BIOPROCESSING STRATEGIES FOR LIPOPEPTIDE BIOSURFACTANT PRODUCTION IN A SUBMERGED FERMENTATION OF *Streptomyces* sp. PBD-410L USING PALM OIL AS CARBON SOURCE

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2020

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

December 2020

ACKNOWLEDGEMENT

Alhamdulillah, thank Allah for His greatness and blessing for the completion of my PhD journey. I am most grateful and appreciative to my supervisor, Assoc. Prof. Ahmad Ramli Mohd Yahya for his invaluable encouragement and mentorship on all fronts, enthusiasm, support, understanding, criticism and patience during this project and the writing of this thesis. All of these would not have been possible without the opportunities and support. It has been a truly amazing journey and most exciting time working under his guidance. I would also like to thank all the technical staff in the School of Biological Sciences, USM especially the laboratory assistants of the Biotechnology Laboratory for providing me with all the assistance which is instrumental to the success of my research project. I am greatly indebted to the Ministry of Higher Education and Universiti Sains Malaysia for My PhD scholarship and Graduate Research Assistant Scheme, respectively, for providing financial assistance during my PhD's education. Special appreciation is given to my lovely mentor, Dr. Shifa for all her guidance, moral support and knowledge throughout this challenging journey. A special thanks and grateful to my cheerful friends, especially to K.Kila, Zurin, Aina, Ikha, Syafiq, Piqah, Dani and Shafiq 2.0 for their cooperation and moral supports that has always brighten up my day. I would like to express my deepest gratitude to my lovely family, Angah, Alang, Jaja and Syahmi for their encouragement, love, prayers and sacrifices throughout my study. To the love of my life, Nazmi, I thank you. You stood by me through thick and thin, never getting bored with me, always giving me advice and support throughout the journey of my life. Last but not least, to my beloved son, Nadeem Aqil, thank you for accompanying 'ibu' and cheer me up every day.

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a	liquid side
$C*$	concentration of oxygen saturation at the gas-liquid interface
C_{crit}	critical dissolve oxygen
C_L	concentration of oxygen saturation in bulk liquid
D	diameter
$\frac{dC_L}{dt}$	accumulation rate of oxygen
E_{24}	emulsification index
k_L	total specific interfacial area
$k_L a$	volumetric oxygen transfer coefficient
N	agitation speed
q_{O_2}	specific oxygen consumption rate
$q_{O_2}X$	oxygen uptake rate
t	time
μ	specific growth rate
μ_{max}	maximum specific growth rate
X	cell concentration
X_{max}	maximum biomass
$Y_{x/s}$	yield of biomass over supplied substrate
$Y_{p/s}$	yield of product over supplied substrate

LIST OF ABBREVIATIONS

CMC	critical micelle concentration
DO	dissolved oxygen
FAME	fatty acid methyl ester
GCMS	gas chromatograph integrated with a mass spectrometer
MATH	microbial adhesion to hydrocarbon
OST	oil spreading technique
OD	optical density
OTR	oxygen transfer rate
OUR	oxygen uptake rate
rpm	rotation per minute
SCA	starch casein agar
STR	stirred-tank reactor
sOUR	specific oxygen uptake rate
vvm	volume per volume per minute

STRATEGI BIOPROSES UNTUK PENGHASILAN BIOSURFAKTAN LIPOPEPTIDA DALAM FERMENTASI TENGGELAM *Streptomyces* sp. PBD-410L MENGGUNAKAN MINYAK SAWIT SEBAGAI SUMBER KARBON

ABSTRAK

Biosurfaktan adalah surfaktan yang diperoleh secara biologi, yang dihasilkan oleh fermentasi kumpulan sel-sel hidup yang pelbagai. *Streptomyces* sp. boleh menjadi calon yang sesuai untuk biosurfaktan yang selamat dan mikrob industri yang berdaya maju kerana sifat tak patogen kepada manusia. Kajian mengenai penambahbaikan penghasilan biosurfaktan melalui manipulasi strategi bioproses dalam reaktor tangki teraduk, terutamanya bagi bakteria berfilamen ini sangat terhad. Kajian ini diperlukan untuk penambahan kemahiran dan pengalaman yang menjurus kepada penghasilan biosurfaktan secara besar-besaran untuk aplikasi perindustrian. Dengan itu, kajian ini memberi tumpuan kepada penilaian merit aspek bioproses dalam meningkatkan penghasilan biosurfaktan oleh bakteria berfilamen. *Streptomyces* sp. PBD-410L, penghasil biosurfaktan jenis lipopeptida, digunakan sebagai model penghasil biosurfaktan berfilamen tak patogen. Penghasilan biosurfaktan lipopeptida telah dikaji dalam fermentasi kelompok dan kelompok bersuap dalam reaktor tangki teraduk 3-L menggunakan minyak kelapa sawit sebagai substrat utama. Penilaian terhadap kadar pemindahan oksigen (OTR) dan kadar pengambilan oksigen (OUR) dalam sistem akues-minyak-gas ini, menunjukkan bahawa oksigen tidak terhad untuk kultur *Streptomyces* sp. PBD-410L pada setiap kadar pengudaraan dan agitasi. Pemindahan oksigen tertinggi dicapai pada kelajuan agitasi dan kadar pengudaraan yang tertinggi, iaitu pada 600 rpm dan 1.0 vvm. Walau bagaimanapun, nilai biojisim

menurun (2.6±0.1 g/L) apabila kultur bioreaktor diaduk pada 600 rpm dan 1.0 vvm. Dalam kultur kelompok, kesan parameter-parameter bioproses, iaitu pengudaraan, pengadukan, ketegangan oksigen terlarut (DO), suhu, dan nisbah C/N telah dikaji untuk meningkatkan pertumbuhan biojisim dan penghasilan biosurfaktan. Penghasilan biosurfaktan lipopeptida maksimum (3.81 g/L) tercapai apabila bioreaktor beroperasi pada kadar pengudaraan 0.5 vvm, kelajuan agitasi 200 rpm dengan ketegangan DO tidak dikawal sepanjang tempoh fermentasi, suhu 37ºC dan nisbah C/N 20. Keadaan terbaik yang diperoleh daripada kultur kelompok telah diadaptasi dalam kultur kelompok bersuap menggunakan strategi-oksigen terlarut pegun untuk meningkatkan lagi penghasilan biosurfaktan lipopeptida. Penghasilan biosurfaktan lipopeptida telah meningkat dari 3.81 ke 5.37 g/L, apabila kultur suapan berkelompok pada kadar suapan permulaan 0.6 mL/h (200 rpm, 0.5 vvm, 37ºC). Gabungan pemendakan amonium sulfat diikuti dengan pengekstrakan etil asetat didapati sebagai teknik perolehan yang terbaik untuk biosurfaktan lipopeptida dengan menunjukkan diameter sebaran minyak terbesar pada 173.33±11.54 mm. Penemuan dalam kajian ini menunjukkan betapa pentingnya strategi bioproses dalam meningkatkan penghasilan biosurfaktan daripada genus *Streptomyces*.

BIOPROCESSING STRATEGIES FOR LIPOPEPTIDE BIOSURFACTANT PRODUCTION IN A SUBMERGED FERMENTATION OF *Streptomyces* sp. PBD-410L USING PALM OIL AS CARBON SOURCE

ABSTRACT

Biosurfactant is a biologically-derived surfactant, produced by fermentation of a heterogeneous group of living cells. *Streptomyces* sp. can be an appropriate candidate for a safe and industrially viable microbe for biosurfactants due to their non-pathogenicity to human. The study on the improvement of biosurfactant production through manipulation of bioprocessing strategies in a stirred-tank bioreactor (STR) particularly on this filamentous bacterium is very limited. This study is a requisite in gaining expertise and experience towards the production of mass production of biosurfactant in industrial applications. Hence, the present study focusses on assessing the merits of the bioprocessing aspects in improving biosurfactant production by filamentous bacterial. *Streptomyces* sp. PBD-410L, a lipopeptide-type biosurfactant producer, was used as a model of a non-pathogenic filamentous biosurfactant producer. The production of lipopeptide biosurfactant was investigated in batch and fed-batch fermentation in a 3-L STR using palm oil as the main substrate. The evaluation of oxygen transfer rate (OTR) and oxygen uptake rate (OUR) in this gas-oil-aqueous system, indicated that oxygen was non-limiting for *Streptomyces* sp. PBD-410L culture at any aeration and agitation rate. The highest oxygen transfer was achieved at the maximum agitation speed and aeration rate, which was at 600 rpm and 1.0 vvm, respectively. Nevertheless, the lowered biomass value (2.6±0.1 g/L) was obtained when the bioreactor culture was agitated at 600 rpm and 1.0 vvm. In batch cultivation, the impact of bioprocessing parameters, namely aeration, agitation,

dissolved oxygen (DO) tension, temperature, and C/N ratio were investigated to improve the biomass growth and lipopeptide biosurfactant production. The maximum lipopeptide biosurfactant production (3.81 g/L) was attained when bioreactor was operated at the aeration rate 0.5 vvm, agitation speed 200 rpm with uncontrolled DO tension throughout fermentation period, temperature of 37ºC and C/N ratio of 20. The best condition obtained from the batch cultivation was adopted in the fed-batch cultivation using DO-stat feeding strategy to further improve the lipopeptide biosurfactant production. The lipopeptide biosurfactant production was enhanced from 3.81 to 5.37 g/L, when fed-batch fermentation was performed at initial feed rate 0.6 mL/h (200 rpm, 0.5 vvm, 37ºC). The combination of the ammonium sulphate precipitation followed by ethyl acetate extraction was found to be the best recovery method for lipopeptide biosurfactant by showing the highest diameter of oil spreading at 173.33±11.54 mm. The finding in this study exemplifies the importance of bioprocessing strategies in enhancing biosurfactant production from genus *Streptomyces*.

CHAPTER 1

INTRODUCTION

1.1 Research background

Surfactants are one of the important classes of chemical products that is often used in our daily routine activities. These products include toothpaste, skincare products, haircare products, cosmetic products, and other pharmaceutical products (Banat *et al.*, 2014; Marchant & Banat, 2012). These surface-active agents are amphiphilic molecules with both hydrophilic and hydrophobic moieties that align themselves at the interface between two liquids of differing degrees of polarities and hydrogen bonding such as oil/water or air/water interfaces. Due to this physical-chemical characteristic, surfactants can lower surface and interfacial tension, subsequently assist solubilization of hydrocarbon in water or water in hydrocarbons in the form of microemulsion. This behaviour makes surfactants versatile for applications in a broad range of industries (Santos *et al.*, 2016). According to Markets and Markets (2016b), the global market for surfactant was estimated to be USD30.64 billion in 2016. It is predicted to continue to increase by approximately USD39.86 billion by 2021. However, due to their manufacturing processes and byproducts that are potentially hazardous or less acceptable to the environment, the current surfactant market is slowly being shared with 'green surfactant' products, known as biosurfactant (Singh *et al.*, 2019).

Biosurfactant is a biologically derived surfactant, produced by a heterogeneous group of living cells including bacteria, yeast and fungi. Similar to synthetic surfactants, the presence of both hydrophilic and hydrophobic moieties makes them one of the important compounds in every industry that deals with multiphase systems.

In fact, biosurfactants play prominent roles, namely in emulsification, dispersion, solubilization, mobilization, wetting, surface tension reduction, formation of micelles and foam formation in various applications in fields like bioremediation, biodegradation, oil recovery, food, pharmaceuticals, and many other applications in different industrial sectors (Banat *et al.*, 2010; Fracchia *et al.*, 2014; Franzetti *et al.*, 2014). In particular, the use of biosurfactants over their chemically synthesized counterpart is more beneficial due to their low toxicity, having specific activity at extreme and wide range of pH, temperature and salinity. More importantly, they are biodegradable and suitable for environment applications such as cleaning of wastewater from oil and heavy metal or organic contaminant removal from contaminated soil (Korayem *et al.*, 2015). Among other biomolecules, biosurfactants stand out as the "multifunctional biomolecules/materials of the 21st century" throughout the world (Olasanmi & Thring, 2018; Santos *et al.*, 2016).

Although biosurfactants have a huge demand in the global market, their industrial-scale production remains challenging. One of the main barriers in the production of biosurfactant is its low yields. There are a few factors that need to be considered to enhance the production processes of biosurfactants, such as the selection of microorganisms, the use of cheaper and renewable substrates and the optimum design of the fermentation system (Marchant & Banat, 2012; Marchant *et al.*, 2014). Diverse structures of biosurfactants have been reported in the literature. They are typically categorized based on the chemical nature and microbial origin. In general, biosurfactants can be divided into five major classes, namely glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate biosurfactants.

Among all biosurfactants, the best-known compounds are glycolipids and lipopeptides that are mainly produced by bacteria namely *Pseudomonas* and *Bacillus* genera and yeasts, such as *Candida* or *Yarrowia* (Mukherjee & Das, 2010; Mulligan, 2005; Vaz *et al.*, 2012). These compounds are in a huge demand in the biosurfactant market due to the attractive functional properties. Nevertheless, the production process of these compounds faces some reservations from consumers due to safety concerns of the microbial producers. The most commonly used biosurfactant-producer, *Pseudomonas* sp., is a human opportunistic pathogen in nature. Hence, it warrants an extensive train of recovery and purification steps during downstream processing (Lyczak *et al.*, 2000; Nickzad *et al.*, 2018). Inevitably, this raises the overall cost of biosurfactant production since the downstream processes account approximately 60-80% of the total production expenditure (Banat *et al.*, 2014).

In order to overcome these predicaments, a few strategies can be implemented, such as selecting biosurfactant producers from non-pathogenic and safe organisms to avoid the pathogenicity concerns. These concerns limit the application in pharmaceutical and food industries. Moreover, a safe producer simplifies the recovery and purification steps during downstream processing as the crude products can be directly used from the fermentation broth, depending on the fields of application (Reis *et al.*, 2013).

Biosurfactant production occurs through fermentation of heterogenous microorganisms using water-soluble and insoluble substrates by *de novo* pathway or assembly from other substrates. In most cases, the carbon source used during fermentation of biosurfactant-producing microorganisms will determine the type and titre of the biosurfactants. Diverse types of carbon sources have been investigated by many researchers to improve the yield of biosurfactants (Banat *et al.*, 2014). Presently,

interest has been increasing in the usage of cheaper and renewable carbon sources such as sugars, molasses, plant oils, oil wastes, starchy substances, lactic whey, distillery wastes and animal fat to minimize the overall production cost (Makkar *et al.*, 2011).

Other than the choice of the carbon source, the development of an efficient and optimized bioprocess, namely through manipulation of the operating parameters (eg. temperature, agitation, aeration, and DO tension) play a crucial role in improving the biosurfactant production. Various fermentation strategies have been employed by researchers to obtain the optimum production processes including batch, fed-batch and continuous fermentation. Accordingly, a number of the production processes, particularly rhamnolipid production from *Pseudomonas* sp, have been patented. However, only a few of them was successfully applied at the industrial-scale production due to low productivity and extensive foam formation during the fermentation (de Kronemberger *et al.*, 2007; Müller *et al.*, 2012; Reis *et al.*, 2013). Therefore, countless efforts are being taken among researchers to further optimize the production processes in the hopes of achieving higher biosurfactant productivity.

1.2 Motivation and scope of study

The genus *Streptomyces* are Gram-positive filamentous bacteria that have been recognized as a prominent source of natural products for industries. Almost 50% of the total number of microbial metabolites are produced from genus *Streptomyces*, particularly antibiotics (van Wezel & McDowall, 2011; Zhou *et al.*, 2018). The characteristics of non-pathogenicity in humans allow the application of the products to a wide range of industrial sector, especially in pharmaceutical, personal care and food industries. One of the main concerns that arise in biosurfactant production is the pathogenicity characteristic held by most of the microbial biosurfactant producers

which limits their exploitation in large-scale industrial processes and applications (Ghasemi *et al.*, 2019). The selection of *Streptomyces* sp. as biosurfactant producers can offer safe biosurfactant products to the market as none of which are known to cause harm to human or the environment. Unlike other microbial biosurfactant fermentation, the cultivation of these filamentous bacteria in large scale bioreactors are much easier to control due to the less excessive foaming.

Presently, the ability of *Streptomyces* sp. in producing biosurfactants has been proven by many researchers using various type of substrates. A number of biosurfactants have been well-characterized and identified, but most of their structures are still not fully determined by researchers (Bhuyan-Pawar *et al.*, 2015; A. Khopade *et al.*, 2012; Lamilla *et al.*, 2018; Manivasagan *et al.*, 2014). In comparison with other microbial biosurfactant producers, the information on the role of bioprocess engineering in improving biosurfactant production, particularly in bioreactor, from this filamentous bacterium is few and far between. The first report on the production of biosurfactant in a STR by this genus was published by our research group (Zambry *et al.*, 2017). The study focussed on the fermentation process, namely the influence of agitation speed on the growth and production of biosurfactant by *Streptomyces* sp. R1 in a 3-L STR using palm oil as the main carbon source. The highest biosurfactant production indicated by surface tension measurement (40.50±0.50 dynes/cm) and E_{24} (67.80±2.0) were attained when the bioreactor culture was agitated at 600 rpm. This study was the first in multiple fronts, such as the use of filamentous bacteria in biosurfactant production, the use of an immiscible substrate as the main carbon source and the study on operating conditions for metabolite production in a multi-phase fermentation broth.

Another strain of *Streptomyces* sp. namely as *Streptomyces* sp. PBD-410L has been isolated from local mangrove sediment by our research group and showed the ability as a good biosurfactant producer (Awang, 2018; Rusly, 2019). The biosurfactant produced by this filamentous bacterium was characterized as a lipopeptide biosurfactant (Rusly, 2019). Recent studies have assessed several types of carbon sources including water-immiscible and miscible substrates on the production of biosurfactant from this filamentous bacterium (Bhaskarani, 2018; Razip, 2019; Rusly, 2019). As anticipated from the experience in our research team, lipopeptide biosurfactant production was greater when using insoluble substrates, namely palm oil (3% v/v), as the main carbon source in the culture medium. Razip (2019) has investigated the type of inoculum (spore and vegetative) and the best medium formulation using full factorial design in shake-flask fermentation for the growth and biosurfactant production from *Streptomyces* sp. PBD-410L. The highest OST and E_{24} recorded from shake-flask fermentation were reported at 70 mm and 62.22%, respectively.

It is important to highlight that a bench-scale bioreactor, commonly stirred-tank reactor (STR), is mainly used for process optimization of any metabolites production before being scaled-up to a larger scale for mass production. Thus, the best medium formulation and condition obtained from shake-flask fermentation of *Streptomyces* sp. PBD-410L was extrapolated to the bench-top STR. As is commonly the case, shake-flask fermentations often do not match those in STR cultivations due to the different hydrodynamic conditions in these two systems. Moreover, being a filamentous organism, operating parameters such as aeration and agitation in the STR system can affect the growth rates and morphology, thus metabolite production.

Hence, the focus of this study is to investigate the lipopeptide biosurfactant production through the manipulation of bioprocessing parameters in a stirred-tank benchtop bioreactor cultivation of *Streptomyces* sp. PBD-410L using palm oil as the main carbon source. The batch cultivation of *Streptomyces* PBD-410L was carried out in shake-flasks and a 3-L STR using the best medium formulation found by (Razip, 2019; Rusly, 2019). Biosurfactant production was estimated based on the measurement of OST and E_{24}. The efficiency of aeration rate and agitation speed introduced in a 3-L STR were evaluated through the determination of oxygen transfer rate (OTR) and oxygen uptake rate (OUR). In batch STR cultivations, the impact of bioprocessing parameters, namely aeration rate, agitation speed, DO tension, temperature, and C/N ratio was investigated to improve the biomass and biosurfactant production. Later, the best condition obtained from the batch cultivation was applied to the fed-batch cultivation using a DO-stat feeding strategy to further enhance the biosurfactant production by this filamentous bacterium. To the best of our knowledge, this research is the first report on the employment of fed-batch fermentation in biosurfactant production from the genus *Streptomyces*.

Due to the lack of previous work on the recovery of biosurfactant from this genus, two techniques that are frequently used in biosurfactant extraction, namely precipitation (acid, zinc sulphate and ammonium sulphate) and solvent extraction (hexane, petroleum ether, chloroform, methyl tert-butyl ether (MTBE), ethyl acetate and methanol were chosen as the recovery methods in this study. The combination of reagents and solvents was assessed to determine the best recovery method for lipopeptide biosurfactant produced by *Streptomyces* sp. PBD-410L.

1.3 Problem statement

1. The cultivation of aerobic filamentous organism, particularly *Streptomyces* sp., in submerged fermentation is very challenging due to low solubility of oxygen in aqueous medium. Thus, it is important to ensure that the oxygen transfer in STR system support enough oxygen for growth of this filamentous bacterium and thus influence the biosurfactant production.
2. STR is a good choice for a bioreactor since it offers better control of homogeneity (as opposed to pneumatically-agitated bioreactors), particularly in fermentation using insoluble substrate as the main carbon source. However, very few studies are available on the optimization of the best operational condition in the STR to produce optimum biosurfactant production from the genus *Streptomyces* sp. It is important to highlight that the cultivation of filamentous bacteria and its metabolite production in STR are greatly influenced by operational condition such as agitation speed and aeration rate.
3. Fed-batch cultivation strategy have been proven by most researchers in improving biosurfactant production. However, none of the research study was conducted on the employment of this mode of fermentation on biosurfactant production from the genus *Streptomyces*. Moreover, the use of palm oil in fed-batch fermentation poses some challenges due to its insolubility in the culture medium. Thus, a proper feeding rate and strategy must be developed to avoid accumulation of residual oil in the culture medium, leading to increased medium viscosity and warranting additional downstream processing.
4. The development of efficient recovery and purification methods will assist in making viable biosurfactant production, increasing its chances in effectively competing with commercial surfactant production. However, to date, the

downstream processes of biosurfactants are still plagued with low purity and product yields. Part of the problem is the large diversity of structures of biosurfactants, which is not amenable to generalisations in downstream processing investigations.

1.4 Research objectives

1. To determine the oxygen transfer in a bench-scale STR system using palm oil as a substrate.
2. To characterize suitable batch fermentation conditions for biosurfactant production by *Streptomyces* sp. PBD-410L in a bench-scale STR.
3. To enhance the biosurfactant production by applying DO-stat feeding strategy.
4. To determine the best recovery method for lipopeptide biosurfactant.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties of biosurfactant

Biosurfactant or microbial surface-active compounds are secondary metabolites with amphipathic molecules that possess both hydrophilic and hydrophobic moieties (Pacwa-Płociniczak *et al.*, 2011)(Figure 2.1). In liquid media, the term "hydrophobic" (water hating or oil-loving) are called as lyophobic and hydrophilic parts (water-loving) as lyophilic (Fracchia *et al.*, 2012). The presence of a polar head and a hydrophobic tail enable this molecule to partition at interfaces: liquid/solid, liquid/gas and liquid/liquid (Figure 2.2). Such traits grant biosurfactants with the ability as agents of emulsification, thickening, solubilization, mobilization, wetting, surface tension reduction, the formation of micelles, dispersing or stabilising and foam formation agent in various biotechnological and industrial applications (Pacwa-Płociniczak *et al.*, 2011; Santos *et al.*, 2016; Satpute *et al.*, 2010).

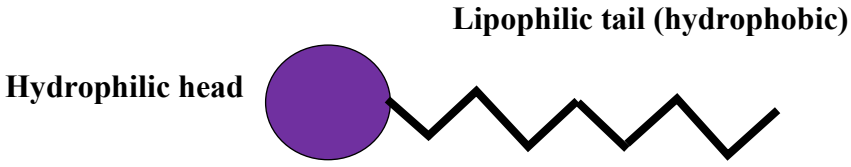


Figure 2.1: A schematic of biosurfactant molecule with hydrophobic and hydrophilic moieties

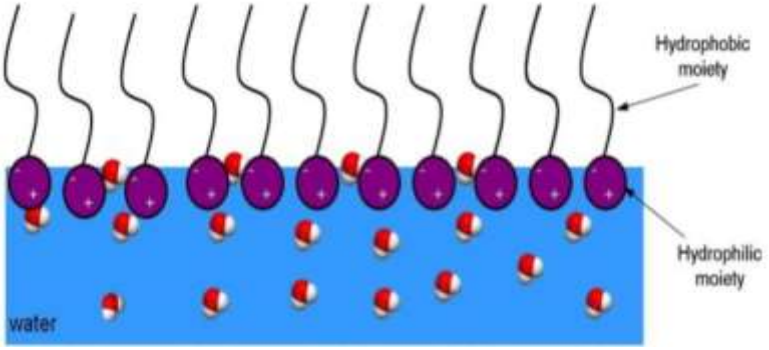


Figure 2.2: Partitioning of biosurfactants molecule at the interface between liquid and air (Pacwa-Płociniczak *et al*., 2011)

There are three major roles that are played by biosurfactants, namely increasing the surface area of hydrophobic substrates, enhancing the bioavailability of hydrophobic substrates through solubilization/desorption and controlling the attachment and removal of microorganisms from the surface (Rosenberg & Ron, 1999; Vijayakumar & Saravanan, 2015). Accordingly, biosurfactants stand out in environmental restoration and have also been characterized as one of the promising and versatile process chemicals (Das & Mukherjee, 2007). Moreover, the extraordinary properties of biosurfactants allow them to find a niche in different industrial sectors, namely pharmaceutical, therapeutics, cosmetics, soaps and detergents, food and beverages, agriculture and removal of heavy metals and oil recovery (Banat *et al.*, 2014; Cameotra *et al.*, 2010; Olasanmi & Thring, 2018).

Unlike chemical surfactants, which are mostly derived from petroleum and oleochemical products, biosurfactants are naturally produced by fermentation of diverse microorganisms such as bacteria, yeast and fungi (Sari *et al.*, 2019). They play significant physiological roles in cellular metabolism, motion and serve as one of the defence mechanisms of some microorganisms. Accordingly, diverse genera, including *Acinetobacter, Alcanivorax, Arthrobacter, Bacillus, Candida, Corynebacterium, Flavobacterium, Lactobacillus, Mycobacterium, Nocardia, Pseudomonas, Rhodococcus, Rhodotorula, Serratia, Streptomyces* and *Thiobacillus*

have been reported to produce heterogeneous classes of biosurfactants as secondary metabolites (Rahman & Gakpe, 2008; Santos *et al.*, 2016; Zhang *et al.*, 2010).

2.2 Substrates for biosurfactant production

The biosurfactant-producing microorganisms can produce biosurfactants using various types of carbon sources including carbohydrate group, oils and fats, and hydrocarbon groups through fermentation. The most common types of carbohydrates that have been used as a carbon source for biosurfactant productions are glucose, fructose, glycerol, starch and mannitol. Among them, glucose is the best carbon source that is commonly reported to give a high yield of biosurfactants as it can easily be metabolized by microorganisms through the direct glycolysis pathway for the generation of energy (Nurfarahin *et al.*, 2018). For instance, *Pseudomonas aeruginosa* MTCC7815 produced higher amount of biosurfactant (3.937 g/L) in the presence of glucose as main carbon source in the fermentation medium instead of others carbon sources (glycerol, fructose and starch) (Tomar & Srinikethan, 2016). In contrast, sucrose was found to be the optimal substrate for the production of surfactin by *Bacillus* sp. although the bacterium could also withstand high glucose concentration in the fermentation medium (Fonseca *et al.*, 2007). A similar finding was observed for other microbial biosurfactant producers like *Streptomyces* sp. B3 where the production of biosurfactant was found higher in the medium containing sucrose, followed by trehalose, dextrose, and fructose (A. Khopade *et al.*, 2012). However, Manivasagan *et al.* (2014) observed contradictory results in the fermentation of *Streptomyces* sp. MAB36 where the production of biosurfactant was the highest with starch as the sole carbon source in the medium.

In many cases, most microorganisms that grow in the presence of hydrophobic substrates, such as fatty acids and vegetable oils tend to secrete these surface-active molecules into the aqueous media (Reis *et al.*, 2013; Thavasi *et al.*, 2011). This attributed the ability of microbial surfactants to act as an oil-in-water emulsifier and hence ease the uptake of poorly soluble substrates across the cell membrane for the growth, metabolism of microorganisms and metabolites production. The presence of a hydrophobic component in the substrate probably will promote the production of a hydrophobic moiety of biosurfactant although this particularly relies on the behaviour and metabolism of the microorganism itself (Nurfarahin *et al.*, 2018). Compared to carbohydrates on a weight-by-weight basis, the oil substrate is vital for the microbial production of secondary metabolite because of their stimulation of bacterial growth and product synthesis, anti-foaming properties and their higher energy content which about 2.4 times the energy of glucose (Efthimiou *et al.*, 2008; Large *et al.*, 1998; Peacock *et al.*, 2003).

The utilization of different types of oil substrates for higher biosurfactant production has been extensively reported from various groups of microbial biosurfactant producers. For example, Müller *et al.* (2012) reported higher biosurfactant production from *Pseudomonas aeruginosa* with the vegetable oil as main carbon source. In another study, Chong and Li (2017) found that the use of water-insoluble carbon source such as vegetable oil, commonly produces higher titre of rhamnolipid production in *Pseudomonas aeruginosa* compared to those grown in water-soluble carbon sources (e.g., glucose). Moreover, the biosurfactant produced by other microbial group namely as *Streptomyces* sp. DPUA 1559 greatly reduced the surface tension of the culture medium from 60 to 27.14 mN/m and 95% emulsification of residual motor oil when cultivated in a mineral medium containing 1% residual

frying soybean oil as the carbon source (Santos *et al.*, 2018). The vegetable oil and other hydrocarbon-based substrates can be described as the most economical and profitable carbon sources for large-scale biosurfactant production especially for *Pseudomonas*, *Bacillus* and *Candida* sp. (Sivapathasekaran & Sen, 2017).

2.2.1 Palm oil as a substrate for biosurfactant production

Palm oil is a typical triacylglycerol which consists of fatty acid and glycerol moieties. Each gram of palm oil contains approximately 0.94 g of the total fatty acids and 0.09 g of glycerol, as determined by GC and enzymatic analysis, respectively (Marsudi *et al.*, 2008). Compared to other vegetable oils, palm oil contains almost equal ratio in composition of saturated and unsaturated fatty acids (Mba *et al.*, 2015). The utilization of palm oil as a carbon source in producing biosurfactants have been well reported by many researchers (Oliveira *et al.*, 2006; Sarachat *et al.*, 2010; Vanavil *et al.*, 2013). For example, Syahriansyah and Hamzah (2016) reported that *Bacillus subtilis* UKMP-4M5 exhibited highest biosurfactant production, quantified with a reduction of surface tension of culture medium at 32.7±0.66 mN/m when grown on a medium supplemented with palm oil as the sole carbon source, compared to those grown in immiscible and other miscible substrates, namely crude oil, palm oil mill effluent, glycerol and molasses, respectively. In another study reported by Sari *et al.* (2018), the carbon source for production of biosurfactant by *Halomonas meridiana* BK-AB4 was changed from olive oil into cheaper and more abundant vegetable oil, which is palm oil.

Triglycerides are believed to be natural inducers of biosurfactant synthesis, which increases lipid solubility and subsequently, improves lipid degradation (Sena *et al.*, 2018). Locally, palm oil is singled out as the favourable choice of carbon sources

for biosurfactant production since it is a popular domestic plant and abundantly available in Southeast Asia. Malaysia is known as the second world largest palm oil producer (MPOB, 2013). The utilization of palm oil in biosurfactant production is a low-cost strategy for the industry as palm oil is the cheapest compared to other commonly used soluble substrates such as glucose and fructose (Nurfarahin *et al.*, 2018; Poomtien *et al.*, 2013; Saharan *et al.*, 2011).

2.3 The value of biosurfactant

Since biosurfactants are biologically produced using organic constituent of carbon sources, they are more compatible with the environment compared to synthetic surfactants that may pose potential dangers to the environment due to their unwilling nature (Roy, 2017). Moreover, despite having similar properties as their chemical counterparts, these biomolecules have a variety of advantages including their ecological acceptability, low toxicity, biodegradability, multi-functionality, effectiveness, stability and activity at high temperatures, extreme pH values and high salinity and also ability to synthesized from renewable and cheaper substrates (George & Jayachandran, 2013; Roy, 2017). These favourable properties provide the motivation for surfactant manufacturers to shift into biosurfactant market. Furthermore, the growing awareness of society for green alternatives and eco-friendly products helps spearhead the growth of biosurfactant market. The biosurfactant market has potential impact in food industry, cosmetics, healthcare, textile, agrochemicals, household detergents, personal care and others. Among these applications, household detergent and personal care products contribute the biggest portion in the biosurfactant market ("World Biosurfactant Market-Opprotunities and Forecast 2019-2026,"). According to Global Market Insights, Inc., approximately

370,000 tons of biosurfactants were needed by industries in 2015. The demand is expected to reach approximately 476, 500 tonnes, worth up to 2.21 billion USD in 2018. By 2023, the demand is estimated to reach nearly 2.69 billion USD (Markets & Markets, 2016a).

2.4 Classification of biosurfactant

Unlike synthetic surfactants which are classified according to the nature of their polar group, biosurfactants are categorized based on their molecular weight, chemical composition, and microbial origin (Sharma *et al.*, 2016; Vijayakumar & Saravanan, 2015). The structure, number and type of biosurfactant are highly determined by the individual microbe in which is synthesized. Among the diverse groups of microorganisms, bacteria are known as the predominant group that produce higher titres of biosurfactants. These microbial biosurfactants typically have amphiphilic structures with the hydrophobic end, either a long-chain fatty acid, hydroxyl fatty acid or α-alkyl-β-hydroxy fatty acid, and a hydrophilic moiety which can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, an alcohol and a carboxylic acid (Desai & Banat, 1997; Santos *et al.*, 2016).

Generally, the diverse structures of biosurfactants can be divided into two main categories based on their molecular weight, namely low-molecular-mass molecules that function effectively to lower surface and interfacial tensions, and high-molecular-mass polymers, which are more effective as emulsion-stabilizing agents. The low-mass surfactants consist of glycolipid, phospholipids, fatty acids, lipopeptide and lipoprotein, while the high-mass surfactants comprise polymeric surfactants and particulate surfactant (Kosaric & Sukan, 2014; Nitschke & Costa, 2007). Besides the grouping by molecular weight, biosurfactants are also categorized based on their five

main chemical compositions namely; glycolipid; lipopeptides and lipoproteins; fatty acids and lipids (phospholipids and neutral lipids); polymeric biosurfactants; and particulate biosurfactants (Fenibo *et al.*, 2019; Rahman & Gakpe, 2008). Table 2.1 lists the major classes of biosurfactants produced by microorganisms.

Another common classification of biosurfactants is by their net charge, which can either be non-ionic, anionic, cationic, and amphoteric biosurfactants (Henkel & Hausmann, 2019). Most of biosurfactants are found as anionic or non-ionic compounds. Only a few are cationic, which often possess higher toxicity (Kosaric & Sukan, 2014). The first microbial biosurfactants introduced into the market were sophorolipids which were very hydrophobic. The next group of biosurfactants that was made commercially available in the market were rhamnolipids which are known to be very hydrophilic. Sophorolipids are used as an ingredient in some cleansing agents (Ecover, Malle, Belgium) while rhamnolipids have been applied as an active substance in the U.S. EPA approved Zonix^{TM} fungicide (Jeneil Biosurfactants Co., Saukville, USA) (Jeneil Biosurfactants Co., Saukville, USA) (Müller *et al.*, 2012; Nguyen *et al.*, 2010; Nguyen & Sabatini, 2011).

According to Kosaric and Sukan (2014), there are more than 2000 distinct biosurfactant structures that have been identified to date, including chemically different families of compounds, and also groups of congeners, that is, structurally closely related compounds with minor structural variations. Among the major classes of biosurfactants, glycolipids and lipopeptides (Figure 2.3) account for the tremendous share of commercially available microbial surfactant and scientific interest due to their high surface activity (Henkel & Hausmann, 2019; Inès & Dhouha, 2015). These microbial biosurfactant groups can serve as antimicrobial, antiadhesive, antitumor and

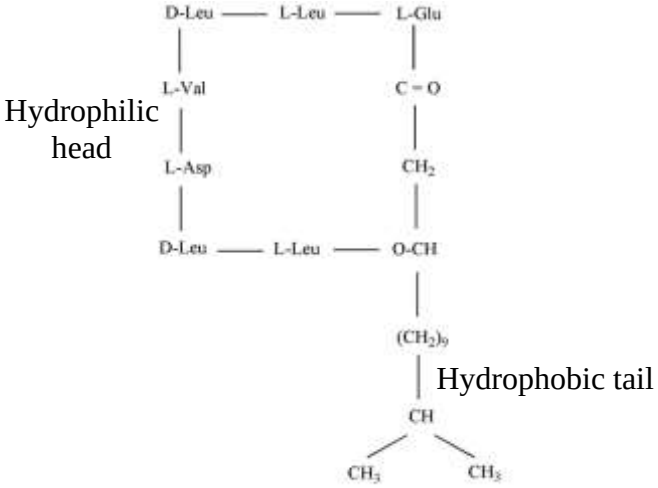
antizoospore agents in the medical and pharmaceutical industries (Banat *et al.*, 2010; Raaijmakers *et al.*, 2010).

Table 2.1: The major classes of biosurfactants and respective producing microorganisms

Class	Type	Microbial sources	References
Glycolipid	Rhamnolipid	*Pseudomonas* sp. *Serratia rubidea*	(Jadhav *et al.*, 2011)
	Sophorolipids	*Candida bombicola* (formerly called *Torulopsis bombicola*) *Candida Apicola* *Candida Bogoriensis*	(Elshafie *et al.*, 2015) (Joshi-Navare & Prabhune, 2013)
	Trehalolipids	*Rhodococcus* sp.	(White *et al.*, 2013)
		Nocardia sp. *Mycobacterium* sp.	(Kügler *et al.*, 2015)
		Aspergillus niger	(Kannahi & Sherley, 2012)
Lipopeptide and lipoprotein	Surfactin	*Bacillus* sp.	(Varadavenkatesan & Murty, 2013)
	Cyclic lipopeptide (pseudofactin)	*Pseudomonas fluorescens* strain BD5	(Janek *et al.*, 2010)
		Streptomyces sp.	(Baltz *et al.*, 2005)
		Penicillium chrysogenum SNP5	(Gautam *et al.*, 2014)
Fatty acid and lipids	Spiculisporic acid Corynomycolic acid	*Penicillium spiculisporum*	(Kosaric & Sukan, 2014)
	Phosphatidylethanolamine	*Rhodococcus erythropolis*	(Stancu, 2015)

Polymeric Biosurfactant	Emulsan	*Acinetobacter calcoaceticus* RAG-1	(Kosaric & Sukan, 2014)
	Liposan	*Acinetobacter radioresistens*KA-53,	(Rahman & Gakpe, 2008)
Particulate Biosurfactant		*Acinetobacter calcoaceticus Cyanobacteria*	(Santos *et al.*, 2016)

(a) Surfactin



(b) Rhamnolipid

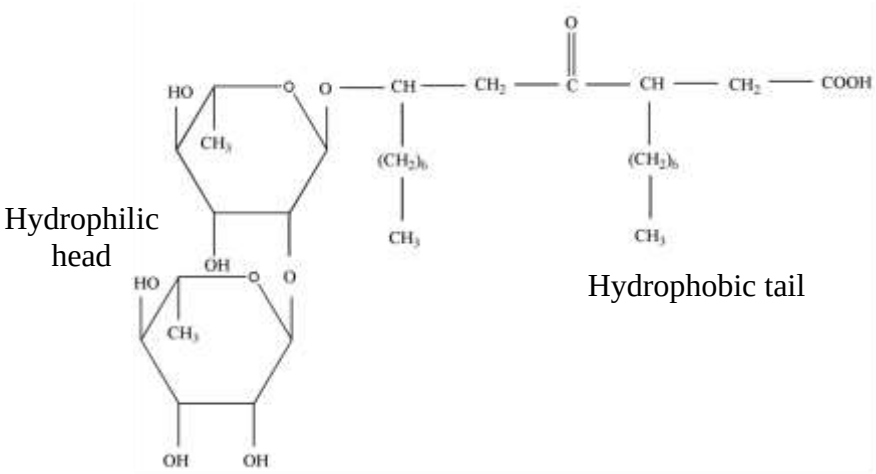
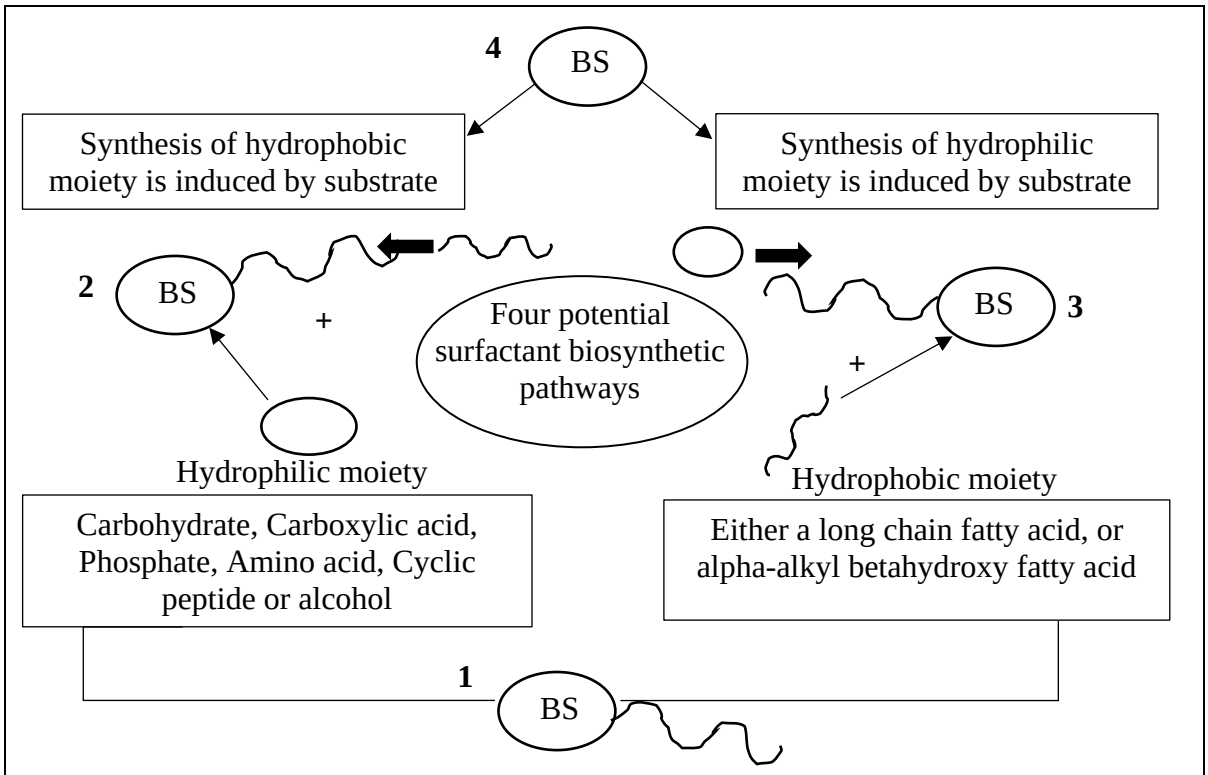


Figure 2.3: The chemical structural of most studied biosurfactants; (a) Surfactin, a lipopeptide biosurfactant; (b) Rhamnolipid, a glycolipid biosurfactant.

2.5 Metabolic pathway of biosurfactant

Since biosurfactants are amphiphilic compounds, every variant of these biomolecules contains both of hydrophilic and hydrophobic moiety. This means that two different metabolic pathways are involved in the biosynthesis of biosurfactant, where one controls the formation of the hydrophilic portion while the other dictates the hydrophobic portion. It is believe that the microorganisms utilized water-soluble substrates like carbohydrate groups to build up the hydrophilic moiety of biosurfactants, while hydrophobic substrates like fats and oils are used to synthesize the hydrophobic portion of biosurfactants (Desai & Banat, 1997; Nurfarahin *et al.*, 2018; Sineriz *et al.*, 2001). In other words, the carbon flux in the biosynthesis of biosurfactant will be regulated by both glycolytic (buildout of hydrophilic moiety) and lipogenic pathways (lipid generation) that are controlled by microbial metabolism (Haritash & Kaushik, 2009).

Figure 2.4 shows four principle possibilities for synthesis of such an amphiphilic molecule: (1) the hydrophilic and hydrophobic moieties are synthesized *de novo* by two independent pathways, followed by their linkages; (2) the hydrophobic moiety is synthesized *de novo* while hydrophilic moiety synthesis is substrate dependent, followed by its linkages; (3) the hydrophilic moiety is synthesized *de novo* while the substrate play a role to induce the hydrophobic moiety and the subsequent linkages; (4) the synthesis of both hydrophilic and hydrophobic moieties are substrate-dependent with subsequent linkages.



Caption:

1. Both independently synthesizes by two *de novo* pathways
2. Hydrophilic moiety by *de novo* pathway and hydrophobic moiety is induced substrate
3. Hydrophobic moiety by *de novo* pathway and hydrophilic moiety is induced substrate
4. Synthesis of both biosurfactant moieties depend upon substrate

*BS: Biosurfactant

Figure 2.4: Metabolic pathway of biosurfactant production by microorganisms (Karlapudi *et al*., 2018)

In most cases, the mechanism of biosurfactant production is highly determined by the types of carbon source present in the culture medium (Sineriz *et al.*, 2001). Figure 2.5 shows the common metabolic pathways involved in the production of biosurfactants using water-soluble substrates. For example, the biosynthesis of biosurfactant using simple carbohydrates like glucose is initiated by transforming glucose to the intermediate, glucose-6-phosphate (G6P) via glycolytic pathway. This part is known as one of the major precursors of carbohydrates found in the hydrophilic moiety of biosurfactant. Accordingly, a series of enzymes are utilized to catalyse G6P

en route to produce numerous forms of hydrophilic moieties in the biosurfactants such as trehalose, sophorose, rhamnose, mannose, and polysaccharide. The formation of the hydrophobic part (lipid) occurs when the glucose is oxidized to pyruvate. Pyruvate is then converted into acetyl-CoA, that makes up malonyl-CoA when combined with oxaloacetate. Afterwards, the oxaloacetate is converted into fatty acids which function as a precursor for lipid production (Hommel & Huse, 1993).

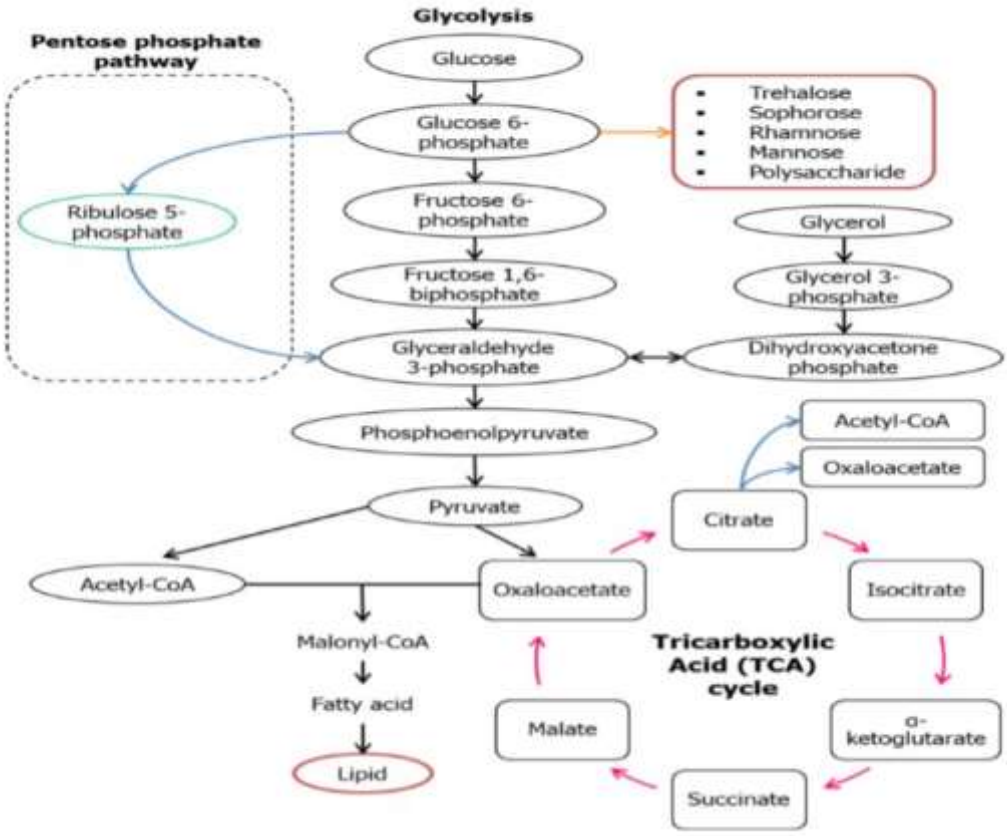


Figure 2.5: Metabolic pathways involved in the production of biosurfactants using water-soluble substrate. Adapted from Korla and Mitra (2014)

Although microorganisms can utilize water-soluble substrates to produce biosurfactant, many studies reported that some microorganisms produced better titres of biosurfactants when cultivated in water-insoluble substrates. Figure 2.6 illustrates the main reaction involved in the synthesis of biosurfactant using hydrocarbon as the

carbon source. In microorganisms that utilize hydrocarbon as the main carbon source for biosurfactant production, the biosynthesis is mainly directed to the lipolytic pathway and gluconeogenesis (the formation of glucose via different hexose precursors). Biosynthesis begins when these microbes go through the lipolytic pathway and gluconeogenesis which leads to the de novo formation of the hydrophobic and hydrophilic moieties via gluconeogenesis. This pathway is the reverse of glycolysis where glucose is produced as the end product. The reactions are catalyzed by a series of enzymes such as hexokinase, pyruvate kinase, and phosphofructokinase-1 that are irreversible. Gluconeogenesis performs with the oxidation of fatty acids to form acetyl-CoA via β-oxidation that will later go into the tricarboxylic acid (TCA) cycle to form pyruvate. Pyruvate is then converted into polysaccharide precursor (G6P) that is involved in a series of an enzyme similar in glycolysis.

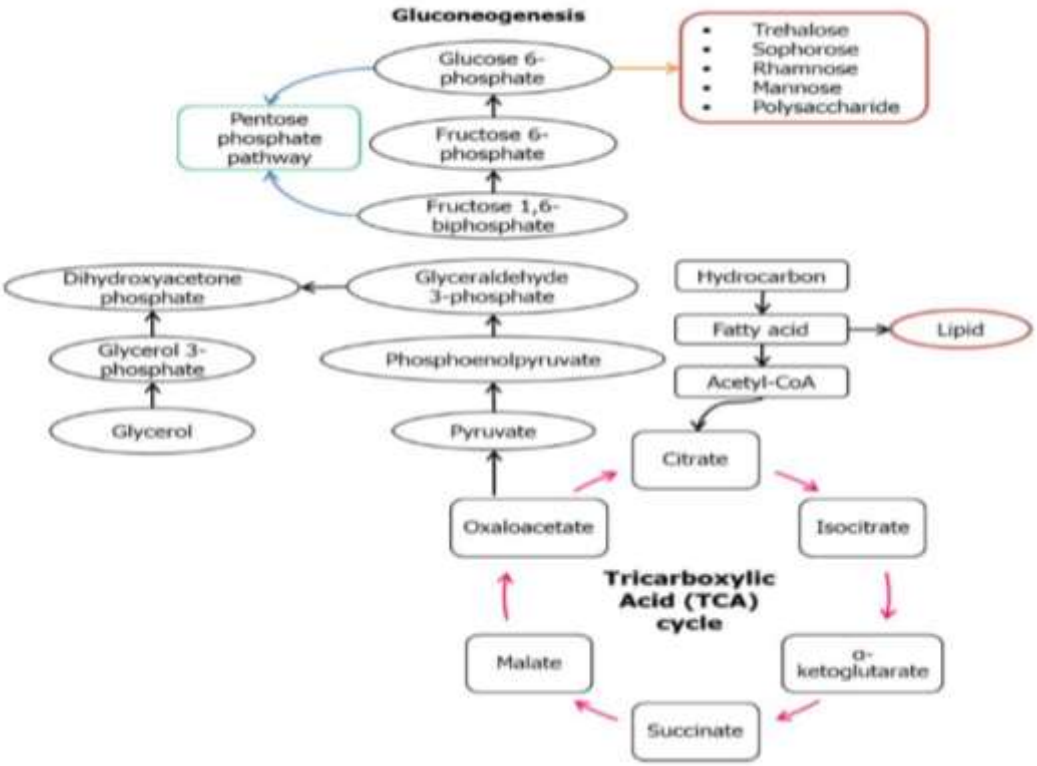


Figure 2.6: Metabolic pathways involved in the production of biosurfactants using water-insoluble substrate. Adapted from Santos *et al*. (2016)

The biosynthetic and regulatory pathways are well studied in some of the microbial biosurfactant producers such as *Pseudomonas* sp. and *Bacillus* sp. which are well-known rhamnolipid and surfactin or subtilisin producers, respectively. The biosynthesis of rhamnolipid is performed by two sequential glycosyl transfer reactions where each reaction is catalyzed by a different rhamnosyltransferase (Burger *et al.*, 1963; Zhu & Rock, 2008). In surfactin production, it is catalyzed non-ribosomally by a large multienzyme peptide synthetase complex called the surfactin synthetase (Das *et al.*, 2008). However, the metabolic pathway of biosurfactant production for most of the other microorganisms are still not clearly understood and vary depending on the microbial strain.

2.6 Pattern of biosurfactant production

Biosurfactants can either be produced extracellularly or remain attached to the cell surface as particulate biosurfactants in aqueous media during the fermentation process. An intracellular biosurfactant benefits the cell with the existence of a membrane lipid structure to promote the transport of insoluble substrates through the membrane. Similarly, an extracellular biosurfactant helps substrate solubilization outside the cell and is usually present as a complex structure of lipids, proteins and carbohydrate (Prabhu & Phale, 2003). Most of the biosurfactants produced by known biosurfactant producers, namely *Pseudomonas* and *Bacillus* sp., are secreted extracellularly to the culture medium during fermentation, causing a decrease in the medium surface tension (Georgiou *et al.*, 1992; Gudiña *et al.*, 2011). On the contrary, trehalose lipid synthesized by *Rhodococcus* is often found bound to the cell envelope (Franzetti *et al.*, 2010).