BIOSURFACTANT PRODUCTION BY IMMOBILIZED *GORDONIA* SP. GY40 CELLS AND APPLICATION FOR PETROLEUM REMOVAL FROM SEAWATER

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การผลิตสารลดแรงตึงผิวชีวภาพโดยเซลล์ตรึงของ *Gordonia* sp. GY40 และการประยุกต์ใช้เพื่อการกำจัดปิโตรเลียมจากน้ำทะเล

นางสาวสุพัตรา ละออรัตนศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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สุพัตรา ละออรัตนศักดิ์ : การผลิตสารลดแรงตึงผิวชีวภาพโดยเซลล์ตรึงของ *Gordonia* sp. GY40 และการประยุกต์ใช้เพื่อการกำจัดปิโตรเลียมจากน้ำทะเล (BIOSURFACTANT PRODUCTION BY IMMOBILIZED *GORDONIA* SP. GY40 CELLS AND APPLICATION FOR PETROLEUM REMOVAL FROM SEAWATER) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. เอกวัล ลือพร้อมชัย, 99 หน้า.

งานวิจัยนี้มีจุดมุ่งหมายเพื่อผลิตสารลดแรงตึงผิวชีวภาพโดยใช้เซลล์ตรึงของ *Gordonia* sp. GY40 และเพื่อใช้สารลดแรงตึงผิวนี้สำหรับเสริมการกำจัดคราบปิโตรเลียมในน้ำทะเล โดย *Gordonia* sp. GY40 เป็นแบคทีเรียผลิตสารลดแรงตึงผิวที่คัดแยกจากดิน จังหวัดชลบุรี ในงานวิจัยก่อนหน้านี้ ในขั้นแรกได้คัดเลือกชนิดของวัสดุตรึงและสารอาหารที่เหมาะสม พบว่าเมื่อตรึง *Gordonia* sp. GY40 บนไคโตซานและมีน้ำมันถั่วเหลือง 2% เป็นแหล่งคาร์บอน *Gordonia* sp. GY40 สามารถลดแรงตึงผิวของอาหารเลี้ยงเชื้อจาก 59.6 เป็น 34.9 mN/m โดยส่วนน้ำใส (supernatant) ที่มีสารลดแรงตึงผิวสามารถก่ออิมัลชั่นกับดีเซลและน้ำมันหล่อลื่นระบบรางเลื่อนเครื่องจักรและสามารถกระจายน้ำมันหล่อลื่นระบบรางเลื่อนเครื่องจักรและน้ำมันเตาได้ 100 และ 97% ตามลำดับ สารลดแรงตึงผิวชีวภาพที่มีประสิทธิภาพการกระจายน้ำมันและการเกิดอิมัลชั่นจะสามารถนำมาใช้เร่งการย่อยสลายทางชีวภาพของปิโตรเลียมในน้ำทะเลได้ นอกจากนี้พบว่าสารลดแรงตึงผิวชีวภาพที่ผลิตได้ไม่มีความเป็นพิษต่อทั้ง *Gordonia* sp. JC11 ที่มีความสามารถย่อยสลายน้ำมันหล่อลื่น และจุลินทรีย์ท้องถิ่นน้ำทะเล จากนั้นนำสารลดแรงตึงผิวชีวภาพที่ผลิตขึ้นมาประยุกต์ใช้ร่วมกับ *Gordonia* sp. JC11 ที่ตรึงบนโฟมพอลิยูรีเธน เพื่อกำจัดน้ำมันเตาปนเปื้อนในน้ำทะเลสังเคราะห์พบว่าระบบนี้สามารถกำจัดน้ำมันเตาที่ความเข้มข้นเริ่มต้น 1,000 มก./ล. ได้ 81% ในเวลา 6 วัน ในขณะที่การใช้เซลล์ตรึงแบคทีเรียอย่างเดียวสามารถกำจัดน้ำมันเตาได้ 70% ต่อมาได้ทำการทดลองในน้ำทะเลที่เก็บจากท่าเรือ จังหวัดชลบุรี บริเวณมาบตาพุด จังหวัดระยอง และบริเวณเกาะเสม็ด จังหวัดระยอง พบว่าการใช้สารลดแรงตึงผิวร่วมกับแบคทีเรียย่อยสลายน้ำมันสามารถกำจัดน้ำมันเตาที่เติมลงในน้ำทะเลได้60-70% ในขณะที่วิธีทางธรรมชาติ (ชุดควบคุม) สามารถกำจัดน้ำมันได้เพียง 26-35%

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : BIOSURFACTANT, IMMOBILIZATION, *GORDONIA* SP., BIOREMEDIATION AGENT, PETROLEUM CONTAMINATED SEAWATER

SUPATTRA LAORRATTANASAK : (BIOSURFACTANT PRODUCTION BY IMMOBILIZED *GORDONIA* SP. GY40 CELLS AND APPLICATION FOR PETROLEUM REMOVAL FROM SEAWATER) ADVISOR: ASST.PROF EKAWAN LUEPROMCHAI, Ph.D., 99 pp.

This research aims to produce biosurfactant by using *Gordonia* sp. GY40 immobilized cells and to apply the biosurfactant for enhancing petroleum removal from seawater. *Gordonia* sp. GY40 was previously isolated from soil in Chonburi province, Thailand and found to efficiently produce biosurfactant. Firstly, immobilization technique and optimum biosurfactant production was studied. When using chitosan flakes as immobilizing matrix and 2% soy-bean oil as carbon source in basal medium, *Gordonia* sp. GY40 could reduce the surface tension of basal medium from 59.6 to 34.9 mN/m. The cell-free supernatant was able to emulsify diesel and slideway oil; as well as, disperse 100 and 97% of slideway oil and diesel in the oil displacement test, respectively. Biosurfactant with dispersion and emulsification activities could be used to enhance the biodegradation of petroleum in seawater. Furthermore, this biosurfactant was not toxic to lubricant-degrading bacteria, *Gordonia* sp. JC11 as well as indigenous seawater microorganisms. After that, biosurfactant was used together with polyurethane foam-immobilized *Gordonia* sp. JC11 to remove fuel oil in synthetic seawater. The system removed 81% of 1,000 ppm fuel oil within 6 day, while the immobilized cells alone removed 70% of the oil. The experiments were later tested with seawater samples from a port in Chonburi province, Mapthaphut, Rayong province, and Samed Island, Rayong province. The application of biosurfactant along with immobilized cells removed 60-70% of fuel oil, while the natural attenuation (control) removed only 26-35%.

Field of Study Environmental Management Student's Signature……

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LIST OF ABBREVIATIONS

CMD	= Critical Micelle Dilution
BM	= Basal Medium
NSW	= Natural Seawater
SDS	= Sodium Dodecyl Sulfate
BSF	= Biosurfactant
BG	= Bottom Glycerol
ST	= Surface Tension

CHAPTER I

INTRODUCTION

STATEMENT OF PROBLEM

Petroleum is fossil fuel consisting of hydrocarbons and spontaneously occurs. The demand of petroleum consumption has been increased around the world because of growing economy. Consequently, petroleum pollutions in the ocean are occurred. Oil contamination in seawater comes from over-sized of ship tank and accidentally spilled (Gentili et al., 2006; Hua, 2006). Hydrocarbons in marine environment had low rate of degradation (Zahed et al., 2010). Fuel oils for shipping and diesel are consisted of hardly degraded components such as branched, cyclic, aliphatic and aromatic hydrocarbons (Nievas et al., 2008). Polycyclic aromatic hydrocarbons (PAHs) are also contaminated in the environment because of petroleum usage. Petroleum can pose high risk to the marine ecosystem; moreover, oil slick can spread on shoreline. General techniques for oil spill remediation such as boom and combustion (physical technique) cannot completely remove oil; besides, using dispersant (chemical technique) could be toxic to aquatic animals.

Bioremediation technique for oil spill situation is interested; because of its cheap and environmental friendly (Si-Zhong et al., 2009). This technique uses oil-degrading bacteria from outside or inside contaminated areas. Variety of bacteria can degrade xenobiotics by using their enzymes. However, bioremediation of petroleum in marine environment is limited by the low solubility of complex hydrocarbons in seawater, which also decrease their bioavailability for bacterial degradation.

Therefore, degradation rate was slow. Consequently, this research aims to improve the biodegradation of hydrocarbons by adding biosurfactant to the contaminated seawater.

Biosurfactant can be produced by variety of bacterial strains and substrates. Many researchers reported different types of biosurfactant from different bacteria. In this study, the biosurfactant was produced from *Gordonia* sp. GY40. Bacterial free cells have been used to produce biosurfactant, however it is difficult to purify and recovery biosurfactant from the culture. Bacterial immobilization, i.e. the formulated bacteria with non-toxic carrier, is an alternative way to improve the biosurfactant production as well as to facilitate its separation from liquid-biomass. Since, various immobilization techniques can be used. The study compared the efficiency of immobilized bacteria from chitosan surface attachment and silica encapsulation on biosurfactant production. Then, this study characterized the biosurfactant properties such as disperse ability, emulsification activity, increasing solubility, and toxicity. The biosurfactant was later applied together with a lubricant-degrading bacterium, *Gordonia* sp. JC11 for removal of fuel oil in seawater. These knowledge will be used for develop a bioaugmentation approach for clean-up petroleum contaminated seawater.

OBJECTIVES

The aims of this study are to produce biosurfactant from immobilized cells and to apply the biosurfactant for enhancing petroleum removal from contaminated seawater. The sub-objectives are as follows:

1. To select an immobilization technique for producing *Gordonia* sp. GY40 immobilized cells.
2. To study the properties of biosurfactant produced by *Gordonia* sp. GY40 immobilized cells.
3. To remove petroleum hydrocarbons from contaminated seawater by using the biosurfactant from *Gordonia* sp. GY40 and a lubricant-degrading bacterium, *Gordonia* sp. JC11.

HYPOTHESES

Gordonia sp. GY40 immobilized cells will produce higher yield of biosurfactant than free cells. In additions, the biosurfactant from *Gordonia* sp. GY40 can be applied with lubricant-degrading bacteria, *Gordonia* sp. JC11 to enhance petroleum removal from seawater.

SCOPES OF THE STUDY

The research was divided into three phases as follows:

Phase 1: Bacterial immobilization and biosurfactant production

Immobilization techniques including chitosan surface attachment and silica encapsulation were set up and compared for biosurfactant production. Surface tension and oil-displacement test were criteria for selection of an immobilization technique for the next experiment. After that, various carbon sources for biosurfactant production were tested by using batch production. There were 3 carbon sources; 1) bottom glycerol which is interesting to be a low-cost renewable substrate, 2) soy-bean oil which can enhance the production of biosurfactant, and 3) glycerol which is a water soluble substrate. In addition, surface topography of the immobilized cells was observed to determine cell attachment and number. Besides, the immobilized cells were repeatedly used and tested for the storage conditions. The benefit from reusing the immobilized cells is to decrease the cost and time of immobilization process.

Phase 2: Study of biosurfactant properties

After selecting immobilization method and carbon source, biosurfactant was produced in 125 mL flask batch experiment. The properties of produced biosurfactant were investigated, namely, critical micelle dilution (CMD), crude biosurfactant determination, ionic charge, oil displacement against various petroleum hydrocarbons, solubilization for fuel oil, emulsification activity, dispersant activity, and toxicity on *Gordonia* sp. JC11 and indigenous seawater microorganisms.

Phase 3: Application of the biosurfactant and *Gordonia* sp. JC11 for petroleum hydrocarbons removal in seawater

GY40 biosurfactant was applied along with lubricating-degrading bacteria, *Gordonia* sp. JC11 to remove fuel oil in seawater by carrying out in batch experiment. *Gordonia* sp. JC11 was immobilized on PUF similar to Chantamalee et al. (2013). The first set of microcosms used synthetic seawater and compared between microcosms containing *Gordonia* sp. JC11 with and without biosurfactant. A suitable treatment and degradation period were selected to test with real seawater collected from three sites. This experiment was carried out to confirm the efficiency of biosurfactant and *Gordonia* sp. JC11 for enhancing fuel oil biodegradation in seawater.

BENEFIT OF THE STUDY

Since the petroleum contamination in seawater is widespread. The remediation techniques are required; however, it needs environmental friendly approach. The application of biosurfactant along with oil-degrading microorganisms is an interesting clean-up approach. Biosurfactant can enhance solubility and bioavailability of petroleum; thereby, the biodegradation rate will increase. Moreover, biosurfactant production could be improved by using immobilized bacterial cells and suitable substrate. Consequently, the addition of oil-degrading bacteria along with biosurfactant would be an efficient and cost-effective approach for remediation of contaminated seawater.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

THEORETICAL BACKGROUNDS:

1. Petroleum contaminated seawater

Petroleum is organic compound that composes of hydrocarbons occurring naturally. Petroleum is originated from organism decay for several hundred million years ago which are scattered both on land and at sea and accumulated with silt and clay in the environment (Venkatachalapathy et al., 2010). Nowadays, fuel consumption is increased due to economic growth. The exploration drilling for petroleum and the amount of petroleum transported by sea have increased in many parts of the world. Therefore, a risk of petroleum contamination in the marine environment is high. In Thailand, the Gulf of Thailand is the center of all industrial and economic growth. It can be seen from various industrial estates around the area. Consequently, the area is contaminated with petroleum hydrocarbons in water (Wattayakorn, 2012).

Petroleum hydrocarbons have complex structure and are hardly degraded because of their complex molecules and assemble of chemicals, e.g., aliphatics, aromatics, and polar compounds (Kim et al., 2013). Oxygen, nitrogen, and sulfur can be found in petroleum molecules and led to different characteristics according to the different types of hydrocarbons. Due to their toxicity to living organisms and potential accumulation in the sediment, a cost-effective remediation technique is required. In

the marine environment, hydrocarbons had a different rate of degradation and some components required long time period (Zahed et al., 2010).

Gulf of Thailand is considered as a risk zone of oil spill, which causes by accidental crashing of boat, oil transfer, and illegally contaminated wastewater discharge (Singkran., 2013). Furthermore, the most serious problem is untreated discharge because the limited wastewater treatment facilities in sea (Cheevaporn and Menasveta., 2003). Wattayakorn (2012) studied the history of gulf of Thailand and showed that the level of petroleum pollution in water increases to medium level after being industrialized country and PAH is presented to be dominant of contamination.

2. Bioremediation of petroleum contamination

Bioremediation, one of several techniques for spilled oil removal is an option that effective, cheap, and less damage to the environment (Si-Zhong et al., 2009). 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. For seawater remediation, biostimulation has been used to activate the native microbial community and bioaugmentation has been also using for enhanced the degradation (Gentili et al., 2006; Cunningham et al., 2004). These techniques used the metabolism from microorganisms to remove the pollutant and change them into simple compounds. The process occurs by itself. Bacterial strains capable of degrading variety of hydrocarbons are as follows;

Table 2. 1 Oil-degrading bacteria and their substrates

Bacteria	Substrate	Reference
Gordonia sp. JC11	Lubricant	Chanthamalee and Luepromchai (2012)
Alcaligenes faecalis	Diesel	Bharali et al. (2011)
Mycobacterium frederiksbergense	PAHs (pyrene, etc.)	Sarma and Pakshirajan (2011)
Gordonia alkanivorans S7	Diesel	Kwapisz et al. (2008)

Majority of 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 synthesized through Tricarboxilic acid cycle and biomass, carbon dioxide, and water are product from this pathway (Chandra et al, 2012).

For the biodegradation, some factors can be affected, such as, physical and chemical composition of hydrocarbon, nutrients, temperature, pH, oxygen, and bioavailability especially for high molecular weight polycyclic aromatic hydrocarbon (PAHs) because it contains four or more benzene rings that hard to degrade (Juhasz and Naidu, 2000; Aktas et al., 2013). Figure 2.1 showed the pathway of aromatic hydrocarbon degradation under aerobic condition. Benzene ring was cleaved by using enzymes from bacteria, e.g., oxygenase, dehydrogenase. Finally, the by-product from this reaction was water and carbon dioxide.

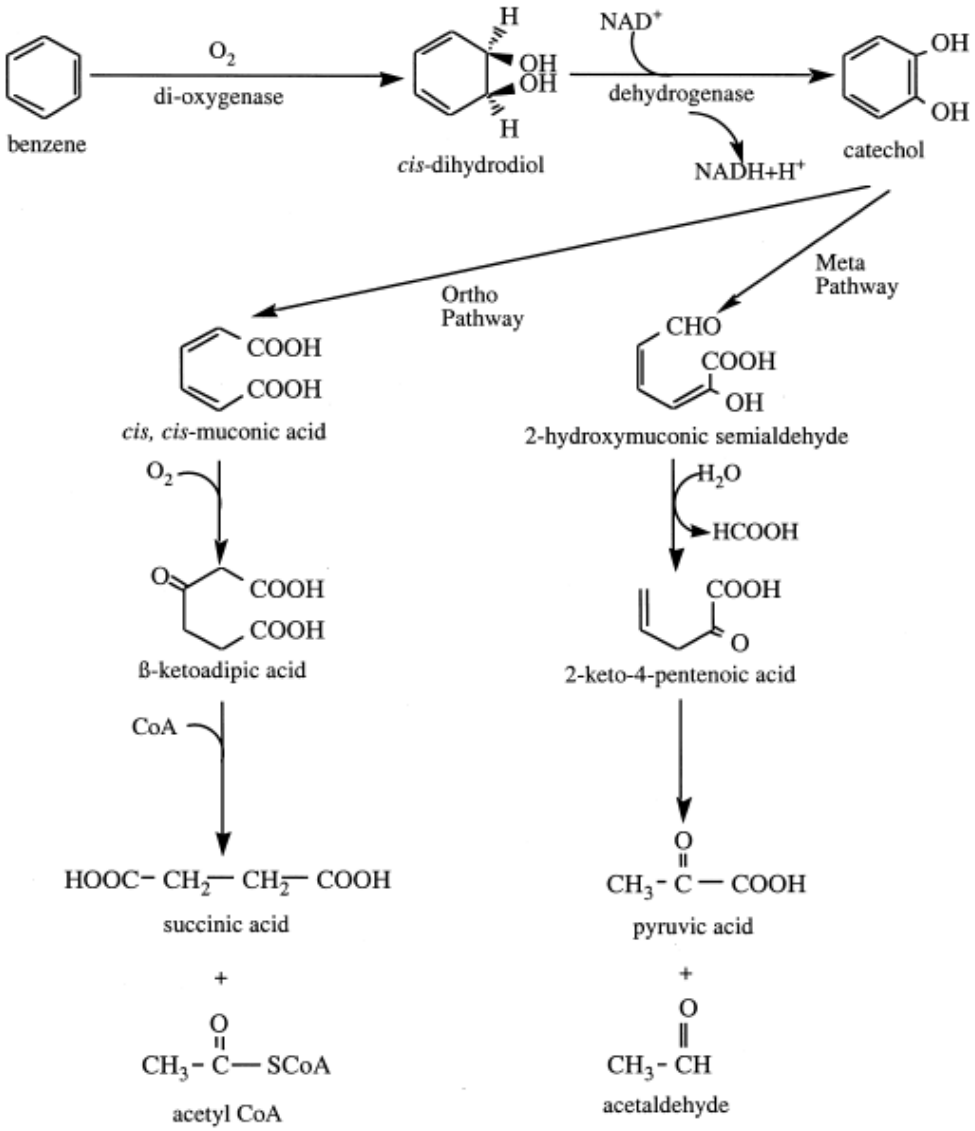

Figure 2. 1 Biodegradation pathway of aromatic hydrocarbons by bacteria (Juhasz and Naidu, 2000)

3. Biosurfactant and biosurfactant production

Biosurfactant is an amphiphilic molecule which produced by some microorganisms for oil dispersion (Saeki et al., 2009). Lower toxicity and higher biodegradability than synthetic surfactant are the reasons why researchers have considerable attention on using biosurfactant (Desai and Banat, 1997). Biosurfactant can increase bioavailability of hydrophobic compounds by allowing hydrophobicity part more easily associated to bacterial cells and thereby promote biodegradation (Mulligan and Gibbs, 2004). This knowledge gave advantages for hydrocarbons removal because it contains water-insoluble part. Bharali et al. (2011) showed that the present of biosurfactant can increase solubilization and enhance emulsification of petroleum hydrocarbon. In addition, some microorganisms can degrade the contaminants together with biosurfactant production. However, the large-scale production of biosurfactant has been restricted due to its high production costs in relation to inefficient bioprocessing method available, poor strain productivity and the need to use expensive substrates (Deleu and Paquot, 2004).

Table 2. 2 Biosurfactant produced by various types of microorganisms and substrates

Microorganisms	**Type of production**	**Substrate**	**Reference**
Bacillus licheniformis TKU004	Batch	Squid pen powder	Chen et al. 2012
Ustilago maydis	Fed-batch reactor	Crude glycerol	Liu et al. 2012
Pseudomonas aeruginosa SP4	Sequencing batch reactor	Glucose	Pansiripat et al.2010
Gordonia sp. BS29	Batch	n-tridecane	Franzetti et al. 2009
Aeromonas spp.	Batch	Crude oil	Ilori et al. 2005
Serratia marcescens	Batch	Glycerol	Ferraz et al. 2002

4. *Gordonia* species

Gordonia sp. has diverse abilities such as degradability of xenobiotic, possibility to synthesis compound which useful in various applications, and ability to produce the associated surface-active compound (Arenskötter et al., 2004).

Table 2. 3 Some strains of biosurfactant-producing *Gordonia* sp.

Strain	Type of surfactant	Application	Reference
Gordonia sp. BS29	Glycolipid	Washing agents for remediation of hydrocarbon-contaminated soils	Franzetti et al. 2009
Gordonia sp. JE-1058	Glycolipid	Bioremediation agent for oil spill clean-up in seawater	Saeki et al. 2009
*Gordonia s*trains FEMS	Glycolipid	Emulsifying agent for hydrocarbon-biodegradation	Franzetti et al. 2008
Gordonia amarae	Glycolipid	Bioremediating sparingly soluble for in- situ contamination	Dogan et al. 2006

Furthermore, many *Gordonia* sp. can degrade the pollutant by using various enzymes and pathways. For example, *Gordonia alkanivorans* can degrade diesel (Ta-Chen et al., 2008) and *Gordonia* sp. JC11 can degrade waste lubricant in seawater (Chanthamalee and Luepromchai., 2012; Chanthamalee et al., 2013). Both *Gordonia* sp. produce exopolysaccharides for biodegradation enhancement.

5. Application of biosurfactant during bioremediation

Bioremediation can be enhanced by two biosurfactant associated processes. The first is increasing of bioavailability of hydrophobic water-soluble substrate since some hydrocarbons have low solubility, and limited the bioavailability to microorganism-degradation. Secondly, increasing of the water-insoluble part in surface area of substrate, when interfacial area between water and oil was blocked, bacterial growth in hydrocarbon is limited. Li and Chen, (2009) described that exceeding of the surfactant at threshold or critical micelle concentration (CMC), hydrophobic part of substrate will aggregate with micelle; consequently, solubilization occurred. Besides, the phenomena of hydrocarbons in micellar phase moving into aqueous phase will take place and bioavailability would increase (Mulligan et al., 2001). Figure 2.2 shown surface tension and interfacial tension are decrease; after that, remained the same when concentration at above CMC. Meanwhile, solubility of substrate gradually increases by solubilization mechanism.

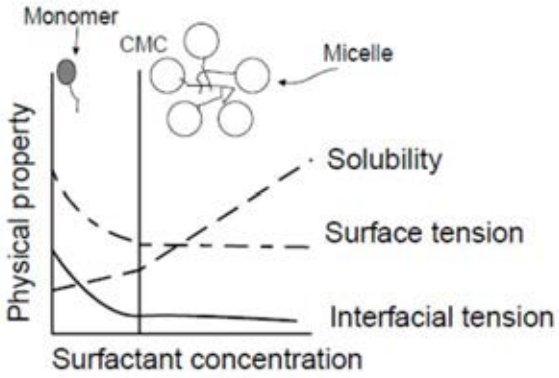

Figure 2. 2 Schematic diagram of the variation of surface tension, interfacial and contaminant solubility with surfactant concentration (Mulligan et al., 2001)

6. Bacterial immobilization

Immobilization is an alternative method that immobilized bacterial inoculum was formulated with constant carrier as protective space; besides, improved activity of cells and survival (Gentili et al., 2006; Hou et al., 2012). The material supports for immobilization are agricultural materials and others. Immobilization is widely available in biotechnology process because of its inexpensive, non-toxic material, effectiveness, tolerance in harsh environment, easy operation, and cell protection (Klein and Ziehr., 1990; Leenen et al., 1996; Kourkoutas et al., 2004). Perspective of immobilized cells over free cells are; prolong activity and stability, increased substrate uptake and yield, reduced the production time, and cost of recovery (Kourkoutas et al., 2004). Besides, competition of indigenous microorganisms can be reduced by immobilization (Lin and Wang, 1991).

Many immobilization methods are available and divided into two main groups, namely, the attachment of microorganisms on to the surface of solid carrier (i.e. adsorption on surface, electrostatic binding with surface, and covalent binding with surface) and the artificial immobilization of microorganisms into supporting material (i.e. entrapment within porous matrices, microencapsulation, and containment behind a barrier) (Figure 2.3). Moreover, several studies were obtained by using variety of supporting carrier (Table 2.4).

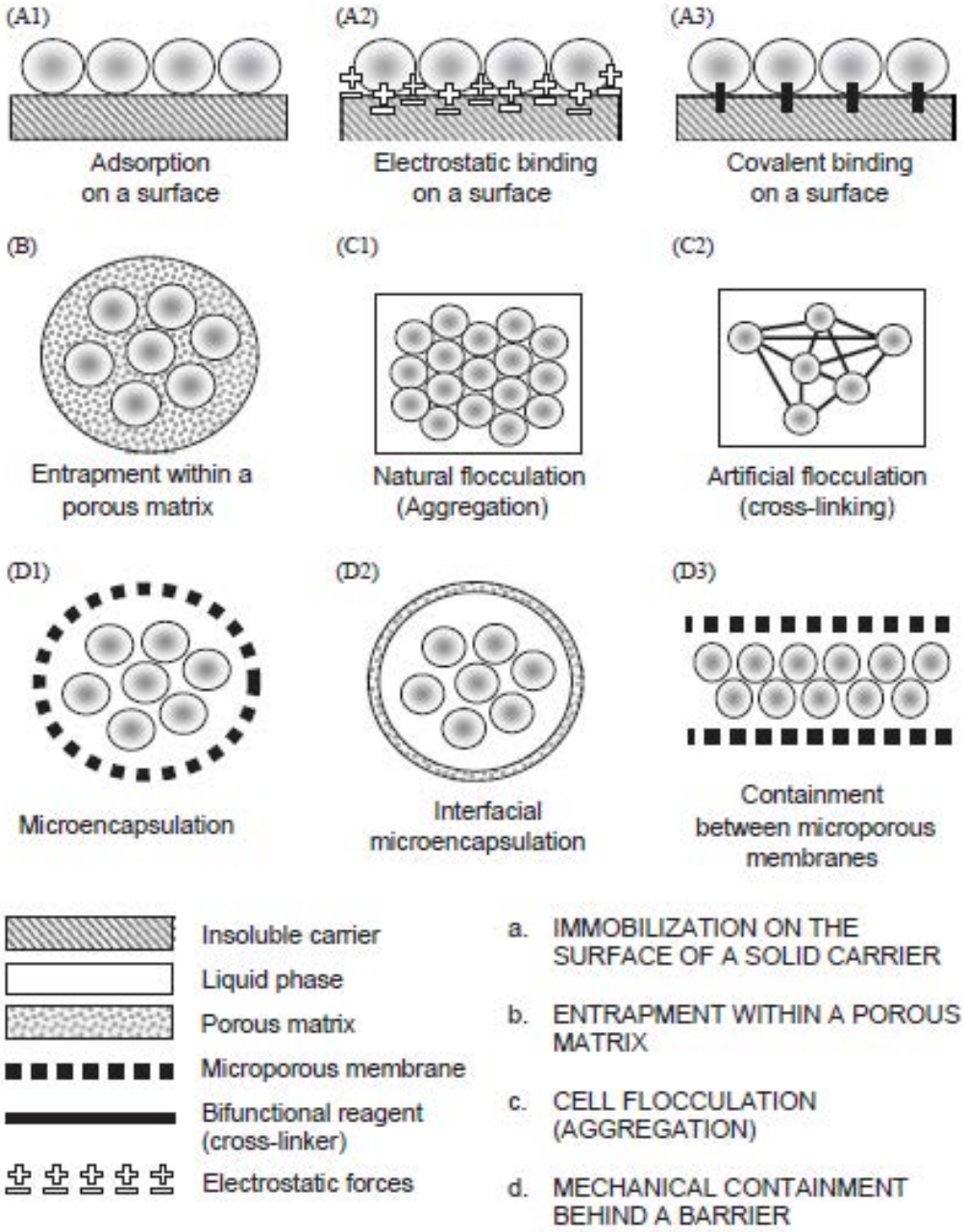

Figure 2. 3 Immobilization techniques (Kourkoutas et al., 2004)

Table 2. 4 Example of immobilization techniques and immobilizing matrices

Surface attachment	**Entrapment within porous matrix**
Chitosan (Khondee et al., 2012)	Silica (Khongkham et al., 2011)
Chitosan membranes (Orrego et al., 2010)	Alginate embedded magnetic (Heyd et al., 2009)
Chitin And Chitosan Flakes (Gentili et al., 2006)	Alginate (Abouseoud et al., 2008)

LITERATURE REVIEWS:

Biodegradation of petroleum in seawater

Recent researchers have found several hydrocarbon degrading microorganisms (Golyshin et al., 2003; Hassanshahian et al., 2012). In addition, the bacterial cells were immobilized on matrices to increase their stability and effectiveness. For example, Lin et al. (2009) isolated psychotropic petroleum-degrading bacterium *Pseudoalteromonas* sp. P29 and its ability to degrade vacuum oil 80-90% at 5°C in 28 days. Hou et al. (2012) isolated *Acinetobacter* sp. F9, marine bacteria to degrade diesel oil and found that 90% of diesel was removed in the second day by the bacterium that immobilized on calcium alginate-chitosan compound membrane in batch-experiment.

In Thailand, Chanthamalee and Luepromchai (2012) studied the petroleum-degrading bacteria which isolated from oil contaminated seawater. *Gordonia* sp. JC11, the highest efficient strain, degraded 25-55 % of 1000 mg/L of total hydrocarbons in lubricants. The bacterium can produce exopolysaccharides which potentially enhance biodegradation. In fact, Satpute et al (2010) reported that exopolysaccharides, produced by various marine bacteria, help them to interact with the hydrocarbon and increase the emulsification (figure 2.4).

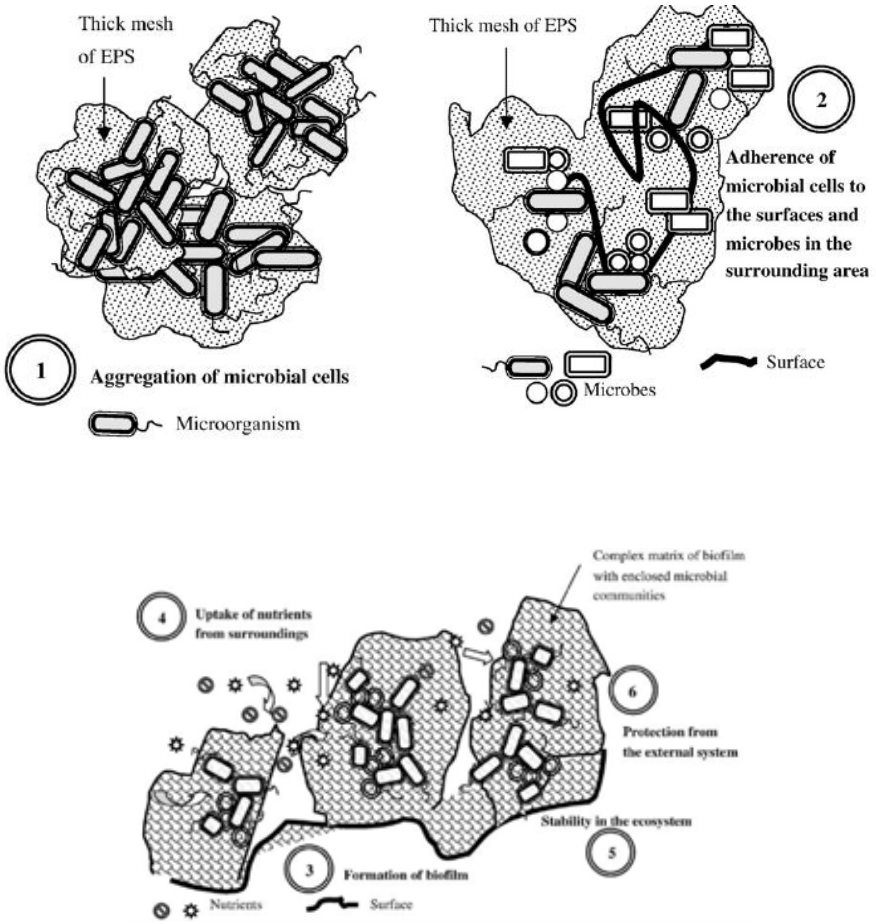

Figure 2. 4 Various roles played by exopolysaccharide (EPS) produced by marine microorganisms in the marine ecosystem (Satpute et al., 2010)

In addition, when immobilized JC11 on polyurethane foam (PUF), it was able to remove 42-56% of 100-1000 mg/L waste lubricant in 5 day (Chanthamalee and Luepromchai, 2012). They suggested that lubricant degrading efficiency was increased because of the high interaction between lubricant and attached cells. However, JC11 cannot degrade the intermediates from degradation of saturates and aromatics. This may cause by the limitation of bioavailability that made hydrocarbon insoluble in water.

Chanthamalee et al (2013) found that natural attenuation of bilge water by indigenous microorganisms was ineffective because of the high amount of oil. Therefore, PUF immobilized *Gordonia* sp. JC11 was applied to enhance oil degradation. Batch-experiment shown PUF-immobilized *Gordonia* sp. JC11 efficiently degraded lubricants in bilge water and PUF could sorb large amount of lubricant. During the operation of a small fishing vessel, the immobilized PUF was later applied as package. However, *Gordonia* sp. JC11 was die-off and the PUF package was sank to the bottom after the initial lubricant removal. Hence, the package should be replaced. Nonetheless, this technique is simple and low-cost, thus it is recommended for increasing efficacy for oil-removal in bioreactor.

Saeki et al. (2009) showed the potential as a bioremediation agent for seawater oil spill remediation in a baffled flask test. The results concluded that crude-oil degradability of the indigenous microorganism in seawater can be stimulated by the biosurfactant (JE-1058 agent). Venosa and Holder (2013) reported that dispersibility of the biosurfactant can help removing oil slick from the water surface.

The main fuels for cargo ships and vehicle are diesel and fuel oil, which consist of branched, cyclic aliphatic and aromatics. The problem from these fuel is from over-sized of cargo ships tank which causing of oil spill in marine environment (Hua, 2006; Nievas et al, 2008). Hence, a way to stimulate the oil spill cleanup was using dispersant followed by microbial treatment because dispersion of oil increases the area available for microbial growth. Microorganisms can attack to the oil-water interface; then, biodegradation of oil droplets was occur and converted to biomass, water and carbon dioxide.

Enhanced biosurfactant production by immobilized bacteria and selected substrates

Recently, many biosurfactant researches are investigated and aimed to reduce production cost, develop production process and improve efficiency. Immobilization method is selected for continuous biosurfactant production and extraction process. Thus, it would lower production cost. From the previous researches, immobilization techniques have been used to produce bacterial inoculum for producing the biosurfactant in difference immobilization carrier and substrate. Abouseound et al. (2008), Heyd (2009), and Onwosi and Odibo (2012) used entrapment technique and calcium alginate as a matrix for *Pseudomonas* sp. to produce the biosurfactant. Immobilized cells are better than free cells during biosurfactant production because they can be easily extracted and used continuously. Yeh et al. (2006), Gancel et al. (2009), and Chtioui et al. (2010) studied the attachment of *Bacillus* sp. on activated carbon, polypropylene foam coating with Fe^{2+}, and polypropylene beads formed with powder activated carbon. The results were higher biosurfactant production from immobilized cells than free cells and the immobilized cells were more stable.

This research aims to immobilize *Gordonia* sp. GY40, a biosurfactant-producing bacterium on chitosan using surface attachment and in silica using encapsulation techniques. Silica- and chitosan-immobilized cells were prepared according to Khongkhaem et al. (2011) and Khondee et al. (2012), respectively. The advantages of attached cells are easily preparation, low diffusion restriction, and stable, while encapsulation gave high porosity and completely separated immobilized cells from the culture medium. The immobilized cells are expected to produce higher yield of biosurfactant than free cells similar to other studies in Table .

Table 2. 5 Biosurfactant yield by free cells and immobilized cells

Strain of bacteria	**Free cell**		**Immobilized cells**		
	Yield (g/L)	**Reference**	**Yield (g/L)**	**Carrier**	**Reference**
Bacillus sp.	0.35	Chen et al. (2013)	6.45	Activated carbon	Yeh et al. (2006)
Pseudomonas aeruginosa	1.65	Pansiripat et al. (2010)	4.2	Cryogels	Christova et al. (2013)
Pseudomonas sp.	4.38	Aparna et al. (2012)	5.6	Calcium aliginate beads	Onwosi and Odibo (2012)

Microorganisms can use hydrocarbon, fatty acids, and carbohydrates separately nor combination for biosurfactant production. Therefore, many studies search for variety of substrates (Ferraz et al. 2002).

To select a substrate for biosurfactant production by the immobilized cells, vegetable oil, glycerol, and bottom glycerol were compared as carbon sources for biosurfactant production. Vegetable oil has been used for enhancing the production of biosurfactant (Ferraz et al., 2002) because Linoleic acid in its composition. Glycerol are water soluble substrate and renewable petroleum substrate; by reasons of, reduced the treatment cost (Silva et al, 2009). It widely used for many industries and also biosurfactant production (Silva et al., 2010). Bottom glycerol is a by-product from bio-diesel production which contained low amount of glycerol, 10% (w/w) from bio-diesel finery and discover capability to produce biosurfactant instantly pure glycerol (Pereira et al, 2013; Lui et al, 2011), thus it might be used to provide carbon source for biosurfactant production.

CHAPTER III

METHODOLOGY

3.1 RESEARCH OVERVIEW

This diagram is a descriptive of research field which divided into three phases, namely, immobilization and biosurfactant production, study of biosurfactant properties, and application of the biosurfactant and *Gordonia* sp. JC11 for petroleum hydrocarbons removal in seawater as shown in figure 3.1.

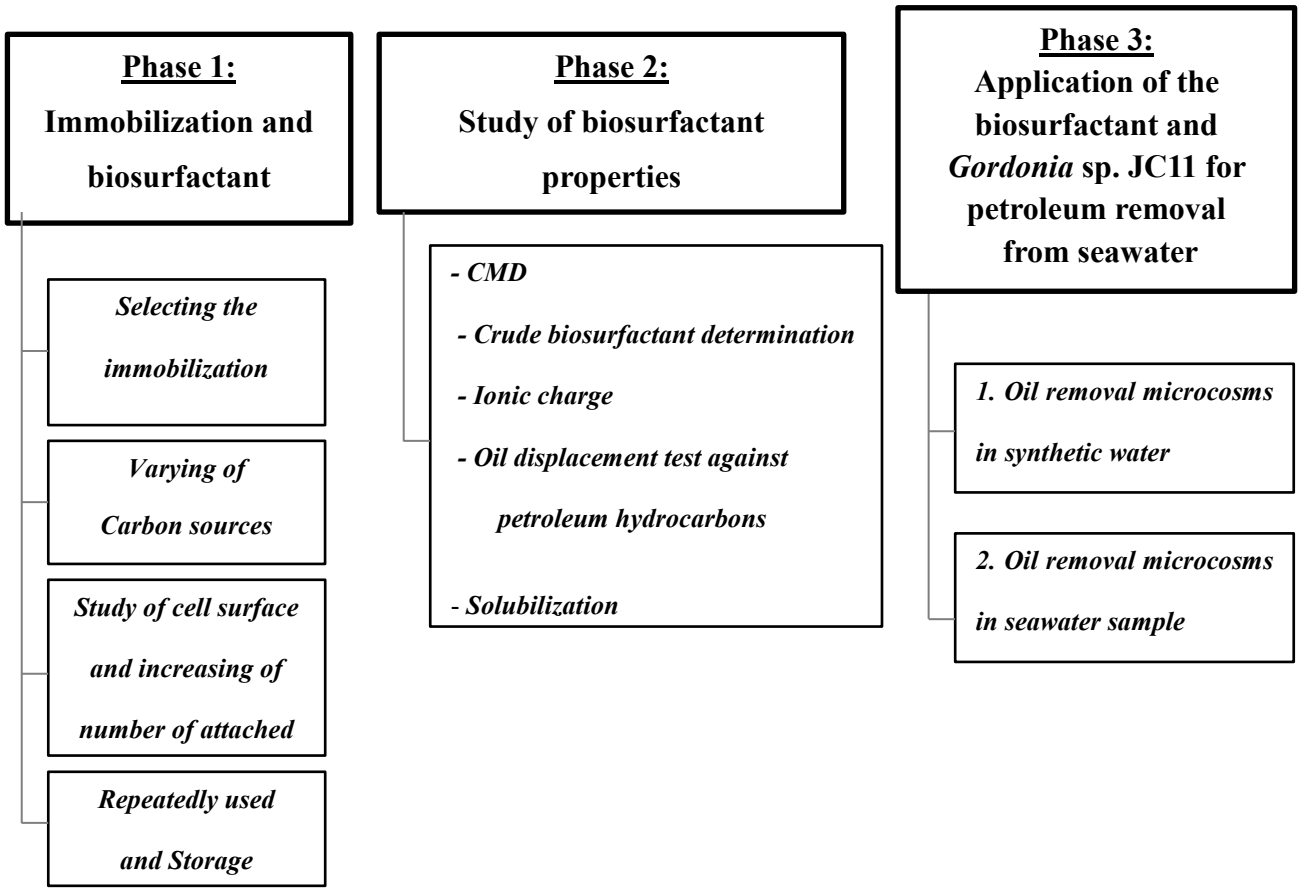

Figure 3. 1 Overview of this research

3.2 MATERIALS

3.2.1 Organisms

Gordonia sp. GY40 is an efficient biosurfactant-producing bacterium. The bacterium was isolated from local soil by Nanthorn Paorach, Department of Microbiology, Faculty of Science, Chulalongkorn University. *Gordonia* sp. GY40 is regularly maintained in basal medium (BM) with 2% bottom glycerol.

Gordonia sp. JC11 was isolated from oil-contaminated seawater by Chanthamalee and Luepromchai (2012). The bacterium is maintained in NSW broth with 100 ppm of fuel oil. For preparation of bacterial inoculum, 25%LB broth was used as substrate and shaken at 200 rpm, room temperature for 3 day. The bacterial cells were collected by centrifugation at 8,000 rpm, 10 min and washed twice with NSS.

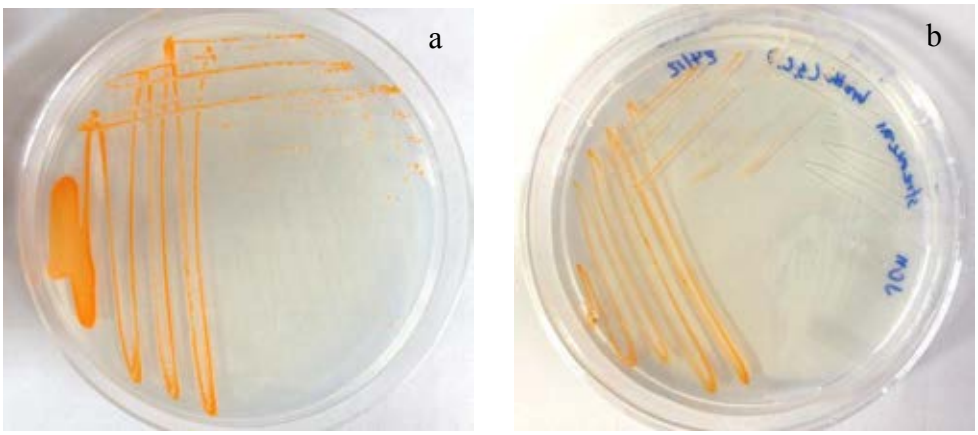

Figure 3. 2 *Gordonia* sp. GY40 (a) and *Gordonia* sp. JC11 on 25% LB agar

3.2.2 Seawater samples

Three seawater samples were collected for applied to oil removal microcosms. Before that, synthetic seawater (Chanthamalee and Luepromchai, 2012) was used in the preliminary study. Seawater samples came from three different sites. The first sample was collected on June 1, 2013 near a port in Bangsan, Chonburi province that may contaminated with ship fuels. The second sample was collected on June 7, 2013 from Maptaphut, Rayong province, where it has high risk of petroleum oil contamination from the industrial estate. The third sample came from Samed Island, Rayong province and collected after the oil spilled on July 27, 2013. The seawater samples were analyzes by methods in Table 3.1.

Table 3. 1 Parameters for measurement the seawater samples

Parameter	**Methods**
pH	pH meter
Satlinity	Reflectometer/Brix meter
Total nitrogen	Macro-Kjeldahl and Colorimetric
Total phosphorus	Ascorbic acid
COD	Potassium Dichromate
Total Peroleum Hydrocarbons (TPH)	Thin Layer Chromatography and Flame Ionization Detection (TLC-FID)

3.2.3 Oil sample

This study used fuel oil as representative of petroleum contaminated in seawater. The Fuel oil provided by PTT Company. Fuel oil is heavy oil blended with various residual from refinery process (Prelec et al., 2013). It can emit nitrogen oxides (NOx), sulphur oxides (SOx), carbon monoxide (CO) and unburned particles from combustion processes. Generally, properties of fuel oils are difference and depend on the composition (Table 3.2).

Table 3. 2 Characteristics of fuel oil (Prelec et al., 2013).

Characteristics	**Average ± SD**
Density at 15°C (kg/m^3)	989.03 ± 6.88
Viscosity at 100°C (mm^2/s)	38.16 ± 4.17
Lower heat value (Mj/kg)	40.14 ± 0.70
Sulphur (%m/m)	2.30 ± 0.02
Coke (Condradson) (%m/m)	14.43 ± 0.88
Asphaltens (%m/m)	7.57 ± 1.96

From the preliminary study, Thin Layer Chromatography and Flame Ionization Detection (TLC-FID) shown the hydrocarbons in fuel oil consisted in fuel oil, namely, saturated hydrocarbon, aromatics, resin, and asphaltene. Resin is polarity high molecular weight composed with O, S, N atoms. Asphaltene, complex molecular hydrocarbon composed of S, O, N, and metals, is important problem in oil industries because it is blocked the crude oil extraction and polluted the environment (Tavassoli et al., 2012).

For study of biosurfactant properties was used Fuel oil and 4 petroleum hydrocarbons, including, diesel oil, slideway oil, crude oil, and waste lubricant (Table 3.3).

Table 3. 3 Petroleum hydrocarbons used in the study

Petroleum	Type	Supplier/Source
Fuel oil	-	PTT Company
Diesel	-	PTT Company
Slideway oil	-	PTT Company
Crude oil	Murban Light Thaioil Co., Ltd.	Thaioil Co., Ltd.
Lubricant	Waste no.1	Fishing boat, Chanthaburi, Thailand

3.3 EXPERIMENTS

PHASE 1: IMMOBILIZATION AND BIOSURFACTANT PRODUCTION

3.3.1 Immobilization techniques

1) Chitosan immobilization (Surface attachment)

In this research used squid pen chitosan from ELAND Corporation LTD. The properties were 1-2 mm size and beta-chitin type. Following Khondee et al (2012), *Gordonia* sp. GY40 which kept on 25%LB agar was added to sterile chitosan (80 g/L) in 25%LB broth by using loop to pick up the colonies of GY40; then, shaken at 200 rpm taken for 3 days, next immobilized cells were washed by using NSS twice times.

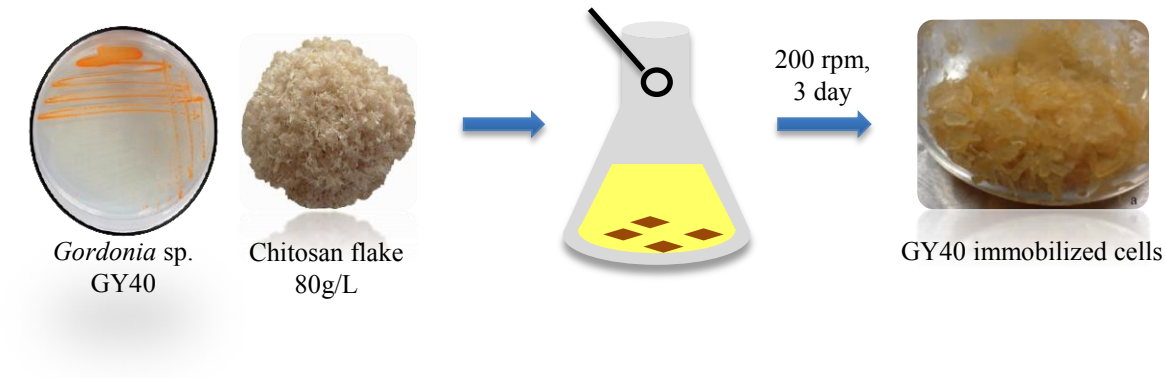

Figure 3. 3 Procedure of chitosan attachment immobilization

2) Silica immobilization (Encapsulation)

Gordonia sp. GY40 was obtained to 2 OD at 600 nm in NSS for culture broth. Silica immobilization used sol-gel method according to Khongkhaem et al. (2011), tetraethoxysilane (TEOS) from Sigma-Aldrich Corporation was mixed with HCl and kept at 4°C, 72 hr then added KOH for adjust pH to 8, finally mixed with culture broth and silica-gel was formed. Silica immobilized cells were cut into 1 cm x1 cm.

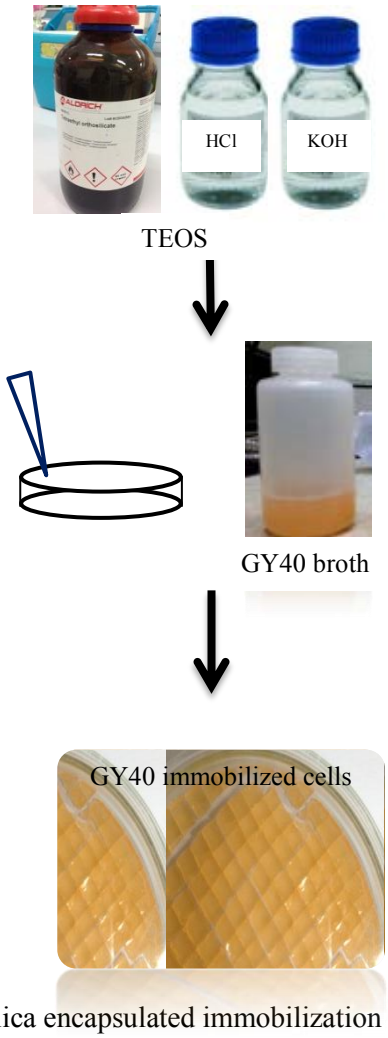

Figure 3. 4 Procedure of silica encapsulated immobilization

3.3.2 Biosurfactant production

The comparison of immobilization techniques were carried out in shake flasks. Briefly, 100 g/L silica immobilized cells or 80 g/L chitosan immobilized cells which had the same initial cell number per gram of immobilized cells were added to Basal medium (see in Appendix A) containing 2% bottom glycerol as a carbon source and 0.75% palm oil as an inducer. Immobilized cells were added to 250 ml Erlenmeyer flask containing 50 ml basal medium and carbon source and shaken at 200 rpm, room temperature for 7 day. The free cells were used as control. After incubation, supernatant was collected after centrifugation at 800 rpm, 10 min for biosurfactant determination, including, surface tension, oil displacement test for fuel oil. Cell number was counted in unit of CFU per gram immobilized cells in 0 day and 7 day.

3.3.3 Effect of carbon source

After selection of a suitable immobilization technique from 1.2, the effect of carbon sources on biosurfactant production was determined. Carbon sources, including, soy-bean oil, palm oil, glycerol, and bottom glycerol (Ferraz *et al*., 2002; Silva et al., 2010; Lui *et al,* 2011) at 2% were added to the basal medium. Immobilized cells was added to 250 ml Erlenmeyer flask containing 50 ml basal medium and carbon source and shaken at 200 rpm, room temperature for 7 day. After incubation, supernatant was collected after centrifugation at 800 rpm, 10 min for biosurfactant determination, including, surface tension, oil displacement test for fuel oil. Cell number was counted in unit of CFU per gram immobilized cells in 0 day and 7 day.

3.3.4 Study of immobilized cells

Immobilized cell was obtained surface topography for observation of bacterial attachment on media by using SEM (Scanning Electron Microscopy). According to Khondee et al (2012) the immobilized cell was taken by JSM-5410LV scanning electron microscope (JEOL). The samples were fixed with 2.5% (v/v) glutaraldehyde and dehydration by sequential ethanol gradients. Then, desiccation with a critical point dryer prior was used for gold coating

3.3.5 Repeating use of the immobilized cells

The immobilized cells were collected to reuse to produce the biosurfactant. Surface tension and cell number in immobilized cells were measured for determined the suitable using time.

3.3.6 Biosurfactant storage

Biosurfactant was tested the suitable period and temperature of storage. The cell-free supernatant was kept at 4°c for 1, 2, 3, 4 weeks, and 2 months. At each time, surface tension was measured for selecting the storage conditions.

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PHASE 2: STUDY OF BIOSURFACTANT PROPERTIES

3.3.7 Study of biosurfactant properties

1) Surface tension and critical micelle dilution (CMD) measurement

The supernatant was diluted with water before measuring surface tension at 25°C by tensiometer (Kruss, K10ST, Germany). Critical micelle concentration was determined from a plot of surface tension versus the supernatant dilution.

2) Crude biosurfactant determination: solvent extraction

The supernatant was extracted with hexane (1:1) to primary remove the residual oil by separating funnel. The pH of the supernatant was later adjusted to 2.0 with 1 M HCl to reduce the biosurfactant solubility. The biosurfactant was extracted with equal volume of chloroform-methanol (2:1). The solvent was then evaporated, and the residue thick yellowish product was dissolved in methanol, filtered and concentrated again using a rotary evaporator. The weight of crude biosurfactant was used to determine biosurfactant production yield.

3) Ionic charge of biosurfactant

According to Daoshan *et al*., (2004), the biosurfactant was mixed with dichloromethane and indicator, dimidium bromide. The changing of indicator color was investigated.

4) Oil displacement test against petroleum hydrocarbons

In this study, various petroleum hydrocarbons, including, slideway oil, diesel, fuel oil, crude oil, and waste lubricant were used. The synthesized seawater was added to petri dish and petroleum oil was dropped on seawater surface. Then, supernatant of biosurfactant was dropped onto the surface of oil. The measurement oil displacement was done by comparing the diameter of biosurfactant with diameter of oil by using followed formula Eq. (1). Sodium Dodecyl Sulfate (SDS) and water were used as positive and negative control respectively.

$$\text{Oil displacement test} = \frac{\text{Diameter of biosurfactant} \times 100}{\text{Diameter of dropped oil}} \qquad \text{Eq. (1)}$$

5) Toxicity of biosurfactant on Gordonia sp. JC11 and seawater microorganisms

The biosurfactant was tested whether it is toxic to *Gordonia* sp. JC11 and seawater microorganism by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) adapted from Bharali et al., (2011). MIC was done by culturing the bacterial strain in LB broth which incubated 37°C, overnight. The diluted culture broth should have 0.2 OD at 600 nm. The autoclaved cell-free supernatant of biosurfactant was diluted by seeded in 96-well plate which added NSS 50 µl as diluents. After that, bacterial inoculum was inoculated and then incubated at 37°C for 24 h. MIC was the lowest concentration of biosurfactant which no visible growth. For seawater sample, 50 µL of seawater was added to well plate which had 50 µL of diluted biosurfactant. Meanwhile, MBC was the highest

dilution of biosurfactant which single colony not appeared in solid media, 25% LB agar for JC11 test and Marine agar for seawater microorganism.

6) Solubilization

Twenty five mL of biosurfactant solution and 100 mg oil sample were shaken at 200 rpm for 1 day and left it stationary. Ten mL of solution layer was extracted by chloroform (ration 1:1); then, TLC-FID was used to analyze the amount of oil. Solubilized efficacy was calculated followed formula Eq. (2).Comparison of biosurfactant activity, SDS was used.

$$\text{Efficacy} = \frac{\text{Concentration of oil in the solution layer x 2.5}}{\text{Total concentration of oil}} \qquad \text{Eq. (2)}$$

7) Emulsification test

The emulsification index was measured following described by Copper and Goldenberg (1987) to determine stability of the biosurfactant. Mixture of 1 mL biosurfactant and 1 mL hydrocarbon in the glass tube was vortexed at high speed for 2 min. After 24 hr, emulsification index (E24) was measured by using as followed formula Eq. (3). Comparison of biosurfactant activity, SDS was used.

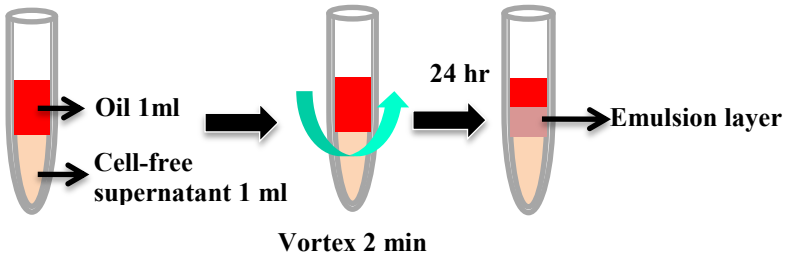

Figure 3. 5 Procedure of emulsification index

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of emulsion layer x 100}}{\text{Total height of mixture}} \qquad \text{Eq. (3)}$$

8) Dispersant activity

BFT for *Gordonia* sp. GY40 biosurfactant was performed adapted from Venosa et al (2002). Briefly, Fuel oil 100 mg will be dispensed onto the surface of 120 mL seawater in 250 mL Erlenmeyer flask; after that, 1 mL biosurfactant was added onto Fuel oil surface. The flask was shaken at 250 rpm for 10 min and left stationary 10 min. The sample was collected 30 ml of sample. The efficacy was analyzed by extracted twice times with chloroform (ratio 1:1), analyzed the residue oil by using TLC-FID and calculated by followed formula Eq. (4). Comparison of biosurfactant activity, SDS was used.

$$\%\ \text{Dispersant Efficacy} = \frac{\text{Mass of dispersed oil x 100}}{\text{Total mass of oil}} \qquad \text{Eq. (4)}$$

PHASE 3: APPLICATION OF THE BIOSURFACTANT AND *GORDONIA* SP. JC11 FOR PETROLEUM REMOVAL FROM SEA WATER

3.3.8 Seawater microcosms

The methods were adapted from Chanthamalee and Luepromchai (2012) and Chanthamalee et al (2013). Application for bioremediation of petroleum oil contaminated seawater was examined in the batch condition by using synthetic seawater and three seawater samples. PUF immobilized *Gordonia* sp. JC11 at 0.1 g and cell-free supernatant (CFS) of biosurfactant at the 0.5 CMD were added to 40 ml synthetic seawater (NSW) or seawater sample and 1,000 mg/L fuel oil (Figure 3.6 and Figure 3.7). The microcosms were shaken at 200 rpm, room temperature for 10 days and the triplicate samples were collected to measure cell growth and residual oil content every 2 day. Besides, the biodegradability of *Gordonia* sp. JC11 microcosm and control will be performed together (Figure 3.6 and Figure 3.7). The residual oil concentration was extracted from the whole flask and analyzed by TLC-FID; in addition, number of bacteria was counted by drop plate technique.

$$\% \text{ Removal} = \frac{(\text{Residual oil day}_0 - \text{Residual oil at day}_x) \times 100}{\text{Residual oil at day}_0} \qquad \text{Eq. (5)}$$

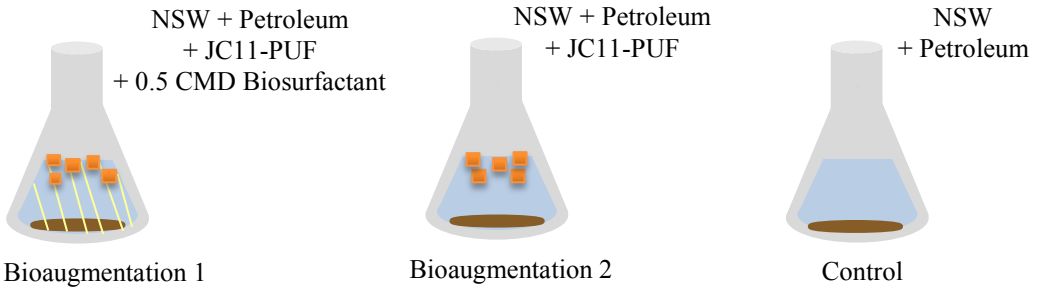

Figure 3. 6 Three experiments of seawater microcosms

Figure 3. 7 *Gordonia* sp. JC11 immobilized on PUF (a). Seawater microcosms, including, JC11-PUF with fuel oil (b1), JC11-PUF with fuel oil and GY40 biosurfactant (b2), and control which only fuel oil (b3).

1) Residual oil extraction

Residual of petroleum was extracted out from NSW 40 mL by chloroform 19 mL and 1 mL of internal standard, steryl alcohol (1-Octadecanol, 99%, Sigma-Aldrich) 6.25 mg/mL chloroform and shaken in separator funnel for 2 min. Then, left stationary 1 min and collected the chloroform phase. One sample was extracted twice times. After that, solvent phase was evaporated until it left 5 mL.

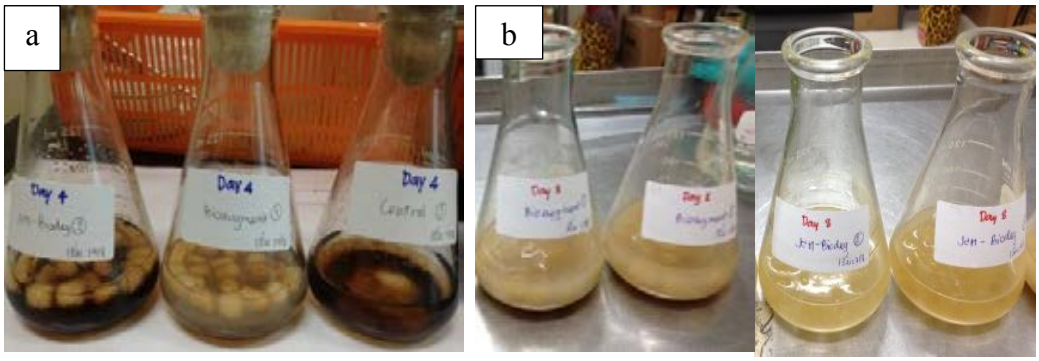

Figure 3. 8 JC11-PUF contained microcosm before extracted (a) and after extracted (b)

2) Thin layer chromatography and flame ionization detection (TLC-FID)

JC11 cells and GY40 biosurfactant altogether with seawater was extracted with an equal amount of chloroform and determined the amount of remaining oil. Stearyl alcohol was used as internal standard. TLC-FID was used for hydrocarbon fraction and TPH determination and carried out according to Maruyama et al. (2003). Briefly, the oil sample was diluted in chloroform and spotted onto silica gel-packed capillary quartz rod (Iatron Laboratories, Japan); then, used consecutive development method employing different solvent systems for fractionation. Scanning the rod used the IatroscanTM MK-6/6S (Mitsubishi Kogaku Iatron, Inc., Japan). Amount of each petroleum hydrocarbon fraction was calculated by comparison the peak area with the internal standard.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 IMMOBILIZATION AND BIOSURFACTANT PRODUCTION

4.1.1 Selection of immobilization techniques

Biosurfactant production by chitosan- and silica-immobilized *Gordonia* sp. GY40 and free cells were compared. The process used 2% bottom glycerol as a carbon source and 0.75% palm oil as an inducer. Figure 4.1 shown *Gordonia* sp. GY40 cells in orange color attached on chitosan (a) or encapsulated in silica (b). The surface tension and oil displacement activity of the produced biosurfactant (in supernatant) were shown in Figure 4.2 and cell numbers of the inoculum were in Table 4.1. During biosurfactant production, chitosan attachment showed the increasing of cell number while silica encapsulation had decreasing cell number. The supernatant from chitosan-immobilized cells had the highest % of oil displacement test with fuel oil at 83.72% and reduced surface tension from 59.6 (basal medium) to 38.9 mN/m. Silica immobilized cells shown lower % of oil displacement test. The oil displacement test was used to indicate the concentration of biosurfactant in supernatant following Bharali et al. (2012)

Concentration of biosurfactant production by 3 techniques in Table 4.1 indicated that immobilization of *Gordonia* sp. GY40 by chitosan attachment produced the highest amount of biosurfactant. Kourkoutas et al, (2004) showed that immobilized cells can uptake substrate in higher level than free cells because of the direct contact between cells and hydrophobic substrate. In this research, the chitosan-

immobilized cells had higher contact with palm oil than silica-immobilized cells because chitosan can sorb oil (Khondee et al., 2012).

Silica encapsulation showed the lowest amount of biosurfactant when using the same conditions with chitosan attachment and free cells. It may cause from the lower transfer of product to solution. Verbelen et al, (2006) concluded that encapsulation technique had limitation of mass transfer, as well as, limitation of internal mass transfer which is the ratio of consumed rate over product by diffusion theory. The results indicated that using chitosan as matrix for bacteria attachment was appropriate for preparing inoculum for biosurfactant production.

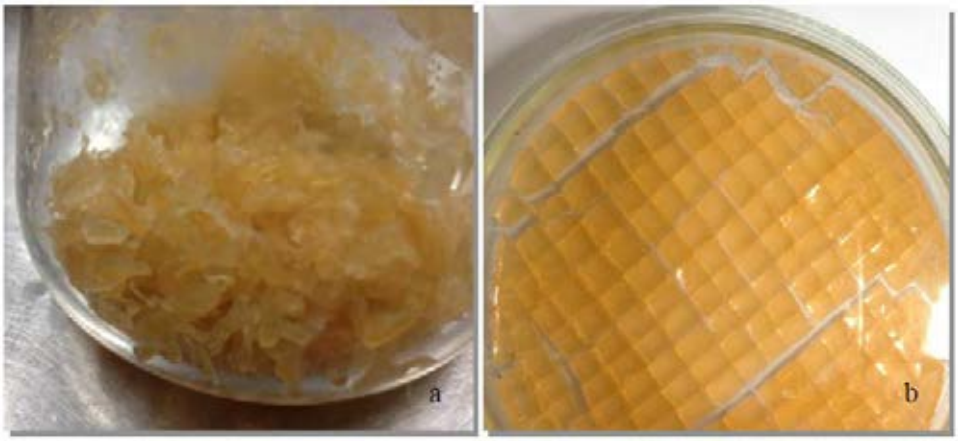

Figure 4.1 *Gordonia* sp. GY40 immobilized on chitosan (a) and in silica (b)

Table 4.1 Cell number of the inoculum before (day 0) and after (day 7) biosurfactant production using 2% bottom glycerol as a carbon source and 0.75% palm oil as an inducer

Type of inoculum	Day 0	Day 7	Yield (g/L)
Silica immobilized cells (CFU/g)	$1.3\pm1.2x10^{7}$	$8.1\pm0x10^{6}$	0.29±0.1
Chitosan immobilized cells (CFU/g)	$2.1\pm1.0x10^{7}$	$4.8\pm0.9\ x10^{8}$	1.16±0.4
Free cells (CFU/ml)	$1.1\pm1.0x10^{7}$	ND	0.41±0.1

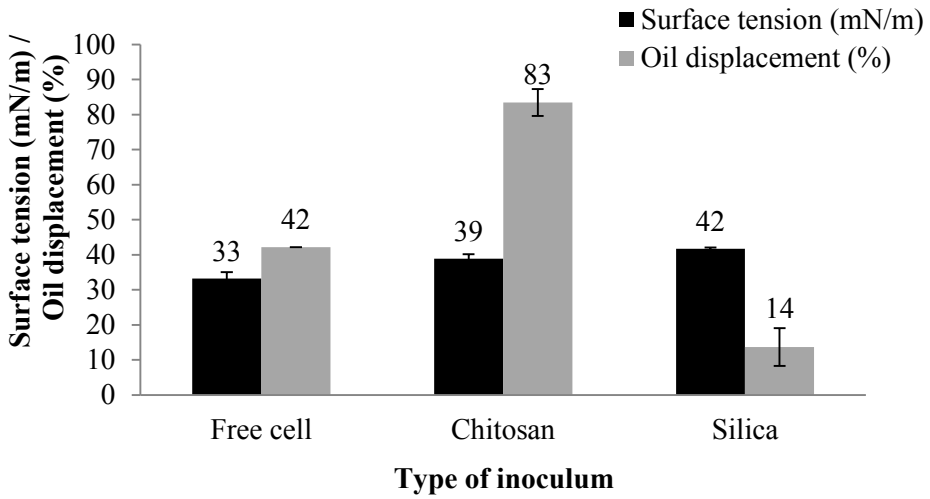

Figure 4. 2 Surface tension (mN/m) and oil displacement test with fuel oil (%) of supernatant of *Gordonia* sp. GY40 in difference technique

4.1.2 Effect of carbon source

The effect of carbon sources including bottom glycerol, soy-bean oil, glycerol, and palm oil on biosurfactant production was studied by chitosan-immobilized cells. Figure 4.3 shown that biosurfactant from 2% soy-bean oil had the highest % oil displacement test with fuel oil at 83.72% and reduced surface tension from 59.6 (basal medium) to 33.86 mN/m. In addition, the increasing cell number per gram chitosan was shown in Table 4.2. The result from palm oil showed the lower % of oil displacement test. This may cause from the amount of free fatty acid, which used as precursor for lipophilic moiety in biosurfactants, is lower in palm oil than in soy-bean oil. Ferraz et al, (2002) found that vegetable oil, such as, sunflower oil, soy-bean oil had linoleic acid which can increase the rate of biosurfactant production. Fatty acids in oil molecule can act as precursor for hydrophobic part of biosurfactant to excrete outside and thereby increase the production amount (Kim et al., 2002). In addition, Ilori et al., (2006) reported that soy-bean oil can be the source of nitrogen for microorganisms. Consequently, 2% soy bean oil was selected for scale-up the biosurfactant production.

Table 4. 2 Cell number per gram chitosan (CFU/g) before (day 0) and after (day 7) biosurfactant production with different carbon sources

Carbon source	Day 0	Day 7	Yield (g/L)
2% Bottom glycerol	$1.3\pm1.2\times10^{7}$	$4.8\pm0.8\times10^{8}$	1.16±0.4
2% Glycerol	$2.9\pm0.1\times10^{7}$	$1.7\pm0.6\times10^{9}$	0.78±0
2% Palm oil	$2.9\pm0.1\times10^{7}$	$5.1\pm1.3\times10^{8}$	0.61±0.1
2% Soy-bean oil	$9.8\pm0.2\times10^{6}$	$5.1\pm1.7\times10^{8}$	1.85±0

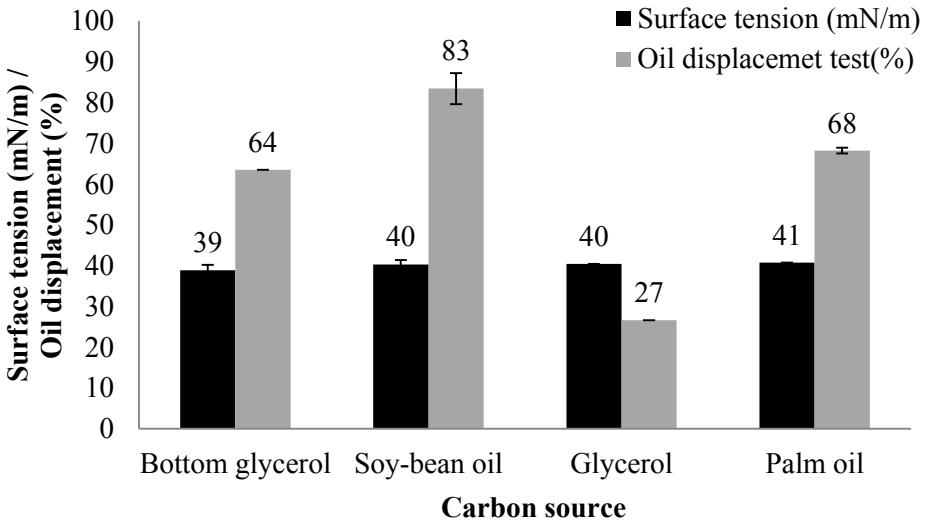

Figure 4. 3 Surface tension and oil displacement test of supernatant from chitosan immobilized cells after biosurfactant production with different carbon sources

4.1.3 SEM photographs of the immobilized cells

After selecting the suitable immobilized carrier for *Gordonia* sp. GY40, the topography of the chitosan surface using SEM was observed. SEM photographs of GY40 immobilized cells compared with chitosan were in Figure 4.4. Squid pen chitosan flakes have roughly surface and many crevices, which promote the attachment of bacterial cells (Khondee et al., 2012).

The attachment of *Gordonia* sp. GY40 cells on chitosan flakes was a suitable technique for bacterial immobilization. Chitosan can protect bacterial cells from the environment effectively (Gentili et al., 2006), avoid the mass transfer limitation, and easy to produce.

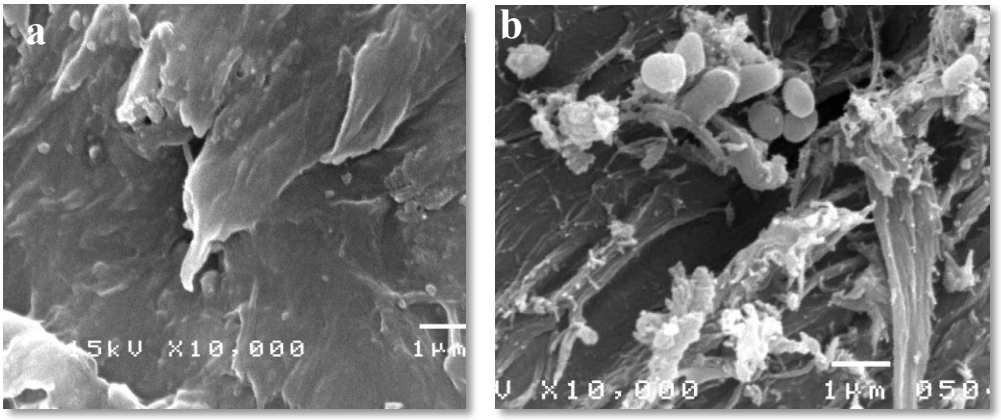

Figure 4. 4 Chitosan surface (a) and *Gordonia* sp. GY40 attached on chitosan (b) when observed by using Electron Scanning microscopic (SEM).

4.1.4 Repeating use of the immobilized cells

Immobilized cells of *Gordonia* sp. GY40 was repeatedly used for biosurfactant production 4 times. The cell number of immobilized cells and surface tension were determined in Figure 4.5. After the first biosurfactant production, the cell number and surface tension were 5.1 x 10^8 CFU/g and 35.77 mN/m, respectively. The second production showed that the cell number increased to 2.44 x 10^9 CFU/g and surface tension reduction also slightly decreased from 23.8 to 22.4 mN/m. It is indicated that chitosan-immobilized cells could be reused for the second time. However, at third and fourth productions, cell numbers were decreased. This result showed that cells were detached from chitosan and led to the increasing of surface tension. This demonstrated that using these immobilized cells were not suitable because of cell detachment from the carrier. To avoid this problem, *Gordonia* sp. GY40 immobilized cells might be applied in bioreactor, for example, Khondee et al, (2012) used airlift bioreactor for chitosan immobilized cells because this type of reactor had low shear force and simple operation

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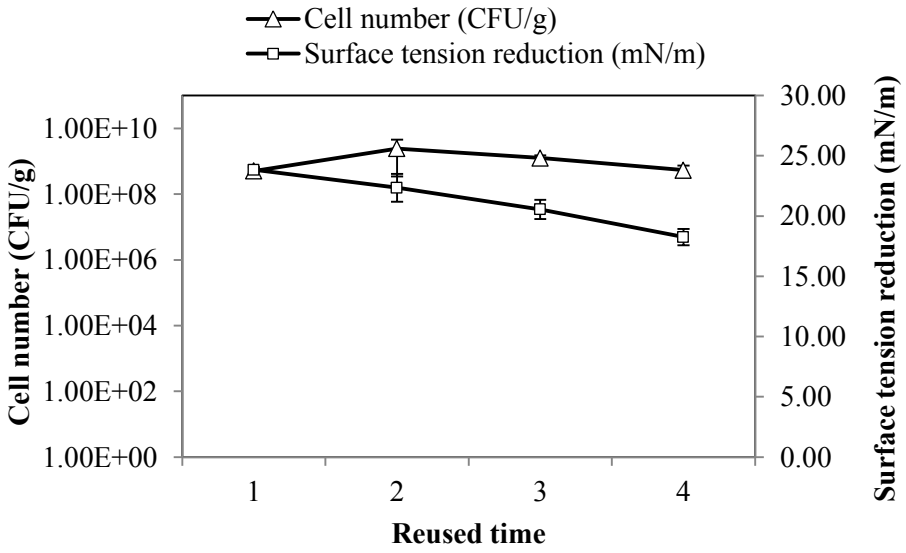

Figure 4. 5 Cell number per gram (CFU/g) and surface tension reduction (from Basal medium 59.6 mN/m) of GY40-chitosan immobilized cells which repeatedly used for biosurfactant production.

4.1.5 Biosurfactant storage

The storage time of biosurfactant was determined by observing the surface tension of supernatant after kept in plastic bottle at 4°C. This condition was chosen because it is easy to do and no need for advance technique. Surface tension was measured along the period of time (Figure 4.6). The results showed that surface tension was not significantly decreased after storage. As a result, this biosurfactant could be maintained for at least 1 month. Further studies could be to vary the time periods and storage conditions.

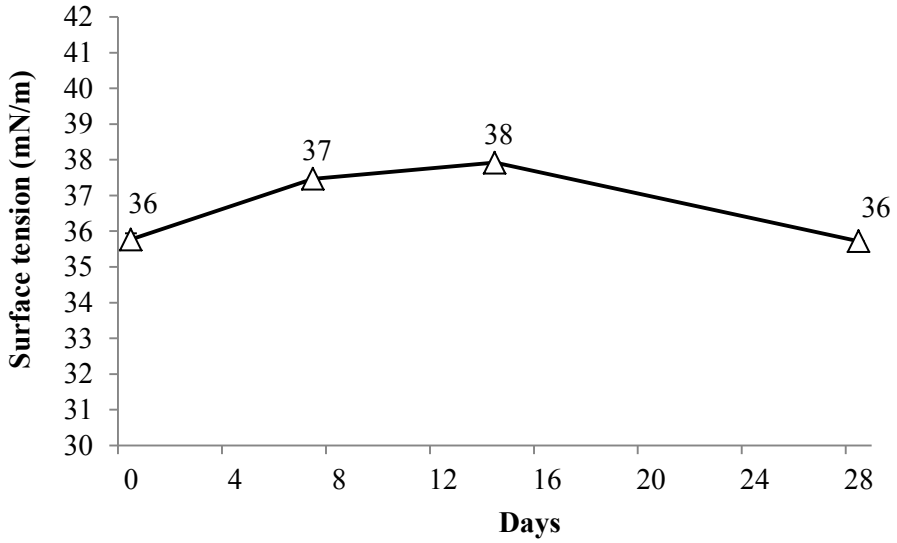

Figure 4. 6 Surface tension of biosurfactant after storage.

Some researchers developed the biosurfactant product with expanded shelf life. For example, Ramnani et al. (2005) purified biosurfactant by using ultrafiltration with 100 kda membrane and found that it had shelf life of 1 year when mixing with sodium sulphate in the retentate.

4.2 PROPERTIES OF BIOSURFACTANT

The properties of biosurfactant was determined from cell-free supernatant, which obtained after cultivating chitosan-immobilized *Gordonia* sp. GY40 in basal medium containing 2% soy-bean oil as a carbon source for 7 days.

4.2.1 **Crude biosurfactant determination, Surface tension, critical micelle dilution (CMD) measurement, and Emulsification activity**

Biosurfactant recovery was done by using chloroform-methanol extraction. The yield was 1.85 g/L, which was higher than free cells at 1.16 g/L when using 2% bottom glycerol and 0.75% palm oil as an inducer. The result showed lower yield of biosurfactant compared with other researches (Table 4.3). However, other researchers used difference carbon sources and different bacteria.

The critical micelle dilution is the lowest concentration of surfactant that monomer form the micelle (Silva et al., 2010). The CMD of the supernatant was 0.25. It was able to reduce surface tension to 34.99 mN/m (Figure 4.7). CMD can show the biosurfactant activity; lower CMD referred to low micellar formation and can display some functions, such as, emulsification and solubilization (Yin et al., 2009).

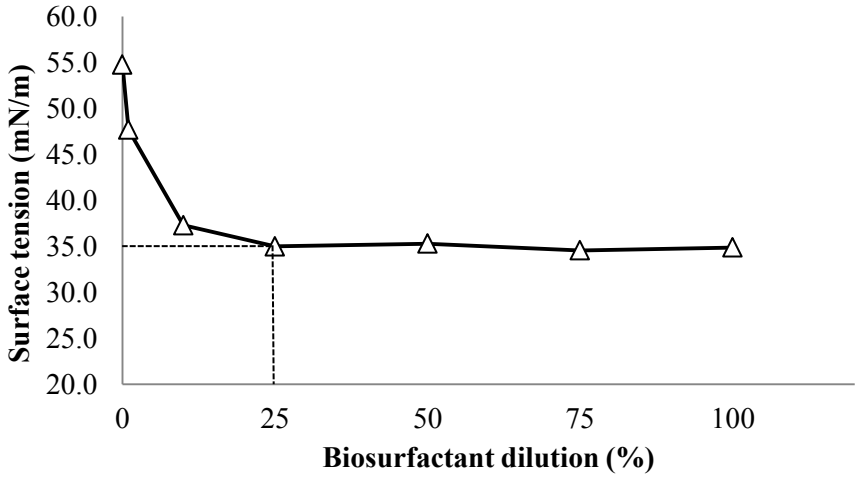

Figure 4. 7 Critical micelle dilution of the biosurfactant (diluted supernatant) from chitosan immobilized cells using 2% of soy-bean oil as a carbon source

Petroleum hydrocarbons, namely, fuel oil, waste lubricant, crude oil, slideway oil, and diesel were used to determine the emulsification activity of biosurfactant (Figure 4.8). Fuel oil, waste lubricant, and crude oil were viscous and black color (Figure 4.9). These characteristics made the emulsify activity test inaccurate because of errors from pipette and observation.

Figure 4. 8 Emulsification test of biosurfactant on fuel oil, waste lubricant, crude oil, slideway oil, and diesel respectively.

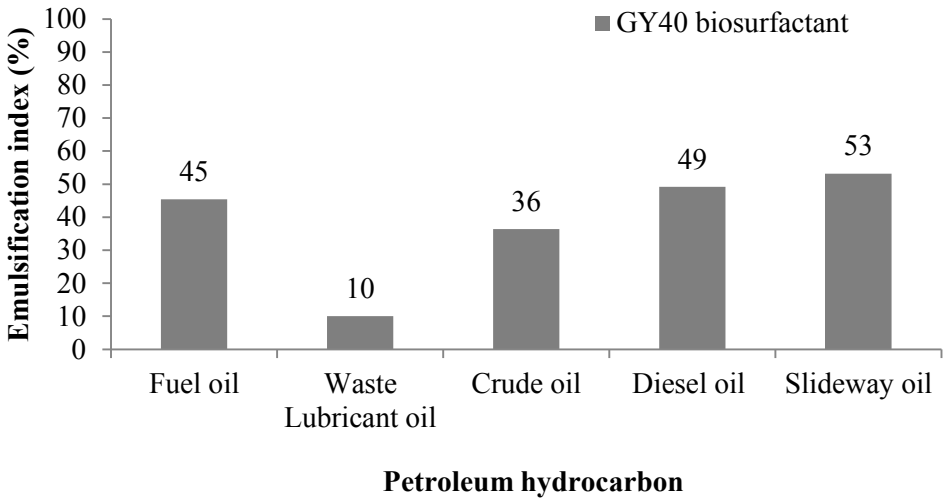

Figure 4. 9 Preliminary study the percentage of emulsification test in difference type of hydrocarbons

After that, slideway oil and diesel were chosen for testing the emulsifying activity of biosurfactant against SDS (commercial surfactant). The results showed that 25% and 100% supernatant of biosurfactant had efficiency to emulsify slideway oil similar to SDS. For diesel oil, 100% biosurfactant had the highest percentage of emulsification activity (Figure4.10). It indicated that GY40 biosurfactant was suitable to emulsify slideway oil and diesel. However, the recommended biosurfactant concentration should be higher for diesel than that for slideway. The results indicated

that biosurfactant and SDS had low ability to emulsify diesel, which may cause from high saturated hydrocarbons in its composition.

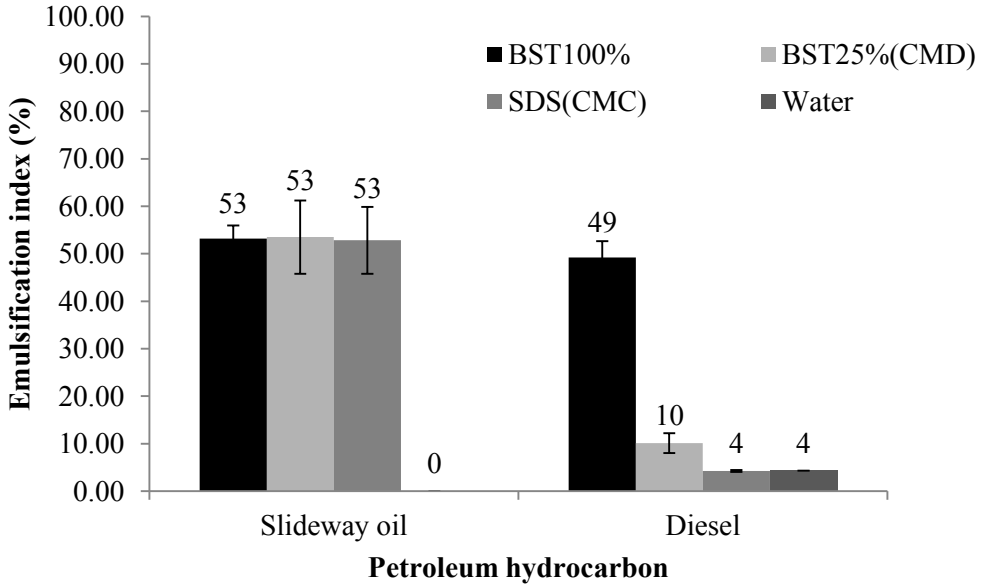

Figure 4. 10 Percentage of emulsification activity of biosurfactant vs. SDS in slideway oil and diesel (BSF = Biosurfactant)

In summary, the chitosan-immobilized GY40 produced biosurfactant at 1.85 g/L. The biosurfactant could decrease surface tension from 59.66 (basal medium) to 34.99 mN/m. The biosurfactant was good for emulsifying slideway oil. The results of biosurfactant yield, CMC, and emulsification index were comparable to other researches (Table 4.3). When using difference carbon source and substrate concentration, biosurfactant concentrations were different (Table 4.3) and depend on biosurfactant producing strains. For example, Saeki et al, (2009) produced biosurfactant from *Gordonia* sp. strain JE-1058 which using 55% n-hexadecane in 5 liter bioreactor and concentration was 69 g/L. These results suggested that bioreactor might be used to increase biosurfactant yield. In addition, the concentration of carbon source and cell number onto the chitosan should be varied. Thereby, the concentration of biosurfactant and emulsification activity would be increased.

Table 4. 3 Comparison of biosurfactant production by various types of bacteria and substrates

Strain	Substrate	Substrate conc.	Surface tension (mN/m)	Yield (g/L)	CMC (mg/L)	E24		Ref
						Oil	%	
Gordonia sp. GY40	Soy-bean oil	2%	35	1.9	-	Diesel	48	This research
Gordonia sp. strain JE-1058	n-hexadecane	55%	-	69	-	-	-	Saeki et al. (2009)
Pseudomonas aeruginosa	Crude oil	2% (w/v)	26	6.0	90	n-hexadecane	80	Saikia et al. (2012)
Selenomonas ruminantium CT2	Molasses	15 g/L	26	5.0	8	-	-	Saimmai et al. (2012)
Rhodococcus ruber	Alkanes	2%	30	-	133	-	-	Zheng et al. (2009)
Alcaligenes faecalis	Diesel	2%	32	9.2	38	Diesel	60-70	Bharali et al. (2011)
Bacillus methylotrophicus	Crude oil	2%(v/v)	28	1.8	-	Crude oil	78	Chandankere et al. (2013)
Serratia marcescens	Crude oil	0.20%	-	9.0	-	-	87	Ibrahim et al, 2013

4.2.2 Ionic charge of biosurfactant

The procedure was described by Daoshan et al. (2004) using two-phase titration. The result shown that GY40 biosurfactant could not change the color after titration to red or blue, which is mean that this biosurfactant was neither anionic nor cationic surfactant (Figure 4.11). Consequently, further study is needed to characterize the charge type of biosurfactant.

Anionic Cationic GY40 biosurfactant

Figure 4. 11 the results from titration using Dimidium Bromide as an indicator, anionic surfactant was red color (a1), cationic surfactant (a2) when using dimidium bromide as an indicator, and GY40 biosurfactant has no changed (b).

4.2.3 Oil displacement test against petroleum hydrocarbons

The percentages of oil displacement by biosurfactant against fuel oil, waste lubricant, crude oil, slideway oil, and diesel were equal to 97.01, 83.43, 47.3, 25.97, and 14.29 %, respectively (Figure 4.12 and 4.13). Crude oil and waste lubricant consist of complex hydrocarbons and some organic compounds that hardly to disperse than slideway oil, fuel oil, and diesel. Except for waste lubricant, the produced biosurfactant and SDS (a commercial anionic surfactant) had similar oil displacement efficiency, which suggested the potential application as dispersant for petroleum.

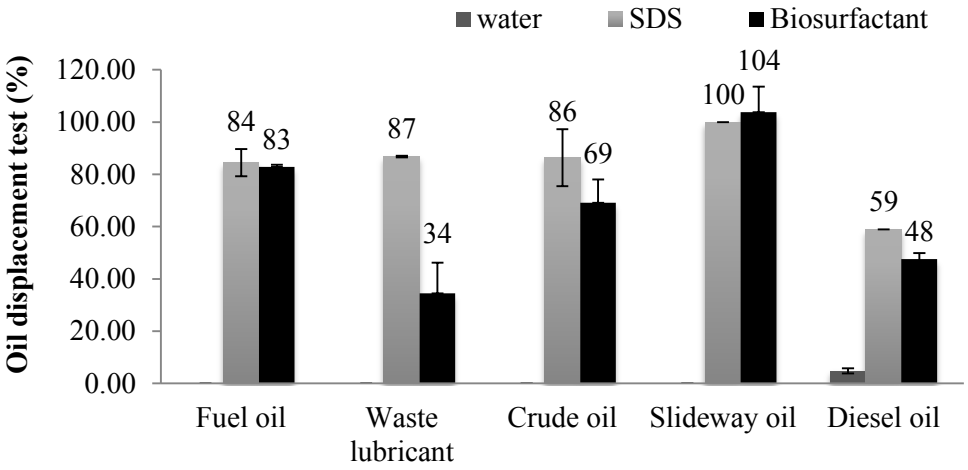

Figure 4. 12 Oil displacement test against various petroleum hydrocarbons of the biosurfactant from chitosan immobilized cells using 2% of soy-bean oil as a carbon source

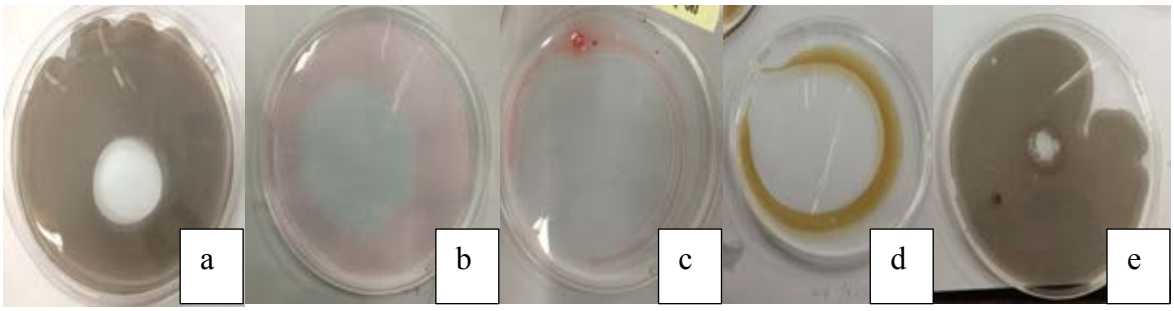

Figure 4. 13 Oil displacement of crude oil (a), diesel (b), slideway oil (c), fuel oil (d), and waste lubricant (e) after adding the biosurfactant

4.2.4 Dispersant evaluation

To evaluate the efficiency of dispersant in synthetic seawater, GY40 biosurfactant (100% supernatant) was used and compared with SDS (commercial surfactant) at CMC. In the preliminary experiment, diluted biosurfactant was used and found that it could not disperse fuel oil. The dispersion requires high concentration of surfactant. The experiment showed that GY40 biosurfactant had slightly lower dispersion efficiency than SDS. Thus, GY40 biosurfactant might be used as a fuel oil dispersant.

The evaluative experiment in this research was adapted for fuel oil sample. However, many factor such as oil composition, type of dispersant, salinity on seawater, sea energy, can influence the effectiveness of dispersant (Fingas, 2011).

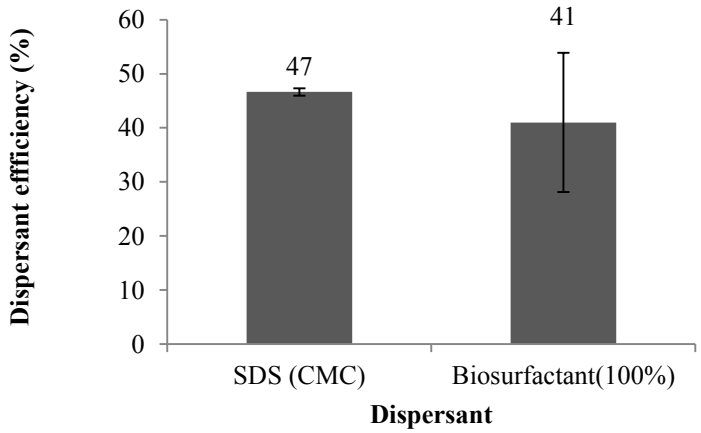

Figure 4. 14 Percentage of dispersant activity of biosurfactant and SDS on fuel oil

4.2.5 Solubilization

To compare the solubilization of fuel oil, diluted GY40 biosurfacant (10%, 25%, and 100%) were compared with SDS, a commercial surfactant. The results found that 100% and 25% (CMC) biosurfactant solubilized fuel oil better than SDS. The results indicated that GY40 biosurfactant has a strong potential to be applied to increase the petroleum solubility and thereby enhance the activity of petroleum-degrading bacteria.

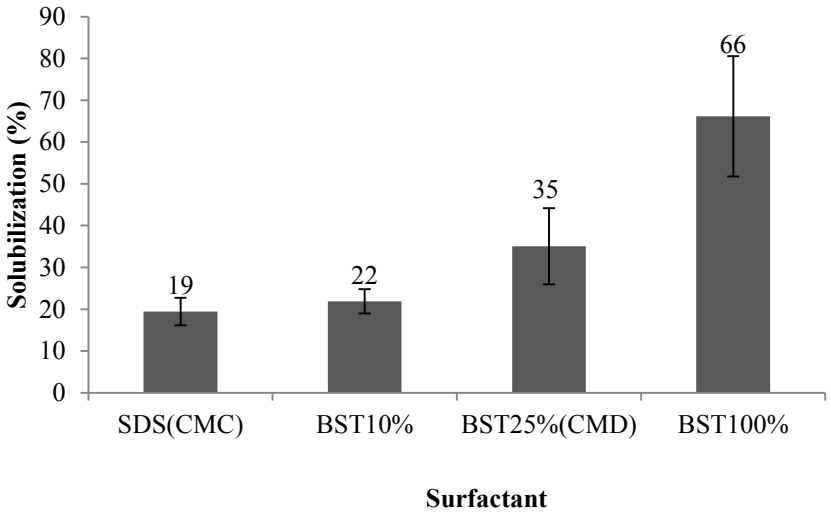

Figure 4. 15 Percentage of solubilization of dilution biosurfactant and SDS on fuel oil (BSF = Biosurfactant)

4.2.6 Toxicity test

The toxicity of GY40 biosurfactant on lubricant-degrading bacteria, *Gordonia* sp. JC11 and microorganisms in seawater sample were demonstrated. GY40 biosurfactant was not toxic to JC11. MIC test showed the turbidity in well plate after adding diluted biosurfactant and JC11. The result was confirmed by MBC test that observed the *Gordonia* sp. JC11 growth (Table 4.4). Besides, positive control with only *Gordonia* sp. JC11 (Figure 4.16 (a1)) and negative control with only LB broth confirmed that no contamination was occurred (Figure 4.16 (a2)).

Table 4. 4 Toxicity test; MIC test of biosurfactant on *Gordonia* sp. JC11

Sample/ concentration (%)	**100**	**50**	**25**	**12**	**6**	**3**	**1**
BSF + JC11	+	+	+	+	+	+	+
Neg. control (LB)	-	-	-	-	-	-	-
Pos. control (JC11)	+	+	+	+	+	+	+

+ growth, - not growth

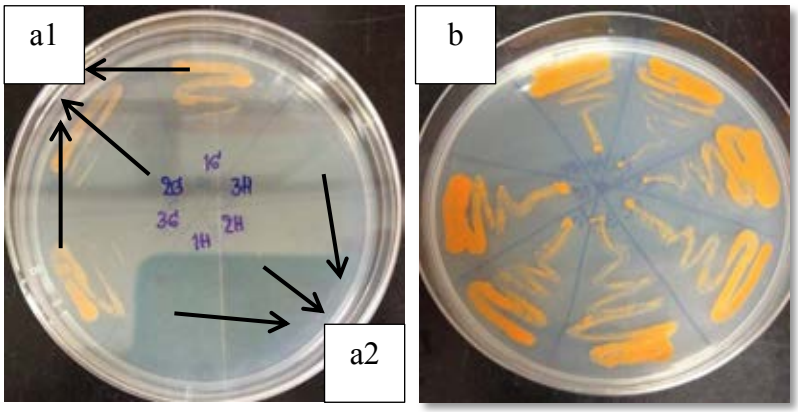

Figure 4. 16 MBC test; *Gordonia* sp. JC11 positive control (a1), LB broth only (negative control) (a2), *Gordonia* sp. JC11 with diluted GY40 biosurfactant(b)

For microorganisms from three seawater samples, including Bangsan, Maptaphut, and Samed Island, the tests were done by comparing biosurfactant, SDS and Dehydol LS9, a commercial nonionic surfactant. The observation from MIC was not clear; therefore, MBC test was selected and tested at CMC concentration. The results showed that microorganisms cannot growth in SDS (Table 4.5), because SDS can cause cell membrane disruption (Filip et al., 1973; Rosety et al., 2001), while LS9

and biosurfactant allowed microorganism to grow (Table 4.5 and Figure 4.17). In general, nonionic surfactant is classified as non- toxic surfactant (Mahale et al, 2012) and biosurfactant generally considered as less-toxic product (Kapadia and Yagnik., 2013). Although, microbial growth from LS9 was lower than from biosurfactant.

Table 4. 5 Toxicity test; MBC test of biosurfactant on seawater microorganisms from three samples (concentration at CMC of SDS 0.01 mM, LS9 0.01 mM, and biosurfactant 25%)

Seawater sample	**Surfactant type**	**Growth**
Bangsan, Chonburi	Biosurfactant	++
	SDS	-
	LS9	+
Maptaphut, Rayong	Biosurfactant	+++
	SDS	-
	LS9	++
Samed island, Rayong	Biosurfactant	+++
	SDS	-
	LS9	+

++ high growth, + growth, - no growth

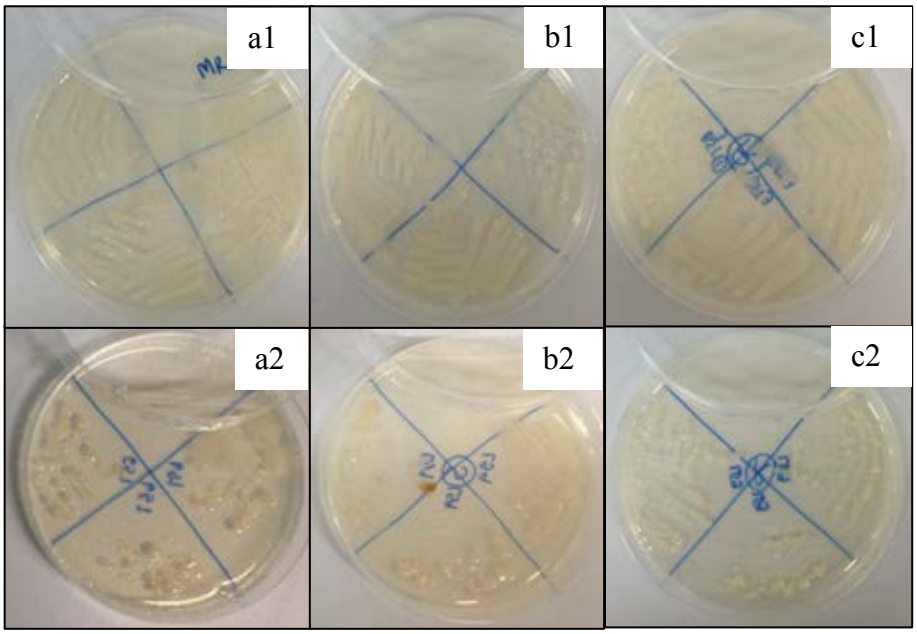

Figure 4. 17 MBC test of biosurfactant (CMC) on seawater microorganism from Bangsan (a1), Maptaphut (b1), Samed Island (c1) and LS9 (CMC) on seawater microorganism from Bangsan (a2), Maptaphut (b2), Samed Island (c2).

4.3 APPLICATION OF THE BIOSURFACTANT AND *GORDONIA* SP. JC11 FOR PETROLEUM REMOVAL FROM SEAWATER

4.3.1 Seawater microcosms: synthetic seawater

The application of biosurfactant for fuel oil removal was carried out in flask-batch experiment. The percentage of oil removal and cell number of *Gordonia* sp. JC11 in polyurethane foam (PUF) were shown in Figure 4.18. The growth of *Gordonia* sp. JC11 in PUF seem to be stable, which confirmed that the biosurfactant was not toxic. However, the number of *Gordonia* sp. JC11 in seawater was not counted. Otherwise, the number of bateria in seawater should increase due to bacterial growth after petroleum consumption. The concentrations of fuel oil in microcosms with JC11-PUF and JC11-PUF with biosurfactant gradually increased along time and dropped after 8 days. The micrococosms with both bacteria and biosurfactant had percent oil removal more than with JC11-PUF only. This may cause from biosurfactant enhanced petroluem solubilization which made more petroleum bioavailability and increasing of oil-degradability. From previous results, biosurfactant solubilized the fuel oil as well as dispersed it into droplets. From Chanthamalee et al (2013), *Gordonia* sp. JC11 immobilized on PUF can remove 53% of 1,000 ppm waste lubricant after 5 days. In this research, JC11-PUF removed 65% of 1000 ppm fuel oil after 10 days and the addition of biosurfactant enhanced fuel oil removal to 71% in the same condition. Bharali et al, (2011) suggested that surfactant can enhance petroleum biodegradation by contact directly with large alkane droplets; then, solubilization was occurred. Hua et al., (2004) found that adding of biosurfactant produced by *Candida antarctica* can increase the biodegradation of crude oil in batch culture from 53 to 74.6 % degradation rate. They suggested that biosurfactant can

increase the rate of growth and degradation. Saeki et al. (2009) demonstrated that adding biosurfactant can form small sized droplets and increased the interfacial; then, bioavailability and activate biodegradation of indigenous microorganisms.

However, using difference type petroleum hydrocarbons can give difference results due to the different compositions of hydrocarbons

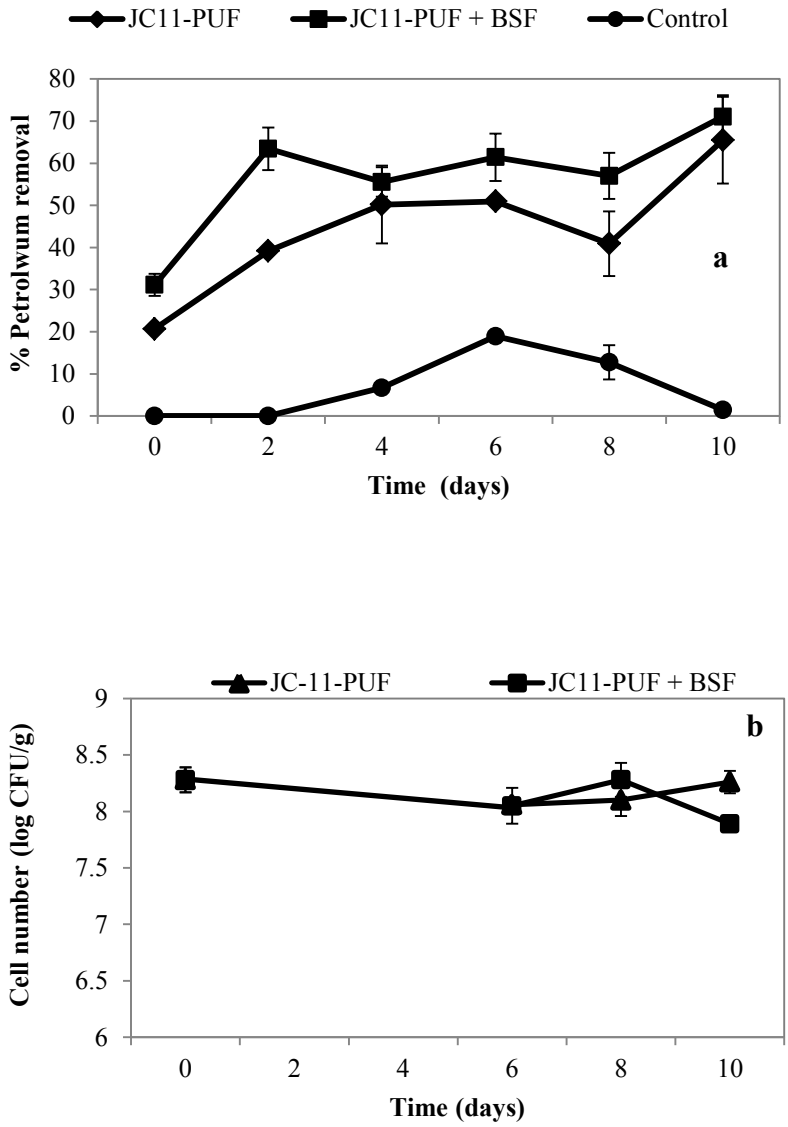

Figure 4. 18 Percentage of fuel oil removal in synthetic seawater (a) and cell number per gram immobilized PUF (b)

4.3.2 Seawater microcosms: seawater samples

For seawater sample, the experiments were done by selecting the suitable condition from synthetic water which was adding 0.5CMD GY40 biosurfactant and JC11-PUF and shaken for 6 days which can remove fuel oil 80.36% (Table B-9, Appendix B). Fuel oil was added to the samplse at 1,000 ppm. The percent petroleum removal were 60%, 76%, and 60% in samples from Bangsan, Maptaphut, and Samed Island, respectively (Figure 4.19). Maptaphut seawater had the highest efficiency to remove petroleum. It might be due to the higher bacterial number in this treatment than the others (Figure 4.20). The control showed 35% oil removal that might be from the activity of indigenous bacteria. Seawater from Bandsan and Samed had percent of oil removal lower than Maptaphut in both control and bioaugmentation treatment. The bacterial cell number in these seawater samples were also lower.

The seawater from three sites had lower oil removal efficiency when compare to synthetic seawater. One reason is the amounts of nitrogen and phosphorous are different which can see in Table 4.6. In synthetic seawater, the amount of nitrogen were 0.1 g/L and yeast extract which support the growth of bacteria was added. On the other hand, seawater from three sites had very low level of nitrogen. Nitrogen is important for aerobic degradation pathway and phosphorus can improve bioremediation in marine environment (Hii et al., 2009; Kwapisz et al., 2008). To apply the biosurfactant in the real environment, the biosurfactant might be sprayed to disperse the oil and the contaminated seawater might be collected for ex-situ remediation. Ex-situ bioremediation will use bioreactor and adding the nutrient; then, the degradation rate will increase. Moreover, increasing the cell number of *Gordonia* sp. JC11 in PUF may be considered.

Table 4.6 Physical properties of seawater samples

Parameters (unit) /Seawater sample	Natural Seawater*	Bangsan, Chonburi	Maptaphut, Rayong	Samed island, Rayong	Standard of seawater*
Date of sampling	-	Jun. 1, 2013	Jun. 7, 2013	Aug. 2, 2013	-
Appearances	Yellow and odorless	Clear and odorless	Yellow and odorless	Clear and odorless	-
pH	7.8	7.69	8.2	8.18	7-8.5
Salinity (%)	3.4	3.4	3.4	3.9	10
Total nitrogen (mg/L)	100	< 0.03	< 0.03	< 0.03	69
Total phosphorus (mg/L)	400	0.2	< 0.1	< 0.1	45
Initial concentration of TPH (mg/L)	-	4.61	3.14	6.44	0.05
COD	3,023	2,070	3,070	1,675	-
Total bacteria (CFU/ml)	-	8.21	8.90	7.13	-
Total petroleum-degrading bacteria (CFU/ml)	-	4.48	4.74	4.53	-

* See in Appendix A

** According to Pollution Control Department, Thailand.

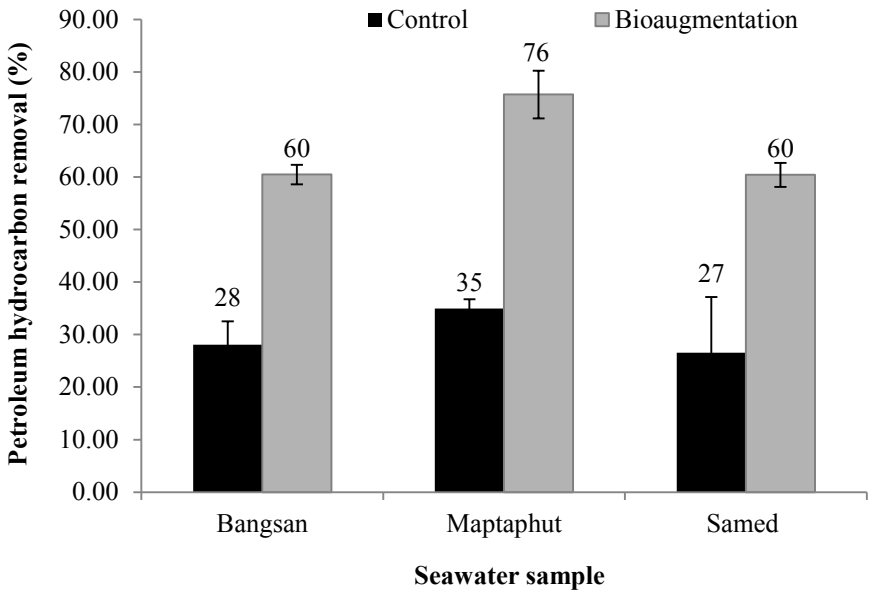

Figure 4. 19 Percentage of fuel oil removal in three seawater samples

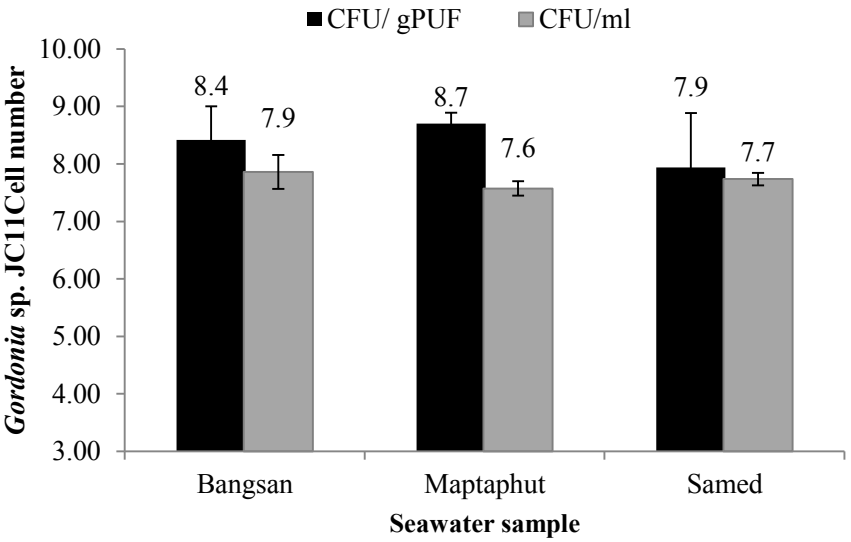

Figure 4. 20 Cell number of *Gordonia* sp. JC11 in three seawater sample after bioaugmentation treatment

CHAPTER V

CONCLUSIONS

5.1 CONCLUSIONS

The problem of petroleum pollutions are wide spread around the world. Consequently, remediation techniques are required. This study aims to apply the biosurfactant from *Gordonia* sp. GY40 together with lubricant-degrading bacteria, *Gordonia* sp. JC11 for petroleum removal in seawater. Moreover, the study tried to increase the biosurfactant yield by immobilizing *Gordonia* sp. GY40 on carrier. Fuel oil was used as a representative of contaminated petroleum in seawater because it is widely used as ship fuels (Nievas et al, 2008).

The research started with selecting a suitable immobilization technique. Silica encapsulation and chitosan attachment were compared by using 2% bottom glycerol and 0.75% palm oil as an inducer in Basal medium for biosurfactant production. During cultivation, the surface tension of media containing silica- and chitosan-immobilized cells was decreased. The cell number on silica was decreased from 10^7 to 10^6 CFU/g, while on chitosan was increased. The supernatant from chitosan-immobilized cells had higher efficiency on oil displacement test than that from silica-immobilized cells. The results indicated that biosurfactant was produced at high concentration from the bacteria after chitosan attachment. After that, carbon sources for biosurfactant production were varied. When 2% soy-bean oil was used, the supernatant could decrease surface tension similar to other substrates, however it gave

the highest efficiency on oil displacement test. Consequently, *Gordonia* sp. GY40 immobilized by chitosan attachment and 2% soy-bean oil were the suitable immobilization technique and carbon source for biosurfactant production, respectively. The chitosan-immobilized cells could be reused at least 1 time and biosurfactant in the supernatant had shelf-life of at least 1 month.

The properties of biosurfactant in supernatant were determined from critical micelle concentration, ionic charge, oil displacement with various petroleum hydrocarbons, dispersant evaluation, solubilization, emulsification index, and microbial toxicity test. The biosurfactant was not anionic or cationic type; therefore, it was potentially non-ionic type. The supernatant was able to reduce surface tension to 34.99 mN/m and crude biosurfactant yield was 1.85 g/L. The critical micelle dilution of the supernatant was 25%. Emulsification index indicated the ability biosurfactant to emulsify diesel oil and slideway oil. Furthermore, oil displacement test on seawater shown that the biosurfactant could displace slideway oil and fuel oil at the highest percentage of 104% and 83% respectively. For dispersibility, biosurfactant had ability similar to SDS. Solubilization test indicated that GY40 biosurfactant could increase solubility of fuel oil which could enhance the biodegradation. The biosurfactant was not toxic to *Gordonia* sp. JC11 and indigenous microorganisms in seawater from Bangsan, Maptaphut, and Samed Island.

The biosurfactant was later applied together with *Gordonia* sp. JC11 which can efficiently degrade lubricant (Chanthamalee and Luepromchai, 2012) in seawater. The microcosms of fuel oil contaminated seawater were carried out under laboratory condition. The results of synthetic seawater show the enhancement of biodegradation

after adding both GY40 biosurfactant and PUF immobilized JC11 for 6 days. The percentages of fuel oil removal were 80.63% in microcosms with both oil-degrading bacteria and biosurfactant, 69.88% in microcosms with only oil-degrading bacteria, and 18.93% in control microcosms. Moreover, microcosms containing three seawater samples showed the effectiveness of PUF immobilized JC11 and GY40 biosurfactant about 60-76%, although the removals were lower than synthetic seawater. It may come from the low level of nitrogen and phosphorous in natural seawater.

In conclusion, biosurfactant production could be optimized by immobilizing *Gordonia* sp. GY40 cells using attachment technique on chitosan flakes and using soy-bean oil as carbon substrate. The produced biosurfactant had high potential for apply during remediation of petroleum hydrocarbons in seawater. In addition, *Gordonia* sp. GY40 biosurfactant worked well with lubricant-degrading bacteria, *Gordonia* sp. JC11 for petroleum removal in seawater. It could speed up the rate of biodegradation. In the future, this biosurfactant may be applied as dispersant for removal of petroleum on seawater surface or as remediation agent for increasing the hydrocarbons solubility and stimulating the activity of petroleum-degrading microorganisms.

5.2 SUGGESTIONS

1) In this study, biosurfactant yield was still lower than other researches. Thus, the cell number of *Gordonia* sp. GY40 per gram of chitosan should be increased. In addition, the production should be operated in a bioreactor to improve biosurfactant yield. In order to obtain higher yield of biosurfactant, the experiment should find the optimum production conditions, i.e. number of initial bacteria and incubation time.

2) The type and charge of biosurfactant should be confirmed by using advance techniques such as, FT-IR mass spectroscopy and HPLC (for characterization of surfactant type) and LC-MS-MS (for characterization of surfactant charge). The understanding of biosurfactant composition is necessary when determining an appropriate application of the produced biosurfactant.

3) An appropriate application of biosurfactant and oil-degrading bacteria during oil spill should be studied. In a mean time, the research recommended a following application procedure. Initially, the size and character of spill must be evaluated before using boom to block the oil spill area. Then, the biosurfactant should be applied to the contained area as oil dispersant. The present of biosurfactant would also increase solubilization of oil for the indigenous bacteria to degrade. In some cases, oil degrading bacteria might be added along with the biosurfactant. Meanwhile, monitoring of oil degradation is needed and should be done in parallel with the rehabilitation of the contaminated area.

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APPENDICES

APPENDIX A

Media

Media

1. Basal medium (per 1 Liter)

NH_4SO_2 7.0 g K_2HPO_4 1.0 g

KH_2PO_4 0.5 g KCl0.1.0 g

$MgSO_4.7H_2O$ 0.5 g $CaCl_2$ 0.01 g

$FeSO_4.7H_2O$ 0.01 g Yeast extract 0.1 g

Trace element 0.05 ml (consisted of 0.26 g H_3BO_3, 0.5 g $CuSO_4.5H_2O$, 0.5 g $MnSO_4.H_2O$, 0.06 g $MoNa_2O_4.2H_2O$, 0.7 g $ZnSO_4.7H_2O$)

Mixed all in 1 liter of distilled water and adjusted pH to 7.5

2. LB broth (Luria-Bertani broth) (per 1 Liter)

Tryptone 10.0 g

Yeast Extract 5.0 g

Sodium Chloride 10.0 g

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

3. Natural Seawater (Higashihara et al., 1978)

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

4. Marine broth (Difco™ Marine Broth 2216)

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		

Suspend 37.4 g of the powder in 1 L of purified water; then, mix throughtly.

APPENDIX B

Data of phase I, II, and III

Table B- 1 Cell number per gram or ml of immobilization techniques

Immobilization Tech.		plate1	plate2	plate3	Aver	AVER	SD
Freecell 1	dilute-4	20	21	16	19	1.14E+07	1.04E+07
	dilute-5	4	5		3		
	dilute-6				0		
Freecell 2	dilute-4	9	10	10	9.6666667		
	dilute-5	3			1		
	dilute-6				0		
Silica Day0	dilute-4	7	9	10	8.6666667	1.33E+07	1.17E+07
	dilute-4	16	16	11	14.333333		
	dilute-5	3			3		
Silica Day7	dilute-3	6	9	12	9	8.12E+06	
	dilute-4				0		
	dilute-5				0		
Chitosan D0	dilute-4	28	29	29	28.666667	9.78E+06	1.69E+05
	dilute-5	3	3		3		
Chitosan D7	dilute-4				0	5.10E+08	1.71E+08
	dilute-5				0		
	dilute-6	11	11	11	11		
	dilute-5		29	29	29		
	dilute-6	4	4	3	3.6666667		
	dilute-5	26	23		24.5		
	dilute-6	4			4		
	dilute-6	4			4		

Table B- 2 Cell number per gram or ml of chitosan-immobilized cells in different carbon source (a) – (d)

(a) 2% Bottom glycerol+ 1.25% palm oil

Chitosan Day 0							
-	**-**	**plate1**	**plate2**	**plate3**	**Aver**	**AVER**	**SD**
1	dilute-4	20	21	16	19.00	2.14E+07	1.04E+07
	dilute-5	4	5		3.00		
	dilute-6				0.00		
2	dilute-4	9	10	10	9.67		
	dilute-5	3			1.00		
	dilute-6				0.00		
3	dilute-4						
	dilute-5	4	5		3.00		
	dilute-6				0.00		
Chitosan Day 7							
-	**-**	**plate1**	**plate2**	**plate3**	**Average**	**AVER**	**SD**
1	dilute-4				0.00	4.78E+08	8.46E+07
	dilute-5	28	24	29	27.00		
	dilute-6	4	5	6	5.00		
2	dilute-4				0.00		
	dilute-5				0.00		
	dilute-6	8	4	3	5.00		
3	dilute-4						
	dilute-5				0.00		
	dilute-6	14	10	9	11.00		
	dilute-7	3			3.00		

(b) 2% Soy-bean oil

Chitosan Day 0							
		plate1	**plate2**	**plate3**	**Aver**	**AVER**	**SD**
1	dilute-4	28	29	29	28.67	9.78E+06	1.69E+05
	dilute-5	3	3		3.00		
Chitosan Day 7							
		plate1	**plate2**	**plate3**	**Average**	**AVER**	**SD**
1	dilute-4				0.00	5.10E+08	1.71E+08
	dilute-5				0.00		
	dilute-6	11	11	11	11.00		
2	dilute-4				0.00		
	dilute-5		29	29	29.00		
	dilute-6	4	4	3	3.67		
3	dilute-4						
	dilute-5	26	23		24.50		
	dilute-6	4			4.00		

(c) 2% Glycerol

Chitosan Day 0							
		plate1	**plate2**	**plate3**	**Aver**	**AVER**	**SD**
1	dilute-4	28	29	29	28.67	2.93E+07	9.43E+03
	dilute-5	3	3		3.00		
	dilute-6						
Chitosan Day 7							
		plate1	**plate2**	**plate3**	**Average**	**AVER**	**SD**
1	dilute-4					1.73E+09	5.66E+08
	dilute-5						
	dilute-6	15	15	10	13.33		
2	dilute-4						
	dilute-5						
	dilute-6	14	24	26	21.33		

(d) 2%Palm oil

Chitosan Day 0							
-	**-**	**plate1**	**plate2**	**plate3**	**Aver**	**AVER**	**SD**
1	dilute-4	28	29	29	28.67	2.93E+07	9.43E+03
	dilute-5	3	3		3.00		
	dilute-6						
Chitosan Day 7							
-	**-**	**plate1**	**plate2**	**plate3**	**Average**	**AVER**	**SD**
1	dilute-4					5.11E+08	1.26E+08
	dilute-5						
	dilute-6	4	6	7	5.67		
2	dilute-4						
	dilute-5						
	dilute-6	4	4	3	3.67		
3	dilute-4						
	dilute-5						
	dilute-6	7	8	3	6.00		
	dilute-7						

Table B- 3 Amount of biosurfactant (yield)

		wt before	wt after	wt	g/L	Average	SD
Free cell	BM+BG2% (1)	3.9572	3.9752	0.018	0.51	**0.41**	0.1
	BM+BG2% (2)	4.176	4.1915	0.0155	0.44		
	BM+BG2% (3)	4.0935	4.1033	0.0098	0.28		
Silica (OD2)	BM+BG2% (1)	4.0613	4.0706	0.0093	0.21	**0.29**	0.1
	BM+BG2% (2)	4.1293	4.1393	0.01	0.22		
	BM+BG2% (3)	4.1355	4.1492	0.0137	0.30		
	BM+BG2% (4)	4.1103	4.1303	0.02	0.44		
Chitosan	BM+2%BG (1)	3.8903	3.9204	0.0301	0.86	**1.16**	0.4
	BM+2%BG (2)	4.1959	4.2312	0.0353	1.01		
	BM+2%BG (3)	3.797	3.8529	0.0559	1.60		
C-sourcce		**wt before**	**wt after**	**wt**	**g/L**	**Average**	**SD**
Bottom glycerol	BM+BG2% (1)	3.8903	3.9204	0.0301	0.86	**1.16**	0.4
	BM+BG2% (2)	4.1959	4.2312	0.0353	1.01		
	BM+BG2% (3)	3.797	3.8529	0.0559	1.60		
Glycerol	BM+gly2% (1)	4.0918	4.1074	0.0156	0.78	**0.78**	
	BM+gly2% (2)			-			
Palm oil	BM+PO2% (1)	18.1725	18.1856	0.0131	0.66	**0.61**	0.1
	BM+PO2% (2)	20.1841	20.1952	0.0111	0.55		
Soy-bean oil	BM+SB2% (1)	18.0647	18.1021	0.0374	1.87	**1.85**	0.0
Lot2	BM+SB2% (2)	18.4156	18.452	0.0364	1.82		

Table B- 4 percentage of Oil displacement test (a) – (b)

(a) Oil displacement test in SDS and different immobilization techniques

Sample	dia. fuel oil	dia. biosurfactant	%	Aver	SD
SDS (Control)	7.8	6.7	85.90	85.77	1.8
SDS (Control)	8	7	87.50		
SDS (Control)	8.7	7.3	83.91		
DI (Control)	8.5	0.1	1.18		
Chitosan 2%BG	8	4.1		69.64	19.9
Chitosan 2%BG	8.6	7.2	83.72		
Chitosan 2%BG	9	5	55.56		
Silica OD2	8.7	0.7	8.05	13.67	5.4
Silica OD2	8.3	1	12.05		
Silica OD2	7	1.4	20.00		
Silica OD2	6.4	1.2	18.75		
Silica OD2	6.3	0.6	9.52		
GY40 Free cell 2%BG	8.3	3.5	42.17	17.22	21.6
GY40 Free cell 2%BG	8.9	0.4	4.49		
GY40 Free cell 2%BG	8	0.4	5.00		

(b) Oil displacement test against different petroleum hydrocarbons

Biosurfactant

petroleum	ODT	ODT	ODT	Aver	SD
Fuel oil	**83.43**	**82.19**	**-**	**82.81**	**0.9**
Waste lubricant	**14.29**	**32.05**	**36.71**	**34.38**	**11.8**
Crude oil	**60.00**	**77.92**	**69.33**	**69.08**	**9.0**
Slideway oil	**97.01**	**99.17**	**115.00**	**103.73**	**9.8**
Diesel	**47.30**	**50.00**	**45.45**	**47.58**	**2.3**

SDS

petroleum	ODT	ODT	ODT	Aver	SD
Fuel oil	**80.77**	**88.16**		**84.47**	**5.23**
Waste lubricant	**87.01**	**86.54**		**86.78**	**0.33**
Crude oil	**78.67**	**94.12**		**86.40**	**10.92**
Slideway oil	**100.00**	**100.00**		**100.00**	**0.00**
Diesel oil	**58.90**			**58.90**	**0.00**

water

petroleum	ODT	ODT	ODT	Aver	SD
Fuel oil	**0.00**	**0.00**		**0.00**	**0.00**
Waste lubricant	**0.00**			**0.00**	**0.00**
Crude oil	**0.00**	**0.00**		**0.00**	**0.00**
Slideway oil	**0.00**	**0.00**		**0.00**	**0.00**
Diesel oil	**4.11**	**5.48**		**4.80**	**0.97**

Table B- 5 Surface tension

(a) surface tension of repeatedly used of chitosan immobilization

	Surface tension	**ST reduction**	**Aver**	**SD**
Reused 2	37.879	21.721	22.35	1.16
	35.915	23.685		
	37.954	21.646		
	Surface tension		**Aver**	**SD**
Reused 3	39.883	19.717	20.55	0.79
	38.311	21.289		
	38.952	20.648		
	Surface tension		**Aver**	**SD**
Reused 4	41.456	18.144	18.25	0.68
	40.618	18.982		
	41.973	17.627		

(b) surface tension of storage times

Storage time	**rep1**	**rep2**	**rep3**	**Aver**	**SD**
0	35.574	35.892	35.843	35.76967	0.171214
7	37.467			37.467	0
14		37.919		37.919	0
28	35.721			35.721	0

Table B- 6 Emulsification test of preliminary study, diesel, slideway oil, SDS, and water

Type of oil	%E24
Fuel oil	45.45
Waste Lubricant oil	10
Crude oil	36.36
Diesel oil	49.21
Slideway oil	53.18

Biosurfactant	Conc.	rep	Emulsion	All	%E24	Aver	SD
Slideway oil		1	1.1	2	55.00	53.18	2.76
	100%	2	1.2	2.2	54.55		
		3	1	2	50.00		
		1	1.3	2.4	54.17	53.50	7.73
	25%	2	1	2.2	45.45		
		3	1.4	2.3	60.87		
Diesel		1	1.1	2.2	50.00	49.21	3.43
	100%	2	1.2	2.3	52.17		
		3	1	2.2	45.45		
		1	0.2	2.2	9.09	10.10	2.09
	25%	2	0.2	2.3	8.70		
		3	0.3	2.4	12.50		

SDS	Conc.	rep	Emulsion	All	%E24	Aver	SD
Slideway oil		1	1	2	50.00	52.83	7.06
	100%	2	1	2.1	47.62		
		3	1.4	2.3	60.87		
Diesel		1	0.1	2.3	4.35	4.23	0.20
	100%	2	0.1	2.3	4.35		
		3	0.1	2.5	4.00		

Water	Conc.	rep	Emulsion	All	%E24	Aver	SD
Slideway oil		1	0	2.2	0	0.00	0.00
	100%						
Diesel		1	0.1	2.3	4.35	4.35	0.00
	100%						

Table B- 7 Dispersant efficacy (%)

	Whole fl + BFT			
		%BFT	**Aver**	**SD**
SDS1	19.80	47.11	**46.64**	**0.67**
SDS2	18.24	46.16		
BSF1	20.30	31.90	**41.00**	**12.86**
BSF2	17.74	50.09		

Table B- 8 Solubilization efficacy (%)

Replication	% Solubilization	Aver	SD	**AVER**	**SD**
SDS11	12.87	17.08	5.95	**19.42**	**3.31**
SDS12	21.29				
SDS21	17.48	21.76	6.06		
SDS22	26.05				
BSF(10)11	8.03			**21.91**	**2.91**
BSF(10)12	7.55				
BSF(10)21	23.96	21.91	2.91		
BSF(10)22	19.85				
BSF(25)11	34.18	41.48	10.32	**35.05**	**9.09**
BSF(25)12	48.77				
BSF(25)21	15.08	28.62			
BSF(25)22	28.62				
BSF(100)11	75.28	76.37	1.55	**66.19**	**14.40**
BSF(100)12	77.47				
BSF(100)21	53.19	56.01	3.99		
BSF(100)22					
BSF(100)23	58.84				

Table B- 9 Percentage of fuel oil removal (a) – (b)

(a) Microcosms: synthetic water

Day	% removal				Control	
	JC11 degradability	SD	Bioaugmentation	SD	Control	SD
0	20.66	0	31.13	2.63	0	0
2	39.18	0	63.43	5.04	0	0
4	56.87	9.24	62.23	3.52	6.68	0
6	69.88	1.08	80.36	5.64	18.93	0
8	53.65	7.66	69.73	5.48	12.74	4.07
10	66.92	10.31	72.48	5.12	1.45	0

(b) Microcosms: seawater samples

	Petrolume hydrocarbon removal (%)			
	Control	SD	Bioaugmentation	SD
Bangsan	28.07	4.47	60.47	1.86
Maptaphut	34.95	1.79	75.70	4.53
Samed	26.55	10.59	60.41	2.27

Table B- 10 Cell number of *Gordonia* sp. JC11 in microcosms (a) – (b)

(a) Microcosms: synthetic water

Day	**JC-11-PUF (LogCFU/g)**	**SD**	**JC-11-PUF + BSF (LogCFU/g)**	**SD**
0	8.28	0.11	8.28	0.11
2	-	-	-	-
4	-	-	-	-
6	8.06	0.04	8.05	0.16
8	8.1	0.14	8.28	0.15
10	8.26	0.1	7.89	0.07

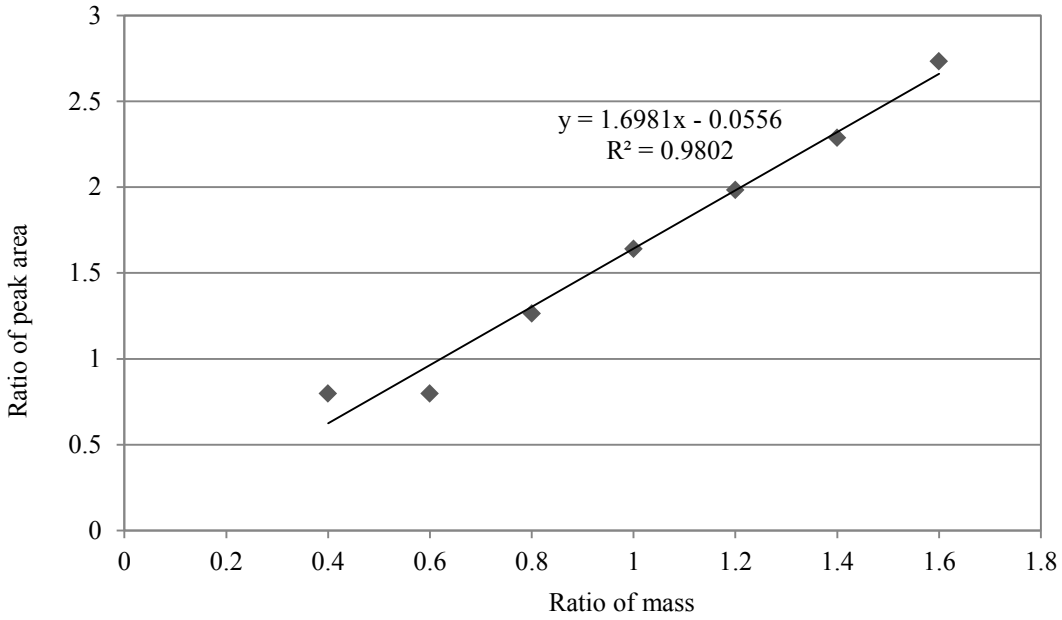
(b) Microcosms: seawater samples

samples	(logCFU/ gPUF)	SD	logCFU/ml	SD
Bangsan	8.42	0.58	7.86	0.30
Maptaphut	8.70	0.19	7.57	0.12
Samed	7.94	0.95	7.74	0.11

APPENDIX C

Standard curve

Figure C- 1 Standard curve of fuel oil by using TLC-FID



BIOGRAPHY

Ms. Supattra Laorrattanasak was born on April 11, 1989 in Bangkok, Thailand. She attended Rittiyawannalai School, Bangkok since 2001-2007. After that, she graduated with a Bachelor of Science in Microbiology from Chulalongkorn University in 2011 and continues to study Master's degree in Environmental Management from International Postgraduate programs in Environmental Management, Chulalongkorn University, since 2011-2013.

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