PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY MANGROVE *Streptomyces* sp. PBD-410L

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UNIVERSITI SAINS MALAYSIA

2019

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

August 2019

ACKNOWLEDGEMENT

In the Name of Allah, the Most Gracious and the Most Merciful.

My full gratitude to Allah S.W.T., The Creator of the universe who paved the path for me to be able to complete my thesis. Peace be upon His Prophet Muhammad S.A.W., the light of Humanity.

First and foremost, I would like to express my sincere and utmost gratitude to my respected supervisor, Associate Professor Dr. Ahmad Ramli Mohd Yahya for his unwavering supports and mentorship throughout this study. Thank you for all the advices, ideas, moral support and patience in guiding me through this project. Your wealth of knowledge in this field is inspiring. I would like to extend my appreciation to my co-supervisor, Dr. Nur Asshifa Md Noh for her advises and opportunities given throughout my journey. A big thanks to my fellow friends, especially Kak Sya, Kak Killa, Aina, Syafiq, Piqah and Dhani also members of #DU872 for the moral supports, assistance and guidance. Thank you for always brightening up my days whenever we were all together. Not to forget a big appreciation to School of Biological Sciences and their staffs for providing an assistance and research facilities for my studies.

A special thank from the bottom of my heart for my irreplaceable beloved family. To my dearest mom and dad, Nozila Ismail and Rusly Ahmad, as well as my sister and brother for their patience and endless supply of unconditional love, moral and financial support. I will always love all of you.

Last but not least, to all valued scientists and everyone who are now reading this thesis, thank you for your interest. May we all be successful in life and hereafter and may Allah the Almighty bless us all. Amen.

Thank you so much!

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LIST OF ABBREVIATIONS

g/L	gram per litre
v/v	volume per volume
w/v	weight per volume
rpm	rotation per minute
g	gram
%	percent
kg	kilogram
L	litre
mL	mililitre
µL	microlitre
µm	micrometre
°C	degree celcius
SCA	Starch casein agar
MSM	Minimal salt medium
M	Molar
NB	Nutrient broth
NA	Nutrient agar
E_{24}	Emulsification index
OST	Oil spreading technique
ST	Surface tension
SEM	Scanning electron microscope
HMDS	Hexamethyldisilazane

PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
R_f	Retention factor
OD	Optical density
GCMS	Gas chromatography mass spectrometry
FAME	Fatty acid methyl ester
h	hour
STR	Stirred tank bioreactor

PENGHASILAN DAN PENCIRIAN BIOSURFAKTAN DIHASILKAN OLEH *Streptomyces* sp. PBD-410L BAKAU

ABSTRAK

Biosurfaktan merupakan sebatian aktif permukaan yang dihasilkan daripada mikrob yang mengurangkan ketegangan permukaan antara fasa cecair dengan kekutuban yang berbeza. Pada masa ini, pengeluar biosurfaktan yang terbaik dilaporkan adalah spesis *Pseudomonas* dan *Bacillus*. Malangnya, mereka dikenali sebagai patogen manusia yang oportunis, dengan sesetengahnya mempunyai rintangan antibiotik, yang menimbulkan kebimbangan. Aktinobakteria filamen boleh menjadi pengeluar biosurfaktan alternative yang lebih selamat. Kajian ini memberi tumpuan kepada pengenalpastian aktinobakteria yang menghasilkan biosurfaktan, pencirian biosurfaktan yang dihasilkan dan kesan parameter fermentasi terhadap penghasilan biosurfaktan. Sebanyak 60 pencilan aktinobakteria daripada habitat bakau disaring untuk penghasilan biosurfaktan dengan menggunakan minyak sawit sebagai sumber karbon utama. Kehadiran biosurfaktan telah disahkan menggunakan pengukuran ketegangan permukaan (ST), teknik sebaran minyak (OST) dan indeks pengemulsian (E_{24}). Pencilan PBD-410L telah dipilih sebagai pengeluar biosurfaktan yang terbaik berdasarkan nilai tertinggi bagi OST (68.0±1.7 mm) dan nilai ketegangan permukaan terendah (39.7±0.6 dynes/cm). Pencilan ini dikenalpasti dan dicirikan sebagai *Streptomyces* sp. PBD-410L, berdasarkan ciri-ciri morfologi dan pengenalpastian molekul. Biosurfaktan ekstrak mentah telah berjaya diperoleh dengan kepekatan 11.04 g/L dengan menggunakan campuran kloroform/methanol (2:1 v/v). Peratusan hasil terhadap minyak kelapa sawit adalah 40.8%, dengan nilai 70.7 mm bagi teknik penyebaran minyak. Penulenan biosurfaktan ekstrak mentah

melalui kromatografi turus menghasilkan 1.06 g/L biosurfaktan separa tulen yang menunjukkan nilai 82.7 mm dalam teknik penyebaran minyak. Ia menunjukkan biosurfaktan separa tulen membentuk 9.5% (b/b) daripada daripada biosurfaktan ekstrak mentah. Hasil daripada ujian biokimia, analisa TLC, GC-MS, FT-IR dan NMR mengenalpasti bahawa biosurfaktan separa tulen sebagai lipopeptida. Lipopeptida yang dihasilkan oleh bakteria berfilamen ini terdiri daripada asid palmitik dan oleik sebagai moieti hidrofobik dan kumpulan peptida sebagai moieti hidrofilik. Kepekatan misel kritikal telah ditentukan sebagai 0.02 g/L dan diuji berkesan pada pelbagai suhu (30-121°C), pH (1-11) dan kemasinan [5-20% (w / v) NaCl]. Keadaan pertumbuhan yang sesuai dan penghasilan lipopeptida telah dikaji. KNO_3 dipilih sebagai sumber nitrogen dalam penghasilan lipopeptida. Dengan menggunakan reka-bentuk eksperimen tiga faktor dua peringkat dalam pengkulturan kelalang goncang menunjukkan nilai tertinggi dalam OST (73.5 mm) dan ketegangan permukaan terendah (35.5 dynes / cm) pada 1.6% i/i minyak kelapa sawit, 4 g/L KNO_3 dan suhu pengeraman 37°C. Keadaan ini telah diekstrapolasi ke bioreaktor tangki teraduk 3.6-L. Bagi memastikan penyebaran minyak yang mencukupi dan meminimumkan pemisahan fasa, kultur telah diaduk pada 300 rpm atau kelajuan hujung pengaduk pada 0.91 ms^{-1} yang memberikan 7.8 ± 0.3 g/L minyak yang diekstrak dari sampel kaldu. Pertumbuhan sel tertinggi dan penghasilan lipopeptida dicapai pada kadar pengadukan 300 rpm, memberikan OST pada 50.7 mm dan nilai E_{24} pada 46.6%. Kesimpulannya, *Streptomyces* sp. PBD-410L daripada habitat bakau telah terbukti menghasilkan biosurfaktan lipopeptide dengan menggunakan kelapa sawit sebagai substrat karbon utama.

PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY MANGROVE *Streptomyces* sp. PBD-410L

ABSTRACT

Biosurfactants are microbially produced surface-active compounds that reduce the surface tension between liquid phases of differing polarity. Presently, the best reported biosurfactant producers are *Pseudomonas* and *Bacillus* spp. Unfortunately, they are known opportunistic human pathogens, with some having antibiotic-resistance, causing grave concerns. Filamentous actinobacteria can be safer alternative biosurfactant producers, with no known human pathogenicity. The present study focused on the identification of biosurfactant-producing actinobacterium, characterization of the biosurfactant produced and the effect of fermentation parameters on biosurfactant production. A total of 60 actinobacterial isolates from mangrove sediments were screened for biosurfactant production using palm oil as the main carbon source. The presence of biosurfactant was confirmed using surface tension (ST) measurement, oil spreading technique (OST) and emulsification index (E_{24}). Isolate PBD-410L was selected as the best biosurfactant producer based on the highest value for OST (68.0±1.7 mm) and the lowest surface tension measurement (39.7±0.6 dynes/cm). This isolate was characterized and tentatively identified as *Streptomyces* sp. PBD-410L, based on morphological characteristics and molecular identification. Crude biosurfactant extract was successfully recovered at a concentration of 11.04 g/L by using chloroform/methanol (2:1 v/v) mixture. The percentage yield on palm oil was 40.8%, giving 70.7 mm diameter in oil spreading technique. Purification of the crude biosurfactant extract through silica gel column chromatography produced a total of 1.06 g/L partially

purified biosurfactant, showing 82.7 mm diameter value in oil spreading technique. This shows that the partially purified biosurfactant made up 9.5% (w/w) of the crude biosurfactant extract. Results from biochemical assays, TLC, GC-MS, Fourier FT-IR and NMR analyses identified the partially purified biosurfactant as a lipopeptide. The lipopeptide produced by this filamentous bacterium comprised palmitic and oleic acids as the hydrophobic moiety and a peptide group as the hydrophilic moiety. Its critical micelle concentration (CMC) was determined to be 0.02 g/L. It was shown to be effective at a wide range of temperature (30-121°C), pH (1-11) and salinity [5-20% (w/v) of NaCl]. The suitable conditions for growth and lipopeptide production have been studied. KNO_3 was selected as a nitrogen source in lipopeptide production. Using a three-factor two-level experimental design in a shake flask cultivation, the highest OST value (73.5 mm) and the lowest surface tension (35.5 dynes/cm) were achieved at 1.6% v/v of palm oil, 4 g/L of KNO_3 and 37°C incubation temperature. This condition was subsequently extrapolated to a 3.6-L bench-top stirred tank bioreactor. To ensure adequate oil dispersion and minimize phase separation, the culture was agitated at 300 rpm or 0.91 m s^{-1} stirrer tip speed, giving 7.8±0.3 g/L of extracted oil from the broth sample. The highest cell growth and lipopeptide production was achieved at the agitation rate of 300 rpm, giving an OST of 50.7 mm and an E_{24} of 46.6%. In conclusion, *Streptomyces* sp. PBD-410L from mangrove habitat has been shown to produce a lipopeptide biosurfactant using palm oil as the main carbon substrate.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Surfactants are one of the most frequently used chemical products in daily life, notably in household and personal care products (Gudina *et al*., 2013; Varjani & Upasani, 2017). They are surface-active compounds with both hydrophilic and hydrophobic moieties that enable them to accumulate at the interphase between two liquids of different degrees of polarities and hydrogen bonding such as oil/water or air/water interfaces. Such characteristics allow them to reduce the surface and interfacial tension between two immiscible liquids, thereby assisting the liquids to mix homogenously (Santos *et al*., 2016). Their unique properties, namely wettability, detergency, emulsion activity, dispersity, stability, and foaming ability, have broad practical applications in diverse industrial sectors such as oil and gas, pharmaceutical, cosmetic, petroleum, and food processing industries (Marchant & Banat, 2012).

Currently, most surfactants that are marketed are synthetically derived (Varjani & Upasani, 2017). Some of their manufacturing processes and by-products are potentially hazardous or are becoming less acceptable to the environment in these environmentally-conscience times. For this reason, the demand of biologically-synthesized surfactants as a replacement for chemically derived surfactants are on the rise (Md, 2012). These natural surfactants are termed as biosurfactants. They are produced by heterogenous groups of living cells. Biosurfactants function just as their chemical counterpart but offer more advantages such as more biodegradable, have low toxicity and environmental friendly. In addition, the unique characteristics such as

high effectiveness at extreme pH, salinity and temperature make biosurfactants to be good substitutes for chemical surfactants, particularly in the food, cosmetic and healthcare industries, industrial cleaning products and agricultural chemicals (Chakraborty *et al*., 2015).

Biosurfactant is a valuable metabolite. The global demand for biosurfactants is increasing through the years. However, there are limitations in their large-scale production. Presently, the best reported biosurfactant producers are from bacterial origin, namely the genera *Pseudomonas* and *Bacillus* (Noh *et al*., 2012; Gudina *et al*., 2013). Unfortunately, these are known opportunistic human pathogens and the antibiotic-resistance ability of some strains is causing grave concerns. These provides the motivation for studying a safer microbial system for biosurfactant production.

Indeed, multiple consecutive steps are required in the recovery process to obtain pure products. This causes the production cost to sky-rocket with downstream processing contributing to almost 70-80% of the total production cost (Desai & Banat, 1997). Furthermore, the fermentation process of biosurfactant-producing bacteria on a large-scale production is challenging due to severe foam formation, an inevitable consequence of elevated concentration of surface active agents.

Filamentous actinobacteria can be an alternative to alleviate these challenges. They offer safer biosurfactant producers, none of which are known to show pathogenicity in human. Among actinobacterial members, *Streptomyces*, a Gram-positive filamentous bacterium, has proven to be a prominent source of natural products for industries (Panjiar *et al*., 2013; Kügler *et al*., 2015). The characteristics of non-pathogenicity in human extends the application of the products to a wide range of industries, especially in pharmaceutical, personal care and food industries (Santos

et al., 2017). Unlike bacterial fermentations, the fermentation of filamentous bacteria in large scales are much less challenging in a number of aspects. There is less excessive foam formation and product separation from biomass is typically much easier, subsequently reducing the product recovery cost. These characteristics emphasize the preferability of filamentous bacteria as a biosurfactant producers.

To date, reports on the ability of actinobacteria as biosurfactant producers are few and far between. In fact, the first report on actinobacterial biosurfactant dates back to merely about 20 years ago. Richter *et al*. (1998) reported the first extracellular hydrophobic peptide surfactant produced by Streptomyces tendae. Since then, glycolipid biosurfactants from *Streptomyces* sp. B3, *Streptomyces matensis*, *Streptomyces* MAB36 and *Streptomyces* sp. ISP2-49E have been studied (Khopade *et al*., 2012a; Kalyani *et al*., 2014b; Manivasagan *et al*., 2014; Yan *et al*., 2014). Nevertheless, most of the reported studies focussed on the isolation, screening and optimization process of the biosurfactant production. The available information on the detail characterization of the type of biosurfactant from this filamentous bacterium are limited.

Therefore, this study was conducted to screen several actinobacterial isolates from mangrove habitat for extracellular biosurfactant production. The mangrove habitat was chosen due to its rich microbial biodiversity, a direct consequence of its large daily variations in environmental conditions such as temperature, salinity, pH, moisture and oxygen level. The isolate showing the highest potential as a biosurfactant-producer was then identified using morphological characteristics and 16S rRNA gene sequence analysis. Then, the structure and properties of the biosurfactant produced by this filamentous bacterium was characterized and identified.

Suitable conditions for growth and biosurfactant production were investigated and extrapolated into a stirred tank bioreactor cultivation.

1.2 Aim and Objectives of Research

The main aim of the present study was to screen for biosurfactant-producing actinobacteria isolated from mangrove habitats. The specific objectives were:

1. To identify biosurfactant produced by an actinobacterium that is isolated from local mangrove.

2. To determine the effect of fermentation parameters on the production of biosurfactant from the isolated actinobacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosurfactant

2.1.1 Properties of Biosurfactant

Biosurfactants are biologically synthesized by various microorganisms including yeast, fungi, filamentous and non-filamentous bacteria (Youssef *et al*., 2004; Khopade *et al*., 2012a). They are surface active compounds that are categorized as secondary metabolites produced by microorganisms. Biosurfactants are typically produced at the late exponential or stationary growth phase (Chakraborty *et al*., 2015). The majority of known biosurfactants are synthesized by microorganisms with water immiscible substrates, but some have been produced with water soluble substrates such as glucose, glycerol and ethanol. Biosurfactants are also known as amphiphilic molecules, comprising two different domains which are hydrophilic head and hydrophobic tail (Kuyukina *et al*., 2015). The hydrophilic portion makes them soluble in water which is composed of amino acids or peptides, anions or cations, or monosaccharides, disaccharides, or polysaccharides. In contrast, the hydrophobic tail may consist of saturated, unsaturated or hydroxylated fatty acids that are insoluble in water.

By having both polar and non-polar domains, biosurfactants have the ability to reduce surface or interfacial tension between fluid phases that have different polarity and degree of hydrogen bonding such as water-oil or water-air interfaces (Seghal Kiran *et al*., 2010). The mechanism of action by biosurfactants to reduce surface and interfacial tension is an adsorption. The biosurfactant will be adsorbed onto the

different phases causing more interaction and mixing of that phases (Uzoigwe *et al*., 2015). The ability of biosurfactant to reduce surface tension of water is generally used as a measure of efficiency of the biosurfactant.

Khopade *et al*. (2012b) reported that the most effective biosurfactant can reduce surface tension of water from 72 dynes/cm surface tension to value ranging between 25-30 dynes/cm. The surface tension correlates with the concentration of the surface-active compound until the critical micelle concentration (CMC) is reached. The CMC is defined as the minimum concentration necessary to initiate micelle formation. Efficient biosurfactants have a low critical micelle concentration, which means that less biosurfactant is required to decrease the surface tension (Ibrahim *et al*., 2013).

Biosurfactants have gained more attention in recent years because they offer several advantages over chemical surfactants. They are low toxicity in nature, therefore, are more preferable for applications in cosmetic and pharmaceutical industries than chemical surfactants. Besides that, they are easily degraded by microorganisms in water or soil. This property makes them suitable to treat oil spills and wastes. Biosurfactants are also compatible to the environment and remain effective at extreme conditions including temperature, pH and salinity (Chakraborty *et al*., 2015). For instance, the lipopeptide produced by *Bacillus licheniformis* JF-2 is stable at 75°C for up to 140 hours. It remains active within a pH range of 5 to 12 and can tolerate high salinity up to 10% w/v NaCl.

In terms of availability, biosurfactants can be produced by microorganisms via fermentation with a vast number of substrates, including oils, hydrocarbons, glycerol and inexpensive renewable substrates like agricultural wastes. Therefore,

biosurfactants gain interest as an eco-friendly alternative to the chemical surfactants (Ismail *et al*., 2013).

2.1.2 Screening for Biosurfactant Producer

The potential of any microorganisms in producing biosurfactant are determined by screening methods. Screening methods can give qualitative and/or quantitative results for biosurfactants production. However, for convenience and speed, qualitative methods are generally sufficient for screening of isolates. Many studies reported the use of different screening methods at a time and suggest their classification as primary, secondary and tertiary screening tools (Varjani *et al*., 2014).

Biosurfactants are known as surface active molecules that have the ability to adsorb to surfaces or interfaces. Based on this, wide arrays of screening method for biosurfactant-producing microorganism are developed including the direct surface and interfacial tension measurements. Other screening methods have been developed that use the interfacial activity of the biosurfactants but do not measure it directly, such as the drop collapse method and the oil spreading method (Walter *et al*., 2010).

The surface tension of water/sample can be measured by using the Du-Nouy ring method. This method is based on measuring the force required to detach a ring or loop of wire from an interface or surface. The detachment force is proportional to the interfacial tension. The Du-Nouy ring method is commonly practised in screening of biosurfactant producers and surface tension reduction is used as a standard method in measuring of surface properties of biosurfactant (Płaza *et al*., 2006; Anyanwu *et al*., 2011; Pereira *et al*., 2013). This method is easy to conduct if measured by an automated tensiometer. However, it is quite challenging to operate the traditional

tensiometer without an automated system because it is time consuming and inconvenient, especially for screening large numbers of isolates (Youssef *et al*., 2004).

Another method of screening for biosurfactants is the oil spreading technique, which is an indirect method. This method involves measuring the diameter of clear zone that appears when a drop of biosurfactant is placed on top of the oil-film (Thavasi *et al*., 2011). In the presence of biosurfactant, the oil layer will be spread on the water surface. Otherwise, the drop of sample will remain beaded on the oil layer due to the hydrophobicity of the oil (Nasr *et al*., 2009). In fact, this diameter is directly proportional to the biosurfactant concentration. The study conducted by Zambry *et al*. (2018) showed the diameter of the clear zone linearly increased with the concentration of crude biosurfactant extract over a concentration range of 0 to 10 g/L. This method is easy to perform and less time-consuming than the surface tension measurement. Many previous studies reported the use of the oil spreading technique to detect the presence of biosurfactant (Youssef *et al*., 2004; Thampayak *et al*., 2008; Thavasi *et al*., 2011; Zambry *et al*., 2017).

The drop collapse method is a quantitative method of detecting biosurfactant. It was first developed by Jain *et al*. (1991). The presence of the biosurfactant is indicated by a collapse and spread of an oil drop. The oil drop disperses because of the reduction of interfacial tension between hydrophillic and hydrophobic surfaces. This method is attractive for detecting biosurfactant because it is sensitive and easy. (Płaza *et al*., 2006; Thavasi *et al*., 2011; Varadavenkatesan & Murty, 2013). However, this method is not recommended for low concentrations of biosurfactant as it is prone to false negatives. A better practice would be to subject the samples that show negative results in drop collapse method to the oil spreading technique to detect lower concentrations of biosurfactant.

The emulsification index (E24) is a qualitative method for detecting biosurfactants, achieved by measuring the amount of the emulsion layer relative to the entire solution. The emulsion layer is formed in the presence of a surfactant when mixing aqueous with hydrocarbon or immiscible liquid. Kerosene was the most popular hydrocarbon used in the emulsification test (Rosenberg *et al*., 1979). Biosurfactants that are able to stabilize the emulsion layer are known as bioemulsifiers. Therefore, it is necessary to include this method in the screening methods for biosurfactant detection in addition to surface tension, oil spreading technique and drop collapsed methods.

2.1.3 Metabolic Pathway of Biosurfactant

In biosurfactant production by microorganisms, different type of substrates in the culture medium would influence the metabolic pathways in biosurfactant synthesis (Weber *et al*., 1992; Desai & Banat, 1997). For example, when using a water-soluble substrate like glucose, the carbon flow is regulated by both lipogenic pathway (lipid generation) and the development of hydrophilic moiety via glycolytic pathway (Haritash & Kaushik, 2009). Figure 2.1 illustrates the metabolic pathways involved in biosurfactant synthesis using water-soluble substrate. Glucose is broken down to form the intermediate (glucose-6-phosphate, G6P). Subsequently, a series of enzymes catalyze G6P on route to synthesize various forms of hydrophilic moieties in the biosurfactant; trehalose, sophorose, rhamnose, mannose and polysaccharide. Meanwhile, the hydrophobic moiety (lipid) is formed also using the carbon substrate but with a slightly different pathway. Glucose is oxidized to pyruvate which is then transformed into acetyl-CoA. Acetyl-CoA is carboxylated to form malonyl-CoA,

which is a precursor to fatty acids, will subsequently converted into fatty acids for lipid production (Nurfarahin *et al*., 2018).

In contrast, when hydrocarbon groups are used as the carbon source in biosurfactant production, the synthesis requires different metabolic pathways, namely the lipolytic pathway and gluconeogenesis (GNG). This leads to hydrophobic moiety production through fatty acid synthesis (FAS II) pathway and synthesizing the hydrophilic moiety de novo through GNG. The GNG pathway is essentially the reverse of glycolysis to produce glucose as the end-product, involving different enzymes, namely hexokinase, pyruvate kinase and phosphofructokinase-1. These reactions are irreversible. GNG begins with the oxidation of fatty acids to form acetyl-CoA through β-oxidation. Then, it enters the tricarboxylic acid (TCA) cycle to form pyruvate and subsequently, converted into G6P. The main reactions involved in the synthesis of biosurfactant using hydrocarbon as the main substrate are shown in Figure 2.2.

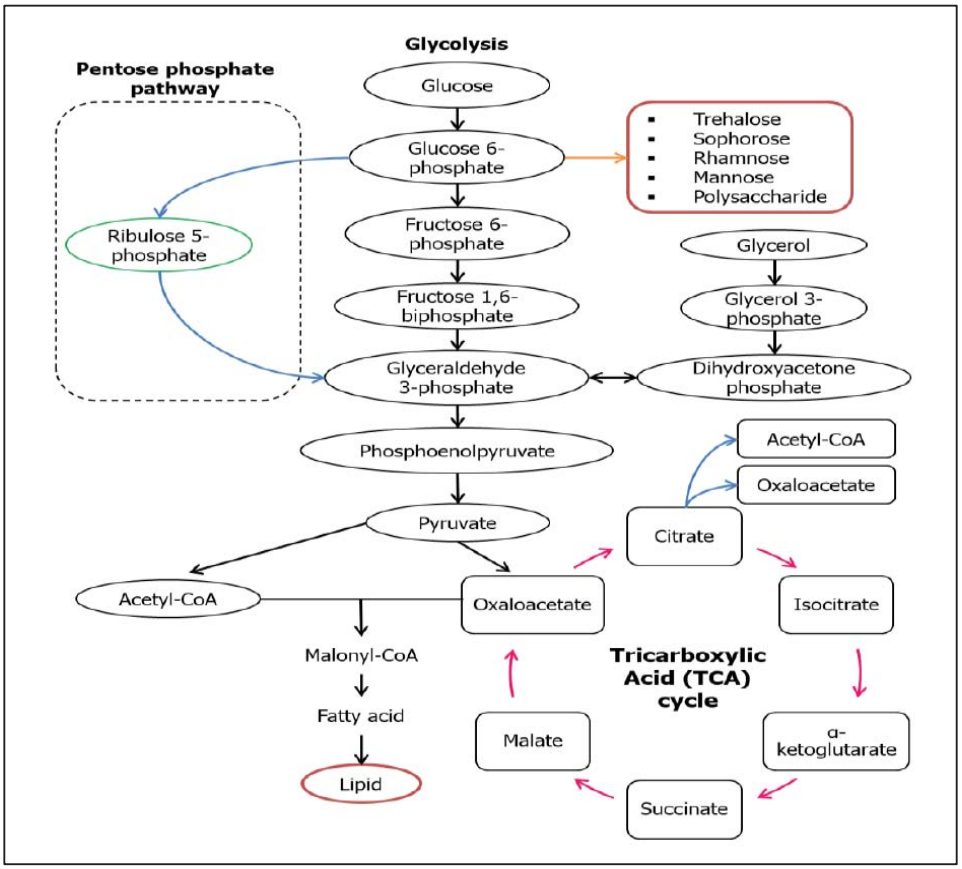

Figure 2.1: Metabolic pathways involved in synthesis of biosurfactant using water-soluble substrate. Adapted from Haritash and Kaushik (2009).

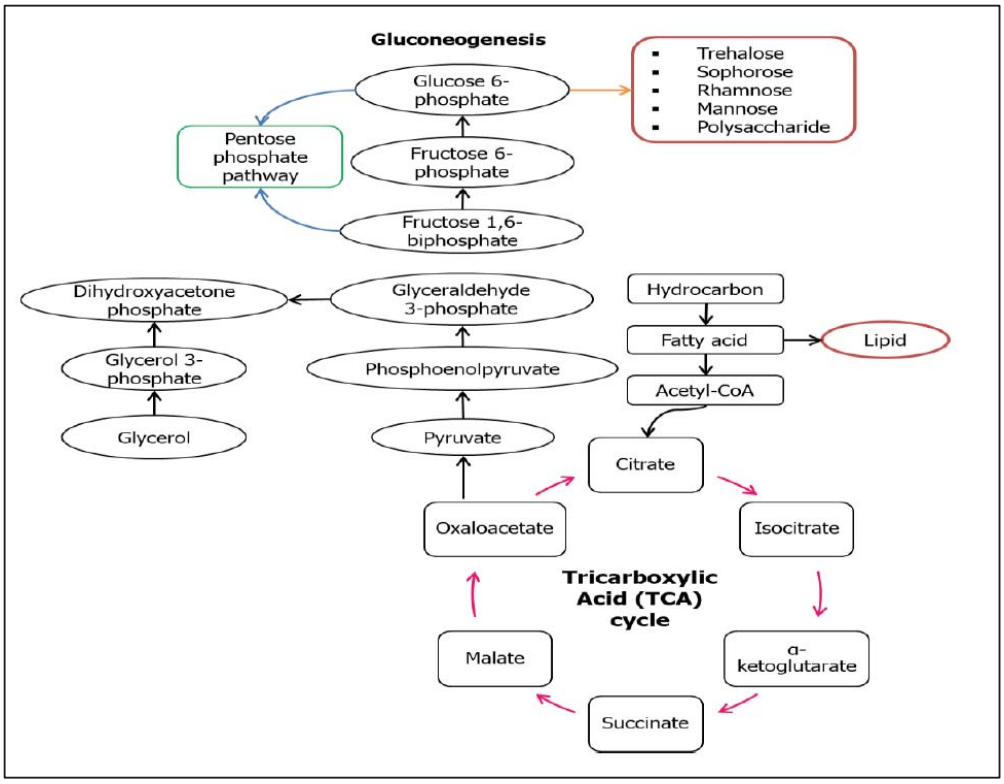

Figure 2.2: Metabolic pathways involved in synthesis of biosurfactant using hydrocarbon substrate. Adapted from Santos *et al*. (2016).

To complete the process of biosurfactant formation, some multienzyme complexes are required after the production of lipid and sugar moieties. To date, the molecular biosynthesis regulation for rhamnolipid (glycolipid type) by *Pseudomonas aeruginosa* and surfactin (lipopeptide type) synthesized by *Bacillus subtilis* were among the earliest biosurfactant metabolic pathways to be decoded (Burger *et al*., 1963). The rhamnolipid production is carried out by two sequential glycosyl transfer reaction that is catalysed by a different rhamnosyltransferase and surfactins production is catalysed non-ribosomally by a large multienzyme peptide synthetase complex known as the surfactin synthetase (Das *et al*., 2008). In the case of lipopeptides, they

are generally synthesized in a ribosome-independent manner with non-ribosomal peptide synthetases (Roongsawang *et al*., 2011).

2.1.4 Classification and Characterization of Biosurfactant

Biosurfactants produced by microorganisms vary in their molecular size and chemical property. Two components that are involved in categorization of biosurfactants are their chemical composition and microbial origin. The hydrophilic portion of the biosurfactant can be an amino acid, carbohydrate, phosphate, cyclic peptide, carboxylic acid or alcohol while the hydrophobic portion may consist of saturated, unsaturated or fatty acids (Khopade *et al*., 2012a).

Several types of biosurfactants have been recovered including lipopeptides and lipoproteins, glycolipids, phospholipids and fatty acids, particulate and polymeric surfactants, depending on the producing microorganisms, raw materials and conditions of the fermentation processes (Makkar *et al*., 2011). Most of them are either anionic or neutral. Only a few are cationic, typically those containing amine groups (Gibbs *et al*., 2004).

The best known biosurfactants are glycolipids and lipopeptides. Traditionally, the most studied glycolipids are rhamnolipids (derived from rhamnose), sophorolipids (derived from sophorose, a disaccharide), trehalolipids (derived from trehalose) and mannosylerythritol lipids (MELs) while lipopeptides are mainly produced by *Bacillus* species. Table 2.1 shows some other biosurfactants that have been reported by researchers.

Table 2.1: Main classes of biosurfactant and some respective producers

No	Type of biosurfactant	Microorganisms	References
1	Glycolipid	*Pseudomonas aeruginosa* USM-AR2	Salwa *et al.* (2009)
		Brevibacterium casei MSA19	Kiran *et al.* (2010)
		Pseudomonas aeruginosa OCD1	Sonali Sahoo *et al.* (2011)
		Yeast *Pseudozyma*	Yamamoto *et al.* (2012)
		Geobacillus Stearothermophilus	Jara *et al.* (2013)
		Streptomyces sp. MAB36	Manivasagan *et al.* (2014)
		Yeast *Starmerella bombicola*	Matsuzawa *et al.* (2015)
		Staphylococcus saprophyticus SBPS 15	Mani *et al.* (2016)
		Wickerhamomyces anomalus CCMA 0358	Souza *et al.* (2017)
		Rhodococcus erythropolis S67	Luong *et al.* (2018)
2	Lipopeptide	*Nocardiopsis alba* MSA10	Gandhimathi *et al.* (2009)
		Pseudomonas fluorescens BD5	Janek *et al.* (2010)
		Bacillus cereus NK1	Sriram *et al.* (2011)
		Paenibacillus polymyxa	Quinn *et al.* (2012)
		Bacillus sp I-15	Ismail *et al.* (2013)
		Bacillus mojavensis A21	Ayed *et al.* (2014)

		Bacillus subtilis	Wei-Chuan Chena *et al.* (2015)
		Bacillus subtilis SPB1	Mnif *et al.* (2016)
		Paenibacillus dendritiformis CN5 strain	Bezza and Chirwa (2017)
		Corynebacterium aquaticum	Martins *et al.* (2018)
3	Phospholipid, neutral lipids and fatty acids	*Micrococcus luteus* BN56	Tuleva *et al.* (2009)
		Rhodococcus sp.	Zaragoza *et al.* (2010)
		Pseudozyma aphidis DSMZ 70725	Onghena *et al.* (2011)
		Klebsiella pneumoniae WMF02	Jamal *et al.* (2012)
		Pseudomonas putida BD2	Janek *et al.* (2013)
		Tsukamurella spumae	Kügler *et al.* (2014)
		Klebsiella pneumoniae H1	Yang *et al.* (2015)
		Staphylococcus hominis	Rajeswari *et al.* (2016)
		Rhodococcus erythropolis M-25	Pi *et al.* (2017)

2.1.5 Chemical Structure of Biosurfactant

To date, the best known biosurfactants are glycolipids and lipopeptides, predominantly those produced by *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively. Glycolipids correspond to a fatty acid in combination with a carbohydrate moiety. Figure 2.3 shows some chemical structure of the most recognized glycolipids. They consist to a group of compounds that differs by the nature of the lipid and carbohydrate moiety (Inès & Dhouha, 2015).

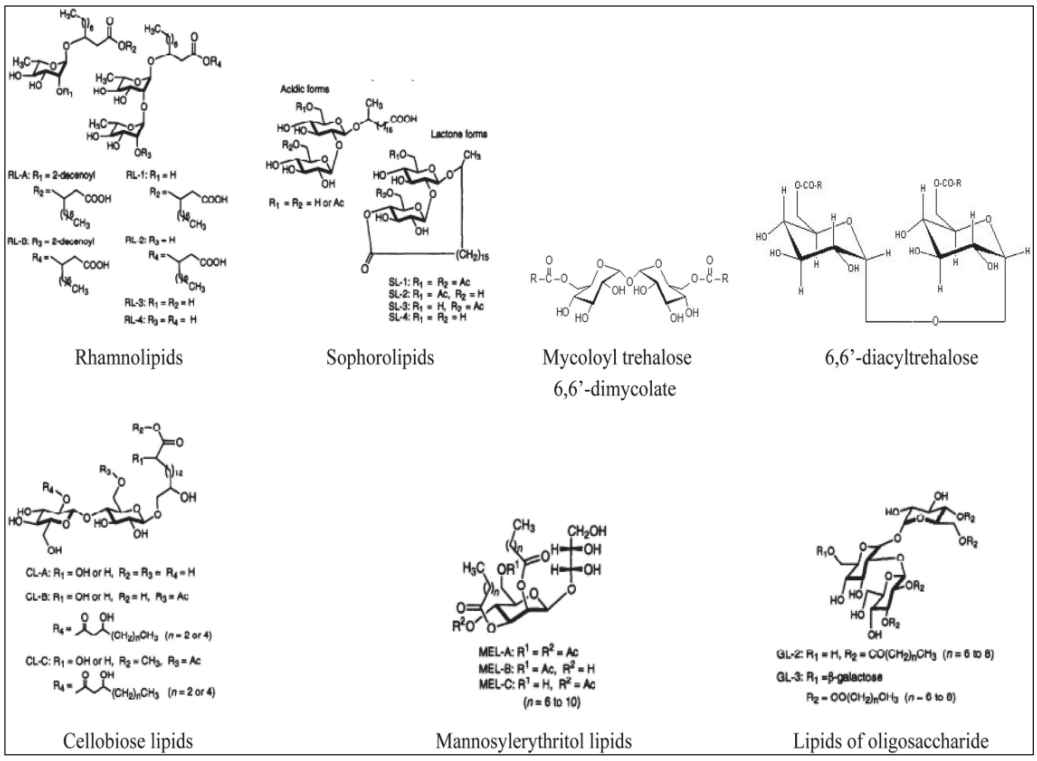

Figure 2.3: Chemical structure of the most recognized glycolipid. Adapted from (Inès & Dhouha, 2015).

Rhamnolipid are the most studied biosurfactants from the glycolipid type. Their production by *Pseudomonas aeruginosa* was first reported by Jarvis and Johnson (1949). They comprised of one or two molecules of rhamnose (hydrophilic head) bound to one or two molecules of β-hydroxy fatty acid (hydrophobic tail) linked together via a glycosidic bond. Figure 2.4 shows the general structure of rhamnolipid with a hydrophilic head and a hydrophobic tail. The most commonly found structures of rhamnolipids are monorhamnolipids and dirhamnolipids. The β-hydroxy fatty acid chains are most commonly saturated or, less abundantly, mono- or polysaturated. Their chain lengths vary from C8 to C16 which are linked to each other through an ester bond (Price *et al*., 2009; Abdel-Mawgoud *et al*., 2011). The best known rhamnolipid congener is α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate, which is typically symbolized as Rha-Rha-C10-C10 as shown in Figure 2.5.

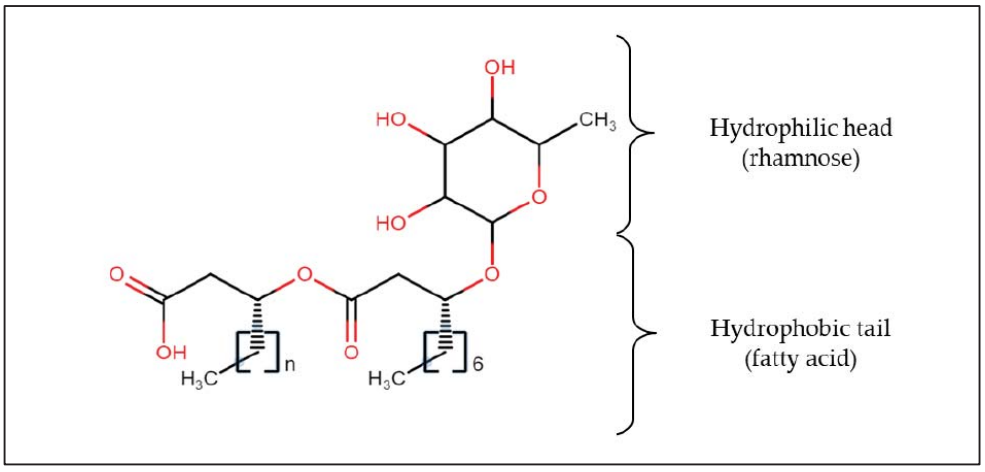

Figure 2.4: Rhamnolipid structure with hydrophilic head and hydrophobic tail. Adapted from Nurfarahin *et al*. (2018).

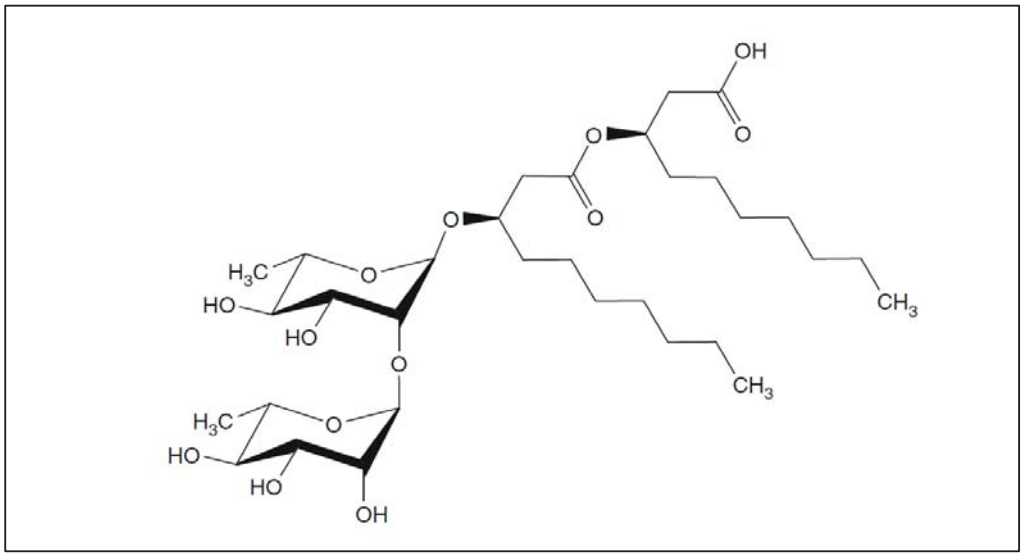

Figure 2.5: Chemical structure of the first identified rhamnolipid; known as Rha-Rha-C10-C10. Many more variations (congeners) have been identified since then. Adapted from Abdel-Mawgoud *et al*. (2011).

Lipopeptides from *Bacillus* spp. are the most widely studied compared to other microorganisms. In structure, lipopeptides contain hydrophobic fatty acids and hydrophilic cyclic peptides. The lipopeptides from *Bacillus* spp. can be divided into three families according to the structure of the cyclic peptides: surfactin, iturin and fengycin (Jiang *et al*., 2014). Surfactin is one of the most powerful biosurfactants from lipopeptide biosurfactant, able to reduce the surface tension of water from 72 to 27 mN/m (Chen *et al*., 2015; Beltran-Gracia *et al*., 2017). It was first identified from the culture medium of Bacillus subtilis that consist of four isomers (Surfactin A-D) (Arima *et al*., 1968). Its chemical structure was determined by Kakinuma *et al*. (1969). Figure 2.6 represents the chemical structure of surfactin that comprises a common peptide loop of seven amino acids (L-asparagine, L-leucine, glutamic acid, L-leucine, L-valine and two D-leucines) with a long hydrophobic fatty acid chain. The peptide is linked via a lactone bond to a β-hydroxyl fatty acid of 13-15 C atoms (Chen *et al*., 2015).

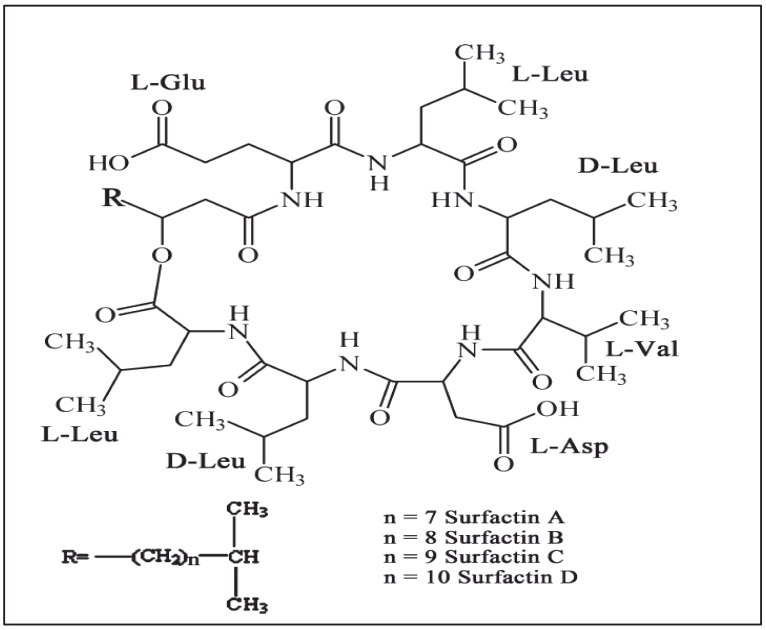

Figure 2.6: The chemical structure of surfactins.

The differences in the sequence of amino acids and number of carbon atoms in the fatty acids means the surfactin contain a variety of structural analogues (Liu *et al*., 2015). It has been reported that 44 compounds of surfactins and their monomethyl and dimethyl esters was produced by *Bacillus subtilis*. These compounds include the known surfactin variants with L-Leu, L-Val, or L-Ile in position 7 of the peptide ring and unknown variants with leucine residues in position 2 and/or 7 replaced by L-Val and L-Ile (Kowall *et al*., 1998).

Besides that, iturin is also a ring lipopeptide. It contains seven amino acids (heptapeptides) linked to a fatty acid (β-amino) chain that can vary from C-14 to C-17 carbon molecules. Figure 2.7 shows the example of cyclic structure of iturin, containing seven amino acids residues attached to a 14-carbon chain (Meena & Kanwar, 2015). Fengycin is another lipopeptide, in addition to surfactin and iturin. The structure of fengycin contains a peptide chain of 10 amino acids linked to a fatty

acid chain. The length of the fatty acid chain can vary from 14 to 17 carbon atoms, thus giving different homologous compounds and isomers. Figure 2.8 represents the cyclic structure of fengycin.

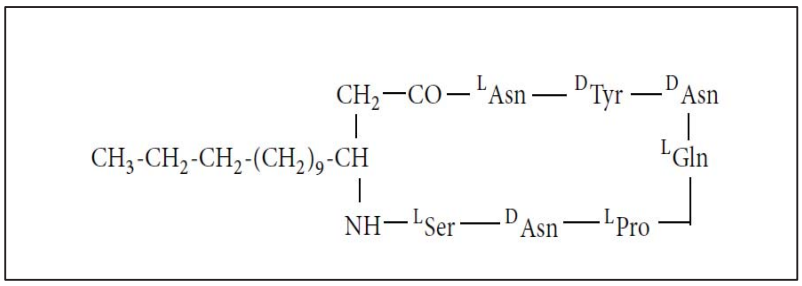

Figure 2.7: Cyclic structure of iturin

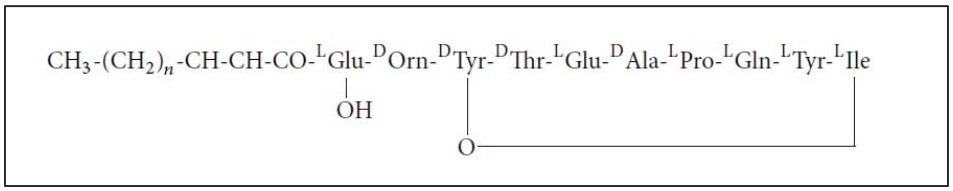

Figure 2.8: The classical structure of fengycins.

2.1.6 Applications of Lipopeptide Biosurfactant

There are several applications of lipopeptide biosurfactant in industries. Lipopeptides have been reported as anti-microbial agents. They show very strong anti-fungal, anti-tumor, anti-fungal and anti-viral properties. As reported by Fracchia *et al*. (2012), a surfactin produced by *Bacillus subtilis* are found to have anti-fungal and anti-bacterial properties. It inhibited the growth of four fungi isolates, namely Botrytis cinereal A258 (about 50% inhibition) followed by *Sclerotina sclerotiorum* K2291 (about 50% inhibition), *Collectotrichum sclerotiorum* A259 (about 40% inhibition) and *Phoma complanata* A233 (about 38% inhibition). Surfactin was tested against *Escherichia coli* in an anti-bacterial activity test, and it was successful to inhibit the growth of 76% of *E. coli* isolates.

Besides that, the high surface and emulsification activities of surfactin are desirable in environmental applications, such as environmental bioremediation and enhanced oil recovery (Whang *et al*., 2008; Deisi Altmajer Vaz *et al*., 2012). It is effective in transporting heavy oil and accelerates the biodegradation of hydrocarbon (Lai *et al*., 2009). Whang *et al*. (2008) found that surfactin (40 mg/L) promotes the production of biomass and the biodegradation of diesel compared to the experiments without the presence of surfactin. In addition to that, Lai *et al*. (2009) revealed that two biosurfactants (rhamnolipid and surfactin) can remove total petroleum hydrocarbon (TPH) from soil with a higher removal efficiency than that of the synthetic surfactants. Pathak and Keharia (2014) reported that a lipopeptide isolated from *Bacillus* sp. GY19 showed that 1-3% lipopeptide solutions had 80-100% oil displacement efficiency with Oman light oil, heavy oil, diesel oil and Arabian light oil. In addition, the lipopeptide solutions were stable at extreme salinity, pH and temperature that could be applied for oil spill remediation.

The cyclic lipopeptides have also been reported as a mosquito larvicidal agent. Mosquitoes serve as vectors for spreading human disease such as malaria, yellow fever, dengue fever, encephalitis, West Nile fever and lymphatic filariasis. Seydlová *et al*. (2011) reported that surfactin-producing *Bacillus subtilis* strain effectively killed the larval and pupal stages of mosquito species such as *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. As some biocontrol agents or insecticides are effective against mosquito pupae, this could be a good tool for applications in malaria control programmes (Geetha *et al*., 2010).

2.2 Actinobacteria

Actinobacteria are prokaryotic bacteria with elongated cells or filaments that usually show some degree of true branching. They share similar characteristics with true bacteria, as shown by their size, chemical composition and biochemical activities. Historically, many mycologists considered them as fungi because of their ability to form branching hyphae at some stage of development. Presently, they are considered as higher, filamentous bacteria (Waksman, 1961; Goodfellow & Williams, 1983). They differ from fungi in terms of their size, spore morphology and cell wall composition. Actinobacteria lack a membrane-bounded nucleus and their cell walls are composed of mucopolysaccharides, with 2% of lipid, while fungi contain membrane-bound nuclei with chitin, cellulose and lignin as their cell wall components (Rangaswami & Bagyaraj, 1998). The absence of a nuclear membrane makes this organism sensitive to lysozyme and common antibacterial agents. The hyphal diameters are much smaller compared to those of fungi and are closer to those of bacteria (Sykes & Skinner, 1973).

Actinobacteria are members of the Gram-positive heterogeneous bacteria with high guanine (G) and cytosine (C) content in their DNA with the GC% of 57-75%. They can produce two types of mycelia; either a single kind designated as substrate or vegetative mycelium, or two kinds, substrate and aerial mycelia (Kokare *et al*., 2004). Actinobacteria can reproduce by sporulation like filamentous fungi. Figure 2.9 shows the life cycle of actinobacteria via sporulation process. However, the comparison between fungi and actinobacteria is only superficial. This is because actinobacteria cells are thin with a chromosome that is organized in a prokaryotic nucleoid and a peptidoglycan cell wall like other bacteria (Barka *et al*., 2016).

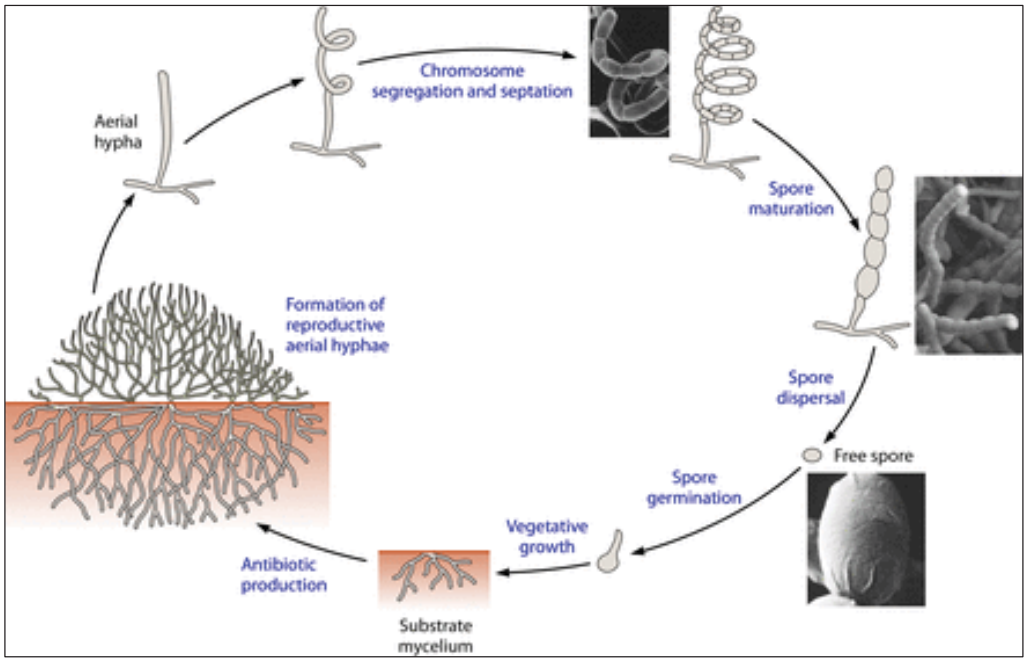

Figure 2.9: The schematic diagram of the life cycle of sporulating actinobacteria. Adapted from Barka *et al.* (2016).

Members of the actinobacteria are widely distributed in both terrestrial and aquatic ecosystems. The abundance of actinobacteria can be found in soil where they are usually present in 105 - 106 colony-forming units per gram of soil and their population increases with decreasing depth of soil (Goodfellow & Williams, 1983). They are sensitive to low pH or acidic condition and grow optimally at a pH range between pH 6.5 to 8.0 (Lee *et al*., 2014). However, rare genera of actinobacteria can be found in alkaline pH arid soils and may contain few *Streptomycete* populations. Alkaliphilic actinobacteria are a valuable source for new products of industrial interest, including enzymes, antimicrobial agents and biosurfactant (Mitsuiki *et al*., 2004).

2.2.1 Classification and Identification of Actinobacteria

The combination of morphological characteristics and molecular methods are important in the classification and identification of actinobacteria. Morphological characterization is based on their colonies, mycelial and spore morphologies. Arifuzzaman *et al*. (2010) suggested that members of actinobacteria be classified in four genera based on colony characteristics. Table 2.2 shows the identification the genera of actinomycetes based on morphological characteristics.