BIOSURFACTANT SCREENING THROUGH METAGENOMIC AND CULTURE-BASED APPROACHES

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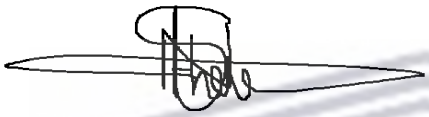
DECLARATION

I, Nombuso Slindile Sithole declare that the thesis titled "***Biosurfactant Screening through Metagenomic and Culture-Based Approaches***" is my work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledge by complete references.

Signature:  Date: 11/November/2022

ABSTRACT

Biosurfactant Screening through Metagenomic and Culture-Based Approaches

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The biosurfactant market is still predominantly represented by the three main products: surfactin, sophorolipids, and rhamnolipids, mainly as a result of the high cost and low yield associated with biosurfactant production. Moreover, only a few novel structures have been discovered in the last decade, due to a lack of interest, low hit rate, and high redundancy, in which the predominant microorganisms producing the same group of biosurfactants are isolated. Hence, there is the obvious need to open up the market with structurally and functionally diverse compounds that have the potential to tailor to specific needs or applications in different industries. In this study, bioprospecting for new biosurfactants was done through the use of easy and well established screening methods employed to screen for surface activity (i) in a metagenomic library and (ii) in an unexplored bacterial strain. A culture-independent approach *via* function-based metagenomic screening was applied in an attempt to uncover the diversity hidden in unculturable microorganisms from the sea. A culture-based approach was also employed to investigate the biosurfactant production potential of the novel *Planococcus* sp. CP5-4 strain, which until this study has never been reported as a potential biosurfactant producer. Employing the high throughput atomized spray screening method, a total of 25 000 and 20 000 clones were screened for biosurfactant activity both in *Escherichia coli* EPI300 and *Pseudomonas putida* MBDI host strains, respectively, and no activity was detected. While the screening methods namely the atomized spray method, drop-collapse test, microplate-grid analysis assay, and emulsification index (EC_{24}) confirmed the *Planococcus* sp. CP5-4 strain as a new biosurfactant producer with an EC_{24} of up to 80.3% ± 3.70. Moreover, the *Planococcus* sp. CP5-4 strain had multiple surface-active extracts detected, and the highest active extract was able to reduce the contact angle (θ°) of water from 104° ± 0.45 to 62.1° ± 3.73 measured through low-bond axisymmetric drop shape analysis (LB-ADSA). The role of the carotenoid biosynthetic pathway in the production of the biosurfactants detected was hypothesized and investigated. The wild type orange *Planococcus* sp. CP5-4 strain and its mutants; one with a truncated *crtP* gene (yellow mutant) and the other without the carotenoid biosynthetic pathway (unpigmented mutant) made it possible to investigate for the first time the relation between the carotenoid biosynthetic pathway and the biosurfactant activity observed. One of the molecules with measurable surface reduction abilities was deduced to be a pigmented C_{30}-carotenoid fatty acid ester produced by the *Planococcus* sp. CP5-4 strain which could be detected *via* Thin Layer Chromatography followed by primuline and α-naphthol [EtOH] H_2SO_4 staining.

KEYWORDS: Biosurfactant, Culture-based, Culture-independent, Metagenomic, Function-based screening, *Planococcus* sp. CP5-4, Carotenoid glycolipid, C_{30}-carotenoid fatty acids ester, Contact angle measure.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Antibiotics and secondary metabolites analysis shell	AntiSMASH
Bacterial Artificial Chromosome	BAC
Base pair	Bp
Basic Logic Alignment search tool	BLAST
Centrifugal force	*x g*
Cetyltrimethylammonium bromide	CTAB
Chloramphenicol Resistance	Cam^R
Colony forming unit	Cfu
Contiguous	Contig
Critical Micelle Concentration	CMC
Degree Celsius	°C
Deoxyribonucleic acid	DNA
Emulsification Capacity after 24 hours	EC_{24}
Environmental DNA	eDNA
Escherichia coli	*E. coli*
Ethylenediaminetetraacetic acid	EDTA
Gas Chromatography Mass Spectrometry	GC-MS
Gram	G
High Molecular weight	HMW
High-performance liquid chromatography	HPLC
Hydrophilic-Lipophilic Balance	HBL
Institute for microbial biotechnology and metagenomics	IMBM
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Kanamycin	Kan
Kilobase pair	Kb
Lambda DNA digested with PstI restriction enzyme	λpstI
Liquid chromatography mass spectrometry	LC-MS
Litre	L
Luria-Bertani broth	LB
Lyso-ornithine Lipid(s)	LOL
Mass Spectrometry	MS
Metagenomic DNA	mDNA
Methyl methane sulfonate	MMS
Microgram per millilitre	µg/mL
Micrograms	µg

Microlitres	µl
Micromolar	µM
Milligram	Mg
Millilitre	mL
Millimolar	mM
Minutes	Min
Molar	M
N-acylated amino acids synthase	(NAS)
National center for biotechnology information	NCBI
Next-Generation Sequencing	NGS
Non-ribosomal peptide synthase	NRPS
Nuclear magnetic resonance	NMR
Oil-in-water emulsion	O/W
Origin of replication	ORI
Ornithine Acyl-ACP N-Acyltransferase gene	*olsB*
Overnight	O/N
Plasmid fosmid	pFos
Polyketide synthase	PKS
Polymerase chain reaction	PCR
Restriction endonuclease	RE
Revolutions per minute	Rpm
Ribonucleic acid	RNA
Ribosomal RNA	rRNA
Sodium dodecyl sulphate	SDS
Thin layer chromatography	TLC
Trademark	TM
Tris-base Acetic acid EDTA	TAE
Tris-hydroxymethyl aminomethane	Tris
Ultraviolet	UV
Ultraviolet-visible spectroscopy	UV/Vis
Volts	V
Volume/volume	v/v
Water-in-oil emulsion	W/O
Weight per volume	w/v

Chapter 1: Literature review

1.1. Introduction

Surfactants are surface-active amphiphilic compounds that reduce the surface tension of liquids or the interfacial tension between two phases (Thies *et al*., 2016). They have diverse applications in food, cosmetics, pharmaceuticals, bioremediation, and agriculture where there are commonly used as detergents, emulsifiers, and wetting and foaming agents (Thies *et al*., 2016; Fenibo *et al*., 2019; Shakeri *et al.,* 2021). Although synthetic surfactants are widely employed in industrial and consumer products, there are several issues associated with their use. It is of great concern that they are produced from non-renewable petrochemical feedstock and depending on their structure they cannot be degraded, thus having a lasting detrimental effect on the environment (Cirelli *et al*., 2008; Thies *et al*., 2016; Sanches *et al*., 2021). This has fuelled the growing interest in the discovery of biosurfactants as an environmentally compatible alternative. Biosurfactants are biologically produced surface-active agents, found abundantly in nature. They are produced by a variety of organisms such as filamentous fungi, bacteria, yeast, and plants; however, great emphasis will be placed on the discovery of biosurfactants of microbial origin (Sanches *et al*., 2021). Biosurfactants have activity that is comparable with or superior to synthetic surfactants (Otzen, 2017). Furthermore, they possess an expansive structural diversity which accounts for the array of diverse physicochemical properties they exhibit (Gutierrez *et al*., 2019; Kubicki *et al*., 2019).

The diversity of biosurfactants is not well represented in the market, furthermore, only a few novel biosurfactant structures have been discovered in the last decade hence the need for prospecting for new biosurfactants (Trindade *et al*., 2021). There are two means generally employed to explore for new biosurfactant structures, which are namely culture-dependent and culture-independent approaches (Gabor *et al.,* 2004). The culture-dependent approach entails screening, isolation, and extraction of biosurfactants from culturable microorganisms. This approach has yielded the largest fraction of the biosurfactants reported in literature to date (Walter *et al*., 2010; Kubicki *et al*., 2019). However, not all microorganisms are culturable. Only an estimated 1% of microorganisms can be cultured using traditional culturing techniques (Bekele *et al*., 2021). This limits the discovery of novel biosurfactants to only a small fraction of what is available in the microbiome (Williams and Trindade, 2017). Therefore, bioprospecting based on methods that look past culturing approaches permits potential access to genes from undiscovered microorganisms and could lead to the discovery of novel structures (Martin *et al*., 2014; Bekele *et al*., 2021). Metagenomics is a culture-independent tool at the forefront of novel biosurfactant discovery and through metagenomics, genes, and gene clusters, based on heterologous expression of metagenomic library clones and/ or sequencing, can be identified (Bekele *et al.*, 2021). Access to novel structures is dependent on the properties being screened for as well as the distinctiveness of the microorganism screened (Trindade *et al*., 2021).

This thesis presents prospecting for novel biosurfactant(s) using both culture-dependent and culture-based approaches. Chapter one covers the introduction of concepts on biosurfactants and highlights them as being good candidates to replace synthetic surfactants based on the physicochemical properties they display. Chapter one also discusses the bottlenecks preventing an accelerated shift towards biosurfactants dominating the market and the challenges associated with bioprospecting for new structures. Furthermore, this chapter includes a broad review of the classification of biosurfactants and an evaluation of well-studied structures, including the relationship

shared between the structure, its functional properties, and its potential applications. Additionally, the review focuses on the approaches used for biosurfactant prospecting by revising a range of well-established screening methods employed to screen, identify, and isolate biosurfactant activity, as well as deliberate on the culture-dependent and culture-independent approaches employed for biodiscovery. Lastly, the chapter briefly evaluates the extraction and purification procedures used in this study to extract and purify putative biosurfactant(s). Chapter two of this study draws more attention to prospecting for novel biosurfactant structures through employing parallel function-based screening of a seawater metagenomic library. The chapter presents reasons for the selection of a seawater metagenomic library and expands on the motivation for utilizing the parallel function-based metagenomic screening method to search for biosurfactant activity. Furthermore, the chapter delves into the methods and materials used, the results obtained, and the conclusions drawn.

Chapter three and four focus on the culture-based approach for the identification of biosurfactants(s) produced by the *Planococcus* sp. CP5-4 strain as well as the investigation of carotenoids as biosurfactant(s) synthesized by the strain, respectively. The role of *Planococcus* species in hydrocarbon degradation and biosurfactant production is communicated in the introduction of Chapter three. Furthermore, the section expands on the reasons for the selection of the novel *Planococcus* sp. CP5-4 strain for biosurfactant prospecting, and briefly elucidates the type of biosurfactant(s) typically synthesized by *Planococcus* species. In this chapter methods and materials used in bioprospecting for surface activity from the *Planococcus* sp. CP5-4 strain are presented, including procedures employed for the preliminary screening for biosurfactant activity, as well as preliminary physicochemical and molecular characterization of the biosurfactant(s) produced by the strain. Chapter four is a continuation of Chapter three; however, it focuses on investigating the correlation between a traced carotenoid biosynthetic pathway and the pigmented extract(s) found to be active in Chapter three. The final chapter five encompasses the general discussion and conclusion drawn from the study as well as the study's limitations and recommendations, suggestions for future work, and concluding remarks dedicated to the whole project. Sections of this thesis were redrafted towards a published chapter titled; "Screening strategies for biosurfactant discovery" (Trindade *et al*., 2021).

1.2. Surfactant evolution: Biosurfactants as the alternative replacements for synthetic surfactants

1.2.1. Soaps as the first description of a surfactant

The earliest evidence of surfactants were soaps, dated back to the 3rd millennium, simply produced from plant ash and animal fats (Konkol and Rasmussen, 2015). However, modern soaps are carboxylate soaps and were first manufactured commercially in 1907 (Konkol and Rasmussen, 2015). Carboxylate soaps are manufactured by saponification of plant or animal oils and free fatty acids which are then neutralized by alkali metal or an organic base such as wood ash containing potassium carbonate (Atiku *et al*., 2014; Konkol and Rasmussen, 2015). Carboxylate soap stayed relevant until 1961 when synthetic surfactants emerged (Konkol and Rasmussen, 2015). This was partly because of the deficiency of the natural oils used to make these soaps (Konkol and Rasmussen,

2015). The disadvantages associated with soaps are that they are not very effective in their activity, as well as not compatible with modern applications, for example, they have poor solubility in cold water, in water with ions such as Ca^{2+}, Mg^{2+}, and Fe^{3+}, and at a pH below 7 (Atiku *et al*., 2014; Kogawa *et al*., 2017). However, they still hold a large fraction of surfactant consumption to date.

1.2.2. Synthetic surfactants as the leading surfactant in the market

Synthetic surfactants represent most of the surfactants found in the market to date and are mostly manufactured through organo-chemical synthesis using petrochemical feedstocks (Gutierrez *et al*., 2019; Sanches *et al*., 2021). There are six major synthetic surfactants in the market, which include linear alkylbenzene sulfonates (LABS), alcohol ethoxylates (AE), surfactant, alcohol ether sulfates (AES), alkylphenol ethoxylates, and alcohol sulfates (AS) (Schramm *et al*., 2003; Myers, 2010; Jackson *et al*., 2015b; Sourav *et al.,* 2015). Chemical surfactants possess significant advantages over their competitors, and this has sustained them as the lead surfactants in the market (Myers, 2010). Cost-effectiveness is one such major advantage, for example, low-priced raw materials such as paraffin and benzene are used as starting materials for their synthesis and this leads to low-cost production at commodity scales (Myers, 2010; Sanches *et al*., 2021). Another advantage is that they have very effective surfactant activity which aligns with modern applications. For example, alcohol ethoxylates are good detergents at low temperatures and alcohol ether sulfates are mild enough to be used in personal care products (Myers, 2010).

Chemical surfactants have a few disadvantages, which has led to the search for alternative surfactants. Even though some chemical surfactants can be produced from renewable feedstock, which includes alcohol sulfates and alcohol ethoxylates, the bulk of their production is from non-renewable petrochemical feedstocks, making them inherently non-biodegradable (Myers, 2010; Chokwe *et al*., 2017; Sanches *et al*., 2021). Furthermore, the biodegradability of intermediate residues is not completely understood whilst there is limited data available on their effect once they enter the environment (Myers, 2010). For example, alkylphenol ethoxylates have slow biodegradation rates and toxic degradation intermediates such as phenol are produced during their biodegradation (Chokwe *et al*., 2017).

1.2.3. Biosurfactants as the environmentally friendly alternative

Biosurfactants are biological surface-active agents produced by both plants and microorganisms. Biosurfactants have been identified from all domains of life; Bacteria, Eukaryota, and Archaea and are thought to be produced by diverse microorganisms (Valenzuela-Reyes *et al*., 2014; Jackson *et al*., 2015b). This literature review will focus on biosurfactants of microbial origin. In recent years the focus on biosurfactants has grown as the favourer of environmentally friendly products has increased (Jackson *et al*., 2015b; Vajayakumar and Saravanan, 2015; Sourav *et al*., 2017; Sanches *et al*., 2021). Furthermore, they are comparable enough to compete with synthetic surfactants, with some having an even better activity (Otzen, 2017; Sanches *et al*., 2021). They have great structural diversity that has led to specific applications that cannot be achieved by chemically synthesized surfactants and they have a good economic potential (Fakruddin, 2012; Vajayakumar and Saravanan, 2015; Singh *et al*., 2018; Williams *et al*., 2019). These advantages will be expansively elaborated on later in this chapter.

1.3. The physiological role of biosurfactant production

Phylogenetic analysis has shown a great diversity between microorganisms that produce biosurfactants (**Figure 1-1**) (Bodour *et al*., 2003; Valenzuela-Reyes *et al*., 2014; Balan *et al*., 2017). However, the genes at the species level are highly conserved, which signifies that the biosurfactant production by different species serves an important survival function for those microorganisms (Bodour *et al*., 2003). Bacterial genera (*Bacillus, Pseudomonas,* and *Acinetobacter*), yeast (*Pseudozyma and Candida*), fungal genera (*Aspergillus* and *Fusarium*) are the predominant producers of the various biosurfactants reported in literature (Fenibo *et al*., 2019). The most common biosurfactants synthesized are surfactin, rhamnolipids, sophorolipids, emulsan, and mannosylerythritol lipids (Santos *et al*., 2016; Fenibo *et al*., 2019).

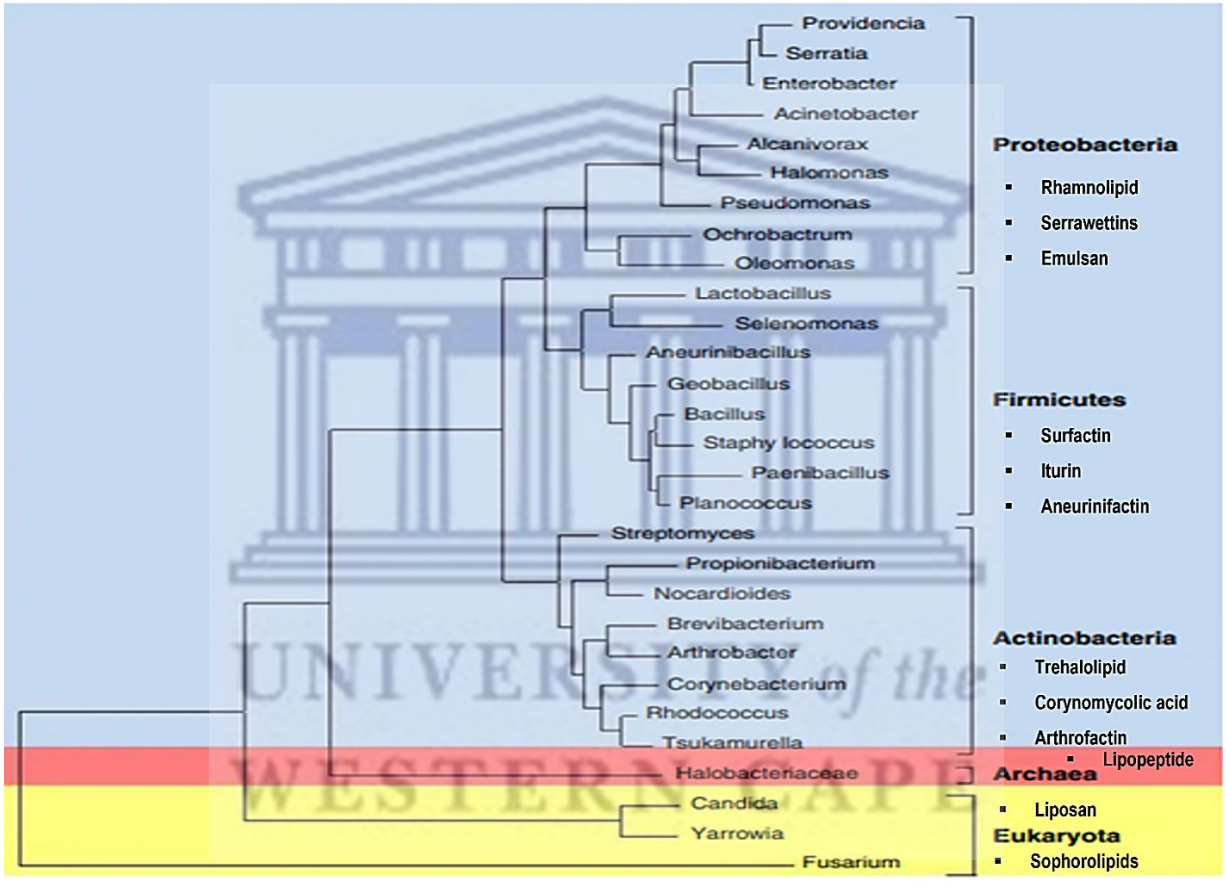


Figure 1-1: Phylogenetic tree showing the taxonomic diversity of biosurfactant-producing microorganisms from all domains of life; Bacteria, Eukaryota, and Archaea, and examples of the type of biosurfactants produced. The picture was adapted from (Jackson *et al*., 2015b).

Microorganisms can produce biosurfactants both intracellularly and/or extracellularly. However, the physiological function of biosurfactants is not well understood but it is greatly influenced by the environmental conditions that the microorganisms are subjected to, consequently giving the bacteria adaptive capabilities to survive in that exact environment (Ron and Rosenberg, 2001; Fakruddin, 2012; Vajayakumar and Saravanan, 2015). This phenomenon is observed where rhamnolipids are produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) due to nutrient deprivation (Bodour *et al*., 2003; Kugler *et al*., 2015). Furthermore, several microorganisms often produce

biosurfactant with antimicrobial activities (Fenibo *et al*. 2019). Biosurfactants are mostly synthesized during the growth of the bacteria on a water-immiscible substrate (Zheng *et al*., 2009). The biosurfactant produced is sometimes involved in regulating the microorganism's cell movements through attachment and detachment from the substrates, as demonstrated by *Acinetobacter calcoaceticus* (*A. calcoaceticus*) (Ron and Rosenberg, 2001; Vajayakumar and Saravanan, 2015). Moreover, biosurfactants are produced as a mechanism to access nutrients by enabling the bioavailability of water-immiscible substrates by increasing their solubility. Additionally, biosurfactants enhance nutrient transportation across the cell membrane to the interior of the cell, for example, *Pseudomonas* spp. also produces rhamnolipids to assist in the diffusion of organic compounds across the cell membrane (Vajayakumar and Saravanan, 2015; Nurfarahin *et al*., 2018). Microorganisms also undergo cell wall structure changes through producing biosurfactants, for example, *Bacillus subtilis* (*B. subtilis*) produces surfactin for maturing body formation, and *Acinetobacter spp*. synthesize lipopolysaccharides such as emulsan to assist in cell wall changes (Bodour *et al*., 2003). Therefore, microorganisms produce biosurfactants to serve several functions including intracellular bioavailability, motility, bacterial pathogenesis, quorum sensing response, antimicrobial activities, and biofilm formation amongst other functions (Fakruddin, 2012; Jackson *et al*., 2015b; Kugler *et al*., 2015; Singh *et al*., 2018).

1.4. Biosurfactant general properties: The need for biosurfactant biodiscovery

There are several unique physicochemical and biochemical properties that make biosurfactants an attractive alternative to synthetic surfactants (Al-Dhabi *et al*., 2020; Bjerk *et al*., 2021). The highlighted properties which will be discussed in this section include surface tension reduction; critical micelle concentration capacity; resilience to a broad pH; temperature; and salinity range; foaming abilities; emulsifying capacity; being environmentally compatible, and bioactive in some instances (Sourav *et al*., 2015; Santos *et al*., 2016; Al-Dhabi *et al*., 2020; Sanches *et al*., 2021). Furthermore, they demonstrate an expansive structural diversity (Estopa, 2017; Bjerk *et al*., 2021; Sanches *et al*., 2021). It is important to note that the biosurfactant properties are highly dependent on the molecular structure (Estopa, 2017; Sourav *et al*., 2017; Williams and Trindade, 2017; Sanches *et al*., 2021). Due to these properties biosurfactants are used as multipurpose compounds. They are employed as surface and interfacial tension reducers, emulsifiers, foaming agents, for dispersion/phase separation, and as solubilizers, wetting and detergency/cleansing agents, lubricants, and antimicrobial products (Sourav *et al*., 2017; Bjerk *et al*., 2021; da Silva *et al*., 2021). As a result, biosurfactants are vastly employed in different markets including the personal care sector, agriculture, pharmaceutical, food industry, biomedicine, materials engineering, bioenergy, and in environmental remediation (da Silva *et al*., 2021; Sanches *et al*., 2021).

1.4.1. Surface and interfacial tension:

The main physicochemical characteristic biosurfactants have is their ability to act on the surfaces or interfaces of liquids (Sourav *et al*., 2015). Hence, surface, and interfacial tension reduction are the commonly measured physical properties of biosurfactants. Surface tension is defined in two ways; as the tension force per unit length exerted by a liquid in all directions at an interface of a solid or another liquid; or it can be explained in terms of energy, as the amount required to decrease the interior forces of bulk liquid molecules and molecules at the interface of the liquid

in contact with other surfaces (Satpute *et al*., 2010; Walter *et al*., 2010; Trindade *et al.*, 2021). Surface tension is measured as the dimensions force/length in the unit dyne/cm or mN/m. At a value of 72 mN/m, water is known to be one of the organic liquids with the highest surface tension value (Walter *et al*., 2010; Trindade *et al.*, 2021). An effective biosurfactant should interrupt the force per unit length of water thus lowering the surface tension of water from 72 to 30 mN/m (Sourav *et al*., 2017; Trindade *et al.*, 2021). Interfacial tension (IFT) is the intermolecular attractive force of the molecule in a liquid (Walter *et al*., 2010; Sourav *et al*., 2017; Trindade *et al.*, 2021). A surface-active biomolecule must lower the interfacial tension for water against *n*-hexadecane from 40 to 1 mN/m (Sourav *et al*., 2017). A surface tension reduction of water to 25 mN/m by surfactin synthesized by *B. subtilis* is reported and surfactin can also lower the interfacial tension of water/hexadecane to less than 1 mN/m. Similarly, *P. aeruginosa* produces rhamnolipids able to decrease the interfacial tension of water/hexadecane to less than 1 mN/m and water's surface tension to 26 mN/m (Vajayakumar and Saravanan, 2015; Fenibo *et al*., 2019).

1.4.2. Critical micelle concentration (CMC):

First denoted by McBain in 1913, CMC is the minimum concentration of biosurfactant at which monomers aggregate to form colloidal-sized molecular clusters called micelles. Micelles vary in shape and size, from small spherical structures to elongated rod-like micelles, and large lamellar or vesicles (Sourav *et al.*, 2017). The efficiency of a biosurfactant is determined by how low its CMC is, the lower the CMC the less product needed to reduce the surface tension and interfacial tension (Rosen, 1978; Trindade *et al.*, 2021). CMC is determined empirically by measuring at which point the reduction of surface tension stops with the increase of biosurfactant concentration (Sourav *et al.*, 2017; Trindade *et al.*, 2021). When the CMC is reached, the biosurfactant aggregates rather than accumulate at the surface and this no longer results in further surface tension reduction even with increased biosurfactant concentration (Sourav *et al.*, 2017; Trindade *et al.*, 2021). The CMCs of biosurfactants are generally found to be lower than that of synthetic surfactants (Vajayakumar and Saravanan, 2015). Compared to synthetic surfactants, biosurfactants have been found to have 10 to 40-fold lower CMC value and are thus considered more effective and efficient (Sourav *et al.,* 2015). The typical range of the CMC for biosurfactants is between 1 to 200 mg/L with a molecular mass range from 500 to 1500 Dalton (Sourav *et al.*, 2015; Santos *et al*., 2016). For example, rhamnolipids, sophorolipids, and trehalolipids range from 10 to 234, 5 to 40, and 4 to 250 mg/L respectively (Estopa, 2017). While the CMC of synthetic surfactants such as sodium dodecyl sulphate (SDS), Trixon X-100, and tween 80 are 2385, 130, and 13 mg/L respectively (Estopa, 2017).

1.4.3. Temperature, pH, and salinity tolerance:

Biosurfactants, particularly those of extremophile origin, can resist extremes of pH, temperature, and salinity (Schultz and Rosado, 2020). For instance, lipopeptides synthesized by *Bacillus licheniformis* Jf-2 can tolerate a pH range of 5-12 and temperatures of 75°C for 140 hours (Fenibo *et al*., 2019). Furthermore, lipopeptides from *B. subtilis* remain active even after being autoclaved at 121°C for 15 min or stored at -18°C for 6 months, their activity is sustained at a pH range of 5-11 and at a 20% NaCl salt concentration (Santos *et al*., 2016; Fenibo *et al*., 2019). Biosurfactants mostly retain their activity at up to 10% salt concentrations, while synthetic surfactants become

inactive at 2% salinity (Santos *et al*., 2016). These qualities are useful for industrial processes as most industrial processes pass through a variety of extreme temperature, salinity, and pH conditions (Roy, 2017).

1.4.4. Emulsification:

Emulsification is the dispersion of one liquid phase in another resulting in a mixing of two immiscible phases. Emulsification is an important characteristic exhibited by biosurfactants and exploited by various cosmetics, food, and pharmaceutical industries (Santos *et al*., 2016). Due to its significance emulsification is amongst the first physicochemical properties measured during bioprospecting. The ability of a biosurfactant to form a stable emulsion with an immiscible liquid for over 24 hours is measured as an emulsification capacity index (EC_{24}) and a percentage represents the unit of measure (Santos *et al*., 2016). A bacterial consortium can achieve an emulsion as high as 95.75% (Sumiardi *et al*., 2018). Emulsan and liposan are prominent bioemulsifiers and are mainly synthesized by *Acinetobacter calcoaceticus* and *Candida lipolytica* respectively (Ron and Rosemberg, 2001). Emulsan is the most powerful stabilizer with the ability to form a stable emulsion at a concentration as low as 0.01 to 0.001% (Ron and Rosemberg, 2001, *Fracchia et al*., 2012). Furthermore, lipopeptides and glycolipids synthesized by fungal strains have been reported to have higher emulsification than most synthetic surfactants such as Triton x-100 (Sanches *et al*., 2021).

1.4.5. Foaming:

Foaming occurs when the surface-active molecule gets concentrated at a gas-liquid interface, which creates air bubbles below the surface of the liquid that are sustained without collapsing (Chen *et al*., 2006; Gong *et al*., 2021). This biosurfactant characteristic is a very valuable addition to the development of cosmetic, detergent, and pharmaceutical products (Chen *et al*., 2006). Furthermore, in foaming, where small bubble size and stable foaming transpire, the foam can be employed for the parting of metal groups from suspensions by mineral processing industries (Chen *et al*., 2006; Gong *et al*., 2021). Biosurfactants with a better foaming ability than synthetic surfactants are reported in literature. For example, surfactin has better foam stability in water at a concentration as low as 0.05 mg/L than the synthetic surfactants SDS and bovine serum albumin (BSA) (Razafindralambo *et al*., 1998; Otzen, 2017). At 100 mg/L concentration surfactin demonstrated 88% foaming ability, whereas BSA at 200 mg/L can only achieve 65% foaming stability (Razafindralambo *et al*., 1998).

1.4.6. Environmentally friendly:

There is an overall growing environmental awareness amongst consumers, and they are demanding sustainable, less toxic, and environmentally friendly products from producers. This has fuelled an interest in the discovery of biosurfactants that are environmentally friendly (Thies *et al*., 2016). This shift is also supported and driven by governmental legislation that demands the use of renewable greener products to meet sustainable goals, plus the demand for structurally diverse biosurfactants tailored to specific industrial functions by the different markets (Maier, 2003). Parameters such as biodegradability, toxicity, carbon footprint, and renewability frame the idea of sustainability (Santos *et al*., 2016).

18 million tons of synthetic surfactants per year are produced globally and are continuously released into the environment and due to the COVID-19 pandemic, the use of surfactants is estimated to increase by 35% per year

(Bjerk *et al*., 2021). The biggest concern is that depending on their structure they cannot be degraded and have a lasting negative effect on the environment (Cirelli *et al*., 2008). Biodegradation tests executed following the organization for economic cooperation and development (OECD) guidelines (301C Modified MITI Test), on sophorolipids from yeast *Candida bombicola,* showed evidence of biodegradation of the biosurfactants just after cultivation (Sourav *et al*., 2017). This is as opposed to the biodegradation signs of the synthetic surfactant Triton X-100 tested against it which became present after 8 days (Sourav *et al*., 2017). Biosurfactants are also more suitable for environmental applications such as bioremediation as they do not accumulate. Therefore, biosurfactants such as sophorolipids have been employed to remove Cochlodinium algae with an efficiency of 90% per 30 minutes treatment (Vajayakumar and Saravanan, 2015; Roy, 2017).

Synthetic surfactants are known to be toxic, for example, during the synthesis of AES synthetic surfactant, the toxic precursor dioxin gets released as a by-product in an ethoxylation process (Myers, 2010). On the other hand, biosurfactants are less toxic, due to their biological origin (Sanches *et al*., 2021). A toxicity and mutagenicity profile of rhamnolipids when compared to synthetic surfactants supports that the former is nontoxic and not mutagenic. This makes it perfect for application in the pharmaceutical, cosmetics, bioremediation, and food industry (Jackson *et al*., 2015b; Vajayakumar and Saravanan, 2015; Sourav *et al*., 2017; Sanches *et al*., 2021). For the same reasons, sophorolipids from *Candida bombicola* and liposan from *Candida lipolytica*, are used in the cosmetics and food industries as emulsifiers (Vajayakumar and Saravanan, 2015). Additionally, microorganisms can use produce or waste as raw material for biosurfactant production (Fakruddin, 2012). Moreover, some biosurfactants are bioactive meaning they exhibit antibacterial, antifungal, antiviral, and anti-tumor effects and therefore can be employed as therapeutic agents (Sanches *et al*., 2021).

1.4.7. Structural diversity:

Biosurfactants have the potential of having greater structural variability or diversity because of the vast genetic diversity represented by the variety of microorganisms that synthesize them (Gutierrez *et al*., 2019). The unique structure sometimes results in distinct physicochemical properties and thus, permits specific functions and industrial applications that cannot be achieved by chemically synthesized surfactants since they are limited to fewer structures (Santos *et al*., 2016; Singh *et al*., 2018; Williams *et al*., 2019; Al-Dhabi *et al*., 2020). This drives the need to invest in increasing the existing biosurfactant portfolio by encouraging the constant seeking of novel biosurfactant structures (Kubicki *et al*., 2019). The need for structural diversity will be visited later in the chapter. The few prominent structures that already cater to the market, will be discussed and important relationships shared between structural diversity, function and application will be highlighted.

1.4.8. Economic potential:

Another equally important driver for biodiscovery is the economic potential that biosurfactants have, stemming from their attractive qualities already discussed. Biosurfactants are one of the most economically valuable biotechnology compounds of the 21st century (Singh *et al*., 2018). Their global market value in 2016 was worth 3.99 billion USD and is set to increase to 5.52 billion USD by 2022 at a 5.6% compound annual growth rate (CAGR) between 2016 and 2022 (Sophie *et al*., 2022). While the bio-based surfactants global market is projected to be worth 17.27 billion

USD by 2022 with a CAGR of 51% between 2017 and 2022 (Sophie *et al*., 2022). Influenced by their stricter green product regulations, countries in Europe, followed by the United States, lead the market. European companies such as Ecover, Evonik, and Henkel are responsible for 53% of the biosurfactant production market (Singh *et al*., 2018; Gutierrez *et al*., 2019; Sanches *et al*., 2021). Other honorable mentions in the markets include the Asian companies Kanebo Cosmetics inc. (Japan) and Allied Carbon Solution co., Ltd (Japan), Holiferm Technology (UK), and Givaudan SA (France) (Twigg *et al*., 2020). However, the market is currently dominated by three main biosurfactants namely surfactin, sophorolipids, and rhamnolipids (Sourav *et al*., 2017; Sanches *et al*., 2021). These biosurfactants are predominantly applied for home and personal care products and in the pharmaceutical sector. For example, sophorolipids and rhamnolipids are used in cleaning, cosmetics, and toiletries products (Twigg *et al*., 2020). The is plenty of evidence that the market is growing, for example, Evonik and Henkel introduced the first rhamnolipid-containing toothpaste in France, Spain, and Italy, and Evonik and Unilever collaborated to introduce the first rhamnolipid-based dishwasher, Quix into the Chile market in 2019 (Twigg *et al*., 2020; Sophie *et al*., 2022). However, there is an obvious need to open up the markets to more diversity and compounds with new properties that will have the potential to tailor to specific needs and, at the same time, appeal to broader markets.

1.5. Bottlenecks preventing an accelerated growth of biosurfactants in the market

There is a gap that exists between biosurfactants that are available and what is available in the market (Walter *et al*., 2010; Muller, 2021; Sanches *et al*., 2021; Trindade *et al*., 2021). Despite all the described advantages that biosurfactants have, several restrictive factors are preventing the shift towards them replacing synthetic surfactants. The main obstacle relates to the high production costs which is caused by the high cost of substrates or feedstocks used in the fermentation process and the downstream extraction and purification requirements which account for 70-80% cost of the production (Santos *et al*., 2016; da Silva *et al*., 2021; Sanches *et al*., 2021). Another major bottleneck pertains to the low yield and productivity of biosurfactants when bioprocessing is vetted (Al-Dhabi *et al*., 2020). Therefore, high production costs combined with low production capacity results in biosurfactants being costly and not easily accessible to diverse markets (Al-Dhabi *et al*., 2020; Muller, 2021). For example, sophorolipids production amount to 500 tons per year and represents the highest biosurfactant production and they are sold at 10 to 15 €/Kg, rhamnolipids at 15 to 20 €/Kg, and mannosylerythritol lipids at 100 to 150 €/Kg (Muller, 2021). Other disadvantages are that most biosurfactants are of pathogenic origin, the low availability of new structures, poor knowledge of the properties of known biosurfactants, and issues associated with patents (Santos *et al*., 2017; Sanches *et al*., 2021).

There is a lot of research and effort invested towards overcoming these limitations and industrial biotechnology companies are devoted to circumventing the limitations inherent to each stage, from discovery to production and application (Sourav *et al*., 2015). However, research is predominantly focused on the engineering of biosynthetic pathways into non-pathogenic host strains (da Silva *et al*., 2021; Muller, 2021). For example, Evonik industries transferred the metabolic pathway of rhamnolipids predominantly synthesized by the pathogenic *Pseudomonas aeruginosa* into a non-pathogenic production host strain. Additionally, key research is also centred around reducing production costs, for example, by avoiding the use of high-cost substrate by utilizing renewable and/or waste

streams for fermentation and by improving downstream processes (Sanches *et al*., 2021). Employing genetic modification to improve strain and their production potential (Sourav *et al*., 2017; Sanches *et al*., 2021). Improving culture conditions to increase yield (Estopa, 2017). There has also been an expansion in exploring other strains, with an increased research focus on yeast strains such as *Torulopsis*, *Pseudozyma*, *Saccharomyces*, R*hodotorula,* and *Kluyveromyces* and fungi strains such as *Aspergillus*, *Ustilago*, *Fusarium*, *Trichoderma*, and *Penicillium* (Estopa, 2017).

This study will focus on solutions needed to be employed to supply the market with new biosurfactants, therefore, contributing to growing the existing biosurfactant portfolio. Only a few novel structures have been discovered in the last decade due to low hit identification and redundancy in which the same microorganisms are isolated and the same biosurfactant structures are identified (Trindade *et al*., 2021). Some of the reasons for the stunted novel structure discovery can be pinpointed to the challenges associated with isolation and screening for new products and even the lack of interest in biosurfactant discovery (Trindade *et al*., 2021). Accessing novel structures relies on the characteristics being screened for and how unique the microorganisms screened are (Trindade *et al*., 2021). Therefore, a focus on the application of different biodiscovery approaches is proposed. In this context, we advocate for isolation and screening methods that look past culturing approaches, such as bioprospecting employing culture-independent metagenomic approaches to access the genes of the 99% of uncultivatable microorganisms. Furthermore, to increase the probability of discovering new structures, the exploration of underexplored extreme environments such as the marine environment and culturing of underrepresented taxa/strains are put forward (Monciardini *et al*., 2014; da Silva *et al*., 2021).

1.6. Classification of biosurfactant: A broad view

A general biosurfactant structure is composed of a polar head group and a non-polar tail group bonded together (Santos *et al*., 2016; Al-Dhabi *et al*., 2020; Sanches *et al*., 2021). In an aqueous solution, the polar group is hydrophilic, and it dissolves in a polar molecule such as water, whereas the non-polar tail group is hydrophobic, and it dissolves in a non-polar solvent such as oil (Santos *et al*., 2016). It is this ability that affords the compound the ability to interact with both the polar and non-polar parts of a two-phased mixture. Biosurfactants are commonly classified by their molecular weight which is defined by the ratio of the non-polar and polar units and by the chemical composition of their structure (Vajayakumar and Saravanan, 2015; Roy, 2017; Kubicki *et al*., 2019).

1.6.1. Molecular weight: High or low molecular weight

Biosurfactants are classified into two categories namely low and high molecular weight (Ron and Rosenberg, 2001). Low molecular weight biosurfactants are known to be more effective at lowering surface and interfacial tension (Sourav *et al*., 2017, Gutierrez *et al*., 2019; Bjerk *et al*., 2021). While high-molecular-weight biosurfactants are more effective at stabilizing emulsion (Ekkers *et al*., 2012; Sourav *et al*., 2017, Gutierrez *et al*., 2019). The hydrophilic-lipophilic balance (HLB) of the two parts is normally used to infer biosurfactant properties, especially if they are of a non-ionic nature (Sourav *et al*., 2017). Ionic biosurfactants are generally hydrophilic, whereas non-ionic surfactants depend on their HLB balance and can, therefore, be either hydrophilic or lipophilic (Sourav *et al*.,

2017). The balance of HLB is known to determine the solubility of biosurfactants depending on the hydrophilic group's ability to attract water and the hydrophobic group's ability to attract oil. A lower HLB promotes the stabilization of water in oil emulsions (W/O), whereas compounds with a high HLB value stabilize oil in water (O/W) emulsions (**Figure 1-2**) (Farn, 2006; Sourav *et al.*, 2017).

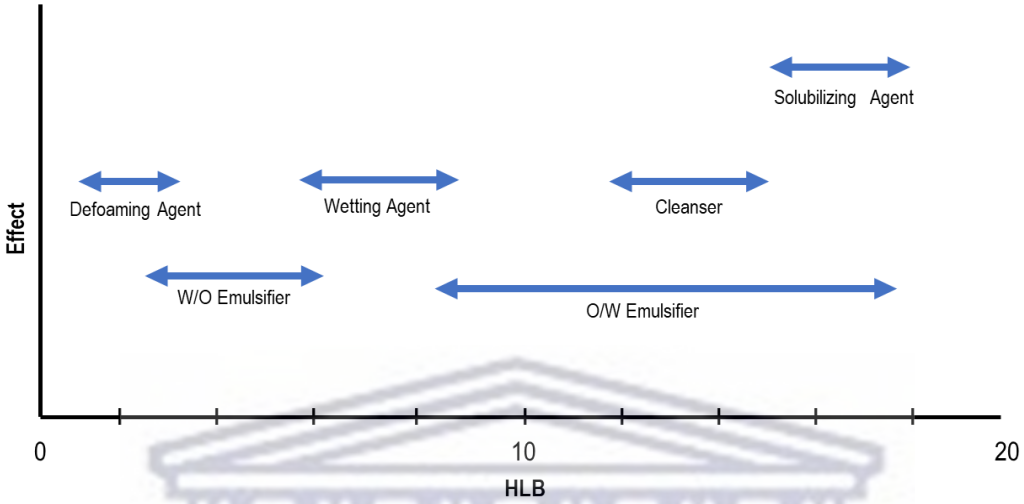


Figure 1-2: Hydrophilic-lipophilic balance (HLB) value used to predict biosurfactant properties and can be used to assign biosurfactant applications. Image adapted from (Sourav *et al.*, 2017).

Griffin was the first to propose a systematic ranking of HLB by assigning a number indicating the balance between the hydrophilic and hydrophobic moiety (Griffin, 1949; 1954; Sourav *et al.*, 2017, Gutierrez *et al.*, 2019). The scale ranking is assigned from 1 to 20, with 1 assigned to the most hydrophilic material and 20 to the most hydrophobic compound (**Figure 1-2**) (Sourav *et al.*, 2017, Gutierrez *et al.*, 2019). Knowing the characteristics efficiently and quickly is crucial in formulation development and HLB is often used as an indicator to choose a specific surface-activity application (**Figure 1-2**) (Sourav *et al.*, 2017, Gutierrez *et al.*, 2019). HLB value can be obtained by dividing the percentage of the hydrophilic moiety by five for non-ionic surfactants (Gadhave *et al.*, 2014):

$$HLB = \frac{E}{5}$$

Where E= Percentage by weight.

For fatty acid esters by using the formula below:

$$HLB = 20\left(1 - \frac{S}{A}\right)$$

Where S = Number of ester saponification and A= Acid number of fatty acids (Gadhave *et al.*, 2014).

For fatty acids with poor saponification data, HLB can be calculated based on the formula (Gadhave *et al.*, 2014):

$$HLB = \frac{E + P}{5}$$

Where E= Percentage by weight of the hydrophilic moiety and P= Weight percentage of the hydrophobic moiety (Gadhave *et al.*, 2014).

Despite the usefulness of HLB as an indicator, it alone is not enough to determine application during formulation development and it is mostly used as a reference value (Sourav *et al.,* 2017, Gutierrez *et al.*, 2019). The other two

good indicators that are applied to infer application are the Cloud point for anionic surfactants and the Krafft point for ionic surfactants (Sourav *et al.*, 2017, Gutierrez *et al.*, 2019).

According to Nguyen and Sabatini (2011), there are four emulsion types, namely type I, type II, type III, and type IV known as Winsor type microemulsions (**Figure 1-3**). Type I microemulsions, the oil in water or O/W microemulsion, the bioemulsifiers encapsulates oil within the water continuous phase (Chai *et al*., 2005; Nguyen and Sabatini, 2011). Type II contrary to Type I, is the water in oil or W/O microemulsion. In which the bioemulsifier encapsulates water in reverse micelles within the oil continuous phase resulting in a two-phase system. However, is also a two-phase system (**Figure 1-3**). This is contrasted by the type III and type IV of the Winsor type microemulsions, which are three-phase and single-phase systems respectively (Chai *et al*., 2005). Type III has both excess oil and water phases and a middle microemulsion phase resulting in an oil-in-water-in-oil microemulsion W/O/W (Chai *et al*., 2005). Type II emulsion types have a positive value of hydrophilic-lipophilic deviation (HLD) indicating a hydrophobic biosurfactant system, while type I has a negative HLD thus indicating a hydrophilic biosurfactant system (Nguyen and Sabatini, 2011). The W/O microemulsions have Hydrophilic-lipophilic balance (HLB) of less than 10 and for the O/W microemulsions the HLB is greater than 10 (Griffin,1954; Sourav *et al.*, 2017; Gutierrez *et al.*, 2019)

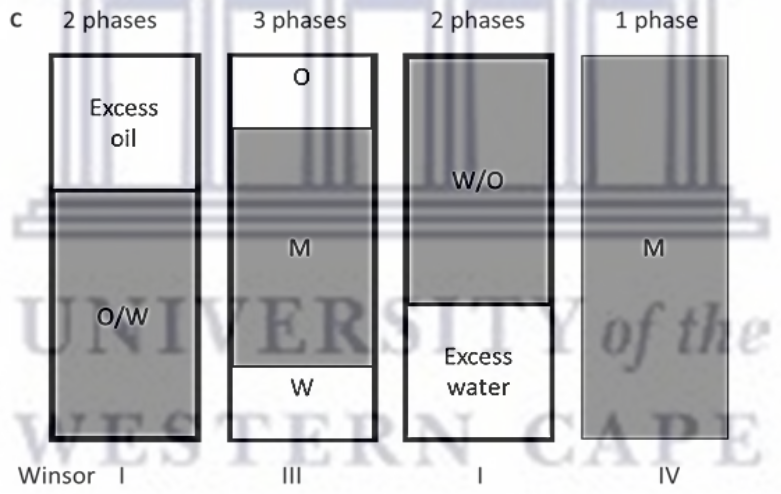


Figure 1-3: Classification of microemulsion equilibria according to Winsor characterisation as type I, type II, type III, and type IV. (**M**) microemulsion. Image adapted from (Chai *et al*., 2005).

1.6.2. Chemical composition of the hydrophilic and hydrophobic unit

As previously discussed, biosurfactants are amphiphilic meaning they have both hydrophobic and hydrophilic moieties which account for their surface activity. Their hydrophilic unit can be comprised of phosphate, small hydroxyl, carboxyl groups, carbohydrates (such as mono-, oligo-, or polysaccharides), or polypeptide moieties (Kubicki *et al*., 2019; Bjerk *et al*., 2021). The hydrophobic units can be unsaturated or saturated hydrocarbon chains of fatty acids, hydroxy fatty acids, fatty alcohols, and α-alkyl or β-hydroxy fatty acids all with varying lengths between 8 and 18 carbon atoms or sterol rings (Kubicki *et al*., 2019). The hydrophobic and hydrophilic parts can either be joined by glycosidic linkage (sugar-hydroxyl fatty acids and sugar-sugar) or ester linkage (including

lactones) with organic and inorganic acids or amide linkage (single and peptide) to form a potent biosurfactant (Sourav *et al*., 2017; Bjerk *et al*., 2021). Separate biosynthetic pathways generally synthesize the hydrophilic and hydrophobic units and the two moieties later combined to form the amphiphilic biomolecule, the synthesis is often dependent on the substrate used during fermentation (Bjerk *et al*., 2021; Sanches *et al*., 2021).

Based on their chemical structure, primarily their hydrophilic head group biosurfactants are mainly divided into five major classes namely glycolipids, lipopeptides, neutral lipids, polymeric compounds, and particulate biosurfactants (**Table 1-1**) (Kubicki *et al*., 2019; Al-Dhabi *et al*., 2020). Classes such as glycolipids, lipopeptides, and phospholipids fall under low molecular weight biosurfactants (LMW), whereas proteins, lipoproteins, lipopolysaccharides, amphipathic polysaccharides, or other complex mixtures of these biopolymers (Lipoheteropolysaccharides) are high-molecular-weight (HMW) biosurfactants (**Table 1-1**) (Sourav *et al*., 2017, Gutierrez *et al*., 2019; Kubicki *et al*., 2019).

Table 1-1: Classification of biosurfactants.

Biosurfactant classification				
Group	**Class**	**Sub-class**	**Microorganism**	**References**
Low molecular weight Biosurfactant	Glycolipids	Rhamnolipids	*P. aeruginosa*	Sanches *et al*., 2021
		Sophorolipids	*Candida bombicola*	Otzen, 2017
		Trehalose-lipids	*Rhodococcus erythropolis*	Zheng *et al*., 2009
		Cellobiose lipids	*Ustilago zeae, Ustilago. maydis*	Roy, 2017
		Mannosylerythritol lipids	*Candida antarctica*	Bjerk *et al*., 2021
	Lipopeptides and Lipoproteins	Surfactin	*Bacillus. Subtilis*	Al-Dhabi *et al*., 2020
		Viscosin	*Pseudomonas Fluorescens*	Roy, 2017
		Peptide-lipid	*Bacillus. Licheniformis*	Shakeri *et al.,* 2021
	Neutral lipids	Fatty acids	*Corynebacterium lepus*	Martin *et al*., 2014
		Neutral lipids	*Nocardia erythropolis*	Shakeri *et al.,* 2021
		Phospholipids	*Thiobacillus thiooxidans*	Martin *et al*., 2014
High molecular weight Biosurfactant	Polymeric surfactants	Emulsan	*Acinetobacter calcoaceticus*	Shakeri *et al.,* 2021
		Biodispersan Alasan	*A. calcoaceticus* *Acinetobacter radioresistens*	Roy, 2017
		Liposan	*Candida lipolytica*	Martin *et al*., 2014
	Particulate surfactants	Vesicles	*Acinetobacter calcoaceticus*	Martin *et al*., 2014
		Whole cells	*Variety of bacteria* such as *Cyanobacteria*	Shakeri *et al.,* 2021

1.7. The relationship between chemical structure, functional property, and application of biosurfactant

Biosurfactants' expansive spectrum of structural diversity is associated with the vast biological and physicochemical properties they possess (Kubicki *et al*., 2019). Currently, there are over 2000 distinct biosurfactants that have been characterized, encompassing compounds from chemically different families with congeners (structurally closely related compounds with minor structural diversity) also included (Kubicki *et al*., 2019). Even minor structural differences are sometimes mirrored in the different physicochemical properties a biosurfactant exhibits (Bjerk *et al*., 2021). For example, mono- and di- rhamnolipids from the same microorganism are found to have two different bioactivities; mono-rhamnolipids have bacteriostatic effects, while di-rhamnolipid have bactericidal effects on *P. aeruginosa* (Kubicki *et al*., 2019). Furthermore, biosurfactants are generally produced as a complex mixture by a single bacterium (Otzen, 2017)

To expand on the need for bioprospecting for new structures this section will provide a deeper picture of the relationship between structure, function, and application. This review will focus on glycolipids and lipopeptides as the two main classes that are currently industrially and economically relevant and that have attracted a lot of research focus as synthetic surfactant substitutes (Martin *et al.*, 2014; Bjerk *et al*., 2021). As previously mentioned, the market is currently dominated by three main biosurfactants from these main classes, namely surfactin, sophorolipids, and rhamnolipids. In addition to these, trehalose lipids and mannosylerythritol (MEL) lipids form part of the best-studied biosurfactants and are mainly reviewed for industrial applications and therefore will also be used in this review as examples to provide a much deeper picture of the state of the development and application of biosurfactants (Walter *et al*., 2010; Williams *et al*., 2019; Kubicki *et al*., 2019; Araujo *et al*., 2020).

1.7.1. Glycolipids

A **glycolipid** surface-active structure is made up of mono- or di- or oligosaccharide moieties attached to a lipid moiety through a glycosidic linkage (Otzen, 2017; Roy, 2017). The hydrophilic part is typical sugars such as glucose, mannose, galactose, glucuronic acid, and rhamnose (Zheng *et al*., 2009; Santos *et al*., 2016; Kubicki *et al*., 2019). The hydrophobic part is composed of saturated or unsaturated fatty acids, hydroxylated fatty acids, or fatty alcohols (Otzen, 2017; Zheng *et al*., 2009). Glycolipids include glucose lipids, cellobiose lipids, polypeptide glycoside, isoprenoid, and carotenoid glycolipid types. The best-explored glycolipids are sophorolipids, mannosylerthritol lipids (MELs), trehalose lipids, and rhamnolipids (Santos *et al*., 2016; Kubicki *et al*., 2019).

1.7.1.1. Sophorolipids

Sophorolipids (SLs) were first described in *Torulopsis apicola,* but they are mainly produced by the yeast related to the *Starmerella* and *Rhodotorula* clade (Kubicki *et al*., 2019; Eras-Muñoz *et al*., 2022). However, studies tend to focus on *Candida bombicola* (Ekkers *et al*., 2012). *Candida bombicola* has a high SL yield of 400g/L making it good for commercial products and applications and this is partly the reason as to why SLs are the second most studied biosurfactants after rhamnolipids. Sophorolipids are composed of a disaccharide sophorose hydrophilic head group (2-O-β-D-glucopyranosyl-D-glucopyranose) linked via a β-glycosidic linkage to predominantly ω- or

(ω-1)-hydroxylated carboxylic acid with between 16 and 18 carbon atoms (**Figure 1-4**) (Roy, 2017; Bjerk *et al*., 2021; Wang *et al*., 2021). The two glucose disaccharides are coupled together by a β-1,2' bond resulting in a hydrophilic sophorose head group (Wang *et al*., 2021). The sophorose can also be acetylated at 6' and/or 6'' positions (Wang *et al*., 2021) (**Figure 1-4**). SLs are generally produced as a mixture of six to nine different acid and lactonic sophorolipids. SLs are considered lactonic if their fatty acid moiety is internally esterified to form a closed or cyclic ring and are acidic if the moiety has a free carboxylic acid at the end as seen in **Figure 1-4** (Bjerk *et al*., 2021; Eras-Muñoz *et al*., 2022; Wang *et al*., 2021). According to da Silva *et al*. (2021) there are more than 20 congeners that SL can manifest as, however, there are a few generally dominant forms. Structural variation can be equated to the length and saturation of the fatty acid unit, the acetylation of the sophorose, lactonization, and where the hydroxyl group is situated in both the lipid portion and the carbohydrates unit esterified with fatty alcohol (da Silva *et al*., 2021).

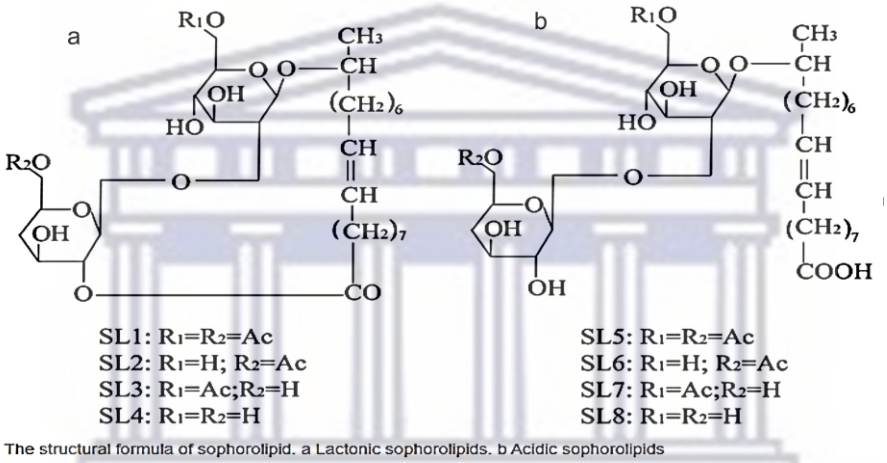


Figure 1-4: Chemical structure of (a) lactonic sophorolipids and (b) acid sophorolipids composed of a disaccharide sophorose hydrophilic head group linked via a β-glycosidic linkage to a fatty acid chain with between 16 and 18 carbons (Wang *et al*., 2021).

The SLs congeners are exploited for many applications because of the variety in their structure, which is reflected in the substantial activities they possess. For example, surface tension reduction is associated with lactonic SLs when compared to acidic SL (da Silva *et al*., 2021). Lower CMC is measured for lactonic SL over acidic forms because of the higher hydrophobicity and lower solubility that lactonic SLs have in water (da Silva *et al*., 2021; Kleinen *et al*., 2022). Lower solubility is also equated to the degree of acetylation of the SL structure (da Silva *et al*., 2021; Kleinen *et al*., 2022). Acidic forms are mainly foam producers. However antimicrobial activity is recognized for both lactonic and acidic SLs and this allows for their application in topical creams and oral hygiene (da Silva *et al*., 2021; Kleinen *et al*., 2022). SLs are generally employed as antimicrobial agents against a broad range of bacterial and fungal pathogens and can be applied in the food industry as emulsifiers, and in the synthesis of copper amongst other uses. SLs are produced by non-pathogenic species, have good surface reduction capabilities between 40-30 mN/m, have CMC as low as 40-100 mg/L, and have HLB values between 10 to 13 which makes them suitable for use as detergents and stabilizers of oil and water emulsions (Kim *et al*., 2020; da Silva *et al*., 2021). Furthermore, SLs are found to be stable at wide pH, temperature, and salinity ranges.

Additionally, SLs have been approved by the food and drug administration (FDA) because of their low cytotoxicity and therefore can be used in the food and pharmaceutical industries (Kim *et al*., 2020). Moreover, chemicals and enzymes can be used to manipulate the structure for a desired functional property. For example, pH changes influences leaching and chelating acids SL's interfacial properties. This is exploited in hydrometallurgical extraction of metals and during a foam flotation process used to separate ions and minerals respectively (da Silva *et al*., 2021).

1.7.1.2. Mannosylerythritol lipids

Mannosylerythritol lipids (MELs) are synthesized by fungal species such as *Ustilago maydis* and *Pseudozyma* sp. and the dominant strain that synthesizes MEL is the fungi *Ustilaginales* (Kubicki *et al*., 2019; da Silva *et al*., 2021). The MELs hydrophobic units are composed of fatty acid molecules with carbon chains between C8–C20 and the hydrophilic unit is composed of a 4-O-D mannopyranosyl-meso-erythritol with numerous forms of acylation and acetylation (**Figure 1-5**) (Banat *et al*., 2010; Fukuoka *et al*., 2016; da Silva *et al*., 2021). Both units are connected via a glycosidic bond to form a potent compound (**Figure 1-5**). Structural variation is manifested according to the microorganism in which the MELs are synthesized as well as the substrate used for their synthesis (Banat *et al*., 2010). For example, MELs produced by *Ustilaginales hordei* are mostly mono-acetylated compared to the ones synthesized by *U. maydis*. Several homologs of MELs are characterized by the variation in the number of acyl groups present on the mannose subunit and /or erythritol length and level of saturation of the fatty acid chain (Fukuoka *et al*., 2016; da Silva *et al*., 2021). The mannitol MEL variant has a greater hydrophobicity than a variant with erythritol residue (Banat *et al*., 2010; da Silva *et al*., 2021). Structure-function relation is mostly a requirement for the characterization of their potential application. It has been found that enzymes with different substrates produce different MELs and therefore, research studies could engineer the production of specific MELs suited for specific applications (Banat *et al*., 2010; da Silva *et al*., 2021).

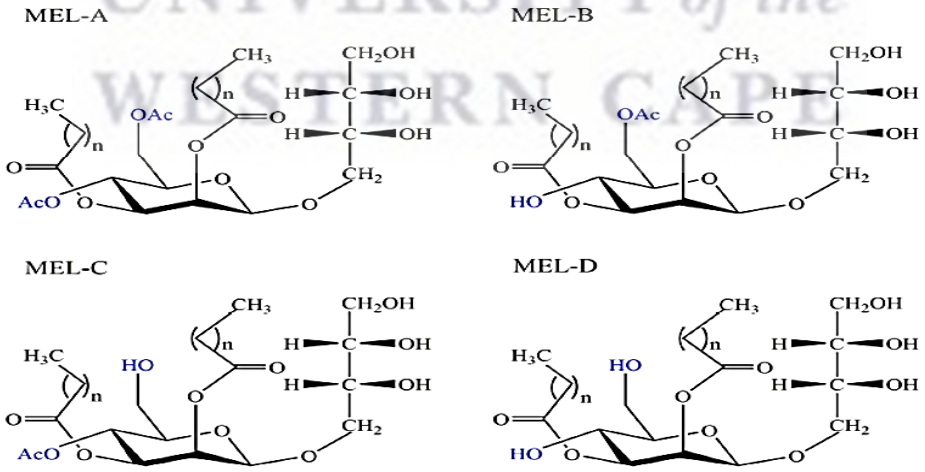


Figure 1-5: Different chemical structures of mannosylerythritol lipids, composed of fatty acid molecules with carbon chains between C8–C20 and the hydrophilic unit is composed of a 4-O-D mannopyranosyl-meso-erythritol, n= 2 ~ 18 (Shu *et al*., 2021).

As previously stated, a variety of MEL homologs exist and are characterized based on the number and location of acetyl groups present (Banat *et al*., 2010; Fukuoka *et al*., 2016). MEL-A is diacetylated at O-4 and O-6 and is the dominant MEL, MEL-B is monoacetylated at O-6, while monoacetylation at O-4 results in MEL-C, MEL-D is deacetylated and triacetylation at O-2, O-4, and O-4 produces MEL-A_2 (Fukuoka *et al*., 2016; da Silva *et al*., 2021). MEL-A synthesized by *Ceriporia lacerate* has endophytic activity and it is therefore utilized in bioremediation (da Silva *et al*., 2021). MEL-A has an incredible surface tension reduction ability it can decrease the surface tension of water to 3 mN/m, as such, they are applied in pharmacological action in drug administration (da Silva *et al*., 2021). MEL-A has a great emulsification capacity for hydrocarbons with an EC_{24} of 78.5%, it is therefore used to remove oil contamination (da Silva *et al*., 2021). MEL-B and C alter the hydrophobicity of solid surfaces and inhibit the germination of *Magnaporthe grisea conidia* and thus they are used in biopesticide formulations (da Silva *et al*., 2021). MELs are employed in cooling and biorefinery industrial processes, where diacetylated MELs prevent accumulation in ice-water slurries by stabilizing small ice particles which prevents the development of bigger crystals (Kubicki *et al*., 2019). Other MEL applications include their use as anti-melanogenic agents for skin lightening amongst a long list of other applications. However, because of the complex production profile of MELs which depend on a variety of factors that are not easily controlled, difficulties when it comes to optimizing and scaling up production are reported (Sophie *et al*., 2022). Nonetheless, companies namely Oleon are interested in these compounds and are investing in the development of the MEL market and production (Sophie *et al*., 2022).

1.7.1.3. Trehalose Lipids

Trehalose Lipids are non-reducing disaccharide trehalose, acylated and linked by an α-α-1,1-glycosidic bond to a long-chained straight or α-branched 3-hydroxy fatty acids (**Figure 1-6**). The hydrophobic moiety, mycolic acids which are hydroxylated branched-chain fatty acids (2-alkyl 3-hydroxy branched-chain fatty acids) usually have between 28 and 54 carbons (**Figure 1-6**) (Estopa, 2017). The number and chain length of the esterified fatty acids ensures variation found in trehalose lipids and therefore their surface properties and biological activity (Estopa, 2017). The length of hydrophobic moiety depends on the type of microorganism it's synthesized from, for example, *Mycobacterium* species have between 60 and 90 carbons, and shorter mycolic acids are found from *Nocardia* which synthesize between 44 to 60 carbons (Estopa, 2017; Kubicki *et al*., 2019; da Silva *et al*., 2021). The structural diversity of the compound is also dependent on the type of strain and growth conditions the producing microorganisms are subjected to. The predominantly studied trehalose lipid is trehalose-6-60 dimcolate, characterized by an α-branched chain mycolic acid esterified at the C6 position at each glucose (Ekkers *et al*., 2012; Roy, 2017; Kubicki *et al*., 2019; da Silva *et al*., 2021). They are synthesized from Actinobacteria like *Mycobacterium*, *Corynebacterial*, *Arthrobacter*, and *Rhodococcus* species (Ekkers *et al*., 2012; Roy, 2017; Kubicki *et al*., 2019; da Silva *et al*., 2021). There are also non-ionic trehalolipids such as trehalose monocrynomycolates, dicorynomycolates, and tricorynomycolates, acylated derivates of trehalose and anionic include trehalose tetraesters and succinoyl trehalolipids (Ekkers *et al*., 2012; Roy, 2017; Kubicki *et al*., 2019; da Silva *et al*., 2021).

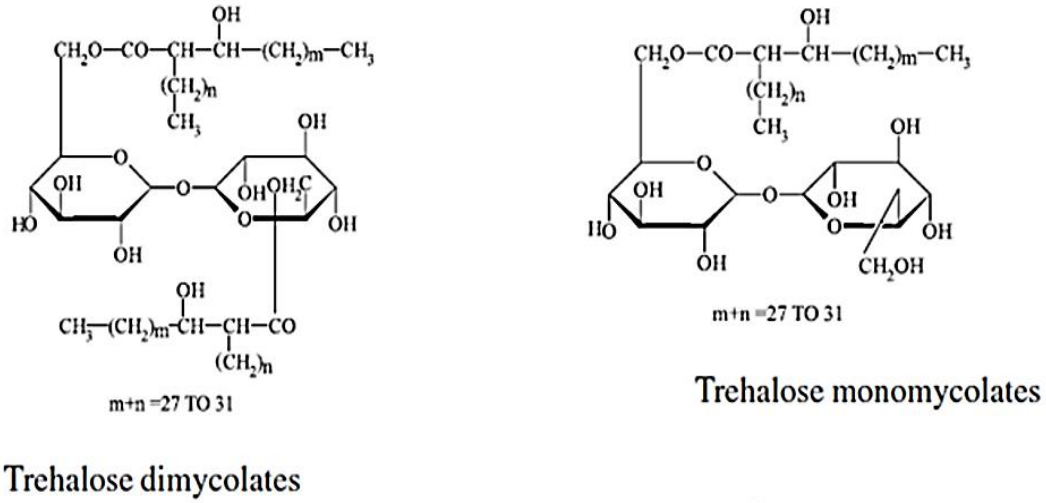


Figure 1-6*:* Chemical structure of trehalose dimycolates and trehalose monomycolates: trehalose linked by an α-α-1,1-glycosidic bond to a long-chained straight or α-branched 3-hydroxy fatty acids (Banat *et al*., 2010).

A trehalose lipid synthesized by *Fusarium fujikuroi* can reduce the surface tension reduction of water to as low as 20.08 mN/m (Kubicki *et al*., 2019; da Silva *et al*., 2021). The typical surface reduction range by trehalose lipids is found to be between 19 to 43 mN/m with the lowest surface tension reduction recorded to be 5 mN/m with CMC as low as 0.7 mg/L. Properties exhibited by trehalose lipids include hydrocarbon solubilizer, bioremediation qualities, emulsifying properties, microbial enhanced oil recovery (MEOR) properties, and other properties suitable for cosmetics and food application (Roy, 2017; Kubicki *et al*., 2019). Furthermore, biomedicine properties have been reported because of the immunomodulatory and anti-tumor activities found, moreover, the lipids have virulence effects against *Mycobacterium tuberculosis*. However, the trehalose lipids are produced intracellularly which means their extraction processes are challenging and require additional steps making the recovery process expensive (Estopa, 2017). This accounts for the slight industrial interest in the biosurfactant produced. Furthermore, most strains that produce the biosurfactant grow slowly, have low titer, and are pathogenic (Estopa, 2017).

1.7.1.4. Rhamnolipids

Rhamnolipids are one of the most important biosurfactants characterized by their excellent physicochemical properties. Rhamnolipids are anionic microbial glycolipids best known from the pathogenic *Pseudomonas aeruginosa* (Bjerk *et al*, 2021; Eras-Muñoz *et al*., 2022). Their structure composition is of one or two α-L (+)-rhamnosyl units commonly coupled to one or two β-hydroxy fatty acid units via a *0*-glycosidic linkage (**Figure 1-7**) (Irfan-Maqsood and Seddiq. 2014). However, ester-linked bonds instead of glycosidic bonds have been reported from *Burttiauxella* sp. and *Serratia marcescens* species isolated from mangrove and coral reefs respectively as well as from other species (Kubicki *et al*., 2019). An α-1,2 glycosidic linkage bonds the two rhamnosyl units and ester bonds between the β-hydroxyl group link adjacent fatty acids to each other to form a di-rhamnolipid (**Figure 1-7**) (Irfan-Maqsood and Seddiq, 2014). Nearly 60 various homologs and congeners have been reported in literature (Bjerk *et al*., 2021). The different rhamnolipid congeners are distinguished by their chain lengths, which can lie between 8 and 16 carbon atoms, rhamnose group (1 or 2), degree of branching, and saturation (0 or 1

double bound) of the two fatty acid chains (Irfan-Maqsood and Seddiq, 2014; Otzen, 2017; Bjerk *et al*., 2021). This difference is detected through the different biophysical properties rhamnolipids display, such as CMC, surface tension, acyl chain fluidity, the structure of aggerates state, and the ability to insert onto the membrane (Otzen, 2017; Bjerk *et al*., 2021). All are dependent on the environmental and growth conditions, and the microorganism (Bjerk *et al*., 2021). For example, *P. aeruginosa* species are found to have shorter alkyl chain rhamnolipids than many of the *Burkholderia* species. While different substrates such as alkenes, citrates, glucose, fructose, and olive oil can be manipulated to produce different properties.

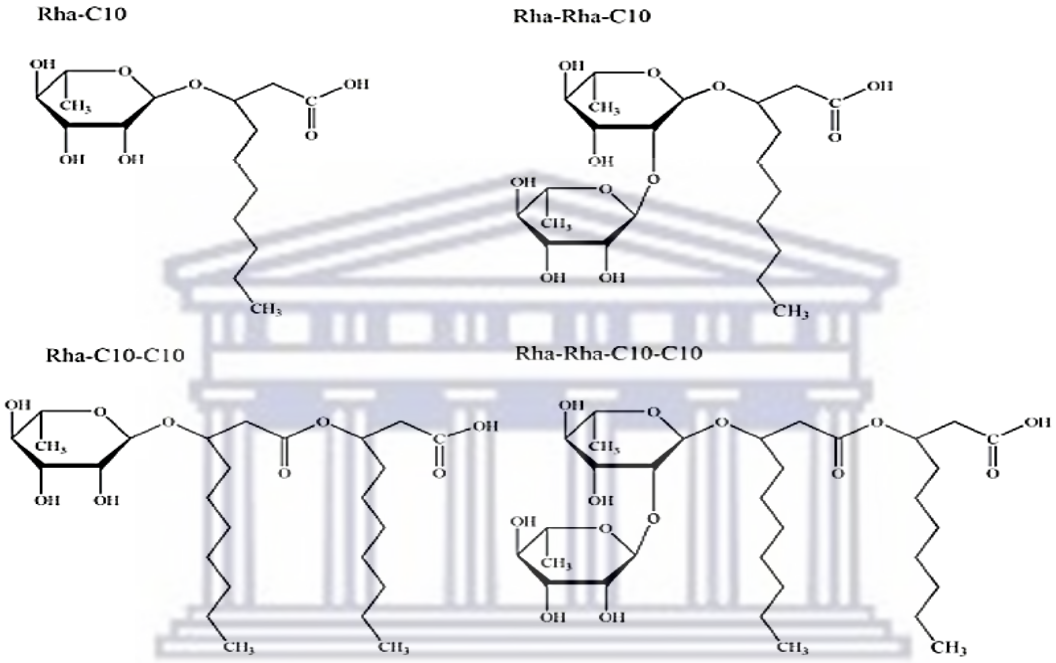


Figure 1-7: Common chemical structure of one or two α-L (+)-rhamnosyl units commonly coupled to one or two β-hydroxy fatty acid units via a *0*-glycosidic linkage (Shu *et al*., 2021).

The well-studied/characterized rhamnolipid producer is *P. aeruginosa*, additionally, other best-known producers include *P. putida*, *P. chlororaphis*, and *P. fluorescens* (Kubicki *et al*., 2019). Rhamnolipid-producing microorganisms belong to a vast phylogeny that are as diverse as their chemical structures. For example, producers are found belonging to Firmicutes, Proteobacteria, Actinobacteria, and Fungi (Roy, 2017; Kubicki *et al*., 2019). Furthermore, microorganisms producing rhamnolipids are isolated from a wide range of environments such as freshwater, soil, oil wells, pathogen biofilms, industrial affluents, surfaces of the leaves, and marine environments (Roy, 2017). The biosynthetic pathway of rhamnolipids was the first to be deciphered (Sanches *et al*., 2021). Most of the research on rhamnolipid is dedicated to overexpressing the biosurfactant in non-pathogenetic hosts such as *E. coli* (Sanches *et al*., 2021; Sophie *et al*., 2022).

1.7.2. Lipopeptides

Lipopeptides are peptide chains, either with a linear hydrophilic head or a lactone ring if they are cyclic, attached via an amide linkage to long-chain fatty acids. Various bacterial genera namely *Pseudomonas*, *Lactobacillus*, *Bacillus*, *Streptomyces*, and *Serratia* are known to synthesize lipopeptides (Kubicki *et al*., 2019). There are more than 100 lipopeptides known, belonging to groups such as surfactin, iturins, fengycin, plipastatin, and karstakins, with surfactin being the most investigated type (Raaijmakers *et al*., 2010; Kourmentza *et al*., 2021).

1.7.2.1. Surfactin

Surfactin is prominently produced by *B. subtilis,* is one of the most effective biosurfactants, and therefore is part of the best-studied and commercialized biosurfactants. This is mainly because they possess excellent surface tension reduction qualities and unique properties that can be equated to the complex chemical structure that lacks clear distribution of polarity and are composed of branched and ring structures (Liu *et al*., 2012; Bjerk *et al*., 2021). Surfactin can reduce the surface tension of water from 72 to 27 mN/m, and the interfacial tension of hexedine/ water from 43 to 1 mN/m. They have excellent CMC measured at 10^{-5} M; this is attributed to their strong self-assembly ability that results in the formation of sphere-like micelles (Liu *et al*., 2015; Bjerk *et al*., 2021). This formation facilitates close packing at the interface and large aggregates even at very low concentrations (Liu *et al*., 2015; Bjerk *et al*., 2021). Surfactin is also highly soluble in water and is known to have significant bioactivity. The prominent examples of this phenomenon are the antibiotics daptomycin and polymyxin β, synthesized from *Streptomyces roseosporus* and *Paenibacillus* polymyxa respectively (Liu *et al*., 2015; Kubicki *et al*., 2019).

Surfactin is a peptide chain formed by seven α-amino acids bonded to a hydroxyl fatty acid by lactone bond to form a cyclic lipopeptide (**Figure 1-8**) (Liu *et al*., 2012; 2015). The diverse chemical structures that surfactin has is characterized by the differences found in the sequence of amino acids and the carbon atoms in the fatty acids and this diversity reverberates onto the various physicochemical properties that surfactin displays (Liu *et al*., 2015). Surface tension reduction abilities increase as the hydrophobicity of the alkyl chain increases (Banat *et a*l., 2010; Liu *et al*., 2015). Small micelles formation is observed for short-chain fatty acids and the opposite is true for longer fatty acids (Banat *et al*., 2010; Liu *et al*., 2015). Lower surface and poor hemolytic activities are measured for linear surfactin while they are improved for cyclic structures (Banat *et al*., 2010; Liu *et al*., 2015). Even when the cyclic structure is opened through saponification, the leaner structure that results from the reaction has reduced surface activity (Banat *et al*., 2010; Liu *et al*., 2015). This phenomenon was reflected in an oil replacement study in which the activity decreased by one-third when leaner surfactin was employed compared to when the parent cyclic surfactin was used (Liu *et al*., 2015). The famous "horse saddle" structure characterized by a distinct polar and a non-polar domain adds an important role to the properties that surfactin displays (Banat *et al*., 2010; Liu *et al*., 2015). Surfactin can achieve emulsification capacity with diesel at EC_{24} of 90%. Due to this physicochemical property, it is mostly applied in the bioremediation of oil spills and oil contaminates and used for microbial enhanced oil recovery (MEOR) (Liu *et al*., 2015). A surfactin isolate *B. subtilis* B30 was found to enhance oil recovery by 17-31% (Liu *et al*., 2015). Furthermore, surfactin is also deemed advantageous due to its properties such as high biodegradability and low toxicity and remains effective at wide temperatures (21-70°C), NaCl salinity (0-10%), and

pH (3-10) ranges (Liu *et al*., 2015). Much of the research focus on surfactins is on their spatial structure, properties, and molecular behavior in an aqueous solution (Liu *et al*., 2015).

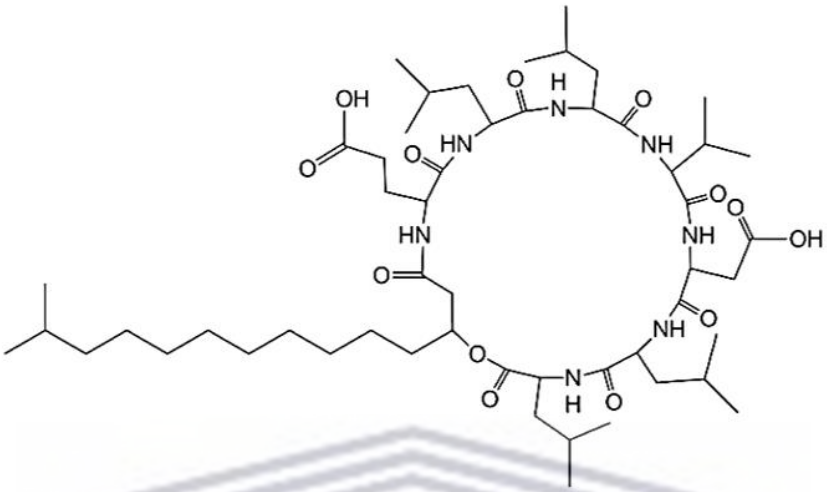


Figure 1-8*:* Chemical structure of cyclic surfactin composed of peptides bonded to hydroxyl fatty acid via a lactone bond (Liu *et al*., 2012).

1.8. Methods for screening biosurfactant activity

A strategic selection of the isolation and screening approaches to be employed in the search for biosurfactant-producing microorganisms is of crucial importance, as it sets the scene for the likelihood and the level of success in the discovery of biosurfactants (Trindade *et al*., 2021). The assessment of the activity constitutes a key component regardless of whether a culture-based or culture-independent approach is employed (Trindade *et al*., 2021). There are a wide variety of well-established and relatively simple screening methods available for screening biosurfactant activity (Walter *et al*., 2010; Mnif and Ghribi, 2015; Trindade *et al*., 2021). As biosurfactants encompass such wide structural diversity, the most commonly used screening methods are based on the physicochemical properties they display (Walter *et al*., 2010; Mnif and Ghribi, 2015; Trindade *et al*., 2021). These methods are mostly dependent on direct and indirect measures of surface and interfacial tension activity, with a few methods that assess specific physicochemical features of specific groups of biosurfactants (Walter *et al*., 2010; Mnif and Ghribi, 2015; Trindade *et al*., 2021). Screening for surface activity, using a single or a combination of assays, can be performed on whole cells, or culture supernatants, where both qualitative and quantitative data can be obtained (Walter *et al*., 2010; Mnif and Ghribi, 2015; Williams and Trindade, 2017; Trindade *et al*., 2021). The principle aim of screening is to identify structurally new biosurfactants with effective surface and interfacial tension reduction, low critical micelle concentration (CMC), high emulsion capacity, good solubility, and the ability to retain activity at a broad pH, salinity, and temperature range (Walter *et al*., 2010; Sourav *et al*., 2017).

This section provides an overview of all the screening methods used. The screening tests are selected based on ease, sensitivity, high-throughput capabilities, and the accessibility of instruments used for screening (Trindade *et al*., 2021). These screening methods are categorized as “Universal”- assays, employed for the initial screening of large numbers or collections for general biosurfactant activity; or, “Targeted”- assays which include either indirect

assays to detect specific biochemical or performance properties, or the more sophisticated direct assays that identify biosurfactants based on physicochemical properties (Trindade *et al*., 2021).

1.8.1. Universal screening assays

Included in this category are screening methods based on an indirect measure of the ability to reduce surface and interfacial tension, which are the phenotypic characteristics shared by all biosurfactants. These assays are easy, rapid, sensitive, normally do not require expensive equipment, and are often suitable for the screening of large numbers of cultured strains or metagenomic library clones. Hence, such assays are typically used as the first line of screening for the isolation of positive biosurfactant producing strains/clones (Thavasi *et al.*, 2011; Trindade *et al*., 2021). The atomized spray method, the drop collapse test, and the microplate-grid analysis assay were employed in this study.

1.8.1.1. The drop collapse test

This method assesses the stability of drops of culture broth, culture supernatant, or solutions of pure biosurfactant when they encounter an oil-coated or hydrophobic surface (**Figure 1-9**) (Jain *et al.*, 1991; Trindade *et al*., 2021). The underlining principle is that the drop collapse occurs as a result of the reduction of interfacial tension within the liquid and the surface tension reduction on the hydrophobic surface caused by biosurfactant activity (Youssef *et al.*, 2004; Saruni *et al*, 2019; Trindade *et al*., 2021). The control drops without biosurfactant activity remain stable and do not collapse due to the hydrophobic surface repelling the polar sample such as water (Trindade *et al*., 2021). The stability of the drop depends on the concentration and surface tension reduction capabilities of the biosurfactant being screened (Trindade *et al*., 2021).

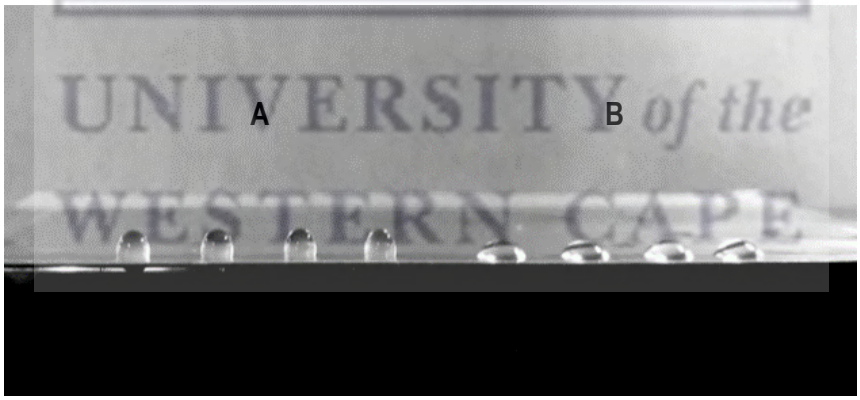


Figure 1-9: The drop collapse test method is used to screen for biosurfactant activity by observing the stability of drops containing biosurfactants on a hydrophobic surface (Clear polyolefin starseal). (**A**) pure water samples without the presence of biosurfactant. (**B**) water with the presence of surfactant resulting in the collapsed droplets. Image by Trindade *et al*., 2021.

One of the advantages of this method is its sensitivity and ease of use, with just a small volume of sample needed and no special equipment required, as little as 10 μL (Youssef *et al*., 2004; Trindade *et al*., 2021). It can be applied in large sample screening, and modifications to enable automated screening in microplates for high throughput screening (Burch *et al.*, 2010; Saruni *et al.*, 2019; Trindade *et al*., 2021). Staining of the droplets is an additional

modification to enhance the visualization of the drops (Mnif and Ghribi, 2015; Trindade *et al*., 2021). Disadvantageously, hydrophobic biosurfactants, and those that possess surface reduction capabilities but do not necessarily result in droplet collapse, cannot be detected using this method (Balan *et al.*, 2017; Trindade *et al*., 2021). Activity relies obviously on a detectable concentration of the compound in the droplet and the hydrophobicity of the applied surface (Balan *et al.*, 2017; Trindade *et al*., 2021). Notably, there is actually no conclusive evidence that links surface tension reduction to the method thus reducing its reliability (Burch *et al.*, 2010; Balan *et al.*, 2017 Trindade *et al*., 2021). Lastly, the method is only qualitative, although it can be adapted to be quantitative for the relative measurement of biosurfactant concentration for pure surfactants by measuring the drop size or the contact angle (Kurniati *et al*., 2019).

1.8.1.2. The atomized spray method

In this assay a thin mist of paraffin is sprayed over bacterial colonies cultivated on agar plates, revealing the formation of droplets as a halo around a biosurfactant producing colony (**Figure 1-10**) (Burch *et al.*, 2010; Trindade *et al*., 2021). The radius of the halo can be measured for a semi-quantitative analysis (Burch *et al.*, 2010; Trindade *et al*., 2021). The method rapidly detects activity and does not require sample preparation, making it suitable for high throughput screening of thousands of colonies at once, and therefore ideal for metagenomic library screening (Williams and Trindade, 2017; Williams *et al.*, 2019; Trindade *et al*., 2021). Furthermore, the atomized spray method (also known as the oil vaporization assay) provides greater sensitivity than the drop collapse method, detecting 10- to100-fold lower biosurfactant concentrations and allows the detection of biosurfactants with low water solubility (Burch *et al.*, 2010; Trindade *et al*., 2021). Due to the versatility and sensitivity of the atomized spray assay, it is considered a superior screening method to most of its indirect screening method counterparts.

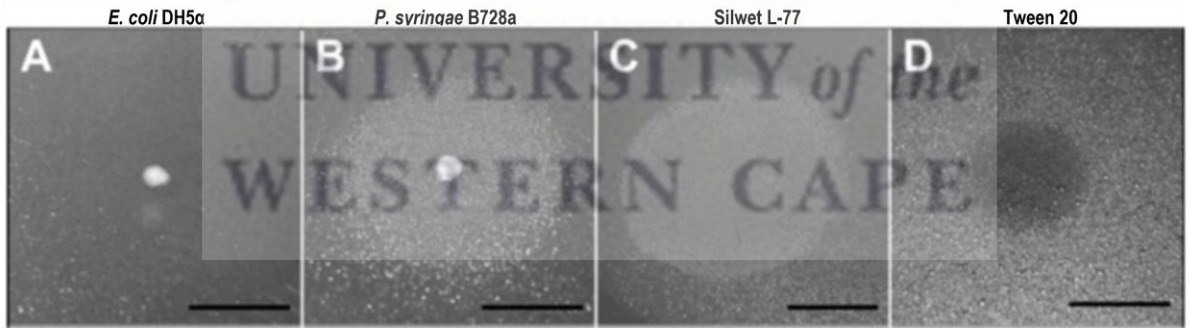


Figure 1-10*:* Atomized spray method employed for biosurfactant activity screening. Positive results manifest as a light-diffractive halo resulting from the formation of droplets surrounding microbial growth on agar. (**A**) A negative result of a non-biosurfactant producer *E. coli* DH5α. (**B**) A biosurfactant producer *Pseudomonas syringae* B728a. (**C**) Surfactant molecules silwet L-77 and (**D**) Tween 20. The image was obtained from (Burch *et al*., 2010) and the scale bar is 10 mm.

1.8.1.3. The microplate-grid analysis assay

The microplate-grid analysis assay has been patented as a qualitative measure to screen culture supernatant for surface tension reduction abilities by Cottingham *et al*. (2004). The assay involves assessing optical changes of

gridded paper placed under the 96-well plate containing the culture supernatant being tested (**Figure 1-11**). Pure water in a hydrophobic well has a flat surface and no optical distortion, whereas the presence of a biosurfactant results in optical distortion (Trindade *et al*., 2021). The optical changes on the surface are brought about by the wetting of the edge of the well, subsequently becoming concave and taking the shape of the diverging lens, causing optical distortion (Trindade *et al*., 2021). The assay is sensitive, rapid, and easy, allowing immediate detection of surface-active compounds from a small volume (100 μL) (Kavuthodi *et al*., 2015). Furthermore, it is suitable for automated high throughput screening and therefore appropriate as a method for function-based metagenomic library screening (Williams and Trindade, 2017; Trindade *et al*., 2021). Proper imaging is required to capture the correct optical distortion to remove subjective bias, however, the sample must be clear with no turbidity or intense color to observe the underlying grid changes (Saruni *et al*., 2019; Trindade *et al*., 2021).

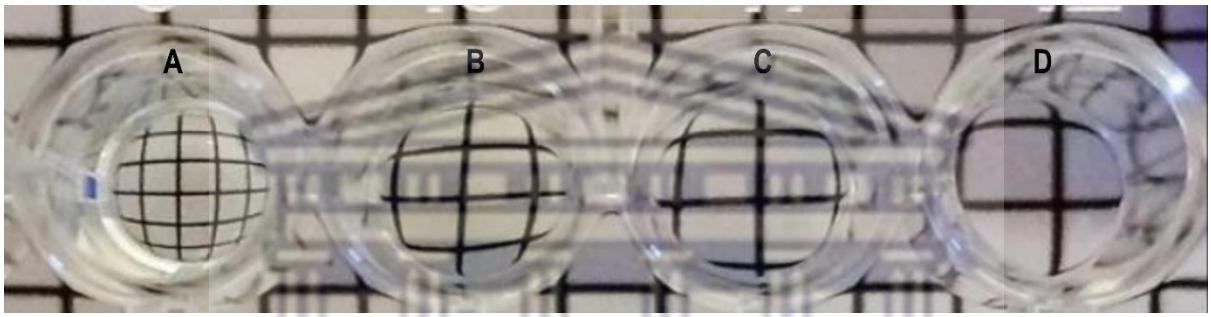


Figure 1-11: The microwell plate assay measures the surface activity of biosurfactants in a solution. (**A**) optical distortion caused by active biosurfactant. (**B**) Culture media. (**C**) Non-active sample. (**D**) Empty well, with a flat surface. Image by Williams, 2010.

1.8.2. Targeted screening assays

The assays described here encompass two parts: methods that screen for specific biochemical or physicochemical characteristics and performance of the biosurfactant; and those that directly measure surface and interfacial tension reduction (Trindade *et al*., 2021). The employed techniques in this study included emulsification after 24 hours, thin layer chromatography techniques, Cetyl trimethylammonium bromide (CTAB)-methylene blue, and low bond axisymmetric drop shape analysis (LB-ADSA).

1.8.2.1. Emulsification after 24 Hours (EC24)

The emulsification capacity index (EC_{24}) measures the ability of an emulsifier to stabilize an emulsion of immiscible liquids over 24 hours after the mixing of two immiscible phases (Cooper and Goldenberg, 1987; Trindade *et al*., 2021). For example, when mixing water and oil, the unstable emulsion divides into separate phases depending on the respective densities. However, in the presence of a biosurfactant with emulsification capabilities the emulsion is stabilized through interfacial tension reduction between the immiscible phases allowing them to readily mix (**Figure 1-12**) (Trindade *et al*., 2021). This method, therefore, assesses the capability of a culture (or supernatant) to form an emulsion with a hydrocarbon such as paraffin, kerosene, and hexadecane after they are mixed (Cooper and Goldenberg, 1987; Trindade *et al*., 2021) and it is mostly utilized to measure the quantity of biosurfactant production (Zheng *et al*., 2009; Trindade *et al*., 2021).

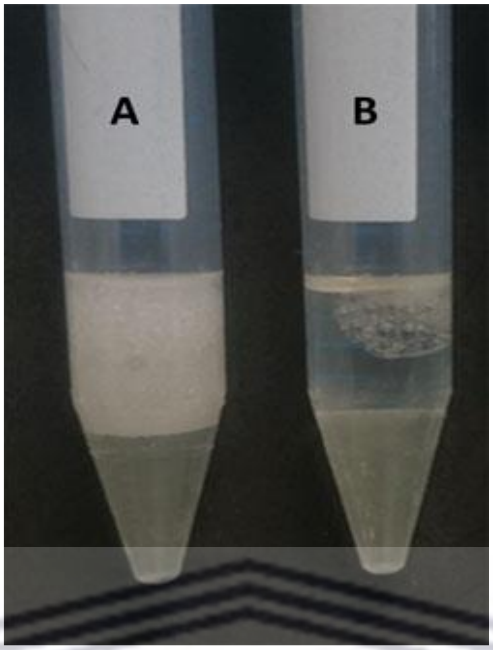


Figure 1-12: Paraffin and culture supernatant emulsification 24 h after mixing. (**A**) Stable emulsion caused by the presence of lyso-ornithine lipid (LOL) biosurfactant in the cell-free culture. (**B**) Two immiscible phases separated as a result of an unstable emulsion. Image by Trindade *et al*., 2021.

The EC_{24} is measured as the height of the emulsification over the total height of the two-phased mixture, 24 hours after mixing by agitation to form the emulsion as represented by **Equation 1-1** (Cooper and Goldenberg 1987). Alternatively, emulsification can be detected by quantifying the turbidity from the emulsion using a turbidimeter (Reddy and Fogler, 1981; Trindade *et al*., 2021).

Equation 1-1: Emulsification capacity index (EC_{24}) measures the ability of an emulsifier to stabilize the emulsion of immiscible liquids after 24 hours.

$$EC24 = \frac{Height\ of\ emulsification}{total\ height\ of\ the\ two\ phased\ mixture\ after\ 24\ hrs}\ x\ 100$$

However, the ability of a biosurfactant to form an emulsion is rarely associated with its surface and interfacial tension reduction potential (Balan *et al*., 2017; Williams and Trindade, 2017; Trindade *et al*., 2021). Therefore, a good emulsion does not necessarily equate to surface and interfacial tension reduction. Conversely, biosurfactants capable of reducing the surface and interfacial tension are not necessarily good emulsifiers (Sourav *et al*., 2017; Trindade *et al*., 2021).

1.8.2.2. Detection of biosurfactant production by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is mainly applied in the characterization of the chemical nature of the produced biosurfactants by using selective reagents and manipulating the polarity of solvents when separating crude extracts on a silica gel plate (Trindade *et al*., 2021). For the detection of functional groups of biosurfactants, different staining reagents can be used, e.g., ninhydrin stains lipopeptides red whereas α-naphthol stains glycolipids purple

(Trindade *et al*., 2021). Hydrophobic moieties like aromatic ring systems or lipid chains can be visualized by applying iodine vapor or primuline respectively (Trindade *et al*., 2021). Therefore, TLC can be used to differentiate mixed compounds, for example, the difference between mono-rhamnolipids and di-rhamnolipids, or between lactonic and acidic sophorolipids congeners when compared to a standard (Trindade *et al*., 2021). This method is not suitable for high-throughput screening (Trindade *et al*., 2021). Preparative TLC are employed to purify and isolate analytes of interest from a mixture (Sherma and Fried, 1987). Preparative TLC plates are characterised by a thick silica-gel layer, which allows for processing of larger quantities of product ranging from 10 mg to greater than 1 g (Sherma and Fried, 1987). Often the substrates to be analysed will be applied as a long streak at the sample application zone of the TLC plate, rather than as separate spots as done on analytical TLC plates (Sherma and Fried, 1987). After development the targeted compounds are recovered by scrapping them from the plate and eluting them in a solvent of choice to be further purified or analysed (Sherma and Fried, 1987).

1.8.2.3. Cetyl trimethylammonium bromide (CTAB)-methylene blue plate assay

The Cetyl trimethylammonium bromide (CTAB)-methylene blue method, also referred to as the "blue agar plate" method, serves as a semi-quantitative assay for the preliminary detection of extracellular anionic surfactants (Siegmund and Wagner, 1991; Trindade *et al*., 2021). The positive detection of biosurfactants on agar containing 0.5 mg/mL CTAB and 0.2 mg/mL methylene blue is seen by the formation of a blue halo surrounding a bacterial colony growing on top of the agar (Siegmund and Wagner, 1991; Fenibo *et al*., 2019; Trindade *et al*., 2021) (**Figure 1-13**). The blue halo is formed through the binding and forming of a complex of the anionic biosurfactant with the cationic surfactant of CTAB (Siegmund and Wagner, 1991; Fenibo *et al*., 2019; Trindade *et al*., 2021). However, not all bacteria can be screened using the method because CTAB is toxic to some bacteria including *E. coli* (Williams and Trindade, 2017; Trindade *et al*., 2021). Nevertheless, another approach can be employed which includes creating equidistant wells onto the agar and inoculating the sample or crude extracts or culture broth to be screened into the wells and incubating at 37°C for 48-72 hours (Fenibo *et al*., 2019; Trindade *et al*., 2021).

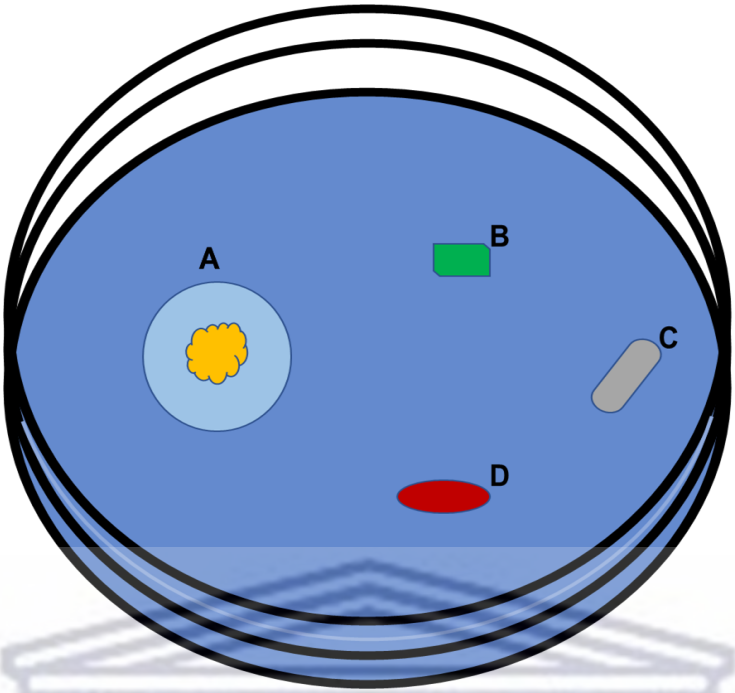


Figure 1-13: Cetyl trimethylammonium bromide (CTAB)-methylene blue method detection of extracellular glycolipids or anionic biosurfactants. (**A**) The positive detection of biosurfactants on agar containing 0.5 mg/mL CTAB and 0.2 mg/mL methylene blue is seen by the formation of a blue halo surrounding a sample. (**B,C,D**) negative results. Image adapted from Fenibo *et al*., (2019).

1.8.2.4. Quantitative screening methods based on the direct measure of the surface and interfacial tension

Tensiometers can be universally used to quantify the activity of any biosurfactant by measuring the change in surface and interfacial tension at their air/water and oil/water interfaces. Force or optical tensiometry are techniques that are commonly used to measure surface and interfacial tension (Hoorfar and Neumann 2004; Lauren, 2017; Trindade *et al*., 2021). Force tensiometry involves a direct measure of the surface or interfacial force exerted on a probe. The optical tensiometry unit measure is calculated from theoretical equations whereby an image profile of a drop or bubble is extracted and fitted to an equation (Hoorfar and Neumann 2004; Lauren, 2017; Trindade *et al*., 2021). The contact angle can be used as an easy optical tensiometry measure to screen for biosurfactant activity and to characterize biosurfactants. There is a shared relationship between a contact angle and the surface tension and interfacial tension reduction abilities of a surface-active compound (Akbari *et al*., 2018). Reduction of a contant angle results from a reduction of the surface and interfacial tension caused by the surface-active compound. Contact angle (θ°) lies between the boundary that separates a liquid and a solid phase, the angle between the outline of the contact angle and the surface of the liquid (**Figure 1-14**) (Yuan and Randall, 2013; Ossila, 2020). The contact angle is used to measure the wettability of a solid by a liquid (Akbari *et al*., 2018). At complete wettability, the contact angle is measured under 0°, and general wettability lies between 0° and 90° and non-wettability at a 90° angle and above (**Figure 1-14**) (Yuan and Randall, 2013; Ossila, 2020). The contact angle is related to the surface tension of the liquid and solid being tested. A goniometer can be used to measure the contact

angle directly or the contact angle can be calculated from drop image analysis (Yuan and Randall, 2013; Ossila, 2020). The are several methods available in literature used to measure contact angle and surface tension and some methods combine both measures (Hoorfar and Neumann 2004; Yuan and Randall, 2013). The available techniques include the pendant drop shape technique, axisymmetric drop shape analysis by profile, and low bond axisymmetric drop shape analysis:

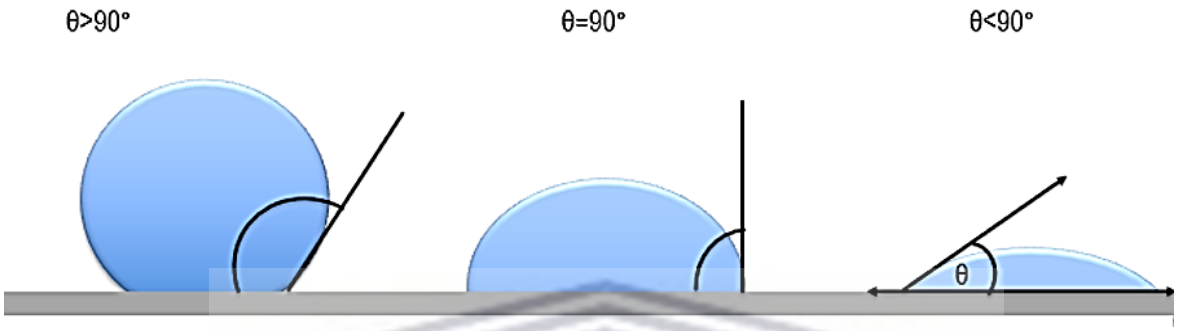


Figure 1-14: The contact angle used to screen for biosurfactant activity and to characterize the biosurfactants produced. Contact angle (θ°) lies between the boundary that separates a liquid and a solid phase, the angle between the outline of the contact angle and the surface of the liquid. The contact angle is used to measure the wettability of a solid by a liquid. At complete wettability, the contact angle is measured under 0°, general wettability lies between 0° and 90° angle, and non-wettability at a 90° angle and above. Image adapted from Ossila, (2020).

1.8.2.4.1. Axisymmetric drop shape analysis by profile (ADSA-P)

Axisymmetric drop shape analysis by profile (ADSA-P) is an optical method, used to simultaneously measure liquid surface tension and contact angle from the profile of a droplet resting on a solid surface (Van der vegt *et al*.,1991). The underlying principle is that the shape of a liquid droplet depends greatly on the liquid surface tension; biosurfactants with low surface tension tend to minimize the surface area of the drop causing the droplet to deviate from a perfectly spherical shape when compared to those with high surface tension, as indicated by the drop collapse test (Van der vegt *et al*., 1991; Walter *et al*., 2010; Mnif and Ghribi, 2015; Berry *et al*., 2015; Trindade *et al*., 2021). The circumference of a liquid on a solid surface is captured as an image and the measurements are subsequently fitted to the capillary Laplace Equation to calculate the surface tension (Berry *et al*., 2015; Trindade *et al*., 2021). The advantage of the ADSA-P is the small volume needed for the drop shape analyses. However, the shortcomings of using this method include the requirements of a camera and software. It involves complex calculations and computational routines, and samples cannot be measured in parallel (Walter *et al*., 2010; Mnif and Ghribi, 2015; Berry *et al*, 2015; Trindade *et al*., 2021).

1.8.2.4.2. Low-bond axisymmetric drop shape analysis (LB-ADSA)

There are less expensive and less complex computational tools or software such as Image J (https//imagej.net/:version1.47vjava1.6.0_20 (32-bits)) that can be used to measure surface tension and contact angle (Stalder *et al*., 2006; Stalder, 2020) Image J analysis software is an optical tensiometric method used to measure the contact angle from a shadow image of a sessile drop and surface and interfacial tension from the

shadow image of a pendant drop (**Figure 1-15**) (Stalder *et al*., 2006; Stalder, 2020). Image J provides two software analyses B-spline snake and low bond axisymmetric drop shape analysis (LB-ADSA) which allows for the calculation of contact angle and surface tension from the liquid drop images (Stalder *et al*., 2006; Stalder, 2020). The software is implemented as a plugin or add-on software for the public domain image processing software Image J and is freely available to use (Stalder *et al*., 2006).

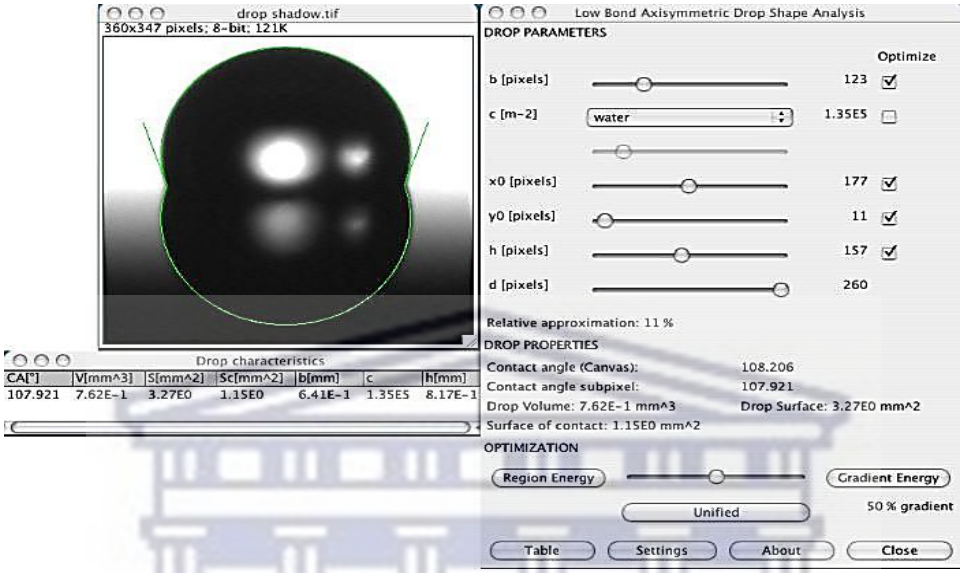


Figure 1-15: Water drop analysis by low bond axisymmetric drop shape analysis (LB-ADSA). The green line indicates the theoretical contour corresponding to the parameters in the dialog to the right (Stalder *et al*., 2006).

LB-ADSA uses an imaging approach and is based on the Young-Laplace equation for measuring contact angles and surface tension (Stalder *et al*., 2006). The first-order approximation of the Young-Laplace equation is fitted onto an image according to photographic image data of axisymmetric sessile drop (Stalder *et al*., 2006; Stalder, 2020). The implementation of the method requires capturing the sessile drop image, followed by the extension of the drop image using a mirror symmetry, and lastly uploading the mirrored symmetric drop image to image J to fit the first-order perturbation of the Laplace equation onto the image (**Figure 1-15**) (Stalder *et al*., 2006; Stalder, 2020). This only works on image J version 1.43 or above. There is a need to ensure that the drop in a picture appears above the substrate and that the method is suitable for drops that are under the force of gravity on a horizontal substrate. The advantage of using the screening method is that a small amount is needed for the drop shape analysis (5-10 µL) (Stalder *et al*., 2006; Walter *et al*., 2010; Mnif and Ghribi, 2015; Stalder, 2020).

1.9. Culture-dependent and culture-independent approaches for biodiscovery

The well-studied biosurfactants have, for the most part, been discovered through isolation from a pure culture of microorganisms making them culture-dependent (Kumar *et al*., 2007; Nayarisseri *et al*., 2018; Gutierrez *et al*., 2019). The application has over the years been criticized for the isolation of small fractions of already known biosurfactant producing species, independent of the environment that they are isolated or cultured from (Gutierrez *et al*., 2019). This has directly brought about limitations in terms of accessibility to the diverse and complex microbial communities in nature. Efforts placed into culture-based approaches are faced with a decrease in novel

discoveries, with more than 50% estimated reduction in the number of new biosurfactant discoveries (Reen *et al*., 2015; Trindade *et al*., 2021). On the other hand, a culture-independent approach employs techniques such as metagenomics that do not require cultivating and screening of pure strains. Therefore, such an approach is able to overcome the low chances of novelty associated with the culture-dependent approach (Martin *et al*., 2014).

1.9.1. Culture-independent approach: Metagenomics

The culture-independent approach has been successful in the discovery of vast numbers of novel natural products such as protease, laccase, oxidoreductase, and biosurfactants and in accessing chemical diversity from not-so-easily cultured environments (Kennedy *et al*., 2011). Metagenomic screening is a culture-independent tool invented with the purpose of accelerating biodiscovery and accessing novel structures beyond the minority of microorganisms that can be cultured (Jackson *et al*., 2015b; Bekele *et al*., 2021). Metagenomics encompasses the biodiscovery of uncultivated microorganisms from complex communities by extracting total environmental DNA (eDNA) directly from the sample environment such as the soil, marine sponge, or gut microflora (Kennedy *et al*., 2011; Thies *et al.*, 2016; Bekele *et al*., 2021). The extracted eDNA is then inserted into an expression vector and transformed into a heterologous host to create metagenomic library clones which are then subjected to either function- or sequence-based screening (Kennedy *et al*., 2011; Thies *et al.*, 2016; Bekele *et al*., 2021). Function-based screening is used to screen for the activity of interest whereas sequence-based screening is applied to determine the gene responsible for the activity found or to find a possible biosynthetic pathway for the surface-active compound (Thies *et al*., 2016; Bekele *et al.*, 2021).

1.9.1.1. Sequence-based screening of the metagenomic library

This study does not focus on sequence-based metagenomic screening; however, a brief review is essential for the bigger picture of what can be achieved by a metagenomic approach and to put into a frame the importance of investing in function-based screening. Sequence-based screening is employed to analyze genes based on sequence similarity and homology and it has been applied to the discovery of biosurfactants (Gill *et al*., 2017). Sequence-based screening of the metagenomic library can be carried out through; gene-targeting employing degenerate PCR primers or hybridization probes that are designed to amplify conserved sequences coding for the biosynthetic gene cluster being investigated, or by a shot-gun approach which entails either sequencing the metagenomic DNA directly or the library clones via next-generation sequencing (NGS) (Kennedy *et al*., 2011; Trindade *et al*., 2015; 2021). NGS is a great means for bioprospecting because it permits rapid identification of large biosynthetic pathway gene clusters and the prediction of the chemical structures subsequently (Trindade *et al*., 2021). Furthermore, with the right bioinformatics and computational systems, sequencing can be performed in an ultra-high-throughput fashion (Trindade *et al*., 2021).

The favor in employing sequence-based screening for the discovery of novel biomolecules is reinforced by the continuous improvement of NGS platforms, including its subsequent scalability, cost reduction, and *in silico* tools developments (Trindade *et al*., 2021). Furthermore, what makes sequence-based screening noteworthy are the publicly accessible databases with enormous metagenomic sequence datasets (Trindade *et al*., 2021). The

datasets embody largely unexploited resources from a variety of organisms from almost every environment possible and genome sequences for over 800 000 bacteria and 4000 fungi are included (Mac Aogáin *et al*., 2019; Trindade *et al*., 2021). Sequence-based screening further takes advantage by enabling *de novo* synthesis of the identified genes and pathways to be cloned into a plasmid vector of choice, through various service providers, while avoiding the sampling and isolation of strains and/ or (meta) genomic DNA and sequencing costs (Hughes and Ellington, 2017; Trindade *et al*., 2021).

Protein coding sequence identification requires assembling genomes and/or contigs based on homology to reference sequence data in carefully selected sequence databases (Trindade *et al*., 2021). Thus, the identification of conserved protein motifs (for example domains/ active sites) or open reading frames (ORFs) is employed through probing algorithms such as basic local alignment search tool (BLAST) (Altschul *et al*., 1990), clusters of orthologous genes (COG) (Tatusov *et al*., 2000), and Kyoto encyclopedia of genes and genomes (KEGG) for similarities (Kanehisa and Goto, 2000; Jackson *et al*., 2015b). However, more than one gene is typically involved in the biosynthesis of secondary metabolites such as biosurfactant, in such cases identification of biosynthetic gene clusters (BGCs) is carried out using the popular antiSMASH tool (antibiotic and secondary metabolite analysis shell) (Blin *et al*., 2019; Trindade *et al*., 2021). This tool has an up-to-date collection of state-of-the-art annotated BGC data, it is easy to use, and it is continuously developed (Trindade *et al*., 2021).

The main disadvantage of employing sequence-based screening is that only known and closely related genetic sequences can be identified. Therefore, without a reference genome and sequence, there is a chance of novel biosurfactant biosynthetic pathways being missed (Martin *et al*., 2014; Williams and Trindade, 2017). However, several genome-guided efforts are being developed to try and minimize this limitation. For instance, not only does antiSMASH identify all BGCs present in the query sequence, but it can also facilitate gross-genome analyses and therefore novel compounds encoded by novel genes can be identified through more complex search processes that can be implemented through graphical query builders (Trindade *et al*., 2021). Furthermore, there are other disadvantages resulting from the accelerated improvement in sequencing technology. Namely, the sequence boom has contributed to vast volumes of genes and genome sequence data (Alves *et al*., 2018). However, this has yielded an increasing gap between what is known in sequence data versus what is characterized as biologically active and of pure compounds, and the latter has a direct contribution to desired applications which is the main objective of bioprospecting for natural products (Alves *et al*., 2018; Trindade *et al*., 2021).

1.9.1.2. Function-based screening of the metagenomic library

As previously stated, biosurfactants are as structurally diverse as the biosynthetic genes/pathways that encode their synthesis (Bodour *et al*., 2003; Valenzuela-Reyes *et al*., 2014; Balan *et al*., 2017). Therefore, this favors the use of function-based screening and is considered the “gold standard” for biodiscovery (Bodour *et al*., 2003). Function-based screening for biosurfactants takes advantage of the various reliable and well established activity screening methods already discussed, which merely detect phenotypes exhibited by biosurfactants. There are three major advantages associated with using function-based screening over sequence-based screening which

are: i) the identification of genes or pathways in a host library by their functional activity, without the need for prior knowledge of the gene sequence (Bodour *et al*., 2003; Walter *et al*., 2010; Lam *et al*., 2015; Trindade *et al*., 2015); ii) consequently minimizing the exclusion of unknown genes and different classes structurally and genetically which can lead to the discovery of novel and diverse biosurfactant structures (Bodour *et al*., 2003; Walter *et al*., 2010; Lam *et al*., 2015); iii) Activity is guaranteed and the success of expression in the chosen heterologous host makes it easier for downstream production and analysis (Bodour *et al*., 2003; Walter *et al*., 2010; Lam *et al*., 2015; Thies *et al*., 2016). Furthermore, depending on the efficiency of the screening method selected, high-throughput screening can be accomplished.

To date, there are only a few biosurfactant discoveries under the belt of metagenomics and heterologous expression. When compared to other environmental biodiscovery efforts, the approach has not contributed much, with only four reports of successful biosurfactant discoveries via function-based metagenomics (Bradly *et al*., 2004; Thies *et al*., 2016; Williams *et al*., 2019, Araujo *et al*., 2020). The first example was a "coincidental" discovery, in which long-chain N-acyl amino acids and palmitoyl putrescine biosurfactants were isolated by screening soil and sediment metagenomic libraries for antimicrobial compounds (Brady *et al*., 2004). This discovery highlights the advantage of using function-based screening, in which novel biosynthetic pathways and activities are discovered without the requirement of sequence homology. However, Thies and colleagues in 2016 reported the first example in literature of a successful function-based screening where biosurfactant activity was specifically assayed, resulting in the identification of long-chain N-acyltyrosine from abattoir wastewater. However, genes responsible for the synthesis of N-acylated amino acids (NAS) have been largely reported in these discoveries, which is not ideal for the expansion of the biosurfactant diversity pool (Williams *et al*., 2019; Trindade *et al*., 2021). Williams and colleagues (2019) predicted that N-acyl amino acids are still going to be identified in future biosurfactant activity screenings, mainly because of their pronounced surface activity and abundance in nature.

A recent study by Araujo *et al*. (2020) perfectly demonstrated the potential of function-based metagenomic screening toward the discovery of completely novel biosurfactants. Through employing function-based screening, they discovered a protein (MBSP1) with emulsification properties from a metagenomic library constructed from a riverbank soil sample. The novel MBSP1 biosurfactant is the only recorded finding that diverges considerably from what has been discovered structurally when a function-based metagenomic approach is employed (Araujo *et al*., 2020). Emulsification and surface activity by the MBSP1 protein serves as the first example of prokaryotic homologs; the known fungal homologs include the hydrophobins and cerato-platanins predominantly (Cox and Hooley, 2009; Pitocchi *et al*., 2020; Araujo *et al*., 2020). Furthermore, although previously unassigned to a function, homologs of MBSP1 are common among Halobacteriaceae (Araujo *et al*., 2020; Trindade *et al*., 2021). Nevertheless, function-based screening is still limited to these few cited examples.

The present degree of success in novel biosurfactant discovery evidently doesn't mirror the technological advancements in metagenomics, the improvements in heterologous host systems, and in high-throughput screening tools, and the availability of metagenomic libraries from diverse environments, which all favor an

increased probability for novel discoveries (Culligan *et al*., 2014; Jackson *et al*., 2015b; Williams *et al*, 2019). The lack of interest in biosurfactant discovery might be a substantial contributing factor in the dearth of novel discovery, particularly since metagenomics has significantly contributed to the discovery of numerous novel enzymes, bioactive and biosynthetic genes or pathways that are of particular interest to the pharmaceutical and food industries (Coughlan *et al*., 2015). Some of the novel enzymes discovered through functional metagenomics include esterase/lipase, β-agarase, cellulase/hemicellulose, protease, amylase, xylanase, and much more from the extensive list (Brandy and Clard, 2000; Coughlan *et al*., 2015; Thies *et al*., 2016). Just to get a glimpse of the difference in quantity when compared to biosurfactant discovery, a total of 332 enzymes were identified from metagenomic libraries over a period of three years, from 2014 to 2017 of which 273 were discovered through functional metagenomics (Berini *et al*., 2017). However, it is important to note that functional enzymes have higher chances of discovery than natural compounds because unlike compounds they usually have direct and powerful detection methods and they are normally easily expressed (Alam *et al*., 2021).

According to Trindade *et al.* (2015), there are two major challenges that limit the potential for function-based metagenomic screening to be fully realised. Firstly, the sequence technology boom, as highlighted in the previous section, because of the easiness, reduced price, and technological advancement in sequencing a lot of research leans towards it. The second challenge is the heterologous expression. The success of function-based screening primarily depends on the ability of the hosts to express the foreign genes, therefore, it is a huge limiting factor (Gabor *et al*., 2004; Craig *et al*, 2010; Penesyan *et al*., 2013, Maruthamuntu *et al*., 2016; Distaso *et al*., 2017; Gill, 2017; Calderon *et al*., 2019; Williams *et al.,* 2019). The heterologous expression challenges are experienced as a result of:

(I) The choice of insert size on the clone has significant influence on heterologous expression and is guided by primary research goals, DNA quality, targeted genes, and screening strategies (Distaso *et al*., 2017). It is recognized that it is easier to express a single active enzyme than an entire biosynthetic pathway (Bekele *et al*., 2021). However, usually entire biosynthetic pathways are needed for most natural product expression (Bekele *et al*., 2021). Secondary metabolites sometimes require massive gene clusters to be expressed. For example, polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) are coded by massive gene clusters made-up of 20 or more genes, which are sometimes distributed between multiple polycistronic transitional units (Reen *et al*., 2015; Trindade *et al*., 2021). Small insert libraries that are less than 10 kb are constructed in plasmids, while larger DNA inserts, with sizes between 25-35 kb, 24-40 kb, and 100-200 kb are constructed in cosmids, fosmids, and bacterial artificial chromosomes (BACs) respectively (Bekele *et al*., 2021). Large insert libraries are required for recovering large gene clusters with complex biochemical pathways and large operons and biosurfactant biosynthetic pathways are found to approach 100 kb in size (Culligan *et al*., 2014; Distaso *et al*., 2017; Bekele *et al*., 2021). This is sometimes difficult to achieve in a single fosmid vector (Williams *et al.*, 2019; Bekele *et al*., 2021). To combat this, libraries are therefore often prepared in BACs, which can accommodate DNA inserts of up to 350 kb and these can be preserved at a low copy number (Bekele *et al*., 2021). However, in practice,

it is hard to achieve libraries of this nature from metagenomic DNA (Bekele *et al*., 2021). Smaller insert libraries are therefore more often constructed and have a high probability of being expressed because they are mostly aligned in an appropriate orientation, however, only pathways that require identification of a biocatalyst encoded by a single gene, or a small operon can be expressed, therefore, excluding expression of large clusters (Culligan *et al*., 2014; Distaso *et al*., 2017). This is reflected in the pathways that have been discovered thus far. Function-based metagenomics identification predominately reports surface-active molecules that can be traced back to the activity of single enzyme or protein, whereas the biosynthesis of the well-known biosurfactants are encoded in operons of more than a few genes or even large BGCs (Reen *et al*., 2015; Trindade *et al*., 2021). These clusters become very hard to express in a heterologous host and require optimal induction conditions (Bekele *et al*., 2021; Trindade *et al*., 2021).

(II) Low chances of gene expression are due to silent genes under laboratory conditions (Gill *et al*., 2017; Trindade *et al*., 2021). Not having the right culturing conditions also lowers the chances of gene expression. It is well documented that the type of nutrients and environmental conditions are responsible for the proteins synthesized by microorganisms which in turn regulate the secondary metabolites expressed (Reen *et al*., 2015; Bekele *et al*., 2021). To achieve expression, therefore, means replicating the biochemistry needed by the microorganism and this is a big challenge, especially for genes from extreme and diverse environments (Reen *et al*., 2015; Bekele *et al*., 2021; Trindade *et al*., 2021). Furthermore, most genes that are expressed in their natural environment are not expressed under laboratory conditions (Schroeckh *et al*., 2009; Reen *et al*., 2015). This is caused by the lack of synergies associated with multifaceted, competitive, and interdependent interactions found in nature (Schroeckh *et al*., 2009; Trindade *et al*., 2021). Moreover, in a heterologous system this is amplified (Schroeckh *et al*., 2009; Reen *et al*., 2015). Factors that contribute to the non-expression of genes in a heterologous system include: the host not meeting the required intermediate compound(s) from the native producer (Kennedy *et al*., 2011; Trindade *et al*., 2021); the absence of *in trans* genetic elements; the fragmentation of previously clustered gene clusters; as well as the lack of post-translational modification processes (Schroeckh *et al*., 2009; Kennedy *et al*., 2011; Trindade *et al*., 2021).

(III) The successful expression of the gene(s) of interest is highly dependent on the ability of the host to recognize the regulatory sites that direct gene expression and to also provide a cellular environment that meets conditions needed for suitable substrate and protein folding (Williams *et al.,* 2019). *Escherichia coli* (*E. coli*) is the general host of choice for the construction and function-based screening of metagenomic libraries because of its ability to express a wide range of genes that belong to different microorganisms, its' fast growth kinetics, the availability of genetic tools, its high transformation efficiency, low cost, as well as its high cell density and the reputable protocols and commercial kits for library creation based on *E. coli* vectors (Culligan *et al.*, 2014; Rosano and Ceccarelli, 2014; Trindade *et al*., 2021). However, *E. coli* is not able to recognize all genetic elements (Distaso *et al*., 2017). Only 40% of genes represented in

an environment are expressed by *E. coli,* while microorganisms such as actinobacteria have approximately 80% of the promoters that are not recognized by *E. coli* due to the large difference of Guanine plus Cytosine (G+C) content (Gabor *et al*., 2004; Distaso *et al*., 2017; Williams *et al*., 2019). Therefore, *E. coli* often cannot express certain genes, particularly those of distantly related organisms. Therefore, exploring hosts like *Streptomyces* or other hosts that have genetic features compatible with a range of marine strains could bypass issues associated with expression such as guanine-cytosine content and avoid problems associated with promoter/ribosome-binding sites recognition or different codon usage amongst others (Gabor *et al*., 2004; Graig et al., 2010; Kubicki *et al*., 2019).

(IV) Parallel function-based screening, in which more than one host is employed for the expression of the metagenomic library can be employed to circumvent some of the described issues (Gabor *et al*., 2004; Craig *et al*., 2010; Williams *et al*., 2019; Trindade *et al*., 2021). The parallel functional screening's objective is to increase the diversity of transcriptional and translational requirements to target as wide a diversity of bacteria taxa as possible (Williams *et al*., 2019; Trindade *et al*., 2021). Parallel function-based screening of a metagenomic library provides a diverse set of the biochemical and regulatory environments to ensure the expression of a large set of genes or pathways from the library, thus increasing the probability of finding a hit (Ekkers *et al*., 2012). As such, host such as *Bacillus*, *Pseudomonas,* and *Streptomyces* have been developed, as they are known biosurfactant producers, therefore, provide the metabolic pool required to synthesise biosurfactant compounds (Jackson *et al*., 2015b).

The parallel function-based metagenomic approach was first successfully applied by Williams and colleagues (2019) who identified a novel biosurfactant from a hydrocarbon-contaminated metagenomic library, which resulted in the isolation of an ornithine acyl-ACP N-acyltransferase and ornithine lipids (Williams *et al*., 2019). The metagenomic fosmid could not confer biosurfactant activity in *E. coli* whereas, the ornithine lipids were produced in *Pseudomonas putida* (Williams *et al*., 2019). Thus, screening for activity in an additional host expanded the range of sequences and compounds that could be detected (Williams *et al*., 2019). However, the expression of the same gene in different hosts can result in different products (Lee *et al*., 2019). For example, the expression of the same N-acyltransferases in different proteobacteria yielded different products (Lee *et al*., 2019; Williams *et al*., 2019; Trindade *et al*., 2021). The expression in *E. coli* yielded extracts containing predominantly N-acyl tyrosine, whereas N-acyl-phenylaline was produced if the genes were expressed in *P. putida* and N-acyl leucin in *P. koreensis* (Lee *et al*., 2019; Williams *et al*., 2019; Trindade *et al*., 2021). Although screening in multiple non-*E. coli* hosts offers such advantages, the transformation efficacy level required is sometimes difficult to meet for non-*E. coli* hosts (Loeschcke and Thies, 2015; Distaso *et al*., 2017).

(V) Even after successful expression, two events can take place to hinder the successful detection of surface activity. The activity can be lost or degraded, before it is detected, or the expressed surface-active

compound can be toxic to the heterologous host resulting in the death of a clone before detection (Ekkers *et al*., 2012; Loeschcke and Thies, 2015). For example, anti-tumor, anti-inflammatory, and anti-protozoan compounds are sometimes secreted and form part of the multi activities of compounds (Ekkers *et al*., 2012; Zobel *et al*., 2015 Weimer *et al*., 2020). Secondary compounds that are toxic to *E. coli* as the initial host consequently result in the loss of the clones carrying the pathway, therefore, shuttling the vector wouldn't mitigate the problem (Loeschcke and Thies., 2015; Zobel *et al*., 2015; Weimer *et al*., 2020). However, certain vectors offer the ability to maintain the library clones at low copy by eliminating the arabinose induction, thus reducing the toxicity and increasing the chances of clone survival, although it suffers the disadvantage of low expression (Zobel *et al*., 2015; Weimer *et al*., 2020). The consequence would be that the screening method might not be sensitive enough to detect the compound if it's concentration is low (Distaso *et al*., 2017; Weimer *et al*., 2020).

1.9.2. Culture-dependent approach for biosurfactant biodiscovery

A culture-dependent approach entails the isolation, screening, and extraction of surface-active compounds from culturable microorganisms (Reen *et al*., 2015). Prior to the era of metagenomics, culture-dependent techniques were employed as the principal method for biosurfactant discovery (Walter *et al*., 2010). However, it is well documented that culture-based approaches are not capable of accessing all that nature has to offer, termed the "Great Plate Count Anomaly" (Staley and Konopka, 1985; Stewart, 2012; Trindade *et al*., 2021). The biggest challenge hindering the full utilization of the culture-dependent approach is that a universal culture medium capable of culturing all microorganisms imaginable does not exist (Stewart, 2012). Biosurfactant producing strains normally exist in a mixed population composed of a multitude of different species and strains with different and unique growth requirements for each bacterium (Stewart, 2012; Gill *et al*., 2017). Those requirements become more difficult to meet for microorganisms from unique and harsh environments (Stewart, 2012; Martin *et al*., 2014). Another limitation is that even after managing to bring microorganisms into a culture, some pathways remain silent under laboratory conditions (Stewart, 2012). However, in biodiscovery, many professionals still advocate for the screening of natural products directly from culturable microorganisms, because most of the hit-to lead programs established for novel compound discoveries report that the chemical diversity offered by genomic libraries is not reflected in the number of quality hits found (Monciardini *et al*., 2014). There is still great value in culturing microorganisms especially for gaining knowledge on the biological and physicochemical aspects of biosurfactants produced and for learning about a microorganism's role in the environment, ecology, and nutrient cycle (Stewart, 2012; Gill *et al*., 2017; Trindade *et al*., 2021). Biosurfactant production and the physiology of the microorganism are interdependent, so culturing bacteria is critical for constructing and operating a stable and resilient high-performance system essential for industrial-scale production (Gill *et al*., 2017).

According to Trindade *et al*., (2021) moving away from culturing microorganisms will likely result in many microorganisms producing biosurfactants remaining undiscovered, complete abandonment of the culture-dependent approach is not feasible. Instead, improving cultural techniques and bioprospecting underrepresented phyla or strains could increase novelty (Stewart, 2012; Martin *et al*., 2014; Reen *et al*., 2015). With the intention of

maximizing the cultivatable fraction of microbial communities, there have been enormous advancements over the years in culture media and cultivation procedures that closely mimic the natural environment in terms of nutrient composition and concentration, pressure, oxygen gradient, pH, and more and these factors aid in the screening and isolation of microorganisms with bioactivities of interest (Martin *et al*., 2014). According to Reen *et al*., (2015) new chemical entities are more likely to be produced and discovered from microbes that are less abundant or not yet discovered. The main microorganisms mostly reported as biosurfactant producers are of the genera *Pseudomonas*, *Candida*, *Bacillus*, *Corynebacterium*, *Rhodococcus,* and *Acinetobacter* (**Figure 1-16**) (Eras-Munoz *et al*., 2022; Guo *et al*., 2022). The consequence of focusing mainly on these genera is the extraction of the same group of biosurfactants, which include rhamnolipids, sophorolipids, surfactin, and mannosylerythritol; with rhamnolipids being the main biosurfactant produced and reported in literature (**Figure 1-16**) (Eras-Munoz *et al*., 2022; Guo *et al*., 2022).

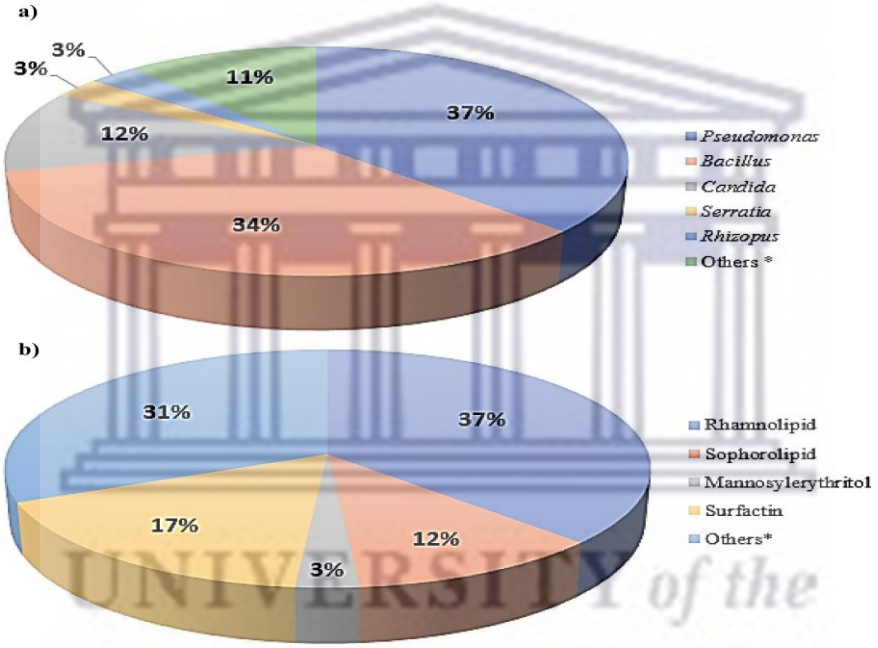


Figure 1-16: A pie chart representing the main (**a**) microorganism and the (**b**) biosurfactant they produced according to studies done between the years 2015 to 2021 reported by Eras-Munoz *et al*., 2022.

There is a higher probability for new biosurfactant discovery if a novel strain is investigated. This study focuses on the evaluation of biosurfactant production by the recently discovered novel *Planococcus* sp. CP5-4 strain isolated from the Cerebos crystallizer salt ponds (Silva-Castro *et al*. 2019). Although *Planococcus s*pecies are known biosurfactant producers, only a few strains and the biosurfactant that they produce have been described to date (Engelhardt *et al*., 2001; Labuzek *et al*., 2003; Kumar *et al*., 2007; Ebrahimipour *et al*., 2014; Yang *et al*., 2018; Waghmode *et al*., 2019). According to the genome taxonomy database (GTDB) classification, *Planococcus* sp. CP5-4 strain is a new species that belongs to the *Planococcus*_B genus (**Figure 1-17**) (Moyo *et al.*, 2021). The *Planococcus*_B genus encompasses strains such as *Planococcus_B* sp. 002833405, *Planococcus_B rifietoensis*, *Planococcus maitriensis* strain S1, *Planococcus plakortidis DSM 23997*, *Planococcus maritimus* DSM 17275, *Planococcus maritimus* MKU009 (Moyo *et al*., 2021), of which *P. maitriensis* has already been reported as a

biosurfactant producer (**Figure 1-17**) (Kumar *et al*., 2007). A new species potentially provides a source of novel biosurfactants and forms the impetus for the investigation presented in Chapter 3 and 4.

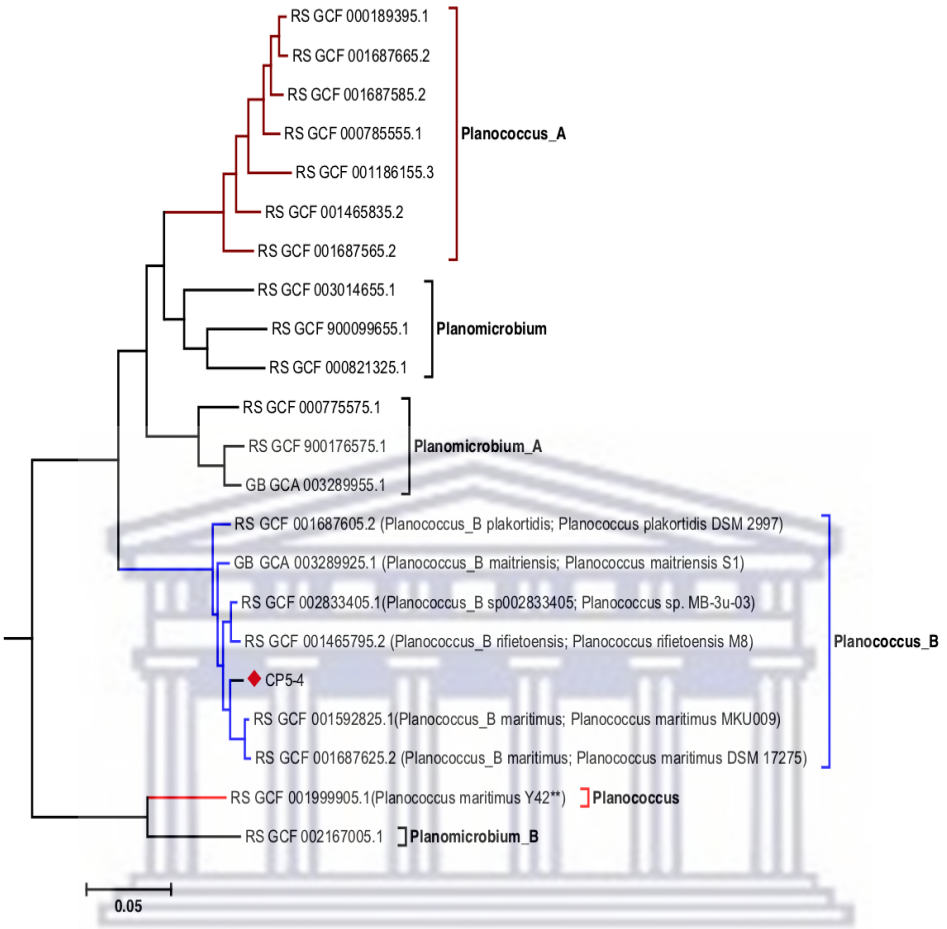


Figure 1-17: The NCBI genome taxonomy database (GTDB) classification of the *Planococcus* genera according to *Planococcus*, *Planococcus*_A, and *Planococcus*_B class. The *Planococcus* sp. CP5-4 strain belongs to the *Planococcus*_B genus (Moyo *et al*., 2022).

1.10. Extraction, and purification of putative biosurfactants

The recommended experimental procedure when investigating biosurfactant production by a strain should follow the following four steps, starting with the extraction of biosurfactant, followed by the detection of biosurfactant activity and the purification of crude product, and lastly structural characterization through techniques such as TLC, nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) (Satpute *et al*., 2010, Smyth *et al*., 2010b). Numerous experimental techniques and methods are adopted when investigating the diverse biosurfactant molecules and this includes a combination of various extraction, purification, detection, and analytical methods (Satpute *et al*., 2010). The ability to extract and purify biosurfactants when investigating the production of potential biosurfactants by a strain/clone is very important (Sanches *et al*., 2021; Smyth *et al*., 2010b). The objective of extracting is to obtain crude extracts free from the aqueous culture medium and other cellular constituents such as proteins, polysaccharides, and small molecules (Hisham *et al.,* 2019). Extraction procedures can range from conventional methods such as centrifugation, solvent extraction, acetone precipitation, ethanol precipitation, and

acid precipitation to more complex methods such as ion exchange, chromatography, adsorption, foam fractionation, lyophilization, ultrafiltration, isoelectric focusing, and dialysis (Satpute *et al.*, 2010; Smyth *et al*., 2010a; 2010b). The choice of the extraction procedure varies according to the growth process and physicochemical properties of the biosurfactants and, as such recovery will depend on solubility (water and or organic solvents are used), ionic charge (chromatography-based method), and location (cell-bound, extracellular, or intracellular production) (Smyth *et al*., 2010a; 2010b). A trial-and-error approach is normally adopted to find an extraction protocol for strains that do not have a standard method available and starting with conventional techniques is the best option when extracting, purifying, and optimizing the yield of biosurfactants produced by a strain. Even the same microorganism of different strains can have different extraction protocols, for example, in a study by Ebrahimipour *et al*. (2014) best extraction procedure for biosurfactant produced by *Planococcus* was alkaline precipitation of cell-free culture supernatant (CFCS) in acetone followed by oil dissolution of precipitate pellet in tetrachloride carbon, while the commonly reported procedure is acid precipitation of supernatant plus solvent extraction of the precipitants. This section will focus on the methods employed for biosurfactant extraction applied in this study.

1.10.1. Acid precipitation

The solubility of biosurfactants is influenced by pH, temperature, ionic strength, composition, and dielectric constant (Smyth *et al*., 2010a). These factors can be manipulated to achieve biosurfactant precipitation and thus biosurfactant extraction. The acid precipitation extraction method is widely used for biosurfactants extraction because it is simple and inexpensive, and the materials used are readily available (Satpute *et al*., 2010). Under acidic conditions below pH 6.5 macromolecular impurities and biosurfactants start to precipitate out, however, the ideal pH for protonation of biosurfactants compound is 2 (Liu *et al*., 2007). After the removal of biomass from the culture through centrifugation, hydrochloric acid is normally added to the CFCS to bring the pH down, the compounds become insoluble in water and precipitate out. Acidification with subsequent incubation at 4°C has been found to enhance product recovery (Kugler *et al*., 2015; Rangarajan and Clarke, 2016). The mixture is then centrifugated further thereafter, and the acidic pellet/precipitate of the insoluble biosurfactant left after centrifugation is collected and extracted further with various solvents such as chloroform, methanol, ethyl acetate, etc. Acid precipitation has been found to have a high recovery factor but low purity when reviewed in literature, therefore, further extraction tries to circumvent the low purity levels, thus, the acid precipitation plus solvent extraction combination method is normally adopted (Smyth *et al*., 2010a). Acid precipitation has been able to recover biosurfactants such as surfactin, lipopeptides, glycolipids, and others (Liu *et al*., 2007; Smyth *et al*., 2010a). However, a disadvantage of employing acid precipitation is that acidification can destroy the structure of the biosurfactant in question, for example, acid precipitation has been found to cause the esterification of lipopeptides (Smyth *et al*., 2010a; Kugler *et al*., 2015). Therefore, to minimize possible structural damages, prior to solvent extraction, the acidic pellet/precipitate can be resuspended in water and pH readjusted thus promoting the natural structure reformation of the biosurfactant being extracted.

1.10.2. Solvent extraction

A two-phase solvent extraction method is also a commonly used method for the extraction of biosurfactants. A variety of solvents can be employed when extracting, although polar solvents or solvent mixtures are normally used to achieve this (Smyth *et al*., 2010a; Kugler *et al*., 2015). The most common solvents used are the combination of chloroform and methanol or polar aprotic solvents, for example, ethanol, ethyl acetate, butanol methyl-tert-butyl, diethyl ether, n-pentene, n-hexane, dichloromethane, chloroform, acetic acid, and acetone (Kugler *et al*., 2015, Satpute *et al.*, 2010). The extraction method is based on the principle that hydrophobic moieties of biosurfactants are soluble in solvents, allowing the extraction and separation from the aqueous phase (Satpute *et al*., 2010). The degree of extraction or biosurfactant recovery depends on the solvent polarity and biosurfactant polarity. Chloroform has been used as a popular solvent, especially for extracting lipids with intermediate polarity and its combination with methanol has led the two combined solvents to be a universal extraction solvent used at diverse ratios (Santos *et al*., 2016). Employing different ratios of chloroform and methanol permits for the variation of the polarity of the extraction solvent to the targeted compounds. In a study by Shah *et al.* (2016) solvent extraction was found to be the best recovery technique against other techniques such as acid precipitation, with solvent extraction being able to recover 7.5 g/L of rhamnolipids from *P. aeruginosa* while acid precipitation was only able to recover 3.5 g/L. However, excessive use of solvents can result in high costs and environmental impact which is one of the disadvantages when using solvents for the extraction of biosurfactants (Satpute *et al.*, 2010). For example, chloroform is a toxic chloro-organic solvent that is harmful to human health and the environment (Santos *et al*., 2016). However, one of the advantages of using solvents is the fact that they are reusable to some extent. Characterization of the synthesized biosurfactant generally follows the extraction and purification process and a combination of analytical, chromatographic, and spectroscopic techniques are normally employed to achieve this (Sanches *et al*., 2021).

1.11. Study motivation and corresponding aims and objectives

Biosurfactants have a significant opportunity to replace chemical surfactants used in diverse markets. Compared to chemical surfactants, biosurfactants are biodegradable and they do not have lasting detrimental ecological effects. They also have vast structural diversity while chemically synthesized surfactants are only limited to few structures. Structural diversity is associated with unique physicochemical and biochemical properties which allows for specific industrial applications. However, the diversity of biosurfactants is not well represented in the market and only a few novel structures have been discovered in the last decade (Trindade *et al*., 2021). Therefore, investing in novel biosurfactant discovery has the potential to increase the number of new biosurfactants that can be offered to the market. The aim of this project is to discover novel biosurfactants through both the culture-independent approach, by employing functional metagenomic screening as a tool; and through a culture-dependent route, where the potential for biosurfactant production by the novel *Planococcus* sp. CP5-4 strain is investigated. Well-established activity screening methods; namely the atomized spray, drop-collapse test, microplate-grid analysis assay, and emulsification index measure, have been selected for the identification and characterization of the biosurfactant compounds produced.

1.11.1. Aims and Objectives

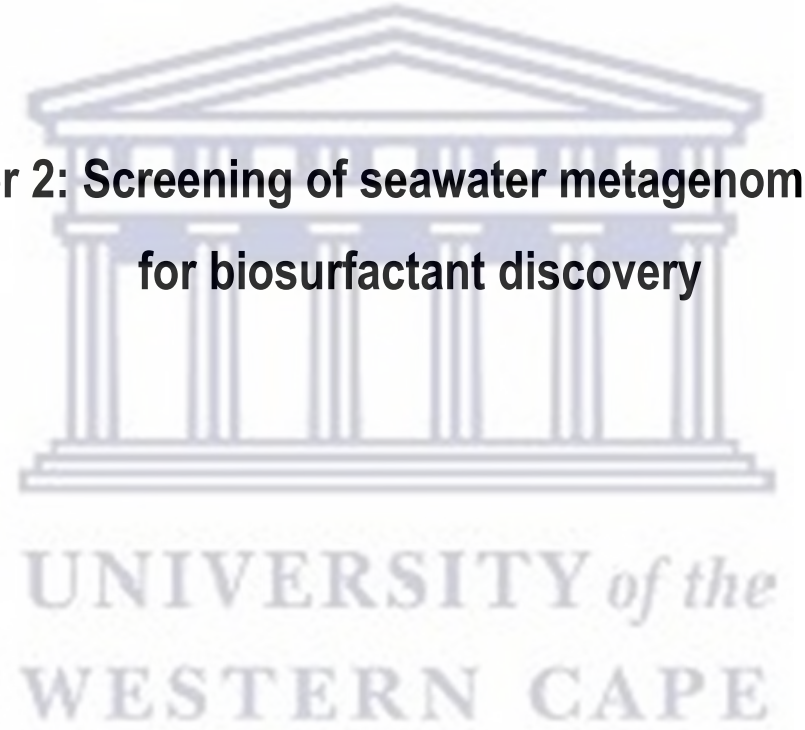
Due to the advantages and limitations described in the literature review for various biodiscovery approaches, the aim of this study was to screen for novel biosurfactants using both functional metagenomic screening and a culture-dependent approach. Presented first is the screening of a seawater metagenomic library in two different heterologous hosts; *E. coil* EPI300 to *P. putida* MBDI. The objectives for this part of the work were as follows**:**

- Determining the library titer to ascertain the number of viable clones present in the aliquoted seawater metagenomic library.
- Employment of the atomised spray method to screen for biosurfactant activity from the seawater metagenomic library through the parallel screening in *E. coil* EPI300 to *P. putida* MBDI.

The second part of the study focused on the biosurfactant production potential of the *Planococcus* sp. CP5-4 strain and to establish the association between the biosurfactant produced by the strain and the synthesis of carotenoids. As such, the objectives of this part of the study were as follows:

- Preliminary screening for biosurfactant activity produced by the *Planococcus* sp. CP5-4 strains.
- Determining the best method for the extraction and partial purification of the putative biosurfactant(s) produced by the *Planococcus* sp. CP5-4 strains.
- Performing preliminary characterization of the biosurfactant(s) produced by the *Planococcus* sp. CP5-4 strains.
- Assessing the contribution of carotenoids to the biosurfactant activity using various extraction, separation, and detection methods

Chapter 2: Screening of seawater metagenomic library for biosurfactant discovery



2.1. Introduction

Microorganisms producing biosurfactants have been identified from diverse environments such as oil fields, soil, and the ocean (Bodour *et al*., 2003; Satpute *et al.*, 2010). However, most of the microorganisms have been isolated from terrestrial sources specifically from hydrocarbon-contaminated environments (Balan *et al.*, 2017). Due to the high rediscovery rate and decreasing number of novel compounds discovered from terrestrial habitats, a shift has occurred over the last decade towards exploring the marine environments (Trindade *et al*., 2015). The marine environment makes up 70% of the Earth's surface and accounts for 80% of life on Earth, most of which has not been explored thus far (Kennedy *et al*., 2011). It is the largest aquatic ecosystem, it harbors enormous phylogenetic and metabolic diversity and has high abundance of microorganisms estimated at 3 x 10^{30}, therefore, it is not surprising that it is considered a good source for biodiscovery (Gutierrez *et al.*, 2019; Kennedy *et al*., 2011). The marine environment contains microorganisms with unique metabolic and physiological properties. According to chemoinformatic studies, 71% of marine natural products are not represented in terrestrial sources and 53% of the products have only been found once (Trindade *et al*., 2015). Harsh, fluctuating, and unique environmental conditions, namely extreme temperature, high pressure, high salinity, unstable pH, limited nutrient availability, and increased UV exposure characterize the marine environment (Bodour *et al*., 2003; Satpute *et al.*, 2010; Martin *et al.*, 2014; Jackson *et al*., 2015b). For example, temperatures ranges can get as low as -2°C and as high as 400°C in hydrothermal vents, the pressure can exceed 100 MPa and trenches depth are measured up to 11 000 m (Kennedy *et al*., 2011; Mullineaux *et al*., 2018; Dayanidhi *et al*., 2020). These conditions act as a selective pressure for the synthesis of unique bioactive compounds with potentially unique physiological and biochemical properties utilized by microorganisms to adapt to these types of environments, thus promoting high levels of structurally diverse compounds (Kennedy *et al*., 2011). The ongoing adaptation to the environment is therefore facilitated by the high frequency of gene transfer between the marine microbial communities which results in high genetic flexibility (Kennedy *et al*., 2011). Seawater alone is estimated to have a gene density of 10^6 per milliliter (Kennedy *et al*., 2011; Trindade *et al*., 2015). Given the assumption that there are an average of 3000 genes per single genome and 40% of those genes have catalytic activity, it is estimated that 1.2 x 10^9 reactions in a sample could be mediated by 3 x 10^9 genes which makes seawater a rich source for structurally diverse compounds to still be found (Kennedy *et al*., 2011).

Over 200 000 structurally diverse natural marine products have been discovered from ocean life forms such as sponges, fish species, sea-cucumbers, sea-hares, worms, bryozoan, corals, algae, aplysia, ascidians, and microorganisms (Martin *et al.*, 2014; Hu *et al*., 2015; Mullineaux *et al*., 2018; Dayanidhi *et al*., 2020). There are several examples found in literature of identified biocatalysts from the marine environment which include biosurfactants, dehalogenase, cellulase, baeyer-Villiger monooxygenase, glycoside, hydrolase, α- amilase, fumarase, esterase, laccase, antibiotics and more (Martin *et al.*, 2014; Hu *et al*., 2015; Mullineaux *et al*., 2018; Dayanidhi *et al*., 2020). From 2011 to 2012 there was a 10% increase in the amount of reported marine compounds because of the increased focus placed on the biodiscovery of natural products from marine microorganisms (Martin *et al.*, 2014). The marine environment has not been vigorously explored for microbial biosurfactants, yet it offers a

diverse group of microbes with numerous natural bioactive compounds of commercial importance, and therefore, serves as a good source for biosurfactant isolation and novel biodiscovery (Wolfgang and Rolf, 2010; Jackson *et al*., 2015b; Gutierrez *et al*., 2019; Waghmode *at el.*, 2020). Approximately 102 marine bacteria have been reported thus far as being able to produce biosurfactants and encompass 43 glycolipids and 59 non-glycolipids (20 lipopeptide, 5 surfactin, 12 glycolipoprotein/lipoprotein, and 22 other non-gycolipids) (Waghmode *et al.*, 2020). Despite such high potential for novel discoveries, one of the main reasons the marine environment has not been explored fully is the difficulties with culturing marine microorganisms. When compared to other environments a 10-fold decrease in culture isolation of seawater bacteria is recorded, therefore, to increase the probability of biodiscovery a culture-independent metagenomic strategy represents a feasible option, especially with its proven success in delivering chemically diverse structures (Trindade *et al*., 2015). In this chapter, a seawater metagenomic library that was previously constructed using the pCCERI fosmid and transformed into *E. coli* EPI300 (Gerda Du Plessis, unpublished), was conjugated into *P. putida* MBDI. The library in both hosts was screened for biosurfactant activity using the paraffin atomized spray method.

2.2. Material and Methods

The overview of the methodological approach employed in his chapter is as follows. Metagenomic DNA extracted from cells collected on a 0.45 µm PVDF filter 500 L open ocean seawater (Cape Town continental shelf), was size selected, randomly sheared, end repaired and ligated in the pCCERI vector. The library was transduced into *E. coli* EPI300 through lambda phage packaging (Gerda Du Plessis, Unpublished). Robotically picked colonies of this seawater library were cultured and conjugated from *E. coli* EPI300 to *P. putida* MBDI. The library in both hosts was screened for biosurfactant activity by the atomised paraffin droplet spray method.

2.2.1. Bacterial strains, and plasmids, used in this study

The bacterial strains and plasmids used in this study are listed in **Table 2-1** and **Table 2-2** Luria-Bertani broth [LB; 1% (w/v) NaCl, 0.5% (w/v) yeast extract, and1% (w/v) Tryptone] and Luria-Bertani agar [LA; LB with 1.5% (w/v) bacteriological agar] supplemented with 12.5 mg/L chloramphenicol and 50 mg/L kanamycin required for selection of the library in *E. coli* EPI300 cultured at 37°C unless stated otherwise (Williams *et al*., 2019). *P. putida* MBDI was cultured in LB broth and/or agar supplemented with 30 mg/L apramycin (Sigma) for the selection of pCCERI fosmid conjugates. M9 media (Appendix: **Table A 1** and **Table A 2**) containing 30 mg/L apramycin and 0.2% benzoate agar as the carbon source was used to culture *P. putida* MBDI exconjugants (Williams *et al*., 2019).

Table 2-1: Bacterial strains used in this study.

Bacterial Strain	Genotype	Source of reference
Escherichia coli EPI300	F^- mcrA Δ (mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ (ara, leu)7697 galU galK λ- rpsL nupG trfA tonA dhfr	Epicenter (Illumina)
Pseudomonas putida MBD1	*P. putida* KT2440 derivative; Kan^r; φC31 *attB* site+	Martinez *et al*., 2004

Table 2-2: Plasmids used in this study.

Plasmid	Description	Source or reference
pCC2FOS™	Commercial cloning vector, Cam^R	Epicenter (Illumina)
pCCERI	A derivative of pCC1FOS, Cam^R, φC31 integrase, *attP*.	Jackson *et al*., 2015a
pER1.3.50.2	No sequence available, derived from pRK2013 with *trfA* gene deleted	Jackson *et al*., 2015a

2.2.2. Determination of the viability of the seawater metagenomic library

To determine the number of viable clones, the metagenomic library (stored at -80°C) was thawed and 10^{-9} serial dilutions were made. An amount of 2 µL from each dilution was spread plated on LB agar with 12.5 mg/L chloramphenicol (Cam^R) and 0.02% L-arabinose. Four replicates of each dilution were plated and incubated at 30°C overnight. Individual colonies growing on the agar plates following incubation were counted, and the average number of colonies per dilution of up to 300 colonies growing on the plate was used to determine the titer of the library, which was calculated according to **Equation 2-1** (William *et al*., 2019).

Equation 2-1: The equation used to calculate the titer of a metagenomic library.

$$\text{Colony-forming unit (CFU/mL)} = \frac{(No.\ of\ colonies)(dilution\ factor)(1000\mu l.ml^{-1})}{volume\ of\ cells\ plated}$$

The number of colony-forming units was used to determine the volume of the library to be plated out on the Q-tray LB agar plates (245 x 245 mm^2) for colony picking using the Q-Pix2^{XT} automated colony picker (Molecular Devices, Queensway, UK).

2.2.3. Picking of *E. coli* clones by a Q-pix2XT automated colony picker

An amount of 750 µL of the 10^{-2} dilution of the seawater metagenomic library was dispensed on a sterile Q-tray containing LB agar supplemented with12.5 mg/L Cam^R and 0.02% L-arabinose. The library was evenly distributed on the agar by carefully adding 15-30 sterile glass beads with a diameter of 5 mm on the surface of the agar, closing the Q-tray lid, and shaking the beads until the surface of the agar had completely absorbed the inoculum (Williams, 2016). The beads were aseptically removed, and the inoculated Q-tray plates were incubated overnight at 37°C. After incubation, individual colonies growing on the Q-tray were transferred into 96-well microtiter plates, where each well contained 200 µL LB media with 12.5 mg/L Cam^R and 0.02% L-arabinose, using the Q-Pix2^{XT} automated colony picker. The inoculated microtiter plates were incubated overnight at 37°C with shaking at 120 rpm before being replicated and gridding onto a Q-tray LB agar plate with 12.5 mg/L Cam^R and 0.02% L-arabinose. The gridded clones were grown overnight at 37°C and screened for biosurfactant activity using the atomized paraffin droplet spray method. The overnight *E. coli* EIP300 clones in 96-well microtiter plates were also used for the conjugation of the seawater metagenomic library into *P. putida* MBDI.

2.2.4. Conjugation of the seawater metagenomic library from *E. coli*-EPI300 into *P. putida* MBDI

The seawater metagenomic library was conjugated from *E. coli* EPI300 to *P. putida* MBDI as described in the work done by Williams (2016). Concurrently to the overnight 96-well microtiter plates culturing of individual *E. coli* EPI300 picked clones, a fresh colony of *P. putida* MBDI was inoculated into LB media containing 50 mg/L kanamycin and incubated overnight at 30°C with shaking at 120 rpm to achieve conjugation. The overnight culture was diluted 20-fold with LB media containing 50 mg/L kanamycin and 10 mM $MgCl_2$. The diluted culture was incubated for 2 hours at 30°C with shaking at 120 rpm. At the end of the incubation period, the culture was heat-shocked at 42°C for 10 minutes. An amount of 200 µL of the culture was thereafter dispensed into a new set of 96-well microtiter plates, this was then followed by dispensing 10 µL culture of each *E. coli* EPI300 clone into the 96-well microtiter plate. The 96-well microtiter plate ended up with a mixture of both *E. coli* EPI300 into *P. putida* MBDI and this was incubated at 30°C overnight without shaking to facilitate conjugation. Following conjugation, *P. putida* MBDI conjugants were selected from the 96-well plate by gridding individual overnight cultures from each well onto Q-trays containing M9-0.2% benzoate (Appendix**: Table A 1** and **Table A 2**) agar, supplemented with 30 mg/L apramycin. The inoculated M9 agar plates were then incubated at 28°C until transconjugants were visible (after 2-3 days of incubation). Approximately 20 000 conjugants were counted, representing 80% efficiency. The *P. putida* MBDI exconjugants gridded on the M9 0.2% benzoate agar plates were replicated again using the Q-Pix2^{XT} automotive colony 96 pin replica stamps on a Q-tray containing LB agar with 30 mg/L apramycin and incubated at 30°C overnight. The 96-well master plates were stored at -80°C with 15% (v/v) sterile glycerol added.

2.2.5. Biosurfactant screening using the paraffin droplet (atomized) spray method

The replicated seawater metagenomics libraries in *P. putida* MBDI and *E. coli* EPI300 were each screened by spraying a mist of atomized paraffin oil as per Burch *et al*. (2010) instructions on the colonies growing on each gridded Q-tray plate. The paraffin oil was atomized using an airbrush Compressor Kit (AirCraft Pneumatic Systems, South Africa) generating between 15 and 20 bar of pressure. Biosurfactant production was confirmed by the formation of a halo around the colonies.

2.2.6. Restriction enzyme digestion for metagenomic library validation

To confirm the integrity and diversity of metagenomic inserts, 40 *E. coli* EPI300 library clones were randomly selected from the replicated Q-tray LB agar plate for restriction analysis, following instruction from Williams (2016). The clones were cultured in 5 mL LB media with 12.5 mg/L chloramphenicol and 0.02% L-arabinose as detailed in **section 2.2.1**. Five microliters from each overnight culture were used to inoculate separate 5 mL volume of fresh LB broth with 12.5 mg/L chloramphenicol and 0.02% L-arabinose, which were incubated at 37°C with shaking at 300 rpm for a period of 12–16 hours. Fosmid DNA was extracted using a modified version of the SDS-based DNA extraction method standardized by Zhou *et al*. (1996). The cells which were pelleted by centrifugation at 5000 x *g* for 10 min at 4°C. The pellets were incubated on ice for 2 min, thereafter, 0.8 mL of cell suspension solution (1M glucose, 1 M Tris-HCl, 0.5 M EDTA, pH 7.5) with 10 mg/mL RNase was added. The solution was mixed and vortexed until the pellet was homogenously resuspended. A volume of 0.8 mL lysis buffer solution (10 M NaOH, 10% SDS) was added to the mixture. The lysis buffer was mixed in by inverting the Eppendorf tube 4-6 times at

room temperature, after which 0.8 mL neutralization buffer (11.5 g glacial acetic acid, 29.28 g potassium acetate, 88.5 mL dH_2O) was added to the lysis reaction and mixed in by inverting the Eppendorf tubes a further 4-6 times before incubating on ice for 15 minutes. The lysis mixture was centrifuged at 11 500 x *g* for 10 min at 4°C. The supernatant was transferred to a fresh Eppendorf tube and fosmid DNA precipitation was carried out by adding 5 volumes of 95% ethanol to the supernatant and placing the Eppendorf tube at -20°C overnight. The fosmid DNA was pelleted by centrifuging the ice-cold mixture at 13 860 x *g* for 15 min at 4°C. After this, the supernatant was decanted from the pellet, which was left to air dry before being resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8). Agarose gel electrophoresis was employed to check the extracted fosmid DNA's quality as follows: 7 µL of DNA sample composed of 1 µL of 5x Qiagen GelPilot® DNA loading dye plus 6 µL fosmid DNA were loaded onto a 1% (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide, parallel to a 1 Kb DNA ladder (New England Biolab, NEB) and electrophoresed for 45 minutes at 90 volts in TAE buffer (Williams, 2016). The fosmid DNA on the gel was visualized using an ultraviolet (UV) transilluminator (Alpha imager 2000, alpha Inno tech, USA) at the end of electrophoresis. The yield of the extracted fosmid DNA was determined using the NanoDrop® ND-1000 Ultraviolet-visible spectroscopy UV-Vis (Thermo Scientific, Wilmington, DE, USA). Restriction digests were carried out in 50 µL containing 1 µg of fosmid DNA, 1 µL (10 U) HindIII (NEB), and 10% (v/v) 10X NEB buffer 2.1 and water. The reaction mix was incubated for 1 hour at 37°C. The restriction digest products were loaded as described above.

2.3. Results and discussion

2.3.1. Assessment of the seawater metagenomic library titer

Before commencing with metagenomic library screening, the library titer was determined to ascertain the number of viable clones present in the aliquoted seawater metagenomic library. The number of colony-forming units (CFU) in the seawater metagenomic library was calculated to be 203 300 CFU/mL. This figure was less than the expected 300 000 CFU/mL, which was previously reported before storage at -80°C (Gerda Du Plessis, unpublished). Approximately 30% of the clones in the original library were no longer viable, thus showing a significant reduction in clone recovery after months of storage at very low temperatures. The significant reduction in titer, might have resulted in a loss of clones with potential biosurfactant activity. Nevertheless, the titer was sufficient to continue with subsequent picking and screening.

2.3.2. Screening of the seawater metagenomic library for biosurfactant activity

A total of 25 000 clones (**Table 2-3**) in *E. coli* EPI300 were picked, cultured, and gridded on selective LB agar and screened for biosurfactant activity, resulting in zero hits being detected. Subsequently, the same clones were conjugated to *P. putida* MBDI and subjected to similar screening. An 80% conjugation efficiency was achieved resulting in 20 000 clones being screened in *P. putida* MBDI; however, no biosurfactant activity was detected. Conjugation efficiency is dependent on extrinsic and intrinsic factors, this might include both biotic and abiotic factors such as mating time, pH, temperature, carbon and metal concentration, donor-to-recipient ratio, cell density,

growth phase and cell physiological state (Alderliesten *et al.*, 2020; Prensky *et al*., 2020). Conjugation requirements that are not met result in the loss of clones that might potentially have the targeted activity.

Table 2-3: Biosurfactants discovered using function-based metagenomic screening.

Publication	Environmental sources	Hosts	Vector	Library size	Insert size	Clones screened	hits	Biosurfactant
Brady *et al*., 2004	Six different soils and one sediment	*E. coli* BL21(DE3)	pCSL12 and pCSLC2 cosmids	N/A	25-35 kb	N/A	11	N-acyl amino acid and palmitoyl putrescine
Thies *et al*., 2016	Abattoir wastewater metagenome	*E. coli* DH10b	pEBP18[30] plasmid	170 000	1-10 Kb	80 000	22	2 Long-chain N-acyl tyrosine
Williams *et al*., 2019	Lake sediment soil contaminated with hydrocarbons	*E. coli* EPI300 and *P. putida* MBDI	pCCERI fosmid	84 000	31 Kb	20 000	1	Ornithine lipid(s) in *P. putida* and Lyso-ornithine lipid(s) (LOL) in *E. coli*
Araujo *et al*., 2020	Soil sample from a riverbank	*E. coli* DH10b	pBC phagemid	N/A	1.4 Kb	1240	1	Metagenomic biosurfactant protein 1 (MBSP1)
This study	Seawater	*E. coli* EPI300 and *P. putida* MBDI	pCCERI fosmid	203 300	32.2 Kb	25 000	0	N/A

There are numerous key factors intricately linked to each other that might influence the probability of finding a hit during biosurfactant discovery when using function-based metagenomic screening (Maruthamuntu *et al*., 2016; Distaso *et al*., 2017). These factors include the library size, the number of clones screened, abundance of the targeted gene, DNA insert size, the source of the environment selected and its captured DNA diversity, selected host-vector system, the detection method used itself, the efficiency of heterologous gene expression in a selected host, etc. and the relevant factors will be expanded on (Penesyan *et al*., 2013; Culligan *et al.*, 2014; Maruthamuntu *et al*., 2016). The incident rate of discovery is greatly influenced by the activity levels of the biosurfactant and the abundance of the target gene in the environmental DNA used to construct the metagenomic library (Gabor *et al*., 2004; Ferrer *et al*., 2016). Some activities are more abundant than others, for example, the incident rate of the industrial relevant enzymes such as esterases/lipases, proteases, phosphatases, amylases, and glycosidases can range from 1 hit for every 11 clones screened (1:11) to 1 hit for every 193 200 clones screened (1:193 200) (Ferrer *et al*., 2016). Acylase enzymes were found at a rate of 1:333, esterase/lipase at 1:17 320, while glycosidases had 1 hit per 31 190 clones in a report by Ferrer *et al*. (2016). The rate depends on the occurrence of the enzyme class of interest per genome (Lam *et al*., 2015; Ferrer *et al*., 2016). In general, the studies reporting biosurfactant screening have low hit rates indicative of low gene abundance (Brady *et al.*, 2004; Trindade *et al*., 2015; Williams *et al.*, 2019). Brady and team (2004) discovered long-chain acyl-amino acids at an incident rate of 1 positive clone within 10 000 to 20 000 clones after screening 7 different metagenomic libraries constructed from different environments (**Table 2-3**). In a study by Williams and colleagues (2019) 20 000 clones (**Table 2-3**) were screened before attaining a positive biosurfactant hit, falling within the hit rate found by Brady *et al*. (2004). The number of

clones screened in this study was 25 000 and 20 000 in *E. coli* EPI300 and *P. putida* MBDI respectively (**Table 2-3**), which was an acceptable coverage for a probable hit in relation to the biosurfactant hit rate found in literature.

The insert size of DNA is one of the factors that influence the incidence of biodiscovery in functional screening (Distaso *et al*., 2017). One positive hit per 1000 clones is expected for compounds of a single gene enzyme (Williams and Trindade, 2017). However, the hit rate can increase to 1 per 10 000 screened clones for larger insert size, as the probability of expression significantly decreases as the size or number of genes in the pathway increases (Penesyan *et al*., 2013; Williams *et al*., 2019). Biosurfactants and most natural compounds are often coded by a large number of genes and depending on vector and the promoter included on the vector the native promoter might not be recognised if the DNA insert is large and further away from vector promoters. In a study done by Thies *et al.* (2016) a total of 22 positive clones were detected after screening 80 000 clones, of which 2 showed positive biosurfactant activity (**Table 2-3**) showing a hit rate of 1 per 3636 clones screened for a 1-10 kb insert size library. Araujo and team (2020) discovered a biosurfactant protein by screening only 1240 clones of a 1.4 kb insert size library (**Table 2-3**). However, a hit rate of 1:20 000 clones for a metagenomic library with a 31 kb insert size was found by Williams and colleagues (2019), demonstrating the influence insert size has on the hit rate (**Table 2-3**). The insert size in this study was 32.2 kb (**Table 2-3**), therefore a failure to obtain a positive hit after screening over 20 000 clones in each host led to the investigation of the diversity of the library as one of the factors that might be responsible for not finding a hit addressed in the next section.

2.3.3. Assessment of the diversity of the seawater metagenomic library through restriction enzyme digestion.

Restriction enzyme digestion was performed on fosmid DNA extracted from 40 randomly selected clones to assess the diversity of the seawater metagenomic library. A variety of restriction patterns were observed demonstrating some degree of molecular diversity of the seawater metagenomic library, for example, in lanes C02 and C06 the fosmid show completely different fingerprints (**Figure 2-1**) suggesting they contain different inserts. However, fosmids in lanes C05 and C06 have similar fingerprints or shared fragments (**Figure 2-1**), suggesting that the library has low diversity, therefore, a low probability of attaining a positive hit for biosurfactant biosynthetic genes. Furthermore, if the activity is associated with rare or low abundance phylotypes in a metagenomic library, a massive DNA library of about 10^6 might be required to adequately represent hundreds of individual genomes found in complex and symbiotic communities (Trindade *et al*., 2015). The low diversity observed for the metagenomic library may have been caused by low coverage as a result of multiple clones of the same clone being picked, the loss of clones reported after clone recovery, or other factors such as the environment selected and how well the diversity of the selected environment was captured. The quality of genome extraction and the cloning processes are responsible for capturing the true diversity of the environment (Kowalchuk *et al*., 2007; Jackson *et al*., 2015a; Distaso *et al*., 2017).

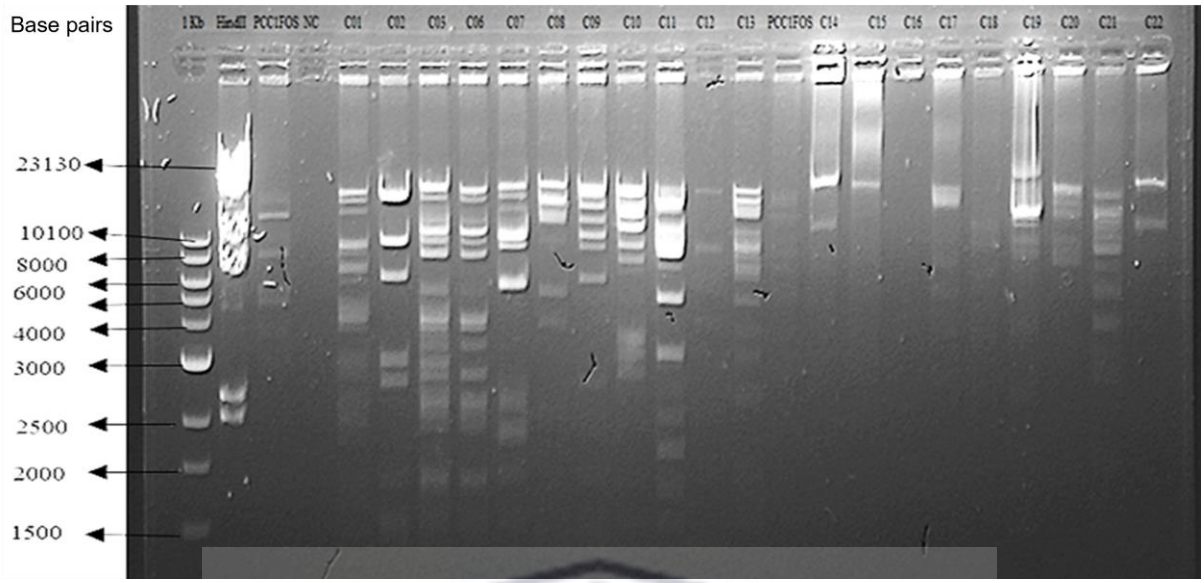


Figure 2-1: A gel representing 40 randomly selected clones used to investigate the diversity of the seawater metagenomic library (**C1**-**C22**). The fosmid DNA extracted from the library clones was digested using HindIII restriction enzyme and analyzed using electrophoresis through a 1% (w/v) agarose gel. The variety in the restriction patterns represents the difference in DNA used to construct the library, while similarities represent a lack of DNA diversity. Lane 1; 1 kb marker, lane 2; λ HindIII DNA marker, and lane 3; positive control pCC1Fos.

Although the metagenomic library screened in this study shared similarities to the one constructed by Williams *et al*. (2019) with respect to the host, vector, approximate insert size, and parallel host function-based screening the main difference was the source of the environmental DNA used to construct these libraries. Gene diversity can be increased by 80% by varying the extraction factors such as sample selection, pre-treatments, and DNA extraction and processing (Lam *et al*., 2015; Ngara and Zhang, 2018). Environmental sample or source influences the diversity captured in a metagenomic library, with some environments being more enriched with bacteria with the targeted protein than others. For example, frequency of clones varies from 1:667 to 1:15 000 for clones with carboxyl-esterase activity when screened under the same conditions, however, from different deep-sea habitats (Ferrer *et al*., 2016). Therefore, other marine habitats might have resulted in better diversity than the selected seawater environment. Studies suggest that marine communities associated with filter-feeding invertebrates and oil pollution are remarkable sources for biosurfactant identification (Kubicki *et al*., 2019). For example, all Demospongiae class sponges within the phylum Porifera normally exceed the bacterial concentration of seawater by two-three orders of magnitude. Therefore, the sponge would be a better source for bioactive secondary metabolites (Hentschel, 2004). Hence, sampling that specific marine environment would have possibly resulted in a much more diverse metagenomic library, while still aligning with the choice of the microbial community chosen for this study that appeals to the exploration of novel and structurally diverse biosurfactants from the underrepresented marine environment.

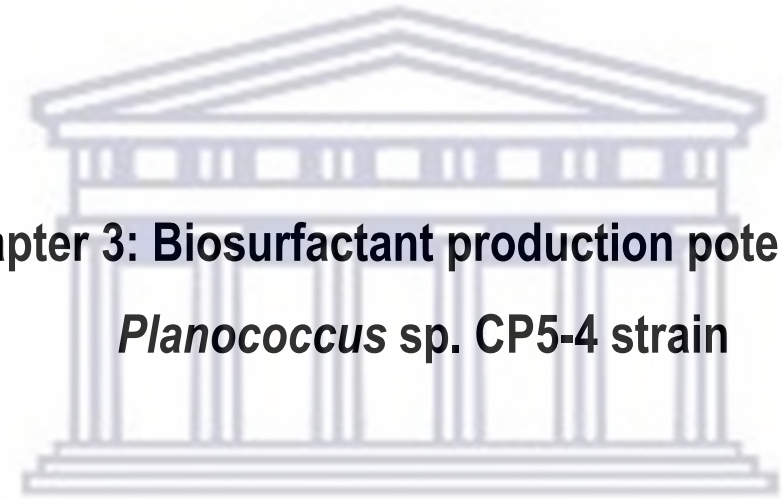
However, specific enrichment can be used to promote the targeted activity from any selected environment (Hentschel, 2004; Maruthamuntu *et al*., 2016). Enrichment entails providing growth conditions mimicking the

application setting that are very favorable for the targeted organism and unfavorable for the competing microorganisms (Walter *et al*., 2010). For the screening of biosurfactant producing microbes, hydrophobic compounds are often used as the sole carbon source in enrichment cultures. The eDNA used for the metagenomic library constructed by Williams *et al*., (2019) is from hydrocarbon contaminated environment, and by Thies *et al,* (2016) is from an abattoir wastewater sample (**Table 2-3**), therefore, both environments favor biosurfactant producing microorganisms, thus an increased chance of the incident of biosurfactant discovery (Ekkers *et al*., 2012). However, the side effect of ecological enhancement is the reduction of diversity through culturing biases. Culturing biases defeat the use of a culture-independent approach with intentions of inclusion and true representation of all genes of the selected community (Ekkers *et al*., 2012). Another disadvantage of enrichment is proliferation of the organisms that depend on the growth or activity of the target microbes, resulting in a false enrichment, and consequently in a reduction of the targeted activity (Ekkers *et al*., 2012). However, this issue can be minimized by refinement of the selective criteria applied (Ekkers *et al*., 2012).

2.4. Conclusion

This study used parallel function-based metagenomic screening to screen a seawater metagenomic library for biosurfactant activity. Prior to screening for biosurfactant activity the library titer was determined. The number of colony-forming units (CFU) in the library was calculated to be 203 300 CFU/mL and this was enough to proceed with screening. Through the atomised spray method, the clones of the seawater metagenomic library were screened for biosurfactant activity in *E. coil* EPI300 first. The clones were thereafter conjugated to *P. putida* MBDI, in which a 80% conjugation efficiency was achieved, which allowed for parallel function-based screening. An automated colony picker assisted in high throughput conjugation and library screening using the atomized spray method. After screening 25 000 and 20 000 clones in *E. coli* EPI300 and *P. putida* MBDI, respectively, there was no biosurfactant activity detected. According to the review made in this chapter the number of clones screened falls under an acceptable coverage for getting a biosurfactant hit (Brady *et al*., 2004; Williams *et al*., 2019). Given the lack of any positive hits (**Table 2-3**), the duplication of the insert in a small subset of randomly selected clones (**Figure 2-1**), and the considerable uncertainties for heterologous expression, it was deemed too risky to continue screening this library, considering the substantial resources used to conduct the parallel functional screening. Even so the marine environment is a promising source for novel biosurfactant discovery, and a culture-independent approach is the best way to access the vast diversity harboured in the marine environment as fermentation of marine microorganisms remains a difficult task (Lui *et al*., 2020). Metagenomic library screening through a function-based method possesses great potential for novel biosurfactant discovery, a potential that cannot be overlooked, and the method is expected to still result in the discovery of novel biosurfactants (Kowalchuk *et al*., 2007). However, as stated by Kowalchuk and colleagues (2007) the screening for biosurfactant activity from a metagenomic library is like finding a needle in the metagenome haystack, there is still a lot of research to be done on this topic and more to be discovered.

Chapter 3: Biosurfactant production potential of *Planococcus* sp. CP5-4 strain



3.1. Introduction

Planococcus are halophilic, psychrotolerant, non-motile, ~1.4 µm cocci (short) shaped, Gram-positive bacteria mainly reported for the synthesis of carotenoid pigments (Waghmode *et al*., 2020; Moyo *et al*., 2022). However, likely due to their halophilic nature they are also known to produce diverse secondary metabolites with diverse activity (Ganapathy *et al*., 2016; Waghmode *et al*., 2019; 2020). As elaborated in Chapter 1 this genus could also represent a good source of novel biosurfactants. In a previous study in the IMBM laboratory (unpublished at the time this project was initiated) the *Planococcus* sp. CP5-4 strain, and two mutants derived from this strain were observed to produce different amounts and texture of foam during shake flask culturing. Foam formation during culturing is one of the phenotypic characteristics of biosurfactant production by a bacterium (Chen *et al*., 2006; Hamzah *et al.,* 2020). The nature of the mutations provided some evidence to suggest that the biosurfactant could be a carotenoid, as has been suggested for other *Planococcus* species namely *Planococcus* sp. *DI* and *Planococcus maritimus* SAMP MCC 3013 (Ebrahimipour *et al*., 2014; Waghmode *et al*., 2019). In this Chapter we investigated *Planococcus* sp. CP5-4's potential as a biosurfactant producer and in Chapter 4 we investigate whether carotenoid synthesis was associated with biosurfactant activity (Moyo *et al*., 2022). As a novel species there was a high probability for new biosurfactant discovery from investigating the novel *Planococcus* sp. CP5-4 strain and is what gave the impetus for this study.

3.2. Materials and methods

3.2.1. Culturing the *Planococcus* sp. CP5-4 strains

The *Planococcus* sp. CP5-4 strain isolated from the Cerebos crystallizer salt ponds in Velddrif, Western Cape, South Africa (S 32°47'10,632, E 18°10'9,499) (Silva-Castro *et al*. 2019). Culturing of the *Planococcus* sp. CP5-4 strains was conducted as described by Silva-Castro *et al*., (2019). Briefly, the strains were revived through streaking onto TSB-salt-sorbitol agar composed of tryptic soy broth (TSB,17 g/L pancreatic digests of casein; 3 g/L enzymatic digests of soya bean; 5 g/L sodium chloride (NaCl); 2.5 g/L dipotassium hydrogen phosphate; 2.5 g/L glucose) supplemented with an additional 5% NaCl and 0.5 M sorbitol (TSB-salt-sorbitol) and with agar (14 g/L agar) added, followed by incubation at 28°C for 72 hours. For subsequent experiments, the *Planococcus* sp. CP5-4 wild type strain and the two mutant strains (yellow and unpigmented phenotypes) (**Figure 3-1**), produced through random chemical mutagenesis of the wild type orange strain using methyl methane sulfonate (MMS) by Moyo *et al*. (2022) were cultured by picking a colony from the agar plate and aseptically inoculating it to 25 mL of TSB-salt-sorbitol broth contained in a 100 mL Erlenmeyer flask. The inoculated broth was incubated at 28°C with shaking at 150 rpm for three days and served as pre-inoculum. The production of biosurfactant from each of the *Planococcus* sp. CP5-4 variants was carried out by inoculating 10% (v/v) of the pre-inoculum culture into fresh TSB-salt-sorbitol broth contained in baffled flasks. The inoculated cultures were then incubated at 28°C for 7 days with shaking at 150 rpm. The foaming properties were noted, where the difference in foam height and foam stability was measured and observed respectively. The head of foam was measured using a ruler from the base of the head of foam, while foam stability was noted by comparing the size and how compact the bubbles were between the strains after leaving the culture to stand for 15 minutes after shaking.

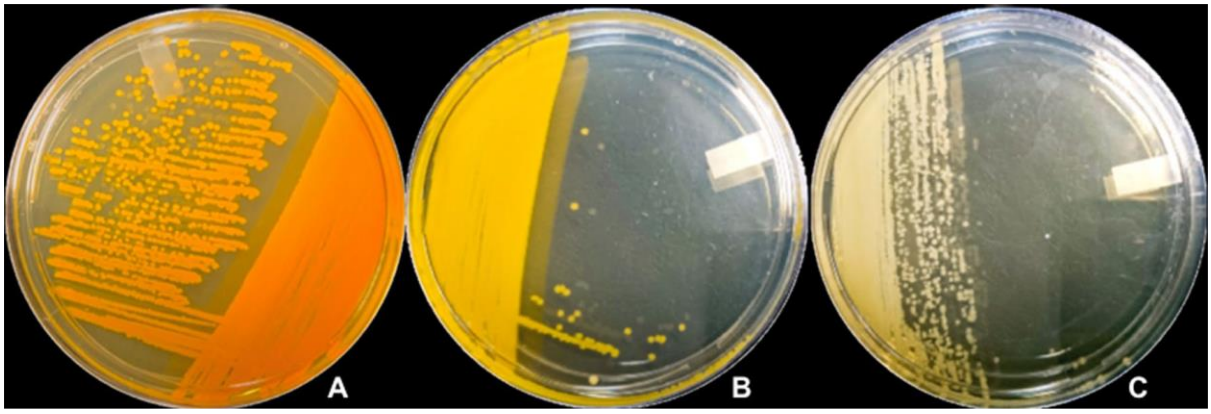


Figure 3-1: *Planococcus* sp. CP5-4 (**A**) wild type strain, and two mutants (**B**) yellow and (**C**) unpigmented phenotypes produced through random chemical mutagenesis of the wild type strain using methyl methane sulfonate (MMS) by Moyo *et al*. (2022).

3.2.2. Acid precipitation extraction method

At the end of the 7-day incubation period, the cultures were centrifuged for 20 minutes at 4629 x *g* in a Beckman Coulter Avanti J26 XPI set at a temperature of 4°C. The Cell-free culture supernatant (CFCS) was then decanted into a new tube and used for biosurfactant extraction. As a control, uninoculated TSB-salt-sorbitol broth was also subjected to the biosurfactant extraction method described. Acid precipitation through the dropwise addition of 32% concentrated HCl to the CFCS was also used for the extraction of biosurfactants as per Smyth *et al*. (2010a). Hydrochloric acid was added to the CFCS until it reached a pH of approximately 2.0. A 6 N sodium hydroxide (NaOH) solution was used to adjust the pH as needed during the extraction process and the acidified CFCS stored at 4°C overnight to promote the precipitation of biosurfactant(s). The precipitate that formed in the chilled acidified CFCS was collected by centrifugation at 1300 x *g* for 30 minutes in a refrigerated Beckman Coulter Avanti J-26 XPI centrifuge set at 4°C. The same precipitate weight was maintained across the variants. The precipitate was redissolved in deionized water and the pH of the acid precipitate in deionized water was adjusted to 7.5 using 2 N NaOH to aid the solubilization of the precipitate. To ensure complete solubilization, the pH adjusted mixture was placed on an orbital shaker set at 60 rpm overnight. All the strains were treated the same unless stated otherwise.

3.2.3. Preliminary screening: Atomized spray test

The atomized spray method by Burch *et al*., (2010) was used to screen for biosurfactant activity from the crude extracts. Briefly, a fine mist of paraffin was sprayed over 10 µL of the dried extract on a TSB agar plate using an Airbrush Compressor Kit (Aircraft Pneumatic Systems, South Africa) producing between 15 and 20 bar of pressure. Biosurfactant activity was viewed indirectly away from the light to detect the appearance of droplet halo formation around the sample being tested and the results were scored against each other, distilled water and 10% w/v SDS synthetic surfactant. To score the activity of the putative biosurfactant tested, the results obtained were compared to the activity of sterile distilled water as the negative control (B) (-) and 10% w/v SDS in distilled water as a positive control (C) (+++). Scoring of (-) to (+++) was given by comparing test samples (Z) to the negative control (B (-))

and the positive control (C (+++)). A "-" score was given if Z ≤ B whereas a "+++" score was given if Z ≥ C, a "+" score was given if (Z>B)≤(C-Z)/2, and a "++" score if (Z>B)>((C-Z)/2)<C.

3.2.4. Physicochemical characterization of the biosurfactant(s) extracts from *Planococcus* sp. CP5-4 strain

Emulsion capacity, Molisch's staining, Cetyltrimethylammonium bromide (CTAB)/ methylene blue agar were used for preliminary physicochemical characterization of the biosurfactant extracts from *Planococcus* sp. CP5-4 strains are as follows:

3.2.4.1. Emulsion capacity of the biosurfactants

The emulsification assay was carried out according to Cooper and Goldenberg, (1987) by mixing through vortexing at high speed two immiscible systems at a ratio of 1:1 (v/v) for 2 minutes. After mixing, the samples were left to stand for more than 24 hours. The principle is that unstable emulsions separated into two separate phases while the biosurfactants-containing samples with emulsification capabilities stabilize the emulsion. Each result is a repeat of 3 experiments unless stated otherwise. The ability of samples to stabilize the emulsion of the two-phased system was calculated using the equation (Sourav *et al.*, 2017) below:

$$EC24 = \frac{Height\ of\ emulsification}{total\ height\ of\ the\ two\ phased\ mixture\ after\ 24\ hrs}\ x\ 100$$

An emulsion capacity (EC_{24}) test was performed by mixing hydrocarbon paraffin with cell-free culture or with biosurfactant(s) extract of the three *Planococcus* sp. CP5-4 strains. The biosurfactant(s) extract used were extracted from 200 mL, 2 L, and 3 L cultures using acid precipitation following protocol from **section 4.2.2.2**. The same weight of precipitate obtained for each strain was thereafter resuspended in 20 mL Milli-Q water and the pH was adjusted to pH 7.5 and then tested for emulsification. The emulsion capacity of the wild type orange extract, extracted through acid precipitation from 3 L CFCS culture was tested in the same manner with engine lubricating oil (motor oil; Sasol multigrade F, SAE 20W-50 (Society of automotive engineers)). The ability of the sample to stabilize the emulsion of the two-phase system was calculated and the stability was monitored for over 10 days.

3.2.4.2. CTAB-methylene blue assay for biosurfactants

The CTAB-methylene blue assay was used to detect extracellular glycolipids or other anionic biosurfactants according to Siegmund and Wagner (1991). The screening was carried out using a well diffusion assay on TSB agar supplemented with 0.5 mg/mL CTAB and 0.2 mg/mL methylene blue (Appendix: **Table A 3**). Equidistant wells were created in the agar and inoculated with 30 µL of the putative biosurfactant(s) extracts (acid precipitate extracts in milli-Q water (pH 7.5)) were tested. The agar plates were then incubated at 28°C for over 3 days. SDS (10% w/v) was used as a positive control sample. Positive detection was revealed by the formation of a blue halo surrounding the well.

3.3. Results and discussion

3.3.1. Preliminary screening for biosurfactant activity from *Planococcus* sp. CP5-4 strains: Head of foam and emulsion capacity

Interest in *Planococcus* sp. CP5-4 strain as a potential biosurfactant producer was conceived after observing differences in the foam production by the two pigmented phenotypes (wild type and yellow strain) versus the unpigmented mutant when cultured (**Figure 3-2**). The foaming properties noted were the difference in foam height, stability, and feature/texture. The wild type orange *Planococcus* sp. CP5-4 strain had the highest head of foam measure at 110 mm, which was stable for over 15 minutes, and had a foam feature characterized by thin compact bubbles (**Figure 3-2**). The yellow mutant *Planococcus* sp. CP5-4 variant had a slightly lower head of foam height that was measured at 88 mm, it was also stable for over 15 minutes, and its foam had a less compact slightly bigger bubble size than that of the wild type *Planococcus* sp. CP5-4 strain (**Figure 3-2**). The unpigmented mutant *Planococcus* sp. CP5-4 variant's foam head was measured at 35 mm in height, it had the biggest bubble sizes compared to the pigmented variants and the foam started to collapse within the first 15 minutes after stopping culture aeration (**Figure 3-2**). The uninoculated TSB growth media had no foam 15 minutes after shaking the flask (**Figure 3-2**).

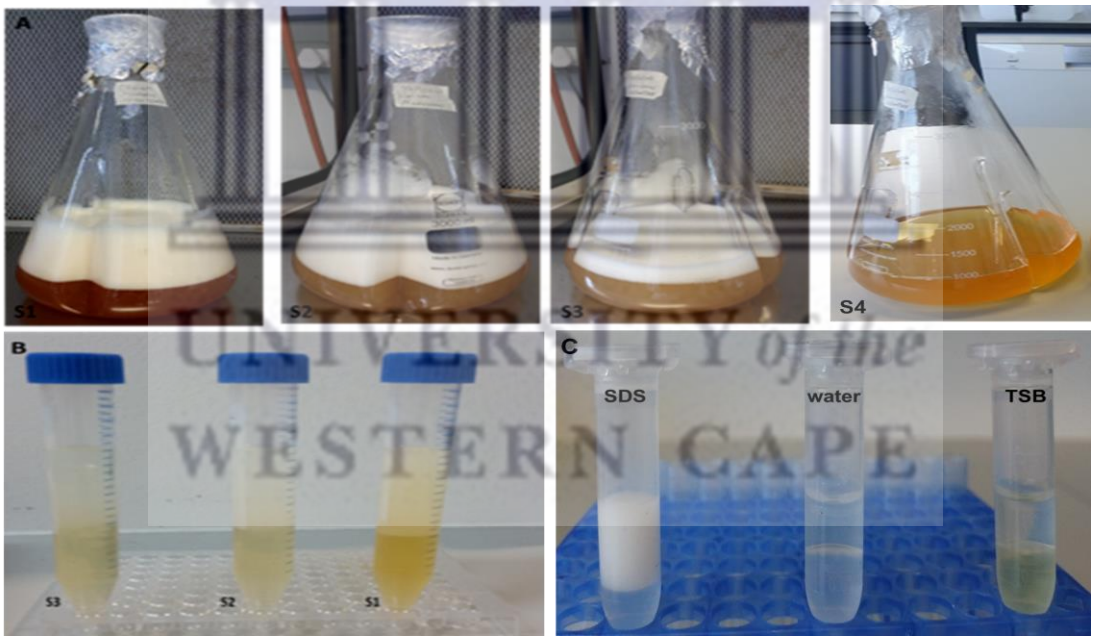


Figure 3-2: **A:** Image illustrating the head of foam of the three strains of *Planococcus* sp. CP5-4 cultured in a baffled flask on a shaker at 150 rpm for 7 days. **B:** Emulsification of paraffin and cell-free culture supernatant of the three strains mixed at 1:1 (v/v) ratio, captured 24 hours after emulsification. **(S1)** wild type orange, **(S2)** yellow mutant, **(S3)** unpigmented mutant *Planococcus* sp. CP5-4 strains, and **(S4)** uninoculated TSB. **C:** Controls; SDS (10% w/v), water, and TSB growth media.

The wild type and yellow mutant *Planococcus* sp. CP5-4 strain had an emulsion capacity (EC_{24}) of 50% ± 0.15 (**Figure 3-2**) and the unpigmented mutant *Planococcus* sp. CP5-4 strain showed a lower EC_{24} at 32.2% ± 0.17. The emulsion was characterized by a hard plug formation. The wild type strain (S3), had the hardest plug, followed

by the yellow mutant with the unpigmented strain displaying a soft-like emulsion out of the three strains (**Figure 3-2**). The positive control SDS 10% (w/v) had an EC_{24} measured at 63.3%, and the negative control samples; Milli-Q water and TSB growth media were not able to form an emulsion (**Figure 3-2**). Expression of certain metabolic pathways can impact bacterial growth substantially, and biosurfactant production is growth dependent for many strains, however, in the Moyo *et al*. (2022) study the mutagenesis did not appear to affect the growth of mutant strains compared with the wild type. Therefore, the difference in cell growth was eliminated as a contributing factor for the observed differences.

3.3.2. Preliminary physicochemical characterization: Head of foam and emulsion capacity of the biosurfactant crude extracts, extracted from the *Planococcus* sp. CP5-4 strains

The extracted crude biosurfactant(s) precipitant was resuspended in water, neutralized, and the flask shaken to determine foam formation ability after extraction (**Figure 3-3**). The wild type *Planococcus* sp. CP5-4 strain had the highest head of foam measured at 30 mm, followed by the yellow mutant *Planococcus* sp. CP5-4 variant with the head of foam measured at 26 mm and the unpigmented mutant *Planococcus* sp. CP5-4 strain had the least head of foam measured at 20 mm in height (**Figure 3-3**). The foaming properties including stability and feature/texture maintained the same patterns observed before extraction, therefore, acid precipitation was able to extract the surface active compounds.

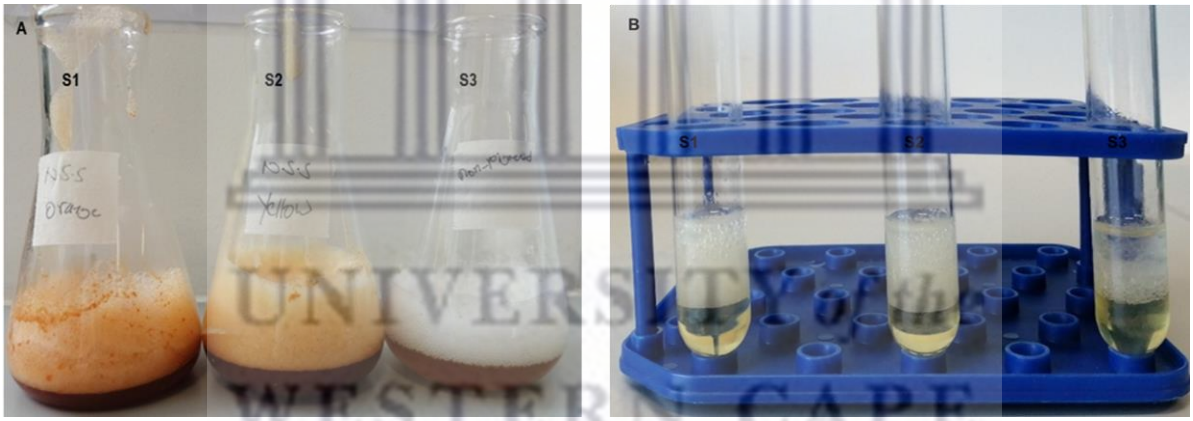


Figure 3-3: Assessing physicochemical properties of extracts from the wild type orange (**S1**), yellow mutant (**S2**), and unpigmented mutant (**S3**) strains of *Planococcus* sp. CP5-4, extracted by acid precipitation method. **A**: Crude extract dissolved in water (pH 7.5), the image was captured 15 minutes after shaking **B**; Emulsification of paraffin and biosurfactant extracts from 200 mL culture mixed at 1:1 (v/v) ratio.

The emulsion capacity (EC_{24}) of the biosurfactant extracted from a 200 mL culture of the wild type orange, yellow, and unpigmented mutants were measured at 60.0% ± 0.94, 57.3% ± 1.90, and 00.0% (was not stable) respectively (**Figure 3-3:B; Table 3-1**). The increase in culturing volume resulted in a higher emulsion capacity percentage, for example the unpigmented mutant strain' EC_{24} at 200 mL was 0.00 ± 0.00%, however, it increased significantly to 52.0 ± 0.81% after culturing at 1 L (**Table 3-1**) (Appendix: Error! Reference source not found.**Figure D 2: Figure D 3**). The emulsion capacity for all biosurfactant extracts extracted from a 1 L culture increased when compared to that of the 1 L cell-free culture from the preliminary screening result. This is due to a concentration increase after

extraction and resuspension at a lower volume, therefore, confirming the extracellular production of biosurfactant(s) by the strain and the extraction abilities of the extraction method used.

Table 3-1: Emulsion capacity (EC_{24}%) results for biosurfactant extracted from different volume cultures of wild type orange, yellow mutant, and unpigmented mutant *Planococcus* sp. CP5-4 strains.

Strain	200 mL	1 L	3 L
Wild type orange	60.0 ± 0.94	63.7 ± 0.94	80.3 ± 3.70
Yellow mutant	57.3 ± 1.90	63.7 ± 0.47	68.0 ± 1.40
Unpigmented mutant	0.00 ± 0.00	52.0 ± 0.81	57.0 ± 0.47

The emulsification activity for the wild type *Planococcus* sp. CP5-4 strain was tested further with motor oil hydrocarbon and successfully formed an emulsion of 92.30% (EC_{24}), which was stable for more than 10 days (**Figure 3-4**). *Planococcus* sp. isolated by Ebrahimipour *et al*. (2014) produced a biosurfactant that had an emulsion capacity of 77% with crude oil. In a study done by Waghmode *et al*. (2019) the biosurfactant synthesized by *P. maritimus* SAMP MCC 3013 formed emulsification with kerosene, diesel, n-hexadecane, toluene, coconut oil, and with castor oil immiscible compounds at 68%, 78%, 64%, 56%, and 53% respectively. The emulsification is influenced by the hydrocarbon source to be emulsified as much as it is determined by the biosurfactant used and density of two immiscible phases is also one of the factors that determine the extent of the stability of the emulsion (Maphosa and Jideanu, 2018). The emulsion represented by the *Planococcus* sp. CP5-4 strain showed evidence of type II, the water in oil or W/O microemulsion.

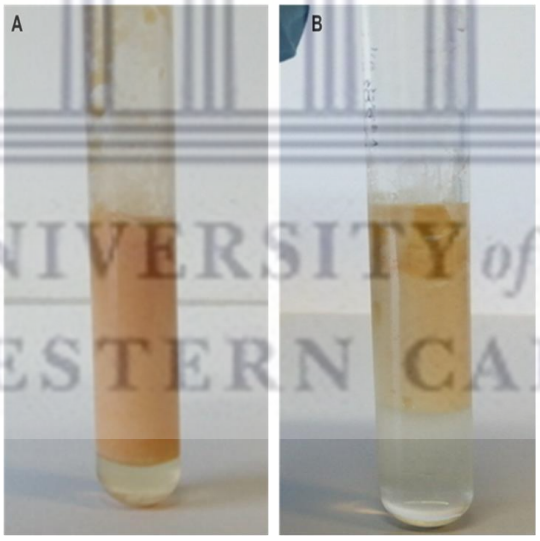
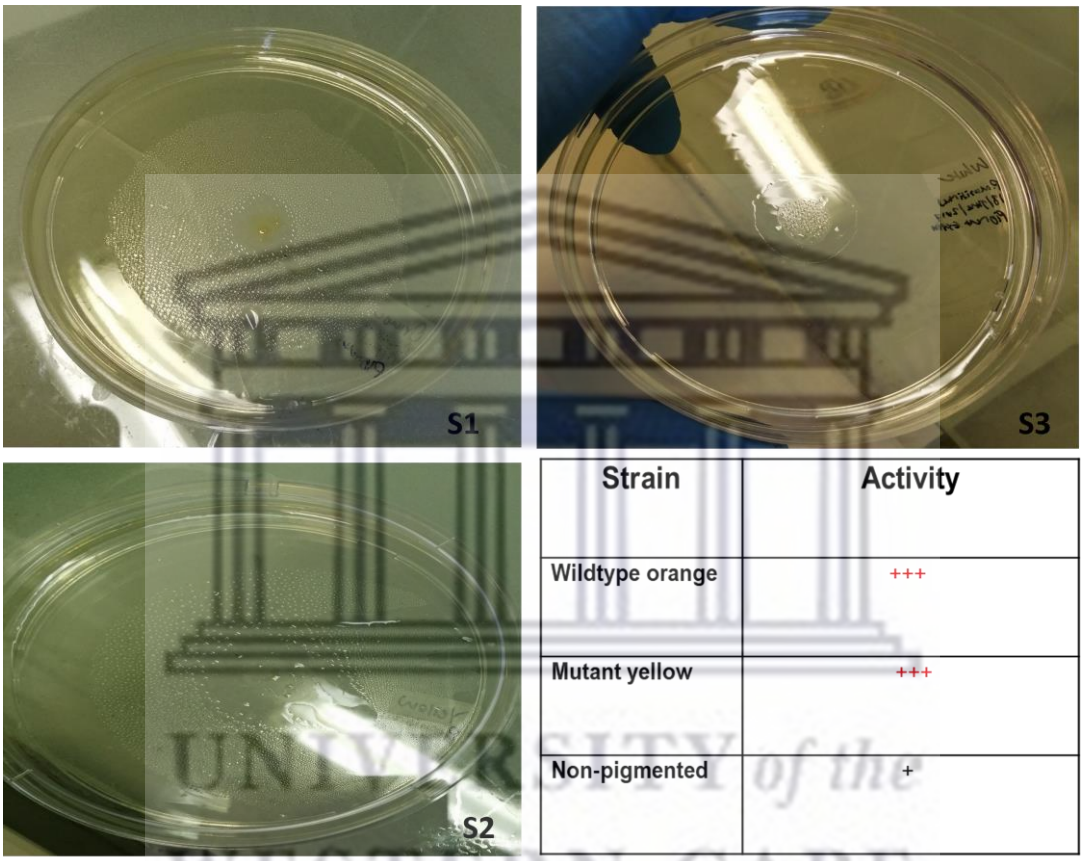


Figure 3-4: Emulsion capacity (EC_{24}%) results for biosurfactant extracted from 3 L volume culture of wild type *Planococcus* sp. CP5-4 strain. Hydrocarbon motor-oil was mixed at a 1:1 (v/v) ratio with **A:** Biosurfactant extracts and **B:** 10% (w/v) SDS and, the EC_{24} is 92.3% and 66.2% respectively.

3.3.3. Preliminary screen of the crude extract for biosurfactant activity: Atomized spray method

The crude extracts extracted *via* pigmented extraction were screened for activity, all the crude extracts from the variants had biosurfactant activity detected with the atomized spray method (**Figure 3-5**). The wild type and yellow mutant *Planococcus* sp. CP5-4 strain extracts displayed significantly higher activity compared to the unpigmented

mutant *Planococcus* sp. CP5-4 strain (**Figure 3-5**). The activity detected is presumed to be connected with the observed colour differences, as strains with pigmentation showed higher activity over the non-pigmented strain. However, the presence of activity for the unpigmented strain confirms further the possibility that *Planococcus* sp. CP5-4 strain may synthesize other biosurfactant compounds that are not associated with the mutations as this is the only known different parameter between the variants. Furthermore, the pigment extraction method was able to extract the surface active compound and other compounds and not exclusively pigmented compounds (Melendez-Martinez *et al*., 2019; Ram *et al*., 2020).



Strain	Activity
Wildtype orange	+++
Mutant yellow	+++
Non-pigmented	+

Figure 3-5: Atomized spray test results of total pigment extract *of Planococcus* sp. CP5-4 strains **S1:** wild type orange, **S2:** yellow mutant, **S3:** unpigmented mutant and a table representing the observed results.

3.3.4. Preliminary molecular screening and structural characterization: Cetyltrimethylammonium (CTAB)/ methylene agar plate method

CTAB/methylene blue agar assay was applied to test for glycolipid production and/or other anionic biosurfactant production according to Siegmund and Wenger (1991). The positive control 10% (w/v) SDS was positive for anionic surfactant, indicated by the blue halo zone formation around the well (**Figure 3-6**). There was no sign of activity for the crude extracts of the three strains (**Figure 3-6**). The absence of the dark blue halo provided evidence that the potential biosurfactants produced by *Planococcus* sp. CP5-4 strains may not be anionic in nature. The results were different from what was reported by Waghmode and team (2019) in which they found that the biosurfactant produced by *Planococcus* SAMP was anionic in nature. Biosurfactants are found to be mostly either anionic or

neutral, but there are cationic biosurfactants and those that are cationic mostly come from amine groups (Santos *et al*., 2016). Waghmode *et al*. (2019) employed a 0.2% ninhydrin staining procedure and confirmed that their extract does not have any amide groups. In this study, the same results were observed when 0.2% ninhydrin was used as a staining reagent, the was no evidence of amino acids thus, pointing to the carbohydrate/sugar and lipid nature of the biosurfactants synthesized by *Planococcus* sp. CP5-4 strain (Appendix: **Figure D 4**). However, the biosurfactant produced might be neutral.

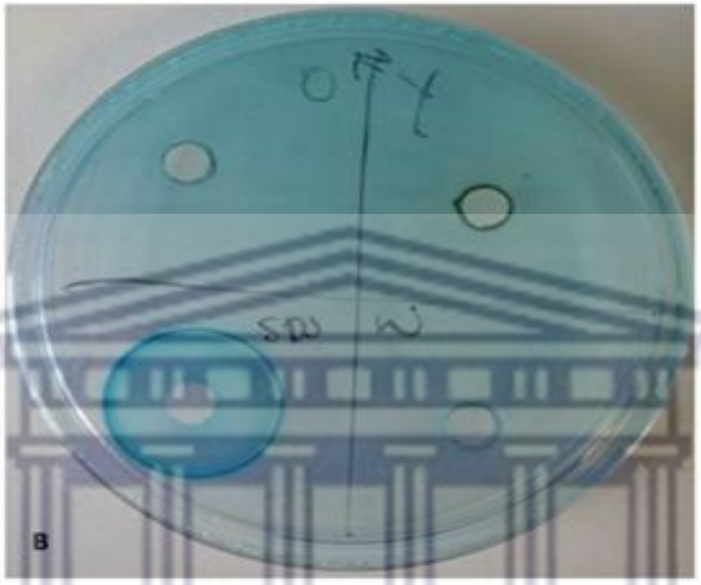


Figure 3-6: Results of the CTAB/methylene agar assay employed for glycolipid and anionic biosurfactant identification. Performed by inoculating the wells with crude biosurfactant extracts of *Planococcus* sp. CP5-4 wild type (**O**), yellow mutant (**Y**), and unpigmented mutant (**W**) strains and SDS (10%, w/v) as the positive control.

3.4. Conclusion

The aim of this chapter was to investigate the biosurfactant production potential of the *Planococcus* sp. CP5-4 wild type (orange) and the mutant strains (yellow and unpigmented phenotypes) using a cultured based approach. Biosurfactant activity was detected after preliminary screening through measuring the head of foam and emulsion capacity and *via* the atomised spray test. The screening methods employed confirmed *Planococcus* sp. CP5-4 as a biosurfactant producer, with measurable activity. The wild type orange *Planococcus* sp. CP5-4 strain displayed the highest activity, followed by the yellow mutant *Planococcus* sp. CP5-4 variant and the unpigmented mutant *Planococcus* sp. CP5-4 variant with the least activity. The difference in activity seemed to be associated with the colour differences between the strains as a result of the mutation. However, the unpigmented strain had measurable activity indicating the possibility that *Planococcus* sp. CP5-4 strain may synthesize other biosurfactant compounds that are not associated with the mutation. Through preliminary characterization of the biosurfactant(s) produced by *Planococcus* sp. CP5-4, our finding revealed that biosurfactants detected might be neutral. The relationship between the pigment synthesised and the biosurfactant produced by the *Planococcus sp.* CP5-4 strain was investigated further and in detail in Chapter 4.

Chapter 4: Identification of carotenoid as biosurfactant(s) produced by *Planococcus* sp. CP5-4

4.1. Introduction

The relationship between hydrocarbon-degradation properties and biosurfactants is well documented, (Patowary *et al*., 2017; Karlapudi *et al*., 2018). *Planococcus* was only known for hydrocarbon degradation and bioremediation properties before their biosurfactant and bioemulsification activities until authors Kumar and colleagues in 2007 reported the production of biosurfactant exopolysaccharide (EPS) by *P. maitriensis* Anita I. The strain produced biosurfactant with the ability to spread oil, emulsify xylene, and had properties equivalent to Triton X and Tween 80, which are well-known mild non-ionic synthetic detergents, therefore, the surface-active compound was suggested for bioremediation, microbial-enhanced oil recovery (MEOR), and cosmetic applications. Since then, several more *Planococcus* strains have been characterised as biosurfactant producers and a timeline of these discoveries is presented in **Table 4-1**. However, the first structural elucidation of a *Planococcus*-produced biosurfactant was only presented in 2019 by Waghmode and colleagues (2019). The biosurfactant synthesized by *Planococcus* maritimus SAMP MCC 3013 degrades the branched n-alkanes found in crude oil (C11 to C33) making it suitable for regulating hydrocarbon-contaminated marine reservoirs (Waghmode *et al*., 2019) (**Table 4-1**). Structural characterization revealed a glycol-carotenoid/-terpenoid (Waghmode *et al*., 2019). Therefore, this was the first study to make the association between a carotenoid glycolipid biosurfactant class in *Planococcus*. As can be gleaned from the summary presented in **Table 4-1** it is evident that *Planococcus* species are producers of biosurfactants with noteworthy physicochemical properties, therefore, their exploration might be a good investment to expand the biosurfactant diversity pool. The biosurfactant production potential of *Planococcus* sp. CP5-4 strain was confirmed in Chapter 3. The nature of the mutations provided some evidence to suggest that the biosurfactant could be a carotenoid, as has been suggested for other *Planococcus* species namely *Planococcus* sp. *DI* and *Planococcus maritimus* SAMP MCC 3013 (Ebrahimipour *et al*., 2014; Waghmode *et al*., 2019). The proposed theory is that the carotenoid(s) synthesised by *Planococcus* sp. CP5-4 is the biosurfactant(s) produced. Carotenoids produced by *Planococcus* species exhibit structural diversity (Kumar *et al*., 2007; Ganapathy *et al*., 2016; Waghmode *et al.*, 2019). Therefore, this presents the opportunity for the discovery of biosurfactants with novel carotenoid chemical structures by studying other *Planococcus* species. In this Chapter we investigate whether the biosurfactant activity is attributed to the carotenoid that was elucidated recently (Moyo *et al*., 2022).

Table 4-1: Timeline for the discovery and assessment of the hydrocarbon degradation, bioremediation, and biosurfactant properties by *Planococcus* species.

Strain	Origin	Findings	Authors
P. alkanoclasticus sp. nov. strain MAE2	The sediment of the intertidal beach.	Biodegradation of branched n-alkanes in crude oil (C11 and C33)	Engelhardt *et al.,* 2001
*P.*sp. strain S5	The sewage treatment plant in Poland.	Degrade aromatic hydrocarbons like salicylate or benzoate and grow well in presence of phenol	Labuzek *et al*., 2003
P. maitriensis Anita I	Coastline seawater of Bhavnagar district, Gujarat India.	Exopolysaccharide (EPS) production and emulsifying efficacy; EPS emulsifies xylene Showed reduction of surface tension (ST) (72 to 46.07 mN/m)	Kumar *et al.,* 2007
Planococcus sp. DI	Oil-contaminated area, Iran	Produce biopolymer, EPS The emulsifying activity of BS demonstrated 77% emulsification activity against crude oil.	Ebrahimipour *et al*., 2014
P. jake 01	Waste activated sludge	Novel biosurfactant producing strain that was applied in sludge disintegration and biogas production	Kavitha *et al*., 2015
P. strain Y42	Oil-contaminated soil, Qinghai-Tibetan Plateau	Has genes responsible for hydrocarbon degradation and thus can be used for bioremediation	Yang *et al*., 2018
P. maritimus SAMP MCC 3013	Coastal seawater, India	Biosurfactant characterization based on physical properties The CMC value of terpene containing biosurfactant was 1.3 mg/mL and reduced surface tension of PBS from 72 to 30 mN/m. Structure: glyco-carotenoid/-terpenoid	Waghmode *et al.,* 2019
Planococcus sp. *XW-1*	Yellow Sea	Produce a glycolipid-type biosurfactant at a low temperature (4°C) using petroleum as the sole carbon source. Biosurfactant produced can degrade crude oil Can reduce the surface tension of water to 26.8 mN/m and CMC value of 60 mg/L.	Guo *et al*., 2022

4.1.1. Amphiphilic carotenoids

Carotenoids are diverse and widespread naturally occurring pigments, responsible for the yellow, orange, or red-color pigmentation found in nature (Ebrahimipour *et al*., 2014; Rodrigo-Banös *et al*, 2015; Ganapathy *et al*., 2016; Vila *et al*, 2018). They are widely distributed in plants, algae, fungi, bacteria, and other eukaryotic organisms (Saini and keum, 2017; Melendez-Martinez *et al*., 2019; Ram *et al*., 2020). As secondary metabolites, they play essential roles in cell adaptability, in protecting cells from oxidative damage and UV radiation, are involved in the regulation of gene expression, and are integrated into the maintenance of membrane fluidity (Ebrahimipour *et al*., 2014; Saini and Keum, 2017). Carotenoids are popularly known for antimicrobial, anti-inflammatory, chemotherapeutic, Vitamin A, antioxidant, and anticancer properties, and because of these properties they have pharmaceutical significance and are used both in the cosmetics and food industries (Shindo *et al*., 2008; Ebrahimipour *et al*., 2014; Vila *et al*, 2018). The basic frame of a carotenoid is found in different modified manners fostered through hydrogenation, isomerization, rearrangement, chain shortening or extension, double bond migration, cyclization, dehydrogenation, introduction of oxygen function, or from a combination of these processes, resulting in a great diversity of the structure (Shindo *et al*., 2008). Furthermore, carotenoids are found free or associated with other molecules which impact some of their properties, typical molecules that carotenoids can be associated with are fatty acids (carotenoid esters), sugar (glycosylated carotenoids), protein (carotenoprotein), or sulphate (Fernandes *et al*., 2018; Melendez-Martinez *et al*., 2019; Silva-Castro *et al*., 2019). The structural diversity is responsible for the numerous biochemical and physiological functions associated with carotenoids.

Almost all naturally occurring carotenoids are classified as hydrophobic with few exceptions such as diacids, sugar esters, and carotenoid sulfates out of the 800 naturally occurring carotenoids reported in the literature (Naess *et al*., 2006; Hada *et al*., 2012; Maoka, 2020). A lot of focus has been placed on hydrophobic compounds with little to no interest in the biosurfactant properties of amphiphilic carotenoids (Naess *et al*., 2006). However, the hydrophobic nature of carotenoids normally limits their bioavailability, thus also their consideration for most applications, such as food colorants and pharmaceuticals, to name two. Reported examples in literature of natural amphiphilic carotenoids with the ability to dissolve in water and that display biosurfactant properties include diacids crocetin (C20:7), norbixin, and isobixin (24:9), crocin, and carotenoid sulfate (Naess *et al*., 2006; Hada *et al*., 2012; Maoka, 2020). However, reports are mostly on sugar esters as the only highly water-soluble carotenoids, while the surface properties and aqueous aggregation of other hydrophilic/amphiphilic carotenoids have not been extensively investigated (Naess *et al*., 2006; Hada *et al*., 2012; Maoka, 2020). Academic curiosity is growing towards hydrophilic carotenoids with biosurfactant activity for practical application in the medicine, cosmetics, textile, and food technology industries (Naess *et al*., 2006; Giani *et al*., 2021). For example, it has been reported that water-soluble/amphiphilic carotenoid antioxidants can react with oxidants in the blood plasma and cell cytosol, while lipid-soluble antioxidants only prevents lipid peroxidation of the cell membrane (Kim *et al*., 2022; Lilwani *et al*., 2022).

4.1.2. Linkage between carotenoid and biosurfactant synthesis in *Planococcus* sp. CP5-4

There are no extensive reports on the biosynthetic genes responsible for carotenoid production by *Planococcus* species and even less evidence is available that support the connection between carotenoid synthesis and biosurfactant production by the genus. The existence of the carotenoid biosynthetic genes is mentioned for *Planococcus rifietoensis* M8^T, *Planococcus halotolerans* SCU63, *Planococcus donghaensis* JH1T, *Planococcus* sp. ANT_H30 and *Planococcus maritimus* MKU009 (Moyo *et al*., 2022). Nevertheless, biosynthetic gene cluster sequence analysis has only been done for *Planococcus maritimus* iso-3, *Planococcus maritimus* SAMP MCC 3013, and *Planococcus faecalis* AJ003 and recently for *Planococcus* sp. CP5-4 (Waghmode *et al.,* 2019; Moyo *et al*., 2022). *Planococcus* sp. CP5-4 produces a novel glycol-C_{30}-carotenoic acid and based on the arrangement of the carotenogenic genes this structure is hypothesised to also be produced by the strains belonging to the *Planococcus* _B genus (**Figure 4-1**) (Moyo *et al*., 2021). Waghmode *et al*. (2019) performed bioinformatic studies with the aim of making the connection between the carotenoid biosynthetic pathway in *Planococcus maritimus* SAMP MCC 3013 and the biosynthesis of the biosurfactant produced (Waghmode *et al*., 2019) (**Table 4-1**). Entire gene sets responsible for the synthesis of a terpenoid using genomic sequence analyses were located and based on the genomic and functional studies it was put forward that the terpenoid might be a backbone for the biosurfactant produced (Waghmode *et al*., 2019). However, an experimental link between the carotenoid pathway and biosurfactant activity has not been established in this genus. Therefore, this chapter focused on confirming, for the first time the involvement of carotenoid biosynthetic pathway in the production of the main biosurfactant by *Planococcus* sp. CP5-4 and the mutation of the wild type strain permits the experimental investigation.

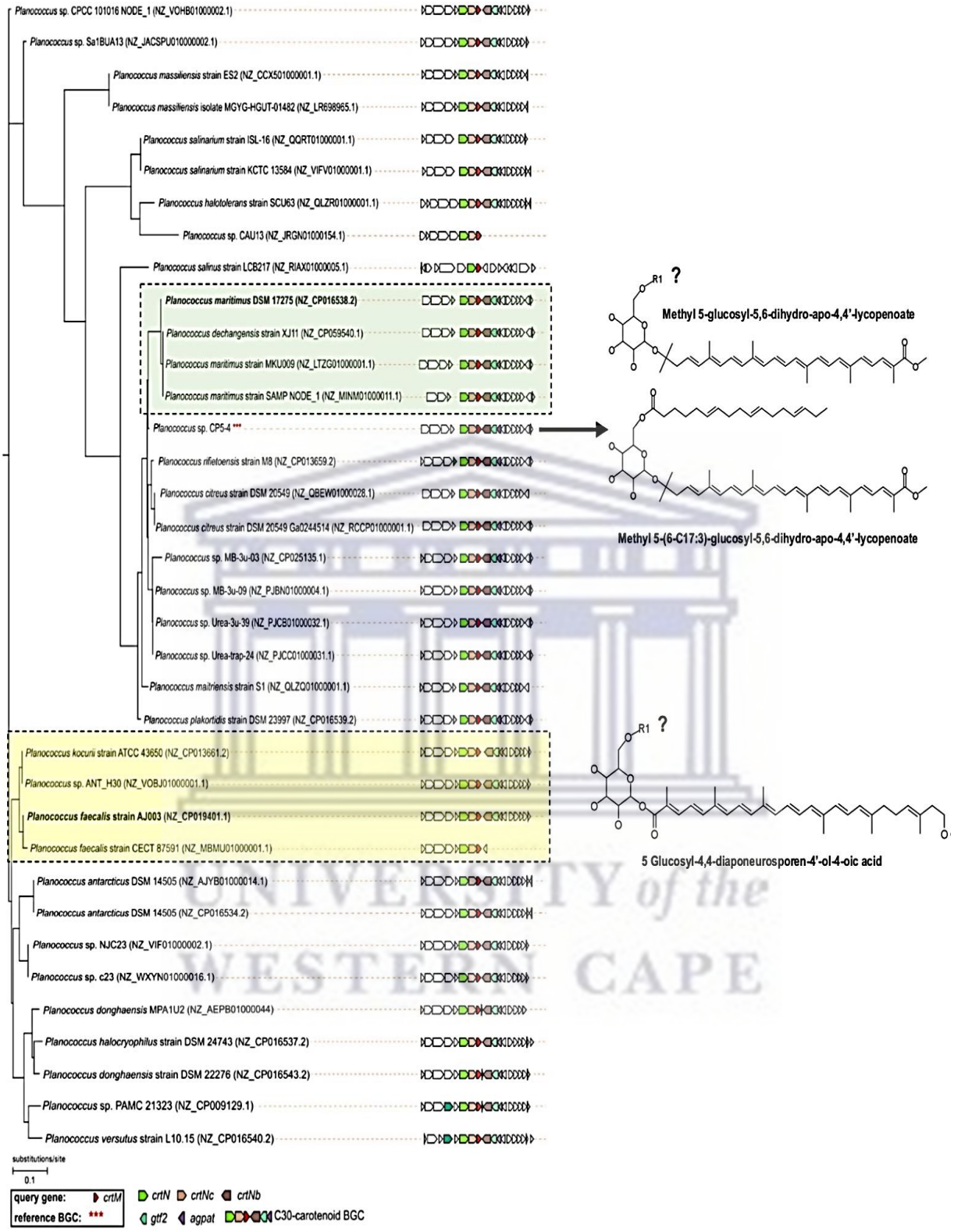


Figure 4-1: Taxonomic classification of *Planococcus* sp. CP5-4 together with pigment biosynthesis gene identification and characterization carried-out by Moyo *et al*., (2022) through genome sequence analysis and compound isolation.

4.2. Materials and methods

4.2.1. Confirmation of carotenoid pathway and its deletion from the *Planococcus* sp. CP5-4 unpigmented strain

The deletion of the carotenoid pathway from the unpigmented *Planococcus* mutant strain was confirmed by performing sequence alignment of *de novo* assembled contigs of the wild type orange and mutant unpigmented *Planococcus* sp. CP5-4 strains. Prior to sequence alignment, the raw Illumina sequence reads for the various *Planococcus* genome sequences generated by Moyo (2021) were paired in CLC genomics workbench ver. 7.5.1 (Qiagen, Denmark, (https://www.qiagenbioinformatics.com). The paired-end reads were trimmed to remove low-quality reads (average base quality <Q20) and adapter sequencing using the following parameters: Quality score = 0.05 max, Ambiguous base = 3, Minimum sequence length = 50 nucleotides, and removal of adapter sequence. The trimmed paired-end read sequences were used for *de novo* assembly with the following parameter set: Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3, Length fraction = 0.5, Similarity = 0.8, Minimum contig length = 1000 bp. Assembled contigs were analyzed using Antibiotics and secondary metabolites analysis shell (antiSMASH) to locate the contig containing the carotenoid biosynthetic gene cluster of the wild type *Planococcus* sp. CP5-4 strain. This contig was annotated using RAST tk *via* the KBase web portal (https://www./kbase.us/; Arkin *et al*., 2018) for gene prediction and functional annotation. Furthermore, the identified gene cluster was uploaded to the minimum information about a biosynthetic gene cluster (MIBiG) database to characterize the secondary metabolite pathways (Medema *et al*., 2015). Moreover, the comparison of the identified pathway to similar regions on other bacterial genomes available on the GenBank database was carried out. The annotated contig was then used as a reference for the alignment of contigs from the unpigmented *Planococcus* sp. CP5-4 mutant strain to confirm the deletion of the carotenoid pathway.

4.2.2. Biosurfactant extraction and purification methods used

The three strains used in study were cultured following instructions in **section 3.2.1**. Five extraction methods were employed to extract biosurfactants from both the CFCS and the cell pellet of each strain. In this study the direct extract obtained immediately following this was termed crude extract. The extraction methods used are detailed in the section below. Two hundred milliliters of the CFCS from the same 1 L culture of each of the *Planococcus* sp. CP5-4 strains were subjected to different extraction methods to assess their efficiency in biosurfactant extraction. Once the best method was established, it was used to extract the biosurfactant from a 1 L volume of each of the cultures to test for the potency of the biosurfactant. As a control, uninoculated TSB-salt-sorbitol broth was also subjected to the biosurfactant extraction methods described below. All the strains were treated the same unless stated otherwise.

4.2.2.1. Ethyl acetate extraction method (liquid-liquid partitioning)

The ethyl acetate extraction method was performed following instructions by Smyth *et al*. (2010a). Ethyl acetate was added at a 1:1 (v/v) ratio to the CFCS contained in a separatory funnel, the solution was mixed by vigorous shaking for 3-4 minutes before the mixture was left to separate for 20 minutes. The bottom aqueous phase was

withdrawn and re-extracted twice more with fresh ethyl acetate. The three, ethyl acetate solvent phases from each extraction were pooled and the solvent fraction evaporated under a vacuum inside a rotary evaporator (IKA rotary evaporator RV 10, Berlin) set at 35°C until completion (Williams *et al*., 2019). The 'oily-gummy' orange-red, yellow, and off-white residues left inside the round-bottomed flasks that contained the biosurfactant extract from the wild type, yellow, and unpigmented mutant *Planococcus* sp. CP5-4 cultures, respectively, were resuspended in 1 mL of methanol. The crude extracts displayed these physical characteristics independent of the extraction method applied. Furthermore, all the crude extracts were resuspended in 1 mL methanol.

4.2.2.2. Acid precipitation extraction method

Acid precipitation through the dropwise addition of 32% concentrated HCl to the CFCS was also used for the extraction of biosurfactants as per Smyth *et al*. (2010a). Hydrochloric acid was added to the CFCS until it reached a pH of approximately 2.0. A 6 N sodium hydroxide (NaOH) solution was used to adjust the pH as needed during the extraction process and the acidified CFCS stored at 4°C overnight to promote the precipitation of biosurfactant(s). The precipitate that formed in the chilled acidified CFCS was collected by centrifugation at 1300 x *g* for 30 minutes in a refrigerated Beckman Coulter Avanti J-26 XPI centrifuge set at 4°C. The three extraction methods described below required acid precipitation as the first step in the extraction procedure. The acid precipitation procedure was done following instructions in **section 3.2.2**. The same precipitate weight was maintained across the strains/variants.

4.2.2.2.1. Acid precipitate dissolved in methanol (solid-liquid extraction)

The precipitate was dissolved in 5 mL of methanol by vortexing for 2 minutes to solubilize the biosurfactant. The undissolved material was removed following centrifugation at 4629 x *g* for 20 minutes and resuspended in methanol again. This process was carried out three times, the methanolic fractions were pooled, and the methanol evaporated until it reached a 1 mL volume in a speedvac set at 40°C and 10.0 mbar.

4.2.2.2.2. Acid precipitation and ethyl acetate extraction, with and without pH adjustments

A combination of the acid precipitation and solvent extraction methods was also used for the extraction of biosurfactant(s) from the CFCS as follows: Acid precipitation from CFCS was first conducted as described in **section 4.2.2.2** and 20 mL of deionized water added to the precipitate, which was then vortexed overnight. Extraction was thereafter carried out by adding ethyl acetate to the mixture at a ratio of 1:1 (v/v) in a separatory funnel. Recovery of the solvent fraction was conducted as described in **section 4.2.2.1**. The second version of this extraction procedure was employed, with the main difference being the pH adjustment (neutralization) step prior to solvent extraction. The pH of the acid precipitate in deionized water was adjusted to 7.5 using 2 N NaOH to aid the solubilization of the precipitate. To ensure complete solubilization, the pH adjusted mixture was placed on an orbital shaker set at 60 rpm overnight before ethyl acetate extraction. All the solvent fractions were pooled and evaporated to completion in a rotary evaporator.

4.2.2.2.3. Acid precipitation and chloroform-methanol extraction (Liquid-liquid partitioning)

A combination of the acid precipitation and solvent extraction methods was also employed for the extraction of biosurfactant(s) from the CFCS by adding an equal volume of a 2:1 (v/v) chloroform-methanol solvent mixture instead of ethyl acetate to the neutralized acid-precipitate dissolved in deionized water (pH 7.5) (Smyth *et al*., 2010a). Recovery of the solvents fraction was carried out as described in **section 4.2.2.1**. The collected solvents were pooled together and evaporated.

4.2.2.3. Pigment extraction method

Following Bligh and Dyer (1959) method for pigment extraction, the cells were pelleted from the culture broth by centrifugation at 6 000 x *g* for 15 min at 4°C, after which, the pelleted cells were collected and washed with sterile water three times to remove traces of the culture broth. The cells were then pelleted once more, the water fraction discarded, and the pellet left to air dry prior to pigment extraction. The pellets were weighed out and kept the same across the variants. A solvent mixture of 7:3 (v/v) acetone and methanol with 0.1% butylated hydroxytoluene (BHT) to prevent oxidation of the carotenoids, was added to the cell pellet, and the mixture vortexed. The homogeneous mixture was subjected to repeated freeze and thaw cycles in liquid nitrogen to lyse the cell and release the pigment. The lysed cell suspension was then centrifuged at 10 000 x *g* for 10 min at 4°C and the pigmented supernatant was collected. The extraction was repeated on the cell pellets until the cells were bleached out. To obtain the crude pigment compounds from the supernatant, the solvent fraction was evaporated using an N_2 gas stream, and the 'oily-gummy' residues were stored at 4°C in a dark place until further processing. The resulting pigmented residue was thereafter resuspended in 2 mL methanol with 0.1% BHT. All extractions were conducted in the dark and the crude extract was kept in foil-wrapped containers at 4°C to avoid photo-oxidation. The resulting residue was thereafter resuspended in 2 mL methanol with 0.1% BHT to be used later.

4.2.3. Spectrophotometric analysis of the pigment crude extract from *Planococcus* sp. CP5-4

The pigment was extracted using the pigment extraction method in **section 4.2.2.3**. The extracted pigment in 2 mL methanol with 0.1% BHT was used to perform spectrophotometric analysis following instructions by Silva-Castro *et al*. (2019). Tentative identification of the extracted pigments was conducted by the UV-Vis absorption spectra from 200-800 nm using a double spectrophotometer (Varian Cary 50 UV-Vis spectrometer).

The total carotenoid content in the extracts was determined by measuring the OD of each of the samples at the λmax of extracted solution and calculated using the equation below.

$$[\text{carotenoid}]\ \mu g/g = \frac{A\ x\ V\ (ml)\ x\ 10^4}{E_{1cm\ x\ P\ (g)}}$$

Where A is the raw absorbance, V is the total extraction volume, P is the sample weight, and E the 134 x10^3 $mM^{-1}cm^{-1}$– β- carotene extinction coefficient.

4.2.4. Characterization of the crude extracts by thin layer chromatography (TLC) analysis

Ten microliters of each of the crude biosurfactant extracts obtained using the five extraction methods described above were analyzed by spotting into 20 x 10 cm silica gel 60 F_{254} TLC plates (Merck) (Smyth *et al*., 2010a). The plates were spotted and left to dry prior to being developed. The TLC plates were developed with solvent systems A; chloroform: methanol: water: acetic acid in the ratio 65:25:4:1; v/v/v/v. The TLC development chambers were each equilibrated for 15 minutes before placing the spotted TLC plates. The solvent front was allowed to ascend to 7 cm away from the point of origin of the TLC plate. After which, the plates were removed and left to air-dry in a fume hood prior to the detection of the separated compound. Lipid groups on the developed TLC plates were detected using primuline by spraying the staining reagent uniformly onto the plates and leaving the plate to dry completely under a fume hood (Williams, 2016). After an hour, the stained plates were viewed under UV excitation at 254 and 366 nm. Primuline was prepared by dissolving solid primuline in 80% (v/v) acetone in water to form 1% (w/v) liquid primuline. Molisch's reagent (α-naphthol [EtOH] H_2SO_4) was used for testing the presence of carbohydrates. To characterize glycolipids and other carbohydrates/ sugars the TLC plates after development were sprayed with 2.4% (w/v) α-naphthol in 10% (v/v) sulphuric acid and 80% (v/v) ethanol (Williams *et al*., 2019). A crude rhamnolipid extract (a gift from Dr. Wesley Williams) was spotted on the TLC plate as a positive control.

4.2.5. Partial purification of biosurfactant(s) extract by TLC technique

As several impurities are co-extracted with the compound of interest, TLC was used to obtain a partially purified biosurfactant(s). One hundred and eighty microliters of each of the crude extracts were applied to the origin of a prep TLC plate and the plates developed in solvent system A. Primuline was sprayed onto a small section of the dried developed TLC plate to identify the lipid fraction. The stained lipid bands were then extrapolated to the unstained portion of the TLC plate. The portion without the primuline stain was extracted by scraping the silica from the plate and solvent re-extraction by resuspending the scrapings into 6 mL of a 2:1 (v/v) chloroform: methanol solvent mixture. The solvent phase was separated from the silica by centrifugation at 500 x *g* for 10 min then withdrawn and transferred to a new tube. A maximum of 3 TLC preparative plates for each strain, were prepared and scraped for each experiment, the number of plates were kept constant across the variants and in this study, we estimated that the extracted compounds were concentrated enough to be detected. The extracts were placed in a Speedvac set to 40°C and 10.0 mbar to evaporate the solvent till dry. The extracted obtained after were considered partially purified in this study. The dried partially purified extracts were successively resuspended in 200 µL of distilled water and stored at room temperature until further use.

4.2.6. Biosurfactant activity testing of partially purified biosurfactant extracts

The partially purified biosurfactant extracts above were screened for biosurfactant activity using the atomized spray method, drop collapse test, micro-plate grid test, and *via* contact angle measure and the activity was scored using the scoring system in **section 3.2.3**: This was done by comparing the diameter of the halo for the atomized spray method, the drop collapse magnitude, and grid distortion caused by active samples. The activity test were performed as described bellow:

4.2.6.1. Atomized spray test

The atomized spray method by Burch *et al*., (2010) was used to screen for biosurfactant activity from the partially purified extracts following instruction in **section 3.2.3**. The diameter of the halo formation was measured. The quadruple diameter (n=4) for each sample was measured therefore giving the average of the results presented.

4.2.6.2. Drop collapse test

The drop collapse test was used to screen for biosurfactant activity of the scrapped and extracted bands. The method was adapted from Jain *et al*. (1991), the activity was tested by placing 10 µL of the sample on top of a hydrophobic 96-well microplate, which was observed for drop collapse after 1 minute. Wide-shaped, flat-top drops were noted as positive results while round more beaded drops were noted as negative results. The relative magnitude of the drop was observed and scored.

4.2.6.3. Microplate-grid analysis test

The microplate-grid analysis assay was used to screen for the surface activity of individual bands from TLC following the method by Vaux and Cottingham, (2007). To test for biosurfactant activity 100 µL of each sample was dispensed in a flat-bottom 96-well plate (Thermo Fisher scientific, Lithuania), activity was noted by assessing the optical changes of grid paper placed underneath the 96-well plate. Distortion of grid paper was noted as a positive result and the degree of distortion was scored.

4.2.7. Solvent system changes for partial purification of pigmented bands

To minimize co-migration and improve the separation and purification of the various compounds detected on TLC plates, 10 microliters of each of the crude biosurfactant extracts using the chloroform methanol extraction method described in **section 4.2.2** (**4.2.2.2.3**) were separated with solvent B in addition to solvent A. This was done following characterization of the crude extracts by thin layer chromatography (TLC) analysis in **section 4.2.4**. The TLC plates were developed with solvent systems A; chloroform: methanol: water: acetic acid in the ratio 65:25:4:1; v/v/v/v and solvent systems B; chloroform: methanol: water: acetic acid in the ratio 130: 30: 4: 1; v/v/v/v to isolate the pigments. All the extracts were partially purified using the preparative TLC as described in **section 4.2.5**. Lipid groups on the developed TLC plates were detected using primuline. The dried partially purified extracts resuspended in 200 µL of distilled water were assayed for biosurfactant activity.

4.2.8. Contact angle measurement

For contact angle measurements, the partially purified extracts of the wild type orange and yellow mutant *Planococcus* sp. CP5-4 strains were obtained from 3 L CFCS of each strain using acid precipitation followed by chloroform-methanol extraction section **4.2.2 (4.2.2.2.2)**. The extracts were resolved on a TLC plate using solvent systems A and B described above. Biosurfactant activity was determined by measuring the contact angle using Low-bond axisymmetric drop shape analysis (LB-ADSA) using Image J (https//imagej.net/:version1.47vjava1.6.0_20 (32-bits).

Several adaptations were made, through a trial-and-error process to set up the parameters that were consistent with every measurement. Ten microliter samples to be tested were placed on top of the self-adhesive seal polyolefin surface (STARLab) on a solid substrate (**Figure 4-2**). The film on top of the substrate was used as a hydrophobic surface and a new adhesive film was used each time. Greyscale photographs were taken of the drops all the while ensuring that a 90° angle was maintained towards the horizontal substrate (**Figure 4-2**). The images were then processed into an asymmetric mirror image using the Mirror lab photo-editing app and the reflected images' contact angle was determined using the image J LB-ADSA software.

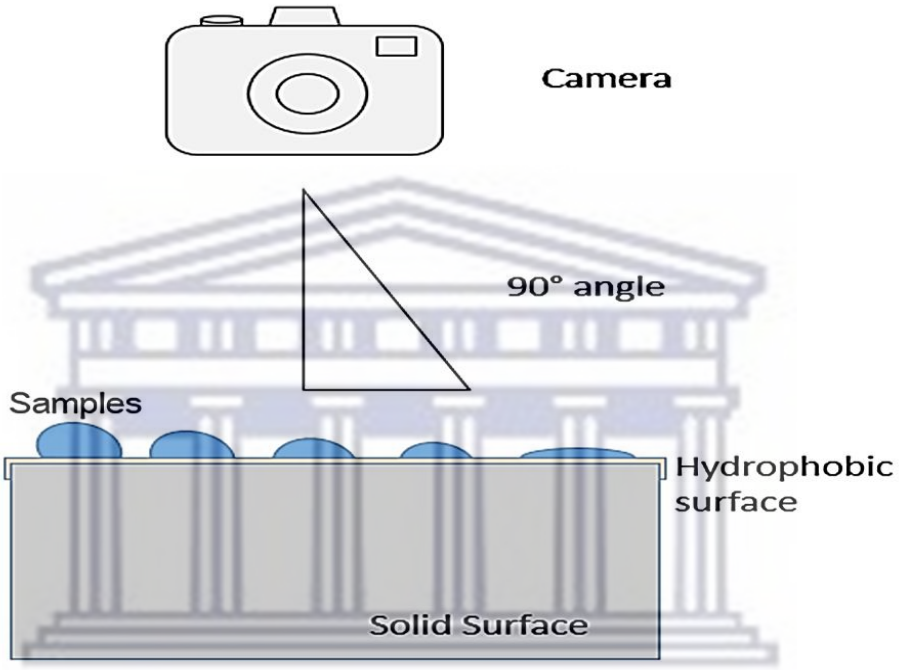


Figure 4-2: Illustration of samples placed on top of the self-adhesive seal polyolefin's hydrophobic surface (STARLab) on a solid substrate and of the process employed to photograph the sessile droplets. Pictures were taken at a 90° angle toward the horizontal substrate for contact angle measurements.

The analysis was implemented using the following steps: the image was loaded into Image J software (http://imagej.nih.gov/ij/download). The plugin LB-ADSA was launched, the correct image scale was set under the setting menu, the liquid capillary construct was selected, and the canvas adjusted roughly to the profile. By first adjusting the apex position, followed by the drop height and then the radius of curvature at the apex. Further details about the algorithm and the underlying physics are available in the PDF paper installed along with the software. To increase the accuracy, an average of quadruple drops for each sample was measured therefore giving the average contact angle as the results presented. All measurements were performed at room temperature (25°C). Distilled water was used as a reference and 10% w/v SDS as a positive control.

4.3. Results and discussion

4.3.1. Confirmation of carotenoid pathway and its deletion from the *Planococcus* sp. CP5-4 unpigmented strain

CLC genomic workbench data processing of the raw Illumina sequence reads resulted in 40 contigs with a length greater than 1000 bp contigs with an N50 value of 378140 bp for the wild type orange *Planococcus* sp. CP5-4 strain, and 35 contigs with an N50 value of 256327 bp for unpigmented mutant *Planococcus* sp. CP5-4 strain. Following antiSMASH analysis a terpene-like secondary metabolite pathway on contig 3 of the wild type *Planococcus* sp. CP5-4 genome sequence was identified as detailed in Moyo *et al*., (2022) (Appendix: **Figure E 2, C**). Contig 3 with the pathway, was aligned against the 35 contigs of the unpigmented mutant *Planococcus* sp. CP5-4 strain resulting in contig 11 of the unpigmented strain having the best match to contig 3 (**Figure 4-3**). The 7.4 Kb carotenoid pathway deletion and missing nucleotide bases of about 146 kb from contig 11 of the unpigmented mutant strain was confirmed when sequences were aligned with that of the wild type *Planococcus* sp. CP5-4 strain (**Figure 4-3**). This equated to the expected size of the deleted carotenoid pathway detected by Moyo *et al*. (2022). The annotated genes of contig 3 of the wild type *Planococcus* sp. CP5-4 strain provided a visual representation of the genes that belong to the contig (**Figure 4-3**). The genes responsible for the biosynthesis of carotenoids were determined to be 4,4'- diapolycopene oxygenase (*crtNb*), all trans-phytoene synthase (*crtB*), diapolycopene oxygenase (*crtNb*), all-trans-zeta-carotene desaturase (*crtI*), Phytoene desaturase (*crtP*) which appeared close together in a cluster and putative glycosyltransferase (*gff2*) which was downstream of the cluster (**Figure 4-3**) this was comparable to what Moyo *et al*. (2022) found in their study. The main activity was observed to be associated with the strain that had presence of the pigmented strains as concluded in Chapter 3, after this analysis we can state that the carotenoid biosynthetic pathway might be indeed involved in biosurfactant synthesised by *Planococcus* sp. CP5-4 strain. The wild type *Planococcus* sp. CP5-4 strain with the intact carotenoid biosynthetic pathway had the highest activity (**Figure 3-3**; **Table 3-1**). According to Moyo *et al*. (2022) the mutagenesis also resulted in the deletion of a 3 Kb sequence and a truncation of a carotenoid oxidase-like methyl oxidase gene (*crtP*) thus producing the yellow mutant.

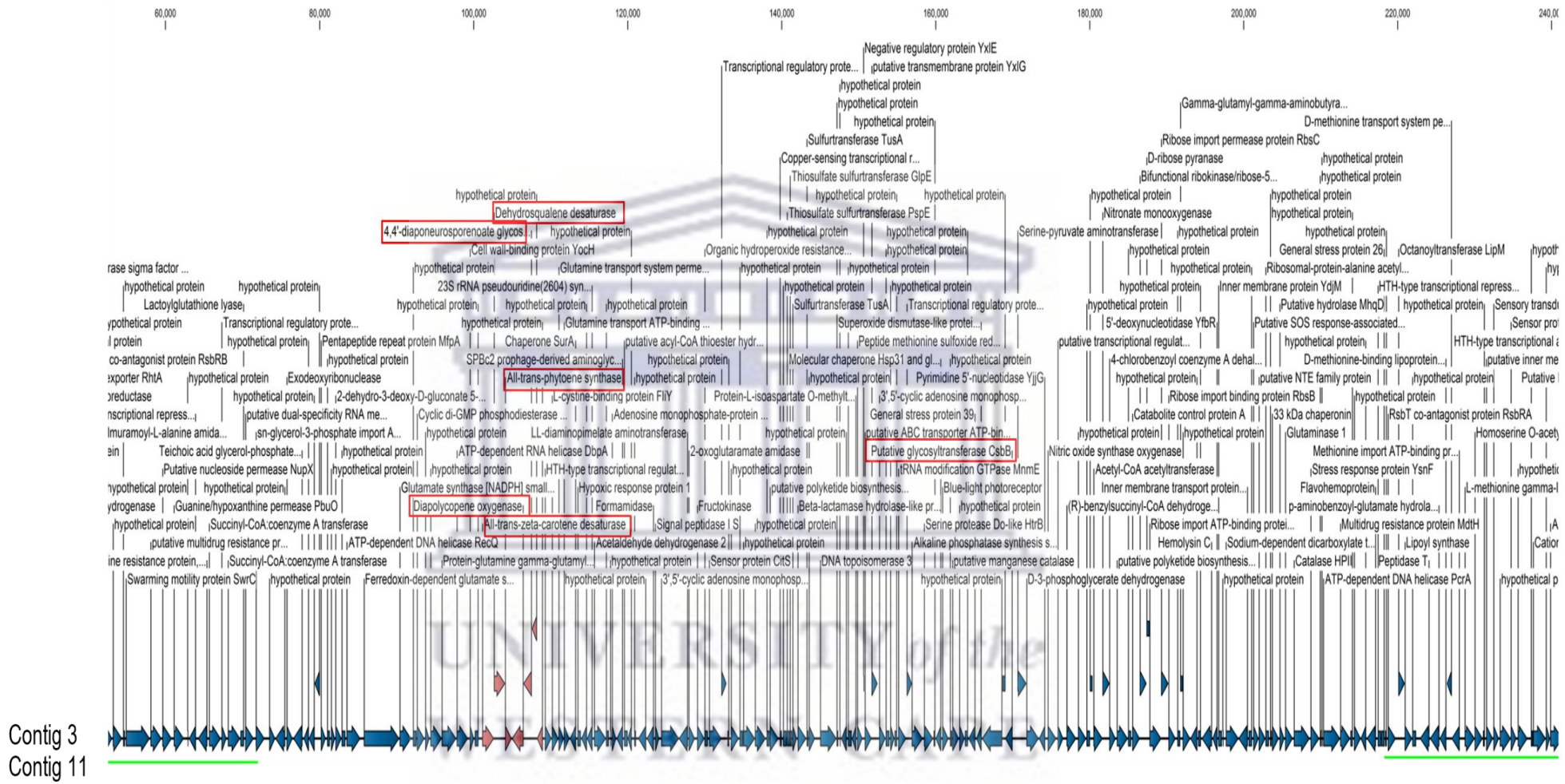


Figure 4-3: **Contig 3** and **contig 11** alignments of the wild type orange and mutant unpigmented *Planococcus* sp. CP5-4 strains respectively. Aligned to highlight the deletion of the carotenoid pathway from the mutant unpigmented strain represented by the 146 Kb gap and the green flanking regions. The red open reading frames (ORFs) are predicted carotenoid biosynthesis genes in wild type genome and blue ORFs other genes not associated with pathway. Contig 3 is annotated and the genes responsible for carotenoid biosynthesis are marked in red

4.3.2. Spectrophotometric analysis of the pigment extract from *Planococcus* sp. CP5-4

The UV-Vis absorption spectra confirmed the presence of the carotenoid pigment *via* the differences in the absorption maximum (λmax) of the extracts as measured by the spectrometer with methanol used as a blank (**Figure 4-4**). The absorption maximum was measured at 468 and 486 nm for wild type and yellow mutant *Planococcus* sp. CP5-4 strains respectively while the unpigmented mutant absorption maximum was not defined/ detected or at 0 nm (**Figure 4-4**). *Planococcus* sp. CP5-4 wild type pigment was found to have an absorption maximum of 475 nm by Silva-Castro *et al*. (2019). The spectrum was characterized by the typical "three-finger" pattern spectrum which is a representative of recognised carotenoid pigments such as beta-carotene, astaxanthin, and lycopene (Chemat-Djenni *et al*., 2010; Takaya *et al*., 2018; Moyo *et al*., 2021). Depending on the carotenoid pigment, the pattern usually displays a maximum absorbance at approximately 450 nm at a blue region and has two shoulder peaks at around 440 to 490 nm and this was found true for the extraction conducted in this study (Mercadante and Egeland, 2004). The approximate content of total carotenoids was calculated to be 191.41 µg/g, and 148.19 µg/g, for wild type orange, and yellow mutant variants respectively and was not defined for the unpigmented mutant variant or at 0 µg/g.

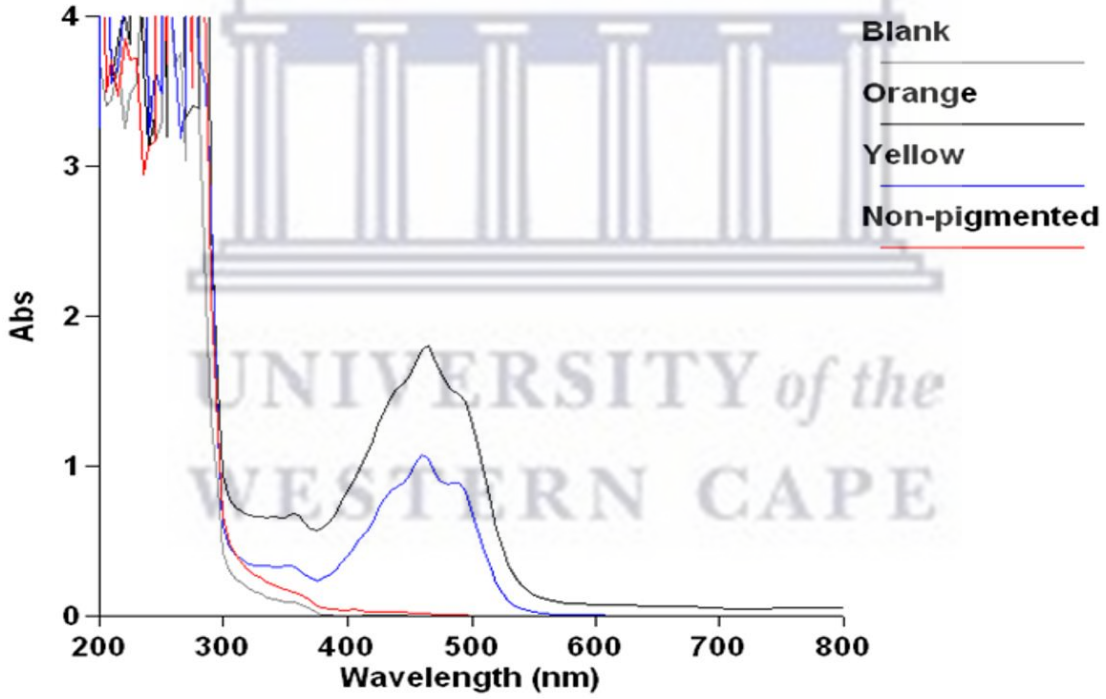


Figure 4-4*:* UV/Vis scanning spectrum of pigment extracts from *Planococcus* sp. CP5-4 wild type orange, yellow mutant, and unpigmented mutant strains, scanned from 200 to 800 nm. UV/Vis absorption spectra confirm the difference between wild type orange, yellow mutant, and unpigmented mutant *Planococcus* sp. CP5-4 variants in terms of absorption maximum (λmax).

4.3.3. Investigating the best extraction method for the extraction of the potential biosurfactant(s) produced by *Planococcus* sp. CP5-4

A two-phase ethyl acetate solvent extraction as one of the commonly used biosurfactant extraction methods was employed as the first choice for extraction. The extraction ability of the method was analyzed on a TLC plate stained with primuline reagent. For each variant, several bands appeared on the plate, and some were stained with primuline such as band 1 of the wild type *Planococcus* sp. CP5-4 strain (**Figure 4-5**). Spots closer to the point of origin represent more polar, less mobile compounds that are potentially bigger in size compared to the spots closer to the solvent front which are less polar compounds that are more mobile and potentially smaller in size. The variants displayed different extract profiles on the TLC plate, this is seen, near the point of origin, where the yellow mutant had two compounds (**A** and **B**) stained with primuline, while the unpigmented mutant had no compound that stained with primuline in this region when viewed under UV light excitation (**Figure 4-5: A**). Each strain extract were developed on a preparative TLC and portion of the TLC plate was sprayed to mark the bands to be scraped and used for the analysis without primuline interference (**Figure 4-5: E,D,E**).

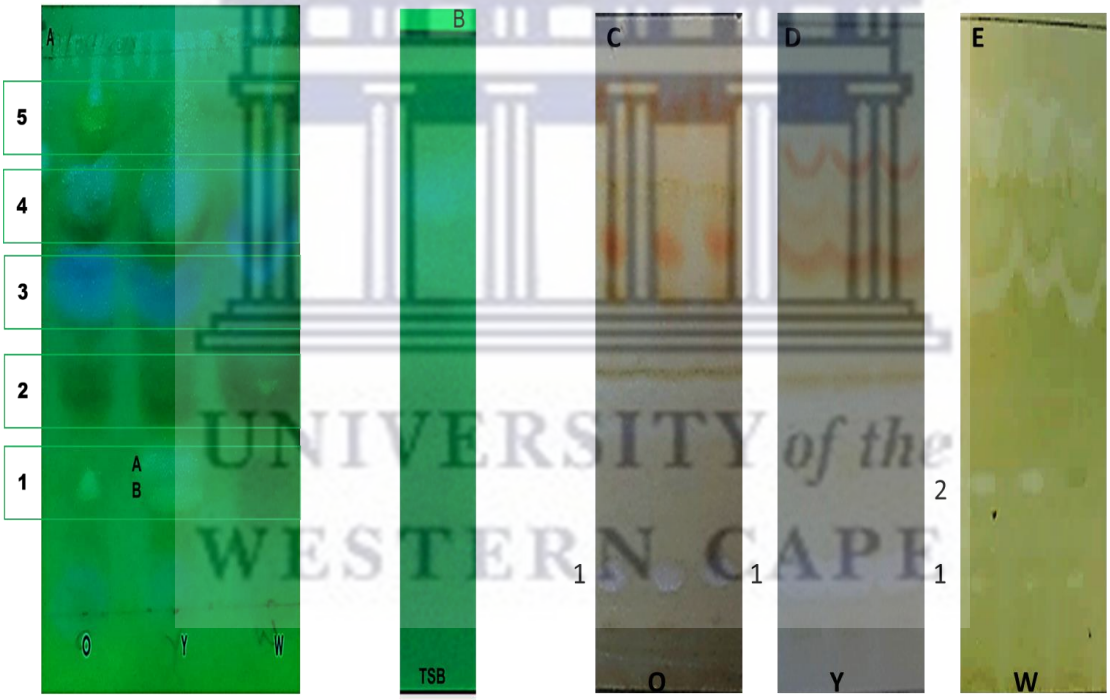


Figure 4-5: Thin-layer chromatography (TLC) analysis of the putative biosurfactant extracts from the (**O**) wild type orange, (**Y**) yellow mutant, and (**W**) unpigmented mutant *Planococcus* sp. CP5-4 strains, the extracts were extracted using ethyl acetate. The TLC plates were developed with a chloroform: methanol: water: acetic acid (65:25:4:1, v/v/v/v) solvent system. **A**: Result image of the extracts on a TLC plate stained with primuline for lipid detection captured under UV light excitation. Each lane donates a band (**1-5**) with a similar Rf value. **B**: Negative control TSB, and **C**-**E**: illustrations of the preparative TLC plates scraped, 3 points on each TLC plate were sprayed with primuline as a marker for where to scrape the bands. The TLC plate were captured under natural lighting.

The atomized spray method was used as the initial method to screen for biosurfactant activity of the extracts scraped from the TLC plates. The activity test results confirmed the extraction ability of ethyl acetate to extract the biosurfactant(s) and further confirmed the strain as a producer. All three *Planococcus* sp. CP5-4 variant showed evidence of biosurfactant activity, detected by a halo formation around the extracts screened (Appendix: **Figure D 5**, **Figure D 6**, **Figure D 7**). Extracts of bands 1 and 2 showed evidence of activity for the wild type strain and a halo diameter of 12.0 ± 0.41 and 8.00 ± 0.82 respectively, band 1 presented the largest halo compared to active bands of the other two mutant variants (**Table 4-2**). Band 1 was the only band that had clear evidence of activity represented by halo formation with a diameter of 10.1 ± 0.24 for the yellow mutant *Planococcus* sp. CP5-4 variant, with other extracted bands showing insignificant results (**Table 4-2**). For the unpigmented mutant *Planococcus* sp. CP5-4 variant, the activity was detected at bands 1 to 3, with diameter measurements of 10.5 ± 0.41, 6.67 ± 0.24, and 6.50 ± 0.41 respectively (**Table 4-2**). Collectively the results from the TLC scraping and activity testing suggest that a variety of surface active compounds are produced by *Planococcus* sp. CP5-4. It is well established that a single bacterial strain is capable of producing different biosurfactants. For example, rhamnolipid is produced as both mono- and di- rhamnolipid from a single *Pseudomonas* sp. strain (Kugler *et al*., 2015). In the case of sophorolipids production, the lactonic and acidic versions are produced by a single *Bacillus* strain (Vajayakumar and Saravanan, 2015; Sanches *et al*., 2021).

Table 4-2: The diameter measurements (mm) of the halo formation around extracts of *Planococcus* sp. CP5-4 strains from the atomized spray test.

Band extract	Wild type orange	Yellow mutant	Unpigmented mutant
1	12.0 ± 0.41	10.1 ± 0.24	10.5 ± 0.41
2	8.00 ± 0.82	0	6.67 ± 0.24
3	0	0	6.50 ± 0.41
4	0	0	0
5	0	0	0

The two-phase ethyl acetate extraction was able to extract surface-active compounds, however, the method also co-extracted other metabolites that were not surface active and not stained with primuline which is a major disadvantage of the protocol, therefore, alternative extraction methods were considered. The assumption that the bands not stained by primuline are not surface-active is not supported, for example, band 3 of the unpigmented mutant *Planococcus* sp. CP5-4 variant showed activity without the presence of primuline staining, however, most of the non-stained bands did not show activity at all. Furthermore, the presence of lipids stained with primuline does not necessary mean that they are exclusively surface-active compounds. The solvent targets all non-polar compounds excreted by the bacterium which will also include other non-surface active compounds, hence, the need to do an activity test for each of the bands. The atomized spray test is one of the standard methods utilized during preliminary screening because of its ease of use and sensitivity, but it is known to be a highly sensitive method mostly utilized for preliminary screening, therefore in literature, other assays have been used to complement it. Given the aforementioned reasons, the next objective was to investigate different extraction

methods with the aim of finding a method that will result in fewer co-extracted impurities and enrich for the targeted putative biosurfactant(s), and to subsequently employ a variety of screening methods to further characterize the biosurfactant activity.

Following extraction using the variety of extraction method described, extracts were analyzed by TLC (**Figure 4-6**). Rhamnolipids had Rf values 0.60 and 0.72, which served as a reference to show positive lipid staining by primuline and the ability of the mobile phase to separate the different compounds (**Figure 4-6:Rhm**). Irrespective of the variant or extraction method employed, there were common bands detected, likely representing common metabolites unrelated to the genome deletion (**Figure 4-6**). There were prominent dark bands labelled as (**7P-**) for extracts from the pigmented strains with Rf values slightly lower and higher than band 7 which were absent in the unpigmented strain (**Figure 4-6**). The dark spots are the pigmented molecules extracted from the pigmented strains, easily visible prior to staining of the TLC plate and show up as dark spots under UV light excitation following primuline staining (**Figure 4-6: A, B, D, E**). However, the pigmentation from the two-phased ethyl acetate extraction when viewed under natural light on the TLC plate is spread-out/smeared and not defined, and under UV excitation it does not appear as defined dark spots (**Figure 4-5**).

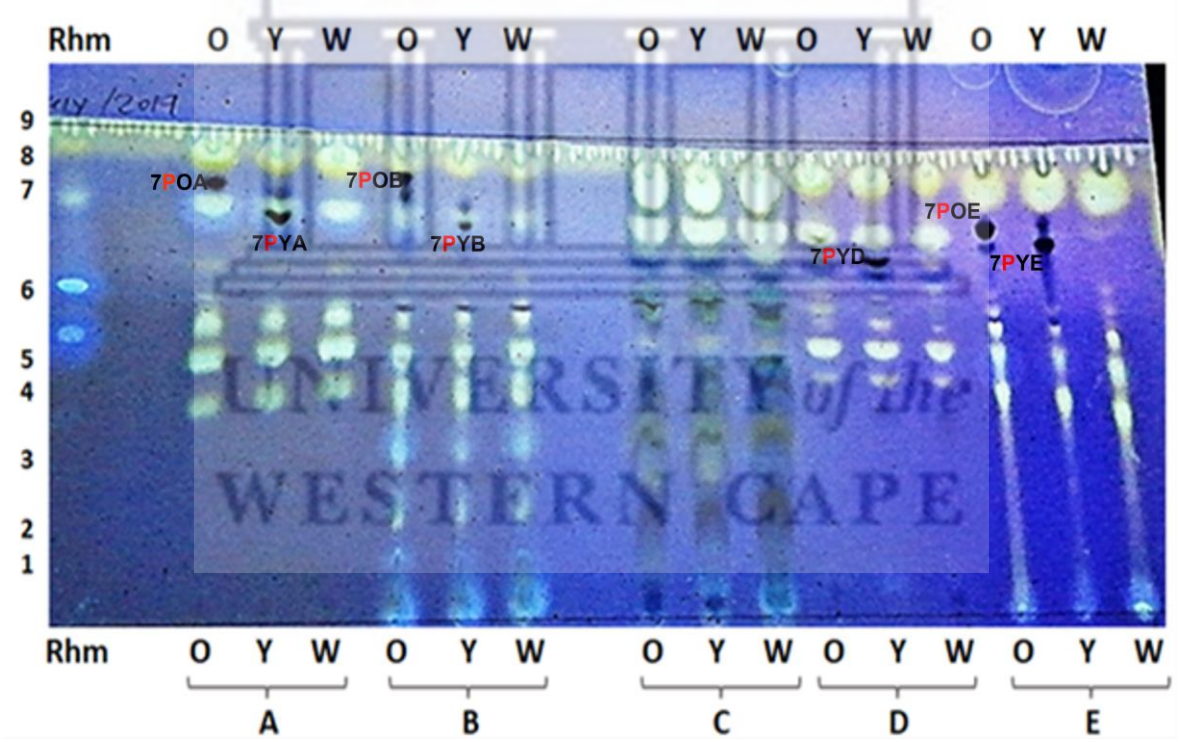


Figure 4-6: TLC analysis of the extracts from the (**O**) wild type orange, (**Y**) yellow mutant, and (**W**) unpigmented mutant *Planococcus* sp. CP5-4 strains. The extracts were prepared from 200 mL culture volume using five extraction methods; **A**: Acid precipitation plus chloroform-methanol (pH7.5), **B**: Acid precipitate dissolved in methanol, **C**: Acid precipitation extraction plus ethyl acetate, **D**: Acid precipitation plus ethyl acetate (pH7.5), **E**: Pigment extraction methods. Developed with chloroform: methanol: water: acetic acid (65:25:4:1, v/v/v/v) solvent system. The plate was captured under UV light excitation after primuline staining for lipid component indintification of the extracts. **Rhm**: rhamnolipids. (**7P-**) Dark spots representing pigmented compounds.

Acid precipitation followed by solvent extraction (chloroform-methanol (1:2, v/v)) was investigated as one of the bio-recovery procedures (**Figure 4-6**). Applying acid precipitation first helps reduce the working volume, therefore, leading to the use of a smaller volume of solvents for subsequent extractions. This method as seen in (**Figure 4-6:A**) was considered the best when compared to ethyl acetate extraction and other extraction methods. This conclusion was drawn because the smeared bands were eliminated indicating that there are fewer co-extracted compounds, instead, only defined primuline-stained extracts were present, which represents less interference. Acid precipitation coupled with solvent extraction has been used to recover biosurfactants with low molecular weight such as glycolipids and lipopeptides from *Pseudomonas* sp. and *Bacillus* sp. respectively (Rangarajan and Clarke, 2016). The ratio of chloroform-methanol used differs depending on the polarity of the targeted compounds, therefore, this procedure should be empirically determined for specific compounds (Hisham *et al*., 2019). Waghmode *et al*. (2019) achieved biosurfactant extraction from *Planococcus maritimus* SAMP MCC 3013 using a 2:1(v/v) ratio of chloroform-methanol.

Acid precipitation without solvent extraction was also investigated as a potential extraction method, to avoid the use of large volumes of solvents (**Figure 4-6:B**). When compared to ethyl acetate extraction, acid precipitation resulted in less smearing and a higher number of primuline-stained bands, which might indicate that much like acid precipitation together with solvent extraction, it was capable of minimizing co-extracted impurities. However, more lipid-stained bands were present in the former method as opposed to the latter (**Figure 4-6**). This may indicate (i) that the extraction method might be better at recovering a variety of biosurfactants/ or other lipid-containing compounds, or (ii) the method extracts higher amounts of each lipid-containing compound present thus they can be detected during TLC analysis or (iii) acid precipitation hydrolyses the lipid-containing compounds into different composites (**Figure 4-6:B**). Acid precipitation alone has been able to recover biosurfactants such as surfactin, lipopeptide, and glycolipids (Rangarajan and Clarke, 2016). The acid precipitation method is also a good alternative, that is easy, inexpensive, and readily available, with less environmental impact (Hisham *et al.,* 2019). However, the disadvantage of employing the method is that acidification can destroy the structure of the biosurfactant in question, for example, acid precipitation has been found to cause the esterification of lipopeptides (Smyth *et al*., 2010a).

Acid precipitation combined with ethyl acetate extraction before and after pH adjustments were also investigated as feasible extraction procedures (**Figure 4-6:C, D**). Before pH adjustment, only bands 7 and 8 were stained with primuline and band 7 appeared to have the largest intensity of compounds compared to all the primuline-stained bands on the TLC plate. However, after pH adjustment, there were more lipid-stained compounds and significantly reduced band smearing demonstrating that pH adjustment may be a necessary step during extraction to reduce co-extracted impurities. A major drawback of using the extraction method is that the pigmented compounds were not present on the TLC plate represented by the absence of dark spots around band 7, except for spot 7PYD (**Figure 4-6:D**). Therefore, the method might not be efficient in extracting the pigment. However, it might be a good procedure for separating the pigment from other biosurfactant-like compounds produced by the bacterium.

The TLC analysis of the pigment-extracted extracts from the cell pellet had similar band patterns exhibited by other extraction methods specifically the acid precipitation combined with chloroform-methanol (**Figure 4-6:E**). The lipid-stained compounds of band 7 near the solvent front were present for all the extraction methods used, however, they were absent when pigment extraction was employed, instead, the pigmented molecules were predominantly present in this region of the TLC for the pigmented extracts and the lipid extract for the unpigmented *Planococcus* sp. CP5-4 strain was not present. This might indicate that band 7 lipid-based compounds are either predominantly secreted into the extracellular environment, or the extraction method (chemicals and ratios used) do not support the extraction of the compound from the cell pellet. The unpigmented variant, expectedly, did not have the pigment spots at all (**Figure 4-6:E**). The pigmented molecule is absent when unpigmented extracts are observed across the different extraction procedures applied. This was the main difference observed between the pigmented variants versus the unpigmented variant extracts, as a result of the mutation(s).

A TLC fingerprint profile was recognized; bands 4,5 6,7 and 8 were present independent of the extraction method used apart from procedures that used ethyl acetate. Bands 4,5 and 6 appeared closer to the point of origin on the TLC plate and bands 7 and 8 appeared closer to the solvent front (**Figure 4-6**). The bands were given names, where bands 4, 5, 6, 7, and 8 were named CP5-4A, CP5-4B, CP5-4C, CP5-4D, and CP5-4E respectively to recognize the bands even when they have a different Rf value. The Rf value of bands was around 0.32, 0.42, 0.54, 0.85, and 0.95 respectively but the values changed slightly with each TLC developed. However, the fingerprint remained constant.

Chemical characterization of biosurfactant produced by *P. maritimus* SAMP MCC 3013 revealed a biosurfactant containing terpene, sugar, and lipid moieties in its structure (Waghmode *et al*., 2019). Ebrahimipour *et al*. (2014) also confirmed a terpene biosurfactant with lipid (fatty acid part), and carbohydrate parts. *Planococcus* species generally produce glycolipids type biosurfactants and exopolysaccharides (Waghmode *et al*., 2020). In this study primuline staining thus far has shown evidence of lipids being one of the structural composites of the active biosurfactants detected as well as evidence of pigmentation for some of the molecules extracted. The sugar moieties were investigated *via* α-naphthol [EtOH] H_2SO_4 staining (**Figure 4-7**). Sulfuric acid is a staining reagent that causes the dehydration of carbohydrates to produce an aldehyde either furfural or other derivatives which then reacts with α-naphthol resulting in the change of color (Ebrahimipour *et al*., 2014) (**Figure 4-7**). The appearance of the color purple, pink, and red indicates a positive reaction (**Figure 4-7**). The positive control rhamnolipids stained positive with purple stain both for mono-and di- rhamnolipid at an Rf value of 0.67 and 0.79 respectively (**Figure 4-7: Rhm**).

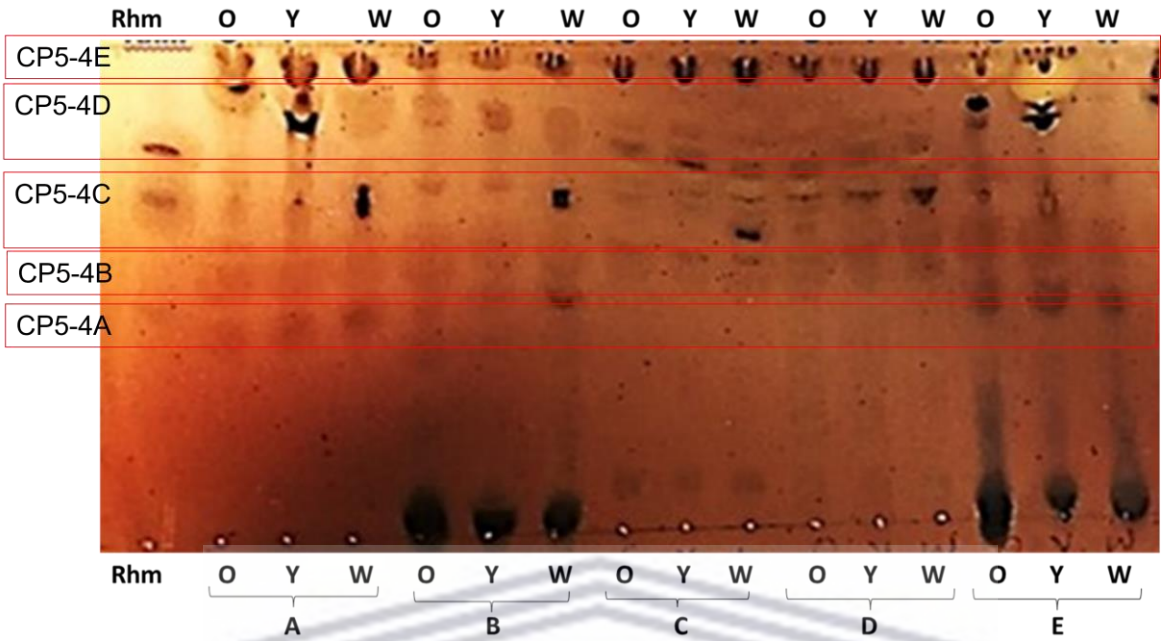


Figure 4-7: A picture of a TLC plate stained with α-naphthol [EtOH] H_2SO_4 reagent for carbohydrate components of the biosurfactant extracts. Extracted from (**O**) wild type orange, (**Y**) yellow mutant, (**W**) unpigmented mutant *Planococcus* sp. CP5-4 strains. Extracted using **A**: acid precipitation plus chloroform-methanol (pH 7.5), **B**: acid precipitate dissolved in methanol, **C**: acid precipitation extraction plus ethyl acetate, **D**: acid precipitation plus ethyl acetate (pH 7.5), and **E**: pigment extraction methods. The plate was developed with solvent system chloroform: methanol: water: acetic acid (65:25:4:1; v/v/v/v). Positive spots displayed the color purple. **Rhm**: rhamnolipids.

Band CP5-4D extracts of both the wild type and yellow mutant *Planococcus* sp. CP5-4 strain had positive results after staining, however, this was only positive for extracts extracted with acid precipitation plus chloroform-methanol as well as pigment-extracted extracts. These extracts were the pigmented molecule. The rest of the extracts belonging to the pigmented strains showed no significant evidence of staining by the reagent. The was also positive evidence of sugar moieties for band CP5-4C extracts of the unpigmented *Planococcus* sp. CP5-4 mutant, however, the same band extracts belonging to the two pigmented strains had negative results. The were traces of positive staining spread through the TLC plate, however, the spots were not as prominent because the compounds might have been slightly below our detection limit.

The extraction protocols selected for further evaluation of biosurfactants activity were the acid-precipitation combined with chloroform-methanol (1:2, v/v) and pigment extraction methods. The acid-precipitation and chloroform-methanol extraction method represented all the named bands, resulted in minimum compound smearing, appeared to have a good amount of the compounds stained with primuline and the method was able to extract the pigmented molecules that were stained with α-naphthol [EtOH] H_2SO_4 reagent. Acid precipitation alone was also an attractive alternative with extra bands namely 1, 2, and 3, however, the method does not include pH adjustment thus the compound structural configuration might be compromised and that might manifest in different ways for each compound present and because of these uncertainties, it was not employed further (**Figure 4-6**).

Pigment extraction was selected for obvious reasons, the pigmented strains had better surface activity, therefore, extraction of the pigment molecule was needed to analyze further its influence in this.

4.3.4. TLC analysis of scaled-up extracts

The previously selected extraction procedures were scaled up to process 1 L cell-free culture supernatant (CFCS). The scaling-up step was employed to improve the concentration of the compounds extracted for detection on the TLC plate and activity testing. As expected, the "fingerprint" was maintained even when a larger volume of CFCS was extracted, for both selected extraction procedures (**Figure 4-8:A, B**). However, there were additional bands which are labelled B1, B4, and B5 on the TLC plate (**Figure 4-8**). This is likely due to the concentration of these bands being higher in the final extracts due to the larger volume of culture being extracted bringing them above our detection limit. The lipid compounds of the CP5-4D band were not as prominent for extracts from the cell pellet, extracted *via* the pigment extraction protocol instead the CP5-4D band was characterized mainly by the dark pigmented spots for the pigmented *Planococcus* sp. CP5-4 strains and minor traces of the lipid molecules for all the variants. The bands detected on the TLC plates were scraped from the plate to obtain partially purified compounds and were screened for activity. The solvent system chloroform: methanol: water: acetic acid (65:25:4:1, v/v/v/v) was found to be the practical developing solvent system for the extracts in this study with minimum co-migration. However, because of the co-migration, the band area was scraped rather than specific Rf value points which was a limiting factor to the approach. For example, the CP5-4D band likely includes more than 1 compound, one of which is the pigment, while the other is a lipid compound, which is also visible in the unpigmented variant showing they co-migrate together (**Figure 4-8:C**). Therefore, the numbering on the plates refers to compounds migrating with a similar Rf and does not necessarily represent a single compound based on the staining observations between the pigmented and the unpigmented variant.

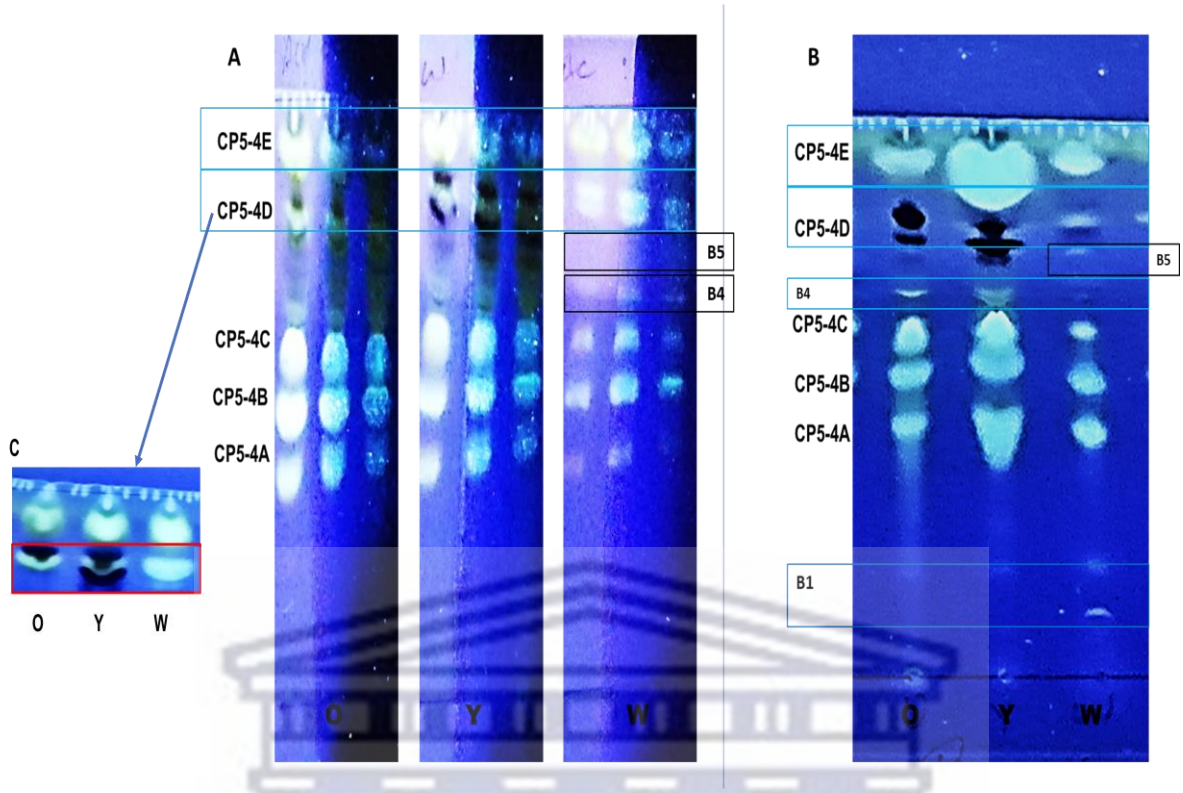


Figure 4-8:TLC analysis of extracts from the wild type orange (**O**), yellow mutant (**Y**), and unpigmented (**W**) mutant *Planococcus* sp. CP5-4 strains, extracted from 1 L culture volume using two different extraction methods. **A**: Acid-precipitation combined with chloroform-methanol (1:2, v/v) and **B**: Pigment extraction methods. Primuline was used to detect lipids and the plate was viewed and captured under UV light excitation. The extracts were developed on the TLC plate with chloroform: methanol: water: acetic acid (65:25:4:1, v/v/v/v) solvent system. **C**: Magnified example of co-migration of the pigment and primuline stained compound in band **CP5-4D**.

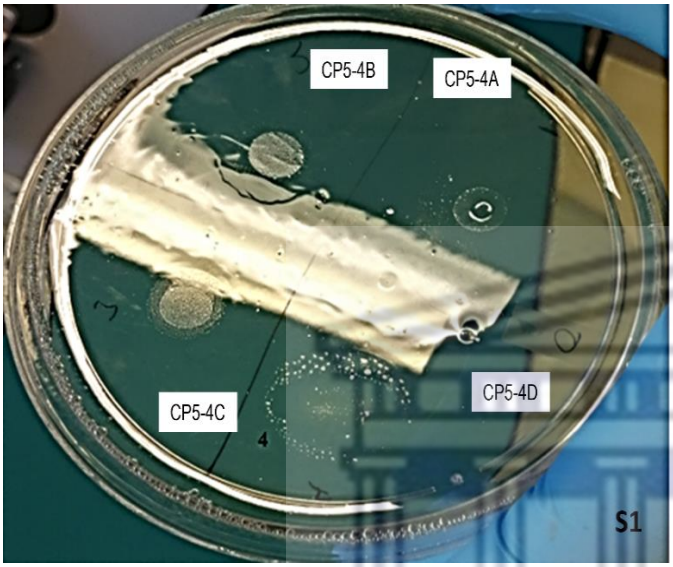
4.3.5. Evaluation of biosurfactant activity from scaled-up (1 L) fermentation

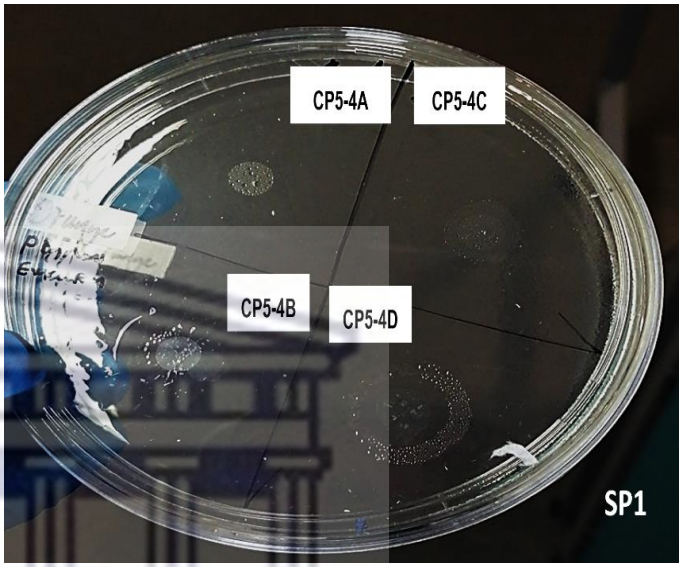
The atomized spray method, drop collapse, and micro-plate grid tests were employed to measure biosurfactant activity of the partially purified biosurfactant extracts scraped from the preparative TLC plates and dissolved in water.

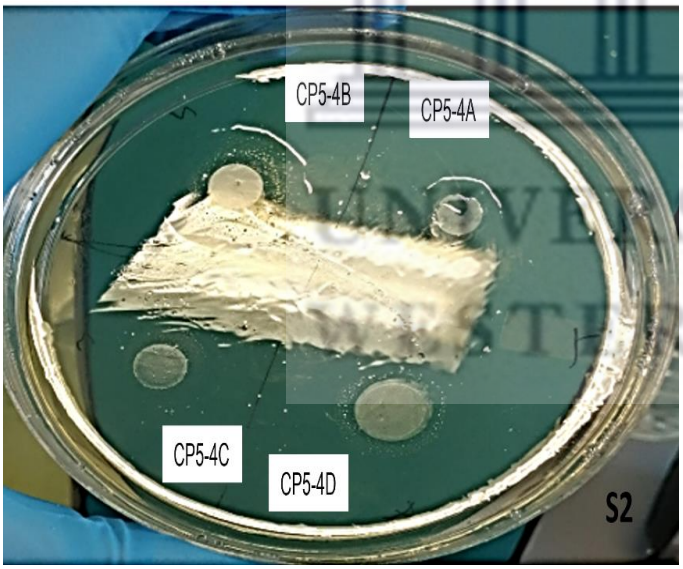
4.3.5.1. Atomized paraffin spray method

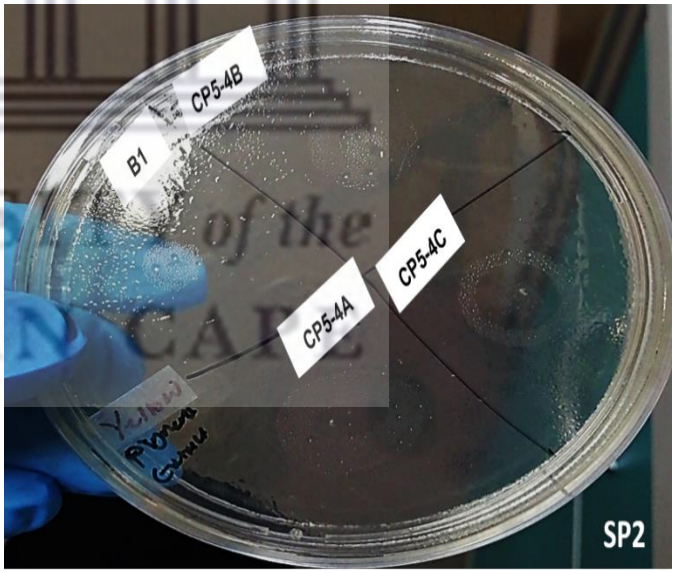
The activity was assessed for extracts of acid-precipitation combined with chloroform-methanol (1:2, v/v) (ACM) and pigment extraction (PE) methods. Multiple surface-active compounds were produced by the *Planococcus* sp. CP5-4 strain; validated by the various surface-active bands observed in (**Figure 4-9**). When screening for biosurfactant activity using the atomized spray method, a halo formation passes as a positive detection for surface activity, however, in this study there was also evidence of a solid-disc formation (**Figure 4-9: S1, CP5-4B**), and this was mainly observed for extracts of ACM (**Figure 4-9**). The difference between the two extraction methods is the source extracted from, which is either the cell-free culture or cell pellet, the type and ratio of the solvents used, and the manipulation of pH for ACM extraction. Therefore, the solid-disc formation might be pH induced precipitation such as in (**Figure 4-9: S2, CP5-4B**), however, the pH was neutralized and measured after to maintain

consistency plus some of the ACM extracts didn't have the disc formation. The absence of the solid-disc formation for the pigment extracts might also indicate that the compound that is responsible for this characteristic is produced only extracellularly or it is not extracted using the PE method, but this difference was also observed for the compounds with same the Rf value. However, these are all assumptions drawn from what was repeatedly observed. Nonetheless, the halo formation was still considered as the detector for biosurfactant activity.









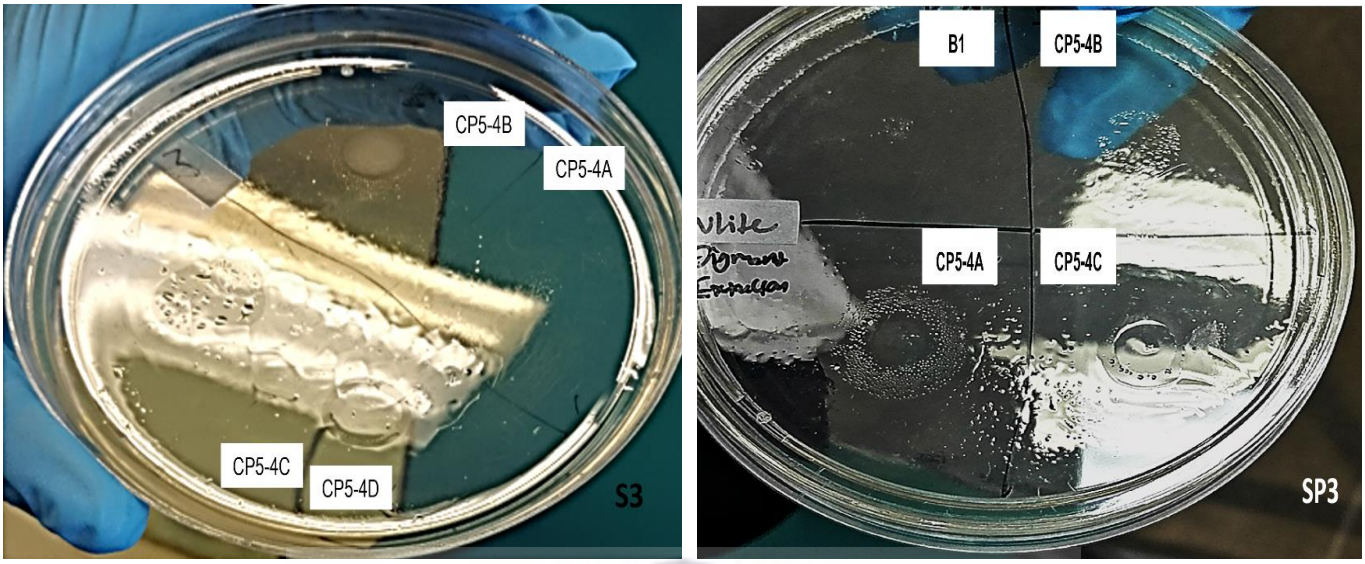


Figure 4-9: Evaluation of surface activity using the atomized spray method for the acid-precipitation combined with chloroform-methanol (1:2, v/v) extract (**S**) and the pigmented extraction method (**SP-**). Extractions were performed from 1 L culture supernatant of the wild type orange (-**1**), yellow mutant (-**2**), and unpigmented mutant (-**3**) *Planococcus* sp. CP5-4 strains. Hallo formation around an extract indicates a positive result for biosurfactant activity. The abbreviation of bands screened is denoted in **section 4.3.3.**

When comparing the activity of the bands extracted using the ACM method, the largest halo formation was measured for the CP5-4B extracts belonging to all the strains/variants (**Table 4-3**). The halo diameter was measured at 17.0 ± 0.82, 23.2 ± 1.31, and 15.5 ± 1.5 mm for wild type orange, yellow mutant, and unpigmented mutant *Planococcus* sp. CP5-4 strains respectively (**Table 4-3**). The CP5-4D extract from the wild type orange and yellow mutant *Planococcus* sp. CP5-4 strains also had large halo formation, measured at 22.0 ± 0.82 and 16.3 ± 0.47 mm respectively (**Table 4-3**). However, the CP5-4D extract displayed a reduced halo size measured at 13.7 ± 0.47 mm for the unpigmented mutant *Planococcus* sp. CP5-4 variant (**Table 4-3**). The difference observed from the TLC plate between the CP5-4D extracts of the pigmented and unpigmented *Planococcus* sp. CP5-4 strains was that the pigmented strains had the pigment and lipid molecules present on the band, and the unpigmented strain had only the lipid molecule (**Figure 4-8**). Halo diameters measured between 10-15 mm for the CP5-4C extract of the wild type *Planococcus* sp. CP5-4 strain, for the CP5-4A and CP5-4C extracts of the yellow *Planococcus* sp. CP5-4 variant, and for the CP5-4A, CP5-4C, and B4 extracts of the unpigmented *Planococcus* sp. CP5-4 variant **(Table 4-3)**. The rest of the bands had halo formation measured below 10 mm or were non-active. After screening pigment-extracted extracts, the largest halo diameter was measured for; CP5-4B and CP5-4D extracts of the wild type *Planococcus* sp. CP5-4 strain (**Table 4-3**); for CP5-4A, CP5-4B, and CP5-4C extracts of the yellow mutant *Planococcus* sp. CP5-4 variant, and for CP5-4A extract of the unpigmented *Planococcus* sp. CP5-4 variant (**Table 4-3**). Most of the extracts left had a halo diameter measured between 10-15 mm (**Table 4-3**). The rest of the screened extracts had an insignificant activity or were non-active (**Table 4-3**).

Table 4-3: A table showing the diameter (mm) measurements of the halo formation around extracts after employing atomised spray test for screening.

Atomised spray test: Diameter (mm)						
	Acid-precipitation and chloroform-methanol			Pigment extraction		
Band extracts	**O**	**Y**	**W**	**O**	**Y**	**W**
B1	0	0	0	N/A	9.00 ± 0.00	3.33 ± 0.47
CP5-4A	9.16 ± 0.623	12.3 ± 0.47	7.00 ± 0.81	6.67 ± 0.47	23.5 ± 1.31	20.3 ± 0.47
CP5-4B	17.0 ± 0.82	23.2 ± 1.31	15.5 ± 1.5	17.3 ± 0.47	17.2 ± 0.62	12.0 ± 0.00
CP5-4C	12.0 ± 0.40	12.5 ± 0.41	14.2 ± 0.85	12.2 ± 0.24	17.7 ± 0.48	13.5 ± 0.41
CP5-4D	22.0 ± 0.82 (P)	16.3 ± 0.47 (P)	13.7 ± 0.46	20.3 ± 0.47 (P)	11.7 ± 0.47 (P)	N/A
CP5-4E	N/A	3.67 ± 0.47	N/A	N/A	N/A	N/A
B4	0	0	11.3 ± 0.1.89	N/A	N/A	N/A
B5	0	0	N/A	0	0	5.33 ± 1.25

The extracts were extracts from the wild type orange (**O**), yellow mutant (**Y**), and unpigmented mutant (**W**) of the Planococcus sp. CP5-4 strains. Black indicates no "-" activity <, purple"1-9 mm" <, green "10-15 mm" < and red "'<15 mm".*p = pigmented band.*N/A and 0 = non-active bands and bands that are not present respectively. The abbreviation of bands screened is denoted in **section 4.3.3.**

The wild type orange bands CP5-4B and CP5-4D had the largest halo diameter measured which was consistent and independent of the extraction method used, moreover, the activity for every other band screened for the wild type *Planococcus* sp. CP5-4 strain had the same diameter range. For CP5-4B extracts a large diameter was measured independent of the strain, except for the unpigmented variant when pigment extraction is employed as the extraction method, however, some of the bands did show significant activity for the unpigmented *Planococcus* sp. CP5-4 variant that is comparable to the two pigmented *Planococcus* sp. CP5-4 strains. Nevertheless, the overall diameter measurements for the unpigmented *Planococcus* sp. CP5-4 variant were generally measured as least in comparison to the pigmented *Planococcus* sp. CP5-4 strains. The yellow *Planococcus* sp. CP5-4 strain had the highest number of bands with large diameter measurements.

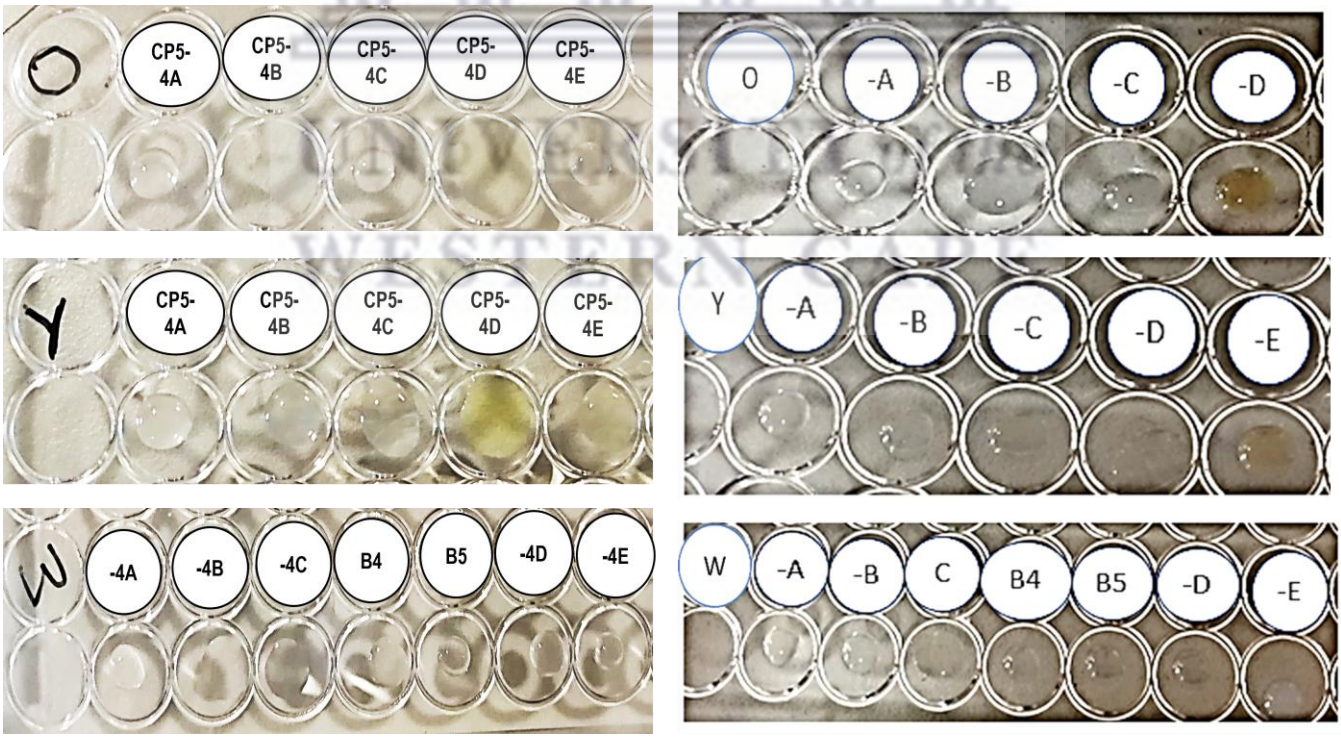
The positive results from both extraction procedures demonstrate that *Planococcus* sp. CP-54 strain might be producing the biosurfactant(s) both extracellularly and intracellularly and/or the production is cell-bound (**Table 4-3**). All the variants were observed to have multiple surface-active compounds, meaning that *Planococcus* sp. CP5-4 produces more than one biosurfactant product, furthermore, the strain was able to synthesize surface active compound(s) independent of the mutation(s). This was supported by the activity observed from the unpigmented *Planococcus* sp. CP5-4 variant extracts. When extracting pigment from the cell pellet by disrupting the cell membrane all that is contained within the cell is extracted including the targeted pigment molecule and other cell-

bound compounds that might be surface-active, which might explain the surface activity of the unpigmented mutant strain even when the pigment extraction method was employed for extraction (Melendez-Martinez *et al*., 2019; Ram *et al*., 2020). However, the wild type orange and yellow mutant extracts displayed significantly larger halo diameter measurements compared to the unpigmented mutant strain extracts. Therefore, the strengthened the correlation already highlighted that exist between biosurfactant activity and the pigment synthesised by the *Planococcus* sp. CP5-4 strains.

4.3.5.2. Drop collapse test

The stability of the drop is dependent on the biosurfactants' concentration, after performing the drop collapse test, drops collapsed completely or spread out for the control sample 10% (w/v) SDS and remained bead up for water (**Figure 4-10**). There were fewer overall active bands found when drop collapse was applied for screening compared to when the atomized spray method was used which is what is normally observed in literature as the drop collapse assay has lower sensitivity. The ACM extracts yielded more activity with drops that collapsed completely compared to the extracts obtained through the pigment extraction method (**Figure 4-10**). The observed difference might be dependent on the extraction method itself, since different extraction procedures will be better at pulling out certain compounds than others, furthermore, extracting from the total supernatant volume versus from the cell pellet is more likely to yield different extract concentrations even of the same compound(s).

Acid-precipitation and chloroform-methanol (1:2, v/v) **Pigment extraction method**



H_2O **10% (w/v) SDS**

Figure 4-10: Images of the drop collapse test results. The partially purified extracts prepared using acid-precipitation combined with chloroform-methanol (1:2, v/v) and pigment extraction methods, extracted from wild type orange (**W**), yellow mutant (**Y**), and unpigmented mutant (**W**) *Planococcus* sp. CP5-4 strains and screened for surface activity by placing sessile drops on a hydrophobic surface. The numbers represent the band extract numbers from the TLC plates. Water (H_2O) was employed as a negative control with a beaded-up drop and 10% (w/v) SDS as a positive control with a spread-out drop. The abbreviation of bands screened is denoted in **section 4.3.3.**

Complete drop collapse was realized for CP5-4B and CP5-4D compounds from the wild type *Planococcus* sp. CP5-4 strain, for CP5-4D extract from yellow *Planococcus* sp. CP5-4 variant and for CP5-4B extract from unpigmented *Planococcus* sp. CP5-4 variant following ACM extraction, therefore, showing a higher concentration of the surface active compound(s) than the rest of the extracts observed (**Figure 4-10**; **Table 4-4**). The completed drop collapse was observed for CP5-4D for the pigmented *Planococcus* sp. CP5-4 strains while the same band had reduced activity for the unpigmented *Planococcus* sp. CP5-4 variant (**Table 4-4**). Complete drop collapse was also observed for CP5-4B from the wild type, for CP5-4C, and CP5-4D from the yellow mutant, and for CP5-4C from the unpigmented mutant *Planococcus* sp. CP5-4 strains of the pigment extracted extracts (**Figure 4-10**; **Table 4-4**). However, the activity for CP5-4D extracts from the wild type *Planococcus* sp. CP5-4 strain was reduced when the pigment-extracted extracts were screened, which altered from the screening results observed up until now. Nevertheless, the same band from the unpigmented molecule showed no activity at all. The reduced activity of the unpigmented *Planococcus* sp. CP5-4 variant validates further that the presence of pigmentation does indeed affect the biosurfactants synthesised by *Planococcus* sp. CP5-4.

Table 4-4: A table representing the drop collapse test results for extracts from the wild type orange (**O**), yellow mutant (**Y**), and unpigmented mutant (**W**) *Planococcus* sp. CP5-4 strains.

Drop collapse test						
	Acid-precipitation and chloroform-methanol extraction			Pigment extraction		
Band extract	**O**	**Y**	**W**	**O**	**Y**	**W**
B1	0	0	0	N/A	N/A	N/A
CP5-4A	N/A	N/A	N/A	N/A	N/A	N/A
CP5-4B	+++	+	+++	+++	++	++
CP5-4C	N/A	++	++	+	+++	+++
CP5-4D	+++ (P)	+++ (P)	+	++ (P)	+++ (P)	N/A
CP5-4E	N/A	+	N/A	N/A	++	N/A
B4	0	0	N/A	N/A	N/A	N/A
B5	0	0	N/A	0	0	N/A

The extracts were extracted using acid-precipitation combined with chloroform-methanol (1:2, v/v) and pigment extraction methods. Black indicates no "-" activity <, purple insignificant "+"<, green average to good "++"< and red excellent ''+++" concentration ranking.*p = pigmented band.*N/A and 0 = non-active bands and bands that are not present respectively. The abbreviation of bands screened is denoted in **section 4.3.3.**

4.3.5.3. Microplate-grid analysis assay

More bands showed activity after screening the extracts with a microplate-grid analysis assay compared to when the drop collapse test was used (**Figure 4-11**). One of the challenges with the grid test was that some samples were foggy, an example being the CP5-4B extract of the wild type *Planococcus* sp. CP5-4 strain (**Figure 4-11**), thus abstracting the grid distortion to be viewed, this issue was also observed for bands extracted with color, this meant that some bands were not well represented making this test the least reliable out of the three activity test employed (**Figure 4-11**).

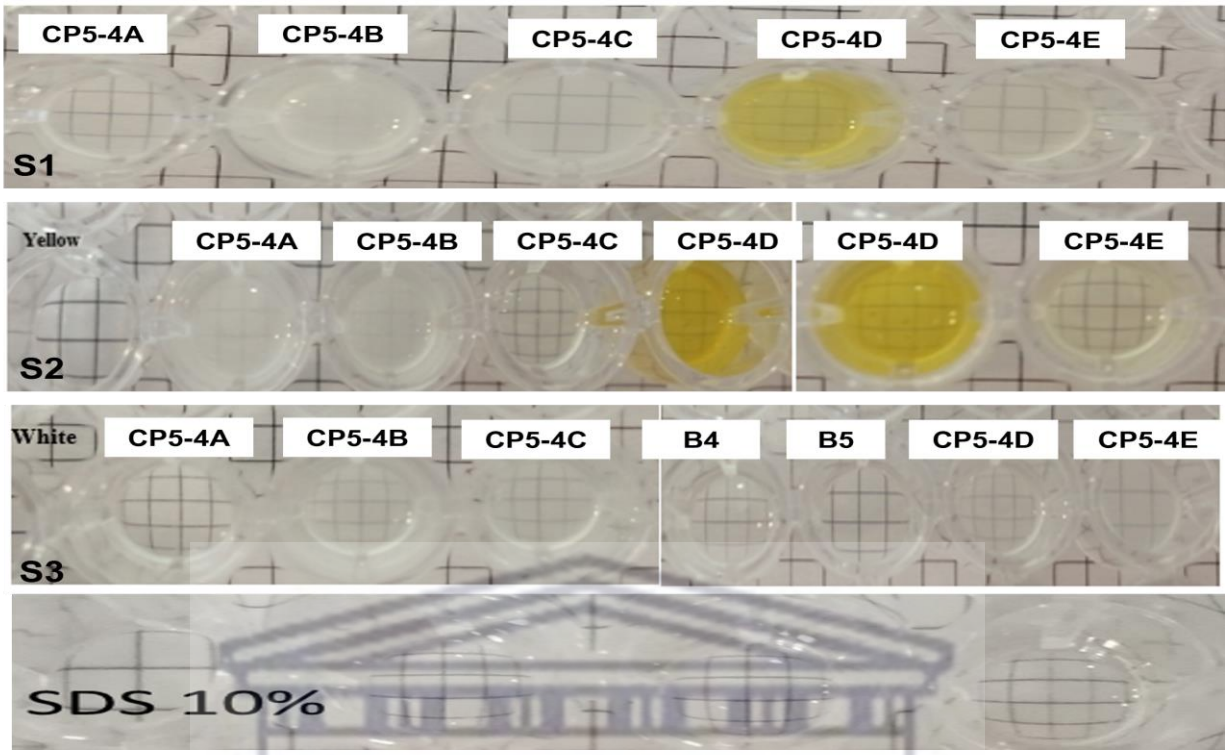


Figure 4-11: Photo of microplate-grid analysis assay results, showing extracts screened for biosurfactant activity. The extracts were extracted from the wild type orange (**S1**), yellow mutant (**S2**), and unpigmented mutant (**S3**) *Planococcus* sp. CP5-4 strains using acid precipitation combined with chloroform-methanol (1:2, v/v). The numbers on the plate represent the band number of the scraped extract developed on a preparative TLC plate. The positive control 10% (w/v) SDS is represented by grid distortion. The abbreviation of bands screened is denoted in **section 4.3.3.**

Through counting the number of squares in the well, significant grid distortion was observed for band CP5-4D extract from the wild type *Planococcus* sp. CP5-4 strain, CP5-4C, CP5-4D, and CP5-4E extracts of the yellow *Planococcus* sp. CP5-4 variant and CP5-4B, CP5-4C, and CP5-4D extracts of the unpigmented *Planococcus* sp. CP5-4 variant (**Table 4-5**). The rest of the bands had insignificant, or no grid distortion detected. The CP5-4D extracts from the wild type and yellow *Planococcus* sp. CP5-4 strains had significant grid-distortion therefore activity which was consistent with the two previously used screening methods. The consistency was also maintained for CP5-4B extracts of the unpigmented *Planococcus* sp. CP5-4 variant (**Table 4-5**). Band CP5-4B extract of the wild type *Planococcus* sp. CP5-4 strain did not show significant distortion indicating low activity which was not consistent with what has been screened thus far and to the CP5-D extract of the strain. The activity intensity is generally similar for these two bands independent of the screening method and extraction procedure employed. Significant distortion was observed for band CP5-4D extract of the unpigmented mutant strain which contradicted the results found from results observed thus far (**Table 4-5**). This might be due to the fogged wells of CP5-4D that the distortion was interfered or the change in pattern might be related to the difference in the method used, the atomized spray test and drop collapse test do not favour the detection of hydrophobic biosurfactants that much, therefore, the grid test might have increased sensitivity to hydrophobic compounds when compared to the

former tests resulting in better detection. Nevertheless, due to the mentioned challenges of employing the grid test, which might have influenced the results the test was not reliable for this study.

Table 4-5: Microplate-grid analysis assay results for biosurfactant activity.

Acid-precipitation and chloroform-methanol			
Band	**O**	**Y**	**W**
B1	0	0	0
CP5-4A	+	+	+
CP5-4B	++	++	+++
CP5-4C	N/A	+++	+++
CP5-4D	+++ (P)	+++ (P)	+++
CP5-4E	N/A	+++	N/A
B4	0	0	++
B5	0	0	N/A

The extracts were extracted from wild type orange (**S1**), yellow mutant (**S2**), and unpigmented mutant (**S3**) *Planococcus* sp. CP5-4 strains, extracted using acid-precipitation and chloroform-methanol (1:2, v/v) extraction method. Black indicates no "-" activity <, purple insignificant "+" activity <, green average to good "++" activity < and red excellent ''+++" activity ranking.*p = pigmented band.*N/A and 0 = non-active bands and bands that are not present respectively. The abbreviation of bands screened is denoted in **section 4.3.3.**

To improve the separation and purification of the various compounds that could contribute to biosurfactant activity we experimented with a second solvent system/solvent system B (S_2; chloroform: methanol: water: acetic acid [130:30:4:1;v/v/v/]) for the separation of compounds on preparative TLC plates. It was thought that the reduced polarity would lead to improved separation of compounds migrating close to the solvent front in the original solvent system/solvent system A (S_1; chloroform: methanol: water: acetic acid [65:30:4:1; v/v/v/v]). This change in the system was found to result in the separation required (**Figure 4-12**). By comparison of panels B and C compared to panel A (**Figure 4-12**) and the TLC plate from (**Figure 4-8**), it can be clearly seen that the extract representing CP5-4D constituted more than one compound which separates more clearly into multiple bands by adjusting the solvent system, represented in panel B and C (**Figure 4-12**). The lipid compound in CP5-4D$_{S2}$ was separated from the pigmented compound in CP5-4D(P) both for the wild type and yellow mutant *Planococcus* sp. CP5-4 strains (**Figure 4-12:B, C**). Therefore, in addition to the named bands CP5-4A, CP5-4B, CP5-4C, CP5-4D, and CP5-4E identified through the TLC analysis presented in (**Figure 4-8**) and panel A, bands CP5-4D(P), CP5-4D$_{S2}$, and CP5-4E$_{S2}$ were differentiated as depicted in (**Figure 4-12**). Especially noticeable was that CP5-4D(P) represented multiple pigmented compound (**Figure 4-12**). Furthermore, the extracts from the yellow *Planococcus* sp. CP5-4 variants seem to have a carotenoid/pigment compound that is not present in the wild type *Planococcus* sp. CP5-4 strain. This pigment might relate to different intermediates in the carotenoid synthesis, the wild type also produces this pigment but at much lower concentrations (**Figure 4-12**:**B**). To further characterize the putative biosurfactant compounds both solvent systems were employed to achieve the best separation and purification for each

compound. This resulted in the highest purity of compounds extracted in this study and in the next section we describe their characteristics using low-bond axisymmetric drop shape analysis.

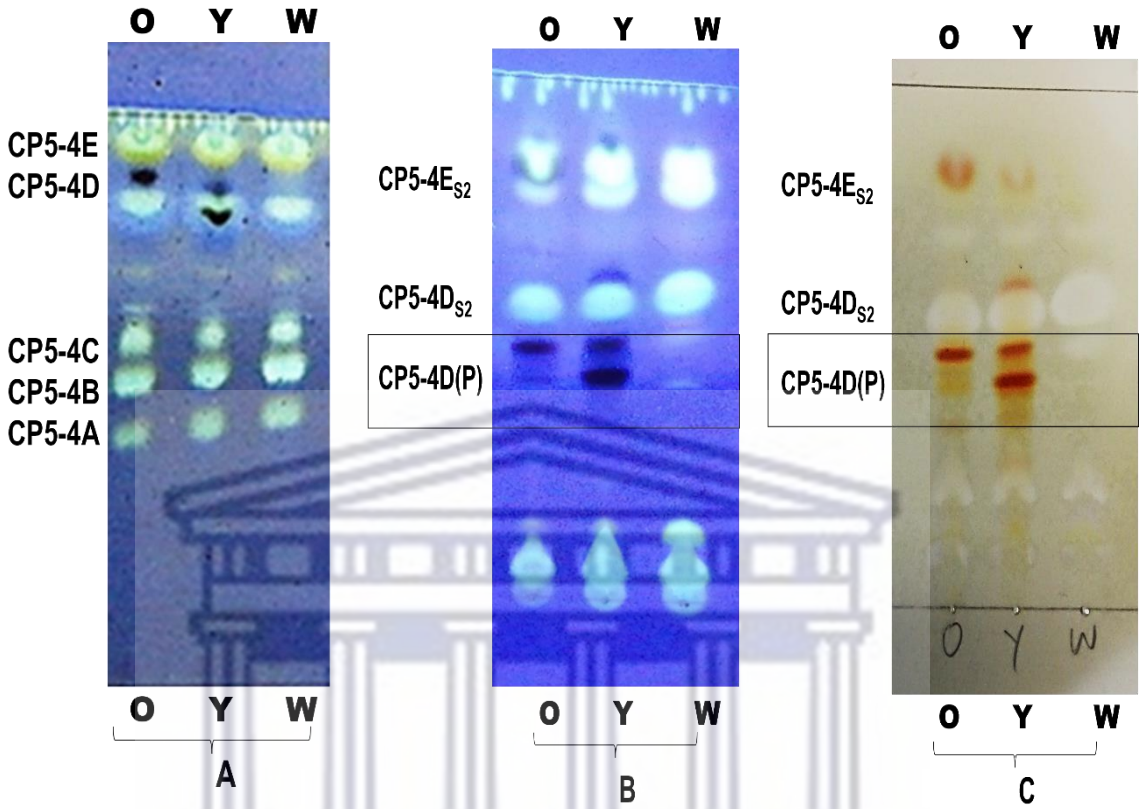


Figure 4-12: Results showing the difference in extract development on TLC plates when two different solvent-developing systems are used to further tease apart the various compounds for better resolution. Acid precipitation plus chloroform-methanol extraction method was employed to obtain extracts from (**O**) wild type orange, (**Y**) yellow mutant, and (**W**) unpigmented mutant *Planococcus* sp. CP5-4 strains. Images of TLC plates developed using solvent system **A**: chloroform: methanol: water: acetic acid [65:30:4:1; v/v/v/v] and **B**: chloroform: methanol: water: acetic acid [130:30:4:1;v/v/v/v] were employed both viewed and captured under UV light excitation after primuline staining. Panel **C**: same plate as panel B viewed and captured under natural light after primuline staining. Primuline was used to detect lipids. The abbreviation of bands screened is denoted in **section 4.3.3.**

4.3.6. Quantitative comparison of surface tension contact angle, measured using low-bond axisymmetric drop shape analysis (LB-ADSA) by Image J

The individual bands were characterized by determining their effect on droplet shape using low-bond axisymmetric drop shape analysis (LB-ADSA), see the example of a contact angle measure below (**Figure 4-13**). The contact angle is related to the surface tension and interfacial tension reduction abilities of a surface-active compound.

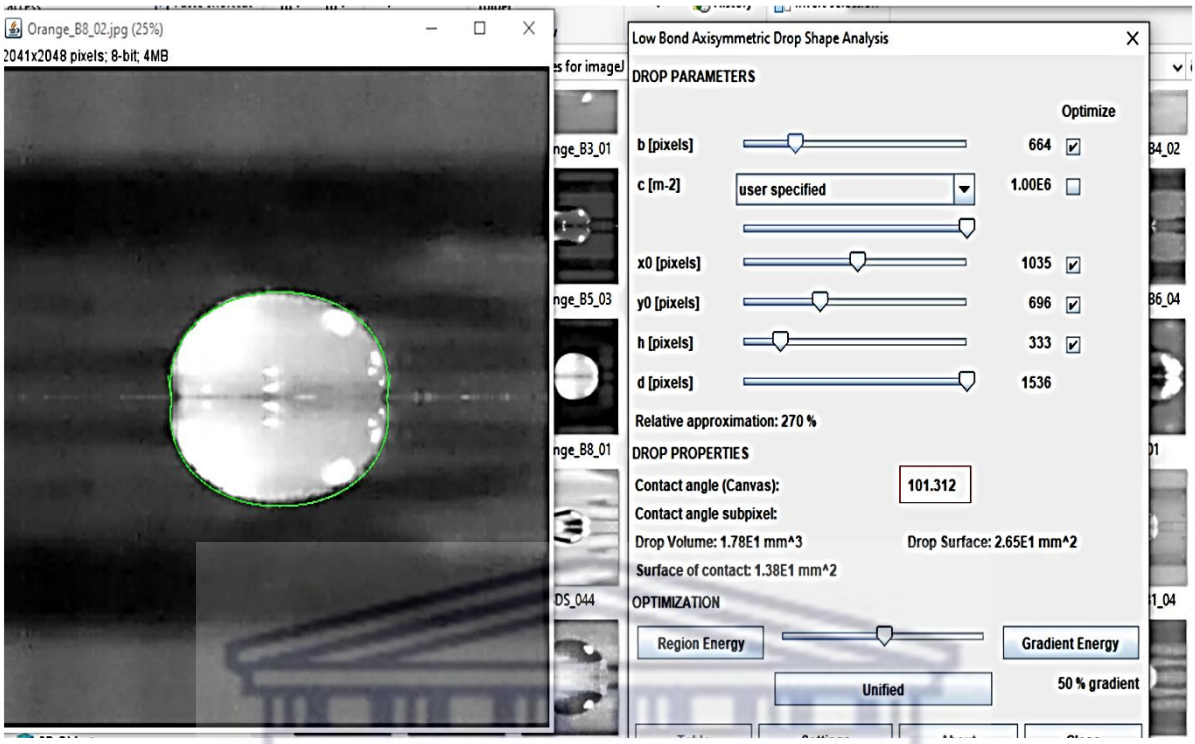
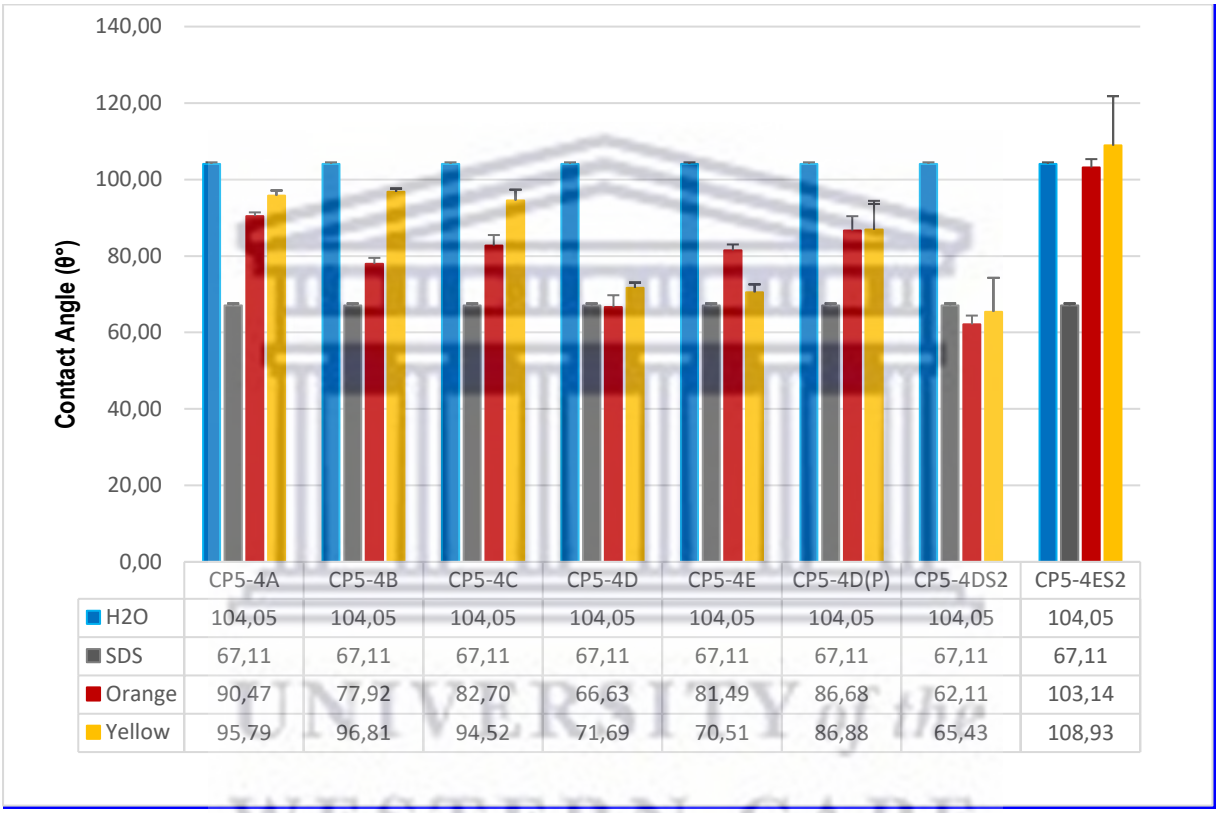


Figure 4-13: Example of the contact angle measurement of the drop collapse image via Image J programming using low-bond axisymmetric drop shape analysis (LB-ADSA).

The contact angle for water was measured at 104° ± 0.45, however, after the addition of 10% (w/v) SDS, it was reduced to 67.1° ± 0.48, which is a 36.89% angle reduction. The CP5-4D and CP5-4D$_{S2}$ extracts from the wild type *Planococcus* sp. CP5 had the lowest contact angles, measured at 66.6° ± 3.11 and 62.1° ± 3.73 respectively, both lower than the angle measured for 10% (w/v) SDS (**Figure 4-14**). The CP5-4D, CP5-4E, and CP5-4D$_{S2}$ extracts from the yellow *Planococcus* sp. CP5 variant had the lowest contact angles for the variant (**Figure 4-14**). The contact angle for CP5-4D$_{S2}$ was 65.4° ± 8.83 which was also lower than the contact angle measured for 10% (w/v) SDS. The yellow mutant *Planococcus* sp. CP5-4 variant had significant activity showing that the deletion of the CrtNb-like apo-4, 4'-terminal methyl oxidase gene confirmed by Moyo *et al*., (2022) does not compromise the amphiphilic structural integrity consequently allowing the synthesized carotenoid to retain the surface activity. However, because of the point deletion on the pathway the yellow *Planococcus* sp. CP5-4 variant had slight physicochemical differences from the wild type *Planococcus* sp. CP5-4 strain. The relatively high surface reduction abilities were detected for CP5-4D extracts from both pigmented *Planococcus* sp. CP5 strains and this was consistent with the rest of the activity tests performed in this study, with the wild type *Planococcus* sp. CP5 strain's extract displaying relatively better surface reduction abilities. The CP5-4D(P) bands with pigmented compounds from the wild type and yellow mutant *Planococcus* sp. CP5-4 strains conferred significant activity, however, after band separation the surface reduction ability of the pigmented molecules was reduced when compared with the activity measured for the CP5-4D extract constituting both the lipid and pigment molecules. The improved surface reduction ability was instead observed for CP5-4D$_{S2}$ indicating that the lipid compound had better surface reduction abilities than the pigmented molecules. However, throughout the activity tests performed in this study lipid the CP5-

4D extract from the unpigmented *Planococcus* sp. CP5-4 variant mostly showed reduced activity when compared to the CP5-4D extract of the pigmented *Planococcus* sp. CP5-4 strains and since the lipid stained compounds of CP5-4D from all the variants share the same Rf value they were thought to be more likely the same compound. Therefore, this observation of improved activity did not correlate with what has been observed about this extract. Nonetheless, it was noted in this study that the extracts containing pigmented compounds were able to reduce the contact angle of water significantly. The presence of the carotenoid biosynthetic pathway producing the carotenoids/pigments on the overall activity when compared to the unpigmented *Planococcus* sp. CP5-4 variant was considered to infer the most significant influence.



	CP5-4A	CP5-4B	CP5-4C	CP5-4D	CP5-4E	CP5-4D(P)	CP5-4DS2	CP5-4ES2
H2O	104,05	104,05	104,05	104,05	104,05	104,05	104,05	104,05
SDS	67,11	67,11	67,11	67,11	67,11	67,11	67,11	67,11
Orange	90,47	77,92	82,70	66,63	81,49	86,68	62,11	103,14
Yellow	95,79	96,81	94,52	71,69	70,51	86,88	65,43	108,93

Figure 4-14: Contact angle measurements of TLC-prepared extracts as detailed in **Figure 4-12** for the wild type orange and yellow mutant strains. The contact angle (θ°) was measured using LB-ADSA. The contact angle measures the wettability of a solid by a liquid; non-wettability ≥ 90° ≤ general wettability ≥ 0°≤ complete wettability. Water (H_2O) was used as a negative control and 10% (w/v) SDS as a positive control. Error bars are expressed as mean ± standard deviation (± SD, n=4), where n represents the number of measurements of contact angle for each sample. The abbreviation of bands screened is denoted in **section 4.3.3.**

The contact angle is dependent on the hydrophobicity of the solid surface and the liquid sample. For instance, the biosurfactant produced by *P. maritimus* SAMP MCC 3013 reduces the contact angle of water on a teflon surface from 125.3° to 103.0° (22.3%), on a parafilm surface from 103.9° to 91.2° (12.7%) and on a glass surface from 22.4° to 20.0° (2.4%) (Waghmode *et al*., 2019). This shows a gradient activity profile of the surfaces from most hydrophobic to least hydrophobic. The hydrophobicity of the parafilm surface is close to that of the polyolefin surface used in this study because water has a contact angle of 104° on both surfaces. The biosurfactants

produced by *Planococcus* sp. CP5-4 exhibited far better surface activity, as most of the extracts achieved a contact angle reduction below the 91.2° measured for *P. maritimus* SAMP MCC 3013. The CP5-4D$_{S2}$ extracts, displayed the highest reduction with a contact angle measurement of 62.1° ± 3.73 (**Figure 4-14**). However, concentration is a big factor in the measured difference, and we do not know the concentration at which we applied the biosurfactant to various assays due to the lack of an appropriate standard.

The CP5-4D and CP5-4D$_{S2}$ extracts were able to reduce the contact angle of water 3 times greater than the glycol-carotenoid/-terpenoid biosurfactant synthesized by *P. maritimus* SAMP MCC 3013. Furthermore, *Planococcus* sp. CP5-4 biosurfactants have slightly improved wetting properties than rhamnolipid when compared to the results obtained from a study done by Gomari and colleagues (2018). The objective of their study was to use rhamnolipids to increase the wettability of carbonate rock, which was grounded to form a calcium carbonate disc. The average contact angle was 108.6° if the disk was washed with water, which was reduced to 73.4° after washing the disc with a rhamnolipids solution (Gomari *et al*., 2018). This reduction difference is slightly lower than what we recorded in our study. Carotenoid biosurfactants from *Planococcus* species reported in literature do display low micelle concentration (CMC) and high surface tension reduction abilities that rank them as comparable to rhamnolipids, surfactin, and sophorolipids (Waghmode *et al*., 2019; Chen *et al*., 2021; Kim *et al*., 2022). The carotenoid biosurfactant produced by *Planococcus* SAMP MCC 3013 was able to reduce the surface tension of PBS from 72 to 30 mN/m and had a CMC value of 1.3 mg/mL (Waghmode *et al*., 2019). The *Planococcus* sp. XW-1 strain can reduce the surface tension of water to 26.8 mN/m and has a CMC value of 60 mg/L (Guo *et al*., 2022). The highly active and efficient rhamnolipids, surfactin, and sophorolipids biosurfactants are found to have a surface tension of 24, 27, and 30-35 mN/m and CMC values of 20, 11, and 150 mg/L, respectively, which is close to the range of what has been measured for carotenoid biosurfactants (Chen *et al*., 2021; Shakeri *et al* 2021; Kim *et al*., 2022). Therefore, *Planococcus* sp. CP5-4 strain is a potentially efficient biosurfactant with promising surface activity.

4.3.7. The carotenoid biosynthetic pathway in the production of biosurfactant by *Planococcus* sp. CP5-4

The main biosurfactant activity seems to be associated with the carotenoids. Bacteria belonging to the *Planococcus* genus mainly synthesize glycosylated carotenoid acid esters carotenoids, characterized by a glucose molecule esterified to both a triterpenoid carotenoid carboxylic acid and a fatty acid (FA) (Shindo *et al*., 2008; 2014). *Planococcus* sp. CP5-4 produces methyl 5-(6-C17:3)-glucosyl-5, 6'-dihydro-apo-4, 4'-lycopenoate (**Figure 4-15**) as the main carotenoid and pigment and the biosynthesis has been elucidated by Moyo *et al*. (2022) (**Figure 4-16**), in which the P450 hydroxylated terminal methylene group is glycosylated by a glucosyltransferase for the synthesis of methyl 5-glucosly-5, 6-dihydro-apo-4, 4'-lycopenoate (**Figure 4-16**). Following this, the polyunsaturated (8Z, 11Z, 14Z) heptadecatrionic fatty acid is acylated by a glycerol acyltransferase to what is synthesised, to give out the final glucosylated C_{30}-carotenoid fatty acids ester compound (**Figure 4-16**).

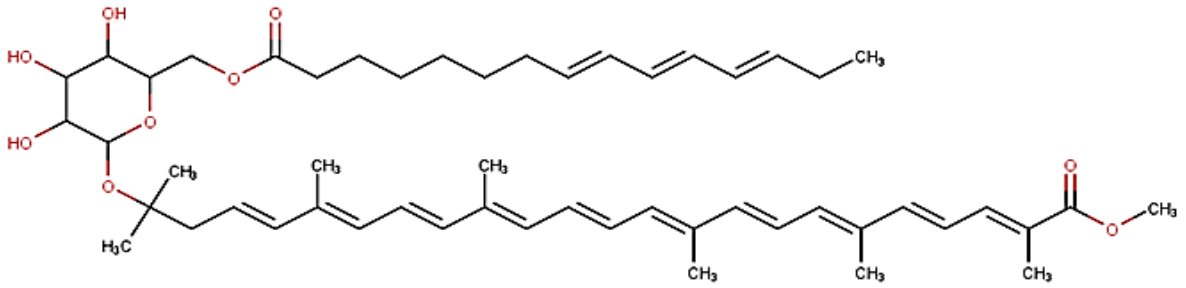


Figure 4-15: The main pigment compound produced by *Planococcus* sp. CP5-4 strain; methyl 5-(6-C17:3)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate (Moyo *et al*., 2022).

Figure 4-16: Biosynthetic cascade showing the synthesis of C_{30}-carotenoid fatty acids ester by *Planococcus* sp. CP5-4 (Moyo *et al*., 2022).

The structural composition of the synthesized C_{30}-glycosylated carotenoid fatty acid ester is composed of two hydrophobic moieties, the C_{30}-carotenoid, and fatty acids both linked to hydrophilic carbohydrate moiety (**Figure 4-15**). This is the basic prerequisite of a potent biosurfactant making the C_{30}-glycosylated carotenoid fatty acid ester an ideal biosurfactant candidate. The CTAB/methylene blue agar assay employed in this study suggested that the potential biosurfactants produced by *Planococcus sp*. CP5-4 strains may not be anionic in nature. This confirmed that none of the biosurfactants synthesized by the strain have glycosidic bonds, therefore, supporting the theory that the linkage between hydrophilic and hydrophobic moieties is an ester bond. The ester bond is supported further by the structural elucidation of the carotenoid synthesized by the *Planococcus sp*. CP5-4 strain as characterized by Moyo *et al*., (2022). This work provides supporting evidence that the methyl 5-(6-C17:3)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoateas characterized by Moyo and colleagues (2022) is one of the biosurfactants synthesized by *Planococcus* sp. CP5-4, which falls under the class of carotenoid glycolipids. This means that the *Planococcus* sp. CP5-4 synthesizes a novel biosurfactant, because the fatty acid C17:3 polyunsaturated fatty acid (8Z, 11Z, 14Z)-heptadecatrionic acid structure is a first to be reported for the *Planococcus* genus (Moyo *et al.*, 2022).

Employing the structure of the C_{30}-carotenoid fatty acids ester biosurfactant, the hydrophilic-lipophilic balance (HLB) value for the compound was calculated to be 4.96, this value predicts the type of emulsions and behavior to be water in oil emulsion and this was confirmed as emulsification by the extracts resulted in a type II Winsor microemulsion, evidently supported in Chapter 3. HLB affects the stability of the emulsion, compounds with low HLB tend to be more hydrophobic because they tend to have more non-polar groups and result in water-in-oil emulsion, whereas compounds with a high-value HLB are dominated by polar groups and form oil-in-water emulsion (Akbari *et al*., 2018). Emulsification is one of the significant biosurfactant functional properties that is heavily exploited by the food and cosmetics industries, mostly using it to control the consistency and texture of products. Emulsions tend to break down with time because of thermodynamic instability (Akbari *et al*., 2018). The physicochemical mechanism namely phase separation, flocculation, Ostwald ripening, gravitational separation, coalescence, and particle coalescence challenge the stability of emulsions (Akbari *et al*., 2018). Therefore, biosurfactants/emulsifiers are used to stabilize emulsions through surface tension reduction of the immiscible phases (Akbari et al., 2018). The *Planococcus* sp. CP5-4 strain biosurfactants were able to stabilize an emulsion between water and paraffin and water and motor oil and had emulsion index measured at 63.7% and 92.30% respectively and can maintain stability for more than 10 days, furthermore, these emulsions are characterized as a dense compacted plug-like water-in-oil emulsion. Therefore, *Planococcus* sp. CP5-4 biosurfactant(s) have the potential to be used to achieve stabilization of heavy creams, lotions, mayonnaise, salad dressing, shampoos, etc., and because of its retained pigmentation it has an added benefit of a natural colorant which is important in making the products appealing to customers, however, this can also be a disadvantage for products that do not require colorants.

In work done by Moyo *et al*., (2021) *Planococcus* sp. CP5-4 strain was able to increase the evaporation rates of industrial brine disposed of in an evaporation pond system by 20% when compared to using synthetic dye. The pigment produced by the strain was found to be the main contributor to the increased evaporation rate, through absorbing and transferring solar energy (Moyo *et al*., 2021). This phenomenon occurs when the pigmented biosurfactant lowers the surface tension of water, leading to a higher saturation vapor pressure thus an increase in evaporation rate (Moyo *et al*., 2021). Therefore, the C_{30}-carotenoid fatty acids ester biosurfactant with its emulsifying abilities and inherent halotolerant nature might have properties suitable to be employed not only for biological treatment of industrial brines but as a part of waste management strategy to manage waste composed of salt, metals, and oils or as an environmentally compatible option for bioremediation of seawater or inland oil spills. The C_{30}-carotenoid fatty acids ester biosurfactant has the potential to be utilized as a unique and tailored solution in the bioremediation/ waste management sector.

Biosurfactants produced by *Planococcus* sp. CP5-4 also have the potential to be used in the oil industry for the bioremediation of hydrocarbon-contaminated environments, in microbial-enhanced oil recovery technology, in the removal and mobilization of oil residues, and in other applications that require emulsification (Santos *et al.*, 2017). It is well documented that *Planococcus* species have properties that can be utilized for bioremediation, for instance, they can withstand heavy metals, have oil-scavenging properties, and *Planococcus* sourced biosurfactants remain stable in harsh environmental conditions and have surfactant and emulsifying abilities that assist in increasing solubility and bioavailability of oils/hydrocarbons thus support their biodegradation (Eras-Munoz *et al*., 2022). This is evidently true as *P. alkanoclasticus* sp. nov. strain MAE2 is able to degrade branched n-alkanes in crude oil (C11 and C33), *Planococcus* maritimus Y42 can utilize crude oil for growth (Engelhardt *et al*., 2001; Yang *et al*., 2018), while *Planococcus P*. strain S5 assists in the degradation of aromatic hydrocarbons like salicylate or benzoate and grows well in the presence of phenol (Labuzek *et al*., 2003). Furthermore, the *Planococcus* DI strain biosurfactant remains stable between temperatures 20-60°C (Ebrahimipour *et al*., 2014), and *Planococcus* sp. XW-1 strain can degrade crude oil at temperatures as low as 4°C (Yang *et al*., 2018; Guo *et al*., 2022).

Carotenoids have a variety of medical properties, they are found to have anticancer, antioxidant, and antiobesity properties (Kim *et al*., 2022; Lilwani *et al*., 2022). The novel carotenoid from the *P. maritimus* MKU009 strain was found to have antioxidant properties (Ganapathy *et al*., 2016). There is also evidence of antioxidant properties, cytotoxic activity, and anti-tubercular activity associated with C_{30}-carotenoid biosurfactants which means it can be used for pharmacological application as well (Waghmode *et al*.,2020; Kim *et al*., 2022). Furthermore, the pharmacological potential of the carotenoid biosurfactant derived from *Planococcus* SAMP MCC 3013 makes C_{30}-carotenoid fatty acids ester biosurfactant a huge interest as an alternative therapy in pharmaceutical industries (Waghmode *et al*.,2020; Lilwani *et al*., 2022).

All of the surface-active compounds and lipid compounds detected on the TLC plate have not been characterized. The compounds with detected activity might be carotenoids, or carotenoid intermediates, or congeners and/or completely different structure compounds to the carotenoids. For example, a search for biomass compounds

conserved in the three *Planococcus* sp. CP5-4 strains was performed by Moyo, (2021) and cardiolipin comparable to one produced by *Bacillus subtilis* was found to be present in all three strains (Appendix: **Table D 1**). As a phospholipid, it is possible for cardiolipin to be extracted by solvents used in this study and to be stained by primuline. Most microorganisms synthesize cardiolipin, in a study done by Pinkas and colleagues, (2020) where they investigated the functional properties of the cardiolipin synthesized by *B. subtilis*, they reported that the cardiolipin's function is to protect the microorganisms' own membrane against permeabilization caused by the biosurfactant it produces i.e., surfactin (Pinkas *et al*., 2020). Surfactin is known as an antimicrobial pore forming biosurfactant and in their study, they found that the cardiolipin amount produced by *B. subtilis* increases during surfactin production (Pinkas *et al*., 2020). Furthermore, when *B. subtilis* with disrupted genes that are responsible for cardiolipin production, it was found that membrane leakage caused by surfactin increased, as well as the degree of lysing (Pinkas *et al*., 2020). Maybe cardiolipin serves the same function for *Planococcus* sp. CP5-4 strain. Nonetheless, it would be of interest to search deeper into other compounds conserved by strain, furthermore, the whole genomic sequence can also assist as a guide for the investigation of the possible compounds responsible for the detected biosurfactant activity. However, *Planococcus* sp. CP5-4 strain might be synthesizing surface active compounds through utilizing other biosynthetic pathways other than the carotenoid biosynthetic pathway.

4.4. Conclusion

This study provided evidence that classifies *Planococcus* sp. CP5-4 strain as a biosurfactant producer with the ability to produce multiple compounds that confer biosurfactant activity. The best method for the extraction and partial purification of the biosurfactant(s) produced by *Planococcus* sp. CP5-4 was investigated and determined to be acid precipitation followed by chloroform-methanol extraction. Multiple extracts were found active after activity screening using the well-established biosurfactant screening tests. Biosurfactants are often produced as a complex mixture, that occur at different ratios (Twigg *et a*l., 2020). The main activity seems to be associated with the pigments molecules synthesised by the carotenoid biosynthetic pathway present in the wild type and yellow pigmented strains. Nevertheless, there were other bands belonging to the unpigmented *Planococcus sp.* CP5-4 variant that were also active and other extracts produced by the wild type and yellow mutant *Planococcus* sp. CP5-4 strain that had significant activity yet were not associated with the pigmented molecules. Employing the second solvent assisted with a better separation of the compounds and isolation of the pigmented molecule extract, as it was observed co-migrating with lipid stained extracts. The use of both solvent systems ensured pigment isolation and aided with a more accurate identification of the compounds that confer activity. Furthermore, once the lipid stained extracts were separated from the pigmented molecule, they showed improved activity, therefore, confirming further the existence of other substantial pathways that might be synthesizing the biosurfactant(s) screened. Contrary the pigmented molecule extracts' activity was reduced; however, the activity remain significant. Consequently, the difference in physicochemical properties and activity measured might be (i) influenced by the difference in the presence and/ ratio of each of the compounds belonging to each variant, for example, the presence of carotenoids seems to contribute to the differences observed between the variants or the difference might be related to the (ii) variance in concentration of each of the compounds produced by each variant. This is

true for bands that had the same Rf value but had differences in screened or measured activity. For example, most of the unpigmented *Planococcus* sp. CP5-4 variant compounds share the same Rf value with compounds of the pigmented *Planococcus* sp. CP5-4 strains, however, had reduced activity. The difference in concentration could be seen when looking at the difference in saturation of the compounds on the TLC plates, indicating that the unpigmented mutant might be producing the compounds at a lower concentration as the amount loaded on preparative TLC plates were kept consistent (Appendix: **Figure D 8**, **Figure D 9**, **Figure D 10**). Through preliminary characterization of the biosurfactant(s) produced by *Planococcus* sp. CP5-4, our finding revealed that biosurfactants detected have lipid and carbohydrate/sugar components and moreover some of active extracts are associated with the pigment synthesized by the strain. Out of the multiple surface active compounds detected, this study was able to confirm one of the active structures to be methyl 5-(6-C17:3)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate. Moreover, for the first time this study presented the biosynthetic pathway of the carotenoid-biosurfactant produced by *Planococcus* sp. CP5-4 strain and highlighted the pathway where the terpenoid is converted into a biosurfactant. Hence, through this work we were able provide evidence showing that the carotenoid(s) synthesised by *Planococcus* sp. CP5-4 strain have biosurfactant activity, thus, supporting the theory put forward.

Chapter 5: General discussion, recommendations, and concluding remarks

5.1. Summary and general discussion

In the last decade only a few novel biosurfactant structures have been discovered (Trindade *et al*., 2021). Therefore, this study aimed at bioprospect for new biosurfactants using both culture-independent and culture-based method for the expansion of biosurfactant diversity:

5.1.1. Culture-independent screening: Function-based metagenomics

Culture-independent screening entailed high-throughput parallel function-based screening of a seawater metagenomic library by employing the atomized spray test. With the assistance of the Q-pix2XT automated colony picker robot a total of 25 000 and 20 000 clones both in *E. coli* EPI300 and *P. putida* MBDI host strains were screened respectively for surface activity, which resulted in zero biosurfactant activity observed. As discussed in previous chapters, it is recognized that the conventional function-based metagenomics approach for biosurfactant discovery has many hurdles, therefore, there is a need for adjustment or other creative tactics that will assist in overcoming the challenges. Some of the considerations that might contribute to culture-independent biosurfactant novel discovery in the future are highlighted below;

Sequence-based metagenomics is intended to bypass some impinging factors associated with function-based metagenomics, such as previously mentioned factors linked with heterologous expression, however, the challenge to recover full-length genes from eDNA and the likeliness of rediscovery remain as undesirable factors in its employment (Zhang *et al*., 2021). The use of both functional and sequence-based metagenomics as parallel approaches toward novel biosurfactant discovery has been suggested and is said to be more likely to increase the probability of identifying novel structures. In silico screening of the metagenomic library followed by heterologous expression and function-based screening is a suggested process of merging the two metagenomic approaches (Trindade *et al*., 2021; Li *et al*., 2022). For example, Li and colleagues (2022) developed a sequence-function-based metagenomics approach for screening large-sized copper resistance genes *copA*. This method was able to bypass biases in gene abundance and gene length associated with conventional functional metagenomics, as *copA* are found in low abundance in nature (Li *et al*., 2022). Their approach combined metagenomic assembly technology (which facilitated the assembling of small DNA fragments into large contig), together with local BLAST analysis, functional genomics, chemical synthesis, and evolutionary trace analysis (Li *et al*., 2022). This led to a total of 175 novel *copA* sequences discovered from 88 metagenomes sourced worldwide (Li *et al*., 2022). The sequences were of distant relation with 55 sequences arising from unknown species and 10 of which were randomly picked and chemically synthesized for functional testing (Li *et al*., 2022). Furthermore, parallel screening was employed as proteins that were not expressed in *E. coli* were expressed in *Ralstonia metallidurans* (Li *et al*., 2022).

Genomic and synthetic biology can be merged to potentially accelerate the biodiscovery of novel structures and consequently lead to the improvement in the discovery of a new generation of biosurfactants (Parages *et al*., 2016; Alves *et al.*, 2018). There is a report on an approach that bypassed heterologous expression by characterizing the biosynthetic pathways directly from sequencing reads by Chevrette and Handelsman, (2020). The identified reads

of the biosynthetic gene clusters (BGC) from shotgun metagenomics were binned for targeted assembly and the entire pathway was reconstructed thereafter (Chevrette and Handelsman, 2020). The reconstructed BGC was then synthesized for expression (Chevrette and Handelsman, 2020). The advantages of this approach included sequencing of longer fragments and discovery even at very low coverage (Chevrette and Handelsman, 2020).

Metagenomics is a tool at the forefront of novel biosurfactant discovery, however, as a branch of the omic technology, it can be employed together with metatranscriptomics, metaproteomics, and metabolomics in the near future. Collectively the omics will assist in understanding better the gene-to-protein and structure-to-function synergies governing biosurfactant productions (Gaur *et al*., 2021; Zhang *et al*., 2021). As we have discussed metagenomic focuses on the discovery of new compounds and enzymes, furthermore, it is also concerned with genetic information of microorganisms from different environments, species composition, and diversity (Gaur *et al*., 2021). On the other hand, metatranscriptomics studies mRNA transcription, therefore, studies genes that are under-expressed and overexpressed in relation to changes in environmental factors, while metaproteomics is a study of protein, thus, assist in gathering real-time information with regards to the genes expressed and metabolomic studies microbial cell's total metabolic content i.e., the metabolites produced (Gaur *et al*., 2021).

A relatively new technological innovation based on metabolomics can be employed as a biosurfactant screening procedure that does not rely on the surface and interfacial tension reduction properties of biosurfactants. Namely the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) capacity, which is a rapid, and reliable tool that can detect variants of a class of compounds (Matos *et al*., 2019). MALDI-TOF-MS is a highly sensitive tool used for characterization by analyzing smooth ionization. The technique achieves this by distinguishing structural differences in a mixture based on the precise measurements of the mass-to-charge ratio which is associated with the molecular weight of compounds (Matos *et al*., 2019, Trindade *et al*., 2021). Furthermore, the structure is ascertained during primary screening, therefore bypassing the purification process (Trindade *et al*., 2021). However, MALDI-TOF-MS has only been practical for the screening of glycolipid-type structures; its innovation still needs to be tested on other structural groups (Matos *et al*., 2019, Trindade *et al*., 2021). Other major drawbacks are that the tool is expensive and requires skilled professionals. Metabolomics was employed to investigate the active fraction of *Rhodococcus* sp. 12R cultivated in 22 different media, this led to the identification of 30 new glycolipid-bearing functional groups, that displayed anticancer and antiviral properties (Gaur *et al*., 2021). Furthermore, the approach was employed to study the physiological state and biochemical activity of numerous marine organisms which led to the identification of surfactin biosurfactant amongst other diverse molecular families that are of industrial importance (Gaur *et al*., 2021). This example highlights that even a non-targeted metabolic approach can be utilized to identify specialized chemistry like biosurfactants more so in non-biosurfactant producers. Expanding the use of complementary omic technologies offers a promising prospect for biosurfactant discovery through culture-independent methods.

5.1.2. Culture-based method: A novel C_{30}-carotenoid fatty acid ester biosurfactant by *Planococcus* sp. CP5-4

In the pursuit of novel biosurfactants we are far from abandoning culture-dependent approaches, as all the culturing options have obviously not been exhausted (Stewart, 2012; Trindade *et al*., 2021). Through a culture-based approach *Planococcus* sp. CP5-4 was confirmed as a new biosurfactant producer and an emulsifier. Furthermore, by comparing the surface activity of the wild type *Planococcus* sp. CP5-4 strain, versus the yellow, and unpigmented mutants we revealed for the first time that the carotenoid biosynthetic pathway is indeed involved in the synthesis of the biosurfactant produced by the strain. Additionally, with the assistance of TLC staining reagents and activity tests, we confirm one of the active extracts screened is a C_{30}-carotenoid fatty acids ester, a pigment produced by the strain. Furthermore, with the backing of liquid chromatography-mass spectrometry (LC-MS) analysis and biosurfactant pathway illustration performed by Moyo and colleagues (2022) we concluded that the biosurfactant produced is a novel methyl 5-(6-C17:3)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate. The physicochemical properties of the biosurfactants produced include surface and interfacial tension reduction, emulsification, foaming, and wetting properties. Such qualities are desirable in different markets and are therefore important characteristics to consider when looking at the practical applications for biosurfactants (Santos *et al*., 2016; Araujo *et al*., 2020). These properties are very important and are exploited in different industries, for example, the biosurfactant has foam formation characterized by small bubble size and stable foam. This property is utilized in mineral processing to separate metal groups from suspensions (Chen *et al*., 2006; Gong *et al*., 2021.

5.2. Limitations of the study and recommendations

The study's major limitations included the inability to generate highly pure extracts and the lack of quantifying each extract, to determine their concentration or yield and ratio which would have facilitated in painting a more accurate picture of the activity screened. Additionally, the concentration of the partially purified extracts obtained from the preparative TLC plates were not standardized because the extracts were too small to weigh and as a result of the lack of a standard protocol, therefore, making the activity test analysis qualitative or semi-quantitative. Furthermore, the inability to do a detailed physicochemical characterization of the biosurfactants produced was another main limitation, because this would have allowed us to accurately define the biosurfactant's added value, by rating the biosurfactants against other biosurfactants. The comparison would have assisted in recognizing whether or not the new biosurfactants have noteworthy properties, that are yet to be described or that are better than what has already been reported. We do recommend that future studies should take interest in overcoming these limitations. Furthermore, other areas of research should include the improvement of bacterial growth and biosurfactant production/yield, isolating and chemically identifying all the compounds contributing to the biosurfactant activity detected, function testing to assign applications and scale up depending on the cost-effective production studies. It is of great importance to invest in understanding the novel C_{30}-carotenoid fatty acids ester biosurfactant and the rest of the biosurfactants produced by *Planococcus* sp. CP5-4 strain in order to evaluate their efficiency and significance and if appropriate to move the biosurfactants from laboratory to industry, as diversity expansion of what is currently offered in the market is the end goal.

5.2.1. Future studies

Optimization of bacterial growth and biosurfactant production: The biggest challenge with *Planococcus* sp. CP5-4 is its slow growth, it takes 3 days to pre-culture and 7 days to grow which is not ideal for production or research. Future studies should include an investigation of parameters that affect growth and production to optimize for a better yield. This can be achieved through employing production phenotypic analysis throughout the growth cycle to measure the best phase to harvest the C_{30}-carotenoid fatty acids ester biosurfactant or other biosurfactant produced. For example, biosurfactant production was found to be growth related for *P. alkanoclasticus* sp. nov. strain MAE2 strain (Engelhardt *et al*., 2001). This analysis can be performed by testing the different environmental parameters that influence bacterial growth and biosurfactant production which include chemical, physical, and nutrient factors. The physical and chemical factors include parameters such as pH, temperature, salinity, aeration and agitation speed, light, divalent cations, and stress conditions (nutrient limitations) (Santos *et al*., 2016; Fakruddin, 2012; Al-Dhabi *et al*., 2020). The nutrient parameters include a carbon source, as the most important factor in growth and production, and vary from species to species (Santos *et al*., 2016; Al-Dhabi *et al*., 2020), nitrogen source as the second most important, carbon: nitrogen ratio and phosphate (Sanches *et al*., 2021). For example, the cell growth rate of *Planococcus* sp. XW-1 was higher when diesel oil and hexadecane were used as carbon sources compared to when pyrene and crude oil are used (Guo *et al*., 2022). Biosurfactant sourced from *Planococcus* sp. DI had the optimal production at a temperature, pH, and NaCl salinity of 40°C, 9, and 0% w/v respectively (Ebrahimipour *et al*., 2014). Therefore, screening of these variables through one variable at a time approach can lead to improved biosurfactant growth and yield by the *Planococcus* sp. CP5-4 strain (Al-Dhabi *et al*., 2020).

Optimization of bacterial growth and biosurfactant production can also be improved through taking advantage of the already known pathway confirmation by Moyo and colleagues (2022). The genomic analysis and pathway confirmation by Moyo and colleagues (2022) provide profound insight that makes it feasible to employ biological and engineering to improve biosurfactant production and control yield. For example, the already traced carotenoid biosynthetic pathway will make it easier in the future to induce the expression of targeted genes. The known pathway can be used to facilitate the extension of synthetic biology-driven concepts, such as developing competent recombinant strain for the heterologous synthesis of the C_{30}-carotenoid fatty acids ester biosurfactant in a fast-growing microorganism such as *Escherichia coli.* A similar objective was recently achieved by Kim and colleagues (2022), they were able to express 4,4'-diaponeurosporene, a C_{30} carotenoid in recombinant *E. coli*. In their study, they found that the core biosynthetic genes that regulate the expression, namely dehydrosqualene synthase (*crtM*) and dehydrosqualene desaturase (*crtN*) can be expressed in *E. coli* and the production of 4,4'-diaponeurosporene was enhanced by 6.1 fold when compared to the production from the wild type strain. As highlighted *E. coli* is a noteworthy recombinant host prospect to do future research on for the improvement of C_{30}-carotenoid fatty acids ester production and yield. For a feasible commercial scale, it is mandatory to meet production yield and costs (Waghmode *et al*., 2020)

High purifying efficacy: A preliminary purification process through extraction and TLC separation does not result in completely purified compounds. Therefore, other purification processes are needed to achieve high-quality purification. Several purification processes can be applied to achieve this such as chromatography using reverse phase C18 columns, high performance liquid chromatography (HPLC), dialysis/lyophilization, medium pressure liquid chromatography (MPLC), column chromatograph using silica gel with the developing system discovered or isoelectric focus to name a few (Fenibo *et al*., 2019).

Predicting the structure details: Information on the molecular structure is important for the classification of the surface-active compound being investigated and for application studies (Twigg *et al*., 2020). TLC analysis was employed for preliminary elucidation of other structural compositions. However, the procedures applied do not provide enough information on structural composition and details nor can be used as good quantification of the amount or titer of each of the compounds present (Twigg *et al*., 2020). Only higher resolution techniques such as spectroscopy can correctly determine the surface-active structures. The techniques usually used for the characterization of biosurfactants are TLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Tandem mass spectrometry (MS/MS), High-performance liquid chromatography-mass spectrometry (HPLC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectroscopy (FTIR) (Fenibo *et al*., 2019; Al-Dhabi *et al*., 2020; Twigg *et al*., 2020). The yellow *Planococcus* sp. CP5 variant produces an "additional" carotenoid compared to the wild type *Planococcus* sp. CP5 strain, which had a different Rf value. Therefore, the structural differences of the pigmented compounds could be investigated by employing the listed tools.

Physicochemical characterization and function testing for assigning the application: There is a need to determine the physicochemical characteristic and activity of each of the purified compounds in relation to their concentration. Biosurfactant-sourced from *Planococcus* species are not well documented and the few reports available in literature have partial physicochemical characterization with Waghmode and colleagues (2019) being the first and only group to give a detailed report on the physicochemical characterization. Therefore, a need to investigate the physicochemical properties of biosurfactants produced by *Planococcus* sp. CP5-4 is well recognized. Furthermore, according to Twigg and colleagues (2020), the phenotypic measure of surface activity using indirect screening techniques is not precise nor accurate enough to cement a compound as a surface active molecule. Some assays can be sensitive to interferences and behavioural changes of surface-active compounds under certain conditions such as pH change, therefore, accurate measures of surface tension activities are required. Moreover, information on properties such as resilience of the biosurfactants to different pH, temperature and salinity range, surface tension (ST), the interfacial tension (IFT), and critical micelle concentration (CMC) are needed to understand the biosurfactant properties and identify appropriate applications of the biosurfactant(s) in various technologies. IFT and ST can be measured using force tensiometer-based methods namely Du Nouy ring, stalagmometric, and Wilhelmy plate methods or an optical-based methods such as pendant drop shape technique and axisymmetric drop shape analysis by profile (ADSA-P) (Mnif and Ghribi 2015). CMC is obtained by plotting surface tension against a series of biosurfactant concentrations to find the CMC point where surface tension does

not change even with the increase in biosurfactant concentrations (Mnif and Ghribi 2015). Based on the results obtained in this study and what is recorded in literature we only inferred some of the properties as well as possible applications of the biosurfactants produced by *Planococcus* sp. CP5-4 strain, however, it would be of great value to do a detailed investigation of the physicochemical features and to do function testing to assign the various applications in the near future of the biosurfactants produced by *Planococcus* sp. CP5-4 strain as a potentially efficient biosurfactant with promising surface activity.

5.3. Concluding remarks

A culture-based approach stands as a great method for biosurfactant discovery and in its continuous use lies plenty of opportunities for the discovery of new classes of biosurfactants or variants of known classes, particularly from unexplored microbial strains. However, in theory, culture-based methods may never overcome the "Great Plate Count Anomaly", no matter the effort placed into improving laboratory techniques. Therefore, culture-independent approaches are required in an effort towards accelerating what can be offered to the economically attractive biosurfactant market by the environment (Stewart, 2012; Zhang *et al*., 2021). Hence, employing more than one of the approaches and exploiting their usefulness for biosurfactant discovery is of key importance and will potentially ensure that all that nature has to offer is realized. Furthermore, implementing collaborative efforts means complex regulatory mechanisms that govern biosurfactant production systems might be understood better. For example, culturing of an organism is required to understand the quorum sensing mechanism, which is linked to microbial growth and protein expression. Application of omics can therefore assist in collecting information from non-culturable microorganisms and proteins not expressed within a system (Jackson *et al*., 2015b; Gaur *et al*., 2021; Zhang *et al*., 2021). In summary, increased research interests, efforts placed in improving culture-based laboratory techniques, the constant innovation in omics technology, and the continuous focus on extreme environments saturated with novel genes will surely deliver novel biosurfactants in future, as we have only scratched the "tip of the iceberg" for biosurfactant discovery.

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Appendix

A. Buffers and media

Table A 1: The base M9 Benzoate media (Espinosa-Urgel *et al*., 2000).

Components	**1000 mL**
Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NH_4Cl	1.0 g
NaCl	0.5 g
Sodium benzoate	2 g
1M $MgSO_4.7H_2O$	1.0 mL
1M $CaCl_2$ solution	100 µL
Agar	12 g

After autoclaving of the based M9 benzoate media, $MgSO_4.7H_20$ and $CaCl_2$ were added with 200 µl/L 1% Ferric Ammonium Citrate (Ortega-González *et al*., 2014) and 2.5 ml/L trace elements were added.

Table A 2: M9 Benzoate agar trace elements.

Component	**mg/L**
HBO_3	300
$ZnCl_2$	50
$MnCl_2.4H_2O$	30
$CoCl_2$	200
CuCl2.2H2O	10
$NiCl_2$	20
$NaMoO_4$	30

Table A 3: CTAB/Methylene blue agar.

Components	**1000 mL**
Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NH_4Cl	1.0 g
NaCl	0.5 g
20% Glucose	10 Ml
1 M $MgSO_4.7H_2O$	1.0 Ml
10 mg/ mL Thiamine-HCl	1.0 Ml

1 M $CaCl_2$	100 µL
$NaNO_3$	0.8 g
Yeast Extract	0.1 g
CTAB	0.2 g
Methylene Blue	5 mg
Agar	12 g

Table A 4: LB media

Components	g/L
Tryptone	10
Yeast extract	5
NaCl	10

Table A 5: LB agar media

Components	g/L
Tryptone	10
Yeast extract	5
NaCl	10
Agar	15

Table A 6: Fosmid extraction solution (100 mL each) utilise 4 mL each for each extraction.

Solution 1	
1M Glucose	5.0 mL
1M Tris-HCl	2.5 mL
0.5M EDTA	2.0 mL
dH2O	90.5 mL
Solution 2	
10 M NaOH	2.0 mL
10% SDS	5.0 mL
dH2O	93 mL
Solution 3	
Glacial acetic acid	11.5 mL
KAC	29.28 g
dH2O	88.5 mL

B. The final concentration of antibiotics used in this study

Table B 1: Buffers and media

Component	20 µL
DNA	10 ug
10 x NE buffer 2.1	2 µL (10% total volume)
HindIII	5 µL {10 unit (1 µL) to cut 1 ug in 50 µL reaction)
H20	20µL – rest of volume

Table B 2: 34mg/ml Chloramphenicol

Chloramphenicol	0.34 g
100% ethanol	10 mL (sterilized through 0.22 µm syringe filter)
Stored @ -20°C	

Table B 3: 20% L-Arabinose

L(+) Arabinose	2 g
Autoclaved distilled water	9 mL (sterilized through 0.20 µm syringe filter)
Stored @ 8°C	

Table B 4: 100 mg/mL Ampicillin stock solution

Ampicillin	0.5 g
96% ethanol	5 mL
Stored @ -20°C	

Table B 5: 50 µg/ml kanamycin make 50mg/ml stock

Kanamycin	0.5 g
Sterile H_2O	10 mL (sterilized through 0.20 µm syringe filter)
Stored @ -20°C	

Table B 6: 50mg/ml apramycin

Apramycin	0.5 g
Sterile H2O	10 mL (sterilized through 0.22 µm syringe filter)
Stored @-20°C	

C. Supplementary results for Chapter 2:

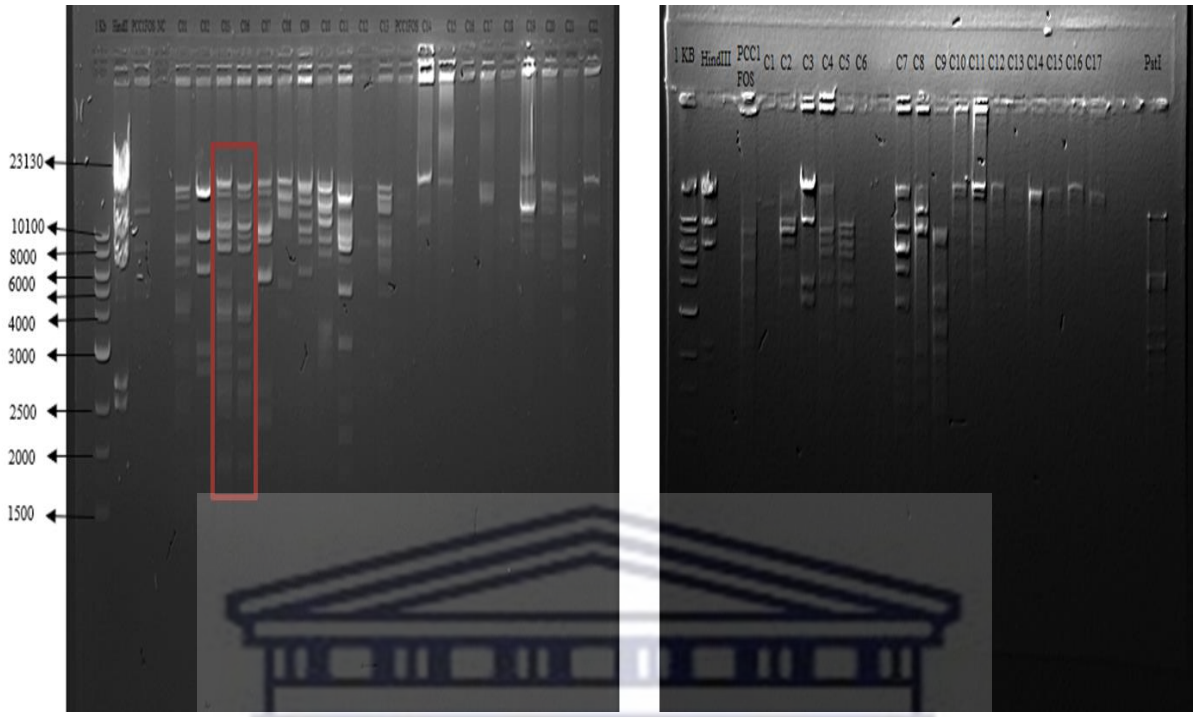

Figure C 1: HindIII restriction enzyme digest: Restriction digest was performed on the extracted colony fosmid DNA and was analyzed by 1% agarose gel electrophoresis. Lane 1; 1 kb marker, lane 2; λ HindIII DNA marker, lane 3; pCC1Fos as a positive control and sample clones labelled as C-, a digest of picked colony fosmid. A total of 40 digests were performed. Lane 25; PstI marker.

D. Supplementary results for Chapter 3:

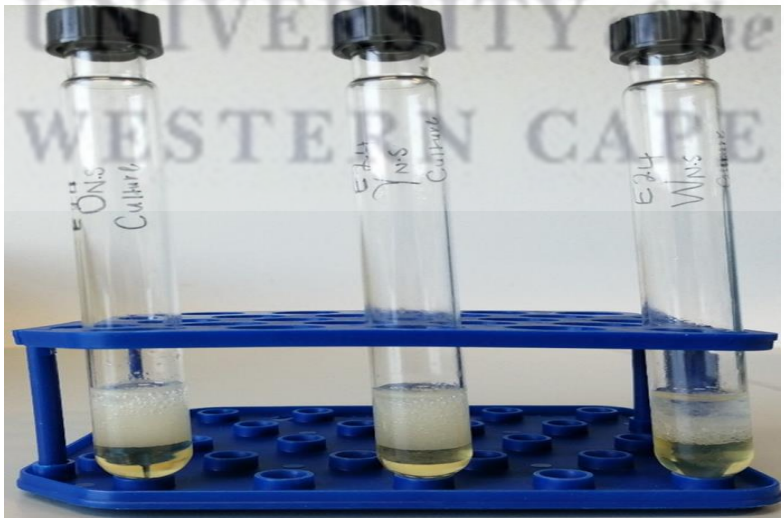

Figure D 1: Photographs of the emulsification of hydrocarbon paraffin and biosurfactant extracts culture mixed at a 1:1 (v/v) ratio. The photo was taken 24 hours after emulsification. Extracted from 200mL wild type orange (**O**), yellow mutant (**Y**), and unpigmented mutant (**W**) *Planococcus* sp. CP5-4 strain culture, extracted by acid precipitation method. The precipitates were re-suspended in water (pH 7.5).

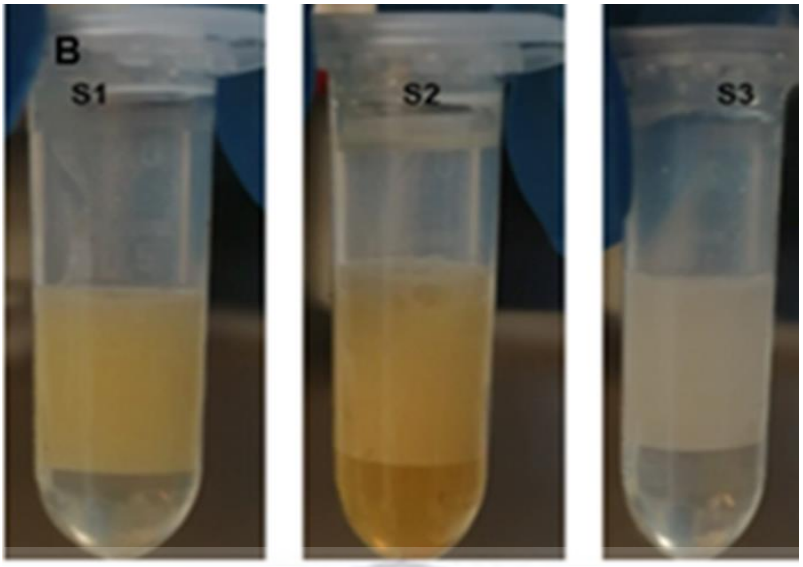

Figure D 2: Photographs of the emulsification of hydrocarbon paraffin and biosurfactant extracts culture mixed at a 1:1 (v/v) ratio. The photo was taken 24 hours after emulsification. Extracted from 1L wild type orange (**S1**), yellow mutant (**S2**), and unpigmented mutant (**S3**) *Planococcus* sp. CP5-4 strain culture, extracted by acid precipitation method. The precipitates were re-suspended in water (pH 7.5).

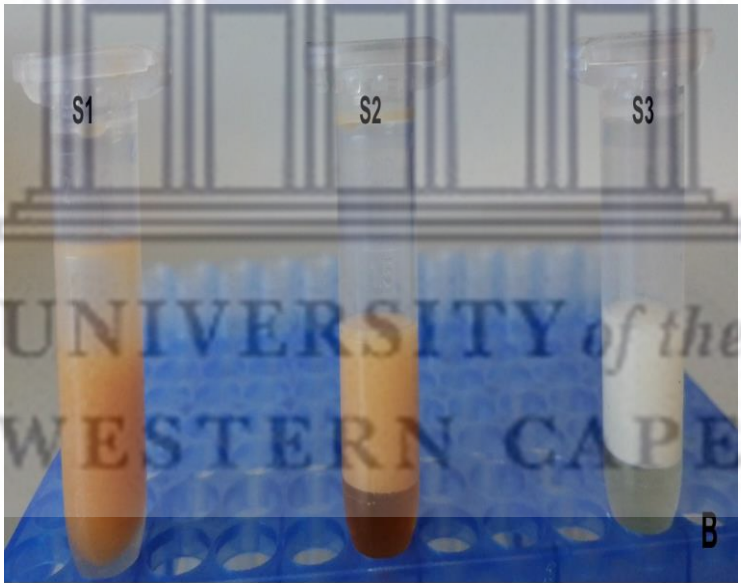

Figure D 3: Photographs of the emulsification of hydrocarbon paraffin and biosurfactant extracts culture mixed at a 1:1 (v/v) ratio. The photo was taken 24 hours after emulsification. Extracted from 3L wild type orange (**S1**), yellow mutant (**S2**), and unpigmented mutant (**S3**) *Planococcus* sp. CP5-4 strain culture, extracted by acid precipitation method. The precipitates were re-suspended in water (pH 7.5).

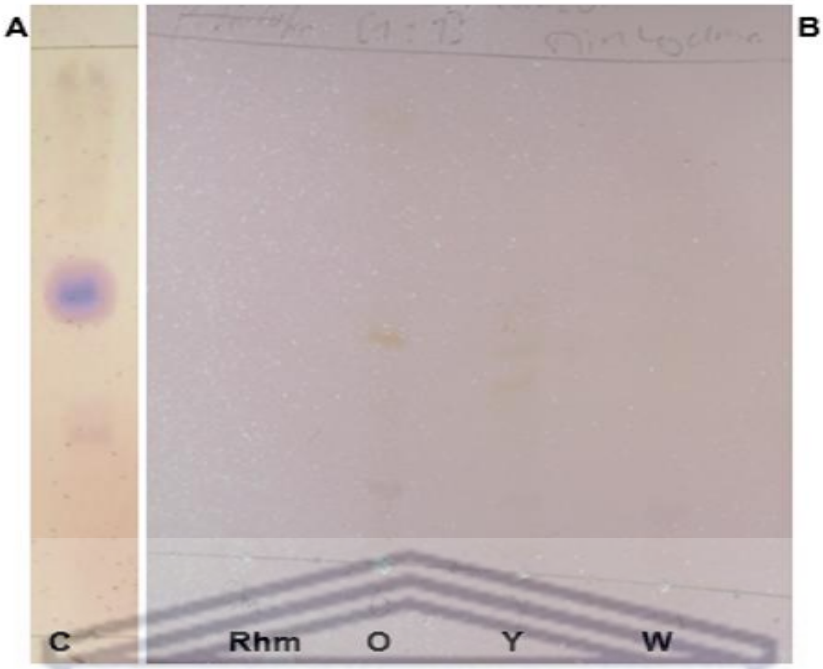

Figure D 4: TLC plate stained with 0.2% ninhydrin staining reagent. The procedure confirmed that the wild type orange (**S1**), yellow mutant (**S2**), and unpigmented mutant (**S3**) *Planococcus* sp. CP5-4 strain extracts do not have any amides groups in this study.

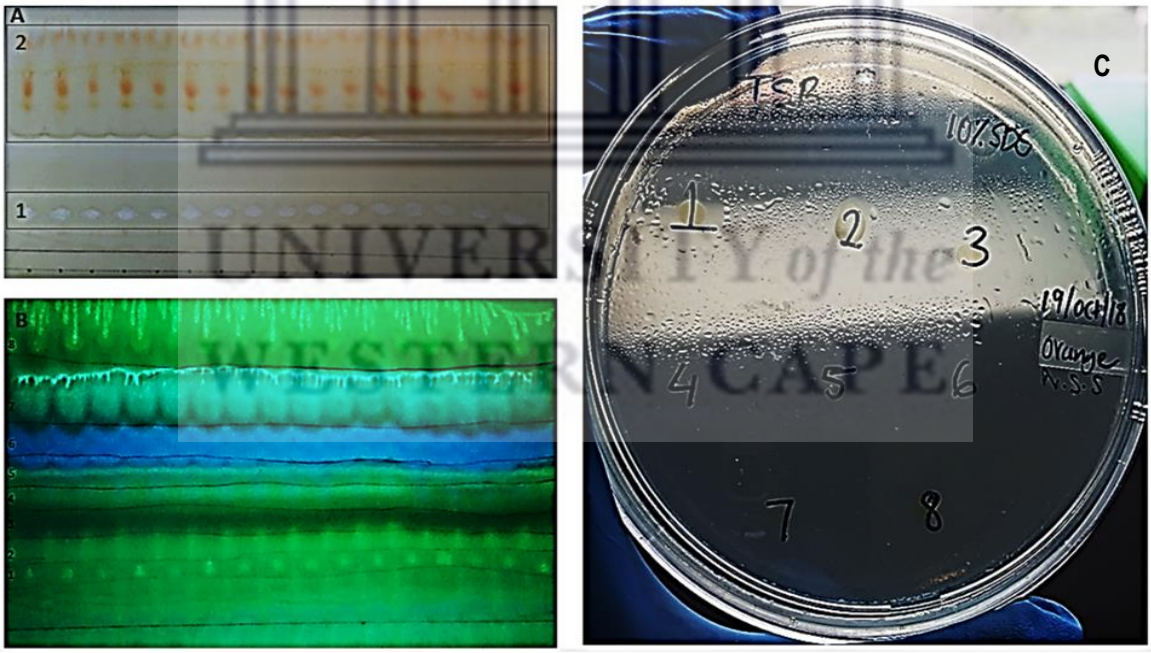

Figure D 5: **A** and **B**: Thin-layer chromatography (TLC) analysis of an extract of potential biosurfactant produced by *Planococcus* sp. CP5-4 wild type orange strain, extracted *via* ethyl acetate solvent extraction method **C**: The halo formation around extracts of the strain measured and recorded after atomized spray test assy. The partially purified extracts were obtained by scraping the TLC plates and extracted with methanol solvents thereafter and was followed by complete drying off of the solvent and re-suspension of the obtained extract in deionized water for activity testing. Hallo formation around an extract indicates a positive result for biosurfactant activity.

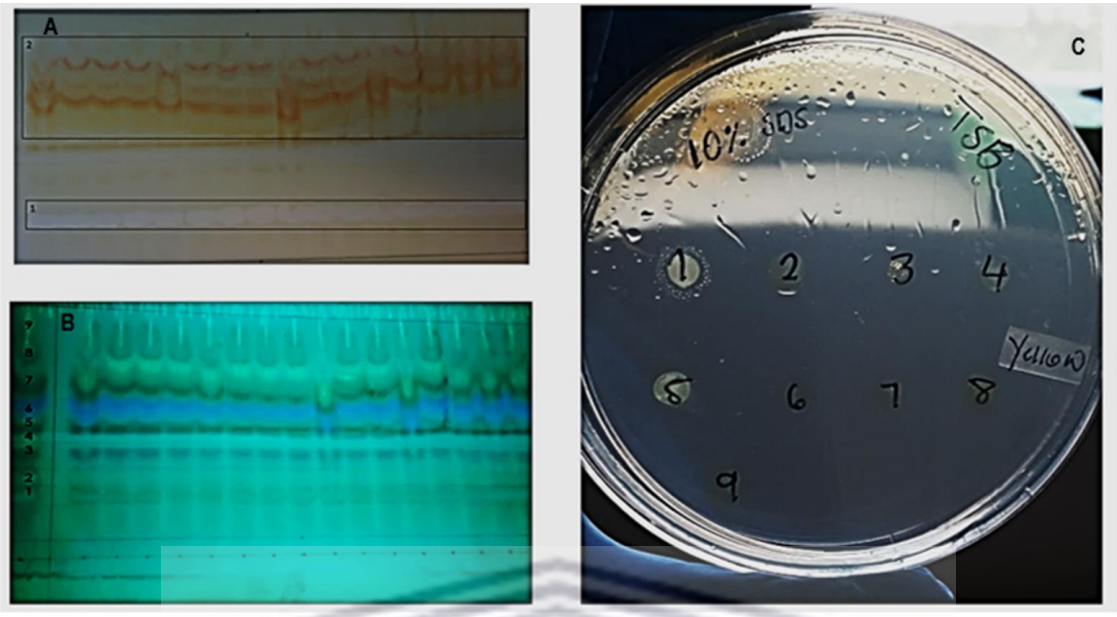

Figure D 6: **A** and **B**: Thin-layer chromatography (TLC) analysis of an extract of potential biosurfactant produced by *Planococcus* sp. CP5-4 yellow mutant strain, extracted via ethyl acetate solvent extraction method **C**: The halo formation around extracts of the strain measured and recorded after atomized spray test assy. The partially purified extracts were obtained by scraping the TLC plates and extracted with methanol solvents thereafter and was followed by complete drying of the solvent and re-suspension of the obtained extract in deionized water for activity testing. Hallo formation around an extract indicates a positive result for biosurfactant activity

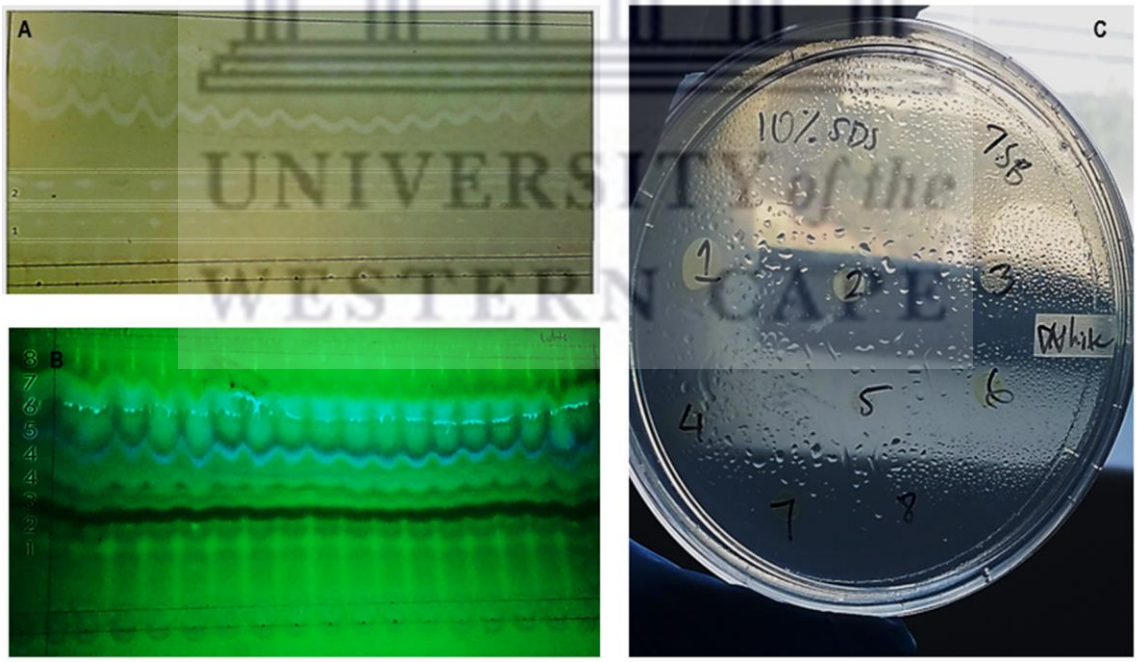

Figure D 7: **A** and **B**: Thin-layer chromatography (TLC) analysis of an extract of potential biosurfactant produced by *Planococcus* sp. CP5-4 unpigmented mutant strain, extracted via ethyl acetate solvent extraction method **C**: The halo formation around extracts of the strain measured and recorded after atomized spray test assy. The partially purified extracts were obtained by scraping the TLC plates and extracted with methanol solvents thereafter

and was followed by complete drying off of the solvent and re-suspension of the obtained extract in deionized water for activity testing. Hallo formation around an extract indicates a positive result for biosurfactant activity.

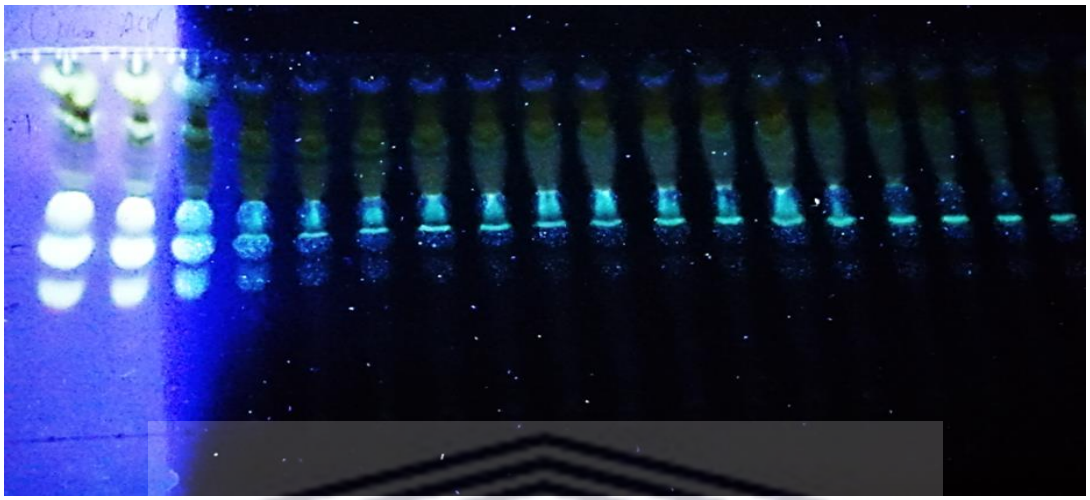

Figure D 8*:* Example of one preparative thin-layer chromatography (TLC) plate used for partial purification of the extracts extracted *via* acid precipitation plus chloroform-methanol (2:1, v/v) extraction method. The plate represent developed Wild type orange *Planococcus* sp. CP5-4 strain extracts. The partially purified extracts were obtained by scraping the TLC plates and extracted with methanol solvents thereafter. After separating the silico particle from solvent, the solvent was dried to completion and re-suspension of the obtained extract in deionized water for activity testing.

Figure D 9*:* Example of one preparative thin-layer chromatography (TLC) plate used for partial purification of the extracts extracted *via* acid precipitation plus chloroform-methanol (2:1, v/v) extraction method. The plate represent developed yellow mutant *Planococcus* sp. CP5-4 strain extracts. The partially purified extracts were obtained by scraping the TLC plates and extracted with methanol solvents thereafter. After separating the silico particle from solvent, the solvent was dried to completion and re-suspension of the obtained extract in deionized water for activity testing.

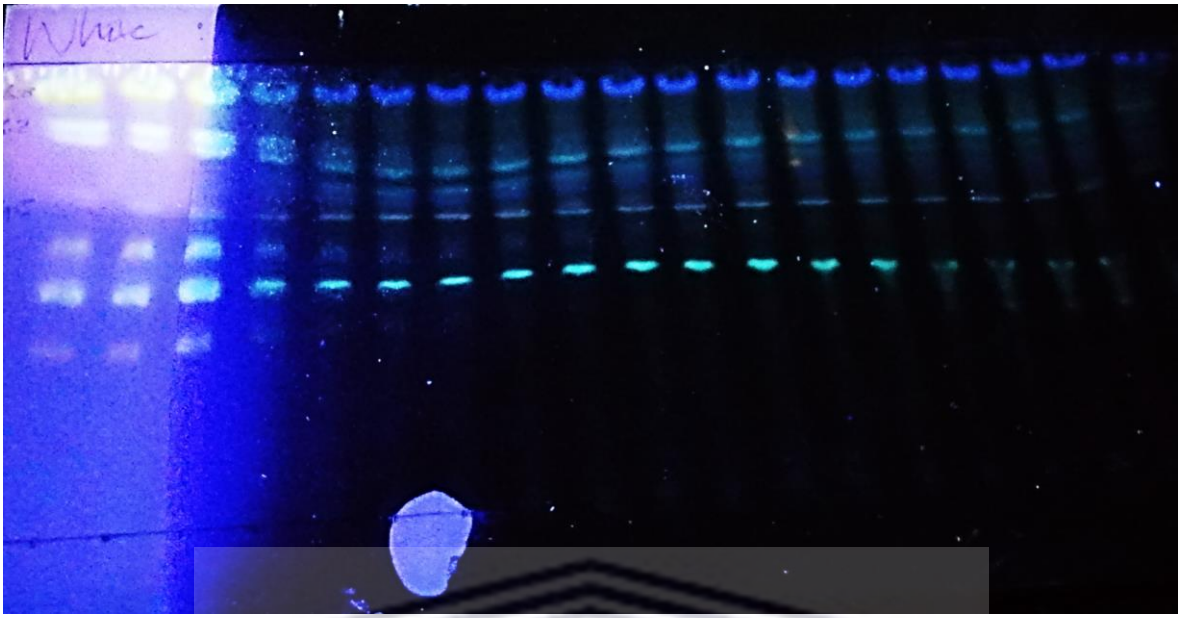

Figure D 10*:* Example of one preparative thin-layer chromatography (TLC) plate used for partial purification of the extracts extracted *via* acid precipitation plus chloroform-methanol (2:1, v/v) extraction method. The plate represent developed unpigmented mutant *Planococcus* sp. CP5-4 strain extracts. The partially purified extracts were obtained by scraping the TLC plates and extracted with methanol solvents thereafter. After separating the silico particle from solvent, the solvent was dried to completion and re-suspension of the obtained extract in deionized water for activity testing.

Table D 1:Biomass compounds conserved in the three *Planococcus* sp. CP5-4 strains (Moyo, 2021).

Compound	Name	Number of models	Conserved	WT	YE	UN
cpd15793	Stearoylcardiolipin (*B. subtilis*)	3	1	Present	Present	Present
cpd15795	Anteisoheptadecano ylcardiolipin (*B. subtilis*)	3	1	Present	Present	Present
cpd15795	Anteisoheptadecano ylcardiolipin (*B. subtilis*)	3	1	Present	Present	Present
cpd15795	Anteisoheptadecano ylcardiolipin (*B.subtilis*)	3	1	Present	Present	Present

E. Supplementary results for Chapter 4:

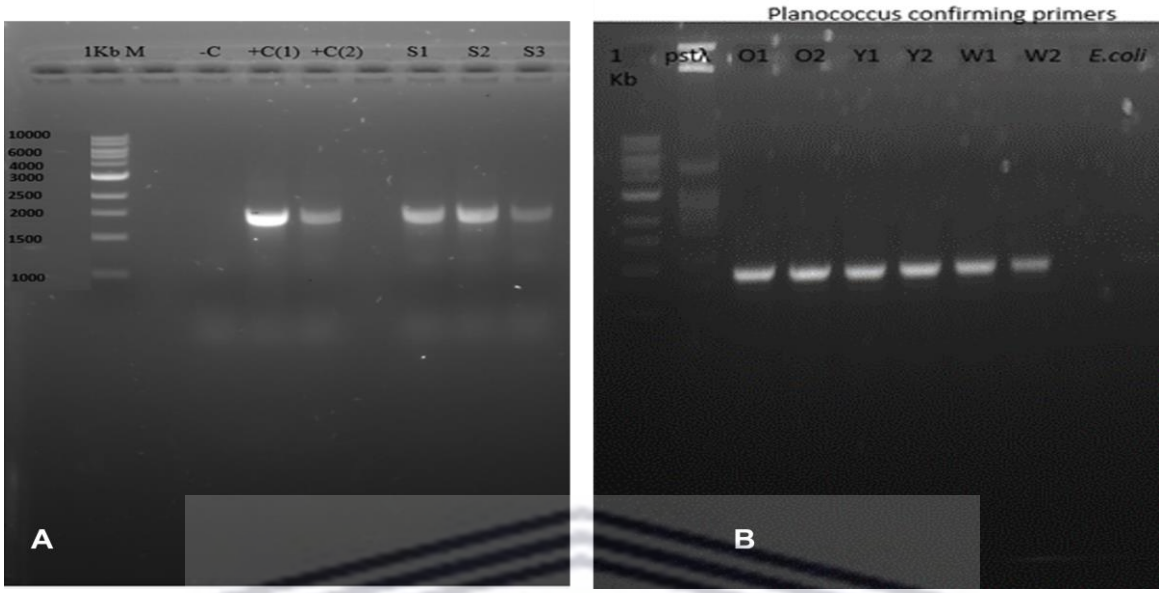

Figure E 1: A: 16s rRNA colony PCR results which were performed by extracting colony DNA. The DNA extracted was analyzed by 1% agarose gel electrophoresis. Lane 1; 1 kb marker, lane +C is a negative control consisting of PCR reaction mix with water, lane +C(1) and +C(2) positive control genomic DNA and colony *E. coli* DNA respectively. Sample **S1**, **S2,** and **S3** are *Planococcus* sp. Cp4-5 wild type orange, yellow, and non-pigmented strains respectively. **B**: PCR amplification using *Planococcus* sp. CP5-4 specific primer to confirm the strain. *E. coli* showed negative results for amplification and amplification was positive for (**O**) wild type, (**Y**) yellow mutant, and (**W**) unpigmented mutant samples. Amplification of the 16S rRNA gene by PCR directly from the colony DNA was used to confirm the *Planococcus* sp. CP5-4 strains/variants. After employing sanger sequencing and analysis of the 16S rRNA gene sequenced, the amplified amplicons from the revived strains confirmed that the strains were indeed *Planococcus* sp. CP5-4.

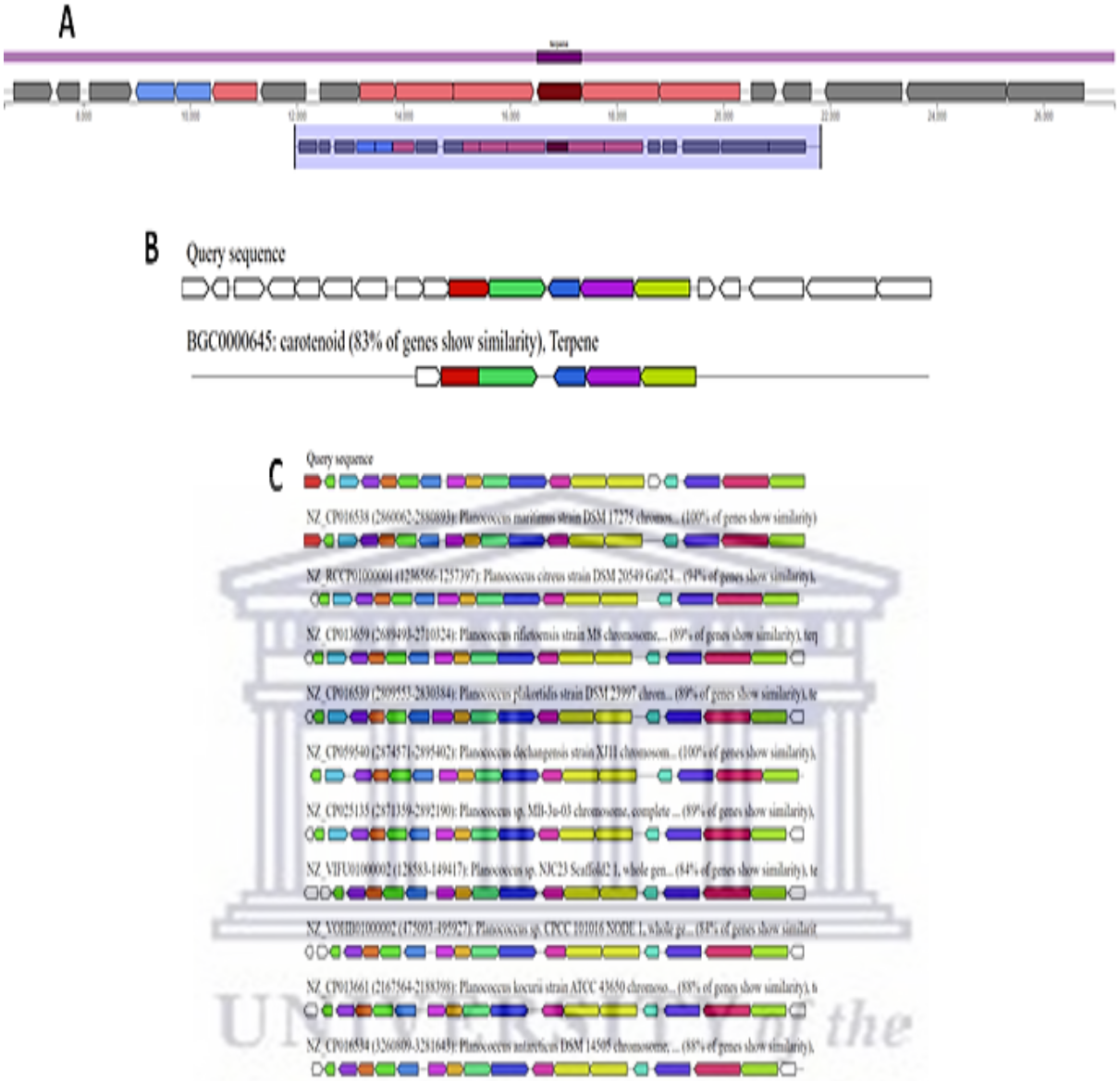

Figure E 2:Identification of a terpene-like secondary metabolite pathway on contig 3 of wild type Planococcus sp. CP5-4 genome sequence by antiSMASH. **B**: Comparison of the identified gene cluster to the MIBiG database of characterized secondary metabolite pathways showing high similarity to the carotenoid pathway from Halobacillus halophilus (BGC0000645). **C**: Comparison of the identified pathway to similar regions on other bacterial genomes available on the GenBank database.