0, 1, 10, 100, 200 mg/L) on *Artemia salina* were assessed and the outcomes displayed in Table 7.9. From this study, *Paenibacillus* sp. D9 BioS displayed no toxicity at the different concentrations to brine shrimp.

**Table 7.9** Mortality of *Artemia salina* in different concentrations of the *Paenibacillus* sp. D9 biosurfactant

| Biosurfactant concentration (mg/L) | The mortality rate of *Artemia salina* |
|---|---|
| 0 | 0 ± 0.0 |
| 1 | 0 ± 0.0 |
| 10 | 1 ± 1.0 |
| 100 | 3 ± 1.0 |
| 200 | 3 ± 0.2 |

Experiments were processed in triplicate and the results are the mean ± standard deviation of three independent experiments

As observed, low mortality was observed for concentration close to CMC (100 mg/L), and CMC (200 mg/L). In the short-term bioassay, there was no sign of lethality towards the *Artemia salina* larvae after 24 h. Similar to this research, da Rocha Junior et al. (2018) showed the non-toxic potential of BioS synthesized from *Candida tropicalis* at different concentrations of 0.25% and 0.5%, respectively. Lipoprotein BioS by *Streptomyces* sp. DPUA1566 did not exhibit any form of mortality at different concentrations of BioS utilized (Santos et al., 2018). In contrast, de França et al. (2015) discovered that the *Bacillus subitillis* exhibited BioS a low death rate (under 20%) when utilized at varying concentrations of 12.5, 25 and 50 mg/L, respectively. The non-toxic effect of *Paenibacillus* sp. D9 BioS proffer its usefulness in different applications relatable to soil and aquatic environments, as the biomolecule was confirmed to be ecological safe and environmentally friendly.

## 7.4 Conclusion

The investigation enhanced *Paenibacillus* sp. D9 BioS economics by the utilization of possible low-cost materials. These outcomes demonstrated the likelihood of waste frying oils (coconut and sunflower) to be utilized as an exceptionally viable substrate for the economic production of *Paenibacillus* sp. D9 BioS. The BioS was successful in dispersing engine oil, with further capability in removing different heavy metals from the environments including contaminated effluents, synthetic wastewater, contaminated sands, and food crops. In addition, *Paenibacillus* sp. D9 BioS can be successful as constituents or as a whole for commercial detergent formulation. The *Paenibacillus* sp. D9 BioS-containing detergent is equivalent to commercial products in removing extreme hydrophilic, and hydrophobic stains. This study confirmed the fundamental prospect of BioS synthesized by *Paenibacillus* sp. D9 in environmental, and biotechnological applications.

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## Conflict of Interest

The authors wish to declare no conflict of interest

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# Chapter 8

## Heterologous expression of Sfp-type phosphopantetheinyl transferase is indispensable in the biosynthesis of lipopeptide biosurfactant.

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ORIGINAL PAPER


# Heterologous Expression of *Sfp*-Type Phosphopantetheinyl Transferase is Indispensable in the Biosynthesis of Lipopeptide Biosurfactant

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**Abstract**
Phosphopantetheinyl transferases are of tremendous enthusiasm inferable from their fundamental parts in activating polyketide, fatty acid, and non-ribosomal peptide synthetase enzymes and additionally an increasing number of biotechnological applications. The present study reports the identification of *sfp* gene from the *Paenibacillus* sp. D9, which encompasses 693 bp encoding a 230-amino acid protein with a molecular weight of 25.3 kDa. The amino acid sequence *Paenibacillus* sp. D9 Sfp revealed more than 90% sequence identity to other Sfp proteins from other *Paenibacillus*. The *sfp* gene was cloned and recovered efficiently using affinity chromatography with maximal specific phosphopantetheinyl transferase activity at an optimal pH of 8.0 and temperature of 30 °C. The enzyme also exhibited stability under a wide-ranging pH and temperature. The presence of $Zn^{2+}$, $Cu^{2+}$, and $Fe^{2+}$ ions improved the enzymatic activity, while other metals such as $Ni^{2+}$, $Co^{2+}$, and $Mg^{2+}$ had inhibitory effects. The introduction of EDTA also displayed no inhibition. Kinetic parameters were obtained having values of 4.52 mg/mL, 35.33 U/mg, 3.64 $s^{-1}$, and 0.104 $mM^{-1}$ $s^{-1}$ for $K_m$, $V_{max}$, $k_{cat}$, and $k_{cat}/K_m$, respectively. The biosurfactant synthesized by the recombinant BioSp was found to be surface active, reducing the surface tension to 33.7 mN/m on the glucose substrate after 5 days of incubation at 37 °C. The recombinant *Escherichia coli* strain also exhibited an improvement in biosurfactant yield (1.11 g/L) when contrasted with 0.52 g/L from *Paenibacillus* sp. D9. High esterase activity of 2.55 IU/mL using *p*-nitrophenyl acetate was observed on the recombinant strain, as the protein connected with the release of the biosurfactant was observed to be an esterase. The characteristics of improved biosurfactant and esterase synthesis by hyper-producing recombinant strain possess numerous values from biotechnology standpoint.

**Keywords** Biosurfactant · Cloned recombinant · Esterase · *Paenibacillus* sp. D9 · Phosphopantetheinyl transferase

## Introduction

There is expanding enthusiasm for discovering new proteins and biosurfactant (BioS) hyper-producing strains for their utilization in modern bioprocess [1, 2]. Because of this intensifying mindfulness on the need to secure the biological community and humankind, there has been an extended enthusiasm for surfactants of microbial origin with high surface properties as conceivable options in contrast to chemically synthesized ones [3]. Additionally, ongoing advances in sustainability science and biotechnology research have empowered the natural synthesis of surfactants. The biological synthesis has high green potentials and utilizes negligible steps and renewable resources [4]. Due to non-toxic effect,

**Heterologous expression of Sfp-type phosphopantetheinyl transferase is indispensable in the biosynthesis of lipopeptide biosurfactant.**

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## Abstract

Phosphopantetheinyl transferases are of importance due to their essential role in activating polyketide, fatty acid, and non-ribosomal peptide synthetase enzymes and additionally an increasing number of biotechnological applications. The present study reports the identification of the *sfp* gene from the *Paenibacillus* sp. D9, which encompasses 693 bp encoding a 230-amino acid protein with a molecular weight of 25.3 kDa. The amino acid sequence *Paenibacillus* sp. D9 Sfp revealed more than 90% sequence identity to other Sfp proteins from other *Paenibacillus*. The *sfp* gene was cloned and Sfp was recovered efficiently using affinity chromatography with maximal specific phosphopantetheinyl transferase activity at an optimal pH of 8.0 and temperature of 30℃. The enzyme also exhibited stability under a wide-range of pH and temperature. The presence of $Zn^{2+}$, $Cu^{2+}$, and $Fe^{2+}$ ions improved the enzymatic activity, while other metals such as $Ni^{2+}$, $Co^{2+}$ and $Mg^{2+}$ had inhibitory effects. The introduction of EDTA also displayed no inhibition. Kinetic parameters were obtained having values of 4.52 mg/mL, 35.33 U/mg, 3.64 $s^{-1}$, and 0.104 $mM^{-1}$ $s^{-1}$ for $K_m$, $V_{max,}$ $k_{cat}$, and $k_{cat}$/Km, respectively. The biosurfactant synthesized by the recombinant BioSp was found to be surface-active, reducing the surface tension to 35.7 mN/m on the glucose substrate after 5 days of incubation at 37℃. The recombinant *E. coli* strain also exhibited an improvement in biosurfactant yield (1.11 g/L) when contrasted with 0.52 g/L from *Paenibacillus* sp. D9. High esterase activity of 2.55 IU/ml using p-nitrophenyl acetate was observed for the recombinant strain, as the protein connected with the release of the biosurfactant was observed to be an esterase. The characteristics of improved biosurfactant and esterase synthesis by hyper-producing recombinant strain possess numerous values from biotechnology standpoint.

## Keywords

Biosurfactant; Cloned recombinant; Esterase; *Paenibacillus* sp. D9; Phosphopantetheinyl transferase

## 8.1 Introduction

There is increasing enthusiasm for discovering new proteins and biosurfactant (BioS) hyper-producing strains for their utilization in modern bioprocess (Sekhon et al., 2011). Because of the mindfulness on the need to secure the biological community, an extended need for surfactants of microbial origin with high surface properties as alternative option to synthetically synthesized ones (Gautam et al., 2014). Due to their non-toxic effect, different biotechnological industries have made BioS producing bacteria important target. BioS might be synthesized from sustainable and less expensive raw materials and are progressively ecologically-friendly (Domingos et al., 2015). BioSs, specifically the 'Lipopeptides' are biomolecules comprising of lipids connected to a peptide, that are small chains of amino acid monomers joined by peptide (amide) bonds. BioSs advantages over synthetic surfactants includes high surface activity, non or low toxicity, biodegradability, and environmental compatibility (Qiu et al., 2014). Although researchers are enthused about replacing synthetic surfactants with BioSs, the genuine problem still lies in the cost of production. Recombinant hyper-producing strains are made with the use of recent genomic sequencing data. This coupled with the utilization of inexpensive carbon substrates enables increasing yields and reducing production costs (Sekhon et al., 2012). According to transparent market research, the global BioS market is presently at US$ 40 million revenues in 2019 and is expected to increase in the next decade (Makkar et al., 2011). In any case, the large-scale industrial utilization of BioS still seems, by all accounts, to be constrained because of high costs of production (Banat et al., 2010; Makkar et al., 2011). To decrease the cost, it is critical to create hyper-producing or mutant strains with improved production yields or with a capacity to specifically deliver specific, successful congeners of BioSs which are a combination of closely related products (Bachmann et al., 2014). A decrease in BioS cost making them monetarily appealing will depend on the improvement of cost-effective procedures including the utilization of inexpensive materials, recovery and higher product yields through genetically engineered microorganism (Jimoh and Lin 2019a). Recombinant, non-pathogenic, and hyper-producing strains are being made with the utilization of most recent genome sequencing advances. The BioS present surface properties of huge responsiveness for biotechnological sectors and are utilized as, emulsifiers, anti-tumoral, antifungals, antibacterial, and antivirals agent (Gelis-Jeanvoine et al., 2016). Lipopeptide biosurfactants have expanding therapeutic, scientific, and biotechnological applications amongst the major classes of BioSs (Anburajan et al., 2016; Jimoh and Lin, 2019a). The BioS biosynthesis genes of genus *Bacillus* have been practically,

functionally, and structurally characterized (Anburajan et al., 2016). However, limited report exists for the genus *Paenibacillus,* hence the need for this research.

*Paenibacillus* sp. D9 is a hydrocarbon-degrading and BioS-producing bacterium that can produce biomolecule that are stable in an extensive array of acidic and alkaline conditions. It is well characterized physiologically, biochemically, but not genetically. *Paenibacillus* sp. D9 was revealed to synthesize biomolecules that comprise another class of *Paenibacilli* lipopeptide (Jimoh and Lin, 2019b). Reports on lipopeptide BioS synthesis by production fermentation focuses generally on bioprocess improvement, for instance, improvement of medium design and culture conditions. Notwithstanding, it ought to be noticed that studies on the microbial synthesis of lipopeptide BioS is still constrained by the low product yields. The general enhancement is yet insufficient to legitimize a business proposition of this kind of BioS. Thus, further improvement of BioS yield through recombinant genetic engineering is essential. Moreover, it is conceivable to build up overproducing hyper-producing, mutant, and recombinant strains for acquiring the greatest profitability.

Phosphopantetheinyl transferases are enzymes essential for the synthesis of numerous compounds including unsaturated fatty acids, polyketide, and non-ribosomal peptide metabolites (Bunet et al., 2014). In this way, *sfp* gene (phosphopantetheinyl transferase) has all the earmarks of being basic and associated with biosynthesis of lipopeptide BioS synthesized by *Paenibacillus* sp. D9. The molecular characterization of the *sfp* gene from *Paenibacillus* sp. D9 will give more understanding into the structural definition associated with BioS biosynthesis. In this scenery, research was undertaken on the characterization, improved synthesis, functional prediction and investigation of phosphopantetheinyl transferase gene from BioS producing P*aenibacillus* sp. D9.

## 8.2 Materials and methods

### *8.2.1 Bacterial strain, growth conditions, plasmids, and DNA*

The plasmid, pET47b was purchased from Sigma Aldrich, USA. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as cloning and expression hosts, respectively. For DNA isolation, a *Paenibacillus* sp. D9 colony was inoculated into 5 mL LB broth and afterward incubated at 30℃, 150 rpm overnight. *Paenibacillus* sp. D9 Genomic DNA was purified as per the manufacturer's instruction using Gene JET genomic DNA purification

kit (Thermo Scientific). The concentration and purity of the DNA was determined using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) at $OD_{260}/OD_{280}$ ratio.

### *8.2.2 Polymerase chain reaction amplification*

The *sfp* gene primers were designed utilizing Snap Gene software according to the total genomic sequences of *Paenibacillus* sp. D9 (GenBank Accession number JZEJ00000000). The PCR mixture consisted of 2 µL of the DNA, 0.5 µM each of the forward and reverse primer, 0.2 U/µL iProof DNA polymerase, 1X of 5X iProof HF buffer, and 2.4 µL of nuclease-free water. The *sfp* gene was amplified utilizing the following primers **5'-*GGA* TCC** AAT GGT GGA GAT TTA CGC GGT CGA GAT CCC TTC CGG-3' (forward primer with an endonuclease site BamHI) and **5'-*CTC* GAG** CCG TCC CAG CCG CTC CTC CAG C-3'(reverse primer with an endonuclease site XhoI) by PCR under the following conditions: an initial denaturation at 98°C for 30 s, 30 cycles (98°C for 10 s, 61°C for 30 s, 72°C for 30 s) and finally, 72°C for 5 min. PCR reaction was done with reference to iProof high fidelity DNA polymerase kit (Bio Rad) using T100™ Thermal Cycler (BIO-RAD). The expected amplified product (693 bp) were separated on 1.2% (w/v) agarose dissolved in 1 × Tris-acetate-EDTA buffer (20 mM acetic acid, 40 mM Tris, and 1 mM EDTA, pH 8.0) stained with 0.5 µg/mL ethidium bromide through electrophoresis. The gel was visualized using a UV transilluminator (SYNGENE BioSys).

### *8.2.3 Cloning, and transformation of the biosurfactant gene*

The PCR amplicon of the *sfp* was extracted under UV light and purified utilizing GeneJet Gel Extraction Kit as indicated by the manufacturer's instruction (Thermo Fisher Scientific). The PCR product was subsequently eluted using 50 µL sterile $dH_2O$. Both PCR amplicon (insert) and pET47b plasmid (GE Health care, USA) were restricted with FastDigest endonucleases, 1 µL of 10 U BamHI and XhoI each (Thermo Fisher Scientific) for directional cloning. The pET47b vector and double digested PCR product were both purified as per manufacturer's instruction utilizing a DNA Clean & Concentrator™ kit (Zymo Research). A ratio of 2:5 of the digested the vector and plasmid were added to reaction mixtures. A rapid DNA Ligation kit (Thermo Fisher Scientific) was used to perform ligation reactions as per standard laboratory procedures and conditions. A successfully cloned plasmid (designated as pET47b-*sfp*) containing the 693 *sfp* gene fragment was obtained. *E. coli* DH5α (Novagen, USA) was used for the mini-preparation of the recombinant strain while *E. coli* BL21 (DE3) was utilized for *sfp* gene expression. The heat shock procedure was used to transform the pET47b-*sfp*

plasmid into *E. coli* BL21 (DE3) pLysS expression system (Sambrook and Russell, 2001). The positive clones containing 50 mg/mL were confirmed by restriction digestion using XhoI and BamHI.

### *8.2.4 Expression and purification of recombinant Sfp protein*

The positive clones were grown in LB broth and analyzed for the expression studies. In brief, 1 mL of an overnight culture containing pET47-*sfp* plasmid was transferred into 100 mL in medium containing kanamycin (50 mg/ml) until the $OD_{600}$ reached 0.6─0.7. This was followed by the introduction of isopropyl-β-D-thiogalactoside (IPTG) at 1 mM final concentration with continuous incubation at 37°C for 4 h. There was another setup for negative control using the pET47 vector following the same protocol. The cells were then harvested by centrifugation at 8000 × *g* at 4°C. A 50 mM Tris buffer, pH 8.0 containing 10 U (1 μg/ml) DNase I, 0.1 M $MgCl_2$, and 1 mM PMSF was used to re-suspend the cell pellet. This was followed by sonication at 50 kHz utilizing an Omni International Sonic Ruptor 400 Ultrasonic homogenizer. The thermal impact was limited by placing the sample on ice. The homogenized sample was exposed to 30s on/30 s off pulses for 6 min at amplitude of 50%. The centrifugation of the lysate was achieved at 10 000 × *g* for 20 min and 30 μL of the supernatant was evaluated for the expression of soluble proteins by SDS-PAGE. The recombinant Sfp protein was purified using a 5.0 mL His Pur Cobalt column (affinity chromatography), (Thermo Scientific). The affinity column was thoroughly washed with de-ionized water (20 mL), followed by column equilibration with 50 mM sodium phosphate buffer (5 × column volumes), pH 7.5 (Buffer A) containing 5 mM imidazole. The column was loaded with recombinant enzyme extract (25 mL), and subsequently washed with Buffer A containing 20 mM imidazole (5-bed volumes). The recombinant Sfp was eluted with Buffer A with 150 mM imidazole at a flow rate of 0.4 mL/min. The eluted active fractions were pooled and dialyzed overnight against Buffer A. The total protein concentration was determined utilizing BCA™ Protein Assay Kit (Thermo Scientific). Amicon Ultra Centrifugal Filter Devices (Millipore) were used to concentrate purified proteins and further characterized as described below.

### *8.2.5 Enzyme assays and characterization*

#### 8.2.5.1 *In vitro* phosphopantetheinyl transferase activity

*In vitro*, phosphopantetheinylation experiments were performed to investigate the capacity of Sfp protein in the crude and purified product using coenzyme A (CoA) kit (Sigma-Aldrich, USA). The reactions contained

20 µM Sfp protein, 10 µL of the coenzyme A substrate mix, 2 µL of conversion enzyme mix CoA (or malonyl-/methylmalonyl-CoA), 2 µL of acyl coenzyme A and coenzyme assay buffer (pH 7.5) in a total reaction volume of 60 µL. The CoA concentration proportional to the phosphopantetheinyl transferase present was determined by enzymatic assay, which resulted in a colorimetric (570 nm) product. The enzyme unit was calculated based on the spectrophotometric based assay results using control as set by the manual as standard. Under standard assay conditions, one unit of phosphopantetheinyl transferase was defined as the amount of enzyme essential to release 1 µmol of acyl coenzyme A $min^{-1}$

#### 8.2.5.2 Esterase activity of purified Sfp phosphopantetheinyl transferase

The esterase activity of the purified enzyme (0.16 mg/mL) was determined using 75 mM phosphate buffer containing 10 mM $MgSO_4$ (pH 7.0) and 100 mM 4-nitrophenyl (pNP) acetate as substrate (Politino et al., 1997). The activity was examined by measuring the increase in optical density at 405 nm after incubation at 37°C for 30 min. One esterase unit was defined as the quantity of enzyme required to release 1 µmol of *p*-nitrophenol per minute with the specific esterase activity expressed as µmol/mg protein/min. The enzyme unit was calculated based on the spectrophotometric based assay using the formula

$A = \varepsilon l c$

Where A = absorbance ($M^{-1}$ $cm^{-1}$), l = cell path length (1 cm), c = absorbance concentration (M) and ε = the molar extinction coefficient.

#### 8.2.5.3 Effect of pH and temperature on the activity of crude and purified Sfp phosphopantetheinyl transferase

To estimate optimal temperature and pH, the relative activity was examined at varied pH and temperature. The optimum pH for phosphopantetheinyl transferase in recombinant *E. coli* BL21 (DE3) and purified Sfp were observed by incubating the enzymes at different pH under the standard assay conditions at 37℃, for 30 minutes. The effect of pH was determined at pH ranging from 4.0 to 10.0, utilizing sodium acetate buffer (pH 4.0-6.0), and coenzyme assay buffer (pH 7.0), and 50 mM Tris-HCl buffer (pH 8.0-10.0). All subsequent enzyme assays were performed at optimum pH. The optimum temperature of the phosphopantetheinyl transferase in recombinant *E. coli* BL21 (DE3) and purified Sfp enzyme were studied at temperatures ranging 25, 30, 37, 42℃ at the optimal pH under the standard assay conditions.

#### 8.2.5.4 Stability of purified Sfp phosphopantetheinyl transferase

The enzyme thermostability was observed by incubating the purified enzyme samples for different incubation temperatures 35, 45, 55, and 65℃ for 30 min before the assay in the absence of substrate. The samples were placed on ice immediately by which the remaining activities of phosphopantetheinyl transferase were measured under standard condition. The enzyme pH stability was determined by maintaining the pure preparation of the purified enzyme samples for 30 min at pH 5-9 in the absence of substrate. Subsequently, the activity of phosphopantetheinyl transferase was determined under standard assay condition. The enzymatic activity without pre-incubation was denoted as 100%.

#### 8.2.5.5 The effects of heavy metals and EDTA on crude and purified Sfp phosphopantetheinyl transferase

The effect of various heavy metal, i.e. $Mg^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Zn^{2+}$, and $Cu^{2+}$ or EDTA (Sigma Aldrich, USA) at 1 mM final concentration was made for corresponding samples on *Paenibacillus* sp. D9 Sfp phosphopantetheinyl transferase. The activity under standard conditions without heavy metal as 100% was compared to the relative activity of Sfp phosphopantetheinyl transferase. All experiments were analysed in three independent biological replicates. The assays were carried out at the indicated concentration at 30ºC for 30 min

#### 8.2.5.6 Determination of kinetic parameters of purified Sfp phosphopantetheinyl transferase

The kinetic studies of *Paenibacillus* sp. D9 Sfp phosphopantetheinyl transferase were assayed in 50 mM Tris-HCl buffer (pH 8.0) at 30ºC. Assays were performed with the increasing substrate concentrations (0-1000 µM) utilized at optimal conditions. The values of maximum reaction velocity ($V_{max}$), Michaelis constant ($K_m$) of the purified Sfp phosphopantetheinyl transferase were perquisite to calculate the turnover number ($k_{cat}$), and catalytic efficiency ($k_{cat}/K_m$) utilizing the Lineweaver and Burk plot. The Lineweaver-Burk plot was defined as reciprocal reaction velocities versus reciprocal substrate concentrations. To limit substrate utilization to below 5%, the reaction time was restricted to 15 min.

### *8.2.6 Molecular characterization and in silico sequence analysis*

The deduced Sfp amino acid sequences were utilized as inquiries in the BLASTP calculation with parameters set as defaults to scan for related proteins accessible from the NCBI pursued by manual changes. A

phylogenetic tree was additionally developed dependent on the amino acid sequences of the *sfp* gene utilizing Clustal X and Mega X software. The phylogenetic tree was developed utilizing the Neighbor-joining technique and bootstrapped 1000 times. Multiple sequence alignment of the sequences that indicated high comparability was aligned utilizing the CLUSTAL-X program. The output alignments were imported into the GeneDoc program (http://www.psc.edu/biomed/genedoc/). The theoretical pI value was predicted using the ProtParam tool (https://web.expasy.org/cgi-bin/protparam/protparam).

### *8.2.7 Sfp expression of the recombinant E. coli strain on different substrates*

For determining the production of BioS, *Paenibacillus* sp. D9 and positive recombinant transformant, viz. *BioSp* were grown on a Bushnell Haas (BH) basal medium containing 2.0% (v/v) of each substrate, viz., waste canola oil, sunflower oil, diesel fuel, *n*-hexadecane, glucose, and glycerol at 37°C, respectively. The BH medium without the addition of carbon source was used as control. The medium utilized for culturing the positive transformant was supplemented with kanamycin. Subsequently growth, BioS activity, and esterase activity were assessed after 5 days of incubation. The increase or decrease in growth was determined by measuring the optical density at 600 nm using UV spectrophotometer (Shimadzu Cooperation, Japan). The production medium was allowed for clear separation of hydrophobic layer containing the substrates and hydrophilic layer containing the bacterial cells. The spectrophotometer was blanked with the medium containing the different substrates during the measurement of $OD_{600}$ value.

The BioS activity and esterase activity were determined using the cell-free supernatant achieved by centrifugation (13,500 × *g*) of the culture fermentation broth.

#### 8.2.7.1 Esterase activity of the recombinant strain

Esterase activity of the recombinant strain was carried out using the cell-free supernatant, 75 mM phosphate buffer containing 10 mM $MgSO_4$ (pH 7.0), and 100 mM 4-nitrophenyl (pNP) acetate as substrate (Politino et al., 1997). The activity was examined by measuring the increase in optical density at 405 nm after incubation at 37°C for 30 min. One esterase unit was defined as the quantity of enzyme required to release 1 µmol of *p*-nitrophenol per minute with the specific esterase activity expressed as µmol/mg protein/min. The enzyme unit was calculated based on the spectrophotometric based assay using the formula

$A = \varepsilon l c$

Where A = absorbance ($M^{-1}$ $cm^{-1}$), b = cell path length (1 cm), c = absorbance concentration (M) and ε = the molar extinction coefficient.

#### 8.2.7.2 Emulsifying activity of the isolated biosurfactant

The cell-free supernatant after IPTG induction was introduced into 10 mL glass tubes containing TM buffer (20 mM Tris-HCl buffer, pH 7.0; 10 mM $MgSO_4$) and 0.04 mL of a 1:1 (vol/vol) of *n*-hexadecane and 2-methylnaphthalene to a final volume of 1.5 mL (Colla et al., 2010; Toren et al., 2001). The tubes were vortexed and left at room temperature for 60 min. One unit of emulsifying activity is defined as the amount of BioS that yielded an optical density $A_{600}$ of 0.1 using a Shimadzu spectrophotometer.

$EA_{o/w}$ = (Absorbance $_{sample}$ – Absorbance $_{blank}$) / 0.1

$EA_{o/w}$ = Emulsifying activity of oil in water

#### 8.2.7.3 Surface tension

A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) with 1.9 cm De Noüy platinum ring at room temperature was used to measure ST. This was carried out using 40 mL cell-free supernatant achieved by centrifuging culture broth at 13,500 × *g* for 20 min (Gudiña et al., 2012). The experiments were produced in average of three independent biological replicates. For proper calibration and to avoid error in instrumentation, ST of distilled water were initially measured. The ST of BH medium supplemented with different substrates were determined as controls (Zhang et al., 2016).

### *8.2.8 Recovery and purification of the recombinant biosurfactant*

The culture was harvested by centrifuging at 13,500 × *g* for 20 min at 4℃ when the BioS activity was observed to be greatest in the supernatant after five days. Acid precipitation method was utilized to partially purify and extract the BioS (Al-Wahaibi et al., 2014). The pH was adjusted to 2.0 using 6 M HCl to precipitate BioS. The solution was kept overnight at 4℃ and the precipitated BioS was collected by centrifuging the solution at 13,500 × *g* for 20 min at 4℃. The collected BioS pellet was dissolved in distilled water and pH was adjusted to 7.0, followed by freeze-drying (VirTis BenchTop Pro, USA). The freeze-dried sample was then evaluated for BioS activity at 550 nm. For evaluating maximum BioS production, purified (lyophilized) powder was evaluated and weighed as g/L. In this case, the isolated BioS was subsequently confirmed for surface properties before its further usage.

### *8.2.9 Statistical analysis*

All the experimental data analyzed were expressed in terms of arithmetic averages obtained from at least three independent biological replicates, with standard deviation **(±)**. The student t-test was ascribed to a 95% confidence level, analyzed using GraphPad Prism 6.

## 8.3 Results

### *8.3.1 Cloning and expression of sfp gene of Paenibacillus* sp. D9

The *Paenibacillus* sp. D9 DNA and pET47b plasmid were isolated and purified (Figure 8.1a). The purity and high concentration of both the DNA and pET47b plasmid were observed as shown (Figure 8.1a). From this result, the PCR amplicon was confirmed as *sfp* gene (693 bp) (Figure 8.1b). The insertion of *sfp* gene into pET47b (Figure 8.1c) was confirmed by restriction digestion using XhoI and BamHI. (Figure 8.1d). The amplified *sfp* gene was inserted into the designation vector pET 47b-*sfp*, further expressed in *E. coli DH5α*, and subsequently *E. coli* BL21 (DE3) pLysS.

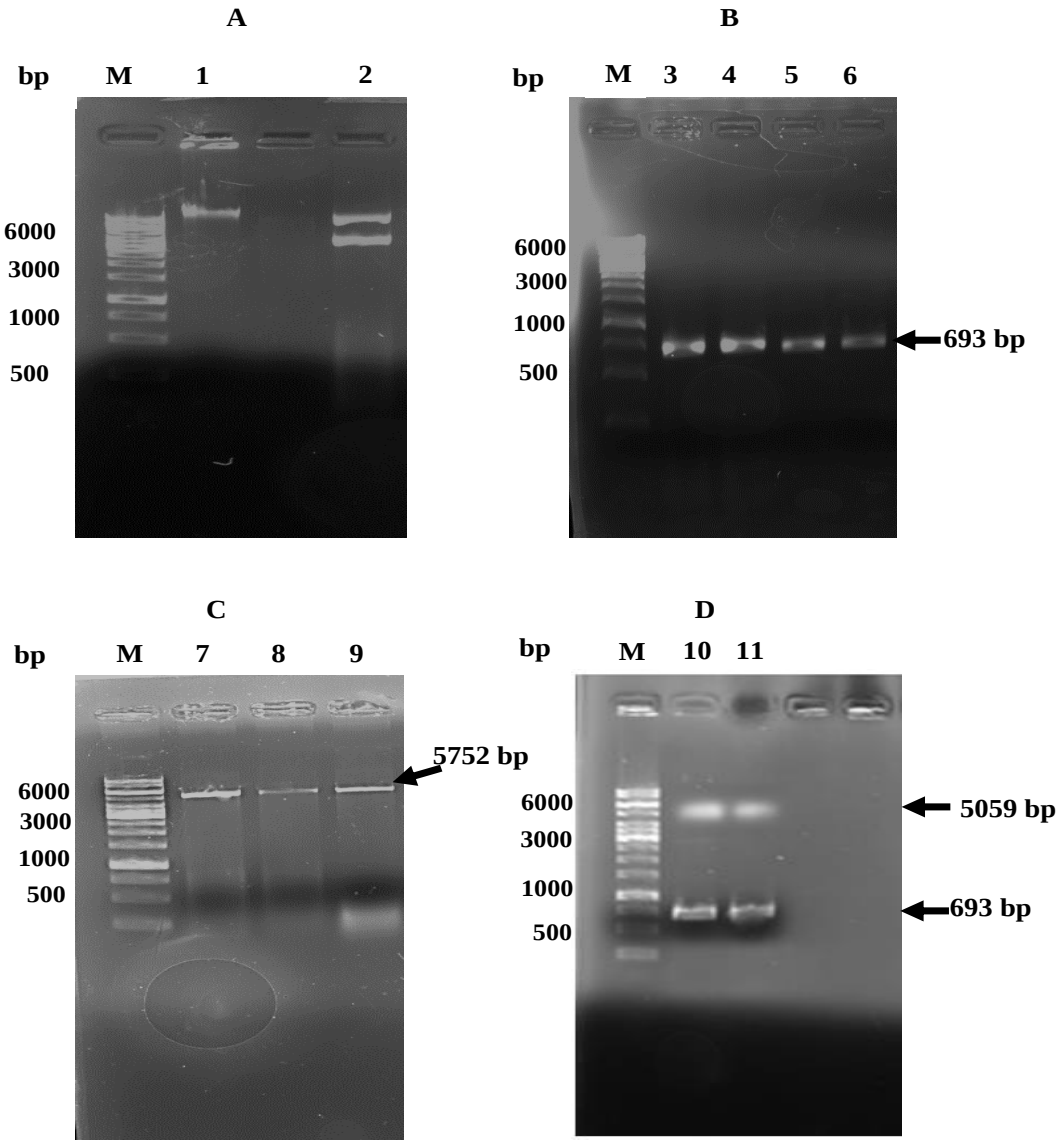



**Figure 8.1** Cloning of *sfp* gene to pET 47b (a) purified *Paenibacillus* sp. D9 DNA, and pET 47b plasmid (b) PCR amplification of *sfp* gene (c) pET47b-*sfp* with insert (d) Presence of *sfp* gene confirmation by restriction digestion using XhoI and BamHI. M: Molecular markers; 1: Purified *Paenibacillus* sp. D9 DNA; 2: pET 47b plasmid; 3-6: *sfp* PCR products; 7-9: pET47b-*sfp* with insert; 10-11: restriction digest of pet 47b and *sfp* gene insert

The protein expression was investigated by separation of protein samples on 10% SDS-PAGE (Figure 8.2). In contrast to the sample before induction, the recombinant Sfp protein was highly expressed in IPTG-induced *E.*

*coli* BL21 (DE3) (Figure 8.2, Lane 2). The predicted weight of the Sfp protein was 25.3 kDa, with the 6x His-tag adding (~2.5 kDa) for a total of ~27.8 kDa (Figure 8.2).

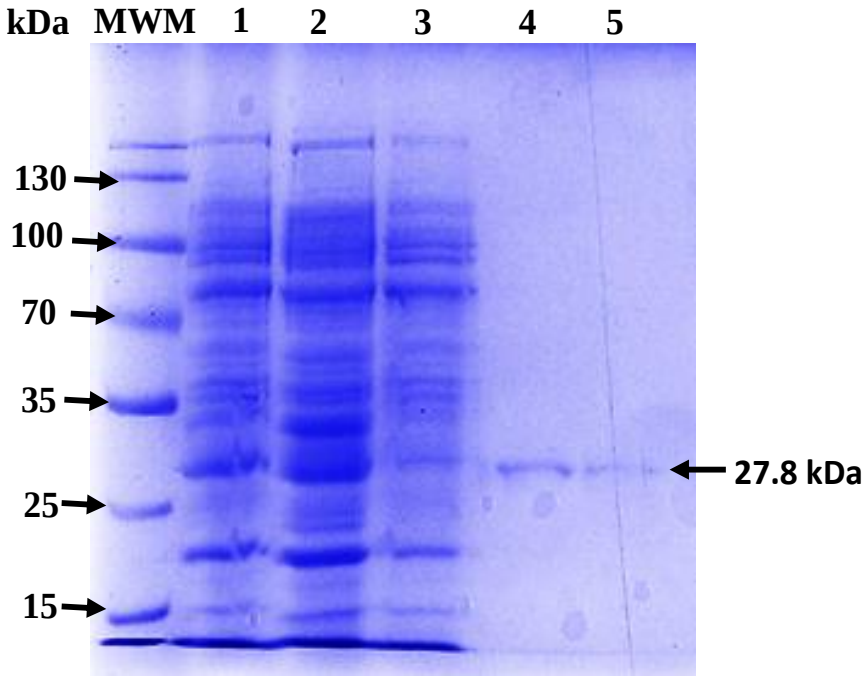



**Figure 8.2** Expression of pET47b-*sfp* recombinant grown at 37°C after 4h IPTG induction and soluble proteins were isolated from cell-free supernatant. MWM: Molecular weight marker (Thermo Fischer Scientific); 1: protein extracts before IPTG induction, control; 2: protein extracts after IPTG induction; 3: Unbound protein; 4-5: Purified Sfp protein.

### *8.3.2 Purification and characterization of the Sfp protein*

To describe the compound biochemically, recombinant Sfp protein was over-expressed and purified from the *E. coli* strain (DE3) pLysS for utilization in *in vitro* assays. Cloned *Paenibacillus* sp. D9 Sfp protein of molecular weight 27.8 kDa was purified to homogeneity using a Cobalt Affinity Chromatography (Figure 8.2, Lanes 4 and 5). Upon incubation of the purified Sfp protein in the presence of coenzyme A substrate, and conversion enzyme mix CoA (or malonyl-/methylmalonyl-CoA), there was increase in the CoA concentrations responsible for the transfer of acyl group (Figure S1, Table S1). As such, purified sfp protein is competent for carrying 4-phosphopantetheinylation of acyl-CoAs.

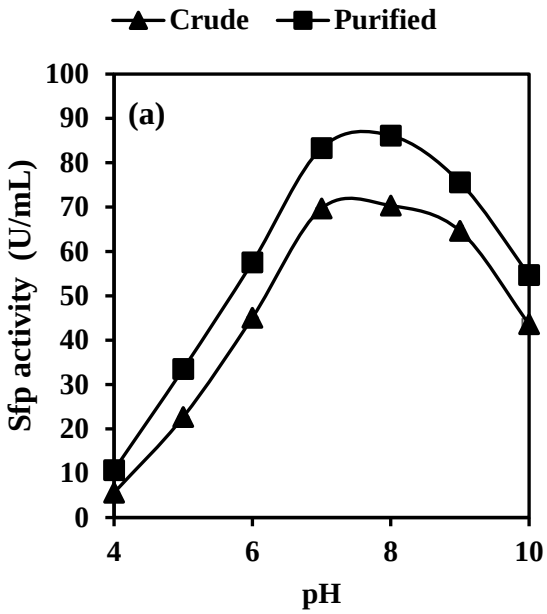
**Table 8.1** Summary of purification of recombinant Sfp protein from *E. coli* transformant

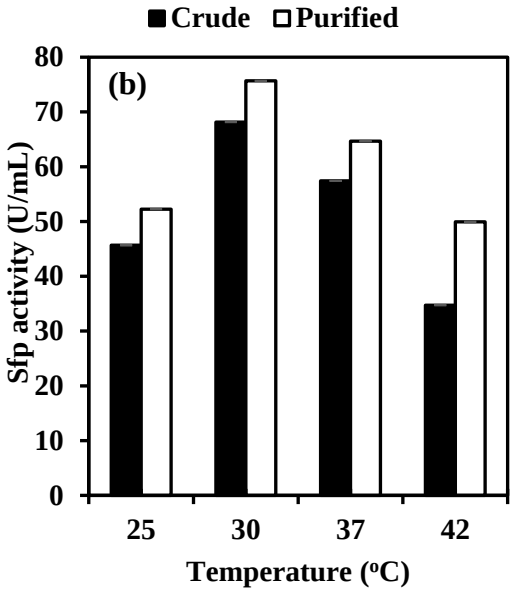
| Purification steps | Fraction volume (ml) | Protein Conc. (mg/mL) | Total protein (mg) | Enzyme activity (U/ml) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|---|---|---|
| Cloned extract | 25 | 0.38 | 9.50 | 10.17 | 254.25 | 26.76 | 100.0 | 1.00 |
| Dialysis | 25 | 0.30 | 7.50 | 10.49 | 262.25 | 34.97 | 103.1 | 1.31 |
| Affinity Chromatography | 2 | 0.16 | 0.32 | 13.94 | 27.88 | 87.13 | 10.6 | 3.26 |

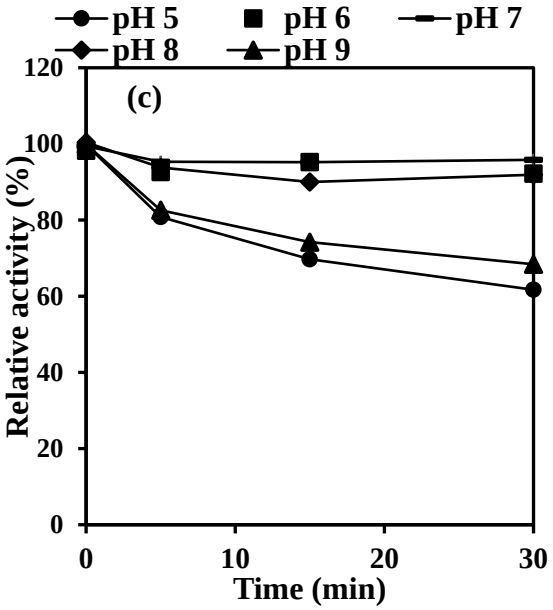
Table 8.1 outlines the enzyme activity and protein concentration of the samples for every purification step. Prior to column chromatography, the specific activity of the crude extract was increased by dialysis with 103% recovery. Subsequently, affinity chromatography was used to purify the Sfp protein to homogeneity with a specific activity of 87.14 U/mg and a 3.26-fold increase in purity.

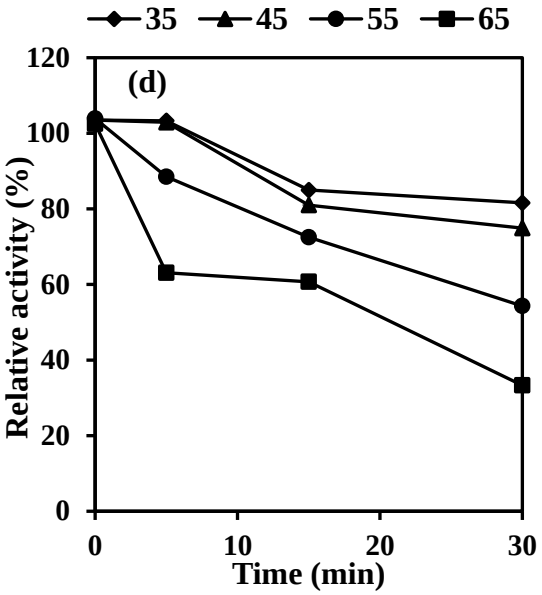
The effect of pH on recombinant *Paenibacillus* sp. D9 Sfp activity was conducted using a selected range of pH, i.e., 4-10. As represented in Figure 8.3a, the crude phosphopantetheinyl transferase showed optimal activity at pH 8 while the purified enzyme possessed a similar pattern with an optimal activity at pH 8. The temperature effects on phosphopantetheinyl transferase were also determined using diverse temperature conditions 25℃, 30℃, 37℃ and 42℃ (Figure 8.3b). The optimal enzyme activity for both the crude and purified phosphopantetheinyl transferase was attained at 30℃. As such, maximum activity of phosphopantetheinyl transferase was attained in the temperature range of 25–42℃.

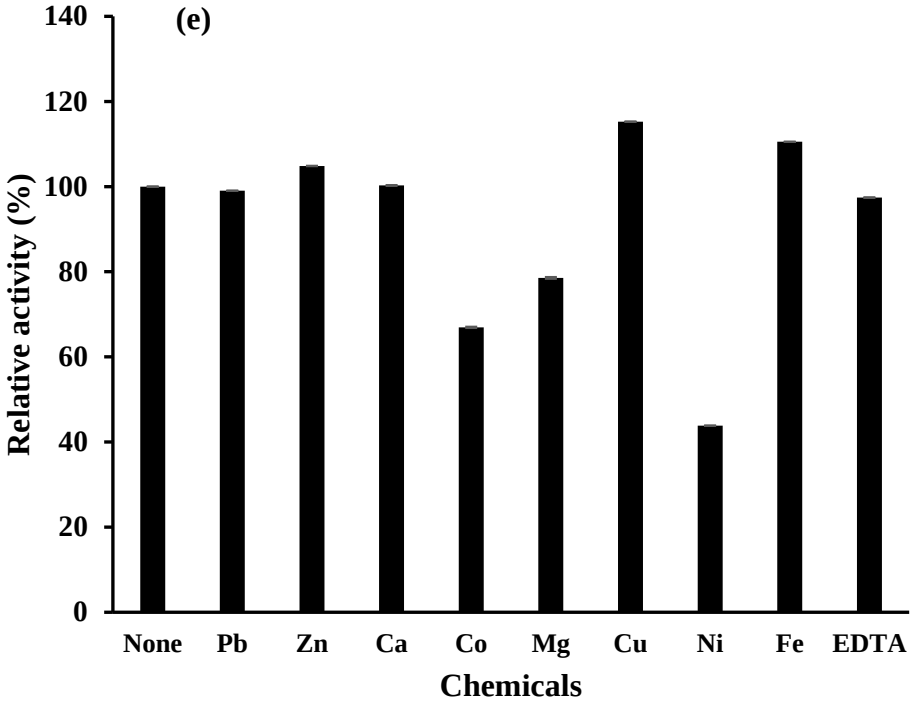
From the pH stability studies, the purified *Paenibacillus* sp. D9 phosphopantetheinyl transferase was stable at pH 6, 7 and 8, retaining at least 90% of its relative activity after 30 min (Figure 8.3c). Furthermore, the purified Sfp was active optimally at 30ºC (at pH 8), and furthermore, in the temperature range 35–55ºC, over 80% of the maximum activity was retained (Figure 8.3d). Subsequently, the enzyme had retained more than 70% of the maximum activity after incubation at 55ºC for 30 min (Figure 8.3d). The enzyme additionally had 60% activity when incubated at 65ºC for 15 min, however expanding the treatment time to 30 min made the enzyme lose practically all activity.


Crude
Purified
(a)
Sfp activity (U/mL)
pH


Crude
Purified
(b)
Sfp activity (U/mL)
Temperature (°C)


pH 5
pH 6
pH 7
pH 8
pH 9
(c)
Relative activity (%)
Time (min)


35
45
55
65
(d)
Relative activity (%)
Time (min)


(e)
140
120
100
80
60
40
20
0
Relative activity (%)
None
Pb
Zn
Ca
Co
Mg
Cu
Ni
Fe
EDTA
Chemicals

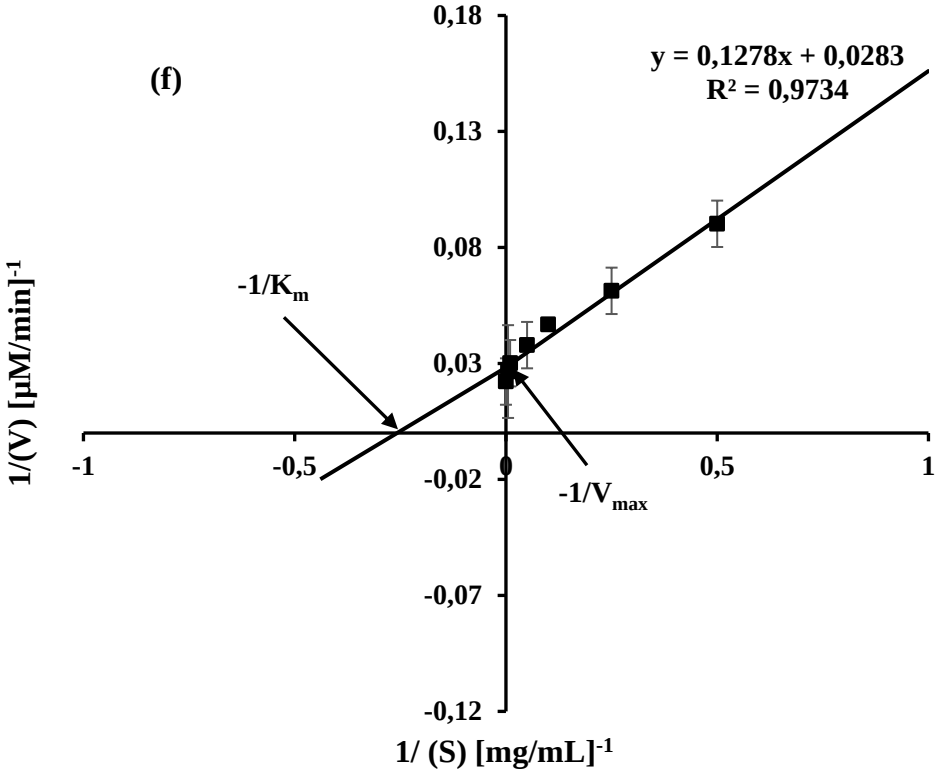

(f)
y = 0,1278x + 0,0283
R² = 0,9734
0,18
0,13
0,08
0,03
-0,02
-0,07
-0,12
-1
-0,5
0
0,5
1
-1/Km
-1/Vmax
1/(V) [µM/min]-1
1/ (S) [mg/mL]-1

**Figure 8.3** Characterization of phosphopantetheinyl transferase (a) effect of different pH; (b) effect of Temperature on cloned crude and purified phosphopantetheinyl transferase from *Paenibacillus* sp. D9 (c) pH stability of phosphopantetheinyl transferase. The enzyme was maintained at pH 5, 6, 7, 8, or 9 for 5, 15, 30 min before the assay (d) Thermostability of phosphopantetheinyl transferase. The enzyme was incubated at 35, 45, 55 or 65$^{o}$C for 5, 15, 30 min prior to the assay (e) Effect of metal chelator and metal ions on Sfp activity. Subsequently, the effect of EDTA and metal ions on Sfp phosphopantetheinyl transferase was resolved at a final concentration of 1 mM. The activity of the enzyme without the introduction of the chemicals was viewed as 100%, while the relative activity of the rest of the estimations was determined (f) The kinetic properties exhibited by purified Sfp phosphopantetheinyl transferase based on the Lineweaver-Burk plot. Values are mean of three independent replicates.

The heavy metals effect was likewise considered in the investigation and fused in the standard assay condition whereby the untreated sample was taken as a control (100%). Figure 8.3e outlines the impact of some metal ions on the activity of the purified *Paenibacillus* sp. D9 Sfp phosphopantetheinyl transferase. From Figure 8.3e, it may be observed that 1mM $Zn^{2+}$, $Cu^{2+}$, and $Fe^{2+}$ increased Sfp phosphopantetheinyl transferase in the enzymatic assay reactions. Also, under the same conditions, $Ni^{2+}$, $Co^{2+}$ and $Mg^{2+}$ inhibited the activity of phosphopantetheinyl transferase, while $Ca^{2+}$ and $Pb^{2+}$ ions had no substantial impact. There was no obvious inhibitory effect of the chelating EDTA on the enzyme activity.

The enzyme was additionally characterized to determine the kinetic parameters such as $K_m$, $V_{max}$, and $k_{cat}$ of the purified Sfp phosphopantetheinyl transferase. Values for $K_m$ and $V_{max}$ were determined from the double-reciprocal plots. The $K_m$ and $V_{max}$ values were 4.52 mg/mL and 35.33 U/mg, respectively (Figure 8.3f), while 3.64 $s^{-1}$, and 0.104 $mM^{-1}$ $s^{-1}$ were obtained for $k_{cat}$ and $k_{cat}/K_m$ respectively.

### *8.3.3 Phylogenetic analysis and molecular characterization of the biosurfactant gene*

The *sfp* gene complete nucleotide and construed amino acid sequence achieved from *Paenibacillus* sp. D9 (GenBank Accession number JZEJ00000000) was highlighted in Figure 8.4. The *sfp* gene sequence was 693 bp and encoded amino acid sequence (230 AAs), including an ATG initiation codon and a TAA termination codon by analysis (Figure 8.4), utilizing online software available.

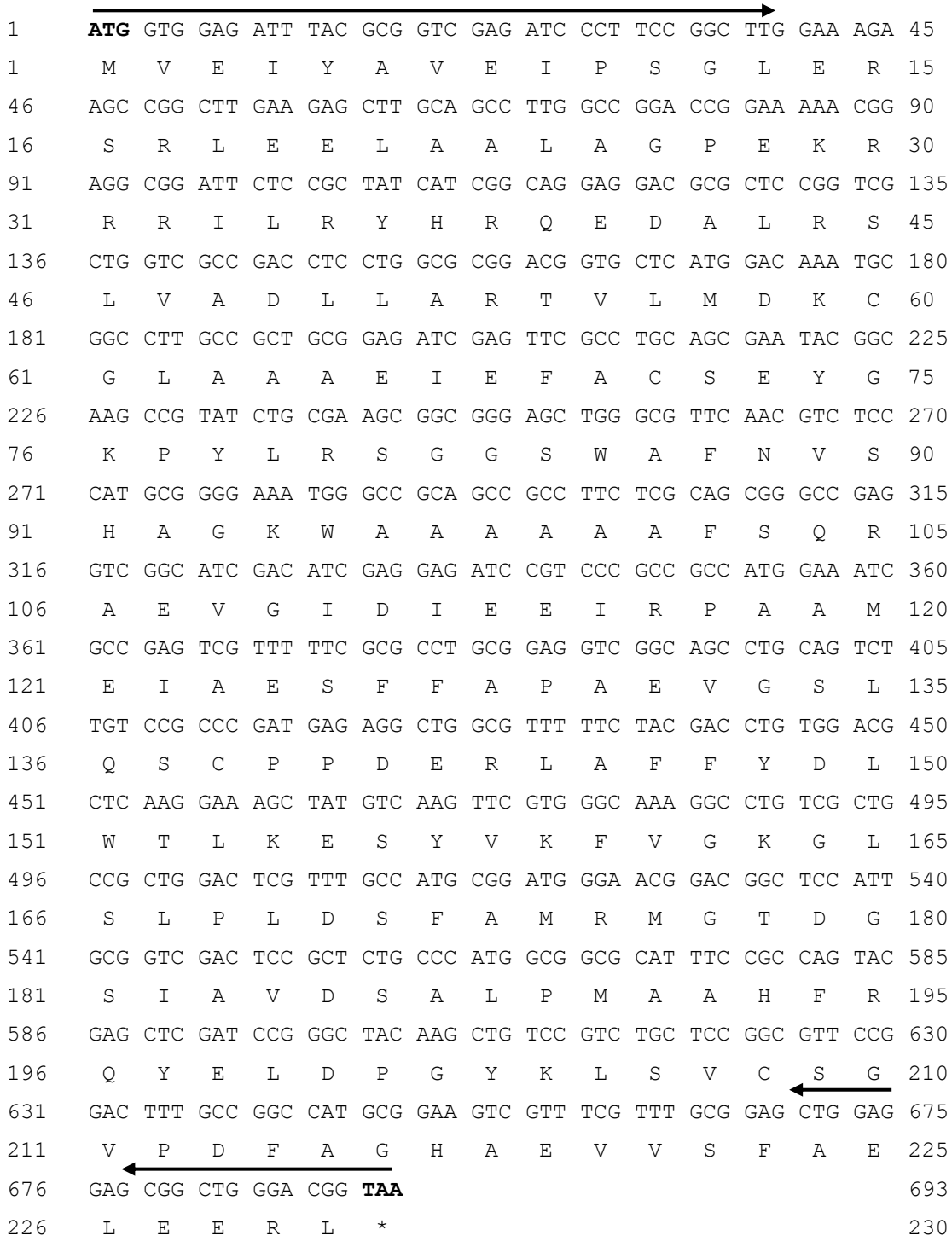



**Figure 8.4** The *sfp* gene complete nucleotide and construed amino acid sequence achieved from *Paenibacillus* sp. D9 (GenBank Accession number JZEJ00000000). The annealing sites utilized in the amplification and cloning of the *sfp* gene from *Paenibacillus* sp. D9 are indicated by the arrows. Start and stop codons are indicated by bold letters.

BLASTP investigation of the concluded protein sequence uncovered 99%, 93%, and 90% similarity to prior detailed Sfp sequences from *Paenibacillus* sp. P22 (Accession No. WP_048746037.1), *Paenibacillus* sp. RU4T (Accession No. SIR11239.1), and *Paenibacillus* sp. RUD330 (Accession No. WP_094248422.1) respectively (Figure 8.5).

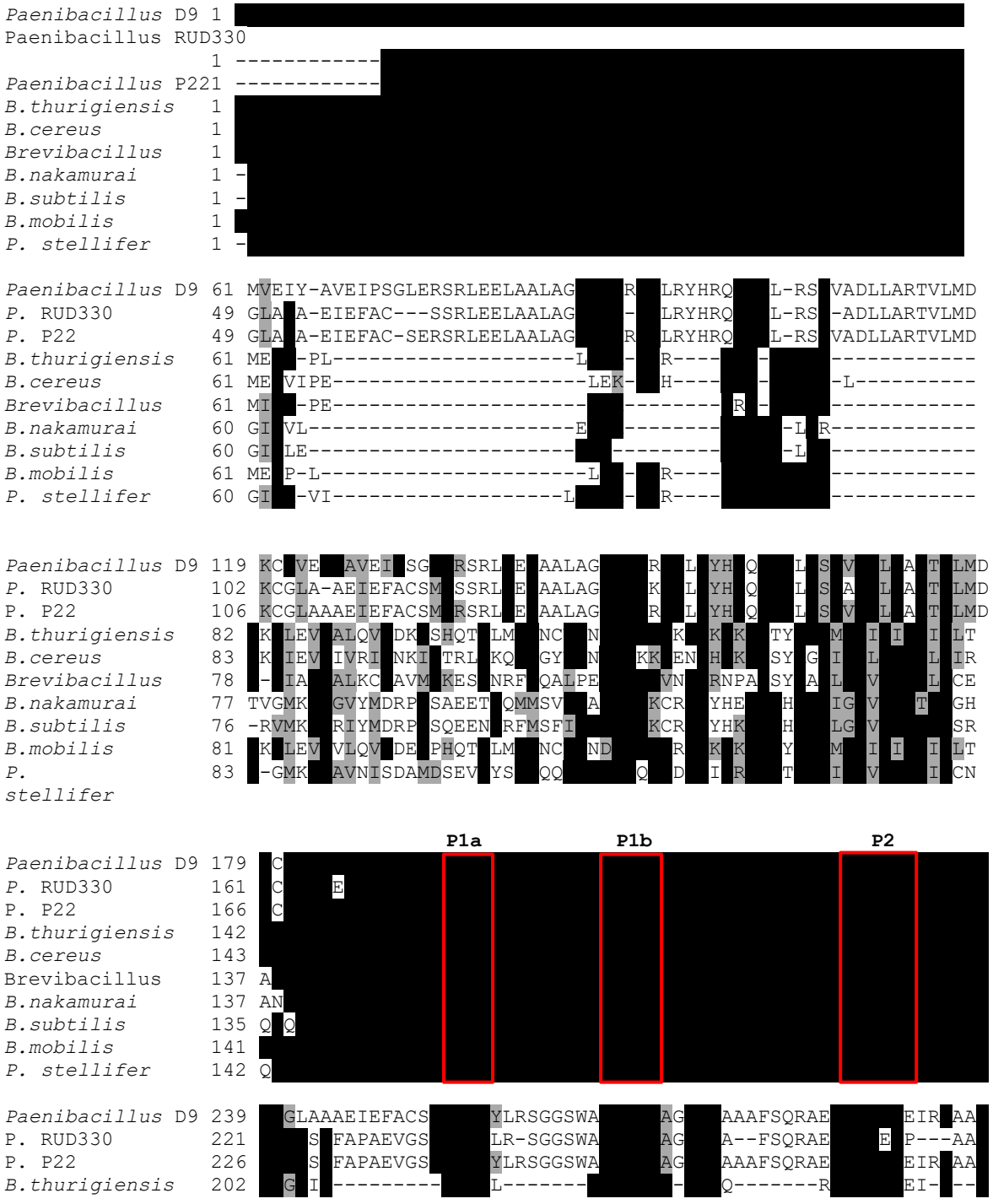



**Figure 8.5** Multiple sequence alignments Sfp protein sequences from different microorganisms. The alignment was achieved utilizing amino acid sequences from D9 (*Paenibacillus* sp. D9), this work; *Paenibacillus* sp. P22, *Paenibacillus* sp. RUD330; *Bacillus thuringiensis*; *Bacillus cereus*; *Brevibacillus brevis*; *Bacillus nakamurai*; *Bacillus subtilis*, *Bacillus mobilis*; and *Paenibacillus stellifer*. The shaded (black) boxes represent the sequence similarity. The red boxes are the conserved domains of the Sfp type PPTase family which are involved in the biosynthesis of non-ribosomal peptides.

The *sfp* type PPTase family conserved domains were recognized in the deduced amino acid sequence from *Paenibacillus* sp. D9. The conserved domains P1a, P1b, P2, and P3 (Figure 8.5), which are associated with the biosynthesis of non-ribosomal peptides, were seen in the Sfp sequence. Additionally, there was revelation of relatedness of this gene based on the phylogenetic tree analysis to earlier reported sequences from *Paenibacillus* sp. P22 (Accession No. WP_048746037.1), and *Paenibacillus* sp. RUD330 (Accession No. WP_094248422.1) (Figure 8.6).

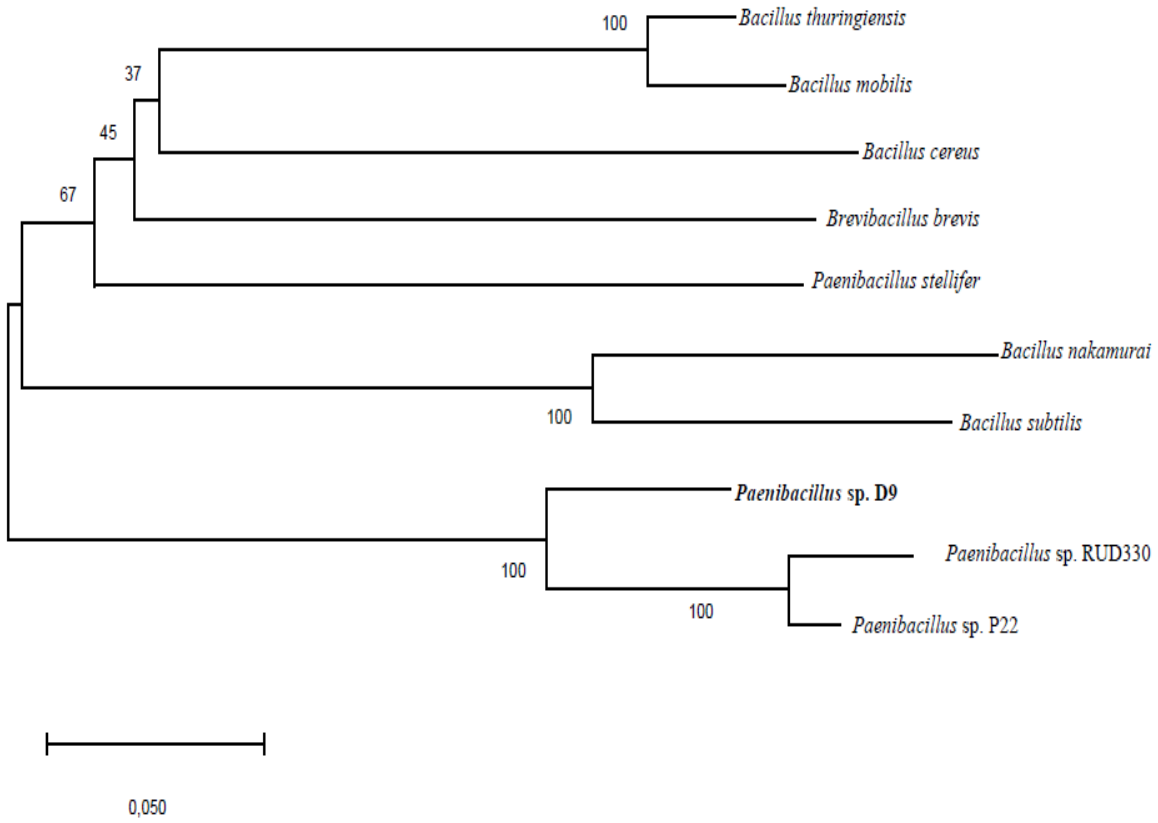



**Figure 8.6** The Phylogenetic tree demonstrating evolutionary relationships of varied bacterial Sfp amino acid sequences. The tree was inferred using the neighbour-joining technique utilizing Mega X. The topology was tried in the bootstrap test (1000 replicates). The Sfp amino acid sequence obtained from the *Paenibacillus* sp. D9 utilized in the study is demonstrated in bond letters. All other reference sequences utilized for the tree development were procured from GenBank.

### *8.3.4 Regulation of biosurfactant production with various substrates on the recombinant strain*

To confirm the significant role played by the *Paenibacillus* sp. D9 *sfp* gene in the biosynthesis of lipopeptide BioS, the recombinant strain was induced with different carbon substrates (Table 8.2). As such, BioS yields, BioS activities, and esterase activities were assessed to confirm to surface activities. From different carbon source tested, glucose concentration with 1.05 g/L led to the greatest BioS yield. It is also imperative to note that sunflower oil and canola which are both hydrophobic oily substrates produced high BioS concentrations of 1.03 g/L, and 0.86 g/L respectively. Hence, there was no significant difference ($p > 0.05$) between the substrates stated above namely glucose, sunflower oil, and canola oil. The outcomes showed significance relative

to control samples with no production of BioS yield discovered. This however, rule out any possibilities of the substrates co-precipitating with the isolated BioS. From the carbon sources utilized for BioS synthesis by the recombinant (*BioSp*) organism in basal medium; glucose, glycerol, sunflower oil, and canola oil produced maximum BioS activity in the range of 2.147 to 1.097 (optical density at 540 nm). Very low BioS activities of 0.122, and 0.157 were observed when grown in the presence of *n*-hexadecane, and diesel fuel owing to inability to utilize these complex hydrocarbons. There was a strong correlation ($r = 0.62$, $p < 0.05$) between BioS synthesis and esterase activity exhibited by recombinant *BioSp* strain on the different substrates. However, the greatest esterase inducer amongst all the substrate was glucose with esterase activity of 2.231 IU/ml followed by sunflower oil, canola oil, and glycerol with esterase activities of 0.238, 0.200, 0.181 IU/ml respectively (Table 8.2). However, there was low production of esterase activities on diesel fuel and *n*-hexadecane respectively.

**Table 8.2** Cell growth, esterase activity, biosurfactant activity, and biosurfactant yield of *E. coli* recombinant (*BioSp*) on different carbon substrates after 5 days of incubation at 37°C

| Substrates | Cell growth $OD_{600}$ | Esterase activity (IU/ml) | Emulsifying activity ($OD_{540}$) | Biosurfactant yield (g/L) |
|---|---|---|---|---|
| Control | 0.05 ± 0.01 | 0.021 ± 0.01 | 0.08 ± 0.02 | 0.03 ± 0.01 |
| Glucose | 2.14 ± 0.01 | 2.231 ± 0.037 | 2.147 ± 0.012 | 1.05 ± 0.07 |
| Glycerol | 1.64 ± 0.03 | 0.181 ± 0.026 | 2.011 ± 0.031 | 0.51 ± 0.07 |
| Diesel fuel | 0.17 ± 0.01 | 0.090 ± 0.013 | 0.157 ± 0.004 | 0.13 ± 0.06 |
| *n*-Hexadecane | 0.28 ± 0.03 | 0.089 ± 0.017 | 0.122 ± 0.011 | 0.13 ± 0.04 |
| Sunflower oil | 0.83 ± 0.01 | 0.238 ± 0.014 | 1.181 ± 0.019 | 1.03 ± 0.10 |
| Canola oil | 0.69 ± 0.01 | 0.200 ± 0.027 | 1.097 ± 0.011 | 0.86 ± 0.02 |

All data points are means ± standard deviation (S.D.) of three independent experiments

*Paenibacillus* sp. D9 and recombinant strain *BioSp* developed on a basal medium containing 2.0% (v/v) glucose at 37°C were assessed for their growth, esterase and BioS activities. High *BioSp* yield of 1.11 g/L was observed in *BioSp* recombinant strain followed by *Paenibacillus* sp. D9, non-induced *Escherichia coli* BL21 DE3 (pLysS), and *Escherichia coli* DH5α. However, the recombinant strain produced esterase and BioS activities

of 2.55 IU/ml and 4.22 in contrast with the parent strain-produced BioS (Table 8.3). There were minimal esterase activities as well as BioS activities observed for both *E. coli* DH5α and *E. coli* BL21 DE3 (pLysS) strains indicating their inability to utilize the carbon substrate. Subsequently, the recombinant strain and parent *Paenibacillus* sp. D9 produced higher BioS and esterase activities when compared to *E. coli* DH5α and *E. coli* BL21 DE3 (pLysS). To further confirm the production of BioS, surface tension was reduced to 35.7 mN/m by the recombinant *BioSp* strain. There was the induction of BioS activity on *Paenibacillus* sp. D9 showing 36.8 mN/m surface tension. The plasmids *Escherichia coli* DH5α, and *Escherichia coli* BL21 DE3 (pLysS), which were utilized as control showed no surface activities on the glucose substrate indicating their inability to produce BioS.

**Table 8.3** Cell growth, surface tension, and biosurfactant yield of *Paenibacillus* sp. D9 and recombinant organism on glucose after 5 days of incubation at 37ºC.

| Strain | Esterase activity (IU/ml) | Biosurfactant activity | Biosurfactant yield (g/L) | Surface tension (mN/m) |
|---|---|---|---|---|
| *Paenibacillus* sp. D9 | 1.72 ± 0.03 | 1.69 ± 0.03 | 0.52 ± 0.07 | 36.8 ± 0.4 |
| *E. coli* DH5α | 0.17 ± 0.02 | 0.15 ± 0.01 | 0.05 ± 0.04 | 61.6 ± 0.2 |
| *E. coli* BL21 | 0.27 ± 0.06 | 0.23 ± 0.01 | 0.08 ± 0.02 | 59.6 ± 0.6 |
| *E. coli BioSp* | 2.55 ± 0.02 | 4.22 ± 0.01 | 1.11 ± 0.04 | 35.7 ± 0.4 |

All data points are means ± standard deviation (S.D.) of three independent experiments

## 8.4 Discussion

*Bacillus* and *Paenibacillus* spp. strains are among the rod-shaped bacteria which are producers of numerous secondary metabolites that have varied functions and structure (Porob et al., 2013). The metabolites synthesized by these genus have distinguished properties with great importance in the biotechnological industries (Devaraja et al., 2013). There has been a report that lipopeptide is produced through a non-ribosomal thiotemplate mechanism (Porob et al., 2013). This investigation is valuable to identify *sfp* presence in genus *Paenibacillus* coupled with little details on the structural synthesis of the lipopeptide group. The *sfp* gene, which codes for a

non-ribosomal peptide synthetase complex is an essential unit of the *srf*A operon. The *sfp* gene encodes phosphopantetheinyl transferase and is essential for the biosynthesis of lipopeptide BioS. From investigating the sequence of *Paenibacillus* sp. D9, the *sfp* gene was 693 bp long and encoded 230 amino acids (Figure 8.4). BLASTP investigation of the nucleic acid sequences uncovered 99% similarity to the *Paenibacillus* sp. P22 (Accession No. WP_048746037.1) *sfp* sequence. The phylogenetic tree dependent on the Sfp amino acid sequence demonstrated arrangements from *Paenibacillus* sp. D9 similar to Sfp protein sequences of *Paenibacillus* sp. P22 and *Paenibacillus* sp. RUD330 (Figure 8.6). This is possibly due to close phylogenetic relatedness of *Paenibacillus* sp. D9 to both *Paenibacillus* sp. P22 and *Paenibacillus* sp. RUD330.

In the lipopeptide biosynthetic gene cluster, the related substrates of Sfp protein are likely the bearer proteins of the lipopeptide non-ribosomal peptide synthetase. The Sfp inactivation has demonstrated that Sfp protein is indispensable in lipopeptide biosynthesis (Bunet et al., 2014). Sfp-type PPTases, show broader substrate preferences and have been associated with secondary metabolism in bacteria (Schimming et al., 2016). This superfamily comprises of two subtypes: the Sfp and ACPS type which is both present in *Paenibacillus* sp. D9 amino acid sequence. The 4'-phosphopantetheine (4'-PP) moiety is transferred by this family from coenzyme A (CoA) to the invariant serine of pfam00550. This study revealed higher activities of Sfp protein (phosphopantetheinyl transferase) on the substrates used for confirmation (esterase and coenzyme A substrate mix). From this study, the *sfp* gene of *Paenibacillus* sp. D9 was successfully cloned into pet-47b vector and expressed in *Escherichia coli* BL21 DE3 (pLysS) after transformation. Interestingly, the specific activity (87.14 U/mg) of the purified phosphopantetheinyl transferase was 3.26-fold than that of the cloned crude extract (Table 8.1).

The results of temperature, pH and stability show the phosphopantetheinyl transferase was active in inclusive range of temperature and pH. These properties will enable its biotechnological applications as most industries usually require enzymes that are functional and stable at high temperatures and pH. The effects of pH, temperature, and heavy metals had similar output on the maximum activity of the purified *Paenibacillus* sp. D9 Sfp phosphopantetheinyl transferase. The S-H group of cysteine bonds reacts with heavy metals, forming a covalent bond with sulfur atom and dislodging the hydrogen ion. Thus, the enzymatic activity is either increased or disrupted (Yadav and Magadum, 2017). A few enzymes, for example, Sfp phosphopantetheinyl transferase require heavy metals as co-factors to be more progressively active. These co-factors are essential in the protein structure required for maximum activity (Gohara and Di Cera, 2016). The three metal ions $Zn^{2+}$,

$Cu^{2+}$, and $Fe^{2+}$ showed a stimulatory effect on purified Sfp phosphopantetheinyl transferase activity. Nevertheless, other metal ions like $Ca^{2+}$ and $Pb^{2+}$ showed no impact on the enzyme activity. Strikingly, Sfp phosphopantetheinyl transferase held practically maximum activity with 1mM EDTA concentration. The outcomes showed that Sfp phosphopantetheinyl transferase was unequivocally impervious to the chelating reagent which is intriguing in that no metal ion is a fundamental requirement for maximum Sfp activity. Also, its stability to the chelating reagents (one of the key ingredients in detergent formulations) is of incredible significance from the biotechnological perspective and future works.

To facilitate scientific research and engineering development, enzyme kinetics inclusive of $K_m$ and $V_{max}$ are significant coefficients, and thus needs to be considered favorably (Yadav and Magadum, 2017). This study discusses exhaustively the kinetic parameters of Sfp phosphopantetheinyl transferase and represents the first report. In this area, the kinetic parameters of the Sfp phosphopantetheinyl transferase characterized above, are critical to understanding the enzyme reaction in controlled metabolism. The low Km of Sfp phosphopantetheinyl transferase indicated high affinity of the enzyme as ½ Vmax was reached with low substrate concentration.

From this study, the BioS gene (*sfp*) was effectively cloned and over-expressed in *BioSp*, demonstrating an increase in BioS and esterase activity than the parent strain. Similar to this research, Khanna et al. (2009) proposed recombinant *Escherichia coli* pSKA clones containing BioS gene *srfA* demonstrated higher esterase and BioS activity with olive oil when contrasted to parent *Bacillus* sp. SK320 strain. In another study, there was over-expression of BioS genes in *BioSa*, *BioSb*, and *BioSc*; displaying a twofold increase in BioS activity than the parent strain. Also, enhanced esterase production was conferred on the recombinant cells as compared to *Bacillus substilis* SK320 (Sekhon et al., 2011). There was identification of *lch*AA gene and significant amount of lichenysin produced in the 53 tested *Bacillus licheniformis* strains (Madslien et al., 2013). Liu et al. (2012) likewise reported surfactin variations from *Bacillus subtilis* TD7 was essentially affected by culture medium with various amino acid supplements. The optimization of major medium components on strain WX02-Psrflch synthesized 2.15 g/L lichenysin, a 16.8-overlay enhancement when contrasted with that of wild strain WX-02 (Qiu et al., 2014). In another report, engineered *Bacillus subtilis* (pHT43comXphrC) strain synthesized 0.14 g/L in comparison to *Bacillus subtilis* after 48 h of culture production while utilizing synthetic wastewater. As such, there was a 6.7-fold increase in surfactin production by the engineered *Bacillus subtilis*

(pHT43comXphrC) strain utilizing engineered wastewater contrasted and that in the wild strain (Jung et al., 2012).

This research demonstrated that the great significance as the recombinant strain could utilize simple carbon sources such as glucose, and glycerol, as well as hydrophobic oily carbon substrates; canola and sunflower oil, respectively (Table 8.2). Cheap substrates, for example, vegetable oils and oil waste have the potential for improving BioS synthesis. Utilizing hyper-producing microbial strains or mutants with high yielding limits and inexpensive low-cost substrates as raw material for the synthesis of BioS has not been improved at a modern level. The utilization of these in-expensive hydrophobic oily carbon substrates for cell growth and BioS synthesis by this recombinant organism becomes important to further reduce the cost of production on a large scale. The designed *E. coli* strain has modern application since it incorporates BioS at high rates and can minimize the complex downstream and purification procedures related to the regular bioprocess. Similar to this research, the recombinant BioS producing strains *BioSa*, *BioSb,* and *BioSc* were able to utilize olive oil which is also a hydrophobic substrate as carbon, respectively, with the highest BioS production of 2.45 g/L observed on *BioSc* (Sekhon et al., 2011). There was confirmation of BioS properties on recombinant *BioSp* strain as revealed by the surface tension activities. The recombinant organism reduced the surface tension of basal BH medium to 35.7 mN/m (Table 8.3) indicating significant production of this surface-active biomolecule. The capacity to decrease surface activity is a critical parameter to assess the nature of surface-active compounds. To support this finding, apoemulsan recombinant-esterase in comparison with fully proteinated emulsan were examined. The outcomes revealed that the esterase-apoemulsan complex was progressively feasible in emulsifying a range of hydrophobic substrates that are usually not emulsified by crude emulsan itself (Sekhon et al., 2012).

The role played by protein (or an enzyme) in the emulsifying action of BioSs is hard to discount. Genetic engineering, therefore, can be done to improve not only BioS surface activity but also its production yield as carried out extensively in this study. Genetically recombinant and engineered organisms can lead to achievement in the bio-production process. Identification of the *sfp* gene encoding 4-phosphopantetheinyl transferase and its crucial function in the synthesis of *Paenibacillus* sp. D9 lipopeptide BioS is novel. The information on the molecular genetics of *Paenibacillus* sp. D9 was utilized to produce recombinant microorganism. Furthermore, the hyper-producing microbial strain from this report was able to grow on cheap substrates producing BioS in high yield as compared to other strains. The results suggest that the successful

expression of the BioS gene enabled the high surface potential of the *BioSp* as compared to the other recombinant strains. As such, there is a difference in properties and advantages attributed to *BioSp* which propose its significance in bioremediation and other biotechnological applications.

## 8.5 Conclusion

A new phosphopantetheinyl transferase with N-terminal sequence was identified from *Paenibacillus* sp. D9. The cloning of the *sfp* gene encoding 4-phosphopantetheinyl transferase is projected to possess several advantages in biotechnological application. This enzyme was confirmed to regulate and play a major role in the biosynthesis of lipopeptide BioS. This research demonstrated a conceivable relationship between BioS and esterase synthesis and first to be reported of any *Paenibacillus* species. The recombinant strain also showed a positive increase in the surface, esterase activity, and BioS yield when compared to the parent strain. BioS-esterase complex by hyper-producing recombinant strain was found to have amazing surface-active properties, which indicates enhanced hydrocarbon biodegradation, and bioremediation.

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Compliance with ethical standards

## Conflict of Interests

The authors wish to declare no conflict of interest

## Human and animal rights and informed consent

This article does not contain any studies with human participants performed by any of the authors.

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# Chapter 9. Conclusions and future perspectives

## 9.1 Conclusion

One of the significant classes of industrial chemicals in terms of production volume are surfactants (Jimoh and Lin, 2019a). Most of these chemically synthesized surfactants may unavoidably end up in the environment after synthesis, or after use. In this context, ecological toxicity, biomagnification, bioaccumulation, and biodegradability of surfactants have become subjects of collective concern. Lipopeptides are biomolecules comprising of a lipid connected to a peptide, that are small chains of amino acid monomers joined by peptide (amide) bonds. Due to improved environmental biocompatibility, and excellent functional properties as compared to chemical surfactants, the market share value of lipopeptide biosurfactant (BioS) in the last five years has expressively increased (Jimoh and Lin, 2019b). The current research was initiated to examine the basic concept associated with BioS synthesis, production optimization, its application implications, as well as the molecular and genetical perspectives. To accomplish these aims, a set of six different experimental procedures were achieved as discussed below.

The experiments in Chapter 3 showed *Paenibacillus* sp. D9 produces high molecular weight lipopeptide compounds by using hydrophobic carbon sources. However, these substrates are quite expensive coupled with the costs required for post-fermentative recovery and purification processes. From this research, *Paenibacillus* sp. D9 was able to withstand toxic hydrophobic compounds, and subsequently producing a novel surface-active agent termed “lipopeptide.” The BioS have effective surface-active properties, and formation of an emulsion with a wide array of hydrocarbons and hydrophobic pollutants. The *Paenibacillus* sp. D9 strain could tolerate high diesel concentrations and a wide range of utilization on different hydrocarbons substrates. A greater production yield was achieved extracellularly with *Paenibacillus* sp. D9. *Paenibacillus* sp. D9 thus produced a high amount of lipopeptide BioS with positive potentiality in biodegradation and bioremediation.

The biodegradation of hydrocarbon is enhanced by the key roles played by biodegradative enzymes. These biodegradative enzymes include alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase, monooxygenase, and esterase (Jauhari et al., 2014; Sekhon et al., 2011). In Chapter 4, *Paenibacillus* sp. D9 produced BioS and synthesized degradative enzymes in the presence of diesel fuel and *n*-hexadecane. These major factors and enzymes which were overly produced along with high cell surface hydrophobicity enhanced the biodegradation of *n*-hexadecane and diesel fuel. The production of enzymes and BioS by *Paenibacillus* sp.

D9 strain led to an increase in effectiveness of biodegradation. Thus, 98.4% of *n*-hexadecane (C16) and 80.2% of diesel fuel (C9-C25) were utilized as source of carbon and energy by *Paenibacillus* sp. D9, corresponding to the high synthesis of alkane hydroxylase, alcohol dehydrogenase, and esterase enzymes accompanied by high BioS activity. Hence, *Paenibacillus* sp. D9 was more vigorous in degradation, and mineralization of hydrophobic pollutants, and could be used to decontaminate HC compounds, oil spills at polluted sites. As such, the correlation between BioS production and biodegradative enzymes in hydrophobic pollutants was confirmed in this research. These outcomes provide new knowledge into the physiology, biodegradative mechanisms and pathways of the genus *Paenibacillu*s.

Research has demonstrated that the synthesis of BioS compounds is basically affected by the culture conditions and in addition nutrient accessibility to the microorganism incorporating the BioSs (Md, 2012). The finding of BioS-synthesizing microorganisms that can be purified under optimum medium conditions and at the same time synthesize substantial amounts of BioS. This would be a method for reducing the monetary hindrances related with the extensive scale production of these biomolecules. From this research, BioS production by *Paenibacillus* sp. D9 increased to 4.11 g/L under optimized conditions from 1.15 g/L in BH medium containing 3.0 % diesel fuel and 1.0 % ammonium sulfate, 4.0mM $MgSO_4$, pH 7.0, temperature 30$^{o}$C and 1.5 mL inoculum size (Chapter 5). From the result, increase in BioS synthesis was favored by the new optimized condition. The product displayed great thermal stability, was able to withstand extreme acidic and alkaline conditions, and demonstrated resilience to changing salt condition, which delineates clear viewpoints for its conceivable use in extreme environmental conditions. These conditions include bioremediation, biodegradation of hydrophobic pollutants, microbial enhanced oil recovery and other biotechnological-related fields. The impact of the synergistic relationship between *Paenibacillus* sp. D9 and the introduction of exogenous *Paenibacillus* sp. D9 lipopeptide BioS on the biodegradation of high molecular weight hydrocarbons and hydrophobic pollutants was investigated (Chapter 5). Thus, the application of introduced *Paenibacillus* sp. D9 BioS increase the removal and dissolvability of these hydrophobic mixtures as opposed to the use of the *Paenibacillus* sp. D9 alone. An increase in diesel fuel biodegradation rate up to 65.1% was observed under the same conditions with the addition of 500 mg/L *Paenibacillus* sp. D9 BioS. There was an increase in biodegradation effectiveness of diesel fuel by the *Paenibacillus* sp. D9 strain when contrasted with the lipopeptide-free inoculated culture, thus exhibiting the potential of *Paenibacillus* sp. D9 BioS for biodegradation and bioremediation.

As the production of high molecular weight lipopeptide from unconventional substrates is not (yet) economically feasible, it would be advantageous if *Paenibacillus* sp. D9 could in some way be required to synthesize BioS utilizing conventional and cheaper medium-chain fatty acids, vegetable oils or even waste frying oils. As such, the potential of *Paenibacillus* sp. D9 to grow on cheap carbon sources such as waste frying oils (canola, sunflower, castor, and coconut) was investigated as alternate substrates as this may prompt a decrease in bioprocess cost (Chapter 6). Economical processes that employ low-cost materials and relatable process parameters are key to successful BioS production and eventually solve the major setback regarding high production cost (e Silva et al., 2014; Rufino et al., 2014). There was a new contribution to knowledge, as the result from this Chapter 6 produced a better production yield of 5.31 g/L. This provides a substantial parameter in the BioS market enabling its availability as a low-cost alternative for future use. The diverse features affecting the BioS synthesis have been considered in recent years, however few of these, used appropriate statistical tools for experimental design and modification (Franzetti et al., 2009). The orthodox method of medium optimization includes the one variable at a time studies approach. This strategy is tedious and leads to negligence in the interaction between factors, with no guarantee in definite determination of the best optimal conditions. The ability of *Paenibacillus* sp. D9 BioS to biodegrade and bioremediate both solid- and liquid-contaminated environments under different conditions was tested. The ecologically-friendly BioS possess a higher removal efficiency from both environments using kinetic and static conditions as compared to chemical surfactant. This BioS has preferable biodegradation proficiency over its manufactured chemical counterpart in realizing the bioremediation of oil-polluted sands. The efficacy of the BioS produced in bio-remediating under different environmental conditions has also not been relatively tested, hence the impact of this study. As such, the lipopeptide BioS produced on low-cost substrate (waste canola oil) is a great contribution to the world BioS market as the problem associated with high cost of production would become a thing of the past.

Now, there has been huge emphasis positioned at the devastating effects and severity of the usage on synthetic surfactants on the environment. This is based on their highly toxic, persistent nature, and non-biodegradable properties (Santos et al., 2016; Sarubbo et al., 2015b). Chapter 7 assess the *Paenibacillus* sp. D9 lipopeptide BioS synthesis in a combination of cheap substrates, functional properties, applicability for varying recent and trending biotechnological processes. Due to high substrates used (10%), different inoculum conditions were varied to ascertain the ability of *Paenibacillus* sp. D9 to withstand selective pressure and concentrations.

Results provided reveal surface tension activities of around 31.7–32.7 mN/m, and maximum *Paenibacillus* sp. D9 BioS yield more than 8 g/L, regardless of the inoculum sizes used. This investigation sheds light on the elective usage of waste cooking oil as a high-vitality source for the synthesis of high-value products. The *Paenibacillus* sp. D9 BioS was successful in dispersing engine oil, with further capability in removing different heavy metals from the environments including contaminated effluents, synthetic wastewater, contaminated sands, and food crops. Still, of great concern to the environment is the high incidence of phosphate and sulfate resulting from contaminated effluents. The BioS including the cell-free supernatant were both efficient in the removal of both phosphate and sulfate rather to the chemical surfactant. Few reports have mentioned the efficient role of BioS in removing heavy metals from polluted effluents (Hidayati and Surtiningsih, 2014; Sarubbo et al., 2015a) This is the first report to show the effective advantage of BioS in removing heavy metal from acid mine drainage contaminated effluents, and different physiochemical parameters such as pH, phosphate, sulfates, and so on. From this study, the lipopeptide BioS also selectively removed cadmium from contaminated vegetables in the order of onion = tomato > cucumber > potato. The BioS eliminated a substantial amount of heavy metal from polluted food samples. Thus, the BioS synthesized could be utilized economically, enabling its usefulness for human health. Chapter 7 further shows the illustrative formulation tests representing the relative washing performances of *Paenibacillus* sp. D9 BioS, SDS, Triton X, commercial detergents, and bio-commercial detergent against sunflower oil-tomato sauce and coffee stains. The removal of stains by BioS-containing detergent is equivalent to manufactured detergent particularly for the removal of hydrophilic and hydrophobic extreme stains. This research proffers an incredible noteworthiness since the *Paenibacillus* sp. D9 BioS considered as a substitute for chemical surfactants was non-toxic and possess higher biodegradability. There are relative low production yields of BioS which subsequently limits its biotechnological applications. As such, there has been an increasing requirement for the enhancement of BioS production through development of hyper-producing recombinant strains or modification by genetic engineering (Willenbacher et al., 2016). Chapter 8 was valuable to recognize the presence of the *sfp* gene in the genus *Paenibacillus* with little report on the structural synthesis of lipopeptide from this group. Interestingly, the specific activity (87.14 U/mg) of the recombinant phosphopantetheinyl transferase was 2.49-fold higher than that of the cloned crude extract. The results of temperature, pH and stability show the phosphopantetheinyl transferase was active in a wide range of temperature and pH. These properties will enable its biotechnological applications as most industries usually require enzymes that are functional and stable at high temperatures and pH. From this study,

the BioS gene (*sfp*) were effectively cloned and overly expressed in *BioSp*, demonstrating an increment in BioS and esterase activity than the parent strain. This research produced great significance as the recombinant strain could utilize simple carbon sources such as glucose, glycerol, as well as hydrophobic oily carbon substrates; canola and sunflower oil respectively. The utilization of in-expensive hydrophobic oily carbon substrates for cell growth and BioS synthesis by this recombinant organism becomes important to further reduce the cost of production on a large scale. The results suggest that the engineered *recombinant* strain has probable biotechnological applications since it synthesizes BioS at high yields and can reduce the complex purification process related with the regular bioprocess.

## 9.2 Future perspectives

Irrespective of the different composition and applications that BioSs had shown, the large-scale industrial mass synthesis of these compounds is the major focus nowadays. Further researches are essential to improve the industrial scale applications with consideration on numerous environmental complexes and factors that limit BioS synthesis and utilization. To encourage field uses of these BioS enhanced innovations, substantial tests are foreseen to consolidate heterogeneities in topographical/hydrological features and in microbial degradation of polluted sites. With the new improvement in this field and spotlight on interdisciplinary research joined with advancements of metabolic, and genetic engineering, the prospects of BioSs will be financially effective. The exploration in this field is progressing quickly and it envelops fields as diverse as, textile, pharmaceutics, cosmetics, oral hygiene, petroleum, wastewater treatment, agriculture, surface science, natural science, and molecular biology.

The invention of current strategies and disclosure of progressively innovative sources are likewise expected in the future. So, there would be a further decrease in costs of production, with continuous discovery of novel BioSs, proper knowledge and understanding of these biomolecules. Molecular methods to screen for BioS producers remain in vacancy, hampered by the way biosynthetic pathways and genes are rationed at species level. Vital bits of knowledge into the extensive variety of BioSs are expected to be accomplished dependent on advances in genomic and proteomic aspects of microbiology sooner rather than later.

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# Appendix

## Supplementary Information Chapter 3

## Production and characterization of lipopeptide biosurfactant producing *Paenibacillus* sp. D9 and its biodegradation of diesel fuel

**Running Title: Lipopeptide biosurfactant producing *Paenibacillus* sp. D9 and its biodegradation of diesel**

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## Supporting Figures

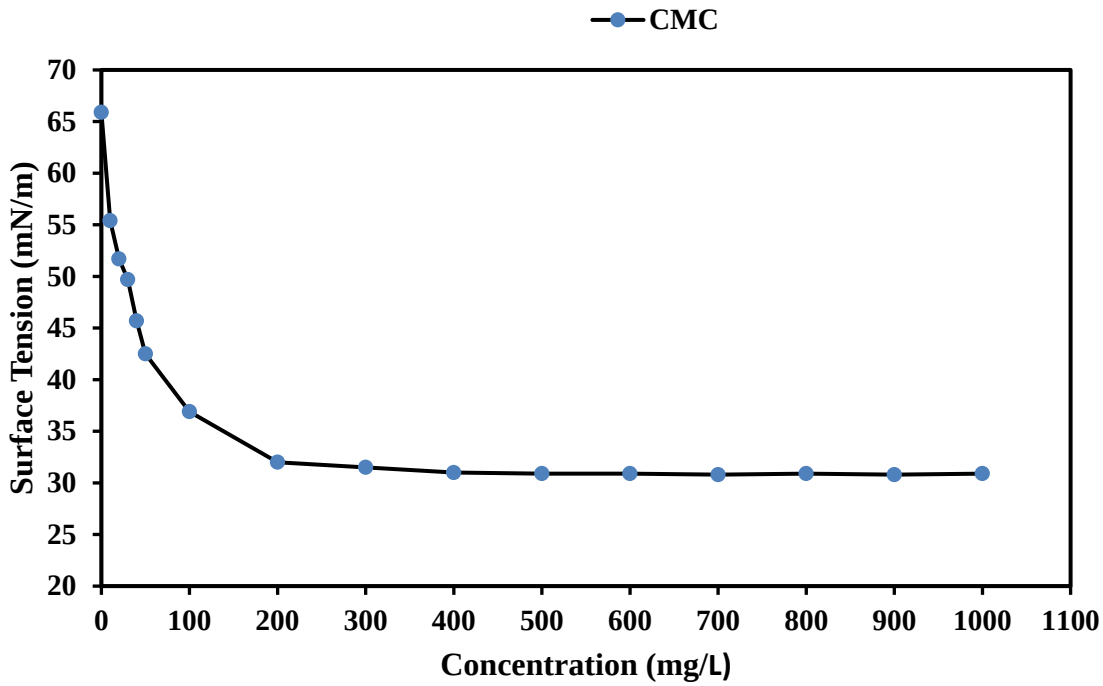


**Figure S1** Surface tension changes of biosurfactant of *Paenibacillus* sp. D9 against different concentration of biosurfactant solution.


**Figure S2** TLC Analysis of purified biosurfactant of *Paenibacillus* sp. D9.

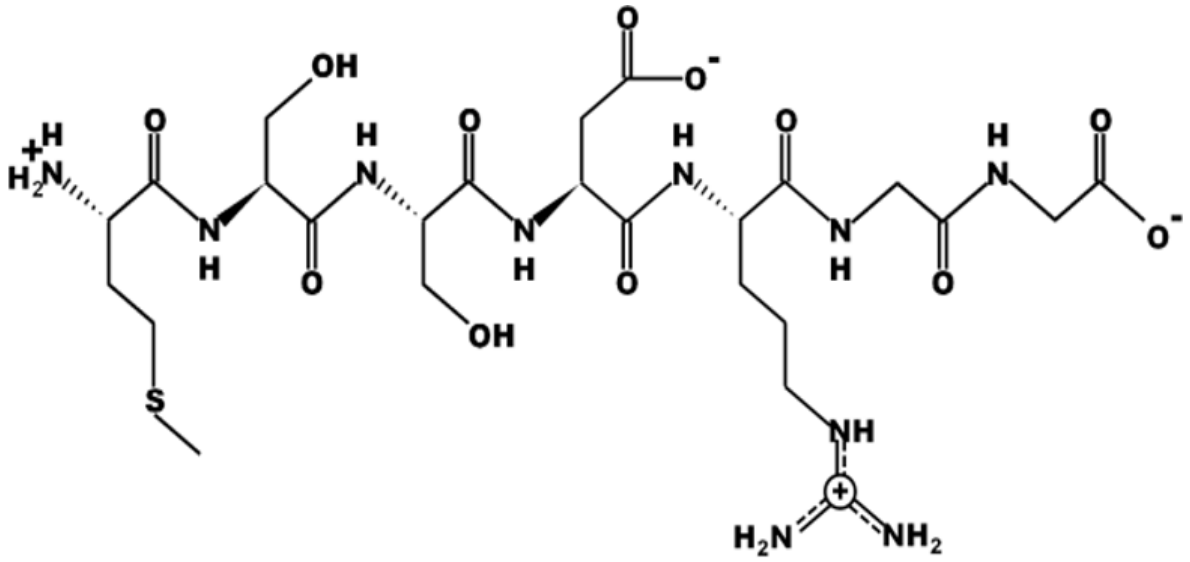

**Figure S3** Peptide structure generated from the purified lipopeptide biosurfactant.

## Supporting Tables

**Table S1** Growth utilization pattern of the strain *Paenibacillus* sp. D9 on hydrocarbon substrates

| Compounds | Growth | Compounds | Growth |
|---|---|---|---|
| *n*-Hexadecane | +++ | Benzoic acid | ++ |
| *n*-Hexane | - | Salicylic acid | ++ |
| *n*-Dodecane | +++ | Phthalic acid | ++ |
| *n*-Tetradecane | +++ | Diesel | +++ |
| Phenol | - | *n*-Paraffin | ++ |
| *n*-Toluene | + | Motor oil | ++ |
| Engine oil | +++ | Cyclohexane | - |
| 1-Nonene | +++ | Tetracosane | + |

**+++ OD600>1.0, ++ OD600> 0.5, + OD600>0.2, − OD600<0.2**

**Table S2** Emulsification index (E24 index) on the increasing order of concentrations of carbon source tested

| **System** | E24 Index **(%)** |
|---|---|
| 1% | 63.3 ± 0.3 |
| 2% | 67.5 ± 0.1 |
| 5% | 69.2 ± 0.2 |
| 10% | 73.3 ± 0.2 |
| Control A | 0.0 ± 0.0 |
| Control B | 61.7 ± 0.2 |

Control A: Distilled water + Diesel concentration

Control B: Tween 80 + Diesel concentration

## Supplementary Information Chapter 7

# Biotechnological applications of *Paenibacillus* sp. D9 lipopeptide biosurfactant produced in low-cost substrates

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## Figures

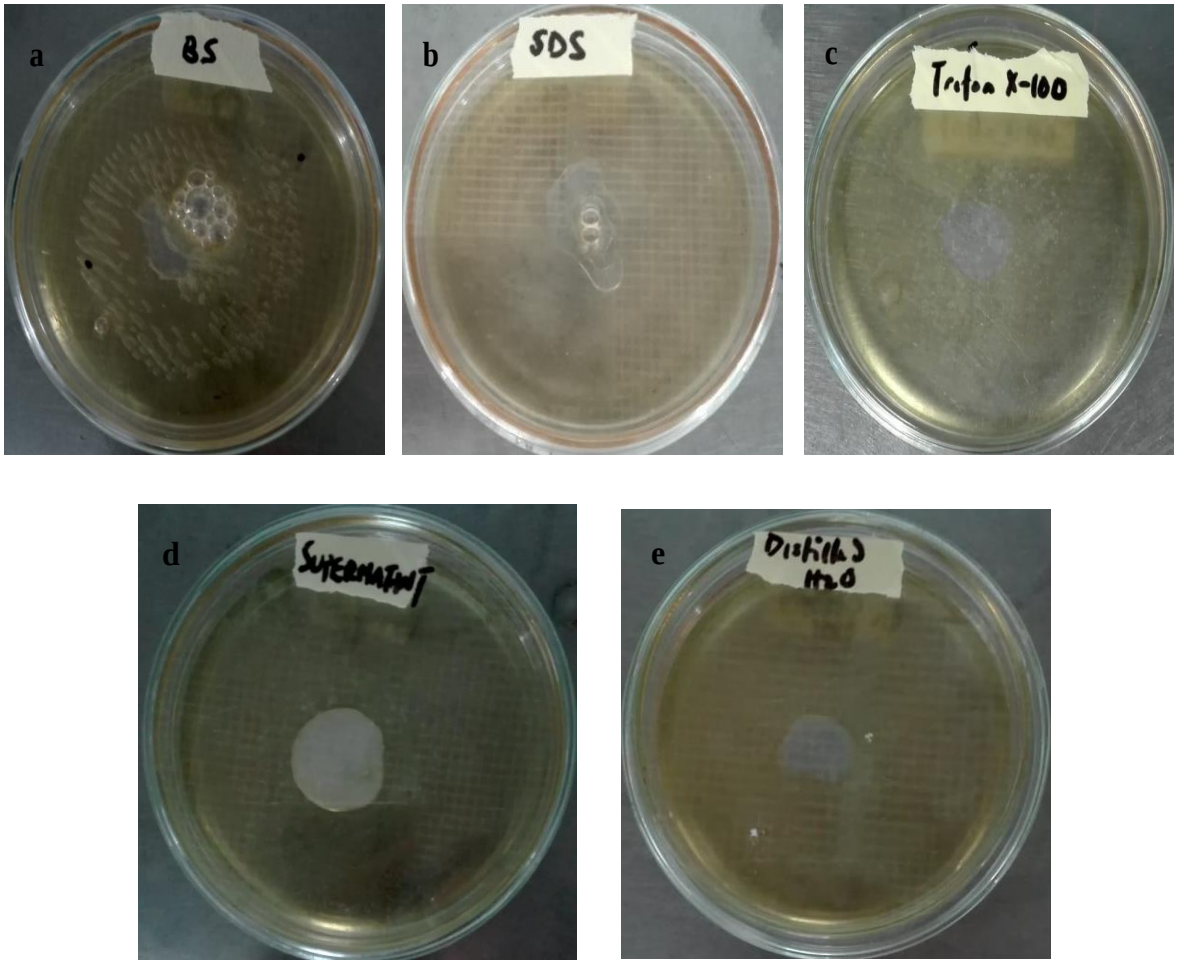


**Figure S1** The illustrations of engine oil dispersion after the addition of biosurfactant (a), chemical surfactants SDS (b), and Triton X-100 (c), supernatant (d), and distilled water (e). The clear zones displayed above indicate the ability to displace oil and are all positive results. The largest and smallest area displaced was obtained in illustration A and E, for biosurfactant and distilled water, respectively.

## Supplementary Information Chapter 8

# S*fp*-type phosphopantetheinyl transferase is indispensable in the biosynthesis of lipopeptide biosurfactant in *Paenibacillus* sp. D9

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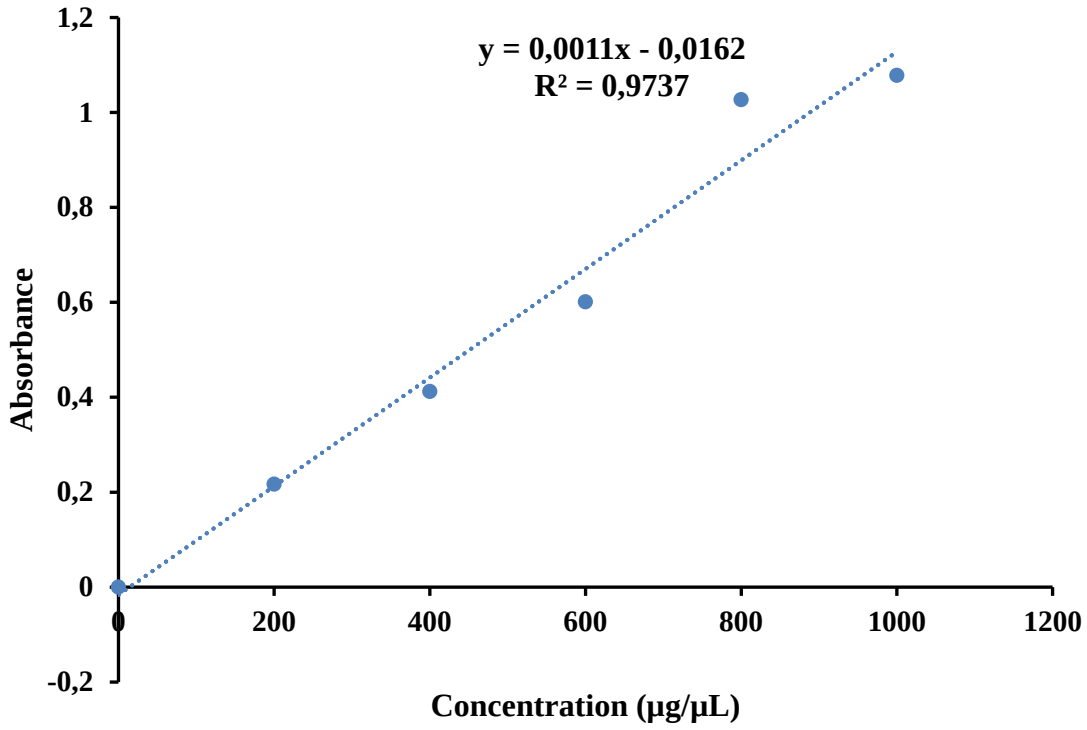


Figure S1 Standard plot of phosphopantetheinyl transferase activity against absorbance

**Table S1**. Coenzyme A concentration activity of purified and crude Sfp phosphopantetheinyl transferase

| $OD_{570nm}$ | OD 1 | OD 2 | OD 3 | Ave | Std | Concentration (U/ml) |
|---|---|---|---|---|---|---|
| Purified Sfp | 1.96 | 1.92 | 1.96 | 1.95 | 0.02 | 1097.46 |
| Crude Sfp | 1.72 | 1.75 | 1.76 | 1.74 | 0.01 | 925.64 |

OD = Optical density