RHAMNOLIPID PRODUCTION BY *Pseudomonas aeruginosa* USM-AR2 AND CONGENER CHARACTERIZATION

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RHAMNOLIPID PRODUCTION BY *Pseudomonas aeruginosa* USM-AR2 AND CONGENER CHARACTERIZATION

by

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LIST OF ABBREVIATIONS

CMC	Critical micelle concentration
$CoCl_2.6H_2O$	Cobalt (II) chloride hexahydrate
$CuSO_4.5H_2O$	Copper (II) sulphate pentahydrate
E_{24}	Emulsification activity
FAS II	Type II fatty acid synthesis pathway
$FeCl_3 \cdot 6H_2O$	Iron (III) chloride hexahydrate
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography mass spectrometry
HAA	3-(3-hydroxyalkanoyloxy) alkanoic acid
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium chloride
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled with mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
$MnSO_4 \cdot H_2O$	Manganese (II) sulphate monohydrate
MSM	Mineral salt medium
$Na_3C_6H_5O_7$	Sodium citrate
$NaNO_3$	Sodium nitrate
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density

OD_{421}	Optical density reading at wavelength of 421nm
OD_{540}	Optical density reading at wavelength of 540nm
OST	Oil spreading test
Q-TOF	Quadrupole Time-of-Flight
R_f	Retention factor
Rha-C_{10}-C_{10}	α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate
Rha-Rha-C_{10}-C_{10}	O-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate
rhl	Rhamnolipid biosynthesis related gene
RhlA	3-hydroxyacyl-ACP:3-hydroxyacyl-ACP-O-3-hydroxy-acyltransferase
RhlB	Rhamnosyltransferase I
RhlC	Rhamnosyltransferase II
RP	Reverse-phase
ST	Surface tension
TLC	Thin layer chromatography
UPLC-PDA	Ultra performance liquid chromatography coupled with photodiode array detector
UV	Ultraviolet
v/v	Volume per volume
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate
CD_3OD	Deuterated methanol
N_2	Nitrogen gas
1D	One-dimensional

2D	Two-dimensional
COSY	Correlation spectroscopy
HSQC	Heteronuclear single quantum coherence
HMBC	Heteronuclear multiple-bond correlation spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance

LIST OF UNITS AND SYMBOLS

%	Percent
°C	Degree Celsius
µL	Microlitre
cm	Centimetre
cm^{-1}	Reciprocal centimetre
Da	Dalton
g/L	Gram per litre
K	Kelvin
L	Litre
L/min	Litre per minute
m/z	Mass to charge ratio
mg/L	Milligram per litre
MHz	Megahertz
mL	Millilitre
mm	Millimetre
mN/m	Millinewton per metre
ppm	Parts per million
psi	Pounds per square inch
psig	Pounds per square in gauge
rpm	Rotation per minute
V	Voltage

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PENGHASILAN RHAMNOLIPID OLEH *Pseudomonas aeruginosa* USM-AR2 DAN PENCIRIAN KONGENER

ABSTRAK

Pseudomonas aeruginosa USM-AR2, strain pencilan tempatan daripada sampel minyak mentah telah menunjukkan penghasilkan rhamnolipid yang tinggi apabila dibiak dalam medium garam mineral (MSM) yang ditambah dengan sisa minyak masak sebagai sumber karbon tunggal. Rhamnolipid yang tergolong dalam kelas biosurfaktan glikolipid merupakan salah satu biosurfaktan yang paling banyak dikaji. Ini disebabkan oleh sifat fizikokimia dan biologinya yang luar biasa, termasuklah mempunyai nilai kepekatan misel kritikal (CMC) yang rendah, keupayaan pengemulsi yang tinggi dan sifat anti-mikrob yang kuat. Rhamnolipid boleh berbentuk mono- atau di-rhamnolipid, dengan setiap kongener dan homolog, mempunyai konstituen asid lemak dengan rantaian dan darjah tepu yang berlainan. Dalam kajian ini, fermentasi rhamnolipid telah dijalankan dalam kelalang goncang (200 rpm, 168 jam, 30°C) dengan 3% (v/v) sisa minyak masak yang ditambah ke dalam MSM sebagai sumber karbon tunggal. Komposisi kongener bagi campuran rhamnolipid yang dihasilkan oleh *P. aeruginosa* USM-AR2 dicirikan oleh kromatografi lapisan nipis (TLC), spektrometri jisim kromatografi cecair (LC-MS), spektrometri jisim tandem kromatografi cecair (LC-MS/MS), spektroskopi inframerah fourier transformasi (FTIR) dan spektroskopi resonans magnetik nuklear (NMR). Analisis TLC menunjukkan kehadiran titik utama atas dan bawah dengan nilai R_f iaitu 0.70 dan 0.41. Analisis FTIR dan NMR seterusnya mengesahkan struktur rhamnolipid yang terdiri

daripada gula rhamnose dan asid lemak. Analisis LC-MS dan LC-MS/MS mendedahkan 14 kongener rhamnolipid, dengan Rha-C_{10}-C_{10} sebagai kongener utama dan kesemua kongener mono-rhamnolipid menyumbang kepada 76.5% daripada jumlah campuran rhamnolipid. Rhamnolipid mempamerkan nilai CMC yang sangat rendah dengan pengurangan ketegangan permukaan air kepada 30 dynes/cm pada 5.0 mg/L. Selain itu, aktiviti permukaan rhamnolipid kekal stabil dalam pelbagai keadaan. Maklumat ini akan membantu dalam memperhalusi protokol pembiakan dan memberikan pandangan tentang potensi aplikasi rhamnolipid yang dijangka bergantung kepada komposisi.

RHAMNOLIPID PRODUCTION BY *Pseudomonas aeruginosa* USM-AR2 AND CONGENER CHARACTERIZATION

ABSTRACT

Pseudomonas aeruginosa USM-AR2, a locally isolated strain from crude oil sample has been shown to produce copious amounts of rhamnolipid when grown in mineral salt medium (MSM) supplemented with waste cooking oil as the sole carbon source. Rhamnolipid, belonging to the class of glycolipid biosurfactant is one of the most extensively studied biosurfactants. This is due to their exceptional physicochemical and biological properties, ranging from having low critical micelle concentration (CMC) value, high emulsifying ability and potent anti-microbial activities. Rhamnolipid can either be mono- or di-rhamnolipid, with each congener and homologue, having a constituent of fatty acids with varying chains and degrees of saturation. In this study, the rhamnolipid fermentation was carried out by shake flask fermentation (200 rpm, 168 hours, 30°C) with 3% (v/v) waste cooking oil supplemented into the MSM as the sole carbon source. The congener composition of rhamnolipid mixtures produced by *P. aeruginosa* USM-AR2 was characterized by thin layer chromatography (TLC), liquid chromatography mass spectrometry (LC-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS), Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. The TLC analysis showed presence of upper and lower major spots with R_f values of 0.70 and 0.41, respectively. The FTIR and NMR analysis further confirmed the structure of rhamnolipid to be composed of rhamnose sugar and

fatty acids. The LC-MS and LC-MS/MS analysis revealed 14 rhamnolipid congeners, with Rha-C_{10}-C_{10} as the main congener and mono-rhamnolipid congeners accounting to 76.5% of the total rhamnolipid mixture. The rhamnolipid exhibited remarkably low CMC with surface tension reduction of water to 30 dynes/cm at 5.0 mg/L. Additionally, the surface activity of the rhamnolipid remained stable across a broad range of conditions. This information will assist in refining cultivation protocols and provide insights into the potential applications of rhamnolipid, which is anticipated, to be composition dependent.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Biosurfactants are biologically derived surface-active compounds, mainly synthesized by microorganisms as secondary metabolites (Ndlovu et al., 2017). Although their production and function for bacterial cells have yet to be fully understood, several physiological functions have been proposed which include to aid in solubilization of insoluble substrates, adhesion to interfaces in natural environment, metal complexation to adapt in their ecological niche as well as to regulate cellular toxicity (Cerqueira dos Santos et al., 2024). Apart from exhibiting remarkable properties such as high surface-active and emulsifying activity, biosurfactants outperform their chemical counterparts in terms of their ecological applicability as a result of their biological origin, which is driving the shift to their usage as an environmentally friendly alternative, especially in this current era of environmental consciousness. These properties have made these surface-active compounds an indispensable component in the modern industries. The anticipated growth for biosurfactants in the global market is projected to reach a compound annual growth rate of over 5.5% from 2020 to 2026, with the market size of rhamnolipids to reach over USD 145 million. This exceeded the forecasted CAGR of chemical surfactants at 5.3% from 2020 to 2027, demonstrating the growing interest in shifting towards the usage of biosurfactants (Pardhi et al., 2022)

Being one of the extensively studied biosurfactants, rhamnolipid produced by *Pseudomonas aeruginosa* was first reported by Bergström et al. in 1946. Shortly after, in

1949, Jarvis and Johnson documented the structure of rhamnolipid to be comprised of two rhamnose moieties linked to two β-hydroxydecanoic acids moieties. Since then, numerous reports have emerged on the structure of rhamnolipid congeners, each showing slight variations. It has become apparent that what was initially described as rhamnolipids are, in actual fact, biosynthesized as a complex mixture consisting of different congeners. These mixtures consisted of either one or two rhamnose sugar molecules linked to a number of β-hydroxy fatty acids of various length as well as degrees of saturation through a glycosidic bond. The rhamnolipid congeners can be classified into mono-rhamnolipid and di-rhamnolipid depending on the number of rhamnose sugar present (Varjani et al., 2021). It has been suggested that the congener composition are highly influenced by the strains and fermentation conditions, in which they can exist simultaneously in a single fermentation process (Rahman et al., 2010).

Pseudomonas aeruginosa USM-AR2, a locally isolated strain from crude oil sample has been shown to produce copious amounts of rhamnolipid when grown in mineral salt medium (MSM) supplemented with water immiscible substrates (Aggo et al., 2023; N. A. Md Noh et al., 2014). However, little information is known on the congener composition of the rhamnolipid produced. It is well documented that variations in the rhamnolipid composition are inevitable, with the congener mixtures differing between each rhamnolipid producing strains and fermentation conditions. This subsequently explains the difference in the physicochemical properties of rhamnolipid especially their critical micelle concentration (CMC) and emulsifying activity (E_{24}). Numerous research efforts have been conducted to characterize and elucidate the structural composition of biosurfactants, with a particular focus on rhamnolipid produced by *P. aeruginosa*. Apart

from understanding the heterogeneity of rhamnolipid which is considered as one of the impeding factors for their commercial and industrial application, it is also crucial to consider their economical upstream and downstream processes to pave way for rhamnolipid as a 'green' multifunctional molecule in conquering the global surfactant market.

1.2 Problem statement

Pseudomonas aeruginosa is known as one of the potent rhamnolipid producer, however the rhamnolipid composition and yield often differs significantly when cultured in medium supplemented with different carbon and nitrogen sources, as well as under different fermentation conditions (Li, 2017). The use of low-cost waste substrate particularly as carbon source in an effort to produce a cost-effective rhamnolipid production has been extensively studied, with increasing number of findings on hydrophobic substrates as the preferred carbon source by rhamnolipid-producing strains (Domínguez Rivera et al., 2019). This has subsequently led to the recent discovery of novel rhamnolipid congeners, with their composition being highly influenced by the source of carbon and fermentation conditions as mentioned previously.

There have been over 60 different rhamnolipid congeners and homologues reported to date, all with varying molecular and structural composition (Tiso et al., 2017). However, good resolution in analytical methods is prevalent for quantifying the rhamnolipid congeners, as well as in characterizing the structural composition these rhamnolipid mixtures. Robust high-throughput chromatographic and spectroscopic analyses, namely the high-performance liquid chromatography coupled with tandem mass spectrometry

(HPLC-MS/MS), ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and liquid chromatography coupled with mass spectrometry (LC-MS), Fourier transform infrared (FTIR) and nuclear magnetic resonance spectroscopy (NMR) have enabled the rhamnolipid mixtures to be identified and quantified (Charles Oluwaseun et al., 2017; Rudden et al., 2015).

It is noteworthy that most of the previous work on the rhamnolipid produced by *P. aeruginosa* USM-AR2 are mainly focused on improving the bioprocessing strategies (Asshifa et al., 2017; N. A. Md Noh et al., 2014; Zulkhifli et al., 2023) and potential application of rhamnolipid as bio-fungicide (Hadi et al., 2022; Mohamad Shukri et al., 2025) and biopesticide (Zaludin et al., 2021), without indication on the rhamnolipid congener composition. Moreover, rhamnolipid have been widely reported for their high stability at wide range of temperature, pH and salinity (Kong et al., 2021), and therefore the stability of rhamnolipid produced by *P. aeruginosa* USM-AR2 deserved to be assessed for their practical application in harsh environment. As the surface-active properties of rhamnolipid is greatly dependent on its composition, identifying and characterizing the structural composition of rhamnolipid congeners will imply further refinement of its suitable application in addition to providing better understanding on the mechanism involved in this application.

1.3 Research hypothesis

Rhamnolipid produced in *P. aeruginosa* USM-AR2 fermentations exists as mixtures of mono-rhamnolipid and di-rhamnolipid congeners. The composition of these rhamnolipid mixtures dictates their physicochemical properties, which will aid in matching the mixtures with their potential applications.

1.4 Research objectives

This study aims to resolve the congener composition of rhamnolipid produced by *P. aeruginosa* USM-AR2 by applying several analytical techniques, namely thin layer chromatography (TLC), LC-MS, LC-MS/MS, FTIR and NMR spectroscopy. The subsequent part of the study will assess the physicochemical properties of rhamnolipid by determining the CMC value, followed by stability study at different conditions by employing the OST, E_{24} and surface tension measurement.

1.4.1 To produce and recover rhamnolipid by *P. aeruginosa* USM-AR2 via shake flask fermentation and solvent extraction, respectively.

1.4.2 To characterize the composition and structure of rhamnolipid congeners produced by *P. aeruginosa* USM-AR2 via chromatographic and spectroscopic analyses.

1.4.3 To study the physicochemical properties of rhamnolipid produced by *P. aeruginos*a USM-AR2.

1.4.4 To assess the stability of rhamnolipid produced by *P. aeruginos*a USM-AR2 at different conditions.

1.5 Significance of study

Findings in this study will assist in refining cultivation protocols as well as providing fundamental insights into the potential applications of rhamnolipid produced *by P. aeruginosa* USM-AR2, which is hypothesized to be dependent on its congener composition. Identifying the composition of the rhamnolipid congeners is crucial as it is greatly influenced by factors such as the rhamnolipid-producing strain and culture condition as motioned previously, in which the physicochemical properties of the rhamnolipid mixture depend on the individual congeners and the ratio of the mono-rhamnolipid to di-rhamnolipid congeners (Liu et al., 2014). For instance, rhamnolipid mixtures with higher mono-rhamnolipid composition have been reported to exhibit lower CMC and better antimicrobial activity, while higher CMC and better emulsifying activity have been displayed by mixtures containing di-rhamnolipid (Das et al., 2014; Tiso et al., 2017).

The use of waste cooking oil as carbon source not only resulted in positive effects on rhamnolipid synthesis and bacterial growth, but also serve as an economic and promising approach in enhancing the biodegradation of such waste (Lan et al., 2015; Sharma et al., 2021; Shi et al., 2021). This is because the production of rhamnolipid will increase the miscibility of waste cooking oil in the medium for substrates uptake, and subsequently accelerating the rate of oil degradation (Sharma et al., 2019). In conclusion, employing waste cooking oil as the carbon source will not only provide a robust energy source to support microbial growth and biotransformation of such wastes into high-value products but also provide an eco-friendly alternative for their disposal (Gautam et al., 2023; George & Jayachandran, 2013).

CHAPTER 2

LITERATURE REVIEW

2.1 Biosurfactants

Surfactants are a group of chemically derived compounds, typically of petrochemical origin, that possess both hydrophobic and hydrophilic moiety in a single molecule as depicted in Figure 2.1, giving rise to their surface-active properties. The occurrence of dual moieties enables these surface-active compounds to effectively reduce surface and interfacial tension of liquids, leading to formation of microemulsions, in which immiscible compounds are solubilized in water or vice-versa (Farias et al., 2021). These properties have made them an indispensable component in the modern industries, leading to several environmental concerns which was associated with their source of origin that pose severe toxicity to the environment (Gautam et al., 2023). This has provided a strong motivation to seek for their environmentally friendly counterparts, with the use of biosurfactant as one of the prospective approaches especially in this era of environmental sustainability.

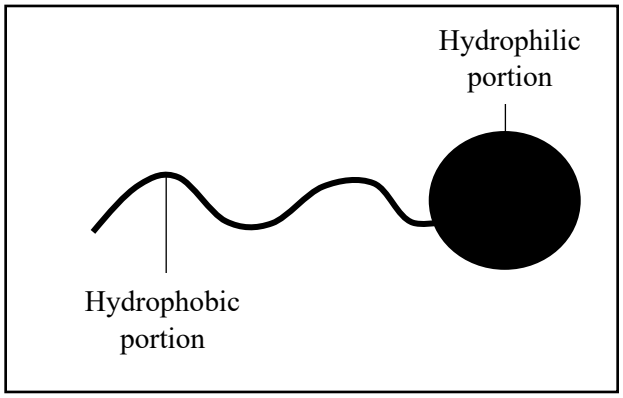

Figure 2.1 The general structure of a surfactant

Biosurfactants are compounds that harbour similar properties as surfactants, but are of microbiological origin such as from bacteria, fungi, and yeast (Marchant & Banat, 2012). Different with surfactants, they can be produced by microorganisms either at the cell surface or excreted extracellularly. Microorganisms have been reported to be the most sustainable source of biosurfactants due to their enormous genetic diversity, apart from being commercially promising (Nikolova & Gutierrez, 2021). Among the most reported biosurfactant producers belong to the genus *Pseudomonas*, *Bacillus*, *Candida*, and *Rhodococcus*. These compounds offer many advantages over their chemically derived counterparts that are currently available in the market. Their prominent traits include having higher biodegradability, lower toxicity, effectiveness in extreme environments, action specificity, and can be synthesized from renewable resources that gives rise to their ecological applicability (Pardhi et al., 2022). Their importance is demonstrated from their massive volumes which is employed in a myriad of applications, ranging from agriculture, bioremediation, detergents, food and beverages, healthcare, and pharmaceuticals (Nikolova & Gutierrez, 2021).

2.1.1 Properties of biosurfactants

The effectiveness of biosurfactants is proven through their capability to lower the surface and interfacial tension at the interface of two immiscible phases such as liquid/air or polar/non-polar liquids interfaces, respectively. Such properties have enabled biosurfactants to fulfil an essential role in detergency, foam formation, emulsification, and oil dispersion, which are the traits desired in the surfactant industries (Sarubbo et al., 2022). A good biosurfactant is described to have the ability in lowering the surface tension of water to less than 35 mN/m from 70 mN/m, and the interfacial tension of water to 1

mN/m from 40 mN/m against n-hexadecane, apart from having low CMC which is considered one of the critical criteria in assessing the efficacy of a surface-active compound as it can directly impact the performance and surface-active performance of a surfactant molecules (Kashif et al., 2022; Santos et al., 2016). The surface tension with unit expressed in dynes/cm or mN/m, refers to the amount of energy required to expand the surface area of a liquid as a result of strong intermolecular forces between water molecules (Nikolova & Gutierrez, 2021).

According to Desai and Banat (1997), CMC refers to the minimum concentration of surfactant required to initiate the formation of micelles, which will subsequently reduce the surface or interfacial of water as depicted in Figure 2.2. Micelles are aggregated structures with the hydrophobic portion oriented towards the inside the surfactant molecule and hydrophilic portion positioned outward, in contact with water. Upon reaching the CMC, further addition of surfactants will no longer result in reduction of surface tension. Surface-active molecules with low CMC are considered to be more favourable for use than those with greater CMC as lower concentration of surfactants are needed to initiate surface-active activities (Farias et al., 2021). For instance, rhamnolipid can undergo aggregation in aqueous solution as a result of bearing a carboxylic group on its terminal end, making it a weak acid (Lebrón-Paler et al., 2006). A majority of biosurfactant possess lower CMC, surface and interfacial tension values compared to their conventional counterparts, making them a more efficient substitute to be used in the similar applications (Jahan et al., 2020).

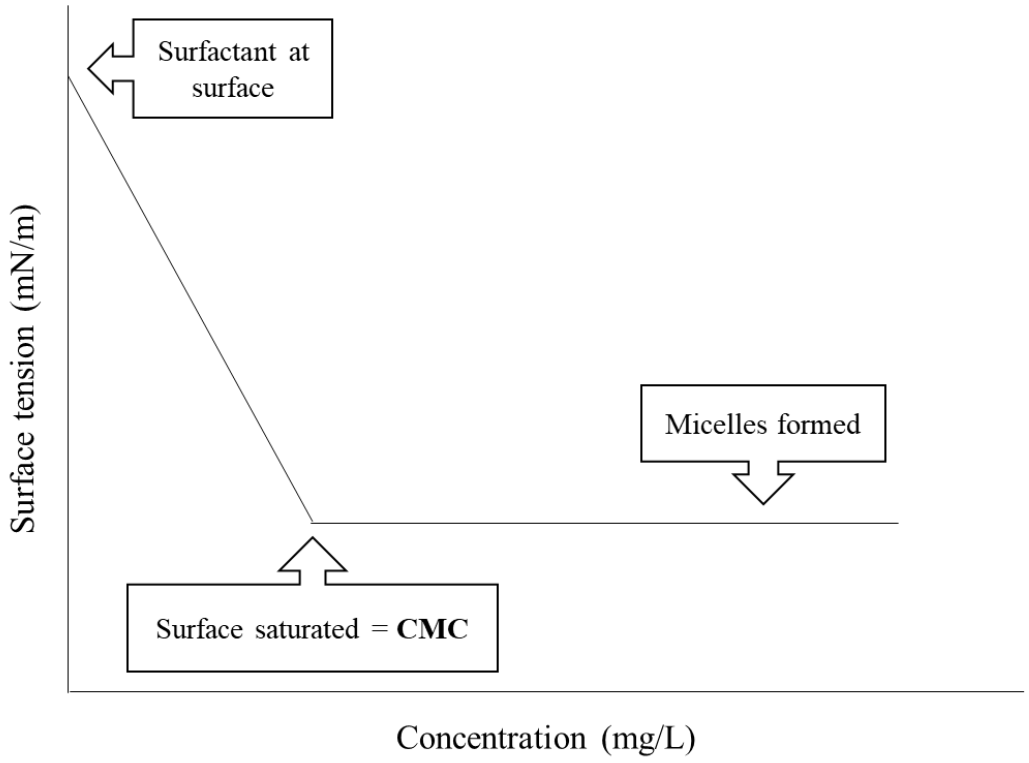

Figure 2.2 Surface tension of surfactant solution with increasing concentration

2.1.2 Classification of biosurfactants

As aforementioned, the immense genetic diversity of biosurfactant producing microorganisms has resulted in the enormous discovery of biosurfactants with promising properties with their production to be either inducible or constitutively. Additionally, their diversity arising from the different functional groups from each class of biosurfactants has also led to substrate specificity as well as in their mode of action (Kashif et al., 2022). However, there are significant difference in each of the biosurfactant produced depending on the strain types and can be categorized into five different classes according to their molecular weight and mechanism of action. These five classes include lipopeptides, glycolipids, fatty acids/neutral lipid as well as polymeric biosurfactants (Drakontis & Amin, 2020a; Vijayakumar & Saravanan, 2015).

The average molecular mass of biosurfactant ranges between 500 to 1500 Da, where biosurfactants are further categorized into low-molecular weight and high-molecular weight surface-active molecules (Sarubbo et al., 2022). The low-molecular weight biosurfactants such as work efficiently in reducing the surface and interfacial tension of the liquid-gas and liquid-liquid interface, respectively. Meanwhile, the high-molecular weight biosurfactants namely lipoproteins and polysaccharides, commonly termed as bio-emulsifiers, function effectively in forming stable water-in-oil or oil-in-water emulsions (Bognolo, 1999). These remarkable properties of biosurfactants makes them an essential element in the surfactant industries, with majority of research focusing on the low-molecular weight biosurfactants. Table 2.1 summarizes common types of biosurfactants, their microbial origins and potential applications.

Table 2.1 Summary of common types of biosurfactants, their microbial origins and potential applications.

Class	Subclasses	Microbial strains	Applications	References
Glycolipid	Rhamnolipid	*P. aeruginosa*	Agriculture, anti-microbial, bioremediation, cleaning products, cosmetics, food, pharmaceuticals	Thakur et al. (2021)
	Sophorolipids	*Candida bambicola*	Agriculture, anti-cancer, anti-microbial, biomedical, bioremediation, cosmetics, food, nano-biotechnology	Pal et al. (2023)
	Manosylerythritol lipids	*Pseudozyma* sp.	Agriculture, cosmetic, nanoparticles, pharmaceuticals	de Andrade et al. (2022), Morita et al. (2015)
	Trehalolipids	*Rhodococcus* sp.	Bioremediation	Andreolli et al. (2023)
Lipopeptide and lipoproteins	Surfactin	*Bacillus subtilis*	Anti-microbial, bioremediation, cosmetics, food, MEOR	Zhen et al. (2023)
	Iturin	*Bacillus amyloliquefaciens*	Anti-fungal	Dang et al. (2019)
	Fengycin	*Bacillus amyloliquefaciens*	Agriculture, anti-microbial	Medeot et al. (2020)
	Viscosin	*Pseudomonas fluorescens*	Anti-biofilm	Bonnichsen et al. (2015)

2.2 Rhamnolipid; the most intensively studied biosurfactant

Being one of the extensively studied biosurfactants as a result of their effective surface-active activity, rhamnolipid produced by *P. aeruginosa* (formerly known as *Pseudomonas pyocyanea*) was first reported by Bergström et al. in 1946 when grown on medium supplemented with glucose (Shu et al., 2021). Rhamnolipid belonging to the glycolipid class of biosurfactant are made up of hydrophilic and hydrophobic moieties consisting of one or two L-rhamnose sugar, connected to one or two, and occasionally, up to three β-hydroxy fatty acids through α-1,2-glycosidic linkage, respectively (Abdel-Mawgoud et al., 2009; Déziel et al., 1999). The production of rhamnolipid in bacteria has been associated with several physiological roles and functions, which include cell adhesion, cell motility, substrate solubilization and utilization, as well as formation and maintenance of biofilm (Esposito et al., 2023). Often, rhamnolipids are synthesized as a complex combination of congeners with each congener having conserved hydrophilic head groups but varies in the chain length of the hydrophobic tail groups (Déziel et al., 1999). These hydrophobic group can comprise of either unsaturated or saturated β-hydroxy fatty acids with 6 to 24 carbons linked together by an ester bond, with majority of studies reporting values ranging from 8 to 16 carbons depending on the rhamnolipid-producing strains (Behrens et al., 2016; Tiso et al., 2017).

The most prevalent congeners produced by *P. aeruginosa* described from previous literatures are α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C_{10}-C_{10}) and O-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}), commonly referred to as mono-rhamnolipid and di-rhamnolipid, respectively (Behrens et al., 2016). However, presence of minor

congeners with odd number chain lengths and saturation of the β-hydroxy fatty acids have also been reported, in which their presence can confer significantly different properties to the rhamnolipid mixture. Figure 2.3 illustrates the general structure of mono-rhamnolipid and di-rhamnolipid congeners where the fatty acids chain length denoted by the letter *m* and *n.* It is well documented that the production of rhamnolipid is influenced by various factors, such as type of strains, type of carbon and nitrogen sources and fermentation parameters. Additionally, these factors also dictate the congener composition of rhamnolipid mixture, and subsequently affecting the physicochemical properties of the final mixture (Desai & Banat, 1997).

Rhamnolipid produced by *P. aeruginosa* have been reported to exhibit lower CMC values compared to synthetic surfactants, which is considered as one of the most important parameters in assessing the activity of surface-active compounds (Chen et al., 2018). Previous findings have documented CMC values between 5 to 200 mg/L for rhamnolipid, with surface tension of water reaching a minimum of 28 mN/m (Thakur et al., 2021). Lower CMC values (5-60 mg/L) have been associated with rhamnolipid mixtures that predominantly composed of mono-rhamnolipid such as Rha-C_{10}-C_{10} (Dyke & Brauer, 1993), whereas intermediate CMC values (40-65 mg/L) have been displayed by mixtures containing di-rhamnolipid such as Rha-Rha-C_{10}-C_{10} (Zhang & Miller, 1992). However, due to inconsistency in terms used to describe the purity of rhamnolipids, it is therefore challenging to determine the purity with any degree of accuracy from these data. A recent by Yi Zhang and colleagues (2022) summarized that the CMC of rhamnolipid was notably influenced by the hydrophobicity of the carbon source, as well as the purity of the rhamnolipid in which impure rhamnolipid will exhibit higher CMC. Additionally,

chemical characterization of rhamnolipids will aid in defining the relationship between the composition and their surface-active behaviours. This is because the ratio and composition of congeners, the size of the hydrophilic head group, the branching and length of the hydrophobic fatty acid chain as well as the presence of unsaturated bonds can greatly influence the CMC values.

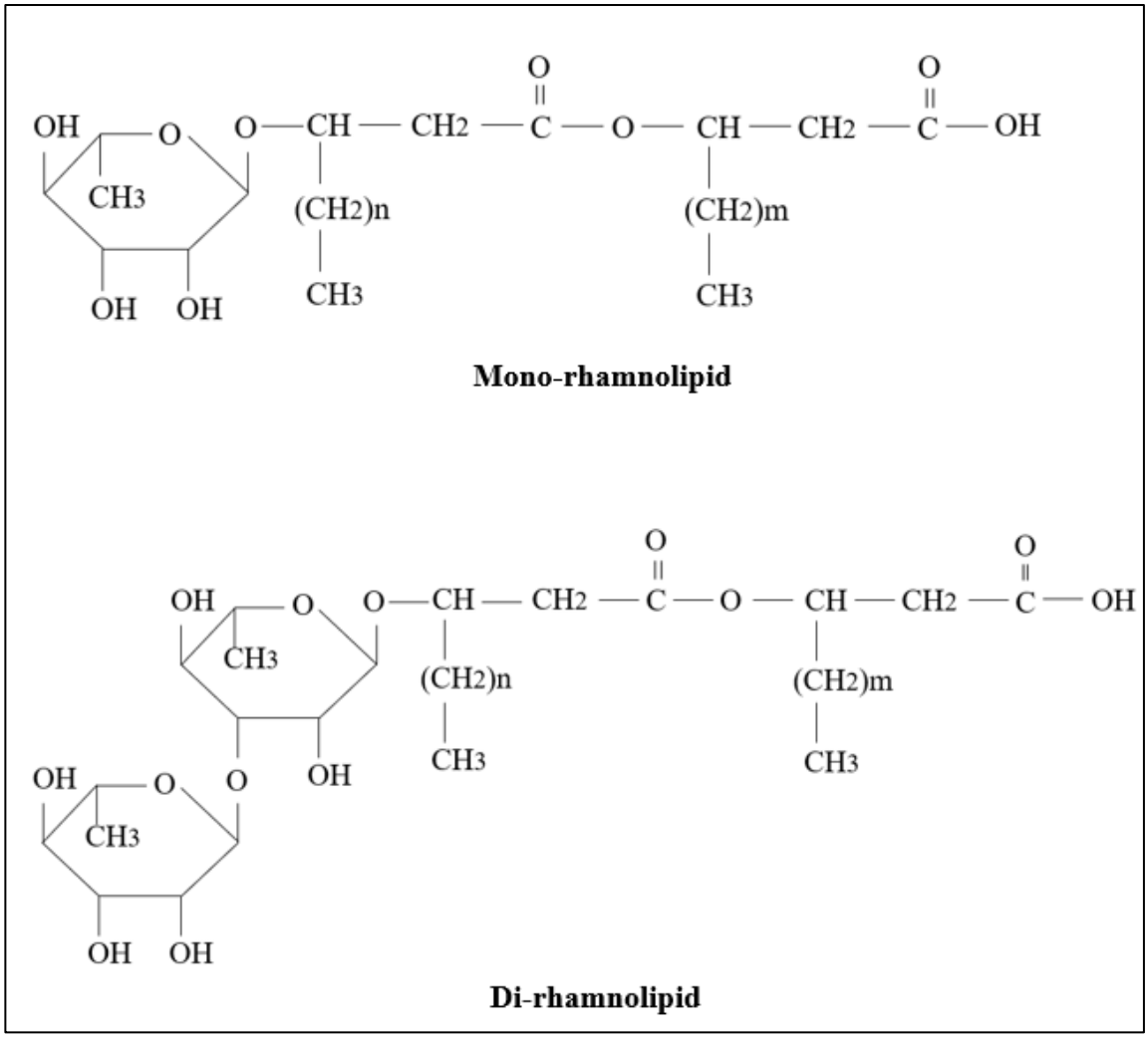

Figure 2.3 General structure of rhamnolipid congeners

2.2.1 Metabolic pathway of rhamnolipid biosynthesis

The biosynthesis of rhamnolipid in *P. aeruginosa* comprises of three unique key enzymes known as 3-hydroxyacyl-ACP:3-hydroxyacyl-ACP O-3-hydroxy-acyltransferase (RhlA), rhamnosyltransferase I (RhlB), rhamnosyltransferase II (RhlC) and a quorum-sensing based regulatory system (Soberón-Chávez et al., 2021). The biosynthesis as illustrated in Figure 2.4 was initiated by the generation of the rhamnolipid precursors, namely the (dTDP)-L-rhamnose and 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA). The sugar moiety is typically generated via the Entner-Doudoroff pathway or gluconeogenesis, depending on the nature of carbon source supplemented into the fermentation medium. The lipid moiety is formed by the esterification of two β-hydroxy fatty acids, catalysed by the acyltransferase RhlA (Eric Déziel et al., 2003; Zhu & Rock, 2008). The discussion of the origin of the β-hydroxy fatty acids remained controversial, as whether they are synthesized from the fatty acid *de novo* synthesis and exclusively being the accepted substrate of RhlA, as previously described by Zhu and Rock (2008), or via the β-oxidation process as described by Zhang and co-workers (2012) as well as Abdel-Mawgoud and colleagues (2014). The HAA molecule and (dTDP)-L-rhamnose molecule will act as the substrate for RhlB to generate mono-rhamnolipids (Wittgens et al., 2017). The subsequent addition of a second dTDP-L-rhamnose molecule by RhlC to the mono-rhamnolipid will yield di-rhamnolipid (Rahim et al., 2001). Additionally, the biosynthesis of rhamnolipid congener with single β-fatty acids chain can also occur from the hydrolysis of the second β-fatty acids chain by two α/β-hydrolases that has yet to be identified (Wittgens et al., 2017).

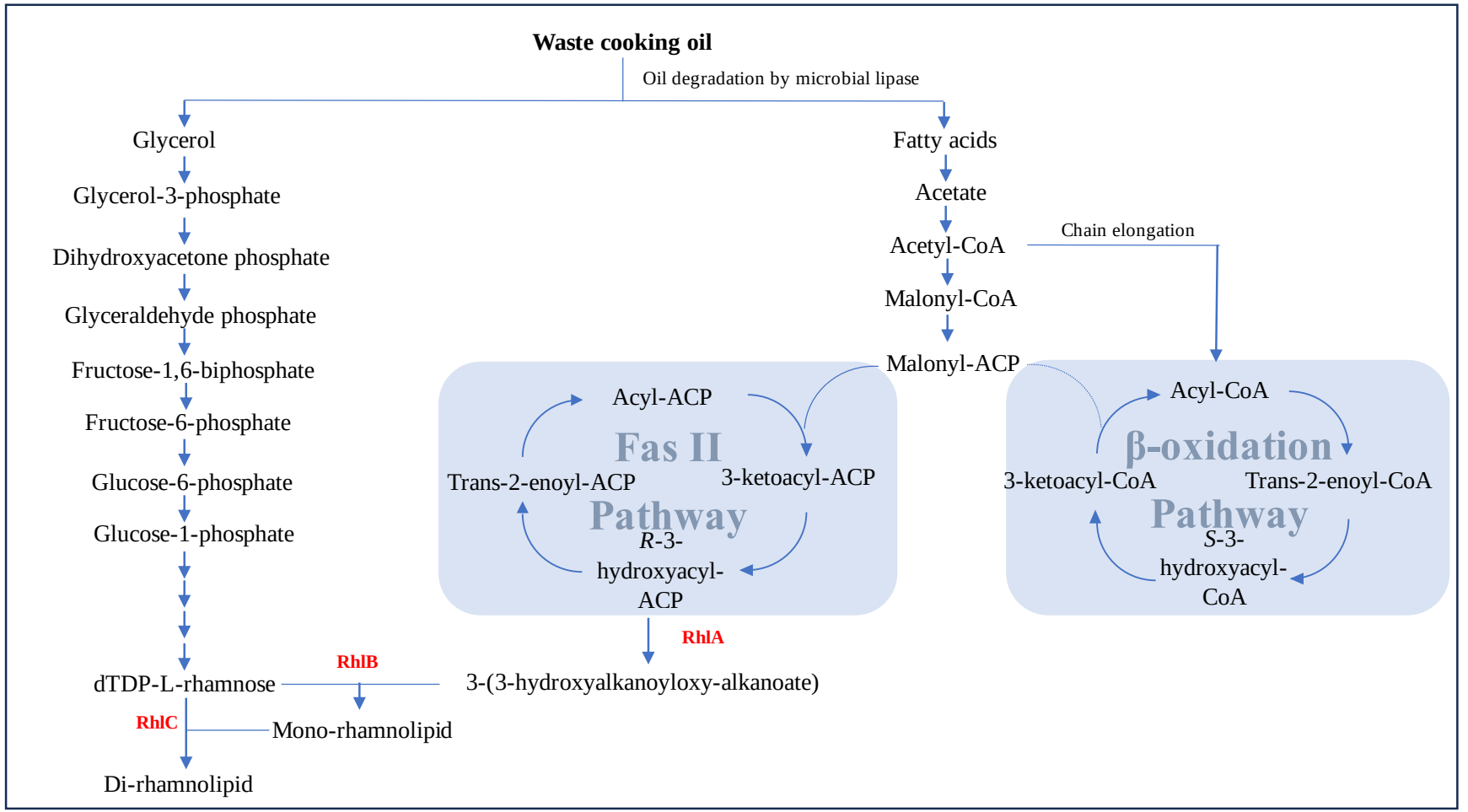

Figure 2.4 Rhamnolipid biosynthesis in *P. aeruginosa*

Interestingly, the *rhl* genes responsible in the biosynthesis of rhamnolipids was found to be exclusive to *Pseudomonas* and *Burkholderia* species. The *rhlA* and *rhlB g*enes are located within a single operon (Ochsner et al., 1996) while the *rhlC* gene is found in a separate operon, encoded together with an upstream gene with unknown function in *P. aeruginosa* (Rahim et al., 2001). On the other hand, the *rhl* genes are located within the same operon in the *Burkholderia* species (Dubeau et al., 2009). Numerous reports have reported the successful introduction and expression of these genes in a number of non-pathogenic host bacteria to synthesize rhamnolipid, and manipulating the expression levels of the RhlB and RhlC enzymes to alter the composition of the rhamnolipid congeners (Chong & Li, 2017). For example, the knockout of *rhlC* gene in *P. aeruginosa* SGΔrhlC resulted in enhanced production of mono-rhamnolipid congeners with high antimicrobial activity against various bacterial and fungal pathogens (Zhao et al., 2021). This strategy is said to be an innovative approach as it will not only aid in the development of rhamnolipids with various structural diversity, but also making it as biomolecule with huge potential of tailor-made properties to suit their demand in multiple industrial applications (Wittgens & Rosenau, 2020). Unfortunately, due to the complexity of the regulatory pathway for rhamnolipid biosynthesis, current approach in enhancing rhamnolipid productions is focused on manipulating the culture conditions instead of improving the technological properties of the rhamnolipid-producing strains (HoŠková et al., 2013; Reis et al., 2011).

2.2.2 Downstream processing of rhamnolipids

As the extraction and recovery of industrially important compound from the fermentation broth can constitute up to 80% of the overall production cost, it is therefore crucial to determine a cost-effective downstream process in the recovery of the desired product (Invally et al., 2019). Typical downstream processing of glycolipid biosurfactants includes separation of cell, disruption of cell, isolation of crude products, concentration, and purification to obtain final products as summarized in Figure 2.5 (Hubert et al., 2012). Despite being one of the most intensively studied biosurfactant, the majority of research was focused on optimising upstream procedures to increase production yield, with little attention paid to downstream processing of rhamnolipid. The major obstacles often lie in the purification of rhamnolipids that are attributed to the nature of rhamnolipids being produced as a complex combination of congeners as well as their low concentration in the culture broth. Furthermore, the use of hydrophobic substrates as carbon source for rhamnolipid production has been reported to further complicate the downstream processing as free fatty acids and other hydrophobic components remained in the fermentation broth, particularly at high rhamnolipid concentration, as a result of the ability of rhamnolipids to emulsify and solubilize hydrophobic compounds in aqueous phase. Nonetheless, these studies have contributed to the development of extraction, purification, and characterization of rhamnolipids.

The rhamnolipid fermentation is typically followed by separation of bacterial cells from the culture broth by centrifugation. As rhamnolipid is an extracellular product, the cell-free supernatant was subjected to multi-step purification process to recover the rhamnolipids. The most common purification technique applied are acid-precipitation and

solvent extraction. A comparative study between different precipitation methods and solvent extraction by Shah et al. (2016) revealed that the latter resulted in higher recovery of rhamnolipid. This is due to acid precipitation being a non-selective process that can result in precipitation of other non-rhamnolipids metabolites present in the culture broth (Invally et al., 2019). The acidification of the supernatant containing rhamnolipid to acidic pH between 2 and 3, will result in the precipitation of rhamnolipids mainly due to their protonation and increased insolubility in aqueous solution. After incubation for several hours at 4°C, the supernatant will be centrifuged and filtered to collect the rhamnolipid precipitate. This purification process is usually followed by solvent extraction technique to remove any remaining hydrophilic compounds before chromatographic analysis. Various solvents and solvent mixtures are employed for liquid-liquid extraction, such as chloroform, ethyl acetate, and chloroform-methanol mixture being the most common ones (S. J. Varjani & Upasani, 2017; Y. Zhang & Miller, 1992). Subsequent evaporation of the solvent will result in the appearance of crude rhamnolipid extract that contain all the rhamnolipid congeners. The crude extract usually appears as viscous sticky oily residue with amber brown colour and fruity odour (Haba et al., 2014).

Often, further purification of the crude extract is required before analysis involving usage of sophisticated analytical instruments as well as applications in pharmaceutical grade by means of filtration and chromatographic separations (Müller et al., 2012). Small sample size can be purified by thin-layer chromatography coated with silica-gel, while a larger sample size will require normal-phase column chromatography embedded with highly polar silica gel eluted with mobile phase containing different solvent mixtures of chloroform, methanol, and water (Satpute et al., 2010). The chromatography was started

by eluting neutral lipids by flushing the column with chloroform. This was followed by eluting the column with a series of chloroform:methanol solvent system with increasing polarity: 50:3 (v/v), 50:5 (v/v), and 50:50 (v/v). The 50:5 (v/v) and 50:50 (v/v) chloroform:methanol mixture was used to elute the mono-rhamnolipid components and di-rhamnolipid components, respectively. Then, partially purified rhamnolipid can be recovered upon complete evaporation of solvents from the collected fractions (Deepika et al., 2015a; George & Jayachandran, 2013; Gogoi et al., 2016). Pure rhamnolipid can be extracted by subjecting dissolving the concentrated fractions on methanol to TLC analysis and developed in a solvent system that will result in optimal separation of congeners based on the number of rhamnose sugar (Oluwaseun et al., 2017; Das et al., 2014).

Alternatively, other type of chromatographic technique such as ion-exchange, hydrophobic interaction, and reverse-phase C8 or C18 column have been applied to further purify rhamnolipid. Often, the usage of reverse phase column chromatography is coupled with mass spectrometry instrument to separate the rhamnolipid mixtures into individual congeners in order to analyse their distribution. The flow of mobile phase will be adjusted accordingly, with the use of water and solvents such as methanol or acetonitrile. The separation of congeners based on the chain length of hydroxy fatty acids can be done either with isocratic or gradient elution system of increasing polarity (El-Housseiny et al., 2020; Sabturani et al., 2016b). Additionally, adsorption chromatography offers a cost-friendly substitute to solvent extraction with the use of normal phase resin and non-polar solvents, as the resins can be used reuse for subsequent purification process and it requires smaller consumption of solvents (Eslami et al., 2020).

Other purification techniques such as membrane filtration and foam fractionation are less frequently applied due to limitation such as inefficient in-situ product removal due to membrane fouling and removal of biomass due to adsorption of cells in foam, respectively (Heyd et al., 2008). This method is however effective in separating and removing small medium components from the culture broth. Next, applying foam fractionation to recover rhamnolipid offers advantages in terms of reduced usage of antifoaming agents to overcome excess foam formation. However, the main concern of such technique is the continuous loss of biomass during fermentation in which the cells are entrapped by the form, where the loss of cells can significantly reduce the production efficiency (Gong et al., 2021). This demands the need for integrated strategy to obtain optimal foaming without compromising the product recovery and purification outcome. Ultimately, the purification process of rhamnolipids often depends on the desired applications where studies focusing on optimizing the downstream process with minimal solvent usage while achieving high product recovery and yield.

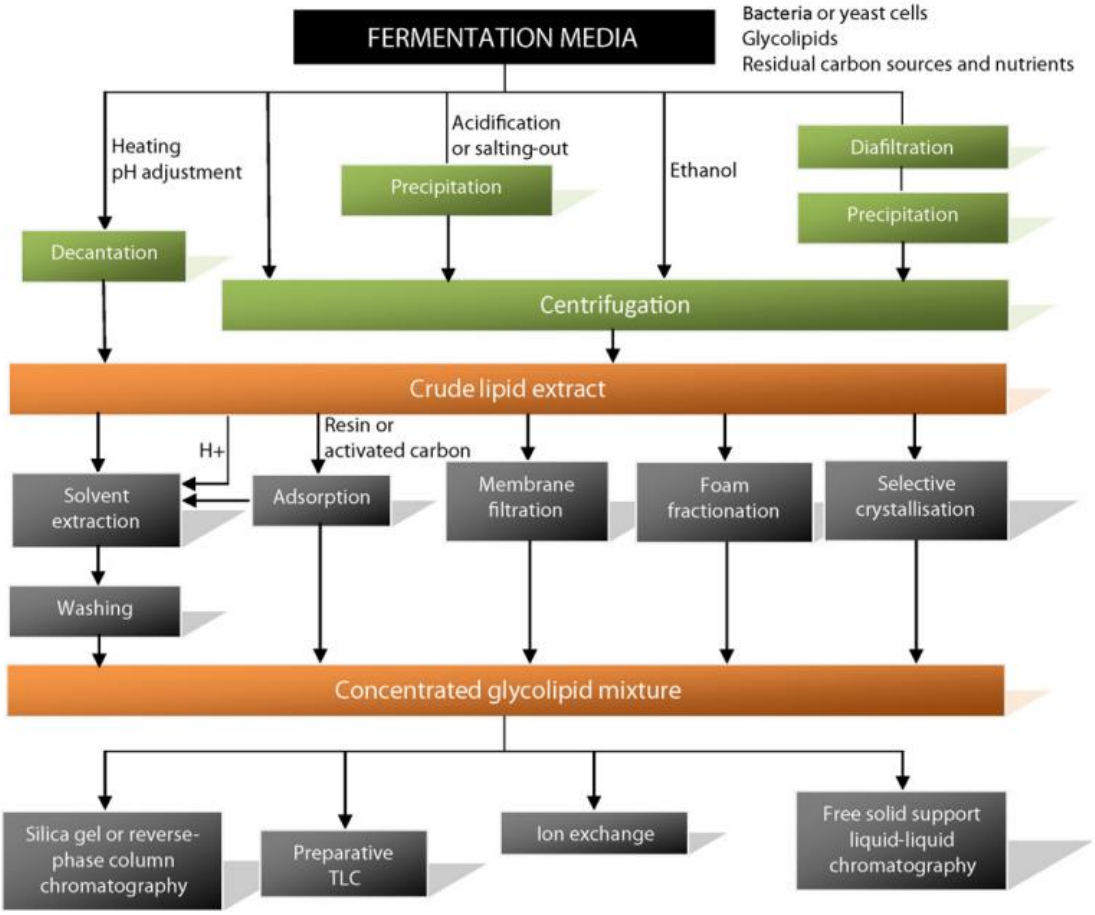

Figure 2.5 Downstream processing of glycolipid biosurfactant (Hubert et al., 2012)

2.2.3 Characterizing the chemical structure of rhamnolipid congeners

All microbial cell factories producing rhamnolipid share a common feature, in which they produced mixture of rhamnolipid congeners that differ in the number of rhamnose sugar and length of the β-hydroxy fatty acids, with one or two predominant congeners (Tiso et al., 2017). As aforementioned, identifying the rhamnolipid congeners is crucial as a slight difference in the congener composition can significantly affect their properties, which will consequently determine their suitable industrial application in various fields (Zhao et al., 2020). Various approaches have been applied to characterize the structural composition of rhamnolipids produced by various strains of *P. aeruginosa* involving simple colorimetric method to advanced and sophisticated chromatographic separation of the congeners, often followed by mass spectrometry as well as spectroscopic analysis to identify the number of rhamnose moiety and the chain length of the fatty acid moiety present in each congener (Eslami et al., 2020).

The quantification of rhamnolipid is commonly determined by using calorimetric method such as anthrone assay and orcinol assay. Both assays are used to estimate the total rhamnolipid concentration as well as to monitor the profile of rhamnolipid production during the fermentation process. However, such methods pose a major limitation in terms of the actual concentration of rhamnolipid in the sample as it is not specific for rhamnose, in which the interference of free sugars from the carbon source in the medium can lead to false overestimation of the actual reading. These approaches, despite being simple and rapid, are not able to distinguish the rhamnolipid congeners as it can only be used to detect the presence of rhamnose sugar moiety of rhamnolipids. Moreover, variations in results