POTENTIAL BIOSURFACTANT-PRODUCING BACTERIA ISOLATED FROM PETROLEUM SLUDGE

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biotechnology)

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ABSTRACT

Biosurfactants are amphiphilic compounds produced by microorganisms with almost similar properties as chemically synthesized surfactants. With amphiphilic properties, these compounds are widely applied as bioemulsifier and enhancer in the bioremediation process. This study aimed to screen potential biosurfactant producers from petroleum sludge. Bacteria were isolated from petroleum sludge samples and screened for biosurfactant activity by analysing Emulsification activity (E_{24}) and Surface Tension of the culture supernatant. Selected isolates were subjected to 16S rRNA analysis while the isolate that showed the highest surface tension reduction was further subjected to the growth study. The effect of carbon and nitrogen source towards surface tension reduction was tested. Then, the biosurfactant produced was partially purified and characterized. Out of the 47 isolates, *Pseudomonas* sp. P15 exhibited among the highest emulsification index (67.41%) and biosurfactant activity by reducing the surface tension measurement as low as 18.92 mN/m. In the bacterial growth study, *Pseudomonas* sp. P15 exhibited the highest surface tension reduction when 2% of used engine oil (UE2) was used as the carbon source. The addition of nitrogen source, 0.5% of yeast extract did not affect the surface tension reduction but increased the bacteria growth and biosurfactant yield by 100-fold (43.37 g/L). After liquid-liquid extraction, the partially purified biosurfactant showed similar R_f value to the rhamnolipid standard in the thin layer chromatography (TLC) and effectively emulsified the hydrocarbon substrate. The finding indicated that *Pseudomonas* sp. P15 isolated from petroleum sludge has potential to produce biosurfactant that can be used in wide range of industrial application such as bioremediation of petroleum contaminated sites and enhance the oil recovery in the petroleum industry.

خلاصة البحث

إن منشطّات السطح الحيوية، أو المواد الفعالة سطحيًّا، هي مركبات مزدوجة تنتجها كائنات دقيقة لها خواص مشابهة تقريبًا لمنشّطات السطح المصنعة كيميائيًّا. وبفضل خصائص الازدواج فإنّ هذه المركبات تستخدم على نطاق واسع كمستحلبات ومحسّنات في عملية المعالجة الحيوية. استهدفت هذه الدراسة فحص مُنتِجات منشّطات السطح الحيوية المحتملة من الحمأة البترولية. تم عزل البكتيريا من عينات الحمأة البترولية وفحص نشاط المواد الفعالة سطحيًّا عن طريق تحليل نشاط الاستحلاب (E24) والتوتر السطحي للسائل الرائق من المزرعة البكتيرية. وخضعت العينات المختارة لتحليل الحمض النووي الريبوسوميّ S16، بينما تمّ تعريض العزلة التي أظهرت أعلى انخفاض في التوتر السطحي لمزيد من دراسة النمو. وتمّ اختبار تأثير كلٍّ من مصدر الكربون والنيتروجين على تقليل التوتر السطحي، وتمّ بعد ذلك التنقية الجزئية وتوصيف منشّط السطح الحيوي المُنتَج. ومن بين 47 عزلة، تم اختيار العزلة P15 كمُنتِج محتمل لمنشّط السطح الحيوي. وأظهرت بكتيريا *Pseudomonas* sp. P15 مؤشر استحلابٍ أعلى (67.41%) ونشاطاً سطحيًّا عن طريق خفض قياس التوتر السطحي إلى 18.92 مل نيوتن/م. وأظهرت *Pseudomonas* sp. P15 في دراسة نموّ البكتيريا أعلى انخفاض في قيمة التوتر السطحي حين تمّ استعمال 2% من زيت المحرك المستخدم (UE2) كمصدر للكربون، ولم تؤثر إضافة مصدر النيتروجين- 0.5% من مستخلص الخميرة- على تقليل التوتر السطحي ولكنّها أدت إلى زيادة نمو البكتريا ومردود منشّط السطح الحيوي بمقدار مئة ضعف (43.37 جم/لتر). وبعد عملية استخلاص السائل أظهر منشّط السطح الحيوي المنقّى جزئيًّا قيمةً لمعامل R_f مماثلةً لقيمة مَرجِع rhamnolipid في كروماتوجرافيا الطبقة الرقيقة (TLC)،كما استَحلب الركيزة الهيدروكربونية بشكلٍ فعال. وأشارت النتائج إلى أنّ بكتيريا *Pseudomonas* sp. P15المعزولة من الحمأة البترولية لديها القدرة على إنتاج منشّط سطحي والذي يمكن استخدامه في مجموعة واسعة من التطبيقات الصناعية مثل المعالجة الحيوية للمواقع الملوثة بالبترول وتعزيز استخلاص النفط في الصناعة البترولية.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology)

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DECLARATION

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SPECIAL DEDICATION,

To my beloved husband

who always cheer me up, bear with all the dramas and lean the ears to hear my stories even though this is not your forte,

To my wonderful parents

who have portrayed their own dedication pursuing journey in their life, encouraged me to do well too in my journey.

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LIST OF SYMBOLS

Cm	Centimetre
g	Gram
g/L	Gram per litre
h	Hour
L	Litre
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mN/m	Dynes
nm	Nanometre
rpm	Revolutions per minute
v	Volume
v/v	Volume per volume
w	Weight
w/v	Weight per volume
%	Percentage
°C	Degree Celsius
µL	Microlitre
µm	Micrometre
±	Plus, minus
+	Plus
x	Times

LIST OF ABBREVIATION

ANOVA	Analysis of Variance
BH	Bushnell Haas
BLAST	Basic Local Alignment Search Tool
CFS	Cell-free Supernatant
CMC	Critical Micelle Concentration
DNA	Deoxyribonucleic Acid
dH_20	Deionized Water
EDTA	Ethylenediaminetetraacetic Acid
E_{24}	Emulsification Index
LB	Luria Broth
MEOR	Microbial Enhanced Oil Recovery
NCBI	National Centre for Biotechnology Information
OD	Optical Density
PCR	Polymerase Chain Reaction
POME	Palm Oil Mill Effluent
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SDS	Sodium Dodecyl Sulphate
SPSS	Statistical Package for The Social Sciences
TLC	Thin Layer Chromatography
et al.	(*et alia*); and others
viz.	(*videlicet*); namely

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Surfactants are amphiphilic compounds that consist of hydrophilic (head) and hydrophobic (tails) end which make it soluble in water, lipids or oil (Migahed & Al-Sabagh, 2009). Due to their properties, these compounds have a speciality in reducing surface and interfacial tension between two liquids which have a different degree of polarity and hydrogen bonds such as oil and water interfaces. The surfactants are widely used in the petroleum industry, food, pharmaceutical, cosmetics and detergent. Surfactants can be classified based on their origin; chemically synthesized surfactants and biological surfactants or called as biosurfactants. There is an increase of interests in biosurfactants production as an alternative to the chemically synthesized surfactants. The highly branched synthetic surfactant's structure makes it hard to degrade and lead to less bioavailability to the environment compared to biosurfactants (Shoeb et al., 2013).

Biosurfactants are amphiphilic compounds produced by microorganisms with almost similar properties as chemically synthesized surfactants (Banat, 1995; Pornsunthorntawee et al., 2008; Yin et al., 2009). Biosurfactants have low toxicity, more biodegradable and environmental-friendly (Ainon et al., 2013; Saisa-Ard et al., 2013). Since biosurfactants have very low critical micelle concentration (CMC) than chemically made surfactants, the biosurfactants are effective at low concentrations and able to reduce the surface and interfacial tensions between water or air, air or oil and water or oil (Migahed & Al-Sabagh, 2009).

Compared to other microorganisms including fungi and yeast, bacteria are known as major biosurfactants producers that extensively produce biosurfactants as secondary metabolites. Among the well-known types biosurfactants produced by biosurfactant producers namely glycolipids, rhamnolipids and surfactin. Rhamnolipid biosurfactants is one of the biosurfactants that commonly produced by *Pseudomonas aeruginosa* (Antoniou et al., 2015; Bharali & Konwar, 2011; Cameotra & Singh, 2008), surfactin by *Bacillus subtilis* (Pereira et al., 2013) and other glycolipids (Noparat et al., 2014). Biosurfactants producer or called as biosurfactant-producing bacteria are likely found in the environment that contains rich hydrocarbon and favourable condition such as oil reservoir (Chandankere et al., 2013), soil (Almansoory et al., 2019; de Faria et al., 2011; Zambry et al., 2017) ocean (Ismail et al., 2018), hot spring (Suthar & Nerurkar, 2016) or wastewater (Affandi et al., 2014). Other than that, microbial resources from palm oil industry such as palm oil mill effluent (POME) (Marajan et al., 2015) and palm oil-contaminated soil (Saisa-ard et al., 2014) are also potential biosurfactant producers due to the presence of carbon source in oil palm products.

Additionally, the biosynthesis of biosurfactants is depending on the fermentation conditions and environmental factors. The biosurfactant-producing bacteria require enough nutrients in terms of carbon and nitrogen sources to generate energy for their growth. As mentioned by Bertrand et al. (2018), carbon and nitrogen sources are the most important parameters for biosurfactants production followed with other physical parameters such as temperature, pH and presences of trace metal. Several types of raw materials, either water-soluble or insoluble substances, have been used as a carbon source in biosurfactants production include crude oil, diesel, oily sludge, glucose, sucrose and glycerol. As mentioned by Chandankere et al. (2013), crude oil was used as a carbon source in biosurfactant production by *Bacillus methylotrophicus*

USTBa. The presence of crude oil in the fermentation process increased the emulsification activity up to 78% and 1.8 g/L of biosurfactants productions. The combination of n-hexadecane as carbon source and urea as nitrogen source in the production of biosurfactants by *Pseudomonas aeruginosa* OBP1 showed 4.8 g/L of maximum yield (Bharali & Konwar, 2011).

1.2 PROBLEM STATEMENT

Biosurfactants are widely used as an alternative to chemically synthesized surfactants. Due to their amphiphilic nature, these compounds are able to alter two different polarities of surfaces such as water/air, oil/air, or oil/water surfaces. With these physicochemical properties, a Japanese chemical company namely Kaneka Corp has developed surfactin biosurfactants to disinfect the polluted area affected by the Fukushima nuclear (Sajna et al., 2015). In Malaysia, the Petrochemical Industry is known as one of the largest sectors that provides essential products such as crude oil and natural gas. However, environmental pollution occurred throughout the petroleum production process such as oil spill, contamination in soils and water. As in conventional methods, chemically synthesized surfactants are used in remediation process. However, the application of chemically synthesized surfactants would create other environmental problems. There is urgency on producing biosurfactants produced by microorganisms to overcome those problems. The application of biosurfactants are profoundly more environmental-friendly and biodegradable. Therefore, this research was carried out to find out new potential biosurfactants producer isolated from petroleum sludge due to lack information on bioprospecting novel biosurfactant compounds produced by bacteria isolated from the contaminated environment.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

To screen potential biosurfactant producers from petroleum sludge.

1.3.2 Specific Objectives

i. To isolate the potential biosurfactant producers from the petroleum sludge.
ii. To screen biosurfactant activity using Emulsification Index (E_{24}) and Surface Tension Measurement.
iii. To determine the effect of carbon and nitrogen sources towards surface tension reduction of the selected biosurfactant producers.
iv. To determine the specific types of biosurfactant produced.

1.4 RESEARCH HYPOTHESIS

i. Isolates that able to strive in the medium containing 2% of crude oil will have biosurfactant activity.
ii. The lower the surface tension measurement the higher the chance that the isolate is a biosurfactant producer.
iii. Different types of carbon and nitrogen source will affect the reduction of surface tension measurement.
iv. The R_f value of the biosurfactant in the TLC will differentiate the type of biosurfactant produced.

1.5 RESEARCH QUESTIONS

i. How many isolates that can be determined as potential biosurfactant-producing bacteria isolated from the petroleum sludge?
ii. How many of the isolates are able to exhibit biosurfactant activity?
iii. Which type of carbon and nitrogen sources that able to reduce surface tension measurement?
iv. What types of biosurfactant produced by the selected biosurfactant producer?

CHAPTER TWO

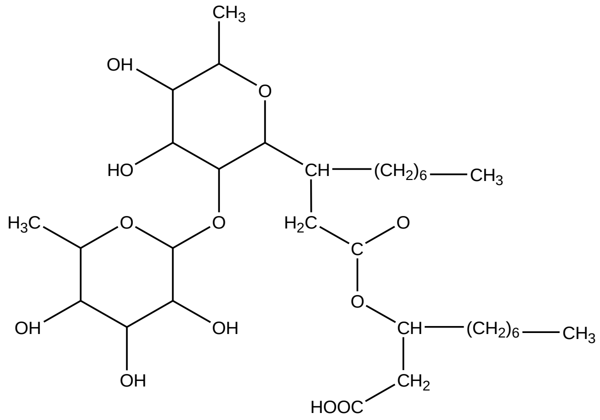
LITERATURE REVIEW

2.1 CLASSIFICATION OF BIOSURFACTANTS

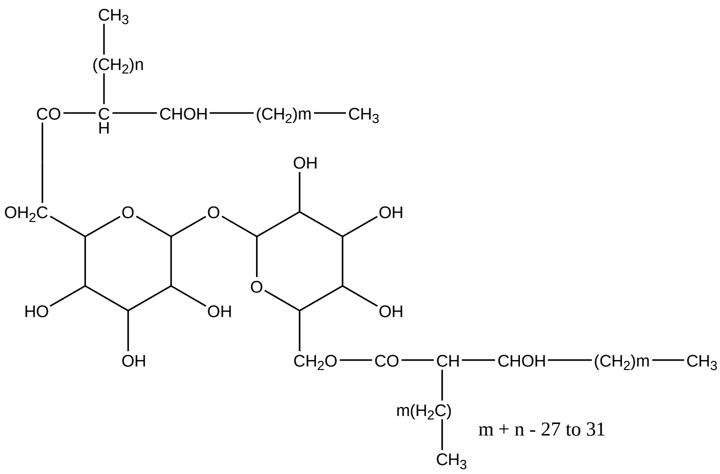
As amphipathic substances, biosurfactants able to adsorb and alters the conditions in or between interfaces. These surfactants produced by microorganisms can be divided into low-molecular-weight molecules and high-molecular-weight polymer based on their chemical properties and types of biosurfactants producers. As mentioned by Ron and Rosenberg (2001), biosurfactants that classified as low-molecular-weight surfactants are generally glycolipids or lipopeptides with simple fatty acids and phospholipids, which is efficiently reduce the surface and interfacial tension. Polymeric surfactants and particulate biosurfactants are high-molecular-weight biopolymers, which is tightly attached towards the surface and these group of biosurfactants can react effectively as emulsion stabilizing agents.

2.1.1 Glycolipids

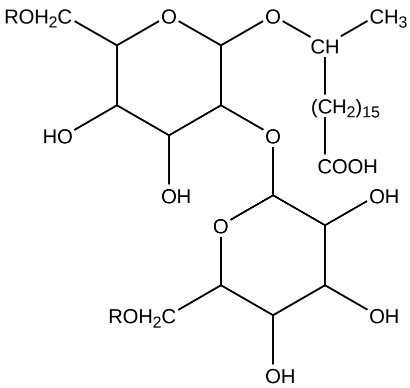
Glycolipids biosurfactant compose of carbohydrates like mono-, di-, tri- and tetrasaccharides that linked with hydroxyls fatty acids or long-chain fatty acids connected with either an ester or ether group (Shoeb et al., 2013; Vandana & Singh, 2018). The best examples of glycolipids are rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol. Figure 2.1 showed the structures of common glycolipids biosurfactant.



(A)



(B)



R = CH_2CO

(C)

Figure 2.1 Structure of Common Glycolipids Biosurfactants. (A) Rhamnolipid. (B) Trehalolipid. (C) Sophorolipid (Patel et al., 2019; Sharma 2016)

One of the most studied glycolipids-biosurfactants are rhamnolipids that consists of one or two molecules of rhamnose sugar which are linked together with β-hydroxydecanoic acids as shown in Figure 2.1(A) (Shoeb et al., 2013). Commonly, rhamnolipids are produced by *Pseudomonas* species that isolated from hydrocarbon-contaminated environments such as *Pseudomonas aeruginosa* from petroleum sludge (Bharali & Konwar, 2011) and *Pseudomonas stutzeri* from Palm Oil Mill Effluent (Fazli & Hertadi, 2019). In another study, it was reported that rhamnolipids were produced by a microbial consortium consist of the Gram-negative *P. aeruginosa* and Gram-positive *Rhodococcus* sp., which facilitated the degradation of hydrocarbon compounds (Cameotra & Singh, 2008). With its speciality in reducing surface and interfacial tension, rhamnolipid biosurfactants were also used to reclaim oil from the reservoir (Yan et al., 2012).

Other microorganisms such as yeasts that belong to genus *Candida* and *Torulopsis* were reported produced glycolipids biosurfactants (Ron & Rosenberg, 2001). These biosurfactants were known as sophorolipids that consist of dimeric carbohydrate sophorose linked through a glycosidic bond to a long-chain hydroxy fatty acid (Figure 2.1 (C)) (Santos et al., 2016). These biosurfactants are bind together by at least six to nine varieties of hydrophobic sophorolipids that are a mixture of free acid form and macrolactones. The structure of sophorolipids can be vary depending on the types of carbon sources. Sophorolipids produced by *Candida bombicola* has potential to be used as antibacterial and antibiofilm agents due to its ability to disrupt the formation of biofilm and prevent the adhesion (De Rienzo et al., 2015).

However Kitamoto et al. (1993), reported that other productive yeast strains known as *Candida antarctica* was producing mannosylerythritol lipids (MEL), yeast glycolipids to exhibit the antimicrobial activity towards Gram-positive bacteria. These yeast strains also called as *Pseudozyma antarctica* isolated from vegetable oils produced mannosylerythritol lipids to facilitate in removal and biodegradation of hydrocarbon (Kitamoto et al., 2001). On the other hand, mannosylerythritol produced by *Saccharomyces cerevisiae* used as bioemulsifier in mayonnaise formulation (Alizadeh-Sani et al., 2018). These biosurfactants were formed by polypeptide chains linked together with short and long mannose bonds.

Trehalose lipids is one of the glycolipids biosurfactant produced by several microorganisms belonged to the mycolates groups including *Mycobacterium, Nocardia* and *Corynebacterium* (Desai & Banat, 1997; Sen, 2010). Trehalolipids contains non-reducing disaccharide trehalose combined at C-6 and C-6 of mycolic acids as elucidated in Figure 2.1 (B) (Shoeb et al., 2013). The structure of trehalose lipids is different depends on the microorganism producer. Mycolic acids are long-chain, α-branched-β-hydroxy fatty acids that found in the cell wall of the mycolata taxon or a group of bacteria which is mostly species of *Mycobacterium* (Franzetti et al., 2010). In other study, *Rhodococcus erythropolis* was reported to be one of the trehalose lipids producers that increase the solubility and biodegradation of phenanthrene (PHE) (Chang et al., 2004).

2.1.2 Lipopeptides

Most well-studied lipopeptides were synthesized by bacterial and actinomycetes species. These lipopeptides antibiotic-like biosurfactants are promising as antimicrobial agents. Lipopeptides surfactants are generally contained of a high amount of cyclic lipopeptides that linked to a fatty acid. The best examples of lipopeptides biosurfactants such as surfactin, iturin, fengysin and lichenysin. Both surfactin and iturin are known