PRODUCTION AND CHARACTERISATION OF NANOEMULSIONS STABILISED BY SOPHOROLIPIDS PRODUCED FROM DIFFERENT SECONDARY SUBSTRATES

By

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Chair : Associate Professor Norhafizah binti Abdullah, PhD
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Sophorolipids (SLs) produced from *Starmerella bombicola* are among the most extensively researched glycolipids biosurfactants. SLs surfactants are preferred over their chemical counterpart due to their intrinsic properties such as high biodegradability, environmentally friendly, low toxicity and excellent surface activities. However, their production is still inferior to the chemical surfactants and their performance as emulsifier are poor. There are also increasing awareness pertaining the usage of natural ingredients in commercial products as a part of sustainable greener alternative effort. Hence, this study aims to use a more affordable raw material to produce SLs which subsequently will be used as a stabiliser for nanoemulsions. Four main secondary substrates were used for production of SLs via fermentation of *S. bombicola*. These included vegetable oils such as refined, bleached and deodorised palm olein (RBD PO), RBD palm kernel olein (RBD PKO), and RBD coconut olein (RBD CO) and new palm-based waste known as fatty acid methyl ester (FAME) waste. These substrates were supplemented at 10 % v/v into the fermentation medium of *S. bombicola*. After cultivation of *S. bombicola* for 8 days, the highest SLs yield of 32.05 g/L was obtained from fermentation using RBD PO followed by RBD PKO, RBD CO and FAME waste at 11.91 g/L, 10.34 g/L and 6.51 g/L respectively. The reason was RBDPO was rich in C16 and C18 fatty acids. Fatty acid chain length of SLs produced from RBD PO and FAME waste were mainly of C16 and C18 while SLs from RBD PKO and RBD CO consisted mainly C12. All SLs produced from the various substrates exhibited surface tension reducing properties of water from 72 mN/m to a range between 33.64 and 36.98 mN/m. Critical micelle concentration values (CMC) of SLs from RBD PO, RBD PKO, RBD CO and FAME waste were 90 mg/L, 120 mg/L, 120 mg/L and 40 mg/L, respectively. The SLs were also able to displace medium chain triglyceride (MCT) oil with a diameter ranging from 2.2 to 3.45 cm. In addition, emulsification activity towards MCT oil shown by the SLs was from 20.45 to 44.70%. SLs were also studied for their antimicrobial activity towards *Bacillus cereus* and *Pseudomonas aeruginosa*. The inhibition zone was determined for *B. cereus* with diameters ranging from 7.70 to 14.54 cm, but no antimicrobial activity was observed for SLs towards *P. aeruginosa*. SLs were then used in the formulation of nanoemulsions blends (coded F1 to F9) in which F1 formulation was with soybean lecithin as sole surfactant, F2 to F5 with SLs as sole surfactant, and F6 to F9 with mixture of SLs and soybean lecithin as the second emulsifier. The nanoemulsions were made using homogenisation and ultrasonication methods. Results showed F1, F6, F7, F8 and F9 exhibited a good stability while F2, F3, F4 and F5 displayed not only unsatisfactory stability but also very large particle size exceeding nano-scale range. Among the formulations, F9 had the smallest average particle size (236 nm) with the zeta potential value of – 61.7 mV. Meanwhile, formulation F6 bigger average particle sizes (391 nm) with zeta potential value of – 75.2 mV. Formulation blends (F6 to F9) exhibited a stable emulsion in which they can withstand thermal processing from 40 ºC to 90 ºC and pH range from pH 3 to pH 9. However, these formulations were susceptible to salt concentration range of 10 mM to 300 mM when phase separations were observed. All formulation blends showed good stability during storage stability at 4 ºC and 25 ºC. At elevated temperature of 40 ºC, only F6 and F9 retained their high stability because their particle sizes remained relatively constant over the period of 30 days. However, changes in the average particle size were observed in F1, F7 and F8 over time. All of the nanoemulsions showed antimicrobial activity towards *B. cereus* and *P. aeruginosa*. Their antioxidant activities are low in the range 37 to 46% with the highest activity observed in F6 at 45.62%. Additionally, they were found to be cytotoxic towards human epithelial colorectal adenocarcinoma (Caco2) cell line with the lowest concentration to inhibit 50% of the cells (IC_{50}) of 35 µg/mL for F1 followed by F7, F8, F9 and F6 with IC values of 65, 70, 85 and 35 µg/mL, respectively. In conclusion, RBDPO was a more superior SLs feedstock while FAME waste was the sustainable substrate for SLs production. The SLs developed in this study exhibit excellent surface properties, CMC, surface-tension reducing abilities and antimicrobial activity. Nonetheless, their poor emulsifying performance forms unstable emulsion when they are used as a sole emulsifier. So, nanoemulsions can be produced using a mixture of SLs and soybean lecithin which showed greater stability towards temperature and pH stress but were unable to withstand low salt concentration. They also exhibited the antimicrobial and cytotoxic activity.

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PENGHASILAN DAN PENCIRIAN NANOEMULSI YANG DISTABILKAN OLEH SOPHOROLIPIDS YANG DIHASILKAN DARIPADA SUBSTRAT SEKUNDER YANG BERBEZA

Oleh

LEOW YEW SENG

Disember 2022

Pengerusi : Profesor Madya Norhafizah binti Abdullah, PhD
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Sophorolipids (SLs) yang dihasilkan daripada *Starmerella bombicola* adalah biosurfaktan glikolipid yang paling banyak dikaji. Surfaktan SLs lebih disukai berbanding surfaktan kimia kerana sifat intrinsiknya seperti kebolehbiodegradan yang tinggi, mesra alam, tahap ketoksikan yang rendah dan aktiviti permukaan yang sangat baik. Walau bagaimanapun, pengeluarannya masih rendah berbanding surfaktan kimia dan prestasinya sebagai pengemulsi adalah lemah Terdapat peningkatan kesedaran berkaitan penggunaan bahan semula jadi dalam produk komersial sebagai sebahagian daripada usaha alternatif yang lebih hijau. Justeru, kajian ini bertujuan untuk menggunakan bahan mentah yang lebih berpatutan untuk penghasilan SLs yang seterusnya akan digunakan sebagai penstabil nanoemulsi. Empat substrat sekunder telah digunakan untuk penghasilan SLs melalui penapaian *S. bombicola*. Mereka ialah minyak sayuran merangkumi olein sawit yang telah ditapiskan, dilunturkan dan dinyahbaukan (RBD PO), olein isirong sawit RBD (RBD PKO), olein kelapa RBD (RBD CO) dan sisa sawit baharu iaitu sisa asid lemak metil ester (FAME). Substrat-substrat ini ditambah pada 10% (v/v) ke dalam media penapaian *S. bombicola*. Setelah pengkulturan *S. bombicola* selama 8 hari, perolehan SLs tertinggi iaitu 32.05 g/L didapati daripada penapaian yang menggunakan RBD PO diikuti oleh RBD PKO, RBD CO dan sisa FAME masing-masing pada 11.91 g/L, 10.34 g/L dan 6.51 g/L. Hal ini demikian kerana RBD PO kaya dengan rantai asid lemak C16 dan C18. Komposisi utama rantai asid lemak SL yang dihasilkan daripada RBD PO dan sisa FAME terdiri daripada C16 dan C18 manakala SLs daripada RBD PKO dan RBD CO terdiri daripada rantai asid lemak C12. Semua SLs yang dihasilkan daripada semua substrat mampu mengurangkan tegangan permukaan air daripada 72 mN/m kepada julat antara 33.64 dan 36.98 mN/m. Nilai kepekatan misel kritikal (CMC) SLs daripada RBD PO, RBD PKO, RBD CO dan sisa FAME ialah 90 mg/L, 120 mg/L, 120 mg/L dan 40 mg/L masing-masing. SLs juga dapat menganjakkan minyak trigliserida rantai sederhana (MCT) dengan diameter antara

2.2 hingga 3.45 cm. Selain itu, aktiviti pengemulsian SLs terhadap minyak MCT yang ditunjukkan oleh SL diperoleh daripada 20.45 hingga 44.70%. SLs juga dikaji untuk aktiviti antimikrob terhadap *Bacillus cereus* dan *Pseudomonas aeruginosa*. Zon perencatan dapat ditentukan untuk *B. cereus* dengan diameter antara 7.70 hingga 14.54 cm tetapi tiada aktiviti antimikrob yang diperhatikan untuk SLs terhadap *P. aeruginosa*. SLs kemudian digunakan dalam formulasi adunan nanoemulsi (berkod F1 hingga F9) dengan formulasi F1 mengaplikasikan lesitin kacang soya sebagai surfaktan tunggal. Untuk, F2 hingga F5, SLs sebagai surfaktan tunggal, digunakan manakala F6 hingga F9 menggunakan campuran SLs dan lesitin kacang soya sebagai pengemulsi kedua. Nanoemulsi dibuat menggunakan kaedah campuran homogenisasi dan ultrasonik. Keputusan menunjukkan F1, F6, F7, F8 dan F9 ada kestabilan yang baik tetapi F2, F3, F4 dan F5 bukan sahaja tidak stabil tetapi juga ada saiz zarah yang sangat besar melebihi julat skala nano. Antara formulasi yang dikaji, F9 mempunyai saiz zarah purata terkecil (236 nm) dengan nilai potensi zeta sebanyak -61.7 mV. Sementara itu, formulasi F6 mempunyai saiz zarah purata yang lebih besar (391 nm) dengan nilai potensi zeta sebanyak -75.2 mV. Campuran formulasi (F6 hingga F9) mempamerkan emulsi yang stabil kerana dapat menahan pemprosesan terma dari 40 ^{o}C hingga 90 ^{o}C dan julat pH dari pH 3 hingga pH 9. Walau bagaimanapun, formulasi ini tidak stabil apabila terdedah kepada julat kepekatan garam dari 10 mM hingga 300 mM kerana fasa pemisahan diperhatikan. Kestabilan adunan formulasi F6 hingga F9 adalah baik apabila disimpan dalam suhu 4 ^{o}C dan 25 ^{o}C. Dalam suhu tinggi 40 ^{o}C, hanya F6 dan F9 mengekalkan kestabilan tingginya kerana saiz zarah kekal sama untuk tempoh 30 hari. Namun demikian, terdapat perubahan dalam saiz zarah purata untuk F1, F7 dan F8 dari semasa ke semasa. Semua nanoemulsi menunjukkan aktiviti antimikrob terhadap *B. cereus* dan *P. aeruginosa*. Aktiviti antioksidan mereka adalah rendah dari julat 37 hingga 46% dengan aktiviti tertinggi diperhatikan dalam F6 pada 45.62%. Selain itu, mereka sitotoksik terhadap adenokarsinoma kolorektal epitelium manusia, garisan sel Caco2 dengan kepekatan terendah untuk menghalang 50% sel (IC_{50}) sebanyak 35 μg/mL untuk F1 diikuti oleh F7, F8, F9 dan F6 dengan nilai IC 65, 70, 85 dan 35 μg/mL, masing-masing. Kesimpulannya, RBDPO ialah substrat SLs yang lebih unggul manakala sisa FAME ialah substrat yang mampan untuk penghasilan SLs. SLs yang diperoleh dalam kajian ini mempamerkan sifat permukaan yang sangat baik, CMC dan kebolehan mengurangkan tegangan permukaan danaktiviti antimikrob. Walau bagaimanapun, prestasi pengemulsi yang lemah membentuk emulsi yang tidak stabil apabila SLs digunakan sebagai pengemulsi tunggal dalam perumusan emulsi. Nanoemulsi boleh dihasilkan menggunakan campuran SL dan lesitin kacang soya yang menunjukkan kestabilan yang lebih tinggi terhadap tekanan suhu dan pH tetapi tidak dapat menahan kepekatan garam yang rendah. Nanoemulsi juga mempamerkan aktiviti antimikrob dan sitotoksik.

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LIST OF ABBREVIATIONS

CO	Coconut oil
FAME waste	Fatty acid methyl ester waste
PKO	Palm kernel oil
PO	Palm oil
RBD CO	Refining, bleached and deodorized coconut olein
RBD PKO	Refining, bleached and deodorized palm kernel olein
RBD PO	Refining, bleached and deodorized palm olein
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrophotometer
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
CMC	Critical micelle concentration
HLB	Hydrophilic-lipophilic balance
HPH	High pressure homogenizer
IFT	Interfacial tension
SCFA	Short chain fatty acids
RSM	Rotor stator mixer
MCFA	Medium chain fatty acids
MCT	Medium chain triglyceride
RI	Refractive index
GMO	Genetically modified organism
BL	Soybean lecithin
O/W	Oil-in-water
PI	Phase inversion

SLs	Sophorolipids
W/O	Water-in-oil
E24	Emulsification index
Caco2	Human epithelial colorectal adenocarcinoma cell line
CO_2	Carbon dioxide
DMEM	Dulbecco’s modified Eagle’s medium
MRC5	Human lung fibroblast
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RPMI	Roswell Park Memorial Institute
DPPH	1,1-diphenyl-2-picryl-hydrazyl
IC_{50}	Concentration to inhibit 50% of the cell
s^{-1}	Per second
min^{-1}	Per minute
h	Hour
nm	Nanometer
µm	Micrometer
mm	Millimeter
cm	Centimeter
ºC	Degree Celsius
rpm	Revolutions per minute
µ	Growth rate
t_d	Doubling time
µg/mL	Microgram per millimeter
mg/mL	Milligram per millimeter
mg/L	Milligram per litre

g/L	Gram per litre
mM	Millimolar
v/v	Volume per volume
wt%	Weight percent
µL	Microlitre
mL	Millilitre
k_{od}	Oil degradation rate
MPa	MegaPascal
mPa.s	Millipascal-second
mV	Millivolt
mN/m	milliNewton per meter
m/z	Mass to charge ratio
µL/well	Microlitre per well
cells/mL	Cells per millilitre

CHAPTER 1

INTRODUCTION

1.1 Research Background

Surfactants are surface active compounds which consist of two different moieties such as hydrophilic head group and lipophilic tail group (Kamal *et al.*, 2017). Their amphiphilic nature enables them to alter the interfacial tension of a liquid by adsorbing themselves to the surface layer of liquid. A group of surfactants in a form of micelle structures, are well-known for their ability to reduce surface tension of solution. Co-surfactants are also used to facilitate surfactants in lowering oil-water interfacial tension more efficiently(Brannon-Peppas, 2018; Czajka *et al.*, 2015). Surfactants can be produced from either natural or synthetic sources. They are widely used in various applications such as agriculture, pharmaceutical, detergent, microbiology and nanotechnology (Sonawane *et al.*, 2015).

Natural surfactants or biosurfactants are amphiphilic molecules produced from microorganisms. As compared to the chemical or synthetic surfactant, they exhibit properties such as biodegradable, environmentally friendly, stable in extreme environmental conditions such salt concentration, pH and temperature and less toxic compared to their chemical counterpart (Ribeiro *et al.*, 2020). There are different types of biosurfactants with sizes ranging from high molecular weight to low molecular weight such as glycolipids, lipopeptides, phospholipids, polysaccharide-protein complexes and polymeric surfactants. Each of this group contains different chemical structures and surface characteristics (Jimoh and Lin, 2019).

Among all the biosurfactants, glycolipids are the most extensively researched. They include sophorolipids, trehalose lipids, mannosylerythritol lipids and rhamnolipids. Their chemical structures consist of hydrophilic carbohydrate-based head group and hydrophobic tail which contains a long chain fatty acid (Mnif and Ghribi, 2016). The most popular glycolipids in the industry is sophorolipids which is produced extracellularly by wide range of non-pathogenic yeast strains such as *Starmerella bombicola*, *Rhodotorula bogoriensis*, *Candida apicola*, *Candida batistae*, *Candida florica* and *Candida kuoi* (Jezierska *et al.*, 2018). For sophorolipids production from these microorganisms, different carbon source such as alkanes, vegetable oils and alcohols have been incorporated in the growing medium (Ashby and Solaiman, 2020).

Among the SLs producer microorganisms, *S. bombicola* has garnered much attention from researchers recently due to its non-pathogenicity, broad range of growth temperatures (4 to 34 °C) and highest SLs productivity up to 100 g/L. This strain was first discovered in the honey of bumblebees in 1970 and later detected in high sugar-fermented vegetable extracts and concentrated grape juice. Its source of origin explains its preference to grow in environment with high sugar content (100 g/L glucose). Its growth can also be supported by consumption of secondary hydrophobic carbon substrates from fatty acids, alkanes, vegetable oils, animal fats and fatty acid methyl esters, and waste feedstocks derived from industrial by-products, waste streams and food waste (De Graeve *et al.*, 2018; Hirata *et al.*, 2021; Qazi *et al.*, 2022; Wang *et al.*, 2019). Therefore, its ability motivated this research to choose *S. bombicola* for production of SLs.

For sophorolipids production, many researchers have used different secondary carbon substrates. They were hydrophobic substrates from plants such as jatropha, karanja, neem, sunflower, rapeseed, coconut, olive and corn oil (Jadhav *et al.*, 2019; Ma *et al.*, 2020; Kim *et al.*, 2021). These raw materials are expensive but the sophorolipids yield obtained was unsatisfactory (≈ 28 g/L). This can hinder their competitiveness with commercial synthetic surfactant (Bajaj and Annapure, 2015). Another vegetable oil explored by Hirata *et al.* (2021) and Razak *et al.* (2015) was palm oil which improved the sophorolipids yield to 49.5 g/L. This oil from palm fruits can decrease the production cost of sophorolipids because they are the cheapest vegetable oil and can be found abundantly in Malaysia (Septevani *et al.*, 2015). Chen *et al.* (2020) and Wongsirichot *et al.* (2021) also mentioned the utilisation of feedstocks derived from industrial and food wastes to lower sophorolipids production costs. This approach also tackles environmental issues related to wastewater and greenhouse gases by converting wastes into value-added products (Liepins *et al.*, 2021).

Another biosurfactant of interest available commercially in market is soybean lecithin derived from soybean. It is categorised as phospholipids consisting of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. These components act as emulsifying, wetting and dispersing medium which enable soybean lecithin to exhibit emulsification properties (Nyankson *et al.*, 2015; Tripathy *et al.*, 2018). Soybean lecithin is inexpensive, readily available and can serve as functional food. It is also classified as natural ingredient, generally regarded as safe which enabled it to be incorporated in food, cosmetic and pharmaceutical industries (List, 2015; Chiplunkar and Pratap, 2017). However, there are low-abundance proteins and antinutrients (raffinose and stachyose) in soybean lecithin which may lead to stomach discomfort and allergic reactions in humans and animals (Ali *et al.*, 2020). Its function as emulsifier is also ineffective in producing stable emulsion due to insufficient steric repulsion between oil droplets (Rocchio *et al.*, 2017a).

Next, biosurfactants have been applied as one of the stabilisers for nanoemulsions by many researchers (Onaizi *et al.*, 2021; Doost *et al.*, 2019). To produce nanoemulsions, a colloidal model consists of two immiscible phases (oil and water) to encapsulate high value bioactive material. It is used to administer active substances through nanosized droplets to the targeted area (Dinshaw *et al.*, 2021). It offers several advantages such enhanced bioavailability, improved solubility and stability of compound and increased antimicrobial activity due their small droplet size for penetration (Saini *et al.*, 2019). So, it has garnered many interests for application in biotechnology, medical and functional food fields (Sasikumar and Kamalasanan, 2017; Shaker *et al.*, 2019; Das *et al.*, 2020). On the other hand, nanoemulsions preparation is a costly process because it requires specific equipment and techniques of formation to obtain reduced droplet size. Suitable surfactant and cosurfactant needs to be selected for nanoemulsions formation since

Ostwald ripening usually occurs in nanoemulsions during storage for a longer period of time. (Pagar and Darekar, 2019).

1.2 Problem Statement

Currently, many commercial synthetic surfactants are still used in pharmaceutical products because they met the criteria as a surface-active agent in established method of production and economy. However, there are concerns on the impact of their application towards consumer's health, environment and delivery of active ingredients (Panda *et al.*, 2020). Alternative option is SLs (surfactant from biological origin). However, to date, SLs remained inferior in the cost of production compared to chemical surfactants (Dolman *et al.*, 2019). Hydrophobic substrates (plant-based fatty acids) which play important role in sophorolipids production have been explored by many researchers. Although oleic acid, sunflower oil and rapeseed oil have been used, sophorolipids produced still cannot compete economically with chemical surfactants (Roelants *et al.*, 2019). Cheaper substrates from renewable sources and industrial wastes are proposed to reduce production costs of sophorolipids. In this study, a new palm-based waste from palm oil refinery wastes of fatty methyl ester production site known as FAME waste was explored. It is usually generated as wastewater which could be detrimental to the environment if left untreated. So, exploration of this waste to produce SLs through waste-to-wealth approach can achieve sustainable waste management and produce valueable bioproduct (Singh *et al.*, 2019; Xu *et al.*, 2019).

Another issue of concern is a market trend on 'clean label' in which stronger preference towards natural ingredients over synthetic additives in commercial products which creates 'clean-label' trend among manufacturers (Berton-Carabin and Schroën, 2019). For 'clean label', attentions on natural surfactant are emphasised owing to their intrinsic properties such as less toxic, biodegradable, environmental friendly and highly tolerant towards extreme environmental conditions as compared to chemical surfactants (Tmáková *et al.*, 2015). Surfactants derived from proteins and polysaccharides are suitable as stabiliser for emulsion but high concentration of these biopolymer-based surfactants is needed to obtain emulsions with small droplet sizes and good stability (Salvia-Trujillo *et al.*, 2016; Zembyla *et al.*, 2020). Another biosurfactants, sophorolipids has been applied by other researchers to form emulsion. Jiménez-Peñalver *et al.* (2020) and Koh *et al.* (2016) was able to obtain emulsion using modified sophorolipids esters, higher concentration of sophorolipids and different homogenisation speeds. However, the droplet size of emulsion was large and increased over time. Thus, another method needs to be selected for producing sophorolipids-stabilised emulsion with finer droplet size and greater stability.

1.3 Objectives

The current study of the research was conducted with two objectives as listed below:

1. to characterise sophorolipids produced from *Starmerella bombicola* culture using different types of vegetable oils and palm-based waste

2. to compare performance and stability of sophorolipids-stabilised nanoemulsions using different formulation blends with soybean lecithin

1.4 Scope of Study

This research was conducted with the aim to produce sophorolipids-stabilised nanoemulsions which used sophorolipids produced by *S. bombicola* from different sources of substrates. The substrates used in the fermentation of *S. bombicola* in shake flasks were palm oil, palm kernel oil, coconut oil and fatty acid methyl waste. After fermentation, sophorolipids were extracted and partially purified to be characterised using Gas Chromotography Flame Ionisation Detector (GC-FID) The physical characteristics of sophorolipids from different substrates were compared respectively. Later, for formation of nanoemulsions, sophorolipids and another commercial biosurfactant, soybean lecithin was incorporated in the formulation blends. The performance and stability of each formulation blends was studied based on their particle size, viscosity, zeta potential, polydispersity index, stability under different environmental stresses (pH, salinity and temperature, storage stability). They were also characterised for their antimicrobial, antioxidant, and cytotoxicity activity.

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