
UNIVERSITI PUTRA MALAYSIA

# PRODUCTION OF RHAMNOLIPID BIOSURFACTANT BY *Pseudomonas aeruginosa* USING SLUDGE PALM OIL AS CARBON SOURCE IN STIRRED TANK BIOREACTOR

NADHIRAH BINTI YAACOB

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By

**NADHIRAH BINTI YAACOB**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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**November 2019**

**Chairman : Professor Arbarkariya B Ariff, PhD**
**Faculty : Biotechnology and Biomolecular Sciences**

Rhamnolipid biosurfactant is a lipid surfactant produced from various carbon sources through fermentation by many species of *Pseudomonas* and *Acinetobacter*. The importance of rhamnolipid biosurfactant is focused on the role of oil recovery in oil and gas industry. Rhamnolipid biosurfactant is an amphipathic structure and has various and unique carbon chain (lipid). Rhamnolipid biosurfactant production is costly due to high cost of fermentation, recovery and purification process. Reduced fermentation cost for rhamnolipid biosurfactant production may be achieved using waste such as sludge palm oil (SPO) as carbon sources, which is abundantly produced by palm oil industry. The possibility of using SPO as carbon source in fermentation by *Pseudomonas aeruginosa* for rhamnolipid production was investigated in this study. The pure strain of *P. aeruginosa* (ATCC 9027) was obtained from Advanced Oleochemical Technology (AOTD), Malaysian Palm Oil Board (MPOB), which was initially purchased from AXON Scientific Sdn. Bhd. Preliminary, the selection of appropriate basal medium for rhamnolipid production by *P. aeruginosa* was carried out in shake flask culture. The effect of SPO, glucose and sodium nitrate concentrations was also investigated in shake flask culture using the selected basal medium. The effect of culture's pH, agitation speed and aeration rate was evaluated in 1.3 L stirred tank bioreactor using the optimal medium obtained from shake flask study. The effect of dissolved oxygen transfer (DOT) on rhamnolipid production was also evaluated in 1.3 L stirred tank bioreactor. The extraction of Rhamnolipid produced by fermentation in stirred tank bioreactor using SPO as carbon source was performed using acid precipitation and solvent extraction methods. Rhamnolipid was purified using TLC and HPLC methods. The LCMS analysis was carried out on the purified rhamnolipid in order to propose the structures. From shake flask experiments, the optimal medium for rhamnolipid production by *P. aeruginosa* (ATCC 9027) was achieved using 16 g/L glucose, 0.2 g/L $NaNO_3$ and with the addition of 0.2% v/v SPO which produced 14 g/L rhamnolipid. The rhamnolipid production using the optimal

medium was 2 times higher as compared to non-optimal medium. In fermentation using stirred tank bioreactor, the optimal cultivation conditions were achieved at initial culture pH 5, temperature of 30°C, agitation speed of 400 rpm and air flow rate at 0.2 vvm, in which the dissolved oxygen tension (DOT) was controlled at 90% saturation throughout the fermentation The maximum rhamnolipid concentration obtained under this optimal fermentation condition was 14.79 g/L, which gave the cell efficiency and overall rhamnolipid productivity of 0.736 g/L and 0.287 g/Lh respectively. The HPLC chromatogram of rhamnolipid produced by *P. aeruginosa* (ATCC 9027) through the fermentation using SPO has four peaks, indicated that different fatty acid chains of mono rhamnolipid and di-rhamnolipid were presence. The analysis of the peaks using liquid chromatography mass spectrophotometer (microOTOF-Q) indicated that peak 1 has m/z value of 649.3824, peak 2 with m/z value of 675.3934, peak 3 has 503.3235 m/z value and peak 4 has value m/z of 677.4108, in which, structure of rhamnolipid biosurfactant for each peak was proposed. Results from this study demonstrated that SPO could be used as the promising raw materials for rhamnolipid production at reduced cost.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENGHASILAN BIOSURFAKTAN RHAMNOLIPID DARI *P. Aeruginosa* MENGGUNAKAN ENAP CEMAR MINYAK SAWIT SEBAGAI SUMBER KARBON DALAM TANGKI BIOREAKTOR BERPENGADUK**

Oleh

**NADHIRAH BINTI YAACOB**

**November 2019**

**Pengerusi : Profesor Arbarkariya B Ariff, PhD**
**Fakulti : Bioteknologi dan Sains Biomolekul**

Biosurfaktan Rhamnolipid adalah surfaktan lipid yang dihasilkan dari pelbagai sumber karbon dalam fermentasi aerobik oleh banyak spesies *Pseudomonas* dan *Acinetobacter*. Kepentingan biosurfaktan rhamnolipid tertumpu pada peranan pemulihan minyak dalam industri minyak dan gas. Biosurfaktan Rhamnolipid mempunyai struktur amphipatik dan rantai karbon yang pelbagai dan unik (lipid). Pengeluaran biosurfaktan Rhamnolipid adalah mahal dalam fermentasi, pemulihan dan proses pengaslian. Oleh itu, kos fermentasi yang tidak mahal diperkenalkan dengan menggunakan sisa minyak sebagai sumber karbon seperti 'Sludge palm oil' (SPO), yang dihasilkan secara banyak dalam industry minyak sawit. Kemungkinan pengeluaran biosurfaktan Rhamnolipid dari *Pseudomonas aeruginosa* yang menggunakan SPO sebagai sumber karbon disiasat dalam kajian ini. Strain tulen *P. aeruginosa* (ATCC 9027) ini telah didapati melalui Advanced Oleochemical Technology (AOTD), MPOB yang dibeli dari AXON Scientific Sdn. Bhd. Penyelidikan awal untuk memilih kadar basal media untuk pengeluaran Rhamnolipid oleh *P. aeruginosa* dilakukan dalam kelalang bergoncang. Kepekatan SPO, kepekatan glukosa dan kepekatan natrium nitrat juga dilakukan dalam kelalang bergoncang menggunakan basal media yang terpilih. Kesan terhadap pH, kelajuan agitasi dan pengudaraan dinilai dikaji di dalam 1.3 L bioreaktor berpengaduk menggunakan media yang optima dari kajian kelalang bergoncang. Strategi pengawalan oksigen terlarut terhadap penghasilan Rhamnolipid dikaji di dalam 1.3L bioreaktor berpengaduk. Kemudian pengekstrakan Rhamnolipid yang dihasilkan di dalam bioreaktor berpengaduk menggunakan SPO sebagai sumber karbon dilakukan menggunakan pemendakan asid dan pengekstrakan pelarut. Rhamnolipid ditulenkan menggunakan kaedah TLC dan HPLC. Seterusnya, analisis LCMS dilakukan untuk mencadangkan struktur biosurfaktan. Dari kelalang bergoncang, media fermentasi yang optimum untuk penghasilan Rhamnolipid dari *P. aeruginosa* (ATCC 9027) telah dicapai menggunakan 16 g/L glukosa, 0.2 g/L sodium nitrik dan 0.2% v/v penambahan

enap cemar minyak sawit telah menghasilkan 14 g/L Rhamnolipid. Produksi Rhamnolipid menggunakan media optimum adalah sebanyak 2 kali ganda berbanding dengan ujikaji kawalan. Dalam fermentasi menggunakan bioreaktor berpengaduk, kondisi optimum telah dicapai dengan pH pada 5, suhu 30°C dan kadar pemutaran 400 rpm dengan 0.2 vvm pengaliran udara, dimana pengawalan oksigen terlarut dikawal pada 90% ketepuan sepanjang fermentasi. Konsentrasi Rhamnolipid maksimum dihasilkan di dalam kondusi ini adalah sebanyak 14.79 g/L, di mana effisiensi sel dan produktiviti keseluruhan adalah 0.736 g/L dan 0.287 g/Lh. HPLC kromatogram Rhamnolipid yang dihasilkan oleh *P. aeruginosa* (ATCC 9027) melalui fermentasi menggunakan SPO mempunyai empat puncak bacaan yang menunjukkan penghasilan rantaian asid lemak yang berbeza. Puncak tersebut dianalisis menggunakan liquid chromatography mass spectrophotometer (microOTOF-Q) menunjukkan puncak 1 mempunyai nilai m/z 649.3824, puncak 2 mempunyai nilai 675.3934 m/z, puncak 3 503.3235 m/z, dan puncak 4 mempunyai 677.4108 m/z, di mana, struktur Rhamnolipid terhasil bagi setiap puncak dicadangkan. Hasil dari kajian ini mendapati bahawa SPO boleh digunakan sebagai sumber karbon dalam penghasilan Rhamnolipid untuk pengurangan kos.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Arbakariya B. Ariff, PhD**
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

**Murni Halim, PhD**
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

**Abdul Rashid Yatim, PhD**
Senior Research Officer
Advance Oleochemical Technology
Malaysian Palm Oil Board
(Member)

**ZALILAH MOHD SHARIFF, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 December 2020

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Name and Matric No: Nadhirah binti Yaacob, GS44030

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# CHAPTER 1

# INTRODUCTION

Chemical compounds that can display surface activity are called surfactants. Surfactants are an amphiphilic molecules that comprise both the hydrophilic and hydrophobic tails. Surfactants have a great variety of the household and industrial applications and an important class of chemical products (Makkar *et al.*, 2011). Synthetic surfactants are used in a variety of applications, including detergents, cosmetics and personal hygiene, food, agriculture, paper and pulp, textiles, and in the oil industry. Though these surfactants have been used in these industries for years, they have brought about environmental concerns due to their aquatic toxicity and slow rate of degradation. As part of the recent green initiative, which is still gaining momentum, much research is being carried out in an attempt to replace these petrochemical products with ones derived from renewable resources (Miller, 2011). Due to this issue, a natural choice processes called biosurfactants have been introduced,

Biosurfactants are natural surface active agents produced by variety of microorganisms such as bacteria and yeast. Biosurfactants have hydrophilic and hydrophobic components in its compound. Biosurfactants are metabolites that can exhibit surface activity and are synthesized by the bacteria, yeast and fungi that are grown on different carbon sources (Miller *et al.*, 2012). Their hydrophilic part is usually composed of sugars, amino acids, or carboxylic acids. The hydrophobic part is typically an aliphatic hydrocarbon chain of fatty acids. These components helps increases the solubility and biodegradation of insoluble compound like hydrocarbon. Biosurfactants could be considered as 'Green Chemicals' because of their biodegradability and biocompatibility (Sadagari, 2013).

Biosurfactants created by microorganisms allow them to grow and reproduce. Fakruddin Md explained that the main physiological role of biosurfactants is to permit microorganisms to grow on water-immiscible substrates by reducing the surface tension at the phase boundary, thus making the substrate more readily available for food uptake and metabolism. Furthermore, another role is involved in biosurfactants synthesis to desorption of cells from the biofilm which helping them to find new environment (Sadagari, 2013).

However, the production of biosurfactants is restricted as the production process is uneconomically that the surfactants from petrochemical sources. Thus in order to interest the industrial, the biosurfactants have a chance in large scale productions when the raw material and process is minimal. Cheap substrates, cheap process, high biosurfactants yields and highly active biosurfactants with the specific properties for the specific applications are the keys of success (Amani *et al.*, 2013). Rhamnolipids are one of the promising biosurfactants known from *Pseudomonas aeruginosa* bacteria. Rhamnolipids are glycolipids compromised one or two rhamnose sugars that

linked to one or two β-hydroxy fatty acids through the glycosidic bond (Hoskova *et al.*, 2015). Abdel-mawgoud *et al.*, (2011) stated that the main rhamnolipid producers are bacteria of the *Pseudomonas* genus, however other species have been reported produce rhamnolipids such as *Acinetobacter calcoaceticus, Pseuxanthomas* sp, *Enterobacter sp.* and *Pantoea* sp.

The rhamnolipid synthesis involves several steps. According to Chong and Li, they reported that precursors for rhamnolipid synthesis are the sugar (dTDP-L-rhamnose) and hydrophobic moieties such as 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA). The sugar part can be synthesized from D-glucose, while the hydrophobic part can be synthesized through the fatty acid synthesis pathway, starting with two carbon units.

Malaysia is pioneer country in oil palm plantation and the palm oil produced is more than sufficient to meet the demand in food industry and it is co friendly products. Palm oil consists of palmitic acid and steric acid which is a less aggressive fatty acid and has short chain of fatty acids. This makes palm oil a potential substrate to be used in the formulation of bisourfactants in fermentation. Sludge palm oil is the floating residual oil which is separated during the early stage of POME discharge. This residual oil is a low grade quality oil, thus making SPO a waste in palm oil production. This oily by product consists of 83.5% triglycerides, 8% diglycerides, 0.5% monoglycerides and 8% free fatty acids which can act as carbon source for microbial growth during biosurfactant production (Chow *et al*., 2002). Previously, biosurfactant production uses expensive pure form of carbon for example glucose, fructose and sucrose and this makes the cost of production high. However Abouseoud *et al.*, (2008); Robert *et al*., (1989) found that production of biosurfactants using water soluble carbon sources such as glycerol, mannitol and ethanol has better production of biosurfactant. The production of biosurfactant was triggered only when all the soluble carbon was consumed and when a water-immiscible hydrocarbon was available (Banat *et al*., 1995). Being water-immiscible, SPO is not only a favourable choice of carbon source for biosurfactant production but also a cost effective source found in abundantly in Malaysia. In this study, SPO was introduced as a novel substrate for rhamnolipid biosurfactant production.

Production of rhamnolipid biosurfactant uses SPO as carbon source is similar to other production of rhamnolipid biosurfactant (Kronemberger *et al.*, 2010; Bhardwaj *et al.*, 2013; Kaskatepe *et al.*, 2015). Optimization of production and cell viability can be achieved with fermentation medium that can support the nutrient requirement of rhamnolipid biosurfactant. Furthermore, a commercial growth medium should be inexpensive ingredients and allow *Pseudomonas aeruginosa*g to reach a maximum cell density in a short amount of time in order for the production cost to be minimized. Moreover, the fermentation broth should allow convenient harvest of cells.

Strategies to improve the rhamnolipid fermentation was identified by fermentation parameters such as basal medium, glucose concentration, $NaNO_3$ concentration, SPO concentration, aeration, pH and agitation. Basal medium, glucose concentration, $NaNO_3$ concentration, SPO concentration was identified using shake flask while

aeration, pH and agitation uses stirred tank bioreactor. Downstream process of this studies involve acid precipitation and solvent extraction. Recovery of rhamnolipid biosurfactant by HPLC and LCMS, and LCMS was used in order to identify the proposed rhamnolipid structure that was produced using SPO.

The overall objective of this study was to evaluate the feasibility of using SPO as substrate for biosurfactant production by *P. aeruginosa*. The specific objectives of this study were:

i) to evaluate the effects of the addition of various concentrations of glucose and nitrogen source ($NaNO_3$) to SPO on the enhancement of the production of rhamnolipid by *P. aeruginosa* in shake flask fermentation.
ii) to investigate the effect of controlling the culture pH during the fermentation on the performance of rhamnolipid production by *P. aeruginosa* in 1.3 L stirred tank bioreactor.
iii) to investigate the effect of agitation and oxygen transfer on the performance of rhamnolipid production by *P. aeruginosa* in 1.3 L stirred tank bioreactor.

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## BIODATA OF STUDENT

The student was born on October 7th, 1992 in Melaka. She completed her Fifth Form at MRSM Alor Gajah, Melaka in 2009. She continued her education at UiTM Puncak Alam in 2010 and completed her 1 year pre-university course in science. In 2011, she pursued her tertiary education in UniKL MICET in the field of Bioprocess Technology and obtained her Bachelor of Technology degree major in Bioprocess Technology in 2015. Later on, she furthered her Master of Science degree in the field of Industrial Biotechnology at Universiti Putra Malaysia, Selangor. She received MPOB scholarship for her Master degree.