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Identification and Characterization of Biosurfactant Producing Bacteria from Sungai Dungun

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DECLARATION

I hereby declare that work embodied in this report is the result of originally on my own work except the excerpts and summaries of which I have explained the source.

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I certify that the report of this final year project entitled "Identification and Characterization of Biosurfactant Producing Bacteria from Sungai Dungun" by Nazmi Bin Mohammad, matric number F15A0099 has been examined and all correction recommendation by examiners have been done for the degree of Bachelor of Applied Science (Bioindustrial Technology), with Honors, Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan.

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Pengenalpastian dan Pencirian Bakteria Pengeluaran Biosurfaktan dari Sungai Dungun

ABSTRAK

Biosurfaktan adalah sebatian amphipilik yang disintesis oleh pelbagai bakteria dan kulat yang dapat mengurangkan ketegangan permukaan dan antara muka. Melalui sifatnya tersendiri, biosurfaktan boleh digunakan sebagai pengganti surfaktan sintetik dalam pelbagai industri seperti industri makanan, kosmetik, industri minyak, industri farmaseutikal dan aplikasi alam sekitar. Dalam kajian ini, objektifnya adalah untuk mengenal pasti dan mencirikan bakteria penghasil biosurfaktan yang dipencilkan daripada Sungai Dungun. 3 pencilan bakteria dipilih untuk dicirikan dalam kajian ini iaitu pencilan DSA 4, DSA 7 dan DWA 9. Pencilan bakteria ditumbuhkan di atas Medium Garam Mineral (MSM) yang ditambah dengan 1% minyak masak. Biosurfaktan yang dihasilkan oleh isolat bakteria ini dicirikan melalui pengujian emulsi, ujian titisan runtuh, ujian penyebaran minyak, ujian keupayaan merosotkan hidrokarbon dan ujian hemolisis darah. Daripada keputusan yang diperhatikan, isolat DWA 9 menunjukkan keupayaan paling tinggi dalam pengeluaran biosurfaktan. Ia menunjukkan keputusan positif bagi ujian titisan runtuh, menunjukkan β-hemolisis dan kawasan penyebaran minyak terluas dalam ujian penyebaran minyak dan juga aktiviti pengemulsian tertinggi dan peratusan degradasi hidrokarbon tertinggi dengan 34.43% dan 54% masing-masing. Bagi pencirian molekul isolat, gen 16S rDNA berjaya diasingkan dari semua pencilan. Walau bagaimanapun, selepas penjujukan gen dan analisis BLAST, hanya isolat DSA 7 dapat dikenal pasti, yang berasal daripada genus *Aeromonas*.

Kata kunci: Biosurfaktan, surfaktan, ujian penyebaran minyak, ujian titisan runtuh dan ujian hemolisis darah.

Identification and Characterization of Biosurfactant Producing Bacteria from Sungai Dungun

ABSTRACT

Biosurfactants are an amphiphilic compounds synthesized by a variety of bacteria and fungi which as the ability to reduce surface and interface tension. Through their distinctive traits, biosurfactants can be used as substitute for synthetic surfactants in various industries such as food, cosmetics, oil industries, pharmaceutical industries and environmental application. In this study, the objective is to identify and characterize biosurfactant producing bacteria isolated from Sungai Dungun. 3 bacterial isolates were chosen to be characterized in this research which is isolates DSA 4, DSA 7 and DWA 9. The bacterial isolates were grown on Mineral Salt Medium (MSM) that was supplemented with 1% cooking oil. Biosurfactant produced by these bacterial isolates were characterized through emulsification assay, drop collapse assay, oil spreading assay, hydrocarbon degrading capability test and blood haemolysis test. From the results observed, isolate DWA 9 showed the greatest capability in the production of biosurfactant. It showed positive result for drop collapse assay, demonstrated β-hemolysis and the widest oil displacement area in oil displacement test and also the highest emulsification activity and hydrocarbon degradation with 34.43 % and 54 % respectively. As for the molecular characterization of the isolates, 16S rDNA gene was successfully isolated from all the isolates. However, after gene sequencing and BLAST analysis, only isolate DSA 7 was able to be identified, which originated from the genus *Aeromonas.*

Keywords: Biosurfactant, surfactant, oil spreading assay, drop collapse assay and blood haemolysis test.

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LIST OF ABBREVIATIONS AND SYMBOLS

MSM	Mineral Salt Medium
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
PCR	Polymerase chain reaction
TSB	Trypticase soy broth
GTE	Glucose-HCl-EDTA
SDS	Sodium dodecyl sulphate
TE	Tris-HCl-EDTA
NaCl	Sodium Chloride
dNTPs	Deoxyribonucleic triphosphate
PAHs	Polycyclic Aromatic Hydrocarbons
˚C	Degree Celcius
µL	Micro Litre
Min	Minute
mg/L	Miligram per litre
Kb	Kilo base
Bp	Base pair

CHAPTER 1

INTRODUCTION

1.1 Research Background

Surfactants are amphiphilic molecules that have the ability to reduce interfacial tension. Due to the compound's ability in reducing surface tension, it can enveloped the cell, blend and break up non-soluble substrates in aqueous solution (Lin., 1996). In the wide variety of industrial processes, synthetic surfactants find its uses in the foaming, wetting, emulsification detergency, dispersing and solubilisation. But chemically synthesis surfactants are slowly replaced by biosurfactants that are produce through microbial activity or enzymatic based. Similar to chemically based surfactants, biosurfactants are also a type of amphiphilic molecules that was produced and covered the cell surface (Gautam & Tyagi., 2006).

Biosurfactant unique traits of high surface activity, its biodegradability and are able to be synthesis by wide range of microorganism have been the main reason why it has been studied greatly in the past years (Chen *et al*., 2007).

A wide variety of microorganism like yeast, bacteria and filamentous fungi can produces biosurfactants through the use of different substances such as oils, sugar and wastes (agro-industrial waste and animal waste). But the most commonly used nutrient to produce biosurfactants are typically carbohydrates and vegetable oils (Rahman *et al.*, 2002). The micelles formed from the amphiphiles molecule have the potential to be

utilized in chemical works where surface active agents or surfactants are used to describe it. The main feature of biosurfactants is that it has two ends both hydrophobic and hydrophilic. The hydrophobic end of the molecule is soluble in water. It consists of long chain made up of fatty acids that is not soluble or less soluble in water. The hydrophilic end consists of carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Rahman *et al.*, 2008)

Biosurfactants have taken the interest of many people as a promising new replacement for the current generation of synthetic surfactants and are anticipated to harbour many possibilities for the future mostly in industrial and also environmental applications (Banat *et al.*, 2000). Despite the growing interest of biosurfactants, economically it cannot match the chemically synthetic surfactants. Extensive research on biosurfactant could lower the costs of production either by increasing the output and accumulation of product. Low cost materials for biosurfactant production is also important for the economic aspect since raw materials take up about 50% for the cost of the final product (Nitschke *et al.*, 2004).

For the past few years, the production of biosurfactant from a variety of microorganisms has been widely studied. There has been a great amount of information involving their properties and types of production. Regardless of having a lot of commercial interesting traits and distinct advantages compared to their chemically synthetic counterparts, the commercial scale of the biosurfactants production has not been grasped properly due to low output and expensive cost in its production. Hence, it is essential that these microbial surfactants are produced in a larger scale with lower cost and high profits. One of the approach is to increase the yield of biosurfactants is by utilizing recombinant hyperproducing strains. Still this new sector of biosurfactant of research is still very new (Mukherjee *et al*., 2006). There is a need to find or create new

type of strain that is able to produce biosurfactants in large quantity and great quality without having to spend a large amount of cost in producing it.

As a result of the pathogenic trait of bacteria like *Bacillus* and *Pseudomonas* species, their applications have been narrowed down. They are not applicable in food industries which use a lot of biosurfactants. But there are few species of yeast that does not exhibit these pathogenic properties, it also are considered as Generally Recognize as Safe (GRAS) status. Some of these strains belong to genera *Candida, Starmerella, Pseudozymaor* or *Yarrowia* (Souza et al., 2017). Because of this, there is a necessity to seek a new types of biosurfactant and bioemulsifiers and the research done on countless other organism that produced biosurfactants have been increasing this past years (Giaouris *et al*., 2012). Through extensive researches done on biosurfactants, and some of the research on biosurfactants are related with bioremediation process (Shaidatul *et al*., 2017). For instance, Pacwa-Płociniczak and fellow researchers was able to isolate new *Pseudomonas sp.* P-1 strain from soil that was contaminated with heavy petroleum hydrocarbon (Pacwa-Płocinicza *et al.*, 2014). According to Henkel and his fellow researchers (2017), there are only a few biosurfactant like sophorolipids, rhamnolipids and mannosylerythritol lipids that have found their uses as an industrial product.

From the researches of biosurfactants, it has been found that the exploitation of biosurfactants could give a great advantage to industries sector in terms of its capability and its positive effects on the environment. Through extensive research biosurfactant can be further refined into a product that can be found in all sectors of the industry.

In this study, 3 bacterial isolates were used which were isolated from water sample of Sungai Dungun. The bacterial isolates are DSA 4, DSA 7, and DWA 9 undergoes identification of biosurfactant producing bacteria and also the characterization of biosurfactant.

1.2 Objectives

The objectives of this study are:

- To identify biosurfactant producing bacteria that were isolated from Sungai Dungun water samples using molecular and physical characterization.
- To characterize the biosurfactant produced by the bacterial isolates using quantitative and qualitative analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Surfactants and Biosurfactants

Surface active agents or more commonly known as surfactants has a wide range of characteristic like the abilities to lower the surface and interfacial tensions between liquids (Al-Araji *et al*., 2007). Moreover, biosurfactants has been observed to improve transport of nutrient across membrane, function in a variety of host-microbe interactions and can serve as biocidal and fungicidal protection to the organism that are producing the biosurfactants (Jennings & Tanner, 2000). Surface tension or SFT can be defined as the force per unit length applied by a liquid which is in contact with solid or other liquid. Other than that, it is also has been considered as the measurement of free energy per unit area correlating with a surface or an interface (Satpute *et al.*, 2010). Meanwhile, biosurfactants are defined as compound from biological sources that shows an elevated surface-active traits (Georgiou *et al*., 1992). Biosurfactants have been suggested to be used as a tool to disintegrate hydrocarbon since the 1960s. The chemically synthesized and commercially available surfactants that are sourced from the un-sustainable oil based commodities is more expensive and pose a great threat to the earth because of the inorganic state of the compound itself (Roy, 2017)

2.2 Structure of Biosurfactants

There are several classifications of biosurfactants, it can be grouped according to chemical structure, function, and also their microbial origin. Biosurfactants have both hydrophobic and hydrophilic moieties part in preference to partition at the interface between contrasting phases like gas, liquid, solid and some liquids with polarities that are different (oil/water and water/oil) and also hydrogen bonding (De Almeida *et al*., 2016).

Biosurfactant's hydrophobic moiety is made up of hydrocarbon chain of a saturated, unsaturated, hydroxylated, or branch fatty acid. While in their hydrophilic moiety part, it can be only as simple as the carboxylate group of fatty acids, amino acids or even phosphate group. These compounds have a part of phospholipids or either has a complex structure like mono-saccharides, disaccharides and polysaccharides of glycolipids and polar side chains and peptide backbone of lipopeptide biosurfactants.

One of the reasons why biosurfactant has a great degree of solubility in water is due to their hydrophilic part. While the ones that is responsible for the capillary activity is the lipophilic part. Both of lipophilic and hydrophilic are bond together with linkage called an ester linkage using both organic and also inorganic acids. The crucial role that is played by the ionization of the functional groups is in the surface activity, particularly if the simple carboxylic acids plays a role in the water-oil interface. The most feasible way to classify biosurfactants is based on their charge or even molecular weight (Siñeriz, F., 2000).

There are two categories of biosurfactants from microbial origin which is low molecular weight (LMW) and also high molecular weight (HMW) (Smyth *et al*., 2010)

2.3 Classification and Chemical Nature of Biosurfactant

Surfactants from chemical origin are commonly categorized based on the nature of their polar group. Meanwhile surfactant of microbial origin can be grouped generally by their chemical composition dictated by separate molecules creating what is known as the hydrophobic and hydrophilic moieties. The main categories for low mass surfactants which comprise of glycolipids, lipopeptides and phospholipids, while high mass surfactants classes comprise of polymeric and particulate and surfactants such as the poly-anionic hetero-polysaccharides which consist of both polysaccharides and also proteins. Biosurfactant produce from microbes can vary through different environment according to the nutrition that the microbes are using. (Manley, 1999).

(a) Glycolipids

Glycolipids consist of either one or two residues of sugar which is glucose and galactose either in α- or β- configuration and are attached together to different lipid backbones. Found mainly in cell membranes of bacteria, fungi, plants and animals. Glycolipids form the backbone of varying metabolic functions and also biological structure like the cell membranes. Glycolipids are used in the emulsification of insoluble substrates in water, elevating surface area and bio-availability of substrates mainly for cellular growth. Glycolipids also take part in mobility of the cell due to the ability reduce interfacial tension when the microbes locates at the interface. Which in turn assist in its search for better environments for growth, reproduction and colonization. Other than that it also mediates in the function of adhesion directly to the

interfaces of its natural environment. Moreover, it can be utilize as an energy source and storage of extra-cellular carbon (Cortés-Sánchez *et al*, 2013)

(i) Rhamnolipids

One of the best known and extensively studied glycolipids is rhamnolipids which is an efficient compound which have one or two molecule of β-hydroxydecanoic acid. Most of the rhamnolipids produced are mostly synthesized by *Pseudomonas spp*. There are two kinds of rhamnolipids produced by *Pseudomonas aeruginosa* in liquid culture which is mono-rhamnolipids and also di-rhamnolipids. A few of the *Pseudomonas* species is only able to produce mono-rhamnolipids while other species can synthesize both mono- and di-rhamnolipids (Kaskatepe & Yildiz, 2016).

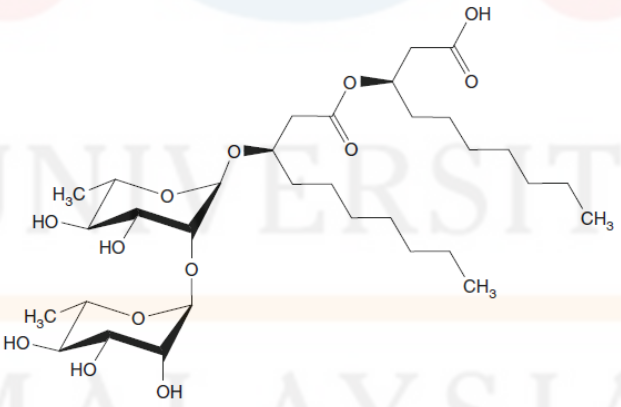


Figure 2.1: Rhamnolipid chemical structure
(Abdel-mawgoud *et al*, 2011)

The rhamnolipids producing *Pseudomonas sp.* can be isolated from a wide range of environment and habitats such as water, soil and even plants. It also seems to have a pathogenic characteristic and an opportunistic pathogen to humans that can cause severe

nosocomial infections. When it is grown under distinct environment conditions this species of bacteria can synthesize rhamnose-containing glycolipids (Maier & Soberón-Chávez, 2000). Rhamnolipids can be used for a wide range of application. One of the notable uses is through bioremediation. In recent year, Rhamnolipids have been observed to have elevated the oil biodegradation effectiveness by about 5.6% (Kiran *et al*, 2016)

Rhamnolipids produced by *P. aeruginosa* also have the capabilities to be an antibacterial compound which was shown by De Rienzo and fellow researchers (2015), the combination of both rhamnolipids and sophorolipids shows a promising effect on the ability to inhibit the growth of bacteria and also their biofilms. Rhamnolipids produced from *P. aeruginosa* also have been reported to be used in nanomaterials capping, stabilizing and dispersant. After undergoing molecular characterization the bacteria used to produce the rhamnolipids shares a 99% resemblance to *P. aeruginosa* which is named CEMS077 strain. The rhamnolipids were then used to produced stable antioxidant ZnO particles which is a type of nanoparticles (Singh *et al*., 2014). In some cases, due to *P. aeruginosa* ability to produce high concentration of rhamnolipids, the gene responsible for the production of rhamnolipids was then inserted into other bacteria. Zhao and his colleagues (2015) managed to insert recombinant plasmid originated from a strain of *P. aeruginosa* into *Pseudomonas stutzeri*. The recombinant strain was used for *in-situ* microbial enhanced oil recovery (MEOR) in oil industry where rhamnolipids is used to help in the extraction of oil but due to *P. aeruginosa* incapability to produce rhamnolipids in oxygen deficient environment, the genes have to be inserted into *P. stutzeri* which is capable of surviving in oxygen deficient environment inside the oil reservoir. The result shown was promising and shows that the ability to produce rhamnolipids can be transferred from one different strain to

another different strain. A variation in time in terms of production of rhamnolipids from *P. aeruginosa* can also produce different types of composition of the rhamnolipids to be used in different applications like bioremediation and oil recovery (de Santana-Filho *et al*., 2014).

(ii) Sophorolipids

Sophorolipids are mainly produce by fungi. Some of the examples of fungi species that produce sophorolipids are *Candida batistae*, *Candida bombicola* and also *Candida sp*. SY16 (Bhardwaj, 2013). Being a surfactant molecule, sophorolipids are amphilic molecules that interact with the phase boundary inside heterogeneous environment. Sophorolipids are made up from hydrophobic fatty acid tail of 16 to 18 atoms of carbon and also a sophorose which is a hydrophilic carbohydrate head. Sophorose is a type of glucose disaccharide with an odd β-1, 2 bonds and is able to be acetylated. There is a wide application of sophorolipids such as a dishwasher that was introduced to the market by a Japanese company called Saraya, they utilize sophorolipids as a cleaning agent. There is also a French compant called Soliance which uses a formulation containing sophorolipids as cosmetic product. Sophorolipids also can be used as a germicide which is applicable for cleaning fruits and vegetable (Van Bogaert et al., 2007).

(iii) Trehalolipids

The trehalolipids is described as a disaccharide that is non-reducing where two units of glucose are linked with α, α-1, 1-glycosidic linkage. Trehalolipids served as a basic component of the glycolipids cell wall usually in *Mycobacteria* and *Corynebacteria*. Trehalolipids that is most observed is the trehalolipids 6, 6' – dimycolate, where it is α-branched-chain mycolic acid which is esterified to the C6 position in each unit of glucose. Various microorganisms that are in mycolates group like *Artrobacter*, *Nocardia*, *Rhodococcus* and also *Gordonia* can synthesize different trehalose containing glycolipids. Trehalolipids application can be seen in the oil industry where it has been observed that trehalolipids that was produced by *Rhodococcus spp* has enhanced the displacement of the crude oil from the cores of the rock by 20%, and also elevated the oil recovery by 30% from sandstones which was studied by utilizing *Nocardia rhodochrus* that was producing the trehalolipids (Ho & Ping, 2008)

(b) Lipopeptide

This class of biosurfactants are made up of lipid tail which linked to the short linear or cyclic oligopeptide. Lipopeptide are mostly synthesized by fungi which include *Aspergillus* sp. and numerous other bacterial genera like *Streptomyces* sp., *Pseudomonas* sp. and *Bacillus* sp. Lipopeptide has been a curiosity for researchers because of their antimicrobial, cytotoxicity, anti-tumour, immunosuppressant and also

their surfactant trait. Among the most studied bacterial producer of lipopeptides are *Pseudomonas* sp. and *Bacillus* sp. (Raaijmakers *et al*, 2010)

(i) Surfactin

Produced by varying strains of *Bacillus subtilis*, surfactin is bacterial cyclic lipopeptide and considered among the most effective biosurfactants. It has the capability to lower the surface tension of water from 72 to 27 mN/ m and at concentration as low as 0.005%. The surfactin structure is comprises of seven amino acids which are bonded together to the carboxyl and hydroxyl group of a 14- carbon fatty acid. It have a few biological activities like the capability to lyse erythrocytes, inhibiting formation of clot, lyse bacterial speroplasts and also protoplasts and inhibiting cyclic 3, 50 monophosphate diesterase (Shaligram & Singhal, 2010).

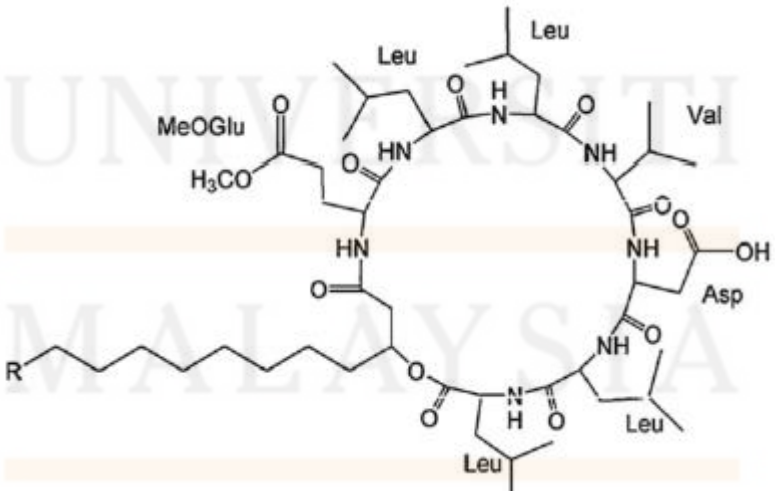


Figure 2.2: Chemical structure of surfactin
(Liu *et al*, 2015)

Other than that, surfactin also has been observed to elevate the surface area of hydrophobic water insoluble growth substrates resulting in higher bioavailability of nutrients and influenced the attachment and detachment of bacterial cells to and from substrates. It also has some function in the formation of biofilm (Seydlová & Svobodová, 2008).

(c) Fatty Acids and Phospholipids

When grown on *n*-alkanes a few species of bacteria and yeast are capable of producing large amount of fatty acids and phospholipid surfactants. There is correlation between the hydrophilic and lipophilic balance and also the length of the hydrocarbon chain in their structures. Strain HO1-N from *Acinetobacter* sp. they produced phosphatidylethanolamine rich vesicles, forming an optically clear micro emulsions of alkanes inside the water. *Rhodococcus erythropolis* produced phosphatidylyethanolamine grown using *n*-alkanes leads to the reduction in interfacial tension between water and hexadecane to less than 1 mN/ m and a critical micelle concentration (CMC) of 30 mg/ L (Manley, 1999). One of the biosurfactants is used as a major structural element of biological membranes which is phospholipids. Being an amphiphilic compound, phospholipids has the ability to adopt multiple molecular assemblies when dispersed in water, like in the form of bilayer vesicles or micelles that confers them unique interfacial traits and making it very interesting in terms of foam and also emulsion stabilization (Pichot *et al.*, 2013). When grown on alkane substrates, phospholipid level of production increases dramatically. For example, *Acinetobacter* sp, HO1-N that was grown in hexadecane has been observed to produce phospholipid rich

extracellular membrane vesicles inside the medium in large quantity (Rosenberg & Ron, 1999)

(d) Polymer Type

Polymeric biosurfactant generally are used a great emulsion stabilizer where in one study, *Acinetobacter calcoaceticus* RAG-1 was used to produce an effective polyanionic amphiphatic heteropolysaccharide bioemuslifier known as emulsan (Rahman & Gakpe, 2008)

(i) Emulsan

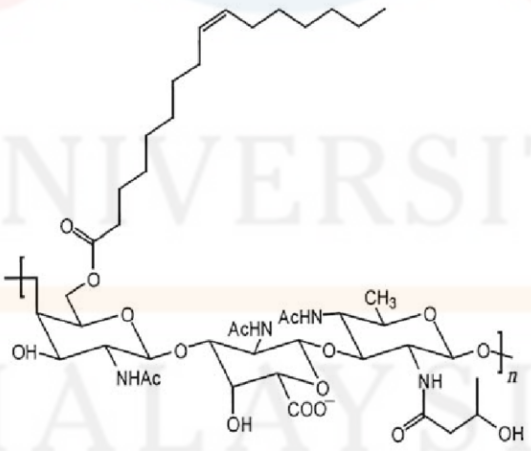


Figure 2.3: Emulsan chemical structure
(Bach & Gutnick, 2005)

Emulsan produced by the bacterium *Acinetobacter calcoaceticus* is a polyanionic lipo heteropolysaccharide bioemulsifier and have proven to be an efficient

emulsifier of oily substances. It has taken interest in cleaning, degreasing and maintenance applications due to its capability to create stable hydrocarbon-in-water emulsion (Amani & Kariminezhad, 2016)

(ii) Alasan

The component of polysaccharide alasan is unique in which that it have covalently bound alanine. The component of protein of alasan seems to play an important role in both the structure and activity of the complex structure (Kaloorazi & Choobari, 2013)

(iii) Liposan

Liposan is usually produced by *Candida lipolytica*, it is an extracellular water-soluble emulsifier and made up of 83% carbohydrate and 17% protein. The carbohydrate part of the liposan is a heteropolysaccharide which consists of glucose, galactose, galactosamine and also galacturonic acid (Kaloorazi & Choobari, 2013)

(iv) Mannoproteins

Mannoproteins are mostly produced by the yeast *Saccharomyces cerevisiae* that made up 35% – 40% of the cell wall. It is a glycoproteins which is often glycosylated

located on the most outer layer of the yeast cell wall in which they are connected to the matrix of amorphous β- 1,3 glucan by covalent bonds. It also has been reported as a great bio-emulsifier and is partially water-soluble components released by the action of β- 1, 3 glucanases (Caridi, 2006)

(e) Particulate Biosurfactants

Particulate biosurfactant plays a crucial role in helping in the alkane uptake by the microbial cells where the extracellular membrane vesicles partition hydrocarbons to create a microemulsion. For instance in the *Acinetobacter* sp. HO1-N vesicles it consisted of protein, phospholipid and also lipopolysaccharide (Erum *et al*, 2013)

2.4 Advantages of Biosurfactants

(a) Biodegradability and Controlled Inactivation of Microbial Surfactants

Some of chemically synthesized and is widely used and sold surfactants like the perfluorinated anionics has certain traits that makes it to be able to resist biodegradation and this caused it accumulated in the nature creating problems for the ecological system. But when compared to the surfactants produce by microorganism which is the same like the rest of products from natural sources, they are more affected to biodegradation by microorganisms that are present inside water and soil (Cameotra *et al*, 2010)

(b) Selectivity for Specific Interfaces

Higher specificity has been observed on biological molecules when compared to materials made from chemicals. Current commercially available and chemically synthesized surfactants did not show great specificity when compared to microbial synthesized surfactant. This trait can be observed in the specificity of emulsan when it acts on the mixture of aliphatic and also aromatic hydrocarbons and other example like *Pseudomonas* PG1 that produce surfactant which has solubilizing factor towards pristine (Cameotra *et al*., 2010)

(c) Surface and Interfacial Activity

At lower concentration, biosurfactants are able to create a lower surface tension which shows a considerable capability and efficiency when compared to the surfactants that are produced chemically. The measurement of efficiency which is the CMC of microbial produced surfactants can range from 1 to 2000 mg/L, considering the interfacial tension between oil or water and surface tension range from 1 and 30 mN/ m (Sarubbo, 2014)

(d) Tolerance towards Temperature, pH and Ionic Strength

Under extreme conditions biosurfactants can still functions without any flaws. Some of the example can be seen in *Bacillus licheniformis* JF-2 which produced lipopeptide that can maintain its stability at the temperature of up to 75˚C for 140 hours

and can also survive at the pH of 5 to 12. High salt concentration also cannot deter biosurfactants from functioning well and can perfectly function at the salt concentration of about 10%, but compared to chemically synthesized it can only withstand 2% of NaCl concentrations (Sarubbo, 2014)

(e) Emulsion Forming

Biosurfactants also have the ability to act as emulsifiers or de-emulsifiers. Emulsion can be described as a heterogeneous system that made up from immiscible liquid that is dispersed into another liquid through the formation of droplets whereby the diameter of it usually more than 0.1 mm. Oil-in-water (o/w) and water-in-oil (w/o) are two basic types of emulsion. Emulsion has low stability, but on the other hand if there is an addition of biosurfactants this can help the emulsion to be stable for a long period of time. This trait can be applied like liposan where it is a water-soluble emulsifier which is produced by *Candida lipolytica*. Liposan produce by *C. lipolytica* is utilized with edible oils in which it creates a stable emulsion (Santos *et al*., 2016).

(f) Low Toxicity

Most of chemically synthesized materials pose health problem to people especially chemical surfactants which cause the haemolytic activity towards human erythrocyte, this problem is lower in microbial synthesized biosurfactant compared to cationic and anionic surfactant. Biosurfactants does not give any adverse effect to many

of the human organs like the heart, lung, liver and kidney and it will not interferes with blood coagulation time. Biosurfactants can also hinder the luminescence of up to 50% of *Vibrius fisheri* which is a lot higher when compared to the chemically synthetic surfactants and had affected the fibrolast activity of the mouse that had a concentration of about 500 times higher that linear alkylbenzene (LAS). The biosurfactant's acute and chronic toxicity is a lot smaller when compared to the chemically synthetic Triton X-100 (Karpenko, 2009)

(g) Availability

To produce the biosurfactants it only needs a cheap and readily available in large quantity of raw materials. The essential carbon that is needed to create the backbone of the biosurfactants can be obtained from hydrocarbons, carbohydrates and also lipids (Kosaric, 1992).

2.5 Substrate for Microbial Production of Biosurfactants

(a) Agro-Industrial Waste

Materials like bran, straw of wheat, hull of soy, corn, rice sugar cane molasses, beet molasses, bagasse of sugarcane, cassava flour and the wastewater produced during the production of respective product are a good example of agro-industrial waste. There are some waste components that have high concentration of starch which is considered as a good substrate like rice water, corn steep liquor, cereals and also pulses processed

water. The main trait of agro-industrial waste is that it can have a very high concentration of carbohydrates and also lipids which in this case could be utilized as a source of carbon needed for growth of microbes which then will produce the biosurfactants needed (Ibrahim *et al*, 2014). The reason why agro-industrial wastes are a good candidate to be used as substrates for the production of biosurfactant is that it is a sustainable and comes from renewable and organic materials. By undergoing bioconversion using the type of waste it can be very good for the economical aspect since it is very low in capital and used lower energy cost to handle, it also reduce pollution of the environment and makes the operation of handling this waste relatively easy (Neboh *et al*, 2016)

(b) Industrial Waste from Animal and Plant Origin

Some of the waste like whey from dairy industry, animal fat and molasses can also be used as a substrate. The use of whey is low in cost and reasonable to be used in biosurfactants fermentation. Whey contains proteins, peptides, amino acids, lipids, minerals and other vitamins which is an essential nutrient to be used as substrate that can helps in a good growth of microbes (Saharan *et al*, 2012). Daniel manages to use whey as a substrate in his research in 1998 to produce sophorolipids that is synthesized by *Candida bombicola*. Lactose contain inside the whey is used as the carbon source for the production of sophorolipids (Daniel *et al*, 1998). The fats and tallow from the processed animal can be acquired from industries of meat processing. In 2007, Felse manage to grow *Candida bombicola* which was used to produce sophorlipids by using fatty acid residues from tallows of animal that was acquired from the waste of meat

industries (Felse *et al*, 2007), Other than that, molasses can also be used as the substrates, molasses is produced by the sugar industry when it manufactures the sugar from sugarcane. In 2006, Rodrigues used molasses as one of the low cost substrate for the production of biosurfactants produced from both *Lactococcus lactis* and also *Streptococcus thermophiles*. The result from the use of molasses as the substrates shows a high yield and productivities of the biosurfactant (Rodrigues *et al*, 2006).

(c) Other Industrial Wastes

Other industrial waste like soap stock also can be utilized as one of the substrate for biosurfactant production. Soap stock can be characterized as a gummy and has an amber-liked colour, it is the by product from the production of oilseed, it occurs when hexane and various other chemical are utilize in the process to extract out and refining the edible from oilseeds. Soap stock has been utilized as a substrate to produce emulsan and also bio-dispersan (Saharan *et al*., 2012). Soapstock was used as the substrate by Benincasa research's in 2004. Soap was used in this case to grow *Pseudomonas aeruginosa* that synthesized rhamnolipids. The result gotten from the research shows a satisfactory production of the rhamnolipids (Benincasa *et al*., 2004)

2.6 Commercial Potential of Biosurfactant

(a) Biosurfactants in Oil Industries

Biosurfactants have a number of advantages compared to chemically synthesized surfactant. Some of the example of it uses is in the microbial enhanced oil recovery (MEOR), cleaning vessels that are contaminated, and also in helping the transports of heavy crude oil through the pipeline (Silva et al., 2014). Figure 2.4 shows a depiction on how the mechanism of MEOR works.

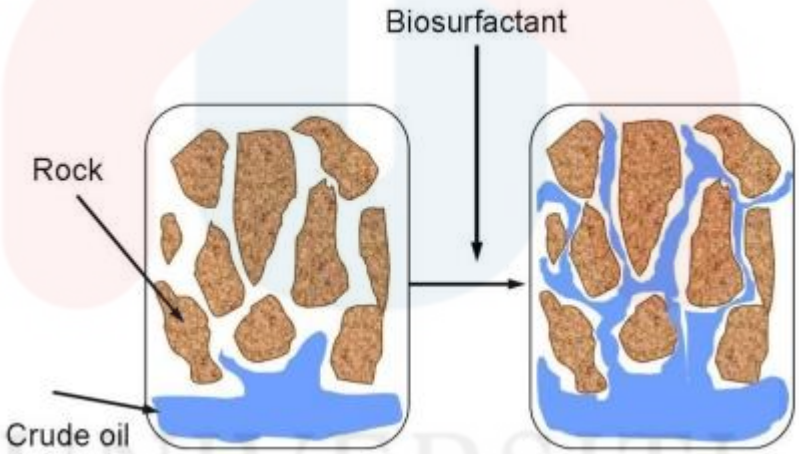


Figure 2.4: Mechanism of enhanced oil recovery by biosurfactants (Pacwa-płociniczak *et al*, 2011)

MEOR works by injecting nutrients and suited anaerobic bacterias which is able to live and thrive under low oxygen or anoxic reservoir condition. The bacteria will produce biosurfactants as one of its metabolic products. It helps to alter the oil properties and its interaction between oil, water and porous media. This will help the oil to be more mobile and moves freely, therefore helps to recovering the oil particularly the ones from depleted and marginal reservoirs. Thus prolong the period of being able to recover oil from the wells (Oku, 2016). Emulsan that were produce by *Acinetobacter*

calcoacetices Rag1 was used to clean contaminated vessels which was studied by Chamanrokh, the result have shown that the emulsan produced from the bacteria is a good example to remove any remaining oils from oil tankers or vessels (Chamanrokh *et al*, 2008)

(b) Biosurfactants in Cosmetic Industries

The most important trait of biosurfactants in cosmetic industries and formulation is the emulsifier functions. This is due to the fact the emulsions have certain capabilities compared to other biosurfactant. Hence, by using emulsifiers it makes them can be applied with ease and not very costly .At the same time it lets the use of liposoluble and also hydrosoluble substances (Vecinoe *et al*., 2017). One of the examples of the use of biosurfactant in cosmetic is shown by Fontan in 2016 where he used the biosurfactant that was extracted from corn steep liquor on the human hair. His results have shown that human hair can absorb the biosurfactant rapidly. This means that biosurfactant can be used in shampoo for hair care and other hair related products (Rincón-Fontán *et al*., 2016)

(c) Biosurfactants in Pharmaceutical Industries

Biosurfactants also have a variety of applications in pharmaceutical sectors. One of the applications is gene delivery. By using cationic liposomes it allows for gene transfection and also lipofection, these methods have high probability in delivering foreign gene to targeted cells without having any unfavourable side effects. These

techniques are applied in the *in vitro* synthesis of pulmonary surfactants for the treatment of premature babies (Sandeep, 2017). Biosurfactants also can be used as immunological adjuvants by using lipopeptides produced by both *Lactococcus lactis* 53 and *Streptococcus thermophilus* which comprise of effective non-toxic and also non-pyrogenic immunological adjuvants when it is combined with traditional antigens. It also functions to stimulate the stem fibroblast metabolism and also immunomodulatory action (Rodrigues *et al*, 2006). Biosurfactant can also be used to extract any intracellular products by permeabilising the cells after fermentation process to recover intracellular products. It does this by aiding the reverse micelle solution which is aimed at the selective permeabilization of *Escherichia coli* to extract penincillin acylase (P. Singh & Cameotra, 2004). The diversity of structure that the biosurfactants have makes them having an antimicrobial property such as it become toxic to cell membrane permeability that closely resembles the effect of detergent (Techaoei *et al*., 2007)

(d) Biosurfactant in Food Industries

Biosurfactants can also be utilized in the food industries as food emulsifiers, antioxidant agents, anti-adhesives and as biofilm removal. In food emulsifiers, amphiphiles that are low in molecular weight plays a crucial role in the stability of liquid emulsions like beverages, dressings, sauces and alcoholic emulsions (Campos et al., 2013). A research done on bioemulsifier produced by *Candida utilis* has been observed to be used as salad dressings and the utilization of rhamnolipids has also been observed to enhance the characteristic of croissants, butter and other frozen pastries. The mannorprotein synthesized by *Saccharomyces cerevisiae* has been studied and it

shows that it can be utilized to stabilize water-oil and producing emulsions which is applicable for the production of mayonnaise, cookies and also ice creams. There are also the yeast from *Candida yalida, Candida utilis, Hansenula anomala, Rrhodotorula graminis, Rhodospiridium dioboyatum* and red alga belonging to *Porphiridium cruentum* and bacteria which belongs to *Klebsiella* sp. and also *Acinetobacter calcoaceticus* which has been studied as producers of extracellular bioemusifiers with better stabilizing activating compared to gum Arabic and also carboxymethyl cellulose (Barros *et al*, 2007). As for antioxidants, mannosylerythritol lipids (MELs) have been reported to have a great antioxidant properties and can be used as anti-aging skin ingredient due to its adaptable interfacial and biochemical characteristic (Takahashi *et al*, 2012)

2.7 Biosurfactant Producing Microorganism

Both prokaryote and eukaryote have the abilities to secrete and produce biosurfactants. In a few of the cases, bacteria only produce the biosurfactant solely at the time of their growth period on hydrophobic substrates such as *Rhodococcus* and *Corynebacterium*. There are also other groups that is able to produce biosurfactants on both water soluble and also hydrophobic substrate like *Pseudomonas aeruginosa* and *T. bombicola* (Gautam & Tyagi, 2006). Two of the biggest and known producer of biosurfactants is from the genera *Pseudomonas* and *Bacillus*. Type of fermenter used to produce the biosurfactants, pH, nutrients, substrates supplied and temperature can influence the composition and yield of the microbial surfactants (Sarubbo, 2014).

2.8 Environmental Applications of Biosurfactants

The most common use of biosurfactant in environmental applications is the bioremediation which can be done in two ways. The first method is done by increasing the bioavailability of the substrate for the microorganisms while the second method involve interaction between the cell surface which elevates the hydrophobicity of the surface which allows hydrophobic substrates to associate with ease with bacterial cells (Mulligan & Gibbs, 2004). In one studies where the biosurfactant's biodegradative properties of *Pseudomonas* sp. LP1 strain tested on crude oil and diesel oil showed that it could metabolize hydrocarbon components of crude oil and diesel oil of about 92.34% and 95.29% respectively (Obayori *et al*., 2009). Soil washing technology also utilized biosurfactant in cleaning up oil in contaminated area. Soil washing technology can defined as chemico-physical traits of biosurfactant rather than their effect on metabolic activities or changes in cell-surface trait of the bacteria itself (Ibrahim *et al.*, 2010)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

(a) Bacteria Strain

Three bacterial isolates which are isolate DSA 4, DSA 7 and DWA 9 were used in this study. All three of the bacterial strains were isolated from the Sungai Dungun water and soil sample. The bacterial isolates were then maintained on mineral salt medium (MSM) agar that was supplemented with 1% (v/v) cooking oil. These bacterial isolates were incubated at 30˚C for 24 hours. The density of the bacterial isolates were checked and the ideal OD was between 0.8 abs – 1.2 abs

(b) Apparatus and Equipment

The equipment that were used are centrifuge, electrophoresis system, thermal cycler, oven, and vortex mixer.

(c) Chemical and Reagent

Chemicals that were used in this study were Gram staining kit (crystal violet, gram iodine, acetone, safrananin), trypticase soy broth (TSB), Glucose-Tris-HCl-EDTA (GTE), sodium dodecyl sulphate (SDS), Tris-HCl-EDTA (TE), phenol, chloroform, isoamyl alcohol, sodium acetate, isopropanol, loading dye, agarose agar, PCR reaction mixture, mineral salt medium (MSM), blood agar, vegetable oil, phenanthrene, sodium chloride (NaCl).

3.2 Methods

(a) Physical characterization of bacterial isolates

(i) Colony Morphology

The colony's colour, margin, size, elevation, texture and shape were observed on the culture plates. Any apparent physical characteristics were noted down. The size of colony can range from large colonies to tiny colonies smaller than 1 mm. Margin means the magnified edge shape. Next, shape of colony can vary from round to irregular to filamentous and also root-like shape. Furthermore there is also colour or chromogenesis of the colonies where there is pigmentation of the colony such as white, buff, red, purple and other colours. Elevation of the colony means, how high does colony rise above the agar. The texture can be observed by how butyrous, viscid, brittle and mucoid the colony can be.

(ii) Gram Staining

These parts of test involved the usage of 4 different chemicals which are crystal violet, iodine, acetone and also safranin. Crystal functioned as the primary stain, iodine as a mordant to fixed the stain, acetone as a decolouriser and also safranin as the counterstain. To properly fix the diluted bacterial suspension onto the already dried and clean glass slide, a loopful of suspension was heat fixed quickly onto the surface of the glass slide through the use of Bunsen burner and was air dried after smearing the suspension onto the slide. The first chemical that was used is the crystal violets where it was poured onto the smeared glass slide and kept it for 1 minute. The glass slide then was rinsed using distilled water. The glass slide then was flooded with gram's iodine and was dried out for 1 minute, after that washed with distilled water. A few drops of acetone were dropped onto the glass slide and kept it only for 30 seconds which then was washed with distilled water. The last chemicals that was used in this step is the safranin where a few drops of it were dropped onto the smear enough to cover the whole smear for 1 minute and was washed with distilled water afterwards. The stained glass slide was observed under a microscope.

(b) Molecular characterization of bacteria isolates

(i) DNA Extraction

A single colony of the isolates was inoculated into 10 ml of trypticase soy broth (TSB). It then was incubated overnight at 37˚C and 150 rpm. After overnight incubation, the culture was centrifuged at 2000 rpm for 15 minutes, 4˚C and the pellet that formed was taken. The pellet was washed using 1 ml of Glucose-Tris-HCl-EDTA (GTE) buffer and undergoes centrifugation at the speed of 10,000 rpm for 5 minutes. In order to resuspend the pellet, 200 µl of GTE buffer was added and the mixture was kept on ice for at least 10 minutes. Before incubating the mixture at 50˚C for about 30 minutes, 50 µl of 25% sodium dodecyl sulphate (SDS) was added into the mixture. After incubation, 200 µl Tris-HCl-EDTA (TE) buffer, 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) were added and the mixture will also be inverted a few times. The mixture again was centrifuged at 10,000 rpm for 15 minutes. The newly formed upper part of the mixture was transferred into a new tube. In order to precipitate the DNA that formed after the centrifugation process, 400 µl sodium acetate and 800 µl of isopropanol were added. After that, the mixture was centrifuged at 10,000 rpm for 5 minutes. The resulting pellet was air dried and dissolved using 50 µl of distilled water. The DNA was kept at - 20˚C.

(ii) Agarose Gel Electrophoresis

1 µl of 6 × loading dye was loaded onto the parafilm. 5 µl of DNA was added and mixed together by pipetting up and down. After the loading dye and DNA were mixed thoroughly, the solution was transferred into the agarose gel well. After all of the gel well was filled without leaking out the solution outside the gel well, then a marker was used to fill the final lane. After closing the gel tank properly, the power source was switched on and the gel was run for 45 minutes at 80 V. After electrophoresis has finished the resulting gel was viewed under a UV transilluminator

(iii) Amplification of 16S rRNA Gene Using Polymerase Chain Reaction (PCR)

50 µl of PCR reaction mixture was prepared by mixing 32.5 µl double distilled water, 5 µl 16S forward primer (2.5 µM), 5 µl 16S reverse primer (2.5 µM), 1 µl DNA template (2.5 µM), 0.5 µl *Taq* polymerase (2.5 µM), 1 µl dNTPs (2.5 µM) and also 5 µl 10 × PCR buffer . The PCR conditions were followed by using the conditions mentioned in Table 3.2. The DNA sequences of the 16S forward and reverse primers are as stated in Table 3.1 below

Table 3.1: DNA sequences of 16S forward and reverse primers

Forward primer (68F)	5'TNA NAC ATG CAA GTC GAR 3'
Reverse primer (1324R)	5' ACG GGC GGT GTG TRC 3'

Table 3.2: PCR conditions for 16S rDNA gene amplifications

Step	Temperature (˚C)	Time of reaction	Cycles
Pre-denaturation	94	4 minutes	1
Denaturation	94	1 minute	30
Annealing	53.8	1 minute	30
Extension	72	1 minutes	30
Final Extension	72	7 minutes	1

(iv) DNA purification

The PCR product were purified using MEGAquick-spin™ Total Fragment DNA Purification Kit and using the protocol as described by the manufacturer

(v) Gene Sequencing

PCR products were sent to First BASE Laboratories Sdn. Bhd. for sequencing reaction. The result was analysed using BLAST analysis at https://blast.ncbi.nlm.nih.gov/Blast.cgi website

(c) Biosurfactant Production Characterization

(i) Inoculum preparation

20 mL MSM broth supplemented with cooking oil was prepared inside a conical flask. Inside a laminar flow, a single loop of bacteria culture was inoculated into the MSM broth. The culture incubated for 24 hours at 30˚C, 150 rpm

(ii) Drop Collapse Assay

This assay was done by carefully putting a single drop of the overnight culture supernatant onto the glass slide that was covered with oil. The change was observed after one minute. When there is changes on the glass slide which is when there is a collapsed of the supernatant and spreading all over on the oil coated surface, this means that there is a presence of biosurfactant. However, if there are no changes or the structure of the still remains after a minute, it means that there is no biosurfactants signifying a negative result. A similar test was also conducted using parafilm. However, it was not coated with oil since the surface is already hydrophobic.

(iii) Blood Haemolytic Test

Bacterial isolates were streaked onto blood agar. The plates were incubated 37˚C for 48 hours. The results of these tests were classified into three observations which are

α, β and γ haemolysis. If there is any haemolytic activity on the plate it means that it is correlating with biosurfactants production. α means a greenish halo is formed that signifies an incomplete haemolysis, β means a clear zone on the plate signifying a complete haemolysis and lastly γ means there is no changes observed on the blood agar signifying no haemolysis occurs.

(iv) Emulsification Test

Emulsification test was conducted to determine the emulsification index (E24%). This test was carried out by adding 2 ml kerosene into 15 ml Falcon tube containing the same amount of overnight bacterial culture. The mixture was vortex for about 2 minutes. Then the tubes were kept in room temperature for 24 hours. The height of the emulsion formed was measured before and after 24 hours to determine the emulsion's stability. To attain a great accuracy for the measurement, this test was repeated 3 times. To be considered as a stable emulsion the E24 must be equal or more than 50%. The formula of emulsification index (E24%) was calculated according to the formula mention below:

$$E_{24} = \frac{Height\ of\ emulsion\ layer}{Height\ of\ total\ solution} \times 100 \qquad (Equation\ 3.1)$$

(v) Oil Spreading Test

10 µl of engine oil was dropped on 40 ml of water inside a petri dish. After that 10 µl of culture supernatant was dropped onto the centre of oil layer. When the presence of biosurfactant is detected there will be a formation of clear zone on the oily layer because of the oil displacement. As for negative control, culture supernatant was replaced with distilled water.

(vi) Hydrocarbon Degrading Capability Test

This test was conducted to determine the capability of the biosurfactants to degrade hydrocarbon. Bacterial isolates were inoculated into MSM broths containing 0.25% (w/v) phenanthrene. To measure the degradation of the hydrocarbon, samples from the culture broths were taken right after 7 days. The samples were centrifuged at the speed of 10,000 rpm for about 20 minutes at the temperature of 4˚C. The resulting pellets are washed with 0.9% (w/v) of NaCl solution. After that, the pellets were dried inside an oven at the temperature of 60˚C to 70˚C to determine the dry weight. Drying of the pellets was continued until there is a constant dry cell weight. The degradation of phenanthrene (%) was calculated according to the formula mention:

$$Phenanthrene\ degradation\ (\%) = \frac{final\ weight\ of\ phenanthrene\ (g)}{Initial\ weight\ of\ phenantherene\ (g)} \times 100 \quad \text{(Equation 3.2)}$$

CHAPTER 4

RESULTS & DISCUSSION

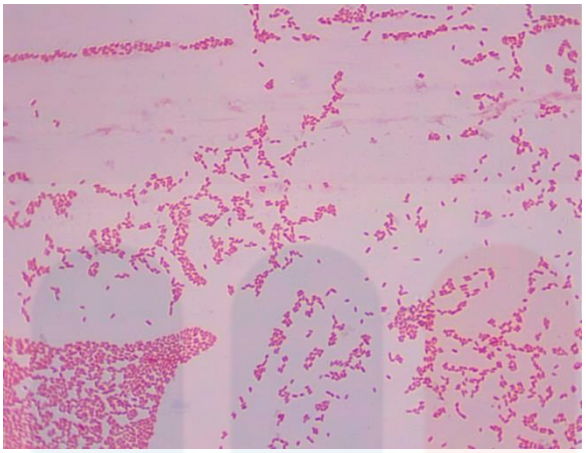
4.1 Identification and classification of bacterial isolates

(a) Gram staining and cellular morphology

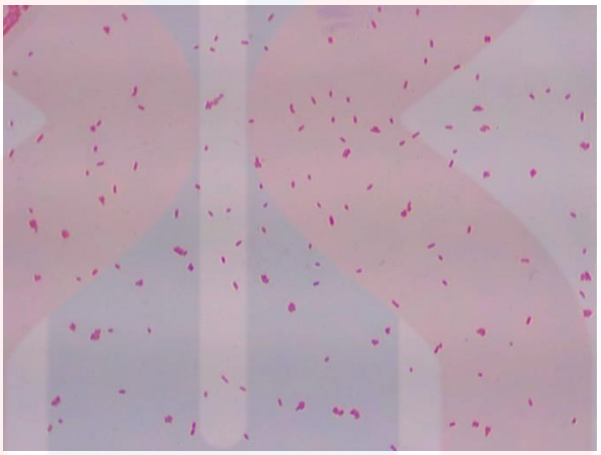
To determine the Gram group of the 3 bacterial strains that were isolated, gram staining was carried out. After staining the isolates were observed using light microscope under 100 × magnification using oil immersions. The shape and colour of the individual cells were observed and determined and can be seen in Figure 4.1. The results are tabulated in Table 4.1.

Table 4.1: Gram staining results for bacterial and cellular morphology of bacterial isolates from Sungai Dungun

Isolates	Microscopic Observation	
	Gram Positive/ Negative	Shape
DSA 4	Negative	Short rod
DSA 7	Negative	Short rod
DWA 9	Negative	Short rod



a) DSA 4



c) DSA 7

d) DWA 9

Figure 4.1: Microscopic observation of bacterial isolates under 100 × magnification. a) DSA4 b) DSA7 c) DWA9

All of the isolates (DSA 4, DSA7, and DWA 9) have shown a red coloured cells which proved that it is a Gram-negative bacteria. The reason why Gram-negative bacteria do not retain the crystal violet is due to the fact that they have thin peptidoglycan layer and also an overlying lipid-protein bilayer also referred to as the outer membrane, in which it can be disturbed by the process of decolourization. Compared to their Gram-positive counterparts who have thicker and approximately impermeable wall that can withstand the effect of decolourization and is made up of peptidoglycan and also secondary polymers (Beveridge & Beveridge, 2009). All of the three isolates is short rod in shape, which is a common among gram-negative bacteria to show rod shape. Since microorganisms that are capable of producing surface active molecules are ubiquitous (present in both land and water) this means production of biosurfactant from the bacterial isolates in this study is feasible. A variety of biosurfactant producing bacteria have been successfully isolated and characterized directly from marine sites that are polluted with hydrocarbon based products (Satpute *et al*, 2010).

Most of biosurfactants produced and utilize in hydrocarbon degradation, mainly rhamnolipids are produced by gram-negative bacteria such as *P. aeruginosa* (Silva, *et al*, 2010). Other than *Psedomonas* sp., there other marine bacteria reported by Maneerat and fellow researchers (2005) namely *Myroides* sp. which is an aerobic, gram-negative, non-motile, rod-shaped bacterium and usually yellow to orange pigmented which was isolated from Songkhla Lake in Thailand. This strain closely resembles the characteristic of DSA 4 isolate. There is also *Antartobacter* sp. which is gram-negative and rod-shaped generally found in marine ecosystem. *Antartobacter* sp. is able to produce emulsifying agent called AE22 (Gutiérrez *et al*, 2007).

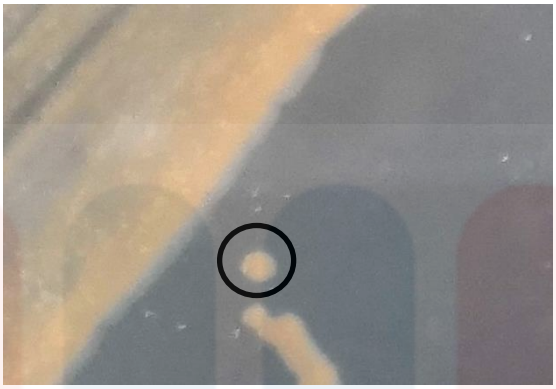
Other strain like *Marinobacter* sp. which is gram-negative and rod-shaped and in one research it shows that the capability to degrade various liquid and solid hydrocarbons (A-mallah & Goutx, 1990).

(b) Colonial morphological observation

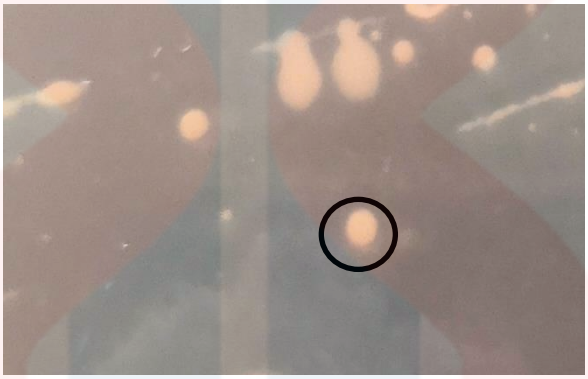
The 3 strains were streaked onto nutrient agar to determine their colony morphology. Colonies grown on agar plates were observed intricately to determine their pigmentation, opacity, margin, elevation, shape, and also their size. The colonies can be seen if Figure 4.2. The colonial morphology of bacterial isolates is shown in Table 4.2

Table 4.2: Colony morphology of bacterial isolates

Isolates	**Morphology**					
	Pigmentation	Opacity	Margin	Elevation	Shape	Size
DSA 4	Pigmented (Orange)	Opaque	Entire	Convex	Circular	Small
DSA 7	Non-pigmented (White)	Opaque	Entire	Pulvinate	Circular	Small
DWA 9	Non-pigmented (Cream)	Opaque	Entire	Convex	Circular	Medium



a) Colony morphology of DSA 4



b) Colony morphology of DWA 9

c) Colony morphology of DSA 7

Figure 4.2: Colony of bacterial isolates grown on nutrient agar. a) DSA 4, b) DWA 9, c) DSA 7

4.2 Production of biosurfactant by bacterial isolates

(a) Emulsification Assay

One of the reliable methods to do quantitative analysis for biosurfactant production is emulsification index (% EI24) (Cooper & Goldenberg, 1987). To determine the emulsification activity of bacterial isolates, 2 equal parts of kerosene and bacterial culture were added inside a test tube and vortexed for 3 minutes. The tubes were left for 24 hours. After 24 hours of incubation, the observation was noted and shown in Figure 4.3 below.

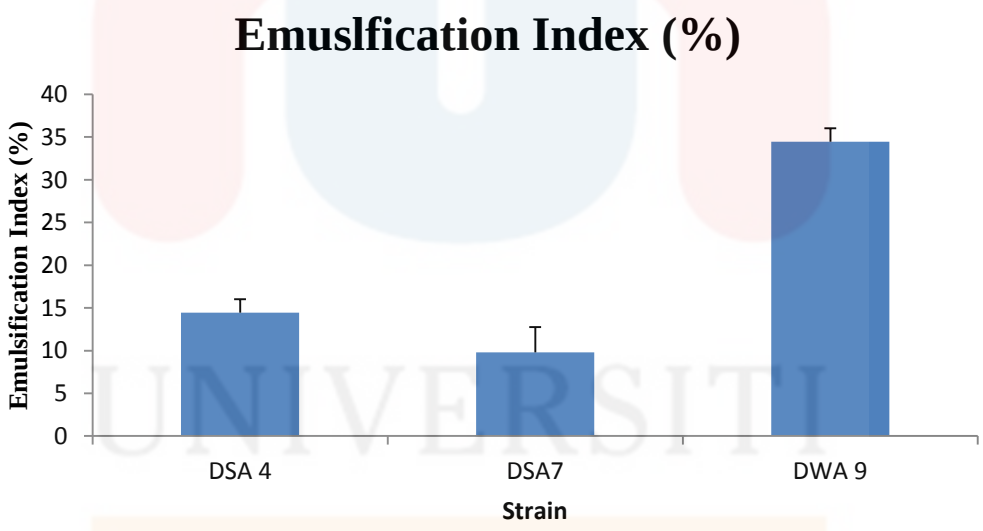
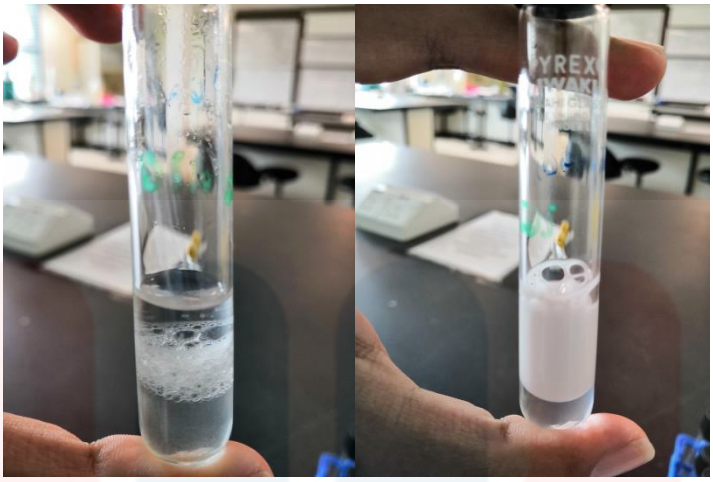


Figure 4.3: Emulsification index (E24) of bacterial isolates

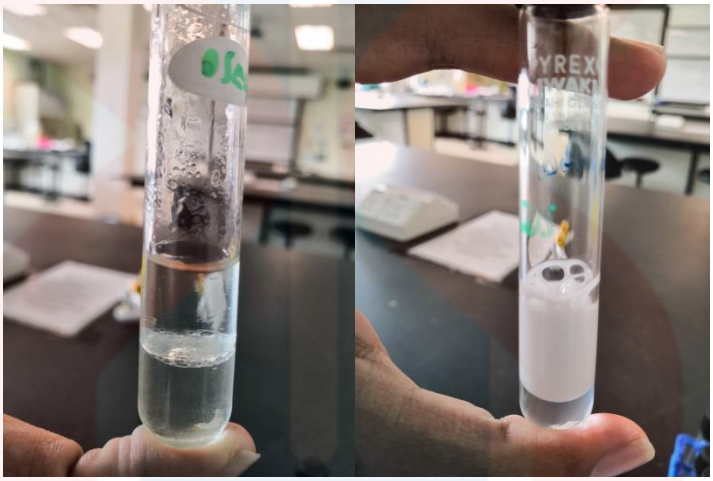
In a study done by Thavasi and fellow researchers (2011), they reported that emulsification assay is not a direct method to screen the production of biosurfactant. In this assay it was believed that the cell free culture broth could contain biosurfactant that will then emulsify the hydrocarbons that is present in the test tube. So taking the statement above and correlating with the result gotten from the assay it shows that DWA 9 strain have the highest emulsification activity with an average of 34.44 % and DSA 4 is the 2nd highest with an average of 14.44 %., DSA 7 has the lowest emulsification activity with only 9.80 % of their average.

In a research done by Janek and fellow researchers (2010), they found out that the biosurfactant produced by *Pseudomonas fluorescens* BD5 called pseudofactin I and pseudofactin II and used glucose as carbon source have a great emulsification activity on aromatic and aliphatic hydrocarbons and some other plant oils. Their emulsifying activity is comparable to synthetic surfactants like Tween 20 and Triton X-100. In one of the oil that they use which is olive oil, the emulsification activity reached 90% and their lowest value is below 60%.

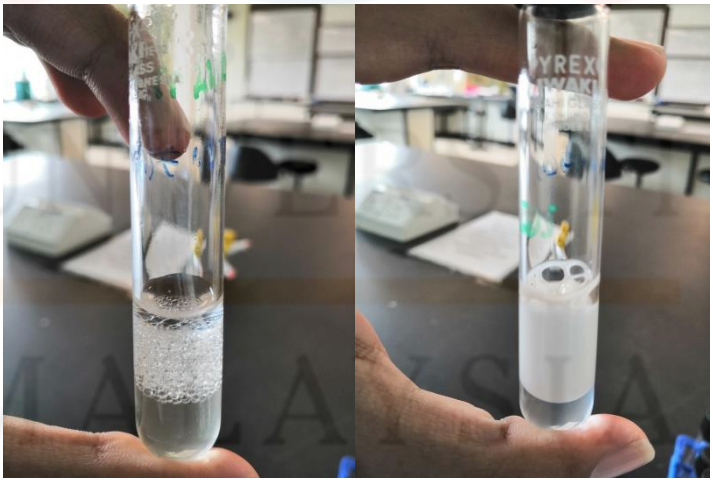
In other research, biosurfactant produce by *Bacillus subtilis* isolates produces a crude biosurfactant which have been observed to have great emulsification properties when sucrose was utilized as a carbon source. Different variation of surfactin in the structure of biosurfactant shows a change in the emulsification activity whereby the surfactin indirectly increases the emulsification activity (Pereira *et al*., 2013)**.** The emulsification activity can be seen in Figure 4.4.



a)



b)



c)

Figure 4.4: Emulsification of bacterial isolates (left) compared to SDS (right) as control.

a) DSA4, b) DSA 7, c) DWA9

(b) Hydrocarbon degrading capability test

To test the strains capability in degrading hydrocarbon, the strain were inoculated into MSM broth supplemented with 0.25% phenanthrene and incubated at room temperature for 7 days. After 7 days of incubation, the final weight of phenanthrene was measured. The percentage of degradation of phenanthrene was calculated using the formula below. The result from the hydrocarbon degradation assay is shown in Figure 4.5.

$$Phenanthrene\ degradation\ (\%) = \frac{final\ weight\ of\ phenanthrene\ (g)}{Initial\ weight\ of\ phenantherene\ (g)} \times 100 \qquad \text{(Equation 4.1)}$$

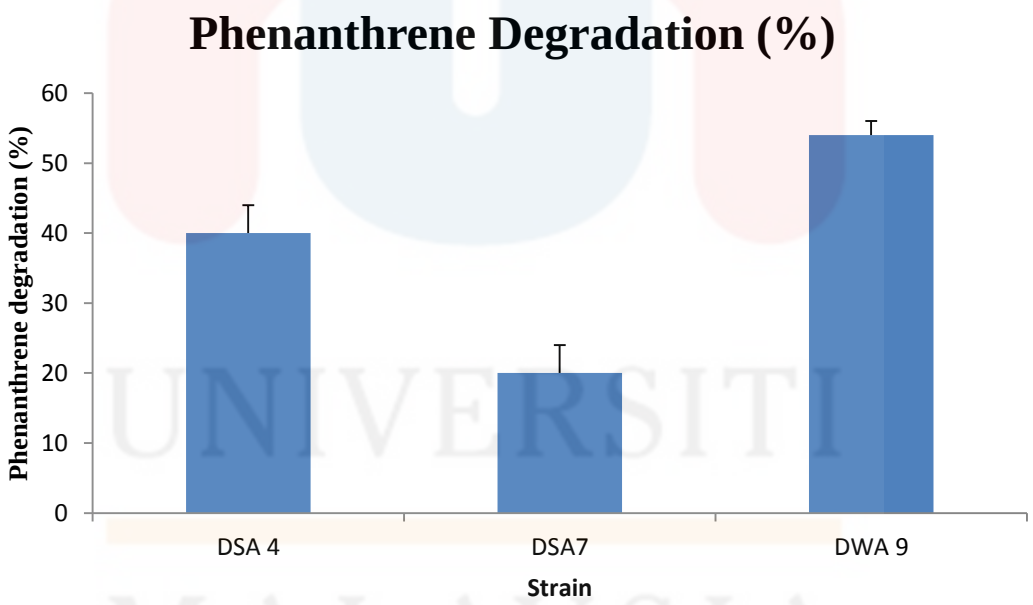


Figure 4.5: Percentage of phenanthrene degradation (%) by bacterial isolates

This test was done through a simple determination in the weight loss of the phenanthrene. The initial weight of the phenanthrene which is 0.25 g will then be compared with the final weight which is after 7 days. From the result shown in Figure 4.6, DWA 9 clearly has the highest phenanthrene degradation of about 54 % in average. DSA 4 is the second highest with 40 % in average. DSA 7 shows the weakest degradation activity with only 20 % in average

Phenantherene is a form of polycyclic aromatic hydrocarbons (PAHs) where researchers have been studying the effect of biosurfactant on PAHs such as phenantherene. One of the way PAHs can enter the environment and contaminated it is through the emissions from combustion process (Akkar & Ockne, 2003)

The interaction and mechanism of phenantherene degradation by biosurfactant have been studied a lot by researchers. One such case is by done by Pan and fellow researchers (2010) who stated that when there is an interaction between phenanthrene and biosurfactant, the reaction between the compound was spontaneous and exothermic increasing the temperature. There is also research done on the degradation phenanthrene through the use of fungi which is *Polyporus* sp. where by the maximum degradation rate was 92% where it was incubated for 30 days at agitated at 120 rpm. The mechanism on how *Polyporus* sp. manage to degrade phenanthrene is where the fungi used phenanthrene as carbon and energy source more specifically through the ring oxidation and ring cleavage processes (Hadibarata & Tachibana, 2010).

(c) Drop collapse assay

This method was introduced by Jain & Lee (1991), to test the stability of the biosurfactant. In this test, two methods were used which is by using parafilm and also glass slide coated with cooking oil. In this assay, MSM broth and SDS were used as negative control and positive control respectively. The result of this test is shown in Table 4.3.

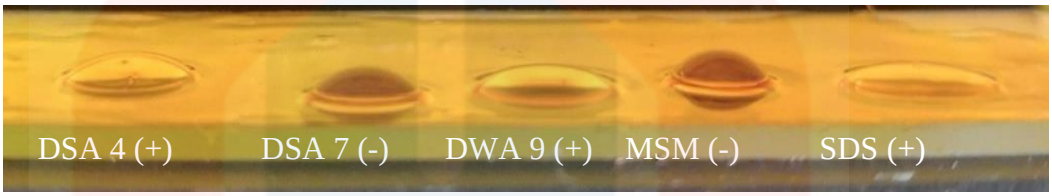
Table 4.3: The result for drop collapse test for bacterial isolates

Strain	Glass slide	Parafilm
SDS (Control)	+	+
MSM (Control)	-	-
DSA 4	+	+
DSA 7	-	-
DSA 9	+	+

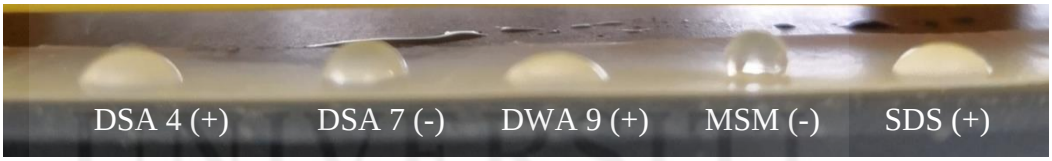
From the result shown in Table 4.5, both of DSA 4 and DSA 7 are considered positive since the structure collapsed proving that they are positive for biosurfactant. According to a research done by Mounira & Abdelhadi (2015), it was concluded that there will be a drop collapse activity for strains which produce extracellular biosurfactants and the strains that do not produce will gives a negative result

This test requires the action of the destabilization of liquid droplets by the biosurfactants. Hence a few drops of the strain supernatant from different culture were place on either the oil coated glass slide or parafilm. In theory, if the supernatant does not contain or contain very low amount of biosurfactants, the water molecules that is

polar in nature were repelled from the hydrophobic surface and thus it structure of the drop will remain stable and result will be negative as shown by isolate DSA 7. But in the presence of biosurfactants, the interfacial tension between the hydrophobic surface and the supernatant drop was weaken causing the drop structure to collapse and spread out as shown by isolate DSA 4 and DWA 9 (Chakrabarti, 2012). Due to hydrophobic part of the parafilm, it can cause droplet from the supernatant to collapse in the presence of biosurfactant, this is the reason why parafilm was also used in this study (Korayem *et al*., 2015). Figure 4.6 below shoes the result of drop collapse assay on glass slide and parafilm.



a) Glass slide



b) Parafilm

Figure 4.6: Drop collapse assay of bacterial isolates on different surface materials. a) Glass slide, b) parafilm

(d) Oil spreading assay

The main purpose of this test is to determine the efficiency of the biosurfactant produced by the bacterial isolate in displacing oil layer as shown in Figure 4.7. In this test, distilled water was added into a petri dish and few drops of engine oil was also added, then 10 µL of the supernatant of bacterial culture from each strain was added. The results was tabulated into Table 4.4.

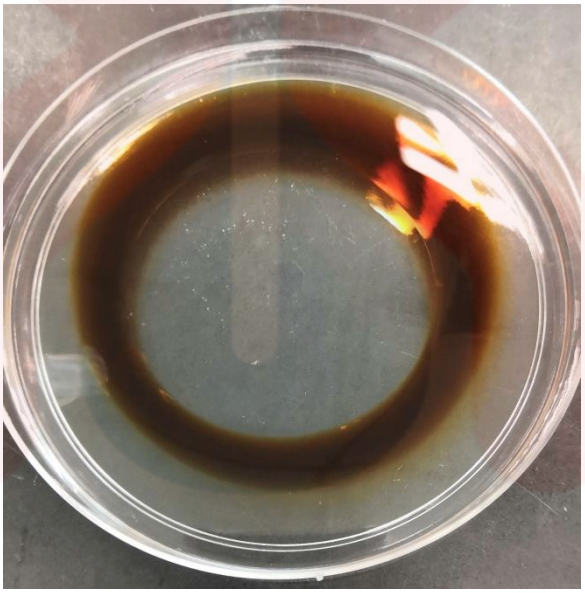


Figure 4.7: Displacement of engine oil by bacterial isolates

Table 4.4: Oil spreading assay of bacterial isolates using engine oil

Isolates	Test	Diameter of clear zone (cm)	Average diameter of clear zone (cm)
DSA 4	1	4.00	4.00
	2	4.00	
	3	4.00	
DSA 7	1	2.50	2.83
	2	3.00	
	3	3.00	
DWA 9	1	4.50	4.67
	2	4.50	
	3	5.00	
SDS (Control)	1	5.00	5.00
	2	5.00	
	3	5.00	

All of the strains show positive result with different diameters of oil displacement. From the result in Table 4.6 it shows that DWA 9 has the greatest displacement of oil in terms of the diameter with an average of 4.6 cm and it is very close to the result of the control which SDS (5.0 cm). While both DSA 4 and DSA 7 has an average of 4.00 cm and 2.83 cm respectively. It was stated that the area of oil that has been displaced by the effect of the supernatant is directly proportional with biosurfactant concentration in the culture medium (Mounira & Abdelhadi, 2015). So based on this statement, it is clearly visible that DWA 9 has the highest concentration of biosurfactant from the 3 strains, DSA 4 the 2nd highest and DSA 7 the lowest.

(e) Blood haemolysis test

In this test, blood agar was utilized to run this test. Bacterial isolates were streaked onto blood agar and incubated overnight at 30˚C. After 24 hours, the blood agar plates were visually checked to see for sign of clear zone around the streaked area. If there were clear zones around the streaked area, it will be evidential that there is a presence of biosurfactant. Using the same method as oil spreading assay, this test will also correlates the diameter of the clear zone with concentrations of the biosurfactant. Meaning that the bigger the diameter of the clear zone, the higher the concentrations of biosurfactant (Zhang, Xu, *et al*, 2012). The result for haemolytic activity on blood agar can be seen in Figure 4.8 and tabulated into Table 4.5.

Table 4.5: Haemolytic activity of isolates from Sungai Dungun samples

Isolates	Haemolysis types
DSA 4	Γ
DSA 7	β
DWA 9	β

a)

b)

c)

Figure 4.8: Haemolytic activity of isolates. a) DSA 4, b) DSA 7, c) DWA 9

From Figure 4.9, it was observed that clear zone was formed around the streaked area of both isolates DSA 7 and DWA 9. The presence of clear zone around the streaked area represents β-haemolysis meaning that the blood agar is fully degraded. Due to the lack of clear zone or greenish discoloration around the streaked area of isolate DSA 4, this represent γ-haemolysis which means that there is no degradation of blood agar.

This part of test was done since it is used widely to characterize biosurfactant and there are also some cases where it is the only method used to screen biosurfactant (Banat, 1993). Some researcher also suggested that this test is an important step in the initial screening of biosurfactant screening (Mulligan & Cooper, 1984). There is also some correlation between the haemolytic activity and also the biosurfactant production, and blood agar is recommended to be utilized as one of the main method to properly determine the presence of biosurfactant (Carrillo, *et al*, 1996).

But, there have been also a few cases where this test does not include potential biosurfactants producer. Youssef and fellow researchers (2004) stated that some of biosurfactant producer can have haemolytic activity and that other compound can also cause the haemolysis. In this test, DSA 4 is considered negative since it does not able to lyse blood agar, but in other test such as oil spreading test, drop collapse test, emulsification test and also the hydrocarbon degradation test it shows positive results. While for DSA 7 which is positive in this test shows mostly shows negative or low result. This proves that blood haemolysis test does not precisely screen biosurfactants producers and is only appropriate to be used as an initial screening method in some cases.

4.3 Molecular characterization of bacteria isolates

The three isolates undergoes molecular characterization through the amplifications and analysis of 16S rDNA gene

Identification of bacterial isolates that is rapid and accurate is an essential task in clinical microbiology and gives and understandings into aetiologies of infectious disease and suitable antibiotic treatment. While normal phenotypic methods are somewhat cheap and allow most of the commonly encountered bacteria to be identified, some bacteria groups are hard to identify, and require special equipment and experts. Additionally, phenotypic approaches depend on the availability of a culture that is pure and relying on subsequent growth characteristics and biochemical profiling. Hence, substantial amount of time is needed for bacteria that grow slowly to be identified. Through the sequencing of 16S rDNA it represents a universal tool that in theory gives solutions to these issues, providing a clear-cut data, even for slow growing and unusual isolates, most of the time within 48 hours, which are reproducible in most laboratories (Woo, *et al*., 2008)

Different from the phenotypic identification in which it can be altered by the presence or absence of non-housekeeping genes or by variability in characters expression, 16S rDNA sequencing give an accurate identification of isolates with unusual phenotypic characteristics. Through this approaches it has given way to identify thermo-tolerant *Campylobacter fetus* strains which is significant discovery of bacteraemia in immunocompromised patients (Woo, *et al*., 2002). Researchers have pointed out the effectiveness of 16S rDNA gene as molecular marker and also a universal indicator to classify unrecognized microorganism, the 16S rDNA are used in

this study. Hence 16S rDNA can give an insight on the evolutionary relatedness among microorganism as suggested by Carl Woese (Singh, 2016)

(a) DNA extraction of bacterial isolates

The bacterial isolates have been shown to be viable samples after undergoing characterization of biosurfactant test. These bacterial isolates were selected for DNA extraction to obtained pure DNA.

The bacterial isolates underwent DNA extraction through the standard phenol-chloroform and alcohol precipitation method. Phenol-chloroform extraction is a type of liquid-liquid extraction whereby is split mixture of molecules based on various solubility of particular molecules in two different immiscible liquid (Zumbo, 1979)

Segregation of biomolecules into a system with two phases relies upon primarily on the physical-chemical properties of the protein itself. Properties like isoelectric, surface hydrophobicity and molar mass. There are also medium properties for instance polymer or surfactant type and concentration, pH, type and concentration of salt. Through the regulation of these factors, partition can be selected to recover the target protein or DNA from culture medium (Mazzola *et al.*, 2008)

As shown in Figure 4.9, there is clearly a visible band on three of the lanes (L2, L3, and L4). This means that the extraction of DNA has been successful. But what make the bands difference is their intensity. As can be seen in L2 (DSA4) and L5 (DWA4), both of these lanes shows the highest intensity by their bright band signifying a high amount of DNA samples, but when compared with L4 (DSA3) the band is not

that bright signifying a lower yield of DNA. The three bacterial isolates have a similar DNA size which can be seen in Figure 4.9.

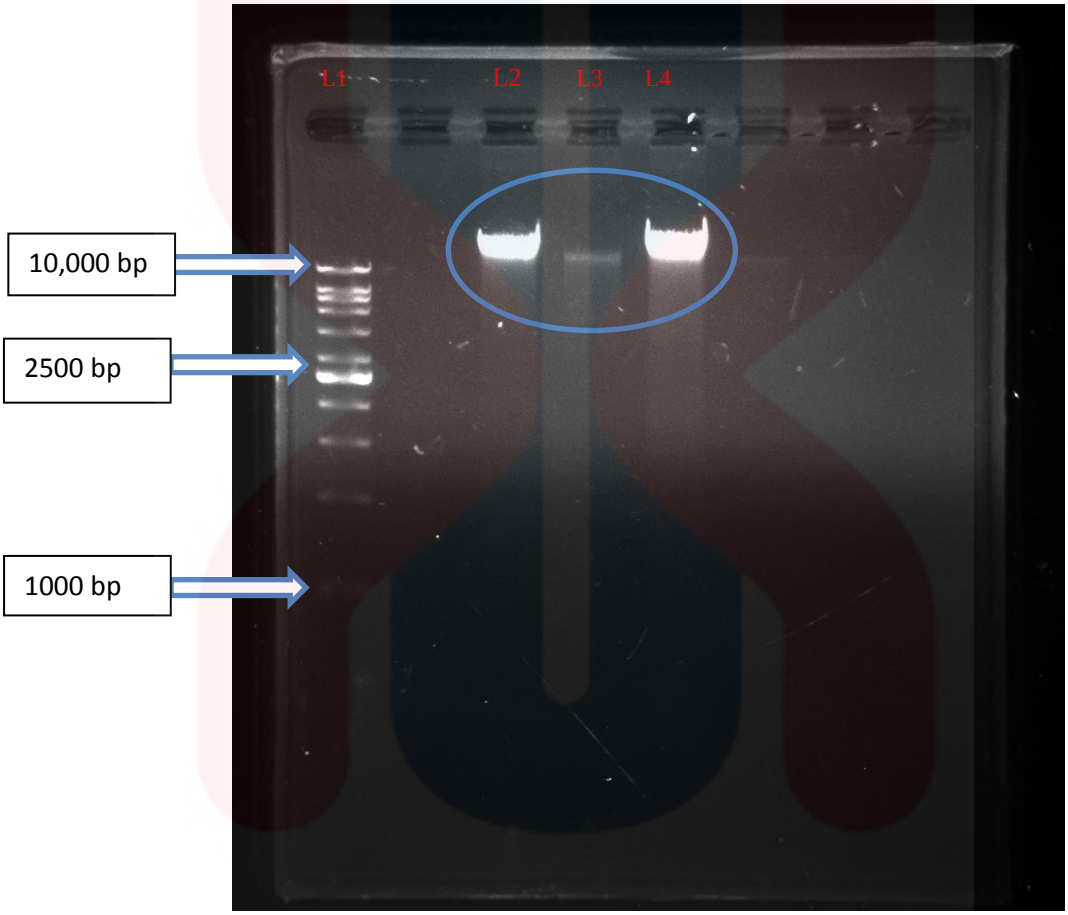


Figure 4.9: Extracted Bacterial DNA after electrophoresis on 1% agarose gel. L1: . L1: VC 1kb DNA ladder (Vivantis), L2: gDNA extracted from isolate DSA 4 .L3: gDNA extracted from isolate DSA 7, L4: gDNA extracted from isolate DWA 9

(b) Amplification of 16S rRNA Gene Using Polymerase Chain Reaction (PCR)

After DNA was successfully extracted from the 3 bacterial isolates and confirmed through agarose gel electhrophoresis, the DNA was used as a template to amplify 16S rDNA gene from all the isolates. Amplification of 16S rDNA was carried out using reverse and forward primer which will give between 1000 bp to 1500 bp of the product. From Figure 4.10 below, it shows the PCR product from the amplification of the 16S rDNA gene after electrophoresis was carried out on 1% agarose gel

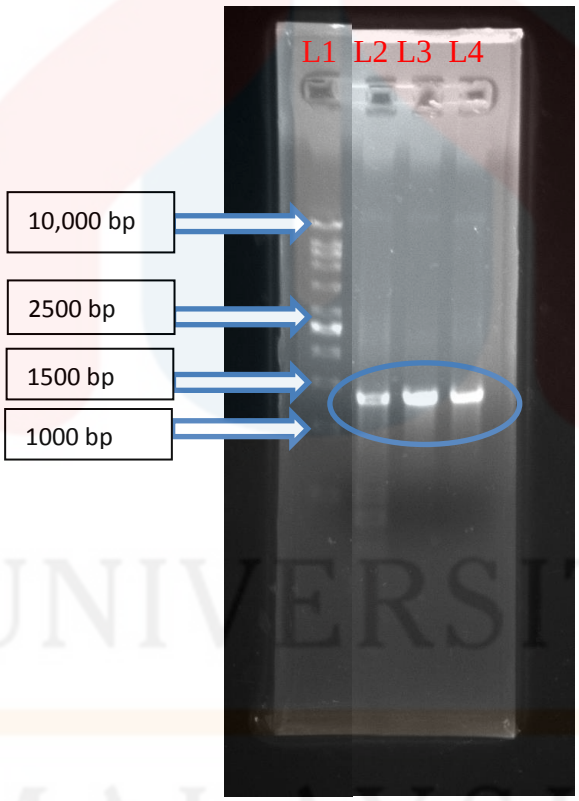


Figure 4.10: PCR products from amplification of 16S rDNA gene of bacterial isolates after electrophoresis on 1% agarose gel. L1: VC 1kb DNA ladder (Vivantis), L2: PCR product amplified from gDNA of isolates DSA 4, L3: PCR product amplified from gDNA of isolates DSA 7, L4: PCR product amplified from gDNA of isolates DWA 9

Figure 4.10 shows the result of amplification of 16S rDNA where all three of the bacterial isolates has been successfully amplified. This can be seen with 3 clearly visible bands on the gel in L2 (DSA4), L3 (DSA7) and L4 (DWA9)

The size of the PCR products are between 1500 bp and 1000 bp which can be seen in Figure 4.10 with DNA ladder showing the size. The actual full size length of the entire 16S rDNA gene sequence is about 1500 bp and it is made up of highly conserved regions which give a broad spectrum, and nine hypervariable regions ranging from V1 to V9 which allows high level of taxonomic discrimination (Hong & Farrance, 2015)

PCR works by following a simple principle. As the name stated, it is a chain reaction where 1 DNA molecule is utilized to make 2 copies and so forth until it is in high amount. This action is executed by a specific protein called *Taq* polymerase which is a taken from thermostable bacteria, in which it bind together individual DNA creating blocks which then form long molecular strands. DNA building blocks like nucleotides that consist of adenine, thymine, cytosine and guanine are needed for polymerase to do this. Primer is also needed and when these three come together in a process it will create similar copies of the templates in higher amount. Different temperature is needed for each component to function that is where thermocycler functions in which it regulates proper temperature for each process (Joshi & Deshpande, 2010)

(c) DNA purification

After the PCR products have been successfully identified by running gel electrophoresis, the PCR products need to be purified to remove any unwanted materials from the targeted DNA. MEGAquick-spin^{TM} Total Fragment DNA Purification Kit was used to purify the PCR product. Electrophoresis has confirmed the successful purification of the PCR product by presence of the bands on the gel.

(d) Gene sequencing

10 µl of the purified DNA was then sent to First BASE Laboratories Sdn. Bhd.to run the sequencing reaction. The sequence sent by them were analysed using BLAST analysis where it showed that only one if the DNA from the bacterial isolates can be compared with other strains in the database. The reason why 16S rDNA- based molecular identification could identify the bacterial isolate is for its universal distribution among bacteria and also the presence of species-specific variable regions (Weisburg *et al*, 1991). Bacterial isolate DSA 7 was able to be identified through gene sequencing and comes from the genus *Aeromonas.* Based on the percentage of similarity, there are two possible bacteria which is either *Aeromonas hydrophila* or *Aeromonas veronii*. The reason for this two bacteria is where they come from, both of these bacteria can be found in fresh water (Macdonald *et al*, 1987), this correlates with sample where this bacterial was taken which is at a fresh water river called Sungai Dungun.

The two other bacterial isolates which are DSA 4 and DWA 9 failed to be identified which is due to their sequence being too short. Their delivery time to the First BASE Laboratories Sdn. Bhd may have caused the DNA sample to be partially denatured causing a shorter sequence. Although DSA 7 has not shown a great result in characterization of biosurfactants, a few research have shown that the genus *Aeromonas* is a great biosurfactant producer able to give a satisfying result in most test of biosurfactant characterization (Hamed *et al*, 2012). Figure 4.11 and Figure 4.12 below shows the result from the BLAST analysis of bacterial isolate DSA 7.

Description	Max score	Total score	Query cover	E value	Ident
Aeromonas hydrophila subsp. hydrophila JCM 3975 gene for 16S ribosomal RNA, partial sequence	1760	1760	93%	0.0	98%
Aeromonas caviae strain BN_2064 16S ribosomal RNA gene, partial sequence	1760	1760	93%	0.0	98%
Aeromonas sp. strain 1709048444G 16S ribosomal RNA gene, partial sequence	1760	1760	93%	0.0	98%
Aeromonas caviae strain R25-6 chromosome, complete genome	1760	17482	93%	0.0	98%
Aeromonas sp. ASNIH1 chromosome, complete genome	1760	17493	93%	0.0	98%

Figure 4.11: BLAST analysis result for isolate DSA7 (forward)

Description	Max score	Total score	Query cover	E value	Ident
Aeromonas hydrophila subsp. dhakensis Consuelo Esteve:ABF145 16S ribosomal RNA gene, partial sequence	1576	1576	89%	0.0	97%
Aeromonas hydrophila strain S23 16S ribosomal RNA genes, partial sequence	1576	1576	89%	0.0	97%
Aeromonas caviae GSH8M-1 DNA, complete genome	1570	15502	89%	0.0	97%
Aeromonas caviae strain QSRB4 16S ribosomal RNA gene, partial sequence	1570	1570	89%	0.0	97%
Aeromonas sp. strain Ah22 16S ribosomal RNA gene, partial sequence	1570	1570	89%	0.0	97%

Figure 4.12: BLAST analysis result for isolate DSA7 (reverse)

CHAPTER 5

CONCLUSION

5.1 Conclusion

3 bacterial isolate taken from Sungai Dungun were used in this study for the characterization of biosurfactant. Bacterial isolates were grown on a MSM agar supplemented with 1% (v/v) cooking oil. To characterize the biosurfactant produced by the isolates, blood haemolysis test, drop collapse test, oil spreading test, emulsification test and hydrocarbon degrading capability test were used.

In blood haemolysis test, isolate DSA 7 and DWA 9 showed positive result with β-haemolysis observed on the blood agar, while isolate DSA 4 showed negative result with Γ-haemolysis observed. In drop collapse test, both DSA 4 and DWA 9 are able to give positive result with the collapse of the supernatant structure on the parafilm and oil coated glass slide while DSA 7 is unable to collapse the structure thus giving negative result.

All 3 bacterial isolates give positive result in oil spreading test with DSA 4 and DWA 9 showed greater activity with the diameter of oil displacement of 4.00 cm and 4.67 cm respectively. DSA 7 only gives 2.83 cm of oil displacement. Next, for emulsification test, only DWA 9 showed a high emulsification activity with an average of 34.44 %, but DSA 4 and DSA 7 only produce 14.44% and 9.80% of emulsification activity. Hydrocarbon degrading test also give positive result for all 3 bacterial isolates

in which DWA 9 showed the highest percentage of hydrocarbon degradation which is 54%.

The bacterial isolates then undergoes molecular characterization by 16S rDNA analysis. 16S rDNA gene was successfully amplified from all the three isolates. However, after running gene sequencing on the amplified product, only 1 isolate was able to be identified which was DSA 7. DSA 7 was identified to be a genus from *Aeromonas*.

5.2 Recommendation

Using different source of sample, like taking the sample from a lake or water sample near oil related industries. Next, Optimizing hydrocarbon degrading capability test by using different polycyclic aromatic hydrocarbon (PAH) like naphthalene, anthracene and pyrene to gives better and clearer result. Moreover, PCR product with a higher concentration of DNA should be sent for sequencing to get better result.

Other screening methods can also be done to improve the result of this study. Methods like testing the surface tension of the biosurfactant using the drop weight method. Moreover, test like foam formation activity can also be carried out where the foam activity is check through the foam stability, height and shape in a graduated cylinder

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Appendices

Table A1: Emulsification index for bacterial strains

Emulsification Index (%)	DSA 4	DSA7	DWA 9
Batch 1	13.33	6.07	33.33
Batch 2	16.67	13.32	33.33
Batch 3	13.33	10	36.67
Average	14.44333	9.796667	34.44333
Standard Deviation	1.574491	2.96329	1.574491

Table A2: Final weight of phenanthrene after 7 days incubation

Final Phenanthrene Weight (g)	Initial Weight (g)	DSA 4 (%)	DSA7 (%)	DWA 9 (%)
Batch 1	0.25	36	16	52
Batch 2	0.25	44	24	56
Average	-	40	20	54
Standard Deviation	-	4	4	2