CHARACTERIZATION OF BACTERIAL STRAINS OF RHAMNOLIPID SURFACTANTS FROM PALM KERNEL CAKE AND ITS APPLICATIONS

BY

SUMATHY VELLO

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Engineering)

Kulliyyah of Engineering
International Islamic University Malaysia

MAY 2020

ABSTRACT

Rhamnolipid is a glycolipid surfactant used in various sectors due to its versatile action. The major problem of rhamnolipid production is an expensive substrate and high production microbial strains. With this in mind, a novel substrate from palm waste, palm kernel cake (PKC) was explored for rhamnolipid production using co-culture to maximize the return. A mixture of seven bacterial population was isolated from PKC and labelled as VS1 to VS7. All the isolates were identified as biosurfactant producers through haemolytic assay, drop collapse, surface tension, oil spreading and emulsification index. However, only VS2, VS3, VS5 and VS7 were rhamnolipid producers. Biochemical analysis and 16S rRNA sequence analysis disclosed that they were *Enterococcus faceium* (VS2), *Pantoea ananatis* LMG 5342 (VS3), *Enterococcus hirae* (VS5) and *Stenotrophomonas maltophilia* K279 (VS7). The selection of co-culture in this investigation was based on the compatibility test with *Pseudomonas aeruginosa* ATCC 9027, a commercial strain. Isolated bacteria *Stenotrophomonas maltophilia* K279 was the most compatible bacteria in this study. Out of the eleven screened factors, four factors, namely sucrose, glucose, $NaNO_3$ and KH_2PO_4, were the most significant components for rhamnolipid production in Plackett Burman experimental design. As PKC functioned as the primary substrate, sucrose was chosen as the co-substrate. One factor at a time (OFAT) experiment showed that PKC (8%), sucrose (4 g/L), $NaNO_3$ (1.4 g/L), KH_2PO_4 (1.3 g/L), temperature (35°C), pH (7) and inoculum size (6%) were the optimum concentrations and conditions required for best rhamnolipid production. Media optimization using Face centered central composite design (FCCCD) showed that sucrose (4.1 g/L), $NaNO_3$ (1.9 g/L) and KH_2PO_4 (1.29 g/L) produced the highest E24 value indicating maximum rhamnolipid production. Process optimization for aeration and agitation in a bioreactor using 2^k factorial design indicated that aeration of 1 vvm and agitation above 250 rpm was suitable for maximum production of rhamnolipid. An increase of 25% in rhamnolipid recovery was recorded with mixed culture compared to using a single strain in a production comparison study. The brown viscous extract showed the presence of mono-rhamnolipid with a R_f value of 0.70 in TLC analysis. The presence of hexadecanoic acid, methyl ester, was the fatty acid detected in GS-MS analysis for our rhamnolipid. Both ^1H NMR and ^{13}C NMR detected the presence of rhamnose ring in the chromatogram. *In vitro* antibacterial experiment showed that rhamnolipid was more potent towards Gram negative bacteria compared to Gram positive bacteria. Likewise, rhamnolipid recovered in this study successfully removed 91.3% (Zn), 91% (Cu) and 90.7% (Fe) at 10 ppm that is common in agriculture soil.

خلاصة البحث

رامنوليبيد (Rhamnolipid) هي دهون سكرية خافضة للتوتر السطحي تستخدم في عدة لقطاعات بسبب تنوع وظائفها. ولكنّ المشكلة الأساسية في إنتاج الرامنوليبيد هي تكلفته العالية وتواجد السلالات الميكروبية التي تنتجه بوفرة. مع أخذ ذلك في الاعتبار، تمّ استكشاف مادّة جديدة من نفايات النخيل ، كعكة نواة النخيل (PKC) لإنتاج الرامنوليبيد باستخدام مبدأ التكافل في نموّ السلالات لزيادة الإنتاج. حيث تم عزل خليط من سبع سلالات بكتيرية من PKC، وتسميتها VS1 إلى VS7. تم تصنيف جميع العزلات كمُنتِجات للعوامل البيولوجية الخافضة للتوتر السطحي من خلال الفحص الانحلالي، سقوط القطرة، التوتر السطحي، انتشار الزيت، ومؤشر الاستحلاب. ومع ذلك، كانت السلالات VS5 ، VS3 ، VS2 و VS7 فقط هي المنتجة للرامانوليبيد. وكشف التحليل البيوكيميائي وتحليل تسلسل الحمض النووي rRNA 16S أنّ هذه السلالات كانت *Enterococcus faceium* (VS2)، *Pantoea ananatis* LMG 5342 (VS3)، *Enterococcus hirae* (VS5)، و *Stenotrophomonas maltophilia* K279 (VS7). واستند اختيار سلالات التكافل على اختبار التوافق مع السلالة التجارية *Pseudomonas aeruginosa* ATCC 9027. وكانت البكتيريا المعزولة *Stenotrophomonas maltophilia* K279 أكثر السلالات توافقًا في هذه الدراسة. من بين أحد عشر عاملًا تم فحصها، كانت أربعة عوامل، وهي السكروز والجلوكوز و $NaNO_3$ و KH_2PO_4 ، هي أهم المكونات لإنتاج رامنوليبيد في التصميم التجريبي Plackett Burman. وبما أن PKC يعمل كركيزة أساسية، فقد تمّ اختيار السكروز كركيزة مساندة. أظهرت دراسة عامل واحد في كل مرة (OFAT) أنّ PKC بنسبة (8%) ، السكروز (4 جم/لتر) ، $NaNO_3$ (1.4 جم/لتر)، KH_2PO_4 (1.3 جم/لتر) ، درجة الحرارة (35 درجة مئوية) ، درجة الحموضة (7.00) و حجم اللقاح (6%) هي التركيزات المثلى والظروف المطلوبة للحصول على أفضل إنتاج رامنوليبيد. وأظهرت دراسة تحسين الإنتاج باستخدام التصميم المركب المركزي (FCCCD) أنّ السكروز (4.1 جم/لتر)، $NaNO_3$ (1.9 جم/لتر) و KH_2PO_4 (1.29 جم/لتر) أنتجت أعلى قيمة E24 مايشير إلى الحد الأقصى من إنتاج رامنوليبيد. أوضحت عملية تحسين التهوية والتحريك في المفاعل الحيوي باستخدام تصميم عامل 2^k أن التهوية بمقدار 1 vvm والتحريك بقيمة أعلى من 250 دورة في الدقيقة، كانت مناسبة لإنتاج الحد الأقصى من رامنوليبيد. وفي دراسة لمقارنة الإنتاج، سُجِلت زيادة بنسبة 25% في عائد الرامنوليبيد باستخدام خليط من سلالات البكتيريا مقارنةً باستخدام سلالة واحدة. وأظهر المستخلص البني اللزج، وجود أحادي الرامنوليبيد بقيمة R_f تبلغ 0.70 في تحليل TLC. وكان حمض الهيكساديكانويك واستر الميثيل ، هي الأحماض الدهنية التي تم الكشف عنها في التحليل الكروماتوجرافي GS-MS لـرامنوليبيد. كشف كلٌّ من ^1H NMR و ^{13}C NMR وجود حلقة رامنوز(rhamnose) في الطيف اللوني. وأظهرت التجارب المخبرية المضادة للبكتيريا أنّ الرامنوليبيد كان أكثر فعالية تجاه البكتيريا السالبة الجرام مقارنة بالبكتيريا الموجبة الجرام. كذلك فإنّ رامنوليبيد المنتج في هذه الدراسة نجح في إزالة 91.3% (Zn) ، 91% (Cu) و 90.7% (Fe) عند 10 جزء من المليون والتي تعتبر شائعة في التربة الزراعية.

APPROVAL PAGE

The thesis of Sumathy Vello has been approved by the following:

_____
Parveen Jamal
Supervisor

_____
Md. Zahangir Alam
Co-Supervisor

_____
Mohammed Saedi Jami
Co-Supervisor

_____
Md. Noor bin Salleh
Co-Supervisor

_____
Wan Mohd Fazli Wan Nawawi
Co-Supervisor

_____
Nassereldeen Ahmad Kabbashi
Internal Examiner

_____
Abdulrahman Hamid Nor
External Examiner

_____
Mohammad Naqib Eishan Jan
Chairman

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Sumathy Vello

Signature ..... Date .....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

ISOLATION AND IDENTIFICATION OF BACTERIAL STRAINS FOR PRODUCTION OF RHAMNOLIPID SURFACTANT FROM PALM KERNEL CAKE AND ITS APPLICATION AS ANTIMICROBIAL AGENT AND HEAVY METAL REMOVER

I declare that the copyright holders of this thesis are jointly owned by the student and IIUM.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Sumathy Vello

……..…….. ……..
Signature Date

ACKNOWLEDGEMENTS

The journey to earning my Ph.D in Engineering could not have been possible without the blessings from the Almighty. I thank God for making me stronger every single day along this long journey and for introducing all beautiful souls into my life who became the pillars of my life.

I am deeply indebted to my supervisor, Prof. Dr. Parveen Jamal for her valuable advice and unwavering guidance. She has always supported and nurtured me with her unparalleled knowledge and insightful suggestions for this research. A big thank you to her for encouragement and patience that cannot be underestimated throughout this project.

I am extremely grateful to all my co-supervisors, Prof. Dr. Md. Zahangir Alam, Prof. Dr. Saedi Jami, Prof. Emeritus Dato' Wira Ir Dr. Md. Noor bin Salleh and Dr. Wan Mohd Fazli Wan Nawawi for their assistance and support throughout my research. This dissertation compilation is not possible without their helpful advice and feedback.

Thanks, should also go to Bro. Naseeruddin (Environmental lab), Br. Aslan (Bioprocess lab), Bro. Hafizul (Instrumental lab) for all their technical help during my lab work.

I would also like to extend my deepest gratitude to my family for being my backbone. Firstly, I thank my late father, Mr. Vello for his profound belief in my abilities to pursue my doctorate and my mother for all her moral support along this journey. Special thanks to my siblings and friends for their confidence in me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
bp	base pair
BLAST	Basic Local Alignment Search Tool
CCD	Central Composite Design
Cd	Cadmium
CFU	Colony Forming Units
CTAB	Cetyltrimethylammonium bromide
DOE	Design of Experiments
DNA	deoxyribonucleic acid
EMB	Eosin Methylene Blue
EOR	Enhanced Oil Recovery
FAME	fatty acid methyl ester
FCCCD	Face Centered Central Composite Design
GCMS	Gas Chromatography-Mass Spectrometry
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HMW	Biosurfactant of High Molecular Weight (HMW)
IIUM	International Islamic University Malaysia
LMW	Low Molecular Weight (LMW)
MEGA	Molecular Evolutionary Genetic Analysis

MIC	Minimum Inhibitory Concentration
MR	Methyl red
NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
OD	Optical density
OFAT	One Factor At a Time
PBD	Plackett-Burman Design
PCR	Polymerase Chain Reaction
PFAD	palm fatty acid distillate
PKC	Palm Kernel Cake
POME	Palm Oil Mill Effluent
Rha	Rhamnose
RLs	Rhamnolipids
rpm	rotation per minute
RSM	Response Surface Methodology
TLC	Thin Layer Chromatography
UPM	University Putra Malaysia
USD	United States dollar
VP	Voges Proskauer
vvm	Volume of air flow per volume of working unit per minute

LIST OF SYMBOLS

mN/m	Millinewton per meter
g/L	gram per liter
ml	Milliliter
µl	Microliter
°C	degree Celsius
h	hour
µg/ml	Microgram per millimeter
µl/ml	Microliter per millimeter
v/v	Volume per volume
mt	Metric tonne
mg/mL	Milligrams per milliliter
nm	Nanometer
λ	lambda
E24	emulsification index
R_f	Retention factor
ppm	Parts per million
cm	centimeter
mm	millimeter
R^2	coefficient of determination
$	dollar

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND STUDY

Surfactant (surface active compound) is an essential compound in our daily lives simply because of its use in various industries. From hygienic need in detergents, bioactive compounds as antibiotics (Rodrigues et al., 2006), emulsifier and additives in food (Fracchia et al., 2012), pesticide for plants and in oil fields for oil recovery shows its versatility. Twenty years ago, the petroleum-based surfactants were conquering these industries. It is necessary to realize that the scenario is offbeat in the current biotechnology era with a substitute, biosurfactant (biologically surface active agent) (Burch et al., 2011). Biosurfactants are primarily synthesized by an extensive range of microorganisms particularly micro and macroscopic ones like bacteria and fungi (Shekhar et al., 2015) are attracting the attention of industrialists due to numerous easy and cost-effective industrial applications. The ability of these molecules to reduce surface tension has made it unique and widely applied in a large number of industries for chemically synthesized surfactants (Pattanathu and Gakpe, 2008). This amphiphilic molecule is biologically biodegradable, and most importantly can be synthesized on renewable resources that make it more preferable over the synthetic ones (Muller et al., 2012).

In times to come, it can be expected to see tremendous advancement in biosurfactant market only due to its utilization in various industries. According to a market survey conducted by Global Market Insight, the industrial trend for biosurfactant consumption is expected to rise to 540-kilotons by 2024 (Anonymous, 2018). Although there are many types of biosurfactants in industrial practise,

rhamnolipid biosurfactant is forecasted to overcome other biosurfactants like sophorolipids, methyl ester and lipopeptides. In 2017, rhamnolipid biosurfactants valued at USD 11.1 million due to its diverse applications. By 2024, the use of rhamnolipids in food processing industry alone is expected to surpass USD 1.8 million (Anonymous, 2018). Holding properties like high emulsifying activity, low minimum surface tension and higher affinity for hydrophobic organic molecules (Colak and Kahraman, 2013) make its function suitable in agriculture, hydrocarbon recovery, household and personal care product (Anonymous, 2018).

From an economic point of view, biosurfactants are not yet competitive with the chemically synthesized surfactants (Radzuan et al., 2018). In the business world investor's objective is always to invest less and expect high returns (Banat et al., 2014). Sadly, in the case of biosurfactant industry, the monetary input is higher than the output due to several reasons. Firstly, the availability and choice of raw material. The limited available substrate with the right composition of nutrient for microorganism’s utilization is one of the significant issues in the biosurfactant industry. Secondly, there is a lack of overproducing microorganisms as this results in low productivity (Gakpe et al., 2007). On the other hand, pricey large-scale production remains as the commonest reported demerits of biosurfactant production. Expensive media components are not returned with high yield concerns people in this business. Along with this, expensive purification is another cost obstacle faced by manufacturers (Rodrigues et al., 2006). Marchant and Banat (2012) reported that the right microorganism selection with the use of renewable substrates and improved fermentation process are the significant areas should be accounted for before producing biosurfactants.

Thus, this scenario justifies why scientists are actively engaged in research on rhamnolipid biosurfactant in the last ten years. Among the different substrates, wastes (food, agriculture and industry) are currently the favourite choice among contemporary researchers (Chong et al., 2017). Correspondingly, agricultural waste recycling is a flourishing trade among businessman today. Since globalization has left a substantial negative impact on the environment, stringent regulatory norms are implemented in most developing countries to substitute synthetic products with bio-based products. Therefore, this justifies why a scientist is more directed towards the green solution. Additionally, people's awareness of the environment and health is winning their choices to bio-based products.

Apart from being a tropical country, Malaysia is also known for its rich biodiversity. For this reason, the agricultural industry is a leading economy booster of the country. The climate and fertile soil support a wide variety of crops on the land. Rubber, oil palm, cocoa, rice and coconut are some of the dominant commercial plants of Malaysia. In South-East Asia, Malaysia is the second biggest palm oil producers, although this oil crop is originally from Africa (Mohd Noor et al., 2017). Palm Kernel Cake (PKC) is a residual waste obtained after oil extraction from palm nut through mechanical pressing (Chong et al., 2008). Investigator Imandi et al. (2010) highlighted that approximately 3 million tonnes of PKC are produced as wastes after oil extraction from palm kernel in Malaysia. As of today, only a portion of PKC is used to make animal feed for cow, cattle, goat and pig as it is rich with carbohydrate, protein, minerals and fatty acids (Boateng et al., 2008). Bioeconomy is one approach encouraged by the government to generate a green economy to lift countries' economy and moving towards zero waste management. There is evidence from the literature that waste from oil processing industry is one promising renewable

substrate to produce biosurfactant. Coconut cake, olive oil mill waste and soya bean cake are some of the reported resources (Banat et al., 2014).

Accordingly, the present study attempts to use PKC to produce rhamnolipid surfactant that is expected to be the next generation biosurfactant. A consortium of bacterial strains was used to produce rhamnolipid as an attempt to increase the yield. Optimization experiment has been emphasized in this study to obtain information on the optimum media and process conditions such as temperature, pH, aeration and agitation for rhamnolipid production. At the end of this study, it is hoped to identify the bacteria that produce rhamnolipid isolated from PKC and its potent antimicrobial properties after testing for antimicrobial properties.

1.2 PROBLEM STATEMENTS AND ITS SIGNIFICANCE

It is known that Malaysia is one of the biggest palm oil producers in the world. As a consequence, the environmental pollution caused by the discharge of organic wastes from palm oil industry represents a considerable risk to the ecosystem. Secondly, the currently practised synthetic surfactants have limited application since they are known to possess toxicity properties. Moreover, they are often applied as mixtures for better performance rather than individual components. Other leading obstacles are related to cost and production. Expensive substrates and downstream processing resulting in overpriced production cost is the main reason why it is challenging to scale down the rate of these biomolecules. Besides, there are very limited substrates with the right balance of nutrients like carbon, nitrogen and phosphorus for optimal growth of microorganisms to produce rhamnolipid. Therefore, this results in a low yield. Sometimes low production yield is due to the difficulty in finding the suitable