OPTIMIZATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY Pseudomonas sp. LM19 USING PALM KERNEL FATTY ACID DISTILLATE

THIO CUI WEN

FBSB 2019 2

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By

THIO CUI WEN

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

January 2019

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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Chair : Assoc. Prof. Phang Lai Yee, PhD
Faculty : Biotechnology and Biomolecular Sciences

Biosurfactants were used in various industries owing to their high biodegradability and low toxicity. However, biosurfactant applications are still limited due to its high production cost and low production. This obstacle can be overcome by optimizing the fermentation condition to increase production yield, while simultaneously exploiting potential low-cost materials as substrate to reduce production cost. In Malaysia, growing production of palm oil and its downstream products resulted in a surplus of low value oleochemical products such as palm kernel fatty acid distillate (PKFAD). Therefore, the first objective in this study was to determine the optimum fermentation conditions for biosurfactant production by indigenous *Pseudomonas* sp. LM19 using PKFAD as carbon source. The second objective was to characterize the biosurfactants produced using analytical and physical properties characterization techniques. Fermentation conditions including the incubation temperature, inoculum size, PKFAD concentration, pH, agitation speed, and incubation time were screened and optimized. Factors ranges were set using the preliminary experiments in a combination of one-factor-at-a-time (OFAT) and two-level full factorial designed experiments based on conditions reported by peer studies. These factors were then optimized using response surface methodology (RSM). A full randomized factorial design with thirty experimental runs was suggested by Design Expert Software to assist the identification of optimum fermentation conditions. Responding variables chosen for both preliminary and optimization experiments were emulsification index (E_{24}) and rhamnolipid concentration quantified via orcinol test. From the study, the optimum conditions obtained from RSM were pH 7.5, 2% (v/v) PKFAD, agitation speed of 170 rpm and 192 h of incubation time with E_{24} of 58.6 ± 0.62% and biosurfactant concentration of 1.6 ± 0.01 g/L. The produced biosurfactant was then characterized to identify the features of biosurfactant. Thin layer chromatography (TLC) indicated that the biosurfactant produced was found to be glycolipid as it showed the presence of both lipid and sugar moieties. Two spots were obtained with Rf values of 0.68 and 0.38, being

mono-rhamnolipid and di-rhamnolipid, respectively. Similar findings were resonated by Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) analysis as both tests complemented each other to confirm the presence of an ester bond linking aliphatic chain to saturated carboxylic group, a trademark functional group in glycolipids. NMR results further revealed the presence of various functional groups present in rhamnolipid molecules, indicating the glycolipid biosurfactant was rhamnolipid in nature. Gas chromatography mass spectrometry (GCMS) recorded the presence of the pseudo-molecular ion, two methyl-esterified fatty acids in the form of β-hydroxy fatty acids. High performance liquid chromatography equipped with quadruple photodiode and evaporative light scattering detector (HPLC-QDa-ELSD) was then used to determine the rhamnolipid congeners. The rhamnolipid produced by *Pseudomonas* sp. LM19 were di-rhamnolipid dominant, containing 57.42% of the sample while the rest were mono-rhamnolipids. Antimicrobial activity and surface tension test were conducted to identify the functional properties of the rhamnolipid produced. Rhamnolipid produced was able to inhibit the growth of *Bacillus subtilis*. The surface tension test indicated that the rhamnolipid produced exhibited a critical micellar concentration (CMC) of approximately 28 mg/L. To conclude, the biosurfactant production by *Pseudomonas* sp. LM19 using PKFAD as substrate was optimized, with its preferred fermentation conditions of pH 7.5, 2% (v/v) PKFAD, agitation speed of 170 rpm and 192 h of incubation time. The biosurfactant produced was extracted and characterized as type of rhamnolipid biosurfactant, containing di-rhamnolipid dominant with C_{10}-C_{10} lipid chains, exhibited CMC of 28 mg/L.

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

PENGOPTIMUMAN DAN PENCIRIAN BIOSURFAKTAN YANG DIHASILKAN OLEH *Pseudomonas* sp. LM19 DENGAN MENGGUNAKAN SUBSTRAT SULINGAN ASID LEMAK KERNEL SAWIT

Oleh

THIO CUI WEN

Januari 2019

Pengerusi : Prof. Madya Phang Lai Yee, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Biosurfaktan telah digunakan dalam pelbagai industri kerana kebolehbiodegradasian yang tinggi dan ketoksikannya yang rendah. Walau bagaimanapun, aplikasi biosurfaktan adalah masih terhad kerana kos pengeluaran yang tinggi dan hasil pengeluaran yang rendah. Halangan ini boleh diatasi dengan mengoptimumkan keadaan fermentasi untuk meningkatkan hasil pengeluaran, pada masa yang sama mengeksploitasi bahan kos rendah yang berpotensi sebagai substrat untuk mengurangkan kos pengeluaran. Di Malaysia, pengeluaran minyak sawit semakin meningkat dan produk sampingannya menyebabkan lebihan produk oleokimia yang bernilai rendah seperti sulingan asid lemak kernel sawit (PKFAD). Oleh itu, objektif pertama kajian ini adalah untuk menentukan keadaan optimum fermentasi untuk pengeluaran biosurfaktan oleh *Pseudomonas* sp. LM19 dengan menggunakan PKFAD sebagai sumber karbon. Objektif kedua adalah untuk mencirikan biosurfaktan yang dihasilkan melalui pencirian sifat analisis dan fizik. Keadaan fermentasi, iaitu suhu inkubasi, saiz inokulum, kepekatan PKFAD, pH, kelajuan penggoncangan, dan masa inkubasi, telah disaring dan dioptimumkan. Faktor julat telah ditetapkan dengan menggunakan kajian awal dalam gabungan satu faktor-pada-satu masa (OFAT) dan eksperimen faktorial dua tahap penuh, direka berdasarkan keadaan yang telah dilaporkan oleh kajian terdahulu. Faktor-faktor ini kemudiannya dioptimumkan dengan menggunakan metodologi sambutan permukaan (RSM). Satu reka bentuk faktorial rawak penuh dengan tiga puluh ujikaji telah dicadangkan oleh perisian *Design Expert* untuk membantu mengenal pasti keadaan fermentasi optimum. Maklum balas yang dipilih bagi kedua-dua eksperimen awal dan eksperimen pengoptimuman ialah indeks pengemulsian (E_{24}) dan kepekatan rhamnolipid yang diukur melalui ujian *orcinol*. Dari kajian ini, keadaan optimum yang diperoleh daripada RSM ialah pH 7.5, 2% (v/v) PKFAD, kelajuan pergolakan 170 rpm dan masa inkubasi 192 j dengan E_{24} 58.6 ± 0.62% dan penghasilan biosurfaktan 1.6 ± 0.01 g/L. Seterusnya, biosurfaktan yang dihasilkan dicirikan untuk mengenal pasti sifat biosurfaktan

tersebut. Kromatografi lapisan nipis (TLC) menunjukkan bahawa biosurfaktan yang dihasilkan ialah glikolipid kerana ia menunjukan kehadiran kedua-dua lipid dan moiet gula. Nilai R_f yang diperoleh ialah masing-masing 0.68 dan 0.38, bagi mono-rhamnolipid dan dwi-rhamnolipid. Penemuan yang serupa telah ditunjuk oleh spektroskopi resonans inframerah transformasi Fourier (FTIR) dan analisis resonans magnetik nuklear (NMR) kerana kedua-dua ujian tersebut adalah saling melengkapi untuk mengesahkan kehadiran ikatan ester yang menghubungkan rantaian alifatik kepada kumpulan karboksilik tepu, sebagai penentu kumpulan berfungsi untuk glikolipid. Keputusan NMR juga menunjukkan kehadiran pelbagai kumpulan berfungsi yang terdapat dalam molekul rhamnolipid, seterusnya menunjukkan biosurfaktan glikolipid adalah bersifat rhamnolipid. Spektrometri jisim kromatografi gas (GCMS) mencatatkan kehadiran ion pseudo-molekul, dua asid lemak metil terester dalam bentuk asid lemak β-hidroksi. Alat kromatografi cecair prestasi tinggi dilengkapi dengan fotodiod kuadrupel dan pengesan penyebaran cahaya sejatan (HPLC-QDa-ELSD) kemudiannya digunakan untuk mengenal pasti kongener rhamnolipid. Rhamnolipid yang dihasilkan oleh *Pseudomonas* sp. LM19 adalah didominasi oleh dwi-rhamnolipid, iaitu 57.42% daripada sampel manakala selebihnya ialah mono-rhamnolipids. Aktiviti antimikrob dan ujian tegangan permukaan telah dijalankan untuk mengenal pasti ciri-ciri fungsi rhamnolipid yang didapati dapt menghalang pertumbuhan *Bacillus subtilis*. Ujian tegangan permukaan menunjukkan bahawa rhamnolipid yang dihasilkan mempamerkan kepekatan misel kritikal (CMC) sekitar 28 mg/L. Kesimpulannya, pengeluaran biosurfaktan oleh *Pseudomonas* sp. LM19 menggunakan PKFAD sebagai substrat telah dioptimumkan dengan keadaan fermentasi pilihan, iaitu pH 7.5, 2% (v/v) PKFAD, kelajuan penggoncangan 170 rpm dan masa inkubasi 192 j. Biosurfaktan yang dihasilkan telah diekstrak dan dicirikan sebagai jenis biosurfaktan rhamnolipid, yang didominansi oleh dwi-rhamnolipid dengan C_{10}-C_{10} rantai lipid, dan menpamerkan CMC sebanyak 28 mg/L.

ACKNOWLEDGEMENTS

I am grateful that I have completed this study. I would like to thank all individuals who have helped, supported and inspired me throughout this study. First and foremost, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Phang Lai Yee for the assistances along with her patience, valuable advice, guidance and supervision throughout the research and studies. Her guidance, encouragement and passion helped me in the research and writing to complete this uphill task. I am really grateful with her supervision which enable the accomplishment of this study.

I would also like to express my thanks to my co-supervisors, Assoc. Prof. Dr. Umi Kalsom Mohd. Shah and Dr. Lim Wen Huei for their insightful comments, suggestions and guidance throughout this study. I would like to further my thanks to Dr. Lim for his continuous supports behind the scenes. Furthermore, I would like to give a thousand of thank to Dr. Voon Phooi Tee and her colleagues, who had given me logistical support throughout the study. I am very appreciative towards the co-operation and commitment with Malaysian Palm Oil Board throughout this research. I also treasured the assistance from all lecturers and staffs from Faculty of Biotechnology and Biomolecular Sciences for helping me directly and indirectly throughout this study.

Next, I would like to express my heartfelt appreciation to Ministry of Higher Education (MOHE) for giving the MyMaster scholarship for my master study and also Fundamental Research Grant Scheme (FRGS) for financial support throughout the research.

I would like to record my deepest gratitude goes to my beloved family for their encouragement, supports and unflagging care and love in my life. Also, I would like to acknowledge my gratefulness to all my friends and colleagues for their opinions and encouragements. Last but not least, I would like to thanks my partner Mr. Ng Ke Hong for his accompany, unlimited support, encouragement, and inspiration me throughout these tough days.

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:

Phang Lai Yee, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Umi Kalsom Md Shah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Lim Wen Huei, PhD
Senior Research Officer
Advanced Oleochemical Technology Division (AOTD)
Malaysian Palm Oil Board (MPOB)
(Member)

____
ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: ______________________________
Name of Member of Supervisory Committee: ______________________________

Signature: ______________________________
Name of Member of Supervisory Committee: ______________________________

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°C	degree Celsius
%	percentage
β	beta
g	gravity force
δ	sigma (chemical shift)
µL	microliter
C	carbon
$CaCl_2$	calcium chloride
cm	centimetre
cm^{-1}	per centimetre
g	gram
g/L	gram per liter
h	hour
KCl	potassium chloride
kDA	kilodaltons
KH_2PO_4	monopotassium phosphate
K_2HPO_4	dipotassium phosphate
L	litre
M	molar
$MgSO_4$	magnesium sulfate
mg	milligram
min	minute
mL	milliliter
mN/m	milli Newton per metre
mm	millimetre
NaCl	sodium chloride

nm	Nano metre
ppm	parts per million
Rha	rhamnose
rpm	revolutions per minute
s	seconds
sp.	species
USD	United States Dollar
v	volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

Surfactants are amphiphilic compound that gathers predominantly at interfaces, thus decreasing the interfacial tensions between two phases, and subsequently form aggregate structures such as micelles. Surfactants are widely incorporated in our lives mainly as detergents. Newer technologies allowed even wider application of surfactants in other industries including cosmetics and medical field. The global demand for surfactants was currently sufficed by several man-made chemical surfactants which are primarily petroleum-based.

One of the foremost controversies arise with the usage of chemical surfactants is its toxicity towards animals, ecosystem and environment. Regular disposal of chemical surfactants on land and into water systems caused harm to the community. For instance, the two main surfactants used in 2000s, alkyl phenol ethoxylates and linear alkylbenzene sulfonates were documented to degrade under normal disposal conditions to its metabolite, nonylphenol, an endocrine disruptor and severe health hazard (Scott & Jones, 2000). As a result, proposals on chemical surfactants usage restrictions were widely discussed. In conjunction with the rising awareness towards the usage of renewable products, development of possible substitutes to these synthetic surfactants has been advocated. Biosurfactants are prime example of such environment friendly options. Biosurfactants have similar characteristics to chemical surfactants, and can be an alternative to chemical surfactant in detergency, medical, cosmetic industry and even bioremediation. According to recent figures, biosurfactant's global market volume is expected to be 476.512 tonnes by 2018. In 2011, biosurfactant's global market value was USD 1.73 billion, and expected to reach USD 2.7 billion by year 2024 (Sekhon et al., 2012; Randhawa & Rahman, 2014).

Biosurfactants are structurally diverse group of surface-active substrates, biologically produced by microorganisms such as bacteria, fungi and yeasts via microbial fermentation processes or by enzymatic syntheses. Biosurfactants are secondary metabolites, in which are normally produced during late log phase and/or the early of stationary phase of the microorganism's growth (Pruthi & Cameotra, 2003). Biosurfactants reduce surface or interfacial tension between two liquids or between a liquid and a solid, a characteristic they shared with their chemical counterparts. The special nature of biosurfactants facilitates its potential to be utilized as detergents, emulsifiers, dispersants, wetting agents, and foaming agents. Biosurfactants also vitally exhibit very low critical micelle concentrations (CMC), allowing biosurfactants to be effective at low concentrations, lower than many chemically made surfactants. Furthermore, as compared to chemical surfactants, biosurfactants tends to have higher biodegradability, lower toxicity, while also being effective at extremes temperature, pH, and high salinity (Muñoz-Torrero et al., 2012). Recently, biosurfactants are gaining much attention because they essentially denote the ecological alternative to their synthetic counterparts.

Biosurfactants mainly consist of two parts of molecules, namely the hydrophobic moiety and the hydrophilic moiety, which allow biosurfactants to be amphiphilic in nature. Henceforth, biosurfactants can be categorised according to the type of moieties consist in its compound. For example, a biosurfactant that consists of sugar and lipid, is grouped under glycolipid, whilst a biosurfactant consist of amino acids and lipids is grouped under lipopeptides. One of the most investigated biosurfactants is rhamnolipids, where its outstanding emulsifying and tension-active properties received much attention. Rhamnolipids are glycolipid biosurfactants comprising of a hydrophobic lipid moiety made up by one to two β-hydroxy fatty acyl molecules, linked to a hydrophilic sugar moiety which consist either one or two L-rhamnose (Abdel-Mawgoud et al., 2011; Caiazza et al., 2005; Liu et al., 2014). Rhamnolipids are secondary metabolite mainly produced by *Pseudomonas* species, with the presence of appropriate carbon source.

Microorganisms require carbon as substrate for cell components construction and energy requirement to produce metabolites. There are two main types of carbon sources that can be used in the microbial fermentation process to produce biosurfactant, namely water-soluble and water-insoluble carbon source. In the case of *Pseudomonas* sp., water-insoluble carbon sources were preferred (Caiazza et al., 2005). This is because insoluble carbon sources are usually rich in lipid contents, adhering to the requirements for biosurfactant production by *Pseudomonas* sp. (Mathur et al., 2016). Being made up of mostly hydrophobic hydrocarbon chains, lipid moiety of biosurfactant could be formed easier when hydrophobic substrate was used. *Pseudomonas* sp. cultivated in water-insoluble carbon containing medium like hydrocarbon are likely to secrete biosurfactant to facilitate uptake of hydrocarbon by emulsifying the hydrocarbon droplet (Stoimenova & Vasileva-Tonkova, 2009). Henceforth, more and more studies focused on the potential usage of various types of water-insoluble carbon sources, like soy bean oil and sunflower oil in producing rhamnolipids surfactant using *Pseudomonas* sp. as the designated microorganism. Similarly, water-insoluble palm-based oleochemicals, palm kernel fatty acid distillate (PKFAD) would in theory exhibit potential as biosurfactant producing carbon source.

PKFAD has similar characteristics with other plant oils in which were made up of mainly free fatty acids. PKFAD can be obtained after the refinery process of crude palm kernel oil via crushing, degumming, bleaching, and deodorization. As residue of the refinery process, PKFAD has low commercial value and is commonly regarded as a by-product of crude palm kernel oil. Conventionally, PKFAD is mainly used as a feedstock for farm animals or even organic fertilizers. Therefore, PKFAD may be a more cost-effective substrate in biosurfactant production as compared to soy bean oil and coconut oil which are common food commodity.

1.2 Problem Statements

Chemical synthetic surfactant cost around $1–3/kg whereas biosurfactant rhamnolipids cost up to $20-25/kg depending on the rhamnolipids fermentation based on volumetric productivity (Chong & Li, 2017; Ebadipour, 2016). The high production cost and low product yield had thus widely hampered the use of biosurfactant in the market. However, owing to the recent global trend in using substances that are more environmentally friendly, the more biodegradable and less toxic biosurfactant were thus being advocated as a viable alternative to chemical surfactants. Since 30% or more of the overall cost was made up by the raw material cost, it is logical to search for cheaper alternatives in the view to reduce the cost of raw materials for production, and subsequently promote affordable biosurfactant applications (Nitschke & Pastore, 2004). Optimization of factors aiming to increase the yield of biosurfactant is equally critical and vital in order to maximize the biosurfactant production at the lowest cost. There are a lot of reports on the biosurfactant production by using low-cost substrates for example, agro-industrial wastes as it normally contains high amount of lipids or sugars which required in the biosurfactant production. These include the oil refinery wastes (Bednarski et al., 2004; Nitschke et al., 2005), fruit wastes (George & Jayachandran, 2009), and soap stock (Benincasa et al., 2004).

In Malaysia, agro-industrial waste in the form of palm based oleochemical products such as PKFAD are available plentifully at low-cost. Palm kernel production obtained 4.85 million tonnes and crude palm kernel oil production reached 2.29 million tonnes in year 2018 (Malaysian Palm Oil Board, 2018). As a palm based oleochemical by-product, PKFAD is biodegradable and renewable allowing it to exhibit a favourable economics profile. High free fatty acids composition in PKFAD also enabled it to be a favourable option to be used as carbon source in the biosurfactant production. Apart from selection of suitable substrate, there are several ways to maximize biosurfactant production which include using effective microorganism, as well as development of optimized bioprocess. Throughout peer reviews, one of the main and well-known biosurfactant producing microorganism is bacterium, which classified under the genus *Pseudomonas*. Low commercial value PKFAD could be a favourable option to be utilized as carbon source for biosurfactant production by *Pseudomonas* sp. since water-insoluble carbon source is one of the preferred substrates of this microorganism. Indigenous *Pseudomonas* sp. LM19 was believed to be able to replicate the biosurfactant production model performed by other reported *Pseudomonas* sp. Characterization of the biosurfactant produced is necessary to further identify the potential applications of the biosurfactant. With the potential of PKFAD as a substrate in biosurfactant production is yet to be explored, there is a chance of increasing the value of PKFAD.

1.3 Objectives

i. To determine optimum fermentation conditions for biosurfactant production by *Pseudomonas* sp. LM19 using Palm Kernel Fatty Acid Distillate (PKFAD) as carbon source.
ii. To characterize biosurfactants produced using analytical techniques (TLC, GCMS, HPLC, FTIR, and NMR).

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