Characterization of biosurfactant from *Bacillus* sp. GY19 and formulation of dispersant for petroleum remediation in seawater

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Environmental Management
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ลักษณะสมบัติของสารลดแรงตึงผิวทางชีวภาพจาก *Bacillus* sp. GY19 และการพัฒนาสูตรสารกระจายคราบน้ำมันเพื่อบำบัดปิโตรเลียมในน้ำทะเล

นางวิชญา รงค์สยามานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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วิชญา รงค์สยามานนท์ : ลักษณะสมบัติของสารลดแรงตึงผิวทางชีวภาพจาก *Bacillus* sp. GY19และการพัฒนาสูตรสารกระจายคราบน้ำมันเพื่อบำบัดปิโตรเลียมในน้ำทะเล (Characterization of biosurfactant from *Bacillus* sp. GY19 and formulation of dispersant for petroleum remediation in seawater) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เอกวัล ลือพร้อมชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. จันทรา ทองคำเภา, Prof. Dr. David A. Sabatini, 227 หน้า.

การรั่วไหลของน้ำมันในทะเลส่งผลกระทบรุนแรงต่อสิ่งแวดล้อม งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาสูตรกระจายคราบน้ำมันที่ปราศจากตัวทำละลาย โดยอาศัยหลักการไฮโดรฟิลิคลิโพฟิลิคดีวิเอชั่น (HLD) หรือความแตกต่างของความชอบน้ำและไม่ชอบน้ำ และใช้สารลดแรงตึงผิวชีวภาพชนิดลิโพเปปไทด์ที่ผลิตจากแบคทีเรีย *Bacillus* sp. GY19 ลิโพเปปไทด์ที่นำมาใช้อยู่ในรูปแบบผง ซึ่งได้จากการทำแห้งแบบเยือกแข็งของสารลดแรงตึงผิวที่สกัดด้วยวิธีทำให้เกิดฟอง ซึ่งพบว่าสารลดแรงตึงผิวชีวภาพในรูปแบบผงมีความสามารถในการทนต่อสภาวะต่างๆ เช่น อุณหภูมิต่างๆ ความเป็นกรด-ด่าง และอิเล็คโตรไลต์ได้ นอกจากนี้ยังมีความเป็นพิษต่ำต่อไรน้ำเค็ม (LC_{50} 2,609 มิลลิกรัมต่อลิตร) และกุ้งขาว (LC_{50} 1,050 มิลลิกรัมต่อลิตร) โดยค่าคุณสมบัติความความโค้งที่พื้นผิว(Cc) ของลิโพเปปไทด์เท่ากับ 4.93 ซึ่งแสดงว่ามีความไม่ชอบน้ำสูงกว่าสารลดแรงตึงผิวสังเคราะห์ชนิดโซเดียมไดเฮกซิลซัลโฟซัคซิเนต (Cc = -0.92) ต่อมาได้พัฒนาสูตรสารกระจายคราบน้ำมันโดยใช้สมการ HLD ในการคำนวณปริมาณสัดส่วนของลิโพเปปไทด์และโซเดียมไดเฮกซิลซัลโฟซัคซิเนตให้สอดคล้องกับค่าความชอบและไม่ชอบน้ำของน้ำมันที่ปนเปื้อนโดยพิจารณาจากค่าเทียบเท่าจำนวนคาร์บอนสายตรง (ค่า EACN) และสอดคล้องต่อค่าความเค็มของแหล่งน้ำทะเล 3.4% จากการคำนวณพบว่าสัดส่วนโมลาร์ของลิโพเปปไทด์จะเพิ่มขึ้นตามน้ำมันที่มีค่าความไม่ชอบน้ำเพิ่มขึ้น โดยส่วนผสมของลิโพเปปไทด์และโซเดียมไดเฮกซิลซัลโฟซัคซิเนตสามารถเกิดไมโครอิมัลชั่นแบบที่ 3 ที่เฉพาะเจาะจงกับสารไฮโดรคาร์บอนและน้ำมันดิบได้ เช่น ของผสมที่ประกอบด้วยลิโพเปปไทด์ 0.025 โมลาร์และโซเดียมไดเฮกซิลซัลโฟซัคซิเนต 0.75 โมลาร์ ในสารละลายโซเดียมคลอไรด์ 3.4% เหมาะสมกับน้ำมันดิบของบงกชไลต์ จากการทดสอบการกระจายน้ำมันและทดสอบการละลาย พบว่าสูตรที่พัฒนาขึ้นสามารถลดแรงตึงระหว่างผิวน้ำมันและเพิ่มการละลายของน้ำมันดิบได้ดีกว่าสารกระจายคราบน้ำมันในท้องตลาดและสารลดแรงตึงผิวชีวภาพชนิดลิโพเปปไทด์เพียงอย่างเดียว และเพื่อให้การกำจัดคราบน้ำมันเป็นไปอย่างสมบูรณ์ ในการทดสอบต่อมาได้นำแบคทีเรียย่อยน้ำมันปิโตรเลียมชนิด *Gordonia* sp. JC11 มาย่อยสลายหยดน้ำมันดิบที่เกิดขึ้นหลังจากการใช้สารกระจายคราบน้ำมันฐานลิโพเปปไทด์ โดยได้ทำการทดสอบในระบบจำลองนิเวศวิทยา 3 มิติ ขนาด 40 ลิตร และ 160 ลิตร เพื่อยืนยันประสิทธิภาพสารกระจายคราบน้ำมันฐานลิโพเปปไทด์ ทั้งในระบบน้ำทะเลสังเคราะห์ และระบบน้ำทะเลธรรมชาติที่เก็บจากท่าเรือในจังหวัดชลบุรี พบว่าระบบที่ใช้สูตรกระจายคราบน้ำมันฐานลิโพเปปไทด์ร่วมกับ *Gordonia* sp. JC11 สามารถกำจัดน้ำมันดิบชนิดบงกชไลต์ได้เร็วกว่าในชุดทดลองที่มีสูตรกระจายคราบน้ำมันเพียงอย่างเดียว ซึ่งสอดคล้องกับจำนวนแบคทีเรียย่อยน้ำมันที่มีเพิ่มขึ้นอีกด้วย จึงสรุปได้ว่าควรมีการใช้สูตรของสารกระจายคราบน้ำมันฐานลิโพเปปไทด์ ตามด้วยการเติม *Gordonia* sp. JC11 สำหรับการบำบัดคราบน้ำมัน

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

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WITCHAYA RONGSAYAMANONT: Characterization of biosurfactant from *Bacillus* sp. GY19 and formulation of dispersant for petroleum remediation in seawater. ADVISOR: ASSOC. PROF. EKAWAN LUEPROMCHAI, Ph.D., CO-ADVISOR: ASST. PROF. CHANTRA TONGCUMPOU, Ph.D., PROF. DR. DAVID A. SABATINI, Ph.D., 227 pp.

Oil spills in seawater have resulted in significant contamination to the environment. This research aimed to formulate a solvent-free dispersant for crude oil spills based on the hydrophilic-lipophilic deviation (HLD) concept and using lipopeptides from *Bacillus* sp. GY19. The lipopeptides were recovered and concentrated from cell-free broth by foam fractionation and freeze-drying, respectively. They had good surface activity under varying temperatures, pH and NaCl levels. Moreover, the lipopeptides had low toxicity to copepods (LC_{50} 2,609 mg/L) and whiteleg shrimp (LC_{50} 1,050 mg/L). The characteristic curvature (Cc) of the lipopeptides showed that they were more hydrophobic (Cc 4.93) than sodium dihexyl sulfosuccinate (SDHS, Cc -0.92). The HLD equation was used to calculate the lipopeptide and the SDHS fractions in the dispersant formulations according to the equivalent alkane carbon number (EACN) of hydrocarbons and seawater salinity. The molar fraction of lipopeptides increased with increasing EACN. The lipopeptide-SDHS mixtures formed microemulsion Type III with specific hydrocarbons and crude oils, for example, a mixture of 0.025 M lipopeptide biosurfactant and 0.075 M sodium dihexyl sulfosuccinate in 3.4 % of NaCl was suitable for Bongkot light crude oil. Oil displacement and baffled flask tests showed that the formulations reduced the interfacial tension and solubilized crude oil in the water column at higher efficiency than commercial dispersants or lipopeptides alone. To complete the oil spill removal, *Gordonia* sp. JC11, a petroleum degrading-bacteria was applied to degrade the crude oil droplets that formed after applying the lipopeptide based dispersant. The 40 L and 160 L 3D-box mesocosm experiments confirmed the efficiency of lipopeptide based for remediation process in both synthetic seawater and natural seawater collecting from a port of Chonburi Province. In the mesocosm with both lipopeptide based dispersant and *Gordonia* sp. JC11, the concentration of crude oil decreased faster than that in the mesocosm with dispersant alone. The results were corresponded with the increasing number of oil degrading bacteria in seawater. In conclusion, the lipopeptide based dispersant should be applied followed by *Gordonia* sp. JC11 for oil spill remediation.

Field of Study: Environmental Management
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Chapter I
INTRODUCTION

1.1 Statement of problem

Crude oil spills in seawater significantly contaminate the ocean and coastal environments. The dispersion of oil spills by natural processes, chemical dispersants and mechanical dispersion can enhance the water accommodated fraction (WAF) of oil and reduce the amount of oil reaching coastal areas (Zeinstra-Helfrich et al., 2015). The application of dispersants is the fastest way to protect vulnerable coasts because they offer the highest maximum oil encounter rate (Prendergast and Gschwend, 2014). In addition, dispersants can enhance hydrocarbon removal from seawater, as seen by increased crude oil biodegradation (Zahed et al., 2011) and pyrene photodegradation (Gong et al., 2015) . Commercial oil dispersants are usually a mixture of 2-3 surfactants and organic solvents. For example, COREXIT dispersants contain 60- 100% hydrocarbons, 1- 5% propylene glycols and 10- 30% organic sulfonic acid salts (NalcoEnvironmentalSolutions, 2014), while Slickgone dispersants contain 60- 70% kerosene and 1-10% sodium dioctylsulfosuccinate (Savron, 2014). The application of commercial dispersants containing both surfactants and solvent can be harmful to marine organisms as well as to microbial communities (Kleindienst et al., 2015) . There is an urgent need to find strategies to formulate environmentally benign oil dispersants, and biosurfactants are considered a candidate for such dispersant formulations (Nyankson et al., 2015).

Biosurfactants from various microorganisms are amphiphilic molecules that have the ability to remove hydrophobic organic compounds (Trellu et al., 2016). They are interesting candidates for petroleum bioremediation because of their ability to enhance hydrocarbon solubility, mobility and biodegradation, and they have low

toxicity and are biodegradable (Mnif and Ghribi, 2015). The formulation of biosurfactant-based dispersants has been reported by Song et al., 2013) who used a uniform design (UD) to optimize the concentration of each ingredient. The best formulation for heavy crude oil contained 9.45% rhamnolipid, 9.75% sophorolipid, 27.25% polysorbate-80, 3.51% sorbeth-40 tetraoleate and 50% ethylene glycol butyl ether. Due to the complexity of this formulation, this study aimed to formulate simple biosurfactant-based dispersants without using organic solvents. In addition, the formulation should correspond to the composition of oil because the type of oil plays an important role in the success of dispersant application (Zeinstra-Helfrich, et al., 2015; Prendergast and Gschwend, 2014).

This study focused on lipopeptide biosurfactants, which have been used to disperse petroleum hydrocarbons. For example, purified surfactin from *Bacillus subtilis* 41651 A1 has a similar dispersant to oil ratio (DOR) as COREXIT when applied to hexane in saline water (Marti et al., 2014) , and lipopeptides in foamate from *Bacillus* sp. GY19 showed 100% oil displacement efficiency with diesel oil and could also disperse Arabian light oil (76-84%) and heavy oil (65-67%) (Khondee et al., 2015). To increase the surface activity of lipopeptides, (Youssef et al., 2007) mixed lipopeptides with a synthetic surfactant to provide the hydrophobic/hydrophilic conditions necessary for lowering the interfacial tension (IFT) against hydrocarbons. In this study, the solvent-free dispersants were formulated by mixing lipopeptides from *Bacillus* sp. GY19 with sodium dihexyl sulfosuccinate (SDHS). Concentrated *Bacillus* sp. GY19 lipopeptides were prepared by freeze-drying the foamate from Khondee et al. (2015), while SDHS was selected due to its low toxicity to aquatic organisms (Franzetti et al., 2006).

The equivalent alkane carbon number (EACN) has been used to characterize the hydrophobicity of alkane-type hydrocarbons and to represent the behavior of complex hydrocarbon mixtures, such as crude oils, which have an EACN in the range of 6-12 (Wan et al., 2014). The hydrophilic–lipophilic deviation (HLD) concept and the

EACN have been applied to design surfactant formulations for various purposes, such as flow assurances, during petroleum production processes (Salager and Forgiarini, 2012) and cold temperature detergency of vegetable oils and fats (Do et al., 2015). However, to the best of our knowledge, this is the first time the HLD concept has been introduced to determine a suitable dispersant formulation. The HLD is an empirical equation based on microemulsion formulation, and it includes parameters that represent the oil polarity, surfactant hydrophobicity, temperature and co-surfactant (alcohol). The general HLD equation for mixed anionic surfactants at room temperature without alcohol is simplified as Eq.1 (Acosta et al., 2008);

$$HLD = \ln(S) - K \times Nc,o + X1(Cc1) + X2(Cc2) \quad \text{Eq. 1}$$

where S is the salinity in the aqueous phase (g/100 mL), K is a constant of the surfactant and Nc,o is the EACN; X_1 and X_2 are the molar fraction of each surfactant and Cc1 and Cc2 are the characteristic curvature (Cc) values of each surfactant.

At HLD = 0, the interaction of the surfactant and water is exactly equal to the interaction between the surfactant and oil, which then exhibits the three-phase behavior of Winsor Type III microemulsions and the lowest IFT (Nguyen and Sabatini, 2011). Because a major role of a dispersant is to enhance natural dispersion by reducing the IFT as well as forming micellar droplets for oil solubilization, a formulation that provides the lowest possible IFT is desirable. Consequently, a lipopeptide based dispersant could be formulated by optimizing the molar ratio of each surfactant to correspond with the EACN of each oil type and the salinity of seawater to achieve HLD=0. The advantages of this approach are simple and quantifiable.

A common feature of crude oil is low water solubility, which poses special problems for those microorganisms capable of utilizing such water-immiscible substrates as source of carbon and energy (Chandran and Das, 2012). Besides increasing

surface area of hydrophobic water-insoluble substrates, dispersant can increase the bioavailability of hydrophobic compounds and lead to increase oil degradation by indigenous and effectiveness petroleum degrading bacteria. Recently, Laorrattanasak et al. (2016) reported that biosurfactant from *Gordonia westfalica* GY40 promoted that ability of *Gordonia* sp. JC11, a petroleum degrading-bacterium isolated from seawater In Thailand on degrading fuel oil in seawater. Biosurfactant from *Gordonia westfalica* GY40 is considered an effective dispersant, however its production yield was low. Therefore, this study, investigated the efficiency of lipopeptide based dispersant along with *Gordonia* sp. JC11 for enhancing petroleum hydrocarbon removal in both synthetic seawater and natural seawater collected from the coastal in Thailand. The experiments were carried out in batch mode using 40 L and 160 L mesocosm tanks for small and medium scale experiments, respectively.

In conclusion, this work was divided into 3 phases as followed;

1. characterization of lipopeptide biosurfactant from *Bacillus* sp. GY19 on their surface properties and physiochemical characteristics

2. formulating of lipopeptide based dispersant by using the HLD concept

3. investigation of the feasibility of lipopeptide based dispersant and oil-degrading bacteria for oil-spill remediation process.

The acquired knowledges from this research were the property of biosurfactant from *Bacillus* sp. GY19 and basic dispersant formulation for remediation process. The applications of the HLD concept and lipopeptides were expected to be a model for formulating solvent-free biosurfactant-based dispersants to clean up crude oil spills. Moreover, the outcome of this research was a formulating principle for other biosurfactants, which could be applied for various organic pollutants. Finally, it would confirm the efficiency of biosurfactant and its dispersant formulation for remediation

process. The process of using biosurfactant will reduce the environmental impacts and lead to sustainable oil spill remediation process in the future.

1.2 Research hypotheses

1. Biosurfactant produced by *Bacillus* sp. GY19 had better surface activity than synthetic surfactant due to its complex structure and had lower toxicity because it was biologically produced from natural based substrates.

2. HLD concept could be used to formulate the lipopeptide based dispersant and would effectively reduce interfacial tension and increase the dispersed efficiency of petroleum hydrocarbons, according to the balancing of hydrophilic and lipophilic of mixtures.

3. Lipopeptide based dispersant could enhance the biodegradability of petroleum-degrading bacteria by increasing petroleum bioavailability.

1.3 Research objectives

The goals of this research were to characterize the lipopeptide biosurfactant produced from *Bacillus* sp. GY19 and to formulate lipopeptide based dispersant for oil spill remediation. To achieve these goals, several objectives are established as follows.

1. To characterize physiochemical, surface properties and toxicity of lipopeptides from *Bacillus* sp. GY19.

2. To formulate a lipopeptide based dispersant using HLD concept to achieve the low interfacial tension and high dispersant efficiency.

3. To study the feasibility of lipopeptide based dispersant in enhancing petroleum oil dispersion and biodegradation in seawater.

1.4 Scope of the study

1. Lipopeptide biosurfactant produced from *Bacillus* sp. GY19 following Khondee et al. (2015) was used.
2. Low toxic and biodegradable synthetic surfactant was selected as minor ingredient in the dispersant formulation.
3. Hexane, decane, and dodecane were chosen as the model of petroleum hydrocarbons because they represent a wide range of petroleum crude oils.
4. Two crude oils including Bongkot light crude oil and Arab light/Arab extra light blend were used to study the efficiency of biosurfactant based dispersant.
5. Whiteleg shrimp and copepods were used as model marine organisms to study the toxicity of lipopeptide based dispersant.
6. The efficiency of biosurfactant based dispersant was tested with synthetic and natural seawater in petri dish, modified baffle flasks and mesocosm tanks.
7. *Gordonia* sp. JC11 isolated from oil-contaminated seawater by Chanthamalee and Luepromchai (2012) was used as a model petroleum-degrading bacterium.

1.5 Experimental framework

The conceptual framework of this study is in Figure 1.1. Initially, the experiments aimed to characterize the properties of biosurfactant powder and to formulate the dispersant by using the HLD concept. The formulation was expected to effectively reduce interfacial tension against hydrocarbons and increase the dispersion effectiveness. Finally, the efficiency of dispersant in enhancing petroleum biodegradation was observed after adding petroleum-degrading bacteria to the treated seawater.

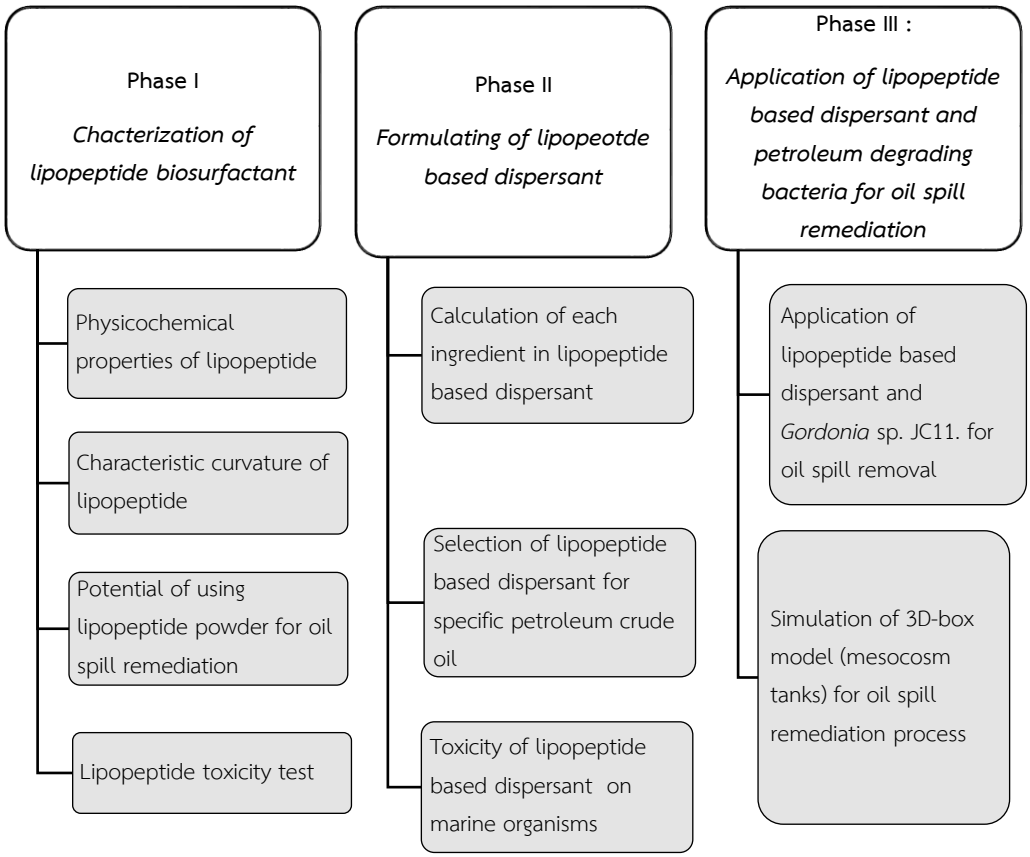


Figure 1.1 Flow chart of the research.

CHAPTER II

THEORETICAL BACKGROUNDS AND LITERATURE REVIEWS

2.1 Theoretical backgrounds

2.1.1 Oil spill

2.1.1.1 Oil spill evidences

The demand of petroleum as an energy source increases with the increasing worldwide industrialization. Oil spill occurs frequently around the world during petroleum exploitation and transportation (Brito et al., 2009 , and Joo et al., 2013). Large amount of oil has been released into the sea and caused devastating effects on the marine environment. The environmental consequences of oil spills are dramatic for marine habitats and relevant biological and human activities (Crescenzi et al., 2002). The large oil spills in the world were shown in Table 2.1

Table 2.1 World history of oil spills from tankers and refineries

Oil spill	**Region**	**Oil spilt (1000t)**	**Treatment**
Lakeview	America (inland)	1200	Containment and accidental fire
Torrey Canyon	UK	119	Boom, Detergent
Amoco Cadiz	France	223	Non-Severe weather
Ixtoc1	Mexico	454-480	Skims, booms, accidental fire, well capped
Castillo de Beliver	S. Africa	252	Diluted dispersants
Exxon Valdez	Alaska	43	Skinners, booms, sorbent. Dispersant, surfactants, solvent, burning, hot water washing
Kuwaiti fires	Kuwait	200000	Burning
Mt Haven	Genoa	144	Burnt for 3 days, solid removal
Deepwater Horizon	Gulf of Mexico	500-585	Fire accidental capping, booms, barrier, skimming dispersant

In Thailand, the marine department reported that there were 9 big oil spill accidences near offshore and marine places as shown in Table 2.2. These big oil spills released over 20,000 liters of crude oil at that time. Most of the oil spills caused during transportation (Marine department, 2017).

Table 2.2 List of large oil spills in Thailand

Year	Type of spilled oil	Location	Amount release
2001	Crude oil	Rayong Province	30 Tons
2002	Fuel oil	Chonburee province	234 Tons
2002	Fuel oil	Chonburee province	210 Tons
2005	Crude oil	Chonburee province	20 Tons
2006	Fuel oil	Rayong Province	20 Tons
2007	Saraline 185V	Trident-16 (Offshore Mobile Drilling Unit)	220 Barrels
2007	Diesel and Fuel oil	Songkhla Province	20 Ton
2008	Fuel oil	Samutprakarn Province	> 40 Tons
2011	Diesel B5	Phuket Province	40 Tons

Source: Marine department (2017)

Unfortunately, oil spills often spread to shorelines and other environmentally sensitive areas and by then, the oil is usually several days old and weathered; it is usually thick, often emulsified, and difficult to eliminate (Pereira et al., 2013). Additionally, Oil spills can have devastating consequences for society; economically, environmentally, and socially. As a result, oil spill accidents have started intense media attention, bringing many sector together for oil spill best practice response and remediation (Broekema, 2016).

2.1.1.2 Hydrocarbons in oil spills

Over the last decade, there are many types of oil spills in the world. Oil types differ from each other in their viscosity, volatility, and toxicity, which can have different effects to the environment. The petroleum crude oil have classified into four type as shown in Table 2.3.

Table 2.3 Types of spill oil and properties

Type of oil	Characteristics
Type 1	Very Light Oils (Gasoline and Jet Fuels) ■ Highly volatile. ■ High concentrations of toxic (soluble) compounds. ■ Localized, severe impacts to water column. ■ No cleanup possible.
Type 2	Light Oils (Diesel, No. 2 Fuel Oil and Light Crudes) ■ Moderately volatile; will leave residue after a few days. ■ Moderate concentrations of toxic (soluble) compounds. ■ Long-term contamination potential. ■ Cleanup can be very effective
Type 3	Medium Oils (Most Crude Oils) ■ About one-third will evaporate within 24 hours. ■ Oil contamination of intertidal areas can be severe and long-term. ■ Oil impacts to waterfowl and fur-bearing mammals can be severe. ■ Cleanup most effective if conducted quickly
Type 4:	Heavy Oils (Heavy Crude Oils, No. 6 Fuel Oil and Bunker C) ■ Long-term contamination of sediments possible ■ Weathers very slowly ■ Shoreline cleanup difficult under all conditions

Source: Office of response and restoration (2017)

The report from ITOF shows that a large proportion (39%) of the oil spills were spills of heavy fuel oil (IFO 380 and above) followed by crude oil, intermediated Fuel oil, Light Fuel/Diesel respectively (Chapman et al., 2007). In 2010, the oil spill in Deepwater Horizon (DWH) released an approximately 4.9 million barrels of South Louisiana sweet crude oil into the Gulf of Mexico (Abbriano et al., 2011), resulting in the largest marine oil spill in U.S. history and perhaps the second largest in the world (Gong et al., 2014). Another big oil spill occurred from ruptured hull in Prince William Sound, Alaska names Exxon Valdez oil spill discharged 11 million gallons of Alaskan North Slope crude oil.

Most of spilled oil contains many fractions of hydrocarbon including saturated n-alkanes, polycyclic aromatic hydrocarbons (PAHs), and their alkylated homologs, with 50% as low-molecular-weight petroleum hydrocarbons (methane and C2–C11 alkanes) (Ryerson et al., 2012). The detection of hydrocarbon component such as PAHs (both parent and alkylated), n-alkanes, xylene (BTEX) and toluene, benzene, and ethylbenzene found significant high concentration in both surface and deepwater samples (Camilli et al., 2010 and Sammarco et al., 2013)

2.1.1.3 Dispersants for oil spill remediation

There are many options available for treating oil pollution, including physical, chemical and biological treatment (Larson, 2010). A candidate technique that widely used to treat oil spill is dispersant. Dispersant are classified from their generation and their type as showed in Table 2.4.

Table 2. 4 History and characteristics of dispersant

Generation and Type	Characteristics of dispersant
First generation	Industrial cleaner and degreaser - high toxic to aquatic organism - short time usage
Second generation (Type I dispersant)	- contain a no or low aromatic hydrocarbon solvents - 15-25% of surfactant mixed with solvent - Required high dose rate (Dispersant to Oil ratio, DOR) between 1:1 and 1 :3 - Low toxicity than first generation - No longer use in many countries
Third generation (Type II, III dispersant)	- contain a blend of 2-3 surfactants, glycol and light petroleum distillate solvents. - 25-65 % surfactant mixed with solvent - Dosage Type II DOR 2:1 to 1:5, Type III DOR 1:5-1:15

Source: This table were modified from The International Tanker Owners Pollution Federation Limited (ITOPF, 2016)

Dispersants from nonionic and anionic surfactants are now the most generally formulated as shown in Table 2.5

Table 2.5 Example of surfactants use as dispersant in oil spill remediation process

Surfactant	Example
Nonionic surfactants	- sorbitan esters of fatty acids - polyalkoxylated sorbitan esters of fatty acids - polyalkoxylated fatty alcohols - polyethylene glycol esters of oleic acid - sorbitan monolaurate - ethoxylated sorbitan trioleate
Anionic surfactants	- salts of dialkyl sulfosuccinates - alkyl benzene sulfonic acid - sodium lauryl sulfate - isopropylamine dodecyl benzene sulfonate - sodium diocty sulfosuccinate

Modified from: (Fiocco and Lewis, 1999)

When dispersant reaches the lower part of the oil slick, the surfactant molecules spread along the oil–water interface and lower the interfacial tension. Small droplets of oil then begin to break away and disperse into the upper zones of the water column. As surfactant is carried off with the oil droplets, additional surfactant in the oil phase replenishes the slick oil–water interface. Consequently, the oil slick gradually depleted as droplets break away and more surfactant reaches the interface. The dispersed oil droplets are stabilized by the surfactant layer which prevents combination and re-surfacing (Figure 2.1).

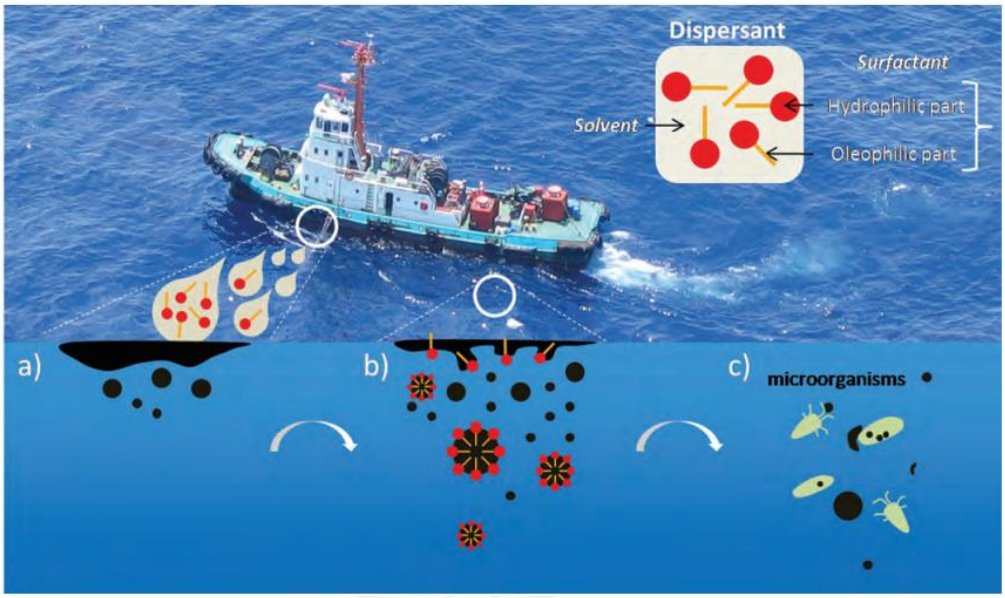


Figure 2.1 Dispersant mechanism of oil spill.

(a) Dispersant contain molecules of surfactant and solvent is applied to oil slick .

(b) The surfactant molecules coalesce with the oil slick and diffuses in to oil, solvent delivers surfactant throughout oil and oil-water interface, and reduce surface tension, making small oil droplets to break away from oil slick.

(c) Oil droplets were dispersed by turbulence and degraded by naturally microorganism (ITOPF, 2016).

There are two major issues associated with the use of dispersants. The first one is dispersant effectiveness and the second one is toxicity of the resulting oil dispersion in the water column (Fingas and Brown, 2011). The effectiveness of dispersant is determined by measuring the amount of oil that puts into the water column and comparing it to the amount of oil that remain on the water surface. The effectiveness was influence by many factors that have been investigated include dispersant-to-oil ratio (DOR), salinity, dispersant characteristics (e.g., hydrophilic-lipophilic balance, surfactant chemical structure, and solvent characteristics), mixing energy, and the

physical-chemical characteristics of the oil (Fingas and Brown, 2011 , Fingas, 2015 , Nedwed et al., 2011 , Chandankere et al., 2014 and Marti et al., 2013).

Many different types of dispersant test procedures and apparatus have been described in literature. At least 35 methods of testing dispersant effectiveness have been developed (Clayton et al., 1993). Three approaches have been used for dispersant applications in these tests. The testing procedures range from simple laboratory tests using shake flasks to complicated tests using pilot scale apparatus with continuous flow. In general, laboratory tests can be classified into four categories (Clayton et al. 1993):

(1) Tank tests with water volumes ranging from 1 to 150 L.

(2) Interfacial surface tension tests measure properties of the treated oil instead of dispersant effectiveness directly.

(3) Flume tests using dispersant for simulating real world conditions of oil spills.

(4) Flask tests that are conducted at a relatively small scale and are now popular used, including the Labofina, Warren Springs, or rotating flask test; the swirling flask test; and the baffled flask test (Sorial et al., 2004, Venosa and Holder, 2007, Venosa et al., 2010)

Other concern of dispersant application is toxicity both of the dispersant itself and the dispersed oil droplets. Early dispersant formulations were essentially solvent based degreasing agents adapted from other uses. These early dispersants proved to be highly toxic to aquatic organisms, resulting in an unfavorable public impression of dispersant use that persists today. Dispersants in use today are much less toxic than early generation dispersants. However, surfactants used in most dispersants are synthetic petroleum-based surfactants; which some of them are toxic and can be accumulated in the environment. Consequently, this study aimed to use biosurfactant for dispersant formulation.

2.1.2 Biosurfactant

2.1.2.1 Definitions

Biosurfactants are amphiphilic compounds containing both hydrophilic and hydrophobic moieties in their structure and most were produced by a wide variety of living microorganisms (Banat, 2000). According to their structural like chemical surfactant and functional diversity making biosurfactants are able to partition at the oil/water interfaces and reduce the interfacial tension (Darvishi et al., 2011) Moreover, biosurfactants are a desirable alternative to synthetic surfactant because of their selectivity, biodegradability, low toxicity and stability at extreme temperatures, pH levels and salt concentrations (Nerurkar et al., 2009). For this reason, biosurfactant have been used in various applications such as food production, pharmaceutical, cosmetic, agricultural, detergent, enhanced oil recovery and remediation of oil spills (Pornsunthorntawee et al., 2009, Nguyen et al., 2010) Biosurfactants are classified into different types e.g. glycolipids, polymeric biosurfactant and lipopeptide, rhamnolipid. The type and their application were shown in Table 2.6

Table 2.6 Biosurfactant application and their producing bacteria

Type	Application	Producing bacteria	References
Glycolipid	Bioremediation crude oil and polycyclic aromatic hydrocarbon in contaminated site	*Bacillus* sp. NO4, *Pseudomonas putida* IR1, *Aeromonas* spp.	(Rizi et al., 2012) (Ilori et al., 2005) (Sadouk et al., 2008) (Saeki et al., 2009)
Rhamnolipid	Bioremediation and petroleum hydrocarbon contaminated site	*Pseudomonas aeruginosa* J4, *Pseudomonas aeruginosa*	(Whang et al., 2008) (Arutchelvi and Doble, 2010) (Nguyen et al., 2010)
Lipopeptide	- Enhance oil recovery - Bioremediation of petroleum hydrocarbon from contaminated site	*Azotobacter chroococcum,* *Azotobacter chroococcum*, *Alcanivorax dieselolei* B-5	(Thavasi et al., 2011) (Thavasi et al., 2011) (Qiao and Shao, 2010)
Surfactin	Enhance oil recovery and biodegradation of hydrocarbon and heavy metal	*Bacillus subtilis* ATCC 21332	(Whang et al., 2008) (Pacwa-Płociniczak et al., 2016)

Biosurfactant generally classified by their chemical structure to low-molecular-weight and high-molecular-weight polymers (Banat et al., 2010). The hydrophobic moiety of biosurfactant is either long chain fatty acid, hydroxy fatty acid, or α-alkyl- β- hydroxy fatty acid and the hydrophilic moiety can be carbohydrate, amino acid, cyclic peptide, phosphate, or carboxylic acid alcohol (Bordoloi and Konwar, 2009).

2.1.2.2 Biosurfactant properties

Surface and interfacial activities including ionic type, surface tension reduction, interfacial tension behavior and emulsification are importance properties for biosurfactant. Most biosurfactant are either anionic or neutral; only a few, such as those containing amine groups, are cationic (Mulligan, 2005). The ionic type of biosurfactant is relevant to the improvement of the biosurfactant efficiency especially in the solubilization capacity.

Several biosurfactants have been reported to have a high surface activity with a low surface tension reduction and low critical micelle concentration (CMC). The critical micelle concentration is the concentration at which surfactant form micelle form (Rosen, 2004). At the concentration below CMC, surfactant expresses as the monomer and had high the surface tension. The increasing in surfactant concentration leads to reduction of surface tension until the concentration reach the CMC as shown in Figure 2.2 (Pacwa-Płociniczak et al., 2011). Another important parameter of surfactant is the interfacial tension between two immiscible phases like oil and water. In the present of biosurfactant, hydrophobic moiety turn to interact with oil phase, in contrast, the hydrophilic moiety heading to the water phase making the combination of oil and water (Figure 2.3). The interfacial tension refers to the force that holds the surface of a particular phase together. The minimal interfacial tension of system means that low forces require to hold the two immiscible phases and the system become one phase (Rosen, 2004).

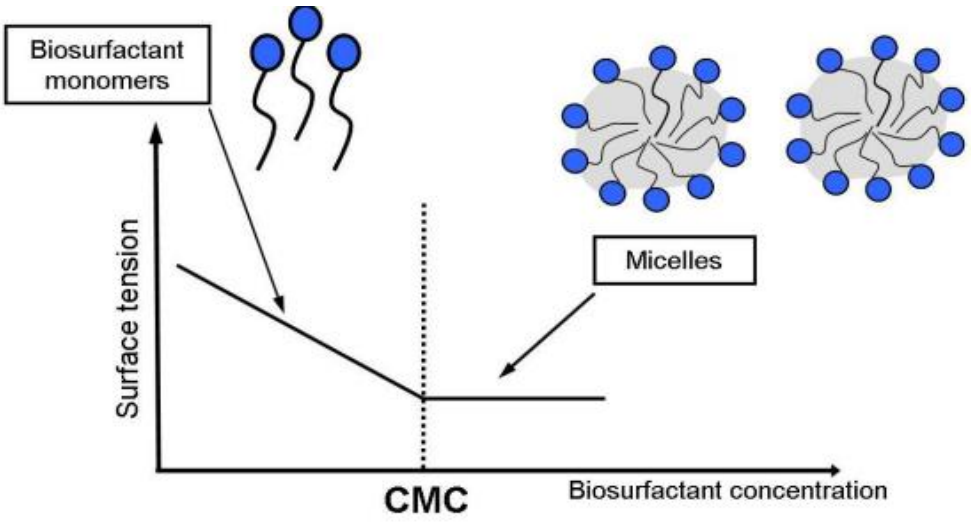


Figure 2.2 The relationship between surface tension, critical micelle concentration and biosurfactant Source : (Pacwa-Plociniczak et al., 2011)

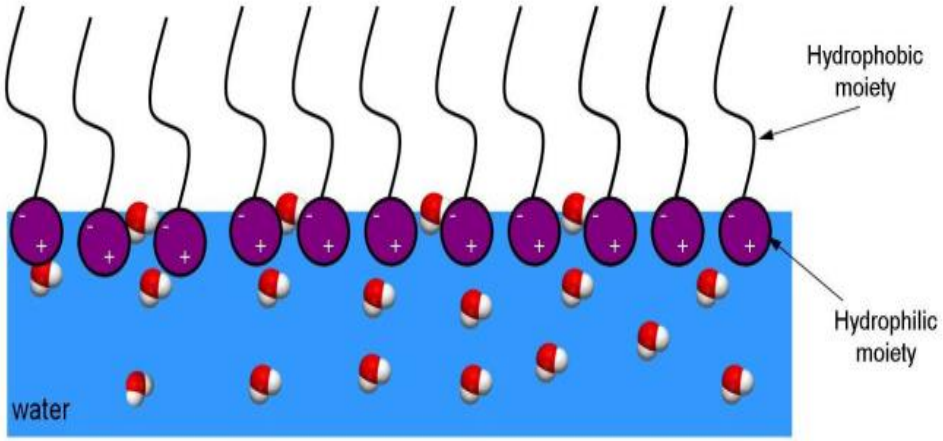


Figure 2.3 Biosurfactant molecules at the interface between liquid and air Source : (Pacwa-Plociniczak et al., 2011)

The properties of surfactants which related to the balance between their hydrophilic and hydrophobic moieties are defined as hydrophilic-lipophilic balance (HLB). Surfactants can be classified according to their Hydrophile-Lipophile portion. The HLB value indicates whether a surfactant will produce a water-in-oil or oil-in-water emulsion. Emulsifiers with a lower HLB value of 3-6 are lipophilic and promote water-

in-oil emulsification, while emulsifiers with higher HLB values between 10 and 18 are more hydrophilic and promote oil-in-water emulsions. A classification based on HLB values has been used to evaluate the suitability of different surfactants for various applications. Usually, HLB value is important in determining oil dispersion effectiveness and oil spill dispersants should traditionally have (HLB) values around 10 which had the same solubility in oil and water (Fingas and Fieldhouse, 2012).

HLB had limited use in the prediction of oil-surfactant-water equilibrium. Therefore, the value of the Characteristic curvature (Cc) value was quantified reflects the tendency of the surfactant to form micelles (negative values of Cc) or reverse micelles (positive values of Cc) in the presence of a reference oil (Acosta et al., 2008). There are many researches on characterization of Cc value of synthetic surfactant while only Nguyen and Sabatini (2011) reported the Cc value of rhamnolipid and sophorolipid as shown in Table 2.7. The information from surfactant characterization is then used for design of the most efficient formulation to improve in many hydrocarbon applications from a formation for the desired application.

Table 2.7 Characteristic curvature of biosurfactant

Biosurfactant	Cc value	Structure	References
Rhamnolipid (Glycolipid)	-1.41	**Head group** - carboxylate group - rhamnosyl **Tails group** - two identical tails of C8 alkyl chain	(Nguyen and Sabatini, 2011)
Sophorolipids (Glycolipid)	4.5	**Head group** -Carbohydrate **Tails group** - fatty acid tail of 16 or 18 carbon atoms	(Nguyen and Sabatini, 2011)

In conclusion, many studies found that biosurfactant produced by the same microorganism with different substrates can have different molecular structures and compositions. From this reason, biosurfactants can have various properties. To achieve the high effectiveness of biosurfactant in oil spill application, the basic properties of each biosurfactant must be characterized.

2.1.3 Enhancement of surfactant efficiency

In general, blends of surfactants are more effective than a single surfactant at a given HLB value (Fiocco and Lewis, 1999). The optimal HLB system could lower the interfacial tension of two immiscible phases and lead to the effectiveness of the dispersant application. The efficiency of surfactant system can enhance by following approaches.

2.1.3.1 Addition of electrolyte

Hydrocarbon solubilization can be enhanced by enlarging an internal volume of micelle which mainly depends on the combining of surfactant monomers to form micelle. Micelle combination is an abundance of surfactant monomer in micelle which depends on monomer structure for different types of biosurfactant. Combining between micelle can increase when the sectional area of hydrophilic head group decrease and hydrophobic tail is increasing (Israelachvili, 1994, Ronsen, 2004). When the electrolyte for example Na^+ is added into anionic surfactant as rhamnolipid-typed biosurfactant; the electrolyte will create linkage between anionic head group of monomer and then reduce repulsion among each monomer at head group (Figure 2.4). Reduced repulsion of monomer brings into decreasing the sectional area of head group and a number of monomer for micelle formation increase or the combination among micelle is increased. Consequently, the internal volume and the solubilization of micelle is increased (Bai et al., 1998)

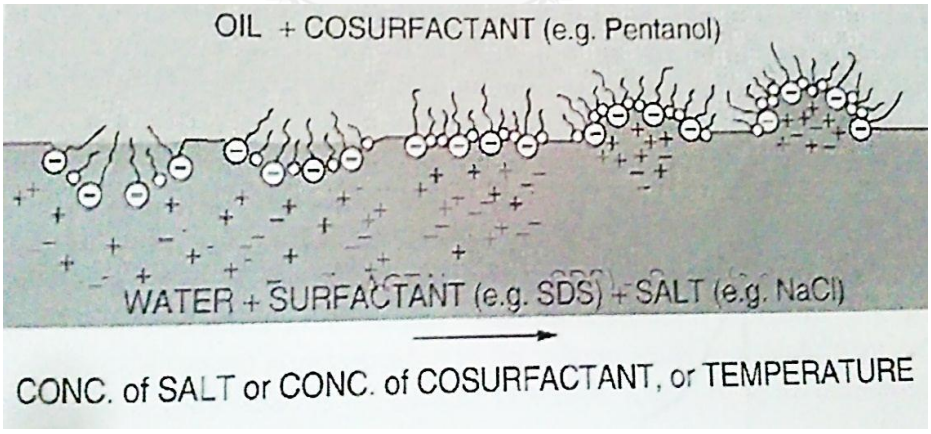


Figure 2.4 Interaction between electrolyte and surfactant (Promod Kumar and Mittal, 1999)

2.1.3.2 Addition of co-surfactant

Co-surfactant is medium-chain alcohol which its EACN is between 3 and 8 such as pentanol, polyethylene glycol, ether, glycerene mono- and di-ester, and etc. When co-surfactant is added to the surfactant system, it will rotate in between surfactant monomers and reduce the repulsion among each monomer at the head group. Consequently, the balance between water and hydrocarbon molecules is improved (Kumar and Mittal, 1999; Baglioni et al., 2001; Acosta, 2007) (Figure 2.5).

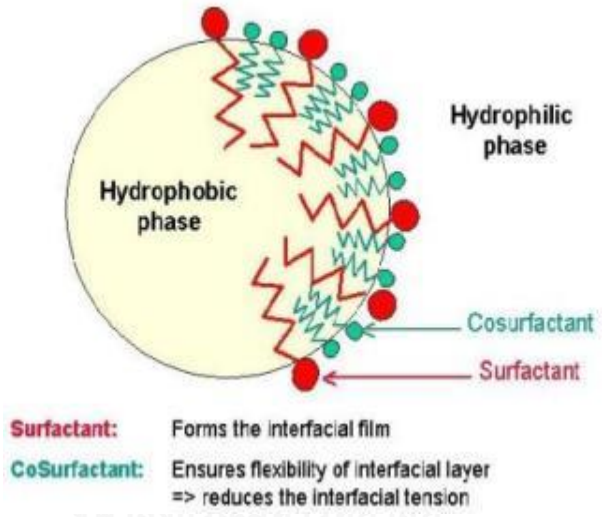


Figure 2.5 Interaction between co-surfactant and surfactant
(Source: http://www.pharmainfo.net)

2.1.3.3 Combination of biosurfactant with synthetic surfactant/biosurfactant

From previous reports, co-surfactant and linker were classified as volatile organic carbon (VOC), so it can have caused environmental impacts. Other strategies to enhance the efficiency of surfactant system are the combination of biosurfactant

with low toxicity synthetic surfactant or another biosurfactant which can adjust the hydrophilic and lipophilic part of surfactant.

Nguyen et al. (2008) investigated the efficiency of rhamnolipid biosurfactant and synthetic surfactant mixtures for improving the interfacial activity of the surfactant system against several light non-aqueous-phase liquids (LNAPLs). Since rhamnolipid biosurfactant is relatively more hydrophilic, the researcher hypothesized that mixtures of rhamnolipid biosurfactants with more hydrophobic synthetic surfactants would produce lower interfacial tensions (IFTs) than an individual rhamnolipid biosurfactant. Three alkyl propoxylated (PO) sulfate synthetic surfactants were individually mixed with the rhamnolipid. As the hydrophobicity of the surfactant mixture approached that of the hydrocarbon, IFT values decreased by one to two orders of magnitude below that achieved with individual surfactants. This work shows that the rhamnolipid has excellent phase behavior at low concentrations and can be used in surfactant mixtures to achieve the low IFT values needed for environmental remediation.

2.1.3.4 Hydrophilic-Lipophilic Deviation (HLD)

The hydrophilic–lipophilic deviation (HLD) concept and the EACN have been applied to design surfactant formulations for various purposes, such as flow assurances, during petroleum production processes (Salager et al. (1979 and 1999) and cold temperature detergency of vegetable oils and fats (Do et al, 2015). The equation of HLD frameworks were showed as following;

For ionic surfactants HLD

$$= \ln(S) - K \times EACN - f(A) + Cc - \alpha T^{*} \Delta T \quad [1]$$

For nonionic surfactant HLD

$$= b^{*}S - K \times EACN - f(A) + Cc - \alpha T^{*} \Delta T \quad [2]$$

Where

S	the salinity in the aqueous phase (g/100 mL)
EACN	the equivalent alkane carbon number of the oil
K, b	an empirical constant depending on the type of surfactant head group
f(A)	the function of the type and concentration of the alcohol used
Cc	the surfactant parameter as the characteristic curvature
ΔT	the difference between the experimental temperature and the reference temperature, which is 25 °C
αT	temperature coefficients

Generally, the model uses the concept of the hydrophilic-lipophilic difference (HLD) to calculate the chemical potential difference of transferring a surfactant from the oil to the aqueous phase; as a function of formulation variables such as type of surfactant, oil, temperature, electrolyte concentration (Acosta and Bhakta, 2008).

The general HLD equation for mixed anionic surfactants at room temperature without alcohol is simplified as Eq.3 (Acosta et al., 2008)

$$HLD = \ln(S) - K \times EACN + X_1(Cc_1) + X_2(Cc_2) \quad [3]$$

where X_1 and X_2 are the molar fraction of each surfactant and Cc1 and Cc2 are the characteristic curvature (Cc) values of each surfactant.

At HLD = 0, the interaction of the surfactant and water is exactly equal to the interaction between the surfactant and oil, which then exhibits the three-phase behavior of Winsor Type III microemulsions and the lowest IFT (Acosta et al., 2008, Witthayapanyanon et al., 2008, Nguyen and Sabatini, 2011).

The advantages of this approach are simple and quantifiable. Therefore, there are many researches using the HLD concept for various applications as shown in Table 2.8.

Table 2.8 Application of surfactant mixed system using HLD concept

Application	Surfactant system	Target oil	Condition	Reference
Oilfield Corrosion Inhibitors in oilfield pipelines	series of anionic (alkoxylated phosphate esters) cationic (alkoxylated amines, Aromatic amines, imidazoline acetates and quaternary amines)	Toluene as a model	Temp.: 25 °C Salinity: 1 - 20 %	Kiran et al. (2014)
Detergency	Mixtures of Anionic Extended Surfactants: - C10–18PO–2EO–$NaSO_4$ - sodium dioctyl sulfosuccinate	Vegetable Oils and Semi-Solid Fats Using: canola, jojoba, coconut and palm kernel oils	Temp.: 10 -30 °C Salinity: 0.05-5 %	Do et al., (2015)
Surfactant flooding	internal olefin sulfonate (IOS) - alkyl benzenesulfonate - alpha olefin sulfonate Alcohol: *Iso*-butylalcohol *sec*-butylalcohol	Several Dead crude oils and surrogate oils	Temp.: 25 °C Salinity: 1.7 – 9.1 %	Jin et al., (2015)

Application	Surfactant system	Target oil	Condition	Reference
Surfactant flooding	Alkane sulfonate (SAS) and sodium dodecyl benzene sulfonate (SDBS)	Heptane	Temp.: 20 °C, 50 °C, 90 °C Salinity: 0- 8 %	Ghosh and Johns (2016)
chemical enhanced oilrecovery (cEOR)	sodium alkyl alkoxy sulfate surfactantsand a sodium alkyl ethoxy sulfate surfactant	crude oil at high viscos	Temp.: 52°C 20 °C, 50 °C, 90 °C Salinity: 30%	Budhathoki et al. (2016)
Predicting solubilisation could apply for Drug delivery Lecithin Food Pharma	Lecithin as surfactant mixed with Peceol Polyglycerol caprylate as a linkers	ethyl caprate	Temp.: 20 °C, 52°C, 50 °C, 90 °C Salinity: 30%	Nouraei and Acosta (2017)

2.1.4 Biodegradation of petroleum hydrocarbons during oil spill remediation

Bioremediation is a process whereby microorganisms degrade and metabolize chemical substances and restore environment quality. It aims to accelerate the natural attenuation process through which microorganisms assimilate organic molecules to cell biomass and produce by-products such carbon dioxide, water and heat (Atlas, 1995). It can be divided into 2 sub-techniques, bioaugmentation which added the effective microorganisms into contaminated site, and biostimulation which stimulated the indigenous microorganisms by adding nutrients. These techniques used microorganisms to remove the pollutant and change them into simple compounds. The degradation process occurs by itself.

A common feature of crude oil is low water solubility, which poses special problems for those microorganisms capable of utilizing such water-immiscible substrates as source of carbon and energy (Chandran and Das, 2012). Surfactants enhance solubilization of contaminants. Biodegradation is therefore enhanced by surfactants due to increasing bioavailability of pollutants. Bioremediation of oil sludge using biosurfactants has been reported as shown in Figure 2.6 (Cameotra and Singh, 2009)

The main mechanism of hydrocarbon biodegradation is occurred under aerobic condition. It starts with intracellular attack to organic pollutant; oxidative process cooperated with oxygen using oxygenases, and peroxidases. The conversion of intermediate can occur step by step and synthesize through tricarboxilic acid cycle, while biomass, carbon dioxide, and water are products from this pathway as shown in Figure 2.7 (Das and Chandran, 2011).

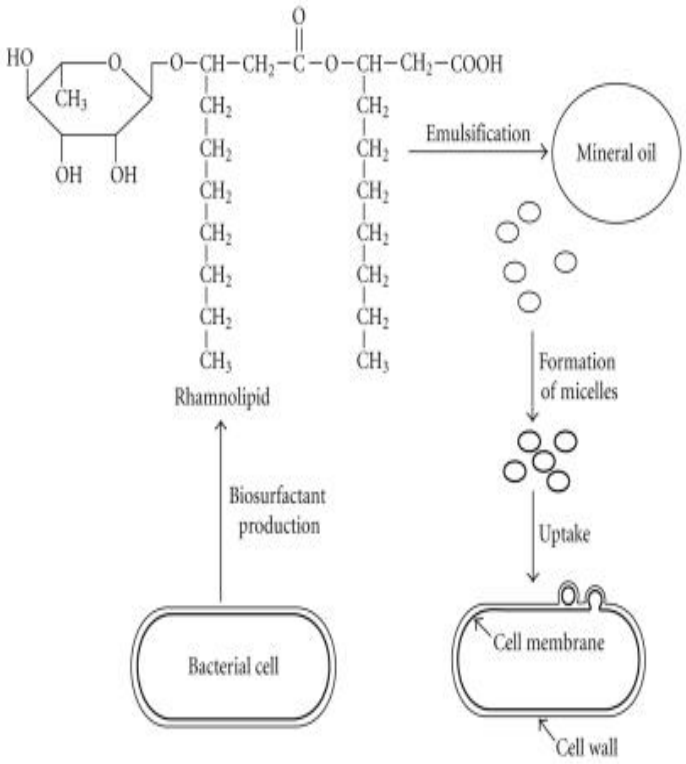


Figure 2.6 Involvement of biosurfactant (rhamnolipid) produced by *Pseudomonas* sp. in the uptake of hydrocarbons (Das and Chandran, 2011)

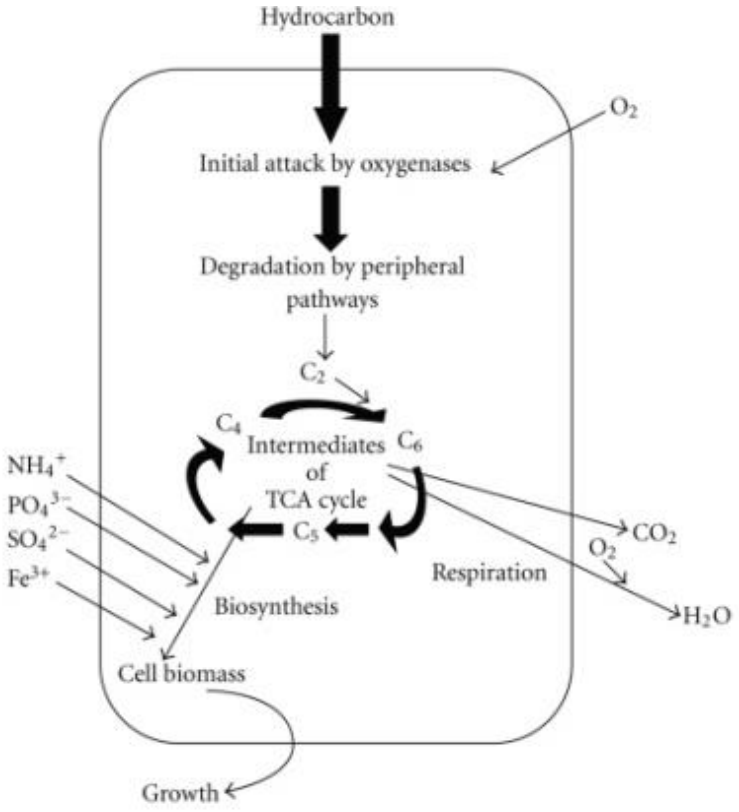


Figure 2.7 The main principle of aerobic degradation of hydrocarbons by microorganisms (Das and Chandran, 2011).

Environmental factors often limit the amount of oil degradation. There are 3 key elements;

i) hydrocarbon in physical type and concentration

ii) abiotic factors including salinity, pH, dissolved oxygen (DO), temperature.

iii)biotic factor referring to the competition with other microbial communities.

2.2 Literature reviews:

2.2.1 Lipopeptide biosurfactant

Lipopeptide biosurfactant produced from *Bacillus* species exhibited good characteristic in lowering the interfacial tension of hydrocarbon which is related to the application of biosurfactant in petroleum industries and remediation. From literature reviews, members of genus *Bacillus* are considered a suitable group for industrial synthesis of biosurfactants because the species within this taxon are well known producers of surface active metabolites. Biosurfactants produced from *Bacillus* species are usually classified as lipopeptide which is a hydrophilic protein moiety (often in cyclic structure) attached to fatty acids. The most popular representative for this group is surfactin (Arima et al., 1968, Das et al., 2009)(Arima et al. 1968; Das et al., 2009) (Figure 2.8). However, the surface activities of biosurfactant produce from *Bacillus* species from literature are varied.

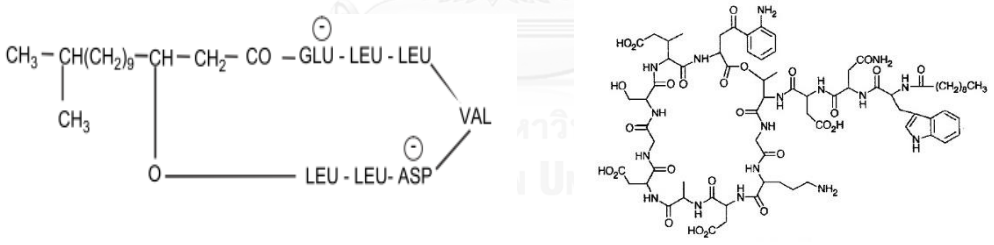


Figure 2.8 Structure of lipopeptide biosurfactant

Lipopeptide biosurfactant produced from *Bacillus* species with difference carbon sources and conditions usually reduce the surface tension of water and some medium broth from 60-72 to 23-42 mN/m and have critical micelle concentrations between 0.001 -1 g/L (Horowitz, 1990, Yakimov et al., 1998, Youssef et al., 2007, Abdel-

Mawgoud et al., 2008, Al-Bahry et al., 2013, Ismail et al., 2013, Chen et al., 2015, De Oliveira et al., 2017).

Youssef et al. (2007) showed that lipopeptide biosurfactant produced from 3 strains of *Bacillus* sp. (*Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. *spizizenii* and *Bacillus mojevenesis*) could reduce the interfacial tension between four hydrocarbons difference in alkane carbon number in range 0.3-2.17 mN/m for toluene, 1.17-3.27 mN/m for hexane, 0.84-3.19 mN/m for decane and 0.86-4.27 mN/m for hexadecane. These the result might confirmed that the lipopeptide had ability to compatible with hydrophobic hydrocarbon which meant that lipopeptide biosurfactant in this study tend to be hydrophobic surfactant. However, researcher concluded that the interfacial tension activity against each hydrocarbon depended on the relative proportions of 3-OH-C14, C15, C16, and C18 in the fatty acid tail of lipopedtide produced from difference *Bacillus* species. Moreover, Nitschke et al. (2010) reported that biosurfactant from *Bacillus subtilis* had its IFT against hexadecane 0.97 mN/m (Nitschke et al., 2010).

There are few researches on the hydrophilic/hydrophobic properties of lipopeptide biosurfactant in term of HLB values (Dehghan-Noudeh et al., 2005, Vaz et al., 2012). The reported HLB of lipopeptide varied in range of 10-21, which is considered as hydrophilic biosurfactant. Dehghan-Noudeh et al. (2005) reported the HLB of lipopeptide biosurfactant produced by *Bacillus subtilis* ATCC 6633 was 21.27 (Dehghan-Noudeh et al., 2005). On the other hand, Vaz et al. (2012) reported that there is no consensus on the HLB of surfactin. Since, it is capable of lowering the surface tension of water to 27 mN/m, they suggested that it may also have an HLB near 10 (Vaz et al., 2012).

From these researches, the characteristics of biosurfactant produced from *Bacillus* species varied. The characterization of biosurfactant produced from new bacterium with different carbon source is necessary before application of the biosurfactant. Therefore, this study investigated the characteristics of biosurfactant produced by *Bacillus* sp. GY19 in waste glycerol based medium containing palm oil.

2.2.2 Formulation of biosurfactant as dispersant

Surfactant blends show high dispersant effectiveness when compared with individual surfactant, which means synergistic agonistic interactions between surfactant molecules. In addition, the mixture of nonionic and ionic surfactants solutions form mixed micelles, which exhibits better efficiency in decreasing oil-water interfacial tensions and lower critical micelle concentration (CMC) than individual components and facilitate the dispersion of the oil droplets (Song et al., 2013). Biosurfactant become more interested to use as the dispersant in oil spill remediation. However, most of the dispersant application uses only biosurfactant in the form of crude extract or concentrated solution (Seaki et al., 2009; Marti et al., 2013). To increase its efficiency, the biosurfactant should be mixed with other surfactant. The mixture of biosurfactant and synthetic surfactant had been studied to optimize the interfacial tension behavior on various hydrocarbons (Nguyen et al. 2008 and Youssef et al., 2007).

Youssef et al. (2007) tested the interfacial activity of biosurfactants from individual bacterial strains and mixtures of biosurfactants from different bacterial strains with and without a synthetic surfactant. The result showed that the interfacial activity against toluene of lipopeptide biosurfactants produced by various *Bacillus* species depended on the relative proportions of 3-OH-C14, C15, C16, and C18 in the fatty acid tail. When mixing lipopeptide biosurfactants with the more hydrophilic, rhamnolipid biosurfactant, the IFT against toluene decreased as the percentage of the

3-OH C14 fatty acid increased in the lipopeptide. Mixtures of lipopeptide biosurfactants with the more hydrophobic synthetic surfactant, C12, C13-8PO SO4Na, were able to produce low IFT against hexane and decane. In general, the researcher found that lipopeptide biosurfactants with a heterogeneous fatty acid composition or mixtures of lipopeptide and rhamnolipid biosurfactants lowered the IFT against hydrophilic NAPLs. Conversely, mixtures of lipopeptide biosurfactants with a more hydrophobic synthetic surfactant lowered the IFT against hydrophobic NAPLs.

Song et al. (2013) developed oil spill dispersants based on two kinds of sorbitol nonionic surfactant (polysorbate 85 and sorbeth-40 tetraoleate), two kinds of glycolipid biosurfactants (rhamnolipid and sophorolipid) and a less toxic solvent (ethylene glycol butyl ether). The dispersant formulation was optimized by uniform design and the HLB values of dispersant were adjusted. The HLB values of formulations with the highest efficiency were 13.37 and 12.49, which were good agreement with the value of oil spill dispersant proposed. Moreover, they studied factors affecting the dispersion efficiency. They found that two dispersants formulation had high dispersion effectiveness (DE) for heavy crude oil at the dispersant-to-oil ratio below 1:25 and the temperature above 5°C.

Athas et al., (2014) studied the combination of two food grade surfactants i.e. lecithin (L), a phospholipid extracted from soybeans, and Tween 80 (T), a surfactant used in many food products. The result found that lecithin and Tween 80 blends show a synergistic effect in emulsion formation while neither L or T is effective on its own. The synergy is maximized at a 60/40 weight ratio of L/T. A comparison of lecithin and Tween 80 blends with Corexit 9500A shows that at a 60/40 weight ratio of L/T created a smaller oil droplets that remained stable to coalescence for a much longer time.

The smaller size and stability of crude oil droplets are believed to be important to their dispersion and eventual microbial degradation in the ocean (Athas et al., 2014).

Do et al. (2015) studied the mixture of extended surfactant (C10–18PO–2EO–NaSO4) and sodium dioctyl sulfosuccinate a hydrophobic twin-tailed surfactant for cleaning vegetable oils and semi-solid fats at cold temperature. The surfactant mixtures showed synergism in detergency performance compared to single surfactant. Moreover, the result that detergency efficiency of the surfactant formulation was greater than 90% at above the oil melting point while at low melting point temperature the performance decreased. Additional, results show that the experimental microemulsion phase behaviors interrelated very well with predictions from the hydrophilic–lipophilic deviation concept or HLD. Therefore, this knowledge had a potential to use for formulating a desire personal care and consumer product.

Recently, Budhathoki et al. (2016) studied the using of HLD concept for design the optimal middle phase microemulsion in high saline brine using hydrophilic lipophilic deviation (HLD) method. The results found that sodium alkyl alkoxy sulfate surfactants and a sodium alkyl ethoxy sulfate surfactant are tested at 52°C for reservoir brine having a total dissolved solid of above 300,000 mg/L. The optimized surfactant formulations show excellent aqueous phase stability, produce an ultra-low-interfacial tension (IFT), and give fast coalescence rates of less than 30 min at reservoir salinity and temperature. In addition, the hydrophilic lipophilic deviation (HLD) method is used to find the optimal surfactant/co-surfactant ratio at the reservoir salinity and temperature. The formulations meet IFT and stability criteria for cEOR process. Finally, the studied suggested that the HLD method is found to be a promising tool for designing microemulsion systems for cEOR applications (Budhathoki et al., 2016).

From the literature review, the formulation of lipopeptide biosurfactant as dispersant had not been studied. Therefore, it is very challenging to formulate the lipopeptide biosurfactant with the low-toxicity synthetic surfactant as dispersant for oil remediation process.

2.2.3. Application of biosurfactant for enhancing petroleum biodegradation

The application of biosurfactants in the remediation of organic compounds, such as hydrocarbons, aims at increasing their bioavailability (biosurfactant-enhanced bioremediation) or mobilizing and removing the contaminants (Banat et al., 2010). The combination of biosurfactant and petroleum degrading bacteria become more interested to enhance the removal and biodegradation of contaminant.

Benincasa (2007) studied the ability of rhamnolipid produced from agroindustrial wastes by *Pseudomonas aeruginosa* to enhance indigenous soil microorganisms on degradation of hydrocarbons under laboratory conditions. They found that 1 mg of biosurfactant/g of soil was the most efficient for the total petroleum hydrocarbon reduction, which reached 85% at the first 20 days in soil microcosms. Moreover, respirometer and microbial analyses showed that the biosurfactant added did not have toxic effects over the microbial populations (Benincasa, 2007).

Saeki et al. (2009) studied the efficiency of spray drying sterilized culture broth containing biosurfactant produced from *Gordonia* sp. strain JE-1058 or JE1058BS in oil spill remediation. Using a baffled flask test developed by the United States Environmental Protection Agency, JE1058BS showed a strong potential to be applied as an oil spill dispersant even in the absence of a solvent. Moreover, crude-oil degradability of the indigenous microorganisms in seawater can be stimulated by the biosurfactant (JE-1058 agent).

Chandankere et al. (2014) produced biosurfactant from *Bacillus methylotrophicus* USTBa which was isolated from hydrocarbon contaminated aqueous medium using crude oil as sole source of carbon. The produced biosurfactant exhibited 90% emulsification activity (EI) on crude oil. Moreover, *Bacillus methylotrophicus* USTBa efficiently degraded different alkanes from crude oil. The biosurfactant did not exhibit inhibitory effect to various vegetables, however strong antibiotic activity against gram positive and gram-negative bacteria was observed. The study suggests application of the USTBa biosurfactant as an appropriate candidate for bioremediation of crude oil contaminants.

Laorrattanasak et al. (2016) studied the application of biosurfactant produced from *Gordonia westfalica* GY40 with an efficient oil-degrading bacterium isolated by Chanthamalee et al. (2013), *Gordonia* sp. JC11 immobilized on polyurethane foam (PUF) on fuel oil degradation. The biosurfactant in a cell-free broth at 0.5× CMD was added along with polyurethane foam-immobilized *Gordonia* sp. JC11 in seawater containing 1 g/L of fuel oil. These systems could remove 81% of initial fuel oil in nutrient seawater medium within 6 days. Moreover, the test performed with three seawater samples collected from Thai coastal area. The addition of both biosurfactant and immobilized *Gordonia* sp. JC11 showed the higher efficiency on fuel oil removal (60–70%) when compared with natural attenuation (26–35 %). They suggested that *G. westfalica* GY40 biosurfactant and *Gordonia* sp. JC11 had a potential for cleaning-up oil spills in seawater.

From the literature review, the combination of biosurfactant and petroleum degrading bacteria is interested in enhancing petroleum hydrocarbon remediation in contaminated site. Therefore, the enhancing of oil spill remediation by lipopeptide based dispersant formulation and petroleum-degrading bacteria was studied in this research.

Chapter III
Characterization of lipopeptide biosurfactant produced from *Bacillus* sp. GY 19

3.1 Introduction

Biosurfactants are amphiphilic compounds containing both hydrophilic and hydrophobic moieties in their structure. According to their structural like chemical surfactant and functional diversity, biosurfactants are able to partition at the oil/water interfaces and reduce the interfacial tension (Davashi et al., 2010). Biosurfactants are interested because of their efficacy as dispersion and remediation agents and their environment-friendly qualities such as low toxicity and high biodegradability (Mulligan, 2005, Saeki et al., 2009 and Marti et al., 2104). However, the biosurfactant structure is more complicated than chemical surfactant, thus it is difficult to predict its physiochemical and surface activities.

One of the best strains in our laboratory is *Bacillus* sp. GY19, which had the highest lipopeptide biosurfactant yield after culturing in bottom glycerol based medium. Khondee et al., (2015) reported that *Bacillus* sp. GY19 biosurfactant in foamate form had high efficiency in the surface tension reduction and could be applied in the EOR application. Most biosurfactants from *Bacillus* species are classified as lipopeptide containing a hydrophilic protein moiety (often in cyclic structure) attached to fatty acids. In general, the surface activity and the interfacial activity of lipopeptides against difference hydrocarbons depended on the relative proportions of carbon number in the fatty acid tail (Youssef et al., 2007). Moreover, lipopeptide biosurfactant produced from different *Bacillus* species, carbon sources and cultured conditions

usually showed the difference physicochemical and surface activities (Youssef et al., 2007; Abdel-Mawgoud et al., 2008; Ismail et al., 2012; Chen et al., 2012; de Oliveira et al., 2012 ; Morita et al., 2012; Al-Bahry et al., 2012 and Khondee et al., 2015). It is therefore important to characterize the properties of lipopeptides from *Bacillus* sp. GY19 before the formulation of lipopeptide based dispersant.

The initial study was performed to compare the properties of biosurfactant samples in the forms of cell-free broth, foamate, crude extract and freeze-dried foamate powder. The preparation of crude extract required solvent which might lead to the high cost and produce more toxic waste from this process. Moreover, the low concentration of biosurfactant in a cell-free broth affects to the high volume required for the application. From the limitation of using crude extract and cell-free broth, Khondee et al. (2015) recovered lipopeptides from *Bacillus* sp. GY19 cell-free broth by a foam fractionation process. This study further concentrated the lipopeptides by freeze-drying of the foamate. The final product was lipopeptide biosurfactant powder, which might be used directly as remediation agent. To confirm that the lipopeptide molecules were effective after freeze-drying process, this study investigated its lipopeptide content, surface tension, critical micelle concentration CMC), solubilization, storage time and stability. Then, relative hydrophilicity/hydrophobicity, characteristic curvature (Cc) value and toxicity of the lipopeptide solution prepared from powder were evaluated. The parameters are important for the formulation of lipopeptide based dispersant in Chapter 4. In addition, the potential of using lipopeptide biosurfactant powder directly for petroleum removal was evaluated.

3.2 Materials and methods

3.2.1 Lipopeptides and chemicals

Bacillus sp. GY19 produces lipopeptides when 10% (v/v) waste glycerol and 1.25% (v/v) palm oil are used as substrates Khondee et al. (2015). The major lipopeptide in this bacterium is surfactin which consists of seven amino acids connected with a fatty acid (C16) (Rau, 2015). In this study, lipopeptides were produced and recovered from cell-free broth by foam fractionation following Khondee et al. (2015). To increase the concentration of lipopeptide molecules, the foamate was freeze-dried with a lyophilizer for 8 hr. The freeze-dried foamate powder contained lipopeptides (50%) and some impurities and nutrients. In general, one liter of foamate with 10.9 g lipopeptides/L yielded approximately 20 g powder with 0.5 g lipopeptides/g. The given concentration of lipopeptide solution in this study represents the concentration of the crude lipopeptides in the samples. For example, 1 g of lipopeptide biosurfactant powder was dissolved in 100 mL of deionized water to prepare a 0.5% (5 g/L) lipopeptide solution.

Sodium dihexyl sulfosuccinate (SDHS) (80% wt) was purchased from Sigma Aldrich. Rhamnolipid solution (R90L, 5% wt) was purchased from AGAE technology to use as a control biosurfactant. Sodium chloride (NaCl, ≥99%) was purchased from Fisher Scientific. Benzene, hexane, decane, dodecane and hexadecane were purchased from Sigma Aldrich, and their EACNs were 0, 6, 10, 12 and 16, respectively. The properties of hydrocarbons were shown in Table 3.1. All other chemicals were of analytical grade. Synthetic seawater was prepared by dissolving 34 g Marinium reef sea salt in 1 L deionized water to achieve salinity of 34 ppt.

Table 3.1 Characteristic of hydrocarbons

Hydrocarbon	EACN	Density (g/cm^3)	Structure
Benzene	0	0.867	
Hexane	6	0.73	
Decane	10	0.78	
Dodecane	12	0.75	
Hexadecane	16	0.66	

3.2.2. Determination of the lipopeptide properties and stability

The concentrated lipopeptide powder was analyzed for lipopeptide content, surface tension, critical micelle concentration (CMC), solubilization and storage time. All measurements were compared with lipopeptides in other forms, including cell-free broth, foamate and crude extract. The lipopeptide content was determined from the weight of the crude lipopeptides, which were extracted using acid precipitation and solvent extraction according to Khondee et al. (2015). The surface tension of the lipopeptide solutions was measured by a digital tensiometer (K10ST, Kruss). The CMC was obtained from the cross section of the plot between surface tension and the concentration of lipopeptides in the sample in g/L.

The solubility of lipopeptides was evaluated by dissolving 140 mg of powder or 70 mg of crude extract in 10 mL of a solvent, such as deionized water, alkaline water (pH 9), methanol, ethanol, dimethyl sulfoxide, acetone, chloroform and hexane. The weight of powder was two times more than the crude extract to achieve an equal

weight of lipopeptides in the mixture. The lipopeptide-solvent mixtures were hand shaken, allowed to stand for 24 h and filtered to collect the remaining solids. The percentages of dissolved lipopeptides were calculated based on the dry weight of residual lipopeptides and the initial sample.

The effect of storage time on the surface activity of the stored samples was determined. The surface tension of a 1xCMC lipopeptide solution prepared from the stored samples should remain the same.

3.2.3 Stability of the lipopeptide powder was investigated under different environmental conditions.

The effect of extreme condition including temperature, pH, and salinity on activity of lipopeptide powder solutions were investigated to definite the ability of lipopeptide biosurfactant to use in the extreme environmental conditions following.

3.2.3.1 Effect of NaCl concentration: The different concentration of NaCl varying from 0-10 % v/v.

3.2.3.2 Effect of pH: The lipopeptide biosurfactant solutions were adjusted to different pH at 2-11 by using 6 NaOH and 6 N HCl.

3.2.3.3 Effect of temperature: The lipopeptide biosurfactant solution was maintained at a constant temperature range of 30-121 °C for 2 hr., and then cooled to room temperature.

The surface tension was measured after incubating the 1xCMC lipopeptide solution according to methods by Laorrattanasak et al. (2016). All analyses were performed in triplicates.

3.2.4 Determination of the relative hydrophobicity/hydrophilicity of lipopeptide biosurfactant

Interfacial tension values against hydrocarbons with different equivalent alkane carbon numbers (EACN) were used to determine the relative hydrophobicity/ hydrophilicity of lipopeptide biosurfactant. The lipopeptide biosurfactant at the concentration 10 g/L were determined the interfacial tension against toluene, hexane, decane and hexadecane which had EACN 1, 6, 10 and 16 respectively. The interfacial tension was measured by using Spinning Drop Tensiometer (model SVT20).

3.2.5 Determination of the lipopeptide Cc value

The Cc values of lipopeptides have not been reported. This study determined the Cc value based on the HLD concept and phase behavior study. To confirm the methodology, the Cc value of rhamnolipid was also investigated and compared to the known Cc values reported by Nguyen and Sabatini (2011). SDHS was used as the reference surfactant, which has a Cc value of -0.92 Nguyen and Sabatini (2011). The Cc value was quantified using the slope between optimum salinity with ln (S*/S*SDHS) and the molar fraction of lipopeptides in the surfactant mixture. The molar mass of the lipopeptide was 1,049 g/mol, which was calculated based on the estimated molecular weight of surfactin with a C16 fatty acid tail. The lipopeptides and SDHS were mixed at different surfactant ratios with a final concentration of 0.1 M. The phase behavior of the mixed surfactants with benzene and at various salinity concentrations was investigated. Briefly, equal volumes of oil and aqueous phase (500 µL each) were placed in 1.5 mL glass tubes (diameter 3 mm). The tubes were hand-shaken for one minute once daily for the first 3 days and then left to equilibrate for 2 weeks Acosta et al. (2008). Microemulsions were visually identified by passing a laser light through the phase Nguyen et al. (2010). The optimum salinity values, S*, were the concentrations of NaCl where microemulsion Type III occurred from the lipopeptide-SDHS-benzene mixture, and S*SDHS were from the mixture of SDHS-benzene. The equilibrium IFT was measured between the excess water and oil phases using a glass

capillary tube and a spinning drop tensiometer (M6500, Grace Instrument) similar to Nguyen et al. (2008).

3.2.6 Determination of lipopeptide toxicity to marine organisms

3.2.6.1 Toxicity to marine organisms

The acute toxicity of the lipopeptides was determined using whiteleg shrimp and copepods. Whiteleg shrimp (*Litopenaeus vannamei*) are important commercial aquatic animals in Thailand (Figure 3.1 (a), and copepods are small crustaceans that are usually used as acute aquatic toxicity indicators (Figure 3.1 (b). Whiteleg shrimp in the post-larva period were obtained from a hatchery, while the adult copepods were isolated from natural seawater and cultured under laboratory conditions.

Figure 3.1 Whiteleg shrimp in post larva stage (a) and adult copepod (b) used as a model to toxicity test

To start the toxicity test, ten shrimp and copepods were separately placed in aerated plastic boxes containing lipopeptides diluted with seawater. The lipopeptide concentrations were 0.5-3,000 mg/L. Nonetheless, the highest potential lipopeptide

level in the water column after application as dispersant was 2.3 mg/L., which was calculated from the application of 7% lipopeptides (formulation 2) in the baffled flask test (in Chapter V). Each concentration was tested in triplicate. The mortality of whiteleg shrimp and copepods were determined under a microscope after 96 h. The median lethal concentration (LC_{50}) of lipopeptides at 96 h was calculated from a regression equation (Y= mortality; X= concentration). The toxicity of lipopeptides was compared with that of Slickgone at the same concentration.

3.2.6.2 Phytotoxicity

The phytotoxicity of the lipopeptide biosurfactant was evaluated in a static test based on seed germination and root elongation of the vegetables tomato, rice, and green bean seed following the methods described by Luna et al. (2013). Solutions of biosurfactant powder were prepared with distilled water at concentrations of ½ the CMC, the CMC and 2x of the CMC. Toxicity was determined in sterilized Petri dishes (1 cm × 10 cm) containing Whatman N° 1 filter paper (Luna et al., 2013). Ten seeds were inoculated in each Petri dish with 5 ml of the test solution at room temperature (Figure 3.2) The phytotoxicity of lipopeptide biosurfactant solution was compared with sodium dodecyl were determined. sulfate (SDS) as the synthesis surfactant. After five days of incubation in the dark, seed germination, root elongation (≥5 mm) and the germination index (a factor of relative seed germination and relative root elongation) were determined as follows:

$$\text{Relative seed germination } (\%) = \frac{\text{number of seeds germinated in the solution}}{\text{number of seeds germinated in the control}} \times 100$$

$$\text{Relative root length } (\%) = \frac{\text{mean root length in the extract}}{\text{mean root length in the control}} \times 100$$

$$\text{Germination index} = \frac{\text{\% of seed germination}}{\text{\% of root growth}} \times 100$$

(a) (b) (c)

Figure 3.2 Ten seeds of Tomato (a), Rice (b) and Green bean (c) for the toxicity test

3.2.6.3 Minimum bactericidal concentration (MBC)

Minimal inhibition and minimal bactericidal concentrations were estimated using an assay carried out in 96 well plates (Andrews, 2001). Tested compounds were solutions of the lipopeptide biosurfactant solution, and synthesis surfactant as SDS, Dehydol LS7TH, Dehydol LS9TH. Each well contained initially 50 µl of 0.85% NaCl solution. 100 µl of each testing solution with the initial concentration of 100 g/l were present in the first well of each row and subsequently 50 µl were pipetted to the next wells, respectively with a multichannel pipette to achieve a dilution row. Two strains of bacteria isolated from environment were used as the

inoculum (Sirirataruengsuk, 2013). The inoculum was prepared by incubated the bacterial in nutrient broth 24 hr. Then the inoculums were centrifuged and washed twice time with 0.85% NaCl and adjusted to an OD_{540} = 0.1. Each test contains one negative control (only sterile NaCl solution, no inoculum, no test solution) and one positive control (NaCl solution and inoculum, no test solution). The inoculums in each well were streak on nutrient agar plate to determine the lowest concentration which bacteria could not grow as the minimum bactericidal concentration (MBC) as shown in Figure 3.3.

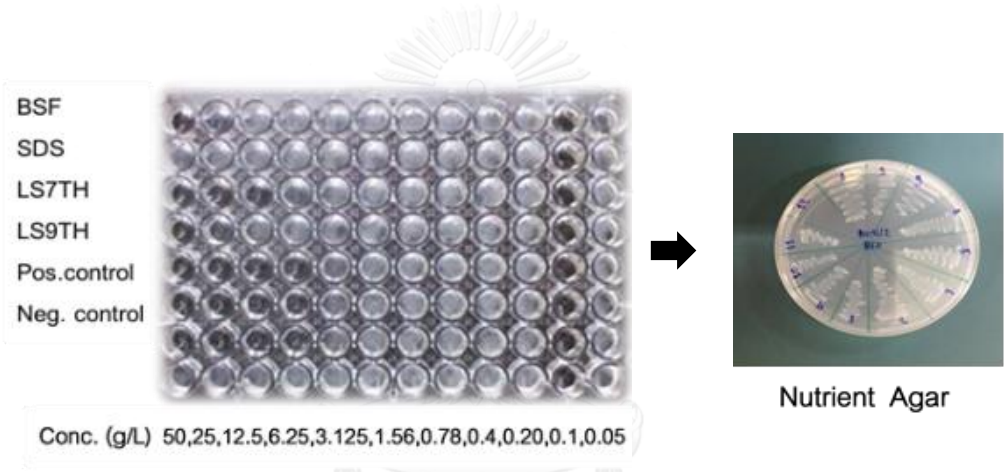


Figure 3.3 The Minimal inhibition and minimal bactericidal test in 96 wells plate for test surfactant (a) and nutrient agar plate to determine the lowest concentration after incubate in 96 wells plate.

3.2.7. Potential of using lipopeptide biosurfactant powder for petroleum removal

3.2.7.1 Oil displacement test

Oil displacement is a method used to determine the diameter of the clear zone, which occurs after adding surfactant-containing solution on an oil-water

interphase. The diameter evaluation allows the surface tension reduction efficiency of a given biosurfactant. The oil displacement test was adapted from Rodrigues et al. (2006) by added 25 ml sea water to a petri dish which is 80 mm in diameter. 8 μl of fuel oil was added to the water surface, followed by the addition of 10 μl of biosurfactant solutions on to the oil surface (Rodrigues et al., 2005). The dispersant to oil ratio (DOR) was 1:0.8.

The diameters of this clearing zone were measured and percentage of oil displacement was calculated. The oil displacement of biosurfactant powder solution was compared with the nonionic chemical surfactant (Dehydol LS9TH), commercial detergent and water.

3.2.7.2 Solubilization test

The fuel oil solubilization was adapted from Laorrattanasak et al. (2016) by adding 100 mg of fuel oil into 25 mL of lipopeptide biosurfactant solution. Then, the sample was shaken at 200 rpm for 24 hr. The amount of fuel oil in the solution was detected by TLC-FID.

3.2.7.3 Sand washing Test

The sand washing study was conducted to observed fuel oil removal with the biosurfactant powder solutions. The sand washing methods described in Urum et al, (2006) was applied for this study. Fuel oil 62.5 mg was added to 3.125 g of Ottawa sand to reach the concentration of fuel oil in sand 20 mg fuel oil/g sand (Urum et al., 2006). Then, the lipopeptide biosurfactant solution was added and the samples were vortex 10 minutes. The amount of fuel oil in the washing solution was determined by TLC-FID.

3.3 Results and discussion

3.3.1 Properties of lipopeptides biosurfactant

The crude lipopeptide extract was brown in color and was sticky as shown in Figure 3.4. Although it had high lipopeptide content, it was slightly soluble in water (Table 3.2). The application and extraction of the crude extract would require the addition of solvent, which would increase the cost and contribute to toxic waste production.

(a) (b) (c)

Figure 3. 4 The form of lipopeptide biosurfactant including cell-free broth (a), foamate (b) and crude extract (c) of lipopeptide biosurfactant

The freeze-drying technique has been introduced to concentrate lipopeptides from cell-free broth after acid precipitation (Vaz et al., 2012 and Al-Bahry et al., 2013). However, the lipopeptides from *Bacillus* sp. GY19 have long fatty acid chains (Khondee et al., 2015) and did not readily precipitate. The lipopeptides were therefore concentrated from the foamate using the freeze-drying technique. The freeze-dried lipopeptides formed a white-brown powder (Figure 3.5) and showed 30-fold and 50-fold higher lipopeptide content than cell-free broth and foamate, respectively. The

CMC values of lipopeptides in the foamate and powder were comparable, with surface tensions of 28-30 mN/m (Table 3.2).

Figure 3.5 Freeze-dried foamate powder

The advantages of the lipopeptide powder were long storage time (Table 3.2). It can be stored in 4 °C more than 2 years and easy to use after storage whereas the foamate solution stored in -20°C more than 1 years. However, it will take a longer time on de-freezing of the solutions. Another advantage of the lipopeptide powder is the ability to dissolve in water making it could prepare at high biosurfactant concentrations with a low toxicity solvent for the desire application (Table 3.3). The process of freeze-dried is a dehydration process worked with freezing the material and then reducing the surrounding pressure to allow the frozen water in the material and forming a solid phase. The process did not remove any nutrient and element from the solution. Therefore, it is easy to re-suspend the powder with the water. While, the crude lipopeptide was extracted by using the organic solvent which then removed some polar fraction out of the crude. Even though, it is more purified than the powder form. But, it is hardly to dissolve in water and easy to dissolve in high polarity toxic organic solvent which will negative affect to the environment. Consequently, the concentrated lipopeptides could be used as water-based ingredients in the dispersant formulations.

Table 3.2 Properties of lipopeptide samples.

Parameter	Cell-free broth	Foamate	Powder	Crude extract
Amount of lipopeptides	6.4 g/L	10.9[a] g/L	0.5 g/g powder	1 g/g extract
CMC[b] (g lipopeptide/L)	1.4	0.3[c]	0.5	1.0
Surface tension at CMC (mN/m), pH 7	28.9±0.6	28.4±0.1	29.8±1.2	30.8±0.7
Compatible solvent[d]	Water	Water	Water and high polarity solvents e.g., methanol	Low polarity solvents e.g., acetone
pH for lipopeptide solubilization in water	7	7	7	> 9
Storage time	3-4 d at 4 ℃ > 6 mo at -20 ℃	3-4 d at 4 ℃ > 1 yr at -20 ℃	> 2 yr at 4 ℃	> 1 yr at 4 ℃

[a]Data from Khondee et al., (2015).

[b]To obtain the CMC, lipopeptide biosurfactant solutions were prepared by dissolving or diluting lipopeptide samples in water at varying concentrations. The CMC value was determined from a plot of the surface tension vs lipopeptide concentrations.

[c]Calculated based on the results of Khondee et al., (2015).

[d]The extent of lipopeptide solubility in various solvents is shown in Table

Table 3.3 Percent of dissolved lipopeptides in high to low polarity solvents.

Solvent	Percent Dissolved (%)	
	Powder	Crude extract
Water	93.05 ± 0.66	34.19 ± 13.86
Alkaline water	97.47 ± 1.35	31.75 ± 2.68
Methanol	86.64 ± 2.52	78.41 ± 9.48
Ethanol	87.75 ± 9.92	95.68 ± 4.16
DMSO	63.78 ± 0.14	39.31 ± 11.16
Acetone	43.75 ± 4.99	96.99 ± 2.68
Chloroform	45.11 ± 3.91	99.43 ± 0.99
Hexane	44.00 ± 2.44	79.23 ±13.98

3.3.2 Effect of temperature, pH and sodium chloride on surface activity

The surface activity of lipopeptides is stable under the temperatures range between 30-121 °C (Figure. 3.6a) and is relatively stable at pH 4-10; at pH 4, the surfactant provides the lowest surface tension at 29.3 mN/m at CMC (Figure 3.6b). Biosurfactants from other *Bacillus* strains also found to be more stable in alkaline rather than acidic conditions due to the acidic nature of lipopeptides (Vaz et al., 2012). The surface activity of lipopeptides is increased in the presence of NaCl (2-10% w/v), where the lowest surface tension is 25.6 mN/m at ≥4% NaCl (Figure 3.6c). This property is similar to those of other anionic biosurfactants. For example, Laorattanasak et al. (2016) reported that the binding of sodium ions and the negatively charged hydrophilic portion of the *Gordonia westfalica* GY40 biosurfactant resulted in lower surface tension due to the enhancement of biosurfactant solubilization and micelle formation. These results indicated that *Bacillus* sp. GY19 lipopeptides are suitable for application in seawater as well as in other environmental conditions.

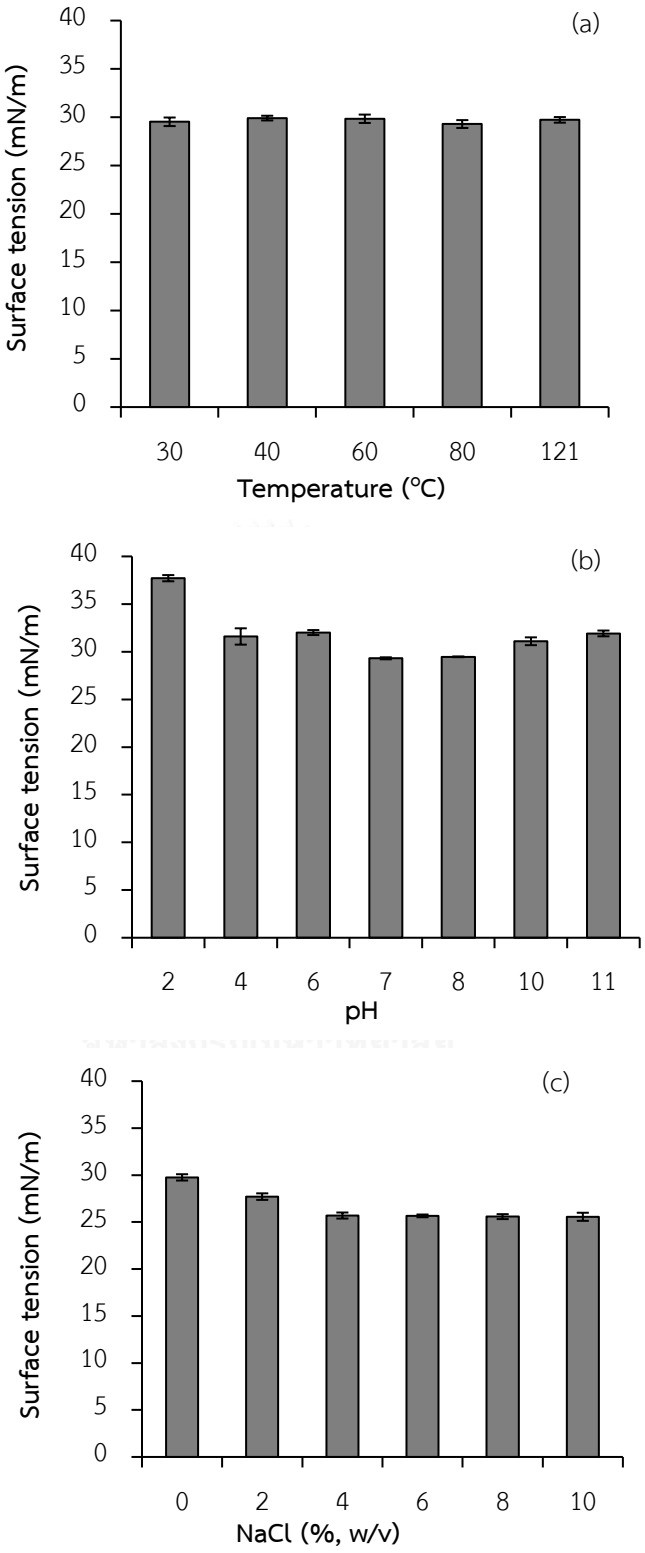


Figure 3.6 The effect of temperature (a), pH (b) and NaCl (c) on the stability of lipopeptides. The concentration of lipopeptides was 0.5 g/L (1xCMC).

3.3.3 Relative hydrophobicity/hydrophilicity of lipopeptide biosurfactant

The relative hydrophobicity/ hydrophilicity of biosurfactant is an important property of biosurfactant by determine the interfacial tension properties. Interfacial tension values against hydrocarbons with different equivalent alkane carbon numbers (EACN) were used to determine the relative hydrophobicity/ hydrophilicity of biosurfactant (Acosta et al., 2005). The hydrophobicity of hydrocarbons increases with the EACN. A surfactant that has its lowest IFT against a hydrocarbon with a low EACN is considered to be relatively hydrophilic (Youssef et al., 2007). The hydrophilic and hydrophobic proportion of biosurfactant can be used to determine the application of specific biosurfactant. In this study, the relative hydrophilic/hydrophobic of lipopeptide biosurfactant solution was determined by measuring the interfacial tension of lipopeptide biosurfactant solution at the concentration 10 g/L against hydrocarbons with varying EACNs ranging from 1 to 16 (toluene, hexane, decane and hexadecane) as shown in Figure 3.7. The result showed that biosurfactant produced from *Bacillus* sp. GY19 had its lowest IFT values against hexadecane (EACN = 16). The IFT values decreased as EACN increased (From 4.14±0.28 – 2.66±0.17 mN/m). The result showed that the lipopeptide biosurfactant was relative more hydrophobic surfactant.

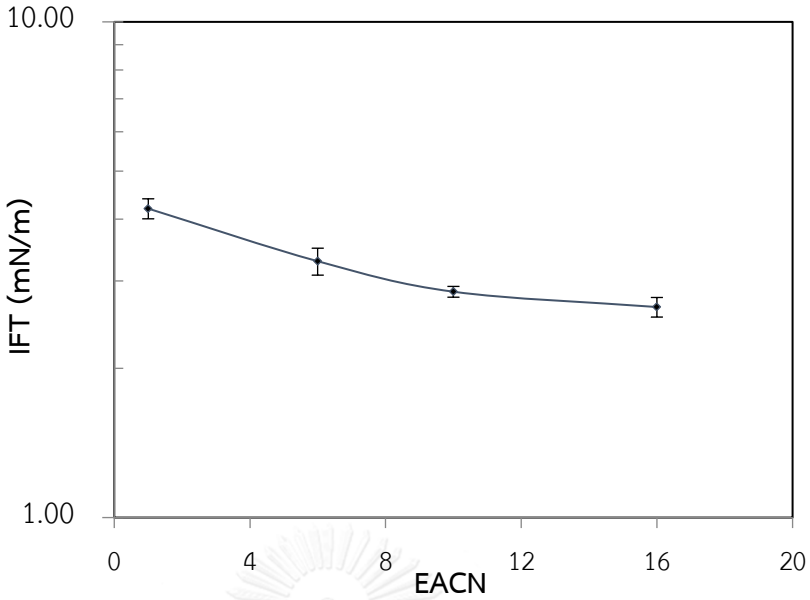


Figure 3.7 Correlation between interfacial tension of lipopeptide biosurfactant against four hydrocarbons difference in hydrophobicity, which represented as equivalent alkane carbon number (EACN) of each hydrocarbon including toluene, hexane, decane, and hexadecane (EACN 1, 6, 10 and 16, respectively).

3.3.4 Characteristic curvature of lipopeptides

To determine the magnitude of hydrophilic-lipophilic nature of lipopeptide biosurfactant, the study characterized its Cc value from phase behavior study and HLD concept. The phase behavior of the lipopeptide-SDHS-benzene mixture at various salinity concentrations showed the transition of the microemulsion from Type I to Types III and II with increasing salinity (Figure 3.8).

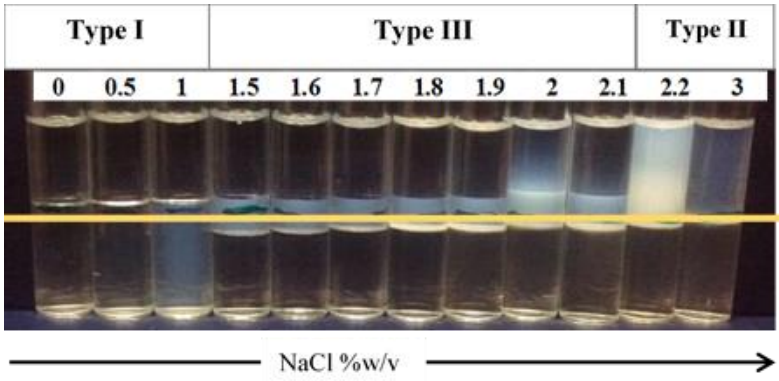


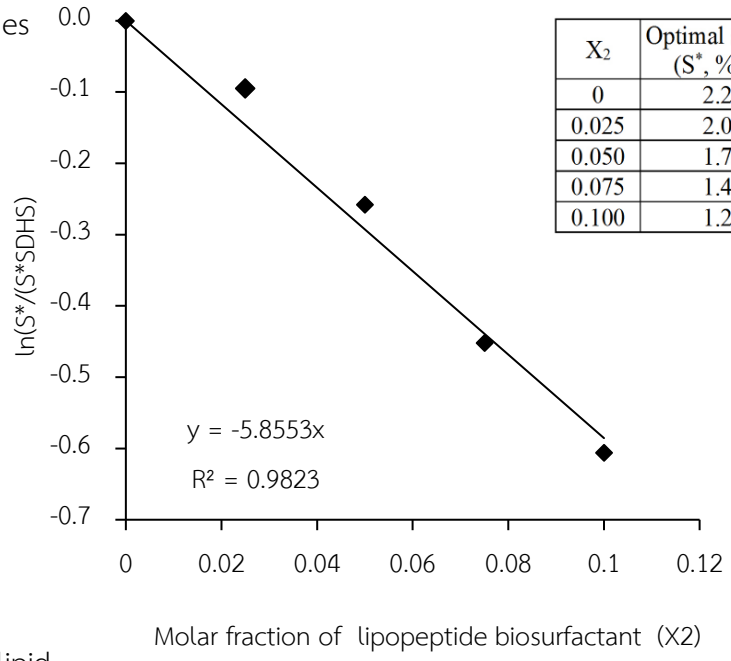
Figure 3.8 Example of microemulsion Type I, II and III during the phase behavior study. The phase scan for lipopeptides with increasing NaCl concentrations (0 - 3%) was conducted with benzene as oil phase at room temperature.

The equilibrium IFT at the optimum salinity of the lipopeptide-SDHS-benzene system was in the range of 0.01-0.02 mN/m, which almost reached the ultralow IFT (<0.01 mN/m). A correlation between optimum salinity, ln (S^*/S^*_{SDHS}) and the molar fractions of lipopeptides in the surfactant mixture is shown in Figure 3.9a. The slope of this plot was -5.8553, which represented the value of Cc_1-Cc_2 according to Acosta et al. (2008). Since the Cc_1 value of SDHS was -0.92, the Cc_2 value for lipopeptides was calculated to be 4.93. The positive value of Cc_2 indicated that the lipopeptide was a hydrophobic surfactant. The Cc value of rhamnolipid (2.5:1 mixture of monorhamnolipid and dirhamnolipid) was characterized using a similar approach.

From Figure 3.9b, the quantified Cc value of rhamnolipid was -1.32, which was comparable to the Cc value of rhamnolipids (1:1 mixture of monorhamnolipid and dirhamnolipid) reported in Nguyen and Sabatini (2011) at -1.41. The Cc values also demonstrated that lipopeptides were more hydrophobic than rhamnolipids, whose hydrophobicity is similar to that of other lipopeptides reported by Youssef et al. (2007).

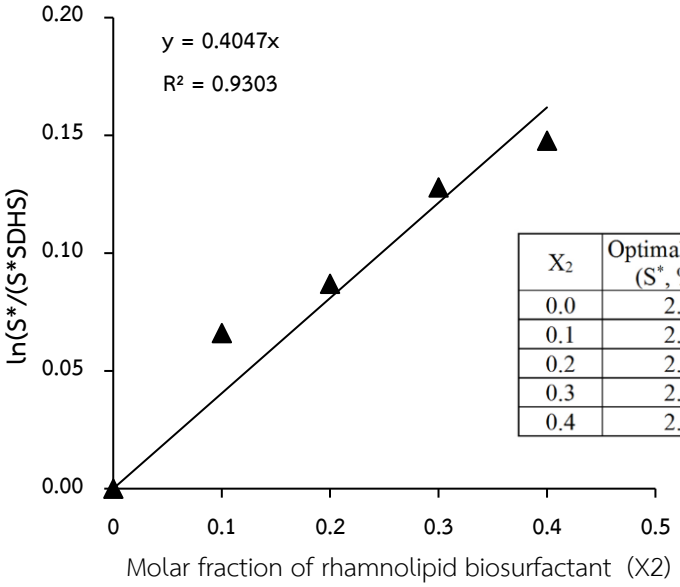
When compared the Cc value of lipopeptide biosurfactant with the sophorolipid biosurfactant contained carbohydrate in head group and fatty acid tail of 16-18 carbon atoms found that the Cc value of both biosurfactant tend to be more hydrophobic. The Cc value of sophorolipid biosurfactant is 4.5 (Nguyen and Sabatini, 2011).

(a) Lipopeptides



X_2	Optimal salinity (S^*, %w/v)
0	2.20
0.025	2.00
0.050	1.70
0.075	1.40
0.100	1.20

(b) Rhamnolipid



X_2	Optimal salinity (S^*, %w/v)
0.0	2.20
0.1	2.35
0.2	2.40
0.3	2.50
0.4	2.55

Figure 3.9 Correlation between the fractions of lipopeptides (a) and rhamnolipid (b) in a 0.1 M SDHS-biosurfactant-benzene microemulsion system with optimal salinity.

3.3.5 Biosurfactant Toxicity

3.3.5.1 Acute toxicity of lipopeptides on marine organisms

The toxicity of biosurfactants from *Bacillus* spp. have been determined with aquatic organisms. For example, the lethal concentration (LC_{50}) of surfactin and fatty acyl-glutamate from *B. subtilis* strains 41651 A1 and 40688 E4 on larval Gulf Killifish, *Fundulus grandis* were 2.5 and 25 mg/L, respectively (Marti et al., 2014). The crude biosurfactant from *B. subtilis* ICA56 have an effective concentration (EC_{50}) of 170 mg/L on a microcrustacean, *Daphnia magna*, which is about 8 times higher than sodium dodecyl sulfate (de Oliveira et l., 2017). This study found that *Bacillus* sp. GY19 lipopeptides were less toxic than Slickgone to both post-larval whiteleg shrimp and adult copepods (Figure 3.15). The Slickgone is one commercial dispersant widely used in for oil spill clean-up. However, Slickgone contained higher amount of hydrocarbons such as kerosene. Therefore, it could increase the toxicity to the marine organisms.

The LC_{50} of lipopeptides for whiteleg shrimp was 1,050 mg/L, and it was 31 mg/L for Slickgone. The toxicity of Slickgone was comparable to that reported by Petpiroon and Chunharat (2005), who reported an LC_{50} for juvenile giant tiger prawn of 32 mg/L. For copepods, the LC_{50} values for lipopeptides and Slickgone were 1,174 and 68 mg/L, respectively. Although, the tested organisms in our study were different from the previous reports and the lipopeptides from various *Bacillus* spp. strains might have different activity. The low toxicity of lipopeptides in our study was likely due to the absence of solvents and other toxic chemicals during the production process. In addition, the lipopeptide powder contained small amounts of remaining nutrients that might support the growth of tested organisms. These results confirmed the potential application of lipopeptides in the marine ecosystem.

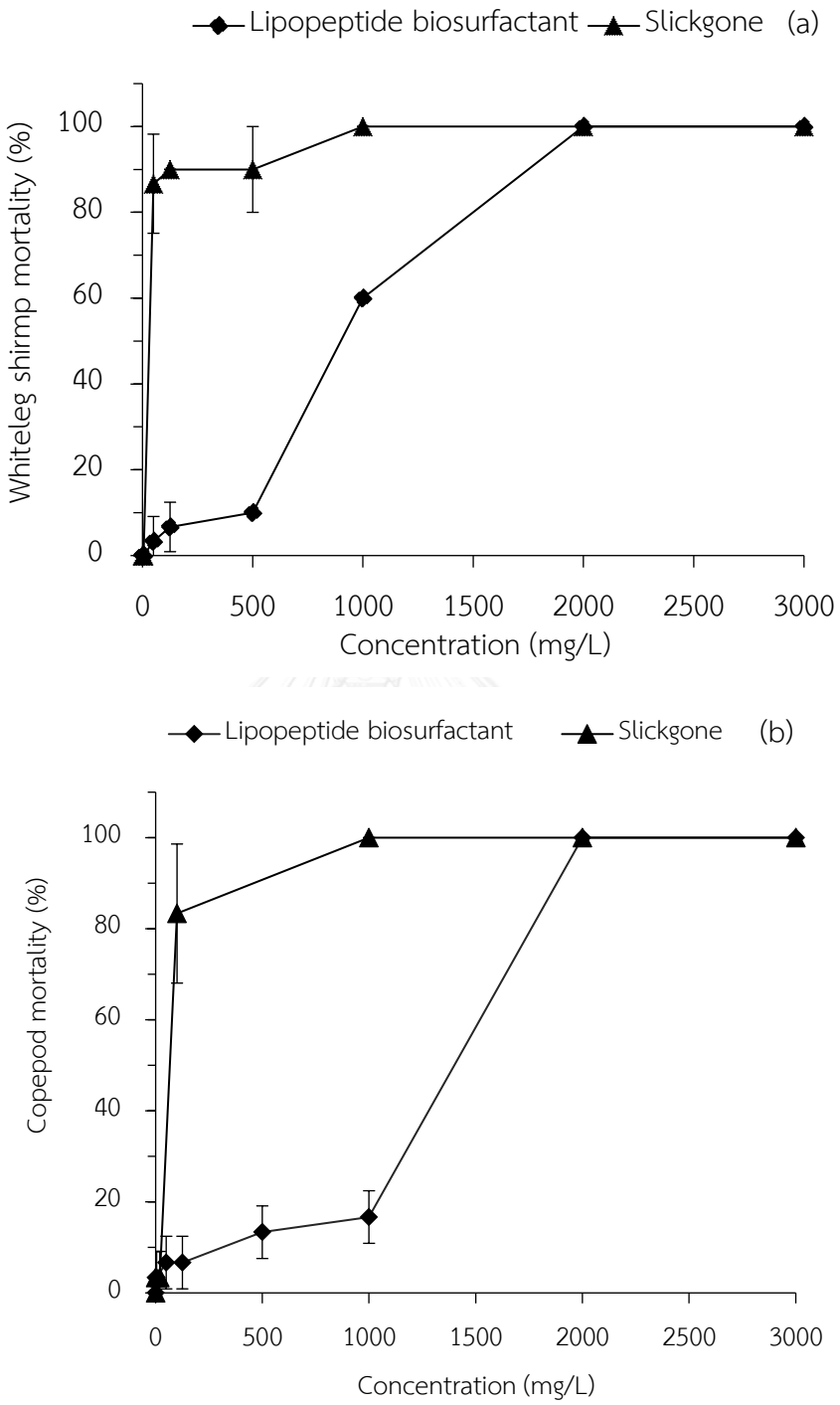


Figure 3.10 Percent mortality of whiteleg shrimp (a) and copepods (b) after a 96-h exposure to lipopeptides or Slickgone.

3.3.5.2 Minimum Bactericidal Concentration (MBC)

In recent years, lipopeptides form the most widely reported class of biosurfactants having antimicrobial action (Rodrigues et al. 2006, Das et al, 2007, and Hajfarajollah et al., 2014). The antimicrobial lipopeptides include fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis* (Vater et al. 2002). The mode of action of these lipopeptides has been proposed to be membrane disruption due to interaction between the cationic polymyxin and the anionic bacterial outer membrane leading to a detergent-like activity (Mnif and Ghribi, 2015). Therefore, in this study the toxicity of lipopeptide biosurfactant from *Bacillus* sp. GY19 on the PAH degrading bacteria also was determined compared to the commercial surfactants.

The minimum bactericidal concentration or MBC was used to determine the lowest concentration of toxic compound that results in more than 99.9% killing of the bacteria inoculums tested. In this study, the lipopeptide biosurfactant at the concentrations range from 0.5 – 50 g/L were preliminary tested with two pyrene-degrading bacteria i.e. PRY 12 and PRY16 isolated locally and compared with synthetic surfactants including SDS, Dehydol LS7 TH and Dehydol LS9 TH.

The result found that lipopeptide biosurfactant and Dehydol LS7Th did not inhibit both of pyrene-degrading bacteria at concentrations lower than 50 g/L whereas Dehydol LS9TH inhibited each bacterium at different concentrations as shown in Table 3. Lipopeptide biosurfactant found to be low toxicity and not effect to the Pyrene-degrading bacteria. These results might from the lipopeptide biosurfactant solution used in the study was made from the solvent-free process. The solution contained some nutrient and element which could supported the bacterial growth. These results were supported by Das et al. (2007) showed that crude (solvent extract) lipopeptide biosurfactant produced from marine *Bacillus circulans* is active against Gram-negative bacteria such as *Proteus vulgaris* and *Alcaligens faecalis* at a very low concentration as low as 10 mg/L by using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods (Das and Mukherjee, 2007).

Moreover, type of bacteria is one factor on the tolerant of lipopeptide biosurfactant concentration in difference degrees. It was supported by the study of Hajfarajollah et al. (2014). that the isolated lipopeptide biosurfactant in various concentrations showed antimicrobial activity against various microbial strains tested in the different degrees. The growth inhibition capability at lipopeptide produced by *P. freudenreichii* concentrations ranging from 50 to 3.2 mg/ml found that it is completely inhibited the growth of *R. erythropolis* at concentration of 25 mg/ml. Even though it is slightly inhibition effect was observed against B. cereus at lipopeptide concentration of at 25 mg/L (Hajfarajollah et al., 2014).

From this study, the lipopeptide biosurfactant produced from *Bacillus* sp. GY19 had good potential to apply with the pyrene-degrading bacteria for environment application

Table 3.4 Minimal Bactericidal concentration (MBC) of various lipopeptide biosurfactant and other surfactants on pyrene-degrading bacteria.

Bacterial strain	**Minimal Bactericidal concentration (MBC)**			
	Lipopeptide biosurfactant (anionic)	**Dehydol LS7TH (nonionic)**	**Dehydol LS9TH (nonionic)**	**SDS (anionic)**
PRY 12*	> 50	>50	>50	>0.4
PRY 16*	> 50	>50	> 12.5	>0.2

* Pyrene degrading bacteria were isolated by Sirirataruengsuk, 2013

3.3.5.3 Phytotoxicity

The phytotoxicity assay of lipopeptide biosurfactant was determined with tomato, rice, and green bean seeds comparing with SDS, a synthetic surfactant. The result shown that all five types of seeds in the SDS test had no growth. It means that SDS in all concentration of 0.5CMC- 2xCMC (0.12, 0.23 and 0.46 g/L) affected the germination of the seeds (Figure 3.11).

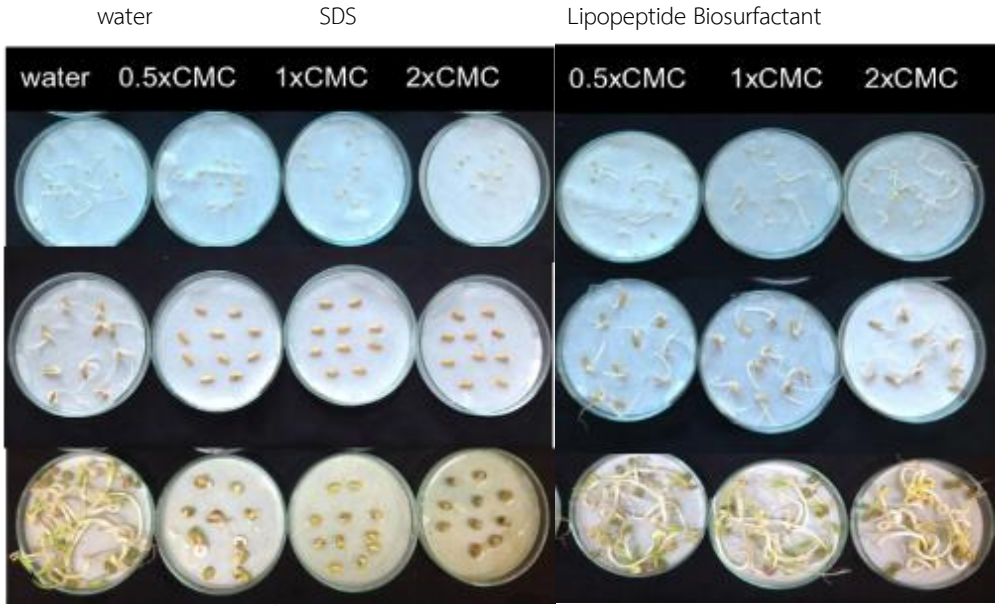


Figure 3. 11 Germination of tomato, rice and green bean seeds in the present of SDS and lipopeptide biosurfactant at the concentration 0.5x – 2x CMC compared to water as control

The germination index (GI) (Figure 3.14), which combines measures of relative seed germination (Figure 3.12) and relative root elongation (Figure 3.13), has been used to evaluate the toxicity of the biosurfactant. The germination index value of 80% has been used as an indicator of the absence of phytotoxicity (Luna et al, 2013). The results indicated that the lipopeptide solutions did not have an inhibitory effect on seed germination or root elongation in tomato, rice and green bean. Moreover, leaf growth and the elongation of secondary roots occurred under all conditions tested

(Figure 3.11). The biosurfactant could promoted the seed germination of all plant. The result was corresponding to the study of Luna et al, (2013) on biosurfactant vegetable inhibition. They found that biosurfactant from *Candida sphaerica* UCP0995 no inhibited the growth of four types of vegetable such as *Brassica oleracea, Solanum gilo, Lactuca sativa L. and Brassica oleracea L*. at the concentration 0.5CMC- 2xCMC (0.125 g/L – 0.5 g/L). The results of phytotoxicity of lipopeptide biosurfactant produced from *Bacillus* sp. GY19 found to be an advantages on the application of biosurfactant in the environment. For example, the use of biosurfactant on petroleum/heavy metal soil washing and the then apply the washed soil in the plant growth in the future seems promising.

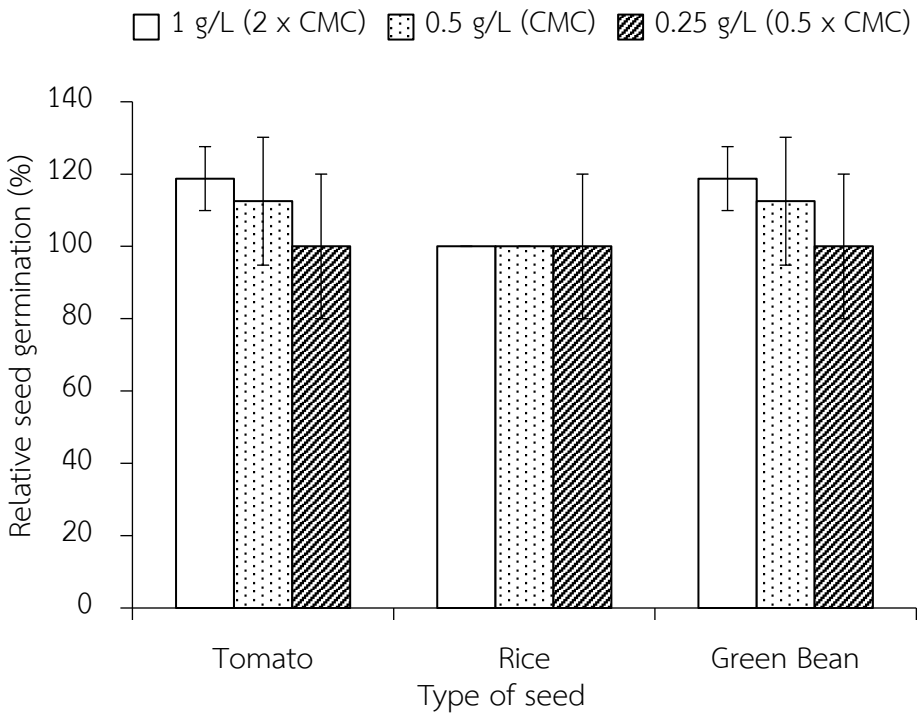


Figure 3.12 Percentage of Relative germination of Tomato, Rice and Grean bean in day 5

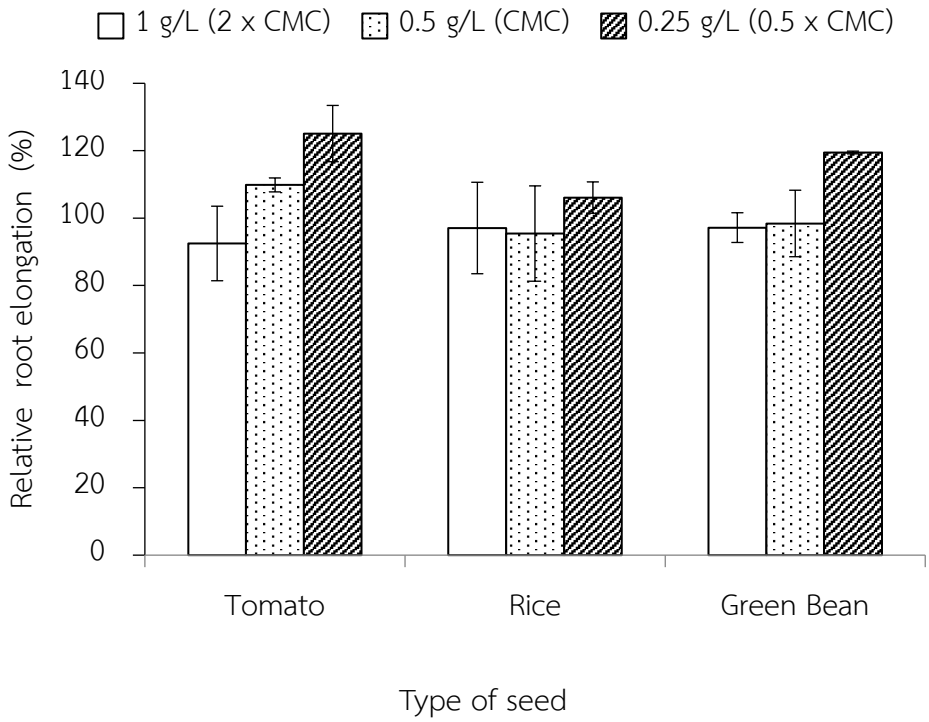


Figure 3.13 Percentage of Relative root elongation of Tomato, Rice and Grean bean in day 5

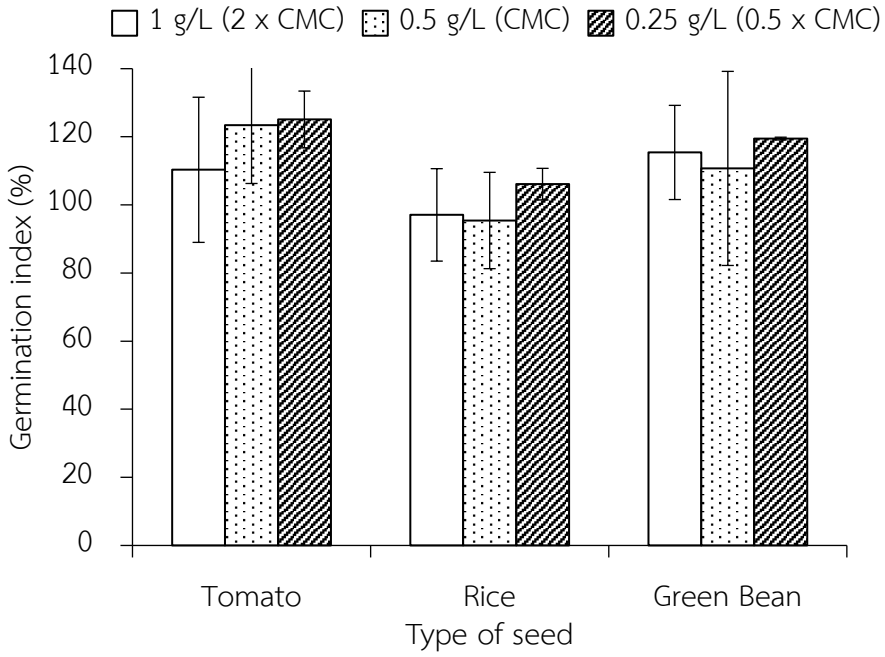


Figure 3.14 percentage Germination Index of Tomato, Rice and Grean bean

From the toxicity results of lipopeptide biosurfactant produced from *Bacillus* sp. GY19 in acute toxicity on marine organism, the inhibition of bacterial and vegetable growth showed a good potential to apply lipopeptide biosurfactant in many applications such as oil spill remediation in aquatic environment and the petroleum remediation processes which is safe to the environment.

3.3.6 Potential of lipopeptide biosurfactant for petroleum removal

The potential of lipopeptide biosurfactant for petroleum removal was observed by oil displacement, oil solubilization and sand washing tests. Fuel oil was used as a model petroleum in this experiment. Fuel oil is considered as more hydrophobic petroleum hydrocarbon and high viscosity (Chao et al. 2012). The results were compared with chemical surfactant (Dehydol LS9TH), commercial detergent and water. The lipopeptide biosurfactant concentration was 5 g/L (10xCMC).

The oil displacement test shown that the lipopeptide biosurfactant could dispersed fuel oil similar to Dehydol LS9TH and commercial detergent (88 - 92 %) (Figure 3.15 and Figure 3.16). When compared the fuel oil displacement efficiencies from cell-free broth produced *Gordonia westfalica* GY40 at the concentration 1.85 g/L (4xCMD) and DOR 1:0.8 found that the oil displacement efficiency was 75–90 % (Laorrattanasak et al., 2016). Even though, at the concentration of lipopeptide from *Bacillus* sp. GY19 was higher than the concentration of *Gordonia westfalica* GY40 but the oil displacement was comparable. It might from the concentration of both surfactant reached the above CMC. Therefore, the surface activity was stable. 92 %) .

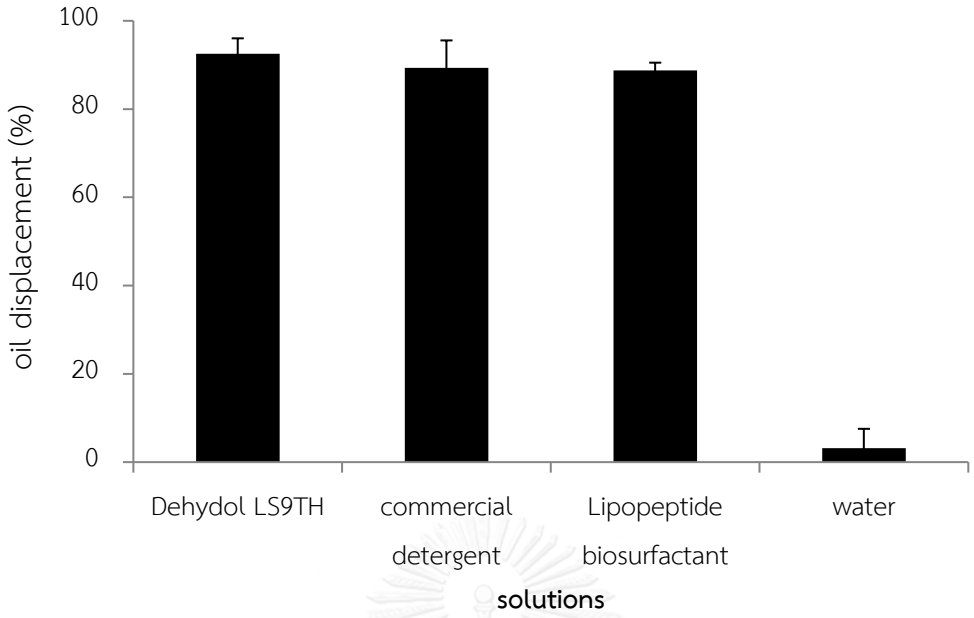


Figure 3. 15 Fuel oil displacement efficiencies of lipopeptide biosurfactant and other solutions.

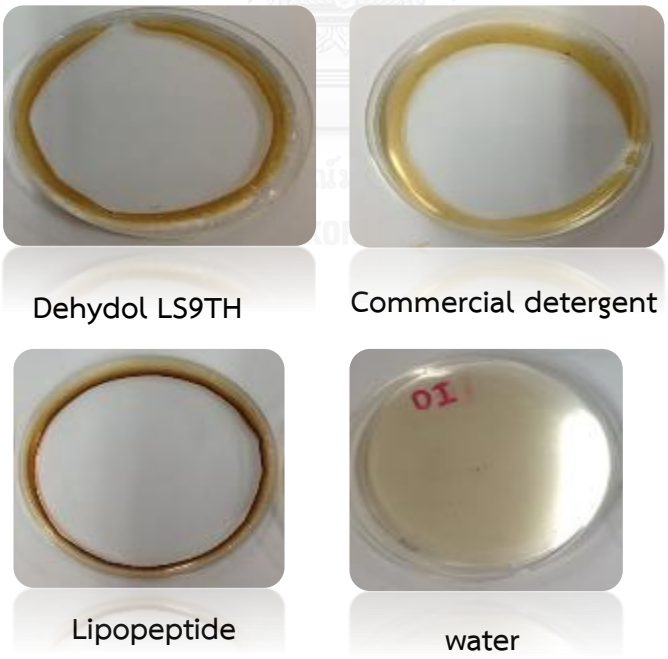


Figure 3.16 Example of fuel oil displacement test results

The results for the fuel oil solubilization and sand washing from Ottawa sand tests by lipopeptide biosurfactant showed the similar performance to those of Dehydol LS9TH but was better than those of commercial detergent.

For the fuel solubilization, only 6.7 % of fuel oil (270 mg L^{-1}) dissolved in in water (Figure 3.17). The lipopeptide biosurfactant at 10x CMC (5 g/L) was able to dissolve fuel oil compared to Dehydol LS9TH 26-27% (1027.2 - 1043 mg/L) from the 4000 mg/ L at the initial concentration. Moreover, it efficiency was higher that commercial detergent 10% (414 mg/L). However, the fuel oil solubilization was about 3 times lower than biosurfactant from *Gordonia westfalica* GY40 (66 %) at 4xCMD (Laorrattanasak et al.,2016).

These results might according from the incompatible of lipopeptide biosurfactant and fuel oil. However, the efficiency of difference petroleum crude oil will different. Mnif et al. (2014) that lipopeptide biosurfactant produced from *B. subtilis* SPB1 showed 87% oil removal efficiency from diesel-contaminated soil. It efficiency was comparable to commercial surfactant (SDS, anionic surfactant and Tween 80, nonionic surfactant). To apply the biosurfactants to specific petroleum oil type would be concern.

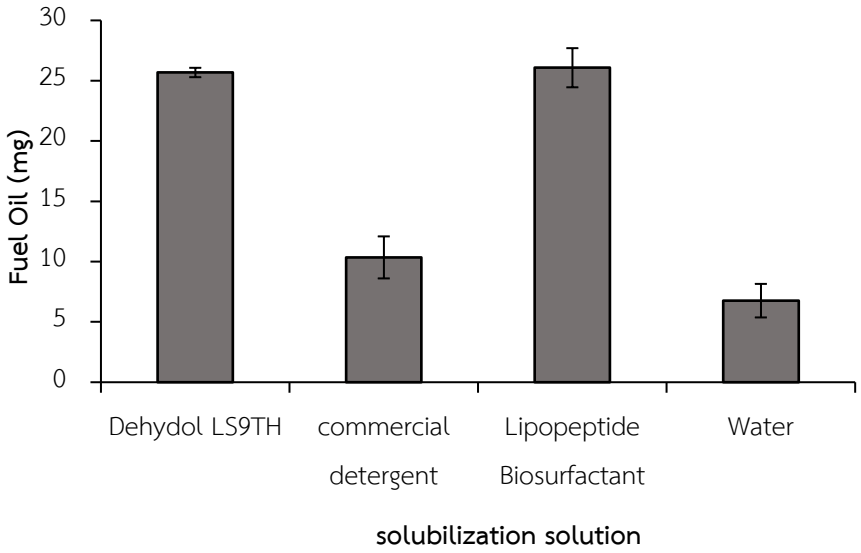


Figure 3. 17 Amounts of solubilized fuel oil in lipopeptide biosurfactant and other solutions

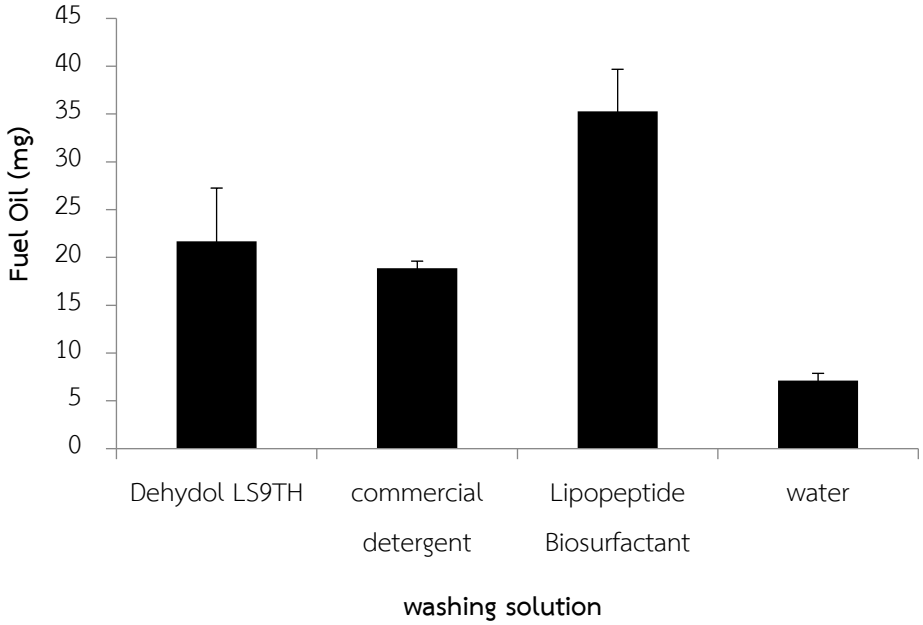


Figure 3.18 Amounts of residual fuel oil in lipopeptide biosurfactant and other washing solutions after used to washed contaminated sand

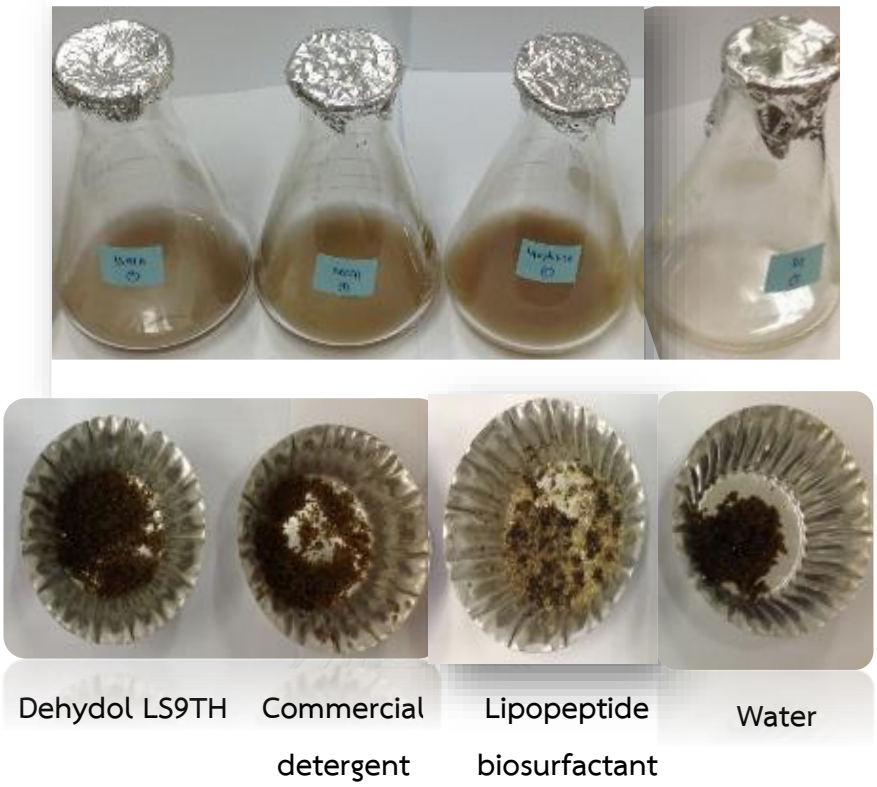


Figure 3.19 Residual oil in washing solutions (above) and sand (below) after the sand washing.

The results indicated that the lipopeptide biosurfactant produced from *Bacillus* sp. GY19 has high potential to be applied for remediation of petroleum contaminated sites.

3.4 Conclusions

The lipopeptide powder concentrated from the foamate using the freeze-drying technique was stable after storage and had good surface activity when compared to other forms of lipopeptide biosurfactant. The lipopeptide powder was solubilized well in water and high polarity solvents such as methanol, and ethanol. It was stable under wide range of temperature (30-121 °C), pH (pH 4-10), and increased in the presence of NaCl (2-10% w/v). The lipopeptide biosurfactant was relatively more hydrophobic as seen from its IFT value against hexadecane (more hydrophobic hydrocarbon), which was lower than that of toluene (more hydrophilic hydrocarbon). To confirm the magnitude of hydrophobicity of lipopeptides, the HLD concept was used to quantify its Cc value and found to equal 4.93. The lipopeptide had no toxic effect on marine organisms (LC_{50} 1050 mg/L and 1174 mg/L for whiteleg shrimp and copepods, respectively.), vegetables (concentration 250 – 1000 mg/L in tomato, rice and green bean) and pyrene-degrading bacteria (concentration above 50 g/L). The lipopeptide biosurfactant showed the ability to disperse fuel oil similar to Dehydol LS9TH and commercial detergent. Moreover, it could solubilize and wash fuel oil similar to Dehydol LS9TH but higher than commercial detergent. In conclusion, the freeze-drying lipopeptide biosurfactant was effective and could be applied as an ingredient for the formulation of solvent-free petroleum dispersant.

Chapter IV

Formulation of lipopeptide based dispersants using HLD concept

4.1 Introduction

Nowadays, there are many researches on the application of biosurfactant for petroleum remediation especially on oil spill remediation. However, most of researches focused on single biosurfactant (Seaki et al., 2009 and Marti et al., 2013), which had lower removal efficiency than commercial dispersants such as Corexit series and Slickgone NS series (Nacol, 2017). The high efficiency of commercial dispersant is due to the present of petroleum based surfactants and organic solvents.

Surfactant blends usually show high dispersant effectiveness when compared with individual surfactant because of the synergistic interactions between surfactant molecules (Al-Sabagh et al., 2007, Song e al., 2013). Nevertheless, not all surfactant compositions are suitable for dispersing spilled oil, and many of the effective ones have the drawbacks of being toxic and/or non-biodegradable. Only few researchers studied the formulation of biosurfactant as dispersant. For example; Song et al. (2013) developed an oil spill dispersant by mixing two glycolipid biosurfactants (rhamnolipid and sophorolipid) with low-toxicity nonionic surfactant and less toxic solvent ethylene glycol butyl ether. The two dispersants had high dispersion effectiveness (DE) for heavy crude oil.

From the results in Chapter III, lipopeptide biosurfactant has potential to apply as a dispersant for oil spill remediation. It was a hydrophobic biosurfactant (Cc 4.93) when compared to sodium dihexyl sulfosuccinate (SDHS) (Cc -0.92). Moreover, the oil dispersion of lipopeptide biosurfactant showed a good efficiency compared to the tested commercial detergent. The major role of a dispersant is to enhance natural

petroleum dispersion via IFT reduction as well as micellar droplet formation for oil solubilization. Consequently, a formulation that provides the lowest possible IFT is desirable. Each type of crude oil has difference characteristic. Therefore, the oil dispersant should be formulated to accommodate different oil type. In this study, the hydrophilic–lipophilic deviation (HLD) concept was first applied to design lipopeptide based dispersant formulation for difference petroleum oil types. EACN was used to define specific crude oil characteristic, which has been reported in the range of 6-12 (Wan et al., 2014).

The use of dispersants is recommended to speedily disperse spilled crude oil into the water column (Fingas, 2001). Eventually, the oil that dispersed into the water column will be reduced and decomposed by microorganisms (Jung et al., 2009). However, the dispersed oil can lead to an increase in toxicity of the chemically enhanced water accommodated hydrocarbon fraction (CEWAF) and the dispersant itself is potentially toxic to aquatic organisms (Couillar, et al., 2004; Lee et al., 2014 and Gardiner et al., 2013). From previous results in Chapter III, the lipopeptide biosurfactant itself has low toxicity to marine organism. However, the mixing with other ingredients might increase toxicity of the lipopeptide based dispersants. Therefore, the optimum amount of lipopeptide based dispersant was measured along with the toxicity of water accommodated fraction (WAF) of oil with marine organism.

Consequently, this part first identified the optimum fractions of lipopeptide biosurfactant and the low toxicity synthetic surfactant using HLD concept. The formulations were carried out and tested with various types of alkane hydrocarbons. Then, the optimum formulations were selected to determine the dispersant efficiency with two petroleum crude oils. Lastly, the acute toxicity of a selected formulation and specific crude oil was tested with the whiteleg shrimp. There were 3 samples including

water accommodated fraction (WAF), chemically enhanced water accommodated hydrocarbon fraction (CEWAF) and lipopeptide based dispersant alone. The applications of the HLD concept and lipopeptides were expected to be a model for formulating solvent-free biosurfactant-based dispersants to clean up crude oil spills and reduce the effect to the marine environment.

4.2 Materials and methods

4.2.1 Lipopeptides and chemicals

Lipopeptide biosurfactant powder from chapter III was used for formulating lipopeptide based dispersant. The molar mass of the lipopeptide was 1,049 g/mol, which was calculated based on the estimated molecular weight of lipopeptide with a C16 fatty acid tail. In general, one liter of foamate with 10.9 g lipopeptides/L yielded approximately 20 g powder with 0.5 g lipopeptides/g. The given concentration of lipopeptide solution in this study represents the concentration of the crude lipopeptides in the samples. For example, 1 g of biosurfactant powder was dissolved in 100 mL of deionized water to prepare a 0.5% (5 g/L) lipopeptide solution.

SDHS (80% wt) was purchased from Sigma Aldrich. Sodium chloride (NaCl, ≥99%) was purchased from Fisher Scientific. Hexane, decane, dodecane were purchased from Sigma Aldrich, and their EACNs were 6, 10, 12, respectively. Two light crude oils, including an Arab light/Arab extra light blend (ARL/AXL) and Bongkot light crude oil (BKC), were obtained from Thai Oil PCL. These crude oils have different oil compositions and properties (Table 4.1). Slickgone, a widely used dispersant in Thailand, was obtained from Thai Oil PCL. All other chemicals were of analytical grade. Synthetic seawater was prepared by dissolving 34 g Marinium reef sea salt in 1 L deionized water to achieve salinity of 34 ppt.

Table 4.1 Characteristics of crude oils.

Crude oil	Hydrocarbon composition (%)				Viscosity	Density
	Saturates	Aromatics	Resin	Asphaltene	(cP)	(g/cm^3)
Arab light/Arab extra light blend (ARL/AXL blend)	31	34	20	15	3.8	0.84
Bongkot light (BKC)	100	-	-	-	1.2	0.64

4.2.2 Formulation of the mixed surfactant system using the HLD concept

The Cc value of the lipopeptides was calculated as 4.93 from Chapter III in section 3.3. 4. To formulate the mixed surfactant system, the Cc values of the lipopeptides and SDHS were used to calculate their molar fractions in the surfactant mixtures. The HLD concept of binary anionic surfactant mixtures can be written as Eq.1 (Acosta et al., 2008). At HLD = 0, the equation could be simplified to calculate the molar fraction of the lipopeptides by fixing S at 3.4% NaCl to represent the seawater salinity and assigning $K_1 = K_2 = 0.19$ as

$$X_1 = (0.19 \times Nc,o - 0.3)/5.85 \qquad \text{Eq.1}$$

Where X_1 is the molar fraction of the lipopeptides; the molar fraction of SDHS can be calculated using $1\text{-}X_1$; and Nc,o is the EACN. The molar fractions of lipopeptides and SDHS for four hydrocarbons with EACNs ranging from 6 to 16 are shown in Table 4.2

Table 4. 2 Molar fractions of lipopeptides and SDHS calculated from the HLD concept

EACN ($N_{C,O}$)	NaCl % (S)	Molar fraction	
		Lipopeptides (X_1)	SDHS (X_2)
6	3.4	0.14	0.86
10	3.4	0.27	0.73
12	3.4	0.34	0.66
16	3.4	0.47	0.53

To confirm the values from the HLD calculation, a phase behavior study and IFT measurements against hexane, decane and dodecane were first determined by varying the lipopeptide molar fractions in the 0.1 M surfactant mixture.

To compare the efficiency of mixed surfactants with single surfactants, such as lipopeptides and SDHS, the molar fractions of lipopeptides and SDHS were fixed at 0.27 and 0.73, respectively, and the total surfactant concentration was increased to 0. 25 M. These molar fractions were formulated for decane, but the mixture was also tested with other hydrocarbons to confirm its specificity.

4.2.3 Determination of lipopeptide-based dispersant efficiencies

The optimal mixed surfactant systems were selected as lipopeptide-based dispersants. A phase behavior study and IFT measurements of these formulations against the ARL/AXL blend and BKC crude oils were performed to confirm their ability to form microemulsion Type III and lower IFT by a specific oil type. Then, oil dispersion and solubilization activities were determined using the oil displacement technique and the modified baffled flask test, respectively.

The efficiencies of the lipopeptide-based dispersants were compared with 5% v/v Slickgone and 7% w/v lipopeptides. These concentrations were selected based on the recommended dose of Slickgone and the highest concentration of lipopeptides in the formulated dispersants. All tests were performed in triplicate. For the oil displacement test, the formulation was dropped onto the surface of the crude oil layer, which was formed by adding 100 µL of crude oil onto 20 mL of synthetic seawater in a Petri dish (diameter of 80 mm). The DOR was varied from 1:2 to 1:200. The diameter of the clear zone on the oil surface was measured to calculate the oil displacement efficiency according to (Bharali et al., 2011).

The baffled flask test is used to determine the ability of dispersant to solubilize crude oil in the water column after adequate mixing (Venosa et al., 2002). Initially, 100 µL of oil was carefully dropped onto the surface of 120 mL synthetic seawater in a baffled flask (Figure 4.1). The formulation (4 µL) was then dispensed onto the center of the oil layer, giving a DOR of 1:25. The flask was placed on an orbital shaker at a rotation speed of 200 rpm for 10 min and then allowed to settle for 20 min. The first 5 mL of sample was drained from the stopcock, and 20 mL of sample was collected for oil extraction. The amount of crude oil was determined by thin layer chromatography and flame ionization detection (TLC-FID) methods, as described in Chanthamalee et al. (2013). The effectiveness was calculated based on the ratio of oil dispersed in the test system to the total oil following Srinivasan et al. (2007).

Figure 4.1 Photograph of the baffle flask for dispersant efficiency modified from Venosa et al. (2002) which had a stopcock at the bottom for taking the sample out.

4.2.4 Acute toxicity test

Oil dispersed following the action of a dispersant can lead to an increase in toxicity of the chemically enhanced water accommodated hydrocarbon fraction (CEWAF). Therefore, acute toxicity of biosurfactant based dispersant and dispersed oil were tested on whiteleg shrimp. There were 3 samples as followed;

1. Water accommodated hydrocarbon fraction (WAF)
2. Chemically enhanced water accommodated hydrocarbon fraction (CEWAF)
3. Lipopeptide based dispersant only

The sample preparation was modified from Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) methodology (Lee et al., 2013).

For water accommodated hydrocarbon fractions (WAFs) of crude oil were prepared using Bongkot light crude oil (BKC) obtained from PTT Public Company Limited. In brief, 700 mL of seawater (34 ppt; fully aerated) and a Teflon-coated stirring bar (2 cm) were placed into a 1-L glass bottle with a silicone tube which place the end of the tube near the bottom for taking a sample. Then, 17.5 mL of Bongkot light crude oil was added (ratio of crude oil: seawater was 1:40).

For chemically enhanced water accommodated hydrocarbon fraction (CEWAF) which consists of Bongkot light crude oil (BKC) and lipopeptide based dispersant. The procedure was done as same as in WAF. After crude oil was added, the lipopeptide based dispersant was added 1.75 ml to achieve the dispersant to oil ratio 1:10 (Dispersant to Oil ration, DOR = 1: 10).

Then, the preparation of saturated dispersant solutions was done by adding 1.75 mL of lipopeptide based dispersants into seawater which was the same amount of dispersant used for the CEWAF. The bottle was covered with a cap, sealed, and placed on a magnetic stirrer plate as shown in Figure 4.2. To avoid the formation of a large vortex and oil droplets, low energy magnetic stirring (150 rpm) was applied for 18 h in a dark box (Figure 4.3). Then, the mixture was allowed to settle for 6 h for the separation of the water and oil phases.

WAF CWAF Lipopeptide based dispersant only

Figure 4.2 Experimental set-up of WAF, CWAF and Lipopeptide based dispersant only.

Figure 4.3 Dark boxes for the set-up of WAF, CWAF and Lipopeptide based dispersant samples

The aqueous layer was drained off and transferred into a clean amber glass bottle and then stored at 4 °C to use as 100 % (v/v) stock solution. For the toxicity test, the samples were diluted with 34 ppt seawater to 80%, 60%, 40%, and 20%. Ten whiteleg shrimp in a separate well was exposed to different concentrations of testing samples (Figure 4.4). Mortalities of whiteleg shrimp at 96 h exposure were examined. The Lethal concentration at 10% and 50% (LC10 and LC50) were computed using probit analysis from SPSS (Finney, 1971).

Figure 4.4 Whiteleg shrimp in Postlarva stage

4.3 Results and discussion

4.3.1 Formulation of lipopeptide-based dispersants using the HLD concept

Since the lipopeptides had a high positive Cc value, SDHS was selected over rhamnolipids due to its lower negative Cc value, which would require a lower molar fraction to reach the optimum HLD. Based on Eq. 2, the molar fractions of lipopeptides in the lipopeptide-SDHS mixtures increased with increasing EACN of the hydrocarbons because the system required more hydrophobic surfactant to balance the hydrophobicity of oil (Table 4.3).

Table 4.3 Molar fractions of lipopeptides and SDHS calculated from the HLD concept

EACN ($N_{C,O}$)	NaCl % (S)	Molar fraction	
		Lipopeptides (X_1)	SDHS (X_2)
6	3.4	0.14	0.86
10	3.4	0.27	0.73
12	3.4	0.34	0.66
16	3.4	0.47	0.53

The IFT values of the 0.1 M surfactant mixture against hexane, decane and dodecane depended on the lipopeptide molar fractions (Figure. 4.4). The lipopeptide-SDHS mixture at a 0.14/0.86 molar fraction gave the lowest IFT (0.09 mN/m) for hexane, which corresponded to the calculated value for hydrocarbons with EACN = 6 in Table 4.3. However, the IFT values for decane and dodecane for all samples were higher than 0.5 mN/m, and increasing the lipopeptide molar fractions slightly decreased the IFT values (Figure. 4.5). These results indicated that the 0.1 M mixture was compatible

with the low EACN hydrocarbons, while the high EACN hydrocarbons probably required higher lipopeptide concentrations.

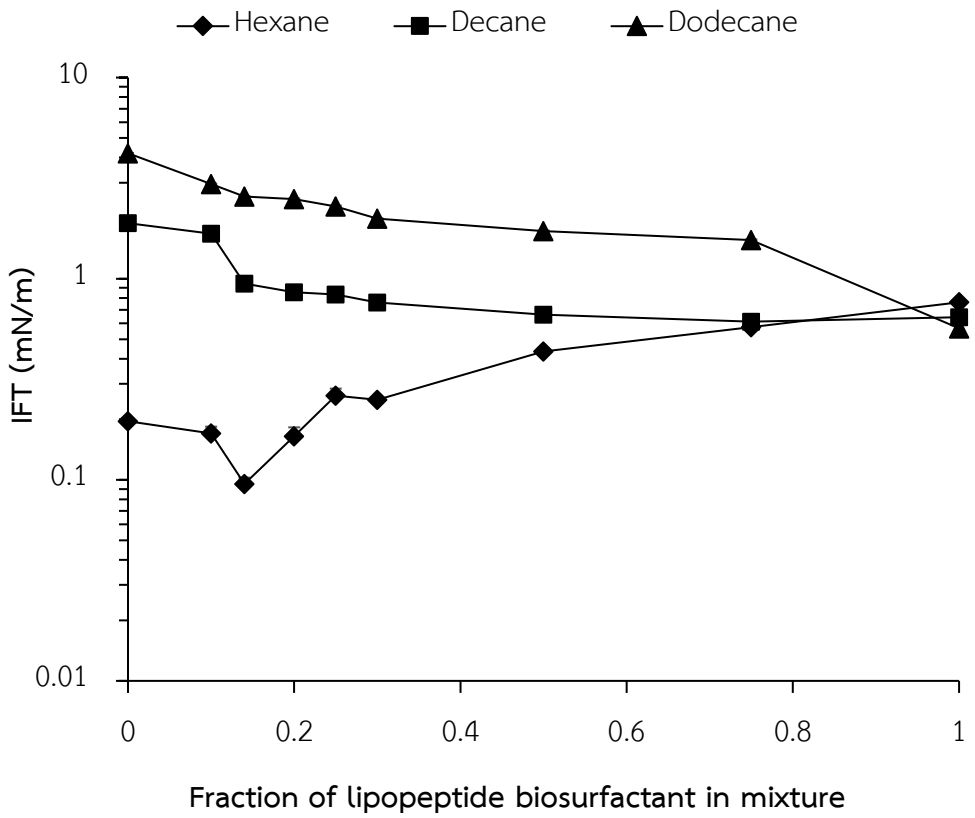


Figure 4.5 The effect of lipopeptide molar fractions on the IFT for various hydrocarbons. The total surfactant concentration was 0.1 M.

When the total surfactant concentration was increased to 0.25 M, the lipopeptide-SDHS mixture at a 0.27/0.73 molar fraction, which was calculated for decane, gave the lowest IFT against decane (0.08 mN/m), followed by dodecane (0.14 mN/m), hexadecane (0.15 mN/m) and hexane (0.31 mN/m) (Figure 4.6). Thus, the calculation using the HLD concept provided a suitable surfactant system for a specific hydrocarbon, but the total surfactant concentration should be increased for the high EACN hydrocarbons. The higher level of surfactant molecules could balance the hydrophobicity between the surfactant system, and more hydrophobic hydrocarbons and resulted in lower IFT. When comparing mixed and single surfactant systems, the

lipopeptide-SDHS mixture had lower IFT than either lipopeptides or SDHS alone for all hydrocarbons (Figure 4.6). The structure of surfactin, a major lipopeptide in *Bacillus* sp. GY19, is bulky and consists of a hydrophobic moiety with a long fatty acid chain and some lipophilic amino acids as well as a hydrophilic moiety with a backbone of the cyclic peptide and two anionic residues (Liu et al., 2015).

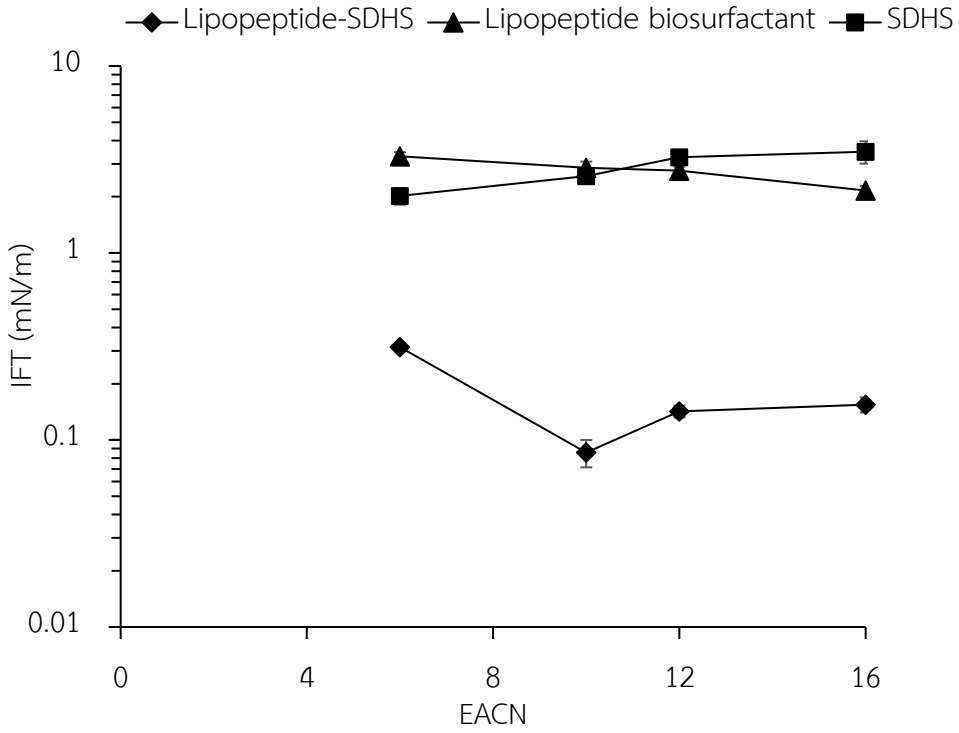


Figure 4. 6 The comparison of single and mixed surfactant systems on the IFT for various hydrocarbons. The total surfactant concentration was 0.25 M. The molar fractions of the lipopeptides and SDHS was 0.27 and 0.73, respectively.

In the system with lipopeptide alone, there would be a repulsion force between negatively charged amino acids on adjacent lipopeptide molecules. The synergistic effect of the lipopeptide-SDHS mixture was likely due to the position of the small SDHS molecule between two lipopeptide molecules. The molecule of SDHS bonded with lipopeptide biosurfactant through the hydrogen bond. The amphiphilic

molecules of the SDHS and lipopeptide immersed in water. The proposed lipopeptide-SDHS structure was showed in Figure 4.7 The arrangement of the lipopeptide-SDHS molecules on the oil-water interface would therefore reduce the IFT and allow the formation of spherical micelles with a high volume of solubilized oil.

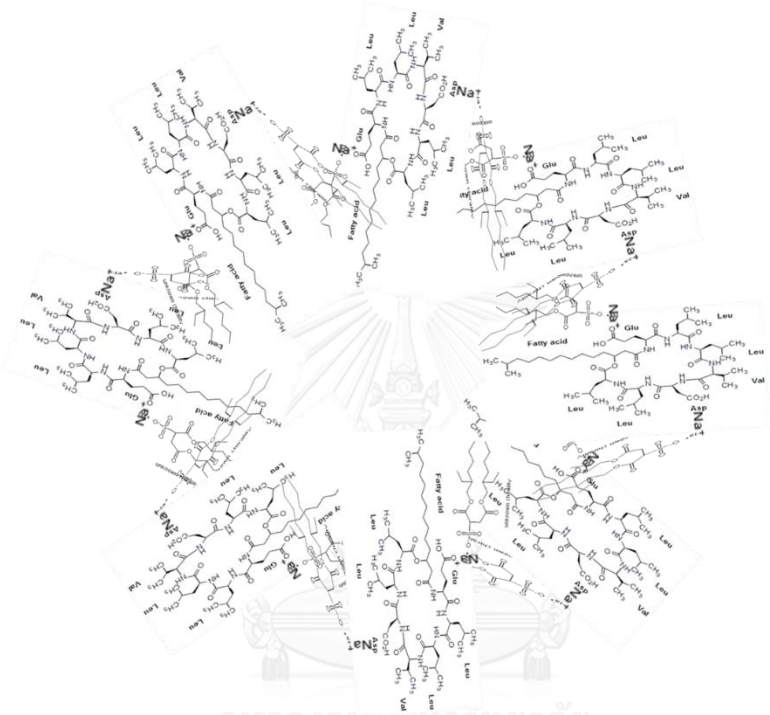


Figure 4.7 Proposed structure of micelle in the mixture.

Consequently, a dispersant for crude oil with known EACN could be formulated by calculating the molar fractions of lipopeptide and SDHS using Eq. 1. The approach was rapid and convenient. Although optimization of the total surfactant concentration might be required for some crude oils, only two more testing formulations would be required for the higher and lower surfactant concentrations. On the other hand, experimentation requires many surfactant formulations. For example, Song et al. (2013) tested 24 dispersant formulations generated from a uniform design method before acquiring a suitable dispersant for heavy crude oil.

Based on the above results, two lipopeptide-based dispersants were formulated as 0.1 M lipopeptide-SDHS mixture at a 0.14/0.86 molar fraction (formulation 1) and 0.25 M lipopeptide-SDHS mixture at a 0.27/0.73 molar fraction (formulation 2) (Table 4.4).

The compositions of Formulation 1 and 2 are shown in Table 4.4. To prepare the lipopeptide based dispersant formulations, the lipopeptides were initially dissolved in saline water (3.4% NaCl) and stirred until they fully dissolved. Then, SDHS was added to the lipopeptide biosurfactant solution and mixed well. Finally, saline water was further added to make up the 100% volume.

Table 4.4 Compositions of the lipopeptide based dispersants.

Dispersant	**Compositions**	
	Molar fraction of surfactants	**Amount of all compositions (%w/v)***
Formulation 1	Lipopeptides 0.14 SDHS 0.86 (Final surfactant concentration = 0.1 M)	Lipopeptides 1.4%, SDHS 2.9% and NaCl 3.4%
Formulation 2	Lipopeptides 0.27 SDHS 0.73 (Final surfactant concentration = 0.25 M)	Lipopeptides 7%, SDHS 6.1% and NaCl 3.4%

* The freeze-dried foamate powder contained 50% (w/w) lipopeptides. Consequently, the given weight of lipopeptides must be multiplied by 2 when preparing the lipopeptide based dispersant from powder. For example, formulation 1 is composed of 2.8 g of lipopeptide powder and 2.9 g of SDHS in 100 mL of saline water.

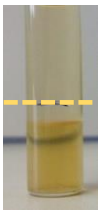
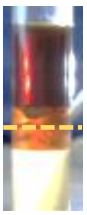
4.3.2 Effectiveness of lipopeptide-based dispersants

The efficiencies of lipopeptide-based dispersants were determined from their abilities to form microemulsion, reduce IFT, and disperse and solubilize crude oil in synthetic seawater. Formulation 1 was expected to work well with BKC crude oil because the oil should have a low EACN (≅ 6) based on the low viscosity and density (Table 4.1). On the other hand, formulation 2 should be compatible with the ARL+AXL blend (EACN ≅ 10-12) due to its higher viscosity and density (Table 4.1).

From phase behavior study, formulation 1 formed microemulsion Type III with hexane, ARL+AXL blend and BKC crude oil, while formulation 2 could formed microemulsion Type III with decane and the ARL+AXL blend and Type II with less hydrophobic hydrocarbon, hexane (Table 4.5). The results were corresponded with data in Figure. 4.8, which showed that high concentrations of lipopeptides as in formulation 2 were required in the system with more hydrophobic hydrocarbons.

On the other hand, the high concentration of lipopeptides was not suitable for less hydrophobic hydrocarbon, thus the formation of microemulsion Type II was occurred in the system containing hexane and formulation 2. All systems with microemulsion Type III had a very low IFT (0.08–0.1 mN/m) (Table 4.5). The IFT of formulation 2 against the ARL+AXL blend was lower than that of formulation 1; as a result, formulation 2 was more appropriate for the ARL+AXL blend. The similar microemulsion Types found from crude oil with relevant EACN hydrocarbons, e.g., the ARL+AXL blend vs decane and the BKC crude oil vs hexane, confirmed that the dispersant formulation could be prepared based on Eq. 1 by using the estimated EACN of each crude oil.

Table 4.5 Microemulsion type and IFT of lipopeptide-based dispersants.

Hydrocarbon/ Crude oil	Formulation 1*		Formulation 2*	
	Microemulsion	IFT (mN/m)	Microemulsion	IFT (mN/m)
Decane		0.9467		0.0857
	No emulsion		**Type III**	
Hexane		0.0954		0.3137
	Type III		**Type II**	
ARL/AXL blend		0.1548		0.0832
	Type III		**Type III**	
BKC		0.1314		0.3084
	Type III		**Type II**	

*Formulation 1 contained 1.4% lipopeptides and 2.9% SDHS, while formulation 2 contained 7% lipopeptides and 6.1% SDHS.

The oil displacement test showed that both formulations could be applied at a low dosage (DOR) for specific crude oils (Figure 4.8). For the ARL/AXL blend, formulation 2 had >90% oil displacement at DOR 1:75, while formulation 1 and Slickgone required DOR 1:10 for the same oil displacement (Figure 4.8a). For BKC crude oil, formulation 1 at DOR 1:50 had almost 100% oil displacement, which was more effective than formulation 2 and Slickgone at the same DOR (Figure. 4.8b). Lipopeptide alone had the lowest % oil displacement for both crude oils (Figure 4.9).

The dispersed oil from both formulations solubilized well in a water column, as seen from the higher % effectiveness in the baffled flask test compared with Slickgone and lipopeptide alone (Figure. 4.9). Formulation 1 had a much higher effectiveness for BKC crude oil (97%) than for the ARL/AXL blend (60%). The effectiveness of formulation 2 was 91% for the ARL/AXL and 81% for the BKC crude oil. These results confirmed the specificity of each formulation and indicated the higher efficiency of these formulations over commercial dispersant and lipopeptide alone.

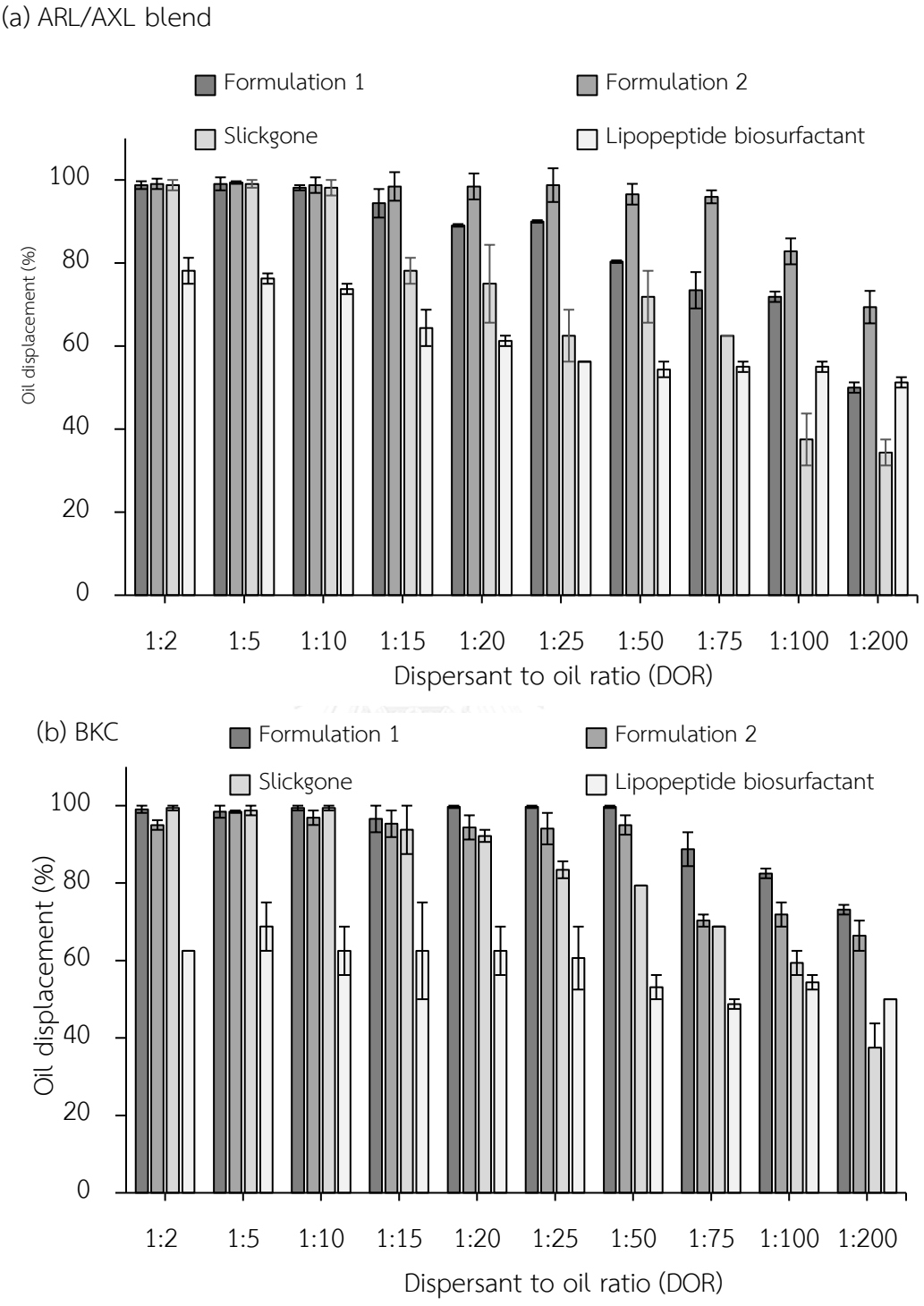


Figure 4.8 Oil displacement test of lipopeptide-based dispersants, Slickgone and lipopeptides against the ARL/AXL blend (a) and BKC crude oil (b). Formulation 1 contained 1.4% lipopeptides and 2.9% SDHS, while formulation 2 contained 7% lipopeptides and 6.1% SDHS.

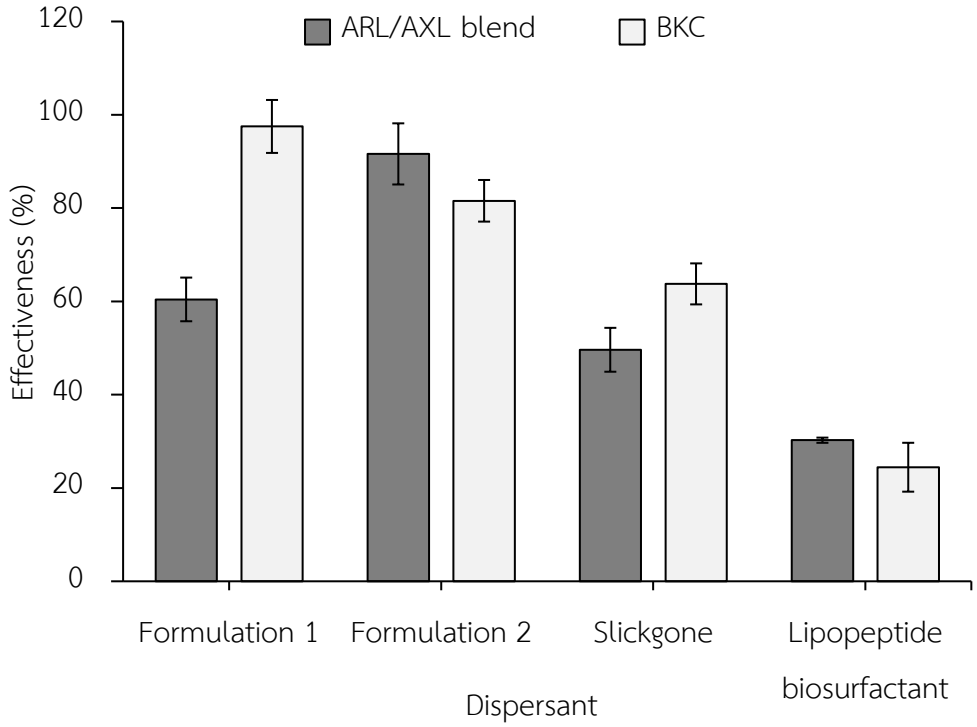


Figure 4.9 Effectiveness of lipopeptide-based dispersants, Slickgone and lipopeptides over crude oils in the baffled flask test. Formulation 1 contained 1.4% lipopeptides and 2.9% SDHS, while formulation 2 contained 7% lipopeptides and 6.1% SDHS.

The high efficiency of lipopeptide-based dispersants can be attributed to their ability to lower the IFT. This will enable the breaking up of oil into smaller droplets (Zeinstra et al., 2015). Then, the oil droplets can be solubilized in the seawater due to the formation of mixed lipopeptides and SDHS micelles. The synergistic effect of mixed surfactants on crude oil dispersion was also found for a mixture of lecithin and Tween-80; however, this formulation requires ethanol as a solvent (Athas et al., 2014).

To the best of our knowledge, this is the first study to formulate an oil dispersant without using a solvent and with only two ingredients. The efficiency of dispersants should be confirmed in a larger experimental setting because the behaviors of the dispersed oil also depend the on sea energy, as demonstrated by a 1000 kL in

situ mesocosm study by Joo et al. (2013). In addition, the biodegradability and toxicity of the dispersed oil should be studied in future research.

4.3.3 Acute toxicity

The previous result found that lipopeptide biosurfactant from *Bacillus* sp. GY19 itself had low toxicity to both of copepod and whiteleg shrimp. However, the lipopeptide based dispersant formulation which consisted of lipopeptide biosurfactant and sodium dihexyl sulfosuccinate might have different acute toxicity on marine organisms.

This study focused on the lipopeptide based dispersant containing lipopeptides 1.4%, SDHS 2.9% and NaCl 3.4%, which had high effectiveness with Bongkot light crude oil. The toxicity test was determined with spilled oil (WAF with Bongkot light crude oil), the lipopeptide based dispersant formulation and the dispersed oil (CEWAF with Bongkot light crude oil and lipopeptide based dispersant) using whiteleg shrimp. Percent mortality of whiteleg shrimp after a 96-h exposure of lipopeptide based dispersant, WAF with Bongkot light crude oil, the lipopeptide based dispersant formulation and the dispersed oil and CEWAF with Bongkot light crude oil and lipopeptide based dispersant was shown in Figure 4.10. The lethal concentration at 10% and 50% was calculated using probit analysis and shown in Table 4.6.

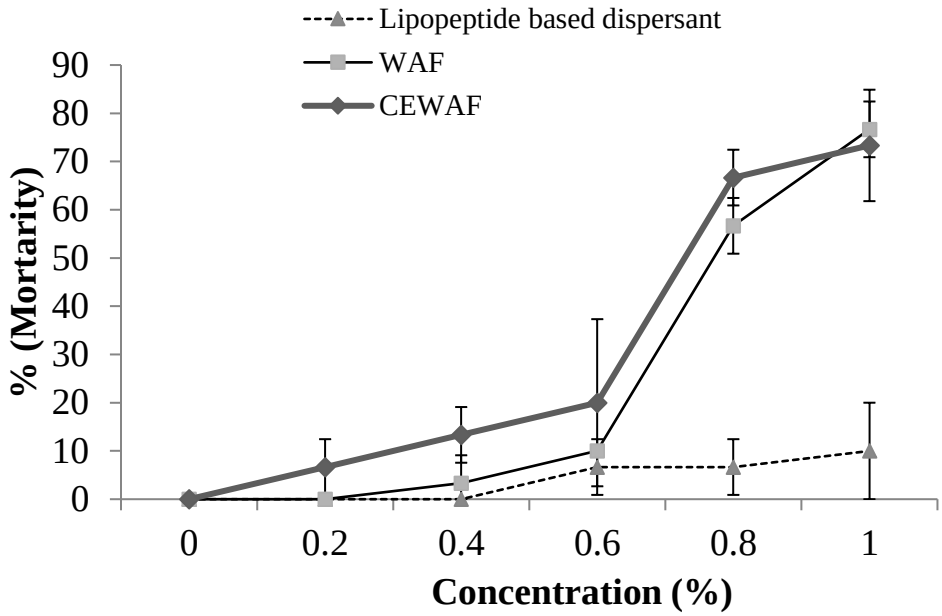


Figure 4.10 Percent mortality of whiteleg shrimp after a 96-h exposure of lipopeptide based dispersant, WAF with Bongkot light crude oil, the lipopeptide based dispersant formulation and the dispersed oil and CEWAF with Bongkot light crude oil and lipopeptide based dispersant

Table 4.6 Lethal concentration at 10 % and 50% (LC10 and LC50) for whiteleg shrimp exposed to various samples at 96 hr.

Samples	LC_{10} (%)	$LC_{50,}$ (%)
Lipopeptide based dispersant	93.7	>100.0
WAF	53.7	81.28
CEWAF	32.0	73.17

The whiteleg shrimp acute toxicity test found that lipopeptide based dispersant was less toxic than WAF and CEWAF. When lipopeptide based dispersant was applied, the molecule of the lipopeptide based dispersant increased the amount of crude oil solubilization in water column as in CWAF. From this reason, the toxicity of CWAF (LC50 73.17%) was slightly higher than WAF (LC_{50} 81.28%). However, most of chemical dispersant such as Corexit series had toxicity to aquatic organism by itself and could increase the toxic from the spilled oil 2-10 times (Couillard et al., 2005, Gardeström et al., 2006, Coelho et al., 2013, Lee et al., 2013)). Therefore, we can confirm that the formulated biosurfactant based dispersant was safe for marine organisms.

4.4 Conclusions

Bacillus sp. GY19 lipopeptides that were prepared from freeze-dried foamate had good surface activity under saline conditions and low toxicity to marine organisms. As a result, they were appropriate ingredients in the oil dispersant. The optimum HLD (HLD = 0) was used to calculate the fractions of lipopeptides and SDHS in the dispersant formulation based on the EACN of hydrocarbons and seawater salinity. All lipopeptide-based dispersants were prepared and applied without any solvent. Due to their synergistic effect, the formulation with a suitable lipopeptide-SDHS molar fraction had higher dispersant effectiveness than lipopeptides alone.

The lipopeptide based dispersant slightly increased the toxicity of dispersed oil to the whiteleg shrimp and this was due to the enhancing of crude oil solubilized in seawater. The extent of toxicity was low when compared to other reported dispersants. Therefore, this result suggested that the lipopeptide based dispersant was applicable for petroleum remediation.

The formulation could be further optimized by increasing the total surfactant concentration for more hydrophobic and heavier crude oil. For seawater with different salinities, the dispersant could be formulated using other NaCl concentrations. In conclusion, the HLD concept can be conveniently applied to formulate environmentally friendly lipopeptide-based dispersants for the clean-up of crude oil spills.

Chapter V

Application of lipopeptide based dispersant along with petroleum degrading bacteria for oil spill remediation

5.1 Introduction

Bioremediation is a process whereby microorganisms degrade and metabolize chemical substances and restore environment quality. It aims to accelerate the natural attenuation process through which microorganisms assimilate organic molecules to cell biomass and produce by-products such carbon dioxide, water and heat (Atlas and Cerniglia, 1995). A common feature of crude oil is low water solubility, which poses special problems for those microorganisms capable of utilizing such water-immiscible substrates as source of carbon and energy (Chandran and Das, 2012). Dispersant containing surfactants molecule could enhance solubilization of crude oil. Biodegradation is therefore enhanced by surfactants due to increasing bioavailability of pollutants (Cameotra and Singh, 2009). The lipopeptide based dispersant formulated from previous experiment (Chapter IV) showed a good ability to dispersed petroleum crude oil in seawater. Moreover, the formulation had low toxicity to the marine organisms.

The main mechanism of hydrocarbon biodegradation is occurred under aerobic condition. It starts with intracellular attack to organic pollutant; oxidative process cooperated with oxygen using oxygenases, and peroxidases. The conversion of intermediate can occur step by step and synthesize through tricarboxilic acid cycle, while biomass, carbon dioxide, and water are products from this pathway (Das and Chandran, 2011). Recently, Laorrattanasak et al. (2016) reported that biosurfactant from

Gordonia westfalica GY40 promoted the activity of *Gordonia* sp. JC11, a petroleum degrading-bacterium on degrading fuel oil in seawater. Therefore, in this study lipopeptide based dispersant was applied with petroleum degrading bacteria, *Gordonia* sp. JC11, to study their efficiency on removal of Bongkot light crude oil. The initial experiment was conducted with contaminated sand in a small-scale experiment. Then, 3D-box oil spill simulation experiment was performed in rectangular glass tanks with 78 L capacity (150 long, 35 deep and 15 wide and 277 L capacity (150 long, 44 deep and 42 wide) for small and medium scale experiments, respectively. The seawater samples were consisted of both synthetic and natural seawater.

The results from this study would confirm the potential application of lipopeptide based dispersant along with added petroleum-degrading bacteria for accelerating petroleum oil removal. In the experiment with natural seawater, the activity of indigenous bacteria on degrading dispersed oil was investigated to determine whether the lipopeptide based dispersant could be applied alone in the seawater after oil spill.

5.2 Materials and methods

5.2.1 Lipopeptide based dispersant and chemicals

The lipopeptide based dispersant used in this experiment was the mixture of lipopeptide biosurfactant 0.025 M and sodium dihexyl sulfosuccinate 0.075 M in 3.4 % of NaCl solution. From the previous results, this lipopeptide based dispersant performed the microemulsion type III and had high dispersion efficiency with Bongkot light crude oil.

Synthetic seawater was prepared by using the sea salt purchased from Mariscience Int'l Co., Ltd. containing all the essential major and minor elements of the sea. The synthetic seawater was prepared at 34 ppt salinity to represent the natural seawater. The natural seawater was collected from Ao Udom port, Amphoe Si Racha, Chonburi Province, Thailand (Figure 5.1). The physical properties of seawater sample used in the 40L and 160 L mesocosms were shown in Table 5.1.

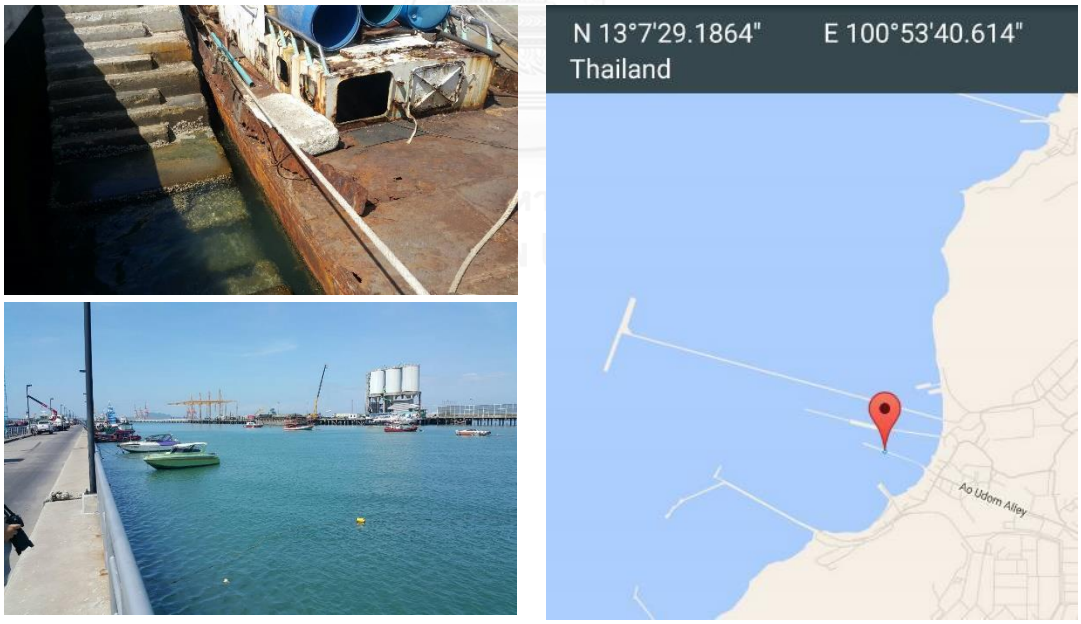


Figure 5.1 The collecting sample point at Ao Udum Port, near Thai oil public company transportation port.

Table 5.1 Physical properties of natural seawater samples for 40 L mesocosms

	40 L		160 L
Parameter	1st	2nd	1st
Date of collecting	11 December 2015	18 September 2016	11 December 2015
Sample condition	Clear and odorless	Clear and odorless	Clear and odorless
Total Nitrogen	0.1 mg/L	<0.1 mg/L	0.1 mg/L
Total Phosphorus	0.12 mg/L	0.56 mg/L	0.12 mg/L
Oil&Grease	< 0.1 mg/L	< 0.1 mg/L	< 0.1 mg/L
Total suspended solid	10.3	10.5 mg/L	10.3
Salinity (ppt)	34	32.5	34
pH	7.87	7.69	7.87

Gordonia sp. JC11 isolated by Chanthamalee and Luepromchai (2012) was prepared by culturing in 25% LB broth for 5 day. The bacteria were applied at 10% volume of 40 L and 160 L seawater in the 3D-box mesocosm tank.

Bongkot light crude oil dyed with Red O oil

Lipopeptide based dispersant

Gordonia sp. JC11

Figure 5. 2 Bongkot light crude oil dyed with Red O oil, Lipopeptide based dispersant and *Gordonia* sp. JC11 stock solution

5.2.2 Application of lipopeptide based dispersant and *Gordonia* sp. JC11 for oil removal from sand in small scale experiment

The experiment was conducted in 125 mL Erlenmeyer flask containing 10 g of sand collected from Pattaya Beach (Figure 5.3). The sand was sterilized before used. Bongkot light crude oil was added at 400 mg to the sand overnight before experiment. Then, the lipopeptide based dispersant was added to the contaminated sand at 1:5 dispersant to oil ratio (DOR) followed by 5 mL of NSW as describe in Tabel A.2. The test was shaken for 30 min at 200 rpm before adding 1 mL of *Gordonia* sp. JC11 stock solution (OD_{600} = 1). The experiment was incubated for 10 days. The samples were collected at day 0, 7 and 10 to analyze for the remaining crude oil and bacteria number.

Figure 5.3 Sand sample from Pattaya Beach

5.2.3 Simulation of 3D-box model (mesocosm tanks) for oil spill remediation process

Oil spill mesocosm experiments were carried out to evaluate the potential of lipopeptide based dispersant and *Gordonia* JC11 on petroleum removal. The 3D-box experiment was performed in rectangular glass tank and was divided into two sizes including 40 L in the 78 L tank capacity (150 long, 35 deep and 15 wide) and 160 L in the 277 L capacity (150 long, 44 deep and 42 wide). The diagram of 3D mesocosm tank experiments was illustrated below (Figure 5.4) and the experiment set up was described in the following section.

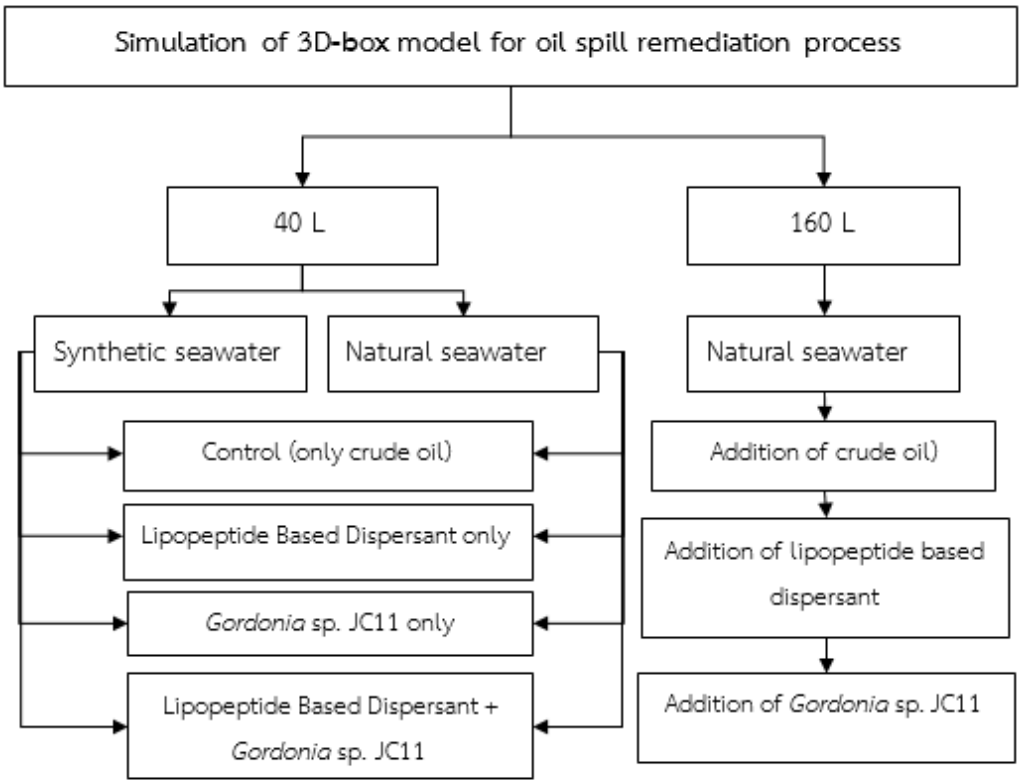


Figure 5. 4 The experimental diagram of simulation of 3D-box model for oil spill remediation process

5.2.3.1 Experimental set up of 40 L mesocosm tank

The mesocosm tank was filled with 5 cm depth sand and 40 L of seawater. The seawater provided in this experiment consisted of synthetic seawater and natural seawater, which were carried out to confirm the activity of lipopeptide based dispersant along with Gordonia sp. JC11 and indigenous bacteria. In every treatment, 9 mL of Bongkot light crude oil was slowly dropped on the surface water. Each treatment was carried out separately as follows;

1. Bongkot light crude oil only: there was only crude oil in the seawater.

2. Lipopeptide based dispersant only: After addition of crude oil, 4.5 mL of lipopeptide based dispersant was sprayed on oil film to achieve the DOR 1:2 for synthetic seawater and DOR 1:5 for natural seawater.

3. *Gordonia* sp. JC11 only: After addition of crude oil, 4 L of *Gordonia* sp. JC11 stock solutions as prepared above was added.

4. Lipopeptide based dispersant and *Gordonia* sp. JC11 : After addition of crude oil, 4.5 mL of lipopeptide based dispersant was sprayed on oil film and 4 L *Gordonia* sp. JC11 stock solutions was added 30 minute later.

The seawater was mixed by a wave generator pump (pump power 1200 l h^{-1}), placed at the one side of the tank. The sampling port was set on the top of the tank to hold the silicone tube at 4 locations including

i. Point 1 which placed near the wave generator the collecting point at 5-depth and 15-depth

ii. Point 2 which placed near another side of the tank and placed far from the wave generator the collecting point at 5-depth and 15-depth. (see Figure 5.5).

The 30 mL seawater sample from each point was taken aseptically, using sterile syringe connected with silicone tube every day for 5-7 days. The samples were analyzed for remaining crude oil by total oil analyzer (Horiba) and number of total bacteria and oil degrading bacteria using MPN method using marine broth (Table A.3 in appendix A).

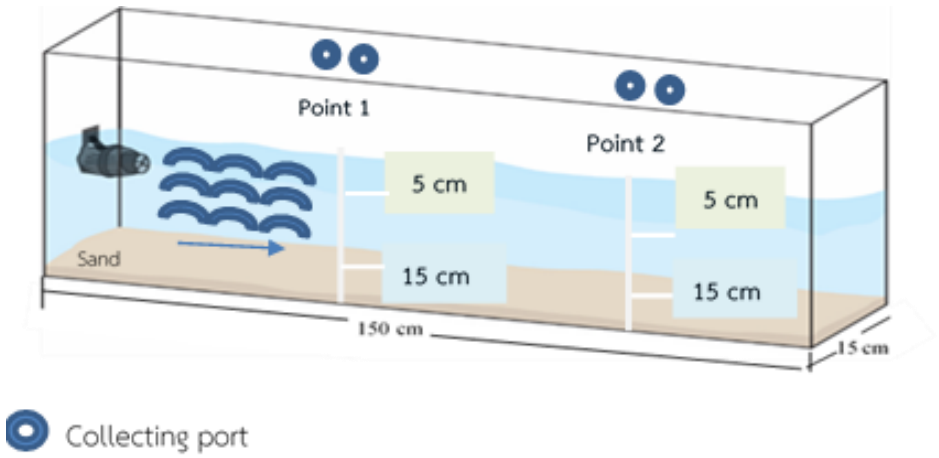


Figure 5. 5 Diagram of the 40 L mesocosm tank as a small-scale experiment used in this study. The collecting points were conduct with silicone tube at 2 points.

5.2.3.2 Experimental set up of 160 L mesocosm tank

To confirm the application of lipopeptide based dispersant along with *Gordonia* sp. JC11, an experiment was carried out in 160 L mesocosm (277 L 3D-box tank) containing natural seawater. Due to the larger tank size, the seawater was mixed by a controllable wave generator, which is a propeller pump model RW15 from Jebao company. It can generate the power pump in the wide range of 1200 -15,000 L h^{-1}. The wave maker was set up at W2 as a continues wave maker and placed at the one side of the tank the speed set up was shown in Figure 5.6.

Figure 5. 6 The eco-propeller pump model RW15 was set up was a W2 as a continuous wave maker and the speed was set at the lowest point (green light) and the power pump was set at the fastest point.

The sampling port was set on the top of the tank to hold the silicone tube for taking a sample as same as in 40 L mesocosm experiment. Bongkot light crude oil was filled into the tank on the surface water 63 mL to achieve the oil layer of 0.01 mm thick. The treatment was described in Table 5.2.

Table 5.2 Time table on applying the lipopeptide based dispersant and *Gordonia* sp. JC11 in the 160 L mesocosm tank.

Time	Treatment
0 hr	Bongkot light crude oil was added at 63 mL on the top of the tank.
18.30 hr	Lipopeptide based dispersant was sprayed on the top of the tank at DOR 1:5 (lipopeptide based dispersant 12.5 mL)
24.5 hr	*Gordonia* sp. JC11 was added at 16 L on the top of the tank to achieve 10% of stock solution.

To monitor the water quality, 30 mL of seawater from 4 locations i.e. point 1 and point 2 at 5-cm and 15-cm depths from surface water (see Figure 5.7) at 0 hr, 6 hr, 18 hr, 19 hr, 24 hr, 24.5 hr, 30 hr, 42 hr, 54 hr, 66 hr, 72 hr, 84 hr, 96 hr, 120 hr, 144 hr, and 168 hr, respectively. The samples were analyzed for remaining crude oil and number of total bacteria and oil degrading bacteria using MPN method as same as in 40 L mesocosm tank (section 5.2.3.1).

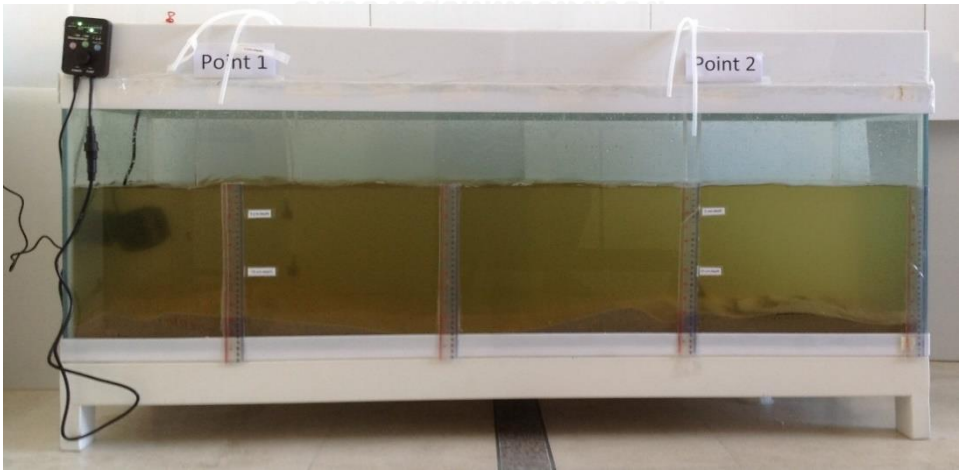


Figure 5.7 The 160 L medium scale mesocosm tank used in this study. The collecting points were conduct with silicone tube at 2 difference positions (point 1 and 2).

5.3 Results and discussion

5.3.1 Application of lipopeptide based dispersant and *Gordonia* sp. JC11 for oil spill removal from sand in small scale experiment

The application of lipopeptide based dispersant was first determined by small scale experiment. Sand washing was performed to study the ability of lipopeptide based dispersant on crude oil removal. From the oil spill accident, crude oil was spread and contaminated both surface seawater and coastal. Therefore, in this studied the Bongkot light crude oil was used as a model to remove from sand collecting from Pattaya beach by using lipopeptide based dispersant and petroleum degrading bacteria, *Gordonia* sp. JC11, on enhancing crude oil removal from sand.

At 30 min-shaking, the ability of lipopeptide based dispersant was on crude oil was observed. The effect of lipopeptide based dispersant in the treatment with lipopeptide based dispersant (lipopeptide based dispersant only and lipopeptide based dispersant + *Gordonia* sp. JC11) was compare to the treatment without adding lipopeptide based dispersant (seawater only as a control and *Gordonia* sp. JC11 only). The remaining crude oil in sand was extracted and shown in Figure 5.8. The dispersant was able to desorp the oil out of sand as seen from the lower amount of remaining oil after 30-min shaking than those in the control and *Gordonia* sp. JC11 only treatments.

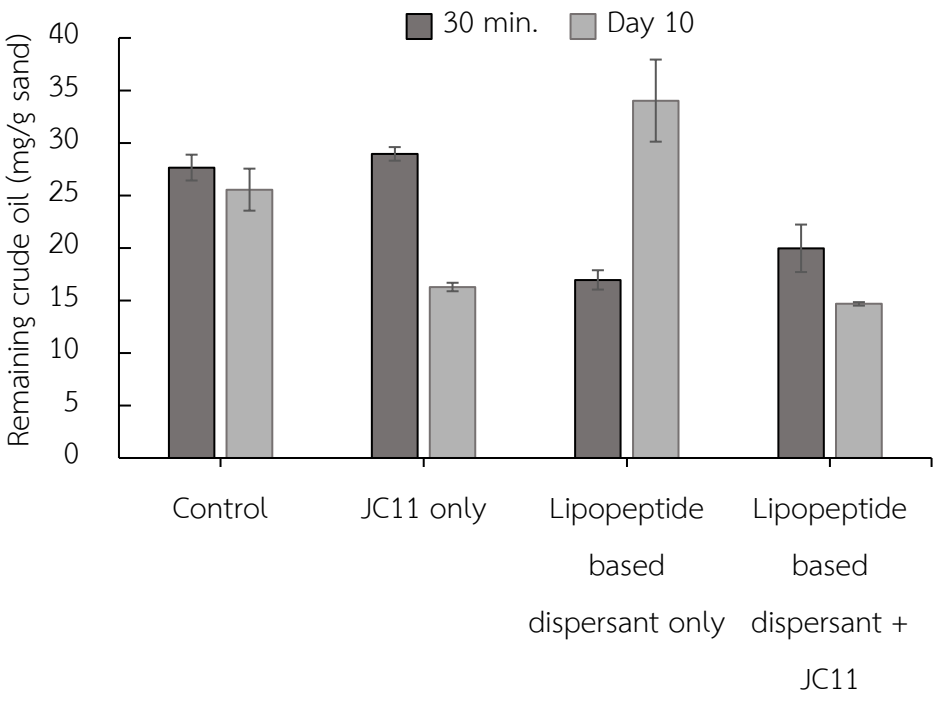


Figure 5. 8 The remaining crude oil in sand after 30-min shaking time and 10-day incubation. The control experiment contained only synthetic seawater.

After 10-day incubation, the result showed that the remaining crude oil in sand with lipopeptide based dispersant and *Gordonia* sp. JC11 (14.67 mg/g) was lower than the control (25.5 mg/g sand) and lipopeptide based dispersant only (34.03 mg/g sand) treatments (Figure 5.8). Without the dispersant, *Gordonia* sp. JC11 had lower oil-degrading activity and slightly more oil was remained (16.28 mg/g). These results indicated that *Gordonia* sp. JC11 played major role in oil degradation and lipopeptide based dispersant could enhance the bacterial activity. Saeki et al. (2009) also found that biosurfactant from spray drying sterilized culture broth of *Gordonia* sp. strain JE-1058 is able to remove crude oil at higher efficiency than seawater alone and increase the oil degradability of the indigenous microorganisms. The high activity of *Gordonia* sp. JC11 in the presence of biosurfactant was similar to Laorrattanasak et al. (2016).

In the treatment with lipopeptide based dispersant only, the amount of oil on day 10 was the highest when compared with other treatments (Figure 5.8). This might be due to the re-sorption of oil back to the sand after incubation. The remaining crude oil increased from 16 mg/g sand after 30-min shaking to 34 mg/g sand on day 10. Sand used in this experiment was a sterile sand which had no indigenous bacteria. Therefore, the biodegradation of crude oil did not occur. Consequently, the lipopeptide based dispersant should be applied along with oil-degrading bacteria for a complete oil removal.

The number of *Gordonia* sp. JC11 in two treatments slightly increased which confirmed bacterial growth after petroleum consumption Figure 5.9. These results indicated that *Gordonia* sp. JC11 had a strong potential to be applied with the lipopeptide based dispersant for clean-up of oil spill in the marine environment. In addition, the oil mobilized by lipopeptide based dispersant could be easily biodegraded by the bacteria.

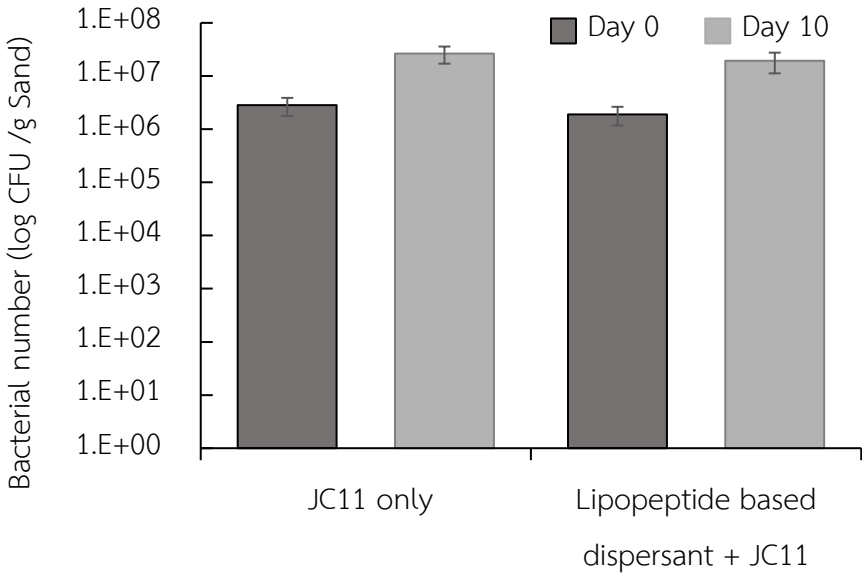


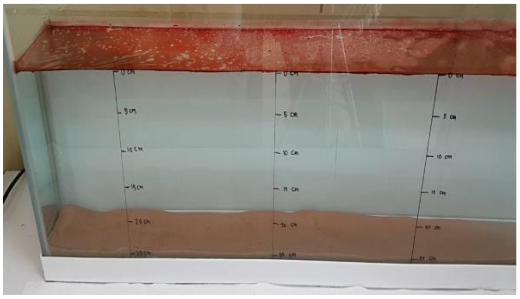
Figure 5.9 The number of *Gordonia* sp. JC11 at day 0 and 10 from the treatment with *Gordonia* sp. JC11 only and lipopeptide based dispersant along with *Gordonia* sp. JC11.

5.3.2 Simulation of 40 L 3D-box model (mesocosm tanks) for oil spill remediation process

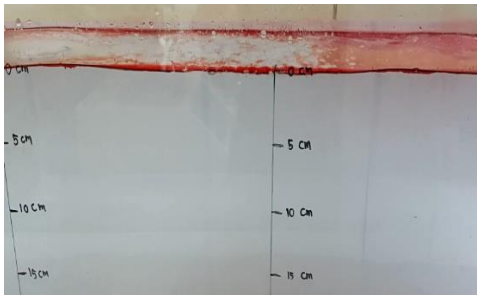
5.3.2.1 Mesocosm experiment using synthetic seawater

The previous experiment found that the lipopeptide based dispersant showed a good performance on crude oil removal from sand when applied along with oil-degrading bacteria. However, the environmental condition such as sea energy may influence on the efficiency of dispersants. The mesocosm experiment is required to simulate the natural phenomena in a laboratory-scale study. The mesocosm experiments were performed in difference scale ranging from 30 L -10, 000 (Gertler et al., 2012, Joo et al., 2013, Hassanshahian et al., 2014). In addition, a mesocosm study is cheaper than a full-scale field study (Joo et al., 2013). Therefore, this study initially confirmed the efficiency of lipopeptide based dispersant in mesocosm experiments with synthetic seawater to avoid the effect of indigenous bacteria.

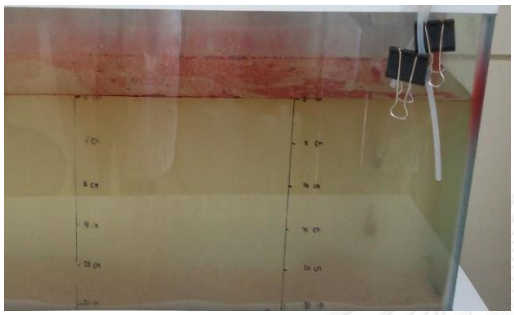
After the lipopeptide based dispersant was sprayed on the oil layer, the oil was dispersed in a wide range and some oil droplets was occurred while there was no oil dispersion in the control experiment. For the experiment with *Gordonia* sp. JC11, the water turned turbid to orange color from the bacterial cells. The characteristic of oil film at the beginning of the treatment was shown in Figure 5.10. These observations was similar to Bao et al. (2012), which used 600 L mesocosm tank and artificial seawater. They reported that the thick layer of crude oil in the control tank remained unchanged while in the biosurfactant and N-series bacteria consortium treatment, the seawater become turbid as a result from the dissolution of crude oil from biosurfactant action and bacteria growth (Bao et al., 2012).



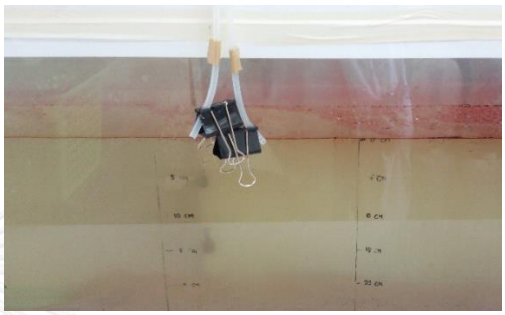
Control (Crude oil only)



Lipopeptide based dispersant only



Gordonia sp. JC11 only



Lipopeptide based dispersant + *Gordonia* sp. JC11

Figure 5.10 Characteristic of oil film in the mesocosm with synthetic seawater at day 0 including (a) crude oil only as a control, (b) lipopeptide based dispersant only, (c) *Gordonia* sp. JC11 only and (d) Lipopeptide based dispersant with *Gordonia* sp. JC11.

The sample at 0^{th} day was collected after adding all the amendments. From the figure 5.11 and 5.12, the remaining crude oil increased over time in all four points of the control experiment. The remaining crude oil was highest at day 7 in all points except in point 1 at 15-depth from the surface of water. This point had higher effect from the wave turbulence and therefore the oil was blown away to less than 5 mg/L. In the treatments with *Gordonia* sp. JC11 only and with lipopeptide based dispersant and *Gordonia* sp. JC11, the remaining crude oil in four points was lower than the control and lipopeptide based dispersant only treatments at all time points (7 day) (Figure 5.11 and Figure 5.12).

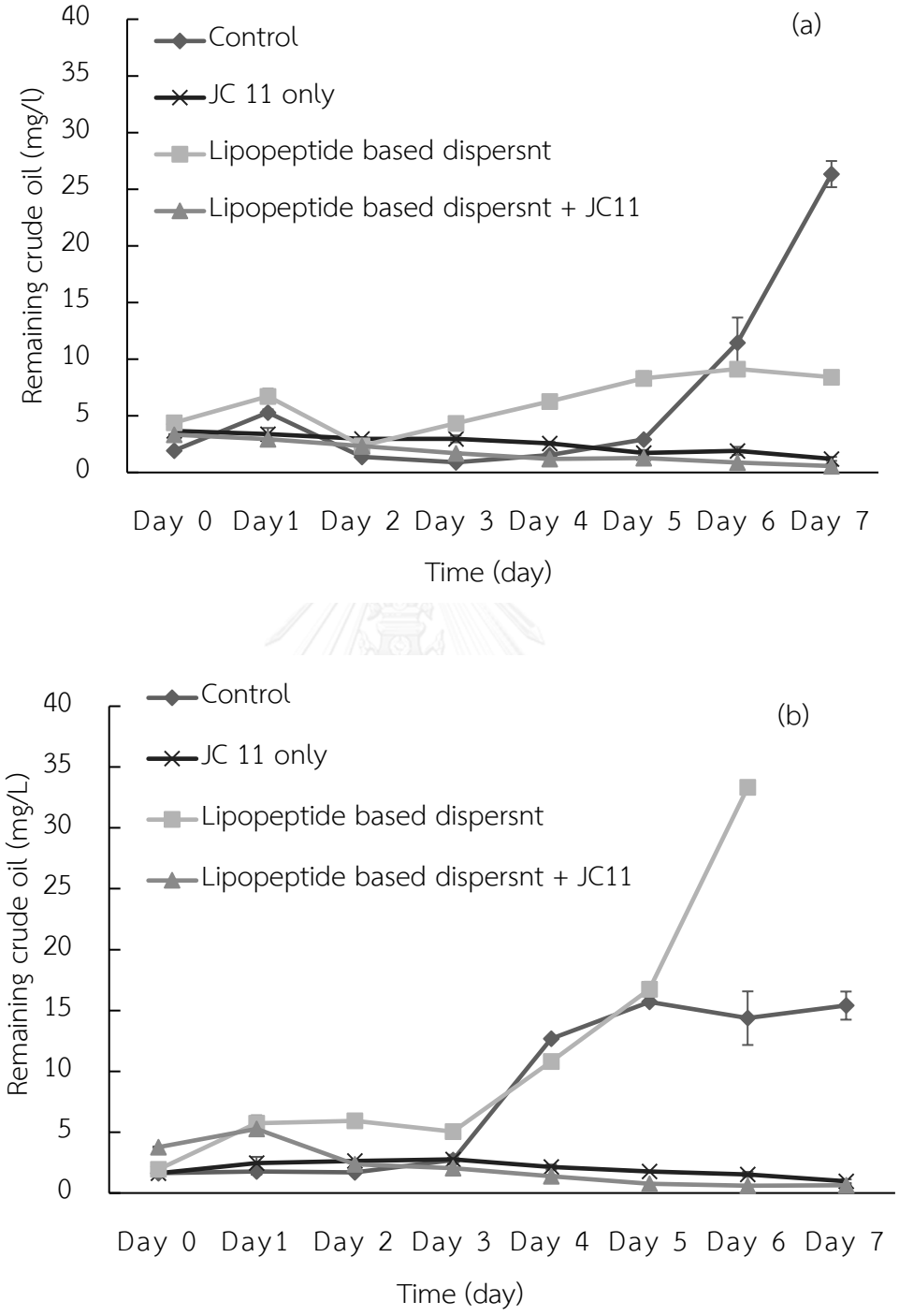


Figure 5.11 The remaining crude oil in sampling point 1 at 5-cm depths (a) and 15-cm depths (b) from the surface of synthetic seawater.

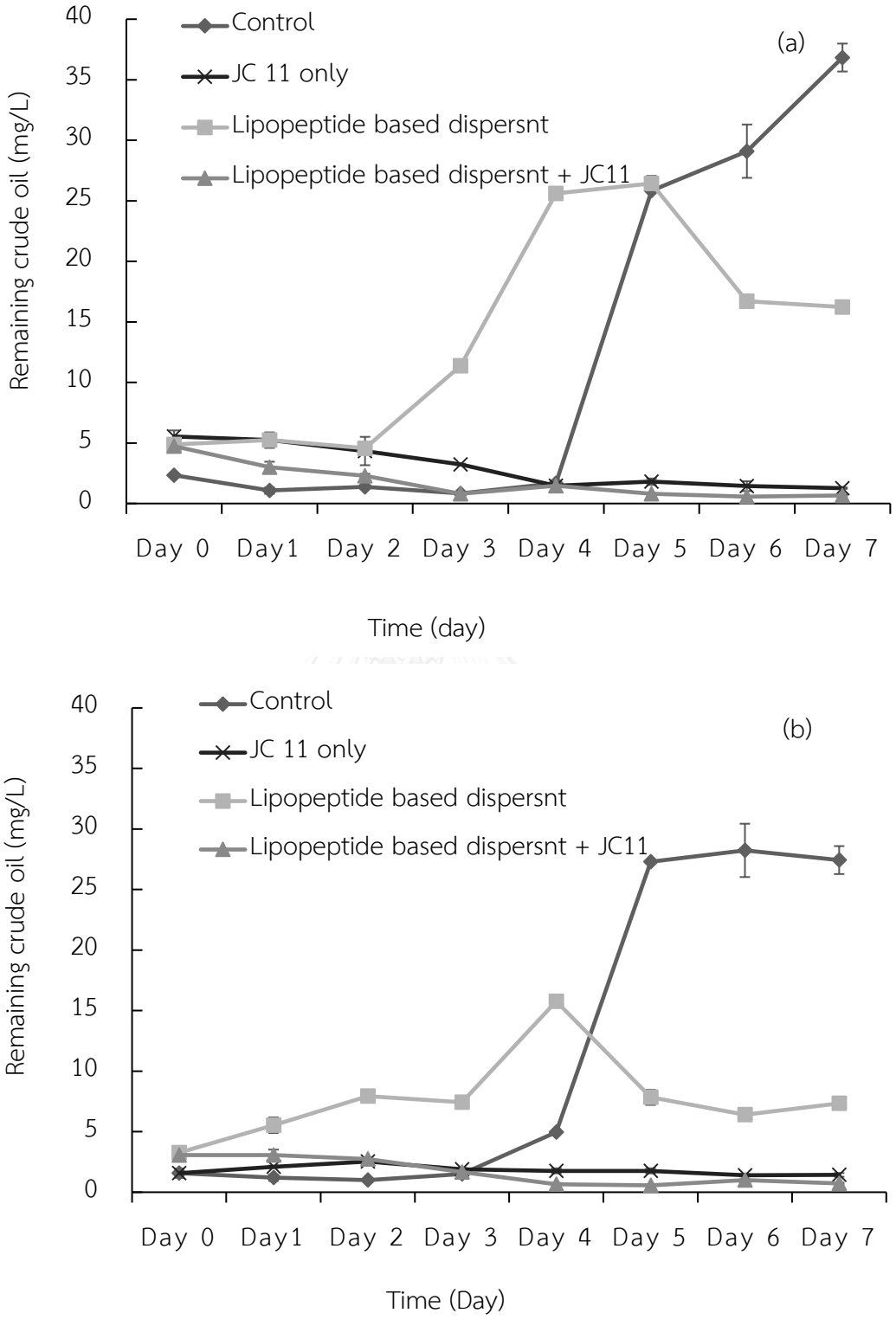
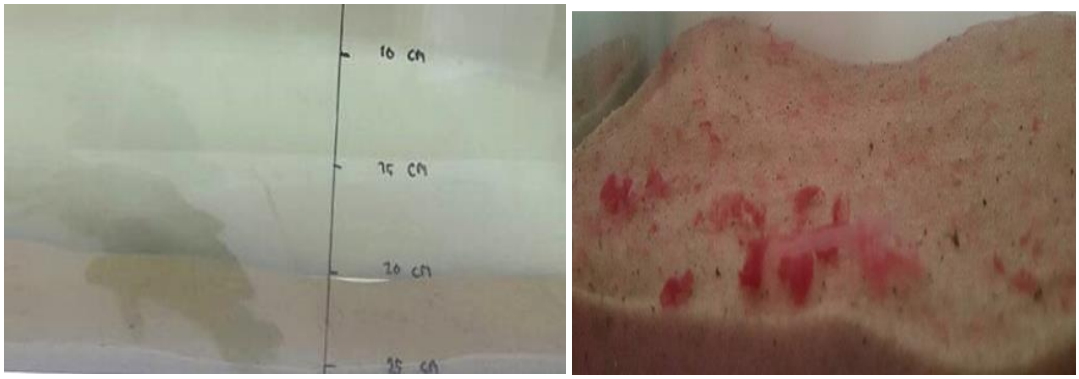


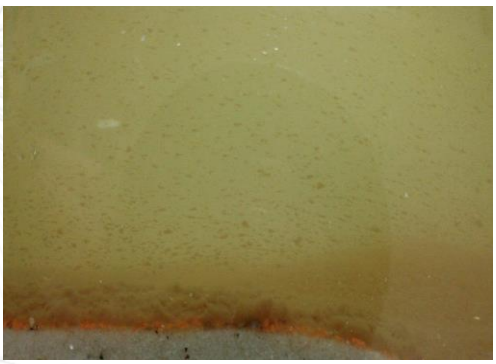
Figure 5.12 The remaining crude oil in sampling point 2 at 5-cm depths (a) and 15-cm depths (b) from the surface of synthetic seawater.

The observation of lipopeptide based dispersant only treatment found that the remaining crude oil in point 1 with more effect from the wave turbulence increased over time until the 5th day (at 5-depth) and 6th day (15-depth). After that, the remaining crude oil decreased, which was corresponded to the residual oil on the sand (Figure 5.13b.). There was an oil film on the bottom of the tank in the treatment with *Gordonia* sp. JC11 only (Figure 5.13c). In the treatment with lipopeptide based dispersant and *Gordonia* sp. JC11 (Figure 5.13d), the residual cells of *Gordonia* sp. JC11 were precipitated as seen from the orange-brown color residues at day 7. The remaining crude oil in lipopeptide based dispersant and *Gordonia* sp. JC11 mesocosm was the lowest in all sampling points.



Control (Crude oil only) (a)

Lipopeptide based dispersant only (b)

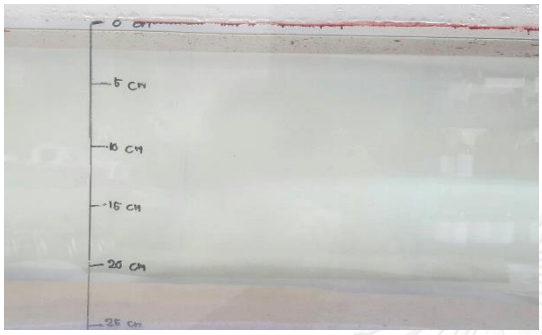


Gordonia sp. JC11 only (c)

Lipopeptide based dispersant + *Gordonia* sp. JC11 (d)

Figure 5.13 The residual of oil on the sand in the treatment with (a) crude oil only as a control (b) lipopeptide based dispersant only (c) *Gordonia* sp. JC11 only and (d) Lipopeptide based dispersant with *Gordonia* sp. JC11 at day 7. All treatments contained synthetic seawater.

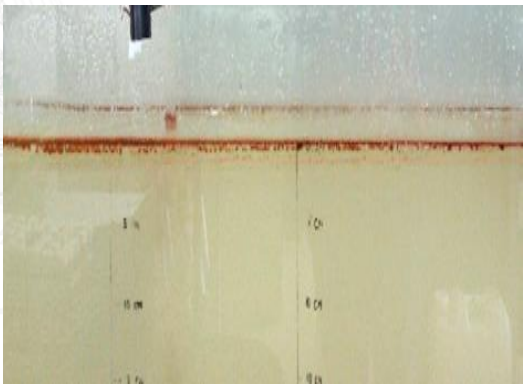
At day 7, the amount of residual crude oil on the water surface at point 1 was lower than that of point 2 which had higher wave turbulence in all treatments. These results indicated that the wave turbulence or the mixing parameter could increase the oil dispersion efficiency (Figure 5.14 and Figure 5.15).



Control (Crude oil only) (a)

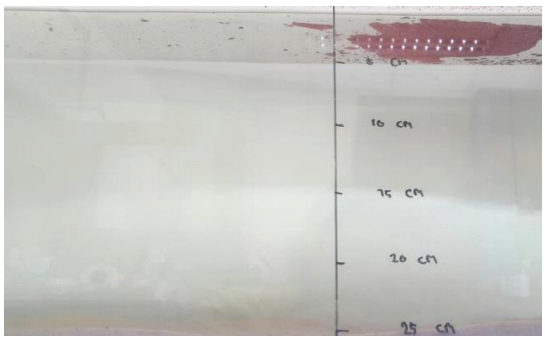
Lipopeptide based dispersant only (b)

Gordonia sp. JC11 only (C)



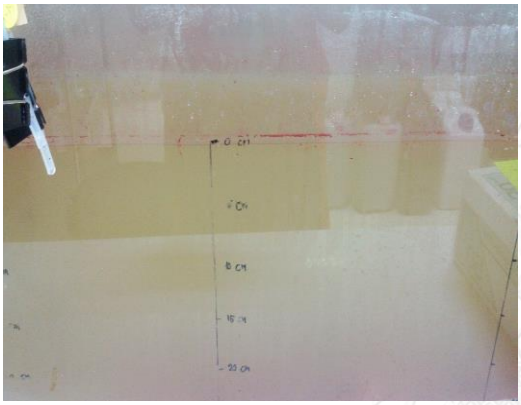
Biosurfactant based dispersant + *Gordonia* sp. JC11 (d)

Figure 5.14 The residual of oil on the surface of synthetic seawater at the point 1 of the treatment with (a) crude oil only as a control, (b) lipopeptide based dispersant only, (c) *Gordonia sp*. JC11 only and (d) Lipopeptide based dispersant with *Gordonia* sp. JC11 at day 7.

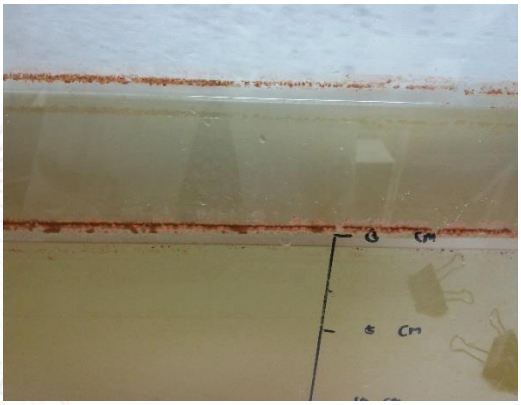


Control (Crude oil only)

Lipopeptide based dispersant



Gordonia sp. JC11 only



Lipopeptide based dispersant + *Gordonia* sp. JC11

Figure 5.15 The residual of oil on the surface of synthetic seawater at the point 2 of the treatment with (a) crude oil only as a control, (b) lipopeptide based dispersant only, (c) *Gordonia sp*. JC11 only and (d) Lipopeptide based dispersant with *Gordonia* sp. JC11 at day 7.

In the treatment with added bacteria, the cell number increased from 1.7 x10^7 – 3 x 10^7 CFU/mL to 6.5 x 10^7- 9.5x 10^7 CFU/mL at day 3 and slightly decreased to 1.7 x 10^6 – 6.5 x 10^7 CFU/mL at day 7 (Figure 5.16). Similar to section 5.3.1, the result indicated that *Gordonia* sp. JC11 degraded crude oil after the application of lipopeptide based dispersant.

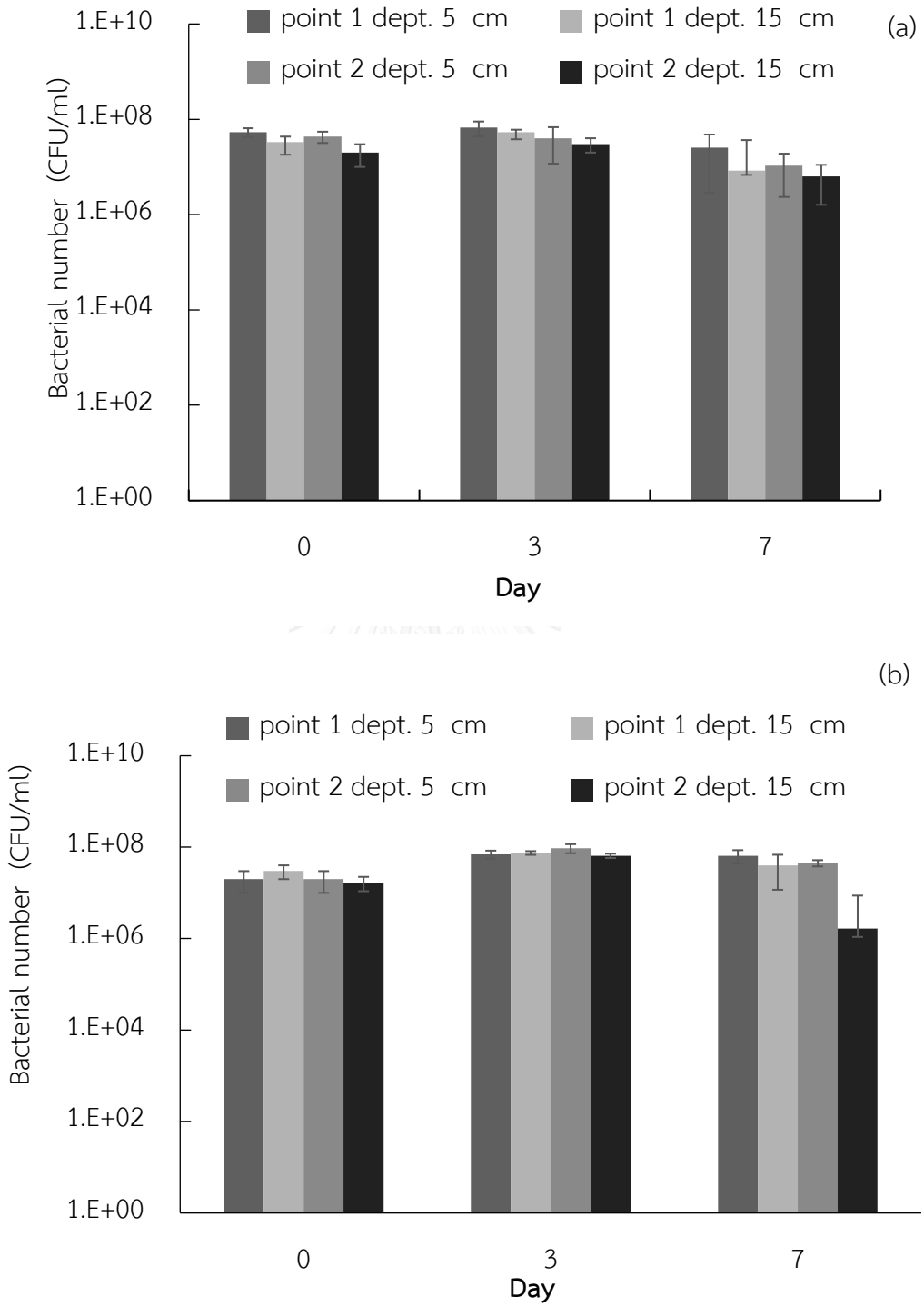


Figure 5.16 The bacteria cell number of *Gordonia* sp. JC11 in synthetic seawater of the treatment with (a) *Gordonia* sp. JC11 only and (b) lipopeptide based dispersant applied along with *Gordonia* sp. JC11.

5.3.2.2 Mesocosm experiment using natural seawater in 40 L mesocosms

At the marine ecosystem, petroleum degradation is principally performed by indigenous microorganisms. Previously, Laorrattanasak et al. (2016) reported that the addition of biosurfactant from *G. westfalica* GY40 increased the ability of *Gordonia* sp. JC11 on fuel oil removal in natural seawater collecting from the Gulf of Thailand Chonburee and Rayong Province. However, the experiment was done in Erlenmeyer flasks which did not account for other environmental factors. Therefore, this study investigated the effect of indigenous bacteria on the application of lipopeptide based dispersant and *Gordonia* sp. JC11 in 40 L mesocosm experiments.

The preliminary mesocosm experiment with natural seawater found that the indigenous bacteria rapidly degraded Bongkot light crude oil when added at 9 mL, which was the amount used in synthetic seawater microcosms (Table D in appendix D). Therefore, this mesocosm experiment was performed by increasing the volume of oil 4 times from the previous experiment (36 mL of crude oil). After an hour, 7.2 mL lipopeptide based dispersant was sprayed to the top of the tank to achieve the dispersant to oil ratio (DOR) 1:5 followed by the addition of *Gordonia* sp. JC11. The samples were taken at 4 different locations to compare the effects of water turbulence and depths from surface water on remaining crude oil for 5 days.

At the position 1 which had more wave turbulence, the remaining crude oil in the treatment with lipopeptide based dispersant only increased over time in both 5 and 15 depth from surface water (Figure 5.17). These results indicated that molecules of surfactant interacted with crude oil and led to the formation of small droplets. At the end of the experiment, crude oil mainly distributed in the water column more than at the surface water when lipopeptide based dispersant + *Gordonia* sp. JC11 were applied (Figure 5.17 and 5.18).

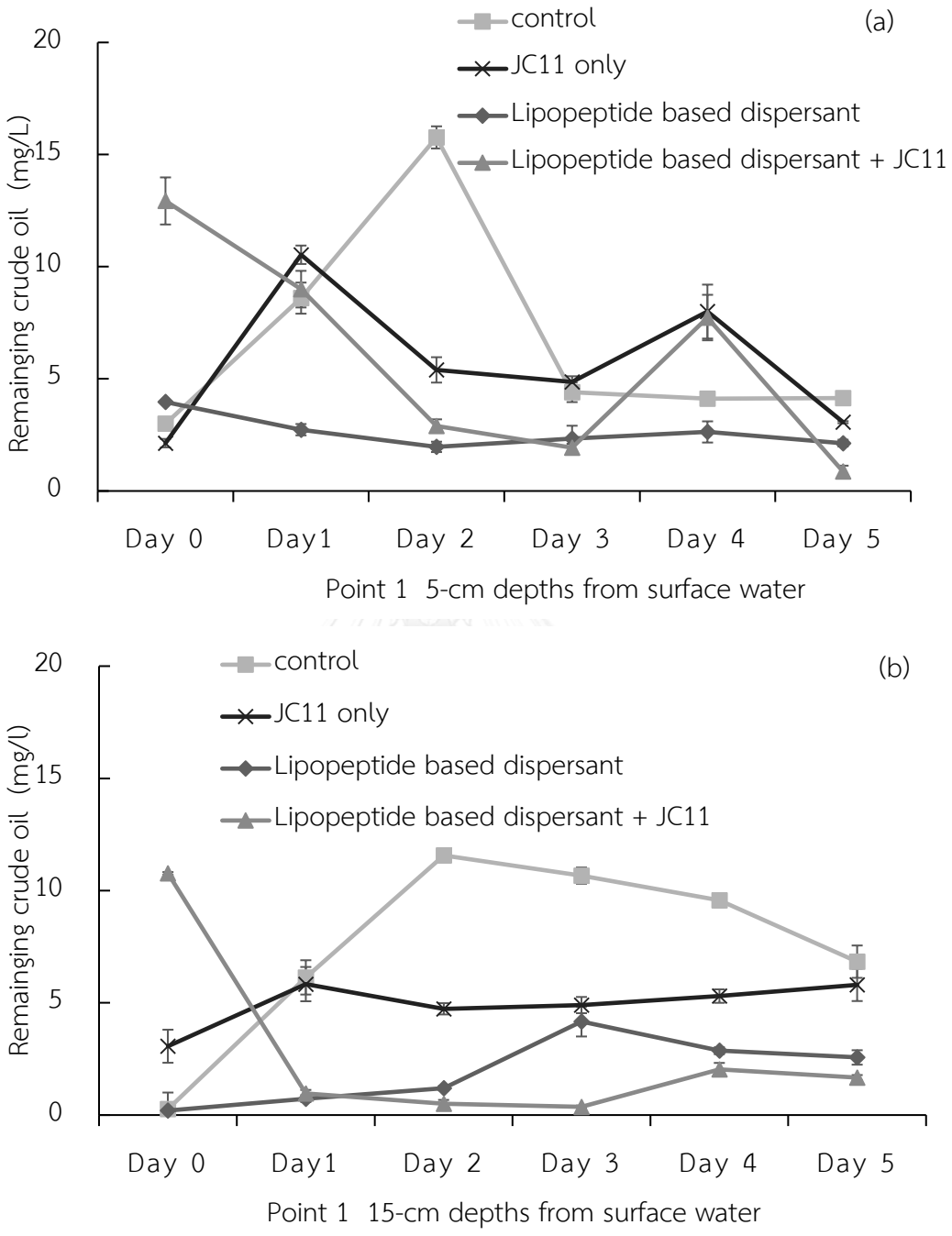
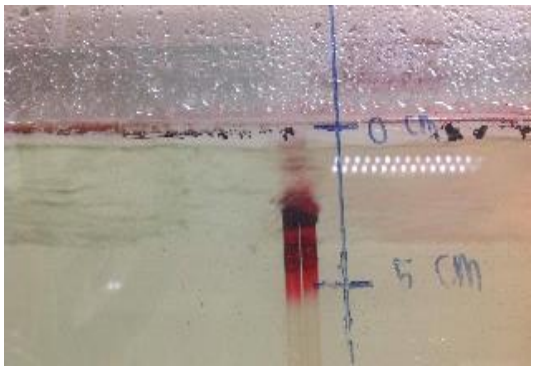
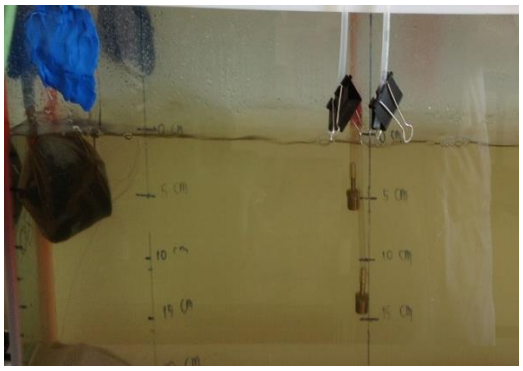


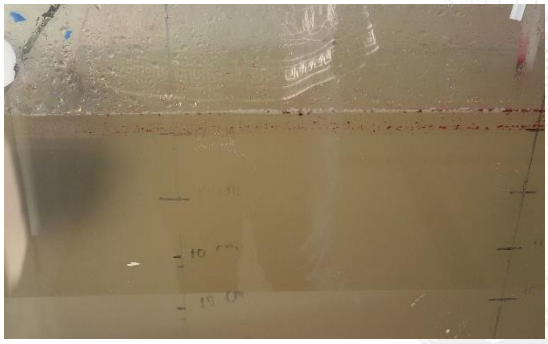
Figure 5. 17 The remaining crude oil in sampling point 1 at 5-cm depths (a) and 15-cm depths (b) from the surface of natural seawater.



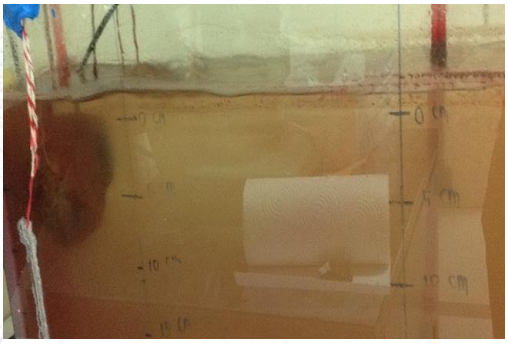
Control (Crude oil only)



Lipopeptide based dispersant only



Gordonia sp. JC11 only



Biosurfactant based dispersant + *Gordonia* sp. JC11

Figure 5.18 Residual oil on the surface of natural seawater at the point 1 of (a) crude oil only as a control (b) lipopeptide based dispersant only (c*) Gordonia* sp. JC11 only and (d) Lipopeptide based dispersant with *Gordonia* sp. JC11 at day 5.

The result at point 2 which had less effect from wave tuburance was shown in Figure 5.19 (a) and 5.19 (b). At the 5 cm dept from surface water, the result was in the same trend of point 1 but the amount of crude oil was more than those in point 1. It was corresponded with the low water turbulance there. The cell number of oil degrading slightly increased and corresponded to the crude oil reduction but the total bacteria remained the same in day 5 (Figure 5.20 (a) and 5.20 (b)). The treatment with with lipopeptide based dispersant and *Gordonia* sp. JC11 had the highest bacterial number at the end of study. By the help of indigenous bacteria and *Gordonia* sp. JC11,

the remaining crude oil was decreased over time corresponding with the increasing of cell number of oil degrading bacteria in four points.

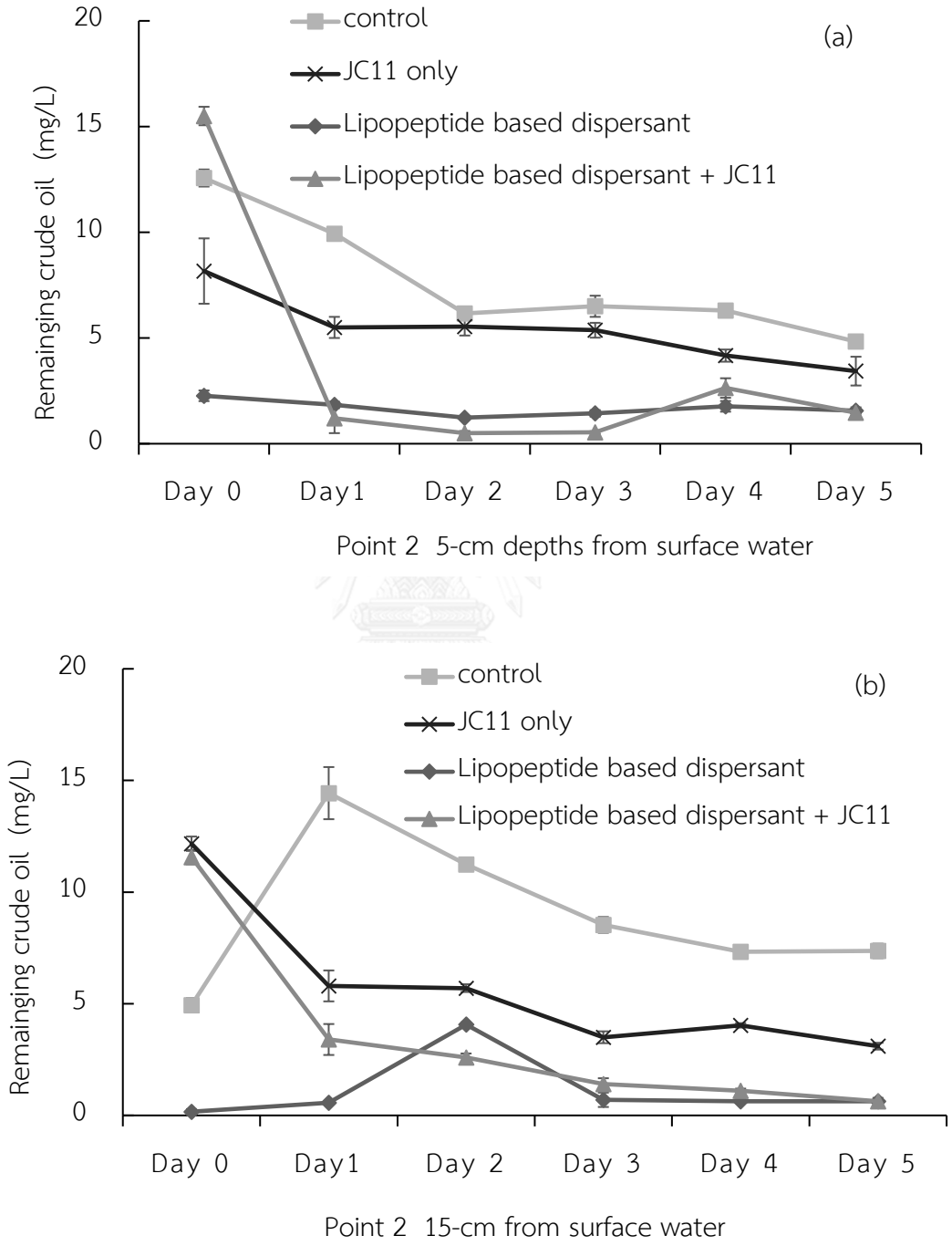


Figure 5.19 The remaining crude oil in sampling point 2 at 5-cm depths (a) and 15-cm depths (b) from the surface of natural seawater.

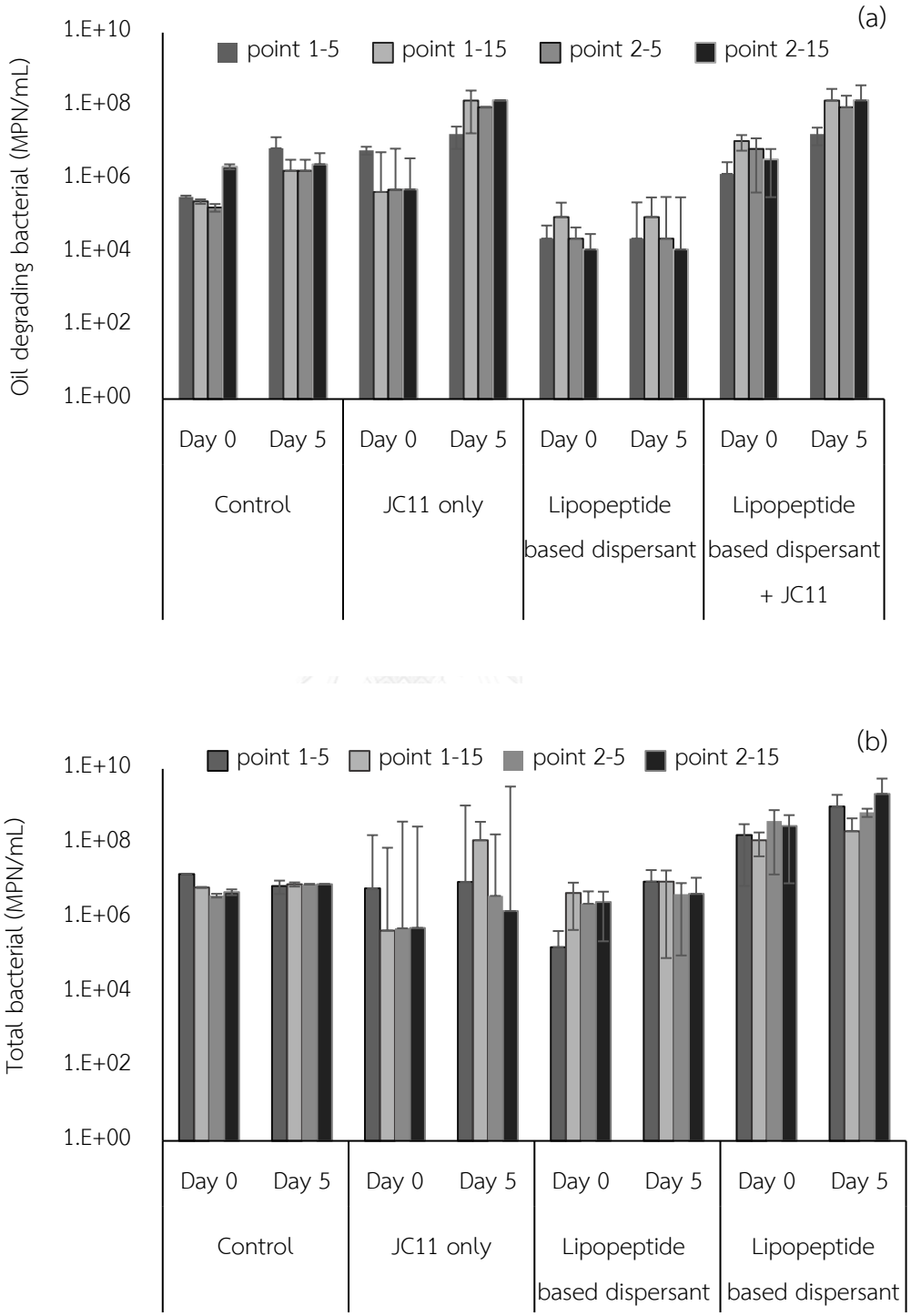
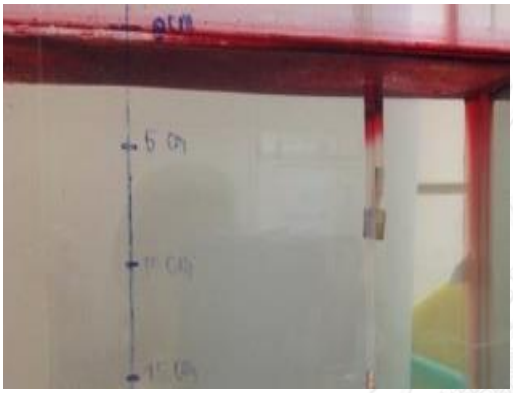
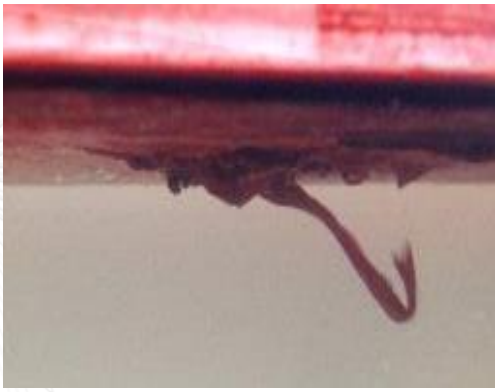


Figure 5. 20 The number of oil degrading bacteria (a) and total bacteria (b) at day 0 and day 5 in sampling point 1 and 2 at 5-cm and 15-cm depths from the surface of natural seawater.

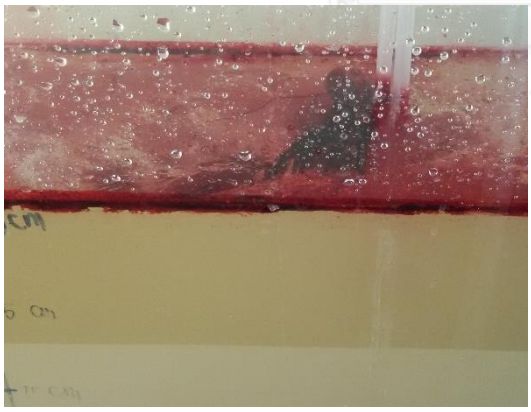
At the end of study (day 5), thick oil film in the treatment with lipopeptide based dispersant only was found near the surface of water (Figure 5.21). The residual oil film look like the emulsion from the interaction of oil slick and surfactant molecules in lipopeptide based dispersant. On the other hand, the treatment with lipopeptide based dispersant and *Gordonia* sp. JC11 showed thinner oil film, which indicated that the dispersed oil was degraded quicker.



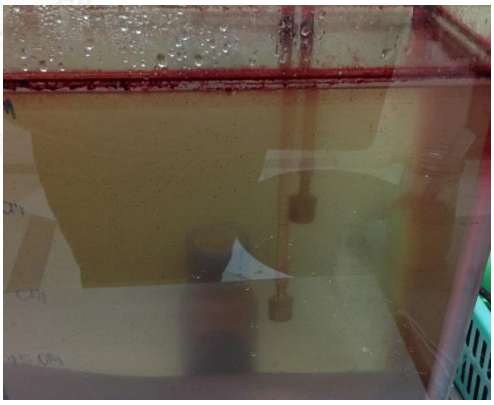
Control (Crude oil only)



Lipopeptide based dispersant only



Gordonia sp. JC11 only



Biosurfactant based dispersant + *Gordonia* sp. JC11

Figure 5. 21 The residual of oil on the surface of natural seawater at the point 2 of each treatment including (a) crude oil only as a control, (b) lipopeptide based dispersant only, (c) *Gordonia* sp. JC11 only and (d) Lipopeptide based dispersant with *Gordonia* sp. JC11 at day 5.

In conclusion, the indigenous bacteria were able to degrade crude oil after the addition of lipopeptide based dispersant but their activities were lower than *Gordonia* sp. JC11. The addition of both lipopeptide based dispersant and *Gordonia* sp. JC11 would lead to a rapid oil removal from natural seawater. The increasing activity of indigenous bacteria was similar to Cappello et al., (2012), which apply the biosurfactant EPS_{2003} obtained from *Acinetobacter calcoaceticus* for enhancing the crude oil removal by the indigenous oil-degrading bacteria in natural seawater. The addition of biosurfactant EPS_{2003} in 70L mesocosm increases the total bacterial abundance, change in the community structure and activity. Consequently, biosurfactant EPS_{2003} can be used for oil slick dispersion and selection of marine hydrocarbon degraders thus increasing bioremediation process Cappello et al., (2012).

5.3.3 Simulation of 160 L 3D-box model (mesocosm tanks) for oil spill remediation process

In this study, the mesocosm tank was scale-up to a medium scale experiment. The 160 L tank was 2 times wider than the 40 L tank but the length and depth were similar. When the continuous wave generator was operated in the tank, the seawater moved farther away and looked more similar to the coastal zone. The natural seawater was collected from Au Udom port, Chonburi Province, Thailand before starting the experiment. The physical properties of seawater was showed in Table 1 and found that the properties were not different from the previous study. After the natural seawater was filled in the tank containing natural sand, the experiment was set up for 48 hr for a stability of the system before adding Bongkot light crude oil.

The amount of crude oil increased rapidly from the beginning to 18 hr in both sampling points as shown in Figure 5.22 and 5.23. At the 18.30 hr, the lipopeptide based dispersant was sprayed on the top of tank, which further increased the amount of crude oil in all points. The results indicated that the crude oil had increased solubilization into the water column due to the interactions with the molecule of

surfactant in the lipopeptide based dispersant. However, the wave probably blown the lipopeptide based dispersant and dispersed oil away so the increasing of crude oil in point 2 was higher than point 1 (high wave turbulence).

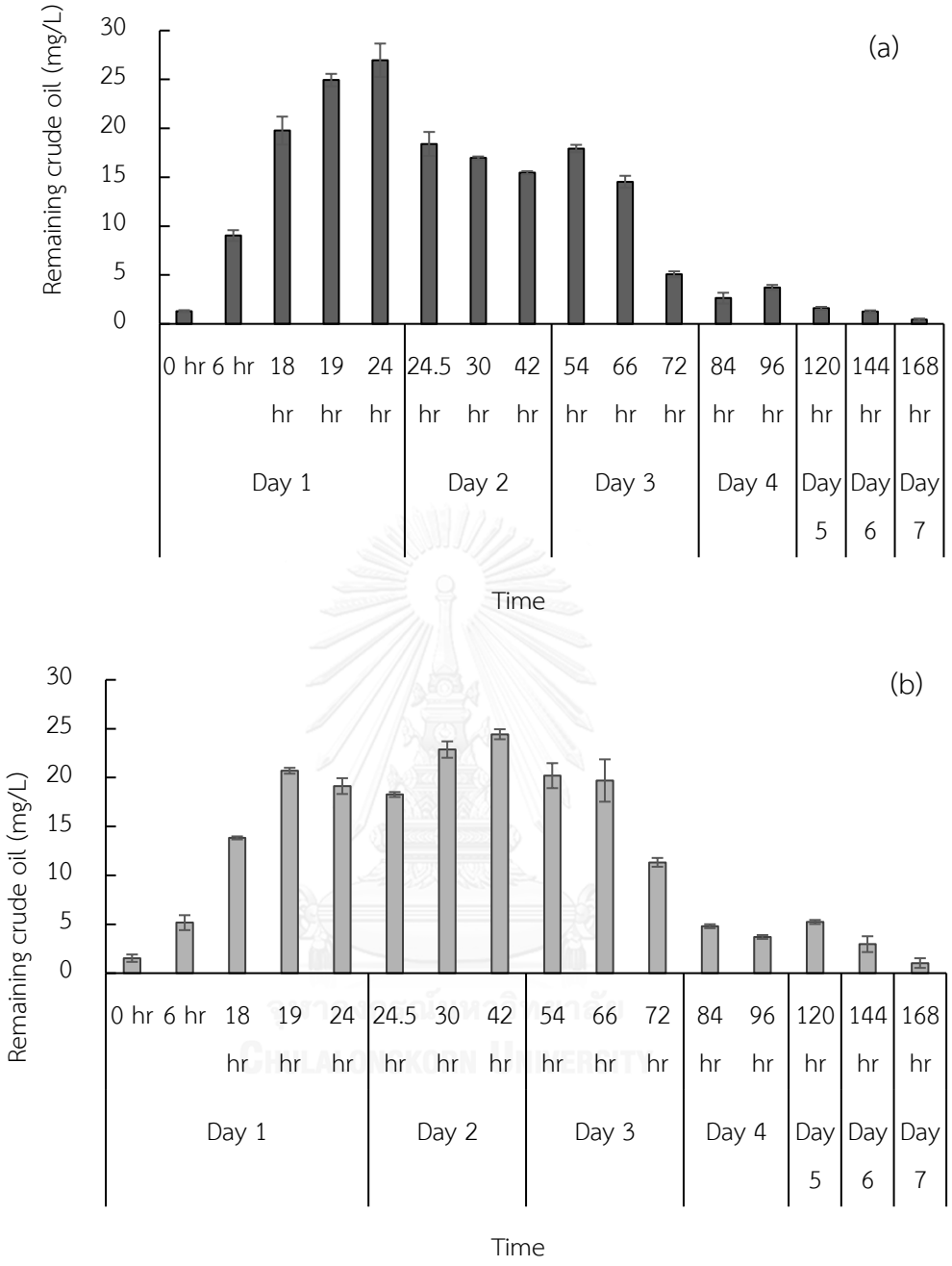


Figure 5.22 The remaining crude oil in sampling point 1 at 5-cm depths (a) and 15-cm depths (b) from the surface of natural seawater during 7 day expperiment of 160 L mesocosm tank. The experiment was followed by adding the crude oil after sample was collected at time 0 hr. Then, the lipopepitde based dispersant was sprayed on after collected sample at 18 hr and *Gordonia* sp. JC11 was added after collected sample at 24 hr.

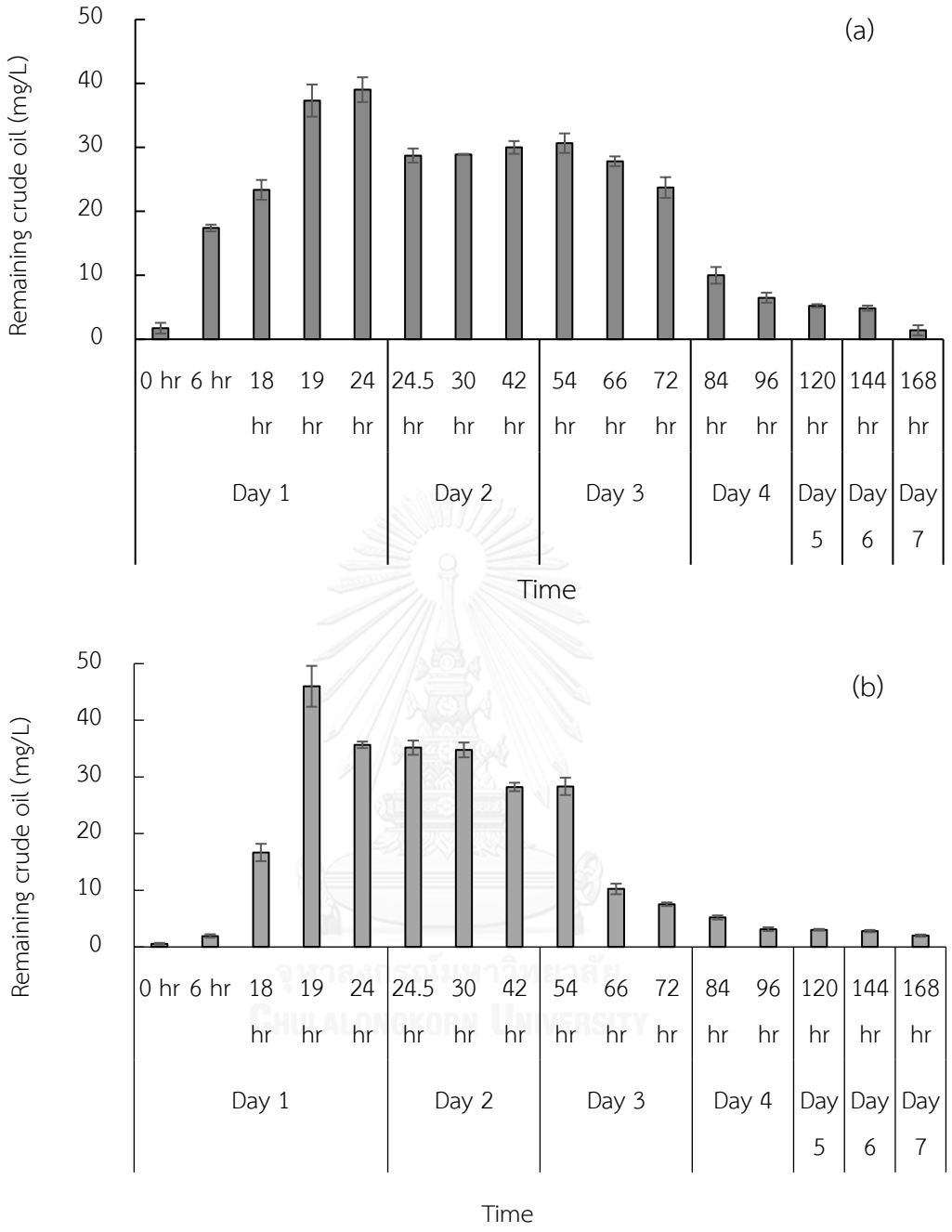
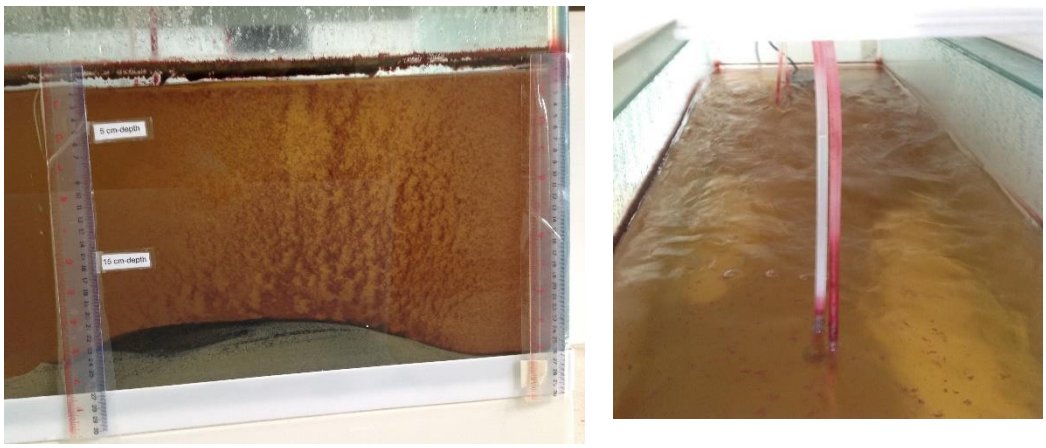


Figure 5.23 The remaining crude oil in sampling point 2 at 5-cm depths (a) and 15-cm depths (b) from the surface of natural seawater during 7 day experiment of 160 L mesocosm tank. The experiment was followed by adding the crude oil after sample was collected at time 0 hr. Then, the lipopepitde based dispersant was sprayed on after collected sample at 18 hr and *Gordonia* sp. JC11 was added after collected sample at 24 hr.

At 24.5 hour (1 day), 16 L of stock solutions of *Gordonia* sp.JC11 was added into the tank. The sample was collected immediately after *Gordonia* sp. JC11 addition. The amount of crude oil in the system slightly decreased over time until day 3 in all four points. After 84 hr, the remaining crude oil in all four points was decreased to lower than 10 mg/L. At the end of the experiment (7 day), the remaining crude oil was decreased to lower than 2 mg/L. The remaining crude oil was comparable to the initial amount of oil in natural seawater.

The characteristic of 160 L mesocosm tank was observed overtime and showed in table 3. Before the lipopeptide based dispersant was sprayed (at 18 hr), the crude oil was solubilized in the tank by the effect of wave maker. After lipopeptide based dispersant was sprayed for 30 minute (at 19 hr), the seawater turned red in both point 1 and point 2. The oil layer on surface water at point 1 was less than point 2 and it was more turbidity than point 2. These phenomena inferred that the surfactant molecules and the power of wave enhanced the solubilization and dispersion of oil.

After *Gordonia* sp. JC11 was added into the mesocosm tank at the 24.5 hr of the experiment, the seawater turned red-orange colour from the oil and bacterial cells (table 3 in 30 hr). After 42 hr, the sample was more turbid and some residual cells were found at point 2 more than point 1. The residual cell thickness increased over time and found to stick at the glass tank. However, from the top view inside the tank (Figure 5.24), the seawater was as clear as the initial seawater. On the surface of natural seawater, there were suspended solids which could be dead bacterial cells.



(a) (b)

Figure 5.24 The suspended solids in the 160 L mesocosm with natural seawater at point 2 from the outside view (a) and inside top view (b) at 166 hr (day 7).

The decreasing of remaining crude oil was corresponded with the cell number of oil degrading bacteria in four points which slightly increased over time (Figure 5.25 and 5.26). However, the total bacteria remained the same in day 5 and slightly decreased over time until day 7. The result of bacterial number fluctuation in this experiment was corresponded with Hassanshahian et al., (2014). The amount of oil-degrading bacteria obtained by MPN method found that the number of oil-degrading bacteria increased rapidly from the beginning to 3rd day and slightly increased until 10th day. After that, the number of oil-degrading bacteria slightly decreased (Hassanshahian et al., 2014). The results from 160 L tank were comparable to the 40 L tank using natural seawater. The lipopeptide based dispersant worked well with both the indigenous petroleum bacteria and the augmented *Gordonia* sp. JC11. Consequently, it could be applied to the real situation of oil spill remediation. However, the application of lipopeptide based dispersant followed by *Gordonia* sp. JC11 was more suitable for oil spill remediation as seen from the lowest amount of remaining oil and the fastest oil removal process.

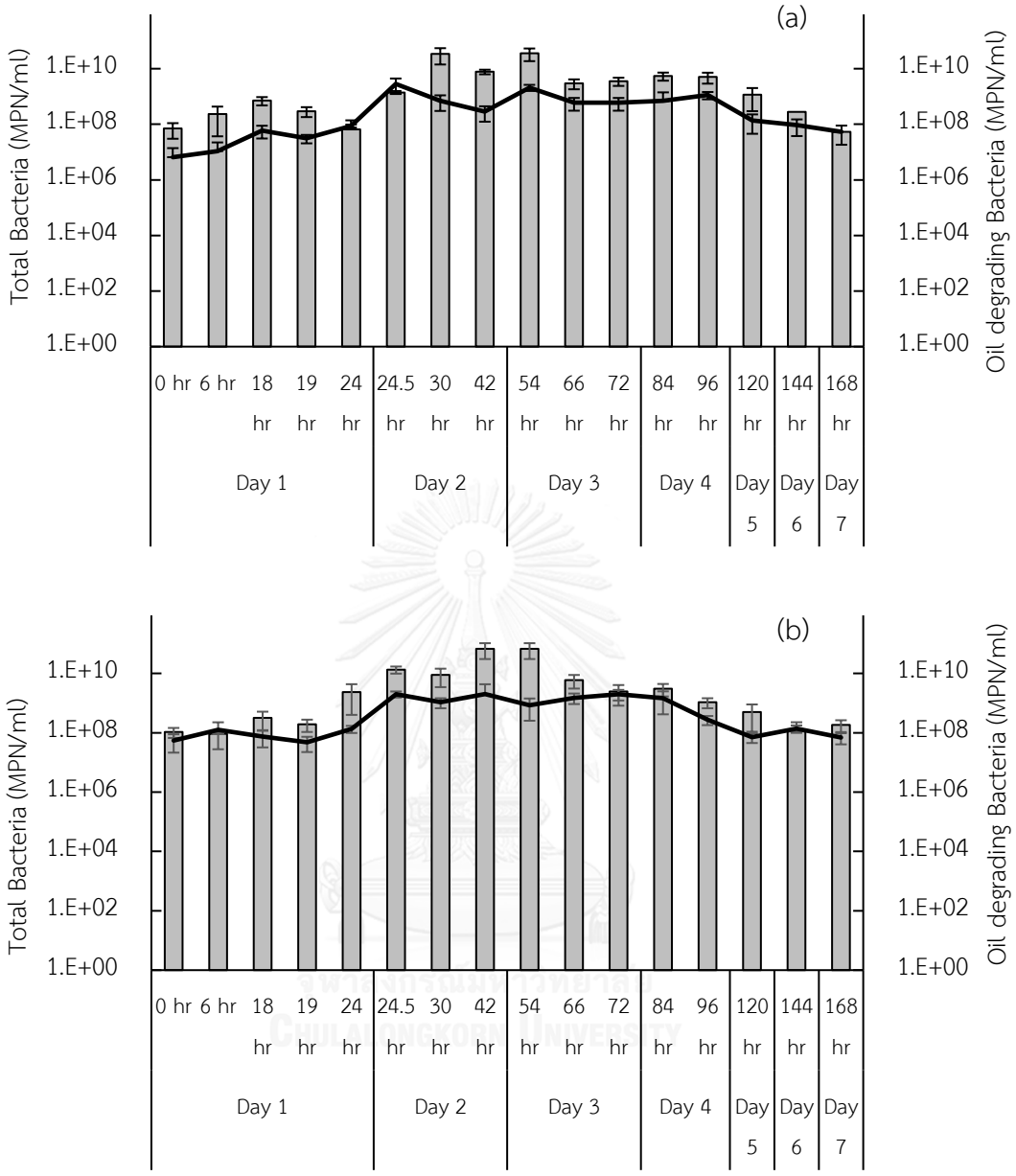


Figure 5.25 The number of total bacteria (bar graph) comparing with the oil degrading bacteria (line graph) in sampling point 1 at 5-cm depths (a) and 15-cm depths (b) from the surface water during 7 day expperiment of medium scale mesocosm tank. The experiment was followed by adding the crude oil after sample was collected at time 0 hr. Then, the lipopepitde based dispersant was sprayed on after collected sample at 18 hr and *Gordonia* sp. JC11 was added after collected sample at 24 hr.

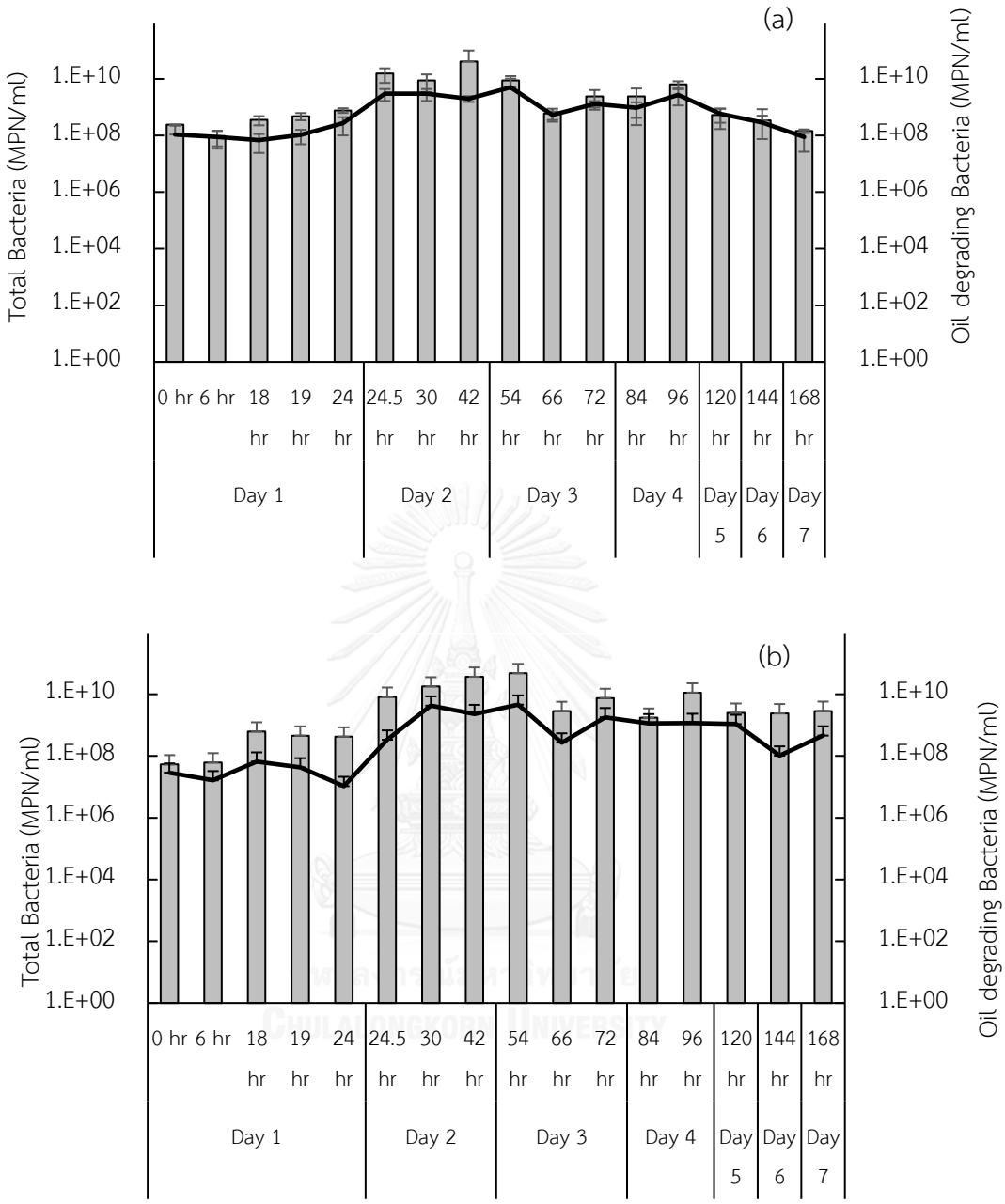
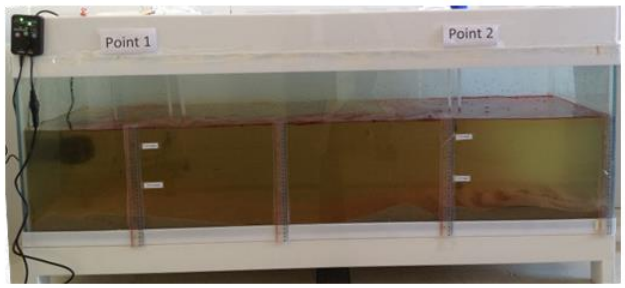
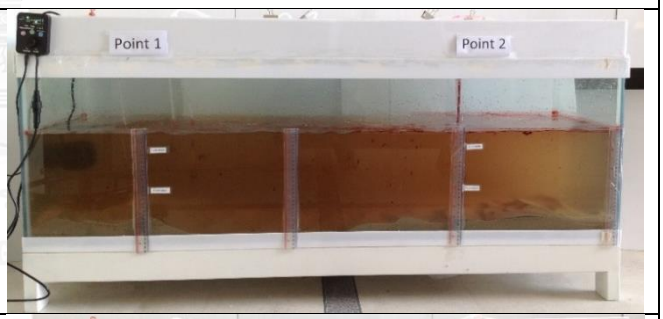
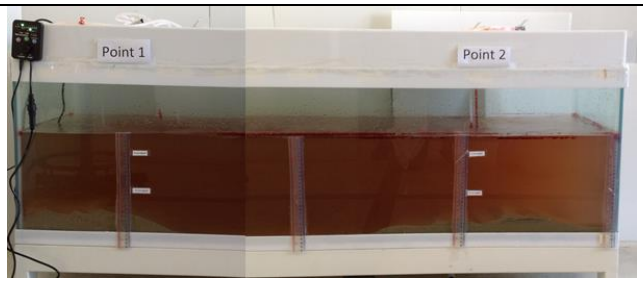
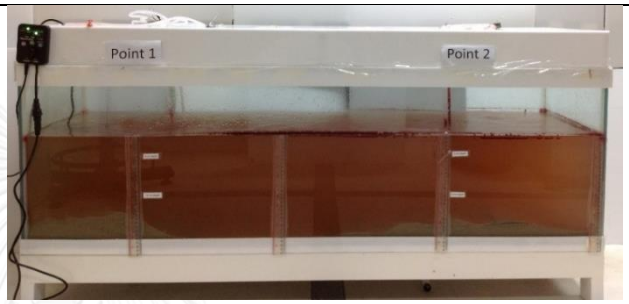
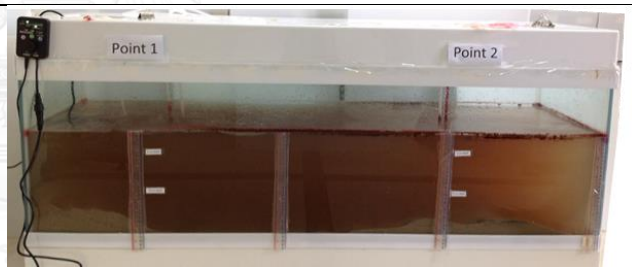
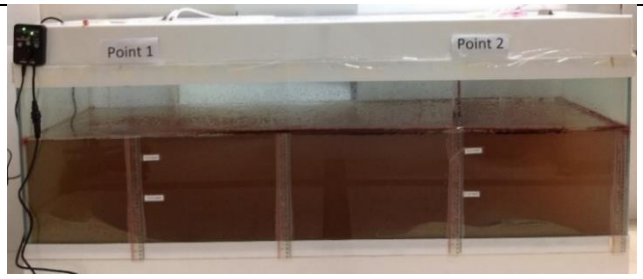
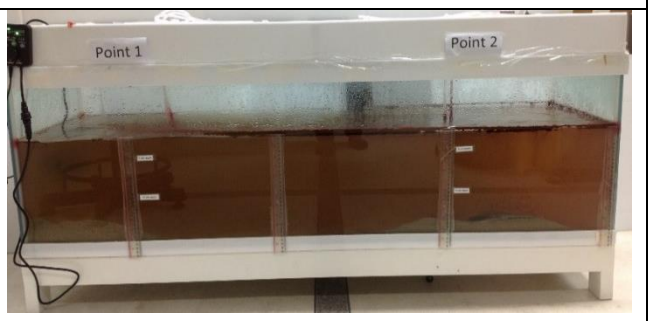
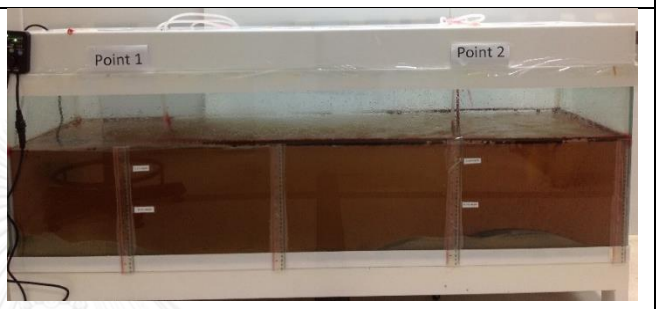
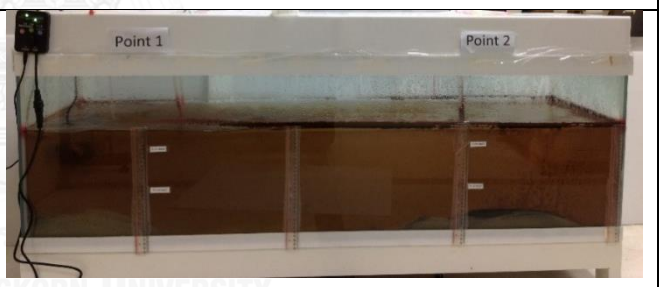
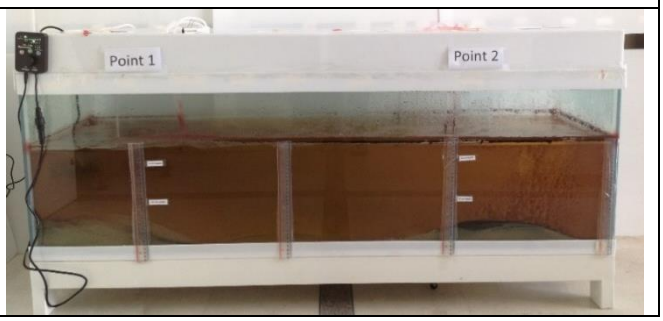


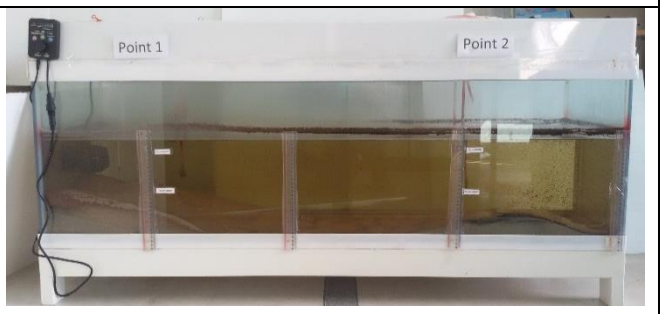
Figure 5.26 The number of total bacteria (bar graph) comparing with the oil degrading bacteria (line graph) in sampling point 1 at 5-cm depths (a) and 15-cm depths (b) from the surface water during 7 day expperiment of medium scale mesocosm tank. The experiment was followed by adding the crude oil after sample was collected at time 0 hr. Then, the lipopepitde based dispersant was sprayed on after collected sample at 18 hr and *Gordonia* sp. JC11 was added after collected sample at 24 hr.

Table 5.3 Characteristic of the mesocosm tank at difference time points.

Time	Experiment	The characteristic of mesocosm tank
	The mesocosm was set-up 48 hr before the experiment start	
0 hr	Bongkot light crude oil was added on the top of the tank	
18 hr	Before lipopepitde based dispersant was added in mesocosm tank	
19 hr	After lippepitde based dispersant was added in mesocosm tank	

Time	Experiment	The characteristic of mesocosm tank
24.5 hr	After *Gordonia* sp.JC11 was added into the mesocosm tank	
30 hr	-	
42 hr	-	
54 hr	-	

Time	Experiment	The characteristic of mesocosm tank
72 hr	-	
96 hr (day 4)	-	
144 hr (day 6)	-	
168 hr (day 7)	-	

Time	Experiment	The characteristic of mesocosm tank
Day 14		

5.4 Conclusions

Lipopeptide based dispersant containing lipopeptides 1.4%, SDHS 2.9% and NaCl 3.4% showed the ability to enhance crude oil washing from sand and increased the efficiency of *Gordonia* sp. JC11 to degrade crude oil. In the presence of lipopeptide based dispersant only, crude oil was re-deposited back to the sand after incubation.

The 3D-box model was performed to evaluate the possibility of applying lipopeptide based dispersant and *Gordonia* sp. JC11 on enhancing crude oil removal from seawater. The experiment was done in two scale of 40 L and 160 L as a small and medium scale experiments. In the mesocosm with both lipopeptide based dispersant and *Gordonia* sp. JC11, the concentration of crude oil decreased faster than that in the mesocosm with dispersant alone. The results were corresponded with the increasing number of oil degrading bacteria in seawater.

The natural seawater mesocosm showed that the indigenous bacteria could enhance the activity of lipopeptide based dispersant and *Gordonia* sp. JC11 for spilled Bongkot light crude oil removal. The result suggested that the lipopeptide based dispersant might be applied alone in the seawater with high number of indigenous oil degrading bacteria. However, the application of lipopeptide based dispersant followed by *Gordonia* sp. JC11 was more suitable for oil spill remediation as seen from the lowest amount of remaining oil and the fastest oil removal process.

In addition, oil spill thickness and wave turbulence affected the efficiency of lipopeptide based dispersant. It is therefore possible to apply the lipopeptide based dispersant for oil spill treatment in the coastal environment.

Chapter VI
Conclusions and Recommendations

6.1 Conclusions

Lipopeptide biosurfactant produced from *Bacillus* sp. GY19 was concentrated from the foamate solution as a powder form. The using of biosurfactant become interested in many applications according from the good surface activity, low toxicity, and tolerant at various extreme environment conditions. The aim of this work was to apply lipopeptide biosurfactant use for oil spill remediation in seawater. Initially, the lipopeptide powder was characterized for the surface activity, the toxicity and a potential application in petroleum remediation.

Lipopeptide biosurfactant showed a good surface activity and was stable after storage when compared to crude lipopeptide biosurfactant form. The lipopeptide powder was solubilized well in water making it easy to apply or use. Moreover, it displayed a stability under wide range of temperature, pH, and increased in the presence of NaCl. The lipopeptide biosurfactant was characterized as an anionic surfactant type and displayed in a more hydrophobic biosurfactant. The HLD concept was used to confirm the magnitude of lipopeptide biosurfactant and the value found to equal 4.93 which tended to be more hydrophobic surfactant. This value was first characterized in this study. The lipopeptide biosurfactant showed low toxicity to marine organisms (whiteleg shrimp and copepod), vegetables (tomato, rice and green bean) and selected pyrene-degrading bacteria. Moreover, the lipopeptide biosurfactant showed the ability to disperse fuel oil similar to Dehydol LS9TH and commercial detergent. It could solubilize and wash fuel oil similar to Dehydol LS9TH but its efficiency was higher than commercial detergent.

From this first part, the freeze-drying lipopeptide biosurfactant was expected as an appropriate ingredient in the oil dispersant. SDHS was selected as another ingredient to formulate the bio-based solvent free dispersant. To achieve the low interfacial tension (IFT) and stable microemulsion droplet, the HLD concept was used to calculate the optimum fraction of lipopeptide and SDHS based on the EACN of

hydrocarbons and seawater salinity. From the HLD calculation, the molar fractions of lipopeptides in the lipopeptide-SDHS mixtures increased with increasing EACN of the hydrocarbons because the system required more hydrophobic surfactant to balance the hydrophobicity of oils. The suitable lipopeptide-SDHS molar fraction showed a higher dispersant effectiveness than lipopeptides alone. At high dispersant to oil ratio, the dispersant effectiveness was comparable to the commercial dispersant. The application of HLD concept for biosurfactant based dispersant formulation was rapid and convenient. However, the lipopeptide based dispersant slightly increased the toxicity of dispersed oil to the whiteleg shrimp and this was due to the enhancing of crude oil solubilized in seawater. When compared to other reported dispersants, the extent of toxicity from lipopeptide based dispersant was minor.

To confirm the efficiency of lipopeptide based dispersant for oil spill remediation, the formulation suitable for Bongkot light crude oil was used along with a petroleum degrading bacteria, *Gordonia* sp. JC11. Lipopeptide based dispersant showed the ability to wash Bongkot light crude oil from sand and worked well with *Gordonia* sp. JC11. The larger scale experiment was performed in small and medium mesocosm tanks. The simulation of synthetic seawater confirmed that *Gordonia* sp. JC11 had increased oil removal efficiency when applied with lipopeptide based dispersant. In natural seawater, the lipopeptide based dispersant enhanced the oil-degrading activity of indigenous bacteria. The results suggested that the lipopeptide based dispersant might be applied alone in the seawater with high number of indigenous oil degrading bacteria. However, the application of lipopeptide based dispersant followed by *Gordonia* sp. JC11 was more suitable for oil spill remediation as seen from the lowest amount of remaining oil and the fastest oil removal process.

6.2 Recommendations for future work

1) There are many biosurfactants produced from local bacterial strains, which could be used to replace synthetic surfactant (i.e. SDHS) in the dispersant. The Cc value of these biosurfactants should be quantified before the selection of biosurfactant.

2) Due to the high production cost of freeze-drying lipopeptide powder, other processes for concentrating the lipopeptides such membrane filtration or spray drying techniques should be used.

3) EACN value of petroleum crude oil is an important parameter for formulating the lipopeptide based dispersant. Therefore, the EACN of target crude oil should be characterized by the HLD concept.

4) The formulating of dispersants by other kinds of biosurfactant or local synthetic surfactant should be investigated to lower the cost of production.

5) Statistical software could be used for formulating of lipopeptide based dispersant by using HLD concept to reduce the number of formulations.

6) The toxicity of lipopeptide-SDHS based dispersant should be determined to confirm the effect on the marine organism.

7) The application of lipopeptide based dispersant should be confirmed in a larger experimental setting to study the effect of environmental conditions such as turbulence, sunlight and temperature and the effect to ecosystem.

6.3 Benefits of the research

Currently, the production of lipopeptide biosurfactant from *Bacillus* sp. GY19 is investigated in large scale. The key challenge of biosurfactant application is to characterize the biosurfactant in terms of physiochemical properties, surface behaviors, and etc. The outcome from this study will be useful for the application of lipopeptide biosurfactant and the basic information could be applied to other biosurfactants in the future.

Another part of this research was on the formulation of biosurfactant mixtures for oil spill remediation. The acquired dispersant formulation is considered as an innovation for remediation of petroleum spill because there is not many researches using biosurfactant. Moreover, the lipopeptide based dispersant was solvent-free and based on HLD concept, which have never been reported. The outcome of this research will be a basic principle for formulating other biosurfactant-based products in the future.

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APPENDIX

APPENDIX A MEDIAS AND STANDARD CURVES

Media

Table A.1 LB broth (Luria-Bertani broth) (per 1 Liter)

Component	Amount
Tryptone	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	10 gram

Suspend/dissolve all in 1 L of purified water, and adjusted pH to 7.0

Table A.2 Natural Seawater (NSW)

Component	Amount
NH_4NO_3	1.0 g
K_2HPO_4	0.02 g
Ferric citrate	0.02 g
Yeast extract	0.5 g

Mixed all and dissolved in 800 ml synthetic seawater, 200 ml distilled water; then, adjusted pH to 7.8

Table A.3 Marine broth (Difco™ Marine Broth 2216)

Component	Amount	Component	Amount
Peptone	5.0 g	Yeast Extract	1.0 g
Ferric Citrate	0.1 g	Sodium Chloride	19.45 g
Magnesium Chloride	5.9 g	Magnesium Sulfate	3.24 g
Calcium Chloride	1.8 g	Potassium Chloride	0.55 g
Sodium Bicarbonate	0.16 g	Potassium Bromide	0.08 g
Strontium Chloride	34.0 mg	Boric Acid	22.0 mg
Sodium Silicate	4.0 mg	Sodium Fluoride	2.4 mg
Ammonium Nitrate	1.6 mg	Disodium Phosphate	8.0 mg

Dissolve the following in 1000 ml of distilled water and adjust pH to 7.6, then, mix thoroughly.

Standard curve of crude oils

Standard curve of crude oils were plotted between ratio of peak area (crude oil/stearyl alcohol) and ratio of mass (crude oil/stearyl alcohol). Total amount of stearyl alcohol used in extraction was 25 mg. The calculation to determine amount of crude oils in sample is follow:

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

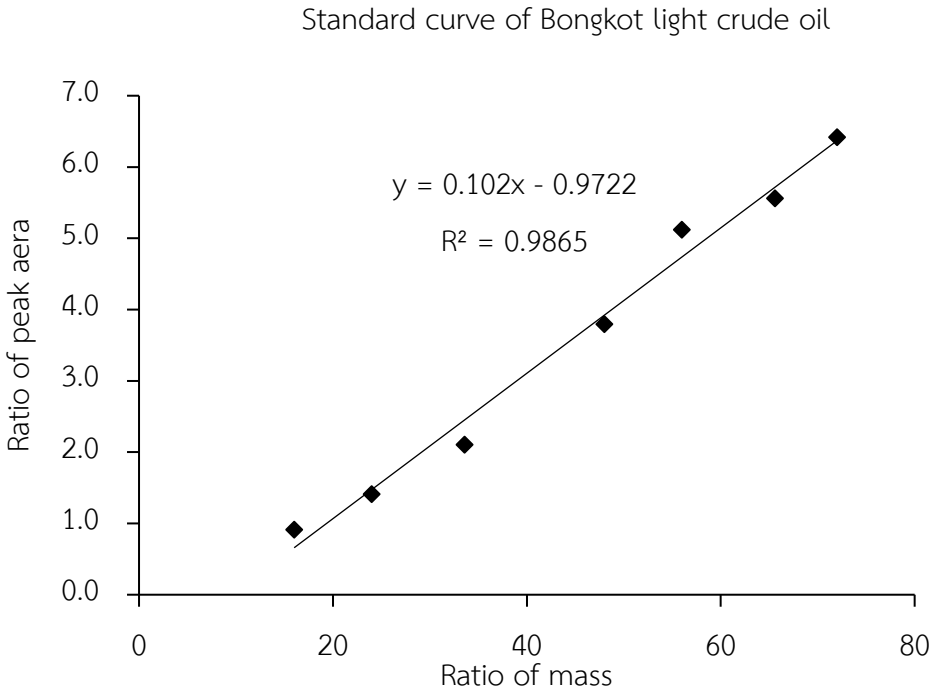


Figure A.1 Standard curve of Bongkot light crude oil from TLC-FID. Each data point was averaged from triple spots on chromatorods.

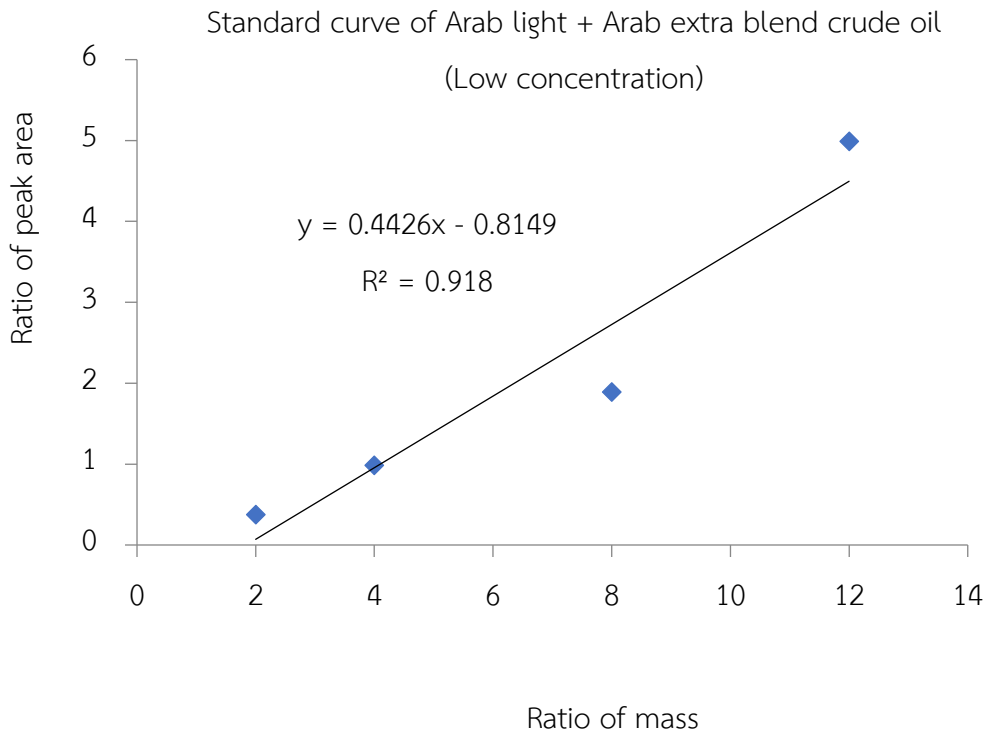


Figure A.2 Standard curve of Arabian light/Arab extra blend crude oil with low from TLC-FID. Each data point was averaged from triple spots on chromatorods.

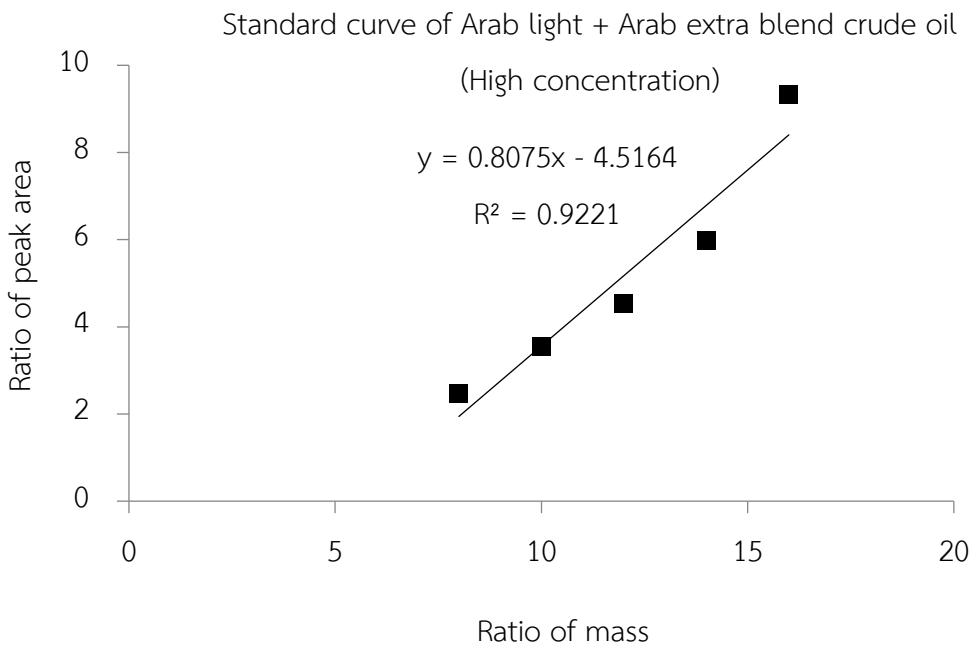


Figure A.3 Standard curve of Arabian light/Arab extra blend crude oil with high range concentration from TLC-FID. Each data point was averaged from triple spots on chromatorods.

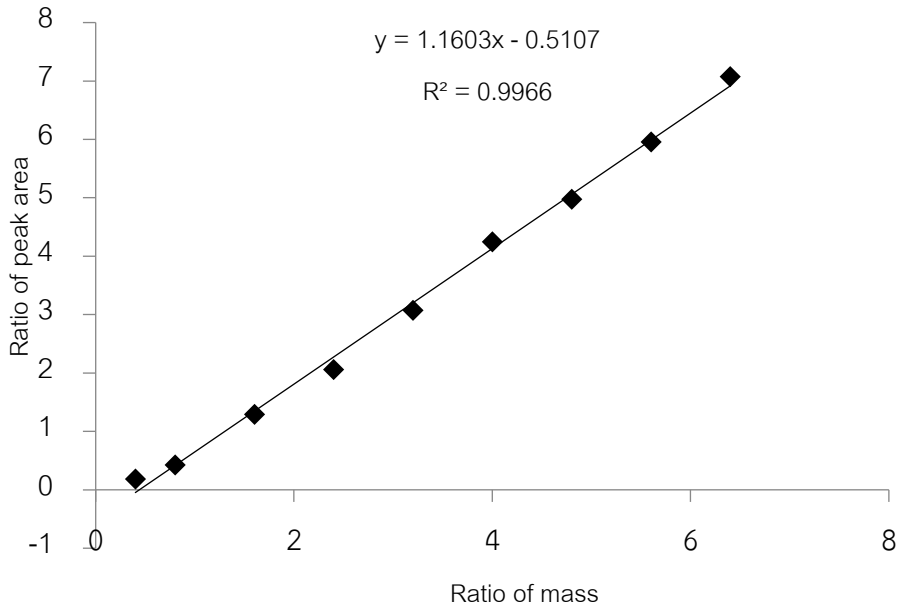
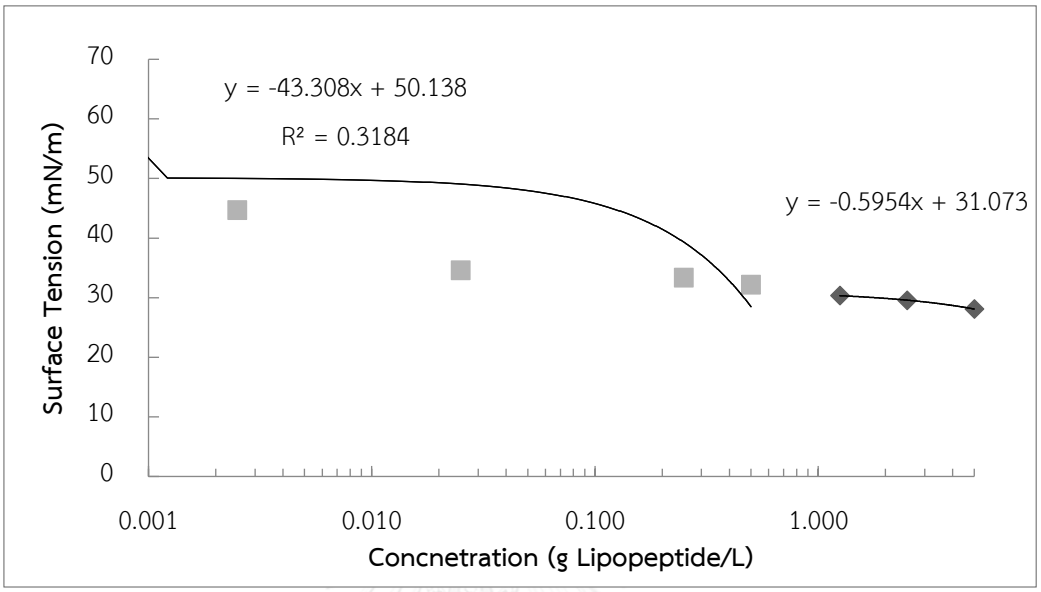


Figure A.4 Standard curve of Fuel oil with low range concentration from TLC-FID. Each data point was averaged from triple spots on chromatorods.

APPENDIX B SUPPLEMENTARY DATA OF CHAPTER III



$$-43.308x + 50.138 = -0.5954x + 31.073$$

$$X = 0.45$$

Figure B.1 CMC of lyophilized lipopeptide biosurfactant

Table B.1 Percent of dissolved freeze-drying lipopeptide biosurfactant in high to low polarity solvents.

Solvent		Mass filter paper (mg)		Residual (mg)	Mass of Lyophilized (mg)		Percent dissolve	AVE	SD
		Before	After		Before	After			
Water	1	164.50	174.90	10.4	160.3	149.9	93.51		
	2	166.20	178.00	11.8	159	147.2	92.58	93.05	0.66
pH 10	1	164.20	169.10	4.9	140.7	135.8	96.52		
	2	149.70	152.00	2.3	145.6	143.3	98.42	97.47	1.35
Methanol	1	147.50	166.10	18.6	160.7	142.1	88.43		
	2	161.90	185.50	23.6	155.9	132.3	84.86	86.64	2.52
Ethanol	1	143.20	151.50	8.3	158.6	150.3	94.77		
	2	161.80	187.00	25.2	130.8	105.6	80.73	87.75	9.92
DMSO	1	145.90	203.50	57.6	158.6	101	63.68		
	2	161.10	216.90	55.8	154.5	98.7	63.88	63.78	0.14
Acetone	1	138.90	216.30	77.4	146.8	69.4	47.28		
	2	162.00	252.20	90.2	150.9	60.7	40.23	43.75	4.99
Chloroform	1	143.70	224.70	81	155.4	74.4	47.88		
	2	160.80	253.40	92.6	160.6	68	42.34	45.11	3.91
Hexane	1	143.00	227.40	84.4	155.5	71.1	45.72		
	2	149.10	238.70	89.6	155.2	65.6	42.27	44.00	2.44

Table B.2 Percent of dissolved crude extract of lipopeptides in high to low polarity solvents.

Solvent	Mass of vial Before (g)	Mass of crude Before (g)	Total mass after dissolve (g)	Residual crude oil (g)	Mass of Dissolve (g)	*Percent dissolve*		
							AVE	*SD*
Water	16.7161	0.0820	16.7781	0.0620	0.0200	24.39		13.86
	16.7895	0.0541	16.8198	0.0303	0.0238	43.99	34.19	
pH 10	16.8264	0.0737	16.8753	0.0489	0.0248	33.65		
	18.7390	0.0844	18.7982	0.0592	0.0252	29.86	31.75	2.68
Methanol	16.7151	0.0618	16.7243	0.0092	0.0526	85.11		
	16.7600	0.0866	16.7845	0.0245	0.0621	71.71	78.41	9.48
Ethanol	16.6790	0.1014	16.6804	0.0014	0.1000	98.62		
	16.7431	0.0702	16.7482	0.0051	0.0651	92.74	95.68	4.16
DMSO	16.7766	0.0858	16.8219	0.0453	0.0405	47.20		11.16
	16.5421	0.0713	16.5910	0.0489	0.0224	31.42	39.31	
Acetone	16.5940	0.1077	16.5952	0.0012	0.1065	98.89		
	18.8054	0.0898	18.8098	0.0044	0.0854	95.10	96.99	2.68
Chloroform	16.6751	0.0754	16.6750	0.0001	0.0755	100.1		
	16.8134	0.0870	16.8145	0.0011	0.0859	98.74	99.43	0.99
Hexane	16.8223	0.0910	16.8322	0.0099	0.0811	89.12		13.98
	16.7607	0.0783	16.7847	0.0240	0.0543	69.35	79.23	

Table B.3 The effect of temperature, pH and NaCl on the stability of lipopeptides. The concentration of lipopeptides was 0.5 g/L (1xCMC).

	Surface tension (mN/m)				
Temperature (°C)	1	2	3	AVG	SD
30	29.220	29.626	30.032	29.626	0.406
40	30.139	29.896	29.653	29.896	0.243
60	29.789	30.215	29.363	29.789	0.426
80	29.369	29.754	28.984	29.369	0.385
121	29.871	30.012	29.729	29.871	0.142
NaCl (%)	1	2		AVG	SD
0	29.862	30.143	29.580	29.862	0.282
2	27.340	27.754	27.547	27.547	0.207
4	25.327	25.919	25.623	25.623	0.296
6	25.815	25.524	25.670	25.670	0.146
8	25.709	25.848	25.570	25.709	0.139
10	25.553	25.983	25.123	25.768	0.304
pH	1	2		AVG	SD
2	37.342	37.572	37.801	37.572	0.230
4	32.242	31.637	31.032	31.637	0.605
6	32.172	32.357	32.542	32.357	0.185
7	29.177	29.251	29.325	29.251	0.074
8	29.487	29.459	29.514	29.487	0.027
10	31.203	30.913	31.493	31.203	0.290
11	32.112	32.326	31.898	32.112	0.214

Table B.4 Interfacial tension of lipopeptide biosurfactant against four hydrocarbons difference in hydrophobicity, which represented as equivalent alkane carbon number (EACN) of each hydrocarbon including toluene, hexane, decane, and hexadecane (EACN 1, 6, 10 and 16, respectively).

		IFT (mN/m)					
Hydrocarbon	EACN	1	2	3	4	AVG	SD
Toluene	1	4.4700	4.2090	4.1000	4.0230	4.20	0.20
Hexane	6	3.5421	3.3521	3.063	3.2011	3.29	0.21
Decane	10	2.867	2.887	2.752	2.9143	2.86	0.07
Hexadecane	16	2.6812	2.8182	2.5943	2.5422	2.66	0.12

Table B.5 The Correlation between the fractions of lipopeptides in 0.1 M SDHS-biosurfactant-benzene microemulsion system at difference molar fraction

AMA 0.1 lipopeptide 0						Equilibrium IFT (mN/m)					
NaCl %	Type	bottom	upper	bottom	upper	total	1	2	3	AVE	SD
1	I	16.5	13.5	0	0	30	0.1840	0.1745	0.1765	0.1784	0.0050
1.7	I	17	13	0	0	30	0.0903	0.0929	0.0912	0.0915	0.0013
2	III	13	11	4	2	30	0.0455	0.0455	0.0444	0.0451	0.0006
2.1	III	13	12	3	2	30	0.0322	0.0322	0.0357	0.0334	0.0021
2.2	III	12.5	12.5	2.5	2.5	30	0.0291	0.0272	0.0279	0.0281	0.0009
2.4	III	11	14	2	3	30	0.0353	0.0353	0.0407	0.0371	0.0031
3	II	13.5	16.5	0	0	30	0.1386	0.1294	0.1324	0.1335	0.0047

AMA 0.0975 lipopeptide 0.0025							Equilibrium IFT (mN/m)				
NaCl %	Type	bottom	upper	bottom	upper	total	1	2	3	AVG	SD
1	I	15.5	14.5	0	0	30	0.249	0.2332	0.2411	0.2411	0.0079
1.5	I	17	13	0	0	30	0.075	0.0739	0.0739	0.0743	0.0007
1.9	III	13	11	3.5	2.5	30	0.029	0.0305	0.0280	0.0292	0.0013
2	III	12.5	12.5	2.5	2.5	30	0.028	0.0292	0.0247	0.0274	0.0024
2.15	III	13	12	2	3	30	0.038	0.0394	0.0369	0.0383	0.0013
2.3	III	13	11.5	2	3.5	30	0.058	0.0562	0.0634	0.0594	0.0037
2.5	II	13	17	0	0	30	0.106	0.1170	0.1045	0.1092	0.0068

AMA 0.095 lipopeptide 0.005					Equilibrium IFT (mN/m)				
bottom	upper	bottom	upper	total	1	2	3	AVG	SD
16	14	0	0	30	0.0891	0.0906	0.0925	0.0907	0.0017
13	12	3.5	1.5	30	0.0504	0.0508	0.0415	0.0475	0.0053
13	12	3	2	30	0.0195	0.0256	0.0276	0.0242	0.0042
12.5	12.5	2.5	2.5	30	0.0195	0.0226	0.0240	0.0220	0.0023
12	12.5	2	3.5	30	0.0232	0.0232	0.0304	0.0256	0.0042
13	12	1.5	3.5	30	0.0412	0.0449	0.0413	0.0425	0.0021
14.5	15.5	0	0	30	0.0653	0.0632	0.0647	0.0644	0.0011

AMA 0.0925 lipopeptide 0.0075						Equilibrium IFT (mN/m)				
Type	bottom	upper	bottom	upper	total	1	2	3	AVG	SD
I	16	14	0	0	30	0.0283	0.0351	0.0312	0.0315	0.0034
III	17	13	0	0	30	0.0321	0.0278	0.0311	0.0304	0.0023
III	12	11.5	4	2.5	30	0.0253	0.0227	0.0166	0.0215	0.0045
III	12.5	12.5	2.5	2.5	30	0.0158	0.0158	0.0157	0.0158	0.0001
III	12	12	2.5	3.5	30	0.0321	0.0278	0.0301	0.0300	0.0022
III	12	12	2	4	30	0.0401	0.0409	0.0413	0.0408	0.0006
II	14	16	0	0	30	0.0411	0.0429	0.0453	0.0431	0.0021

Table B.6 Percent mortality of copepods after a 96-h exposure to lipopeptides and Slickgone.

Conc. (mg/L)	Lipopeptide biosurfactant							
	Dead (96 hr)			% Dead (96 hr)				
	1	2	3	1	2	3	Aver	SD
0	0	0	0	0	0	0	0.00	0.00
0.5	1	0	0	10	0	0	3.33	5.77
5	1	0	0	10	0	0	3.33	5.77
50	1	1	0	10	10	0	6.67	5.77
125	1	1	0	10	10	0	6.67	5.77
500	2	1	1	20	10	10	13.33	5.77
1000	2	2	1	20	20	10	16.67	5.77
2000	10	10	10	100	100	100	100.00	0.00
3000	10	10	10	100	100	100	100.00	0.00
5000	10	10	10	100	100	100	100.00	0.00

Conc. (mg/L)	Slickgone NS type 2/3							
	Dead (96 hr)			% Dead (96 hr)				
	1	2	3	1	2	3	Aver	SD
0	0	0	0	0	0	0	0.00	0.00
0.5	0	0	1	0	0	10	3.33	5.77
50	1	0	0	10	0	0	3.33	5.77
125	8	10	7	80	100	70	83.33	15.28
500	10	10	10	100	100	100	100.00	0.00
1000	10	10	10	100	100	100	100.00	0.00
2000	10	10	10	100	100	100	100.00	0.00
3000	10	10	10	100	100	100	100.00	0.00
5000	10	10	10	100	100	100	100.00	0.00

Table B.7 Percent mortality of whiteleg shrimp after a 96-h exposure to lipopeptides and Slickgone.

Conc. (mg/L)	Lipopeptide biosurfactant							
	Dead (96 hr)			% Dead (96 hr)				
	1	2	3	1	2	3	AVE	SD
0	0	0	-	0	0	-	0.00	0.00
0.5	0	0	0	0	0	0.00	0.00	0.00
5	0	0	0	0	0	0.00	0.00	0.00
50	1	0	0	10	0	0.00	3.33	5.77
125	0	1	1	0	10	10.00	6.67	5.77
500	1	1	1	10	10	10.00	10.00	0.00
1000	6	6	6	60	60	60.00	60.00	0.00
2000	10	10	10	100	100	100.00	100.00	0.00
3000	10	10	10	100	100	100.00	100.00	0.00

Conc. (mg/L)	Slickgone NS type 2/3							
	Dead (96 hr)			% Dead (96 hr)s				
	1	2	3	1	2	3	AVE	SD
0	0	0	-	0.00	0.00	0.00	0.00	0.00
0.5	0	0	0	0.00	0.00	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00	0.00	0.00
50	10	8	8	100.00	80.00	80.00	86.67	11.55
125	9	9	9	90.00	90.00	90.00	90.00	0.00
500	9	10	8	90.00	100.00	80.00	90.00	10.00
1000	10	10	10	100.00	100.00	100.00	100.00	0.00
2000	10	10	10	100.00	100.00	100.00	100.00	0.00
3000	10	10	10	100.00	100.00	100.00	100.00	0.00

Table B.8 Root length of Tomato in day 5

sample seed	surfactant	conc.	Root length (cm)											
			day 5										AVG	SD
			1	2	3	4	5	6	7	8	9	10		
Tomato	SDS	2xCMC	No growth											
		2xCMC												
		1xCMC												
		1xCMC												
		0.5xCMC												
		0.5xCMC												
	BSF	2xCMC	25	35	35	25	45	30	35	40	35	30	33.5	5.9
		2xCMC	30	40	35	38	30	41	30	30	35	0	30.9	11.1
		1xCMC	40	35	30	42	45	30	30	45	25	40	36.2	6.8
		1xCMC	40	35	35	45	50	40	45	35	40	41	40.6	4.7
		0.5xCMC	50	50	30	60	30	50	40	40	43	44	43.7	8.9
		0.5xCMC	45	35	50	35	50	43	40	50	43	40	43.1	5.4
	DI		42	45	60	65	53	50	50	45	50	50	51.0	6.6
			35	50	55	45	45	50	35	50	45	45	45.5	6.1

Table B.9 Root length of Rice in day 5

sample seed	surfactant	conc.	Root length (cm)											
			day 5										AVG	SD
			1	2	3	4	5	6	7	8	9	10		
Rice	SDS	2xCMC	No growth											
		2xCMC												
		1xCMC												
		1xCMC												
		0.5xCMC												
		0.5xCMC												
	BSF	2xCMC	50	50	55	50	45	46	40	52	40	40	46.8	5.2
		2xCMC	55	50	50	55	47	50	45	50	45	65	51.2	5.7
		1xCMC	60	45	40	40	45	45	45	44	55	38	45.7	6.5
		1xCMC	40	40	70	40	50	45	60	54	65	42	50.6	10.6
		0.5xCMC	65	55	45	60	60	50	50	55	55	55	55.0	5.5
		0.5xCMC	50	60	45	50	60	60	55	45	50	50	52.5	5.6
	DI		60	55	55	60	45	60	45	55	50	50	53.5	5.5
			50	50	70	60	50	45	60	40	40	15	48.0	14.2

Table B.10 Root length Grean bean in day 5

sample seed	surfactant	conc.	Root length (cm)											
			day 5											
			1	2	3	4	5	6	7	8	9	10	AVG	SD
Green Bean	SDS	2xCMC	No growth											
		2xCMC												
		1xCMC												
		1xCMC												
		0.5xCMC												
		0.5xCMC												
	BSF	2xCMC	25	35	35	25	45	30	35	40	35	30	33.5	5.9
		2xCMC	30	40	35	38	30	41	30	30	35		34.3	4.3
		1xCMC	30	35	30	42	45	30	30	45	25	40	35.2	6.9
		1xCMC	30	35	35	45	50	22	45	5	33	30	33.0	12.3
		0.5xCMC	50	50	30	60	30	50	40	10	40	40	40.0	13.4
		0.5xCMC	45	35	50	35	50	43	40	50	45	45	43.8	5.4
	DI		42	45	60	65	32	15	50	25	40	40	41.4	14.3
			35	50	55	45	15	50	35	50	40	40	41.5	11.0

Table B.11 Fuel oil displacement efficiencies of lipopeptide biosurfactant and other solutions.

Solution	Diameter (cm) Before			Diameter (cm) After			% oil displacement			AVG	SD
	1	2	3	1	2	3	1	2	3		
Dehydol LS9TH	8.0	8.0	8.0	7.2	7.6	7.4	90.0	95.0	92.5	92.50	2.50
commercial detergent	8	8	8	7.5	6.8	7.1	93.8	85.0	88.8	89.17	4.39
Biosurfactant	8.0	8.0	8.0	7.2	7.0	7.1	90.0	87.5	88.8	88.75	1.25
water	8.0	8.0	8.0	0.1	0.3	0.3	1.3	3.8	3.8	2.92	1.44

Table B.12 Amounts of solubilized fuel oil in lipopeptide biosurfactant and other solutions

Lipopeptide Biosrufactant												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVE	SD
	Saturated	Aromatic	Resin	Asphalt ene	Total	Stearyl alc.						
1	300	50553	4422	8263	63538	25979	2.4457	1.6677	12.5	20.8464		
2	225	58152	3856	2626	64859	25541	2.5394	1.7484	12.5	21.8554		
3	550	58072	2422	3478	64522	20858	3.0934	2.2259	12.5	27.8236		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	25.22	3.66
	Saturated	Aromatic	Resin	Asphalt ene	Total	Stearyl alc.						
1		18556	4806	7591	30953	12729	2.4317	2.5359	12.5	31.6986		
2	2692	11918	4008	5715	24333	14628	1.6635	1.8738	12.5	23.4223		
3	2902	12045	5619	5282	25848	17804	1.4518	1.6914	12.5	21.1423		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphalt ene	Total	Stearyl alc.						
1	186	13889	10590	5726	30391	15119	2.0101	2.1726	12.5	27.1570		
2	179	11740	18282		30201	14973	2.0170	2.1785	12.5	27.2314		
3	704	11361	9620	8578	30263	16050	1.8855	2.0652	12.5	25.8149		

Dehydol LS9TH												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVE	SD
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	2412	40847	19836		63095	21532	2.9303	2.0853	12.5	26.0664	25.05	1.55
2	18587	41392	1246	1208	62433	22441	2.7821	1.9576	12.5	24.4699		
3	177	45930	20747	664	67518	23324	2.8948	2.0547	12.5	25.6839		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	169	29794	13666	2650	46279	14950	3.0956	2.2278	12.5	27.8472		
2	1366	48383	435	21389	71573	25920	2.7613	1.9397	12.5	24.2459		
3	430	48190	16809	6370	71799	24730	2.9033	2.0621	12.5	25.7758		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	200	25453	12121	3000	40774	14932	2.7306	1.9133	12.5	23.9156		
2	1000	39032	393	20211	60636	21343	2.8410	2.0084	12.5	25.1048		
3	200	45021	14542	6543	66306	25642	2.5858	1.7884	12.5	22.3556		

Water												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVE	SD
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1			1835	1478	3313	25015	0.1324	0.5543	12.5	6.9286	6.30	0.45
2			874		874	25983	0.0336	0.4691	12.5	5.8642		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	953	553			1506	24272	0.0620	0.4936	12.5	6.1702		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	1012	601			1613	24272	0.0665	0.4974	12.5	6.2177		

Commercial detergent												
Replication	Peak area						Ratio of peak area (y)	Ratio of mass (x) (x=y/slop	Amount of stearyl added	Total oil (mg) (x*12.5)	AVE	SD
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	757	1124	2753	1783	6417	21828	0.2940	0.6935	12.5	8.6689	10.36	1.92
2	268	386	268	1834	2756	23013	0.1198	0.5434	12.5	6.7920		
3	434	2678	2569		5681	22185	0.2561	0.6608	12.5	8.2605		
Replication	Peak area						Ratio of peak area (y)	Ratio of mass (x) (x=y/slop	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	3161	240	161	8956	12518	21556	0.5807	0.9406	12.5	11.7580		
2	2796	241	7390	1461	11888	20376	0.5834	0.9430	12.5	11.7872		
3	2537	399	6302	1933	11171	21116	0.5290	0.8961	12.5	11.2011		
Replication	Peak area						Ratio of peak area (y)	Ratio of mass (x) (x=y/slop	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	3201	251	152	8956	12560	21556	0.5827	0.9423	12.5	11.7789		
2	3000	230	7298	1461	11989	20376	0.5884	0.9472	12.5	11.8406		
3	2493	400	6210	1933	11036	21116	0.5226	0.8906	12.5	11.1322		

Table B.13 Amounts of residual fuel oil in lipopeptide biosurfactant and other washing solutions after used to washed contaminated sand

Dehydol LS9TH												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVG	SD
	Saturated	Aromatic	Resin	Asphalten e	Total	Stearyl alc.						
1	133	3243	20537	2001	25914	21308	1.2162	1.4883	12.5	18.60		
2	2889	2721	208	20249	26067	20597	1.2656	1.5309	12.5	19.14		
3	2501	2344	1393	20691	26929	20406	1.3197	1.5775	12.5	19.72		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	21.70	5.57
	Saturated	Aromatic	Resin	Asphalten e	Total	Stearyl alc.						
1	1085	659	19766	29352	50862	20016	2.5411	2.6302	12.5	32.88		
2	577	3940	23345	203	28065	23105	1.2147	1.4870	12.5	18.59		
3	167	4266	22203	1213	27849	19054	1.4616	1.6998	12.5	21.25		
Replication							Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphalten e	Total	Stearyl alc.						
1	4065	2774	18598	1960	27397	18085	1.5149	1.7458	12.5	21.82		
2	4964	175	22002	1004	28145	18227	1.5441	1.7710	12.5	22.14		

Lipopeptide biosurfactant												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVG	SD
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	5670	43966		381	50017	19925	2.5103	2.6036	12.5	32.5451		
2	5993	50255	848	1590	58686	22243	2.6384	2.7140	12.5	33.9255		
3	5253	44472	837	1785	52347	19490	2.6858	2.7549	12.5	34.4366		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	3092	36721	742	1289	41844	16058	2.6058	2.6859	12.5	33.5743		
2	3419	43279	242	1146	48086	18389	2.6149	2.6938	12.5	33.6727	35.29	4.40
3	3082	42222	149	1326	46779	18974	2.4654	2.5650	12.5	32.0620		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	10940	45201	964	292	57397	21176	2.7105	2.7762	12.5	34.7020		
2	10635	42336	820	1056	54847	19321	2.8387	2.8867	12.5	36.0836		
3	11496	48567	1086	2468	63617	16683	3.8133	3.7266	12.5	46.5826		

Commercial Detergent												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVG	SD
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	7828	512	9773	4372	22485	17845	1.2600	1.5261	12.5	19.08	18.87	0.74
2	11588	601	12422	989	25600	22415	1.1421	1.4245	12.5	17.81		
3	12196	573	13497	2126	28392	23517	1.2073	1.4806	12.5	18.51		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	11894	1178	8822	3846	25740	19845	1.2971	1.5580	12.5	19.48		
2	16908	1394	12997	5246	36545	30214	1.2095	1.4826	12.5	18.53		
3	17355	1621	15255	1867	36098	27104	1.3318	1.5880	12.5	19.85		
Replication							Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	6617	823	4600	2800	14840	17363	0.8547	1.1768	12.5	14.71		
2	7763	853	5407	4339	18362	22212	0.8267	1.1526	12.5	14.41		
3	8921	802	6488	3180	19391	24742	0.7837	1.1156	12.5	13.94		

Water												
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)	AVG	SD
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	702	491	390	1279	2862	25357	0.1129	0.5374	12.5	6.72	7.11	0.77
2	240	521	598	373	1732	21022	0.0824	0.5112	12.5	6.39		
3	1311	1414	432		3157	25066	0.1259	0.5487	12.5	6.86		
Replication	Peak area						Ratio of peak area (y) (y=total/stea	Ratio of mass (x) (x=y/slope)	Amount of stearyl added	Total oil (mg) (x*12.5)		
	Saturated	Aromatic	Resin	Asphaltene	Total	Stearyl alc.						
1	128	1262	905	667	2962	21667	0.1367	0.5580	12.5	6.9745		
2	522	1667	1425	264	3878	25407	0.1526	0.5717	12.5	7.1462		
3	3550	1191	199	1379	6319	22079	0.2862	0.6868	12.5	8.5851		

APPENDIX C SUPPLEMENTARY DATA OF CHAPTER IV

Table C.1 The effect of lipopeptide molar fractions on the IFT for various hydrocarbons. The total surfactant concentration was 0.1 M.

Molar Fraction		IFT (mN/m2)		
AMA	Lipopeptide	Hexane	Decane	Dodecane
0.1	0	0.1985	1.854	4.214
		0.1899	1.954	4.198
		0.1987	1.854	4.1985
AVE		0.1957	1.8873	4.2035
SD		0.0050	0.0577	0.0091
0.09	0.01	0.1543	1.687	2.9524
		0.1643	1.654	2.9776
		0.1732	1.684	2.9402
		0.1872		2.9005
AVE		0.1698	1.6750	2.9567
SD		0.0140	0.0182	0.0191
0.086	0.014	0.1061	0.9787	2.547
		0.0962	0.8745	2.5478
		0.0869	0.987	2.621
		0.0914		2.541
		0.0962		
AVE		0.0954	0.9467	2.5642
SD		0.0071	0.0627	0.0380
0.08	0.02	0.1541	**0.857**	2.487
		0.15441	**0.8547**	2.548
		0.185		2.421
AVE		0.1645	0.8559	2.4853
SD		0.0178	0.0016	0.0635

The C.1 effect of lipopeptide molar fractions on the IFT for various hydrocarbons. The total surfactant concentration was 0.1 M. **(Cont'.)**

Molar Fraction		IFT (mN/m2)		
AMA	Lipopeptide	Hexane	Decane	Dodecane
0.075	0.025	0.241	0.8321	2.2012
		0.2584	0.8324	2.314
		0.2854	0.841	2.345
AVE		0.2616	0.8352	2.2867
SD		0.0224	0.0051	0.0757
0.07	0.03	0.252	0.745	1.987
		0.245	0.7574	1.9958
		0.252	0.784	1.987
AVE		0.2497	0.7621	1.9899
SD		0.0040	0.0199	0.0051
0.05	0.05	0.421	0.647	1.748
		0.451	0.6547	1.654
		0.4325	0.6841	1.761
AVE		0.4348	0.6619	1.7210
SD		0.0151	0.0196	0.0584
0	0.7	0.5847	0.6214	1.524
		0.5745	0.6014	1.624
		0.5624	0.611	1.521
AVE		0.5739	0.6113	1.5563
SD		0.0112	0.0100	0.0586
0	1	0.7854	0.6214	0.541
		0.754	0.654	0.584
		0.7489	0.6541	0.5748
AVE		0.7628	0.6432	0.5666
SD		0.0198	0.0189	0.0226

Table C. 2 The comparison of single and mixed surfactant systems on the IFT for various hydrocarbons. The total surfactant concentration was 0.25 M.

Formulation	Hydrocarbon		IFT Lipopeptide biosurfactant Mixtures					
		EACN	1	2	3	4	AVG	SD
Final conc. 0.25	Hexane	6	0.3335	0.3210	0.2865	0.3137	0.3137	0.0172
	Decane	10	0.0894	0.1011	0.0762	0.0762	0.0857	0.0143
	Dodecane	12	0.1180	0.1481	0.1480	0.1550	0.1423	0.0104
	Hexadecane	16	0.1432	0.1543	0.1563	0.1653	0.1548	0.0143
	Crude oil ARL + AXL		0.0683	0.08920	0.08920	0.08540	0.0830	0.0086
	Bong kot Crude oil		0.301	0.3262	0.298	0.2903	0.3039	0.0134

Hydrocarbon		IFT Pure lipopeptide biosurfactant					
	EACN	1	2	3	4	AVG	SD
Toluen	1	4.4773	4.2091	4.103	4.023	4.203	0.198
Haxane	6	3.5421	3.3521	3.063	3.2011	3.290	0.206
Decane	10	2.867	2.887	2.752	2.9143	2.855	0.071
Haxadecane	16	2.6812	2.8182	2.5943	2.5422	2.659	0.121

Hydrocarbon		IFT Pure SDHS alone					
	EACN	1	2	3	4	AVG	SD
Haxane	6	1.932	2.1092	2.212	1.832	2.021	0.171
Decane	10	2.712	2.821	2.421	2.3431	2.574	0.229
Dodecane	12	3.2121	3.1032	3.4321	3.281	3.257	0.138
Haxadecane	16	3.3421	3.421	3.5415	3.6211	3.481	0.124

Table C.3 Oil displacement test of lipopeptide-based dispersants against the ARL/AXL blend.

DOR	Crude oil							
	ARL + AXL							
	Formulation 1							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	before	after	% oil displacement		
1:2	8	8.00	100	8	7.8	97.5	98.75	1.25
1:5	8	8.00	100	8	7.85	98.125	99.06	0.9375
1:10	8	8.00	100	8	7.7	96.25	98.13	1.875
1:15	8	7.75	96.875	8	7.35	91.875	94.38	2.5
1:20	8	7.00	87.5	8	7.25	90.625	89.06	1.5625
1:25	8	7.45	93.125	8	6.95	86.875	90.00	3.125
1:50	8	6.60	82.5	8	6.25	78.125	80.31	2.1875
1:75	8	6.00	75	8	5.75	71.875	73.44	1.5625
1:100	8	5.85	73.125	8	5.65	70.625	71.88	1.25
1:200	8	4.00	50	8	4	50	50.00	0

Table C.4 Oil displacement test of lipopeptide-based dispersants against the ARL/AXL.

DOR	Crude oil							
	ARL + AXL							
	Formulation 2							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	Before (cm)	After (cm)	% oil displacement		
1:2	8	8.00	100	8	7.85	98.12	99.06	0.93
1:5	8	7.90	98.75	8	8	100	99.37	0.62
1:10	8	7.95	99.375	8	7.85	98.12	98.75	0.62
1:15	8	8.00	100	8	7.75	96.87	98.43	1.56
1:20	8	7.75	96.875	8	8	100	98.43	1.56
1:25	8	8.00	100	8	7.8	97.5	98.75	1.25
1:50	8	7.95	99.375	8	7.5	93.75	96.56	2.81
1:75	8	7.55	94.375	8	7.8	97.5	95.93	1.56
1:100	8	7.25	90.625	8	6	75	82.81	7.81
1:200	8	5.50	68.75	8	5.6	70	69.37	0.62

Table C.5 Oil displacement test of lipopeptide against the ARL/AXL blend.

DOR	Crude oil							
	ARL + AXL							
	Lipopeptide biosurfactant only							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	Before (cm)	After (cm)	% oil displacement		
1:2	8	6.00	75	8	6.5	81.25	78.13	3.125
1:5	8	6.00	75	8	6.2	77.5	76.25	1.25
1:10	8	6.00	75	8	5.8	72.5	73.75	1.25
1:15	8	4.80	60	8	5.5	68.75	64.38	4.375
1:20	8	5.00	62.5	8	4.8	60	61.25	1.25
1:25	8	4.50	56.25	8	4.5	56.25	56.25	0
1:50	8	4.50	56.25	8	4.2	52.5	54.38	1.875
1:75	8	4.50	56.25	8	4.3	53.75	55.00	1.25
1:100	8	4.50	56.25	8	4.3	53.75	55.00	1.25
1:200	8	4.20	52.5	8	4	50	51.25	1.25

Table C.6 Oil displacement test of Slickgone against the ARL/AXL blend.

DOR	Crude oil							
	ARL + AXL							
	Slickgone							
	1			2			AVG	SD
	before	after	% oil displacement	before	after	% oil displacement		
1:02	8	8.00	100.0	8	7.80	97.5	98.75	1.25
1:05	8	8.00	100.0	8	7.85	98.1	99.06	0.9375
1:10	8	8.00	100.0	8	7.70	96.3	98.13	1.875
1:15	8	6.00	75.0	8	6.50	81.3	78.13	3.125
1:20	8	5.25	65.6	8	6.75	84.4	75.00	9.375
1:25	8	5.50	68.8	8	4.50	56.3	62.50	6.25
1:50	8	5.25	65.6	8	6.25	78.1	71.88	6.25
. 1 : 75	8	5.00	62.5	8	5.00	62.5	62.50	0
.1 : 100	8	2.50	31.3	8	3.50	43.8	37.50	6.25
.1 : 200	8	2.50	31.3	8	3.00	37.5	34.38	3.125

Table C.7 Oil displacement test of lipopeptide-based dispersants against the BKC.

DOR	Crude oil							
	BKC							
	Formulation 1							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	Before (cm)	After (cm)	% oil displacement		
1:2	8	8	100	8	7.85	98.125	99.06	0.938
1:5	8	8	100	8	7.75	96.875	98.44	1.563
1:10	8	8	100	8	7.9	98.75	99.38	0.625
1:15	8	8	100	8	7.45	93.125	96.56	3.438
1:20	8	8	100	8	7.95	99.375	99.69	0.313
1:25	8	8	100	8	7.95	99.375	99.69	0.313
1:50	8	8	100	8	7.95	99.375	99.69	0.313
1:75	8	6.75	84.375	8	7.45	93.125	88.75	4.375
1:100	8	6.5	81.25	8	6.7	83.75	82.50	1.250
1:200	8	5.75	71.875	8	5.95	74.375	73.13	1.250

Table C.8 Oil displacement test of lipopeptide-based dispersants against the BKC crude oil.

DOR	Crude oil							
	BKC							
	Formulation 2							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	Before (cm)	After (cm)	% oil displacement		
1:2	8	7.5	93.75	8	7.7	96.25	95	1.25
1:5	8	7.85	98.12	8	7.9	98.75	98.43	0.31
1:10	8	7.9	98.75	8	7.6	95	96.87	1.88
1:15	8	7.9	98.75	8	7.35	91.875	95.31	3.44
1:20	8	7.8	97.5	8	7.3	91.25	94.37	3.13
1:25	8	7.85	98.125	8	7.2	90	94.06	4.06
1:50	8	7.8	97.5	8	7.4	92.5	95	2.50
1:75	8	5.5	68.75	8	5.75	71.875	70.31	1.56
1:100	8	5.5	68.75	8	6	75	71.87	3.13
1:200	8	5.625	70.31	8	5	62.5	66.40	3.91

Table C.9 Oil displacement test of lipopeptide against the BKC crude oil.

DOR	Crude oil							
	BKC							
	Lipopeptide biosurfactant							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	Before (cm)	After (cm)	% oil displacement		
1:2	8	5.00	62.5	8	5	62.5	62.50	0
1:5	8	5.00	62.5	8	6	75	68.75	6.25
1:10	8	4.50	56.25	8	5.5	68.75	62.50	6.25
1:15	8	6.00	75	8	4	50	62.50	12.5
1:20	8	5.50	68.75	8	4.5	56.25	62.50	6.25
1:25	8	5.50	68.75	8	4.2	52.5	60.63	8.125
1:50	8	4.00	50	8	4.5	56.25	53.13	3.125
1:75	8	4.00	50	8	3.8	47.5	48.75	1.25
1:100	8	4.50	56.25	8	4.2	52.5	54.38	1.875
1:200	8	4.00	50	8	4	50	50.00	0

Table C.10 Oil displacement test of Slickgone against the BKC crude oil.

DOR	Crude oil							
	BCK							
	Slickgone							
	1			2			AVG	SD
	Before (cm)	After (cm)	% oil displacement	Before (cm)	After (cm)	% oil displacement		
1:2	8	8.00	100	8	7.90	98.75	99.38	0.62
1:5	8	8.00	100	8	7.80	97.5	98.75	1.25
1:10	8	8.00	100	8	7.90	98.75	99.38	0.66
1:15	8	8.00	100	8	7.00	87.5	93.75	6.25
1:20	8	7.25	90.62	8	7.50	93.75	92.19	1.56
1:25	8	6.85	85.62	8	6.50	81.25	83.44	2.18
1:50	8	6.35	79.37	8	6.35	79.37	79.38	0
1:75	8	5.50	68.75	8	5.50	68.75	68.75	0
1:100	8	4.50	56.25	8	5.00	62.5	59.38	3.16
1:200	8	2.50	31.25	8	3.50	43.75	37.50	6.25

Table C.11 Effectiveness of lipopeptide-based dispersants, Slickgone and lipopeptides over crude oils in the baffled flask test.

	amount of crude oil from TLC	amount of oil in 120 ml seawater		Mass of initial crude oil	Efficiency	AVE	SD
	mg	mg	g	g	%		
Formulation 1 (F-1)	9.00	54.00	0.054	0.084	64.29		
	7.73	46.38	0.046	0.084	55.21		
	8.65	51.87	0.051	0.084	61.75	60.42	4.68
Formulation 2 (F-2)	11.97	71.79	0.071	0.084	85.46		
	12.72	76.31	0.076	0.084	90.85		
	13.79	82.74	0.082	0.084	98.50	91.61	6.55
Slickgone NS type 2/3	6.31	37.85	0.037	0.084	45.06		
	7.35	44.09	0.044	0.084	52.49		
	6.86	41.17	0.041	0.084	49.01		
	7.85	47.10	0.047	0.084	56.08		
	6.36	38.18	0.038	0.084	45.46	49.63	4.70
Lipopeptide biosurfactant	4.17	25.02	0.025	0.084	29.78		
	4.21	25.26	0.025	0.084	30.07		
	4.32	25.92	0.025	0.084	30.8643	30.24	0.56

Table C.12 Oil displacement test of lipopeptide-based dispersants, Slickgone and lipopeptides against BKC crude oil

	amount of crude oil from TLC	amount of oil in 120 ml seawater		Mass of initial crude oil	Efficiency %		
	mg	mg	g	g		AVE	SD
Formulation 1	9.80	58.80	0.0588	0.064	91.88		
	10.10	60.60	0.0606	0.064	94.69		
	11.20	67.20	0.0672	0.064	105.00		
	10.50	63.00	0.063	0.064	98.44	97.50	5.68
Formulation 2 (F-2)	11.97	71.79	0.0516	0.064	80.625		
	12.72	76.32	0.0504	0.064	78.75		
	13.79	82.75	0.0564	0.064	88.125		
	15.18	91.11	0.0504	0.064	78.75	91.61	6.55
Slickgone NS type 2/3	6.31	37.85	0.0438	0.064	45.07		
	7.35	44.10	0.0402	0.064	52.50		
	6.86	41.18	0.042	0.064	49.02		
	7.85	47.11	0.0372	0.064	56.08		
	6.36	38.19	0.0438	0.064	45.46	49.63	4.70
Lipopeptide biosurfactant	2.43	14.58	0.01458	0.064	22.78		
	3.21	19.26	0.01926	0.064	30.09		
	2.87	17.23	0.017226	0.064	26.92		
	1.92	11.52	0.01152	0.064	18.00	24.45	5.24

Table C.13 Population of whiteleg shrimp after a 96-h exposure of lipopeptide based dispersant, WAF with Bongkot light crude oil, the lipopeptide based dispersant formulation and the dispersed oil and CEWAF with Bongkot light crude oil and lipopeptide based dispersant

Lipopeptide based dispersant								
	Amount of shrimp dead			**% Mortarity**				
Conc.	**1.0**	**2.0**	**3.0**	**1.0**	**2.0**	**3.0**	Aver	SD
0	0	0	0	0	0	0	0.0	0.0
20	0	0	0	0	0	0	0.0	0.0
40	0	0	0	0	0	0	0.0	0.0
60	1	0	1	10	0	10	6.7	5.8
80	0	1	1	0	10	10	6.7	5.8
100	0	2	1	0	20	10	10.0	10.0

WAF								
	Amount of shrimp dead			**% Mortarity**				
Conc.	**1.0**	**2.0**	**3.0**	**1.0**	**2.0**	**3.0**	**Aver**	**SD**
0	0	0	0	0	0	0	0.0	0.0
20	0	0	0	0	0	0	0.0	0.0
40	0	1	0	0	10	0	3.3	5.8
60	1	1	1	10	10	10	10.0	0.0
80	5	6	6	50	60	60	56.7	5.8
100	8	7	8	80	70	80	76.7	5.8

CWAF								
	Amount of shrimp dead			**% Mortarity**				
Conc.	**1.0**	**2.0**	**3.0**	**1.0**	**2.0**	**3.0**	Aver	SD
0	0	0	0	0	0	0	0.0	0.0
20	0	0	0	0	0	0	0.0	0.0
40	0	1	1	0	10	10	6.7	5.8
60	1	1	2	10	10	20	13.3	5.8
80	0	3	3	0	30	30	20.0	17.3
100	7	7	6	70	70	60	66.7	5.8

APPENDIX D SUPPLEMENTARY DATA OF CHAPTER V

APPENDIX D SUPPLEMENTARY DATA OF CHAPTER IV

Table D.1 The remaining crude oil in sand after 30-min shaking time and 10-day incubation. The control experiment contained only synthetic seawater.

	30 min. after shaking				
Treatment	mg crude oil/gram sand				
	1	2	3	AVE	SD
Crude oil only	27.64	28.89	26.43	27.65	1.23
Gordonia sp. JC11 only	28.62	29.70	28.55	28.96	0.65
Lipopeptide based dispersant	0.00	16.31	17.61	11.30	9.81
lipopeptide based dispersant + *Gordonia* sp. JC11	17.63	20.13	22.14	19.97	2.26
Day 10					
Treatment	mg crude oil/gram sand				
	1	2	3	AVE	SD
Crude oil only	33.25	35.87		34.56	1.85
Gordonia sp. JC11 only	21.88	21.59	21.55	21.67	0.18
Lipopeptide based dispersant	39.41	37.91	36.08	37.80	1.67
lipopeptide based dispersant + *Gordonia* sp. JC11	17.71	17.74	18.91	18.12	0.69

Table D.2 The number of *Gordonia* sp. JC11 at day 0 and 10 from the treatment with *Gordonia* sp. JC11 only.

Crude oil + *Gordonia* sp. JC11					
Day 0					
Colony dilution 3	CFU/ml	gram sand (g)	CFU/gram sand	AVE	SD
15	1.E+06	0	4.E+06		
12	1.E+06	0	3.E+06		
10	1.E+06	1	2.E+06	3.E+06	1.E+06
Day 10					
Colony dilution 5	CFU/ml	gram sand	CFU/gram sand	AVE	SD
1	1.00E+07	0.568	1.76E+07		
2	2.00E+07	0.784	2.55E+07		
2	2.00E+07	0.551	3.63E+07	2.65E+07	9.38E+06

Table D.3 The number of *Gordonia* sp. JC11 at day 0 and 10 from the treatment with *Gordonia* sp. JC11 only and lipopeptide based dispersant along with *Gordonia* sp. JC11.

Crude oil + *Lipopeptide based dispersant* + *Gordonia* sp. JC11					
Day 0					
Colony dilution 3	CFU/ml	gram sand (g)	CFU/gram sand	AVE	SD
7	7.00E+05	0.575	1.22E+06		
10	1.00E+06	0.546	1.83E+06		
11	1.10E+06	0.412	2.67E+06	1.91E+06	7.29E+05
Day 10					
Colony dilution 5	CFU/ml	gram sand	CFU/gram sand	AVE	SD
1	1.00E+07	0.545	1.83E+07		
1	1.00E+07	0.85	1.18E+07		
2	2.00E+07	0.715	2.80E+07	1.94E+07	8.15E+06

Table D.4 The remaining crude oil in sampling point 1 at 5-cm depths and 15-cm depths from the surface of natural seawater. This experiment was done with the 9 mL of Bongkot light crude oil only as a control.

Point 1 5-depth					
	Natural Seawater				
	1	2	3	AVE	SD
Day 0	3.9	4	4	3.967	0.058
Day1	3	2.5	2.7	2.733	0.252
Day 2	1.7	2.1	2.1	1.967	0.231
Day 3	3	2	2	2.333	0.577
Day 4	3	2.8	2.1	2.633	0.473
Day 5	2	2.3	2.1	2.133	0.153
Point 1 15-depth					
	Natural Seawater				
	1	2	3	AVE	SD
Day 0	0.3	0.2	0.1	0.20	0.10
Day1	0.9	0.7	0.6	0.73	0.15
Day 2	1.3	1.2	1.1	1.20	0.10
Day 3	3.4	4.5	4.6	4.17	0.67
Day 4	2.7	2.9	3	2.87	0.15
Day 5	2.2	2.7	2.8	2.57	0.32

Table D.5 The remaining crude oil in sampling Point 2 at 5-cm depths and 15-cm depths from the surface of natural seawater. This experiment was done with the 9 mL of Bongkot light crude oil only as a control.

Point 2 5-depth					
	Natural Seawater				
	1	2	3	AVE	SD
Day 0	2	2.5	2.3	2.27	0.252
Day1	1.8	2	1.7	1.83	0.153
Day 2	1.3	1.2	1.2	1.23	0.058
Day 3	1.3	1.6	1.4	1.43	0.153
Day 4	1.5	1.8	2	1.77	0.252
Day 5	1.6	1.7	1.4	1.57	0.153

Point 2 15-depth					
	Natural Seawater				
	1	2	3	AVE	SD
Day 0	0.3	0.1	0.1	0.17	0.12
Day1	0.6	0.6	0.5	0.57	0.06
Day 2	3.7	4.3	4.2	4.07	0.32
Day 3	0.7	0.8	0.6	0.70	0.10
Day 4	0.6	0.6	0.7	0.63	0.06
Day 5	0.6	0.6	0.7	0.63	0.06

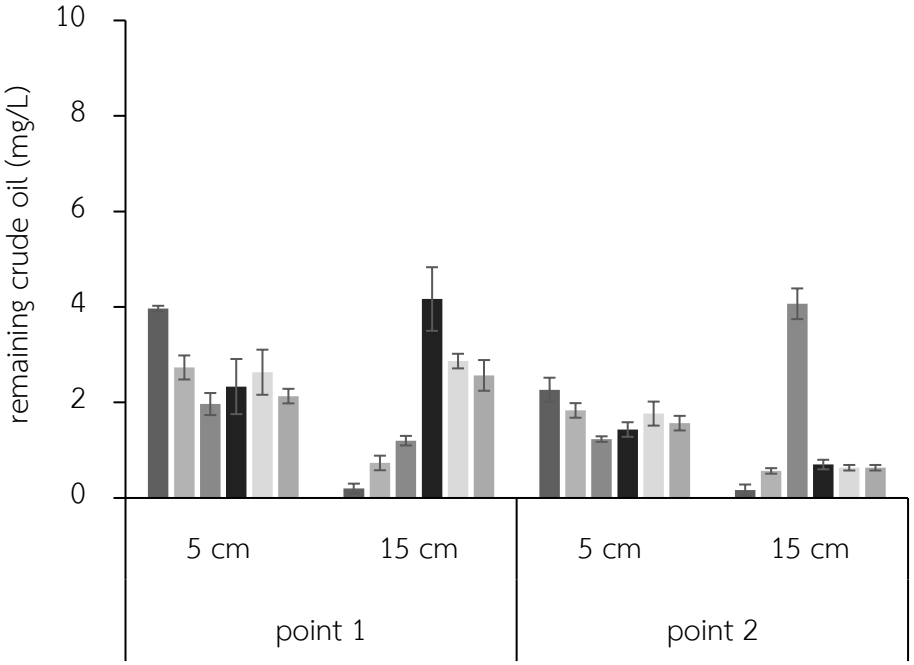


Figure D.1 The remaining crude oil in sampling point 1 and Point 2 at 5-cm depths and 15-cm depths from the surface of natural seawater. This experiment was done with the 9 mL of Bongkot light crude oil only as a control.

Table D.7 The remaining crude oil in four sampling points for crude oil only treatment of natural seawater.

Point 1-5	Remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	2.9	3.1	3	3.000	0.100
Day1	9	9	7.8	8.600	0.693
Day 2	16	15.2	16.1	15.767	0.493
Day 3	3.9	4.6	4.7	4.400	0.436
Day 4	4.13	4.1	4.1	4.110	0.017
Day 5	4.12	4.2	4.12	4.147	0.046
Point 1-15	Remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	0.1	0.5	0.2	0.3	0.208
Day1	5.9	6.1	6.4	6.1	0.252
Day 2	12	11	11.7	11.6	0.513
Day 3	10.4	11	10.6	10.7	0.306
Day 4	9.2	10.1	9.4	9.56	0.473
Day 5	6	7.2	7.3	6.83	0.723
Point 2-5	Remaining crude oil (mg/L)				
	1.0	2.0	3.0	AVE	SD
Day 0	13.0	12.5	12.2	12.56	0.40
Day1	10.2	9.9	9.7	9.93	0.25
Day 2	6.0	6.2	6.3	6.16	0.15
Day 3	7.0	6.5	6.0	6.50	0.50
Day 4	6.0	6.5	6.4	6.30	0.26
Day 5	5.0	4.7	4.8	4.83	0.15
Point 2-15	Remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	5.3	4.7	4.8	4.933	0.321
Day1	13.4	14.2	15.7	14.433	1.168
Day 2	11.4	11.3	11	11.233	0.208
Day 3	8.9	8.5	8.2	8.533	0.351
Day 4	7.6	7.2	7.2	7.333	0.231
Day 5	7.5	7	7.6	7.367	0.321

Table D. 8The remaining crude oil in four sampling points for Lipopeptide based dispersant treatment of natural seawater.

1-5	remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	3.9	4	4	3.97	0.06
Day1	3	2.5	2.7	2.73	0.25
Day 2	1.7	2.1	2.1	1.97	0.23
Day 3	3	2	2	2.33	0.58
Day 4	3	2.8	2.1	2.63	0.47
Day 5	2	2.3	2.1	2.13	0.15
1-15	remainig crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	0.3	0.2	0.1	0.20	0.10
Day1	0.9	0.7	0.6	0.73	0.15
Day 2	1.3	1.2	1.1	1.20	0.10
Day 3	3.4	4.5	4.6	4.17	0.67
Day 4	2.7	2.9	3	2.87	0.15
Day 5	2.2	2.7	2.8	2.57	0.32
	remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	2	2.5	2.3	2.27	0.25
Day1	1.8	2	1.7	1.83	0.15
Day 2	1.3	1.2	1.2	1.23	0.06
Day 3	1.3	1.6	1.4	1.43	0.15
Day 4	1.5	1.8	2	1.77	0.25
Day 5	1.6	1.7	1.4	1.57	0.15
	remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	0.3	0.1	0.1	0.17	0.12
Day1	0.6	0.6	0.5	0.57	0.06
Day 2	3.7	4.3	4.2	4.07	0.10
Day 3	0.7	0.8	0.6	0.70	0.32
Day 4	0.6	0.6	0.7	0.63	0.06
Day 5	0.6	0.6	0.7	0.63	0.06

Table D.9 The remaining crude oil in four sampling points for *Gordonia* sp. JC11 treatment of natural seawater.

1-5	remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	2	2.4	2	2.133	0.189
Day1	10	11	10.6	10.533	0.411
Day 2	5	6.2	5	5.400	0.566
Day 3	4.6	5.2	4.8	4.867	0.249
Day 4	6.3	8.9	8.8	8.000	1.203
Day 5	3	3.1	3.1	3.067	0.047
1-15	remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	3	2.2	4	3.067	0.736
Day1	6.5	5	6	5.83	0.76
Day 2	4.5	5	4.7	4.73	0.25
Day 3	4.5	5.2	5	4.90	0.36
Day 4	5.6	5	5.3	5.30	0.30
Day 5	5	6	6.4	5.80	0.72
2-5	remaining crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	6	9	9.5	8.17	1.55
Day1	6	5	5.5	5.50	0.50
Day 2	5.2	5.4	6	5.53	0.42
Day 3	5.4	5	5.7	5.37	0.35
Day 4	4	4	4.5	4.17	0.29
Day 5	3.2	2.9	4.2	3.43	0.68
2-15	remainig crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	11.2	12.0	13.3	12.2	1.1
Day1	5.0	6.4	6.0	5.8	0.7
Day 2	5.3	5.3	6.5	5.7	0.7
Day 3	3.0	3.5	4.0	3.5	0.5
Day 4	4.0	3.9	4.2	4.0	0.2
Day 5	2.5	2.8	4.0	3.1	0.8

Table D.10 The remaining crude oil in four sampling points for Lipopeptide based dispersant + *Gordonia* sp. JC11 treatment of natural seawater.

	remainig crude oil (mg/L)				
1-5	1	2	3	AVE	SD
Day 0	14.4	12	12.4	12.93	1.05
Day1	10	8	9	9.0	0.81
Day 2	2.5	3.2	3	2.90	0.29
Day 3	2	2.1	1.7	1.93	0.17
Day 4	6.3	8.4	8.5	7.73	1.01
Day 5	1	0.5	1.1	0.86	0.26
	remainig crude oil (mg/L)				
1-15	1	2	3	AVE	SD
Day 0	10.8	10.7	10.8	10.77	0.06
Day1	1.1	1	0.8	0.97	0.15
Day 2	0.4	0.4	0.7	0.50	0.17
Day 3	0.4	0.3	0.4	0.37	0.06
Day 4	2.2	1.7	2.2	2.03	0.29
Day 5	1.6	1.6	1.8	1.67	0.12
2-5	remainig crude oil (mg/L)				
	1	2	3	AVE	SD
Day 0	16	15.3	15.2	15.50	0.436
Day1	0.7	0.9	2	1.20	0.700
Day 2	0.4	0.5	0.6	0.50	0.100
Day 3	0.6	0.5	0.5	0.53	0.058
Day 4	2.1	2.9	2.9	2.63	0.462
Day 5	1.5	1.6	1.3	1.47	0.153
remainig crude oil (mg/L)					
2-15	1	2	3	AVE	SD
Day 0	11.20	11.70	11.80	11.57	0.32
Day1	3.00	4.20	3.00	3.40	0.69
Day 2	2.80	2.50	2.50	2.60	0.17
Day 3	1.10	1.60	1.50	1.40	0.26
Day 4	1.00	1.10	1.20	1.10	0.10
Day 5	0.60	0.50	0.80	0.63	0.15

Table D.11 The number of oil degrading bacteria and total bacteria at day 0 and day 5 in sampling point 1 and 2 at 5-cm and 15-cm depths from the surface of natural seawater of control treatment.

oil degrading bacteria (MPN/mL)				
	point 1-5	point 1-15	point 2-5	point 2-15
Day 0	3.40E+05	2.50E+05	2.10E+05	2.40E+06
	3.50E+05	2.80E+05	1.30E+05	2.40E+06
	3.00E+05	2.10E+05	1.70E+05	1.80E+06
AVE	3.30E+05	2.47E+05	1.70E+05	2.20E+06
SD	2.16E+04	2.87E+04	3.27E+04	2.83E+05
Day 5	7.30E+06	1.80E+06	1.90E+06	2.65E+06
	5.90E+06	1.70E+06	1.50E+06	2.20E+06
	7.80E+06	1.60E+06	1.70E+06	2.85E+06
AVE	7.00E+06	1.70E+06	1.70E+06	2.57E+06
SD	8.04E+05	8.16E+04	1.63E+05	2.72E+05
Total bacterial (MPN/mL)				
Day 0	6.93E+06	8.00E+06	6.00E+06	5.40E+06
	7.00E+06	7.60E+06	5.53E+06	3.80E+06
	6.89E+06	7.80E+06	5.10E+06	4.00E+06
AVE	6.94E+06	7.80E+06	5.54E+06	4.40E+06
SD	4.55E+04	1.63E+05	3.68E+05	7.12E+05
Day 5	1.55E+07	6.50E+06	3.80E+06	4.90E+06
	1.75E+07	7.50E+06	4.00E+06	5.00E+06
	1.20E+07	5.50E+06	4.00E+06	4.80E+06
AVE	1.50E+07	6.50E+06	3.93E+06	4.90E+06
SD	2.27E+06	8.16E+05	9.43E+04	8.16E+04

Table D.12 The number of oil degrading bacteria and total bacteria at day 0 and day 5 in sampling point 1 and 2 at 5-cm and 15-cm depths from the surface of natural seawater of Lipopeptide based dispersant only

oil degrading bacteria (MPN/mL)				
	point 1-5	point 1-15	point 2-5	point 2-15
Day 0	5.90E+04	2.50E+05	5.00E+04	1.20E+03
	4.00E+03	1.50E+04	2.00E+03	3.50E+04
	9.00E+03	1.50E+04	2.00E+04	1.50E+02
AVE	2.40E+04	9.33E+04	2.40E+04	1.21E+04
SD	2.48E+04	1.11E+05	1.98E+04	1.62E+04
Day 5	2.10E+05	2.30E+05	6.00E+05	6.00E+03
	2.10E+03	3.00E+02	3.50E+03	6.10E+05
	4.20E+05	4.50E+05	2.10E+05	2.00E+05
AVE	2.11E+05	2.27E+05	2.71E+05	2.72E+05
SD	1.71E+05	1.84E+05	2.47E+05	2.52E+05

Total bacterial (MPN/mL)

Day 0	4.80E+05	8.00E+06	4.30E+06	4.30E+06
	1.50E+03	5.80E+06	4.30E+05	930000
	1.50E+03	1.00E+03		
AVE	1.61E+05	4.60E+06	2.37E+06	2.62E+06
SD	2.26E+05	3.37E+06	1.94E+06	1.69E+06
Day 5	2.00E+07	2.00E+07	3.00E+06	1.30E+07
	9.00E+05	3.50E+06	9.00E+06	1.00E+05
	7.00E+06	4.50E+06	9.00E+05	5.00E+04
AVE	9.30E+06	9.33E+06	4.30E+06	4.38E+06
SD	7.97E+06	7.55E+06	3.43E+06	6.09E+06

Table D.13 The number of oil degrading bacteria and total bacteria at day 0 and day 5 in sampling point 1 and 2 at 5-cm and 15-cm depths from the surface of natural seawater of *Gordonia* sp .JC11

oil degrading bacteria (MPN/mL)				
	point 1-5	point 1-15	point 2-5	point 2-15
Day 0	3.50E+06	1.10E+06	8.00E+05	6.10E+05
	9.00E+06	2.00E+05	4.00E+05	1.80E+05
	6.00E+06	5.00E+04	3.50E+05	8.00E+05
AVE	6.17E+06	4.50E+05	5.17E+05	5.30E+05
SD	2.25E+06	4.64E+05	2.01E+05	2.59E+05
Day 5	2.10E+07	7.40E+07	2.10E+06	2.90E+05
	2.30E+06	2.60E+08	5.60E+06	3.80E+06
	4.30E+06	2.90E+07		4.30E+05
AVE	9.20E+06	1.21E+08	3.85E+06	1.51E+06
SD	8.38E+06	1.00E+08	1.75E+06	1.62E+06
	Total bacterial (MPN/mL)			
Day 0	2.90E+08	9.00E+07	6.40E+08	2.70E+08
	7.20E+07	3.80E+08	3.80E+07	1.00E+08
	6.40E+07	2.30E+07	2.10E+08	2.10E+07
AVE	1.42E+08	1.64E+08	2.96E+08	1.30E+08
SD	1.05E+08	1.55E+08	2.53E+08	1.04E+08
Day 5	2.60E+08	3.80E+06	7.40E+08	4.00E+08
	6.40E+08	1.40E+08	2.10E+08	3.00E+09
	4.50E+07	1.50E+08	9.30E+08	4.30E+08
AVE	3.15E+08	9.79E+07	6.27E+08	1.28E+09
SD	2.46E+08	6.67E+07	3.05E+08	1.22E+09

Table D.14 The number of oil degrading bacteria (a) and total bacteria (b) at day 0 and day 5 in sampling point 1 and 2 at 5-cm and 15-cm depths from the surface of natural seawater of lipopeptide based dispersant + *Gordonia* sp .JC11

oil degrading bacteria (MPN/mL)				
	point 1-5	point 1-15	point 2-5	point 2-15
Day 0	1.20E+06	1.10E+07	6.80E+06	2.70E+06
	5.00E+03	6.00E+06	4.00E+05	7.00E+06
	3.00E+06	1.60E+07	1.30E+07	8.00E+05
AVE	1.40E+06	1.10E+07	6.73E+06	3.50E+06
SD	1.23E+06	4.08E+06	5.14E+06	2.59E+06
Day 5	1.80E+07	9.00E+05	9.20E+06	4.50E+06
	8.00E+06	3.00E+08	6.70E+07	4.00E+08
	2.50E+07	1.20E+08	2.00E+08	2.00E+07
AVE	1.70E+07	1.40E+08	9.21E+07	1.42E+08
SD	6.98E+06	1.23E+08	7.99E+07	1.83E+08
	Total bacterial (MPN/mL)			
Day 0	3.50E+08	1.10E+08	8.00E+08	6.10E+08
	9.00E+07	2.00E+08	4.00E+07	1.80E+08
	6.00E+07	5.00E+07	3.50E+08	8.00E+07
AVE	1.67E+08	1.20E+08	3.97E+08	2.90E+08
SD	1.30E+08	6.16E+07	3.12E+08	2.30E+08
Day 5	2.10E+09	8.00E+06	8.00E+08	2.00E+08
	7.60E+08	1.30E+08	5.60E+08	6.00E+09
	8.00E+07	5.00E+08		2.00E+08
AVE	9.80E+08	2.13E+08	6.80E+08	2.13E+09
SD	8.39E+08	2.09E+08	1.20E+08	2.73E+09

Table D.15 The remaining crude oil in sampling point 1 at 5-cm depths from the surface of natural seawater during 7 day expperiment of 160 L mesocosm tank.

		Point 1-5				
Day		1	2	3	AVE	SD
	0 hr	1.4	1.3	1.2	1.30	0.10
	6 hr	8.4	9.4	9.3	9.03	0.55
	18 hr	20.1	21.0	18.2	19.77	1.43
	19 hr	24.2	25.3	25.3	24.93	0.64
Day 1	24 hr	25.7	26.3	28.9	26.97	1.70
	24.5 hr	19.3	17.0	18.9	18.40	1.23
	30 hr	16.8	17	17.1	16.97	0.15
Day 2	42 hr	15.6	15.5	15.3	15.47	0.15
	54 hr	17.5	18.2	18.1	17.93	0.38
	66 hr	15.2	14.4	14	14.53	0.61
Day 3	72 hr	5	5.4	4.8	5.07	0.31
	84 hr	2	3	2.9	2.63	0.55
Day 4	96 hr	4	3.7	3.5	3.73	0.25
Day 5	120 hr	1.5	1.7		1.60	0.14
Day 6	144 hr	1.2	1.2	1.4	1.27	0.12
Day 7	168 hr	0.5	0.5	0.3	0.43	0.12

Table D.16 The remaining crude oil in sampling point 1 at 15-cm depths from the surface of natural seawater during 7 day expperiment of 160 L mesocosm tank.

		Point 1-15				
Day		1	2	3	AVE	SD
	0 hr	1.1	1.7	1.8	1.53	0.38
	6 hr	4.5	5.0	6.0	5.17	0.76
	18 hr	13.8	14.0	13.7	13.83	0.15
	19 hr	20.4	20.7	21	20.70	0.30
Day 1	24 hr	19	18.4	20	19.13	0.81
	24.5 hr	18.0	18.5	18.3	18.27	0.25
	30 hr	21.9	23.3	23.4	22.87	0.84
Day 2	42 hr	25	24.3	24	24.43	0.51
	54 hr	21.3	18.8	20.5	20.20	1.28
	66 hr	17.2	20.9	21	19.70	2.17
Day 3	72 hr	11.3	11.8	10.9	11.33	0.45
	84 hr	4.8	4.6	5	4.80	0.20
Day 4	96 hr	3.9	3.7	3.5	3.70	0.20
Day 5	120 hr	5.4	5.3	5	5.23	0.21
Day 6	144 hr	3.9	2.5	2.5	2.97	0.81
Day 7	168 hr	1.6	0.8	0.7	1.03	0.49

Table D.17The remaining crude oil in sampling point 2 at 5-cm depths from the surface of natural seawater during 7 day expperiment of 160 L mesocosm tank.

		Point 2-5				
Day		1	2	3	AVE	SD
	0 hr	2.7	1.3	1.2	1.73	0.84
	6 hr	17.0	18.0	17.2	17.40	0.53
	18 hr	24.9	23.4	21.8	23.37	1.55
	19 hr	35.0	37.0	40.0	37.33	2.52
Day 1	24 hr	40	40.3	36.8	39.03	1.94
	24.5 hr	30.0	28.2	28	28.73	1.10
	30 hr	28.8	28.9	29	28.90	0.10
Day 2	42 hr	29	31	30	30.00	1.00
	54 hr	32	31	29	30.67	1.53
	66 hr	28.5	27	28	27.83	0.76
Day 3	72 hr	22	24	25.2	23.73	1.62
	84 hr	8.7	11.3	10	10.00	1.30
Day 4	96 hr	7	6.9	5.6	6.50	0.78
Day 5	120 hr	5	5.5	5.2	5.23	0.25
Day 6	144 hr	5.3	4.5	4.8	4.87	0.40
Day 7	168 hr	2.3	1.1	0.8	1.40	0.79

Table D.18 The remaining crude oil in sampling point 2 at 15-cm depths from the surface of natural seawater during 7 day expperiment of 160 L mesocosm tank.

		Point 2-15				
Day		1	2	3	AVE	SD
	0 hr	0.4	0.6	0.7	0.57	0.15
	6 hr	1.6	2.2	2.0	1.93	0.31
	18 hr	18.0	15.0	17.0	16.67	1.53
	19 hr	45.0	50.0	43.0	46.00	3.61
Day 1	24 hr	35	36	36	35.67	0.58
	24.5 hr	35	36.5	34	35.17	1.26
	30 hr	33.3	35.2	35.8	34.77	1.31
Day 2	42 hr	28.2	29.0	27.5	28.23	0.75
	54 hr	30	27	28	28.33	1.53
	66 hr	11.3	9.8	9.6	10.23	0.93
Day 3	72 hr	7.9	7.4	7.3	7.53	0.32
	84 hr	5.5	4.8	5.3	5.20	0.36
Day 4	96 hr	3.5	3	2.9	3.13	0.32
Day 5	120 hr	3	2.9	3.2	3.03	0.15
Day 6	144 hr	2.8	2.6	3	2.80	0.20
Day 7	168 hr	1.8	2.2	2	2.00	0.20

Table D.19 The number of total bacteria in sampling point 1 at 5-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Total Bacteria (MPN/ml)					
Day	Time (Hr)	Point 1-5				
		1	2	3	AVE	SD
Day 1	0 hr	9.30E+07	2.40E+07	9.30E+07	7.00E+07	3.98E+07
	6 hr	9.30E+07	4.60E+08	1.50E+08	2.34E+08	1.98E+08
	18 hr	7.50E+08	4.60E+08	9.30E+08	7.13E+08	2.37E+08
	19 hr	2.30E+08	4.30E+08	2.30E+08	2.97E+08	1.15E+08
	24 hr	1.50E+08	2.30E+07	2.30E+07	6.53E+07	7.33E+07
Day 2	24.5 hr	1.20E+09	1.50E+09	1.50E+09	1.40E+09	1.73E+08
	30 hr	4.60E+10	4.61E+10	1.10E+10	3.44E+10	2.02E+10
	42 hr	9.30E+09	7.50E+09	6.40E+09	7.73E+09	1.46E+09
Day 3	54 hr	4.60E+10	4.60E+10	1.60E+10	3.60E+10	1.73E+10
	66 hr	4.30E+09	2.30E+09	2.30E+09	2.97E+09	1.15E+09
	72 hr	2.30E+09	4.60E+09	3.80E+09	3.57E+09	1.17E+09
Day 4	84 hr	4.60E+09	7.50E+09	4.30E+09	5.47E+09	1.77E+09
	96 hr	7.50E+09	3.80E+09	3.80E+09	5.03E+09	2.14E+09
Day 5	120 hr	4.30E+08	9.30E+08	2.10E+09	1.15E+09	8.57E+08
Day 6	144 hr	2.30E+08	3.80E+08	2.30E+08	2.80E+08	8.66E+07
Day 7	168 hr	2.30E+07	4.30E+07	0.00E+00	2.20E+07	2.15E+07

Table D.20 The number of total bacteria in sampling point 1 at 15-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Total Bacteria (MPN/ml)					
Day	Time (Hr)	Point 1-15				
		1	2	3	AVE	SD
Day 1	0 hr	9.30E+07	1.50E+08	7.50E+07	1.06E+08	3.92E+07
	6 hr	9.30E+07	1.00E+07	9.30E+07	6.53E+07	4.79E+07
	18 hr	4.30E+08	1.00E+07	4.30E+08	2.90E+08	2.42E+08
	19 hr	9.30E+07	2.40E+08	2.40E+08	1.91E+08	8.49E+07
	24 hr	4.30E+09	2.40E+09	3.80E+08	2.36E+09	1.96E+09
Day 2	24.5 hr	9.30E+09	1.60E+10	1.50E+10	1.34E+10	3.61E+09
	30 hr	1.50E+10	4.30E+09	7.50E+09	8.93E+09	5.49E+09
	42 hr	4.60E+10	1.10E+11	4.60E+10	6.73E+10	3.70E+10
Day 3	54 hr	1.10E+11	4.60E+10	4.60E+10	6.73E+10	3.70E+10
	66 hr	4.30E+09	4.30E+09	9.30E+09	5.97E+09	2.89E+09
	72 hr	1.50E+09	1.50E+09	4.30E+09	2.43E+09	1.62E+09
Day 4	84 hr	4.60E+09	2.40E+09	2.10E+09	3.03E+09	1.37E+09
	96 hr	7.50E+08	1.50E+09	9.30E+08	1.06E+09	3.92E+08
Day 5	120 hr	4.30E+08	9.30E+08	1.50E+08	5.03E+08	3.95E+08
Day 6	144 hr	2.30E+07	7.50E+07	2.40E+08	1.13E+08	1.13E+08
Day 7	168 hr	2.30E+08	9.30E+07	2.30E+08	1.84E+08	7.91E+07

Table D.21 The number of total bacteria in sampling point 2 at 5-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Total Bacteria (MPN/ml)					
Day	Time (Hr)	Point 2-5				
		1	2	3	AVE	SD
Day 1	0 hr	4.30E+01	2.40E+08	2.40E+08	1.60E+08	1.39E+08
	6 hr	4.30E+07	0.00E+00	1.50E+08	6.43E+07	7.72E+07
	18 hr	4.30E+08	0.00E+00	2.10E+08	2.13E+08	2.15E+08
	19 hr	6.40E+08	1.00E+06	3.80E+08	3.40E+08	3.21E+08
	24 hr	6.40E+08	1.00E+06	7.50E+08	4.64E+08	4.04E+08
Day 2	24.5 hr	2.40E+10	1.00E+08	1.50E+10	1.30E+10	1.21E+10
	30 hr	1.50E+10	1.00E+08	7.50E+09	7.53E+09	7.45E+09
	42 hr	1.10E+10	1.00E+08	1.10E+11	4.04E+10	6.06E+10
Day 3	54 hr	1.10E+10	1.00E+08	1.10E+10	7.37E+09	6.29E+09
	66 hr	4.30E+08	1.00E+08	4.30E+08	3.20E+08	1.91E+08
	72 hr	1.50E+09	1.00E+08	1.50E+09	1.03E+09	8.08E+08
Day 4	84 hr	4.60E+09	1.00E+08	2.40E+08	1.65E+09	2.56E+09
	96 hr	7.50E+09	1.00E+08	4.30E+09	3.97E+09	3.71E+09
Day 5	120 hr	4.30E+08	1.00E+08	2.30E+08	2.53E+08	1.66E+08
Day 6	144 hr	4.30E+07	1.00E+07	9.30E+08	3.28E+08	5.22E+08
Day 7	168 hr	1.50E+08	1.00E+08	1.20E+08	1.23E+08	2.52E+07

Table D.22The number of total bacteria in sampling point 2 at 15-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Total Bacteria (MPN/ml)					
Day	Time (Hr)	Point 2-15				
		1	2	3	AVE	SD
	0 hr	4.30E+07	6.40E+09	1.50E+08	2.20E+09	3.64E+09
	6 hr	4.30E+07	4.30E+07	1.50E+08	7.87E+07	6.18E+07
	18 hr	4.30E+08	1.50E+09	4.30E+08	7.87E+08	6.18E+08
	19 hr	1.60E+08	1.20E+08	9.30E+08	4.03E+08	4.57E+08
Day 1	24 hr	9.30E+08	2.40E+08	1.60E+08	4.43E+08	4.23E+08
	24.5 hr	2.40E+10	7.50E+09	1.50E+10	1.55E+10	8.26E+09
	30 hr	4.60E+10	1.50E+10	4.60E+10	3.57E+10	1.79E+10
Day 2	42 hr	4.60E+10	1.10E+11	4.60E+10	6.73E+10	3.70E+10
	54 hr	1.10E+11	2.40E+10	2.90E+10	5.43E+10	4.83E+10
	66 hr	1.50E+10	9.30E+09	1.20E+10	1.21E+10	2.85E+09
Day 3	72 hr	4.30E+08	1.50E+10	4.30E+09	6.58E+09	7.55E+09
	84 hr	2.40E+10	2.40E+10	2.10E+10	2.30E+10	1.73E+09
Day 4	96 hr	2.40E+10	2.30E+09	7.50E+09	1.13E+10	1.13E+10
Day 5	120 hr	9.30E+09	4.30E+09	6.40E+09	6.67E+09	2.51E+09
Day 6	144 hr	4.30E+08	4.60E+09	4.30E+08	1.82E+09	2.41E+09
Day 7	168 hr	9.30E+09	4.30E+09	4.30E+09	5.97E+09	2.89E+09

Table D.23 The number of oil degrading bacteria in sampling point 1 at 5-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Oil degrading Bacteria (MPN/ml)					
Day	Time (Hr)	Point 1-5				
		1	2	3	AVE	SD
	0 hr	1.50E+07	2.30E+06	2.30E+06	6.53E+06	7.33E+06
	6 hr	2.40E+07	4.30E+06	2.40E+07	1.74E+07	1.14E+07
	18 hr	9.30E+07	4.30E+07	4.30E+07	5.97E+07	2.89E+07
	19 hr	4.30E+07	2.50E+07	2.50E+07	3.10E+07	1.04E+07
Day 1	24 hr	9.30E+07	7.50E+07	9.30E+07	8.70E+07	1.04E+07
	24.5 hr	4.60E+09	2.40E+09	1.50E+09	2.83E+09	1.59E+09
	30 hr	2.40E+08	9.30E+08	9.30E+08	7.00E+08	3.98E+08
Day 2	42 hr	2.40E+08	4.60E+08	1.50E+08	2.83E+08	1.59E+08
	54 hr	2.40E+09	2.40E+09	1.50E+09	2.10E+09	5.20E+08
	66 hr	4.30E+08	4.30E+08	9.30E+08	5.97E+08	2.89E+08
Day 3	72 hr	9.30E+08	4.30E+08	4.30E+08	5.97E+08	2.89E+08
	84 hr	1.50E+09	4.30E+08	1.50E+08	6.93E+08	7.12E+08
Day 4	96 hr	9.30E+08	9.30E+08	1.50E+09	1.12E+09	3.29E+08
Day 5	120 hr	2.40E+08	9.30E+07	7.50E+07	1.36E+08	9.05E+07
Day 6	144 hr	1.50E+08	3.80E+07	9.30E+07	9.37E+07	5.60E+07
Day 7	168 hr	4.60E+07	2.30E+07	9.30E+07	5.40E+07	3.57E+07

Table D.24 The number of oil degrading bacteria in sampling point 1 at 15-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Oil degrading bacteria (MPN/ml)					
Day	Time (Hr)	Point 1-15				
		1	2	3	AVE	SD
	0 hr	9.30E+07	4.30E+07	2.90E+07	5.50E+07	3.36E+07
	6 hr	2.40E+08	6.40E+07	2.40E+08	1.81E+08	1.02E+08
	18 hr	1.20E+08	6.40E+07	3.80E+07	7.40E+07	4.19E+07
	19 hr	2.50E+07	4.30E+07	7.50E+07	4.77E+07	2.53E+07
Day 1	24 hr	1.60E+08	9.30E+07	1.50E+08	1.34E+08	3.61E+07
	24.5 hr	2.10E+09	1.50E+09	2.40E+09	2.00E+09	4.58E+08
	30 hr	1.50E+09	9.30E+08	7.50E+08	1.06E+09	3.92E+08
Day 2	42 hr	1.20E+09	4.60E+09	2.30E+08	2.01E+09	2.29E+09
	54 hr	3.80E+08	6.40E+08	1.50E+09	8.40E+08	5.86E+08
	66 hr	2.10E+09	1.50E+09	9.30E+08	1.51E+09	5.85E+08
Day 3	72 hr	1.60E+09	1.50E+09	2.90E+09	2.00E+09	7.81E+08
	84 hr	9.30E+01	2.40E+09	3.50E+08	9.17E+08	1.30E+09
Day 4	96 hr	3.80E+08	2.30E+08	2.10E+08	2.73E+08	9.29E+07
Day 5	120 hr	9.30E+07	7.50E+07	4.30E+07	7.03E+07	2.53E+07
Day 6	144 hr	1.60E+08	9.30E+07	1.60E+08	1.38E+08	3.87E+07
Day 7	168 hr	3.80E+07	9.30E+07	7.50E+07	6.87E+07	2.80E+07

Table D.25 The number of oil degrading bacteria in sampling point 2 at 5-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Oil degrading Bacteria (MPN/ml)					
Day	Time (Hr)	Point 2-5				
		1	2	3	AVE	SD
Day 1	0 hr	2.40E+08	4.30E+07	4.30E+07	1.09E+08	1.14E+08
	6 hr	7.50E+07	1.50E+08	7.50E+07	1.00E+08	4.33E+07
	18 hr	4.30E+07	4.30E+07	1.20E+08	6.87E+07	4.45E+07
	19 hr	1.20E+08	1.50E+08	4.30E+07	1.04E+08	5.52E+07
	24 hr	2.40E+08	4.60E+08	1.20E+08	2.73E+08	1.72E+08
Day 2	24.5 hr	2.40E+09	4.60E+09	2.10E+09	3.03E+09	1.37E+09
	30 hr	2.40E+09	4.60E+09	2.10E+09	3.03E+09	1.37E+09
	42 hr	2.10E+09	2.40E+09	1.50E+09	2.00E+09	4.58E+08
Day 3	54 hr	2.10E+09	1.10E+10	2.10E+09	5.07E+09	5.14E+09
	66 hr	4.30E+08	4.30E+08	7.40E+08	5.33E+08	1.79E+08
	72 hr	1.50E+09	9.30E+08	1.50E+09	1.31E+09	3.29E+08
Day 4	84 hr	9.30E+08	4.30E+08	1.50E+09	9.53E+08	5.35E+08
	96 hr	1.60E+09	4.60E+09	2.10E+09	2.77E+09	1.61E+09
Day 5	120 hr	9.30E+08	3.90E+08	4.30E+08	5.83E+08	3.01E+08
Day 6	144 hr	4.30E+07	1.60E+08	6.40E+07	8.90E+07	6.24E+07
Day 7	168 hr	4.30E+07	1.50E+08	9.30E+07	9.53E+07	5.35E+07

Table D.26 The number of oil degrading bacteria in sampling point 2 at 15-cm depths from the surface water during 7 day expperiment of medium scale mesocosm tank.

	Oil degrading bacteria (MPN/ml)					
		Point 2-15				
Day	Time (Hr)	1	2	3	AVE	SD
	0 hr	4.30E+07	4.30E+07	9.30E+07	5.97E+07	2.89E+07
	6 hr	6.40E+07	7.50E+07	6.40E+07	6.77E+07	6.35E+06
	18 hr	1.50E+08	3.50E+07	3.80E+07	7.43E+07	6.55E+07
	19 hr	1.60E+08	1.20E+08	7.50E+07	1.18E+08	4.25E+07
Day 1	24 hr	4.30E+07	3.90E+07	2.30E+07	3.50E+07	1.06E+07
	24.5 hr	1.20E+09	9.30E+08	1.60E+09	1.24E+09	3.37E+08
	30 hr	4.60E+09	1.10E+10	2.90E+09	6.17E+09	4.27E+09
Day 2	42 hr	9.30E+08	4.60E+08	4.60E+09	2.00E+09	2.27E+09
	54 hr	4.60E+09	1.10E+10	2.10E+09	5.90E+09	4.59E+09
	66 hr	9.30E+08	4.60E+08	4.60E+08	6.17E+08	2.71E+08
Day 3	72 hr	4.60E+09	1.50E+09	1.50E+09	2.53E+09	1.79E+09
	84 hr	2.40E+09	4.30E+08	2.40E+09	1.74E+09	1.14E+09
Day 4	96 hr	2.40E+09	2.40E+09	3.80E+08	1.73E+09	1.17E+09
Day 5	120 hr	2.50E+09	9.30E+08	4.30E+08	1.29E+09	1.08E+09
Day 6	144 hr	2.40E+08	9.30E+07	4.30E+07	1.25E+08	1.02E+08
Day 7	168 hr	2.10E+08	1.10E+09	4.60E+08	5.90E+08	4.59E+08

APPENDIX E JOURNAL PUBLICATIONS AND CONFERENCES

JOURNAL PUBLICATIONS

Rongsayamanont W, Soonglerdsongpha S, Khondee N, Pinyakong O, Tongcumpou C, Sabatini DA & Luepromchai E (2017) Formulation of crude oil spill dispersants based on the HLD concept and using a lipopeptide biosurfactant. Journal of Hazardous Materials 334: 168-177.

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VITA

Mrs. Witchaya (Kaewtip) Rongsayamanont was born on July 19, 1984 in Phatthalung, Thailand. She attended Stree Phatthalung School in Phatthalung and graduated in 2002. She graduated with a second-class honors in Bachelor degree of Science in Environmental Science and Technology from Faculty of Environmental and Resource Studies of Mahidol University, Thailand. Later, she pursued her master's degree study in the international Program in Environmental Management, Center of Excellence on Hazardous Substance Management (HSM) Graduate School, Chulalongkorn University, Thailand since 2007-2009. After that she started her Ph.D. in the same program in 2011 and complete her Doctoral degree in Environmental Management in 2016.

Her publications, conferences, and awards were presented in Appendix E.