EVALUATION OF BIOSURFACTANT TOXICITY USING PHYTOTOXICITY ASSAY

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF BIOTECHNOLOGY (HONOURS)

FACULTY OF HEALTHAND LIFE SCIENCES
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI, MALAYSIA

2017

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ACKNOWLEDGEMENT

It had been an interesting and challenging for me to complete on this thesis. First of all, I would like to thank my parents who had supported me financially in INTI International University. Next, I would like to say thank to my friends for being there always to cherish and comfort whenever I stumbled. Without them, I would not have managed to reach this point of my studies. Next, I would like to thank all the lecturers and lab assistants who had assisted me in this project. I appreciated their act of kindness and willingness to share knowledge, expertise ideas with me whenever I seek help from them.

Last but not least, I would like to thank my supervisor, Dr. Wong Kok Kee, for assisting and guiding me during this whole project. Thank you for being patience and have never turned down me whenever I needed help and advice. I am thankful for all the time and efforts to correct my work endlessly and clarify my weakness to improve my writing skills and technique so that I could finish this thesis successfully.

ABSTRACT

The inoculum of *Pseudomonas* sp. was revived from stock culture in glycerol and inoculate into containing minimal salt BH media supplemented with 1% (v/v) *n*-hexadecane as sole carbon source. The culture was incubated for 7 days to produce the biosurfactants. The calculated biomass of biosurfactant production per gram (dried weight) of bacterial cells were 15.3 g biosurfactant/g cell biomass. The biosurfactants produced was characterized as glycolipid since positive result was obtained in sugar assay and negative result was obtained in protein assay. The ability of biosurfactant to emulsify the engine oil was found equally strong ($p<0.05$) as the commercial chemical surfactant, Triton X. Seeds of mung beans and fenugreeks were exposed to 0.6 g/L of biosurfactant to assess the phytotoxicity of biosurfactants. The length of roots and shoots of mung bean and fenugreek exposed to biosurfactant showed no significant differences when compared to seeds exposed to deionized water (negative control), but were 6-8 times longer ($p<0.05$) compared to roots and shoots exposed to Triton X (positive control). The germination index value of mung bean and fenugreek exposed to biosurfactant was 6 and 8 times respectively higher than seeds exposed to Triton X. Hence, the biosurfactant was found to be non-toxic and exhibit no inhibitory effect on the growth of mung bean and fenugreek. Mung bean was more sensitive to biosurfactant when compared to fenugreek based on the GI value obtained. Mung bean could be a better agent for evaluating the toxicity of other biosurfactant.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

d	day
°C	degree Celsius
GI	Germination Index
g/L	gram per litre
kDa	kilodalton
LAS	Linear AlkylbenzeneSulphonate
BH	Bushnell-Haas media
MDa	Megadalton
mg/L	milligram per litre
mg/mL	Milligram per millilitre
CFU/mL	Colony-Forming Units Per Milliliter
μL	microlitre
TLC	Thin Layer Chromatography
NaOCl	Sodium Hypochlorite
OD	Optical Density
nm	nanometer
mL	millilitre
min	minute
hr	hour
NB	Nutrient Broth
OECD	Organisation for Economic Cooperation and Development
%	percent
rpm	revolutions per minute

CHAPTER 1

INTRODUCTION

Biosurfactants are compounds produced and secreted on the cell surfaces of microorganisms. Hence, they are also known as surface active biomolecules (Sudhanshu, Arumugam & Tangavel, 2014). Biosurfactants are amphiphilic, which contain both hydrophilic and hydrophobic moieties. The polar head of biosurfactant may appear in different varieties like phosphate, carbohydrate or amino acid while the nonpolar tail is a hydrocarbon chain (Sreeremya, 2017). Due to this amphiphilic structure, the interfacial and surface tensions between solids, gases and liquids can be reduced and this allows the two phases to disperse and mix easily (Somasundaran & Zhang, 2001).

Different microorganisms produce different types of biosurfactants that can be categorized according to their microbial origin and chemical properties. Biosurfactants are known to be secreted by various bacteria and fungi (Varadavenkatesan & Murty, 2013). Most of the bacterial biosurfactants are glycolipid, followed by lipopeptides and surfactin. Glycolipids consist of sugar moieties linked to hydroxyaliphatic acids or long chain aliphatic acids by an ester group. Lipopeptides are biosurfactants in which a lipid is attached to a polypeptide chain. Surfactin is a cyclic biosurfactant, which composed of amino acid ring structure attached to a fatty acid chain. Fungi produce mostly glycolipid in the form of sophorolipids (Vijayakumar & Saravanan, 2015). Sophorolipids composed of dimeric carbohydrate sophorose coupled to a long-chain hydroxyl fatty acid via glycosidic linkage (Rashmi & Suresh, 2015).

Due to the amphiphilic nature and structure varieties, biosurfactants are applied in various industries like agriculture, medicine, food and cosmetic (Nikhil, Rohit, Shikha,Vaijayanti & Jaspal, 2016). In agriculture, biosurfactants can increase the bioavailability of nutrients and eliminate pathogens from plants. Biosurfactants also can be used to improve the quality of soil through soil remediation (Sachdev & Cameotra, 2013). In the field of medicine, biosurfactants exerted antifungal, antiviral and antibacterial activities that were used as therapeutics agents to combat many diseases (Rodrigues, Banat, Teixeira & Oliveira, 2006). In food industry, biosurfactants are added into food as wetting agents, dispersants, solubilizers and emulsifiers in products like dairy food, margarine and baked goods which contain oils and fats (Ranasalva, Sunil & Poovarasan, 2014). In cosmetic industry, the biosurfactants are used to produce many products like conditioners and shampoos due to their properties as emulsifier, moisturizer and cleaner. For an example, the sodium surfactin which is extremely hydrophilic can be applied to a broad range of skincare products like creams and lotions in combination with other substances (Kanebo, n.d.).

The majority of currently used surfactants in industries are produced by chemical means and are different with the biosurfactants which naturally produced by microorganisms. According to current researches, the synthetic surfactant is more toxic than the biosurfactant. The synthetic surfactants are non-biodegradable and end up in environment after use whereas the biosurfactants are biodegradable and will not have adverse impact on the environment (Makkar & Rockne, 2003). However, biosurfactants have been reported to be toxic, as demonstrated by biosurfactants produced by *L. mesenteroides* that exert cytotoxicity against mammary cell line by decreasing the cell viability (Salman, Al Marjan & Ghani, 2016). Hence, the toxicity of biosurfactant needs to be evaluated if the biosurfactants are to replace synthetic surfactant in cosmetic, food and pharmaceutical industry. The objective of this study was (1) to evaluate the toxicity of biosurfactant produced by *Pseudomonas* sp. using phytotoxicity assay, and (2) to compare the sensitivity between mung bean and fenugreek to assay biosurfactant phytotoxicity.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOSURFACTANT

Biosurfactants are chemical compounds that are synthesized by the microorganisms and secreted on the surfaces of the microbial cells or extracellularly (Desai & Banat, 1997). Structure wise, biosurfactants are amphiphilic molecules that possesses both hydrophobic and hydrophilic moiety. The hydrophobic moiety usually is a long chain fatty acid while the hydrophilic moiety can be phosphate, carbohydrate, amino acid or other compounds (Płaza, Chojniak, & Banat, 2014).Due to this amphiphilic property, the biosurfactants can accumulate between fluid phases, and reduces the interfacial and surface tensions of the fluid (Karanth, Deo & Veenanadig, 1999).

2.2 TYPES OF BIOSURFACTANT

The biosurfactants are mainly categorized by their molecular weight and chemical composition (Nikhil, Rohit, Shikha, Vaijayanti & Jaspal, 2016). Low molecular weight biosurfactants generally composed of simple fatty acids, sugars and functional groups like carboxylic acids (Uzoigwe, Burgess, Ennis, & Rahman, 2015). The low molecular weight biosurfactants are usually with a molecular mass from 1 to 2 kDa (Ramkrishna, 2010). The most commonly low molecular weight biosurfactants are the glycolipids in which its molecular weight is 1060.341 Da. Glycolipid consists of β-hydroxy fatty acids linked to different sugars and the lipopeptides which consist of fatty acids linked to cycloheptapeptides attached to amino acids.

High molecular weight biosurfactants are sometimes called bioemulsifiers, because this group of biosurfactant consists of complex mixtures of lipopolysaccharides, heteropolysaccharides, proteins and lipoproteins, with a molecular mass higher than 1 MDa (Ramkrishna, 2010). The bioemulsifiers can emulsify two immiscible liquids efficiently similar to biosurfactants but in contrast are less effective to reduce the surface tension. Thus, the bioemulsifiers can be said to have emulsifying activity only and not the surface activity (Uzoigwe, Burgess, Ennis, & Rahman, 2015). Examples of bioemulsifiers are polymeric surfactant such as emulsan with a heteropolysaccharide backbone covalently linked to fatty acids, and the particulate surfactants secreted in vesicle form by *Acinetobacter* sp. composed of lipopolysaccharide, phospholipids and protein. Table 2.1 shows the classification based on the molecular weight.

Table 2.1 Classification of biosurfactants based on molecular weight

Types	Descriptions	Examples
Low Molecular Weight	lower the interfacial and surface tension at water/air interfaces.	• glycolipids • lipopeptides
High Molecular Weight	bioemulsan. emulsify and stabilize oil in water emulsion	• polymeric surfactant • particulate surfactant

Source: Adapted from Nikhil, Rohit, Shikha, Vaijayanti & Jaspal (2016).

Furthermore, the diversity of biosurfactants is also dependent on the chemical structures. According to their chemical composition, biosurfactant can be classified into four groups which are glycolipids, lipopeptides, polymeric and particulate surfactants.

Biosurfactant from the glycolipids are molecules mainly consist of carbohydrate linked to long chain fatty acid and it can be further classified into sophorolipid, rhamnolipid and trehalose lipid (Mnif & Ghribi, 2016). The lipopeptides are biosurfactant consisting of a lipid group thatis linked to polypeptide chain (Shoeb, Akhlaq, Badar, Akhter & Imtiaz, 2013). Polymeric biosurfactants like emulsan that consists of heteropolysaccharide backbone that covalently links to fatty acids and alasan that consists of proteins, alanine and polysaccharides can act as emulsifier that stabilize oil particles during process of bioremediation (Uzoigwe, Burgess, Ennis, & Rahman, 2015). Particulate surfactants are the extracellular membrane vesicles which contain higher phospholipid and polysaccharide than the outer membrane of the microbes (Desai & Banat, 1997), such as aminolipid secreted by *Serratia marcescens* (Matsuyama, Murakami, Fujita & Yano, 1986). Table 2.2 shows the classification based on the chemical composition of biosurfactants and the structural differences as in Figure 2.1.

Table 2.2 Classification of biosurfactants based on chemical composition

Types of Biosurfactants	Chemical composition
Sophorolipid	Dimeric carbohydrate sophorose linking to long chain hydroxyl fatty acid and hydrophobic sophorosides.
Rhamnolipid	Rhamnose linking to molecules of β-hydroxydecanoic acid.
Trehalose Lipid	Two units of glucose link in α,α-1,1- glycosidic linkage.
Lipopeptide	Cyclic lipopeptides linking to a fatty acid
Polymeric Biosurfactant	Polymeric heterosaccharide containing proteins.
Particulate Biosurfactant	Extracellular vesicles consisted of phosphatidyl group link to cardiolipin, acylphosphatidyl glycerol, and triacyllysocardiolipin

Source: Adapted from Nikhil, Rohit, Shikha, Vaijayanti & Jaspal (2016)

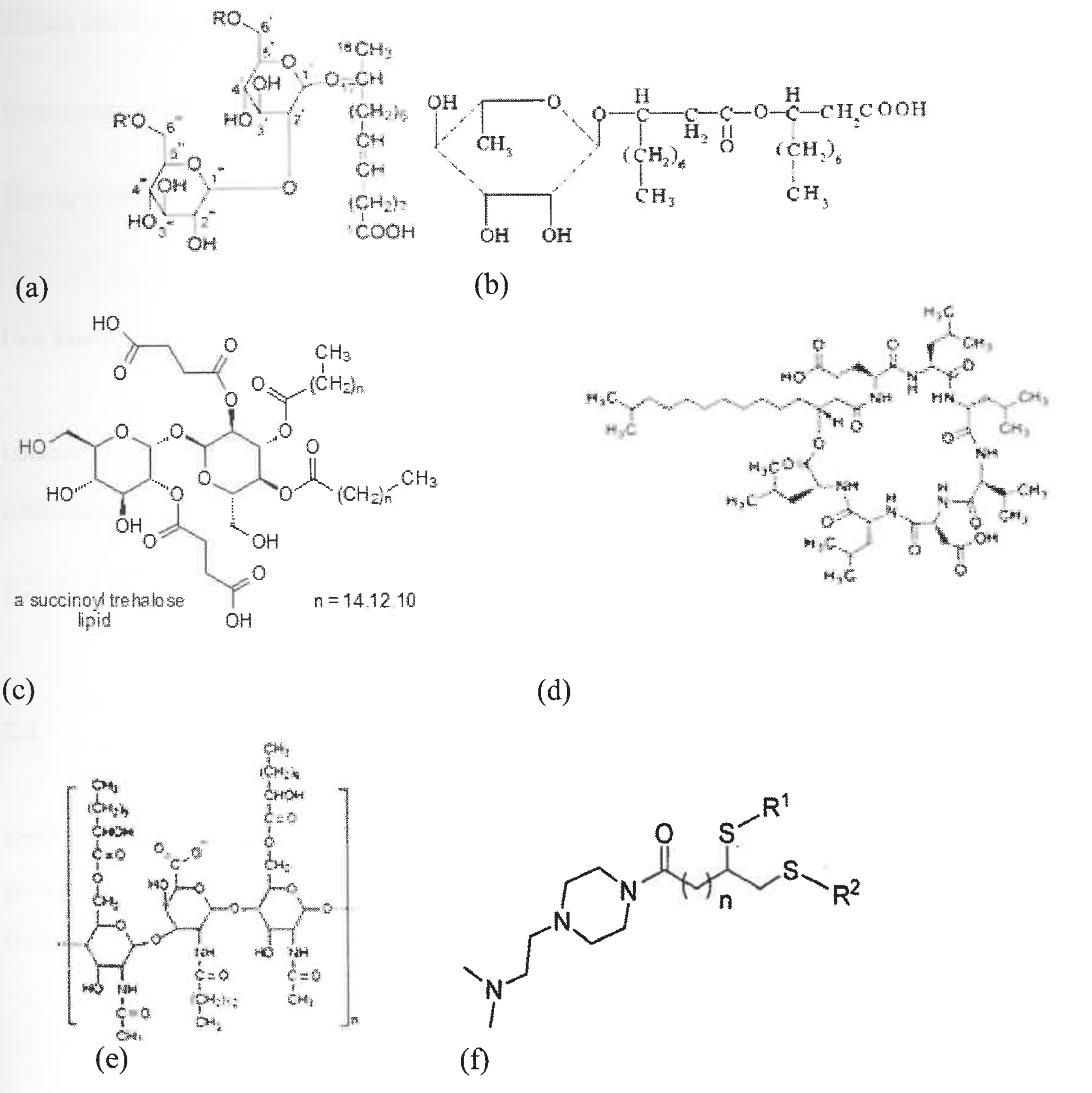

Figure 2.1 Structural differences of (a) Sophorolipid (Nattaya & Mayuree, 2009), (b) Rhamnolipid (Dahrazma, Mulligan, & Nieh, 2008), (c) Trehalose Lipid (Christie, 2017), (d) Lipopeptide- Surfactin (Silva, Soares, Lima & Santana 2015), (e) Polymeric biosurfactant- Emulsan (Christie, 2017), (f) Particulate biosurfactant (Pavel, Linxian, Gary, Yi & David, 2011)