SCREENING FOR BIOSURFACTANT PRODUCTION AMONGST AEROBIC ENDOSPORE FORMING BACTERIA ISOLATED FROM MFABENI PEATLAND SEDIMENT CORE

BY

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Dr. C.H. Hunter (Supervisor)

Date:

DECLARATION

I, Folasade Abimbola Adu, declare that:

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

ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

iii) this dissertation does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons;

iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a) their words have been re-written, but the general information attributed to them has been referenced;

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ABSTRACT

Biosurfactants are surface-active agents that possess amphiphilic properties, which gives them the ability to reduce surface and interfacial tensions. They are produced by a wide range of microbes and are recognized for their industrial and environmental applications. Several classes of biosurfactant have been characterized, with lipopeptide production by members of the genus *Bacillus* being recognized as being significant. Endospore-formers within the order *Bacillales* can be considered to be a potential source of novel biosurfactants. A study was undertaken with the aim of screening for biosurfactant activity amongst aerobic endospore-forming bacteria (AEFB) isolated from Mfabeni peatland, St Lucia, KwaZulu-Natal, South Africa. This site has functioned continuously as a wetland since before the Holocene (>48 000 years) and represents an important ecosystem that has not been explored from a microbiological perspective. The isolates screened in this study were previously isolated from sections of a sediment core, which were radiocarbon dated from ca. 589 – 37,906 cal years BP. Eighty-two isolates were screened for biosurfactant activity using the hemolysis, drop collapse, and oil spreading assays. The oil-spreading assay was found to be the best method for assaying biosurfactant activity based on ease of use, sensitivity, and ability to give a clear difference between positive and negative results. Approximately 87% of isolates were judged to exhibit biosurfactant activity using this screening method. Isolates were further evaluated to determine the effect of pH (3.0 – 10.0), temperature (35^{o} – 100^{o}C) and salinity (0.5 – 15 %) on biosurfactant stability in cell-free culture supernatants (Tryptic soy broth). A surfactin producing isolate, *Bacillus velezensis* R16, was included in the assays for comparative purposes. Biosurfactant activity remained fairly thermostable in most instances over the temperature range tested. Under acidic conditions (pH 3 and 5.5) it was evident from the controls that the constituents of the TSB culture medium interfered with the oil spreading assay and no valid conclusions could be made. At pH 7 – 10 biosurfactant activity remained mostly consistent. Increasing salinity concentration had the most significant effect on biosurfactant activity leading to decreases in oil displacement activity for a number of the isolates. Eight isolates (viz., SAB19, SAB42, SAC15, SAC18, SAD5, SAD17, SAD18, and SAD23) exhibited promising biosurfactant activity over the different environmental parameters tested and were selected for further characterization and identification.

Emulsification (E24) efficiency tests using sunflower seed oil and paraffin oil ranged from 19.5% up to 61.85%. Using a Du-Nouy tensiometer it was established that isolates were able to reduce surface tension of culture medium from 57.3 mN/m to between 44.7 and 30.6 mN/m. Surfactant lipopeptides were extracted from isolates cultured in Landy medium, using acid precipitation followed by methanol extraction. Extracts were partially purified using thin layer chromatography (TLC) and hydrophobic fractions were characterized using liquid chromatography in conjunction with electrospray-ionization time-of-flight mass spectrometry. The mass peaks detected by the UPLC-ESI-TOF MS were identified based on comparison to surfactin and iturin standards as well as a lipopeptide profile obtained from the *B. velezensis* R16 reference strain. All of the Isolates produced surfactin homologs as well as a hydrophobic compound (*m/z* 1326.1) that was putatively assigned as a precursor of the antibiotic Plantazolicin (PZN). A number of isolates also produced homologs of iturin/bacillomycin and/or fengycin lipopeptides. REP-PCR genomic fingerprinting allowed isolates to be differentiated at the strain level, with several groups of closely related strains being distinguished. Taxonomic classification revealed that the isolates could be separated into two genera namely *Bacillus* and *Brevibacillus*. The *Bacillus* spp. isolates showed high levels of 16S rRNA gene sequence similarity (>99%) to members of the "*B.amyloliquefaciens* Operational Group" of related organisms; whereas the *Brevibacillus* isolates showed high levels of sequence similarity (>99%) to strains of *Brev. brevis* and *Brev. formosus.* Promising biosurfactant producers were isolated amongst the AEFB isolates screened in this study. However, novel biosurfactant was not identified.

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INTRODUCTION

Biosurfactants are surface-active compounds that are able to reduce surface and interfacial tensions (Desai and Banat, 1997; Mulligan, 2005). Their scope of application includes use as wetting agents, emulsifiers, foaming agents, detergents and dispersants. In recent years they have gained favor over synthetic surfactants because of their ecological acceptability due to reduced toxicity, higher biodegradability and stability over a range of environmental conditions (Desai and Banat, 1997; Lima *et al*., 2011). Increasingly, biosurfactants are being recognized for their industrial and environmental applications, which include bioremediation, decontamination of manufacturing wastes, clearing of oil spills, and microbial enhanced oil recovery (Joshi *et al*., 2012).

Biosurfactants are produced by a range of bacteria and fungi with several classes of biosurfactant compounds having been distinguished (Mata-Sandoval *et al.,* 1999; Mata-Sandoval *et al.,* 2001; Chen *et al.,* 2007a). Various classes or categories of biosurfactant are recognized, which include lipopeptides, mycolic acid, glycolipids, lipopolysaccharides, and phospholipids (Smyth *et al.,* 2010). The interest in biosurfactants has resulted in a growing number of studies that are focused on screening for novel biosurfactant compound producers, screening for strains with enhanced biosurfactant production capabilities, and, screening for biosurfactant compounds that can function over a wide range of environmental conditions.

Wetlands and peatlands are ecosystems which support a diverse array of aerobic and anaerobic microbial communities that play important roles in the recycling of organic matter. Mfabeni Peatland, located within the St. Lucia Wetland Park, KwaZulu-Natal is regarded as one of the most significant peatland ecoregions in South Africa (Grundling *et al*., 2013). The diversity and functioning of microbes within this ecosystem is largely unexplored and represents an untapped source of microbial diversity with potential biotechnological applications (Naidoo, 2017). Aerobic endospore-forming bacteria (AEFB) are of special interest due to their association with the production of industrially significant enzymes and bioactive chemicals such as antibiotics, biopesticides and biosurfactants (Couto *et al*., 2015). Peatlands are associated with an accumulation of partially degraded plant material that builds up over time as the result of anoxic

conditions that arise under waterlogged conditions. Under these conditions endospores from AEFB may become trapped within layers of accumulating organic material. Peatland sediment therefore, has the potential to serve as an archival record of AEFB diversity from this environment (Naidoo, 2017).

A study was undertaken with the aim of screening for biosurfactant activity amongst a phylogenetically diverse collection of aerobic endospore-forming bacteria (AEFB) isolated previously from sections of a sediment core taken from Mfabeni peatland. These samples were taken from radiocarbon dated sections of the core which were dated from ca. 589 to 37,906 cal years BP.

The objectives of the study were:

i) To screen for biosurfactant production amongst aerobic endospore forming bacteria from a subset of isolates obtained from a Mfabeni peatland sediment core;
ii) To determine the effect of environmental parameters namely, temperature, pH, and salt concentration on the activity and efficacy of biosurfactants produced by AEFB;
iii) To extract, partially purify and characterize biosurfactant compound(s) produced by selected AEFB using acid precipitation followed by methanol extraction, Thin Layer Chromatography (TLC) and reverse phase Ultra Performance Liquid Chromatography (UPLC) used in conjunction with Electrospray Ionization-Mass Spectrometry (ESI-MS);
iv) To differentiate and classify selected AEFB isolates using REP-PCR genomic fingerprinting and 16S rRNA gene sequence analysis.

This dissertation has been divided into four chapters. Chapter one is a literature review that provides an overview of biosurfactants, specifically focusing on those produced by *Bacillus* spp.. Chapters two and three cover specific aspects of the research undertaken in the study and are each presented in the format of an independent scientific paper. Chapter four provides a summary of the major findings and highlights their significance.

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Microbial surfactants, or biosurfactants, are surface active, amphiphilic, low molecular weight compounds produced by a wide range of different microorganisms (Banat, 1995; Padmavathi and Pandian, 2014). Biosurfactants act by lessening the interfacial and surface tensions between particles in two liquids or between a liquid and a solid (Banat *et al.,* 2000). Lipopeptides, fatty acids, polysaccharides, glycolipids, phospholipids, lipoproteins, neutral lipids, and polymerics are examples of biosurfactants that have been distinguished (Neu, 1996; Muthusamy *et al.,* 2008). Biosurfactants are favored over synthetic surfactants because of their unique properties such as reduced toxicity, higher biodegradability, ecological acceptability, and stability under adverse conditions (Desai and Banat, 1997; Lima *et al*., 2011).

Biosurfactants are increasingly regarded as having commercial value due to a range of potential applications such as; acting as wetting agents, emulsifiers, foaming agents, detergents, and dispersants. They are applicable in a wide range of industries including cosmetics, petroleum, food processing, agricultural, and pharmaceutical industries (Banat *et al*., 2000). They are also suitable for use in oil recovery and in the management and remediation of contaminated sites (Banat, 1995; Al-Sulaimani *et al*., 2011). They have also been investigated for various biomedical applications as well; for example, as antiviral, antifungal, antibacterial, and anti-adhesive agents against a range of drug resistant pathogens (Gudiña *et al.,* 2010; Luna *et al.,* 2011).

Aerobic endospore-forming bacteria (AEFB) are Gram positive, rod-shaped organisms, suited to culturing on a large scale and are mostly non-pathogenic (Fritze, 2004; Neves *et al*., 2007). They are amongst the diverse group of microorganisms that produce biosurfactants (Al-Bahry *et al*., 2013; Couto *et al*., 2015). This group of bacteria include members of the genus *Bacillus* and are regarded as a promising group of bacteria because of their biotechnological potential (Mandic-Mulec and Prosser, 2011; Al-Bahry *et al*., 2013; Couto *et al*., 2015).

Lipopeptides are one of the most widely studied classes of biosurfactant associated with AEFB. Structurally, they are made up of a lipid tail joined to a short linear or cyclic oligopeptide. In addition to their surfactant properties, they are known for their antimicrobial, antitumor, cytotoxic, and immunosuppressant properties (Cameotra and Makkar, 2004; Donadio *et al.,* 2007; Gross and Loper, 2009; Pirri *et al.,* 2009). Surfactin, a lipopeptide synthesized by *Bacillus subtilis,* is regarded as one of the most effective biosurfactant discovered to date (Arima *et al.,* 1968; Gudiña *et al.,* 2013). Other examples of lipopeptides produced by *B. subtilis* include fengycin, mycosubtilin, iturin, and bacillomycin (Vater *et al.,* 2002).

Considering the advantages that microbial surfactants have over artificial surfactants, there is a need to screen and identify novel biosurfactant producers, and also identify AEFB strains that have enhanced biosurfactant production capabilities.

1.2 PRODUCERS OF BIOSURFACTANTS: - AEROBIC ENDOSPORE-FORMING BACTERIA (AEFB)

The genus *Bacillus* was established in 1872 by Cohn and is amongst the first set of bacteria to be described. They have been isolated from various environments, which includes soil, sea water, dust, and even ocean basin cores (Singh *et al*., 2007; Wilson *et al*., 2008; Aislabie *et al*., 2009; Teixeira *et al*., 2010; Vollú *et al*., 2014). The ability of AEFB to produce spores makes isolation, cultivation, and maintaining them in the laboratory relatively easy. Dormant endospores are readily dispersed, this allows them to be distributed widely and colonize a wide range of habitats (Wipat and Harwood, 1999).

AEFB have adapted to a broad range of environmental conditions and are able to inhabit diverse habitats (Nicholson *et al.,* 2000). For example, members of the *Halobacillus, Thermobacillus,* and *Psychrobacillus* genera have been isolated from halophilic, thermophilic, and psychrophilic areas respectively (Brandes *et al.,* 2011). AEFB have also been isolated from various environments like salt marshes, marine sediment, thermal acid waters, marine sponges, glaciers, wetlands, volcanic soil, and geothermal vents (Margesin and Miteva, 2011; Phelan *et al.,* 2012; Sonalkar *et al.,* 2014; Aanniz *et al.,* 2015). Many of these strains have traits like proteolytic, amylolytic, and

antimicrobial activities which are of biotechnological interest (Phelan *et al.,* 2012; Aanniz *et al.,* 2015).

AEFB are of special biotechnological interest because of their ability to produce industrially significant enzymes and bioactive chemicals, their ability to degrade a range of pollutants, as well as their use as biopesticides. Biosurfactants are among the most significant bioactive chemicals synthesized by AEFB (Couto *et al*., 2015). Biosurfactants are normally produced as secondary metabolites during late exponential growth or stationary phase (Mahdy *et al*., 2012). They may be retained intracellularly or excreted extracellularly into the surrounding medium (Kazim *et al*., 2017). The biosurfactants produced assists in making insoluble substrates available to the microorganism through solubilization and desorption (Viramontes-Ramos *et al*., 2010; Mahdy *et al*., 2012). They also enhance the surface area of hydrophobic surfaces and adjust the joining or removal of microorganisms to surfaces (Saravanan and Vijayakumar, 2015).

Additionally, biosurfactants have been linked to a range of functions that include environmental remediation, and biological control (Ron and Rosenberg, 2001; Mandic-Mulec and Prosser, 2011). Among the biosurfactants produced by the AEFB, surfactin and lichenysin are two well-studied lipopeptide surfactants made by *B. subtilis* and *B. licheniformis* respectively (Sekhon *et al.,* 2012). These compounds are able to function under extreme temperature (e.g. 4°C to 100°C) and pH (e.g. 3 to 10) conditions; these properties have allowed them to be used in a range of diverse applications (Rodrigues *et al.,* 2006; Jacques, 2011; Kaloorazi and Choobari, 2013).

1.3 ECOLOGICAL ROLE OF BIOSURFACTANTS

Biosurfactants have been reported to have a range of ecological roles, which may be specific to the bacteria producing the surfactant (Raaijmakers *et al*., 2010). Some of these roles are discussed in the following sections.

1.3.1 BIOFILM FORMATION

Biofilms are dense collections of bacterial cells that form on surfaces as a result of cell division and multiplication. The bacterial cells release proteins that form slime materials which keeps the biofilm together (Stewart and Franklin, 2008). Within biofilms, bacteria are shielded from harsh

environmental conditions such as exposure to antibiotics. Lipopeptide biosurfactants synthesized by *Bacillus* species have been associated with biofilm formation and attachment to surfaces. It has been suggested that biosurfactants enhance the circulation of oxygen and food by maintaining liquid-filled channels in biofilms (Davey *et al.,* 2003; Klausen *et al.,* 2003). Hofemeister *et al*. (2004) demonstrated that *B. subtilis* strain AI/3 needed surfactin to be able to develop pellicles, and biofilms.

1.3.2 MOTILITY

Twitching, swarming, and swimming are the main ways by which bacteria are able to move on surfaces (Henrichsen, 1972). During the swarming and swimming mode of movement, bacteria merge their flagella, and are thrust forward as a result of the rotation of the flagella (Harshey, 2003). Bacterial cells locomote in clusters for swarming but singly in the case of swimming (Raaijmakers *et al*., 2010). Biosurfactants can influence cell movement due to their ability to modify the thickness of surface layers (Lindow and Brandl, 2003; Raaijmakers *et al*., 2010). It is believed that lipopeptides participate in cell aggregation and in the coordination of their movement (Raaijmakers *et al*., 2010). They also assist their producers in moving to locations on plant surface that are rich in nutrient by acting as wettability agents (Lindow and Brandl, 2003; Nielsen *et al.,* 2005).

1.3.3 ANTAGONISM

It has been observed that microorganisms which produce lipopeptide biosurfactants have better competitive benefits compared to other microorganisms (Raaijmakers *et al*., 2010). These biosurfactants inhibit growth and cause lysis of a wide array of microorganisms such as oomycetes, viruses, fungi, and bacteria (Raaijmakers *et al*., 2010). Surfactin has been found to deactivate viruses through the disruption of the viral constituents. It also inhibits various animal and human pathogens by forming pores in their membranes causing cellular disruption (Vollenbroich *et al.,* 1997; Huang *et al.,* 2006). Lipopeptides are also effective against fungi; for instance, fengycin is known for its antifungal activity against *Botrytis cinerea* and *Fusarium graminearum* (Romero *et al.,* 2007; Wang *et al.,* 2007) while iturin is effective against *Rhizoctonia solani* and *Penicillium roqueforti* (Yu *et al.,* 2002; Chitarra *et al.,* 2003).

1.4 TYPES OF BIOSURFACTANTS

Biosurfactants have been classified based on the kind of charge present on each moiety which includes; positively charged cationic biosurfactants, negatively charged anionic surfactants, amphoteric surfactants, and non-ionic biosurfactants (Ginkel, 1989; Rahman and Gakpe, 2008). Biosurfactants have also been categorized based on their molecular weight and chemical composition. These include low molecular weight and high molecular weight biosurfactant compounds. The low molecular weight compounds are generally active at reducing interfacial and surface tensions; the most widely studied of which are the lipopeptides and glycolipids (Rahman and Gakpe, 2008). Examples of lipopeptides include surfactin, iturin, fengycin, and bacillomycin, while the glycolipids comprise the sophorolipids, rhamnolipids, and trehalolipids (Ron and Rosenberg, 2001). High molecular weight compounds, such as bioemulsan, are the most efficient stabilizing agents and are effective in steadying oil in water colloids (Dastgheib *et al.,* 2008; Salihu *et al.,* 2009). In this literature review, the focus is on lipopeptides because they are one of the most widely studied classes of biosurfactant associated with *Bacillus* species.

1.4.1 LIPOPEPTIDES

Lipopeptides are a well-known group of biosurfactants synthesized by *Bacillus* species (Perfumo *et al.,* 2010). They exhibit wide-ranging activities which include antimicrobial, antifungal, and antitumoral characteristics (Donadio *et al.,* 2007; Perfumo *et al.,* 2010). Lipopeptides have the ability to form pores in cell membranes, which causes membrane imbalance and death in sensitive organisms (Bender *et al.,* 1999; Baltz, 2009).

Lipopeptides are made up of a cyclic oligopeptide moiety attached to a lipid tail (Stein, 2005; Perfumo *et al.,* 2010; Raaijmakers *et al.,* 2010). These lipopeptides have been divided into four classes namely; surfactins, iturins, fengycins, and kurstakins (Hathout *et al.,* 2000; Ongena and Jacques, 2008). Surfactins consists of surfactin, esperin, pumilacidin, and lichenysin, whereas the Iturin family consists of mycosubtilin, iturin A, A_L, and C, and bacillomycin D, F, L, and LC. The fengycin family consists of fengycin A and B, and plipastatin A and B (Ongena and Jacques, 2008). A fourth class- the kurstakins were originally isolated from strains of *B. thuringiensi*s and little is

known about their ecological significance or applicability (Hathout *et al.,* 2000; Béchet *et al.,* 2012).

1.4.1.1 SURFACTIN

Surfactin is a cyclic lipoheptapeptide connected to a hydrophobic β-hydroxy fatty acid side chain (Figure 1.1) (Seydlová *et al.,* 2011). Based on the different amino acid sequence, surfactin has been categorized into A, B, and C groups (Rodrigues *et al.,* 2006; Korenblum *et al.,* 2012). Normally, several surfactin isoforms with fatty acid side chains of varying carbon chain length (13 to 15 carbon) can be found together within a mixture of various peptide variants in cells (Tang *et al.,* 2007). The structure and composition of the fatty acid side chain and the amino acids in surfactin molecule depends on both the culture conditions and the producer strain (Seydlová *et al.,* 2011). Surfactins are known to possess antiviral, antifungal, antimicrobial, antibacterial, and antitumor abilities which makes them of interest for use in medical, and environmental applications (Kim *et al.,* 1998; Ahimou *et al.,* 2000; Nitschke and Costa, 2007; Mulligan, 2009; Banat *et al.,* 2010).

Surfactants, such as surfactin can be used as detergents and soaps (Heerklotz *et al*., 2004; Dufour *et al*., 2005). Several mechanisms have been proposed to describe the molecular mechanism involved in the disruptive action of surfactants (Deleu *et al*., 2003). In the first instance, surfactin is thought to act as a cation-carrier that is able to carry cations through an organic barrier. The fatty acid portion of surfactin penetrates a phospholipid bilayer to interact with the acyl chain of phospholipid. The heptapeptide headgroup aligns with the polar head region of the phospholipids. When a cation, such as calcium ion forms a complex with the surfactin molecule, it causes a 'flip-flop' response whereby the surfactin reorientates itself across the lipid bilayer (Heerklotz *et al*., 2004). The second explanation is that surfactin form pores, or cationic channels across a phospholipid membrane. This then leads to an osmotic imbalance, which causes disruption of the lipid membrane (Deleu *et al*., 2003). The third hypothesis describes the detergent effect of surfactin being a result of its fatty acid chain inserting and causing disruption or disorganization of the phospholipid bilayer (le Maire *et al*., 2000). This effects membrane

permeability and can lead to membrane solubilization. The formation of mixed micelles can also occur (Kragh-Hansen *et al*., 1998).

Surfactins are synthesized by a surfactin synthetase enzyme, which is a non-ribosomal peptide synthetase (Hue *et al*., 2001). Surfactin is considered to be a potent biosurfactant. At a concentration of 20 µM, it is able to lower the surface tension of water from 72 mN/m to 27 mN/m (Yeh *et al*., 2005). Water tension is lowered by the surfactant molecule occupying the intermolecular space between water molecules; and reducing the forces of attraction between these molecules. This creates a more fluid solution, which increases the wetting ability of the water (Dufour *et al*., 2005).

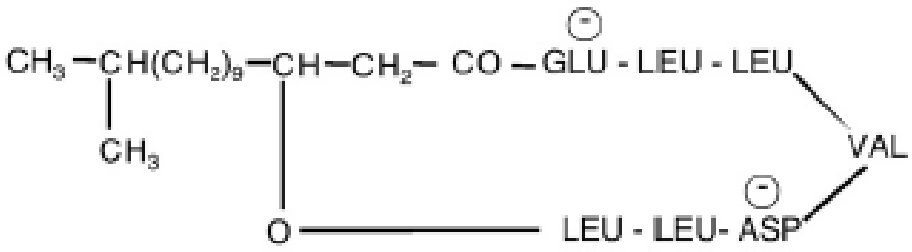

Figure 1.1: Cyclic structure of surfactin. It contains fatty acid chain with 13 to 15 carbon length (Mulligan, 2009)

1.4.1.2 ITURIN

Iturin is a cyclic heptapeptide connected to a β-amino fatty acid chain of varied length, ranging from 14 to 17 carbon (Figure 1.2) (Tsuge *et al.,* 2001; Meena and Kanwar, 2015). It is produced by a number of *Bacillus* spp. that fall within the *B. subtilis* complex of closely related taxa (Tsuge *et al.,* 2001). They are potent antifungal agents which makes them potential candidates for use as biopesticides (Vater *et al*., 2002; Romero *et al*., 2007; Pecci *et al*., 2010). The cytoplasmic membrane of the fungi is penetrated by the hydrophobic part of iturin which causes pore formation and leads to osmotic disruption of the cell cytoplasm (Stein, 2005).

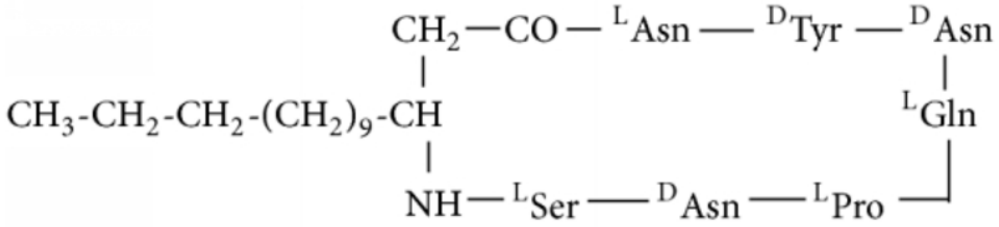

Figure 1.2: Cyclic structure of Iturin. It contains fatty acid chain with 14 to 17 carbon length (Meena and Kanwar, 2015)

1.4.1.3 FENGYCIN

Fengycin is a cyclic lipodecapeptide which is connected to a β-hydroxy fatty acid of varying chain length (16 to 19 carbons) (Steller and Vater, 2000; Wei *et al*., 2010). Fengycin is an antifungal agent and is particularly effective against filamentous fungi (Steller and Vater, 2000; Deleu *et al.,* 2008). Two groups of fengycin have been distinguished, Fengycin A and Fengycin B. Fengycin A possesses the amino acid alanine (Ala) at position 6 of the oligopeptide structure while Fengycin B has valine (Val) at this position (Steller and Vater, 2000; Meena and Kanwar, 2015).

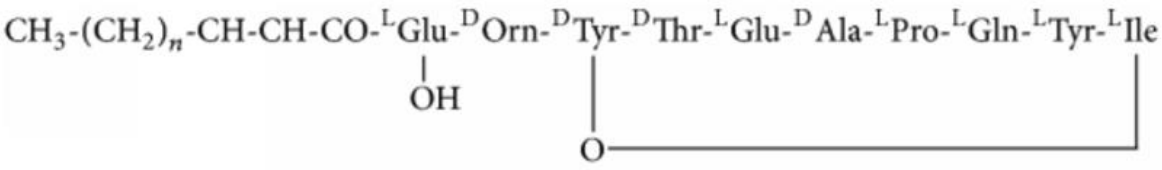

Figure 1.3: Cyclic structure of fengycin. It contains fatty acid chain with 16 to 19 carbon length (Meena and Kanwar, 2015)

1.5 FACTORS AFFECTING PRODUCTION OF BIOSURFACTANTS

Environmental, and nutritional factors are the main factors which affect the production of biosurfactants (Rahman and Gakpe, 2008). Not only do these factors determine the quantity of biosurfactant produced but they can also influence the type produced (Salihu *et al.,* 2009).

1.5.1 ENVIRONMENTAL FACTORS

Environmental factors like pH, temperature, salinity, oxygen availability, and growth conditions impact production of biosurfactant (Desai and Banat, 1997; Rahman *et al.,* 2002; Ilori *et al.,* 2005; Raza *et al.,* 2007). For instance, oxygen transfer was found to be one of the major limiting factors for scaling up production of surfactin in *B. subtilis* (Sheppard and Cooper, 1990). The presence or absence of glucose as a carbon source in a growth medium can also determine the availability and amount of biosurfactant produced (Walter *et al*., 2010). *Bacillus* species have been reported to be able to produce biosurfactants at temperatures up to 100°C, pH up to 10, and at salinity concentrations up to 10% (Desai and Banat, 1997; Echigo *et al*., 2005; Márquez *et al*., 2011; Md, 2012; Varadavenkatesan and Murty, 2013; Liang *et al*., 2017).

For application in microbial enhanced oil recovery (MEOR), surfactant producing bacteria must have the capacity to withstand inhospitable conditions such as low oxygen, high temperature, salinity, and pressure (Couto *et al*., 2015). Alternatively, they should produce stable biosurfactant compounds that can function under a range of adverse conditions. Biosurfactant producers which meet these criteria have the potential to be used in enhancing bioremediation and in the clean-up of oil spills (Sekhon *et al*., 2012).

1.5.2 NUTRITIONAL FACTORS

Several carbon substrates have been used for biosurfactant production such as glycerol, glucose, and crude oil (Desai and Banat, 1997; Ilori *et al.,* 2005; Rahman and Gakpe, 2008). The kind, and amount of biosurfactant produced depends largely on the concentration of the carbon source used (Zajic and Donaldson, 1985; Rahman and Gakpe, 2008; Raza *et al.,* 2007). For example, *Pseudomonas aeruginosa* produced higher yields of biosurfactant (100 – 165 mg g^{-1}) when supplied with long chain alcohols, corn oil, and lard compared to succinate acid and glucose, which produced lower yields (12 – 36 mg g^{-1}) (Mata-Sandoval *et al.,* 2001). This isolate was also able to produce rhamnolipid from a range of carbon substrates, which include C11 and C12 alkanes, olive oil, glycerol, succinate, glucose, and fructose (Rahman and Gakpe, 2008). In a study carried out by Saharan *et al*. (2011), biosurfactant production by *B. subtilis* MTCC 2423 was

enhanced after supplementing culture media with beef extract, sucrose, sodium pyruvate, and glucose as carbon source.

1.6 APPLICATIONS OF BIOSURFACTANTS

Biosurfactants are useful to man and have found application in a wide range of industries due to their emulsification, foaming, surface tension reduction, lubrication, and moisture retention properties (Desai and Banat, 1997; Barros *et al*., 2008; Banat *et al*., 2010; Damasceno *et al.,* 2012). Currently, the need for surfactants has largely been met by synthetic surfactants which in many instances are not only harmful but are also often non-biodegradable; these limitations have heightened the demand for natural surfactants.

1.6.1 BIOSURFACTANTS IN MEDICINE

The establishment of bacterial biofilms on surfaces are potentially important sources of nosocomial infections (Singh and Cameotra, 2004). Significantly, bacteria found in biofilms can exhibit increased tolerance to antibiotics; they are also effective in avoiding host defense mechanism. Factors such as swarming, motility, and biofilm formation are important determinants of the ability of a bacterium to colonize surfaces. Biosurfactants such as surfactin have anti-adhesive properties, which hinders the binding of pathogenic microorganisms to surfaces or infection sites. Surfactin has been shown to reduce the amount of biofilm formed by strains of *Escherichia coli*, *Salmonella enterica*, and *Proteus mirabilis* (Seydlová and Svobodová, 2008). In controlled laboratory studies, coating catheters by running them through a solution of surfactin was found to reduce biofilm formation. The coating of catheters with surfactin has been proposed as a means to limit biofilm development by potential pathogens (Seydlová and Svobodová, 2008).

Surfactin has also been identified as an anti-inflammatory agent because of its ability to hinder inflammation in eukaryotic cells, which occurs as a result of the interaction between these cells and lipopolysaccharide (Seydlová and Svobodová, 2008; Kim *et al*., 2006). However, intravascular use of surfactin is not recommended due to its non-specific cytotoxic effect on cell membranes

(Seydlová and Svobodová, 2008). Concentrations below 25 µM were not found to be disruptive to cell membrane (Seydlová and Svobodová, 2008).

1.6.2 BIOSURFACTANTS AND MICROBIAL CONSORTIA

It has been shown that the use of microbial consortia can improve biodegradation efficacy compared to monocultures (Kadali *et al.,* 2012). This has been attributed to mutually beneficial interactions, which can have a positive influence on growth and survivability (Sampath *et al.,* 2012). For example, the addition of rhamnolipid biosurfactants has been shown to improve the biodegradation efficiency of slow degrading consortia. Conversely, biosurfactants can have an opposite effect whereby they cause a significant decrease in the biodegradation rate associated with fast degrading consortia (Owsianiak *et al*., 2009). This phenomenon is attributed to differences in substrate uptake modes. In instances where hydrocarbon uptake occurred directly from the aqueous phase, the addition of surfactants was thought to increase solubilization of hydrocarbons and improve the biodegradation. Conversely, in instance where high initial rates of biodegradation of hydrocarbons occurred at the interface boundary with a biofilm, the introduction of surfactants could potentially restrict the contact between microorganisms and substrates, thereby negatively effecting biodegradation rate (Owsianiak *et al.,* 2009).

1.6.3 BIOSURFACTANTS IN AGRICULTURE

Several biosurfactants such as iturin A, fengycin, and surfactin demonstrate antimicrobial activity against fungal and bacterial plant pathogens and can be regarded as promising biocontrol agents suited to sustainable agricultural practices (Sachdev *et al*., 2013). For instance; surfactants produced by *Bacillus* spp. have been reported to show biocontrol actions against several plant pathogens, which include *Dickeya* and *Pectobacterium* spp. which cause soft rots, *Colletotrichum gloeosporioides* which causes anthracnose on papaya leaves and *Fusarium* spp. which cause damping off of vegetable seedlings (Kim *et al.,* 2010; Velho *et al.,* 2011; Eddouaouda *et al.,* 2012; Krzyzanowska *et al.,* 2012).

Biosurfactants can also be applied to improve antagonistic action of microorganisms and their associated microbial products (Jazzar and Hammad, 2003; Kim *et al*., 2004). For example, weed species have been eliminated by spraying the plants with a combination of biosurfactants (0.2%)

and *Myrothecium verrucaria* (2.0 x 10^7 conidia ml^{-1} at 300 L ha^{-1}) (Boyette *et al*., 2002; Hoagland *et al*., 2007). Thus, these microbial surfactants and/or their producers are potential eco-friendly alternatives for environmentally hazardous chemical insecticides and pesticides used in agriculture (Sachdev *et al*., 2013).

1.6.4 BIOSURFACTANTS IN FOOD INDUSTRY

In the food industry, emulsification is of great importance in influencing the solubilization of odor producing compounds, as well as the texture and consistency of food products (Radhakrishnan *et al.,* 2011). Emulsifiers control clustered globules and keeps activated systems steady so as to stabilize emulsions (Nitschke and Costa, 2007; Patino *et al.,* 2008). An emulsion is a varied system comprising of an immiscible liquid that is distributed into another liquid, in droplet form (Nitschke and Costa, 2007). Biosurfactants are able to improve the consistency of such systems by decreasing the interfacial tension, which in turn decreases the surface energy between different phases (Nitschke and Costa, 2007; Muthusamy *et al.,* 2008). These surfactants can also alter rheological properties and influence the shelf life of products (Nitschke and Silva, 2018).

The emulsification index also known as E24 is a fast and qualitative method used to ascertain the emulsifying properties of a biosurfactant (Desai and Banat, 1997). In a recent study done by Mnif *et al*. (2012), the addition of a lipopeptide biosurfactant (0.075% concentration) was found to improve the volume and structure of bread crumbs when compared to soya lecithin. The biosurfactant also decreased staling and multiplication of microbes as well as improve texture of the bread after eight days (Mnif *et al*., 2012). Addition of 0.1% lipopeptide has also been reported to improve the adhesive and cohesive texture of cookie dough (Zouari *et al*., 2016).

1.7 PRODUCTION OF BIOSURFACTANTS

Although biosurfactants have many industrial benefits and attributes compared to their chemical counterparts, producing these on an industrial scale has not been attempted fully because of high costs (Deleu and Paquot, 2004). The use of renewable, low-cost waste materials such as molasses, cassava wastewater, grape pomace, starch rich wastes and wastes from oil refineries have been investigated as potential solutions to this challenge (Makkar and Cameotra, 2002; Nitschke and Pastore, 2006; Rivera *et al.,* 2007; Sobrinho *et al.,* 2008; Saharan *et al*., 2011).

Dubey and Juwarkar (2001) were able to produce biosurfactant from *Pseudomonas aeruginosa* using a synthetic medium supplemented with hexadecane and glucose. Other low-cost materials, which are also easily assessible include vegetable oils and their wastes e.g. soybean and sunflower, marine oils, and tallow (Pekin *et al.,* 2005). These oils are efficient in promoting the production of biosurfactant during microbial growth (Rahman *et al.,* 2002; Bednarski *et al*., 2004).

Potatoes are a major source of starch like substances and have been used for biosurfactant production (Saharan *et al*., 2011). They also provide a good source of vitamins, sulphur, and nitrogen for growth (Saharan *et al.,* 2011). For example, *B. subtilis* 21332 is able to produce surfactin when cultured on potato wastewater whether it was either supplemented with trace element or not (Thompson *et al*., 2000; Noah *et al*., 2005). Carbohydrate rich effluents from cassava industries have also been shown to induce surfactin production in *B. subtilis* (Nitschke and Pastore, 2006).

1.8 OPTIMIZATION OF BIOSURFACTANT PRODUCTION

The optimization of biosurfactant production is an area of study that has received growing attention in recent year (Sarubbo *et al*., 2001; Sahoo *et al*., 2011; Liu *et al*., 2012; Kazim *et al.*, 2017; Moshtagh *et al*., 2018). The type, quantity, and quality of biosurfactant produced is dependent on a range of cultural conditions, which include; temperature, agitation, pH, aeration, nitrogen source, metal ion concentration, as well as the type of carbon sources used (Saharan *et al.,* 2011). The process conditions for biosurfactant production also need to be improved in order to increase yields to make production commercially viable (Dubey and Juwarkar, 2001; Hewald *et al.,* 2005; Saharan *et al.,* 2011). For example, addition of iron (2.0 mM) and manganese (0.2 mM) to a growth medium was found to enhance biosurfactant production (up to 4.8 g/l) by *B. subtilis* (Gudiña *et al*., 2015).

Changing one variable at a time while keeping the others constant has been used as a traditional way of optimizing bioprocesses (Saharan *et al.,* 2011). More recently, response surface methodology (RSM), a statistical optimization tool has been developed for process optimization (Saharan *et al.,* 2011). This tool explores the relationships between selected explanatory variants

and one or several response variables (Saharan *et al.,* 2011). RSM has been used to optimize inoculum, pitching rates, substrate concentration as well as environmental conditions in order to improve surfactin production by *B. subtilis* (Sen and Swaminathan, 2004). It has also been used for the enhancement of biosurfactant production by *Pseudomonas aeruginosa* AT10, *Lactococcus lactis, Streptococcus thermophilus,* and *B. licheniformis* (Abalos *et al.,* 2002; Rodrigues *et al.,* 2006). Optimization methods assist industries in selecting and formulating the best mixture of inexpensive substrates for media production and in employing the most beneficial environmental conditions for enhanced production of biosurfactant (Saharan *et al.,* 2011).

1.9 METHODS FOR DETECTING BIOSURFACTANT PRODUCTION

A number of screening methods have been developed to detect biosurfactant production amongst microorganisms (Walter *et al*., 2010). Assays such as the Du-Nouy ring tensiometer assay, axisymmetric drop shape assay, and pendant drop shape assay measure the surface and interfacial tensions of biosurfactants directly; whereas the drop collapse, oil spreading, emulsification capacity, hydrophobic interaction chromatography (HIC) and microplate assays give an indirect measurement of surfactant production (Walter *et al.,* 2010). Hemolysis and cetyltrimethylammonium bromide (CTAB) assays are regarded as specialized screening methods that are used to screen for specific types of surfactant and they are not appropriate for a general biosurfactant screening approaches (Walter *et al.,* 2010).

1.9.1 OIL SPREADING ASSAY

The oil spreading assay, also known as oil displacement test, relies on the displacement of oil by the biosurfactant (Morikawa *et al.,* 2000). A fixed volume of oil is added to distilled water in a petri dish and an aliquot of cell-free supernatant containing the suspected biosurfactant is then added to the middle of the oil droplet (Morikawa *et al.,* 2000; Walter *et al.,* 2010). Displacement of the oil as indicated by a zone of clearing is considered to be a positive indication of the presence of surfactant compounds (Morikawa *et al.,* 2000; Walter *et al.,* 2010). The diameter of the displacement corresponds to the level of biosurfactant activity (Morikawa *et al.,* 2000; Walter *et al.,* 2010). The oil spreading method is simple, fast, requires a small amount of sample and does not require specialized equipment (Płaza *et al.,* 2006). It is also sufficiently sensitive to

detect biosurfactant activity at low concentrations. Several studies have used the oil spreading technique as a reliable means to screen for biosurfactant compound production amongst various microorganisms (Youssef *et al.,* 2004; Płaza *et al.,* 2006).

1.9.2 DROP COLLAPSE ASSAY

The drop collapse assay relies on changes in interfacial tension that occurs to a drop of liquid when exposed to surfactant compounds (Jain *et al*., 1991). A drop of surfactant containing supernatant is added onto a surface already coated with oil. If the supernatant contains surfactant, the drop collapses, increasing in diameter due to a reduction in interfacial tension between the drop and the surface. In the absence of a surfactant, the drop remains stable. This method is simple and fast and does not require large amount of sample.

1.9.3 EMULSIFICATION CAPACITY ASSAY

The emulsification capacity assay, also known as the E24 test, relies on the ability of a surfactant molecule to form a stable emulsion (Cooper and Goldenberg, 1987). This assay is carried out to determine the emulsification property of surfactant compounds. It involves the formation of micelles where hydrophobic liquids become dispersed within hydrophilic liquids (e.g. water), thereby forming an emulsion of two substances, which are normally immiscible (Banat, 1995). Cell-free supernatant is added to oil and vortexed for 2 minutes, after which the mixture is left to stand for 24 h and then the height of the emulsion layer is measured (Cooper and Goldenberg, 1987; Walter *et al.,* 2010). The E24 test determines emulsification activity and correlates to the surfactant concentration. It is calculated by determining the ratio of the height of emulsion layer and the total height of liquid (Cooper and Goldenberg, 1987). The equation used is shown below:

$$E24 = \frac{height\ of\ emulsion\ (hemulsion)}{total\ height\ of\ liquid\ (htotal)} \times 100\%$$

1.9.4 MICROPLATE ASSAY

The microplate assay was developed as a qualitative assay that is used to detect the presence of surfactant compounds and relies on the property of biosurfactants being able to cause visual

distortion in cell-free supernatants (Vaux and Cottingham, 2007; Walter *et al*., 2010)). Cell-free supernatant samples are added to a 96- micro-well plate and then placed on a sheet of grid paper. Each well is viewed from above to determine whether there is biosurfactant present which results in a concave surface, distorting the image of the grid. The microplate test is simple, fast, sensitive, and allows for an instantaneous detection of surface active compounds (Chen *et al.,* 2007b). The method uses small amount of sample, making it suitable for automated high throughput screening (Chen *et al.,* 2007b).

1.9.5 DU-NOUY RING TENSIOMETER ASSAY

The Du-Nouy ring method quantifies the force needed to separate a platinum wire loop from a liquid surface interface (Tadros, 2005). The force exerted is directly proportional to the interfacial tension and is quantified using an automated tensiometer (Walter *et al.,* 2010). The Du-Nouy Ring test has been used extensively to screen microbes for biosurfactant production (Cooper and Goldenberg, 1987; Bodour and Miller-Maier, 1998; Rahman *et al*., 2002; Płaza *et al*., 2006). A culture is regarded as a promising surfactant producer if it is able to reduce the surface tension of a liquid medium to 40 mN/m or less (Cooper and Goldenberg, 1987). Willumsen and Karlson (1996) defined a good biosurfactant producer as a culture that can reduce the surface tension of a growth medium by $\geq$20 mN/m compared to a distilled water control. Although this method is accurate and relatively easy to use, it requires specialized equipment and is limited in that it is unable to measure different samples simultaneously (Walter *et al.,* 2010). Another restriction of this method is that relatively large sample volumes are needed for analysis; these samples may also need to be diluted due to a limited concentration range that can be analyzed (Bodour and Miller-Maier, 1998).

1.9.6 STALAGMOMETRIC ASSAY

The stalagmometric assay is an assay that uses a Traube stalagmometer to measure the surface tension of a liquid (Dilmohamud *et al.,* 2005). A Traube stalagmometer is an instrument that allows for the consistent formation of uniform volumes of liquid droplets and subjects them to gravitational force within a modified capillary tube. The number of drops that fall per unit of volume is compared to a control liquid (i.e. water) of known density and surface tension, in order

to establish the sample density. This information is then used to calculate the surface tension of the sample according to the following equation:

$$\sigma_L = \frac{\sigma_W \cdot N_W \cdot \rho_L}{N_L \cdot \rho_W}$$

Where σ_L is surface tension of the liquid to be examined, σW is the surface tension of the water, N_L is the number of liquid drops from the sample, Nw is the number of water drops, PL is density of the sample, and Pw is density of water (Dilmohamud *et al*., 2005). The disadvantage of this test is that only consecutive measurement can be carried out (Walter *et al.,* 2010).

1.9.7 HEMOLYSIS

Some biosurfactants are able to lyse erythrocytes and this principle is used to detect surfactant using the hemolysis assay (Mulligan *et al.,* 1984). Cell cultures of bacteria are grown on sheep blood agar for 48 hours. A positive result, which is indicated by zone of clearance around the growth is observed for strains that can lyse blood cells. The hemolysis assay is usually used as an initial screening for isolates that can produce biosurfactants (Schulz *et al.,* 1991; Youssef *et al.,* 2004; Płaza *et al.,* 2006). One of the restrictions of this method is that it is not precise, as there are enzymes that can also lyse blood cells. Another limitation is that diffusion of the biosurfactant may be restricted due to physicochemical constraints of the compound; which can hinder the formation of clear zones (Jain *et al.,* 1991). Some studies have demonstrated that certain biosurfactants do not exhibit any hemolytic activity at all and that the method lacks specificity (Schulz *et al*., 1991; Youssef *et al*., 2004; Plaza *et al*., 2006). Hence, this method is prone to giving false negative and false positive results. Mulligan *et al*. (1984) suggested that the blood agar method should only be used as a preliminary screening method and, ideally, should be backed up by other techniques based on surface activity measurements.

1.9.8 CTAB AGAR PLATE

The cetyltrimethylammonium bromide (CTAB) agar plate method is a convenient screening method used for detecting anionic biosurfactants (Siegmund and Wagner, 1991). The organisms

under investigation are grown on a CTAB agar plate comprising of methylene blue and cetyltrimethylammonium bromide. Microorganisms that produce anionic biosurfactants, displace the cationic CTAB which results in dark blue zones forming around the colonies (Walter *et al.,* 2010). This assay has been adopted to evaluate biosurfactant production under various culture conditions such as different temperatures or substrates that are added directly to the agar plate (Walter *et al.,* 2010). A major disadvantage of the CTAB assay is that it is potentially harmful to some microorganism and could hinder growth (Walter *et al.,* 2010). Siegmund and Wagner (1991) have suggested that CTAB could be substituted by another cationic surfactant in order to overcome these limitations.

1.10 METHODS FOR IDENTIFYING AND CHARACTERIZING BIOSURFACTANTS

Various techniques and methods have been developed over the years to identify and characterize biosurfactant compounds ranging from simple colorimetric assays to more complex sequencing and mass spectrometry approaches (Smyth *et al*., 2010). Based on size and structural complexity, low molecular weight biosurfactants tend to be easier to characterize compared to biopolymers. Mass spectrometry is used extensively to characterize novel biosurfactant compounds. After isolating and purifying the surfactant compounds, a mass spectrometer can then be used to determine the molecular mass of the biosurfactant. Other techniques such as peptide sequence determination and fatty acid analysis can then follow (Smyth *et al.,* 2010).

1.10.1 EXTRACTION OF LIPOPEPTIDE BIOSURFACTANTS

Biosurfactants need to be extracted so as to obtain a crude extract (Smyth *et al.,* 2010). This is done by centrifuging the cell culture followed by acid precipitation, after which the crude biosurfactant is extracted using a suitable solvent (Vater *et al.,* 2002).

1.10.2 PURIFICATION OF BIOSURFACTANTS

Crude extracts containing biosurfactants need to be further purified because some impurities might have been extracted along with the targeted biosurfactant. Various approaches or

methods have been used for purifying lipopeptides. These include thin layer chromatography (TLC), and liquid chromatography.

1.10.2.1 THIN LAYER CHROMATOGRAPHY (TLC)

Crude extract is dissolved in suitable solvent and spotted onto the bottom of a TLC plate such as a silica gel 60 plate (Symmank *et al.,* 2002; Vater *et al.,* 2002). After drying, the plate is developed in a suitable solvent mixture and the compounds are allowed to resolve (Symmank *et al.,* 2002). Subsequently, the plate is dried, and separated compounds are detected using various approaches. In some instances, plates are sprayed with 5% sulphuric acid and placed in the oven to visualize spots (Symmank *et al.,* 2002). Alternatively, plates may be viewed under ultraviolet (UV) illumination to detect compounds or even sprayed with atomized water to detect hydrophobic regions associated with amphiphilic compounds

1.10.2.2 HPLC-UV/HPLC-MS

One of the best techniques used for the detection and purification of lipopeptides is high performance liquid chromatography (HPLC) (Aguilar, 2004). Reversed phase chromatography is mostly used because it separates the individual peptides according to their polarity (Symmank *et al.,* 2002; Aguilar, 2004). Separated peaks are then detected by UV absorbance and can be pooled into fraction collectors for further characterization (Aguilar, 2004). The molecular mass of each peak can be detected when a mass spectrometer (MS) is coupled to the HPLC (Aguilar, 2004).

1.10.2.3 MATRIX ASSISTED LASER DESORPTION/IONIZATION-TIME OF FLIGHT (MALDI-TOF) MASS SPECTROMETRY

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a mass spectrometry ionization method which can be used to detect intact compounds (Smyth *et al.,* 2010). This method is effective in identifying and characterizing the secondary metabolites of microorganisms (Erhard *et al*., 1997; Leenders *et al*., 1999). MALDI-TOF MS has been used in different studies to detect lipopeptides (Vater *et al*., 2002; Hotta *et al*., 2011; Debois *et al*., 2014; Mandic-Mulec *et al*., 2015; Ndlovu, 2017). The samples to be examined are added to a matrix and allowed to dry (Smyth *et al.,* 2010). After this, a laser with several grades of gaseous ions

which gives off energy are released. This can then be distinguished in a Time of Flight (TOF) analyzer and observed. The molecular mass of purified lipopeptide biosurfactant and high molecular weight biosurfactant are detected using MALDI-TOF MS. It can also be used for analyzing peptide compounds obtained from protease digestion (Smyth *et al.,* 2010). MALDI-TOF MS has been reported to be a suitable method for screening unknown microorganisms in order to make novel bioactive compounds of interest available to industries (Vater *et al*., 2002).

1.11 CONCLUSION

Biosurfactants have gained increased recognition over time as a result of their amphiphilic properties which confers them with the ability to reduce surface tension. This capability makes them useful in several industries like pharmaceutical, petroleum, agricultural, and food industries amongst others. They are also preferred over synthetic surfactants because they are potentially non-toxic, environmentally friendly, and easily degradable. Screening for, and identifying biosurfactant producing bacteria is warranted in order to search for novel biosurfactant compound producers, to identify strains with enhanced biosurfactant production capabilities, and, to identify biosurfactant compounds that can tolerate extreme conditions.

CHAPTER TWO

PRELIMINARY SCREENING OF BIOSURFACTANT ACTIVITY AMONGST AEROBIC ENDOSPORE-FORMING BACTERIA ISOLATED FROM MFABENI PEATLAND, SOUTH AFRICA

2.1 INTRODUCTION

Aerobic endospore-forming bacteria (AEFB) are a group of Gram-positive, rod shaped, bacteria that fall within the order *Bacillales* in the phylum *Firmicutes* (Fritze, 2004; Singh *et al.,* 2007). AEFB consist of at least twenty-two genera and includes over 200 species (Fritze, 2004; Singh *et al.,* 2007). Characteristically, this group of bacteria form endospores, which helps them to resist unfavorable environmental conditions such as extreme temperatures, arid conditions, and nutrient deficiency (Singh *et al.,* 2007). Members of the AEFB group are recognized for their ability to produce compounds that are of biotechnological interest; examples include antibiotics, biopesticides, plant growth promoters, and biosurfactants (Couto *et al*., 2015).

Biosurfactants are compounds which possess both hydrophobic and hydrophilic properties, which enables them to reduce surface tension at the surface and interface of immiscible liquids, thereby facilitating proper mixing (Desai and Banat, 1997; Mulligan, 2005). They exhibit a number of attributes such as reducing surface tension, providing lubrication, and, acting as wetting and foaming agents (Gautam and Tyagi, 2006; Franzetti *et al.,* 2010). Generally, biosurfactants are considered to be environmentally friendly since they can be degraded by microorganisms and have lower toxicity compared to their chemical counterparts (Desai and Banat, 1997; Kosaric, 2001; Mohan *et al.,* 2006). Biosurfactants have a range of potential environmental applications, which include bioremediation, decontamination of manufacturing wastes, clearing of oil spills, and microbial enhanced oil recovery (Joshi *et al.,* 2012). Ideally, in order to be effective, biosurfactants need to be able to withstand and function over a wide range of environmental

conditions. For this reason, screening for biosurfactant activity amongst AEFB isolates from diverse environments is warranted (Walter *et al.,* 2010).

A wide range of screening methods have been developed to detect biosurfactant production; these include the Du-nouy tensiometer ring method, pendant drop shape technique, stalagmometric method, hemolysis assay, axisymmetric drop shape analysis, drop collapse assay, oil spreading assay, emulsification assay, cell surface hydrophobicity testing, and bacterial adhesion to hydrocarbons assay (BATH) (Walter *et al.,* 2010). Each method varies in terms of ease of use, equipment constraints, sensitivity and whether the data is qualitative and/or quantitative in nature. In this study simple screening methods that allow large numbers of isolate samples to be processed were used and evaluated.

Peatlands are microbiologically diverse environments which experience an accumulation of organic material over time (Gorham *et al*., 2001). The degradation of organic material within this environment supports a diverse array of microorganisms, including AEFB, which may be of biotechnological interest. Mfabeni peatland was therefore chosen as the study site because microbiologically, it has not been explored and, thus, could be a source of novel biosurfactant producers.

The aim of this study was to screen for biosurfactant production amongst a phylogenetically diverse collection of AEFB isolates obtained from Mfabeni peatland, St Lucia, KwaZulu- Natal. A subsequent aim was to determine the effect of environmental parameters namely, temperature, pH, and salinity on biosurfactant activity.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial isolates

The AEFB isolates used in this study were obtained from a culture collection housed within the discipline of Microbiology, School of Life Sciences, University of KwaZulu- Natal (UKZN). The isolates had been revived and isolated from a sediment core taken from Mfabeni peatland, St Lucia, KwaZulu-Natal (Naidoo, 2017). A total of eighty-two isolates were selected for screening purposes (Table 2.1). Isolates were obtained as cryopreserved master-cultures stored at -80℃. The isolates had been isolated from five sample depths (12- 344 cm), which were radiocarbon

dated and were found to range from ca. 589 – 37,906 cal years BP (Table 2.1). The isolates had previously been distinguished genotypically based on REP-PCR fingerprinting (Naidoo, 2017). A strain of *Bacillus velezensis*- R16, which is a known biosurfactant producer was used as a positive control during the course of the biosurfactant screening (Hunter, 2016).

Table 2.1: AEFB isolates from Mfabeni peatland selected for biosurfactant screening

Sample	Depth (cm)	Number of isolates	Strain
A (ca. 589 cal years BP)	12	31	SAA1, SAA5, SAA7, SAA8, SAA9, SAA16, SAA21, SAA23, SAA24, SAA25, SAA28, SAA32, SAA34, SAA35, SAA37, SAA39, SAA42, SAA43, SAA63, SAA66, SAA79, SAA85, SAA91, SAA93, SAA98, SAA101, SAA107, SAA108, SAA109, SAA110, SAA114
B (ca. 1,964 cal years BP)	21	23	SAB1, SAB2, SAB6, SAB7, SAB10, SAB11, SAB14, SAB19, SAB20, SAB23, SAB24, SAB30, SAB35, SAB42, SAB45, SAB46, SAB50, SAB51, SAB52, SAB53, SAB54, SAB59, SAB63
C (ca. 17,568 cal years BP)	89	12	SAC1, SAC2, SAC13, SAC15, SAC16, SAC18, SAC19, SAC20, SAC24, SAC25, SAC26, SAC27
D (ca. 33,328 cal years BP)	237	15	SAD1, SAD3, SAD4, SAD5, SAD9, SAD10, SAD12, SAD17, SAD18, SAD21, SAD23, SAD34, SAD41, SAD45, SAD47
E (ca. 37,906 cal years BP)	344	1	SAE10

2.2.2 Subculturing of AEFB

Isolates were revived from frozen 20% (v/v) glycerol stock cultures and streaked on to sterile 10% Tryptone Soy Agar (TSA) plates supplemented with 7.5 g/L bacteriological agar. Inoculated plates

were incubated at 30℃ for 48 h. After growth, the plates were checked for purity and discrete single colonies were sub-cultured on 10% TSA plates and agar slants. The discrete colonies were also transferred into 20% (v/v) glycerol for long term storage at -80℃.

2.2.3 Screening for biosurfactant activity

Isolates were cultured in 20 mL sterile Tryptone Soy Broth (TSB) for 48 h in an orbital shaker incubator (120 rpm) at 30℃. A 1 mL sample of each broth was centrifuged at 12,000 rpm for 5 min in microfuge tubes (1.5 mL), after which the cell-free supernatants were decanted into 1.5 mL microfuge tubes and stored at -20℃ until needed for biosurfactant activity screening.

2.2.3.1 Hemolysis test

The hemolysis test was carried out using 5% (v/v) sheep blood agar plates according to the method of Mulligan *et al*. (1984). Fresh sheep blood (50 mL) was added to a 950 mL of sterile molten TSA and gently mixed to enhance homogeneity and avoid the formation of bubbles. Isolates were sub-cultured for 48 h on 10% TSA before inoculation onto blood agar plates, where each colony was spot inoculated using sterile 1 mL micropipette tips (Greiner bio-one). The plates were incubated at 30℃ for 48 h, after which the plates were visually assessed for signs of hemolysis i.e. the formation of clear zones around the bacterial spots. Inoculated spots on the blood agar plate which did not exhibit hemolysis were regarded to be non-biosurfactant producers.

2.2.3.2 Drop-collapse test

The Drop-collapse test was conducted as described by Jain *et al*. (1991), with some modifications. Ten microliters of cell-free supernatant (Section 2.2.3) was added to 15 µL of water placed on a flat sheet of parafilm (parafilm M- 992, Bemis flexible packaging). After one minute each drop was examined to determine whether it had retained its raised state or whether it had collapsed. The diameter of each drop was measured using Vernier calipers. A standard commercial surfactant- Triton-X (10 µL) was used as a positive control; while sterile uninoculated broth was used as negative control. Each test was done in duplicate and the means calculated.

2.2.3.3 Oil-spreading test

Oil spreading analysis was carried out according to the method of Morikawa *et al*. (2000). A 200 µL sample of engine oil (GTX 20W- 50, Castrol South Africa) was added to 20 mL of distilled water in a Petri dish. Thereafter, 100 µL cell-free supernatant (Section 2.2.3) was added directly on to the center of the oil to determine the oil displacement effect. The mixture was observed for zones of oil displacement after 30 seconds and the zone diameters were measured using a Vernier caliper. Distilled water and uninoculated broth served as negative controls while Triton-X and cell-free supernatant from isolate *B. velezensis* R16 were used as positive controls. The tests were done in duplicate and the means calculated.

2.2.4 Determination of the effect of environmental parameters on biosurfactants produced

Isolates which demonstrated biosurfactant activity were selected for further testing to determine the effects of environmental variables, namely temperature, pH, and salinity on biosurfactant activity and stability.

2.2.4.1 Effect of temperature on biosurfactant activity

Samples of cell-free supernatant (200 µL) (Section 2.2.3) were heated at four temperatures 35℃, 55℃, 75℃, and 100℃ for 15 mins using a heating block (Vacutec model OPR-HB-100). Oil spreading assays were carried out as described previously (Section 2.2.3.3) to determine the effect of temperature on the biosurfactant activity. Uninoculated broth was used as negative control, while cell-free supernatant of isolate *B. velezensis* R16 served as the positive control. Each test was carried out in duplicate and the means calculated.

2.2.4.2 Effect of pH on biosurfactant activity

To determine the effect of pH on biosurfactant activity, cell-free supernatant (200 µL) were combined with different buffer solutions (200 µL) to achieve a range of final pH values, namely pH 3, 5.5, 7, 8.5, and 10. Citrate buffer was used for pH values 3 and 5.5, Tris buffer was used for pH values 7 and 8.5 and Bicarbonate-carbonate buffer was used for pH 10 respectively. The different volumes of each buffer used in achieving the pH ranges are presented in Appendix A. The oil spreading assay was used to determine the effect of pH values on biosurfactant activity. Uninoculated broth was used as a negative control while cell-free supernatant from isolate *B.*

velezensis R16 was used as a positive control. The tests were done in duplicate and the means calculated.

2.2.4.3 Effect of salinity on biosurfactant activity

Cell-free supernatants (200 µL) of each isolate were added to 200 µL of NaCl solutions to achieve final salt concentrations of 0.5, 2.5, 5, 10, and 15% (w/v) respectively. Uninoculated broth was used as a negative control while cell-free supernatant from isolate *B. velezensis* R16 was used as a positive control. The oil spreading assay was then used to determine the effect of the different salt concentrations on the biosurfactant activity. The tests were done in duplicate and the means calculated.

2.2.4.4 Statistical analysis

A general linear model (GLM) was used to run an Analysis of Variance (ANOVA) on the results using SAS software (V.9.4). Where ANOVA yielded significant results ($P< 0.0001$), the Duncan Multiple Range test was applied (5% probability level) to separate the means.

2.2.5 Emulsification index test

The emulsification index test E24 was carried out as described by Cooper and Goldenberg (1987). Two different hydrocarbons were used namely; sunflower seed vegetable oil, and paraffin oil. Five milliliters of cell-free supernatant were added to 5 mL sunflower seed vegetable oil and paraffin oil in a test tube and vortexed for 5 mins. After which the test tubes were left to stand for 24 h and the height of emulsion layer was measured. The tests were done in duplicate and isolate *B. velezensis* R16 was used as a positive control, while uninoculated broth was used as negative control. The E24 was calculated thus

$$\text{E24} = \frac{h\ emulsion}{h\ total} \text{ x } 100\%$$

E24 is an indirect method of measuring biosurfactant concentration, $h\ emulsion$ is the height of emulsion formed after 24 h, and $h\ total$ is the total height of liquid (Cooper and Goldenberg, 1987).

2.2.6 Surface tension measurement

The ability of biosurfactants produced by selected AEFB isolates to reduce surface tension was determined using a Du-Nouy tensiometer. Analysis of Cell-free supernatant (50 mL) (section 2.2.3) was undertaken by Department of Chemistry, Durban University of Technology (DUT) for analysis. Uninoculated broth was used as a negative control and isolate *B. velezensis* R16 was used as a positive control.

2.3 RESULTS

2.3.1 Preliminary screening for biosurfactant production amongst AEFB isolates

Of the eighty-two isolates screened, 65.85% tested positive for the hemolysis assay, 87.80% tested positive for the oil spreading assay, and 95.12% tested positive for the drop collapse assay. Isolates which showed zones of clearing on the blood agar plates for the hemolysis assay were recorded as positive, while those that did not show zones of clearing were recorded as negative for biosurfactant activity (Table 2.2). For the drop-collapse assay, Triton X-100 and isolate *B. velezensis* R16- controls both gave readings of 5.5 mm whereas uninoculated broth- control gave reading of 4.0 mm. Therefore, drop collapse assays with diameters which measured above 4.0 mm were recorded as positive for biosurfactant activity (Table 2.2). For the oil spreading assay, Triton X-100 and *B. velezensis* R16 both displaced the oil with mean diameter measurements of 85 and 83 mm respectively. The broth and control did not show any oil displacement (ca. 20 mm diameter). Therefore, isolates which showed zones of displacement from ≤ 20 mm were recorded as negative for biosurfactant activity, zones ranging from > 20 to < 40 mm were recorded as low biosurfactant activity, ≥ 40 to < 60 mm as moderate biosurfactant activity and ≥ 60 to 85 mm as high biosurfactant activity. The relative distribution of biosurfactant activity amongst the AEFB isolates is shown in Figure 2.1. Ten isolates did not exhibit biosurfactant activity, twenty-two showed low activity, thirty-seven showed moderate activity, and thirteen showed high biosurfactant activity.

Based on sensitivity, the ability to visualize clear differences between positive and negative controls, and the semi-quantitative nature of the analysis, the oil spreading assay was judged to

be the best method for assaying biosurfactant production and therefore was used to further determine the effect of environmental variables on biosurfactant activity.

Table 2.2: Results showing biosurfactant activity amongst AEFB isolates

		Percentage of isolates positive for biosurfactant activity		
Sample depths	**Number of isolates**	**Hemolysis test**	**Drop collapse test**	**Oil spreading test**
A	31	54.84	100	93.55
B	23	86.96	86.96	78.26
C	12	83.33	100	100
D	15	40	93.33	86.67
E	1	100	100	100
Positive control-R16		Positive	5.5 mm	85 mm
Negative control-Uninoculated broth		Negative	4.0 mm	20 mm

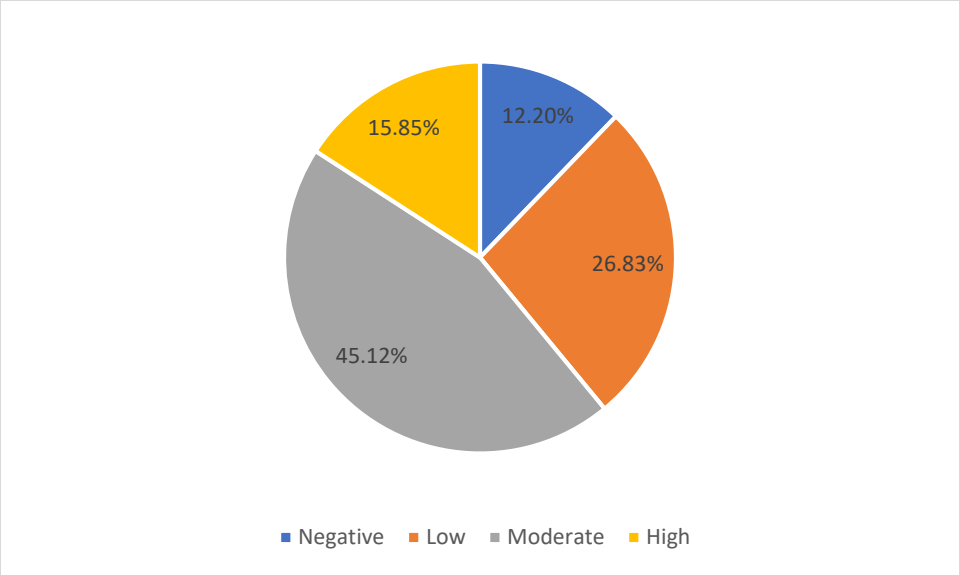

Figure 2.1: Pie chart illustrating the response of AEFB isolates to oil spreading assay. Zones of oil displacement ranging from ≤ 20 mm represent a negative biosurfactant response, > 20 to < 40 mm low biosurfactant activity, ≥ 40 to < 60 mm moderate biosurfactant activity, and ≥ 60 to 85 mm high biosurfactant activity.

2.3.2 Effect of environmental parameters on the activity of biosurfactant produced by AEFB

Seventy-two isolates that displayed biosurfactant activity in the preliminary oil spreading assay were tested to determine the effect of environmental parameters namely; temperature, pH, and salinity on their biosurfactant activity. ANOVA analysis of each of the environmental parameters tested yielded data results that were found to be statistically significant ($P< 0.0001$) (Table 2.3). The Duncan Multiple Range test was then applied to separate the means (5% probability level) (Appendix B). For ease of visualization and comparing the data, the results of the oil displacement assays have been presented graphically as mean values, comparing isolates from each sample set (Figures 2.2 – 2.13).

Table 2.3: Summary of ANOVA analysis of environmental parameters influencing biosurfactant activity

			Temperature		
	35°C	**55°C**	**75°C**	**100°C**	
P - value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
CV	11.828	9.945693	7.554116	9.054245	
R^2	0.971813	0.981918	0.991756	0.985474	
			pH		
	3	**5.5**	**7**	**8.5**	**10**
P - value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CV	4.589569	6.697646	14.35434	15.10394	16.59596
R^2	0.941608	0.904043	0.977101	0.981576	0.988408
			Salinity		
	0.5%	**2.5%**	**5%**	**10%**	**15%**
P - value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CV	21.53308	21.53308	19.03057	23.519	31.65505
R^2	0.980464	0.980464	0.987418	0.978315	0.972755

2.3.2.1 Effect of temperature on biosurfactant activity

Cell-free supernatant were exposed to temperatures of 35℃, 55℃, 75℃, and 100℃. The uninoculated broth controls showed mean oil displacement measurements ranging from 27.5 mm to 15 mm over the 35℃ to 100℃ temperature range (Figure 2.2 – 2.5). Isolate *B. velezensis* R16 control showed oil displacement ranging from 75.5 mm to 69.5 mm over the same temperature range.

None of the isolates from Sample A showed biosurfactant activity as high as the *B. velezensis* R16 control (Figure 2.2). Both the positive and the negative controls showed slight reduction in zones of oil displacement with increasing temperature. At 35°C and 55°C, oil displacement appeared fairly consistent. An increase in temperature to 75°C and 100°C resulted in a slight reduction in

zones of oil displacement observed (Figure 2.2). This trend was apparent for some of the isolates e.g. SAA23, SAA35, SAA37, SAA79, SAA108, and SAA110 but was not consistent for all the isolates. A number of isolates e.g. SAA1, SAA7, SAA9, SAA16, SAA34, SAA42, SAA63, and SAA98 exhibited their largest zone of oil displacement at 55°C but were found to be significantly different from the R16 control ($P < 0.0001$) (Appendix B).

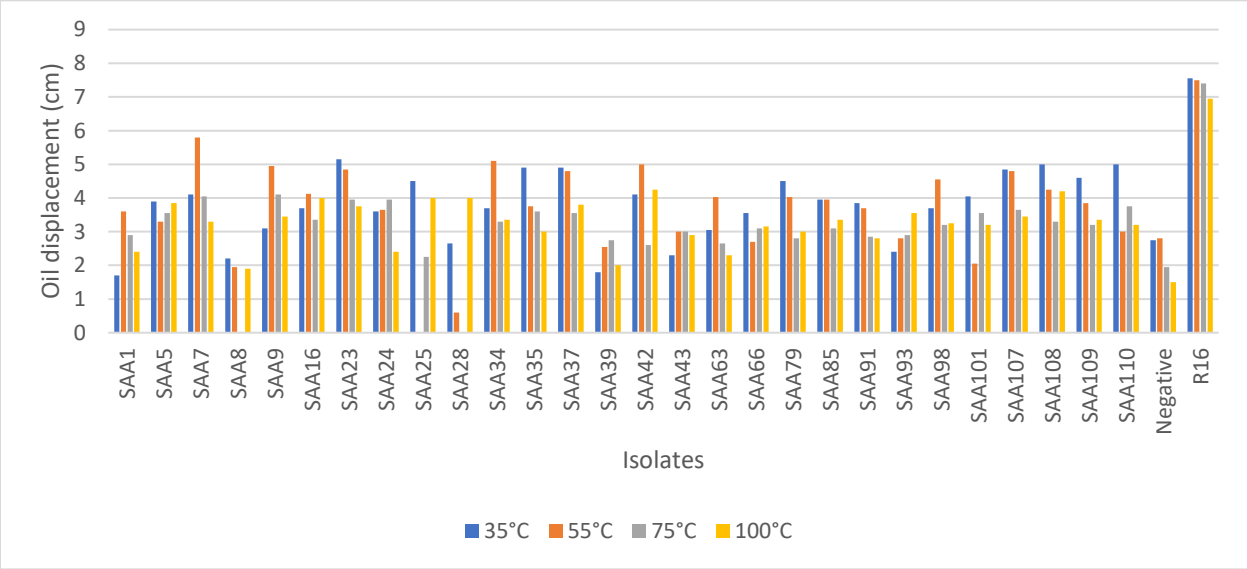

Figure 2.2: Effect of temperature on the biosurfactant activity of AEFB isolates cultured from Sample A (12 cm) of Mfabeni peatland determined using the oil spreading assay. Mean oil displacement values are presented (n = 2)

Figure 2.3 shows the effect of temperature on biosurfactant activity of Sample B isolates. Again, none of these isolates showed biosurfactant activity as high as the *B. velezensis* R16 control. Isolates SAB7, SAB46, and SAB53 did not exhibit oil displacement activity at 100°C and 75°C respectively. Oil displacement activity for isolates SAB6, SAB10, SAB11, and SAB54 appeared fairly consistent over the temperature ranges tested although they were still significantly less than the positive control ($P < 0.0001$) (Appendix B). Some isolates e.g. SAB19, SAB23, SAB30, SAB42, SAB46, and SAB51 showed highest biosurfactant activity at temperature 55°C (Figure 2.3).

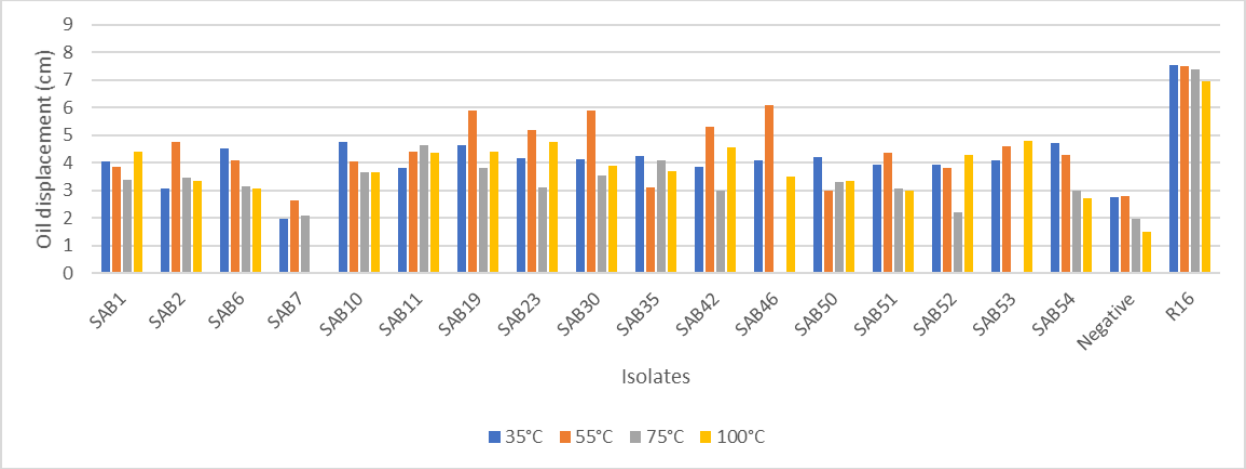

Figure 2.3: Effect of temperature on the biosurfactant activity of AEFB isolates cultured from Sample B (21 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

All the isolates obtained from Sample C showed zones of oil displacement that were significantly greater than the negative control ($P < 0.0001$) (Appendix B). Most isolates showed consistency in biosurfactant activity over the different temperature ranges tested (Figure 2.4). Isolates SAC15, SAC16, SAC18, and SAC19 exhibited oil displacement activity which are closely approximated to the values obtained for the *B. velezensis* R16 control (Figure 2.4).

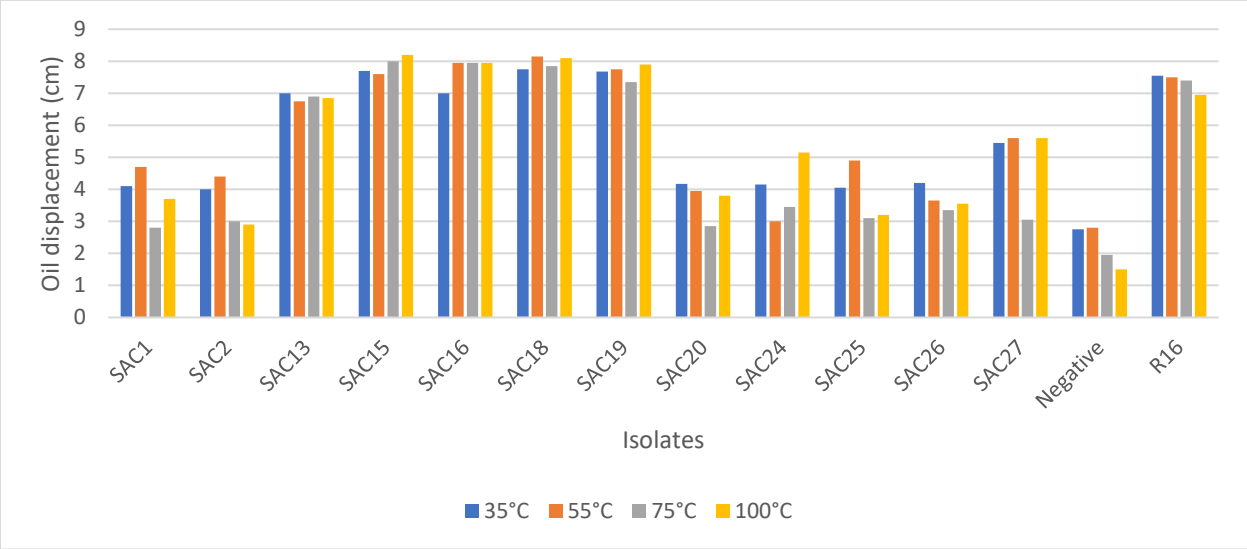

Figure 2.4: Effect of temperature on the biosurfactant activity of AEFB isolates cultured from Sample C (89 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

Isolates SAD12, SAD17, SAD18, and SAD23 showed oil displacement values as high as and higher than the *B. velezensis* R16 control ($P < 0.0001$) (Figure 2.5). Isolates SAD5, SAD10, SAD23, and SAE10 showed oil displacement that was highest at temperature 100°C (Figure 2.5). SAD5, SAD9, and SAD10 showed intermediate biosurfactant activity but not as high as the *B. velezensis* R16 control.

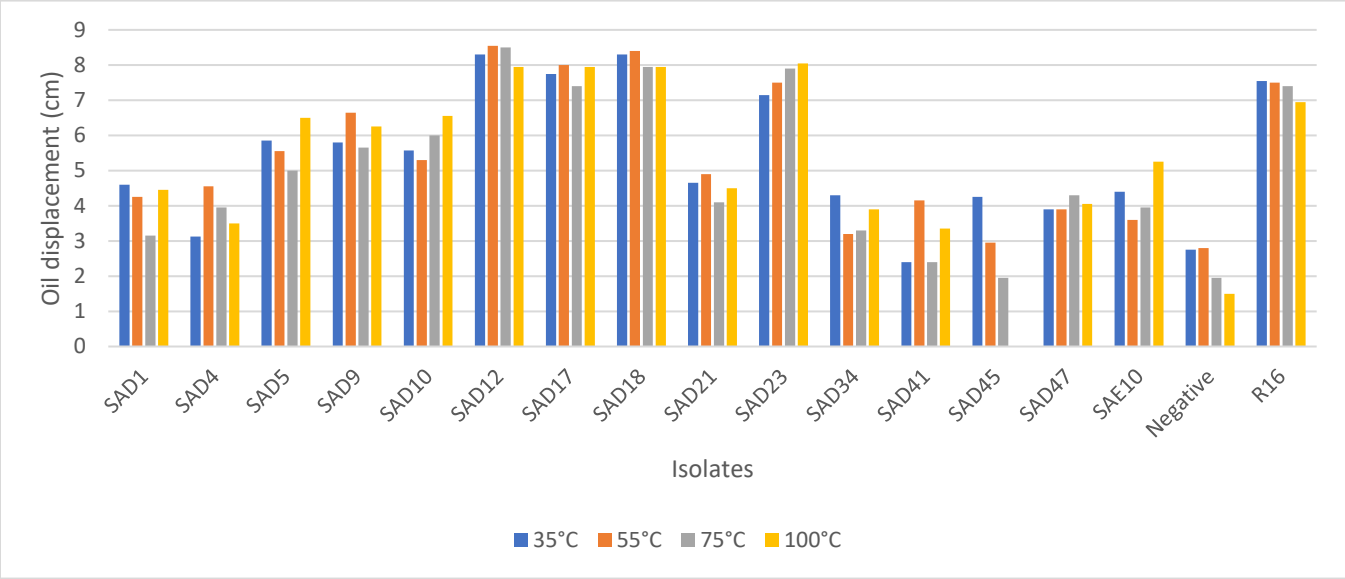

Figure 2.5: Effect of temperature on the biosurfactant activity of AEFB isolates cultured from Sample D and E (237 and 344 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

2.3.2.2 Effect of pH on biosurfactant activity

Acidic conditions (pH 3 and 5.5) were found to have a major significant impact on the negative control whereby the oil displacement values obtained were significantly greater than pH 7, 8.5, and 10 values ($P < 0.0001$) (Figure 2.6 – 2.9). It is, therefore, difficult to ascribe pH differences between isolates and controls in these pH ranges. However, from sample C and D, a number of

isolates, (SAC13, SAC15, SAC18, SAD12, SAD17, SAD18, and SAD23) showed similar activity to the results obtained for the temperature ranges tested (Figure 2.8 – 2.9).

For the pH 7, 8.5, and 10 activity assays, it was observed that most of the isolates showed better activity at pH 7 and decreased with an increase in pH (Figure 2.8 – 2.9). Some isolates (SAA93, SAB30, SAB52, SAD4, and SAD5) including the R16 control were found to show their highest activity at pH 8.5 (Figure 2.8 – 2.9). Isolates SAA8, SAA23, SAB11, SAB14, and SAB63 did not show activity at pH 7 while SAA28, SAB20, SAB24, SAB45, and SAB59 did not show activity for pH 7, 8.5, and 10.

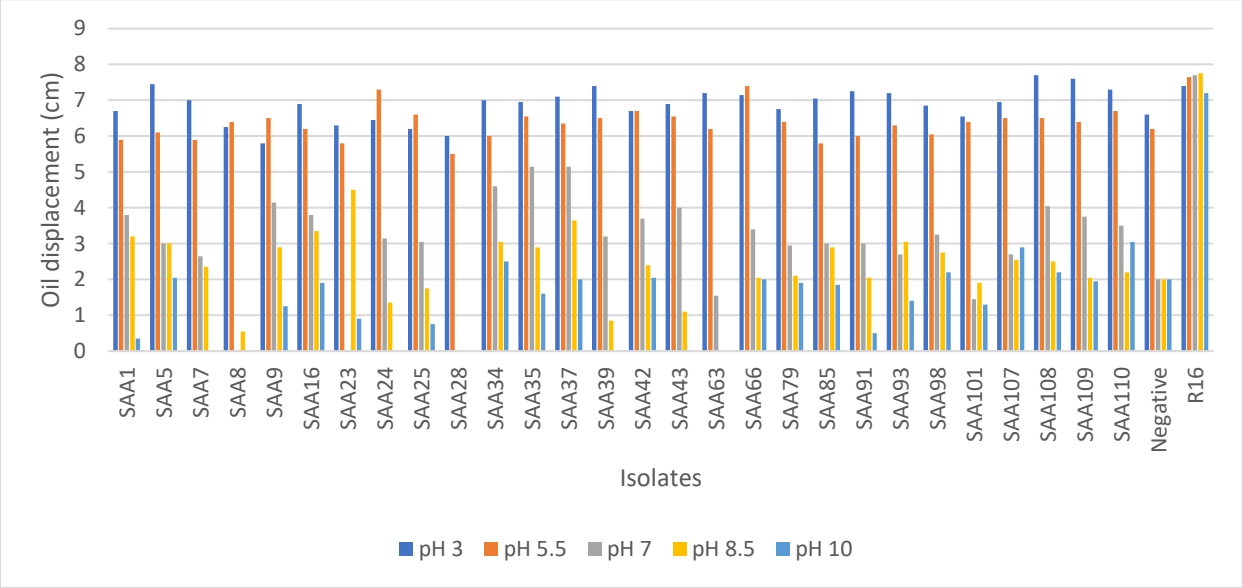

Figure 2.6: Effect of pH on the biosurfactant activity of AEFB isolates cultured from Sample A (12 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

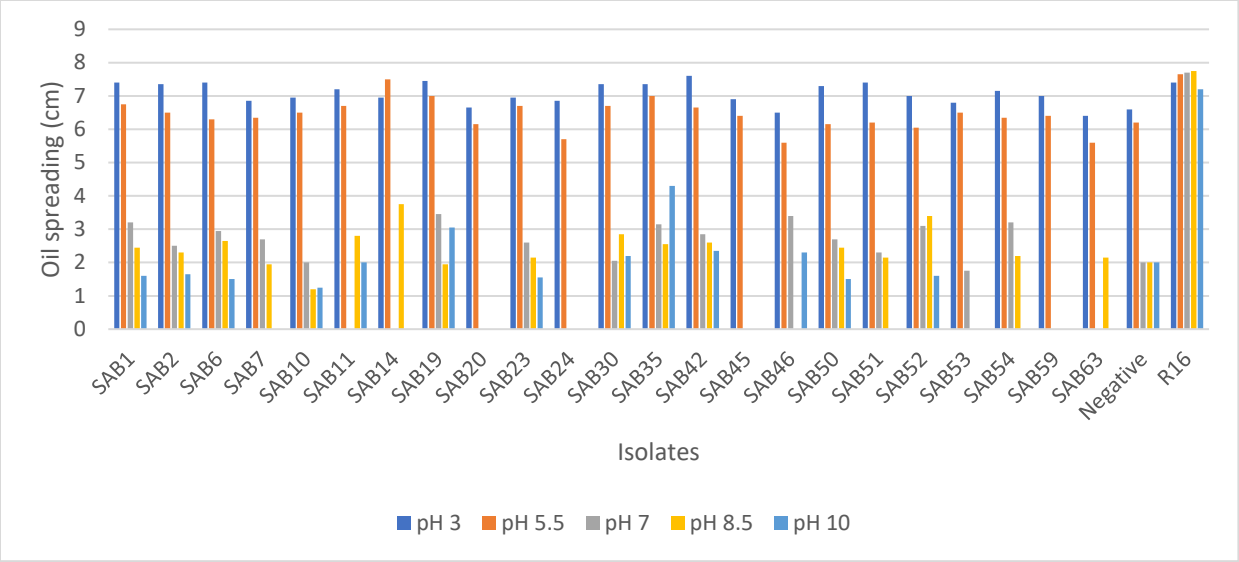

Figure 2.7: Effect of pH on the biosurfactant activity of AEFB isolates cultured from Sample B (21 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n =2)

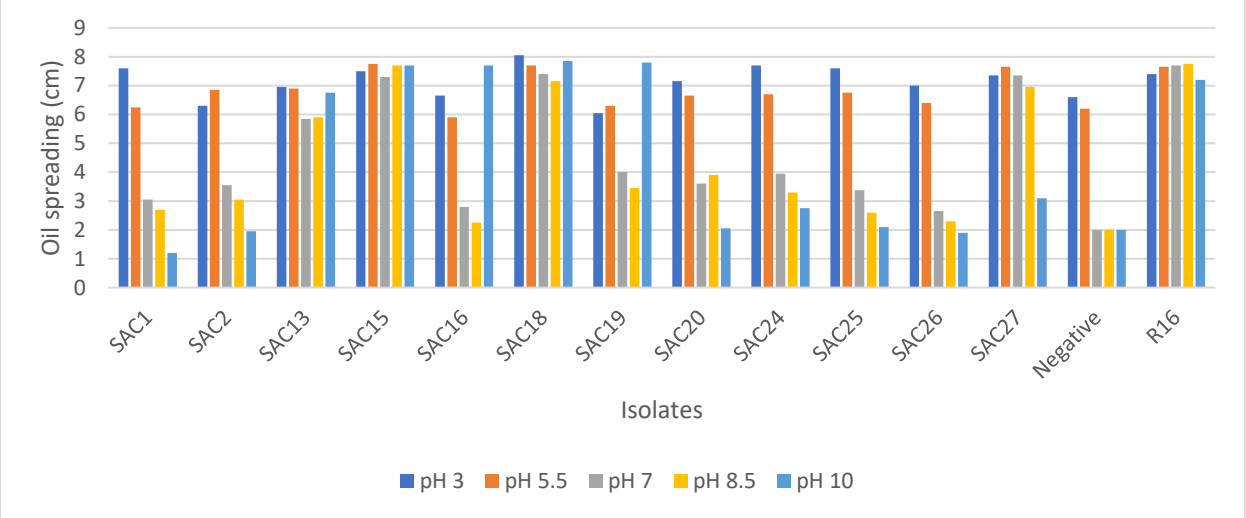

Figure 2.8: Effect of pH on the biosurfactant activity of AEFB isolates cultured from Sample C (89 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n =2)

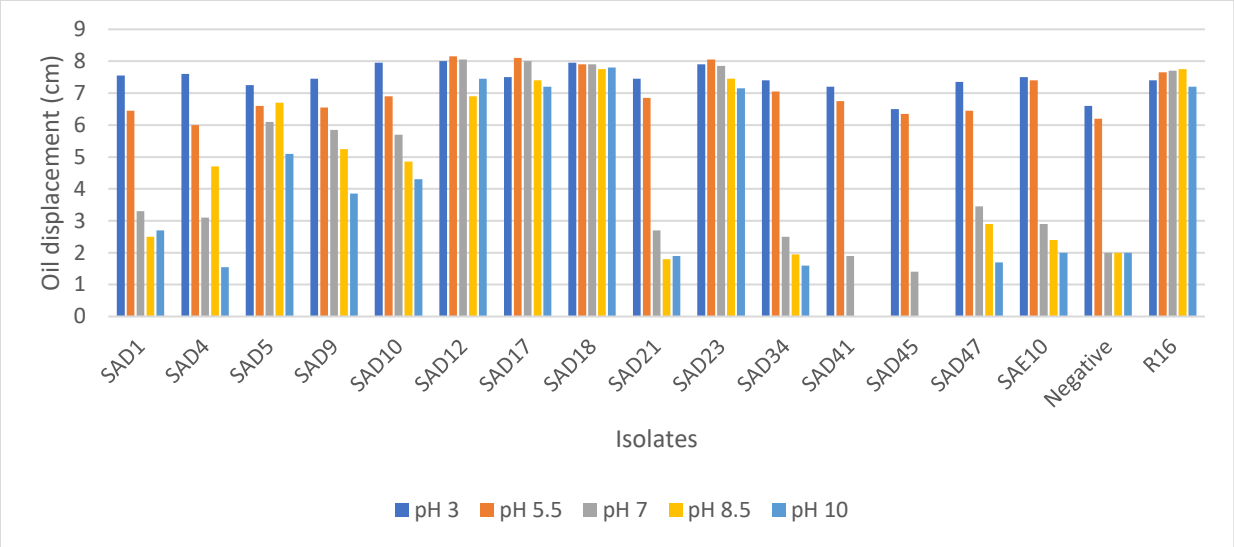

Figure 2.9: Effect of pH on the biosurfactant activity of AEFB isolates cultured from Sample D and E (237 and 344 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n =2)

2.3.2.3 Effect of salinity on biosurfactant activity

Generally, 0.5% salinity gave the most consistent results. Increase in salt concentration resulted in varied changes in oil displacement responses of most of the isolates e.g. isolates SAA5, SAA7, SAA9, SAA16, SAA23, SAA24, SAA25, SAA34, SAA35, SAA37, SAA39, SAA43, SAA63, SAA91, SAA93, SAA107, SAA108, SAA109, and SAA110. For the different salt concentrations tested, the negative control showed no oil displacement activity (ca. 20 mm) while the *B. velezensis* R16 control showed oil displacement measurement ranging from 76 mm to 71 mm over the 0.5% to 15% salinity ranges. None of the Sample A isolates showed as high oil displacement activity as the R16 control (Figure 2.10). In some instances, increase in salinity led to decrease or loss of oil displacement ability e.g. SAA8, SAA28, SAA98, and SAA101.

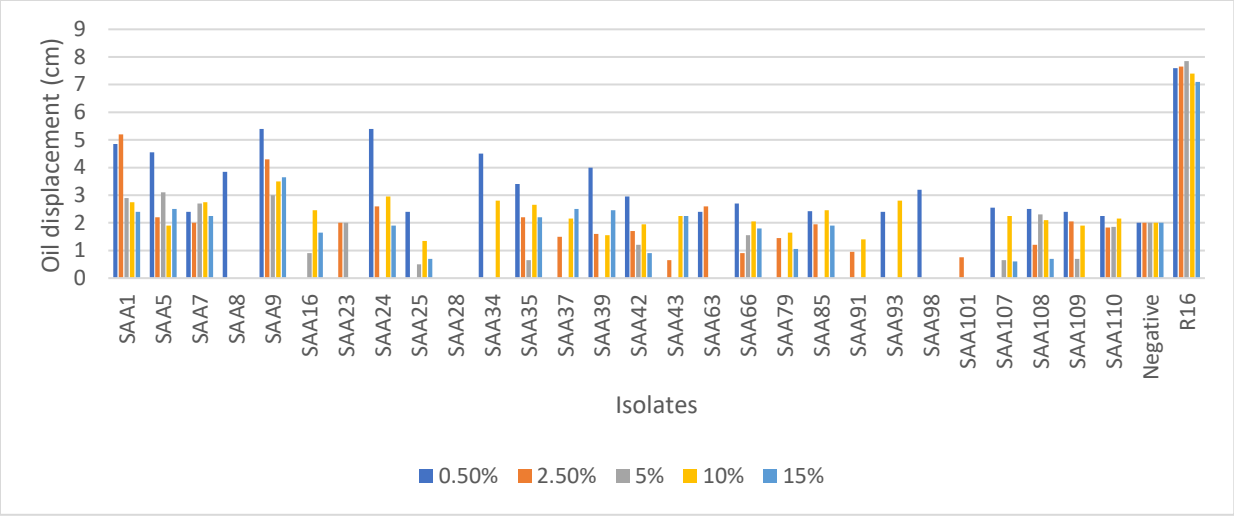

Figure 2.10: Effect of salinity on the biosurfactant activity of AEFB isolates cultured from Sample A (12 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

For Figure 2.11, isolate SAB35 showed biosurfactant activity for all the different salt concentrations tested while most of the other isolates exhibited loss of oil displacement activity e.g. SAB2, SAB6, SAB7, SAB10, SAB19, SAB23, SAB30, SAB42, SAB46, SAB50, SAB51, SAB52, SAB53, and SAB54. None of the isolates showed activity as high as the R16 control. Several of the isolates e.g. SAB1, SAB2, SAB10, SAB19, SAB35, SAB46, SAB51, and SAB52 showed highest activity at 0.5% salinity but which were significantly lesser than the positive control ($P < 0.0001$) (Appendix B). Oil displacement activity of isolate SAB1 decreased with increase salinity (Figure 2.11).

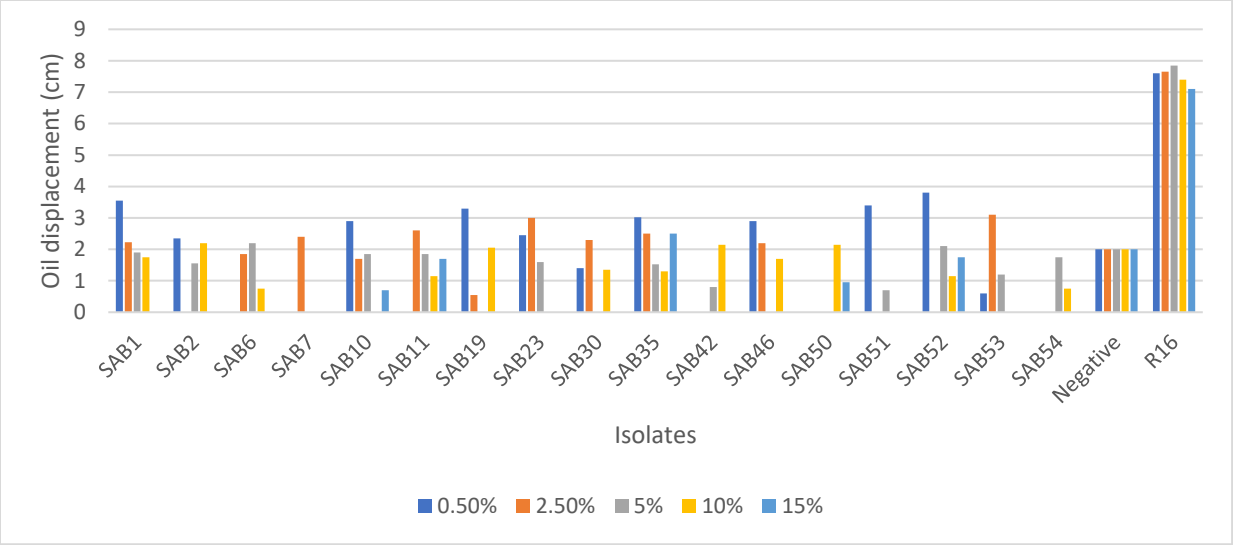

Figure 2.11: Effect of salinity on the biosurfactant activity of AEFB isolates cultured from Sample B (21 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

Some isolates from Samples C and D- SAC15, SAC18, SAC27, SAD12, SAD17, SAD18, and SAD23 showed consistently high oil displacement readings and the impact of increasing salt concentration was relatively minor (Figure 2.12 – 2.13). Isolate SAC18 showed activity that was significantly higher than the positive control at 0.5% salinity ($P < 0.0001$) (Appendix B). Again, isolates SAC1, SAC2, SAC16, SAC19, SAC20, SAC24, and SAC26 showed varied changes and loss of activity over the different salt concentrations (Figure 2.12).

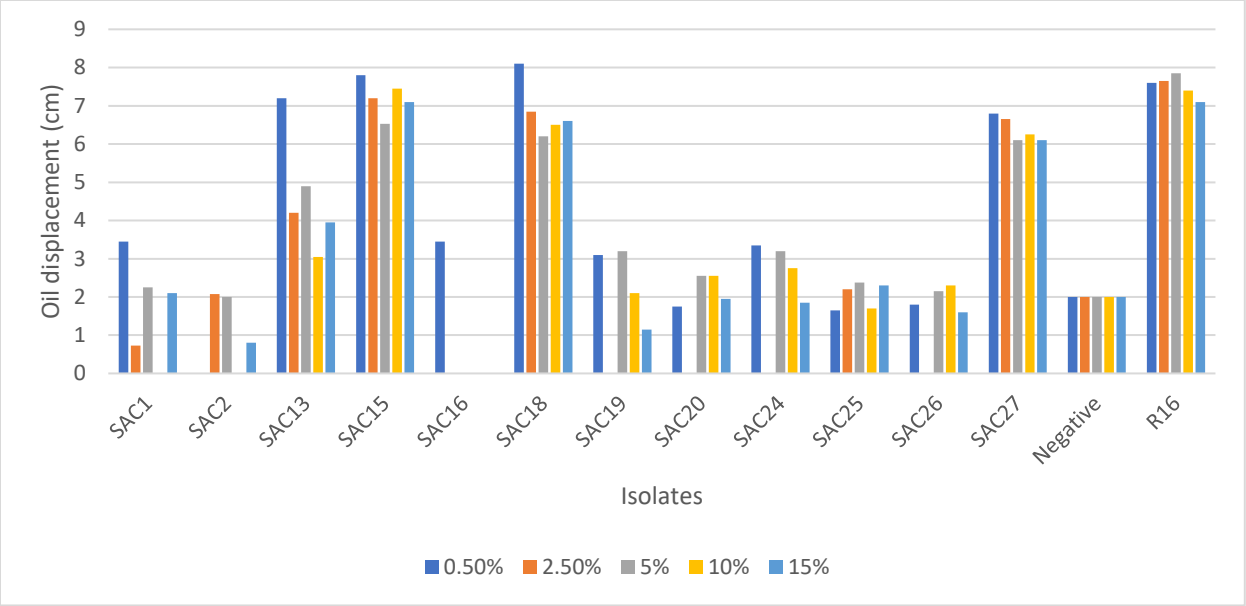

Figure 2.12: Effect of salinity on the biosurfactant activity of AEFB isolates cultured from Sample C (89 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

Isolates SAD9, SAD10, SAD17, SAD18, and SAD23 showed lowest activity at 15% salt concentration and were significantly lower than the B. velezensis R16 control ($P < 0.0001$) (Appendix B). Isolates SAD34, SAD41, SAD45, SAD47, and SAE10 did not show biosurfactant activity at 10% and 15% (Figure 2.13). The oil displacement activity exhibited by SAD12, SAD17, and SAD18 were significantly higher than the R16 control but this was not consistent across the salt concentrations tested ($P < 0.0001$) (Appendix B).

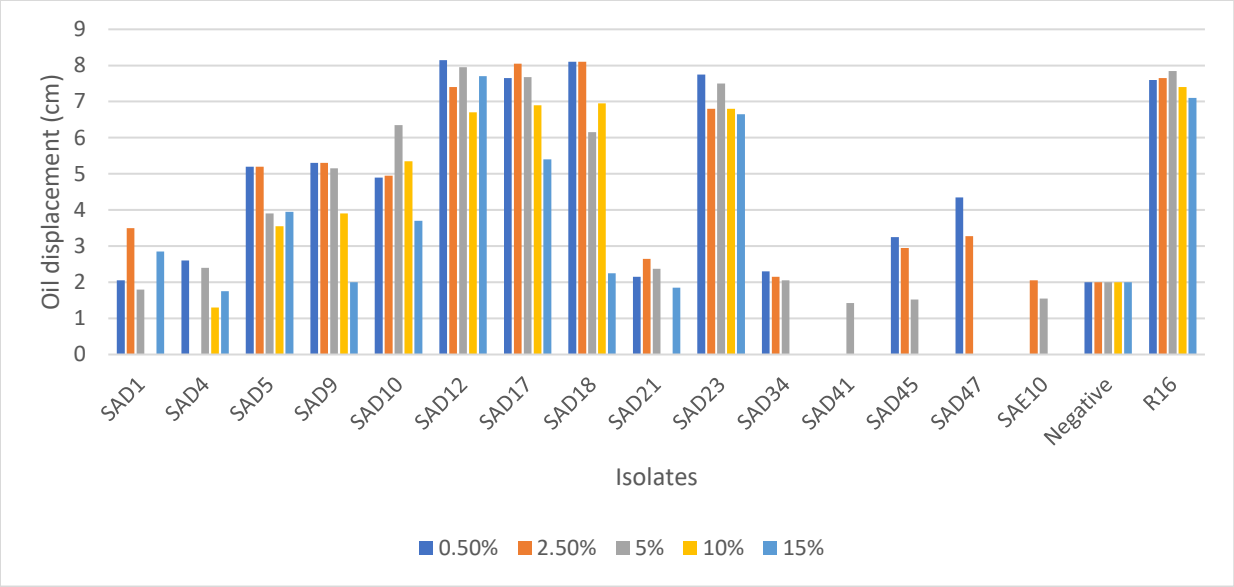

Figure 2.13: Effect of salinity on the biosurfactant activity of AEFB isolates cultured from Sample D and E (237 and 344 cm) of Mfabeni peatland determined using oil spreading assay. Mean oil displacement values are presented (n = 2)

2.3.3 Emulsification index (E24) assay

AEFB isolates which demonstrated the greatest displacement in the oil spreading assays namely; SAB19, SAB42, SAC15, SAC18, SAD5, SAD17, SAD18, and SAD23 were selected for further testing for their ability to emulsify oil (Section 2.2.5). No emulsion was observed for the uninoculated broth (negative control) (Figure 2.14). The *B. velezensis* R16 control exhibited an emulsion index of 39.23% for the vegetable oil and 54.38% for paraffin. Table 2.4 shows the result obtained for the E24 assay of the selected AEFB isolates. The highest emulsion index was obtained for isolates SAB19 and SAB42 (Table 2.4).

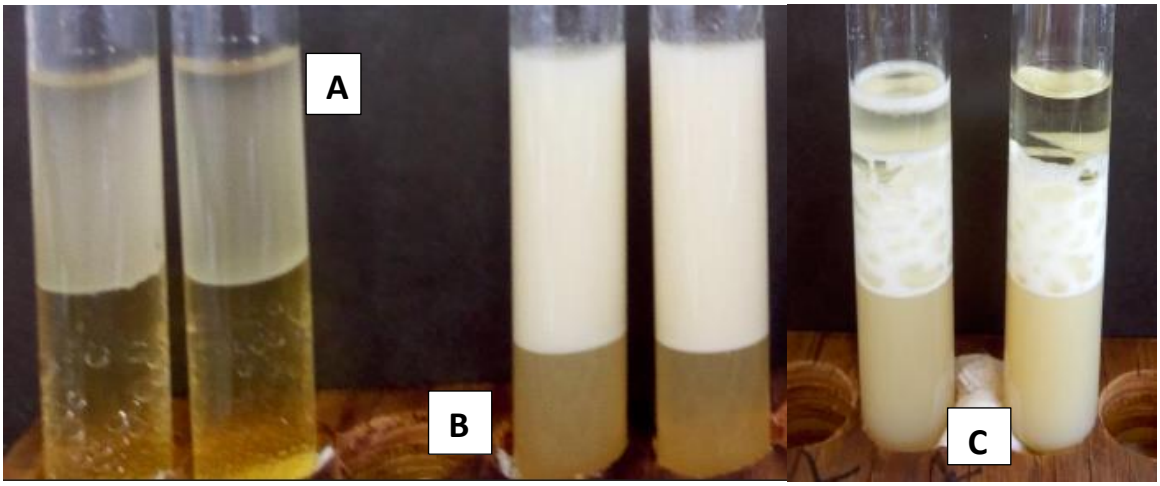

Figure 2.14: The emulsification (E24) index observed for the uninoculated broth (negative) control (A), Isolate SAB19 (B) and the *B. velezensis* R16 control (C) using vegetable seed oil

Table 2.4: Results showing emulsification (E24) index of selected AEFB isolates

	Emulsification (E24) index	
Isolates	**Vegetable seed oil**	**Paraffin**
Positive control- R16	39.23%	54.38%
Negative control- Uninoculated broth	-	-
SAB19	61.85%	59.5%
SAB42	61.11%	69.27%
SAC15	23.30%	54.69%
SAC18	30.77%	19.53%
SAD5	53.85%	56.76%
SAD17	51.59%	57.02%
SAD18	39.55%	55.78%
SAD23	43.32%	48.70%

2.3.4 Surface tension measurement

Eight isolates namely; SAB19, SAB42, SAC15, SAC18, SAD5, SAD17, SAD18, and SAD23 were tested for their ability to reduce surface tension. The surface tension reading for the uninoculated broth was 57.3 mN/m. All the isolates tested were positive for reducing surface tension. The R16 control demonstrated the greatest reduction in surface tension (29.6 mN/m), while isolates SAC18, SAC15, SAD17, SAD18, SAD23, and SAD5 reduced TSB surface tension to 30.9, 30.8, 30.7, 30.6, 31.6 and 32.2 mN/m respectively. Isolates SAB19 and SAB42 exhibited the lowest reduction in surface tension with 44.3 and 44.7 mN/m respectively (see Appendix C for certificate of analysis).

2.4 Discussion

Wetlands represent microbiologically diverse environments, which support bacteria with a wide range of metabolic capabilities. The microbes found in such environments play an important role in the degradation of plant materials and in the biogeochemical cycling of essential elements. The Mfabeni peatland has functioned continuously as a wetland for approximately 50,000 years (Meadows, 2015; Naidoo, 2017). This environment is not well characterized from a microbiological standpoint and could harbor microorganism that are of biotechnological interest. AEFB isolated from ancient sediment core were selected for screening purposes in this study.

Biosurfactant production amongst AEFB was evaluated using three assays namely blood agar hemolysis assay, a water drop-collapse assay, and the oil spreading assay (Table 2.2). The hemolysis assay is a qualitative assay used for the detection of biosurfactant producers. Biosurfactants interfere with the cell membrane of red blood cells, leading to hemolysis of the red blood cells, which in turn is observed as a zone of clearing on the blood agar plate. When an isolate shows a zone of inhibition on a blood agar plate, it suggests that such isolate is a biosurfactant producer (Mulligan *et al*., 1984; Yonebayashi *et al*., 2000). It has been suggested that the hemolysis assay can give false positive results and therefore should be used alongside other assays (Youssef *et al*., 2004). In this study, the hemolysis assay proved to be the least sensitive of the biosurfactant activity assays tested (Table 2.2).

Although the drop collapse assay is considered to be a qualitative assay, it can also be used in a semi-quantitative manner (Bodour and Miller-Maier, 1998). When a liquid droplet containing biosurfactant is placed on a hydrophobic surface, the surfactant reduces the surface tension present on the hydrophobic surface causing the droplet to collapse. The concentration of the biosurfactant determines the stability of the droplet (Walter *et al*., 2010). This assay has been reported to have high sensitivity because it is concentration dependent. In this study the drop collapse assay proved to be the most sensitive of the screening assays used as it detected the highest levels of biosurfactant activity amongst isolates. However, the differences between the negative control and biosurfactant positive results differed by only a few millimeters; it was, therefore, difficult to distinguish quantitative differences between isolates that were positive for biosurfactant production.

The oil spreading assay is considered to be a semi-quantitative assay because the extent of oil spreading can be directly correlated to the concentration of biosurfactant produced (Walter *et al*., 2010). This assay works by measuring the zones of oil displacement observed after a liquid containing biosurfactant is added onto the center of an oil droplet suspended in water (Morikawa *et al*., 2000). The biosurfactant reduces the interfacial tension of the hydrophobic surface, thereby causing a dispersion of the oil on the water surface, which in turn leads to zone of clearing (Morikawa *et al*., 2000; Walter *et al*., 2010). Approximately eighty-eight percent of isolates assayed tested positive for oil displacement. From the results it was observed that biosurfactant activity could be differentiated into low, medium, and high levels of activity. Most isolates exhibited low or medium levels of activity. Approximately 16% of isolates were rated to be high level producers (Figure 2.1). These isolates showed levels of oil displacement similar to those achieved with the *B. velezensis* positive control. Youssef *et al*. (2004) noted that the drop collapse and oil spreading assays yielded results that could be closely correlated, which supports our findings. The oil spreading assay was selected over the other two assays to determine the effect of environmental parameters on biosurfactant activity because of its ease of use, sensitivity, and the clear distinction between the positive and the negative response.

Further assays were carried out to determine the effect of temperature, pH, and salinity on the biosurfactant activity of the isolates. Most of the isolates were able to maintain their oil

displacement activity over the temperature ranges tested. This result indicated that the biosurfactant compounds showed a degree of thermostability over the temperature ranges tested. This could be due to the amphiphilic nature of the lipopeptide biosurfactants produced. These findings support those of Sharma *et al*. (2018) who found that strains of *B. amyloliquefaciens* and *B. subtilis* retained their biosurfactant activity at temperatures ranging from 4°C to 100°C. Several studies have shown that lipopeptides such as surfactin produced by *Bacillus* species are thermostable at temperatures up to 100°C for 30 mins (Varadavenkatesan and Murty, 2013). Interestingly, 33% of the AEFB isolates screened showed highest biosurfactant activity at 55°C. It is possible that isolates which did not exhibit oil displacement activity at 75°C and 100°C experienced denaturation at these temperature ranges.

At acidic pH (3 and 5.5), the broth control exhibited high levels of oil displacement which was inconsistent with the activity readings obtained for pH 7 to 10. An explanation could be that the acidic conditions interfered with the ionic nature of the proteins in the broth causing them to mimic biosurfactant activity. As a result of this finding, the high zones of oil displacement observed for all the isolates at acidic pH could not be attributed to biosurfactant activity and were considered to be inconclusive (Figure 2.6 – 2.9). Notwithstanding, from the pH oil spreading assays, several isolates sourced from Samples C and D showed elevated levels of biosurfactant activity that was consistent with the *B. velezensis* control over all the pH ranges tested. These isolates were consistent with those that performed well in the preliminary screening and the temperature testing assays

Of the three parameters tested, salinity had the most significant effect on biosurfactant activity. Increase in salt concentration led to decrease in oil displacement activity for some of the isolates. Even with the best performing isolates including the positive control, there appeared to be reduction in activity. The most consistent results were observed at 0.5% salinity (Figure 2.10 – 2.13). Although some studies such as Couto *et al*. (2015) have shown that lipopeptides produced by *Bacillus* species can withstand salt concentration of up to 20%, it was not the case in this study. This could be because increased salinity had an effect on the fatty acid or carbon atoms present in the chemical structure of lipopeptide which in turn led to a decrease or loss in oil displacement activity.

The ability of the biosurfactants produced by these isolates to withstand the different environmental parameters could be because of their chemical structure and microbial origin (Banat *et al*., 2010). Lipopeptide biosurfactants produced by *Bacillus* species such as *B. licheniformis* and *B. subtilis* are known to remain potent under extreme temperature, pH, and salinity and as such are regarded as useful for various activities such as bioremediation and enhanced oil recovery (Sekhon *et al*., 2012; Kaloorazi *et al*., 2013; Varadavenkatesan and Murty, 2013; Elazzazy *et al*., 2014; Chen *et al*., 2015). It is possible that the isolates from the C and D sample depths were able to exhibit exceptional biosurfactant activities as high as the *B. velezensis* R16 control because having stayed in an undisturbed wetland for years have developed the ability to express molecular components which enables them to thrive irrespective of extreme environmental conditions.

The isolates which showed the highest levels of biosurfactant activity and stability were further tested for their ability to reduce surface tension and emulsification capacity. The surface tension measurement was carried out using the Du-Nouy ring method (Tadros, 2005; Chen *et al*., 2007). This method works by measuring the force needed to separate a ring or plate from a liquid surface (in the case of this study - nutrient broth) (Tadros, 2005). Biosurfactant producers are considered to be significant if they are able to reduce surface tension below 40 mN/m (Desai and Banat, 1997; Soberón-Chávez and Maier, 2011; Bezza and Chirwa, 2015). The isolates evaluated in this study were able to reduce surface tension to between 44.7 to 30.6 mN/m whilst the *B. velezensis* R16 control reduced surface tension to 29.6 mN/m (Section 2.3.4). Surfactin at a concentration of 20 µM produced in culture media has been reported to be able to reduce surface tension of water from 72 to 27 mN/m (Cooper and Goldenberg, 1987; Banat, 1993; Chen et al., 2015).

The Emulsification index (E24) is a method that measures the ability of biosurfactants to form stable emulsion by causing the mixing of immiscible liquids and is used as a way of determining biosurfactant emulsification capacity (Inès and Dhouha, 2015; Ndlovu, 2017). The ability of biosurfactants to form stable emulsion is an important feature useful in diverse industries such as cosmetics, food, pharmaceuticals, and oil recovery (Ndlovu, 2017). The E24 index was carried out using different oils to determine the emulsion formation of the test isolates for the oils.

Comparing the results of the vegetable seed oil with the paraffin oil, six out of the eight isolates exhibited a higher percentage of E24 for the paraffin oil. This may be because paraffin contains a mixture of higher alkanes. The ability of the lipopeptide compounds to emulsify paraffin is a pointer that they may be suitable candidates for hydrocarbon degradation amongst other functions.

It was expected that the isolates which reduced surface tension better would exhibit high emulsification indices, but it was not always the case (Section 2.3.3). This outcome is not out of place as it has been shown that the ability of an isolate to form a stable emulsion does not necessarily correlate with the ability to reduce surface tension (Van Dyke *et al*., 1993; Willumsen and Karlson, 1997; Plaza *et al*., 2006).

2.5 Conclusion

The different assays used confirmed that biosurfactant producing AEFB were present amongst the isolates screened. Drop-collapse assay appeared to be the most sensitive of the assays used, while the oil spreading assay was found to be more efficient in showing a clear difference between positives and negatives. The biosurfactant activity appeared to be wide spread amongst the isolates. However, the proportion of isolates with enhanced biosurfactant activity was much less; only eight isolates sourced from Sample C and D showed levels of activity consistent with the lipopeptide producing reference strain *B. velezensis* R16. Selected isolates showed enhanced activity over a range of environmental parameters. These isolates were then selected for further testing to determine their emulsification capacity and their ability to reduce surface tension. Isolates which exhibited high emulsification capacity showed lower ability to reduce surface tension.

CHAPTER THREE

CHARACTERIZATION OF BIOSURFACTANT COMPOUNDS PRODUCED BY SELECTED AEROBIC ENDOSPORE-FORMING BACTERIA ISOLATES FROM MFABENI PEATLAND

3.1 INTRODUCTION

Aerobic endospore-forming bacteria (AEFB) comprise a collection of bacteria capable of forming resistant endospores at the onset of unfavorable conditions; these include conditions such as a lack of nutrients, a build-up of toxic metabolites, and desiccation (Fritze, 2004; Henriques and Moran, 2007). Representatives of this group of bacteria have been isolated from diverse environments such as Antarctic soils (Pearce *et al*., 2012; Vollú *et al*., 2014); human gastrointestinal tracts (Fakhry *et al*., 2008); and marine sediments (Chen *et al*., 2017) amongst others. In this study, AEFB isolates from Mfabeni peatland, a wetland environment were screened for biosurfactant activity. The purpose of the study was to screen for biosurfactant production amongst AEFB and select promising biosurfactant isolates for further characterization.

Biosurfactants have gained attention over the years because of their surface-active ability which makes them useful in various industries including agricultural, pharmaceutical, cosmetics, food, and bioremediation industries (Lai *et al*., 2009; Pacwa-Plociniczak *et al*., 2011; de Franca *et al*., 2015). Biosurfactants are preferred over synthetic chemical surfactants because they are potentially non-toxic, easily degradable, environmentally friendly and may be able to withstand extreme salinity, temperature, and pH (Yang *et al*., 2015; Moro *et al*., 2018).

Members of the genus *Bacillus* belonging to the AEFB group, are known for their ability to produce a range of bioactive compounds (Katz and Demain, 1977; Stein, 2005; Ongena and Jacques, 2008). Many of these bioactive compounds are amphiphilic in nature and have biosurfactant properties (Roongsawang *et al.,* 2002). An important class of such compounds are the lipopeptides, which are secondary metabolites that appear to be produced mainly by

members of the *Bacillus subtilis* cluster of related taxa (Roongsawang *et al*., 2002; Singh and Cameotra, 2004; Sun *et al*., 2006; Meena *et al*., 2018). Surfactin, fengycin, and iturin are the three main types of lipopeptide associated with this group (Ongena and Jacques, 2008; Geissler *et al*., 2017; Moro *et al*., 2018). Kurstakins are a lesser known group of lipopeptides produced by strains of *B. cereus* and *B. thuringiensis* (Béchet *et al*., 2012).

In addition to their biosurfactant properties lipopeptides are recognized for a range of activities, which include antibacterial, antifungal, insecticidal and antiviral properties (Arima *et al.,* 1968; Winkelmann *et al.,* 1983; Kracht *et al.,* 1999; Geetha *et al.,* 2010). Surfactin is the most widely studied of these lipopeptide compounds and is currently regarded as the most potent of all the lipopeptide biosurfactant described to date (Vater *et al.,* 2002). It is known to lower the surface tension of water from 72 to 27 mN/m and also exhibits antibacterial activity (Vollenbroich *et al.,* 1997; Ongena and Jacques, 2008; Kim *et al.,* 2010).

Various methods have been used to characterize and identify biosurfactant compounds. Examples include Gas chromatography mass spectrometry (GC-MS), Ultra performance liquid chromatography (UPLC), Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and Fast atom bombardment mass spectrometry (FAB-MS) (Makkar and Cameotra, 1998; Leenders *et al.,* 1999; Satpute *et al.,* 2010; Tredgold, 2015). The acid precipitation method has also been successfully used to extract and partially purify lipopeptide compounds from *Bacillus* species (Vater *et al*., 2002; Hsieh *et al*., 2008; Hunter, 2016).

Methods such as repetitive element polymerase chain reaction (REP-PCR), random amplification of polymorphic DNA PCR (RAPD-PCR), pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and 16S rRNA gene sequence analysis are examples of tools used for differentiating between, and identifying bacterial species (Versalovic *et al*., 1994; Klima *et al*., 2010; Munday *et al*., 2013; Garcia *et al*., 2015). REP-PCR is a fingerprinting method that uses a primer set that binds with a repetitive DNA sequence commonly found within bacterial genomes (Ndlovu, 2017). When target sequences are amplified the gene fragments produce a strain specific fingerprint that can be observed as banding patterns through gel electrophoresis (Ndlovu, 2017). REP-PCR has been successfully used to distinguish and identify biosurfactant

producers amongst different bacterial genomes (Bodour *et al*., 2003; Tran *et al*., 2008; Ndlovu, 2017). 16S rRNA gene sequence analysis is a benchmark identification technique that allows for genus and species level delineation and classification (Mandic-Mulec *et al*., 2015). It is also used for the determination of phylogenetic relationship between closely related aerobic endospore-forming bacteria species (Agbobatinkpo *et al*., 2013).

The aim of this study was to characterize biosurfactant compounds produced by selected AEFB isolates from Mfabeni peatland. REP-PCR and 16S rRNA gene sequence analysis were used to distinguish and classify AEFB isolates. Crude biosurfactant extracts were analyzed using TLC and UPLC in conjunction with electrospray ionization- time of flight mass spectrometry (ESI-TOF MS) to identify the biosurfactant compounds produced.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial isolates

Eight AEFB isolates SAB19, SAB42, SAC15, SAC18, SAD5, SAD17, SAD18, and SAD23 were selected for further characterization based on their surfactant properties (Chapter two). *Bacillus velezensis* R16, a known biosurfactant producer, was used as a reference strain during this study (Hunter, 2016). The cultures of these isolates were maintained on 10% TSA and TSB as described previously (Section 2.2.3).

3.2.2 Biosurfactant production and acid precipitation

Isolates were sub-cultured on 10% TSA plates and incubated for 24 h at 30℃. Single colonies from each of the isolates were then transferred into 10 mL TSB and cultured for 24 h at 30℃ in a rotary shaker (120 rpm) (MRC Laboratory Equipment, Israel). One milliliter from each culture was inoculated into 10 mL Landy medium (glucose, 15 g; L-glutamic acid, 5 g; $MgSO_4 \bullet 7H_2O$, 1.02 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MnSO_4 \bullet H2O$, 5 mg; $CuSO_4 \bullet 5H_2O$, 0.16 mg; and, $FeSO_4 \bullet 7H_2O$, 150 µg; 1000 mL distilled water; pH 6.0) (Mckeen *et al*., 1986) and cultured for 24 h under the same conditions described previously. Ten milliliters of each Landy medium culture were then transferred into 100 mL Landy medium and cultured as described above for 72 h. The culture media were then

centrifuged at 12,000 × g for 30 min at 4℃ to pellet cellular biomass (JA-10 rotor, Avanti J-26XPI, Beckman, USA). Biosurfactants produced were extracted from cell-free supernatant through acid precipitation, which was done by adjusting the pH of the supernatant to pH 2.0 using 1N HCl (Vater *et al*., 2002). The supernatant was kept overnight at 4℃ before undergoing a centrifugation step (12,000 x g for 30 min at 4 ℃), afterwhich the supernatant was removed, and the precipitate extracted twice with methanol (2 x 2.5 ml). These methanol extracts were stored at -20℃ for future use. To check the efficiency of extraction, the extracts were tested for their surfactant ability using oil spreading assay (Section 2.2.3.3).

3.2.3 Thin layer chromatography (TLC)

TLC Silica Gel DC 60 F_{254} aluminum plates (5x10 cm) (Merck, Darmstadt, Germany) were used to observe and separate the methanol extracts. Solvent mixture (70:30 ($^{v}/_{v}$)- propan-1-ol: water) was poured into TLC tank not more than 1 cm from the bottom of the tank and covered for 20 min to allow a saturation of the atmosphere in the tank (Brenner *et al*., 1965; Hunter, 2016). Methanol extracts (20 µL) were spot inoculated onto the TLC plates and separated using the solvent mixture. The developed plates were viewed using Ultra Violet (UV) illumination to detect separated compounds, and by spraying with atomized water to detect hydrophobic regions. The retention factor (Rf) values of the separated compounds were calculated according to the formula established by Razafindralambo *et al*. (1993)

After viewing the TLC plates under UV, they were dried with a hairdryer to remove solvent traces and then sprayed with distilled water to detect hydrophobic regions which are indicative of amphiphilic biosurfactant compounds. The hydrophobic regions were then scraped off the plates using clean glass slides and transferred into 1.5 mL microfuge tubes. Methanol (500 µL) was added to each tube followed by vortexing for 2 min and allowing to stand overnight to enhance the extraction of the biosurfactant compounds. Thereafter, the microfuge tubes were centrifuged (12,000 X g for 5 min) to separate the silica gel scrapings from the methanol extracts. The extracts were kept at -20℃ for further use.

3.2.4 Ultra Performance Liquid Chromatography (UPLC) in conjunction with mass spectrometry (MS)

The crude methanol extracts of each isolate, as well as selected hydrophobic regions scraped off TLC plates, were further characterized using reverse-phase Ultra Performance Liquid Chromatography (UPLC) in conjunction with electrospray ionization-mass spectrometry (ESI-MS). Surfactin and iturin A standards (10 µg/mL) were included as reference standards. Lipopeptide extract obtained from *B. velezensis* R16 was also used as a reference control. This isolate has been previously characterized and reported to produce lipopeptide homologs; surfactin, bacillomycin, and fengycin (Hunter, 2016). The extracts (5 µL) were loaded onto a Waters Acquity BEH C_{18} column (2.1 × 100 mm, particle size of 1.7 µm, 35°C) and were separated under Ultra Performance Liquid Chromatography conditions (UPLC, Waters Acquity, Milford, MA, USA). Two solvents 0.2% ($^{v}/_{v}$) acetic acid and methanol were used for the separation at a 0.35 ml min^{-1} flow rate. The UPLC system was originally run isocratically for 30 mins with the two solvents at ratio 9: 1, thereafter, there was a 10% to 100% methanol increase over a 30 – 38 mins period before resuming back to the original isocratic run. Electrospray ionization- mass spectrometry detected eluted compounds and their molecular weight were determined using a Waters LCT Premier, Time-of-Flight mass spectrometer system fitted with an electrospray ionization source (Waters). UPLC and ESI-MS analysis was performed at the Sasol Mass Spectrometry Laboratory, Discipline of Chemistry, University of KwaZulu-Natal, Pietermaritzburg campus, South Africa.

3.2.5 Extraction of template DNA for Polymerase Chain Reaction (PCR)

AEFB isolates were sub-cultured on 10% TSA plates and incubated for 24 h at 30°C. Single colonies of each isolate were picked-off using a sterile inoculating loop and transferred into 200 µL Tris-EDTA (TE) buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) in 2 mL microfuge tubes. The tubes were vortexed to homogenize the content and then heated at 95°C for 15 mins in a dry heating block (Accublock™, Labnet International, Inc., USA). All tubes were then centrifuged at 10 000 x g for 1 min (Heal Force Neofuge 13) and the supernatant was stored at -20°C for future use.

3.2.6 Repetitive extragenic palindromic-Polymerase Chain Reaction (REP-PCR)

REP-PCR fingerprinting was performed according to the method described by Urzi *et al*. (2001), using a BOX-A1R primer. KAPA 2G Robust PCR kit reagents (KAPA Biosystems, Inc, U.S.A) were used to run the PCR reactions. Each reaction contained 1X buffer, 1.5 mM MgCl2, 0.2 mM dNTP, 0.4 µM primer (Table 3.1), 0.5 U Taq polymerase, 1 µL of template DNA, and nuclease-free water (Promega, Madison, U.S.A) to bring the volume up to 25 µL. *Bacillus velezensis* R16 which had been previously amplified using this PCR kit was used as a positive standard, while a negative standard containing nuclease-free water (1 µL) with no template DNA was also included.

The temperature profile for the reaction had an initial denaturation at 95℃ for 5 mins, followed by 35 cycles consisting of denaturation at 95℃ for 1 min, annealing at 45℃ for 1 min and extension at 72℃ for 2 mins. A final extension of 72℃ for 10 mins was included at the end of the PCR cycles. PCR reactions were performed using a G-storm GS1 thermal cycler (G-storm, Sommerset, U.K). PCR reaction samples were separated and visualized by electrophoresis using a 1.5% ($^{w}/_{v}$) agarose gel. One microliter of 6 x DNA loading dye (Thermo scientific) was mixed with 5 µL of each PCR amplicon sample prior to loading and each gel was then run at 80 V for 80 mins (BG-Power 300, BayGene, Vacutec, China) in 1 x Tris-Borate-Ethylenediaminetetraacetic acid (TBE) buffer (89 mM Tris base, 89 mM Boric acid and 2 mM EDTA with pH 8.3). A 1 kb DNA ladder (Thermo-Scientific Gene Ruler) was included in each gel to estimate band sizes. SYBR™ safe dye (X1) (Invitrogen, USA) was used to stain the gels and then viewed using ultraviolet illumination. Gel images were captured using Genesnap™ software (Syngene v 7.0.9, England).

3.2.7 16S rRNA gene amplification

Gene amplification of selected AEFB isolates was carried out according to the method of Ström *et al.* (2002) and Tzuc *et al.* (2014). PCR reactions were carried out in a G-storm GS1 thermal cycler (G-storm, Sommerset, U.K) using 25 µL reaction volumes consisting of 2.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of DNA polymerase (2G Robust, KAPA), 1X buffer, 0.4 µM of each primer (Table 3.1), and 2 µL of template DNA (section 3.2.5). The final reaction volume was made up to 25 µL using nuclease-free water (Promega). Template DNA from *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7 was included as a positive control. The negative standard contained

nuclease-free water (2 µL) and had no template DNA. The temperature profile used included initial denaturation at 94℃ for 5 mins, followed by 30 cycles consisting of a denaturation at 94℃ for 30 secs, annealing at 54℃ for 30 secs, and an extension step at 72℃ for 80 secs. A final extension at 72℃ for 5 mins was included. Presence of amplified DNA from each PCR reaction was confirmed by agarose (1.5% $^{w}/_{v}$) gel electrophoresis, performed as described previously (Section 3.2.6).

Table 3.1: Sequence of the primers (BOX-A1R) used for REP-PCR and 16S rRNA reactions

Primers	Sequence (5' – 3')	Reference
REP-PCR	CTACGGCAAGGCGACGCTGACG	Versalovic *et al*., 1994
16S rRNA Forward primer	AGAGTTTGATCCTGGCTC	Ström *et al*., 2002
16S rRNA Reverse primer	CGGGAACGTATTCACCG	Ström *et al*., 2002

3.2.8 16S rRNA gene sequencing analysis

The resulting 16S rRNA gene fragment amplification products were sent to Inqaba Biotech™ (Hatfield Pretoria, South Africa) for sequencing using an ABI 3130XL sequence analyzer (Applied Biosystems). Amplicons were first purified using Wizard PCR Prep Kits (Promega) prior to sequencing with ABI PRISM Dye Terminator Cycle Sequencing Kit. Sequencing reads were made using both forward and reverse primers.

3.2.9 16S rRNA gene sequence phylogenetic analysis

Chromas Lite software (version 2.6.2) was used to edit the sequence chromatograms from the sequencing reads of each isolate. Alignment of the sequence data and generation of consensus sequences for each isolate was undertaken using Bioedit software (version 7.2.5.0) (Hall, 1999). Consensus sequences were saved in the FASTA format and then compared to 16S rRNA gene sequences deposited in the GenBank database (https://blast.ncbi.nlm.nih.gov/genbank) using the Megablast search algorithm to detect highly similar sequences. The GenBank search was

limited to type and reference strains within the 16S rRNA gene sequence database. Phylogenetic trees were created using 16S rRNA gene sequence data to infer the evolutionary relationship of the selected AEFB isolates. Mega software (version 7.0.2.1) was used to construct phylogenetic tree using Neighbor-Joining and Maximum-Likelihood methods (Tamura *et al.,* 2013). The Tamura-Nei substitution model was used to evaluate genetic distance for the Neighbor-Joining analysis and corroborated using Bootstrap analysis based on 1000 replications (Felsenstein, 1985). The Kimura 2-parameter substitution model was used to evaluate the Maximum-Likelihood analysis method and estimate evolutionary distances by means of bootstrap values based on 1000 replicates. Sequences were first aligned using a MUSCLE alignment algorithm (Edgar, 2004). Missing data and all nucleotide basepair gaps were excluded from pairwise sequence comparisons. 16S rRNA gene sequence data of phylogenetically related taxa was included for comparative purposes. Rooting of trees was achieved using 16S rRNA gene sequence of *Clostridium beijerinckii* JCM 8026 as an outgroup.

3.3 Results

3.3.1 Acid precipitation and methanol extract

The extracted fractions of all the selected isolates (Section 3.2.2) exhibited biosurfactant activity when tested using the oil spreading assay. This indicated that the acid precipitation extraction step was successful in each instance.

3.3.2 TLC separation and analysis

Crude biosurfactant extracts obtained from culture via acid precipitation and methanol extraction were analysed using TLC. Figure 3.1 shows the TLC hydrophobic band profiles observed for *B. velezensis* R16 and the selected AEFB isolates when a propan-1-ol: water (70:30) solvent mix was used. Rf values for the hydrophobic regions were calculated (Table 3.2). These regions were considered to be amphiphilic compounds, which are associated with biosurfactant properties. Some of the hydrophobic regions observed are not clear in Figure 3.1 as the image was captured when the TLC plate was still partially wet. The zones visualized have been marked in the image. For *B. velezensis* R16, 5 hydrophobic regions were distinguished (Table 3.2). The Rf

values obtained for R16 corresponded with those established and identified in a previous study by Hunter (2016) (Appendix D).

Table 3.2: Rf values obtained for the hydrophobic region of the selected AEFB isolates visualized using TLC

Isolate	Rf values				
B. velezensis R16	0.89	0.81	0.74	0.64	0.33
SAB19	0.85				
SAB42	0.85	0.15			
SAC15	0.89	0.13			
SAC18	085	0.17			
SAD5	0.85	0.12			
SAD17	0.81	0.71	0.59	0.17	
SAD18	0.88	0.75	0.13		
SAD23	0.87	0.77	0.72		

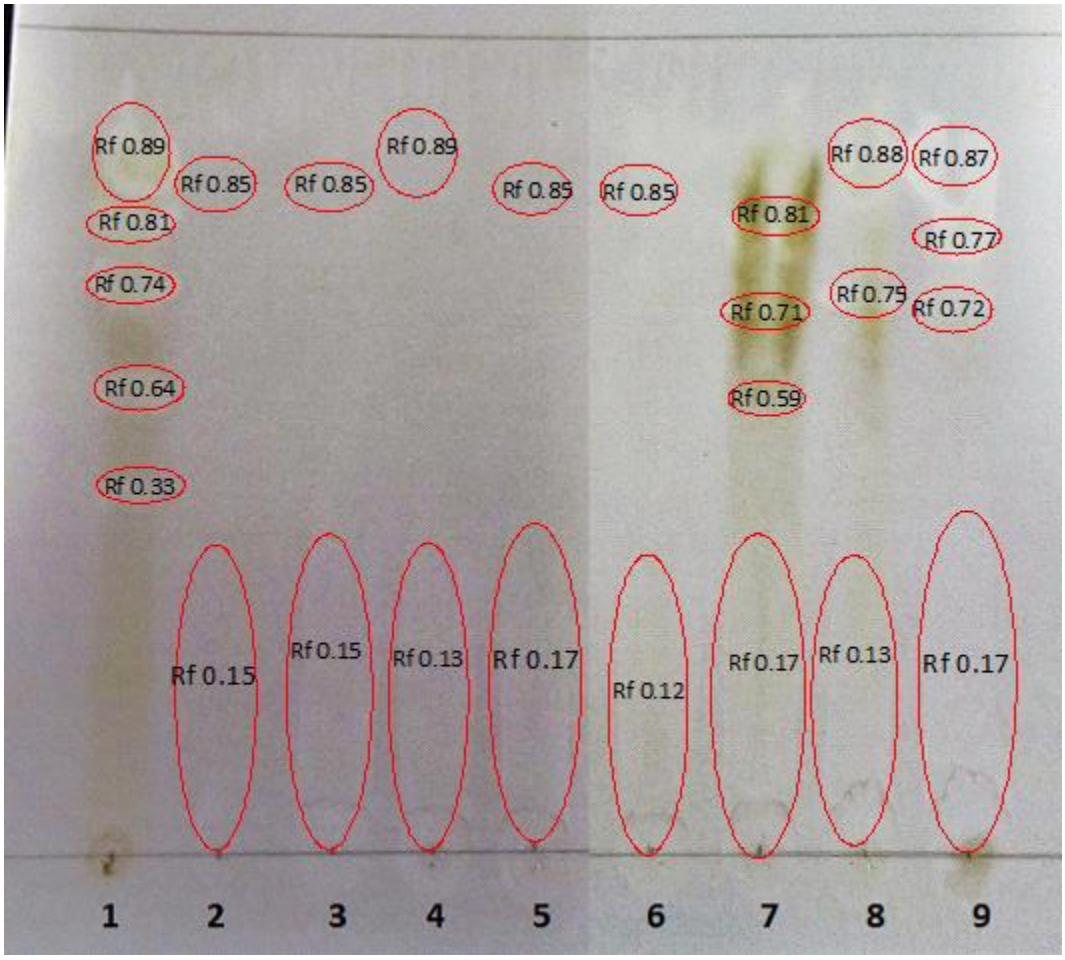

Figure 3.1: Thin layer chromatography of methanol extracts demonstrating hydrophobic region associated with AEFB isolates: 1- *B. velezensis* R16 control; 2- SAB19; 3- SAB42; 4- SAC15; 5- SAC18; 6- SAD5; 7- SAD17; 8- SAD18; and, 9- SAD23. Compound bands were detected by spraying the surface with distilled water to detect hydrophobic regions.

3.3.3 Mass Peaks detected by UPLC in conjunction with ESI-TOF MS

The methanol extract of each selected AEFB isolate was analyzed by UPLC ESI-TOF MS and the mass peaks at different elution times for each isolate were evaluated. Figure 3.2 shows the chromatogram for the crude methanol extract of the *B. velezensis* R16 control. The mass peaks eluted by R16 control were found to be consistent with those reported in a previous study by Hunter (2016). The mass peaks of the selected AEFB isolates were identified and assigned by comparing them with those reported in Hunter (2016) (Appendix D) as well as the mass peaks obtained for the surfactin and iturin A standards (Appendix E).

The peaks labelled A, B, and C in the chromatogram represent homologs of bacillomycin, fengycin, and surfactin lipopeptide compounds respectively. Figures 3.3 – 3.5 shows the mass peaks of major representative lipopeptide homologs eluted.

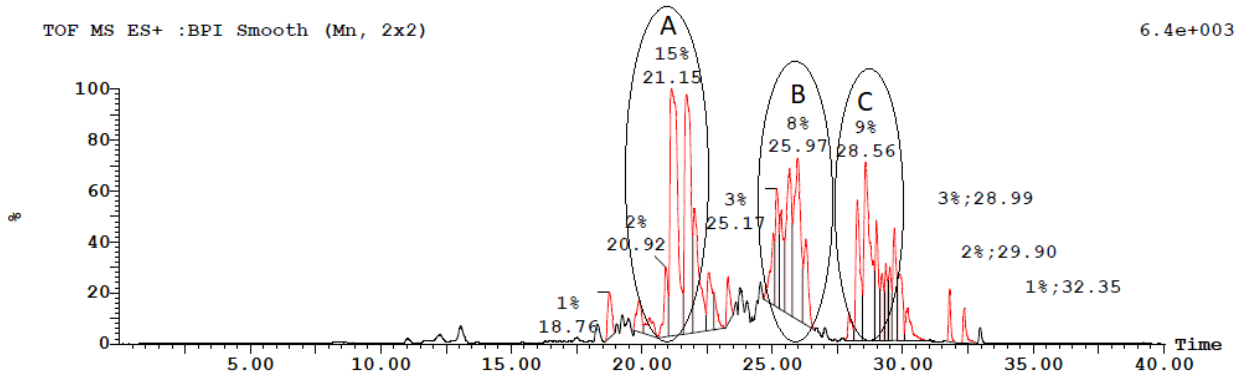

Figure 3.2: UPLC-ESI-TOF MS chromatogram of crude extract from *B. velezensis* R16 control. Portions labelled A, B, and C are representatives of lipopeptide compounds. Peak A- bacillomycin, peak B- fengycin, and peak C- surfactin

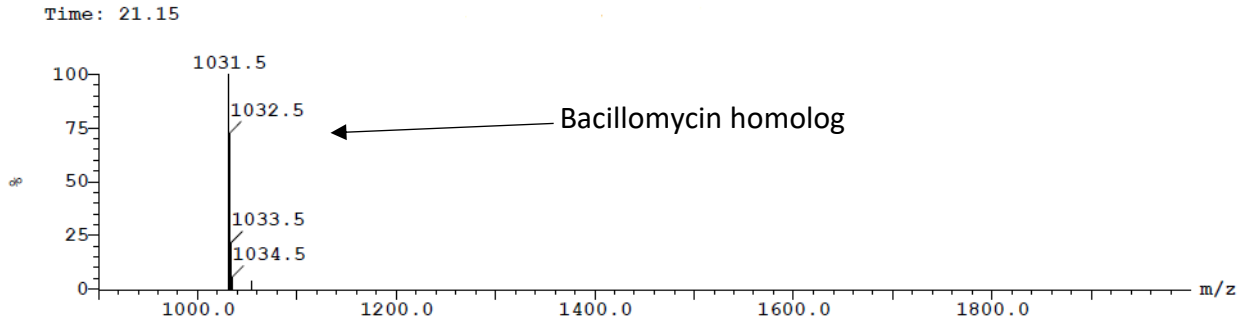

Figure 3.3: Mass peak of peak A which eluted at time 21.15 with m/z 1031.5 represents a bacillomycin homolog

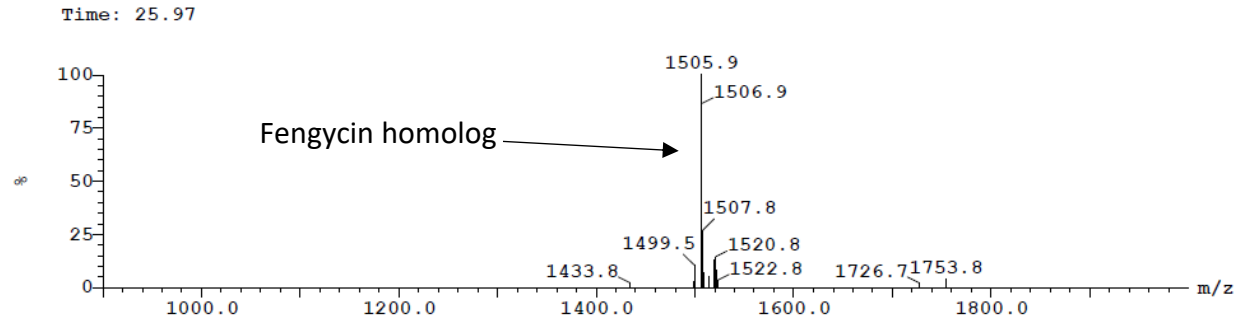

Figure 3.4: Mass peak of peak B which eluted at time 25.97 with m/z 1505.9 represents a fengycin homolog

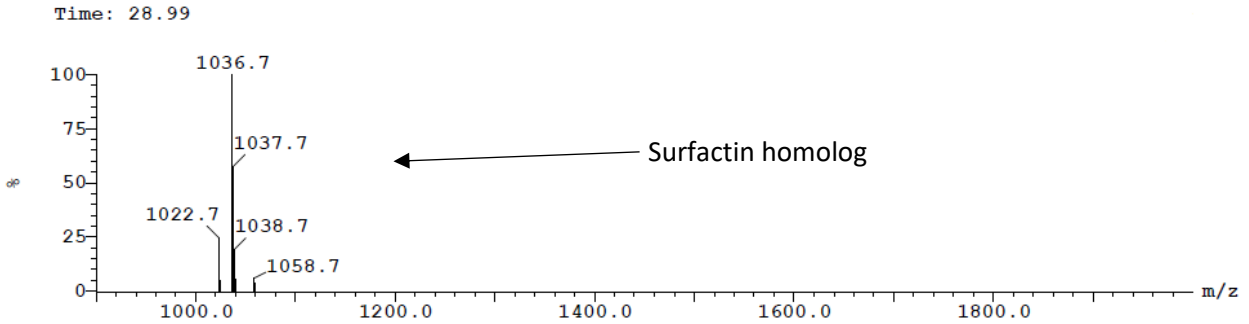

Figure 3.5: Mass peak of peak C which eluted at time 28.99 with m/z 1036.7 represents a surfactin homolog

Figure 3.6 – 3.13 shows the chromatogram obtained for the crude extract of each of the selected isolates. The *B. velezensis* R16 control had more prominent mass peaks compared to isolate SAB19, SAB42, SAC15, SAC18, and SAD5 while isolates SAD17, SAD18, and SAD23 showed as much prominent mass peaks as the control.

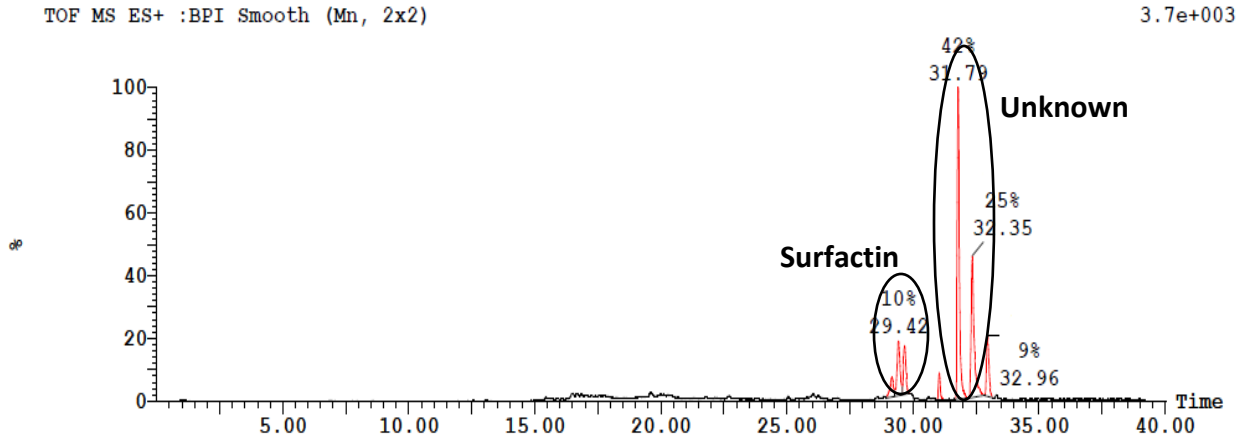

Figure 3.6: Chromatogram of the crude extract for isolate SAB19 obtained using UPLC ESI-TOF MS

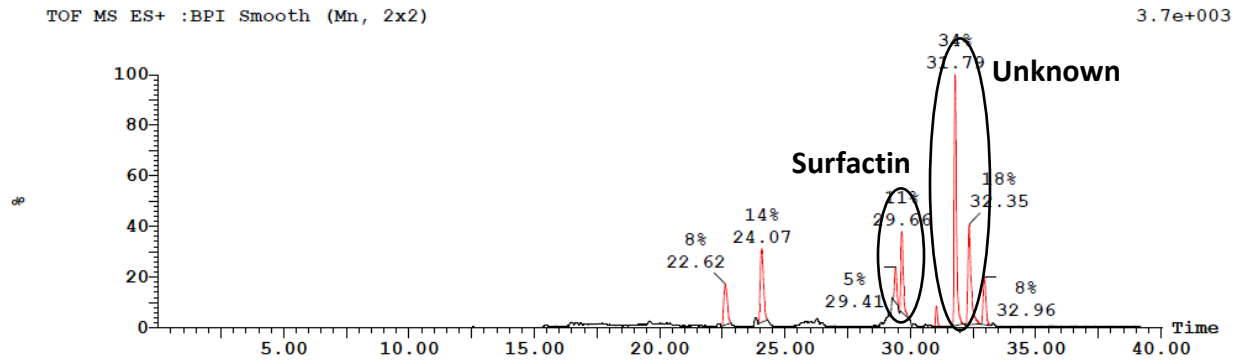

Figure 3.7: Chromatogram of the crude extract for isolate SAB42 obtained using UPLC ESI-TOF MS

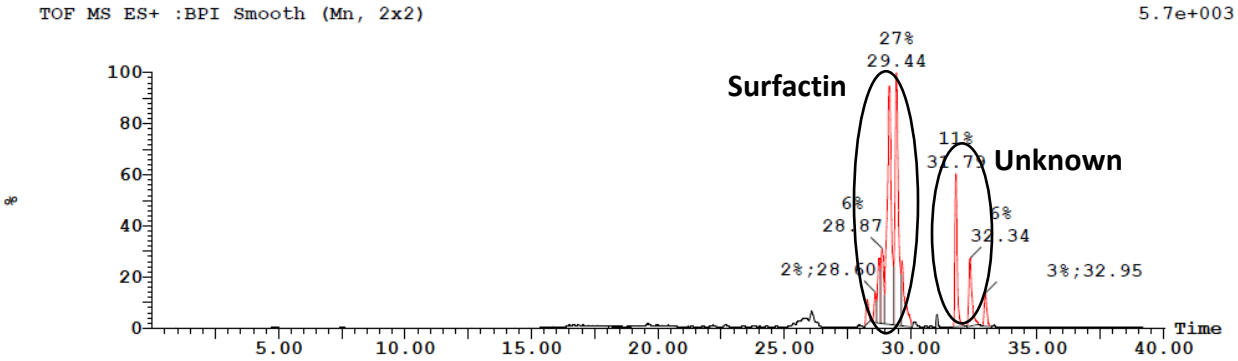

Figure 3.8: Chromatogram of the crude extract for isolate SAC15 obtained using UPLC ESI-TOF MS

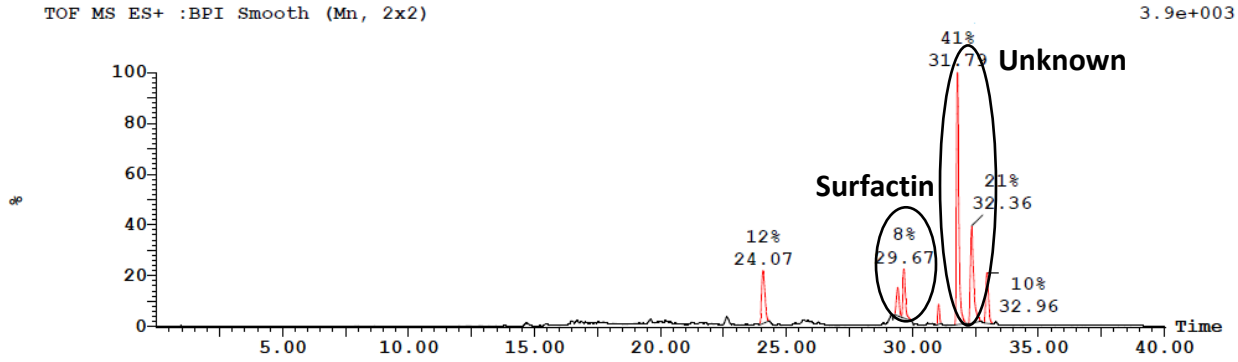

Figure 3.9: Chromatogram of the crude extract for isolate SAC18 obtained using UPLC ESI-TOF MS

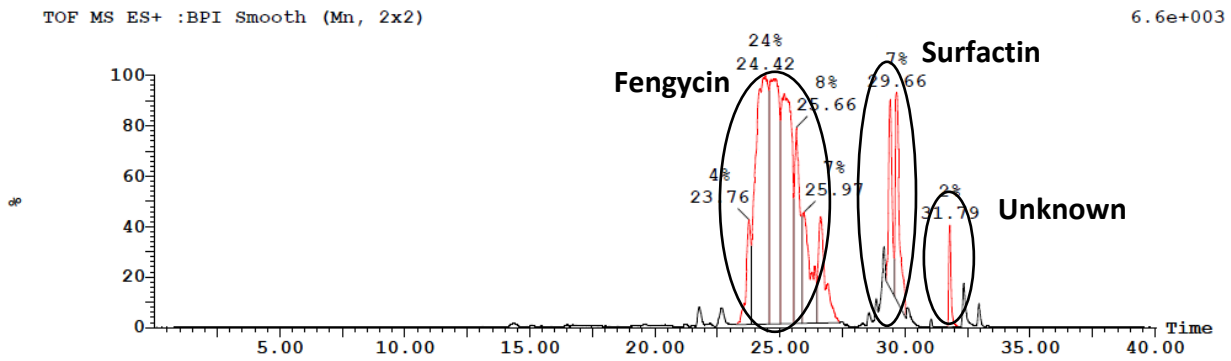

Figure 3.10: Chromatogram of the crude extract for isolate SAD5 obtained using UPLC ESI-TOF MS

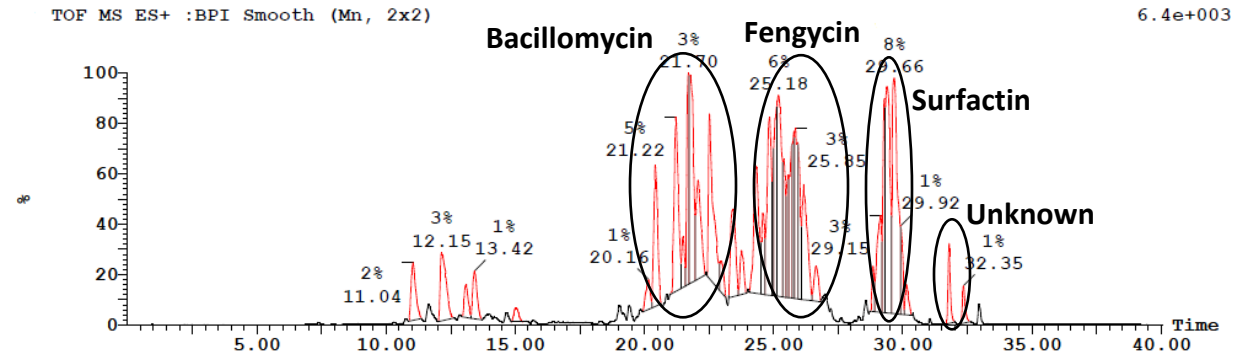

Figure 3.11: Chromatogram of the crude extract for isolate SAD17 obtained using UPLC ESI-TOF MS

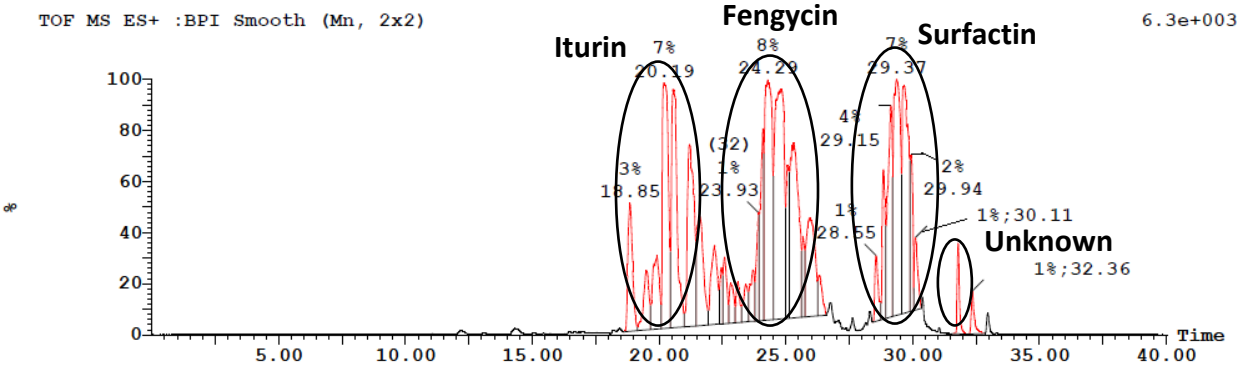

Figure 3.12: Chromatogram of the crude extract for isolate SAD18 obtained using UPLC ESI-TOF MS

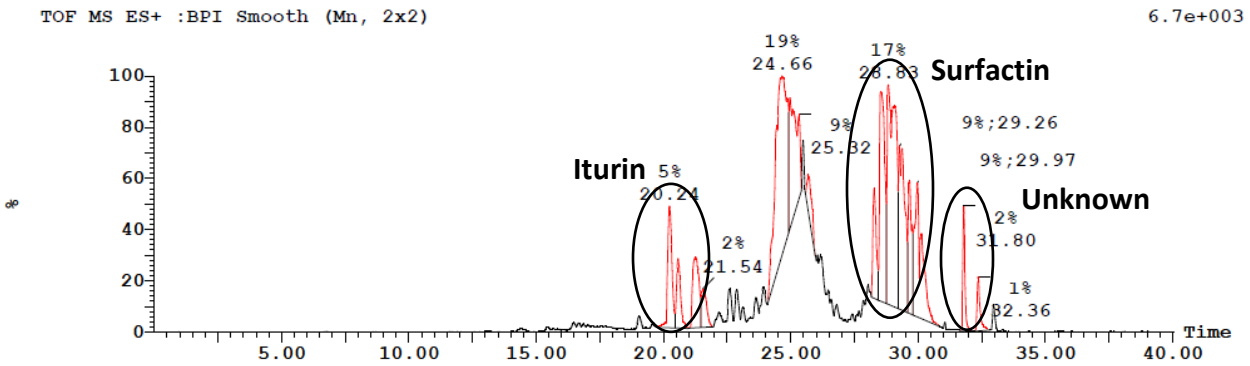

Figure 3.13: Chromatogram of the crude extract for isolate SAD23 obtained using UPLC ESI-TOF MS

The different types of lipopeptide compounds, homologs, mass peaks (m/z) and theoretical values (m/z) identified for each of the AEFB isolates are listed in Table 3.3. The amphiphilic compounds with these values have previously been identified to be lipopeptide compounds with sodium adducts (Hunter, 2016). Most of the isolates produced different homologs of surfactin. Isolate SAD17 produced surfactin, bacillomycin, and fengycin, SAD18 produced iturin, fengycin, and surfactin, SAD23 produced iturin and surfactin, SAD5 produced surfactin and fengycin, while isolates SAB19, SAB42, SAC15, and SAC18 produced only surfactin homologs.

Table 3.3: Detection of Lipopeptide compounds produced by AEFB isolates identified by UPLC-ESI-TOF MS analysis of methanol extracts from TLC crude extracts

Lipopeptide	Mass peak (m/z)	Theoretical values (m/z)	Homolog	Isolate
Surfactin	1022.7	1217.7	C14 $(M + H)^+$	SAC15, SAD17, SAD18, SAD23
	1036.7	1230.7	C15 $(M + H)^+$	SAB19, SAB42, SAC15, SAD17, SAD18, SAD23
	1050.7	1243.7	C16 $(M + H)^+$	SAB19, SAB42, SAC15, SAC18, SAD5, SAD17, SAD18, SAD23
	1064.7	1256.7	C17 $(M + H)^+$	SAB42, SAC18, SAD5, SAD17, SAD18, SAD23
Iturin	1043.5	1224.6	C14 $(M + H)^+$	SAD18, SAD23
	1079.5	1237.6	C15 $(M + H)^+$	SAD18
	1071.7	1250.6	C16 $(M + H)^+$	SAD23
Bacillomycin	1031.5	1170.5	C14 $(M + H)^+$	SAD17
	1045.5	1183.5	C15 $(M + H)^+$	SAD17
	1059.5	1196.5	C16 $(M + H)^+$	SAD17
Fengycin	1463.8	1670.9	Ala-6-C16 $(M + H)^+$	SAD5
	1477.8	1683.9	Ala-6-C17 $(M + H)^+$	SAD18
	1491.7	1670.9	Val-6-C16 $(M + H)^+$	SAD5, SAD18
	1505.8		Val-6-C17 $(M + H)^+$	SAD17

Each mass peak assignment was done based on standards (surfactin and iturin A) and values reported in the literature (Hunter, 2016)

After comparing and assigning the mass peaks obtained for each isolate with the R16 control, it was observed that an unidentified peak that corresponded to the hydrophobic bands with Rf 0.12 – 0.17 was present in all the isolates (Table 3.4). Figure 3.14 shows a representation of the chromatogram and mass peak of the unknown compound associated with fraction (Rf 0.12 – 0.17) scraped from a TLC plate.

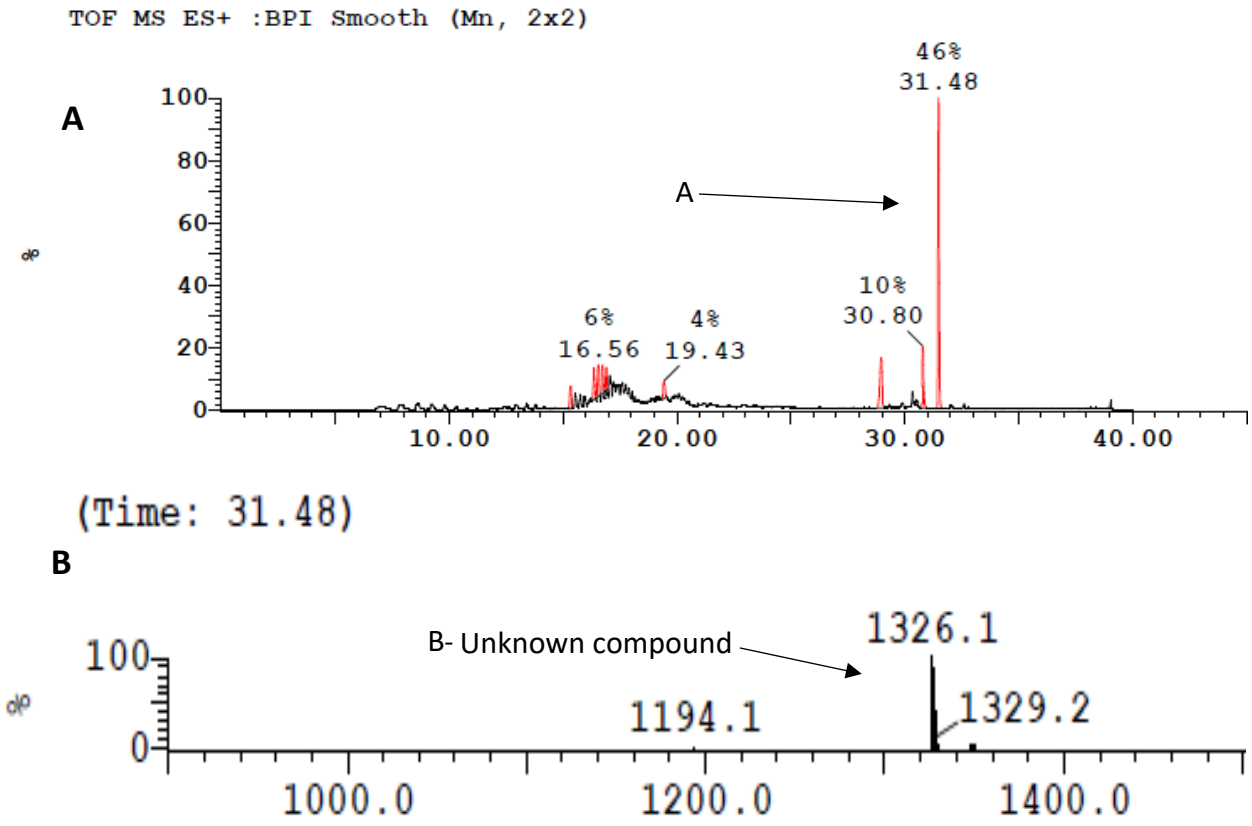

Figure 3.14: Chromatogram (A) and mass peak (B) of hydrophobic fraction (Rf 0.12 – 0.17) from TLC.

Table 3.4: Unidentified peak associated with the hydrophobic region (Rf 0.12 – 0.17) from the scraped fraction of TLC plates

Isolates	Time eluted	Mass Peak (m/z)
SAB42	31:48	1326.1
SAC15	31:47	1326.1
SAD5	31:49	1326.1
SAD17	31:47	1326.2
SAD18	31:49	1326.1
SAD23	31:49	1326.1

3.3.4 Determination of AEFB diversity using Rep-PCR

Figure 3.15 shows the REP-PCR fingerprint profiles obtained for the selected AEFB isolates and the *B. velezensis* R16 control. R16 demonstrated a unique banding profile, as did isolates SAB42, SAB19, and SAC18. Isolates SAD18 and SAD23 showed similar banding patterns based on band presence and intensity. SAC15 also has a similar banding profile although band intensities are different. Isolates SAD5 and SAD17 showed similar banding patterns to one another. With the exception of SAC15, the banding profiles were sample specific.

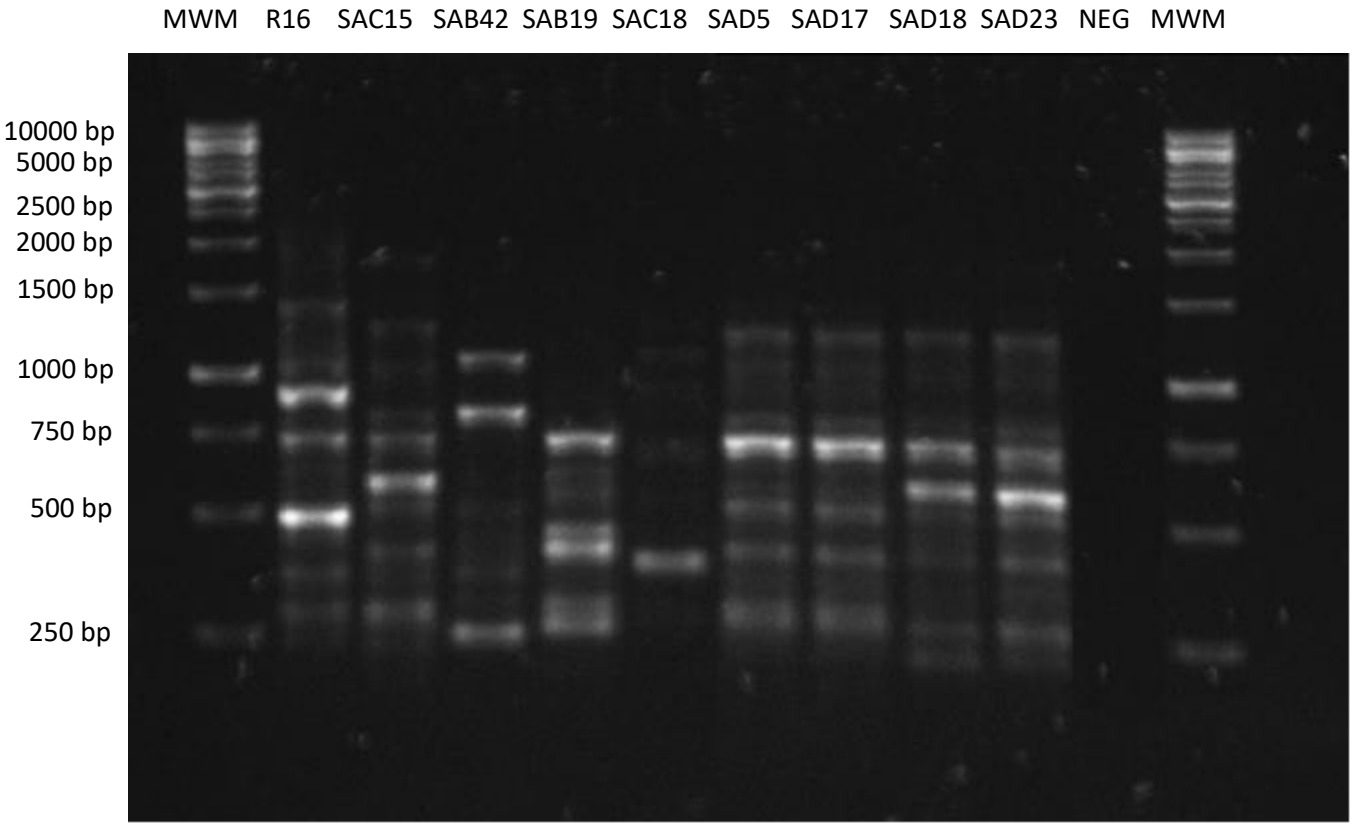

Figure 3.15: Agarose gel (1.5%) electrophoresis image comparing REP-PCR fingerprints of eight AEFB isolates from an ancient Mfabeni peatland sediment core. MWM: 1 kbp DNA ladder; R16-Positive control; Neg: DNA-free control.

3.3.5 Determination of AEFB diversity using 16S PCR amplification

The diversity amongst the AEFB isolates were further evaluated with the 16S rRNA gene sequence PCR amplification (Appendix F) and a phylogenetic analysis was done to determine the evolutionary relationship between the isolates. Each of the test isolates produced product that was approximately 1300 bp.

The neighbor joining and maximum likelihood phylogenetic trees (Figures 3.17 and 3.18) showed similar topologies for both phylogenetic trees. Isolates SAC15, SAC18, SAD17, SAD18, SAD23, and SAD5 could be assigned to the genus *Bacillus* whereas SAB19, and SAB42 could be grouped with members of the genus *Brevibacillus*. The isolates classified as *Bacillus* spp. showed high sequence similarity to members of the *B. subtilis* cluster of related organisms.

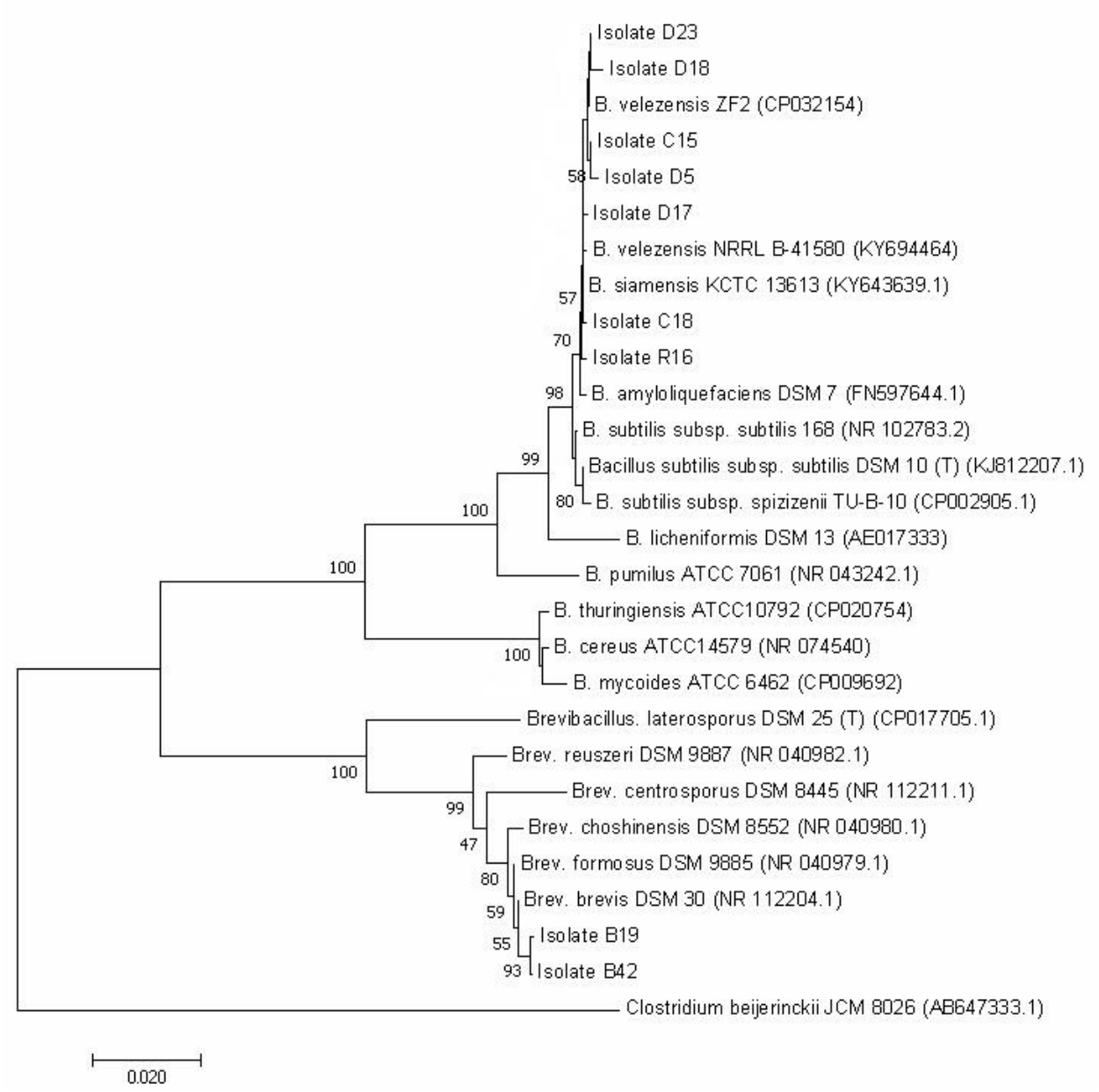

Figure 3.17: The evolutionary history was inferred using the neighbor joining method based on the Tamura-Nei substitution model (Saitou and Nei, 1987). Molecular phylogenetic analysis of AEFB isolates using neighbor joining method. The scale bar corresponds to 0.020 nucleotide substitutions per sequence positions. Abbreviations: B= *Bacillus*; Brev. = *Brevibacillus*.

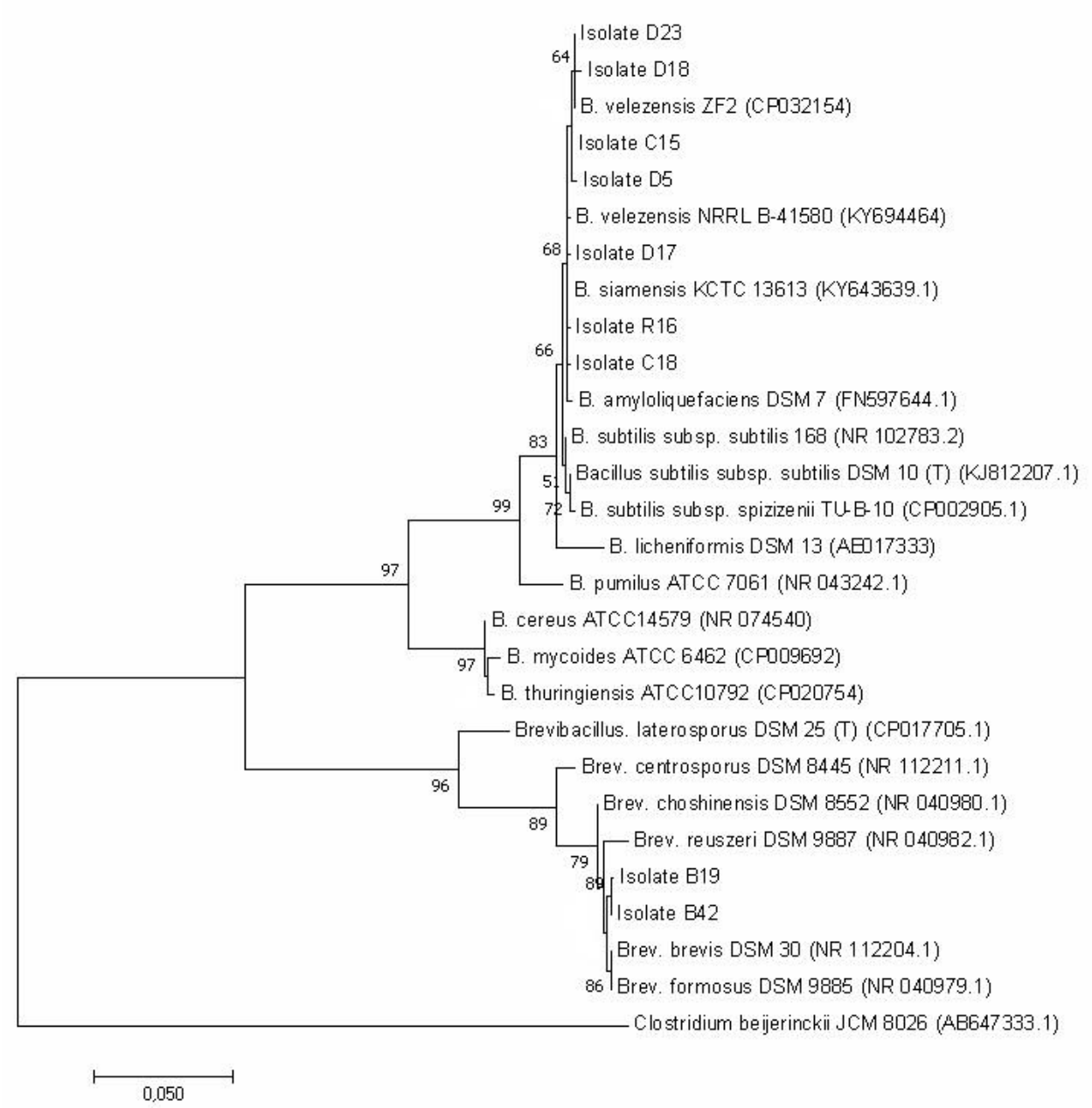

Figure 3.18: Molecular phylogenetic analysis of AEFB isolates by maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980; Kumar et al., 2016). The scale bar corresponds to 0.050 nucleotide substitutions per sequence positions. Abbreviations: B= *Bacillus*; Brev. = *Brevibacillus*.

3.4 Discussion

Bacteria species belonging to the genus *Bacillus* such as *B. subtilis*, *B. atrophaeus*, *B. licheniformis*, *B. pumilus*, *B. amyloliquefaciens*, and *B. velezensis* are commonly associated with lipopeptide biosurfactant production (Morikawa *et al.,* 1992; Koumoutsi *et al.,* 2004; Singh and Cameotra, 2004; Sun *et al.,* 2006; Ongena and Jacques, 2008; Hunter, 2016). Lipopeptides have both hydrophilic and hydrophobic parts which confers their biosurfactant properties (Ongena and Jacques, 2008; Chen *et al*., 2017). They are widely regarded as being environmentally friendly and possess a broad range of antimicrobial spectra. Some have been shown to be active against plant diseases and exhibit powerful antitumor and antiviral activities (Banat *et al*., 2000; Kosaric, 2001; Yang *et al*., 2006; Romero *et al*., 2007).

Several types of lipopeptide such as surfactin, iturin, fengycin, lichenysin, polymyxin A, and daptomycin have been distinguished (Jones, 1949; Roongsawang *et al*., 2002; Ongena and Jacques, 2008; Kim *et al*., 2010; Stankovic *et al*., 2012; Nakhate *et al*., 2013). Of these, the most important appears to be the iturins, surfactins, and fengycins (Ongena and Jacques 2008, Chen *et al.,* 2017). Generally, it has been found that a higher percentage of biosurfactant producing *Bacillus* strains produce only one type of lipopeptide, while a fewer number can produce two or more (Romero *et al.,* 2007, Pecci *et al.,* 2010; Chen *et al*., 2017). Lipopeptides are of great application in various areas such as environmental, agricultural, medical, and food industries (Banat *et al.,* 2000; Nakhate *et al*., 2013; Joshi *et al.,* 2016). The aim of this study, therefore, was to characterize and identify biosurfactant producers and compounds produced by selected AEFB isolates revived from an ancient sediment core taken from Mfabeni peatland.

The isolates and biosurfactant compounds characterized and identified in this study were selected based on their superior biosurfactant activities (Chapter two). These isolates produced lipopeptide biosurfactants which have been previously associated with members of the genus *Bacillus* (Roongsawang *et al*., 2002; Ongena and Jacques, 2008; Hunter, 2016). The reference strain *B. velezensis* R16 has been previously identified as a lipopeptide producer and, therefore, was used as a reference standard to confirm the extraction methods used and to match and assign the mass spectra of peaks from chromatogram of the AEFB isolates (Hunter, 2016).

An acid precipitation method was used to extract lipopeptide compounds produced by the selected AEFB isolates from the culture broth. This method proved effective as the extracted compounds from each isolate exhibited biosurfactant activity after testing using the oil spreading assay (Section 3.2.2). It has been successfully used for lipopeptide extraction in various studies (Vater *et al*., 2002; Wei *et al*., 2010; Hunter, 2016; Farias *et al*., 2017; Liang *et al*., 2017).

For partial purification and preliminary identification of the biosurfactant compounds produced, methanol extract of acid precipitate from cell-free supernatant of each isolate was separated on TLC plates (Section 3.2.3). The solvent mixture (propan-1-ol: water) was able to separate the biosurfactant compounds. Hydrophobic regions were observed on the plates after spraying with atomized water, which corroborates the findings of Hunter (2016). One limitation encountered with the spraying of water was that the visualization of hydrophobic regions was temporary, and they disappeared very rapidly and therefore needed to be noted before the plates were completely dried.

The crude methanol extract of each AEFB isolate was successfully resolved by UPLC-ESI-TOF MS. The mass peaks observed in each chromatogram were well separated (Figure 3.6 to 3.13). The test isolates were positive for lipopeptide biosurfactant production, with different classes of lipopeptide namely, surfactin, iturin, bacillomycin, and fengycin being identified (Table 3.3). These compounds are produced as non-ribosomally synthesized lipopeptides. During this process, fatty acid side chains of varying length are produced, which are resolved as several peaks grouped into homologs of related compounds. Surfactin is the most potent of the lipopeptide compounds (Yeh *et al*., 2005; Chen *et al*., 2015). Fengycin and iturin lipopeptides have also been reported to be potent antifungal agents (Steller *et al*., 1999; Steller and Vater, 2000; Wei *et al*., 2010).

In addition to the mass peaks that could be assigned to lipopeptide compounds, each test isolate produced an unknown compound with the mass peak mz 1326.1, which exhibited hydrophobic properties when resolved by TLC. A search of the literature revealed that this mass peak has been previously linked to a precursor of an antibiotic called plantazolicin (PZN) produced by *B. amyloliquefaciens* FZB42 (Lee *et al*., 2013). PZN is a ribosomally synthesized and post-

translationally modified peptide (RiPP), which has been classified as a linear azole-containing peptide (LAP) and a thiazole/oxazole-modified microcin (TOMM) (Arnison *et al*., 2013). Interestingly, this antibiotic exhibits selective antibacterial activity towards *B. anthracis* by heightening the transient weakness present in the cell membrane of the microorganism, which in turn leads to membrane depolarization (Molohon *et al*., 2011; Lee *et al*., 2013; Molohon *et al*., 2016).

Differentiation and molecular identification of the selected isolates was done through REP-PCR and 16S rRNA gene sequence analysis. REP-PCR can distinguish isolates at the strain level while 16S rRNA gene sequence analysis can be used to classify unknown isolates to the genus and species level (Bodour *et al*., 2003; Agbobatinkpo *et al*., 2013; Naidoo, 2017; Ndlovu, 2017). From the findings, several related strains could be distinguished. The phylogenetic analysis was done through neighbor joining and maximum likelihood methods and phylogenetic trees were constructed (Figure 3.17 and 3.18). The topology for both trees are similar, which increases the confidence in the evolutionary relationships that are inferred.

Isolates SAB19 and SAB42 are grouped within the *Brevibacillus* genus. Although their REP-PCR fingerprint profiles differed significantly to one another they showed high levels of 16S rRNA gene sequence similarity to strains of *Brev. brevis* and *Brev. formosus*. The lipopeptide profiles of these two strains were very similar, they were both found to produce surfactin homologs, though in low amounts relative to a hydrophobic “PZN precursor” compound that was co-extracted during the acid precipitation extraction step (Figure 3.6 and 3.7). Several studies have linked *Brevibacillus* strains to surfactin production, which supports the findings of this study (Haddad *et al*., 2008; Reddy *et al*., 2010; Mnif *et al*., 2011). Strains of *Brevibacillus linens* have also been reported to produce PZN-like molecules, which is also consistent with this study’s findings (Molohon *et al*., 2011). PZN has been reported to be hydrophobic (Scholz *et al*., 2011) and it stands to reason that the “PZN-precursor” compound would also display hydrophobic properties as evidenced from TLC separation of the lipopeptide crude extract (Figure 3.1).

Of the isolates selected for further characterization SAB19 and SAB42 showed the lowest reduction in surface tension (44.3 and 44.7 mN/m respectively) (Section 2.3.4); but interestingly,

exhibited the highest emulsification indices (59.5 - 69.27%) after 24 h (Section 2.3.3). These findings may be attributed to the low levels of surfactin produced and could indicate the emulsification properties of the PZN-precursor compound. Further characterization of the biosurfactant nature and properties of the "PZN-precursor" is therefore warranted.

Isolates SAC15, SAC18, SAD5, SAD17, SAD18, and SAD23 all showed very similar 16S rRNA gene sequences and grouped within the *B. subtilis* complex of closely related taxa (Figure 3.17 and 3.18). These isolates showed very high levels of sequence similarity to strains of *B. velezensis* and *B. siamensis*. Both of these species are grouped within the *B. amyloliquefaciens* clade, otherwise known as the "*B.amyloliquefaciens* Operational Group", which is a sub-division that falls within the *B. subtilis* related taxa (Fan *et al*., 2017). Strains of both these species are commonly isolated from plant associated habitats and are associated with lipopeptide production.

All of these Isolates were found to produce surfactin; whereas, the ability to synthesize other lipopeptides such as fengycin, iturin and bacillomycin appeared to be more variable amongst strains. The ability of these isolates to reduce surface tensions were very similar (30.6 - 32.2 mN/m) (Section 2.3.4), which suggests that surfactin production was a major determinant in influencing surface tension rather than the other lipopeptides present.

From REP-PCR fingerprinting it was possible to distinguish several groupings of related strains amongst the isolates. In some instances, related strains (e.g. SAD18 and SAD23) displayed very similar lipopeptide profiles (Figures 3.14 and 3.15); in other instances, strains such as SAD5 and SAD17 varied in their ability to produce bacillomycin homologs (Figures 3.10 and 3.11). These findings illustrated that inter- and intra-strain variability in lipopeptide production was found amongst the isolates screened. It is speculated that intra-strain differences are most likely to arise from gene inactivation through point mutation rather than from cultural limitations.

A general observation was that isolates that produced all three classes of lipopeptide namely, surfactin, fengycin and iturin/bacillomycin, consistently performed the best in all of the oil spreading assays that were undertaken. It would be interesting to determine whether this phenomenon is a quantitative effect or a qualitative one; due to the presence of different

lipopeptides. Another area to be explored would be to focus on optimizing cultural conditions for lipopeptide production in order to maximize lipopeptide yields.

The AEFB isolates used in this study were revived from an ancient peatland sediment core that had been radio carbon dated to more than 30 000 cal years BP (Naidoo, 2017). The findings from this study suggest that the ability to synthesize potent biosurfactant compounds such as surfactin was present in isolates revived from some of the oldest samples processed. Further phylogenetic analysis of surfactin synthetase genes and/or other house-keeping genes from these isolates would be of interest to compare with "modern" strains in order to determine possible evolutionary changes amongst strains. Molecular clock analysis could also be used as a means to verify that these isolates were in fact ancient in origin and not carryover contaminants from the coring and sampling process.

3.5 Conclusion

From this study it was found that selected AEFB isolates revived from an ancient sediment core taken from Mfabeni peatland demonstrated significant biosurfactant activity. Characterization of the lipopeptide extracts revealed that homologs of surfactin, fengycin, and iturin/bacillomycin were prominent amongst the compounds identified. Surfactin was produced by each isolate - albeit at different relative abundancies. A peak putatively assigned to a precursor of the antibiotic PZN was also identified and associated with each isolate. REP-PCR allowed isolates to be differentiated at the strain level, with several groups of closely related strains being distinguished. Taxonomic classification revealed that the isolate could be separated into two genera namely *Bacillus* and *Brevibacillus*. Interestingly, the isolates from both genera were able to produce surfactin and the PZN-like precursor.

CHAPTER FOUR

GENERAL OVERVIEW

4.1 Summary of findings

Mfabeni Peatland is one of the oldest active peatland regions in Southern Africa (Grundling *et al*., 2013). It is a biologically diverse ecosystem that supports a wide range of plant and animal life. Microorganisms contribute significantly to the productivity of wetland systems and play an important role in the cycling of carbon and other nutrients (Gorham *et al*., 2001). Conditions within Mfabeni peatland are moderately acidic and it experiences fluctuating salinity levels due to seasonal water fluctuations and water infiltration from surrounding costal dune systems (Grundling *et al*., 2013; Naidoo, 2017). Physico-chemical conditions such as these impacts bacterial community structure and functionality. It stands to reason that microbes adapted to this environment would also produce bioactive compounds that tolerate the prevailing conditions. Since the diversity and functioning of microbes within this ecosystem is largely unexplored it represents an untapped source of microbial diversity with potential biotechnological applications.

A recent study found that genotypically distinct populations of aerobic endospore-forming bacteria (AEFB) were prevalent within a sediment core sample taken from Mfabeni peatland (Naidoo, 2017). Members of the AEFB group are recognized for their ability to produce compounds that are of biotechnological interest (Wipat and Harwood, 1999; Mandic-Mulec and Prosser 2011). In this study, AEFB isolates from Mfabeni peatland were screened for biosurfactant activity. Increasingly, biosurfactants are being recognized for their industrial and environmental applications and have gained favour over synthetic surfactants because of their ecological acceptability (Muthusamy *et al*., 2008; Mulligan, 2009; Banat *et al*., 2010; Ławniczak *et al*., 2013). The purpose of the study, therefore, was to select promising biosurfactant producing isolates for further characterization.

Isolates were screened for their ability to produce biosurfactants using the oil spreading assay, hemolysis assay, and drop collapse assay. Isolates which demonstrated biosurfactant activity were subjected to different environmental parameters i.e. temperature, pH, and salinity to determine their effect on biosurfactant activity. Surface tension measurement and emulsification (E24) index were determined for selected isolates that showed promising biosurfactant activity levels. These isolates were further identified through 16S rRNA gene sequencing and the biosurfactant compound(s) extracted and characterized through acid precipitation, TLC, and UPLC ESI-TOF MS.

From this study it was established that:

- Most of the AEFB isolates screened demonstrated biosurfactant activity. This result was not unexpected since biosurfactant production contributes to an organism's ecological fitness and can play an important role in motility, biofilm formation, antagonism and nutrient solubilization.
- The three assays used for the preliminary screening were found to be relatively simple to use and did not require any special equipment. Of the three, the hemolysis assay was found to be the least sensitive, whereas the oil spreading assay was judged to be the best because it is a semi-quantitative method, which gives a direct correlation between the concentration of the biosurfactant produced and the extent of the oil displacement.
- The physicochemical parameters (viz., temperature, pH, and salinity) tested had varying effects on the biosurfactant activity. Most of the isolates were able to maintain their activity over the different temperature ranges tested. Thirty-three percent of the isolates showed highest activity at temperature 55°C. For salinity, varied results were observed. At pH 3 and 5.5, the negative control exhibited oil displacement. Therefore, the high biosurfactant activity showed by the isolates at low pH had to be viewed with caution and could not be recorded as significant.
- Eight isolates (viz., SAB19, SAB42, SAC15, SAC18, SAD5, SAD17, SAD18, and SAD23) showed significantly higher levels of biosurfactant activity for each of the environmental parameters tested and were selected for further evaluation and characterization. Surface

tension measurements showed that the isolates were able to reduce surface tension of TSB medium from 57.3 mN/m to between 30.6 mN/m to 44.7 mN/m. Emulsification (E24) efficiency tests using sunflower seed oil and paraffin oil ranged from 19.5% up to 61.85%. Interestingly, isolates which exhibited the highest emulsification stability (viz., SAB19, SAB42) showed the least capacity for lowering surface tension. This finding is not out of place as it has been reported that surface activity of isolates does not necessarily correlate with their emulsification capacity (Walter *et al*., 2010).

- All of the selected isolates produced lipopeptide compounds when cultured on Landy medium. Biosurfactant compounds were successfully extracted from each culture medium using an acid precipitation step. UPLC ESI-TOF MS anlysis of extracts revealed that all of the Isolates produced surfactin homologs as well as a hydrophobic compound (*m/z* 1326.1) that was putatively assigned as a precursor of the antibiotic Plantazolicin (PZN). A number of isolates also produced homologs of iturin/bacillomycin and/or fengycin lipopeptides.
- REP-PCR and 16S rRNA gene sequence analysis were found to be effective tools in distinguishing and identifying the genetic diversity amongst the AEFB isolates. Several groups of closely related strains were distinguished. Taxonomic classification revealed that the isolates could be separated into two genera namely *Bacillus* and *Brevibacillus*. The assignment of strains at species level proved to be difficult due to matches having high levels of similarity (≥99%) to several closely related species. The *Bacillus* spp. strains fell within the "*B.amyloliquefaciens* Operational Group", a sub-group within the clade that makes up the *B. subtilis* complex of related taxa. The *Brevibacillus* spp. strains matched closely to strains of *Brev. brevis* and *Brev. formosus.* For future studies a Multi-locus sequence typing (MLST) approach targeting various housekeeping genes could be used to characterize isolates to species level.

4.2 Future studies

From this study it is evident that biosurfactant production is prevalent amongst AEFB isolated from Mfabeni peatland sediment. In terms of biosurfactant activity the best performing isolates were all found to be surfactin producers. This result is consistent with reports in the literature that indicate that surfactin is one of the most potent biosurfactant known to man. Interestingly, surfactin production was associated with two AEFB genera. Since the isolates screened in this study were revived from an ancient core it would be interesting to look at the evolutionary relationships between genes associated with lipopeptide synthesis and compare them to "modern" reference strains. The quantification and optimization of biosurfactant production is another area that needs to be explored. Although no novel biosurfactant compounds were identified amongst the top performing isolates it is feasible that some could be found amongst the second tier AEFB isolates that did not perform as well. Further examination of the biosurfactants produced by these isolates is therefore warranted.

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APPENDIX A: Measurement for the different buffer solutions used for pH adjustments

Buffer	**Solutions used**	**pH and amount of solution used**
Citrate buffer (100 mL)	Solution A- 0.1 M citric acid monohydrate Solution B- 0.1 M trisodium citrate dihydrate	pH 3- 82 mL of solution A was mixed with 18 mL of solution B pH 5.5- 25.5 mL of solution A was mixed with 74.5 mL solution B
Tris buffer (200 mL)	Solution A- tris base Solution B- 1 M HCl	pH 7- 2.423 g of solution A mixed with 18.59 mL of solution B pH 8.5- 2.423 g of solution A was mixed with 5.88 mL solution B
Bicarbonate carbonate buffer (100 mL)	Solution A- 0.1 M sodium bicarbonate Solution B- 0.1 M sodium carbonate decahydrate	pH 10- 40 mL of solution A was mixed with 60 mL of solution B

Figure 1: Table showing the different volumes of buffer solutions used in achieving pH ranges for pH test

APPENDIX B: ANOVA analysis for the effect of temperature on biosurfactant activity

A

ANOVA analysis for effect of temperature on biosurfactant activity									
Isolates	**Mean/Dunc**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Isolates**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**
	Temp 35 °C	**Temp 55 °C**	**Temp 75 °C**	**Temp 100 °C**		**Temp 35 °C**	**Temp 55 °C**	**Temp 75 °C**	**Temp 100 °C**
SAA1	17.00^{QRS}	36.00^{VACZBUYXW}	29.00^{QSRTP}	24.00^{WYXV}	SAB42	38.50^{GLNJKIMH}	53.00^{KIHJGL}	30.00^{QSRTOP}	45.50^{EHGF}
SAA5	39.00^{GLJKIMH}	33.00^{VACZBDYXW}	30.50^{QSRNOP}	38.50^{RIPJOHNQMLK}	SAB45				
SAA7	40.50^{GLJKIMH}	58.00^{IHFG}	40.50^{JHI}	33.00^{RPUOTQS}	SAB46	41.00^{GLJKIMH}	61.00^{EFG}	0.00^{Z}	35.00^{RPUOTNQMLS}
SAA8	11.00^{TRS}	19.50^{FG}	0.00^{Z}	9.50^{A}	SAB50	42.25^{GLJKIH}	30.00^{ACZBDYE}	33.00^{LQRNOPM}	33.50^{RPUOTNQS}
SAA9	30.50^{ONPM}	49.50^{KIHOJMNL}	41.00^{GHI}	34.50^{RPUOTNQMS}	SAB51	39.50^{GLJKIMH}	43.50^{TSRQPOUMNL}	30.50^{QSRNOP}	30.00^{RWUTVS}
SAA16	37.00^{OLNJKIM}	41.25^{TVSRQPOUNW}	33.50^{LQKRNOPM}	40.00^{IPJOHNGMLK}	SAB52	39.25^{GLJKIMH}	38.00^{TVSZUYXW}	22.00^{WXYV}	43.00^{IJHGLK}
SAA21					SAB53	40.75^{GLJKIMH}	46.00^{TKSRQPOUMNL}	0.00^{Z}	48.00^{EGF}
SAA23	46.50^{GFJKIEH}	48.50^{KIQPOJMNL}	39.50^{JKHI}	37.50^{RIPJOHNQMLKS}	SAB54	47.00^{GDFJKIEH}	42.75^{TVSRQPOUMN}	30.00^{QSRTOP}	27.00^{WUXV}
SAA24	36.00^{OLNJKM}	36.50^{VAZBUYXW}	39.50^{JKHI}	24.00^{WYXV}	SAB59				
SAA25	43.50^{GFJKIH}	0.00^{I}	22.50^{UWXYV}	40.00^{IPJOHNGMLK}	SAB63				
SAA28	26.25^{OQP}	6.00^{HI}	0.00^{Z}	40.00^{IPJOHNGMLK}	SAC1	41.00^{GLJKIMH}	47.00^{TKSRQPOJMNL}	28.00^{UQSRTV}	37.00^{RIPJONQMLKS}
SAA32					SAC2	40.00^{GLJKIMH}	44.00^{TSRQPOUMNL}	30.00^{QSRTOP}	29.00^{WUTVS}
SAA34	37.00^{OLNJKIM}	51.00^{KIHJMNL}	33.00^{LQRNOPM}	33.50^{RPUOTNQS}	SAC13	70.00^{B}	67.50^{ECD}	69.00^{D}	68.50^{B}
SAA35	49.00^{GDFCEH}	37.50^{TVAZUYXW}	36.00^{IJKNOIM}	30.00^{RWUTVS}	SAC15	77.00^{BA}	76.00^{BC}	80.00^{A}	81.50^{A}
SAA37	49.00^{GDFCEH}	48.00^{KRQPOJMNL}	35.50^{IJKNOIM}	38.00^{RIPJOHNQMLK}	SAC16	70.00^{B}	79.50^{BA}	79.50^{BA}	79.00^{A}
SAA39	18.00^{QRS}	25.50^{FDE}	27.50^{USRTV}	20.00^{YXZ}	SAC18	77.50^{BA}	81.50^{BA}	78.50^{BAC}	81.00^{A}
SAA42	41.00^{GLJKIMH}	50.00^{KIHOJMNL}	26.00^{UWSTV}	42.50^{IJHGMLK}	SAC19	76.75^{BA}	77.50^{BA}	73.50^{DC}	79.00^{A}
SAA43	23.00^{QP}	30.00^{ACZBDYE}	30.00^{QSRTOP}	29.00^{WUTVS}	SAC20	41.75^{GLJKIMH}	39.50^{TVSRQPUYXW}	28.50^{UQSRTP}	38.00^{RIPJOHNQMLK}
SAA63	30.50^{ONPM}	40.25^{TVSRQPOUXW}	26.50^{UWSTV}	23.00^{WYX}	SAC24	41.50^{GLJKIMH}	30.00^{ACZBDYE}	34.50^{IJKNOPM}	51.50^{EDF}
SAA66	35.50^{OLNKM}	27.00^{CFBDE}	31.00^{QSRNOP}	31.50^{RPUTQVS}	SAC25	40.50^{GLJKIMH}	49.00^{KIPOJMNL}	31.00^{QSRNOP}	32.00^{RPUOTQVS}
SAA79	45.00^{GFJKIEH}	40.25^{TVSRQPOUXW}	28.00^{UQSRTV}	30.00^{RWUTVS}	SAC26	42.00^{GLJKIMH}	36.50^{VAZBUYXW}	33.50^{LQKRNOPM}	35.50^{RPUOTNQMLKS}
SAA85	39.50^{GLJKIMH}	39.50^{TVSRQPUYXW}	31.00^{QSRNOP}	33.50^{RPUOTNQS}	SAC27	54.50^{DFCE}	56.00^{IHJG}	30.50^{QSRNOP}	56.00^{CD}
SAA91	38.50^{GLNJKIMH}	37.00^{VAZUYXW}	28.50^{UQSRTP}	28.00^{WUTXV}	SAD1	46.00^{GFJKIEH}	42.50^{TVSRQPOUMN}	31.50^{QSRNOPM}	44.50^{IJHGF}
SAA93	23.00^{QP}	28.00^{ACFBDE}	29.00^{QSRTP}	35.50^{RPUOTNQMLKS}	SAD3				
SAA98	37.00^{OLNJKIM}	45.50^{TSRQPOUMNL}	32.00^{LQSRNOPM}	32.50^{RPUOTQS}	SAD4	31.25^{OLNPM}	45.50^{TSRQPOUMNL}	39.50^{JKHI}	35.00^{RPUOTNQMLS}
SAA101	40.50^{GLJKIMH}	20.50^{FGE}	35.50^{IJKNOIM}	32.00^{RPUOTQVS}	SAD5	58.50^{C}	55.50^{KIHJG}	50.00^{F}	65.00^{B}
SAA107	48.50^{GDFCIEH}	48.00^{KRQPOJMNL}	36.50^{IJKNIM}	34.50^{RPUOTNQMS}	SAD9	58.00^{DC}	66.50^{EFD}	56.50^{E}	62.50^{CB}
SAA108	50.00^{GDFCE}	42.50^{TVSRQPOUMN}	33.00^{LQRNOPM}	33.50^{RPUOTNQS}	SAD10	55.75^{DCE}	53.00^{KIHJGL}	60.00^{E}	65.50^{B}
SAA109	46.00^{GFJKIEH}	38.50^{TVSRZUYXW}	32.00^{LQSRNOPM}	32.00^{RPUOTQVS}	SAD12	83.00^{A}	85.50^{A}	83.00^{A}	79.50^{A}
SAA110	50.00^{GDFCE}	30.00^{ACZBDYE}	37.50^{IJKHIM}	42.00^{IJHNGMLK}	SAD17	77.50^{BA}	80.00^{BA}	74.00^{BDC}	79.50^{A}
SAA114					SAD18	83.00^{A}	84.00^{BA}	79.00^{BAC}	78.50^{A}
SAB1	40.50^{GLJKIMH}	38.50^{TVSRZUYXW}	34.00^{LQKNOPM}	44.00^{IJHGF}	SAD21	46.50^{GFJKIEH}	49.00^{KIPOJMNL}	41.00^{GHI}	45.00^{IEHGF}
SAB2	30.50^{ONPM}	47.00^{KSRQPOJMNL}	34.50^{IJKNOPM}	33.50^{RPUOTNQS}	SAD23	71.50^{B}	75.00^{BCD}	79.00^{BAC}	80.00^{A}
SAB6	45.00^{GFJKIEH}	41.00^{TVSRQPOUW}	31.50^{QSRNOPM}	30.50^{RWUTQVS}	SAD34	43.00^{GJKIH}	32.00^{ACZBDYXW}	33.00^{LQRNOPM}	39.00^{IPJOHNQMLK}
SAB7	19.50^{QR}	26.50^{CFDE}	21.00^{WXY}	0.00^{B}	SAD41	24.00^{QP}	41.50^{TVSRQPOUNW}	24.00^{UWXTV}	33.50^{RPUOTNQS}
SAB10	47.50^{GDFJCIEH}	40.50^{TVSRQPOUXW}	36.50^{IJKNIM}	36.50^{RPJOTNQMLKS}	SAD45	42.50^{GLJKIH}	29.50^{ACZBDE}	19.50^{XY}	0.00^{B}
SAB11	38.00^{LNJKIMH}	44.40^{TSRQPOUMNL}	46.50^{GF}	43.50^{IJHGFK}	SAD47	39.00^{GLJKIMH}	39.00^{TVSRQZUYXW}	43.00^{GH}	40.50^{IJOHNGMLK}
SAB14					SAE10	46.50^{GFJKIEH}	36.00^{VACZBUYXW}	39.50^{JKHI}	52.50^{ED}
SAB19	46.50^{GFJKIEH}	59.00^{EHFG}	38.00^{IJKHI}	44.00^{IJHGF}	Pos-R16	75.50^{BA}	75.00^{BCD}	74.00^{BDC}	69.50^{B}
SAB20					Negative	27.50^{OQNP}	28.00^{ACFBDE}	19.50^{XY}	15.00^{AZ}
SAB23	41.50^{GLJKIMH}	52.00^{KIHJMGL}	31.00^{QSRNOP}	47.50^{EGF}	P-value	<0.0001	<0.0001	<0.0001	<0.0001
SAB24					CV	11.828	9.945693	7.554116	9.054245
SAB30	41.25^{GLJKIMH}	59.00^{EHFG}	35.50^{IJKNOIM}	39.00^{IPJOHNQMLK}	R2	0.971813	0.981918	0.991756	0.985474
SAB35	42.50^{GLJKIH}	31.00^{ACZBDYX}	41.00^{GHI}	37.00^{RIPJONQMLKS}					

APPENDIX B cont'd: ANOVA analysis for the effect of pH on biosurfactant activity

B

ANOVA analysis for effect of pH on biosurfactant activity												
Isolates	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Isolates**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**
	pH 3	**pH 5.5**	**pH 7**	**pH 8.5**	**pH 10**		**pH 3**	**pH 5.5**	**pH 7**	**pH 8.5**	**pH 10**
SAA1	67.00^{KRJOIQHNPML}	59.00^{OMKNQLP}	38.00^{HEGDFI}	32.00^{KJINGMHL}	3.50^{QR}	SAB42	76.00^{EBDACF}	66.50^{EMKINHJGLF}	28.50^{OHNPLKMJQI}	26.00^{KQJONPMRL}	23.50^{IFHEGJ}
SAA5	74.50^{EBIDHAGCF}	61.00^{OMKNJQLP}	30.00^{OHNPLGKMJFI}	30.00^{KJOINGMHL}	20.50^{IMHKLGJ}	SAB45					
SAA7	70.00^{KEJOIQHNGPMFL}	59.00^{OMKNQLP}	26.50^{ONPLKMJQ}	23.50^{QONPMRL}	0.00^{R}	SAB46	65.00^{ROQNTPMSL}	56.00^{ONQP}	34.00^{HELGKMJFI}	0.00^{Y}	23.00^{IFHEGJ}
SAA8	62.50^{RQTPS}	64.00^{OMKINHJL}	0.00^{U}	5.50^{YX}	0.00^{R}	SAB50	73.00^{KEJBIDHAGCFL}	61.50^{OMKNJQLP}	27.00^{ONPLKMJQI}	24.50^{KQJONPMRL}	15.00^{MOKLNJ}
SAA9	58.00^{T}	65.00^{OMKINHJGL}	41.50^{ED}	29.00^{KJOINPMHL}	12.50^{MOPLN}	SAB51	74.00^{EJBIDHAGCF}	62.00^{OMKNJQLP}	23.00^{OSNPRMQT}	21.50^{0QSOTPR}	0.00^{R}
SAA16	69.00^{KEJOIQHNGPMFL}	62.00^{OMKNJQLP}	38.00^{HEGDFI}	33.50^{KJIGHL}	9.50^{QOPN}	SAB52	70.00^{KEJOIQHNGPMFL}	60.50^{OMKNJQLP}	31.00^{OHNELGKMJFI}	34.00^{KJIGH}	16.00^{MKLNJ}
SAA21						SAB53	68.50^{KJOIQHNGPMFL}	65.00^{OMKINHJGL}	17.50^{SRQT}	0.00^{Y}	0.00^{R}
SAA23	63.00^{ROQTPS}	58.00^{OMNQLP}	0.00^{U}	45.00^{FE}	9.00^{QOPN}	SAB54	71.50^{KEJIDHNGCMFL}	63.50^{OMKINHJL}	32.00^{HELGKMJFI}	22.00^{QSONPR}	0.00^{R}
SAA24	64.50^{ROQNTPMS}	73.00^{EBIDHAGCF}	31.50^{HNELGKMJFI}	13.50^{USTWXV}	0.00^{R}	SAB59					
SAA25	62.00^{RQTS}	66.00^{MKINHJGLF}	30.50^{OHNELGKMJFI}	17.50^{USTWRV}	7.50^{QOPR}	SAB63					
SAA28	60.00^{RTS}	55.00^{OQP}	0.00^{U}	0.00^{Y}	0.00^{R}	SAC1	76.00^{EBDACF}	62.50^{OMKINJQLP}	30.50^{OHNELGKMJFI}	27.00^{KQJOINPMRL}	12.00^{MOPN}
SAA32						SAC2	63.00^{ROQTPS}	68.50^{EKIDHJGLF}	35.50^{HELGKDJFI}	30.50^{KJOINGMHL}	19.50^{IMHKLGJ}
SAA34	70.00^{KEJOIQHNGPMFL}	65.00^{OMKINHJGL}	46.00^{CD}	30.50^{KJOINGMHL}	25.00^{IFHEG}	SAC13	69.50^{KEJOIQHNGPMFL}	69.00^{EKIDHJGCF}	58.50^{B}	59.00^{DC}	67.50^{B}
SAA35	69.50^{KEJOIQHNGPMFL}	65.50^{OMKINHJGL}	51.50^{CB}	34.00^{KJIGH}	16.00^{MKLNJ}	SAC15	75.00^{EBDHAGCF}	77.50^{BDAC}	73.00^{A}	77.00^{A}	77.00^{A}
SAA37	71.00^{KEJOIDHNGMFL}	63.50^{OMKINHJL}	51.50^{CB}	36.50^{FIGH}	20.00^{IMHKLGJ}	SAC16	66.50^{KRJOIQNPML}	59.00^{OMKNQLP}	28.00^{ONPLKMJQI}	22.50^{QSONPR}	77.00^{A}
SAA39	74.00^{EJBIDHAGCF}	65.00^{OMKINHJGL}	32.00^{HELGKMJFI}	8.50^{YWX}	0.00^{R}	SAC18	80.50^{A}	77.00^{EBDAC}	74.00^{A}	71.50^{BA}	78.50^{A}
SAA42	67.00^{KRJOIQHNPML}	67.00^{EMKIDHJGLF}	37.00^{HEGKDJFI}	24.00^{KQONPMRL}	20.50^{IMHKLGJ}	SAC19	60.50^{RTS}	63.00^{OMKINJLP}	40.00^{EGDF}	34.50^{JIGH}	78.00^{A}
SAA43	69.00^{KEJOIQHNGPMFL}	65.50^{OMKINHJGL}	40.00^{EGDF}	11.00^{UWXV}	0.00^{R}	SAC20	71.50^{KEJIDHNGCMFL}	66.50^{EMKINHJGLF}	36.00^{HELGKDJFI}	39.00^{FG}	20.50^{IMHKLGJ}
SAA63	72.00^{KEJBIDHNGCMFL}	62.00^{OMKNJQLP}	15.50^{SRT}	0.00^{Y}	0.00^{R}	SAC24	77.00^{EBDAC}	67.00^{EMKIDHJGLF}	39.50^{HEGDF}	33.00^{KJIGMHL}	27.50^{FEG}
SAA66	71.50^{KEJIDHNGCMFL}	74.00^{EBDHAGCF}	34.00^{HELGKMJFI}	20.50^{UQSOTPR}	20.00^{IMHKLGJ}	SAC25	76.00^{EBDACF}	67.50^{EMKIDHJGLF}	33.75^{HELGKMJFI}	26.00^{KQJONPMRL}	21.00^{IFHKLGJ}
SAA79	67.50^{KRJOIQHNGPML}	64.00^{OMKINHJL}	29.50^{OHNPLGKMJFI}	21.00^{QSOTPR}	19.00^{IMHKLGJ}	SAC26	70.00^{KEJOIQHNGPMFL}	64.00^{OMKINHJL}	26.50^{ONPLKMJQ}	23.00^{QSONPMR}	19.00^{IMHKLGJ}
SAA85	70.50^{KEJOIHNGPMFL}	58.00^{OMNQLP}	30.00^{OHNPLGKMJFI}	29.00^{KJOINPMHL}	18.50^{IMHKLJ}	SAC27	73.50^{KEJBIDHAGCF}	76.50^{EBDACF}	73.50^{A}	69.50^{BA}	31.00^{E}
SAA91	72.50^{KEJBIDHAGCMFL}	60.00^{OMKNJQLP}	30.00^{OHNPLGKMJFI}	10.00^{WXV}	5.00^{QPR}	SAD1	75.50^{EBDAGCF}	64.50^{OMKINHJGL}	33.00^{HELGKMJFI}	25.00^{KQJONPMRL}	27.00^{FHEG}
SAA93	72.00^{KEJBIDHNGCMFL}	63.00^{OMKINJLP}	27.00^{ONPLKMJQI}	30.50^{KJOINGMHL}	14.00^{MOKLN}	SAD3					
SAA98	68.50^{KJOIQHNGPMFL}	60.50^{OMKNJQLP}	32.50^{HELGKMJFI}	27.50^{KQJOINPMRL}	22.00^{IFHKGJ}	SAD4	76.00^{EBDACF}	60.00^{OMKNJQLP}	31.00^{OHNELGKMJFI}	48.50^{E}	15.50^{MOKLNJ}
SAA101	65.50^{KROQNTPMSL}	64.00^{OMKINHJL}	14.50ST	19.00^{UQSTPRV}	13.00^{MOPLN}	SAD5	72.50^{KEJBIDHAGCMFL}	66.00^{MKINHJGLF}	61.00^{B}	67.00^{BC}	51.00^{C}
SAA107	69.50^{KEJOIQHNGPMFL}	65.00^{OMKINHJGL}	27.00^{ONPLKMJQI}	25.50^{KQJONPMRL}	29.00^{FE}	SAD9	74.50^{EBIDHAGCF}	65.50^{OMKINHJGL}	58.50^{B}	52.50^{DE}	38.50^{D}
SAA108	77.00^{EBDAC}	65.00^{OMKINHJGL}	40.50^{EDF}	25.00^{KQJONPMRL}	22.00^{IFHKGJ}	SAD10	79.50^{BAC}	69.00^{EKIDHJGCF}	57.00^{B}	48.50^{E}	43.00^{D}
SAA109	76.00^{EBDACF}	64.00^{OMKINHJL}	37.50^{HEGDJFI}	20.50^{UQSOTPR}	19.50^{IMHKLGJ}	SAD12	80.00^{BA}	81.50^{A}	80.50^{A}	69.00^{BA}	74.50^{BA}
SAA110	73.00^{KEJBIDHAGCFL}	67.00^{EMKIDHJGLF}	35.00^{HELGKJFI}	11.00^{UWXV}	30.50^{E}	SAD17	75.00^{EBDHAGCF}	81.00^{A}	80.00^{A}	74.00^{BA}	72.00^{BA}
SAA114						SAD18	79.50^{BAC}	79.00^{BAC}	79.00^{A}	77.50^{A}	78.00^{A}
SAB1	74.00^{EJBIDHAGCF}	67.50^{EMKIDHJGLF}	32.00^{HELGKMJFI}	24.50^{KQJONPMRL}	16.00^{MKLNJ}	SAD21	74.50^{EBIDHAGCF}	68.50^{EKIDHJGLF}	27.00^{ONPLKMJQI}	18.00^{UQSTRV}	19.00^{IMHKLGJ}
SAB2	73.50^{KEJBIDHAGCF}	65.00^{OMKINHJGL}	25.00^{OSNPLRMQ}	23.00^{QSONPMR}	16.50^{IMKLNJ}	SAD23	79.00^{BDAC}	80.50^{BA}	78.50^{A}	74.50^{BA}	71.50^{BA}
SAB6	74.00^{EJBIDHAGCF}	63.00^{OMKINJLP}	29.50^{OHNPLGKMJFI}	26.50^{KQJONPMRL}	15.00^{MOKLNJ}	SAD34	74.00^{EJBIDHAGCF}	70.50^{EBIDHJGCF}	25.00^{OSNPLRMQ}	19.50^{UQSTPRV}	16.00^{MKLNJ}
SAB7	68.50^{KJOIQHNGPMFL}	63.50^{OMKINHJL}	27.00^{ONPLKMJQI}	19.50^{UQSTPRV}	0.00^{R}	SAD41	72.00^{KEJBIDHNGCMFL}	67.50^{EMKIDHJGLF}	19.00^{SPRQT}	0.00^{Y}	0.00^{R}
SAB10	69.50^{KEJOIQHNGPMFL}	65.00^{OMKINHJGL}	20.00^{OSPRQT}	12.00^{UTWXV}	12.50^{MOPLN}	SAD45	65.00^{ROQNTPMSL}	63.50^{OMKINHJL}	14.00^{T}	0.00^{Y}	0.00^{R}
SAB11	72.00^{KEJBIDHNGCMFL}	67.00^{EMKIDHJGLF}	0.00^{U}	28.00^{KQJOINPMHL}	20.00^{IMHKLGJ}	SAD47	73.50^{KEJBIDHAGCF}	64.50^{OMKINHJGL}	34.50^{HELGKJFI}	29.00^{KJOINPMHL}	17.00^{IMKLNJ}
SAB14						SAE10	75.00^{EBDHAGCF}	74.00^{EBDHAGCF}	29.00^{OHNPLGKMJI}	24.00^{KQJONPMRL}	20.00^{IMHKLGJ}
SAB19	74.50^{EBIDHAGCF}	70.00^{EIDHJGCF}	34.50^{HELGKJFI}	19.50^{UQSTPRV}	30.50^{E}	Pos-R16	74.00^{EJBIDHAGCF}	76.50^{EBDACF}	77.00^{A}	77.50^{A}	72.00^{BA}
SAB20						Negative	66.00^{KRJOQNPMSL}	62.00^{OMKNJQLP}	0.00^{U}	0.00^{Y}	0.00^{R}
SAB23	69.50^{KEJOIQHNGPMFL}	67.00^{EMKIDHJGLF}	26.00^{ONPLRKMQ}	21.50^{QSOTPR}	15.50^{MOKLNJ}	P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SAB24						CV	4.589569	6.697646	14.35434	15.10394	16.59596
SAB30	73.50^{KEJBIDHAGCF}	67.00^{EMKIDHJGLF}	20.50^{OSNPRQT}	28.50^{KQJOINPMHL}	22.00^{IFHKGJ}	R2	0.941608	0.904043	0.977101	0.981576	0.988408
SAB35	73.50^{KEJBIDHAGCF}	70.00^{EIDHJGCF}	31.50^{HNELGKMJFI}	25.50^{KQJONPMRL}	43.00^{D}						

APPENDIX B cont'd: ANOVA analysis for the effect of salinity on biosurfactant activity

C

ANOVA analysis for effect of salinity on biosurfactant activity											
Isolates	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Isolates**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**	**Mean/Duncan**
	0.50%	**2.50%**	**5.00%**	**10.00%**	**15.00%**		**0.50%**	**2.50%**	**5.00%**	**10.00%**	**15.00%**
SAA1	52.00^{vw}	48.50^{tu}	29.00^{EHFG}	27.50^{IEHGF}	24.00^{GFH}	SAB42	0.00^{a}	0.00^{a}	8.00^{QPRS}	$21.50^{ILNHMGKFJ}$	0.00^{P}
SAA5	$22.00^{fghijklmn}$	45.00^{st}	31.00^{EF}	$19.00^{ILNHMGKJ}$	25.00^{GF}	SAB45					
SAA7	$20.00^{efghijkl}$	24.00^{fghijk}	27.00^{IEHFG}	27.50^{IEHGF}	22.50^{GIFH}	SAB46	$22.00^{fghijklmno}$	29.00^{ijklmn}	0.00^{S}	$17.00^{ILNMOKJ}$	0.00^{P}
SAA8	0.00^{a}	38.50^{pqr}	0.00^{S}	0.00^{P}	0.00^{P}	SAB50	0.00^{a}	0.00^{a}	0.00^{S}	$21.50^{ILNHMGKFJ}$	9.50^{POMNLK}
SAA9	43.00^{tuv}	54.00^{u}	30.00^{EFG}	35.00^{ED}	36.50^{ED}	SAB51	0.00^{a}	34.00^{nopq}	7.00^{QRS}	0.00^{P}	0.00^{P}
SAA16	0.00^{a}	0.00^{a}	9.00^{QPR}	$24.50^{ILHGKFJ}$	$16.50^{JGIMNLHK}$	SAB52	0.00^{a}	38.00^{pqr}	21.00^{IKHMLJ}	11.50^{NO}	$17.50^{IGIMFLHK}$
SAA21						SAB53	31.00^{mnopqr}	6.00^{a}	12.00^{QPRO}	0.00^{P}	0.00^{P}
SAA23	$20.00^{efghijkl}$	0.00^{a}	$20.00^{IKMNLOJ}$	0.00^{P}	0.00^{P}	SAB54	0.00^{a}	0.00^{a}	17.50^{KMNLOJ}	7.50^{PO}	0.00^{P}
SAA24	$26.00^{hijklmnopqr}$	54.00^{u}	0.00^{S}	29.50^{EGF}	$19.00^{JGIFLHK}$	SAB59					
SAA25	0.00^{a}	24.00^{fghijk}	5.00^{RS}	13.50^{NMO}	7.00^{POMN}	SAB63					
SAA28	0.00^{a}	0.00^{a}	0.00^{S}	0.00^{P}	0.00^{P}	SAC1	7.25^{abc}	34.50^{nopq}	$22.50^{IKHMNLGJ}$	0.00^{P}	21.00^{IGIFH}
SAA32						SAC2	$20.75^{fghijklm}$	0.00^{ab}	$20.00^{IKMNLOJ}$	0.00^{P}	8.00^{POMNL}
SAA34	0.00^{a}	45.00^{st}	0.00^{S}	28.00^{EHGF}	0.00^{P}	SAC13	42.00^{stu}	72.00^{vw}	49.00^{C}	30.50^{EDF}	39.50^{D}
SAA35	$22.00^{fghijklmn}$	34.00^{nopq}	6.50^{QRS}	26.50^{IEHGFJ}	22.00^{GIFH}	SAC15	72.00^{xy}	78.00^{wx}	65.25^{B}	74.50^{A}	71.00^{BA}
SAA37	$15.00^{bcdefgh}$	0.00^{a}	0.00^{S}	$21.50^{ILNHMGKFJ}$	25.00^{GF}	SAC16	0.00^{a}	34.50^{nopq}	0.00^{S}	0.00^{P}	0.00^{P}
SAA39	$16.00^{cdefghi}$	40.00^{qrs}	0.00^{S}	15.50^{LNMOK}	24.50^{GFH}	SAC18	68.50^{x}	81.00^{x}	62.00^{B}	65.00^{BA}	66.00^{B}
SAA42	$17.00^{cdefghi}$	29.50^{jklmno}	12.00^{QPRO}	$19.50^{ILNHMGKJ}$	9.00^{POMNLK}	SAC19	0.00^{a}	31.00^{lmno}	32.00^{ED}	$21.00^{ILNHMGKFJ}$	$11.50^{IPOIMNLK}$
SAA43	6.50^{abc}	0.00^{a}	0.00^{S}	$22.50^{ILHMGKFJ}$	22.50^{GIFH}	SAC20	0.00^{a}	17.50^{cde}	25.50^{IEHFGJ}	$25.50^{IEHGKFJ}$	19.50^{IGIFHK}
SAA63	$26.00^{hijklmnopqr}$	24.00^{fghijk}	0.00^{S}	0.00^{P}	0.00^{P}	SAC24	0.00^{a}	33.50^{nop}	32.00^{ED}	27.50^{IEHGF}	$18.50^{IGIFLHK}$
SAA66	9.00^{abcd}	27.00^{hijklm}	15.50^{PMNLO}	$20.50^{ILNHMGKFJ}$	$18.00^{JGIMFLHK}$	SAC25	$22.00^{fghijklmno}$	16.50^{cd}	$23.75^{IKHFLGJ}$	$17.00^{ILNMOKJ}$	24.00^{GFH}
SAA79	14.50^{bcdefg}	0.00^{a}	0.00^{S}	16.50^{LNMOKJ}	$10.50^{JPOMNLK}$	SAC26	0.00^{a}	18.00^{cdef}	$21.50^{IKHMNLJ}$	$23.00^{ILHMGKFJ}$	$16.00^{IGIMNLHK}$
SAA85	$19.50^{defghijkl}$	24.25^{fghijk}	0.00^{S}	$24.50^{ILHGKFJ}$	$19.00^{JGIFLHK}$	SAC27	66.50^{x}	68.00^{v}	61.00^{B}	62.50^{B}	61.00^{BC}
SAA91	9.50^{abcde}	0.00^{a}	0.00^{S}	14.00^{LNMO}	0.00^{P}	SAD1	35.00^{rst}	20.50^{defg}	18.00^{KMNLOJ}	0.00^{P}	28.50^{EF}
SAA93	0.00^{a}	24.00^{fghijk}	0.00^{S}	28.00^{EHGF}	0.00^{P}	SAD3					
SAA98	0.00^{a}	33.00^{mnop}	0.00^{S}	0.00^{P}	0.00^{P}	SAD4	0.00^{a}	26.00^{ghijkl}	24.00^{IKHFGJ}	13.00^{NMO}	$17.50^{IGIMFLHK}$
SAA101	7.50^{abc}	0.00^{a}	0.00^{S}	0.00^{P}	0.00^{P}	SAD5	52.00^{vw}	52.00^{u}	39.00^{D}	35.50^{ED}	39.50^{D}
SAA107	0.00^{a}	25.50^{ghijkl}	6.50^{QRS}	$22.50^{ILHMGKFJ}$	6.00^{PON}	SAD9	53.00^{w}	53.00^{u}	51.50^{C}	39.00^{D}	20.00^{IGIFHK}
SAA108	12.00^{bcdef}	29.00^{ijklmn}	$23.25^{IKHMFLGJ}$	$22.50^{ILHMGKFJ}$	7.00^{POMN}	SAD10	49.50^{uvw}	49.00^{tu}	65.00^{B}	53.50^{C}	37.00^{ED}
SAA109	$20.50^{fghijklm}$	24.00^{fghijk}	7.00^{QRS}	$19.00^{ILNHMGKJ}$	0.00^{P}	SAD12	74.00^{xy}	81.50^{x}	79.50^{A}	67.00^{BA}	77.00^{A}
SAA110	18.25^{defghi}	22.50^{defgh}	18.50^{KMNLOJ}	$21.50^{ILNHMGKFJ}$	0.00^{P}	SAD17	80.50^{y}	76.50^{wx}	76.75^{A}	69.00^{BA}	54.00^{C}
SAA114						SAD18	81.00^{y}	81.00^{x}	61.50^{B}	69.50^{BA}	24.00^{GFH}
SAB1	$22.25^{fghijklmnop}$	35.50^{opq}	$19.00^{IKMNLOJ}$	$17.50^{ILNHMOKJ}$	0.00^{P}	SAD21	$26.50^{ijklmnopqr}$	21.50^{defgh}	$23.75^{IKHFLGJ}$	0.00^{P}	$13.50^{IOIMNLHK}$
SAB2	0.00^{a}	23.50^{efghij}	15.50^{PMNLO}	$22.00^{ILNHMGKFJ}$	0.00^{P}	SAD23	68.00^{x}	77.50^{wx}	75.00^{A}	68.00^{BA}	67.50^{BA}
SAB6	$18.50^{defghijk}$	0.00^{a}	$22.00^{IKHMNLGJ}$	0.00^{P}	0.00^{P}	SAD34	$21.50^{fghijklm}$	23.00^{efghi}	20.05^{IKMNLJ}	0.00^{P}	0.00^{P}
SAB7	$24.00^{ghijklmnopq}$	0.00^{a}	0.00^{S}	0.00^{P}	0.00^{P}	SAD41	0.00^{a}	0.00^{ab}	14.25^{QPNO}	0.00^{P}	0.00^{P}
SAB10	$17.00^{cdefghi}$	29.00^{ijklmn}	18.50^{KMNLOJ}	0.00^{P}	7.00^{POMN}	SAD45	$29.50^{ilmnopqr}$	32.50^{mnop}	15.25^{PMNO}	0.00^{P}	0.00^{P}
SAB11	$26.00^{hijklmnopqr}$	0.00^{a}	18.50^{KMNLOJ}	11.50^{NO}	$17.00^{JGIMNLHK}$	SAD47	32.75^{npqrs}	43.50^{rst}	0.00^{S}	0.00^{P}	0.00^{P}
SAB14						SAE10	$20.50^{fghijklm}$	0.00^{ab}	15.50^{PMNLO}	0.00^{P}	0.00^{P}
SAB19	5.50^{ab}	33.00^{mnop}	0.00^{S}	$20.50^{ILNHMGKFJ}$	0.00^{P}	Pos-R16	76.50^{xy}	76.00^{wx}	78.50^{A}	74.00^{A}	71.00^{BA}
SAB20						Negative	0.00^{a}	0.00^{a}	0.00^{S}	0.00^{P}	0.00^{P}
SAB23	$30.00^{lmnopqr}$	24.50^{ghijk}	16.00^{KPMNLO}	0.00^{P}	0.00^{P}	P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SAB24						CV	21.53308	21.53308	19.03057	23.519	31.65505
SAB30	$23.00^{fghijklmnopq}$	14.00^{c}	0.00^{S}	13.50^{NMO}	0.00^{P}	R2	0.980464	0.980464	0.987418	0.978315	0.972755
SAB35	$25.00^{ghijklmnopqr}$	30.25^{klmno}	15.25^{PMNO}	13.00^{NMO}	25.00^{GF}						

Figure 2: Mean and ANOVA table for the different environmental parameters tested. Table labelled A contains the mean values and ANOVA analysis for temperature, B contains the mean values and ANOVA analysis for pH, and C contains for salt concentration

APPENDIX C: Certificate of analysis for surface tension measurement of selected AEFB isolates

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DURBAN UNIVERSITY of TECHNOLOGY

Certificate of Analysis
Chemical

Name:	Dr C Hunter	**REPORT: DUT 18/025**
Client:	UKZN - Pmb	
Address:	King Edward Avenue Scottsville	
Telephone:	031-260-1111	Fax: 031-2607781
Reference		DUT 18/025
Client Reference		P/O: A495142

Sample ID	Surface Tension analysis in mN/m
Un-inoculated broth negative control	57.3
SAB19 Supernatant	44.3
SAB42 Supernatant	44.7
SAC15 Supernatant	30.8
SAC18 Supernatant	30.9
SAD5 Supernatant	32.2
SAD17 Supernatant	30.7
SAD18 Supernatant	30.6
SAD23 Supernatant	31.6
R16-positive control Supernatant	29.6

Signed: _____ Date : 19/07/2018
Jimmy Chetty- Analytical Chemist

Figure 3: Values obtained for the surface tension measurement of the selected AEFB isolates by Durban University of Technology

Appendix D: Retention factor (R_f) values and mass peak assignment of lipopeptide compounds extracted from *B. velezensis* R16 determined using UPLC-ESI-TOF MS

Fraction	Mass peak (m/z)	Assignment
Rf 0.88	1008.67, 1030.65	C13-surfactin $(M + H)^+$
	1022.68, 1044.66	C14-surfactin $(M + H)^+$
	1036.69, 1058.66	C15-surfactin $(M + H)^+$
	1050.72, 1072.7	C16-surfactin $(M + H)^+$
	1064.73, 1086.72	C17-surfactin $(M + H)^+$
Rf 0.74	1031.55, 1053.52	C14-bacillomycin $(M + H)^+$
	1045.56, 1067.52	C15-bacillomycin $(M + H)^+$
	1059.58, 1081.50	C16-bacillomycin $(M + H)^+$
	1073.6, 1095.57	C17-bacillomycin $(M + H)^+$
Rf 0.64	1477.84, 1499.82	Ala-6-C17 fengycin $(M + H)^+$
	1491.85, 1513.84	Val-6-C16 fengycin $(M + H)^+$
	1505.87, 1527.85	Val-6-C17 fengycin $(M + H)^+$
Rf 0.53	1449.80, 1471.78	Ala-6-C15 fengycin $(M + H)^+$
	1463.82, 1485.80	Ala-6-C16 fengycin $(M + H)^+$
	1477.83, 1499.82	Ala-6-C17 fengycin $(M + H)^+$

Each mass peak assignment was done based on standards (surfactin and iturin) and values reported in the literature (Hunter, 2016)

APPENDIX E: UPLC ESI-TOF MS chromatograms and mass peaks commercial iturin and surfactin standards

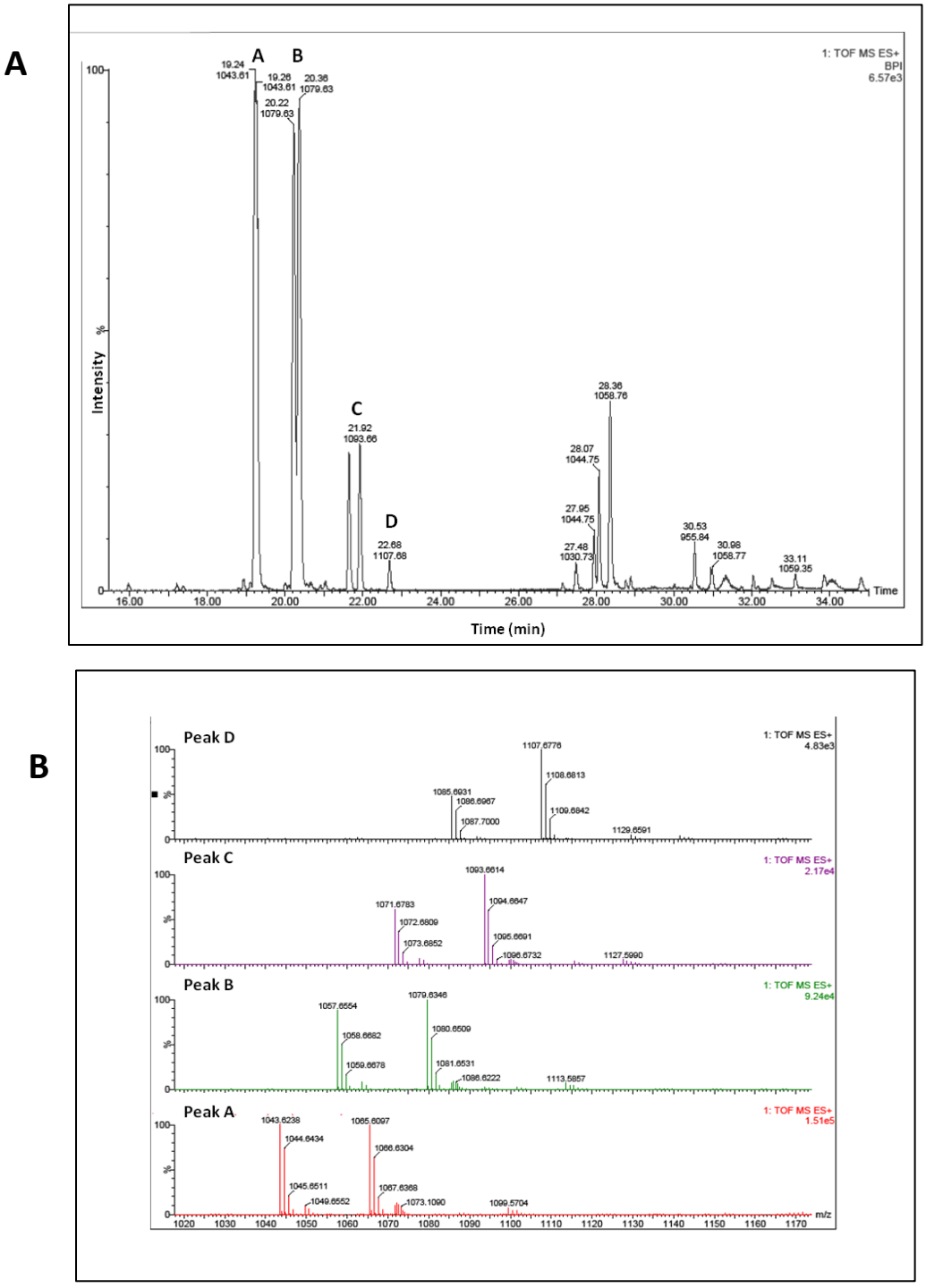

Figure 4: UPLC-ESI-TOF MS chromatogram (A) and mass peaks eluted (B) for iturin isoforms present in a commercial iturin standard (Sigma-Aldrich)

APPENDIX E cont'd

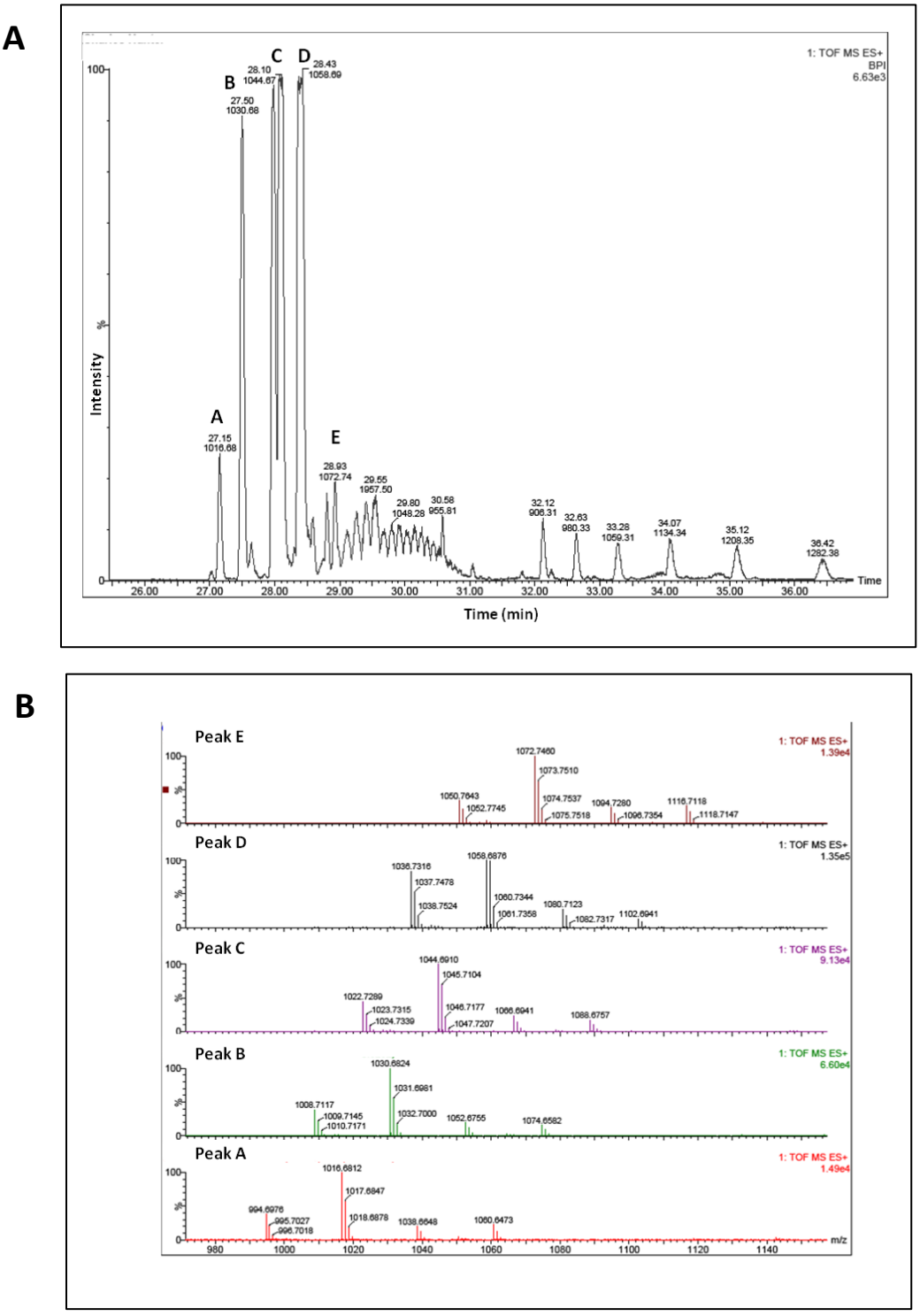

Figure 4: UPLC-ESI-TOF MS chromatogram (A) and mass peaks eluted (B) for surfactin isoforms present in a commercial surfactin standard (Sigma-Aldrich)

APPENDIX F: 16S rRNA gene sequence PCR amplification of selected AEFB isolates

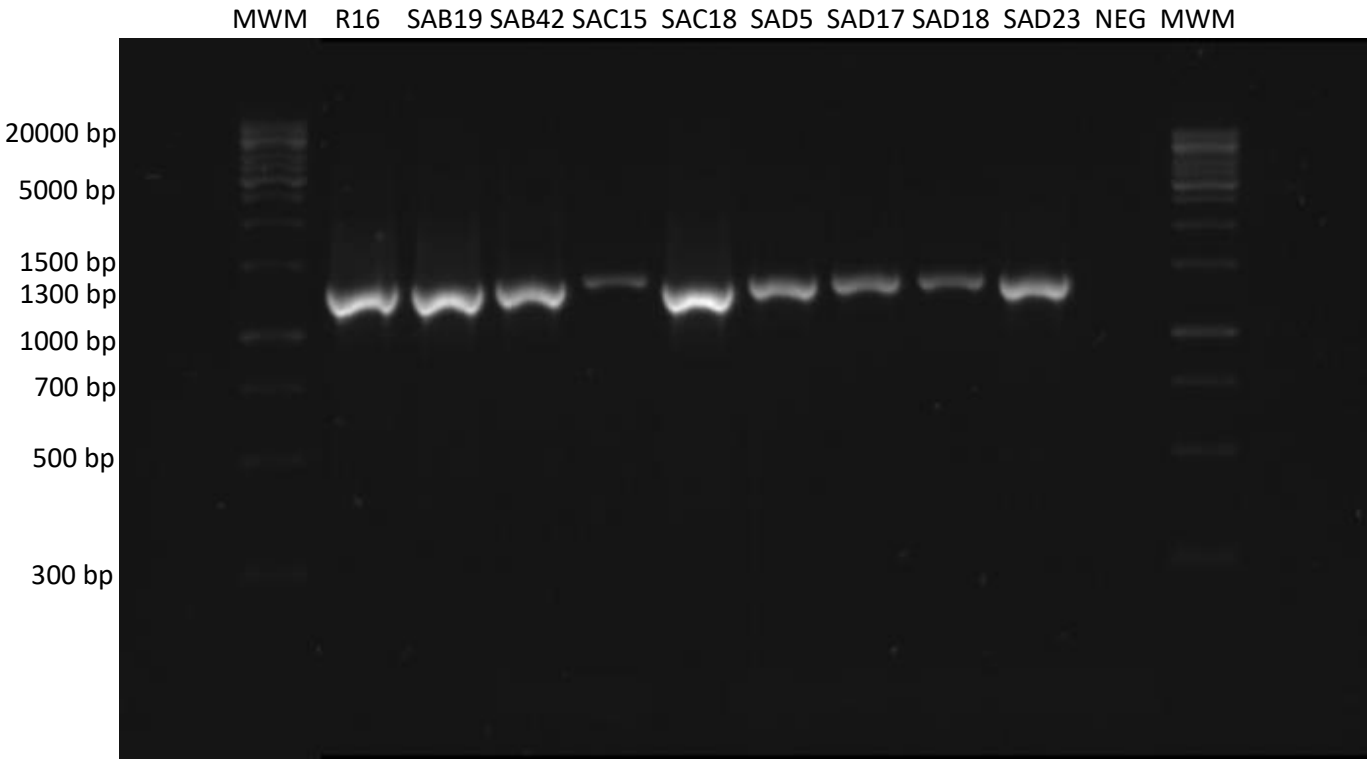

Figure 5: Agarose gel (1.5%) electrophoresis image indicating 16S rRNA gene sequencing PCR amplification products of eight AEFB isolates from an ancient Mfabeni peatland sediment core.