PRODUCTION OF BIOSURFACTANT BY *ACHOMOBACTER* SP. GY30 FROM WASTE GLYCEROL AND POTENTIAL APPLICATION FOR VEGETABLE OIL EXTRACTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Environmental Management
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การผลิตสารลดแรงตึงผิวจาก *Achromobacter* sp. GY30 โดยใช้ของเสียกลีเซอรอล และศักยภาพภาพการประยุกต์ใช้เพื่อการสกัดน้ำมันพืช

นางสาวจิณณพัต ชัยสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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Thesis	PRODUCTION OF BIOSURFACTANT BY *ACHROMOBACTER* SP. GY30 FROM WASTE GLYCEROL AND POTENTIAL APPLICATION FOR VEGETAL OIL EXTRACTION
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Field of Study	Environmental Management
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จิณณพัต ชัยสิทธิ์: การผลิตสารลดแรงตึงผิวจาก *Achromobacter* sp. GY30 โดยใช้ของเสียกลีเซอรอลและศักยภาพการประยุกต์ใช้เพื่อการสกัดน้ำมันพืช (PRODUCTION OF BIOSURFACTANT BY *ACHROMOBACTER* SP.GY30 FROM WASTE GLYCEROL AND POTENTIAL APPLICATION FOR VEGETABLE OIL EXTRACTION.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. อรฤทัย ภิญญาคง, 116 หน้า.

โดยทั่วไปการสกัดน้ำมันพืชจากเมล็ดพืชจะใช้การสกัดด้วยเฮกเซน อย่างไรก็ตามเฮกเซนเป็นสารระเหย และมีความเป็นพิษต่อมนุษย์และสิ่งแวดล้อม ดังนั้นเพื่อลดการใช้เฮกเซน วิธีทางเลือกในการสกัดน้ำมันได้ถูกพัฒนาขึ้น ยกตัวอย่างเช่น กระบวนการสกัดโดยใช้น้ำ (aqueous extraction process, AEP) อย่างไรก็ตามการสกัดโดยวิธีนี้ซึ่งใช้น้ำเพียงอย่างเดียว ให้ประสิทธิภาพการสกัดที่ต่ำ สารลดแรงตึงผิวชีวภาพจึงถูกนำมาใช้เพื่อเพิ่มปริมาณการสกัดน้ำมัน ถึงแม้ว่าการใช้สารลดแรงตึงผิวชีวภาพจะเพิ่มขึ้นทุกปี ยังพบข้อจำกัดเกี่ยวกับต้นทุนการผลิตที่สูงและให้ผลิตที่ต่ำ ในการศึกษานี้ แบคทีเรียที่มีประสิทธิภาพในการผลิตสารลดแรงตึงผิวชีวภาพได้ถูกคัดแยกและคัดเลือก เพื่อศึกษา องค์ประกอบที่เหมาะสมของอาหารเลี้ยงเชื้อ จากนั้นศึกษาศักยภาพการประยุกต์ใช้ในการสกัดน้ำมันพืช ผลการทดลองพบว่า *Achromobacter* sp. GY30 ผลิตอาหารเลี้ยงเชื้อที่มีค่าแรงตึงผิว (surface tension) และค่าแรงตึงผิวระหว่างพื้นที่ประจัน (interfacial tension) ที่ต่ำ สามารถเกิดกิจกรรมอิมัลชันกับน้ำมันพืชได้สูง และให้ค่าการหลุดออกของน้ำมันที่สูง (oil detachment, %) ดังนั้นจึงถูกเลือกเป็นแบคทีเรียที่มีประสิทธิภาพในการผลิตสารลดแรงตึงผิวชีวภาพ จากนั้น ศึกษาองค์ประกอบสารอาหารที่เหมาะสมต่อสายพันธุ์ GY30 ในการผลิตสารลดแรงตึงผิวชีวภาพ ปัจจัยที่ศึกษา ได้แก่ ความเข้มข้นของของเสียกลีเซอรอลชนิดและความเข้มข้นของแหล่งไนโตรเจน ค่า C/N และการเติมพรีเคอเซอ โดยใช้การศึกษาทีละตัวแปร และการใช้แบบจำลองการทดลอง สำหรับการศึกษาทีละตัวแปรพบว่า การผลิตสารลดแรงตึงผิวชีวภาพเพิ่มขึ้น 2 เท่า เมื่อใช้ของเสียกลีเซอรอล 5% (w/v) $NaNO_3$ 0.4% (w/v) และเติมน้ำมันปาล์ม 0.1% (v/v) อย่างไรก็ตามองค์ประกอบของอาหารเลี้ยงเชื้อที่เหมาะที่สุดคือการใช้ ของเสียกลีเซอรอล 7% (w/v) $NaNO_3$ 0.4% (w/v) และเติมน้ำมันปาล์ม 0.01% (v/v) ซึ่งมี ค่า C/N เท่ากับ 42 โดยสภาวะนี้ได้จากการศึกษาโดยใช้แบบจำลองทางสถิติ ซึ่งสภาวะนี้ผลิตสารลดแรงตึงผิวชีวภาพได้สูงสุด 0.79 g/l นอกจากนี้ยังพบว่าสารลดแรงตึงผิวชีวภาพจากการทดลองนี้เพิ่มสูงขึ้นจากการทดลองก่อนหน้าถึง 20 เปอร์เซนต์ จากนั้นศึกษาศักยภาพการประยุกต์สารลดแรงตึงผิวชีวภาพจากสายพันธ์ GY30 ในการสกัดน้ำมันพืช ผลการทดลองพบว่า ปริมาณน้ำมันที่มากที่สุดที่หลุดออกจากเมล็ดพืชคิดเป็น 64% เมื่อใช้น้ำเลี้ยงเชื้อที่ปราศจากเซลล์ นอกจากนั้น ยังพบว่า การใช้สารละลาย ที่มีสารลดแรงตึงผิวชีวภาพอย่างหยาบ น้ำมันเนื้อในปาล์มที่ได้อยู่ในรูปของน้ำมันอิสระ ซึ่งเป็นสิ่งที่ต้องการในการสกัดน้ำมัน จากศึกษาปริมาณกรดไขมันอิสระ พบว่า น้ำมันที่สกัดได้โดยวิธีนี้มีคุณสมบัติเทียบเคียงกับน้ำมันที่สกัดได้โดยใช้เฮกเซน และยังมีคุณสมบัติดีกว่าน้ำมันที่ได้จากการฮีบ ดังนั้น การใช้สารละลายที่มีสารลดแรงตึงผิวชีวภาพอย่างหยาบในการสกัดสำหรับสกัดน้ำมันเนื้อในปาล์ม เป็นวิธีทางเลือกที่น่าสนในเพื่อลดการใช้เฮกเซน

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5387507020: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS: BIOSURFACTANT / *ACHROMOBACTER* SP. / SURFACE TENSION / WASTE GLYCEROL / VEGETABLE OIL EXTRACTION

JINNAPAT CHAIYASIT: PRODUCTION OF BIOSURFACTANT BY *ACHROMOBACTER* SP.GY30 FROM WASTE GLYCEROL AND POTENTIAL APPLICATION FOR VEGETABLE OIL EXTRACTION. ADVISOR: ASST. PROF. ONRUTHAI PINYAKONG, Ph.D., 116 pp.

Vegetable oil is commonly extracted from oilseeds by solvent extraction with hexane. However, hexane is volatile and toxic to human and environment. To reduce the use of hexane, alternative methods of oil extraction have been developed such as aqueous extraction process (AEP). Nevertheless, AEP by using water alone has low oil extraction efficiency. Biosurfactant can be used to improve the oil extraction yield in AEP. Although the use of biosurfactants has been increasing over the year, their large scale production and application are limited with high production cost and low productivity. In this study, an effective biosurfant-producing bacterium was isolated and selected for investigated their optimum medium composition and examined for their potential application in vegetable oil extraction. *Achromobacter* sp. GY30 which produced low surface and interfacial tension of medium, high E_{24} (%) with vegetable oil, and high oil detachment (%), was selected as an effective biosurfactant-producing bacterium. Strain GY30 was then determined for its optimum culture medium composition for biosurfactant production. The parameters affected biosurfactant production; waste glycerol concentration, nitrogen source and concentration, C/N ratio, and precursor supplementation, were evaluated by using classical method (study one factor at a time) and experimental design. From using the classical method, increasing biosurfactant yield of 2 fold was observed when using 5% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.1% (v/v) palm oil. The best culture medium composition of 7% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.01% (v/v) palm oil, correlated to C/N ratio of 42, was obtained when using experimental design. It produced high biosurfactant yield of 0.79 (g/l). Moreover, it could enhance productivity 20% from the previous experiment. Then, the potential application of biosurfactant from strain GY30 in vegetable oil extraction was studied. The highest oil detachment of 60% was obtained when using cell-free broth. In addition, it was found that isolated biosurfactant solution could extract palm kernel oil in free oil form which is favorable in the oil extraction process. For free fatty acid content, it was found that extracted oil from this method was comparable to those of the hexane method and much better than those of mechanical pressing. Thus, the use of isolated biosurfactant in aqueous-based method for palm kernel oil extraction was of the interest alternative method for reducing the use of hexane.

Field of Study: Environmental Management Student's Signature: .....

Academic Year: 2011 Advisor's Signature: .....

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NOMENCLATURE

A_{600}	Absorbance at wavelength 600 nanometer
g	Gram
LB	Luria-Bertani
mN/M	Millineton per meter
ml	Millilitre
mM	Millimolar
M	Molar
O.D.	Optical Density
N	Normal

CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Vegetable oil is conventionally extracted from oilseeds by using mechanical pressing alone or combines with solvent extraction in order to achieve high oil extraction yield. Solvent extraction with hexane is commonly used in large scale production due to it give high oil extraction efficiency more than 95% (Rosenthal *et al.*, 1996). However, hexane is volatile and toxic to both human and environment and it requires expensive equipment to handle. Moreover, hexane from vegetable oil production processes has been recognized by US Environmental Protection Agency to be the major sources of a single hazardous air pollutant (HAP) (EPA, 2001). Based on environmental point of view, alternative technologies such as aqueous extraction process (AEP) have been investigated to reduce the use of hexane.

Aqueous extraction process (AEP) has believed to be as a cleaner, cheaper and safer alternative technology for oil extraction (Rosenthal *et al*., 1996). However, AEP by using water alone has the limitation of lower oil extraction efficiency (less than 70%) and de-emulsion treatment is required to be conducted when emulsion are formed (Rosenthal *et al.*, 1996). In addition, AEP requires high temperature (50-60°C) and high water/solid ratio (20/1 to 30/1) which are undesirable in application (Do and Sabatini, 2010). To solve the problem, the use of biosurfactants can enhance the oil extraction yield in AEP.

Biosurfactants are surface-active agents which produced by various microorganisms. They consist of two parts-a hydrophilic moiety and a hydrophobic moiety. They have many advantages over synthetic counterparts including lower toxicity, biodegradability, better environmental compatibility, high selectivity and specific activity at extreme condition, and can be produced from renewable substrates

(Georgiou *et al*., 1992; Benincasa, 2007; Muthusamy *et al.*, 2008; Lotfabad *et al.*, 2009; Lima *et al.*, 2011). Moreover, they can be applied in many applications especially in environmental application (Pacwa-Plociniczak *et al.*, 2011). Although biosurfactants have been widely used, the big challenge about their commercialization with high production cost and low productivities still exists (Banat *et al.*, 2010).

Biosurfactant production can be affected by many factors such as carbon and nitrogen source and concentration, carbon–nitrogen ratio, and the availability of nutrient (Lotfabad *et al.*, 2009; Roldan-Carrillo *et al.*, 2011). Moreover, the media composition is also play an important role in production yield. Besides, using renewable resources as substrate is one of the strategies to minimize production cost and obtain the maximum biosurfactant yield as well (Mukherjee *et al.*, 2006). Waste glycerol is waste from biodiesel production process. It has been received much attention due to the rise of biodiesel production over the year produced large amount of waste glycerol (Morita *et al.*, 2007).

This research therefore used waste glycerol as substrate to produce biosurfactant from an effective biosurfactant-producing bacterium. Along with media optimization by changing one variable at a time and using experimental design were applied. Then, potential application of biosurfactant for vegetable oil extraction was studied by using aqueous biosurfactant-based extraction and determined in term of oil detachment (%) compared to extracted oil by hexane method.

1.2 Objectives

The main object of this study was to product biosurfactant from an effective biosurfactant-producing bacterium by using waste glycerol as substrate and study potential application of biosurfactant on vegetable oil extraction by using aqueous biosurfactant-based extraction. The specific objectives are:

1.2.1. To isolate and screen biosurfactant-producing bacteria by using waste glycerol, slop oil or soybean oil as substrate.

1.2.2. To investigate the optimal condition of biosurfactant production.

1.2.3. To reduce the use of hexane by using aqueous biosurfactant-based extraction.

1.3 Hypothesis

Optimization of media component for biosurfactant production can obtain high biosurfactant yield. Moreover, using industrial waste such as waste glycerol as substrate in biosurfactant production may reduce waste disposal. Furthermore, using aqueous biosurfactant-based extraction can reduce the use of hexane in vegetable oil extraction.

1.4 Scope of work

This research was divided into three phases as follow:

Phase 1: Screening and isolation of an efficient biosurfactant-producing bacterium.

In this phase biosurfactant-producing bacteria from vegetable samples were enriched by using waste glycerol, slop oil, and soybean oil as substrate. Then, biosurfactant-producing bacteria were screened based on emulsification activity with vegetable oil and they were isolated to obtain pure cultures. Next, selected biosurfactant-producing bacteria from laboratory library combined with isolated strains from previous step were cultivated and selected again based on their carbon source (waste glycerol, slop oil, and soybean oil), surface tension (ST, mN/m) lower than 35 mN/m, and emulsification with vegetable oil (%). Then, supernatant of selected strains were determined for interfacial tension (IFT, mN/m) with vegetable oil (jatropha and palm oil) by using tensiometer with ring and 2-3 of the bacterial strains which have the lowest interfacial tension were chosen for testing oil detachment (%). A bacterial strain which achieved the highest oil detachment was obtained for studying production of its biosurfactant in the further step.

Phase 2: Production of biosurfactant by selected bacterium.

In term of reducing production cost, waste glycerol was considered to be used as substrate for biosurfactant production.

An effective biosurfactant-producing bacterium was chosen and studied effect of various carbon concentrations, nitrogen sources and concentrations, precursor supplementations by using classical method (varied one factor at a time). Then, response surface methodology (RSM) was applied using a two-level full factorial design with three variables; carbon concentration, nitrogen concentration, and precursor supplementation. Biosurfactant yield was used as the response variable to obtain the optimal condition and high biosurfactant yield. Next, properties of biosurfactant were investigated in term of surface and interfacial tension (mN/m), emulsification activity, critical micelle concentration (CMC), and identification of ionic charge of crude biosurfactant.

Phase 3: Application of the biosurfactant in vegetable oil extraction by using aqueous-biosurfactant based extraction.

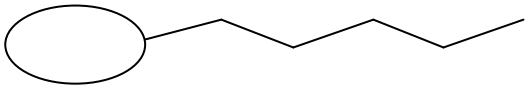
Oil kernel seeds were prepared and extracted by using cell-free broth or the solution of the crude biosurfactant lower CMC, at CMC, and above CMC. Oil detachment (%) of each condition was calculated compare to oil extracted using hexane method. Then, extracted oil using hexane and aqueous biosurfactant-based extraction method in this study as well as extracted oil received from Suksomboon Palm Oil Industry, using mechanical pressing, were examined oil clarity, color, and free fatty acids existing.

CHAPTER II

LITERATURE REVIEW

2.1 Biosurfactants

Biosurfactants are amphiphilic compounds produced by various microorganisms. They compose of a hydrophilic head (carbohydrate, amino acid, phosphate, or cyclic peptide) and a hydrophobic tail (saturated, unsaturated and hydroxylated fatty acids or fatty alcohol) (Lang, 2002). Biosurfactants have many advantages over synthetic counterparts include lower toxicity, biodegradability (Lima *et al.*, 2011), better environmental compatibility (Georgiou *et al.*, 1992), high selectivity and specific activity at extreme temperatures, pH, and salinity (Muthusamy *et al*., 2008; Lotfabad *et al*., 2009), and can be produced from renewable substrates (Benincasa, 2007).



Hydrophilic head Hydrophobic tail

Fig. 2.1 Biosurfactant structure (Pacwa-Płociniczak *et al.*, 2011)

2.2 Classification of biosurfactants

Biosurfactants can be classified into many groups based on their chemical composition, properties, molecular weight, mode of action and microbial origin. According to molecular weight, they can be divided into low molecular-mass biosurfactants (glycolipids, lipopeptides and phospholipids) and high molecular-mass polymers such as polymeric and particulate surfactants (Rosenberg and Ron, 1999; Pacwa-Płociniczak *et al*., 2011). Moreover, the ionic charge of biosurfactants depends

on their hydrophilic head group. Most biosurfactants are either anionic or neutral (Muthusamy *et al*., 2008). Using biosurfactants can be lower surface and interfacial tension to promote mobilization or solubilization (low molecular-mass), or used as effective emulsion-stabilizing agents to enhance emulsification (high molecular-mass) (Fig. 2.2).

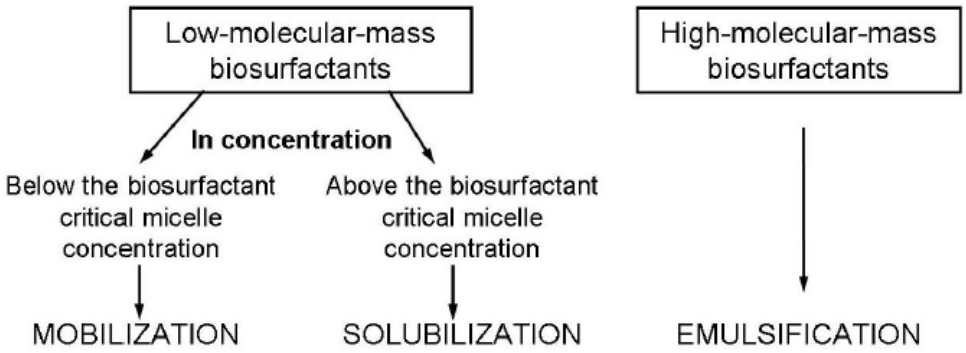


Fig. 2.2 Mechanisms depend on the molecular mass and concentration of biosurfactants (Pacwa-Płociniczak *et al*., 2011)

2.3 Properties of biosurfactant

2.3.1.1 Surface and interfacial tension

Owing to biosurfactants are surface-active agents, they have both hydrophilic and hydrophobic parts, allowing them to favorable present at interface of two immiscible phases (air–water and oil–water). Accumulation of biosurfactants reduce repulsive force between two dislike phases resulting in reduction of surface (liquid-air) or interfacial (liquid-liquid) tension and allowing these two phases to mix and interact easily. Surface and interfacial tensions are measured in term of mN/m or dyne/cm. Generally, the ability to lower surface and interfacial tensions is used to be the criteria to determine the effective biosurfactant (Soberon-Chavez and Maier, 2011). The effective biosurfactant can reduce the surface tension between water and

air from 72 to 35 mN/m and the interfacial tension between water and *n*-hexadecane from 40 to 1 mN/m (Desai and Banat, 1997; Soberon-Chavez and Maier, 2011).

2.3.2 Emulsification

Biosurfactants have ability to emulsify water immiscible compounds, normally oil. Emulsions are thermodynamically stable dispersion of water and oil. They contain tiny particles of one liquid suspended in another. Emulsification activity is determined in term of emulsification index (E_{24}, %) by measure height of emulsion divided by height of total mixture after 24 hours (Yin *et al*, 2009). E_{24} could be used as reliable factor to determine the quantity of biosurfactant (Pal *et al.*, 2009)

2.3.3 Critical micelle concentration (CMC)

Addition of biosurfactants decrease surface tension until a certain point which surface tension cannot be reduced anymore. At this point, biosurfactant monomers are formed micelle and concentration of biosurfactant is called critical micelle concentration (CMC) (Fig. 2.3). The CMC is generally used to determine the efficiency of biosurfactant. An efficient biosurfactant has low CMC, which meaning low biosurfactant concentration is required to reduce surface tension (Desai and Banat, 1997).

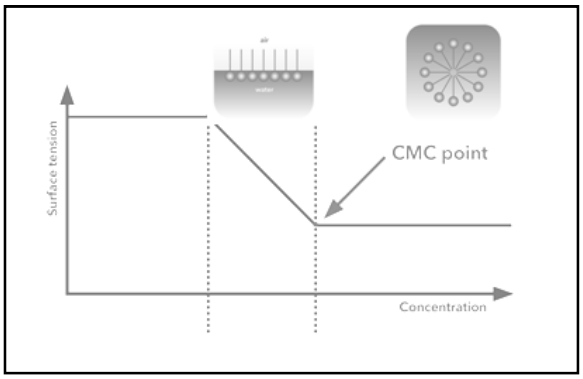


Fig. 2.3 The relationship between biosufactant concentrations, surface tension reduction, and formation of micelles (adapted from http://www.attension.com/critical-micelle-concentration.aspx)

2.4 Biosurfactant production

Owing to vast advantages of biosurfactants and their potential applications in many fields especially in environmental application, the use of biosurfactants have been increasing over the year (Fig. 2.4). Although biosurfactants are widely used in many applications, the drawbacks of biosurfactant production still exist with high cost production and low biosurfactant yield (Mukherjee *et al.*, 2006)

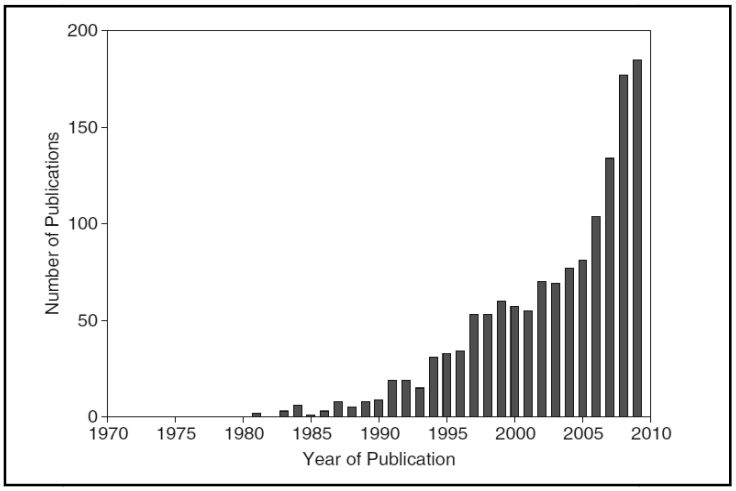


Fig. 2.4 The number of biosurfactant publications obtained from a year by year. (Soberon-Chavez and Maier, 2011)

2.4.1 Low-cost substrate

However, it has been reported that raw materials account for 10–30% of the total production cost in most biotechnological processes (Muthusamy *et al*., 2008). Thus, using renewable resources, low-cost raw materials, or industrial waste as substrate (Table 2.1) in biosurfactant production to reduce the production cost has been received much attention from many researchers.

Nitschke and Pastore (2006) studied biosurfactant produced by *Bacillus subtilis* LB5a using cassava wastewater as substrate. They found that the biosurfactant could reduce surface tension of medium to 26.6 mN/m and crude biosurfactant yield of 3 g/l was obtained after 48 hours. Benincasa, in 2007, also studied rhamnolipid

produced by *Pseudomonas aeruginosa* LBI growing on soapstock as the substrate. According to Thavasi *et al.* (2011), *Lactobacillus delbrueckii* cultured by using peanut oil cake as the carbon source and found that maximum biosurfactant concentration of 5.35 mg/ml was obtained after 144 hours of incubation.

Table 2.1 Use of inexpensive raw materials for the production of biosurfactants by various microbial strains (adapted from Muthusamy *et al*., 2008)

Low cost or waste raw material	Biosurfactant type	Producer microbial strain	yield (g/l)	Reference
Rapeseed oil	Rhamnolipids	*Pseudomonas* sp. DSM 2874	45	Trummler *et al*., 2003
Babassu oil	Sophorolipids	*Candida lipolytica* IA 1055	11.72	Vance-Harrop *et al*., 2003
Turkish corn oil	Sophorolipids	*Candida bombicola* ATCC 22214	400	Pekin *et al*., 2005
Sunflower and soybean oil	Rhamnolipid	*Pseudomonas aeruginosa* DS10–129	4.31	Rahman *et al*., 2002
Sunflower oil	Lipopeptide	*Serratia marcescens*	2.98	Rahman *et al*., 2002
Oil refinery waste	Glycolipids	*Candida antarctica,Candida apicola*	10.5	Deshpande and Daniels, 1995
Curd whey and distillery waste	Rhamnolipid	*Pseudomonas aeruginosa* BS2	0.92	Dubey and Juwarkar, 2004
Potato process effluents	Lipopeptide	*Bacillus subtilis*	2.7	Noah *et al*., 2005

2.4.1.1 Soybean oil

Soybean oil is the major vegetable oil produced in the world. In 1998, it accounted for 80–90% of total edible oil consumption in the US (Gunstone, 2002). Soybean oil is a lipidic carbon source and has a high content of linoleic acid which was suggested for the increase in biosurfactant production by some microorganisms (Ferraz *et al.*, 2002). Moreover, several researchs have shown that soybean oil can be

an effective low cost raw material for production of biosurfactant (Kitamoto *et al.* 1993; Kim *et al.*, 2006; Thaniyavarn *et al.*, 2008; Abdel-Mawgoud *et al*., 2009).

Kitamoto *et al.* (1993) studied surface active properties and antimicrobial activities of two kinds of mannosylerythritol lipids (MEL-A and B) produced by *Candida antarctica* T-34 when grown on soybean oil. The result showed, at critical micelle concentrations (CMCs), MEL reduced the surface tension and the interfacial tension against *n*-tetradecane to 28 and 2 mN/m , respectively.

Thaniyavarn *et al.* (2008) reported biosurfactant production by *Pichiaanomala* PY1 grown on various carbon and nitrogen sources. They found that the optimum condition for the sophorolipid production was obtained when using 4% soybean oil as carbon source culturing at 30°C, pH 5.5 for 7 days. The surfact tension of medium decreased to 28 mN/m under this cultivation condition.

Rahman *et al.* (2002) also used soybean oil as substrate for biosurfactant production by *Pseudomonas aeruginosa* DS10-129. They found that 4.31 g/l of rhamnolipid was obtained after culturing 288 hours.

Another study was Abdel-Mawgoud *et al.* (2009), They cultured *Pseudomonas aeruginosa* isolate Bs20 for rhamnolipid production using a mineral salts medium with soybean oil as the carbon source. The biosurfactant could lower the surface tension of water to 30 mN/m at the concentration about 13.4 mg/l. Moreove, it presented emulsifying capacity and thermo and halo tolerance properties which could be applied in bioremediation of hydrocarbon-contaminated sites or in the petroleum industry.

However, using soybean oil as substract for biosurfatant production producted coexisting by-products such as free fatty acids and mono- or diacylglycerols which is obstructive the isolation of biosurfactant (Morita *et al.*, 2007)

2.4.1.2 Industrial waste

The use of industiral waste such as waste glycerol (WG) and slop oil (SO) as substrate in biosurfactant prodution is of the interest as well because not only it can economize the production cost but it is also reduce amount of waste. WG is waste from biodiesel production process (Fig. 2.5). It has been received much attention due to the rise of biodiesel production over the year cause accumulation of waste (Fig. 2.6). Moreover, WG which is water-soluble carbon source can be easily used by many microorganisms. While SO is waste from petroleum refining process, petrochemical manufacturing, and oil spilled.

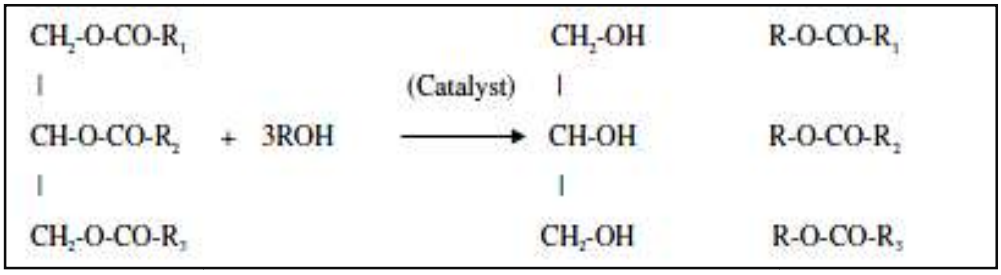


Fig. 2.5 Transesterification of triglyceride and alcohol (Leung *et al*., 2010)

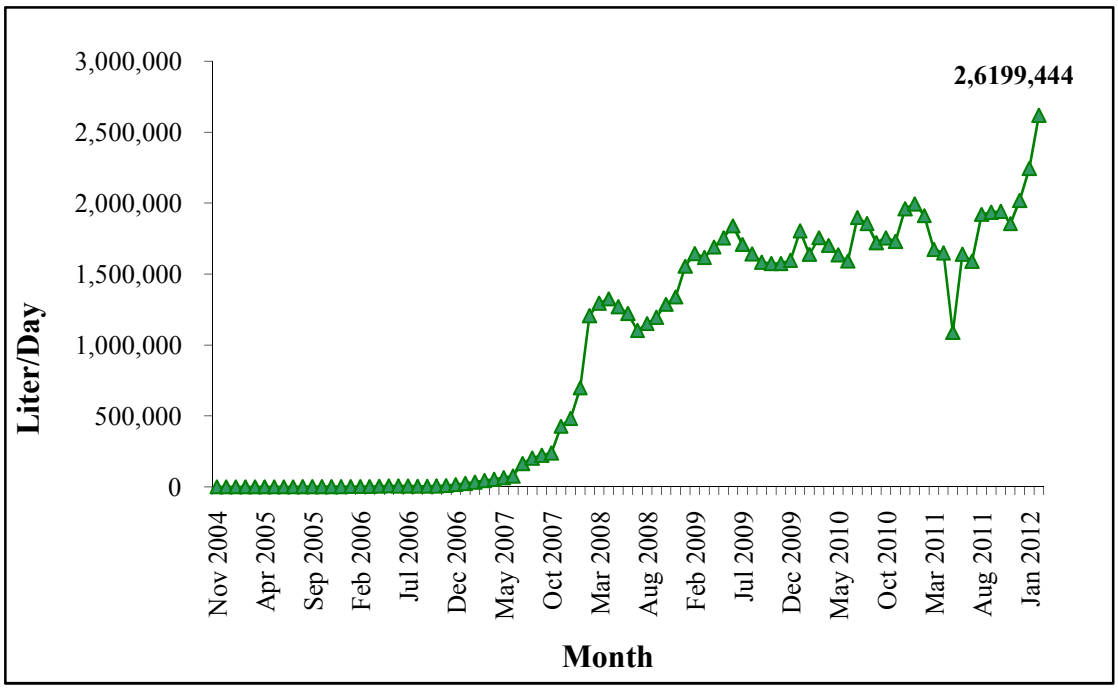


Fig. 2.6 Consumption of biodiesel (B100) in Thailand over nine years (Department of Alternative energy Development and Efficiency, Ministry of Energy)

1.) Waste glycerol

Waste glycerol (WG) is byproduct from transesterification in biodiesel industry. WG has different impurities and glycerol concentration depend on the chemical used and the biodiesel production process. Normally, WG contains glycerol, methanol, salt, free fatty acid, alkali-catalyst, and soap (Thompson and He, 2006; Pyle, 2008; Leung *et al.*, 2010).

The utilization of WG in biosurfactant production has been received much attention by many authors because it can reduce production cost and also amount of waste in the same time. Lui *et al.* (2011) investigated bioconversion of crude glycerol received from alkali-catalyzed transesterification of waste cooking oil with methanol. They found that *Ustilago maydis*could could produce high biosurfactant of 32.1 g/l when culturing in synthetic medium (MinCG) containing 50 g/l crude glycerol and 20.3 mg/l ammonium citrate as the carbon and nitrogen sources, at pH 4 and 30˚C. Furthermore, they observed that the present of methanol at 2% or over could inhibit

cell growth and production of glycolipids. Thus, the autoclave of crude glycerol must be required to remove methanol.

Another example of the use of waste glycerol as energy source is de Sousa *et al*. (2011). They studied the use of a co-product of biodiesel production (transesterification of castor bean oil by methanol in alkaline medium (NaOH)) as carbon source for biosurfactant production by *P. aeruginosa* MSIC02. The strain MSIC02 was grown in mineral medium containing 5% (w/v) of different carbon sources; crude glycerin (obtained after methanol removing by evaporation), hydrolyzed glycerin (prepared by acid hydrolysis of crude glycerin), soybean oil, or castor oil) and using 2.4 g/l of nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$ or peptone). The result revealed that using hydrolyzed glycerin and $NaNO_3$ as carbon and nitrogen source gave the highest rhamnolipid concentration of 1269.79 mg/l.

2.) Slop oil

Slop oil (SO) is waste oil from petroleum activities along with oil spill accidents. It consists of at least 240 hydrocarbon components which are 54% of C5 to C11 and the rest of C12 to C23. It is inflammability and toxicity (Dave *et al.*, 1994).

The biodegradability of slop oil by microorganism was reported by Dave *et al.* (1994). They isolated bacteria from slop oil and active sludge contaminated soil from petrochemical industry. They observed that 7 out of 22 isolated strains have ability to grow and degrade slop oil. When testing these isolated strains in liquid medium, they found that each of 7 strains could degrade slop oil about 40 %, and mixture of 7 strains could degrade slop oil lower than 50%. They also studied degradation of slop oil in contaminated soil by using mixed strains and found that slop oil could be degraded to 70% after 30 days. The germination and growth of wheat seedlings in contaminated soil was examined. They revealed that slop oil was phytotoxic to wheat seedling, as observed by delayed and decreased germination and growth when grew in sterile contaminated soil. However, they found that bioaugmentation of contaminated soil with a mixed bacterial culture could decrease phytotoxicity of slop oil. Up to 80%

of slop oil could be degraded after 30 days when wheat seedling grew in the contaminated soil with bioaugmentation of mixed bacterial culture.

However, the study of slop oil conversion into useful compounds (bio-based material) or using as substrate for biosurfactant production has not been reported yet.

2.4.2 Factors impacted biosurfactant production

For improvement of biosurfactant yield, there was evidence that the culture medium composition plays an important role in the biosurfactant production by microorganisms (Silva *et al*., 2010). The quality and quantity of biosurfactant can be affected by many factors such as carbon and nitrogen source and concentration, and C/N ratio (Mukherjee *et al.*, 2006).

2.4.2.1 Carbon source and concentration

Many water-soluble carbon sources such as glucose, mannitol, and ethanol as well as water-immiscible substrates such as *n*-alkanes and olive oil were used for biosurfactant production (Desai and Banat, 1997; Lafabad *et al.*, 2009).

Robert *et al.* (1989) studied effect of various carbon sources for biosurfactant production by *Pseudomonas aeruginasa* 44T1. They applied 2% (w/v) of different carbon sources; glucose, fructose, sodium acetate, sodium succinate, sodium pyruvate, sodium citrate, glycerol, mannitol, olive oil, and *n*-alkanes C10 to C16, into 100 ml mineral salt medium. The culture media were shaken at 200 rpm and incubated at 30°C. Using *n*-alkanes as a carbon source, the significant amount of rhamnolipid was produced only when strain 44T1 was grown with C12 *n*-alkanes and low amount of biosurfactant received when grew on C11 *n*-alkanes. They also found that strain 44T1 was able to grow and produce biosurfactant when cultured on glycerol, mannitol and glucose. However, the highest biosurfactant of 7.65 g/1 with a production yield of 38.2% was obtained when using olive oil as carbon source.

Abouseoud *et al.* (2008) also examined effect of different carbon source on biosurfactant production by *Pseudomonas fluorescens* Migula 1895-DSMZ, and measured in term of surface tension (ST, mN/m) and emulsifying index (E_{24}). The bacterial strain was grown in mineral salt medium with different carbon sources; *n*-hexadecane (2%, w/v), olive oil (2%, w/v), and glucose (20 g/l), and using NH_4Cl as nitrogen source. They found that using olive oil as carbon, gave the best result (ST of 32 mN/m and E_{24} of 65%).

2.4.2.2 Nitrogen source and concentration

Nitrogen source and concentration play a critical role on the type and concentration of the biosurfactant production (Silva *et al*., 2010).

Anna *et al.* (2002) assessed the potential production of biosurfactant from *Pseudomonas aeruginosa* PA1 by using glycerol as substrate. Different nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$, and CH_4N_2O) was studied. They found that, the highest ramnolipid of 3.16 g/l was obtained when using $NaNO_3$ as nitrogen source, corresponding to C/N ratio of 60.

Silva *et al.* (2010) observed the most efficient nitrogen source for biosurfactant production from *Pseudomonas aeruginosa* UCP0992 grown on glycerol. The nitrogen sources were evaluated at 0.2%; $NaNO_3$, NH_4NO_3, urea, $(NH_4)_2SO_4$, peptone, yeast extract and corn steep liquor. They found that $NaNO_3$ was the most efficient nitrogen sources. The use of 0.6% $NaNO_3$ in medium containing 3% glycerol showed the best result and produced crude biosurfactant of 8 g/l after 96 hours.

2.4.2.3 C/N ratio

Another factor that affected the performance biosurfactant production was the ratio C/N (Santos *et al*., 2002).

Wu *et al.* (2008) investigated the influence of C/N ration on rhamnolipid production by *Pseudomonas aeruginosa* EM1 using glycerol and $NaNO_3$ as carbon and nitrogen source. The results indicated that the highest ramnolipid yield of 7.5 g/l was achieved when using C/N ratio of 52. Moreover, it was observed that a C/N ratio of 6.5 to 52 gave the similar productivity, but rhamnolipid production was rapidly decreased when the C/N ratio was too high (130).

In 2009, Lotfabad *et al.* studied effect of various C/N ratios on biosurfactant produced by *Pseudomonas aeruginosa* MR01 isolated from oil excavation areas in Iran. Different C/N ratios of 10/1, 20/1, 30/1, 40/1, and 50/1 were applied and evaluated in term of biosurfactant yield (g/l), dry cell weight (DCW, g/l), and surface tension of medium (mN/m). They found that using glucose and $(NH_4)_2SO_4$ as carbon and nitrogen sources respectively, C/N of 10 to 30 gave the similar results, while the biosurfactant yield slightly decrease when the C/N ratio increased from 40 to 50. The maximum biosurfactant production and DCW up to 0.84 g/l and 1.65 g/l, respectively, were obtained using C/N ratio of 20.

2.4.2.4 Supplementation of precursor

Besides adjusting of carbon and nitrogen source and concentration as well as C/N ratio, precursor supplementation is one of the factors which can improve biosurfactant yield. It is well known that water-insoluble stimulate biosurfactant production (Stuwer *et al.*, 1987; Ferraz *et al.*, 2002).

Cooper and Paddock (1984) produced glycolipid from *Torulopsis bombicola* ATCC 22214 in the standard medium and using two types of carbon sources (glucose and safflower oil). They found that small amount of glycolipid was obtained when glucose or safflower oil presented alone. However, when *T. bombicola* cultured with glucose first and then added safflower oil, the biosurfactant yield was increased to 70 g/l.

Ferraz *et al.* (2002) also studied biosurfactant production by two *Serratia marcescens* strains (strain LB006 and 0710) cultured in minimal culture medium

supplemented with vegetable oils (soybean, olive, castor, sunflower, and coconut fat). The result showed that addition of sunflower oil in culture medium of LB006 strain gave the best results (reduce surface tension of the culture medium from 52.70 to 29.75 mN/m) with a critical micelle dilution CMD^{-1} and CMD^{-2} of 36.69 and 51.41 mN/m, respectively.

Moreover, supplementation of amino acid can be used to improved biosurfactant production. Lotfabad *et al.* (2009) reported that yield of biosurfactant produced by *Pseudomonas aeruginosa* MR01 increase from 1.4 g/l to 2.1 g/l when isoleusin was added. However, the use of amino acid supplementary was confined in environmental application due to its cost and economic return.

2.4.3 Experimental design

Optimization of biosurfactant production by experimental design techniques has been extensively studied by many researchers.

Pal *et al.* (2009) studied media optimization for biosurfactant production from *Rhodococcus erythropolis* MTCC 2794. They found that the biosurfactant yield increased 3.5-fold when using artificial neural network coupled with genetic algorithm (ANN-GA), while the use of surface response methodology (RSM) gave a 5-fold enhancement in biosurfactant production.

Kiran *et al.* (2010) also studied optimization of biosurfactant production from *Brevibacteriu maureum* MSA13 under solid state culture (SSC) by using agro-industrial and industrial waste as substrate. RSM-based experiments were applied in this study, and they found that the optimization of biosurfactant production using experimental model could increase 3 fold over the original isolate under SSC condition.

Roldan-Carrillo *et al.* (2011) focused on the interaction among the C/N, C/Mg and C/Fe ratios on biosurfactant production by *Serratia marcescens* SmSA using a mineral medium containing glucose as the carbon source. A Box-Behnken

experimental design (type of response surface methodology, RSM) were used to receive the maximum biosurfactant production and evaluated the factors affected biosurfactant production. Surface tension test was used as response variable. The oil spreading technique was also applied to confirm biosurfactant property. The best treatment was C/N = 5, C/Fe = 26,000 and C/Mg = 30; it reduced surface tension of medium to 33 mN/m and produced high clear zone of oil displacement of 1.1cm. The response surface analysis also revealed that the interaction between C/N and C/Mg were the most factors affected the surface tension reduction and biosurfactant production.

2.5 Applications of biosurfactants

Using of synthetic surfactants can produce the derivatives, which probably lead to environmental problem and be costly to cleanup. Thus, the alternative environmentally friendly compounds like biosurfactants have been paid attention. Biosurfactants, which produced by a wide variety of microorganisms, have advantages over synthetic counterparts such as lower toxicity and environmental compatibility (Desai and Banat, 1997). Hence, they are widespread used in many fields including petroleum, environmental cleanup, food, biological, agricultural, bioprocessing, and cosmetics industry. The use of biosurfactants in many industries and their role are depicted in Table 2.2.

The use of biosufactants in environmental application has been coughing the interest by many authors (Mulligan, 2005; Singh *et al*., 2007; Banat *et al*., 2010; Pacwa-Płociniczak *et al*., 2011; Soberon-Chavez and Maier, 2011). Environmental applications of biosurfactants include biodegradation/bioremediation of contaminant substances such as petroleum hydrocarbon, polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons, soil washing technology, microbial enhanced oil recovery (MEOR). Biosurfactants produced by microorganisms such as *Pseudomonas aeruginosa* has been reported that it has ability to enhance biodegradation of hexadecane (Noordman *et al*., 2000). The efficiency of biosurfactant from *Pseudomonas aeruginosa* UCP099 could enhance oil recovery from artificially contaminated sand with diesel was investigated (Silva *et al*., 2010).

Table 2.2 The various applications of biosurfactants (Singh *et al.*, 2007)

Industry	Application	Role of biosurfactants
Petroleum	Enhanced oil recovery	Improving oil drainage into well bore, stimulating release of oil entrapped by capillaries, wetting of solid surfaces, reduction of oil viscosity and oil pour point, lowering of interfacial tension, dissolving of oil
	De-emulsification	De-emulsification of oil emulsions, oil solubilization, viscosity reduction, wetting agent
Environmental	Bioremediation	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration
	Soil remediation and flushing	Emulsification through adherence to hydrocarbons, dispersion, foaming agent, detergent, soil flushing
Food	Emulsification and de-emulsification	Emulsifier, solubilizer, demulsifier, suspension, wetting, foaming, defoaming, thickener, lubricating agent
	Functional ingredient	Interaction with lipids, proteins and carbohydrates, protecting agent
Biological	Microbiological	Physiological behaviour such as cell mobility, cell communication, nutrient accession, cell–cell competition, plant and animal pathogenesis
	Pharmaceuticals and therapeutics	Antibacterial, antifungal, antiviral agents, adhesive agents, immunomodulatory molecules, vaccines, gene therapy
Agricultural	Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypovirulence
Bioprocessing	Downstream processing	Biocatalysis in aqueous two-phase systems and microemulsions, biotransformations, recovery of intracellular products, enhanced production of extracellular enzymes and fermentation products
Cosmetic	Health and beauty products	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action

In this study we focus on the use of biosurfactants in aqueous-base method for vegetable oil extraction application to reduce the use of hexane.

2.6 Vegetable oil extraction method

The conventional method for oil extraction oilseed in small and medium scale is mechanical pressing. However, this method cannot extract the oil inside the seed. Thus, in large-scale oil extraction production, solvent extraction with hexane has been involved to recover the oil. It is possible to achieve oil yield in excess of 95% (Rosenthal *et al*., 1996). Recently, using hexane has been considered in environmental point of view because it is volatile and toxic in both human and environment (EPA, 2001). Hence, alternative technologies have been developed in order to eliminate the use of hexane including aqueous-based extraction (AEP) (Pan *et al*., 2002), enzyme-assisted aqueous extraction (Shah *et al*., 2005), and aqueous surfactant-based extraction (Kadioglu *et al*., 2010). The oil extraction efficiency of these methods is depicted in table 2.3.

Table 2.3 The oil extraction efficiency (%) from different extraction methods

Extraction method	Oil yield (%)	References
Conventional method		
Mechanical pressing (Hydraulic presses)	67-74	Kwasi, 2002
Solvent extraction (Hexane)	95-99	Rosenthal *et al*., 1996
Alternative method		
Aqueous-based extraction (AEP)	38	Shah *et al.*, 2005
Enzyme-assisted aqueous extraction	80-90	de Moura *et al*., 2008
Aqueous surfactant-based extraction	90-95	Do and Sabatini, 2010

Nevertheless, using of biosurfactants in aqueous-based extraction for vegetable oil extraction is paid attention by many authors (Nguyen and Sabatini, 2009; Nguyen *et al*., 2010) according to their many potential properties over synthetic surfactants such as less toxicity. The use of biosurfactants can enhance the oil extraction yield in AEP which using water alone. In oil extraction mechanism, using biosurfactant can reduce repulsive force of two dislike phases by lowering the interfacial tension. Resulting in breaking of oil trapped in the insoluble matrix into droplets and making them possible to be liberated from the matrix (Do and Sabatini, 2010).

2.6.1 Aqueous extraction process (AEP)

The AEP has been studied and believed to be a cleaner, cheaper, and safer process than using hexane. However, this method has its own drawbacks include low oil extraction efficiency (less than 70%) and de-emulsion treatment is needed to be conducted when emulsion are formed (Rosenthal *et al*., 1996). In addition, AEP requires high temperature (50-60°C) and high water/solid ratio (20/1 to 30/1) which are undesirable in application (Do and Sabatini, 2010).

2.6.2 Enzyme-assisted aqueous extraction

In order to enhance oil recovery base on compatible environment issue, many oil extraction techniques including enzyme-assisted aqueous extraction and aqueous surfactant-based extraction have been developed (Naksuk *et al*., 2009)

In 2002, Sharma *et al.* used a commercial mixture of three proteases, which Protizyme TM was predominant, in aqueous enzymatic extraction of peanut oil. The result found that oil recovery of 86–92% was achieved with optimal conditions; enzyme concentration of 2.5% (w/w) in 10 g of peanut seeds, pH 4.0, 40°C, incubation 18 hours, and shaking at 80 rpm.

In the same way, de Moura *et al.* (2008) studied the use of two proteases, Protex 6L (P6L) and Protex 7L (P7L) on the oil and protein extraction yield from soybeans using enzyme-assisted aqueous extraction process (EAEP). Protex 6L was found to be more effective than Protex 7L. By using 0.5% Protex 6L obtained oil and protein extraction yields of 96 and 85%, respectively. Moreover, cream de-emulsification has been estimated by enzymatic and pH treatment. It was revealed that when using 2.5% Protex 6L and pH 4.5 the cream obtained after EAEP can be de-emulsified.

Nevertheless, enzyme-assisted aqueous extraction also has its own limitations such as each type of enzyme specific on a certain type of compound, thus, an efficient extraction system require the combination of enzyme at least 3 types, the enzyme cost, and requirement of long incubating time and high temperature which both are undesirable in application (Do and Sabatini, 2010).

2.6.3 Aqueous surfactant-based extraction

Aqueous surfactant-based has been interested because surfactants are non-toxic substance and require low energy since the process can be carried out at room temperature. Moreover, it offers better crude oil quality in term of free fatty acid when compare with hexane extraction (Do and Sabatini, 2010).

Kadioglu *et al.* (2010) developed an aqueous surfactant-based extraction system for extraction of corn oil. Anionic extended surfactants used in this study were sodium linear-alkyl polypropoxylated polyethoxylated sulfates ($C_{12,14}$–P_{10}–E_2–SO_4Na and C_{10}–P_{18}–E_2–SO_4Na). Surfactant and salinity concentration affected oil extraction process were examined.The result showed that about 83% of the sum of free oil and oil-in-water emulsion was obtained when using condition of 0.4% $C_{12,14}$–P_{10}–E_2–SO_4Na, 1% NaCl, a solid/liquid ratio of 1/10, and shaking at room temperature for 45 min. Furthermore, the chemical compositions of oil extracted from this system were the same as oil extracted from hexane.

By adjusting parameters (grain size of oilseed, contact time, and solid/liquid ratio) that impacted oil extraction process and the use of combination surfactants have been evaluated on increasing of oil extraction efficiency. Naksuk *et al.* (2009) investigated the mixed surfactant solutions in order to provide an ultralow interfacial tension for the palm kernel oil extraction. The mixed surfactant consisted of 3 wt% Comperlan KD, nonionic surfactant, and either 0.1 wt% Alfoterra145-5PO (system A) or 145-8PO (system B), as anionic extended surfactant, to produce an ultralow interfacial tension 0.0197 and 0.0359 mN/m, respectively. The effect of process parameter such as NaCl concentration, grain size, oilseed loading, and contact time have been examined. The mixed surfactant systems A and B gave the extraction efficiency of 93.99 and 94.13%, respectively, with the optimum condition of 10% NaCl, ground seed size 0.212-0.425 mm, 1 g loading per 10 ml of surfactant solution, and contact time of 30 min. The quality of oil obtained from these mix surfactant systems and hexane extraction are similar.

2.6.4 Aqueous biosurfactant-based extraction

However, to discover more environmentally friendly oil extraction technique, using of biosufactant can be challenging of the alternative oil extraction method. Nguyen *et al.* (2010) studied biodiesel production of peanut oil via diesel-based reverse-micellar microemulsions extraction based on the “likes dissolve likes”. Biosurfactants used in the system were rhamnolipid (JBR) and sophorolipid (SPL). The result showed that the extraction efficiency up to 95% was achieved at room temperature, contact time of 10 minutes. However, this process produced oil dissolved in continuous phase which suits for biodiesel production process. Nguyen and Sabatini (2011) also studied characterization and emulsification properties of rhamnolipid and sophorolipid. They found that rhamnolipid and sophorolipid was relatively hydrophilic and hydrophobic, respectively, as compared to synthetic surfactants. In addition, the mixture of these two biosurfactants was able to produce microemulsions for a wide range of oils.

Thus, this study aimed to product biosurfactant by using industrial waste such as waste glycerol as substrate to reduce production cost and waste deposal in the same time. In addition, optimization of culture media composition using a single-factor experiment and experimental design were applied to achieve high biosurfactant yield by adjusting carbon and nitrogen source and concentration, and precursor supplementation. Moreover, we aimed to fulfill green concept of reducing the use of hexane in vegetable oil extraction by using aqueous biosurfactant-based extraction method which is quit a new method and has not been reported yet.

CHAPTER III

METHODOLOGY

3.1 Research overview

The methodology of this research was divided into three parts. First, screening and isolation of an efficient biosurfactant-producing bacterium. In this step, an effective bacterial strain was obtained to study biosurfactant production in the further step. Second part, production of biosurfactant by selected bacterium. Culture media component was adjusted to obtain an optimum condition which produced the maximum biosurfactant yield. The last part was application of the biosurfactant in vegetable oil extraction by using aqueous biosurfactant-based extraction. Difference concentrations of biosurfactant solution were applied into the system. Qualification of extracted oil from both hexane and aqueous biosurfactant-based extraction method were studied and compared in term of oil clarity, color, and free fatty acids existing. Flowchart of the research was illustrated in Figure 3.1.

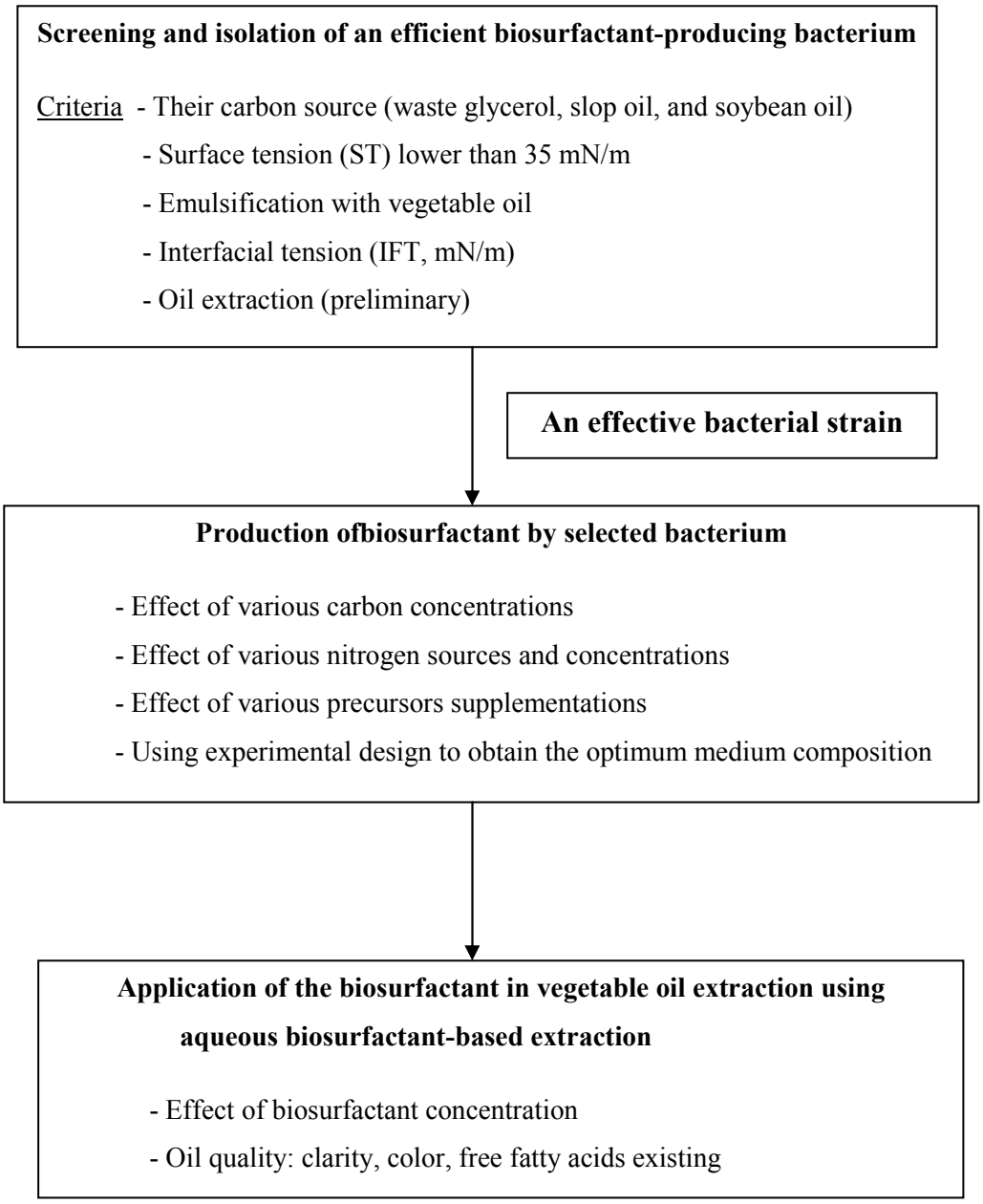


Fig. 3.1 Flow chart of the research

3.2 Material

3.2.1 Chemicals

All chemicals were analytical grade.

1. Waste glycerol was obtained from Thai Oleochemical Co., LTD (TOL), Thailand
2. Yeast extract was obtained from Bio Springer, France
3. Tryptone was obtained from Difco Laboratories, USA
4. Beef extract was obtained from RCI Labscan Limited, Thailand
5. Sodium chloride (NaCl) was obtained from Merck, Germany
6. Sodium nitrate ($NaNO_3$) was obtained from Merck, Germany
7. Magnesium sulfate ($MgSO_4.7H_2O$) was obtained from Merck, Germany
8. Potassium chloride (KCl) was obtained from Merck, Germany
9. Ammonium nitrate (NH_4NO_3) was obtained from J.T. Baker, USA
10. Ammonium sulfate $(NH_4)_2SO_4$ was obtained from Merck, Germany
11. Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck, Germany
12. Dipotassium hydrogen phosphate (K_2HPO_4) was obtained from Merck, Germany
13. Calcium chloride ($CaCl_2$) was obtained from Merck, Germany
14. Iron (II) sulfate ($FeSO_4.7H_2O$) was obtained from Merck, Germany
15. Boric acid (H_3BO_3) was obtained from Merck, Germany
16. Copper (II) sulfate ($CuSO_4.5H_2O$) was obtained from Carlo ERBA, France

17. Manganese sulfate ($MnSO_4.H_2O$) was obtained from Merck, Germany
18. Sodium molybdate ($MoNa_2O_4.2H_2O$) was obtained from May & Baker LTD Degenham, UK
19. Zinc Sulfate ($ZnSO_4.7H_2O$) was obtained from Carlo ERBA, France
20. Hydrochloric acid (HCl) was obtained fromMerck, Germany
21. Sodium hydroxide (NaOH) was obtained from Merck, Germany
22. Bacto agar was obtained from Difco, USA
23. Glycerol was obtained from Research organics, Inc., USA
24. Chloroform was obtained from RCI Labscan, Thailand
25. n-Hexane was obtained from Merck, Germany
26. Methanol was obtained from Merck, Germany
27. Soybean oil was obtained from Grape band, Thailand
28. Refined Palm olein from pericarp was obtained from Morakot, Thailand
28. Aerosol OT (AOT) or sodium bis(2-ethylhexyl) sulfosuccinate was obtained from Sigma-Aldrich, USA

3.2.2 Equipments

1. Rotary vacuum evaporator, model CCA-1110, EYELA, Japan
2. ISSCO laminar flow, International Scientific Supply, Japan
3. Hot air oven, model D06063, Memmert, Germany
4. Oven, Contherm Scientific, New Zealand
5. Filter papers, pour size 0.11 μm, Whatman^{TM}, UK

6. Vortex mixer, model Genie 2, Scientific Industries, USA

7. Centrifuge, model 6500, Kubota, Japan

8. Avanti^{TM} centrifuge, model J-30I, Beckman coulter, USA

9. Spectronic 20 Genesys, model 4001/1, Spectonic Unicam, USA

10. Micropipette (20 µl, 200 µl, 1 ml, 5 ml, and 10 ml), Gilson, France

11. pH meter, Seven Easy sereis, Mettler Toledo, Switzerland

12. Tensiometer, DCAT11, dataphysics, Geramany

13. Balance, model P2002-S and AG285, Mettler Toledo, Switzerland

14. Centrifugal vaporizer, model CVE-2000, EYELA, Japan

15. Autoclave, model ES-315 and SS-315, Tomy, Japan

16. Shaker, model INNONA 2300, New Brunswick Scientific, USA

17. Sieve size No. 10 and No. 40, Retsch, Germany

18. Incubator, model SLI 1000 ND, EYELA, Japan

19. Magnetic stirrer with heating, Model C-MAG HS7, IKA®, Germany

3.2.3 Vegetable samples

Jatropha seeds and jatropha kernel oil used in this research were obtained from Thai Jatropha Oil Co., Ltd. Crude Palm kernel oil was donated from Suksomboon Palm Oil Industry. Palm samples including palm kernel seed, palm fruit, palm kernel shell, palm fiber, decenter cake, coarse kernel meal, and fine kernel meal were supported by Faculty of Engineering, Prince of Songkla University. All vegetable samples were stored at 4°C upon receipt.

3.3 Screening and isolation of an efficient biosurfactant-producing bacterium

3.3.1 Enrichment of biosurfactant-producing bacteria

Vegetable samples (jatropha seed, palm kernel seed, palm fruit, palm shell, palm fiber, decenter cake, coarse kernel meal, and fine kernel meal) 5 g were added into 125-ml flask containing 50 ml basal medium (BM) consisting of (per liter): $NaNO_3$, 7 g, K_2HPO_4, 1 g, KH_2PO_4, 0.5 g, KCl, 0.1 g, $MgSO_4.7H_2O$, 0.5 g, $CaCl_2$, 0.01, $FeSO_4.7H_2O$, 0.1 g which has already autoclaved. A trace element solution composts of (per liter): H_3BO_3, 0.26 g, $CuSO_4.5H_2O$, 0.5 g, $MnSO_4.H_2O$, 0.5 g, $MoNa_2O_4.2H_2O$, 0.06 g, $ZnSO_4.7H_2O$, 0.7 g was autoclaved and added into the medium. Then 3% (v/v) of waste glycerol, slop oil, or soybean oil was used as carbon source. The cultures were shaken at 200 rpm, room temperature for 7 days. Turbidity of culture media was observed and compared to the control. Then, turbid media were transferred into fresh media which has already added with their substrate. The transferred step was repeated 3 to 4 times to enrich biosurfactant-producing bacteria.

3.3.2 Screening and isolation of biosurfactant-producing bacteria

Culture media 1.5 ml from step 1.1 were transferred into eppendorf tube and centrifuged at 8,000 rpm for 20 min. to collect cell-free supernatant. Emulsification activity (adapted from Cooper and Goldenberg, 1987) was evaluated by transferring 1 ml of supernatant into screw cap tube and then equal volume of vegetable oil was added (soybean, jatropha, and palm oil). The solution was mixed vigorously for 2 min and left it stand for 24 hour. After that, emulsification index (E_{24} (%)) was calculated follow the formula below.

$$E_{24} = h_E / h_T \times 100 \qquad (3.1)$$

h_E is height of emulsion, h_T is total height

Culture media which could emulsify vegetable oil were selected and then (50 µl) spread onto BM agar which has smeared with its substrate. The selected samples

were incubated at 30°C until bacterial colonies were observed. Pure culture of each colony was obtained by repetitive streaking onto Luria-Bertani (LB). Morphology and Gram stain tests of pure bacterial strains were carried out.

3.3.3 Cultivation and selection of biosurfactant-producing bacteria

Biosurfactant-producing bacteria from laboratory library were also selected based on their carbon source (waste glycerol, slop oil, and soybean oil), surface tension (ST) of medium lower than 35 mN/m and ability to emulsify with vegetable oil. Then, these selected strains and the isolated strains from previous step were cultured on 50 ml BM with different carbon sources; waste glycerol, slop oil, or soybean oil by shaking at 200 rpm, room temperature for 7 days. Next, cell-free supernatant was collected by centrifugation at 8,000 rpm for 20 min. Supernatant was measured surface tension with a tensiometer using Wilhelmy plate (mN/m) and E_{24} (%). Cell pellets were washed with distilled water and dried by heating, for measuring dry cell weight (g/l), until constant weight was obtained. The experiment was carried out with three replications.

Biosurfactant-producing bacteria from laboratory library and the isolation experiment were selected again base on their activity to reduce surface tension of media lower than 35 mN/m and ability to emulsify vegetable oils (soybean oil, jatropha oil, or palm oil). Then, supernatant of selected strains were determined for interfacial tension (mN/m) with vegetable oil (jatropha and palm oil), by using tensiometer with ring and measured oil detachment (%).

3.3.4 Oil extraction (preliminary)

To find an effective bacterial strain for studying biosurfactant production in next step, preliminary oil extraction experiment was conducted.

3.3.4.1 Oilseeds preparation

Oilseeds (jatropha and palm seed) were dehulled and oven-dried at 85°C for 2 hours. Dried oil kernel seeds were ground and sieved to the particle size between 0.425-2.00 mm by using sieve size No. 40 and No.10 (ASTM).

3.3.4.2 Hexane extraction

Hexane extraction method was adapted from Kadioglu *et al.* (2010), oil kernel seed (1 g) was weighed into a 125-ml flask and 10 ml of hexane was added. The mixture was shaken horizontally at 200 rpm, room temperature for 30 min. The condition was done in triplicate. Then, the slurry was centrifuged at 3,500 rpm for 20 min. The hexane phase was removed with a pasture pipette and put into a pre-weighed glass tube. Hexane was completely evaporated at 70°C and the remaining oil was weighed. The amount of oil extracted by the hexane extraction method was evaluated as the total oil present in oilseed by following equation.

$$\text{Total oil present in oilseed (\%)} = W_{\text{extracted oil}}/W_{\text{oilseed}} \text{x } 100 \qquad (3.2)$$

$W_{\text{extracted oil}}$ is weight of extracted oil, W_{oilseed} is weight of oilseed

3.3.4.3 Vegetable oil extraction (adapted from Do and Sabatini, 2010)

The optimum condition for vegetable oil extraction was selected following Naksuk *et al.* (2009). Oil kernel seed (1 g) was put into 10 ml of biosurfactant solution* (1/10 of solid/liquid ratio) in a 125-ml flask. Then, the mixture was shaken horizontally at 200 rpm, room temperature for 30 min. The mixture was carried out in triplicate. Then, the mixture was centrifuged at 3,500 rpm for 20 min to let free oil phase separate on top and allow residue meal to settle. Aqueous solution with oil was removed with pipette and put into a pre-weighted glass tube. The remaining particles were washed with 10 ml of distilled water twice. The slurry was centrifuged at 3,500

rpm for 10 min. Next, water was removed with pipette. After that, the residual meal was dried at 85°C overnight. Hexane (10 ml) was added and shaken at 200 rpm for 15 min. Then, the slurry was centrifuged at 3500 rpm for 10 min. The hexane phase was removed with pipette and put into a pre-weighed glass tube. Hexane was evaporated at 70°C and the remaining oil was weighed. Detached oil by this method was compared to the total oil content extraction by hexane and calculated oil detachment (%) by the following equation. In this step, extraction by surfactant-based using anionic surfactant (AOT) and water alone were also carried out.

* Cell-free broth was used for aqueous biosurfactant-based extraction, 2.5 mM Aerosol OT (AOT, anionic surfactant) was used for aqueous surfactant-based extraction.

$$\%\text{Oil detachment} = (W_{hexane} - W_{remaining\ oil})/W_{hexane} \times 100 \qquad (3.3)$$

$W_{remaining\ oil}$ is weight of remaining oil (g/g kernel) by aqueous biosurfactant-based extraction

W_{hexane} is weight of oil (g/g kernel) by hexane

A bacterial strain which achieved the highest oil detachment was selected for studying production of its biosurfactant in the further step. Moreover, in term of reducing production cost, waste glycerol was considered to be used as substrate for biosurfactant production.

3.4 Production of biosurfactant by selected biosurfactant-producing bacterium

3.4.1 Effect of various carbon concentrations

A 2% cell suspension of 1 optical density at 600 nm (OD_{600}), corresponding to inoculums of 10^6 CFU/ml, was inoculated into 250-ml flask containing 100 ml productive medium component (per liter): glucose, 1 g, beef extraction, 0.5 g,

K_2HPO_4, 3.3 g, KH_2PO_4, 0.14 g, $NaNO_3$, 5 g, NaCl, 0.04 g, $FeSO_4$, 0.1 g, with waste glycerol as a sole carbon source. The concentration of waste glycerol was adjusted to 2, 5, 7, and 12 % (w/v). The culture media were shaken at 200 rpm, room temperature for 5 days. Then, the samples were centrifuged to collect cell-free supernatant at 8,000 rpm for 20 min. Supernatant was measured surface tension (mN/m), and biosurfactant concentration (g/l). Cell pellet was determined dry cell weigh (g/l).

For waste glycerol preparation:

Waste glycerol was dissolved in distilled water in ratio of 2/1. Then, waste glycerol solution was filtrated by using filter papers, pours size 0.11 μm and cleaved before use. Organic compound presented in waste glycerol was calculated from soluble COD (mg/l) and the calculation was shown in appendix C.

3.4.2 Effect of various nitrogen sources and concentration

Using the selected waste glycerol concentration, the following nitrogen sources were evaluated: $NaNO_3$, $(NH_4)_2SO_4$, and NH_4NO_3 and nitrogen concentrations were adjusted to be 0.1, 0.2, 0.4, 0.6% (w/v). Cell-free supernatant and cell pellet was collected separately by centrifugation at 8,000 rpm for 20 min. Next, the surface tension (mN/m) and biosurfactant concentration (g/l) of supernatant were measured. Cell pellets were tested for dry cell weight (g/l).

3.4.3 Effect of various precursors supplementation

The most appropriate waste glycerol concentration and nitrogen source and concentration was used in this step. Vegetable oil (soybean or palm oil) was used to induce the biosurfactant production (Cooper and Paddock, 1984). After 48 hours of cultivation, 0.1 and 1% (v/v) of each type of oil was added into culture medium. At the end of cultivation, culture medium was centrifuged at 8,000 rpm for 20 min. Cell-free supernatant was measured for the surface tension (mN/m) and biosurfactant concentration (g/l) and cell pellets were tested dry cell weight (g/l).

3.4.4 Experimental design

A two-level full factorial design was developed with three variables; carbon concentration, nitrogen concentration, and precursor supplementation, at low and high level (-1 and +1). This experiment required 8 runs (Table 3.1). Biosurfactant yield was used as the response variable. All runs were done in three replicates. Moreover, response surface methodology (RSM) was applied in this research to study the main effects and the interaction between the factors; which affected the biosurfactant production.

Table 3.1 A 2^3 two-level full factorial experimental design

Run	**Coded variables**			**Uncoded variables**		
	X1	**X2**	**X3**	**Carbon concentration**	**Nitrogen concentration**	**Precursor supplementation**
1	-1	-1	-1	5	0.4	0.1
2	1	-1	-1	7	0.4	0.1
3	-1	1	-1	5	0.6	0.1
4	1	1	-1	7	0.6	0.1
5	-1	-1	1	5	0.4	1
6	1	-1	1	7	0.4	1
7	-1	1	1	5	0.6	1
8	1	1	1	7	0.6	1

3.4.5 Biosurfactant isolation

Biosurfactant isolation method 1 was previously used in step 3.4.1 to 3.4.2. Then, 2nd method of extraction crude biosurfactant was applied to improve biosurfactant yield.

Method 1 (adapted from de Sousa *et al.*, 2011):

Biosurfactants were extracted from cell-free broth by using a liquid/liquid extraction, chloroform/methanol (65/15, v/v), in ratio of cell-free broth/solvent equal to 3/1. Then, the mixture was shaken at 200 rpm, room temperature for 2 hours. Phase separation was done in funnel, collect the below phase (crude biosurfactant). Solvent was evaporated to obtain crude biosurfactant.

Method 2 (adapted from Silva *et al.*, 2010):

Biosurfactants was extracted from cell-free broth by adjusting pH to 2 with 6M HCl and stored at 4°C overnight. Then, equal volume of chloroform/methanol (2/1, v/v) was added and shaken at 200 rpm, room temperature for 30 min. The mixer was settled in funnel to allowed phase separation. The below phase was collected and evaporated at 60°C using a rotary evaporator. The sticky yellow brown product was obtained (crude biosurfactant). Then, dissolved this product with methanol and evaporated again at 45°C to receive crude biosurfactant.

3.4.6 Properties of biosurfactant

Surface and interfacial tension (against jatroha or palm oil) reduction of cell-free supernatant collected by centrifugation at 8,000 rpm for 20 min were measured by using tensiometer with plate and ring, respectively.

Emulsification activity with vegetable oil (soybean, jatropha, and palm oil) was measured and calculated for E_{24} (%) following step 3.2.2 and equation (1).

The critical micelle concentration (CMC) was evaluated by measuring surface tension of serially dilution of crude biosurfactant solution. The constant surface

tension was obtained when the biosurfactant concentrations reach CMC. The value of CMC was determined by plotting surface tension against surfactant concentration (log graph). The CMC value was tested to be mg/l of crude biosurfactant.

Crude biosurfactant ionic charge was determined by titration method (Liu *et al*., 2004). This method could classify and calculate for ionic concentration of biosurfactant. Briefly, crude biosurfactant solution 45 ml, which was known exact concentration, was put into a 250-ml Erlemeyer flask. Then, 25 ml of distilled water, 15 ml of Dichloromethane (DCM), and 10 ml of Dimidium Bromide/Disulphine Blue (indicator) were added into the solution. The solution was shaken vigorously for 30 seconds. Then, the sample was settled to allowed phase separation (If crude biosurfactant is anionic charge, the below phase will change into pink color.). Next, the sample was titrated with 1 mM Cetyl Trimethyl Ammonium Bromide (CTAB, cationic surfactant) and shaken for 15 sec. (If the below phase has anionic charge property, the pink color will change into colorless at the end point). The concentration of anionic crude biosurfactant was calculated by the following equation.

$$M = \frac{V_{CTAB} \times C_{CTAB}}{V_{sample}} \qquad (3.4)$$

M is concentration of crude biosurfactant ionic charge

V_{CTAB} is volume of cationic surfactant (CTAB) at end point (ml)

C_{CTAB} is concentration of cationic surfactant (CTAB) at end point (mM)

V_{sample} is volume of crude biosurfactant (ml)

3.5 Vegetable oil extraction

3.5.1 Aqueous biosurfactant-based extraction

All biosurfactant solutions and conditions were prepared follow step 3.3.4.1 and 3.3.4.3**.**

Oil from oil kernel seed (palm kernel seed, 1 g) was extracted by using 10 ml cell-free broth or the solution of the crude biosurfactant at CMC, ten times higher than its CMC, and five times lower than its CMC to study effect of various biosurfactant concentrations on oil extraction efficiency. Then, the sample was shaken horizontally at 200 rpm, room temperature for 30 min. After shaking, the extracted oil was separated by centrifugation at 3,500 rpm for 20 min. The remaining oil was extracted by using 10 ml of hexane and calculated for oil detachment (%) compared to oil extracted using hexane method following equation (3). The IFT of each biosurfactant concentration was also investigated by using tensiometer with ring method.

3.5.2 Oil quality

The crude oil quality from aqueous biosurfactant-based system was measured and compared to those of the extracted oil from hexane extraction. The parameters were chosen for measure the quality of extracted oil including oil clarity, color, and fatty acids existing. Clarity and color of extracted oil by aqueous biosurfactant-based system were determined by visual observation and compared to oil extracted by using hexane. Free fatty acids existing were determined by titration method AOCS Official Method Ca 5a-40. Briefly, 1 ml of sample was put into a 250-ml flask. Then, 100 ml of 95% ethyl alcohol and 2 ml of phenolphthalein indicator solution was added. The sample was titrated with standard sodium hydroxide, 1.0 N, and shaken vigorously until the appearance of the first permanent pink color of the same intensity as that of the neutralized alcohol before the addition of the sample (the color must persist for 30

sec.) was observed. Then, the percentage of free fatty acid was calculated as oleic, lauric, and palmitic acid following equation 3.5, 3.6, and 3.7 respectively.

$$\text{Free fatty acids as oleic, \%} = \frac{\text{ml of alkali x N x 28.2}}{\text{mass, g of sample}} \quad (3.5)$$

$$\text{Free fatty acids as lauric, \%} = \frac{\text{ml of alkali x N x 20}}{\text{mass, g of sample}} \quad (3.6)$$

$$\text{Free fatty acids as palmitic, \%} = \frac{\text{ml of alkali x N x 25.2}}{\text{mass, g of sample}} \quad (3.7)$$

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening and isolation of an efficient biosurfactant-producing bacterium

4.1.1 Enrichment of biosurfactant-producing bacteria

Biosurfactant-producing bacteria were enriched from vegetable samples (5 g): jatropha seed, palm kernel seed, palm fruit, palm shell, palm fiber, decenter cake, coarse kernel meal, and fine kernel meal, by culturing in 50 ml basal medium containing trace element and using 3% (v/v) of waste glycerol, slop oil, or soybean oil as carbon source. The sample was subsequently transferred into fresh medium when bacterial growth was observed. The growth of culture was determined by increasing of turbidity compared to the control. After the transferred step was repeated 3 to 4 times, it found that the turbidity of culture media from every vegetable samples were increased. Then, culture media were screened for biosurfactant-producing bacteria base on their ability to emulsify vegetable oils (soybean, jatropha, and palm oil) in term of E_{24} (%). Similar to Lotfabad *et al.* (2009) study, they used emulsification index (E_{24}) to examine the ability of biosurfactant-containing broth by using several hydrocarbons.

4.1.2 Screening and isolation of biosurfactant-producing bacteria

The enrichment culture from jatropha seed, palm kernel seed, palm fruit, palm shell, palm fiber, decenter cake, and fine kernel meal using waste glycerol or soybean oil as carbon source had ability to emulsify vegetable oil in range of 25 to 60% (E_{24}). These samples were expected to have biosurfactant-producing bacteria. To isolate bacterial strains, the selected samples were spread (50 µl) on BM agar which has smeared with their substrate and then incubated at 30°C until bacterial colonies were observed. Several different types of bacterial colonies were appeared. Then, each bacterial colony was purified by repetitive streaking onto Luria-Bertani (LB) to obtain pure isolated strains. Twenty-seven bacterial strains were obtained and examined for their morphology and Gram stain tests and the results are shown in Table 4.1.

Table 4.1 Colony characteristics of isolated bacterial strains from vegetable samples

Sample	Substrate (3%)	Bacterial strain	Colony morphology	Gram stain
1. Jatropha	WG	JBG1	Circular shape, yellow color, smooth surface, convex	Gram negative, rod
		JBG2	Irregular shape, yellow color, undulate edge, dry surface, raised	Gram positive, rod
		JBG3	Circular shape, orange color, entire edge, dry surface, flat	Gram negative, rod
		JBG4	Circular shape, white color, entire edge, smooth surface,	Gram positive, cocci
		JBG5	Irregular shape, white color, rough surface. raised	Gram negative, rod
	SB	JSB1	Circular shape, white color, dry surface, raised	Gram negative, rod
		JSB2	Irregular shape, light yellow color, undulate edge, dry and rough surface, flat	Gram negative, rod
		JSB3	Circular shape, yellow color, entire edge, smooth surface, convex	Gram positive, rod
2. Palm kernel	WG	PBG1	Circular shape, white color, smooth surface, convex	Gram positive, cocci
		PBG2	Circular shape, yellow color, smooth and wet surface, convex	Gram negative, cocci

WG = Waste glycerol, SB = Soybean oil

Table 4.1 Colony characteristics of isolated bacterial strains from vegetable samples (cont.)

Sample	Substrate (3%)	Bacteria strain	Colony morphology	Gram stain
2. Palm kernel	SB	PSB1	Circular shape, white color, entire edge, smooth surface, convex	Gram positive, cocci
		PSB2	Circular shape, white color, entire edge, smooth surface, convex	Gram positive, cocci
3. Mesocarp	WG	MBG1	Circular shape, light yellow color, entire edge, smooth surface, convex	Gram positive, rod
		MBG2	Circular shape, white color, entire edge, smooth surface, convex	Gram negative, cocci
	SB	MSB1	Circular shape, white color, entire edge, smooth surface, convex	Gram negative, cocci
4. Palm kernel shell	SB	PSSB1	Circular shape, light yellow color, entire edge, smooth surface, convex	Gram negative, cocci
		PSSB2	Circular shape, white color, entire edge, smooth surface, convex	Gram positive, cocci
		PSSB3	Circular shape, light yellow color, entire edge, dry surface, raised	Gram positive, cocci
5. Palm fiber	SB	FSB1	Circular shape, white color, entire edge, smooth and wet surface, convex	Gram positive, cocci
		FSB2	Circular shape, white color, entire edge, smooth and wet surface, convex	Gram positive, cocci
		FSB3	Circular shape, white color, entire edge, smooth and wet surface, convex	Gram negative, cocci
6. Decenter cake	SB	DSB1	Circular shape, white color, entire edge, wet surface, convex	Gram negative, rod
		DSB2	Irregular shape, yellow color, curled edge, wet surface, raised	Gram positive, cocci
7. Fine kernel meal	WG	FMBG	Irregular shape, white color, smooth and wet surface, convex	Gram negative, cocci
	SB	FMSB1	Circular shape, white color, entire edge, smooth surface, convex	Gram positive, cocci
		FMSB2	Circular shape, white color, entire edge, smooth and wet surface, convex	Gram negative, cocci
		FMSB3	Circular shape, yellow color, entire edge, smooth surface, convex	Gram positive, cocci

WG = Waste glycerol, SB = Soybean oil

4.1.3 Cultivation and selection of biosurfactant-producing bacteria

From the previous step, it was found that culture media containing slop oil as carbon source did not appropriate for culturing biosurfactant-producing bacteria since no significant emulsification activity was observed from every vegetable samples. Moreover, slop oil is inflammability and toxicity (Dave *et al.*, 1994). Thus, waste glycerol and soybean oil were used as carbon source for further culturing bacterial strains.

To isolate the bacterial strains capable of biosurfactant production, twenty-seven bacterial strains obtained from previous step were inoculated in 50 ml of basal media supplemented with 3% (v/v) of waste glycerol or soybean oil and shaken at 200 rpm, room temperature for 7 days. The cell-free supernatant was collected by centrifugation and measured for surface tension using tensiometer with Wilhelmy plate method (mN/m) and E_{24} (%). Cell pellets were measured for dry cell weight (g/l) and the results are shown in Table 4.2.

Table 4.2 The isolated bacterial strains cultured in basal media with waste glycerol or soybean oil as carbon source and their properties

Bacterial strain	Carbon source (3%)	Surface tension (mN/m) at 25°C	E_{24} (SB, %)	E_{24} (J, %)	E_{24} (P, %)	Dry cell weight (g/l)
1. JBG1	WG	50.45±0.79	NE	NE	NE	2.29±0.09
	SB	31.43±0.61	2.5	46.5	44	6.94±0.73
2. JBG2	WG	47.35±1.61	NE	NE	NE	4.53±0.07
	SB	53.91±1.93	NE	NE	NE	2.28±0.91
3. JBG3	WG	51.75±5.60	NE	NE	NE	1.19±0.06
	SB	57.80±6.29	NE	NE	NE	3.33±2.03
4. JBG4	WG	48.64±0.66	NE	NE	NE	0.39±0.04
	SB	49.53±5.50	NE	NE	NE	1.84±0.11
5. JBG5	WG	46.80±0.80	NE	NE	NE	0.86±0.34
	SB	56.01±4.36	NE	NE	NE	2.57±0.96
6. JSB1	WG	42.64±0.97	NE	NE	NE	0.68±0.02
	SB	44.02±0.25	18	8	6	11.05±1.90

WG = Waste glycerol, SB = Soybean oil, J = Jatropha oil, P = Palm oil, - = Not Emulsion

Table 4.2 The isolated bacterial strains cultured in basal media with waste glycerol or soybean oil as carbon source and their properties (cont.)

Bacterial strain	Carbon source (3%)	Surface tension (mN/m) at 25°C	E_{24} (SB, %)	E_{24} (J, %)	E_{24} (P, %)	Dry cell weight (g/l)
7. JSB2	WG	45.35±1.49	NE	NE	NE	0.76±0.05
	SB	29.91±1.00	37	43	39	9.78±1.74
8. JSB3	WG	46.22±1.31	NE	NE	NE	1.67±0.10
	SB	53.01±5.02	NE	NE	NE	2.25±0.87
9. PBG1	WG	42.11±8.00	NE	NE	NE	1.52±1.96
	SB	50.13±1.28	NE	NE	NE	2.31±1.10
10. PBG2	WG	48.07±1.92	4.5	53	56	1.17±0.05
	SB	56.10±7.17	NE	NE	NE	1.25±0.35
11. PSB1	WG	46.47±0.49	NE	NE	NE	0.59±0.10
	SB	49.09±4.65	NE	NE	NE	2.92±0.45
12. PSB2	WG	45.08±4.65	3	33	29	3.60±1.91
	SB	48.70±1.32	NE	22	NE	3.78±0.78
13. MBG1	WG	45.52±6.13	NE	NE	NE	0.53±0.17
	SB	56.43±12.14	NE	NE	NE	2.05±0.28
14. MBG2	WG	43.23±0.42	NE	NE	NE	0.71±0.26
	SB	49.24±4.92	NE	NE	NE	2.24±0.66
15. MSB1	WG	46.46±1.55	NE	NE	NE	0.90±0.08
	SB	56.17±5.23	NE	NE	NE	4.06±1.23
16. PSSB1	WG	41.58±2.36	NE	NE	NE	0.50±0.22
	SB	41.17±6.87	2.4	2.5	NE	4.98±1.22
17. PSSB2	WG	41.58±0.40	NE	NE	2.4	0.52±0.04
	SB	50.34±0.79	NE	16	9.5	1.81±1.29
18. PSSB3	WG	43.81±0.91	NE	NE	2.4	0.93±0.24
	SB	39.77±1.81	NE	NE	NE	13.35±2.66
19. FSB1	WG	47.36±2.63	11	2	7	0.33±0.11
	SB	49.32±0.47	NE	NE	NE	2.90±0.45
20. FSB2	WG	47.99±1.09	4	11	NE	2.40±1.05
	SB	47.66±4.58	NE	NE	NE	2.64±0.74
21. FSB3	WG	44.82±1.56	NE	NE	23	0.41±0.15
	SB	50.06±3.20	NE	NE	NE	3.03±1.16
22. DSB1	WG	39.93±0.93	5	NE	2	1.26±0.13
	SB	52.16±11.93	NE	NE	NE	3.12±0.92
23. DSB2	WG	41.50±0.74	NE	NE	NE	0.51±0.02
	SB	47.83±2.58	NE	NE	NE	1.44±0.48

WG = Waste glycerol, SB = Soybean oil, J = Jatropha oil, P = Palm oil, NE = Not Emulsion

Table 4.2 The isolated bacterial strains cultured in basal media with waste glycerol or soybean oil as carbon source and their properties (cont.)

Bacterial strain	Carbon source (3%)	Surface tension (mN/m) at 25°C	E_{24} (SB, %)	E_{24} (J, %)	E_{24} (P, %)	Dry cell weight (g/l)
24. FMBG	WG	43.63±1.30	19	37	54	0.58±0.08
	SB	53.20±5.56	48	7	6	2.83±0.66
25. FMSB1	WG	35.53±8.56	NE	NE	NE	1.99±0.55
	SB	36.99±3.73	NE	NE	NE	13.74±3.39
26. FMSB2	WG	45.01±0.86	NE	NE	2.3	0.50±0.03
	SB	52.64±4.39	NE	NE	1	1.78±0.79
27. FMSB3	WG	47.71±0.09	NE	NE	NE	3.80±0.40
	SB	47.06±0.70	NE	NE	NE	2.54±1.17
Control	WG	44.45±1.02	-	-	-	-
	SB	58.10±6.72	-	-	-	-

WG = Waste glycerol, SB = Soybean oil, J = Jatropha oil, P = Palm oil, NE = Not Emulsion,

As a result two bacterial strains; JBG1 and JSB2, grown on soybean oil had the capacity to reduce surface tension of medium lower than 35 mN/m (31.43 and 29.91 mN/m respectively), which were referred as the effective biosurfactant-producing bacteria (Desai and Banat, 1997; Soberon-Chavez and Maier, 2011). Moreover, these two strains had high emulsification activity with vegetable oil in range of 35 to 50%. Thus, bacterial strains JBG1 and JSB2 using soybean oil as carbon source were chosen and determined for interfacial tension (IFT, mN/m) and preliminary oil extraction (%) in the further step.

Furthermore, biosurfactant-producing bacteria from laboratory library were also considered to select the effective biosurfactant-producing bacteria. Based on their capacity to grow by using different type of oil (glycerol, lard, or soybean oil) as substrate, surface tension (ST) of medium lower than 35 mN/m and ability to emulsify with vegetable oil. Among 52 biosurfactant-producing bacteria from laboratory library, six bacterial strains were chosen and shown in Table 4.3.

Table 4.3 The effective biosurfactant-producing bacteria selected from library laboratory and their properties

Bacteria	**Source**	**Substrate**	**Surface tension (mN/m)**	**E_{24} (SB, %)**
Bacillus sp. GY17	Soil	0.5% Glycerol	29	75
Achromobacter sp. GY30	Soil	5% Glycerol	28	66
Cellulosimicrobium sp. GY33	Soil	5% Glycerol	33	62
Stenotrophomonas sp. LP1	Greased trap water	3% Lard	27	NE
Alcaligenes sp. LS	Greased trap water	3% Soybean oil	28	NE
Rhodococcus sp. CALSB1	Waste food	3% Soybean oil	31	NE

NE = Not Emulsion, SB = Soybean oil

Then, these selected strains were cultured on 50 ml BM with different carbon sources; waste glycerol, slop oil, or soybean oil relied on the previous data and then shaking at 200 rpm, room temperature for 7 days. Cell-free supernatant was collected by centrifugation and cell pellet was measured for dry cell weight (g/l). Supernatant was measured surface tension with a tensiometer using plate (mN/m) and E_{24} (%) with vegetable oil (soybean, jatropha, and palm oil). The results are presented in Table 4.4.

Table 4.4 The effective biosurfactant-producing bacteria selected from laboratory library cultured in basal media supplemented with waste glycerol, slop oil, or soybean oil as carbon source and their properties

Bacteria	**Carbon source (3%)**	**Surface tension (mN/m) at 25°C**	**E_{24} (SB, %)**	**E_{24} (J, %)**	**E_{24} (P, %)**	**Dry cell weight (g/l)**
Bacillus sp. GY17	WG	29.44±0.13	36	45	64	1.05± 0.03
Achromobacter sp. GY30	WG	29.45±0.08	32	41	39	1.04±0.06
Cellulosimicrobium sp. GY33	WG	41.12±0.37	NE	NE	NE	2.41±0.01
Stenotrophomonas sp. LP1	SB	43.21±7.98	NE	NE	NE	2.10±0.84
Alcaligenes sp. LS	SB	29.28±1.80	47	52	78	4.69±0.22
Rhodococcus sp. CALSB1	SO	36.68±0.79	NE	NE	NE	0.26±0.02

SB = Soybean oil, J = Jatropha oil, P = Palm oil, NE = Not Emulsion

Biosurfactant-producing bacteria (6 strains) from laboratory library were selected again base on their activity to reduce surface tension of media lower than 35 mN/m and ability to emulsify vegetable oils (soybean oil, jatropha oil, or palm oil). Three strains including *Bacillus* sp. GY17, *Achromobacter* sp. GY30 (using waste glycerol as carbon source) and *Alcaligenes* sp. LS (using soybean oil as carbon source) were chosen. Thus, the most effective biosurfactant-producing bacteria obtained from isolation experiment and laboratory library were bacterial strain JBG1, JSB2, *Bacillus* sp. GY17, *Achromobacter* sp. GY30, and *Alcaligenes* sp. LS. Next step, cell-free broth of strains JBG1, JSB2, GY17, GY30, and LS were determined for IFT (mN/m) with vegetable oil (jatropha and palm oil) by using tensiometer with ring method (Table 4.5) and further tasting for preliminary vegetable oil extraction.

Table 4.5 Interfacial tension (mN/m) between cell-free broth of the effective biosurfactant-producing bacteria selected from isolation experiment and laboratory library and vegetable oil

Bacteria	**Carbon source (3%)**	**Interfacial tension (IFT, mN/m) at 25°C**	
		Jatropha oil	**Palm oil**
Water	-	31.07±3.42	39.19±0.11
Bacillus sp. GY17	WG	ND	1.15±0.25
Achromobacter sp. GY30	WG	ND	0.78±0.01
Alcaligenes sp. LS	SB	1.49±0.36	1.56±0.02
Bacterium strain JBG1	SB	1.53±0.26	1.99±1.09
Bacterium strain JSB2	SB	4.09±1.03	4.61±0.45

WG = Waste glycerol, SB = Soybean oil, ND = Not detected

The limitation of measurement IFT (mN/m) by using tensiometer with ring method is 1 mN/m (Operating manual DCAT, 2005). Moreover, only clear and well separation of immiscible two phases could detect by this method. Cell-free broth of strain GY17 and GY30 produced a bit emulsion when adding jatropha oil, so thus their IFT could not be detected.

4.1.4 Oil extraction (preliminary)

To find an effective bacterial strain for studying biosurfactant production in next step, preliminary oil extraction experiment was conducted. Aqueous biosurfactant-based extraction method was applied and determined as alternative method for vegetable oil extraction. The amount of oil extracted by aqueous biosurfant-based extraction was calculated in term of oil detachment (%) and compared to conventional extraction method by using hexane.

4.1.3.1 Hexane extraction

By using hexane extraction method, the total amount of oil present in jatropha and palm kernel seed were 44-53 and 46-48%wt respectively and similar to the amount of oil reported by previous study (Makkar *et al.*, 1997) at 43-59%wt for jatropha kernel and 48-59%wt for palm kernel (Gunstone, 2002).

4.1.3.2 Vegetable oil extraction

In this step, oil extraction by surfactant-based using anionic surfactant (AOT) and water alone were also carried out.

The result shown that high jatropha kernel oil detachments were 45.28 and 39.62%, respectively, when using cell-free broth of LS and GY30. In addition high palm kernel oil detachments were 62.76 and 61.70%, respectively, when using cell free-broth of GY17 and GY30 (Fig. 4.1).

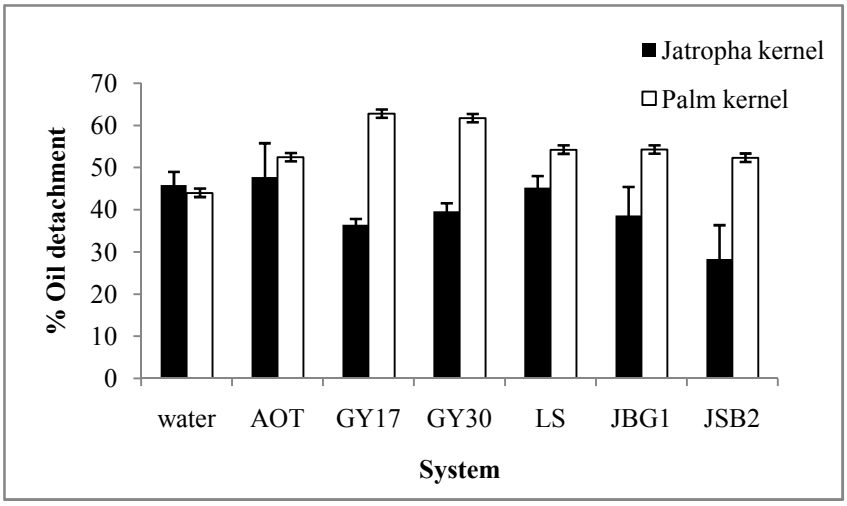


Fig. 4.1 %Oil detachment by using aqueous biosurfactant-based extraction compared to the total oil content extraction by hexane extraction (AOT = Aerosol OT)

It could be concluded that biosurfactant produced from bacterial strains LS, GY17, and GY30 had the most potential and suit for the vegetable oil extraction application. In order to select an efficient strain for biosurfactant production, the carbon source of each bacterial strain should be considered. Waste glycerol (WG) is byproduct from biodiesel production process; it could be used as carbon source for growing microorganism (de Sousa *et al.*, 2011). The use of WG as substrate has been paid much attention by many researchers because it could reduce the production cost and waste deposal in the same time. Lui *et al.* (2011) showed that *Ustilago maydis* had ability to use crude glycerol received from alkali-catalyzed transesterification of waste cooking oil with methanol as carbon source to produce biosurfactant. Similar to de Sousa *et al.* (2011), they also studied the effect of a co-product of biodiesel production as carbon source on the biosurfactant production by *P. aeruginosa* MSIC02. Different carbon sources; crude glycerin (obtained after methanol removing by evaporation), hydrolyzed glycerin (prepared by acid hydrolysis of crude glycerin), soybean oil, and castor oil) were applied. They found that hydrolyzed glycerin was the best carbon source for cell growth and production of biosurfactants. Although, strain GY17 and GY30 could grow on WG, strain GY30 gave high jatropha kernel oil detachment (%) and similar amount of palm kernel oil detachment (%). Moreover, biosurfactant production from *Achromobacter* species had not been examined yet. Hence, *Achromobacter* sp. GY30 was of interest and chosen for further study in optimization of biosurfactant production to obtain the maximum biosurfactant yield in vegetable oil extraction application.

4.2 Production of biosurfactant by selected biosurfactant-producing bacterium

There was evidence that medium composition plays an important role on biosurfactant production by microorganisms (Mukherjee *et al.*, 2006). Especially, carbon and nitrogen type and concentration have been reported to affect in both quality and quantity of biosurfactants (Das *et al.*, 2009). Furthermore, several researches have been indicated that addition of insoluble precursors could increase biosurfactant yield (Desai and Banat, 1997; Ferraz *et al.*, 2002; Mukherjee *et al.*, 2006). Thus, carbon concentration, nitrogen source and concentration as well as precursor supplementation were investigated for biosurfactant production from *Achromobacter* sp. GY30.

4.2.1 Effect of carbon concentration on biosurfactant production

The concentrations of waste glycerol (WG) were adjusted to be 2, 5, 7, and 12% (w/v). Cell-free broth and cell pellet were collected separately by centrifugation and cell-free broth was determined for the surface tension, emulsification index (E_{24}), and biosurfactant yield. Cell pellet was used to measure dry cell weigh. The results are performed in Table 4.6.

Table 4.6 Effect of various waste glycerol concentrations on biosurfactant production by *Achromobacter* sp. GY30 grown in productive medium during 5 days in terms of surface tension, growth, E_{24}, and biosurfactant yield

Waste glycerol concentration (%, w/v)	Surface tension (mN/m) at 25°C		E_{24} (%)			Dry cell weight (g/l)	Yield (g/l)
	Control	Sample	SB	J	P		
2	37.47±0.25	29.37±0.14	20	64	64	0.32±0.03	0.06 ± 0.01
5	35.64±0.02	30.28±0.07	39	59	55	1.59±0.40	0.09 ± 0.35
7	35.50±0.21	31.14±0.30	59	7	50	1.52±0.06	0.15 ± 0.02
12	35.36±0.60	34.74±1.11	NE	NE	NE	0.14±0.003	0.49 ± 0.08

SB = Soybean oil, J = Jatropha oil, P = Palm oil, and NE = Not emulsion

Although biosurfactant yield was increased when increasing WG concentration, the high surface tension and no emulsification activity were obtained when using 12% WG as carbon source for culturing GY30. Surface tension and emulsification activity (E_{24}) are commonly used to indicate surface activity property (Das *et al.*, 2009; Silva *et al.*, 2010). Thus, using WG concentration at 12% (w/v) was not appropriate for biosurfactant production from GY30.

However, when strain GY30 was grown on 2, 5, and 7% WG low surface tensions at 29, 30 and 31 mN/m, respectively was observed. E_{24} over 50% with vegetable oil were also obtained when using these WG concentrations. Moreover, when dry cell weight of GY30 was compared, we found that using 5 and 7% WG as carbon source obtained high dry cell weight at 1.59 and 1.52 g/l, respectively. Base on surface tension reduction, dry cell weight, E_{24}, and biosurfactant yield, using 5 and 7% WG concentrations as carbon source trend to be the most potential WG concentrations for growing GY30 to receive high production yield. Nevertheless, to confirm the high biosurfactant yield, cell-free broth containing biosurfactant of 5 and 7% WG were diluted to be 10^{-1} and 10^{-2} times and then measured for their surface tension. The results are shown in Table 4.7.

Table 4.7 Surface tension at 10^{-1} and 10^{-2} time dilutions of cell-free broth from 5 and 7% waste glycerol

Waste glycerol concentration (%, w/v)	Surface tension (mN/m) at 25°C	
	Dilution 10^{-1}	10^{-2}
5	36.00±0.23	48.50±0.49
7	37.07±0.58	70.32±0.62

The result revealed that surface tension of dilution 10^{-2} of cell-free broth from 5% WG was 48.50 mN/m which was lower than those of 7% WG (70.32 mN/m). Hence, 5% WG seem to produce high biosurfactant yield and was chosen for further study.

4.2.2 Effect of nitrogen sources and concentration on biosurfactant production

Using the most appropriate waste glycerol concentration (5%, w/v) from the previous step, a various nitrogen source and concentration including C/N ratio were investigated. Three types of the most commonly used nitrogen source were applied based on literature review; $NaNO_3$, $(NH_4)_2SO_4$, and NH_4NO_3 (Franzetti *et al*., 2009; Yataghene *et al.*, 2009; Silva *et al.*, 2010) and selected the best nitrogen source for biosurfactant production. Then, appropriate nitrogen concentrations and C/N ratios of selected nitrogen were studied in term of surface tension (mN/m) and biosurfactant concentration (g/l) by measuring cell-free supernatant and dry cell weight (g/l) by using cell pellets. The results are presented in Table 4.8.

Table 4.8 Effect of various nitrogen sources and concentrations and C/N ratio on biosurfactant production by *Achromobacter* sp. GY30 grown on productive medium using 5% waste glycerol during 5 days in terms of surface tension, growth, and biosurfactant yield

Nitrogen		**C/N**	**Surface tension (mN/m) at 25°C**	**Dry cell weight (g/l)**	**Yield (g/l)***	
Type	**Concentration (%, w/v)**				**1st method**	**2nd method**
$NaNO_3$	0.1	120	31.18±0.30	1.95±0.15	0.13±0.06	-
	0.2	60	30.69±0.34	1.26±0.36	0.15±0.01	-
	0.4	30	30.18±0.19	1.52±0.09	0.16±0.01	0.34±0.06
	0.6	20	29.86±0.03	1.59±0.70	0.13±0.01	-
$(NH_4)_2SO_4$	0.1	120	43.90±0.36	1.77±0.10	0.03±0.00	-
	0.2	60	44.24±0.87	1.72±0.15	0.04± 0.00	-
	0.4	30	44.78±0.38	1.76±0.10	0.03±0.00	-
	0.6	20	41.18±0.48	1.61±0.10	0.04±0.00	-
NH_4NO_3	0.1	120	32.41±1.62	0.76±0.27	0.10±0.02	-
	0.2	60	40.96±1.23	0.75±0.46	0.12±0.00	-
	0.4	30	40.50±0.40	0.44±0.01	0.12±0.04	-
	0.6	20	39.68±0.33	0.42±0.01	0.11±0.03	-

*1st method was crude biosurfactant extracted by using biosurfactant isolation method 1: (adapted from de Sousa *et al.*, 2011)

Biosurfactants were isolated from cell-free broth by using a liquid/liquid extraction, chloroform/methanol (65/15, v/v), in ratio of cell-free broth/solvent equal to 3/1. Then, the mixture was shaken at 200 rpm, room temperature for 2 hours. Phase separation was done in funnel, collect the below phase (crude biosurfactant). Solvent was evaporated to obtain crude biosurfactant.

2nd method was crude biosurfactant extracted by using biosurfactant isolation method 2: (adapted from Silva *et al.*, 2010)

Biosurfactants was isolated from cell-free broth by adjusting pH to 2 with 6M HCl and stored at 4°C overnight. Then, equal volume of

chloroform/methanol (2/1, v/v) was added and shaken at 200 rpm, room temperature for 30 min. The mixer was settled in funnel to allowed phase separation. The below phase was collected and evaporated at 60°C using a rotary evaporator. The sticky yellow brown product was obtained (crude biosurfactant). Then, dissolved this product with methanol and evaporated again at 45°C to receive crude biosurfactant.

It was found that $NaNO_3$ gave the best result of low surface tension (between 29 to 31 mN/m), high dry cell weight (over 1 g/l), and high biosurfactant yield (about 0.15 g/l). Similar to Lotfabad *et al.* (2009) work, they studied effect of various nitrogen sources; $NaNO_3$, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, urea, yeast, and peptone, on the biosurfactant produced by *Pseudomonas aeruginosa* MR01 grown on minimal salt medium containing glucose. They found that the highest biosurfactant yield (0.98 g/l) was received when using $NaNO_3$ as a nitrogen source. Mulligan and Gibbs (1989) explained role of nitrogen source on biosurfactant produced by *P. aeruginosa*. Briefly, during the synthesis of biosurfactant, the nitrogen limitation may promote lipid accumulation which is the rate-determining factor. When comparing the use of nitrate and ammonia, the adsorption of nitrate as a nitrogen source is slower than ammonia, this step simulate a nitrogen-limiting condition which is preferable for biosurfactant production.

The biosurfactant yield obtained from this experiment was increased about 1.5 fold when compared to the previous step (step 4.2.1). Table 4.8 also showed that using $(NH_4)_2SO_4$ as nitrogen source could help cell growth but was not used for biosurfactant production. This observation was consistent with the finding in Silva *et al.* (2010) study. However, biosurfactant yield about 0.1 g/l was observed with low dry cell weight when using NH_4NO_3 as nitrogen source. Thus, in this step $NaNO_3$ was selected as nitrogen source for biosurfactant production and used for the next experiment.

Another perspective to improve biosurfactant productivity was the C/N ratio. In this experiment, waste glycerol concentration was fixed at 5% (w/v), $NaNO_3$ was selected from the previous experiment and used as nitrogen source, concentration of

$NaNO_3$ was adjusted to be 0.1, 0.2, 0.4, and 0.6% (w/v) corresponding to C/N ratio of 120, 60, 30, and 20. The best results of low surface tension (30 mN/m), high dry cell weight (1.52 g/l), and high biosurfactant yield (0.16 g/l) were obtained at concentration of 0.4% (w/v) which C/N ratio equal to 30 (Table 4.8). Moreover, this $NaNO_3$ concentration could promote in term of both cell growth and biosurfactant production. However, it should be noted that C/N ratio in rang of 20 to 120 could also give high biosurfactant yield. The results obtained in this experiment were agreed with Lotfabad *et al.* (2009) work, they found that using C/N ration in range of 10 to 30, high biosurfactant yields, high dry cell weigh, and low surface tension were obtained. Wu *et al.* (2008) also found that the C/N ratio in rang of 6 to 53, *Pseudomonas aeruginosa* EM1could produce the high biosurfactant yield when using glucose and $NaNO_3$ as carbon and nitrogen source.

Hence, addition of $NaNO_3$ as nitrogen source with a concentration of 0.4% (w/v) which C/N ratio equal to 30 was chosen for studying in the further step.

However, it was found that even biosurfactant productivity was improved when adjusting nitrogen source and concentration including C/N ratio, small amount of the biosurfactant yield was obtained. To enhance biosurfactant yield, an effective downstream recovery process should be considered (Banat *et al*., 2010). The 2^{nd} method of biosurfactant recovery was developed (adapted from Silva *et al*., 2010). It is a combination of acid precipitation and solvent extraction. Using acid precipitation, the biosurfactants are prepared in form that can be easily recovered from the culture medium by solvent extraction (Sarachat *et al*., 2010).

Nevertheless, the biosurfactant yield of the selected condition; 5% (w/v) waste glycerol and 0.4% $NaNO_3$ as carbon and nitrogen source and concentration, was extracted by using new biosurfactant recovery to estimate the biosurfactant yield. It was found that the biosurfactant yield of 0.34 g/l was received (Table 4.8). The result performed that this yield was 2 fold of that obtained from using 1^{st} biosurfactant recovery method. Thus, from further step the 2^{nd} method of biosurfactant recovery was applied to extract crude biosurfactant.

4.2.3 Effect of precursors supplementation on biosurfactant production

Using the selected waste glycerol concentration and selected nitrogen source and concentration with appropriate C/N ratio**,** the addition of biosurfactant precursors to the growth medium was investigated. Vegetable oils (soybean and palm oil) were used as a precursor in the study. Each type of oil, 0.1% (v/v), was added into culture medium after 48 hours of cultivation. Then, Cell-free supernatant was collected and measured for the surface tension (mN/m) and biosurfactant concentration (g/l) and cell pellets were tested dry cell weight (g/l). The results are showed in Table 4.9.

Table 4.9 Effect of various precursors supplementation on biosurfactant production by *Achromobacter* sp. GY30 grown on productive medium using 5% waste glycerol and 0.4% $NaNO_3$ during 5 days in terms of surface tension, growth, and biosurfactant yield

Precursor type	**Concentration (%, v/v)**	**Surface tension (mN/m) at 25°C**	**Dry cell weight (g/l)**	**Biosurfactant yield (g/l)**
Palm oil	0.1	28.83 ±	1.61 ± 0.22	0.64 ± 0.03
Soybean oil	0.1	29.56 ± 0.56	1.01 ± 0.51	0.62 ± 0.08

The supplementation culture medium with vegetable oil showed that both palm and soybean oil could reduce surface tension. However, using palm oil produced slightly higher dry cell weight and biosurfactant yield than soybean oil. Supplementation with palm oil could also increase biosurfactant yield about 2 fold from the previous step. Thus, palm oil was selected for further experiments.

Stuwer *et al*. (1987) studied the influence of addition vegetable oils on biosurfactant produced by *Torulopsis apicola* IMET. They observed that biosurfactant yield was increased about 3 fold when sunflower oil was supplemented in culture medium containing glucose and $NaNO_3$ as carbon and nitrogen source. Ferraz *et al.* (2002) also suggested that fatty acid in vegetable oil stimulated the biosurfactant production by *Serratia marcescens* sp. LB006.

4.2.4 Experimental design

The previous optimization of medium composition is the classical method by changing one factor at a time, while the interaction among these factors was not examined. Moreover, this method is time consuming. To solve these limitations, statistical experimental design like response surface methodology (RSM) was utilized to determine interactions between the factors and prediction the optimal medium composition. A two-level full factorial design (type of RSM) was developed in this study with three variables; carbon and nitrogen concentration, and precursor supplementation, at low and high level (-1 and +1). The concentration input value of three factors was selected based on our previous tests and shown in Table 4.10. Biosurfactant yield was used as respond variable.

Table 4.10 Results of 2^3 two-level full factorial experimental design using the strain GY30

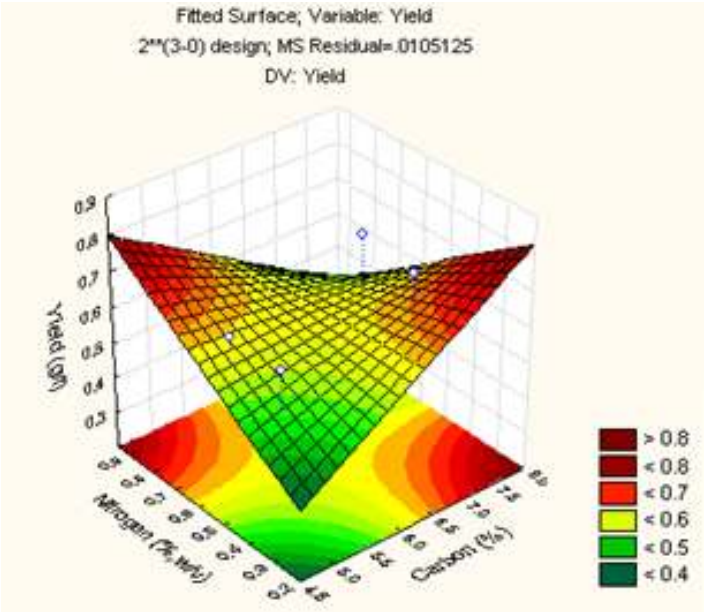
Run no.	**Carbon (%) X_1**	**Nitrogen (%, w/v) X_2**	**Palm oil (%, v/v) X_3**	**Yield (g/l) Z**	**C/N**
1	5	0.4	0.1	0.64 ± 0.03	30
2	7	0.4	0.1	0.74 ±0.11	42
3	5	0.6	0.1	0.64 ±0.04	20
4	7	0.6	0.1	0.76 ±0.12	28
5	5	0.4	1	0.44 ±0.00	30
6	7	0.4	1	0.57 ±0.06	42
7	5	0.6	1	0.58 ±0.09	20
8	7	0.6	1	0.45 ±0.02	28

The result showed that biosurfactant yield in range of 0.43 to 0.76 g/l was received with different runs. It was observed that run number 4 gave the highest biosurfactant yield of 0.76 g/l. According to the response values obtained from the experimental design (Table 4.10), a second-order equation was generated following equation (4.1) with $R^2 = 0.89600594$:

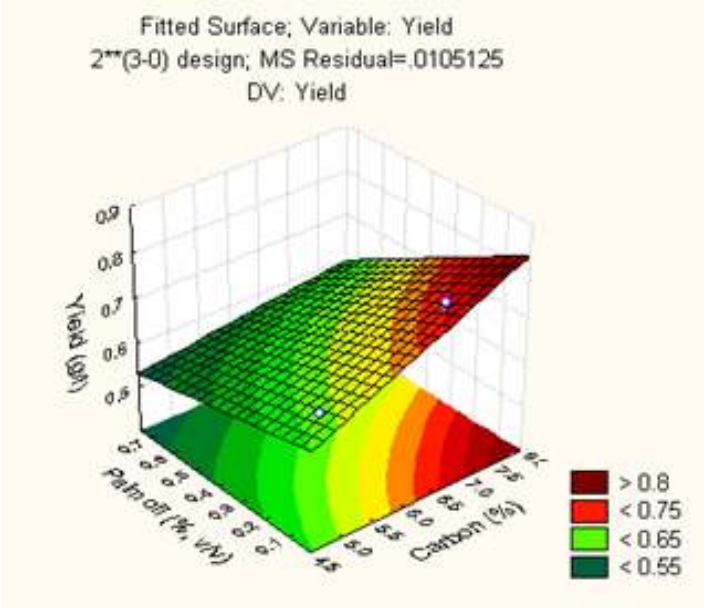
$$\text{Yield} = 0.50 - 0.31(X_1X_2) - 0.058(X_1X_3) + 0.03(X_2X_3) + 0.02(X_1)^2 + 1.92(X_2)^2 + 0.12(X_3)^2 \quad (4.1)$$

Where X_1, X_2, and X_3 were concentration of waste glycerol, $NaNO_3$, and palm oil, respectively.

The respond surface analysis was studied by using two factors, while the third factor was fixed. The three-dimensional response surfaces were plotted in Fig. 4.2A and B based on equation 4.1. Regarding to Fig 4.2A, waste glycerol and $NaNO_3$ concentration affected on biosurfactant yield. High biosurfactant yield was obtained when using high waste glycerol concentration (gather than 6.5%, w/v) with low $NaNO_3$ concentration (lower than 2%, w/v) or low waste glycerol concentration (lower than 5%, w/v) with high $NaNO_3$ concentration (gather than 0.7%, w/v). Fig 4.2B also showed that supplementation culture medium with palm oil slightly affected biosurfactant yield. Thus, little palm oil supplementation (lower than 0.1%, v/v) could be used to obtain high biosurfactant yield.



(A)



(B)

Fig 4.2 Response surface analysis (A) Effect of waste glycerol and $NaNO_3$ concentration (B) Effect of palm oil and waste glycerol concentration, on biosurfactant yield from strain GY30

Base on results from Fig 4.2A and B, the conditions which predicted to give high biosurfactant yield were selected and presented in Table 4.11. These conditions were cultured in laboratory and examined for actual amount of biosurfactant. Then, the actual and predicted amount of biosurfactant were compared and chosen the optimum condition which produced maximum biosurfactant.

Table 4.11 Prediction condition analysis on biosurfactant yield

Condition				**C/N**	**Surface tension (mN/m) at 25°C**		**Dry cell weight (g/l)**	**Yield (g/l)**	
No.	**Waste glycerol (%, w/v)**	**$NaNO_3$ (%, w/v)**	**Palm oil %, v/v)**		**Control**	**Sample**		**Predict value**	**Actual value**
1	2	0.8	0.01	6.6	37.47± 0.25	30.77± 0.28	0.22± 0.02	1.30	0.17± 0.02
2	3	0.8	0.01	9	38.03± 0.57	30.67± 0.02	0.38± 0.02	1.14	0.21± 0.01
3	5	0.8	0.01	15	40.85± 0.64	29.64± 0.42	0.89± 0.05	0.92	0.35± 0.04
4	6.5	0.1	0.01	157	36.71± 0.10	29.96± 0.55	0.91± 0.09	1.07	0.40± 0.04
5	7	0.1	0.01	169	37.49± 0.60	29.12± 0.22	1.09± 0.20	1.14	0.40± 0.08
6	7	0.4	-	42	35.86± 0.62	30.13± 0.36	0.36± 0.05	0.73	0.40± 0.02
7	7	0.4	0.01	42	38.30± 0.89	28.90± 1.28	0.52± 0.13	0.81	0.79± 0.03

The C/N ratio was reported as an extremely sensitive parameter influencing the biosurfactant production (Santos *et. al*, 2002; de Rosa *et al.*, 2010). It was observed that condition no. 7 of using 7% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.01% (v/v) palm oil gave the highest biosurfactant yield of 0.79 g/l. The experimental design provided the optimum condition which could enhance the biosurfactant production by *Achromobacter* sp. GY30 from 0.64 g/l (single-factor experiments) to 0.79 g/l. The C/N ratio of this optimum condition was 42, which was within the range of optimal C/N ratio (20 to 120) determined in single-factor experiments. Although condition no. 6 which C/N ratio was also 42 but addition of palm oil was neglected, small amount of biosurfactant yield was obtained and much lower than the predict value. This finding is in agreement with that observed in our single-factor studies. However, the design experiment indicated that small amount of palm oil could stimulate higher biosurfactant production. For condition no.1 to 5, it was found that the C/N ratios were out of optimum range (20 to 120). Condition no. 1 to 3 with low C/N ratio (6, 9, and 15 respectively) gave biosurfactant yield lower than prediction, which implied the culture medium containing high nitrogen did not promote biosurfactant production by strain GY30. Silva *et al*. (2010) reported that an excess of the nitrogen concentration (low C/N ratio) was only used as the substrate to the synthesis of cellular material and was not utilized for the biosurfactant production. In contrast, high C/N ratios over optimum range also limited bacterial growth, the cellular metabolism along with the production of productivity. However, it depended on bacterial strain. Condition no. 4 and 5 of C/N ratio higher than 120 (156 and168 respectively) also gave poor biosurfactant yield. The result was correlated with the literature, Lotfabad *et al*. (2009) found that using C/N ratio over 40 (optimum C/N ratio was 10 to 30) for culturing *Pseudomonas aeruginosa* MR01, dramatically decreased biosurfactant yield was obtained. Wu *et al*. (2008) also observed that the C/N ratio of 6.5 to 52, the similar productivity was received, but significantly decreased in biosurfactant yield was observed when the C/N ratio increased to 130.

Nevertheless, it was observed that over biosurfactant yield was predicted by experiment design and predict and actual value of some conditions did not correlated.

It might due to other parameters which could affect biosurfactant production by strain GY30; such as effect of nutrient and environmental condition, were not determined in this experiment. Roldán-Carrillo *et al*. (2011) suggested that each variables of C/N, C/Fe, and C/Mg ratios as well as the interaction among these variables had a significant effect on the reduction of surface tension (from 57 to 33 mN/m) and therefore on biosurfactant production by *Serratia marcescens* SmSA. Silva *et al*. (2010) found that biosurfactant yield produced by *Pseudomonas aeruginosa* UCP0992 was increased from 5.5 to 8 g/l when adjusting fermentation conditions; incubation temperature, aeration, and agitation speeds. Moreover, by using two-level full factorial design (8 runs) in this study might not enough to predict the whole factors affected biosurfactant production from strain GY30. Thus, increasing more experimental design by using other types of RSM such as Box-Behnken design (15 runs) (Roldán-Carrillo *et al*., 2011) or central composite design (20 runs) (Wu *et al*., 2008) might improve accuracy of the experimental model. Besides, the error of predict value might occur from using different batch of waste glycerol as carbon source for optimization culture medium composition. Thus, using the same lot of waste glycerol should be concerned in the further study to prevent the error from this factor.

Summary, optimization culture medium composition for biosurfactant produced by *Achromobacter* sp. GY30 by using classical method (changing one factor at a time) gave the optimum condition of using 5% (w/v) waste glycerol, 0.4% (w/v) NaNO3, and 0.1% (v/v) palm oil. In this step, a 2-fold enhancement in biosurfactant was obtained (from 0.34 to 0.64 g/l). Then, experimental design was applied to improve the biosurfactant yield and predicted the best optimum condition. It was found that the best optimum condition was using of 7% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.01% (v/v) palm oil, correlated to C/N ratio of 42. This optimum condition increased biosurfactant 20% (from 0.64 to 0.79 g/l). Although the use of experimental design did not offer high biosurfactant yield improvement, it suggested that low amount of palm oil could be used to stimulate higher biosurfactant yield. The biosurfactant obtained from this optimum condition was studied for their properties in the further experiment.

4.2.5 Properties of biosurfactant

Properties of the cell-free broth containing the biosurfactant from *Achromobacter* sp. GY30 cultivated in productive medium with 7% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.01% (v/v) palm oil during 5 days were measured in term of surface tension, interfacial tension, emulsification activity with vegetable oil (soybean, jatropha, and palm oil), critical micelle concentration (CMC) and identification of ionic charge of crude biosurfactant. Crude biosufactant solution was prepared by dissolved crude biosurfactant with distilled water.

The surface tension of medium was reduced from 38 to 29 mN/m and the interfacial tension of 2.22 mN/m was obtained when testing with palm oil. Emulsification index (E_{24}, %) between cell-free broth containing the biosurfactant from strain GY30 and soybean, jatropha and palm oil were 14, 52, and 60 respectively.

For CMC determination, surface tensions against the crude biosurfactant concentrations from strain GY30 were plotted (Fig. 4.3). The surface tension of water was gradually decreased with increasing biosurfactant concentration from 70 to 31 mN/m, with a biosurfactant concentration of 53.2 mg/l. The result indicated that strain GY30 produced efficient biosurfactants due to less biosurfactants were required to decrease surface tension (Sarubbo *et al*., 2006).

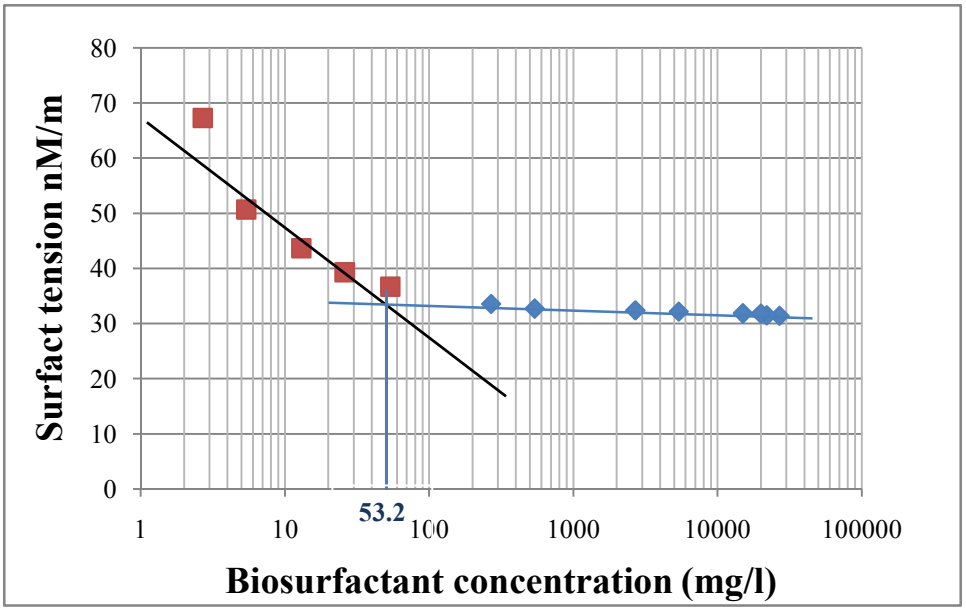


Fig 4.3 A critical micelle concentration of crude biosurfactant produced by *Achromobacter* sp. GY30 cultivated in productive medium contained 7% bottom glycerol, 0.4% $NaNO_3$, and supplemented with 0.1% palm oil during 5 days (at 25°C)

Determination of crude biosurfactant ionic charge is the important property owing to this property can be used to improve the efficiency of biosurfactant for applying in many fields. Different ionic charge used different method to improve its efficiency for example solubility of ionic charge biosurfactant can be improved by adding electrolyte (Rosen, 2004).

After mixing 53.2 mg/l of crude biosurfactant solution with distilled water, Dichloromethane (DCM), and indicator (Dimidium Bromide/Disulphine Blue) and shacking vigorously for 30 sec., the below phase was presented the pink color (Fig.4.4). It was indicated that crude biosurfactant was anionic charge. Next, this solution was titrated with 1 mM Cetyl Trimethyl Ammonium Bromide (CTAB, cationic surfactant) until the colorless color of the below phase was observed (end point). Then concentration of this anionic charge was calculated. The result found that 4.4 ml CTAB was used to reach the end point. Thus, concentration of anionic of crude biosurfactant from strain GY30 was calculated to be 0.098 mM.

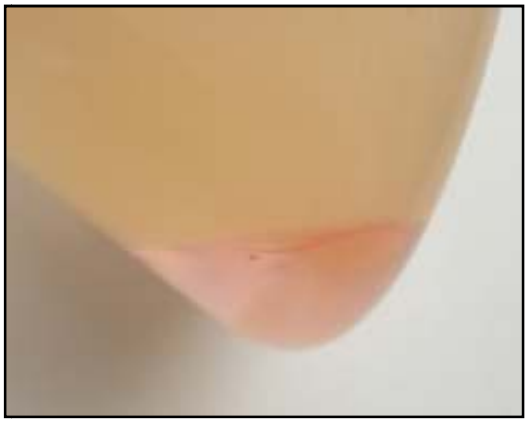


Fig 4.4 Characteristic of crude biosurfactant solution produced by *Achromobacter* sp. GY30, the color of the below phase changed into pink color after adding of indicator

4.3 Vegetable oil extraction

In the previous study (step 4.1.3.2), biosurfactant produced by strain GY30 showed high potential on palm kernel oil extraction. Hence, palm kernel seed was selected and studied in this oil extraction step.

4.3.1 Aqueous biosurfactant-based extraction

Palm kernel seed (1 g) was extracted for palm kernel oil by using 10 ml of the following system: cell-free broth, the solution of crude biosurfactant at CMC (53.2 mg/l), ten times higher than its CMC (532 mg/l), and five times lower than its CMC (10.6 mg/l). Palm kernel oil extraction by surfactant-based using anionic surfactant (AOT) and water alone were also carried out. The concentration of AOT at CMC (0.11% wt) was used. The extracted oil from this step was compared to the total amount of oil extracted by using hexane method and calculated in term of oil detachment (%). The results are performed in Fig. 4.5. In addition, IFT between each system and palm oil was investigated by using tensiometer with ring method. The results are shown in Table 4.12.

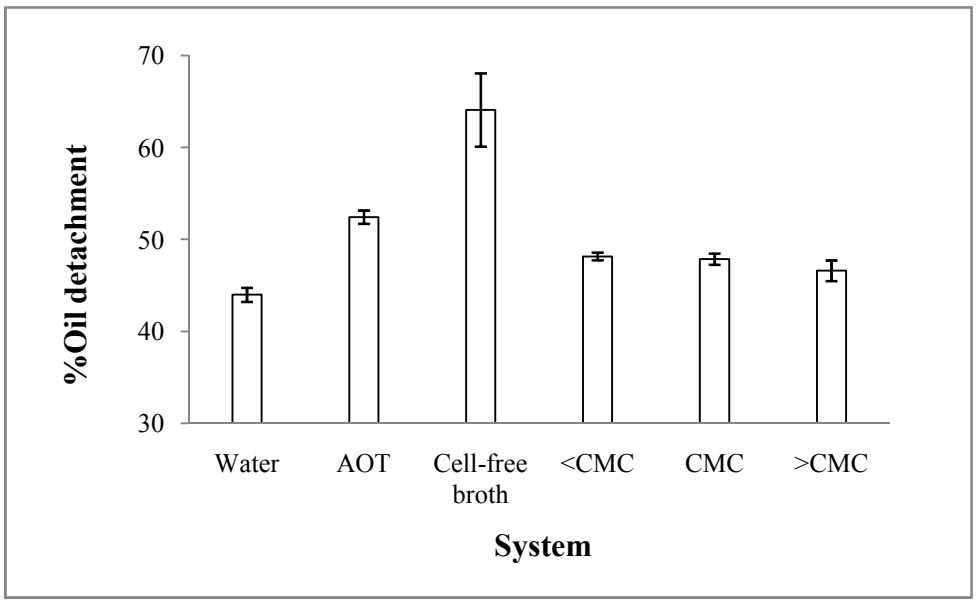


Fig 4.5 Oil detachment (%) of palm kernel using various biosurfactant concentrations on aqueous biosurfactant-based extraction compared to the total content extraction by hexane extraction method

Table 4.12 IFT between biosurfactant solutions and palm oil

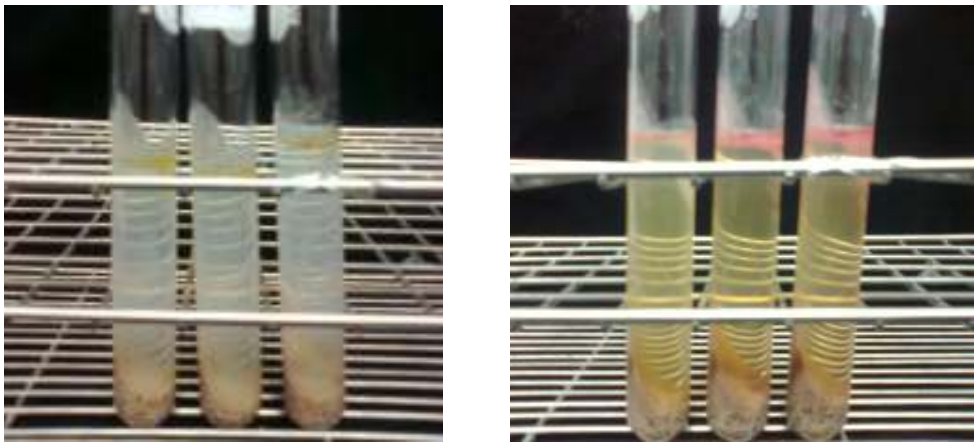
System	IFT (mN/m) at 25°C
water	39.19 ± 0.11
Cell-free broth	2.22 ± 1.02
Crude biosurfactant solution	
below CMC (532 mg/l)	30.89 ± 3.98
at CMC (53.2 mg/l)	37.80 ± 0.16
above CMC (10.6 mg/l)	35.89 ± 3.40

The role of biosurfactant in oil extraction is the biosurfactant monomer accumulation at the interface between water and vegetable oil in crush oilseed resulting in reduction of repulsive force between water and oil interface (IFT). Breaking of attached oil in oilseed into droplets and making them possible to be released from the particle (Kadioglu *et al*., 2010). Wang *et al.* (2009) also reported that oil attached in oilseed can be effectively extracted into liquid phase when using surface-active agent.

As seen in Fig. 4.5 oil detachment (%) in every system was higher than the system that using water alone. It was suggested that biosurfactant from strain GY30 had potential application in palm kernel oil extraction. The lowest oil detachment of palm kernel oil of 44% was obtained when using water alone. Similar to Do and Sabatini study, they extracted peanut oil by using water and anionic extended-surfactants (0.15 wt% C16-10.7POsulfate, 0.15 wt% C12-14PO-2EOsulfate, and 0.15 wt% C10-18PO-2EOsulfate) at optimum salt concentrations. They found that the lowest fraction of peanut oil extracted of 40% was obtained when using water alone.

When comparing among different crude biosurfactant concentrations; below, at, and above CMC, used in oil extraction system, the biosurfactant concentration at below CMC was expected to produce high oil detachment (%) due to it contained high amount of biosurfactant monomers. Whereas other concentrations; at and above CMC, biosurfactant monomers are preferable formed micelle. However, no significant difference oil detachment (%) was observed when using these biosurfactant concentrations (Fig. 4.5). The IFT results also showed the same trend of high IFT values of these biosurfactant concentrations, while a bit lower IFT value was obtained by using below CMC biosurfactant solution (Table 4.12). The advantage of using crude biosurfactant was the system produced extracted oil in form of free oil phase which is desirable in the extraction process (Fig. 4.6A). Nevertheless, the drawback of this method which is low oil extraction yield can be further improved by the addition of optimum electrolyte into the system or using of mix biosurfactant (Naksuk *et al.*, 2009).

The results also showed that the highest oil detachment of 64.10% (Fig. 4.5) which produced the lowest IFT of 2.22 mN/m (Table 4.12) was obtained when using cell-free broth. It might be due to cell-free broth contained many electrolytes: NaCl, K_2HPO_4, and KH_2PO_4, and waste glycerol component (carbon source) might contain soap which help produced low IFT value and promoted high oil extraction yield. According to Naksuk *et al*. (2009), the addition of NaCl (electrolyte) in range of 0 to 20% wt in mixed extended surfactant system (mix 3% Comperlan KD and 0.1% Alfoterra145-5PO) for palm kernel oil extraction was studied. They found that IFT value was reduced from 1 to 0.01 mN/m when NaCl concentration was increased to 17.5% wt. Although cell-free broth gave high oil detachment (%), the system produced emulsion-like phase which is undesirable in the oil extraction application and need the addition step to recover the oil (Fig. 4.6B).



(A) (B)

Fig. 4.6 Palm kernel oil extracted by aqueous biosurfactant-based extraction (A) using below CMC of crude biosufactant solution (B) using cell-free broth

Thus, using of crude biosurfactant solution in aqueous biosurfactant-based method for extraction of palm kernel oil was of the interest alternative method for reducing the use of hexane due to the system produced free oil phase which is favorable of the oil extraction process. Then, extracted oil from this method was examined for oil quality compare to extracted oil from hexane method.

For determination of residual oil remaining in palm kernel, however, by shaken residual palm kernel with hexane, then removed and evaporated the hexane phase at 70°C, and weighed remaining oil might give the over weight of remaining oil (low oil detachment, %) due to the residual of hexane or water (from aqueous biosurfactant-based extraction) might be not completely eliminated from remaining oil by evaporation. Thus for further study, Soxhlet extraction method (hexane) should be applied for precisely result of oil residual analysis including the determination of total oil present in oilseed.

4.3.2 Oil quality

Crude palm kernel (CPK) oil extracted by using hexane and aqueous biosurfactant-based method (crude biosurfactant) in this study was examined for oil quality in term of clarity, color, and free fatty acids content. CPK oil received from Suksomboon Palm Oil Industry which extracted by mechanical pressing was also compared. Moreover, the oil recovery (%) of mechanical pressing, hexane method (literature), and aqueous biosurfactant-based method received from this study were compared. The results are shown in Table 4.13.

Table 4.13 Comparison of the qualities of palm kernel oil extraction by mechanical pressing, hexane and aqueous biosurfactant-based extraction method

Parameters	**Mechanical pressing**	**Hexane method**	**Aqueous biosurfactant-based method**
Oil recovery (%wt)	67-74*	95-99**	~50-60***
Color	Clear yellow	Clear yellow	Clear yellow
State of liberated oil	-	Free oil	Free oil
Free fatty acid (%wt)			
Oleic	1.34±0.09	10.58±0.20	12.33±0.87
Lauric	1.05±0.06	7.50±0.14	8.31±0.62
Palmitic	1.47±0.09	9.60±0.18	10.64±0.78

* Hydraulic presses, Kwasi, 2002

** Rosenthal et al., 1996

***In this research (step 4.3.1 Fig. 4.5)

From Table 4.13, the visual appearance; color and clarity, of extracted oil from different oil extraction methods were similar (clear yellow). Moreover, both oil extraction method; hexane and aqueous biosurfactant-based, produced free oil phase which was satisfactory for this application. Free fatty acid content (%, wt) of the extracted palm kernel oil was examined by titration method (AOCS) and expressed as oleic, lauric, and palmitic (%) following the equation 3.5-3.7 in step 3.5.2. It was found that free fatty acid content (FFA, %) of extracted oil from hexane and biosurfactant-based method were higher than extracted oil from pressing method. Moreover, palm kernel oil extracted by biosurfactant-based method has a slightly higher content of free fatty acid than hexane method.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Vegetable oil is commonly extracted by solvent extraction with hexane. However, hexane is volatile and toxic in both human and environment. Thus, alternative approaches for oil extraction such as aqueous extraction process (AEP) have been evaluated to replace the use of hexane. Nevertheless, AEP by using water alone produce low oil extraction yield. The use of biosurfactant can improve the oil extraction yield in AEP. Although biosurfactant is widely used due to their vast advantages such as low toxicity and their potential application in many fields, their large scale production and application are limited by high production cost and low productivity.

This research aimed to screen an effective biosurfactant-producing bacterium and optimize its culture medium composition for biosurfactant production by using waste glycerol as substrate. The potential use of biosurfactant in aqueous-based extraction for vegetable oil extraction was also evaluated.

Biosurfactant-producing bacteria were enriched and isolated from vegetable samples by using waste glycerol, slop oil, and soybean oil as substrate. The bacterial strains which had capability to produce biosurfactant from this step combined with biosurfactant-producing bacteria from laboratory culture collection were selected based on their carbon source (waste glycerol, slop oil, and soybean oil), surface tension (ST, mN/m) lower than 35 mN/m, emulsification with vegetable oil (%), interfacial tension (IFT, mN/m), and oil extraction (preliminary, %). *Achromobacter* sp. GY30 gave low surface and interfacial tension, high E_{24} (%) with soybean, jatropha, and palm oil, and high oil detachment (%). Thus, strain GY30 was determined as an effective biosurfactant-producing bacterium and selected to study for its optimum culture medium composition.

For biosurfactant production experiment, the parameters affected biosurfactant production; carbon concentration, nitrogen source and concentration, C/N ratio, and precursor supplementation, were evaluated. From the classical method by changing one factor at a time, increasing biosurfactant yield of 2 fold was obtained by using 5% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.1% (v/v) palm oil. Then, the experimental design (RSM) was applied to improve biosurfactant yield from the previous experiment and to predict the best culture medium composition. The best condition of 7% (w/v) waste glycerol, 0.4% (w/v) $NaNO_3$, and supplemented with 0.01% (v/v) palm oil, correlated to C/N ratio of 42, was obtained; it produced high biosurfactant yield of 0.79 (g/l). Using experimental design could enhance productivity 20% from single-factor experiments. Although experimental design did not improve much biosurfactant yield, it offered the valuable suggestion that low amount of palm oil could be used to increase biosurfactant yield. Based on this suggestion the use of palm oil could be reduced 10 times. The properties of biosurfactant obtained from this optimum condition were studied in the further experiment. The surface and interfacial tension of medium was reduced to 29 and 2.22 mN/m (with palm oil). The E_{24} of soybean, jatropha and palm oil were 14, 52, and 60% respectively. Moreover, a CMC of 53.2 mg/l was obtained.

For oil extraction application, the use of biosurfactant in different forms; cell-free broth and crude biosurfactant concentrations (below, at, above CMC), were studied by using aqueous-based extraction method. Biosurfactant from strain GY30 showed the potential use in palm kernel oil extraction. The highest oil detachment of 64% was obtained when using cell-free broth. Nevertheless, the system produced emulsion-like phase which is undesirable in oil extraction process and need the addition step to recover the oil. Using crude biosurfactant solution could produce extracted palm kernel oil in free oil form in every concentration which is favorable in this application. Thus, the use of crude biosurfactant solution in aqueous biosurfactant-based method for extraction of palm kernel oil was of the interest alternative method for reducing the use of hexane. However, low oil yield extracted from this method could overcome by study the optimum electrolyte for the system or using of mix biosurfactant (Naksuk *et al.*, 2009).

The last section, oil quality of crude palm kernel oil extracted from three different methods; hexane and aqueous biosurfactant-based method by using crude biosurfactant in this research and mechanical pressing from Suksomboon Palm Oil Industry were evaluated for their clarity, color, and free fatty acids content. The results showed the similar observation of the visual appearance; color and clarity, of crude palm kernel oil extracted from these different methods. For Free fatty acid content (%, wt) analysis, the oil extracted from biosurfactant-based method was comparable to those of the hexane method and much better than those of mechanical pressing.

5.2 Recommendations

1. The other parameters affect biosurfactant production such as effect of various nutrients and cultivation condition (Guerra-Santos *et al*., 1986; Silva *et al*., 2010) should be further investigated.

2. Increasing more experimental runs in experimental design by using other types of RSM such as Box-Behnken design (15 runs) might improve the accuracy of this tool (Roldán-Carrillo *et al*., 2011).

3. Characterization of crude biosurfactant from *Achromobacter* sp. GY30 should be further studied for more understanding of biosurfactant synthesis and easier to improve their potential use in oil extraction application.

4. For oil extraction experiment, the optimum electrolyte of the oil extraction system which produced low interfacial tension (mN/m) should be studied.

5. The parameters which affected oil extraction process for example affect of grain size, contact time, oil kernel seed load, and biosurfactant concentration should be examined.

6. The oilseeds sample should not be stored for a long time before used in the experiment. Due to oilseed might dry and make the oil sticker attach in the oilseed which is hard to extract.

7. The oil extracted efficiency of biosurfactant-based method can be improved by addition of co-surfactant or linker (Do *et al*., 2009), mixing with chemical or biosurfactant, or adjusting temperature.

8. For determination of oil content in oilseed including oil residual analysis, hexane extraction by Soxhlet method should be applied to reduce effect of residual hexane and water present in extracted or remaining oil.

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APPENDICES

APPENDIX A

Media Preparation

Luria Bertani (LB) broth

Tryptone	10	g
Yeast extracts	5	g
NaCl	5	g
Dissolved in distilled water to	1,000	ml

Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

LB agar

Add 15 g of agar to LB broth 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

APPENDIX B

CHEMICALS

0.1 N NaOH

1 N NaOH	10	ml
Distilled water	90	ml

6 N HCl

12 N HCl	21.90	ml
Distilled water	78.10	ml

70% Ethanol

95% Ethanol	737	ml
Distilled water	263	ml

APPENDIX C

Waste glycerol properties and preparation

Table C-1 Characteristics of waste glycerol (sample 07-03-2011)

Parameter	Waste Glyceol (lot 07-03-2011)		
	BG Lab	**Central Lab**	**SGS**
pH	3.33		
Alkalinity			
TVA	142		
Total COD	1,000,600	1,069,400	953,600
Soluble COD (mg/l)	978,000	996,408	896,000
BOD		462,667	
TKN		144	
Total Phosphate		212	502
Sodium		17,510	29,176
Potassium		43,856	43,300
Chloride		14	44
Sulfate	32,361	22,700	24,400
Sulfide		22	8
Oil&Grease		ND	
Solids content:			
TS	127,890		
SS	3,043		
DS	124,847		
TVS	82,487		
VSS	2,960		

Fig. C-1 Stock waste glycerol solution (66.67%)

Waste glycerol preparation

Stock waste glycerol solution was prepared by dissolving wasted glycerol in distilled water in ratio of 2/1 consistent with concentration of 66.67% and then filtrated by using filter papers.

5% Waste glycerol (1000 ml)

Stock waste glycerol (66.67%)	75	ml
Distilled water	925	ml

Organic compound presented in waste glycerol

Density of waste glycerol = 1,253 g/l

Soluble COD = 996,408 mg/l

5% Waste glycerol (100 ml)

Stock waste glycerol (66.67%)	7.5	ml
Distilled water	92.5	ml

1 ml of solution had waste glycerol 2 g

7.5 ml of solution had waste glycerol 15 g

Density of waste glycerol = 1,253 g/l = 15 g/ x ml

x ml = 0.012 L

1 L of waste glycerol had dissolved organic compound 996.408 g

0.012 L of waste glycerol had dissolved organic compound 11.96 g

APPENDIX D

RAW DATA

Table D-1 Preliminary E24 of vegetable sample

Sample	**Substrate (3%)**	**E_{24}** Soybean oil	**E_{24}** Jatropha oil	**E_{24}** Palm oil
1. Jatropha	WG	15.79	54.76	47.62
	SO	-	-	+
	SB	2.63	2.38	21.05
2. Palm kernel	WG	5.26	20.51	12.82
	SO	-	-	+
	SB	30	12.5	23.08
3. Mesocarp	WG	1.05	52.40	65.12
	SO	-	-	+
	SB	19.05	23.81	36.36
4. Palm kernel shell	WG	13.16	0.48	20
	SO	-	-	+
	SB	28.95	52.58	60

WG = Waste glycerol, SO = Slop oil, SB = Soybean oil

- = Not Emulsion, + = Partial emulsion but cannot calculate

Table D-1 Preliminary E24 of vegetable sample (cont.)

Sample	**Substrate (3%)**	**E_{24}** Soybean oil	**E_{24}** Jatropha oil	**E_{24}** Palm oil
5. Palm fiber	WG	1.58	10	10.53
	SO	-	-	2.38
	SB	0.51	25.64	45.24
6. Decenter cake	WG	-	-	-
	SO	-	-	+
	SB	25	14.28	41.86
7. Coarse kernel meal	WG	-	-	-
	SO	-	-	+
	SB	2.50	10	13.64
8. Fine kernel meal	WG	-	33.33	23.81
	SO	-	-	-
	SB	4.88	14.28	27.29

WG = Waste glycerol, SO = Slop oil, SB = Soybean oil

- = Not Emulsion, + = Partial emulsion but cannot calculate

Table D-2 Colony of isolated biosurfactant-producing bacteria

Sample	Substrate (3%)	Bacterial strain	Colony picture
1. Jatropha	WG	JBG1	
		JBG2	
		JBG3	
		JBG4	
		JBG5	
	SB	JSB1	

WG = Waste glycerol, SB = Soybean oil

Table D-2 Colony of isolated biosurfactant-producing bacteria (cont.)

Sample	Substrate (3%)	Bacteria lstrain	Colony picture
1. Jatropha	SB	JSB2	
		JSB3	
2. Palm kernel	WG	PBG1	
		PBG2	
	SB	PSB1	
		PSB2	

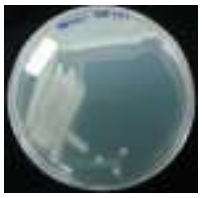
WG = Waste glycerol, SB = Soybean oil

Table D-2 Colony of isolated biosurfactant-producing bacteria (cont.)

Sample	Substrate (3%)	Bacterial strain	Colony picture
3. Mesocarp	WG	MBG1	
		MBG2	
	SB	MSB1	
4. Palm kernel shell	SB	PSSB1	
		PSSB2	
		PSSB3	

WG = Waste glycerol, SB = Soybean oil

Table D-2 Colony of isolated biosurfactant-producing bacteria (cont.)

Sample	Substrate (3%)	Bacterial strain	Colony picture
5. Palm fiber	SB	FSB1	
		FSB2	
		FSB3	
6. Decenter cake	SB	DSB1	
		DSB2	

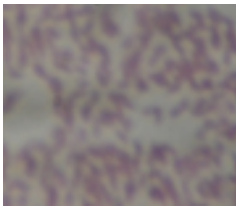
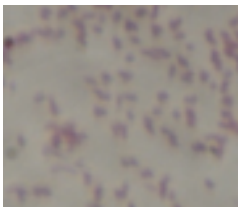
WG = Waste glycerol, SB = Soybean oil

Table D-2 Colony of isolated biosurfactant-producing bacteria (cont.)

Sample	**Substrate (3%)**	**Bacterial strain**	**Colony picture**
7. Fine kernel meal	WG	FMBG	
	SB	FMSB1	
		FMSB2	
		FMSB3	

WG = Waste glycerol, SB = Soybean oil

Table D-3 Gram stain of the effective biosurfactant-producing bacteria

Sample	**Substrate (3%)**	**Bacterial strain**	**Gram stain**	
Jatropha	WG	JBG1	Gram negative, rod	
	SB	JSB2	Gram negative, rod	

WG = Waste glycerol, SB = Soybean oil

Table D-4 Properties of selected biosurfatant-producing bacteria from laboratoty library

Bacteria	**Carbon source (3%)**	**Triplicates**	**Surface tension (mN/m)**	**E_{24} (SB, %)**	**E_{24} (J, %)**	**E_{24} (P, %)**	**Dry cell weight (g/l)**
Bacillus sp. GY17	WG	1	29.30	36	45	64	1.028
		2	29.44				1.030
		3	29.57				1.088
		Av.	29.44				1.049
		SD	0.13				0.03
Achromobacter sp. GY30	WG	1	29.39	32	41	39	0.978
		2	29.42				1.082,
		3	29.55				1.066
		Av.	29.45				1.04
		SD	0.08				0.06
Cellulosimicrobium sp. GY33	WG	1	41.00	NE	NE	NE	2.412
		2	40.81				2.400
		3	41.53				2.407
		Av.	41.12				2.406
		SD	0.37				0.01
Stenotrophomonas sp. LP1	SB	1	48.877	NE	NE	NE	2.832
		2	34.074				1.148
		3	46.667				1.996
		Av.	43.21				2.103
		SD	7.98				0.84
Alcaligenes sp. LS	SB	1	27.219	47	52	78	4.926
		2	30.55				4.486
		3	30.08				4.666
		Av.	29.28				4.693
		SD	1.80				0.22
Rhodococcus sp. CALSB1	SO	1	37.58	NE	NE	NE	0.274
		2	36.36				0.240
		3	36.10				0.271
		Av.	36.68				0.257
		SD	0.79				0.02

Table D-5 Interfacial tension of selected biosurfactant-producing bacteria selected from vegetable sample and laboratory library

Bacteria	**Carbon source (3%)**	**Triplicates**	**Interfacial tension (IFT, mN/m)**	
			Jatropha oil	**Palm oil**
Water	-	1	28.658	39.198
		2	33.488	39.074
		Av.	31.07	39.19
		SD	3.42	0.11
Bacillus sp. GY17	WG	1	ND	1.149
		2		1.5
		Av.		1.149
		SD		0.25
Achromobacter sp. GY30	WG	1	ND	0.769
		2		0.787
		Av.		0.78
		SD		0.01
Alcaligenes sp. LS	SB	1	1.300	1.544
		2	1.667	1.569
		Av.	1.49	1.560
		SD	0.36	0.02
Bacterium strain JBG1	SB	1	1.490	1.219
		2	2.000	2.760
		Av.	1.53	1.99
		SD	0.26	1.09
Bacterium strain JSB2	SB	1	3.365	4.286
		2	4.818	4.929
		Av.	4.09	4.61
		SD	1.03	0.45

WG = Waste glycerol, SB = Soybean oil, ND = Not detected

Table D-6 Oil detachment (%) by aqueous biosurfactant-based extraction method using cell-free broths

System	Triplicates	Oil detachment (%)	
		Jatropha kernel	**Palm kernel**
Water	1	48.08	44.84
	2	43.66	43.32
	3	-	43.81
	Av.	45.87	43.99
	SD	3.12	0.78
AOT	1	39.25	43.64
	2	55.29	45.68
	3	48.17	43.23
	Av.	47.75	52.44
	SD	8.04	1.31
Cell-free broth of GY17	1	39.62	59.57
	2	41.51	65.96
	3	33.96	-
	Av.	36.48	62.76
	SD	3.93	4.52
Cell-free broth of GY30	1	41.51	65.96
	2	37.74	61.7
	3	39.62	57.45
	Av.	39.62	61.7
	SD	1.88	4.26
Cell-free broth of LS	1	43.4	57.45
	2	47.17	51.06
	3	-	-
	Av.	45.28	54.23
	SD	2.66	4.52

AOT = Aerosol OT (anionic surfactant)

Table D-6 Oil detachment (%) by aqueous biosurfactant-based extraction method using cell-free broths

System	Triplicates	Oil detachment (%)	
		Jatropha kernel	**Palm kernel**
Cell-free broth of JBG1	1	33.96	51.06
	2	43.4	57.45
	3	-	57.45
	Av.	38.68	54.26
	SD	6.68	3.69
Cell-free broth of JSB2	1	22.64	48.42
	2	33.96	59.57
	3	-	48.94
	Av.	28.3	52.31
	SD	8.00	6.29

Table D-7 Effect of various waste glycerol concentrations on biosurfactant production by *Achromobacter* sp. GY30 inter of ST, DCW, E_{24}, and biosurfactant yield

Waste glycerol concentration (%, w/v)	Tripli-cates	Surface tension (mN/m)		E_{24} (%)			Dry cell weight (g/l)	Yield (g/l)
		Control	Sample	SB	J	P		
2	1	37.725	29.473	20	64	64	0.291	0.067
	2	37.222	29.213				0.346	0.065
	3	37.467	29.413				0.329	0.049
	Av.	37.47	29.37				0.32	0.06
	SD	0.25	0.14				0.03	0.01
5	1	35.669	30.333	39	59	55	1.928,	0.098
	2	35.638	30.206				1.143	0.700
	3	35.626	30.308				1.713	0.093
	Av.	35.64	30.28				1.59	0.09
	SD	0.02	0.07				0.40	0.01
7	1	35.545,	30.88	59	7	50	1.568	0.162
	2	35.275	31.405				1.55	0.131
	3	35.685	31.413				1.457	0.170
	Av.	35.50	31.14				1.52	0.15
	SD	0.21	0.30				0.06	0.02
12	1	35.217	35.374	NE	NE	NE	0.142	0.509
	2	35.327	33.459				0.137	0.553
	3	35.531	35.392				0.136	0.400
	Av.	35.36	34.74				0.14	0.49
	SD	0.6	1.11				0.003	0.08

SB = Soybean oil, J = Jatropha oil, P = Palm oil, and NE = Not emulsion

Table D-8 Effect of $NaNO_3$ and concentrations on biosurfactant production by *Achromobacter* sp. GY30 inter of ST, DCW, E_{24}, and biosurfactant yield

Nitrogen		**Tripli-cates**	**C/N**	**Surface tension (mN/m)**	**Dry cell weight (g/l)**	**Yield (g/l)**	
Type	**Concentration (%, w/v)**					**1st method**	**2nd method**
$NaNO_3$	0.1	1	120	31.517	1.891	0.178	-
		2		31.039	2.116	0.141	-
		3		30.97	1.842	0.069	-
		Av.		31.2	1.95	0.13	-
		SD		0.30	0.15	0. 06	-
	0.2	1	60	31.07	1.891	0.196	-
		2		30.575	2.116	0.144	-
		3		30.419	1.842	0.16	-
		Av.		30.8	1.26	0.15	-
		SD		0.34	0.36	0.01	-
	0.4	1	30	30.293	1.483	0.168	0.32
		2		30.275	1.621	0.148	0.40
		3		29.958	1.445	0.163	0.29
		Av.		30.2	1.52	0.16	0.34
		SD		0.19	0.09	0.01	0.06
	0.6	1	20	29.837	1.184	0.122	-
		2		29.896	1.195	0.137	-
		3		29.859	2.396	0.0987	-
		Av.		29.9	1.59	0.13	-
		SD		0.03	0.70	0.02	-

Table D-8 Effect of $NaNO_3$ and concentrations on biosurfactant production by *Achromobacter* sp. GY30 inter of ST, DCW, E_{24}, and biosurfactant yield

Nitrogen		**Tripli-cates**	**C/N**	**Surface tension (mN/m)**	**Dry cell weight (g/l)**	**Yield (g/l)**	
Type	**Concentration (%, w/v)**					**1st method**	**2nd method**
$(NH_4)_2SO_4$	0.1	1	120	44.004	1.886	0.0397	-
		2		44.197	1.728	0.032	-
		3		43.501	1.685	0.0293	-
		Av.		43.90	1.77	0.03	-
		SD		0.36	0.10	0.00	-
	0.2	1	60	44.689	1.838	0.038	-
		2		44.793	1.771	0.03	-
		3		43.227	1.551	0.037	-
		Av.		44.24	1.72	0.04	-
		SD		0.87	0.15	0.00	-
	0.4	1	30	45.181	1.881	0.034	-
		2		44.748	1.698	0.0293	-
		3		44.406	1.707	0.0323	-
		Av.		44.78	1.76	0.03	-
		SD		0.38	0.10	0.00	-
	0.6	1	20	41.353	1.632	0.045	-
		2		41.555	1.7	0.041	-
		3		40.639	1.511	0.036	-
		Av.		41.18	1.61	0.04	-
		SD		0.48	0.10	0.00	-

Table D-8 Effect of $NaNO_3$ and concentrations on biosurfactant production by *Achromobacter* sp. GY30 inter of ST, DCW, E_{24}, and biosurfactant yield

Nitrogen		**Tripli-cates**	**C/N**	**Surface tension (mN/m)**	**Dry cell weight (g/l)**	**Yield (g/l)**	
Type	**Concentration (%, w/v)**					**1st method**	**2nd method**
NH_4NO_3	0.1	1	120	32.511	0.929	0.12	-
		2		33.986	0.908	0.085	-
		3		30.745	0.453	0.096	-
		Av.		32.41	0.76	0.10	-
		SD		1.62	0.27	0.02	-
	0.2	1	60	41.936	0.511	0.106	-
		2		41.372	1.285	0.113	-
		3		39.58	0.463	0.103	-
		Av.		40.96	0.75	0.12	-
		SD		1.23	0.46	0.00	-
	0.4	1	30	40.82	0.449	0.101	-
		2		40.05	0.446	0.075	-
		3		40.64	0.428	0.146	-
		Av.		40.50	0.44	0.12	-
		SD		0.40	0.01	0.04	-
	0.6	1	20	39.8	0.428	0.104	-
		2		39.3	0.427	0.172	-
		3		39.92	0.408	0.127	-
		Av.		39.68	0.42	0.11	-
		SD		0.33	0.01	0.03	-

Table D-9 various precursors supplementation on biosurfactant production by *Achromobacter* sp. GY30 inter of ST, DCW, E_{24}, and biosurfactant yield

Precursor type	**Concen-tration (%, v/v)**	**Tripli-cates**	**Surface tension (mN/m) at 25°C**	**Dry cell weight (g/l)**	**Yield (g/l)**
Palm oil	0.1	1	28.965	1.65	0.67
		2	28.072	1.81	0.64
		3	29.445	1.38	0.61
		Av.	30.20	1.61	0.64
		SD	0.13	0.22	0.03
Soybean oil	0.1	1	29.454	1.608	0.541
		2	29.065	0.71	0.706
		3	30.16	0.726	0.633
		Av.	29.56	1.01	0.62
		SD	0.56	0.51	0.08

Table D-10 2^3 two-level full factorial experimental design using the strain GY30 and biosurfactant yield as respond value

Run no.	**Carbon (%) X_1**	**Nitrogen (%, w/v) X_2**	**Palm oil (%, v/v) X_3**	**Tripli-cates**	**Yield (g/l) Z**	**C/N**
1	5	0.4	0.1	1	0.67	30
				2	0.64	
				3	0.61	
				Av.	0.64	
				SD	0.03	
2	7	0.4	0.1	1	0.62	42
				2	0.84	
				3	0.77	
				Av.	0.74	
				SD	0.11	

Table D-10 2^3 two-level full factorial experimental design using the strain GY30 and biosurfactant yield as respond value

Run no.	**Carbon (%) X_1**	**Nitrogen (%, w/v) X_2**	**Palm oil (%, v/v) X_3**	**Tripli-cates**	**Yield (g/l) Z**	**C/N**
3	5	0.6	0.1	1	0.68	20
				2	0.72	
				3	0.52	
				Av.	0.64	
				SD	0.04	
4	7	0.6	0.1	1	0.8	28
				2	0.62	
				3	0.85	
				Av.	0.76	
				SD	0.12	
5	5	0.4	1	1	0.44	30
				2	0.44	
				3	0.44	
				Av.	0.44	
				SD	0.00	
6	7	0.4	1	1	0.52	42
				2	0.64	
				3	0.55	
				Av.	0.57	
				SD	0.06	
7	5	0.6	1	1	0.63	20
				2	0.48	
				3	0.64	
				Av.	0.58	
				SD	0.09	
8	7	0.6	1	1	0.48	28
				2	0.44	
				3	0.44	
				Av.	0.45	
				SD	0.02	

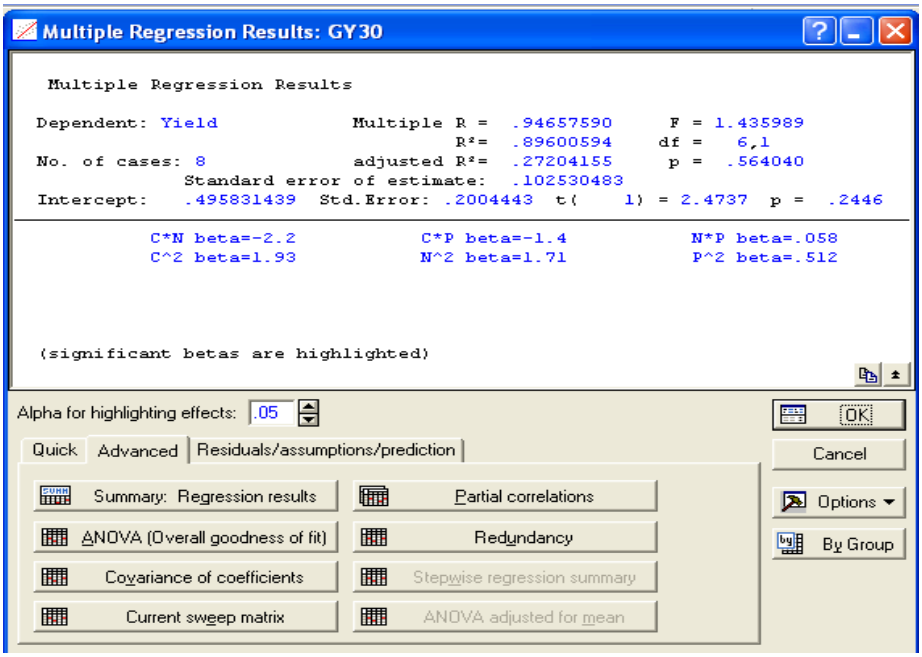


Fig. D-1 Multiple regression result by uesing 2^3 full factorial faction experiment

Regression Summary for Dependent Variable: Yield (GY30)
R= .94657590 R²= .89600594 Adjusted R²= .27204155
F(6,1)=1.4360 p<.56404 Std.Error of estimate: .10253

N=8	Beta	Std.Err. of Beta	B	Std.Err. of B	t(1)	p-level
Intercept			0.495831	0.200444	2.473662	0.244572
C*N	-2.18898	2.539219	-0.312500	0.362500	-0.862069	0.547071
C*P	-1.44884	2.000782	-0.058333	0.080556	-0.724138	0.601003
N*P	0.05831	1.690915	0.027778	0.805556	0.034483	0.978056
C^2	1.93118	1.690915	0.018090	0.015840	1.142091	0.457833
N^2	1.71001	2.000782	1.922222	2.249073	0.854673	0.549782
P^2	0.51152	2.539219	0.116162	0.576632	0.201448	0.873448

Fig. D-2 Regression summary for biosurfactant yield

Table D-11 Prediction conditions and their properties compared actual value

Condition				C/N	Triplicates	Surface tension (mN/m) at 25°C		Dry cell weight (g/l)	Yield (g/l)	
No.	WG (%, w/v)	$NaNO_3$ (%, w/v)	Palm oil %, v/v)			Control	Sample		Predict value	Actual value
1	2	0.8	0.01	6.6	1	37.725	31.011	0.193	1.30	0.146 ,
					2	37.222	30.826	0.226		0.189
					3	37.467	30.468	0.23		0.174
					Av.	37.47	30.77	0.22		0.17
					SD	0.25	0.28	0.02		0.02
2	3	0.8	0.01	9	1	37.38	30.718	0.391	1.14	0.202
					2	38.245	30.639	0.36		0.200
					3	38.465	30.667	0.383		0.211
					Av.	38.03	30.67	0.38		0.21
					SD	0.57	0.02	0.02		0.01
3	5	0.8	0.01	15	1	41.086	29.207	0.835	0.92	0.3384
					2	41.35	29.689	0.916		0.4228
					3	40.128	30.034	0.931		-
					Av.	40.85	29.64	0.89		0.35
					SD	0.64	0.42	0.05		0.04
4	6.5	0.1	0.01	157	1	36.679	29.926	1.009	1.07	0.3896
					2	36.824	29.423	0.887		0.3628
					3	36.622	30.53	0.829		0.438
					Av.	36.71	29.96	0.91		0.40
					SD	0.10	0.55	0.09		0.04
5	7	0.1	0.01	169	1	37.731	29.13	0.993	1.14	0.353
					2	36.814	29.33	1.318		0.354
					3	37.938	28.90	0.962		0.501
					Av.	37.49	29.12	1.09		0.40
					SD	0.60	0.22	0.20		0.08

Table D-11 Prediction conditions and their properties compared actual value

Condition				C/N	Triplicates	Surface tension (mN/m) at 25°C		Dry cell weight (g/l)	Yield (g/l)	
No.	WG (%, w/v)	$NaNO_3$ (%, w/v)	Palm oil %, v/v)			Control	Sample		Predict value	Actual value
6	7	0.4	-	42	1	35.61	30.04	0.31	0.73	0.426
					2	35.39	29.82	0.37		0.39
					3	36.56	30.52	0.40		0.4
					Av.	35.86	30.13	0.36		0.40
					SD	0.62	0.36	0.05		0.02
7	7	0.4	0.01	42	1	38.06	30.37	0.672	0.81	0.808
					2	36.31	28.28	0.416		0.768
					3	37.43	28.05	0.483		-
					Av.	38.30	28.90	0.52		0.79
					SD	0.89	1.28	0.13		0.03

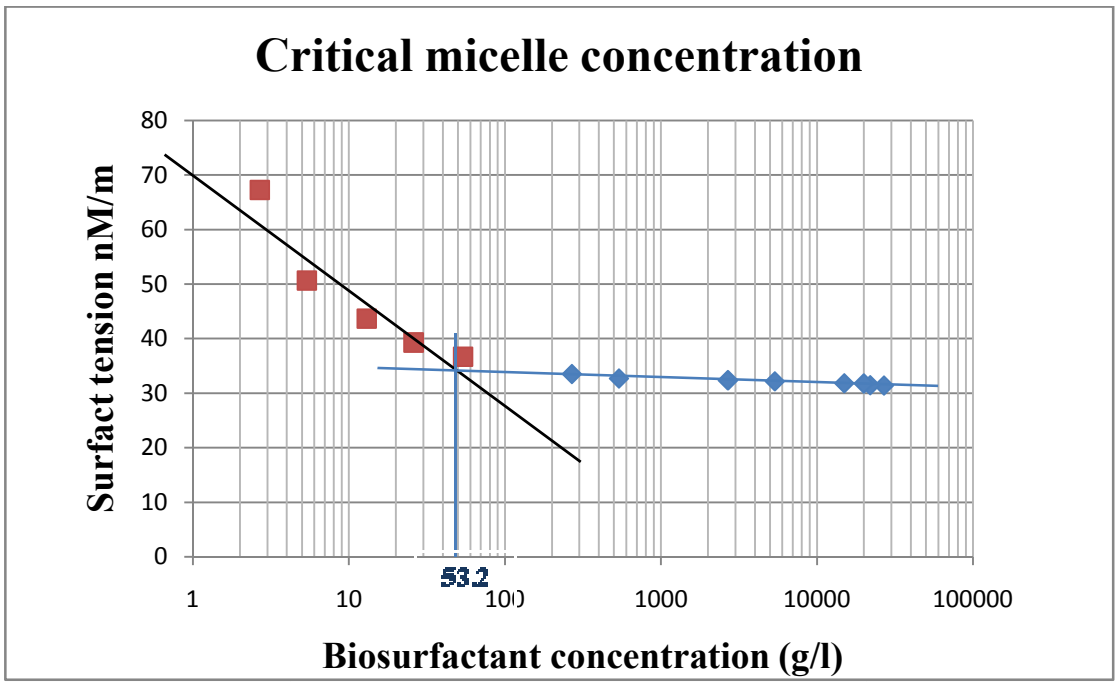


Fig. D-3 Log graph of critical micelle concentration

Table D-12 Oil detachment (%) of palm kernel by using aqueous biosurfactant-based extraction method

System	Triplicates	Oil detachment (%)
Water	1	44.84
	2	43.32
	3	43.81
	Av.	43.99
	SD	0.78
AOT	1	53.25
	2	52.22
	3	51.84
	Av.	52.44
	SD	0.73
Cell-free broth	1	65.99
	2	68.65
	3	60.82
	Av.	64.10
	SD	3.98
Crude biosurfactant solution		
<CMC	1	48.56
	2	47.73
	3	48.18
	Av.	48.16
	SD	0.42
CMC	1	47.2
	2	48.02
	3	48.4
	Av.	47.87
	SD	0.61

Table D-12 Oil detachment (%) of palm kernel by using aqueous biosurfactant-based extraction method

System	Triplicates	Oil detachment (%)
>CMC	1	45.36,
	2	46.93
	3	47.53
	Av.	46.61
	SD	1.12

Table C-13 The IFT value of biosurfactant solutions against palm oil

System	Triplicates	IFT (mN/m) at25◦C
water	1	39.198
	2	39.302
	3	39.074
	Av.	39.19
	SD	0.11
Cell-free broth	1	2.947
	2	1.504
	Av.	2.22
	SD	1.02
Crude biosurfactant solution		
below CMC (532 mg/l)	1	28.073
	2	33.707
	Av.	30.89
	SD	3.98

Table D-13 The IFT value of biosurfactant solutions against palm oil

System	Triplicates	IFT (mN/m) at25◦C
at CMC (53.2 mg/l)	1	37.911
	2	37.679
	Av.	37.80
	SD	0.16
above CMC (10.6 mg/l)	1	38.29
	2	33.49
	Av.	35.89
	SD	3.40

Table D-14 Extracted oil quality

Parameters	Mechanical pressing		Hexane method	Aqueous biosurfactant-based method
Oil recovery (%wt)	67-74*		95-99**	~50-60***
Color	Clear yellow		Clear yellow	Clear yellow
State of liberated oil	-		Free oil	Free oil
Free fatty acid (%wt)	Triplicates			
Oleic	1	1.42	10.72	12.33
	2	1.42	10.43	11.1
	3	1.58	-	-
	Av.	1.34	10.58	12.33
	SD	0.09	0.20	0.87
Lauric	1	1.01	7.6	8.75
	2	1.01	7.4	7.87
	3	1.12	-	-
	Av.	1.05	7.50	8.31
	SD	0.06	0.14	0.62
Palmitic	1	1.29	9.73,]	11.19
	2	1.29	9.47	10.08
	3	1.44	-	-
	Av.	1.47	9.60	10.64
	SD	0.09	0.18	0.78

BIOGRAPHY

Miss Jinnapat Chaiyasit was born on July 26th, 1985 in Udonthani province, Thailand. She attended Udonpittayanukool School in Udonthani and graduated in 2004. She received Bachelor's Degree in Biochemistry, Faculty of science, Chulalongkorn University in 2007. She pursued her Master degree study in International Postgraduate Programs in Environmental and Hazardous Waste Management, Chulalongkorn University, Bangkok, Thailand in May 2010. She finished Master Degree of Science in Environmental Management in 2011.