Anti-adhesive and anti-biofilm potential of extra-cellular biosurfactants produced by mycolate actinobacteria of the suborder *Corynebacterineae* against biofilm-forming human pathogens

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Abstract

Microbial biosurfactants, amphipathic surface-active compounds, are promising candidates for the prevention and control of clinical biofilms and unlike their chemical counterparts exhibit low toxicity and are biodegradable. Members of mycolic acid-containing actinobacteria (MACA), notably of suborder *Corynebacterineae* produce various types of biosurfactants, some of which have anti-adhesion and anti-biofilm properties but investigations to date are limited, perhaps because some species produce mycolates that play a role in pathogenicity. Yet, the MACA group encompasses a great number of species and genera with diverse metabolic capabilities and more extensive study may therefore yield biosurfactants, both known and novel, with effective anti-biofilm properties. Thus, this work aimed to establish how widespread biosurfactant production is amongst diverse MACA, investigate the anti-adhesion and anti-biofilm properties of extracellular biosurfactants from representative species, establish the relationship between biosurfactant production and growth during batch cultivation, and investigate the chemical properties of biosurfactants with anti-biofilm properties. Production of both cell-associated and extra-cellular biosurfactants, determined by various physico-chemical assays, was found to be widely distributed in species across the nine genera investigated, including genera not previously reported for biosurfactant production (*Millisia* and *Williamsia*). Reduction in surface tension (ST) to ≤40mN/m using the de Noüy ring method was reported in 90/94 strains when screened as whole cell broths (WCB) and 12 strains in cell-free supernatants (CFS) during growth on hydrophobic hexadecane (2% v/v) as the sole carbon source. Only weak negative correlations were observed between de Noüy ST and other surface tension-based screening assays: drop collapse (DC) (r_s = -0.087) and oil spreading (OS) (r_s = -0.125). Weak to moderate correlations were observed between all other assays revealing that the physico-chemical properties of biosurfactants can vary between MACA strains, and that screening assays vary in their predictability of biosurfactant production. There was no correlation between hydrophobicity and mycolic acid chain length (based on genus assessment).

Extracellular crude biosurfactants extract from eleven MACA were screened for the ability to prevent cell adhesion, inhibit biofilm formation, and disrupt mature biofilms formed by various bacterial strains. All crude biosurfactant extracts inhibited *Klebsiella pneumonia* NCIMB 8865 (24-45%) biofilms and distrupt pre-formed biofilms of *Pseudomonas aeruginosa* ENU 18 (20-53%), and some demonstrated antibiofilm properties against other pathogens. Further study of glycolipid-containing extracts from three strains, *Rhodococcus ruber* IEGM 231^{T}, *Rhodococcus yunnanensis* DSM 44837^{T} and *Williamsia muralis* N1261^{T}, revealed ≤55% anti-adhesion and ≤80% inhibition of *K. pneumonia* NCIMB 8865 biofilms and ≤28% anti-adhesion, ≤57% inhibition and ≤20% disruption of preformed biofilms with *P. aeruginosa* ENU 18. Glycolipid-containing extract from these strains showed similar and in some cases, higher potency confirming the glycolipid were the bioactive components in the glycolipid-containing extracts. Biosurfactant synthesis was cell-associated until the end of exponential growth (~96 h) after which the compounds were released extra-cellularly during stationary phase. The critical cell micelle dilution of cell-free supernatants ranged from 1:100 to 1:500. Glycolipid-containing extract were identified as rhamnolipids by thin MALDI-TOF-MS. This is the first report of anti-biofilm activity by glycolipid biosurfactants produced by *R. yunnanensis* DSM 44837^{T} and *W. muralis* N1261^{Tsp} and paves the way for further investigation. This study demonstrates that extra-cellular glycolipids produced by diverse MACA possess antibiofilm activities that could be applied to the development of novel bio-based coatings for clinical surfaces and devices. This work also highlights the MACA group as a potentially rich source for further novel biosurfactants, which may have anti-biofilm properties.

Declaration

I declare that this thesis is a result of my own independent work and all the work presented in it was undertaken and written by me. I also declare that no part of this work has been submitted for any other degree or professional qualification.

Janki Hiren Hodar Date 06 /03/2024

Dedication

To my beloved husband, parents, sister, and in-laws who have been supportive throughout my studies and my decisions in life.

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expertise in analytical techniques for biosurfactant characterisation and interpretation of the result.

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LIST OF ABBREVIATIONS AND ACRONYMS

BATH	bacterial adhesion to hydrocarbons
$CaCl_2$	calcium chloride
CFS	cell-free supernatant
CMC	critical micelle concentration
CMD	critical micelle dilution
CSH	cell surface hydrophobicity
CTAB	cetyl trimethyl ammonium bromide
DC	drop collapse
DSM	Deutsche Sammlung von Mikroorganismen
EA	emulsification activity
EI	emulsification index
ENU	Edinburgh Napier University
EU ml^{-1}	emulsification activity per millilitre
$FeSO_4 \cdot 7H_2O$	ferrous sulfate heptahydrate
KCl	potassium chloride
K_2HPO_4	dipotassium phosphate
KH_2PO_4	dihydrogen phosphate
MACA	mycolic acid-containing actinobacteria
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate
mN m^{-1}	millinewton/meter
MSM	mineral salts medium
NaCl	Sodium chloride
$NaNO_3$	sodium nitrate
NCIMB	National collections of industrial food and marine bacteria
$(NH_4)_2SO_4$	ammonium sulfate
OS	oil spreading
r_s	significant correlation
ST	surface Tension
WCB	whole cell broth
WHO	World Health Organisation

Chapter 1. Introduction

1.1 Background

In recent years, biosurfactants, which are natural surface-active compounds, have emerged as a promising alternative approach to biofilm control (Dusane *et al*., 2010; Rivardo *et al*., 2009). This is due to their ability to deter biofilm-forming microorganisms from adhering to surfaces, inhibit biofilm growth, and disperse pre-existing biofilms (Kiran *et al*. 2010; Kuyukina *et al*., 2016; Pradhan *et al*., 2013). In addition, biosurfactants offer a range of advantages over their synthetic counterparts currently in use, including low toxicity, functionality across a wide range of salinity, pH and temperature, biodegradability, a low critical micelle concentration, substantial structural diversity, and the capacity for sustainable production using low-cost substrates (Makkar *et al*., 2011; Pacwa-Plociniczak *et al*., 2011; Rosa *et al*., 2015).

A variety of microorganisms, including mycolic acid-containing actinobacteria (MACA), are well-documented producers of biosurfactants (Fenibo *et al*., 2019b) but to date, research and development has largely focused on their application in environmental remediation and oil recovery. However, in recent studies, biosurfactants produced by some members of this group have been shown to possess both antimicrobial and anti-biofilm properties against various bacterial and fungal pathogens. However, these studies are limited to just a few select strains. Yet, the MACA encompasses a large and diverse group of species and there are likely to be additional strains, both known and undiscovered, with the potential to produce biosurfactants with anti-biofilm properties.

Biofilm formation poses a significant and pressing challenge in clinical settings, particularly since the emergence of antimicrobial-resistant strains and the tendency of these to colonise medical devices, leading to infections that are difficult to treat. Biofilms are implicated in approximately 50% of nosocomial infections in patients with indwelling medical devices (Piozzi *et al*., 2004; Wu *et al*., 2015), and can exhibit up to a 1000-fold increase in antibiotic resistance (Brandl *et al*., 2008; Wu *et al*., 2015). The diminishing effectiveness of currently available antibiotics and biocides used to combat biofilms has intensified

research efforts aimed at discovering alternative treatments. There is a growing interest in the development of anti-biofilm compounds that are non-toxic and do not lead to drug resistance in biofilm-forming microorganisms, particularly when used routinely (Hentzer & Givskov, 2003). This study will therefore focus on exploring the antibiofilm potential of biosurfactants produced by diverse MACA species.

1.2. Biosurfactants

Biosurfactants, biologically derived surface-active molecules, are predominantly produced by microorganisms including bacteria, fungi, and yeast. The bacterial species *Acinetobacter calcoaceticus*, *Bacillus subtilis,* and *Pseudomonas aeruginosa* and the yeast *Candida albicans*, are among the most well-studies species (Fenibo *et al*., 2019a). Biosurfactants produced by microorganisms may be bound to the cell surface or secreted extracellularly (Mao *et al*., 2015) for gliding, mobility, swarming, substrate uptake, and adhesion of biofilms (Deleu & Paquot, 2004). In biofilm formation, they help bacterial adhesion to surfaces by modification of the cell surface hydrophobicity. They also aid microbial cell detachment from surfaces by abrasion, sloughing, and erosion (Quadriya *et al*., 2018).

These molecules are characterised by their amphiphilic nature, possessing both hydrophobic (apolar) and hydrophilic (polar) moieties that allow them to partition at various phases (oil/water, water/air, oil/air) by reducing the surface and interfacial tensions (Bognolo, 1999). The hydrophobic moiety consists of long carbon chain fatty acids, which may be saturated or unsaturated hydrocarbon chains, while the hydrophilic moiety can contain phosphate groups, amino acids, carbohydrates, peptides, carboxyl acids, or alcohols, (Santos *et al*., 2016). Biosurfactants are broadly grouped as either low-weight surface-active compounds or as high molecular-weight bioemulsifiers (Nikolova & Gutierrez, 2021). They can also be categorised according to ionic charge (neutral or non-ionic, anionic, cationic) or chemical composition and structure. There are several classes of biosurfactants, including fatty acids, glycolipids such as neutral lipids, lipopeptides, lipoproteins, phospholipids, rhamnolipids, sophorolipids, and trehalose lipids (Saravanan & Vijayakuma, 2015).

The exploration of biosurfactants began in the 1960s; however, their use has expanded significantly in recent years and global demand is rising (Cerqueira *et al*., 2011; Silva *et al*., 2014). Given their structural diversity and functional properties biosurfactants have a wide range of applications in various industries, health care, and the environment (Singh & Cameotra, 2004; Souza *et al*., 2014; Zhao *et al.,* 2016) (Fig 1.1). These compounds are versatile and multifunctional and have carved out a distinct niche in the commercial market owing to their biodegradability and eco-friendly characteristics (Shekhar *et al*., 2015). Biosurfactants are useful in the implementation of sustainable industrial processes, as these can be produced from renewable resources (Marchant & Banat, 2012; Santos *et al*., 2016). This rise in interest is reflected by an increase in biosurfactant-related patents, from around 250 in 2006 to more than 850 in 2019 (European Patent Office, 2019). The biosurfactant global market has been predicted to grow to 5.52 billion US dollars in 2022 up from 4.20 billion dollars in 2017, representing an annual growth rate of 5.6% (Markets & Markets, 2017).

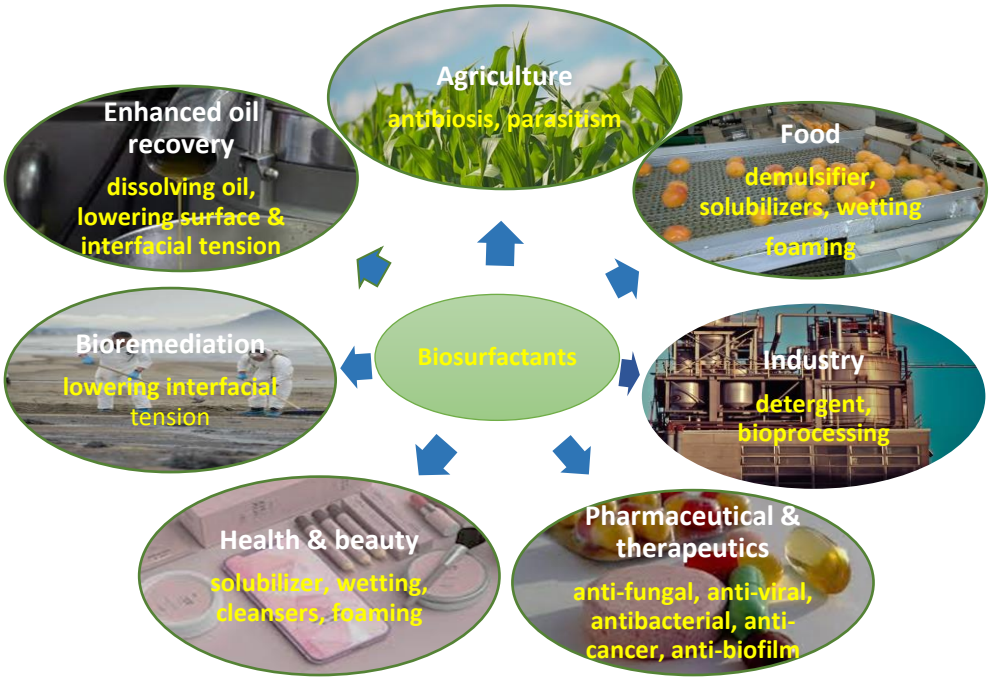


Fig 1.1. Applications of biosurfactants in various industries.

1.3. Mycolic acid-containing actinobacteria of the suborder *Corynebacterineae*

The mycolic acid-containing actinobacteria (MACA) are Gram-positive bacteria that range from motile to non-motile, aerobic to anaerobic, and spore to non-spore-forming (Barka *et al*., 2015). Cell morphology also varies from simple rods and cocci to more complex life cycles with some strains forming un-branched or branched filaments that fragment into pleomorphic forms. However, only a few species produce aerial hyphae or substrate mycelia. Colonies growing on agar plates are normally visible within 2-7 days of inoculation although slow-growing mycobacteria take considerably longer. Some species are acid-fast or acid-alcohol-fast (Kim *et al*., 2013). Members of this group are predominantly saprophytic breaking down plant and animal debris in the process of decomposition and undertake important functions in the ecosystem. However, a small number of species such as *Corynebacterium diphtheria* and M*ycobacterium tuberculosis,* are important human and animal pathogens and some others are considered opportunistic pathogens (LoBue *et al*., 2010; Sangal & Hoskisson 2013; Sharma *et al*., 2019).

The mycolic acid-containing actinobacteria (MACA) form a phylogenetically cohesive group within the suborder *Corynebacterineae* based on 16S rRNA gene sequence analysis. This group is large, currently encompasses over 400 species classified in 15 genera, namely *Corynebacterium* (Lehmann & Neumann 1896), *Dietzia* (Rainey *et al*., 1995), *Gordonia* (Stackebrandt *et al.*, 1988), *Hoyosella* (Jurado *et al*., 2009), *Lawsonella* (Bell *et al.,* 2016), *Millisia* (Soddell *et al*., 2006a), *Mycobacterium* (Lehmann & Neumann 1896), *Nocardia* (Trevisan, 1889a), *Rhodococcus* (Zopf, 1891), *Segniliparus* (Butler *et al*., 2005), *Skermania* (Chun *et al*., 1997), *Smaragdicoccus* (Adachi *et al*., 2007), *Tomitella* (Katayama *et al*., 2010), *Tsukamurella* (Collins *et al*., 1988) and *Williamsia* (Kampfer *et al.,* 1999).

Most species in the suborder synthesise mycolic acids, high molecular weight 3-hydroxy fatty acids with a long alkyl branch in the 2-position. These represent the major lipid constituents of the cell envelope in these organisms where they are attached to the cell wall arabinogalactan to form a barrier that contributes

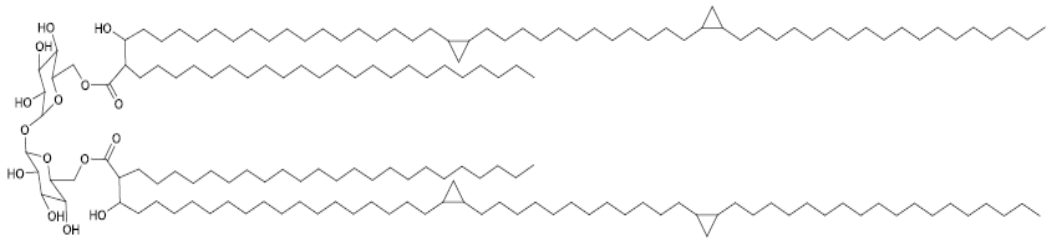
to cell permeability and resilience and conveys hydrophobicity to the cell surface (Bendinger *et al*., 1993; Marrakchi *et al*., 2014; Vilchèze, 2020). Mycolic acids vary in the number of carbons on the 2-alkyl-branch across genera from C_{22}-C_{38} in corynebacteria to C_{60}–C_{90} in mycobacteria (Table 1.1) (Marrakchi *et al*., 2014). The presence of mycolic acids is a synapomorphic trait unique to this suborder (Embley & Stackebrandt, 1994) although over the course of evolution, some members have lost the ability to produce mycolic acids including several species of the genera *Corynebacterium* and *Hoyosella* (Baek *et al*., 2018). Cell-bound mycolic acids can act as biosurfactants, their presence and length contributing to cell surface hydrophobicity (CSH) (Blackall & Marshall, 1989a) and surface-active properties (Lee *et al*., 2005). Hence, the presence of mycolic acids must be taken into consideration when screening members of the *Corynebacterineae* for biosurfactant production.

1.3.1. Biosurfactants produced by mycolic acid-containing actinobacteria. Various members of the suborder *Corynebacterineae* have been reported to synthesise cell-bound and extra-cellular biosurfactants, including members of the genera *Corynebacterium, Dietzia, Gordonia, Mycobacterium, Nocardia*, and *Tsukamurella* (Kugler *et al*., 2015). Biosurfactants produced by members of this group include lipopeptides, oligosaccharide lipids, polymeric glycolipids, terpenoid glycosides, trehalose corynemycolates, trehalose mycolates and dimycolates, and trehalose lipid esters (Kugler *et al*., 2015). Structures of the different types of biosurfactants produced by MACA are shown in Figure 1.2., page 7.

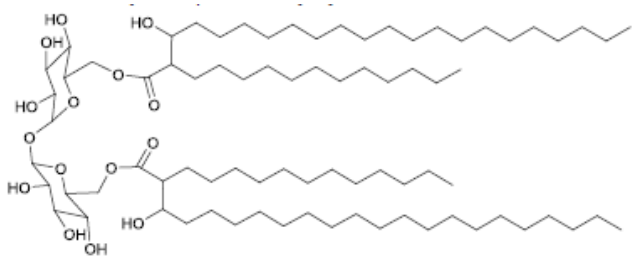
Glycolipids, consisting of a carbohydrate moiety linked to fatty acids, are the most common group of biosurfactants produced by MACA (Mnif & Ghribi, 2016). These are characterised by high structural diversity and depending on the type of carbohydrate moiety, may be grouped as cellobiose lipids, diglycosyl diglycerides, galactosyl-diglyceride, lipomannosyl-mannitols, lipomannans, and lipoarabinomannanes, mannosylerythritol lipids, monoacylglycerol, rhamnose lipids, sophorose lipids, and trehalose lipids (Kitamoto *et al*., 2002).

Table 1.1. Chemotaxonomic features of genera classified in the suborder *Corynebacterineae* (adapted from Goodfellow & Jones, 2015).

Mycolic and corynemycolic containing trehalose lipids

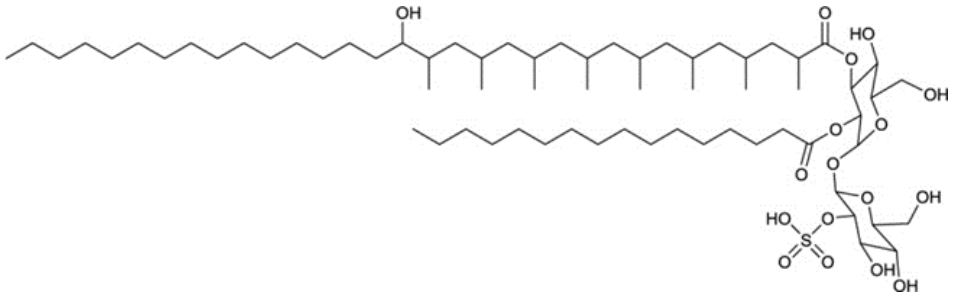


Trehalose dimycolate produced by *Mycobacterium tuberculosis*

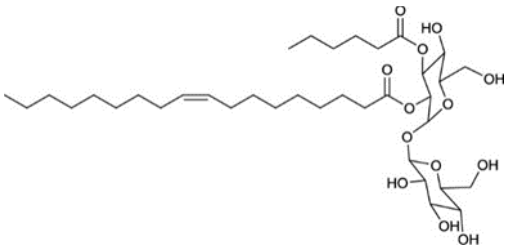


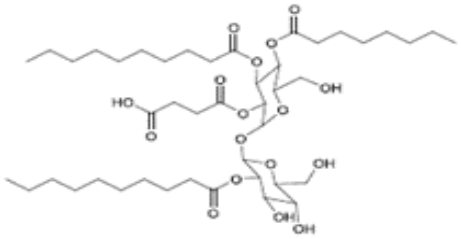
Trehalose dicorynemycolate produced by *Rhodococcus erythropolis*

Trehalose lipid esters



Diacetylated trehalose sulfolipid produced by *Mycobacterium tuberculosis*

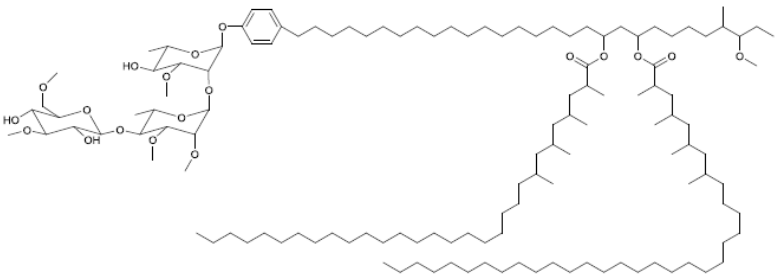




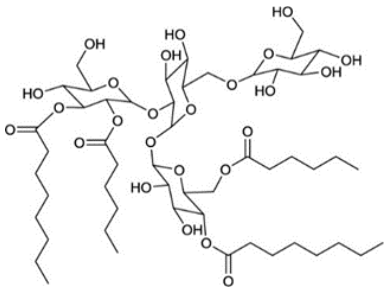
Succinic trehalose tetra-ester produced by *Nocardia farcinia*

Trehalose diester produced by *Tsukamurella spumae*

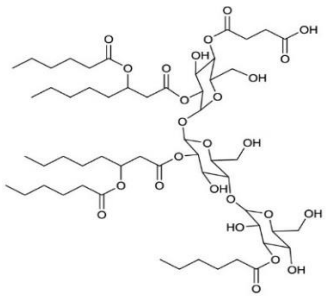
Oligosaccharide lipids.



Methylated dirhamnose/glucose phenol phtiocerol named phenolic glycolipid of *Mycobacterium leprae* (Brennan,1989)

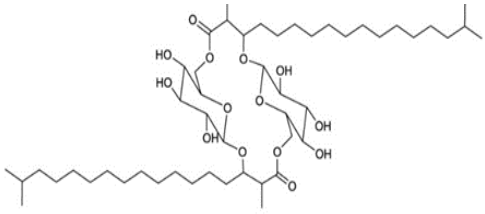


Tetrasaccharide lipid from *Tsukamurella tyrosinosolvens*



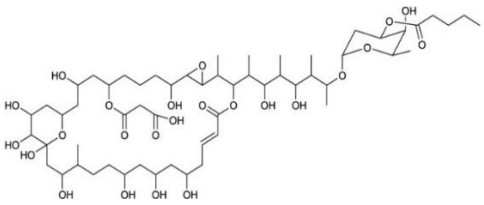
Succinic trisaccharide lipid from *Rhodococcus fascians*

Macrocyclic dilactones



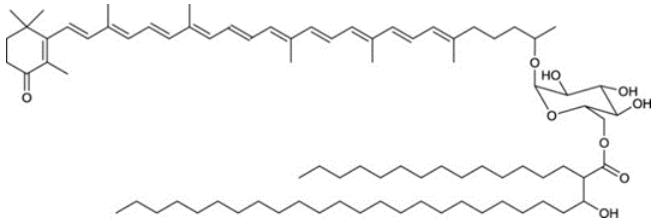
Glucolypsin A produced by *Nocardia vaccinii*

Macrocyclic glycosides



Brasilinoide A produced by *Nocardia brasiliensis*

Terpene-containing biosurfactants



Carotenoid glycoside esterified with a rhodococcus type mycolic acid produced by *Rhodococcus rhodochrous*

Figure 1.2. Types and key structural features of biosurfactants produced by mycolic acid-containing actinobacteria (adapted from Kugler *et al*., 2015).

Glycolipids have various interesting functional properties such as emulsification and de-emulsification, foaming, surface, and interfacial tension reduction, solubilisation, and mobilisation abilities. They are potential candidates in biomedicine and therapeutics because of their reported anti-carcinogenic, antimicrobial, antiviral, haemolytic, and immune-modulating activities (Kitamoto *et al*., 2002).

Trehalose-containing glycolipids have been studied in most detail. Several structural types have been reported including mono-, di- and tri-corynemycolates which have been characterised in species such as *Rhodococcus erythropoiesis*, *R. ruber,* and *R. wratislaviensis* (Tuleva *et al*., 2008) and trehalose di-nocardiomycolates which have been characterised for *R. opacus* (Kugler *et al*., 2015). The mycobacterial trehalose mycolates or di-mycolates (cord factors) have been thoroughly investigated given their role as modulators of mycobacterial pathogenesis and host immune response (Sakamoto *et al*., 2013; Shao *et al*., 2011). Rhamnolipid biosurfactants have also been reported to be produced by a handful of MACA strains including *Dietzia maris* (Wang *et al*., 2014b) and *R. fascians* (Gesheva *et al.*, 2010).

1.3.2. Applications of biosurfactants from mycolic acid-containing actinobacteria.

Biosurfactants produced by members of the genus *Rhodococcus* and other MACA have primarily been explored for their potential environmental applications, particularly in the fields of hydrocarbon remediation and oil recovery (Ivshina *et al*., 2016; Saeki *et al*., 2009; Vyas *et al*., 2011; Wang *et al*., 2014). However, these versatile compounds hold promise for a wide array of applications across many sectors (Fig 1.3). Several biosurfactants produced by MACA exhibit interesting biomedical properties, including anti-inflammatory, antioxidant, anti-tumour, anti-microbial, anti-viral, and wound healing (Table 1.2, page 11).

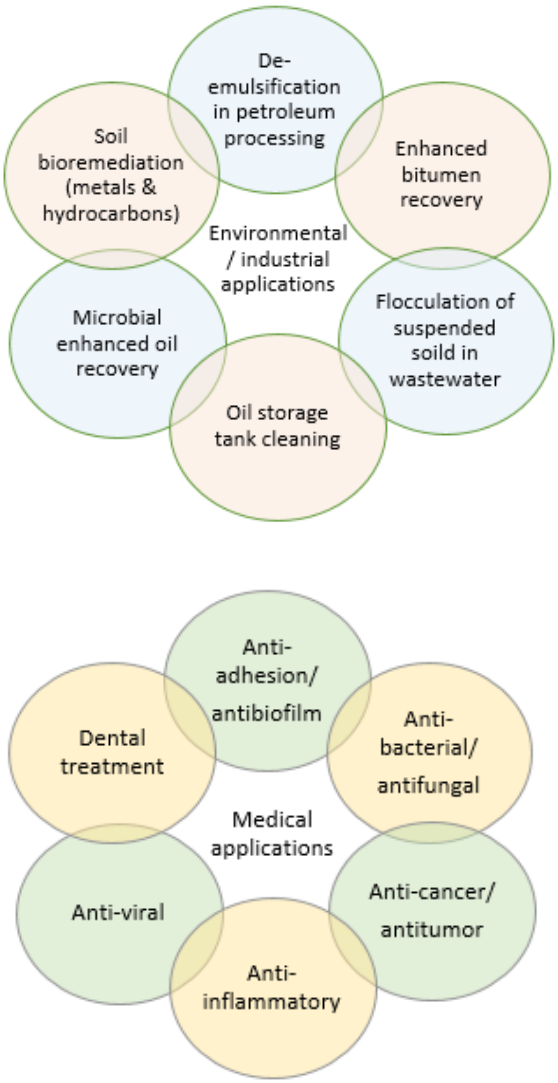


Fig 1.3. Potential medical and environmental applications of biosurfactants from mycolic acid-containing actinobacteria (Adapted from Stainsby *et al*., 2022).

In addition, a small number of studies conducted recently also reveal that biosurfactants produced by strains belonging to the genera *Corynebacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* demonstrate promising anti-microbial and anti-biofilm activities against pathogenic microorganisms (Janek *et al*., 2018; Kuyunika *et al*., 2016; Mikami *et al*., 2000; Pirog *et al*., 2014, Vollbrecht *et al*., 1999).

Table 1.2. Biomedical properties of biosurfactants from mycolic acid-containing actinobacteria.

Strain	Biosurfactant	Biomedical properties	Reference
Corynebacterium xerosis NS5	Coryxin (lipopeptide)	Antibacterial activity against gram-positive *Staphylococcus aureus* and *Streptococcus mutans* and gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* strains.	Dalili *et al.,* 2015
Nocardia brasiliensis IFM-0406	Brasilinolide	Antifungal against *Aspergillis, Candida, Cryptococcus,* and *Paecilomyces* species.	Mikami *et al*., 2000
Nocardia farcinica BN26	Trehalose lipid	Cytotoxic effects on human tumor cell lines BV-173 and SKW-3, and to a lesser extent, HL-60. Mediated cell death by the induction of partial apoptotic DNA laddering.	Christova *et al*., 2015
Rhodococcus sp. I2R	Extracellular complex of glycolipids	Antiviral activity against Herpes simplex virus 1 (HSV-1) and human coronavirus HCoV-OC43. Anti-proliferation activity against human prostatic carcinoma cell line PC3	Palma *et al*., 2021
Rhodococcus sp.	Rhamnolipid	Antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Salmonella enterica, Staphylococcus aureus*, *Listeria monocytogenes*.	Undabarrena, 2016
Rhodococcus sp. TB-42	Analogues of succinoyl trehalose lipid-3 (STL-3)	Inhibited growth and induced the differentiation of human HL-60 promyelocytic leukaemia cell line	Sudo *et al*., 2000
Rhodococcus erythropolis IMB Ac-5017 (EK-1)	Trehalose mono- and di-mycolates; neutral lipids; phospholipids	Antibacterial activity against *B. subtilis and S. aureus, E. coli, Pseudomonas sp, and* antifungal activity against *C. albicans, C. utilis, and C. tropicalis.*	Pirog *et al*., 2014

Strain	Biosurfactant	Biomedical properties	Reference
Rhodococcus erythropolis SD-74	Purified succinoyl trehalose lipid (STL-1)	*In vitro* induction of human monocytoid leukemic cell line U937 differentiation and cytotoxicity against human lung carcinoma cell line A549. *In vitro* induction of human promyelocytic leukaemia (HL60) cell line differentiation into monocytes and inhibition of protein kinase C.	Isoda *et al*., 1995 Isoda *et al*., 1997
Rhodococcus erythropolis	Glycolipid	Antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, and* antifungal activity against *Aspergillus niger, Aspergillus flavus.*	Abdel-megeed *et al*., 2011
Rhodococcus fascians BD8	Trehalose lipid	Antimicrobial activity against *Vibrio harveyi* and *Proteus vulgaris*, and partial inhibition of other gram-positive and negative bacteria and fungus *Candida albicans*.	Janek *et al*., 2018
Rhodococcus ruber IEGM 231	Mixture of trehalose dimycolate (TDM), diacyltrehalose and monoacyltrehalose	*In vitro* induction of IL-1β, IL-6, and TNF-α cytokine secretion by human monocytes *In vitro* induction of Th1-polarizing factors IL-12 and IL-18 by human mononuclear cells and monocytes and reactive oxygen species (ROS) by peripheral blood leukocytes.	Kuyukina *et al*., 2007 Baeva *et al*., 2014
Rhodococcus ruber IEGM 231	Glycolipid Monoacyltrehalose fraction (MAT)	*In vivo* induction of IL-1β by mouse peritoneal macrophages. *In vivo* suppression of bactericidal activity and proinflammatory cytokine IL-1β of mouse peritoneal macrophages, anti¬body production by splenocytes and stimulates the production of IL-10.	Gein *et al*., 2018, 2020
Tsukamurella tyrosinosolvens DSM 44370	Oligosaccharide lipids	Antibacterial activity against *Bacillus megaterium, E. coli, and Ustilago violacea.*	Vollbrecht *et al*. 1999

1.4. Microbial biofilms

Biofilm formation is an evolutionary adaptation by bacteria to allow survival in unfavourable environmental conditions, such as periods of starvation and desiccation (Dang & Lovell, 2016; Ikuma *et al*., 2013). During biofilm formation, which is a complex, multi-step process (Fig 1.4), a community of microorganisms adheres to living or abiotic surfaces (Stoodley *et al*., 2002) where they exhibit altered phenotypes and a unique architectural arrangement. Within the forming biofilm, cells communicate with one another through quorum sensors that secrete autoinducer substances (AI) into the surrounding environment, thereby triggering the upregulation of biofilm development and maturation (Sturbelle *et al*., 2015).

Fig 1.4. The five main stages in biofilm formation: 1. biofilm formation begins with an initial reversible attachment where bacterial cells attach to the surface through appendages like pilli, flagella, and curli. 2. Irreversible attachment where cells form exopolymeric materials that help them to affix more securely on the surface. 3. Micro-colonies form and the biofilm begins to mature. 4. Biofilms form a three-dimensional structure where cells are packed in clusters with channels running between them. 5. Biofilm disperses cells to initiate new biofilm formation (Hollmann *et al*., 2022).

Biofilms can have both beneficial and detrimental effects. In various industries, biofilms are harnessed for productive purposes such as sewage treatment, filtration of surface water for drinking, electricity generation in microbial fuel cells (Wang *et al*., 2013, 2014a), metal dissolution in the bioleaching industry (Vera *et al*., 2013) and oil contamination removal from marine systems (Martins *et al*., 2008). However, biofilm formation can also be detrimental, for example, causing surface corrosion, frictional resistance of fluids, water pipeline

blockage and sensor malfunction, and product contamination (Kumar & Anand, 1998; Qian *et al*., 2007). Biofilms are of particular concern in health-care settings where infections manifesting from biofilms can be devastating, leading to severe symptoms and death (Auler *et al*., 2010; Srey *et al*., 2013).

1.4.1. Clinical biofilms. In clinical settings, such as care homes and hospitals, biofilms form on various surfaces such as indwelling medical devices, tubing on medical equipment, and wet or damp areas such as taps and sinks (Hall-Stoodley *et al*., 2004; Lindsay & Von Holy, 2006) and can persist over extended periods. Biofilm infections cause several clinical challenges, which include chronic inflammation, impaired wound healing, and the dissemination of community-acquired diseases (Stewart *et al*., 2012). Their formation on indwelling devices can lead to infections associated with urinary and venous catheters, peritoneal dialysis catheters, pacemakers, prosthetic joints intrauterine devices, and contact lenses. Direct biofilm infections in host tissues result in infections such as chronic otitis media, chronic osteomyelitis, chronic pneumonia in cystic fibrosis patients, chronic prostatitis, gingivitis, palindromic urinary tract infection, and endocarditis (Donlan, 2001).

In 2019, healthcare spending related to biofilms had a significant economic impact. In the United Kingdom, it resulted in direct costs of $7.2 billion, while globally, it accounted for $387 billion, constituting 5% of the total global healthcare expenditure. This substantial expenditure directly influences global health-related economic activity, which amounted to $7,800 billion (National Biofilm Innovation Centre, 2020). The prevalence of catheter-associated urinary tract infections affects approximately 150 million individuals worldwide each year. In England, biofilm formation on artificial pacemakers and prosthetic cardiac valves is a concern, with around 1% occurrence for new devices and 3% for replacement devices (National Biofilm Innovation Centre, 2020).

1.4.2. Clinically important biofilm-forming pathogens. Clinically significant pathogens capable of forming biofilms encompass a diverse array of both Gram-positive and Gram-negative bacteria. Among the most prevalent biofilm-forming species are *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella*

pneumoniae, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus viridans* (Chen *et al*., 2013). ESKAPE pathogens (Rice, 2008), which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, are recognised as the leading causes of nosocomial infections globally. These species exhibit multidrug resistance and are difficult to prevent and treat using conventional therapeutics (Meade *et al*., 2021). The ESKAPE pathogens are also recognised on the World Health Organisation (WHO) list of global priority pathogens that urgently require novel antimicrobial agents to treat them. This list encompasses bacteria belonging to 12 families, categorised as critical, high, or medium according to their risk level, with a substantial number responsible for biofilm-related infections (WHO, 2017).

The most critical group includes carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and carbapenem-resistant, ESBL-producing *Enterobacteriaceae*. These bacteria cause bloodstream infections and pneumonia and have developed resistance to numerous antibiotics, including third-generation cephalosporins. Members of the *Enterobacteriaceae*, such as *E. coli* and *Klebsiella* spp. are the most common causes of catheter-associated urinary tract infections (CAUTI), while *Pseudomonas aeruginosa* is the primary pathogen causing ventilator-associated pneumonia (McGuffie *et al*., 2016).

Escherichia coli, in addition to CAUTI, is responsible for other device-associated infections on prosthetic grafts, joints, shunts, and intravascular catheters (Fey, 2010). Key virulence factors of *E. coli* include surface factors like type 1 fimbriae, P fimbriae, flagella, capsular lipopolysaccharides, and outer membrane proteins, which can contribute to biofilm formation (Shah *et al*., 2019; Sharma *et al*., 2016). *Klebsiella pneumoniae*, another WHO-listed pathogen, has emerged as a global multidrug-resistant hospital-acquired pathogen with limited treatment options (Paczosa & Mecsas, 2016). Biofilm formation is an essential step in the pathogenicity of this species (Wu *et al*., 2011), and it can be found on the skin, in soft tissues, the bladder, and lungs (Piperaki *et al*.,

2017). Capsular polysaccharides, type 1 and type 3 pili, are major virulence factors contributing to *K. pneumoniae* pathogenesis (Li *et al*., 2014; Murphy & Clegg, 2012; Wood *et al*., 2009).

Pseudomonas aeruginosa, also a critical-list pathogen, is a common hospital-acquired pathogen responsible for severe opportunistic infections and a global challenge due to the rise of multidrug-resistant strains (Moreira *et al*., 2013; Pachori *et al*., 2019). The ability of *P. aeruginosa* to form biofilms by using attachment organelles (flagella and type IV pili) is a key factor in acute and chronic infections.

Other antibiotic-resistant species on the WHO high-risk list include methicillin-resistant *Staphylococcus epidermidis* and *S. aureus* (MRSA) which are the major cause of nosocomial infections, responsible for two-thirds of all indwelling device-related infections (Khatoon *et al*., 2018). They frequently form biofilms on a range of medical implants and devices, including cardiovascular devices, prosthetic heart valves, cardiac pacemakers, in-dwelling catheters, contact lenses, cerebrospinal fluid shunts, joint replacements, and intravascular lines (Donlan & Costerton, 2002; Hall-Stoodley *et al*., 2004; Lázaro-Díez *et al*., 2016). A series of surface proteins facilitate initial attachment, followed by the expression of protein-based exopolysaccharides that aggregate staphylococcal cells, while surfactant peptides give rise to the three-dimensional structure of biofilms (Otto, 2008). Biofilm formation by enterococci is responsible for 25% of all catheter-associated urinary tract infections, wound infections, and endocarditis (Ch'ng *et al*., 2019). The expression of pili on the cell surface facilitates cell adhesion and is considered the trigger of biofilm formation on various biotic and abiotic surfaces (Costerton, 2001; Mandlik *et al*., 2008).

Given the widespread distribution of diseases attributed to biofilm-forming strains, addressing the importance of biofilms in the prevention and treatment of many chronic diseases has become a paramount concern.

1.4.3. Challenges in the treatment of clinical biofilms.

Current approaches to treat infections include antibiotic administration, the application of antibiotic coatings on medical devices, and the use of biocides to clean surfaces. However, the excessive and inappropriate use of antibiotics and biocides can exert selective pressure on bacteria, promoting the survival and proliferation of resistant strains, in turn making it difficult to treat medical device-related infections (Gupta *et al*., 2016). Biofilms are more resistant to antibiotic monotherapy (single-agent antibiotic therapy), necessitating the use of combination therapy (Herrmann *et al*., 2010) in which the combined action of two or more antibiotics can lead to a synergistic effect and reduce the development of resistance (Bari *et al*., 2023; Broussou *et al*., 2018; Vazquez-Grande & Kumar, 2015).

Another strategy involves the use of biocides to prevent the growth of biofilms on medical instrument surfaces and in the hospital environment (Donaghy *et al*., 2019; Fraise *et al*., 2002; Maillard, 2005; Wesgate *et al*., 2016). Biocides have a broad spectrum of activity and multiple cellular targets. Their exact mechanisms of action are difficult to establish, however, given their simultaneous impact on various cellular constituents. Biocides can disrupt critical cellular functions, such as replication, transcription, protein synthesis, general metabolism, and membrane integrity, leading to the leakage of intracellular components (Schulte *et al*., 2004). However, the over-use of biocides also leads to increased bacterial resistance and indeed cross-resistance to unrelated antimicrobials including antibiotics (Maillard, 2018).

Biofilms act as a barrier protecting cells from chemical perturbation such as biocides and antibiotics, by limiting their penetration into the biofilm matrix (Yan & Bassler, 2019). Biofilm-forming bacteria can exhibit increased resistance to antibiotics and biocides through various protection mechanisms including extracellular polymeric substance (EPS), extracellular DNA (eDNA), and antibiotic-degrading enzyme accumulation which in combination with variations in growth, stress response, persistent cells, altered genotype, and genetic diversity are responsible for the frequent failure of biofilm treatments

(Bueno, 2014; Paharik & Horswill, 2016). The penetration and interaction of antibiotics within the inner layers of biofilms are affected by the extracellular polymeric matrix (EPS), which functions as a protective barrier. This complex matrix consists of various anionic and cationic molecules, including proteins, glycoproteins, and glycolipids (Nadell *et al*., 2015). Additionally, the movement of antimicrobial substances is limited by adsorption sites within the matrix, such as the glycocalyx layer, an integral component of the EPS. This layer can adsorb up to 25% of the antibacterial molecules (Sugano *et al*., 2016).

Within the matrix, extracellular DNA (eDNA) also acts as a barrier by physically interacting with antimicrobials and sequestering these away from their intended cellular targets thereby reducing their efficacy (Chiang *et al*., 2013). Moreover, eDNA is involved in the horizontal transfer of antibiotic-resistance genes (Bae, Oh & Jeon, 2014) and the accumulation of enzymes responsible for the degradation of antibiotics within the matrix (Bagge *et al*., 2004). The presence of eDNA can significantly increase biofilm resistance to antimicrobial agents. One such mechanism involves modifications to the outer membrane through the chelation of cations, such as magnesium ions. Since eDNA is anionic, this interaction lowers the magnesium concentration, thereby inducing signal molecules that confer resistance (Johnson *et al*., 2012; Lewenza, 2013; Wilton *et al*., 2015).

The efficacy of antimicrobial treatment is also hampered by factors such as growth rate, stress responses, and the presence of persistent cells. Most antibiotics target specific macromolecules produced during cell growth. However, many microorganisms within biofilms are slow-growing or metabolically inactive cells therefore production of these macromolecules is limited and consequently, the effectiveness of antibiotics is diminished (Dincer *et al*., 2020). Persistent cells, which represent a reversible transformation of a small subpopulation of bacteria into slow-growing variants, are produced in response to endogenous stressors like exposure to antibiotics and oxidative stress. These cells are highly resistant to antibiotic-induced cell death due to their lower metabolic rate (Öner, 2013). Bacteria are also equipped with a range of stress responses to adapt to changes in their environment, including

variations in temperature, water activity, oxidative stress, and nutrient deprivation (Driffield *et al*., 2008; Li *et al*., 2020; Stewart, 2012). These stress responses can impact antimicrobial susceptibility by affecting the processes targeted by antimicrobials (Poole, 2012).

Efflux pumps, membrane proteins responsible for exporting harmful substances from bacterial cells, play a significant role in conferring antibiotic resistance in some pathogens. When these efflux pumps are overproduced, it can result in multidrug resistance (Blanco *et al*., 2016; Bolla *et al*., 2011; Singh *et al*., 2017). These pumps downregulate porin production in various pathogenic bacteria, which slows down the diffusion of hydrophilic solutes, thereby reducing transmembrane diffusion of lipophilic solutes (Pagès *et al*., 2008).

Horizontal gene transfer mechanisms, such as conjugation and transformation, can facilitate the spread of antibiotic-resistance genes through the biofilm community (Juhas, 2015; Mah, 2012; Rodríguez-Rojas *et al*., 2013). Modulating quorum sensing signaling, which is integral for cell-to-cell communication, can ultimately lead to changes in gene expression that lead to antibiotic resistance (Tang & Zhang, 2014). Extracellular DNA, proteins, and other biofilm matrix components can act as mutagens contributing to increased mutation frequency and influencing the acquisition of resistance. Slow-growing bacterial cells in biofilms remain viable for extended periods, which allows for the accumulation of genetic mutations. Prolonged exposure to stressful conditions, including antibiotic exposure, can lead to the accumulation of resistance-conferring mutations (Wilton *et al*., 2015).

Biofilm resistance to biocides and antibiotics presents a significant global health challenge therefore new and effective approaches that prevent and remove biofilm-forming pathogens, including antibacterial-resistant strains, that develop in clinical settings are urgently needed (Khan *et al*., 2021; Roy, 2018).

1.5. Biosurfactants from mycolic acid-containing actinobacteria (MACA) with anti-biofilm properties.

Biosurfactants exhibit diverse mechanisms to prevent, inhibit, or disrupt biofilms (Busscher *et al*., 1997; Meylheuc *et al*., 2001; Monteiro *et al*., 2011). They can increase membrane permeability, causing membrane disruption, cell lysis, and loss of metabolites. These compounds can also modify the properties of surfaces, such as reducing the hydrophobicity of the substratum surface, to prevent/reduce bacterial adhesion (Janek *et al*., 2012), interfere with cell-to-cell communication (Irie *et al*., 2005; Rasmussen & Givskov, 2006; Valle *et al*., 2006), create voids within biofilms, and alter the architectural arrangement of bacteria within the biofilm structure (Boles *et al*., 2005). Some biosurfactants are also known to down-regulate gene expression associated with biofilm formation (Salehi *et al*., 2014).

The treatment of surfaces with biosurfactants, therefore, offers a potential approach to prevent colonisation by pathogenic microorganisms, and hence biofilm formation. Biosurfactants have also been reported to disrupt established biofilms including those of clinically significant pathogens (Banat *et al*., 2014), and could be used for example to clean and flush medical equipment. Rhamnolipids, for example, have been shown to disrupt the membrane of cells by acting as an inverted-cone shaped molecule extending a positive curvature in the cell membrane (Ortiz *et al*., 2010) and interacting with the membrane phospholipids, resulting in an alteration in the acyl chain and disturbing the integrity of the bilayer membrane (Sánchez *et al*., 2009). Sophorolipids and trehalose lipids have a similar mechanism that leads to destabilization, change in permeability, loss of membrane functions, and structural changes (Joshi-Navare & Prabhune, 2013; Zaragoza *et al*., 2009).

Various glycolipid and lipopeptide biosurfactants synthesised by actinobacteria have been shown to display anti-adhesion and/or anti-biofilm properties against other biofilm-forming bacteria including clinically significant pathogens, as exemplified in Table 1.3.

Table 1.3. Biosurfactants produced by actinobacteria that have antimicrobial and/or anti-biofilm activity.

Actinobacterium	**Biosurfactant type**	**Anti-biofilm/antimicrobial targets**	**Reference**
Actinobacteria strain MSA3	lipopeptide	Anti-biofilm activity against *Staphylococcus aureus.*	*Kiran et al., 2017*
Brevibacterium casei MSA19	glycolipid	Anti-biofilm activity against *Escherichia coli, Pseudomonas aeruginosa,* and *Vibrio spp.*	Kiran *et al*., 2010
Brevibacterium aureum MSA13	lipopeptide	Broad-spectrum antibiotic activity with a most profound effect against *Candida albicans.*	Seghal Kiran *et al*., 2010
Nocardiopsis alba MSA10	lipopeptide	Anti-biofilm activity against *Vibrio harveyi*. Antimicrobial activity against *Enterococcus faecalis, Bacillus subtilis, and C. albicans*.	Selvin *et al*., 2016 Gandhimathi *et al*., 2009
Streptomyces B3	glycolipid	Anti-microbial activity against *B. subtilis, E. coli, S. aureus, P. aeruginosa,* and *C. albicans*.	Khopade *et al*., 2012
Streptomyces sp. MAB36	glycolipid	Antimicrobial activity against *B. megaterium*, *B.cereus*, *S. aureus*, *E. faecalis, Shigella dysenteriae, S. boydii, C. albicans,* and *Aspergillus niger.*	Manivasagan *et al*., 2014
Streptomyces amritsarensis	lipopeptide	Antimicrobial activity against various gram-positive and gram-negative bacteria.	Sharma *et al*., 2014
Streptomyces matensis PLS-1	rhamnolipid	Antibacterial compounds against unicellular Gram-positive and gram-negative.	Kalyani *et al*., 2014
Streptomyces roseosporus	cyclic lipopeptide	Antibacterial activity against gram-positive and negative species especially methicillin-resistant *S. aureus* (MRSA)	Kirkpatrick *et al*., 2003; Nguyen *et al*., 2006

Indeed, the branched cyclic lipopeptide 'daptomycin' obtained from *Streptomyces roseosporus* has reached commercial use under the name Cubicin. It has bactericidal activity against clinically relevant Gram-positive bacteria such as penicillin-resistant *Streptococcus pneumonia,* vancomycin-resistant enterococci, coagulase-negative staphylococci, and methicillin-resistant *Staphylococcus aureus* (Tally *et al*., 1999).

The reduced toxicity of biosurfactants in comparison to chemical surfactants, combined with their effectiveness across a broad spectrum of environmental conditions makes them particularly promising anti-biofilms agents (Rodrigues *et al*., 2006a, b, 2007; Dusane *et al.* 2010; Rivardo *et al*. 2009) as they could reduce reliance on toxic and non-biodegradable biocide compounds and overcome some challenges associated with resistance.

Over the past decade, a handful of studies on glycolipid biosurfactants from MACA species*,* have shown these to have promising anti-adhesion and anti-biofilm properties against Gram-positive and negative bacteria, as well as yeast and fungi (Table 1.4) (Dalili *et al*., 2015; Janek *et al*., 2018; Kugler *et al.,* 2015; Pirog *et al*., 2014). Trehalolipid biosurfactants from *R. ruber* IEGM 231 were found to reduce the adhesion of actively growing Gram-positive and Gram-negative bacteria by 30 to 76% (Kuyukina *et al.*, 2016). A trehalose lipid produced by *R. fascians* BD8 demonstrated anti-adhesive properties against both bacteria and yeast on a polystyrene surface and silicone urethral catheters (Janek *et al*., 2018). Meanwhile, a lipopeptide biosurfactant produced by *C. xerosis* NS5 decreased the adhesion of biofilm-forming strains on plastic tissue culture plates by 50 to 82% (Dalili *et al.,* 2015). A biosurfactant mix including trehalose di-acetates and di-mycolates from *N. vaccinii* IMB B-7405 reduced the adhesion of bacteria by 35–75%, yeast by 80–85%, and micromycetes by 40–50%. This biosurfactant mix prevented biofilm formation on various surfaces including catheters, dentures, plastic, polyvinyl chloride, tiles, and steel (Pirog *et al*., 2014).

Table 1.4. Biosurfactants with anti-biofilm properties produced by mycolic acid-containing actinobacteria.

Strain(s)	Surfactant(s)	Property	Target strains	Reference(s)
Corynebacterium xerosis NS5	Coryxin (lipopeptide)	Anti-adhesion and Disruption	*Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli,* and *Pseudomonas aeruginosa*.	Dalili *et al.,* 2015
Nocardia vaccinii IMB B7405 (K-8)	Complex amino lipids; neutral lipids trehalose di-acelates and di-mycolates	Anti-adhesion	*E. coli, Proteus vulgaris, P. aeruginosa* and *Enterobacter cloaceae, Candida albicans* on silicon urogenital catheters. *C. albicans* and *E. coli* on treated acrylic dental material. *Bacillus subtilis* and *Aspergillis niger* when coated on various abiotic substrates.	Pirog *et al*., 2014
Rhodococcus erythropolis IMB Ac-5017 (EK-1)	Complex of trehalose mono- and di-mycolates; neutral lipids (cetyl alcohol, palmitic acid, methyl ether of n-pentadecanoic acid, mycolic acids); phospholipids (phosphatidylglycerol, phosphatidylethanol-amine)	Anti-adhesion	Gram-negative bacteria and *C. albicans* on silicon urogenital catheters. *B. subtilis* on various abiotic substrates. *C. albicans* and *E. coli* on acrylic dental material. *S. aureus* and *P. aeruginosa* on plastic and steel.	Pirog *et al*., 2014
Rhodococcus fascians BD8	Trehalose lipid	Anti-adhesion	Various Gram-positive and negative strains and *C. albicans* on polystyrene. *Enterococcus hirae* and *E. faecalis, E. coli,* and *C. albicans* on glass, polystyrene, and silicone urethral catheters.	Janek *et al*., 2018
Rhodococcus ruber IEGM 231	Trehalolipid	Anti-adhesion	*Bacillus subtilis, Corynebacterium glutamicum, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens,* and *Brevibacterium linens* on polystyrene.	Kuyukina *et al.,* 2016

While existing studies in this area are limited and each focused on just a single strain, it is important to note that glycolipid production is common amongst members of this large and metabolically diverse group with various structural types reported. Hence, there is the potential to identify further strains from within this group that produce glycolipid biosurfactants with attractive anti-biofilm properties.

1.6. Approaches to screening for microbial biosurfactants.

The drive to discover novel biosurfactants necessitates robust, sensitive, and high throughput methods to screen microorganisms for production. Biosurfactants may be separated from crude cell extracts using adsorption chromatography and purified and characterised using chemical analytical methods such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance spectroscopy (NMR) (Patil & Pratap, 2018; Varjani & Upasani, 2017). However, these approaches are complex and time-consuming and do not lend themselves to high-throughput screening. More commonly, initial screening is undertaken using indirect assays that measure one or more physio-chemical properties of biosurfactants, such as cell surface hydrophobicity, the capacity to emulsify hydrocarbons, haemolytic or lipolytic activity, surface activity, and wetting properties (Pacwa-Plociniczak *et al*., 2011). Commonly applied qualitative and semi-quantitative methods used to test microorganisms for biosurfactant production along with their reported advantages and limitations are detailed in Table 1.5.

Screening assays are normally conducted on microorganisms in pure cultures either in liquid or on solid agar, but some may allow testing of environmental strains growing on primary isolation plates without sub-culture (Burch *et al*., 2010). Various tests can screen for both extracellular and cell-bound forms, and this has been found to be most effective when strains are cultivated in the presence of hydrophobic substrates (Walter *et al.,* 2013). Biosurfactant production by microorganisms appears to be influenced by environmental conditions, whereby biosurfactants facilitate the uptake and biodegradation of hydrophobic substrates (Kugler *et al*., 2015).

Table 1.5. Physico-chemical methods used to screen microorganisms for biosurfactant production.

Screening Assay	Reference	Type of method	Reported advantages	Reported disadvantages
Property detected: anionic surfactants				
Cetyl trimethyl ammonium bromide (CTAB) agar plate method	Siegmund & Wagner, 1991	Semi-quantitative	Easy to perform, different cultivation conditions can be applied directly e.g., substrates or temperature, and can be transferred to liquid culture conditions.	Specific for anionic biosurfactants and inhibits the growth of some microorganisms (Twigg *et al*., 2020).
Property detected: Cell surface hydrophobicity				
Microbial/Bacterial adhesion to hydrocarbons (MATH/BATH) assay	Chao *et al*., 2014	Quantitative	A simple photometric assay (Panjiar *et al*., 2015)	Bacterial cells may act as biosurfactants due to their hydrophobic cell surface.
Replica Plate Assay	Rosenberg *et al*., 1981	Qualitative	Simultaneously screen for biosurfactant production in many strains on readily available materials and identification and isolation of potential strains can be combined in one step.	Bacterial strain adherence to polystyrene is affected due to growth conditions like media composition, aeration, pH, temperature, and cell age (Pruthi & Cameotra 1997).
Hydrophobic Interaction Chromatography	Smyth *et al*., 1978	Qualitative	Screening and isolation of potential strains can be combined in one step.	Not reported.
Salt aggregation	Lindahl *et al*., 1981	Qualitative	A simple method providing a good estimation of the degree of cell surface hydrophobicity (Pruthi & Cameotra, 1997).	Not reported.
Property detected: Emulsification				
Emulsification assay	Rosenberg *et al*., 1979	Quantitative	Reliable, simple, and low-cost.	Gives only an indication of the presence of biosurfactants (Ariech &Guechi, 2015).
Emulsification Index	Cooper & Goldenberg, 1987	Quantitative	Reliable, simple, and low-cost. (Bonilla *et al*., 2005)	Gives only an indication of the presence of biosurfactants.

Screening Assay	Reference	Type of method	Reported advantages	Reported disadvantages
Property detected: Haemolytic activity				
Blood agar lysis	Mulligan *et al.*, 1984	Qualitative	Simple and easy method (Afshar *et al*.,2008).	Not all biosurfactants have haemolytic activity (Schulz *et al*., 1991) and lysis of blood cells can be caused by other microbial compounds e.g.virulence factors, hence poor specificity (Plaza *et al*., 2006). Biosurfactants that diffuse poorly in agar may not lyse blood cells (Thavasi *et al*., 2011; Youssef *et al*., 2004).
Property detected: Lipopeptides				
Bromophenol blue assay	Ong & Wu, 2018	Quantitative	Easy to perform, instant results, small sample volumes required, no toxic chemicals, and suitable for high-throughput screening.	Not reported.
Property detected: Surface activity				
Oil spreading	Morikawa *et al*., 2000	Qualitative and Quantitative	Simple, easy to perform, rapid, and reliable (Plaza *et al.,* 2006), can be used to detect low activity and low biosurfactant levels (Tugrul *et al*., 2005)	Not reported.
Titled glass slide test	Persson & Moli, 1987	Qualitative	Easy to perform.	Must be supported by secondary screening (Trindade *et al*., 2021).
Surface / interfacial tension measurement	Tadros, 2005	Quantitative	Straightforward (Lin, 1996) and gives a strong indication of biosurfactant production.	Time-consuming, requires specialised equipment and large sample volumes, not easily adapted to high-throughput and quantitative measurement restricted to concentration ranges below the critical micelle concentration (Bodour & Miller-Maier, 1998).

Screening Assay	Reference	Type of method	Reported advantages	Reported disadvantages
Hydrocarbon overlay agar assay	Morikawa, 1992	Qualitative	Simple to perform.	Cannot be applied to strains that do not degrade hydrocarbons (Trindade *et al*., 2021).
Penetration Assay	Maczek *et al*., 2007	Qualitative	Simple to perform and can be applied in high throughput screening.	Not reported.
Property detected: De-/Wetting				
Drop collapse /Modified drop collapse test	Jain *et al*., 1991; Bodour & Miller-Maier, 1998	Qualitative and Quantitative	Simple, easy to perform, rapid, and reliable, small sample volumes required (Plaza *et al.,* 2006).	Not sensitive, and often cannot detect low concentrations of biosurfactants (Youssef *et al.,* 2004).
Microplate assay	Chen *et al*., 2007	Qualitative	Easy to perform, instant results, small sample volumes required, reliable, and suitable for high-throughput screening.	Not reported.
Atomized oil assay	Burch *et al*., 2010	Qualitative	Can be performed directly on many colonies simultaneously, does not require preparation of cultures and supernatants, is sensitive, and allows high-throughput screening.	Highly hydrophilic surfactants can modify oil droplets to give false-positive results, and the nutrient medium used can preclude from detection of surfactants by some strains that require specific conditions for surfactant production (Burch, 2011).

The presence of hydrophobic substrates including hexadecane and n-tetradecane has been shown to upregulate genes involved in the biosynthesis of rhamnolipids, as observed in *Dietzia maris* (Wang *et al*., 2014b). It is not surprising therefore that biosurfactant-producing microorganisms are prevalent in various environments such as soil or water contaminated with hydrophobic organic compounds e.g., refinery wastes, oils, etc. (Walter *et al*., 2010).

For laboratory studies on biosurfactant production, strains are typically cultivated using a mineral salt medium containing essential trace elements supplemented with a hydrocarbon substrate as the sole carbon source. These substrates can include crude oil, diesel, n-alkanes, hexadecane, paraffin, polyaromatic hydrocarbons (PAHs), or vegetable oils like olive oil or rapeseed oil. These substrates may be incorporated into the liquid or solid medium, spread across the agar surface, or soaked onto a filter in the lid of Petri dishes (Domingues *et al*., 2013). The alkane, hexadecane, commonly found in the environment, is a natural substrate for MACA strains and has been shown to yield 50% exolipids, based on weight, in *Rhodococcus* strains (Uchida *et al*. 1989). A concentration of 2% v/v has proven effective for promoting growth and biosurfactant production in various species, including *Dietzia maris* (Nakano *et al*., 2011), *Gordonia amicalis* and *G. terrae* (Franzetti *et al*., 2007), *Rhodococcus ruber* (Philp *et al*.,), *Rhodococcus* spp. Shavandi *et al*., 2011), and *Tsukamurella pseudospumae* and *T. spumae* (Kugler *et al*., 2015). This standardisation allows for meaningful comparisons across different studies.

Most approaches to detect biosurfactant production rely on the measurement of surface and/or interfacial tension reduction (Walter *et al.*, 2013), and several quantitative methods are available (Table 1.6.).

Table 1.6. Quantitative methods for measuring surface and interfacial tension.

Method	Principle	Disadvantages
Du-Noüy-Ring (Tadros, 2005)	Measures the force required to detach a wire ring or loop from an interface or surface using a tensiometer.	• Requires specialised equipment. • Multiple samples cannot be measured simultaneously. • Millilitres volume of sample required for analysis. • Restricted range of concentration that can be analysed without dilution. (Bodour & Miller-Maier, 1998)
Traube stalagmometer (Dilmohamud *et al*., 2005)	A broad flattened tip pipette forms large reproducible-sized droplets, which finally drop under the action of gravity. The number of drops per volume, the density of the sample, and the surface tension to the control (water) determines the surface tension of the sample.	• Samples cannot be measured in parallel. • Prone to variability due to fast drop formation that allows complete adsorption of the surfactants to the newly generated drop, and hence this method is not recommended. (Plaza *et al*., 2006)
The pendant drop shape technique (Tadros, 2005)	An optical method for measuring the interfacial tension where a drop of liquid is allowed to hang from the end of a capillary that adopts an equilibrium profile that is a unique function of the tube radius, the interfacial tension, density, and the gravitational field.	• Multiple samples cannot be measured in parallel.
Axisymmetric Drop Shape Analysis by Profile ((ADSA-P; Van der Vegt *et al*., 1991)	An optical method for measuring surface tension is based on the principle that the shape of the liquid droplet depends greatly on the surface tension of the liquid. Low surface tension liquid droplets deviate more than a perfectly spherical shape droplet with high surface tension.	• Special cameras and software are required. • The calculation of the surface tension is complex. • Multiple samples cannot be measured in parallel.

The du Noüy ring method considered accurate, is most frequently used (Pornsunthorntawee *et al*., 2008; Walter *et al.*, 2013). This assay measures the force (Newton per Meter; mN m^{-1}) required to detach a wire ring or loop that is attached to a microbalance sensor head from the surface of a liquid or the interface between two immiscible phases using a tensiometer (Tadros, 2005; Fig 1.5). The detachment force is proportional to the surface tension.

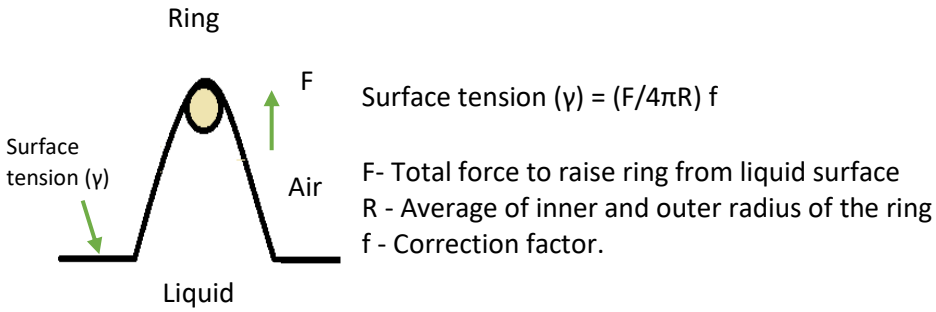


Fig 1.5. Calculation of surface tension and the correction factor for the du Noüy ring method.

The surface tension decreases with increasing surfactant concentration. However, beyond a certain concentration, termed the critical micelle concentration (CMC), biosurfactant molecules associate to form micelles, and no further reduction in surface tension is observed so any further increase in biosurfactant concentration cannot be detected (Fig 1.6).

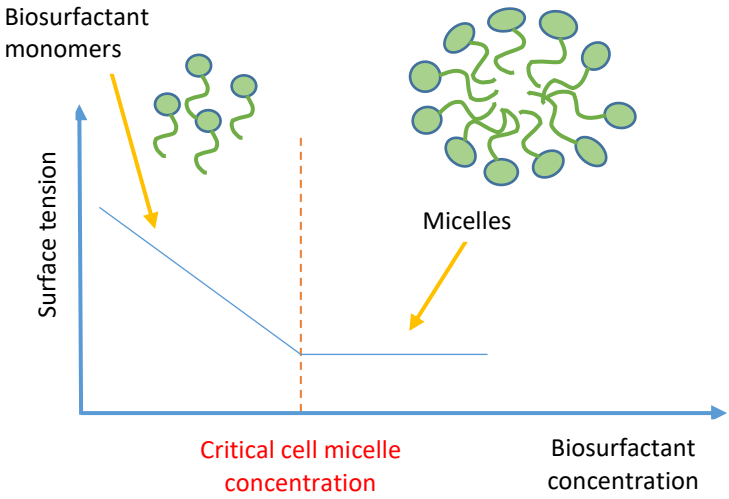


Fig 1.6. Relationship between biosurfactant concentration, surface tension, and the formation of micelles.

The oil spreading (OS) assay, alternatively known as the oil displacement assay (Morikawa *et al.*, 2000), also relies on the surface-active properties of biosurfactants. This method is straightforward, requiring no specialised equipment, unlike the du Noüy ring method and other techniques detailed in Table 1.6, page 29). In this assay, the addition of biosurfactant(s) displaces a layer of oil on the water's surface, creating a distinct clearance zone. The assay is usually applied qualitatively, although the diameter of the clear zone has demonstrated a linear relationship with surfactant concentration in *Bacillus*

spp. (Youssef *et al*., 2004) and has also been employed semi-quantitatively. The OS assay has been used to test various MACA strains for biosurfactant production (Table 1.7).

The drop collapse (DC) assay, conducted qualitatively, is also straightforward and relies on the capacity of biosurfactants to destabilise and cause the collapse of a liquid droplet (Jain *et al*., 1991). Modifications using multi-well microtitre plates have enabled the simultaneous screening of numerous strains (Chen *et al*., 2007; Satpute *et al*., 2008). This method has been employed to screen strains of *Gordonia* and *Rhodococcus* (Table 1.7).

Emulsification is another physicochemical property that may be exploited to screen for biosurfactant production. Bio-emulsifying surfactants stabilise emulsions, which consist of microscopic droplets of one liquid within another immiscible liquid, such as oil droplets in water, by reducing the interfacial tension (Desai & Banat, 1997; Ron & Rosenberg, 2001). The electrically charged hydrophilic ends of the biosurfactant surround the droplets in the internal phase, preventing them from coalescing and forming larger droplets. The droplets behave like electrically charged particles, causing them to repel each other. The emulsification capacity of a surface-active compound can be measured by the quantitative emulsification index (EI) assay (Cooper & Goldenberg, 1987) which measures the ability of the emulsion to remain stable over a defined period (Boye *et al*., 2010).

Alternatively, the emulsification activity (EA) assay (Rosenberg *et al*., 1979) measures the amount of oil emulsified per unit of biosurfactant with one unit of emulsification activity defined as the quantity of biosurfactant required to form an emulsion with an absorbance of 1.0 when read at 540 nm wavelength (Cirigliano & Carman, 1984; Lima *et al.*, 1997). Studies investigating biosurfactant and bioemulsifier production among MACA strains frequently employ the EI assay (Table 1.7). It is worth noting that not all biosurfactants can produce emulsions although all bioemulsifiers are considered biosurfactants (Viramontes-Ramos *et al*., 2010).

Table 1.7. Screening methods used to detect biosurfactant production by mycolic acid-containing actinobacteria.

Physico-chemical property	**Screening method**	**MACA species**	**Reference**
Cell-surface hydrophobicity	Microbial/Bacterial adhesion to hydrocarbons (MATH/BATH)	*Dietzia maris*	Nakano *et al.,* 2011
		Gordonia alkanivorans	Mohebali *et al.,* 2007
		Nocardia otitidiscaviarum	Vyas & Dave, 2011
		Rhodococcus fascians	*Gesheva et al.,* 2010
Emulsification	Emulsification assay	*Gordonia amicalis*	Jackisch-Matsuura *et al*., 2014
	Emulsification Index	*Dietzia* spp.	Hvidsten *et al*., 2017; Nakano *et al.,* 2011
		Gordonia spp.	Franzetti *et al*., 2008; Kurniati *et al*., 2016
		Nocardia spp.	Vyas & Dave, 2011
		Rhodococcus sp.	Suryanti *et al*., 2016, Gesheva *et al*., 2010, Tuleva *et al*., 2008
		Tsukamurella spp.	Kügler *et al*., 2014
Surface activity	Oil spreading	*Dietzia* spp.	Hvidsten *et al*., 2017
		Gordonia spp.	Franzetti *et al*., 2008
		Rhodococcus erythropolis	Peng *et al*., 2007
	Drop collapse/ modified drop collapse	*Gordonia westfalica*	Laorrattanasak *et al*., 2016
		Rhodococcus erythropolis	Peng *et al*., 2007
	Surface & interfacial tension measurement	*Dietzia* sp.	Nakano *et al.,* 2011; Hvidsten *et al*., 2017
		Gordonia spp.	Franzetti *et al*., 2008; Laorrattanasak *et al*., 2016; Kurniati *et al*., 2016
		Nocardia sp.	Vyas & Dave, 2011
		Rhodococcus spp.	Zheng *et al*., 2009; Peng *et al*., 2007; Tuleva *et al*., 2008
		Tsukamurella spp.	Kügler *et al*., 2014

The cetyl trimethyl ammonium bromide (CTAB) method (Siegmund & Wagner, 1991) can be used to detect anionic surfactants (Sidkey *et al*., 2016). Test strains are grown on light blue mineral salts agar containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue. Anionic biosurfactants secreted by the microorganism, form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue, and thus productive colonies are surrounded by dark blue halos. This method has not been applied to screen MACA strains although they are known to produce anionic glycolipids therefore this method, if found reliable, would be useful for confirming the presence of anionic biosurfactants at the initial screening stage. A similar recommendation to use the CTAB method at the initial stage was made by Sidkey *et al.* (2016).

Cell surface hydrophobicity (CSH) has been reported to indicate biosurfactant production (Pruthi & Cameotra, 1997) and is used as an indirect test for this. Several methods are available to measure CSH, including contact angle measurement (Smits *et al*., 2003), hydrophobic interaction chromatography (HIC; Smyth *et al*., 1978), microsphere adhesion to cells (Hazen & Hazen, 1987), microbial/bacterial adherence to hydrocarbons (MATH/BATH) method (Chao *et al*., 2014; Rosenberg *et al*., 1980), replica plate assay (Rosenberg, 1981) and the salt aggregation test (Ljungh & Wadström, 1982). The MATH/BATH assay is most frequently used and measures CSH based on the adherence of microbial cells to hydrocarbon droplets. When cells are agitated with a continuous aqueous phase and oil, those cells that are hydrophobic will attach to the oil and are removed from the aqueous phase. The resulting decrease in the turbidity of the aqueous phase, measured by spectrophotometry, correlates to the hydrophobicity of the cell culture (Rosenberg *et al*., 1980). This is converted to a percentage adhesion to hydrocarbon or percentage CSH value by comparison to a control without oil. Kaczorek *et al*. (2016) proposed that cells with hydrophobicity values of 0 to 30% should be considered hydrophilic, those with values greater than 30% as having a mix of hydrophilic and hydrophobic properties, whilst cells with CSH values greater than 60% be considered as hydrophobic. This assay has been used to screen for biosurfactant production

in various MACA including *Dietzia*, *Gordonia,* and *Rhodococcus* species (Table 1.7, page 32).

1.7. Extraction and purification of biosurfactants from mycolic acid-containing actinobacteria.

Screening assays indicate whether a given strain produces cell-bound and/or extra-cellular biosurfactants but offer limited information on the specific chemical characteristics of the biosurfactant(s) produced. Extraction and purification of biosurfactants allow for more detailed characterisation of the chemical and physical properties, chemical structures, and behaviour of biosurfactants. It is also important to isolate biosurfactants responsible for specific biological activities, such as antimicrobial or anti-adhesive properties. Purification also allows for accurate quantification, which is important for determining the concentration of active surfactant molecules in a sample.

The recovery of biosurfactants from bacteria largely depends on their location i.e., cell-bound, extracellular, or intracellular, as well as their water solubility and ionic charge. Crude biosurfactant extracts which may contain mixtures of different biosurfactant compounds can be recovered from cell cultures (cell-bound and extra-cellular biosurfactants) or cell-free broth (extra-cellular biosurfactants only) and subsequently purified using a variety of methods. Commonly used biosurfactant extraction methods include acid or ammonium sulphate precipitation, centrifugation, crystallization, foam fractionation, ion exchange columns, organic solvent extraction, and ultrafiltration.

Solvent extraction is typically used to extract glycolipid biosurfactants from MACA strains (Konishi *et al*., 2014, Kugler *et al*., 2015; Zargar *et al*., 2022). The solvents are added to the culture or cell-free supernatant, and the mixture is agitated and then separated. This approach has been used to extract glycolipids including rhamnolipids and trehalose lipids (Desai & Banat, 1997; Smyth *et al*., 2009). Commonly used solvents include the polar aprotic solvents ethyl acetate and methyl tert-butyl ether (MTBE), dichloromethane, or varying ratios of chloroform-methanol or MTBE–chloroform (Table 1.8).

Table 1.8. Solvent mixes used to extract biosurfactants from mycolic acid-containing actinobacteria.

Method	**Strain(s)**	**References**
Chloroform-methanol	*Dietzia maris* WR-3	Nakano *et al*., 2011
	Gordonia amicalis DRM 190-07	Jackisch-Matsuura *et al*., 2014
	G. westfalica GY40	Laorrattanasak *et al.,* 2016
	Nocardia otitidiscaviarum MTCC 6471	Vyas & Dave 2011
	N. vaccinii IMV B-7405	Pirog *et al*., 2015
	Rhodococcus sp. NJ2	Kumari *et al*., 2012
	R. fascians A-3	Gesheva *et al*., 2010
	R. erythropolis IMV Ac-5017	Pirog *et al*., 2015
Methyl tert-butyl ether (MTBE)	*Rhodococcus sp. PML026*	White *et al*., 2013
	R. erythropolis 3C-9	Peng *et al*., 2007
	R. erythropolis MTCC 2794	Patil & Pratap, 2018
	R. ruber IEGM 231	Kuyunika *et al*., 2001
	R. wratislaviensis BN38	Tuleva *et al*., 2008
Ethyl acetate	*Tsukamurella spumae* and *T. pseudospumae*	Kügler *et al.*, 2014

A chloroform-methanol mixture is often used to extract glycolipids, with the ratio changed to adjust the polarity to help target the extractable components (Jackisch-Matsuura *et al*., 2014; Kyunika *et al*., 2001; Laorrattanasak *et al*., 2016; Vyas & Dave 2011). Rhamnolipid glycolipids can be extracted using chloroform-methanol (2:1, v/v) or ethyl acetate (Schenk *et al*., 1995; Trummler *et al*., 2003), while the solvents used for trehalose glycolipids include MTBE, ethyl acetate and chloroform-methanol (2:1, v/v) (Kuyukina *et al*., 2001; Marqués, *et al*., 2009).

Acid precipitation method have been used to extract rhamnolipids (Haba *et al*., 2000; Smyth *et al*., 2009) and trehalose lipids (Rapp *et al*., 1979) and are easy and inexpensive. Solvent extraction coupled with an acidification step to reduce water solubility can help improve the extraction yield (Konishi *et al*., 2014; Passeri *et al*., 1990). Acid precipitation followed by extraction by n-hexane and

chloroform-methanol mixture has been used for trehalose lipid extraction (Banat *et al*., 2010). Biosurfactants from MACA including *Nocardia otitidiscaviarum* MTCC (Vyas & Dave, 2011), *Gordonia westfalica* GY40 (Laorrattanasak *et al*., 2016), *Rhodococcus* sp. NJ2 (Kumari *et al*., 2012) and *Rhodococcus* sp. HL-6 (Tian *et al*., 2016) has been successfully recovered by acid precipitation followed by chloroform-methanol (2:1 v/v).

The extracted biosurfactants can be further purified using various chromatography-based methods. Thin layer chromatography (TLC), silica gel chromatography (SGC), high-performance liquid chromatography (HPLC), size exclusion chromatography, and ion-exchange chromatography have all been used to purify biosurfactants produced by mycolic-acid-containing actinobacteria (Table 1.9). These allow purification based on molecular weight, solubility, and ionic properties, and provide highly purified products compared to other methods (Venkataraman *et al*., 2021).

Various other techniques have been used to characterise and identify glycolipids biosurfactant compounds such as Gas chromatography-mass spectrometry (GC–MS; Peng *et al*., 2007; Zargar *et al*., 2022), Liquid chromatography-mass spectroscopy with electrospray ionization (LC-MS-ESI; Arutchelvi & Doble 2010; Varjani & Upasani, 2016) and High performance liquid chromatography-mass spectrometry (HPLC-MS; Dardouri *et al*., 2021; George & Jayachandran, 2013; Pathania & Jana 2020). Other techniques used are Fourier transform infrared spectroscopy (FTIR; Arutchelvi & Doble 2010; Frankfater *et al*., 2020; Khademolhosseini *et al*., 2019; Varjani & Upasani, 2016), Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF; Sato *et al*., 2019; Shreve & Makula, 2019), Nuclear magnetic resonance spectroscopy (NMR; Khademolhosseini *et al*., 2019; Varjani & Upasani, 2016) and Tandem mass spectrometry (MS/MS; Hoskova *et al*., 2013).

Table 1.9. Methods for purification of biosurfactants extracted from mycolic acid-containing actinobacteria.

Method	Strain(s)	References
Liquid chromatography (LC)	*Tsukamurella spumae* (DSM44113, DSM44114) and *T. pseudospumae* (DSM44117)	Kügler *et al*., 2014
Thin layer chromatography (TLC)	*Gordonia amicalis* DRM 190-07 *Rhodococcus sp.* PML026	Jackisch-Matsuura *et al*., 2014 White *et al*., 2013
Silica gel chromatography (SGC)	*R. ruber* IEGM 231 *R. wratislaviensis* BN38	Kuyunika *et al*., 2001 Tuleva *et al*., 2008
SGC and high-performance liquid chromatography (HPLC)	*Dietzia maris* As-13-3	Wang *et al*., 2014b
SGC and TLC	*D. maris* WR-3 *R. erythropolis* 3C-9 *R. erythropolis* MTCC 2794	Nakano *et al*., 2011 Peng *et al*., 2007 Patil & Pratap, 2018

1.8. Research Rationale

Biofilms pose a significant clinical challenge, given their capacity to induce a wide array of severe and recalcitrant infections (Vestby *et al*., 2020). There is an urgent demand for novel anti-biofilm agents capable of preventing the adherence and development of biofilms on various surfaces and dispersing pre-existing biofilms effectively. Amongst various candidates for investigation as new anti-biofilm agents, biosurfactants have proven to be effective against a broad range of clinical pathogens. Further, their biodegradability and low toxicity confer several advantages over other candidates and indeed their chemical surfactant counterparts (Makkar & Cameotra 2002; Patel & Kharawala 2022; Shekhar *et al*., 2015). Whilst several biosurfactants have been shown to

affect microbial biofilms, there is a considerable drive to discover additional natural biosurfactants with enhanced anti-biofilm effects against an extended range of target pathogens, and with improved productivities and yields (Paraszkiewicz *et al*., 2021).

Whilst genomics-based approaches offer the potential to engineer strains with desired attributes and to optimise the bioactive compounds of interest, there are critical knowledge gaps and technological limitations that hinder their application in the near future. There are various challenges with respect to biological platforms (biological design principles, genetically tractable organisms, minimal cell, and in vitro systems), computational tools and bioinformatics (databases and information standards,) as well as methods and technology (analytical tools, DNA synthesis, and assembly and genome-scale engineering tools) (Kelley *et al*., 2014). In the meantime, natural biosurfactants remain the focus for research.

The mycolic acid-containing actinobacteria (MACA) of the suborder Corynebacterineae are a significant source of new bioactive compounds (Azman *et al*., 2017) including biosurfactants (Kugler *et al*., 2015) and recent studies have shown that glycolipid compounds have potential application as anti-biofilm agents (Janek *et al*., 2018; Kiran *et al*., 2010, 2017). However, the strains studied to date represent only a fraction of the species within the suborder Corynebacterineae. Further, new species are frequently described, many of which might harbour previously undiscovered biosurfactants. Thus, this group of actinobacteria holds the potential to yield many novel biosurfactants that could find application as anti-adhesion and anti-biofilm agents. Despite this promise, the exploration of this group remains limited to date.

The current study will explore a large collection of MACA species for their ability to synthesise both cell-bound and extra-cellular biosurfactants. A variety of qualitative and quantitative indirect assays that measure various physicochemical properties of biosurfactants will be employed and evaluated for their suitability in screening members of the mycolic acid-containing actinobacteria. This will be important because mycolic acids have been

reported to influence cell surface hydrophobicity and may possess inherent biosurfactant properties, potentially affecting the results of screening assays and introducing the possibility of false positives and negatives.

Strains found to produce extra-cellular biosurfactants will undergo further assessment for their potential anti-adhesion and/or anti-biofilm properties against clinically relevant bacteria. The selection of representative clinical strains will be based on their ability to form biofilms under static growth conditions and will include both Gram-negative and Gram-positive species with known antibiotic resistance profiles. The extracellular biosurfactant extracts demonstrating promising anti-adhesion and/or anti-biofilm effects against one or more biofilm-forming bacteria will be further purified and the constituent glycolipid biosurfactants characterised by chemical analytical methods. The further purified extracts will then be assessed to determine if their anti-biofilm properties remain.

1.9. Research Aims and Objectives

This program of research aims to investigate diverse mycolic acid-containing actinobacteria for their ability to produce extracellular biosurfactants and to test these for anti-adhesion, biofilm inhibition, and/or biofilm disruption properties against biofilms formed by clinically significant pathogenic bacteria.

The main objectives of this research are to:

1. Evaluate mycolic acid-containing actinobacteria (MACA) belonging to various genera for cell-bound and/or extra-cellular biosurfactant production using screening assays based on different physico-chemical properties.
2. Determine a reliable biosurfactant screening approach for MACA strains, taking into consideration the performance of and agreement between various screening assays, and any potential impacts of mycolic acid chain length and cell surface hydrophobicity on the test results.
3. Determine the anti-biofilm effects of extracellular biosurfactants from selected MACA strains against clinically significant biofilm-forming bacteria using quantitative static biofilm assays.

4. Isolate the glycolipid components from extra-cellular biosurfactant extracts recovered from selected MACA strains and determine the chemical composition by chemical-analytical techniques.
5. Determine the anti-biofilm properties of the extra-cellular glycolipids recovered from selected MACA strains.

Chapter 2. Screening actinobacteria of the suborder *Corynebacterineae* for biosurfactant production.

2.1. Introduction

While biosurfactants with antibiofilm properties have been reported for various bacterial groups such *Bacillus*, *Lactobacillus, Pseudomonas* and *Staphylococcus* (Gudiña *et al*., 2015; Janek *et al*., 2012; Mani *et al*., 2016; Rivardo *et al*., 2009; Sambanthamoorthy *et al*., 2014) some of these microorganisms are opportunistic pathogens and limited productivity of most restricts their widespread adoption and commercialisation. Biosurfactants produced by some members of the mycolic acid-containing actinobacteria (MACA) in the suborder *Corynebacterineae* also possess anti-biofilm and anti-microbial activities (Chapter 1, Table 1.4., page 23) (Dalili *et al*., 2015; Janek *et al*., 2018; Kugler *et al.,* 2015; Pirog *et al*., 2014). This group is large and expanding (Goodfellow & Jones, 2015) other species are likely capable of producing biosurfactants, both known and novel, with anti-biofilm properties.

Any survey of this group requires reliable and effective screening assays for cell-bound and extra-cellular biosurfactant production. Various qualitative and semi-quantitative assays are available (refer to Chapter 1, Tables 1.5 & 1.6, page 25 & 29) several of which have been used to test MACA strains (as exemplified in Table 1.7, page 32). However, given the indirect nature and limitations associated with some of these screening methods, no one assay is considered definitive (Satpute *et al*., 2008; Walter *et al*., 2013; Sidkey *et al*., 2016) therefore studies tend to utilise several assays (Plaza *et al.,* 2006; Satpute *et al.,* 2008; Sidkey *et al.,* 2016; Thavasi *et al*., 2011; Youssef *et al*., 2004).

Despite this, few studies have focused on the evaluation of multiple assays and on establishing correlations between these and even less have included a substantial set of test strains (Youssef *et al.*, 2004; Thavasi *et al*., 2011). A handful of other studies has relied on small numbers of test strains, which are often unidentified isolates (Afshar *et al*., 2008; Ariech & Guechi, 2015; Plaza *et al*., 2006; Varjani *et al*., 2007). Studies on actinobacteria and mycolic acid-

containing species specifically are lacking in the literature. Volchenko *et al.* (2007) compared three assays using a small number of *Gordonia*, *Nocardia,* and *Rhodococcus* strains. In the only other study focused on actinobacteria, Loganathener *et al*. (2010) attempted to correlate the results of various assays on three putative *Streptomyces* spp. The reliability of individual assays and correlations between assays appear to vary between these studies and between bacterial groups.

Biosurfactants are structurally diverse, and strains can produce various classes and congeners (Tiso *et al*., 2017) with potentially distinct physico-chemical properties (Rocha *et al*., 2020). Hence, in the current study, it will be important to include multiple assays to mitigate individual assay limitations and minimise the risk of false results. Additionally, the inclusion of diverse species and genera is important given the unique cell wall chemistry of this group, specifically the presence of cell-bound and detachable mycolic acids of varying lengths. These compounds may influence cell surface hydrophobicity (CSH) (Blackall & Marshall, 1989b), upon which some biosurfactant assays are based.

Mycolic acids with longer carbon chain lengths are generally hydrophobic whilst shorter mycolic acids are hydrophilic (Bendinger *et al.*, 1993) which may influence the degree of cell surface hydrophobicity (Mori *et al*., 1988). This might suggest that *Corynebacterium* species with shorter mycolic acids (carbon chain length C_{22}-C_{38}) have lower CSH than *Mycobacterium* species with longer mycolic acids (chain length C_{60}-C_{90}). Hence, when employing screening assays based on CSH, the results may be affected by strain mycolic acid length, potentially differing between genera.

Additionally, mycolic acids can exhibit surface-active properties (Lee *et al.,* 2005), and whilst most are cell-bound, some form the lipid component of extracellular trehalose lipids, particularly following growth on alkanes or related hydrophobic substrates (Ahmadi-Ashtiani *et al*., 2020; Cooper *et al*., 1980). Given their biosurfactant-like characteristics, mycolic acids may elicit positive results in surface tension-based assays for both cell-bound and extra-cellular biosurfactants (Lee *et al*., 2005). Hence, MACA strains with longer-chain

mycolic acids could potentially reduce surface tension more than those with shorter-chain mycolic acids.

Further, the ability to emulsify different carbon sources appears associated with mycolic acid chain length (Lee *et al*., 2005) implying that the choice of hydrophobic substrate may influence emulsification assay outcomes for MACA strains. The presence of mycolic acids and the substrate used for growth must therefore be considered when selecting appropriate screening strategies and when interpreting the results of surface tension and emulsification-based biosurfactant tests. It may be the case that mycolic acids 'mask' the presence of other surfactants or indeed be the only surface-active compounds present. However, none of the aspects discussed above have been investigated in any detail to date.

Edinburgh Napier University holds a large collection of well-characterised type strains of validly described *Corynebacterineae* species, encompassing nine phylogenetically related genera. Biosurfactant production has previously been reported for several species in the collection (e.g., Kugler *et al*., 2014, 2015; Kuyukina *et al*., 2001, as indicated in Table 2.1, page 46), but not necessarily the type strains held. Most species in the collection have not been investigated for biosurfactant production previously. Furthermore, very few studies have explored the potential antibiofilm properties of biosurfactants produced by these strains. Hence, this culture collection offers a unique opportunity for a large-scale survey of surface-active compounds within a phylogenetically coherent yet metabolically diverse group. The collection also provides a valuable resource for the potential discovery of novel biosurfactants with anti-biofilm properties.

In this study, various standard biosurfactant-screening assays will be applied to this collection of MACA strains, and evaluated for their sensitivity, reproducibility, and easy-of-use. This is important given that biosurfactant screening results can differ based on assay type, and correlations between assays appear to vary with the bacterial group(s) under study. Existing studies on this have not explored assay correlations amongst the *Corynebacterineae*. In addition, the relationships between biosurfactant production, the presence

and chain length of mycolic acids, and cell surface hydrophobicity remain poorly understood. The presence of cell-bound and extra-cellular mycolic acid compounds may mask the detection of other biosurfactants. Further, mycolic acid presence and chain length may also influence cell surface hydrophobicity and emulsification, potentially impacting the usefulness and/or reliability of screening assays based on these properties. Given that mycolic acid chain length (and genus assignment) has been reported for all strains in the culture collection, comparative analysis against cell surface hydrophobicity and emulsification under standard growth conditions can be done to determine any correlations between these factors biosurfactant test results. Evaluating assay reliability, correlations, and any influence of mycolic acids on results interpretation will guide future investigations of biosurfactant production amongst known and novel *Corynebacterineae* strains.

2.1.1. Study Aim and Objectives.

The primary aims of the present study are to investigate diverse mycolic acid-containing actinobacteria for the ability to produce biosurfactants and to determine the most reliable screening strategy for the detection of biosurfactant production by members of this group.

The specific objectives are to:

1. Screen diverse mycolic-acid containing actinobacteria for the production of cell-bound and/or extra-cellular biosurfactants using various indirect physico-chemical assays.
2. Establish correlations between different biosurfactant screening assay results and other properties unique to this bacterial group including cell surface hydrophobicity, mycolic acid chain length, and genus assignment.
3. Evaluate the assays employed to determine the most effective and reliable approach for future biosurfactant screening of mycolic acid-containing actinobacteria.
4. Select suitable test strains that produce extra-cellular biosurfactants to further investigate of biosurfactants with anti-biofilm properties.

2.2. Materials and Methods

2.2.1. Test Strains.

Ninety-four strains, representing 91 validly described species of the suborder *Corynebacterineae*, and belonging to the mycolic acid-containing genera *Dietzia* (13 strains), *Gordonia* (30 strains), *Millisia* (1 strain), *Mycobacterium* (6 strains), *Nocardia* (5 strains), *Rhodococcus* (25 strains), *Tsukamurella* (8 strains) and *Williamsia* (4 strains), along with non-mycolate species of the genus *Corynebacterium* (2 strains) (Table 2.1) were investigated in the present study. All strains were held in the Edinburgh Napier University (ENU) culture collection and originally purchased from the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) or donated from collections at Northumbria and Newcastle Universities.

2.2.2. Maintenance and long-term preservation of cultures.

Biomass of each strain was maintained on the nutrient-rich complex medium, Tryptic Soy Agar (TSA; Sigma-Aldrich, Dorset, UK) prepared by suspending 40g of dehydrated media in 1 litre of distilled water and sterilizing at 121°C for 15 minutes. The TSA plates were prepared by pouring 20ml of cooled medium (~50°C), into sterile petri dishes (150mm × 15mm) and allowing it to solidify at room temperature. Replicate glycerol stocks (50% v/v) were prepared as a source of working inocula and for long-term storage. Glycerol suspensions were prepared by scraping 1 - 2 loopfuls of single colonies grown on solid agar at 30°C for 4 days and emulsifying in 1.5 ml glycerol solution in 2.5 ml cryo-vials to obtain an even cell suspension before storage at -80°C. To obtain a working inoculum, glycerol stocks were thawed to room temperature, and a loopful (~10µl) of suspension was aseptically cross-streaked onto TSA plates. Inoculated plates were incubated at 30°C for 3 - 4 days or until visible colonies appeared.

Table 2.1. Actinobacterial strains belonging to the suborder *Corynebacterineae* included in the present study.

Strain code	Designation	Source/strain histories
Genus *Corynebacterium*		
N1278^{T}	*Corynebacterium amycolatum* Collins *et al*., 1988^{VP}	NCBI 43765^{T}, human skin, immunosuppressed patients, New Zealand.
DSM 8821^{T}	*Corynebacterium otitidis* (Funke *et al*., 1994^{VP}) Baek *et al*., 2018	*Corynebacterium otitidis* (Funke *et al*., 1994) Baek *et al*., 2018; ATCC 51513^{T}; G. Funke, GF 234/92, middle ear fluid from otitis media patient, Zürich, Switzerland.
Genus *Dietzia*		
DSM 45334^{T}	*Dietzia aerolata* Kämpfer *et al*., 2010^{VP}	NCBI 595984^{T}, air from a duck barn, Berlin in Germany.
DSM 45698^{T}	*Dietzia alimentaria* Kim *et al*., 2011^{VP}	JCM 16360^{T}, traditional salt-fermented seafood made of clam jeotgal, Korea (south), Republic of Sokcho.
DSM 45140^{T}	*Dietzia cercidiphylii* Li *et al*., 2008a^{VP}	NCBI 498199^{T}, surface-sterilized root sample of *Cercidiphyllum japonicum*, China Yunnan Province.
DSM 44904^{T}	**Dietzia cinnamea* (Nesterenko *et al.,* 1982*)* Rainey *et al.,* 1995^{VP} emend. Nouioui *et al*., 2018	*Rhodococcus maris* (ex Harrison 1929) Nesterenko *et al*., 1982, *Dietzia cinnamea Yassin et al., 2006* emend. Nouioui *et al*., 2018, NCBI 321318^{T}, a perianal swab from a patient with bone marrow transplantation, Germany.
DSM 447481^{T}	*Dietzia dagingensis* Nazina *et al*., 2003	263^{T}, formation water of the Daging oil field, Heilongjian province, China, Asia.
DSM 44907^{T}	*Dietzia kunjamensis* Mayilraj *et al*., 2006^{VL} emend. Nouioui *et al*., 2018	*Dietzia kunjamensis* subsp. *kunjamensis* (Mayilraj *et al.,* 2006) Nouioui *et al.,* 2018, JCM 13325^{T}, soil, cold desert of the Himalayas, India.
DSM45074^{T}	*Dietzia lutea* Li *et al*., 2011^{VL}	KCTC 19232^{T}, desert soil, Egypt.
DSM43672^{T}	**Dietzia maris* (Nesterenko *et al*., 1982) Rainey *et al*., 1995^{VP} emend. Nouioui *et al*., 2018	*Rhodococcus maris* (ex Harrison 1929) Nesterenko *et al*., 1982, *Dietzia cinnamea* Yassin *et al*., 2006 emend. Nouioui *et al*., 2018, ATCC 35013^{T}, soil.
DSM 44860^{T}	*Dietzia natronolimnea* Duckworth *et al*., 1999^{VL}	JCM 11417^{T}, soda lake, East Africa.
DSM 44961^{T}	*Dietzia papillomatosis* Jones *et al*., 2008^{VP}	NCIMB 14145^{T}, skin scrapings of a 17-year-old boy with C-reactive protein, United Kingdom.
DSM 44820^{T}	*Dietzia psychralcaliphila* Yumoto *et al*., 2002^{VP}	NCIMB 13777^{T}, water (6°C, pH7).
DSM 45139^{T}	*Dietzia kunjamensis subsp. schimae* (Li *et al*., 2008b) Nouioui *et al.,* 2018^{VP}	*Dietzia schimae* (Li *et al*., 2008b), JCM 16003^{T}, isolated from the stem of the plant Schima, China, Yunnan.
DSM 45568^{T}	*Dietzia timorensis* Yassin *et al*., 2010^{VP}	JCM 18295^{T}, soil, West Timor, Kupang, Indonesia.
Genus *Gordonia*		
N934^{T}	*Gordonia aichiensis* (Tsukamura 1983) Klatte *et al.,* 1994c^{VP}	DSM 43978^{T}, sputum, Obu, Japan.

Strain code	Designation	Source/strain histories
DSM 44369^{T}	**Gordonia alkanivorans* Kummer *et al*., 1999^{VP}	*Gordonia nitida* Yoon *et al*., 2000, JCM 10677^{T}, tar and phenol contaminated soil, Rosnitz, Thuringia, Germany.
DSM 43392^{T}	**Gordonia amarae* Lechevalier & Lechevalier 1974) Klatte *et al*., 1994^{VP} emend. Nouioui *et al*., 2018	*Nocardia amarae* Lechevalier & Lechevalier 1974, ATCC 27808^{T}, isolated from activated sludge, Florida.
DSM 43391	**Gordonia amarae* Lechevalier & Lechevalier 1974) Klatte *et al*., 1994^{VP} emend. Nouioui *et al*., 2018	*Nocardia amarae* Lechevalier & Lechevalier 1974, IMET 7517, isolated from activated sludge.
DSM 46078	**Gordonia amarae* Lechevalier & Lechevalier 1974) Klatte *et al*., 1994^{VP} emend. Nouioui *et al*., 2018	*Nocardia amarae* Lechevalier & Lechevalier 1974, IMET 7516, isolated from abnormal foam, Riverdale, Miami, United States of America.
DSM 44461^{T}	**Gordonia amicalis* Kim *et al*., 2000^{VP} emend. Nouioui *et al*., 2018	CIP 108824^{T}, isolated from garden soil in the vicinity of Perm, Russia.
DSM 44811^{T}	*Gordonia araii* Kageyama *et al*., 2006^{VP} emend. Nouioui *et al*., 2018	JCM 12131^{T}, isolated from human sputum, Japan.
DSM 43247^{T}	**Gordonia bronchialis* (Tsukamura 1971) Stackebrandt *et al*., 1989^{VP} emend. Nouioui *et al*., 2018	*Rhodococcus bronchialis* (Tsukamura 1971) Tsukamura 1974, ATCC 25592^{T}, isolated from the sputum of a woman with a cavitary disease of both upper lungs.
DSM 45229^{T}	**Gordonia cholesterolivorans* Drzyzga *et al*., 2009^{VP}	Chol-3^{T}, CECT 7408^{T}, isolated from sewage sludge from a sewage treatment plant, Ciudad Real, Spain.
DSM 44981^{T}	*Gordonia defluvii* Soddell *et al*., 2006b^{VP}	J4^{T}, NCIMB 14149^{T}, isolated from activated sludge foams, Brimbank Park, Victoria, Australia.
DSM 44462^{T}	**Gordonia desulfuricans* Kim *et al*., 1999^{VP} emend. Nouioui *et al*., 2018	213E^{T}, NCIMB 40816^{T}, soil in the vicinity of an oil shale spoil heap, West Lothian, Scotland, UK.
DSM 44810^{T}	*Gordonia effusa* Kageyama *et al*., 2006^{VP} emend. Nouioui *et al*., 2018	JCM 12130^{T}, human sputum, Japan.
DSM 44140^{T}	*Gordonia hirsuta* Klatte *et al*., 1996^{VP}	K 718a^{T}, JCM 10105^{T}, biofilter of an animal rendering plant, Lower Saxony, Germany.
DSM 45298^{T}	*Gordonia humi* Kämpfer *et al*., 2011^{VP}	CCM 7727^{T}, soil attached to a spawn used for growing *Agaricus brasiliensis*, Taiwan.
DSM 44015^{T}	**Gordonia hydrophobica* Bendinger *et al*., 1995^{VP} emend. Nouioui *et al*., 2018	ATCC 700089^{T}, compost biofilter for waste gas purification of animal rendering plant emissions, Lingen, Germany.
DSM 45085^{T}	*Gordonia lacunae* Le Roes *et al*., 2009^{VL} emend. Nouioui *et al*., 2018	JCM 18296^{T}, soil, Plettenberg Bay, South Africa.
DSM 45064^{T}	*Gordonia malaquae* Yassin *et al*., 2007^{VP} emend. Nouioui *et al*., 2018	CCUG 53555^{T}, sludge of wastewater treatment plant, Taichung Industrial Park, Taichung City, Taiwan.
DSM 44568^{T}	*Gordonia namibiensis* Brandão *et al*., 2002^{VL}	NCIMB 13800^{T}, soil, Namibia, Kalahari.

Strain code	Designation	Source/strain histories
DSM 45646^{T}	*Gordonia neofelifaecis* Liu *et al*., 2011^{VP} emend. Nouioui *et al*., 2018.	AD-6^{T}, JCM 31031^{T}, faeces of a clouded leopard (*Neofelis nebulosa*), Sichuan Province, China.
DSM 44499^{T}	*Gordonia nitida* Yoon *et al*., 2000^{VP}	*Gordonia alkanivorans* Kummer *et al.,* 1999, KCCM 80004^{T}, industrial wastewater, Democratic People's Republic of Taegu, Korea (north).
DSM 44809^{T}	*Gordonia otitidis* Iida *et al*., 2005^{VP}	JCM 12355^{T}, human, Japan.
DSM 44604^{T}	*Gordonia paraffinivorans* Xue *et al*., 2003^{VP}	JCM 12461^{T}, production wellhead water sample, Daqing oilfield, China.
DSM 44302^{T}	**Gordonia polyisoprenivorans* Linos *et al*., 1999^{VP} emend. Nouioui *et al*., 2018	ATCC BAA-14^{T}, fouling tire water on a farmer's field, Northrhine-Westfalia, Münster, Germany.
DSM 43197^{T}	**Gordonia rubropertincta* (Hefferan 1904) Stackebrandt *et al*., 1989^{VP} emend. Nouioui *et al*., 2018	*Rhodococcus rubropertinctus* (Hefferan 1904) Tsukamura 1974 emend. Mordarski *et al*., 1980, *Rhodococcus corallinus* (Bergey *et al*., 1923) Goodfellow & Alderson 1977, ATCC 14352^{T}, soil, Japan.
DSM 44576^{T}	*Gordonia shiwensis* Kim *et al*., 2003^{VP}	NRRL B-24155^{T}, sulphur particle, Republic of Korea, Korea (south).
DSM 44455^{T}	*Gordonia sinesedis* Maldonado *et al*., 2003^{VL}	J72^{T}, NCIMB 13802^{T}, soil, near the river Thames.
DSM 44995^{T}	*Gordonia soli* Shen *et al*., 2006^{VP}	CC-AB07^{T}, BCRC 16810^{T}, soil, Taiwan.
DSM 43896^{T}	*Gordonia sputi* (Tsukamura 1978) Stackebrandt *et al*., 1989 ^{VL} emend. Tsukamura 1991 emend. Riegel *et al*., 1994	*Rhodococcus chubuensis* Tsukamura 1983, *Rhodococcus obuensis* Tsukamura 1983; *Rhodococcus sputi* (ex Tsukamura 1978) Tsukamura & Yano 1985, ATCC 29627^{T}, sputum of a patient with pulmonary disease, Obu, Japan.
N659^{T}	*Gordonia terrae* (Tsukamura 1971) Stackebrandt *et al*., 1989 ^{VP} emend. Nouioui *et al*., 2018	*Rhodococcus terrae* (Tsukamura 1971) Tsukamura 1974, DSM 43249^{T}, soil, Obu, Japan, 3612.
DSM 44215^{T}	**Gordonia westfalica* Linos *et al*., 2002^{VP} emend. Nouioui *et al*., 2018	Kb 2^{T}, JCM 11757^{T}, fouling tire water on a farmer's field, Münster, Germany.
Genus *Millisia*		
J81^{T}	*Millisia brevis* Soddell *et al*., 2006a^{VP}	DSM 44463^{T}, activated sludge, Bendigo, Victoria, Australia.
Genus *Mycobacterium*†		
L948^{T}	*Mycobacterium* (*Mycobacteriodes*) *abscessus* (Moore and Frerichs 1953) Gupta *et al*., 2018^{VP}	*Mycobacterium chelonei subsp. abscessus* (Moore & Frerichs 1953) Kubica *et al*., 1972, *Mycobacterium abscessus subsp. abscessus* (Moore and Frerichs 1953) Leao *et al*., 2011 emend. Tortoli *et al*., 2016), DSM 44196^{T}, human knee infection presenting with abscess-like lesions.
M401^{T}	*Mycobacterium* (*Mycolicibacterium*) *aurum* (Tsukamura 1966) Gupta *et al*., 2018^{AL}	*Mycobacterium aurum* Tsukamura 1966 emend. Nouioui *et al*., 2018, DSM 43999^{T}, soil.

Strain code	Designation	Source/strain histories
N294^{T}	**Mycobacterium* (*Mycolicibacterium*) *fortuitum subsp. Fortuitum* (da Costa Cruz 1938) Gupta *et al*., 2018^{AL}	*Mycobacterium fortuitum subsp. fortuitum* (da Costa Cruz 1938) Tsukamura *et al*., 1986; *Mycobacterium fortuitum* da Costa Cruz 1938 emend. Nouioui *et al*., 2018, *Mycolicibacterium fortuitum subsp. fortuitum* (da Costa Cruz 1938) Gupta *et al.,* 2018; DSM 46621^{T}, cold abscess, human.
M206^{T}	*Mycobacterium* (*Mycolicibacterium*) *peregrinum* (Kusunoki & Ezaki 1992) Gupta *et al*., 2018^{VP}	*Mycobacterium peregrinum* (Bojalil *et al*., 1962) Kusunoki & Ezaki 1992 emend. Nouioui *et al*., 2018, DSM 43271^{T}, bronchial aspirate of child.
N290^{T}	** Mycobacterium* (*Mycolicibacterium*) *phlei* (Lehmann & Neumann 1899) Gupta *et al*., 2018^{AL}	*Mycobacterium phlei* Lehmann & Neumann 1899 emend. Nouioui *et al.,* 2018, DSM 43239^{T}, Rome.
N292^{T}	** Mycobacterium* (*Mycolicibacterium*) *smegmatis* (Trevisan 1889a) Gupta *et al*., 2018^{VP}	*Mycobacterium smegmatis* (Trevisan 1889a) Lehmann & Neumann 1899 emend. Nouioui *et al*., 2018; DSM 43756^{T}; M. Ridell, strain GA 735; NCTC 8159^{T} R. E. Gordon.
Genus *Nocardia*		
N317^{T}	**Nocardia asteroides* (Eppinger 1891) Blanchard 1896^{VP} emend. Nouioui *et al.,* 2018	ATCC 19247^{T}; R.E. Gordon, 727: L.Ajello, M170-6; W.M. Burman, PSA 165.
N318^{T}	**Nocardia brasiliensis* (Lindenberg 1909) Pinoy 1913 ^{VP} emend. Nouioui *et al*., 2018	DSM 43758^{T}, R.E. Gordon, IMRU 845; J. Schneidau, Jr. 381; A. Batista, 631, strain 337.
N671^{T}	**Nocardia farcinica* Trevisan 1889b^{AL}	DSM 43665^{T}; M. Ridell, strain GA 919; M. Goodfellow, strain M 258; M.P. Lechevalier; ATCC (Nocardia asteroides); R.E. Gordon; Madura foot.
N1243	**Nocardia farcinica* Trevisan 1889b^{AL}	R.J. Seviour, Biotechnology Research Centre, La Trobe University, Bendigo, Victoria, Australia; strain GD1.
N1158^{T}	**Nocardia otitidiscaviarum* Snijders 1924^{AL}	DSM 43242T^{T}, E.P. Snijders (Nocardia caviae); infected middle ear of guinea pigs.
Genus *Rhodococcus*		
DSM 45380^{T}	*Rhodococcus artemisiae* Zhao *et al*., 2012^{VP}	YIM 65754^{T}, CCTCC AA 209042^{T}, a surface-sterilized stem of *Artemisia annua* L, Yunnan province, China.
DSM 44587^{T}	*Rhodococcus baikonurensis* Li *et al*., 2004^{VP}	A1-22^{T}, JCM 11411^{T}; air in Russian space station MIR.
DSM 45141^{T}	*Rhodococcus cercidiphylii* Li *et al*., 2012^{VL}	YIM 65003^{T}, CCTCC AB 207160^{T}, surface-sterilized leaf sample of *Cercidiphyllum japonicum*, Yunnan, China.
DSM 43347^{T}	*Rhodococcus coprophilus* Rowbotham & Cross 1979^{VP} emend. Nouioui *et al*., 2018	N744^{T}; ATCC 29080^{T}, lake mud.
DSM 43066^{T}	**Rhodococcus erythropolis* (Gray & Thornton 1928) Goodfellow & Alderson 1979 emend. Nouioui *et al*., 2018^{VP}	*Nocardia calcarea* (Metcalfe & Brown, 1957), *Arthrobacter picolinophilus* (Tate & Ensign, 1974); ATCC 25544^{T}, soil.
DSM 20669^{T}	**Rhodococcus fascians* (Tilford 1936) Goodfellow 1984 emend. Nouioui *et al*., 2018^{VP}	*Corynebacterium fascians* (Tilford 1936) Dowson 1942,*Rhodococcus luteus* (ex Söhngen 1913) Nesterenko *et al*. 1982; DSM 20669^{T}, Fasciation of sweet peas, *Lathyrus odoratus*.

Strain code	Designation	Source/strain histories
DSM 43954^{T}	*Rhodococcus globerulus* Goodfellow *et al*., 1985 emend. Nouioui *et al*., 2018^{VL}	N544^{T}, ATCC 25714^{T}, soil.
DSM 44689^{T}	*Rhodococcus gordoniae* Jones *et al*., 2004 emend. Nouioui *et al.,* 2018^{VP}	W4937^{T}; NCTC 13296^{T}; human lung, United States of America.
DSM 46766^{T}	*Rhodococcus jialingiae* Wang *et al.,* 2010^{VP}	*Rhodococcus qingshengii* Xu *et al*., 2007 emend. Nouioui *et al*., 2018; *Rhodococcus enclensis* Dastager *et al*., 2014 emend. Nouioui *et al*., 2018, seawater-ice sample, Spitzbergen, Norway.
DSM 44719^{T}	**Rhodococcus jostii* Takeuchi *et al*., 2002^{VP}	NBRC 16295^{T}; femur in the grave, Czech Republic.
DSM 44908^{T}	*Rhodococcus kroppenstdetii* Mayilraj *et al*., 2006^{VP}	K07-23^{T}; JCM 13011^{T}, soil, India, Himalaya.
DSM 45001^{T}	*Rhodococcus kunmingensis* (Wang *et al*., 2008) Nouioui *et al*., 2018^{VP}	*Rhodococcus kunmingensis* Wang *et al*., 2008; KCTC 19149^{T}, rhizosphere soil, Yunnan Province, China.
DSM 44675^{T}	*Rhodococcus maanshanensis* Zhang *et al*., 2002^{VP} emend. Nouioui *et al*., 2018	JCM 11374^{T}, soil, Maanshan Mountain, Anhui Province, China.
DSM 43752^{T}	*Rhodococcus marinonascens* Helmke & Weyland 1984^{VP}	ATCC 35653^{T}; marine sediment.
DSM 43205^{T}	**Rhodococcus opacus* Klatte *et al*., 1995^{VL}	ATCC 51881^{T}, soil sample, Tübingen-Derendingen, Germany.
DSM 44240^{T}	**Rhodococcus percolatus* Briglia *et al*., 1996^{VP}	JCM 10087^{T}, contaminated sludge and sediment samples, Finland.
DSM 44812^{T}	**Rhodococcus phenolicus* Rehfuss and Urban 2006^{VL} emend. Nouioui *et al*., 2018	G2P ^{T}, JCM 14914^{T}, biological wastewater processor, Johnson Space Center, Texas, United States of America.
DSM 44555^{T}	**Rhodococcus pyridinivorans* Yoon *et al.,* 2000 ^{VP} emend. Nouioui *et al.,* 2018	KCCM 80005^{T}, industrial wastewater, Korea (North), Democratic People's Republic.
ATCC 35071^{T}	**Rhodococcus rhodnii* Goodfellow & Alderson 1979 ^{AL} emend. Nouioui *et al.,* 2018	N445^{T}, DSM 43336^{T}, reduviid bug, *Rhodnius prolixus*.
DSM 43241^{T}	**Rhodococcus rhodochrous* (Zopf 1891) Tsukamura 1974 emend. Nouioui *et al*., 2018.	*Rhodococcus roseus* (ex Grotenfelt 1889) Tsukamura *et al*., 1991, ATCC 13808^{T}, R.E. Gordon, 372.
DSM 44892^{T}	*Rhodococcus triatomae* Yassin 2005 ^{VP} emend. Nouioui *et al.,* 2018	CCUG 50202^{T}, blood-sucking bug Triatome, University, Bonn, Germany.
DSM 44783^{T}	*Rhodococcus tukisamuensis* Matsuyama *et al*., 2003^{VP} emend. Nouioui *et al*., 2018	JCM 11308^{T}, H. Matsuyama; Mb8, soil, Sapporo City, Japan.
DSM 44107^{T}	**Rhodococcus wrastislaviesis* (Goodfellow *et al*., 1995) Goodfellow *et al*., 2002^{VP} emend. Nouioui *et al.,* 2018	*Tsukamurella wratislaviensis* Goodfellow *et al*., 1995; ATCC 51786^{T}, soil.

Strain code	Designation	Source/strain histories
DSM 44837^{T}	*Rhodococcus yunnanensis* Zhang *et al*., 2005^{VP}	JCM 13366^{T}, Forest soil, Yunnan Province, China.
DSM 44108^{T}	*Rhodococcus zopfii* Stoecker *et al*., 1994^{VP} emend. Nouioui *et al*., 2018	ATCC 51349^{T}, bioreactor, University Washington, United States of America.
Genus *Tsukamurella*		
DSM 44067^{T}	*Tsukamurella inchonensis Yassin et al., 1995*^{VP}	N1238^{T}, blood cultures from a patient who had ingested hydrochloric acid, Inchon, Republic of Korea.
DSM 20162^{T}	**Tsukamurella paurometabola* (Steinhaus 1941) Collins *et al*., 1988^{VP} emend. Nouioui *et al.,* 2018	*Corynebacterium paurometabolum* Steinhaus 1941, *Rhodococcus aurantiacus* (ex Tsukamura & Mizuno 1971), Tsukamura and Yano 1985, *Tsukamurella paurometabolum* (Steinhaus 1941) Collins *et al*., 1988; ATCC 8368^{T}, mycetomes and ovaries of bed bugs (*Cimex lectularius*).
DSM 44118^{T}	**Tsukamurella pseudospumae* Nam *et al*., 2004^{VP} emend. Teng *et al*., 2016	*Tsukamurella sunchonensis* Seong *et al.,* 2008 emend. Nouioui *et al.,* 2018, JCM 13375^{T}, foam on the surface of aeration tanks in an activated-sludge-treatment plant, England.
DSM 44142^{T}	**Tsukamurella. pulmonis* Yassin *et al.,* 1996^{VP} emend. Teng *et al.,* 2016	*Tsukamurella spongiae* Olson *et al.,* 2007 emend. Nouioui *et al.,* 2018; JCM 10111^{T}, sputum of a 92-year-old woman with lung tuberculosis, Gauting, Munich, Germany.
DSM 45046^{T}	*Tsukamurella soli* Weon *et al*., 2010^{VP}	KACC 20764^{T}, forest soil, Halla mountain, Republic of Jeju island, Korea (south).
DSM 44113^{T}	**Tsukamurella spumae* Nam *et al*., 2003^{VL}	N1171^{T}; activated sludge foam, Stoke Bardolph Water Reclamation Works, Nottinghamshire, UK.
DSM 44573^{T}	*Tsukamurella strandjordii* Kattar *et al*., 2002^{VL}	ATCC BAA-173^{T}; blood from a 5-year-old girl with acute mycelogenous leukemia.
DSM 45335^{T}	*Tsukamurella sunchonensis* Seong *et al*., 2008^{VL} emend. Nouioui *et al.,* 2018	JCM 15929^{T}, activated sludge, Republic of Suncheon, Korea (south).
Genus *Williamsia*		
DSM 45372^{T}	*Williamsia faeni* Jones *et al*., 2010^{VP}	N1350^{T}, soil from a hay meadow plot, Cockle Park Experimental Farm, Northumberland, United Kingdom.
DSM 44693^{T}	*Williamsia maris* Stach *et al*., 2004^{VP} emend. Nouioui *et al*., 2018	JCM 12070^{T}, sediment, Sea of Japan, Japan.
N1261^{Tsp}	*Williamsia muralis* Kämpfer *et al.,* 1999^{VP}	DSM 44343^{T}, water-damaged indoor building material of a children's day care centre; Finland.
DSM 45037^{T}	*Williamsia serinedens* Yassin *et al*. 2007^{VP} emend. Nouioui *et al.,* 2018	CCUG 53151^{T}, a soil sample from an oil-contaminated site, Chyai County, Taiwan.

^{AL}: Cited in the approved lists of bacterial names; ^{T}: type strain; ^{Tsp}: type species of the genus; ^{VP}: validly published, ^{VL}: validation list. *: species reported to produce biosurfactants. †, strains assigned to the genus *Mycobacterium* have subsequently been formally reassigned to *Mycolicibacterium* or *Mycobacteriodes* (Oren & Garrity, 2018).

2.2.3. Micro-morphology and colony characteristics.

Air-dried smears prepared by mixing small colonies, which appeared after 3 – 5 days of growth on TSA plates with water on a glass slide, and allowing the smear to dry at room temperature, were Gram-stained (Smith & Hussey, 2005) to check for purity. Stained preparations were viewed by bright-field microscopy under oil immersion using the x100 objective (Olympus BX51). Cell shape, the presence of hyphae, and primary and secondary branching were noted. The colony features of the test strains were examined by eye and using a binocular zoom plate microscope (Leica Stereo Microscope MDG41) using a PLANAPO 1.0X objective and colony size, shape, elevation, margin, pigmentation, surface appearance, and the development of substrate mycelium and aerial hyphae noted. Colony and micro-morphological features of each strain were compared to published formal descriptions (Goodfellow *et al*., 2012) and recorded.

2.2.4. Biosurfactant screening methods.

2.2.4.1. Culture medium and bacterial growth.

The test strains were grown aerobically in 500 ml Erlenmeyer flasks with 100 ml sterile mineral salt medium (MSM; Kuyukina *et al*., 2001). The mineral salt medium was prepared by dissolving 2.0g K_2HPO_4; 2.0g KH_2PO_4; 2.0g $(NH_4)_2SO_4$; 1.0g NaCl; 0.5g $MgSO_4 \cdot 7H_2O$; 0.01g $CaCl_2$; 0.01g $FeSO_4 \cdot 7H_2O$; 1.0g $NaNO_3$, 0.1g KCl, and 0.1g yeast extract in 1 litre of distilled water and sterilized by autoclaving at 121°C for 15mins. The MSM was supplemented with a sole carbon source, either hexadecane (2% v/v; Sigma-Aldrich, Dorset, UK) added aseptically to sterilized MSM or glucose (2% w/v; Sigma-Aldrich, Dorset, UK) added to MSM prior to medium sterilization. Flasks were inoculated with a starter culture (2% v/v) taken from Tryptic Soy Broth (TSB) grown for 3 days at 30°C with rotary shaking (New Brunswick shaking incubator) at 180 rpm and incubated under the same conditions for up to 14 days.

2.2.4.2. Preparation of Cell-Free Supernatant (CFS). Cell-free supernatant (CFS) was separated from cultivated whole cell broth (WCB) by centrifugation at 13,000× *g* for 10 mins followed by filtration of the supernatant through

0.22µm pore size filter paper (Millipore) to remove cells. Both WCB and CFS samples from each strain were screened for biosurfactant production using the assays described below. All assays were performed in triplicate and mean values and standard deviations were determined where appropriate. Purified biosurfactant (90% rhamnolipids 1 mg ml^{-1}; Sigma Aldrich, UK) was used as the positive control, and distilled water as the negative control unless otherwise stated.

2.2.4.3. Drop collapse test. This test was performed following the procedure described by (Chen *et al*., 2007) with further minor modifications. Aliquots (2 µl) of light mineral oil (Sigma-Aldrich, Dorset, UK) were added as discrete drops to the surface of a 96-well microtitre plate (polystyrene, flat bottomed, and treated and equilibrated for 1 h at room temperature, before adding 5µl of WCB or CFS from each test culture to the surface of each oil drop. The shape and size of each oil drop were observed after 1 min using grid paper placed underneath the microtitre plate. An optical distortion of the grid is observed when water (negative control) is added to a well whereas the presence of biosurfactant in the test sample leads to surface tension reduction causing the drop to collapse, removing the optical distortion effect. The WCB and CFS samples from each strain were scored positive for biosurfactant production when the flattened drops with a normal grid pattern below the microplate were observed, and scored as + or ++, corresponding to partial or complete drop collapse. While convex drops with the optical distortion of the grid pattern were scored as negative (-) for the absence of biosurfactant.

2.2.4.4. Oil spreading method. Oil spreading was performed as described by Morikawa *et al.* (2000) with minor modifications. Water (20 ml) coloured with bromothymol blue (100 µl, to observe oil spreading) was added to a petri dish (90 mm diam.) and overlaid uniformly with 20 µl of mineral oil. A sample of WCB or CFS (10 µl) was then added to the oil surface and the presence of biosurfactant was observed by oil displacement. An arbitrary scoring system based on the diameter of the petri plate was used to record results as - (no

displacement); +, zone of clearance 1 - 8.7 cm diameter; ++, zone of clearance ≥8.8cm diameter.

2.2.4.5. Emulsification Assay (EA). This assay was performed according to the method described by Rosenberg *et al*. (1979). Cell-free supernatant was screened by adding 4 ml to mineral oil (1 ml) in a screw-capped test tube, vortex-shaken for 2 min, and the emulsion left undisturbed for 1 h at room temperature to allow the aqueous and oil phases to separate. The absorbance of the aqueous phase was measured in a spectrophotometer (Jenway 6300) at OD_{600} nm. Uninoculated MSM broth was used as a blank. One emulsification unit (emulsification activity per ml [EU ml^{-1}]) is equal to an absorbance of 0.01 at 600nm. Water replaced CFS for the negative control and Triton X-100 (Sigma Aldrich, UK) replaced CFS as the positive control.

2.2.4.6. Emulsification Index (EI_{24}). Equal volumes (2 ml each) of mineral oil, water, and the WCB or CFS were added to a graduated screw-capped test tube, and the total height of the liquid was measured. The tube was vortexed for 1 min and allowed to stand for 24h at room temperature before measuring the height of the stable middle emulsion layer. The percent emulsification was calculated using the formula (Satpute *et al*., 2008):

$$\text{Emulsification index } EI_{24}\ (\%) = \frac{\text{height of the emulsification layer}}{\text{total height of the solution}} \times 100$$

2.2.4.7. Surface tension measurement. Surface tension measurement of WCB and CFS from each strain was determined by force tensiometer (Automatic tensiometer TD 3, Germany), using the du Noüy ring method (Pornsunthorntawee *et al*., 2008). Each sample (15 ml) was equilibrated for 5 mins at room temperature in a glass dish before measuring the surface tension in triplicate. The surface tension was measured at the ambient temperature of ~20-22°C every 10-16 seconds until the equilibrium value was achieved. The ring was cleaned using acetone to remove surface-active impurities and flamed after testing each sample. Rhamnolipid (1 mg ml^{-1}) and distilled water were used as standards. The machine was calibrated before the start of the experiment using a fixed weight of 500mg.

2.2.4.8. CTAB (cetyltrimethylammonium bromide) assay. The CTAB assay was performed according to Siegmund & Wagner (1991). Mineral blue agar plates were prepared using mineral salt agar medium supplemented with 0.5 mg l^{-1} cetyltrimethylammonium bromide (cationic surfactant; Millipore, UK), 0.05 mg l^{-1} methylene blue (basic dye; Sigma-Aldrich, UK), and 2% (v/v) hexadecane as the sole carbon source. WCB or CFS (100 µl) samples were loaded to 4mm diam. wells aseptically cut into the mineral blue agar plates, which were then incubated at 30°C for 48 - 72 hrs. The negative control wells contained water while the rhamnolipid (1 mg ml^{-1}) standard was used as a positive control. A dark blue halo around an inoculated well was considered positive for the presence of anionic biosurfactant(s) while no blue halo zone was considered negative.

2.2.5. Bacterial adherence to hydrocarbon (BATH) assay.

Cell surface hydrophobicity was determined by the bacterial cell adherence to hydrocarbon (BATH) assay (Chao *et al*., 2014) with some modifications. After growth in MSM with hexadecane (2% v/v) or glucose (2% w/v) as the sole carbon source, cell pellets from each culture broth were collected by centrifugation at 13,000× g for 10 mins, washed twice with buffered salt solution (g l^{-1}, 16.9 K_2HPO_4, and 7.3 KH_2PO_4), resuspended in buffer and diluted to an OD_{600nm} of 0.5. Each washed cell suspension (4 ml) was added to a clean test tube with 200 µl of mineral oil and vortex-shaken for 1 min before standing for 1 h to allow the oil and aqueous phases to separate. *Rhodococcus ruber* IEGM 231^T, known to adhere to hydrocarbons (Philp *et al*., 2002), was used as a positive control. Absorbance (OD_{600nm}) of the aqueous phase was measured in a spectrophotometer (Jenway 6300) using buffer as a blank. The percentage of cells attached to the oil was calculated using the formula:

% of bacterial cell adherence = [1 - (OD_{shaken} with oil/$OD_{original}$)] x 100.

Where,

OD_{shaken} with oil is the OD of the mixture containing cells and oil and,

$OD_{original}$ is the OD of the cell suspension in the buffer solution (before mixing with mineral oil)

2.2.6. Data analysis.

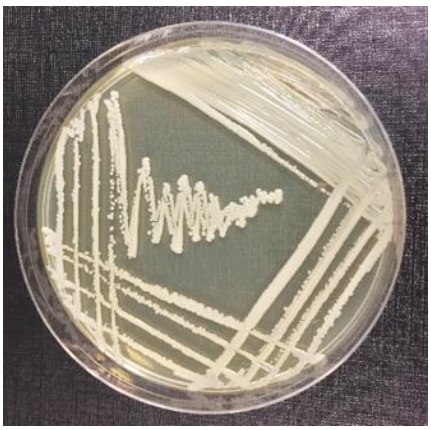
All tests were performed in triplicate and presented as mean or mean ± standard deviations using Excel. Spearman rank correlation coefficient was conducted using SPSS® Statistics v26.0 to determine correlations between screening assays. The correlation coefficient, R_s, ranged between -1.000 (very strong negative correlation) and 1.000 (very strong positive correlation). SPSS was also used to create box and whisker plots for datasets where the box represents the interquartile range (25-75%), divided by a horizontal line that represents the median. A cross on the box plot indicates the mean. The whisker lines indicate variability outside the upper and lower quartiles. Outliers are plotted as individual dots above or below and in line with the whiskers. Significance between datasets was tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

2.3. Results

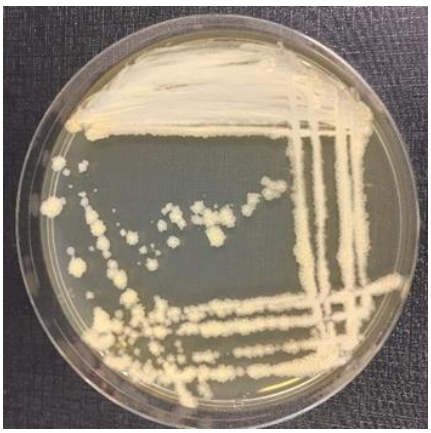
In the present study, 94 actinobacteria belonging to 91 validly described species of the suborder *Corynebacterineae*, and assigned to nine different genera, were selected to screen for biosurfactant production. These test strains possess mycolic acids located in the cell wall apart from two strains *Corynebacterium amycolatum* N1278^T and *Corynebacterium* (*Turicella*) *otitidis* DSM 8821^T. Thirty-six strains belong to species that have previously been reported to produce biosurfactants (as indicated in Table 2.1, page 46) although, in this study, most are the type strains of the species and not necessarily the strain(s) investigated in published studies. The ability to produce biosurfactants amongst strains of the remaining species was not known.

2.3.1. Colony, micromorphology, and staining characteristics.

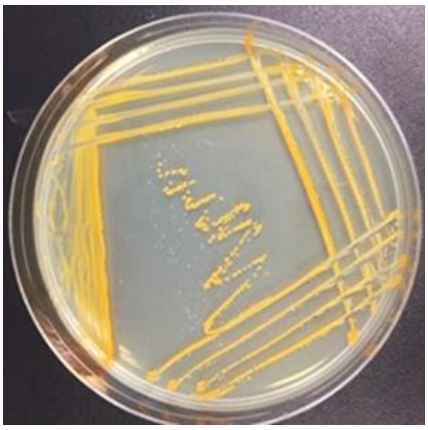
All 94 test strains were non-motile, Gram-positive rods and/or coccoid cells, or pleomorphic cells and many formed rudimentary filaments after growth on TSA plates incubated at 30°C for 3 - 5 days. Observation of colonial characteristics, including features such as colour, shape, texture, margins, elevation, and presence of substrate/surface and aerial mycelia, revealed a wide diversity of colony types as exemplified in Fig 2.1. The colony and micro-morphological features noted for each strain agreed with formal descriptions obtained from species description papers and Bergey's Manual of Systematic Bacteriology (Volume 5, 2012).



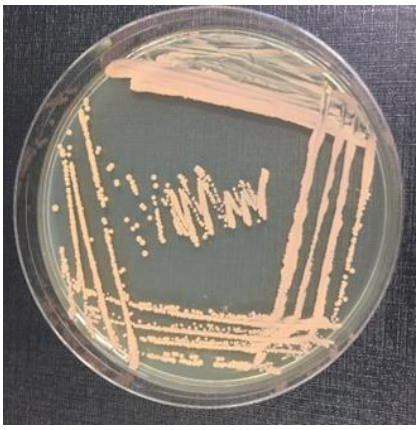
Corynebacterium amycolatum N1278^{T}



Corynebacterium otitidis DSM 8821^{T}

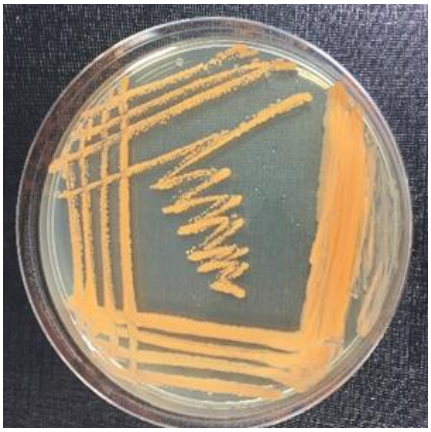


Dietzia papillomatosis DSM44961^{T}

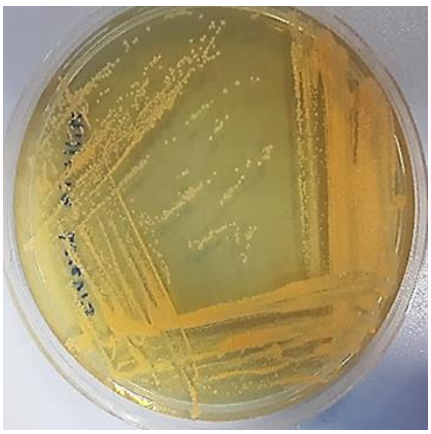


Gordonia polyisoprenivorans DSM 44302^{T}

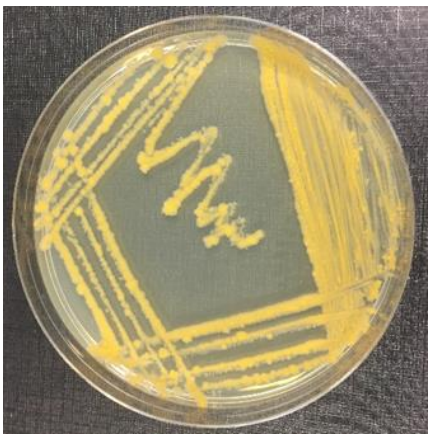
Gordonia araii DSM 44811^{T}



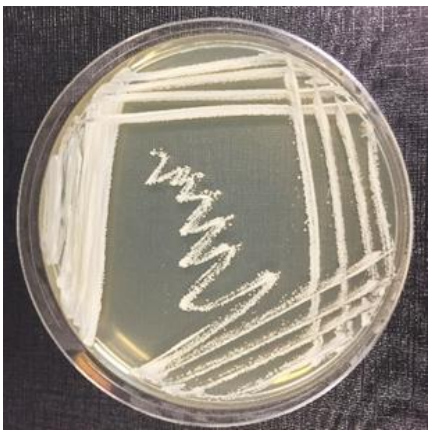
Gordonia nitida DSM 44499^{T}



Millisia brevis J81^{T}

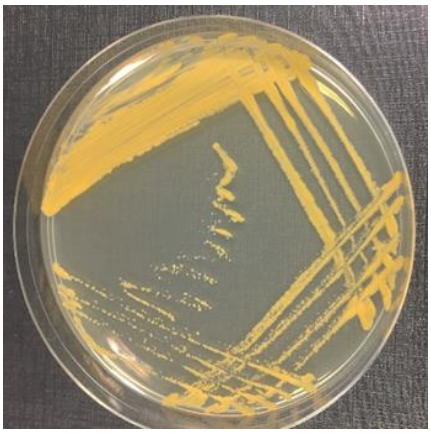


Mycobacterium aurum M401^{T}

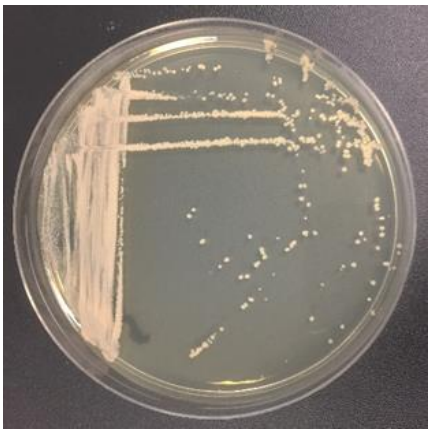


Nocardia farcinica N671^{T}

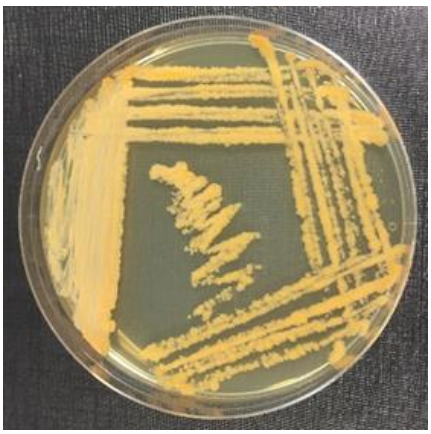
Rhodococcus kunmingensis DSM 45001^{T}



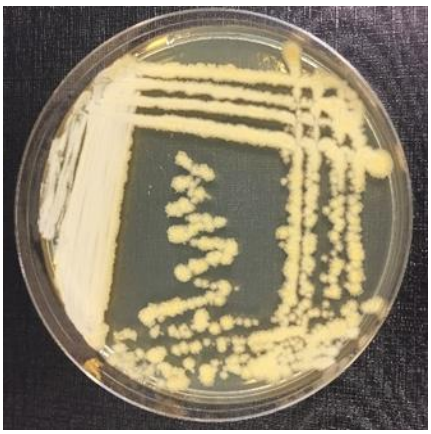
Rhodococcus cercidiphylii DSM 45141^{T}



Rhodococcus yunnanensis DSM 44837^{T}



Tsukamurella sunchonensis DSM 45335^{T}



Williamsia muralis N1261^{Tsp}

Figure 2.1. Actinobacterial test strains representative of each genus included in the present study, growing on tryptone soy agar (TSA) plates after 3-5 days of incubation at 30°C. Strains possess diverse colony characteristics on TSA plates from pinpoint (1mm diam.) to larger circular or irregular colonies (≤7mm diam.), with entire or feathered edges, flat-raised, dome, umbonate or irregular elevation, smooth or folded surfaces, most without aerial hyphae, and either a translucent, opaque, matt or shiny appearance whilst the texture may be mucoid, butterous or friable and the colour ranges from cream-white to pale yellow, pink, orange, and red-orange.

2.3.2. Growth and biosurfactant production on hydrophilic and hydrophobic carbon sources.

In this study, strains belonging to six species previously reported to produce biosurfactants were included in a pilot study to compare biosurfactant production in liquid mineral salts medium (MSM) containing either a hydrophilic (2% w/v glucose) or hydrophobic (2% v/v hexadecane) sole carbon source. These strains were *Dietzia cinnamea* DSM 44904^T, *D. maris* DSM 43672^T, *Gordonia westfalica* DSM 44215^T, *Nocardia otitidiscaviarum* N1158^T, *Rhodococcus erythropolis* DSM 43066^T, and *Tsukamurella pseudospumae* DSM 44118^T (Cia *et al*., 2015; Kavyanifard *et al*., 2016; Kugler *et al*., 2014; Laorrattanasak *et al*., 2016; Nakano *et al*., 2011; Vyas & Dave, 2011;). All strains, incubated at 30°C and 180rpm, grew as dense homogenous cell suspensions when grown in wholly aqueous MSM with glucose (2% w/v), and the supernatants were readily separated from cell biomass for biosurfactant testing. In comparison, the strains also grew well in the biphasic MSM with hexadecane (2% v/v). However, for some strains, non-homogeneous dispersion of cells was observed with biomass partitioned in the upper hydrophobic layer with or without additional turbid growth in the lower liquid medium.

Cell-free supernatants (CFS) extracted from the culture broths were screened for extra-cellular biosurfactant production on days 3, 7, and 14 for strains grown on both substrates using the qualitative Drop Collapse (DC) and Oil Spreading (OS) assays and the quantitative Emulsification Assay (EA) and Emulsification Index (EI_{24}) assays. These methods have been previously reported to be reliable for biosurfactant screening and hence were used in this study (1.6, page 24). Cell-surface hydrophobicity values, were determined from whole cell broths (WCB) using the BATH assay.

Cell-free supernatant (CFS) screened for biosurfactant production using the Drop collapse (DC) and Oil spreading (OS) assays are provided in Table 2.2. Four of the six strains were positive for DC after 3 days and remained positive for up to 14 days of cultivation in MSM with either carbon source. *Rhodococcus erythropolis* only tested positive after 14 days of incubation when grown on glucose whereas *Gordonia westfalica* tested negative for DC when grown on both carbon sources.

Table 2.2. Biosurfactants produced by actinobacterial test strains detected in cell-free supernatants by Drop Collapse and Oil Spreading assays and cell surface hydrophobicity determined from whole cell-broth by the Bacterial adherence to hydrocarbon assay(BATH) assay after 3, 7 and 14 days growth in Mineral Salt Medium (MSM) supplemented with 2% glucose (G) or 2% hexadecane (H) as the sole carbon source.

Test strain	Incubation period (d)	[a]Drop collapse		[b]Oil spreading		[c]BATH (%)	
		MSM + G	MSM + H	MSM + G	MSM + H	MSM + G	MSM + H
Dietzia cinnamea DSM 44904^T	3	+	+	-	+	27.03 ± 2.75	50.65 ± 0.01
	7	+	+	-	+	7.79 ± 5.473	57.89 ± 0.02
	14	+	+	+	+	3.77 ± 0.27	77.81 ± 0.01
Dietzia maris DSM 43672^T	3	+	+	-	+	44.49 ±3.30	51.52 ± 0.02
	7	+	+	-	+	8.61 ± 2.13	25.18 ± 0.01
	14	+	+	+	+	8.67 ± 2.50	31.30 ± 0.02
Gordonia westfalica DSM 44215^T	3	-	-	-	-	9.20 ± 0.48	54.32 ± 0.00
	7	-	-	-	-	7.02 ± 1.14	55.33 ± 0.01
	14	-	-	-	-	5.47 ± 2.82	7.16 ± 0.03
Nocardia otitidiscaviarum N1158^T	3	+	+	-	-	31.53 ± 3.89	46.67 ± 0.00
	7	+	+	-	-	5.17 ± 0.19	8.17 ± 0.01
	14	+	++	-	-	6.61 ± 0.63	14.94 ± 0.02
Rhodococcus erythropolis DSM 43066^T	3	-	+	-	+	17.03 ± 0.19	74.68 ± 0.01
	7	-	+	-	+	28.33 ± 2.66	51.63 ± 0.03
	14	+	+	+	+	14.43 ± 2.82	36.52 ± 0.04
Tsukamurella pseudospumae DSM 44118^T	3	+	+	-	+	0.69 ± 1.97	43.06 ± 0.02
	7	+	+	-	+	0.82 ± 2.02	18.05 ± 0.02
	14	+	+	+	+	0.66 ± 1.17	17.32 ± 0.04

[a], Drop collapse assay: '-' no activity, '+' partial drop collapse, '++' complete drop collapse after 1 min; [b], Oil spreading assay: '-' no activity, '+' oil spreading with a clear zone 1.0-8.8 cm diam, '++' oil spreading with a clear zone ≥8.8 cm diam; [c] Bacterial adherence to hydrocarbon assay (%). Data expressed is a mean ± std dev of three replicates for BATH assay, DC and OS.

By contrast, the four strains showed no OS activity when grown on glucose until 14 days of cultivation while the same strains were positive after only 3 days of growth on hexadecane and remained positive until 14 days. Two strains, *G. westfalica* and *N. otitidiscaviarum,* tested negative for OS on both carbon sources. No emulsification activity was observed in any CFS samples with emulsification assay (EA) values recorded as ≤0.0163 EU ml^{-1} whereas the positive control (Triton-X) value was 2.8 ± 0.05 EU ml^{-1}, and Emulsification Index (EI_{24}) values were ≤2%, after growth on either carbon source (data not shown). The Emulsification Assay (EA) showed greater standard deviation and hence was only used for the pilot study and was not included in the subsequent larger-scale screening study.

Cell-surface hydrophobicity determined as percentage adhesion to hydrocarbon (mineral oil) varied with test strain, length of incubation, and carbon source (Table 2.2., page 61). Generally, percentage adhesion to hydrocarbon values were highest after 3 days of incubation and decreased significantly after this time. Hydrophobicity values were generally much higher when strains were grown on hexadecane rather than glucose. Adhesion to hydrocarbon values for *Tsukamurella pseudospumae* grown on glucose were negligible throughout incubation. Interestingly, the highest cell surface hydrophobicity value was observed for *Dietzia cinnamea* and this increased over the course of growth on hexadecane from 50.65 to 77.81%.

Hence, all six previously reported biosurfactant producers were found to produce greater biosurfactants with hexadecane as a carbon source compared to glucose.

2.3.3. Screening actinobacteria for cell-associated and extracellular biosurfactant production.

The ninety-four actinobacteria strains were screened for biosurfactant production after 7- and 14-day growth in MSM supplemented with hexadecane (2% v/v) as the sole carbon source. Five biosurfactant screening methods were utilised and evaluated: Drop collapse (DC), Oil spreading (OS), Emulsification index (EI_{24}), Surface tension (ST; du Noüy ring method), and the Cetyl

trimethylammonium bromide (CTAB) plate method for anionic biosurfactants. Whole-cell broths (WCB) were screened for the combined presence of cell-bound and extra-cellular biosurfactants, whereas cell-free supernatants (CFS) were screened for extra-cellular (freely released) biosurfactants only. In addition to these tests, cell surface hydrophobicity was determined by the Bacterial Adhesion to Hydrocarbon (BATH) assay using WCB samples. Sterile distilled water acted as the negative control and purified rhamnolipid (1mg ml^{-1}) as the positive control unless otherwise stated. The following sections report on the results of each biosurfactant assay.

2.3.3.1. Drop collapse. The drop-collapse assay was used qualitatively with results interpreted and scored as shown in Fig 2.2. When the negative control was added, the oil droplet remained intact and the grid below distorted, and thus scored -, whilst the positive control resulted in full drop collapse and was scored ++. Drop collapse activity was widely distributed amongst the test strains (Table 2.3 and Appendix 7.1., page 264) with 87 out of 94 (92.6%) scoring positive in at least one sample whilst the remaining 7 strains (7.4%) scored negative in WCB and CFS samples after both 7- and 14-days incubation, including six gordoniae and one *Mycobacterium* strain. Good agreement between triplicate tests was observed for both positive and negative results Most strains produced only a partial (+) oil drop collapse whilst most full drop collapse (++) scores (12/94) were recorded for WCB samples from *Rhodococcus* strains screened at 14 days (Appendix 7.1., page 264).

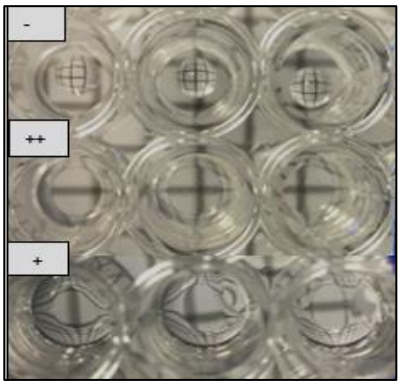


Fig 2.2. Interpretation of qualitative Drop Collapse (DC) assay results (Chen *et al*., 2007) using a 96-well microplate with underlying gridded paper. Convex drops with distortion of the grid pattern were scored '-' no drop collapse; flattened drops with normal grid pattern below were scored '++' for full drop collapse; partial distortion of the grid was scored '+' for partial drop collapse.

Table 2.3. The frequency of strains in each genus which scored positive for Drop Collapse (DC) when whole-cell broths (WCB) and cell-free supernatants (CFS) were screened after 7 and 14 days of growth in Mineral Salt Medium with hexadecane (2%, v/v) at 30°C with rotary shaking at 180 rpm.

Genus (no. test strains)	Response level	No. positive for Drop Collapse* (% of test strain positive)			
		Day 7		Day 14	
		WCB	CFS	WCB	CFS
Corynebacterium (2)		1 (50)	1 (50)	2 (100)	1 (50)
	+	1	1	1	1
	++	0	0	1	0
Dietzia (13)		12 (92)	11 (85)	13 (100)	13 (100)
	+	12	11	13	13
	++	0	0	0	0
Gordonia (30)		5 (17)	5 (17)	21 (70)	16 (52)
	+	5	5	21	16
	++	0	0	0	0
Millisia (1)		1 (100)	1 (100)	1 (100)	1 (100)
	+	1	1	1	1
	++	0	0	0	0
Mycobacterium (6)		5 (83)	4 (67)	4 (67)	4 (67)
	+	4	4	4	4
	++	1	0	0	0
Nocardia (5)		4 (80)	5 (100)	5 (100)	5 (100)
	+	4	4	4	5
	++	0	1	1	0
Rhodococcus (25)		6 (24)	11 (44)	25 (100)	15 (60)
	+	2	10	16	11
	++	4	1	9	3
Tsukamurella (8)		5 (62)	8 (100)	6 (75)	7 (88)
	+	4	6	5	7
	++	1	2	1	0
Williamsia (4)		4 (100)	4 (100)	4 (100)	3 (75)
	+	4	4	4	4
	++	0	0	0	0
Total (94)		**43 (46)**	**50 (53)**	**81 (86)**	**65 (69)**
Positive only in WCB/ CFS		**7 (7)**	**14 (15)**	**20 (21)**	**4 (4)**
Positive in both WCB + CFS		**36 (38)**		**61 (65)**	

* Positive strains scored either + or ++; 0 indicates no response level in that category; water was the negative control and purified rhamnolipid was the positive control. All strains were tested in triplicate.

After incubation for 7 days, 45.7% (43/94) strains in WCB and 53.2% (50/94) strains in CFS scored positive for full or partial oil drop collapse (Table 2.3., page 64). Thirty-six strains (38%) tested positive in both WCB and CFS samples whilst 7 strains (7%), belonging to various genera, tested positive in WCB but not CFS indicating the production of cell-bound biosurfactants only. Surprisingly, fourteen strains (15%), again from various genera, only tested positive in the CFS samples (Table 2.3).

After incubation for 14 days, the number of strains that scored positive for full or partial drop-collapse increased in both the WCB (81/94; 86%) and CFS (65/94; 69%) samples (Table 2.3., page 64). The increase in the frequency of positive scores in WCB and CFS samples was mainly attributable to *Gordonia* and *Rhodococcus* strains. Sixty-one strains tested positive in both WCB and CFS samples whilst 18 strains tested positive in WCB but not CFS and four strains tested positive only in the CFS samples (Table 2.3., page 64).

Mycobacterium peregrinum M6^T was the only strain to score positive in WCB after 7 days but negative after 14 days whilst *Gordonia nitida* DSM 44499^T alone scored positive in CFS after 7 days but negative after 14 days. Thirteen strains from various genera only scored positive in WCB samples after 7 or 14 days and not in the CFS indicating the production of cell-bound biosurfactants only. Five strains, including three gordoniae and two tsukamurellae, only scored positive for biosurfactant production in CFS samples throughout the incubation period.

2.3.3.2. Oil spreading assay. The oil spreading technique was applied qualitatively to detect biosurfactant production using the following scoring system: '-' for no oil displacement; '+' for zones of clearance 1-8.7 cm diam. and '++' for zones of clearance ≥8.8cm diam. The negative control did not displace mineral oil (-), whilst the positive control created a zone of clearance ≥8.8cm diam. and was scored ++. Positive results (+ or ++) were widely distributed amongst the test strains (Table 2.4) with 96% (90 /94) scored positive in at least one sample type (WCB or CSF). Good agreement was found between replicate test results.

Table 2.4. Frequency of strains in each genus scored positive for oil spreading (OS) when whole-cell broths (WCB) and cell-free supernatants (CFS) were screened after 7 and 14 days of growth in Mineral Salt Medium with hexadecane (2%, v/v) at 30°C with rotary shaking at 180 rpm.

Genus (no. test strains)	Response level	No. positive for Oil Spreading* (% of test strain positive)			
		Day 7		Day 14	
		WCB	CFS	WCB	CFS
Corynebacterium (2)		2 (100)	1 (50)	2 (100)	1 (50)
	+	1	1	1	0
	++	1	0	1	1
Dietzia (13)		11 (85)	11 (85)	13 (100)	13 (100)
	+	7	9	9	11
	++	4	2	4	2
Gordonia (30)		8 (27)	5 (17)	26 (87)	19 (63)
	+	6	5	24	19
	++	2	0	2	0
Millisia (1)		1 (100)	1 (100)	1 (100)	1 (100)
	+	0	1	0	1
	++	1	0	1	0
Mycobacterium (6)		5 (83)	3 (50)	6 (100)	5 (83)
	+	3	3	3	3
	++	2	0	3	2
Nocardia (5)		5 (100)	3 (60)	5 (100)	3 (30)
	+	2	3	3	1
	++	3	0	2	2
Rhodococcus (25)		24 (96)	24 (96)	25 (100)	25 (100)
	+	7	22	17	22
	++	17	2	8	3
Tsukamurella (8)		8 (100)	8 (100)	7 (88)	7 (88)
	+	4	7	7	6
	++	4	1	0	1
Williamsia (4)		4 (100)	3 (75)	4 (100)	3 (75)
	+	2	3	2	3
	++	2	0	2	0
Total (94)		**68 (72)**	**59 (63)**	**89 (95)**	**77 (82)**
Positive only in WCB/CFS		**9 (10)**	**0 (0)**	**14 (15)**	**2 (2)**
Positive in both WCB + CFS		**59 (63)**		**75 (80)**	

* Positive strains scored either + or ++; 0 indicates no response level in that category; water was the negative control and purified rhamnolipid was a positive control. All strains were tested in triplicate.

After incubation for 7 days, 72% (68/94) strains in WCB and 62% (59/94) strains in CFS strains scored positive for full or partial oil spreading (+ or ++) (Table 2.4., page 66). Fifty-nine strains tested positive in both WCB and CFS while nine tested positive in WCB but not CFS. No strains were scored positive in CFS alone (Table 2.4., page 66). Twenty-six strains (27.7%) scored negative in both the WCB and corresponding CFS.

After 14 days of incubation, the number of strains that scored positive for either full or partial oil spreading increased in both the WCB (89/94; 94.7%) and CFS (77/94; 81.9%). Seventy-five strains tested positive in both WCB and CFS samples, 14 strains tested positive in WCB but not CFS, and two strains, *Gordonia hydrophobica* DSM 44015^T and *Tsukamurella strandjordii* DSM 44573^T, tested positive in the CFS samples only (Table 2.4., page 66). Three strains scored negative for both WCB and CFS.

The WCB samples gave rise to more '++' (oil spreading ≥8.8cm diameter) scores than CFS samples, most notably amongst *Rhodococcus* and *Tsukamurella* strains (Table 2.4., page 66). When screening WCB samples, only 2 strains scored positive at 7 but not at 14 days, whereas 22 strains only scored positive after 14 days of incubation. Similarly, in CFS samples, 3 strains only scored positive at 7 days and 22 strains only scored positive at 14 days, (data not shown, refer to Appendix 7.1., page 264).

Notable was the increase in the number of *Gordonia* strains that achieved partial oil spreading (+) in both WCB and CFS samples after 14 days compared to 7 days of incubation (Table 2.4., page 66). The majority of strains belonging to *Rhodococcus* (24/25), *Tsukamurella* (7/8), Dietzia (11/13), and *Millisia* (1/1) were positive for both WBC and CFS samples on both days 7 and 14. Eleven strains from various genera only scored positive in WCB samples after 7 or 14 days and not in the CFS indicating the production of cell-bound biosurfactants only.

2.3.3.3. Emulsification Index (EI_{24}). Whole-cell broth (WCB) and cell-free supernatant (CFS) samples from triplicate cultures were tested for the ability to form stable emulsions with mineral oil and water. The height of the emulsion layer as a percentage of the total solution height was taken as the EI_{24} value. Interpretation of EI_{24} values varies in the literature with no set cut-off value for positive and negative results. However, an EI_{24} value of 30% or higher is commonly reported as a 'good' emulsification activity (Varjani *et al*., 2007). In this study EI_{24} values ≥30% were categorised as positive for 'high emulsification', whereas EI_{24} values 11-29% were considered as 'low emulsification' and EI_{24} values 1-10% as 'weak emulsification'.

Mean EI_{24} measurements from triplicate samples for all 94 strains are detailed in Appendix 7.1. (page 264). The positive control, purified rhamnolipid, gave high mean EI_{24} values of ~40% when tested alongside cultures at both incubation times. No emulsion was observed for the negative control. The distribution of positive and negative scores for the test strains is shown by category in Appendix 7.2 (page 277) with most strains producing low (11-29%) or weak (1-10%) EI24 indices in WCB and CFS samples after 7 and 14 days. Only 16 out of 94 (17%) strains produced high EI_{24} indices of ≥30% in WCB after 7 or 14 days (Fig. 2.3), of which 3 strains, *Rhodococcus kunmingensis* DSM 45001^T, *R. percolatus* DSM 44240^T and *Williamsia serinedens* DSM 45037^T, achieved high EI_{24} indices on both days (Fig 2.3). After incubation for 7 days, 11 strains (11.7%) formed 'high' emulsions (EI_{24} values ≥30%) (Table 2.5., page 70), predominantly members of the genus *Tsukamurella* (Fig. 2.3). WCB EI_{24} values across the 94 strains ranged from 2.5 ± 0.0 to 40.5 ± 0.0% with a mean of 11.1% (Fig. 2.4., page 71). CFS samples produced significantly lower emulsification indices ($p<0.05$) (Fig. 2.4., page 71); no strains produced 'high' emulsion and only two produced 'low' emulsions (Table 2.5., page 70).

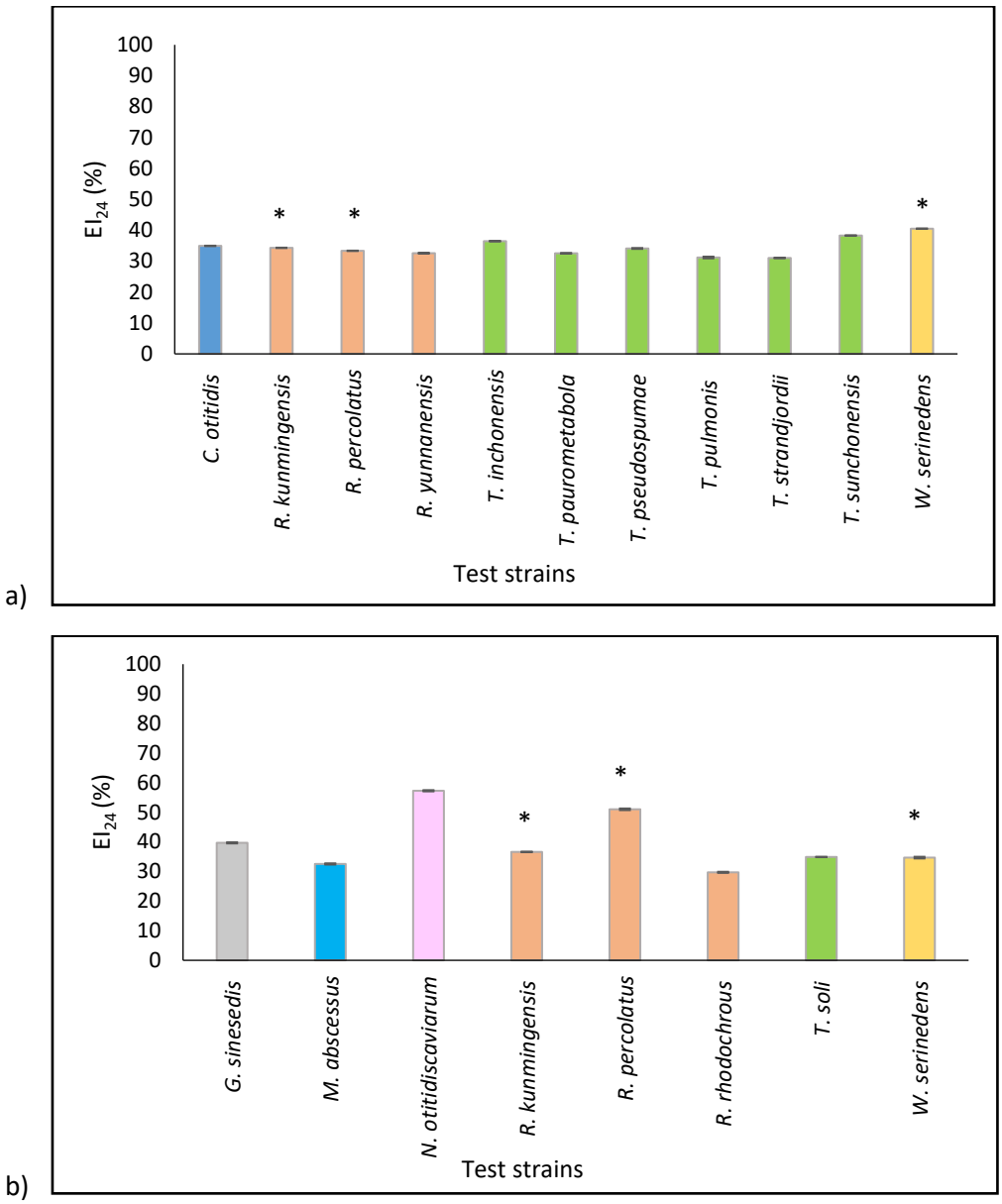


Fig. 2.3. Actinobacterial strains that formed stable emulsions with EI_{24} values ≥30% in whole-cell broths (WCBs) after incubation for a) 7 days and b) 14 days in MSM with 2% (v/v) hexadecane at 30°C and 180 rpm. Strains were tested in triplicate and mean values shown. *Strains showing high emulsification activity on both days.

After 14 days, only 8 strains (8.5%), distributed across 6 genera, achieved WCB EI_{24} values ≥30% (Table 2.5), the strain identities are shown in Fig 2.3. WCB EI_{24} values across the 94 strains ranged from 2.6 ± 0.0 to 57.3 ± 0.21% (mean 11.6%) and were not significantly different from those measured in WCB samples after 7 days of incubation (Fig. 2.4., page 71). Again, the CFS samples produced significantly lower EI_{24} values ($p<0.05$), with none of the strains produced high emulsification indices, although the number of strains producing 'weak' emulsification (EI_{24} values 1-10%) increased slightly from 19 to 22 on day 14 (Appendix 7.2., page 277).

Table 2.5. Frequency of strains in each genus scored positive for 'high' or 'low' Emulsification Index (EI_{24}) values in whole-cell broths (WCB) and cell-free supernatants (CFS) mixed with mineral oil after cultivation for 7 or 14 days in Mineral Salt Medium with hexadecane (2%, v/v) at 30°C with rotary shaking at 180 rpm.

Genus (no. test strains)		No. positive for Emulsification (EI_{24})* (% of test strain positive)			
		Day 7		Day 14	
		EI_{24} ≥30%	EI_{24} 11-29%	EI_{24} ≥30%	EI_{24} 11-29%
Corynebacterium (2)	WCB	1 (50)	0	0	0
	CFS	0	0	0	0
Dietzia (13)	WCB	0	9 (69)	0	13 (100)
	CFS	0	0	0	0
Gordonia (30)	WCB	0	15 (50)	1 (3)	3 (10)
	CFS	0	0	0	3 (10)
Millisia (1)	WCB	0	1 (100)	0	1 (100)
	CFS	0	0	0	0
Mycobacterium (6)	WCB	0	1 (17)	1 (17)	0
	CFS	0	0	0	0
Nocardia (5)	WCB	0	2 (40)	1 (20)	1 (20)
	CFS	0	0	0	0
Rhodococcus (25)	WCB	3 (12)	15 (60)	3 (12)	12 (48)
	CFS	0	0	0	0
Tsukamurella (8)	WCB	6 (76)	0	1 (12)	0
	CFS	0	1 (12)	0	0
Williamsia (4)	WCB	1 (25)	0	1 (25)	1 (25)
	CFS	0	1 (25)	0	0
Total (94)	**WCB**	**11 (12)**	**43 (46)**	**8 (9)**	**31 (33)**
	CFS	**0 (0)**	**2 (2)**	**0 (0)**	**3 (3)**

* EI_{24} values ≥30% were catergorised as 'high emulsification', EI_{24} values 11-29% were categorised as 'low emulsification'; 0 shows no EI_{24} value in that category; all strains were tested in triplicate; water was the negative control and purified rhamnolipid was a positive control.

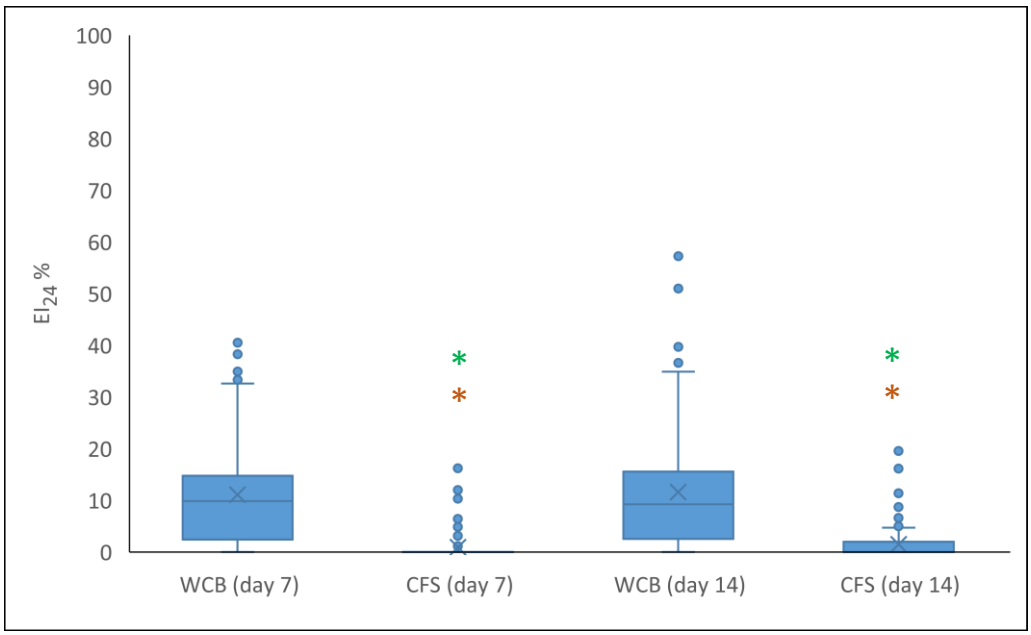


Fig.2.4. Range of Emulsification Index (E_{24}) values for the 94 actinobacterial strains determined for whole-cell broth (WCB) and cell-free supernatant (CFS) samples after growth in MSM supplemented with hexadecane (2% v/v) for 7 and 14 days. Each box represents the upper and lower quartile, with the mean (x) and median (__) values. Data falling outside the Q1 – Q3 range, denoted by the whiskers, are plotted as outliers. The negative control was distilled water. EI_{24} (%) = α / β ×100, where α and β represent the height of the emulsified layer and the total height, respectively. Significance difference between * WCB day 7 and other datasets, *WCB day 14 and other datasets tested by one-way ANOVA (Analysis of variance) followed by post hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

The highest EI_{24} value produced by CFS (16.2%) after 7 days was recorded for *Tsukamurella pulmonis*, which also gave a high EI_{24} index (31.2%) in the corresponding WCB. A similar observation was made for *Gordonia sinesedis* (CFS EI_{24} 16.2%) after 14 days and a strong EI_{24} was observed in the WCB. However, these were the exceptions with no relationship observed between high EI_{24} in WCB and EI_{24} in CFS samples for other strains ($p<0.05$).

2.3.3.4. Surface Tension (du Noüy ring method). The 94 test strains were screened for surface tension (ST) reduction using the du Noüy ring method after 7 and 14 days of incubation. Surface tension values measured from whole-cell broths (WCB) and cell-free supernatants (CFS) were categorised as high ST reduction (<40 mN m^{-1}), moderate ST reduction (40-50 mN m^{-1}), or weak to no ST reduction (>50-72 mN m^{-1}) according to the categories proposed by Thavasi *et al*. (2011). The ST values of WCB and CFS samples for all 94 strains are given in Appendix 7.1. (page 264). Distilled water readings of 68-72 mN m^{-1} were recorded at an ambient temperature of~20-22°C. The positive control, purified rhamnolipid (1mg ml^{-1}), reduced the ST of water to a mean value of 28.07 ±0.19 mN m^{-1}.

Surface tension values for WCB screened after 7 days ranged from 60.48 ± 0.14 mN m^{-1} recorded for *Gordonia defluvii* DSM 44981^T down to 20.26±0.07 mN m^{-1} for the amycolate strain *Corynebacterium otididis* DSM 8821^T with a mean value of 28.3 ± 5.5 mN m^{-1} (Fig. 2.5). Ninety strains (95.7%) reduced ST to <40 mN m^{-1} (Table 2.6., page 74). Values for the corresponding CFS samples ranged from 69.91±0.05 mN m^{-1} (*Dietzia lietea* DSM 45074) down to 24.58±0.03 mN m^{-1} (*Tsukamurella paurometabola* DSM 20162^T) with a mean significantly higher that for the WCB cultures (52.7 ± 12.4 mN m^{-1}) (Fig. 2.5). Only 12 strains (12.8%), gave surface tension <40 mN m^{-1} in the CFS (Table 2.6., page 74). Strains belonging to various genera, notably *Gordonia* and *Tsukamurella*, reduced the ST of both WCB and CFS samples cultivated for 7 days to <40 mN m^{-1} (Table 2.6., page 74).

After 14 days of cultivation, ST values for WCB's were not significantly different from those recorded on day 7, ranging from 70.23±0.06 (*Rhodococcus artemisiae* DSM 45380^T) down to 22.59±0.07 mN m^{-1} again recorded for *C. otididis* DSM 8821^T (mean ST for all strains 30 ± 7.5 mN m^{-1}) (Fig. 2.5). Eighty-six strains (91%) reduced ST to <40 mN m^{-1} (Table 2.6., page 74). Five strains capable of reducing ST to <40 mN m^{-1} after 7 days no longer did so after 14 days whilst only one strain, *Gordonia defluvii* DSM44981^T, reduced ST to <40 mN m^{-1} only after 14 days.

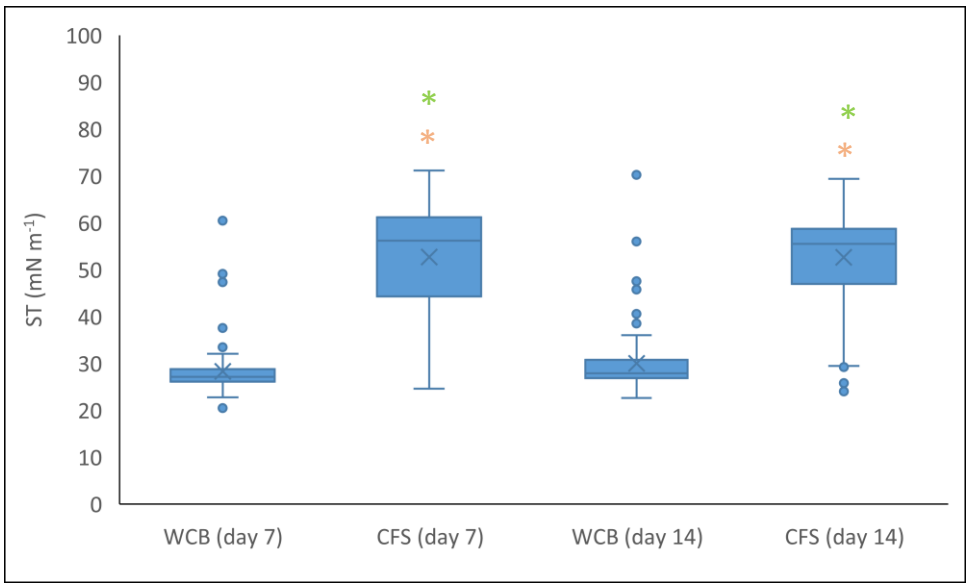


Fig.2.5. Range of Surface Tension (ST mN m^{-1}) values for the 94 actinobacterial strains determined for triplicate samples by the du Noüy ring method for whole-cell broths (WCB) and cell-free supernatants (CFS) after growth in MSM supplemented with hexadecane (2% v/v) for 7 and 14 days. Each box represents the upper and lower quartile, with mean (x) and median (_) values. Data falling outside the Q1 – Q3 range, denoted by the whiskers, are plotted as outliers. The negative control was distilled water. Significant difference between * WCB day 7 and other datasets, *WCB day 14 and other datasets tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

The corresponding CFS samples were significantly higher than those recorded for days 7 and 14 for WCB samples (Appendix 7.3., page 277). Values ranged from 69.40±0.15 mN m^{-1} (*Dietzia papillomatosis* DSM 44961^T) down to 24.05±0.01 mN m^{-1} (*T. strandjordii* DSM 44573^T) with a mean of 52.6 ± 11.0 mN m^{-1}. Only fourteen (15%) strains reduced ST to <40 mN m^{-1} (Table 2.6). Three strains, *Nocardia asteroides* N317^T, *N. farcinica* N1243, and *Rhodococcus triatomae* DSM 44892^T only reduced ST in CFS to below 40 mN m^{-1} after 14 days.

Twelve strains belonging to several genera reduced ST to <40 mN m^{-1} in both WCB and corresponding CFS samples taken on both days 7 and 14 of cultivation. Strains belonging to *Dietzia*, *Millisia,* and *Mycobacterium* did not appear to produce extracellular biosurfactants capable of reducing the CFS to an ST below the 40 mN m^{-1} threshold.

Table 2.6. Frequency of strains in each genus capable of high surface tension reduction (<40 mN m^{-1}) when whole-cell broths (WCB) and cell-free supernatants (CFS) were screened using the du Noüy ring method after cultivation for 7 or 14 days in Mineral Salt Medium with hexadecane (2% v/v) at 30°C with rotary shaking at 180 rpm.

Genus (no. test strains)	No (%) of test strain positive for the reduction in surface tension (<40 mN m^{-1})			
	Day 7		Day 14	
	WCB	CFS	WCB	CFS
Corynebacterium (2)	2 (100)	1 (50)	2 (100)	1 (50)
Dietzia (13)	13 (100)	0 (0)	13 (100)	0 (0)
Gordonia (30)	28 (93)	8 (27)	27 (90)	1 (3)
Millisia (1)	1 (100)	0 (0)	1 (100)	0 (0)
Mycobacterium (6)	6 (100)	0 (6)	4 (67)	0 (0)
Nocardia (5)	5 (100)	1 (20)	5 (100)	3 (60)
Rhodococcus (25)	25 (100)	4 (16)	24 (96)	2 (8)
Tsukamurella (8)	6 (75)	5 (62)	6 (75)	5 (62)
Williamsia (4)	4 (100)	2 (50)	4 (100)	2 (50)
Total (94)	**90 (96)**	**12 (13)**	**86 (92)**	**14 (15)**

2.3.3.5. Anionic biosurfactants (CTAB assay). The test strains were screened for anionic biosurfactant production by the qualitative CTAB assay as whole-cell broths (WCB) and cell-free supernatants (CFS) with dark blue halos produced on blue mineral salts agar scored as positive (Fig 2.6). After cultivation for 7 days, 57 strains (60.6%) scored positive for anionic surfactants in WCB samples but no extra-cellular anionic surfactants were detected in the corresponding CFS samples.

By comparison, after 14 days, the number of strains that scored positive in WCB increased to 82 (87.2%) (Table 2.7) and this increase was observed across all genera (Table 2.7). Further, the strains positive on day 7 remained positive until day 14. In addition, 24 strains (25.5%), including representatives of all genera except *Dietzia* and *Willamsia*, tested positive in CFS samples by day 14 (Table 2.7).

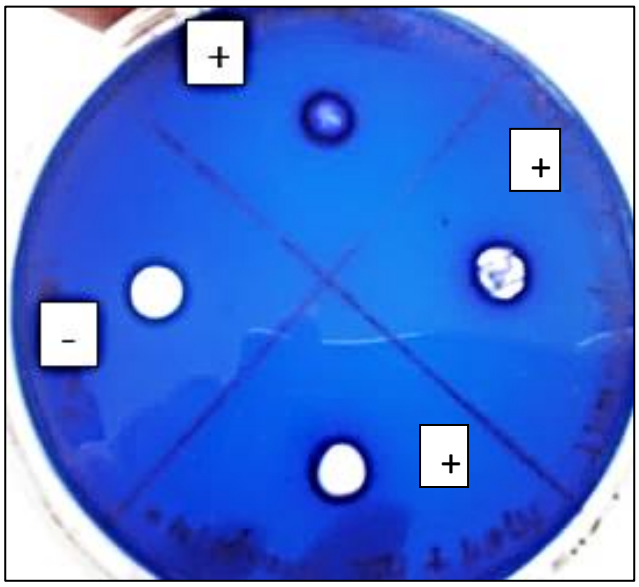


Fig.2.6. Interpretation of the CTAB (cetyltrimethylammonium bromide) plate assay for detection of anionic biosurfactants (Siegmund & Wagner, 1991): a dark blue halo around inoculated whole cell broth or cell-free supernatant is scored '+'; absence of a dark blue halo is scored '-'.

Table 2.7. The frequency of strains in each genus scored positive for anionic biosurfactants using the CTAB assay when whole-cell broths (WCB) and cell-free supernatants (CFS) were screened after 7 and 14 days of growth in Mineral Salt Medium with hexadecane (2%, v/v) at 30°C with rotary shaking at 180 rpm.

Genus (no. test strains)	No. positive for CTAB* (% of test strain positive)			
	Day 7		Day 14	
	WCB	CFS	WCB	CFS
Corynebacterium (2)	0 (0)	0 (0)	1 (50)	1 (50)
Dietzia (13)	5 (38)	0 (0)	11 (85)	0 (0)
Gordonia (30)	19 (63)	0 (0)	25 (83)	6 (20)
Millisia (1)	0 (0)	0 (0)	1 (100)	1 (100)
Mycobacterium (6)	4 (67)	0 (0)	6 (100)	1 (17)
Nocardia (5)	4 (80)	0 (0)	5 (100)	4 (80)
Rhodococcus (25)	20 (80)	0 (0)	24 (96)	11 (44)
Tsukamurella (8)	4 (50)	0 (0)	7 (88)	1 (13)
Williamsia (4)	1 (25)	0 (0)	2 (50)	0 (0)
Total (94)	**57 (61)**	**0 (0)**	**82 (87)**	**25 (27)**

CTAB, cetyl trimethylammonium bromide; MSM, Mineral Salts Medium. *All strains were tested in triplicate; the negative control was water and the positive control purified rhamnolipid.

2.3.4. Cell surface hydrophobicity (BATH assay).

The BATH assay was performed on whole cell broths to measure cell surface hydrophobicity (CSH) of the 94 actinobacteria, after cultivation for 7 and 14 days. The percentage adhesion values were categorised as follows: high cell adhesion (≥90 %); good cell adhesion (60 to 89%); moderate cell adhesion (40 to 59%) and no cell adhesion (hydrophilic cells) (<40%) using the threshold values proposed by Thavasi *et al*. (2011). The positive control strain, *Rhodococcus ruber* IEGM 231^{T}, showed cell adherence of 74.18 ± 0.05 %.

After incubation for 7 days, 72 out of 93 strains were categorised as positive for the BATH assay as they formed stable emulsions with mineral oil and achieved CSH values ≥40%. (Table 2.8). Positive responses ranged from 41.99 ± 0.01% measured for *Mycobacterium aurum* M401^{T} to 93.72± 0.01% for *Gordonia paraffinivorans* DSM 44604^{T} (mean for all strains 72.5 ± 14.2%). Similarly, after 14 days, 71 strains tested positive, with CSH values in the range 40.1 ± 0.03% for *Gordonia sputi* DSM 43896^{T} to 95.1 ± 0.02% for *Tsukamurella spumae* DSM 44113^{T} (mean 73.2 ± 15.3%).

The distribution of test strains in each CSH category was similar on days 7 and 14 (Table 2.8) although the identity of the strains assigned to each changed. A total of 16 and 14 strains showed moderate cell adhesion (CSH 40-59%) after 7 and 14 days, respectively, with only 4 strains positive in this category on both days. The number of strains with good cell adhesion (CSH 60-89%) increased from 49 on day 7 to 51 on day 14, of which only 29 were assigned to this category on both days. (Appendix 7.1., page 264). Seven strains scored high cell adhesion (CSH ≥90%) on day 7 and this dropped to 6 at 14 days (Table 2.8), of which only 2 were assigned this category on both days.

High CSH values were attributed to *Dietzia*, *Gordonia*, *Rhodococcus,* and *Tsukamurella* strains (Table 2.8). Other notable observations include the increase in CSH values between days 7 and 14 from 'moderate' to 'good' for several mycobacterial strains and from 'good' to 'high' for several tsukamurellae. *Corynebacterium and Millisia* strains were negative for CSH on both days whilst only 1 *Williamsia* strain scored positive (day 7 only).

Table 2.8. The frequency of actinobacterial strains in each genus scored positive for cell surface hydrophobicity using the Bacterial Adhesion to Hydrocarbon (BATH) assay with mineral oil, after cultivation for 7 or 14 days in Mineral Salt Medium with hexadecane (2%, v/v) at 30°C with rotary shaking at 180 rpm.

Genus (no. test strains)	No. strains assigned to each CSH (%) range* (% of positive test strains)					
	Day 7			Day 14		
	>90%	60-89%	40-59%	>90%	60-89%	40-59%
Corynebacterium (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dietzia (13)	1 (7.5)	7 (54)	4 (31)	0 (0)	8 (62)	4 (31)
Gordonia (30)	5 (17)	20 (67)	3 (10)	2 (7)	20 (67)	3 (10)
Millisia (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Mycobacterium (6)	0 (0)	1 (17)	4 (67)	0 (0)	5 (83)	1 (17)
Nocardia (5)	0 (0)	2 (40)	1 (20)	0 (0)	2 (40)	1 (20)
Rhodococcus (25)	2 (8)	13 (52)	4 (16)	1 (4)	15 (60)	5 (20)
Tsukamurella (8)	0 (0)	4 (50)	0 (0)	3 (38)	0 (0)	1 (13)
Williamsia (4)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)
Total (94)	**7 (7)**	**49 (52)**	**16 (17)**	**6 (6)**	**51 (54)**	**14 (15)**

*Categories: high cell adhesion (≥90%); good cell adhesion (60 to 89%) and moderate cell adhesion (40 to 59%), using the threshold values proposed by Thavasi *et al*., (2011).

The differences in measured CSH values between days 7 and 14 are apparent when the strains are grouped according to genus (Fig, 2.7). Generally, the mean and median CSH% value for strains assigned to each genus did not change significantly between days 7 and 14 apart from *Mycobacterium* ($p < 0.05$) (Fig 2.7). A decrease in mean CSH% between 7 and 14 days was observed for strains belonging to *Corynebacterium* and *Gordonia* but this was not significant ($p>0.05$). *Williamsia* was the only genus to record a significantly lower mean CSH% value (17.7 ± 0.01 %) on day 14 than on day 7 (30.2 ± 0.01%).

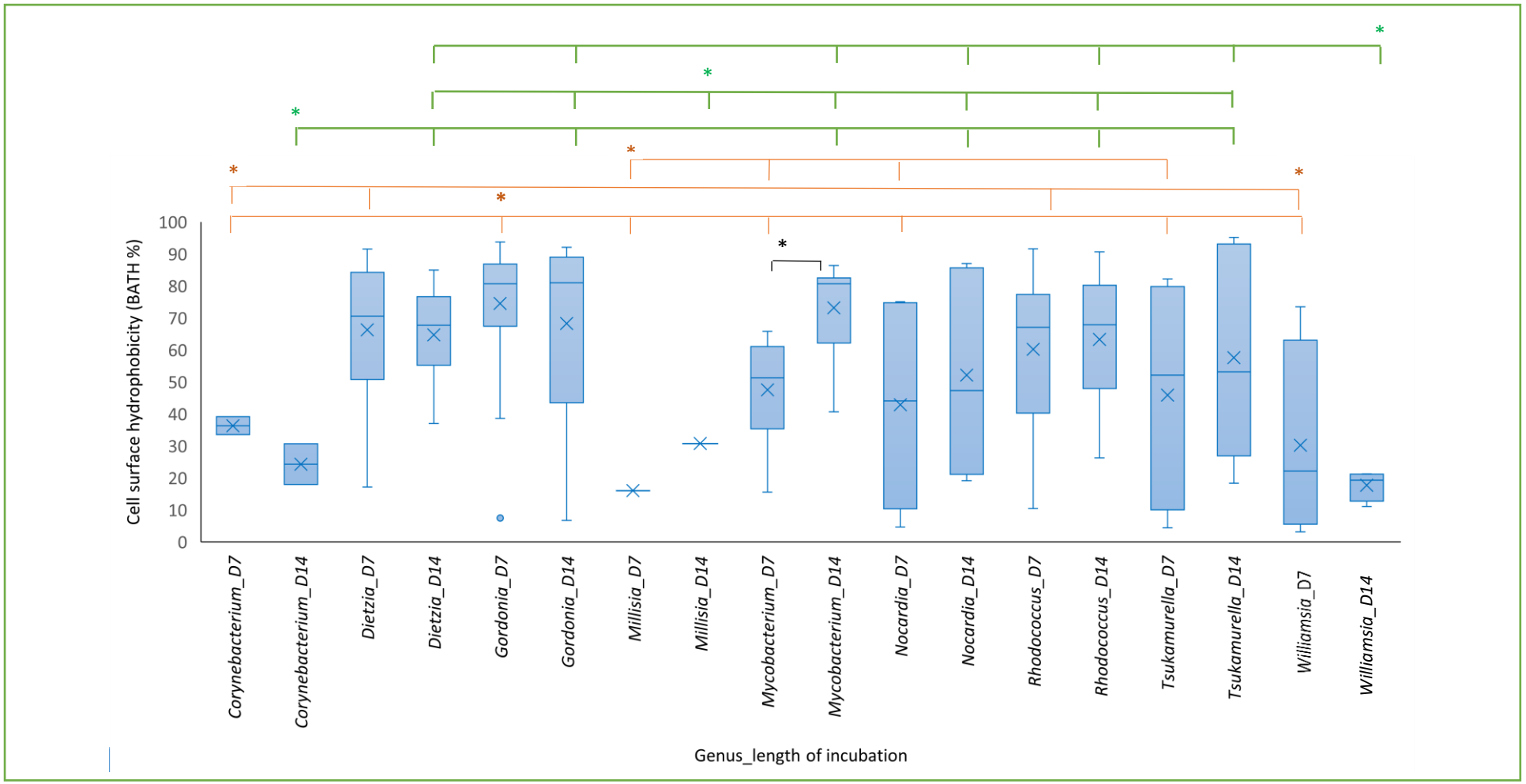


Fig. 2.7. The range of cell surface hydrophobicity values (CSH%) for 93 strains belonging to each genus, as determined by the BATH assay using mineral oil after growth in MSM supplemented with hexadecane (2% v/v) for 7 and 14 days. Each box represents the upper and lower quartile, with mean (x) and median (_) values. Data falling outside the Q1 – Q3 range, denoted by the whiskers, are plotted as outliers. Distilled water (DW) was employed as a negative control. Significance differences between genera on *day 7, * on day 14, and *between day 7 and 14 were tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

When screened after 7 days of incubation, strains assigned to the genus *Gordonia* achieved the highest mean CSH% value (74.5%± 18.5%), significantly higher ($p<0.05$) than those determined for the other genera, except *Dietzia* and *Rhodococcus*. The mean CSH% values for *Corynebacterium* and *Williamsia* were significantly lower than for *Rhodococcus* and *Dietzia* ($p<0.05$) and the mean CSH% for *Millisia* was significantly lower than for *Mycobacterium, Nocardia,* and *Tsukamurella* ($p<0.05$).

On day 14, strains assigned to the genus *Mycobacterium* achieved the highest mean CSH% value (73.12% ± 16.86%). The mean CSH% for *Corynebacterium*, *Millisia,* and *Williamsia* were not statistically different ($p > 0.05$) from each other, but all three were significantly lower than all other genera ($p < 0.05$). No other significant differences between genera were observed and no relationship between mycolic acid chain length and CSH% values was noted (Appendix 7.4., page 278).

2.3.5. Comparison of response rates and correlations between biosurfactant screening assays.

The positive or negative response rate of individual strains to different biosurfactant assays in whole cell broth (WCB) and cell-free supernatant (CFS) either on day 7/ 14 is shown in Table 2.9. All 94 strains tested positive for total biosurfactants (WCB) only or total biosurfactants and extracellular biosurfactants (CFS) in one or more screening assays.

The frequency of positive results varied with the screening assay applied, the number of strains that scored positive for 2 or more assays decreased as the number of assays compared increased (Appendix 7.5., page 279). It was also noted that there was greater agreement across multiple assays when cultures were screened on day 14 than on day 7 in both WCB and CFS. In the case of WCB samples, all 94 test strains scored positive for any two assays, while 88 scored positive across 4 assays, and this dropped to 51 across all 6 assays (Appendix 7.5., page 279). By comparison, the CFS samples from 76 strains tested positive for any 2 assays but only 15 strains scored positive over any 4 assays.

The surface tension (ST) assay gave rise to the highest number of positive strains when WCB samples were tested on both days 7 and 14 with 90 and 86 strains reducing ST to <40 mN m^{-1} whereas the Oil spreading (OS) assay gave rise to the highest number of positive strains in CFS samples on both days (Table 2.10., page 88). The length of incubation also affected the frequency of positive scores for each assay, and differences were most marked for WCB samples tested by the Drop collapse (DC), OS, and Cetyltrimethylammonium bromide (CTAB) assays (Table 2.10., page 88). The Emulsification Index (EI_{24}), bacterial adherence to hydrocarbons assay (BATH), and ST assays had more similar positive response rates on days 7 and 14. Importantly, the most similar positive scores were observed for WCB's after incubation for 14 days. In tests on CFS samples, only the DC and OS assays gave rise to high positive results and each provided similar scores on day 7 and again on day 14 (Table 2.10., page 88).

Table 2.9. Positive or negative response rate of individual strains to a) the six different biosurfactant assays used to screen whole-cell broths and b) the five assays used to screen cell-free supernatants either on 7/14 days of incubation in MSM with 2% (v/v) hexadecane at 30°C and 180 rpm. Category of positive strains includes + or ++ for drop collapse (DC), oil spreading (OS), + for cetyl trimethylammonium bromide (CTAB) assay and emulsification index (EI_{24}), surface tension (ST) <50 mN m-1 and bacterial adherence to hydrocarbons assay (BATH) >40% (moderate and high).

Actinobacterial Strains	Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Cntrl +ve	+	+	+	+	+	+	+	+	+	+	+
Cntrl –ve	-	-	-	-	-	-	-	-	-	-	-
Corynebacterium amycolatum N 1278^T	+	+	-	+	+	+	-	-	-	-	-
Corynebacterium otitidis DSM 8821^T	+	+	+	+	-	-	+	+	-	+	-
Dietzia aerolata DSM45334^T	+	+	+	+	+	+	+	+	-	-	-
Dietzia alimentaria DSM45698^T	+	+	+	+	+	+	+	+	-	+	-
Dietzia cercidiphylii DSM 45140^T	+	+	+	+	+	+	+	+	-	+	-
Dietzia cinnamea DSM 44904^T	+	+	+	+	+	+	+	+	-	-	-
Dietzia dagingensis DSM 447481^T	+	+	+	+	+	+	+	+	-	-	-
Dietzia kunjamensis DSM 44907^T	+	+	+	+	-	+	+	+	-	-	-
Dietzia lutea DSM45074^T	+	+	+	+	+	+	+	+	+	-	-
Dietzia maris DSM43672^T	+	+	+	+	+	+	+	+	-	-	-
Dietzia natronolimnea DSM 44860^T	+	+	+	+	+	+	+	+	-	-	-
Dietzia papillomatosis DSM44961^T	+	+	+	+	-	+	+	+	-	-	-
Dietzia psychralcaliphila DSM 44820^T	+	+	+	+	+	+	+	+	-	-	-

Actinobacterial Strains	Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Dietzia schimea DSM 45139^T	+	+	+	+	+	+	+	+	-	-	-
Dietzia timorensis DSM 45568^T	+	+	+	+	+	+	+	+	+	-	-
Gordonia aichiensis N934^T	-	+	+	+	-	+	-	-	-	-	-
Gordonia alkanivorans DSM 44369^T	-	+	+	+	+	+	-	-	-	-	-
Gordonia amarae DSM43392^T	-	+	+	+	-	+	-	-	-	-	-
Gordonia amarae DSM43392^T	-	+	+	+	-	+	-	-	-	-	-
Gordonia amarae DSM46078^T	-	+	+	+	+	+	-	+	+	-	-
Gordonia amarae DSM43391^T	+	+	+	+	-	+	+	+	+	-	-
Gordonia amicalis DSM 44461^T	+	+	+	+	-	-	+	+	+	-	-
Gordonia araii DSM 44811^T	-	-	-	+	-	+	-	-	-	-	-
Gordonia bronchialis DSM 43247^T	+	+	+	+	+	+	+	+	-	-	-
Gordonia cholesterolivorans DSM45229^T	-	+	+	+	+	+	+	+	+	-	-
Gordonia defluvii DSM44981^T	-	-	-	+	+	+	-	-	-	-	-
Gordonia desulfuricans DSM 44462^T	+	+	+	+	+	+	+	-	+	+	-
Gordonia effusa DSM 44810^T	+	+	+	+	+	+	+	+	+	-	+
Gordonia hirsuta DSM 44140^T	+	+	+	+	+	+	+	+	+	-	-
Gordonia humi DSM45298^T	+	+	+	+	+	+	-	+	-	-	-
Gordonia hydrophobica DSM 44015^T	+	-	+	+	+	+	-	+	-	-	-
Gordonia lacunae DSM45085^T	+	+	+	+	+	+	+	+	-	-	+

Actinobacterial Strains	Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Gordonia malaquae DSM 45064^T	-	-	-	+	+	+	-	-	-	+	-
Gordonia namibiensis DSM 44568^T	+	+	+	+	+	+	+	+	-	+	-
Gordonia neofelifaecis DSM45646^T	+	+	+	+	+	+	-	-	-	-	+
Gordonia nitida DSM 44499^T	-	+	+	+	+	+	+	-	-	-	-
Gordonia otitidis DSM 44809^T	-	+	-	+	-	+	+	+	-	-	-
Gordonia paraffinivorans DSM 44604^T	+	+	+	+	+	+	+	+	-	-	-
Gordonia polyisoprenivorans DSM 44302^T	+	+	+	+	+	+	-	-	-	-	+
Gordonia rubropertincta DSM 43197^T	+	+	+	+	+	+	+	+	-	-	-
Gordonia shiwensis DSM 44576^T	+	+	+	+	+	+	+	+	+	-	+
Gordonia sinesedis DSM 44455^T	+	+	+	+	+	+	-	+	+	+	-
Gordonia soli DSM 44995^T	+	+	+	+	+	+	+	+	+	-	-
Gordonia sputi DSM 43896^T	+	+	+	+	+	+	+	+	-	-	+
Gordonia terrae N659^T	+	+	+	+	+	+	+	+	-	-	-
Gordonia westfalica DSM 44215^T	+	+	+	+	+	+	-	+	+	-	-
Millisia brevis J81^T	+	+	+	+	+	+	+	+	-	+	+
Mycobacteriodes abscessus L948^T	+	+	+	+	+	+	+	+	-	-	-
Mycolicibacterium aurum M 401^T	+	+	+	+	+	+	+	+	-	-	-

Actinobacterial Strains	Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Mycolicibacterium fortuitum subsp. Fortuitum N 294^T	+	+	+	+	+	+	+	+	-	-	-
Mycolicibacterium peregrinum M 206^T	+	+	+	+	+	+	-	+	-	-	-
Mycolicibacterium phlei N 290^T	+	+	+	+	+	+	+	+	-	-	+
Mycolicibacterium smegmatis N 292^T	-	+	+	+	+	+	-	-	-	-	-
Nocardia asteroides N 317^T	+	+	-	+	+	+	+	-	-	+	+
Nocardia brasiliensis N 318^T	+	+	+	+	+	+	+	+	+	-	+
Nocardia farcinica N 671^T	+	+	+	+	+	+	+	+	+	-	+
Nocardia farcinica N1243	+	+	+	+	+	+	+	+	+	+	+
Nocardia otitidiscaviarum N 1158^T	+	+	+	+	+	+	+	-	-	+	-
Rhodococcus artemisiae DSM 45380^T	+	+	+	+	+	+	+	+	+	-	+
Rhodococcus baikonurensis DSM 44587^T	+	+	+	+	+	+	+	+	+	+	-
Rhodococcus cercidiphylii DSM 45141^T	+	+	+	+	+	+	+	+	+	+	-
Rhodococcus coprophilus DSM 43347^T	+	+	+	+	+	+	+	+	+	+	-
Rhodococcus erythropolis DSM 43066^T	+	+	+	+	+	+	+	+	+	-	+
Rhodococcus fascians DSM 20669^T	+	+	+	+	+	+	+	+	-	-	+
Rhodococcus globerulus DSM 43954^T	+	+	+	+	-	+	+	+	+	+	-
Rhodococcus gordoniae DSM 44689^T	+	+	+	+	+	+	+	+	-	-	+

Actinobacterial Strains	Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Rhodococcus jialingiae DSM 46766^T	+	+	+	+	+	+	+	+	-	-	+
Rhodococcus jostii DSM 44719^T	+	+	+	+	+	+	+	+	-	-	+
Rhodococcus kroppenstdetii DSM 44908^T	+	+	+	+	+	+	+	+	+	-	-
Rhodococcus kunmingensis DSM 45001^T	+	+	+	+	+	+	+	+	-	-	-
Rhodococcus maanshanensis DSM 44675^T	+	+	+	+	+	+	-	+	+	-	+
Rhodococcus marinonascens DSM 43752^T	+	+	+	+	+	+	+	+	-	+	-
Rhodococcus opacus DSM 43205^T	+	+	+	+	+	+	+	+	-	-	+
Rhodococcus percolatus DSM 44240^T	+	+	+	+	+	+	-	+	-	+	-
Rhodococcus phenolicus DSM 44812^T	+	+	+	+	+	+	+	+	+	-	-
Rhodococcus pyridinivorans DSM 44555^T	+	++	+	+	+	+	+	+	+	-	-
Rhodococcus rhodnii ATCC 35071^T	+	+	+	+	+	+	+	+	-	-	-
Rhodococcus rhodochrous DSM 43241^T	+	+	+	+	+	+	+	+	+	-	-
Rhodococcus triatomae DSM 44892^T	+	+	+	+	+	+	-	+	-	+	+
Rhodococcus tukisamuensis DSM 44783^T	+	+	+	+	+	+	-	+	+	-	+
Rhodococcus wrastislaviesis DSM 44107^T	+	+	+	+	+	+	+	+	+	-	+

Actinobacterial Strains	Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Rhodococcus yunnanensis DSM 44837^T	+	+	+	+	+	+	+	+	-	+	-
Rhodococcus zopfii DSM 44108^T	+	+	+	+	+	+	+	+	+	+	-
Tsukamurella inchonensis DSM 44067^T	+	+	+	+	+	+	+	+	+	-	-
Tsukamurella paurometabola DSM 20162^T	+	+	+	+	+	+	+	+	-	+	-
Tsukamurella pseudospumae DSM 44118^T	+	+	+	+	+	+	+	+	-	+	-
Tsukamurella. pulmonis DSM 44142^T	+	+	+	+	+	+	+	+	+	+	-
Tsukamurella soli DSM 45046^T	-	+	+	+	+	+	+	+	-	-	+
Tsukamurella spumae DSM44113^T	-	+	+	+	+	+	+	+	-	-	-
Tsukamurella strandjordii DSM 44573^T	+	+	+	+	+	-	+	+	-	+	-
Tsukamurella sunchonensis DSM45335	+	+	+	+	-	-	+	+	-	+	-
Williamsia faeni DSM 45372^T	+	+	+	+	-	-	+	+	+	-	-
Williamsia maris DSM 44693^T	+	+	+	+	+	-	+	+	-	-	-
Williamsia muralis N1261^{Tsp}	+	+	+	+	-	+	+	+	+	+	-
Williamsia serinedens DSM 45037^T	+	+	+	+	-	-	+	+	-	+	-

Table 2.10. Comparison of positive results for the detection of biosurfactant production using the drop collapse, oil spreading and emulsification index assays, surface tension measurement, cetyltrimethylammonium bromide (CTAB) and/or bacterial adherence to hydrocarbons assay (BATH) assay amongst 94 mycolic acid-containing actinobacteria screened as whole-cell broths and as cell-free after either 7- or 14-days growth in MSM medium supplemented with hexadecane (2% v/v) at 30°C with rotary shaking at 180 rpm.

Screening method	Days incubation	Whole-cell broth				Cell-free supernatant			
		No of positive strains	Response level	No of strains with each response level	No of weak/negative responses	No of positive strains	Response level	No of strains with each response level	No of weak/negative responses
Drop collapse [a]	7	43	++	6	51	50	++	4	44
			+	37			+	46	
	14	81	++	12	13	65	++	3	29
			+	69			+	62	
Oil Spreading [b]	7	68	++	36	26	59	++	5	35
			+	32			+	54	
	14	89	++	23	5	77	++	11	17
			+	66			+	66	
Emulsification Index (%) [c]	7	73	< 30	62			< 30	21	
			≥ 30	11	21	21	≥ 30	0	73
	14	87	< 30	79			< 30	25	
			≥ 30	8	7	25	≥ 30	0	69
Surface Tension (mN m^{-1}) [d]	7	93	< 40	90	1	13	< 40	12	81
			40-50	3			40-50	1	
	14	91	< 40	86	3	29	< 40	14	65
			40-50	5			40-50	15	
CTAB assay [e]	7	60	+	60	34	0	+	0	94
	14	87	+	87	7	26	+	26	68
BATH assay (%) [f]	7	72	40-89	65	22				
			≥ 90	7					
	14	71	40-89	65	23				
			≥ 90	6					

[a], Drop collapse assay: '++'- complete drop collapse, '+'- partial drop collapse after 1 minute of sample addition. [b], Oil spreading assay: '+' - oil spreading with a clear zone diam. of 1.0-8.8 cm, '++' - oil spreading with a clear zone diam. of ≥8.8cm. [c] Emulsification Index (%), [d] Surface tension reduction (mN m^{-1}),[e] CTAB, cetyl trimethylammonium bromide and [f] BATH (%), cell surface hydrophobicity; Values are the number of positive strains in that category.

Spearman's correlation coefficient was used to determine any correlations between the six assays (Appendix 7.6., page 280) based on whole data sets of WCB and CFS results after strain cultivation for 7 and 14 days and subsets grouped by culture sample (WCB and CFS) and length of incubation. Surprisingly, no strong correlations were observed between any of the assays. However, a weak positive correlation was found between the qualitative OS and DC assays (r_s = 0.35) and in particular the CFS samples on days 7 and 14 (r_s = 0.47, 0.40).

The OS assay showed a moderate correlation with the emulsification index (EI_{24}) assay (r_s = 0.45) with the strongest correlation observed for the WCB samples on day 7 (r_s = 0.56). Correlation coefficients between DC and EI_{24} data were weaker (r_s = 0.21) with the highest correlation found for WCB (day 14) data (r_s = 0.34) (Appendix 7.6., page 280). The ST assay correlated negatively with other assays. However, these correlations were very weak (maximum -0.24 with oil spreading). The BATH and CTAB assays did not correlate with other assays.

2.4. Discussion

As the demand for microbial-derived surface-active compounds is increasing, the search for novel biosurfactants will benefit from rapid qualitative and quantitative, high-throughput screening methods that are reliable, reproducible, and easy to use. This is particularly needed in the case of the mycolic acid-containing actinobacteria (MACA) as these biosurfactant producers have complex cell wall structures due to the presence of mycolic acids, which confer cell surface hydrophobicity (Blackall & Marshall, 1989b) and display biosurfactant-like properties (surface active) in themselves (Lee *et al*., 2005). No large-scale studies have been conducted to explore biosurfactant production across the diverse genera of the *Corynebacterineae* with most member species yet to be investigated. An initial pilot study was carried out to identify a suitable carbon source and incubation time for biosurfactant production in MACA and to identify suitable screening assays for large-scale study. Subsequently, a large and diverse collection of well-characterised MACA were screened for cell-bound and/or extra-cellular biosurfactant production using six different assays. All six screening assays were used to screen 94 MACA strains as the pilot study did not point to any assays other than EA that were unreliable and would warrant exclusion from the main study. Moreover, as strains are diverse and might expect them to respond differently to the same test hence wanted to compare results to a test by a large number of strains and look for any patterns based on genus (and perhaps mycolic acid length). In addition, these assays which rely on a number of different physico-chemical properties of biosurfactants, including cell surface hydrophobicity, emulsification, surface tension reduction, wetting properties, and anionic molecules, were evaluated to determine the most efficient and reliable screening strategy.

2.4.1. Production of cell-bound and extra-cellular biosurfactants amongst mycolic acid-containing actinobacteria.

All 94 MACA strains scored positive for the production of cell-bound biosurfactants but only 29 strains scored positive for extra-cellular biosurfactants, in one or more screening assays. The WCB samples were

considered to contain both cell-bound and extracellular (free) biosurfactants, if present, whilst the CFS samples were assumed to contain only extra-cellular biosurfactants. This is a really promising result but the variation in test results between assays created a rather complex picture. Surface tension reduction (du Nouy ring method) is perhaps considered the most definitive test for the confirmation of extra-cellular biosurfactant production as this method is quantitative and reproducible and has been recommended in several previous studies (Plaza *et al*., 2006; Sidkey *et al*., 2016; Youssef *et al*., 2004). When this criterion was applied, only 12 out of the 29 strains were confidently identified as extracellular biosurfactant producers based on a reduction in ST to ≤40 mN m^{-1} in cell-free supernatants at both sampling times (days 7 and 14). The identification of relatively few extra-cellular biosurfactant-producing strains is perhaps to be expected, as *Rhodococcus* species at least, are mainly reported to produce biosurfactants bound to the cell envelope (Kyunika *et al*., 2001; Franzetti *et al*., 2010) with only a few strains known to release extracellular biosurfactants (Kim *et al*. 1990; Ristau & Wagner, 1983).

Four of these 12 strains have previously been reported to produce extracellular biosurfactants: *Nocardia otitidiscaviarum* (Vyas & Dave, 2011)*, Tsukamurella paurometabola* (Gibson *et al*., 2004)*, Tsukamurella pseudospumae* (Kügler *et al*., 2014) and *Tsukamurella pulmonis* (Pasciak *et al*., 2010a). A fifth, *Corynebacterium otitidis*, has been reported to produce cell-bound biosurfactants only (Gilleron *et al*., 2005). The other seven strains confirmed as extracellular biosurfactant producers in this study, namely *Gordonia desulfuricans, Rhodococcus baikonurensis*, *R. yunnanensis, Tsukamurella strandjordii, T. sunchonensis, Williamsia muralis,* and *Willimsia serinedens* have not previously been reported to produce biosurfactants, other than cell-bound trehalose di-mycolates which are common amongst all the mycolic acid-containing actinobacteria. The identification of new MACA biosurfactant-producers holds promise for the discovery of novel surface-active compounds. Further, the observation that extra-cellular biosurfactants are produced by strains from various genera strongly indicates that exploring diverse *Corynebacterineae* for biosurfactant production is worthwhile.

The production of extra-cellular biosurfactants appeared to be time-dependent as the frequency of positive scores was higher on day 14 than on day 7 for all 5 screening assays. *Nocardia* strains in particular only tested positive after 14 days of cultivation. This is in agreement with the previous observation that the mechanism of production, retention, and release of biosurfactants are related to incubation time (Bredholt *et al.,* 2002). Indeed, several strains, for example, *Nocardia asteroides* N317^{T}, *N. farcinica* N1243, and *Rhodococcus triatomae* DSM 44892^{T} only released biosurfactants into the growth medium beyond day 7 with a positive result for ST reduction in cell-free supernatant samples only recorded at day 14. This observation suggests that while biosurfactants may be released into the culture medium during the growth phase, the release continues beyond this time into the stationary phase and under growth-limiting conditions (Rapp *et al*., 1979). Studies on *Rhodococcus erythropolis* found that extracellular release of biosurfactants only occurred under specific growth-limiting conditions (Kim *et al*., 1990; Ristau & Wagner 1983) while *R. opacus* released biosurfactants during growth when n-alkane was being consumed (Kim *et al.,* 1990). White *et al.* (2013) reported that extracellular biosurfactants produced by *Rhodococcus* strain PML026 only reached maximum levels after 20 days of cultivation, during the stationary phase. Previous studies on *Nocardia* strains have also demonstrated that the release of extracellular biosurfactants is dependent on growth stage but this is strain dependent. *Nocardia otitidiscaviarum* MTCC 6471 released maximum quantities of extracellular biosurfactant at the latter stages of growth while *N. vaccinii* IMV B-7405 produced these earlier in growth (Vyas & Dave, 2011; Pirog *et al*., 2015).

As in DC and OS methods, few positive results were only seen with CFS and not with WCB. This is difficult to explain as CFS is derived from WCB and therefore we would expect to detect any biosurfactant in the WCB. It may be a technical error or perhaps reflect the insensitivity of the assay; scores are determined by eye and perhaps these samples were borderline between – and + scores. Alternatively, the presence of other unknown components associated with cells present in the WCB samples could possibly impede the effects of the biosurfactants present.

The selection of day 7 and day 14 as time points for screening biosurfactant production in the present study was based on observations made during a pilot-scale study and standardised for all strains to facilitate testing of large numbers of strains. However, it is recognised that the rate of growth on hexadecane, the relationship between growth and extra-cellular biosurfactant production, and therefore the optimum time point to detect maximum production will not be the same for all strains. It is possible therefore that some slower-growing strains such as mycobacteria may not produce detectable levels of extracellular biosurfactants until beyond 14 days of incubation. Equally, the release of extra-cellular biosurfactants during exponential growth may be missed for faster-growing strains that have entered the stationary phase much earlier than day 7.

The growth medium containing hexadecane as a carbon source was found to be better than the medium containing glucose for actinobacteria biosurfactant production in the pilot study, as more strains tested positive when grown on hydrophobic hexadecane than on glucose. A study by Peng *et al.* (2007) found a similar absence of biosurfactant production when glucose was used as a carbon source for the extracellular biosurfactant-producing strain *Rhodococcus erythropolis* (3C-9 strain) and the use of n-alkanes (C_{14}–C_{36}) showed two types of biosurfactant production. Philip *et al.* (2002) in their study, found *R. ruber* IEGM 231 to produce the highest yield of biosurfactant per unit biomass in hexadecane when compared with different n-alkane carbon sources. Moreover, Nakano *et al.* (2011) also found *Dietzia maris* WR-3 to produce biosurfactant in hexadecane observed by reduction of surface tension to the lowest level (31 mN m^{-1}) compared to D-glucose on which it was unable to produce biosurfactant. Further, in the pilot study a significant difference in growth pattern was also seen in minimal salt medium (MSM) containing glucose (2% v/v) and in hexadecane (2% v/v) with dense growth in the glucose-containing medium while in the biphasic MSM (hydrocarbon/water) with hexadecane (2% v/v), biomass partitioned into the upper hydrophobic layer with or without additional turbid growth in the lower liquid medium. A previous study on *Rhodococcus* species showed a similar growth pattern of rapid accumulation of biomass within a water-insoluble phase, and subsequent turbid

growth in the aqueous phase (White *et al*., 2013). This nature of growth in hydrocarbon substrate is due to the oxygen take-up, which is the first step in hydrocarbon degradation for increased contact between cell and hydrocarbon droplets and enhanced level of biosurfactant production, which tends to solubilize more in the oil phase rather than the aqueous phase causing the cells to aggregate at the hydrophobic layer. Biosurfactant reduces the surface tension between hydrocarbon-water systems causing greater dispersion of water-insoluble substrate (n-alkane) in the aqueous phase due to their amphipathic properties resulting in higher interaction of cells with solubilized hydrocarbon droplets much smaller than the cells and rapid uptake of hydrocarbon into the cells. Hence, leading to the transfer of cells into the aqueous phase resulting in turbid growth (Das *et al.*, 2011; Haferburg *et al*., 1986). The biosurfactants produced by the cells are either cell surface-associated, retained on the outer surface, or excreted to facilitate the attachment and transport of alkanes by increasing cell contact at the aqueous-hydrocarbon interface (Wagner *et al*., 1983).

The batch culture used for biosurfactant production in this study had the advantage of being simple, suitable for identifying an appropriate carbon source for mycolic-acid containing actinobacterial biosurfactant production, and cost-effective for large-scale screening of biosurfactant producers. However, it was seen that the batch cultivation resulted in the degradation of the culture over longer incubation times due to a decline in the carbon and nutrient levels and the accumulation of byproducts in the culture flask. The build-up of byproducts changes culture conditions causing differences in growth rates between strains meaning that this may affect some strains more than others by the end of 14 day cultivation. In some, it may trigger the release of biosurfactants extra-cellularly later or earlier. As seen in this study, most of the screening assays were positive by day 14 for extracellular biosurfactant production. Moreover, the limited volume used in the batch cultivation results in low product yield. Abacha *et al*. (2016) and Zambry *et al*. (2021) reported a similarly positive and negative impact of batch culture for biosurfactant production where batch culture had the advantage of being simple, with a low risk of external contamination as no

further additions were required, more cost-effective during initial screening, process development and optimization of medium composition for biosurfactants production. However, batch cultivation being a closed system consequentially came with some limitations such as the use of high initial substrate concentrations, accumulation of undesired by-products, and lower overall productivity due to nutrient depletion over its long operating time.

The observations from this screening study highlight the importance of cultivation conditions for the production and detection of biosurfactants. Given that the MACA is a metabolically diverse group it may be the case that additional strains are capable of producing extra-cellular biosurfactants under different growth conditions to those selected in the present study. Indeed, changing the carbon substrate and other physico-chemical parameters could also alter the composition and yield of extra-cellular biosurfactant compounds.

2.4.2. Evaluation of the biosurfactant screening assays.

Previous studies have identified correlations between different biosurfactant screening assays but very few of these have considered mycolic acid-containing bacteria. This group of organisms is unique in that the presence of mycolic acids on the cell wall can confer hydrophobicity to the cell surface as well as surface-active properties. Hence, the results of physico-chemical assays based on cell surface hydrophobicity (CSH) and surface tension (ST), and correlations between these assays, may well be influenced by the presence of mycolic acids and differ from observations reported in other studies.

Assays based on ST in the present study included the qualitative OS and DC assays and the quantitative du Nouy ring method. The ST assay correlated negatively with DC and OS assays which is expected as the presence of biosurfactants is indicated by a reduction in ST values. Indeed, all three resulted in high frequencies of positive results; however, there were discrepancies between assays for some test strains indicating false positive and false negative results. Thus, only a weak positive correlation was found between DC with OS (r_s 0.345). Ariech & Guechi *et al.* (2015) and Youssef *et al.* (2004) reported higher positive correlations for DC and OS (r_s = 0.94, 0.864) which may be due

to the use of a small no of strains in Ariech & Guechi *et al.* (2015) study or use of a large number of strains belonging to same species in Youssef *et al.* (2004) study. Most of the strains that scored positive for the OS assay were also able to reduce ST however, the negative correlation was weak (r_s -0.125). Previous studies have reported stronger negative correlations between the OS and ST methods (rs = 0.959, -0.971) (Ariech & Guechi *et al*., 2015; Youssef *et al*., 2004). A negligible negative correlation was observed between the DC and ST (r_s -0.085) in the current study.

The cetyltrimethyl ammonium bromide (CTAB) assay which detects glycolipid biosurfactants was included in the screening study as MACA strains are known to mainly produce glycolipid biosurfactants (Kugler *et al*., 2015). Sidkey *et al.* (2016) recommended the use of the CTAB assay for initial screening for glycolipid surfactants along with the DC and OS methods. Interestingly, glycolipid surfactants detected in the present study were predominantly cell-associated although several strains appeared to release these extracellularly after extended incubation (day 14). The CTAB assay only showed weak correlations with the other assays.

Most biosurfactants that stabilise emulsions are called bioemulsifiers (Uzoigwe *et al.,* 2015). The production of cell-bound bio-emulsifiers was widely distributed amongst the MACA strains, as determined by the ability to emulsify mineral oil in the Emulsification Index assay (EI_{24}). However, lower numbers of strains produced extra-cellular bioemulsifiers suggesting that these remain cell-bound during growth on hexadecane. When the arbitrary categories for emulsification recommended by Varjani *et al*. (2007) were applied, only two strains, *Nocardia otitidiscaviarum* N1158^T and *Rhodococcus percolatus* DSM 44240^T achieved high emulsification (EI_{24} indices >50%) and only in whole cell broths. Vyas & Dave (2011) reported good emulsification ability (> 40%) by *N. otitidiscaviarum* strain MTCC 6471^T.

Defining categories for emulsification capability based on EI_{24} indices is useful for identifying good bioemulsifiers. However, interpretation of EI_{24} values varies in the literature with no set cut-off for positive and negative results. Bosch *et al*. (1988) defined a stable emulsion as the ability to maintain 50% of

the original emulsion height while Chen *et al*. (2007) referred to 40%. In this study, an emulsification index of 30% or higher was considered to indicate biosurfactants with good emulsification properties (Varjani *et al*., 2007) as this has been frequently applied in the literature. However, it is often difficult to compare results with studies as various oils, assay periods, and temperatures are used as well as cut-off values depending on the intended application of the biosurfactants, leading to different conclusions regarding the emulsification abilities of test strains. In the present study, a negative correlation between EI_{24} and ST (lower ST values indicate a positive response) and positive correlations between EI_{24}, OS, and DC were observed. Ariech & Guechi *et al.* (2015) also found a negative correlation between EI_{24} and ST measurements and positive correlations between EI_{24}, OS, and DC. Other studies have also suggested this method for reliable biosurfactant/bioemulsifier screening (Thavasi *et al*., 2011; Loganathan *et al*., 2010; Satpute *et al*., 2008).

Cell surface hydrophobicity (CSH) is commonly used as a biosurfactant screening method. In this study, high CSH values were observed for *Dietzia, Gordonia, Rhodococcus,* and *Tsukamurella* strains. However, no significant correlations were observed between CSH and other screening assays thus CSH was unrelated to surface tension reduction and emulsification properties. Indeed, some test organisms such as *Corynebacterium* and *Millisia* strains that showed no CSH were in fact positive for other screening assays. Previous studies have reported negative correlations between CSH and ST, and positive correlations with the EI assay (Volchenko *et al.*, 2007; Thavasi *et al*., 2011). This assay should be used in combination with other assays as hydrophobicity may be caused by cell wall components other than biosurfactants, in the case of test strains in the present study, this could be mycolic acids, leading to false positive results (Thavasi *et al.,* 2011). Hydrophobic cells may also act as biosurfactants themselves (Hommel, 1994). Hence, this method may be useful to indicate the presence of surface-active compounds in whole-cell broths but cannot be used to detect extra-cellular biosurfactants.

Further, there is very little data in the literature relating to the influence of mycolic acid chain length on ST and EI_{24} except for CSH. The presence of mycolic

acids did not appear to influence assay results with no correlations observed between mycolic acid chain length (based on genus assignment) and cell surface hydrophobicity or surface tension. Stratton *et al*. (2002) similarly found a similar lack of correlation between mycolic acid chain length and CSH when three *Rhodococcus* species were studied, and the results suggested that mycolic acids do not contribute to CSH. Sunairi *et al*. (1997) found a similar effect with mycolic acid chain length showing no relation to CSH. Hence, the fact that the mycolic acid chain length range overlaps between genera and the fact that chain length varies for any one group/strain based on growth conditions did not affect the biosurfactant screening of strains.

However, the observation was based on genus assignment, no actual chain length was measured across various strains in each genus it lacks the link between mycolic acid chain length with CSH and ST. Apart from mycolic acid, the carbon source has been known to affect the cell wall lipids of mycolata. Especially growth on hydrophobic substrates is also known to increase cell wall hydrophobicity (Sokolovská *et al*., 2003; Wick *et al*., 2002).

All the screening methods were found to be reproducible when applied to biosurfactant screening by mycolic acid-containing actinobacteria with a low coefficient of variation ($p<0.05$) with replicate analyses. However, this study also confirmed that reliance on one biosurfactant screening method is not sufficient to detect biosurfactant production by mycolic acid-containing actinobacteria. Instead, a combination of assays is required. This is not surprising given that qualitative assays may be insensitive to small quantities of biosurfactant and those reliant on observation with the eye may be prone to error. In addition, the assays rely on different physico-chemical properties therefore depending on the mix and chemical nature of the biosurfactant compounds produced by any one organism, different assays may be more or less suitable for detection. The use of multiple assays helps to reduce the chances of false-positive and false-negative results. The Drop Collapse (DC) and Oil Spreading (OS) assays are simple, rapid, and enable the preliminary screening of high numbers of strains. These are therefore useful as an initial screen before applying more involved quantitative assay methods and readily

detected biosurfactant production amongst MACA strains in the present study. However, given the low correlation between these assays application of both rather than one is advised.

The EI_{24} assay is more involved than the DC and OS assays and high throughput is not possible. It did not prove particularly useful for the detection of extra-cellular biosurfactants amongst the MACA test strains, and it would appear that bioemulsifiers were mainly cell-bound. However, this assay is useful for the detection of cell-bound biosurfactants and perhaps more useful if bioemulsification is the key property sought. The BATH assay is also more involved and did not correlate with any other assays in the present study. The measurement of ST is time-consuming, and the equipment only allows testing of one organism at a time making it difficult to screen large numbers of strains hence it should only be used as a confirmatory test for strains scored positive by other methods. Previous studies have also suggested the use of more than one screening method for the initial screening of biosurfactant production and recommended the use of DC, OS, and EI_{24} methods for initial screening followed by final confirmation using ST measurement (Chen *et al*., 2007; Plaza *et al*., 2006; Satpute *et al.*, 2008; Sidkey *et al*., 2016; Thavasai *et al.*, 2011; Youssef *et al*., 2004).

2.4.3. Selection of test strains for investigation of biosurfactant anti-biofilm properties.

Most of the strains screened in this study merit further investigation, not only for biosurfactants with anti-biofilm activities but also for a range of other potential properties. However, such studies first require extraction and isolation of the biosurfactants, and as this is a time-consuming process, the selection of a smaller number of strains is necessary. The main criterion for the selection of strains for further investigation was the ability to produce extracellular biosurfactants. This is because product recovery is more challenging, expensive, and time-consuming when biosurfactants of interest are cell-bound, which in turn limits commercial-scale application (Cappelletti *et al.,* 2020). In this study, surface tension (ST) reduction to <40mN m^{-1} in the cell-free supernatant (CFS) after days 7 and 14 of culture incubation was taken as

confirmation of extra-cellular biosurfactant production. Strains belonging to the genera *Dietzia, Gordonia, Mycobacterium,* and *Millisia* did not achieve ST values as low as <40 mN m^{-1} in CFS samples and were therefore excluded from further investigation although several scored positive for extra-cellular surfactants by one or more other screening assays and merit future study at some stage. A further criterion for strain selection was high emulsification (EI_{24}) in CFS samples as surfactants having both bioemulsifier and surface tension reduction properties have been known to have wide applications. Based on these criteria, various corynebacteria, nocardiae, rhodococci, tsukamurellae, and williamsiae have been identified to study the anti-biofilm properties of extra-cellular biosurfactants. This group includes *Rhodococcus erythropolis* DSM 43066^T as trehalose glycolipid biosurfactants from several other strains of this species that have been extensively characterised and shown to possess diverse properties with the potential biomedical application (Kuyukina *et al*., 2016). The next study will focus on the extraction and anti-biofilm testing of extra-cellular biosurfactants against clinically relevant biofilm-forming bacteria.

2.5. Conclusion

This is the first study to explore such a large and diverse group of *Corynebacterineae* strains for biosurfactant production. Most of these can synthesise cell-bound biosurfactants and many also release extra-cellular biosurfactants when cultivated on the hydrophobic substrate hexadecane. To the best of our knowledge, this is the first report of biosurfactant synthesis in many of these strains and notably the first to report this capability in strains belonging to the genera *Millisia* and *Williamsia*. Comparison and evaluation of various screening assays show that whilst they are highly reproducible, false-positive and false-negative results arising from qualitative assays, in particular, necessitate the use of multiple assays and quantitative measurement of surface tension is the most reliable assay. No clear relationship was observed between cell surface hydrophobicity and mycolic acid chain length (based on genus assignment) and biosurfactant production. However, cell surface hydrophobicity was of limited value as an assay to detect cell-bound biosurfactants and along with the emulsification index assay of little value for

detecting extracellular biosurfactants. Several extra-cellular biosurfactant-producing strains have been confidently identified and will be further investigated for potential anti-biofilm properties.

Chapter 3. Anti-biofilm properties of extra-cellular biosurfactants produced by mycolic acid-containing actinobacteria against clinically relevant pathogenic bacteria

3.1. Introduction

Biofilm formation on both biotic and abiotic surfaces, including medical implants, poses a significant threat to human health (Auler *et al*., 2010; Kumar & Anand, 1998; Srey *et al*., 2013; Qian *et al*., 2007). It is associated with various chronic and medical device-related infections that often exhibit resistance to biocides and antibiotics, resulting in difficult-to-treat infections (Donlan, 2001; Stewart *et al*., 2012). Consequently, the healthcare sector faces a significant challenge in preventing and managing biofilms and there is a growing demand for innovative strategies to prevent, disrupt, and eliminate biofilm formation (Khan *et al*., 2021; Roy, 2018).

The National Institute of Health (United States) reports that biofilm formation is responsible for 65% - 80% of all microbial and chronic infections (Jamal *et al*., 2018). The most life-threatening bacteria involved in healthcare infections are the ESKAPE pathogens (Rice, 2008) with those exhibiting multidrug resistance responsible for the most severe hospital-acquired infections (Meade *et al*., 2021). These pathogens are particularly difficult to prevent and treat (van Kleef *et al*., 2013). For this reason, the ESKAPE pathogens are also included on the World Health Organisation's priority pathogens list (WHO, 2017) signifying a global effort to discover new antimicrobial and biocidal agents to combat these biofilm-forming organisms.

As the detailed study of biofilm development and physiology has become possible, including insights into multicellular communication, differentiation, rudimentary fluid transport systems, and internal architecture (Girard *et al*., 2010; Leis *et al*., 2005), strategies to prevent biofilm formation and eradicate established biofilms at various developmental stages have emerged. These approaches may target the prevention of microbial adhesion to surfaces, inhibition of biofilm growth, or disruption of mature biofilms (Kalia & Purohit,

2011). Promisingly, biosurfactants have been shown to display effective anti-adhesion, inhibition, and disruption activities against a diverse array of biofilm-forming pathogens (Kiran *et al*., 2010; Kuyukina *et al*., 2016; Pradhan *et al*., 2013).

Biosurfactants can prevent the adhesion of cells to surfaces through various mechanisms. These compounds have natural cleaning properties that can reduce the accumulation of organic matter and debris on surfaces, making conditions less favourable for biofilm development (Haddaji *et al*., 2022). Cell adhesion is dependent on the physical and chemical properties of the surface including hydrophobicity, charge, and polymeric properties (Treter *et al*., 2014). Biosurfactants can modify the hydrophobic or hydrophilic properties of surfaces, changing how bacteria interact with the surface and consequently their ability to adhere to it (Banat *et al*., 2010). By binding to available surface sites, biosurfactants also reduce the space and opportunities for pathogen cells to attach (Friedlander *et al*., 2019). Biosurfactants can also attach to the cell membrane, modify cell-surface properties, and reduce outer membrane hydrophobicity and adhesion properties, acting as a barrier between the cell and the surface (Das *et al*., 2009).

Where microbial cells have already adhered to a surface, biosurfactants can facilitate their detachment and dispersal, inhibiting the development of stable biofilms. Loss of cell membrane integrity has been reported as a mechanism of biofilm inhibition activity (Gudiña *et al*., 2013). This process may involve the insertion of biosurfactants into the lipid bilayer of the cell membrane, resulting in the formation of transmembrane pores. This in turn leads to increased cell wall permeability, the release of lipopolysaccharide molecules, and ultimately, the breakdown of cell integrity (Rivardo *et al*., 2009; Sotirova *et al*., 2008). In addition, biosurfactants can interfere with quorum sensing, which can impede the coordinated growth of biofilms (Yan *et al*., 2019) and modify the expression of virulence genes ((Ibacache–Quiroga *et al*., 2013). Biosurfactants have been shown to limit the growth of planktonic bacteria during the exponential phase. This suggests that these compounds have an influence on normal cell division, which could also contribute to the inhibition of biofilm formation (Díaz de

Rienzo *et al*., 2016a; Kim *et al*., 1998; Lotfabad *et al*., 2010). Where biofilms are established, biosurfactants can also cause dissociation of the biofilm matrix, which comprises exopolysaccharides (EPS), extracellular DNA (eDNA), and proteins (Boles *et al*., 2005; Flemming & Wingender, 2010; Satpute *et al.,* 2016; Whitchurch *et al*., 2002) making it less stable and more susceptible to degradation.

The anti-biofilm properties of biosurfactants, in combination with their low toxicity and biodegradability, make them suitable for preventing and managing biofilm formation by various means, for example as anti-adhesive coating agents on surfaces prone to biofilm formation, such as urinary catheters (Mishra *et al*., 2020). Biosurfactants can be added to wound care products and topical treatments and as an antiseptic for flushing/wash devices in contact with human tissue e.g. catheters (Gupta *et al*., 2017; Lydon *et al*., 2017; Stipcevic *et al*., 2006). Incorporating biosurfactants into disinfectant cleaning solutions can help break down and remove biofilm growth from various environments and medical devices such as prosthetic implants (Singh *et al*., 2018).

Several mycolic acid-containing actinobacteria of the suborder *Corynebacterineae* produce biosurfactants, most notably trehalose-containing glycolipids, that exhibit effective anti-adhesion (Janek *et al*., 2018; Kuyukina *et al.*, 2016) and anti-biofilm (Dalili *et al.,* 2015; Kiran *et al.,* 2010) activities. However, this actinobacterial group remains underexplored as a source of further novel anti-biofilm biosurfactants. The study reported in Chapter 2 confirmed that a diverse range of MACA species, belonging to several genera, produce extracellular and/or cell-bound biosurfactants when grown in liquid culture with hexadecane as a sole source of carbon. Strains that produced extra-cellular biosurfactants are of particular interest as these often accumulate in higher concentrations in the culture medium than cell-bound forms and are readily accessible for extraction and analysis. Cell-bound biosurfactants instead require additional steps for extraction to ensure that these compounds are separated from other cellular components (López-Prieto *et al*., 2021; Vecino *et al*., 2015)

This study will investigate the anti-biofilm properties of crude extracellular biosurfactant extracts produced by eleven selected mycolic acid-containing actinobacteria screened in the previous chapter. Strains to be investigated were selected based on their ability to produce extra-cellular biosurfactants after both 7- and 14-days of growth, determined by the reduction of surface tension (ST) to <40mN/m in cell-free supernatants, in addition to testing positive in one or more other screening assays (Chapter 2, Appendix 7.1., page 267). Two additional strains, selected based on interesting responses to other physico-chemical screening assays, will also be investigated: *Rhodococcus percolatus* (ST 56 - 46mN/m) which produced >50% emulsification in the EI_{24} assay and *R. cercidiphylii* (ST 42 -51mN/m) which had a cell surface hydrophobicity of 91% in the BATH. In addition, *R. erythropolis* (ST 51 - 58mN/m) will be studied as biosurfactants produced by members of this species have been widely reported in the literature which will allow for comparison of findings. Lastly, *Rhodococcus ruber* IEGM 231^T, previously reported to produce biosurfactants with anti-adhesion properties (Kuyukina *et al*., 2016) will be included as a positive control and as a benchmark against which the effectiveness of biosurfactant extracts from the other test strains will be compared. Biosurfactants will be extracted in crude form after growth on hexadecane (2% v/v) and assayed for anti-adhesion, biofilm growth inhibition, and pre-formed biofilm disruption properties using a modified closed *in vitro* microtitre assay system (Lebeaux *et al*., 2013). A range of clinically relevant Gram-positive and Gram-negative bacteria identified on the WHO priority pathogen list will be assessed for their ability to form biofilms. Strains capable of biofilm formation will be challenged with the biosurfactant extracts. *Staphylococcus epidermidis* ATCC 35984, a known biofilm producer (Okajima *et al*., 2006), will be used as a positive control for the biofilm formation study.

3.1.1. Study Aim and Objectives

This study aims to assess extracellular biosurfactant extracts produced from different *Corynebacterineae* species for their potential to inhibit adhesion and biofilm formation and disrupt established biofilms formed by clinically significant bacteria.

The specific objectives of the study are to:

1. Establish an effective method to extract, detect, and classify extracellular biosurfactant extracts from diverse MACA species prior to further study.
2. Assess the biofilm-forming capabilities of various clinically relevant Gram-positive and Gram-negative pathogens, including those with known antibiotic resistance profiles, using a microtitre plate system.
3. Determine the anti-adhesion, biofilm inhibition, and biofilm disruption abilities of extracellular biosurfactant extracts against biofilm-forming pathogens using optimised quantitative microtitre plate assays.
4. Assess and compare the efficacy of biosurfactant extracts derived from different *Corynebacterineae* species to identify promising candidates for further characterisation.

3.2. Materials and Methods

3.2.1. Test strains. The eleven test strains included in the present study are listed in Table 3.1.

3.2.2. Extraction and detection of crude biosurfactants.

3.2.2.1. Biosurfactant extraction and recovery. Starter cultures were prepared by inoculating shake flasks containing 100 ml Tryptic Soy broth (TSB) with a single loopful of pure culture followed by incubation at 30°C with rotary shaking at 180 rpm for 72hrs. Subsequently, each starter culture was then used to inoculate (2% v/v) 1L volumes of mineral salts medium (MSM; section 2.2.4.1., page 52) supplemented with 2% v/v hexadecane (Sigma-Aldrich) in 3L Erlenmeyer flasks which were incubated at 30°C with rotary shaking at 180 rpm for 14 days. After incubation, bacterial cells were removed from the cultures by centrifugation (High-Speed Centrifuge Avanti J-26XP, Beckman Coulter) at 13,000 x *g* and 4°C, for 20mins. The supernatants were adjusted to pH 2 with 3M hydrochloric acid (HCL) and incubated at 4°C overnight, to precipitate extracellular biosurfactants. The quantity of HCL required for pH adjustment varied with each culture. Following acid precipitation, 800-900ml of chloroform-methanol (2:1, v/v) was added to each supernatant, and the mixtures were incubated at 180rpm and 30˚C for 2h to extract the biosurfactants. The white viscous layer (middle layer) containing crude biosurfactant extract was collected using a separating funnel leaving behind an excess of solvent (chloroform-methanol) and MSM medium. The remaining chloroform-methanol in the white viscous layer was evaporated using a rotatory evaporator (BUCHI Rotavapor R-114) at 30˚C for 15-30 mins depending on the extract collected. Each crude biosurfactant extract was weighed (g/L culture, dry weight), checked for surface activity by the oil spreading assay (section 2.2.4.4, page 53) and surface tension measurement (section 2.2.4.7, page 54) and stored at 4°C until further use.

Table 3.1. Actinobacterial strains included in the present study.

Actinobacterial strain	Strain Code	Abbreviated code for biosurfactant (B) extracts
Corynebacterium otitidis	DSM 8821^{T}	BCoti
Rhodococcus ruber (positive control)	IEGM 231	BRrub
Rhodococcus cercidiphylii	DSM 45141^{T}	BRcer
Rhodococcus erythropolis	DSM 43066^{T}	BRery
Rhodococcus percolatus	DSM 44240^{T}	BRper
Rhodococcus yunnanensis	DSM 44837^{T}	BRyun
Tsukamurella pseudospumae	DSM 44118^{T}	BTpse
Tsukamurella. pulmonis	DSM 44142^{T}	BTpul
Tsukamurella strandjordii	DSM 44573^{T}	BTstr
Williamsia muralis	N1261^{Tsp}	BWmur
Williamsia serinedens	DSM 45037^{T}	BWser

^{T}- type strain; ^{TSP}- type species.

3.2.2.2 Thin Layer Chromatography (TLC) for biosurfactant detection. The presence and purity of biosurfactants in the cell extracts was determined by thin-layer-chromatography (TLC). The crude biosurfactant extracts (0.01g ml^{-1}) were dissolved in chloroform-methanol (2:1 v/v) and a 2µl aliquot of each was applied as a spot on a line marked 1 cm from the base of a 20x20 cm aluminium-backed silica gel 60A plate (Sigma Aldrich, UK). Purified rhamnolipid standard (90% AGAE rhamnolipid; Sigma Aldrich, UK) was added as a positive control to each plate. The plates were developed by ascending one-dimensional TLC in glass development tanks containing chloroform-methanol-water (65:25:4 by volume; Franzetti *et al*., 2010), or chloroform-methanol-acetic acid (65:15:2 by volume; Gesheva *et al*., 2010) as the solvent system. Each solvent mix was prepared on the day of use and allowed to equilibrate in a glass tank lined with Whatman filter paper for approximately 1 hour. Each TLC plate was then placed upright in a tank and developed until the solvent front had migrated to ~75% of the height of the plate and this front was marked and measured. The plates were air-dried to remove solvents and sprayed evenly with freshly prepared orcinol reagent (0.1% w/v) in 5% v/v sulphuric acid or p-anisaldehyde reagent (acetic acid-p-anisaldehyde-sulphuric acid (100:1:2 v/v; Kugler *et al*., 2014) to detect glycolipids, and heated at 110°C for 5 mins to develop. Biosurfactants

were identified by brown-purple spots with orcinol spray or green spots with p-anisaldehyde spray (Franzetti *et al*., 2010). The retention factor (R_f) of spots identified as glycolipids was calculated as follows:

$$R_f \text{ (Retention factor)} = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent front}}$$

3.2.3. Formation of static biofilms in microtitre plates.

3.2.3.1. Test strains and culture conditions. Fourteen strains (Table 3.2) were tested for the ability to form biofilms in microtitre plate wells when cultivated in Brain Heart Infusion (BHI), Luria Bertani (LB), and Tryptic Soy Broth (TSB), over a 72h period. The strains were sub-cultured from glycerol (50%, v/v) stocks held at -80°C in the Edinburgh Napier University (ENU) culture collection onto tryptic soy agar (TSA) (Sigma-Aldrich, Dorset, UK) and incubated at 30°C (Jencons-WTC binder Incubator) for 24 to 48hrs. *Staphylococcus epidermidis* ATCC 35984, ordered from Leibniz-Institute DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was first reactivated from dried biomass in tryptic soy broth (TSB; Sigma Aldrich, UK) incubated at 30°C in an orbital shaking incubator and 180rpm for 24 h before sub-culture to TSA plates to obtain isolated colonies.

3.2.3.2. Biofilm formation. A single colony of each strain was inoculated in TSB broth (10 ml), and incubated at 30°C and 180rpm for 24 h before adjusting the optical density (OD_{595nm} λ) using TSB to 0.2 (equivalent to 10^5 cfu ml^{-1} as determined by viable plate count). After brief vortexing, each adjusted culture (100 µl) was transferred to wells of a sterile 96-well flat-bottomed polystyrene microtitre plate (Sigma, UK). The uninoculated culture broth was added as a sterility control and *Streptococcus epidermidis* ATCC 35984 was included as a positive biofilm control (Gill *et al*., 2005). Plates were incubated static for 24, 48, and 72h at 30°C (Jencons-WTC binder) and turbidity of the wells was measured at 595nm in a microplate spectrophotometer (FLUOstar Omega).

Table 3.2. Clinically relevant strains tested for the ability to form biofilms.

Biofilm test strain	Strain code	Antibiotic sensitive /resistant	Hazard level according to WHO
Enterococcus faecalis	NCIMB 7432	Vancomycin-sensitive	
**Enterococcus faecalis*	ATCC 51299	Vancomycin-resistant (Van-B phenotype)	High
Enterococcus faecium	ENU pj1	Vancomycin-sensitive	
**Enterococcus faecium*	ERI 2	Va ncomycin and teicoplanin resistant (Van-A phenotype)	High
Escherichia coli	ATCC 47055	ESBL-sensitive	
**Escherichia coli*	ERI 39	ESBL-resistant	Critical
Klebsiella pneumoniae	NCIMB 8865	Carbapenem -sensitive	
**Klebsiella pneumoniae*	ERI 44	Carbapenem-resistant	Critical
Pseudomonas aeruginosa	ENU 18	sensitive	
Pseudomonas aeruginosa	ENU 19	sensitive	Critical
**Staphylococcus aureus*	NCTC 6571	Methicillin-sensitive	
**Staphylococcus aureus*	SMRL/ 14/0440	Methicillin-resistant	High
Staphylococcus epidermidis	ENU IL-42	Methicillin-sensitive	
Staphylococcus epidermidis	ENU IL-43	Methicillin-resistant	High

ERI - Edinburgh Royal Infirmary; ENU- Edinburgh Napier University; ESBL- extended-spectrum beta-lactamases; MRSA- methicillin-resistant *Staphylococcus aureus*; Van- vancomycin-resistant *Enterococci*. *- clinical origin strains; ENU pj1 - Environmental isolate, Isolated from river almond; ENU IL-42 and 43 - Colonising strain from a healthy volunteer.

3.2.3.3. Biofilm measurement using crystal violet. Biofilms formed in the microtitre plates were measured using the crystal violet (CV) staining method with some minor modifications such as time, temperature, and solubilisation of CV (O'Toole, 2011). After incubation and growth measurement, the culture medium was removed by inverting the plate and tapping drying on tissue paper. The plates were then washed three times with distilled water to remove loosely attached cells and air-dried for 30 mins. A 100 µl volume of 0.1% (w/v) crystal violet (Sigma Aldrich, UK) solution was added to each well and left to stand for 30 mins at room temperature (20-22°C). The dye was then removed by inverting the plate, and the wells were washed again three times with distilled water and allowed to dry for 30 mins. For quantitative analysis of biofilm formation, the wells were de-stained with 200µl of 95% (v/v) ethanol (Sigma Aldrich, UK) on a shaking platform at room temperature for 30 mins. A 100 µl volume from each well was transferred to a clean flat-bottomed 96 well microplate and the absorbance was measured at OD_{595nm} in the microplate

reader. Assays were performed with 4 technical replicates for all test strains and controls and three biological replicates performed on different days. Biofilm formation was calculated using the formula (Kadurugamuwa *et al*., 2003):

Biofilm formation (OD_{595nm}) = Average absorbance of the test well - Average absorbance of control well (uninoculated culture broth).

Strains were selected for anti-biofilm experiments based on their ability to form biofilms 75% or greater than those formed by *S. epidermidis* ATCC 35984, the positive control strain, based on comparison of optical density measurements (OD_{595nm}) of eluted and destained solutions.

3.2.4. Anti-biofilm assays using crystal violet.

Crude biosurfactant extracts from the 11 actinobacterial strains were dissolved in sterile distilled water at a concentration of 10mg ml^{-1}, sonicated for 1 min in a sonicating water bath, and vortexed to obtain homogeneous samples prior to performing the anti-biofilm assays. All assays were performed with 4 technical replicates for each biosurfactant extract tested against each strain, and 3 biological replicates conducted on separate days.

3.2.4.1. Anti-adhesion assay. The anti-adhesion activities of the biosurfactant extracts were determined in 96-well flat-bottomed polystyrene microplates as previously described (Ivshina *et al.,* 2013). A 100 µl volume of biosurfactant extract solution was added to each well and the absorbance was measured at 595nmλ in a microplate reader (FLUOstar Omega). Each plate was incubated at 30°C overnight to bind the biosurfactant extract to the wells and any unattached biosurfactant extract was discarded by inverting the plates. The starter inocula for selected biofilm-forming strains (Table 3.2, page 110) were prepared as described in 3.2.3.2 above. A 100 µl volume of bacterial suspension was added to each coated well and plates with uncoated wells (negative control) and absorbance was read again at 595nmλ at time zero (t0). The plates were then incubated static at 30°C for 24-72 hrs depending on the optimum time required for each strain to establish a biofilm (determined using the method in section 3.2.3). After incubation, the turbidity of the wells at t24-t72

was determined at 595nmλ before applying the crystal violet staining method described in section 3.2.3.3 to quantify biofilm formation. Final optical density readings (595nmλ) were measured after de-staining. The percentage of cells adhered to wells coated with biosurfactant extract compared to controls (biosurfactant extract coated only) was calculated by using the formula 3.2.4.4.

3.2.4.2. Biofilm inhibition assay. Biofilm inhibition was determined according to the method described by Costa *et al*. (2018) with some minor modifications such as the volume of the sample, incubation time and temperature, and crystal violet staining method. The starter inoculum for biofilm-forming isolates studied was prepared as described in section 3.2.3.2. Equal volumes (100µl) of biosurfactant extract and the bacterial suspension were added simultaneously to each well of a flat-bottomed polystyrene microplate. The optical density at time t0 was measured at 595nmλ using a microplate reader (FLUOstar Omega) prior to static incubation at 30°C for 24-72hrs for biofilm formation, depending on the strain. The control wells contained bacterial suspensions without biosurfactant extract. After incubation, the optical density was measured at 595nmλ, and the medium was discarded to remove loosely attached bacterial cells before washing three times with sterile distilled water. Biofilms were quantified using the crystal violet stain method (3.2.3.3) with de-stained plates read at 595nmλ. The percentage of biofilm inhibition was measured using the formula in 3.2.4.4.

3.2.4.3. Biofilm disruption assay. The ability to disrupt mature biofilms was tested using the method described by Giri *et al*. (2019) with minor modifications to sample volume, incubation time and temperature, and crystal violet staining. The starter inocula for biofilm-forming isolates were prepared as described above. The wells of flat-bottomed polystyrene microplates were filled with 100µl of bacterial suspension and incubated static at 30°C for 24-72hrs for biofilm formation, depending on the clinical strain. After incubation, absorbance was measured at 595nmλ in a microplate reader (FLUOstar Omega), and unattached bacterial cells were then removed by inverting the plate and washing three times with sterile distilled water. A 100µl volume of biosurfactant extract was added to each well. The control wells were filled with

sterile sonicated water. Absorbance readings (595nmλ) were taken at t0 and again after static incubation for 24 h at 30°C. The liquid was discarded, the wells washed with sterile distilled water, and the remaining biofilm quantified using the crystal violet stain method (3.2.3.3) with de-stained plates read at 595nmλ. The percentage of bacterial eradication (disruption) by biosurfactant extract was calculated using the formula in section 3.2.4.4.

3.2.4.4. Calculation of biofilm anti-adhesion, biofilm growth inhibition, and disruption activity. Results were blank corrected by subtracting the average absorbance from control wells (media only) from the average absorbance of the test wells before determining the percentage of anti-biofilm activity.

The mean (four technical replicates) absorbance value was used for determining the percentage anti-adhesion, inhibition, and disruption of biofilm by biosurfactant extract using the following formula:

Microbial adhesion/inhibition/biofilm disruption (%) = [1 - (OD_{595} sample*/ OD_{595} control$^{\#}$)] × 100

*ODsample is the optical density of the well with a biosurfactant extract concentration and bacteria suspension.

ODcontrol is the optical density of the bacteria suspension with no biosurfactant extract.

3.2.5. Triphenyl tetrazolium chloride (TTC) assays.

3.2.5.1. TTC assay optimisation. In addition to the crystal violet assay which stains biomass, selected anti-biofilm assays were undertaken using the tetrazolium dye 2,3,5-triphenyl tetrazolium chloride (TTC) which measures metabolically active cells instead. First, to determine the optimum conditions for the TTC assay, culture inoculum load for biofilm development and the wavelength at which absorbance was measured, were tested. The tests were performed with 4 technical replicates and two biological replicates, on different days.

2, 3, 5-triphenyl tetrazolium chloride (TTC) (Sigma Aldrich, UK) was prepared in TSB medium (according to the required concentrations), filter sterilised

through 0.22 µm pore size cellulose acetate filters and stored in the dark at 4°C until use. All biofilm measurement assays were performed using the test strains *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumoniae* NCIMB 8865. Cultures grown in TSB for 24 h at 30°C with shaking at 180 rpm, were adjusted to the required OD_{595} values (OD_{595} 0.1 – 0.8) (Jenway 6300 spectrophotometer) using fresh TSB broth. To prepare the biofilms, 100 µl of each suspension was inoculated to the wells of a flat-bottomed 96-well microplate before static incubation at 37 °C for 24h. The medium in the wells was then discarded by inverting the plate and the wells were washed three times with distilled water. Then, TTC solution at the required concentration(s) (100µl) was added to the wells and incubated static for a further 20 h at 30°C. After incubation, the TTC was discarded by inverting the plate, and the wells were again washed three times with distilled water. The metabolized TTC dye was suspended in 200 µl of methanol, and the plates rotated at low speed on an orbital shaker at room temperature for 30 min before transferring 100µl from each well to a clean microtitre plate (Haney *et al*., 2021). Absorbance readings were measured in a microplate reader at wavelengths 450 and 490nm (Sabaeifard *et al*., 2014).

3.2.5.2. Anti-biofilm assays using TTC. Prior to testing the biosurfactant extracts, the optimum TTC concentration (% w/v) for the anti-biofilm assays was confirmed in a series of biofilm inhibition and biofilm disruption assays using a range of TTC concentrations and commercially available non-ionic surfactants Tween 80 and Triton X-100 (Sigma Aldrich, UK). Two-fold dilutions of each surfactant were prepared directly in 96 well flat-bottomed microplates using sterile distilled water. The assays were conducted using *Pseudomonas aeruginosa* ENU 18 at the optimum inoculum size, according to the methods described in sections 3.2.4.2 and 3.2.4.3, and biofilms quantified by TTC, as described in section 3.2.5.1.

Anti-biofilm (anti-adhesion, biofilm inhibition, and biofilm disruption) assays using selected biosurfactant extracts were conducted as detailed in sections 3.2.4.1 to 3.2.4.4, using the optimum culture inoculum load, and biofilms quantified using the TTC dye method described in 3.2.5.1. with optimised TTC

concentration. Crystal violet assays were performed in parallel to the TTC assays to compare the measurement of biofilm formation based on biomass staining versus formazan production by metabolically active cells, respectively. All assay was performed with four technical replicates and two biological replicates were done on different days.

3.2.6. Data analyses

All assays were performed in three biological replicates (except for biosurfactant extracts that did not show anti-biofilm activity in the first biological replicate determined using crystal violet staining) and expressed as mean ± standard deviation (SD) which was calculated using Excel. Tukey's One-way analysis of variance (ANOVA) with post-hoc correction (SPSS) was used to evaluate the significance of the difference ($p<0.05$). Median EC_{50} values (effective concentration to reduce biomass by 50%) for biocides were statistically determined using PriProbit (v 1.62 software) designed to analyse sigmoidal dose-response curve data.

3.3. Results

In the present study, extracellular biosurfactants from 11 actinobacterial species of the suborder *Corynebacterineae* were recovered as crude extracts and their physico-chemical nature investigated before testing their anti-biofilm properties against various clinically relevant bacteria. Anti-biofilm assays, including anti-adhesion, biofilm inhibition, and biofilm disruption, were undertaken using closed microplate assay systems and resting cells. Biofilm measurement was carried out using crystal violet (CV) staining and subsequently, those biosurfactant extracts that demonstrated the highest anti-biofilm activities were tested using an optimised tetrazolium dye (TTC) assay that measures metabolically active cells. The efficacy of the different biosurfactant extracts was compared to that of *Rhodococcus ruber* IEGM 213^T previously reported to produce trehalolipid biosurfactants that inhibit bacterial adhesion.

3.3.1. Detection of biosurfactants extracted from actinobacteria.

Crude biosurfactant extracts were recovered from the 11 actinobacterial strains using acid precipitation and solvent extraction methods. The white viscous middle layer produced from each cell-free supernatant (exemplified in Fig 3.1a), expected to contain mixtures of biosurfactants, was isolated and the solvents evaporated to leave a dried powder (Fig. 3.1b). Extract yields from liquid cultures ranged from 0.4 - 0.6 g l^{-1} dry weight.

All crude extracts tested positive for surface active properties using the oil spreading (OS) test and reduced the surface tension (ST) of water to ≤ 30 mN m^{-1} by the du Noüy ring method (data not shown). These ST reduction values were equivalent to those determined for the cell-free supernatants recovered from growing cultures (Chapter 2, Appendix 7.I., page 264). In the case of *Rhodococcus* strains (Table 3.1., page 108) the ST values were lower (≤ 30mN/m) than those measured for cell-free supernatants (51-58 mN/m) after 14 days incubation.

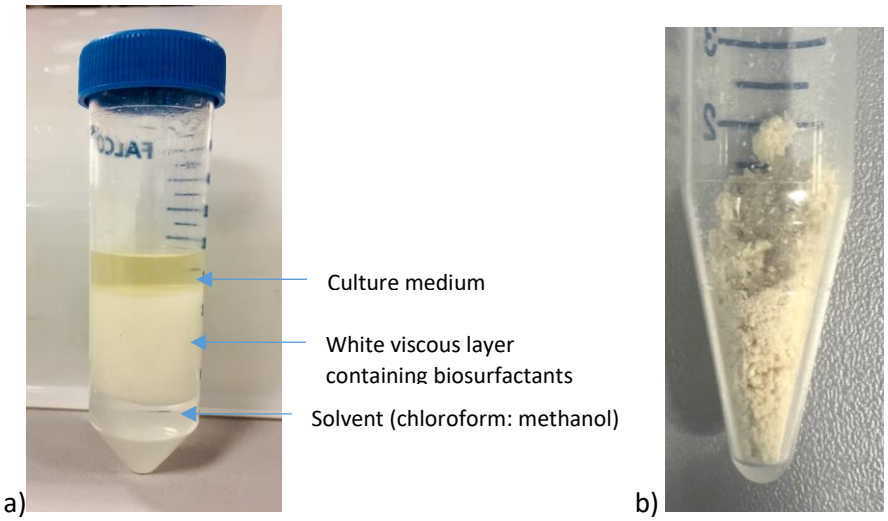


Fig 3.1. Crude biosurfactant extract from *Rhodococcus ruber* IEGM 231^{T} as a) the white viscous (middle) layer obtained after acid precipitation and solvent extraction, and b) as a dried powder after solvent evaporation.

Thin-layer chromatography was employed to confirm the presence of biosurfactants and detect glycolipids in crude extracts. Initially, various solvent-spray reagent combinations were tested, using a purified rhamnolipid standard (glycolipid biosurfactant), to ensure clear separation and resolution of biosurfactants. Two solvent systems gave rise to clearly visible, discrete rhamnolipid spots on TLC plates with measurable retention factors (R_f) and orcinol reagent detected glycolipids with all solvent systems tested (Table 3.3.)

Table 3.3. The effects of different solvent systems and developing reagents on the mobility and detection of purified rhamnolipid after ascending one-dimensional thin-layer chromatography.

Developing reagent	Solvent system			
	Chloroform: methanol (2:1)	Chloroform - methanol-acetic acid (65:15:2 v/v/v)	Chloroform-methanol:-water (65:25:4 v/v/v)	Chloroform-methanol-water (85:15:2 v/v/v)
Orcinol	R_f 0.92	Smudged spot	R_f 0.77	Faint smudged spot
p- anisaldehyde	R_f 0.92	Smudged spot	R_f 0.77	No spots detected

The presence of glycolipids was confirmed in biosurfactant extracts from all 11 test strains as revealed by the presence of brown-purple spots on TLC plates run using chloroform-methanol-water (65:25:4 v/v/v) or chloroform-methanol-acetic acid (65:15:2 v/v/v) as the solvent system. Ten strains produced a single spot whilst *Rhodococcus ruber* IEGM 213^T gave rise to two spots, one with the same R_f value as the rhamnolipid standard (Table 3.4). Biosurfactant extracts produced R_f values in the range 0.68 to 0.81, except for those from *Corynebacterium otitidis* DSM 8821^T (BCoti) which gave rise to a spot with a lower R_f value (0.64), *R. yunnanensis* DSM 44837^T (BRyun) which had the lowest R_f of 0.31 and *Williamsia muralis* N1261^{Tsp} (BWmur) which gave a spot closer to the solvent front (R_f 0.85) (Fig 3.2).

Table 3.4. Retention factor (R_f) values for glycolipids detected in crude biosurfactant extracts from actinobacterial test strains on one-dimension thin layer chromatograms sprayed with orcinol reagent.

Actinobacterial strain	Strain Code	Retention factor (R_f)
Rhamnolipid (glycolipid standard)		0. 77
Corynebacterium otitidis	DSM 8821^T	0.64
Rhodococcus cercidiphylii	DSM 45141^T	0.72
Rhodococcus erythropolis	DSM 43066^T	0.71
Rhodococcus percolates	DSM 44240^T	0.68
Rhodococcus ruber	IEGM 231^T	0.69, 0.77
Rhodococcus yunnanensis	DSM 44837^T	0.31
Tsukamurella pseudospumae	DSM 44118^T	0.77
Tsukamurella pulmonis	DSM 44142^T	0.71
Tsukamurella strandjordii	DSM 44573^T	0.72
Williamsia muralis	N1261^{Tsp}	0.85
Williamsia serinedens	DSM 45037^T	0.68

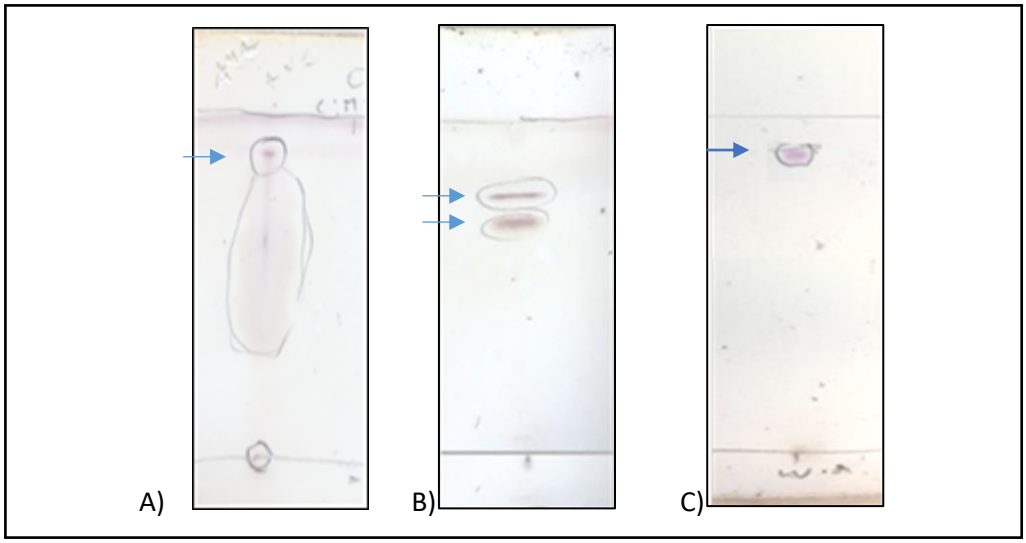


Fig 3.2. One-dimensional thin-layer-chromatography of (A) Rhamnolipid standard and acid precipitated-solvent extracted glycolipids from (B) *Rhodococcus ruber* IEGM 231^T; (C) *Williamsia muralis* N1261^{Tsp}. The glycolipids were separated using chloroform-methanol-water (65:25:4 by volume) and for *Williamsia* sp. chloroform-methanol-acetic acid (65:15:2 v/v/v) as the solvent system and revealed by heating at 110°C for 5 minutes after spraying with ornicol reagent.

3.3.2. Biofilm-forming capabilities amongst the clinically relevant bacteria.

Several optimisation experiments were conducted to establish optimal growth conditions for biofilm formation in flat-bottomed microplate wells.

3.3.2.1. Effect of medium type and fixation step on static biofilm formation.

Initially, three clinical strains, including *Staphylococcus epidermidis* ATCC 35984, a known biofilm-producer (Okajima *et al*., 2006) serving as a reference strain, *Escherichia coli* ATCC 47055, and *Klebsiella pneumoniae* NCIMB 8865, were subjected to biofilm formation assays after cultivation in nutrient-rich Luria Bertani (LB) medium and nutrient-limited defined M9 medium. Growth was measured spectrophotometrically following the removal of crystal violet dye at 24 and 48 h. These strains demonstrated measurable biofilm formation in LB medium but not in M9 medium (p <0.05) (Fig. 3.3). Additionally, adding a biofilm fixation step using cold methanol (5 min at -20°C) before crystal violet staining revealed no significant difference ($p > 0.05$) in biofilm formation between fixed and unfixed samples after 24 hours of incubation in LB medium. However, after 48 h incubation, the fixation step significantly reduced biofilm measurements for two strains, *S. epidermidis* ATCC 35984 and *E. coli* ATCC 47055 ($p<0.05$) (Fig. 3.3). Consequently, the fixation step was omitted from subsequent experiments to avoid biomass loss.

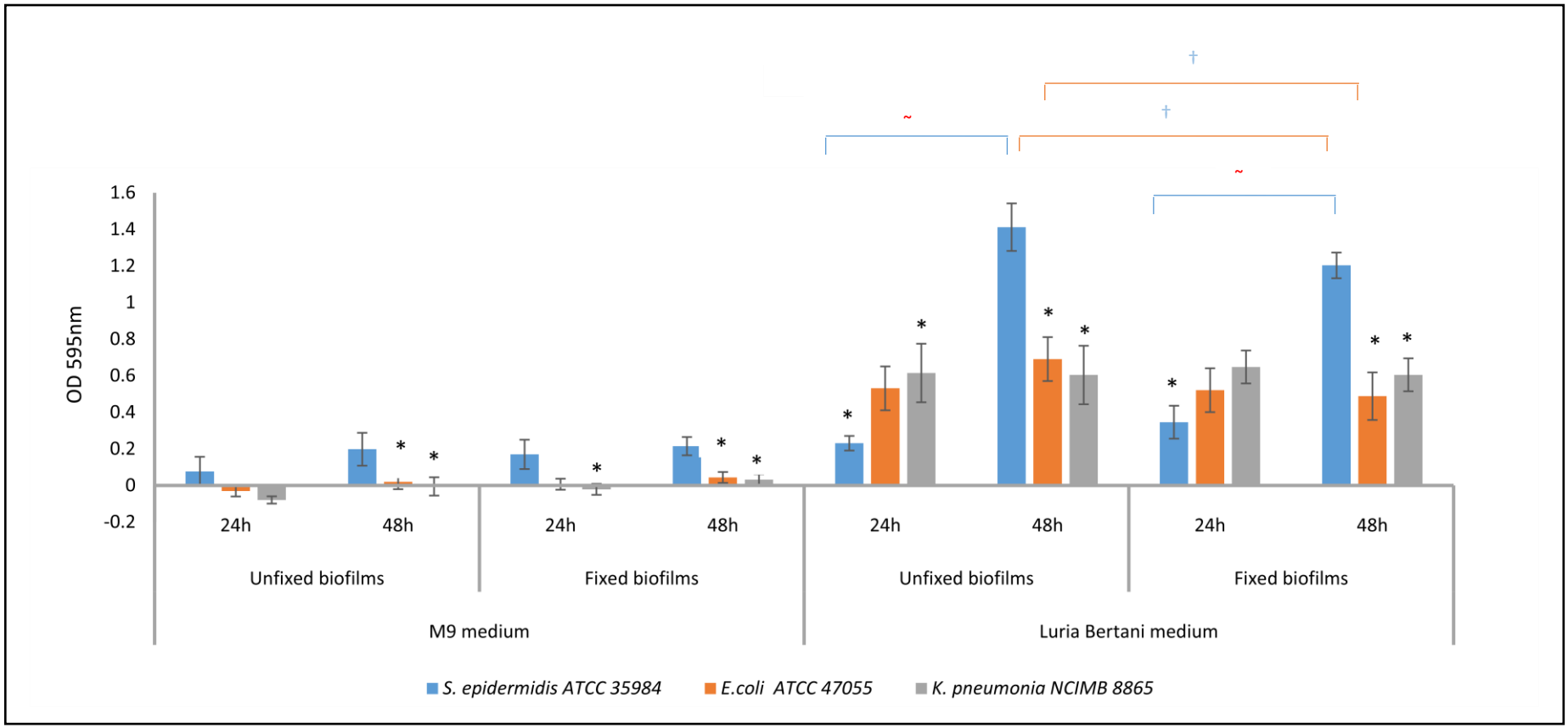


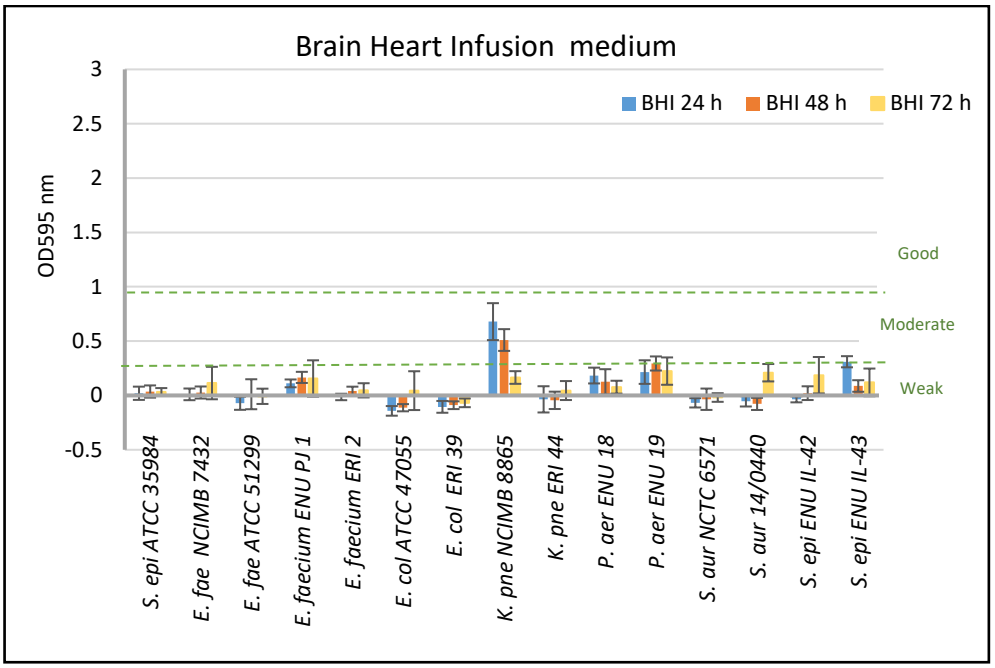
Fig. 3.3. Biofilm development by *Staphylococcus epidermidis* ATCC 35984 (positive biofilm-forming control), *Escherichia coli* ATCC 47055, and *Klebsiella pneumoniae* NCIMB 8865 on growth media M9 and Luria Bertani (LB) after 24 and 48h incubation as measured by crystal violet staining using fixed and unfixed biofilms. Data are expressed as the mean ± standard deviation from three biological replicates. A statistically significant difference between †unfixed and methanol fixed biofilms, ˜ 24h and 48h,* M9 and LB medium (at one particular time or biofilms) tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

3.3.2.2. Effects of incubation time and nutrient-rich medium on static biofilm formation. All 14 clinically relevant test strains (Table 3.3, page 110) were tested for their biofilm-forming capabilities in microplates during cultivation in three different nutrient-rich media, Brain Heart Infusion (BHI), Luria Bertani (LB), and Trypic Soy Broth (TSB), over 72h. The reference strain, *Staphylococcus epidermidis* ATCC 35984 formed good biofilms in LB medium after 48 and 72h ($OD_{595} \geq 1.2$) and in TSB but only after 72h (0.95 OD_{595nm}) whereas it did not form biofilms when grown in BHI medium (Fig 3.4a). Biofilm development of *S. epidermidis* ATCC 35984 in LB was therefore used as a reference against which to compare the biofilm-forming abilities of the other strains (Toledo *et al*., 2001). Biofilm formation was categorised as good (≥75%, $OD_{595} \geq 0.9$), moderate (25%-75%, OD_{595} 30 - 0.89), weak (<25%, $OD_{595} < 0.3$), or as no biofilm formation ($OD_{595} < 0$) depending on the percentage value of OD_{595} readings compared to *S. epidermidis* ATCC 35984 reading (Table 3.5, page 135).

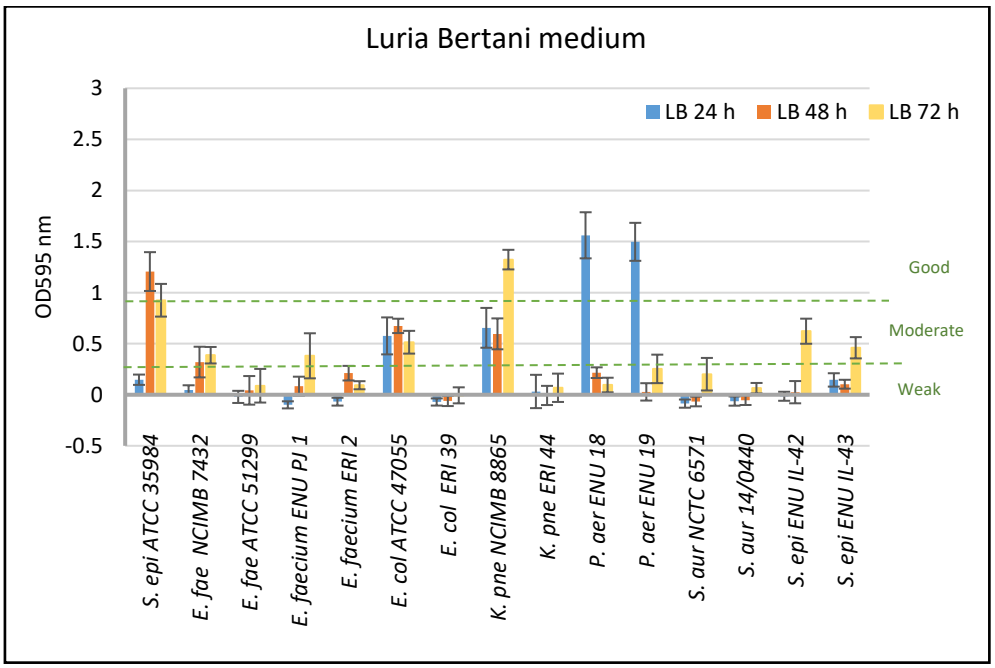
Significant variations ($p < 0.05$) in biofilm formation were observed amongst the test strains, depending on the growth medium and length of incubation (Table 3.5., page 135). In a BHI medium, most strains either formed weak biofilms or did not form them at all ($OD_{595} \leq 0.30$) after 72h except for *Klebsiella pneumoniae* NCIMB 8865*,* which produced a moderate biofilm ($p > 0.05$) at 24 and 48 hours (Fig. 3.4a). By comparison, LB and TSB supported better biofilm formation, with most strains exhibiting consistent growth patterns and weak to moderate biofilm development (Fig. 3.4 b,c). While the optimal medium for biofilm formation varied by strain, only three strains formed good biofilms in LB, whereas five formed good biofilms in TSB (≥75% biofilm formation compared to the reference strain *S. epidermidis* ATCC 35984).

One test strain, *Escherichia coli* ERI 39 did not form biofilms in any of the media, while several strains, including all *Enterococcus faecalis*, *E. faecium,* and *Staphylococcus aureus* strains, only formed weak biofilms (Table 3.5., page 125). These strains were therefore excluded from subsequent studies.

a)


Brain Heart Infusion medium
BHI 24 h
BHI 48 h
BHI 72 h
OD595 nm
3
2.5
2
1.5
1
0.5
0
-0.5
Good
Moderate
Weak
S. epi ATCC 35984
E. fae NCIMB 7432
E. fae ATCC 51299
E. faecium ENU PJ 1
E. faecium ERI 2
E. col ATCC 47055
E. col ERI 39
K. pne NCIMB 8865
K. pne ERI 44
P. aer ENU 18
P. aer ENU 19
S. aur NCTC 6571
S. aur 14/0440
S. epi ENU IL-42
S. epi ENU IL-43

b)


Luria Bertani medium
LB 24 h
LB 48 h
LB 72 h
OD595 nm
3
2.5
2
1.5
1
0.5
0
-0.5
Good
Moderate
Weak
S. epi ATCC 35984
E. fae NCIMB 7432
E. fae ATCC 51299
E. faecium ENU PJ 1
E. faecium ERI 2
E. col ATCC 47055
E. col ERI 39
K. pne NCIMB 8865
K. pne ERI 44
P. aer ENU 18
P. aer ENU 19
S. aur NCTC 6571
S. aur 14/0440
S. epi ENU IL-42
S. epi ENU IL-43

c)

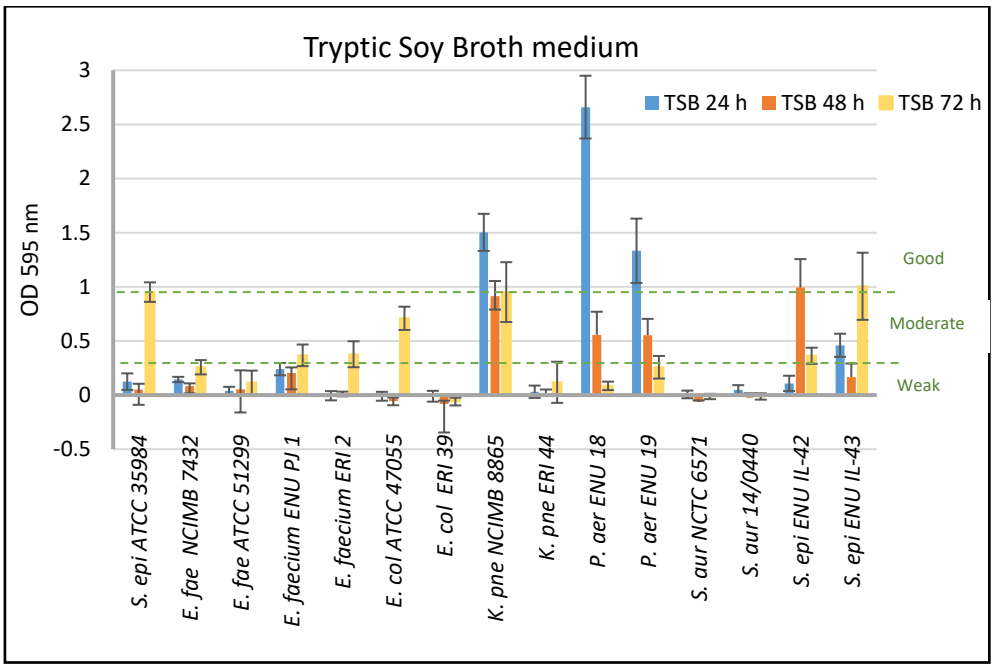


Fig. 3.4. Biofilm formation by clinically relevant bacterial strains in a) Brain Heart Infusion, b) Luria Bertani, and c) Tryptic Soy Broth over 24, 48, and 72h incubation in 96-well microtitre plates after staining with crystal violet (OD_{595} nm). Biofilm formation was compared with the maximum biofilm formed by *S. epidermidis* ATCC 35984 (biofilm formation control) in Luria Bertani medium at 48h (OD_{595nm} 1.2). Category cut-offs; green dash line (--). Categories of biofilm formation; Good biofilm formation: >75% of positive control (OD_{595} ≥0.9); Moderate biofilm formation: 75% - 25% biomass of positive control (OD_{595} 0.30 - 0.89); Weak biofilm formation: <25% biomass of positive control (OD_{595} <0.3); no biofilm formation (OD_{595} 0), Data are expressed as the mean ± standard deviation from three biological replicates.

Six strains were selected to assess the anti-biofilm effects of the crude biosurfactant extracts: *Klebsiella pneumoniae* NCIMB 8865*,* and *Pseudomonas aeruginosa* strains ENU18 and ENU 19*,* which formed good biofilms in LB and TSB (p>0.05), and *S. epidermidis* ENU IL-43 that formed good biofilms in TSB (OD_{595} 1.2)(Table 3.5). *Escherichia coli* ATCC 47055 (ESBL-sensitive), which displayed moderate biofilm formation in LB and TSB (OD_{595} 0.71, p>0.05)(Table 3.5) was chosen due to its classification as a 'critical' antibiotic-resistant bacterium on the WHO global priority pathogen list (World Health Organization, 2017). Finally, *S. epidermidis* ATCC 35984 was included as the reference strain for biofilm formation and one of clinical concern.

All biofilm strains were subsequently cultivated in TSB medium for 24h (*K. pneumoniae* NCIMB 8865*, P. aeruginosa* ENU18*, P. aeruginosa* ENU 19*)* or 72h (*S. epidermidis* ATCC 35984, and *S. epidermidis* ENU IL-43 *and E.coli* ATCC 47055) for maximum biofilm formation.

Table 3.5. Biofilm formation by clinically relevant test strains in different nutrient rich media during 24h, 48h, and 72h incubation, compared to the established biofilm-forming strain *S. epidermidis ATCC 35984*.

	LB			BHI			TSB		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72h
Enterococcus faecalis NCIMB 7432	W˜	M*	M*	W	W	W	W	W	W
Enterococcus faecalis ATCC 51299	-	W	W	-	W	-	W	W	W
Enterococcus faecium ENU PJ 1	-	-	M	W	W	W*	W	W	M˜
Enterococcus faecium ERI 2	-	-	W	-	W	W	-	W	M˜*
Escherichia coli ATCC 47055	M	M	M	-	-	W*	-	-	M
Escherichia coli ERI 39	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae NCIMB 8865	M	M	G˜	M	M	W˜*	G*	G*	G
Klebsiella pneumoniae ERI 44	W	-	W	-	-	W	W	-	W
Pseudomonas aeruginosa ENU 18	G˜	W	W	W*	W	W	G˜	M˜*	W˜
Pseudomonas aeruginosa ENU 19	G˜	W	W	W*	W	W	G˜	M˜*	W˜
Staphylococcus aureus NCTC 6571	-	-	W	-	-	-	W	-	-
Staphylococcus aureus 14/0440	-	-	W	-	-	W	W	-	-
Staphylococcus epidermidis ENU IL-42	-	W	M˜	-	W	W*	W˜	G˜*	M˜
Staphylococcus epidermidis ENU IL-43	W*	W	M˜*	M˜	W	W*	M˜	W˜	G˜*

Staphylococcus epidermidis ATCC 35984 was used as a positive biofilm-forming control forming good biofilms in LB medium at 48 and 72h ($OD_{595} \geq 1.2$) and used as a reference against which to compare the biofilm-forming abilities of the other strains. W- weak (<25%, $OD_{595} < 0.3$); M- moderate (25%-75%, OD_{595} 0.30 - 0.89); G- good (> 75%, $OD_{595} \geq 0.9$); -, no biofilm formation ($OD_{595} < 0$); LB- Luria Bertani; BHI- Brain Heart Infusion; TSB- Tryptic Soy broth. A statistically significant difference between ˜ 24h, 48h, and 72h,*LB, BHI, and TSB medium (at one particular time) were tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

3.3.3. Anti-biofilm effects of biosurfactant extracts on biofilm-forming bacteria determined by crystal violet (CV) staining.

The eleven crude extracts containing biosurfactants were tested for their anti-adhesion, biofilm inhibition, and biofilm disruption activities against the six selected biofilm-forming strains. *Rhodococcus ruber* IEGM 231^{T} was included as a reference strain known to reduce bacterial adhesion (Kuyukina *et al*., 2016) to assess the efficacy of the other 10 biosurfactant extracts. All extracts tested exhibited anti-biofilm activity in one or more assay types (anti-adhesion, inhibition, and disruption) but only against specific biofilm-forming strains (Appendix 7.7., page 281). Six crude extracts (from *R. cercidiphylii*, *R. erythropolis*, *R. percolatus*, *R. ruber*, *T. pulmonis*, and *W. muralis*) demonstrated all three anti-biofilm properties (anti-adhesion, inhibition, and disruption).

3.3.3.1. Anti-adhesion properties of biosurfactant extracts (CV). Crude biosurfactant extracts pre-coated on polystyrene flat-bottomed wells were tested for the ability to prevent or reduce bacterial attachment of biofilm-forming strains. Biofilm formation relative to the negative control biofilms formed without pre-coated biosurfactant was determined by staining with crystal violet (CV) staining. Six biosurfactant extracts reduced adhesion in one biofilm-forming strain (Fig. 3.5, $p>0.05$ biological replicates). The biosurfactant extract from *R. ruber* (BRrub) reduced the adhesion of *Klebsiella pneumonaie* NCIMB 8865 by 30.1 ± 3.4% and the extracts from *R. cercidiphylii* (BRcer) and *R. percolatus* (BRper) achieved similar effects ($p>0.05$). Only the extract from *Williamsia muralis* (BWmur) reduced adhesion by *Escherichia coli* ATCC 47055 (13.9%).

However, the biofilm-forming strains showed increased adhesion in the presence of one or more biosurfactant extracts compared to the control biofilms (Fig. 3.6., page 128).

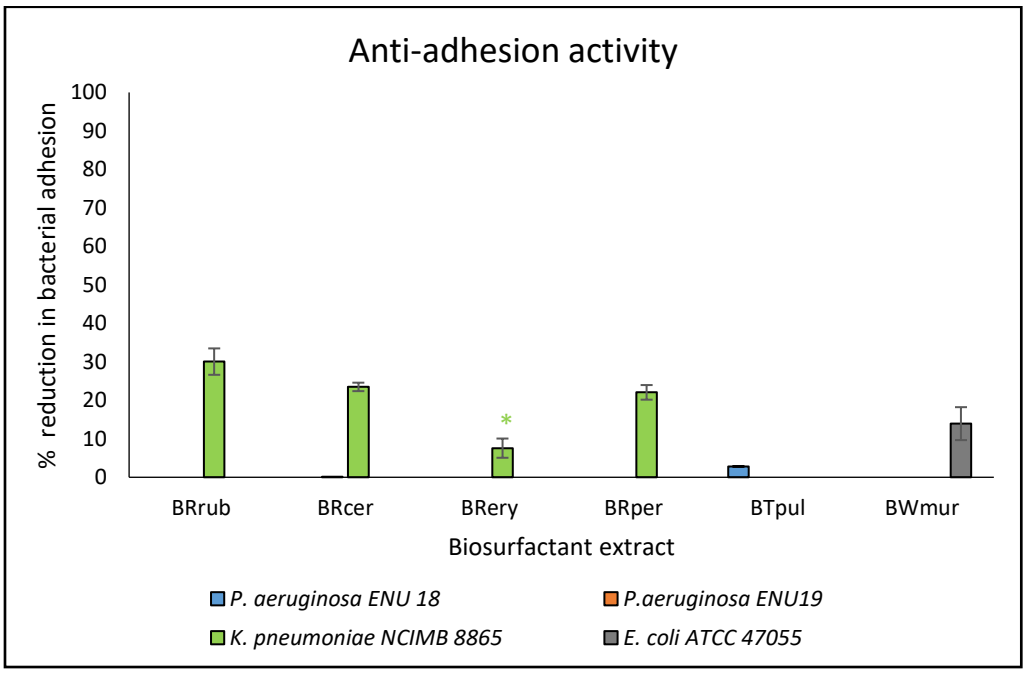


Fig. 3.5 Biosurfactant extracts from *Corynebacterineae* strains that reduced bacteria adhesion to pre-coated wells on polystyrene plates, measured by crystal violet staining and spectrophotometry (OD_{595nm}), with the percent reduction (%) calculated relative to the negative control (biofilm formation on an uncoated plate taken as 100%). *Rhodococcus ruber* IEGM 231 served as a reference strain. Data are expressed as mean ± standard deviation from three biological replicates.*Statistically significant difference between extracts with the highest anti-adhesion activity (BRrub, BRcer, BRper) and other extracts tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

This increase ranged from 1.4% above that of the control for *K. pneumoniae* NCIMB 8865 grown in the presence of the *Corynebacterium otitidis* biosurfactant extract (BCoti) to 93.2% for S*taphylococcus epidermidis ATCC 3598* with the BCoti extract. Biofilm formation for S*. epidermidis ATCC 3598* increased in the presence of 10 other crude biosurfactant extracts ranging from 2.8 - 54.6% above the control. Similar increases in biofilm formation were observed for other tested strains: *P. aeruginosa* ENU 18 (1.6% to 82.1%), *P. aeruginosa* ENU 19 (4.1% to 65.1%), *K. pneumoniae* NCIMB 8865 (4.1% to 52.3%), *E. coli* ATCC 47055 (17.4% to 75.2%), and *S. epidermidi*s ENU IL43 (9.6% to 79.0%). Only the biosurfactant extract from *Williamsia serinedens* (BWser) did not lead to a significant increase (i.e. to <3%) in cell adhesion by the biofilm-forming strains.

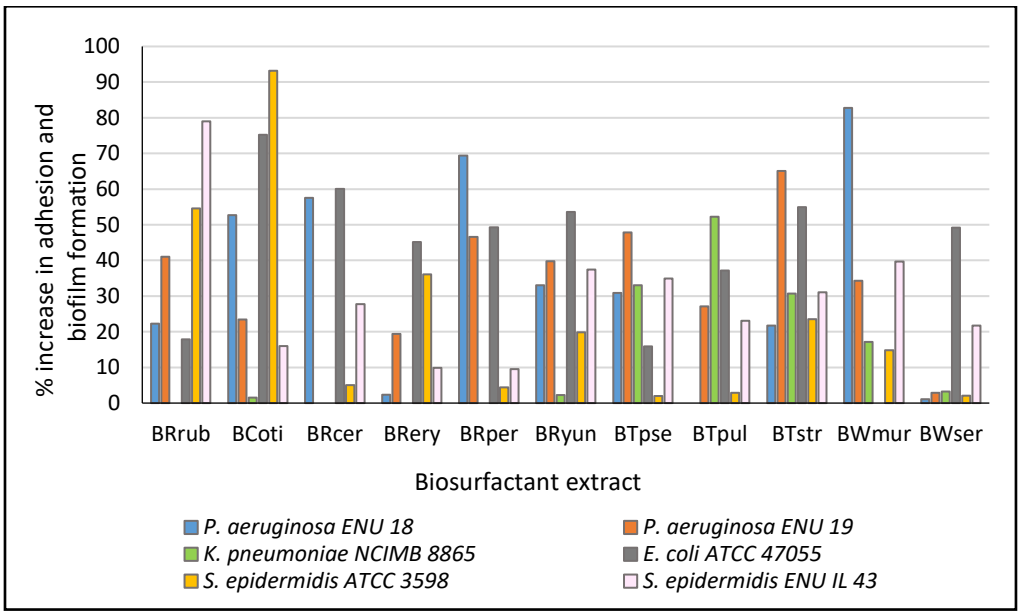


Fig 3.6. Percentage increase (%) in bacterial adhesion and biofilm formation on polystyrene plates pre-coated with biosurfactant extracts from *Corynebacterineae* strains, as determined using crystal violet staining and spectrophotometry (OD_{595nm}). The percent increase (%) in bacterial adhesion to the surface was calculated using the negative control (biofilm formation on the uncoated plate). *Rhodococcus ruber* IEGM 231 was used as a reference strain (known to reduce microbial adhesion on pre-coated polystyrene). Data expressed as the mean ± standard deviation from four technical replicates.

3.3.3.2. Biofilm inhibition properties of biosurfactant extracts (CV). Crude biosurfactant extracts were tested for the ability to inhibit the growth of biofilm-forming strains by adding both to 96-well polystyrene plates prior to incubation. Any inhibition of biofilm formation was determined by comparison with negative controls without the addition of biosurfactant extract after CV staining. All the crude biosurfactant extracts inhibited biofilm-formation by one or more of the clinically relevant test strains (Fig. 3.7, $p>0.05$ biological replicates). Inhibition was observed in five of the six biofilm-forming bacteria. All the extracts had some inhibitory effect on *K. pneumoniae* NCIMB 8865 biofilm formation (25- 50% inhibition) with the highest effects observed for *R. ruber* extract (BRrub), *R. erythropolis* (BRery), and *R. cercidiphylii* (BRcer) extracts, ($p>0.05$). *Escherichia coli* ATCC 47055 biofilm formation was inhibited by biosurfactant extracts from *Williamsia* strains (BWmur and BWser) and *C. otididis* (BCoti). *Pseudomonas aeruginosa* ENU 18 biofilm formation was only significantly inhibited by BRyun from *R. yunnanensis* (~27%) ($p<0.05$). No biofilm inhibition was observed for S. *epidermidi*s ENU IL43 (Fig. 3.7).

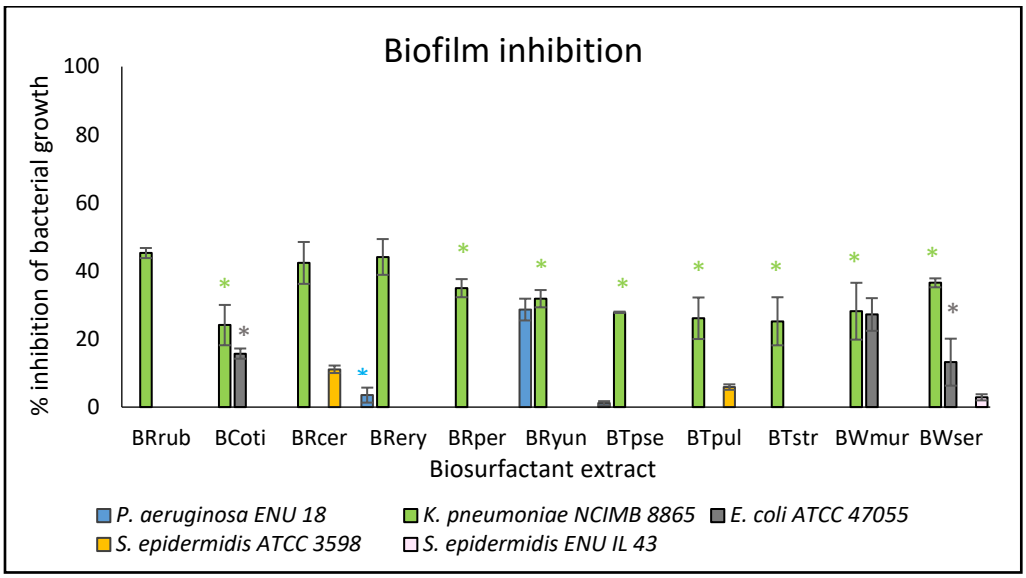


Fig. 3.7. Biosurfactant extracts from *Corynebacterineae* strains that inhibited bacterial biofilm growth on polystyrene plates, measured by crystal violet staining and spectrophotometry (OD595nm), with the percent inhibition (%) calculated relative to the negative control (biofilm formation in absence of biosurfactant extract taken as 100%). *Rhodococcus ruber* IEGM 231 was used as a positive control. Data expressed as the mean ± standard deviation from three biological replicates. *Statistically significant difference between extracts with the highest inhibition activity (* BRRub, BRcer, BRery against *K. pneumoniae*, * BWmur against *E.coli,* and *BRyun against *P. aeruginosa* ENU 18) and other extracts against one particular strain tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

An apparent increase in biofilm formation compared to untreated controls was observed for five of the six test strains exposed to one or more of the biosurfactant extract (Fig. 3.8). This increase varied and ranged from 1.8% observed for *Staphylococcus epidermidi*s ATCC 3598 grown with the *W. serinedens* extract (BWser) to 81.1% above that of the control for *P. aeruginosa* ENU 18 in the presence of the *R. cercidiphylii* extract (BRcer). Biofilm formation by *P. aeruginosa* ENU 18 increased in the presence of 7 biosurfactant extracts, ranging from 8.7 - 62.6% above the control. Similar increases were observed for *P. aeruginosa* ENU 19 (14.2 - 86.5%), *E. coli* (2.9 - 50.9%), S. *epidermidi*s ATCC 3598 (1.8- 60.3%), and of S. *epidermidi*s ENU IL43 (8.3-36.9%). No apparent stimulation of biofilm formation was observed for *K. pneumoniae* NCIMB 8865.

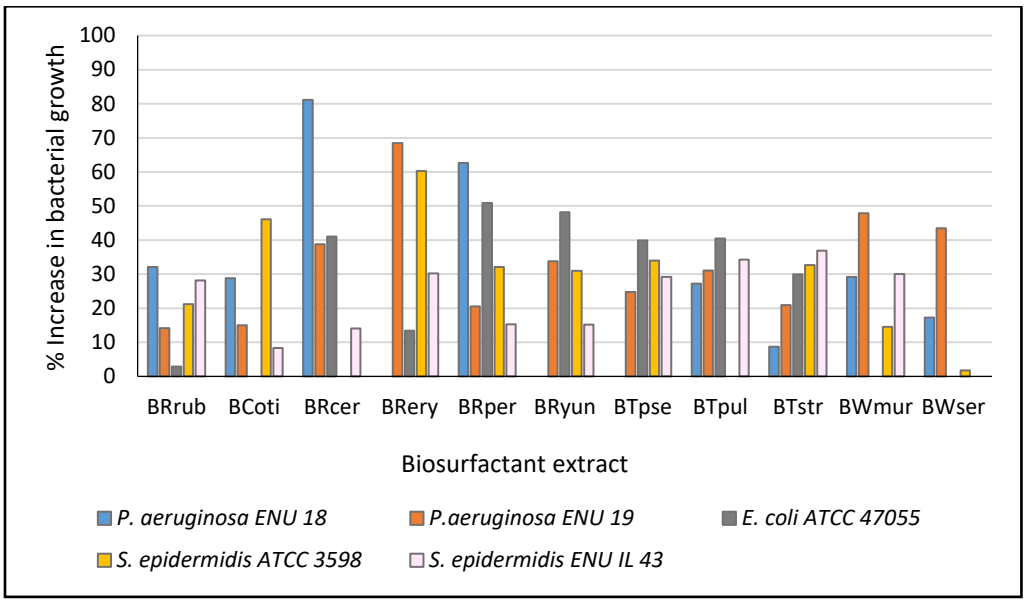


Fig 3.8. Percent increase (%) in biofilm formation on polystyrene plates in the presence of crude biosurfactant extract from *Corynebacterineae* strains, determined by crystal violet staining and spectrophotometry (OD_{595nm}). The percent increase (%) in biofilm formation compared to the control biofilms growing in the absence of biosurfactant extract. *Rhodococcus ruber* IEGM 231 was used as a positive control. Data expressed as mean ± standard deviation from four technical replicates.

3.3.3.3. Biofilm disruption properties of biosurfactant extracts (CV). Crude biosurfactant extracts were tested for the ability to disrupt pre-formed biofilms grown in 96-well polystyrene plates by comparison with untreated pre-formed biofilms after CV staining. All biosurfactant extracts disrupted established biofilms formed by one or more test strains (Fig. 3.9, $p>0.05$ biological replicates). Only, the *T. strandjordii* biosurfactant extract (BTstr) exhibited a degree of biofilm disruption against all strains (ranging from 11 - 44%, $p<0.05$). All crude biosurfactant extracts disrupted pre-formed *Pseudomonas aeruginosa* ENU 18 biofilms (7-53%) with the highest disruption observed with the *R. yunnanensis* (BRyun), *T. pseudospumae* (BTpse), and *W. muralis* (BWmur) extracts ($p>0.05$). *Escherichia coli* ATCC 47055 biofilms were disrupted the most (31.0 ± 0.1%) when treated with BRyun extract ($p<0.05$), while *Staphylococcus epidermidis* ENU IL43 biofilms were most disrupted (51.9 ± 0.7%) when treated with *R. erythropolis* extract (BRery). Biofilms of *Klebsiella pneumoniae* NCIMB 8865, *Pseudomonas aeruginosa* ENU 19, and *Staphylococcus epidermidis* ATCC 3598 were only disrupted by <25% when treated with the various biosurfactant extracts (Fig. 3.9).

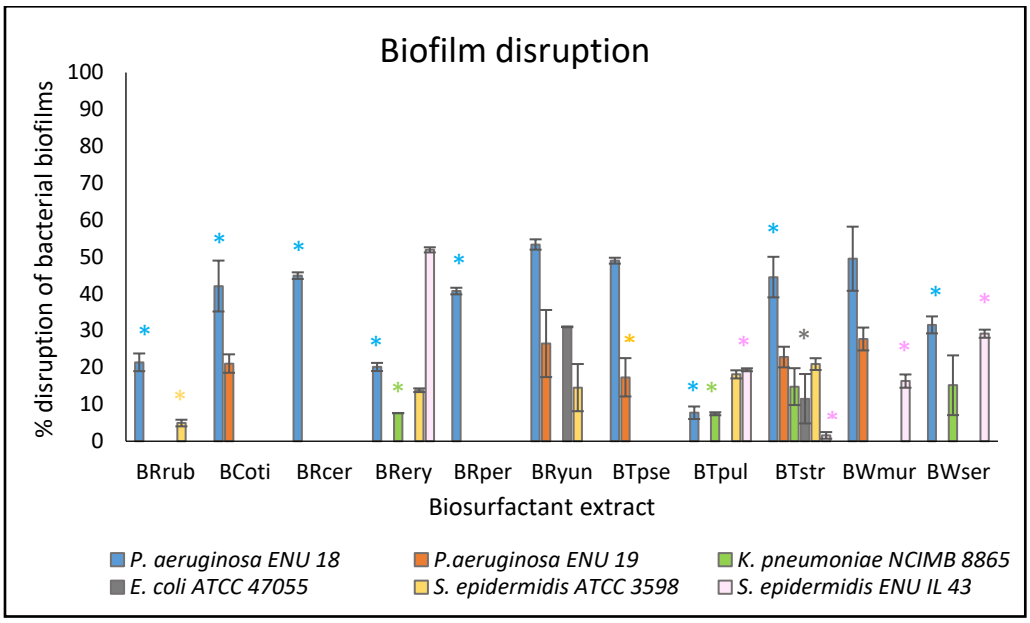


Fig. 3.9. Biosurfactant-mediated disruption of pre-formed bacterial biofilms by crude biosurfactant extracts from *Corynebacterineae* strains, measured by crystal violet staining and spectrophotometry (OD595nm) with the percent disruption (%) calculated relative to the control (untreated pre-formed biofilms taken as 100%). *Rhodococcus ruber* IEGM 231 was used as a positive control. Data expressed as the mean ± standard deviation from three biological replicates. *Statistically significant difference between highest disruption activity (**P. aeruginosa* ENU 18,* *P. aeruginosa* ENU 19, **K. pneumoniae*, * *E.coli*, * *S. epidermidis* ATCC 3598,* *S. epidermidis* ENU IL43) and other extracts against one particular strain tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

An increase in the size of pre-formed biofilms compared to untreated control biofilms was observed for five of six strains in the presence of one or more biosurfactant extract (Fig. 3.10). The increase ranged from 2.7% by *S. epidermidi*s ENU IL43 exposed to the *R. percolates* extract (BRper) to 78.6% by *E. coli* exposed to the *R. erythropolis e*xtract (BRery). For *E. coli* ATCC47055, the size of pre-formed biofilms increased by 1.8 - 72.8% after exposure to eight other biosurfactant extracts. Similarly, biofilms formed by *P. aeruginosa* ENU 19 increased by 10.6 - 39.2 %, *Klebsiella pneumonaie* by 5 - 43.5%, S. *epidermidi*s ATCC 3598 by 3.3 - 42.3%, and *S. epidermidi*s ENU IL43 by 2.7 – 62.7%. Biofilms formed by *P. aeruginosa* ENU 19 were not stimulated to grow further in the presence of crude biosurfactant extracts.

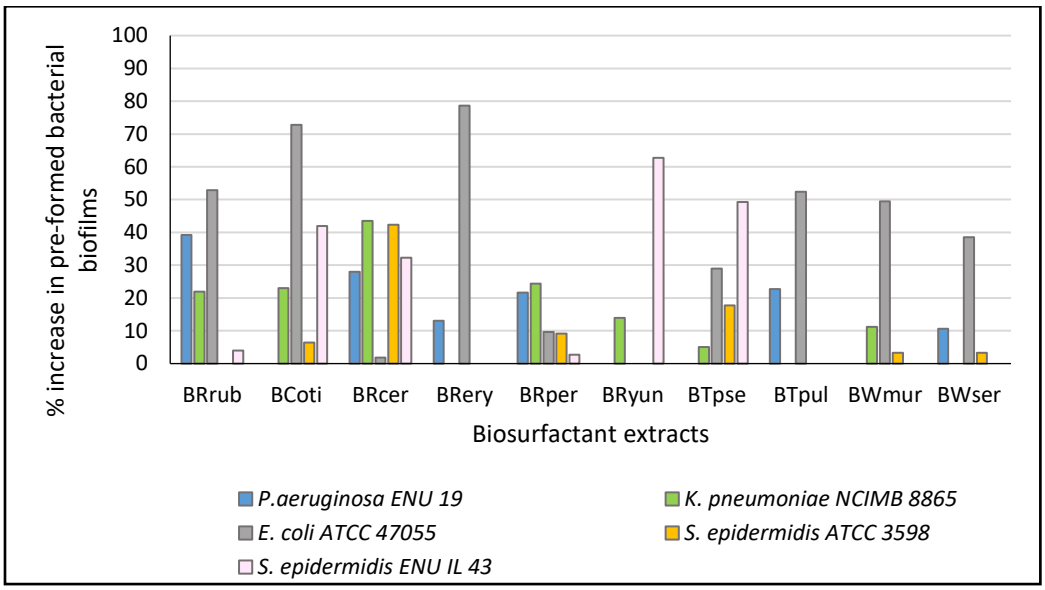
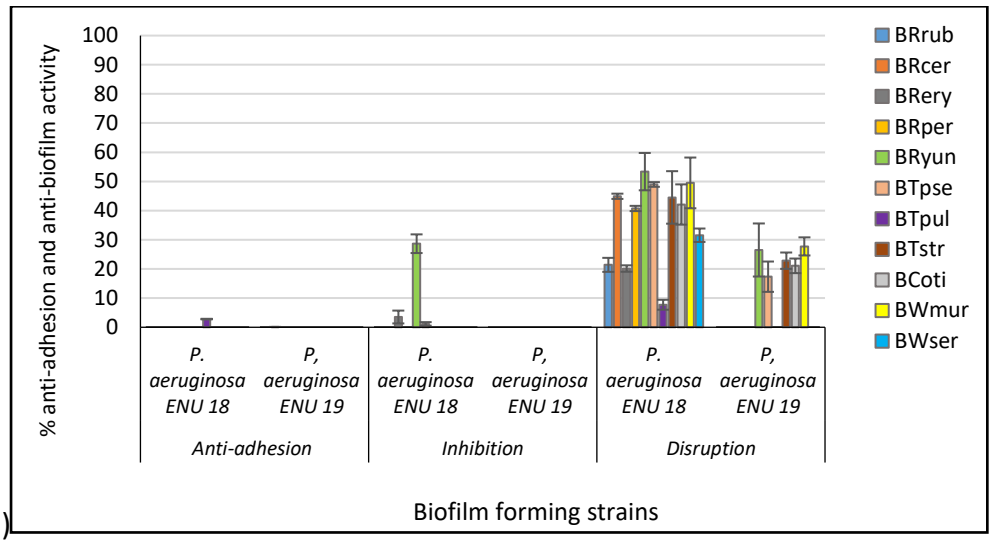


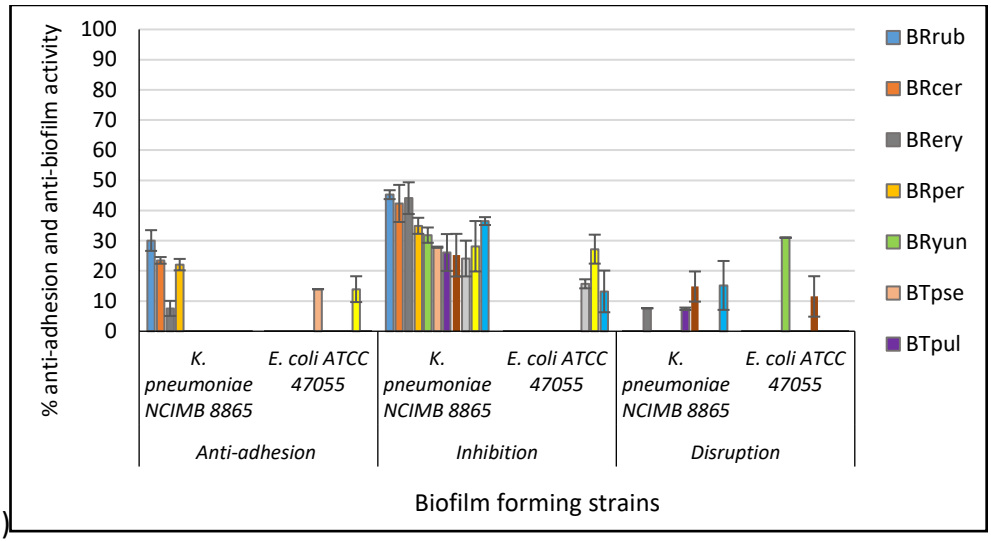
Fig 3.10. Percent increase (%) in pre-formed biofilms on polystyrene plates in the presence of crude biosurfactant extracts from *Corynebacterineae* strains, determined by crystal violet staining and spectrophotometry (OD_{595nm}). The percent increase (%) in biofilm formation compared to the control biofilms not exposed to crude biosurfactant extract Taken as 100%). *Rhodococcus ruber* IEGM 231 was used as a positive control. Data expressed as the mean ± standard deviation from four technical replicates.

3.3.3.4. Susceptibilities of the biofilm-forming strains to biosurfactant extracts.

Biofilm-forming strains showed varying susceptibility to the crude biosurfactant extracts, with considerable differences depending on the anti-biofilm assay applied *Pseudomonas aeruginosa* strains ENU 18 and ENU 19 exhibited reduced adherence to polystyrene wells with several biosurfactant extracts but biofilm inhibition and disruption were negligible for these strains (Fig. 3.11a). *Klebsiella pneumonaie* NCIMB 8865 was susceptible to all the biosurfactant extracts during biofilm formation (inhibition assays), but the effects were low in other assays (Fig. 3.11b). Moderate dispersal of pre-formed biofilms of reference strain *Staphylococcus epidermidis* ATCC 3589 and *S. epidermidis* ENU IL-43 was achieved in the presence of several biosurfactant extracts (Fig. 3.11c). However, these strains were resistant to anti-adhesion and biofilm inhibition. *Escherichia coli* ATCC 47055 showed minor susceptibility to a few extracts across all three assays, with low percentages of inhibition.



a)



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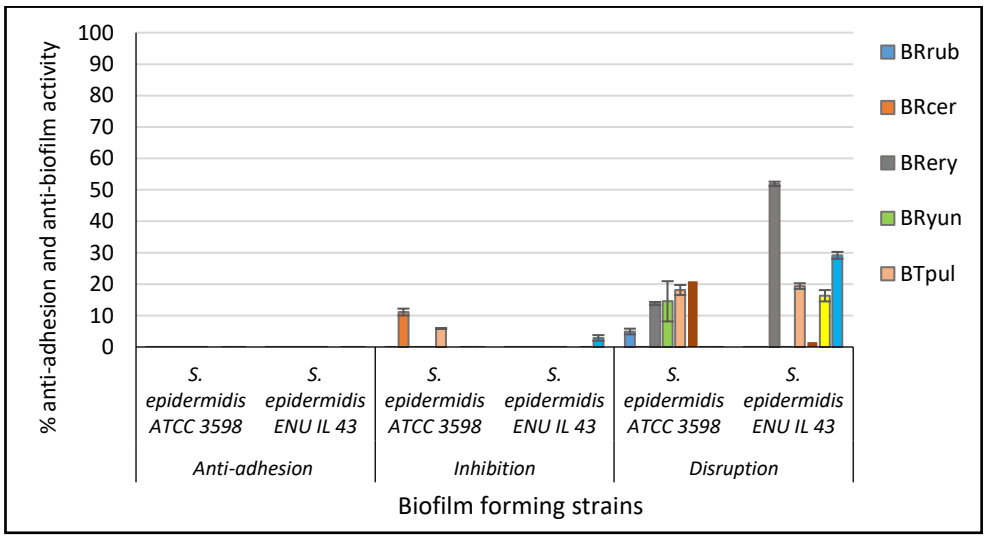


c)

Fig. 3.11. Anti-adhesion, inhibition, and disruption of a) *Pseudomonas aeruginosa* strains ENU 18 and ENU 19, b) *Klebsiella pneumonie* NCIMB 8865 and *Escherichia* coli ATCC 47055, and c) *Staphylococcus epidermidis* strains ATCC 3598 and ENU IL 43 by crude actinobacterial biosurfactant extracts measured using crystal violet staining and spectrophotometry(OD_{595nm}). *Rhodcoccus ruber* IEGM 231 was used as a reference strain known for anti-adhesion and anti-biofilm properties. Data expressed as the mean ± standard deviation from three biological replicates.

3.3.4. Anti-biofilm effects of crude biosurfactant extracts determined using 2,3,5-Triphenyl Tetrazolium Chloride (TTC) dye.

Whilst the crude biosurfactant extracts demonstrated anti-adhesion and anti-biofilm properties against several bacteria, the crystal violet (CV) assay results also indicated potential stimulation of biofilm growth. To address this, selected crude extracts were tested for their anti-biofilm properties using TTC dye, which specifically measures metabolically active cells. This was done to determine whether the observed increase in biofilm formation was due to an artifact of the CV stain.

3.3.4.1. Optimisation of the TTC assay to quantify biofilms. Prior to conducting the anti-biofilm assays, optimisation studies were carried out to assess the effects of parameters reported to influence biofilm formation and measurement: strain, and starting inoculum size (0.1 - 0.8 OD; Kim *et al*., 2009); TTC concentration (0.05, 0.1, and 0.2%, w/v; Sabaeifard *et al*., 2014; Kowalczuk, Ginalska *et al.,* 2012; Haney *et al*., 2021; Klancniketal *et al.,* 2014) and spectrophotometric wavelength (450nm and 490nm; Sabaeifard *et al*., 2014). In each case, the quantification of active cells within biofilms by the TTC assay was compared with indirect biofilm measurements obtained by crystal violet (CV; at 595 nm wavelength).

Generally, *K. pneumoniae* formed better biofilms than *P. aeruginosa*, as determined by higher OD readings across all assay combinations (Figs. 3.12 and 3.13). Significantly higher biofilm measurements resulted from assays using 0.2% w/v than those using 0.05 or 0.1% w/v TTC ($p<0.05$) at most inoculum densities and this was most pronounced for *K. pneumoniae* biofilms (Fig. 3.12a). Starting inoculum size had no significant effect on *P. aeruginosa* biofilm development although variations were more obvious at 0.2% w/v TTC (Fig. 3.13a). However, significant differences were observed for *K. pneumoniae* biofilms formed using starting inocula of 0.1, 0.7 and 0.8 ($p>0.05$) (Fig. 3.12a). Biofilm measurements with TTC at 450 and 490nm wavelengths remained consistent, with the exception of *K. pneumoniae* biofilms with 0.2% w/v TTC, which showed higher values at 490nm ($p<0.05$) for all inoculum densities (Fig 3.12a).

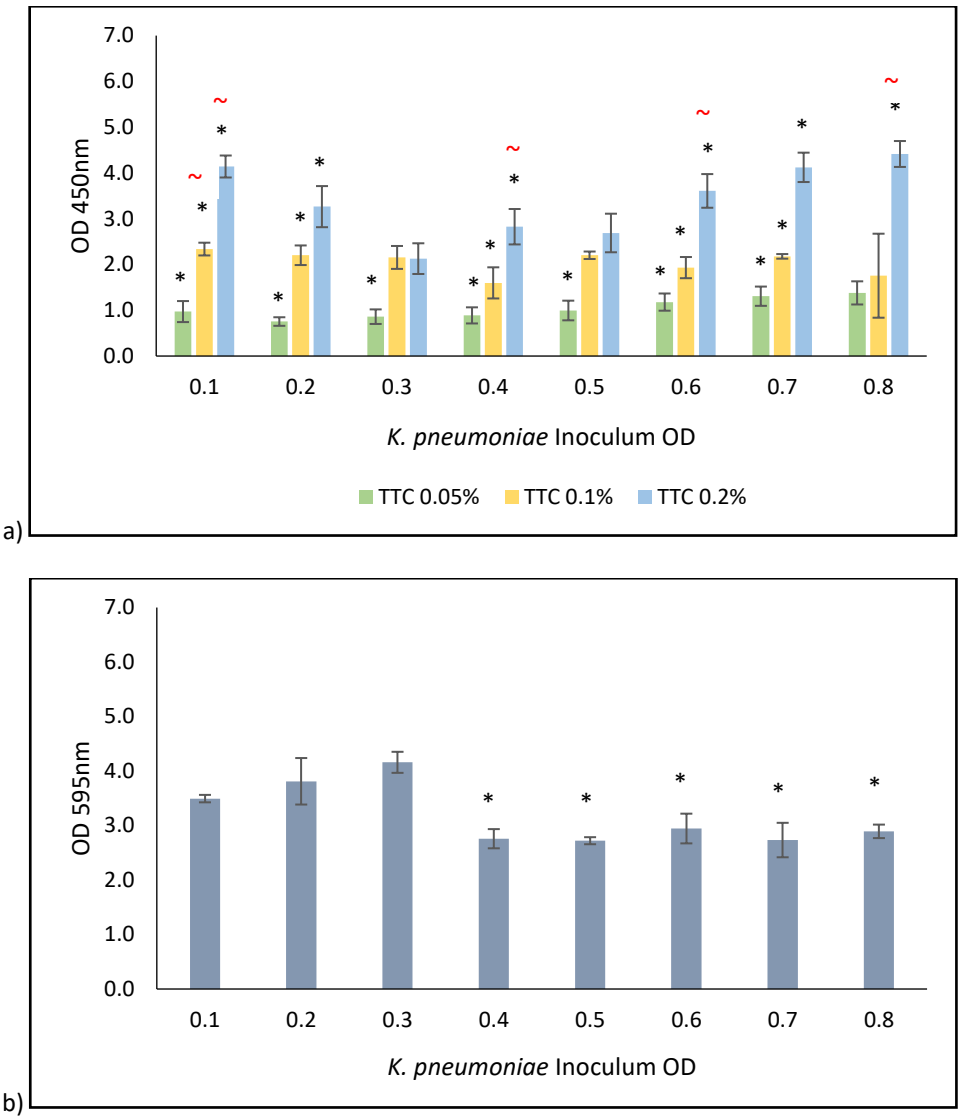
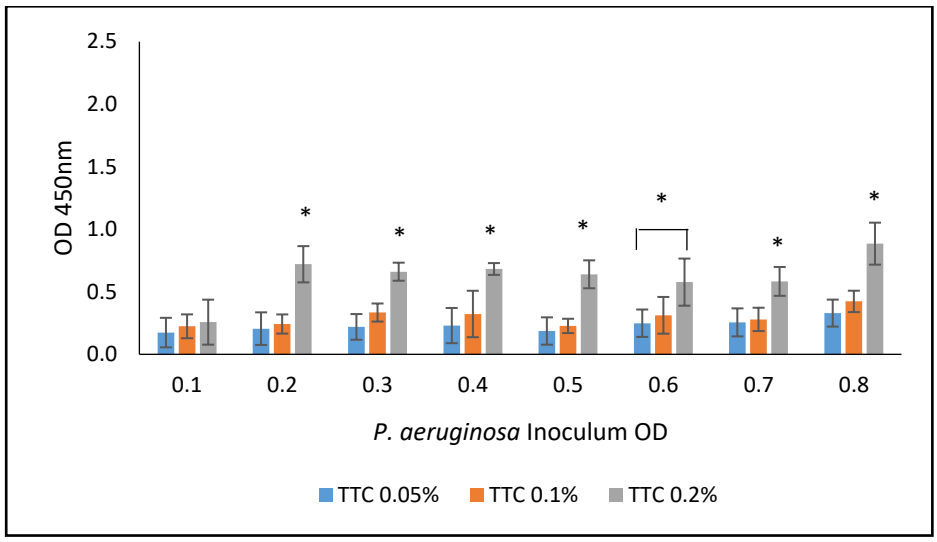


Fig. 3.12. Biofilm development by *Klebsiella pneumoniae* using varying starting inocula (OD_{595} 0.1-0.8) measured by a), cellular metabolic activity using TTC concentrations (0.05%, 0.1%, and 0.2% w/v) and spectrophotometric wavelengths 450nm and b) crystal violet (CV) staining measured at 595nm wavelength. Data represent the mean ± standard deviation of two biological replicates. One-way ANOVA with post-hoc Tukey HSD was used to calculate the statistical difference (*) between TTC concentrations at one particular inoculum OD and between larger biofilm formation than those arising from all other starting inocula measured using CV (*$p<0.05$.). ~ indicates a significant difference with TTC measurements at 490nm wavelength.



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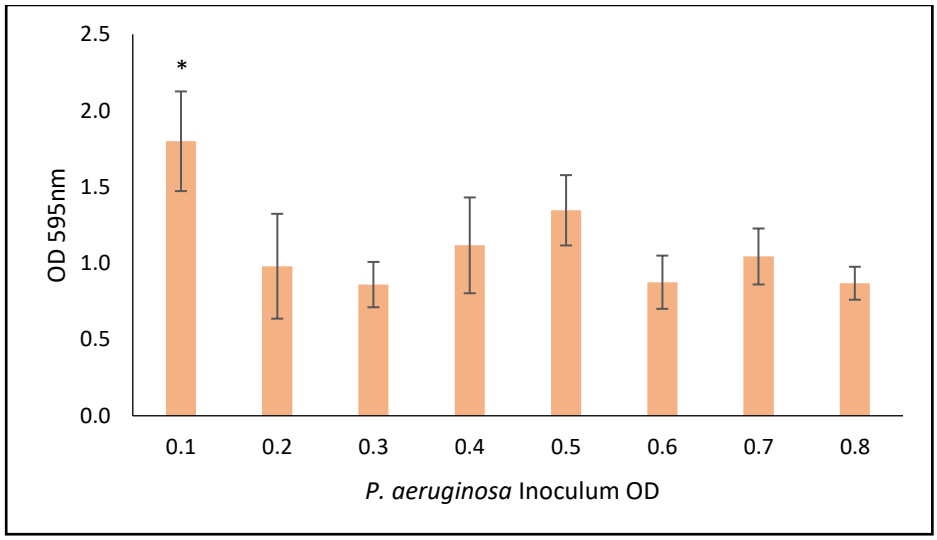


b)

Fig. 3.13. Biofilm development by *Pseudomonas aeruginosa* using varying starting inocula (OD_{595} 0.1-0.8) measured by a), cellular metabolic activity using TTC concentrations (0.05%, 0.1%, and 0.2% w/v) and spectrophotometric wavelengths 450nm (and 490nm) and b) crystal violet (CV) staining measured at 595nm wavelength. Data represent the mean ± standard deviation of two biological replicates. One-way ANOVA with post-hoc Tukey HSD was used to calculate the statistical difference (*) between TTC concentrations at one particular inoculum OD and between larger biofilm formation than those arising from all other starting inocula measured using CV (*$p<0.05$.)

More variation between *P. aeruginosa* biofilms formed with different inocula was observed with CV staining. Biofilms initiated with the lowest inoculum (0.1 OD) were larger ($p<0.05$) than those arising from all other starting inocula (Fig 3.13b). In contrast, significantly larger *K. pneumoniae* biofilms were observed with CV staining when using a starting inoculum of 0.3 OD ($p<0.05$) and biofilms initiated using starting inocula of 0.1 and 0.2 OD were significantly higher than those formed at OD 0.4 and above (Fig 3.12b). Biofilms formed at most inoculum sizes were significantly larger when measured using 0.2% w/v TTC compared with CV dye ($p<0.05$) although measurements were at different wavelengths.

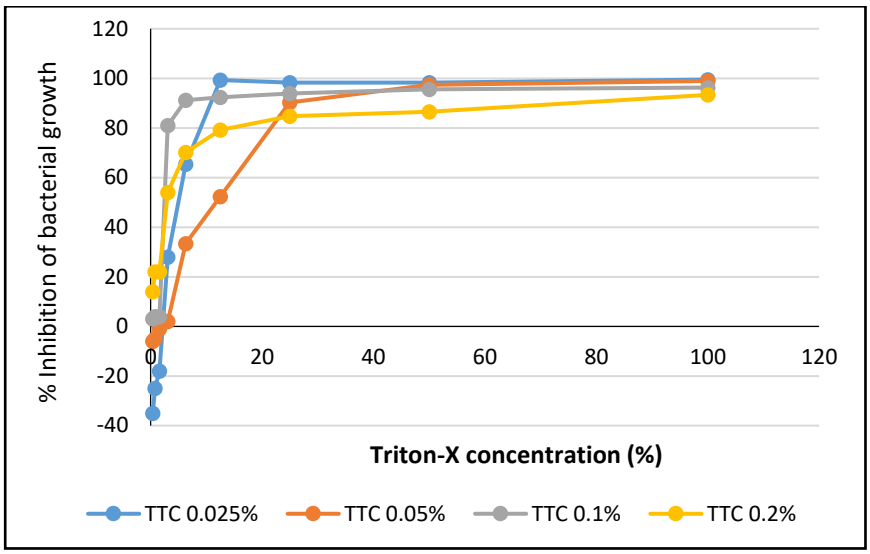
3.3.4.1.1. Effects of TTC concentration on cell viability measurement in biofilms inhibition assays using commercial anionic surfactants Triton X-100 and Tween 80.

Biofilm inhibition assays were conducted on *P. aeruginosa* ENU 18 using the surfactant detergents Triton X-100 and Tween 80 at a range of concentrations and the effect of TTC concentration on biofilm measurement was examined using 450nm wavelength readings.

The inhibition of *P. aeruginosa* displayed a typical sigmoidal dose-response curve with Triton X-100, but variations in the dose-response range were noted across assays with different TTC concentrations (Fig. 3.14). Consequently, the calculated median EC_{50} values varied with different TTC concentrations (Table 3.6., page 139). The median EC_{50} values determined at 0.1 and 0.2% w/v TTC (2.11, 3.34% v/v) were not significantly different, whereas they were higher at 0.05% TTC and at the lowest (0.025% w/v), the median EC_{50} could not be confidently estimated, as the fiducial limits were not determined.

Tween 80 displayed lower biofilm inhibition, with similar dose-response patterns (Fig. 3.14) and consistent EC_{50} values (0.36, 0.21%) (Table 3.6) observed at 0.05 and 0.1% w/v TTC. Minimal inhibition was observed at the lowest TTC concentration (0.025% w/v) and the EC_{50} value could not be determined. At the highest TTC concentration (0.2% w/v) biofilm inhibition was only detected at Tween 80 concentrations ≥1.6% v/v, the EC_{50} was much higher (34.38%) and no confidence could be placed in this (large fiducial limits).

a)



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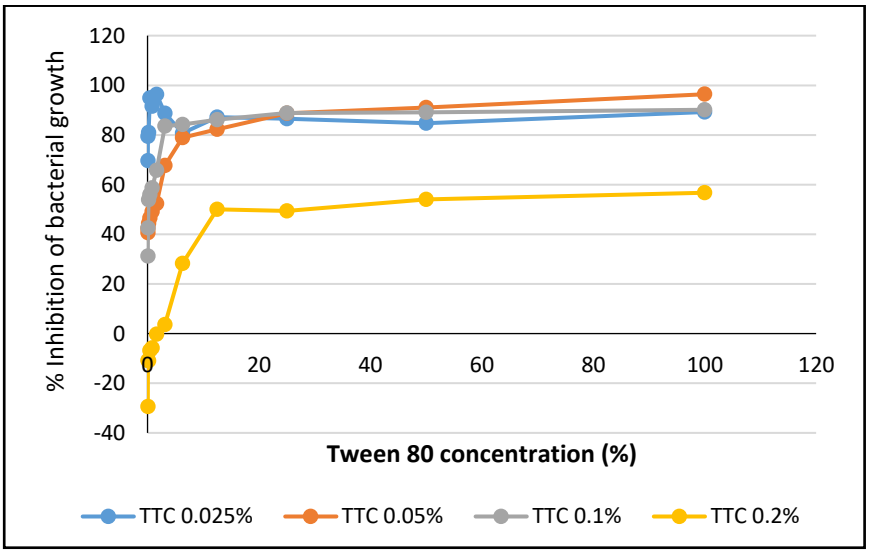


Fig. 3.14 Dose-response curves for inhibition of *P. aeruginosa* ENU 18 biofilm growth under static conditions in the presence of surfactants: a) Triton X-100 and b) Tween 80, at various concentrations (v/v), determined using TTC dye at different concentrations (% w/v) and spectrophotometric measurement at 450nm wavelength. Data expressed as the mean ± standard deviation of two biological replicates.

Table 3.6. Median EC_{50} (half effective concentration) values for surfactants Triton X-100 and Tween 80 in *P. aeruginosa* ENU 18 biofilm inhibition assays using 2,3,5-triphenyl tetrazolium chloride (TTC) viability stain applied at different concentrations (0.025 – 0.2% w/v) and measured at 450 nm wavelength.

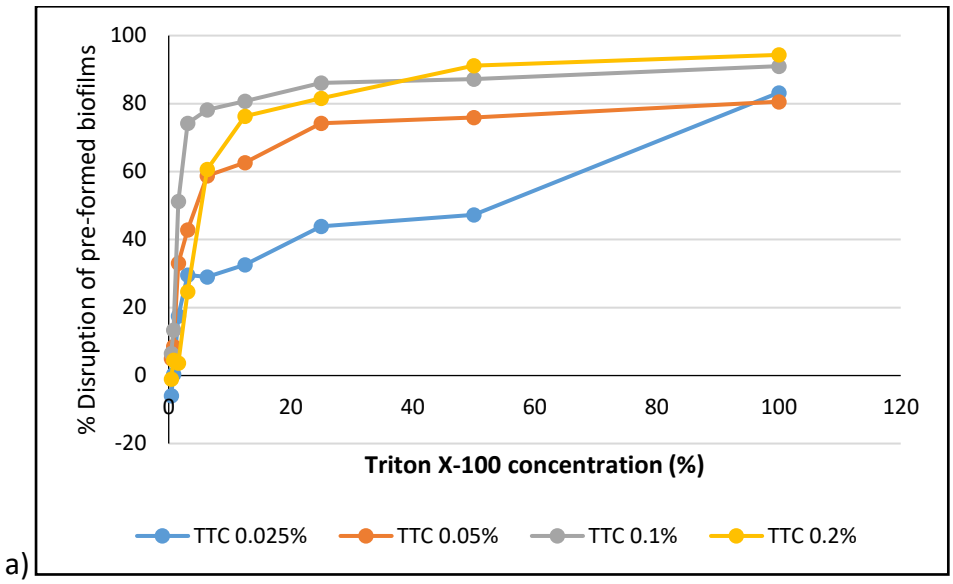
	Biocide EC_{50} (% v/v)			
	TTC (% w/v) used for biofilm measurement			
	0.025	0.05	0.1	0.2
Triton X-100	**6.24** (ND)	**10.66** (9.58/11.87)	**2.11** (0.77/4.49)	**3.34** (2.74/4.05)
Tween 80	**ND** (ND)	**0.36** (0.19/0.59)	**0.21** (0.13/0.30)	**34.38** (21.06/68.22)

EC_{50} -Surfactant Median Inhibition concentration; ND, not determinable; OD, optical density; 0.95 fiducial limits in brackets.

3.3.4.1.2. Effects of TTC concentration on cell viability measurement in biofilms disruption assays using commercial anionic surfactants Triton X-100 and Tween 80.

The effects of TTC concentration were also observed in biofilm disruption assays conducted on *P. aeruginosa* ENU 18 using Triton X-100 and Tween 80 at known concentrations. Again, 0.1% w/v TTC was identified as optimal for biofilm disruption experiments. No significant differences were observed when comparing biofilm quantification at 450nm and 490nm (data not shown) and 450nm was used for further studies. Consequently, this concentration was adopted as the standard for all subsequent anti-biofilm assays involving the crude biosurfactant extracts.

Disruption of established *P. aeruginosa* biofilms again followed a typical sigmoidal dose-response curve with Triton X-100. Variations in the dose-response were observed when biofilms were measured using different TTC concentrations (Fig. 3.15). Triton X-100 median EC_{50} values were similar when determined at 0.05, 0.1%, and 0.2% w/v TTC (6.89, 2.53%, 7.10% v/v). The EC_{50} value obtained using the lowest TTC concentration (0.025% w/v) was significantly higher (26.22% v/v) (Table 3.7, page 141), consistent with the findings in the Tween 80 inhibition assay.



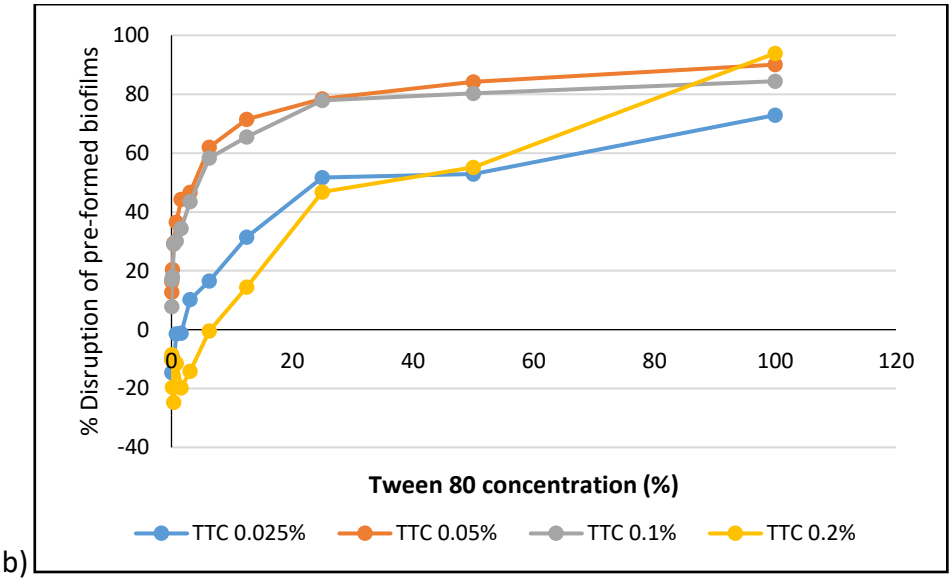


Fig. 3.15 Dose-response curves for disruption of *P. aeruginosa* ENU 18 biofilm growth under static conditions in the presence of surfactants: a) Triton X-100 and b) Tween 80, at various concentrations (v/v), determined using TTC dye at different concentrations (% w/v) and spectrophotometric measurement at 450nm wavelength. Data expressed as the mean ± standard deviation of two biological replicates.

Tween 80 achieved greater disruption of established *P. aeruginosa* biofilms with consistent dose-response patterns observed for assays using 0.05 and 0.1% w/v TTC. As a full range of responses were observed with the detergent concentrations applied, confidence could be placed in the Tween-80 EC_{50} values using 0.05 and 0.1% w/v TTC (0.79, 3.49% v/v). At the lowest and highest TTC concentrations (0.025% and 0.2% w/v), similar dose-response patterns were observed, indicating complete biofilm disruption at ≥1.6% and 6.3% v/v Tween 80, respectively, resulting in significantly higher EC_{50} values (28.98, 33.40% v/v).

Table 3.7. Median EC_{50} (half effective concentration) values for surfactants Triton X-100 and Tween 80 in *P. aeruginosa* ENU 18 biofilm disruption assays using 2,3,5-triphenyl tetrazolium chloride (TTC) viability stain applied at different concentrations (0.025 – 0.2% w/v) and measured at 450 nm wavelength.

	Biocide EC_{50} (% v/v)			
	TTC (% w/v) used for biofilm measurement			
	0.025	0.05	0.1	0.2
Triton X-100	**26.22** (14.61/61.57)	**6.89** (4.44/10.79)	**2.53** (1.13/4.73)	**7.10** (5.16/9.78)
Tween 80	**28.98** (14.13/91.24)	**0.79** (0.55/1.10)	**3.49** (2.72/4.52)	**33.40** (29.82/37.59)

EC_{50}-Surfactant Median Inhibition concentration; OD, optical density; `0.95 fiducial limits` in brackets.

3.3.4.2. Anti-adhesion and anti-biofilm effects of biosurfactant extracts against *P. aeruginosa* ENU 18 and *K. pneumoniae* NCIMB 8865 determined using the TTC assay.

Based on their performance in the anti-biofilm tests with crystal violet (CV), crude biosurfactant extracts from *Rhodococcus ruber* (BRrub), *R. yunnanensis* (BRyun), and *Williamsia muralis* (BWmur) underwent further testing using the TTC assay (0.1% w/v and 450nm λ) alongside CV staining for comparison. The tests were conducted on biofilm-forming strains *P. aeruginosa* ENU 18 and *K. pneumoniae* NCIMB 8865 as these were more susceptible than other biofilm-formers to biofilm disruption and biofilm inhibition, respectively.

Anti-adhesion activity of biosurfactant extracts using TTC. *Klebsiella pneumoniae* NCIMB 8865 exhibited a 45-55% reduction in adhesion to polystyrene wells, compared to untreated controls ($p < 0.05$), when measured by the TTC assay (Fig. 3.16). The CV assay showed significantly lower percent reductions in adhesion for all three biosurfactant extract coatings, and the *R. yunnanensis* extract (BRyun) had no anti-adhesion effects against this strain (Appendix 7.8, page 282).

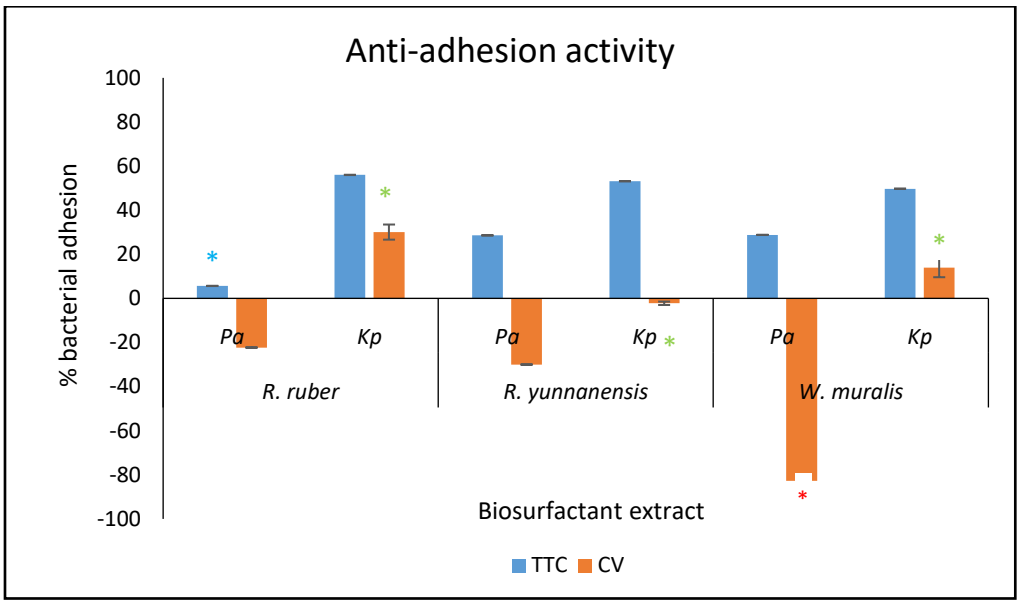


Fig. 3.16. The anti-adhesion effects of biosurfactant extracts against *P. aeruginosa* (Pa) and *K. pneumoniae* (Kp) when added to pre-formed biofilms in polystyrene microplates, quantified using TTC dye (2,3,5-triphenyl-tetrazolium chloride, 0.1% v/v) and spectrophotometry at 450nm and using CV (crystal violet) dye at 595nm. The percent (%) reduction in active cells in biofilms was calculated using untreated controls (biofilm formation on an uncoated plate) considered as 100% biofilm formation. Data are expressed as the mean ± standard deviation from three biological replicates. *Statistically significant difference between the anti-adhesion activity of biosurfactant extracts against a specific strain and measuring dye (* *Pa* TTC **Pa* CV **Kp* TTC **Kp* CV), tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

Adhesion of *P. aeruginosa* ENU 18 was significantly reduced compared to untreated controls when added to polystyrene wells pre-coated with the *R. yunnanensis* (BRyun) and *W. muralis* (BWmur) crude extracts, but not with the *R. ruber* extract (BRrub) ($p<0.05$), when measured by the TTC assay ($p < 0.05$). By comparison, no anti-adhesion activity ($p<0.05$) against *P. aeruginosa* was observed when assayed with CV dye and the measurements for all three extracts were higher than for untreated controls.

Biofilm inhibition activity of biosurfactant extracts using TTC. The three crude biosurfactant extracts exhibited strong biofilm inhibition against *K. pneumoniae* NCIMB 8865 when grown statically (76 - 84%) ($p<0.05$), using the TTC assay (Fig 3.17). These percent inhibition values were significantly higher, often more than twice as high, as those determined using the CV assay ($p<0.05$).

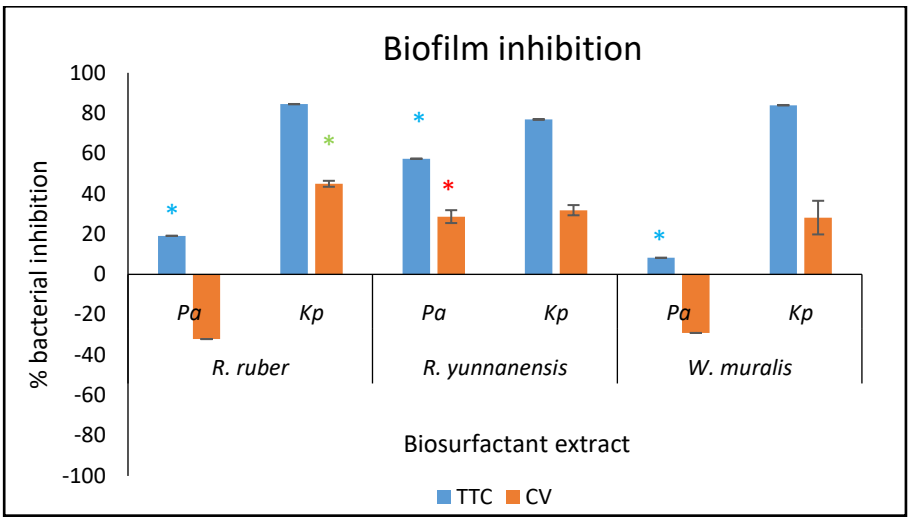


Fig. 3.17. The biofilm inhibition effects of three biosurfactant extracts from *Corynebacterineae* strains against *P. aeruginosa* (Pa) and *K. pneumoniae* (Kp) during growth in wells of polystyrene microplates, quantified using TTC dye (2,3,5-triphenyl-tetrazolium chloride, 0.1% v/v) and spectrophotometry at 450nm and CV (crystal violet) dye at 595nm. The percent (%) inhibition of biofilms was calculated using untreated controls (biofilm formation in the absence of biosurfactant extract) considered as 100% biofilm formation. Data are expressed as the mean ± standard deviation from three biological replicates. *Statistically significant difference between inhibition activity of biosurfactant extracts against a specific strain and measuring dye (* *Pa* TTC *Pa* CV *Kp* TTC *Kp* CV), tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

Inhibition of *P. aeruginosa* ENU 18 biofilms by the biosurfactant extracts was less pronounced (~29 - 57%) but remained significant compared to untreated controls (Appendix 7.8., page 282). Notably, BRyun exhibited higher inhibition against *P. aeruginosa* than BRrub and BWmur extracts ($p < 0.05$).

In CV assays, biofilm inhibition by the BRyun e xtract against *P. aeruginosa* ENU 18 was lower than in the TTC assay ($p<0.05$). No inhibition activity was observed for the BRrub and Bwmur extracts and instead, the measurements were higher than those of untreated controls (as indicated by negative inhibition values).

Biofilm disruption activity of biosurfactant extracts using TTC. Weak to negligible biofilm disruption was observed when pre-formed *K. pneumoniae* NCIMB 8865 and *P. aeruginosa* ENU 18 biofilms were treated with the three biosurfactants extract when measured by the TTC assay (Fig 3.18). Exposure to the BRyun extract resulted in the highest biofilm disruption in *Klebsiella pneumoniae* NCIMB 8865 (17.9 ± 0.08 %) and although this effect was weak, this was significantly higher than for the other extracts ($p<0.05$).

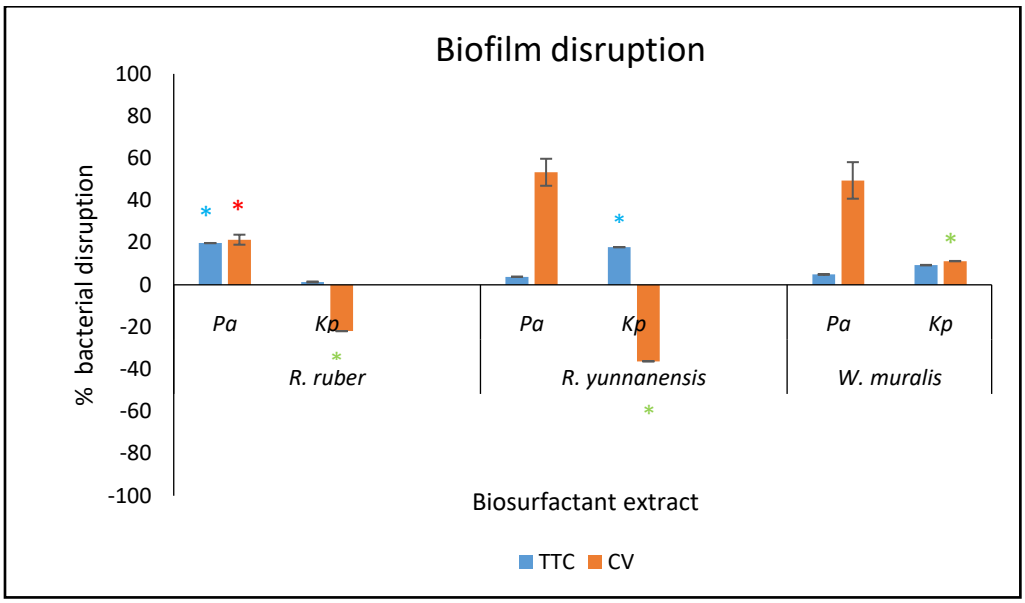


Fig. 3.18. The biofilm disruption effects of three biosurfactant extracts from *Corynebacterineae* strains on *P. aeruginosa* (Pa) and *K. pneumoniae* (Kp) when added to pre-formed biofilms in polystyrene microplates, quantified using TTC dye (2,3,5-triphenyl-tetrazolium chloride, 0.1% v/v) and spectrophotometry at 450nm and CV (crystal violet) dye at 595nm. The percent (%) disruption of biofilms was calculated using the untreated controls (untreated pre-formed biofilms) considered as intact (100%) biofilms. Data are expressed as the mean ± standard deviation from three biological replicates. *Statistically significant difference between disruption activity of biosurfactant extracts against a specific strain and measuring dye (* *Pa* TTC *Pa* CV *Kp* TTC *Kp* CV), tested by one-way ANOVA (Analysis of variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) Test ($p < 0.05$).

The CV assay gave measurements for the BRrub and BRyun extracts higher than those of untreated controls as indicated by negative disruption values (Appendix 7.8., page 282). *Klebsiella pneumoniae* disruption caused by the BWmur extract did not significantly differ when measured by the TTC and CV assays.

3.4. Discussion

Many mycolic-acid-producing actinobacteria produce biosurfactants but few studies have considered their anti-biofilm properties, and none have focused exclusively on extracellular biosurfactants (Dalili *et al.,* 2015; Janek *et al* 2018., Kuyukina *et al*., 2016; Pirog *et al*., 2014). This study focussed on the recovery of extracellular biosurfactant extract from eleven actinobacteria and testing the anti-biofilm properties against six clinically important bacteria using anti-adherence, biofilm inhibition, and biofilm disruption assays with a crystal violet staining method. Subsequently, TTC assay was used to confirm the promising anti-biofilm activities of three selected biosurfactant extracts.

3.4.1. Recovery and characterisation of extracellular biosurfactant extracts.

Downstream recovery of products from bioprocesses is a major consideration in full-scale commercial production of bioactive compounds. In the case of biosurfactant recovery, this can be technically challenging, time-consuming, and expensive, and result in low yields thereby prohibiting commercial application (Cappelletti *et al*., 2020, Dolman et al., 2019; Mukherjee *et al*., 2006). This limitation should be considered when searching for biosurfactants for potential commercial applications. Extracellular biosurfactants are more readily recovered from growth medium than cell-bound biosurfactants, therefore in the present study, only extracellular biosurfactant extracts were investigated. These were readily extracted from cell-free supernatants (CFS) using a solvent extraction method (chloroform-methanol 2:1 v/v) after separation from biomass grown on hexadecane for 14 days. This is considered an efficient method for extraction as it removes protein and helps in the concentration as well as purification and is an established method for recovery of biosurfactants from mycolic acid-containing actinobacteria (Varjani & Upasani, 2016).

A reasonable quantity of extracellular extracts possibly containing biosurfactants (~0.4 and 0.6 g L^{-1}) were recovered for eleven actinobacteria strains. These extracellular extracts were not pure samples or not only glycolipid complexes and may contain other biosurfactant types or compounds

hence, a direct comparison with other studies cannot be made to compare the yield of biosurfactants. However, other studies reported yields of biosurfactants from related strains grown on various hydrophobic substrates. Ciapina *et al.* (2006) recovered 0.1 g L^{-1} biosurfactant from *Rhodococcus erythropolis* after 7 days of growth on hexadecane as the sole carbon source. Jackisch-Matsuura *et al.* (2014) recovered 0.53 g L^{-1} of biosurfactant from *Gordonia amicalis* after 3 days of growth on 1% diesel while Vyas & Dave (2011) recovered 0.4 g L^{-1} of glycolipid biosurfactants from *Nocardia otitidiscaviarum* grown on crude oil and Kugler *et al.* (2014) extracted 1.28 g L^{-1} of glycolipid biosurfactant from *Tsukamurella* strains after 4 days growth on sunflower oil. The presence of surface-active compounds in the extracts was confirmed by the oil spreading assay and measurement of surface tension (ST) reduction revealed lower values (<30mN/m) than those recorded for cell-free supernatants (40-50 mN/m). This indicates that the extraction process enhances the purity and increases the concentration of extra-cellular biosurfactants. A similar observation was made by El-Sheshtawy & Doheim (2014) where cell-free supernatant reduced ST to 46mN/m while the crude biosurfactant extract reduced the ST further to <32 MN/m.

In addition to the biological properties of the biosurfactant-producing strains, various other factors including carbon source, cultivation conditions, length of incubation will affect the quantity, chemical structure, and physiochemical properties of the biosurfactant produced (Rahman & Gakpe, 2008; Velioglu & Urek, 2016; Roy, 2018). In the present study, hexadecane was found to be a suitable carbon source for biosurfactant production. This substrate has been used effectively to study biosurfactant production by various mycolic acid-containing actinobacteria, notably *Rhodococcus* spp. (Ciapina *et al*. 2006; Franzetti *et al*., 2010; Philp *et al*., 2002). However, the non-homogenous nature of growth that results from using a bi-phasic medium can be problematic not only for screening assays and quantification for growth but also for biosurfactant recovery (Nurfarahin *et al*., 2018; Zambry *et al*., 2017). In this study, all strains, regardless of the rate of growth, were cultivated for 14 days prior to biosurfactant extraction. This incubation time was selected based on

the results of various screening assays employed in Chapter 2 which revealed positive scores for CFS samples at this time point. However, the period of incubation required to achieve maximum quantities of extracellular biosurfactant may differ between strains depending on growth rate and whether the biosurfactant production is growth associated or not. Whilst, this standardised incubation time worked well for this study, any scale-up of production would require optimisation for each strain.

The extra-cellular biosurfactant extracts recovered from the actinobacteria potentially contain a mixture of biosurfactant types. However, as actinobacteria are known to produce predominantly glycolipid biosurfactants (Kugler *et al*., 2015) this study focused on the recovery of these compounds. The range of R_f values determined for the glycolipid spots detected may indicate the presence of various glycolipid types amongst the test strains. Indeed, glycolipid complexes were detected in all the biosurfactant extracts using thin layer chromatography and comparison with a standard glycolipid (R90 rhamnolipid) indicated that several strains may be producing extra-cellular rhamnose-containing glycolipids. Other glycolipids biosurfactant standards were not easily available to purchase, which would have resulted in detecting different types of glycolipids in the extract.

Rhamnose-containing biosurfactants have previously only been detected in a handful of mycolic acid-containing actinobacteria including *Rhodococcus fascians* strain A-3 (Gesheva *et al*., 2010) and *Nocardia otitidiscaviarum* MTCC 647 (Vyas and Dave, 2011). Hence, the mycolic acid-containing group may be a promising source of rhamnolipids apart from more commonly associated *Pseudomonas* and *Burkholderia* species (Abdel-Mawgoud *et al*., 2010).

In this study, reagents specific for glycolipids, , namely orcinol (0.1% in 5% sulphuric acid; Patil & Pratap, 2018) and p-anisaldehyde (acetic acid: p-anisaldehyde: sulphuric acid (100:1:2 v/v; Kugler *et al*., 2014), were tested in combination with 4 solvent systems (Chloroform: methanol- 2:1 v/v, chloroform–methanol-water - 65:25:4 v/v/v, chloroform–methanol-water - 85:15:2 v/v/v and chloroform-methanol-acetic acid - 65:15:2 v/v/v, commonly

reported to separate biosurfactant mixtures from mycolate actinobacteria. The retention factor (R_f) and appearance of reference rhamnolipid spots on TLC plates were affected by the solvent system and reagent applied. The Orcinol reagent was the most reliable as it detected the rhamnolipid with all solvent systems tested. The chloroform–methanol-water (65:25:4 v/v/v) was found to be a suitable solvent system for separating glycolipids from the actinobacterial test strains by thin layer chromatography (TLC) as other combinations did not clearly resolve the rhamnolipid spot and/or gave Rf values close to 1 whereby the spot and the solvent front travelled close together. This solvent mix has been used effectively to separate and detect glycolipid biosurfactants from actinobacteria in previous studies (Kugler *et al*., 2014; Kuyunika *et al*., 2001; Zheng *et al*., 2009). The exception was the extract from *Williamsia muralis* for which chloroform-methanol-acetic acid (65:15:2 v/v/v) was found to be optimal. The required solvent composition for optimal separation depends upon the composition of the glycolipid mixture present; hence, this suggests that the glycolipids detected from *W. muralis* differ from those produced by the other actinobacterial test strains.

3.4.2. Identification of biofilm-formation in clinically relevant bacteria.

In the present study, various gram-positive and gram-negative bacterial strains of clinical relevance (ESKAPE pathogens and priority pathogens on the World Health Organisation list, 2017) were investigated for their ability to form biofilms in static polystyrene microplates, as this is the closed system selected for testing biosurfactant anti-biofilm properties. *In vitro,* static biofilm formation was confirmed for six of the fifteen clinically relevant bacterial tested including *Escherichia coli* ATCC 47055, *Klebsiella pneumoniae* NCIMB 8865, *Pseudomonas aeruginosa* ENU 18, *P. aeruginosa* ENU 19, *Staphylococcus epidermidis* ENU IL-42 and *S. epidermidis* ENU IL-43. *Staphylococcus epidermidis* ATCC 35984 is an emerging pathogen and a known biofilm-producer (Okajima *et al*., 2006) which has been widely investigated in antibiofilm studies (Abdalsadi, 2018; Ceresa *et al*., 2019; Khalid *et al*., 2019; Sambanthamoorthy *et al*., 2014). Hence, this organism was used to benchmark biofilm production by the other strains. The ability to form biofilms was shown

to vary between the six strains depending on the culture medium and length of incubation.

It was seen that the growth medium had a significant influence on biofilm formation. Biofilm formation was better for all six strains in nutrient-rich complex media (Luria Bertani, LB, and Tryptone soy broth, TSB) than in the defined minimal medium M9. Previous studies on similar strains have reported good biofilm formation in nutrient-rich media including LB, TSB, and Brain Heart Infusion (BHI) medium (Hanvey *et al*. 2018; Knobloch *et al.* 2002, Prakash *et al.*, 2017; Pratt & Kolter, 1998; Rohde *et al.,* 2010). However, other studies have reported better biofilm formation in minimal media such as M9 and M63 than in nutrient-rich media (Naves *et al*., 2008; Skyberg *et al*., 2007). Both minimal and nutrient-rich medium has been commonly used in biofilm studies (Adamus-Białek *et al*., 2015; Naves *et al*., 2008; Pratt & Kolter 1998) which have demonstrated that the medium composition has a large impact on biofilm growth (Dassanayake *et al*., 2020; Haney *et al*., 2018). The medium most suited for biofilm formation likely depends on the type of strain and its nutrient requirements. Incubation time was also shown to influence biofilm formation and to be strain dependent; two strains formed good biofilms after 24 h while the other 4 formed good biofilms after a longer incubation of 72h. It is difficult to compare these observations to other studies due to differences in the strains utilised, cultivation conditions, and methodology. However, it is important to establish the optimum incubation time for individual strains prior to undertaking anti-biofilm assays.

Several other factors may influence the evaluation of biofilm formation such as the plate washing procedures, the use of a single microtitre plate or transfer to a second plate prior to measurement, the use of rowing versus resting cells, and the mathematical formula to quantify bacterial biofilm ability (Naves et al., 2008). Some studies advocate the use of a fixative to preserve the bacteria attached to the surface and thereby reduce variability caused by loss of biofilm during the staining process (Kwasny & Opperman, 2010). However, in the present study, using methanol as a fixative resulted in a significant reduction in biofilms formed by *S. epidermidis* ATCC 35984 and *E. coli* ATCC 47055. It is likely

that these strains were damaged by fixation, causing detachment from the microplate wells. Cell fixation by methanol and acetone has been reported to damage membrane lipids and cause cell shrinkage resulting in detachment of cells from surfaces (Chao & Zhang, 2011; Hoetelmans *et al*. 2001; St-Laurent *et al*., 2006).

No standard protocols are in universal use for *in vitro* biofilm formation and anti-biofilm assays although various protocols have been published (e.g. Haney *et al*., 2018; Merritt *et al*., 2005; Stepanoviç *et al*., 2007). However, it is important that experimental conditions are standardised in individual studies as far as possible to allow confident interpretation of results.

3.4.3. Anti-biofilm potential of extracellular biosurfactants extracts produced by *Corynebacterineae* strains.

The extra-cellular biosurfactant extracts of 10 mg ml^{-1} concentration from all eleven-actinobacterial strains possessed some anti-biofilm activity against one or more biofilm-forming test strains. The 10 mg ml^{-1} concentration was selected based on the finding of Kuyukina et al. (2016) whereby this concentration of biosurfactant extract from *Rhodococcus ruber* IEGM 231^T showed significant anti-adhesive activity against biofilm-forming bacteria. This result supports the argument that the *Corynebacterineae* is a rich and under-exploited source of biosurfactants with potential application as anti-biofilm agents.

Biosurfactant extracts (10 mg ml^{-1}) from *Rhodococcus ruber IEGM* 231^T and *R. cercidiphylii* showed the highest anti-adhesion (30%) and inhibition activity (45%) against *Klebsiella pneumonaie* NCIMB 8865 compared to other extract studied. The anti-adhesion and inhibition activity in the present study were similar or different to those reported for actinobacteria-derived biosurfactants from other bacterial species against strains of the same biofilm-forming species or different species. Biosurfactant extracts from *R. ruber* IEGM 231^T is known to prevent the adhesion of both Gram-positive and Gram-negative bacteria (30 -76%) at 10-100 mg l^{-1} concentration. Biofilms of *Escherichia coli* K-12 required a higher concentration of 10 mg l^{-1} to prevent 49% of biofilms adhesion while even at a lower concentration (1mg l^{-1}) the extract was effective in preventing 30% of *Pseudomonas fluorescence* NCIMB 9046 biofilms (Kyunika *et al*., 2016).

Meanwhile, at a lower concentration of 0.5 mg l^{-1}, *Rhodococcus fascians* BD8 biosurfactant reduced the adhesion of Gram-negative bacteria by 70–95% and Gram-positive bacteria by 41–44% (Janek *et al*., 2018). The high anti-adhesion activity was seen against various *E.coli* strains (62-70%), followed by *Enterococcus faecalis* strains (43-60%), and *Staphylococcus epidermidis* KCTC 1917 (41%). However, a higher concentration of *Nocardia vaccinii* IMB B-7405 and *R. erythropolis* IMB Ac-5017 biosurfactants (0.3- 0.05 mg mL^{-1}) was required to prevent *E. coli* IEM-1 biofilms (42%, 54%) and *P aeruginosa* P-55 biofilms (52 , 93%) (Pirog *et al*., 2014). Biosurfactants are able to modify the physico-chemical properties of the surface (hydrophobicity), varying the cell adhesion ability through less cell surface hydrophobicity, inhibiting the electron transport chain thus restricting cellular energy demand (Satpute *et al*., 2016) and causing biofilm inhibition.

Further, in the study, the highest disruption activity was attributed to biosurfactant extract from *R. yunnanensis*, *Tsukamurella pseudospumae,* and *Williamsia muralis* that disrupted 50% of *P. aeruginosa* ENU 18 while biosurfactant of *R. erythropolis* disrupted 51% of *Staphylococcus epidermidis* ENU IL43 biofilms. A previous study on biosurfactant produced by actinobacterium *Brevibacterium casei* MSA19 was found to disrupt *P. aeruginosa* MTCC 2453, and *K. pneumoniae* PC3 biofilms at 1.5µg mL^{-1} concentration (Kiran *et al*., 2010). Meanwhile, biosurfactants from *Staphylococcus lentus* at 18 mg ml^{-1} concentration disrupted *P. aerugi*nosa MTCC 2295 biofilms by 81.7 ± 0.59% (Hamza *et al*., 2017). The disruption ability of biosurfactants is due to their ability to cause detachment of cells through abrasion, and erosion by creating pores and ion channels in lipid bilayer membranes affecting the porosity and integrity of the membranes (Patel *et al*., 2021; Quadriya *et al*., 2018).

Particularly interesting was the susceptibility of biofilm-forming strains *Klebsiella pneumoniae* NCIMB 8865 and *Pseudomonas aeruginosa* ENU 18 biofilms that were affected by all the biosurfactant extracts. It is not known why these strains were less resistant than other strains tested. Biofilm of *P. aeruginosa* ATCC 9027 has previously been reported to be disrupted (30%) by

actinobacteria *Corynebacterium xerosis* NS5 biosurfactants (Dalili *et al*., 2015). However, as the studies on the anti-biofilm activity of actinobacteria biosurfactants against *K. pneumoniae* and *P. aeruginosa* biofilms are limited this study shows the ability of various actinobacteria species to have activity against these biofilm-forming strains, which are of concern in clinical settings due to wide range of infection associated with their biofilm.

Anti-biofilm activity of glycolipid biosurfactants is reported to interfere with *P. aeruginosa* in both cell-cell interactions and cell–substratum interactions causing the removal of extracellular polymeric substances (EPS) and destruction of microcolonies (Davey *et al*., 2003; Díaz *et al*., 2016b; Schooling *et al*., 2004). K*lebsiella pneumoniae* produces extracellular structures, including capsules and fimbriae, which play an important role in biofilm formation along with the signalling molecules that allow bacterial communication (Ribeiro *et al*., 2016). The biosurfactant extracts cause alteration to these structures or cell-to-cell signals reducing the biofilm formation of *K. pneumoniae* (Ribeiro *et al*., 2016; Schroll *et al*., 2010).

Although promising anti-biofilm results were obtained against some pathogens, other pathogens were found to be unaffected. This may be due to the different chemical composition of biosurfactants, critical cell micelle concertation, biofilm structures, biofilm formation stage, biofilm size, etc. varying between the different species of microorganisms (Nikolova & Gutierrez, 2021), which presumably may have different influence on the anti-biofilm effects. Moreover, the cell membrane of bacteria also influences a bacterium's ability to adhere to and form biofilms and presumably affects their susceptibility to biosurfactants. The outer membrane of Gram-negative bacteria contains lipopolysaccharides (LPS) that can slow the passive diffusion of hydrophobic compounds, while narrow pores limit by size the penetration of hydrophilic drugs (Zgurskaya *et al*., 2015). *Enterobacteriaceae* family such as *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp are known to produce extended-spectrum β-lactamase (ESBLs) and class A β-lactamases enzymes that can hydrolyze anti-microbial agent and become resistant to inhibition (Breijyeh *et al*., 2020; Paterson, 2006). In *P. aeruginosa* overexpression of efflux pumps

and decreasing outer membrane permeability makes them survive the treatment (Breijyeh *et al*., 2020). Meanwhile, in biofilms of Gram-positive *S. epidermidis* nutrient limitation and slow growth, and formation of persistent cells, EPS matrix can affect their susceptibility to treatments (Chen *et al*., 2019).

The findings of the anti-biofilm studies revealed some limitations with the crystal violet assay. Anti-biofilm assays conducted using CV quantification indicated an apparent stimulatory effect by the biosurfactant extracts on several biofilm-forming bacteria. However, this observation was considered an artefact most likely due to a lack of specificity which means that CV stain can bind to the whole biofilm matrix including exopolysaccharides (EPS) and proteins in addition to cellular biomass (Castro *et al.,* 2022; Latka *et al*., 2020). In addition, as this stain cannot differentiate between live and dead cells, cell debris will also contribute to biofilm measurement. Methods such as BacLight™ live/dead staining, colony forming units the calculation of bacterial plating efficiency and Vital fluorescence staining can be used to detect cell debris that contribute in biofilm measurement (Hannig *et al*., 2013; Netuschil *et a*l., 2014). Consequently, CV may overestimate the quantity of biofilm biomass. In the anti-biofilm assays, the inclusion of untreated controls would be expected to account for this. However, the apparent increase in biofilm size may reflect an increase in the production and release of exopolysaccharide material instead of biomass during the assay. In turn, this may be a protective response during exposure to the anti-microbial agent (Yin *et al*., 2019). Clinical isolates of *Staphylococcus epidermidis* and *Haemophilus influenza* when treated with antibiotics were found to increase EPS in biofilms by upregulating genes involved in EPS production (Rachid *et al*., 2000; Wu *et al.,* 2014). Hence, it becomes more problematic when the microorganism produces a higher level of extracellular polymeric substances (Skogman *et al*., 2012), and is poorly suited to evaluating the killing of biofilm cells (Peeters *et al*., 2008). Kuyukina *et al*., (2016) observed a similar stimulatory effect in anti-adhesion assays on biosurfactants from *R. ruber* IEGM 231^{T} while Sabaeifard *et al.* (2014) when determining the effects of antibiotics against *P. aeruginosa* PAO1 biofilms when CV staining was employed. Sabaeifard *et al.* (2014) believed that since the CV is

attached to the EPS of biofilm it can even detect the cell-free EPS suggesting CV assay is not reliable and the assay results need to be confirmed with another assay, such as the use of vital dye. The potential increase in EPS, which may be the cause of greater CV staining, can be quantified using the colorimetric procedure for the determination of sugar by the phenol-sulfuric method and proteins by Bradford method (Ruas-Madiedo, P. & de los Reyes-Gavilán, C., 2005).

Given the non-specific nature of CV, it is desirable to pair this assay with another method for biofilm quantification. Viable plate counts are time-consuming and require large volumes of consumables and microtitre plate methods are therefore preferable. In this study, the metabolic dye TTC was selected as it is rapid, reliable, low-cost, and non-toxic to cells (Sabaeifard *et al*., 2014). The TTC optimisation studies revealed biofilm measurements are generally smaller using TTC than CV supporting the argument that the latter measures additional biofilm matrix materials and overestimates biofilm size, although this appears to be strain dependent. Moreover, crystal violet staining was found to be less reproducible with higher deviations from the mean than TTC dye. However, the effects of TTC concentration are strain dependent and must be further optimised for *K. pneumoniae* and *P. aeruginosa* prior to anti-biofilm testing de Celis *et al*., (2022) and Peeters *et al*. (2008) also noted large variations between replicates with the CV assay and that metabolic dyes such as TTC were more reliable.

Enhanced anti-biofilm properties were observed for the three extracellular biosurfactant extracts when tested using TTC compared to CV. Indeed, some assays that revealed no anti-biofilm effects using CV were found to be positive using TTC. These findings indicate that the CV method can underestimate and mask anti-biofilm properties and it may well be the case that the other extracts not tested yet by TTC possess better antibiofilm activities than revealed by CV in the present study. However, the CV assay measured higher disruption than the TTC assay. These results are more difficult to interpret but indicate that the CV assay is more reliable for disruption rather than anti-adhesion and inhibition assays, for *P. aeruginosa* ENU 18 at least.

Whilst the TTC assay proved to be reproducible and sensitive in the current study, considerable optimisation was required prior to use. Based on inhibition and disruption dose-response curves and EC_{50} values determined for commercial surfactants, it was decided that a concentration of 0.1% w/v TTC was most suitable for subsequent anti-biofilm assays. The EC_{50} estimations ultimately depend on the suitability of detergent concentrations applied and whether these give a full range of biofilm responses. However, given that, full dose responses were observed and EC_{50} values determined using higher concentrations, it may be concluded that the lowest and highest concentrations under and over-estimated biofilm measurements, respectively. The variability observed in the dose-response curves and median EC_{50} value with TTC concentration applied to demonstrate that this is an important variable to optimise for anti-biofilm assays. The use of TTC has been reported to be toxic to bacteria at high concentrations and hence identifying a suitable concentration is important. Sabaeifard *et al*. (2014) also found 0.1% w/v TTC suitable for detecting *P. aeruginosa* biofilms compared to 0.4, 0.2, and 0.02% concentrations. Both 450nm and 490nm wavelengths were found to be suitable for measuring TTC although some significant differences were observed in some optimisation assays, generally, no one proved more reliable. Previous studies suggest that no one TTC wavelength is suitable since the ability of biofilm formation, the biofilm growth period, the ability to reduce tetrazolium salts and many other known and unknown parameters may affect assay results (Gabrielson *et al*., 2002; Knezevic & Petrovic, 2008; Rahman *et al*., 2004; Sabaeifard *et al*., 2014; Shakeri *et al*., 2007).

It must be noted that quantifying bacteria in biofilms using metabolic assays has some limitations. Bacteria in biofilms may have lower metabolic rates than their planktonic counterparts and not all viable cells may provide a measurable reduction of the dye to formazan. Further, the proportion of viable cells in the total biofilm biomass is unknown, therefore it is not known to what degree the viable cells represent the whole biofilm mass (Welch *et al*., 2012).

3.4.4. Selection of extra-cellular biosurfactant extracts for further characterisation.

Extracellular biosurfactant extracts from six *Corynebacterineae* strains (*R. cercidiphylii*, *R. erythropolis, R. percolatus*, *R. ruber, T. pulmonis,* and *W. muralis*) demonstrated all three anti-biofilm properties (anti-adherence, biofilm inhibition, and biofilm disruption) against one or more biofilm-forming bacterial strains when tested using the crystal violet (CV) assays. Apart from *R. ruber* IEGM 231^{T}, none of these actinobacteria have previously been reported to possess anti-biofilm activity hence these findings are novel. The biosurfactant extract from *R. ruber* (BRub) had the highest anti-adhesion (30.1 ± 3.4%) and inhibition (44.1 ± 1.4%) activities (against *Klebsiella pneumoniae* NCIMB 8865 biofilms) although other extracts performed equally well against this and other strains. The biosurfactant extracts from *R. yunnanensis* (BRyun) and *W. muralis* (BWmur) showed the highest disruption activity (53.4 ± 6.4%, 49.5 ± 8.7%), in this case, against *P. aeruginosa* ENU 18, and these strains were therefore selected for further study.

Testing the biosurfactant extracts from *R. ruber, R. yunnanensis*, and *W. muralis* for anti-biofilm activity using the metabolic dye 2,3,5-Triphenyl Tetrazolium Chloride (TTC) confirmed these properties. All three extracts demonstrated even higher anti-adhesion (45 - 55%) and biofilm inhibition activity (76 - 84%) activity against *K. pneumoniae* NCIMB 8865 using TTC. In addition, the *R. yunnanensis* extract (BRyun) was also shown to inhibit the biofilm formation of *P. aeruginosa* ENU 18 by 57%. The disruption capabilities of the biosurfactant extract however appeared lower based on the TTC assay (BRrub disrupted pre-formed *P. aeruginosa* ENU 18 biofilms by 20% and BRyun and BWmur disrupted *K. pneumoniae* NCIMB 8865 biofilms by <17%).

Overall, these results are promising albeit that they are based on tests using crude biosurfactant extracts. Indeed, several other anti-biofilm studies have used crude extracts of total (cell-bound and extracellular) or extracellular biosurfactants (Hamza, *et al*., 2017; Javadi *et al*., 2021; Karlapudi *et al*., 2020; Patel *et al*., 2021), or indeed utilised cell-free supernatants directly (Padmavathi, *et al*., 2014 Sambanthamoorthy *et al*., 2014). However, it must be

noted that these crude extracts may contain other compounds which could possess anti-biofilm properties therefore it is not possible to conclude that it is the extra-cellular biosurfactants that are wholly responsible for the effects observed in this study. It will therefore be necessary to extract one or more biosurfactant components from these crude extracts and undertake further anti-biofilm tests to confirm that the effects observed here are attributable to biosurfactants. Three strains will therefore be further investigated to identify and isolate biosurfactant components from the crude extracts for further antibiofilm testing.

3.5. Conclusions

A reasonable quantity of extra-cellular crude biosurfactant extract were recovered from all the test strains and these extracts contain glycolipids, some of which may be rhamnolipid in nature based on their relative mobilities on thin layer-chromatograms. Extra-cellular crude biosurfactant extracts from several *Corynebacterineae* strains possess anti-biofilm properties. Three actinobacterial strains, *Rhodococcus ruber* IEGM 231^{T}*, R. yunnanensis* DSM 44837^{T}*,* and *Williamsia muralis* N1261^{Tsp} produce extra-cellular biosurfactant extracts with strong anti-biofilm effects (anti-adhesion, inhibition, and disruption) against *P. aeruginosa* ENU 18 and *K. pneumoniae* NCIMB 8865. This is the first report of biosurfactant extracts with anti-biofilm properties from *R. yunnanensis* and *W. muralis* and the first to report biofilm inhibition effects by extra-cellular crude biosurfactant extract from *R. ruber*. Biosurfactant extracts from additional strains possess anti-biofilm properties and additional properties may have been masked when using the crystal violet assay in the present study. This stain does not discriminate biomass from exopolysaccharides which likely led to the false impression in several of the anti-biofilm assays that biosurfactant extracts stimulate cell adherence and biofilm formation. The results of the TTC based anti-biofilm assays did not reveal any stimulatory effects in the presence of biosurfactants, supporting this interpretation. This assay, which quantifies metabolically active cells only, is, therefore, more reliable than the CV assay for anti-biofilm studies. However, the TTC assay requires considerable optimisation for use with different biofilm-forming strains.

Chapter 4. Production, characterisation, and anti-biofilm properties of extra-cellular glycolipid- containing biosurfactants from the actinobacteria *Rhodococcus ruber*, *R. yunnanensis,* and *Williamsia muralis*.

4.1. Introduction

The preceding chapters established the production of cell-bound and extracellular biosurfactants amongst diverse mycolic acid-containing actinobacteria (MACA) while growing on hexadecane. Additionally, crude extracellular biosurfactant extracts from various MACA strains exhibited anti-biofilm effects against selected pathogens. Given their ease of recovery from bacterial cultures, extracellular biosurfactants are preferred over cell-bound biosurfactants, which require more complex separation procedures. However, it was noted that the production of extra-cellular biosurfactants varied between strains and was influenced by the duration of incubation (7 or 14 days).

During batch cultivation, biosurfactant production patterns vary depending on the bacterial strain and the type of biosurfactant(s) synthesised. Biosurfactant production can be categorised as non-growth, growth-associated, or mixed growth-associated (Desai & Banat, 1997; Nitschke & Pastore, 2004). Growth-associated biosurfactant production is demonstrated by a direct relationship between biosurfactant concentration and increased cellular biomass, occurring during exponential growth. Biosurfactant production during both exponential and stationary phases is termed mixed growth-associated production. Non-growth-associated biosurfactant production occurs during the stationary phase or at zero growth (lag phase) (Sivapathasekaran & Sen, 2017). Examining the kinetics of growth and biosurfactant production can distinguish the nature of biosurfactants produced (cell-bound or extracellular), provide insights into the growth and metabolism of test strains, and inform optimisation of growth conditions for maximal yields of biosurfactants (Ibrahim *et al*., 2020).

In this study, three test strains observed to have the most effective anti-biofilm activities, namely *Rhodococcus ruber* IEGM 231^{T}, *Rhodococcus yunnanensis*

DSM 44837^{T}, and *Williamsia muralis* N1261^{Tsp} will be further investigated. The detailed relationships between growth and biosurfactant production (both cell-bound and extra-cellular) have not yet been established for any of these strains. Previously, biosurfactant production by *R. ruber* IEGM 231^{T} was reported to be growth-associated during batch cultivation on hexadecane, commencing early in the exponential phase presumably to enable substrate uptake and utilisation (Philp *et al*., 2002). However, the study only measured surface tension reduction in whole cell broths and did not specifically monitor extracellular biosurfactant production. The current study therefore will establish the relationships between bacterial growth and biosurfactant production using various quantitative screening assays to account for biosurfactants with different physico-chemical properties. In addition, biosurfactant production will be assessed in both whole cell broth (WCB) samples, measuring total biosurfactants, and cell-free supernatant (CFS) samples, measuring only extracellular biosurfactants secreted into the culture broths.

In the previous chapter, anti-biofilm studies were conducted on total crude extracts of extra-cellular biosurfactants, which were found to contain glycolipids, although these extracts likely consist of a mixture of various biosurfactant compounds. As glycolipids, notably trehalose lipids, produced by MACA have previously been reported to demonstrate anti-adhesion and/or anti-biofilm properties (Pirog *et al*., 2014; Janek *et al*., 2018) these compounds will be investigated further in this study. There have been no studies to date on the anti-biofilm properties of glycolipids or indeed any biosurfactants produced by *R. yunnanensis* or *W. muralis*. The anti-adhesion properties of crude trehalose lipids for *R. ruber* IEGM 231^{T} have been studied in some detail (Kuyukina *et al*., 2016) but not biofilm inhibition or disruption.

Glycolipid- containing biosurfactants will be isolated from the total extracts of extra-cellular biosurfactants examined previously. These glycolipids, expected to be complex and comprised of multiple types and congeners, will be analysed using matrix-assisted laser desorption/ionization-time-of-flight, time-of-flight mass spectrometry (MALDI-TOF-TOF). This technique, which allows the identification of intact compounds and can be used to determine the full

molecular mass of biosurfactants, has been used previously to characterise glycolipids from MACA strains (Kai *et al*., 2007; Kügler *et al*., 2014). The anti-biofilm properties of the glycolipid- containing extract, including anti-adhesion, biofilm inhibition, and biofilm disruption, will be assessed against biofilm-forming strains *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumonia* NCIMB 8865. These strains were shown to be susceptible to the total extra-cellular biosurfactant extracts in the previous study. The anti-biofilm properties of the glycolipid- containing extract will be compared to those found for the total crude biosurfactant extracts, to determine whether the glycolipids are responsible for or contribute to the previously observed anti-biofilm effects.

4.1.1. Study Aim and Objectives

This study aims to establish the pattern of biosurfactant production during the growth of three mycolic acid-containing actinobacteria with promising anti-biofilm properties and to further investigate the chemical, physico-chemical, and anti-biofilm properties of extra-cellular glycolipid- containing extract.

The specific objectives of the study are to:

1. Determine the pattern of cell-associated and extra-cellular biosurfactant production by the test strains during batch cultivation on hexadecane using selected indirect physico-chemical assays.
2. Isolate the glycolipid components from the total extracellular crude biosurfactant extracts recovered from the test strains and characterise these using chemical-analytical techniques.
3. Assess the anti-biofilm efficacy of the glycolipid- containing extract against clinically relevant strains, evaluating their potency compared to the whole crude biosurfactant extracts in the previous study.

4.2. Materials and Methods

4.2.1. Cultivation of test strains.

The test strains *Rhodococcus ruber* IEGM 231^{T}, *Rhodococcus yunnanensis* DSM 44837^{T}, and *Williamsia muralis* N1261^{Tsp} were grown as starter cultures according to the method detailed in section 3.2.2.1., page 107.

4.2.2. Growth and biosurfactant production on hexadecane.

Growth and biosurfactant production during batch cultivation was studied in 3L Erlenmeyer flasks containing 1.5L working volumes of sterile mineral salts medium (MSM; section 2.2.4.1., page 52) supplemented with hexadecane (2% v/v; Sigma-Aldrich, Dorset, UK) as the sole carbon source. The flasks were inoculated with a starter culture (2% v/v) and incubated at 30°C with shaking at 180 rpm. Broth samples (50ml) were collected every 24h to determine bacterial growth and biosurfactant production.

4.2.2.1. Bacterial growth measurement. Bacterial growth was measured as biomass (dry weight), turbidity, and viable plate counts. For biomass measurement, a 20ml sample of culture broth was centrifuged at 13000×g (High-Speed Centrifuge, Avanti J-26XP) for 10 mins, washed twice in buffer solution (gl^{-1}, 16.9 K_2HPO_4, and 7.3 KH_2PO_4) and the pellet added to a pre-weighed glass plate and dried to a constant weight in an oven set at 60°C (Jencons-WTC binder). The turbidity of the culture broth was measured at 600 nm wavelength (OD_{600} nm) in a microtitre plate reader (FLUOstar Omega) with the mineral salt medium as the blank. To determine viable plate counts, ten-fold serial dilutions of the culture broth were prepared in 0.85% w/v NaCl and spread as 100µl volumes onto tryptic soy agar (TSA) plates in triplicate. The plates were incubated at 30˚C for 24h and the dilutions giving rise to 30-300 colonies on replicate plates were used to calculate the mean colony-forming units in the culture broth as follows:

$$\text{Colony forming units (cfu ml}^{-1}) = \frac{\text{mean no. of colonies x dilution factor}}{\text{volume of culture plated}}$$

4.2.2.2. Biosurfactant detection. Biosurfactant production was measured over time using whole cell broth (WCB) and cell-free supernatant (CFS) samples apart from cell surface hydrophobicity which was determined by the bacterial cell adherence to hydrocarbon (BATH) assay using WCB samples only (section 2.2.5., page 55). The emulsification index (EI_{24} %) was measured according to the method detailed in section 2.2.4.6 (page 54). Surface tension (ST) measurement and the critical micelle dilution (CMD) were determined by tensiometer (Automatic tensiometer TD 3, Germany), using the du Noüy ring method (section 2.2.4.7, page 54). To determine the CMD, serial dilutions of the WCB and CFS samples were prepared in a mineral salts medium without hexadecane and ST readings were taken.

4.2.3. Isolation of glycolipid biosurfactants by thick layer chromatography.

Thick Layer chromatography 20cm × 20cm glass plates of 1mm depth (Kieselgel 60 GF_{254} silica; Sigma Aldrich, UK) were dried and activated in an oven at 60°C for 24h before use. Glycolipid components were separated and detected by thin layer chromatography (TLC) (section 3.2.2.2, page 108). In the case of *Williamsia muralis* extract, chloroform methanol-acetic acid (65:15:2, v/v/v), was used as the solvent system. Replicate plates were run for each biosurfactant extract and one plate was sprayed with ornicol reagent (0.1%, w/v) as detailed in section 3.2.2.2 (page 108) to determine the position (relative front; R_f) of glycolipid compounds. This Rf was used to identify and mark the position of the compounds on replicate plates without ornicol reagent. The marked silica bands were scraped off to isolate the glycolipids which were then eluted with chloroform-methanol (2:1 v/v) and filtered using a sintered funnel. The solvent was then evaporated to dryness, the samples re-dissolved in chloroform-methanol (2:1 v/v) and filtered through Whatman Filter Paper (No 1), and again evaporated to dryness. The isolated glycolipid-containing samples were dissolved in water and sonicated for 1 min using a sonicating water bath (Langford Ultrasonics water bath), and vortexed for 1 min prior to further analysis. The presence of the biosurfactants was confirmed by TLC (section 3.2.2.2, page 108) and by measuring surface activity using the oil spreading method (OS; section 2.2.4.4., page 53).

4.2.4. Growth inhibition determined by the agar well diffusion method.

The anti-bacterial activities of both the biosurfactant extracts prepared in the previous study (section 3.2.4., page 111) and the isolated glycolipids (section 4.2.3) were tested against *Pseudomonas aeruginosa* R222 and *Klebsiella pneumonia* NCIMB 8865 by the agar well diffusion method (Magaldi *et al*., 2004). The starter culture was prepared as described in 3.2.3.2 (page 109) and the turbidity of each culture was measured by spectrophotometry(Jenway 6300) and back diluted to 0.5 McFarland (0.08 OD_{600} nm) in TSB. The standardised cultures were swabbed across the entire surface of tryptone soya agar (TSA) plates using sterile cotton swabs. Wells were then punched into the plates using a sterile cork borer (6mm diam.) and filled with 20µl crude biosurfactant extract or the extracts with isolated glycolipid biosurfactants. Chemical non-ionic surfactants Tween 80 and Triton-X 100 were used neat (10%) for comparison purposes and water acted as the negative control. The plates were incubated at 30°C for 24h and the diameter of the zones of inhibition was measured in millimetres (mm) after subtracting the well diameter.

4.2.5. Anti-biofilm properties of the glycolipid- containing extract.

The anti-biofilm properties of the extracts containing glycolipid biosurfactants were tested against the biofilm-forming strains *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumonia* NCIMB 8865 by the anti-adhesion, biofilm inhibition, and biofilm disruption assays described in Chapter 3 (sections 3.2.4.1, 3.2.4.2, and 3.2.4.3, respectively, page 111 and 112). Initial starter cultures of the biofilm-forming strains were prepared according to section 3.2.3.2 (page 109). The extracts with glycolipid biosurfactant samples were tested neat and as two-fold dilutions prepared in sterile distilled water up to a 1:10 dilution. Biofilms were quantified using 0.1% w/v 2, 3, 5-triphenyl tetrazolium chloride (TTC) to detect metabolically active cells, and the biofilms were quantified by optical density measurement at 450 nm wavelength using a microplate reader (FLUOstar Omega). The percentage of bacterial adhesion, biofilm inhibition, and biofilm disruption was calculated according to the formula in section 3.2.4.4 (page 113) using appropriate blanks.

4.2.6. Characterisation of glycolipid- containing extract by matrix assisted laser desorption ionization-time of flight mass spectrophotometry (MALDI-TOF-MS).

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) analysis of the glycolipids- containing extract was carried out using a Bruker UltrafleXtreme MALDI-TOF/TOF instrument at the National Mass Spectrometry Facility (NMSF) at Swansea University. Initially, the R90 Rhamnolipids standard (Sigma Aldrich, UK) was used to identify a suitable matrix and conditions for the identification of glycolipids in the actinobacterial samples. Once optimised, each glycolipid- containing extract sample (10mg ml^{-1}) was dissolved in isopropyl alcohol-methyl cyanide (MeCN) (1:10, v/v) and mixed with a matrix solution of di-hydroxybenzoic acid (DHB)-2, 6-Dihydroxyacetophenone (DHAP) (9:1, v/v) in MeCN: H_2O (1:1, v/v) for analysis. Data acquisition was performed in the reflector positive ion and negative ion modes. Mass spectra analysed ranged from 300-4000 mass units and for each mass spectrum, 5000 laser shots over the whole spot were accumulated.

4.2.7. Statistical analysis.

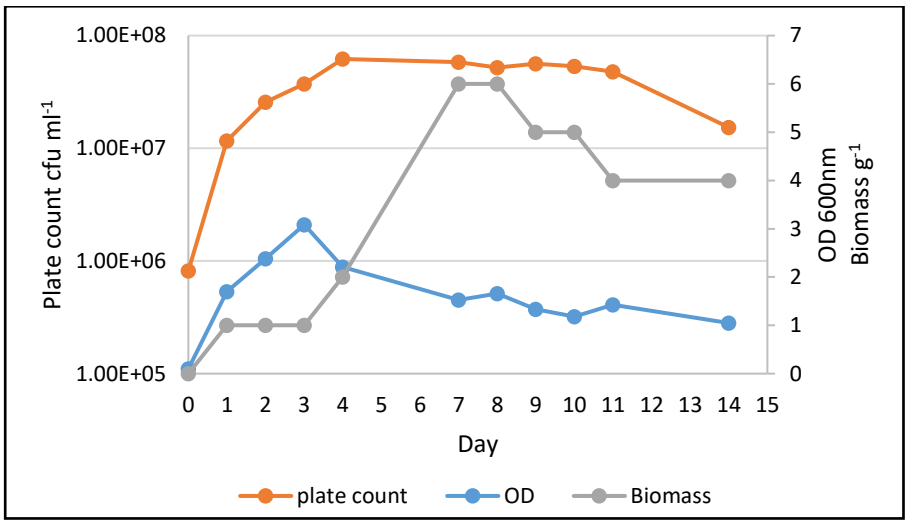
All assays were performed in three biological replicates and the results were expressed as mean ± standard deviation (SD) calculated using Excel. Spearman's correlation coefficients (r_s) were determined using SPSS (v26). The Spearman rank correlation coefficient (r_s) ranges from -1 (strong negative correlation) to +1 (strong positive correlation). Tukey's One-way analysis of variance (ANOVA) with post-hoc correction (SPSS v26) was used to evaluate the significance of the difference ($p<0.05$).

4.3. Results

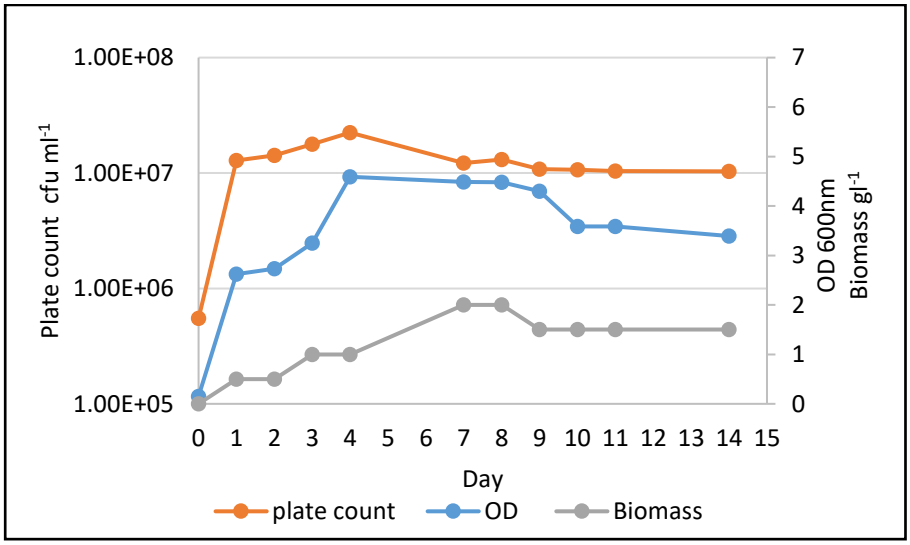
4.3.1. Comparison of methods used to measure growth during batch cultivation on hexadecane.

Growth by the test strains *R. ruber* IEGM 231^{T}*, R. yunnanensis* DSM 44837^{T,} and *W. muralis* N1261^{Tsp} was measured during batch cultivation in minimal salt medium (MSM) with hexadecane (2% v/v) as the sole carbon source, over 14 days of incubation. Three methods were used to determine growth: viable plate counts (cfu ml^{-1}), biomass dry weight (g l^{-1}), and turbidity by reading absorbance (OD_{600}nm). Based on viable plate counts, all strains exhibited a short lag phase (not detected with measurements at 24 h intervals) before rapidly entering the exponential phase lasting 1-3 days depending on the strain, followed by a discernible growth deceleration phase lasting 2-3 days (Fig 4.1). The maximum growth, as determined by viable plate counts, was reached after 4 days, and this corresponded to the highest OD_{600}nm readings obtained for two strains: *R. yunnanensis* DSM 44837^{T} (2.24E+07 cfu ml^{-1}; 4.590 ± 0.24 OD_{600}) and *W. muralis* N1261^{Tsp} (1.91E+07 cfu/ml^{-1}; 1.735 ± 0.08 OD_{600}). While the highest viable plate count for *Rhodococcus ruber* IEGM 231^{T} was recorded on day 4 (6.20E+07 cfu ml^{-1}), the highest turbidity was measured on day 3 (3.086 ± 0.03 OD_{600}). Maximum growth was followed by a long stationary phase lasting until the end of the incubation period, with a decline in growth only observed for *R. ruber* IEGM 231^{T} (after day 11). Only weak positive correlations were found between turbidity readings and viable plate count, with the highest correlation observed for *R. yunnanensis* and *W. muralis* strain (r_s = 0.31, 0.32).

a)



b)



c)

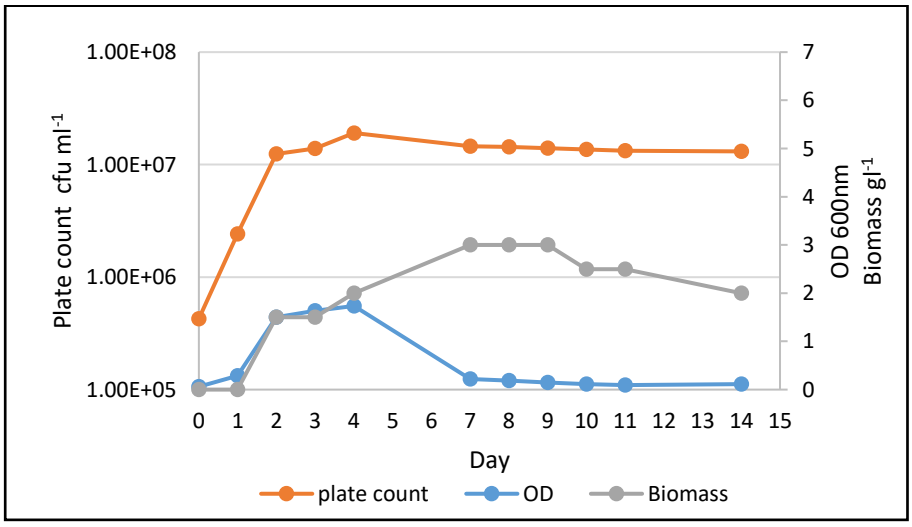


Fig. 4.1. Growth profiles for a) *Rhodococcus ruber* IEGM 231^{T}, b) *R. yunnanensis* DSM 44837^{T}, and c) *Williamsia muralis* N1261^{Tsp} determined by viable plate counts (cfu ml^{-1}), optical density (OD_{600}nm) and biomass dry weight (g L^{-1}) during 14 days of batch cultivation in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C with orbital shaking at 180 rpm. Data are expressed as the mean ± standard deviation of three technical replicates.

Based on the dry weight biomass (g l^{-1}) method, the three actinobacterial strains reached the end of the exponential phase and maximum biomass on day 7 (*R. ruber* 6g l^{-1}; *R. yunnanensis* 2g $l^{-1,}$ and *W. muralis* 3g l^{l-1}) (refer to Appendix 7.9, 7.10, 7.11, page 283-285). However, Spearman's correlation coefficient (r_s) showed moderate positive correlations between viable plate count and biomass dry weight data for the three test strains (r_s = 0.69 – 0.75). Based on these observations viable plate counts were selected for determining the pattern of biosurfactant production during batch cultivation.

4.3.2. Biosurfactant production during batch cultivation on hexadecane determined using indirect physico-chemical assays.

Biosurfactant production in the whole cell-broth (WCB) and cell-free supernatant (CFS) was measured daily during batch growth of *R. ruber* IEGM 231^T*, R. yunnanensis* DSM $44837^{T,}$ *and W. muralis* $N1261^{Tsp}$. The presence of biosurfactants was determined indirectly by cell surface hydrophobicity (CSH), emulsification index (EI_{24}), surface tension (ST), and critical micelle dilution (CMD) measurements. Different relationships between strain growth and biosurfactant production were observed depending on the physico-chemical property measured. Data were not normally distributed (Kolmogorov–Smirnov test; $p<0.05$) hence Spearman's coefficient was used to identify any correlations between growth and biosurfactant production.

4.3.2.1. Biosurfactant production and critical micelle dilutions during growth determined by surface tension reduction. The surface tension (ST) of the un-inoculated growth medium, measured by the de Noüy ring method, was 72 ± 0.04 mN m^{-1}. Immediately after inoculation with the actinobacterial test strains, the ST values of the whole cell broths dropped to around 55 mN m^{-1} and further to minima of <30 mN m^{-1} after 24 h (Fig. 4.2), and they remained around this value until the end of the cultivation period, except for *R. ruber* IEGM 231^T which rose to ~40mN m^{-1}. Weak to strong negative correlations were observed between WCB surface tension and growth measurements determined by viable plate counts (r_s = -0.13 to -0.87) and culture turbidity (r_s = -0.41 to -0.94).

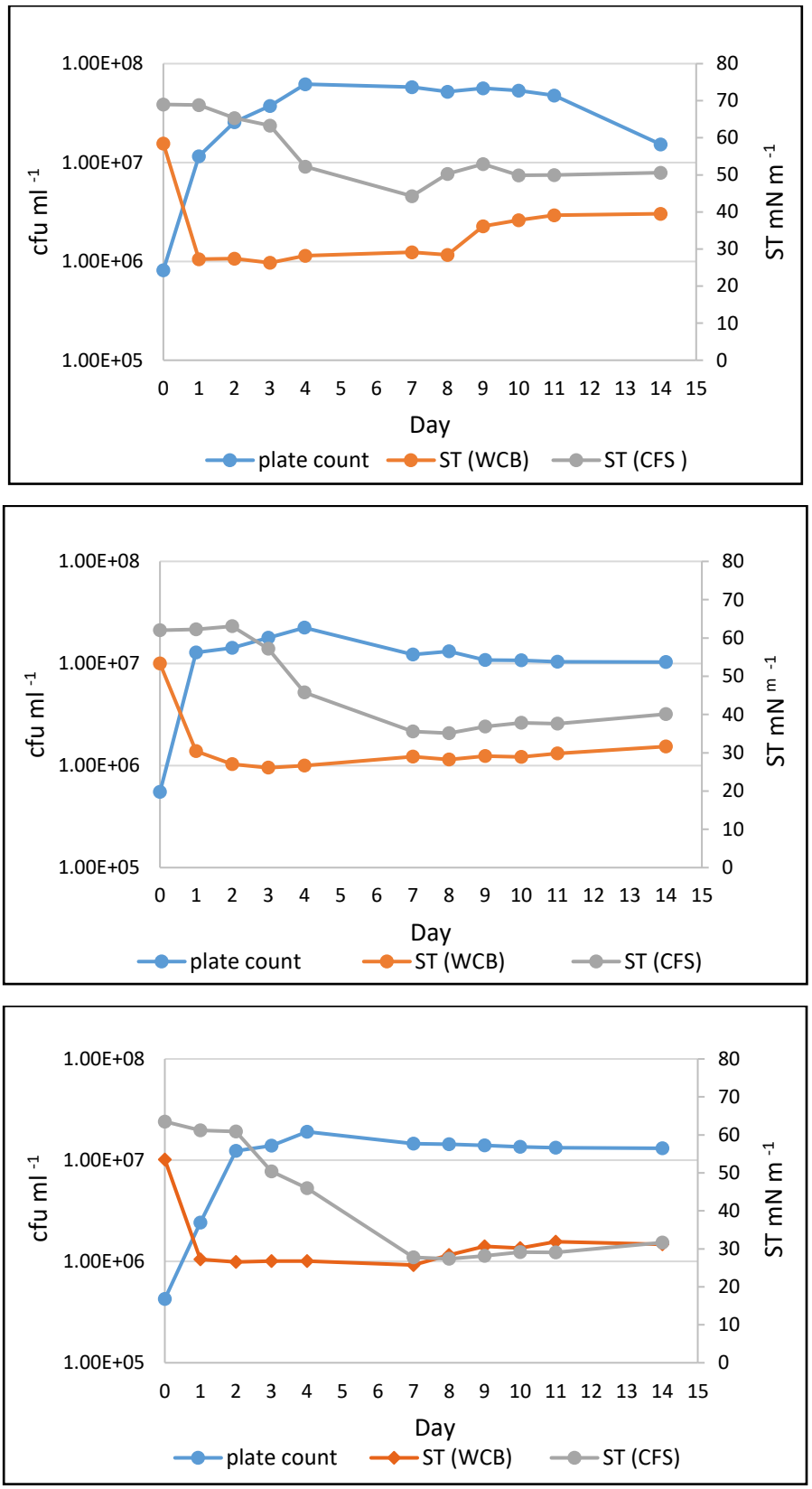


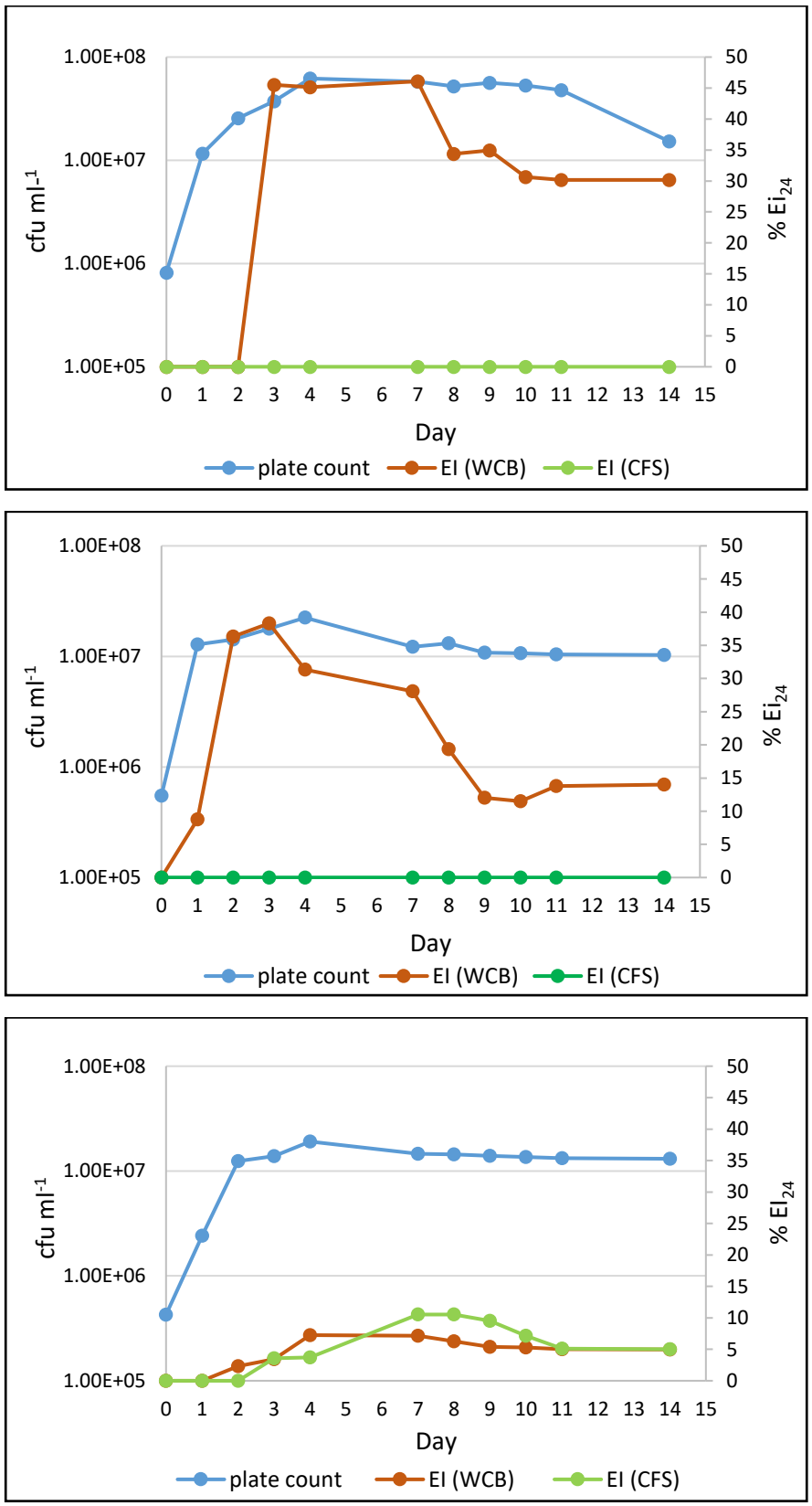
Fig. 4.2. Relationships between growth determined by viable plate counts (cfu ml^{-1}) and surface tension (ST) reduction measured in whole cell broths (WCB, total biosurfactants) and cell-free supernatants (CFS, extra-cellular surfactants only) for a) *Rhodococcus ruber* IEGM 231^{T}, b) *R. yunnanensis* DSM 44837^{T} and, c) *Williamsia muralis* N1261^{Tsp} during batch cultivation for 14 days in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C with orbital shaking (180 rpm).. Data are expressed as the mean ± standard deviation of three technical replicates.

An increase in total biosurfactant concentration was observed in the WCB samples during growth as determined by critical micelle dilution (CMD) values. After incubation for 1 day, the CMD value for *R. yunnanensis* DSM 44837^T was 1:50 and for *R. ruber* IEGM 231^T and *W. muralis* N1261^{Tsp} 1:100. The CMD values then increased and reached maxima on day 4; 1:500 for *R. ruber* IEGM 231^T, 1:200 for *W. muralis* N1261^{Tsp} and 1:100 for *R. yunnanensis* DSM 44837^{T}and remained same thereafter.

The surface tension of cell-free supernatant (CFS) samples from all three strains was between 63-69 mN m^{-1} immediately after inoculation and remained steady until day 2 after which values lowered further reaching a minima of 30 - 50 mN m^{-1} by day 7. After this time, ST values remained constant until the end of cultivation. *Williamsia muralis* N1261^{Tsp} achieved the greatest reduction in surface tension in both WCB and CFS samples (~30 mN m^{-1}).

An increase in extracellular biosurfactant concentration was observed in the CFS during growth deceleration and early stationary phase as determined by critical micelle dilution (CMD) values. On day 2 the CMD was 1:10 and this increased to a maximum of 1:100 for *R. ruber* IEGM 231^T and *R. yunnanensis*, and 1:150 for *W. muralis*, on day 7, after which no further changes in CMD were observed. Moderate to strong negative correlations were observed between CFS surface tension and growth measurements determined by viable plate counts (r_s = -0.66 to -0.71) and biomass dry weight (r_s = -0.86 to -0.98).

4.3.2.2. Biosurfactant production during growth determined by emulsification. The mean emulsification index (EI_{24}) values for whole cell broth (WCB) followed a similar pattern to that of growth by *Rhodococcus ruber* IEGM 231^T and *R. yunnanensis* DSM 44837^T. After a short lag period, emulsification increased during the exponential phase reaching maximum values (EI_{24} 45.53 ± 0.12% and 38.31 ± 0.12%, respectively) by day 3 (Fig. 4.3). Emulsification readings remained steady for *R. ruber* IEGM 231^T between 3 and 7 days of incubation (EI_{24} 45-46%), then dropped to ~ EI_{24} 30% until the end of incubation.

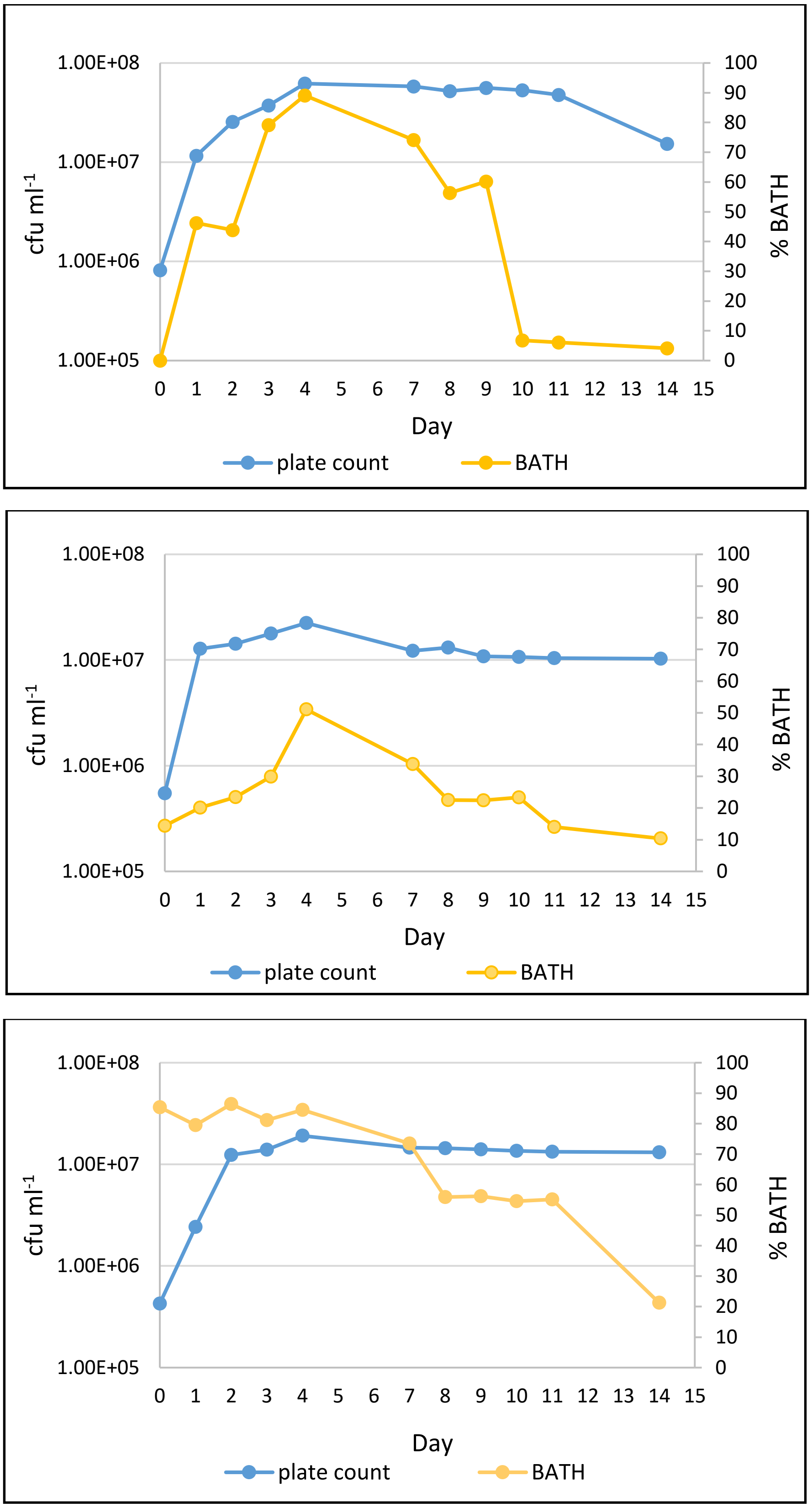


4.3. Relationships between growth measured by viable plate counts (cfu ml^{-1}) and emulsification (% EI_{24}) measured in whole cell broths (WCB, total biosurfactants) and cell-free supernatants (CFS, extra-cellular surfactants only) for a) *Rhodococcus ruber* IEGM 231^{T}, b) *R. yunnanensis* DSM 44837^{T} and, c) *Williamsia muralis* N1261^{Tsp} during batch cultivation for 14 days in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C with orbital shaking (180 rpm) . Data are expressed as the mean ± standard deviation of three technical replicates.

Emulsification readings for *R. yunnanensis* DSM 44837^T WCB declined after day 3 reaching EI_{24} of ~10% by the end of the experiment. Strong positive correlations were observed between emulsification and viable plate counts (r_s = 0.81, 0.74). For *W. muralis* N1261^{Tsp} however, only weak EI_{24} values (<10%) were observed for WCB samples although the changes followed a similar pattern to growth and a strong correlation (r_s = 0.94) was observed between emulsification and viable plate count.

No emulsification was detected in the CFS samples for *R. ruber* IEGM 231^T and *R. yunnanensis* DSM 44837^T throughout the batch cultivation indicating that no extra-cellular emulsifying surfactants were produced. *Williamsia muralis* N1261^{Tsp} produced only weak emulsifications (<10%) with the highest measurements on day 7.

4.3.2.3. Biosurfactant production during growth determined by cell surface hydrophobicity. Mean cell surface hydrophobicity (CSH) measurements by the BATH assay, determined for *Rhodococcus ruber* IEGM 231^T and *R. yunnanensis* DSM 44837^T whole cell broth (WCB) samples, were low at the early stages of growth and increased during exponential growth of reaching maximum values (89.10 ± 0.% and 51.07 ± 0.06%, respectively) on day 4 when maximum viable counts were also observed (Fig. 4.4). These strains exhibited a decrease in CSH during the stationary phase with values dropping rapidly by day 10 and near to zero by day 14 (Appendix 7.9, 7.10, 7.11, page 283-285). The CSH of *Williamsia muralis* N1261^{Tsp} was high (~80%) at the start of batch cultivation and remained so until day four when the maximum viable plate counts (cfu ml^{-1}) were recorded before dropping gradually to moderate values (40 to 59 %; mixed hydrophobic and hydrophilic properties) by day 8 and ~20% (hydrophilic cells) by day 14. Spearman's correlation coefficient (r_s) only showed a weak positive correlation with viable plate count data (r_s = 0.26).



4.4. Relationships between growth measured by viable plate counts (cfu ml^{-1}) and cell surface hydrophobicity determined from whole cell broths using the BATH assay for a) *Rhodococcus ruber* IEGM 231^{T}, b) *R. yunnanensis* DSM 44837^{T} and, c) *Williamsia muralis* N1261^{Tsp} during batch cultivation for 14 days in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C with orbital shaking (180 rpm) . Data are expressed as the mean ± standard deviation of three technical replicates.

4.3.3. Isolation of glycolipid-containing extracts by thick layer chromatography.

In this study, the glycolipid- containing extract wee isolated from crude total biosurfactant extracts from *Rhodococcus ruber* IEGM 231^{T}*, R. yunnanensis DSM 44837^{T,}* and *Williamsia muralis* N1261^{Tsp} by preparative thick layer chromatography. The presence of glycolipids in these extracts was confirmed by TLC and ornicol reagent which revealed either a single discrete brown-purple spot or in the case of *R. ruber*, two discrete spots (data not shown) with similar R_f values to the solvent extracted biosurfactant samples obtained in Chapter 3 (section 3.3.1., page 116). The glycolipid-containing extracts isolated from all three strains tested positive (++) for the oil spreading assay confirming surface tension properties. The crude glycolipid extracts were then investigated for their growth inhibition and anti-biofilm properties.

4.3.4. Growth inhibition by crude biosurfactant and glycolipid-containing extracts.

Crude biosurfactant extracts (prepared in the previous study) and crude glycolipid-containing extracts from *Rhodococcus ruber* IEGM 231^{T}*, R. yunnanensis* DSM 44837^{T} and *Williamsia muralis* N1261^{Tsp} were tested for their ability to inhibit the growth of *Pseudomonas aeruginosa* R222 and *Klebsiella pneumoniae* NCIMB 8865 by the agar well diffusion method. Some inhibition of lawn growth was observed for both the crude biosurfactant extracts and glycolipid-containing extracts from all three actinobacteria but less than the commercial chemical surfactants Triton X-100 and Tween 80 (Table 4.1). The glycolipid-containing extract from *R. ruber* IEGM 231^{T} exhibited the greatest inhibitory activity of all the biosurfactant samples, creating a mean zone of inhibition of 6 ± 0.01mm against *Pseudomonas aeruginosa* R222.

Table 4.1. Growth inhibition activity of crude biosurfactant and glycolipid-containing extracts against biofilm-forming strains using the agar well diffusion metho.

Bio/surfactant	Zone of inhibition (mm)	
	Pseudomonas aeruginosa R222	*Klebsiella pneumonia* NCIMB 8865
Triton X-100 (commercial surfactant)	9 ± 0.01	8 ± 0.02
Tween 80 (commercial surfactant)	8 ± 0.02	6 ± 0.03
Sterile water (negative control)	0 ± 0.00	0 ± 0.00
Rhodococcus ruber IEGM 231^{T}		
Biosurfactant extract	5 ± 0.05	3 ± 0.02
glycolipid-containing extract	6 ± 0.01	4 ± 0.01
Rhodococcus yunnanensis DSM 44837^{T}		
Biosurfactant extract	3 ± 0.02	4 ± 0.03
glycolipid-containing extract	2 ± 0.01	3 ± 0.01
Williamsia muralis N1261^{Tsp}		
Biosurfactant extract	2 ± 0.04	3 ± 0.06
glycolipid-containing extract	2 ± 0.01	4 ± 0.02

4.3.5. Anti-biofilm effects of glycolipids-containing extracts on biofilm-forming bacteria.

The anti-adhesion, inhibition, and disruption properties of the extracts containing glycolipid complexes from *Rhodococcus ruber* IEGM 231^{T}*, R. yunnanensis* DSM 44837^{T,} and *Williamsia muralis* N1261^{Tsp} were tested against the biofilm-forming strains *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumoniae* NCIMB 8865 in static polystyrene microplates using the 2,3,5-TTC - triphenyl tetrazolium chloride (TTC) assay to quantify metabolically active cells in biofilms.

4.3.5.1. Anti-adhesion effects of glycolipid-containing extracts. When applied neat, the extracts containing glycolipid complexes from *R. ruber* IEGM 231^{T} and *W. muralis* N1261^{Tsp} showed similar ($p > 0.05$) anti-adhesion activity against *K. pneumoniae* NCIMB 8865 resting cells (mean reduction of 54-58%) while the extract from *R. yunnanensis* DSM 44837^{T} had significantly lower anti-adhesion

activity (mean 32.9 ± 0.08%)(Fig. 4.5a, page 177). The glycolipids-containing extracts from all three strains were less effective against *P. aeruginosa* ENU 18 and had similar anti-adhesion profiles ($p<0.05$). The extract from *W. muralis* had the highest anti-adhesion activity (mean 39.6 ± 0.06%) while those from *R. yunnanensis* DSM 44837^T and *R. ruber* IEGM 231^T reduced cell adhesion by 30 ± 0.1% and 22 ± 0.12%, respectively (Fig. 4.6, page 178). A marked reduction in anti-adhesion activity (potency) was observed when the glycolipid-containing extracts were diluted 1:2, with minimal activity against *K. pneumoniae* NCIMB 8865 at the 1:6 dilutions (<2% reduction in adhesion) and against *P. aeruginosa* ENU 18 at the 1:4 dilutions (<7%), below which anti-adhesion activity was absent.

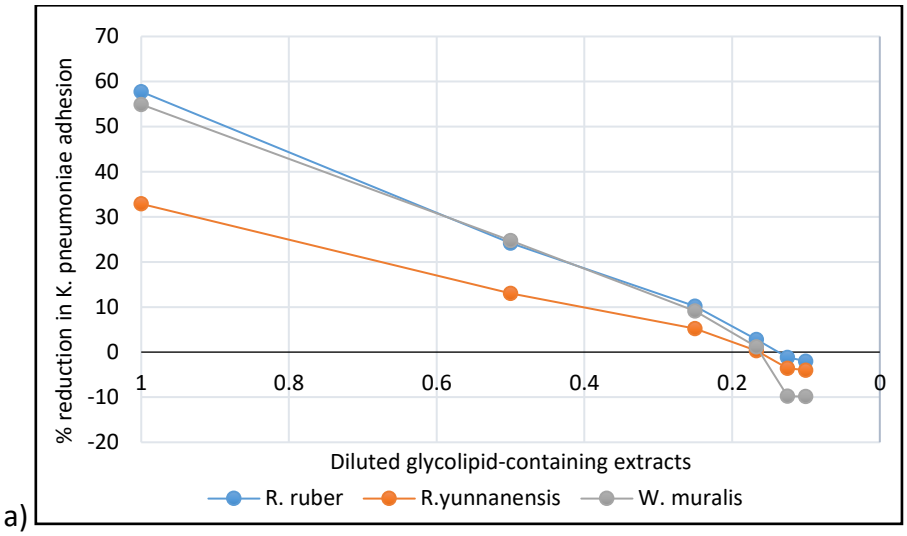
The anti-adhesion activities of the glycolipid-containing extracts were not significantly different ($p>0.05$) from those observed for the total biosurfactant extracts studied in Chapter 3 (Fig. 4.7a-c, page 179). Indeed, the glycolipid-containing extract from *R. ruber* reduced the adhesion of *P. aeruginosa* ENU 18 cells by 22.1 ± 0.12% compared to only 5.7 ± 0.06% by the total crude biosurfactant extract, and this difference was significant (Fig. 4.7a., page 179). The exception was the *R. yunnanensis* DSM 44837^T iglycolipid-containing extract which only reduced the adhesion of *K. pneumoniae* NCIMB 8865 cells by 32.9 ± 0.08 % compared to 53.1 ± 0.07 % achieved by the total crude biosurfactant extract (Fig. 4.7b, 179 and Appendix 7.12., 286).

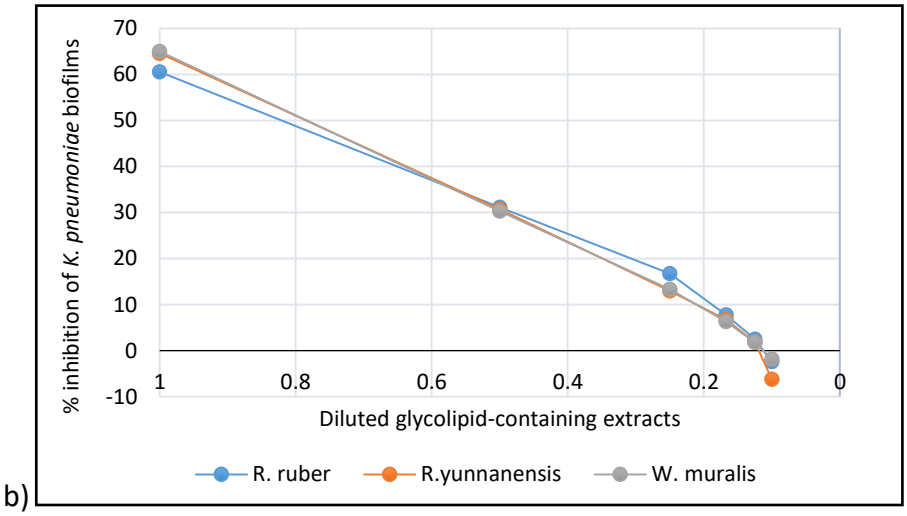
4.3.5.2. Biofilm inhibition effects of the glycolipid-containing extracts. The development of *K. pneumoniae* NCIMB 8865 static biofilms was inhibited to a similar extent (~60.6 ± 0.24 - 65 ± 0.04%) by all three glycolipid-containing extracts(undiluted) with no significant differences between these ($p>0.05$) (Fig 4.5b, page 177). The growth inhibition profiles of *P. aeruginosa* ENU 18 exposed to the extracts from the three strains were similar although the undiluted extract from *R. ruber* IEGM 231^T showed the most inhibition (53.6 ± 0.08%), while those produced by *R. yunnanensis* DSM 44837^T and *W. muralis* N1261^{Tsp} achieved 43.2 ± 0.06% and 35.52 ± 0.04%, respectively ($p<0.05$) (Fig 4.6b, page 178). Biofilm inhibition activity decreased when the glycolipid-containing extracts were diluted, although potency was higher than for anti-adhesion with

minimal activity observed at the 1:8 dilutions (<2% biofilm inhibition) against *K. pneumoniae* and at the 1:6 dilutions (<4%) against *P. aeruginosa*, below which no inhibition occurred.

Although these extracts demonstrated high inhibition activity against *K. pneumoniae* NCIMB 8865 the values were significantly lower ($p>0.05$) than observed for the corresponding total crude biosurfactant extracts (mean ~76.9 ± 0.11- 84.5 ± 0.09%) (Fig. 4.7a-c., page 179). This was also the case for the *R. yunnanensis* DSM 44837^T glycolipid-containing extract against *P. aeruginosa* ENU 18 (Fig. 4.7b, page 179). By contrast, however, the glycolipid-containing extracts from *R. ruber* and *W. muralis* showed significantly higher inhibition (19.1 ± 0.07% and 8.2 ± 0.07%, respectively) against *P. aeruginosa* ENU 18 compared to the corresponding total crude biosurfactant extracts, albeit still low values.

4.3.5.3. Biofilm disruption effects of the glycolipid-containing extracts. The glycolipid-containing extracts from all three test strains showed the weakest effects when exposed to *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumoniae* NCIMB 8865 as pre-formed biofilms. Undiluted extracts from *R. yunnanensis* DSM 44837^T disrupted static *K. pneumoniae* biofilms by (27.4 ± 0.3%) while those from the other strains achieved only weak disruption (<12%)(Fig 4.5c., page 177). The undiluted glycolipid-containing extract from *R. ruber* IEGM 231^T disrupted pre-formed biofilms of *P. aeruginosa* ENU 18 by 25.9 ± 0.15% while those from the other strains only achieved <8% disruption (Fig. 4.6c, page 178). The potency of the extracts was low with minimal activity observed at the 1:4 dilutions for the *R. yunnanensis* extract against *K. pneumoniae* NCIMB 8865 and the *R. ruber* extract against *P. aeruginosa* ENU 18 (<5% disruption). No significant differences were found between the disruption activities of the glycolipid-containing extracts and the total crude biosurfactant extracts (Fig. 4.7a-c., page 179).





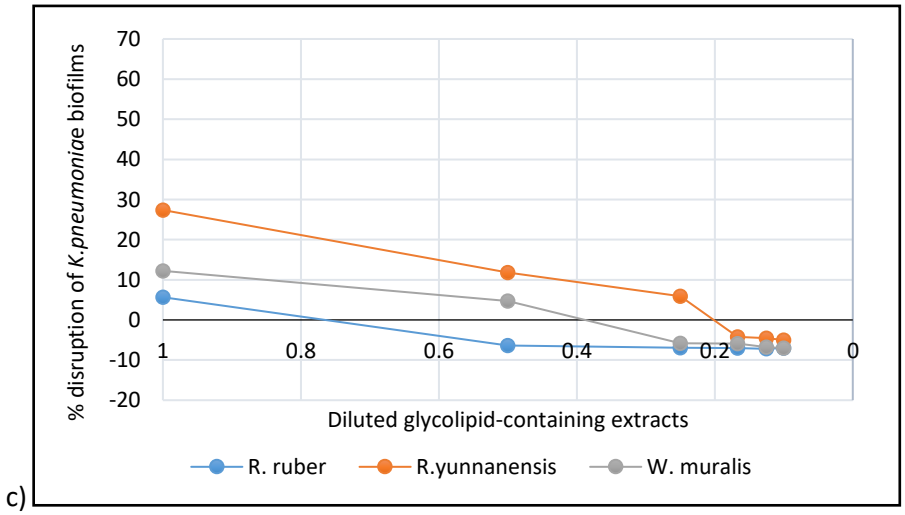
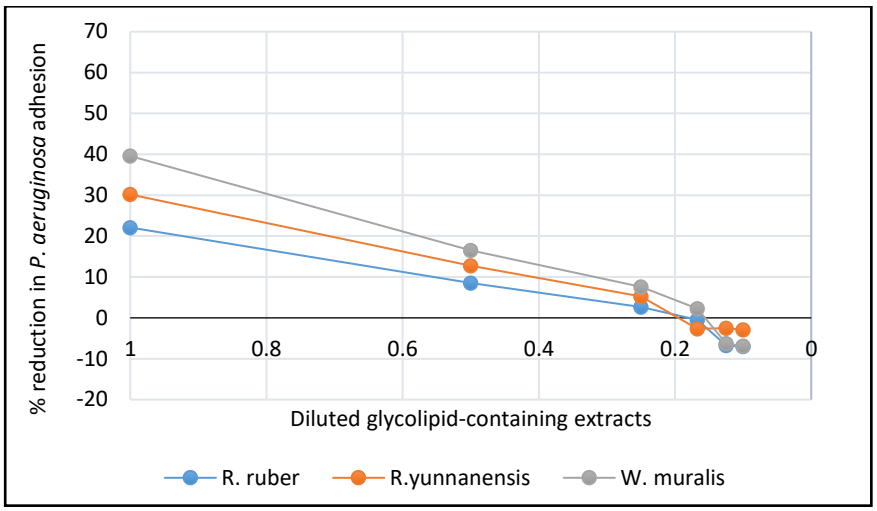
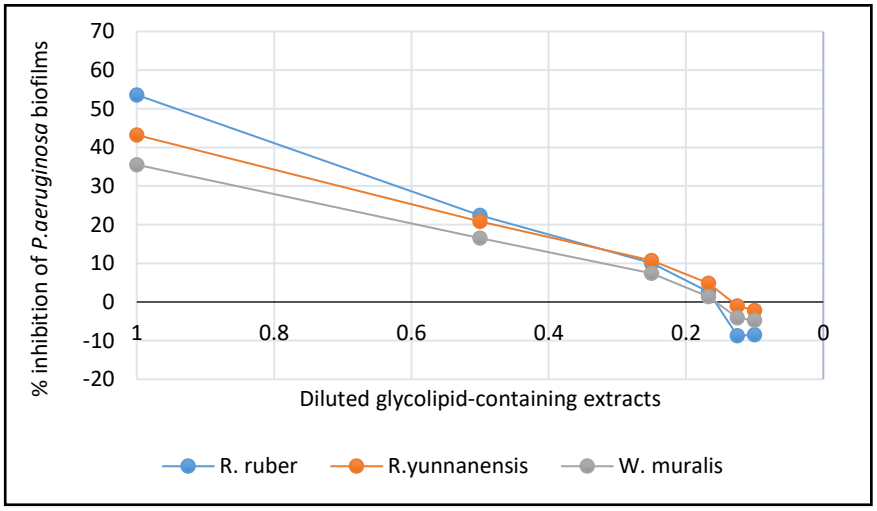


Fig. 4.5. The effects of neat and diluted glycolipid-containing extracts from *Rhodococcus ruber* IEGM 231^{T}, *R. yunnanensis* DSM 44837^{T} and *W. muralis* N1261^{Tsp} on a) adhesion, b) inhibition and c) disruption of *Klebsiella pneumoniae* NCIMB 8865 biofilms in polystyrene microplate wells, quantified using 0.1% w/v 2,3,5-triphenyl tetrazolium chloride (TTC) dye measured at 450nm wavelength. The untreated negative controls were taken as 100% biofilm formation to calculate the percent adhesion, inhibition, and disruption. Data expressed as the mean ± standard deviation of three biological replicates.

a)



b)



c)

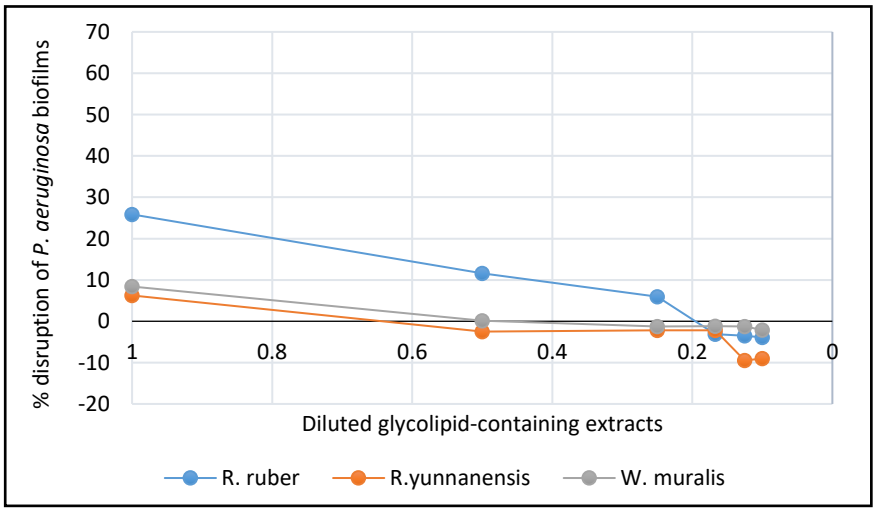


Fig. 4.6. The effects of neat and diluted glycolipid-containing extracts from *Rhodococcus ruber* IEGM 231^{T}, *R. yunnanensis* DSM 44837^{T,} and *W. muralis* N1261^{Tsp} on a) adhesion, b) inhibition, and c) disruption of *Pseudomonas aeruginosa* ENU 18 biofilms in polystyrene microplate wells, quantified using 0.1% w/v 2,3,5-triphenyl tetrazolium chloride (TTC) dye measured at 450nm wavelength. The untreated negative controls were taken as 100% biofilm formation to calculate the percent adhesion, inhibition, and disruption. Data expressed as the mean ± standard deviation of three biological replicates.

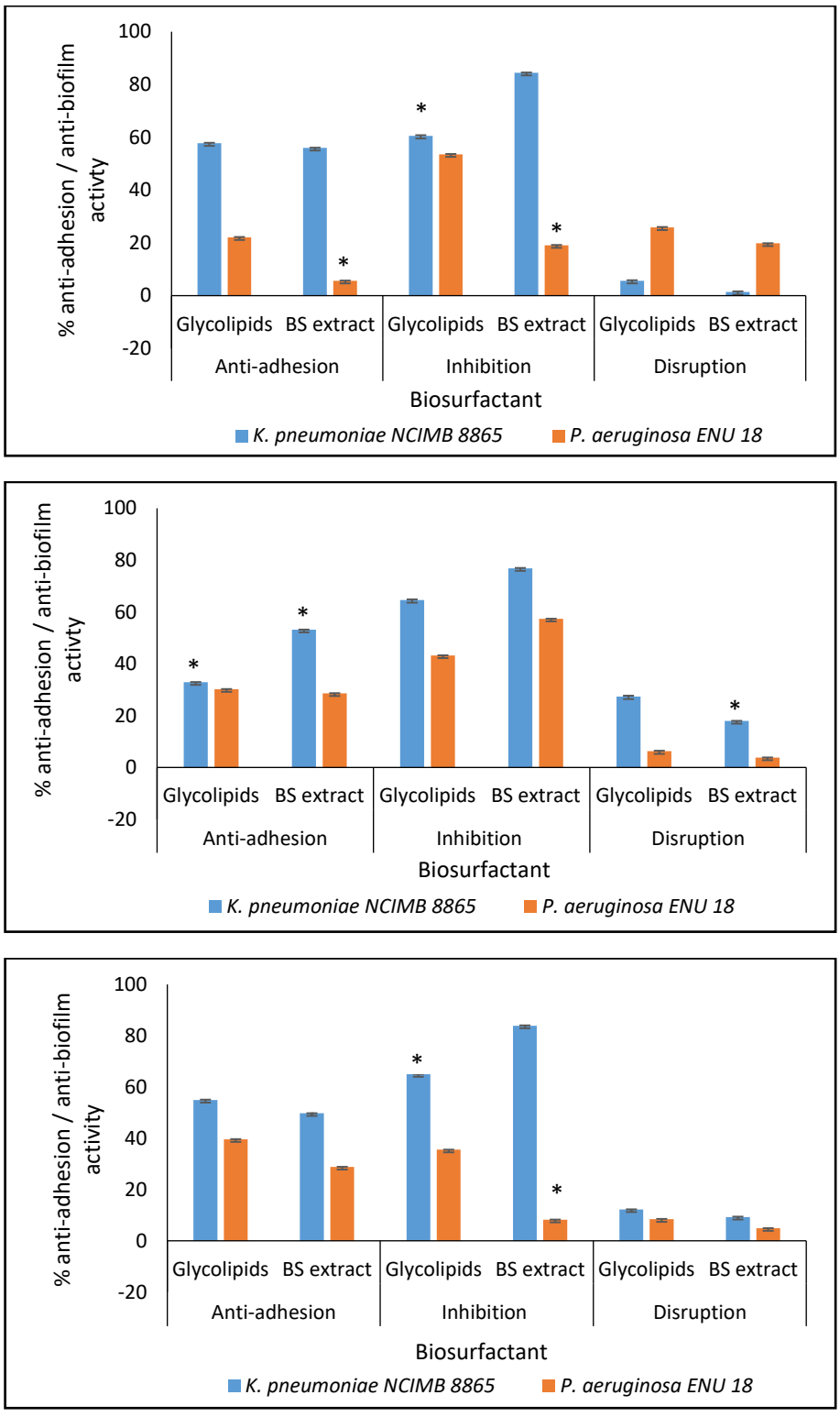


Fig. 4.7. Comparison of the anti-biofilm effects of total crude biosurfactant extracts and glycolipid-containing extracts from a) *Rhodococcus ruber* IEGM 231^{T}, b) *R. yunnanensis* DSM 44837^{T,} and c) *Williamsia muralis* N1261^{Tsp} against *Klebsiella pneumoniae* NCIMB 8865 and *Pseudomonas aeruginosa* ENU 18 static biofilms quantified using 0.1% w/v 2,3,5-triphenyl tetrazolium chloride (TTC) dye measured at 450nm wavelength. The untreated negative controls were taken as 100% biofilm formation to calculate the percent adhesion, inhibition, and disruption. Data expressed as the mean ± standard deviation of three biological replicates. *, statistical difference ($p<0.05$) between isolated glycolipids and crude biosurfactant extracts at one anti-biofilm activity.

4.3.6. Characterisation of actinobacterial glycolipid-containing extract by MALDI-TOF.

Prior to characterising the isolated glycolipid-containing extracts from the three test strains, the R90 rhamnolipid standard from *Pseudomonas aeruginosa* (Sigma Aldrich, UK) was analysed using MALDI-TOF-TOF to identify the most suitable matrix and conditions. Data acquisition was performed in the reflector positive ion and negative ion modes after dissolving in three separate matrices, 9-Amino Acridine (9-AA in 3:2 IPA: MeCN), 9:1 mixture of DHB: DHAP (in MeCN: H_2O), and α-hydroxycinnamic acid (CHCA; in 70% MeCN: 5% formic acid).

The positive ion analysis was most informative with a multitude of ions observed from 400 to 1400 Da. The sodium $[M+Na+]^+$, disodium $[M+2Na-H]^{+,}$ and potassium $[M+K]^+$ adduct ions were found to be common when analysed using different matrices which is generally the case with MALDI-TOF analysis of glycolipids. The spectra generated from the positive ion analyses using 9-AA and DHB matrices were more informative than those generated using CHCA (Appendix 7.13, 7.14., page 287-288). Metastable ions, observed as broad, unresolved ions due to decomposition in the field-free region, were observed in the spectra using CHCA as well as a few identifiable rhamnolipids and many other unidentified peaks. The negative ion data were not informative, with polymeric species from *m/*z 366Da observed in 9-AA, ions that may represent +Na and +K adducts of a component of molecular weight 308 and perhaps DHB adducts at *m/*z 499 and 649.

The glycolipid-containing extracts from *Rhodococcus ruber, R. yunnanensis,* and *Williamsia muralis* N1261^{Tsp} were analysed using DHB as the preferred matrix. As with previous data, positive ion spectra were more informative than negative ion and so were processed more fully. These analyses confirmed the presence of several ions identified as mono-rhamnolipid and di-rhamnolipid moieties although the samples were complex (Table 4.2 and Appendix 7.15, 7.16, 7.17, page 291-3295). The three test strains gave rise to similar rhamnolipid profiles: the predominant molecules were mono-Rha-C_{10}-$C_{12:1}$ (*m/z* 553/569, $[M+Na]^+$/ $[M+K]^+$] and di-Rha-C_{12} (*m/z* 553, $[M-H+2Na]^+$ for all three test strains (Table 4.2).

Table 4.2 Rhamnolipid molecular ions observed in the glycolipid-containing extracts from the actinobacterial strains isolated by thick layer chromatography, by positive ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) analysis with a mass range of 300-4000 mass units.

Rhamnolipid compound	**Formula**	**Adduct**			**Glycolipid producing strains**		
		M+Na	**M-H+2Na**	**M+K**	***R. ruber* IEGM 231^{T}**	***R. yunnanensis* DSM 44837^{T}**	***W. muralis* N1261^{Tsp}**
Dokdolipid C	$C_{30}H_{56}O_{12}$	631	653	647	Weak 653	×	631/647
Rha-C_{8}-$C_{10:1}$	$C_{24}H_{42}O_{9}$	497	519	513	×	519	×
Rha-C_{10}-$C_{12:1}$	$C_{28}H_{50}O_{9}$	553	575	569	553/569	553/569	553/569
Rha-Rha-C_{12}	$C_{24}H_{44}O_{11}$	531	553	547	553	553	531/553
Rha-Rha-C_{8}-C_{10}	$C_{30}H_{54}O_{13}$	645	667	661	645/661	×	×
Rha-Rha-C_{10}-C_{10}	$C_{32}H_{58}O_{13}$	673	695	689	×	×	Weak 689
Rha-Rha-C_{10}-C_{12}	$C_{34}H_{62}O_{13}$	701	723	717	701	701/723	701/723
Rha-Rha-C_{10}-C_{12}	$C_{34}H_{60}O_{13}$			715	×	×	715
3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid	$C_{36}H_{64}O_{10}$	679	701	695	679/701	701	701
Rha-Rha-C_{14}-C_{14}	$C_{40}H_{74}O_{13}$	785	807	801	Weak 785	×	×
Rha-Rha-C_{14}-C_{16}	$C_{42}H_{78}O_{13}$	813	835	829	×	829	835

Abbreviations: M+Na, sodium adduct; M-H+2Na, disodium adduct; M+K, potassium adduct; Rha, rhamnolipid.

Additional minor ions identified as di-Rha-$C_{10\text{-}C12}$ (*m/z* 701, $[M+Na]^+$) and 3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid (*m/z* 701, $[M-H+2Na]^+$) were also produced by all three test strains. The glycolipid-containing extracts from *Rhodococcus ruber* IEGM 231^T contained a mixture of five rhamnolipid congeners with two additional weak ion peaks and unlike the other strains included Rha-Rha-C_8-C_{10} and a weak ion peak identified as Rha-Rha-C_{14}-C_{14}. Similarly, the *R. yunnanensis* DSM 44837^T glycolipid-containing extracts produced 5 rhamnolipid congeners and one additional weak ion peak only found in this strain (Rha-Rha-C_{14}-C_{16}). Six rhamnolipid congeners were detected in the *Williamsia muralis* $N1261^{Tsp}$ glycolipid-containing extrac twhich uniquely contained weak amounts of Rha-Rha-C_{10} and Rha-Rha-C_{10}-C_{10}. (Table 4.2., page 181). The remaining undefined peak could not be assigned to the glycolipid maps.

4.4. Discussion

This study established the relationships between batch growth on hexadecane and extracellular biosurfactant production by the mycolic acid-containing actinobacteria *Rhodococcus ruber* IEGM 231^{T}, *Rhodococcus yunnanensis* DSM 44837^{T,} and *Williamsia muralis* N1261^{Tsp}. Further, extracellular glycolipid-containing extracts were isolated from each strain to characterise the component compounds by MALDI-TOF-MS and to determine the growth inhibition and anti-biofilm properties against clinically relevant biofilm-forming strains.

4.4.1. Biosurfactant production during batch growth.

Accurate growth measurement proved technically challenging in this study largely due to the nature of growth on hexadecane, which led to the non-homogeneous dispersion of cells. Indeed, it is the production of surface-active compounds and the increase in cell surface hydrophobicity to facilitate the uptake of the hydrophobic substrate that are largely responsible for this phenomenon. Biosurfactants alter the cell surface hydrophobicity and reduce interfacial tension to enable access and adherence to hydrophobic substrates thereby enabling efficient transport of hydrophobic substances across the cell membrane (Krasowska & Sigler, 2014). Indeed, Philp *et al*. (2002) suggested that the production of biosurfactants by rhodococci is a pre-requisite for growth on n-alkanes such as hexadecane to increase the bioavailability and solubility of the hydrophobic organic compound. This may lead to effective emulsification of oil in liquid with the hydrophobic substrate distributed as tiny droplets in the culture medium, allowing for homogenous growth. However, more often, emulsification is limited or negligible, and instead, cells must gather at the interface between the immiscible liquids to gain access to the upper hydrophobic layer, leading to non-homogeneous growth in the culture medium.

In this study, the biomass tended to flocculate and partition in the upper hydrophobic layer and adhered to the inner glass walls of the flasks, resulting in less turbid growth in the lower liquid medium. Despite efforts to distribute

growth evenly prior to sampling, likely, measurement of growth by biomass dry weight (g l^{-1}), turbidity (OD_{600}nm), and viable plate counts (expressed as cfu ml^{-1}) were inaccurate. Quite different batch growth curves were plotted using each of these methods and this was reflected in the weak correlations.

The differences observed between viable plate counts and turbidity measurements are not uncommon and most likely explained by the nature of cellular growth by the test strains: *R. ruber* IEGM 231^T forms branching mycelia that fragment to shorter rods and cocci as it transitions through different growth phases (Goodfellow & Alderson, 1977), *R. yunnanensis* DSM 44837^T forms hyphae that fragment into short rods and coccobacilli (Zhang *et al*., 2005) and, *W. muralis* is pleomorphic forming rod-shaped and coccoid cells (Kämpfer *et al*., 1999). Optical density measurements rely on the homogenous distribution of a single cell type, whilst viable plate counts, assume each colony to have arisen from a single cell whereas in this study it is likely that various cell types, including hyphae, were present and indeed may have clumped together, leading to inaccurate measurements. Dry weight biomass (g l^{-1}) provided only crude measurement of growth and did not discern changes in growth that were observed with the other two methods. This is most likely because the working volume of the cultures limited the sample volume that could be used to determine biomass weight.

Other researchers have noted this actinomycetal type growth to be problematic while studying mycolic acid-containing actinobacteria. Philp *et al*. (2002) monitored the growth of *R. ruber* IEGM 231^T by biomass dry weight, which included a washing step with petroleum ether to remove adhered hexadecane and biosurfactants followed by filtration to ensure only cells remained. This was not undertaken in the current study and may partly account for the lack of accuracy found with biomass dry weight measurements. Kügler *et al.* (2014) found the measurement of absolute dry cell mass rather than broth dry cell mass was more accurate due to the attachment of cells to the inside of the growth vessel during the cultivation of *Tsukamurella* strains. However, this requires larger culture volumes but would appear to be the best approach

alongside a biomass washing step to enable accurate determination of batch growth kinetics.

Nevertheless, viable plate counts gave rise to normal batch growth curves and better-discerned change in growth over time than optical density or biomass dry weight. Based on viable count data, total biosurfactant synthesis was found to be growth-associated as determined by surface tension reduction, with cell-bound biosurfactants produced during the exponential phase and released as extracellular biosurfactants as growth decelerated and entered the stationary phase. Previous studies have reported rhodococci to majorly produce cell-associated biosurfactants with some strains able to release a small amount extracellularly (Kim *et al*., 1990; Ristau & Wagner 1983). This mechanism of biosurfactant production is considered common amongst alkanotrophic rhodococci (Ivshina *et al*.,1998). Philip *et al*. (2002) made a similar observation when *R. ruber* IEGM 231^{T} was grown on hexadecane, with surface tension reduced maximally to 27.6 mN m^{-1} mid-way through exponential growth. It should be noted that the sampling intervals in the current study were only every 24 h and given the short lag and rapid exponential phases that occurred within the first 48 h, the precise course of growth during this period was not captured in detail. If repeated, these studies would include more frequent sampling intervals.

The initial cell-bound nature of biosurfactant production shown by surface tension measurement was supported by the observed increase in cell surface hydrophobicity (CSH), if this is taken as an indirect measure of cell-bound surfactants. Previous studies with *Rhodococcus* strains reported a similar increase in CSH during exponential growth utilising water-soluble substrates, followed by a decrease in the stationary phase showing the ability of cells to regulate CSH according to the growth (Bredholt *et al*., 2002; Cappelletti *et al*., 2019; Whyte *et al*., 1999).

These results also indicate the production of cell-bound growth-associated bioemulsifiers as evidenced by the increase in EI_{24} values during exponential growth. Indeed, a positive correlation was observed between growth with CSH

and EI_{24} measurements. Further, the strongest positive correlation for emulsification in the CFS was observed with biomass dry weight (r_s = 0.97), perhaps because the latter reached a maximum on day 7 for this strain. Bioemulsifiers are typically of higher molecular weight than biosurfactants and can efficiently emulsify two immiscible liquids but in contrast to biosurfactants, they are less effective at surface tension reduction (Uzoigwe *et al*., 2015). However, in this study, the bioemulsifiers do not appear to be released into the surrounding medium but instead, remain cell-associated as evidenced by low or zero EI_{24} readings in cell-free supernatants throughout incubation. No emulsification activity occurred in the CFS samples for *R. ruber* and *R. yunnanensis* while only weak (~10%) emulsification was observed in CFS samples from *W. muralis*. It can be concluded therefore that the extracellular biosurfactants produced by the test strains showed surface tension reduction properties but lacked emulsification properties. Bell *et al.* (1998) and Zeng *et al.* (2009) noticed a similar result for *Rhodococcus* strains, reporting an increase in CSH and emulsification with increasing cell density and that biosurfactants mostly remained attached to the cell wall. *Rhodococcus* spp. growing on hexadecane showed similar cell-associated emulsification activities in studies by Hafesburg *et al*. (1986) and Philip *et al*. (2002). The production of bioemulsifiers likely aids in the attachment of cells to hydrophobic substrates and the transport across the cell membrane.

The critical micelle dilution (CMD), defined as the reciprocal of the dilution at which a sharp increase in surface tension is observed in a sample, is a useful parameter to determine changes in biosurfactant concentration once the maximum reduction in surface tension has been reached. However, the CDM only provides an indication of the biosurfactant concentration. In the present study, the CDM values measured over the cultivation period represented changes in total (WCB) or extracellular biosurfactant (CFS) amounts, but the actual concentrations were unknown. The results revealed that maximum total biosurfactant accumulation, determined from WCB samples, coincided with the maximum biomass accumulation at the end of the exponential phase confirming that production was growth associated. To the contrary, extra-

cellular biosurfactants were found to accumulate to a maximum at day 7 and there was no correlation between growth and biosurfactant accumulation as determined by surface tension reduction, in cell-free supernatants. This confirmed that extra-cellular biosurfactant production was non-growth associated and instead, these components were released during deceleration and stationary phase. In addition, t

This is the first study of biosurfactant production during batch cultivation of *R. yunnanensis.* The results for *Rhodococcus ruber* IEGM 231^{T} were in accordance with the previous study by Philip *et al.* (2002) which reported that this strain achieved a maximum CMD of 1:100 after 2 days of growth and this remained constant until the termination of the experiment at day 4. In the present study, this strain achieved a CMD of 1:100 until day 4, and reached maximum of 1:500 after growth for 7 days in a medium containing hexadecane. Hence, a longer incubation time in the present study was found to be suitable for increasing biosurfactant production. The higher CMD values obtained for the WCB compared to the CFS samples indicate higher concentrations of total biosurfactants than extracellular biosurfactants were produced by the rhodococcal test strains. In turn, this suggests only partial release of biosurfactants during the stationary phase whilst some remained cell-bound. Interestingly, *Williamsia muralis* provided higher CMD values in cell-free supernatants than the other strains (1:150) yet the lowest value (1:100) in whole-cell broth. This suggests that this strain releases a greater proportion of biosurfactants extracellularly. It is evident from these experiments that the length of incubation is critical to ensure maximum recovery of extra-cellular biosurfactants. No other studies have investigated extra-cellular biosurfactant production during growth of *Williamsia* species to date. The findings of this study demonstrate that the test strains must be grown beyond the exponential phase into the stationary phase to ensure maximum biosurfactant accumulation.

4.4.2. The production of extra-cellular rhamnolipid glycolipids as determined by MALDI-TOF-MS.

Biosurfactants produced by actinobacteria include mainly trehalose-comprising surfactants, other non-trehalose-containing glycolipids and lipopeptides but also other rarer actinobacterial surfactants. Glycolipids encompass a range of compounds including trehalose lipids, trehalose lipid mycolic acid esters, oligosaccharide lipids, and other non-trehalose lipids including rhamnolipids (Kügler *et al*., 2015). In this study, the three test strains were found to produce glycolipids, and analysis of extra-cellular biosurfactant extracts containing glycolipid complexes, using MALDI-TOF MS revealed the presence of mixtures of mono- and di-rhamnolipid congeners.

Rhamnolipids contain one (mono-) or two (di-) rhamnose sugars linked to β-hydroxylated fatty acid chains. Approximately 60 congeners have been reported, which vary in the chain length, number of rhamnose molecules, and degree of unsaturation for the fatty acid chains (Abdel-Mawgoud *et al*., 2010). Medium composition and cultivation conditions are known to alter the composition and physico-chemical properties of the rhamnolipid congeners produced (Abdel-Mawgoud *et al*., 2014; Hošková *et al*., 2015; Paulino *et al*., 2016). Rhamnolipids have various useful properties such as emulsification, surface activity, and wetting ability (Abdel-Mawgoud *et al*., 2010) and have also been reported with anti-biofilm properties (Dusane *et al*., 2011, 2012; De Rienzo & Martin, 2016; Irie *et al*., 2005).

This is the first study to report the production of rhamnolipids by *Rhodococcus ruber* IEGM 231^{T} although production has been reported for a different strain from this species (*R. ruber* TES III; Kumari, *et al*., 2022). Previous studies on *R. ruber* IEGM 231^{T} have reported on trehalose-containing glycolipids (Philp *et al*., 2002; Kuyukina *et al*., 2001, 2016). This study is also the first to report rhamnolipid production by *Rhodococcus yunnanensis* DSM 44837^{T} and *Williamsia muralis* N1261^{Tsp} although these have been reported for other mycolic acid-containing actinobacteria including *Dietzia maris* which produced di-rhamnolipid after 10 days of growth on hexadecane (Wang *et al*., 2014), *Nocardia otitidiscaviarum* MTCC 647 after 7 days of growth in crude oil (Vyas &

Dave, 2011) and *Rhodococcus fascians* strain A-3 when grown in glucose and kerosene (Gesheva *et al.,* 2010). However, rhamnolipid production is predominantly associated with *Pseudomonas* and *Burkholderia* species and these have been more thoroughly investigated in the literature (Abdel-Mawgoud *et al*., 2010).

The purified R90 rhamnolipid standard was found to be unsuitable as an analytical standard as it was very complex and exhibited too much heterogeneity with respect to +Na and +K adduction to allow identification of every individual mono- and di-rhamnolipid species with confidence, though several were clearly detected in all positive ion spectra using each of the three matrices. Nevertheless, it allowed the determination of the most suitable conditions for analysis of the glycolipid complexes isolated from the test strains *Rhodococcus ruber, R. yunnanensis, and Williamsia muralis*.

MALDI-TOF analysis was used in this study, as this method does not require highly purified samples for detection. This in turn, makes sample preparation relatively straightforward compared to other chemical analytical techniques which require highly purified samples (Elbehiry *et al*., 2022). Moreover, MALDI-TOF can indicate the type of biosurfactant present along with the purity of the sample saving time before more highly advanced chemical analytical methods are used (Zompra *et al*., 2022). In this study, the biosurfactant extracts were separated by preparatory thick layer chromatography which does not guarantee purification of glycolipids. It must be recognised therefore that these samples also contain other compounds, both biosurfactants and possibly non-biosurfactants. The samples, therefore, were not expected to be pure and MALDI-TOF was the most appropriate next step in determining the constituents of these extracts through chemical analytical techniques.

MALDI-TOF analysis was able to identify various congeners of rhamnolipids from the extracts containing glycolipid mixtures and did not require highly purified samples for analysis. Indeed, it was evident from the MALDI-TOF results that other unresolved components were present. Other workers have also utilised MALDI-TOF successfully to detect glycolipids in crude extracts with

high precision with no need to fractionate and purify the detected compounds (Kurtzman *et al*., 2010; Sato *et al*., 2019). However, in this study, the spectra obtained suggest that additional purification and analyses are required to fully elucidate the composition of the extracellular isolated glycolipid complex from each test strain either by using MALDI-TOF or one or more additional chemical analytical techniques such as nuclear magnetic resonance spectroscopy (Patil *et al*., 2018) which is considered the gold-standard for structural elucidation of unknown biosurfactants.

4.4.3. Growth inhibition and anti-biofilm properties of the extra-cellular glycolipid-containing extracts.

The results of the well diffusion assay indicate that glycolipid-containing extracts isolated from *Rhodococcus ruber, R. yunnanensis,* and *Williamsia muralis* exhibited only weak or no growth inhibition activity against *Klebsiella pneumoniae* NCIMB 8865 and *Pseudomonas aeruginosa* ENU 18. In comparison, the non-ionic commercial surfactants Triton-X and Tween 80 produced larger zones of inhibition (ZOI). Both Tween 80 and the glycolipid-containing extract from *R. ruber* achieved the same ZOI. The inhibition activity was determined using non-ionic surfactants Triton X and Tween 80 used for comparison as chemical surfactants, which showed true inhibition and therefore used as interpretation to study inhibition activity.

It is anticipated that these chemical surfactants would possess antibacterial properties against clinical strains; however, this was not confirmed and therefore these were not appropriate positive controls. Consequently, while this study demonstrates a similar, albeit weaker, effect of biosurfactant extracts compared to chemical surfactants and similar ZOIs for total and glycolipid-containing extracts, the absence of a positive control means that it is not possible to differentiate between positive and negative ZOI's.

A previous study by Kuyukina and co-workers (2016) reported a similar lack of anti-bacterial activity by *R. ruber* IEGM 231^{T} against gram-positive and gram-negative bacteria determined in 96-well flat-bottomed polystyrene microplate using different biosurfactant concentration while the biosurfactant showed

anti-adhesion activity. The workers suggested that different biosurfactant concentrations can form different structures on the surface, which surrounds the cells and thus do not affect bacterial cell viability. Zhao *et al*. (2022) reported a weak antimicrobial activity of rhamnolpid against Gram-negative bacteria using agar well diffusion and turbidimetric methods. Díaz De Rienzo reported no antimicrobial activity of rhamnolipid against *P. aeruginosa* studied when compared to sodium dodecyl sulphate (SDS) using the agar well method. Although they looked at different concentrations and did MIC tests in liquid culture for comparison. However, Ndlovu *et al*. (2017) reported rhamnolipid antimicrobial activity against *Klebsiella pneumonia* strains detected using the agar disc method.

The well diffusion assay seems to be more sensitive to diffusion issues of non-polar compounds (King *et al*., 2008, Klancnik *et al*., 2010). While it does not significantly impede the migration of biosurfactants in comparison to liquids, the mobility of biosurfactants in agar may be somewhat slower than in liquid media because they need to diffuse through the agar matrix. The extent to which agar affects the migration of biosurfactants probably depends on several factors, including the concentration of agar in the medium and the chemical properties of the biosurfactants involved. Further, the concentration of glycolipid–containing extract, the volume of extract in the well hole, the density of inoculum, and incubation temperature are some other factors that influence the results with the well-diffusion method (Eloff, 2019). Therefore, this assay may not have been ideal for studying antimicrobial properties.

Only a few studies have reported the production of biosurfactants by mycolic acid-containing actinobacteria with anti-biofilm activities (Pirog *et al*., 2014; Kuyukina *et al*., 2016; Janek *et al*., 2018). These studies found glycolipid biosurfactants, trehalose lipids, to prevent adhesion of biofilm-forming Gram-positive and Gram-negative bacteria, yeast, and micromycetes. In the present study, the extracts containing extra-cellular glycolipids from all three strains demonstrated significant anti-adhesion and inhibition activity (up to ~60%), and to a lesser degree biofilm disruption activity (up to ~25%) against *K. pneumoniae* NCIMB 8865 and *P. aeruginosa* ENU 18, compared to untreated

controls. It is also important to note that the extracts containing glycolipids performed similarly to the total crude biosurfactant extracts studied in the previous chapter and indeed performed better in some assays. This suggests that the major active agents were likely glycolipids although the possibility that some other, as yet undetermined component was responsible, cannot be excluded. As in case with *R. yunnanensis* DSM 44837T glycolipid-containing extract which reduced adhesion of *K. pneumoniae* NCIMB 8865 compared to the total crude biosurfactant extract, which suggests that this test strain produced biosurfactants in addition to the glycolipids with anti-adhesion properties. This is the first report of all three anti-biofilm properties (anti-adhesion, biofilm inhibition, and disruption) by *R. ruber* IEGM 231^{T}. This is also the first report of anti-biofilm activity by extra-cellular biosurfactants produced by *R. yunnanensis* and *Williamsia muralis*.

The anti-biofilm activity of biosurfactants is attributed to the changes they cause in the molecular and physiological characteristics of biofilms. Changes in the surface properties (hydrophobicity, surface charge) of the bacterial cells, or indeed the polystyrene surface of the microplate wells, are thought to be responsible for the prevention of adhesion (Kyunika *et al*., 2016). The anti-adhesion and anti-biofilm properties could also be explained by the ability of biosurfactants to reduce the surface tension between the biofilm and substratum on which they form, thus, dispersing the biofilm (Satpute *et al*., 2019). Biosurfactants can cause pores and ion channels in lipid bilayer membranes affecting the porosity and integrity of the membranes, and causing detachment of cells through abrasion, and erosion (Patel *et al*., 2021; Quadriya *et al.*, 2018). Rhamnolipid biosurfactants specifically have been known to increase cell hydrophobicity, reduce the lipopolysaccharide content in membranes, disturb surface morphology, and alter membrane proteins leading to biofilm inhibition and disruption (Sánchez *et al*., 2009; Ortiz *et al*., 2010).

The anti-adhesion and inhibition activities determined in the present study were similar to or even higher than those reported for glycolipids biosurfactants derived from other bacterial species against strains of the same biofilm-forming species. For example, glycolipids produced by *Lactobacillus*

rhamnosus reduced adhesion of the pathogenic *P. aeruginosa* strain MTCC 741 by 53% at aconcentration of 25 mg ml^{-1} (Patel *et al.,* 2021) while at only 50 µg ml^{-1} concentration of glycoplids (di-rhamnolipids) from *Lysinibacillus* sp. BV152.1 50% inhibited *P. aeruginosa* PAO1 biofilm formation (Aleksic *et al*., 2017). Meanwhile, glycolipids derived from *L. acedophillus and L. pentoususat* reduced *K. pneumonia* ATCC 10031 cell adhesion by 69% and inhibited biofilm growth by 76% at only 0.25 mg ml^{-1} (Abdalsadiq Nagea, 2018) while at higher concentration of 32 mg ml^{-1} purified biosurfactant from L*. rhamnosus* inhibited 24% *K. pneumonaie* biofilms (Abdulsattar, 2014). It should be noted however that these studies were carried out using crystal violet staining and therefore may not be directly comparable to the results determined by TTC dye.

While the anti-biofilm effects of *R. yunnanensis* and *W. muralis* derived biosurfactants have not been reported previously, *Rhodococcus* ruber IEGM 231^T is known to prevent adhesion of various gram-positive and gram-negative bacterial strains at concentrations of 0.1-100 mg l^{-1} (Kuyukina *et al*., 2016). The study reported the highest anti-adhesion activity against *Pseudomonas fluorescence* NCIMB 9046 (11–30 %) *and Escherichia coli* K-12 (34–42 %) at concentrations of 0.1-1 mg l^{-1}. Hence, glycolipid- containing extract from *R. ruber* had anti-adhesion activity on *E.coli* while the anti-adhesion activity against *Pseudomonas* species was similar to that reported in this study. However, a direct comparison cannot be made with other studies as the extract were not pure.

The test biosurfactant extract did not perform so well against established biofilms formed by *K. pneumoniae* NCIMB 8865 and *P. aeruginosa* ENU 18 achieving <25% disruption. However, the ability of the glycolipid-containing extract from *R. ruber* IEGM 231^T to disrupt *P. aeruginosa* biofilms by ~25% is comparable to that reported for the cyclo-lipid peptide produced by *Corynebacterium xerosis* NS5 which achieved 30% disruption of pre-formed biofilms of *P. aeruginosa* (Dalili *et al*., 2015). The biofilm disruption properties of *R. ruber* biosurfactants have not been previously reported. By comparisons, Patel *et al*. (2021) reported that lactic acid bacteria (LAB)-derived glycolipids at 25 mg ml^{-1} caused 59% disruption of pre-formed *P. aeruginosa* MTCC 741

biofilms, while a higher disruption activity of 90.5% was reported against *P. aeruginosa* PAO1 biofilms at 100 µg ml^{-1} glycolipid biosurfactant from *Serratia marcescens* (Dusane *et al*., 2011). Similar to this study, a lower disruption activity against *K. pneumonia* biofilm (<20%) was reported from biosurfactant (a mixture of carbohydrates, lipids, and proteins) of *Lactobacillus jensenii* P6A and *L. gasseri* P65 at 100 µg ml^{-1} (Morais *et al.,* 2017). However, glycolipid biosurfactant from *Shewanella algae* B12 at 20 µg ml^{-1} showed a higher disruption activity against the preformed biofilms of *P. aeruginosa* (92%) and *K. pneumoniae* (87%).

As expected, the potency of the isolated glycolipid complexes reduced with dilution in water thus the anti-biofilm activity is concentration-dependent. Janek *et al*. (2018) also found that the anti-adhesion effects of biosurfactants from *Rhodococcus fascians* BD8 against microbial pathogens were concentration-dependent. However, Kuyukina *et al.* (2016) noted that the anti-adhesive activity of biosurfactants from *R. ruber* IEGM 231^{T} was not strongly concentration-dependent although this suggests very high concentrations were recovered from this organism. A threshold concentration of biosurfactant is required to saturate the polystyrene surface beyond any further increase will not affect the polystyrene surface properties, in turn resulting in no further increase in anti-adhesion activity. Thus at concentrations higher than the threshold, no concentration-dependent effects will be observed. In the present study, the decrease in anti-adhesion activity with decreasing concentration suggests that the threshold concentration had not been reached and that the surfaces of the polystyrene wells were not saturated thus the hydrophobic characteristics of the polystyrene surfaces changes with concentration. The concentration depended anti-adhesion activity was explained by Das *et al.* (2014) in their study with rhamnolipid biosurfactants in which the proportion of mono- to di-rhamnolipids changed upon dilution leading to increased polarity and decreased anti-microbial activity. This suggests that hydrophobicity plays an important role in anti-biofilm effects.

These findings suggest that the glycolipids-containing extract produced by the three test strains are largely responsible for the anti-adhesion and biofilm

disruption activities observed for the total biosurfactant extracts investigated in Chapter 3 as similar levels of activity were observed for most assays with increased activity in some instances. In the case of biofilm inhibition, this is not so clear-cut, and it is possible that other biosurfactants present in the total extracts also contributed to biofilm inhibition. In cases where the glycolipids-containing extract achieved greater effect than the total extracts, this may be due to potency or the removal of other compounds that affected glycolipid properties and/or biofilm development. However, the use of total biosurfactant extract proves effective as a primary screen to detect anti-biofilm activity. This is advantageous in the large-scale search for effective biosurfactants as crude extraction is straightforward and reasonably practical when investigating numerous bacterial strains. The isolation and purification of component compounds from crude extracts however is more time-consuming and technically involved and may require the application of various chemical analytical techniques that are not so readily available. Purification also results in low yields, in turn limiting the extent to which useful properties can be further investigated. In the present study, a low quantity of the extra-cellular glycolipids-containing extract restricted the range of anti-biofilm testing possible and precluded the study of adherence to different substrate materials, poly-microbial biofilms, and combinations or 'cocktails' of biosurfactants as originally planned. Hubert *et al*., 2012 also noted low recovery of glycolipid compounds (milligram quantities) and that the process is laborious. The low concentration of glycolipids recovered after purification is one of the major challenges not only in the research of biosurfactants but also in full-scale commercial production.

In summary, the glycolipid-containing complexes showed good anti-adhesion and biofilm-inhibition against *K. pneumoniae* NCIMB 8865 and *P. aeruginosa* ENU 18 and warrant further investigation to determine the effectiveness against growing cells in addition to resting (static) cells and to establish the mechanisms of action. However, the growth of each test strain will need to be optimised to maximise the recovery of extra-cellular biosurfactants thereby allowing more extensive chemical analysis and investigation of their anti-

biofilm properties. These findings add to those of previous studies on MACA-derived biosurfactants and support the case that this group deserves further investigation for effective anti-biofilm agents.

4.5. Conclusions.

Growth-associated biosurfactant production was observed for all three mycolic acid-containing actinobacteria, and these were mainly cell-bound. The rhodococci strains produced cell-bound bioemulsifiers but *W. muralis* did not. Production of extracellular surfactants was non-growth associated, increasing from the early stationary phase, likely through the release of cell-bound surfactants produced during exponential growth. This is the first study to report the production of extra-cellular rhamnolipids by *R. ruber, R. yunnanensis,* and *W. muralis.* Analysis by MALDI-TOF-MS revealed the presence of several mono and di-rhamnolipid congeners. However, the glycolipids- containing extract were complex mixtures indicating that still further purification and analyses are required to fully elucidate the nature and structure of all the biosurfactant compounds present. Further analysis of the extracted biosurfactants may also reveal the presence of additional chemical groups. Promisingly, the glycolipid-containing extracts isolated by thick layer chromatography demonstrated anti-adhesion, biofilm inhibition, and to a lesser extent dispersion activity against biofilm-forming strains *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumonia* NCIMB 8865. The performance of these extracts was comparable to that observed for the total biosurfactant extracts in most of the anti-biofilm assays. This points to glycolipid components being the major active agents responsible for the anti-biofilm effects, although other compounds may contribute. This is the first report of anti-biofilm activity by biosurfactants produced by *R. yunnanensis* DSM 44837^{T} and *Williamsia muralis* N1261^{Tsp} and paves the way for further investigation.

Chapter 5. Synthesis

Mycolic acid-containing actinobacteria (MACA) of the suborder *Corynebacterineae* synthesise a multitude of biosurfactants that have been investigated for their potential commercial exploitation in various contexts (Peng *et al*., 2014; Stainsby *et al*., 2021). Primarily environmental remediation and petroleum oil recovery (Ivshina *et al*., 2016; Saeki *et al*., 2009; Vyas *et al*., 2011). Recent research involving a small number of strains points to various possible biomedical applications (Christova *et al*., 2015; Mnif *et al*., 2015; Sudo *et al*., 2000), including the prevention and treatment of clinical biofilms (Dalili *et al*., 2015; Janek *et al*., 2018; Kyunika *et al*., 2016; Pirog *et al*., 2014). However, many more known biosurfactant-producing species in the suborder *Corynebacterineae* await investigation. Further, membership in this suborder has expanded significantly over the past 20 years with many new species and genera yet to be investigated for the ability to produce biosurfactants. As such, unknown numbers of actinobacteria in the suborder *Corynebacterineae* may produce both known and novel biosurfactants with the ability to prevent or remove biofilms formed by human pathogens.

Hence, this study set out to identify mycolic acid-containing actinobacteria that produce extra-cellular biosurfactants and to test these for anti-adhesion, biofilm inhibition, and/or biofilm disruption properties against biofilm-forming clinically relevant bacteria. A collection of 94 strains belonging to nine different genera of the suborder *Corynebacterineae* were screened for cell-bound and extra-cellular biosurfactants using various in-direct assays based on the physicochemical properties of biosurfactants. Subsequently, the readily recoverable extra-cellular biosurfactant complexes from eleven strains were tested for anti-adhesion and anti-biofilm effects on biofilm-forming bacteria belonging to various gram-positive and gram-negative species of clinical concern. Three strains found to have good anti-biofilm properties against two pathogens, namely *Klebsiella pneumonaie* and *Pseudomonas aeruginosa*, were studied further. Glycolipid-containing extracts from crude extra-cellular biosurfactants were characterised to identify the compounds present, and the physico-chemical and anti-biofilm properties were confirmed. Finally, the

relationship between growth and biosurfactant production under batch growth conditions was determined for these strains.

5.1. Main research findings.

All 94 *Corynebacteriales* actinobacterial strains were found to produce cell-bound biosurfactants when grown on hydrophobic hexadecane (2% v/v) as the sole carbon source. Extra-cellular biosurfactant production was not universal under the growth conditions imposed but was observed in several strains belonging to various genera. This was in accordance with previous knowledge of biosurfactant production by *Rhodococcus* and related genera (Franzetti *et al*., 2010; Kyunika *et al*., 2001) with only a few strains reported to release extracellular biosurfactants (Kim *et al*., 1990; Ristau & Wagner 1983). In the present study, 12 strains released biosurfactants capable of reducing surface tension (ST) to ≤40 mN m^{-1} over the cultivation period (up to 14 days). Seven of these strains, namely *Gordonia desulfuricans, Rhodococcus baikonurensis, R. yunnanensis, Tsukamurella strandjordii, T. sunchonensis, Williamsia muralis,* and *W. serinedens*, have not previously been reported to produce extra-cellular biosurfactants. To the best of our knowledge, this work provides the first report of biosurfactant production in 58 strains and any by members of the genera *Millisia* and *Williamsia*. The observation that extra-cellular biosurfactants are produced by various mycolic acid-containing actinobacteria strongly indicates that exploring diverse *Corynebacterineae* for biosurfactant production is worthwhile.

This is the first study to report the application and evaluation of different screening assays to biosurfactant detection amongst diverse mycolate actinobacteria. Six in-direct assays were found to be suitable for screening the test strains for cell-bound and extra-cellular biosurfactant production; drop collapse (DC), oil spreading (OS), emulsification index (EI_{24}), du Noüy surface tension measurement (ST), cetyltrimethyl ammonium bromide (CTAB) and bacterial adherence to hydrocarbons (BATH) assays. All the assays showed a low coefficient of variation with replicate analyses and apart from the du Noüy ST assay were straightforward and quick to perform. The du Noüy assay was

considered definitive for biosurfactant production but was time-consuming. However, variations in the frequency of positive results were observed between assays and with the length of incubation, with few strong correlations found between assays. Hence, reliance on one method is not sufficient, and instead, a combination of assays is required to measure different physico-chemical properties of biosurfactants. The presence of mycolic acids (based on genus assignment) did not appear to influence assay results with no correlations observed between mycolic acid chain length (based on genus assignment) and cell surface hydrophobicity or surface tension.

The hydrophilic substrate glucose supported homogenous growth by the test strains while the hydrophobic substrate hexadecane appeared to result in greater biosurfactant production. However, many of the test strains exhibited non-homogeneous growth on this with biomass clumping and partitioning in the upper hydrophobic layer, which in turn may have interfered with the application and interpretation of some assays, notably the Bacterial Adherence to Hydrocarbon (BATH) and Emulsification Index (EI_{24}) assays. The production of extra-cellular biosurfactants appeared to be time-dependent as the frequency of positive scores was higher on day 14 than on day 7. *Nocardia* strains in particular only tested positive for screening assays after 14 days of cultivation. It was recognised that for all the strains, the relationship between growth and extra-cellular biosurfactant production, and therefore the optimum time point to detect maximum production will not be the same. Therefore, it may be the case that additional strains capable of producing extra-cellular biosurfactants under different growth conditions to those selected in the present study may have been missed for extra-cellular biosurfactant production.

Extract possibly containing glycolipids were readily extracted from cell-free supernatants (CFS) using an acid precipitation and solvent extraction (chloroform-methanol 2:1 v/v) method after separation from biomass grown on n-hexadecane for 14 days. The quantity of extract possibly containing glycolipids recovered were ~0.4 and 0.6 g L^{-1} and would have been scaled up with optimization study.

Crude biosurfactant extract were purified by thick-layer chromatography and the presence of glycolipid were confirmed using thin layer chromatography (TLC) with orcinol detection using rhamnolipid (R90) standard. The limitation of this extraction approach is recognised, and the glycolipid-containing extract could potentially also contain other compounds (both biosurfactant and non-biosurfactant in nature). If other biosurfactant standards had been available it would have resulted in detecting different types of glycolipids in the extract. The retention factor (R_f) and appearance of reference rhamnolipid spots on TLC plates were affected by the solvent system and reagent applied. The Orcinol reagent was the most reliable as it detected the rhamnolipid with all solvent systems tested. The chloroform–methanol-water (65:25:4 v/v/v) was found to be a suitable solvent system for separating glycolipids from the actinobacterial test strains and chloroform-methanol-acetic acid (65:15:2 v/v/v) for *Williamsia muralis* by thin layer chromatography.

In vitro, static biofilm formation was confirmed for six of the fifteen clinically relevant bacterial tested including *Escherichia coli* ATCC 47055, *Klebsiella pneumoniae* NCIMB 8865, *Pseudomonas aeruginosa* ENU 18, *P. aeruginosa* ENU 19, *Staphylococcus epidermidis* ENU IL-42 and *S. epidermidis* ENU IL-43. The strains that didn't show biofilm formation might have required different growth conditions for biofilm formation than the one used in the current study. The ability to form biofilms was shown to vary depending on the culture medium and length of incubation. Biofilm formation was better in nutrient-rich complex media (Luria Bertani, LB and Tryptone soy broth, TSB) than in the defined minimal medium M9. This was in accordance with previous studies on similar strains that reported good biofilm formation in nutrient-rich media LB and TSB (Hanvey *et al.,* 2018; Knobloch *et al*., 2002, Prakash *et al*., 2017; Pratt & Kolter, 1998; Rohde *et al*., 2010). Incubation time also influenced biofilm formation with strains forming good biofilms between 24h and 72h. The methanol fixative step used before biofilm staining resulted in a significant reduction in biofilms formed by *S. epidermidis* ATCC 35984 and *E. coli* ATCC 47055 and hence this step was not performed for further studies.

Crude biosurfactant extracts from eleven strains tested at a concentration of 10mg ml^{-1} displayed reduce adhesion and biofilm formation and/or disrupted established biofilms formed by gram-positive bacteria *Enterococcus faecalis* (NCIMB 7432 & ATCC 51299), *Enterococcus faecium* (ENU pj1 & ERI 2), *Staphylococcus aureus* (NCTC 6571 & SMRL/ 14/0440) and negative bacteria *Escherichia coli* (ATCC 47055 & ERI 39), *Klebsiella pneumonia* (NCIMB 8865 & ERI 44*), Pseudomonas aeruginosa* (ENU 18 & ENU 19) and *Staphylococcus epidermidis* (ENU IL-42 & ENU IL-43) in static biofilm assay using crystal violet staining (CV). These biofilm-forming bacteria are of clinical importance such as those belonging to ESKAPE pathogens and on the World Health Organisation antibiotic-resistant priority pathogens list.

Although promising anti-biofilm results were obtained against some pathogens, other pathogens were found to be unaffected and it may well be the case that the other extracts not tested by 2,3,5-triphenyl-tetrazolium chloride (TTC) assay may possess better antibiofilm activities than revealed by CV in the present study. Optimised assays utilising the reducing dye 2,3,5-triphenyl tetrazolium chloride (TTC) which is based on metabolically active cells were found to be more reliable than those using crystal violet staining which was found to overestimate and mask the anti-biofilm properties and was less reproducible with higher deviations. However, the TTC assay requires considerable optimisation for use with different biofilm-forming strains. Based on inhibition and disruption dose-response curves and EC_{50} values determined for commercial surfactants, a concentration of 0.1% w/v TTC was found most suitable for anti-biofilm assays.

The greatest effects were seen for the glycolipid-containing extracts recovered from *Rhodococcus ruber* IEGM 231^T, *Rhodococcus yunnanensis* DSM $44837^{T,}$ and *Williamsia muralis* $N1261^T$ against *P. aeruginosa* ENU 18 and *K. pneumoniae* NCIMB 8865 using TTC assay. As the studies on the anti-biofilm activity of actinobacteria biosurfactants against *K. pneumoniae* and *P. aeruginosa* biofilms are limited this study shows the ability of various actinobacteria species to have activity against these biofilm-forming strains, which are of concern in clinical settings. The TTC assays confirmed that the

biosurfactant extracts exhibited the highest anti-adhesion, biofilm inhibition (up to ~60%), and to a lesser extent biofilm dispersion activity (up to ~25%) against the gram-negative biofilm-formers *Pseudomonas aeruginosa* ENU 18 and *Klebsiella pneumoniae* NCIMB 8865. Similar levels of activity were observed for the extracellular glycolipid-containing extract isolated from the crude biosurfactant extracts. This result supports the argument that *Corynebacterineae* is a rich and under-exploited source of biosurfactants with potential application as anti-biofilm agents. Hence, the use of crude biosurfactant extracts proves effective as a primary screen to detect anti-biofilm activity.

The glycolipids-containing extract were found to contain major amounts of rhamnolipids including several mono and di-rhamnolipid congeners when analysed by MALDI-TOF-MS but likely contained other compounds. This is the first report of extracellular rhamnolipid production and the first demonstration of anti-adhesion, biofilm inhibition, and biofilm disruption properties in extracellular glycolipids released by *R. ruber*, *R. yunnanensis* and *W. muralis*. The MALDI-TOF-MS analysis spectra obtained in this study, suggested additional purification and analyses to fully elucidate the composition of the extracellular glycolipid- containing extract from each test strain either by using MALDI-TOF or one or more additional chemical analytical techniques.

During batch cultivation of these strains on hexadecane, extracellular biosurfactant production was non-growth associated, with maximum accumulation in the early stationary phase. This was mainly due to the release of cell-bound surfactants synthesised during exponential growth. The sampling interval in the current study were only every 24 h and given the short lag and rapid exponential phases that occurred within the first 48 h, the precise course of growth during this period was not captured in detail. It was also noted that the strains must be grown beyond the exponential phase into the stationary phase to ensure maximum accumulation.

Accurate growth measurement proved technically challenging in this study largely due to the nature of growth on n-hexadecane, which led to the non-

homogeneous dispersion of cells. Despite efforts to distribute growth evenly prior to sampling, it is likely that measurement of growth by biomass dry weight turbidity, and viable plate counts were not accurate. Quite different batch growth curves were plotted using each of these methods and this was reflected in the weak correlations. Viable plate counts gave rise to normal batch growth curves and better-discerned change in growth over time than biomass dry weight.

5.2. Future Work.

The overall aim of this programme of research was achieved with several extra-cellular biosurfactants from mycolate actinobacteria found to have good anti-adhesion and antibiofilm effects against clinical strains. The findings of this work reveal that further investigation of biosurfactants from mycolic acid-containing actinobacteria (MACA) for anti-adhesion and anti-biofilm properties is warranted. Further, the findings add to growing evidence that biosurfactants can be used effectively to prevent, reduce, or eradicate biofilms formed by human pathogens. However, several aspects of the current research require further development, and there remain some challenges to overcome. The following areas are highlighted for future work:

1. *Development of a rapid, high-throughput screen for biosurfactant production*.

Screening with multiple screening assays is time-consuming and hence should be reduced to one or two assays that are high throughput and quantitative. Recently developed Victoria blue assay is recommended as a high-throughput method for potential identification and quantification of biosurfactants directly in culture supernatant samples which exploits the surface activity of the biosurfactants should be validated and evaluated further which can be a promising new screening method for screening biosurfactants (Kubicki *et al*., 2020).

2. *Improving extra-cellular biosurfactant yield*. The current study focussed on extra-cellular biosurfactants due to their ease of recovery from the growth medium. The quantity of crude glycolipid-containing extract obtained only

allowed testing of their anti-biofilm properties. Strategies such as the use of different and low-cost carbon or nitrogen source (oil waste, plant-derived oil, raw materials, lactic whey, and distillery waste), adjusting culture conditions (carbon and nitrogen source), and environmental factors (such as pH, temperature, agitation, salinity, and oxygen) have been employed to increase the yield. Optimized process methods have allowed the increase of glycolipids production quantities in MACA. At a high concentration of phosphate buffer and neutral pH conditions, the production of succinoyl trehalose lipids in *R. erythropolis* SD-74 achieved a yield of up to 40 g/L (Uchida *et al*., 1989). Meanwhile the biosurfactant production in *Rhodococcus* spp. MTCC 2574 was increased by 3.4-folds (3.2 g/l to 10.9 g/l) using mannitol as a carbon source, yeast extract, meat peptone as an organics nitrogen source, and hexadecane as an inducer (Mutalik *et al*., 2008). Franzetti *et al.* (2008) used an experimental design technique to increase the 5-fold production of cell-bound glycolipids of *Gordonia* sp. BS29 during the exponential phase by step-wise approach.

Moreover, the extracellular biosurfactant production was non-growth associated in the three test strains but different MACA will have different growth rates so may need to determine the growth/biosurfactant kinetics for each strain to establish optimum incubation for maximum extracellular biosurfactant accumulation. Hence, optimization studies will help to recover higher quantities and efficient production costs.

3. *Robust separation, purification, and full structural characterisation of compounds in the extra-cellular glycolipid-containing extract*.

MALDI-TOF-MS analysis indicated the presence of unresolved components, and hence not all glycolipid types were identified. The spectra obtained suggest additional purification and analyses to fully elucidate the composition of the extracellular glycolipid-containing extract. The samples can go through a second step of purification either by using thin layer chromatography (TLC), silica gel chromatography, high-performance liquid chromatography (HPLC), size exclusion chromatography, and ion-exchange chromatography that have been used to purify biosurfactants produced by mycolic-acid containing actinobacteria (Venkataraman *et al*., 2021). The purified sample can further be

analysed to fully elucidate the composition either by using MALDI-TOF or one or more additional chemical analytical techniques such as nuclear magnetic resonance spectroscopy (Patil *et al*., 2018) considered the gold standard for structural elucidation of unknown biosurfactants.

4. Comprehensive anti-adhesion and anti-biofilm testing.

As the anti-adhesion and anti-biofilm activity using 2,3,5-triphenyl tetrazolium chloride (TTC) were only studied on three MACA glycolipid-containing extracts that showed the highest activity when quantified using crystal violet (CV) assay, the TTC assay can be extended over the remaining eight MACA extract that had low or no activity by CV assay to confirm their true properties. This would then need to isolate glycolipid components from these strains if showed activity against biofilm-forming strains, purify extract to test glycolipid components for anti-adhesion and anti-biofilm properties, and further characterize the glycolipid mixture.

Due to small quantity of biosurfactant recovered limited the anti-adhesion and anti-biofilm studies which can be extended to examine anti-biofilm as effects on different surfaces of relevance to the clinical setting e.g. silicon catheter tubes, investigate effects on growing rather than static cells, testing on poly microbial biofilm, concentration-dependent study and widen the selection of biofilm-forming strains to all ESKAPE pathogens. Rhamnolipids biosurfactants have been found to be effective in removing biofilm biomass of dual-species biofilms(*Candida albicans–Staphylococcus aureus and C. albicans–S. epidermidis*) from polystyrene (94 and 95%) and silicone (93 and 90%) surface (Ceresa *et al*., 2021). Rhamnolipid-coated medical grade polydimethylsiloxane (PDMS) was able to reduce dual-species culture with *C. albicans*, *S. aureus,* and *S. epidermidis* by 97.95% and 98.74% (Dardouri *et al*., 2022). Kuyukina *et al*. (2016) reported the higher biofilm-preventing properties of Rhodococcus ruber IEGM 231^{T} biosurfactant against growing bacteria rather than resting cells. Therefore, a comprehensive anti-adhesion and anti-biofilm study of MACA biosurfactants can be carried out to further strengthen their properties.

Exploring the anti-biofilm potential of biosurfactants as mixtures or combined with other molecules e.g. notably metal nanoparticles or liposomes to examine synergistic effects/ability to enhance antibiofilm activity. Liposomes encapsulated with lipopeptide biosurfactant abetted copper nanoparticles were able to avert the biofilm-associated pathogenesis of methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Kannan *et al*., 2021) while chitosan rhamnolipid nanoparticles were able to disperse *S. aureus* DSM 1104 and *S. epidermidis* biofilms (Marangon *et al*., 2020). While, a mixture of biosurfactant surfactin and an alkaline lipase removes *Listeria innocua* ATCC 33093 biofilm from stainless steel coupons (Pereira *et al*., 2011). However, MACA biosurfactants are not been exploited in this context where they can have a higher combined effect against biofilms.

5. *Biosurfactant toxicity and biocompatability testing*.

Biosurfactants claim to be safer than their chemical counterparts. If to be applied in clinical settings where may come into contact with skin or in the case of urinary tract devices flushed internally, then it needs to be sure that they are non-toxic. So next step on this path would be in vitro toxicity assays of biosurfactant extracts. The cytotoxicity profile of the biosurfactants has been done in human skin and liver cell models using the alamarblue and propidium iodide assays (Voulgaridou, et al., 2021), on fibroblast cell line by a reduction of the MTS tetrazolium compound (Rodríguez-López *et al*., 2020). The results can then be compared with the cytotoxicity limit established by the International Organization for Standardization (ISO) guidelines ISO. Hence, this study will help to provide evidence about the promising use of the actinobacteria-derived biosurfactant in clinical settings and supports their future exploitation.

Chapter 6. References

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Chapter 7 Appendices

Appendix 7.1. Screening of ninety-four mycolic acid-containing actinobacteria strains including two non-mycolic acid-containing actinobacteria for biosurfactant production using six different screening assays on day 7 and day 14 using whole-cell broth and cell-free supernatant grown in minimal salt media containing 2% hexadecane.

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Cntrl +ve	++	++	40.99 ± 0.11	28.07 ±0.19	+	74.18 ± 0.05						++	++	40.37 ±0.09	29.00 ±0.19	+	74.10 ± 0.03					
Cntrl -ve	-	-	0.00	71.02 ±0.20	-	-						-	-	0.00	72.89 ±0.20	-	-					
Corynebacterium amycolatum N 1278^T	+	++	0.00	37.53 ± 0.22	-	33.47 ±0.00	-	-	0.00	67.51 ±0.11	-	++	+	0.00	28.40 ±0.07	+	30.63 ±0.00	-	-	0.00	66.22 ±0.14	-
Corynebacterium otitidis DSM 8821^T	-	+	34.94 ±0.07	20.46 ±0.07	-		+	+	0.00	25.53 ±0.03	-	+	++	3.58 ±0.07	22.59 ±0.07	-		+	++	0.00	26.13 ±0.08	-
Dietzia aerolata DSM45334^T	+	++	15.20 ± 0.21	28.04 ±0.17	-	49.66 ±0.00	+	+	0.00	63.33 ±0.20	-	+	+	10.95 ±0.00	27.22 ±0.05	+	56.64 ±0.08	+	+	0.00	67.58 ±0.17	-
Dietzia alimentaria DSM45698^T	+	++	11.09 ±0.21	27.07 ±0.08	+	82.38 ±0.03	+	++	0.00	53.38 ±0.16	-	+	++	23.86 ±0.00	28.33 ±0.08	+	84.92 ±0.06	+	+	0.00	47.13 ±0.11	-

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Dietzia cercidiphylii DSM 45140^T	-	+	13.90 ±0.07	27.49 ±0.08	-	91.48 ±0.02	+	+	0.00	56.87 ±0.22	-	+	++	24.41 ±0.07	30.64 ±0.09	+	76.64 ±0.07	+	+	0.00	44.37 ±0.21	-
Dietzia cinnamea DSM 44904^T	+	++	0.00	28.74 ±0.06	+	17.11 ±0.04	+	+	0.00	68.60 ±0.07	-	+	+	12.66 ±0.00	27.72 ±0.05	+	75.43 ±0.05	+	+	0.00	64.83 ±0.27	-
Dietzia dagingensis DSM 447481^T	+	+	14.58 ±0.00	29.11 ±0.05	-	85.36 ±0.01	+	+	0.00	53.46 ±0.25	-	+	-	15.16 ±0.14	38.52 ±0.21	+	57.25 ±0.06	+	++	0.00	57.50 ±0.22	-
Dietzia kunjamensis DSM 44907^T	+	-	19.22 ±0.07	26.32 ±0.04	-	83.07 ±0.02	-	-	0.00	68.21 ±0.15	-	+	+	20.61 ±0.28	28.71 ±0.26	-	62.60 ±0.04	+	+	0.00	66.83 ±0.24	-
Dietzia lutea DSM45074^T	+	+	6.91 ±0.07	26.58 ±0.18	-	87.06 ±0.02	+	+	0.00	69.91 ±0.05	-	+	+	26.74 ±0.28	30.80 ±0.09	+	36.97 ±0.03	+	+	4.65 ±0.00	55.58 ±0.20	-
Dietzia maris DSM43672^T	+	+	13.07 ±0.07	25.56 ±0.05	+	55.57 ±0.03	+	+	0.00	62.18 ±0.16	-	+	+	24.76 ±0.07	27.28 ±0.36	+	69.21 ±0.00	+	+	0.00	54.09 ±0.28	-
Dietzia natronolimnea DSM 44860^T	+	++	14.09 ±0.21	26.91 ±0.06	-	48.85 ±0.02	+	++	0.00	67.09 ±0.27	-	+	++	16.59 ±0.14	27.30 ±0.06	+	44.71 ±0.02	+	+	0.00	67.89 ±0.18	-
Dietzia papillomatosis DSM44961^T	+	-	9.82 ±0.14	26.09 ±0.06	-	70.53 ±0.07	-	-	0.00	61.37 ±0.20	-	+	+	19.81 ±0.07	32.46 ±0.07	-	67.72 ±0.09	+	+	0.00	69.40 ±0.15	-

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Dietzia psychralcaliphila DSM 44820^T	+	+	13.60 ±0.07	30.56 ±0.05	-	51.73 ±0.02	+	+	0.00	57.96 ±0.25	-	+	++	13.55 ±0.07	29.95 ±0.09	+	76.57 ±0.09	+	++	0.00	57.60 ±0.20	-
Dietzia schimea DSM 45139^T	+	+	13.57 ±0.07	29.20 ±0.16	+	71.33 ±0.03	+	+	0.00	51.23 ±0.28	-	+	+	12.50 ±0.07	33.05 ±0.06	+	53.71 ±0.04	+	+	0.00	65.84 ±0.25	-
Dietzia timorensis DSM 45568^T	+	+	10.28 ±0.00	30.32 ±0.07	+	66.67 ±0.02	+	+	1.25 ±0.07	53.13 ±0.25	-	+	+	14.21 ±0.07	33.53 ±0.07	+	78.61 ±0.03	+	+	0.00	49.56 ±0.23	-
Gordonia aichiensis N934^T	-	-	0	28.20 ±0.09	-	90.30 ±0.03	-	-	0	58.49 ±0.16	-	-	+	4.44 ±0.00	32.44 ±0.23	+	88.43 ±0.02	-	-	0	57.96 ±0.16	-
Gordonia alkanivorans DSM 44369^T	-	-	5.56 ±0.07	27.09 ±0.19	-	68.15 ±0.04	-	-	0	62.02 ±0.08	-	-	+	9.43 ±0.07	31.20 ±0.22	+	78.61 ±0.03	-	-	0	61.11 ±0.25	-
Gordonia amarae DSM43392^T	-	-	0	24.11 ±0.03	-	85.10 ±0.03	-	-	0	64.55 ±0.26	-	-	+	2.35 ±0.00	57.28 ±0.26	-	83.92 ±0.01	-	-	0	65.65 ±0.17	-
Gordonia amarae DSM43392^T	-	-	0	24.11 ±0.03	-	85.10 ±0.03	-	-	0	64.55 ±0.26	-	-	+	2.35 ±0.00	57.28 ±0.26	-	83.92 ±0.01	-	-	0	65.65 ±0.17	-
Gordonia amarae DSM46078^T	-	-	4.32 ±0.14	25.05 ±0.10	-	91.21 ±0.00	-	-	0	68.13 ±0.07	-	+	+	10.33 ±0.07	25.90 ±0.05	+	89.27 ±0.03	-	+	2.17 ±0.07	58.58 ±0.21	-

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Gordonia amarae DSM43391^T	-	-	0	25.05 ±0.07	-	81.44 ±0.02	-	-	0	58.93 ±0.24	-	+	+	9.76 ±0.00	28.17 ±0.25	-	86.98 ±0.07	+	+	4 ±0.14	56.76 ±0.15	-
Gordonia amicalis DSM 44461^T	-	++	6.67 ±0.00	25.30 ±0.12	-	7.42 ±0.03	+	+	0	65.78 ±0.08	-	+	+	2.44 ±0.00	27.48 ±0.06	-	11.99 ±0.03	+	-	2.38 ±0.07	53.28 ±0.25	-
Gordonia araii DSM 44811^T	-	-	0	48.45 ±0.08	-	89.90 ±0.04	-	-	0	68.22 ±0.07	-	-	-	0	47.66 ±0.30	-	92.04 ±0.02	-	-	0	66.83 ±0.15	-
Gordonia bronchialis DSM 43247^T	-	++	10.26 ±0.14	26.73 ±0.06	+	89.90 ±0.04	-	-	0	59.57 ±0.24	-	+	++	17.64 ±0.07	31.18 ±0.07	+	92.04 ±0.02	+	+	0	58.68 ±0.24	-
Gordonia cholesterolivorans DSM45229^T	-	-	0	25.99 ±0.06	+	74.66 ±0.03	-	-	0	60.59 ±0.28	-	-	+	2.23 ±0.00	28.10 ±0.14	+	88.98 ±0.03	+	+	3.85 ±0.14	57.33 ±0.17	-
Gordonia defluvii DSM44981^T	-	-	0	60.48 ±0.14	-	38.45 ±0.03	-	-	0	69.08 ±0.18	-	-	-	0	31.20 ±0.09	+	70.90 ±0.02	-	-	0	57.39 ±0.11	-
Gordonia desulfuricans DSM 44462^T	+	-	0	27.79 ±0.15	+	62.99 ±0.04	-	-	0	37.10 ±0.02	-	+	+	18.23 ±0.21	25.27 ±0.06	+	82.42 ±0.02	+	-	19.57 ±0.24	34.28 ±0.08	-
Gordonia effusa DSM 44810^T	+	+	0	25.37 ±0.12	+	90.37 ±0.03	+	-	0	66.43 ±0.09	-	+	+	2.53 ±0.00	25.98 ±0.06	+	76.77 ±0.05	+	+	2.27 ±0.07	58.04 ±0.16	+

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Gordonia hirsuta DSM 44140^T	-	+	11.51 ±0.00	28.51 ±0.22	+	84.76 ±0.04	-	+	0	56.22 ±0.27	-	+	+	19.70 ±0.00	32.44 ±0.12	+	6.67 ±0.02	+	+	1.92 ±0.07	54.90 ±0.26	-
Gordonia humi DSM45298^T	-	-	2.36 ±0.00	26.11 ±0.08	+	84.18 ±0.06	-	-	0	54.74 ±0.23	-	+	+	2.35 ±0.00	28.27 ±0.23	+	89.24 ±0.02	-	+	0	55.40 ±0.22	-
Gordonia hydrophobica DSM 44015^T	-	-	0	26.20 ±0.15	+	70.74 ±0.16	-	-	0	67.94 ±0.09	-	+	-	2.33 ±0.00	29.63 ±0.04	+	83.30 ±0.02	-	+	0	51.51 ±0.14	-
Gordonia lacunae DSM45085^T	+	+	2.25 ±0.00	26.55 ±0.11	+	81.67 ±0.05	+	+	0	69.20 ±0.13	-	+	+	2.33 ±0.00	27.25 ±0.10	+	89.06 ±0.02	+	+	0	68.30 ±0.10	+
Gordonia malaquae DSM 45064^T	-	-	0	26.24 ±0.03	-	81.19 ±0.03	-	-	0	50.24 ±0.06	-	-	-	0	41.73 ±0.06	+	44.16 ±0.03	-	-	0	44.39 ±0.12	-
Gordonia namibiensis DSM 44568^T	+	+	9.88 ±0.07	25.36 ±0.07	+	50.72 ±0.01	-	+	0	57.36 ±0.10	-	+	++	9.65 ±0.00	26.89 ±0.11	+	41.47 ±0.02	+	+	0	48.17 ±0.16	-
Gordonia neofelifaecis DSM45646^T	-	-	0	27.48 ±0.09	+	85.75 ±0.03	-	-	0	65.01 ±0.09	-	+	+	2.30 ±0.00	34.33 ±0.17	+	79.61 ±0.06	-	-	0	55.19 ±0.14	+

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Gordonia nitida DSM 44499^T	-	+	7.06 ±0.00	27.11 ±0.17	+	76.68 ±0.07	+	-	0	55.34 ±0.26	-	-	+	2.33 ±0.00	28.44 ±0.12	+	88.93 ±0.02	-	-	0	54.60 ±0.25	-
Gordonia otitidis DSM 44809^T	-	-	0	26.08 ±0.06	-	80.19 ±0.08	-	-	0	57.72 ±0.14	-	-	+	0	30.32 ±0.07	-	91.91 ±0.02	+	+	0	53.48 ±0.08	-
Gordonia paraffinivorans DSM 44604^T	-	-	5.83 ±0.07	25.83 ±0.07	+	93.72 ±0.01	+	-	0	60.59 ±0.27	-	+	+	5.70 ±0.07	25.82 ±0.06	+	89.96 ±0.04	+	+	0	59.91 ±0.25	-
Gordonia polyisoprenivorans DSM 44302^T	-	-	8.19 ±0.21	26.14 ±0.07	-	78.87 ±0.02	-	-	0	61.92 ±0.20	-	+	+	4.60 ±0.00	27.66 ±0.21	+	74.87 ±0.08	-	-	0	62.92 ±0.23	+
Gordonia rubropertincta DSM 43197^T	-	+	8.75 ±0.07	25.63 ±0.06	+	68.85 ±0.10	-	+	0	64.93 ±0.18	-	+	+	7.41 ±0.00	30.87 ±0.10	+	13.35 ±0.04	+	+	0	62.56 ±0.20	-
Gordonia shiwensis DSM 44576^T	-	-	0	25.78 ±0.11	+	86.26 ±0.03	-	-	0	59.81 ±0.27	-	+	+	2.30 ±0.00	30.73 ±0.08	+	85.57 ±0.03	+	+	4.35 ±0.14	57.92 ±0.26	+
Gordonia sinesedis DSM 44455^T	-	-	7.41 ±0.00	26.14 ±0.02	+	59.71 ±0.04	-	-	10.35 ±0.07	45.11 ±0.12	-	+	+	39.68 ±0.21	25.37 ±0.10	+	29.47 ±0.01	-	+	16.14 ±0.14	44.89 ±0.20	-
Gordonia soli DSM 44995^T	+	-	8.68 ±0.14	27.89 ±0.07	+	52.90 ±0.12	-	-	0	65.05 ±0.05	-	+	+	9.23 ±0.00	32.53 ±0.11	+	29.47 ±0.01	+	+	2.08 ±0.07	58.02 ±0.30	-

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Gordonia sputi DSM 43896^T	-	-	9.44 ±0.00	27.15 ±0.07	+	88.63 ±0.01	-	-	0	57.08 ±0.27	-	+	+	9.78 ±0.07	36.05 ±0.07	+	39.94 ±0.01	+	+	0	56.40 ±0.30	+
Gordonia terrae N659^T	-	-	2.57 ±0.00	24.58 ±0.07	+	76.39 ±0.09	-	-	0	69.26 ±0.25	-	+	+	2.31 ±0.00	26.87 ±0.08	+	77.87 ±0.06	+	+	0	64.89 ±0.17	-
Gordonia westfalica DSM 44215^T	-	-	10.95 ±0.07	27.16 ±0.24	+	67.99 ±0.00	-	-	0	69.46 ±0.05	-	+	+	7.44 ±0.14	24.60 ±0.18	+	20.63 ±0.01	-	+	11.36 ±0.35	51.48 ±0.23	-
Millisia brevis J81^T	+	++	12.51 ±0.00	27.05 ±0.19	-	16.06 ±0.03	+	+	0.00	56.00 ±0.23	-	+	++	15.83 ±0.07	26.00 ±0.10	+	30.76 ±0.03	+	+	0.00	42.32 ±0.18	+
Mycobacteriodes abscessus L948^T	+	++	16.03 ±0.07	26.31 ±0.12	+	15.53 ±0.02	+	+	0	61.05 ±0.26	-	+	++	32.54 ±0.21	24.28 ±0.09	+	40.66 ±0.03	+	++	0	59.83 ±0.25	-
Mycolicibacterium aurum M 401^T	++	+	3.54 ±0.07	28.14 ±0.04	-	41.99 ±0.01	+	-	0	64.39 ±0.25	-	+	++	4.83 ±0.00	26.97 ±0.02	+	81.13 ±0.03	+	++	0	57.47 ±0.16	-
Mycolicibacterium fortuitum subsp. Fortuitum N 294^T	+	++	2.50 ±0.00	29.10 ±0.05	+	65.65 ±0.01	+	+	0	66.42 ±0.13	-	+	++	2.39 ±0.00	27.93 ±0.17	+	69.27 ±0.01	+	+	0	63.95 ±0.21	-
Mycolicibacterium peregrinum M 206^T	+	+	0	27.51 ±0.15	+	59.38 ±0.01	-	-	0	68.32 ±0.15	-	-	+	6.35 ±0.07	40.56 ±0.08	+	86.33 ±0.04	-	+	0	66.06 ±0.18	-

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Mycolicibacterium phlei N 290^T	+	+	0	30.32 ±0.07	-	58.65 ±0.01	+	+	0	62.04 ±0.26	-	+	+	7.56 ±0.14	47.50 ±0.07	+	81.18 ±0.05	+	+	0	60.45 ±0.25	+
Mycolicibacterium smegmatis N 292^T	-	-	0	26.16 ±0.24	+	43.83 ±0.01	-	-	0	58.57 ±0.28	-	-	+	2.56 ±0.00	27.35 ±0.08	+	80.14 ±0.05	-	-	0	55.58 ±0.21	-
Nocardia asteroides N 317^T	-	+	0	27.71 ±0.06	-	4.61 ±0.01	+	-	0	64.09 ±0.28	-	+	+	0	27.62 ±0.27	+	22.99 ±0.02	+	-	0	39.92 ±0.26	+
Nocardia brasiliensis N 318^T	+	++	16.99 ±0.14	33.47 ±0.09	+	74.40 ±0.09	+	+	2.47 ±0.00	71.15 ±0.01	-	++	++	13.39 ±0.07	28.09 ±0.08	+	84.24 ±0.03	+	++	2.57 ±0.00	51.30 ±0.19	+
Nocardia farcinica N 671^T	+	++	7.63 ±0.14	32.04 ±0.25	+	44.03 ±0.02	+	+	2.64 ±0.00	69.43 ±0.23	-	+	+	2.60 ±0.00	26.85 ±0.06	+	47.29 ±0.03	+	+	0	62.51 ±0.23	+
Nocardia farcinica N1243	+	++	10.01 ±0.00	29.20 ±0.18	+	75.08 ±0.03	+	+	2.50 ±0.00	63.03 ±0.25	-	+	+	6.16 ±0.07	26.40 ±0.10	+	87.00 ±0.00	+	++	0	38.44 ±0.11	+
Nocardia otitidiscaviarum N 1158^T	+	+	11.58 ±0.07	22.76 ±0.01	+	16.11 ±0.00	++	-	0	26.48 ±0.26	-	+	++	57.26 ±0.21	22.68 ±0.03	+	19.13 ±0.01	+	-	0	27.04 ±0.22	-
Rhodococcus artemisiae DSM 45380^T	-	++	21.63 ±0.00	26.47 ±0.10	-	45.69 ±0.01	-	++	2.17 ±0.07	68.27 ±0.14	-	++	++	9.98 ±0.07	70.23 ±0.06	+	34.01 ±0.02	++	++	6.6 ±0.00	58.33 ±0.27	+

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Rhodococcus baikonurensis DSM 44587^T	-	++	11.83 ±0.07	25.12 ±0.22	+	13.90 ±3.90	-	+	0	38.31 ±0.19	-	++	+	10.68 ±0.00	23.12 ±0.05	+	90.65 ±0.03	++	+	9.25 ±0.14	32.38 ±0.07	-
Rhodococcus cercidiphylii DSM 45141^T	-	++	16.01 ±0.14	28.49 ±0.22	+	91.55 ±0.00	-	++	3.57 ±0.14	51.16 ±0.15	-	++	++	17.26 ±0.42	27.90 ±0.26	+	34.06 ±0.00	+	+	0	42.12 ±0.10	-
Rhodococcus coprophilus DSM 43347^T	-	++	11.23 ±0.07	28.84 ±0.02	+	65.62 ±0.01	-	+	6.43 ±0.07	56.14 ±0.04	-	++	++	30 ±0.85	29.75 ±0.25	+	59.05 ±0.05	+	+	4.73 ±0.14	45.94 ±0.26	-
Rhodococcus erythropolis DSM 43066^T	++	+	13.67 ±0.06	29.32 ±0.22	+	78.51 ±0.00	+	+	0	55.75 ±0.19	-	+	++	11.17 ±0.07	27.50 ±0.13	+	67.91 ±0.10	-	+	2.5 ±0.00	51.35 ±0.22	+
Rhodococcus fascians DSM 20669^T	-	++	19.05 ±0.14	27.55 ±0.17	+	90.71 ±0.00	+	+	0	59.53 ±0.16	-	+	+	7.06 ±0.00	27.54 ±0.16	+	88.55 ±0.02	-	+	0	57.69 ±0.29	+
Rhodococcus globerulus DSM 43954^T	++	+	11.59 ±0.06	28.53 ±0.09	-	76.10 ±0.00	+	+	1.25 ±0.07	56.29 ±0.25	-	+	++	13.34 ±0.00	29.29 ±0.04	-	53.98 ±0.06	-	+	5 ±0.00	47.39 ±0.17	-
Rhodococcus gordoniae DSM 44689^T	+	++	12.5 ±0.21	26.88 ±0.17	+	83.58 ±0.01	+	+	0	58.77 ±0.19	-	++	+	10.93 ±0.07	28.05 ±0.09	+	84.87 ±0.06	+	+	0	56.61 ±0.21	+

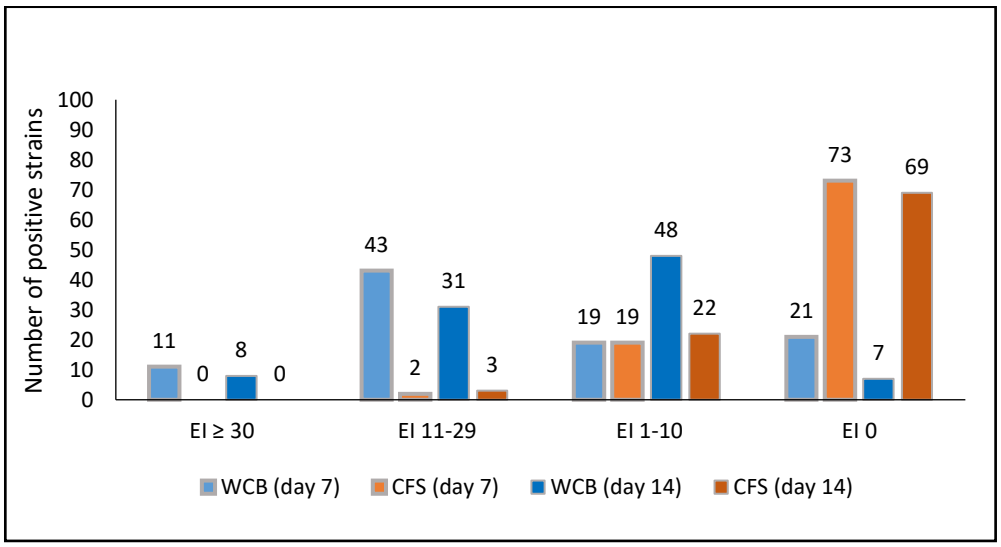
Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Rhodococcus jialingiae DSM 46766^T	-	++	7.23 ±0.00	27.00 ±0.21	+	72.21 ±0.07	+	+	0	53.76 ±0.24	-	++	+	5.9 ±0.07	27.34 ±0.20	+	60.74 ±0.02	+	+	0	52.98 ±0.30	+
Rhodococcus jostii DSM 44719^T	-	++	16.45 ±0.14	27.25 ±0.26	+	71.28 ±0.01	+	+	0	56.79 ±0.19	-	+	+	7.98 ±0.07	27.12 ±0.13	+	80.38 ±0.03	++	+	2.28 ±0.00	50.83 ±0.25	+
Rhodococcus kroppenstdetii DSM 44908^T	-	++	17.16 ±0.28	27.07 ±0.05	-	43.47 ±0.05	-	+	6.27 ±0.07	67.09 ±0.14	-	++	+	19.62 ±0.14	26.10 ±0.19	+	26.21 ±0.01	+	+	4.6 ±0.14	56.69 ±0.29	-
Rhodococcus kunmingensis DSM 45001^T	-	++	34.31 ±0.07	30.66 ±0.12	+	50.39 ±0.01	-	+	0	59.87 ±0.10	-	+	+	36.6 ±0.07	30.19 ±0.08	+	60.90 ±0.04	+	+	0	56.19 ±0.27	-
Rhodococcus maanshanensis DSM 44675^T	-	++	10.47 ±0.07	27.74 ±0.13	+	74.77 ±0.02	-	+	4.88 ±0.00	55.76 ±0.21	-	+	+	9.21 ±0.14	27.16 ±0.13	+	41.03 ±0.01	-	+	0	51.30 ±0.18	+
Rhodococcus marinonascens DSM 43752^T	-	-	0	26.81 ±0.03	+	80.93 ±0.04	-	-	0	64.12 ±0.03	-	+	+	2.44 ±0.00	30.30 ±0.28	+	72.39 ±0.08	+	+	0	44.03 ±0.10	-
Rhodococcus opacus DSM 43205^T	-	+	20.47 ±0.07	27.18 ±0.19	+	85.88 ±0.02	-	+	0	55.82 ±0.21	-	+	++	11.63 ±0.00	27.34 ±0.10	+	80.05 ±0.05	+	++	0	51.09 ±0.24	+

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Rhodococcus percolatus DSM 44240^T	-	++	33.35 ±0.07	30.07 ±0.13	+	66.94 ±0.06	-	+	0	56.85 ±0.08	-	++	+	51.01 ±0.28	29.54 ±0.10	+	74.34 ±0.05	-	+	0	46.50 ±0.27	-
Rhodococcus phenolicus DSM 44812^T	-	++	9.41 ±0.00	29.21 ±0.36	+	36.95 ±0.03	+	+	2.08 ±0.07	64.42 ±0.14	-	++	+	10.1 ±0.07	29.55 ±0.25	+	46.07 ±0.06	+	+	0	59.47 ±0.12	-
Rhodococcus pyridinivorans DSM 44555^T	+	++	9.94 ±0.07	27.74 ±0.15	+	33.96 ±0.03	+	+	3.14 ±0.00	63.98 ±0.18	-	+	+	13.1 ±0.35	29.11 ±0.17	+	46.07 ±0.06	-	+	0	54.50 ±0.18	-
Rhodococcus rhodnii ATCC 35071^T	-	+	10.53 ±0.00	26.13 ±0.16	+	64.81 ±0.01	-	+	0	65.20 ±0.22	-	+	+	11.16 ±0.00	26.95 ±0.02	+	34.30 ±0.02	+	+	0	58.55 ±0.26	-
Rhodococcus rhodochrous DSM 43241^T	++	+	8.38 ±0.15	28.65 ±0.23	+	10.42 ±0.01	++	+	1.16 ±0.07	56.39 ±0.26	-	+	++	29.71 ±0.19	28.56 ±0.29	+	82.52 ±0.02	-	+	9.01 ±0.07	57.08 ±0.07	-
Rhodococcus triatomae DSM 44892^T	-	+	4.47 ±0.14	26.55 ±0.17	+	57.44 ±0.01	-	+	0	52.58 ±0.28	-	+	+	5.97 ±0.07	25.85 ±0.07	+	85.27 ±0.02	-	+	0	40.79 ±0.18	+
Rhodococcus tukisamuensis DSM 44783^T	-	++	24.58 ±0.21	27.02 ±0.21	-	68.25 ±0.01	-	+	2.27 ±0.07	54.20 ±0.18	-	+	+	15.51 ±0.07	27.21 ±0.09	+	49.78 ±0.00	-	+	0	54.90 ±0.17	+

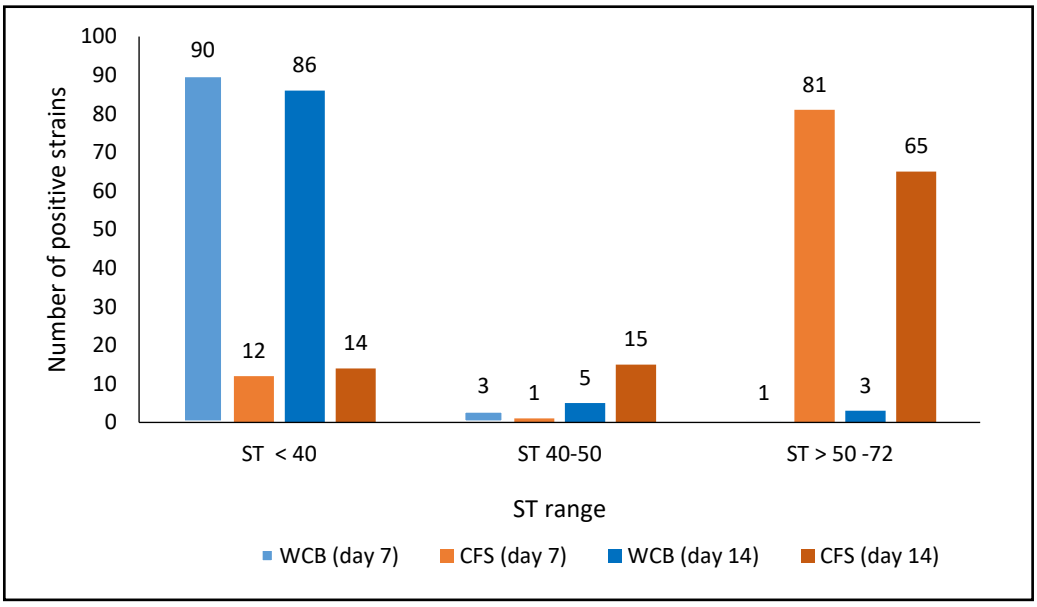
Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Rhodococcus wrastislaviesis DSM 44107^{T}	++	+	19.48 ±0.06	30.77 ±0.16	+	35.30 ±0.01	+	+	1.35 ±0.07	55.64 ±0.20	-	+	++	18.31 ±0.35	28.61 ±0.10	+	67.85 ±0.07	-	++	8.72 ±0.07	49.69 ±0.23	+
Rhodococcus yunnanensis DSM 44837^{T}	-	++	32.58 ±0.05	27.40 ±0.06	+	31.80 ±0.01	-	+	0	37.00 ±0.05	-	+	+	8.7 ±0.14	26.12 ±0.18	+	67.85 ±0.07	+	+	0	37.95 ±0.12	-
Rhodococcus zopfii DSM 44108^{T}	-	++	10.98 ±0.14	29.66 ±0.22	-	72.24 ±0.01	+	+	1.25 ±0.07	55.94 ±0.21	-	+	+	8.33 ±0.07	27.29 ±0.03	+	69.70 ±0.01	+	+	3.69 ±0.07	49.51 ±0.17	-
Tsukamurella inchonensis DSM 44067^{T}	++	++	36.47 ±0.07	47.30 ±0.12	-	35.20 ±0.01	++	+	2.47 ±0.00	55.25 ±0.08	-	+	+	3.69 ±0.07	45.74 ±0.13	+	32.24 ±0.00	+	+	0	53.71 ±0.19	-
Tsukamurella paurometabola DSM 20162^{T}	+	+	32.55 ±0.07	22.74 ±0.05	+	74.31 ±0.07	+	+	0	24.58 ±0.03	-	+	+	1.16 ±0.07	23.11 ±0.05	+	93.26 ±0.03	+	+	0	24.90 ±0.17	-
Tsukamurella pseudospumae DSM 44118^{T}	-	++	34.11 ±0.14	29.38 ±0.05	+	81.60 ±0.04	+	+	0	36.46 ±0.21	-	+	+	0	24.08 ±0.22	+	92.40 ±0.02	-	-	0	37.02 ±0.29	-
Tsukamurella. pulmonis DSM 44142^{T}	+	++	31.19 ±0.28	24.15 ±0.06	+	4.26 ±0.00	+	+	16.23 ±0.07	36.23 ±0.05	-	++	+	8.49 ±0.07	24.32 ±0.08	+	73.92 ±0.03	+	++	2.47 ±0.00	25.82 ±0.25	-

Actinobacterial Strains	Day 7											Day 14										
	Whole-cell broth						Cell-free supernatant					Whole-cell broth						Cell-free supernatant				
	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB	DC	OS	% EI	ST (mN m^{-1})	CTAB	BATH (%)	DC	OS	% EI	ST (mN m^{-1})	CTAB
Tsukamurella soli DSM 45046^{T}	-	+	0	26.40 ±0.29	-	69.02 ±0.05	+	+	0	65.95 ±0.28	-	-	+	34.90 ±0.00	24.40 ±0.14	+	26.11 ±0.02	+	+	0	50.51 ±0.25	+
Tsukamurella spumae DSM44113^{T}	-	+	2.47 ±0.00	49.07 ±0.18	-	82.16 ±0.02	+	+	0	60.64 ±0.16	-	-	+	1.28 ±0.07	55.98 ±0.21	+	95.14 ±0.02	+	+	0	61.09 ±0.27	-
Tsukamurella strandjordii DSM 44573^{T}	+	++	31.03 ±0.07	23.72 ±0.05	+	9.66 ±0.00	++	++	0	25.35 ±0.07	-	+	-	6.14 ±0.07	23.80 ±0.12	+	29.20 ±0.01	+	+	0	24.05 ±0.01	-
Tsukamurella sunchonensis DSM45335	+	+	38.29 ±0.07	25.20 ±0.05	-	10.64 ±0.01	+	+	0	27.34 ±0.10	-	+	+	2.47 ±0.00	23.34 ±0.10	-	18.28 ±0.02	+	+	0	25.70 ±0.23	-
Williamsia faeni DSM 45372^{T}	+	+	7.32 ±0.00	29.05 ±0.03	-	31.55 ±0.01	+	+	1.25 ±0.07	61.06 ±0.24	-	+	+	15.00 ±0.00	31.29 ±0.09	+	17.81 ±0.01	+	-	0.00	54.87 ±0.23	-
Williamsia maris DSM 44693^{T}	+	+	6.67 ±0.00	28.07 ±0.19	+	12.73 ±0.02	+	+	0.00	56.52 ±0.22	-	+	+	6.16 ±0.07	27.44 ±0.14	+	20.76 ±0.01	-	+	0.00	57.97 ±0.28	-
Williamsia muralis N1261^{Tsp}	+	++	4.95 ±0.00	23.75 ±0.04	-	73.46 ±0.04	+	-	11.98 ±0.07	26.57 ±0.00	-	+	++	2.60 ±0.00	23.80 ±0.03	-	21.26 ±0.06	+	+	1.32 ±0.07	29.25 ±0.18	-
Williamsia serinedens DSM 45037^{T}	+	++	40.51 ±0.00	24.98 ±0.18	-	39.13 ±0.03	+	+	0.00	35.84 ±0.03	-	+	++	34.68 ±0.29	27.23 ±0.26	-	17.94 ±0.01	+	+	0.00	29.45 ±0.45	-

Appendix 7.2. Frequency of actinobacterial strains (total no. 94) scored positive and negative for Emulsification Index (EI24) scores by category for whole-cell broths (WCBs) and cell-free supernatants (CFS) screened after 7 and 14 days grown in MSM with 2% (v/v) hexadecane at 30°C and 180 rpm. Strains were tested in triplicate. High (EI24 ≥30%); low (EI24 11-29%); weak emulsification (EI24 ≤10%); no emulsification (EI24 0%).



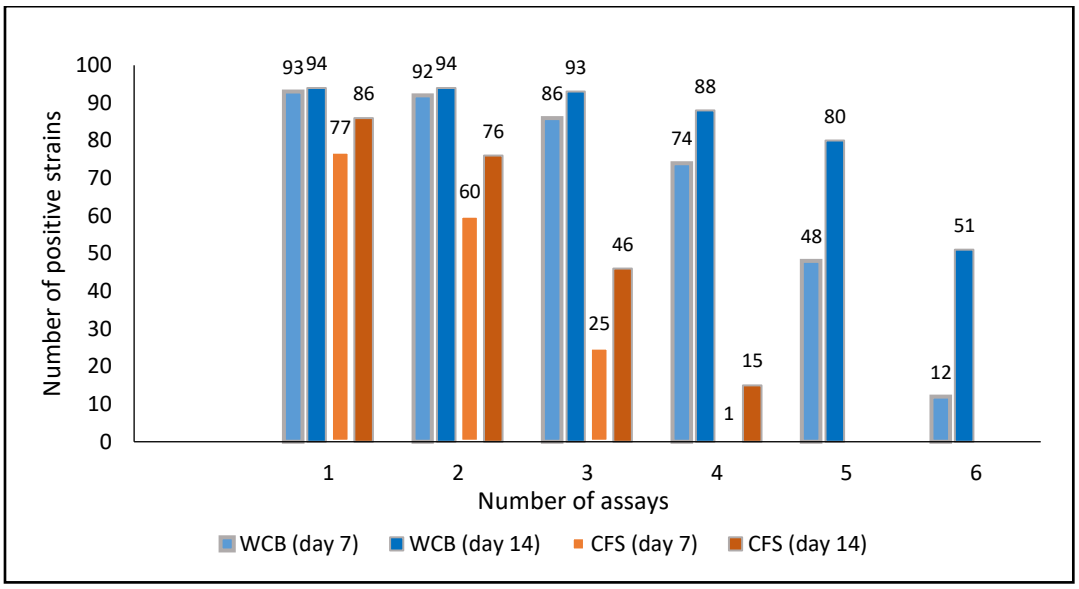
Appendix 7.3. Frequency of actinobacterial strains (total no. 94) scored positive and negative for surface tension (ST mN m-1) scores by category for whole-cell broths (WCBs) and cell-free supernatants (CFS) screened after 7 and 14 days grown in MSM with 2% (v/v) hexadecane at 30°C and 180 rpm. Strains were tested in triplicate. High surface tension reduction (ST <40 mN m-1); moderate surface tension reduction (40-50 mN m-1); and weak to no surface tension reduction (ST > 50-72 mN m-1).



Appendix 7.4. Relation between cell surface hydrophobicity (CSH) and actinobacterial strains with different mycolic-chain lengths measured using the Bacterial Adhesion to Hydrocarbon (BATH) assay with mineral oil, after cultivation for 7 or 14 days in Mineral Salt Medium with hexadecane (2%, v/v).

Genus	**Mycolic acid chain length range**	**CSH% range (min-max)**		**Mean values**	
		Day 7	**Day 14**	**Day 7**	**Day 14**
Corynebacterium (2)	22 - 38	33.5 - 39.1	17.9 – 30.6	36.3 ± 4.0	24.3 ± 9.0
Dietzia (13)	34 - 38	17.1 – 91.5	37.0 – 84.9	66.2 ± 21.1	64.7 ± 14.3
Gordonia (30)	46 - 66	3.11 – 82.2	6.67 – 95.1	74.51 ± 18.5	68.23 ± 27.52
Millisia (1)	44 - 52	16.06	30.76	16.06 ± 0.00	30.76 ± 0.00
Mycobacterium (6)	60 - 90	15.53 – 65.75	40.66 – 86.33	47.53 ± 18.26	73.12 ± 16.86
Nocardia (5)	48 - 60	4.61 - 75.08	19.13 – 87.00	42.85 ± 32.45	52.13 ± 32.44
Rhodococcus (25)	30 - 54	10.42 – 91.55	26.21 – 90.65	60.15 ± 23.09	63.25 ± 18.99
Tsukamurella (8)	64 - 78	4.37 – 82.16	18.28 – 95.14	45.89 ± 32.16	57.57 ± 33.53
Williamsia (4)	50 - 56	3.11- 73.46	11.03 – 21.26	30.21± 31.016	17.71 ± 4.71

Appendix 7.5. The number of actinobacterial strains that tested positive for 1 or more biosurfactant screening assays [from a combination of drop collapse (DC), oil spreading (OS), emulsification index (EI_{24}), surface tension (ST), cetyl trimethylammonium bromide (CTAB), and/or bacterial adherence to hydrocarbons assay (BATH)] when screening whole-cell broths (WCB) and cell-free supernatants (CFS) after growth in MSM with 2% (v/v) hexadecane at 30°C and 180 rpm for 7 and 14 days. Category of positive strains includes + or ++ for DC, OS, + for CTAB assay and EI_{24}, ST <50 nM/m and BATH >40% (moderate and high).



Appendix 7.6. Statistical correlations between the different biosurfactant screening methods were determined using both whole-cell broth (WCB) and cell-free supernatant (CFS) values for days 7 and 14.

Screening assays	Sample (length of incubation)	Spearman rank correlation coefficient (r_s)				
		Drop Collapse	Oil Spreading	Emulsification Index	Surface Tension	CTAB
Drop Collapse		1				
Oil Spreading	WCB (day 7)	0.217	1			
	WCB (day 14)	0.274	1			
	CFS (day 7)	0.466	1			
	CFS (day 14)	0.402	1			
	WCB + CFS (day7 +14)	0.345	1			
Emulsification Index	WCB (day 7)	0.197	0.556	1		
	WCB (day 14)	0.342	0.421	1		
	CFS (day 7)	0.133	0.288	1		
	CFS (day 14)	0.087	0.158	1		
	WCB + CFS (day7 +14)	0.207	0.452	1		
Surface Tension	WCB (day 7)	0.185	0.239	0.042	1	
	WCB (day 14)	-0.229	-0.171	-0.043	1	
	CFS (day 7)	-0.018	-0.116	-0.082	1	
	CFS (day 14)	-0.083	-0.069	-0.177	1	
	WCB + CFS (day7 +14)	-0.087	-0.125	-0.195	1	
Cetyl trimethylammonium bromide (CTAB) assay	WCB (day 7)	-0.15	0.107	0.067	-0.03	1
	WCB (day 14)	0.177	-0.056	0.098	-0.024	1
	CFS (day 7)	-	-	-	-	1
	CFS (day 14)	0.014	0.157	0.024	0.037	1
	WCB + CFS (day7 +14)	0.145	0.247	0.060	-0.006	1
Bacterial adherence to hydrocarbons (BATH) assay	WCB (day 7)	-0.218	-0.289	-0.180	-0.163	0.053
	WCB (day 14)	-0.177	-0.080	-0.294	0.079	0.116
	WCB (day7 +14)	-0.240	-0.201	-0.243	-0.005	0.070

Correlation catergory: 0.00 to 0.19- very weak, 0.20 to 0.39-weak, 0.40 to 0.69 –moderate, 0.70 to 0.89 - strong and 0.90 to 1.00 -very strong correlation. - = no correlation was seen as no strain tested positive in the assay.

Appendix 7.7. Effect of biosurfactant extract to prevent adhesion, inhibit biofilm growth, and disrupt pre-formed biofilms of clinically relevant biofilm-forming strains on polystyrene plate studied using crystal violet staining.

	% Anti-adhesion			% Inhibition					% Disruption					
Biosurfactant Extract	*P. aeruginosa* ENU 18	*K. pneumoniae* NCIMB 8865	*E.coli* ATCC 47055	*P. aeruginosa* ENU 18	*K. pneumoniae* NCIMB 8865	*E.coli* ATCC 47055	*S. epidermidis* ATCC 3598	*S. epidermidis* ENU IL 43	*P. aeruginosa* ENU 18	*P. aeruginosa* ENU 19	*K. pneumoniae* NCIMB 8865	*E.coli* ATCC 47055	*S. epidermidis* ATCC 3598	*S. epidermidis* ENU IL 43
BRrub		30.1 ± 3.4			45.3 ± 5.5				21.4 ± 2.4				4.9 ± 0.9	
BRcer		23.5 ± 0.4			42.4 ± 6.2		11.1 ± 1.1		44.9 ± 0.9					
BRery		7.6 ± 2.5		3.5 ± 2.2	44.1 ± 1.4				20.1 ± 1.1		7.62 ± 0.5		13.8 ± 0.5	51.9 ± 0.7
BRper		22.1 ± 11.1			34.9 ± 2.7				40.7 ± 0.9					
BRyun				28.7 ± 3.2	31.8 ± 2.5				53.4 ± 6.4	26.5 ± 9.1		31.0 ± 0.1	14.5 ± 6.4	
BTpse				1.2 ± 0.6	27.8 ± 0.2				48.9 ± 0.8	17.3 ± 5.2				
BTpul	2.8 ± 0.9				26.1 ± 6.1		5.9 ± 0.8		7.7 ±1.7		7.45 ± 0.4		18.1 ± 1.1	19.4 ± 0.4
BTstr					25.2 ± 7.1				44.5 ± 9.0	22.8 ± 2.8	14.2 ± 5.0	11.5 ± 6.7	20.9 ± 1.6	1.6 ± 0.9
BTuoti					24.1 ± 5.9	15.7 ± 1.5			42.1 ± 6.9	21.1 ± 2.5				
BWmur			13.9 ± 4.3		28.2 ± 8.4	27.2 ± 4.8			49.5 ± 8.7	27.7 ± 3.1				16.3 ± 1.8
BWser					36.5 ± 1.3	13.2 ± 6.9		2.88 ± 0.9	31.6 ± 2.3		15.2 ± 8.1			29.7 ± 1.1

Note: Blank spaces indicate either no anti-biofilm activity or an apparent increase in adhesion or biofilm formation compared to the controls. Data expressed as the mean % values ± standard deviation from three biological replicates.

Appendix 7.8. Mean values for anti-adhesion, inhibition, and disruption activity of *Rhodococcus ruber, R. yunnanensis, and Willimisia muralis* biosurfactant extracts against *Pseudomonas. aeruginosa* ENU 18 and *Klebsiella pneumoniae* NCIMB 8865 static biofilms quantified using 2,3,5-TTC - Triphenyltetrazolium chloride (TTC) assay measured at 450 and 490 nm and crystal violet (CV) assay at 590nm.

Biosurfactant extract	Biofilm forming strain	Anti -adhesion			Inhibition			Dispersal		
		TTC		CV	TTC		CV	TTC		CV
		OD 450nm	OD 490nm	590nm	OD 450nm	OD 490nm	590nm	OD 450nm	OD 490nm	590nm
Rhodococcus ruber	*Pa*	5.7 ± 0.06	6.0 ± 0.06	-22.1 ± 0.06	19.1 ± 0.07	16.4 ± 0.07	-32.1 ± 0.01	19.8 ± 0.06	26.7 ± 0.07	21.4 ± 2.37
	Kp	55.9 ± 0.07	53.1 ± 0.07	30.1 ± 3.43	84.5 ± 0.09	82.4 ± 0.16	44.1 ± 1.48	1.4 ± 0.22	1.2 ± 0.10	-21.9 ± 0.04
Rhodococcus. yunnanensis	*Pa*	28.6 ± 0.08	24.2 ± 0.10	-30 ± 0.02	57.4 ± 0.02	58.4 ± 0.01	28.7 ± 3.2	3.8 ± 0.05	6.0 ± 0.06	53.4 ± 6.4
	Kp	53.1 ± 0.07	51.6 ± 0.11	-2.2 ± 0.77	76.9 ± 0.11	74.5 ± 0.32	31.8 ± 2.54	17.9 ± 0.08	16.5 ± 0.18	-36.3 ± 0.04
Willimsia muralis	*Pa*	28.8 ± 0.06	27.0 ± 0.08	-82.8 ± 0.05	8.2 ± 0.07	11.9 ± 0.05	-29.1 ± 0.01	4.9 ± 0.04	9.1 ± 0.07	49.5 ± 8.66
	Kp	49.7 ± 0.05	47.2 ± 0.07	13.9 ± 4.3	83.9 ± 0.12	82.5 ± 0.15	28.2 ± 8.35	9.3 ± 0.12	9.25 ± 0.14	11.2 ± 0.09

CV- crystal violet; Kp: *Klebsiella pneumoniae* NCIMB 8865; ; OD: optical density; *Pa*: *Pseudomonas aeruginosa* ENU 18; TTC: 2,3,5-TTC - Triphenyltetrazolium chloride.

Appendix 7.9. Growth and biosurfactant production measurements for *Rhodococcus ruber* IEGM 231^{T} recorded during batch cultivation in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C and 180 rpm over 14 days.

Day	Biomass measurement			Biosurfactant measurement				
	OD (600nm)	Plate count (cfu ml^{-1})	Biomass (g L^{-1})	ST WCB (mN m^{-1})	ST CFS (mN m^{-1})	BATH WCB (%)	EI_{24} WCB (%)	EI_{24} CFS (%)
0	0 ± 0.00	8.16E+05 ± 0.30	0	58.47 ± 0.12	69.00 ± 0.19	0 ± 0.00	0 ± 0.00	0
1	1.697 ± 0.00	1.16E+07 ± 0.29	1	27.26 ± 0.02	68.82 ± 0.05	46.19 ± 0.00	0 ± 0.00	0
2	2.378 ± 0.09	2.56E+07 ± 0.32	1	27.39 ± 0.09	65.31 ±0.07	43.87 ± 0.00	0 ± 0.00	0
3	3.086 ± 0.03	3.73E+07 ± 0.36	1	26.29 ±0.13	63.27 ± 0.23	79.13 ± 0.03	45.53 ± 0.12	0
4	2.211 ± 0.04	6.20E+07 ± 0.08	2	28.22 ± 0.2	52.23 ± 0.07	89.10 ± 0.01	45.16 ± 0.14	0
7	1.526 ± 0.04	5.80E+07 ± 0.05	6	29.11 ±0.04	44.28 ± 0.09	74.18 ± 0.05	46.08 ± 0.25	0
8	1.657 ± 0.01	5.20E+07 ± 0.25	6	28.43 ± 0.06	50.27 ± 0.11	56.36 ± 0.20	34.39 ± 0.13	0
9	1.336 ± 0.15	5.63E+07 ± 0.24	5	36.14 ± 0.15	52.96 ± 0.03	60.22 ± 0.05	34.95 ± 0.09	0
10	1.181 ± 0.06	5.33E+07 ± 0.30	5	37.76 ± 0.10	49.86 ± 0.12	6.83 ± 0.20	30.63 ± 0.05	0
11	1.427 ± 0.06	4.78E+07 ± 0.28	4	39.16 ± 0.24	49.98 ± 0.04	6.14 ± 0.01	30.18 ± 0.22	0
14	1.046 ± 0.06	1.53E+07 ± 0.26	4	39.5 ± 0.04	50.58 ± 0.08	4.10 ± 0.03	30.16 ± 0.24	0

Abbreviations: BATH, bacterial adherence to hydrocarbon assay; CFS, cell-free supernatant; EI_{24}, emulsification index; OD, optical density; ST, surface tension; WCB, whole cell broth.

Appendix 7.10. Growth and biosurfactant production measurements for *Rhodococcus yunnanensis* DSM 44837^T recorded during batch cultivation in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C and 180 rpm over 14 days.

Day	Biomass measurement			Biosurfactant measurement				
	OD (600nm)	Plate count (cfu ml^{-1})	Biomass (g L^{-1})	ST WCB (mN m^{-1})	ST CFS (mN m^{-1})	BATH WCB (%)	EI_{24} WCB (%)	EI_{24} CFS (%)
0	0.153 ± 0.05	5.50E+05 ± 0.34	0	53.32 ± 0.04	62.00 ± 0.16	14.38 ± 0.04	0 ± 0.00	0
1	2.620 ±0.07	1.28E+07 ± 0.27	0.5	30.43 ±0.15	62.24 ± 0.27	20.07 ± 0.01	8.78 ± 0.29	0
2	2.732 ±0.03	1.42E+07 ± 0.20	0.5	27.02 ± 0.06	63.05 ± 0.29	23.43 ± 0.01	36.31 ± 0.21	0
3	3.251 ±0.13	1.78E+07 ± 0.20	1	26.09 ± 0.01	57.2 ± 0.07	29.89 ± 0.05	38.31 ± 0.12	0
4	4.590 ±0.24	2.24E+07 ± 0.15	1	26.68 ± 0.04	45.74 ± 0.19	51.07 ± 0.06	31.35 ± 0.70	0
7	4.487 ±0.07	1.22E+07 ± 0.20	2	28.99 ± 0.24	35.58 ± 0.07	33.84 ± 0.02	28.08 ± 0.09	0
8	4.477 ±0.20	1.31E+07 ± 0.25	2	28.25 ± 0.22	35.12 ± 0.15	22.47 ± 0.00	19.33 ± 0.25	0
9	4.300 ±0.10	1.08E+07 ± 0.16	1.5	29.16 ± 0.17	36.86 ± 0.15	22.45 ± 0.01	12.02 ± 0.15	0
10	3.583 ±0.11	1.07E+07 ± 0.30	1.5	28.88 ± 0.09	37.85 ± 0.09	23.31 ± 0.01	11.51 ± 0.19	0
11	3.583 ±0.11	1.04E+07 ± 0.30	1.5	29.84 ± 0.14	37.61 ± 0.20	14.04 ± 0.02	13.79 ± 0.08	0
14	3.394 ±0.26	1.03E+07 ± 0.21	1.5	31.65 ± 0.17	40.09 ± 0.25	10.38 ± 0.03	14.02 ± 0.07	0

Abbreviations: BATH, bacterial adherence to hydrocarbon assay; CFS, cell-free supernatant; EI_{24}, emulsification index; OD, optical density; ST, surface tension; WCB, whole cell broth.

Appendix 7.11. Growth and biosurfactant production measurements for *Williamsia muralis* N1261^{Tsp} recorded during batch cultivation in mineral salts medium supplemented with hexadecane (2%, v/v) at 30°C and 180 rpm over 14 days.

Day	Biomass measurement			Biosurfactant measurement				
	OD (600nm)	Plate count (cfu ml^{-1})	Biomass (g L^{-1})	ST WCB (mN m^{-1})	ST CFS (mN m^{-1})	BATH WCB (%)	EI_{24} WCB (%)	EI_{24} CFS (%)
0	0.060 ±0.02	4.27E+05 ± 0.25	0	53.52 ± 0.17	63.52 ± 0.17	85 ± 0.02	0.00 ± 0.02	0 ± 0.0
1	0.288 ±0.03	2.42E+06 ± 0.30	0	27.26 ± 0.12	61.22 ± 0.07	80 ± 0.01	0.00 ± 0.01	0 ± 0.0
2	1.504 ±0.02	1.24E+07 ± 0.36	1.5	26.52 ± 0.26	60.92 ± 0.28	86 ± 0.02	2.33 ± 0.02	0 ± 0.15
3	1.636 ± 0.05	1.39E+07 ± 0.30	1.5	26.74 ± 0.26	50.41 ± 0.16	81 ± 0.02	3.44 ± 0.02	3.57 ± 0.24
4	1.735 ± 0.08	1.91E+07 ± 0.31	2	26.74 ± 0.25	46 ± 0.28	85 ±0.01	7.26 ± 0.01	3.70 ± 0.22
7	0.217 ± 0.03	1.46E+07 ± 0.24	3	25.74 ± 0.25	27.76 ± 0.19	73 ± 0.04	7.16 ± 0.04	10.53 ± 0.18
8	0.185 ± 0.08	1.44E+07 ± 0.28	3	28.37 ± 0.18	27.36 ± 0.17	56 ± 0.02	6.26 ± 0.02	10.53 ± 0.28
9	0.144 ± 0.03	1.40E+07 ± 0.15	3	30.64 ± 0.25	28.16 ± 0.14	56 ± 0.02	5.37 ± 0.02	9.53 ± 0.16
10	0.115 ± 0.03	1.36E+07 ± 0.35	2.5	30.21 ± 0.11	29.15 ± 0.22	55 ± 0.04	5.30 ± 0.04	7.14 ± 0.21
11	0.095 ± 0.05	1.33E+07 ± 0.25	2.5	31.91 ± 0.05	29.03 ± 0.22	55 ± 0.04	5.01 ± 0.04	5.11 ± 0.04
14	0.110 ±0.02	1.31E+07 ± 0.26	2	31.17 ± 0.06	31.63 ± 0.29	21 ± 0.06	4.95 ± 0.06	5.01 ± 0.13

Abbreviations: BATH, bacterial adherence to hydrocarbon assay; CFS, cell-free supernatant; EI_{24}, emulsification index; OD, optical density; ST, surface tension; WCB, whole cell broth.

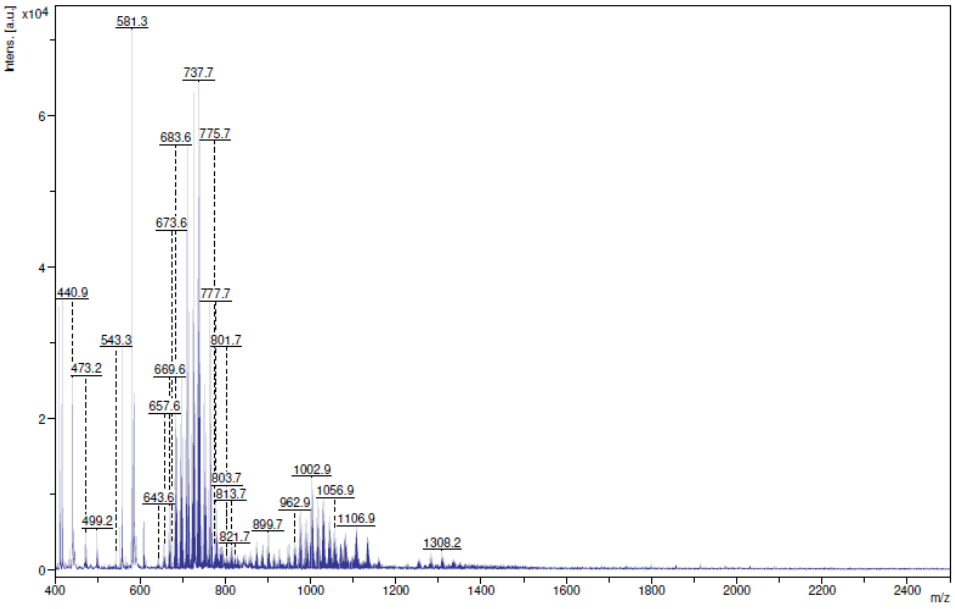
Appendix 7.12. Percent reduction in adhesion, biofilm inhibition, and pre-formed biofilm disruption determined for biosurfactant extracts and isolated glycolipids from actinobacteria in polystyrene microplate assays with quantification using 2,3,5-triphenyl tetrazolium chloride (TTC) and 450nm wavelength.

Actinobacterial strain	Biofilm strain	Anti-adhesion		Biofilm Inhibition		Biofilm Disruption	
		Glycolipids	Biosurfactant extract	Glycolipids	Biosurfactant extract	Glycolipids	Biosurfactant extract
Rhodococcus ruber IEGM 231^{T}	*Kp*	57.8 ± 0.16	55.9 ± 0.07	60.6 ± 0.24	84.5 ± 0.09	5.7 ± 0.18	1.4 ± 0.22
	Pa	22.1 ± 0.12	5.7 ± 0.06	53.6 ± 0.08	19.1 ± 0.07	25.9 ± 0.15	19.8 ± 0.06
Rhodococcus yunnanensis DSM 44837^{T}	*Kp*	32.9 ± 0.08	53.1 ± 0.07	64.6 ±0.26	76.9 ± 0.11	27.4 ± 0.3	17.9 ± 0.08
	Pa	30.2 ±0.08	28.6 ±0.08	43.2 ± 0.06	57.4 ± 0.02	6.3 ± 0.2	3.8 ± 0.05
Williamsia muralis N1261^{Tsp}	*Kp*	54.9 ± 0.05	49.7 ± 0.05	65 ± 0.04	83.9 ± 0.12	12.2 ± 0.5	9.3 ± 0.12
	Pa	39.6 ± 0.06	28.8 ± 0.06	35.5 ± 0.04	8.2 ± 0.07	8.4 ± 0.13	4.9 ± 0.04

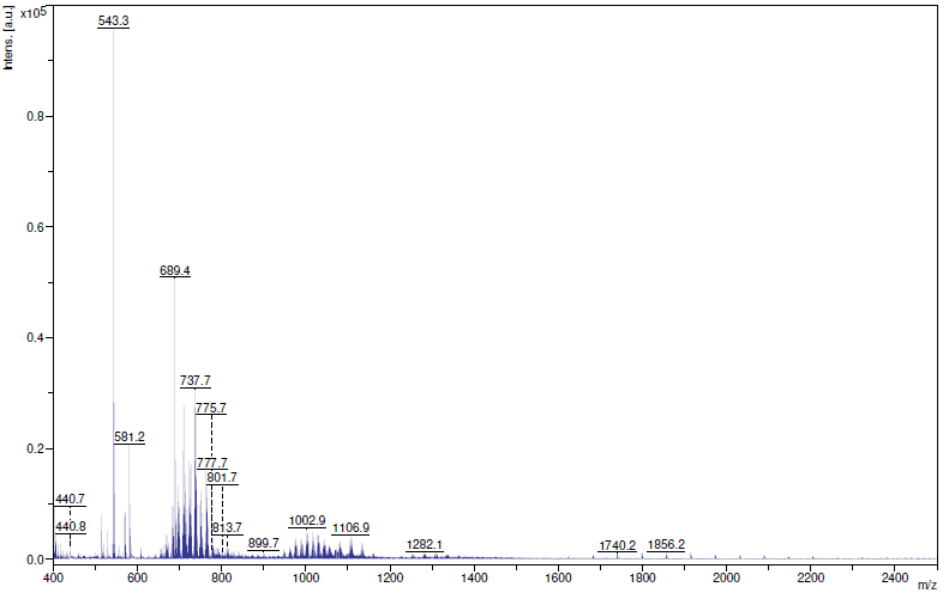
Abbreviations: *Kp*, *Klebsiella pneumoniae* NCIMB 8865; *Pa*, *Pseudomonas aeruginosa* ENU 18.

Appendix 7.13. Mass spectra of purified R90 rhamnolipid biosurfactant standard produced when analysed by positive ion matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) using a) 9-amino acridine (9-AA) and b) di-hydroxybenzoic acid (DHB) matrices analysed from mass range 300-4000 mass units. m/z denotes the mass-to-charge ratio.

a)



b)



Appendix 7.14. Mass peaks and corresponding rhamnolipid compounds detected in purified R90 rhamnolipid biosurfactant standard analysed in positive ion matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) using 9-amino acridine (9-AA) and di-hydroxybenzoic acid (DHB) matrices from a mass range of 300-4000 mass unit.

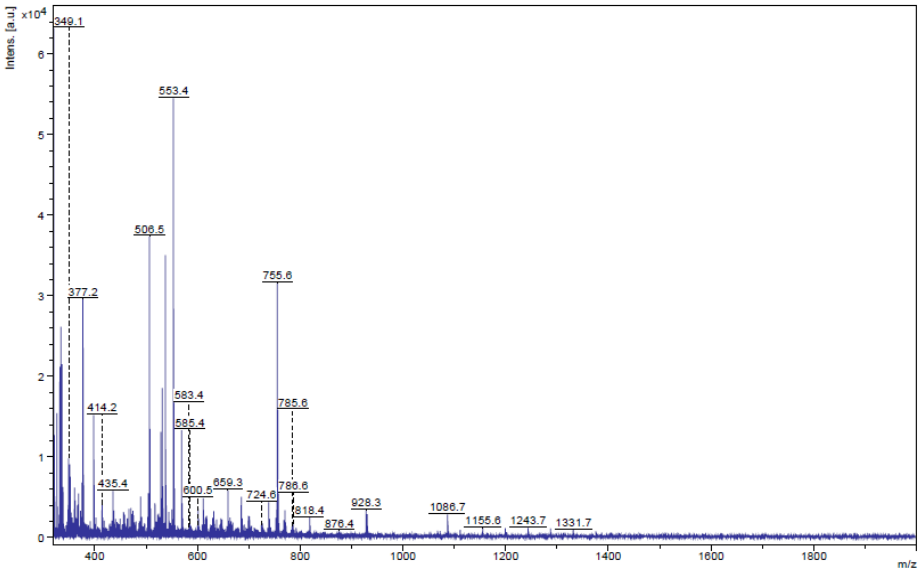
Common Name from LIPID MAPS	Formula	Adduct	Matrix		Adduct	Matrix		Adduct	Matrix	
		[M+Na]+	DHB+	9-AA +	[M-H+2Na]+	DHB +	9-AA +	[M+K]+	DHB +	9AA +
3-O-alpha-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid + ISOMERS	$C_{26}H_{48}O_9$	527.3190			549.3009			543.2930		
3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid	$C_{36}H_{64}O_{10}$	679.4392			701.4211			695.4132		
3-O-(alpha-L-rhamnopyranosyl-(1-2)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoyl-3-hydroxydecanoic acid	$C_{32}H_{58}O_{13}$	673.3769			695.3588			689.3509		
3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl-(1-2)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid	$C_{42}H_{74}O_{14}$	825.4971			847.4790			841.4711		
3-O-alpha-L-rhamnopyranosyl-3-hydroxyoctanoyl-3-hydroxydecanoic acid	$C_{24}H_{44}O_9$	499.2877			521.2696			515.2617		
3-O-alpha-L-rhamnopyranosyl-3-hydroxyundecanoyl-3-hydroxydecanoic acid	$C_{27}H_{50}O_9$	541.3347			563.3166			557.3087		
3-O-alpha-L-rhamnopyranosyl-3-hydroxydodecanoyl-3-hydroxydecanoic acid	$C_{28}H_{52}O_9$	555.3503			577.3322			571.3243		
3-O-alpha-L-rhamnopyranosyl-3-hydroxy-7Z-dodecenoyl-3-hydroxydecanoic acid	$C_{28}H_{50}O_9$	553.3347			575.3166			569.3087		

Common Name from LIPD MAPS	Formula	Adduct	Matrix		Adduct	Matrix		Adduct	Matrix	
		[M+Na]+	DHB+	9-AA +	[M-H+2Na]+	DHB+	9-AA +	[M+K]+	DHB+	9-AA +
3-O-alpha-L-rhamnopyranosyl-3-hydroxy-5Z-dodecenoyl-3-hydroxydecanoic acid	$C_{28}H_{50}O_9$	553.3347			575.3166			569.3087		
3-O-alpha-L-rhamnopyranosyl-3-hydroxy-5Z-tetradecenoyl-3-hydroxydecanoic acid	$C_{30}H_{54}O_9$	581.3660			603.3479			597.3400		
2-O-alpha-L-rhamnopyranosyl-alpha-L-rhamnopyranosyl-beta-hydroxytetradecanoyl-beta-hydroxytetradecanoate	$C_{40}H_{74}O_{13}$	785.5021			807.4840			801.4761		
Dokdolipid A	$C_{24}H_{46}O_8$	485.3085			507.2904			501.2825		
Dokdolipid B	$C_{24}H_{44}O_8$	483.2928			505.2747			499.2668		
Dokdolipid C	$C_{30}H_{56}O_{12}$	631.3664			653.3483			647.3404		
Rha-C14:2	$C_{20}H_{34}O_7$	409.2197			431.2016			425.1937		
Rha-C8-C8	$C_{22}H_{40}O_9$	471.2564			493.2383			487.2304		
Rha-C8-C10:1	$C_{24}H_{42}O_9$	497.2721			519.2540			513.2461		
Rha-C10-C10:1	$C_{26}H_{46}O_9$	525.3034			547.2853			541.2774		
Rha-C10-C12:1	$C_{28}H_{50}O_9$	553.3347			575.3166			569.3087		
Rha-C14-C14	$C_{34}H_{64}O_9$	639.4442			661.4261			655.4182		
Rha-C14-C16	$C_{36}H_{68}O_9$	667.4755			689.4574			683.4495		
Rha-C16-C16	$C_{38}H_{72}O_9$	695.5068			717.4887			711.4808		

Common Name from LIPD MAPS	Formula	Adduct	Matrix		Adduct	Matrix		Adduct	Matrix	
		[M+Na]+	DHB+	9-AA +	[M-H+2Na]+	DHB+	9-AA +	[M+K]+	DHB+	9-AA +
Rha-Rha-C8	$C_{20}H_{36}O_{11}$	475.2150			497.1969			491.1890		
Rha-Rha-C10	$C_{22}H_{40}O_{11}$	503.2463			525.2282			519.2203		
Rha-Rha-C12	$C_{24}H_{44}O_{11}$	531.2776			553.2595			547.2516		
Rha-Rha-C14	$C_{26}H_{48}O_{11}$	559.3089			581.2908			575.2829		
Rha-Rha-C8-C10	$C_{30}H_{54}O_{13}$	645.3456			667.3275			661.3196		
Rha-Rha-C10-C10:1	$C_{32}H_{56}O_{13}$	671.3613			693.3432			687.3353		
Rha-Rha-C10-C12:1	$C_{34}H_{60}O_{13}$	699.3926			721.3745			715.3666		
Rha-Rha-C10-C12	$C_{34}H_{62}O_{13}$	701.4082			723.3901			717.3822		
Rha-Rha-C10-C14:1	$C_{34}H_{64}O_{13}$	703.4239			725.4058			719.3979		
Rha-Rha-C12-C12	$C_{36}H_{66}O_{13}$	729.4395			751.4214			745.4135		
Rha-Rha-C14-C12	$C_{38}H_{70}O_{13}$	757.4708			779.4527			773.4448		
Rha-Rha- C14-C16	$C_{42}H_{78}O_{13}$	813.5334			835.5153		?	829.5074		?
Rha-Rha-C16-C16	$C_{44}H_{82}O_{13}$	841.5647			863.5466			857.5387		
Rha-Rha-C14-C14-C14	$C_{54}H_{100}O_{15}$	1011.6954			1033.6773			1027.6694		
Rha-Rha-C10-C10-CH3	$C_{33}H_{60}O_{13}$	687.3926			709.3745			703.3666		

Not observed Weak Observed

Appendix 7.15. Mass spectra of *Rhodococcus ruber* IEGM 231^{T} glycolipid complexes a) overall spectra, b), c) and d) breakdown of spectra, isolated by solvent extraction and thick-layer chromatography when analysed by positive ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) with a di-hydroxybenzoic acid (DHB) matrix and a mass range 300-4000 mass units. m/z denotes the mass-to-charge ratio.



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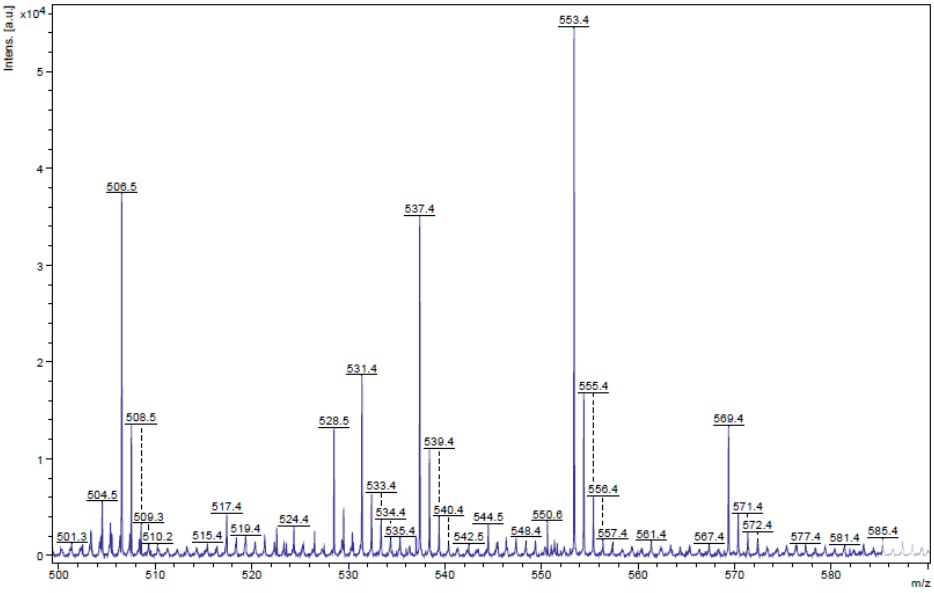

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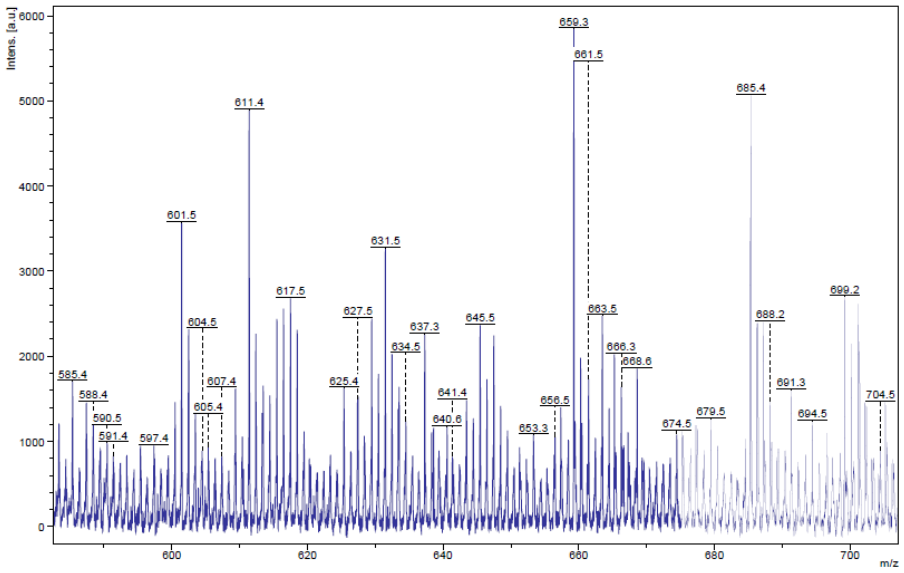
Intens. [a.u.]
6000
5000
4000
3000
2000
1000
0
659.3
661.5
611.4
685.4
601.5
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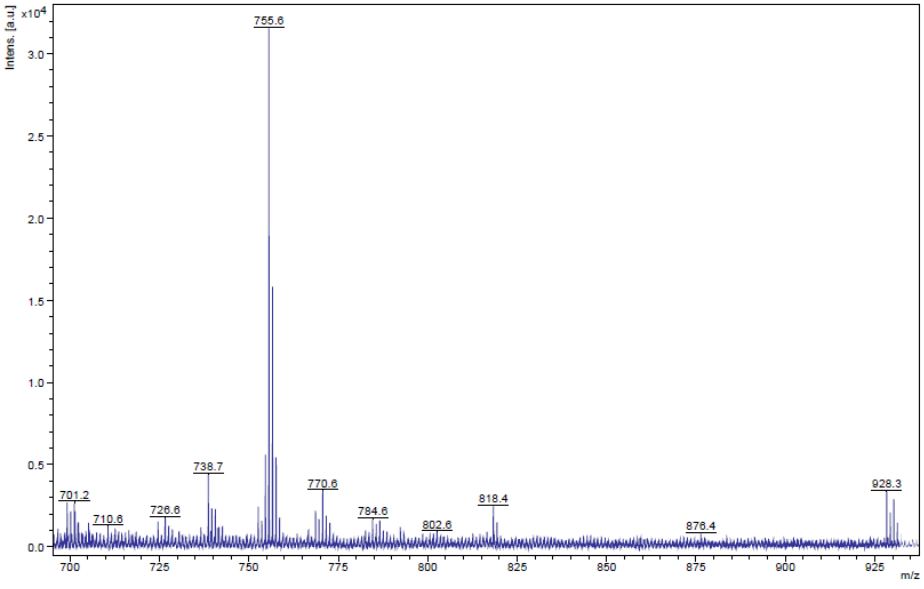
699.2
627.5
663.5
688.2
604.5
637.3
645.5
634.5
666.3
668.6
585.4
607.4
625.4
641.4
656.5
691.3
704.5
588.4
606.4
640.6
653.3
679.5
694.5
590.5
674.5
591.4
597.4
600
620
640
660
680
700
m/z

c)


Intens. [a.u.]
x10^4
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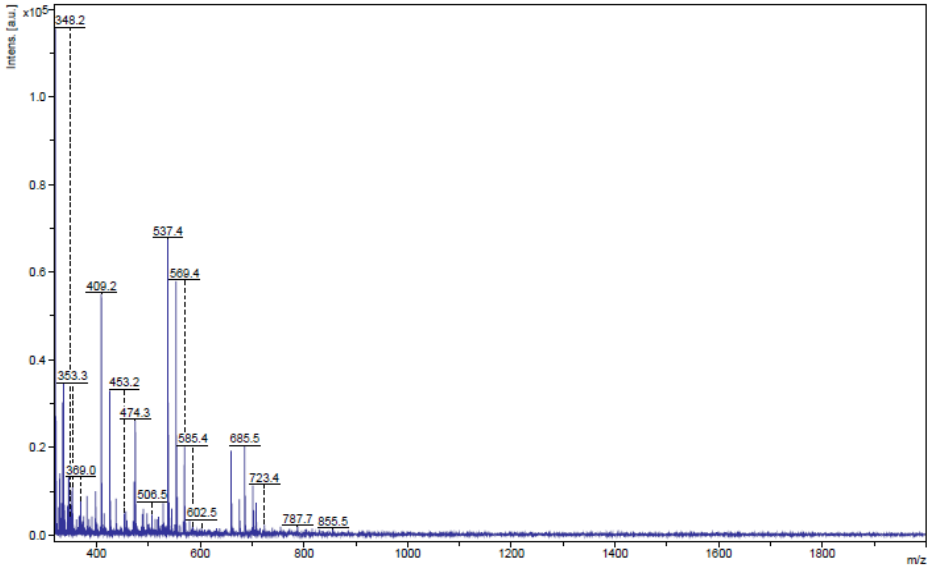
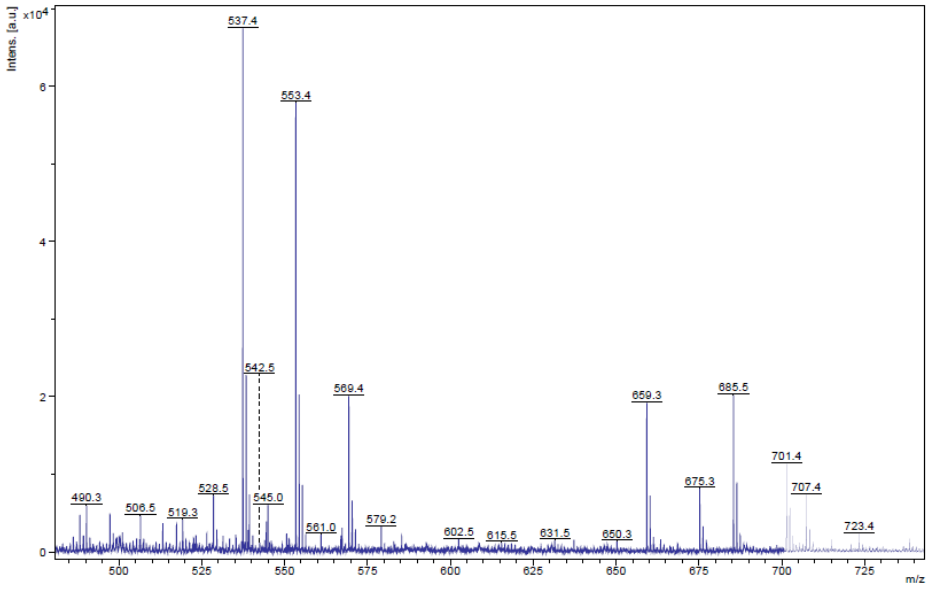
2.5
2.0
1.5
1.0
0.5
0.0
755.6
738.7
770.6
701.2
710.6
726.6
784.6
818.4
802.6
876.4
928.3
700
725
750
775
800
825
850
875
900
925
m/z

d)

Appendix 7.16. Mass spectra of *Rhodococcus yunnanensis* DSM 44837^T glycolipid complexes a) overall spectra, b) and c) breakdown of spectra, isolated by solvent extraction and thick-layer chromatography when analysed by positive ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) with a di-hydroxybenzoic acid (DHB) matrix and a mass range 300-4000 mass units. m/z denotes the mass-to-charge ratio.

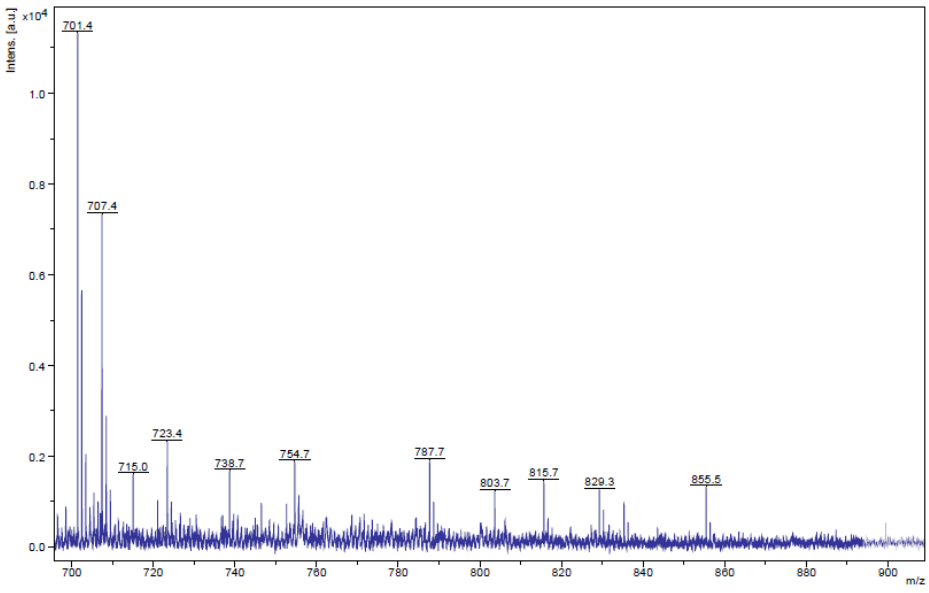


a)



b)

c)


Intens. [a.u.]
x10^4
1.0
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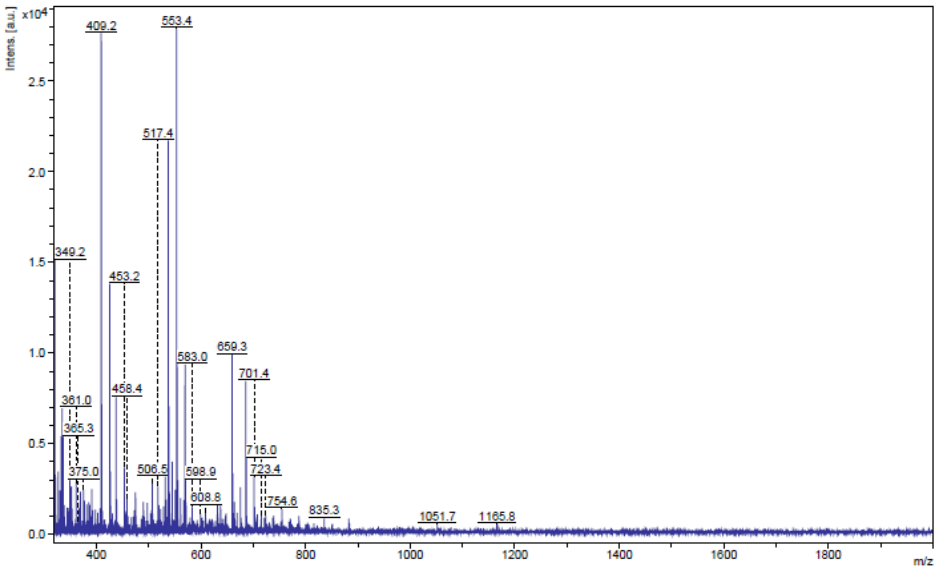
0.6
0.4
0.2
0.0
701.4
707.4
715.0
723.4
738.7
754.7
787.7
803.7
815.7
829.3
855.5
700
720
740
760
780
800
820
840
860
880
900
m/z

Appendix 7.17. Mass spectra of *Williamsia muralis* N1261^{Tsp} glycolipid complexes a) overall spectra b) and c) breakdown of spectra, isolated by solvent extraction and thick-layer chromatography when analysed by positive ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) with a di-hydroxybenzoic acid (DHB) matrix and a mass range 300-4000 mass units. m/z denotes the mass-to-charge ratio.

a)



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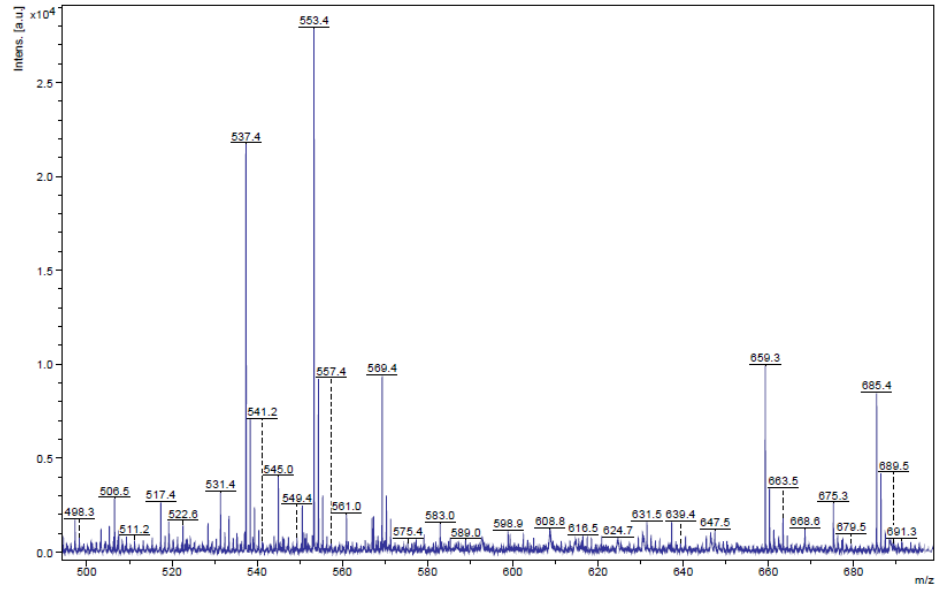
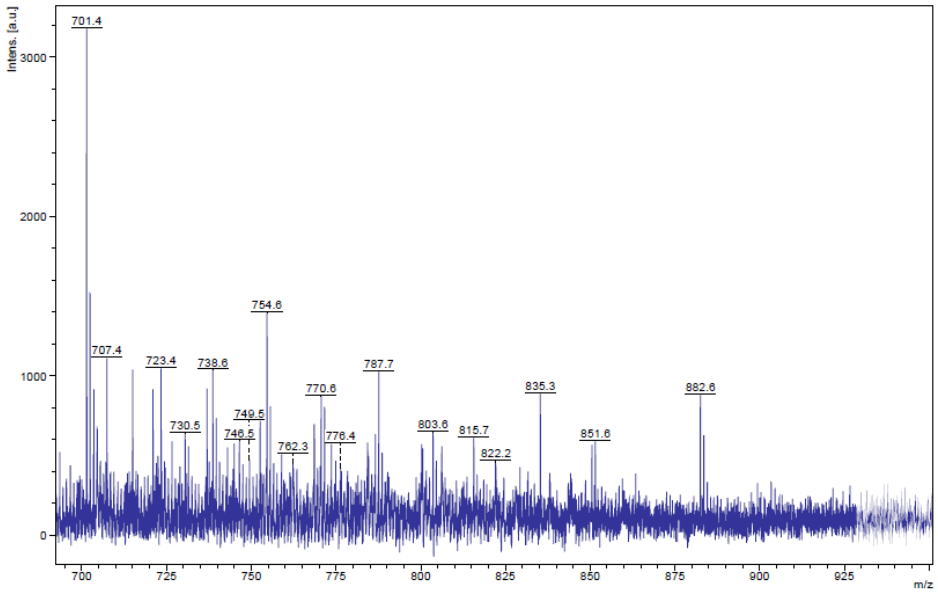


c)


Intens. [a.u.]
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730.5
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746.5
762.3
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787.7
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815.7
822.2
835.3
851.6
882.6
700
725
750
775
800
825
850
875
900
925
m/z