CRUDE OIL REMOVAL BY *Sphingobium* sp. MO2-4 AND
Bacillus megaterium TL01-2 IMMOBILIZED ON AQUAPOROUS GEL

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การกำจัดน้ำมันดิบโดย *Sphingobium* sp. MO2-4 และ *Bacillus megaterium* TL01-2 ตรึงบนอควาพอรัสเจล

นายอดิสรรค์ รังษีหิรัญรัตน์

Thesis Title	CRUDE OIL REMOVAL BY *Sphingobium* sp. MO2-4 AND *Bacillus megaterium* TL01-2 IMMOBILIZED ON AQUAPOROUS GEL
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อดิสรรค์ รังษีหิรัญรัตน์ : การกำจัดน้ำมันดิบโดย *Sphingobium* sp. MO2-4 และ *Bacillus megaterium* TL01-2 ตรึงบนอควาพอรัสเจล. (CRUDE OIL REMOVAL BY *Sphingobium* sp. MO2-4 AND *Bacillus megaterium* TL01-2 IMMOBILIZED ON AQUAPOROUS GEL) อ. ที่ปรึกษาหลัก : รศ. ดร.อรฤทัย ภิญญาคง, อ.ที่ปรึกษาร่วม : ดร.ชุติวรรณ เดชสกุลวัฒนา

       ผลิตภัณฑ์จากปิโตรเลียมเป็นแหล่งพลังงานสำคัญที่ใช้ขับเคลื่อนเศรษฐกิจโลก จึงส่งผลให้เกิดการแสวงหาน้ำมันปิโตรเลียมดิบเพื่อการผลิตและการใช้งานมากขึ้น ซึ่งกิจกรรมต่างๆ เหล่านี้ส่งผลให้ความเสี่ยงของการรั่วไหลของน้ำมันดิบในสิ่งแวดล้อมทางทะเลเพิ่มสูงขึ้น เพื่อเตรียมพร้อมสำหรับปัญหาดังกล่าว ในงานวิจัยนี้ แบคทีเรียจากฟองน้ำทะเลที่ไม่ก่อโรค 2 สายพันธุ์ได้แก่ *Sphingobium* sp. MO2-4 ที่มีความสามารถในการย่อยสลายน้ำมันดิบ น้ำมันดีเซล และน้ำมันเตา และ *Bacillus megaterium* TL01-2 ที่มีความสามารถในการย่อยสลายน้ำมันดิบ มีความไม่ชอบน้ำของผนังเซลล์สูง สามารถทนต่อโลหะหนักได้ และสามารถสร้างไบโอฟิล์ม ถูกนำมาพัฒนาเป็นแบคทีเรียผสม และตรึงบนอควาพอรัสเจลเพื่อใช้สำหรับการกำจัดน้ำมันดิบในน้ำทะเล การทดสอบความสามารถในการกำจัดน้ำมันดิบจากน้ำทะเลจริงของแบคทีเรียตรึงถูกแบ่งออกเป็น 3 กลุ่ม ตามรูปแบบการตรึงแบคทีเรีย เมื่อใช้ความเข้มข้นเริ่มต้นของน้ำมันดิบ 2,000 มิลลิกรัมต่อลิตร พบว่าการตรึงแบคทีเรียแบบผสมด้วยอัตราส่วนปริมาณหัวเชื้อ 2:1 (MO2-4:TL01-2) สามารถกำจัดน้ำมันดิบจากน้ำทะเลได้สูงสุดที่ 1,757 มิลลิกรัมต่อลิตร โดย 1,427 มิลลิกรัมต่อลิตร ถูกย่อยสลายภายใน 7 วัน ของการทดลอง จากผลข้างต้นการตรึงแบคทีเรียด้วยอัตราส่วนดังกล่าวจึงถูกใช้สำหรับการทดลองแบบกึ่งต่อเนื่องและระบบจำลองคลื่น การทดลองแบบกึ่งต่อเนื่องแสดงให้เห็นว่าแบคทีเรียตรึงสามารถกำจัดน้ำมันดิบได้มีประสิทธิภาพมากกว่าแบคทีเรียอิสระ นอกจากนี้การตรวจนับแบคทีเรียแบบ viable plate count และการใช้กล้องจุลทรรศน์แบบส่องกราดยังยืนยันการคงอยู่ของแบคทีเรียตรึงบนอควาพอรัสเจลตลอดการทดลอง ในขั้นสุดท้ายระบบจำลองคลื่นที่มีน้ำทะเล 20 ลิตร ถูกใช้เพื่อจำลองการกำจัดน้ำมันดิบในเหตุการณ์น้ำมันรั่วไหลในสิ่งแวดล้อมทางทะเล ผลการทดลองพบว่าแบคทีเรียตรึงสามารถกำจัดน้ำมันดิบได้มากกว่า 90% ภายใน 7 วันของการทดลอง และเมื่อเปรียบเทียบปริมาณน้ำมันดิบคงเหลือในอควาพอรัสเจลของชุดแบคทีเรียตรึงและชุดอควาพอรัสเจลปลอดเชื้อพบว่าแบคทีเรียตรึงสามารถย่อยสลายน้ำมันดิบได้ 72% จากผลการทดลองทั้งหมดแสดงให้เห็นว่าแบคทีเรียตรึงมีแนวโน้มที่จะสามารถนำไปใช้กำจัดน้ำมันดิบได้ในสิ่งแวดล้อมทางทะเลได้

สาขาวิชา   การจัดการสารอันตรายและสิ่งแวดล้อม   ลายมือชื่อนิสิต ....

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KEYWORD: Bioremediation, Immobilized bacteria, Crude oil removal, Bacterial co-culture

Adisan   Rungsihiranrut   :   CRUDE OIL REMOVAL BY *Sphingobium* sp.   MO2-4 AND   *Bacillus megaterium* TL01-2 IMMOBILIZED ON AQUAPOROUS GEL. Advisor: Assoc. Prof. Onruthai Pinyakong, Ph.D. Co-advisor: Chutiwan Dechsakulwatana, Ph.D.

Petroleum products are the major energy sources that drive global economy. The exploration and exploitation of crude petroleum oil increase the potential risk for oil spills in marine ecosystem. In this work, two non-pathogenic sponge-associated bacteria were developed as immobilized bacterial co-culture for crude oil removal from seawater. Two bacteria were *Spingobium* sp. MO2-4 that could degrade crude oil, diesel oil, and fuel oil, and *Bacillus megaterium* TL01-2 that could degrade crude oil, possessed high cell surface hydrophobicity, could tolerate heavy metal, and was able to form biofilm.. The modified polyurethane foam, aquaporous gel (AQ), was used as carrier for the bacteria. The crude oil removal from actual seawater by immobilized bacteria was carried out in three groups based on immobilization process. With the initial crude oil concentration of 2,000 mg/L, the result showed that immobilization of the bacteria as co-culture using 2:1 (MO2-4:TL01-2) inoculum volume ratio yielded the best result with 1,757 mg/L of crude oil removed and 1,427 mg/L was degraded within 7 days, thus the ratio was chosen for further use in the semi-continuous system and wave simulator tank. The semi-continuous system study revealed the better performance of immobilized bacteria over the free cell; in addition, the immobilized bacteria survivability throughout the experiment was confirmed by viable plate count and Scanning Electron Microscope (SEM). The wave simulator tank containing 20 L seawater was used to simulate the removal of oil spill by immobilized bacteria in marine environment. The result revealed that the immobilized bacteria could remove more than 90% of crude oil within 7 days. Furthermore, when compared the amount of crude oil accumulated in AQs from the immobilized bacteria treatment and sterilized AQs treatment at the end of experiment; the result showed that the immobilized bacteria could degrade 72% of the absorbed crude oil. Accordingly, these results suggested that the immobilized bacteria is a promising tool for bioremediation of crude oil-contaminated marine environment.

Field of Study:	Hazardous Substance and Environmental Management	Student's Signature ....
Academic Year:	2018	Advisor's Signature ....
		Co-advisor's Signature ....

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CHAPTER I

INTRODUCTION

1.1 State of problem

Many offshore oil and gas exploration and exploitation have been conducted around the world in order to keep up with the growing demand for energy. Crude petroleum oil or crude oil extracted from beneath the sea floor is transported through the ocean mainly by oil tanker, upon refined its yielded many products such as diesel oil and fuel oil, which act as the main energy source for various sector. Consequently, these activities can cause a severe case of oil spill in the marine ecosystem (Tornero and Hanke, 2016).

Crude oil is a complex mixture of saturated hydrocarbons, aromatic, resin, asphaltene, and a small amount of metals. Most fraction of the crude oil, especially polycyclic aromatic hydrocarbons (PAHs) and heavy metals are highly toxic because of their carcinogenic and immunotoxic properties (Desforges et al., 2016; Meckenstock et al., 2016). These pollutants are persisting in environment owing to its low solubility and high hydrophobicity; in addition, it can easily accumulate in organism fat tissue and magnify through the food chain (Mitra et al., 2012; Sanganyado et al., 2018). Petroleum hydrocarbons are priority pollutant that must be removed from environment as soon as possible (USEPA, 2014).

In an effort to remove crude oil from the marine environment, multiple techniques could be employed in the area. According to the most recent USEPA guideline, 2001, physical treatments such as booming and skimming, and chemical treatments such as using dispersant, are used as primary response to an oil spill event. These treatments can remove large portion of oil but hardly achieve complete cleanup. Therefore, bioremediation is used as follow up treatment for more thorough and complete removal. Bioremediation utilized the microbial activity to break down hazardous compounds into non-toxic or less toxic form, it is considered as cost-effective and environmentally safe method (Haritash and Kaushik, 2009). Hydrocarbon-degrading bacteria can be found throughout the marine environment; to date, many researchers were able to isolate the hydrocarbon-

degrading bacteria from the oil-contaminated marine samples (Deng et al., 2014; Oyehan and Al-Thukair, 2017). Even so, no research has addressed the marine sponges as source of hydrocarbon-degrading bacteria. Marine sponges are susceptible to the contaminant in seawater and often used as bioindicator for pollutant in the area (Batista et al., 2013; Illuminati et al., 2016). Marine sponges are host for diverse community of microorganism (Kamke et al., 2010); thus make them potentially harbor the effective hydrocarbon-degrading bacteria.

Nonetheless, in natural environment, there are many factors that hinder the bioremediation process such as fluctuation of physical factor, multiple contaminants, and the indigenous microorganism (Cipullo et al., 2018). To solve the problem, cell immobilization can be used to improve the microbial survivability in harsh environment (Bayat et al., 2015). Cell immobilization is the restriction of microbial movement on the support material, which acts like a protection for the cells. The support material formed using polyurethane is a well-known carrier because it is non-toxic, inexpensive, and easy to access (H. Li et al., 2013). Aquaporous gel is a modified polyurethane structure that increases biomass loading capacity, and durability; it is a new support material designed especially for treating contaminated water (Nisshinbo Chemical Inc., Japan). Besides the support material, bacterial biofilm is another factor that improves the immobilization efficiency and preventing cell leak (Ławniczak et al., 2011). Furthermore, using more than one type of bacteria has been proofed to be more effective than using a single strain (Isaac et al., 2015; Tao et al., 2017). Bacterial consortium can work synergistically by providing more metabolic pathways for contaminant degradation (Tzintzun-Camacho et al., 2012); additionally, some strains can produce a useful metabolite such as biosurfactant that aid other strains by reducing the surface tension and increase bioavailability of hydrocarbons (Barin et al., 2014). Moreover, biosurfactant can also increase cell surface hydrophobicity (CSH), which is one of the characteristic that help bacteria adhere to hydrocarbons molecule; thus enhanced the degradation (Zhao et al., 2011). Despite that, Thailand Ministry of Public Health, 2017 considered many well-known hydrocarbon-degrading bacteria such as *Pseudomonas aeruginosa*, *Ochrobactrum*

sp., and *Mycobacterium* sp., as pathogens, which make them unable to be used for environmental application. Hence, constructing an effective hydrocarbon-degrading bacterial consortium must be carefully assessed. Recently, Microbial Technology for Marine Pollution Treatment Research Unit, Chulalongkorn University was able to isolate nineteen hydrocarbon-degrading bacteria from marine sponge samples provided by Burapha University; under the project "Isolation and characterization of petroleum hydrocarbon-degrading bacteria capable of resistant to heavy metal from marine sponge in the eastern coast of the gulf of Thailand and development of ready-to- use bacteria for bioremediation". Among them *Sphingobium* sp. MO4-2, and *Bacillus megaterium* TL01-2 were chosen based on their crude oil degradation activity and non-pathogenic trait for further development.

Accordingly, this research aims to study the petroleum hydrocarbon degradation efficiency, biosurfactant production potential and cell hydrophobicity, heavy metals resistance, and biofilm formation of sponge-associated bacteria, *Sphingobium* sp. MO4-2, and *Bacillus megaterium* TL01-2, as single strain and co-culture. *Sphingobium* sp. MO2-4 was isolated from marine sponge *Chalinula* sp. collected from Mun Island, Rayong Province; *Bacillus megaterium* TL01-2 was isolated from marine sponge *Clathria reinwardti* collected from Thalu Island, Chompon Province. The two strains were previously isolated along with others seventeen strains from marine sponge samples from across The Gulf of Thailand in 2015. Despite the other strains possessing the ability to degrade crude oil, only two were not listed on Thai Ministry of Public Health list of pathogenic bacteria, 2017, which is the important criteria for environmental application. Therefore, the strains MO2-4 and TL01-2 were selected for development of immobilized cell for crude oil removal from seawater and further test in the wave generator tank to simulate the oil spill scenario.

1.2 Objective

1.2.1. To investigate the characteristic of *Spingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 as individual strain and co-culture.

1.2.2. To develop a suitable immobilization approach for both MO2-4 and TL01-2 and test the crude oil removal efficiency of by immobilized cells.

1.3 Hypotheses

1.3.1. Petroleum hydrocarbon degradation can be enhanced with co-culturing technique.

1.3.2. Crude oil removal efficiency in seawater of immobilized bacteria is better than its free cells.

1.4 Scope of this study

The research was divided into three phases

1.4.1 Characteristic of hydrocarbon-degrading sponge-associated bacteria

Characteristic of hydrocarbon-degrading sponge-associated bacteria including degradation of various types of petroleum hydrocarbon, biosurfactant production potential and cell surface hydrophobicity, heavy metal resistance, and biofilm formation were determined to identify the capability of the bacteria as individual strain and co-culture.

1.4.2 Development of immobilized bacteria

The biomass loading capacity was conducted to find the shortest time for immobilization that yield highest number of bacteria attached on support. After acquired the suitable time for immobilization, the crude oil removal from seawater by immobilized bacteria experiment was carried out. The experiment was divided into three groups based on immobilization approaches. The immobilization approach that yielded the best result was selected for semi-continuous experiment to compare the removal efficiency with its free cell counterpart.

1.4.3 Wave simulator tank

The immobilized bacteria crude oil removal efficiency was then tested in 40 L wave simulator tank with 20 L seawater and 3 Kg synthetic sand to simulate the oil spill scenario and compared with natural attenuation experiment.

1.5 Flow chart of this study

1. Hydrocarbon-degrading sponge-associated bacteria characteristic as individual strain and co-culture.

- Petroleum hydrocarbon degradation
- Biosurfactant production potential and cell surface hydrophobicity
- Heavy metal resistance
- Biofilm formation

2. Immobilized bacteria

- Biomass loading capacity
- Shortest time with highest amount of bacteria attached to the support.
- Crude oil removal from seawater
- Divided into 3 groups based on immobilization approach.
  - Separately immobilize then pooled together
  - Immobilize together with 1:1 (MO2-4:TL01-2) inoculum
  - Immobilize together with 2:1 (MO2-4:TL01-2) inoculum volume

Semi-continuous experiment
Immobilization approach that yielded highest removal efficiency.

3. Wave simulator tank
40 L tank with 20 L seawater and 3 Kg

CHAPTER II

LITERATURE REVIEW

2.1 Crude petroleum oil

Crude petroleum oil is a mixture of hydrocarbons and non-hydrocarbons. Generally, crude petroleum oil consists of 83-87% carbon, 10-14% hydrogen, 0.05-1.5% oxygen, 0.05-6% sulfur, and a trace of metals (<1000 mg/l) (Logeshwaran et al., 2018). These compounds can be classified into four fractions: aliphatic hydrocarbons, aromatic hydrocarbon, resins, and asphaltene as shown in Fig. 2.1. The composition ratio is varied depend on the geological setting (McMillan et al., 2016).
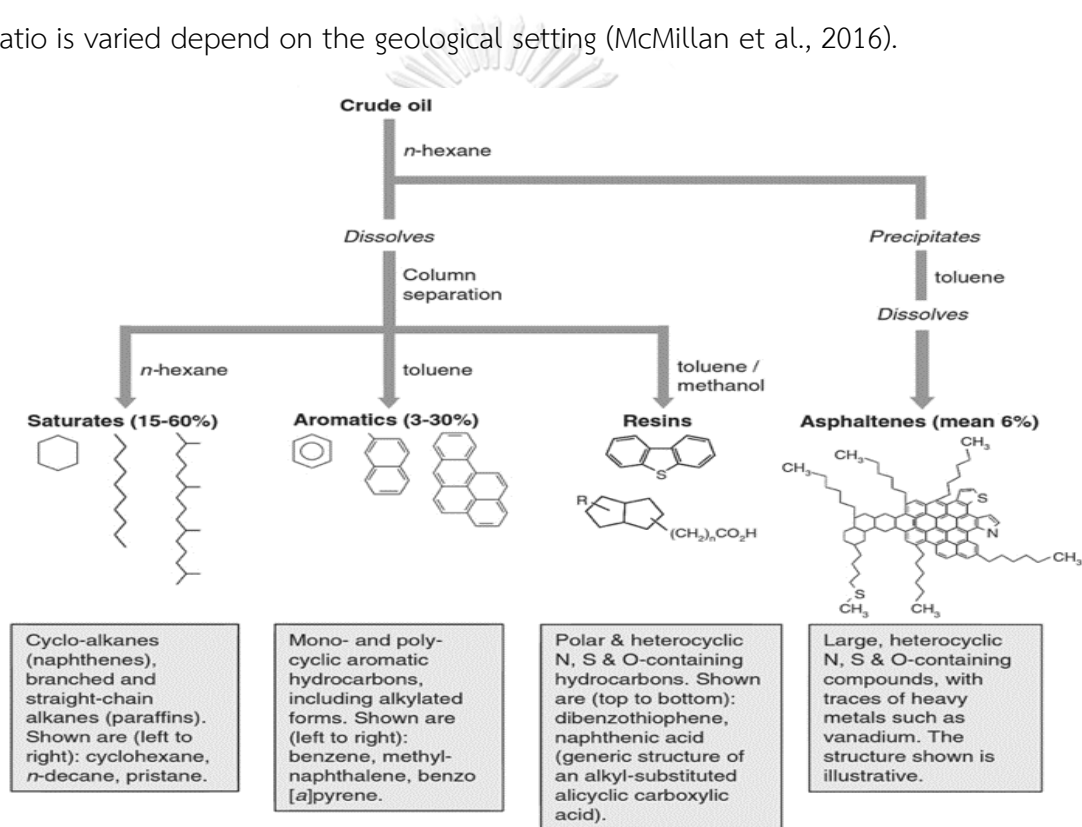
Fig. 2.1 Crude oil component

(McGenity, 2014)

Aliphatic hydrocarbons can be saturated or unsaturated hydrocarbons; these compounds are categorized according to their structure such as alkanes, branched alkanes, and cycloalkanes. However, n-alkanes (straight chain) are considered as the largest portion in crude oil. The phase of n-alkane can be divided based on the number of carbons in the chain; C1-C4 are considered as gas, C5 -C10 are volatile

liquid, C10-C16 are semi-volatile liquid, and carbon atoms greater than 16 are non-volatile liquid and solid (Brewer et al., 2013).

Aromatic hydrocarbons are ringed hydrocarbons, which have benzene as a base molecule; they can be divided into two main groups, which are monocyclic and polycyclic. Monocyclic aromatic hydrocarbons contain one aromatic ring with or without substitution; the compounds in this group are benzene, toluene, ethylbenzene and xylenes or BTEX, which are essential components in gasoline (Ali Khan et al., 2017). Polycyclic aromatic hydrocarbons or PAHs are two or more fused benzene rings. Those with two or three rings are considered as low molecular weight PAHs (LMW) such as phenanthrene, and those with four or more are high molecular weight PAHs (HMW) such as pyrene (Abdel-Shafy and Mansour, 2016). There are 16 PAHs listed by United States Environmental Protection Agency (US (USEPA, 2014)EPA) as possible human carcinogen and priority pollutant in the environment (Fig. 2.2).
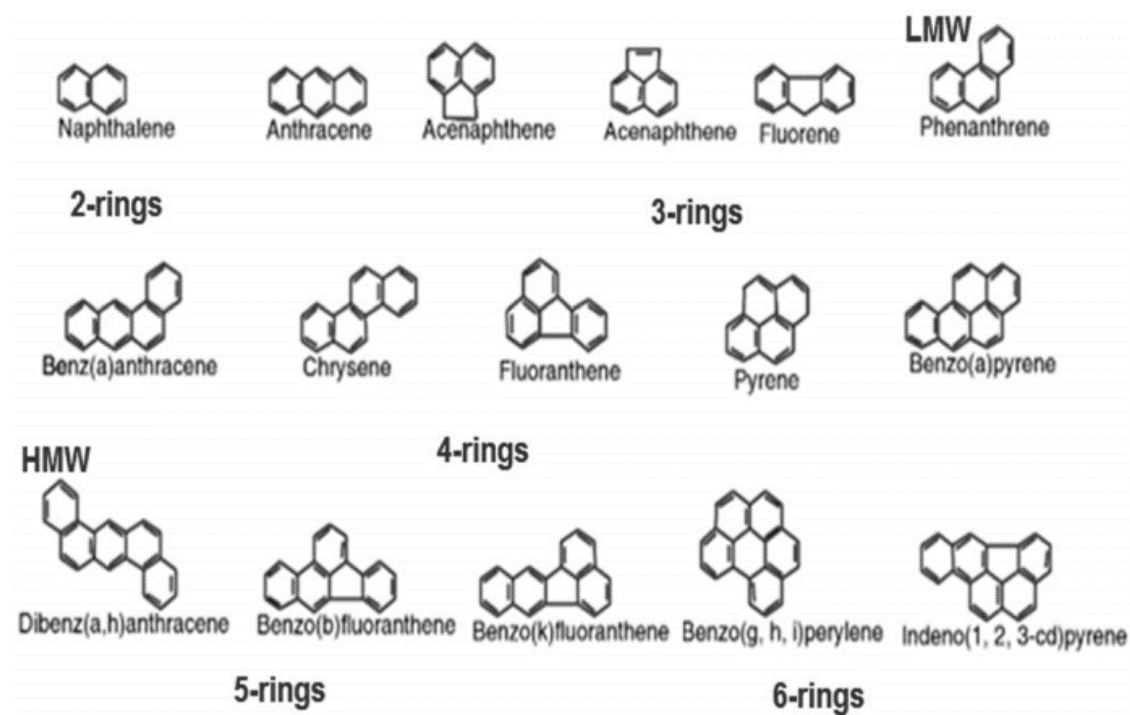
Fig. 2.2 Chemical structure of the 16 PAHs on the USEPA priority pollutant list
(Koshlaf and Ball, 2017).

Resins and asphaltenes are polar hydrocarbon formed by nitrogen, sulfur, oxygen, and metals. Both compounds contain aromatic rings and long alkyl chain; however, asphaltenes are heavier and have more complex molecular structure (Fig. 2.3). Therefore, asphaltenes present in crude oil as colloid and resins act as peptizing agent to disperse asphaltenes and promoting stability of crude oil (Chandra et al., 2013; Varjani and Upasani, 2017).
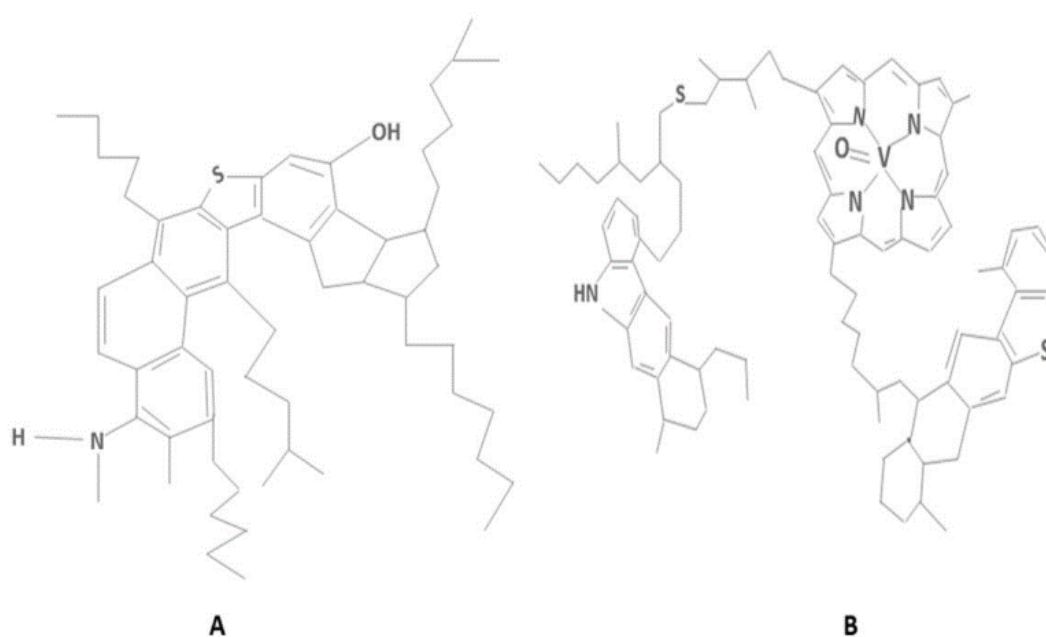
Fig. 2.3 General chemical structure of A) resin and B) asphaltenes (Koshlaf and Ball, 2017).

2.1.1 Crude petroleum oil usage

Prior any use, crude oil must pass through refining process, which is called fractional distillation, to separate each fraction based on their boiling point. The crude oil entered the distillation column and was treated with high temperature, the high molecular weight hydrocarbon will condense first, and the low molecular weight hydrocarbon will condense later as shown in Fig. 2.4 (Alhajji and Demirel, 2015). After refined, the crude oil yielded various products based on their chemical component such as petroleum gas, diesel oil, and fuel oil, which act as main energy sources for many industry (Table 2.1).
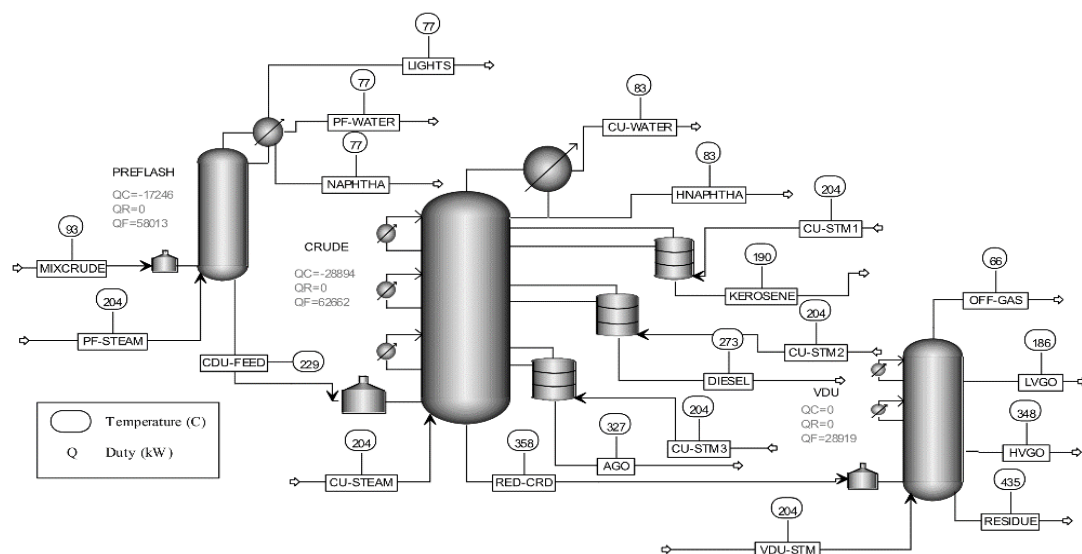
Fig. 2.4 Crude oil refining process. The number indicate the temperature (°C) used. AGO, automotive gas oil; HVGO, heavy vacuum gas oil; LVGO, light vacuum gas oil; PF-STEAM, preflash steam; CU-STEAM, crude unit steam; VDU-STM, vacuum distillation unit steam (Alhajji and Demirel, 2015).

Table 2.1 Type of petroleum products from crude oil refining process

Hydrocarbon range	Products	Boiling point (°C)
C2-C4	Light gases	-90 to 1
C4-C10	Gasoline	-1 to 200
C4-C11	Naphthas	-1 to 205
C9-C14	Jet fuel	150-255
C11-C14	Kerosene	205-255
C11-C16	Diesel fuel	205-290
C14-C18	Light gas oil	255-315
C18-C28	Heavy gas oil	315-425
C18-C36	Wax	315-500
>C25	Lubricating oil	>400
C28-C55	Vaccum gas oil	425-600
>C55	Residuum	>600

Source: Logeshwaran et al., 2018

2.1.2 Crude oil in environment

The increasing demand and the lack of supply of global oil and gas (IEA Oil Market Report, 2018) caused the increase number of oil and gas exploration, extraction, refining and transportation activities. The spillage during drilling and waste stream (produced water) generated during off-shore oil and gas exploration operation are considered as the largest sources of contaminant entering the sea by human (Bakke et al., 2013). After the extraction, crude oil main mode of transportation is through oil tanker; oil tanker accidents accounted up to 15% of all the oil entering the ocean every year (Tornero and Hanke, 2016). Table 2.2 shows major worldwide crude oil spillage event since 1978 with more than millions gallon of crude oil entering the ocean (Lim et al., 2016). There are more than hundred recorded oil spills in marine ecosystem in Thailand since 2004 as show in Fig. 2.5.

Table 2.2 Top major oil spills around the world since 1978

Date	Amount (million gallons)	Name	Location
16 March 1978	68.7	Amoco Cadiz	Brittany coast, France
3 June 1979	140	Ixtoc 1 oil well	Gulf of Mexico
19 July 1979	9	Atlantic Empress oil spill	Caribbean Sea
10 February 1983	80	Nowruz oil field	Gulf of Persian
6 August 1983	78.5	Castillo de Bellver oil spill	Table Bay, South Africa
10 November 1988	43	Odyssey oil spill	North Atlantic
23 January 1991	240-336	Gulf war	Gulf of Persian
28 May 1991	80	ABT Summer	Angola, Africa
11 April 1991	45	M/T Haven Tanker oil spill	Mediterranean sea
20 April 2010	210	Deepwater Horizon	Gulf of Mexico

Source: Lim et al., 2016
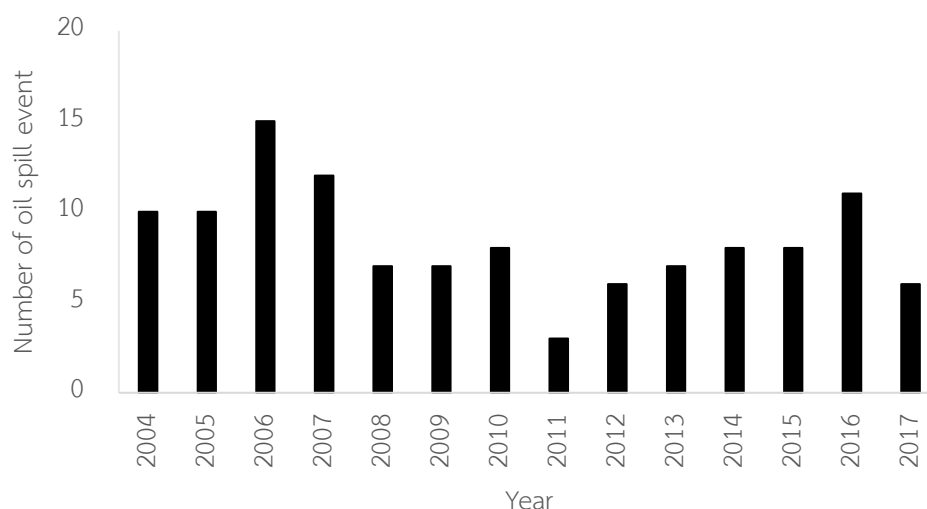
Fig. 2.5 Frequency of marine oil spill recorded in Thailand
(Marine Department, 2018)

2.1.3 Effect of crude oil contamination

Humans and animals can expose to the hydrocarbon compounds by three main routes; dermal contract (skin), inhalation (respiratory), and ingestion (food consumption) (Veyrand et al., 2013; Q. Yang et al., 2015). Many components of crude oil are persistence and highly toxic; therefore, exposure to these compounds can cause severe damage to ecosystem and organisms.

Acute exposure to hydrocarbon compounds is usually came from oil spillage; the damage is proportionally to the concentration. There are many reports on the acute toxicity of hydrocarbon compounds to marine organisms such as Gerger and Weber, 2015 reported that acute exposure to PAHs, regardless of the routes, of adult zebrafish (Danio rerio) results in cardiorespiratory dysfunction. Johansen and Esbaugh, 2017 also found that acute exposure of coastal marine fish to the compounds results in impair cardiorespiratory function and swim performance.

Chronic exposure from leakage, seepage, or waste discharge can cause long-term adverse health effect. Brown-Peterson and colleague, 2015 reported that chronic exposure to petroleum oil-contaminated sediment of juvenile Southern flounder causes severe health effect on multiple biologic levels, such as hepatic

intravascular congestion, and lamellar epithelial proliferation in gill tissues; moreover, Bai and colleague, 2017 reported that chronic exposure to PAHs by inhalation causes DNA damage and genomic instability in lung epithelial cells.

Heavy metals can also be found in crude oil and petroleum refinery waste; unlike other organic component in crude oil, heavy metals cannot be chemically or biologically degraded (Asghari et al., 2013). Heavy metals, even in small amounts can be dangerous to not only humans, but also animals and plants. It can cause dysfunction in many systems in the body, such as renal, kidney, nervous, respiratory, (Cai and Calisi, 2016). Table 2.3 shows effect of several heavy metals to human and the US EPA regulatory limit of heavy metals in the environment. Additionally, a presence of metals can cause a fluctuation of the bacterial community in certain area (Du et al., 2018) and compromise microbial activity in the ecosystem (Guo et al., 2017). Johnston and Roberts, 2009, reported that around 30 - 50% of marine organism species richness was reduced in the marine ecosystem due to the contamination of heavy metals. Montenegro and colleague, 2017, found that in the presence of copper in sediment, the hydrocarbons removal efficiency of the indigenous microorganism was reduced from 39 to 25%.

Table 2.3 Effect and US EPA regulatory limit of heavy metals

Metals	US EPA regulatory limit (mg/L)	Effect
Cd	5.00	Carcinogenic, mutagenic, endocrine disruptor, lung damage and fragile bones
Cr	0.10	Hair loss
Cu	1.30	Brain and kidney damage, liver cirrhosis and chronic anemia, stomach and intestine irritation
Hg	2.0	Autoimmune diseases, depression, drowsiness, fatigue, hair loss, insomnia, loss of memory, restlessness, disturbance of vision, tremors, temper outbursts, brain damage, lung and kidney failure

Table 2.3 Effect and US EPA regulatory limit of heavy metals (Con.)

Metals	US EPA regulatory limit (mg/L)	Effect
Ni	0.20 (WHO permissible limit)	Allergic skin diseases such as itching, cancer of the lungs, nose, sinuses, throat through continuous inhalation, immunotoxic, neurotoxic, genotoxic, affects fertility, hair loss
Pb	15	Excess exposure in children causes impaired development, reduced intelligence, short-term memory loss, disabilities in learning and coordination problems, risk of cardiovascular disease
Zn	0.5	Dizziness and fatigue

Source: Dixit et al., 2015

2.2 Bioremediation

In an effort to clean up crude oil contamination, physical and chemical processes are used such as recovery, dispersion, absorption, or burn the oil. These types of methods are quick but require extensive labor and heavy machinery. Moreover, the result of these methods alone may yield more toxic byproduct (Chandra et al., 2013). Therefore, biological method or bioremediation is often used along with these techniques to improve the removal efficiency. Bioremediation is believed to be the most effective technique to clean up the contaminant. The technique uses activities of microorganisms to break down the hazardous compound or degrade them into less toxic form (Fig. 2.6); in addition, it is cost effective and ecologically friendly (Adams et al., 2015; Das and Chandran, 2010).
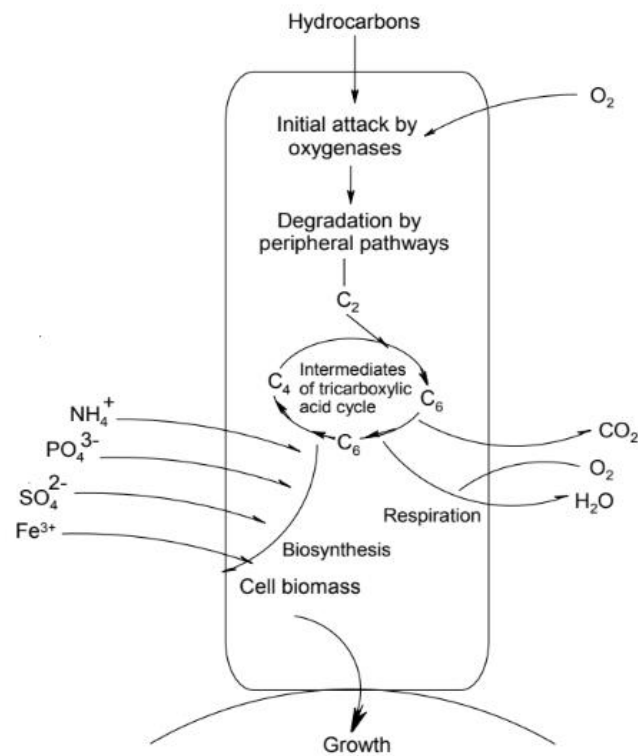
Fig. 2.6 Main principle of aerobic degradation of hydrocarbon compounds
(Olajire and Essien, 2014)

Bioremediation has three main approaches, which are natural attenuation, biostimulation, and bioaugmentation.

Natural attenuation is monitored natural recovery method, which requires no action. In some sites, the indigenous microorganisms already possess the ability to degrade contaminant compounds, thus taking no action is a more cost-effective option. However, a comprehensive review and thoughtful decision must be made before choosing this method (Perelo, 2010).

Biostimulation is the adjustment of environmental factors to promote the growth and activity of indigenous microorganisms such as low nutrient content. Ponsin et al., 2014 reported a successful biosimulation by injecting nitrogen and phosphate into benzene-contaminated groundwater. Biosurfactant is another agent added into the site, as it is non-toxic and biodegradable; and helps promoting oil

degradation efficiency by dispersing the oil, which increases oil bioavailability (Lim et al., 2016).

Bioaugmentation is done by introducing new microorganism with specific catabolic activities into the indigenous population (Abdulsalam et al., 2011). However, single strain bacterium may not have enough metabolic activity to degrade oil components. Therefore, many studies are focused on combining various types of microorganisms to improve the efficiency (Auffret et al., 2015; Mandalaywala and Trivedi, 2016; Poi et al., 2017).

2.3 Hydrocarbon-degrading bacteria

In bioremediation, the key factor is the microorganism itself. Hydrocarbon-degrading bacteria can be found throughout the environment, especially the hydrocarbon-contaminated environment, as numerous of researches have been conducted in order to enrich and isolate effective hydrocarbon-degrading bacteria from various different places (Table 2.4).

2.3.1 Sponge-associated bacteria as hydrocarbon-degrading bacteria source

Every year, large amount of petroleum hydrocarbons enters the marine ecosystem by both human activities and natural occurrence. For that reason, many studies found out that sponge could be used as bioindicator for the contamination of various substances (Batista et al., 2013; Bauvais et al., 2015; Selvin et al., 2009). Sponges are invertebrate at bottom of the sea floor; their feeding behavior is called filter feeder. By filtrate water column above the body, sponge can trap up to 80% of suspended particle within 24 hours (Milanese et al., 2003). The only mean of defense sponge has against the pollutants are microorganisms associated with them because more than 50% of sponge biomass are microorganisms (Webster and Taylor, 2012). Sponge harbors extremely diverse bacterial community within its body and core (Schmitt et al., 2012); moreover, sponge-associated microorganisms are known for their ability to produce bioactive compound as secondary metabolite such as biosurfactant, which is useful in hydrocarbon bioremediation (Dhasayan et al., 2015;

Kiran et al., 2014; Rizzo, Syldatk, et al., 2018). However, there has been no research report the ability to degrade hydrocarbon compounds of marine sponge-associated bacteria.

Table 2.4 Example of crude oil-degrading bacteria and its source

Bacterial	Crude oil (%v/v)	Degradation (%)	Days	Source	Reference
Corynebacterium sp. PG-Z	1	82	7	Oil polluted sediment	Hassanshahian et al., 2014
Acinetobacter sp. BW-1	2	75	8	Bilge water	Cappello et al., 2012
Pseudomonas aeruginosa Z41	0.5	46.40	7	Oil contaminated field	Zhang et al., 2012
Pseudomonas sp. BP10	2	60.60	30	Oil contaminated site	Kumari et al. 2012
Streptomyces parvus B7	1	82	20	Contaminated soil	Parthipan et al. 2018

Nonetheless, Microbial Technology for Marine Pollution Treatment Research Unit, Chulalongkorn University was able to isolate nineteen hydrocarbon-degrading bacteria from marine sponge samples provided by Institute of Marine Science, Burapha University. All nineteen bacterial strains were tested for their ability to degrade 2,000 mg/L crude oil and the result was reported in the project's report under the title "Isolation and characterization of petroleum hydrocarbon-degrading bacteria capable of resistant to heavy metal from marine sponge in the eastern coast of the gulf of Thailand and development of ready-to- use bacteria for bioremediation" in 2016 (Fig. 2.7)
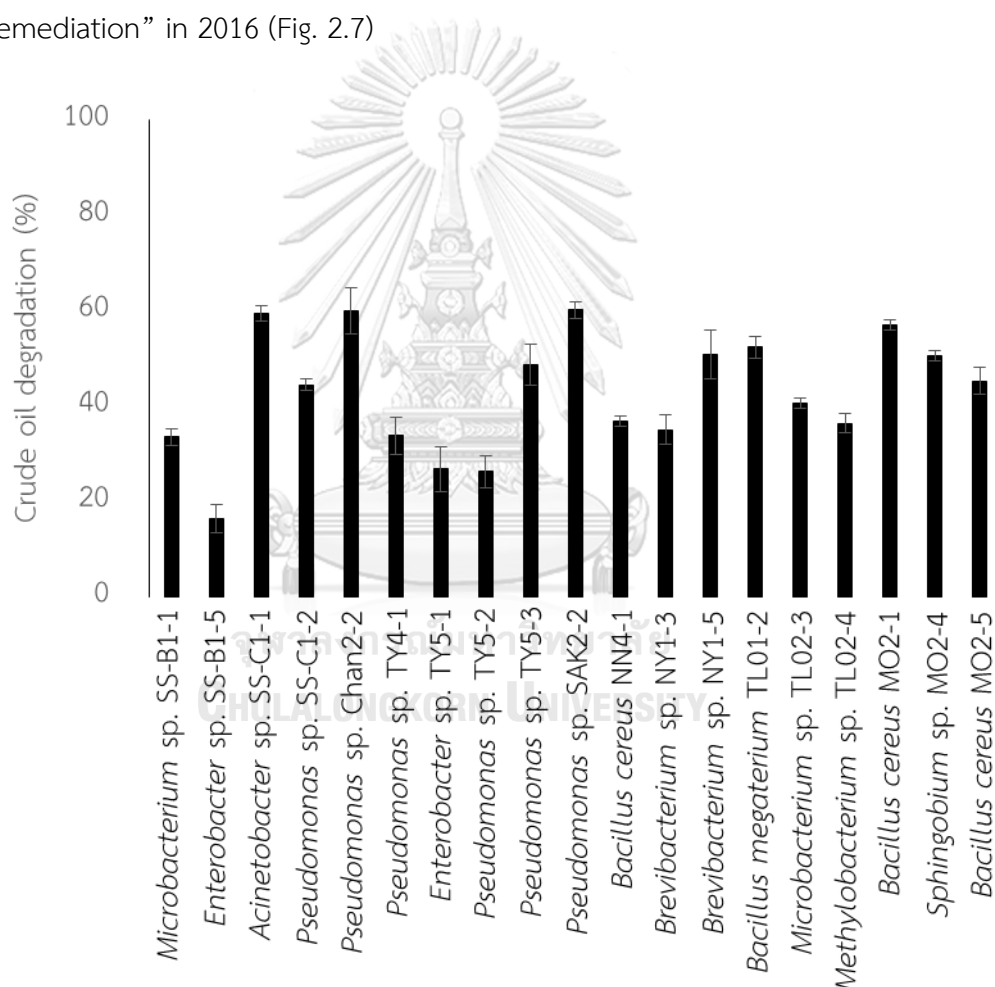
Fig. 2.7 Crude oil degradation efficiency of sponge-associated bacteria

In order to develop an applicable biotechnology for environment works, the use of non-pathogenic bacteria is more preferable. Among them, based on degradation activity and non-pathogenic trait (Thai Ministry of Public Health, 2017),

the strain MO2-4, which was identified as *Sphingobium* sp., was selected for further study. The bacterium *Sphingobium* sp. MO2-4 was isolated from marine sponge *Chalinula* sp. collected from Mun Island, Rayong province and was deposited in the culture collection of Department of Microbiology, Faculty of Science, Chulalongkorn University under the codes MSCU_0843. Briefly, It was found that the strain MO2-4 could degrade board range of substrates as shows in Table 2.5 and capable of surviving in heavy metal 100 µg/L including copper, nickel, zinc, and iron with 60-80% survivability rate. The result was also reported in the project's report mention above.

Table 2.5 *Sphingobium* sp. MO2-4 hydrocarbon degradation efficiency

Substrates	Concentration (mg/L)	Days	Degradation efficiency (%)
Crude oil	2,000	7	50.53±1.03
	4,000	14	65.69±0.95
	8,000	21	47.17±3.36
Diesel oil	2,000	7	52.54±2.72
	4,000	14	54.16±2.07
	8,000	21	43.72±0.25
Fuel oil	2,000	7	54.18±0.77
	4,000	14	29.35±1.09
	8,000	21	34.07±2.07
Tetradecane	500	7	71.47±1.67
Phenanthrene	50	7	32.67±0.4
Pyrene	50	7	25.16±2.04
Phenanthrene + Tetradecane	50 + 500	7	30.72±0.43 28.57±5.16

To further enhance both degradation activity and survivability many methods can be used such as co-culturing and immobilization. In this research, the bacterial strain TL01-2, which was identified as *Bacillus megaterium* and could degrade crude oil 2,000 mg/L for 52.30% within 7 days, was selected, based on the same criteria and non-antagonistic activity, to create effective co-culture with *Sphingobium* sp. MO2-4. The bacterium *Bacillus megaterium* TL01-2 was isolated from marine sponge *Clathria reinwardti*, collected from Thalu Island, Chumphon province, and was deposited in the same culture collection under the code MSCU_0853. The strain TL01-2 ability to degrade other hydrocarbons was tested in this work.

2.3.2 Biosurfactant production

There are still other traits of bacteria that can help enhance hydrocarbon degradation efficiency such as biosurfactant production. Biosurfactants are amphiphilic compounds containing both hydrophobic fraction and hydrophilic fraction as shows in Fig. 2.8 (Karlapudi et al., 2018). They can be categorized based on their chemical composition; the low molecular weight biosurfactants are effective for lowering surface tension, while high molecular weight biosurfactants are good at stabilizing oil-in-water emulsion (Fig. 2.9). Biosurfactants promote degradation of hydrophobic compounds, such as oil, by reduce the surface tension and increase area of contact for microbe, thus increased bioavailability of insoluble compounds (Fig. 2.10) (Souza et al., 2014). There are many records of degradation of hydrocarbons aided by additional of biosurfactant or uses of biosurfactant producing bacteria (Ali Khan et al., 2017; Lee et al., 2018; Suganthi et al., 2018)
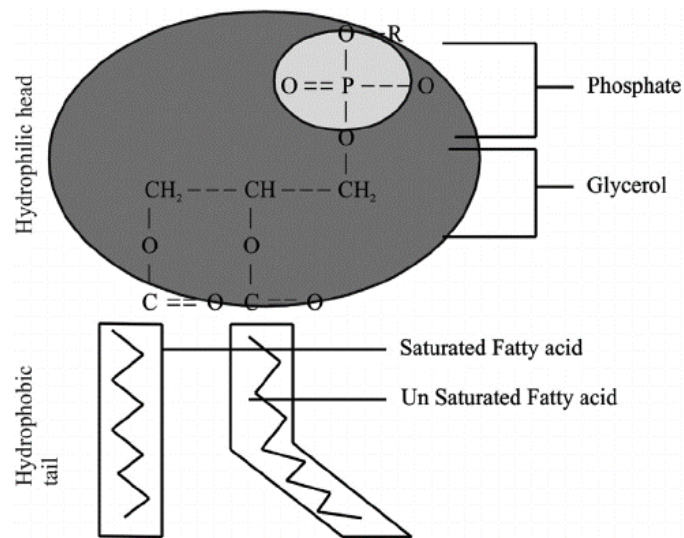
Fig. 2.8 Structure of phospholipid biosurfactant

(Karlapudi et al., 2018)
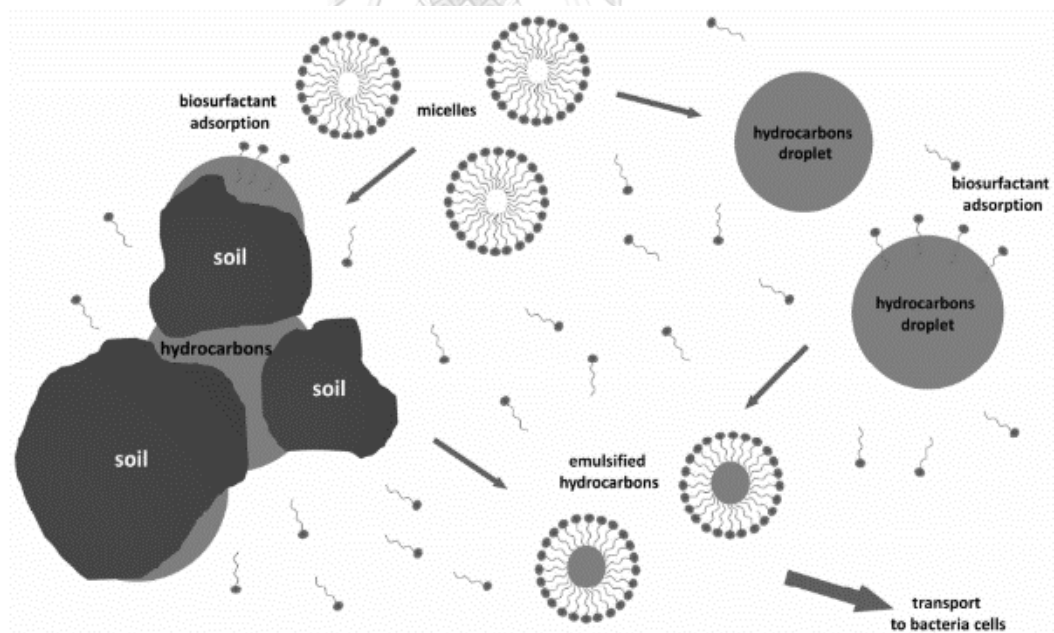
Fig. 2.9 Interaction between biosurfactants and hydrocarbon molecule

(Kaczorek et al., 2018)
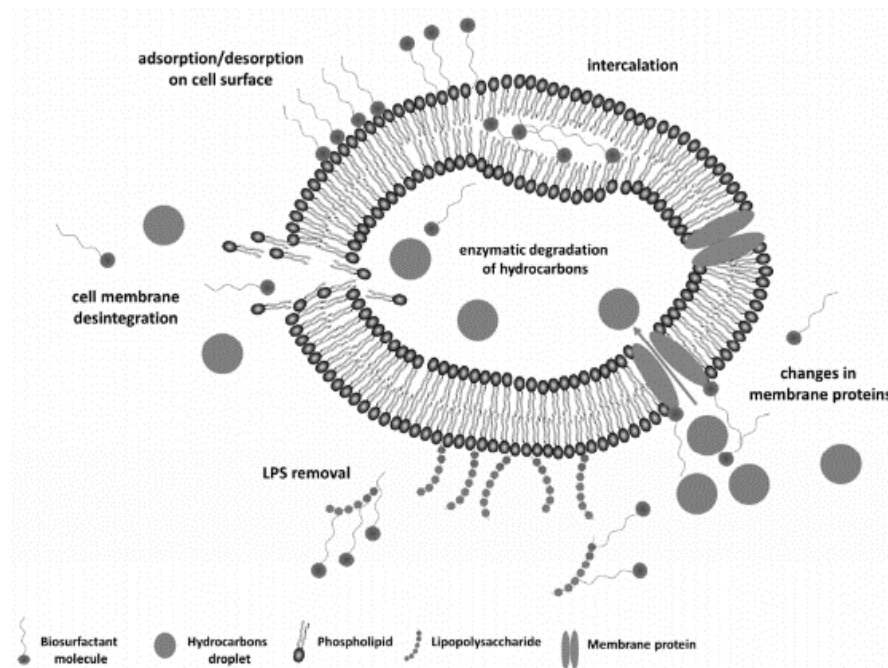
Fig. 2.10 Interaction between biosurfactant and bacterial cells

(Kaczorek et al., 2018)

2.3.2 Cell surface hydrophobicity

Cell surface hydrophobicity of bacteria also plays an important role in hydrocarbon degradation. High cell surface hydrophobicity enhances the adherence between bacteria and hydrophobic compounds results in increased bioavailability (Chao et al., 2014). The research by Obuekwe and colleague in 2009 demonstrated that there is a relationship between high cell surface hydrophobicity and high hydrocarbon degradation efficiency using salt-aggregation test and polystyrene binding assay.

2.4 Bacterial co-culture

Crude oil is a complex mixture of multiple hydrocarbons; therefore, using only single strain of bacteria is often not effective enough to completely remove crude oil from the environment (Brenner et al., 2008). It required synergistic interaction of multiple types of bacteria in order to achieve the goal; bacterial consortium can work synergistically by providing more metabolic pathway for contaminant degradation (Tzintzun-Camacho et al., 2012). Recently, many researchers used the co-culture technique in order to improve hydrocarbon removal efficiency by putting effective bacteria and biosurfactant producing bacteria together.

Isaac and colleague, 2015, formed the consortium comprises of four strains, *Pseudomonas monteilii* P26 and *Pseudomonas* sp. N3 that completely degraded 0.1 mM of naphthalene and degraded 65% and 79% of 0.1 mM phenanthrene after 48 hours, respectively, *Rhodococcus* sp, F27 and *Gordonia* sp. H19 that can degrade pyrene around 5-10% after 21 days. The result showed that the consortium could degrade mixture of naphthalene, phenanthrene and pyrene 0.1 mM each for 100, 100, and 42% within 14 days respectively.

Rizzo and co-worker in 2018, found that by co-culturing biosurfactant producing bacteria *Joostella* sp. A8, which can degrade diesel oil 2 %(v/v) for 26.8% in 20 days, with *Alcanivorax* sp. A53 and with *Pseudomonas* sp. A6, which can degrade diesel oil 2 %(v/v) for 52.7 and 38.2% within 20 days respectively, the co-culture could degrade 2 %(v/v) almost completely within 20 days.

Chen and colleague, 2017, constructed bacterial co-culture of 5 strains bacteria including *Exiguobacterium* sp. ASW-1, *Pseudomonas aeruginosa* ASW-2, *Alcaligenes* sp. ASW-3, *Alcaligenes* sp. ASS-1, and *Bacillus* sp. ASS-2, which each individual strain could degrade 1% (w/v) crude oil ranged from 50-65% in 7 days. The co-culture could achieve higher degradation percentage of 1% (w/v) within the same period of time at 75%.

2.5 Immobilization

In contrast with laboratory scale experiment, environmental conditions cannot be controlled on site. There are many factors influencing bioremediation efficiency such as temperature, type of pollutant, salinity, and nutrient (Varjani and Upasani, 2017). Therefore, increase the survival rate and activity of microorganisms to ensure the remediation efficiency is necessary. In order to do so, cell immobilization can be used to minimize the environmental effect to bacteria cell. By restricting bacteria movement on support material, immobilizations not only provide protection for the cells but also prevent cell washout, which is the major problem in the marine environment (Shen et al., 2015). There are many methods for cell immobilization including adsorption, electrostatic binding, covalent binding, aggregation, cross-linking, entrapment, and encapsulation as shown in Fig. 2.11 (Dzionek et al., 2016).
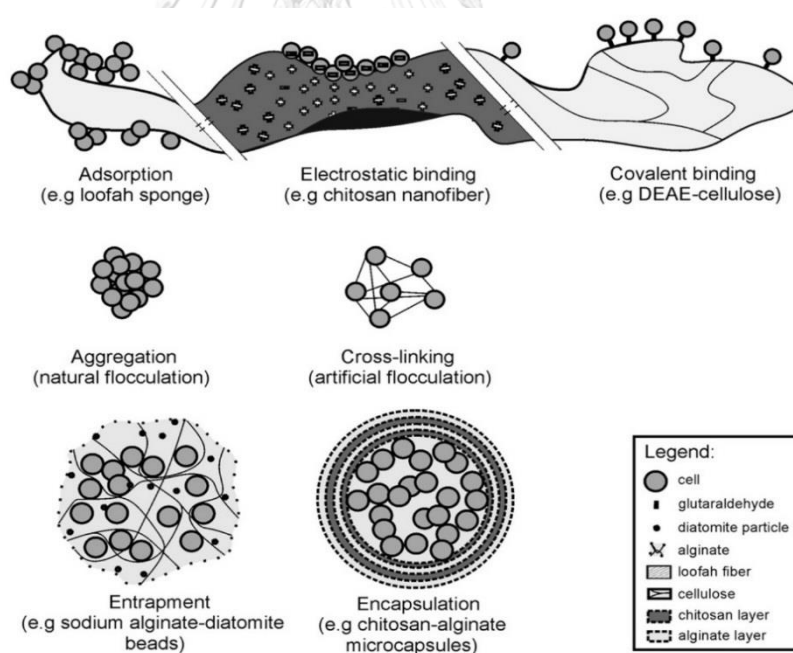
Fig. 2.11 Types of immobilization

(Dzionek et al., 2016)

Each immobilization technique has their own advantage and disadvantage based on their immobilization process. The benefit and drawback of each commonly used immobilization technique are listed in Table 2.6.

Table 2.6 Mechanism, advantage, and disadvantage of immobilization method

Methods	Immobilize mechanism	Advantage	Disadvantage
Adsorption	Physical adherence of cell on solid support	-Mild reaction -Easy to operate -Inexpensive -Fast -Not required chemical additive	-The interaction between cell and support is weak force that may cause the release of immobilized cell
Electrostatic binding	Work similar to adsorption by the supports were required to be washed with buffer to promote the hydrophilic surface that can bind with negatively charged cell surface	-Quick -Strong binding force prevent cell leaking -This method often used with enzyme immobilization	-Require chemical additive to activate the support surface -Activated supports are often toxic to cells that will lower bacterial viability and activity
Covalent binding	Use binding agent to form the covalent bond between bacteria surface and activated inorganic support	-Strong binding force prevent cell leaking -This method often used with enzyme immobilization	-Require chemical additive to activate the support surface -Activated supports are often toxic to cells; it will cause the lower bacterial viability and activity

Table 2.6 Mechanism, advantage, and disadvantage of immobilization method (Con.)

Methods	Immobilize mechanism	Advantage	Disadvantage
Entrapment	Capturing the cell inside support matrix, usually used polymer material or hallow fiber	-Fast -Inexpensive -Mild reaction -Irreversible bind that prevent cell leak, while allow nutrients and metabolite transfer.	-The immobilization process often damages the supports -Diffusion limitation of nutrient and metabolite -Deactivation during immobilization process -Low loading capacity -Difficult to operate
Encapsulation	Envelop the bacteria within spherical selective semi-permeable membrane, thus separate the bacteria from external environment	-Offer the best protective among others methods -Prevent cell leak	-Limited permeability often effect growth of bacteria

Sources: Bayat et al., 2015; Dzionek et al., 2015

There were many researches had proofed that immobilization is a useful tool for hydrocarbon removal.

Alessandrello et al., 2017, reported that co-culture of bacterial strains *Pseudominas monteilii* P26 and *Gordonia* sp. H19 immobilized on polyurethane foam (PUF) could remove 1 g/mL of crude oil for 75% within 7 days. Furthermore, they found that after stored the immobilized bacteria at 4, 15, and 30°C for 2 months; the immobilized bacteria still maintain their degradation activity.

Lin and co-worker, 2014, compared *Acinetobacter* sp. HC8-3S immobilized on cotton fibers to degrade 1 g/mL crude oil in different pH ranged 4.6-9.6 with its free-cell. Within 5 days of incubation, the result showed that at pH 5.6-8.6 both free-cell bacteria and immobilized bacteria could maintain their degradation activity at above 60% but when pH drop below 4.6 the free-cell bacteria could not degrade crude oil more than 10%, while the immobilized bacteria could degrade more than 30% of crude oil.

Wang and colleague, 2015, immobilized the bacteria *Pseudomonas* sp. ODB-1, *Brevundimonas* sp. ODB-2 and ODB-3 on expanded graphite as individual strain and separately tested for the degradation efficiency of 0.5% (v/v) diesel oil in 3.38% sea salt solution. The result showed that all three immobilized bacteria could degrade more than 85% of diesel oil within 6 days, while their free cell could degrade only 40-60%.

Due to its many advantages and a few drawbacks, the adsorption immobilization technique was chosen for this work. Support material is an essential part of adsorption immobilization, the type of support used can affect the efficiency of bioremediation and number of bacteria attracted; The ideal carrier must be non-toxic, non-biodegradable, high cell mass loading capacity, long shelf life, inexpensive, easy to separate from the environment, easy to access and regenerate (Bayat et al., a015). With the properties mentioned, polyurethane is a popular material used to synthesize carrier for bacteria. Nie et al., 2016, reported that out of three support material including, corncob, polyurethane foam (PUF), and wood chips, chosen to immobilized *Pseudomonas aeruginosa* NY3; PUF yielded the highest biomass loading capacity. Furthermore, the immobilized bacteria NY3 on PUF could remove

approximately 90% of 2 g/L crude oil within 40 days. Aquaporous gel (Fig. 2.12), according to the manufacturer Nisshinbo cooperation, Japan, is a modified polyurethane foam, which enhances immobilization efficiency and durability; it is more durable and holds more biomass than normal polyurethane foam. In addition, aquaporous gel is a low-cost material with the price of 35 baht (approximately 1 USD) per kilogram, which make the aquaporous gel not only environmentally-safe, but also easy to access.

Fig. 2.12 Aquaporous gel physical appearance

2.5.1 Bacterial biofilm

Biofilm is regarded as the community of bacteria bind together in the matrix of extracellular polymeric substances (EPS) attached on solid surface (Satpathy et al., 2016). Biofilm formed by bacteria can improve the attachment between the cells and support (Eberl et al., 2017), and solved the major problem for the adsorption immobilization technique (Table 2.4), which is the weak attachment force between support and the bacteria that may cause the leak of bacteria. Others than improve the attachment of bacteria to the support, biofilm also offer additional protection to the cells and promote the interaction between each bacterium in the coated matrix (Røder et al., 2016). Flemming et al., 2016 described the biofilm of bacteria as "emergent properties" where new properties of bacteria are exhibited in the biofilm that are not observed in free-cell bacteria as show Fig. 2.13.
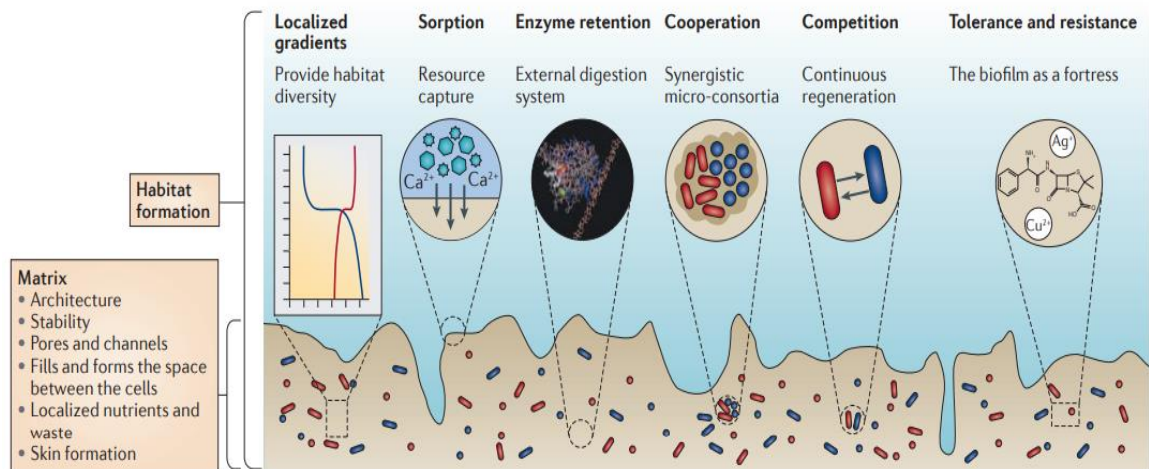
Fig. 2.13 Bacterial biofilm properties and benefit

(Flemming et al., 2016)

Mangwani and co-worker, 2016, compared the degradation efficiency of phenanthrene, which is one of crude oil components, of ten bacteria in its free-cell form and biofilm form. They found that the biofilm form of nine out of ten bacteria could degrade 100 mg/L phenanthrene significantly better than its free-cell form within 7 days. The highest degradation improvement was Pseudomonas aeruginosa N6P6 where the free-cell form could degrade 100 mg/L phenanthrene around 40%, while the biofilm form could degrade around 85% within 7 days.

CHAPTER III

METHODOLOGY

3.1 Types of equipment

1. Autoclave, Kakusan, Japan.

2. Centrifuge model 1920, Kubota, Japan.

3. Dynamic contact angle meter and tensiometer model DCAT21, Future Digital Scientific Corp., USA.

4. Deep freezer -20 $^\circ$C model MDF-U332, Sanyo Electric, Japan.

5. Deep freezer -80 $^\circ$C model ULT1786, Forma Scientific, USA.

6. Erlenmeyer flask 125mL, 250 mL, and 500 mL; Pyrex, USA.

7. Gas Chromatography-Flame Ionization Detector (GC-FID) model 6890N equipped with 320 μm × 30 m HP5 column coated with 5% of phenyl methyl ciloxane (0.25 μm), Agilent Technologies, USA.

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

9. ISSCO laminar flow model HT-122.5, International Scientific Supply, USA.

10. pH meter model 240, Corning, USA.

11. PTFE filters 0.2 μm, Chrom Tech, USA.

12. Scanning Electron Microscope model JSM-IT300LV, Jeol Ltd., Japan equipped with energy dispersive X-ray spectrometer, Oxford Instruments, UK.

13. Spectrophotometer, Thermo Spectronic, USA.

14. Multilabel Reader model 2030, PerkinElmer, Finland.

3.2 Chemicals*

1. Ammonium nitrate (NH_4NO_3), Merk, Germany.

2. Arabian light/Arabian extra light blended crude oil, PPT group co.th, Thailand.

3. Cadmium Nitrate ($Cd(NO_3)_2$), Fluka Chemika, Germany.

4. Chloroform, RCI Labscan, Thailand.

5. Cobalt Chloride ($CoCl_2.6H_2O$), Fluka Chemika, Germany.

6. Copper Nitrate ($Cu(NO_3)_2.2.5H_2O$), Fluka Chemika, Germany.

7. Diesel oil, PPT group co.th, Thailand.

8. Di-potassium hydrogen phosphate (K_2HPO_4), Merk, Germany.

9. Dipotassium chromate ($K_2Cr_2O_7$), Fluka Chemika, Germany.

10. Ethanol, Merck, Germany.

11. Ferric Citrate ($C_6H_5O_7Fe.5H_2O$), BDH Chemicals, UK.

12. Ferric Sulfate ($Fe(SO_4)_3.H_2O$), Fluka Chemika.

13. Fuel oil, PPT group co.th, Thailand.

14. Glycerol ($C_3H_8O_3$), Research Organics, USA.

15. Hexane, RCI Labscan, Thailand.

16. Lead (II) sulfate ($PbSO_4$), Fluka Chemika, Germany.

17. Methanol, Merck, Germany.

18. Mercury Chloride ($HgCl_2$), Fluka Chemika, Germany.

19. Nickel Chloride ($NiCl_2.6H_2O$), Fluka Chemika, Germany.

20. Phenanthrene, Sigma, USA.

21. Pyrene, Sigma, USA.

22. Sodium Chloride (NaCl), Merck, Germany.

23. Tetradecane, Fluka, Germany.

24. Yeast extract, Difco, USA.

25. Zobell marine broth, HiMedia, India

26. Zinc Sulfate ($ZnSO_4.7H_2O$), Fluka Chemika, Germany.

*All reagents used were analytical grade.

3.3 Procedure

3.3.1 Bacterial inoculum preparation

The bacteria used in this study were isolated from marine sponge samples from the eastern coast of the Gulf of Thailand. *Sphingobium* sp. MO2-4 was isolated from marine sponge *Chalinula* sp. and *Bacillus megaterium* TL01-2 was isolated from marine sponge *Clathria reinwardti*; both bacteria were deposited in the culture collection of Department of Microbiology, Faculty of Science, Chulalongkorn University under the codes MSCU_0843 and MSCU_0853 respectively. The bacteria were cultured separately in 250 mL Erlenmeyer flask containing 125 mL 0.25X Zobell's Marine broth and were incubated on rotary shaker 200 rpm at room temperature (27-30°C) for 1 day. After incubation, the cells were collected by centrifugation at 8,000 rpm, 4°C for 10 min; the cells were then washed twice

using0.85% (w/v) NaCl and were suspended in 0.85% (w/v) NaCl. The inoculum concentration was determined by viable plate count on Zobell's Marine agar and adjusted using 0.85% (w/v) NaCl to the final concentration of 10^9 CFU/mL.

3.3.2 Characterization of petroleum hydrocarbon-degrading bacteria

3.3.2.1 Petroleum hydrocarbon degradation efficiency

The bacterial inoculum 0.5 mL from 3.3.1 was added into 4.5 mL Nutrient Seawater medium (NSM; Appendix I), for co-culture 1:1 volume of MO2-4 and TL01-2 bacterial inoculum was mixed together prior the adding; the final concentration of the bacteria in the medium was 10^8 CFU/mL. The substrates used in this experiment were crude oil (Arab light + Arab extra light blended), diesel oil, and fuel oil at the concentration of 2,000; 4,000; 8,000 mg/L, phenanthrene 50 mg/L, pyrene 50 mg/L, tetradecane 500 mg/L and mixture of phenanthrene and tetradecane 25 mg/L and 500 mg/L separately. All treatments incubation time were 7 days, except for petroleum oils 4,000 and 8,000 mg/L, which were 14 and 21 days respectively. The control treatment was NSM containing substrate mentioned above without bacteria. The experiment was done in triplicate.

3.3.2.2 Hydrocarbon extraction from NSM medium

The hydrocarbons residue was extracted from the NSM medium after incubation using 1:1 volume of *n*-hexane:medium. Hexane was added into the medium and mixed by vortex mixer at maximum speed for 2 min; the solvent phase was then separated and evaporated at room temperature; the extraction process was repeated twice.

3.3.2.3 Hydrocarbons residue analysis by GC-FID

The analysis of crude oil and fuel oil residue was performed by GC-FID with the following condition.

HP-5 column	0.32 mm × 30 m, inner column was coated with 5% of phenyl methyl ciloxane (0.25 μm)

Column temperature	initial 40 °C for 2 min, ramp-up 40°C to 300°C at 10°C/min
Carrier gas	He
Flow rate of carrier gas	2.1 mL/min
Detector temperature	320°C
Mode	Split
Injection volume	1 μL

The analysis of diesel oil residue was performed with the following condition.

HP-5 column	0.32 mm × 30 m, inner column was coated with 5% of phenyl methyl ciloxane (0.25 μm)
Column temperature	initial 80 °C for 2 min, ramp-up 40°C to 300°C at 10°C/min
Carrier gas	He
Flow rate of carrier gas	1.7 mL/min
Detector temperature	320°C
Mode	Split
Injection volume	1 μL

The analysis of PAHs and tetradecane residue was performed with the following condition.

HP-5 column	0.32 mm × 30 m, inner column was coated with 5% of phenyl methyl ciloxane (0.25 μm)

Column temperature	initial 80 °C for 2 min, ramp-up 40°C to 300°C at 10°C/min
Carrier gas	He
Flow rate of carrier gas	1.7 mL/min
Detector temperature	320°C
Mode	Splitless
Injection volume	1 µL

The peak area obtained was used to calculate the degradation percentage using the following formula:

% degradation = (Peak area of control − Peak area of samples) × 100
                                    Peak area of control

3.3.2.4 Biosurfactant production potential and cell surface hydrophobicity

The methods used to test for biosurfactant productivity in this study were modified from Thavasi et al., 2011. The bacterial inoculum 5 mL from 3.3.1 was added into 45 mL Productive medium (Appendix I) supplemented with 2,000 mg/L crude oil and 2% (v/v) soil bean oil separately. The culture was then incubated on rotary shaker 200 rpm, room temperature for 7 days. The experiment was done in triplicate. After incubation, the cell pellet and supernatant were separated by centrifugation 8,000 rpm, 4°C for 10 min.

3.3.2.4.1 Surface tension measurement

The surface tension of supernatant from the previous step was measured using Dynamic contact angle meter and tensiometer model DCAT21 (Future Digital Scientific Corp., USA).

3.3.2.4.2 Oil displacement test

The oil displacement was tested in seawater using 1:2 ratio of crude oil: supernatant. Crude oil 20 μL was added into petri dish containing 25 mL seawater. Supernatant 10 μL was dropped into the middle of crude oil layer, then the diameter of clearing zone was measured.

$$\%displacement = \frac{\text{Diameter of clear zone}}{\text{Diameter of crude oil layer}} \times 100$$

3.3.2.4.3 Cell surface hydrophobicity test

The cell pellets from 3.3.2.4 were washed twice with Phosphate Urea Magnesium buffer (PUM; Appendix I) and were suspended in PUM buffer. The optical density (OD) of the cell suspension was adjusted to 1 at 400nm wavelength using Phosphate Urea Magnesium buffer; the cell suspension 4 mL was then mixed with 1 mL hexadecane by vortex mixer at maximum speed for 1 min and let stand for 30 min. The OD of aqueous phase was then measured by spectrophotometer at 400 nm wavelength with PUM buffer as blank. The experiment was done in triplicate. The cell surface hydrophobicity was calculated using the following formula:

$$\% \text{ hydrophobicity} = [1 - (OD_{[final]}/OD_{[initial]})] \times 1$$

3.3.2.5 Heavy metal resistance

The heavy metal resistance test used the following metals: Hg ($HgCl_2$), Zn ($ZnSO_4.7H_2O$), Fe ($Fe(SO_4)_3.H_2O$), Pb (O_4PbS), Ni ($NiCl_2.6H_2O$), Co ($CoCl_2.6H_2O$), Cd ($Cd(NO_3)_2$), Cr ($K_2Cr_2O_7$), Cu ($Cu(NO_3)_2.2.5H_2O$). The bacterial inoculum 5 mL from 3.3.1 was added into 45 mL NSM containing 100 μg/mL of each heavy metal separately; for mixed culture, 1:1 of each bacterial inoculum was mixed together prior the adding. The culture was incubated on rotary shaker 200 rpm at room temperature for 3 days. The control treatment was NSM containing bacteria without heavy metal. The experiment was done in triplicate. The survival rate of the bacteria was determined using viable plate count on Zobell's marine agar and calculated using the following formula:

$$\text{Survivability} = \frac{\text{Number of bacteria in treatment}}{\text{Number of bacteria in control}} \times 100$$

3.3.2.6 Biofilm formation

Biofilm formation test were carried out according to Coffey et al., 2014. The bacterial inoculum 100 μL from 3.3.1 was added into 900 μL 0.25X Zobell's marine broth. The culture broth 120 μL was added into 96-well plate for seven wells. The culture was then incubated at room temperature for 7 days, the sample were collected at day 3 and 7. After incubation, the wells were washed with tap water then stained with crystals violet for 15 min and de-stained with 95% ethanol for 15 min. The ethanol was then transferred into new 96-well plate and the absorbance of ethanol was measured using Multilabel Reader model 2030 (PerkinElmer, Finland). The control treatment was 0.25X Zobell's marine broth without bacteria. The result was interpreted according to Coffey et al., 2014:

$$OD_{(treatment)} \leq OD_{(control)} \quad = \quad \text{No biofilm formation}$$
$$OD_{(treatment)} \geq OD_{(control)} \quad = \quad \text{Weak biofilm formation}$$
$$OD_{(treatment)} \geq 2*OD_{(control)} \quad = \quad \text{Moderate biofilm formation}$$
$$OD_{(treatment)} \geq 4*OD_{(control)} \quad = \quad \text{Strong biofilm formation}$$

3.3.3 Immobilization

3.3.3.1 Biomass loading capacity of aquaporous gel

The AQ 1.5 g was added into 250-mL Erlenmeyer flask containing 100 mL 0.25X Zobell's Marine broth. The bacterial inoculum 10 mL from 3.3.1 was then inoculated; the culture was incubated on rotary shaker 200 rpm at room temperature for 4 days. After incubation, the AQs were separated from liquid culture and were washed with PUM buffer. The AQs were submerged in 100 mL PUM buffer and were sonicated for 2 min twice to extract the immobilized bacteria. The numbers of bacteria were quantified by viable plate count on Zobell's marine agar.

Samples of AQs on day 2 and 4 were separated and used to observe the immobilized bacteria on AQ by scanning electron microscopy (JSM-IT300LV; Jeol Ltd., Japan) equipped with energy dispersive X-ray spectrometer (EDS; Oxford Instruments, United Kingdom).

3.3.3.2 Crude oil removal by immobilized bacteria

The immobilization process was carried out in the same manner as 3.3.3.1 using the incubation time that gave the highest number of bacteria attached on AQs from 3.3.3.1. The experiment was divided into three groups based on immobilization approach. Group A, both bacterial strains were immobilized separately in the different flask with the initial cell concentration of 10^8 CFU/mL in the culture media, then pooled together afterward with 1:1 AQ weight ratio. Group B, the bacterial strains were immobilized as the co-culture in the same flask with 1:1 inoculum volume ratio, the initial cell concentration of the co-culture in the culture media was 10^8 CFU/mL. Due to the faster growth rate of the strain TL01-2, it may occupy most of the surface area of AQs; therefore, higher number of MO2-4 was used. In group C, both bacterial strains were immobilized as the co-culture in the same flask with 2:1 (MO2-4:TL01-2) inoculum volume ratio, the initial cell concentration of the co-culture in the media was 10^8 CFU/mL. The immobilized bacterial co-culture, group B and group C, were also observed under SEM after immobilization process. After incubation, the AQs were washed with PUM buffer prior transferred into 250-mL Erlenmeyer flask containing 100 mL sterilized seawater supplemented with 2,000 mg/L crude oil, the initial concentration of the immobilized bacteria in the seawater was 10^8 CFU/treatment and the total AQ weight was 1 g. The seawater used in this experiment was collected from Bang Sean beach, Chonburi, Thailand (22.37±0.38 ppt salinity). The control treatments used in this experiment were sterilized seawater and sterilized seawater containing AQs without immobilized bacteria. All treatments were incubated at room temperature, 200 rpm for 7 days. The sample was collected at day 0, 1, 3, and 7. All of the AQs were then separated from seawater and the crude oil was extracted from both seawater and AQs using *n*-hexane following 3.3.2.2 and the remaining crude oil was analyzed following 3.3.2.3. The removal efficiency of

each treatment was calculated using the following formula and compared with crude oil concentration standard curve (Appendix C):

Crude oil removal:

Crude oil in sterilized seawater $_{[Day\ 0]}$ − Crude oil in seawater of each treatment $_{[Day\ n^*]}$

Overall crude oil remaining in the system:

Crude oil remaining in seawater $_{[Day\ n]}$ + Crude oil remaining in AQs $_{[Day\ n]}$

Crude oil degradation:

Overall crude oil remaining $_{[Sterilized\ AQs]}$ − Overall crude oil remaining $_{[immobilized\ bacteria]}$

Crude oil absorption:

Crude oil removal − Crude oil degraded − Abiotic loss

*n: sampling date 0, 1, 3, or 7

3.3.3.3 Crude oil removal in semi-continuous experiment

The removal efficiency of immobilized bacteria and free cell bacteria were compared in the semi-continuous system. The immobilization approach from 3.3.3.2 that yielded the highest crude oil removal efficiency was selected for this experiment. The immobilized bacteria and free cell bacteria was added into 100 mL sterilized seawater supplemented with 50 crude oil. The final concentration of bacteria in the medium for both immobilized bacteria and free cell bacteria were 10^8 CFU/treatment. The culture was incubated on rotary shaker 200 rpm at room temperature for 35 days; the crude oil 50 mg was inoculated into the system every 7 days of incubation (the final concentration was 2,500 mg). The sample was collected at day 0, 7, 14, 21, 28, and 35. The control treatments were sterilized seawater containing crude oil without bacteria and sterilized seawater containing crude oil with sterilize aquaporous gel. The experiment was done in triplicate. At the end of the

experiment day 35, the immobilized bacteria were observed under SEM to confirm the attachment of bacteria cells. The crude oil extraction and analysis was done following 3.3.2.2 and 3.3.2.3. The removal efficiency of each treatment was calculated in the same manner as 3.3.3.2 and the growth of free cell bacteria were measured using viable plate count by spread plate on Zobell's Marine agar, while the growth of immobilized bacteria were done following 3.3.3.1.

3.3.4 Crude oil removal in wave simulator tank

The immobilization approach from 3.3.3.2 that yielded the highest crude oil removal efficiency was selected for this experiment. The experiment was conducted in 40 L glass tank with 50 cm length, 20 cm width, and 40 cm depth show in Fig. 3.1. Seawater 20 L and synthetic sand 3 Kg were added into the tank equipped with wave generator 3 watts, and the crude oil 1,000 mg/L was introduced into the tank. The immobilized bacteria 5 g/L was put inside the nylon net and added into the system at the marked point shown in Fig. 3.1. The incubation time was 28 days at room temperature; the control treatments were the tank containing 20 L of seawater and 3 Kg of synthetic sand without immobilized bacteria and the tank containing the same volume of seawater and synthetic sand with 1 g/L sterilized aquaporous gel in nylon net. The sample was collected at two different depth from nine location marked in Fig. 3.1. Seawater 10 mL and were taken aseptically at each sampling point on day 0, 7, 14, 28. The samples collected from each point grouped in the rectangular were pooled together; the total seawater sample was 60 mL/group, 180 mL/treatment. At the end of the experiment, day 28, the immobilized bacteria were taken aseptically. The number of total bacteria from each sample group was determined using MPN method following Johnsen et al., 2002. The crude oil residue in the each sample and aquaprous gel was extracted and analyzed following 3.3.2.2 and 3.3.2.3.

(A)

(B)

(C)
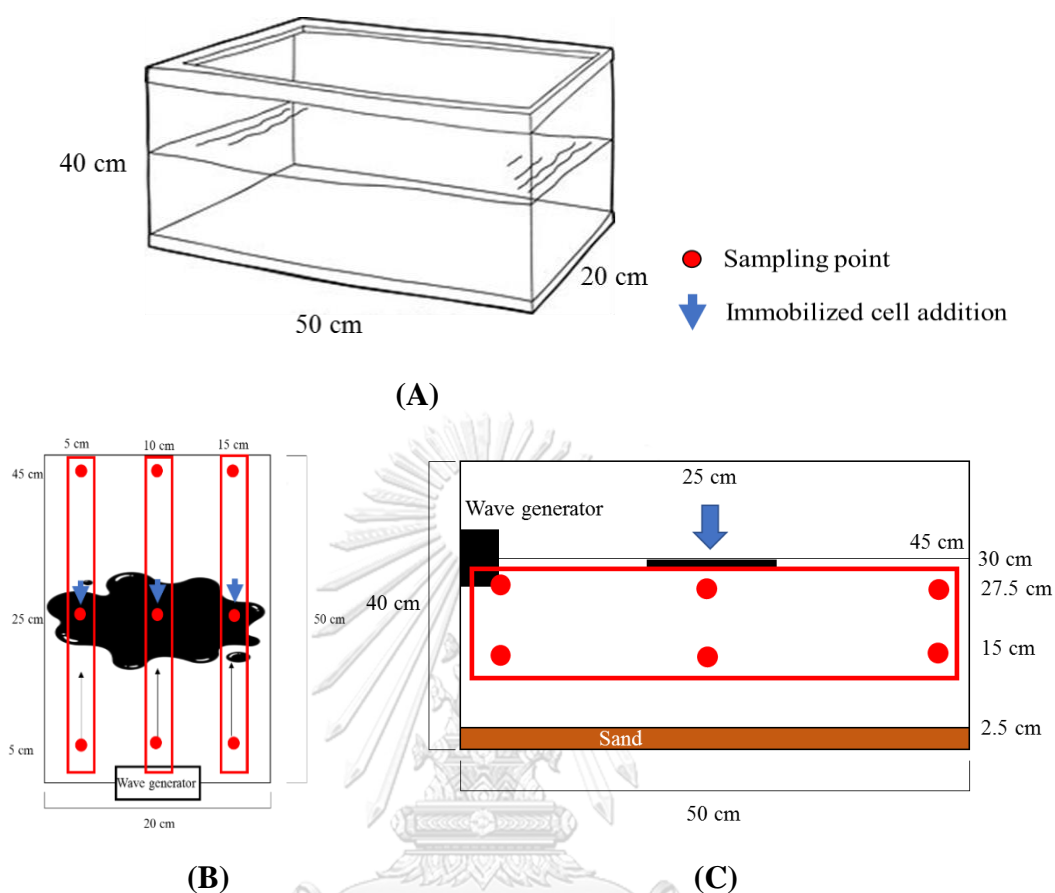
Fig. 3.1 Schematic of wave simulator tank (A) overall structure, (B), top view of wave simulator tank and sampling location (C), longitudinal-section of wave simulator tank and sampling location.

Each group represents one replicate from the wave simulator tank; the total was three replicates. The crude oil removal from sea water sample was calculated using the following formula:

$$\text{Crude oil removal} = \frac{\text{Total peak area}_{[NA^*]} - \text{Total peak area}_{[IM^*]}}{\text{Total peak area}_{[NA]}}$$

*NA: Natural attenuation; IM: Immobilized bacteria treatment

3.3.5 Statistical analysis

The differences between results in each treatment were compared using One-way ANOVA and independent t-test followed by Duncan's multiple range test at 95% confidence interval on SPSS 23.0 for Windows.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characteristic of hydrocarbon-degrading bacteria

The hydrocarbon-degrading bacteria used in this study were *Sphingobium* sp. MO-4, which was isolated from marine sponge *Chalinula* sp. and *Bacillus megaterium* TL01-2, which was isolated from marine sponge *Clathria reinwardti*; both bacteria were deposited in the culture collection of Department of Microbiology, Faculty of Science, Chulalongkorn University under the codes MSCU_0843 and MSCU_0853 respectively.

4.1.1 Biosurfactant production potential and cell surface hydrophobicity (CSH)

The hydrocarbon biodegradation could be enhanced by biosurfactant produced by bacteria; moreover, addition of biosurfactant-producing bacteria was proofed to help increase hydrocarbon degradation efficiency of indigenous bacteria (Mnif et al., 2015). The biosurfactant productivity of both strains, *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2, were tested in productive media with 2 % (v/v) soybean oil and 2,000 mg/L crude oil separately. It was found that both strains could lower the surface tension of productive media from 50.65 mN/m to around 38 mN/m when using 2 % (v/v) soybean oil as a substrate, while slightly decrease of surface tension was observed when using crude oil as substrate for both strains (Table 4.1). The similar result was observed in the oil displacement test, the percentage of oil displacement of the strain MO2-4 and TL01-2 from the 2% (v/v) soy bean oil culture was higher with the percentage of 14.04 and 12.27%, respectively, whereas the crude oil culture was 13.09 and 5.78%, respectively. On the others hand, the strain TL01-2 showed high CSH at 66 and 67 % when using 2% (v/v) soybean oil and 2,000 mg/L crude oil as substrate respectively (Table 4.1).

They had been many researches and reviews indicated that biosurfactant produced by microorganism is a crucial factor that can enhance the biodegradation of hydrocarbon compound; it help reduce the surface tension and promote the interaction between cell and hydrocarbon molecule (Souza et al., 2014; Chen et al.,

2015). It had been reported that the genus *Bacillus* are capable of producing biosurfactant. *Bacillus licheniformis* Y-1 was isolated from oil field; after optimization, the biosurfactant produced by the bacterium could lower the surface tension of water from 74.66 to 27.26 mN/m (Liu et al., 2016). Moreover, the bacterium *Bacillus megaterium* TL01-2 exhibited high cell surface hydrophobicity, which is a useful characteristic that can help the bacterial cell adhere to hydrocarbon molecule, thus initiated the degradation process (Obuekwe et al., 2009). Although there is no report on the biosurfactant production of the bacteria in the genus *Sphingobium*, the bacterium *Sphingobium* sp. MO2-4 in this study showed the potential for biosurfactant productivity.

Table 4.1 Biosurfactant productivity and cell surface hydrophobicity of both *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2

Treatments	Substrates	Surface tension (mN/m)	Oil displacement (%)	Cell surface hydrophobicity (%)
Control (Productive media)	-	50.65 ± 0.40^c	0.00	-
Sphingobium sp. MO2-4	Crude oil	$41.60\pm2.95^{a,b}$	13.09 ± 3.20^a	49.61 ± 1.59^b
	Soybean oil	38.12 ± 0.82^a	14.04 ± 2.20^a	40.98 ± 1.13^c
Bacillus megaterium TL01-2	Crude oil	$45.06\pm2.88^{b,c}$	5.78 ± 2.12^b	67.18 ± 2.49^a
	Soybean oil	38.54 ± 3.75^a	12.27 ± 2.67^a	66.18 ± 1.64^a

*Alphabet indicated the significant different at 95% confidence interval.

**Surface tension of water at 72.00 Nm/m was used as blank.

4.1.2 Heavy metal resistance

The heavy metal resistance test of both strains, MO2-4 and TL01-2, was done in NSM medium with 100 µg/L of 9 heavy metals separately including Hg, Zn, Fe, Pb, Ni, Co, Cd, Cr, and Cu. The result showed that the strain TL01-2 could survive in all types of metal tested with more than 80% survivability, while the strain MO2-4 could only survive in Cu, Ni, Zn, and Fe with 60-80% survivability and could not survive in Co, Cd, and Cr as shown in Fig. 4.1. Heavy metals can be found as a part of crude oil depends on the origin (McMillan et al., 2016), it is highly toxic even in a small amount. To remediate the environment contaminated with crude oil, the bacteria should be able to tolerate to heavy metal. Oyetibo et al., 2017 used the heavy metal resistant *Bacillus subtillis* to degrade crude oil in the presence of Ni and Co; due to no metals absorbed on cell surface, they suggested that the bacilli used extracellular detoxification to prevent heavy metal from entering the cell. Moreover, Wang et al., 2018, demonstrated that *Bacillus megaterium* H3 could immobilized Cd ion in soil and decreased bioavailability of Cd to plant. Chen et al., 2016, showed that *Sphingobium* sp. PHE-SPH could tolerate Cu and small amount of Cd, they also found that the metals were accumulated within cytoplasm of *Sphingobium* sp. PHE-SPH cell, which is the reason where *Sphingobium* sp. could only tolerate heavy metal to the certain amount.
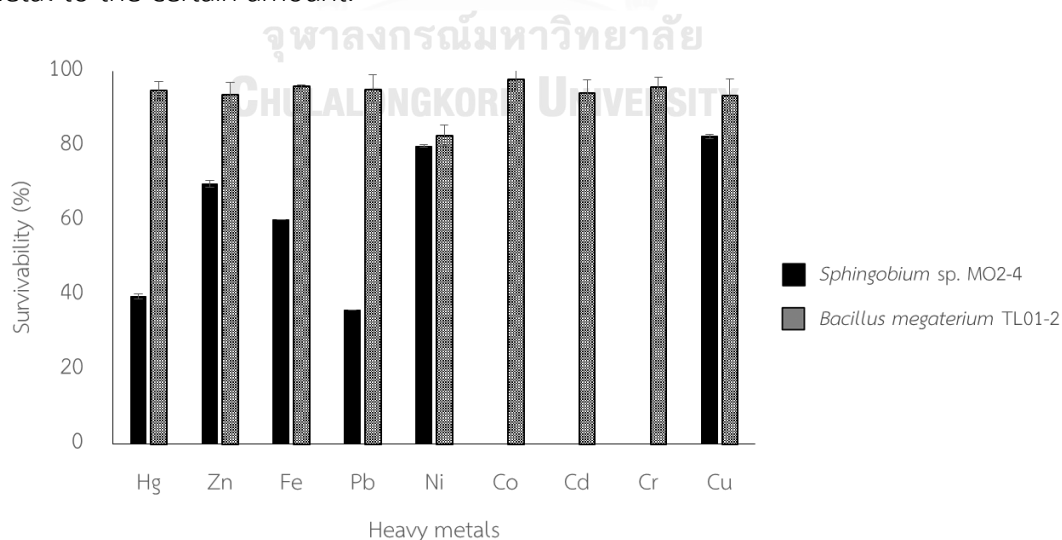
Fig. 4.1 Bacteria survivability in NSW containing 100 µg/L heavy metal after 3 days of incubation

4.1.3 Biofilm formation

Bacterial biofilm plays important role in bacterial survivability in the environment, it offers protection for the cells as well as improves the attachment of the cells on solid surface (Eberl et al., 2016). In this study, the biofilm formation of both *Sphingobium* MO2-4 and *Bacillus megaterium* TL01-2 was determined using crystal violet staining assay. The result showed that the bacterium *Bacillus megaterium* TL01-2 could form moderate to strong biofilm within 3 days of incubation; however, when prolonged the incubation time to 7 days the biofilm formed was dramatically decreased (Fig. 4.2). On the other hand, the bacterium *Sphingobium* sp. MO2-4 showed no sign of biofilm formation for both day 3 and 7 (Fig. 4.2). There were many reports associated genus *Bacillus* with the ability to form biofilm (Elumalai et al., 2019; Parthipan et al., 2017; J. Zhang et al., 2018). Other than protection for the cells mentioned above, biofilm could also promote interaction between members of the matrix (Roder et al., 2016). Therefore, a presence of biofilm forming bacteria in a co-culture could be beneficial for immobilized bacteria development.
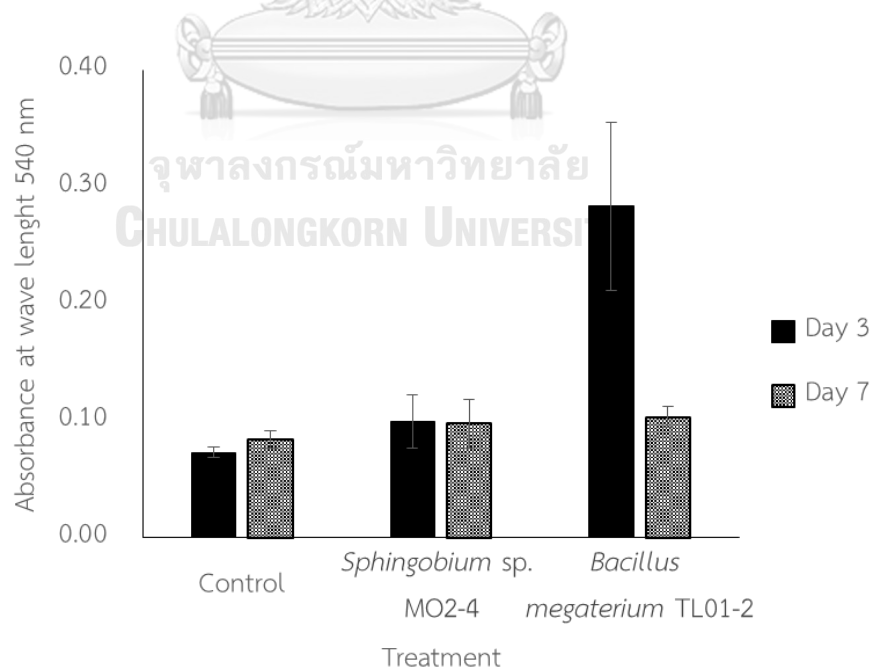
Fig. 4.2 Crystals violet staining assay for biofilm formation test

4.1.4 Hydrocarbon degradation efficiency

The degradation efficiency of various types of hydrocarbon by the strain MO2-4 was previously characterized (Table 2.5). In this study, the strain TL01-2 was tested using the same substrates including 2,000; 4,000; 8,000 mg/L crude oil, diesel oil, fuel oil, 50 mg/L phenanthrene, 50 mg/L pyrene, 500 mg/L tetradecane and the mixture of 25 and 500 mg/L phenanthrene and tetradecane respectively. The result showed that, even though, the strain TL01-2 could degrade petroleum oils lower than the strain MO2-4, it shows slightly higher degradation for phenanthrene and significantly higher for pyrene as shown in Fig. 4.3. Bacteria in the genus *Sphingobium* and *Bacillus* were usually isolated from hydrocarbon contaminated environment. There were several reports of both genus able to degrade petroleum oils, such as *Sphingobium* sp. P2 (Khondee et al., 2015), *Bacillus* sp. AKS2 (Chettri et al., 2016), *Bacillus thuringiensis* B3, and *Bacillus cereus* B6 (Raju et al., 2017) as well as PAHs, for example *Sphingobium* sp. FB3 (Fu et al., 2014), *Sphingobium* sp. KK22 (Maeda et al., 2013), *Bacillus megaterium* YB3 (Meena et al., 2016), and *Bacillus simplex and Bacillus pumilus* (Rabodonirina et al., 2019).

Crude oil is a complex mixture of hydrocarbon; to effectively remove crude oil from the environment, the co-culture of different types of bacteria have been shown to have the potential to be more effective than using individual bacteria (Dellagnezze et al., 2016; Tao et al., 2017). Therefore in this work, the hydrocarbon degradation activity of the co-culture of strains MO2-4 and TL01-2 was investigated using the substrates mentioned above. It was found that the co-culture of MO2-4 and TL01-2 could improve some of the hydrocarbon degradation efficiency. The notable improvement was found in 2,000 mg/L crude oil and diesel oil 4,000 mg/L fuel oil, 500 mg/L tetradecane and the mixture of phenanthrene and tetradecane as shown in Fig. 4.3. Even though the co-culture could not improve the degradation of all hydrocarbons, it still showed more potential than using individual strain. Furthermore, the biosurfactant productivity of the co-culture was also tested; the co-culture percent oil displacement was higher than the individual strain as show in Table 4.2. The enhancement of hydrocarbon degradation by co-culture was reported in many works. For example, the co-culture of *Bacillus* sp. TCOB-4 and *Castellaniella*

sp. TCOB-5 could enhance the crude oil degradation (x. Li et al., 2016) and the co-culture of *Joostella* sp. A8 and *Alcanivorax* sp. A53 could achieved higher diesel oil degradation than its individual strain (Rizzo et al., 2019). Nonetheless, the hydrocarbon degradation improvement of the co-culture was heavily depended on the relationship between members and their roles in the co-culture population (Kamyabi et al., 2017; R. Yang et al., 2019).
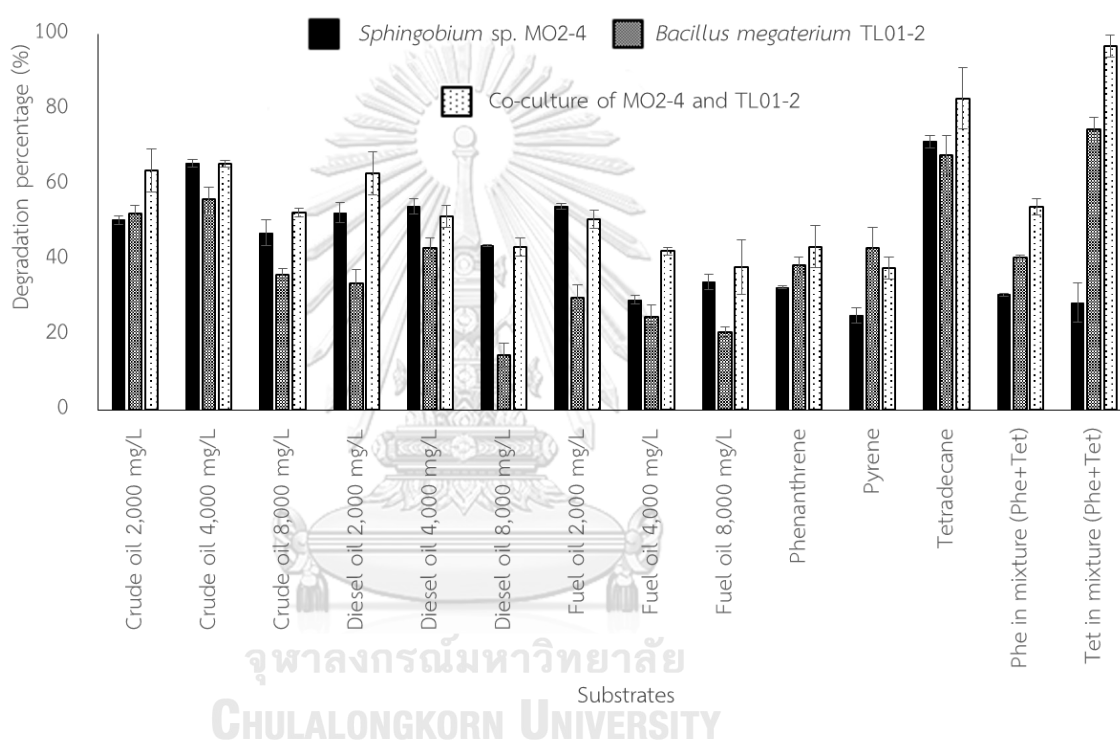
Fig. 4.3 Hydrocarbon degradation of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 as individual strain and co-culture. All treatments incubation time were 7 days, except for petroleum oils 4,000 and 8,000 mg/L, which were 14 and 21 days respectively.

Table 4.2 Biosurfactant productivity and oil displacement of co-culture and individual bacteria

Bacteria	Substrates	*Surface tension reduction (mN/m)	Oil displacement (%)
Sphingobium sp. MO2-4	Crude oil	9.05±2.98[b]	13.09±3.20[c]
	Soybean oil	12.53±0.91[a,b]	14.04±2.20[c]
Bacillus megaterium TL01-2	Crude oil	5.59±2.91[c]	5.78±2.12[d]
	Soybean oil	12.11±3.77[b]	12.27±2.67[c]
Co-culture of MO2-4 and TL01-2	Crude oil	8.77±0.98[b]	24.63±1.83[b]
	Soybean oil	15.14±0.13[a]	42.12±5.77[a]

*Surface tension reduction: surface tension of control – surface tension of treatment

** Alphabet indicated the significant different at 95% confidence interval.

In summary, the bacterium *Sphingobium* sp. MO2-4 showed board hydrocarbon degradation activity, while *Bacillus megaterium* TL01-2 possessed others characteristics that could assist the degradation process such as heavy metal resistance and high cell surface hydrophobicity. Moreover, the co-culture of both *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 could improve the degradation efficiency of some hydrocarbons. In addition, *Bacillus megaterium* TL01-2 could form the biofilm, which could help in the immobilization process. Furthermore, both *Sphingobium* sp. MO2-4 and *Bacillus megaterium* T01-2 were not listed in the pathogenic microorganism list by Thai Ministry of Public Health, 2017. Therefore, the co-culture of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 was used for the development of ready-to-use immobilized bacteria.

4.2 Development of immobilized bacteria

4.2.1 Biomass loading capacity

Biomass loading capacity of aquaporous gel (AQ) for both bacteria was determined to find the highest amount of bacteria attached on AQ in the shortest amount of time. The result yielded revealed that the number of bacteria attached on AQs from the first to fourth day was similar at around 8 Log CFU/gAQ as shown in Fig 4.4. Moreover, the immobilized bacteria on the second day of immobilization were observed under scanning electron microscope (SEM) and the images are shown in Fig. 4.5. The SEM profile showed the differences between cells size, *Bacillus megaterium* TL01-2 cell size is bigger than *Sphingobium* sp. MO2-4. The shortest time, which was one day incubation time for both bacteria, was selected for the next experiment.

(A)

(B)
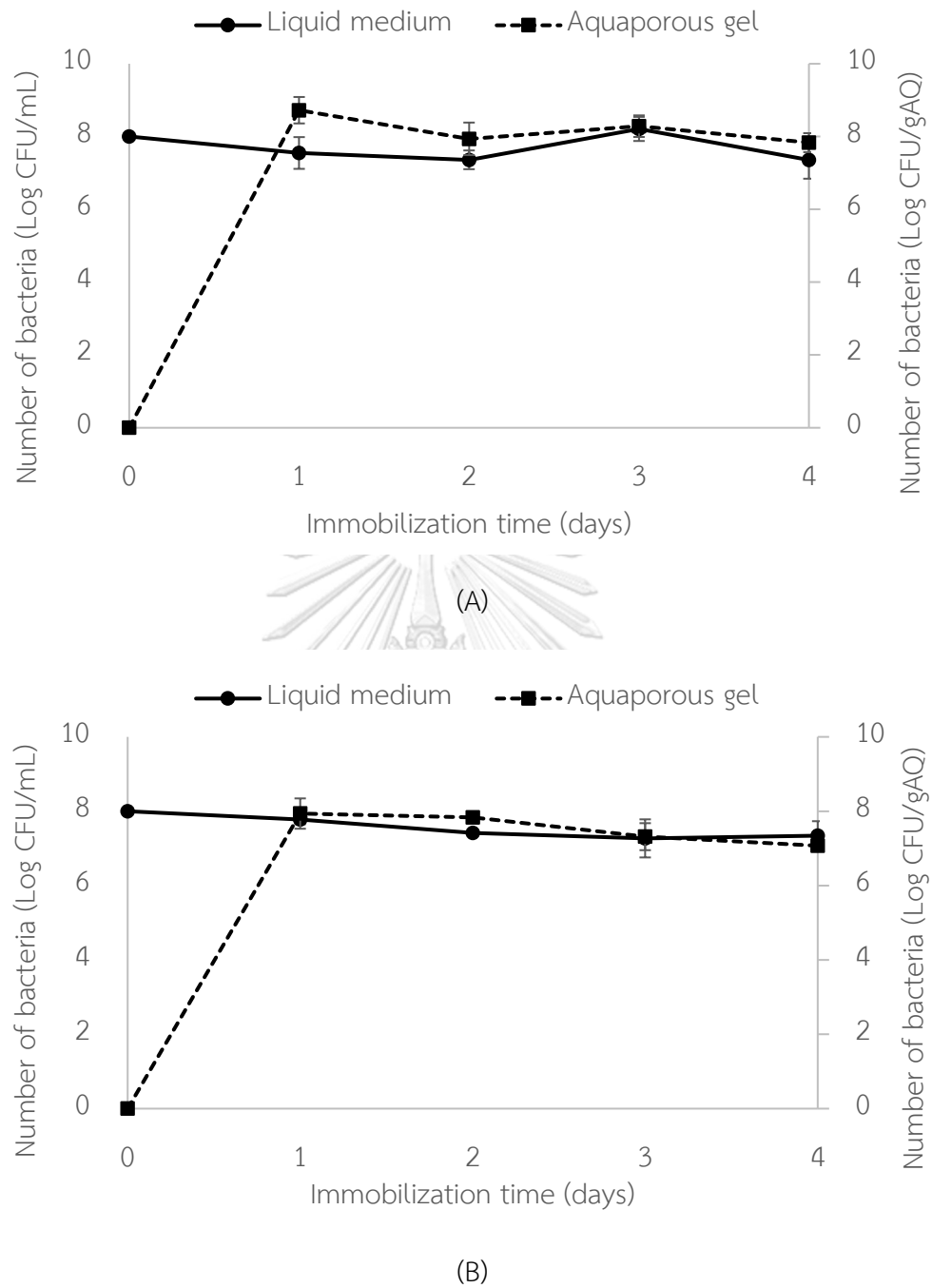
Fig. 4.4 Number of bacteria in liquid medium and AQ after each day of

immobilization

(A), *Sphingobium* sp. MO2-4, (B), *Bacillus megaterium* TL01-2

(A)   (B)   (C)
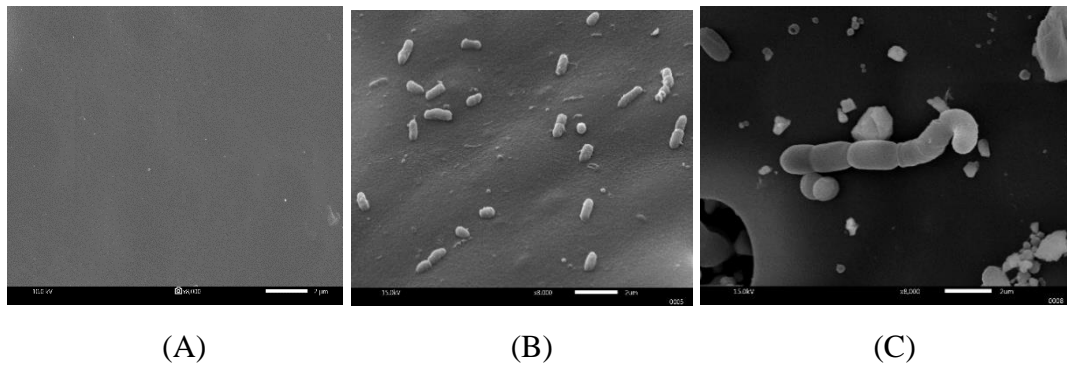
Fig. 4.5 SEM profile at 8,000X magnified power of (A) Sterilized AQ, and immobilized bacteria on AQ, (B) *Sphingobium* sp. MO2-4, (C) *Bacillus megaterium* TL01-2

4.2.2 Co-culture immobilization

After acquired the suitable time for immobilization of individual strain, the co-culture immobilization was conducted. This experiment was divided into 2 groups, in the first group, the bacteria *Sphongobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 were immobilized together in the same flask using the inoculum volume of 1:1 (MO2-4:TL01-2). Owning to the faster growth rate and bigger cell size of *Bacillus megaterium* TL01-2 it may occupied most of AQ surface area before *Sphingobium* sp. MO2-4. Therefore in the second group, the higher inoculum volume of *Sphingobium* sp. MO2-4 was used; the inoculum volume was adjusted to 2:1 (MO2-4:TL01-2). After incubation for one day, the result showed that *Bacillus megaterium* TL01-2 was able to attach on AQ at around 8 Log CFU/gAQ on both groups, while the number of *Sphingobium* sp. MO2-4 attached on AQ in the 2:1 (MO2-4:TL01-2) inoculum volume ratio treatment was higher than the 1:1 (MO2-4:TL01-2) inoculum volume ratio treatment (Table 4.3). The SEM profile also agreed with the viable plate count result, Fig. 4.6C showed the SEM profile of AQ from 2:1 (MO2-4:TL01-2) inoculum ratio treatment, which revealed higher number of *Sphingobium* sp. MO2-4 than the AQ from 1:1 (MO2-4:TL01-2) inoculum ratio treatment (Fig. 4.6B).

Table 4.3 Number of bacteria attached on AQs

Treatment	*Sphingobium* sp. MO2-4 (Log CFU/gAQ)	*Bacillus megaterium* TL01-2 (Log CFU/gAQ)
The 1:1 inoculum volume (MO2-4:TL01-2) ratio	8.92±0.03	8.29±0.09
The 2:1 inoculum volume (MO2-4:TL01-2) ratio	9.20±0.02	8.34±0.08

(A)   (B)   (C)
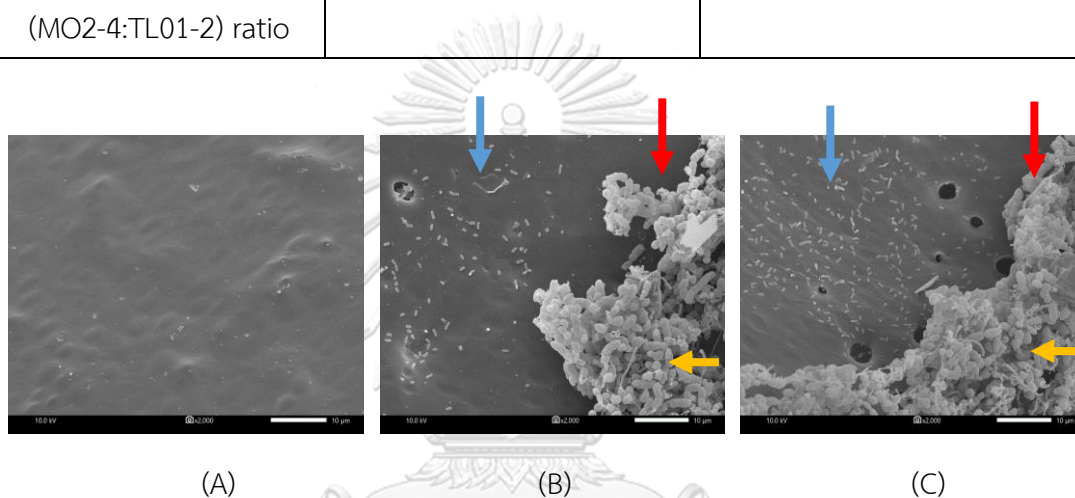
Fig. 4.6 The SEM profile at 2,000X magnified power of (A) Sterilized AQ, and immobilized bacteria on AQ, (B) The 1:1 (MO2-4:TL01-2) inoculum volume immobilization, and (C) The 2:1 (MO2-4:TL01-2) inoculum volume immobilization; where blue arrows are MO2-4, red arrows are TL01-2 and yellow arrows are biofilm.

4.2.3 Crude oil removal from seawater

The immobilized bacteria were tested for their ability to remove 2,000 mg/L crude oil from seawater (salinity: 22 ppt). This experiment was divided into 3 groups based on the immobilization approach. Group A was the individually immobilized the bacteria in the different flask then pooled together with 1:1 AQ weight. Group B was the 1:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization approach. Group C was the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization approach. After incubation, in each day (Fig. 4.7-4.9), the sterilized AQs treatment showed the

crude oil absorption by AQs; it took 7 days for the sterilized AQs to absorb almost all 2,000 mg/L crude oil as shown in Fig. 4.10A, while the immobilized bacteria treatment showed lower crude oil accumulated in AQs, which suggested that biodegradation by the immobilized bacteria took place (Fig. 4.10B). It was cleared that the immobilized bacteria could remove crude oil from seawater; however, each group of immobilized bacteria performed differently (Fig. 4.11). It was found that group A, the individually immobilized the bacteria, could remove lower amount of crude oil when compared to group B and group C, which were the immobilization as co-culture (Table 4.4); even though, the number of bacteria was maintained throughout the experiment in all treatments (Fig. 4.12). The possible explanation for this phenomenon was that when immobilized as co-culture in group B and group C, the biofilm formed by *Bacillus megaterium* TL01-2 could engulf *Sphingobium* sp. MO2-4, which was confirmed by the SEM profile (Fig. 4.6), and offer additional protection against the absorbed crude oil toxicity and salinity of seawater. On the other hand, when individually immobilized the bacteria in group A, *Sphingobium* sp. MO2-4, which was the main hydrocarbon degrader in the co-culture, could not gain benefit from the biofilm produced by *Bacillus megaterium* TL01-2, and thus lower the removal efficiency.

There were many reports stated the benefit of biofilm to bacterial cell. Zhang et al., 2015 reported that biofilm could accumulate hydrocarbons and enhance bioavailability of poorly soluble hydrocarbons. Moreover, the biofilm can promote the interaction between each species within the matrix as well as increase nutrient mass transfer efficiency in the system (Roder et al., 2016; Flemming et al., 2016). Hence, when immobilized individually not all bacteria can gain the benefit mentioned; resulted in dropped of degradation efficiency.

Group A, individually immobilized the bacteria then pooled together with 1:1 AQ weight ratio

Group B, the 1:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization

Group C, the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization

Fig. 4.7 Day 1 of incubation of each group, where the left-most flask was control followed by sterilized AQs and the right-most flask was immobilized bacteria.

Group A, individually immobilized the bacteria then pooled together with 1:1 AQ weight ratio

Group B, the 1:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization

Group C, the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization

Fig. 4.8 Day 3 of incubation of each group, where the left-most flask was control followed by sterilized AQs and the right-most flask was immobilized bacteria.

Group A, individually immobilized the bacteria then pooled together with 1:1 AQ weight ratio

Group B, the 1:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization

Group C, the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization

Fig. 4.9 Day 7 of incubation of each group, where the left-most flask was control followed by sterilized AQs and the right-most flask was immobilized bacteria.
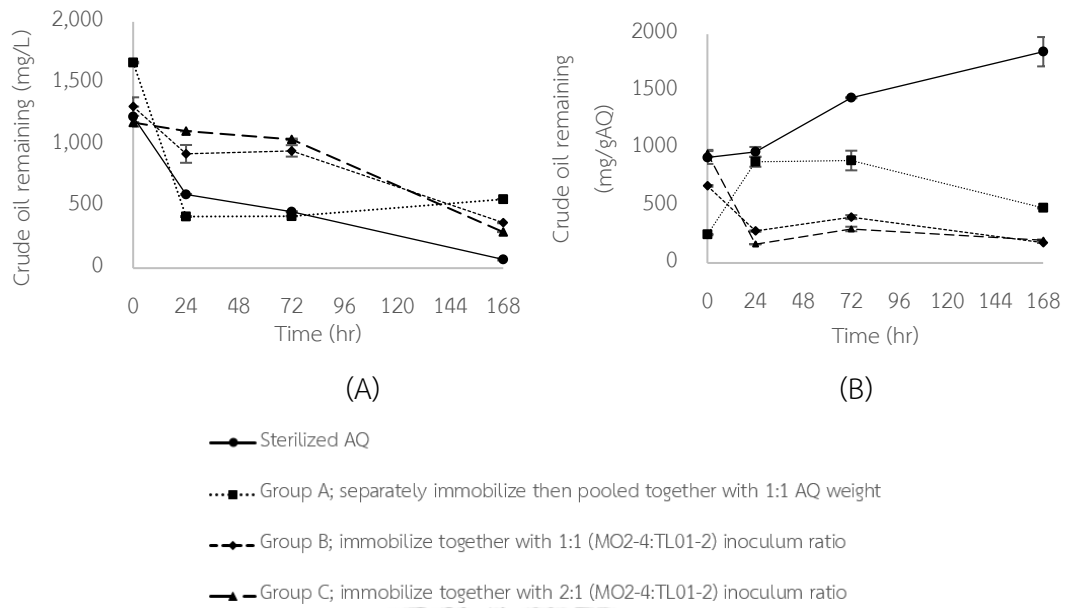
Fig. 4.10 Crude oil remaining in (A) Seawater, (B) AQs
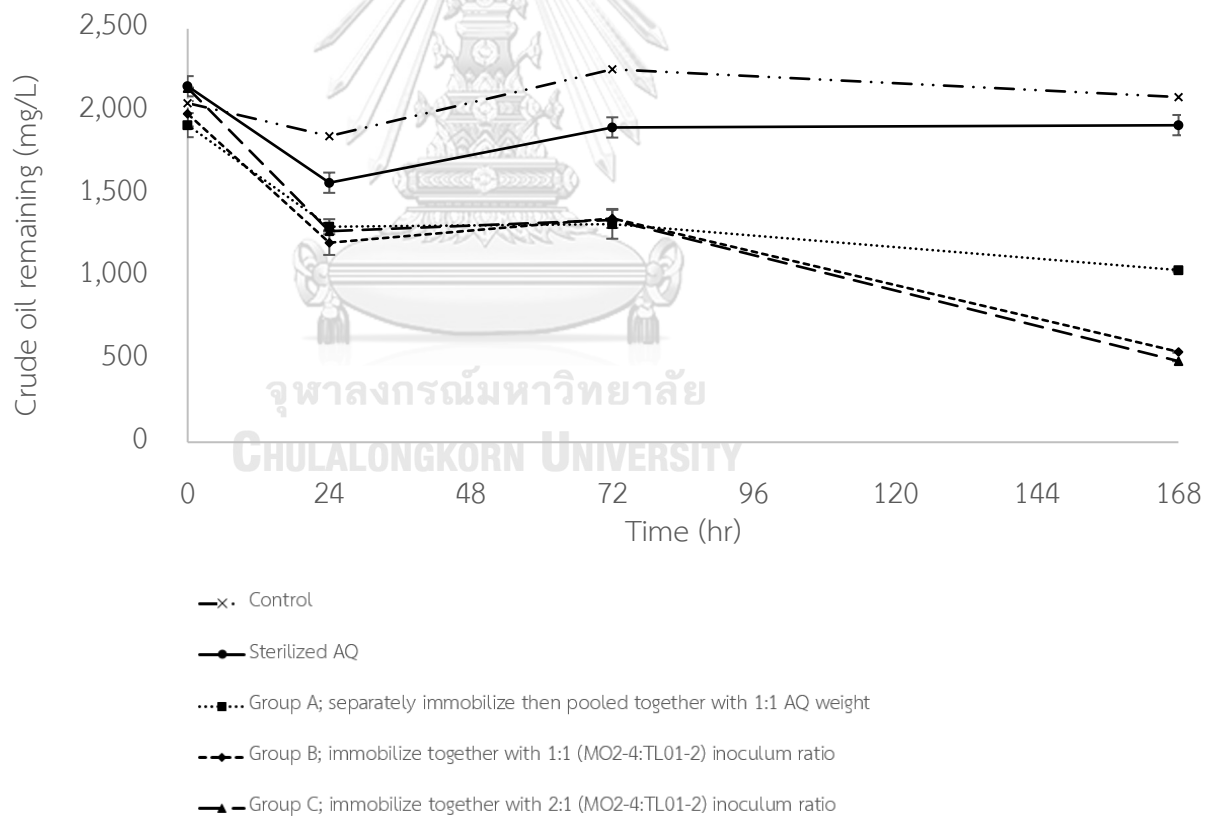
Fig. 4.11 Overall crude oil remaining in the system

Table 4.4 Crude oil removal and degradation in each treatment

Treatments	Total crude oil removal (mg/L)	Absorption (mg/gAQ)	Degradation (mg/L)
Sterilized AQs	1,979.40±22.70[a]	1,846.90±12.85[a]	-
Group A; the individually immobilization	1,490.00±0.55[b]	480.80±0.84[b]	876.70±0.52[a]
Group B; the 1:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization	1,680.70±0.47[c]	177.40±0.56[c]	1,370.80±0.16[b]
Group C; the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization	1,757.00±0.12[c]	197.40.±0.16[c]	1,427.10±0.81[b]

- : no data; alphabet indicated the significant different at 95% confidence interval.

Abiotic loss: 132.5±26.08 mg/L
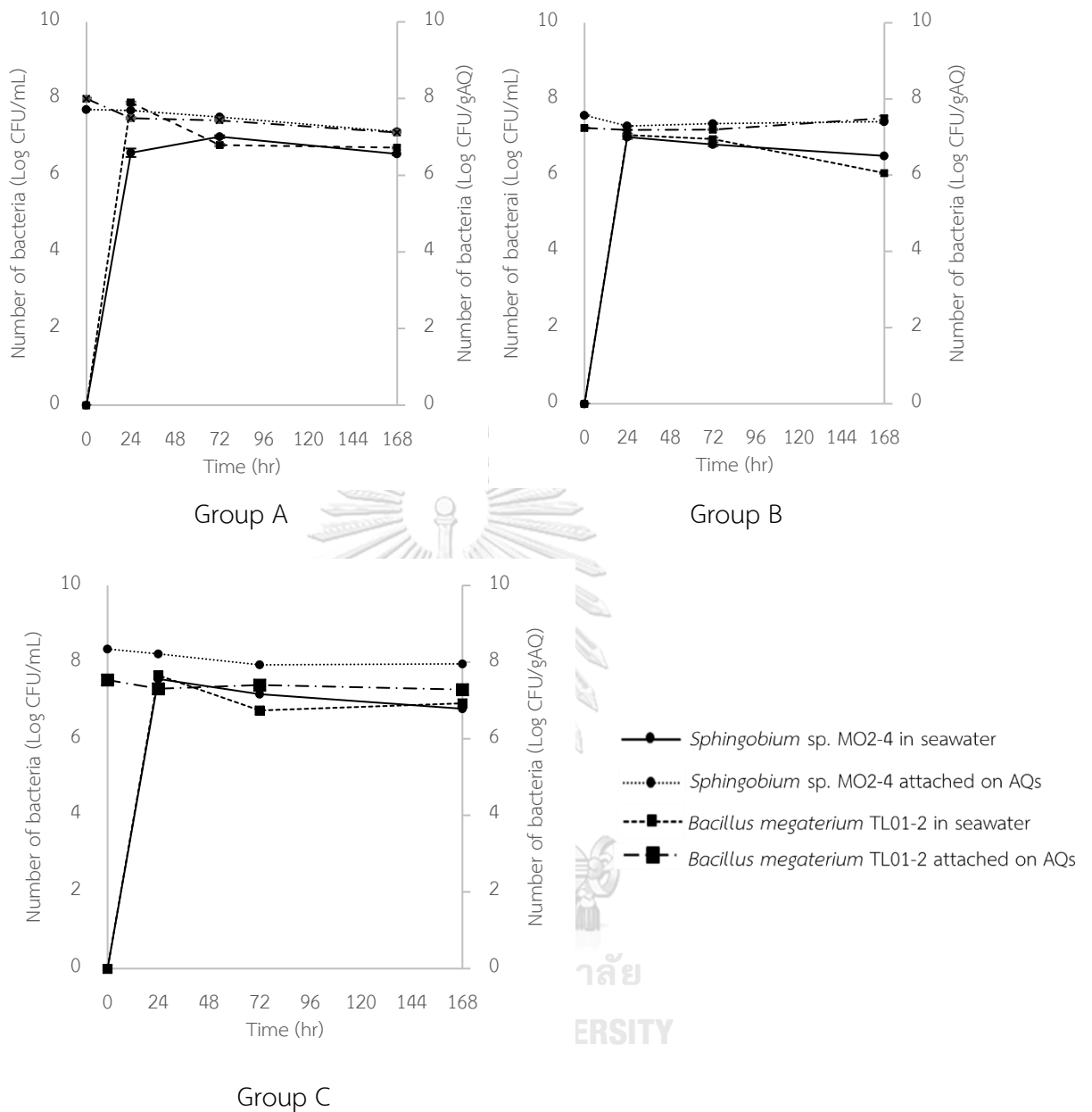
Fig. 4.12 The number of bacteria in the seawater and on AQs of group A, the individually immobilization treatment, group B, the 1:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization treatment, and group C, the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization treatment

Chen et al., 2017 found that the inoculum volume of each member of the co-culture could effect the crude oil degradation efficiency of free cell bacteria depended on the role of that member. However, to our knowledge, there is no

reseacrh investigating the effect of different immobilization approaches of the co-culture before. In conclusion, even though the individually immobilization maybe easier to control the amount of bacteria attached to the support material, but it could lost the benefit of co-culture immobilization especially when there is biofilm forming bacteria present in the system, which can significantly affected thier performance. According to the immobilization and crude oil removal results, group C, which was the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization approach, showed the most promising potential by having high removal efficiency and high number of MO2-4, which was the main degrader of the co-culture, attached on AQs. Therefore, group C was selected for the next experiment.

4.2.4 Semi-continuous crude oil removal experiment

The crude oil removal efficiency of immobilized bacteria, the 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization, and its free cell bacteria were compared. Crude oil 500 mg/L was introduced to the system every 7 days for 35 days. Fig. 4.13 showed the flask after incubation in each week. The result showed that the crude oil removal efficiency of immobilized bacteria was better than its free cell (Fig. 4.14), the removal efficiency may dropped on day 21 but it still performed significantly better than its free cell counter part. At the end of the experiment, the attachment of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 on AQs was confirmed by SEM profile (Fig. 4.15) as well as viable plate count (Fig. 4.16), which revealed that the number of bacteria attached on AQs was maintain throughout the experiment.

(A)
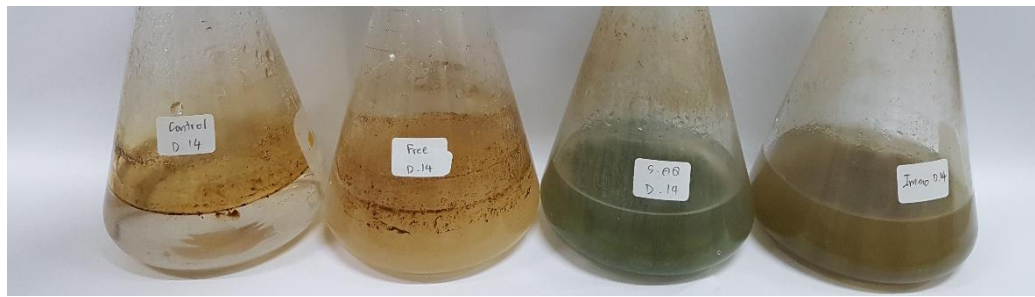
(B)

(C)
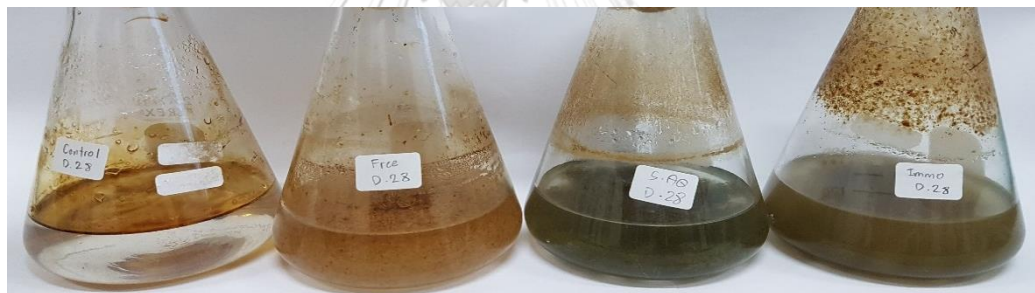
(D)

(E)

Fig. 4.13 Semi-continuous crude oil removal experiment on (A) day 7, (B) day 14, (C) day 21, (D) day 28, and (E) day 35; where the left-most flask is control followed by free cell bacteria, sterilized AQs and the right-most flask was immobilized bacteria.
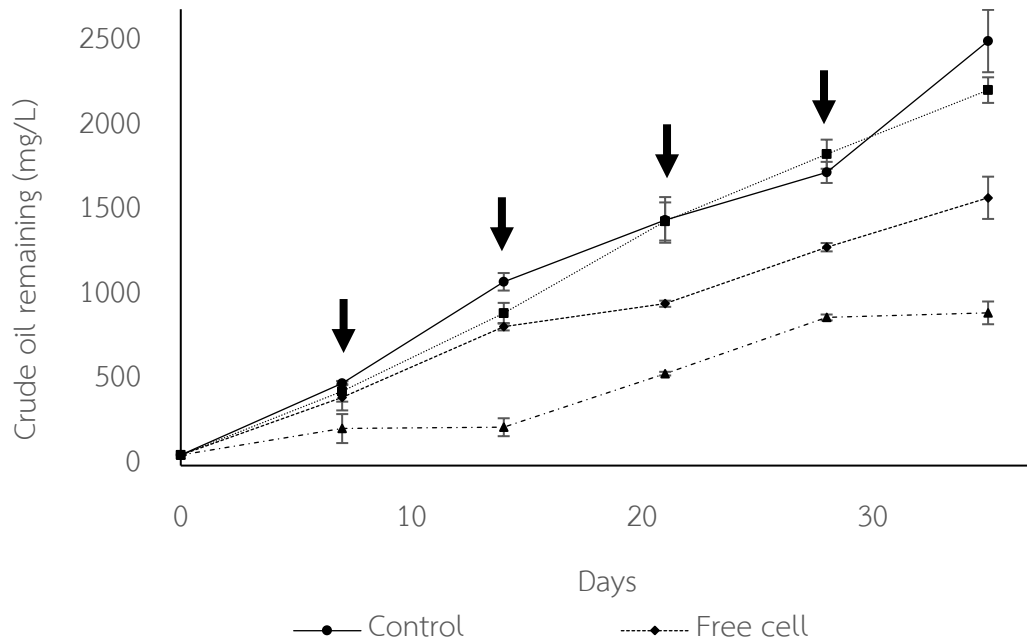
Fig. 4.14 Crude oil remaining in the system of semi-continuous experiment; the arrows indicated the point where 500 mg/L crude oil was spiked into the system.

(A)   (B)
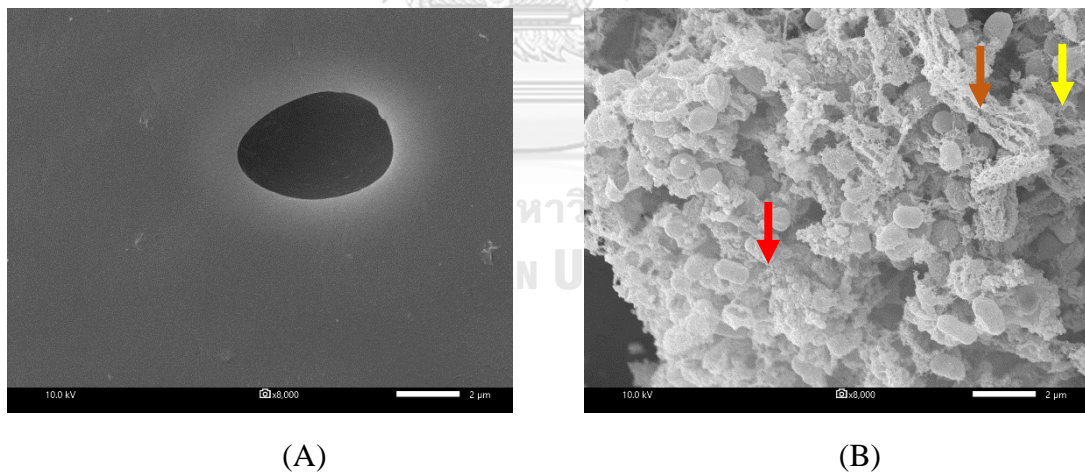
Fig. 4.15 The SEM image at 8,000X magnified power of (A) sterilized AQs, and (B) immobilized bacteria on AQ at the end of experiment; brown arrow shows *Sphingobium* sp. MO2-4, red arrow shows *Bacillus megaterium* TL01-2, and yellow arrow shows the biofilm.

(A)

(B)
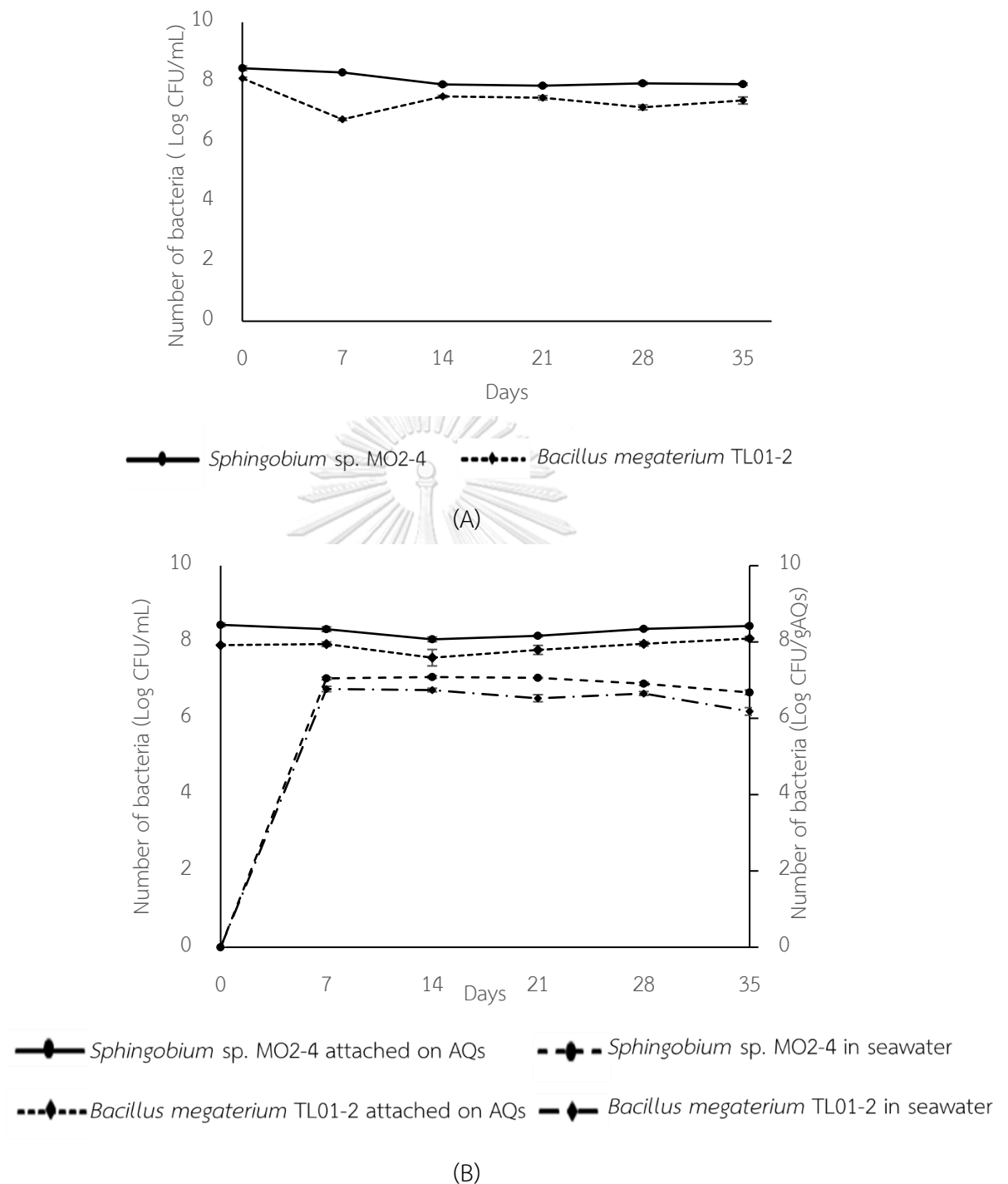
Fig. 4.16 The number of bacteria in the system (A), free cell bacteria, and (B),
immobilized bacteria

The similar result was also reported by Nopcharoenkul et al., 2013 where the
immobilized bacteria *Pseudoxanthomonas* sp. RN402 was able to remove diesel oil
better than its free cell in the semi-continuous system that spiked 200 mg/L diesel

oil every 3 days for 15 days. The result also agreed with another report by Muangchinda et al., 2018 where the immobilized *Serratia* sp. W4-01 could remove diesel oil significantly better than its free cell in the semi-continuous system where 200 mg/L diesel oil was added every 2 days for 14 days. To date, many researchers have confirmed that immobilization can enhance hydrocarbon removal efficiency. For example, *Acinetobacter venetianus* immobilized on modified bamboo charcoal could degrade 94% of 200 mg/L diesel oil compared to its free cell at 80% within 3 days (Y. Chen et al., 2016). Kee et al., 2015 also reported that the immobilized bacterial consortium could degrade 1% v/v crude oil up to 68%, while its free cell could degrade only 47.7% within 8 weeks. Besides protection for the cells, the support material also plays important role in the removal of hydrocarbon. Wang et al., 2015 reported the synergistic interaction between the support material and the bacteria where the support would adsorb the hydrocarbons, which made it easier for the bacteria to access, thus increased the bioavailability of hydrocarbons. This similar interaction was also observed in the previous experiment where most of the crude absorbed by AQs was degraded by *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2. According to the result, the immobilized bacteria of 2:1 (MO2-4:TL01-2) inoculum volume co-culture immobilization showed positive trend in all experiments. Therefore to further develop toward ready-to-use bacteria for environmental application, the larger scale experiment was conducted in 40 L wave simulator tank.

4.3 Crude oil removal from wave simulator tank

The experiment was done in 40 L glass tank containing 20 L seawater and 3 Kg synthetic sand and. The bacteria *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 were immobilized as co-culture using with 2:1 (MO2-4:TL01-2) inoculum volume ratio and put inside the nylon net, then attached to the ceiling of the tank. After that, crude oil 1,000 mg/L was added into the system. The tank operated using 1.5 watt wave generator and the experiment was lasted for 28 days. The initial bacteria concentration was 10^8 CFU/gAQ. In each week, total of 180 mL seawater sample was collected from the tank at different locations. Fig. 4.17 showed the tank

at each of the sampling date. The result of remaining crude oil in the seawater sample collected was shown in table 4.5, while the crude oil removal and number of total bacteria in the immobilized bacteria compared to natural attenuation treatment were shown in Fig. 4.18 and Fig. 4.19.
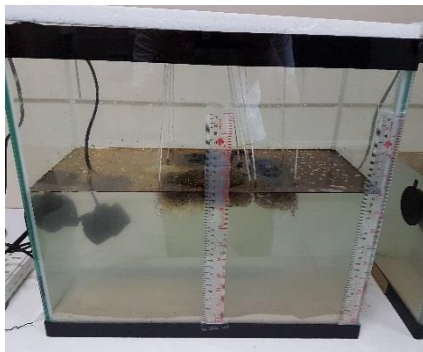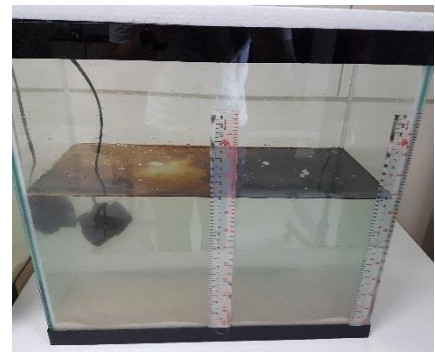
Immobilized bacteria treatment   Natural attenuation treatment

(A)
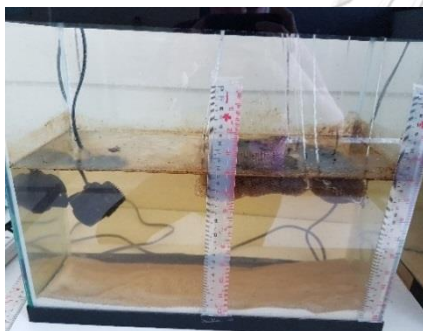
Immobilized bacteria treatment   Natural attenuation treatment

(B)
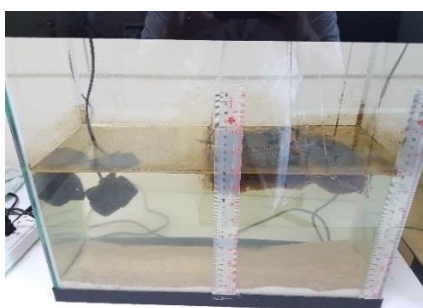
Immobilized bacteria treatment   Natural attenuation treatment

(C)
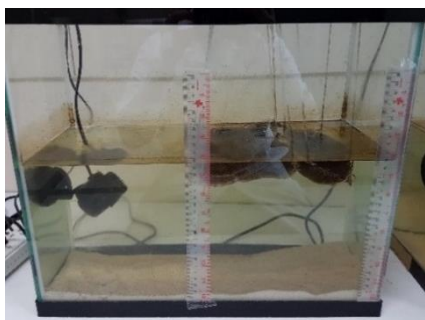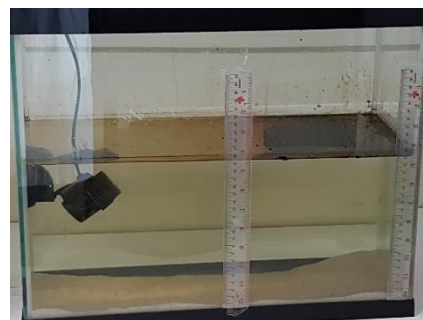
Immobilized bacteria treatment   Natural attenuation treatment

(D)
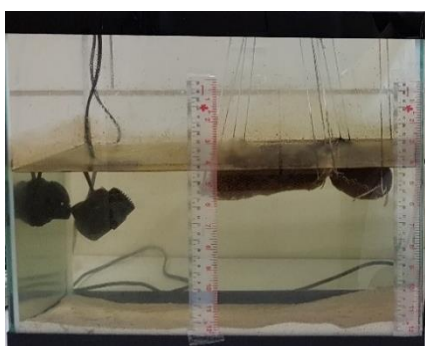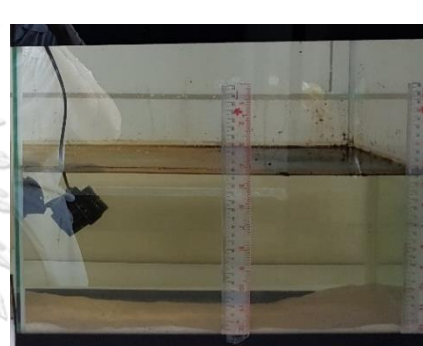
Immobilized bacteria treatment   Natural attenuation treatment

(E)

Fig. 4.17 Wave simulator tank at each day of the experiment (A) Day 0, (B) Day 7, (C) Day 14, (D) Day 21, and (C) Day 28

Table 4.5 Crude oil remaining in the seawater sample collected from the tank

Time	Total peak area	
	Natural attenuation treatment	Immobilized bacteria treatment
Day 0	2,024.03±281.83	1,702.87±596.37
Day 7	14,266.80±2435.32	781.18±253.61
Day 14	12,637.77±4579.37	829.97±432.27
Day 21	18,572.73±9924.73	793.87±104.61
Day 28	19,418.67±2246.86	1,415.90±26.07
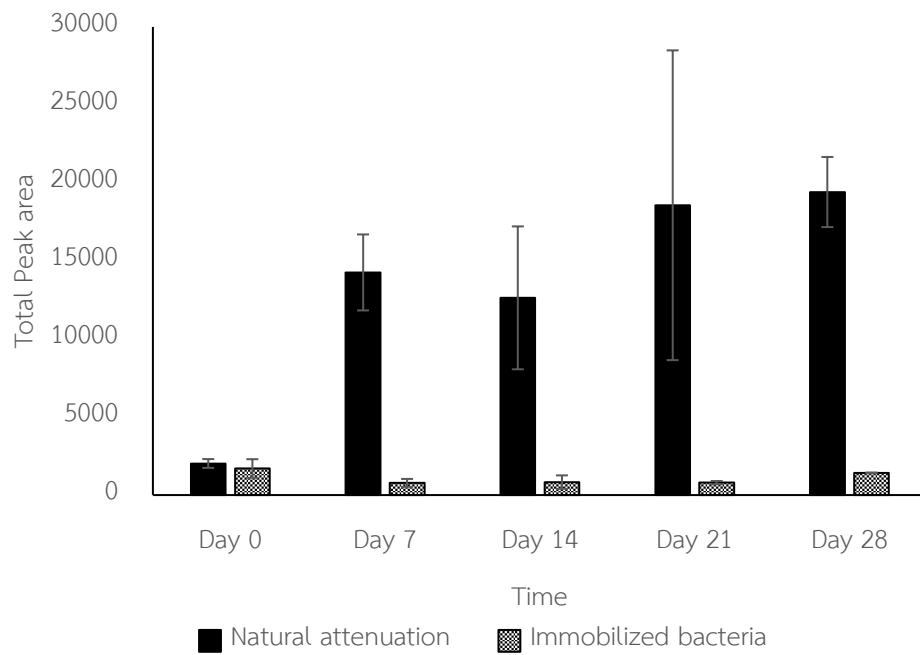
Fig. 4.18 Crude oil remaining in the seawater sample of the immobilized bacteria treatment
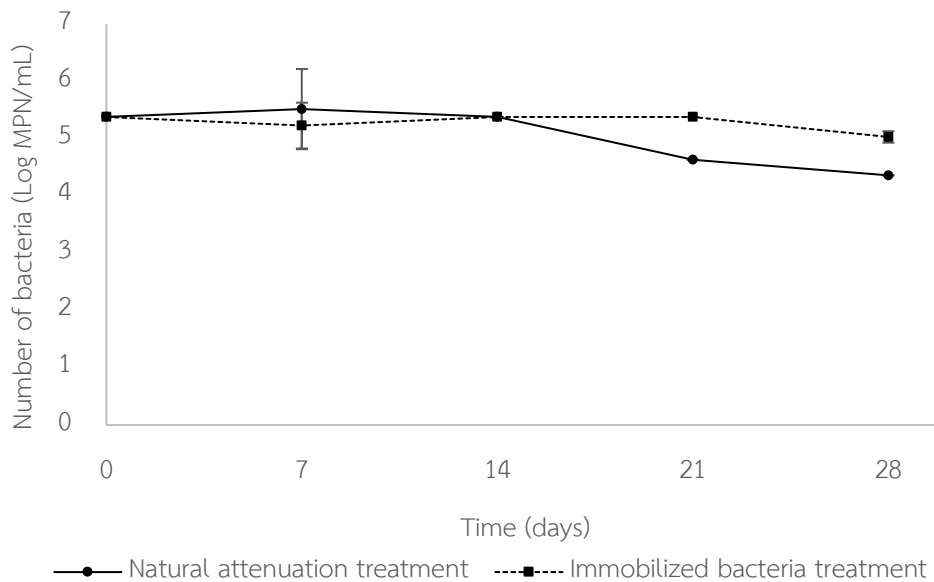
Fig. 4.19 Number of total bacteria in the seawater sample

The different of total peak area between day 0 and others date in natural attenutation treatment was from the sampling procedure. At day 0 the oil layer was

thin and spreaded over the surface of seawater as show in Fig. 4.17A; resulted in less amount of oil collected. However, at the following sampling date, crude oil was pushed to one side and concentrated into thick layers; which made it easier to draw out large amount of oil at the concentrated sampling point. This was similar to the response technique for marine oil spill, where after the blooms were deployed to contain and concentrated the oil into thick layers, the skimmers were then used to siphoned out most of the spill at the concentrated point (Mapelli et al., 2017).

The result revealed that immobilized bacteria could remove most of the crude oil since day 7 of the experiment with more than 90% of oil removed from seawater sample compared to the natural attenuation treatment (Fig. 4.18). In addition, the total bacterial number of the natural attenuation treatment was dropped at the end of experiment; while the total bacterial number of the immobilized bacteria treatment was maintianed througout the experiment (Fig. 4.19). This may indicated the reduction of oil toxicity due to the removal by immobilized bacteria.

In order to confirm the biodegradation by immobilized bacteria, the follow-up experiment was conducted using sterilized aquaporous gel and all the same parameter of immobilized bacteria treatment. The amount of crude oil accumulated in aquaporous gel from each treatment at the end of experiment was showed in table 4.6.

Table 4.6 Crude oil remaining in aquaporous gel at day 28

Crude oil remaining in aquaporous gel (Total peak area)	
Sterilized aquaporous gel treatment	Immobilized bacteria treatment
2,615,790±654,534[a]	730,866.67±121,795[b]

*Alphabet indicated the significant different at 95% confidence interval.

The amount of crude oil absorbed by aquaporous gel from the immobilized bacteria treatment was significantly lower than the sterilized aquaporous gel treatment. The result agreed with the previous small scale experiment with more than 70% of crude oil degraded. Althought the number of bacteria was dropped to 10^6 MPN/gAQ, the immobilized bacteria still maintianed its ability to degrade crude oil. Finally, this experiment proofed that the immobilized bacteria is a promising tool for marine oil spill clean-up and future reseach is needed to improve the removal effieincy.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Crude oil is a complex mixture of hydrocarbons that can cause both acute and chronic damage to living organism and environment. In all continent, oil spillage is a major threat to the marine ecosystem and needed to be dealt with as soon as possible. Therefore, this study aimed to develop a ready-to-use immobilized bacteria in order to response to the marine oil spill event. In this work, the immobilized bacteria was developed using the co-culture of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 immobilized on aquaporous gel. The bacteria *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 were isolated from marine sponge sample collected from the Gulf of Thailand; both bacteria were able to degrade crude oil 2,000 mg/L for approximately 50% within 7 days. As an individual strain, both *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 showed potential for biosrufactant productivity by lower the surface tension of productive media from 50 mN/m^3 to around 37 mN/m^3. After characterization and various hydrocarbon degradation test, it was found that *Sphingobium* sp. MO2-4 was able to degrade several types of petroleum oils including crude oil, diesel oil, and fuel oil; while *Bacillus megaterium* TL01-2 showed high heavy metal resistance, high cell surface hydrophobicity, and strong biofilm formation activity. Moreover, the co-culturing of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 was showed to improve some of the hydrocarbon degradation efficiency. With the promising characteristic, non-anatagonistic activity, and non-pathogenic trait; the co-culture of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 was developed into immobilized bacteria using aquaporous gel as carrier. Three immobilization approaches based on immobilization process were tested; Group A, individually immobilized each bacteria then pulled together with 1:1 aqporous gel weight. Group B, immobilized the bacteria together as co-culture in the same flask with 1:1 (MO2-4:TL01-2) inoculum volume. Group C, immobilized the bacteria together as co-culture in the same flask with 2:1 (MO2-4:TL01-2) inoculum volume. After crude oil removal test, it was found

that the immobilized as co-culture approaches, group B and group C, showed higher removal efficiency regardless of inoculum ratio. It is possible that when immobilized as co-culture in the same flask the biofilm formed by *Bacillus megaterium* TL01-2 could provide the protection for *Sphingobium* sp. MO2-4 as well. Therefore, group C, the immobilized as co-culture with 2:1 (MO2-4:TL01-2) inoculum size was chosen for the semi-continuous experiment due to the higher amount of *Sphingobium* sp. MO2-4, which act as the main degrader. In the semi-continuous experiment crude oil 500 mg/L was introduced to the system every week for 5 weeks, it was confirmed that the immobilized bacteria could perform significantly better than its free cell by maintained the degradation efficiency for 3 weeks. The immobilized bacteria was then tested in a wave simulator tank with 20 L seawater, 3 Kg synthetic sand, 1.5 watt wave generator, and 1,000 mg/L crude oil. After 28 days of experiment, the immobilized bacteria was able to remove more than 90% of crude oil from seawater and more than 70% of crude oil was degraded. In the end, these results demonstrated that the immobilized co-culture of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 on aquaporous gel is a promising tool for marine oil spill removal.

5.2 Recommendations for future work

5.2.1 Reusability of immobilized bacteria should be tested to find the limitation and durability of immobilized bacteria

5.2.2 Effect of dispersant and fertilizer should be tested because in the real scenario dispersant and fertilizer often used to facilitate to oil spill removal.

5.2.3 The shelf-life and storage condition of the immobilized bacteria should be studied

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APPEXNDIX A

MEDIA AND SOLUTION

Marine Broth (Zobell Marine Broth 2216, HiMedia, India)

Zobell Marine Broth powder	40.25	g

Dissolve in 1,000 mL distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C for 15 min.

Marine agar

Zobell Marine Broth powder	40.25	g
Agar powder	2.00	% (w/v)

Dissolve Zobell Marine Broth powder in 1,000 mL distilled water prior adding agar powder, then sterilized by autoclave at 15 pound per square inch, 121°C for 15 min.

Nutrient Seawater Medium (NSM; Muangchinda et al., 2018)

NH_4NO_3	1.00	g
K_2HPO_4	0.02	g
Ferric citrate	0.02	g
Yeast extracts	0.50	g
Seawater	200	mL

Dissolve all component in 800 mL distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C for 15 min.

Productive medium (Khondee et al., 2015)

Glucose	1.00	g
Beef extract	0.50	g
K_2HPO_4	3.30	g
$NaNO_3$	0.14	g

NH4NO$_3$	3.30	g
NaCl	0.04	g
FeSO$_4$.7H$_2$O	0.10	g

Dissolve in 1,000 mL distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C for 15 min.

PAH stock solution 10,000 mg/L

PAH	500	mg
Dimethylformamide	50	mL

Dissolve PAH in dimethylformamide then filtrate through sterilized 0.2μm PTFE. The solution was then stored at -20°C

0.85% NaCl

NaCl	8.50	g
Distilled water	1,000	mL

The solution was sterilized by autoclave at 15 pound per square inch, 121°C for 15 min.

Phosphate-Urea-Magnesium sulfate buffer (PUM buffer)

K$_2$HPO$_4$	16.90	g
KH$_2$PO$_4$	7.30	g
Urea (CH$_4$N$_2$O)	1.80	g
MgSO$_4$.7H$_2$O	0.20	g

Dissolve all component in 1,000 mL distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C for 15 min.

APPENDIX B

PHASE I SUPPLEMENTARY DATA

Table B-1 Biosurfactant production potential of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2

Substrate	Bacteria	Method	Triplicates			Average
			1	2	3	
Crude oil	*Sphingobium* sp. MO2-4	Oil displacement (%)	8.57	15.56	15.15	13.09±3.2
		Surface tension (mN/m^2)	42.30	37.69	44.82	41.60±2.95
	Bacillus megaterium TL01-2	Oil displacement (%)	5.88	8.33	3.125	5.78±2.13
		Surface tension (mN/m^2)	47.47	41.02	46.71	45.06±2.87
Soy bean oil	*Sphingobium* sp. MO2-4	Oil displacement (%)	11.11	16.39	14.65	14.05±2.20
		Surface tension (mN/m^2)	39.28	37.48	37.59	38.11±0.82
	Bacillus megaterium TL01-2	Oil displacement (%)	15.63	12.12	9.09	12.28±2.67
		Surface tension (mN/m^2)	38.54	46.49	38.54	41.19±3.75
Control	Sterilized productive media	Oil displacement (%)	0	0	0	0
		Surface tension (mN/m^2)	50.49	51.20	50.27	50.65±0.39

Table B-2 Cell surface hydrophobicity of *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2

Substrate	Bacteria	Initial aqueous absorbance	Average	Hexadecane treated aqueous absorbance	Average	Cell surface hydrophobicity (%)
Crude oil	*Sphingobium* sp. MO2-4	1.06	1.03 ±0.02	0.54	0.52 ±0.02	49.61 ±1.59
		1.01		0.50		
		1.02		0.52		
	Bacillus megaterium TL01-2	0.92	0.99 ±0.04	0.30	0.32 ±0.02	67.13 ±2.49
		1.01		0.32		
		1.03		0.36		
Soy bean oil	*Sphingobium* sp. MO2-4	1.10	1.05 ±0.04	0.63	0.62 ±0.01	40.98 ±1.13
		1.00		0.62		
		1.04		0.60		
	Bacillus megaterium TL01-2	0.98	1.02 ±0.03	0.36	0.34 ±0.02	66.09 ±1.65

Table B-3 *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 survivability in 100 µg/L heavy metal in 3 days

Heavy metals	*Sphingobium* MO2-4 survivability (%)	*Bacillus megaterium* TL01-2 survivability (%)
Hg	39.66±0.75	94.85±3.23
Zn	69.92±0.90	93.75±0.34
Fe	60.31±0	95.96±3.82
Pb	36.00±0	95.21±2.87
Ni	80.01±0.30	82.76±2.99
Co	0	97.94±3.47
Cd	0	94.2±2.63
Cr	0	95.76±4.31
Cu	82.55±0.60	93.60±1.91

Table B-4 Crystal violets staining assay for biofilm formation by *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 at day 3

Treatment	Absorbance (replicates)							Average
	1	2	3	4	5	6	7	
Control (sterilized medium)	0.077	0.078	0.076	0.074	0.069	0.069	0.069	0.073[a]±0.004
Sphingobium sp. MO2-4	0.126	0.090	0.090	0.081	0.084	0.137	0.087	0.099[a]±0.022
Bacillus megaterium TL01-2	0.254	0.395	0.334	0.295	0.299	0.231	0.174	0.283[b]±0.072

*Alphabets indicated the significant different at 95% confidence interval.

Table B-5 Crystal violets staining assay for biofilm formation by *Sphingobium* sp. MO2-4 and *Bacillus megaterium* TL01-2 at day 7

Treatment	Absorbance (replicates)							Average
	1	2	3	4	5	6	7	
Control (sterilized medium)	0.073	0.078	0.081	0.086	0.087	0.088	0.095	0.084[a]±0.007
Sphingobium sp. MO2-4	0.113	0.077	0.083	0.092	0.090	0.088	0.0138	0.097[a]±0.021
Bacillus megaterium TL01-2	0.094	0.097	0.096	0.101	0.107	0.105	0.120	0.103[a]±0.001

*Alphabets indicated the significant different at 95% confidence interval.

Table B-6 Hydrocarbon degradation efficiency of *Sphingobium* sp. MO2-4, *Bacillus megaterium* TL01-2 and the co-culture

Substrate	Concentration (mg/L)	Day	*Sphingobium* sp. MO2-4 degradation (%)	*Bacillus megaterium* TL01-2 degradation (%)	Co-culture degradation (%)
Crude oil	2,000	7	50.53±1.03[a]	52.30±2.19[a]	63.75±5.67[b]
	4,000	14	65.69±0.95[a]	56.06±3.33[b]	65.47±0.85[a]
	8,000	21	47.17±3.36[b]	36.10±1.52[c]	52.59±1.12[a]
Diesel	2,000	7	52.54±2.72[b]	33.67±3.77[c]	62.88±5.72[a]
	4,000	14	54.16±2.07[a]	43.20±2.65[b]	51.51±2.84[a]
	8,000	21	43.72±0.25[a]	14.73±3.18[b]	43.41±2.35[a]
Fuel	2,000	7	54.18±0.77[a]	29.97±3.25[b]	50.78±2.38[a]
	4,000	14	29.35±1.09[b]	24.90±2.98[c]	42.30±1.00[a]
	8,000	21	34.07±2.07[a]	20.80±1.34[b]	38.09±7.29[a]
Tetradecane	500	7	71.47±1.67[b]	67.81±5.22[b]	82.95±5.57[a]
Phenanthrene	50	7	32.67±0.4[c]	38.60±2.22[b]	43.49±2.86[a]
Pyrene	50	7	25.16±2.04[b]	43.14±5.56[a]	37.76±8.11[a]
Phenanthrene + Tetradecane	25 + 500	7	30.72±0.43[c] 28.57±5.16[c]	40.53±0.69[b] 74.82±3.1[b]	54.09±2.11[a] 96.92±2.87[a]

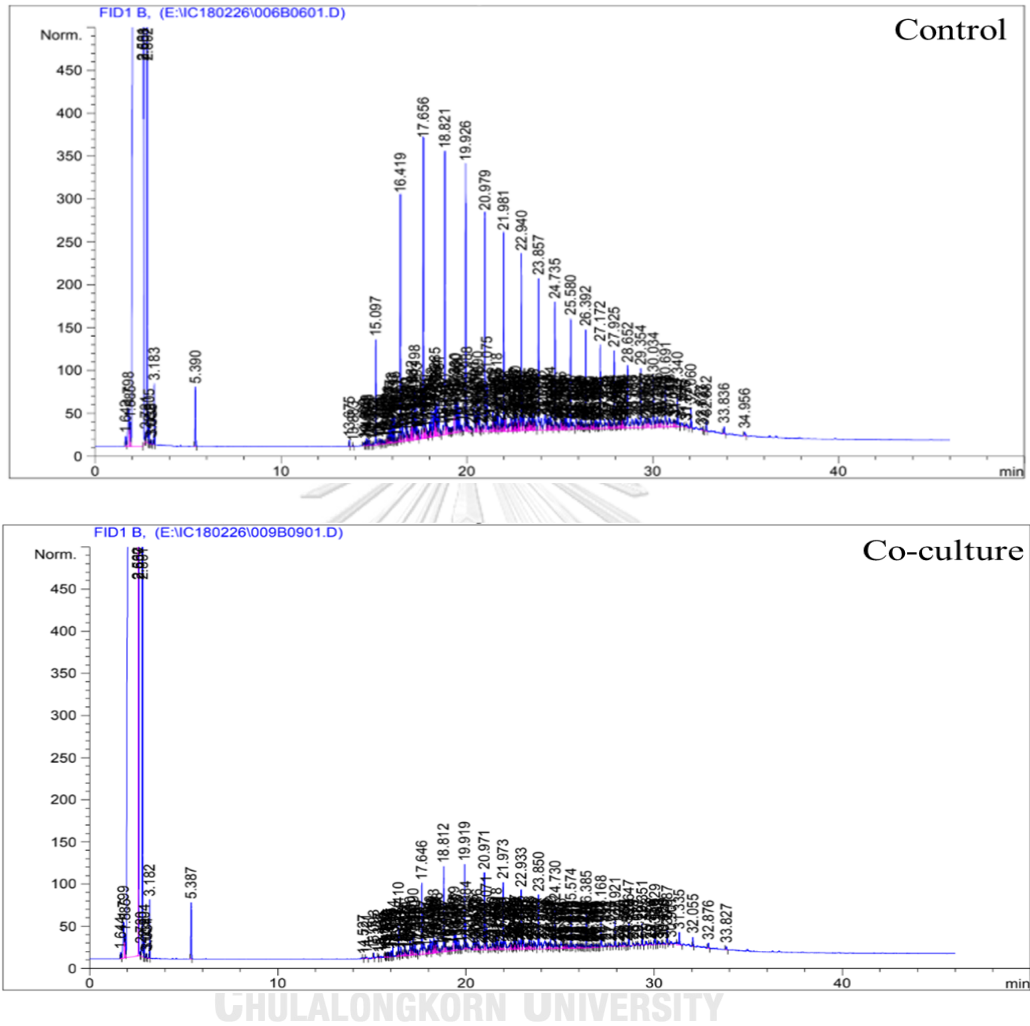*Alphabets indicated the significant different at 95% confidence interval.

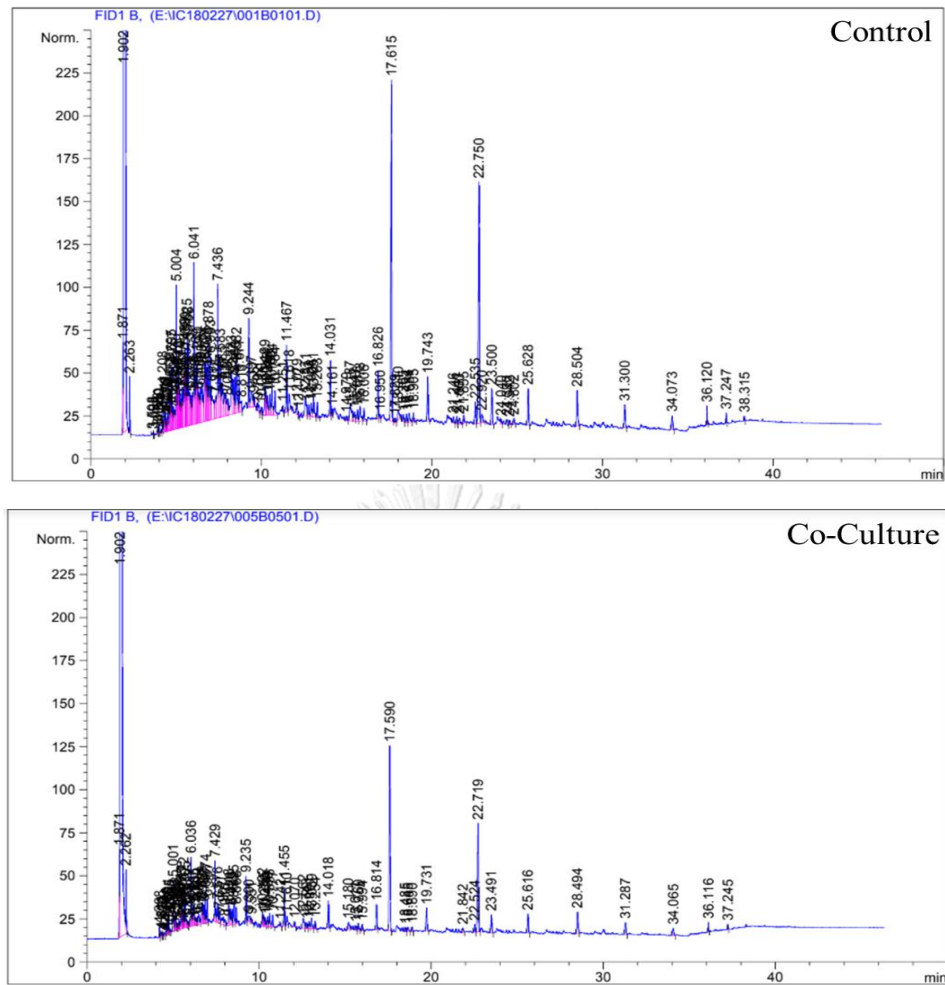**Fig. B-1** Chromatogram of 2,000 mg/L crude oil degradation by the co-culture at day 7

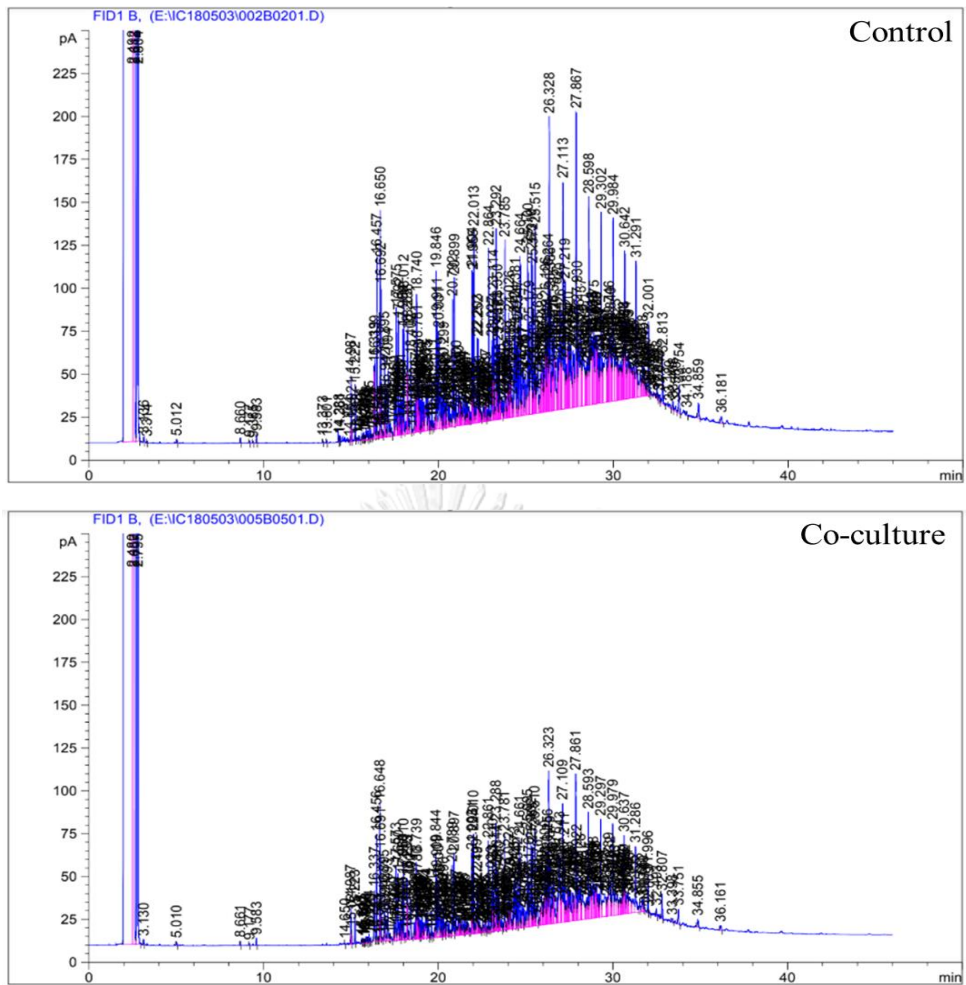**Fig. B-2** Chromatogram of 2,000 mg/L diesel oil degradation by the co-culture at day 7

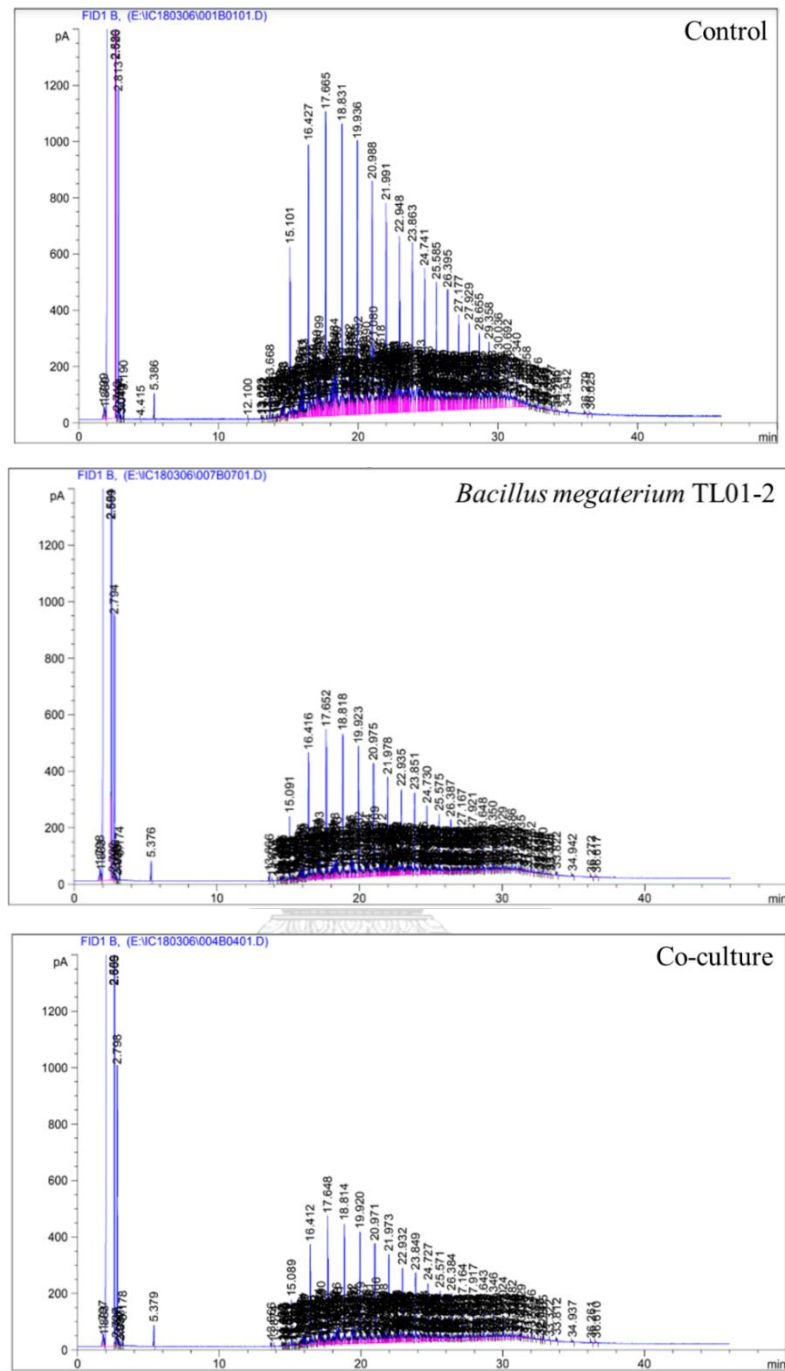**Fig.B-3** Chromatogram of 2,000 mg/L fuel oil degradation by the co-culture at day 7
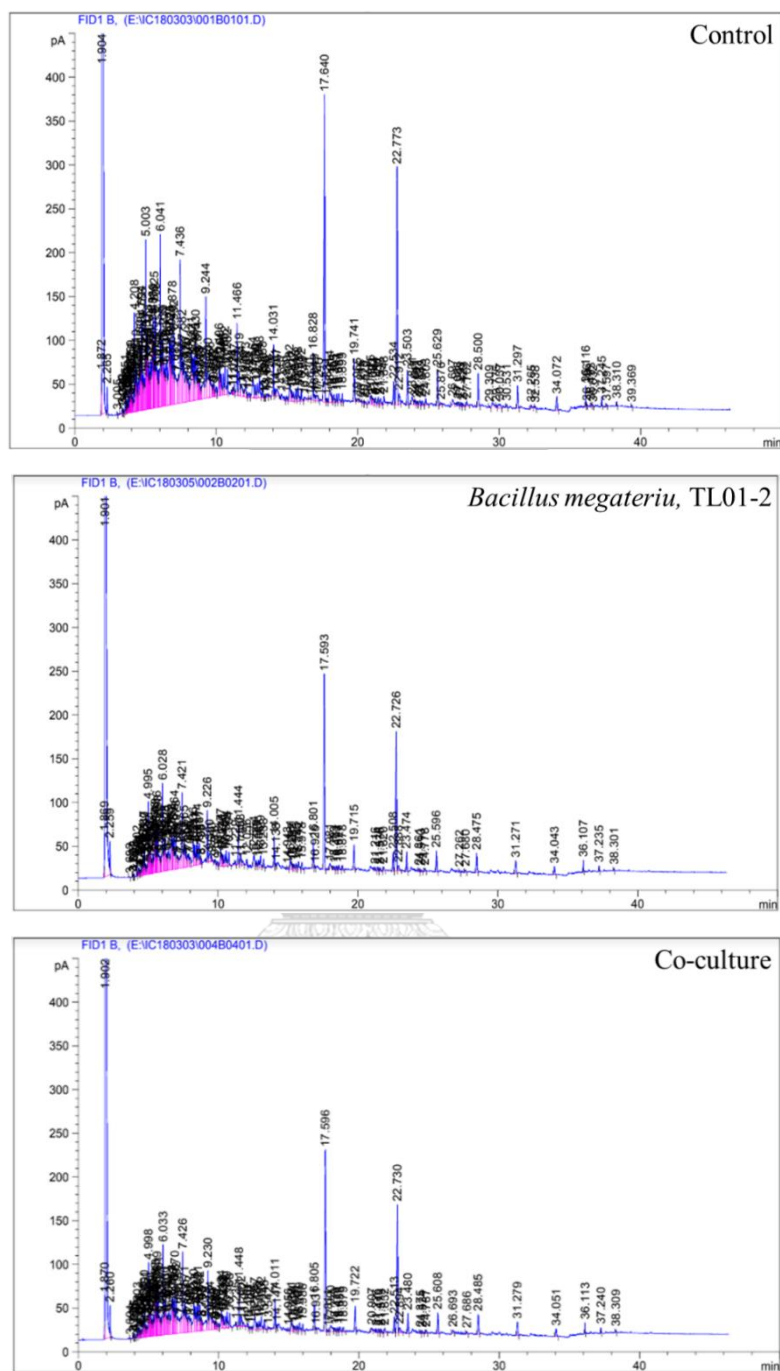
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Fig. B-4 Chromatogram of 4,000 mg/L crude oil degradation by *Bacillus megaterium* TL01-2 and the co-culture at day 14

Fig. B-5 Chromatogram of 4,000 mg/L diesel oil degradation by *Bacillus megaterium*
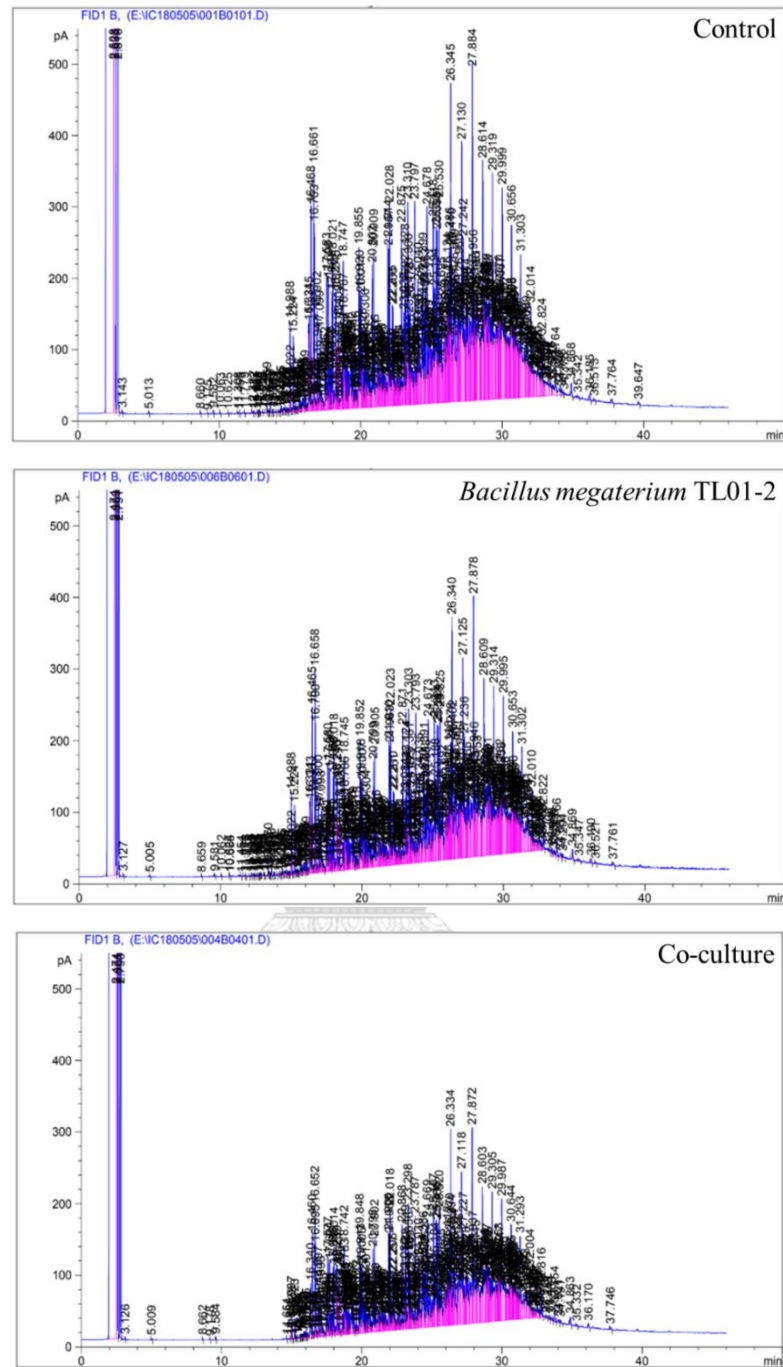TL01-2 and the co-culture at day 14

Fig. B-6 Chromatogram of 4,000 mg/L fuel oil degradation by *Bacillus megaterium* TL01-2 and the co-culture at day 14
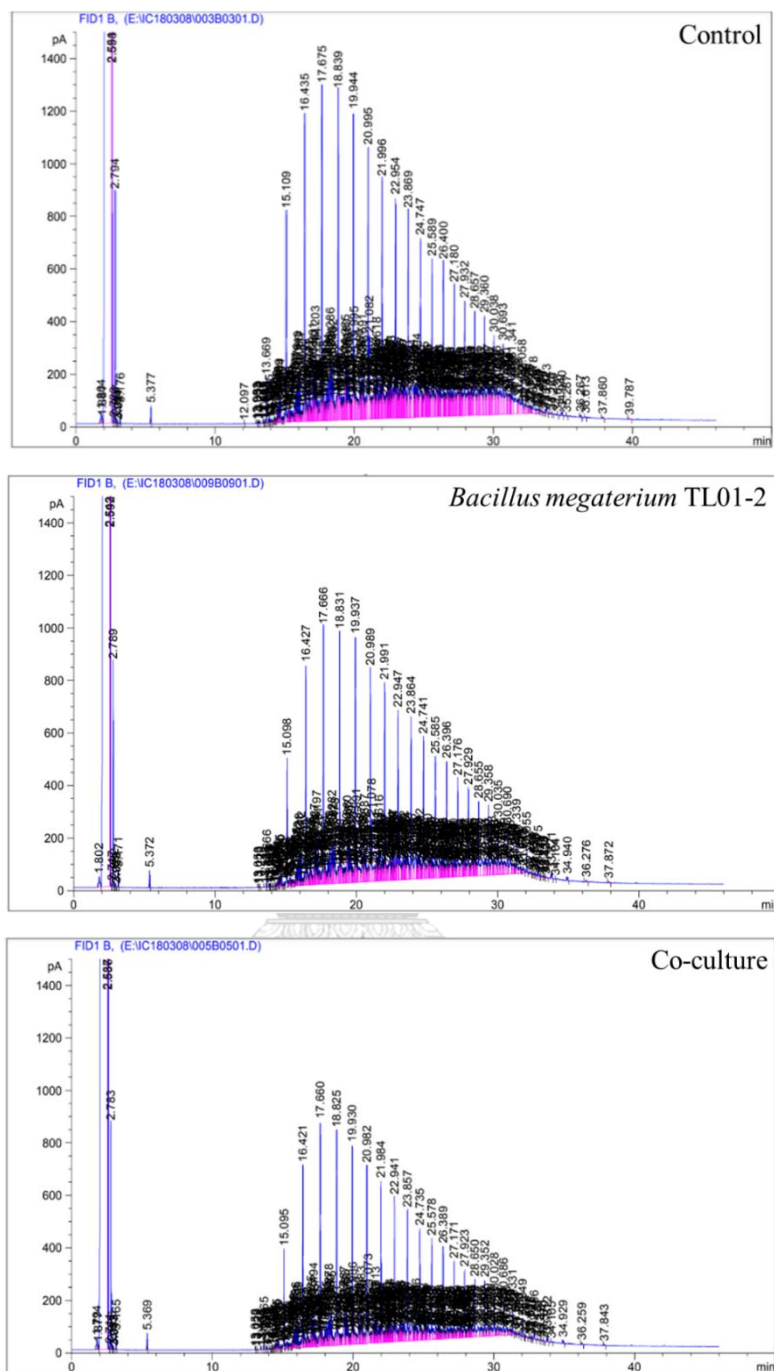
Fig. B-7 Chromatogram of 8,000 mg/L crude oil degradation by *Bacillus megaterium*
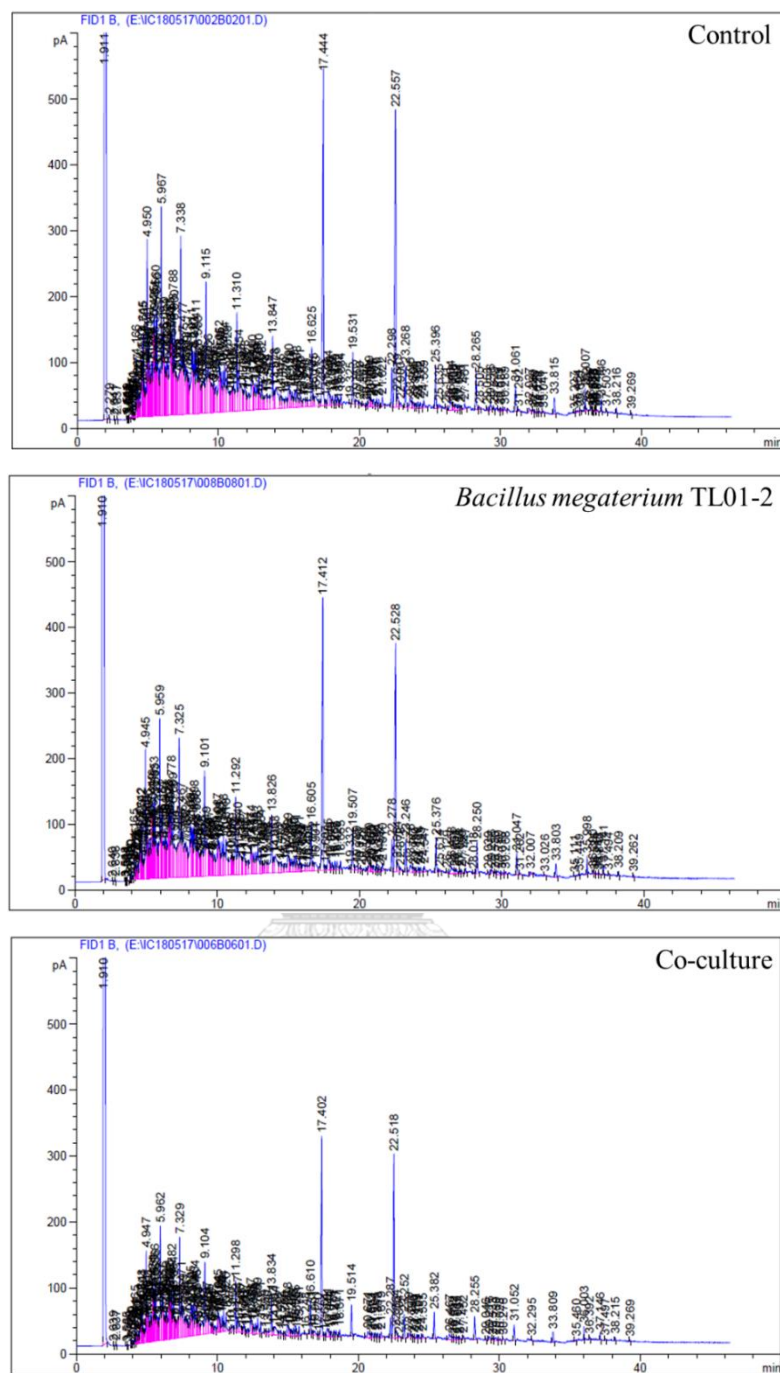TL01-2 and the co-culture at day 21

Fig. B-8 Chromatogram of 8,000 mg/L diesel oil degradation by *Bacillus megaterium*
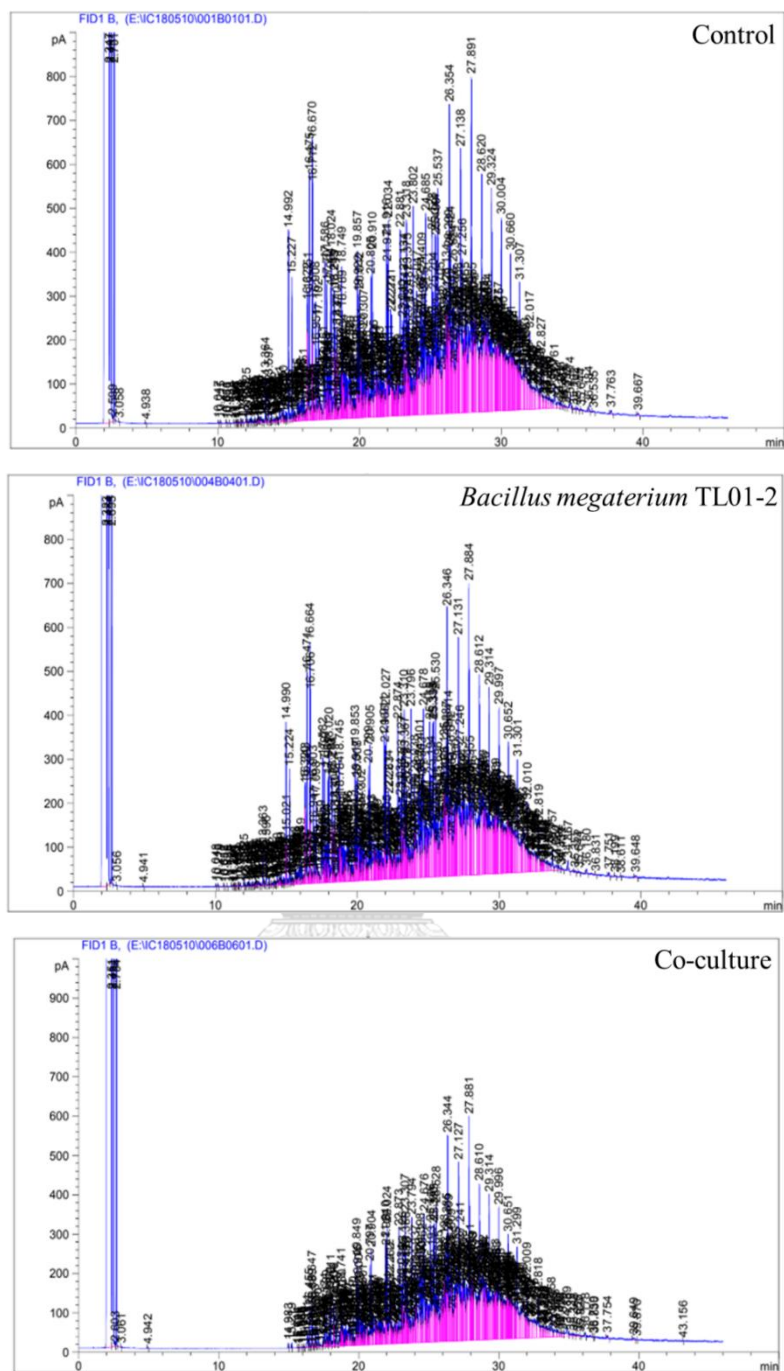TL01-2 and the co-culture at day 21

Fig. B-9 Chromatogram of 8,000 mg/L fuel oil degradation by *Bacillus megaterium*
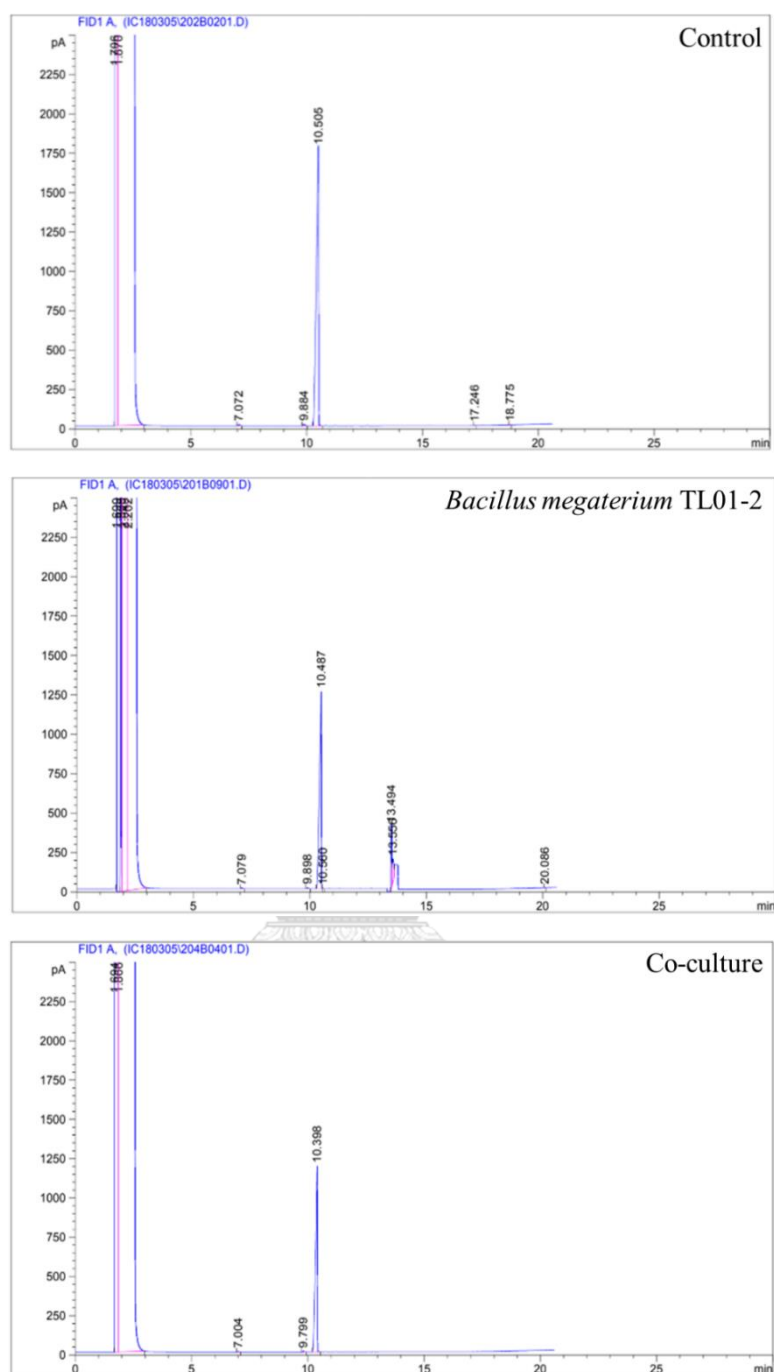
TL01-2 and the co-culture at day 21

Fig. B-10 Chromatogram of 50 mg/L phenanthrene degradation by *Bacillus megaterium* TL01-2 and the co-culture at day 7
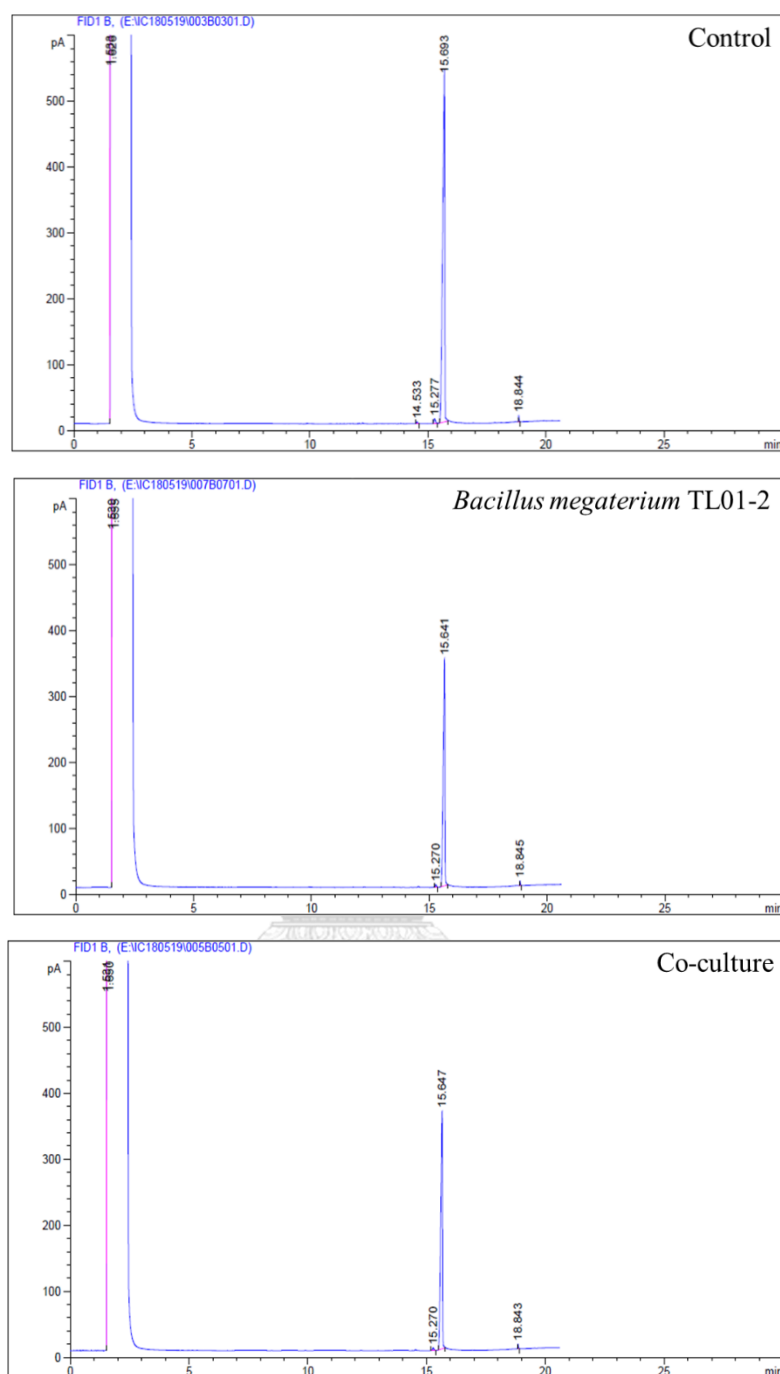
Fig. B-11 Chromatogram of 50 mg/L pyrene degradation by *Bacillus megaterium* TL01-2 and the co-culture at day 7

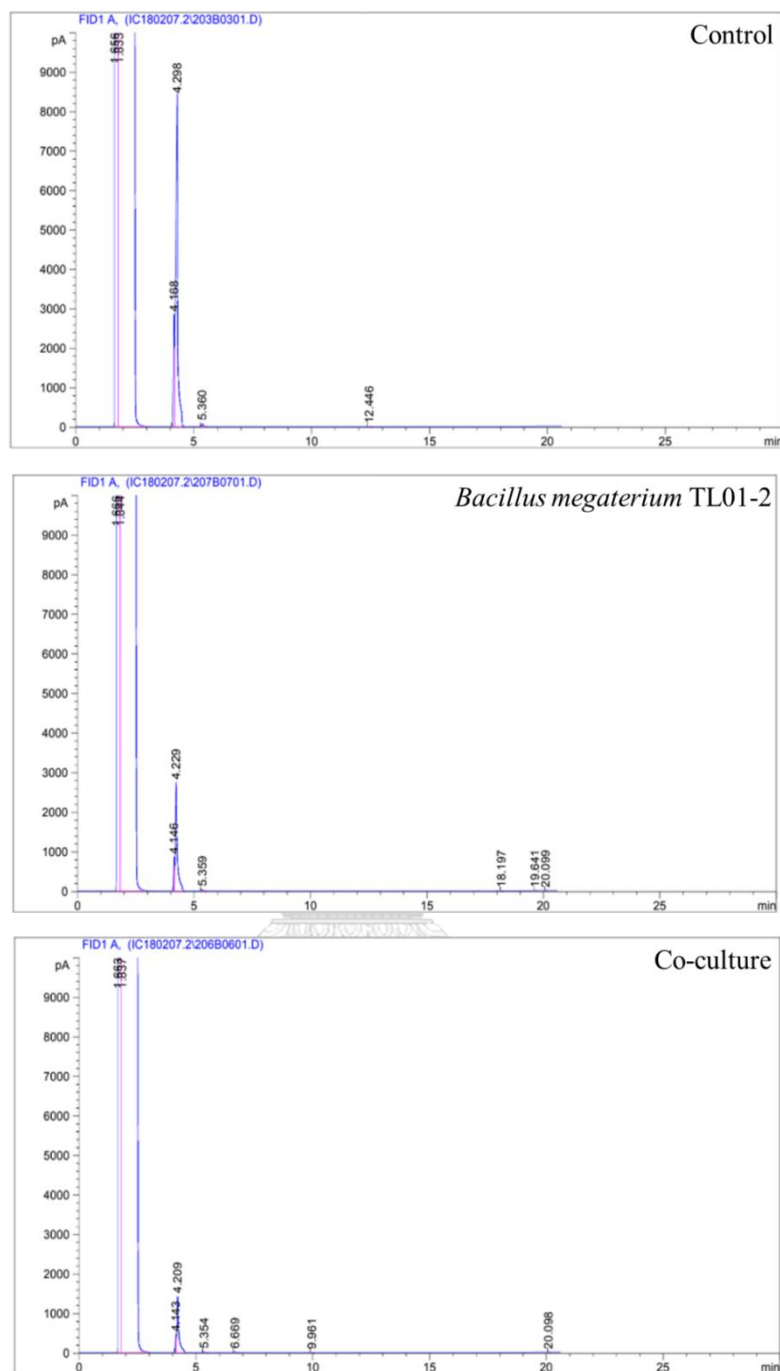**Fig. B-12** Chromatogram of 500 mg/L tetradecane degradation by *Bacillus megaterium* TL01-2 and the co-culture at day 7
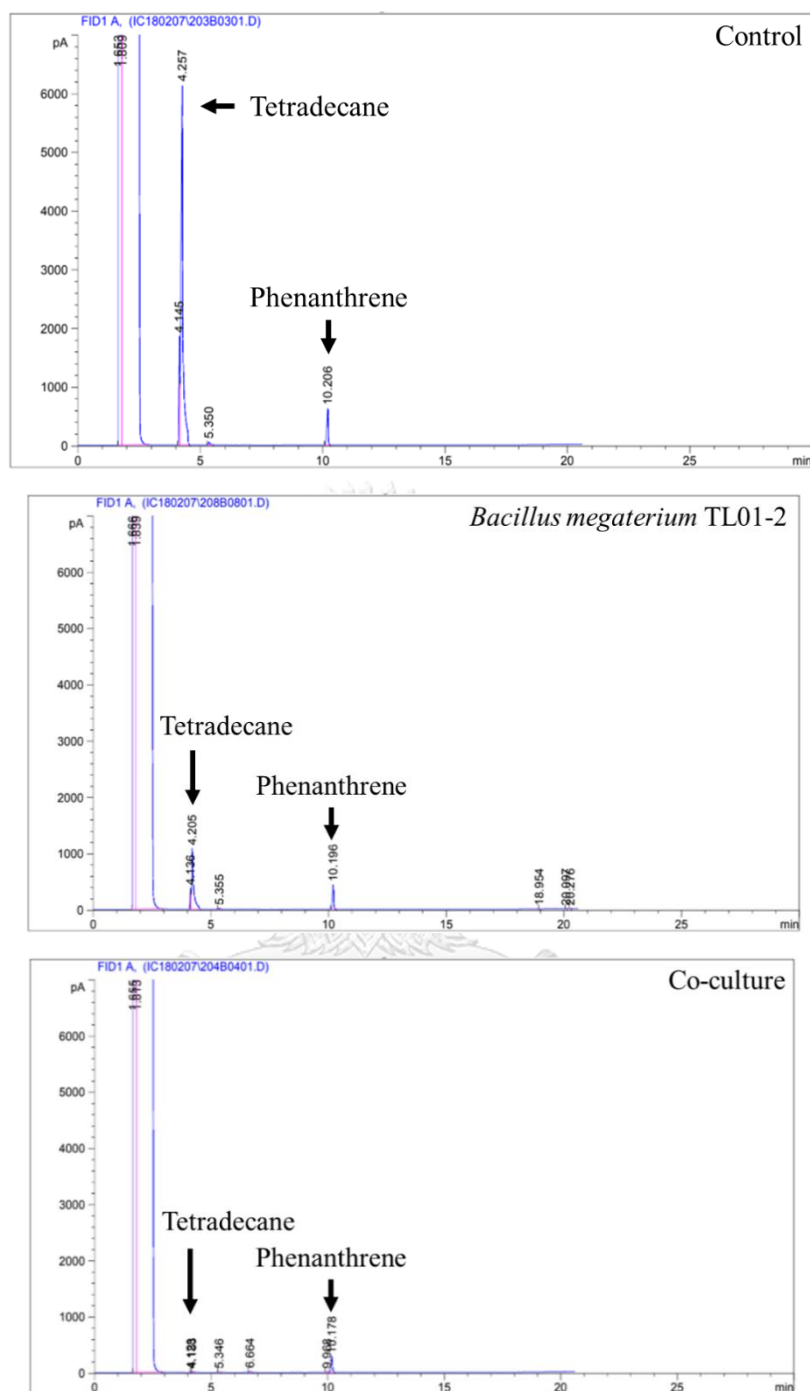
Fig. B-13 Chromatogram of mixture of 500 mg/L tetradecane and 25 mg/L phenanthrene degradation by *Bacillus megaterium* TL01-2 and the co-culture at day7

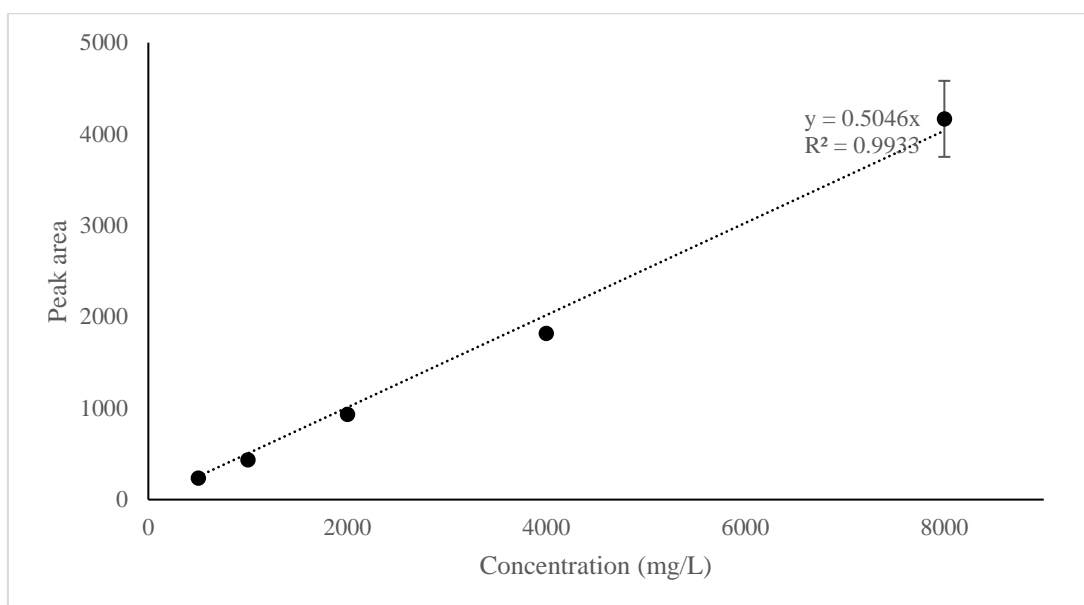# APPENDIX C

PHASE II SUPPLEMENTARY DATA

Fig. C-1 Crude oil standard curve

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Table C-1 Crude oil removal from seawater by immobilized bacteria

Treatment	Location	Crude oil remaining (mg/L)			
		Day 0	Day 1	Day 3	Day7
Control (sterilized seawater)	Seawater	2,050.04± 187.69	1,851.26 ±49.32	2,254.94 ±96.24	2,087.15± 145.42
Sterilized AQs	Seawater	1,230.42± 81.45	599.17 ±3.58	458.76 ±3.73	70.64 ±7.99
	AQs	922.06 ±42.63	971.68 ±42.63	1,444.62 ±5.25	1,846.92± 128.26
	Total	2,152.48± 61.30	1,560.18 ±45.62	1,903.38 ±5.33	1,917.56± 127.78
Group A, Separately immobilize then pooled together with 1:1 AQs weight	Seawater	1,669.67± 6.87	418.95± 0.94	422.10± 1.19	559.98±0. 55
	AQs	247.84 ±5.52	884.21 ±43.23	895.65 ±85.93	480.91 ±5.17
	Total	1,917.51± 72.96	1,303.17 ±45.52	1,317.75 ±87.33	1,040.89± 8.45
Group B, The 1:1 (MO2-4:TL01-2) inoculum volume ratio co-culture immobilization	Seawater	1,312.09± 75.29	927.83 ±46.39	952.15 ±46.39	369.26 ±4.72
	AQs	673.87 ±7.51	279.05 ±5.98	400.98 ±17.34	177.59 ±1.62
	Total	1,985.96± 76.77	1,206.88 ±74.38	1,353.13 ±57.54	546.85 ±5.60
Group C, The 2:1 (MO2-4:TL01-2) inoculum volume ratio co-culture immobilization	Seawater	1,182.21± 1.32	1,112.10 ±0.56	1,045.99 ±6.43	293.00 ±0.16
	AQs	959.73 ±28.93	163.87 ±1.27	297.11 ±19.74	197.47 ±8.06
	Total	2,141.94± 3.16	1,275.97 ±0.65	1,343.10 ±6.50	490.46 ±0.12

Semi-continuous system was initially done using 2,000 mg/L crude oil as starting concentration and spiked with 2,000 mg/L crude oil every week for 5 weeks; the final concentration of crude oil was 10,000 mg/L. The result was shown in Fig. C-2; the concentration of crude oil was too high for both immobilized bacteria and free-cell bacteria and the result could not be clearly interpret. Therefore, the concentration of crude oil was adjusted to 500 mg/L and spiked with 500 mg/L crude oil every week for 5 weeks.
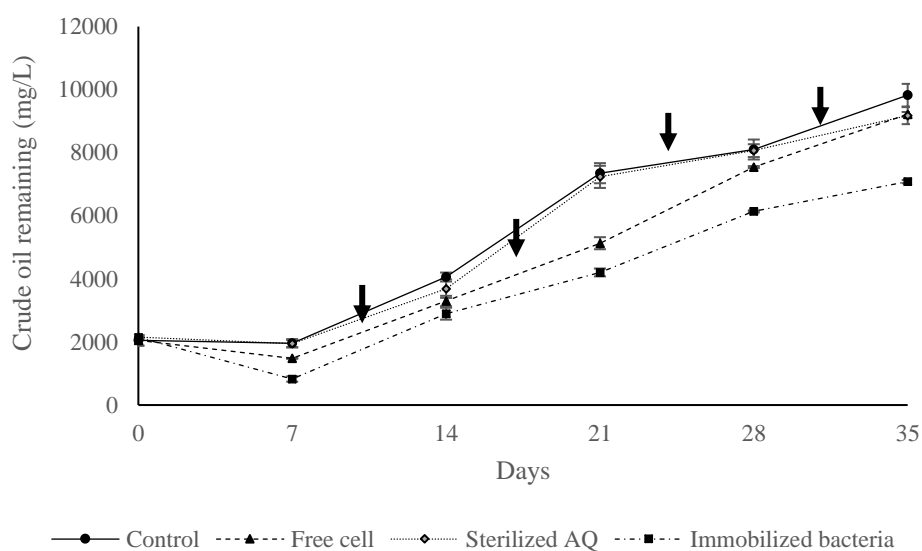

Fig. C-1 Crude oil remaining in the system with the initial crude oil concentration of 2,000 mg/L; arrows indicated the day where 2,000 mg/L crude oil was spiked into the system.

Table C-2 Semi-continuous system with the initial crude oil concentration at 500 mg/L. Crude oil 500 mg/L was spiked into the system every week for 5 weeks.

Treatment	Location	Crude oil remaining (mg/L)					
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Control (Sterilized seawater)	Seawater	726.57 ±49.87	486.97 ±10.65	1,087.34 ±51.81	1,453.42 ±135.13	1,753.46 ±62.43	2,511.71 ±184.50
Free-cell bacteria, 2:1 (MO2-4:TL01-2) inoculum ratio	Seawater	421.24 ±55.61	404.19 ±1.54	821.40 ±4.32	957.51 ±18.20	1,325.47 ±24.69	1,584.27 ±12.50
Sterilized AQs	Seawater	377.33 ±7.69	155.28 ±2.00	244.95 ±7.60	137.44 ±3.22	204.39 ±15.55	229.26 ±4.92
	AQs	248.67 ±2.7	284.18 ±4.12	656.31 ±112.92	1,448.89 ±109.52	1,731.78 ±70.57	1,992.14 ±70.80
	Total	626.01 ±10.58	439.46 ±6.13	901.26 ±114.44	1,586.33 ±112.74	1,936.18 ±86.12	2,221.41 ±75.72
Immobilized bacteria, the 2:1 (MO2-4:TL01-2) inoculum volume ratio co-culture immobilization	Seawater	271.97 ±35.99	165.49 ±1.45	157.07 ±2.49	427.52 ±4.49	642.52 ±16.26	816.00 ±66.89
	AQs	133.45 ±10.45	53.40 ±0.25	69.90 ±2.81	111.65 ±6.33	233.88 ±5.25	87.50 ±0.46
	Total	405.41 ±46.44	218.88 ±1.69	226.97 ±5.30	539.17 ±10.83	876.40 ±17.30	903.49 ±67.36

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PUBLICATION   Immobilization of sponge-associated bacteria Sphingobium
                             sp. MO2-4 and Bacillus megaterium TL01-2 on aquaporous
                             gel for crude oil removal in seawater.
                             16S metagenomic analysis reveals adaptability of a mixed-
                             PAH-degrading consortium isolated from crude oil-
                             contaminated seawater to changing environmental
                             conditions.