

**PRODUCTION OF BIOSURFACTANT BY LOCALLY ISOLATED  
BACTERIA FROM PETROCHEMICAL WASTE**

**RUZNIZA BINTI MOHD ZAWAWI**

**UNIVERSITI TEKNOLOGI MALAYSIA**

PRODUCTION OF BIOSURFACTANT BY LOCALLY ISOLATED BACTERIA  
FROM PETROCHEMICAL WASTE

RUZNIZA BINTI MOHD ZAWAWI

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## ABSTRACT

Ten bacterial strains previously isolated from petrochemical wastes were selected for the screening of biosurfactant producer(s), via four different methods; (i) surface tension measurements, (ii) blood hemolysis test, (iii) drop-collapsing test, and (iv) bacterial adherence to hydrocarbon (BATH) test. Two isolates coded AB-Cr1 and ETL-Cr1 identified as *Actinobacillus* sp. and *Aeromonas* sp., respectively were chosen to be the best candidates for biosurfactant production. Biosurfactant productions by both isolates were found to be growth-associated in all conditions tested. Biosurfactant production in glucose/crude oil medium (7.18-8.26 g/L) was found similar to that observed in crude oil-free medium (6.33-8.76 g/L). The production of biosurfactant was also studied in a fermentor using isolate AB-Cr1, as a factor of temperature, initial glucose concentration, pH and initial nitrogen concentration. The highest production of 12.45 g/L was obtained with AB-Cr1 grown in medium (pH 7) supplemented with 25mM  $\text{NH}_4\text{NO}_3$  as nitrogen source and 3mM glucose as carbon source, incubated at 37°C under non-pH controlled strategy. TLC and FTIR characterization of crude biosurfactant produced by both isolates in medium supplemented or not with crude oil indicated the presence of lipoprotein and non-aromatic glycolipid types of biosurfactant. GC-MS analysis of fatty acid methyl esters indicated the presence of pentadecanoic acid in crude biosurfactant from both isolates as well as octadecanoic and heptadecanoic acid in the biosurfactant produced by AB-Cr1 and ETL-Cr1, respectively. The CMC of the biosurfactant produced in the presence and absence of crude oil were approximately (g/L) 1.0 and 0.1 for AB-Cr1, and 1.2 and 0.2 for ETL-Cr1, respectively. The biosurfactants were found capable of producing a relatively stable emulsion with hydrocarbon at pH 10. It was also found stable at various pHs (3.0-13.0 and 5.0-9.0) for AB-Cr1 and ETL-Cr1, respectively and thermostable for 1 hour at 100°C, based on the value of surface tension.

## ABSTRAK

Sepuluh strain bakteria yang telah dipencilkan dari sisa petrokimia telah dipilih untuk penyingkapan bakteria penghasil-biosurfaktan, melalui empat kaedah; (i) pengukuran ketegangan permukaan, (ii) ujian hemolisis darah, (iii) ujian keruntuhan titisan, dan (iv) ujian pelekatan bakteria kepada hidrokarbon. Dua strain, AB-Cr1 dan ETL-Cr1 dikenalpasti masing-masing sebagai *Actinobacillus* sp. dan *Aeromonas* sp., telah dipilih sebagai bakteria yang paling berpotensi menghasilkan biosurfaktan. Penghasilan biosurfaktan oleh kedua-dua strain didapati bergantung kepada pertumbuhan sel dalam semua keadaan ujian. Penghasilan biosurfaktan di dalam medium glukosa/minyak mentah (7.18-8.26 g/L) didapati serupa dengan medium tanpa minyak mentah (6.33-8.76 g/L). Penghasilan biosurfaktan oleh strain AB-Cr1 juga telah dijalankan di dalam fermenter terhadap faktor suhu, kepekatan awal glukosa, pH dan kepekatan awal nitrogen. Penghasilan maksimum sebanyak 12.45 g/L didapati oleh AB-Cr1 di dalam media (pH 7) yang mengandungi 25mM  $\text{NH}_4\text{NO}_3$  sebagai sumber nitrogen dan 3mM glukosa sebagai sumber karbon, pada suhu 37°C tanpa kawalan pH. Pencirian biosurfaktan mentah bagi kedua-dua strain melalui kaedah TLC dan FTIR menunjukkan kehadiran biosurfaktan jenis lipoprotein dan glikolipid bukan aromatic. Analisis GC-MS terhadap metil ester asid lemak menunjukkan kehadiran asid pentadekanoik di dalam ekstrak biosurfaktan mentah bagi kedua-dua strain dan juga asid oktadekanoik dan heptadekanoik di dalam biosurfaktan yang masing-masing dihasilkan oleh AB-Cr1 dan ETL-Cr1. Nilai CMC bagi biosurfaktan yang dihasilkan dengan dan tanpa minyak mentah adalah masing-masing (g/L) 1.0 dan 0.1 bagi AB-Cr1, dan 1.2 dan 0.2 bagi ETL-Cr1. Biosurfaktan ini berupaya menghasilkan emulsi yang stabil terhadap hidrokarbon pada pH10. Ia juga didapati stabil pada pelbagai pH (3.0-13.0 dan 5.0-9.0) bagi AB-Cr1 and ETL-Cr1, masing-masing dan stabil terhadap haba selama 1 jam pada 100°C, berdasarkan nilai ketegangan permukaan.

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## LIST OF ABBREVIATIONS

mN/m	-	Milinewton per meter
g/L	-	Gram per litre
mL	-	Mililitre
°C	-	Degree Celsius
rpm	-	Rotation per minute
nm	-	Nanometer
w/v	-	Weight per volume
v/v	-	Volume per volume
CMC	-	Critical Micelle Concentration
$\mu$	-	Specific growth rate
$P_{\max}$	-	Maximum product concentration
$X_{\max}$	-	Maximum biomass concentration
$Y_{p/s}$	-	Product yield coefficient (g product formed per g substrate utilized)
$Y_{p/x}$	-	Product yield coefficient (g product formed per g biomass formed)
$Y_{x/s}$	-	Biomass yield coefficient (g biomass formed per g substrate utilized)
<i>et al.</i>	-	And friends
sp.	-	Species
h	-	Hour
$\text{NH}_4\text{NO}_3$	-	Ammonium nitrate
HCl	-	Hydrochloric acid
$K_d$	-	Decay constant

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## CHAPTER 1

### INTRODUCTION

#### 1.1 General Overview: Surfactant and Biosurfactant

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface [Mulligan, 2004]. They contain a hydrophobic moiety with little affinity for the bulk medium and a hydrophilic portion that is attracted to the bulk medium. Surfactants have been used industrially as adhesives, deemulsifiers, flocculating, wetting and forming agents, lubricants and penetrants [Mulligan and Gibbs, 1993].

Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily [Bodour and Miller-Maier, 2002]. Due to the presence of surfactant, less work is required to bring a molecule to the surface and the surface tension is reduced. The ability to reduce surface tension is a major characteristic of surfactant. It is obvious that their surface and membrane-active properties play an important role in the expression of their activities.

Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives, and a number of other consumer and industrial products. They constitute an important class of industrial chemicals widely used in almost every sector of modern industry. The total surfactant production has exceeded 2.5 million tones in 2002 [Deleu and Paquot, 2004] for many purposes such as polymers, lubricants and

solvents. The growth rate is related to the world demand in detergents since this sector uses over 50% of surfactant production [Deleu and Paquot, 2004].

From the total surfactants output, about 54% of them is consumed as household or laundry detergents, with only 32% destined for industrial use [Cameotra and Makkar, 1998]. Almost all surfactants currently in use are chemically derived from petroleum. The choice of surfactant is based on product cost. Generally, surfactants has been extensively used to save energy and consequently energy cost. For example, the new generation of detergents wash effectively at much lower temperatures, resulting in significant energy saving. Physicochemical behavior, charge-type, solubility and adsorption behavior are some of the most important selection criteria for surfactants [Mulligan, 2004].

However, as many industry and research organizations concern to the environmental approach, they are currently attempted to find new ways of producing surfactants. There are two new strategic approaches that are taken into account in developing new surfactant, which are i) the impact of the surfactant to the environment and ii) the functionalities of the surface-active molecules. Synthetic surfactants exhibit a low rate of biodegradation and a high potential to aquatic toxicity. For these reasons, biosurfactants are seen to be the promising alternative for many purposes even though their performance could be slightly inferior or their prices are more expensive.

Biosurfactant is a structurally diverse group of surface-active molecule synthesized by microorganisms. Their capability of reducing surface and interfacial tension with low toxicity and high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants [Banat *et al.*, 2000]. Hester (2001) from the Technical Insights estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of \$US200 million. However, up to now, biosurfactants is still unable to compete with the chemically synthesized surfactants in the surfactant market. This could be due to their high production costs in relation to inefficient bioprocessing method available, poor strain productivity and the need to use expensive substrates [Cameotra and Makkar, 1998; Deleu and Paquot, 2004].

The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential

applications in such areas as the environmental protection. The uniqueness with unusual structural diversity, the possibility of cost-effective ex-situ production and their biodegradability are some of the properties that make biosurfactant a promising choice for use in environmental application [Hua *et al.*, 2003].

Initial focus of industrial interest towards biosurfactants concentrates on the microbial production of surfactants, cosurfactants and so on for the application on microbial-enhanced oil recovery (MEOR) [Kosaric *et al.*, 1987]. The applications of biosurfactants however, are still currently remained at the developmental stage of industrial level. The development of biosurfactant application in industries has focused mainly on high biosurfactant production yield and the production of highly active biosurfactants with specific properties for specific applications.

Majority of surfactants produced today is of petrochemical origin beside of the renewable resources like fats and oils [Deleu and Paquot, 2004]. Amongst the renewable raw materials, oleochemical products represent half of the total surfactant production. The petrochemical industry is one of the important sector in Malaysia, with investments totaling RM28 billion as at the end of 2002 [Mida Malaysia]. Exxon Mobil is one of the multinational petrochemical companies that work in collaboration with Malaysia's national petroleum company, Petronas. This collaboration clearly make Malaysia as a potential country as an investment location for petrochemical industries.

Unfortunately, industrial wastewater from petroleum-related industries has been identified as one of the major source of pollution in Malaysia. The biodegradation of petroleum pollutant and its related compound is limited by poor availability to the microorganisms, due to their hydrophobicity and low aqueous solubility. This suggested that by applying biosurfactants to influence the bioavailability of the contaminant, can possibly enhancing the solubility of these compounds. Due to their biodegradability and low toxicity, they are in demand to be use in remediation technologies [Mulligan, 2004].

At present, biosurfactants plays an important application in petroleum-related industries which is use in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges [Daziel *et al.*, 1996, Bertrand *et al.*, 1994]. These industries are known to be the potential target for the application of these compounds. This is due to the ability

of biosurfactant-producing microorganisms to use petroleum or its' products as substrates as well as the properties of the biosurfactant which required less rigorous testing than chemical surfactant [Cooper, 1986].

To date, there are numbers of reports on the synthesis of various types of biosurfactants by microorganisms using water-soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates [Desai and Banat, 1997]. Petroleum-related industry was found to be one of the industries that have a great potential in producing a microorganism that may produced biosurfactants. Hence, there could probably be a potential chance of producing biosurfactants using locally isolated bacteria originated from petrochemical wastes or other wastewater available in this country. It has been focused here that improving the method of biosurfactant production and characterizing the major properties of the biosurfactant are highly important in the commercial application of biosurfactant.

## **1.2 Scope and Objectives of the Current Project**

The present study focused on studying the production of biosurfactant by bacteria isolated from petrochemical wastes. Ten bacterial isolates were screened for potential biosurfactant producer(s) and two of them were found able to produce biosurfactant by various screening methods. It was therefore of interest to characterize these bacteria and study their ability to produce biosurfactant.

The major part of this thesis describes research into the production of biosurfactant by these bacteria in various conditions tested. The study was initiated with basic identification based on cellular and colony morphologies, followed biochemical characteristics of these bacteria. The study on production of biosurfactant by these isolates was initiated by optimizing the growth of the potential biosurfactant producers as the factor of several parameters such as initial glucose concentration, initial pH and incubating temperature. The ability of these bacteria to produce biosurfactant as single and mix bacterial cultures, in medium supplemented with glucose and/or crude oil were then studied using the optimum growth conditions. Optimization of biosurfactant production by the best biosurfactant producer was further studied in bioreactor as a factor of temperature, initial glucose



concentration, pH and initial nitrogen concentration. This study was also sought to the preliminary characterization of the crude biosurfactant produced by means of their physicochemical properties. Characterization studies included emulsification activity, critical micelle concentration (CMC), stability test, thin layer chromatography (TLC), fourier transform infrared (FTIR) and gas chromatography-mass spectrometry (GC-MS) analyses.

In general, the objective of this research is to study the biosurfactant production by microbial fermentation process and characterized the crude biosurfactant in order to determine their physicochemical properties. Therefore, this study is conducted with the specific objectives:

- § To screen and characterize the potential biosurfactant-producing microbes from petrochemical waste samples.
- § To optimize the biosurfactant production in terms of productivity and the yield of biosurfactants from the substrates.
- § To characterize the crude biosurfactant produced by the bacterial isolates.

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