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By

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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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January 2014

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Faculty: **Engineering**

Production of biosurfactant is gaining popularity in current surfactant industries, as they used low cost and renewable raw materials such as plant or microbial-based. However, recovery of biosurfactants was hampered by solvent toxicity and high amount of waste generated from multi-steps recovery processes. Poor quality of final products due to the homogeneous mixture of acidic (carboxyl) and lactonic (ester) forms also created a problem for surfactant industries, thus made their application limit to general cleaning purposes. The lactonic form of biosurfactant has wider and higher value applications for medical and pharmaceutical industries. This study was conducted to evaluate the potential of using palm olein, a cheaper and readily available raw material for production of sophorolipids biosurfactant (SLs). Two routes for recovery of SLs were compared, which are conventional solvent extraction and non-solvent extraction via salting out method. The characteristic of SLs was investigated via chromatographic and spectrometric techniques to elucidate the structure of lactonic-acidic SLs.

After seven days fermentation, 68.9 g/L SLs was produced from yeast culture *Starmerella bombicola* (ATCC 22214) supplemented with glucose and palm olein as a primary and secondary carbon source, respectively. The two routes of recovery were assessed, namely Route 1 (conventional solvent extraction method) and Route 2 (non-solvent extraction via salting-out method). In Route 2, two types of salts ($Al_2(SO_4)_3$ and $(NH_4)_2SO_4$,) were compared. Route 1 gave the acceptable SLs production and productivity at 53 g/L and 15.2 g/L.day, respectively. Route 2 with $Al_2(SO_4)_3$ gave better production and productivity at 96 g/L and 13.7 g/L.day, respectively. Route 2 with $(NH_4)_2SO_4$ gave superior production and productivity at 99 g/L and 25 g/L.day, respectively. Overall, the ascending order of waste water generated was Route 2 $(NH_4)_2SO_{4)}$ > Route 2 ($Al_2(SO_4)_3$ > Route 1, with 2.4 L > 3 L > 5.3 L, respectively.

The qualitative analysis by TLC plate showed these SLs were present in the form of acidic and lactonic mixture. Analysis by FTIR showed that active functional groups such as lactone, carboxylic acids and ester were present in all recovered SLs samples. As judged by liquid chromatography analysis, Route 2 successfully reduced acidic content into lactonic-rich SLs. This was elucidated by mass spectrometric analysis as the dominant structure was C18:1 diacetylated lactonic SLs, with a molecular mass of 688 g/mol. The physical functionality of SLs as a surface-active agent was proven by its ability to reduce surface tension of water from 72 to 33 mNm^{-1} and having a high critical micellar concentration (CMC) value, 190 mg/L. In conclusion, SLs produced from palm olein was successfully recovered through non-solvent extraction route at high productivity rate and revealed obtained at high-lactonic contents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk Ijazah Master Sains

PENILAIAN KE ATAS SOPHOROLIPID YANG DIHASILKAN OLEH *Starmerella bombicola* DENGAN MENGGUNAKAN SAWIT BERASASKAN OLEIN SEBAGAI SUMBER KARBON

Oleh

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Penghasilan biosurfaktan semakin mendapat perhatian di dalam industri surfaktan pada masa kini berikutan penggunaan bahan mentah yang murah dan boleh diperbaharui seperti berasaskan tumbuhan dan mikrob. Walaubagaimanapun, proses pemulihan menghadapi kesukaran berikutan penggunaan bahan-bahan pelarut yang toksik dalam setiap langkah pemulihan yang seterusnya mengeluarkan jumlah air sisa yang tinggi. Kandungan biosurfaktan yang terdiri daripada campuran homogen yang berasid (karboksil) dan laktonik (ester) juga telah menimbulkan masalah bagi industri surfaktan kerana kualiti produk akhir yang rendah dan seterusnya menghadkan aplikasi biosurfaktan kepada produk pembersihan. Biosurfaktan dalam bentuk laktonik mempunyai aplikasi yang lebih meluas dan bernilai tinggi terutama kepada industri perubatan dan farmaseutikal. Kajian ini dijalankan bagi menilai potensi penggunaan olein sawit, bahan mentah yang lebih murah dan mudah didapati untuk menghasilkan sophorolipids biosurfaktan (SLs). Dua kaedah pemulihan telah dibandingkan, iaitu pengekstrakan menggunakan pelarut konvensional dan pengekstrakan menggunakan bahan bukan pelarut iaitu melalui kaedah pengasinan keluar. Ciri-ciri SLs telah disiasat melalui teknik kromatografi dan spektrometrik bagi mengenalpasti struktur berasid-lactonik SLs.

Selepas tujuh hari penapaian, 68.9 g/L SLs telah dihasilkan daripada kultur yis *Starmerella bombicola* (ATCC 22214), dengan penambahan glukosa dan olein sawit sebagai karbon pertama dan kedua. Dua kaedah pemulihan telah dinilai iaitu Kaedah 1 (pengekstrakan menggunakan pelarut konvensional) dan Kaedah 2 (pengekstrakan menggunakan bahan bukan pelarut iaitu melalui pengasinan keluar). Bagi Kaedah 2, dua jenis garam ($Al_2 (SO_4)_3$ dan $(NH_4)_2SO_4$) telah dibandingkan. Penghasilan SLs daripada Kaedah 1 adalah 53 g/L dan produktiviti adalah 15.2 g/L.hari. Kaedah 2 dengan $Al_2 (SO_4)_3$ menghasilkan SLs yang lebih baik iaitu 96 g/L SLs dengan produktiviti sebanyak 13.7 g/L.hari. Kaedah 2 dengan $(NH_4)_2SO_4$ pula berjaya menghasilkan SLs dalam kuantiti yang paling banyak iaitu 99 g/L

dan produktiviti sebanyak 25 g/L.hari. Bagi kadar keseluruhan pengeluaran air sisa secara turutan menaik adalah Kaedah 2 ($Al_2 (SO_4)_3$) > Kaedah 2 ($(NH_4)_2SO_4$) > Kaedah 1 dimana jumlahnya adalah 2.4 L > 3 L > 5.3 L.

Ramalan kualitatif pada plat TLC pula menunjukkan kesemua sampel SLs terdiri daripada campuran berasid dan laktonik. Analisis FTIR pula menunjukkan kesemua sampel SLs yang dihasilkan daripada olein sawit mempunyai kesemua kumpulan berfungsi aktif iaitu lakton, asid karboksilik dan ester. Menurut analisis kromatografi cecair, Kaedah 2 berjaya mengurangkan kandungan berasid SLs kepada kandungan kaya laktonik. Spektrometrik jisim menjelaskan struktur yang dominan adalah C18:1, struktur diacetilated laktonik, dengan jisim molekul 688 g/mol. Fungsi fizikal SLs sebagai agen aktif permukaan juga diperakui berdasarkan keupayaan mengurangkan ketegangan permukaan air daripada 72 ke 33 mNm^{-1} serta mempunyai kepekatan misel kritikal (CMC) yang tinggi iaitu 190 mg/L. Kesimpulannya, SLs yang dihasilkan daripada olein sawit berjaya dipulihkan melalui kaedah pengekstrakan menggunakan bahan bukan pelarut dengan kadar produktiviti yang tinggi dan berjaya dibuktikan mempunyai kandungan laktonik yang tinggi.

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I certify that a Thesis Examination Committee has met on January 2014 to conduct the final examination of Nurul Nadhirah Binti Abdul Razak on her thesis entitled "Evaluation of Sophorolipids Produced by *Starmerella bombicola* using Palm-Based Olein as Carbon Source" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

SLs	Sophorolipids
RBD palm olein	Refining, bleached and deodorising palm olein
CPO	Crude Palm Oil
$Al_2 (SO_4)_3$	Aluminium sulphate
$(NH_4)_2SO_4$	Ammonium sulphate
PBS	Potassium Phosphate Buffer
cm^{-1}	Per centimetre
µm	Micrometer
nm	Nanometre
mm	Millimetre
µL	Microlitre
g/L	Gram per litre
mg/L	Milligram per litre
g/L.day	Gram per litre per day
v/v	Volume per volume
w/v	Weight per volume
mM	Millimolar
N	Normality
ºC	Degree Celsius
h^{-1}	Per hour
rpm	Revolutions per minute
μ_{max}	Maximum growth rate
t_d	Doubling time
$Y_{X/S}$	Yield of cell mass
$Y_{P/S}$	Product yield
Da	Dalton
R_f	Retention factor
RT	Retention time
MW	Molecular mass / molecular weight
m/z	Mass to charge ratio
mNm^{-1}	Millinewtons per meter
mL/min	Millilitre per minute
µV/min	Microvoltage per minute
µV/sec	Microvoltage per second
TLC	Thin Layer Chromatography
FTIR	Fourier-transform infrared spectroscopy
HPLC	High Performance Liquid Chromatography
ELSD	Evaporative Light Scattering Detector
LCMS	Liquid Chromatography Mass Spectrometry
ESI	Electron spray ionisation
CMC	Critical micelle concentration

CHAPTER 1

INTRODUCTION

1.1 Research background

Surfactants are amphiphilic compounds containing both hydrophilic and hydrophobic moieties (Soberón-Chávez & Maier, 2011). They function by lowering the interfacial tension between two fluids that have different degrees of polarity via partitioning themselves preferentially at the interface between these fluids, thus lead the formation of an ordered molecular layer or also known as micelles (Mukherjee & Das, 2010). This unique interfacial behaviour of surfactants confers an excellent characteristic in various industrial applications.

To date, substitution of petroleum-based surfactant with plant or microbial-based surfactants in oleochemicals industries is desirable to promote a greener product. This will promote reduction of NOx, hydrocarbons and carbon monoxide emission (Mandal & Jayanthi, 2011). It is also a global agenda to replace of products from petroleum-based counterpart with renewable alternative. Surfactants derived from a biological origin was namely as biosurfactant and exhibit better properties than the petroleum-based counterpart such as higher biodegradability, better environmental compatibility, higher foaming capacity, higher activity at extreme temperatures, pH levels and salinity; and lower toxicity (Campos-Takaki *et al.*, 2010).

Majority of biosurfactants producers reported are of bacterial origin including *Pseudomonas* sp. and *Burkholderia* sp. for rhamnolipids production, and *Bacillus* sp. for surfactin production (Soberón-Chávez & Maier, 2011). However, these bacterial strains exhibit a pathogenic behaviour and thus restricted their large-scale application and may not be suitable for human consumption. As an alternative, the use of 'generally regarded as safe' strain of yeast such as *Candida* sp., *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are desirable since these strains are proven as safe, non-toxic or non-pathogenic to both humans and animals (Amaral *et al.*, 2010). Most yeast surfactants have been identified as glycolipids biosurfactants.

Glycolipids biosurfactants are carbohydrates attached with long-chain aliphatic acids or hydroxyaliphatic acids. The most interesting glycolipids studied are the sophorolipids (SLs), which produced in a form of extracellular oily secretions that are heavier than water (Van Bogaert & Soetaert, 2011). Several SLs producers reported are *Candida bombicola* (former *Torulopsis bombicola*), *Candida apicola* (former *Torulopsis magnolia*), *Wickerhamiella domericqiae* and newly found was *Starmerella bombicola* (Price *et al.*, 2012). SLs production was reported in batch cultures using several types of carbon substrates, including synthetic (alkenes) or natural source (vegetable oils).

Presently, study on the production of SLs was the aim of improving the yield with using vegetable oils supplied along with glucose as carbon sources (Wadekar *et al.*, 2012b). Unfortunately, these productions need the use of relatively expensive raw materials such as rapeseed oil (Fleurackers, 2006). Even though palm oil was the most promising renewable resources for various industrial applications, the report on the use as a carbon source still lacks. Palm oil is well known as one of the most important traded agricultural products and mostly used in food manufacturing including margarine, deep-fat frying, shortening and ice creams (Edem, 2002; Lim, 2012). With advancement in technology, palm oil product also acceptable for inedible application and to-date it was widely used for producing surfactants, cosmetic products, bio-diesel fuels and biodegradable polymers (Abdul Khalil *et al.*, 2011; Lim, 2012; Mandal & Jayanthi, 2011).

Even high yield of SLs was obtained using cheaper raw material; the production process was pronounced incomplete as without an efficient and economical means of the downstream process. Typically, those downstream processing often involved unit operations that consumed high amount of solvents, which are toxic in nature. The cost for downstream processing of biological-based products reported account for 60% of the total production costs (Saharan *et al.*, 2012). Desirable process improvement can be achieved by reducing the consumption of toxic materials, reducing the amount of waste generated without affecting product yield and overall production cost.

1.2 Problem statement

Due to some limitation including technical and economic reasons, SLs biosurfactants have not been studied extensively for commercial use. The only commercially available biosurfactants are rhamnolipids and surfactin (Soberón-Chávez & Maier, 2011; Vaz *et al.*, 2012), while for SLs the commercial existing product was from oleic acid (Ribeiro *et al.*, 2012) and expensive vegetable oil, rapeseed oil (Kotland *et al.*, 2013). The issue was rising due to production of SLs using a complex composition of culture medium and the recovery strategy was limited to the solvent extraction method. Although solvent extraction method offers a simple separation of biosurfactant from impurities (Gao *et al.*, 2013; Kim *et al.*, 2009), they generate large amounts of organic wastes, which impractical for larger scale operation. Cheap and less toxic solvents have been successfully used in recovering other types of biosurfactants such as rhamnolipid via precipitation method (Schenk *et al.*, 1995).

Another limitation with the recovered SLs was they exhibit in a mixed form of acidic and lactonic contents. Separation of the polar acidic SLs from the less polar lactonic SLs was reported earlier by using a selective crystallisation in buffer solutions (Hu and Ju, 2001). However, intensive studies have reported on having the acidic form alone (Baccile *et al.*, 2013) due to their surface lowering properties made SLs acceptable for cleaning technologies and global industries, including the Japanese company Saraya (Van Bogaert *et al.*, 2007) and Belgium company Ecover (Develter and Fleurackers, 2008). The SLs in an acidic form was not stable as they allowed for further modification into a stable lactonic form (Hubert *et al*., 2012; Ma *et al*., 2012). In contrast, having SLs in lactone and acetylated form will give an advantage to the medical and pharmaceutical industries due to their properties of having the strongest inhibitory effect and antimicrobial activity of certain yeast and Gram-positive bacteria (Van Bogaert *et al*., 2007). Hence, further improvement and development of efficient recovery strategies for SLs are necessary to minimise the environmental hazards and make them being chosen for extensive applications.

1.3 Objectives

Briefly, this present study is carried out with 2 objectives as follow:

1. To investigate and compare the conventional solvent extraction with non-solvent extraction routes for the recovery of sophorolipids biosurfactant produced by a yeast culture *Starmerella bombicola* (ATCC 22214), supplemented with refined bleached deodorised (RBD) palm olein.
2. To characterise and quantify the recovered sophorolipids using different chromatographic and spectrometric techniques.

1.4 Scope of work

The scope of this study was focused on improving downstream processing with aim to reduce the consumption of organic solvents in the recovery of SLs biosurfactant. RBD palm olein was used as the secondary carbon substrate, added in the fermentation of *S.bombicola* grown in a shake flask. Fermentation method was adopted from the optimised conditions established by another colleague in the same research group. Upon harvesting, SLs was subjected to the recovery process that consists of extraction, concentration and purification steps. Two routes for recovery of SLs were compared, which are recovery through conventional solvent extraction (Route 1) and recovery through non-solvent extraction (Route 2). For Route 2, two types of salts are compared: $Al_2(SO_4)_3$ versus $(NH_4)_2SO_4$. For each route of recovery, production, yield, productivity and the amount of waste generated was assessed. Discussion was drawn based on product quality and quantity, as well as the overall economic evaluation of the recovery process from costing to the environmental awareness point of view. The second objective of this study was on characterisation via profiling and quantifying the SLs product itself, aiming to obtain the lactonic and acidic compounds of SLs. For this, the tools used were Thin Layer Chromatography (TLC), Fourier Transformation Infrared (FTIR), High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometer (LC-MS) techniques. The physical characteristic of these recovered palm-based SLs was compared accordingly.

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