EFFICIENCY OF BIOSURFACTANT FROM *Bacillus* sp. GY19 ON ENHANCEMENT OF PYRENE SOLUBILIZATION AND BIODEGRADATION

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ประสิทธิภาพสารลดแรงตึงผิวชีวภาพจาก *Bacillus* sp. GY19 ในการเพิ่มความสามารถการละลายและการย่อยสลายไพรีน

นางสาววริษฐา ดุละลัมพะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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Thesis Title	EFFICIENCY OF BIOSURFACTANT FROM *Bacillus* sp. GY19 ON ENHANCEMENT OF PYRENE SOLUBILIZATION AND BIODEGRADATION
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WARITHA TULALAMBA: (EFFICIENCY OF BIOSURFACTANT FROM *Bacillus* sp. GY19 ON ENHANCEMENT OF PYRENE SOLUBILIZATION AND BIODEGRADATION). ADVISOR: ASST. PROF ONRUTHAI PINYAKONG, Ph.D., 86 pp.

The purpose of this study was to investigate efficiency of *Bacillus* sp. GY19 and its biosurfactant on enhancing of pyrene bioavailability and biodegradation. Firstly, six biosurfactant-producing bacterial screened strains were examined for efficiency of biosurfactant production by using several substrates as carbon source. *Bacillus* sp. GY19 was selected based on five criteria including; surface tension reduction of culture media (29 mN/m), emulsification activity with crude oil and diesel oil (56% and 64%, respectively), high biomass (1.8 g/l), capability to use bottom glycerol as carbon source and the highest crude oil solubilization property of cultivated broth by using bottom glycerol as carbon source (2,062 mg/l). Further study showed that cell-free broth containing biosurfactant of GY19 exhibited the property to solubilize naphthalene, acenaphthene, phenanthrene, fluoranthene and pyrene at 247, 41, 40, 12.6 and 3.6 mg/l, respectively. However, strain GY19 exhibited low ability to degrade 100 mg/l of phenanthrene, fluoranthene, pyrene and 0.5% (v/v) of crude oil and could not grow and produce biosurfactant in liquid media. Moreover, the GY19 cells also exhibited low adherence (3.5%) to hexadecane as measured by the BATH assay. Crude biosurfactant extracted by liquid-liquid extraction was therefore used in the studying on enhancement of pyrene biodegradation. The increasing concentration of crude biosurfactant resulted in high solubilization capability of PAHs in which 5 g/l of crude biosurfactant exhibited the highest solubilization of phenanthrene, fluoranthene and pyrene with 174, 449 and 522 mg/l, respectively. However, concentration at 5 g/l of crude biosurfactant exhibited toxicity to *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY which were used as pyrene-degrading bacteria in this study. Hence, concentration at 1 g/l (above CMC) of crude biosurfactant was applied for enhancing pyrene degradation by strain RN402 and strain PCY in liquid medium. In enhancing pyrene biodegradation by crude biosurfactant experiment, the toxicity of crude biosurfactant mainly inhibited pyrene degradation for strain RN402 and strain PCY in form of free cells. On the other hand, immobilization of strain RN402 exhibited positive result at 500 mg/l of pyrene degradation while crude biosurfactant still exhibited toxicity to immobilized strain RN402. In conclusion, biosurfactant from *Bacillus* sp. strain GY19 using bottom glycerol as carbon source also exhibited high solubilization of PAHs and crude oil. However, it should be studied on other concentrations of crude biosurfactant on enhancing PAHs bioavailability and biodegradation. Furthermore, solubilization process can be further studied for applying in industrial as a detergent for cleaning oil or combining solubilization-biodegradation process to eliminate PAHs and crude oil.

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

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

วริษฐา ดุละลัมพะ : ประสิทธิภาพสารลดแรงตึงผิวชีวภาพจาก *Bacillus* sp. GY19 ในการเพิ่มความสามารถการละลายและการย่อยสลายไพรีน (Efficiency of biosurfactant from *Bacillus* sp. GY19 on enhancement of pyrene solubilization and biodegradation) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. อรฤทัย ภิญญาคง, 86 หน้า.

วิทยานิพนธ์นี้มีจุดประสงค์เพื่อศึกษาประสิทธิภาพของ *Bacillus* sp. GY19 และสารลดแรงตึงผิวชีวภาพของมันในการเพิ่มความสามารถการถูกนำไปใช้และการย่อยสลายไพรีน ซึ่งแบคทีเรียที่มีความสามารถในการผลิตสารลดแรงตึงผิวชีวภาพ 6 สายพันธุ์ที่ถูกคัดกรองแล้วนั้นถูกนำมาศึกษาประสิทธิภาพในการผลิตสารลดแรงตึงผิวชีวภาพโดยแปรผันชนิดของแหล่งคาร์บอน จากการใช้ 5 หลักเกณฑ์ ดังนี้ การลดค่าแรงตึงผิวของอาหารเลี้ยงเชื้อ (29 มิลลินิวตันต่อเมตร) สมบัติการเกิดอีมัลชั่นกับน้ำมันดิบและน้ำมันดีเซล (56% และ 64%, ตามลำดับ) ให้น้ำหนักเซลล์แห้งสูง (1.8 กรัมต่อลิตร) ความสามารถในการใช้ bottom glycerol เป็นแหล่งคาร์บอนและความสามารถของน้ำเลี้ยงเชื้อที่มีสารลดแรงตึงผิวชีวภาพในการละลายน้ำมันดิบ (2,062 มิลลิกรัมต่อลิตร) พบว่า *Bacillus* sp. GY19 ถูกคัดเลือกเพื่อนำมาศึกษาในงานวิจัยนี้ต่อไป ในการศึกษาเพิ่มเติมพบว่าน้ำเลี้ยงเชื้อที่มีสารลดแรงตึงผิวชีวภาพของแบคทีเรียสายพันธุ์ GY19 แสดงคุณสมบัติในการละลายแนพธาลีน อะซีแนพธีน ฟีแนนทรีน ฟลูออรีนธีนและไพรีน อย่างไรก็ตาม *Bacillus* sp. GY19 มีความความสามารถในการย่อยสลายฟีแนนทรีน ฟลูออแรนธีน ไพรีนที่ความเข้มข้น 100 มิลลิกรัมต่อลิตร และน้ำมันดิบที่ความเข้มข้น 0.5% (v/v) ได้ต่ำและไม่สามารถเจริญและสร้างสารลดแรงตึงผิวชีวภาพได้ นอกจากนี้มีความสามารถในการเกิดไฮโดรโฟบิกกับเฮกซะเดกเคนต่ำ (3.5%) การศึกษาประสิทธิภาพของสารลดแรงตึงผิวชีวภาพในการช่วยเพิ่มความสามรถการย่อยสลายไพรีนได้เลือกใช้สารลดแรงตึงผิวชีวภาพผลิตจาก *Bacillus* sp. GY19 โดยใช้ในรูปสารสกัดหยาบซึ่งทำการสกัดโดยใช้ตัวทำละลาย ซึ่งการเพิ่มความเข้มข้นของสารสกัดหยาบช่วยเพิ่มความสามารถการละลายของของ ฟีแนนทรีน ฟลูออแรนธีนและไพรีนและละลายได้มากที่สุดถึง 174, 449 และ 522 มิลลิกรัมต่อลิตรตามลำดับ ที่ความเข้มข้นของสารสกัดหยาบ 5 กรัมต่อลิตรในทางกลับกันความเข้มข้นนี้กลับมีความเป็นพิษต่อ *Pseudoxanthomonas* sp. RN402 และ *Novosphingobium* sp. PCY แบคทีเรียย่อยสลายไพรีนที่ใช้ในงานวิจัยนี้ ดังนั้นจึงเลือกใช้ความเข้มข้นสารสกัดหยาบที่ 1 กรัมต่อลิตร เพื่อศึกษาความสามารถการเพิ่มการย่อยสลายไพรีนในอาหารเลี้ยงเชื้อเหลวในรูปของเซลล์อิสระของแบคทีเรียสายพันธุ์ RN402 และสายพันธุ์ PCY สารสกัดหยาบและไพรีนที่ถูกละลายมีผลเป็นพิษและยับยั้งความสามารถการย่อยสลายไพรีน อย่างไรก็ตามในรูปเซลล์ตรึงของแบคทีเรียสายพันธุ์ RN402 สามารถย่อยสลายไพรีนได้ที่ความเข้มข้น 500 มิลลิกรัมต่อลิตร ในขณะที่สารสกัดหยาบและไพรีนที่ถูกละลายยังคงเป็นพิษต่อเซลล์ตรึงของแบคทีเรียสายพันธุ์ RN402 งานวิจัยนี้แสดงให้เห็นว่าสารลดแรงตึงผิวชีวภาพจาก *Bacillus* sp. strain GY19 โดยใช้ bottom glycerol เป็นแหล่งคาร์บอนนั้นสามารถละลายพอลิไซคลิกอะโรมาติไฮโดรคาร์บอนและน้ำมันดิบได้ดี อย่างไรก็ตามการศึกษาความสามารถการเพิ่มการย่อยสลายพอลิไซคลิกอะโรมาติไฮโดรคาร์บอนควรศึกษาการใช้ความเข้มข้นของสารสกัดหยาบที่ความเข้มข้นอื่น นอกจากนี้สามารถศึกษาเพิ่มเติมถึงกระบวนการการละลายเพื่อประยุกต์ใช้ในการชะน้ำมันในอุตสาหกรรมหรือในกระบวนการละลายและการย่อยสลายเพื่อใช้ในการกำจัด พอลิไซคลิกอะโรมาติไฮโดรคาร์บอนและน้ำมันดิบได้ต่อไป

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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CHAPTER I

INTRODUCTION

1.1 Statement of problem

Crude oil or petroleum oil is found in deep beneath the earth's surface which can be stored in barrels for future refinement. Generally, crude oil is made up of hydrocarbon compounds and the main hydrocarbons including Aliphatic, Alicyclic and Polycyclic Aromatic Hydrocarbons (PAHs). PAHs are the most toxic hydrocarbons in crude oil. The properties of PAHs are being toxic and carcinogenic to living organisms. In addition, 6-side carbon rings of PAHs are strong bonds and ability to prolong breakdown by natural processes. Generally, a sequence of petroleum components in decreasing order of biodegradability is presented as following: *n*-alkanes > branched-chain alkanes > branched alkanes > low-molecular-weight *n*-alkyl aromatic > monoaromatics > cyclic alkanes > polynuclear aromatics > asphaltenes (Huesemann, 1995). Due to their properties, PAHs are mostly insoluble in water, high hydrophobicity and have complex structures. Hence, PAHs can be accumulated in the environment such as water, soil and sediment, etc. and have limited availability to biodegradation.

PAHs can be removed by many mechanisms such as volatilization, photolysis and biodegradation. Biodegradation is a major PAHs removing process. Biodegradation is a mechanism which is responsible for ecology recovery for removing PAHs from the environment. PAH degradation efficiency is based on environmental conditions, number and type of microorganisms, nature and the chemical structure of PAHs (Haritash and Kaushik, 2009). Lu et al. (2011) reviewed the outline of the current knowledge on biodegradation and found that one important factor affecting biodegradation of PAHs is the presence of biosurfactant. Biosurfactants have been suggested as a promising technique for enhancing remediation of PAHs.

Biosurfactants are amphiphilic compounds which can be produced by various microorganisms. For bioremediation approach, biosurfactants can reduce surface tension and interfacial tension by accumulating at the interface of immiscible fluids and increase the solubility and mobility of PAHs (Ron and Rosenberge, 2002; Milligan, 2005). Biosurfactants are soluble in water at low concentration and with increased concentration, they can form micelle. The concentration which biosurfactants begin to form micelle is called critical micelle concentration (CMC). At concentration beyond CMC, it is shown that biosurfactants can solubilize petroleum hydrocarbons (Bordoloi and Konwar, 2009). Furthermore, many reports revealed that biosurfactants have a potential to enhance solubility of petroleum hydrocarbons and lead to the increase of bioavailability and biodegradation of PAHs (Bordoloi and Konwar, 2009; Gottfried et al., 2010). Yu et al. (2011) showed that solubilization of phenanthrene and pyrene was increased with the increasing concentration of rhamnolipid biosurfactant.

In this study, biosurfactant was introduced for enhancing bioavailability and biodegradation of pyrene in liquid medium. Six biosurfactant-producing bacteria were previously isolated by using particular substrates. *Cellulosimicrobium* sp. GY33, *Achromobacter* sp. GY30 and *Bacillus* sp. GY19 were isolated from soil by using glycerol as a carbon source. *Alcaligenes* sp. LS was isolated from greased trap water by using soybean oil as a carbon source. *Enterobacter* sp. W3-02 and *Rhodococcus* sp. SSP2 were isolated from ballast water and sludge by using lubricating oil as a carbon source, respectively. For this study, one potential strain and substrate was selected using two criteria: i) efficiency of biosurfactant production by using five substrates as carbon sources and ii) efficiency of its cell-free broth containing biosurfactant to solubilize of crude oil. In this study, *Bacillus* sp. GY19 was selected as a potential strain and used bottom glycerol as its carbon source as potential substrate. Then, *Bacillus* sp. GY19 and its biosurfactant were studied about the properties. For studying biosurfactant efficiency on enhancement of pyrene degradation in liquid medium, biosurfactant from *Bacillus* sp. GY19 was used in form of crude extracted biosurfactant for controlling the concentration to use. Hence, crude extracted biosurfactant was investigated in PAHs solubilization and toxicity on the survival of

Pseudoxanthomonas sp. RN402 and *Novosphingobium* sp. PCY which were used as pyrene-degrading bacteria in this study. Then, crude biosurfactant was investigated for the efficiency on enhancing pyrene bioavailability and biodegradation in liquid medium.

1.2 Objectives

The main objectives of this study are selecting the efficient biosurfactant-producing bacteria and investigate the efficiency of its biosurfactant on enhancing PAH biodegradation in liquid medium. Specific objectives are:

1.2.1 To select the efficient biosurfactant-producing bacteria.

1.2.2 To examine the properties of biosurfactant-producing bacteria and its biosurfactant.

1.2.3 To investigate the efficiency of biosurfactant on enhancing PAH biodegradation in liquid medium.

1.3 Hypothesis

Biosurfactant-producing bacteria as well as its biosurfactant can enhance biodegradation of PAH in liquid medium.

1.4 Scope of the study

This study focused on 3 parts:

1.4.1 Selection of biosurfactant-producing bacteria

The six screened biosurfactant-producing bacterial strains were selected for one potential strain and substrate by investigating in two experiments as following:

1.4.1.1 The efficiency of biosurfactant production by using five substrates including: bottom glycerol, crude oil, diesel oil, lubricating oil and slop oil.

1.4.1.2 Crude oil solubilization by cell-free broth containing biosurfactant

Criteria for selecting: - Using bottom glycerol as carbon source
- Surface tension reduction ($\leq$ 35 mN/m)
- Emulsification activity (E24) with crude oil, diesel oil or lubricating oil ($\geq$ 50%)

- High biomass ($\geq$ 1 g/l)
- High solubilization of crude oil

By using these criteria, one potential biosurfactant-producing bacteria and substrate were selected.

1.4.2 Study of properties of selected strain and its cell-free broth containing biosurfactant

The potential selected strain and its cell-free broth containing biosurfactant from culture using potential substrate as carbon source were studied for their properties as following:

1.4.2.1 PAHs solubilization assay by using cell-free broth containing biosurfactant

1.4.2.2 Bacterial adherence to hydrocarbons (BATH) assay

1.4.2.3 PAHs and crude oil biodegradation assay

1.4.3 Study of activity of crude biosurfactant for enhancing pyrene biodegradation in liquid medium

In this study, biosurfactant from selected strain by using potential substrate as carbon source was extracted for investigating in four experiments as following:

1.4.3.1 PAHs solubilization assay by using crude biosurfactant

1.4.3.2 Toxicity assay

1.4.3.3 Activity of crude biosurfactant on enhancing pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY in form of free cell in liquid medium

1.4.3.4 Activity of crude biosurfactant on enhancing pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 in form of immobilized cell in liquid medium

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Polycyclic aromatic hydrocarbons (PAHs)

2.1.1 Crude oil and PAHs

Crude oil or petroleum oil is found in deep beneath the earth's surface which can be stored in barrels for future refinement. Generally, crude oil is made up of hydrocarbon compounds. The major hydrocarbons found in crude oil are Aliphatic, Alicyclic and Polycyclic aromatic hydrocarbons (PAHs). PAHs are the most toxic hydrocarbons found in crude oil. The structures of PAHs are consisted of two benzene rings or more in liner, angular or cluster structural arrangement (Bamforth and Singleton, 2005). They are produced from two major sources including natural sources (such as forest, rangeland fires, oil seeps, volcanic eruptions and exudates from trees) and anthropogenic sources (such as burning of fossil fuel, coal tar, wood garbage refuse, used lubricating oil and petroleum discharge) (Haritash and Kaushik, 2009). PAHs are ubiquitous in natural environment and also are contributors of several environment contaminations including air (Bamforth and Singleton, 2005), water (Miguel et al., 2008), soil (Li et al., 2007), mangrove sediment (Ke et al., 2002), etc.

2.1.2 Properties of PAHs

Generally, a sequence of petroleum components in decreasing order of biodegradability is presented as following (Huesemann, 1995): *n*-alkanes > branched-chain alkanes > branched alkanes > low-molecular-weight *n*-alkyl aromatic > monoaromatics > cyclic alkanes > polynuclear aromatics > asphaltenes. Physical and chemical properties of PAHs vary with the number of rings and their molecular

weight. In chemical properties, PAHs have high molecular weight, low water solubility, high the octanol/water partition coefficient indicates high hydrophobicity and have complex chemical structures that tend to make them poorly biodegradable (Table 2.1). According to the U. S. Environmental Protection Agency and other health and environmental agencies' classification, several PAHs are known as human carcinogen. Furthermore, PAHs are determined in biological effect as toxic, mutagenic substances and have potential to get into human food chain (Mrozik et al., 2003). According to Yuste et al. (2000) studied on characterization of bacterial strains that could grow on high molecular mass residues of crude oil. Oil residues are complex mixture of high molecular mass compounds including saturated, aromatic and PAHs. In this study, they found that the isolated strains can degrade long-chain-length alkanes efficiently, but not PAHs. Moreover, PAHs can be absorbed into the soil components in difficulty to be removed, degraded from the contaminated site and have limited bioavailability for biodegradation (Calvo et al., 2008).

Table 2.1: The formula and physicochemical properties of some studied PAHs

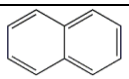
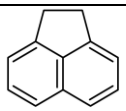
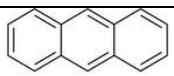
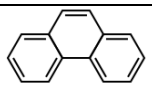
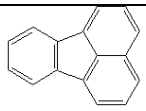
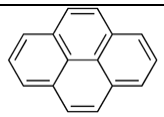
Compound	Molecular Structure	Molecular weight (g/mol)	Water solubility at 25 °C (mg/L)	Log K_{ow}	Reference
Naphthalene		128.2	34.4	3.36	Verchueren, 1996
Acenaphthene		154.21	1.93	3.98	ATSDR, 1995
Anthracene		178.2	0.076	4.45	ATSDR, 1995
Phenanthrene		178.2	1.2	4.45	ATSDR, 1995

Table 2.1: The formula and physicochemical properties of some studied PAHs (cont.)

Compound	Molecular Structure	Molecular weight (g/mol)	Water solubility at 25 °C (mg/L)	Log K_{ow}	Reference
Fluoranthene		202.26	0.20-0.26	4.90	ATSDR, 1995
Pyrene		202.3	0.077	4.88	ATSDR, 1995

2.1.3 PAH-degrading bacteria

Naturally, there are relationships among microorganisms, contaminants and environments. Bioremediation is a mechanism to treat contaminants in environment by using of microorganisms to detoxify and degrade hazardous chemicals in environment such as soil, water and mangrove sediment. Bioremediation has many advantages such as cheap, simple, complete contaminants transformation and can be used *in situ* or *ex situ* treatment. There are many technologies of bioremediation such as phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, rhizofiltration, bioaugmentation and biostimulation (Vidali, 2001). Nowadays, PAH-degrading bacteria have been isolated from various PAHs and petroleum contaminated-environments for degrading PAHs. According to PAHs-degrading bacterial listed in Table 2.2, there are various PAHs-degrading bacteria that can enhance bioremediation of PAHs.

Table 2.2: PAHs-degrading bacteria

Microorganism	**PAHs degradation capability**	**Reference**
Rhodococcus sp., *Acinetobacter* sp., *Pseudomonas* sp.	fluorene, phenanthrene	Yu et al. (2004)
Mycobacterium sp., *Sphingomonas* sp., *Terrabacter* sp., *Rhodococcus* sp.	three- and four- rings	Zhou et al. (2007)
Pseudomonas stutzeri ZP2	phenanthrene	Zhao et al. (2008)
Pseudoxanthomonas sp. RN402, *Diaphorobacter* sp. KOTLB	phenanthrene, fluoranthene, pyrene	Klankeo et al. (2009)
Sphingomonas spp., *Novosphingobium* spp., *Mycobacterium* spp., *Pseudomonas* sp., *Rhodococcus* spp., *Paracoccus* sp.	phenanthrene, fluoranthrene, pyrene	Guo et al. (2010)

Klankeo et al. (2009) reported the isolation of two new PAHs-degrading bacteria including *Pseudoxanthomonas* sp. RN402 and *Diaphorobacter* sp. KOTLB from soil. *Pseudoxanthomonas* sp. RN402 has ability to utilize pyrene as a carbon source which can degrade 99% of 100 mg/l in 16 days. Furthermore, strains RN402 can degrade 100 mg/l of phenanthrene almost completely in 8 days and degrade 55% of 100 mg/l of fluoranthene in 24 days. This study also indicated the presence of *nidA* gene which is a large subunit of the terminal dioxygenase for pyrene degradation in strain RN402. In 2011, Nopcharoenkul et al. examined the application of ready-to-use bacteria in form of liquid formulation of *Pseudoxanthomonas* sp. RN402 for pyrene-contaminated soil remediation. The result showed that bioaugmentation of RN402 could completely degrade pyrene of 300 mg/kg in soil microcosms within 4 weeks.

Furthermore, Wanwasan Wongwongsee (2011) also reported a new strain of PAHs-degrading bacteria which is *Novosphingobium* sp. PCY. The strain PCY has ability to completely degrade 100 mg/l of phenanthrene and pyrene within 18 and 21 days, respectively. Moreover, PCR amplification with the *nidA*- specific primers revealed the presence of a pyrene dioxygenase gene in strain PCY. This result indicated that the amino acid sequence of PCR product showed 91% identity to the NidA, an alpha subunit of ring-hydroxylating dioxygenase, of PAH-degrading *Mycobacterium* sp. py136.

2.1.4 PAHs biodegradation

Bacterial degradation of PAHs under aerobic condition begins with the addition of the both atoms of oxygen molecule to the aromatic ring by dioxygenase system to produce *cis*-dihydrodiol. Then, these intermediates are cleaved by *ortho*- or *meta*- cleavage pathways and transformed to tricarboxylic acid (TCA) cycle (Kelly et al., 1991). Aromatic ring dioxygenases which are important enzyme for degrading PAHs are multicomponent enzyme consisting of an electron-transport chain (ferredoxin, ferredoxin reductase) and terminal dioxygenase. Terminal dioxygenase composes of two subunits which are alpha and beta subunit. The alpha subunit is catalytic component and contains of two conserved regions; the [Fe_2-S_2] Rieske center and the mononuclear iron-containing catalytic domain (Ferrero et al., 2002; Parales et al., 2000) and PAHs degradation pathway as shown in Fig 2.1.

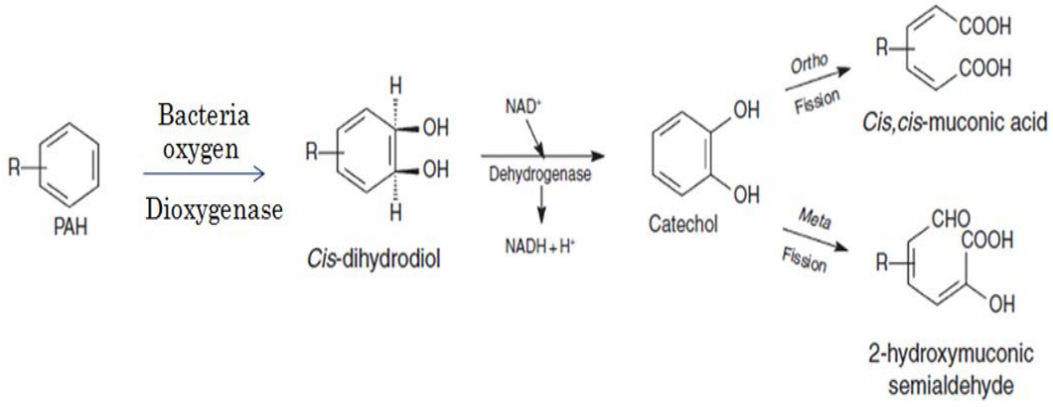


Fig 2.1: PAHs degradation pathway by PAH-degrading bacteria

Pyrene degradation was focused in this study. Pyrene is fused of four bezene rings and it is classified as high molecular weight PAH. Thus, pyrene is hard degraded and accumulated in the environment long terms. However, there are many reports that could isolate pyrene-degrading bacteria and could use it for pyrene biodegradation. Example which listed in Table 2.3 is shown PAHs-degrading bactetria for degrading pyrene and its efficiency of pyrene degradation.

Table 2.3: Pyrene-degrading bactetria and its efficiency of pyrene degradation

Microorganism	Biodegradation efficiency			Reference
	Initial pyrene concentration (mg/l)	Pyrene degradation (%)	Pyrene degradation time (day)	
Mycobacterium sp. 6PY1	200	100	32	Cottin and Merlin (2007)
Novosphingobium sp. PCY	100	100	21	Promchat Chareanpat (2008)
Bacillus cereus Py5 *Bacillus megaterium* PY6	50	92.1	21	Lin and Cai (2008)
Enterobacter sp. 12J1	5	83.8	7	Sheng et al. (2008)

Table 2.3: Pyrene-degrading bactetria and its efficiency of pyrene degradation (cont)

Microorganism	Biodegradation efficiency			Reference
	Initial pyrene concentration (mg/l)	Pyrene degradation (%)	Pyrene degradation time (day)	
Pseudoxanthomonas sp. RN402 *Diaphorobacter* sp. KOTLB	100 100	99 99	16 16	Klankeo et al. (2009)
Sphingomonas, *Mycobacterium*, *Rhodococcuc*, *Paracoccus*, *Pseudomonas*	10	100	14	Guo et al. (2010)
Mycobacterium sp. A1-PYR, *Sphingomonas* sp. PheB4	5,000	50	7	Zhong et al. (2010)
Bacillus vallismortis JY3A	150	90.5	15	Ling et al. (2011)

2.1.5 Immobilized cells for PAHs biodegradation

The limitation of free cell for degrading pollutants is bacterial cells may contact with pollutants directly and suffered from toxic pollutants (Suttinum, 2008). Immobilization of bacterial strain is one technique to solve this problem. Immobilization is one of optional to immobilization of cells to the bedding material. The advantages of immobilized bacterial strain over the conventional free cells are

higher tolerance to high concentration of toxic chemicals and higher cell density resulting in higher rates of biodegradation. There are several efficient methods to immobilize cells as following (Cohen, 2001):

1) Attachment or adsorption on solid carrier surfaces

Cells immobilized on a solid carrier by using electrostatic forces or covalent bonding between the cell membrane and the carrier (Fig 2.2).

2) Entrapment on encapsulation within a porous matrix

Microorganisms are trapped within polymer matrix. The porosity of polymer matrix could prevent leakage of cells (Fig 2.2).

3) Membrane separation

Usually, membrane is ultrafiltration membranes. This method, porous membrane could separate the cells from the toxic liquid outside as barrier. However, the membranes could let substrate to penetrate to the cells.

4) Covalent bonding and covalent crosslinking

The cell's surface and different ligands on the bedding material are combined by using covalent bonds.

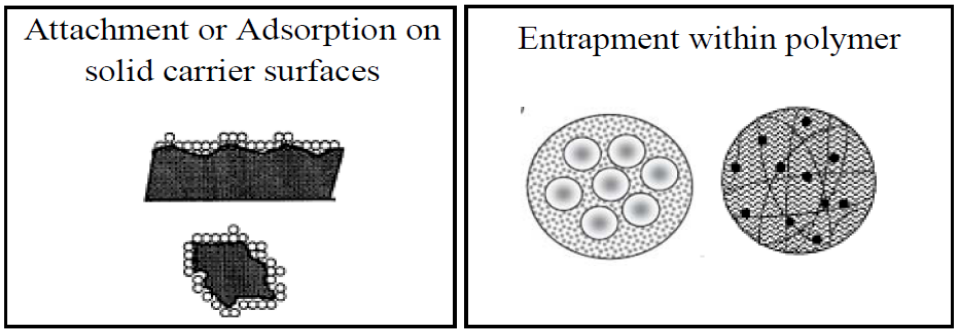


Fig 2.2: Basic methods for cell immobilization (Pilkington et al., 1998)

Many reports showed that immobilization of cells exhibited efficiency to degrade PAHs at high concentration. For example, Tao et al. (2009) studied efficiency of phenanthrene degradation by *Sphingomonas* sp. GY2B cell immobilized in calcium alginate gel beads in mineral salt medium (MSM) and artificial seawater (AS). They found that immobilized strain GY2B exhibited high efficiency of 100 mg/l of

phenanthrene degradation with 98.8% in both of MSM and AS which was higher than those of free cells.

2.2 Biosurfactant

2.2.1 Introduction

Biosurfactants or microbial surfactants are surface active compounds that are produced on living cells surfaces or extracted extracellular, mostly produced by various microorganisms such as bacteria, yeasts and fungi. Biosurfactants are amphiphilic compounds containing hydrophobic and hydrophilic moieties (Fig 2.3).

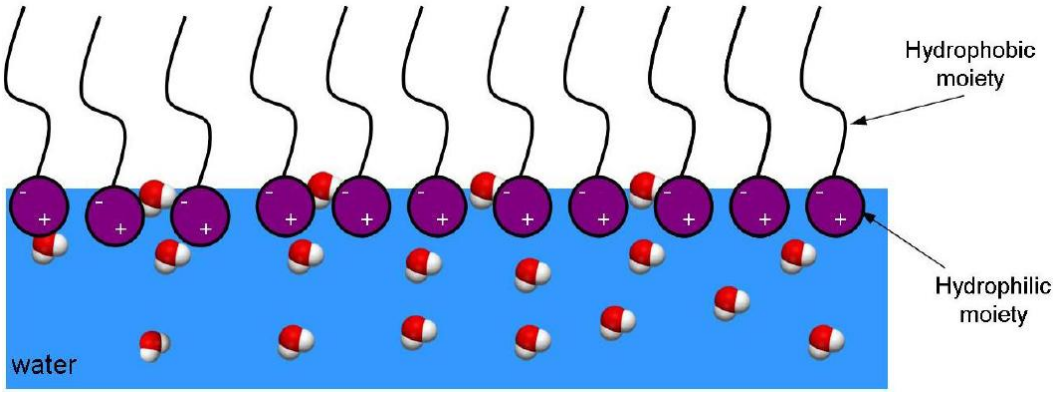


Fig 2.3: Basic structure of biosurfactant (Pacwa-Plociniczak et al., 2011)

There are many structures of biosurfactant based on their compositions, molecular weight, physic chemical properties and microbial origin (Muthusamy et al., 2008). Microorganisms are able to make two different types of biosurfactant based on their molecular weight. The first type is low molecular weight biosurfactants which are efficiently lower surface tension and interfacial tension such as rhamnolipid, trehalolipid and sorpholipid. Another type is high molecular weight biosurfactants which is less effective in reducing interfacial tension such as lipopeptide, lipoprotein and mixture of biopolymer (Calvo et al., 2008). Generally, biosurfactants are classified in five major groups including glycolipids, lipopeptides (lipoprotein), fatty acids, polymeric biosurfactants and particulate biosurfactants (Table 2.4).

Table 2.4: Major biosurfactant classes and microorganisms involved

Biosurfactant		Microorganism	Reference
Group	Class		
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa* MTCC7812, MTCC7814, MTCC7815, MTCC8163, MTCC8165	Bordoloi and Konwar (2009)
		Pseudomonas aeruginosa J4	Whang et al. (2007)
	Trehalolipids	*Corynebactettrium kutscheri*	Thavisi. (2007)
	Sophorolipid	*Candida bombicola*	Daverey and Pakshirajan (2011)
Lipopetide	Surfactin	*Bacillus subtilis* ATCC 21332	Whang et al. (2007)
		Bacillus megaterium	Thavisi. (2008)
	Lichenysin	*Bacillus licheniformis* R2	Joshi et al. (2008)
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Gerson and Zajic (1978)
	Spiculisporic acid	*Penicillium spiculisporum*	Ishigami et al. (1983)
	Phosphatidylethan-olamine	*Rhodococcus erythropolis* MTCC 2794	Pal et al. (2009)

Table 2.4: Major biosurfactant classes and microorganisms involved (cont.)

Biosurfactant		Microorganism	Reference
Group	Class		
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus* RAG-1	Zosim et al. (1982)
	Alasan	*Acinetobacter radioresistens* KA-53	Toren et al. (2001)
	Biodispersan	*Acinetobacter calcoaceticus* A2	Rosenberg et al. (1988)
	Liposan	*Candida lipolytica* UCP 0988	Rufino et al. (2011)

2.2.2 Properties of biosurfactant

The advantages of the biosurfactant involved in bioremediation are increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic water-insoluble substrates (Ron and Roseberg, 2002).

2.2.2.1 Increasing the surface area of hydrophobic water-insoluble substrates

Surface tension is the force between liquid molecule and air, while interfacial tension is the force between two liquid molecules. Generally, the surface tension of water is 72 mN/m and the interfacial tension of water/hexadecane is 40 mN/m. A good biosurfactant can reduce the surface tension of water lower than 35 mN/m and interfacial tension to 1 mN/m (Muthusamy et al., 2008). Biosurfactant is soluble in water at low concentration and when concentration is increased, they can form

micelle. The concentration which biosurfactant begins to form micelle is called critical micelle concentration (Bordoloi and Konwar, 2009).

2.2.2.2 Increasing the bioavailability of hydrophobic water-insoluble substrates

The limit of solubility of water-insoluble substrates in water is the most important factor for using these substrates as carbon and energy source of microorganisms. Hydrocarbons such as PAHs are low water solubility. Therefore, hydrocarbons are low availability to microorganisms. Since biosurfactant are amphiphilic compounds which can enhance the bioavailability of hydrophobic compounds by increasing bioavailability of these substrates and decreasing hydrophobicity of the surface, these hydrophobic substrates are able to interact more easily with the bacterial cells in the presence of biosurfactant (Pacwa-Plociniczak et al., 2011). For example, the uptake of PAHs and crude oil by five biosurfactant-producing bacterial strains belonging to *Pseudomonas aeruginosa*: MTCC7812, MTCC7814, MTCC7815, MTCC8163 and MTCC8165 were significantly increased on the addition of biosurfactant. This indicated that biosurfactant could enhance bioavailability of PAHs and crude oil (Bordoloi and Konwar, 2009).

2.2.3 Biosurfactant production by bacteria

In 2010, Slydatk and Hausmann gave the reason on the limit of biosurfactants production in industrial which including the use of expensive substrates, limited product concentration, low yields and formation of products are mixture rather than pure compounds. Nowadays, biosurfactants have not yet been commercialized production due to low production yields and high purification cost. The use of the alternative substrates such as industrial wastes is an attractive strategy for economical biosurfactant production. Bottom glycerol or waste glycerol is a by-product of biodiesel production. It is produced at 10% (v/v) of the total biodiesel production by transesterification of fatty acid derived from vegetable oils and animal fats (Willke

and Vorlop, 2004). Bottom glycerol is isolated from biodiesel production which is contaminated by other chemicals such as methanol, salt, water, fatty acid and soap. Faria et al. (2011) described the production of surfactin biosurfactant by the *Bacillus subtilis* LSFM-05 using waste glycerol as the sole source of carbon. The yield of biosurfactant reached a maximum value of 1.38 g/l at 60 h with surface tension of 31.2 mN/m. Furthermore, biosurfactant-producing bacteria can utilize waste oils such as vegetable oil and waste lubricating oil which are low-cost carbon sources for production of biosurfactant as shown in Table 2.5.

Table 2.5: Substrates used as a carbon source for production of biosurfactant

Biosurfactant-producing bacteria	Type of biosurfactant	substrate	Yield of biosurfactant (g/l)	Reference
Pseudomonas aeruginosa LBI	rhamnolipid	Sunflower-oil refining waste (sunflower, soybean, olive oil, soapstock)	4.9, 4.8, 5.3 and 4.8	Benincasa and Accorsini (2007)
Pseudomonas aeruginosa S6	rhamnolipid	petroleum	0.18	Yin et al. (2009)
Lactobacillus delbrueckii	glycolipid	Peanut oil cake	5.35	Thavasi et al. (2010)
Pseudomonas fluorescens	rhamnolipid	Olive oil	2	Abouseoud et al. (2010)
Bacillus subtilis LSFM-05	surfactin	Raw glycerol	1.38	Faria et al. (2011)

2.2.4 Utilizing biosurfactants in many industrials

Since biosurfactants have owing their unique properties such as higher biodegradability, low toxicity, environmental friendly, non-hazardous and effective (Pacwa- Plociniczak et al., 2011); so, biosurfactants have gained importance in many industrial applications (Table 2.6).

Table 2.6: Industrial application of biosurfactant (Singh et al., 2007)

Industry	Application	Role of biosurfactant
Petroleum	• Enhance oil recovery (EOR) • De-emulsification	Improving oil drainage into well bore, stimulating release of oil entrapped by capillaries, wetting of solid surfaces, reduction of oil viscosity and oil pour point, lowering of interfacial tension, dissolved of oil De-emulsification of oil emulsions, oil solubilization, viscosity reduction, wetting agent
Environmental	• Bioremediation • soil and water, soil washing, soil flushing	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration Emulsification through adherence to hydrocarbons, dispersion, foaming agent, detergent, soil flushing
Food	• Emulsification and de-emulsification	Emulsifier, solubilizer, demulsifier, suspension, wetting, foaming, defoaming. Thickener, lubricating agent

Table 2.6: Industrial application of biosurfactant (Singh et al., 2007) (cont.)

Industry	Application	Role of biosurfactant
Biological	• Microbiological	Physiological behavior such as cell mobility, cell communication, nutrient accession, cell-cell competition, plant and animal pathogenesis
	• Pharmaceuticals and therapeutics	Antibacterial, antifungal, antiviral agents, adhesive agents, immunomodulatory molecules, vaccine, gene therapy
Agricultural	• Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypovirulence
Bioprosessing	• Downstream processing	Biocatalysis in aqueous two-phase systems and microemulsions, biotransformation, recovery of intracellular products, enhanced production of extracellular enzymes and fermentation products
Cosmetic	• Health and beauty products	Emulsifier, foaming agents, solubilizer, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action

2.2.5 Role of biosurfactants in biodegradation process

The role of biosurfactants in enhancement of hydrocarbon biodegradation involves in three mechanisms including mobilization, solubilization and emulsification (Fig 2.4). Each mechanism depends on the concentration and molecular-mass of biosurfactants. The mobilization mechanism occurs when concentration of biosurfactant is below critical micelle concentration (CMC) in which biosurfactant will reduce surface tension and interfacial tension, increase the contact angle and decrease the capillary force holding hydrocarbon and soil together. The solubilization mechanism occurs at concentration beyond the CMC, biosurfactant will form micelle, increase the solubility of hydrocarbon by hydrophobic ends of biosurfactant molecule connect together inside the micelle while the hydrophilic heads exposed to the aqueous phase on the exterior. The emulsification mechanism occurs by high-molecular-mass biosurfactant. In emulsion, there are very small droplets of hydrocarbons such as fat and oil (Pacwa-Plociniczak et al., 2011). Biosurfactants are often applied as an additive to stimulate bioremediation and removal of hydrocarbons from environments.

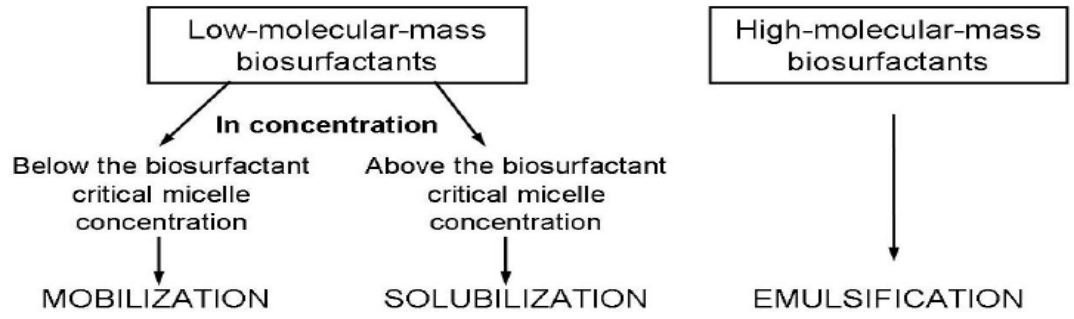


Fig 2.4: Mechanisms of hydrocarbons removal by biosurfactant depending on their concentration and molecular mass (Rosenberg and Ron, 1999; Uram and Pekdemir, 2004)

2.2.6 PAHs solubilization enhancement by biosurfactant

According to Rahman et al. (2002), low solubilization of hydrocarbons is the most problem for bioremediation of PAHs. Since PAHs are mostly insoluble in water and their bioavailability is limited. Moreover, PAHs can be adsorped into the soil

particle as a long-term source of contamination. The solubillization step is very important for removal of organic contaminants from the soil particles, especially, for low water solubility compounds like PAHs (Santanu, 2007). Biosurfactant are amphiphilic compounds produced by various microorganisms. Biosurfactant can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble compound (Ron and Rosenberg, 2002; Mulligan, 2005). Therefore, addition of biosurfactant is a feasible approach to enhance solubilization, bioavailability and biodegradation of PAHs. (Bordoloi and Konwar, 2009; Gottfried et al., 2010). Das et al. (2008) evaluated the efficiency of biosurfactant produced by a marine bacterial strain. In this study, marine biosurfactant-producing strain *Bacillus circulans* could not utilize anthracene as the sole source of carbon, but it could utilize anthracene in supplemented with glycerol as a carbon source. The growth and biosurfactant production in anthracene supplemented with glycerol-containing medium (AGlyMSM) has higher than those in a normal glycerol medium. The results of PAHs solubilization test showed the decrease of free anthracene concentration with an increasing concentration of biosurfactant. This indicated that biosurfactant can trap PAHs molecules in its micellar structure to increase its solubility and bioavailability. In 2011, Yu et al. investigated the solubilization of phenanthrene and pyrene by rhamnolipid biosurfactant. The results showed that both of PAHs were increased in solubilization with increasing of the concentration of biosurfactant. In the mixed systems, the solubility of phenanthrene was lower than in a single PAH system. Micellar concentration in the mixed system was higher than in those the single system. This could be the result from the micelle-PAHs interaction and competitive solubilization between phenanthrene and pyrene.

2.2.7 Biodegradation of biosurfactant

In addition, the properties of biosurfactant is not only emulsion forming, but also environment friendly, low toxicity, surface and interface activity, chemical diversity and effectiveness at an extreme temperature, pH and ionic strength (Pacwa-Plociniczak et al., 2011) Furthermore, biosurfactants are high biodegradability.

Microorganisms may use biosurfactants as carbon and energy source as compared to synthetic surfactants which are persistent in environment. According to Lima et al. (2010), their study evaluated biodegradability of different bacteria (three bacteria: *A. haemolyticus* LBBMA 53, *A. baumanni* LBBMA ES11 and *Pseudomonas* sp. LBBMA 101B) in liquid medium and soil microcosms on biodegradation of biosurfactant (six biosurfactants: surfactin, iturin, fengycin, glycolipid, arthrofactin and flavolipids) and synthetic surfactant (SDS).They found that mixed cultures could use the biosurfactants as substrates in soil microcosms, but there was no significant degradation of the synthetic surfactant SDS. Synthetic surfactant SDS exhibited stability in soil microcosms and low degradation that risk in accumulating in the environment. So, this study indicated that biosurfactants are more suitable for environment application than the synthetic surfactant SDS. For these reasons, biosurfactants have gained importance in the field of environmental bioremediation (Muthusamy et al., 2008).

2.2.8 Application of biosurfactant on enhancement of solubilization

Since the addition of biosurfactant is a feasible approach to enhance solubilization of hydrophobic compounds such as PAHs and oil. Many reports have shown concerning about utility of using biosurfactant in PAHs and oil solubilization. Shin et al. (2006) examined the combined solubilization-biodegradation process with biosurfactant to remediate phenanthrene-contaminated soil. They found that applying solubilization step by flushing process exhibited high phenanthrene removal efficiency from soil. Moreover, the residual biosurfactant after flusing process exhibited nontoxic to the phenanthrene degrader. In addition, solubilization and emulsion of biosurfactant are important factors to enhance oil removal. In 2004, Wei et al. examined the feasibility of biosurfactants for the removal of oil from used polypropylene nonwoven sorbent. They found that biosurfactant have potential for recycling the used sorbent.

2.2.9 Application of biosurfactant on enhancement of bioavailability and biodegradation

Biosurfactants have been reported as enhancers of hydrocarbon biodegradation in liquid medium, water, marine water, soil slurries and soil microcosms.

Liquid medium

Twenty-one strains of indigenous hydrocarbon-degrading bacteria and biosurfactant-producing bacteria are belonging to genera *Bacillus* (8 strains), *Pseudomonas* (3 strains), *Halomonas* (4 strains), *Pseudoalteromonas* (1 strain), *Brevibacterium* (2 strains) and *Marinobacter* (1 strain) were isolated from seawater, sediments and fuel samples extracted from Prestige wreck (400 m deep). In addition, this study evaluated the effect of different bioemulsifiers on bacterial growth. The supplementation of hydrocarbon culture medium with bioemulsifiers (S22-BE, S24-BE and AD2-BE) clearly stimulated the growth of strains *Bacillus* sp. S25, *Brevibacterium* sp. S28 and *Pseudomonas* sp. S29 and also enhanced the ability to degraded alkanes, alkenes and aromatic compounds in marine synthetic medium (Uad et al., 2010).

Water

In 2007, Whang et al. investigated potential application of two biosurfactants (surfactin and rhamnolipid) for enhancing bioremediation of diesel-contaminated water. The results showed that the addition of rhamnolipid from 0 to 80 mg/l significantly increase biomass growth and diesel biodegradation percentage from 1000 to 2500 mg VSS/L and 40-100%, respectively. The addition 40 mg/l of surfactin can enhance biomass growth (2500 mg VSS/L) as well as diesel biodegradation percentage (94%).

Marine water

McKew et al. (2007) investigated the effect of bioaugmentation for bioremediation of crude oil in marine water, additionally compared with biostimulation via nutrient (N and P) and/or rhamnolipid biosurfactant amendment. The results showed that the addition of rhamnolipid had slightly effect on

biodegradation, but in combination with nutrient addition, *n*-alkane degradation significantly increase to 59%.

Soil slurries

The role of salicylate and biosurfactant on phenanthrene degradation in batch soil slurries was investigated which *Pseudomonas putida* ATCC 17484 was used to as phenanthrene-degrading microorganism. In batch soil slurries, adding small amount of rhamnolipid biosurfactant (0.25 g/L) can significantly increase in total removal of 500 mg/kg of phenanthrene (Gottfried et al., 2010).

Soil

Many researchers have studied on enhancing bioremediation of PAHs-contaminated soil in the presence of biosurfactants for application in remediation process (Providenti et al., 1995; Calvo et al., 2002; Uram et al, 2004). In 2001, Naim Kosaric investigated the effect of biosurfactant in eliminating of polycyclic aromatics from the crude oil-contaminated soil. The results showed that the addition of biosurfactant trehalose-5,5´-dicorynomycolates could complete the elimination of crude oil within 6 months. Thus, the addition of biosurfactant can stimulate biodegradation rate. Hickey et al. (2006) investigated the effect of biosurfactant JBR on fluoranthene degradation by *Pseudomonas aeruginosa* PA-10. They found that the addition of biosurfactant can increase fluoranthene degradation rate in the inoculated microcosm which resulting in significant removal of up to 45±5% of 200 mg/kg of PAHs after 28 days. The effect of lower molecular weight of PAHs is not significantly different in the rate of fluoranthene removal. This study also showed that biosurfactant JBR could effectively increase the solubility of PAHs and desorption from soil. Therefore, biosurfactant can be applied as an enhancer in bioremediation technology to be applied in cleaning up soil in contaminated area.

CHAPTER III

METHODOLOGY

3.1 Research overview

The research was divided into 3 parts. The first part was the selection of biosurfactant-producing bacteria. From the first part, *Bacillus* sp. GY19 was selected for further study. The second part was the study of properties of *Bacillus* sp. GY19 and its cell-free broth containing biosurfactant. According to the results in the second part, crude biosurfactant from *Bacillus* sp. GY19 was used to study in the third and the fourth parts. The last part was the study of activity of crude biosurfactant for enhancing biodegradation of pyrene in liquid medium. The research flow chart was illustrated in Fig 3.1.

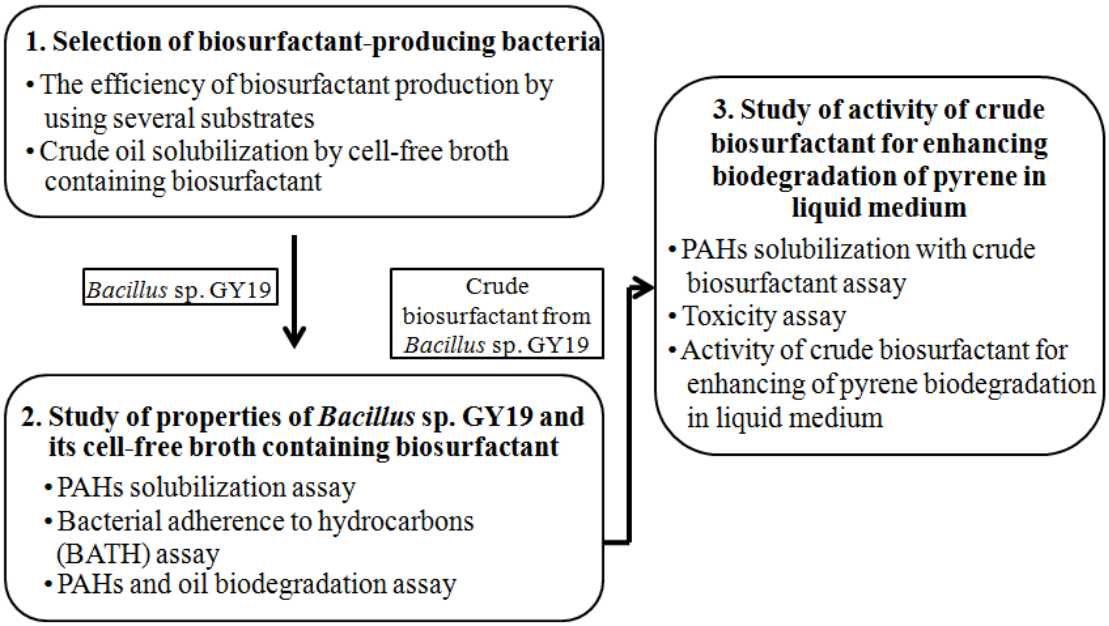


Fig 3.1: Flow chart of the research

3.2 Chemicals and equipments

3.2.1 Chemicals

1. Bottom glycerol was obtained from Thai Oleochemical Company Limited (TOL), Thailand
2. Crude oil was obtained from Petroleum Authority of Thailand (PTT), Thailand
3. Diesel oil was obtained from Petroleum Authority of Thailand (PTT), Thailand
4. Lubricating oil was obtained from Petroleum Authority of Thailand (PTT), Thailand
5. Slop oil was obtained from PTT Aromatics and Refining Public Company Limited (PTTAR), Thailand
6. Hydrochloric acid (HCl) was purchased from BDH Chemical, Australia
7. Tryptone was purchased from Difco Laboratories, USA
8. Yeast extract was purchased from Difco Laboratories, USA
9. Fluoranthene was purchased from Kanto Chemical Co., INC, Japan
10. Phenanthrene was purchased from Sigma, USA
11. Acenaphthene was purchased from Wako, Japan
12. Ferrous chloride ($FeCl_3 \cdot 6H_2O$) was purchased from Merck, Germany
13. Ethyl acetate (C_2H_5) was purchased from Merck, Germany
14. Calcium chloride ($CaCl_2 \cdot 2H_2O$) was purchased from Merck, Germany
15. Sodium chloride (NaCl) was purchased from Merck, Germany
16. Sodium sulfate anhydrous (anhydrous Na_2SO_4) was purchased from Merck, Germany
17. Dimethyl formmamide (DMF) was purchased from BIOBASIC, INC. Japan
18. Pyrene was purchased from Sigma, USA
19. Fluorene was purchased from Wako, Japan

20. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) was purchased from Carlo ERBA, France

21. Dichloromethane was purchased from Merck, Germany

22. Methanol was purchased from Fisher Scientific, UK

23. Chloroform (HPLC grade) was purchased from Lab-Scan, Ireland

3.2.2 Equipments

1. Rotary vacuum evaporator, EYELA, Japan

2. ISSCO laminar flow, International Scientific Supply, Japan

3. Incubator (30 °C), model BE800, Memmert, Germany

4. Hot air oven, model D06063, Memmert, Germany

5. Filter paper pour size 0.22 µm, Micropore, USA

6. Water bath, model digital water bath SB-100, EYELA, Japan

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

8. Autoclave, Kakusan, Japan

9. Spectrophotometer, PTP-1 Peltier System, PerkinElmer instruments, USA

10. Micropipette (2, 10, 20, 200, 1,000, and 5,000 µl), Gilson, France

11. pH meter, model 240, Corning, USA

12. TLC-FID, model IATROSCAN™ MK-6s, Mitsubishi Kaguku Iatron. Inc., Japan

13. Spectrophotometer, model UV-160A, Shimadzu, Japan

14. Tensiometer DCAT11, Dataphysics, Germany

15. Incubator 30 °C, BE800, Memmert, Germany

16. High Performance Liquid Chromatography LC10ADVP, Shimadzu, Japan

    - an inertsill® ODS column (4.6 x 150 mm)

    - UV Detector at 275 nm

3.3 Procedure

3.3.1 Selection of biosurfactant-producing bacteria

Biosurfactant-producing bacteria from laboratory library were previously isolated by particular enrichment substrates and were screened for this study by using four criteria including 1) surface tension reduction in rage from 27 to 33 mN/m, 2) emulsification activity (E24) with vegetable oil or diesel oil more than 50%, 3) oil spreading test more than 50 cm^2 and 4) capability of biosurfactant-producing bacteria to use oil or glycerol as carbon source for producing biosurfactant. The details of six screened bacteria are shown in Table 3.1.

Table 3.1: Surface tension, E24, oil spreading and carbon source of screened biosurfactant-producing bacteria

Bacteria isolates	Bacterial source	Carbon source	Surface tension (mN/m)	E24 (%)		Oil spreading (cm^2)
				Vegetable oil	Diesel oil	
Cellulosimicrobium sp. GY33	Soil	Glycerol 5%	33	62	NE	56.75
Alcaligenes sp. LS	Greased trap water	Soybean oil	27	NE	64.28	59.45
Achromobacter sp. GY30	Soil	Glycerol 5%	28	66	6	38.46
Enterobacter sp. W3-02	Ballast water	Lubricant 3%	28	NE	NE	0.20
Rhodococcus sp. SSP2	Sludge	Lubricant 2%	33	NE	NE	3.14
Bacillus sp. GY19	Soil	Glycerol 0.5%	29	50	NE	19.63

Note: NE : Not Emulsified, Information source: PTT's report (Onruthai et al., 2010)

3.3.1.1 The efficiency of biosurfactant production by using several substrates

Six biosurfactant-producing bacterial strains shown in Table 3.2 were used in this study. Basal medium [BM 1l: $NaNO_3$ 7g, K_2HPO_4 1g, KH_2PO_4 0.5g, KCl 0.1g, $MgSO_4 \cdot 7H_2O$ 0.5g, $CaCl_2$ 0.01g, $FeSO_4 \cdot 7H_2O$ 0.1g, yeast extract 0.1g and pH 7.5] supplemented with either 3% (v/v) of bottom glycerol, crude oil, diesel oil, lubricating oil or slop oil as the sole source of carbon (Table 3.2) was used for culturing bacteria. The cultures were incubated in shaker at 200 rpm for 7 days. Culture samples were centrifuged at 8,000 rpm for 20 min to remove cells. Supernatants were submitted to surface activity measurement as mentioned in 3.3.1.1.1 and emulsification activity measurement as mentioned in 3.3.1.1.2 (Yin et al., 2009). The bacterial dry biomass was used for determining the growth of bacteria.

Table 3.2: The types of substrates for used to produce biosurfactant

Type of substrate	Company	%Volume (v/v)
Bottom glycerol	TOL	3%
Crude oil	PTT	3%
Diesel oil	PTT DELTA-X EURO III/PTT	3%
Lubricating oil	Performa synthetic/ PTT	3%
Slop oil	PTTAR	3%

3.3.1.1.1 Surface tension measurement

The cell-free broths containing biosurfactant were determined surface tension with a tensiometer by using Wilhelmy Plate method.

3.3.1.1.2 Emulsification activity measurement

Each of the cell-free broth containing biosurfactant 1 ml was mixed with 1 ml of hydrocarbons solution such as bottom glycerol, crude oil, diesel oil, lubricating oil

or slop oil in screw-capped glass tube. These mixtures were mixed by vortex for 2 min and left at room temperature for overnight. The change of volume between two phases was measured (E_{24}) by equation (Abouseoud et al., 2007; Priya and Ushara, 2009).

$$E_{24} = \frac{H_{emulsion}}{H_{total}} \times 100$$

When; E_{24} (emulsification stability)
$H_{emulsion}$ (height of emulsion)
H_{total} (height of total solution)

3.3.1.2 Crude oil solubilization by cell-free broth containing biosurfactant

The solubilization experiments were performed in 125 ml flasks. These flasks contained the mixture of crude oil and each of cell-free broth containing biosurfactant as mentioned in 3.3.1.1. Distilled water and basal medium were used instead of cell-free broth containing biosurfactant in the control experiments. Incubation was done in shaker at 200 rpm for 24 h and left at room temperature until clearly separation between crude oil and aqueous phase were observed. Crude oil solubilized in aqueous phase were extracted and analyzed by using thin layer chromatography-flame ionization detector (TLC-FID) as mentioned in 3.3.1.3 (Maruyama et al., 2003).

3.3.1.3 Crude oil extraction and quantification

Crude oil extraction was done by using chloroform as a solvent. Crude oil solubilized in aqueous phase was extracted by adding 4 ml of chloroform and then 1 ml of 6.25 ml stearyl alcohol was added as an internal standard. After shaking at 200 rpm for 30 min, chloroform phase was taken into new tube and evaporated until final volume was about 1 ml of chloroform.

The quantification of crude oil solubilzed was analyzed by using TLC-FID. The 1 µl of crude oil extracted was spotted on a clean chromarod by using microdispenser. Then, spotted chromarod was soaked in solvent tank which to

increase polarity for separating saturated and aromatic hydrocarbon from resin/asphaltenes as shown in Table 3.3.

Table 3.3: The solvents for separating elements of crude oil

Solvent	Time (min)	Distance (cm)
n-hexane	25	10
Dichloromethane (DCM)	12	6.5
	5	4
DCM:methanol (95:5, v/v)	1	1

Finally, the chromarod was dried in a rod dryer at 60 °C for 1 min. The conditions for analyzing crude oil solubilized was operated under following; 30 s/scan speed, 160 ml/min of hydrogen flow rate and retention time of saturated, aromatic, stearyl alcohol, resin and asphaltene were approximately 0.13, 0.24, 0.35, 0.42 and 0.47 min, respectively. Amount of crude oil solubilized was calculated by comparing with a standard curve of crude oil.

3.3.2 Study of properties of *Bacillus* sp. GY19 and its cell-free broth containing biosurfactant

In this step, *Bacillus* sp. GY19 culturing by using bottom glycerol as a carbon source was selected by using four criteria based on the results from above experiments including 1.) surface tension reduction of culture media is 29 mN/m, 2.) emulsification activity with crude oil and diesel oil are 56% and 64%, respectively, 3.) high biomass up to 1.8 g/l and 4.) its cell-free broth containing biosurfactant exhibited the highest crude oil solubilization with 2,062 mg/l. Then, *Bacillus* sp. GY19 was examined for three properties including 1.) bacterial adherence to hydrocarbons (BATH) assay, 2.) PAHs and oil biodegradation assay. The last property, its cell-free broth containing from culture using bottom glycerol as a carbon source was examined for PAHs solubilization activity.

3.3.2.1 PAHs solubilization assay

The excess amount of PAHs was separately added to each 1.5 ml tube containing the cell-free broth containing biosurfactant which produce from *Bacillus* sp. GY19 by using bottom glycerol as a carbon source. The samples were equilibrated for a period of 48 h on shaker (Previous experimental results showed 48 h to be sufficient for reaching solubilization and partitioning equilibrium under mixing). To separate the non-dissolved PAHs, these samples were centrifuged at 5,000 rpm for 30 min. Then, the aliquot of dissolved PAHs in the cell-free broth containing biosurfactant was diluted with methanol. The concentration of dissolved naphthalene, acenaphthene, phenanthrene, fluoranthene and pyrene was analyzed by spectrophotometer PTP-1 Peltier System at 220, 290, 254, 235 and 273 nm wavelengths (Wei et al., 2011).

3.3.2.2 Bacterial adherence to hydrocarbons (BATH) assay

The bacterial cell of *Bacillus* sp. GY19 was cultivated in Luria-Bertani (LB) broth for 18 h and centrifuged at 10,000 rpm for 30 min to collect cells. Sterile phosphate-urea-magnesium sulfate buffer [buffer 1l: K_2HPO_4 16.9g, KH_2PO_4 7.3g, urea 1.8g, $MgSO_4 \cdot 7H_2O$ 0.2g and pH 7.1] was used for washing the cells twice. The bacterial cells were suspended in the same buffer and measure the initial optical density (OD_{400}) ~ 1.0. The cells suspension 4 ml was mixed with 1 ml of hexadecane in a screw-capped glass tube. This mixture was mixed by vortex for 1 min and left at room temperature for 30 min for phase separation. The aqueous phase was measured the final optical density (OD_{400}) and calculated the adherence (%) to hexadecane by equation (Rosenberg et al., 1980).

$$\text{Hydrophobicity} = 1 - \frac{A_f}{A_i} \times 100\%$$

When; A_i (initial optical density)

A_f (final optical density)

3.3.2.3 PAHs and oil biodegradation assay

The inoculums of *Bacillus* sp. GY19 were prepared by culturing the cell in 0.1x LB medium by shaking at 200 rpm for 24 h and centrifuged at 8,000 rpm for 10 min to harvest the cells. Then, the cells were washed twice in 0.85% NaCl and suspended in the same solution. The cells concentration were adjusted to the optical density of 1.0 at 600 nm by using 0.85% NaCl and resting cells was done by shaking at 200 rpm for 24 h. A cells suspension was added in carbon free mineral medium [CFMM 1l: NH_4NO_3 3g, KH_2PO_4 2.2g, $Na_2HPO_4 \cdot 12H_2O$ 0.8g and pH7] supplemented with phenanthrene (100 mg/l), fluoranthene (100 mg/l), pyrene (100 mg/l) or crude oil (0.5%, v/v) and incubated at 30 °C at 200 rpm. Triplicate samples were collected at 14 days to examine the remaining PAHs by high performance liquid chromatography (HPLC) as mentioned in 3.3.2.5. The residual crude oil concentration was extracted and examined by TLC-FID (as in 3.3.1.3) (Maruyama et al., 2003). Determination the growth of bacteria was done by the viable plate count technique on

LB agar plate as mentioned in 3.3.2.4. Surface tension of media was measured by tensiometer to determine the production of biosurfactant (as in 3.3.1.1.1).

3.3.2.4 Total cell count in PAHs and crude oil biodegradation assay

The viable plate count technique was determined the growth of bacteria. The cells were suspended in 0.85% NaCl and then 10 µl of serial ten-fold dilution (10^{-2} – 10^{-7}) was dropped on 0.25x LB agar plate. The plate was incubated at 30 °C for 2 days and the number of bacterial strain was calculated in logCFU per 1 ml of artificial medium.

3.3.2.5 PAHs extraction and quantification

Remaining PAHs in culture samples was extracted by using ethyl acetate. The 5 ml of each culture samples were extracted twice with 5 ml of ethyl acetate in tube. Then, ethyl acetate phase was taken into new tube and dried with anhydrous sodium

sulfate. Finally, ethyl acetate phase was evaporated and final volume was about 1 ml of methanol.

Residual PAHs was analyzed by using high performance liquid chromatography (HPLC) operated under the following conditions: an inertsill® ODS column (4.6 x 150 mm) maintained at 40 °C; the mobile phase was methanol:water (80:20 v/v); a flow rate of 1.0 ml/min, and UV detector was set at 275 nm (Klankeo et al., 2009).

3.3.3 Study of activity of crude biosurfactant for enhancing biodegradation of pyrene in liquid medium

3.3.3.1 Culture condition and biosurfactant production

Optimal condition such as type and concentration of carbon source was used to produce biosurfatant by *Bacillus* sp. GY19. The strain GY19 was cultured in productive medium [1l: glucose 1g, beef extract 0.5g, K_2HPO_4 3.3g, KH_2PO_4 0.14g, $NaNO_3$ 5g, NaCl 0.04g and $FeSO_4$ 0.1g] supplemented with 10% (v/v) of bottom glycerol as a carbon source. The culture was incubated at 30 °C at 200 rpm for 5 days.

3.3.3.2 Biosurfactant extraction

Crude biosurfactant was extracted from the culture media after removing cells by centrifugation at 8,000 rpm for 15 min. The recovery of crude biosurfactant was performed by a liquid-liquid extraction by using chloroform:methanol (65:15, v/v) (adapted from Patel and Desai, 1997). Then, crude extracted biosurfactant solution was prepared in Phosphate Bufferd Saline (PBS) (Vaz et al., 2011).

3.3.3.3 PAHs solubilization with crude biosurfactant assay

The excess amount of PAHs was separately added to each 1.5 ml tube containing the cell-free broth containing each different concentration of crude

biosurfactant (0.25, 0.5, 0.7, 1, 3 and 5 g/l). The samples were equilibrated for a period of 48 h on shaker (Previous experimental results showed that 48 h was sufficient for reaching solubilization and partitioning equilibrium under mixing). To separate the non-dissolved PAHs, these samples were centrifuged at 5,000 rpm for 30 min. Then, the aliquot of dissolved PAHs in the cell-free broth containing biosurfactant was diluted with methanol. The concentration of dissolved phenanthrene, fluoranthene and pyrene was analyzed by spectrophotometer PTP-1 Peltier System at 254, 235 and 273 nm wavelengths (Wei et al., 2011).

3.3.3.4 Toxicity assay

In this study, the toxicity of crude biosurfactant on the survival of *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY was investigated. The bacterial cells were cultured in sterilized 0.25x LB that inoculated with 1% (v/v) inoculums containing 10^6 CFU/ml of *Pseudoxanthomonas* sp. RN402 or *Novosphingobium* sp. PCY (Table 3.4) supplemented with crude biosurfactant. The effect of crude biosurfactant concentrations on the survival of *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY was evaluated with different concentrations of crude biosurfactant (0.5, 0.7, 1, 3 and 5 g/l). The culture media was incubated in shaker at 200 rpm for 1 and 7 day for culturing *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY, respectively. Triplicate samples were examined the survival of bacterial cells by the viable plate count technique on LB agar plate (as in 3.3.2.4).

Table 3.4: PAHs-degrading bacteria used for degrading pyrene in this study

Bacterial strain	Culture collection code	Environment source	PAHs degradation capability	Reference
Pseudoxanthomonas sp. RN402	TISTR 2059	Soil from roadside area of Ratchdumnern Road in Bangkok, Thailand	phenanthrene, fluoranthene, pyrene	Klankeo et al. (2009)
Novosphingobium sp. PCY	TISTR 2007	Mangrove sediment from conservation center and mangrove ecosystem in Phetchaburi, Thailand	phenanthrene, pyrene	Promchat Chareanpat (2008)

3.3.3.5 Activity of crude biosurfactant for enhancing of pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY in form of free cell in liquid medium

The study of activity of crude biosurfactant for enhancing of pyrene biodegradation by *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY in form of free cell in liquid medium experiment was conducted in three different sets:

Set 1: Bacterial strain + Pyrene

Set 2: Bacterial strain + Pyrene + Crude biosurfactant

Set 3: Control (without bacteria and crude biosurfactant)

The inoculums were prepared the same as mentioned in 3.3.2.3. The test tubes containing 5 ml of sterilized CFMM were inoculated with 1% (v/v) inoculums containing 10^7 CFU/ml of *Pseudoxanthomonas* sp. RN402 or *Novosphingobium* sp. PCY. In set of *Pseudoxanthomonas* sp. RN402, each of CEMM culture was supplemented with 100, 500 and 1,000 mg/l of pyrene and triplicate samples were taken at days 0, 4, 8 and 28. In set of *Novosphingobium* sp. PCY, each of CEMM culture was supplemented with 100, 300 and 500 mg/l of pyrene and triplicate samples were taken at days 0, 4, 8, 12, 16 and 20. All culture samples in each set were incubated at 30 °C in shaker at 200 rpm and samples were taken to examine the remaining pyrene concentration by HPLC (as in 3.3.2.5) and growth of bacteria by the viable plate count technique on 0.25x LB agar plate (as in 3.3.2.4) (Thavasi et al., 2010).

3.3.3.6 Activity of crude biosurfactant for enhancing of pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 in form of immobilized cells in liquid medium

The study of activity of crude biosurfactant for enhancing of pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 in form of immobilized cell in liquid medium experiment was conducted in three different sets:

Set 1: Bacterial strain + Pyrene

Set 2: Bacterial strain + Pyrene + Crude biosurfactant

Set 3: Control (without bacteria and crude biosurfactant)

In this experiment, *Pseudoxanthomonas* sp. RN402 was immobilized by using adsorption on solid carrier surfaces method. The immobilized bacterial strain was prepared by culturing in 0.1x LB with sterilized plastic pellets for 6 days. The properties of plastic pellets were shown in Table 3.5. Then, immobilized cells with sterilized plastic pellets 5 g were added in 125-ml Erlenmeyer flask containing 50 ml of sterilized CFMM supplemented with 500 mg/l of pyrene. All cultures samples in each set were incubated at 30 °C in shaker at 200 rpm and samples were taken at day

0, 7, 14 and 21 to examine remaining pyrene concentration by HPLC (as in 3.3.2.5) and growth of bacteria number per gram of dried plastic pellets by the viable plate count technique on 0.25x LB agar plate as mentioned in 3.3.3.6.1.

Table 3.5: The properties of plastic pellets used in this study

Plastic pellets	Properties
Structure	
Code name	BCN-009
Specific surface	836 m^2/m^3
Protected surface	494 m^2/m^3
Specific weight	165 kg/m^3
Material	High density polyethylene (HDPE)
Density	0.95

3.3.3.6.3 Total cell count in carrier material

The immobilized cells in 1 g of dried plastic pellets were suspended in 0.85% NaCl by vortexing for 2 min, then sonicating for 2 min and immediately vigorously mixed by vortexing for 2 min in duplicate. The 10 µl of serial ten-fold dilution (10^{-2} – 10^{-7}) of cell suspension was dropped on 0.25x LB agar plate. The plate was incubated at 30 °C for 2 days and the number of bacterial strain was calculated in log CFU per total weight of plastic pellet.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Selection of biosurfactant-producing bacteria

The selection of biosurfactant-producing bacteria was done by using two criteria including the efficiency of biosurfactant production by using several substrates and crude oil solubilization by its cell-free broth containing biosurfactant.

4.1.1 The efficiency of biosurfactant production by using five substrates

Six biosurfactant-producing bacterial strains (Table 4.1) were screened from laboratory library based on four criteria including 1) surface tension reduction (27 to 33 mN/m), 2) emulsification activity (E24) with vegetable oil or diesel oil ($\geq$ 50%), 3) oil spreading test ($\geq$ 50 cm^2) and 4) capability of biosurfactant-producing bacteria to use oil or glycerol as carbon source for producing biosurfactant. Then, continued from the six screened strains, potential strains and substrates were selected by examining for efficiency to produce biosurfactant by using 3% (v/v) of bottom glycerol , crude oil, diesel oil, lubricating oil or slop oil as carbon source. For selecting potential strains and substrates in this experiment, three criteria were used to indicate the efficiency of biosurfactant production. First was surface tension reduction which could indicate that biosurfactant has potential to reduce surface tension in media. Second was emulsification activity which could indicate that biosurfactant can form microemulsions and hydrocarbons can solubilize in water. The last was biomass which can be used to indicate that bacterial strain could utilize substrates as a carbon source for growing (Shavandi et al., 2010; Amani et al., 2012).

The results as shown in Table 4.1 indicated that six screened strains could utilize most substrates as its carbon source. While, the using of slop oil as carbon source exhibited toxicity to these strains because they gave very low biomass in range of 0 – 0.7 g/l. However, cell-free broth containing biosurfactant from each culture showed different efficiency in surface tension reduction and emulsification activity. In this study, 13 potential strains and substrates (* in Table 4.1) were selected for further

experiment based on four criteria including surface tension reduction (≤ 40 mN/m), emulsification activity with crude oil, diesel oil or lubricating oil (≥50%), high biomass production (≥1g/l) or capability to use bottom glycerol as carbon source.

Table 4.1: Surface tension, E24 of cell-free broth and bacterial dry biomass grown in medium supplemented with particular carbon source

Bacterial isolates	Carbon source 3% (v/v)	Surface tension (mN/m)	E24 (%)			Biomass (g/l)
			Crude oil	Diesel oil	Lubricant	
Cellulosimicrobium sp. GY33	Bottom glycerol*	47±2.3	43	NE	88	3.8±1.2
	Crude oil	52±0.6	36	NE	5	1.1
	Diesel oil*	39±5.5	18	NE	11	3.1±0.4
	Lubricant	43±2.3	NE	NE	6	3.0
	Slop oil	37±0.1	NE	NE	21	0.5
Alcaligenes sp. LS	Bottom glycerol	48±0.03	45	NE	19	2.0
	Crude oil	54±0.03	36	5	16	0.4
	Diesel oil*	37±4.4	55	NE	22	1.3±1.6
	Lubricant*	34±3.7	67	NE	37	2.8±0.9
	Slop oil	36±0.03	43	NE	16	0.2
Achromobacter sp. GY30	Bottom glycerol*	29±1.0	53	62	12	1.7±0.7
	Crude oil*	35±2.1	57	5	11	0.8±0.1
	Diesel oil	38±0.03	28	NE	15	4.3
	Lubricant*	39±1.7	41	NE	10	1.0±0.7
	Slop oil	35±0.03	NE	NE	6	0.4

Table 4.1: Surface tension, E24 of cell-free broth and bacterial dry biomass grown in medium supplemented with particular carbon source (cont.)

Bacterial isolates	Carbon source 3% (v/v)	Surface tension (mN/m)	E24 (%)			Biomass (g/l)
			Crude oil	Diesel oil	Lubricant	
Enterobacter sp. W3-02	Bottom glycerol*	38±1.9	48	NE	39	0.6±0.1
	Crude oil	55±0.03	43	NE	17	0.8
	Diesel oil	31±0.03	30	NE	26	0.4
	Lubricant*	42±6.1	43	NE	16	2.3±2.3
	Slop oil	37±0.02	38	NE	21	0.2
Rhodococcus sp. SSP2	Bottom glycerol	46±0.9	52	NE	82	1.1±0.2
	Crude oil	42±9.1	71	38	35	2.3±1.5
	Diesel oil	32±0.03	78	64	47	0.2
	Lubricant*	34±2.6	67	NE	28	1.1±0.4
	Slop oil	36±0.03	68	NE	18	ND
Bacillus sp. GY19	Bottom glycerol*	29±0.4	56	64	10	1.8±0.1
	Crude oil*	34±0.3	NE	NE	29	1.5±0.6
	Diesel oil*	37±1.8	NE	NE	14	1.4±0.2
	Lubricant	52±3.9	NE	NE	38	2.4
	Slop oil	33±0.3	NE	NE	48	0.7

Note : NE: Not Emulsified, ND: Not Determined, (*) : Potential strains and substrates were selected for further experiments

4.1.2 Crude oil solubilization by cell-free broth containing biosurfactant

The efficiency of crude oil solubilization was the criteria used to select the best strain of biosurfactant-producing bacteria and substrate from the potential strains and substrates (* in Table 4.1). The efficiency of crude oil solubilization was determined by using thin layer chromatography with flame ionization detector (TLC-FID) and data obtained were presented in Fig 4.1.

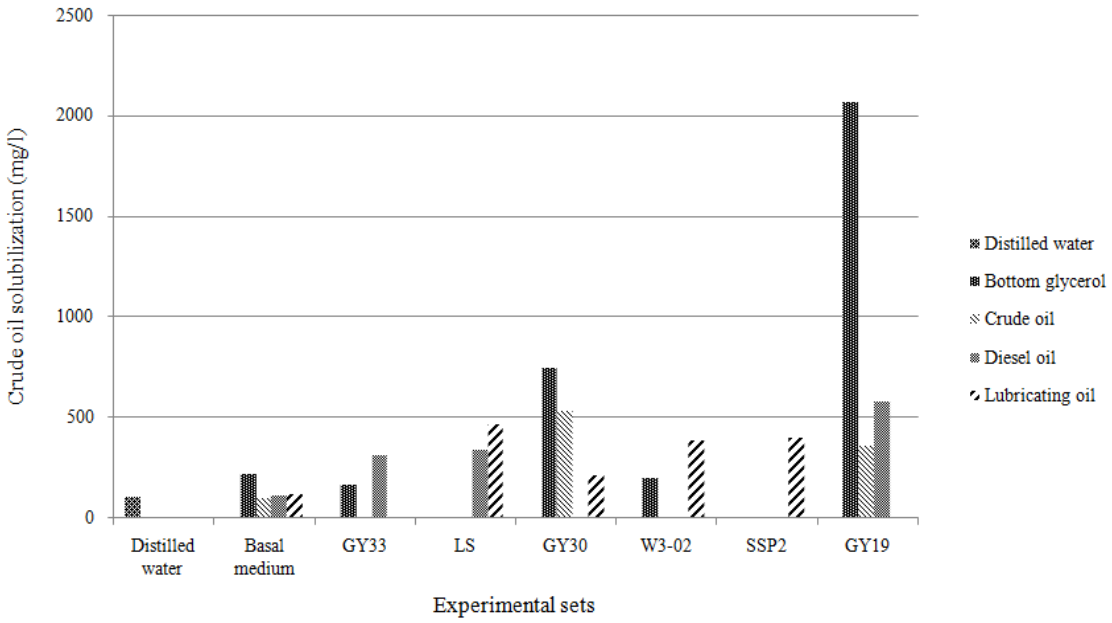


Fig 4.1: Crude oil solubilization by cell-free broth containing biosurfactant of 13 potential strain and substrate

The results showed that cell-free broth containing biosurfactant of *Bacillus* sp. GY19 exhibited the highest crude oil solubilization as compared to those of other bacteria and controls. Especially, cell-free broth containing biosurfactant from culture using bottom glycerol as carbon source from *Bacillus* sp. GY19 exhibited the highest solubilization of crude oil with 2,062 mg/l. Previous reported by Bordoloi and Konwar (2009), they examined solubilization of PAHs and crude oil by biosurfactant produced by *Pseudomonas aeruginosa* MTCC7815, MTCC7812, MTCC7814, MTCC8163 and MTCC8165. Their results also showed that biosurfactants enhance solubilization of phenanthrene, pyrene and crude oil. Since crude oil contains complex mixture of PAHs and this experiment showed that biosurfactant of *Bacillus* sp. GY19

could significantly enhance the solubilization of crude oil. Therefore, cell-free broth containing biosurfactant of *Bacillus* sp. GY19 using bottom glycerol as carbon source might contain large amount of efficient biosurfactant and can be also used to solubilize PAHs in the further experiment.

4.2 Study of properties of *Bacillus* sp. GY19 and its cell-free broth containing biosurfactant

From previous experiments, the result showed that *Bacillus* sp. GY19 could use bottom glycerol as carbon source and has high biomass (1.8 g/l). Moreover, its cell-free broth containing biosurfactant from culture using bottom glycerol as carbon provide the most surface tension reduction (29 mN/m), emulsification activity with crude oil and diesel oil (56% and 64%) and the highest crude oil solubilization (2,062 mg/l). Hence, *Bacillus* sp. GY19 and its cell-free borth containing biosurfactant by using bottom glycerol as carbon source were selected for further study.

4.2.1 PAHs solubilization

PAHs solubilization assay was done by measuring the apparent solubility of naphthalene, acenaphthene, phenanthrene, fluoranthene and pyrene with 5,000 mg/l concentration and the results were showed in Fig 4.2.

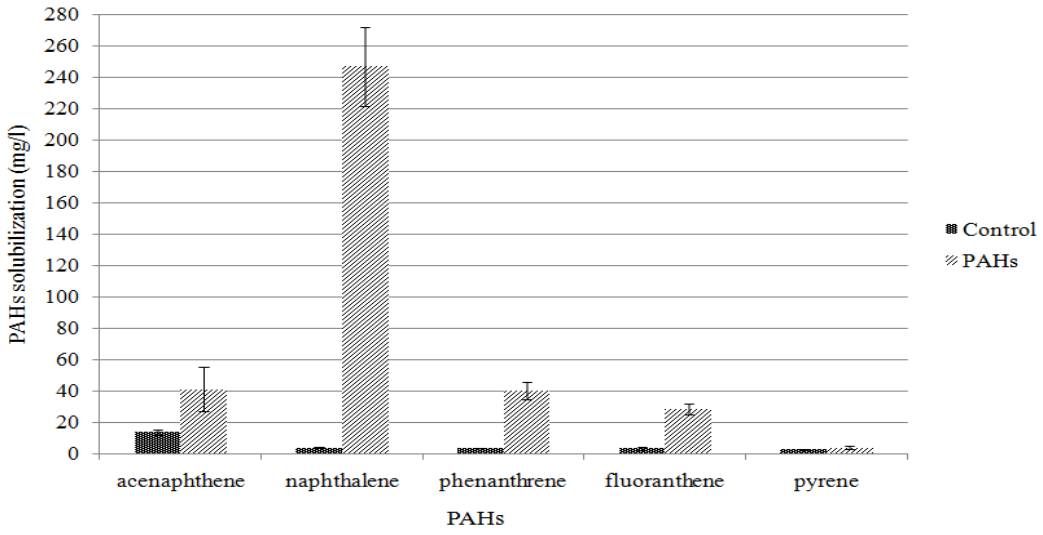


Fig 4.2: Solubilization of PAHs with cell-free broth containing biosurfactant of *Bacillus* sp. GY19 using bottom glycerol as a carbon source

The results showed that cell-free broth containing biosurfactant exhibited the highest solubilization of naphthalene with 247 mg/l. On the other hand, cell-free broth containing biosurfactant exhibited very low solubilization of acenaphthene, phenanthrene, fluoranthene and pyrene only 41, 40 12.6 and 3.6 mg/l, respectively. According to the property of water solubility of naphthalene, acenaphthene, phenanthrene, fluoranthene and pyrene are 34.4, 1.93, 1.2, 0.2 and 0.07 mg/l, respectively (ATSDR, 1995). The results in this experiment were corresponded with water solubility each type of PAHs. However, as the results from experiment 4.1.2, cell-free broth containing biosurfactant exhibited high solubilization of crude oil as compare to PAHs exhibited low solubilization. Since some polar compounds like resins and asphaltenes of crude oil could dissolve in water (Narve Aske, 2002). Furthermore, cell-free broth containing biosurfactant exhibited solubilization efficiency of crude oil by increasing the contact of non-aqueous components such as aromatic compounds in crude oil. Thus, cell-free broth containing biosurfactant exhibited high solubilization of crude oil.

4.2.2 Bacterial adherence to hydrocarbons (BATH)

Bacillus sp. GY19 cells grown on Luria-Bertani (LB) broth exhibited low adherence (3.5%) to hexadecane as measured by the BATH assay. The result indicated that strain GY19 has low cell surface hydrophobicity. Cell surface property is an important factor used for determining the rate of hydrophobic substrates degradation (Pijanowska et al., 2007). As shown in this experiment, strain GY19 exhibited low cell surface hydrophobicity and might also exhibit low degradation of hydrophobic substrates such as PAHs and oil which was studied in the next experiment.

4.2.3 PAHs and crude oil biodegradation

This experiment was performed to examine the efficient of *Bacillus* sp. GY19 on degradation and biosurfactant production by using phenanthrene, fluoranthene, pyrene and crude oil as a carbon source. The results showed that *Bacillus* sp. GY19 had low ability to degrade 100 mg/l of phenanthrene, fluoranthene, pyrene and 0.5%

(v/v) of crude oil and the increasing of total cell number could not be observed by culturing in carbon free mineral medium (CFMM) after 14-day incubation as shown in Fig 4.3 and Fig 4.4, respectively.

These results can be explained by the reason described by Lu et al. (2011) that the various numbers of PAHs-degrading bacteria were isolated from PAHs-contaminated environment (Brito et al., 2006; Chang et al., 2008; Musat et al., 2009). These PAHs-degrading bacteria contain genes encoding PAH-catabolic enzymes specific for PAHs degradation. However, *Bacillus* sp. GY19 was isolated from soil by using glycerol as carbon source. Hence, strain GY19 might have no enzyme for degradation of PAHs and oil. Moreover, low ability to degrade hydrophobic substrates of this strain was also directly correlated with its low cell surface hydrophobicity (as shown in experiment 4.2.2).

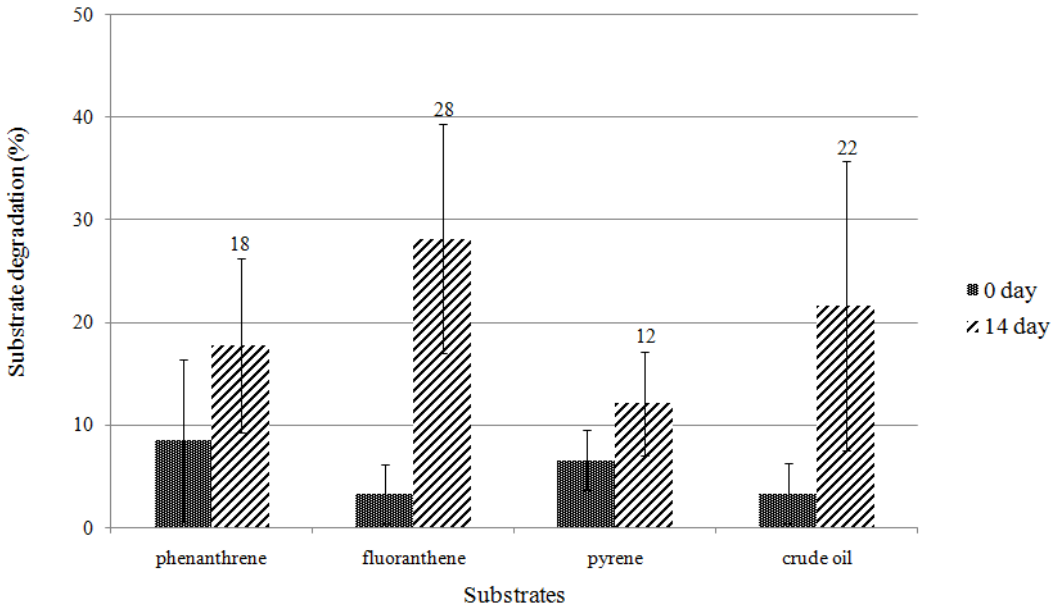


Fig 4.3: Capability degradation (%) of 100 mg/l of each PAHs and 0.5% (v/v) of crude oil by *Bacillus* sp.GY19 in CFMM after 14-day incubation

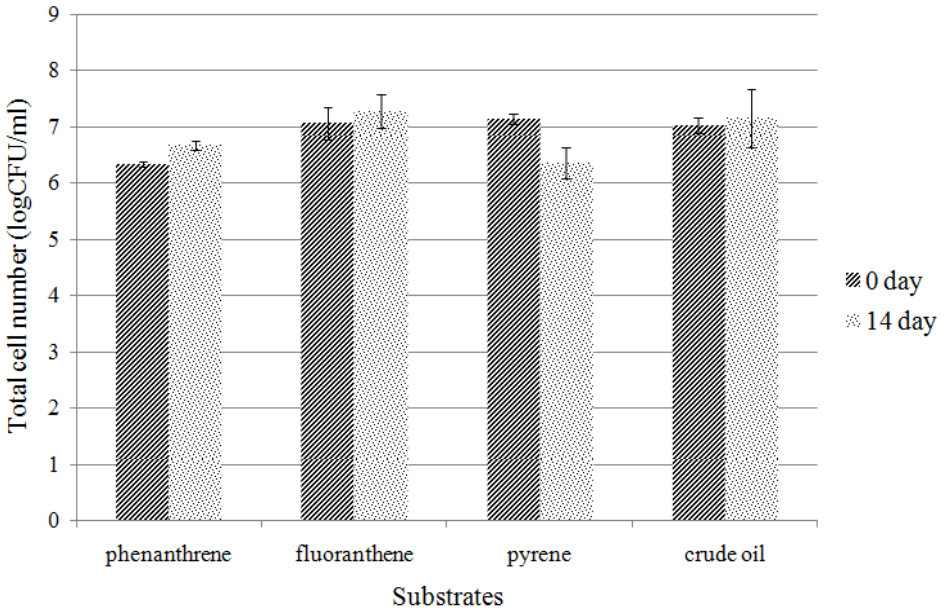


Fig 4.4: Bacterial numbers of *Bacillus* sp. GY19 from degrading100 mg/l of each PAHs and 0.5% (v/v) of crude oil in CFMM after 14-day incubation

Furthermore, *Bacillus* sp. GY19 could not utilize these substrates as its carbon source to produce biosurfactant since surface tension reduction of each media could not be observed as shown in Table 4.2.

Table 4.2: Surface tension in CFMM supplemented with PAHs or crude oil as carbon source by *Bacillus* sp. GY19 at 14-day incubation

Substrates	Surface tension (mN/m)	
	CFMM	CFMM + strain GY19
Phenanthrene	65.495±4.34	67.630±1.72
Fluoranthene	55.416±6.82	54.141±7.22
Pyrene	63.865±7.59	67.671±0.83
Crude oil	64.589±2.85	58.734±1.10

The results indicated that *Bacillus* sp. GY19 could not produce biosurfactant by using PAHs as carbon source since strain GY19 have no enzyme to degrade and

utilize PAHs. In case of crude oil, strain GY19 could not utilize and produce biosurfactant by using 0.5% (v/v) of crude oil as carbon source. However, results in experiment 4.1.1 showed that strain GY19 could utilize and produce biosurfactant by using 3% (v/v) of crude oil as substrate. According to Franzetti et al. (2009) investigated that cultivation factor affecting the production biosurfactant by *Gordonia* sp. BS29. They found that concentration of carbon source was an important factor for producing biosurfactant. Therefore, the concentration of crude oil in this experiment might be too low to be used as carbon source.

4.3 Study of activity of crude biosurfactant for enhancing biodegradation of pyrene in liquid medium

In this experiment, biosurfactant from *Bacillus* sp. GY19 was used in form of crude extracted biosurfactant. As the results in experiment 4.2.1, cell-free broth containing biosurfactant exhibited low solubilization of PAHs since it might contain small amount of biosurfactant. Moreover, the remaining bottom glycerol in cell-free broth containing biosurfactant might cause some error in this experiment by being used as carbon source. Therefore, crude extracted biosurfactant was used in this experiment due to it could provide higher efficiency with larger amount and controllable concentration of biosurfactant.

Biosurfactant production of *Bacillus* sp. GY19 was done by culturing the strain in productive medium supplemented of bottom glycerol as carbon source with 10% (v/v) which is the most optimized concentration to produce the highest both biosurfactant yield and growth of strain GY19 (Luepromchai et al., 2011 from PTT's report). Then, crude biosurfactant was extracted by liquid-liquid extraction and prepared in phosphate buffer saline (PBS).

4.3.1 PAHs solubilization with crude biosurfactant

PAHs solubilization assay was done by measuring the apparent solubility of phenanthrene, fluoranthene and pyrene with concentration of crude biosurfactant solution in range of 0.25, 0.5, 0.7(CMC) (Luepromchai et al., 2011 from PTT's report) 1, 3 and 5 g/l. The results are shown in Fig 4.5.

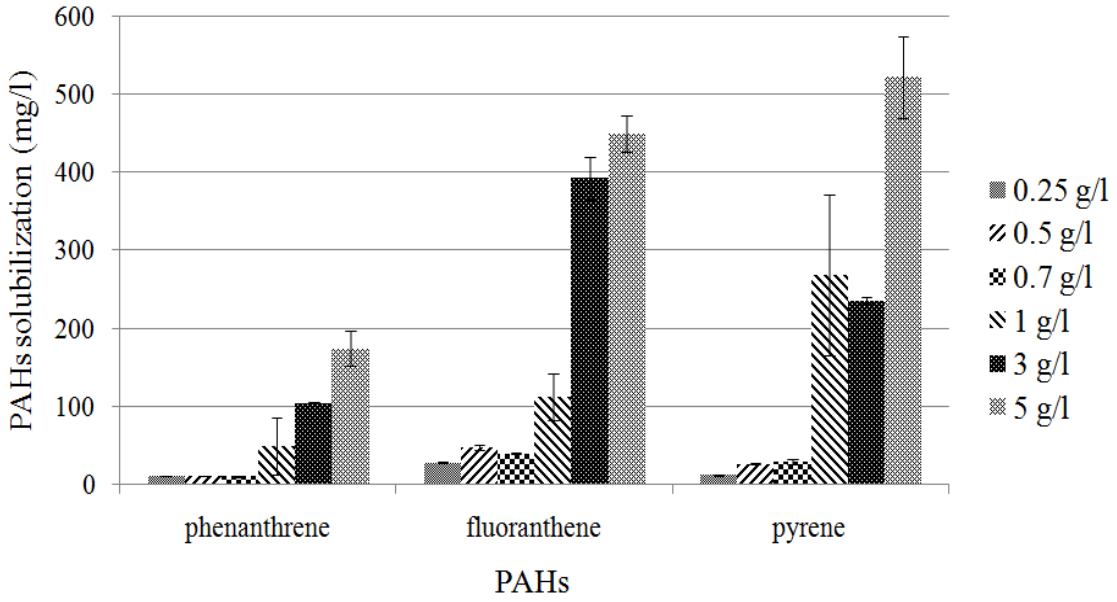


Fig 4.5: Solubilization of PAHs with crude biosurfactant produced by *Bacillus* sp. GY19

The results showed that an increasing concentration of crude biosurfactant resulted in higher solubilization capability of phenanthrene, fluoranthene and pyrene. By increasing concentration of crude biosurfactant ranging from 0.25-5 g/l, solubility of phenanthrene, fluoranthene and pyrene increased by 11-174 mg/l, 29-449 mg/l and 12-522 mg/l, respectively. Previous report by Das and Mukherjee (2006) which investigated the increasing concentration of crude biosurfactant produced by *Bacillus subtilis* DM-04, *Pseudomonas aeruginosa* NM ranging from 0-0.5 g/l to solubilize PAHs. They found that solubilization capability of phenanthrene, anthracene and pyrene increased by 0.5-1.1 mg/l, 0.04-1.1 mg/l and 0.13-1.2 mg/l, respectively. Thus, crude biosurfactant from *Bacillus* sp. GY19 has higher PAHs solubilization efficiency than those of previous report. The increased solubility of PAHs might be important for enhancing bioavailability and biodegradation of pyrene in further experiment.

4.3.2 Toxicity assay

In this study, pyrene-degrading bacteria were used for studying the effectiveness of crude biosurfactant of *Bacillus* sp. GY19 for enhancing of pyrene biodegradation in liquid medium in the next experiment. Therefore, pyrene-degrading bacteria should be considered about the effect of crude biosurfactant of *Bacillus* sp. GY19 on survival. In this experiment, the effect of crude biosurfactant concentration on the survival of *Pseudoxanthomonas* sp. RN402 was evaluated with different concentrations at 0.5, 0.7(CMC), 1, 3 and 5 g/l of crude biosurfactant by culturing in 0.25x LB for 24 h. The results are shown in Fig 4.6.

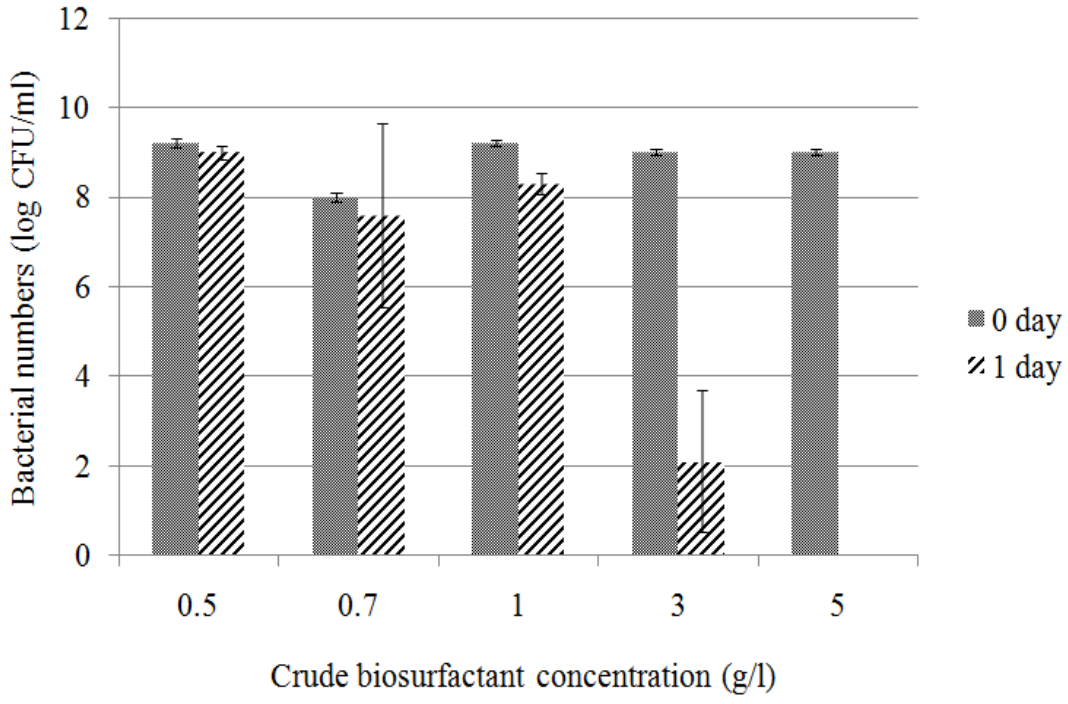


Fig 4.6: The effect of different concentration at 0.5, 0.7, 1, 3 and 5 g/l of crude biosurfactant on survival of *Pseudoxanthomonas* sp. RN402 by culturing in 0.25x LB for 24 h

According to the results shown in Fig 4.6, increasing concentration of crude biosurfactant resulted in decrease of the survival cells of *Pseudoxanthomonas* sp. RN402 after 24-h incubation. Especially, concentration at 5 g/l of crude biosurfactant showed toxic effect by inhibitory the growth of *Pseudoxanthomonas* sp. strain

RN402. These results can be explained by the reason in studying of Shin et al. (2005). They investigated the effect of different concentration (0, 0.025, 0.05, 0.1, 0.15 and 0.24 g/l) of rhamnolipid biosurfactant on phenanthrene biodegradation and cell growth of *Sphingomonas* sp. 3Y and *Paenibacillus* sp. 4-3. Their results showed significant toxic effect to strain 3Y even lower than CMC (0.0577 g/l), but nontoxic to strain 4-3. Their research summarized that the effect of biosurfactant on survival of bacteria was depended on the bacterial strain.

Hence, in this experiment pyrene-degrading *Novosphingobium* sp. PCY was also used for examination of its survival in the presence of crude biosurfactant of strain GY19. The effect of crude biosurfactant concentration on the survival of *Novosphingobium* sp. PCY was evaluated with different concentrations at 0.5, 0.7(CMC), 1, 3 and 5 g/l of crude biosurfactant by culturing in 0.25x LB. For strain PCY, the culture samples were incubated for 7 days since culturing for 24 h might be too short time to monitor the effect of crude biosurfactant on survival of bacterial strain. Furthermore, 7 days incubation was to ensure that bacterial strain could survive when cultured for the long time (20 days) in the next experiment. The results are shown in Fig 4.7.

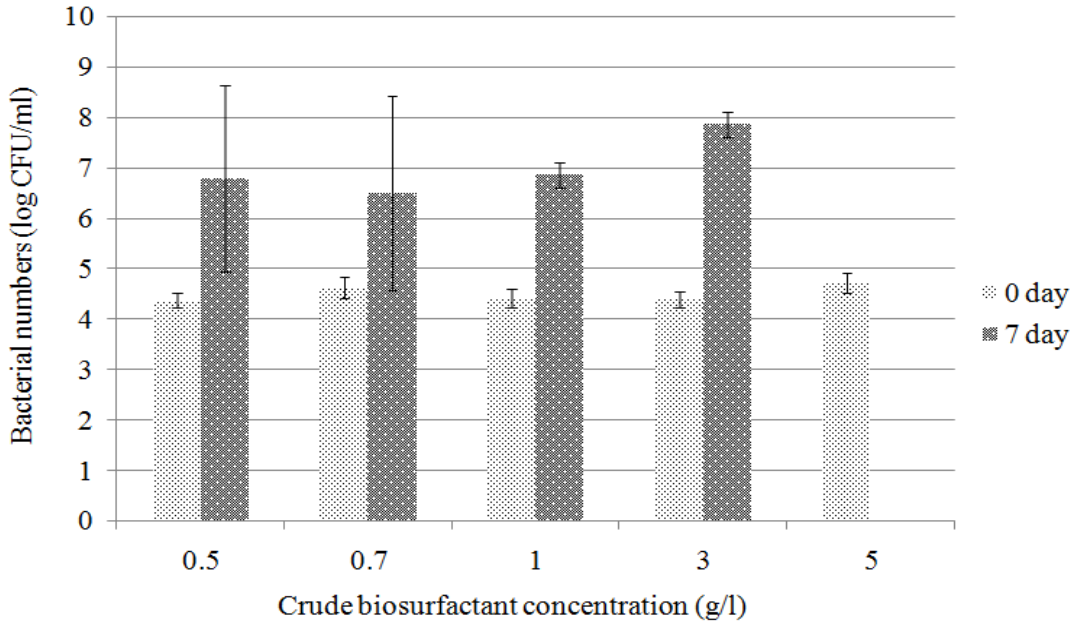


Fig 4.7: The effect of different concentration at 0.5, 0.7, 1, 3 and 5 g/l of crude biosurfactant on survival of *Novosphingobium* sp. PCY by culturing in 0.25x LB for 7 days

By increasing concentration ranging from 0.5-3 g/l of crude biosurfactant resulted in nontoxic to strain PCY by increasing the bacterial numbers after 7-day incubation. On the other hand, results showed significant toxic effect by inhibitory the growth to strain PCY at concentration 5 g/l of crude biosurfactant. The results indicated that crude biosurfactant showed significant nontoxic effect on toward strain PCY, but showed toxic toward strain RN402. However, high concentration (5 g/l) of crude biosurfactant exhibited toxicity to both of strains. These results indicated that the effect of crude biosurfactant on the survival of *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY were depended on the strain as referenced of Shin et al. (2005). Hence, the toxicity of biosurfactant to bacterial strain should be a critical factor for the effectiveness of biosurfactant addition and should be considered carefully for using with bacterial strains.

4.3.3 Activity of crude biosurfactant for enhancing pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY in form of free cell in liquid medium

According to the results in experiments 4.3.1 and 4.3.2, 1 g/l concentration of crude biosurfactant from *Bacillus* sp. GY19 showed high soilubilization of pyrene with 268 mg/l and nontoxic to PAH-degrading bacteria which were used in this study. Thus, at 1 g/l concentration (above CMC) of crude biosurfactant was used in this study.

The study of crude biosurfactant for enhancing of pyrene biodegradation by free cell of *Pseudoxanthomonas* sp. RN402 was examined in CFMM. Each CFMM culture was supplemented with various concentrations at 100, 500 and 1,000 mg/l of pyrene and triplicated samples were taken at days 0, 4, 8 and 28. The results are shown in Fig 4.8(a), (b) and (c), respectively.

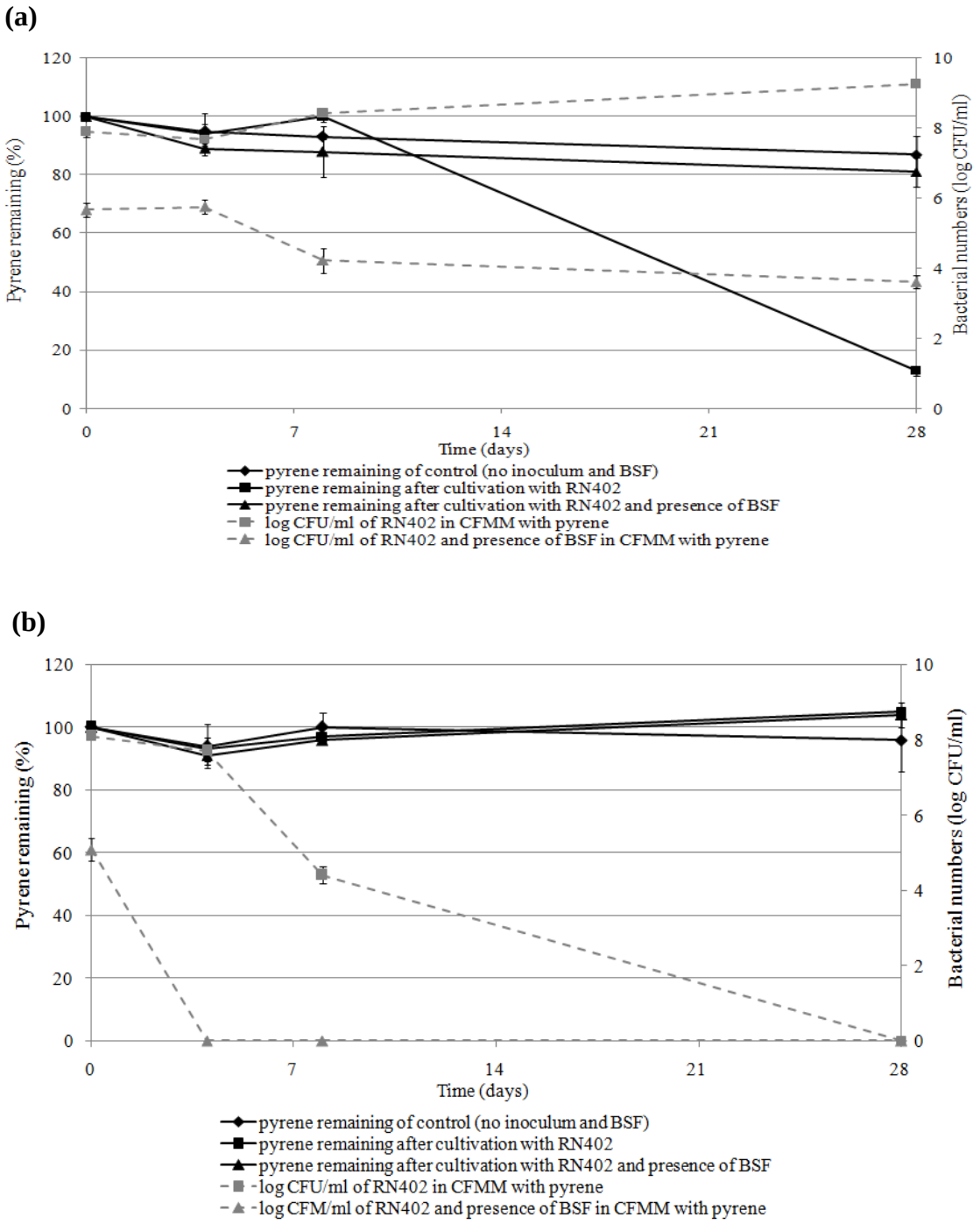


Fig 4.8: Pyrene remaining (%) degraded by *Pseudoxanthomonas* sp. RN402 in CFMM with pyrene at (a) an initial concentration of 100 mg/l, (b) 500 mg/l and (c) 1,000 mg/l in the presence and absence of crude biosurfactant at concentration of 1 g/l

(c)

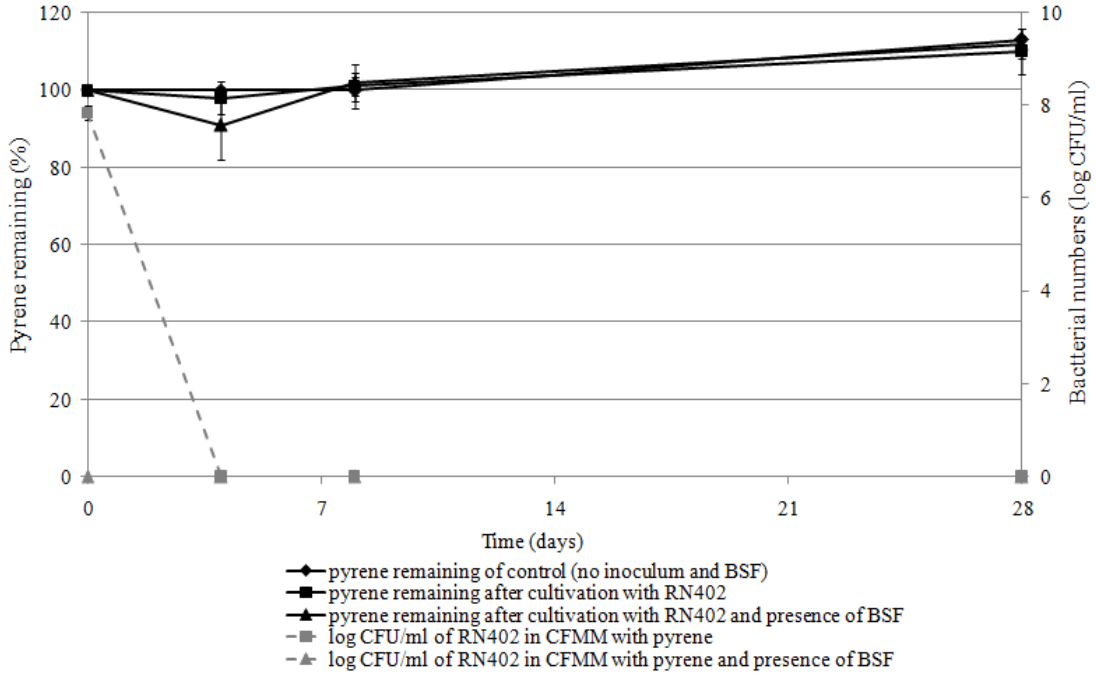


Fig 4.8: Pyrene remaining (%) degraded by *Pseudoxanthomonas* sp. RN402 in CFMM with pyrene at (a) an initial concentration of 100 mg/l, (b) 500 mg/l and (c) 1,000 mg/l in the presence and absence of crude biosurfactant at concentration of 1 g/l (cont.)

The results were found that at concentration 100 mg/l of pyrene, the addition of crude biosurfactant could not enhance pyrene degradation by *Paseudoxanthomonas* sp. RN402 in form of free cell which exhibited 81% for pyrene remaining as compared with those of the absence of crude biosurfactant with 13% of pyrene remaining after 28-day incubation. As the same trend, at concentration 500 and 1,000 mg/l of pyrene, both of treatments in the absence and presence of crude biosurfactant could not enhance pyrene degradation by strain RN402 in form of free cell. Reasons could be explained by monitoring from the growth of bacterial strain.

The effect of crude biosurfactant addition to the cell growth of strain RN402 in form of free cell when pyrene was used as carbon source was also monitored in this experiment. At concentration 100, 500 and 1,000 mg/l of pyrene, crude biosurfactant exhibited significantly toxicity to strain RN402 in form of free cell by affecting initial cell number. Reducing bacterial number of initial cell was observed as compared to

those in the treatment without the addition of crude biosurfactant. After 28-day incubation with 100 mg/l of pyrene, bacterial number of free cell of strain RN402 were increased to maximum at 9.7 log CFU/ml since pyrene was used as carbon source. While the bacterial numbers were decreased in presence of crude biosurfactant due to toxicity of crude biosurfactant. This amount of bacteria may too small to degrade pyrene at this concentration. Furthermore, at concentration 500 and 1,000 mg/l of pyrene, bacterial number exhibited as same trend that the excess amount of pyrene and crude biosurfactant could be toxic and inhibit the growth of strain RN402 in form of free cell.

Due to crude biosurfactant was nontoxic to *Novosphingobium* sp. PCY after culturing 7 days as shown in experiment 4.3.2. Thus, *Novosphingobium* sp. PCY in form of free cell was used to study the effect of crude biosurfactant addition to enhance pyrene degradation in this study. Furthermore, high concentrations of pyrene were exhibited toxic to strain RN402. So in this experiment, concentration of pyrene was reduced to 100, 300 and 500 mg/l for degrading by *Novosphingobium* sp. PCY in CFMM by culturing 20 days. The results are shown in Fig 4.9(a), (b) and (c), respectively.

(a)

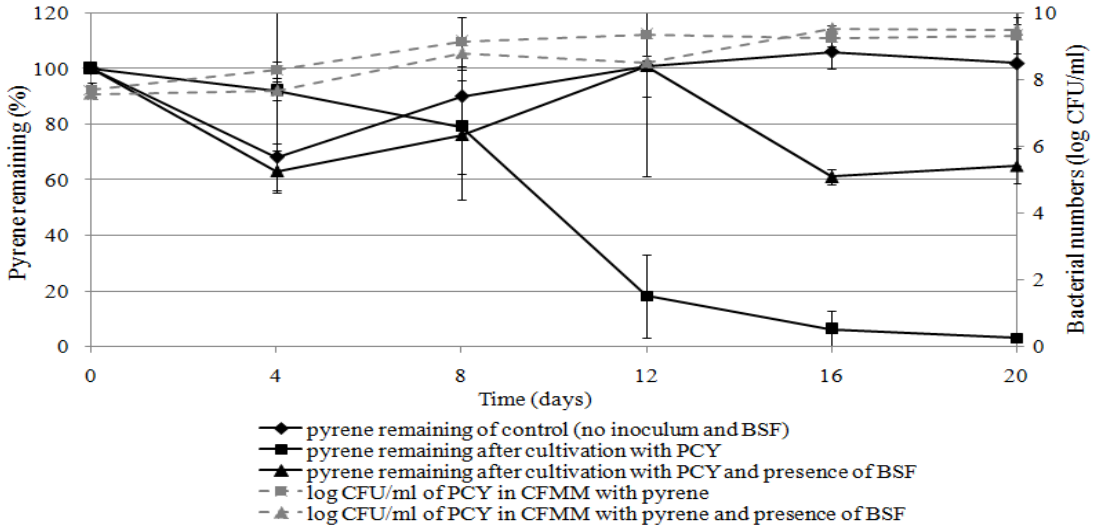
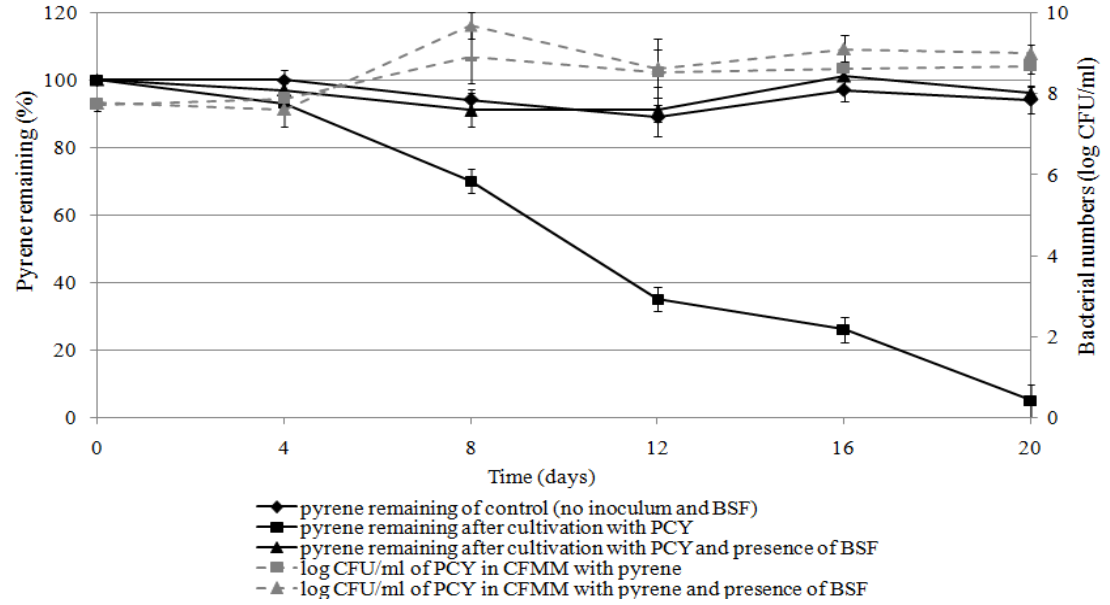


Fig 4.9: Pyrene remaining (%) degraded by *Novosphingobium* sp. PCY in CFMM with pyrene at (a) an initial concentration of 100 mg/l, (b) 300 mg/l and (c) 500 mg/l in the presence and absence of crude biosurfactant at concentration of 1 g/l

(b)



(c)

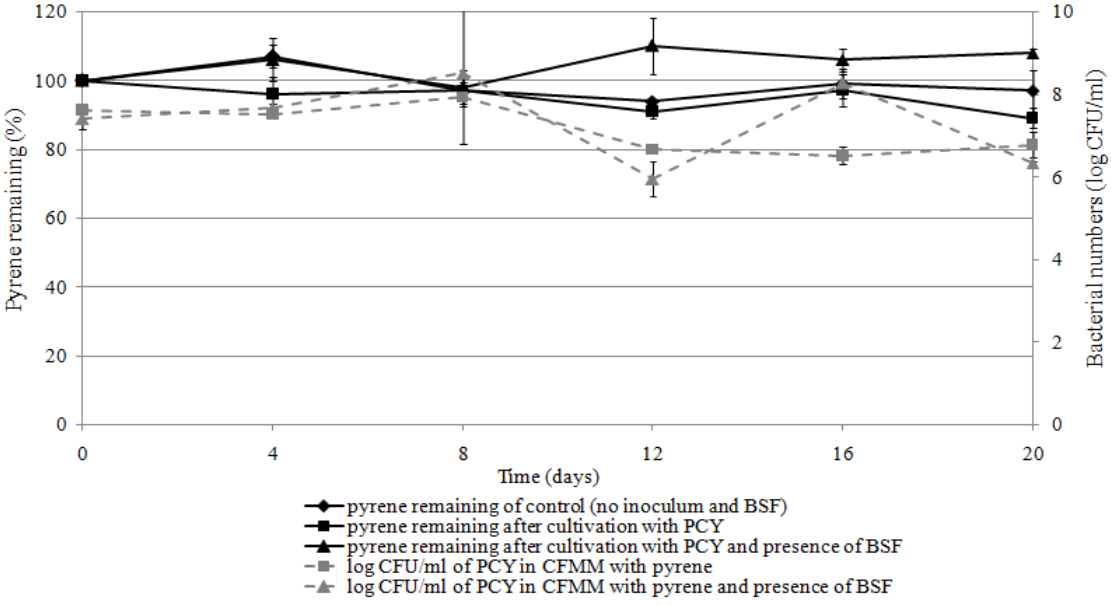


Fig 4.9: Pyrene remaining (%) degraded by *Novosphingobium* sp. PCY in CFMM with pyrene at (a) an initial concentration of 100 mg/l, (b) 300 mg/l and (c) 500 mg/l in the presence and absence of crude biosurfactant at concentration of 1 g/l (cont.)

At concentration of 100 and 300 mg/l of pyrene, the results indicated that crude biosurfactant could not enhance pyrene degradation by *Novosphingobium* sp. PCY in form of free cell. As compared with the treatment without addition of crude biosurfactant, the strain PCY could utilize pyrene at 100 and 300 mg/l to reach 3% and 5% of pyrene remaining after 20-day incubation, respectively. However, bacterial number in both treatments in the presence and absence of crude biosurfactant were increased. The reason might be explained that strain PCY might can utilize both pyrene and crude biosurfactant as carbon source. On the other hand, at concentration 500 mg/l of pyrene, on pyrene degradation by strain PCY in form of free cell was not observed in both treatments of presence and absence of crude biosurfactant. As a result of high concentration of pyrene and crude biosurfactant might exhibited toxicity to bacterial strain.

From all of these results, crude biosurfactant from *Bacillus* sp. GY19 could be toxic to *Pseudoxanthomonas* sp. RN402 in form of free cell and it showed less efficient for enhancing pyrene degradation of both *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY. According to Jin et al. (2007) examined the effect of concentration, polar/ionic head group and structure of surfactants on biodegradation of phenanthrene in aqueous phase. They found that affecting of biosurfactant on PAHs degradation depended on biosurfactant concentration, head group type and structure which the toxicity could reduce by increasing of chain length of biosurfactant. Similar to this experiment, the type and chemical structure of biosurfactant might be toxic to strain RN402. Hence, the type and chemical structure of biosurfactant from *Bacillus* sp. GY19 should be studied for further research.

4.3.4 Activity of crude biosurfactant for enhancing of pyrene biodegradation with *Pseudoxanthomonas* sp. RN402 in form of immobilized cell in liquid medium

To improve pyrene biodegradation from experiment 4.3.3, the immobilized cells were applied in this study. The limitation of free cell for degrading pollutants is bacterial cells may contact with pollutants directly which may be suffered from toxic pollutants (Suttinum, 2008). From the results in experiment 4.3.3, crude biosurfactant

from *Bacillus* sp. GY19 and high concentration at 500 mg/l of pyrene showed toxicity to *Pseudoxanthomonas* sp. RN402 in form of free cell. To protect bacteria strain from critical environment, plastic pellets were applied as solid material for producing immobilized *Pseudoxanthomonas* sp. RN402 inoculum. Enhancement of pyrene degradation by immobilized cells was examined with high concentration 500 mg/l of pyrene and was compared between with and without addition of biosurfactant by culturing for 21 days. The result is shown in Fig 4.10.

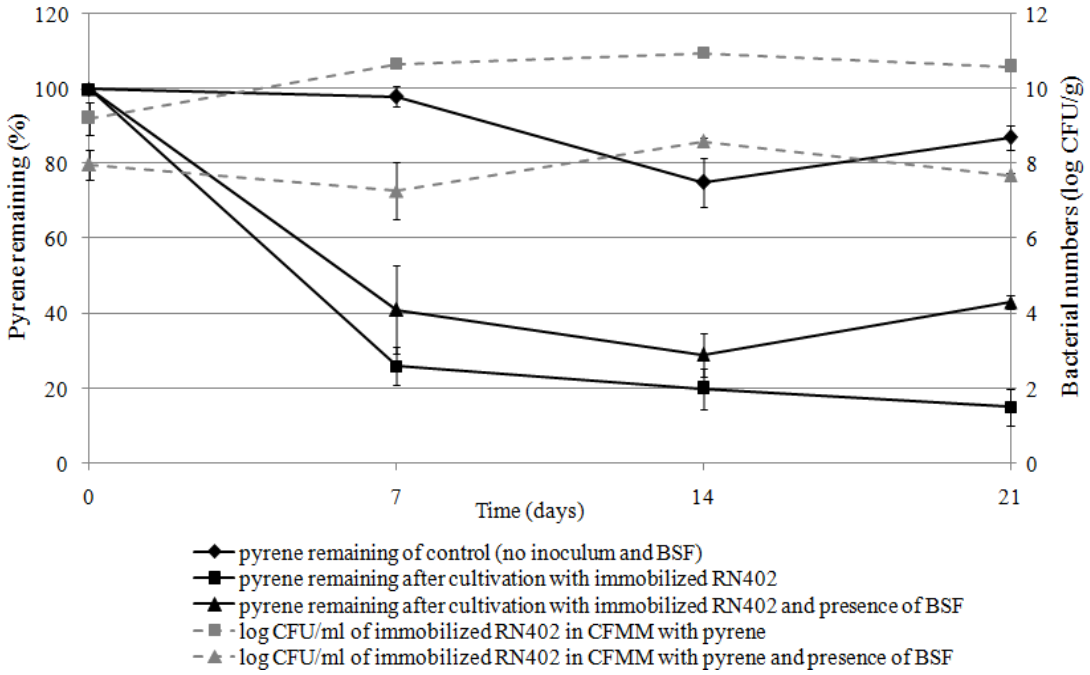


Fig 4.10: Pyrene remaining (%) degraded by immobilized *Pseudoxanthomonas* sp. RN402 in CFMM with pyrene concrentration of 500 mg/l in the presence and absence of crude biosurfactant at concentration of 1 g/l

As compared with the results in previous experiment shown in Fig 4.8(b), at concentration 500 mg/l of pyrene, both of treatments in the absence and presence of crude biosurfactant could not enhance pyrene degradation by strain RN402 in form of free cell. Because of above at CMC of crude biosurfactant and high concentration of pyrene could be toxic to bacterial strain. While in this experiment, the results indicated that the immobilization of *Pseudoxanthomonas* sp. RN402 could protect bacterial cells from toxicity of high concentration of pyrene, but could not protect

toxicity from crude biosurfactant. Moreover, the results revealed that immobilized strain RN402 could degrade pyrene to obtain the remaining pyrene with 15% of initial concentration and the increasing of the growth after 21-day incubation. However, the addition of biosurfactant could not enhance pyrene biodegradation by immobilized cells of strain RN402 as compared with those of treatment with the absence of crude biosurfactant.

In 2010, Sarma and Pakshirajan investigated the immobilization of *Mycobacterium frederiksbergense* with calcium alginate in form of beads for pyrene degradation and enhance bioavailability by adding 1% (w/v) of Tween 80 surfactant. The results found that immobilization of *M. frederiksbergense* could protect cells from toxicity of the surfactant and high concentration of pyrene (1,000 mg/l) which exhibited degradation rate of 250 mg/l. Similarly, this experiment also indicated that immobilization of strain RN402 could improve pyrene degradation at high concentration but could not protect toxicity from the biosurfactant. Furthermore, crude biosurfactant could not also enhance pyrene degradation.

All these results in experiment 4.3.3 and 4.3.4, the addition of 1 g/l (above CMC) concentration of crude biosurfactant of *Bacillus* sp. GY19 exhibited toxicity to both *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY. The cause of toxicity is combining effects of crude biosurfactant which are concentration of crude biosurfactant at above CMC and pyrene solubilization capability at concentration 1 g/l of crude biosurfactant. In 2009, Li et al. reviewed about the toxicity of biosurfactant. Biosurfactant molecules at above CMC could form micelles which can be toxic to bacterial strain by mixing with lipid membrane of cells. As the results, cell membranes could be solubilized and lead to lysis of cell. Moreover, the biosurfactant might enhance bioavailability of pyrene, the excess amount of solubilized pyrene was exhibiting toxicity to both *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY. So, in order to avoid this effect, appropriate concentration of biosurfactant should be considered and should be further studied.

Therefore, the toxicity of biosurfactant to bacterial strains should be considered when biosurfactant is applied on enhancing PAHs biodegradation in environment. However, biosurfactant could be naturally degraded in the environment

by microbial, which is one advantage. According to Lima et al. (2010) examined biodegradability of different biosurfactants in liquid medium and soil microcosms. They found that all different biosurfactants could be degraded by each of pure cultures in liquid medium and mixed culture in soil microcosms. Moreover, their research indicated that biosurfactants are more suitable for environmental applications than synthesized surfactants.

CHAPTER V

CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

Polycyclic aromatic hydrocarbons (PAHs) are the most toxic hydrocarbons found in crude oil. Pyrene is a high molecular weight PAH which consists of four fused benzene rings. Since pyrene is insoluble in water, their bioavailability is limited for biodegradation and will be accumulated in the environment by long time. Hence, solubilization step is very important for degrading of high hydrophobic compound such as PAHs. Biosurfactants are amphiphilic compounds produced by various microorganisms. Moreover, biosurfactants can reduce surface tension and increase the solubility of hydrophobic compound. Therefore, biosurfactants have a potential to enhance solubility of PAHs and lead to the increase of bioavailability and biodegradation. Thus, the aim of this study was to investigate the efficiency of biosurfactant from the potential strain and substrate on enhancing pyrene solubilization and biodegradation in liquid medium.

From six biosurfactant-producing bacterial screened strains, *Bacillus* sp. GY19 was selected based on five criteria. *Bacillus* sp. GY19 could grow on 3% (v/v) of bottom glycerol and gave high biomass (1.8 g/l). In addition, its cell-free broth containing biosurfactant provided the most surface tension reduction (29 mN/m), showed emulsification activity with crude oil and diesel oil (56% and 64%) and exhibited the highest solubilization of crude oil (2,062 mg/l).

In the study of the properties, cell-free broth containing biosurfactant of *Bacillus* sp. GY19 from culture using bottom glycerol as carbon source exhibited ability to solubilize acenaphthene, naphthalene, phenanthrene, fluoranthene and pyrene. However, strain GY19 exhibited low cell surface hydrophobicity (3.5%). Moreover, strain GY19 also exhibited low ability to degrade 100 mg/l of phenanthrene, fluoranthene and pyrene and 0.5% (v/v) of crude oil and could not

produce biosurfactant by using these substrates as carbon source. Therefore, biosurfactant from *Bacillus* sp. GY19 showed potential to be applied in enhancing pyrene biodegradation since biosurfactant had ability of solubilization enhancement.

For the application, crude biosurfactant was extracted by liquid-liquid extraction and prepared in phosphate buffer saline (PBS). The study revealed that increasing concentration of crude biosurfactant resulted in higher solubilization capability of PAHs. Especially, at 5 g/l concentration of crude biosurfactant resulted in the highest solubilization of phenanthrene, fluoranthene and pyrene with 174, 449 and 522 mg/l, respectively. However, the toxicity assay which was performed in 0.25x LB with various concentrations at 0.5, 0.7, 1, 3 and 5 g/l of crude biosurfactant. At 5 g/l concentration of crude biosurfactant was found to be toxic by inhibitory the growth of pyrene-degrading *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY after culturing for 24 h and 7 days, respectively. Hence, pyrene biodegradation experiments were performed to investigate the efficiency of crude biosurfactant at concentration of 1 g/l (above CMC) on enhancement of pyrene biodegradation in liquid medium (CFMM). Crude biosurfactant and pyrene might exhibit combined toxicity by inhibiting pyrene degrading capability of *Pseudoxanthomonas* sp. RN402 in both form of free cell and immobilized cell and *Novosphingobium* sp. PCY in form of free cell. Furthermore, crude biosurfactant might enhance bioavailability of pyrene, the excess amount of solubilized pyrene then exhibited toxicity to bacterial strain.

In conclusion, this study demonstrated that biosurfactant from *Bacillus* sp. GY19 showed high potential to solubilize PAHs and crude oil. However, biosurfactant could not enhance pyrene biodegradation by *Pseudoxanthomonas* sp. RN402 and *Novosphingobium* sp. PCY since exhibited toxicity to both strains. The limitation of biosurfactant from *Bacillus* sp. GY19 concentration and using of the process in which solubilization and biodegradation occur separately should be considered on enhancing PAHs biodegradation. Moreover, based on high ability to solubilize crude oil and PAHs, the biosurfactant should be further studied to apply in other approaches which exemplified in the next topic.

5.2 Suggestions and recommendations for further study

There are many limitation factors for using biosurfactant from *Bacillus* sp. GY19 on enhancing PAHs biodegradation. The using biosurfactant in concentration above the critical micelle concentration (CMC) which might be toxic to bacterial strain since reduction of biodegradation was monitored (Volkering et al., 1995). Thus, the concentration of biosurfactant should be considered and should be studied at CMC or lower CMC for enhancing PAHs degradation in liquid medium. Moreover, to improve the enhancing PAHs bioderadation by biosurfactant from *Bacillus* sp. GY19, the solubilization and biodegradation step should be separated for reducing the affecting of toxicity from PAHs solubilized on efficiency of PAHs biodegradation ability and the growth of pyrene-degrading bacteria (Shin et al., 2006). Furthermore, high solubilization of crude oil by biosurfactant from *Bacillus* sp. GY19 was also exhibited in this study. Biosurfactant could be studied for applying to use in industrial as a detergent such as cleaning oil tank. There is an alternative way to apply solibilization process for using in industrial (Wei et al., 2004).

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APPENDICES

APPENDIX A

Media Preparation

Basal medium (BM)

A.	$NaNO_3$	7	g
	KH_2PO_4	0.5	g
	K_2HPO_4	1	g
	KCl	0.1	g
	$MgSO_4 \cdot 7H_2O$	0.5	g
	$CaCl_2$	0.01	g
	$FeSO_4 \cdot 7H_2O$	0.1	g
	Yeast extracts	0.1	g
	Deionized water	to 1,000	ml

Adjust pH to 7.5. By autoclaving with pressure 15 lb//inch^2 at 121°C for 15 min

B. Trace minerals

H_3BO_4	0.26	g
$CuSO_4.5H_2O$	0.5	g
$MnSO_4 \cdot H_2O$	0.5	g
$MoNa_2O_4 \cdot 2H_2O$	0.06	g
$ZnSO_4.7H_2O$	0.7	g

Sterilize by filtering through 0.2 µm PTFE

Carbon free mineral medium (CFMM)

A.	NH_4NO_3	3	g
	KH_2PO_4	2.2	g

$Na_2HPO_4 \cdot 12H_2O$	0.8	g
Deionized water	to 1,000	ml

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

B. Trace minerals

$FeCl_3 \cdot 6H_2O$	0.05	g
$CaCl_2 \cdot 2H_2O$	0.05	g
$MgSO_4 \cdot 7H_2O$	0.1	g

Sterilize by filtering through 0.2 µm PTFE

Liria Bertani (LB) broth

Tryptone	10	g
Yeast extracts	5	g
NaCl	5	g
Deionized water	to 1,000	ml

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

LB agar

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

APPENDIX B

Chemical

1 N NaOH

NaOH	4	g
Deionized water	100	ml

1 N HCl

12 N HCl	8.33	ml
Deionized water	91.67	ml

0.85% NaCl

NaCl	0.85	g
Deionized water	100	ml

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

PAHs solution: including Acanaphthene, naphthalene, Phenanthrene, Fluoranthene and Pyrene (10,000 mg/l)

PAHs	0.1	g
Dimethylformamide (DMF)	10	ml

Sterilize by filtering through 0.2 µm PTFE

APPENDIX C

1. Crude oil calibration curve

The calibration curve was plotted between ratio of area (crude oil/stearyl alcohol) and ratio of concentration (crude oil/stearyl alcohol).

Amount of crude oil = Ratio of concentration x Amount of stearyl alcolhol

Ratio of concentration (x) = Ratio of area (y)/0.530

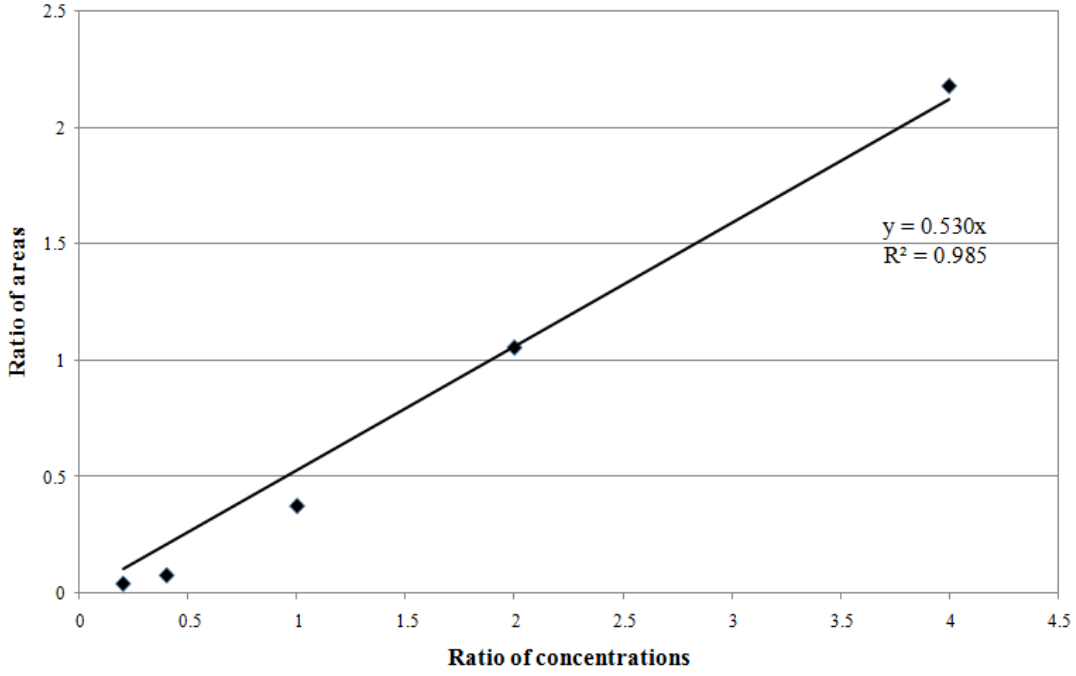

Fig C-1: Standard curve of crude oil from TLC-FID. Each data point was averaged from triple spots on Chromatorods

2. PAHs calibration curve

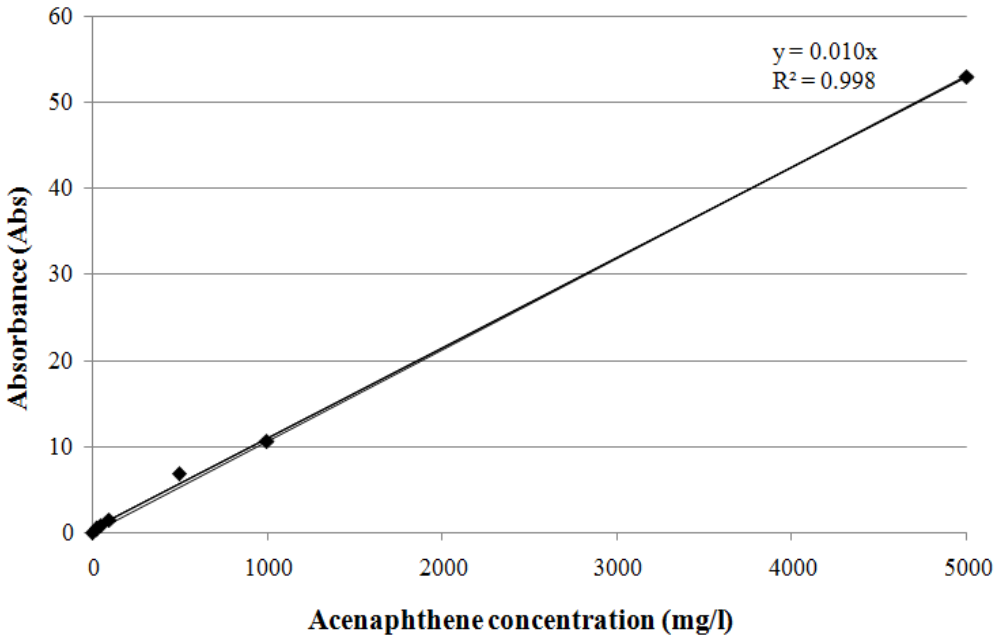

Fig C-2: Standard curve of acenaphthene from Spectrophotometer for determining acenaphthene solubilization by cell-free broth containing biosurfactant and crude biosurfactant

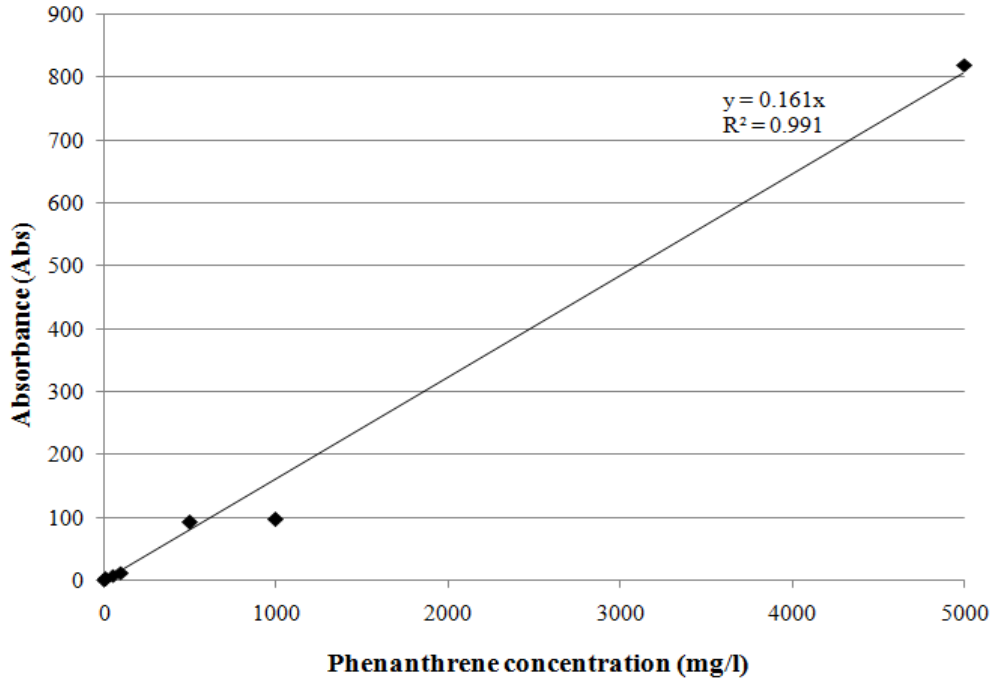

Fig C-3: Standard curve of acenaphthene from Spectrophotometer for determining phenanthrene solubilization by cell-free broth containing biosurfactant and crude biosurfactant

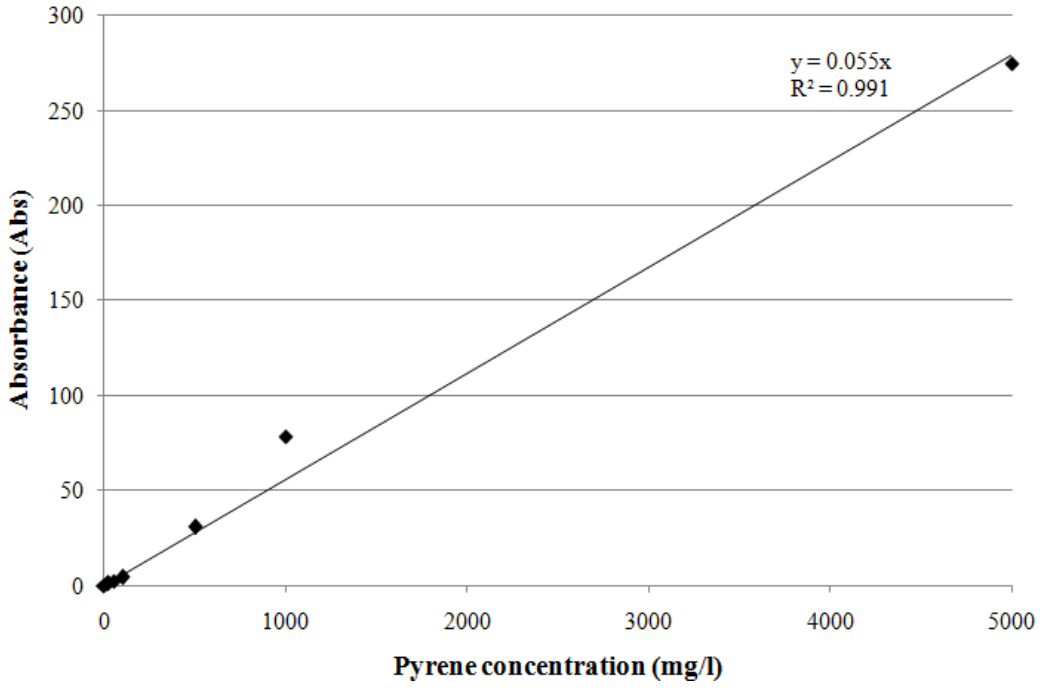

Fig C-4: Standard curve of acenaphthene from Spectrophotometer for determining pyrene solubilization by cell-free broth containing biosurfactant and crude biosurfactant

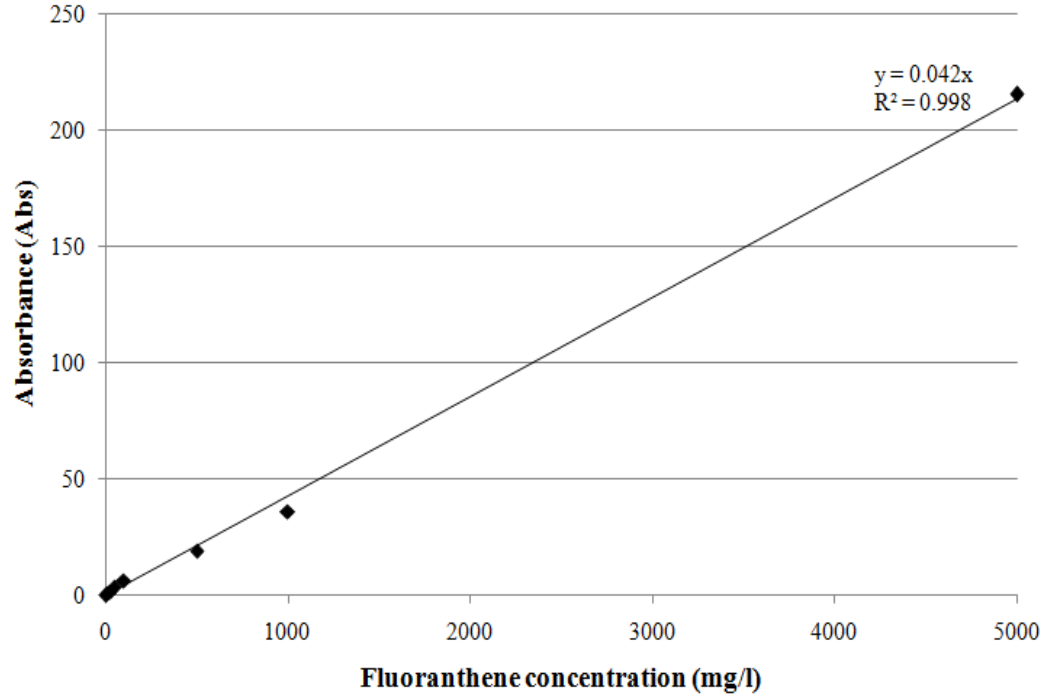

Fig C-5: Standard curve of acenaphthene from Spectrophotometer for determining fluoranthene solubilization by cell-free broth containing biosurfactant and crude biosurfactant

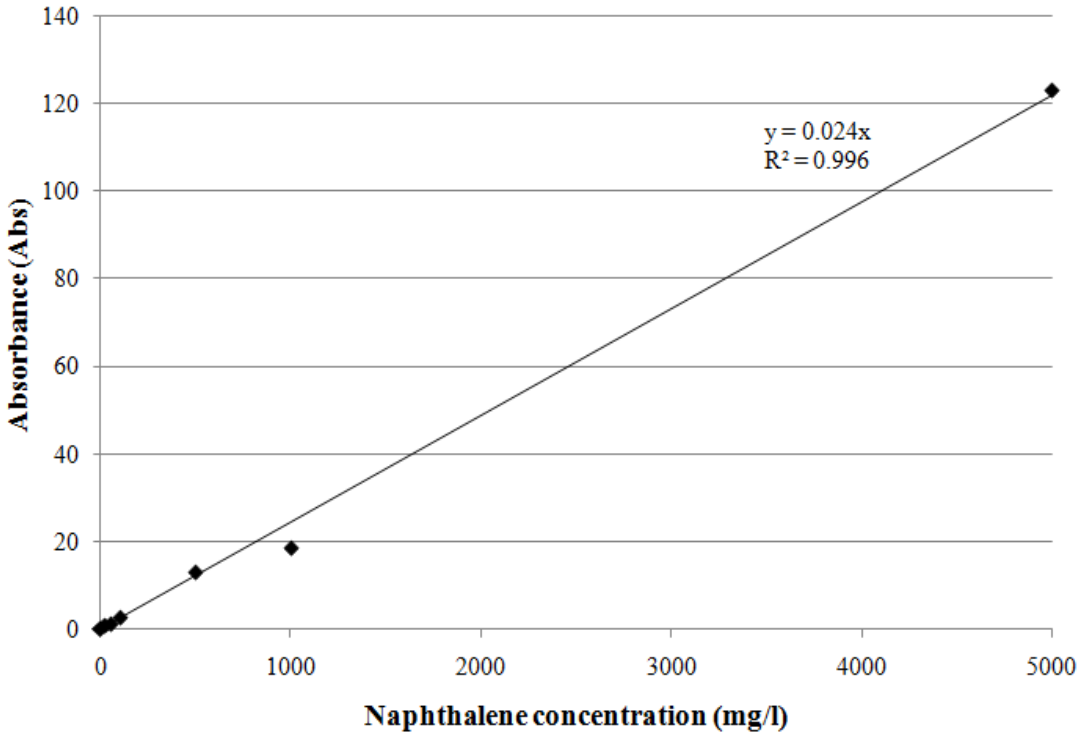

Fig C-6: Standard curve of acenaphthene from Spectrophotometer for determining naphthalene solubilization by cell-free broth containing biosurfactant and crude biosurfactant

APPENDIX D

Raw Data

Table D-1: Amount of crude oil solubilized in cell-free broth containing biosurfactant of 13 potential strains and substrates using TLC-FID

Bacterial isolates	Carbon source 3% (v/v)	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of steryl alcohol	Total peak area/Peak area of stearyl alcohol	Amount of total crude oil
Distilled water		494	306	800	10028	0.0798	103.0265
		456	0	456	8712	0.0523	96.5630
		483	0	483	9576	0.0504	114.8201
Basal medium	Bottom glycerol	579	656	1235	11070	0.1116	197.1232
		1090	639	1729	10634	0.1626	246.8590
		1183	0	1183	11374	0.1040	221.7572
	Crude oil	805	0	805	9878	0.0815	111.3927
		218	723	941	9471	0.0994	107.5338
		251	0	251	8839	0.0284	67.6974
	Diesel oil	185	913	1098	10490	0.1047	139.0885
		421	0	421	9785	0.0429	82.8495
		598	0	598	9512	0.0629	116.9207
	Lubricating oil	101	0	101	11545	0.0088	111.3061
		635	0	635	10279	0.0617	137.4206
		859	0	859	9101	0.0943	199.9510
Cellulosimicrobium sp. GY33	Bottom glycerol	1523	849	2372	16655	0.1424	153.8114
		850	0	850	103399	0.0822	84.6912
		1538	755	2293	957	0.2303	262.7352
	Diesel oil	1817	1144	2961	8771	0.3376	313.0638
		1256	930	2186	13849	0.1578	398.0545
		1426	297	1723	12682	0.1358	228.0731
Alcaligenes sp. LS	Diesel oil	1402	581	1983	9441	0.2100	274.3821
		1638	495	2133	6757	0.3157	451.0760
		1599	515	2114	7216	0.2929	279.9250

Table D-1: Amount of crude oil solubilized in cell-free broth containing biosurfactant of 13 potential strains and substrates using TLC-FID (cont)

Bacterial isolates	Carbon source 3% (v/v)	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of steryl alcohol	Total peak area/Peak area of stearyl alcohol	Amount of total crude oil
Alcaligenes sp. LS	Lubricating oil	739 1500 1680	272 138 599	1011 1638 2279	2613 8942 5631	0.3869 0.1831 0.4047	539.6304 255.1827 601.7334
Achromobacter sp. GY30	Bottom glycerol	2878 1980 1453	865 747 744	3743 2727 2197	5840 5689 5056	0.6409 0.4793 0.4345	743.5272 843.7564 652.2977
	Crude oil	1820 2190 397	0 0 335	1820 2190 732	7191 7761 8262	0.2531 0.2821 0.0886	528.8754 579.0057 478.7453
	Lubricating oil	346 484 932	229 782 257	575 1266 1189	6122 7336 4096	0.0939 0.1726 0.2903	189.9690 211.2817 232.5946
Enterobacter sp. W3-02	Bottom glycerol	639 808 887	463 419 0	1102 1227 887	7359 6294 3157	0.1497 0.1949 0.2809	137.7572 195.7734 253.7899
	Lubricating oil	1411 1474 605	114 332 0	1525 1806 605	6940 7928 1532	0.2197 0.2278 0.3949	328.5557 386.9031 445.2507
Rhodococcus sp. SSP2	Lubricating oil	1120 471 799	0 863 394	1120 1334 1193	2500 2835 5632	0.4480 0.4705 0.2118	395.0696 533.8798 256.2596
Bacillus sp. GY19	Bottom glycerol	8182 7303 8414	1639 5147 6507	9821 12450 14921	15563 15620 17010	0.6310 0.7971 0.8771	1213.412 2063.793 2914.174
	Crude oil	3562 2127 2150	2333 1193 0	5895 3320 2150	18220 15728 15152	0.3235 0.2111 0.1419	421.5315 391.3849 252.2965
	Diesel oil	2937 4992 2626	1717 2636 1424	4654 7628 4050	15236 17542 16491	0.3055 0.4348 0.2455	413.8102 935.1753 394.1205

Table D-2: Amount of crude oil remaining degraded by *Bacillus* sp.GY19 in CFMM after 14-day incubation

Incubation time (day)	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of steryl alcohol	Total peak area/Peak area of stearyl alcohol	Amount of total crude oil
0	21174 24708 19921	18684 11384 7906	29858 36092 27827	5416 6335 6548	5.5129 5.6793 4.2497	8760.0476 8851.5078 6880.6204
14	7302 5421 22569	9385 9752 24740	16687 15164 47309	6459 5829 7681	2.5835 2.6015 6.1592	5046.1822 5650.8500 7119.0070

Table D-3: Concentration of solubilized phenanthrene (mg/l) by crude biosurfactant

Crude biosurfactant concentration (g/l)	Absorbance of phenanthrene			PAHs concentration (mg/l)	SD
	1	2	3		
0.25	1.8600 1.7894 1.7784	1.8754 1.8181 1.8063	1.8840 1.8196 1.7946	11.6344 11.2362 11.1372	0.2631
0.5	1.7189 1.6417 1.6801	1.8101 1.6982 1.7143	1.8375 1.7233 1.7228	11.1108 10.4828 10.5946	0.3349
0.7	1.4583 1.8698 1.7319	1.5886 1.9383 1.7283	1.7897 1.9398 1.7519	10.0136 11.9004 10.7911	0.9482
1	13.9530 6.8290 3.9010	14.9840 6.1600 2.6060	14.4980 6.0940 2.6660	89.9275 39.5093 18.9917	36.5031
3	16.4270 16.6950 17.9540	16.8520 16.0070 16.9960	17.0180 15.8330 16.8010	104.1346 100.4865 105.2816	2.5038
5	23.5770 27.2480 34.4040	24.5490 29.6970 28.6930	24.2910 29.0210 30.9800	149.9317 177.9834 194.7764	22.6567

Table D-4: Concentration of solubilized fluoranthene (mg/l) by crude biosurfactant

Crude biosurfactant concentration (g/l)	Absorbance of fluoranthene			PAHs concentration (mg/l)	SD
	1	2	3		
0.25	1.1874 1.1877 1.2562	1.2018 1.1561 1.2417	1.2223 1.1216 1.2308	28.6625 27.5032 29.5929	1.0469
0.5	2.0941 2.0093 1.8641	2.1436 2.002 1.8957	2.1491 1.9981 1.9044	50.6889 47.6794 44.9540	2.8686
0.7	1.6703 1.6823 1.5891	1.6885 1.7414 1.6669	1.6997 1.7196 1.7167	40.1468 40.8198 39.4659	0.6770
1	5.0920 6.4150 3.3690	5.0580 5.5770 3.2450	5.0780 5.2860 3.2920	120.8571 137.1270 78.6191	30.1992
3	18.7370 16.4330 15.5020	17.3430 15.8420 15.6660	17.2060 15.9000 15.6040	422.9048 382.3413 371.2063	27.2094
5	18.2460 19.3510 20.2560	17.4390 18.9360 19.0460	17.5920 19.0460 19.6870	422.8333 455.0238 468.1667	23.3241

Table D-5: Concentration of solubilized pyrene (mg/l) by crude biosurfactant

Crude biosurfactant concentration (g/l)	Absorbance of pyrene			PAHs concentration (mg/l)	SD
	1	2	3		
0.25	0.6374 0.6466 0.7520	0.6250 0.6500 0.7489	0.6212 0.6504 0.7538	11.4158 11.8000 13.6649	1.2030
0.5	1.3888 1.3611 1.4854	1.4626 1.4332 1.4683	1.4495 1.4318 1.5240	26.0661 25.6127 27.1376	0.78303
0.7	1.7016 1.5665 1.4157	1.8005 1.6027 1.4565	1.8357 1.6171 1.4485	32.3503 29.0079 26.1861	3.0857
1	11.2270 20.6850 13.9860	11.2100 21.5820 11.0430	11.2160 21.2600 10.2980	203.9576 386.5455 214.1030	102.6139
3	13.5710 13.3980 12.8270	13.0530 12.9060 12.6930	12.9450 12.7630 12.6280	239.8121 236.7697 231.2000	4.3674
5	25.5810 30.2490 28.9560	25.9810 29.7890 33.9540	24.9220 28.7780 29.9200	463.5394 538.2788 562.6061	51.6269

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1) Waritha Tulalamba, Nanthorn Paorach, Wanwasan Wongwongsee, Natthariga Laothamteep, Ekawan Luepromchai and Onruthai Pinyakong (2012). Biosurfactant-Producing Bacteria for Enhancing Solubility of Petroleum Hydrocarbons. *14th Asia Pacific Confederation of Chemical Engineering Congress (APCChE 2012)*, Singapore, 21th-24th Feb, 2012.

2) Waritha Tulalamba, Ekawan Luepromchai, Chalermchai Ruangchainikorm, Suwat Soonglerdsongpha, Chatvalee Kalambaheti and Onruthai Pinyakong (2012). Efficiency of biosurfactant from Bacillus sp. GY19 for enhancing solubilization of polycyclic aromatic hydrocarbons. *The 4th AUN/SEED-Net Regional Conference on Biotechnology: EMERGING BIOTECHNILOGY FOR GREEN ENGINEERING*, Bangkok, THAILAND, 26th-27th Jan, 2012.