Utilization of oil mill effluents as alternative substrate for biosurfactant production by *Bacillus* sp. GY19 and its application in crude oil contaminated soil washing

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การใช้ของเสียจากกระบวนการผลิตน้ำมันพืชเพื่อเป็นสารตั้งต้นในการผลิตสารลดแรงตึงผิวชีวภาพโดย *Bacillus* sp. GY19 และการประยุกต์ใช้ในการชะล้างดินปนเปื้อนน้ำมันดิบ

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Environmental Management
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ชวิศา วิชัยดิษฐ์ : การใช้ของเสียจากกระบวนการผลิตน้ำมันพืชเพื่อเป็นสารตั้งต้นในการผลิตสารลดแรงตึงผิวชีวภาพโดย *Bacillus* sp. GY19 และการประยุกต์ใช้ในการชะล้างดินปนเปื้อนน้ำมันดิบ (Utilization of oil mill effluents as alternative substrate for biosurfactant production by *Bacillus* sp. GY19 and its application in crude oil contaminated soil washing) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.อรฤทัย ภิญญาคง, 95 หน้า.

งานวิจัยนี้ศึกษาการผลิตสารลดแรงตึงผิวชีวภาพด้วยเซลล์ตรึง *Bacillus* sp. GY19 บนไคโตซาน โดยใช้ของเสียจากกระบวนการผลิตน้ำมันพืชเป็นสารตั้งต้น ได้แก่ ของเสียจากกระบวนการผลิตน้ำมันปาล์ม และน้ำมันถั่วเหลือง ทั้งนี้เนื่องจากประเทศไทยเป็นผู้ผลิตน้ำมันปาล์มอันดับที่สามของโลก และการผลิตน้ำมันถั่วเหลืองยังมีบทบาทสำคัญต่อการผลิตน้ำมันพืชที่ใช้ภายในประเทศ ดังนั้น การใช้ของเสียดังกล่าวเป็นสารตั้งต้นสำหรับการผลิตสารลดแรงตึงผิวชีวภาพ จึงเป็นการลดปริมาณการเกิดของเสียและเป็นการนำของเสียมาก่อให้เกิดประโยชน์ อย่างไรก็ตาม ผลการศึกษาการใช้ของเสียจากกระบวนการผลิตน้ำมันปาล์ม พบว่า ผลิตสารลดแรงตึงผิวชีวภาพได้ในปริมาณน้อย เนื่องจากของเสียดังกล่าวอาจมีองค์ประกอบของสารประกอบฟีนอล ซึ่งเป็นพิษ และอาจส่งผลกระทบกับใช้สารตั้งต้นของแบคทีเรีย ในขณะที่เมื่อใช้ของเสียจากกระบวนการผลิตน้ำมันถั่วเหลืองเป็นสารตั้งต้น สามารถผลิตสารลดแรงตึงผิวชีวภาพได้ 4.37 กรัม/ลิตร และยังพบว่าเมื่อใช้ความเข้มข้นของของเสียจากกระบวนการผลิตน้ำมันถั่วเหลืองที่ 20% (w/v) เซลล์ตรึงสามารถผลิตสารลดแรงตึงผิวชีวภาพได้ดีที่สุด คือ 0.0365 กรัมต่อลิตรต่อชั่วโมง นอกจากนี้สารลดแรงตึงผิวชีวภาพที่ผลิตได้ยังมีประสิทธิภาพในการลดแรงตึงผิวของอาหารที่ใช้ผลิตจาก 64 เหลือ 40 มิลลินิวตันต่อเมตร และก่อให้เกิดอิมัลชั่นต่อน้ำมันดีเซลสูงถึง 65% เมื่อทดสอบค่าความเข้มข้นเริ่มต้นที่ไมเซลล์จะก่อตัวหลังจากสารลดแรงตึงผิวชีวภาพดังกล่าวถูกชะผ่านดิน (Apparent CMC) พบว่า ความเข้มข้นของสารลดแรงตึงผิวชีวภาพมีผลต่อการชะล้างดินปนเปื้อนน้ำมันดิบ ดังนั้นจึงเพิ่มความเข้มข้นของสารลดแรงตึงผิวชีวภาพโดยวิธีการแยกโฟมและการทำแห้ง ซึ่งผลจากการชะล้างดินที่ปนเปื้อนน้ำมันดิบที่ความเข้มข้น 48 มิลลิกรัมน้ำมันดิบต่อกรัมของดิน พบว่า สารลดแรงตึงผิวชีวภาพที่ความเข้มข้น 8.43 กรัมต่อลิตร สามารถชะล้างน้ำมันดิบออกจากดินตะกอนร่วนได้ 36.33 มิลลิกรัมน้ำมันดิบต่อกรัมของดิน ซึ่งมีประสิทธิภาพใกล้เคียงกับสารลดแรงตึงผิวชีวภาพทางการค้า เช่น SDS และ Tween 80 ที่ความเข้มข้น 0.5 กรัมต่อลิตร ดังนั้นสรุปได้ว่า *Bacillus* sp. GY19 สามารถใช้ของเสียจากกระบวนผลิตน้ำมันถั่วเหลืองเป็นสารตั้งต้นทางเลือกในการผลิตสารลดแรงตึงผิวชีวภาพ และยังมีประสิทธิภาพที่ดีต่อการทดสอบคุณสมบัติของสารลดแรงตึงผิวชีวภาพอีกด้วย

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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CHAWISA WICHAIDIT: Utilization of oil mill effluents as alternative substrate for biosurfactant production by *Bacillus* sp. GY19 and its application in crude oil contaminated soil washing. ADVISOR: ASST. PROF. ONRUTHAI PINYAKONG, Ph.D., 95 pp.

In order to reduce the cost of biosurfactant production, wastes from vegetable oil processes were used as alternative substrate for chitosan immobilized *Bacillus* sp. GY19. Palm oil mill effluent and soy molasses were interesting as alternative substrate since the production of palm oil in Thailand is rated as 3rd rank of world market and soybean oil production also plays an important role in vegetable oil production in the country. Utilization of palm oil mill effluent resulted in small amount of crude biosurfactant produced with no activity of surface active agent shown. It was probably the palm oil mill effluent contained toxic phenolic compounds that affected production activity of bacteria. Meanwhile, utilization of soy molasses gave 4.37 g/l of crude biosurfactant with good activity of surface active agent. The determination of optimal condition and concentration of soy molasses as substrate revealed that 20% (w/v) of soy molasses gave the highest crude biosurfactant produced with productivity rate about 0.0365 g/l/h. Moreover, the produced biosurfactant could reduce the surface tension of medium from 64 to less than 40 mN/m and caused emulsification against diesel oil over 65%. Foam fractionation and freeze-dried biosurfactant were set to increase the concentration of biosurfactant above ACMC. The result of crude oil contaminated soil washing found that the biosurfactant from freeze-dried lyophilized with 8.43 g/l could wash crude oil out from the silt loam soil about 36.33 mg crude oil/g soil, comparable to 0.5 g/l of SDS and Tween 80. So, *Bacillus* sp. GY19 could utilize soy molasses as alternative substrate with good surface active agent produced.

Field of Study: Environmental Management Student's Signature .....
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CHAPTER 1
INTRODUCTION

1.1 Statement of problem

Biosurfactants are amphiphilic compounds produced extracellularly or as a part of cell membrane by living organism such as bacteria, yeasts and fungi from utilization of various substrates including sugars, oils, alkanes, among others (Mulligan, 2005). Biosurfactants contain hydrophilic head (mainly mono-, di-, or polysaccharide, carboxylic acid, amino acid, or peptide) and hydrophobic tail (usually be saturated, unsaturated, or hydroxylated fatty acids) (Nguyen et al., 2008). Accordingly, biosurfactant must be able to dissolve, at least partially, in both water and water-immiscible liquid, thereby affect surface tension and enabling mixing or solubilization; emulsification (Makkar et al., 2011). The ability to reduce surface and interfacial tensions of biosurfactant occurred by the accumulation of immiscible fluids at the interface, thus increasing in solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compound (Singh et al., 2007).

In spite of numerous advantages of biosurfactant, the problem related with large scale and cheap production still exists and is the major problem with economic competitiveness. Moreover, the reasons for limited use of biosurfactants in industrial scale are the use of expensive substrate, limited product concentrations, low yield produced and the formation of product mixtures rather than pure compounds (Syldatk & Hausmann, 2010). Even large scale of biosurfactant productions, most of them has not reached the satisfactory economical level with their low yield produced Additional, to recovery downstream process and purify microbial surfactant, high cost input is required (Rodrigues et al., 2006). These have led to concentrated efforts during the past decade, focusing on minimization of production costs in order to facilitate wider commercial use of biosurfactants.

To achieve the cost effectiveness and economical biosurfactant production, the key parameters in concerned are higher yield produced and lower production costs. Many alternative substrates have been suggested as substrate for economical biosurfactant production such as bottom glycerol, waste from biodiesel production, and other potential substrates which are agro-based industrial waste e.g., vegetable oil industries waste, frying oil waste or diary and sugar industry waste. Moreover, using low cost substrates including various agriculture waste that rich in organic pollutants and raw substrate with negligible or no value were also suggested as attractive strategies for economical biosurfactant production (Makkar et al., 2011).

Palm oil industry is one of the major agro industries in Thailand. Palm oil mill effluent (POME) is produced large amount from the production processes and it is the most significant pollutant from palm oil mill (Poh & Chong, 2009). POME is an oily wastewater that causes many serious environmental problems. Treatment and disposal of this kind of waste quite challenge the contributors because weather physical or chemical treatment processes have been designed, the problem of chemical residuals and total suspended solids are still remain (Karim et al., 2011). This oily product consists of glycerides and free fatty acids which can act as carbon source for microbial growth during biosurfactant production. Furthermore, the essential amino acid and minerals from palm oil fibrous can be also found in the residual oil which contains nitrogen compound that facilitates the growth of organism also (Chow & Ho, 2002).

Soybean molasses, low value co-product from soybean oil processing, is an attractive feedstock for biosurfactant production. Increasing amount of agricultural wastes from soy cultivation is becoming available as a raw material for utilization in biosurfactant production (Saharan et al., 2011). Since, it has high content in fermentable sugar that useful for sustaining microbial growth (Solaiman et al., 2007). Soybean molasses plays an important role on economic growth of many products such as soy protein-based foods and drinks. Differentiate in saccharide and protein containing in soybean molasses may attempt the opportunity of biosurfactant production as it served for carbon and nitrogen source.

Beside the development of economical biosurfactant production, the kinetic of biosurfactant production from utilization of oil mill effluent was investigated in this study in order to get optimal concentration and condition for biosurfactant production. Measuring biosurfactant production and cell growth during fermentation process together with substrates conversion indicated the microbial activity thus; yield of production occurred. Biosurfactant production dependent on the substrate composition and the concentration of media interact with other complex nutrients affect the kinetic of biosurfactant production. Moreover, C:N ratio also plays an important role in the production process as the major substrate for biosurfactant production. Some reviews showed that the biomass production from kinetic study and substrate utilization along with the fermentation process required for the growth of microorganisms are the most crucial parameters for production processes (Banat et al., 2014). Not only carbon and nitrogen source, the other nutrients might affect the production activity also. To achieve the cost effective biosurfactant production, by-product from vegetable oil industry was selected to study the kinetic of biosurfactant production in order to get the optimum concentration and condition for economical biosurfactant production.

Soil contaminated with petroleum or organic pollutants are always treated both *ex situ* and *in situ*. The *ex situ* techniques such as soil washing, which is getting more interest despite that soil excavation is necessary (Khalladi et al., 2009). To avoid the soil excavation many approaches and techniques are still being developed to be more cost-effective (Huguenot et al., 2015). Biosurfactants could help promoting solubilization or immobilization by their amphiphilic properties that are useful for mobilization of hydrophobic compounds that sorbed onto the soil particles. The application of economical produced biosurfactant has also posed by enhancing removal of oil from soil, using the concentration of produced biosurfactant above apparent critical micelle concentration (ACMC) in soil to determine the effectiveness of biosurfactant produced in crude oil contaminated soil washing.

Bacillus sp. GY19, bacterium strain isolated from soil, found to be an effective strain for biosurfactant production. It was immobilized with squid pen chitosan to enhance in potential biosurfactant production when comparing to free cell hence,

increases in cell stability, easier in extraction processes, reusable and enable in continuous production processes also. Bottom glycerol-based medium was used as substrate for biosurfactant production. Moreover, addition of fatty acid such as palm oil has found increase in amount of crude biosurfactant produced. (Khondee et al., 2015). In case of others substrate could be used for biosurfactant production by *Bacillus* sp. GY19. Then, this research is set up to find an appropriate economical substrate and optimization the condition in order to get the highest yield of biosurfactant produce.

In conclusion, this research was divided into 3 phases: i) production of biosurfactant by utilization of alternative substrates, ii) determine optimal concentration and condition of oil mill effluent by kinetic study on biosurfactant production and iii) investigating the potential application of produced biosurfactant in crude oil contaminated soil washing. The expected outcome from this study is to select the most suitable alternative substrate with optimal concentration and condition for *Bacillus* sp. GY19 and end with the environmental application of crude oil contaminated soil washing.

1.2 Objectives

The main objectives of this study are using oil mill effluents as alternative substrate for biosurfactant production in following detailed objectives are listed

1. To select suitable oil mill effluent as alternative substrate for biosurfactant production
2. To determine optimal concentration and condition of the suitable oil mill effluent by kinetic study of biosurfactant production
3. To apply biosurfactant produced from oil mill effluent for crude oil contaminated soil washing

1.3 Hypotheses

1. Oil mill effluents can be used as alternative substrate for biosurfactant production and the potential of produced biosurfactant is effective as compared to biosurfactant produced from bottom glycerol. Consider to the criteria of surface active such as surface tension reduction etc.
2. Bacteria can use carbon source in oil mill effluent as major substrate for biosurfactant production
3. Produced biosurfactant has potential in crude oil contaminated soil washing

1.4 Scope of study

1. Finding the most effective commercial vegetable oil as substrate for biosurfactant production by *Bacillus* sp. GY19

3% (v/v) of different vegetable oils e.g., palm oil, olive oil, corn oil, sunflower oil, soybean oil and rice bran oil was used as substrate comparing with bottom glycerol at the same concentration. Since, each vegetable oil has different fatty acid components then, the potential of biosurfactant production by bacteria might different also. After each substrate was used for biosurfactant production, the produced biosurfactant was tested for the effectiveness such as surface tension, oil displacement, emulsification index, crude biosurfactant concentration and critical micelle concentration. Criteria to choose the most effective biosurfactant are producing the highest in crude biosurfactant and the best efficiency of biosurfactant also shown. Then two of the substrate were selected their waste from the production processes for economical biosurfactant production. Then, oil mill effluents were used as an alternative substrate for biosurfactants production by *Bacillus* sp. GY19. The most suitable substrate, which the highest concentration of crude biosurfactant can be achieved and good activities of surfactant are shown, was selected for study on the kinetic biosurfactant production.

2. Studying the kinetic of biosurfactant production from utilization of oil mill effluents as alternative substrate

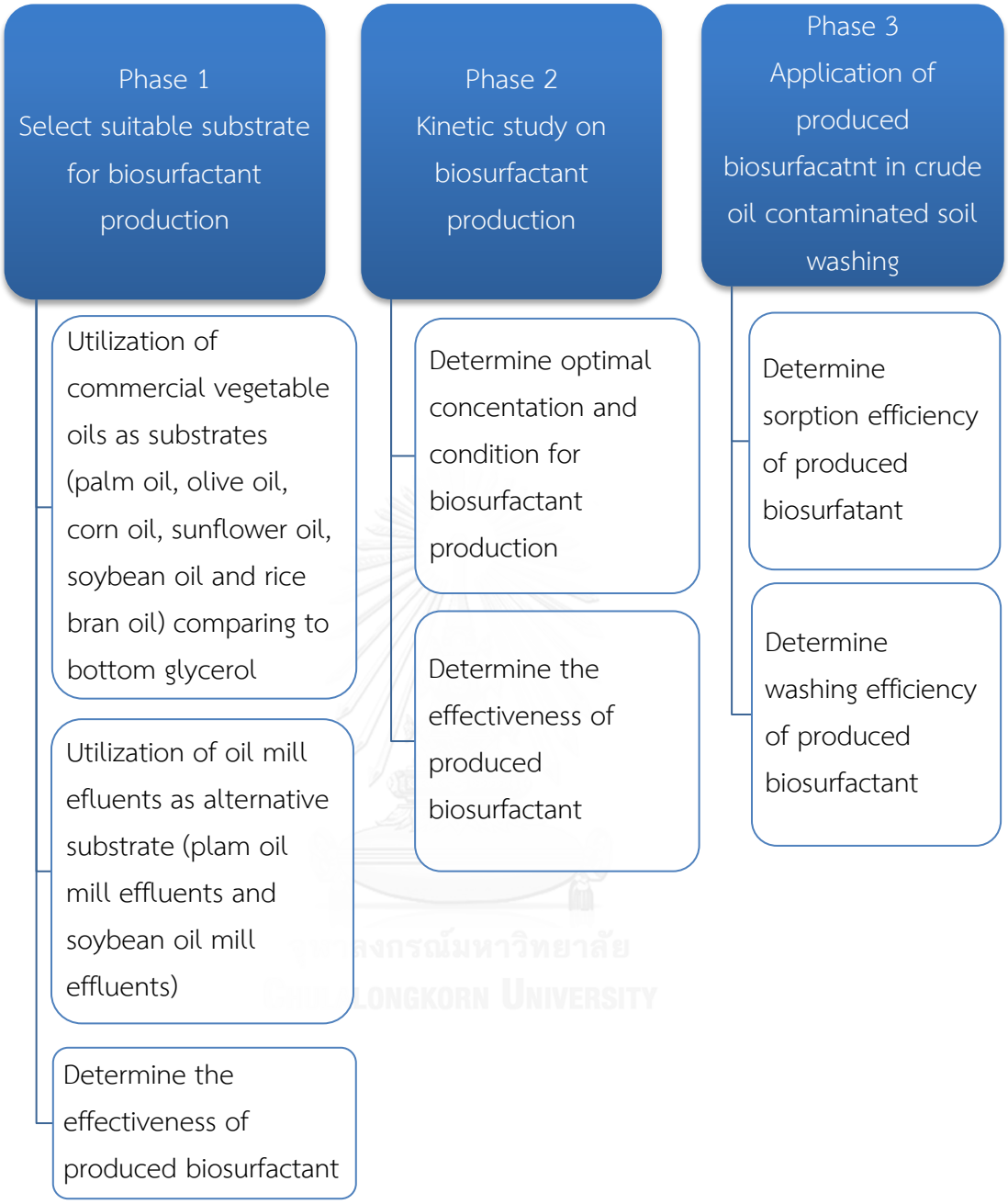
The change in component of carbon, nitrogen and glycerides in the medium to be biosurfactant was studied in each time interval until no increasing in surfactant activities or no change in the cell growth. Produced biosurfactant was tested for the effectiveness such as surface tension, oil displacement, emulsification index, crude biosurfactant concentration and critical micelle concentration. At the time of the highest yield achieved (production per substrate utilization), the condition was selected to be an appropriate time and concentration and used the produced biosurfactant at this condition for soil sorption test and crude oil contaminated soil washing.

3. Studying the efficiency of produced biosurfactant in soil sorption and washing potential of crude oil contaminated soils

The concentration of produced biosurfactant was increased in the form of foamate solutions and freeze-dried biosurfactant comparing with Tween 80 and SDS to determine crude oil contaminated soil washing potential. The concentration at apparent critical micelle concentration (ACMC) in each soil was considered.

1.5 Experimental framework

The conceptual framework of this study was to develop the economical biosurfactant production by finding the most appropriate condition with suitable concentration of substrate. Moreover, the produced biosurfactant was applied in washing potential of crude oil contaminated soil.


Phase 1
Select suitable substrate for biosurfactant production
Utilization of commercial vegetable oils as substrates (palm oil, olive oil, corn oil, sunflower oil, soybean oil and rice bran oil) comparing to bottom glycerol
Utilization of oil mill efluents as alternative substrate (plam oil mill effluents and soybean oil mill effluents)
Determine the effectiveness of produced biosurfactant
Phase 2
Kinetic study on biosurfactant production
Determine optimal concentation and condition for biosurfactant production
Determine the effectiveness of produced biosurfactant
Phase 3
Application of produced biosurfacatnt in crude oil contaminated soil washing
Determine sorption efficiency of produced biosurfatant
Determine washing efficiency of produced biosurfactant

CHAPTER 2
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Theoretical background

2.1.1 Biosurfactant

Biosurfactants are surface active agents that produced by microorganisms. Biosurfactant have the characteristic property of reducing the surface and interfacial tensions using the same mechanisms as chemical surfactants because of its structure consist of hydrophilic head and hydrophobic tail. Biosurfactants are generally the microbial metabolites with a typical amphiphilic structure. Hydrophobic moiety is a long-chain fatty acid, hydroxyl fatty acid, or α-alkyl-β-hydroxy fatty acid while hydrophilic moiety can be carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or alcohol. Synthesis of amphiphilic moiety depends on the substrate used and their linkage is also possible. Both lipid and peptide have been found to be directly synthesized from carbohydrate (Nitschke & Pastore, 2006)

Biosurfactants are classified as the different on the basic of their biochemical nature and microbial species that producing them. Biosurfactants are mainly classified into two classes: low molecular weight surface active agents called biosurfactant (lipopeptide, glycolipids) and bioemulsifier (high molecular weight surface active agents) (Saharan et al., 2011). High molecular weight biosurfactants are more effective as emulsion stabilizing agents; whereas, low molecular weight biosurfactants are efficiently lower surface and interfacial tension (Ron & Rosenberg, 2002). The stabilizing emulsions of high molecular weight biosurfactants increase the surface area available for bacterial biodegradation. The great potential for reducing surface and interfacial tension and forming micelles of low molecular weight biosurfactant increase the bioavailability of contaminants for degrading microorganisms by the partition of contaminants into the micelles cores.

Bacillus sp. GY19 that used in this study was previously used bottom glycerol (portion product from biodiesel production) as a carbon source while free fatty acid

from palm oil used as a precursor for lipophilic moiety. *Bacillus* GY 19 was immobilized with squid pen chitosan to increase cell stability, get easy in extraction process, reusable and enable for continuous production process (Khondee et al., 2015). *Bacillus* sp. was found produce lipopeptide biosurfactant since, the types of microbial surfactant are commonly differentiated on the basic of their biochemical nature and the microbial species producing them (Makkar et al., 2011). Previous research claimed about the problem of foaming in conventional bioreactor while free cell was using then, to solve the problem immobilization of cell can be promoting helping since chitosan has the adsorption property and flocculation ability. Using squid pen chitosan to immobilize *Bacillus subtilis* GY19 found in good cell bounding because the force between positive charge of chitosan and negative charge of cell wall. Scanning electron microscopy (SEM) showed the highest roughness on squid pen chitosan comparing with crap shell and shrimp shell so the highest attachment can be achieved on squid pen chitosan. The result of cell attach on chitosan flakes found to be 10^9 CFU/g and remained for 3 days. Therefore, squid pen chitosan was selected as the best chitosan for *Bacillus subtilis* GY19 immobilization (Khondee et al., 2015).

The strain of *Bacillus* has been found produce lipopeptides and lipoproteins biosurfactants because of their biochemical that produce cyclic structure biosurfactant which consists of hydrophilic peptide (usually between 7 and 10 amino acids long) linked to hydrophobic fatty acid (Smyth et al., 2010). Lipopeptide biosurfactants have gained increasing of interested due to their high surface activities and antimicrobial potential (Wang et al., 2007). Peptides and amino acid containing lipids post remarkable surface active properties by peptide containing lipids exhibit biosurfactant activities. Structural of lipopeptides is hydrophllic moiety containing the cyclic of 7 to 10 amino acid groups and hydrophobic moiety composes portions of lipid. The first lipopeptides produces was surfactin from *B.subtilis* ATCC21332 with ability to reduce surface tension from 72 to 27 mN/m at the low concentration of 0.005% (Arima et al., 1968). Some studies claimed that this type of biosurfactant can be observed by lysing of red blood cell, which is also led to the development of quickly method for screening biosurfactant producing microbes (Kosaric, 1993). The

first carried out of lipopeptide structure analysis found that surfactant of *Bacillus licheniformis* 86 is a mixer lipopeptides containing seven amino acids per molecule while, lipid portion is composed of 8 to 9 methylene groups and a mixture of linear and branched tails (Arima et al., 1968)

To determine the effectiveness of biosurfactants the concentration of biosurfactant forming micelles is considered as critical micelle concentration (CMC) CMC also remarkable as biosurfactants efficiency thus, the lower the CMC, the less biosurfactants needed to reduce surface tension. Above CMC means no further surface tension reduction can be achieved (Fig1). This is due to a variety of weak chemical interactions between the nonpolar and polar moieties of the molecules, as a conclusion, the CMC strongly depends on the structure of the surfactant molecules (Maier, 2003; Soberon & Maier, 2011).

Remarkably low CMCs have been reported of biosurfactants e.g. <1 mM to 10 mM for rhamnolipid mixtures, depending on the ionic strength of the solution (Lebron-Paler, 2008). Increasing in pH makes CMC increases due to deprotonation of the rhamnolipid. Apparent CMC was measured in this study due to the ability of biosurfactant absorb onto the soil.

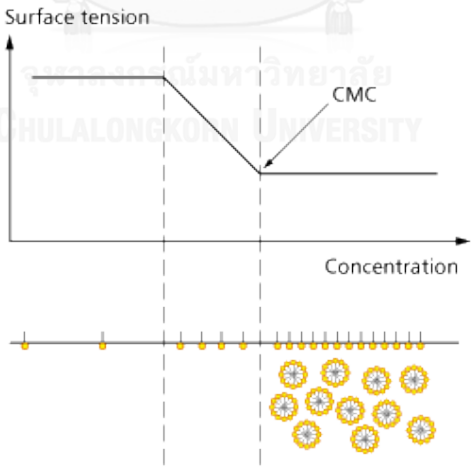


Figure 1 Effect of surfactant concentration on surface tension reduction (Joy, 2003)

2.1.2 Substrate for biosurfactant production

Bottom glycerol, waste from biodiesel production, has been interested as an alternative substrate for biosurfactant production because the world biodiesel production is increasing in every years (Hoogeveen et al., 2009). Thus, the over production and disposal of waste glycerol occurred. Using bottom glycerol for biosurfactant production is one of alternative ways. Anyway, many researches also have used bottom glycerol for alternative energy production such as methane, hydrogen etc., which are cause satisfactory in both economical and production efficiency (Wulf et al., 2006). Using other potential substrates, which are agro-based industrial waste, for biosurfactant production such as single or mixed substrate of vegetable oil processing industries, by-products of vegetable oil industries, vegetable oil industries waste, frying oil waste or dairy and sugar industry waste, were also suggested as attractive strategies for economical biosurfactant production. Moreover, using of low cost substrates including various agriculture products, by-products from industries, and waste materials as alternative substrate apart from traditional carbon and nitrogen source. Industrial or municipal waste that rich in organic pollutants and raw substrates with negligible or no value were also suggested for biosurfactant production (Makkar et al., 2011). Agro-industrial waste contains high amount of carbohydrates, lipids and hence, can be used as carbon source for microbial growth. However, the problems of considering suitable waste material with right balance of nutrients for cell growth and product accumulation associated with the effects of constituents on the properties of final product still exist.

2.1.3 Potential and economical substrate for biosurfactant production in Thailand

Potential substrates for biosurfactant production which are cost effective have been surveyed by many researches. Similarly, usable product from agro industrial waste is therefore a feasible and favorable option (Makkar & Cameotra, 2002). Vegetable oil industries also generate great amount of wastes and their disposal is a serious problem (Karim et al., 2011). Moreover, these kinds of potential substrate are

effective towards enhancing sustainability and resource recovery that becoming the problem in developing countries.

From the global volumetric consumption of vegetable oils show that palm oil (60.50), soybean oil (46.48), canola oil (26.63), sunflower oil (15.45) and other (23.89) in the unit of millions metric tons, respectively (Statista, 2015). Since, canola and sunflower oils are not the main agriculture products in Thailand. The effluent from palm oil and soybean oil are selected to be used as agro based industrial substrate compared with commercial vegetable oils for biosurfactant production.

2.1.3.1 Palm oil mill effluent

Palm oil industry is one of the major agro industries in Thailand. Palm oil mill effluent (POME) was produced large amount from the production processes and is the most significant pollutant from palm oil mill (Poh & Chong, 2009). POME is an oily wastewater that causes many serious environmental problems. Treatment and disposal of this kind of waste quite challenge the contributors because weather physical or chemical treatment processes have been designed, the problem of chemical residuals and total suspended solids are still remain (Karim et al., 2011). Moreover, this oily product from palm oil production consists of 83.5% triglyceride, 8% di glycerides, 0.5% mono glycerides and 8% free fatty acids which can act as carbon source for microbial growth during biosurfactant production (Chow & Ho, 2002). Furthermore, the essential amino acid and minerals from the palm oil fibrous can be also found in the residual oil which contains nitrogen compound that facilitates the growth of organisms also.

Production crude palm oil in Thailand is rated as 3rd rank of world market (USDA, 2015). Both input and output sides of palm oil production process contribute environmental effects such as an input require high amount of water and energy but output generates large quantity of wastewater and solid waste (Chavalparit et al., 2006). Approximately 952 liter of palm oil produced from 1 rai of palm harvested. Typically, 1 ton of crude palm oil production requires about 5 – 7.5 tons of water; over 50% of which ends up as POME (Fig2. Palm oil production processes). The

characteristic of POME is viscous, brownish liquid containing about 95 -96% water, 0.6 – 0.7% oil, 4 – 5% total solids, acidic with pH about 4 – 5 and high temperature 80 - 90°C (Bala et al., 2014). As previous mentioned POME still contains some amount of residual oil from production process so it can act as a carbon source for any fermentation including biosurfactant. Table1 and 2 show the components of saturated and unsaturated in each vegetable oil, which could be used as carbon source for biosurfactant production, adapted from (Zambiazi et al., 2007).

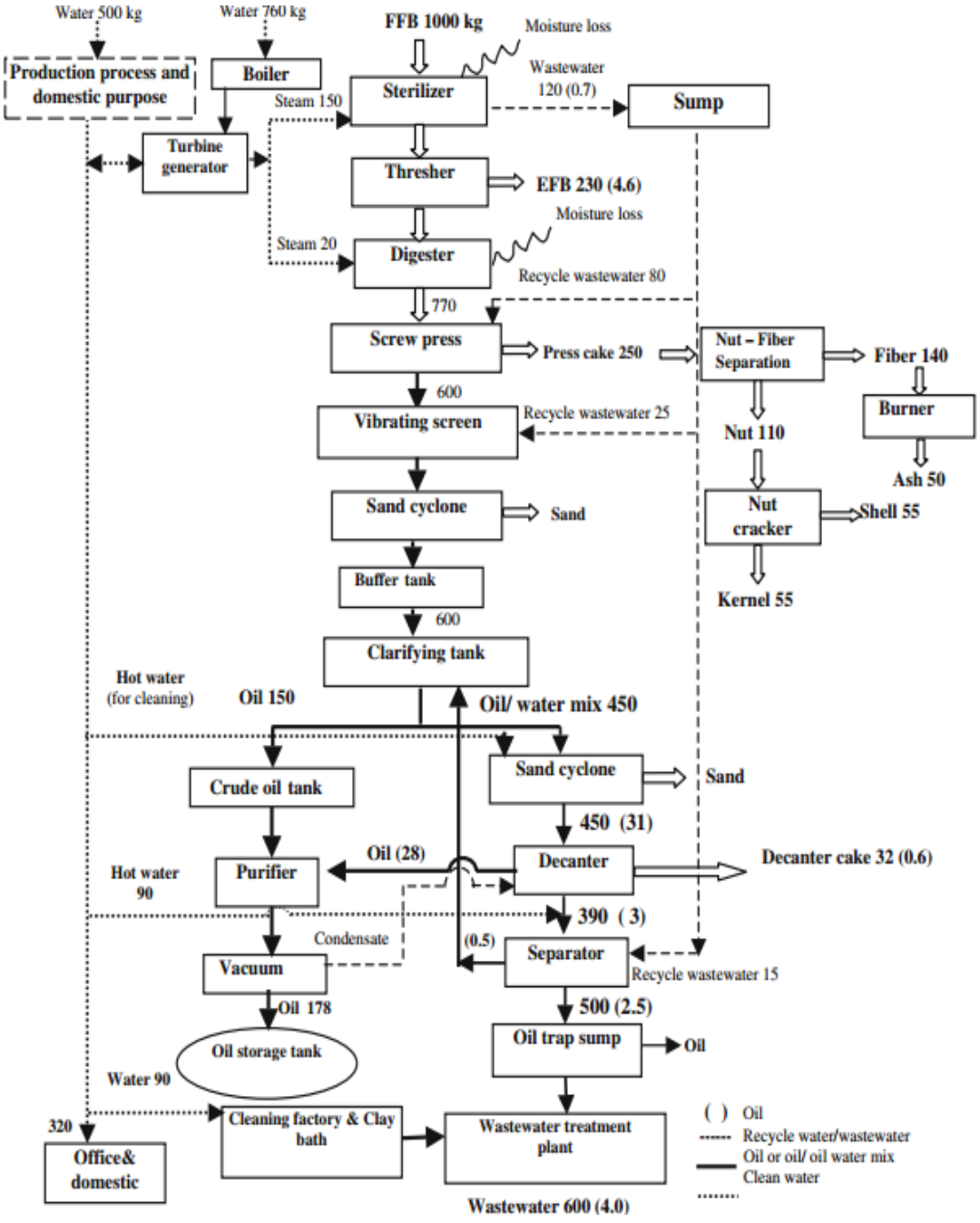


Figure 2 Palm oil production process (Chavalparit et al., 2006)

Table 1 Saturated fatty acid components containing in vegetable oils (Zambiazi et al., 2007)

Oils	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{17}	C_{18}	C_{20}	C_{22}	C_{24}	Total
Canola	-	-	-	0.06	3.75	0.04	1.87	0.64	0.35	0.27	6.98
Corn	-	-	-	-	10.34	0.07	2.04	0.44	0.31	0.26	13.46
Coconut	6.38	5.56	45.46	18.82	10.08	-	4.31	0.08	-	-	90.69
Olive	-	-	-	-	10.84	0.14	3.59	0.50	0.15	0.06	15.28
Palm	-	-	-	1.12	42.70	0.11	4.55	0.39	0.58	0.06	49.45
Rice bran	-	-	-	0.29	14.24		2.13	0.75	0.33	0.48	18.22
Soybean	-	-	-	0.06	9.63	0.11	4.38	0.35	0.67	0.24	15.12

Table 2 Unsaturated fatty acid components containing in vegetable (Zambiazi et al., 2007)

Oils	$C_{16:1}$	$C_{17:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{20:2}$	$C_{22:1}$	$C_{22:2}$	$C_{24:1}$	MUFA	TUFA
Canola	0.21	-	62.41	20.12	8.37	1.54	0.11	-	-	0.26	64.42	28.60
Corn	-	-	25.54	59.27	1.07	0.37	0.09	-	-	0.20	26.11	60.43
Coconut	-	-	7.45	1.80	-	0.06	-	-	-	-	7.51	1.80
Olive	0.92	0.21	75.55	7.01	0.66	0.32	-	-	0.05	-	77.00	7.72
Palm	-	0.06	39.37	10.62	0.21	0.17	-	-	-	0.06	39.66	10.83
Rice bran	-	-	43.87	36.28	0.99	0.64	-	-	-	-	44.51	37.27
Soybean	0.04	0.01	23.44	52.92	7.60	0.36	0.12	-	-	0.07	23.92	60.64

*MUFA is mono unsaturated fatty and TUFA is total unsaturated fatty acid

2.1.3.2 Soybean oil mill effluent

Soybean molasses, low value co-product from soybean oil processing, is an attractive feedstock for biosurfactant production. Increasing amount of agricultural wastes from soy cultivation is becoming available as a raw material for utilization in biosurfactant production (Saharan et al., 2011). Since, it has high content in fermentable sugar that useful for sustaining microbial growth (Solaiman et al., 2007). Soybean molasses plays an important role on economic growth of many products such as soy protein based foods and drinks. Containing high amount of carbohydrate (30% v/v) and others component of soluble carbohydrate; glucose, arabinose, sucrose, raffinose, stachyose and minor of monosaccharides make that soy molasses could be used in the fermentation processes (Solaiman et al., 2004). Differentiate in saccharide and protein containing in soybean molasses may attempt the opportunity of biosurfactant production as it served for carbon and nitrogen source.

Soybean molasses quite interesting as an alternative carbon source since, the second vegetable oil production in Thailand is soybean oil. So, palm oil and soybean oil affect directly on the economic growth of vegetable oil production. Soybean oil production generated great amount of wastewater including soap stock and dry sludge from cyclone precipitation (Fig3. Soy molasses produced from de-oil soybean meal). Soybean molasses is high potential in fermentable carbohydrate (30% w/v) and about 60% of total solids are valuable feed stock for microbial fermentation (Solaiman et al., 2004). Soybean molasses become an attractive feedstock because it is low value co-product from soybean processing but high in fermentable sugar content useful for sustainable microbial growth. The major saccharides component in soy molasses are glucose, arabinose, sucrose, raffinose, stachyose and other minor oligosaccharides. The different in saccharides could help influent the yield and the structure of fermentation products.

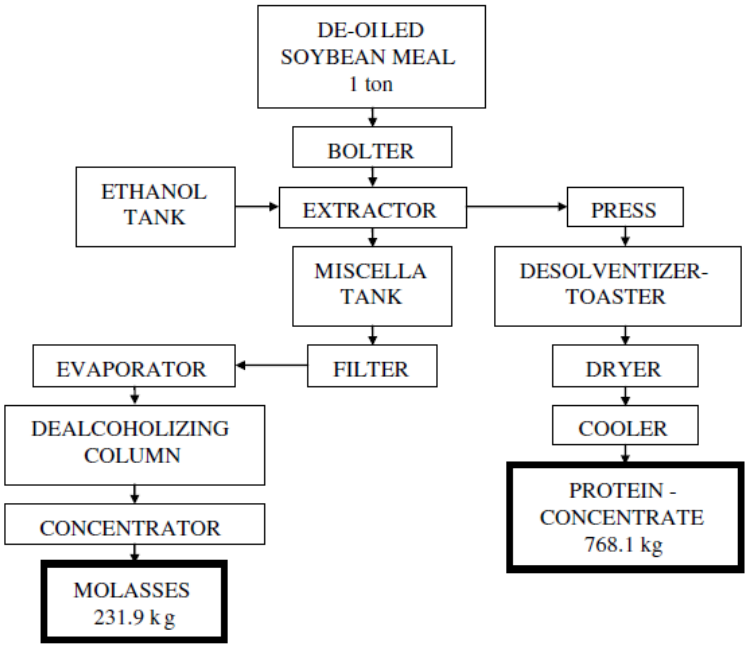


Figure 3 Soy molasses produced from de-oil soybean meal (Siqueira et al., 2008).

2.1.4 Kinetics study on biosurfactant production

Beside the development of economical biosurfactant production the kinetic on biosurfactant production from oil mill effluent was investigated in this study. Measuring biosurfactant production and cell growth during fermentation process together with substrates conversion indicated the microbial activity thus; yield of production and yield of cell growth. Biosurfactant production depends on the substrate composition and the concentrations of media interact with other complex nutrients that affect the kinetic of biosurfactant production. The ratio of carbon and nitrogen also plays an important role in the production process as the major substrate for biosurfactant production. There has been reported that C:N about 18:1 is enhance in biosurfactant production (Guerra-Santos et al., 1984). Moreover, the limitation of Nitrogen has been reported to enhance the production and found that C:N ratio about 22:1 was the best ratio in lowering surface tension to 25.5 mN/m (Abu-Ruwaida et al., 1991). Some reviews showed that the biomass production from kinetic study and substrate utilization along with the fermentation process required

for growth of organisms are the most crucial parameters for production processes (Banat et al., 2014). Moreover carbon and nitrogen source, other nutrients might affect the production activity also. To achieve the cost effective of biosurfactant production the by-product from vegetable oil industries were selected to study the kinetic of biosurfactant production especially soybean molasses. Soybean molasses or soy molasses contains a number of carbohydrates such as sucrose, dextrose, fructose, raffinose, pinitol, stachyose, and verbascose in addition to fat, flavonoids, protein, and minerals (Qureshi et al., 2001).

2.1.5 Application of biosurfactant in soil washing

Application of biosurfactants in enhancing oil recovery has been studied in many researches. It also suggested that one feasible way to treat contaminated soil is bioremediation, which utilized the natural degradative ability of plants or microorganisms, usually fungi or bacteria, to convert contaminants into less toxic compounds, or even ideally carbon dioxide and water (Lai et al., 2009). Anyway to reduce the risk of contaminants in soil posed by spilling has also done by soil washing potential (API., 1979). Soil washing process has been used for remediate many superfund sites contaminated with petroleum hydrocarbons or even their by-products (USEPA, 1995). Clean up technologies used in soil washing are based on bioremediation principle and the use of physiochemical treatment in washing of contaminated soil. There has been a growing interested in using surfactants in environmental remediation (Urum et al., 2004). Enhancing removal of oil from soil, using both higher and lower concentration than CMC has been posed (Mulligan, 2005). Lower CMC mobilization due to the lowering of interfacial tension between oil and water, that interfacial tension lowering ability of surfactant in oil-water system, causes the reduction in capillary force that holds between soil and oil. Higher CMC, where surfactant cluster together and start forming dynamic aggregates known as micelles, so this must lead to solubility occurred (Urum et al., 2003) (Fig4.). Physical characteristic such as density, temperature, surface and interfacial tension of oil, surfactant and soil system also affect the mechanisms of bioremediation and

biodegradation. The difficulty in bioremediation once is mass transfer that limit the contact of microbes thus the poor biodegradation efficiency (Paria, 2008). Hydrocarbons, less water soluble, attach with soil particles also limit rate of mass transfer. Hence the key parameter of transportation the contaminants to aqueous bulk phase was suggested (Mihelcic et al., 1993).

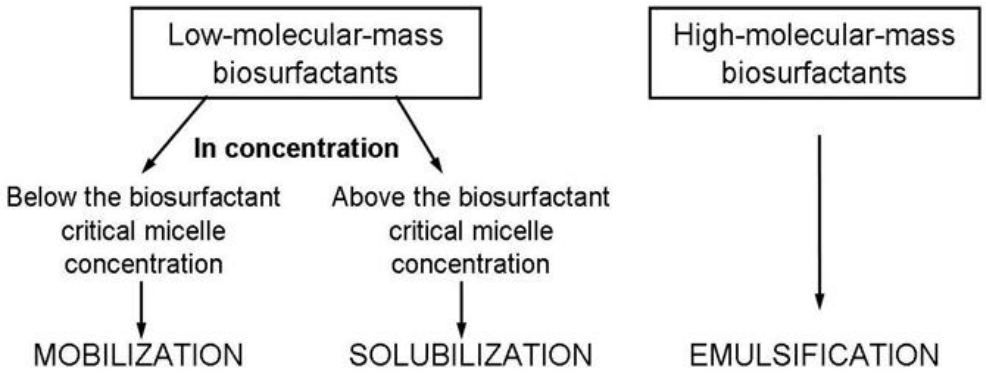


Figure 4 Mechanisms of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration (Matvyeyeva et al., 2014)

2.2 Literature reviews

2.2.1 Biosurfactant production from utilization of vegetable oil

Biosurfactant can be produced from many kinds of substrate that can utilized by bacteria strain such as *P. aeruginosa* A41, isolated strain from seawater, was able to grown in defined medium containing 2% vegetable oil or fatty acid as a carbon source. The result found that the yield steadily increased even after stationary phase. The surface tension of the medium was lowered from 55-70 mN/m to about 27.8-30 mN/m in every carbon source. However, the types of carbon sources have found effect on biosurfactant yield. The yield of rhamnolipid found to be 6.58, 2.91 and 2.93 g/l when olive oil, palm oil and coconut oil was used, respectively. Among them, biosurfactant obtained from palm oil was the best in lowering surface tension (Thaniyavarn et al., 2006).

Two strains of *Serratia marcescens* were grown on minimal culture medium supplemented with vegetable oils to stimulate biosurfactant production. The results showed a decrease in surface tension of the culture medium without oil from 64.54 to 29.57, with a critical micelle dilution (CMD(-1)) and CMD(-2) of 41.77 and 68.92 mN/m, respectively. Sunflower oil gave the best results of 29.75 mN/m with CMD(-1) and CMD(-2) about 36.69 and 51.41 mN/m, respectively. Sunflower oil contains about 60% of linoleic acid. The addition of linoleic acid decreased the surface tension from 53.70 to 28.39, with a CMD(-1) of 29.72 and CMD(-2) of 37.97, suggesting that this fatty acid stimulates the biosurfactant production by the LB006 strain. In addition, the crude precipitate surfactant reduced the surface tension of water from 72.00 to 28.70 mN/m. These results suggest that the sunflower oil's linoleic acid was responsible for the increase in biosurfactant production by the LB006 strain (Ferraz et al., 2002).

2.2.2 Biosurfactant production from utilization of palm oil mill effluent

A biosurfactant-producing bacterium, *Ochrobactrum anthropic* 2/3, was isolated from mangrove sediment and found to be a potential biosurfactant producer. The highest biosurfactant production (4.52 g/l) was obtained when the cells were grown on a minimal salt medium containing 25 % (v/v) palm oil decanter cake and 1 % (w/v) commercial monosodium glutamate as carbon and nitrogen sources, respectively. After microbial cultivation at 30 °C in an optimized medium for 96 h, the biosurfactant produced was found to reduce the surface tension of pure water to 25.0 mN/m with critical micelle concentrations of 8.0 mg/l. It is an effective surfactant at very low concentrations over a wide range of temperatures, pH and salt concentrations. The biosurfactant obtained was confirmed as a glycolipid type biosurfactant (Noparat et al., 2014).

The study of palm oil mill effluent as a promising substrate for biosurfactant production, the potential strains of bacteria were isolated from various hydrocarbon-contaminated soils and screened for biosurfactant production by drop collapse method and surface tension measurements. Out of 26 isolates of bacteria, *Nevskia ramose* NA3 showed the highest bacterial growth with the highest surface tension reduction of 27.2 mN/m. The Plackett-Burman experimental design was employed to determine the important nutritional requirements for biosurfactant production. Six out of 11 factors of the production medium were found to significantly affect the production of biosurfactant. $FeCl_2$ and $NaNO_3$ had a direct proportional correlation with the biosurfactant production. Commercial sugar, glucose, K_2HPO_4 and $MgCl_2$ showed inversely proportional relationship with biosurfactant production in the selected experimental range (Chooklin et al., 2013).

New genera of bacteria that have ability to produce biosurfactant from palm oil contaminated industrial sites along with palm oil effluent, palm oil decanter cake, have also been isolated and those new strain are named *Buttiauxella, Comamonas, Halobacterium, Haloplanus,* and *Sinorhizobium* (Saimmai et al., 2012).

2.2.3 Biosurfactant production from utilization of soy molasses

There are many form of soybean waste that can be used as substrate for biosurfactant production such as soybean oil wastewater (soap stock), soybean oil sludge (molasses). The study of alternative low-cost substrates for rhamnolipids production by *Pseudomonas aeruginosa* LBI strain, the wastes obtained from soybean, cottonseed, babassu, palm, and corn oil refinery were used. The result found that soybean soapstock waste was the best substrate, generating 11.7 g/l of rhamnolipids with a surface tension of 26.9 mN/m, a critical micelle concentration of 51.5 mg/L, and a production yield of 75% (Nitschke et al., 2005).

Sophorolipids (SLs) were produced from *Candida bombicola* using soy molasses and oleic acid as co-substrates. The purified SLs were obtained at 21 g/l. The major SL constituent (81% relative abundance) of the product mixture contains an oleoyl chain (Solaiman et al., 2004) . In 2007, same authors also grew the same strain on soybean molasses as both carbon and nitrogen source with oleic acid added and the yield found to be 53 g/l of purified sophorolipids per liter of starting culture volume. The study demonstrated for the first time the usefulness of the low-value soy molasses as a combined nitrogen- and carbon-source for SL production at a reduced cost (Solaiman et al., 2007).

Not only palm oil or soybean oil mill effluent that can be used as substrate. *P. aeruginosa* 47T2 has also grown in olive oil mill effluent, which is a major waste problem in Spain, the result found the possibility of using oily waste by bacteria (Mercadé et al., 1993).

Isolated *P. aeruginosa* LB1 from soap stock of sunflower oil processing and found the ability to produce 15.9 g/l of rhamnolipids (Benincasa et al., 2002).

Variation of oily waste substrates such as soybean, cotton seed, babasu, palm and corn oil refinery and discovered of the highest rhamnolipids produce was achieved when using soybean soap stock as substrate (Nitschke et al., 2005).

Utilization of mixed waste from peanut oil cake and waste motor lubricant oil, the results can confirm the capability of using waste substrates by *Bacillus megaterium, Azotobacter chroococcum, Corynebacterium kutscheri* and

Lactobacillus delbrueckii demonstrated the using of peanut oil cake as substrate the biosurfactant produced from *Lactobacillus delbrueckii* achieved about 5.35 mg/ml (Thavasi et al., 2008).

Another studies use frying oil wastes as substrate such as the utilization of residual sunflower oil frying waste for biosurfactant production by *Rhodococcus erythropolis*. Their approach was to achieve the cheaper substrate for glycolipids production and with only 3% of sunflower oil frying waste they got glycolipids with high surface activity and emulsification capability. These demonstrate the possibility of using lipophilic waste as novel substrate for biosurfactant production (Sadouk et al., 2008).

Table 3 Review of alternative substrates for biosurfactant production

Organism	Renewable substrate	Biosurfactant type	Application	Reference
B. subtilis ATCC 21332; *B. subtilis* LB5	Cassava flour wastewater	Lipopeptide	-	(Nitschke & Pastore, 2004, 2006)
B. subtilis	Potato casava	Surfactin	Environmental remediation and oil recovery	(Noah et al., 2002)
B. subtilis	Potato casava	Surfactin	Environmental remediation and oil recovery	(Noah et al., 2005)
B. subtilis	Potato waste	Surfactin	Environmental remediation and oil recovery	(Thompson et al., 2000)
B. subtilis	Potato waste	Surfactin	Removal of starch particulates	(Thompson et al., 2001)
B. subtilis ATCC 21332	Potato waste	Surfactin	-	(Fox & Bala, 2000)

Organism	Renewable substrate	Biosurfactant type	Application	Reference
C. bombicola ATCC 22214	Turkish corn oil and honey	sophorolipids	-	(Pekin et al., 2005)
C. lipolytica IA1055	Babassu oil	New bioemulsifier: carbohydrate, lipid, protein	-	(Vance-Harrop et al., 2003)
C. bombicola	Soy molasses-based medium	Sophorolipids	-	(Solaiman et al., 2004; Solaiman et al., 2007)
C. lipolytica	Industrial residue	Biosurfactant	Oil recovery	(R. Rufino et al., 2007)
C. lipolytica	Canola oil	Biosurfactant	-	(Sarubbo et al., 2007)
Candida sp. SY16 95 45	Soybean oil	Mannosylerythritol lipid	-	(Kim et al., 2006)

Organism	Renewable substrate	Biosurfactant type	Application	Reference
Corynebacterium kutscheri	Waste motor lubricant oil	Biosurfactant	Bioremediation	(Thavasi et al., 2007)
P. aeruginosa LB1	Oil wastes	Rhamnolipid	-	(Nitschke et al., 2005)
P. aeruginosa	Molasses	Rhamnolipid	-	(Raza et al., 2007)
P. aeruginosa AT10	Soybean oil refinery wastes	Rhamnolipid	-	(Abalos et al., 2001)
P. aeruginosa GR9-119	Sunflower and soybean oil	Rhamnolipid	-	(Rahman et al., 2002)
P. aeruginosa BS2	Distillery and whey waste	Rhamnolipid	-	(Dubey & Juwarkar, 2001)

Organism	Renewable substrate	Biosurfactant type	Application	Reference
P. aeruginosa BS2	Curd whey and distiller	rhamnolipid	-	(Dubey & Juwarkar, 2004)
P. aeruginosa BS2	Fermented distillery wastewater	rhamnolipid	-	(Dubey et al., 2005)
P. aeruginosa LB1	LB1 soapstock	Rhamnolipid	-	(Benincasa et al., 2002)
P. aeruginosa LB1	LB1 soapstock	Rhamnolipid	Bioremediation	(Benincasa et al., 2004)
Pseudomonas sp.	Used olive, sunflower oil	Rhamnolipid	-	(Haba et al., 2000)

Organism	Renewable substrate	Biosurfactant type	Application	Reference
P. aeruginosa	Vegetable oil refinery wastes	Biosurfactant	-	(Raza et al., 2007)
P. aeruginosa FR	Palm oil	Biosurfactants	-	(F. J. Oliveira et al., 2006)
Pseudomonas sp. DSM 2874	Rapeseed oil	Mixture of four types of glycolipids (rhamnolipid 1-4), L-(+)-rhamnose and (R,R)-3-(3-hydroxydecanoyloxy)	-	(Trummler et al., 2003)

Organism	Renewable substrate	Biosurfactant type	Application	Reference
Trichosporon montevideense CLOA72	Dairy industry effluents	Glycolipid	-	(Monteiro et al., 2009)
Yeast	Oil refinery waste	Glucolipids	-	(Bednarski et al., 2004)

2.2.4 Optimization of biosurfactant production by kinetic study

P. aeruginosa UCP0992 was cultivated on various of carbon and nitrogen (source and concentration) the results suggested that the relationship between biosurfactant production, cell growth, consumption of substrate, emulsification, surface tension reduction, hexadecane, and other substrate utilization seemed to be parallel together (Silva et al., 2010). The kinetics of biomass and biosurfactant production along with substrate utilization and fermentation duration required for organism to grow is the most crucial parameters for commercial production process (Banat et al., 2014).

The strain *Pseudomonas aeruginosa* J4 was grown in petrochemical wastewater with variation of concentration. The authors observed that high nitrogen content in medium limits the biosurfactant production because of unbalance between carbon and nitrogen (Wei et al., 2005).

Study the kinetic of biosurfactant production of *B. subtilis* LAMI005 grown in clarified cashew apple juice. Measuring of varied total reducing sugar from clarified cashew apple juice in fermentation representatives as carbon source utilization when fix the concentration of nitrogen as 1 g/l $(NH_4)_2SO_4$ (Oliveira et al., 2013)

Using soybean molasses as sole carbon and nitrogen source for kinetic study, Carbon source consists in soybean molasses are mono and poly saccharides with the minor component of oligosaccharides. Since, the source of soybean molasses comes from the process of vegetable oil production then soybean molasses itself might contains some amount of free fatty acid, mainly are 54% linoleic acid, 28% oleic acid (Salunkhe, 1992). Therefore, detection of sucrose, raffinose and strachyose, which are main components in soybean molasses, decreasing in an interval times presents as utilization of sugar. Decreasing in nitrate to nitrite and ammonia can be observed the respiration of the strain when the time of fermentation increases. Measuring the reduction of oleic acid, main fatty acid, can be interpreted as utilization of fatty acid as carbon source to promoting biosurfactant production.

2.2.5 Application of biosurfactant in soil washing

Biosurfactants could help promoting solubilization or immobilization by their amphiphilic properties that are useful for mobilization of hydrophobic compounds sorbed onto soil particles. Tween® 80 or Polyoxyethylene (20) sorbitan monooleate, non-ionic surfactant, widely used in soil remediation mainly cleanup hydrocarbons (Pacwa-Płociniczak et al., 2011). Tween® 80 interesting as candidate to deal with hydrocarbons since it first use more than 20 years ago (Laha & Luthy, 1992). Compared to other surfactants the chemical characteristic with low cost and low toxicity on soil microorganisms brings to the great interest for soil remediation (Bautista et al., 2009). Comparing the use of biosurfactant with commercial biosurfactant like Tween® 80 in phenanthrene contaminated soil washing found that Tween® 80 gave higher efficiency in soil washing than biosurfactant produced by *Sapindus saponin* moreover, using organo bentonite to remove phenanthrene from washed solution has been used (Zhou et al., 2013). Another study has done on using biosurfactant produced by *Candida lipolytica* to remove petroleum derivative by adsorb to soil the researchers claimed that produced biosurfactant has potential to decontamination processes of petroleum derivates (Rufino et al., 2013).

CHAPTER 3
METHODOLOGY

3.1 Biosurfactant-producing bacterium

Biosurfactant producing bacterium identified as *Bacillus* sp. GY19 was isolated from planted soil sample using glycerol based medium (Khondee et al., 2015). It was maintained on 25% (w/v) Luria-Bertani agar (Horowitz et al., 1990) and subculture monthly.

3.2 Inoculum preparation

Single colony of bacterium from LB agar was transferred to 25% LB medium and shaken for 24 hours. Then 2% of fresh medium inoculum was used as microorganism for biosurfactant production.

3.3 Chitosan immobilization

Squid pen chitosan was purchased from ELAND Corporation, Ltd and was used for bacteria immobilization. Type of chitin source of quid pen chitosan is beta-chitin with amino group aligned with the OH and CH_2OH groups. After that 2% (v/v) of fresh medium was added to 25% LB medium (adjusted pH to 6) containing 80 g/l chitosan then the medium was shaken for 3 days to get chitosan immobilized cell. The number of cell attach on chitosan was counted by plate count method.

3.4 Substrate used for biosurfactant production

Commercial vegetable oils are derived from general vegetable oil supplier. Palm oil mill effluent derived from southern palm oil Co., Ltd and soybean oil mill effluents were derived from Thai vegetable oil public Co., Ltd. and was added to productive medium as a substrate for biosurfactant production.

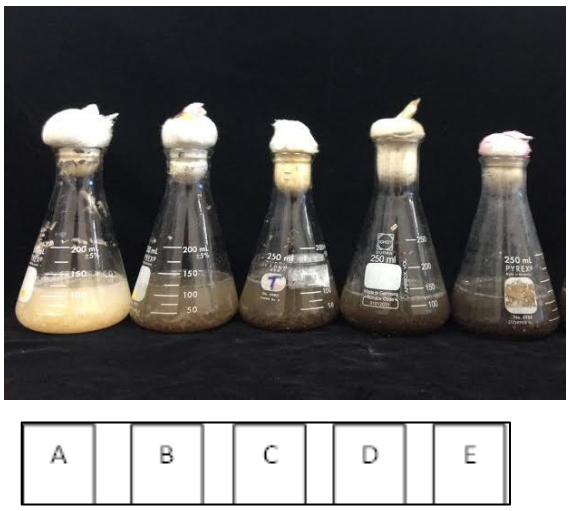


Figure 5 The concentrations of soy molasses used as substrate for biosurfactant production (A) 0%, (B) 10%, (C) 20%, (D) 30% and (E) 50%

3.5 Media for biosurfactant production

Productive medium use for biosurfactant production, which consists of 1 g glucose, 0.5 g beef extract, 3.3 g K_2HPO_4, 0.14 g KH_2PO_4, 0.2 g $NaNO_3$, 3.3 g NH_4NO_3, 0.04 g NaCl, 0.1 g $FeSO_4$ $7H_2O$ was purchased from Sigma-Aldrich Co.LLC.as analytical grade (Nawawi et al., 2010). After that each percent of substrate for biosurfactant production was added (following the scope of this study) then 1 liter of distilled water was added. The media were sterilized at 110°C for 10 min.

3.6 Production of biosurfactant

The 250 ml flask containing 8 g chitosan immobilized cell in 100 ml of productive medium with substrate was shaken for 5 days following previously published protocol (Khondee et al., 2015). After 5 days of production, the culture medium was centrifuged at 8000 rpm for 20 min to get cell-free culture medium then the supernatant was extracted with equal volume of hexane to get rid of the excess oil then analyzed for surface tension, oil displacement, emulsification index. The results were compared to killed immobilized cell, which is chitosan containing in the medium with no cell added.

3.7 Crude biosurfactant extraction

Culture medium from biosurfactant production was separated an excess substrate and immobilized cell out by centrifugation. Then, the obtained cell-free broth was extracted with hexane to remove the excess oil. It was adjusted to a pH of 2 using 6 N HCl and let it precipitate at 4°C overnight. Then solvent extraction in a shaking funnel was performed, using chloroform/methanol (2:1) at a ratio of solvent to broth equal to 1:1 for three times. The chloroform/methanol (lower) phase was collected and evaporated. Once the solvent was evaporated, methanol was added to re-dissolve the residual viscous dark brown product was weighted as crude biosurfactant.

3.8 Analytical methods of biosurfactant effectiveness

3.8.1 Surface tension

The surface tension was measured by tensiometer (Kruss K6, Germeny) at 25°C using plate method.

3.8.2 Emulsification index

Emulsification index was measured by aliquot volume of cell-free medium and diesel oil mixed together by vortexing for 3 min. It was settle for 24 hours then the height of emulsions was measured

3.8.3 Oil displacement

Oil displacement was carried out in petri plat by adding 5 µl of cell-free culture medium into 20 ml distilled water containing 10 µl of crude oil on the surface. The diameter of oil spread was measured.

3.8.4 Critical micelle concentration

The supernatant was diluted to the concentration lowering its crude biosurfactant concentration. Then the diluted broth was measured for surface tension. Relationship between surface tension and concentration of crude biosurfactant in broths represent as critical micelle concentration.

3.9 Analytical methods for substrate utilization on kinetic study

3.9.1 Carbohydrate composition

Carbohydrate composition was measured by phenol-sulfuric method. Briefly, 250 µl of sample added with 125 µl of 80% phenol solution after that conc. Sulfuric acid was added for 625 µl. then, mixed together and left at room temperature for 10 min. the sample was measured by spectrophotometer at 540 nm (Masuko et al., 2005).

3.9.2 Nitrogen composition

Nitrate and Nitrite concentrations were measured by an assay based on the reduction of nitrate, by reduced NADPH, to nitrite in the presence of the enzyme nitrate reductase. The decrease in NADPH concentration was measured by means of its absorbance at 540 nm (Miranda et al., 2001).

3.9.3 Glyceride composition

mono-, di- and tri-glycerides were measured by Thin Layer Chromatography (Flame ionization detection). Detection each chromatrograms of glycerides composition from retention time (Fig 6). Using stationary phase of 3% boric acid impregnated CHROMAROD-SIII and mobile phase 1st as Chloroform 100%, 2nd Chloroform: Methanol/Ammonia (8:2). Gas flow rate of H_2 160 ml, air flow rate 2.0 l.min, scanning speed 30 sec/scan and iatrocorder attenuation 16

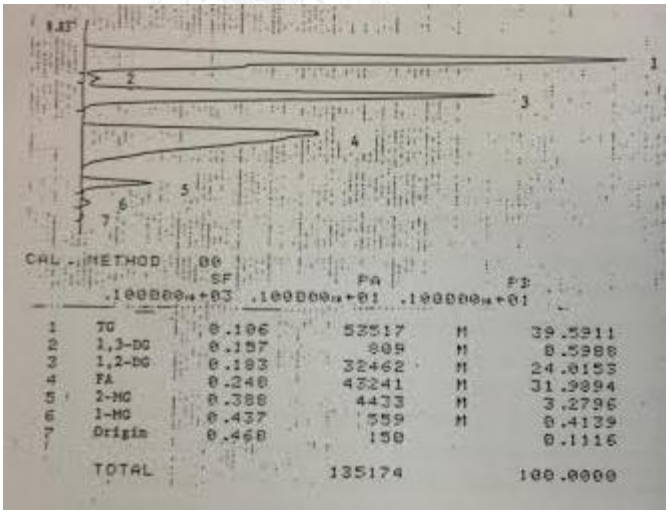


Figure 6 TLC chromatograms of glyceride compositions

3.10 Soil sorption from aqueous solution of produced biosurfactant

Three different types of sediment, originated from Chao Praya River, Thailand (Table 4). These three types of soil were used to test the sorption behavior of the biosurfactant in aqueous solutions. Figure 7 shows the sources of sediment collected from Chao Praya River.

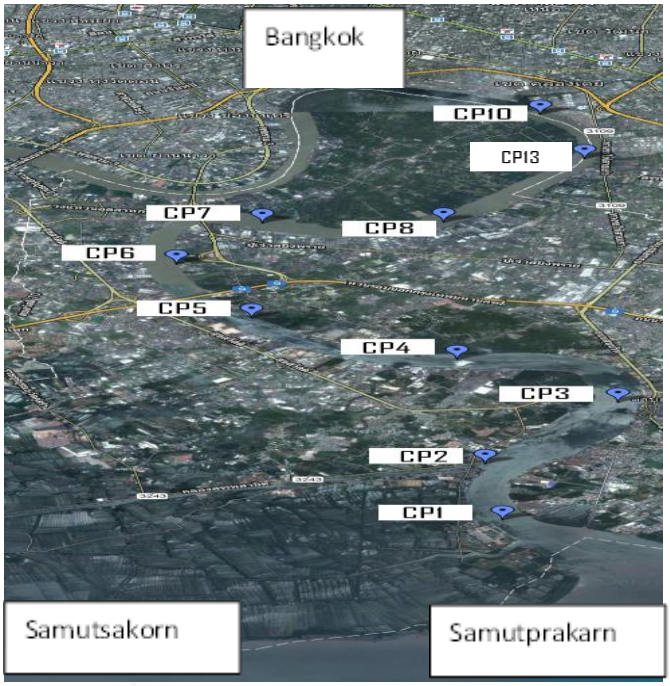


Figure 7 Sources of sediments collected from Chao Praya River

*CP5, CP10 and CP13 sediments were used to study biosurfactant sorption efficiency and crude oil contaminated soil washing

Table 4 Properties of soil samples for sorption study and crude oil contaminated soil washing

soil	pH	EC	TC	OC	IC	Total N	Total P	Active P	Total S	Active S	Sand	Silt	Clay
		ms/cm	%			ppm					%		
CP05	7.35	0.54	2.44	2.34	0.098	0.21	406	578	3663	1021	6.8	39	55
CP10	6.70	1927	4.59	4.40	0.191	0.32	1387	1278	5245	2431	21	52	27
CP13	6.83	0.52	2.86	2.79	0.074	0.20	1001	1298	4024	666	20	34	46

*EC is electrolytic conductivity

TC is total carbon

IC is inorganic carbon

3.11 Soil washing potential from foamate solution of produced surfactant

Soil washing and soil sorption experimental was done as described in previously published protocol (Franzetti et al., 2012). Two grams of sediment was spiked with crude oil (5% w/w). Crude oil was diluted into n-hexane before adding to the sediment, to reach a homogeneous mixture of the crude oil with the sediment surface. Solvent was evaporated for 2 days. Soil washing was performed by using foamate solutions and freeze-dried biosurfactant at different concentrations comparing to Tween 80 (1500mg/l, 2000mg/l), SDS solution (1500mg/l and 2000 mg/l) and water. Parameters such as pH, Temperature and sediment type were constant. After the soil washing process (mixing, and shaking 30min) sediment was centrifuged and rinsed with water. After drying, the residual oil was analyzed by extracting the sediment 3 times with 10ml of n-hexane and measuring the total petroleum hydrocarbon by TLC-FID (Khondee et al., 2015).

3.11.1 Foam fractionation

To increase the concentration of biosurfactant in broth the supernatant was mixed with air and transported through a column (60cm) to obtain a foamate (Khondee et al., 2015).

Figure 8 Foam fractionation technique used to increase the concentration of produced biosurfactant

3.11.2 Freeze-Dried biosurfactant

Foamate solution obtained from foam fractionation was performed Freeze-Dried lyophilized in order to increase the concentration of crude biosurfactant (Hoogmoed et al., 2000). Then, the amount of dried biosurfactant was dissolved in DI water to obtain the concentration of biosurfactant and use in crude oil contaminated soil washing.

Figure 9 Freeze-dried lyophilization used to increase the concentration of produced biosurfactant

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Biosurfactant production from utilization of vegetable oils

Productive medium containing 2% (v/v) of each vegetable oil e.g., palm oil, olive oil, corn oil, sunflower oil, soybean oil and rice bran oil was used as substrate for biosurfactant production in order to find the most appropriate fatty acid for *Bacillus* sp. GY 19 to produce biosurfactant. The results were compared to killed-immobilized cell due to the effect of chitosan on substrate used.

The efficiency of produced biosurfactant in surface tension reduction of productive medium have found when bottom glycerol and palm oil was used as substrate since, they could reduce the surface tension of the medium from 64 mN/m to less than 40 mN/m while other substrates used reduced surface tension only about 40 mN/m (Fig 10A). Comparing to killed-immobilized cell that the surface tension reduced as well when chitosan added, which could mean chitosan also had an effect on surface tension reduction. The related result found when palm oil was used as substrate since not only reduced well in surface tension of medium but also gave high emulsification index about 82% (Fig 10B). However, the ability to cause emulsification against diesel oil found only when bottom glycerol was used as substrate (Fig 11A). Since bottom glycerol, waste from biodiesel production, might contains some amount of soap which is also by-product from biodiesel production that affects more in emulsion layer of emulsification activity than vegetable oils used (Silva et al., 2010). Anyway, the reason that bottom glycerol used as substrate showed high in emulsification activity comparing to killed-immobilized cell (Fig 11A), due to the surface active agent produced from bottom glycerol reduced well in surface tension of medium (Fig. 10A). Result from crude biosurfactant produced found that crude biosurfactant achieved about 5.09, 4.60, 3.78 and 3.01 g/l when corn oil, olive oil, palm oil and soybean oil was used, respectively (Fig 11B). Critical micelle dilution of biosurfactant showed that produced biosurfactant from corn oil,

bottom glycerol and palm oil can be diluted up to 5, 2 and 1 times and remained in surface tension reduction about 44.8, 35.3 and 35.5 mN/m, respectively. It can be concluded that biosurfactant that produced from corn oil, bottom glycerol and palm oil have high ability as surface active substance. Together with high amount of crude biosurfactant achieved from these substrates demonstrated the production activity of biosurfactant also.

Each vegetable oil contains different component of fatty acid so, utilization of fatty acid by bacteria will affect the production of biosurfactant that the favorable fatty acid depends on the microbe utilization (Zambiazi et al., 2007). From the results, it seemed that palm oil was the most favorable fatty acid utilized by bacteria since, high amount of crude biosurfactant produced and activity to reduce surface tension of medium also shown. Containing high amount of total saturated fatty acid in palm affected on the efficiency of produced biosurfactant since the results showed better in surface tension reduction, oil displacement and crude biosurfactant produced.

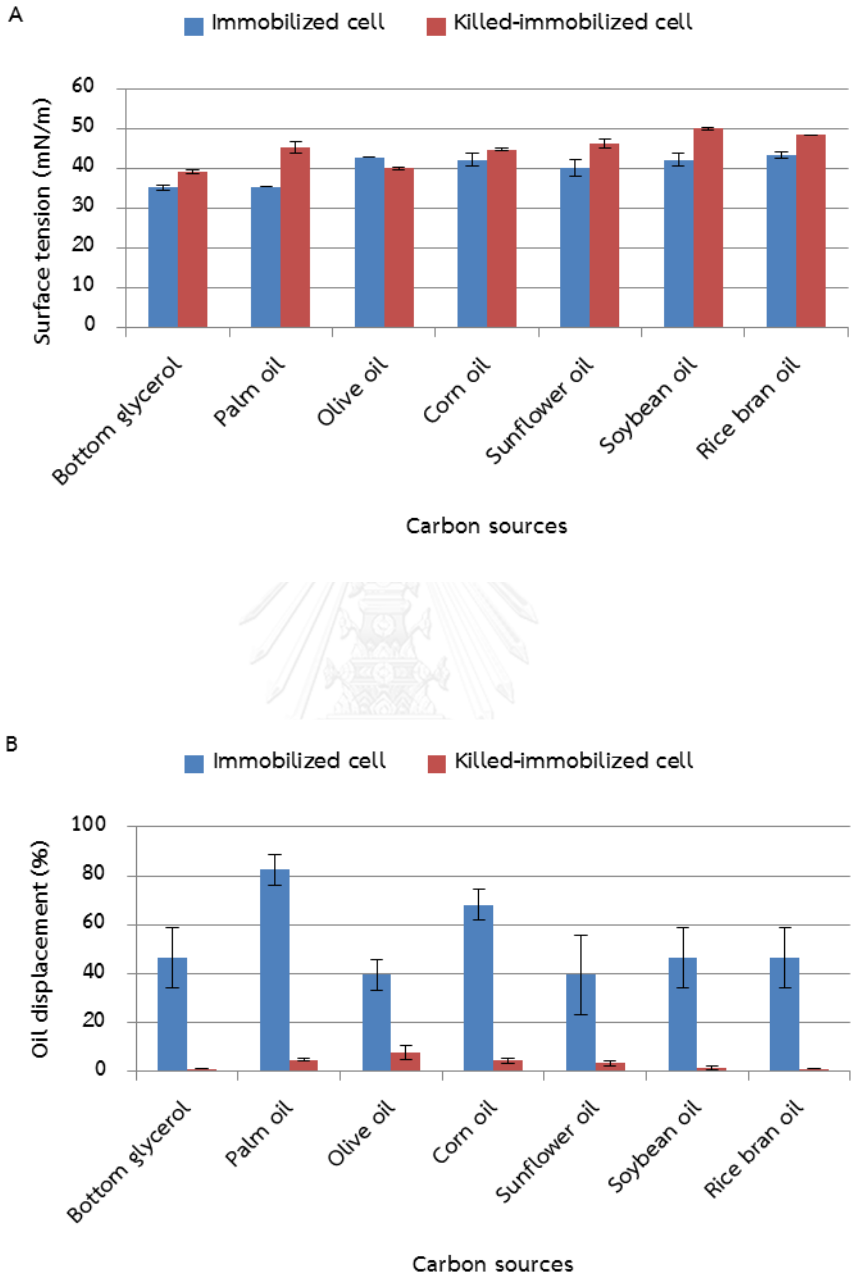


Figure 10 Effect of vegetable oils used as substrate on (A) surface tension reduction and (B) oil displacement

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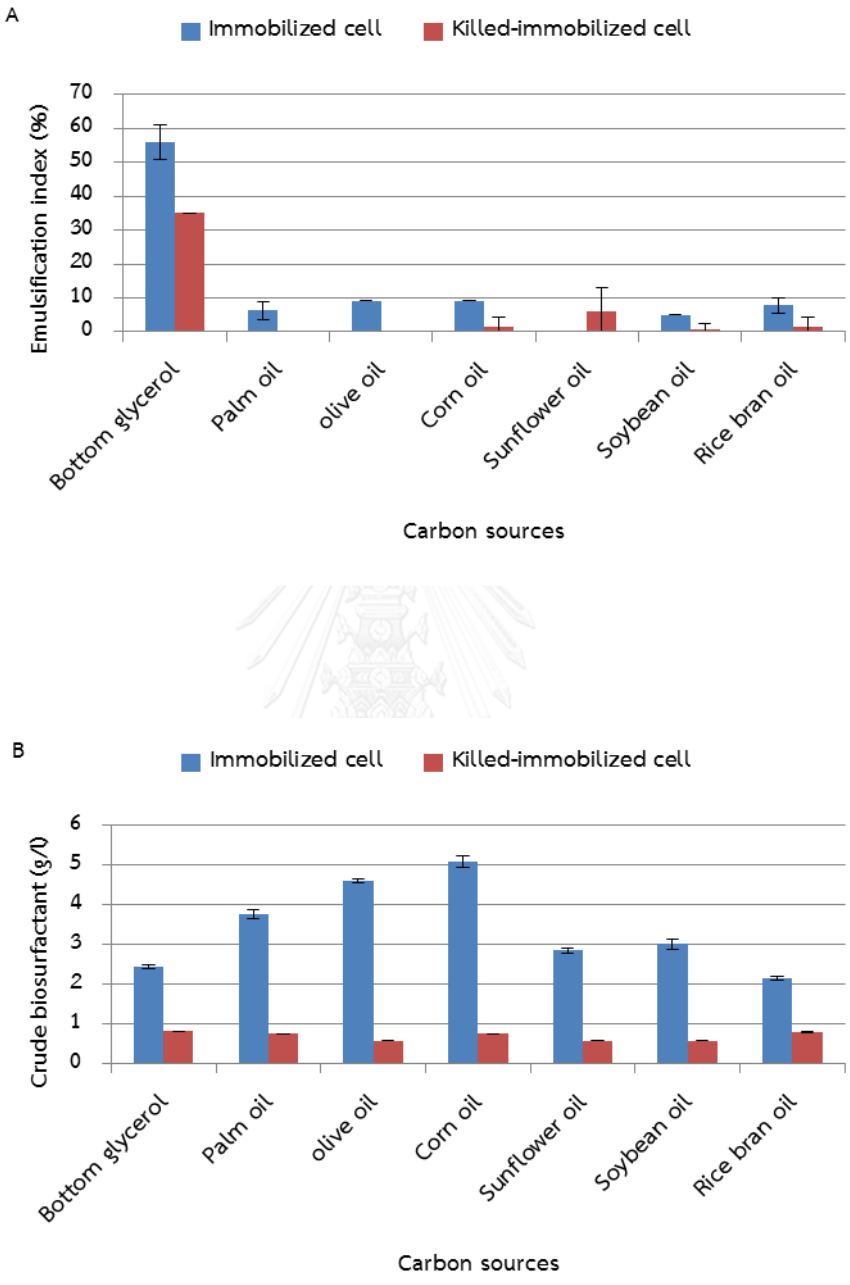

Figure 11 Effect of vegetable oils used as substrate on (A) emulsification index and (B) crude biosurfactant produced.

In order to reduce the cost of biosurfactant production, utilization of agro-based industrial waste was quite interesting since the production processes of oil mill generate the waste that contains some amount of fatty acid or other components that can be used as carbon source. Then, waste from oil mill effluent was considered to be another substrate for economical biosurfactant production. From the results of corn oil, olive oil, palm oil and soybean oil showed high amount of crude biosurfactant production were considered. Anyway, corn oil and olive oil are not main agriculture products in Thailand so waste from palm oil and soybean oil production were used as alternative substrates for further biosurfactant production.

4.2 Biosurfactant production from utilization of oil mill effluents

4.2.1 Biosurfactant production from utilization of palm oil mill effluent

The concentration of POME was varied into 0, 20, 40, 60, 80 and 100% (w/v) of productive medium. Result showed that cell growth started from 10^9 CFU/g chitosan and remained about 10^7 to 10^8 CFU/g chitosan in all concentrations of POME, which is mean there might be some detachment of cell out from the chitosan (Fig 12A). Surface tension reduction has found when 20, 40 and 60% of POME were used as substrate that they could reduce surface tension from 64 to 40.33, 42.67 and 39.67 mN/m ,respectively. (Fig 12B). However, comparing to killed-immobilized cell that could reduce the surface tension of medium to about 53 mN/m ,so immobilized cell seemed to have low ability to reduce in surface tension. Anyway, crude biosurfactant produced found increasing when initial concentration of substrate was increased these due to the viscosity characteristic of POME that might affected on crude biosurfactant extraction since less amount of crude biosurfactant detected (Table 5). The result from critical micelle dilution demonstrated that biosurfactant produced from 20 and 40% POME could dilute up to 8 times dilution and still remained in surface tension reduction (Fig 13B and 13C). Since less amount of crude biosurfactant produced and no activity of oil displacement and emulsification activity showed when POME was used as substrate (Table 5). Then, other potential substrates were considered.

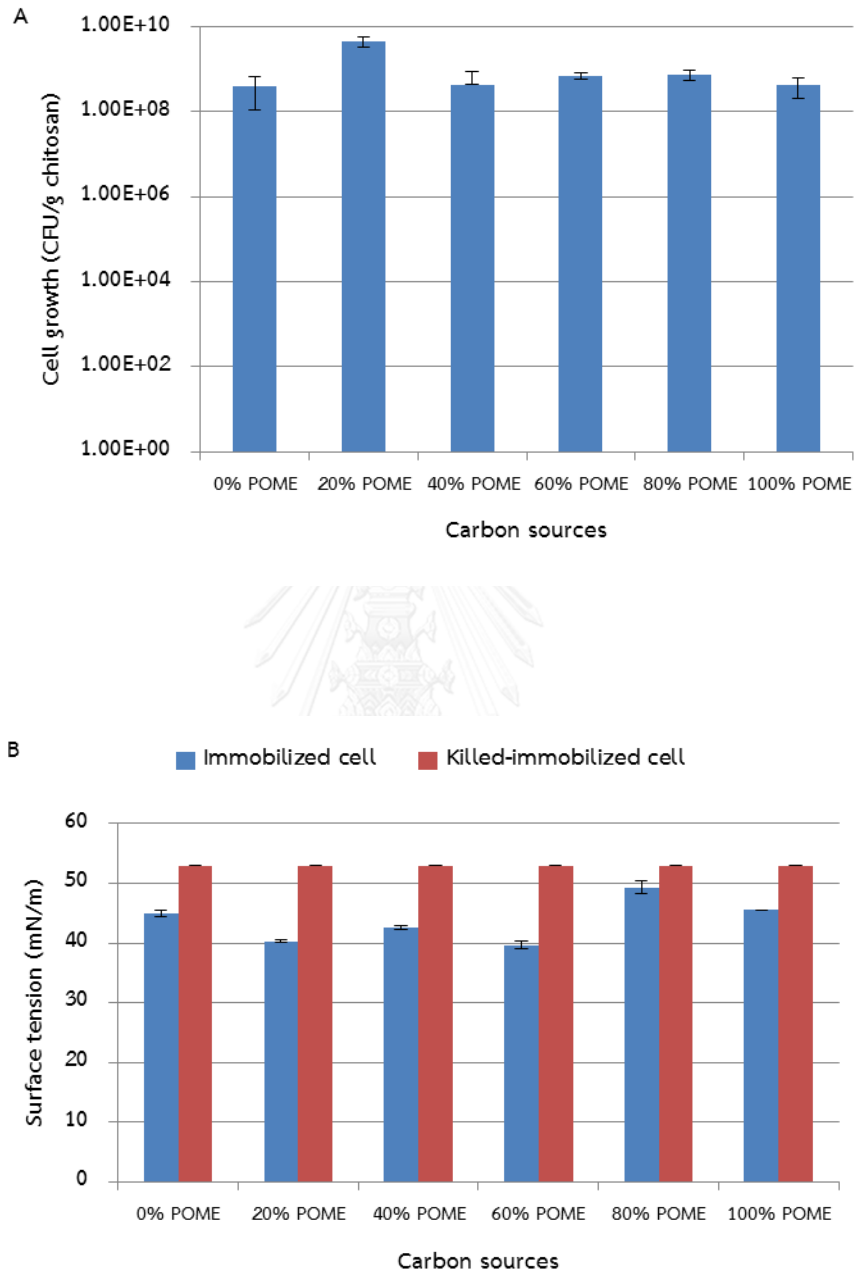

Figure 12 Effect of POME used as substrate on (A) cell growth and (B) surface tension.

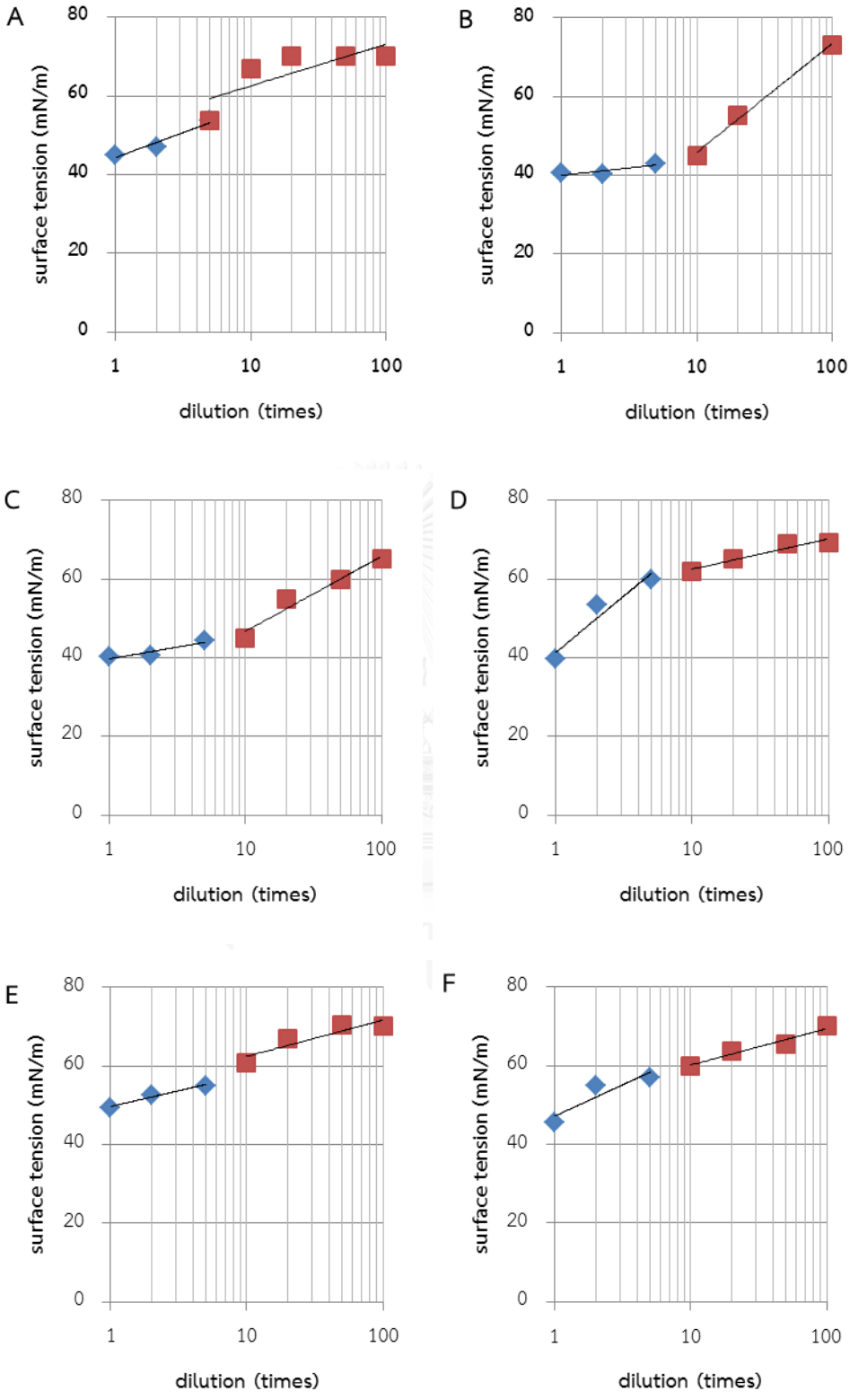

Figure 13 Critical micelle dilution of biosurfactant produced from (A) 0%POME, (B) 20% POME, (C) 40% POME, (D) 60% POME, 80% POME and (F) 100% POME

Table 5 Biosurfactant production from utilization of each concentration of POME

POME (%v/v)	Surface tension (mN/m)	Crude biosurfactant (g/l)	Critical micelle dilution (times dilution)
0	58 ± 0.5	0.09 ± 0.00	1
20	40.33 ± 0.29	0.22 ± 0.01	8
40	42.46 ± 0.29	0.29 ± 0.01	8
60	39.67 ± 0.58	0.26 ± 0.01	1
80	49.33 ± 1.15	0.29 ± 0.01	1
100	45.50 ± 0.00	0.41 ± 0.01	1

Comparing to other studies that their bacterial strain could produce biosurfactant such as *Ochrobactrum anthropic* 2/3 was grown in 25% palm oil decanter cake and produced 4.52 g/l of biosurfactant (Noparat et al., 2014). *Nevskia ramose* NA3 was grown in POME and resulted in highest surface tension reduction about 25 mN/m (Chooklin et al., 2013). While *Bacillus* sp. GY 19 produced less amount of crude biosurfactant when POME was used, this might due to the toxic phenolic compound containing in POME that inhibited the utilization of substrate. Since there is the study claimed about the concentration of phenolic compound containing in POME usually between 100 – 500 ppm, which could affect substrate utilization by bacteria (Alam et al., 2006). Although, there was no chance in cell number after 5 days of production, but the activity of emulsification and oil displacement could not be detected demonstrates that no surface active agent produced.

There have been reported about removal of phenolic compound in POME that the initial concentration of phenol in POME could be up to 500 mg/l and activated carbons was used to adsorb the toxicity (Alam et al., 2006). Another study used *Thermoanaerobacterium* for hydrogen production and phenol removal from POME, the initial concentration of phenol started from 100-1000 mg/l. The result showed that at 400 mg/l of phenol the strain could remove the phenol to 65% (Mamimin et al., 2012).

Treatment of phenolic compound in POME required time and monetary which are not cost reduction for biosurfactant production. Then, others potential substrate was used as an alternative substrate for biosurfactant production.

4.2.2 Biosurfactant production from utilization of soy molasses

Soybean molasses or soy molasses ,dried-sludge from soybean oil processing, was used as a substrate for biosurfactant production. Variation the concentration of soy molasses in productive medium to 0, 20 and 50% (w/v) then, performed biosurfactant production for 5 days. The result showed that bacterial cell concentration strated from 10^9 CFU/g chitosan and remianed about 10^8 CFU/g chitosan after biosurfactant produced in all concentrations (Fig 14A). These might due to the detachment of bacterial cell from chitosan. Surface tension reduction showed when 20 and 50% soy molasses was used, which is 40.67 and 44.33 mN/m ,respectively (Fig 14B). Comparing to killed-immobilized cell that 20% soy molasses used, the immobilized cell seemed to decrease better in surface tension than 50% soy molasses used (Fig 14B). Emulsification activity and oil displacement were shown in the same way, that is emulsification caused about 83.3% and 60.6 % when 20 and 50% soy molassed was used ,respectively. Oil displacement occurred about 46.6 % and 35.8 % when 20 and 50% soy molassed was used, respectively (Table 6). These demonstrated that 20% soy molasses seemd to be an appropriate concentration for biosurfactant production by *Bacillus* sp. GY19. Together with crude biosurfactant achieved to 4.33 g/l when 20% soy molasses was used, while 50% soy molasses gave 3.33 g/l crude biosurfactant (Fig 15A). Moreover, the biosurfactant produced from 20% soy molasses could dilute up to 4 times dilution and maintain in surface tension reduction (Table 6) which is accord to the amount of crude biosurfactant produced. Thus, 20% soy molasses gave both in ability of surface active substance and amount of crude biosurfactant produced.

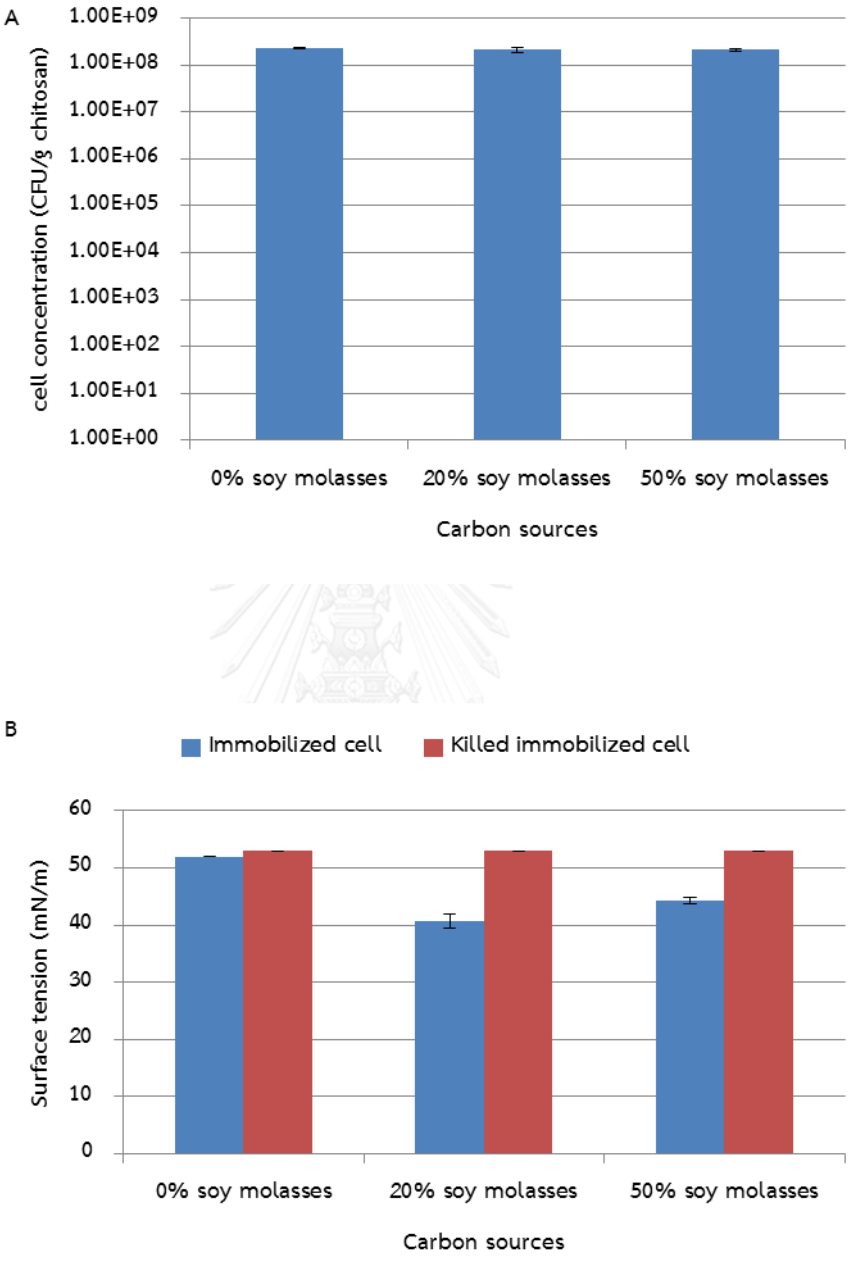

Figure 14 Effect of soy molasses used as substrate for biosurfactant production on (A) cell growth and (B) surface tension

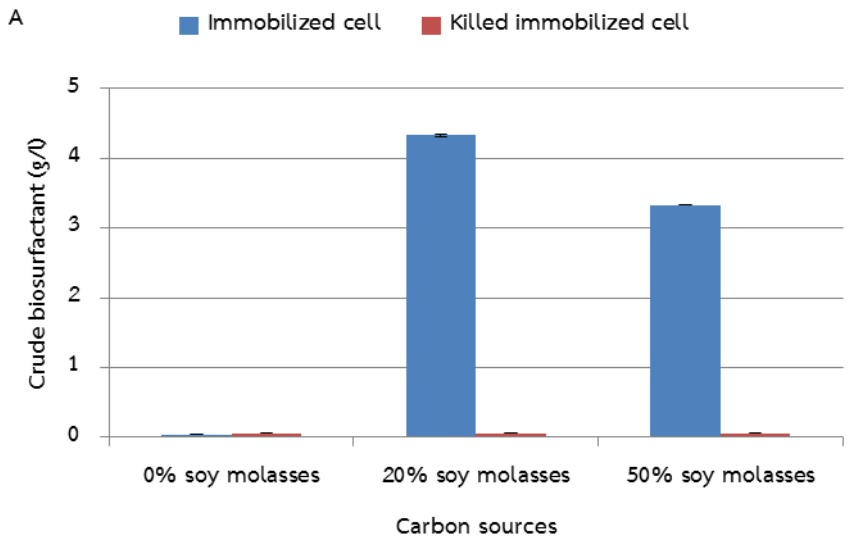

Figure 15 Effect of soy molasses used as substrate for biosurfactant production on (A) crude biosurfactant produced.

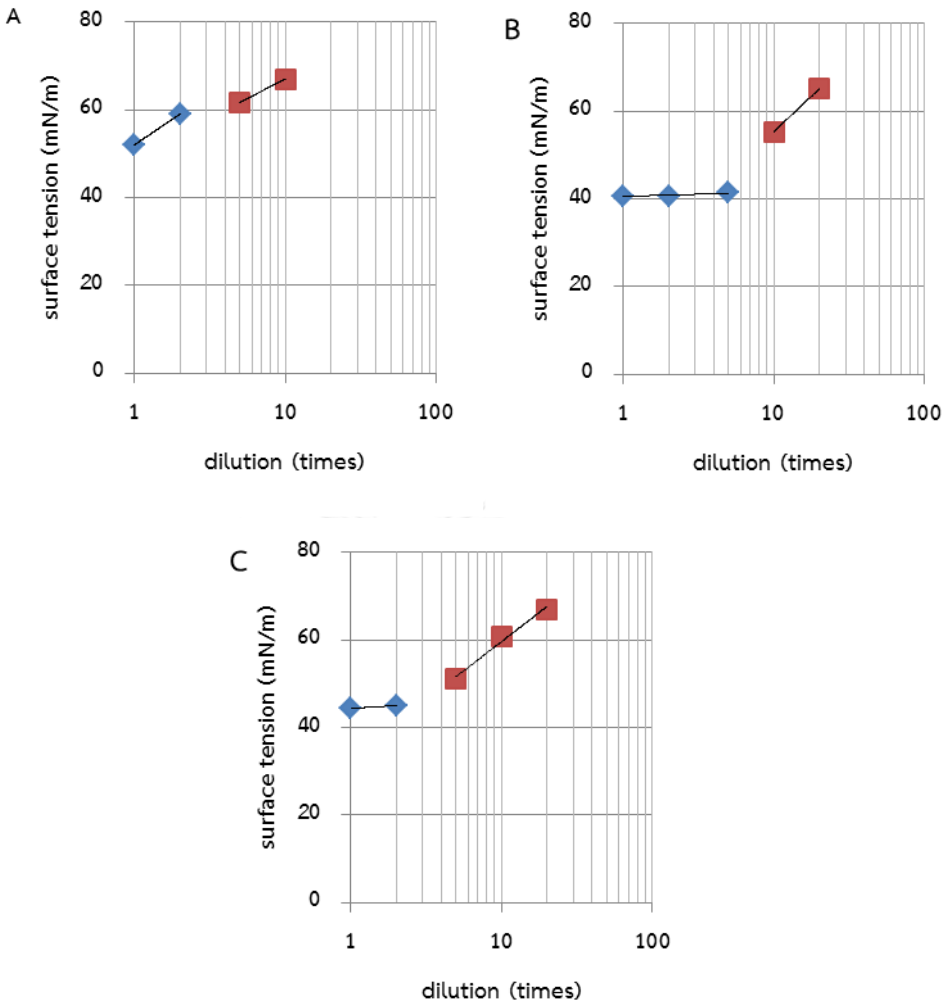

Figure 16 Critical micelle dilution of biosurfactant produced from (A) 0% soy molasses, (B) 20% soy molasses and (C) 50% soy molasses

Table 6 Biosurfactant production from utilization of each concentration of soy molasses

Soy molasses (%w/v)	Surface tension (mN/m)	Emulsification index (%)	Oil displacement (%)	Crude biosurfactant (g/l)	Critical micelle dilution (times dilution)
0	52 ± 0.00	0.00 ± 0.00	0± 0.00	0.00 ± 0.00	0
20	40.67 ± 1.15	83.33 ± 6.94	46.59 ± 6.21	4.33 ± 0.01	4
50	44.33 ± 0.57	60.61 ± 6.94	35.84 ± 6.21	3.33 ± 0.00	3

Comparing to previous research that used *Bacillus* sp. GY 19 to produce biosurfactant, when only 2% bottom glycerol was used as substrate with no inducer added they got 2.6 g/l of crude biosurfactant and when 2% bottom glycerol added with 1.25% palm oil as inducer, the amount of crude biosurfactant achieved about 4.5 g/l.

Soy molasses was interesting as the alternative substrate for biosurfactant production by *Bacillus* sp. GY19 since, high amount of crude biosurfactant produced and efficiency of produced biosurfactant also shown. Then, to get the optimal concentration and condition for biosurfactant production from soy molasses utilization, the kinetic study on biosurfactant production was performed.

4.3 Kinetic of biosurfactant production from utilization of soy molasses

Previously, 0, 20 and 50% (w/v) soy molasses in productive medium were used to find an appropriate concentration for biosurfactant production. The result showed that when 20% soy molasses was used, the highest crude biosurfactant achieved. In order to find the right balance of carbon and nitrogen source for soy molasses used as substrate for biosurfactant production. Then, 10, 20 and 30% (w/v) was selected as substrate concentration to study the kinetic production of biosurfactant produced by *Bacillus* sp. GY19

Variation the concentrations of soy molasses for kinetic study on biosurfactant production to get the most suitable concentration and condition, the result of cell

concentration found that all concentrations of substrate used did not affect much in cell decreasing. The initial cell concentration started from 10^9 CFU/g and decreased to 10^8 CFU/g in 48 hours after that the cell concentration remained constant until 240 hours of production, which indicated the bacterial cell could utilization substrate and maintain in cell concentration moreover, the biosurfactant that produced from the production process could be utilized as carbon source by bacteria itself. So the number of cell attached on chitosan did not decrease (Fig 17A). Surface tension seemed to reduce at 72 hours in every concentrations but 20% soy molasses is the most slightly decreased in surface tension followed by 30% and 10% soy molasses, respectively (Fig 17B). Surface tension reduced approximately 40 mN/m when 20% molasses was used from 120 hours until 240 hours of production. Oil displacement has found in the same concentration that 20% soy molasses caused the highest oil displacement about 42.28 % at 120 hours followed by 30% and 10% (Fig 18A). Emulsification index of 20% soy molasses also gave high that resulted about 70.5 % at 120 hours also (Fig 18B). Crude biosurfactant also achieved well when 20% soy molasses was used, which is achieved about 4.37 g/l at 120 hours (Fig 19). thus, 20% soy molasses seemed to be the most appropriate concentration for *Bacillus* sp. GY19 to produce biosurfactant. The reason when increasing the initial concentration of soy molasses to 50% found the decreasing in crude biosurfactant produced. These due to the limitation of nitrogen compound containing in substrate affected the utilization of substrate and the production of biosurfactant.

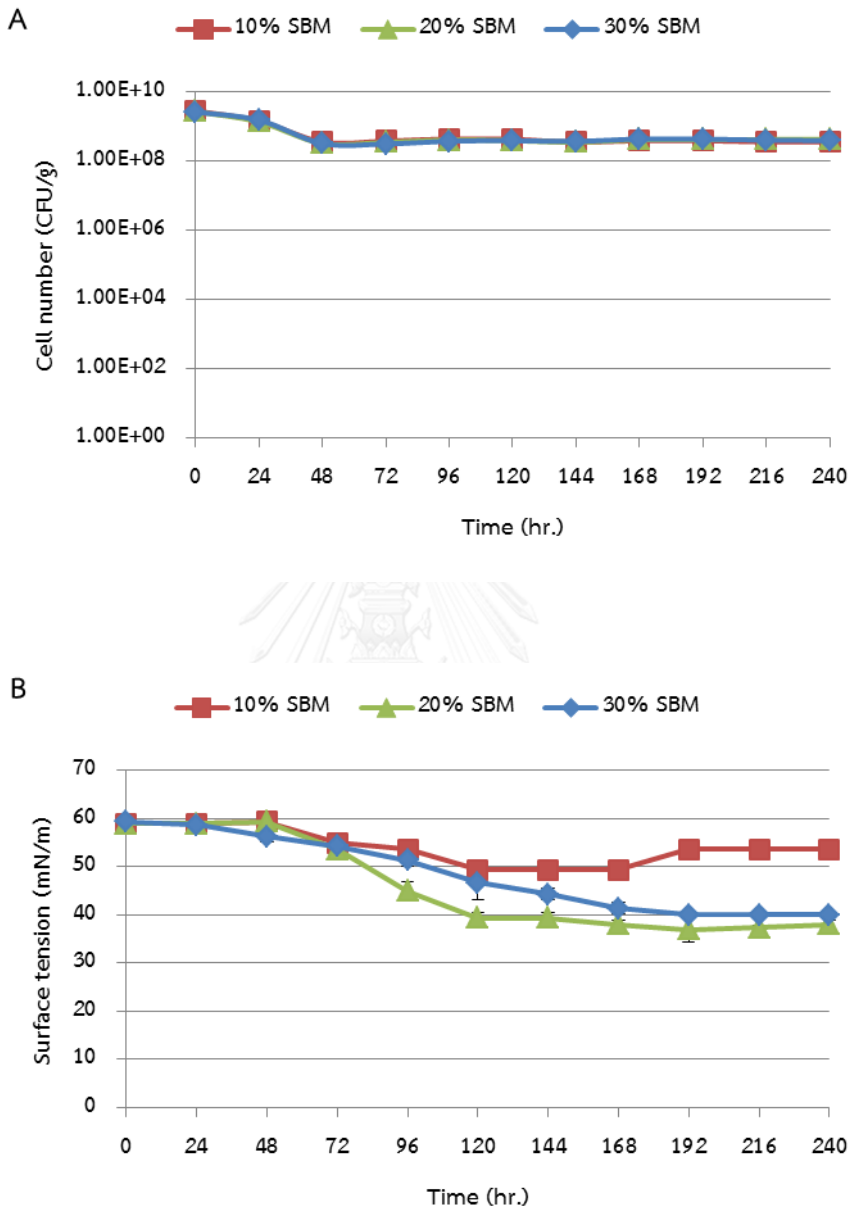

Figure 17 Kinetic study on soy molasses used as substrate for biosurfactant production on (A) cell growth and (B) surface tension

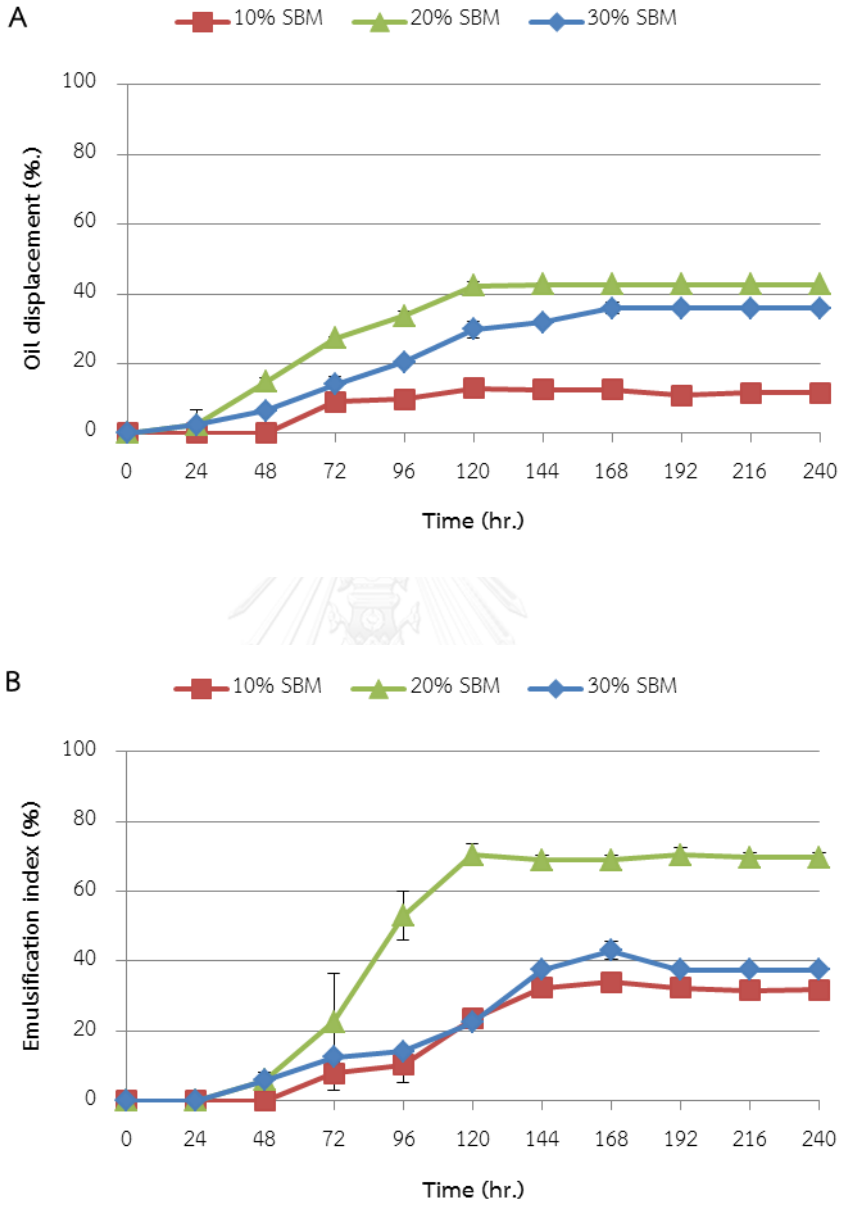

Figure 18 Kinetic study on soy molasses used as substrate for biosurfactant production on (C) oil displacement and (D) emulsification index

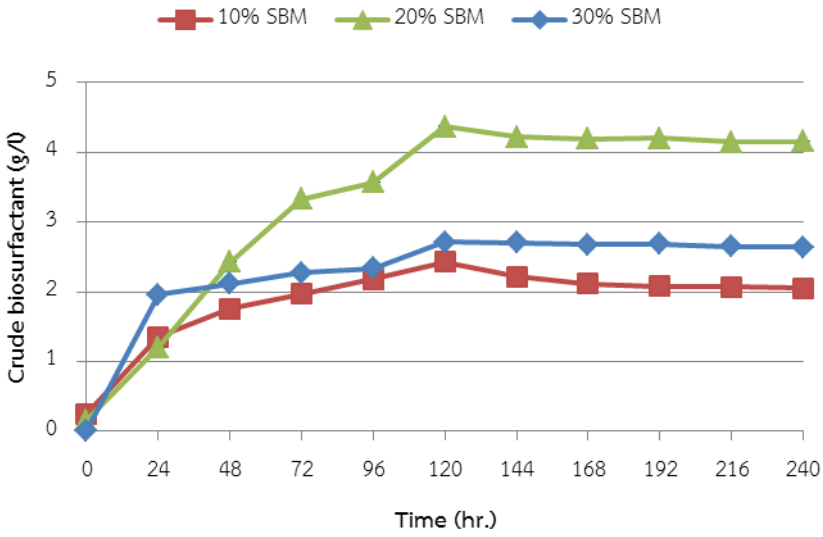

Figure 19 Kinetic study on soy molasses used as substrate for biosurfactant production on crude biosurfactant produced

When study the rate of biosurfactant produced on substrate utilization found that at 120 hours was the time that the maximum yield occurred. Comparing each concentration of substrate found that 20% soy molasses gave the highest yield about 0.061 gram biosurfactant produced per gram substrate used. Moreover, the yield of biosurfactant production could calculated from the amount of biosurfactant produced per amount of fatty acid utilization but since the determination of fatty acids found in small amount affect to too high yield from calculation. Thus, the yield of biosurfactant production on kinetic study was calculated from the amount of crude biosurfactant produced per amount of substrate used. Volumetric productivity of crude biosurfactant (Pp) and volumetric substrate utilization (Ps) also achieved well at 20% soy molasses used which are 0.036 and 1.61 g/l h, respectively (Table 7). The kinetic study on yield of crude biosurfactant per substrate utilization showed the activity of bacterial cell to produced biosurfactant. The production of biosurfactant seemed to start from 24 hr. in every concentration but 20% soy molasses could maintain the production activity until 120 hours make that the highest yield achieved (Fig 20). Even though the high volumetric productivity rate and substrate utilization

showed in 24 hours of production (Fig 21 A and B), but the ratio of biosurfactant produced per substrate used was low make that low yield occurred at this point.

Table 7 Biosurfactant production from *Bacillus* sp. GY19 utilization of each concentration of soy molasses at 120 hours of production

Substrate	Yield (g biosurfactant/g substrate used)	*Pp (g/l h)	Ps (g/l h)
10%SBM	0.024387	0.02025	0.436296
20%SBM	0.061071429	0.036417	1.612222
30%SBM	0.016683	0.022583	1.247037

*Pp is volumetric productivity rate

**Ps is volumetric substrate utilization

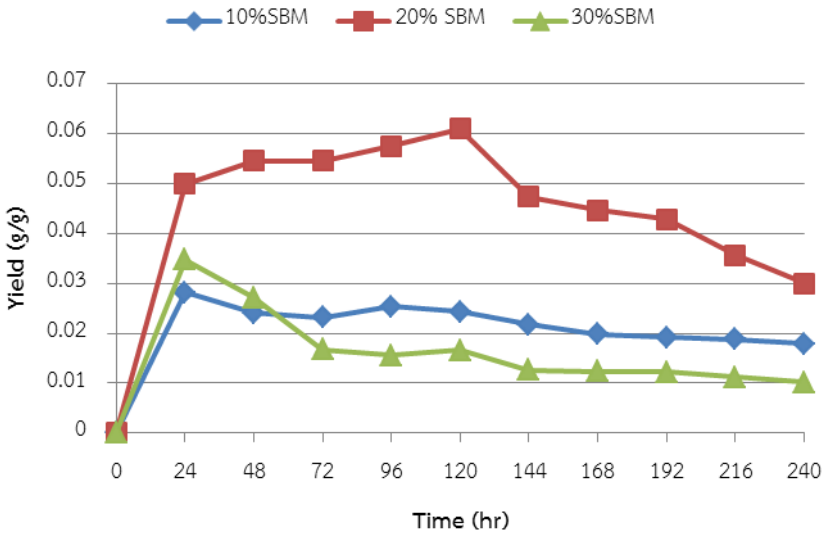

Figure 20 Kinetic study on yield of crude biosurfactant produced per substrate utilization

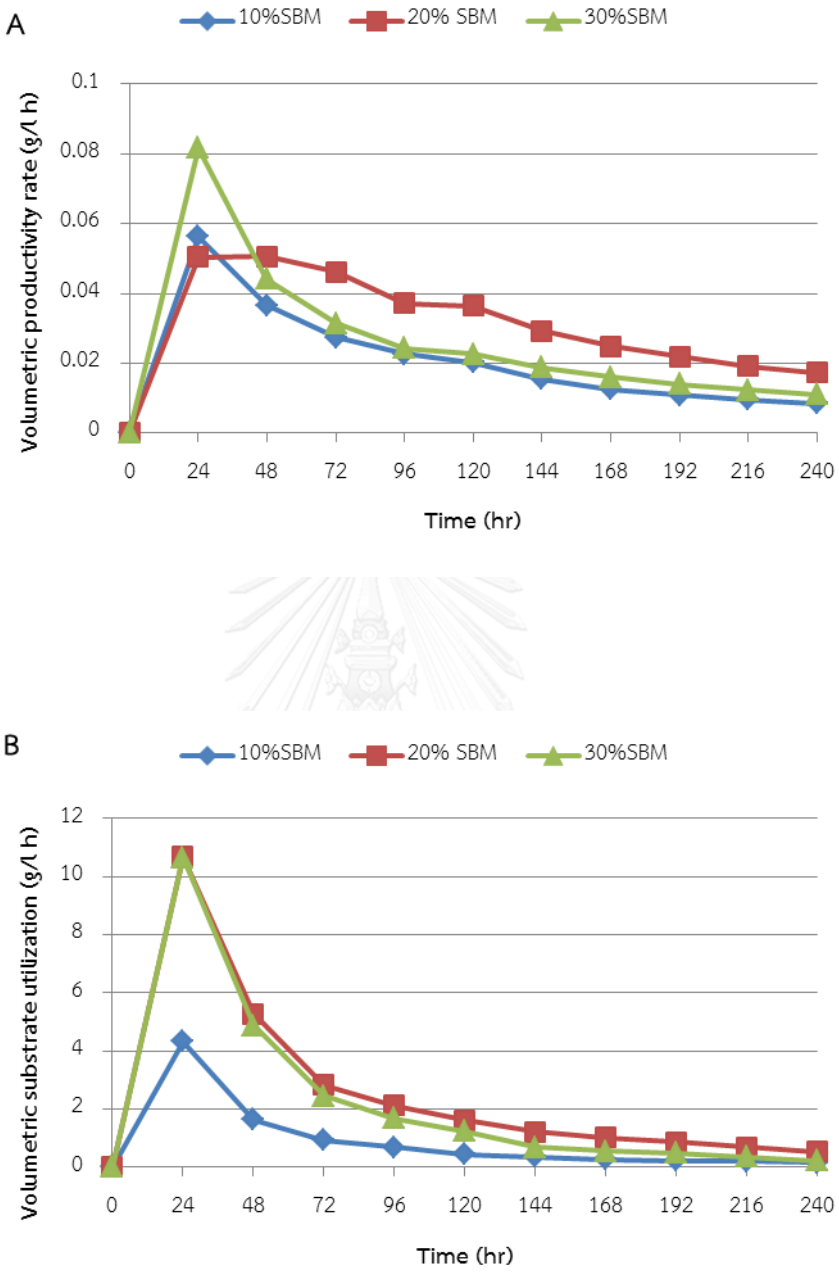

Figure 21 Kinetic study on (A) volumetric productivity rate and (B) volumetric substrate utilization

Substrate utilization was determined in this study and the main substrate of soy molasses to be used by bacteria is carbohydrate. Measuring carbohydrate concentration in the form of glucose presented as carbon source in this study. The result showed that carbohydrate concentration decreased after 24 hours of production and continues decrease until the end of production, which could indicate that carbohydrate utilization as substrate could transform to be biosurfactant or substrate for cell growth (Fig 22).

Not only main substrate utilization was studied on kinetic of biosurfactant production other source of substrate in the medium was determined also such as nitrogen source and fatty acid. Nitrogen source was measured by nitrate nitrite transformation. At 20% soy molasses, which is the best concentration for biosurfactant production found that nitrate concentration slightly decrease in 120 hours of production and nitrite concentration sharply increase after 120 hour which is mean most of nitrate in the medium was transformed to be nitrite at this point so, after 120 hour it might lack of nitrogen compound in the medium makes that maximum yield achieved at 120 hours (Fig 23).

Determination of fatty acid in the medium containing 20% soy molasses found that less amount of fatty acid presented. Most of fatty acids found are mono glycerides which is slightly decrease all the time of production (Fig 24). It can be indicated that fatty acid in the medium does not plays an important role on these biosurfactant production but if there are any fatty acid added to induce the production of biosurfactant, the crude biosurfactant might achieved higher than this study.

In conclusion, kinetic study on soy molasses used as substrate for biosurfactant production found that 20% soy molasses gave the highest yield of biosurfactant production per substrate utilization at 120 hours. So, this optimal concentration and condition was selected to produce economical biosurfactant in order to determine the performance of biosurfactant sorption in soils and study the washing potential of produced biosurfactant in crude oil contaminated soil washing.

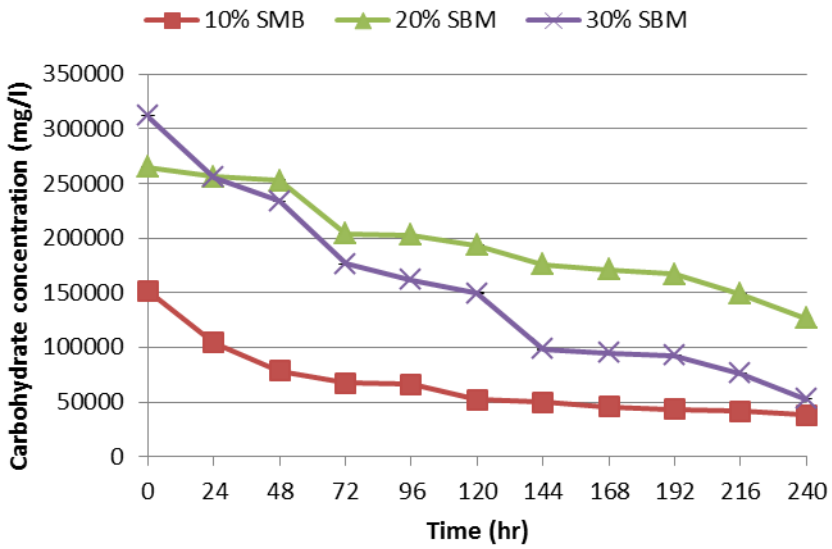

Figure 22 Carbohydrate concentration as substrate for biosurfactant production on kinetic study

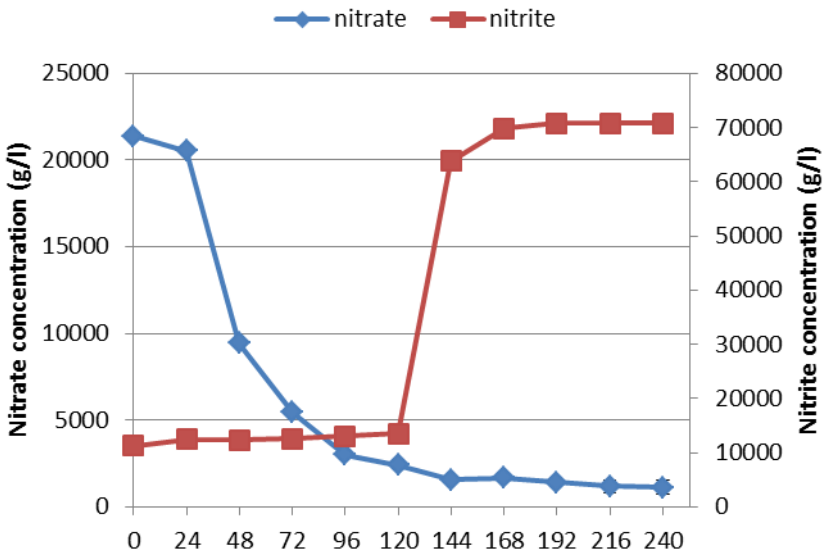

Figure 23 Nitrate reductions when 20% soy molasses was used as substrate for biosurfactant production

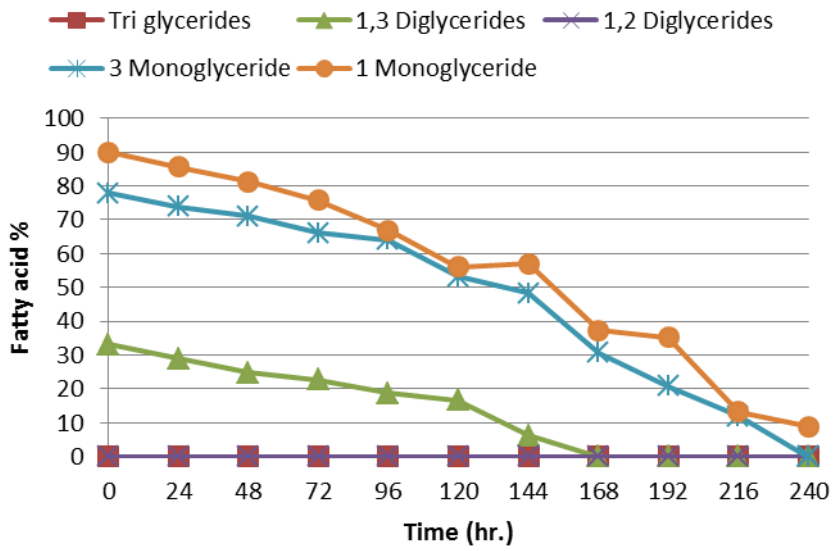

Figure 24 Fatty acids reductions when 20% soy molasses was used as substrate for biosurfactant production

4.4 Performance of produced biosurfactant sorption to soil study

Sorption efficiency of biosurfactant on soil was determined by critical micelle concentration of biosurfactant after the produced biosurfactant was left in each soil for 2 days. Biosurfactant production using optimal concentration and condition of soy molasses as substrate found that crude biosurfactant could achieve 4.37 g/l with critical micelle concentration about 1.21 g/l.

Studying the critical micelle dilution in soil water system showed the times dilution of biosurfactant after rinsed out of soil. The highest CMD showed when CP10 (3.39) followed by CP13 (1.65) and CP5 (1.17), was used respectively (Fig 25 A, B and C).CMD in soil water system indicated the times dilution of produced surfactant after rinsed through the soils. High CMD means high in ability to be diluted and still remained in surface tension reduction. When the produced biosurfactant was rinsed through soils resulted in apparent critical micelle dilution (ACMC) about 3.74, 1.29 and 2.65 g/l for soil sample CP5, CP10 and CP13, respectively (Table 8). The high ACMC resulted in the high sorption capacity of biosurfactant in soil. Then, from the result, crude biosurfactant could adsorp well on CP5 and CP13 thus, CP10 was the easiest contaminated soil to be washed In order to wash the soil with crude oil contaminated, the ACMC have to be considered since the mechanism of biosurfactant divided by the concentration of surfactant such as below the CMC resulted in the mechanism of mobilization and above the CMC means the mechanism of sulobilization. Then, ACMC is the lowest concentration that micelle can be formed in each soils. So, to wash crude oil contaminated soil by the mechanism of solubilization the concentrations of produced biosurfactant above ACMC were considered.

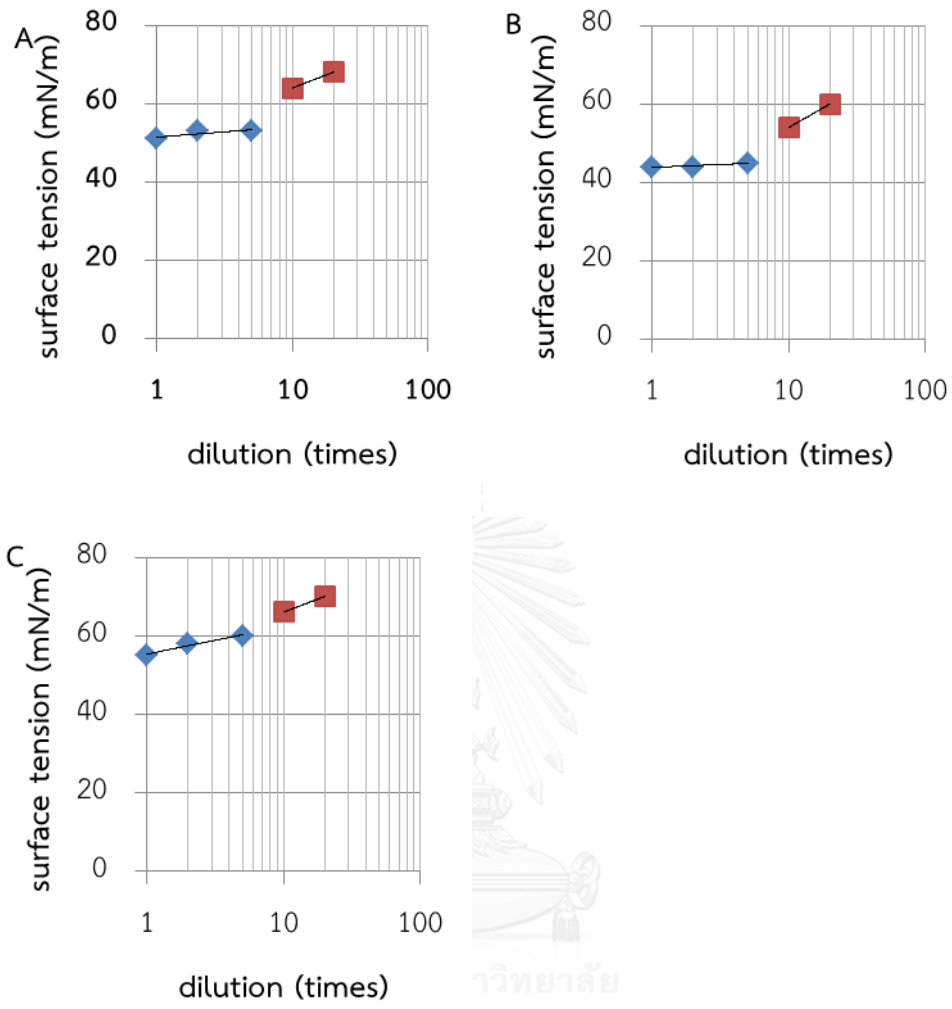

Figure 25 Critical micelle dilution at soil water system of (A) CP5, (B) CP10 and (C) CP13

Table 8 Apparent critical micelle concentration (ACMC) of biosurfactant solution in soil sorption study

Soil	Biosurfactant concentration in medium (g/l)	Biosurfactant concentration of medium at CMC (g/l)	CMD at soil-water system (dilution factor)	Apparent CMC in soil (g/l)
CP5	4.37	1.21	1.17x	3.74
CP10			3.39x	1.29
CP13			1.65x	2.65

Due to the produced biosurfactant has less ability to be diluted since, it can be diluted just only 1.17, 3.39 and 1.65 times dilution on CP5, CP10 and CP 13, respectively (Table 8). Thus, the concentration higher than ACMC for soil washing in this study was divided into (1) biosurfactant solution with 4.37 g/l concentration, (2) foamate solution of biosurfactant with 5.22 g/l concentration and (3) freeze-dried biosurfactant with 8.43 g/l concentration.

Comparing to previous research that biosurfactant was produced from bottom glycerol added with palm oil as inducer found that crude biosurfactant achieved about 10.9 g/l and it can be diluted up to 21 times dilution and still remains in surface tension reduction (Khondee et al., 2015). Then, biosurfactant produced from their study could dilute and maintain the concentration higher than ACMC.

4.5 Performance of produced biosurfactant in crude oil contaminated soil washing

Sediment samples used in this study are CP5, CP10 and CP13, which contain mainly 55% clay, 52% silt and 46% clay, respectively. The concentrations of biosurfactant higher than ACMC were used such as the concentration of biosurfactant obtained from medium (4.37 g/l), the concentration of foamate solution obtained from foam fractionation (5.22 g/l) and the concentration of freeze-dried biosurfactant obtained from lyophilization technique (8.43 g/l).

The result from crude oil contaminated soil washing showed that biosurfactant at the concentration of 4.33 and 5.22 g/l could not wash crude oil from CP5 when compare to DI water (Fig 25A). Theses due to the high ACMC needed in CP5 (Table 8), which required 3.74 g/l, so the concentration of solution and foamate might not appropriate for solubilization mechanism.

The concentration of 4.37 g/l solution, 5.22 g/l foamate and 8.43 g/l freeze-dried biosurfactant used in crude oil contaminated soil washing in CP10 found that increasing in biosurfactant concentration the less crude oil remaining was observed (Fig 25 B). So, increasing the concentration of biosurfactant by freeze-dried gave the

highest concentration of biosurfactant about 8.43 g/l. moreover, the lowest ACMC presented in CP10 indicate as the ability of surfactant to form micelle (Table 8).

The same result from CP5 also observed when CP 13 was used that the concentration of 4.33 and 5.22 g/l could not wash crude oil from CP13 when compare to DI water (Fig 27). Theses also due to the high ACMC needed in CP13 but lower than CP5 (Table 8),

The result from crude oil contaminated soil washing demonstrated that the smallest amount of crude oil remaining in soil found when CP10 was used since, CP10 has high amount in silt which is the easiest rinsing could obtain. The amount of crude oil remaining in soil CP10 found to be 33, 24.67, 11.67 mg oil/g soil from biosurfactant solution, foamate solution and freeze-dried biosurfactant used, respectively (Fig 26B). Meanwhile, other soils less ability to wash crude oil shown these might be because high content in clay presented, which has less porous than silt loam to attach with biosurfactant make that it hard to rinse. Moreover, the data of ACMC demonstrated about sorption capacity of biosurfactant in soil that CP5 and CP13 required high concentration of produced biosurfactant to form micelle on each soil (Table 8)

Comparing to the previous research that uses bottom glycerol as substrate added with palm oil as inducer and crude oil remaining in soil found to be 4.54 mg/g soil. Because of the highest crude biosurfactant achieved about 10.9 g/l and it could dilute up to 21 times dilution make that crude biosurfactant from bottom glycerol added with palm oil has higher surface active activity than crude biosurfactant produced from this research.

Another research used 3% pure glycerol as substrate for biosurfactant production by *Bacillus* sp. GY19, the concentration of crude biosurfactant at 2g/l gave the highest efficiency in crude oil removal from sandy clay loam, which could remove the oil to 90.79%.

Washing potential of commercial surfactants, which are SDS and Tween 80, found that at 0.5 g/l of SDS and Tween 80 the efficiency of crude oil contaminated washing was around 10 mg/g soil in every soil samples. While, increasing the concentration of

commercial surfactant to 1 g/l, crude oil remaining decreased to about 5 mg/g soil (Fig 26 A, B and C).

In conclusion, in order to produce biosurfactant from the utilization of soy molasses even though, it has small CMD. Increasing the concentration of biosurfactant by foam fractionation and freeze-dried biosurfactant have been done and found that the concentration of produced biosurfactant increased to 5.22 and 8.43 g/l of foamate and freeze-dried biosurfactant ,respectively. Thus, the potential of crude oil contaminated soil washing occurred.

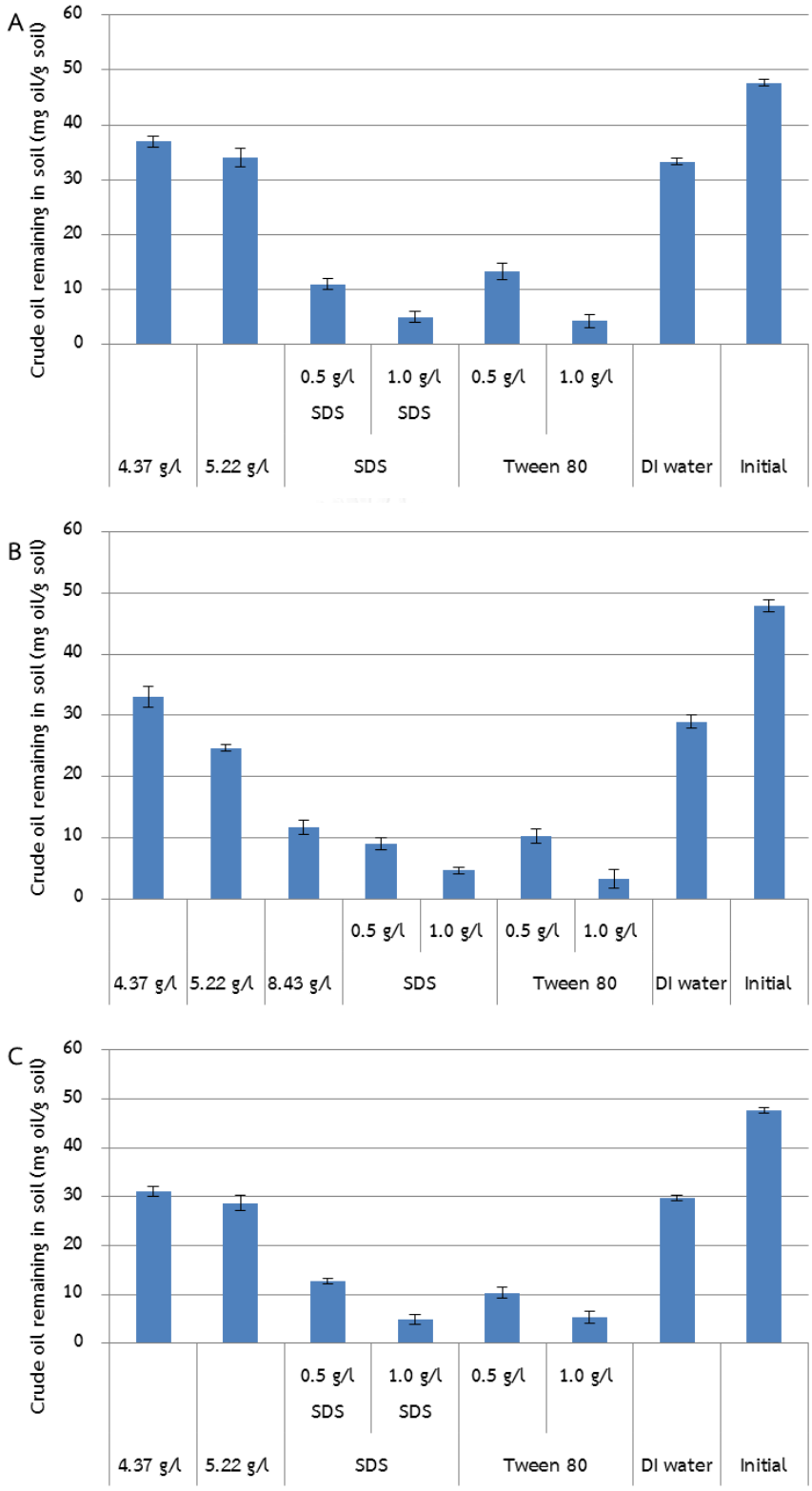

Figure 26 Amount crude oil remaining in soil after washed by each concentrations of biosurfactant in (A) CP5, (B) CP10 and (C) CP13

CHAPTER 5
CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

In order to reduce the cost of biosurfactant production, many alternative substrates have been reviewed such as commercial vegetable oils and vegetable oil mill effluents. The results from 2% vegetable oil utilized as substrate for biosurfactant production found that corn oil, olive oil, palm oil and soybean oil achieved well in crude biosurfactant produced. However corn oil and olive oil is not main agriculture product in Thailand then palm oil and soybean oil mill effluents were selected as alternative substrate for biosurfactant production. Utilization of palm oil mill effluent resulted in small amount of crude biosurfactant produced. Moreover, there was no surface active activity shown. These might due to the toxicity of phenolic compound in palm oil mill effluent that affects the production of biosurfactant. Another alternative substrate considered is soy molasses, waste from soybean oil processing. The result from utilization of 20% soy molasses found to be an appropriate concentration that the crude biosurfactant can be produced to 4.33 g/l. In addition, to find the optimal condition and concentration of biosurfactant produced from soy molasses. 10, 20 and 30% of soy molasses were used and resulted in the highest crude biosurfactant achieved when 20% soy molasses was used. The maximum production activity rate of biosurfactant found to be 0.0365g/l/h of 20% soy molasses at 120 hours.

Sorption efficiency of soils were determined in this study and found that the produced biosurfactant from soy molasses could not be diluted to maintain the concentration higher ACMC. Then, increasing the concentration of biosurfactant is required. Foam fractionation technique and freezed-dried lyophilization is needed. The result from crude oil contaminated soil washing found that the highest efficiency when freeze-dried biosurfactant was used, whuch the concentration of 8.43 g/l that it can get rid of crude oil in soil about 36.33 mg crude oil/g soil.

5.2 Recommendation for future work

Based on this study, some recommendations for future study are proposed as follows; first of all, in order to increase the concentration of crude biosurfactant produced from utilization of soy molasses some inducer might be added because soy molasses that used in this study contains small amount of fatty acid, which could facilitate the production of biosurfactant.

Even though, the crude biosurfactant achieved from the optimal concentration and condition of soy molasses is low and low CMD showed. Thus, this economical biosurfactant could be used as mixed with commercial biosurfactant as cheaper formulation cost. There has been reported that the mixing between anionic biosurfactant with some electrolyte (Na^{+}, $Ca2^{+}$, Mg^{2+}) could increase in solubilization of NAPL into micelle and also reduce critical micelle concentration of biosurfactant also (Helvacı et al., 2004). Addition of Hydrophilic-Lipophilic Linker such as fatty acids, alcohols, and amines could also helping in NAPL solubilization (Acosta et al., 2007).

In the application of crude oil contaminated soil washing, the concentration of produced biosurfactant at lower ACMC should be considered. Since, there must affect better on mobilization mechanism than solubilization mechanism. Due to the low efficiency of biosurfactant solution and foamate solution these might due to the deposition of oil solubilized micelle back to soil particle (Khondee et al., 2015)

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APPENDIX

APPENDIX A STANDARD CURVES

A.1 Standard curve of Arabian light crude oil

Standard curve of Arabian light crude oil was divided into two ranges: high concentration range and low concentration range. The calibration curve was plotted between ratio of area (lubricant oil/stearyl alcohol) and ratio of mass (lubricant oil/stearyl alcohol). Total amount of stearyl alcohol used in extraction was 25 mg. The calculation to determine amount of Arabian light crude oil in sample is follow:

Amount of crude oil (mg) = (Peak area o sample/Peak area of stearyl)
x (Mass of stearyl//Slope)

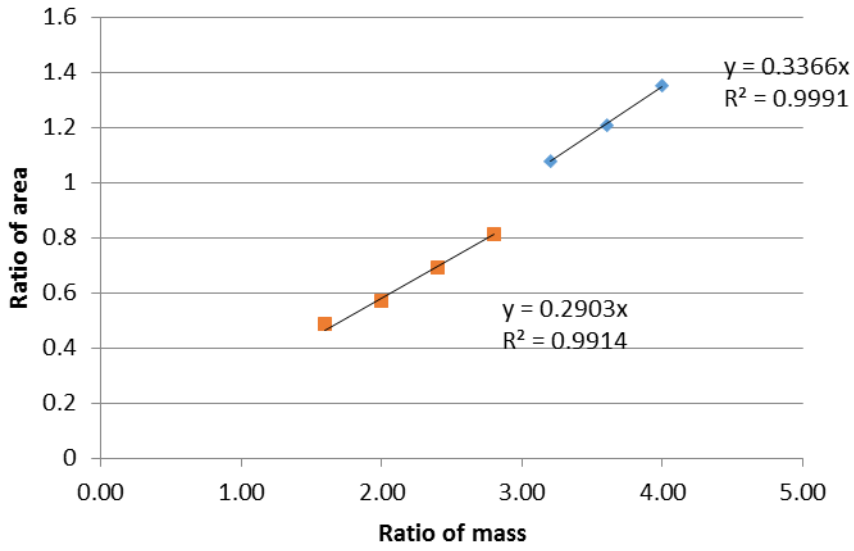

Figure A 1 Standard curve of Arabian light crude oil from TLC-FID. Each data point was averaged from triple spots on chomarods

A.2 Standard curve of carbohydrate

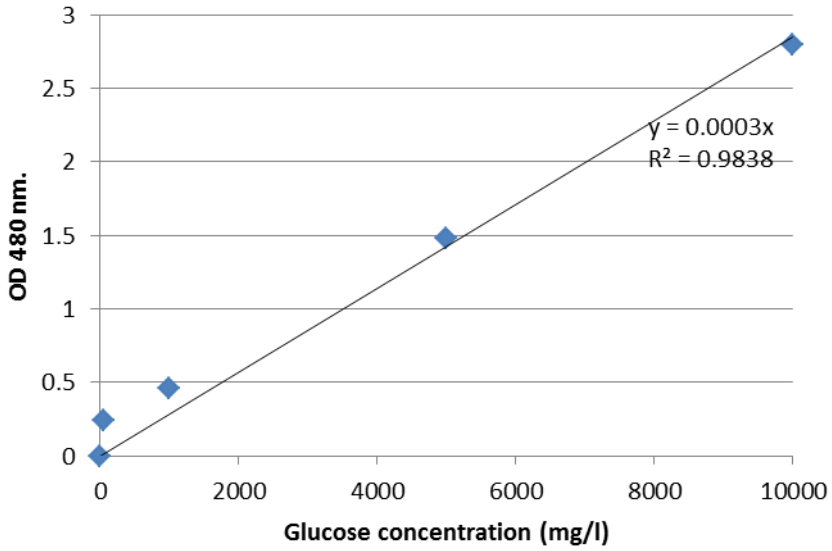

Figure A 2 Standard curve of carbohydrate from spectrophotometer. Each data point was averaged from triple measurement.

A.3 Standard curve of nitrate

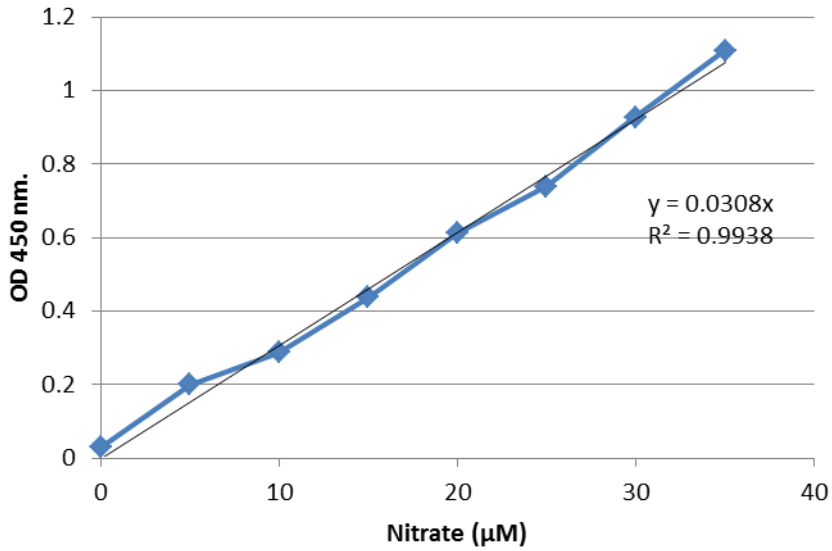

Figure A 3 Standard curve of nitrate from spectrophotometer. Each data point was averaged from triple measurement

A.4 Standard curve of nitrite

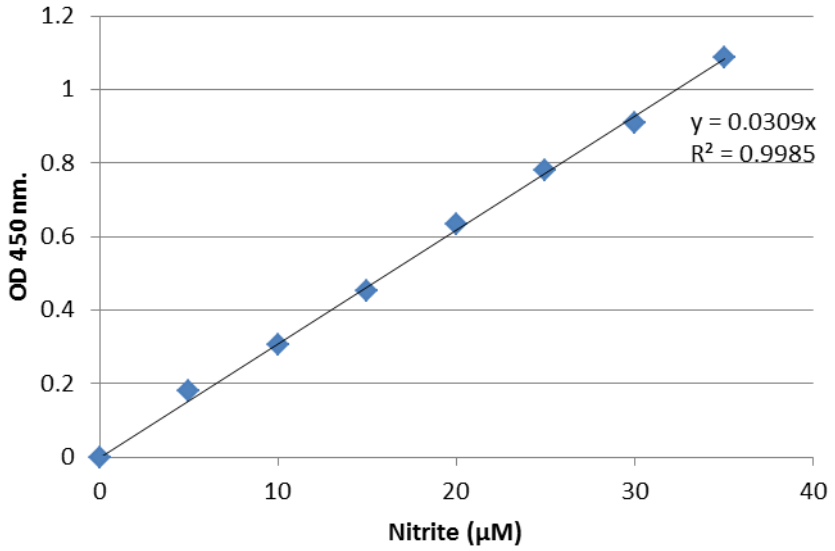

Figure A 4 Standard curve of nitrite from spectrophotometer. Each data point was averaged from triple measurement

APPENDIX B SUPPLYMENTARY DATA OF CHAPTER 4

Table B 1 Surface tension of productive medium from each 2% substrate utilization

media used	Immobilized cell	SD
Bottom glycerol	35.33333	0.57735
Palm oil	35.5	0
Olive oil	43	0
Corn oil	42.33333	1.527525
Sunflower oil	40.33333	2.081666
Soybean oil	42.33333	1.527525
Rice bran oil	43.5	0.866025

Table B 2 Emulsification activity of produced biosurfactant from each 2% substrate utilization

media used	Immobilized cell	SD
Bottom glycerol	55.96491	5.271923
Palm oil	6.212121	2.503441
olive oil	9.090909	0
Corn oil	9.090909	0
Sunflower oil	0	0
Soybean oil	5	0
Rice bran oil	7.727273	2.361887

Table B 3 Oil displacement of produced biosurfactant from each 2% substrate utilization

media used	Immobilized cell	SD
Bottom glycerol	46.59498	12.41613
Palm oil	82.43728	6.208067
Olive oil	39.42652	6.208067
Corn oil	68.10036	6.208067
Sunflower oil	39.42652	16.425
Soybean oil	46.59498	12.41613
Rice bran oil	46.59498	12.41613

Table B 4 Crude biosurfactant produced from 2% of each substrate utilization

media used	Immobilized cell	SD
Bottom glycerol	2.44	0.054148
Palm oil	3.78	0.115
olive oil	4.606667	0.062501
Corn oil	5.086667	0.150736
Sunflower oil	2.853333	0.063948
Soybean oil	3.013333	0.125827
Rice bran oil	2.14	0.048125

Table B 5 Critical micelle concentration of crude biosurfactant produced from 2% of each substrate utilization

substrate	aln1	b1	bin2	b2	delt ab	delta ln	Ln(x)	CMD
palm	35.5	0	2.8259	34.349	-34.349	-32.6741	1.051261	1
corn	0.2404	42.333	8.0555	29.282	13.051	7.8151	1.669972	5
olive	0.2404	43	0.3886	46.041	-3.041	0.1482	-20.5196	0
soybean	0	0	5.7888	44.477	-44.477	5.7888	-7.68328	0
sunflower	0	0	5.4805	46.25	-46.25	5.4805	-8.43901	0
rice bran	0	0	4.2682	53.312	-53.312	4.2682	-12.4905	0
bottom gl	35.333	0	5.1319	20.028	-20.028	-30.2011	0.663155	2

Table B 6 Surface tension of productive medium from each concentration of POME

media used	Immobilized cell	SD
0% POME	45	0.5
20% POME	40.33333	0.288675
40% POME	42.66667	0.288675
60% POME	39.66667	0.57735
80% POME	49.33333	1.154701
100% POME	45.5	0

Table B 7 Crude biosurfactant produced from each concentration of POME

media used	Immobilized cell	std
0% POME	0.08666	0.000577
20% POME	0.22	0.005196
40% POME	0.286666	0.01097
60% POME	0.26	0.006083
80% POME	0.29334	0.005774
100% POME	0.41334	0.006429

Table B 8 Cell number attached on chitosan after 5 days of production from each concentration of POME

	day 0	day 5			avr	std
0% POME	1.70E+09	2.40E+08	2.30E+08	7.30E+08	4.00E+08	2.86E+08
20% POME		3.40E+09	5.90E+09	4.40E+09	4.57E+09	1.26E+09
40% POME		9.40E+08	1.30E+08	2.30E+08	4.33E+08	4.42E+08
60% POME		8.10E+08	7.50E+08	5.50E+08	7.03E+08	1.36E+08
80% POME		9.80E+08	6.50E+08	6.00E+08	7.43E+08	2.06E+08
100% POM		3.40E+08	6.70E+08	2.70E+08	4.27E+08	2.14E+08

Table B 9 Critical micelle concentration of crude biosurfactant produced from each concentration of POME

	aln1	b1	bin2	b2	delt ab	delta ln	Ln(x)	CMD
0%POME	5.4838	44.347	4.566	52.047	-7.7	-0.9178	8.389627	1
20%POME	1.6338	39.968	11.952	18.323	21.645	10.3182	2.09775	8
40%POME	2.5741	39.747	8.2444	27.775	11.972	5.6703	2.111352	8
60%POME	12.354	41.518	3.3462	54.776	-13.258	-9.0078	1.471836	1
80%POME	3.4699	49.67	3.9966	53.196	-3.526	0.5267	-6.69451	1
100%POM	1.741833	31.06567	-8.83907	33.06933	-2.00367	-10.5809	0.189366	1

Table B 10 Surface tension of productive medium from each concentration of soy molasses

media used	average	SD
0% soy molasses	52	0
20% soy molasses	40.66667	1.154701
50% soy molasses	44.33333	0.57735

Table B 11 Emulsification activity of productive medium from each concentration of soy molasses

media used	average	SD
0% SL	0	0
20% SL	83.33333	6.943297
50% SL	60.60606	6.943297

Table B 12 Oil displacement of productive medium from each concentration of soy molasses

media used	average	SD
0% SL	0	0
20% SL	46.59498	6.208067
50% SL	35.84229	6.208067

Table B 13 Crude biosurfactant produced from each concentration of soy molasses

media used	average	SD
0% SL	0.04	0.001
20% SL	4.33	0.012767
50% SL	3.33	0.002

Table B 14 Cell number attached on chitosan after 5 days of production from each concentration of soy molasses

media used	day 0	day 5			average	SD
0% soy molasses	2.3E+09	2.4E+08	2.3E+08	2.3E+08	2.33E+08	5773503
20% soy molasses		2E+08	2E+08	2.3E+08	2.1E+08	17320508
50% soy molasses		2E+08	2E+08	2.3E+08	2.1E+08	17320508

Table B 15 Critical micelle concentration of crude biosurfactant produced from each concentration of soy molasses

	aln1	b1	bin2	b2	delt ab	delta ln	Ln(x)	CMD
0%soy mo	10.099	52	7.6944	49.283	2.717	-2.4046	-1.12992	0
20%soy m	0.4306	40.558	13.946	23.221	17.337	13.5154	1.282759	4
50% soy m	0.9618	44.333	11.542	32.98	11.353	10.5802	1.073042	3

Table B 16 Crude oil contaminated soil washing potential in CP5

CP5		mg oil/ g soil			average	SD
4.37 g/l solution		37	38	36	37	1
5.22 g/l foamate		32	35	35	34	1.732051
SDS	0.5 g/l SDS	11	12	10	11	1
	1.0 g/l SDS	4	5	6	5	1
Tween 80	0.5 g/l	12	15	13	13.33333	1.527525
	1.0 g/l	5	5	3	4.333333	1.154701
DI		33	34	33	33.33333	0.57735
Initial		48	48	47	47.66667	0.57735

Table B 17 Crude oil contaminated soil washing potential **in CP10**

CP10		mg oil/ g soil			average	SD
4.37 g/l solution		32	32	35	33	1.732051
5.22 g/l foamate		25	25	24	24.66667	0.57735
8.43 g/l freeze-dried		11	13	11	11.66667	1.154701
SDS	0.5 g/l	10	9	8	9	1
	1.0 g/l	5	5	4	4.666667	0.57735
Tween 80	0.5 g/l	11	9	11	10.33333	1.154701
	1.0 g/l	3	5	2	3.333333	1.527525
DI		29	28	30	29	1
Initial		49	48	47	48	1

Table B 18 Crude oil contaminated soil washing potential **in CP13**

CP13		**mg oil/ g soil**			**average**	**SD**
4.37 g/l solution		32	31	30	31	1
5.22 g/l foamate		29	30	27	28.66667	1.527525
SDS	0.5 g/l SDS	13	12	13	12.66667	0.57735
	1.0 g/l SDS	5	4	6	5	1
Tween 80	0.5 g/l	11	9	11	10.33333	1.154701
	1.0 g/l	6	6	4	5.333333	1.154701
DI		29	30	30	29.66667	0.57735
Initial		48	48	47	47.66667	0.57735

VITA

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