UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF OPTIMIZED PALM FATTY ACID DISTILLATE-CONTAINING MEDIUM FOR BIOSURFACTANT PRODUCTION BY *Pseudomonas* sp. LM19

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FBSB 2019 16

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By

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

May 2019

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

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May 2019

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

The issues regarding biosurfactants arose from the utilization of costly substrate with low product yield leads to the overall production cost to be pricey. Hence, the development of the biosurfactant production medium using a cheap substrate through a combination of various approaches could aid in solving these issues. On the other hand, free fatty acid (FFA)-containing substrates are well-known substrates in the formation of biosurfactants. Since palm fatty acid distillate (PFAD) is considerably cheap, readily available from the mill and contained a significant amount of FFA, it could be one of the potential substrates for biosurfactant production. Therefore, the objective of this study was to develop the PFAD containing medium for biosurfactant production by *Pseudomonas* sp. LM19 using the combination of traditional and statistical approaches. The process parameters of selected medium were screened and optimized, accordingly using One-Factor-At-Time (OFAT), Plackett Burman Design (PBD) and response surface methodology (RSM) to enhance biosurfactant production and the cell growth. The process parameters of selected modified Bushnell-Haas (BH) medium were further screened in which 6% (v/v) of inoculum size, 1% (v/v) of PFAD and 1.5 g/L of yeast extract have resulted in 2.63 folds increment of biosurfactant production with final emulsification index (E24) of 59.62%. Through PBD, four significant factors were identified; KH_2PO_4, yeast extract, $MgSO_4.7H_2O$, sodium-EDTA which affecting both biosurfactants productivity and cell number. The optimized production medium containing 1.148% (v/v) PFAD; 4.054 g/L KH_2PO_4; 1.30 g/L yeast extract; 0.023 g/L sodium-EDTA; 1.057 g/L $MgSO_4.7H_2O$; 0.75 g/L K_2HPO_4; 0.20 g/L $CaCl_2.2H_2O$; 0.080 g/L $FeCl_3.6H_2O$ resulted in 3.55 folds of increment in responses, biosurfactant productivity (0.3463 g/L/day) with $8.5 \pm 0.47\times10^9$ CFU/mL of cell generation after seven days of incubation compared to unoptimized production media. The experimental value generated for both responses were almost similar to the predicted RSM value. On the other hand, a majority about 54.18% of dirhamnolipid ($RRC_{10}C_{10}$) and 39.94% of monorhamnolipid ($RC_{10}C_{10}$) were identified in the

optimized medium. Above all, manipulation of palm oil by-products such as PFAD proved to be a feasible substrate to increase biosurfactant production. The combination of both traditional and statistical design for the development of biosurfactant production medium could enhance biosurfactant productivity with optimal cell concentration. These ideas may aid in reducing overall production cost and promote their applications for various industries.

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

PEMBANGUNAN MEDIA YANG OPTIMUM MENGANDUNGI SISA PENYULINGAN MINYAK KELAPA SAWIT UNTUK PENGHASILAN BIOSURFAKTAN OLEH *Pseudomonas* sp. LM19

Oleh

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Isu berkaitan biosurfaktan melibatkan penggunaan substrat yang mahal dengan hasil produk yang rendah mengakibatkan keseluruhan kos penghasilan meningkat. Oleh itu, formulasi medium untuk penghasilan biosurfaktan dengan menggunakan substrat yang lebih murah mungkin dapat membantu menyelesaikan isu ini. Selain itu, substrat yang mengandungi asid lemak bebas (ALB) sangat diketahui umum dalam membantu penghasilan biosurfaktan. Oleh kerana sisa sulingan asid lemak kelapa sawit (SSALKS) adalah murah, mudah didapati di kilang dan mengandungi jumlah ALB yang ketara, ia berpotensi untuk dijadikan sebagai salah satu substrat untuk digunakan di dalam penghasilan biosurfaktan. Oleh itu, objektif kajian ini adalah untuk membangunkan medium yang mengandungi SSALKS bagi penghasilan biosurfaktan oleh spesis *Pseudomonas* LM19 dengan menggabungkan pendekatan tradisional dan statistik. Parameter proses bagi medium terpilih telah ditapis dan dioptimumkan, dengan menggunakan Satu Faktor Pada Masa (SFPM), Reka Bentuk Plackett Burman (RBPB) dan Metodologi Permukaan Tindak Balas (MPTB) untuk meningkatkan pengeluaran biosurfaktan dan pertumbuhan sel. Parameter proses bagi medium Bushnell-Haas (BH) yang diubah suai telah ditapis di mana 6% (v/v) saiz inokulum, 1% (v/v) SSALKS dan 1.5 g/L ektrak ragi menyebabkan peningkatan sebanyak 2.63 kali ganda dalam penghasilan biosurfaktan. Melalui RBPB, empat faktor penting telah dikenalpasti iaitu KH_2PO_4, ekstrak yis, $MgSO_4.7H_2O$ dan sodium-EDTA di mana telah memberi kesan kepada produktiviti biosurfaktan dan nombor sel. Medium pengeluaran yang telah dioptimumkan mengandungi 1.148% (v/v) SSALKS; 4.054 g/L KH_2PO_4; 1.30 g/L ektrak ragi; 0.023 g/L sodium-EDTA; 1.057 g/L $MgSO_4.7H_2O$; 0.75 g/L K_2HPO_4; 0.20 g/L $CaCl_2.2H_2O$; 0.080 g/L $FeCl_3.6H_2O$ mengakibatkan peningkatan sebanyak 3.55 kali ganda terhadap kedua-dua tindak balas yang terlibat iaitu produktiviti biosurfaktan (0.3463 g/L/hari) bersama penghasilan sel sebanyak $8.5 \pm 0.47\times10^9$ CFU/mL selepas tujuh hari inkubasi berbanding dengan medium pengeluaran yang masih belum dioptimumkan lagi. Nilai eksperimen untuk kedua-dua tindak balas hampir sama dengan nilai yang diramalkan oleh MPTB. Oleh yang demikian, pemanipulasian produk sampingan

dari kelapa sawit seperti SSALKS sebagai substrat terbukti berkesan dalam meningkatkan pengeluaran biosurfaktan. Gabungan kedua-dua reka bentuk tradisional dan statistik dalam pembangunan medium pengeluaran untuk biosurfaktan mampu meningkatkan produktiviti biosurfaktan dengan kepekatan sel yang optimum. Idea ini dapat membantu mengurangkan kos pengeluaran secara keseluruhan dan menggalakkan penggunaannya dalam pelbagai bidang industri.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful.

Alhamdulillah, all praises to graciously the Almighty for always being there for me through thick and thin. Special thanks to my supervisor, Assoc. Prof. Dr. Phang Lai Yee for introducing this topic, providing the guidance and patience for teaching me throughout this project period that was about three years. It is difficult to put into words how grateful I am. My gratitude is also extended to my co-supervisor, Dr. Mohd Shamzi Mohamed for sharing his knowledge and expertise with me, which otherwise life would have been tougher for me to finish up my thesis throughout this work. Their help for giving positive comments, suggestions and encouraging me during the project is one of the major factors that make this project success.

My appreciation also goes to Madam Aluyah and Madam Ina, laboratory assistance from Faculty of Biotechnology and Biomolecular Sciences for their kind assistance in using some of the laboratory equipment. Without them, I might not be able to prepare my materials and chemical substances in a shorter time. I am greatly indebted also to other postgraduate students and technical staff from the Department of Bioprocess Technology for guiding me with the laboratory work and giving some others advice and guidance during this project.

Last but not least, my deepest gratitude goes to my beloved parents, Abdul Hamid bin Anjang Mohd Ali and Zaidah bt Mohd Rashid including all my sister and brothers for their prayers, encouragement and endless support since the start of this project until the end. To all my valued friends, words just could not describe how much I cherished your companionship, assistance and encouragement all the way through. It is really amazing what you had done to enrich my soul and for you had always believed in me more than I did myself. For my last word, thank u so much.

…May Allah bless us always…

I certify that an Examination Committee has met on the **23rd May 2019** to conduct the final examination of **Nurfarahin binti Abdul Hamid** on her thesis entitled **"Development of Optimized Palm Fatty Acid Distillate-Containing Medium for Biosurfactant Production by *Pseudomonas* sp. LM19 "** in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15th March 1998. The Committee recommends that the student be awarded the degree of Master of Science.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
°C	Degree celsius
%	Percentage
L	Litre
EDTA	Ethylenediaminetetraacetic acid
g	Gram
M	Molar
PFAD	Palm fatty acid distillate
CPO	Crude palm oil
OFAT	One-factor-at-time
PBD	Plackett-Burman design
RSM	Response Surface Methodology
CCD	Central Composite Design
FFA	Free fatty acids
CSL	Corn steep liquor
GC-MS	Gas chromatography-mass spectrometry
HPLC	High Performance Liquid Chromatography

CHAPTER 1

INTRODUCTION

1.1 Background of the study

In recent years, biosurfactants have generated almost USD 24 million of revenues in 2009 and are predicted to reach USD 2.8 billion in 2023 (Randhawa and Rahman, 2014). They provide various advantages over synthetic surfactants (petroleum-based) in term of biodegradability, level of toxicity, high specificity, low irritancy, and compatibility with human skin (Banat *et al*., 2000; Silva *et al*., 2014a). In addition, they also play some important roles in various industries. Biosurfactants are employed in environmental control as they have high efficiency in industrial emulsions management, oil spillage control and bioremediation of polluted soil (Das and Chandran, 2011). Other than that, they are also being applied in agricultural industry as an enhancer for plant-microbe interaction, plant growth promoter, heavy metal remover in plant soil and for wiping out the plant pathogen (Sachdev and Cameotra, 2013). In the food industry, biosurfactants are used as additives due to their antimicrobial, anti-adhesive activities and biofilm control during food processing (Mnif and Ghribi, 2016).

The issues arise when the use of expensive substrate, low end-product titer, formation of a numerous by-products as opposed to a singular desired biosurfactants, and overpriced steps in the purification of biosurfactants which have led to ramping up of their price (Syldatk and Hausmann, 2010) and allowing synthetic surfactant to be a more favorable option. The cost of high-volume low-value biosurfactants was around USD2-3/kg, which was 20-30% more than the cost of chemical surfactants (Gusmao *et al*., 2010). Several strategies have been attempted for the past years to tackle the above-mentioned issues. Among these were the application of cheaper feedstocks (Cavalcante Fai *et al*. 2015; Mariano *et al*., 2008), application of various mathematical design and modelling software in improvement of overall production process (Xiaoke *et al*., 2015; Amodu *et al*., 2014), genetic modification of microbe itself to further enhance their secondary metabolites' production, i.e., focusing on biosurfactants rather than other primary metabolites (Martins Das Neves *et al*., 2007) and the development of more effective methods in biosurfactants recovery such as foam fractionation, adsorption-desorption on polystyrene resins, ultrafiltration and ion exchange chromatography (Winterburn *et al*., 2011; Reis *et al*., 2013).

The issue concerning overpricing in relation to biosurfactants production could be incredibly improved with the use of readily available, renewable, low-cost nutrients as a fermentation feedstock (Campos *et al*., 2014). Food and agricultural wastes which contain a high amount of free fatty acid (FFA) could be the potential *Candida*tes to be used as the substrate for biosurfactants production. Wastes or by-products containing a high portion of FFA such as waste frying oil (Hasanizadeh *et* *al*., 2017), aquaculture wastewater (Pepi *et al*., 2013), and vegetable fat waste (Gusmao *et al*., 2010) displayed the capability as a suitable substrate for various biosurfactants producer. Through this practice, environmental and economic value can be sustained by adding value to the by-products generated by the industry for biosurfactants production. These are readily available and also cheaper compared to the usage of the pure substrate; e.g., glucose (Sigma, USD 45.30/kg – as quoted in 2019).

Malaysia has reclaimed its position as the world's major exporter of palm oil to China for the past few years ago and this inevitably causing a tremendous amount of palm oil by-products to be generated. Malaysia's palm oil industry is the fourth largest contributor to the national economy and currently accounts for 8% of the national GNI per capita (Nazren Radzuan *et al*., 2017). Production of crude palm oil (CPO) had reached 29×10^6 tons in 2015, resulting in 0.95×10^6 tons of palm fatty acid distillate (PFAD) generated as by-product following refining process that includes degumming, bleaching and deodorization. PFAD exists as a light brown solid at room temperature and turns into brownish liquid following the heating process (Gapor, 2010). It contains more than 70% of FFA (mainly palmitic and oleic acids) which make it ideal for soap industry, animal feed industry and as raw materials for oleochemicals industries (Ping and Yusoff, 2009). Besides that, attempts had been made to yield higher end-value product like biodiesel and extracts of vitamin E from PFAD (Malvade and Satpute, 2013; Chu *et al*., 2004). As such there might be a possible route leading to another higher end-value product, e.g. biosurfactants, by utilizing PFAD as a carbon source in the production medium since it contains a high amount of FFA, that proven favorable for biosurfactant production (Patil *et al*., 2014; Felse *et al*., 2007).

A well-known biosurfactants producer; *Pseudomonas* spp. can assimilate diverse substrates having a wide spectrum of FFA such as vegetable oil refinery waste (Raza *et al*., 2007), olive oil mill waste (Moya Ramírez *et al*., 2015) and palm oil (Oliveira *et al*., 2006). Through previous studies, it proved that *Pseudomonas* spp. is a well-known genus of bacteria and widely studied as a good biosurfactants producer especially rhamnolipids which utilizes a wide range of substrate. It was hypothesized that the local isolate namely *Pseudomonas* sp. LM19 can assimilate FFA-based substrate like PFAD based on a previous study (Thio, 2009). However, the biosurfactant production performance by this isolate using PFAD as the substrate is yet to be explored.

Various strategies involving traditional and statistical approaches in optimizing the biosurfactants production conditions could actually increase their yield. Previously, traditional approach like One-Factor-At-Time (OFAT) method is one of the well-known techniques used to investigate the range of parameters involved in biosurfactants production process, followed by statistical screening and optimization approaches such as factorial design and response surface methodology (RSM) to establish the best condition producing the highest possible amount of biosurfactants (Nickzad *et al*., 2018; Jamal *et al*., 2012). Once the optimized conditions are established, time course profiling of optimized conditions can be studied to assist further process development on a larger scale for industrial application (Zajšek and Goršek, 2010). Additionally, the identification of biosurfactants classes and their congeners produced are also very important to determine their specific function, as different classes of biosurfactants could be better suited to specific industries.

1.2 Objectives of the study

Thus, the objectives of this study were:

1. To select a suitable production medium and to screen the process parameter involved using OFAT in biosurfactant production by *Pseudomonas* sp. LM19.
2. To screen the significant nutritional factors in the production medium for biosurfactant production by *Pseudomonas* sp. LM19 using Plackett Burman Design (PBD).
3. To optimize the biosurfactant production medium by *Pseudomonas* sp. LM19 using Response Surface Methodology (RSM) and to identify the class of biosurfactant and its congeners.

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BIODATA OF STUDENT

Nurfarahin binti Abdul Hamid was born on March 3, 1993 in Teluk Intan, Perak, Malaysia and live in Kampong Gajah, Perak, Malaysia. She received her primary education in Sekolah Kebangsaan Selat Pulau, Kampong Gajah, Perak and later continued her secondary study in Gopeng Integrated Boarding School, Gopeng, Perak in pure science stream. In 2011, she continued her degree foundation study in Perak Matriculation College and graduated around April 2012. Late 2012, she pursued her study at Universiti Putra Malaysia, Serdang and received scholarship from Public Service Department Malaysia (PSD) and finished her Bachelor Degree of Biotechnology after four years studied there. Later, on September 2016, she started her Master Degree in the field of Industrial Biotechnology at Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences in Universiti Putra Malaysia. She received fellowship from Graduate Research Fellowship (GRF), UPM for two years. During her master study, she attended several symposiums as poster presenter. In December 2016, she attended her first symposium, The 33rd MSM Symposium 2016 in Ramada Plaza, Melaka as second poster presenter. She presented her work as in poster entitled "Palm-Based Oleochemicals as Substrate for Biosurfactants Production by *Pseudomonas* sp. LM19 in Different Production Media" at second symposium, Bioprocess and Biomanufacturing Symposium in Universiti Sains Malaysia, Penang.

LIST OF PUBLICATIONS

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