Bioprospecting for Novel Biosurfactants and Biosurfactant Producing Bacteria in Wastewater

by

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DECLARATION

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This dissertation includes one original paper published in a peer-reviewed journal and three unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation (research chapters) indicating the nature and extent of the contributions of co-authors.

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SUMMARY

Biosurfactants are surface active amphiphilic compounds, synthesised by numerous bacteria, fungi and yeast. They are known to exhibit broad spectrum antimicrobial activity and are currently applied as antimicrobial agents, antiadhesives, foaming agents, emulsifiers etc. in the cosmetic, food, pharmaceutical and biotechnology industries. The primary aim of the study was thus to bioprospect for novel biosurfactants and biosurfactant-producing bacteria in a wastewater treatment plant (WWTP). Wastewater was selected as it is a suitable environment for the growth of diverse microorganisms and the presence of numerous organic and inorganic contaminants were postulated to enable the flourishing of biosurfactant-producing microorganisms. Chapter 1 then outlined literature pertaining to biofurfactants, their characterisation and mode of action, amongst many other topics.

Chapter 2 of this study focused on the distribution and diversity of biosurfactant-producing bacteria isolated from wastewater. Wastewater samples were collected from various points of the Stellenbosch WWTP and culturable isolates were screened for possible biosurfactant production using the oil spreading and drop collapse methods. Surface tension and emulsification activities were then used for the partial characterisation of the produced biosurfactant compounds. Thirty-two of the 667 bacterial isolates were regarded as biosurfactant producers and were classified into the Aeromonadaceae, Bacillaceae, Enterobacteriaceae, Gordoniaceae and the Pseudomonadaceae families using 16S rRNA analysis. *Bacillus* and *Pseudomonas* were among the most dominant genera, which constituted 21.8% (7/32) and 12.5% (4/32) of all isolates, respectively. High surface tension reduction of the growth medium (71.1 mN/m) was also observed for the *Bacillus* ST34 (34.4 mN/m) and the *Pseudomonas* ST5 (32.3 mN/m) isolates. In addition, the *Bacillus* ST34 and *Pseudomonas* ST5 isolates tested positive for the *sfp* and *rhlB* genes involved in the biosynthesis of surfactin and rhamnolipid biosurfactants. While numerous studies have reported on the isolation of biosurfactant-producing bacteria from contaminated soil and terrestrial environments, the current study indicated that municipal wastewater could be exploited for the isolation of diverse biosurfactant-producing bacterial strains.

In chapter 3, 32 biosurfactant-producing isolates were then genotypically differentiated utilising repetitive element PCRs (rep PCRs) [targeting the repetitive extragenic palindromic (REP) and the BOX element sequences]. This molecular differentiation was performed as the genetic diversity amongst bacterial species is known to produce different concentrations and proportions of various homologues of biomolecules such as biosurfactants and antibiotics. With the use of the conventional PCR assays, some of the isolates were identified as *Bacillus subtilis* (n = 4), *Aeromonas hydrophila* (n = 3) and *Bacillus amyloliquefaciens* (n = 2), amongst others. These bacterial species were genotypically differentiated into four, three and two sub-species (strains), respectively, utilising rep PCRs. The BOX AIR and REP primers utilised for rep PCR in the current

study thus provided a powerful tool to discriminate between biosurfactant-producing bacterial isolates identified as the same species.

Chapter 4 focused on the characterisation and antimicrobial activity of the biosurfactant extracts produced by the isolates *B. amyloliquefaciens* ST34 and *Pseudomonas aeruginosa* ST5. Crude biosurfactants from ST34 and ST5 culture broth were extracted using solvent extraction based methods. Thereafter, the high resolution ultra-performance liquid chromatography (UPLC) coupled to electrospray ionisation mass spectrometry (ESI-MS) method, developed in the current study, was utilised to characterise the produced compounds. Results indicated that *B. amyloliquefaciens* ST34 primarily produced the C_{13}, C_{14}, C_{15} and C_{16} surfactin analogues when grown on mineral salt medium (MSM) supplemented with glycerol. For *P. aeruginosa* ST5, high resolution ESI-MS linked to UPLC confirmed the presence of dirhamnolipid congeners, specifically Rha-Rha-C_{10}-C_{10} as well as monorhamnolipid congeners, specifically Rha-C_{10}-C_{10}. The crude surfactin and rhamnolipid extracts were also assessed for their antimicrobial activities and displayed significant antimicrobial activity against a broad spectrum of opportunistic and pathogenic microorganisms, including antibiotic resistant *Staphylococcus aureus* and *Escherichia coli* strains.

The quantitative and qualitative effects of various substrates utilised for the surfactin and rhamnolipid production by *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5 strains, respectively, were assessed in chapter 5. For *B. amyloliquefaciens* ST34, maximum biosurfactant production was observed in the MSM supplemented with fructose (28 mg/L). In addition, four surfactin analogues were produced by *B. amyloliquefaciens* ST34 using the different substrates, however, the Srf2-4 (C_{13}-$_{15}$ surfactins) were the most dominant in all the *B. amyloliquefaciens* ST34 extracts. For *P. aeruginosa* ST5, maximum biosurfactant production was observed in the MSM supplemented with glucose (307 mg/mL). In addition, six rhamnolipid congeners were produced by *P. aeruginosa* ST5 using the different substrates, however, similar to results obtained in Chapter four, the dRL2 (Rha-Rha-C_{10}-C_{10}) and mRL2 (Rha-C_{10}-C_{10}) were the most abundant compounds produced in all *P. aeruginosa* ST5 extracts.

OPSOMMING

Biosurfaktante is oppervlak aktiewe amfifiliese verbindings, gesintetiseer deur talle bakterieë, swamme en giste. Hierdie verbindings is bekend vir hul breë spektrum antimikrobiese aktiwiteit en word tans gebruik as antimikrobiese middels, emulsifiseerders, surfaktant agente, ens. in die kosmetiese, kos, farmaseutiese en biotegnologie-industrieë. Die primêre doel van hierdie studie was dus om te bioprospekteer vir nuwe biosurfaktante en biosurfaktant-produserende bakterieë teenwoordig in 'n riool-suiweringsaanleg. Afvalwater is gekies omdat dit as 'n geskikte omgewing dien vir die groei van diverse mikro-organismes en daar word gepostuleer dat die teenwoordigheid van talle organiese en anorganiese stowwe die biosurfaktant-produseerende mikro-organismes laat floreer. Hoofstuk een was uiteengesit met literatuur wat betrekking hou tot, onder andere, biosurfaktante, hul karakterisering en metode van werking.

Hoofstuk twee fokus op die diversiteit en verspreiding van biosurfaktant-produserende bakterieë wat vanuit afvalwater geïsoleer is. Om hierdie doelwit te bereik, is afvalwatermonsters by verskeie punte van die Stellenbosch rioolsuiweringaanleg geneem en groeibare isolate is getoets vir moontlike biosurfaktant produksie met behulp van die olie- verspreiding- en die druppel-ineenstortings- metodes. Oppervlakspanning en emulsifiseringsaktiwiteit is daarna gebruik vir die gedeeltelike karakterisering van die geproduseerde biosurfaktante. Twee-en-dertig van die 667 bakteriese isolate is geïdentifiseer as biosurfaktant produseerders en is geklassifiseer in die Aeromonadaceae, Bacillaceae, Enterobacteriaceae, Gordoniaceae en Pseudomonadaceae families, met behulp van 16S rRNS analises. *Bacillus* en *Pseudomonas* is geïdentifiseer as die mees dominante genera, met 21.8% (7/32) en 12.5% (4/32) van die isolate wat onderskeidelik tot hierdie genera behoort. `n Groot vermindering in die oppervlakspanning van die groeimedium (71.1 mN/m) is waargeneem vir die *Bacillus* ST34 (34.4 mN/m) en die *Pseudomonas* ST5 (32.3 mN/m) isolate. Die *Bacillus* ST34 en *Pseudomonas* ST5 isolate het verder ook positief getoets vir die '*sfp*' en '*rhlB'* gene wat betrokke is by die biosintese van surfaktien en rhamnolipied biosurfaktante. Terwyl talle studies verslag doen oor die isolasie van biosurfaktant produserende bakterieë uit besmette grond en landelike omgewings, dui die huidige studie aan dat munisipale afvalwater gebruik kan word vir die isolasie van diverse biosurfaktant produseerende bakteriese stamme

In Hoofstuk drie is hierdie 32 biosurfaktant-produseerende isolate verder geïdentifiseer (tot op spesie vlak) met behulp van genus en spesie spesifieke polimerase kettingreaksies (PKR). Bakteriese isolate wat as dieselfde spesie geïdentifiseer is, is genotipies onderskei deur gebruik te maak van herhalende element PKRs [gerig op die herhalende ekstrageniese palindromiese (HEP) en die "BOX" element DNS volgordes]. Hierdie molekulêre differensiasies is uitgevoer omdat die genetiese diversiteit onder bakteriese spesies kan lei tot die produksie van verskillende konsentrasies en proporsies van verskeie homoloë van biomolekules soos biosurfaktante en

antibiotika. Met die gebruik van konvensionele PKR toetse, is sommige van die isolate geïdentifiseer as ondere andere *Bacillus subtilis* (n = 4), *Aeromonas hydrophila* (n = 3) en *Bacillus amyloliquefaciens* (n = 2). Hierdie bakteriese spesies is genotipies onderverdeel, met behulp van herhalende element PKRs, in vier, drie en twee sub-spesies (stamme), onderskeidelik. Die 'BOX AIR' en 'REP' inleiers wat gebruik is vir die herhalende element PKRs in die huidige studie, is dus 'n kragtige toepassing wat gebruik kan word om te onderskei tussen biosurfaktant-produseerende bakteriese isolate, wat as dieselfde spesie geïdentifiseer is.

Hoofstuk vier het gehandel oor die karakterisering en antimikrobiese aktiwiteit van die biosurfaktant ekstrakte wat deur die *Bacillus amyloliquefaciens* ST34 en *Pseudomonas aeruginosa* ST5 isolate geproduseer is. Ru-biosurfaktante wat deur die ST34 en ST5 isolate geproduseer is, is vanuit die vloeibare medium geisoleer met behulp van oplosmiddel-ekstraksie metodes. Daarna is hoë resolusie ultra-verrigting vloeistofchromatografie gekoppel aan elektrosproei-ionisasie massaspektrometrie (ESI-MS) (waarvoor 'n metode in die huidige studie ontwikkel is) gebruik om die geproduseerde verbindings te karakteriseer. Die resultate het aangedui dat *B. amyloliquefaciens* ST34 hoofsaaklik die K_{13}, K_{14}, K_{15} en K_{16} surfaktien analoë produseer wanneer dit op`n minerale sout medium, waarby gliserol gevoeg is, gegroei word. Vir *P. aeruginosa* ST5 is die hoë resolusie ultra-verrigting vloeistofchromatografie gekoppel aan ESI-MS gebruik om die teenwoordigheid van dirhamnolipied verwante produkte, spesifiek Rha-Rha-K_{10}-K_{10}, asook monorhamnolipied verwante produkte, spesifiek Rha-K_{10}-K_{10}, te bevestig. Die ru-surfaktien en rhamnolipied ekstrakies is ook geëvalueer vir hul antimikrobiese aktiwiteite en het beduidende antimikrobiese aktiwiteit teen 'n wye verskeidenheid opportunistiese en patogeniese mikro-organismes, insluitende antibiotika weerstandige *Staphylococcus aureus* en *Escherichia coli* stamme, getoon.

Die kwantitatiewe en kwalitatiewe effek van verskeie substrate wat gebruik is vir die produksie van surfaktien en rhamnolipiede deur *B. amyloliquefaciens* ST34 en *P. aeruginosa* ST5 stamme, onderskeidelik, is in Hoofstuk vyf geëvalueer. Vir *B. amyloliquefaciens* ST34, is maksimale biosurfaktant produksie waargeneem in die minerale sout medium wat met fruktose aangevul is (28 mg/L). Daarbenewens is *B. amyloliquefaciens* ST34 daartoe instaat om vier surfaktien analoë te produseer deur gebruik te maak van verskillende substrate. Die Srf2-4 (K_{13-15} surfaktiene) is egter steeds die mees dominante verbindings in al die ekstrakte van die *B. amyloliquefaciens* ST34 stam gewees. Vir *P. aeruginosa* ST5 is maksimale biosurfaktant produksie waargeneem in die minerale sout medium wat met glukose aangevul is (307 mg/L). Daarbenewens is ses rhamnolipied verwante produkte deur *P. aeruginos*a ST5 geproduseer deur gebruik te maak van verskillende substrate. Die dRL2 (RHA-RHA-K_{10}-K_{10}) en mRL2 (RHA-K_{10}-K_{10}) was egter steeds die mees algemene verbindings wat in al die ekstrakte van die *P. aeruginosa* ST5 stam geproduseer is.

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LIST OF ABBREVIATIONS AND ACRONYMS

$[2M-H+Na]^+$	sodiated dimers
$[M+H]^+$	singly charged protonated ion species
$[M+Na]^+$	singly charged sodium adduct
A	adenylating domain
ACP	acyl carrier protein
Asp	aspartic acid
ATCC	American type culture collection
Amu	Atomic mass units
bamC	bacillomycin C
BSA	bovine serum albumin
C	condensation domain
C4-HSL	butanoyl-homo-serine lactone
Da	daltons
dRL	dirhamnolipid
E	epimerisation domain
EI	emulsification index
ESI-MS	electro spray ionisation mass spectrometry
Glu	glutamic acid
HAAs	3-(3-hydroxyalkanoyloxy) alkanoic acids
HPLC	high performance liquid chromatography
LC	liquid chromatography
LC/MS/MS	liquid chromatography coupled with tandem mass spectrometry
LPS	lipopolysaccharide
m/z	mass/charge ratio
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MHA	Mueller Hinton agar
mN/m	millinewton per metre
mRL	monorhamnolipid
MS	mass spectrometry
MSM	mineral salt medium
NRPS	non-ribosomal peptide synthetase
ORF	open reading frame
PCP	peptidyl carrier protein
rep PCR	repetitive element polymerase chain reaction
REP	repetitive extragenic palindromic
Rha	rhamnose
Rha-C_{10}-C_{10}	α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate
Rha-C_{10}-C_8	α-L-rhamnopyranosyl-β- hydroxydecanoyl -β- hydroxyoctanoate
Rha-C_8-C_{10}	α-L-rhamnopyranosyl-β- hydroxyoctanonyl-β-hydroxydecanoate
Rha-Rha-C_{10}-C_{10}	α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate
Rha-Rha-C_{10}-C_8	α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxyoctanoate
Rha-Rha-C_8-C_{10}	α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxyoctanonyl-β-hydroxydecanoate
rhlB	rhamnosyltransferase subunit B
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
Sfp	surfactin 4'-phosphopantetheinyl gene
T	thiolation domain
TFA	trifluoroacetic acid
UPLC	ultra performance liquid chromatography
WWTP	wastewater treatment plant

Chapter 1: Literature Review

(UK spelling is employed)

1.1. General introduction

Antimicrobial agents are among the most powerful bioactive molecules produced by microorganisms (Levy, 1998) and the discovery of antibiotic compounds was considered one of the greatest achievements of the twentieth century. Since their discovery, a variety of broad and narrow spectrum antimicrobial agents have been used worldwide in agriculture, human medicine and industry, to destroy or inhibit the proliferation of undesirable microorganisms. However, the misuse and overuse of antibiotic compounds for either direct or indirect benefits to humans and elsewhere, has seen the proliferation of bacterial species which have developed increased resistance to these agents (Mann, 2005). Metagenomic and functional studies indicate that in many bacterial species, antibiotic resistance is an inherent trait (Bernier & Surette, 2013). However, medical and non-medical uses of antibiotics have accelerated the development of antibiotic resistance within bacterial communities associated with animals, humans, plants and the natural environment (Bernier & Surette, 2013). Numerous studies have also reported on the occurrence and increase of various antibiotic resistant bacteria in various environments such as wastewater (Odjadjare et al. 2012; Yakobi, 2016) surface water sources (Zhang et al. 2009; Khan et al. 2013; Bréchet et al. 2014) and drinking water (Talukdar et al. 2013). In addition, resistant microorganisms are regularly detected in humans, cattle, chickens and pigs (Carlet et al. 2012; Tadesse et al. 2012; Shakya et al. 2013). There is thus cause for concern regarding public health. The problem is exacerbated as microbial resistance genes can be transferred among genera and species by horizontal gene transfer, conjugation, transduction and transformation. Moreover, the rapid increase in the emergence of multi-drug resistant microorganisms has given rise to infections that are responsive only to a limited consortium of last resort drugs. Of further concern is that the prospects for the development of new effective drugs are limited (Lammie & Hughes, 2016). The development of new strategies which includes the discovery of novel antimicrobial compounds is thus a priority. Such compounds could either replace antibiotics (due to their various antibacterial mechanisms) or be used in conjunction with antibiotics. It is also possible that novel antimicrobial compounds could limit the spread of antibiotic resistant genes (Sen, 2010).

Biosurfactants are surface active amphiphilic compounds of biological origin, synthesised by specific bacteria, fungi and yeasts (Mulligan, 2005). These compounds are known to exhibit broad spectrum antimicrobial activity and different classes of biosurfactants are being utilised by the agricultural, oil, food, cosmetic, biotechnological and pharmaceutical industries (Kachholz & Schlingman, 1987; Hood & Zottola, 1995; Rosenberg & Ron, 1999; Dembitsky, 2004; Rodrigues et al. 2006a; Piljac et al. 2008; do Valle Gomes & Nitschke, 2012). Once synthesised by the microorganism, the biosurfactants are either secreted extracellularly or are partially attached to the membrane of the cell. The latter arrangement commonly occurs when the microorganism is cultured in water-insoluble substrates. Intracellular biosurfactants are hypothesised to be used for

gene and nutrient uptake, to assist host cells in the neutralisation of toxic elements by sequestration, to aid in cell differentiation and finally to facilitate the storage of energy and carbon (van Hamme et al. 2006). Biosurfactants reduce surface tension at the phase boundary of a water-insoluble substrate, thus rendering the substrate available for nutrient uptake and metabolism by the producing organism (Fakruddin, 2012). In addition, biosurfactants enable microorganisms to move along an interface (liquid-liquid, liquid-solid, liquid-air) more easily. This is as a result of a reduction in surface tension between the different phases, thus aiding in the motility of organisms in potentially hostile environments (van Hamme et al. 2006).

Biosurfactants are composed of biological-chemical complexes that include a wide range of molecules such as fatty and dicarboyxlic acids, fatty acid amides, lactones, alkylglycosides, phospholipids, glycolipids, lipopeptides and sugar molecules. The molecular components of the biosurfactant are divided into hydrophobic and hydrophilic moieties (Sen, 2010). The hydrophobic moiety usually consists of saturated or unsaturated long-chain fatty acids, while the hydrophilic moiety is made up of anions, cations, amino acids or polysaccharides (Desai & Banat, 1997). Biosurfactants are classified into different groups, dependent on their inherent chemical structure and the identity of the microorganism that synthesises these compounds (Ron & Rosenberg, 2001). The major classes of biosurfactant compounds include glycolipids, lipopeptides, phospholipids, polymeric compounds and neutral lipids (Desai & Banat, 1997). Microbially synthesised surfactants (biosurfactants) have advantages over their synthetic counterparts. These include a low toxicity, high selectivity and specificity of action at extreme pH and temperatures, and extensive foaming properties. Biosurfactants are also readily degraded; thus they are considered to be environmentally friendly (Mohan et al. 2006; Hirata et al. 2009; Chrzanowski et al. 2012).

Competition for survival within a microbial community is one of the principal factors driving the synthesis and secretion of antimicrobial biosurfactants by microorganisms (van Hamme et al. 2006) and the antimicrobial properties of biosurfactants depend on various mechanisms to inactivate target organisms. The manner in which the inactivation occurs is also different from the antimicrobial actions associated with conventional antibiotics (Banat et al. 2010). Biosurfactants primarily destroy microbial cells by directly disrupting the integrity of the plasma membrane or cell wall. The magnitude of such damage to the cell boundary makes it difficult for any target organism to develop resistance to the biosurfactant (Sang & Blecha, 2008; Yount & Yeaman, 2013). For example, lipopeptides create pores in the cell membrane of the target organism, creating an imbalance in the movement of ions both into and out of the microbial cell which is lethal to the damaged cell (Baltz, 2009). In addition, lipopeptide biosurfactant compounds produced by *Bacillus* species specifically display growth inhibitory and lytic effects against a broad spectrum of microorganisms. These include Gram-negative and Gram-positive bacteria, fungi and certain viruses (Abalos et al. 2001; Jenssen et al. 2006; van Hamme et al. 2006; Mandal et al. 2013).

Glycolipid-based biosurfactants such as rhamnolipids primarily produced by *Pseudomonas* species also display algicidal, anti-amoebal and zoosporicidal properties. These lipid compounds have also been reported to effectively kill various bacteria as well as fungi and certain viruses (Soberón-Chávez et al. 2005; Banat et al. 2010; Miao et al. 2015; Soltani Dashtbozorg et al. 2016).

Few of the biosurfactant-producing microorganisms are however, used on an industrial scale for the mass production of biosurfactants. This is due to challenges associated with the production of large quantities of these compounds; in particular, difficulties are experienced when attempting to culture isolated microorganisms under normal fermentation conditions. A further challenge to the production of biosurfactants is that the type of biosurfactant produced depends on the culture conditions (incubation temperature and agitation speed) as well as the macro- and micronutrients available to the microorganism synthesising the product (Fakruddin, 2012). However, bioprospecting offers a cost-effective means for the isolation of biosurfactant-producing organisms that can be readily cultured under standard microbial growth conditions, while simultaneously producing large quantities of biosurfactants. Furthermore, bioprospecting makes possible the discovery of a variety of bacterial strains that produce diverse biosurfactants displaying antimicrobial properties against numerous microbes. One attribute of such compounds is that they could be used to supplement or replace current antimicrobial chemotherapies.

The primary aim of the current project was to screen wastewater for biosurfactant-producing bacterial strains and any associated biosurfactant compounds. Wastewater was selected as it offers a readily available nutrient rich environmental source known to support diverse viable bacterial communities, some of which could produce biosurfactants. Wastewater samples were collected from various points at a local wastewater treatment plant. Thereafter, bacterial isolates were cultured, identified and screened for biosurfactant production. Screening was done by using indirect conventional methods such as the drop collapse and oil spreading techniques (refer to section 1.4). All bacterial isolates found to exhibit positive surfactant production were then preliminarily characterised by measuring the surface tension (Du Nouy tensiometer) and emulsification index of any potential biosurfactant produced (refer to section 1.4) (against kerosene, mineral oil and sunflower oil). All selected biosurfactant-producing isolates were identified by using genus and species specific polymerase chain reaction (PCR). To discriminate among species, repetitive element PCR was used (refer to section 1.3). The antimicrobial activity of crude biosurfactant extracts (obtained from two biosurfactant-producing isolates) was screened against selected Gram-positive and Gram-negative bacteria and pathogenic fungi (including yeast). The biosurfactant compounds produced by selected microbes were characterised by means of electrospray ionisation-mass spectrometry and liquid chromatography coupled with mass spectrometry (LC/MS) (refer to section 1.5). In addition, the influence of different carbon

sources (glucose, fructose, sucrose, glycerol, kerosene, diesel and sunflower) on the microbial production of biosurfactants were assessed.

1.2. Biosurfactants

Biosurfactants are secondary metabolites synthesised by actively growing and/or resting microbial cells (bacteria, fungi and yeast). The compounds can be secreted into the external environment, form part of the cell membrane or they may be metabolised within the cell (Van Delden & Iglewski, 1998; Ron & Rosenberg, 2001; Mulligan, 2005). They are non-ribosomally synthesised compounds that display noticeable emulsification and surface activities. This is due to their structure which consists of both hydrophilic and hydrophobic moieties. Biosurfactants form a diverse group of biomolecules with molecular weights ranging from 500 Da to 1 000 kDa (Choi et al. 1996; van Hamme et al. 2006). Based on their chemical composition and microbial origin, biosurfactants have been classified into different groups. There are five major classes which include glycolipids, lipopeptides, phospholipids, polymeric compounds and neutral lipids (Sen, 2010). The different chemical compositions of various biosurfactants contribute to their unique physico-chemical attributes. Generally, they all show diverse emulsification, interfacial and surface tension properties. Certain biosurfactant compounds are required by the producing microorganism for solubilisation of hydrocarbon compounds, and for the formation of biofilms. Some of these compounds can also enhance the motility of microbial cells. In addition, biosurfactants exhibit antiadhesive, anticarcinogenic and antimicrobial properties. Thus they are versatile compounds and therefore have numerous applications in the cosmetic, food, pharmaceutical and oil industries. They are also effectively used for environmental bioremediation (Benincasa et al. 2004; Mulligan, 2005; Rodrigues et al. 2006a; Mulligan et al. 2014).

1.3. Biosurfactant-producing microorganisms

Microbial species reported to produce biosurfactant compounds include certain unicellular eukaryotes and various Gram-negative and Gram-positive bacterial genera such as *Bacillus*, *Burkholderia*, *Flavobacterium*, *Pseudomonas* and others. These microorganisms inhabit a variety of environments however, their isolation from primarily uncontaminated and undisturbed environments such as natural soils and marine environments has been reported (Bodour et al. 2003; Thavasi et al. 2011). In addition, numerous studies have indicated that polluted environments such as those contaminated with oil, as well as wastewater treatment plants, yield increased numbers and diversity of biosurfactant-producing microorganisms (Bodour & Miller-Maier, 1998; Bento et al. 2005; Ndlovu et al. 2016).

Previous studies frequently reported that microorganisms synthesise and secrete biosurfactants for various purposes. These include for the facilitation of nutrient uptake, bacterial cell motility and biofilm formation. In addition, it is known that within a diverse microbial community indigenous to

a particular habitat, biosurfactant producers have a competitive advantage over non-producing microorganisms (Raaijmakers et al. 2010; Chrzanowski et al. 2012). Furthermore, biosurfactants can exert an impact on the behaviour of microbes. This is observed in cell to cell communication and competition among bacteria, the progress of animal and plant pathogenesis and the assembly of fungal fruiting bodies (Ishigami & Suzuki, 1997; Van Delden & Iglewski, 1998; Peypoux et al. 1999; van Hamme et al. 2006; Walter et al. 2010).

The *Pseudomonas* genus is composed of 191 species which include *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Euzéby, 1997). The genus belongs to the family Pseudomonadaceae and all pseudomonads are Gram-negative. *Pseudomonas* species are obligates aerobes and demonstrate a highly diverse metabolism. Thus the bacterium is able to colonise many different aerobic niches. Strains of *Pseudomonas* species have been isolated from a number of environments which include soils contaminated by petroleum based compounds (MacElwee et al. 1990). In environments where there are high densities of microbial cells, quorum sensing leads to an amensalism interaction, commonly reported for *P. aeruginosa*. Biosurfactant compounds are thus produced by *P. aeruginosa* to inhibit the development of other competing taxonomic groups. The production of biosurfactant compounds by *Pseudomonas* is dependent on the physiological status of the cell. An example is the elevated production of rhamnolipids by *Pseudomonas* induced by the depletion of nutrients during the stationary phase of growth (Lang & Wullbrandt, 1999; Clarke et al. 2010).

Bacillus species are rod-shaped, Gram-positive bacterium that belong to the phylum Firmicutes. They are obligate aerobes or facultative anaerobes (depending on the species) and occur in various natural environments either as free-living cells or as parasites. The genus is known for the production of intracellular oval endospores which are formed when environmental conditions become unfavourable. Species within *Bacillus* are reputed to be closely related (Rooney et al. 2009). *Bacillus amyloliquefaciens* is one of the most characterised species, as it has been used widely as a model organism for research into the production of various metabolites including biosurfactant compounds (Schallmey et al. 2004). Various *B. subtilis* strains have also been reported to produce biosurfactant compounds used frequently by various industries (Peypoux et al. 1999; Bodour et al. 2003; Hsieh et al. 2004; Rodrigues et al. 2006a; Walter et al. 2010). The production of lipopeptide biosurfactants by *Bacillus* species which include *B. subtilis* and *B. amyloliquefaciens,* occurs during the exponential and stationary phases of growth. The surfactants function to emulsify substrates and/or to facilitate the uptake of substrates through pores in the bacterial cell membrane (Peypoux et al. 1999; Mulligan et al. 2014). *Bacillus cereus*, *B. amyloliquefaciens*, *B. subtilis*, *B. brevis* and *B. licheniformis* were also reported to synthesise antimicrobial biosurfactants when cultured in media containing hydrocarbons (Bodour et al. 2003; Rodrigues et al. 2006a).

Serratia are Gram-negative facultatively anaerobic bacteria. There are a number of species, of which *Serratia marcescens* is the most widely studied. *Serratia marcescens* was originally considered to be a non-pathogenic saprophytic microorganism and was used as a biological marker because of its easily distinguishable unique red colonies (Hejazi & Falkiner, 1997). Certain extracellular metabolites which include prodigiosin and various enzymes such as chitinase, chloroperoxidase, lipase and protein HasA, all of which facilitate virulence factors, are unique to *S. marcescens*. *Serratia marcescens* has also been reported to produce an extracelullar compound, serrawettin. This compound is a surfactant which facilitates motility in cells lacking flagella (Matsuyama et al. 1995; 2011).

1.3.1. Identification of biosurfactant-producing microoganisms

Microorganisms are ubiquitous in the environment and many secrete biosurfactant compounds in order to facilitate the bioavailability of nutrients, aid in cell movement and enhance protection when environmental conditions become unfavourable (Fakruddin, 2012). However, in the natural environment, microorganisms occur predominantly as mixed populations of various species and strains. Pure cultures of bacteria are then essential for the analysis of cell characteristics and/or properties. The initial step required to isolate microorganisms from the environment as pure cultures thus involves culturing microrganisms in general, on differential or selective growth media. It is only once this has been achieved, that pure cultures can be identified, characterised and screened for biosurfactant production. This approach has been applied successfully by various authors for the isolation, identification and screening of potential biosurfactant-producing microorganisms from diverse environments (Bodour et al. 2003; Bento et al. 2005; Ben Belgacem et al. 2015).

With the use of the conventional PCR, biosurfactant-producing bacterial genera have been classified into species based on their genetic diversity (Bodour et al. 2003; Hsieh et al. 2004). However, conventional PCR does not differentiate or distinguish among closely related species or strains. Certain molecular fingerprinting techniques such as pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST) and the repetitive element polymerase chain reaction (rep PCR), have been developed and utilised for the differentiation of various bacterial species (Versalovic et al. 1994; Klima et al. 2010; Ma et al. 2011; da Silva & Valicente, 2013; Munday et al. 2013; Taylor et al. 2014; García et al. 2015). The rep PCR technique in particular has been applied successfully to differentiate among the genomes of several bacterial strains isolated from diverse environments and classified as biosurfactant producers (Bodour et al. 2003; Tran et al. 2008). The method incorporates the use of a specific set of primers that bind to repetitive DNA sequences in the genome. This enables amplification of different regions of the genome, and creates discrete DNA fragments which resolve as specific banding patterns or

fingerprints, visualised by using gel electrophoresis. The DNA fingerprint is unique to an individual bacterial strain or clone. Repetitive element PCR is more cost-effective than PFGE and the primers used in this technique are homologous to the repetitive sequences of the families of the REP, ERIC and BOX DNA sequences (Baldy-Chudzik & Stosik, 2005).

The ERIC sequences are repetitive DNA sequences restricted to transcribed regions of the genome, either in the upstream or downstream regions of the open reading frames (ORF) or within intergenic regions of the polycistronic operons (Hulton et al. 1991). These are imperfect 124-127 base pair (bp) long palindromes which are highly conserved and have been used to differentiate between enteric bacterial species (Wilson & Sharp, 2006; Fendri et al. 2013).

Prokaryote genomes have highly conserved repeated DNA sequences such as the BOX element that is situated in noncoding regions dispersed throughout the chromosome (van Belkum et al. 1998; van Belkum & Hermans, 2001). The BOX element consists of three distinct regions namely boxA, boxB and boxC, which are 59, 45 and 50 bp long, respectively (van Belkum & Hermans, 2001). The presence of multiple copies of the BOX element on the genome provide useful targets that can be used to discriminate closely related species (van Belkum & Hermans, 2001).

Repetitive extragenic palindromic (REP) sequences consist of palindromic sequences that vary in length from 21 to 65 bp and are detected in the extragenic regions of certain bacterial genomes (Tobes & Pareja, 2006). A single bacterial genome has in excess of 100 copies (Nunvar et al. 2010). The origin and function of REP sequences in bacterial genomes is not completely elucidated, but various functions are ascribed to these elements. These include the provision of binding sites for DNA polymerases and DNA gyrase, acting as mRNA stabilisers and they are thought to be implicated in the expression of genes. It is also suggested that the elements act as integration host factors. In aditition, repetitive extragenic palindromic units are presumed to enhance folding of various G-C rich DNA regions into stem loops. These unique elements of prokaryote DNA make molecular fingerprinting analyses possible. The REP fragments and binding sites share similar DNA characteristics including palindromic structure and size and are located at multiple sites within the extragenic regions of bacterial genomes.

The rep PCR was used by Bodour et al. (2003) to compare biosurfactant-producing microbial isolates from a selected environment and analyse a mixture of surfactants produced by each organism when grown under identical culture conditions. The authors showed that the *Pseudomonas* isolates *P. aeruginosa* ATCC 9027 and *P. aeruginosa* IGB83 both produced rhamnolipids; however, the chemical structures of these compounds were distinct. *Pseudomonas aeruginosa* ATCC 9027 produced only monorhamnolipids, while *P. aeruginosa* IGB83 produced a mixture of mono- and dirhamnolipid, all of which displayed different physico-chemical properties. Another study conducted by Mukherjee and Das (2005) reported on the production of

different isoforms and quantities of surfactin compounds produced by *B. subtilis* strains (DM-03 and DM-04) which also displayed varying degrees of antimicrobial activity against test microbes. It is therefore apparent that various strains of *Bacillus*, *Pseudomonas* and other microbial species can constituently produce different biosurfactant congeners and homologues, which often display different physico-chemical properties in combination to the physico-chemical properties observed in individual congeners.

1.4. Physico-chemical properties of biosurfactants and methods utilised for biosurfactant production screening

Biosurfactants are microbial metabolites of low and high molecular weights and are composed of sugars, amino acids, fatty acids and functional groups such as carboxylic acids. They are produced by many microorganisms and are structurally a diverse group of compounds, primarily catergorised into glycolipids, lipopeptides, lipoproteins, lipopolysaccharides and phospholipids (Walter et al. 2010; Thavasi et al. 2011). High throughput methods for the rapid and reliable screening and selection of numerous potential biosurfactant-producing microoganisms are essential for the discovery of new biosurfactants and/or biosurfactant-producing strains. Methods used for the initial general screening of biosurfactant production are therefore based on some of the easily detectable physical effects exerted by these biomolecules. The physico-chemical properties of biosurfactant compounds are important for their functionality and also facilitate the screening for their presence in a culture medium.

Biosurfactants are known for their excellent surface activity which serves a number of purposes. These include decreasing the surface and interfacial tension between different phases (liquid-air, liquid-liquid and liquid-solid) which contribute to the low critical micelle concentration (CMC) of these compounds and their propensity to form stable emulsions. The ability to lower surface and interfacial tension is caused by the adsorption of the biosurfactant to different phases. This results in more interaction and mixing of dissimilar phases which functions to solubilise hydrophobic substrates (Satpute et al. 2010; Walter et al. 2010; Uzoigwe et al. 2015). The screening methods commonly employed for biosurfactant production assessment are thus based on their interfacial or surface activity (Walter et al. 2010). In addition, the emulsification and foaming activities of biosurfactant compounds are also measured.

1.4.1. Emulsification activity

Emulsification is a functional property of biosurfactants which refers to the dispersion of one liquid phase in another, causing the mixing of two immiscible liquids (Inès & Dhouha, 2015). Measuring the emulsification activity is one of the indirect methods used for screening possible biosurfactant production by microorganisms. This method was first described by Panchal and Zajic (1978), and it requires mixing of an equal volume of a hydrocarbon-based compound (kerosene is the

commonly used oil) with the sample and subsequently placing the mixture at an ambient temperature for 24 hours. As indicated in **Figure 1.1** (**B**) an emulsion is formed when an emulsifying agent such as a biosurfactant is present. The emulisification index (EI) is calculated by using the equation:

$$Emulsification\ index\ (E24)\% = \frac{Height\ of\ the\ emulsion\ layer}{Total\ height\ of\ the\ solution}\ X\ 100 \qquad \text{Equation 1}$$

Emulsification activity is an important characterisitic of biosurfactant compounds and is exploited by various industries. This includes the production of water/oil emulsions for the cosmetics, food and particularly, the pharmaceutical industries. Certain metabolites such as bioemulsifiers (e.g. emulsan and liposan) are secreted by various strains of *Acinetobacter calcoaticus* and *Candida lypolitica.* These two bioemulsifiers have a higher emulsification activity when compared with low molecular weight biosurfactants synthesised from hydrocarbon-based compounds (Satpute et al. 2010; Uzoigwe et al. 2015).

The polymeric biosurfactants, including emulsan and liposan, have a high molecular weight of up to 1 000 kDa and show tensile strength and resistance to shearing. These inherent properties contribute to their exceptional emulsifying properties (Desai & Banat, 1997). Emulsan is able to form stable emulsions at concentrations as low as 0.001%, and is regarded as the most powerful emulsion stabiliser. However, biosurfactants with lower molecular weights (lipopeptides and glycolipid based biosurfactants) which vary between 500 – 1 500 Da, are also known to form stable emulsions (Kim et al. 1997; Benincasa et al. 2004).

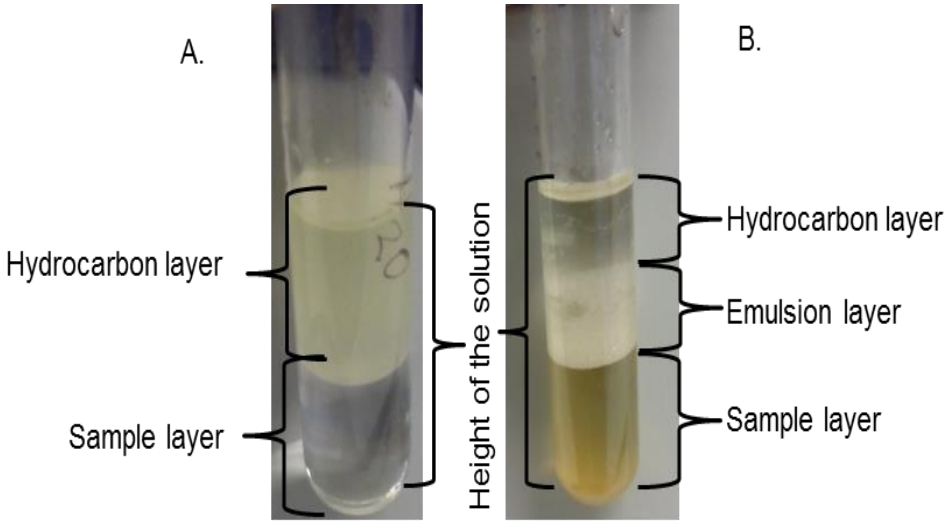

Figure 1.1 An illustration of: A) no emulsion formed between a bacterial culture sample and diesel oil after 24 hours at room temperature and B) an emulsion formed between a bacterial culture sample and diesel oil after 24 hours at room temperature

In a study conducted by Benincasa et al. (2004), rhamnolipids of biological origin formed stable emulsions for up to 21 days when mixed with 15% (m/v) aqueous solution and castor oil

(EI = 67%), benzene (EI = 60%), almond oil (EI = 83%) and crude oil (EI = 75%). Surfactin, which is a low molecular weight biosurfactant, also forms stable emulsions with n-hexadecane and 2-methylnaphthalene (Kim et al. 1997). Research groups have thus reported on the successful application of the emulsification index as a means of assessing the production of biosurfactant biomolecules by various microorganisms isolated from diverse environments (Desai & Banat, 1997; Kim et al. 1997; Das et al. 2008a; Ben Belgacem et al. 2015; Ndlovu et al. 2016).

1.4.2. Surface and interfacial tension

Microorganisms are greatly influenced by interfacial phenomena, particularly when the cell wall or membrane of these organisms interacts with the external environment. This stimulates the synthesis of biosurfactants that are used by the microorganisms to accommodate challenges associated with the interface. In microbial biofilms, surface films and aggregates, it has been reported that interfacial tensions occur more often, with < 0.1% of microbes existing as planktonic cells experiencing less tension in their environment (Nickel & Ladd, 1986). Interfacial phases control the transfer of nutrients, waste and quorum sensing signalling molecules, which is explained by the host-microbe interactions (natural flora and pathogens). The interfacial properties of biosurfactants then influence the growth of microbes as follows; sequestration of toxic metabolites, pH buffering as well as reducing or increasing the availability of substrates for nutrient uptake (van Hamme et al. 2006).

Surface tension is a force per unit length that is exerted by a liquid in contact with another liquid or solid. It can also be considered as a measure of the free energy per unit area that is associated with an interface or surface (Satpute et al. 2010). As illustrated in **Figure 1.2** (**A**), water molecules are bound together by cohesive forces that create surface tension. Biosurfactants are microbially synthesised molecules that display surface activity and are able to adsorb to interfaces or surfaces (Inès & Dhouha, 2015). The surface tension of water is calculated as 72 mN/m, and when a surfactant is added to water (**Figure 1.2 B**) this value is reduced (Satpute et al. 2010). An effective biosurfactant compound should reduce the surface tension of water (72 mN/m) to approximately 35 mN/m (Abdel-Mawgoud et al. 2010; Soberón-Chávez, 2010). The biosurfactant surfactin has been reported to be one of the most effective surfactants and reduces the surface tension of water from 72 to 27 mN/m (Cooper & Goldenberg, 1987; Banat, 1993). In a review article by Mulligan (2005), the surfactin biosurfactant was reported to decrease the surface tension of water to 25 mN/m and the interfacial tension of water/hexadecane was reduced from 40 mN/m to 1 mN/m. Another well-characterised biosurfactant, rhamnolipid, primarily produced by *P. aeruginosa,* reduces the surface tension of water to approximately 30 mN/m and the interfacial tension of water/oil from 43 mN/m to approximately 1 mN/m (Dusane et al. 2010).

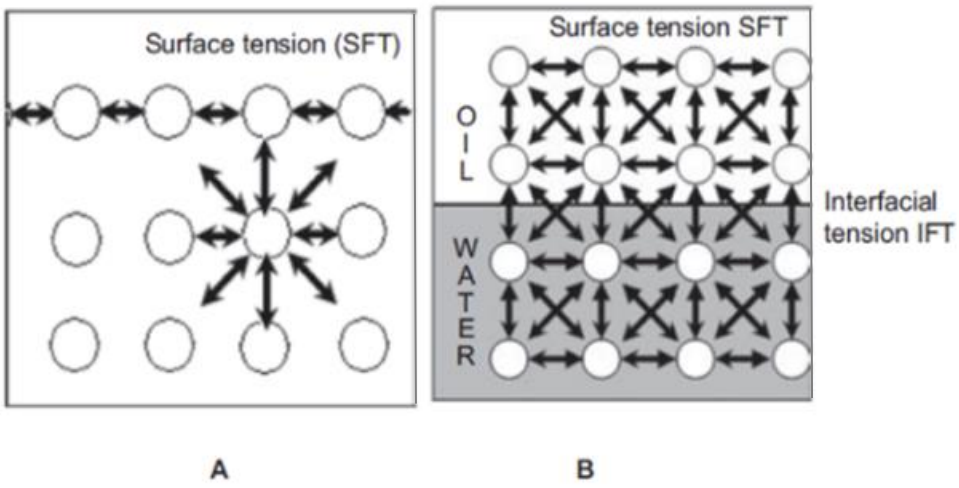

Figure 1.2 Illustration of the surface and interfacial tension A) surface tension of water, B) Surface tension of oil in water with a biosurfactant compound and the effect of interfacial tension of water and oil [adopted from Satpute et al. (2010)]

The direct measurement of the surface tension in a nutrient broth used for culturing microorganims is usually carried out by using a du Nouy tensiometer (**Figure 1.3**). This method is reported to be precise and various studies have shown that the measurement of surface tension is a reliable method for the detection of biosurfactant compound production (Bodour et al. 2003; Youssef et al. 2004; Salihu et al. 2009). Satpute et al. (2010) stated that biosurfactants can have both emulsification and surface tension reduction activities.

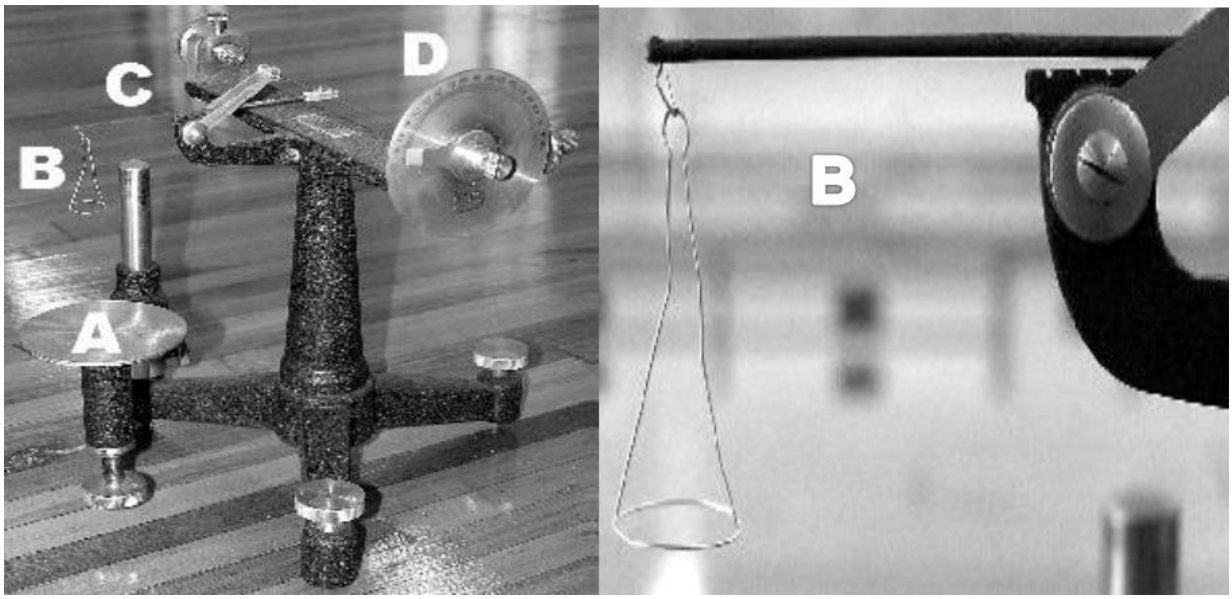

Figure 1.3 The Du Nouy tensiometer used for the measurement of surfactant surface tension (with permission from the Department of Chemistry, Physical Chemistry 324 Practical Guide 2014, Stellenbosch University); A – sample platform; B –platinum ring; C and D – scale adjustment and value readings, respectively.

Bacterial cells rely on mobility for continued growth and reproduction. Motility is facilitated by complex signalling and sensing systems in response to changes in external environmental factors. These include light, pH, redox potential, nutrients, toxic substrates or internal cues

(changes in energy levels and proton motive force) (Peypoux et al. 1999; Singh & Cameotra, 2004; van Hamme et al. 2006). When microbial cells occur at an interface, biosurfactant compounds are usually secreted to facilitate cell movement by reducing the surface and interfacial tension. This enhances the swarming ability of microbes. *Serratia marcescens* has been reported to depend on the production of serrawettins (nonionic biosurfactants) for surface movement as well as for movement on water-repelling surfaces (Matsuyama & Nakagawa, 1996). The rhamnolipid biosurfactant precursors 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) produced by *P. aeruginosa* also facilitate swarming motility in the absence of rhamnolipids (Deziel et al. 2003).

1.4.2.1. Drop collapse method

The drop collapse technique is a qualitative method commonly used to measure the surface tension of liquids. The method relies on the destabilisation of liquid droplets by surfactants, described as surface active compounds (Walter et al. 2010). The presence of biosurfactants causes the liquid droplets to spread or collapse over a hydrophobic surface due to the interfacial tension between the liquid drop and the hydrophobic surface (Hsieh et al. 2004; Walter et al. 2010). The drop collapse method was developed by Jain et al. (1991) for the detection of surfactant production by various microorganisms. After growth of microorganisms in culture media, a drop of the broth culture (secreted and membrane bound biosurfactants) or cell free supernatant (secreted biosurfactant compounds) is used for biosurfactant production screening (Walter et al. 2010). The drop of culture is placed on a surface coated with oil and if the liquid suspension contains biosurfactant biomolecules, the drop will spread or collapse over a surface coated with a hydrocarbon liquid (Walter et al. 2010). However, if there are no biosurfactants present in the culture sample, the drop remains stable as it is repelled by the hydrophobic surface. The surfactant concentration determines the stability of the liquid drop, which implies that this method could also be used for the indirect quantification of pure biosurfactant biomolecules by measuring the size of the drop (Bodour & Miller-Maer, 1998; Bodour et al. 2003). This assay is easy to perform, rapid and does not require specialised expensive equipment. In addition, a minute volume of sample is required. This technique has been applied for the screening of biosurfactant production by microorganisms isolated from different environments (Bodour et al. 2003; Batista et al. 2006; Płaza et al. 2006; Thavasi et al. 2011; Ibrahim et al. 2013; Ben Belgacem et al. 2015; Ndlovu et al. 2016). However, Satpute et al. (2008) and Walter et al. (2010) stated that the drop collapse assay displays a relatively low sensitivity, particularly in samples containing low concentrations of surfactants. This is because a high concentration of surface active compounds is required to be present in a sample for an observable collapse of the drop on a hydrophobic based oil or surface.

1.4.2.2. Oil spreading technique

The oil spreading assay is another method which can be used to screen for biosurfactant production. It was described by Morikawa et al. (2000) during a study of the structure function relationship of the biosurfactant biomolecules, arthrofactin and surfactin. The method requires that ten microlitres of hydrophobic based oil is added to the surface of water (approximately 40 ml) in a petri dish such that a thin layer of oil is formed. Ten microlitres of cell free culture or cell suspension are then placed carefully in the centre of the oil layer. If a biosurfactant is present in the culture, the oil is displaced and a zone of clearing is observed. The diameter of this clearing zone on the oil surface usually correlates positively with the surfactant activity, also known as the oil displacement activity. Similar to the drop collapse method, it is easy to perform, rapid, no specialised equipment is required and only a small volume of sample is required. A number of research groups have indicated that the oil spreading technique is a reliable means of detecting biosurfactant production by various microorganisms (Hsieh et al. 2004; Youssef et al. 2004; Płaza et al. 2006; Thavasi et al. 2011; Zhang et al. 2012; Ben Belgacem et al. 2015).

1.4.3. Foaming activity

Foaming is a property displayed by amphipathic biosurfactants. Biosurfactant compounds are usually concentrated at the gas-liquid interface. Generally, the foam is created when the air bubbles are created underneath the surface of the liquid and are maintained without collapsing. This property of biosurfactant compounds makes them attractive as additives for the development of products such as cosmetics, detergents and pharmaceuticals (Razafindralambo et al. 1996; Mulligan, 2005). Small bubble size and stable foams created by biosurfactant compounds are also of importance in the mineral processing industry for the separation of metal groups from suspensions. In a study conducted by Razafindralambo et al. (1996), surfactin exhibited a better foam stability in milliQ water at concentrations as low as 0.05 mg/L when compared with common commercial surfactants such as bovine serum albumin (BSA) and sodium dodecyl sulphate (SDS). When the concentration was increased to 100 mg/L, the foaming ability of surfactin increased to 88%. However, at the same concentration, SDS exhibited no residual foaming, and BSA at a concentration of 200 mg/L showed a foaming ability of 65%. Thus, surfactin indicated a higher surface activity overall compared to BSA and SDS.

1.5. Characterisation of biosurfactant compounds

Advancement in technology over recent decades has introduced new techniques used for the identification and characterisation of biosurfactant molecules. While a variety of methods are utilised to classify and characterise the biosurfactant compounds produced by a wide range of microorganisms, mass spectrometry (MS) coupled with various chromatographic methods is the most widely used technique. Mass spectrometry identifies the chemical bonds and structures of

biosurfactant compounds. The method also measures the quality and quantity of the biosurfactants present (Mulligan et al. 2014). Mass spectrometers are comprised of three principal parts viz. an ion source, a molecular mass analyser and a detector (Downard, 2004). When a sample is loaded into a mass spectrometer it is first vapourised. Thus volatile samples can be introduced directly into the apparatus, whereas non-volatile samples must first be dissolved in volatile solvents. The sample is then ionised and passes through an electromagnetic field. Based on their charge and mass, the ionised particles separate before finally reaching the detector. The electronic signal is amplified, and conveyed to a computer where it is recorded as a series of chromatograms/peaks. In this manner the overall quality of the compounds produced, as well as the quantity of each ion, can be assessed (Mulligan et al. 2014).

Liquid chromatography coupled with electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) is a highly sensitive method that enables the fingerprinting of low concentrations of metabolites within a crude extract originating from natural sources. It is one of the techniques currently used by various research groups for the characterisation of biosurfactant biomolecules and has been shown to be advantageous by minimising the erroneous identification of a compound. The method saves energy, money and time required for the screening and identification of novel bioactive biosurfactant compounds. Previous studies have utilised this technique successfully to distinguish between different fengycin homologues produced by *Bacillus subtilis* strains (Wang et al. 2004; Hu et al. 2007). Furthermore the method enables the elucidation of the amino acid sequence of the peptidic moiety of natural and hydrolysed fengycins (Deleu et al. 2008). An investigation carried out by Pereira et al. (2012), on rhamnolipids produced by *P. aeruginosa* strains, illustrated that MS coupled with electrospray ionisation provided an accurate and rapid characterisation of these biosurfactants. In addition, Pecci et al. (2010) and Caldeira et al. (2011) successfully identified different lipopeptide compounds produced by *Bacillus* species and partially characterised their chemical composition. Electrospray ionisation has also been used to ionise various biosurfactant-based compounds prior to the analysis of their molecular mass (Benincasa et al. 2004; Déziel et al. 1999; Haba et al. 2003; Monteiro et al. 2007). These studies highlight the use of tandem mass spectrometry as a powerful tool to analyse complex compounds such as biosurfactants. It also permits efficient discrimination among different homologues and isoforms within a mixture of compounds.

Due to its soft ionisation abilities, the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) coupled with mass spectrometry also enables identification of intact biosurfactant compounds (Smyth et al. 2010). Although the MALDI-TOF analyses are costly, previous studies indicated that it is rapid and sensitive, providing high resolution information for the structural characterisation of biosurfactant compounds (Vater et al. 2002; Singh et al. 2014; Sharma et al. 2015). Sharma et al. (2015) successfully used the HPLC and MALDI-TOF for the characterisation of an antimicrobial lipopeptide-based biosurfactant compound produced by *Bacillus pumilis*.

1.6. Different classes of biosurfactant biomolecules

1.6.1. Lipopeptides

Lipopeptide biosurfactants are biological surface active compounds that are widely synthesised by *Bacillus* species. They are composed of a short linear or cyclic heptapeptides or decapeptides linked to fatty acids of varying length (saturated and unsaturated) that act as the hydrophobic moiety (Mandal et al. 2013). In addition, they are low molecular weight (900–2 000 Da) compounds that display diverse and complex chemical structures. Lipopeptides are associated with various biological activities and as such, they are suitable for use in a variety of relevant industries (Raaijmakers et al. 2010; Marchant & Banat, 2012). The fatty acid portion of lipopeptides can assume linear, anteiso or iso branches and this further contributes to their remarkable structural heterogeneity. The structure of lipopeptide biomolecules (surfactins and iturins produced by *Bacillus* species) has been determined through the use of different techniques which include electrospray ionisation coupled with mass spectrometry (ESI-MS) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

1.6.1.1. Lipopeptides produced by *Bacillus* species

Fengycin, bacillomycin, iturin and surfactin are lipopeptide biosurfactant families primarily produced by *Bacillus* species and they display a wide range of activities (Li et al. 2012). Lipopeptides are non-ribosomally synthesised and have received widespread interest due to their versatile applications in industry. Although they differ in chemical structure, they have similar peptide lengths. The peptides are composed of varying amino acid residues found at specific locations within the hydrophilic moiety. Several homologues exist for each lipopeptide variant due to the varying lengths and isomers of the fatty acid chain which confer considerable structural heterogeneity (Ongena & Jacques, 2008). The surfactin family encompasses the esperin, lichenysin, pumilacidin and other groups of surfactin, all of which display variant heptapeptide portions linked to β-hydroxyl fatty acids (C_{12}-C_{16}).

1.6.1.2. Surfactin family

The first sufactin was isolated in 1968 from a *B. subtilis* broth culture (Arima et al. 1968). Surfactins are lipopeptides of low molecular weights ranging from 980 to 1 060 Da. The compound consists of a cyclic lipopeptide composed of a heptapeptide (Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu) linked to a β-hydroxy fatty acid chain comprised of 12 to 16 carbon atoms (Sullivan, 1998; Seydlová et al. 2011). The differences in hydrophobic chain length and the sequence of amino acid residues in the hydrophilic moiety makes the existence of several homologues possible. For example, a single *B. subtilis* strain has been shown to produce different homologues of C_{13}-C_{15}. A few basic structures are presented in **Figure 1.4** (Shaligram & Singhal, 2010). In addition, other forms of

surfactin which have varying amino acids at positions 2, 4 and 7 of the hydrophilic moiety have been reported. All surfactin forms display a negative charge at neutral pH due to the presence of glutamate and aspartate. This contributes to the ability of the compound to act as a cation-carrier and also to display pore forming tendencies (Singh & Cameotra, 2004).

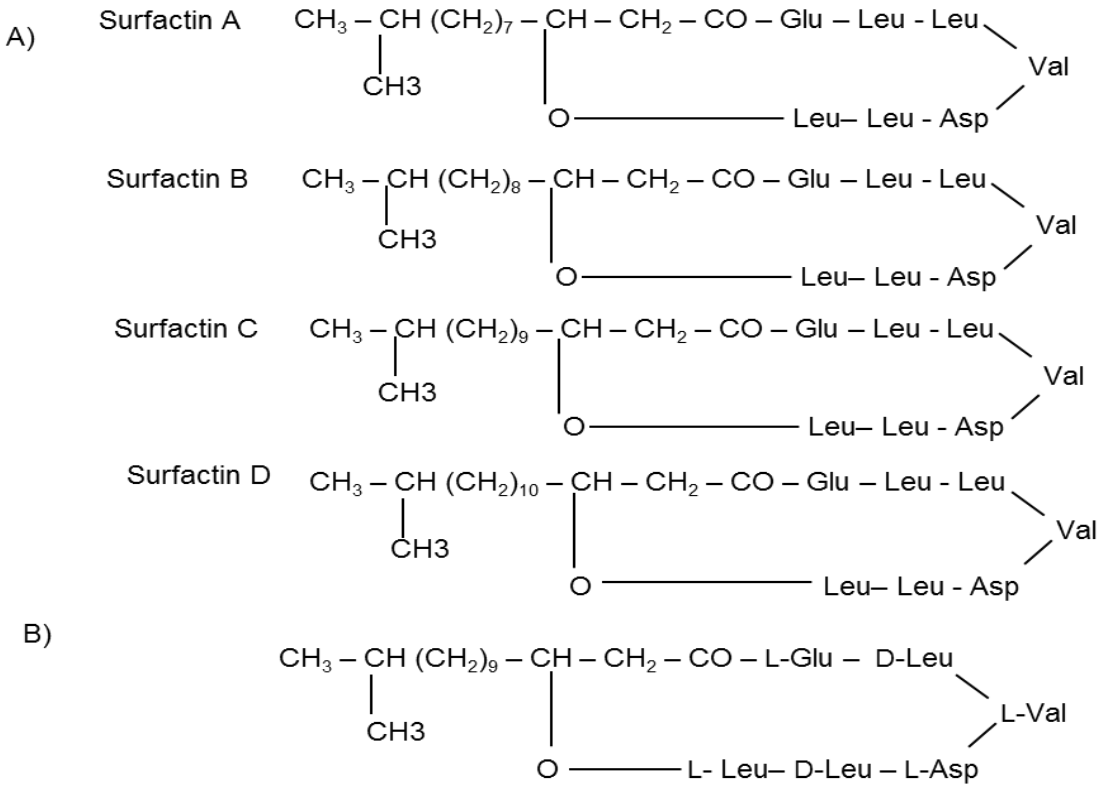

Figure 1.4 Chemical isoform structures of A) various surfactins [adopted from Mulligan et al. (2014)]; and B) a lipohexapeptide form of surfactin [adapted from Shaligram & Singhal, (2010)].

1.6.1.3. Iturin family

Iturin comprises the second family of the lipopeptide compounds produced by certain *B. subtilis* strains and other closely related *Bacillus* species such as *B. amyloliquefaciens*. Iturin was first isolated from a soil sample collected in Ituri, Zaïre (now known as the Democratic Republic of Congo). The structure was similar to that of all of iturin lipopeptides subsequently isolated from *B. subtilis* strains (Sen, 2010). Within the iturin family, iturin A and D, bacillomycin D, F and L, and mycosubtilin are the major groups and are composed of a lipoheptapeptide moiety linked to a β-amino fatty acid chain of varying length (C_{14}-C_{17}) (Walia & Cameotra, 2015). The hydroxyl fatty acid chain can have a linear, anteiso or isoform conformation. The fatty acid chain may be saturated or unsaturated and can show a combination of saturated and unsaturated hydroxyl forms. The β-amino nature of this group is responsible for amide bond formation between the C-terminal group and the fatty acid chain, yielding a macrolactam structure (Raaijmakers et al. 2010).

1.6.1.4. Fengycin family

Fengycin is a cyclic lipodecapeptide containing a β-hydroxy fatty acid with a side-chain consisting of a varying number of carbon atoms. The general chemical structure is illustrated in **Figure 1.5** (Steller & Vater, 2000).

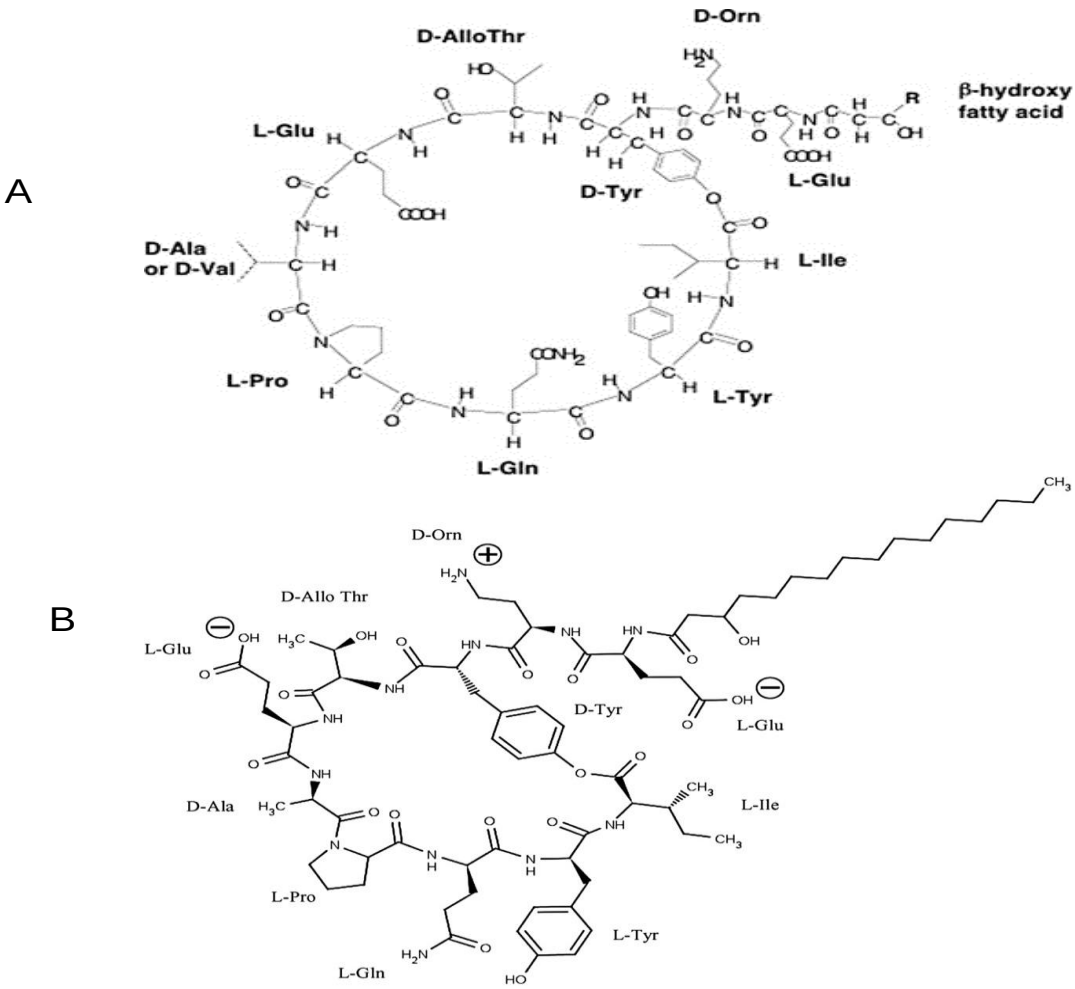

Figure 1.5 The primary structure (A) of Fengycin [adopted from Deleu et al. (2005)]. Chemical structure of fengycin A (B) with a *β*-hydroxy fatty acid chain of 16 carbon atoms. The signs "+" and "–" indicate the possible positive and negative charges, depending on the pH [adopted from Eeman et al. (2009)].

Fengycin occurs as a mixture of isoforms that vary in the branching and length of the β-hydroxy fatty acid moiety as well as the amino acid composition of the peptide ring (Loeffler et al. 1986; Raaijmakers et al. 2010). It is comprised mainly of the fengycin A and B groups. These compounds are also referred to as pliplastatins when the Tyr9 is D-configured (Raaijmakers et al. 2010). The primary structure of fengycin consists of 1 D-Ala, 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr, and 1 D-Orn. However, in fengycin B, D-Ala is replaced by D-Val (**Figure 1.5**). The β-hydroxy fatty acid moiety of both analogs is variable, as fatty acids have been identified as anteiso-pentadecanoic acid (ai-C_{15}), iso-hexadecanoic acid (i-C_{16}) and n-hexadecanoic acid (n-C_{16}). Furthermore evidence suggests that saturated and unsaturated residues of up to C_{18} occur (Loeffler et al. 1986; Steller & Vater, 2000).

1.6.1.5. Biosynthesis and gene regulation of lipopeptide compounds

The lipopeptide biosurfactants are composed of a variable hydrophilic peptide moiety linked to a hydrophobic hydroxyl fatty acid moiety. These compounds are produced commonly by *Bacillus* species (Roongsawang et al. 2010). Lipopeptides are synthesised by means of multistep processes mediated by various non-ribosomal peptide synthetase (NRPS) enzymes which catalyse the condensation and selection of amino acid residues to yield various metabolites. The synthesis of these peptides by multi-modular NRPS requires the assembly of an excess of 300 different precursors to form cyclic or linear structures. These structures consist of a mixture of non proteinogenic amino acids such as β-amino acids, L-amino acids or D-amino acids, or a combination of L- and D-amino acids and hydroxyl groups. The NRPSs are composed of colinear modular structures corresponding to the amino acid sequence within the peptide moiety, where each module is a building block for the stepwise incorporation of amino acids (Gross & Loper, 2009). The NRPS modules are subdivided into the initiation module that consists predominantly of the adenylating (A) domain required for the selection and activation of amino acids, and the thiolation (T) or peptidyl carrier protein (PCP) domain, responsible for the thioesterification of the activated amino acid. However, the initiation module also contains a condensation domain (C) that catalyses the N-acylation of the first amino acid incorporated into the lipopeptide moiety. This therefore facilitates the linking of the fatty acid moiety to the polypeptide moiety (Konz et al. 1999; Roongsawang et al. 2005). Elongation modules consist of domains A, T and C, where the C domain catalyses peptide bond formation between two adjacent amino acids. The three domains (A, T, C) of the elongation module catalyse the formation of a lipopeptide. The final stage of peptide synthesis is a termination process caused by hydrolysis and is catalysed by thioesterase. The latter enzyme at times is also responsible for the cyclisation of a mature peptide moiety (Schwarzer et al. 2001; Samel et al. 2006). A type II thioesterase repairs the NRPS systems.

The final cyclic form of the peptide molecule has been reported to be more stable and active than the corresponding intermediate linear peptide form, and is known to be necessary for the interaction with a target compound. In addition, an epimerisation (E) domain reported to occur in *Bacillus* species (Peypoux et al. 1999; Zhu & Rock, 2008) converts amino acids to D-isomers, and is associated with the modules that incorporate the D-amino acids into the peptide (Roongsawang et al. 2010). However, in an earlier study conducted by Roongsawang et al. (2003), no epimerisation domain module was reported in the NRPSs required for the biosynthesis of lipopeptides by *Pseudomonas* species. The authors postulated that external racemases (isomerase enzymes) functioned in the configuration of the D form of the amino acids which occur in lipopeptides. Metabolite profiles for the lipopeptides produced by *Bacillus* and *Pseudomonas* species have revealed that a single strain can produce representatives of various lipopeptide families, as well as different structural analogues of each lipopeptide. This was demonstrated in

previous studies, where *B. subtilis* and *P. fluorescens* produced 12 and eight analogues of surfactin and massetolide, respectively (Kowall et al. 1998; de Bruijn et al. 2008).

Gene expression for surfactin production in *Bacillus* species is reported to be cell density dependent and occurs predominantly in the exponential and stationary phases of bacterial growth. However, the biosynthesis of fengycins and iturins is primarily associated with the late stationary phase. The peptide synthetases required for the production of surfactin are encoded by four open reading frames (ORF) in the *srfA* operon, which is approximately 27 kb in length. The ORF SrfAA encodes synthetases required for the activation and addition of Glu, Leu and D-Leu; SrfAB encodes synthetases which catalyse the activation and addition of Val, Asp, and D-Leu; SrfAC encodes the synthetase which activates Leu and the thioesterase type 1 motif necessary for peptide termination (refer **Figure 1.6)**. The SrfAD ORF is located terminally and encodes for thioesterase type II required for the lactonisation process and not necessarily for the biosynthesis of surfactin. Downstream of the *SrfA* operon is the surfactin 4'-phosphopantetheinyl (*sfp*) gene encoding the phosphopantetheinyl transferase necessary for the activation of the surfactin synthetase (Sullivan, 1998; Das et al. 2008a).

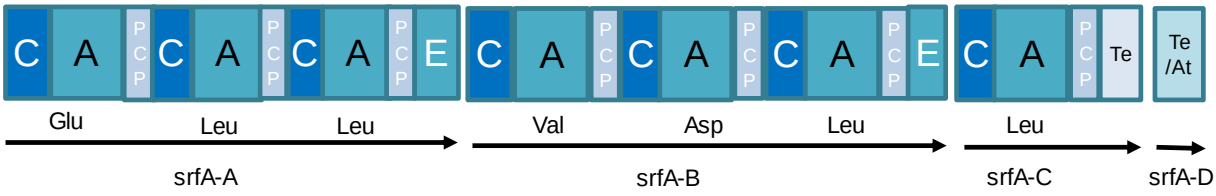

Figure 1.6 Operons of surfactin synthetases. Schematic representation of operon (ORF, domains of NRPSs and amino acid incorporated by the different modules) encoding catalytic machinery responsible for the biosynthesis of the surfactin family of lipopeptides produced by *Bacillus subtilis* [Adapted from Ongena & Jacques, (2008)].

During quorum sensing, the two component system ComA/ComP regulates the expression of *srfA* (**Figure 1.7**). When the cell-density dependent peptide pheromone ComX reaches a particular threshold concentration, membrane histidine-kinase ComP activates the ComA response regulator that binds to the promoter region of the *SrfA* operon and phosphatase RapC, thereby initiating transcription (Roggiani & Dubnau, 1993; Yakimov & Golyshin, 1997). A second pathway for regulation of the biosynthesis of surfactin (expression of *SrfA*) involves the intracellular expression of the pheromone peptides (Phr) - *B. subtilis* encodes for eight of these [PhrA, PhrC (CSF), PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK] -and 11 aspartyl-phosphate phosphatase proteins (RapA to RapK) as illustrated in **Figure 1.7** (Roongsawang et al. 2010). The Phr peptides inhibit the activity of the co-transcribed Rap proteins. The PhrC concentration is directly proportional to the activity of RapC, which is required for the dephosphorylation of ComA (Cosby et al. 1998). However a high intracellular concentration of PhrC represses the synthesis of surfactin, and PhrC production is dependent on the permease SpoOK which transports this peptide across the membrane (Sullivan, 1998). A two-way process is involved in *srfA* gene

expression, and one process is dependent on low concentrations of RapC. This enhances the availability of phosphorylated ComA, which binds to the promoter region thereby triggering transcription. Expression of the *srfA* gene is further regulated by other transcriptional regulators such as DegU or the PerR regulatory factors, as well as repressor proteins such as AbrB and GTP sensor CodY (Raaijmakers et al. 2010).

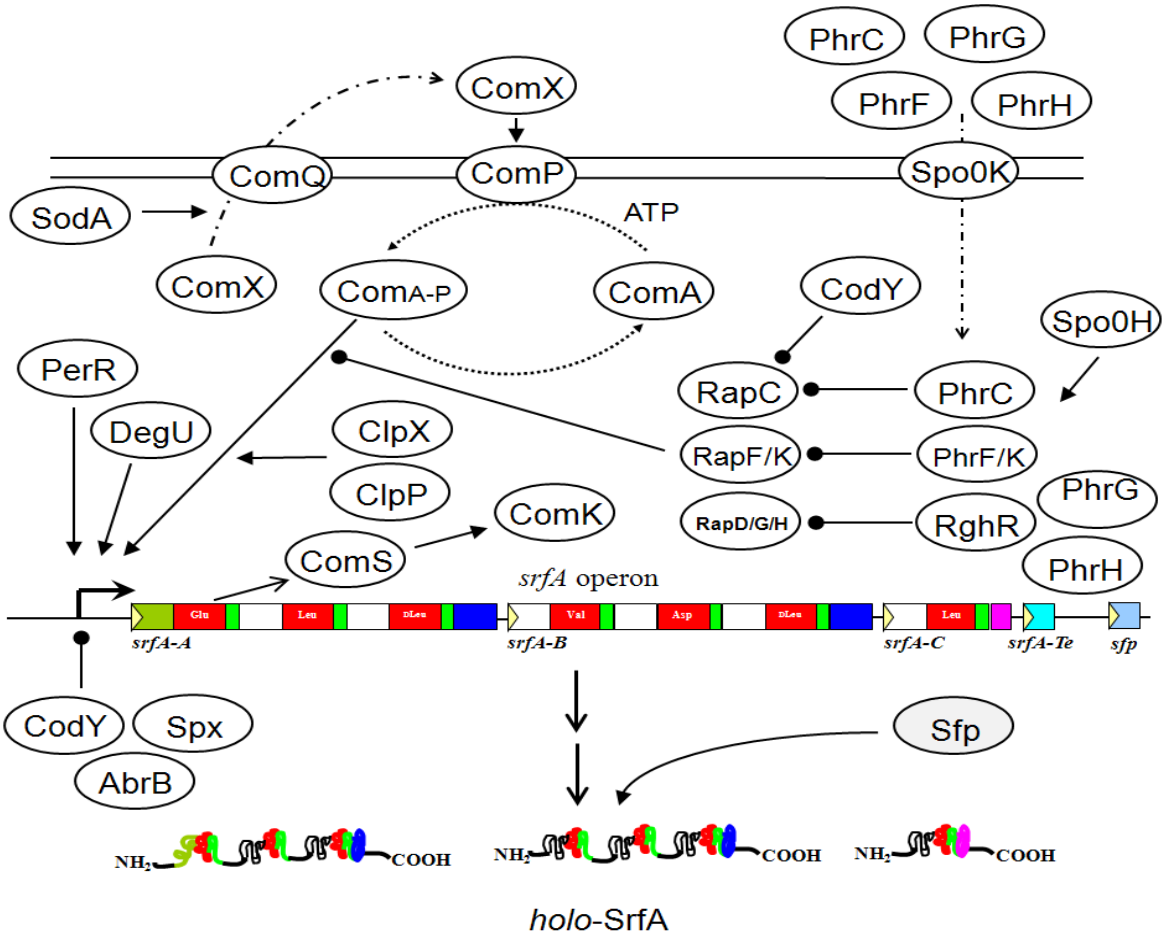

Figure 1.7 A model for gene regulation for the biosynthesis of surfactin by *Bacillus* species. The close-head arrows indicate positive regulation and closed circles indicate negative regulation [Adopted from Roongsawang et al. (2010)].

The iturin family of lipopeptide biosurfactants consists of bacillomycin, iturin and mycosubtilin, primarily synthesised non-ribosomally by *Bacillus* species. All the members of the iturin family are manufactured during the stationary phase of microbial growth (Koumoutsi et al., 2007; Singh et al. 2014). The NRPS gene cluster of this family, required for the synthesis of lipopeptides, is composed of four large ORF gene clusters [bacillomycin D (*bam/bmy*), iturin A (*itu*), mycosubtilin (*myc*)] encoding multifunctional hybrid enzymes in turn required for the synthesis of fatty acid chains, amino acid transfers (aminotransferase) and peptides (peptide synthetase) (Duitman et al. 1999; Moyne et al. 2001; Tsuge et al. 2001; Koumoutsi et al. 2004). The *bmy* and *bam* gene clusters are reported to be similar in *B. amyloliquefaciens* FZB42 and *B. subtilis* AU195 respectively. Insight into the biosynthesis of the members of iturin family is limited but a study

conducted by Koumoutsi et al. (2007) identified a few factors, in addition to those found in the surfactin operon, that are required for the production of bacillomycin D. In that study, *B. amyloliquefaciens* FZB42 was used as the test bacterium.

The synthesis of the lipopeptide bacillomycin, produced by *Bacillus* species, is controlled by the *bmy* operon and uses components of the AbrB system that are similar to those occuring in the surfactin operon. The synthesis is activated during the early stages of the stationary phase of microbial growth by interacting with the DegU factor. The DegQ regulatory protein activated by ComA is also required for the complete expression of the bacillomycin genes (Roongsawang et al. 2010).

Similarly, the production of mycosubtilin is controlled by the AbrB system, encoded by gene clusters in the *myc* operon, and is dependent on the expression of *ComA*, which is in turn regulated by quorum sensing. In addition to factors necessary for the activation of the AbrB system (**Figure 1.7)**, supplementary enzymes are active in the synthetic process and are located in the first ORF for *fenF.* These encode malonyl-CoA transacylase and the second ORF *mycA* encodes acyl-CoA ligase, acyl carrier protein (ACP) and β-ketoacyl synthetase (Hansen et al. 2007; Roongsawang et al. 2010). The sigma H factor and Spo0H also influence the expression of the *myc* operon. The expression of the *bmy* gene is dependent on the sigma A factor which is controlled by a small regulatory protein DegQ as well as ComA. The DegU binds directly to two sites located upstream of the *bmy* promoter (Das et al. 2008b; Raaijmakers et al. 2010) thereby enhancing the production of mycosubtilin.

1.6.1.6. Antimicrobial mode of action of lipopeptides

Lipopeptides are the most well characterised biosurfactant compounds and research has indicated that these compounds use different mechanisms to destroy target microorganisms (Vollenbroich et al. 1997; Makovitzki et al. 2006; Qi et al. 2010; Raaijmakers et al. 2010; Yao et al. 2012; Mandal et al. 2013). The amphipathic nature of the lipopeptide class of biosurfactants permits binding to the lipid (hydrophobic) and the phospholipid (hydrophilic) regions of the bacterial cell membrane. In addition, both the electrostatic charge of the hydrophilic moiety and the length of the lipid contribute extensively to the antimicrobial activity of the lipopeptides (Maget-Dana & Ptak, 1995). These factors facilitate the binding of the lipopeptide to negatively charged lipopolysaccharides and lipoteichoic acid of Gram-negative and Gram-positive bacteria cell boundaries, respectively (Jenssen et al. 2006). In fungi, lipopeptides bind either to the negatively charged membrane phosphatidylinositol (PI) or the polybranched (1, 3)-D-glucan in the cell wall. Lipopeptides accumulate on the surface of the microbial cells (bacteria and fungi) until a threshold concentration is reached, whereafter they permeate the membrane leading to its disintegration, induced by a detergent-like mechanism (Yao et al. 2012). This disintegration is hypothesised to

occur by the formation of pores in the cell membrane of microbial cells thus inducing an increased influx of Ca^{2+} and H^{+} into the cells (Thrane et al. 1999).

The presence of Ca^{2+} ions in environment of the target microbial cell increases the potency of surfactin by promoting the formation of surfactin-Ca^{2+} complexes. This creates surfactin dimers that enhance the synthesis of ion-conducting channels (Maget-Dana & Ptak, 1995). The surfactin-Ca^{2+} complexes are believed to slot into the phospholipid bilayer, thereby forming ion-conducting channels through which intracellular contents are discharged. A study conducted by Carrillo et al. (2003) found that surfactin introduces stress in model lipid membranes by disrupting the stability of the phospholipid bilayer. The stress was attributed to an observed increase in the surface tension of the model membrane. This increase was mediated by the lipid chain of the surfactin inserted into the phospholipid bilayer thus leading to seepage of the intracellular contents from the cell (Heerklotz & Seelig, 2007; Deleu et al. 2008).

Various surfactin groups are reported to inactivate and lyse enveloped viruses and mycoplasmas by means of a physico-chemical interaction of the membrane-active surfactant with the lipid envelope of the virus/mycoplasmas. This causes a loss of proteins from the targeted microorganism. As these proteins are required for adsorption to and/or penetration into the host cell, loss of cellular or viral activity is inevitable (Vollenbroich et al. 1997; Huang et al. 2006). Tendulkar et al. (2007) demonstrated further antifungal properties of surfactin where the compound caused hyphal swelling and subsequent inactivation of the rice pathogen *Magnaporthe grisea*. However, surfactin does not only target the membrane. For example, in a study conducted by Qi et al. (2010), an antifungal lipopeptide produced by *B. amyloliquefaciens* induced apoptosis when it bound to ATPases on the mitochondrial membrane within a cell.

Surfactin is reported to mediate a change in the morphology of a tumour cell. This change induces fragmentation of DNA and increases the loss of polarity in the plasma membrane. Ultimately this causes apoptosis of the cancerous cell (Kim et al. 2007). In a previous study conducted by Kameda et al. (1974), a surfactin compound obtained from *Bacillus natto* KMD 2311 isolated from straw samples in Japan, exhibited cytolytic activity against Ehrlich's ascites carcinoma and human colon cancer cells. Another surfactin, WH1fungin, produced by *B. amyloliquefaciens*, was reported to induce apoptosis at low concentrations (6.25 µg/ml) and at high concentrations (25-50 µg/ml) caused pore formation in fungal cell walls (Qi et al. 2010). WH1fungin inhibits the activity of glucan synthase, causing a decrease in the levels of callose (a component of fungal cell wall) production. It is also suggested that WH1fungin could bind to the mitochondrial membrane ATPase thereby reducing metabolic activities within a fungal cell.

Fengycin exhibits a negative effect on intact model biomembranes when the lipopeptide, at low concentrations (~10 µM) is inserted into the membranes. At higher concentrations (~133 µM) fengycin facilitates agglomeration of the cell membrane leading to seepage of the intracellular

contents (Deleu et al. 2008). At these concentrations fengycin lipopeptides formed micelles in the model membranes. This indicates a solubilisation of the phospholipid bilayer into the extracellular medium and the target cell is destroyed (Heerklotz & Seelig, 2001; Deleu et al. 2008).

The lipopeptide iturin is reported to exhibit extensive antifungal properties, due to a marked interaction with the phospholipid bilayer, ultimately lysing the cell (Vater et al. 2002; Romero et al. 2007; Lichtenberg et al. 2013). Iturin A is an antifungal compound that disrupts the plasma membrane of yeast cells by forming small vesicles and causing aggregation of intramembranous particles. Its mode of action depends on the release of electrolytes and high molecular mass products which eventually cause degradation of the plasma membrane (Thimon et al. 1995). In addition, iturin A is reported to traverse the cell wall and disrupt the cell membrane by the formation of small vesicles which aggregate into small intramembranous molecules. It is also possible that iturin A may enter the cell and interfere with the nuclear membrane (Thimon et al. 1995). Iturin displays an affinity for Na^+, K^+ and rubidium (Rb^+) and for this reason the biosurfactant has potential for the bioremediation of environments contaminated by heavy metals (Mulligan et al. 2001; Rautenbach et al. 2001).

Lipopeptides provide unique mechanisms for the destruction of microbes, generally by causing damage to the cell membrane. This damage is lethal, complex, extensive and difficult to repair. This contrasts markedly with the action of conventional antibiotic compounds that target specific microbial biochemical processes such as DNA replication or the functionality of enzymes, that can eventually be circumvented by microbes through the development of resistance (Raaijmakers et al. 2010). Furthermore, the alternation of L/D isoforms of amino acids in the peptide moiety which provides stability to the compounds, also enhances resistance of lipopeptides to degradation by proteolytic enzymes secreted by target microorganisms (Raaijmakers et al. 2010; Mandal et al. 2013). Various lipopeptides have subsequently been applied to counter the effects of microbial pathogens in the medical and agricultural fields.

1.6.2. Glycolipids

Glycolipids are a class of biosurfactants composed of a hydrophilic moiety made up of mono-, di-, tri- or tetra-saccharide carbohydrates, particularly galactose or glucose. These are attached to different (chain length) hydrophobic moieties which form a lipid backbone. Similar compounds are also found in the form of diacylglycerol glycosides, glucosylceramides and sterylglycosides attached to various phospholipid bilayer backbones of molecules which occur in animals, bacteria, fungi and plants (de Jesus Cortes-Sanchez et al. 2013).

Understanding the structure and function of glycolipids in microbial cells has made large-scale biosynthesis of these compounds possible, thus permitting exploitation of their antimicrobial properties. Biosynthesis of glycolipid biosurfactants is dependent on the polarity of the carbon

source, as this affects the mechanism used to produce the biosurfactant. The primary routes employed to produce this biosurfactant by microorganisms are; (1) both fractions of the compound are synthesised independently from the substrate, (2) the hydrophobic moiety is synthesised directly from the hydrophobic carbon source, but synthesis of the sugar molecule is *de novo*, or (3) some of the glycolipid is directly derived from the carbon source but the lipid is synthesised *de novo* (Soberón-Chávez & Maier, 2011; de Jesus Cortes-Sanchez et al. 2013).

The major fraction of the glycolipid compound is comprised of a hydrophobic moiety and is the product of the β-oxidation of a hydrophobic carbon source, which in turn determines the polarity of a glycolipid compound (de Jesus Cortes-Sanchez et al. 2013). The most common glycolipid-based biosurfactants include mannosylerythritol lipids, sophorolipids and trehalolipids. The dominant group, rhamnolipids, is primarily produced by *Pseudomonas* species, particularly *P. aeruginosa*. The current study focuses on rhamnolipids because of their extensive surface, emulsification and antimicrobial properties. Together, these render this biosurfactant as most promising for use in the bioremediation of various contaminants (metals and microorganisms) and for various applications in the food, agricultural and medical industries.

1.6.2.1. Rhamnolipids

Rhamnolipids are well-known glycolipid biosurfactants, which are reported to be primarily produced by *P. aeruginosa* as secondary metabolites (Bodour et al. 2003; Hsieh et al. 2004; Sen, 2010). First discovered in 1946, a compound produced by *Pseudomonas pyocyanea* (now known as *P. aeruginosa*) was described as an oily glycolipid. This oily compound was then called pyolipic acid because of its chemical composition of L-rhamnose and β-hydroxydecanoic acid (Bergstrom et al. 1947; Jarvis & Johnson, 1949; Hauser & Karnovsky, 1954). The chemical structure was further elucidated by Edwards and Hayashi (1965) who described the rhamnolipids as glycosides with a simple chemical structure consisting of one (monorhamnolipids) or two (dirhamnolipids) rhamnose sugars linked to lipid moieties by an O-glycosidic linkage. The hydrophilic moiety of the rhamnolipid is thus composed of single or double L-rhamnose sugars linked to one another by an α-1,2-glycosidic bond. The hydrophobic moiety primarily consists of one or two, but in rare cases three (Andrä et al. 2006) β-hydroxy fatty acid chains that may be saturated or unsaturated (mono to polyunsaturated) and have varying lengths of C_8 to C_{16}. These groups are linked to one another by an ester bond between the β-hydroxyl group of the distal (relative to the glycosidic bond) chain and the carboxyl group of the proximal chain (**Figure 1.8**). In most cases, the carboxyl group of the distal hydroxyl fatty acid chain remains free, with the exception of a few that tend to be esterified with short alkyl groups such as methyl groups (Abdel-Mawgoud et al. 2010). The aforementioned structural properties have created a large pool of rhamnolipid compounds produced by various microorganisms in diverse environments. The molecular weights of these compounds range from approximately 302 to 989 Da. Abdel-Mawgoud

et al. (2010) collated information on rhamnolipid chemical structures and showed that more than 60 rhamnolipid congeners and homologues produced by various microbial species (strains of *P. aeruginosa*, *Pseudomonas chlororaphis* and *Burkholderia*) have been described (Abalos et al. 2001; Benincasa et al. 2004; Gunther et al. 2005; 2006; Abdel-Mawgoud et al. 2009; Dubeau et al. 2009).

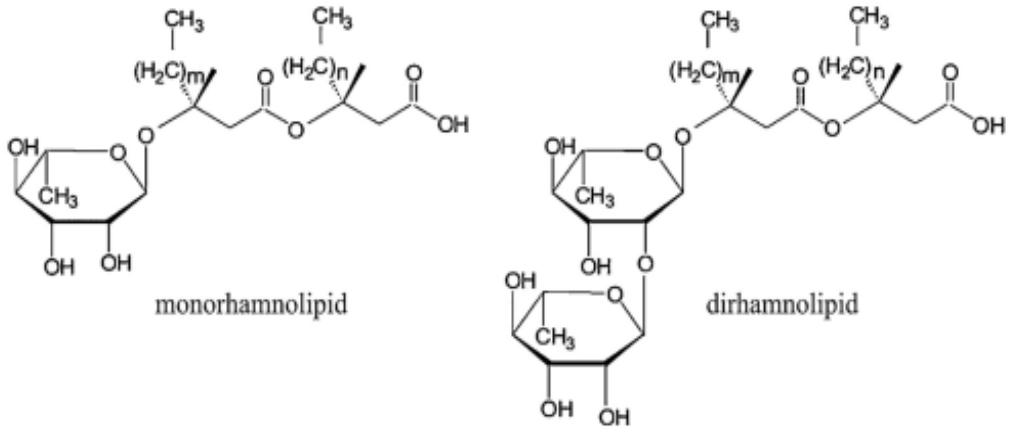

Figure 1.8 General basic structures of typical rhamnolipids [adopted from Dobler et al. (2016)]

The most abundant rhamnolipid compounds described are the a-L-rhamnopyranosyl-a-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}) (**Figure 1.8**), a-L-rhamnopyranosyl-a-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C_{10}) and the monorhamnolipid homologues Rha- C_{10}-C_{10} **(Figure 1.8**) and Rha-C_{10} (Abdel-Mawgoud et al. 2010; Dobler et al. 2016). Arino et al. (1996) described the rhamnolipid composition present in a *P. aeruginosa* batch culture to be 67% di-rhamno-di-lipid, 9% di-rhamno-monolipid, 22% mono-rhamno-di-lipid and less than 3% mono-rhamno-mono-lipid, which influenced the overall physico-chemical properties of the rhamnolipid mixture (Thaniyavarn et al. 2006).

1.6.2.2. Biosynthesis and regulation for rhamnolipid production

Rhamnose (L-Rha) is a major component of the lipopolysaccharide (LPS) core of the outer membrane of various strains of *Pseudomonas* species, as well as the biosurfactant rhamnolipid primarily produced by *P. aeruginosa* (Rahim et al. 2000). The carbon used for the synthesis of rhamnose is derived from sources such as glycerol, mannitol, vegetable oils and ethanol, among others (Chen et al. 2007). Studies have indicated that glycolipids produced from carbohydrates (as the main carbon source) are simpler to separate and purify when compared with the production of glycolipids originating from oil based compounds (Dubey et al. 2005; Banat et al. 2014). Commercial large-scale production of biosurfactant rhamnolipids is carried out at Jeneil Biotech INC in USA (Geys et al. 2014).

Rhamnolipid biosynthesis by *P. aeruginosa* occurs in consecutive steps of glycosyl transfer reactions catalysed by different rhamnosyl-transferases, yielding separate activated precursor

hydrophilic and hydrophobic moieties. These are then dimerised by the rhamnosyl-transferases and other enzymes (Soberón-Chávez et al. 2005). In a liquid medium containing hydrocarbons or carbohydrates as the main carbon source, certain *P. aeruginosa* strains produce two main compounds, namely a monorhamnolipid (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and a dirhamnolipid (rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate). The production of rhamnolipids by *P. aeruginosa* is tightly regulated by a quorum sensing mechanism, in response to both environmental stress and nutritional factors (Déziel et al. 2003; Reis et al. 2011; Geys et al. 2014).

The quorum sensing system of *P. aeruginosa* consists of the *rhlI* and *rhlR* genes encoding the N-butyrylhomoserine lactone autoinducer synthase and transcriptional activator protein RhlR respectively, which regulate the synthetic *rhlA, rhlB* and *rhlC* genes used for the biosynthesis of monorhamnolipids and dirhamnolipids (Ochsner et al. 1994; Soberón-Chávez, 2004). The *rhlA* and *rhlB* genes are located in a single operon and *rhlC* is located on the chromosome of *P. aeruginosa*. The activated rhamnose moiety utilised as a subtrate for both mono- and dirhamnolipids is dependent on the RmlBCAD pathway encoded by the *rmlBCAD* operon, and the catalytic activity of the enzyme algC. To synthesise the precursor rhamnose sugar (**Figure 1.9**), the algC catalyses the synthesis of D-glucose-1-phosphate from a normal D-glucose molecule, which is then converted to dTDP-D-glucose by RmlA. The RmlB further converts the dTDP-D-glucose to dTDP-4-oxo-6-deoxyl-D-glucose, which in turn is converted to dTDP-6-deoxyl-L-deoxyl-4-rhamnose by RmlC. The RmlD finally converts the dTDP-6-deoxy-L-deoxyl-4-rhamnose to dTDP-L-rhamnose. The latter is a substrate for the rhamnosyl-transferases RhlB and RhlC that synthesise mono- and dirhamnolipids compounds, respectively. It has been hypothesised that the RhlG enzyme is responsible for redirecting fatty acid synthesis intermediates into the rhamnolipid pathway as it exhibits similar characteristics to the FabG enzyme (Miller et al. 2006) and nicotinamide adenine dinucleotide phosphate (NADP) dependent ketoacyl reductases.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent ketoacyl reductase RhlG enzyme catalyses the synthesis of 3-hydroxyacyl-ACP from 3-ketoacyl-ACP. The RhlA enzyme, which is loosely bound to the inner membrane of the cell (Rahim et al. 2001) then catalyses the synthesis of 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs). It remains unclear as to whether RhlA transports the rhamnosyl-transferase precursor substrates or is involved in the stabilisation of the RhlB enzyme (Soberón-Chávez et al. 2005; Leitermann et al. 2010). The 3-(3-hydroxyalkanoyloxy) alkanoates (HAA) and dTDP-L-rhamnose compounds are used as subtrates for the production of monorhamnolipid, catalysed by RhlB. The synthesised monorhamnolipids together with dTDP-L-rhamnose, are precursor compounds for the production of dirhamnolipids, a reaction catalysed by the RhlC enzyme that is loosely bound to the cell membrane (Rahim et al. 2001).

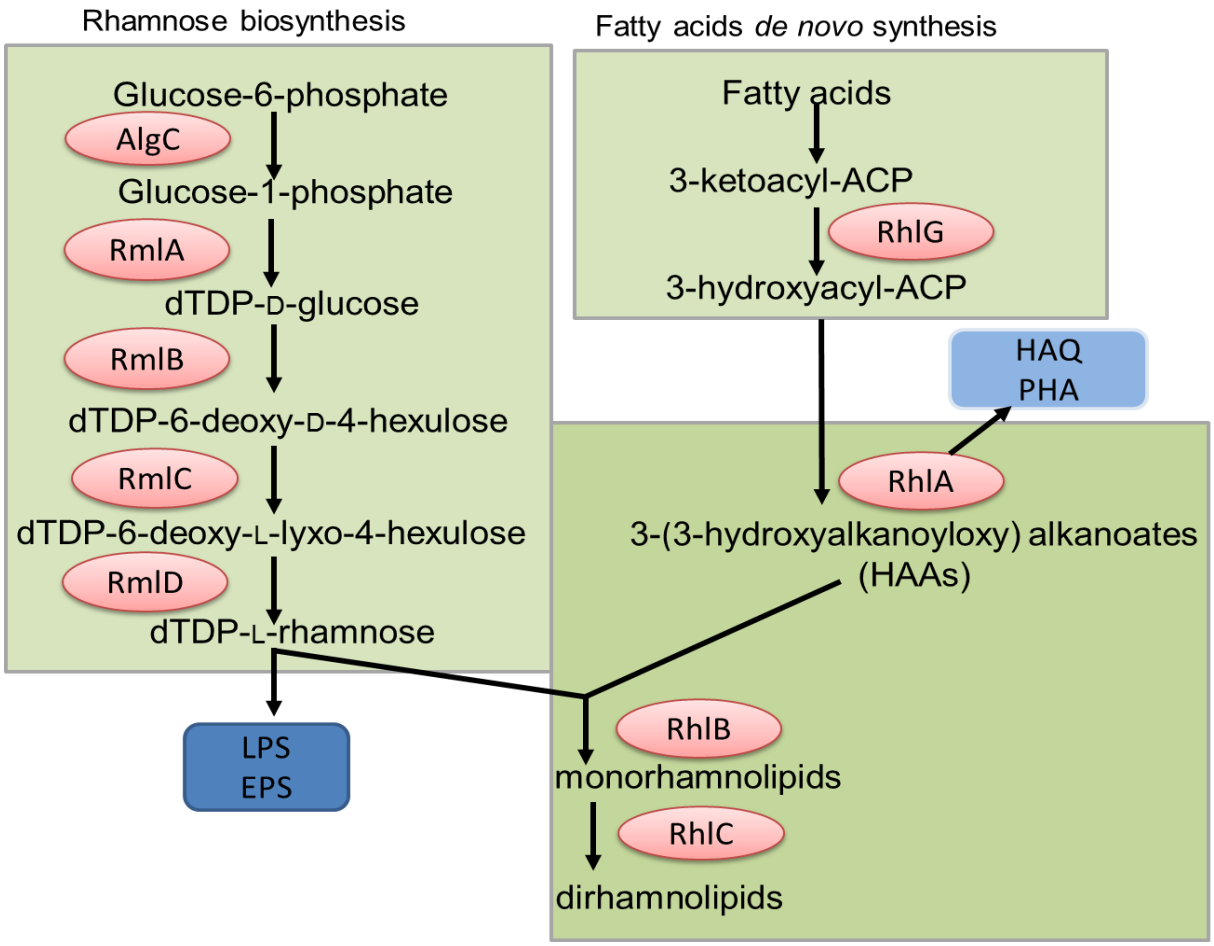

Figure 1.9 Biosynthesis pathway of monorhamnolipid and dirhamnolipid in *Pseudomonas aeruginosa* [adapted from Dobler et al. (2016)].

The *rhlR* and *rhlI* genes that encode for transcriptional regulatory proteins (associated with the quorum sensing system) are clustered together with the *rhlA* and *rhlB* genes in the same operon (*rhlAB* operon). These code for the first rhamnosyl-transferase involved in the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) in order to form monorhamnolipids (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) (Soberón-Chávez et al. 2005). The *rhlC* gene coding for the RhlC rhamnosyltransferase is located on another operon within the genome and it catalyses the synthesis of dirhamnolipids (rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) (Ochsner et al. 1994; Abdel-Mawgoud et al. 2010). The RhlR, coupled with the butanoyl-homo-serine lactone (C4-HSL) inducer molecule, initiates the transcription of *rhlAB*, but supresses transcription when not attached to its autoinducer (Soberón-Chávez et al. 2005) in minimal medium. In addition, not only is the C4-HSL autoinducer molecule bound to the RhlR involved in the activation of *rhlAB* expression; it also requires expression of the RpoS sigma factor (δS) which occurs when grown in minimal medium (Medina et al. 2003). The different locations of the *RhlA, RhlB* and *RhlC* genes within the genome are postulated to create different concentrations and delayed expression of the rhamnosyltranferase enzymes synthesis. This results in variable stochiometric ratios of the mono- and dirhamnolipids obtained

(Abdel-Mawgoud et al. 2010). In addition, the concurrent production of HAAs and rhamnolipids also leads to these compounds being co-extracted (Lépine et al. 2002).

1.6.2.3. Antimicrobial mode of action of rhamnolipids

Rhamnolipids have structures and properties similar to those of detergents and are reported to intercalate in the membrane phospholipid bilayer thereby facilitating the permeability of the membrane and flow of metabolites (Sotirova et al. 2008). The intercalation alters the structure and function of the phospholipid bilayer through the interruption of the protein conformation. Thus transport and energy generation are disrupted and the process is lethal to various Gram-positive bacteria (Banat et al. 2010). In addition, rhamnolipids display algicidal, anti-amoebal, fungicidal, antiviral and zoosporicidal properties (Soberón-Chávez et al. 2005; Banat et al. 2010). Currently, there are no studies that clarify the mechanisms for anti-zoospore activity (inhibition of the spore formation by various fungal, yeast or protozoan organisms) of the rhamnolipids. However research conducted by Miao et al. (2015) and Soltani Dashtbozorg et al. (2016) reported on the anti-zoospore properties of certain rhamnolipids against *Phytophthora sojae* spores. Abalos et al. (2001) showed that a rhamnolipid mixture consisting of up to seven homologues obtained from *P. aeruginosa* AT10 exhibited excellent antifungal activity against *Aspergillus niger*, *Gliocadium virens, Chaetomium globosum, Penicillium crysogeum* and *Rhizoctonia solani.* Antibacterial activity of the rhamnolipid mixtures was also observed against the *Esherichia coli* and *S. marcescens* strains screened.

Purified rhamnolipids produced by *P. aeruginosa* have also been shown to chelate and form stable complexes with numerous metal ions [aluminium (Al^{3+}), copper (Cu^{2+}), lead (Pb^{2+}), cadmium (Cd^{2+}), zinc (Zn^{2+}), iron (Fe^{3+}), mercury (Hg^{2+}), calcium (Ca^{2+}), cobalt (Co^{2+}), nickel (Ni^{2+}), manganese (Mn^{2+}), magnesium ($Mg^{2+)}$, and potassium (K^{+})] (Ochoa-Loza et al. 2001). A study conducted by Ochoa-Loza et al. (2001) demonstrated the possible application of rhamnolipid biosurfactants for the bioremediation of various metals present in surface water and wastewater. In a study conducted by Sandrin et al. (2000), an exogenous rhamnolipid was found to reduce the Cd^{2+} toxicity towards an actively growing *Burkholderia* sp. by sequestration of the rhamnolipid with cadmium. It was also observed that the lipolysaccharide (LPS) layer of *Burkholderia* was removed from the cell surface. After this removal, the uptake of Cd^{2+} decreased as there was an overall lowering of the negative charge of the membrane. It was subsequently suggested that certain bacterial species in metal contaminated sites could produce biosurfactants that could intercalate with metal ions to counter their toxicity. Therefore these biosurfactants have potential as bioremediants.

A study conducted by Stipcevic et al. (2006) investigated the effect of dirhamnolipids on the healing process of a cutaneous wound in Sprague-Dawley rats. The eucerin ointment was mixed

with dirhamnolipid and applied topically over 5% of the body of the mice. It was found that 0.1% of the dirhamnolipid ointment accelerated wound closure which occurred by day 21 of treatment, when compared with a control group treated with Dulbecco's sodium phosphate buffered saline (termed the vehicle-treated group). After failure of standard wound therapy treatment, a 0.1% dirhamnolipid ointment was used on a human patient with a decubitus ulcer (Piljac et al. 2008). The ointment was administered directly to the wound area and the wound healed completely by day 48 of treatment. These two cases demonstrate the possible application of dirhamnolipid for the successful treatment of wounds and decubitus ulcers. Tatjana and Goran (2007) also demonstrated wound healing abilities of rhamnolipids after an organ transplant. In addition, a study performed by Thanomsub et al. (2007) showed that two rhamnolipid compounds [L-rhamnosyl-L-rhamnosyl-β-hydroxyldecanoyl-β-hydroxydecanoate (Rha–Rha-C_{10}-C_{10}) and L-rhamnopyranosyl-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydodecanoate (Rha-Rha-C_{10}-C_{12})] at a concentration of 0.78–50 µg/mL, produced by *P. aeruginosa* B189, displayed no cytotoxic activity against a vero cell line. In addition, no inhibitory activity against human oral epidermoid carcinoma and lung cancer cell lines was observed. Furthermore the Rha-Rha-C_{10}-C_{10} and Rha-Rha-C_{10}–C_{12} compounds were found to inhibit human breast cancer and insect cell lines at concentrations of 6.25 µg/mL and 50 µg/mL, respectively (Thanomsub et al. 2007).

1.7. Possible antimicrobial resistance mechanisms displayed against biosurfactants

Glycolipids and lipopeptides exhibit diverse characteristics and are currently applied in several industries (cosmetic, food and pharmaceutical) as antimicrobial, emulsifying and surfactant agents (Mandal et al. 2013). Due to an alarming increase in the proliferation of multi-drug resistant microorganisms, future large-scale production and application of these molecules as alternative antimicrobials, particularly by the pharmaceutical industry, is crucial. Surfactin in particular has been reported to have antibiotic, anticlotting and antiviral activities (van Hamme et al. 2006). Since antimicrobial peptides form part of the human's natural antimicrobial defence system (first line of defense mechanism against pathogens), the probability that these compounds will cause undesirable side effects is low. This contrasts markedly with the many undesirable side-effects of conventional antibiotics. Furthermore, as referred to in the foregoing, the development of microbial resistance by susceptible microbes to the action of biosurfactants is unlikely. Currently there are very few studies reporting on the development of such microbial resistance (Martin et al. 1995; Nybroe & Sørensen, 2004; Jenssen et al. 2006; Gruenheid & Le Moual, 2012; Rautenbach et al. 2012; Sumi et al. 2014).

However, while certain classes of biosurfactant compounds such as the lipopeptide, glycolipids and sophorolipids have been reported to display antimicrobial properties, not all of these compounds have a broad spectrum of antimicrobial activity. There are bacteria which display an inherent (as opposed to acquired) resistance to biosurfactants. For example, the apparent lack of

activity against Gram-negative bacteria has been ascribed to the presence of the outer membrane that prevents the interaction of the biosurfactant with the phospholipid membrane bi-layer (Nybroe & Sørensen, 2004). In addition, Gram-negative bacteria tend to be resistant to antimicrobial lipopeptide biosurfactants due to the secretion or membrane-localisation of proteolytic enzymes that could convert active lipopeptides into inactive forms (Gruenheid & Le Moual, 2012). Moreover, different types of lipopolysaccharides (capsule polysaccharides, biofilm-forming exopolysaccharides and O-polysaccharide) in the bacterial cell envelope bind to antimicrobial lipopeptides and in so doing prevent the lipopeptides from reaching the cell membrane. Lipopolysaccharides also modify the outer membrane of Gram-negative bacterial cells, and antimicrobial lipopeptides are possibly pumped into or out of the cell by means of ATP-binding cassette (ABC) transporters and resistance modulation-division efflux pump families (Sumi et al. 2014).

For the low G+C content Gram-positive bacteria, antimicrobial resistance to peptides is often facilitated by the resistance modules, which consists of an ABC transporter that acts as a sensor and detoxification system to confer resistance. For example, the BceRS-BceAB module is associated with the resistance of *B. subtilis* to the biosurfactants bacitracin and mersacidin (Kallenberg et al. 2013). Rautenbach et al. (2012) indicated that the antimicrobial lipopeptide surfactin produced by *B. subtilis,* acted as a detoxifying agent to protect the producer organism from the lytic activity of a gramicidin S, a linear peptide produced by *Aneurinibacillus migulanus.* After combining the anionic surfactin and the cationic gramicidin S, an inactive complex between the two compounds was noted. This complex supported resistance to gramicidin S and was observed after analysis by means of circular dichroism and electrospray mass spectrometry.

1.8. Production and applications of glycolipids and lipopeptides

Biosurfactants are versatile compounds that can be used for diverse applications, including bioremediation, as antimicrobials in the medical field, for enhancement of mineral processing, to increase the recovery of oils in the petrochemical industries and for various purposes in the food industry (Fakruddin, 2012). These biocompounds have many advantages over their chemical synthetic counterparts. However there remain numerous hurdles to overcome in order to harness the activity of microorganisms for cost-effective, large-scale production of biosurfactants. The selection of the microbial strains, the type of substrates used, and fermentation technology all play a crucial part in the production of biosurfactants (Marchant et al. 2014).

1.8.1. Nutrient sources utilised for biosurfactant production

The biosynthesis of biosurfactant compounds (glycolipids and lipopeptides) occurs on water insoluble substrates by *de novo* pathways, which vary in different microorganisms. Many bacterial strains produce a mixture of biosurfactant homologues and isoforms, which are influenced by the

type of carbon source present in the growth media (Sen, 1997). The selection of a cost-effective substrate to produce biosurfactants is particularly crucial for large-scale production. A review by Banat et al. (2014) indicated numerous bacterial species have been used to produce a variety of biosurfactants. Cost-effective carbon (water insoluble and soluble) substrates such as blended gasoline, ethanol, wheat bran, palm oil, hydrocarbons such as heptadecane and hexadecane, have been used for the process. Arima et al. (1968) showed that complex growth media, including Luria Bertani and Nutrient broths yielded approximately 100 mg/L of surfactin (regarded as a low yield). Yeh et al (2005) showed that a *B. subtilis* strain produced up to 3 300 mg/L of surfactin when cultured on a mineral salt medium. Wei et al (2004) proved that a defined medium was more effective for the production of surfactin by *Bacillus* species than were complex media. The defined media (mineral salt medium supplemented with a carbon source) is composed of various nitrogen sources including organic ammonium oxalate, urea, yeast extract, peptone, tryptone and corn steep liquor. Inorganic sources included are sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium bromide, ammonium carbonate, and ammonium sulphate (Abdel-Mawgoud et al. 2010). Trace elements commonly used in defined minimal media used for biosurfactant production include Mg^{2+}, K^{+}, Mn^{2+} and Fe^{2+}. These act as major cofactors for the multi-enzyme systems associated with biosynthetic pathways of biosurfactant production (Sen & Swaminathan, 2004; Wei et al. 2007; Chen et al. 2015).

Different types of carbon sources are reported to markedly influence the concentration of biosurfactant compounds produced. In addition, relevant published research has emphasised the effect the carbon source has on the type of biosurfactant compounds synthesised by a specific microbial strain (Bonmatin et al. 2003; Das et al. 2009; Singh et al. 2014). The defined media are thus supplemented with various carbon sources such as glucose, mineral oil, and sucrose among others. A study conducted by Kim et al. (1997) assessed the use of emulsified n-hexadecane, soybean oil and glucose to produce a lipopeptide biosurfactant using *B. subtilis* C9. Results indicated that the lipopeptide biosurfactant was produced only when glucose was used as a carbon source. In addition, a study conducted by Yeh et al. (2005) highlighted the importance of the concentration of the carbon source on the production of biosurfactant compounds. These authors showed that the use of high concentrations (50-60 g/L) of glucose as the principal carbon source for the production of surfactin by a *B. subtilis* strain reduced the pH of the growth medium. This negatively affected surfactin production; optimum production was obtained at concentrations of 20-30 g/L glucose. Sim et al. (1997) investigated the effect of vegetable oils (canola and soybean oils) and glucose for rhamnolipid biosurfactant production by *P. aeruginosa* UW-1. Results obtained showed that there was a 10-12 fold increase in the quantity of rhamnolipid produced when a vegetable oil rather than glucose was used as the primary carbon source. Thaniyavarn et al. (2006) investigated the production of biosurfactants using *P. aeruginosa* A41 obtained from seawater. The microbe was cultured either in a vegetable oil (olive, palm and

coconut oils) or a fatty acid (lauric, myristic, palmitic, stearic, oleic or linoleic acids) as the main carbon source. Different rhamnolipid concentrations of 2.91, 2.93 and 6.58 g/L were obtained with the palm, coconut and olive oils, respectively. In the case of the fatty acid substrates, the rhamnolipid concentration ranged from 0.26 g/L (palmitic acid) to 4.99 g/L (linoleic acid). However, the rhamnolipid obtained when *P. aeruginosa* UW-1 was cultured in in fatty acids had shorter chain lengths and caused a high oil displacement activity when compared with yields obtained when vegetable oil was used (Thaniyavarn et al. 2006). The authors concluded that cost-effective production of industrial volumes of rhamnolipid was likely when using *P. aeruginosa* UW-1 isolates cultured using palm oil as the carbon source.

1.8.2. Cost-effective extraction and purification methods

The use of cost-effective substrates is encouraged for the production of specific biosurfactants on an industrial scale. However, the strategies for extraction and purification of biosurfactant compounds account for most of the production costs and these vary according to the microbial growth production process and the physico-chemical properties of the biosurfactant(s) produced (Shaligram & Singhal, 2010). In addition, when different production processes are used, there is always a risk of contamination from unwanted fermentation by-products and methods used should effectively recover only the biosurfactant compounds. Conventional methods employed for the extraction of biosurfactants are dependent on the ionic charge (chromatography based methods), solubility properties (whether water and/or organic solvents are used) and lastly, the location (cell bound, extracellular or intracellular) of the synthesised biosurfactant compounds with respect to the producing organism (Satpute et al. 2010).

Biosurfactant Extraction: Acid precipitation is a cost-effective and simple method to recover extracellular biosurfactants compounds such as lipopeptides, glycolipids, sophorolipids and others (Satpute et al. 2010). Surfactin produced by *Bacillus* species is purified from the cell-free supernatant, which is obtained by centrifugation of the culture medium to remove bacterial cells and other large contaminants. This latter step is possible as surfactin biosurfactants are extracellular metabolites. Hydrochloric acid is then added to decrease the pH of the cell-free supernatant to approximately 2, which is ideal for the protonation of biosurfactant compounds. This renders the biosurfactant compounds insoluble in water and they precipitate (Mukherjee et al. 2006). Thereafter, the mixture is again centrifuged and the resultant pellet is dried under vacuum and further extracted using various solvents (methanol, acetonitrile, chloroform, ethyl acetate, etc.) (Haba et al. 2000; Thaniyavarn et al. 2003; Nitschke & Pastore, 2006; Smyth et al. 2010).

Biosurfactant Purification: The recovery of biosurfactants from aqueous media has been performed using liquid membrane (pertraction) processes (Dimitrov et al. 2008). Surfactin biosurfactants were successfully extracted from slightly acidic media (pH 5.65–6.05) by batch

pertraction in a rotating disc contactor and by using n-heptane as the liquid membrane. The acidity of the solution affected the process efficiency. After four hours of pertraction, recoveries of approximately 83% and 97% surfactin were obtained at pH values of 6.05 and 5.65 respectively. The effect of pH was also observed on surfactin extraction from aqueous media by the use of non-polar solvents such as n-heptane and n-octane (Dimitrov et al. 2008). It was suggested that the high extraction yields obtained from both acidic and basic broth culture media, when compared with the reduced yields obtained with neutral culture media, could be attributed to the different conformations of the surfactin secreted into the media. In neutral medium, the surfactin exhibited extensive hydrophobicity thought to be caused by the formation of β-sheet micelles thus causing low extraction yields. In constrast, when secreted into acidic or basic media, the surfactin conformation was that of α-helices. The α-helix conformation of the surfactin biosurfactants was thought to expose more of the compound surfaces to organic solvents, thus causing the extraction of higher concentrations. It is apparent that to prevent loss of biosurfactant compounds during downstream recovery processing, sensitive methods must be used to ensure maximum recovery of the compounds of interest.

It should also be noted that most methods used to date for downstream processes are very costly and for success, require highly skilled personnel. For example chromatographic methods that are commonly used in research laboratories for the purification and analysis of biosurfactant compounds (Satpute et al. 2010) are expensive and require highly trained personnel. This presents a challenge for upscaling production, as the downstream processes may incur unacceptable costs. To reduce costs, it would thus be advantageous to use bacterial strains that produce a single congener of a biosurfactant as this would simplify purification of the biosurfactant from the culture media.

1.9. Applications of biosurfactant compounds

The application of biologically active biosurfactant compounds in industries has advantages over chemically synthesised surfactants, as the former can be produced from renewable feed stocks, are less toxic and exhibit a higher efficiency at low concentrations (Desai & Banat, 1997). In addition, biosurfactants have enhanced foaming capacity and function optimally in a variety of environments (Desai & Banat, 1997; Mulligan, 2005). A few of the currently used applications as well as possible future applications of selected biosurfactant compounds in various fields will be briefly reviewed.

Cosmetics: Lipopeptide-based surfactants have moisturising and anti-wrinkle properties. They also display low cytotoxic effects in human cells. For these reasons, they have been used as additives in dermatological products (Mandal et al. 2013). The low critical micelle concentration of surfactin in particular makes it a suitable compound for dermatological applications. It is also reported to be less cytotoxic to mammalian cells and is thus proven safe for application on human

skin. Several derivatives of surfactin are thus used as additives in selected dermatological and cleansing cosmetic products (Kanlayavattanakul & Lourith, 2010). A few USA-based cosmetic companies have also developed lipopeptide-formulated dermatological products that enhance collagen and elastin production, which are reputed to prevent ageing and maintain the healthy physiology of skin cells (Mandal et al. 2013).

Food Industry: There is a developing awareness among consumers against the use of artificial chemicals as additives or preservatives in food products. The unique properties of naturally produced biosurfactant compounds have thus led to their widespread application as food emulsifiers or demulsifiers, antimicrobials and antiadhesive agents, solubilisers, wetting and foaming agents (Banat et al. 2000; Singh & Cameotra, 2004; Nitschke & Costa, 2007). In dairy food products such as ice creams and soft cheeses, the addition of emulsifiers improves the creaminess and texture, particularly in low fat products (Kachholz & Schlingman, 1987; Rosenberg & Ron, 1999). Lipopeptides are used as emulsifiers of raw materials. Surfactin is also used to stabilise raw materials and to maintain food texture and volume. A study conducted by Shepherd et al. (1995) then reported on the successful use of an extracellular biosurfactant compound produced by *Candida utilis* in salad dressing formulations. Food manufactures use low pH preservatives to suppress food spoilage. Ring structured lipopeptides could thus be applied as alternative preservatives as they display notable antimicrobial properties and are not susceptible to proteases. The chemical ring peptide structures of the iturin, fengycin and surfactin families in particular contribute markedly to resistance to proteases (Mandal et al. 2013). Moreover, Gandhi and Skebba (2011) demonstrated that the addition of 0.1% of pure rhamnolipid in formulations for croissants and muffins improved stability of moisture content and texture. As a result, the shelf life of the product was improved.

Medical and Pharmaceutical Industry: Numerous microbal species have developed multi-drug resistance to commercially available antibiotics. This has led to renewed interest in the development of novel antimicrobial compounds with broad spectrum antimicrobial activity.

Daptomycin was one of the first lipopeptide-based antimictobials approved for clinical use in 2003 and it has subsequently been widely used for the treatment of complicated skin and skin-structural infections (Lee et al. 2006). It is a lipopeptide-based antimicrobial compound which shows potent bactericidal activity against clinically relevant bacterial pathogens that include coagulase-negative *Staphylococcus*, methicillin resistant *S. aureus*, glycopeptide intermediate susceptible *S. aureus*, vancomycin resistant enterococci and penicillin resistant *Streptococcus pneumoniae* (Tally et al. 1999).

The lipopeptide compound polymyxin(s) synthesised by *Bacillus polymyxa* exhibits antibacterial activity, particularly against Gram-negative bacteria (Evans et al. 1999; Gales et al. 2001). This antibacterial activity stimulated the development of the antibiotics polymyxin B and E (colistin)

from naturally produced polymyxins. Currently, these compounds are used as a last resort therapeutic option for multi-drug resistant Gram-negative bacteria (Velkov et al. 2014).

Caspofungin is a semi-synthetic antifungal compound derived from a lipopeptide biosurfactant. It exhibits marked *in vitro* antifungal activity against *Aspergillus fumigatus*, *A. flavus*, *Candida albicans* (including fluconazole-resistant strains), *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* and other *Candida* species. Caspofungin inhibits the synthesis of the fungal cell wall by preventing activity of β (l,3)-D-glucan-synthase (Deresinski & Stevens, 2003). This mode of antifungal action is unique to the compound, particularly when compared with commonly used synthetic antifungal agents. The latter include the polyenes, azoles, allylamines, and flucytosine, all of which inhibit cytochrome P450 enzyme activity (Carrillo-Munoz et al. 2006). Caspofungin represents a novel class of lipopeptide-based parenteral antifungal agents which can be prescribed for the treatment of patients who are intolerant of other antifungal therapies. Caspofungin was commercialised in the USA in the early 2000s and is currently in production (Rybowicz & Gurk-Turner, 2002).

Bioremediation: Bioremediation is the use of biological processes to remove or neutralise pollutants from a contaminated environment. Certain microorganisms including *P. aeruginosa*, *Candida bombicola* and *B. subtilis,* metabolise crude oil and hydrocarbons as sole carbon sources for the production of biosurfactants. This offers an adjunct for oil spill clean-up (Mulligan, 2005).

In their review, Maier and Soberon-Chavez (2000) indicated that the addition of rhamnolipid to environments can enhance the degradation of hydrocarbon-based contaminants. The latter compunds include hexadecane, tetradecane, pristine, creosote and hydrocarbon mixtures. Rhamnolipids added to liquid systems enhance the breakdown of hexadecane, octadecane, n-paraffin and phenanthrene. It has been reported that a rhamnolipid concentration of 300 mg/L increases mineralisation of octadecane to 20% (Zhang & Miller, 1992). In another study Churchill et al. (1995) showed that mixing rhamnolipid with a fertiliser (Inipol EAp-22) enhanced biodegradation of aromatic and aliphatic compounds in both the aqueous phase and in soil reactors. In a series of bench-scale experiments, Whang et al. (2008) investigated the use of surfactin and rhamnolipid to enhance the biodegradation of diesel-contaminated soil and water. Addition of the two biosurfactant compounds and their producer organisms (*P. aeruginosa* J4 and *B. subtilis* ATCC 21332) to the test systems indicated that microbial growth was stimulated. This was linked to an increased efficiency and rate of diesel biodegradation. In particular, rhamnolipid concentrations between 0 and 80 mg/L markedly increased microbial growth. Associated with this increase was an escalation in diesel biodegradation. In test systems containing 1 000 and 2 500 mg volatile suspended solids per litre, biodegradation increased to 40% and 100%, respectively.

Certain biosurfactant compounds have the ability to form complexes with heavy metals (refer to section 1.6.2.3). An example is the addition of rhamnolipid (>890 µM) to a mineral salt medium containing 89 µM cadmium. The biosurfactant eliminated the toxicity of Cd^{2+}. It is thought that this was achieved by means of two different mechanisms. One was by forming a Cd^{2+}-rhamnolipid complex and the other related to an interaction of rhamnolipid with the bacterial cell surface, thereby altering cadmium uptake by the cell (Sandrin et al. 2000; Ron & Rosenberg, 2001; Juwarkar et al. 2007). Dahrazma and Mulligan (2007) evaluated the performance of rhamnolipid for the removal of heavy metals (copper, nickel and zinc) from sediments obtained from the Lachine Canal in Canada in a continuous flow configuration (to simulate a remediation technique). After the addition of only 0.5% (v/v) rhamnolipid to the sediments, up to 37% copper, 27% nickel and 13% zinc were removed. Furthermore, after 1% sodium hydroxide was added to a 0.5% rhamnolipid solution, a further increase (4-fold) was reported for the removal of heavy metals from the system. The potential of a rhamnolipid biosurfactant for the removal of Cd^{2+} and Pb from artificially contaminated soil samples was evaluated by Juwarkar et al. (2007). That study indicated that the dirhamnolipid compounds screened removed free Cd^{2+} and Pb from the soils tested. In addition, weakly bound metal forms of Cd^{2+} and Pb were also extracted from the system. These trends were not noted in the aqueous control systems without biosurfactant.

Anti-fouling: Biofilms play a major part in the pathogenesis of certain bacterial infections. Examples include hospital acquired infections caused by *Staphylococcus* species, *Salmonella typhimurium*, *Salmonella enterica*, *E. coli* and *Proteus mirabilis*. These bacteria form biofilms on catheters and are known to cause cystic fibrosis, pneumonia and endocarditis (Rodrigues et al. 2006b; de Jesus Cortes-Sanchez et al. 2013). As well as the microbial constituents of a biofilm, extracellular products secreted by the organisms occur within the film matrix (Hood & Zottola, 1995). The formation of bacterial biofilms on various surfaces used in the food industry and in hospital environments constitutes a potential source of contamination and can cause food spoilage and disease. Even single cells of *Salmonella* and *Listeria monocytogenes* can initiate the formation of a well developed biofilm. Therefore in the food processing industry, there is zero tolerance of these pathogens. In order to prepare and preserve quality safe food products, it is essential that adherence of microorganisms to food contact surfaces is prevented (Hood & Zottola, 1995).

Rhamnolipids have prevented the formation of biofilms and also disrupted established biofilm structures. Due to their inherent surface activity, the biosurfactant prevents microorganisms from adhering to surfaces (Kuiper et al. 2004; Singh & Cameotra, 2004; Boles et al. 2005; Rodrigues et al. 2006a). A study conducted by Mireles et al. (2001) indicated that surfactin decreased biofilm formation by *E. coli, S. enterica*, *S. typhimurium* and *P. mirabilis* in vinyl urethral catheters and on polyvinyl chloride (PVC) plates. Moreover, research indicated that surfactin both prevented the attachment of microbial cells to surfaces and also disrupted existing biofilms (Raaijmakers et al.

2010). Do Valle Gomes and Nitschke (2012) preconditioned polystrene surfaces with individual and mixed cultures of *S. aureus*, *S. enteriditis* and *L. monocytogens*. These authors showed that 0.25% surfactin and 1% rhamnolipid reduced the biofilms created by these organisms. In addition, reconditioning of polytetrafluoroethylene (PTFE) and stainless steel surfaces with a biosurfactant obtained from *P. fluorescens* prevented biofilm formation by *L. monocytogens* L028 (Kralova & Sjöblom, 2009).

1.10. Project aims

Biosurfactants are comprised of diverse compounds of low and high molecular weight and these biomolecules are variously classified as glycolipids, lipopeptides, sophorolipids and lipopolysaccharides, amongst others. The discovery of new biosurfactant-producing microorganisms that can be cultured readily under optimal growth conditions and simultaneously produce substantial quantities of biosurfactants would be a means of reducing the economic barriers associated with large-scale production of these biomolecules. From the foregoing, it is apparent that biosurfactants offer various industries a number of attractive uses. For example some biosurfactants exhibit novel and effective comprehensive antimicrobial control mechanisms to which microbes do not easily develop resistance. This is in marked contrast to the use of conventional antibiotic therapy to control unwanted microorganisms where antibiotic resistance has reached alarming proportions. Furthermore, biosurfactants have potential as effective environmental bioremediants.

Biosurfactants and biosurfactant-producing microorganisms have been isolated from many diverse environments. A wastewater treatment plant (WWTP) was thus selected as the study site, as a wide range of bacteria are present at the various stages of treatment (Hashimoto et al. 2014; Drury et al. 2013). Furthermore, numerous organic and inorganic contaminants, present in the receiving influent could favour biosurfactant production (Shon et al. 2006). The primary aim of the research project was thus to bioprospect for novel biosurfactants and biosurfactant-producing bacteria at various points of a wastewater treatment plant. The aims of the research project were achieved as follows:

1. Distribution and diversity of biosurfactant-producing bacteria in a wastewater treatment plant (published in Environmental Science and Pollution Research): Water samples were systematically collected from the influent, post biological trickling filter treatment (biological trickling filter samples), post membrane bioreactor treatment (aeration tank samples) and post chlorine treatment (effluent) samples of a municipal WWTP. Samples were subjected to various microbial culturing techniques to obtain morphologically distinct isolates. The isolates were then screened for biosurfactant production using conventional methods (drop collapse and oil spreading methods) and potential biosurfactants were partially characterised by measuring the surface tension and emulsification activity. All the biosurfactant-producing

isolates were identified by means of bacterial 16S rRNA gene analysis. The distribution and diversity of the biosurfactant-producing bacteria throughout the WWTP were evaluated using the Shannon-Weaver and Simpson's indices. In addition, each isolate was screened for the presence of rhamnosyltransferase subunit B (*rhlB*), surfactin 4'-phosphopantetheinyl transferase (*sfp*), iturin A synthetase A (*ituA*) and bacillomycin C (*bamC*) genes involved in the biosynthesis of rhamnolipid, surfactin, iturin and bacillomycin biosurfactants respectively. The correlation of total petroleum hydrocarbon-based compound concentrations with the number of isolates obtained in each sample was also determined.

2. Repetitive element PCR for the identification of biosurfactant-producing bacteria isolated from a wastewater treatment plant: From Chapter two, 32 bacterial isolates were considered to be biosurfactant-producing and they were therefore identified to species level (using genus and species specific primers). The same isolates were further differentiated by means of repetitive element polymerase chain reactions (rep PCRs) designed to target the repetitive extragenic palindromic (REP) and the BOX element DNA sequences.

3. Characterisation and antimicrobial activity of crude biosurfactant compounds produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* Isolated from a Wastewater Treatment Plant (submitted to Applied Microbiology and Biotechnology): This aim was achieved by obtaining crude extracellular biosurfactant compounds from the *Bacillus amyloliquefaciens* ST34 and *Pseudomonas aeruginosa* ST5 strains grown on mineral salt medium supplemented with glycerol, using acid-precipitation followed by the solvent extraction method. A method for the characterisation of the crude extracts was designed using standard lipopeptides and glycolipids purchased from Sigma (USA). Thereafter, characterisation of the solvent extracts was performed using an optimised method for the electrospray ionisation mass spectrometry (ESI-MS) and the ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS). Moreover, various opportunistic, pathogenic and antibiotic resistant bacteria as well as fungal strains were then utilised for the assessment of the antimicrobial activity of the crude biosurfactant extracts obtained from the respective isolates.

4. Variants of Lipopeptides and Glycolipids Produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* in Different Substrates (submitted to Applied Microbiology and Biotechnology): The efficiency of biosurfactant production by each bacterium (ST34 and ST5) when cultured on mineral salt medium containing water miscible (glucose, fructose, glycerol and sucrose) or water immiscible (kerosene, diesel and sunflower oil) substrates was determined by means of the high throughput method described by Vosloo et al. (2013). The ESI-MS and UPLC-MS techniques were used to characterise the crude biosurfactant compounds and their respective concentrations. In addition, using the same methods, the purity of biosurfactant compounds and biosurfactant congeners produced by each isolate were

determined. The ideal carbon sources required by each microorganism for maximum yields of biosurfactant compound were identified.

1.11. References

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Distribution and diversity of biosurfactant-producing bacteria in a wastewater treatment plant

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Abstract

The distribution and diversity of culturable biosurfactant-producing bacteria was investigated in a wastewater treatment plant (WWTP) using the Shannon and Simpson's indices. Twenty wastewater samples were analysed and from 667 isolates obtained, 32 were classified as biosurfactant producers as they reduced the surface tension of the culture medium (71.1 mN/m), with the lowest value of 32.1 mN/m observed. Certain isolates also formed stable emulsions with diesel, kerosene and mineral oils. The 16S rRNA analysis classified the biosurfactant producers into the Aeromonadaceae, Bacillaceae, Enterobacteriaceae, Gordoniaceae and the Pseudomonadaceae families. In addition, numerous isolates carried the surfactin 4'-phosphopantetheinyl transferase (*sfp*), rhamnosyltransferase subunit B (*rhlB*) and bacillomycin C (*bamC*) genes involved in the biosynthesis of surfactin, rhamnolipid and bacillomycin, respectively. While, biosurfactant-producing bacteria were found at all sampling points in the WWTP, the Simpson's diversity (1 – *D*) and the Shannon-Weaver (H) indices revealed an increase in bacterial diversity in the influent samples (0.8356 and 2.08), followed by the effluent (0.8 and 1.6094) and then the biological trickling filter (0.7901 and 1.6770) samples. Numerous biosurfactant-producing bacteria belonging to diverse genera are thus present throughout a WWTP.

Keywords: Biosurfactant-producing bacteria; Wastewater; Shannon's index; Simpson's index; Evenness

2.1. Introduction

Biosurfactants are surface-active amphiphilic compounds produced by certain strains of bacteria, fungi and yeasts and can be secreted into the surrounding environment or form part of the cell membrane of the producer (Ron and Rosenburg 2001; Mulligan 2005). A wide range of biosurfactants have been shown to display various properties, which include, emulsification and surface activity, antiadhesive as well as antimicrobial activities (Razafindralambo et al. 1996; Mukherjee et al. 2006; Rodrigues et al. 2006; Shoeb et al. 2013).

The biosurfactants' unique properties contributes to the survival and growth of biosurfactant-producing microorganisms in diverse environments (Bodour et al. 2003; Chrzanowski et al. 2009; Sen 2010; Thavasi et al. 2011). Numerous studies have also reported on the predominant isolation of biosurfactant-producing microorganisms from sites contaminated by heavy metals and hydrophobic organic compounds (Bodour and Miller-Maier 1998; Bodour et al. 2003; Tabatabaee et al. 2005; Walter et al. 2010). It was then hypothesised that in metal-contaminated environments, membrane bound biosurfactants facilitate the uptake of exogenous genetic material and protect the microbial cells from toxic elements possibly by sequestration, as well as enhance cell differentiation (Van Hamme et al. 2006). In contrast, environments that contain high levels of hydrophobic compounds have been reported to trigger the secretion of biosurfactants, which then aid in the reduction of surface tension at the phase boundary, thus allowing microorganisms to move along an interface more easily as well as increasing the bioavailability of nutrients and the metabolism of the organism (Van Hamme et al. 2006; Dusane et al. 2010; Fakruddin 2012). Certain biosurfactants have also been found to protect the producers from being grazed upon by protozoan species (Jousset et al. 2006; Van Hamme et al., 2006).

High population densities of microbial cells are known to secrete secondary metabolites that could be required for cell-to-cell communication by quorum sensing (Dusane et al. 2010; Chrzanowski et al. 2012a, b). Research has then shown that biosurfactant compounds synthesised by certain organisms (mostly *Pseudomonas* and *Bacillus* species), intensify intracellular communication, thus assisting the microorganisms in surviving in habitats colonised by many other microbial species (Kuiper et al. 2004; Chrzanowski et al. 2012a, b). Certain biosurfactant compounds have also been reported to display antimicrobial properties that inhibit other taxonomic groups, allowing the biosurfactant producers to freely grow and proliferate in the environment(Sheppard et al. 1991; Benincasa et al. 2004; Das et al. 2008). Moreover, biosurfactant-producing species may secrete toxins as well as biosurfactant compounds that facilitate the formation of biofilms as a defence strategy (Van Hamme et al. 2006; Dusane et al. 2010; Raaijmakers et al. 2010).

Biosurfactants are thus considered versatile, resilient compounds and they have been earmarked for numerous applications in biotechnology for the bioremediation of metals and hydrocarbon-

based compounds (Mulligan 2005; Juwarkar et al. 2007; 2008; Chrzanowski et al. 2009; Ławniczak et al. 2013), as antimicrobials and antiadhesives in the medical field (Rodrigues et al. 2006; Stipcevic et al. 2006; Piljac and Piljac 2007; Piljac et al. 2008), in the production of cosmetics (Lourith and Kanlayavattanakul 2009), for increased recovery of oils in the petrochemical industries (Lazar et al. 2009; Al-Bahry et al. 2013) and as emulsifiers of raw materials as well as to maintain the stability and texture of food stuffs (Fakruddin 2012; Mandal et al. 2013).

The physico-chemical properties of biosurfactants, their low toxicity, high specificity and ability to function in a wide range of environmental conditions when compared to their synthetic counterparts (Mulligan 2005; Banat et al. 2010), have thus led to an increased exploration of various environments in search for novel biosurfactant-producing microbial strains and biosurfactant compounds. A wastewater treatment plant (WWTP) was selected as the study site, as a wide range of bacteria are present at the various stages of treatment (Hashimoto et al. 2014; Drury et al. 2013 and numerous organic and inorganic contaminants, present in the receiving influent, could favour biosurfactant production (Shon et al. 2006). The objective of this study were thus to systematically isolate biosurfactant-producing bacterial strains from the influent, post biological trickling filter treatment (biological trickling filter samples), post membrane bioreactor treatment (aeration tank samples) and the post chlorine treatment (effluent) samples of a municipal WWTP, using culturing techniques and evaluate their distribution and diversity using the Shannon-Weaver and Simpson's indices. In addition, each isolate was screened for the presence of rhamnosyltransferase subunit B (*rhlB*), surfactin 4'-phosphopantetheinyl transferase (*sfp*), iturin A synthetase A (*ituA*) and bacillomycin C (*bamC*) genes involved in the biosynthesis of rhamnolipid, surfactin, iturin and bacillomycin biosurfactants, respectively. The correlation of total petroleum hydrocarbon-based compounds concentrations with the number of isolates obtained in each sample was also assessed.

2.2. Materials and methods

2.2.1. Sampling sites and collection of wastewater samples

The Stellenbosch WWTP (GPS co-ordinates: -33.943505, 18.824584), which receives influent from general households, agricultural and animal farms, pharmaceutical companies, food industries, etc. in South Africa, was selected as the study site. Four sampling sites (**Fig. 2.1**) were selected based on the different stages of the treatment system used to remove contaminants and the possibility of survival of different microorganisms at that particular treatment stage. Five sampling sessions were conducted (9 July 2014, 24 July 2014, 21 August 2014, 16 September 2014, 2 October 2014), with a total of 20 wastewater samples collected overall at the influent (n = 5), biological trickling filters (n = 5), aeration tank (n = 5) and the effluent (n = 5) points

(**Fig. 2.1**). Samples were collected by immersing a 2 l sterile Schott bottle into the wastewater. The samples were transported to the laboratory on ice to maintain a temperature below 4 ºC.

Fig. 2.1. Stellenbosch wastewater treatment plant with sampled sites indicated by A: Influent point; B: Biological trickling filter; C: Aeration tank; D: Effluent point (GPS co-ordinates -33.943505, 18.824584)

2.2.2. General culturing of wastewater samples

Wastewater samples were serially diluted (10^{-1} – 10^{-4}) and 100 µl of each dilution was spread plated onto Cetrimide agar (CA) base (Biolab Diagnostic, South Africa), cereus selective agar (CSA) base (Merck, Germany) supplemented with egg yolk emulsion (Sigma-Aldrich, USA) and nutrient agar (NA) (Biolab diagnostic, South Africa), with the plates incubated at 35–37 ºC for 24 h. Cetrimide agar base was used for the isolation of *Pseudomonas* species, CSA supplemented with egg yolk emulsion for the isolation of *Bacillus* species. The non-selective NA was also used for isolation of heterotrophic bacteria directly from wastewater samples. After incubation all plates were observed for growth and well isolated colonies (approximately 100 colonies per sampling session), exhibiting typical *Pseudomonas* species (yellow-green to blue-green) characteristics on CA, *Bacillus* species (blue colonies with precipitate and straw coloured) characteristics on CSA and morphologically distinct colonies on NA, were re-streaked on NA plates at least three times to obtain pure cultures.

2.2.3. Culturing conditions and screening for biosurfactant-producing isolates

After culturing of all bacterial isolates, the purified single colonies were then subjected to biosurfactant screening as previously described by Bodour et al. (2003) with some modifications. Briefly, single colonies were inoculated into 5-ml mineral salt medium (MSM) containing 2 % main carbon and energy source (glucose or glycerol). The MSM solution A (per litre) was composed of 2.5 g of $NaNO_3$, 0.4 g of $MgSO_4.7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05 g of $CaCl_2.2H_2O$ and 10 ml of Phosphoric acid (85 %, pH 7.2). Solution B (per litre) was composed of 0.5 g of $FeSO_4.7H_2O$, 1.5 g of $ZnSO_4.7H_2O$, 1.5 g of $MnSO_4.2H_2O$, 0.3 g of K_3BO_3, 0.15 g of $CuSO_4.5H_2O$ and 0.1 g of $NaMnO_4.2H_2O$. One millilitre of solution B and 20 ml of glucose or glycerol were added to 1 l of solution A (Bodour et al. 2003). The test tube broth cultures were then incubated aerobically in a 200-rpm shaker at 30 ºC for 48 and 96 h, and the cell-free supernatant was obtained by centrifugation of the culture broth at 10,000 rpm for 20 min at 4 ºC. The cell-free supernatant was then analysed for the presence of surface-active compounds by using the drop collapse and oil spreading methods.

2.2.4. Drop collapse method

The single colonies obtained were all subjected to the drop collapse method as previously described by Bodour et al. (2003) after 48 h and 96 h of incubation. A thin layer of mineral oil (Sigma-Aldrich, USA), was applied to each well of a 96-well micro plate (Thermo Scientific, Lithuania) and was left to equilibrate at room temperature for approximately 12-24 h. Five microlitres of the cell-free supernatant was then applied to the centre of each well. If the cell-free supernatant drop collapsed and spread, it was recorded as positive for the presence of biosurfactant compounds, while if the drop remained raised, it was recorded as negative for the presence of biosurfactants. All isolates were tested in triplicate, with the MSM used as a negative control.

2.2.5. Oil spreading method

The cell-free supernatant obtained from purified single colonies were also subjected to the oil spreading method as previously described by Youssef et al. (2004) and Silva et al. (2010). Ten microlitres of sunflower oil was added to the surface of distilled water (40 ml) in a 90-mm petri dish, so that a thin layer of oil was formed. Ten microlitres of cell-free supernatant was then gently placed in the centre of the oil layer. If a biosurfactant compound was present in the cell-free supernatant, the oil was displaced and a clearing zone was formed. Bacterial isolates that were positive for biosurfactant production using the oil spreading method were then further subjected to the emulsification test and surface tension measurements.

2.2.6. Physico-chemical characterisation

2.2.6.1. Emulsification capacity assay

The bacterial isolate seed culture was first prepared as described in " culturing conditions and screening for biosurfactant-producing isolates" section. Five millilitres of actively growing bacterial cells were inoculated into a 100 ml MSM in a 500 ml baffled flask as described by Bodour et al. (2003) and incubated at 200 rpm for 5 to 7 days at 30 ºC. The whole culture was then centrifuged at 10,000 rpm at 4 ºC for 20 min to remove microbial biomass. The emulsification index (E_{24}) of each cell-free supernatant was determined by adding 2 ml of diesel, kerosene or mineral oils, respectively, to an equal amount of cell-free supernatant, followed by vortexing for approximately 2 min. The mixture was left to stand for 24 h at room temperature and the E_{24} for each substrate was calculated using equation 1:

$$\text{Emulsification index } (E_{24})\ \% = \frac{\text{Height of the emulsion layer X 100}}{\text{Total height of the solution}} \quad \text{.....(1)}$$

2.2.6.2. Surface tension measurement

Broth cultures (100 ml) of the biosurfactant-producing bacteria were first incubated at 200-rpm for 5 to 7 days at 30 ºC, and the cell-free supernatant was then tested for its ability to reduce surface tension using the Du Nouy ring tensiometer as previously described by Youssef et al. (2004). The surface tension of the biosurfactants in the cell-free supernatants were measured at room temperature however, for the validity of the measurements, the calibration was performed using distilled water, and then, the surface tension for sterile MSM was measured before measuring each sample. All samples were measured at least three times, and an average value was used to express the surface tension of the sample.

2.2.7. Molecular analysis of biosurfactant-producing bacteria

2.2.7.1. Genomic DNA extraction

The extraction and purification of genomic DNA were performed using the boiling method as described by Ndlovu et al. (2015). Where low DNA concentrations were obtained, the ZR Soil microbe DNA miniPrep™ kit (Zymo Research, USA) was used. For the boiling method briefly, the pure cultures of the bacterial strains were grown on NA at 37 °C for 18-24 h. Thereafter, a single colony was inoculated into Luria Bertani (LB) broth and incubated for 18-24 h at 37 °C. One millilitre of broth aliquot was centrifuged at 10,000 rpm for 10 min, the supernatant discarded, the pellet re-suspended in 100 µl of sterile double-distilled water and boiled in a water bath for 15 min at 95 °C. The suspension was then cooled on ice for 10 min and, centrifuged at 10,000 rpm

for 5 min and the supernatant containing the DNA transferred into a sterile 1.5 ml Eppendorf tube. Deoxyribonucleic acid samples were then stored at -20 °C for PCR analysis.

2.2.7.2. 16S rRNA polymerase chain reaction analysis

Amplification of the 16S ribosomal RNA (rRNA) conserved region was performed as previously described by Rawlings (1995) using the universal primers fDD2 (5' CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG 3') and rPP2 (5' CCAAGCTTCTAGACGGITACCTTGTTACGACTT 3'). The reaction mixture contained 1× PCR Green GoTaq Flexi buffer (Promega, USA), 2 mM $MgCl_2$, 0.1 µM deoxynucleoside triphosphate (dNTP) mixture (Thermo Scientific, Lithuania), 0.5 µM of each primer, 1.5 U GoTaq G2 DNA polymerase (Promega, USA) and 2 µl of template DNA and was then made up to a final volume of 50 µl using sterile nuclease-free water. Amplification was performed using the T100™ thermal cycler (Bio-Rad Laboratories, Netherlands) and the PCR cycling parameters consisted of initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 90 s, and then a single final extension step of 72 °C for 5 min. The 1 600-bp PCR product was visualised on a 1.0% agarose gel stained with ethidium bromide (0.5 µg/ml). The amplified PCR products were purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) as per manufacturer's instructions and were sequenced in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems, USA) at the Central Analytical Facility (CAF), Stellenbosch University (Stellenbosch, South Africa).

2.2.7.3. Detection of genes involved in biosynthesis of biosurfactants

The *rhlB*, *sfp, ituA* and *bamC* genes involved in the biosynthesis of rhamnolipid, surfactin, iturin and bacillomycin biosurfactants, respectively, were screened for in all the biosurfactant-producing isolates. The primer sequences used to amplify each target gene using PCR assays are indicated in **Table 2.1**. The conventional PCR for the detection of *rhlB* gene was performed in a total volume of 50 µl containing 1× PCR Green GoTaq Flexi buffer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer (Kpd1 and Kpd2), 5% Dimethyl sulfoxide (DMSO), 1.5 U of GoTaq G2 DNA Polymerase and 5 µl of template DNA. The reaction mixture for the detection of *sfp, ituA* and *bamC* genes consisted of a 1× PCR Green GoTaq Flexi buffer, 0.2 mM dNTP mix, 1.5 U of GoTaq G2 DNA Polymerase, 1.75 mM $MgCl_2$ (*sfp* gene), 2 mM $MgCl_2$ (*ituA* and *bamC* genes), 0.4 µM of each primer (SRFP-F1 and SRFP-R1; BACC1-F and BACC1-R), 0.5 µM of each primer (ITUA1-F and ITUP2-R) and 5 µl of template DNA. All reaction mixtures were made up to a final volume of 50 µl with sterile nuclease free water. The PCRs were performed using a T100™ thermal cycler, with the cycling conditions adapted from previous studies as outlined in **Table 2.1**. The DNA extracted from *Pseudomonas aeruginosa* PAO1 (*rhlB* gene) and *Bacillus amyloliquefaciens* AD2 (*sfp, ituA* and *bamC* genes) were used as positive controls in the PCR assays.

Table 2.1. Primer sequences and PCR cycling conditions used for the detection of biosurfactant target genes.

Gene	Primer name	Primer sequences (5'-3')	PCR cycling conditions	Product size	Reference
rhlB	Kpd1 Kpd2	GCCCACGACCAGTTCGAC CATCCCCCTCCCTATGAC	94ºC for 2 min; 30 cycles: 94ºC for 15 s, 54ºC for 15 s and 72ºC for 15 s; 72ºC for 2 min.	226 bp	Adopted from Bodour et al. (2003)
sfp	SRFP-F1 SRFP-R1	ATGAAGATTTACGGAATTTA TTATAAAAGCTCTTCGTACG	94ºC for 3 min; 30 cycles: 94ºC for 1 min, 46ºC for 30 s, 72ºC for 1 min; 72ºC for 5 min.	675 bp	Adapted from Hsieh et al. (2004)
ituA	ITUP1-F ITUP2-R	AGCTTAGGGAACAATTGTCATCG GGGCTTC TCAGATAGGCCGCCATATCGGAA TGATTCG	94°C for 3 min; 35 cycles: 94°C for 1 min, 60°C for 30 s, 72°C for 2 min 30 s; 72°C for 5 min.	2 kb	Adapted from Tsuge et al. (2005)
bamC	BACC1F BACC1R	GAAGGACACGGCAGAGAGTC CGCTGATGACTGTTCATGCT	94°C for 3 min; 35 cycles: 94°C for 1 min, 60°C for 30 s, 72°C for 1 min 45 s; 72°C for 5 min.	875 bp	Adapted from Ramarathnam et al. (2007)

The PCR products were analysed by electrophoresis using a 1.5% agarose gel (stained with 0.5 µg/ml ethidium bromide), and the presence of the 226, 675, 875 and 2 000-bp DNA bands was considered indicative of the presence of the *rhlB*, *sfp, bamC and ituA* genes, respectively. All positive PCR products were then purified and concentrated using the DNA Clean & Concentrator™-5 Kit as per manufacturer's instructions. The cleaned PCR products were sequenced in accordance with the BigDye Terminator Version 3.1 Sequencing Kit at CAF in Stellenbosch University. The obtained sequences were analysed using the online Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, to find the closest match of local similarity of the isolates to the online international database in GenBank, EMBL, DDBJ and PDB sequence data (Altschul et al. 1997).

2.2.8. Total petroleum hydrocarbon based compounds analysis in wastewater samples

Analysis of total petroleum hydrocarbon based compounds was conducted on the wastewater samples where the biosurfactant-producing bacteria were isolated from (described in section "sampling sites and collection of wastewater"). Thus, a specific identifier namely, a code/number was assigned to these samples [collected in July 2014 (influent to effluent samples) up to samples collected in October 2014 (influent to effluent)] (**Table 2.4**). Sample preparation was conducted as previously reported by Wang et al. (2002), with slight modifications. Approximately 200 ml of wastewater sample was spiked with 100 µl of 100 µg/ml 1-tetradecene (Sigma-Aldrich, USA) and was left to stand for 15 min. The samples were then successively extracted four times (each time, the sample was shaken for approximately 3 min) using 50 ml of dichloromethane (Merck, Germany), with periodic venting to release pressure. Thereafter samples were concentrated to approximately 40 ml using a Buchi Rotavapor R-114 (Buchi Labortechnik AG, Switzerland), and the extracts were further concentrated using the anhydrous sodium sulphate (Merck, Germany) to remove excess water. The concentrated sample extracts were sent to CAF at Stellenbosch

University for analysis using a Gas Chromatography coupled with Mass Spectrometry (GC-MS, model 6890N), coupled to a Agilent technologies inert XL EI/CI Mass Selective Detector (MSD, model 5975B) (Agilent Technologies Inc., USA). A calibration standard used for total petroleum hydrocarbon-based compounds (TPH mix 1) was obtained from Sigma-Aldrich (USA), and it was composed of a mixture of n-alkane compounds ranging from C_{10} – C_{28} (decane, docosane, dodecane, eicosane, hexacosane, hexadecane, octacosane, octadecane, tetracosane and tetradecane).

2.2.9. Statistical analysis

Statistical analysis was performed as previously outlined by Olapade and Ronk (2015). The genera of all biosurfactant-producing bacteria were classified into operational taxonomic units (OTUs) using the 16S rRNA gene sequence data with identity values ranging from 91% to 99%. The OTUs were also evaluated using several species diversity indices. The; Simpson's index (D) was calculated using equation 2:

Simpson's index (D) = $\frac{\Sigma\, n(n-1)}{N(N-1)}$.....(2)

Where; n = the number of isolates belonging to a particular genus and N = the total number of all isolates of all genera

The Simpson's index of diversity (1 – D) and the Simpson's (reciprocal) index (1/D) were also determined for the isolates obtained in each sampling point within the WWTP. The Shannon-Weaver index (H) was calculated using equation 3:

Shannon-Weaver (H) = Σ(PlnP).....(3)

Where; P = the number of isolates belonging to a particular genus

The evenness index (E) was calculated using equation 4:

Evenness index (E) = H/ H_{max}.....(4)

Where; H is the Shannon-Weaver index and H_{max} is the maximum diversity possible.
All calculations were performed using Microsoft excel 2010.

2.3. Results

2.3.1. Screening for biosurfactant-production

Twenty samples were collected at various sampling points from Stellenbosch WWTP and were screened for the presence of biosurfactant-producing bacterial isolates. The initial screening of well-isolated colonies yielded a total of 667 bacterial isolates [206 from site A, 180 from site B,

142 from site C and 139 from site D **(Fig. 2.1**)]. Bacterial isolates were grown in 5 ml of MSM (supplemented with 2 % glucose or glycerol) broth for 48 and 96 h at 37 ºC in a 200-rpm shaker.

For efficient detection of biosurfactant producers, combinations of screening methods were utilised. Of the 667 bacterial isolates screened for using the oil spreading technique, 32 isolates (4.8 %) (**Table 2.2**) showed oil displacement. The drop collapse technique was also utilised to confirm the production of biosurfactants in all the 667 isolates, however, out of the 32 isolates that were positive using the oil spreading technique, only 29 isolates (4.3 %) tested positive using the drop collapse method and three isolates (ST 2, ST 20 and ST 21) tested negative under the conditions utilised in the study (**Table 2.2**).

Thirty-two of the microbial isolates were considered biosurfactant-producing microorganisms. The majority of the biosurfactant-producing microbial isolates were obtained from the influent samples (46.9 %), followed by the biological trickling filters samples (28.1 %) and the effluent samples (15.6%), the least number of potential biosurfactant-producing bacteria was obtained from the aeration tank samples (9.4 %). However, out of a total of five samples collected in the aeration tank, only one sample yielded three biosurfactant-producing isolates, and two of the samples collected from the effluent yielded a total of five isolates.

2.3.2. Physico-chemical characterisation

2.3.2.1. Emulsification capacity assay

The 32 isolates that were regarded as biosurfactant producers were tested for their ability to emulsify diesel, kerosene and mineral oils in order to determine their emulsification activity (**Table 2.2**). The overall emulsion index ± standard deviation ranged from 0 to 90 ± 0.71, 0 to 77.3 ± 1.0, 0 to 29.7 ± 0.42 %, with mineral oil, kerosene and diesel as substrates, respectively. Low emulsification indices were obtained with diesel as a substrate, with no emulsions forming with 21.9 % of the samples. In addition, when mineral oil was used as a substrate for the emulsion assay, 28.1 % of the samples did not form any emulsions. Isolate ST 14 did not emulsify the diesel and mineral oils, and a very low emulsion index (8 ± 1.4 %) was obtained with kerosene as a substrate, while the highest emulsification index was observed for ST 3 (90 %) with mineral oil as a substrate. This isolate (ST 3) recorded an E_{24} of 45.5 ± 0.7% using kerosene, but no emulsification was observed with diesel as a substrate. The majority of the isolates were however, able to form stable emulsions with at least two of the oils utilised as substrates in the current study (**Table 2.2**).

Table 2.2. Identification and characterisation profiles of bacterial isolates producing surface active compounds

Isolate	Genebank accession no.	% ID	*Site	**SFT (mN/m)	***E_{24} (%) (D) ± SD	***E_{24} (%) (MO) ± SD	***E_{24} (%) (K) ± SD	ˣ*bamC*	ˣ*ituA*	ˣ*sfp*	ˣ*rhlB*
ST 1	*Shewanella* (NR_116732)	99	A	60	14.7 ± 0.28	0	0	-	-	-	-
ST 2	*Aeromonas* (CP007567)	99	A	65	0	29 ± 0.71	0	-	-	-	-
ST 3	*Kluyvera* (AM933754)	99	A	34.3	0	90 ± 0.71	45.5 ± 0.7	-	-	-	-
ST 4	*Pseudomonas* (EU140959.1)	99	B	39	11.4 ± 0.71	20.8 ± 0.28	8 ± 2.8	-	-	-	+
ST 5	*Pseudomonas* (JQ659980)	99	B	32.3	14.3 ± 0.42	77.8 ± 0.42	75 ± 2.8	-	-	-	+
ST 6	*Pseudomonas* (CP003190)	99	B	55.5	11.1 ± 0.57	0	8 ± 0.0	-	-	-	-
ST 7	*Providencia* (AB680422)	97	A	52.9	9 ± 1.41	29 ± 1.41	54.5 ± 0.7	-	-	-	-
ST 8	*Alkalimonas* (KJ841884)	99	A	54.7	0	30.4 ± 0.99	43.5 ± 4.9	-	-	-	-
ST 9	*Aeromonas* (LN624814)	99	B	56.9	6 ± 0.71	8.7 ± 0.42	60.9 ± 3.0	-	-	-	-
ST 11	*Bacillus* (KM083098)	95	A	37	29.7 ± 0.42	41.7 ± 0.42	45.5 ± 0.7	-	-	+	-
ST 12	*Citrobacter* (KM515969)	96	A	57.4	7.4 ± 1.27	16.7 ± 0.28	22.7 ± 0.4	-	-	-	-
ST 13	*Serratia* (CP013046)	98	A	62	15 ± 0.71	0	20 ± 0.0	-	-	-	-
ST 14	*Aeromonas* (GU204971)	93	A	57.8	0	0	8 ± 1.4	-	-	-	-
ST 15	*Klebsiella* (JX435602)	97	A	53	3.3 ± 0.42	0	62.5 ± 0.7	-	-	-	-
ST 17	*Kluyvera* (NR_024883)	93	D	52	16.7 ± 0.42	0	20 ± 0.0	-	-	-	-
ST 18	*Bacillus (CP007607)*	99	D	67.9	6.2 ± 0.28	30 ± 0	8 ± 1.4	-	-	-	-

*Site- A - Influent point, B - Biological trickling filters, C - Aeration tank, D - Effluent point, **SFT – Surface tension, ***E_{24} – Emulsification index, (D) – Diesel, (MO) – Mineral oil, (K) – Kerosene, SD – Standard deviation, ˣ- – Not detected, ˣ+ – Detected

Table 2.2. (Continued). Identification and characterisation profiles of bacterial isolates producing surface active compounds

Isolate	Genebank accession no.	% ID	*Site	**SFT (mN/m)	***E_{24} (%) (D) ± SD	***E_{24} (%) (MO) ± SD	***E_{24} (%) (K) ± SD	ˣ*bamC*	ˣ*ituA*	ˣ*sfp*	ˣ*rhlB*
ST 19	*Pseudomonas (*AP014522)	97	D	33.7	12.1 ± 1.56	14.3 ± 0.28	72.7 ± 0.4	-	-	-	+
ST 20	*Enterobacter* (CP007546)	95	D	52	11.4 ± 0.14	0	16 ± 1.4	-	-	-	-
ST 21	*Raoultella* (KF938668)	96	D	66	5.3 ± 0.28	0	28 ± 1.4	-	-	-	-
ST 22	*Proteus* (EF091150.1)	92	A	34.2	5.7 ± 0.28	16.7 ± 0.42	64 ± 1.4	-	-	-	-
ST 23	*Klebsiella* (CP007731)	95	A	57.9	5.2 ± 0.85	0	8.7 ± 0.4	-	-	-	-
ST 24	*Bacillus* (JQ361054)	98	B	37.6	10 ± 0.71	22.2 ± 0.28	64 ± 1.4	-	-	+	-
ST 25	*Bacillus* (HE774679)	96	B	36.2	16.7 ± 0.28	60 ± 1.41	73.9 ± 0.1	+	+	+	-
ST 26	*Escherichia* (KJ803896)	98	A	32.8	14.7 ± 0.42	30 ± 1.41	20 ± 1.4	-	-	-	-
ST 27	*Aeromonas* (LN624814)	96	A	35	10 ± 0.85	5.4 ± 0.28	27 ± 0.0	-	-	-	-
ST 28	*Aeromonas* (KC904096)	95	A	34.4	0	32 ± 1.41	8.7 ± 0.4	-	-	-	-
ST 29	*Serratia* (FJ897467)	98	B	33.3	8.6 ± 0.28	12.5 ± 0.71	69.6 ± 0.6	-	+	+	-
ST 30	*Kluyvera* (AM933754)	91	B	54.6	5.7 ± 0.14	9.5 ± 0.71	17.8 ± 0.3	-	-	-	-
ST 31	*Gordonia* (JQ658422)	96	B	32.5	0	7.7 ± 0.28	53.6 ± 0.6	-	-	-	-
ST 32	*Bacillus* (CP007800)	98	C	33.2	0	47.4 ± 0.57	56.5 ± 0.7	-	-	+	-
ST 33	*Bacillus* (CP006881)	98	C	32.1	12.5 ± 0.0	25 ± 1.41	66.7 ± 1.8	-	-	+	-
ST 34	*Bacillus* (GU250448)	99	C	34.4	23.7 ± 0.14	14.3 ± 0.42	77.3 ± 1.0	+	+	+	-

*Site- A - Influent point, B - Biological trickling filters, C - Aeration tank, D - Effluent point, **SFT – Surface tension, ***E_{24} – Emulsification index, (D) – Diesel, (MO) – Mineral oil, (K) – Kerosene, SD – Standard deviation, ˣ- – Not detected, ˣ+ – Detected

2.3.2.2. Surface tension measurement

All biosurfactant producers reduced the surface tension of sterile MSM (71.1 mN/m) (**Table 2.2**). Overall, the lowest surface tension reduction of up to 67.9 mN/m was obtained for isolate ST 18 (effluent sample), while the highest reduction of surface tension of up to 32.1 mN/m was observed for ST 33 (aeration tank sample) (**Table 2.2**). High surface tension reductions were also observed for the other two isolates obtained in the aeration tank samples, namely ST 32 and ST 34 with a surface tension reduction of 33.2 and 34.4 mN/m recorded, respectively. In addition, a high surface tension reduction of 32.5 mN/m was also observed for isolate ST 31 (effluent sample). For the isolates obtained in the influent, biological trickling filter and effluent samples, the observed values for surface tension reduction ranged from 65 mN/m (ST 2) to 34.4 mN/m (ST 28), 56.9 mN/m (ST 9) to 32.5 mN/m (ST 31) and 67.9 mN/m (ST 18) to 52 mN/m (ST 20 and 17), respectively.

2.3.3. Molecular analysis of biosurfactant-producing bacteria

The 32 biosurfactant-producing bacteria were further subjected to 16S rRNA PCR analysis and were grouped into three classes, namely Actinobacteria, Gammaproteobacteria and the Firmutes. Of the 46.9 % (15) biosurfactant producers isolated from the influent samples, 60 % (9) were identified as belonging to family of Enterobacteriaceae, 33.3 % (5) belonged to the Aeromonadaceae family and one isolate belonged to the Bacillaceae family. Of the 28 % (9) of the bacterial isolates obtained from the biological trickling filter samples, isolates that belonged to the Enterobacteriaceae and Bacillaceae families each constituted approximately 22.2 % (2), while the Aeromonadaceae and Gordoniaceae families constituted 11.1 % (1) each, and the Pseudomonadaceae family was the most dominant, making up 33 % (3) of all isolates. For the aeration tank samples, all isolates (3) belonged to the Bacillaceae family, while for the effluent sample isolates (5), 60 % belonged to the Enterobacteriaceae family.

Fig. 2.2 illustrates the overall frequency distribution of genera within the WWTP at various sampling locations. The *Aeromonas* genus accounted for approximately 26.6 % (4/15) of all bacteria isolated from the influent samples, with the *Klebsiella* genus accounting for 22.2 % (2/9) of all the Enterobacteriaceae detected in the influent samples, while the remainder of the genera (*Serratia*, *Shewanella, Klyuvera, Providencia, Alkalimonas, Escherichia, Proteus* and *Citrobacter*) accounted for approximately 11.1 % (1/9) each. From the biological trickling filter samples, the *Pseudomonas* genus accounted for 33 % (3/9) of the isolates, followed by the *Bacillus* genus (22 %, 2/9), while *Aeromonas, Kluyvera, Serratia* and *Gordonia* each accounted for approximately 11 % (1/9). All isolates (3) obtained from the aeration tank samples belonged to the *Bacillus* genus. Comparatively, isolates obtained from the effluent samples were dominated by the members of the Enterobacteriaceae family (60 %, 3/5), with *Kluyvera, Raoultella* and

Enterobacter detected. In addition, the non Enterobacteriaceae isolates belonging to the genera *Bacillus, and Pseudomonas,* accounted for 20 % (1/5) of the isolates obtained, respectively.

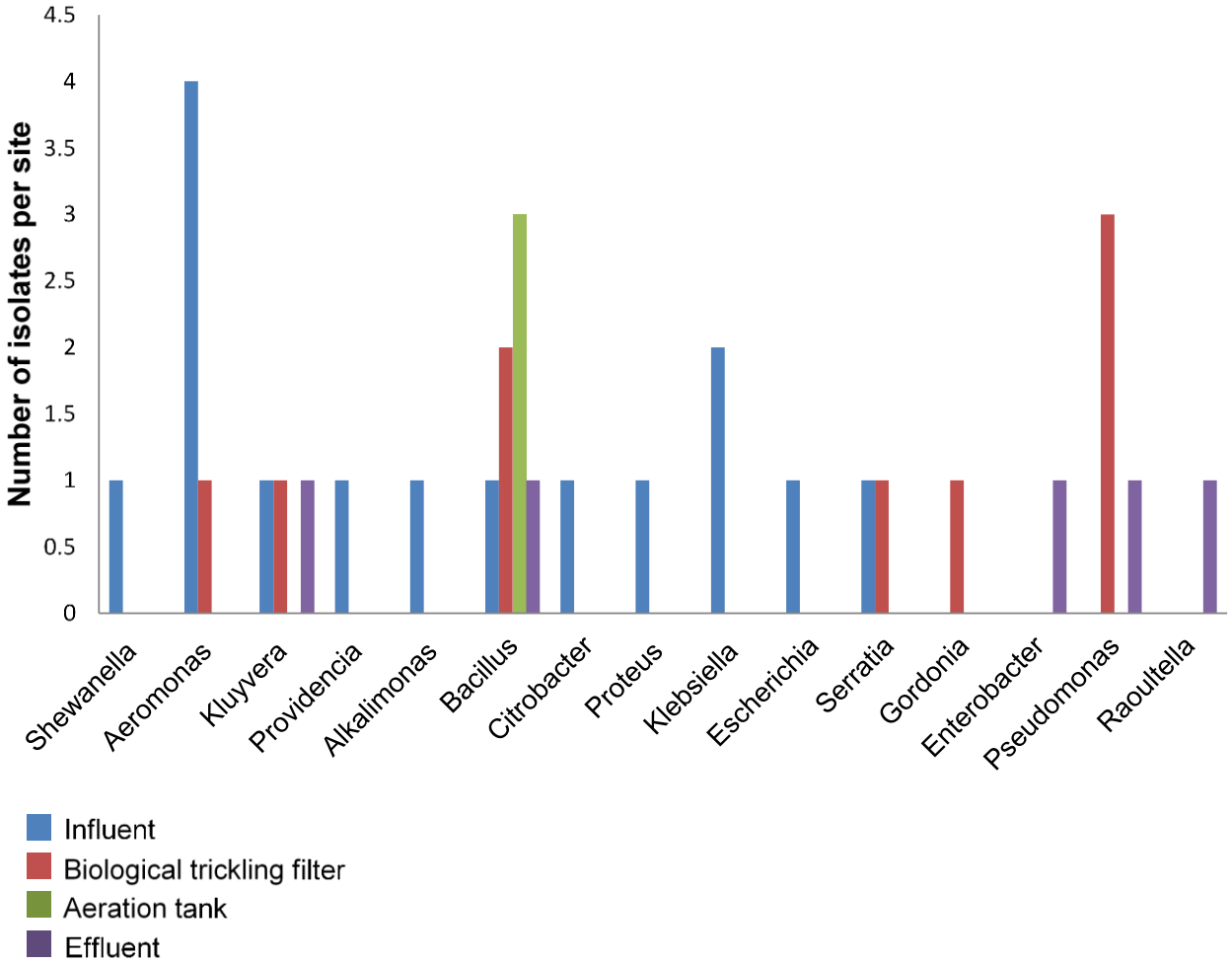

Fig. 2.2. The number of isolates of the respective genera (biosurfactant-producing bacterial isolates; n = 32) detected at the various locations of the wastewater treatment plant

The microbial diversity indices were calculated based on the 16S rRNA gene sequence data to measure the biodiversity of the isolated biosurfactant producers from the different sites of the WWTP. The influent samples contained the highest number of biosurfactant-producing bacteria (15), while the least number of isolates was obtained from the aeration tank samples (3). Results for the diversity measurement based on the Simpson's diversity index (1 - *D*) revealed that overall the influent samples yielded the highest diversity (0.8356), followed by the effluent (0.8000) samples and the biological trickling filter samples (0.7901) (**Table 2.3**). The Simpson's (reciprocal) index (1/*D*) also revealed that the influent samples recorded a higher diversity index of 6.08, while the effluent and biological trickling filter samples recorded indices of 5 and 4.76, respectively. There was no microbial diversity in the aeration tank samples as only one genus was isolated. The microbial diversity was further calculated using the Shannon-Weaver index (*H*), and the influent samples again recorded the highest diversity (2.08), while the effluent and biological trickling filter samples recorded indices of 1.6094 and 1.6770, respectively.

Table 2.3. Diversity indices between communities of putative biosurfactant-producing isolates obtained in samples collected at different sites of the wastewater treatment plant

Sampling point	N	G	*D*	1/*D*	1 – *D*	*H*	*E*	%Coverage
Influent	15	10	0.1644	6.08	0.8356	2.08	0.9033	46.9
Biological trickling filter	9	6	0.2099	4.76	0.7901	1.677	0.9359	28.1
Aeration tank	3	1	1	1	0	0	0	9.4
Effluent	5	3	0.2	5	0.8	1.6094	1	15.6

N = number of isolates; G = number of genera; *D* = Simpson's index; 1/*D* = Simpson's reciprocal index; 1 - *D* = Simpson's index of diversity; *H* = Shannon Weaver; *E* = Evenness.

The Shannon-Weaver index value for the isolates obtained from the biological trickling filter samples was higher when compared to the effluent samples, as more isolates were obtained at this sampling point. The bacterial isolates were then analysed for genus evenness (*E*) within the WWTP, and the effluent samples recorded a higher evenness (1), followed by biological trickling filter (0.9359) and influent samples (0.9033), respectively. However, the isolates obtained from the effluent only constituted approximately 15.6 % of the total isolates, while the biological trickling filter and influent samples constituted 28.1 and 46.9 % of the total isolates, respectively.

2.3.4. Screening for selected genes involved in biosynthesis of biosurfactants

The 32 biosurfactant producers were screened for the presence of *bamC, ituA, rhlB and sfp* genes using gene-specific primers as previously described by Hsieh *et al.* (2004). The PCR results revealed the presence of the 675-bp PCR product corresponding to an *sfp* gene amplicon predominantly in the *Bacillus* species (ST 11, ST 24, ST 25, ST 32, ST 33 and ST 34), as well as in ST 29 identified as *Serratia sp.* (**Table 2.2**). Sequencing of the amplified *sfp* gene revealed a 99 % identity to the *Bacillus subtilis sfp* gene (accession no. X63158, X65610, EU146076, EU146075 and AF233756) or *B. amyloliquefaciens sfp* gene (accession no. JN086145).

The PCR results revealed amplification of the *rhlB* gene in *Pseudomonas* isolates (ST 4, ST 5 and ST 19) as identified by the 16S rRNA analysis (**Table 2.2**). After DNA sequencing, all the isolates that were positive for the *rhlB* gene, displayed 100 % similarity to the *P. aeruginosa* rhamnosyltransferase chain A (*rhlA*), rhamnosyltransferase chain B (*rhlB*) genes (KC008608) or *P. aeruginosa* (*rhlB*) gene involved in the biosynthesis of rhamnolipid biosurfactant. All isolates were further screened for the presence of *bamC* and *ituA* genes, and isolates ST 25 and ST 34 were both positive for these genes, which are involved in the biosynthesis of bacilloymcin and iturin biosurfactants, respectively (**Table 2.2**). The *ituA* gene was further detected in isolate ST 29 (*Serratia* sp.). After DNA sequencing, all the *ituA* gene positive isolates displayed 97 % similarity to the DNA sequences of the *B. amyloliquefaciens* strain Q-426 bamCBAD and

fenEDCBA gene clusters (JQ271536). The *bamC* gene-positive isolate showed 99 % similarity to the *B. subtilis* bacillomycin C operon (AY137375).

2.3.5. Detection of total petroleum hydrocarbon based compounds

Quantitative assessment of certain total petroleum hydrocarbon-based compounds (alkanes ranging from C_{10} to C_{28}) (widely associated with the presence of Gram-negative biosurfactant-producing bacteria) was conducted, on samples where the biosurfactant-producing bacteria were isolated from, using Gas Chromatography (GC). Most of the total petroleum hydrocarbon-based compounds (decane, docosane, eicosane, hexacosane, hexadecane, octacosane and octadecane) were not detected in the samples collected from the various points at the WWTP, during this study. The lowest concentration of 1.070 µg/ml to the highest concentration of 1.372 µg/ml of total petroleum hydrocarbon-based compounds (dodecane, tetracosane and tetradecane) was recorded in the biological trickling filter samples 9 and 4, respectively (**Table 2.4**). Dodecane, tetracosane and tetradecane were detected throughout the sampling period ranging from 0.303 to 0.584 µg/ml. Dodecane was detected at an average of 0.311 µg/ml and ranged from 0.303 (aeration tank sample two) to 0.357 µg/ml (biological trickling filter sample four). The tetracosane was detected at an average of 0.384 µg/ml and ranged from 0.315 (biological trickling filter sample nine) to 0.454 µg/ml (biological trickling filter sample four), while tetradecane was detected at an average of 0.523 µg/ml and it ranged from 0.446 (influent sample 10) to 0.584 µg/ml (influent sample one) (**Table 2.4**).

Table 2.4. Concentrations of total hydrocarbon-based compounds in wastewater samples with the corresponding number of biosurfactant-producing isolates obtained in the sample.

[a]Site (Sampling Date)	Sample Number	Dodecane (µg/ml)	Tetracosane (µg/ml)	Tetradecane (µg/ml)	Total (µg/ml)	No. of isolates obtained
A (7 July 2014)	1	0.304	0.374	0.584	1.262	3
C (7 July 2014)	2	0.303	0.347	0.495	1.145	3
A (24 July 2014)	3	0.308	0.395	0.512	1.216	2
B (24 July 2014)	4	0.357	0.454	0.560	1.372	4
A (21 August 2014)	5	0.305	0.418	0.551	1.274	3
B (21 August 2014)	6	0.304	0.428	0.529	1.261	1
D (21 August 2014)	7	0.305	0.358	0.536	1.199	5
A (16 September 2014)	8	0.309	0.402	0.514	1.225	2
B (16 September 2014)	9	0.305	0.315	0.451	1.070	2
A (2 October 2014)	10	0.310	0.321	0.446	1.077	3
B (2 October 2014)	11	0.315	0.416	0.578	1.310	3

[a]Site: Site- A - Influent point, B - Biological trickling filters, C - Aeration tank, D - Effluent point

2.4. Discussion

Secondary microbial metabolites that display surface and emulsification activities are classified as biosurfactant molecules. These molecules reduce interfacial and surface tension in both hydrocarbon mixtures and aqueous solutions, implying that they can be utilised as potential agents for the bioremediation of hydrocarbon-contaminated environments and for the prevention and disruption of biofilms on various surfaces (Mulligan 2005; Raaijmakers et al. 2010) The ability of certain microbial species to secrete various biosurfactants allows for the primary screening of biosurfactant production, which can be characterised by the changes in surface activities on various hydrocarbon-based compounds (Banat 1993; Youssef et al. 2004; Bento et al. 2005). Thirty-two isolates were classified as biosurfactant-producing organisms using the drop collapse and the oil spreading techniques as preliminary screening assays.

Further characterisation of the thirty-two isolates was performed for surface tension and emulsification activities. Biosurfactant-producing microbial candidates are expected to reduce the surface tension of water to approximately 35 mN/m (Desai and Banat 1997; Soberón-Chávez and Maier 2011). In the current study, the surface tension was reduced to between 67.9 and 32.1 mN/m (lowest surface tension recorded) by the monoculture isolates. *Bacillus* species are widely studied for their biosurfactant production and in the current study all the *Bacillus* species isolated (except isolate ST 18) significantly reduced the surface tension and showed increased emulsification indices. Similarly, Bento et al. (2005) reported that a consortium of four *Bacillus* species, which were isolated from diesel-contaminated soil samples, reduced the surface tension of water to 41.4 mN/m in a culture medium containing diesel as the main carbon source.

Another approach employed in the current study for the characterisation of biosurfactant production was the estimation of the emulsification index, which was assayed using three different hydrocarbon-based compounds as substrates. After 24 h, emulsification activity values of up to 90 % were observed, with isolates forming stable emulsions with at least two of the oils utilised as substrates (**Table 2.2**). Thus, the majority of isolates produced biosurfactants with emulsification activity and they have the potential to be utilised for the bioremediation of various inorganic or hydrocarbon compounds within a WWTP. Many studies have also been performed on the isolation of hydrocarbon degrading *Pseudomonas* species. Four *Pseudomonas* isolates, were obtained in the current study, and they showed high emulsification indices when kerosene was used as a substrate. Isolate ST 3 (identified as *Klyuvera*) also showed potential as a biosurfactant-producing organism with high hydrocarbon-degrading ability, due to the high emulsification index with mineral oil and a moderate value of 45.5 % with kerosene obtained.

A wide range of physiological and metabolic factors are required for microorganisms to secrete biosurfactants within the natural ecosystem (van Hamme et al. 2006). The presence of toxic

molecules (e.g. heavy metals) and insoluble compounds, the low pH, and the wide range of microbial species present in a WWTP could lead to the secretion of different biosurfactant compounds that assist these organisms in surviving. In addition, various microorganisms secrete biosurfactants during their growth in water-insoluble substrates and these biosurfactants are found to aid in the motility of microorganisms in hostile environments by their ability to reduce the surface tension between phases, allowing microorganisms to move along an interface more easily (Van Hamme et al. 2006; Soberón-Chávez and Maier 2011; Fakruddin 2012). In the current study, screening for the *bamC, rhlB, sfp* and *ituA* genes that are involved for the biosynthesis of bacillomycin, rhamnolipid, surfactin and iturin biosurfactants, respectively, was performed. These biosurfactants belong to the lipopeptide (bacillomycin, surfactin and iturin) and glycolipid (rhamnolipid) groups and facilitate the motility and biofilm formation on various surfaces as survival strategies and act as antagonists towards microorganisms they co-habitat with (Chrzanowski et al. 2009; Raaijmakers et al. 2010). Previous studies have reported the identification of the *bamC* and *ituA* genes in *Bacillus* species or other closely related Gram-positive bacteria (Hsieh et al. 2004; Tsuge et al. 2005; Ramarathnam et al. 2007; Soberón-Chávez and Maier 2011; Stankovic et al. 2012). In the current study, the *ituA* gene was detected in *Bacillus* spp. (ST 25 and ST34) and *Serratia* sp. (ST 29). In addition, the *sfp* gene, which is involved in the biosynthesis of surfactin and has been reported to display antimicrobial properties against a wide range of microorganisms (Das et al. 2008), was also detected in numerous *Bacillus* spp. and *Serratia* sp. (ST 29). Isolates ST 25 (*Bacillus*) and ST34 (*Bacillus*) were the only isolates found to be carrying the *sfp*, *ituA* and the *bamC* genes involved in biosynthesis of surfactin, iturin and bacillomycin biosurfactants, respectively, which have been reported to display antimicrobial activities against a wide range of microorganisms (Vollenbroich et al. 1997; Bonmatin et al. 2003; Seydlová et al. 2008). These *Bacillus* isolates (ST 25 and ST 34) thus produce a mixture of biosurfactants possibly promoting their survival in the wastewater environment (Kowall et al. 1998; Peypoux et al. 1999; Arguelles-Arias et al. 2009).

Many contaminants such as hydrocarbon-based compounds and carbohydrates, acts as the main carbon source to various microorganisms, but as the wastewater flows from the influent, biological trickling filters through to the effluent, the treatment processes remove these contaminants. Accordingly, the majority of biosurfactant-producing isolates were obtained in the influent samples, as this was the sampling point with no treatment processes, and it received a wide range of contaminants (including some hydrocarbon-based compounds that were not screened for in this study) and microbial populations from various sources. There was a numerical dominance (**Table 2.3**) of biosurfactant producers belonging to the Enterobacteriaceae family (46.9 %), which was expected as most of these microorganisms are naturally found in water, soil and any other man-made environments. The Bacillaceae family was the second dominant (21.9 %) group isolated from the various points of the WWTP. Overall, the highest Simpson's diversity index was

obtained for the influent point (1 - *D* = 0.8356), which implies high bacterial diversity of biosurfactant-producing isolates at this sampling point, while the lowest diversity was recorded for the aeration tank (1 - *D* = 0) samples. The difference in bacterial species diversity obtained in the current study, at the different stages of the WWTP, could however, be associated with the pollutants and the type of treatment employed at each sample collection site.

2.5. Conclusion

Municipal wastewater inhabits a rich microbial flora, with harsh environmental conditions and the presence of various contaminants, including heavy metals, toxic micro-contaminants and hydrocarbon-based compounds. This implies that municipal wastewater can be a source of novel biosurfactant-producing bacteria and in the current study 32 biosurfactant-producing bacteria were obtained from various points of the WWTP, with the majority of the isolates obtained at the influent point. It is possible that other biosurfactant-producing bacteria could have been present in the wastewater samples analysed, but they did not express their biosurfactant-producing genes under the conditions utilised in the current study. Of particular interest was the presence of the *sfp*, *ituA* and the *bamC* genes involved in biosynthesis of surfactin, iturin and bacillomycin biosurfactants, respectively, in two of the *Bacillus* isolates (ST 25 and ST 34) as well as the detection of *sfp* and *ituA* genes in *Serratia* sp. (ST 29). Significantly, the discovery of novel biosurfactant-producing bacterial strains that can easily be cultured under normal fermentation conditions, while producing large quantities of biosurfactants, and finding novel biosurfactants, can overcome the economic barriers for large-scale production of these surface-active molecules. Further research will focus on the detailed structural elucidation of the biosurfactant compounds produced by these isolates and the assessment of their antimicrobial activity, as well as the ability of these isolates to degrade hydrocarbon-based compounds.

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Chapter 3:

(UK spelling is employed)

Repetitive element PCR for the identification of biosurfactant-producing bacteria isolated from a wastewater treatment plant

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Abstract

Thirty-two microbial isolates, classified as biosurfactant-producing bacteria by the drop collapse and oil spreading methods, surface tension and emulsification of certain oils, were isolated from various points of a wastewater treatment plant. The aim of the current study was to identify the biosurfactant-producing isolates to the species level and differentiate between isolates belonging to the same species utilising repetitive element polymerase chain reactions (rep PCRs) [targeting the repetitive extragenic palindromic (REP) and the BOX element sequences]. Conventional PCR identified *Bacillus subtilis* (n = 4) and *Aeromonas hydrophila* (n = 3) as the dominant bacterial species isolated. The rep PCR utilising primers (BOX AIR, REP1R-1 and REP2-1) to amplify the BOX element and REP sequences then successfully discriminated between isolates classified as the same species, by producing unique DNA banding profiles. Based on the results obtained, certain biosurfactant-producing *B. subtilis*, *A. hydrophila* and *Klebsiella pneumoniae* isolates were genotypically differentiated into four, three and two sub-species (strains), respectively. The use of conventional PCR targeting the conserved regions within each genus may thus not be indicative of the genetic diversity amongst bacterial species that are known to produce different concentrations and proportions of various homologs of biomolecules such as biosurfactants and antibiotics.

Keywords: rep PCR; BOX element; REP sequences; Genotypic characterisation; Biosurfactants

3.1. Introduction

Microbial species reported to produce biosurfactant compounds include certain unicellular eukaryotes and various Gram-negative and Gram-positive bacterial genera such as *Acinetobacter*, *Bacillus*, *Burkholderia, Flavobacterium* and *Pseudomonas,* amongst others. These microorganisms are ubiquitous in the environment and they secrete biosurfactant compounds to facilitate in the bioavailability of nutrients, aid in the movement of microorganisms and allow for protection against harsh environmental conditions (Fakruddin, 2012). Numerous biosurfactants have also previously been reported to display antimicrobial properties, which could be of great value in the medical and pharmaceutical industries, as they have been found to damage and lyse cell membranes, which effectively kills various bacterial species as well as fungi, yeasts and certain viruses (Banat et al. 2010). It has also been reported that biosurfactant compounds exist as different congeners, which display different properties (physico-chemical, antimicrobial, emulsification, surface tension) and various strains of a single species of a microorganism could in fact produce different ratios of biosurfactant mixtures under the same growth conditions (Banat et al. 2014). It is thus crucial that the genetic diversity of biosurfactant-producing species be further elucidated as various biosurfactant compounds may be produced by different microbial strains of a particular species (Bodour et al. 2003; Mukherjee & Das, 2005; Swaathy et al. 2014).

With the use of the conventional polymerase chain reaction (PCR), the biosurfactant-producing bacterial genera have been further classified into species based on their genetic diversity (Bodour et al. 2003; Hsieh et al. 2004). However, conventional PCR lacks the ability to differentiate or distinguish between different strains of a particular species or subspecies. Certain molecular fingerprinting techniques such as the pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST) and repetitive element polymerase chain reaction (rep PCR) have thus been developed and utilised for the discrimination between various bacterial species (Versalovic et al. 1994; Klima et al. 2010; Ma et al. 2011; da Silva & Valicente, 2013; Munday et al. 2013; Taylor et al. 2014; García et al. 2015).

The rep PCR technique in particular, has successfully been employed to differentiate between the genomes of several bacterial isolates classified as biosurfactant producers (Bodour et al. 2003; Tran et al. 2008). A study conducted by Bodour et al. (2003) then indicated that an improved discrimination between bacterial isolates was obtained using rep PCR analysis (16 unique isolates) in comparison to 16S rRNA analysis (10 unique isolates). In addition, these microorganisms were reported to produce different biosurfactant mixtures, which displayed varying surface tension values. A study conducted by Tran et al. (2008) then showed that *Pseudomonas* isolates obtained from the rhizosphere of black pepper displayed substantial

genotypic diversity and produced the same biosurfactant compounds. However, different antimicrobial activities of the biosurfactants were observed against *Phytophthora capsici.*

The rep PCR targets extragenic and intergenic repeated DNA sequences distributed throughout the entire genome of an organism, generating various numbers and sizes of DNA fragments that are strain specific (Stern et al. 1984). It has been reported that these repetitive DNA sequences cover up to 1% of the total genome (Stern et al. 1984). In order to differentiate between environmental isolates, numerous primer sets, which include the enterobacterial repetitive intergenic consensus (ERIC) sequences (Batzke et al. 2007), the BOX element Taylor et al. 2014; Ma et al. 2011; da Silva & Valicente, 2013) and repetitive extragenic palindromic (REP) sequences (Castro et al. 2004; Albufera et al. 2009; Taylor et al. 2014; Ma et al. 2011), have been utilised for rep PCR assays to amplify the highly conserved repetitive DNA sequences.

The ERIC sequences are repetitive DNA sequences restricted to transcribed regions of the genome, either in the upstream/downstream region of the open reading frames or within intergenic regions of the polycistronic operons (Hulton et al. 1991). These are imperfect 124-127 base pairs (bp) long palindromes which are highly conserved and have been used to differentiate between enteric bacterial species (Wilson & Sharp 2006; Fendri et al. 2013).

Certain prokaryotes have been reported to carry the highly conserved repeat BOX element, which is mostly situated in the noncoding regions and dispersed throughout the genomes (van Belkum & Hermans, 2001). The BOX element consists of three distinct regions namely boxA, boxB and boxC, which are 59, 45 and 50 bp long, respectively (van Belkum & Hermans, 2001). The presence of multiple copies of the BOX elements then provide useful targets that can be utilised to discriminate between closely related bacterial species (van Belkum & Hermans, 2001; Versalovic et al. 1994).

The REP sequences vary in length from 21 to 65 bp and are detected in the extragenic space of certain bacterial genomes (Tobes & Pareja, 2006), with each bacterial genome carrying more than 100 copies (Martin et al. 1992; Nunvar et al. 2010). In addition, the REP fragments and binding sites share similar characteristics such as palindromic structure, size and are located at multiple sites within extragenic spaces of bacterial genomes. Previous studies have utilised two or more of the REP primer sets with varying degrees of success when differentiating between bacteria analysed (Batzke et al. 2007; Valerio et al. 2009; Ma et al. 2011; da Silva & Valicente, 2013).

Bacterial isolates were previously obtained from a wastewater treatment plant (WWTP) (Stellenbosch, Western Cape, South Africa GPS co-ordinates: -33.943505, 18.824584) (Ndlovu et al. 2016). These isolates were confirmed to be biosurfactant producers by utilising the drop

collapse and oil spreading methods, surface tension measurements and the emulsification of three different oil based compounds (diesel, kerosene and mineral oil). Thirty-two of the bacterial isolates were regarded as biosurfactant-producing isolates, and were identified by 16S rRNA gene analysis as *Aeromonas* sp., *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp., amongst others, with the majority of the isolates classified to the genus level. The aim of the current study was thus to identify and classify the biosurfactant-producing bacterial isolates to species level using conventional PCR and then discriminate between isolates of the same species using rep PCR (BOX AIR and REP primers) analyses.

3.2. Materials and methods

3.2.1. Genomic DNA extraction

The extraction and purification of genomic DNA from each biosurfactant-producing isolate was performed using the boiling method as described by Ndlovu et al. (2015). Where low DNA concentrations were obtained, the ZR Soil Microbe DNA miniPrep™ kit (Zymo Research, USA) was utilised. For the boiling method, pure cultures of the bacterial isolates were grown on Nutrient Agar (NA) at 37°C for 18-24 hours. Thereafter, a single colony was inoculated into Luria Bertani (LB) broth and incubated for 18-24 hours at 37°C. The 18-24 hours bacterial broth culture (1 mL) was then centrifuged at 10 000 rpm for 10 minutes, the supernatant was discarded and the pellet was re-suspended in 100 µL of sterile double distilled water and boiled in a water bath for 15 minutes at 95°C. The suspension was cooled on ice for 10 minutes, followed by centrifugation at 10 000 rpm for 10 minutes and the supernatant containing the DNA was transferred into a sterile 1.5 mL eppendorf tube. Deoxyribonucleic acid samples were then stored at -20°C until analysis using PCR.

3.2.2. Identification of biosurfactant-producing bacteria

Biosurfactant-producing isolates utilised in the current study were previously identified to genus level using 16S rRNA gene analysis (Ndlovu et al. 2016). Conventional PCR, using genus or species specific primers, was then conducted for amplification of the conserved regions within each genus or species, respectively (**Table 3.1**).

Table 3.1 Primer Sequences and PCR cycling conditions

Bacteria	Primer name	Primer sequences (5'-3')	PCR cycling conditions	Gene (size/bp)	Reference
Gordonia	G268F G1096R	CGACCTGAGAGGGTGATCG ATAACCCGCTGGCAATACAG	94ºC for 5 minutes; 30 cycles of 94ºC for 1 minute, 58ºC for 30 seconds and 72ºC for 1 minute; 72ºC for 7 minutes.	16S rDNA (829)	Adopted from Shen and Young (2005)
Bacillus	p-gyrAF p-gyrAR	CAGTCAGGAAATGCGTACGTCCTT CAAGGTAATGCTCCAGGCATTGCT	94ºC for 3 minutes; 30 cycles of 94ºC for 1 minute, 46ºC for 30 seconds and 72ºC for 1 minute; 72ºC for 5 minutes.	*GyrA* (928)	Adapted from Chun and Bae (2000); Rooney et al. (2009)
E. coli	PhoF PhoR	GTGACAAAAGCCCGGACACCATAAATGCCT TACACTGTCATTACGTTGCGGATTTGGCGT	94°C for 2 minutes; 35 cycles of 94°C for 1 minutes, 55°C for 1 minute and 72°C for 1 minute; 72°C for 5 minutes.	*PhoA* (903)	Adapted from Kong et al. (1999)
S. marcescens	FluxS1 RluxS2	GCTGGAACACCTGTTCGC ATGTAGAAACCGGTGCGG	94°C for 5 minutes; 45 cycles of 94°C for 45 seconds, 58°C for 30 seconds and 72°C for 15 seconds; 72°C for 10 minutes.	*lux* (102)	Adapted from (Zhu et al. (2008)
Aeromonas spp	Aero-F Aero-R	TGTCGGSGATGACATGGAYGTG CCAGTTCCAGTCCCACCACTTCA	94°C for 2 minutes; 35 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 2.5 minutes; 72°C for 5 minutes	Aerolysin (720)	Adopted from Kong et al. (2002)
Pseudomonas spp.	PA-GS-F PA-GS-R	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	95°C for 2 minutes; 25 cycles of 94°C for 20 seconds, 54°C for 20 seconds and 72°C for 40 seconds; 72°C for 5 minutes	16S rRNA (618)	Adapted from Spilker et al. (2004)
Klebsiella spp.	gryA-F gyrA-C	CGCGTACTATACGCCATGAACGTA ACCGTTGATCACTTCGGTCAGG	95°Cfor 3 minutes; 35 cycles of 94°C for 1 minute, 50°C for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes	Gyrase A gene (383)	Adopted from Brisse and Verhoef (2001)

The reaction volumes consisted of 1× PCR Green GoTaq Flexi buffer, 1.5 U GoTaq® G2 DNA polymerase, 0.1 mM of the deoxynucleoside triphosphate (dNTP) mix (for *Klebsiella, Pseudomonas, Aeromonas*), 0.2 mM dNTP mix (for *Bacillus, Gordonia, Enterobacteriaceae, Escherichia, Serratia, Shewanella*), 2 mM $MgCl_2$ (for *Aeromonas, Bacillus, Gordonia, Enterobacteriaceae, Klebsiella, Pseudomonas, Shewanella)*, 1.5 mM $MgCl_2$ (for *Escherichia, Serratia*), 0.2 µM primers (PhoF, PhoR), 0.3 µM primers (PF1, PR1), 0.4 µM PCR primers (G268F, G1096R, She211f and She1259), 0.5 µM PCR primers (En-lsu-3-F, En-lsu-3-R, PA-GS-F, PA-GS-R, FluxS1, FluxS2), 0.8 µM PCR primers (p-gyrAF, p-gyrAR), template DNA volumes used were 2 µL (*Bacillus, Gordonia, Escherichia, Shewanella*) and 5 µL (*Aeromonas, Enterobacteriaceae, Klebsiella, Pseudomonas, Serratia,*). All reaction mixtures were made up to a final volume of 50 µL with sterile nuclease free water.

For each reaction, a negative control consisting of sterile nuclease free water and a positive DNA control were included. The bacterial strains used as positive controls were *Escherichia coli* ATCC 437371, *Pseudomonas aeruginosa* PAO1, *Bacillus subtilis* ATCC 6051, *Klebsiella pneumoniae* ATCC 10031 and *Serratia marcescens* ATCC 14756. Amplification was performed using the T100™ thermal cycler (Biorad Laboratories, Netherlands), with the PCR programs outlined in **Table 3.1**. The PCR products were visualised on a 1.5% and 2% agarose gel stained with ethidium bromide (0.5 µg/mL). The amplified PCR products were purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) as per manufacturer's instructions and were sequenced in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems, USA) at the Central Analytical Facility (CAF), Stellenbosch University (Stellenbosch, South Africa). The obtained sequences were analysed using the online Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, to find the closest match of local similarity of the isolates to the online international database in GenBank, EMBL, DDBJ and PDB sequence data (Altschul et al. 1997).

3.2.3. Rep PCR analysis of biosurfactant-producing isolates

The DNA fingerprints of the biosurfactant-producing isolates were determined using the rep PCR analysis as previously described by Versalovic et al. (1994) and Bodour et al. (2003) using the primers REP1R-1 (5' IIIICGICGICATCIGGC 3') and REP2-1 (5' ICGICTTATCIGGCCTAC 3') targeting the REP sequences. The reaction mixture contained 1× Green GoTaq® reaction buffer (Promega, USA), 0.5 µM of each primer, 0.4 mM dNTP mix (Thermo Scientific, Lithuania), 3 mM $MgCl_2$, 2 U of GoTaq® G2 DNA Polymerase (Promega, USA), 2 µL of template DNA. All reaction mixtures were made up to a final volume of 50 µL using sterile nuclease free water. Amplification was performed using the T100™ thermal cycler and the PCR cycling parameters consisted of 41 cycles consisting of 94°C for 1 minute, 47°C for 1 minute and 72°C for 2 minutes.

The rep PCR analysis was also performed using the BOX AIR (5′-CTACGGCAAGGCGACGCTGACG-3′) primer targeting the BOX element as previously conducted by Ma et al. (2011), with slight modifications. The reaction mixture consisted of 1× Green GoTaq® reaction buffer, 1.25 U of GoTaq® G2 DNA Polymerase, 0.2 mM dNTP mix, 3 mM $MgCl_2$, 5% dimethyl sulfoxide (DMSO), 2 µM of the primer, 2 µL template DNA. The final reaction volume was adjusted to 50 µL with sterile nuclease free water. Amplification was performed using the T100™ thermal cycler and the PCR cycling parameters were composed of 30 cycles consisting of 94°C for 30 seconds, 92°C for 30 seconds, 50°C for 1 minute and 65°C for 8 minutes.

The rep PCR DNA fingerprints obtained by the BOX AIR, REP1R-1 and REP2-1 primers were analysed on a 2% agarose gel (20 cm long) stained with 0.5 µg/mL ethidium bromide; and was run for 3.5 hours at 90 V. The DNA bands were visualised through a UV illumination and images were captured with the MiniBIS Pro (Bio-Imaging Systems). Computer assisted analysis of the produced DNA fingerprints was performed using the AzureSpot software version 13.2 (Azure Biosystems, USA). Molecular sizes of the DNA bands were compared to a 1 kb plus DNA ladder (ThermoFischer Scientific, USA) using the AzureSpot software, which also generated the retardation factor (Rf) values of each DNA band for each bacterial isolate. The Rf value is defined as the measurement of the DNA band position along a lane, and is calculated by measuring the distance travelled by the DNA band divided by the distance of the lane (Tourlomousis et al. 2010). The Rf values range from 0 (the top of the lane) to 1 (the bottom of the lane).

3.3. Results

3.3.1. Identification of the biosurfactant-producing isolates

All biosurfactant-producing bacterial isolates were identified to species level using genus or species specific primer sets (**Table 3.2**). The *Bacillus* isolates (preliminary identification by the 16S rRNA analysis; Ndlovu et al. 2016) were identified as *B. subtilis* (Isolates: ST11, ST24, ST32 and ST33), *B. amyloliquefaciens* (Isolates: ST25 and ST34), while isolate ST18 was identified as *B. cereus*. The *Pseudomonas* isolates (preliminary identification by the 16S rRNA analysis; Ndlovu et al. 2016) were then classified as *P. protogens* (Isolates: ST6 and ST19), *P. lundesis* (Isolate: ST 4) and *P. aeruginosa* (Isolate: ST5) using PCR targeting the conserved 16S rRNA region within the *Pseudomonas* genus. The *Aeromonas* isolates were identified as *A. media* (ST2), *A. cavae* (Isolate: ST27), *and A. hydrophila* (Isolates: ST9, ST14 and ST28). Other isolates identified to the species level included ST13 and 29, which were both identified as *S. marcescens*, ST26 which was identified as *Escherichia coli* and ST15 and ST23, which were both identified as *K. pneumoniae*.

Table 3.2 Molecular identification of biosurfactant-producing microbial isolates

Isolate	Genebank accession no.	% ID*
ST1	*Shewanella putrefaciens* (KC607511.1)	97
ST2	*Aeromonas media* WS (CP007567)	99
ST3	*Enterobacter asburiae* (CP012162.1)	98
ST4	*Pseudomonas lundesis* (EU140959.1)	99
ST5	*Pseudomonas aeruginosa* (KR911837.1)	100
ST6	*Pseudomonas protegens* (KJ742553)	99
ST7	*Providencia stuartti* (CP008920.1)	99
ST8	*Alkalimonas collagenimarina* (KJ841884)	99
ST9	*Aeromonas hydrophila* (CP006579.1)	91
ST11	*Bacillus subtilis* (JQ715854.1)	99
ST12	*Citrobacter freundii* (CP0116571.1)	98
ST13	*Serratia marcescens* (CP013046)	98
ST14	*Aeromonas hydrophila* (CP006870.1)	98
ST15	*Klebsiella pneumoniae* (AY301158.1)	98
ST17	*Kluyvera georgiana* (NR_024883)	93
ST18	*Bacillus cereus* (KP940382)	99
ST19	*Pseudomonas protegens* (KJ742553)	99
ST20	*Enterobacter cloacae* (CP012162.1)	99
ST21	*Klebsiella oxytoca* (CP011636.1)	99
ST22	*Proteus mirabilis (*AM942759.1)	97
ST23	*Klebsiella pneumoniae* (CP011624.1)	99
ST24	*Bacillus subtilis* (CP009749)	98
ST25	*Bacillus amyloliquefaciens* (KJ833588.1)	96
ST26	*Escherichia coli* (CP011938.1)	96
ST27	*Aeromonas caviae* (LN624814)	96
ST28	*Aeromonas hydrophila* (JX512374)	98
ST29	*Serratia marcescens* (CP011642.1)	98
ST30	*Kluyvera cryocrescens* (AM933754)	91
ST31	*Gordonia alkaliphila* (NR 109437.1)	98
ST32	*Bacillus subtilis* (CP011882.1)	99
ST33	*Bacillus subtilis* (CP011882.1)	99
ST34	*Bacillus amyloliquefaciens* (KJ833588.1)	99

3.3.2. Rep PCR typing and variability of the isolates

The DNA fingerprints of the 32 biosurfactant-producing bacterial species were generated using the REP1R-1 and REP2-1 primers to amplify the REP sequences, while the BOX AIR primer was utilised to amplify the repeat BOX element sequences. The Rf values for each DNA band were generated and selected Rf values of bacterial isolates that consisted of two or more species are presented in **Table 3.3** (REP primer set) and **Table 3.4** (BOX AIR primer). The agarose gel images of representative bacterial isolates analysed by rep PCR are presented in **Figure 3.1** (REP1R-1 and REP2-1 primer set) and **Figure 3.2** (BOX AIR primer).

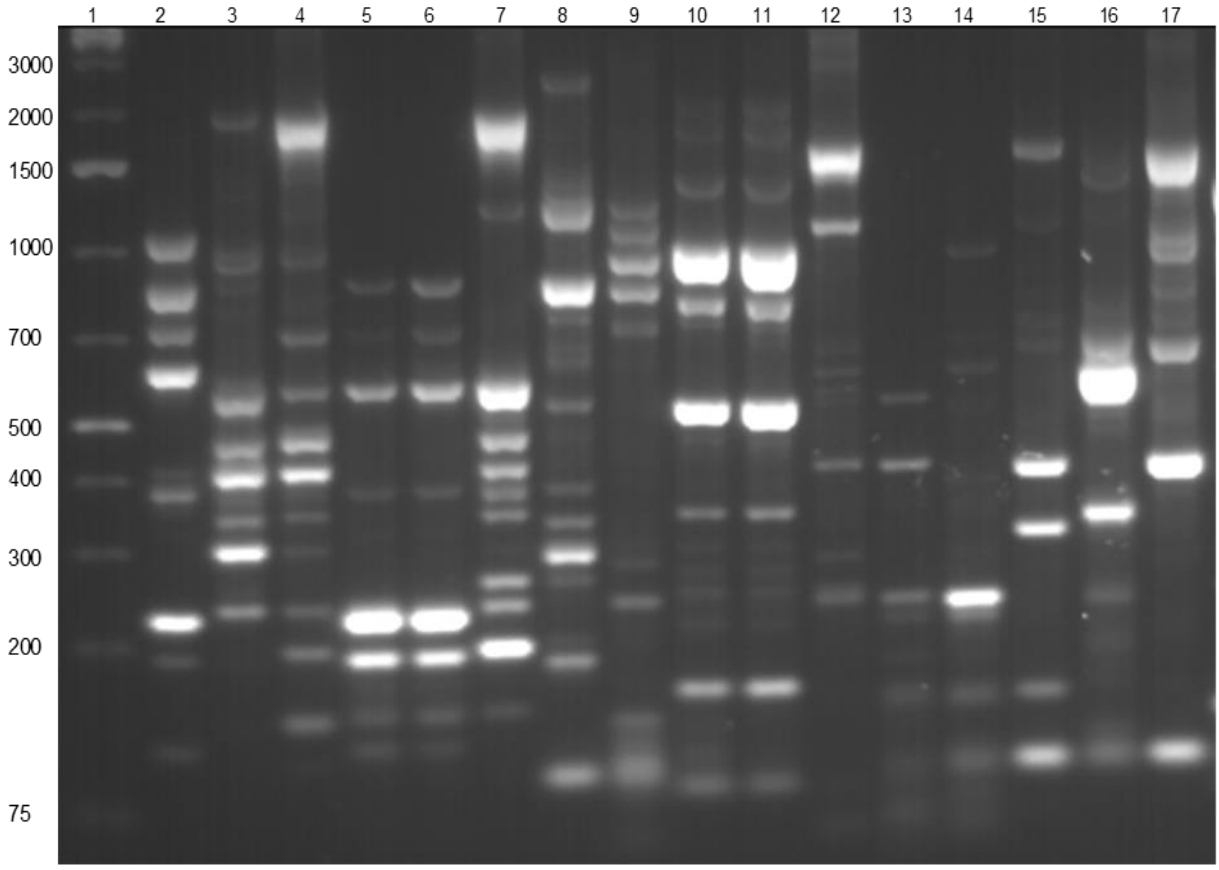

Figure 3.1 Illustration of the Rep PCR (REP1R-1 and REP2-1 primers) results of representative biosurfactant-producing isolates. Lane 1: Molecular Weight Marker (Generuler™ 1 kb plus DNA ladder, Fermentas); lane 2: *B. subtilis* (ST11); lane 3: *B. subtilis* (ST24); lane 4: *B. amyloliquefaciens* (ST25); lane 5: *B. subtilis* (ST32); lane 6: *B. subtilis* (ST33); lane 7: *B. amyloliquefaciens* (ST34); lane 8: *P. lundesis* (ST4); lane 9: *P. aeruginosa* (ST5); lane 10: *P. protegens* (ST6); lane 11: *P. protegens* (ST19); lane 12: *Shewanella putrefaciens* (ST1);lane 13: *Aeromonas media* (ST2); lane 14: *A. hydrophila* (ST9); lane 15: *A. hydrophila* (ST14); lane 16: *Aeromonas caviae* (ST27); lane 17: *A. hydrophila* (ST28)

3.3.3. Repetitive extragenic palindromic sequence based PCR analysis

A total of 25 DNA bands representing different Rf values were detected for the different bacterial species analysed using the REP1R-1 and REP2-1 primer set. While four of the isolates, identified

as *B. subtilis* (ST11, ST24, ST32 and ST33) by conventional PCR (gel image in **Figure 3.1**), produced Rf values ranging from 0.003 to 0.578 (**Table 3.3**), isolates ST32 and ST33 displayed similar DNA banding profiles, with band 10 absent in ST32. However, while isolates ST11 and ST24 shared common DNA bands 14, 17 and 20, overall they displayed different DNA banding profiles from each other as well as isolate ST32 and ST33 (**Table 3.3**).

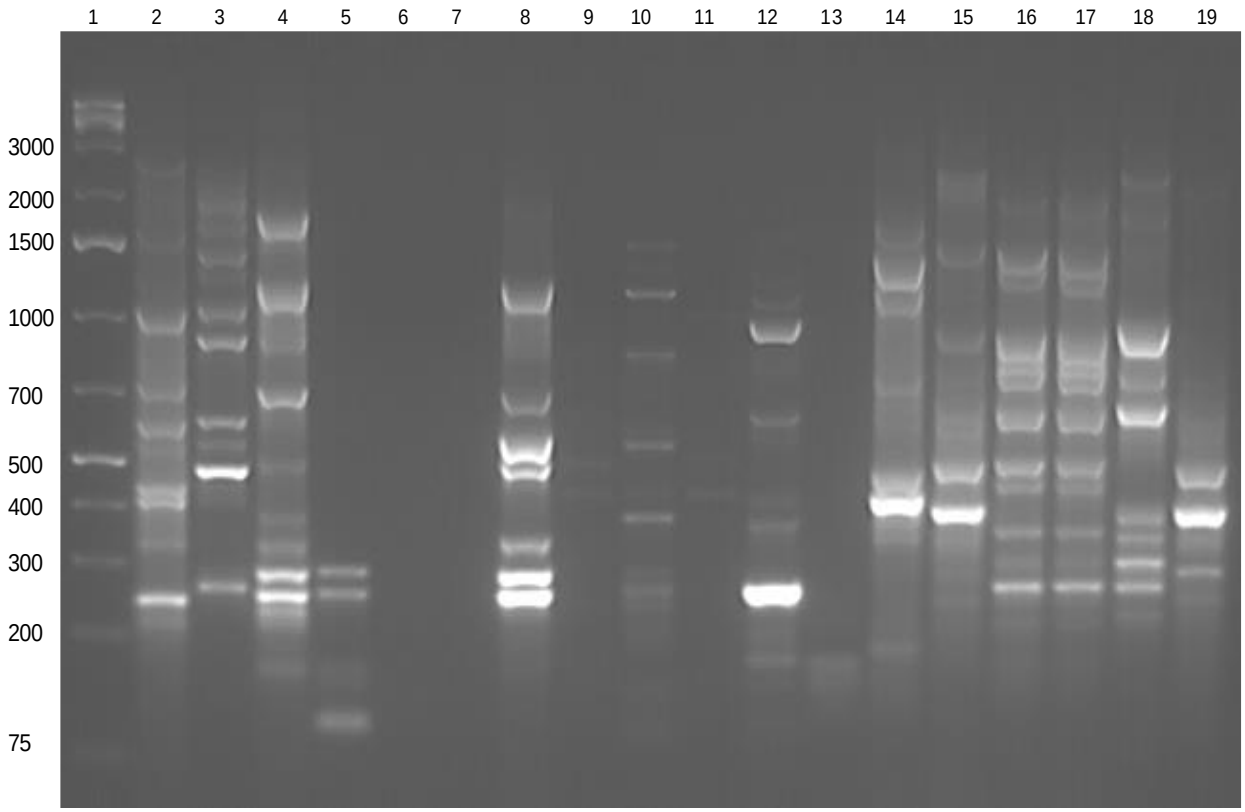

Figure 3.2 Illustration of the Rep PCR (BOX AIR primer) results of representative biosurfactant-producing isolates. Lane 1: Molecular Weight Marker (GenerulerTM 1 kb plus DNA ladder, Fermentas); lane 2: *Serratia marcescens* (ST29); lane 3: *B. subtilis* (ST11); lane 4: *B. subtilis* (ST24); lane 5: *B. amyloliquefaciens* (ST25); lane 6: *B. subtilis* (ST32); lane 7: *B. subtilis* (ST33); lane 8: *B. amyloliquefaciens* (ST34); lane 9: *Aeromonas media* (ST2); lane 10: *Aeromonas hydrophila* (ST9); lane 11: *A. hydrophila* (ST14); lane 12: *Aeromonas caviae* (ST27); lane 13: *A. hydrophila* (ST28); lane 14: *P. lundesis* (ST4); lane 15: *P. aeruginosa* (ST5); lane 16: *P. protegens* (ST6); lane 17: *P. protegens* (ST19)

Isolates ST24, ST32 and ST33 did however, share common DNA bands 9 and 13, which were not observed in isolate ST 11. Two isolates identified as *B. amyloliquefaciens* (ST25 and ST34) produced 12 DNA bands each. Similar DNA banding profiles were produced by these isolates, however, ST34 did not produce DNA bands 8, 10 and 18, while ST25 did not produce DNA bands 6, 16 and 19. The *P. protogens* (ST6 and ST19) isolates also displayed similar DNA banding patterns and Rf values that ranged from of 0.088 to 0.607.

Table 3.3 A representation of the rep PCR (REP primer set) assay performed for biosurfactant-producing isolates obtained from a wastewater treatment plant. The cells with numbers indicate the Rf values for each DNA band that correspond to the reference DNA band number in the first column.

Reference DNA Band	*B. subtilis*				*B. amyloliquefaciens*		*P. protogens*		*P. lundensis*	*P. aeruginosa*	*A. hydrophila*			*A. media*	*A. caviae*
	ST 11	ST 24	ST 32	ST 33	ST 25	ST 34	ST 6	ST 19	ST 4	ST 5	ST 9	ST 14	ST 28	ST 2	ST 27
	Lane 2	Lane 3	Lane 5	Lane 6	Lane 4	Lane 7	Lane 10	Lane 11	Lane 8	Lane 9	Lane 14	Lane 15	Lane 17	Lane 13	Lane 16
1					0.003	0.004									
2													0.014		
3															
4		0.073							0.045						
5					0.084	0.084		0.088				0.1			
6						0.146	0.13	0.134	0.153	0.148			0.117		0.125
7										0.167			0.174		
8		0.189			0.183		0.185	0.188		0.19	0.18				0.185
9		0.206	0.205	0.207					0.212	0.213			0.215		
10				0.245	0.247		0.223	0.226	0.232	0.24					
11									0.266			0.256	0.264		
12											0.274				
13		0.301	0.291	0.291	0.291	0.291	0.306	0.308	0.302				0.313	0.297	0.284
14	0.321	0.338			0.332	0.331	0.329								
15		0.36			0.356	0.354					0.361	0.353	0.351	0.351	0.358
16	0.377		0.371	0.37		0.372			0.368						
17	0.407	0.393			0.389	0.389	0.389	0.39	0.395			0.403			0.39
18		0.418			0.417		0.416		0.423	0.428					
19						0.443	0.436	0.436	0.442						
20	0.465	0.465			0.466	0.462	0.453	0.452		0.46	0.458			0.458	0.458
21			0.475	0.472			0.478							0.472	0.474
22	0.498		0.505	0.504	0.499	0.497	0.53	0.53	0.507		0.536	0.532		0.534	
23	0.544		0.551	0.551	0.556	0.547				0.556					
24			0.578	0.577							0.589	0.586	0.583		0.584
25							0.607	0.606	0.599	0.595				0.629	
~Total	6	9	7	8	12	12	12	10	12	9	6	6	8	6	8
Total (%)	24	36	28	32	48	48	48	40	48	36	24	24	32	24	32

~Total – Total number of DNA bands

Isolates ST6 and ST19 generated 12 and 10 DNA bands, respectively (**Table 3.3**), with band 5 not generated by ST6, while bands 14, 18 and 21 were not generated by ST19. As expected, the rep PCR results using the REP primers for bacterial isolate ST5 (*Pseudomonas aeruginosa*) and ST4 (*Pseudomonas lundensis*) revealed different DNA banding patterns and Rf values from each other. Similarly, isolates ST2 (*A. media*) and ST27 (*A. caviae*) produced different DNA banding patterns to each other as well as to the *A. hydrophila* isolates (ST9, ST14 and ST28). While the three biosurfactant-producing isolates identified as *A. hydrophila* (ST9, ST14 and ST28), also showed variability in the DNA bands produced by the REP primers, similar DNA bands were produced at positions 15 and 24. In addition, the *K. pneumoniae* isolates produced 12 (ST23) and 14 (ST15) DNA bands, with eight common DNA bands (6, 10, 11, 13, 17, 18, 21 and 23) obtained, while the rest of the bands varied from each other (results not shown).

No DNA bands were generated for the isolates identified as *S. marcescens* (ST13 and ST29) using the REP primers, while *Enterobacter asburiae* isolates ST3 and ST20 generated 7 and 14 DNA bands, respectively, with common bands 12, 18, 19, 20 and 24 generated (results not shown). The remainder of the isolates including ST 1 (*Shewanella putrefaciens*), ST 2 (A. media), ST7 (*Providencia stuartti*), ST8 (*Alkalimonas collagenimarina*), ST26 (*Escherichia coli*), amongst others, also displayed variability in the DNA banding pattern revealed by different Rf values obtained (results not shown).

3.3.4. BOX element sequence based PCR analysis

The rep PCR results obtained using the BOX AIR primer for the 32 biosurfactant-producing bacterial isolates yielded a total of 16 unique DNA bands with Rf values ranging from 0.205 to 0.643 (**Table 3.4**). For the analysis of the *B. subtilis* isolates, no DNA bands were visible on the agarose gel (**Figure 3.2**) for isolates ST32 and ST33, hence no Rf values were obtained after analysis of the generated image using the Azurespot software. However, the *B. subtilis* isolates ST11 and ST24 generated common DNA bands 2, 5, 6, 11 and 15 with similar Rf values, while the rest of the DNA bands had different Rf values as indicated in **Table 3.4**. The B. amyloliquefaciens isolates ST25 and ST34 produced 3 and 7 bands, respectively, with only two common DNA bands 14 (Rf: 0.521; 0.524) and 15 (Rf: 0.539; 0.541) produced.

Table 3.4 represents the rep PCR results obtained using the BOX AIR primer, with the *A. hydrophila* species (ST9, ST14 and ST28) displaying variability in the DNA banding patterns as observed by the different Rf values. However, ST9 and ST14 produced a similar DNA band 12 with Rf values of 0.478 and 0.457, respectively. In addition, the Rf values for the *P. protogens* isolates (ST6 and ST19) ranged from 0.275 to 0.563.

Table 3.4 A representation of the rep PCR (BOX AIR primer) assay performed for biosurfactant-producing isolates obtained from a wastewater treatment plant. The cells with numbers indicate the Rf values for each DNA band that correspond to the reference DNA band number in the first column.

Reference DNA Band	*S. marcescens*	*B. subtilis*		*B. amyloliquefaciens*		*P. protogens*		*P. lundensis*	*P. aeruginosa*	*A. hydrophila*		
	ST 29	ST 11	ST 24	ST 25	ST 34	ST 6	ST 19	ST 4	ST 5	ST 9	ST 14	ST 28
	Lane 2	Lane 3	Lane 4	Lane 5	Lane 8	Lane 16	Lane 17	Lane 14	Lane 15	Lane 10	Lane 11	Lane 13
1		0.223							0.205			
2		0.239	0.243					0.255		0.255		
3		0.266				0.275	0.279		0.268			
4						0.285	0.291	0.287		0.294		
5		0.311	0.303		0.305			0.305				
6	0.321	0.334	0.336			0.346	0.351		0.336	0.343		
7						0.357	0.359					
8	0.377		0.383		0.388	0.369	0.372	0.375				
9	0.407	0.399				0.4	0.402		0.398			
10		0.418			0.423					0.417		
11		0.438	0.435		0.44	0.437	0.439		0.443			
12	0.465		0.478			0.452	0.453	0.465	0.472	0.478	0.457	
13	0.498		0.502		0.501	0.489	0.489					
14			0.524	0.521	0.525	0.514						
15	0.544	0.534	0.541	0.539	0.541	0.533	0.533		0.545	0.536		
16			0.6	0.643		0.563		0.583				0.597
~Total	6	9	10	3	7	12	10	6	7	6	1	1
Total (%)	37.5	56.3	62.5	18.8	43.8	75	62.5	37.5	43.8	37.5	6.3	6.3

~Total – Total number of DNA bands

The DNA bands 18 (Rf: 0.514) and 20 (Rf: 0.563) were not produced in isolate ST19, while the other DNA bands were similar to those of ST6. The *S. marcescens* isolate ST29 generated 6 DNA bands (**Table 3.4**) and no DNA bands were generated for isolate ST13, while the *E. asburiae* isolate ST3 and ST20 generated 9 and 7 DNA bands, respectively, with common bands 14, 15 and 17 produced (results not shown). The remainder of the isolates that belonged to different species produced DNA bands unique from each other for the rep PCR results obtained using the BOX AIR primer.

3.4. Discussion

In the current study, conventional PCR was utilised to identify 32 biosurfactant-producing isolates to species level. *Bacillus subtilis* was the most dominant species (12.5%) isolated, followed by *A. hydrophila* (9.4%), as well as *B. amyloliquefaciens, E. asburiae*, *S. marcescens*, *P. protogens*, *K. pneumoniae*, which each contributed 6.3% (n = 2) of the total isolates obtained (**Table 3.2**). The remainder of the isolates were identified as single species. Overall, the results obtained using genus and species specific primers were in agreement with the 16S rRNA gene sequence results obtained in a previous study (Ndlovu et al. 2016), with the exception of isolates ST21 and ST3, which were previously identified as *Raoultella* and *Klyuvera* sp., however in the current study these isolates were identified as *Klebsiella oxytoca* and *Enterobacter asburiae*, respectively. The DNA sequences of *Klebsiella* and *Raoutella* are very similar and therefore misclassification of *Klebsiella oxytoca* using the 16S rRNA gene has been previously reported (de Jong et al. 2013).

The use of conventional PCR is a more advanced method that is employed by many researchers to identify numerous bacteria, including biosurfactant-producing bacteria isolated from diverse environments (Jang et al. 2013; Kim, 2014; Ben Belgacem et al. 2015; Sharma et al. 2015). It is however, important to differentiate or discriminate between biosurfactant-producing bacterial species as it has been reported that microbial isolates belonging to the same species, but different strains, may synthesise different concentrations and congeners of surface active compounds, which include biosurfactant compounds (Swaathy et al. 2014). In the current study, all the isolates were then subjected to rep PCR using the BOX AIR and REP primers to discriminate between isolates belonging to the same species. Visual comparison of the DNA banding profiles (**Figure 3.1** and **Figure 3.2**) as well as the Rf values of the DNA bands produced (**Tables 3.3** and **3.4**), showed that the patterns of isolates belonging to the same genera had a limited degree of similarity with both rep PCRs. Overall, however the REP primer sets yielded more DNA bands when compared to the BOX AIR primer, which is an indication of a higher discriminatory power. It should also be noted that the REP primers produced complex DNA banding patterns for all the bacterial isolates analysed with the exception of *S. marcescens* isolates ST13 and ST29, where no DNA bands were obtained. In contrast, however, the BOX AIR primer yielded 6 DNA bands for isolate ST29 and no DNA bands were produced for isolate ST13 even after repeating the rep

PCR analysis. Comparatively, while similar banding profiles were obtained for isolates ST32 and ST33 (*B. subtilis*) using the REP primers sets, no DNA bands were produced for these isolates using the BOX AIR primer, while the other *B. subtilis* isolates (ST11 and ST24) yielded different DNA banding patterns from each other using both BOX AIR and REP primers. The rep PCR results for *B. subtilis* and *S. marcescens* thus indicated that these biosurfactant-producing isolates possibly belonged to different strains or sub-species and highlights the discriminatory power of rep PCR (BOX AIR and REP primers) for the typing of isolates. The results of the current study are also in agreement with a previous study conducted by Versalovic et al. (1991), were they successfully differentiated between strains of *B. subtilis* using rep PCR with primers targeting the REP sequences.

For the discrimination of *A. hydrophila* (ST9, ST14, ST28) and *K. pneumoniae* (ST15, ST23) isolates, the BOX AIR and REP primers utilised for rep PCR produced different DNA banding profiles within the same species, implying that the repetitive units are situated at different locations within the genome, therefore these isolates are genetically different. The *P. protogens* isolates ST6 and ST19 produced similar DNA banding patterns using the REP primers, and this was also observed when the BOX AIR primer was used. However, the BOX AIR primer yielded more DNA bands for the *P. protogens* isolates when compared to the REP primer set, while for the *B. subtilis* isolates, an increased number of DNA bands were produced by the REP primers, which has previously been proven as a more discriminative tool to distinguish between closely related *Bacillus* species (Taylor et al. 2014). Results obtained in the current study thus highlights that the *B. subtilis* genomes carry more of the REP units than the BOX element units, while the *P. protogens* carries more BOX element units. As expected the isolates that were identified as different species displayed unique DNA banding profiles using both rep PCR assays (Albufera et al. 2009; Brisse & Verhoef, 2001; da Silva & Valicente, 2013; Taylor et al. 2014). Different DNA banding profiles produced by the rep PCR for the same species in this study, indicated that the repeat DNA sequences within each species are located at different positions within the genome as previously reported by Tobes and Pareja (2006). The origin and function of repetitive sequences (REP and BOX elements) have been found to be located in close proximity to genes proposed to be binding sites for DNA polymerases, DNA gyrase, mRNA stabilisers and gene-expression and to act as integration host factors, suggesting that these sequences could be involved in controlling gene expression (van Belkum et al. 1998).

The naturally produced biosurfactants are composed of different biosurfactant congeners and homologues as observed in various strains of *Bacillus* and *Pseudomonas* species (Bodour et al. 2003; Mukherjee & Das, 2005; Trans et al. 2008). In addition, the mixture of congeners may display different physico-chemical properties in combination, which can be very different from the physico-chemical properties observed in individual congeners. The *A. hydrophila* isolates ST14 and ST28 displayed different DNA fingerprints using both rep PCR primer sets. In addition, their

cell free supernatants reduced the surface tension of water (72 mN/m) to 57.8 and 34.4 mN/m, respectively, which could indicate that ST14 produces less active biosurfactant compounds, while ST28 may produce highly active biosurfactant compounds. In a study conducted by Bodour et al. (2003), two *B. subtilis* isolates HAZ2 and GA1-2, differed by only one base in their 16S rRNA gene, however, they displayed different DNA fingerprints after analysis by rep PCR (employing the REP primer set). The surface tension of the biosurfactants produced by isolates HAZ2 and GA1-2 were also significantly different.

In the current study conventional PCR grouped the thirty-two biosurfactant-producing isolates (Ndlovu et al. 2016) into twenty-two species. However, the *B. subtilis*, *A. hydrophila*, *B. amyloliquefaciens and P. protogens* isolates, amongst others, were then further sub-divided into four, three and two isolates (*B. amyloliquefaciens and P. protogens*), respectively, by the rep PCR, possibly indicating that each of these isolates were a unique sub-species/strain, with a possibility of producing different mixtures of biosurfactant congeners/isoforms. The rep PCR has previously been employed by Bodour et al. (2003) and results indicated that the *Pseudomonas* isolates *P. aeruginosa* ATCC 9027 and *P. aeruginosa* IGB83 produced different DNA fingerprints and while both produced rhamnolipids, the chemical structures of these compounds were distinct. *Pseudomonas aeruginosa* ATCC 9027 produced only monorhamnolipids, while *P. aeruginosa* IGB83 produced a mixture of mono- and dirhamnolipid, which display different physico-chemical properties. Another study conducted by Mukherjee and Das (2005) reported on the production of chemically different surfactin compounds produced by *B. subtilis* strains (DM-03 and DM-04), which also displayed different degrees of activity against test microbes.

3.5. Conclusion

The current study highlights the comparative evaluation of the rep PCR (employing the BOX AIR and REP primers) in combination with genus or species specific PCR, to characterise and elucidate the diversity of biosurfactant-producing bacterial species. Overall, the REP primers (amplifying the REP DNA sequences) generated more DNA bands when compared to the BOX AIR primer (amplifying the repetitive BOX element sequences). A significant genetic diversity was identified among *B. subtilis* isolates, using the REP primers, as more DNA bands were generated, while the *P. protogens* and *S. marcescens* strains, were both better discriminated by the BOX AIR primer. The BOX AIR and REP primers utilised for rep PCR in the current study thus provided a powerful tool to discriminate between biosurfactant-producing bacterial isolates identified as the same species. Further research will focus on the detailed structural elucidation of the biosurfactant compounds produced by these isolates and the assessment of their antimicrobial activity, as well as the ability of these isolates to degrade hydrocarbon based compounds.

Authors' contributions:

TN and WK conceived and designed the experiments. TN performed the experiments and analysed data. WK acquired funding for the study. WK and SK contributed reagents/materials/analysis tools. TN and WK wrote the paper. All authors edited the drafts of the manuscript and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4:

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Characterisation and antimicrobial activity of biosurfactant extracts produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* isolated from a wastewater treatment plant

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Abstract

Biosurfactants are unique secondary metabolites, synthesised non-ribosomally by certain bacteria, fungi and yeast and exhibit applications as antimicrobial agents in the medical and food industries. Naturally produced glycolipids and lipopeptides are found as a mixture of congeners, which increases their antimicrobial potency. Sensitive technologies, such as liquid chromatography coupled to mass spectrometry, enable the fingerprinting of different biosurfactant congeners within a naturally produced crude extract. *Bacillus amyloliquefaciens* ST34 and *Pseudomonas aeruginosa* ST5, isolated from wastewater, were screened for biosurfactant production. The biosurfactant compounds were solvent extracted and characterised using ultra-performance liquid chromatography (UPLC) coupled to electrospray ionisation mass spectrometry (ESI-MS). Results indicated that *B. amyloliquefaciens* ST34 produced C_{13}, C_{14}, C_{15} and C_{16} surfactin analogues and their identity were confirmed by high resolution ESI-MS and UPLC-MS. In the crude extract obtained from *P. aeruginosa* ST5, high resolution ESI-MS linked to UPLC-MS confirmed the presence of di- and monorhamnolipid congeners, specifically Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10}, Rha-Rha-C_8-C_{10}/Rha-Rha-C_{10}-C_8 and Rha-C_8-C_{10}/Rha-C_{10}-C_8, as well as Rha-Rha-C_{12}-C_{10}/Rha-Rha-C_{10}-C_{12} and Rha-C_{12}-C_{10}/Rha-C_{10}-C_{12}. The surfactin and rhamnolipid extracts also retained pronounced antimicrobial activity against a panel of pathogenic microorganisms, including antibiotic resistant *Staphylococcus aureus* and *Escherichia coli* strains and the pathogenic yeast *Candida albicans*. The rapid solvent extraction combined with UPLC-MS of the crude samples is a simple and powerful technique to provide fast, sensitive and highly specific data on the characterisation of the biosurfactant compounds. In addition, the crude surfactin and rhamnolipid extracts produced by *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5, respectively, retained significant antimicrobial activity against a broad spectrum of opportunistic and pathogenic microorganisms, including antibiotic resistant strains.

Keywords: *Bacillus amyloliquefaciens* ST34; *Pseudomonas aeruginosa* ST5; surfactin; rhamnolipid; UPLC-MS, ESI-MS

4.1. Introduction

Biosurfactants are secondary metabolites that are non-ribosomally synthesised by actively growing and/or resting microbial cells (bacteria, fungi and yeast) (Van Delden and Iglewski 1998; Ron and Rosenberg 2001; Mulligan 2005). They have been classified into different groups based on their chemical composition and microbial origin and they are divided into five major classes which include glycolipids, lipopeptides, phospholipids, polymeric compounds and neutral lipids (Ron and Rosenberg 2001; Sen 2010). While they have been extensively applied in bioremediation, industrial emulsification and enhanced oil recovery (Banat et al. 2014), certain biosurfactant compounds have also been reported to display multipurpose biomedical and therapeutic properties, which include applications as antiadhesives, anticarcinogens and antimicrobials (Benincasa et al. 2004; Mulligan 2005; Rodrigues et al. 2006; Mulligan et al. 2014).

Glycolipids and lipopeptides constitute the most widely studied groups of biosurfactant compounds displaying broad spectrum antimicrobial activity and are currently applied in several fields (cosmetic, food and pharmaceutical industries) as antimicrobial, emulsifying and surfactant agents (Mandal et al. 2013). The glycolipid based biosurfactants include mannosylerythritol lipids, sophorolipids, trehalolipids and the most dominant group rhamnolipids, that are primarily produced by *Pseudomonas* species, particularly *Pseudomonas aeruginosa* strains. Rhamnolipids consist of one or two rhamnose residues in their hydrophilic moiety linked to one, two or three hydroxyl fatty acid chains of varying lengths (eight to 22 carbons) (Déziel et al. 1999; Gunther et al. 2005).

The lipopeptides generally contain similar peptide chains (short linear or cyclic structures). The hydrophilic moiety is composed of amino acid residues varying only at specific residues and is linked to varying lengths (saturated and unsaturated) of fatty acids that act as the hydrophobic moiety (Makovitzki et al. 2006; Raaijmakers et al. 2010; Yao et al. 2012; Mandal et al. 2013). Lipopeptides are widely produced by *Bacillus* species and they consist of bacillomycins, fengycins, iturins, mycosubtilins as well as the widely studied lipopeptide, surfactin (Ongena and Jacques 2008; Raaijmakers et al. 2010; Sansinenea and Ortiz 2011; Chen et al. 2015; Inès and Dhouha 2015). Surfactin is a cyclic heptapeptide consisting of hydrophobic and negatively charged amino acids with a chiral sequence LLDLLDL linked to hydroxyl fatty acyl residue of between 12 to 16 carbon atoms (Seydlová and Svobodová 2008).

Several isoforms and analogues exist for the naturally produced glycolipids and lipopeptides, which is why they exhibit significant structural heterogeneity (Benincasa et al. 2004; Ongena and Jacques 2008). A variety of methods are utilised to classify and characterise the biosurfactant compounds produced by a range of microorganisms. While mass spectrometry (MS) coupled with various chromatographic methods are the most widely used, with liquid chromatography coupled

to electrospray ionisation mass spectrometry (ESI-MS) a highly sensitive method that enables the fingerprinting of low concentrations of metabolites within a crude extract produced using natural sources. In addition, mass spectrometry is a powerful tool to utilise for analysing complex compounds such as biosurfactants and can efficiently discriminate between different analogues and isoforms within a mixture of compounds. Moreover, the biosurfactant congeners display different physico-chemical properties in combination, which can differ from the physico-chemical properties observed in individual congeners (Bonmatin et al. 2003). A study conducted by Kracht et al. (1999) indicated that surfactin molecules (produced by *Bacillus subtilis* OKB 105) with 13 carbon atoms in their hydrophobic moiety exhibited low antiviral activity, while the surfactin isoform with 15 carbon atoms displayed the highest antiviral activity. In addition, the presence of a single negative charge also contributed to an increased antiviral activity. Studies have indicated that the microbial strains utilised for glycolipid or lipopeptide production have an influence on the yield and composition of the compounds synthesised, which in turn has an effect on their antimicrobial activity (Déziel et al. 1999; Hošková et al. 2013).

The antimicrobial property of biosurfactants rely on different mechanisms to destroy target organisms as compared to conventional antibiotics (Banat et al. 2010) and they primarily destroy bacterial cells by directly disrupting the integrity of the plasma membrane or cell wall (Sang and Blecha 2008; Yount and Yeaman 2013). Most of the glycolipid and lipopeptide based biosurfactant compounds displaying antimicrobial properties, were extracted from microorganisms isolated from marine, terrestrial and sites contaminated by hydrocarbon based compounds (Abalos et al. 2001; Das et al. 2008; Sharma et al. 2014; 2015). Currently there is limited research on biosurfactant compounds produced by bacterial strains isolated from wastewater.

The current study focused on the purification and characterisation of antimicrobial glycolipid and lipopeptide biosurfactant compounds respectively, produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) ST5 and *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) ST34 strains that were isolated from a local wastewater treatment plant. This aim was achieved by obtaining crude biosurfactant compounds from the *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5 strains grown on mineral salt medium (supplemented with glycerol) as well as nutrient agar, using acid-precipitation followed by a rapid solvent extraction method. A method with ESI-MS coupled with ultraperformance liquid chromatography (UPLC), denoted UPLC-MS, was developed for the characterisation of the biosurfactant extracts by using commercially available lipopeptides and glycolipids as standards. Chemical characterisation of the crude extracts was performed using the optimised UPLC-MS method. Finally, various opportunistic, pathogenic and antibiotic resistant bacteria and fungal strains were utilised for the assessment of the antimicrobial activity of the crude biosurfactant extracts obtained from the respective isolates.

4.2. Materials and methods

4.2.1. Bacterial isolates, media composition and biosurfactant production conditions

Biosurfactant-producing bacteria were isolated from wastewater samples collected from Stellenbosch wastewater treatment plant in the Western Cape, South Africa (GPS co-ordinates: -33.943505, 18.824584) as described by Ndlovu et al. (2016). The bacterial isolates ST34, identified as *B. amyloliquefaciens* (collection number SARCC 696 at the South African Rhizobium Culture Collection) and ST5, identified as *P. aeruginosa* (collection number SARCC 697 at the South African Rhizobium Culture Collection), using molecular characterisation (Ndlovu et al. 2016), were utilised in the current study for biosurfactant production. Henceforth the *B. amyloliquefaciens* and *P. aeruginosa* isolates will be referred to by their code identifiers, ST34 and ST5, respectively. The bacterial cultures were maintained in 40 % glycerol at -80 ºC. An inoculum of the glycerol stock of ST34 and ST5 was streaked onto a nutrient agar (NA) plate which was incubated for 18-24 hrs at 37 ºC. A single colony from each respective NA culture was then used to inoculate 5 mL sterile mineral salt medium (MSM) to prepare seed cultures. The MSM utilised for biosurfactant production was composed of the following: 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.02 % $MgSO_4.7H_2O$, 0.002 % $CaCl_2.2H_2O$, 0.005 % $FeCl_3.6H_2O$ and 0.2 % $NaNO_3$ and 3 % glycerol as the main carbon and energy source, with the pH of the medium adjusted to 6.8 (Silva et al. 2010). The cultivation conditions for preparation of the seed culture were 30 ºC, at 200 rpm with an incubation time of 18-24 hrs. After seed culture preparation, a 2 % cell suspension of 0.7 optical density (OD) at 600 nm, which corresponded to approximately 10^7 colony forming units (CFU) mL^{-1}, was inoculated into 500 mL baffled flasks containing 100 mL MSM. The broth cultures were incubated on a 200 rpm orbital shaker (MRCLAB, London, UK) for 120 hrs at 30 ºC.

4.2.2. Extraction and partial purification of the biosurfactants

The crude biosurfactant compounds produced by ST34 and ST5 were obtained from the culture supernatant by a combination of acid and solvent extraction methods. Briefly, after 5 days of culturing the isolates in glycerol MSM, the culture (100 mL) was centrifuged at 11305 x g for 30 min at 4 ºC to remove microbial cells. The presence of surface active compounds in the supernatant was then verified using the oil spreading method as previously described by Ndlovu et al. (2016). Thereafter the supernatants were acidified to a pH of approximately 2 using hydrochloric acid (HCl, Merck, Darmstadt, Germany) as previously described by Das et al. (2008) and were stored overnight at 4 ºC in order to precipitate the biosurfactant compounds. The precipitate was then harvested by centrifugation at 11305 x g for 30 min at 4 ºC, and the pellet was washed with 50 mL of analytical quality water (prepared through a MilliQ system from Millipore, Billerica, USA), with the pH adjusted to 7.5 (Das et al. 2008). The respective insoluble fraction was then lyophilised and dissolved in 15 % (*v/v*) methanol (Merck, Darmstadt, Germany)

(crude extracts obtained from ST34 and ST5), transferred into analytically weighed sterile vials and lyophilised again. The extracts (ST34 and ST5) were analytically weighed and dissolved in 15 % methanol to obtain a 1.00 mg mL^{-1} concentration, which was used for the characterisation and antimicrobial analysis (**Tables 4.1** and **4.2**). The methanol soluble fractions were lyophilised, further extracted using 70 % acetonitrile and then lyophilised again. The extracts (ST34 and ST5) were analytically weighed and dissolved in 15 % acetonitrile to obtain a 1.00 mg mL^{-1} concentration for analysis using the UPLC-ESI/MS.

The ST34 and ST5 isolates were also cultured in duplicate on NA plates and NA slants (10 mL test tube) for approximately 5 days at 30 ºC. Five millilitres of 70 % acetonitrile (Romil, Cambridge, UK) was added to the NA plate cultures, which were then placed on a Bio dancer (New Brunswick Scientific, Enfield, USA) shaker at a speed of 5 rpm for approximately 5 min. The acetonitrile mixture was decanted into a sterile McCartney bottle. For the NA slant cultures, 5 mL of 70 % acetonitrile was added to the test tube, the culture was vortexed for approximately 2 min, where after the acetonitrile mixture was decanted into a sterile McCartney bottle. The lyophilised acetonitrile extracts obtained from NA plates and slants were then suspended in 1 mL sterile analytical quality water, the soluble supernatant was removed and the insoluble fractions were lyophilised and weighed analytically. After weighing, the extracts were dissolved in 15 % acetonitrile to obtain a 1.00 mg mL^{-1} concentration, which was used for the characterisation of the biosurfactants produced by each bacterial strain.

4.2.3. Analysis with ultra-performance liquid chromatography linked to electrospray ionisation mass spectrometry

Mass spectrometry analyses were done in the LCMS Central Analytical Facility at Stellenbosch University. A Waters Quadrupole Time-of-Flight Synapt G2 (Waters Corporation, Miliford, USA) mass spectrometer was utilised for the ESI-MS and was coupled to an Acquity UPLC for the UPLC-MS analysis of the biosurfactant extracts. Three microlitres of the standards and acetonitrile soluble extracts (glycerol-MSM) obtained from ST34 and ST5 at 1.00 mg mL^{-1} were directly injected into a Z spray electrospray ionisation source for direct mass analysis. The identities of the biosurfactant compounds were confirmed with high resolution mass spectrometry by comparing it with the mass/charge ratio (*m/z*) obtained for bacillomycin, fengycin and mycosubtilin (LipoFabrik, Lille, France) and iturin A, surfactin and rhamnolipid (Sigma-Aldrich, St. Louis, USA) as standards.

For UPLC-MS analysis 3 µL sample of each standard, extracts obtained from glycerol-MSM liquid culture, NA surface culture in a petri-dish and NA slant cultures in test tubes was injected and separated on an UPLC C18 reverse-phase analytical column (Acquity UPLC® HSS T3, 1.8 µm particle size, 2.1 x 150 mm, Waters corporation, Dublin, Ireland) at a flow rate of 0.300 mL min^{-1}

using a 0.1 % formic acid (A) to acetonitrile (B) gradient (60 % A from 0 to 0.5 min for loading, gradient was from 40 to 95 % B from 0.5 to 11 min and then 95 to 40 % B from 15 to 18 min). The UPLC-MS profiles of the biosurfactant compounds were compared to those obtained for bacillomycin, fengycin, iturin A, surfactin, rhamnolipid and mycosubtilin standards.

For both direct ESI-MS and UPLC-MS analyses, the analytes were subjected to a capillary voltage of 3 kV, cone voltage of 15 V and a source temperature of 120 ºC. Data acquisition in the positive mode was performed by MS scanning a second analyser through the *m/z* range of 200-3000 Da and the data was thereafter analysed using Masslynx software version 4.1 (Waters Corporation, Milford, USA).

4.2.4. Determination of antimicrobial activity: agar disc susceptibility test

The antimicrobial activity of the extracts obtained from ST34 and ST5, was analysed against various actively growing targets [from American Type Culture Collection (ATCC)], environmental and clinical Gram-positive and Gram-negative microbial strains (**Table 4.1**) as well as fungal strains (**Table 4.2**) on Mueller Hinton agar (MHA) (Merck, Darmstadt, Germany). The bacterial environmental strains were isolated by our research group from rainwater tanks and surface water (Plankenburg River, Stellenbosch, South Africa), while the clinical strains were obtained from laboratories in the Department of Microbiology at Stellenbosch University (Stellenbosch, South Africa). Fungal strains isolated from surface water (Benadé et al. 2016) and clinical samples were obtained from the Environmental Biotechnology laboratory in the Department of Microbiology were also included as antimicrobial test strains against ST34 and ST5 extracts. Briefly, the crude biosurfactant extracts were dissolved in 15 % (*v/v*) methanol and were filtered through a 0.22 µm low protein binding non-pyrogenic syringe filter (Pall Life Sciences, Ann Arbor, USA). A 100 µL overnight culture of the test microbial isolates (**Tables 4.1** and **4.2**), which had been grown in Luria Bertani broth (Merck, Darmstadt, Germany), was then spread plated onto the MHA to create a microbial lawn. Thereafter, using sterile tweezers, 6 mm filter paper discs (Oxoid, Basingstoke, UK) were placed onto the lawn and 50 µL of the biosurfactant extract (1.00 mg mL^{-1}), obtained from either ST34 or ST5, were pipetted directly onto the filter paper in order to create an antimicrobial disc. The antimicrobial tests were performed with a negative control (MHA plus test bacterial strain) and two positive controls (MHA plus pure surfactin and rhamnolipid purchased from Sigma, USA). All tests were performed in triplicate. All the MHA plates were then incubated at 37 ºC for 24-48 hrs where after the diameter of the zone of inhibition around the inoculated paper disc was measured (Das et al. 2008).

Table 4.1 Antibacterial activity of the biosurfactant extracts (1.00 mg mL^{-1}) against a panel of Gram-negative and Gram-positive bacterial isolates as determined by agar disc diffusion method

Organism (strain number)	Source	Antibacterial inhibition zone diameter (mm) ± SD	
Gram-negative target organism		**Surfactin extract**	**Rhamnolipid extract**
Escherichia coli (ATCC 417373)	ATCC	13 ± 0	13.5 ± 0.4
E. coli (ATCC 13706)	ATCC	10 ± 0	29.3 ± 0.9
Enteroinvasive *E. coli* (ATCC 43892)	ATCC	15 ± 0	22.7 ± 2.1
[G]Enteropathogenic *E. coli* (B170)	ATCC	18.3 ± 0.5	20.3 ± 0.5
Enterohaemorhagic *E. coli* (O157:H7)	ATCC	13.7 ± 0.5	13.7 ± 0.5
Enterotoxigenic *E. coli* (H10407)	ATCC	17.7 ± 1.2	13 ± 0
Enteroaggregative *E. coli* (3591-87)	ATCC	12.3 ± 0.5	24.3 ± 1.2
Klebsiella pneumoniae (ATCC 10031)	ATCC	14 ± 1.6	13.5 ± 0.5
*Salmonella typhimurium (*ATCC 14028)	ATCC	25.3 ± 1.2	20.3 ± 0.5
Serratia marcescens (ATCC 13880)	ATCC	12.7 ± 0.9	14 ± 0
K. pneumoniae (P2)	Clinical	13 ± 0.8	11.7 ± 0.9
K. pneumoniae (P3)	Clinical	13.3 ± 0.2	8.3 ± 0.5
Salmonella enterica (SE19)	Environment	12.5 ± 0.5	14 ± 0
Acinetobacter sp. (F1S6)	Environment	12.3 ± 0.5	13 ± 1.4
Serratia sp. (SM14)	Environment	11.7 ± 0.9	14.3 ± 1.2
Serratia sp. (L8)	Environment	12.5 ± 0.5	9.8 ± 0.8
Enterobacter sp. (E11)	Environment	11.3 ± 0.5	13 ± 0.8
Enterobacter sp. (E22)	Environment	14.2 ± 0.6	13 ± 0.8
E. coli (K4CCA)	Environment	14.5 ± 0.5	17.7 ± 1.9
K. pneumoniae (k2a)	Environment	15.3 ± 0.5	13.7 ± 0.5
Gram-positive target organism			
[O]*Staphylococcus aureus* (ATCC 25923)	ATCC	14.7 ± 0.5	13.7 ± 0.5
B. cereus (ATCC 10876)	ATCC	10.3 ± 0.5	13 ± 0.8
B. cereus (LMG 13569)	ATCC	13 ± 0.8	17 ± 1.4
Enterococcus faecalis (S1)	Clinical	18.7± 0.9	10.7 ± 0.5
Enterococcus faecalis (S2)	Clinical	18.3 ± 1.2	21.7 ± 2.4
[G,O,P,T]MRSA *(Xen 30)*	Clinical	15.3 ± 0.5	13.3 ± 0.5
Bacillus cereus (ST18)	Environment	inactive	22.3 ± 0.9
Enterococcus sp. (C513)	Environment	12.3 ± 0.5	15.7 ± 0.5
Micrococcus sp. (AQ4S2)	Environment	14 ± 0	14 ± 1
S. aureus (C2)	Environment	11.5 ± 0.5	14 ± 0
S. aureus (C3)	Environment	12 ± 0	11 ± 0

Values are the means ± standard deviations (SD) of triplicate measurements; ATCC – American Type Culture Collection, [O] – resistant to Oxacillin, [G] – resistant to Gentamicin, [T] – resistant to Tetracycline, [P] – resistant to Penicillin G

Table 4.2 *In vitro* antifungal activity of the surfactin and rhamnolipid biosurfactant extracts (1.00 mg mL^{-1}) against a panel of clinical and environmental fungal isolates as determined by by agar disc diffusion method

Organism	Antifungal zone diameter (mm)	
	Surfactin extract	**Rhamnolipid extract**
#*Cryptococcus neoformans* CAB1063	inactive	13 ± 0.8
#*Cryptococcus neoformans* CAB1067	11.7 ± 0.5	14.3 ± 3.3
#*Cryptococcus neoformans* CAB1055	15.3 ± 0.5	11.3 ± 0.9
#*Candida albicans* 8911	13.3 ± 0.5	14.7 ± 0.5
#*Candida albicans* 8912	13.3 ± 0.5	11.7 ± 0.5
**Cryptococcus neoformans* CAB1034	inactive	18.3 ± 0.8
**Cryptococcus neoformans* CAB831	11.7 ± 1.7	15.3 ± 1.9
**Cryptococcus neoformans* CAB842	12.3 ± 0.9	inactive
**Cryptococcus neoformans* CAB844	15.3 ± 1.2	16.7 ± 1.7
**Candida albicans 1085*	inactive	14 ± 0.8

Clinical strain, * Environmental strain

4.2.5. Statistical analysis

The diameters of the zones of inhibition produced by the ST34 and ST5 extracts against various microbial strains analysed in the current study, were expressed as mean values ± standard deviation. The Student's t-test was then utilised to determine the statistical significant difference between the diameters of the zones of inhibition between the extracts produced by ST34 and ST5, respectively, against the test bacterial and fungal strains. The P values less than 0.05 ($p < 0.05$) were considered significant.

4.3. Results

4.3.1. Direct ESI-MS analysis for solvent extracted biosurfactant compounds produced by ST34

Solvent extracts of the glycerol-MSM liquid culture obtained from ST34 were subjected to direct infusion using positive mode ESI-MS in order to determine the accurate molecular mass (compound identity) for the solvent extracted biosurfactant compounds. The spectra of the possible biosurfactant compounds produced by ST34 were compared to the surfactin, mycosubtilin, bacitracin, iturin A and fengycin standards. However, it only corresponded with the profile observed for the surfactin standard, hence only the results for surfactin standard are depicted in **Fig. 4.1**. In the ESI-MS spectrum of the ST34 extract from glycerol MSM, a cluster of *m/z* peaks with a difference of approximately 14 or 22 or 28 atomic mass units (amu) in their molecular ion species were detected, revealing five groups of analogue molecules (**Fig. 4.1**). The spectra in positive mode showed the main groups of molecular ions at *m/z* 994.65, 1008.66,

1022.68, and 1036.69 which corresponded to the protonated singly charged species $[M+H]^+$ (**Fig. 4.1, Table 4.3**). Their corresponding sodium adducts $[M+Na]^+$ were also detected at *m/z* 1016.63, 1030.64, 1044.65 and 1058.66 (**Fig. 4.1a, Table 4.3**). For the standard surfactin, the spectra in the positive mode displayed the main groups of molecular ions at *m/z* 1008.66, 1022.68 and 1036.66 which corresponded to the protonated singly charged species $[M+H]^+$ (**Fig. 4.1c, Table 4.3**). Their sodium adducts $[M+Na]^+$ were also detected at *m/z* 1044.66 and 1058.68 *m/z* molecules.

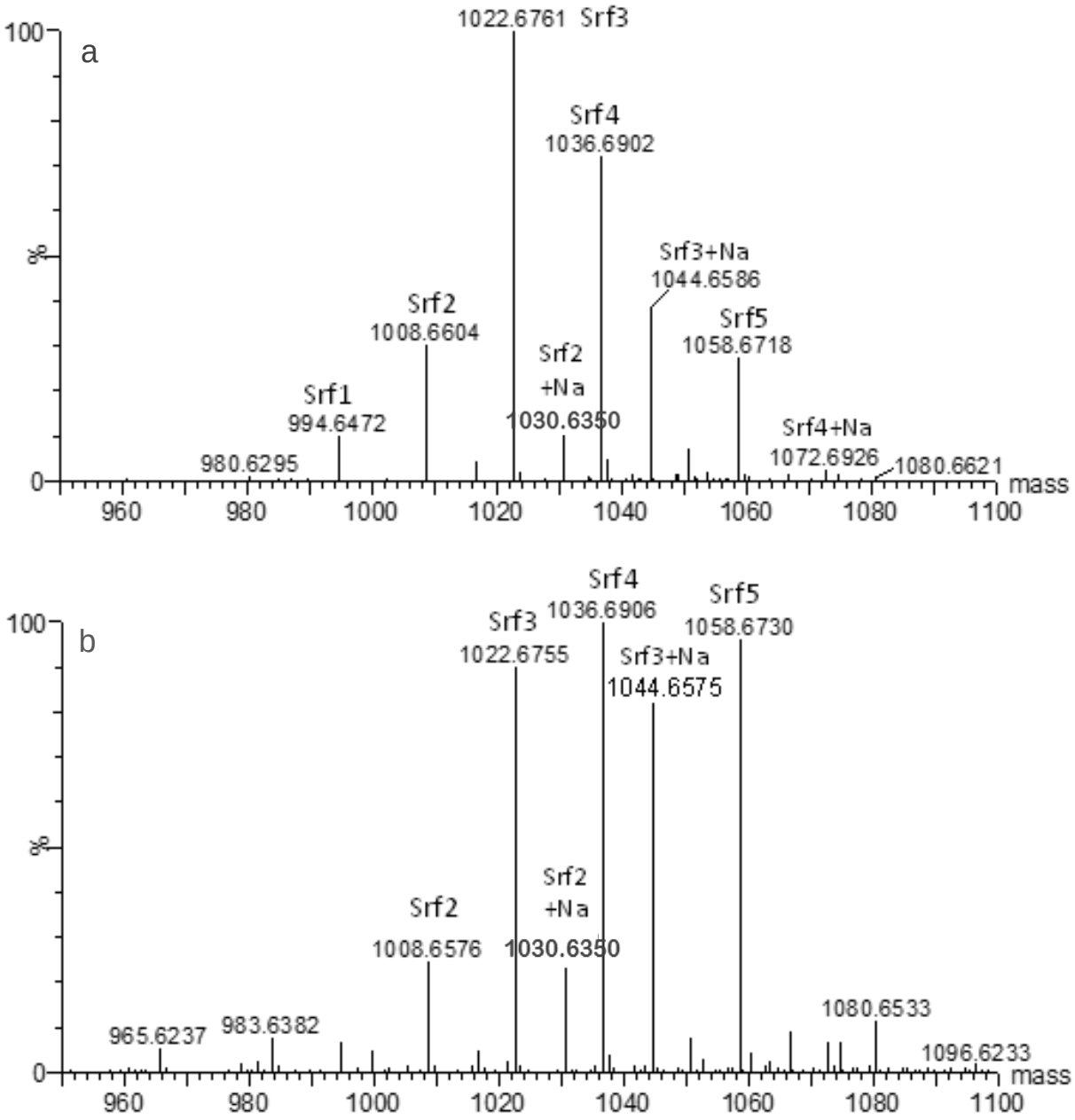

Fig. 4.1 ESI-MS analysis of the ST34 glycerol-MSM extract (A) and surfactin standard (B). The positive mass spectrum generated with MaxEnt 3 is shown. The indicated masses are $[M_r+H]$ = *m/z* values of singly charged species. Refer to **Table 4.3** for identities of Srf 1-5 and expected *m/z* and M_r values.

The singly charged protonated molecular species $[M+H]^+$ at *m/z* 994.6, 1008.7, 1022.7 and 1036.7 and their corresponding singly charged sodiated molecules $[M+Na]^+$ (1016.6, 1030.6, 1044.7 and 1058.7) all differed by 14 or 28 amu (**Table 4.3**). The detected high resolution M_r values (ppm<10) of the possible surfactin analogues in the ST34 extract corresponded to that of the C_{13}, C_{14}, C_{15} and C_{16} surfactin analogues (Srf1-5) in a standard surfactin, confirming their identity (**Fig. 4.1, Table 4.3**).

Table 4.3 Summary of the detected surfactin lipopeptides extracted from cultures of *B. amyloliquefaciens* ST34, as detected using high resolution mass spectrometry (<10 ppm). Their proposed chemical structures, theoretical (Theor) and experimental (Exp) M_r and monoisotopic *m/z* values, as well as observed UPLC retention times for representative examples are given.

Surfactin group (Abbr)	UPLC Rt (min)#	Characterised and proposed* peptide sequences in surfactin group	Mono-isotopic Exp/Theor M_r	Protonated specie Exp/Theor *m/z*	Sodiated specie Exp/Theor *m/z*
Surfactin 1 (Srf1)	10.6; 11.2	cyclo[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	993.6376	994.6472	1016.6265
		cyclo[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	993.6403	994.6481	1016.6190
Surfactin 2 (Srf2)	11.0; 11.2; 11.9	cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**] cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	1007.6521	1008.6604	1030.6350
		cyclo-[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**] *cyclo-[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo-[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1007.6552	1008.6596	1030.6328
Surfactin 3 (Srf3)	11.6; 11.7; 12.3	cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**] cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	1021.6693	1022.6780	1044.6586
		cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**] *cyclo-[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo-[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1021.6715	1022.6752	1044.6494
Surfactin 4 (Srf4)	12.1; 12.2	cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1035.6819	1036.6902	1058.6718
		*cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1035.6881	1036.6909	1058.6662
Surfactin 5 (Srf5)	12.6; 12.7	cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1049.6992	1050.7120	1072.6926
		*cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1049.7032	1050.7066	1072.6886

#UPLC Retention time of main peaks corresponding to the group's *m/z* value

4.3.2. ESMS and UPLC-MS analysis of solvent extracted biosurfactant compounds produced by ST34

An optimised UPLC-MS method was employed to analyse the lipopeptide biosurfactant extract obtained from ST34 cultured in glycerol-MSM (ST34LC) is shown in **Fig. 4.2b** (compared with the surfactin standard; **Fig. 4.2a**). The UPLC-MS profiles of the biosurfactant compounds produced

by ST34 corresponded very well with the profile observed for the surfactin standard (**Fig. 4.2a**). Surface culture on NA (ST34NA) in test tubes (ST34NA-TSC) and petri dishes (ST34NA-PDC) were also utilised to produce biosurfactants by ST34, in order to increase the probability of detecting lipopeptides in/on different growth media. As the NA cultures were extracted with 70 % acetonitrile, the ST34LC was further extracted with 70 % acetonitrile (ST34LC-AE) and analysed. The comparative UPLC-MS profiles of the extracts are shown in **Fig. 4.2**. The UPLC-MS profiles of the surfactin standard and the extracts produced by ST34 showed significant peaks at retention times between 10 and 13 min.

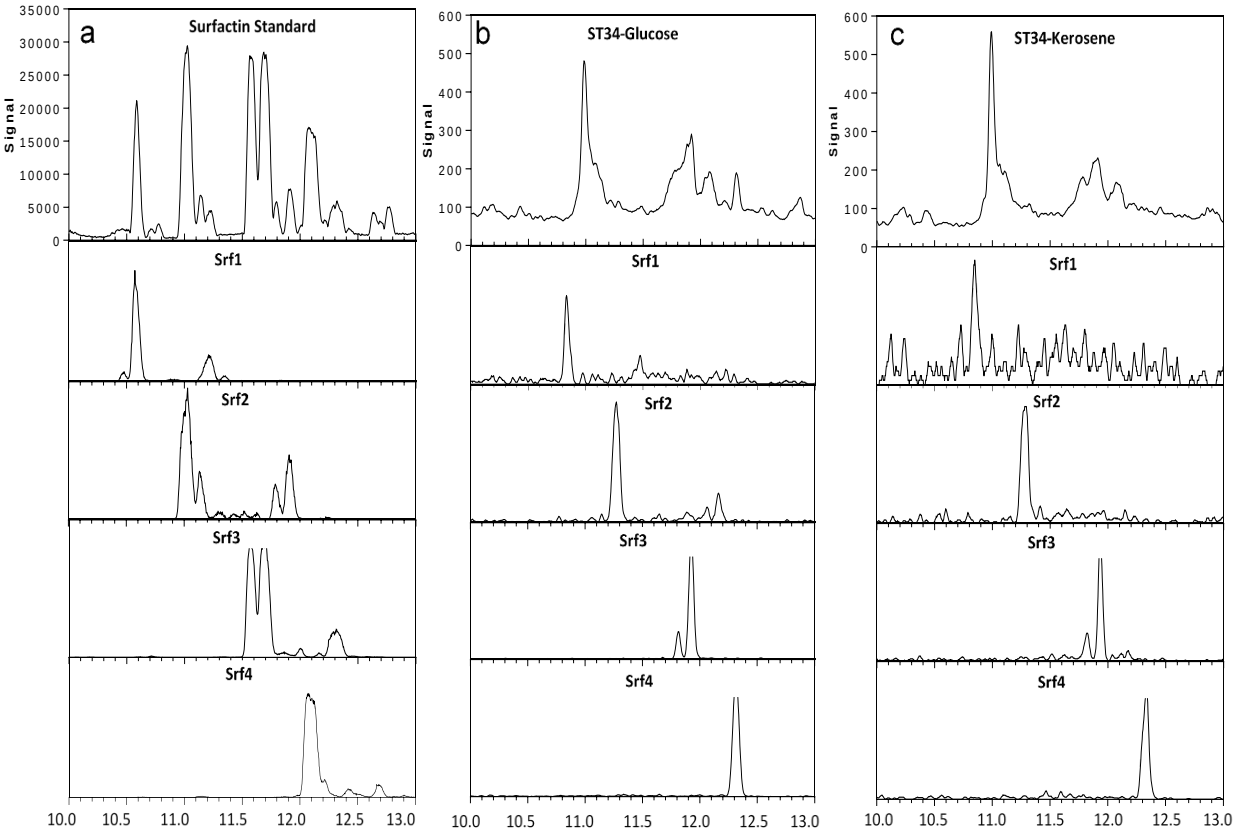

Fig. 4.2 UPLC-MS profiles of surfactin standard (A), ST34 glycerol-MSM liquid culture (ST34LC) extract (B) and ST34 nutrient agar surface culture (ST34NA) (C) showing the five major surfactin groups. The top row profiles show the signal of positive molecular ions detected between 10 and 13 mins. Note the difference in Y axis which are a direct indication of amounts. The profiles below each top row spectrum show the extracted spectra of the five surfactin groups with Srf1 = *m/z* 994.6, Srf2 = m/z 1008.7; Srf3 = *m/z* 1022.7, Srf4 = *m/z* 1035.7 and Srf5 = *m/z* 1050.7

From basic reverse-phase chromatography principles, it is expected that the surfactin species with the longer fatty acyl chains will elute at a later retention time (Rt) from the C18 matrix. This was indeed the case, with the sequence of surfactin groups eluted as follows, surfactin 1 (Srf1) (Rt 10.6; 11.2 min), Srf2 (Rt 11.0, 11.2, 11.9 min), Srf3 (Rt 11.6, 11.7, 12.3 min) Srf4 (Rt 12.1, 12.2 min) and Srf5 (Rt 12.6, 12.7 min) (**Fig. 4.2**; **Table 4.3**). In the surfactin groups, Ile/Leu analogues will elute closer or together, while the slightly smaller and less hydrophobic Val

analogues will elute earlier. It should be noted that the peptide identities within specific surfactin groups were not fully explored as it was beyond the scope of the study. However, this UPLC-MS methodology has the potential to be extended to include tandem mass spectrometry and ion mobility on the Synapt G2 in future studies conducted on these surfactin analogues. For the glycerol-MSM culture extracts, five peaks/peak clusters were observed on the UPLC-MS profile which corresponded to five surfactin groups. The five surfactin groups (Srf1, Srf2, Srf3, Srf4 and Srf5) exhibited similar retention times as the surfactin standard (**Fig. 4.2b**). As indicated, the ST34 was also cultivated in NA in order to increase the probability of detecting the produced biosurfactant compounds. The extracted UPLC-MS profiles for the NA extracts showed major peaks which corresponded to Srf2, Srf3 and Srf4, while traces of Srf1 and Srf5 surfactin analogues were also detected (**Fig. 4.2c**).

A detailed analysis of some of the major peaks in the UPLC-MS profiles of the ST34LC extract (glycerol-MSM culture extract) revealed that these peaks contained both the protonated molecular species, as well as the sodiated species of the surfactin group (**Fig. 4.3**). The ST34LC extract produced two major peaks at 11.0 and 11.7 min. The peak at 11.0 min corresponded to the lipopeptides in the Srf3 group which yielded a surfactin analogue with M_r of 1021.67 (expected M_r of 1021.67) and its sodium adduct at 1044.65 (expected M_r of 1044.65) (**Fig. 4.3, Table 4.3**). It was also observed that next to the main peak (11.0 min, **Fig. 4.2b**) obtained in the ST34 glycerol extract (ST34LC), were two peaks at 11.2 and 11.9 min that corresponded to the Srf2 (**Fig. 4.2b, Table 4.3**). The peaks at 11.2 and 11.9 min both corresponded to the surfactin analogues with M_r of 1007.65, which existed with their sodium adducts with M_r of 1030.64. The other major peak for the ST34LC extract was observed at 11.7 min and corresponded to the Srf3 group that showed an analogue with M_r of 1021.67 (expected M_r of 1021.67) and its sodium adduct at 1044.65 (expected M_r of 1044.65) (**Fig. 4.3, Table 4.3**). A detailed analysis of the major peak observed for the ST34NA extract (NA = petri dish and test tube slant cultures; **Fig. 4.2b**) was then observed at 12.1 min. The peak corresponded to the Srf4 group which yielded a surfactin analogue with M_r of 1035.68 (expected M_r of 1035.69) (**Fig. 4.3, Table 4.3**).

From the accurate M_r values and corresponding UPLC profiles it was then concluded that the ST34 extract contained all five surfactin groups (**Fig. 4.3, Table 4.3**). After further comparison with all the commercial standards utilised in the current study (not shown), results showed that surfactin was the only lipopeptide biosurfactant detected in the ST34 extracts obtained from the glycerol-MSM and NA using the production conditions (glycerol-MSM, temperature and the agitation speed) applied in the current study.

The relative abundance of each surfactin group within the complex surfactin lipopeptides in the ST34 extract obtained from the glycerol-MSM and NA cultures was inferred from the M_r extracted chromatograms by combining the peak areas of each surfactin group eluting between 10 and 13

min. The relative content for each surfactin group is illustrated in **Fig. 4.4**, and it showed that the Srf1 and Srf5 groups were below 5 % relative abundance in the ST34 extracts obtained from the glycerol-MSM and the NA media (both the test tube slant and petri dish cultures).

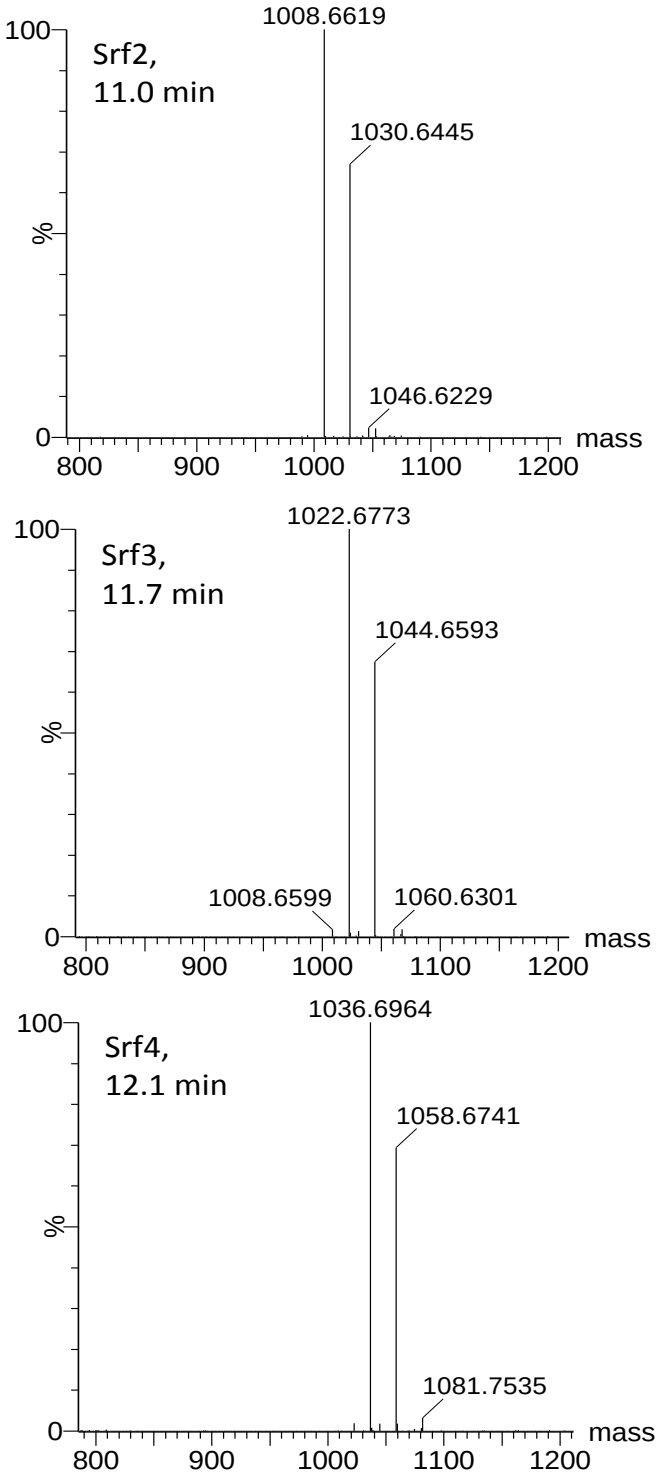

Fig. 4.3 Examples of the ESI-MS mass spectra of three major surfactin groups detected with UPLC-MS. The positive mass spectrum generated with MaxEnt 3 is shown. The indicated masses are [M_r+H] = *m/z* values of singly charged species. Refer to **Table 4.3** for identities of Srf1-5 and expected *m/z* and M_r values.

The Srf2, Srf3 and Srf4 were the main surfactin groups detected in the ST34 extracts illustrated in **Fig. 4.4**. The NA test tube slant culture produced the Srf4 group in higher concentrations, with a relative abundance of approximately 60 % (**Fig. 4.4**). In contrast, the glycerol-MSM liquid cultures produced the Srf3 in higher concentrations, with a relative abundance of approximately 43 % (**Fig. 4.4**).

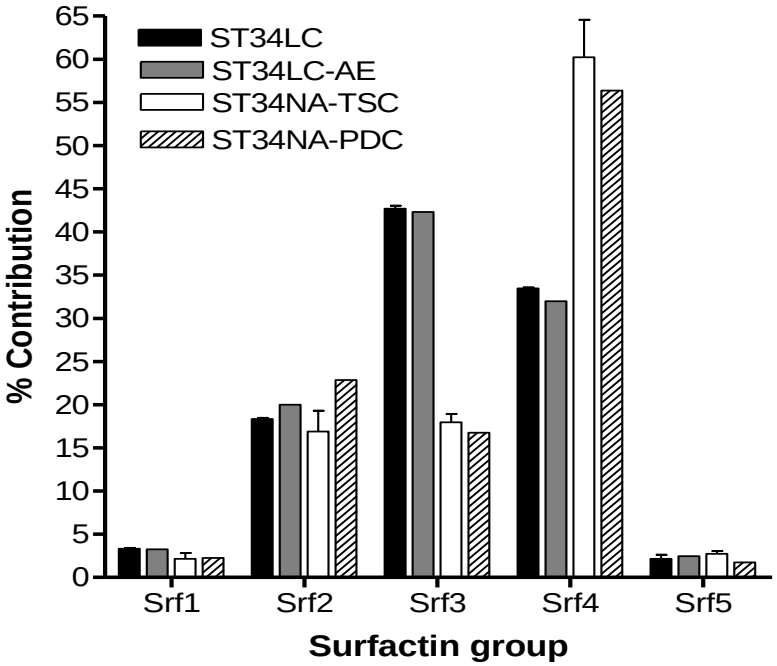

Fig. 4.4 Comparison of the different culture extracts showing the relative contribution of each of the surfactin groups in the biosurfactant extracts. The contribution was calculated from UPLC profiles, with the assumption that all the surfactin species has similar ion responses. ST34LC = glycerol-MSM culture extract, ST34LC-AE = 70% acetonitrile extract of ST34LC, ST34NA-TSC = NA test tube slant culture extract, ST34NA-PDC = NA petri dish culture extract.

4.3.3. Direct ESI-MS analysis of solvent extracted biosurfactant compounds produced by ST5

Solvent extracts of the glycerol-MSM liquid culture obtained from ST5 were subjected to direct infusion using the positive ESI-MS in order to determine the accurate molecular mass (compound identity) for the solvent extracted biosurfactant compounds. The spectra of the possible biosurfactant compounds produced were compared to the rhamnolipid, surfactin, mycosubtilin, bacitracin, iturin A and fengycin standards. However, it only corresponded with the profile observed for the rhamnolipid standard, hence only the results for rhamnolipid standard are depicted in **Fig. 4.5**. In the positive mode ESI-MS for the ST5 extract obtained from the glycerol-MSM ST5 culture we observed a series of sodiated singly charged ions at *m/z* values of 673.38, 645.35, 527.32 and 499.29 (**Fig. 4.5**, **Table 4.4**). Corresponding sodiated dimers $[2M-H+Na]^+$ at *m/z*, 1323.77, and 975.59 (**Fig. 4.5**) were also generally detected. For the standard rhamnolipid, the spectra in positive mode showed on major of rhamnolipid with molecular ions at *m/z* 651.40, 673.38 and 1323.77, which corresponded to the singly charged species, $[M+H]^+$ and $[M+Na]^+$ as

well as sodiated dimer (**Fig. 4.5**). While analysing the full ion spectrum of the rhamnolipid standard, a series of ions of *m/z* values corresponding to the fragment or molecular ions of the 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) were also observed (results not shown). These HAAs were also detected with the rhamnolipid congeners with *m*/*z* values of 331.2, 359.3 and 387.3, which correspond to protonated $[M+H]^+$ molecular ions of a HAA containing one 3-hydroxydecanoate (C_{10}) and one 3-hydroxyoctanoate (C_8) moiety, two C_{10} moieties and one C_{10} and one 3-hydroxydodecanoate (C_{12}) moiety, respectively, were the most abundant (refer to discussion below and **Fig. 4.6d**).

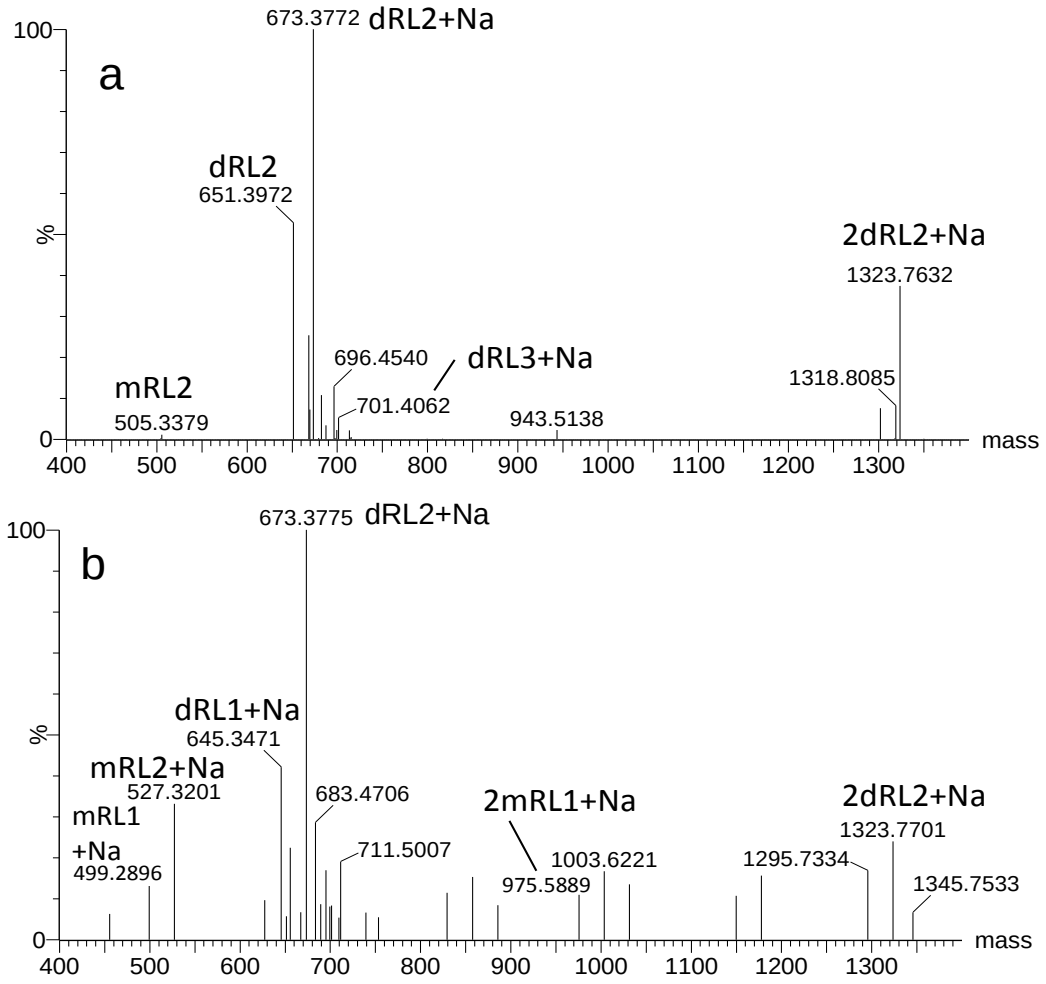

Fig. 4.5 ESI-MS analysis of the ST54 glycerol-MSM extract (a) and rhamnolipid standard (b). The positive mass spectrum generated with MaxEnt 3 is shown. The indicated masses are [M_r+H] = *m/z* values of singly charged species. Refer to Table 4 for identities of RL 1-4 and expected *m/z* and M_r values.

The molecular mass of the possible rhamnolipid congeners detected in the ST5 extract were then determined from the molecular ions observed (**Fig. 4.5, Table 4.4**). The ST5 extract showed singly charged sodiated molecular species $[M+Na]^+$ at *m/z* 645.35, 673.38, 701.41, 499.29, 527.32, 555.35 (**Fig. 4.5**), which is in agreement with M_r of the dirhamnolipids Rha-Rha-C_8-C_{10}/Rha-Rha-C_{10}-C_8 (dRL1), Rha-Rha-C_{10}-C_{10} (dRL2), and Rha-Rha-C_{12}-C_{10}/

Rha-Rha-C_{10}-C_{12} and monorhamnolipids, Rha-C_8-C_{10}/Rha-C_{10}-C_8 (mRL1), Rha-C_{10}-C_{10} (mRL2) and Rha-C_{10}-C_{12}/Rha-C_{12}-C_{10} (mRL3), respectively (**Table 4.4**). Moreover, the *m*/*z* values at 331.25, 359.28 and 387.32, which corresponded to protonated $[M+H]^+$ molecular ions of a HAA containing C_{10}-C_8/C_8-C_{10}, C_{10}-C_{10} and C_{10}-C_{12}/C_{12}-C_{10} moieties, respectively were detected in the ST5 extract (refer to discussion below and **Fig. 4.6**).

Table 4.4 Summary of the rhamnolipids extracted from cultures of *P. aeruginosa* (ST5), as detected with high resolution mass spectrometry (<10 ppm). Their proposed chemical structures, theoretical (Theor) and experimental (Exp) M_r and monoisotopic *m/z* values, as well as observed UPLC retention times for representative examples are given.

Rhamnolipid group (Abbr)	UPLC Rt (min)#	Proposed structures of rhamnolipids	Mono-isotopic Exp/Theor M_r	Protonated specie Exp/Theor *m/z*	Sodiated specie Exp/Theor *m/z*	Sodiated dimeric specie Exp/Theor *m/z*
mRL1	7.23	Rha-C_8-C_{10} Rha-C_{10}-C_8	476.3047 476.2985	477.3089 477.3063	499.2896 499.2883	975.5889 975.5868
dRL1	6.32 6.45	Rha-Rha-C_8-C_{10} Rha-Rha-C_{10}-C_8	622.3576 622.3564	623.3654 623.3642	645.3471 645.3462	1267.7074 1267.7026
mRL2	8.77 8.84	Rha-C_{10}-C_{10}	504.3305 504.3298	505.3383 505.3376	527.3201 527.3196	1031.6501 1031.6494
dRL2	7.84 7.97	Rha-Rha-C_{10}-C_{10}	650.3894 650.3877	651.3972 651.3955	673.3772 673.3775	1323.7701 1323.7652
mRL3	10.32	Rha-C_{12}-C_{10} Rha-C_{10}-C_{12}	532.3640 532.3611	533.3700 533.3689	555.3546 555.3509	1087.7201 1087.7120
dRL3	9.40 9.46	Rha-Rha-C_{12}-C_{10} Rha-Rha-C_{10}-C_{12}	678.4177 678.4190	679.4285 679.4268	701.4114 701.4088	1379.8352 1379.8278

#UPLC Retention time of main peaks corresponding to the group's *m/z* value

4.3.4. ESI-MS and UPLC-MS analysis of solvent extracted biosurfactant compounds produced by ST5

As the chromatographic separation in UPLC-MS analyses limits the interference of counter ions it is more likely to detect more rhamnolipid species in both the rhamnolipid standard and ST5 culture extracts. Our UPLC-MS method was therefore also used to analyse the glycolipid biosurfactant extract obtained from ST5 cultured in glycerol-MSM (ST5LC) (**Fig. 4.6b**). Surface cultures on NA in test tubes were also utilised to produce biosurfactants by ST5, in order to increase the probability of detecting glycolipids on different media (**Fig. 4.6c**).

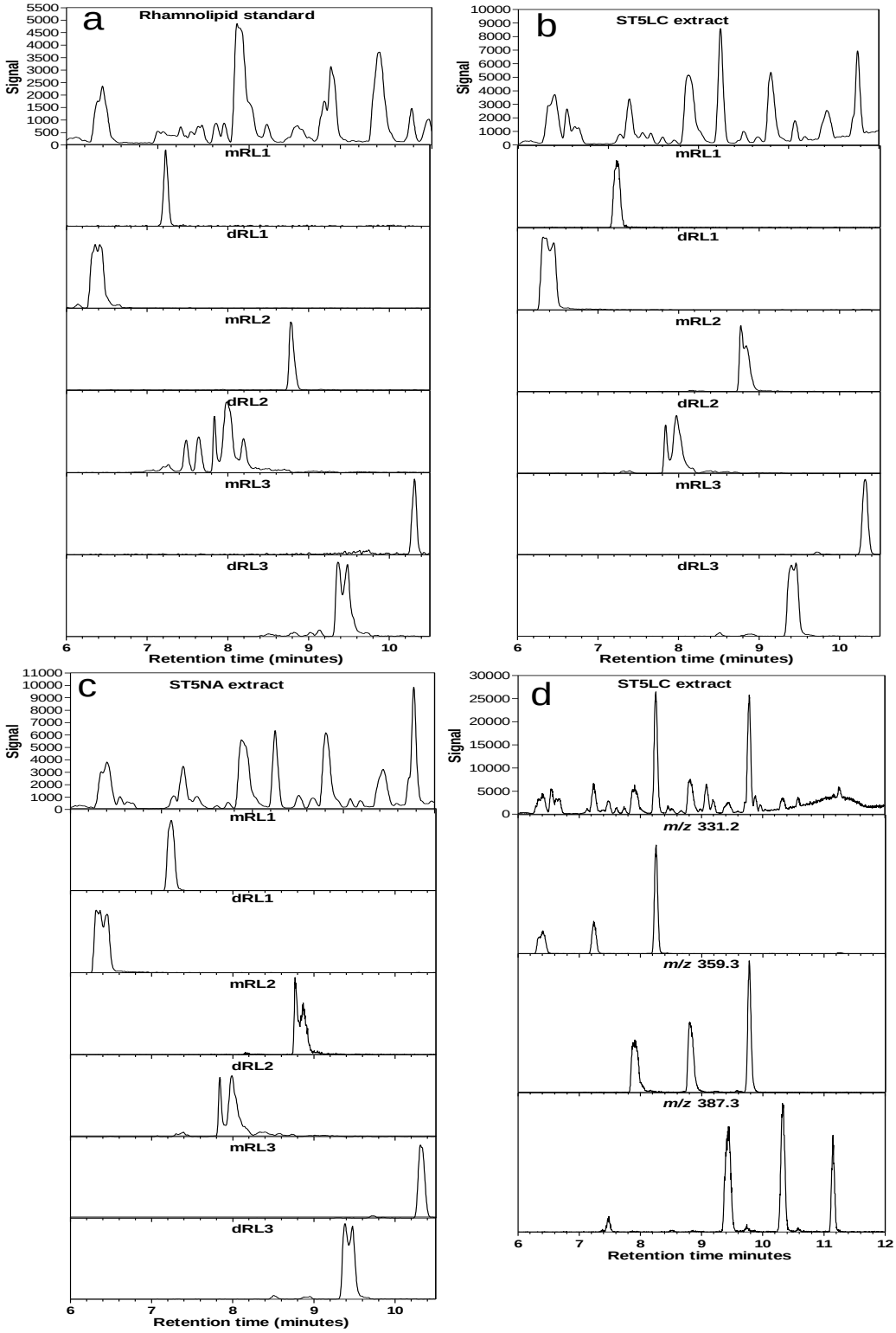

Fig. 4.6 UPLC-MS profiles of rhamnolipid standard (a), ST5 glycerol-MSM liquid culture (ST5LC) extract (b) and ST5 nutrient agar surface culture (ST5NA) (c) showing the four major rhamnolipid groups. The top row profiles show the signal of positive molecular ions detected between 6 and 10 mins. The profiles below each top row spectrum show the extracted spectra of the five rhamnolipid groups with RL1 = *m/z* 673.4, RL2 = m/z 645.3; RL3 = *m/z* 527.3 and RL4 = *m/z* 499.3. Profiles in D show the three types of HHAs (*m/z* 331.2, 359.3 and 387.3), either as precursors (third eluting peak) or fragments (first two eluting peaks) found in the ST5LC extract

The chromatographic profiles of the possible biosurfactant compounds produced were compared to the standards and analysis of results revealed that the profile obtained for ST5 only corresponded with the profile of the rhamnolipid standard (**Fig. 4.5a**). The comparative UPLC-MS profiles of the rhamnolipid standard and the extracts produced by ST5 exhibited significant peaks at retention times between 6 and 10.5 min. From basic reverse-phase chromatography principles, it is expected that the rhamnolipid species composed of two rhamnose and shorter HAA chain(s) will be eluted first, while the rhamnolipid with one rhamnose sugar and longer HAA chain(s) will elute later from the C18 matrix. This principle was observed for the rhamnolipid compounds produced by the ST5 strain, with the dirhamnolipids in the groups (dRL1, dRL2 and dRL3) eluting first and monorhamnolipid (mRL1, mRL2 and mRL3) eluting second in each group (**Fig. 4.5, Table 4.4**).

For the glycerol-MSM culture extract, six peaks/peak clusters were observed in the UPLC-MS profile which corresponded to six rhamnolipid groups. The six rhamnolipid groups from ST5 liquid culture extracts yielded identical retention times and *m/z* values (**Fig. 4.5b**) to those of the rhamnolipid standard (**Fig. 4.5a**). The ST5 extract obtained from the NA in a test tube (not shown) also displayed the same major peaks which corresponded to dirhamnolipids (dRL1, dRL2 and dRL3) and their monorhamnolipids (mRL1, mRL2 and mRL3) (**Table 4**).

A more detailed analysis of some of the major peaks in the UPLC-MS profiles revealed that these peaks contained the free rhamnolipid congener, protonated and sodiated molecular species (**Fig. 4.7**). For example, the peak at 7.9 min corresponded to one of the glycolipid dRL2 group that showed a rhamnolipid congener with M_r of 650.39 (expected M_r of 650.39), the protonated ion at 651.40 (expected *m/z* of 651.40) and its sodium adduct at *m/z* 673.38 (expected *m/z* of 673.38) (**Fig. 4.7a**). The peak at 8.7 min corresponded to the corresponding mRL2 rhamnolipid congener with a M_r of 504.33 (expected M_r of 504.33), with its protonated species at *m/z* of 505.34 (expected *m/z* of 505.34 Da) and its sodium adduct at *m/z* 527.32 (expected *m/z* of 527.32) (**Fig. 4.7b**). The spectra for the monorhamnolipid mRL1 and its dirhamnolipid dRl1 is shown in **Fig. 4.7c** and **d.** Furthermore, the protonated and sodiated HAA fragments of C_{10}-C_8/C_8-$C_{10,}$ were also detected in the rhamnolipid mRL1 and dRL1 peaks (*m/z* 331.2 and 353.2) and HAA fragments of C_{10}-C_{10} in the mRL2 and dRL2 congener peaks (*m/z* 359.3 and 381.3). Refer to **Fig. 4.6d** for the UPLC-MS profiles showing the detection of these major HAAs with *m/z* values of 331.2 and 359.3 in ST5LC extract.

The peak at 10.3 min corresponded to the mRL3 monorhamnolipid congener with a protonated molecular species at M_r 532.36 (expected M_r of 532.36), with its sodium adduct at *m/z* of 555.35 (expected 555.35). A dirhamnolipid Rha-Rha-C_{10}-C_{12} or Rha-Rha-C_{12}-C_{10} was also produced and was observed at 9.4 min at *m/z* 701,41 (expected 701.41) (**Fig. 4.6, Table 4.4**). This identity of the lipid moiety was confirmed by the detection of the hydroxyl fragment of C_{10}-C_{12}/C_{12}-C_{10} at *m/z*

of 387.31 in the RL3 congeners. Refer to **Fig. 4.6d** for the UPLC-MS profiles showing the detection of the C_{10}-C_{12}/C_{12}-C_{10} HAA ions with *m/z* at 387.3 in ST5LC extracts and the rhamnolipid standard.

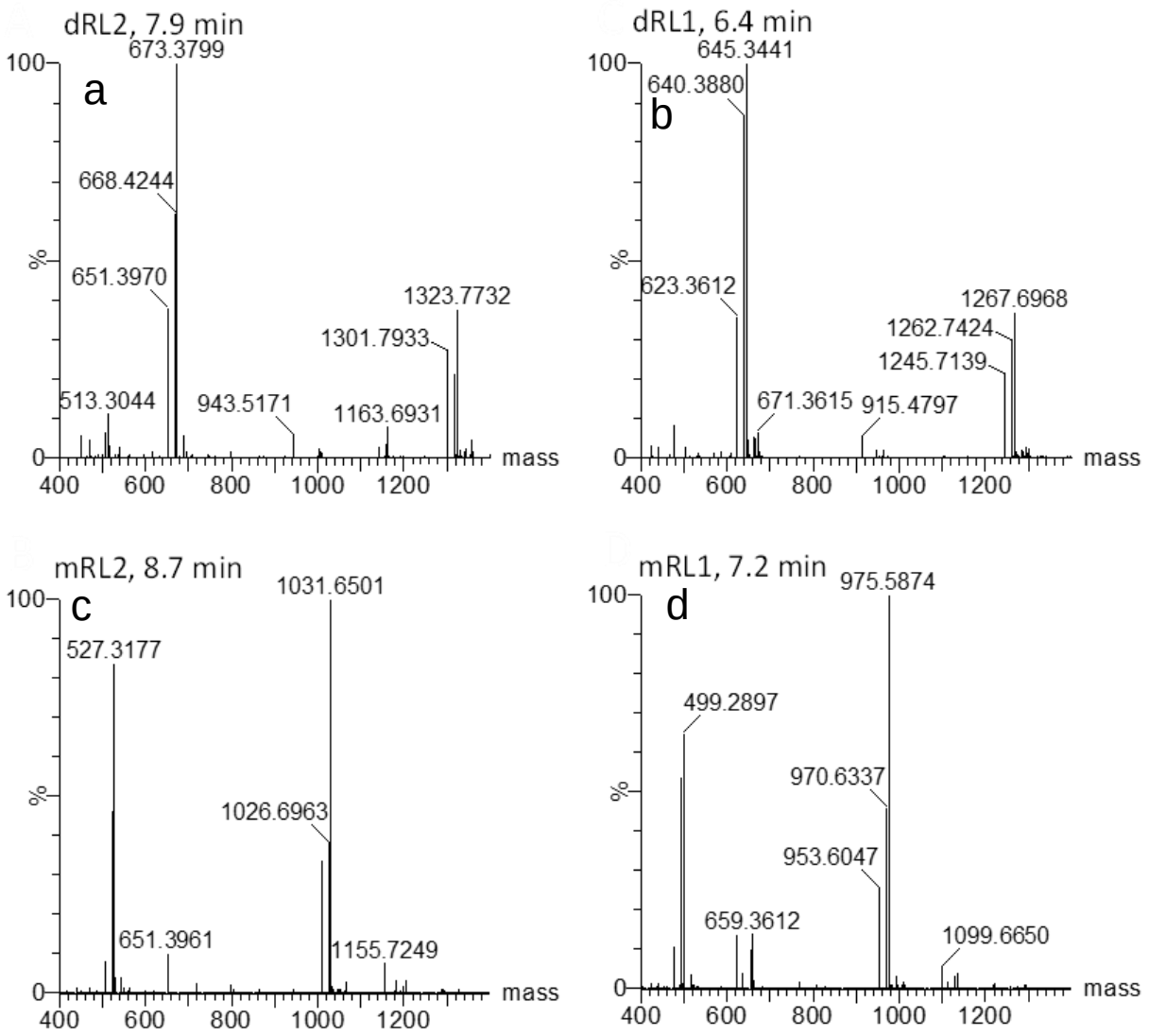

Fig. 4.7 Examples of the ESI-MS mass spectra of major rhamnolipid groups detected with UPLC-MS. Mass spectra were generated with MaxEnt 3. Refer to **Table 4.4** for identities of RL1-4 and expected *m/z* and M_r values

Dimers of the sodiated $[2M-H+Na]^+$ dirhamnolipid Rha-Rha-C_{10}-C_{10} (dRL2) and monorhamnolipid Rha-C_{10}-C_{10} (mRL2) were observed at *m/z* 1323.77 and 1031.65, respectively (**Table 4.4**). Dimers of the sodiated $[2M-H+Na]^+$ dirhamnolipid Rha-Rha-C_{10}-C_8/Rha-Rha-C_8-C_{10} (dRL1) and monorhamnolipid Rha-C_{10}-C_8/Rha-C_8-C_{10} (mRL1) were observed at *m/z* 1267.71 and 975.59, respectively. Similarly, the sodiated dimers of the RL3 group was also detected (**Table 4.4**). The glycerol-MSM and NA cultures of ST5 lead to the production of similar rhamnolipid profiles (**Fig. 4.6**). A total of six rhamnolipid groups (mRL1-3 and dRL1-3) were identified in both the rhamnolipid standard and ST5 culture extracts with high resolution ESI-MS (ppm<10) and their proposed structures are presented in **Table 4.4**.

4.3.5. Antimicrobial activity of biosurfactant extracts

The antimicrobial activity of the identified surfactin and rhamnolipid extracts, produced by ST34 and ST5, respectively, were analysed against various actively growing reference (ATCC), environmental and clinical Gram-positive and Gram-negative bacterial strains (**Table 4.1**) as well as fungal strains (**Table 4.2**). This was achieved by utilising an agar disc susceptibility modified method as outlined in Das et al. (2008). The zones of inhibition produced by each biosurfactant extract against each microbial strain used as a test organism, were recorded. Representative images illustrating the antibacterial and antifungal activity are depicted in **Appendix A (Fig. A1-A3)**.

4.3.6. Antimicrobial activity of ST34 extract

The extracts of strain ST34 were tested against Gram-negative reference (ATCC) strains (n = 10), as well as environmental (n = 8) and clinical (n = 2) strains. Overall, antibacterial activity was observed against all the Gram-negative bacteria (100 %) analysed as test organisms (**Table 4.1**), with varying diameters for the zones of inhibition recorded. For the ten Gram-negative reference strains, the ST34 extracts displayed the lowest zone of inhibition of 10 mm against *E. coli* ATCC 13706 and the highest zone of inhibition against *Salmonella typhimurium* ATCC 14028 at 25.3 ± 1.2 mm (**Table 4.1**). An average zone of inhibition of 15.2 ± 0.6 mm was observed for the reference strains. The ST34 extract was also tested against environmental strains (**Table 4.1**), with the smallest zone of inhibition (9.8 ± 0.8 mm) observed against the *Serratia* sp. L8 strain and the largest zone of inhibition (17.7 ± 1.9 mm) observed against the *E. coli* K4CCA strain. An average zone of inhibition of 13 ± 0.6 mm was observed for all environmental strains. Furthermore, the ST34 extracts displayed zones of inhibition of 13 ± 0.8 and 13.3 ± 0.2 mm against the two clinical *K. pneumoniae* strains (P2 and P3), respectively.

The ST34 extract was then tested against Gram-positive reference strains (n = 3), as well as environmental (n = 5) and clinical (n = 3) strains. Overall, antibacterial activity was observed against 90.1 % of the Gram-positive bacteria analysed as test organisms (**Table 4.1**), with varying diameters for the zones of inhibition recorded. All the reference strains displayed sensitivity against the extract produced by ST34, where the smallest zone of inhibition (10.3 ± 0.5 mm) was observed for *B. cereus* ATCC 10876 and the largest zone of inhibition (14.7 ± 0.5 mm) was observed for *Staphylococcus aureus* ATCC 25923. An average zone of inhibition of 12.7 ± 0.6 mm was observed for the reference strains. For the five environmental strains utilised, the surfactin extract displayed no zone of inhibition against *B. cereus* ST18, while the largest zone of inhibition (14 mm) was obtained against *Micrococcus* sp. AQ4S2. An average zone of inhibition against environmental Gram-positive bacterial strains was observed at 10 ± 0.2 mm. The ST34 extract was also tested against the clinical strains, which displayed the smallest zone of inhibition of

15.3 ± 0.5 mm against *S. aureus* (MRSA) Xen 30, while the largest zone of inhibition was observed at 18.7 ± 0.9 mm against *E. faecalis S2*. An average zone of inhibition of 17.4 ± 0.9 mm was observed for the clinical strains.

Five clinical and five environmental fungal strains were utilised as test organisms for the antimicrobial assessment of solvent extracted from ST34 (**Table 4.2**). The ST34 extract exhibited pronounced antifungal activity against 80 % (4/5) of the clinical strains tested. No antifungal activity was observed against *Cryptococcus neoformans* 1063, while the largest zone of inhibition of 15.3 ± 0.5 mm was observed for C. *neoformans* CAB1055. An average zone of inhibition of 10.7 ± 0.4 mm was observed for the clinical strains. The ST34 extract also displayed antifungal activity against 60 % (3/5) of the environmental fungal isolates utilised in the current study. No zone of inhibition was observed for *C. neoformans* CAB1034 and *Candida albicans* 1085 and the largest zone of inhibition of 15.3 ± 1.2 mm was observed for the *Cryptococcus neoformans* CAB844 environmental strain. An average zone of inhibition of 7.9 ± 0.8 mm was observed for the environmental fungal strains.

4.3.7. Antimicrobial activity of ST5 extract

The extract of strain ST5 was tested against the Gram-negative reference (ATCC) (n = 10), environmental (n = 8) and clinical (n = 2) strains. Overall, antibacterial activity was observed against all the Gram-negative bacterial (100 %) strains analysed as test organisms (**Table 4.1**), with varying diameters for the zones of inhibition recorded. For the reference strains, the ST5 extract displayed the smallest zone of inhibition (13 mm) against Enterotoxigenic *E. coli* H10407, while the largest zone of inhibition (29.3 ± 0.9 mm) was observed against *E. coli* ATCC 13706. An average zone of inhibition of 18.5 ± 0.7 mm was obtained against the reference strains. For the environmental strains, the ST5 extract produced the smallest zone of inhibition of 9.8 ± 0.8 mm against the *Serratia sp. L8* strain, while the largest zone of inhibition of 17.7 ± 1.9 mm was recorded against *E. coli* K4CCA. The average zone of inhibition against the environmental strains was 13.6 ± 0.9 mm. Furthermore, the ST5 extracts displayed zones of inhibition of 8.3 ± 0.5 mm and 11.7 ± 0.9 mm against the two clinical *K. pneumoniae* strains (P2 and P3), respectively.

The ST5 extract was also tested against Gram-positive reference (n = 3), environmental (n = 5) and clinical (n = 3) strains. Overall, antibacterial activity was observed against all the Gram-positive bacterial (100 %) strains analysed as test organisms (**Table 4.1**), with varying diameters for the zones of inhibition recorded. For the reference strains, the smallest zone of inhibition of 13.0 ± 0.8 mm was recorded for *B. cereus* ATCC 10876, while the largest zone of inhibition of 17 ± 1.4 mm was recorded for *B. cereus* LMG 13569. An average zone of inhibition of 14.6 ± 0.9 mm was obtained. For the Gram-positive environmental strains, the smallest zone

of inhibition of 11 mm was recorded for *S. aureus* C3, while the largest zone of inhibition (22.3 ± 0.9 mm) was observed against *B. cereus* ST18. An average zone of inhibition of 15.4 ± 0.9 mm was obtained against the environmental Gram-positive strains. The ST5 extract also displayed activity against all clinical strains, with the smallest zone of inhibition of 10.7 ± 0.5 mm recorded for *E. faecalis* S1 and the largest zone of inhibition of 21.7 ± 2.4 mm recorded for *E. faecalis* S2. The average zone of inhibition produced by the ST5 extract against the clinical strains was 15.2 ± 1.1 mm.

Five clinical and five environmental fungal strains were utilised as test organisms for the antimicrobial assessment of solvent extracted from ST5 (**Table 4.2**). The ST5 extract displayed antifungal activity against 100 % (5/5) of the clinical strains tested. The smallest zone of inhibition of 11.3 ± 0.9 mm was observed for *C. neoformans* CAB 1055 and the largest zone of inhibition (14.7 ± 0.5 mm) was obtained against *C. albicans* 8911 strain. An average zone of inhibition by the ST5 extract against the clinical strains was recorded as 13 ± 1.2 mm. The ST5 extract then displayed 80 % (4/5) antifungal activity against the environmental fungal strains. No zone of inhibition was observed against *C. neoformans* CAB842, and the largest zone of inhibition (18 ± 0.8 mm) was observed against *C. neoformans* 1034. An average zone of inhibition of 12 ± 1 mm was observed for the ST5 extract against the environmental fungal strains.

4.4. Discussion

Bacteria, fungi and yeast producing biosurfactant compounds, which display broad spectrum antimicrobial properties, are usually isolated from diverse terrestrial environments such as the rhizosphere, contaminated soils and hydrocarbon polluted water sources (Bento et al. 2005; Pornsunthorntawee et al. 2008). Initial analysis then indicated that the two bacterial strains ST34 (*B. amyloliquefaciens*) and ST5 (*P. aeruginosa*) isolated from wastewater, produced biosurfactants (Ndlovu et al. 2016). The current study thus focused on the partial purification and characterisation of the antimicrobial lipopeptide and glycolipid biosurfactant compounds produced by ST34 and ST5, respectively. The extracts obtained from the ST34 and ST5 cultures were characterised using a method that was developed in the current study for use with the UPLC-MS analysis, which facilitated the successful detection and separation of different analogues of the surfactin (ST34) and rhamnolipids (ST5) produced by the respective strains.

The solvent extracts obtained from the *B. amyloliquefaciens* ST34 strain was confirmed to contain surfactin lipopeptides, in which the structural surfactin analogues with a mass difference of 14 or 28 amu. These differences are consistent with a CH_2 moiety correlating either to a Val to Ile/Leu modification or longer/branched fatty acyl chain (CH_2-CH_2 moiety). The UPLC-MS separation successfully differentiated between the surfactin analogues in the same mixture, which were identified as C_{13}, C_{14}, C_{15} and C_{16} surfactin analogues (Srf1-5 groups) (**Table 4.3**). The different

groups were observed to have two or more retention times even though they displayed the same *m/z* and M_r values. The Ile containing peptides possibly elute at a slightly different Rt to those substituted with Leu due to the slight differences in their hydrophobicity (Yang et al. 2015), for example Srf4 eluted at 12.1 and 12.2 min (**Table 4.3**). Within each of the five surfactin groups obtained for the ST34 strain, two or more surfactin analogues were detected. The Srf4 group was the most dominant with a relative abundance of approximately 60 % in the ST34 NA culture extracts, while the Srf3 and Srf4 groups were observed at approximately 43 and 33 %, respectively, in the glycerol-MSM culture extracts. Results obtained in the current study are comparable to a study conducted by Pecci et al. (2010), when they successfully identified different surfactin (C_{13} (Srf3), C_{14} (Srf4) and C_{15} (Srf5) surfactins), fengycin A and B analogues compounds produced by *Bacillus licheniformis* V9T14. The authors utilised the LC-ESI-MS/MS for the separation and partial characterisation of the surfactin analogues and fengycin isoforms, as well as the relative percentage content of each compound.

The solvent extracts obtained from the ST5 strain were confirmed to be a mixture of rhamnolipid congeners of monorhamnolipids (Rha-C_{12}-C_{10}/Rha-C_{10}-$C_{12;}$ Rha-C_{10}-C_{10}; Rha-C_{10}-C_8/Rha-C_{10}-C_8) and dirhamnolipids (Rha-Rha-C_{12}-C_{10}/RhaRha-C_{10}-C_{12}; Rha-Rha-C_{10}-C_{10}; Rha-Rha-C_{10}-C_8/ Rha-Rha-C_{10}-C_8). These results are in agreement with a study conducted by Pantazaki et al. (2011), where similar rhamnolipid congeners were detected. Additionally, the detected HAAs in the current study could either be intermediates in rhamnolipid biosynthesis or rhamnolipid fragments obtained by cleavage in the ESI-MS of the rhamnosyl group (hydrophilic moiety) (Lepine et al. 2003). A study conducted by Pereira et al. (2012) on rhamnolipids produced by *P. aeruginosa* strains also illustrated that MS coupled with electrospray ionisation provided an accurate and rapid characterisation of the monorhamnolipids [Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12:1}$] and dirhamnolipids [Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{10}-C_{12}]. Itoh et al. (1971) then produced a mixture of monorhamnolipid (Rha-C_{10}-C_{10}) and dirhamnolipid (Rha-Rha-C_{10}-C_{10}) congeners using the *P. aeruginosa* KY 4025 strain, which they purified and separated using the HPLC based method to obtain individual rhamnolipids. Moreover, they showed that individual rhamnolipids (Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}) displayed pronounced antibacterial activity against both Gram-negative and Gram-positive strains, including a multi-drug resistant *E. coli* strain.

The antimicrobial activity of the surfactin and rhamnolipid congeners produced by ST34 and ST5 against various reference, environmental and clinical bacterial and fungal strains was then determined. Results indicated that both extracts displayed 100 % antibacterial activity against the Gram-negative bacteria analysed (**Table 4.1**). However, based on the average zones of inhibition, the surfactin extract (ST34) exhibited an increased antibacterial activity against the clinical strains (average zone of inhibition of 13.2 ± 0.5 mm), while the rhamnolipid extract (ST5) produced noticeable activity (average zone of inhibition of 18.5 ± 0.7 mm) against the reference target

strains. No significant difference between the surfactin and rhamnolipid extract's antibacterial activity against the Gram-negative environmental ($p = 0.58$) and reference ($p = 0.17$) bacterial strains, respectively, was however observed. In addition, the surfactin extract displayed a higher antibacterial activity against the Gram-positive clinical strains (average zone of inhibition 17.4 ± 0.9 mm), while the rhamnolipid extracts produced an increased antibacterial activity against the Gram-positive environmental (average zone of inhibition 15.4 ± 0.5 mm) and reference strains (average zone of inhibition 14.6 ± 0.9 mm). However, the two tailed t-test showed that there was no significant difference between the zones of inhibition obtained against the clinical ($p = 0.56$) and environmental ($p = 0.12$) Gram-positive strains, respectively for the surfactin and rhamnolipid extracts. Moreover, the surfactin and rhamnolipid extracts displayed no significant difference ($p = 0.34$) between the zones of inhibition obtained against the reference Gram-positive strains. Of particular interest was the sizeable zone of inhibition (22.3 ± 0.9 mm) recorded for the rhamnolipid extract against the *B. cereus* ST18, which was seemingly resistant to the surfactin extract as no zone of inhibition was recorded.

Research has indicated that approximately 5 % of the genome of most *Bacillus* species encodes for the synthesis of antimicrobial compounds (Stein, 2005). Of these structurally diverse antimicrobial compounds, approximately two dozen have been characterised, with the cyclic lipopeptides of three families fengycin, iturin and surfactin displaying antifungal and antibacterial properties (Mandal et al. 2013). Surfactin exhibits an antimicrobial mechanism by accumulating on the surface of the microbial cell (bacteria and fungi) until a threshold concentration is reached. Thereafter they permeate the membrane leading to its disintegration by a detergent-like mechanism (Yao et al. 2012). This disintegration is hypothesised to occur by the formation of pores in the cell membrane of microbial cells thus inducing an increased influx of Ca^{2+} and H^{+} into the cells (Thrane et al. 1999). Comparatively, rhamnolipids have structures and properties similar to that of detergents and have been reported to intercalate into the membrane phospholipid bilayer thereby facilitating the permeability of the membrane and flow of metabolites (Sotirova et al. 2008). The structure and function of the phospholipid bilayer is thus altered, effectively interrupting protein conformation, transport and energy generation, which eventually leads to cell death.

It should be noted that of the 31 bacterial strains analysed in the current study, three strains were resistant to various classes of antibiotics [Enteropathogenic *E. coli* B170 resistant to gentamicin, *S. aureus* ATCC 25923 resistant to oxacillin, *S. aureus* Xen 30 resistant to methicillin, gentamicin, oxacillin and tetracycline (**Table 4.1**)]. The results obtained in the current study indicated that these strains were sensitive to both the surfactin and rhamnolipid extracts produced by ST34 and ST5, respectively. Moreover, 90 % of the fungal strains analysed in the current study were susceptible to the rhamnolipid extract, while only 70 % of the fungal strains were susceptible to the surfactin extract. However, after performing a two-tailed t-test analysis, no significant

difference (p = 0.183) between the zone of inhibition of surfactin and rhamnolipid extracts against the fungal strains analysed was obtained. Yoshida et al. (2001), then showed that the cell free supernatant (containing surfactin) of *B. amyloliquefaciens* RC-2, isolated from healthy Mulberry leaves, strongly inhibited the growth of 44 % and 40 % of bacteria and fungi isolates, respectively. In a study conducted by Sun et al. (2006), a *B. amyloliquefaciens* ES-2 isolate was also shown to produce antimicrobial lipopeptide compounds (fengycins and surfactins), which demonstrated antimicrobial activity against a total of 37 microorganisms (including *E. coli*, *S. aureus* and *B. cereus*). In a study conducted by Abalos et al. (2001), a rhamnolipid mixture that consisted of Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{10}-C_{12}, then displayed broad spectrum antimicrobial activity against a wide range of organisms, including *C. albicans, S. marcescens, B. cereus* and *S. aureus* strains.

In the current study the optimised UPLC-MS method was successfully employed to characterise the extracted surfactin and rhamnolipid mixtures produced by the *B. amyloliquefaciens* ST34 and *P. aeruginosa* (ST5) isolates in liquid and on agar media. The *B. amyloliquefaciens* ST34 strain produced a mixture of surfactin analogues (Srf1-5), which have a synergistic effect on inhibiting bacterial and fungal growth. The most abundant surfactin groups were Srf4>Srf3>Srf2 with minor contributions by Srf 1 and Srf 5. The Rha-C_{10}-C_{10} and Rha-C_{10}-C_8 or Rha-C_{10}-C_8 were the most abundant monorhamnolipids in the extracts, while the Rha-Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_8 or Rha-Rha-C_{10}-C_8 were the most abundant dirhamnolipids produced by the *P. aeruginosa* ST5 strain. In this context, the results indicate that our rapid extraction and UPLC-MS method can be a simple and powerful technique to provide fast, sensitive and accurate identification of a variety biosurfactant compounds synthesised by microbial strains. In addition, pronounced antimicrobial activity against diverse microorganisms, including antibiotic resistant *S. aureus* and *E. coli,* as well as the fungal pathogens *C. albicans* and *C. neoformans* was retained by both the surfactin and rhamnolipid extracts. The two biosurfactant-producing strains isolated from wastewater thus show potential for large-scale production of various analogues/congeners of the surfactin and rhamnolipid biosurfactant compounds for utilisation in the medical and food industries as antimicrobial agents.

Authors' contributions:

TN, WK, and MR conceived and designed the experiments. TN performed the experiments and analysed the antimicrobial activity data. TN and MR analysed the ESI-MS and UPLC-MS data. JAV supervised the experiments for solvent extraction of biosurfactants and sample preparation for UPLC-MS analysis. WK acquired funding for the study. WK and SK contributed reagents/materials/analysis tools. TN, WK and MR wrote the paper. All authors edited the drafts of the manuscript and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

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

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Chapter 5:

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Variants of lipopeptides and glycolipids produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* in different substrates

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Abstract

The quantitative and qualitative effect of water immiscible and miscible carbon-rich substrates on the production of the biosurfactants, surfactin and rhamnolipids, by *Bacillus amyloliquefaciens* ST34 and *Pseudomonas aeruginosa* ST5, respectively, was analysed. A small-scale high throughput 96 deep-well micro-culture method was utilised to cultivate the two strains in mineral salt medium (MSM) supplemented with the water miscible (glucose, glycerol, fructose and sucrose) and water immiscible carbon sources (diesel, kerosene and sunflower oil) under the same growth conditions. The biosurfactants produced by the two strains were isolated by acid precipitation followed by an organic solvent extraction. Ultra-performance liquid chromatography coupled to electrospray ionisation mass spectrometry was utilised to analyse yields and characterise the biosurfactant variants. For *B. amyloliquefaciens* ST34, maximum surfactin production was observed in the MSM supplemented with fructose (28 mg L^{-1}). In addition, four surfactin analogues were produced by ST34 using the different substrates, however, the C_{13}-$_{15}$ surfactins were dominant in all extracts. For *P. aeruginosa* ST5, maximum rhamnolipid production was observed in the MSM supplemented with glucose (307 mg L^{-1}). In addition, six rhamnolipid congeners were produced by ST5 using different substrates, however, Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} were the most abundant in all extracts. This study highlights that the carbon sources utilised influences the yield and analogues/congeners of surfactin and rhamnolipids produced by *B. amyloliquefaciens* and *P. aeruginosa*, respectively. Additionally, glucose and fructose were suitable substrates for rhamnolipid and surfactin, produced by *P. aeruginosa* ST5 and *B. amyloliquefaciens* ST34, which can be exploited for bioremediation or as antimicrobial agents.

Keywords: surfactin; rhamnolipid; *Bacillus amyloliquefaciens* ST34; *Pseudomonas aeruginosa* ST5; carbon sources; UPLC-MS

5.1. Introduction

Biosurfactants are an important class of microbially synthesised compounds that have been extensively researched due to their diverse biological properties and functions (Van Hamme et al. 2006; Gudiña et al. 2013; Kiran et al. 2016). Moreover, owing to their low toxicity and biodegradable nature, they exhibit potential for various commercial applications as environmentally friendly alternatives to synthetic surfactants (Nitschke and Costa 2006). Lipopeptides and glycolipids, in particular, have been exploited for their potential to serve as antimicrobial, antiadhesive, antitumor and antizoospore agents in the medical and pharmaceutical industries (Banat et al. 2010; Raaijmakers et al. 2010).

Lipopeptides are synthesised by means of a multistep pathway mediated by various non-ribosomal peptide synthetase (NRPS) enzymes which catalyse the condensation and selection of amino acid residues to yield various metabolites. Gene expression for surfactin production in *Bacillus* species is reported to be cell density dependent and occurs predominantly in the late exponential and stationary phases of bacterial growth (Gross and Loper 2009). Structural diversity of the lipopeptides then ranges from the varying composition and length of the hydrophobic moiety to the type, number and the configuration of the amino acid present in the hydrophilic moiety (Roongsawang et al. 2010). The lipopeptide structural diversity can significantly influence their biological and physicochemical properties (Bonmatin et al. 2003; Das et al. 2009; Singh et al. 2014), however, lipopeptides are not generally utilised for large-scale commercial production due to the high costs (substrates and downstream processes) associated with their production.

The most effective glycolipids, with strong emulsification and surface activities as well as antimicrobial and antiadhesive properties are rhamnolipids. They are primarily produced by *Pseudomonas aeruginosa* strains as the most prominent secondary metabolite (Syldatk et al. 1985). Rhamnolipid biosynthesis by *P. aeruginosa* occurs in consecutive steps of glycosyl transfer reactions catalysed by different rhamnosyl-transferases, yielding separate activated precursor hydrophilic (mono- or dirhamnose) and hydrophobic (hydoxyfatty acids) moieties. These are then dimerised by the rhamnosyl-transferases and other enzymes (Soberón-Chávez et al. 2005). The production of rhamnolipids by *P. aeruginosa* is tightly regulated by a quorum sensing mechanism, in response to both environmental stress and nutritional factors (Déziel et al. 2003; Reis et al. 2011; Geys et al. 2014). The microbially produced rhamnolipid mixtures display varying properties that depend on the type and proportion of the homologs, which differ, based on the bacterial strain used, culture conditions, medium composition and the type of carbon source used for growth (Abalos et al. 2001; Déziel et al. 1999; Das et al. 2009; Singh et al. 2014).

The selection of a cost-effective substrate to produce biosurfactants is thus particularly crucial for large-scale production, as different types of carbon sources are reported to markedly influence the concentration of biosurfactant compounds produced. In addition, relevant published research has emphasised the effect the carbon source has on the specific congeners/homologues of biosurfactants synthesised by a specific microbial strain (Bonmatin et al. 2003; Das et al. 2009; Singh et al. 2014). A study conducted by Kim et al. (1997) assessed the use of emulsified n-hexadecane, soybean oil and glucose to produce a lipopeptide biosurfactant using a *B. subtilis* C9 strain. Results indicated that the lipopeptide biosurfactant was produced only when glucose was used as a carbon source. Thaniyavarn et al. (2006) also investigated the production of biosurfactants using *P. aeruginosa* A41 isolated from seawater. The microbe was cultured either in a vegetable oil (olive, palm and coconut oils) or a fatty acid (lauric, myristic, palmitic, stearic, oleic or linoleic acids) as the main carbon source. Different rhamnolipid concentrations of 2.91, 2.93 and 6.58 g L^{-1} were obtained with the palm, coconut and olive oils, respectively. In the case of the fatty acid substrates, the rhamnolipid concentration ranged from 0.26 g L^{-1} (palmitic acid) to 4.99 g L^{-1} (linoleic acid). However, the rhamnolipid obtained when *P. aeruginosa* UW-1 was cultured in fatty acids had shorter chain lengths and caused a high oil displacement activity when compared with yields obtained when vegetable oil was used (Thaniyavarn et al. 2006). The authors then concluded that cost-effective production of industrial volumes of rhamnolipid was possible when using *P. aeruginosa* UW-1 isolates cultured using palm oil as the carbon source.

The primary aim of the current study was to assess the quantitative and qualitative effects of different carbon sources have on the production of rhamnolipid and surfactin by *Pseudomonas aeruginosa* (ST5) and *Bacillus amyloliquefaciens* (ST34), respectively. This objective was achieved by culturing each bacterial strain on mineral salt medium (MSM) supplemented with water miscible (glucose, glycerol, fructose and sucrose) or water immiscible carbon substrates (diesel, kerosene and sunflower oil) using the high throughput production method as previously described by Vosloo et al. (2013). Ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) was then used to characterise the crude biosurfactant compounds and determine their respective approximate concentrations. The ideal carbon sources required by each microorganism for maximum yields and diversity of biosurfactant compounds were identified.

5.2. Materials and methods

5.2.1. Pre-culturing of biosurfactant producing isolates

Biosurfactant producing bacteria were isolated from wastewater samples collected from Stellenbosch wastewater treatment plant in the Western Cape, South Africa (GPS co-ordinates: -33.943505, 18.824584) as described by Ndlovu et al. (2016). The bacterial isolates ST34,

identified as *B. amyloliquefaciens* (collection number SARCC 696 at the South African Rhizobium Culture Collection) and ST5, identified as *P. aeruginosa* (collection number SARCC 697 at the South African Rhizobium Culture Collection), using molecular characterisation (Ndlovu et al. 2016), were utilised in the current study. Henceforth the *B. amyloliquefaciens* and *P. aeruginosa* isolates will be referred to by their code identifiers, ST34 and ST5, respectively. Utilising a UPLC-MS method, the ST34 and ST5 strains have previously been shown to produce surfactin and rhamnolipid biosurfactants, respectively (Ndlovu et al. unpublished data). The ST34 and ST5 bacterial strains were thus utilised in the current study to assess the effect of MSM supplemented with various water immiscible and miscible substrates as sole carbon sources for the production of various surfactin analogues and rhamnolipid congeners.

The bacterial isolates were maintained in 40 % glycerol at -80 ºC. A loopful of the glycerol stock of each isolate was transferred onto nutrient agar, streaked and incubated at 37 ºC for 18-24 hrs. Single colonies were inoculated onto 5 mL Luria Bertani (LB) broth, and incubated at 37 ºC for 18-24 hrs. This inoculum was used as a seed culture to inoculate the MSM that was supplemented with various carbon sources (diesel, fructose, glucose, glycerol, kerosene, sucrose and sunflower oil).

5.2.2. High throughput 96 deep-well production of biosurfactants

The high throughput 96 deep-well plate production method was adapted from a previous study conducted by Vosloo et al. (2013). Mineral salt medium was prepared as previously described by Silva et al. (2010) and was supplemented with various substrates as sole carbon sources as follows: 3 % diesel (Total South Africa, Johannesburg, South Africa), 3 % D(-) fructose (Saarchem (Pty) LTD, Johannesburg, South Africa), 3 % D(+) monohydrate glucose (Kimix chemicals and lab suppliers cc, Cape Town, South Africa), 3 % glycerol (Merck, Darmstadt, Germany), 3 % kerosene (Sigma-Aldrich, St. Louis, USA), 3 % sucrose (Merck chemicals, Johannesburg, South Africa) and 3 % sunflower oil (SPAR South Africa (Pty) LTD, Pinetown, South Africa). Aliquots of 50 µL of the overnight culture broth of each bacterium (ST34 or ST5) were then pipetted into the wells (in triplicate) of the sterile 96 deep-well plate containing 500 µL of MSM supplemented with 3 % of the respective substrates utilised as sole carbon sources. The 96 deep-well plates were sealed and were incubated for 120 hrs at 30 ºC on an orbital shaker (MRCLAB, London, UK) (Vosloo et al. 2013).

The solvent extraction of biosurfactant compounds produced by ST34 and ST5 was conducted as outlined in Vosloo et al. (2013). The ST34 and ST5 strains cultured in the respective carbon sources in the 96 deep-well plate were acidified with concentrated hydrochloric acid (HCl, Merck, Darmstadt, Germany) to a pH of approximately 4 and were allowed to stand at ambient temperature for 24 hrs. Thereafter, the 96 deep-well plates were centrifuged at 2 200 × *g* for

60 min, the pellets were re-suspended in 200 µL of 100 % acetonitrile (Romil, Cambridge, UK) and were sonicated for 15 min. A further 200 µL of analytical quality water (prepared through a MilliQ system from Millipore, Billerica, USA) was added to each well, the plates were sonicated for 15 min and then centrifuged at 2 200 × *g* for 30 min. Respective supernatants were then transferred into analytically weighed vials, lyophilised and the mass for each extract was analytically determined. Extracts were then dissolved in 70 % (v/v) acetonitrile to 10.00 mg mL^{-1}, centrifuged at 8 600 × *g* for 10 min to remove particulates and a ten times dilution was performed using analytical quality water to obtain a final concentration of 1.00 mg mL^{-1}. Extracts were subsequently analysed using electrospray ionisation mass spectrometry (ESI-MS) and UPLC-MS.

5.2.3. Analysis with ultra-performance liquid chromatography linked to mass spectrometry

Mass spectrometry analyses were conducted in the LCMS Central Analytical Facility at Stellenbosch University. A Waters Quadrupole Time-of-Flight Synapt G2 (Waters Corporation, Milford, USA) mass spectrometer was utilised for the ESI-MS and was coupled to an Acquity UPLC for the UPLC-MS analysis of the biosurfactant extracts. All extracts were subjected to UPLC-MS analysis. Briefly, 3 µL sample (each extract obtained from MSM supplemented with different substrates as sole carbon sources) was separated on an Acquity UPLC C18 reverse-phase analytical column (Acquity UPLC® HSS T3, 1.8 µm particle size, 2.1 x 150 mm, Waters corporation, Dublin, Ireland) at a flow rate of 0.300 mL min^{-1} using a 0.1 % formic acid (A) to acetonitrile (B) gradient [60 % (A) from 0 to 0.5 min for loading, gradient was from 40 to 95 % (B) from 0.5 to 11 min and then 95 to 40 % (B) from 15 to 18 min]. The UPLC-MS profiles of the biosurfactant compounds were compared to those obtained for surfactin and rhamnolipid standards (Sigma-Aldrich, St. Louis, USA). The approximate yields of the surfactin and rhamnolipid compounds in the solvent extracts obtained from the ST34 and ST5 cultures, respectively, were also determined using the surfactin and rhamnolipid standards (concentration of 1.00 mg/mL).

The analytes were subjected to a capillary voltage of 3 kV, cone voltage of 15 V and a source temperature of 120 ºC. Data acquisition in the positive mode was performed by MS scanning a second analyser through the *m/z* range of 200-3000 and the data was thereafter analysed using MassLynx software version 4.1 SCN 714 (Waters Corporation, Milford, USA).

5.2.4. Statistical analysis

The yield of surfactin and rhamnolipids produced by ST34 and ST5 strains, respectively, grown in the different substrates were expressed as mean values ± standard error of mean. The one-

way analysis of variance (ANOVA) was then utilised to determine the statistical difference in the yield of surfactin and rhamnolipids produced by ST34 and ST5, respectively, when grown on various substrates as sole carbon sources. GraphPad Prism software version 7.02 (GraphPad Software, Inc. San Diego, USA) was utilised to perform one-way ANOVA. The data was considered statistically significant if $p < 0.05$.

5.3. Results

The *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5 strains utilised in the current study, were previously shown to produce surfactin and rhamnolipids, respectively when cultivated in MSM supplemented with glycerol as a sole carbon source (Ndlovu et al. unpublished data). In the current study, the production profile of surfactin and rhamnolipids by the ST34 and ST5 strains, respectively, when cultured in MSM supplemented with a variety of alternative carbon sources was assessed.

5.3.1. Effect of carbon source on the surfactin production by *Bacillus amyloliquefaciens* ST34

A small-scale high throughput method (96 deep-well plate) was utilised to culture the ST34 strain in MSM supplemented with different water immiscible (diesel, kerosene and sunflower oil) and water miscible (glycerol, glucose, fructose and sucrose) substrates (Vosloo et al. 2013). The extracts were obtained from the ST34 MSM cultures (96 deep-well plates) by the solvent (acetonitrile) extraction method and were subjected to UPLC-MS analysis.

For all the ST34 extracts (obtained from MSM supplemented with different carbon-rich substrates), the ion spectra in positive mode showed the main surfactins with molecular ions at *m/z* 1008.66, 1022.68 and 1036.69, which corresponded to the protonated singly charged species $[M+H]^+$ (**Appendix B Fig. S1, S2**, **Table 5.1**). The ion spectra in positive mode also showed the minor surfactin group at *m/z* 994.65 (results not shown). Within the spectrum, singly charged protonated molecular species $[M+H]^+$ of each type of surfactin differed by a mass of 21.99 atomic mass units (amu) and this difference was consistent with the expected singly charged sodiated molecules $[M+Na]^+$ observed at *m/z* 1016.63, 1030.64, 1044.66 and 1058.68 (**Table 5.1**). The observed relative molecular mass (M_r) values of the four groups of molecules corresponded to that of the different surfactin groups denoted Srf1-4 (**Appendix B Fig. S1, S2**, **Table 5.1**), which corresponded to the expected M_r values of known surfactin analogues (**Table 5.1**). The UPLC-MS profiles of the surfactin standard and the extracts produced by ST34 showed significant peaks at retention times between 10 and 13 min, which correlated well with results obtained in Ndlovu et al. (unpublished data). In the current study the surfactin groups then eluted as follows, surfactin group 1 (Srf1) (Rt 10.7, 10.8, 11.5, 11.6 min), Srf2 (Rt 11.3, 11.4, 12.1, 12.2 min), Srf3

(Rt 11.8, 11.9, 12 min) and Srf4 (Rt 12.4 min) (**Fig. 5.1**, **Table 5.1**). Examples of the UPLC-MS profiles of representative of the ST34 extracts are shown in **Fig. 5.1**.

Table 5.1. Summary of the surfactins extracted from *B. amyloliquefaciens* ST34, as detected with high resolution mass spectrometry (<10ppm). The proposed chemical structures, theoretical (Theor) and experimental (Exp) M_r and monoisotopic *m/z* values, as well as observed UPLC retention times for representative examples are provided.

Surfactin group (Abbr)	Rt (min) #	Characterised and proposed* peptide sequences in surfactin group	Mono-isotopic Exp/Theor M_r	Protonated species Exp/Theor *m/z*	Sodiated species Exp/Theor *m/z*
Surfactin 1 (Srf1)	10.7; 10.8; 11.5; 11.6	cyclo[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	993.6376	994.6512	1016.6265
		cyclo[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	993.6403	994.6481	1016.6259
Surfactin 2 (Srf2)	11.3; 11.4; 12.1; 12.2	cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**] cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**] cyclo-[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**]	1007.6565	1008.6644	1030.6390
		cyclo[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**] *cyclo-[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo-[(**$C_{13}H_{24}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1007.6552	1008.6596	1030.6416
Surfactin 3 (Srf3)	11.8; 11.9; 12.0	cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**] cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Val**]	1021.6693	1022.6780	1044.6627
		cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**] *cyclo-[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo-[(**$C_{14}H_{26}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1021.6715	1022.6752	1044.6572
Surfactin 4 (Srf4)	12.4	cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1035.6819	1036.6898	1058.6818
		*cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] cyclo[(**$C_{15}H_{28}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1035.6881	1036.6909	1058.6729
Surfactin 5 (Srf5)	-	cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Leu**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	ND	ND	ND
		*cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Leu**] *cyclo[(**$C_{16}H_{30}O_2$**)-L-Glu-**L-Ile**-D-Leu-L-Val-L-Asp-L-Leu-**L-Ile**]	1049.7032	1050.7066	1072.6886

#UPLC Retention time of main peaks corresponding to the groups *m/z* values, ND – Not detected

For the extracts obtained from the ST34 cultivated in MSM supplemented with the water miscible substrates (glucose, fructose, sucrose and glycerol) and water immiscible substrates (diesel, kerosene and sunflower oil), four major peaks/peak clusters were observed on the UPLC-MS profile, which corresponded to the four surfactin groups (Srf1-4) (**Table 5.1**).

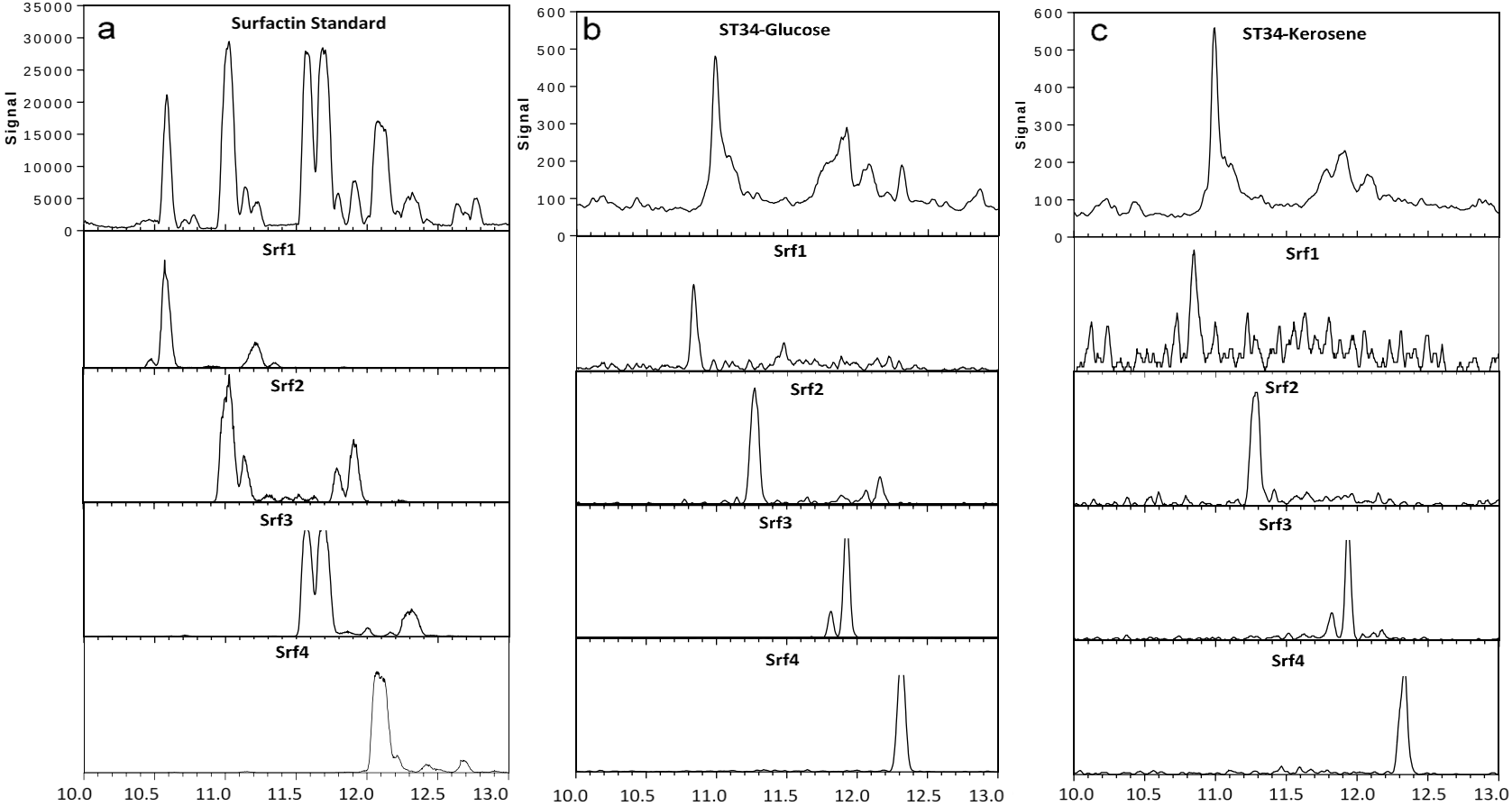

Fig. 5.1. UPLC-MS profiles of the surfactin standard (a); ST34 Glucose-MSM extract (b); ST34 Kerosene-MSM extract (c). The top row profiles depict the signal of positive molecular ions detected between 10 and 13 min. Note the difference in Y axis which are a direct indication of amounts. The profiles below each top row spectrum show the extracted spectra of the four surfactin groups with Srf1 = *m/z* 994.65, Srf2 = m/z 1008.66; Srf3 = *m/z* 1022.68 and Srf4 = *m/z* 1036.69

5.3.2. Relative quantification of surfactin in ST34 extracts

The approximate yields of the surfactin compounds in the solvent extracts obtained from the ST34 cultures were determined using the surfactin standard. This was achieved by totalling the ionisation intensities of all the protonated [M+H]+ surfactin groups (Srf1-4) detected in standard surfactin (**Table 5.2**), which was assumed equal to 1.00 mg mL^{-1} for comparative purposes only, as the absolute purity of the surfactin standard is unknown. The signal intensity of each surfactin group was then utilised to determine the concentration of the respective individual surfactin groups in the ST34 extracts relative to that in the characterised standard surfactin (**Table 5.2**). The ST34 extracts were divided into two groups based on the different type of substrate (water miscible and immiscible) utilised as a source of carbon for the growth and production of surfactin by the ST34 strain.

Table 5.2. Comparison of the approximate quantities of each surfactin group and the total surfactin production profile by *B. amyloliquefaciens* ST34 grown in mineral salt medium supplemented with different substrates as sole carbon sources. Each value represents the average of three culture extracts with standard error of the mean (SEM).

Carbon substrate	Surfactin groups (mg L^{-1})				Total surfactin mg L^{-1} culture
	Srf1	Srf2	Srf3	Srf4	
Diesel	0.8 ± 0.2	1.4 ± 0.3	1.5 ± 0.4	2.3 ± 0.7	6.0 ± 1.6
Kerosene	0.3 ± 0.1	0.9 ± 0.5	1.0 ± 0.6	1.8 ± 1.2	4.1 ± 2.3
Sunflower oil	0.3 ± 0.04	0.7 ± 0.3	0.9 ± 0.5	1.4 ± 1.0	3.3 ± 1.9
Fructose	1.1 ± 0.7	4.4 ± 3.0	11.2 ± 8.6	11 ± 3.9	28 ± 16
Glucose	0.4 ± 0.1	0.7 ± 0.3	1.5 ± 0.9	1.2 ± 0.6	3.7 ± 1.9
Glycerol	0.4 ± 0.1	1.1 ± 0.3	1.2 ± 0.4	1.6 ± 0.4	4.3 ± 1.2
Sucrose	0.5 ± 0.1	1.3 ± 0.1	3.4 ± 0.9	2.3 ± 1.0	7.6 ± 2.0
Surfactin standard	**215.09**	**400.82**	**318.45**	**58.74**	**1000***

*Total concentration of standard surfactin include concentration of the other surfactin variants observed at 6.9 mg L^{-1}

For the water immiscible substrates (diesel, sunflower oil and kerosene), the ST34 strain produced the highest total surfactin of 6.0 ± 1.6 mg L^{-1} in the extract obtained from the MSM supplemented with diesel, while the lowest concentration of 3.3 ± 1.9 mg L^{-1} was observed in the MSM supplemented with sunflower oil (**Table 5.2**).

The relative abundance of each surfactin group within the complex surfactin lipopeptides in the ST34 extract obtained from the MSM supplemented with water immiscible substrate cultures was inferred from the extracted chromatograms by combining the peak areas of each surfactin group eluting between 10 and 13 min.

The relative contribution for each surfactin group in an extract is illustrated in **Fig. 5.2a**, which indicated that the Srf1 group was below 15 % abundance in all three ST34 extracts obtained from

the water immiscible substrates. The Srf2, Srf3 and Srf4 were the main surfactin groups detected in the ST34 extracts obtained for the water immiscible substrates as illustrated in **Fig. 5.2a**. The Srf4 group containing a longer branched fatty acyl chain (C_{15}), was produced in higher quantities, with a relative abundance of 37, 42 and 43 % in the ST34 extracts obtained from the diesel, sunflower oil and the kerosene, respectively (**Fig. 5.2a**). The total surfactin concentration of the Srf4 group then corresponded to 2.3 ± 0.7, 1.4 ± 1.0 and 1.8 ± 1.2 mg L^{-1}, in the ST34 extracts obtained from the MSM supplemented with diesel, sunflower oil and kerosene, respectively (**Table 5.2**).

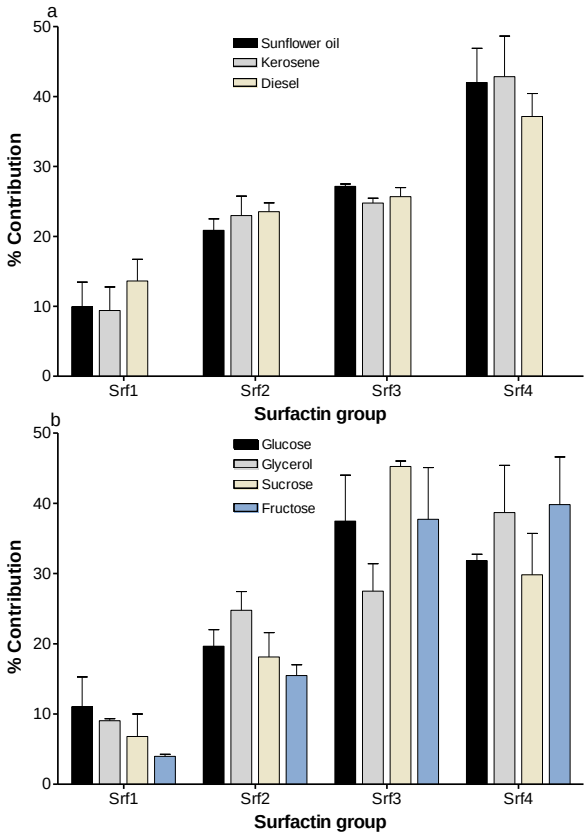

Fig. 5.2. Comparison of the extracts obtained from ST34 cultivated in mineral salt medium supplemented with (a) water immiscible substrates and (b) water miscible substrates, showing the relative contribution of each of the surfactin groups in the biosurfactant extracts. The contribution was calculated from UPLC profiles, with the assumption that all the surfactin species have similar ion responses. Each bar represents the average of three culture extracts with standard error of the mean (SEM)

For the water miscible substrates (glucose, glycerol, fructose and sucrose), the ST34 strain produced the highest total surfactin of 28 ± 16 mg L^{-1} in the extract obtained from the MSM supplemented with fructose, while the lowest concentration of 3.7 ± 1.9 mg L^{-1} was obtained in the MSM supplemented with glucose (**Table 5.2**).

The surfactin Srf1 group constituted approximately 11 % relative abundance of the total surfactin produced by the ST34 strain grown in water miscible substrates (glucose, glycerol, fructose and

sucrose) (**Fig. 5.2b**). Similar to the results obtained for the water immiscible substrates, Srf2, Srf3 and Srf4 were the main surfactin groups detected in the ST34 extracts obtained in the water miscible substrates as illustrated in **Fig. 5.2b**. The highest relative abundance of the Srf2 group (25 %) was obtained in the glycerol extract, while the fructose extract yielded a 16 % relative abundance (**Fig. 5.2b**, **Table 5.2**). For the Srf3 group, the highest relative abundance of 44 % was observed in the sucrose extract, while the lowest abundance of 28 % was observed in the glycerol extract. The Srf4 group then showed a relative abundance of 39, 37, 32, and 30 % in the ST34 extracts obtained from the fructose, glycerol, glucose and sucrose, respectively (**Fig. 5.2b**). The total surfactin concentration of the Srf4 group then corresponded to 11 ± 3.9, 1.6 ± 0.4, 1.2 ± 0.6 and 2.3 ± 1.0 mg L^{-1}, in the ST34 extracts obtained from the MSM supplemented with fructose, glycerol, glucose and sucrose, respectively (**Table 5.2**).

Statistical analysis was performed to determine if there was any significant difference between the surfactin yields when ST34 was grown in MSM supplemented with the different substrates. ANOVA analysis then indicated that no significant difference was observed between the surfactin quantities produced by the ST34 cultivated in MSM supplemented with water immiscible substrates [diesel vs kerosene ($p = 0.99$), diesel vs sunflower ($p = 0.95$) and kerosene vs sunflower oil ($p > 0.99$)]. For the water miscible substrates, ANOVA analysis also indicated no significant difference in the quantities of surfactin produced by ST34 grown in glucose, glycerol and sucrose [glucose vs glycerol ($p > 0.99$), glucose vs sucrose ($p = 0.89$) and glycerol vs sucrose ($p = 0.95$)]. However, a significant difference in the concentration of surfactin in the fructose extracts was obtained when compared to the other water miscible substrates [fructose vs glucose ($p < 0.0001$), fructose vs glycerol ($p < 0.0001$) and fructose vs sucrose ($p < 0.0001$)].

5.3.3. Effect of carbon source on the rhamnolipid production by *Pseudomonas aeruginosa* ST5

The small-scale high throughput method (96 deep-well plate) was also utilised to culture the ST5 strain in MSM supplemented with different water immiscible (diesel, kerosene and sunflower oil) and water miscible (glycerol, glucose, fructose and sucrose) substrates (Vosloo et al. 2013). The extracts were obtained from the ST5 MSM cultures in the 96 deep-well plates by the solvent (acetonitrile) extraction method and were subjected to ESI linked to UPLC-MS analysis. For all the ST5 extracts (obtained from MSM supplemented with different substrates), the ion spectra in positive mode showed the main groups of molecular ions at *m/z* 477.31, 505.34, 533.37, 623.37, 651.4 and 679.43, which corresponded to the protonated $[M+H]^+$ molecular species of known rhamnolipids (**Table 5.3** and **Appendix B Fig. S3**). Corresponding sodium adduct $[M+Na]^+$ molecular ions were also observed at *m/z* 499.29, 645.35, 527.32, 673.38, 555.35 and 701.41. The singly charged protonated $[M+H]^+$ molecular species differed by a mass of 21.99 amu with

the singly charged sodiated $[M+Na]^+$ species of the rhamnolipids (**Appendix B Fig. S3**). This was consistent in all the ST5 extracts, as well as in the rhamnolipid standard (**Table 5.3**).

Table 5.3. Summary of the rhamnolipids extracted from cultures of *P. aeruginosa* ST5, as detected with high resolution mass spectrometry (<10 ppm). The proposed chemical structures, theoretical (Theor) and experimental (Exp) M_r and monoisotopic *m/z* values, as well as observed UPLC retention times for representative examples are provided.

Rhamnolipid group (Abbr)	UPLC Rt (min)#	Proposed structures of rhamnolipids	Mono-isotopic Exp/Theor M_r	Protonated species Exp/Theor *m/z*	Sodiated species Exp/Theor *m/z*	Sodiated dimeric species Exp/Theor *m/z*
mRL1	7.46	Rha-C_8-C_{10} Rha-C_{10}-C_8	476.3047 476.2985	477.3089 477.3063	499.2896 499.2883	975.5889 975.5868
dRL1	6.6 6.5	Rha-Rha-C_8-C_{10} Rha-Rha-C_{10}-C_8	622.3576 622.3564	623.3654 623.3642	645.3471 645.3462	1267.7074 1267.7026
mRL2	9.03	Rha-C_{10}-C_{10}	504.3305 504.3298	505.3383 505.3376	527.3201 527.3196	1031.6501 1031.6494
dRL2	7.69, 7.85, 8.07, 8.25, 8.42	Rha-Rha-C_{10}-C_{10}	650.3894 650.3877	651.3972 651.3955	673.3772 673.3775	1323.7701 1323.7652
mRL3	10.56	Rha-C_{12}-C_{10} Rha-C_{10}-C_{12}	532.3640 532.3611	533.3700 533.3689	555.3546 555.3509	1087.7201 1087.7120
dRL3	9.6 9.7	Rha-Rha-C_{12}-C_{10} Rha-Rha-C_{10}-C_{12}	678.4177 678.4190	679.4285 679.4268	701.4114 701.4088	1379.8352 1379.8278

#UPLC Retention time of main peaks corresponding to the group's *m/z* value

The rhamnolipid congeners detected in the culture extracts were also present in the rhamnolipid standard, which previously facilitated the identification of the congeners produced by the ST5 strain when grown in MSM supplemented with glycerol (Ndlovu et al. unpublished data). Examples of the UPLC-MS profiles of the ST5 extracts from supplemented cultures are shown in **Fig. 5.3**.

The ST5 extracts showed the most dominant singly charged sodiated $[M+Na]^+$ molecular species at *m/z* 645.35, 673.38, 701.41, 499.29, 527.32, 555.35, which is in agreement with that of the dirhamnolipids Rha-Rha-C_8-C_{10}/Rha-Rha-C_{10}-C_8 (dRL1), Rha-Rha-C_{10}-C_{10} (dRL2), and Rha-Rha-C_{12}-C_{10}/Rha-Rha-C_{10}-C_{12} (dRL3) and monorhamnolipids, Rha-C_8-C_{10}/Rha-C_{10}-C_8 (mRL1), Rha-C_{10}-C_{10} (mRL2) and Rha-C_{12}-C_{10}/Rha-C_{10}-C_{12} (mRL3), respectively (**Table 5.3**).

Extracts obtained from the ST5 strain grown in MSM supplemented with water miscible substrates (glucose, glycerol and fructose) produced six major peaks observed on the UPLC-MS profile (**Fig. 5.3**, **Table 5.3**). The sucrose MSM extract however, only produced five significant peaks, which corresponded to dRL1-3 and mRL2 and 3. In comparison, the extracts obtained from the ST5

strain grown in MSM supplemented with diesel, kerosene and sunflower MSM extracts produced two (dRL2 and mRL2), five (dRL1-dRL3 and mRL1 and mRL2) and six (all rhamnolipid groups) peaks, respectively (results not shown).

The UPLC-MS profiles of the rhamnolipid standard and the extracts produced by ST5 showed significant peaks at retention times between 6 and 11 min (**Fig. 5.3**) and correlated with results obtained as outlined in Ndlovu et al. (unpublished data). In this study, the rhamnolipid groups eluted as follows, dirhamnolipid group 1 (dRL1) (Rt 6.6 and 6.5 min), 2 (dRL2) (Rt 7.69, 7.85, 8.07, 8.25 and 8.42 min), 3 (dRL3) (Rt 9.6 and 9.7 min) and monorhamnolipid group 1 (mRL1) (Rt 7.46 min), 2 (mRL2) (9.03 min) and 3 (mRL3) (Rt 10.56) (**Fig. 5.3**, **Table 5.3**).

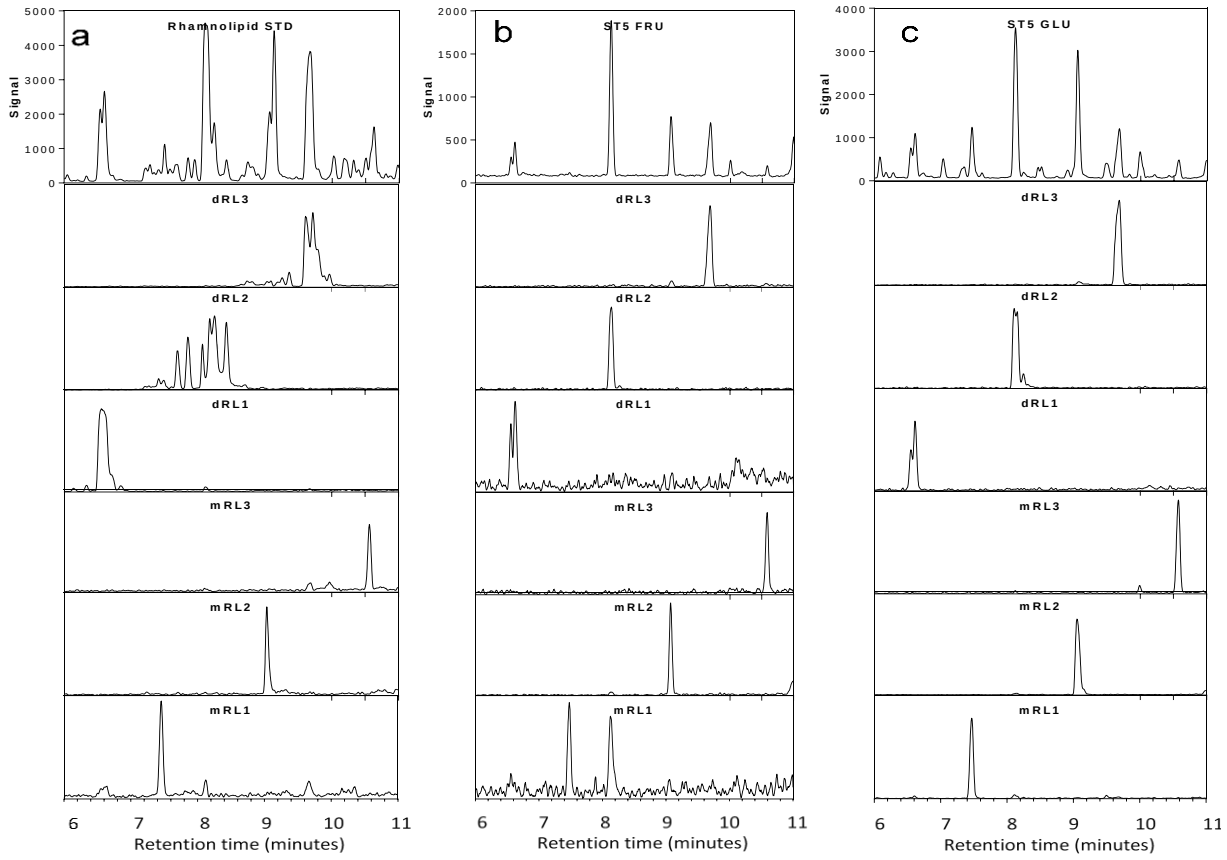

Fig. 5.3. UPLC-MS profiles of rhamnolipid standard (a); ST5 Fructose-MSM extract (b); ST5 Glucose-MSM extract (c). The top row profiles show the signal of positive molecular ions detected between 6 and 11 min. Note the difference in Y axis which is a direct indication of amounts. The profiles below each top row spectrum show the extracted spectra of the rhamnolipid group

5.3.4. Relative quantification of rhamnolipid groups in ST5 extracts

The approximate yields of the rhamnolipid compounds in the solvent extracts obtained from the ST5 cultures were determined using the rhamnolipid standard. This was achieved by totalling the ionisation intensities of all the sodiated $[M+Na]^+$ rhamnolipid groups (dRL1-3 and mRL1-3) detected in the standard rhamnolipid, which was assumed as 1.00 mg mL^{-1} for comparative

purposes only, as the absolute purity of the rhamnolipid standard is unknown. The relative ionisation intensity of each rhamnolipid group in the standard rhamnolipid was then utilised to determine the concentration of their respective individual rhamnolipid group detected in the ST5 extracts (**Table 5.4**).

Table 5.4. Comparison of the approximate quantities of each rhamnolipid group and the total rhamnolipid production profile by *P. aeruginosa* ST5 grown in mineral salt medium supplemented with different substrates as sole carbon sources. Each value represents the average of three culture extracts with standard error of the mean (SEM).

Carbon substrate	Rhamnolipid groups (mg L^{-1})						Total* rhamnolipid mg L^{-1} culture
	dRL3	dRL2	dRL1	mRL3	mRL2	mRL1	
Diesel	0	38 ± 34	0	0	18 ± 15	0	56 ± 49
Kerosene	19 ± 1.2	38 ± 9.7	0	7.4 ± 13	40 ± 3.3	0.01 ± 0.0	104 ± 6.8
Sunflower oil	16 ± 3.2	25 ± 3.6	35 ± 0.5	16 ± 13	27 ± 4.3	0.5 ± 0.5	119 ± 37
Fructose	40 ± 9.5	57 ± 6.7	15 ± 26	17 ± 15	43 ± 8.7	26 ± 4.4	199 ± 57
Glucose	68 ± 32	66 ± 25	20 ± 34	43 ± 18	75 ± 34	36 ± 21	307 ± 147
Glycerol	63 ± 49	60 ± 38	16 ± 28	39 ± 37	71 ± 40	18 ± 16	267 ± 202
Sucrose	10 ± 9.9	21 ± 6.7	5.8 ± 10	7.1 ± 12	28 ± 12	0	72 ± 50
RL Standard	**224.4**	**176**	**99.2**	**316.8**	**142.9**	**40.7**	**1000**

* Approximate values relative to detected signal in 1.00 mg L^{-1} rhamnolipid standard

The approximate concentration of the total rhamnolipids produced by the ST5 strain grown in the water immiscible substrates ranged from 56 ± 49 (diesel-MSM extract) to 119 ± 37 mg L^{-1} (sunflower oil-MSM extract) (**Table 5.4**). The sunflower-MSM extract contained all six rhamnolipid groups, with dRL1 produced at 35 ± 0.5 mg L^{-1}, which corresponded to a relative abundance of 29 % (**Fig. 5.4**). In contrast, the other two water immiscible extracts (diesel and kerosene) predominantly produced the dRL2 and mRL2 rhamnolipid groups, as indicated in **Fig. 5.4a** and **Table 5.4**.

For the water miscible substrates (glycerol, glucose, fructose and sucrose), the ST5 strain produced the highest total rhamnolipid of 307 ± 147 mg L^{-1} in the glucose-MSM extract, while the lowest concentration of 72 ± 50 mg L^{-1} was observed in the sucrose-MSM extract (**Table 5.4**). The abundance of each rhamnolipid group in the various ST5 extracts also varied, with the dRL2 and mRL2 groups constituting above 21 % relative abundance each.

The highest relative abundance of the mRL2 (39 %) was observed in the sucrose MSM extract however, the mRL1 group was not detected in this extract. The dRL1 and mRL1 groups were the least abundant and they were observed at less than 8 and 12 %, respectively in the total

rhamnolipids produced by the ST5 strain (**Fig. 5.4b**). Overall, the dRL2 and mRL2 were the dominant rhamnolipid groups produced in water miscible extracts as indicated in **Fig. 5.4b**.

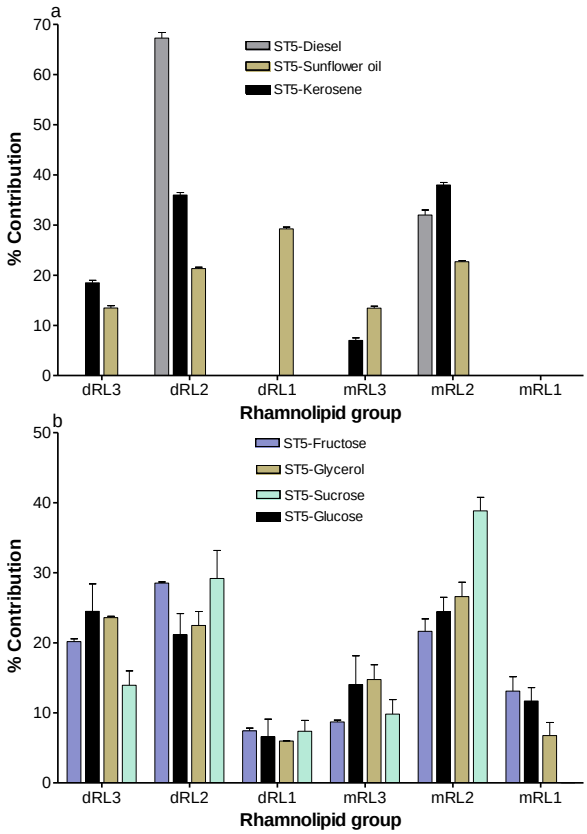

Fig. 5.4. Comparison of the extracts obtained from ST5 cultivated in mineral salt medium supplemented with (a) water immiscible substrates and (b) water miscible substrates, showing the relative contribution of each of the rhamnolipid groups in the biosurfactant extracts. The contribution was calculated from UPLC profiles, with the assumption that all the rhamnolipid species have similar ion responses

Statistical analysis was performed to determine if there was any significant difference between the rhamnolipid yields when ST5 was grown in MSM supplemented with different substrates. ANOVA analysis then indicated that no significant difference was observed between the rhamnolipid yields produced by the ST5 cultivated in MSM supplemented with water immiscible substrates [diesel vs kerosene ($p = 0.0997$), diesel vs sunflower ($p > 0.0991$) and kerosene vs sunflower oil ($p = 0.998$)]. ANOVA analysis also indicated no significant difference in the total rhamnolipid produced by ST5 grown in MSM supplemented with certain water miscible substrates [glucose vs glycerol ($p = 0.9552$), fructose vs glycerol ($p = 0.6461$) and fructose vs glucose ($p = 0.01269$)]. However, as sucrose yielded the lowest concentration of rhamnolipid overall, a significant difference in the quantity of rhamnolipid produced in the sucrose extracts was thus obtained when compared to the other water miscible substrates [fructose vs sucrose ($p = 0.0407$), glucose vs sucrose ($p < 0.0001$), glycerol vs sucrose ($p = 0.0002$)].

5.4. Discussion

The biosynthesis of biosurfactant compounds (glycolipids and lipopeptides) occurs on water immiscible and miscible substrates by *de novo* pathways, which vary in different microbial strains. Many bacterial strains produce a mixture of biosurfactant analogues and congeners, which are also influenced by the type of substrate used as a sole carbon source in the growth media (Sen, 1997). In a previous study, it was indicated that the two bacterial strains ST34 (*B. amyloliquefaciens*) and ST5 (*P. aeruginosa*), isolated from wastewater, carry the *sfp* and *rhl* genes involved in the biosynthesis of surfactin and rhamnolipid, respectively (Ndlovu et al. 2016). The ST34 and ST5 strains were then confirmed to produce (extracellularly) various surfactin groups (Srf1-5) and rhamnolipid congeners, respectively, when grown in MSM supplemented with glycerol (Ndlovu et al. unpublished data). Further analysis indicated that the ST34 produced five surfactin groups (Srf1-5) that were assigned to various surfactin analogues, while the ST5 produced the dirhamnolipids (Rha-Rha-C_{10}-C_{10} and Rha-Rha-C_8-C_{10}/Rha-C_{10}-$C_{8)}$ and monorhamnolipids ($_($Rha-C_{10}-C_{10} and Rha-C_8-C_{10}/Rha-C_{10}-C_8), as detected by the UPLC-ESI-MS method (Ndlovu et al. unpublished data). The current study thus focused on the assessment of the surfactin and rhamnolipid production profile by the ST34 and ST5 strains, respectively, using a small-scale high throughput 96 deep-well plate method (Vosloo et al. 2013). To achieve this, the deep-well plate was inoculated with MSM supplemented with different water miscible and immiscible substrates as sole carbon sources. The solvent extraction method was then utilised to obtain extracts from the ST34 and ST5 cultures, which were characterised using UPLC-MS analysis. In addition, the UPLC-MS analysis facilitated the separation and approximate quantification of each surfactin group produced by the *B. amyloliquefaciens* ST34 and the rhamnolipid groups produced by the *P. aeruginosa* ST5. The ESI-MS data obtained in the current study then facilitated the accurate determination of the surfactin and rhamnolipid molecular masses in the biosurfactant crude extracts.

The solvent extracts obtained from the *B. amyloliquefaciens* ST34 grown in MSM supplemented with different substrates confirmed the extracellular production of four surfactin groups (Srf1-4), which displayed a range of ion spectra as analysed using the UPLC-MS data. This difference was due to the varying fatty acid residues in the surfactin compounds and the relative abundance of each surfactin group fluctuating in each extract analysed. All water immiscible substrates (diesel, kerosene and sunflower oil) were utilised by the ST34 strain as a sole carbon source and while four major peaks were observed, only three major surfactin groups Srf2-4 were produced, which corresponded to the C_{13}-C_{15} surfactin analogues. The ST34 strain yielded a higher relative abundance of the Srf4 group when grown in kerosene and sunflower MSM, with a lower abundance observed for the diesel MSM extract. This could be due to the longer chain reduced carbons in the substrates that are available as precursors for longer branched fatty acyl residues.

The Srf1 group was detected at the lowest relative abundance in the three water immiscible MSM extracts, however, the diesel MSM extract yielded slightly higher quantities of the Srf1 group compared to the sunflower and kerosene MSM extracts. In a previous study conducted by Khondee et al. (2015) a vegetable oil (palm oil) was utilised to produce a lipopeptide biosurfactant by a *Bacillus sp*. GY19. This was one of the first studies to use water immiscible substrates to increase lipopeptide production by a *Bacillus* strain and the authors indicated that an increase in the concentration of the lipopeptide was obtained when the waste glycerol together with the palm oil were used in the fermentation production process (Khondee et al. 2015).

Supplementation of the MSM with water miscible substrates yielded four surfactin groups (Srf1-4) by the ST34 strain. However, in all substrates, three major surfactin groups (Srf2-4 corresponding to C_{13}-C_{15} surfactin analogues) displayed a relative higher abundance in the total surfactin mixture as shown by the UPLC-MS data obtained for the ST34 extracts. The sucrose-MSM extract produced the highest relative abundance of the Srf3 surfactin group, while the fructose-MSM extract yielded the highest abundance of the Srf4 group. In comparison, the glycerol-MSM extract yielded the highest abundance of the Srf2 group. The lipopeptide group with the shortest fatty acid tail, Srf1, was the least abundant in all the ST34 extracts supplemented with water miscible substrates, however, the glucose MSM extracts produced slightly higher relative abundance of the Srf1 group. This result confirms that the growth medium influences the type as well as the various analogues of the biosurfactant produced. In the current study, it was however noted that the water miscible substrates produced comparable yields of surfactin to the water immiscible substrates, with the exception of the fructose MSM extract that yielded significantly higher quantities of total surfactin (28 ± 16 mg L^{-1}). A previous study by Singh et al. (2014) indicated that the carbon source has a significant influence on the type of lipopeptides produced by *B. amyloliquefaciens* AR2. The strain AR2 produced a mixture of fengycin, iturin and surfactin variants. However, the use of sucrose and glycerol as the sole carbon sources allowed for the production of specifically the Srf2 and Srf3 surfactin groups. A study conducted by Thaniyavarn et al. (2003), indicated that *Bacillus licheniformis* grown in nutrient yeast potato dextrose medium produced five surfactin homologues as detected by LC-MS analysis. The surfactin C_{13} (Srf1), surfactin C_{13} (Srf2), surfactin C_{14} (Srf3), surfactin C_{15} (Srf4) and surfactin C_{16} (Srf5) were produced by the *B. licheniformis* F2.2. Arutchelvi et al. (2009), also utilised glucose-MSM to produce surfactin by *Bacillus subtilis* YB7, with the C_{13} and C_{14} surfactin analogues (Srf2 and Srf3) primarily produced.

The solvent extracts obtained from the *P. aeruginosa* ST5 grown in MSM supplemented with different substrates confirmed the extracellular production of six rhamnolipid groups (dRL1-3 and mRL1-3). All the water immiscible substrates (diesel, kerosene and sunflower oil) were utilised by the ST5 strain as a sole carbon source and produced two major rhamnolipid groups dRL2 and

mRL2 which corresponded to the Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} congeners, respectively. This in agreement with previous research where *P. aeruginosa* strains predominantly producing the Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} congeners when grown in immiscible substrates (Déziel et al. 1999; Haba et al. 2003; Raza et al. 2009; Saikia et al. 2014). The ST5 strain then produced a highest relative abundance of the mRL2 group when grown in diesel MSM, with the highest abundance of the dRL2 group observed in the kerosene MSM extract. It should be noted that the diesel MSM extract only produced dRL2 and mRL2, while the six groups of rhamnolipid were detected in the sunflower oil MSM extracts.

Supplementation of the MSM with water miscible substrates also yielded all six rhamnolipid groups (dRL1-3 and mRL1-3) by the ST5 strain. However, in all miscible substrate extracts, two major rhamnolipid groups (dRL2 and mRL2) displayed relative higher abundance in the total rhamnolipid mixture as shown by the UPLC-MS data obtained for the ST5 extracts. The highest total rhamnolipid produced by ST5 strain was observed in the glucose-MSM extract (307 ± 147 mg L^{-1}), followed by the glycerol-MSM extract (267 ± 202 mg L^{-1}). Glycerol is the substrate most widely utilised for rhamnolipid production by *P. aeruginosa* strains (Rahman et al. 2002; Price et al. 2009; Rooney et al. 2009; Samadi et al. 2012; Rudden et al. 2015), however, results obtained in the current study indicate that glycerol and glucose produced the same rhamnolipid congeners at approximately similar concentrations. This is however in agreement with a study conducted by Rudden et al. (2015), where they indicated a similar trend in rhamnolipid congeners produced by the *P. aeruginosa* strain when grown in glycerol and glucose. Furthermore, the 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) (C_{10}-C_{12}/C_{12}-C_{10}, C_{10}-C_8/C_8-C_{10} and C_{10}-C_{10}) were detected in the ST5 extracts, as these compounds are precursors for the synthesis of Rha-Rha-C_{10}-C_{12}/Rha-Rha-C_{10}-C_{12}, Rha-C_{10}-C_{12}/Rha-C_{12}-C_{10}, Rha-Rha-C_{10}-C_8/ Rha-Rha-C_8-C_{10}, Rha-C_{10}-C_8/Rha-Rha-C_8-C_{10}, Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10}, respectively. A previous study by Müller and Hausmann (2011) also indicated that the distribution of rhamnolipid congeners is dependent on the strain and culture stage. The monorhamnolipid congeners are predominantly produced at the early stationary phase, while the dirhamnolipid are predominantly synthesised towards the end of stationary phase.

Surfactin and rhamnolipid production by *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5, respectively, is significantly influenced by the substrate used as sole carbon source. Mineral salt medium supplemented with different water immiscible (diesel, kerosene and sunflower oil) and water miscible substrates (glucose, sucrose, glycerol and fructose) not only influenced the surfactin and rhamnolipid yield, but also the relative abundance of each surfactin analogue and rhamnolipid congener. The results indicated that higher yields of surfactins and rhamnolipids were produced by the ST34 and ST5 strains when fructose and glucose, respectively, were utilised as the sole carbon sources. The current study thus highlights the importance of the carbon source

for the production of surfactin and rhamnolipid yield as well as for the variation in the analogues and possible congeners produced by the ST34 and ST5 strains, respectively. Future studies will focus on the full characterisation of each surfactin analogue and rhamnolipid congener to determine the configuration changes such as the types and sequences of amino acids in the lactone ring and the isomers of the fatty acid moiety. Moreover, these strains could be exploited for their production of different surfactin analogues and rhamnolipid congeners, which could be applied for microbial biocontrol or production of antimicrobial agents for application in bioremediation strategies.

Authors' contributions:

TN and WK conceived the project and MR contributed to the experimental design. TN performed the experiments. TN and MR analysed the ESI-MS and UPLC-MS data. WK acquired funding for the study. WK, SK and MR contributed reagents/materials/analysis tools. TN, WK and MR wrote the paper. All authors edited the drafts of the manuscript and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

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

5.5. References

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Chapter 6:

General Conclusions and Recommendations

(UK spelling is employed)

General Conclusions and Recommendations

Biosurfactants are secondary metabolites synthesised by actively growing and/or resting microbial cells (bacteria, fungi and yeast) (Mulligan, 2005). They are classified into different groups based on their inherent chemical structure and the identity of the microorganisms that synthesise them (Ron & Rosenberg, 2001). These compounds are known to exhibit broad spectrum antimicrobial activity and different classes of biosurfactants are utilised by the agricultural, oil, food, cosmetic, biotechnological and pharmaceutical industries as additives, emulsifiers, antiadhesives and foaming agents (Hood & Zottola, 1995; Rosenberg & Ron, 1999; Dembitsky, 2004; Rodrigues et al. 2006; Piljac et al. 2008; do Valle Gomes & Nitschke, 2012). In addition, certain biosurfactants exhibit antimicrobial control mechanisms, to which microorganisms do not easily develop resistance. This is in marked contrast to the use of conventional antimicrobial therapy where various pathogenic microorganisms display significant antibiotic resistance.

Lipopeptide biosurfactants, primarily produced by various *Bacillus* species, are the most well characterised biosurfactant compounds and research has indicated that these compounds use different mechanisms to destroy target microorganisms (Vollenbroich et al. 1997; Makovitzki et al. 2006; Raaijmakers et al. 2010; Mandal et al. 2013). For example, lipopeptides provide unique mechanisms for the destruction of microbes, generally by causing damage to the cell membrane. Surfactins are well-known cyclic lipopeptides that are non-ribosomally synthesised by various strains of *Bacillus* species as secondary metabolites (Heerklotz & Seelig, 2007; Deleu et al. 2008). The glycolipids are another group of biosurfactants which intercalate into the membrane phospholipid bilayer, thereby disrupting the permeabilising effect of the membrane and instigating the leakage of metabolites out of the cell (Sotirova et al. 2008). Rhamnolipids are well-known glycolipid biosurfactants, which are reported to be primarily produced by *P. aeruginosa* as secondary metabolites (Bodour et al. 2003; Hsieh et al. 2004).

Research has indicated that the production of biosurfactant compounds is mainly influenced by the culture conditions (incubation temperature and agitation speed) as well as the macro- and micronutrients available to the microorganism synthesising the biosurfactants (Fakruddin, 2012). The discovery of biosurfactant-producing microorganisms that can be readily cultured under optimal growth conditions and simultaneously produce substantial quantities of biosurfactants, would be a means of reducing the economic barriers associated with the large-scale production of these biomolecules. Numerous studies have reported on the predominant isolation of biosurfactant-producing microorganisms from sites contaminated by heavy metals and hydrophobic organic compounds (Bodour and Miller-Maier 1998; Bodour et al. 2003; Tabatabaee et al. 2005; Walter et al. 2010). In the current study, a wastewater treatment plant (WWTP) was selected as the study site, as a wide range of bacteria are present at the various stages of

treatment (Hashimoto et al. 2014; Drury et al. 2013). Furthermore, numerous organic and inorganic contaminants present in the receiving influent could favour biosurfactant production (Shon et al. 2006). The primary aim of the research project was thus to bioprospect for novel biosurfactants and biosurfactant-producing bacteria at various points of a WWTP.

The first objective of this study (Chapter 2, published in Environmental Science and Pollution Research) was to systematically isolate biosurfactant-producing bacterial strains from the wastewater samples collected in the: influent (n = 5), post biological trickling filter treatment (biological trickling filter samples) (n = 5), post membrane bioreactor treatment (aeration tank samples) (n = 5) and the post chlorine treatment (effluent) (n = 5) points within the Stellenbosch WWTP. The bacterial strains were isolated from these wastewater samples using culture based techniques. The diversity and distribution of the isolates throughout the WWTP was also evaluated using the Shannon-Weaver and Simpson's indices. Six hundred and sixty-seven isolates were then obtained from the twenty wastewater samples collected during the current study, with 32 isolates identified as prospective biosurfactant producers utilising the drop collapse and oil spreading techniques as preliminary screening assays. Secondary microbial metabolites that display surface and emulsification activities are classified as biosurfactant molecules (Desai & Banat, 1997). The amphiphilic (composed of hydrophilic and hydrophobic moieties) nature of biosurfactant compounds allow them to exhibit excellent surface tension reduction and emulsification capabilities (Satpute et al. 2010). For example, the surface tension of water is calculated as 72 mN/m, and when a surfactant is added to water, this value is reduced (Satpute et al. 2010). The ability to lower surface tension is caused by the adsorption of the biosurfactant compounds to different phases (liquid-air, liquid-liquid and liquid-solid) (Satpute et al. 2010; Walter et al. 2010). Emulsification is another functional property of biosurfactants which refers to the dispersion of one liquid phase into another, causing the mixing of two immiscible liquids (Inès & Dhouha, 2015). Measuring the emulsification activity of a microbial cell free culture is thus one of the indirect methods used for screening possible biosurfactant production by microorganisms. Satpute et al. (2010) then stated that biosurfactants can have both emulsification and surface tension reduction activities. In the current study, the 32 isolates then all reduced the surface tension of the culture medium (observed at final value of 71.1 mN/m), with the lowest value of 32.1 mN/m observed (ST33 – *Bacillus* isolate). Emulsification activity values of up to 90% were also observed, with isolates forming stable emulsions with at least two of the hydrocarbon based compounds utilised as substrates (kerosene, diesel and mineral oil). The two isolates ST34 and ST5 that displayed high surface tension and emulsification activity, where surface tension values of 34.4 and 32.3 mN/m and emulsification activity of kerosene to 77.3 ± 1.0 and 75 ± 2.8%, respectively, were observed, were thus selected for further investigation (Chapters 4 and 5).

In order to identify the primary families and genera, the 32 prospective biosurfactant-producing isolates were then characterised using 16S rRNA conventional PCR. A dominance of biosurfactant producers belonging to the Enterobacteriaceae family (46.9%) was observed. This was expected as most of the Enterobacteriaceae occur naturally in water, soil and any man-made environments. The Bacillaceae family was the second dominant (21.9%) group isolated from the various points of the WWTP. Overall, the highest Simpson's diversity index was obtained for the influent point (1-D = 0.8356) samples, while the lowest diversity was recorded for the aeration tank (1-D = 0) samples. The results thus indicated high bacterial diversity of biosurfactant-producing isolates at the influent site, which could be due to a wide range of contaminants entering the WWTP at this point. Moreover, lower diversity at certain stages of the WWTP could be explained by the fact that as the wastewater flows from the influent point to the biological trickling filters and through to the aeration tanks, various treatment processes are utilised to remove numerous contaminants and microorganisms from the water, thereby also reducing the number of biosurfactant-producing organisms. Biosurfactant gene specific PCR was then utilised to screen each isolate for the presence of rhamnosyltransferase subunit B (*rhlB*), surfactin 4'-phosphopantetheinyl transferase (*sfp*), iturin A synthetase A (*ituA*) and bacillomycin C (*bamC*) genes involved in the biosynthesis of rhamnolipid, surfactin, iturin and bacillomycin biosurfactants, respectively. One of the *Bacillus* (ST34) isolates then carried the *sfp*, *bamC* and *ituA* genes involved in lipopeptide biosynthesis, while the *Pseudomonas* (ST5) isolate carried the *rhlB* gene involved in the biosynthesis of rhamnolipids. In the current study, a *Serratia* sp. (ST29) that carried the *ituA* and *sfp* genes, involved in the biosynthesis of the iturin and surfactin biosurfactants was also isolated. The *ituA* and *sfp* genes are generally reported to be associated with *Bacillus* species and to the best of our knowledge, no studies indicating the presence of these genes in *Serratia* spp. have been reported. While, numerous studies have reported on the predominant isolation of biosurfactant-producing microorganisms from various contaminated environments (Bodour & Miller-Maier 1998; Bodour et al. 2003; Tabatabaee et al. 2005; Walter et al. 2010), results obtained in Chapter 2, however highlight that the municipal wastewater may also serve as a source of biosurfactant-producing bacteria as 32 prospective biosurfactant-producing bacterial strains were obtained from various points of the wastewater treatment plant.

Bacterial species reported to produce biosurfactant compounds include various Gram-negative and Gram-positive genera such as *Acinetobacter*, *Bacillus*, *Burkholderia, Flavobacterium* and *Pseudomonas,* amongst others. It has also been reported that biosurfactant compounds exist as different homologues, which display different properties (physico-chemical, antimicrobial, emulsification and surface tension reduction activities). Various strains of a single species of a microorganism could in fact produce different ratios of biosurfactant mixtures under the same growth conditions (Banat et al. 2014). It is thus crucial that the genetic diversity of biosurfactant-producing species be further elucidated as various biosurfactant compounds may be produced

by different strains of a particular microbial species (Bodour et al. 2003; Mukherjee & Das, 2005). The second objective of this study (Chapter 3) was thus to identify the biosurfactant-producing isolates to the species level and differentiate between isolates belonging to the same species utilising repetitive element polymerase chain reactions (rep PCRs) [targeting the repetitive extragenic palindromic (REP) and the BOX element sequences].

The 32 prospective biosurfactant producers (obtained in Chapter 2) were then successfully characterised using genus and species specific conventional PCR assays. *Bacillus subtilis* was the most dominant species (12.5%) isolated, followed by *Aeromonas hydrophila* (9.4%), as well as *Bacillus amyloliquefaciens*, *Enterobacter asburiae, Serratia marcescens, Pseudomonas protogens* and *Klebsiella pneumoniae,* which each contributed 6.3% (n = 2) of the total isolates obtained. The *B. subtilis*, *A. hydrophila*, *B. amyloliquefaciens and P. protogens* isolates, amongst others, were then further sub-divided into four, three and two sub-species (*B. amyloliquefaciens, P. protogens* and *S. marcescens*), respectively, by the rep PCR, possibly indicating that each of these isolates were a unique strain, with a possibility of producing different mixtures of biosurfactant homologues. A significant genetic diversity was also identified among the *B. subtilis* isolates, using the REP primers, while increased strain discrimination was obtained for the *P. protogens* and *S. marcescens* strains, when utilising the BOX AIR primer. The results signify the occurrence of a variety of repetitive units within microbial species, therefore, for better microbial discrimination, a combination of the two sets of primers should be utilised. Results obtained in the current study confirmed that the use of conventional PCR, targeting the conserved regions within each genus, may not be indicative of the genetic diversity amongst bacterial species that are known to produce different concentrations and proportions of various homologs of biomolecules such as biosurfactants and antibiotics. In contrast, rep PCR has previously been employed to successfully differentiate between the genomes of several bacterial isolates classified as biosurfactant producers (Bodour et al. 2003; Tran et al. 2008). For example, Bodour et al. (2003) indicated that the *Pseudomonas* isolates *P. aeruginosa* ATCC 9027 and *P. aeruginosa* IGB83 produced different DNA fingerprints and while both produced rhamnolipids, the chemical structures of these compounds were distinct. *Pseudomonas aeruginosa* ATCC 9027 produced only monorhamnolipids, while *P. aeruginosa* IGB83 produced a mixture of mono- and dirhamnolipid, which displayed different physico-chemical properties. The BOX AIR and REP primers utilised for rep PCR in the current study thus provided a powerful tool to discriminate between biosurfactant-producing bacterial isolates identified as the same species.

A variety of methods are utilised to classify and characterise the biosurfactant compounds produced by a range of microorganisms. While mass spectrometry (MS) coupled with various chromatographic methods are the most widely used, liquid chromatography coupled to electrospray ionisation mass spectrometry (ESI-MS) is a highly sensitive method that enables the

fingerprinting of low concentrations of metabolites within a crude extract produced using natural sources. The third objective of the study (Chapter 4, submitted to AMB Express) was thus focused on the partial purification and characterisation of the antimicrobial lipopeptide and glycolipid biosurfactant compounds produced by *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5, respectively using ultra-performance liquid chromatography linked to mass spectrometry (UPLC-MS) analysis. The two strains were cultivated in mineral salt medium (MSM) supplemented with glycerol, which has been reported to produce significant concentrations of biosurfactant compounds by various bacterial strains (Bodour et al. 2003; Silva et al. 2010).

A solvent (using acetonitrile) extraction method was developed and optimised in the current study to obtain the crude biosurfactant compound mixture from the glycerol MSM and nutrient agar cultures (utilised in order to increase the probability of detecting lipopeptides/glycolipids on different growth media). The respective extracts were then characterised using a method that was developed and optimised in the current study for high resolution UPLC-MS analysis. The optimised UPLC-MS method facilitated the successful detection and separation of different analogues of surfactin (ST34) and rhamnolipids (ST5) produced by the respective strains. Results indicated that the *B. amyloliquefaciens* ST34 strain primarily produced five surfactin groups Srf1-5, which corresponded to the C_{13} (Srf1 and Srf2), C_{14} (Srf3), C_{15} (Srf4) and C_{16} (Srf5) surfactin analogues when grown on MSM supplemented with glycerol. The different surfactin groups were observed to have two or more retention times even though they displayed the same *m/z* and M_r values. The Ile containing peptides possibly elute at a slightly different retention time (Rt) to those substituted with Leu due to the slight differences in their hydrophobicity (Yang et al. 2015), for example in the current study Srf4 eluted at 12.1 and 12.2 minutes. Within each of the five surfactin groups obtained for the ST34 strain, two or more surfactin analogues were thus detected.

The UPLC-MS method that was developed and optimised in the current study was also employed for the characterisation of extracts obtained from *P. aeruginosa* ST5. The solvent extracts obtained from the ST5 strain were confirmed to be a mixture of six different rhamnolipid groups. The UPLC-MS data confirmed the presence of dirhamnolipid congeners, specifically Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{12}-C_{10}/Rha-Rha-C_{10}-C_{12} and Rha-Rha-C_8-C_{10}/Rha-Rha-C_{10}-C_8, as well as monorhamnolipid congeners, specifically Rha-C_{10}-C_{10}, Rha-C_{12}-C_{10}/Rha-C_{10}-C_{12} and Rha-C_8-C_{10}/Rha-C_{10}-C_8 in the ST5 extracts. The results thus indicate that the developed rapid solvent extraction method and UPLC-MS method was a simple and powerful technique to provide fast, sensitive and accurate identification of surfactins and rhamnolipids synthesised by *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5 strains, respectively.

The discovery of biosurfactant compounds that display a broad spectrum of antimicrobial activity is a priority, due to the increase of antibiotic resistant bacteria in various environments (Zhang et al. 2009; Khan et al. 2013). These compounds could either replace antibiotics (due to their various

antimicrobial mechanisms) or be used in conjunction with antibiotics to limit the spread of opportunistic, pathogenic microorganisms and antibiotic resistant genes. The crude surfactin and rhamnolipid extracts (obtained in Chapter 4) were thus also assessed for their antimicrobial activities. The results from the antimicrobial assays indicated that the crude extracts displayed significant antimicrobial activity against a broad spectrum of opportunistic and pathogenic microorganisms (including *Salmonella enterica, Staphylococcus aureus, Klebsiella pneumoniae*, *Cryptococcus neoformans*, among others). The antibacterial activity of rhamnolipid and surfactin extracts was observed against all the Gram-negative bacteria (100%) analysed as test organisms. While not significant, the surfactin extract displayed a higher antibacterial activity against the Gram-positive clinical strains (average zone of inhibition 17.4 ± 0.9 mm), while the rhamnolipid extracts produced an increased antibacterial activity against the Gram-positive environmental (average zone of inhibition 15.4 ± 0.5 mm) and reference strains (average zone of inhibition 14.6 ± 0.9 mm). Overall, pronounced antimicrobial activity by both the surfactin and rhamnolipid extracts against a panel of microorganisms that include reference (isolates obtained from the American Type Culture Collection), surface- and rainwater isolates as well as clinical isolates, including the antibiotic resistant *S. aureus* and *E. coli,* as well as the fungal pathogens *C. albicans* and *C. neoformans* was recorded. Similarly, a previous study by Yoshida et al. (2001), indicated that the cell free supernatant (containing surfactin) of *B. amyloliquefaciens* RC-2, isolated from healthy Mulberry leaves, strongly inhibited the growth of various bacterial and fungal isolates. A similar trend was observed in another study conducted by Sun et al. (2006), where a *B. amyloliquefaciens* ES-2 isolate produced antimicrobial lipopeptide compounds (fengycins and surfactins), which demonstrated antimicrobial activity against a total of 37 microorganisms (including *E. coli*, *S. aureus* and *B. cereus*). The two biosurfactant-producing strains isolated from wastewater thus exhibit potential for large-scale production of various analogues/congeners of the surfactin and rhamnolipid biosurfactant compounds for utilisation in the medical and food industries as antimicrobial agents such as use for surgical equipment sterilisation or cleaning agents.

The selection of a cost-effective substrate to produce biosurfactants is crucial for their large-scale production as different types of carbon sources are reported to markedly influence the concentration of the produced biosurfactant compounds. In addition, relevant published research has emphasised the effect the carbon source has on the type of biosurfactant compounds synthesised by a specific microbial strain (Bonmatin et al. 2003; Das et al. 2009; Singh et al. 2014). The fourth objective of the current study (Chapter 5, submitted to AMB Express) was thus to assess the quantitative and qualitative effect of water miscible (glucose, fructose, glycerol and sucrose) and water immiscible (diesel, kerosene and sunflower oil) substrates as sole carbon sources on the production profile of rhamnolipid and surfactin by *P. aeruginosa* ST5 and *B. amyloliquefaciens* ST34 strains, respectively. A small-scale high throughput 96-deep well plate

method (Vosloo et al. 2013) was utilised to cultivate the two strains in mineral salt medium supplemented with the water miscible and water immiscible substrates under the same growth conditions.

The ST34 strain cultivated in MSM supplemented with water immiscible substrates as a sole carbon source yielded three major Srf2-4 surfactin groups (Srf1 produced at lower yields). These groups corresponded to the C_{13}-C_{15} surfactin analogues. Among the water immiscible substrates, diesel MSM extract produced the highest total surfactin concentration of 6.0 ± 1.6 mg/L, indicating that it was a better carbon source, while the sunflower oil MSM extract yielded the lowest total surfactin concentration of 3.3 ± 1.9 mg/L. Mineral salt medium supplemented with water miscible substrates yielded four Srf1-4 surfactin groups by the ST34 strain. However, three major surfactin groups (Srf2-4 corresponding to C_{13}-C_{15} surfactin analogues) displayed a relative higher abundance in the total surfactin mixture as shown by the UPLC-MS data obtained for the ST34 extracts for all the miscible substrates utilised. The fructose-MSM (28 mg/L) extract produced the highest relative abundance of the Srf1-4 surfactin groups. In comparison, the glycerol-MSM (4.3 mg/L) and glucose-MSM (3.7 mg/L) extracts produced similar concentrations of the Srf1-4, while the sucrose MSM extracts produced more Srf3 when compared to the latter extracts. This result confirms that the growth medium influences the type and concentrations of the various analogues of the biosurfactant produced. It should be noted, that the results obtained in Chapter 5 were similar to the results obtained in Chapter 4, for the ST34 extract. While, five groups (corresponding to five analogues) of surfactins were produced using the glycerol-MSM extract in Chapter 4, the Srf2-4 were also dominant, while the Srf1 and Srf5 were produced in lower quantities.

In the current study, it was also noted that the ST34 strain cultivated in MSM supplemented with water miscible substrates produced comparable yields of surfactin to the water immiscible substrates, with the exception of the fructose MSM extract, where significantly higher quantities of total surfactin (28 ± 16 mg/L) were obtained. Moreover, MSM supplemented with different water immiscible and water miscible substrates not only influenced the surfactin yield, but also the relative abundance of each surfactin analogue (C_{13}-C_{15}). The results of the current study were comparable to those of Thaniyavarn et al. (2003), where they indicated that *Bacillus licheniformis* F2.2 grown in nutrient yeast potato dextrose medium produced five surfactin homologues namely, surfactin C_{13} (Srf1), surfactin C_{13} (Srf2), surfactin C_{14} (Srf3), surfactin C_{15} (Srf4) and surfactin C_{16}.

The ST5 strain cultivated in MSM supplemented with water miscible and immiscible substrates produced six rhamnolipid groups (dRL1-3 and mRL1-3), with the exception of the diesel MSM, kerosene-MSM and sucrose-MSM extracts, where only two and five groups were detected, respectively (Table 5.5). The rhamnolipid groups corresponded to dirhamnolipid congeners, specifically Rha-Rha-C_{10}-C_{10} (dRL2), Rha-Rha-C_{12}-C_{10}/Rha-Rha-C_{10}-C_{12} (dRL3) and

Rha-Rha-C_8-C_{10}/Rha-Rha-C_{10}-C_8 (dRL1), as well as monorhamnolipid congeners, specifically Rha-C_{10}-C_{10} (mRL2), Rha-C_{12}-C_{10}/Rha-C_{10}-C_{12} (mRL3) and Rha-C_8-C_{10}/Rha-C_{10}-C_8 (mRL1). Among the water immiscible substrates used as a source of carbon for the ST5 strain to produce rhamnolipid congeners, sunflower oil was a better substrate as higher quantity of total rhamnolipid (119 ± 37 mg/L) was obtained. For the water miscible substrates, glucose was a better substrate as a yield of 307 ± 147 mg/L of total rhamnolipid was obtained for the ST5 extract. Literature indicates that glycerol is the substrate most widely utilised for rhamnolipid production by *P. aeruginosa* strains (Rahman et al. 2002; Rooney et al. 2009; Price et al. 2009; Samadi et al. 2012; Rudden et al. 2015), however, results obtained in the current study indicated that the ST5 strain produced higher rhamnolipid quantities in MSM supplemented with glucose. However, statistical analysis indicated no significant difference in the total rhamnolipid quantities produced by ST5 when grown in MSM supplemented with glycerol or glucose. This implies that glycerol and glucose are both suitable substrates for the rhamnolipid production by the *P. aeruginosa* ST5 strain.

While numerous studies have reported on the isolation of biosurfactant-producing bacteria from contaminated soil and terrestrial environments, the current study indicated that municipal wastewater may be exploited for the isolation of diverse biosurfactant-producing bacterial strains. As indicated, a novel *Serratia* sp. (ST29) that carried the *ituA* and *sfp* genes, involved in the biosynthesis of the iturin and surfactin biosurfactants was isolated and characterised in the current study. However, no significant yields of biosurfactant were produced by the ST29 isolate when grown under the culture conditions utilised in the current study. Therefore, different growth conditions (medium composition, incubation temperature, time and agitation speed) should be utilised in future research to optimise the production of biosurfactant compounds from this strain. In addition, two biosurfactant-producing strains (*B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5) isolated from wastewater exhibit potential for the large-scale production of various analogues/congeners of the surfactin and rhamnolipid biosurfactant compounds, respectively. These compounds may be utilised in the medical and food industries as antimicrobial agents and as antifouling agents. These bacterial strains were further manipulated for their production of biosurfactant variants with results indicating that MSM supplemented with fructose and glucose yielded the highest concentrations of surfactin and rhamnolipids, for the *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5 strains, respectively. Future studies will involve the utilisation of MSM supplemented with fructose and glucose as a sole carbon source to produce optimum yields of surfactin and rhamnolipids by the *B. amyloliquefaciens* ST34 and *P. aeruginosa* ST5 strains, respectively for biotechnological application. Future research will also focus on the utilisation of the rhamnolipid and surfactin extracts as coating agents on various polymeric-based materials widely utilised in the water industry (for example, water storage containers), to prevent the

formation of biofilms on these surfaces. The stability, antifouling and antimicrobial potential of the material coated with the two biosurfactants, will be determined.

A new ultra-performance liquid chromatography method was also developed in the current study. This method could be utilised for the analysis and separation of surfactin analogues and rhamnolipid congeners, which could be utilised in future research studies for the characterisation of surfactin and rhamnolipids produced by various other bacterial strains. However, the developed method showed limited application for the quantification of rhamnolipids and therefore further optimisation experiments should be performed. This includes the isolation of the biosurfactant compounds from the growth medium and reducing the level of impurities such as the 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), as well as the use of UPLC tandem mass spectrometry in the multiple reaction monitoring mode for analysis, which has been reported (Rudden et al. 2015) to be a more sensitive and specific method for the quantification of rhamnolipid compounds.

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APPENDICES

APPENDIX A

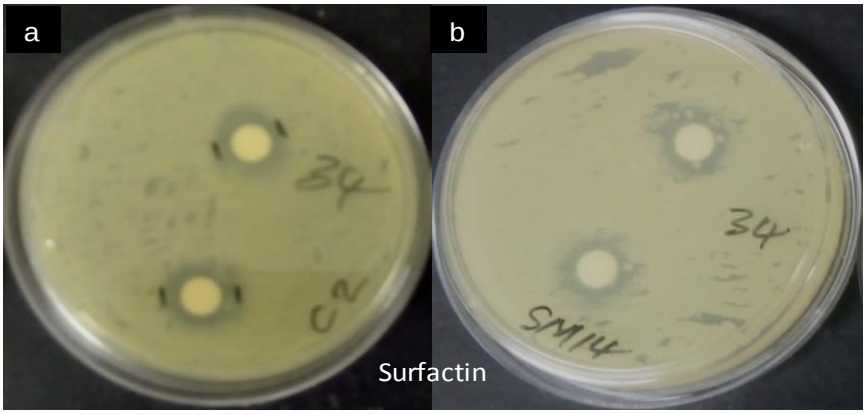

Fig. A1 Antibacterial activity of surfactin extract against a) *Staphylococcus aerues* C2 and b) *Serratia sp.* SM14

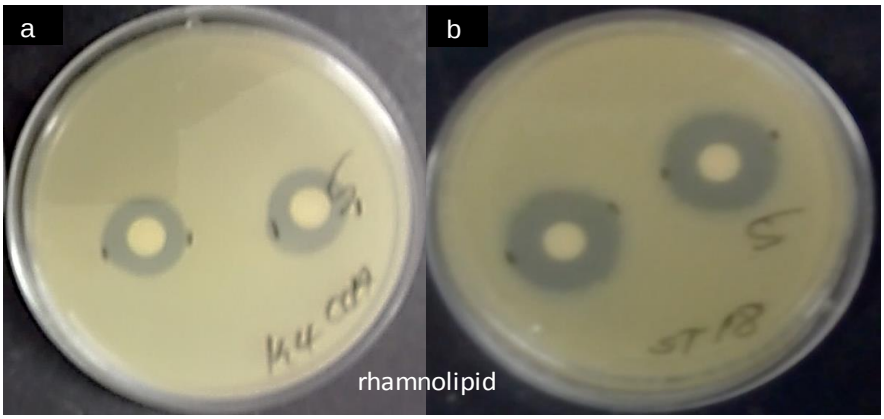

Fig. A2 Antibacterial activity of rhamnolipid extract against a) *Escherichia coli* K4CCA and b) *Bacillus cereus* ST18

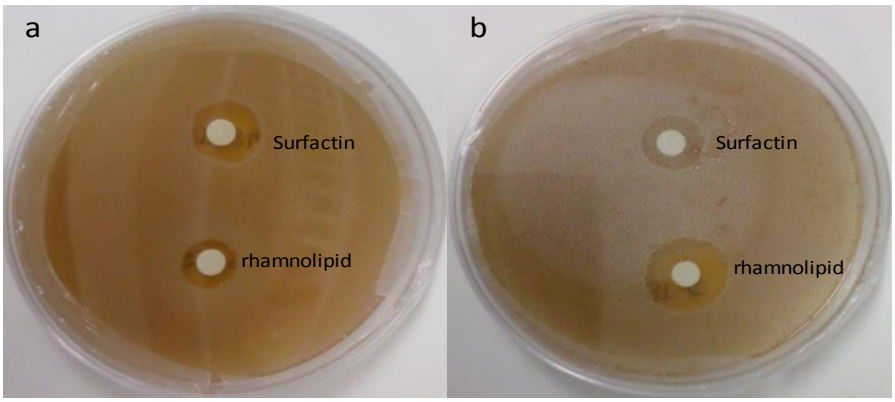

Fig. A3 Antifungal activity of rhamnolipid and surfactin extracts against a) *Cryptococcus neoformans* CAB1055 and b) *Candida albicans* 8911

APPENDIX B

AMB Express

SUPPORTING INFORMATION

Variants of lipopeptides and glycolipids produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* in different substrates

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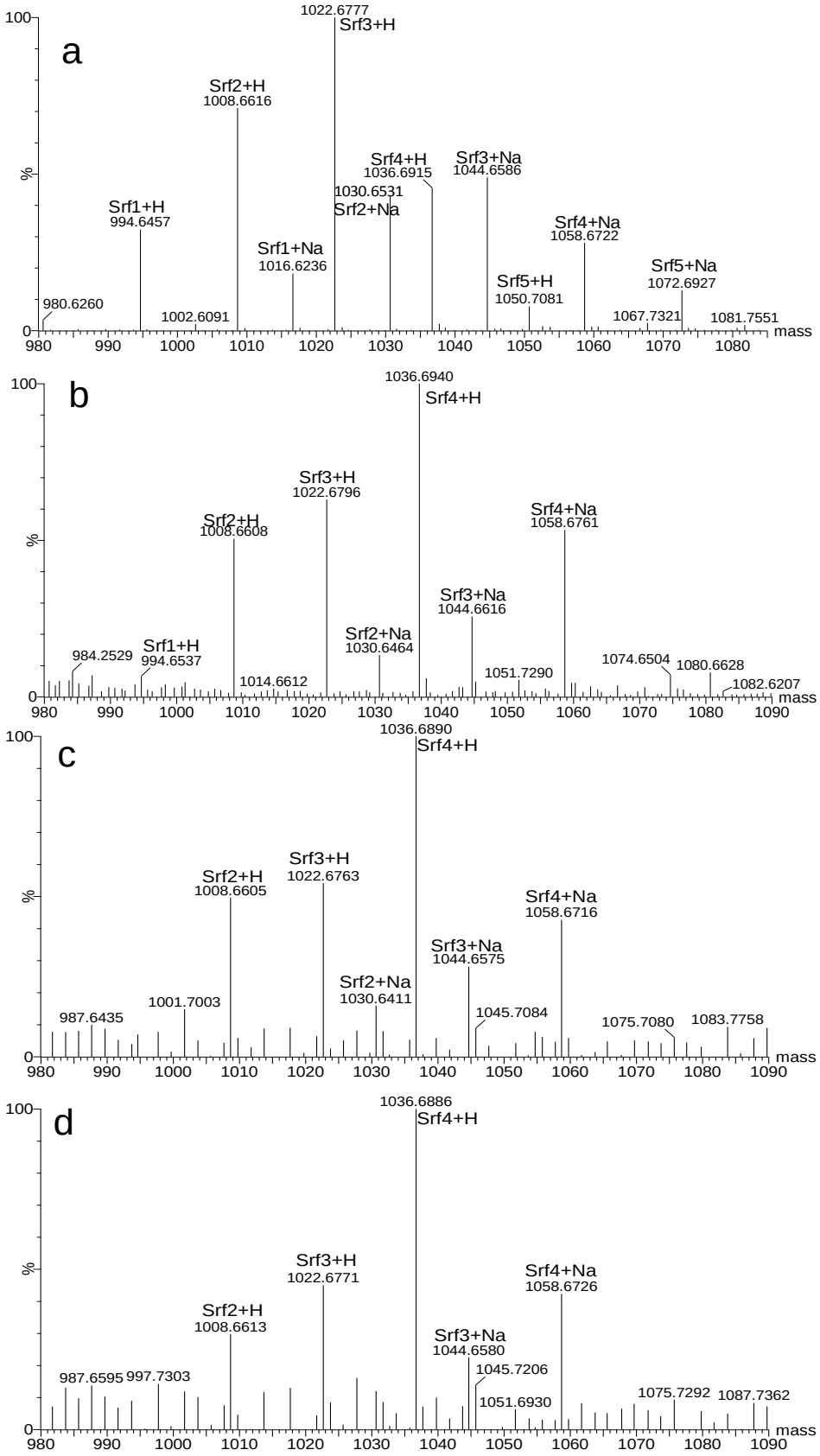

Fig. S1 The ESI-MS total ion mass spectra of the surfactin standard (a), the solvent extracted surfactin lipopeptide produced by *B. amyloliquefaciens* ST34 while growing on mineral salt medium supplemented with diesel (b), kerosene (c) and sunflower oil (d).). The positive mass spectrum generated with MaxEnt 3 is shown. The indicated masses are [M_r + H] and [M_r + H +Na] = *m/z* values of singly charged species.

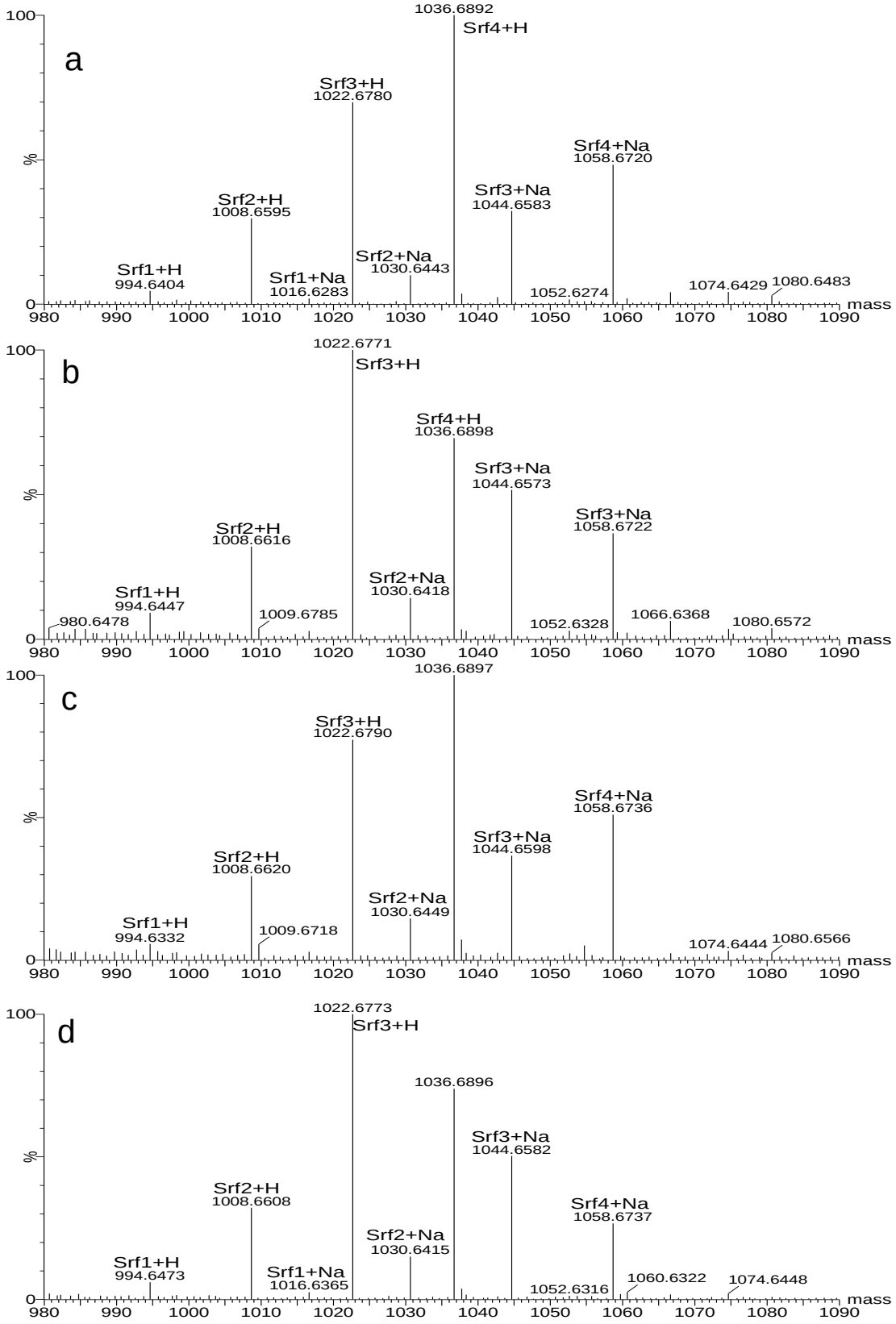

Fig. S2 The ESI-MS total ion mass spectra of the solvent extracted surfactin lipopeptide produced by *B. amyloliquefaciens* ST34 while growing on mineral salt medium supplemented with fructose (a), glucose (b), glycerol (c) and sucrose (d).). The positive mass spectrum generated with MaxEnt 3 is shown. The indicated masses are [M_r + H] and [M_r + H +Na] = *m/z* values of singly charged species.

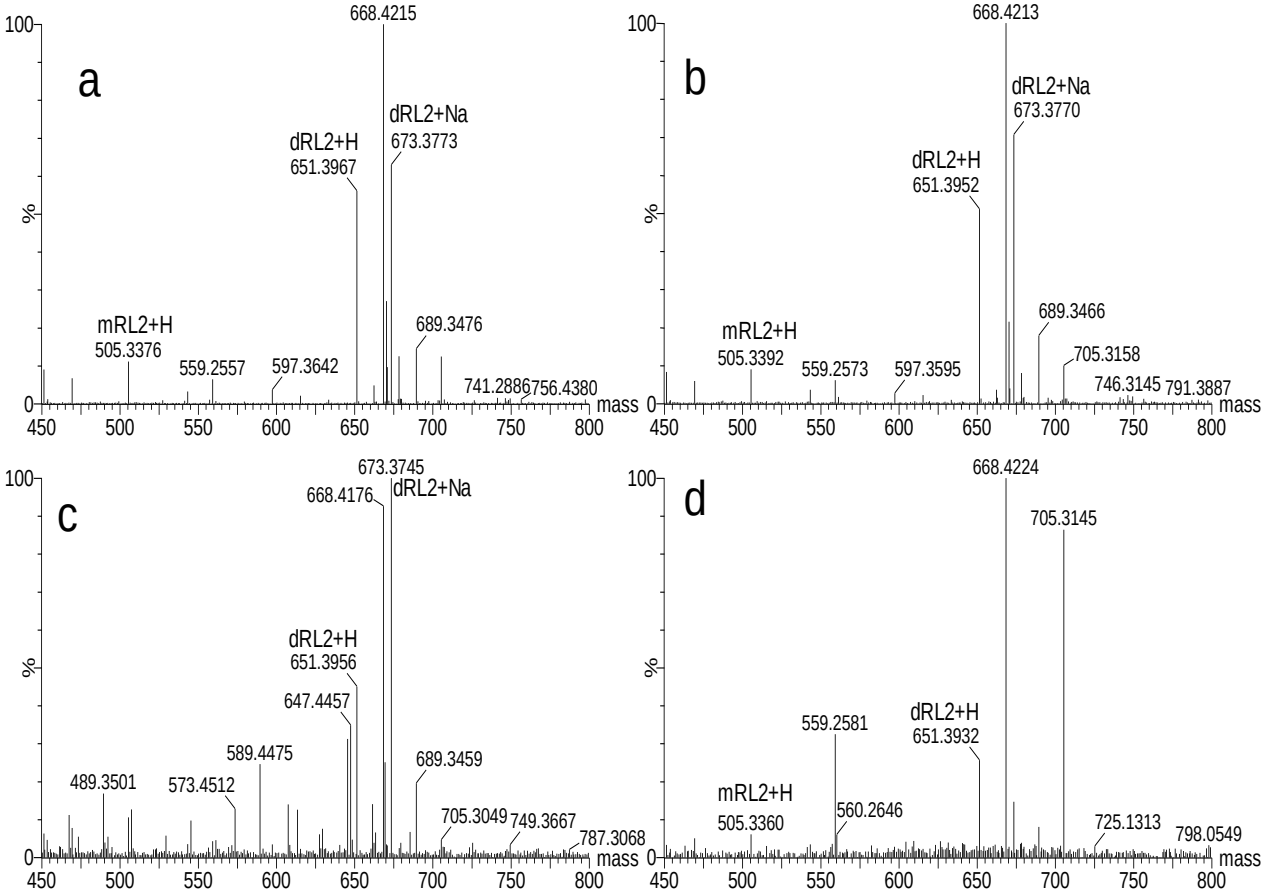

Fig. S3 UPLC-MS ion mass spectra obtained at the chromatogram peak observed at 8.1 minutes for the solvent extracted rhamnolipid glycolipid produced by *P. aeruginosa* ST5 while growing on mineral salt medium supplemented with glycerol (a), kerosene (b), sunflower oil (c) and diesel (d). The positive mass spectrum generated with MaxEnt 3 is shown. The indicated masses are [M_r+H] and [M_r+Na] = *m/z* values of singly charged species