FORMULATION OF DEFINED CONSORTIA ISOLATED FROM SEDIMENTS OF

THE CHAO PHRAYA AND THA CHIN RIVERS FOR REMOVAL OF PETROLEUM

HYDROCARBONS

Miss Duangporn Polrit

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Hazardous Substance and

Environmental Management

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2016

สูตรแบคทีเรียผสมที่คัดแยกได้จากดินตะกอนแม่น้ำเจ้าพระยาและแม่น้ำท่าจีนสำหรับกำจัด
สารปิโตรเลียมไฮโดรคาร์บอน

นางสาวดวงพร พลฤทธิ์

Thesis Title	FORMULATION OF DEFINED CONSORTIA ISOLATED FROM SEDIMENTS OF THE CHAO PHRAYA AND THA CHIN RIVERS FOR REMOVAL OF PETROLEUM HYDROCARBONS
By	Miss Duangporn Polrit
Field of Study	Hazardous Substance and Environmental Management
Thesis Advisor	Associate Professor Onruthai Pinyakong, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

....Dean of the Graduate School

(Associate Professor Sunait Chutintaranond, Ph.D.)

THESIS COMMITTEE

....Chairman

(Associate Professor Tawan Limpiyakorn, Ph.D.)

....Thesis Advisor

(Associate Professor Onruthai Pinyakong, Ph.D.)

....Examiner

(Associate Professor Ekawan Luepromchai, Ph.D.)

....Examiner

(Parinda Thayanukul, Ph.D.)

....External Examiner

(Verawat Champreda, Ph.D.)

ดวงพร พลฤทธิ์ : สูตรแบคทีเรียผสมที่คัดแยกได้จากดินตะกอนแม่น้ำเจ้าพระยาและแม่น้ำท่าจีนสำหรับ กำจัดสารปิโตรเลียมไฮโดรคาร์บอน (FORMULATION OF DEFINED CONSORTIA ISOLATED FROM SEDIMENTS OF THE CHAO PHRAYA AND THA CHIN RIVERS FOR REMOVAL OF PETROLEUM HYDROCARBONS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อรฤทัย ภิญญาคง, 208 หน้า.

ปัจจุบันประเทศไทยมีปัญหาการปนเปื้อนน้ำมันในสิ่งแวดล้อมในหลายพื้นที่ รวมถึงแม่น้ำเจ้าพระยาและ แม่น้ำท่าจีน ซึ่งเป็นแม่น้ำสายหลักสำหรับการคมนาคมขนส่งทางน้ำ จึงมีโอกาสเกิดเหตุการณ์น้ำมันรั่วได้ โดยน้ำมัน ที่ใช้สำหรับเดินเรือส่วนใหญ่คือ น้ำมันเตา (fuel oil) ซึ่งเป็นสารผสมที่ประกอบด้วย พอลิไซคลิกอะโรมาติก ไฮโดรคาร์บอน (Polycyclic Aromatic Hydrocarbons; PAHs) อะลิฟาติก แอสฟาลทีน และเรซิน ดังนั้นงานวิจัย นี้จึงมุ่งเน้นพัฒนาสูตรแบคทีเรียผสมโดยคัดเลือกแบคทีเรียที่มีประสิทธิภาพในการย่อยสลายสารประกอบ ไฮโดรคาร์บอน สามารถผลิตสารลดแรงตึงผิว และมีไฮโดรโฟบิกของผนังเซลล์สูง เพื่อใช้สำหรับกำจัดน้ำมัน ปิโตรเลียมได้อย่างมีประสิทธิภาพ ในงานวิจัยนี้สามารถคัดแยกแบคทีเรียที่มีความสามารถในการย่อยสลาย PAHs และ อะลิฟาติกที่แตกต่างกันได้ 8 สายพันธุ์ จากนั้นคัดเลือกแบคทีเรียที่มีประสิทธิภาพสูง 3 สายพันธุ์ ได้แก่ *Mycobacterium* sp. J101 ซึ่งสามารถย่อยสลาย PAHs มวลโมเลกุลต่ำ มวลโมเลกุลสูง และอะลิฟาติก *Rhodococcus ruber* S103 ซึ่งสามารถย่อยสลายอะลิฟาติกได้ดีและรวดเร็ว อีกทั้งมีเปอร์เซนต์ไฮโดรโฟบิกของ ผนังเซลล์สูง และ *Mycobacterium* sp. Y502 ซึ่งสามารถย่อย PAHs มวลโมเลกุลใหญ่ได้ดีและมีแนวโน้มสามารถ ผลิตสารลดแรงตึงผิวชีวภาพได้ แบคทีเรียเดี่ยวสายพันธุ์ S103 J101 และ Y502 สามารถย่อยสลายน้ำมันเตาความ เข้มข้น 2,000 มก./ล. ในอาหารเลี้ยงเชื้อเหลวได้ประมาณ 20% ในขณะที่แบคทีเรียกลุ่มที่ประกอบด้วยแบคทีเรีย เดี่ยว 3 สายพันธุ์ ย่อยสลายน้ำมันเตาได้ถึง 41% ในระยะเวลา 7 วัน และเมื่อนำแบคทีเรียกลุ่มนี้มาตรึงบน bio-balls พบว่าแบคทีเรียกลุ่มตรึงมีประสิทธิภาพในการกำจัดน้ำมันเตาในอาหารเลี้ยงเชื้อเหลว และในน้ำจากแม่น้ำ เจ้าพระยาโดยอาศัยบทบาทจากการดูดซับของวัสดุตรึงและประสิทธิภาพการย่อยสลายน้ำมันของกลุ่มแบคทีเรีย และเมื่อวิเคราะห์ปริมาณน้ำมันที่ถูกดูดซับบนวัสดุตรึงที่มีและไม่มีแบคทีเรีย ผลการทดลองพบว่า ความเข้มข้น น้ำมันบนวัสดุตรึงที่มีกลุ่มแบคทีเรีย (816 มก./กรัม วัสดุตรึง) เหลืออยู่น้อยกว่าความเข้มข้นน้ำมันบนวัสดุตรึงที่ไม่มี แบคทีเรีย (1,523 มก/กรัม วัสดุตรึง) โดยกลุ่มแบคทีเรียตรึงสามารถย่อยสลายน้ำมันเตาได้ 46% ในระยะเวลา 15 วัน ขณะที่ไม่พบการย่อยสลายในชุดที่ไม่มีแบคทีเรีย นอกจากนี้ การวิเคราะห์แอมพลิคอนของยีน 16S rRNA และ การตรวจนับจุลินทรีย์ด้วยวิธี viable plate count พบว่ากลุ่มแบคทีเรียตรึงสามารถอยู่รอดตลอดระยะเวลาการ ทดลองได้ ดังนั้นผลการทดลองเหล่านี้แสดงให้เห็นว่ากลุ่มแบคทีเรียตรึงมีแนวโน้มที่จะนำไปประยุกต์ใช้บำบัด สิ่งแวดล้อมที่ปนเปื้อนน้ำมันปิโตรเลียมได้

สาขาวิชา   การจัดการสารอันตรายและสิ่งแวดล้อม   ลายมือชื่อนิสิต _____

ปีการศึกษา 2559   ลายมือชื่อ อ.ที่ปรึกษาหลัก _____

5787515320 : MAJOR HAZARDOUS SUBSTANCE AND ENVIRONMENTAL MANAGEMENT

KEYWORDS: PETROLEUM HYDROCARBONS, DEFINED CONSORTIUM, IMMOBILIZATION, BIOAUGMENTATION

DUANGPORN POLRIT: FORMULATION OF DEFINED CONSORTIA ISOLATED FROM SEDIMENTS OF THE CHAO PHRAYA AND THA CHIN RIVERS FOR REMOVAL OF PETROLEUM HYDROCARBONS. ADVISOR: ASSOC. PROF. ONRUTHAI PINYAKONG, Ph.D., 208 pp.

Recently, Thailand faces petroleum contamination in various environments including Chao Phraya and Tha Chin Rivers, which are the main water transportation resulting in oil spill events on the rivers. The oil used in cargo ship is fuel oil which is the mixtures consisting of polycyclic aromatic hydrocarbons (PAHs), aliphatic, asphalthene, and resin. Therefore, this study aimed to formulate the defined bacterial consortium from the selected effective hydrocarbon-degrading and biosurfactant-producing bacteria which having high cell surface hydrophobicity for removal of petroleum oil. In this study, eight strains having different ability to degrade PAHs and aliphatic compounds were obtained and then three high effective bacteria were selected. *Mycobacterium* sp. J101 had ability to degrade low- and high-molecular weight PAHs and aliphatic compounds. *Rhodococcus ruber* S103 degraded aliphatic compounds and had high cell surface hydrophobicity and *Mycobacterium* sp. Y502 degraded high molecular weight PAHs as well as showed potential to produce biosurfactants. These three individual strains, S103, J101, and Y502 could degrade approximately 20% of fuel oil at 2,000 mg L^{-1} in liquid cultivation within 7 days. While, the defined consortium composed of these three strains degraded fuel oil at 41%. When this defined consortium was immobilized on bio-balls, the immobilized defined consortium had fuel oil removal efficiency in carbon free mineral medium (CFMM) and freshwater from Chao Phraya river by adsorption ability of bio-balls and biodegradation efficiency of defined consortium. When the fuel oil remaining on bio-balls with and without defined consortium was analyzed, it was found that the concentration of fuel oil on bio-ball having defined consortium (816 mg g^{-1} bio-ball) was lower than the concentration of fuel oil on sterilized bio-ball (1,523 mg g^{-1} bio-ball). The immobilized defined consortium was able to degrade 46% of fuel oil within 15 days, whereas no degradation was observed in the experiment without bacteria. Furthermore, the result of 16S rRNA gene amplicon sequencing analysis and viable plate count technique found that immobilized defined consortium could survive throughout the experimental period. Consequently, these results indicated that the immobilized defined consortium had a potential to apply for bioremediation of petroleum oil contaminated in the environment.

Field of Study: Hazardous Substance and   Student's Signature ----------------------------------

Environmental Management   Advisor's Signature ----------------------------------

Academic Year: 2016

ACKNOWLEDGEMENTS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Nowadays, petroleum hydrocarbons are widely used in transportation, industry, and agriculture because of the rapid increasing population with industrialization and economic development. Thailand, therefore, confronts petroleum contamination problems in various ecosystems including the Chao Phraya and Tha Chin Rivers, which are the country's main water transportation. Oil spill accidents have occurred in both rivers; for example, diesel and fuel oil spilled in Chao Phraya estuary in 1974 and 1996 caused by a collision of tanker and container vessel (Wattayakorn, 2012). Recently, petroleum oil has also contaminated both rivers; for instance, in 2014, diesel fuel was spilled onto the Chao Phraya River with the oil slick covering a radius of about 40 meters from the capsized boat (Mthai, 2012).

In general, petroleum hydrocarbon used on cargo ships is fuel oil, which consists of 15% saturates, 62% aromatics, 15% asphaltene, and 8% resin. Because of their physical properties and chemical composition, long-chain aliphatic and polycyclic aromatic hydrocarbons (PAHs) are present in the residual hydrocarbons of the petroleum products that accumulate in the sediment. PAHs, thus, are used as a parameter in assessing the relative potential of petroleum products to cause long-term effects (Grimwood, 2001). It is due to their toxic, mutagenic, and carcinogenic potential. They also persist in the environment because of low solubility, high hydrophobicity, and complex structure. In addition, there have been reported that fuel oil is toxic to aquatic organisms such as fish embryo and the human lung (Adams et al., 2014; Oeder et al., 2015).

There are many approaches to remediate petroleum hydrocarbon-contaminated sites including physical, chemical, and biological treatments. One of the popular processes for environmental treatment is bioremediation because it is effective, simple, economic, and eco-friendly (Mao et al., 2012; Thapa et al., 2012).

Bioremediation is an approach to transform the pollutants to less hazardous chemicals using microorganisms including algae, bacteria, yeast, and fungi (Ward et al., 2003). This study is interested in bioaugmentation that uses selected bacteria for removal of petroleum hydrocarbons because it is non-toxic, cheap, and suitable to treat various contaminated sites (Semrany et al., 2012). Due to their properties, PAHs are adsorbed strongly to particulate matter and accumulate in bottom sediments (Isaac et al., 2013). Hence, effective hydrocarbon-degrading bacteria can be isolated from sediments, which are the accumulation source of recalcitrant toxic pollutants. Sediments also have a high diversity of microorganisms because they receive the deposition of microorganisms and organic matters (Zinger et al., 2011). The examples of hydrocarbon-degrading bacteria isolated from the sediment are *Mycobacterium* sp. (Ho et al., 2000), *Rhodococcus* sp. (Song et al., 2011), *Streptomyces* sp. (Balachandran et al., 2012), *Pseudomonas* sp. and *Sphingomonas* sp. (Pedetta et al., 2013).

Recently, there has been growing interest in the use of defined consortia for the treatment of petroleum-contaminated sites. The ideal defined consortia should provide multiple metabolic capacities and the number of catabolic pathway available for contaminant biodegradation, ability to degrade a wider substrate and shorter lag period in the degradation (He et al., 2013; Mikesková et al., 2012). For example, Jasmine and Mukherji (2015) selected bacteria based on their aliphatic and aromatic hydrocarbon degradation abilities. The results demonstrated that defined consortium (*Sphingomonas* sp. AS1, *Ochrobacterium* sp. BSW, and *Burkholderia* sp. HN1) degraded 0.5% (w/v) of extracted oil at 71.5% within 30 days. According to Isaac (2015), five strains that had the ability to degrade low and high molecular weight of PAHs and produce biosurfactant were selected to formulate the consortia. It was found that all consortia reached 100% efficiency in phenanthrene and naphthalene degradation at 0.1 mM each in 48 h. They also degraded pyrene more than monocultures; for example, defined consortium (*Pseudomonas monteilii* P26, *Pseudomonas* sp. N3, *Gordonia* sp. H19, and *Rhodococcus* sp. F27) removed 0.1 mM of phenanthrene and naphthalene at 100% in 2 days and of o.1 mM of pyrene at 42% in 21 days. Whereas, individual strain P26, N3, H19 and F27 could degrade less

than 10%. Moreover, many researchers found that PAH-degrading bacteria could produce the biosurfactant; for instance, a new strain of *Brevibacillus* sp. PDM-3 was able to degrade 93% of phenanthrene (250 mg L^{-1}) in 144 h with the production of the biosurfactant, which started from 60 h increased up to 144 h. The surface tension decrease from 47 to 23 mN m^{-1} in 144 h (Reddy et al., 2010). These findings suggested that biosurfactant might enhance the bioavailability of hydrophobic compounds to bacteria and then increase biodegradation ability. Consequently, the criteria for selection of bacteria are important to formulate defined consortia that degrade components of petroleum oil. The properties of bacteria reconstituted hydrocarbon-degrading defined consortia are the abilities to degrade low and high molecular weight PAHs, degrade aliphatics as well as produce biosurfactant. Additionally, aerobic bacteria that can degrade components of oil must have specific genes including dioxygenase and monooxygenase encoding genes (Cébron et al., 2008; Kohno et al., 2002). The information on genetic mechanisms of biodegradation of oil is necessary to design efficient and predictable remediation procedures.

For environmental applications, free cell and immobilized cell have been used for biodegradation in contaminated sites. Nevertheless, there are many research reported that the immobilized cell has a higher efficacy of petroleum hydrocarbon degradation than the free cell (Huang et al., 2016; Khondee et al., 2012; Lin et al., 2014; Partovinia and Naeimpoor, 2014; Quek et al., 2006). Immobilization technique provides high biomass, cell reuse and economic cost of the process. This technique additionally provides suitable microenvironmental conditions, protection against shear damage, improve genetic stability, high resistance to toxic substances, pH, temperature, solvents and heavy metals, and decline of maturation time for some products (Bayat et al., 2015). The cell immobilization has differently used forms including adsorption, covalent binding, entrapment, and encapsulation. Moreover, the selection of carrier is very important in immobilization such as non-toxicity, high cell mass loading capacity, long shelf life, low cost, and the quality of being easy to handle and regenerate. Interestingly, bio-ball or plastic pellet was used in biodegradation of petroleum hydrocarbons because they are durable, lightweight, capable of floating, and non-toxic. Due to its property, bio-ball is suitable for use as a

carrier for treatment of fuel oil around the port. Nopcharoenkul et al. (2013) showed that immobilized cell using polyethylene plastic pellets (bio-ball) as a carrier not only tolerated higher substrate concentrations but also maintained their biodegradation capabilities. Currently, immobilization technique mostly uses monoculture (Lin et al., 2015; Satchanska et al., 2015; Tao et al., 2010) but there are a few mixed cultures of immobilization technique. Therefore, this study aims to formulate defined consortia from effective petroleum hydrocarbon-degrading and biosurfactant-producing bacteria isolated from the sediments of Chao Phraya and Tha Chin Rivers for removal of petroleum hydrocarbons and examine the possibility of the immobilized defined consortium for removal of fuel oil, commonly used on cargo ships, in fresh water from Chao Phraya River.

1.2 Objectives

1.2.1 To formulate defined consortia from effective petroleum hydrocarbon-degrading and biosurfactant-producing bacteria isolated from the sediments of Chao Phraya and Tha Chin Rivers for removal of petroleum hydrocarbons.

1.2.2 To study the possibility of immobilized defined consortium for removal of fuel oil in fresh-water from Chao Phraya River.

1.3 Hypothesis

1.3.1 Pure cultures isolated from the sediments of Chao Phraya and Tha Chin Rivers can degrade various substrates including PAHs, aliphatic, and oils.

1.3.2 Defined consortia composed of PAH-, alkane-, oil-degrading-, and biosurfactant-producing bacteria can more efficiently remove petroleum hydrocarbons than pure cultures.

1.3.3 The immobilized defined consortium had a potential to remove fuel oil by both adsorption and biodegradation activity.

1.4 Scope of this study

The research was divided into three phases as follows:

1.4.1 Isolation of PAH-degrading bacteria from the Chao Phraya and Tha Chin River sediments and their biodegradation potential

PAH-degrading bacteria was isolated and examined their biodegradation in order to obtain PAH-degrading bacteria and study their biodegradation. To determine substrate specificity of each bacterium, different substrates (four PAHs, and two aliphatic) were used as carbon source. Then, biosurfactant test was detected by four different methods namely hemolytic activity, emulsification index, surface tension, and oil displacement and hydrophobicity of bacterial cell was also tested. After that, 3 efficient hydrocarbon-degrading and biosurfactant-producing bacteria were selected and investigated the biodegradation of petroleum oils including fuel oil, crude oil, and diesel oil.

1.4.2 Formulation of defined consortia for petroleum degradation

To formulate the defined consortia from effectively PAH-degrading bacteria for petroleum degradation, three strains having a potential to degrade PAHs, aliphatics, and oil and produce biosurfactant were formulated as defined consortia; four consortia. Four consortia were then determined the biodegradation of fuel oil, diesel oil, pyrene, and docosane representing complex compound, high molecular weight PAH, and long-chain aliphatic. The highest effective defined consortium was chosen for further study. Additionally, genes involved in petroleum hydrocarbon degradation were detected to confirm their function.

1.4.3 Immobilization of defined consortium after formulation

In order to apply to the contaminated site, the highest effective defined consortium was immobilized on polyethylene plastic pellets. An immobilized defined consortium was not only determined the appropriate immobilization time but also examined the biodegradation of fuel oil in fresh water from the Chao Phraya River. In addition, the immobilized defined consortium was monitored by 16S metagenomic analysis in order to know whether the defined consortium survive in freshwater or not.

1.5 Flow chart of this study

1. Isolation and identification of hydrocarbon-degrading bacteria from the sediments of the Chao Phraya and Tha Chin Rivers (use mixture of PAHs as model)

Examination of PAH degradation

Analysis of substrate specificity (i.e. four PAHs and two aliphatics)

Examination of biosurfactant activity and hydrophobicity of bacterial cell

Eight high effective bacteria

3 various substrate-degrading and biosurfactant-producing bacteria

Examination of biodegradation of petroleum oils (fuel oil, crude oil, and diesel oil)

2. Formulation of defined consortia

Examination of biodegradation of fuel oil, diesel oil, pyrene, and docosane

Detection of genes involved in petroleum hydrocarbon degradation

Chosen highest effective defined consortium

3. Immobilization of a defined consortium after formulation

Examination of biodegradation of fuel oil by immobilized the defined consortium in CFMM medium supplemented with fuel oil

Examination of biodegradation of fuel oil by immobilized defined consortium in freshwater from Chao Phraya River

Monitoring the defined consortium by 16S rRNA gene amplicon sequencing analysis and viable plate count technique

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Petroleum contamination in the Chao Phraya and Tha Chin Rivers

Petroleum hydrocarbon is widely used as an energy source in most industrial and developing country. For these reasons, it leads to oil spill accidents that enter the aquatic environments by human activity such as extraction, transportation, refining, storage, and utilization of petroleum (Kvenvolden and Cooper, 2003). Thailand also faces petroleum contamination in various environments, including the Chao Phraya and Tha Chin Rivers. Both rivers are the main water transportation resulting in frequent oil spills. Furthermore, the Chao Phraya River flows through the major industrial property, and the Tha Chin River passes residential and agricultural area. There are over 200 oil spill accidents in Thai water during 1974 – 2009 as shown in Figure 2.1. Table 2.1 shows the major accidents of the oil spill in the Gulf of Thailand during 1974 – 2009. In addition, petroleum oil has been spilled in both rivers; for example, gasoline spilled onto the Chao Phraya River (Mthai, 2012). In 2014, diesel fuel oil spilled into the Chao Phraya River covered a radius of 40 meters from capsized boat (National News Bureau of Thailand, 2015). The yacht that contained 1,500-liter diesel sank in the harbor of the Riverside Bangkok Hotel and the oil slick was observed (Khaosod, 2015). As a consequence of frequent oil spills, the level of petroleum contamination in Thai waters is low to medium level when compared with more urbanized and industrialized countries. The concentration ranges of petroleum hydrocarbons found in the water and sediments in the rivers are shown in Table 2.2.
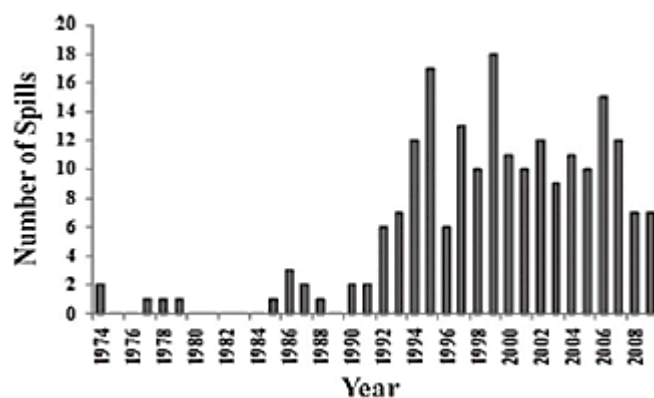
Figure 2.1 Frequency of oil spill events in Gulf of Thailand during 1974 – 2009 (Marine department, 2009)

Table 2.1 The major accidents of the oil spill in the Gulf of Thailand during 1974 – 2009 (Wattayakorn, 2012).

Date	Oil type	Volume (tonnes)	Location	Cause
10 Apr 1974	Diesel & fuel oil	2100	Chao Phraya River mouth	Collision of tanker (Visahakit) and container vessel
29 May 1977	Unknown	300	Chao Phraya River mouth	Collision of tanker (Vachira) and container vessel
1979	Fuel oil	300	Koh Sichang, Chonburi	Grounding due to fire (Sun Flower)
16 Jan 1996	Fuel oil	200	Chao Phraya River mouth	Collision of container vessels

Table 2.1 The major accidents of the oil spill in the Gulf of Thailand during 1974 – 2009 (Wattayakorn, 2012) (continued).

Date	Oil type	Volume (tonnes)	Location	Cause
15 Jan 2002	Diesel oil	240	Sattahip, Chonburi	Grounding of tanker (Eastern Fortitude)
17 Dec 2002	Fuel oil	210	Laemchabang, Chonburi	Collision of container vessel and tanker (Sky Ace)
26 Dec 2004	Fuel oil	150	Koh Larn, Pattaya, Chonburi	Grounding of tanker (Dragon1)

Table 2.2 Concentration ranges of petroleum hydrocarbons in water and river sediments (Wattayakorn, 2012).

Study areas	Water ($\mu g\ l^{-1}$) chrysene equivalent	Water ($\mu g\ l^{-1}$ dw) chrysene equivalent	References
Chao Phraya estuary	2.87 – 43.8 (12.1±10.6)	n.d.	Nokyoo, 1995
Tha Chin estuary	0.93 – 4.25 (2.07±0.88)	4.84 – 151.2 (52.8±37.4)	Sunwanich, 1991; Sunwanich & Wattayakorn, 1991; Wattayakorn & Sunwanich, 1992

Table 2.2 Concentration ranges of petroleum hydrocarbons in water and river sediments (Wattayakorn, 2012).

Study areas	Water (µg l^{-1}) chrysene equivalent	Water (µg l^{-1} dw) chrysene equivalent	References
Chao Phraya estuary Tha Chin estuary	0.19 – 0.80 0.26 – 0.55	n.d.	Chartkittikulwong, 1996; Onodera et al., 1987
Chao Phraya Tha Chin	0.21 – 0.43 0.15 – 0.34	0.72 – 1.57 0.86 – 1.18	Makjun & Ponmanee, 2010

Most petroleum hydrocarbons used for cargo ships are fuel oil leading to contamination of such oil in aquatic environments. The main route of entry to the aquatic environment is accidental spillage during storage, transportation, and use; therefore, the fuel oils are of special concern. The fuel oil is the mixtures that contain polycyclic aromatic hydrocarbons (PAHs), metals, and other compounds. For example, the composition of the fuel oil obtained from PTT Public Company analyzed by TLC-FID analysis consists of 15% saturates, 62% aromatics, 15% asphaltene, and 8% resin. These components of fuel oil including long-chain aliphatic and PAHs appear in the aquatic environments through accidents, spills or leaks because of its persistence and recalcitrance (Grimwood, 2001; Ou et al., 2004).

2.2 Component of fuel oil

    2.2.1 Aliphatic hydrocarbons

Aliphatic hydrocarbons are composed of two different types including straight and saturated carbon chain starting from C6 – C40 that contains odd and even carbon numbers indicating natural and anthropogenic hydrocarbon sources. However, *n*-alkanes are a predominant component of the aliphatic hydrocarbon, which is a low soluble and high hydrophobic compound. In scientific literature, *n*-alkane (linear alkane) has been mentioned as short-chain (C1 to C4), medium-chain (C5 to C9), long-chain (C10 to C17), and very long-chain *n*-alkanes (more than C18) (Matsui et al., 2014). Moreover, there have been reported that these aliphatic hydrocarbons are residual petroleum hydrocarbons and persist in the environment. Furthermore, United Nations Environmental Program (UNEP, 1995) introduced guidelines to identify the levels of harmful (>10 µg g^{-1}) and harmless (<10 µg g^{-1}) aliphatic hydrocarbons in the marine sedimentary environment.

    2.2.2 Polycyclic aromatic hydrocarbons (PAHs)

The fuel oils contain several types of monocyclic and polycyclic aromatic hydrocarbons, PAHs, which are composed of two or more fused benzene rings. PAHs are ubiquitously present in residual fuels because they are less volatile and soluble compounds, which will either adsorb to suspended solid or subsequently settle in the sediments (Grimwood, 2001). Furthermore, they are widely distributed in the environments including soil, sediment, air, and water (Ravindra et al., 2008; Wilcke et al., 2014). They are formed from any incomplete combustion of fossil fuels, coal liquefaction and gasification process (Khalil et al., 2006; Lundstedt et al., 2007). PAHs were additionally found in water and sediments of Chao Phraya and Tha Chin rivers because both of rivers are the main transport routes resulting in the often oil accidents (Wattayakorn, 2012). PAHs, which are found in Chao Phraya and Tha Chin Rivers, are acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene (Boonyatumanond et al., 2006). Total PAH concentrations contaminated in water and sediments of the Chao Phraya and Tha Chin Rivers were 2.47 to 1710 ng L^{-1} and 0.03

to 10.7 mg g^{-1}, respectively. In addition, each PAH has different properties due to their Octanol – Water Partition Coefficient; log K_{ow} (3.4 – 7) and Octanol – Carbon Partition Coefficient; log K_{oc} (3.11 – 6.14) (Jonsson et al., 2007). It indicates that they are low solubility and high hydrophobicity resulting in the accumulation and attachment of solid particle including soil and sediment (Zhang and Tao, 2009). Moreover, Purcaro et al. (2013) reported that the European Scientific Committee on Food (SCF) identified 15 PAHs as both carcinogenic and genotoxic (Table 2.3).

Table 2.3 Name, structure, and molecular weight of PAHs by the Environmental Protection Agency (EPA) and the European Union (EU) (Purcaro et al., 2013)
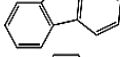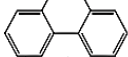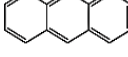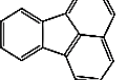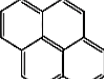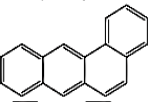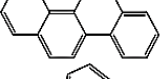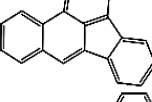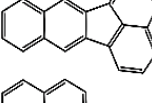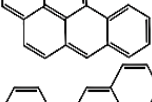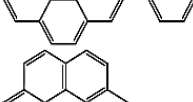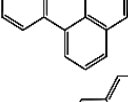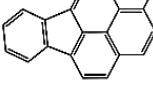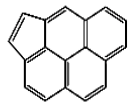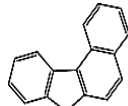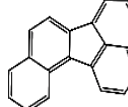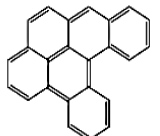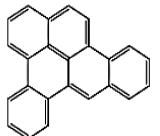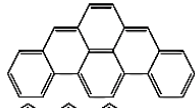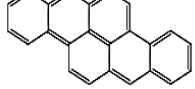
Compounds	Abbreviation	mw	Formula
EPA PRIORITY			
Naphthalene	Na	128	
Acenaphtene	Ac	154	
Acenaphthylene	Ap	152	
Fluorene	F	166	
Phenanthrene	Pa	178	
Anthracene	A	178	
Fluoranthene	Fl	202	
Pyrene	P	202	
EPA/EU PRIORITY			
Benz[*a*]anthracene	BaA	228	
Chrysene	Ch	228	
Benzo[*b*]fluoranthene	BbF	252	
Benzo[*k*]fluoranthene	BkF	252	
Benzo[*a*]pyrene	BaP	252	
Dibenz[*a,h*]anthracene	DBahA	278	
Benzo[*g,h,i*]perylene	BghiP	276	
Indeno[1,2,3-*cd*]pirene	IP	276	

Table 2.3 Name, structure, and molecular weight of PAHs by the Environmental Protection Agency (EPA) and the European Union (EU) (Continued) (Purcaro et al., 2013)

Compounds EU PRIORITY	Abbreviation	mw	Formula
Cyclopenta[c,d]pyrene	CPP	226	
Benzo[c]fluorene	BcF	216	
5-methylchrysene	5MeCh	242	
Benzo[j]fluoranthene	BjF	252	
Dibenzo[a,l]pyrene	DBalP	302	
Dibenzo[a,e]pyrene	DBaeP	302	
Dibenzo[a,i]pyrene	DBaiP	302	
Dibenzo[a,h]pyrene	DBahP	302	

2.3 Toxicity of fuel oil and their petroleum hydrocarbon fractions

Among petrochemical products, fuel oils are of special concern because they are widespread in aquatic ecosystems and highly toxic to aquatic organisms. The fuel oil consists of complex and various components that result in volatile and recalcitrant substances. The residual petroleum products accumulate in the soils and sediments because some chemicals in petroleum oil strongly adsorb to soil and sediment particles and have high hydrophobicity. Thus, the amounts of toxic contaminant and their toxicity depend on the time, dispersal process, and

weathering process. These residues have resulted in negative impacts on aquatic ecosystem including health effects on wildlife and human.

There are several researches studying the effect of fuel oil on aquatic organisms due to the bioaccumulation of fuel oil in the aquatic ecosystems. For example, Alonso-Alvarez et al. (2007) studied the effect of fuel oil on wild yellow-legged gulls that are the uppermost trophic level of the marine food chain. They fed with heavy fuel oil from the Prestige oil spill. The result showed that heavy fuel oil reduced glucose and inorganic phosphorus levels in plasma of gulls, as well as a trend to significantly reduced creatinine values when compared with control gulls fed only with vehicle (i.e. vegetable oil). The lower glucose levels in P-gulls may suggest a decrease in food intake.

In addition, Chao et al. (2012) evaluated the toxicity of four fuel oil including F120, F180, F380, and No.-20 by exposing the marine microalgae *Chlorella* spp. (Chlorophyta) and *Skeletonema coastatum* (Bacillariophyta). The results from bioassay showed that F180 water accommodated fraction (WAF) was the most toxic to both microalgae. In addition, F120 and F380 were especially toxic to marine diatom.

Similarly, Martin et al. (2014) found that H6303 and H6303W fuel oil was chronically toxic to rainbow trout fish embryos followed by H7102 and MESA. Moreover, it was found that the heavy fuel oil is toxic to fish more than crude oil. The alkyl PAH concentrations in oil also explain the rank order of toxicity. Hence, the heavy fuel oil to sink and spawning shoals caused a long-term risk of the fish embryo (developing fish) because PAHs were continually released from the heavy fuel oil.

Furthermore, Oeder et al. (2015) found that fuel oil particles were directly deposited on lung cells, changed in the cellular system, and especially induced the transcription of primary and secondary inflammation markers (IL-8, IL-6, and IL-1).

2.4 Bioremediation

One of the promising technologies to reclaim petroleum-contaminated sites is bioremediation, using microorganisms against algae, bacteria, yeast and fungi to transform or detoxify toxic pollutant to less hazardous chemicals. It is due to high efficiency, eco-friendliness, and low cost (Das and Chandran, 2010). Bioremediation has three main approaches (Perelo, 2010; Yu et al., 2005):

(1) Natural attenuation utilizes intrinsic degradation capacities of the autochthonous microbial population to degrade contaminant. The advantage is the ability to avoid damaging the ecologically sensitive habitat. Nevertheless, this method often takes a long time to complete because population size of native microorganism is low.

(2) Biostimulation, supplying additional nutrients, which affect the growth of indigenous population, is a strategy to promote biodegradation.

(3) Bioaugmentation, introducing appropriate bacteria (a versatile microorganism or an efficient consortium), is a possible mean to enhance biodegradation of contaminants. Several papers indicate that the bioaugmentation is a promising technique to solve toxic substrates and enhance removal efficacy (Asquith et al., 2012; Wu et al., 2008). Interestingly, it has been reported that it is suitable to treat water and sediment contaminated by chemicals as an alternative method (Semrany et al., 2012). For instance, Ruberto et al. (2003) observed the response of the indigenous soil bacteria to the presence of gas-oil in microcosm. The initial concentration of gas-oil was 14,380 µg g^{-1} (dry weight). Indigenous bacteria removed 35% of the total hydrocarbon concentration (THC) while bioaugmentation with *Acinetobacter* sp. strain B2-2 caused a decrease of 65% in 51 days. The results showed that B2-2 strain, bioaugmented bacterium, could improve the biodegradation efficacy representing a valuable alternative tool.

2.5 Use of defined consortia for bioremediation

In general, bacteria are isolated from contaminated areas that are the target for treatment. Most researchers often isolate the bacteria from hydrocarbon-contaminated sediments to obtain effective bacteria degrading those contaminants. The bacteria are cultured in the proper condition by enrichment technique for increasing the amount of hydrocarbon-degrading bacteria. Effective hydrocarbon-degrading bacteria can be isolated from sediments, which are the accumulation source of recalcitrant toxic pollutants. Sediments also have a high diversity of microorganisms. There are many types of research on isolating hydrocarbon-degrading bacteria from petroleum-contaminated sediment shown in Table 2.4.

Table 2.4 Hydrocarbon-degrading bacteria

Bacteria	Area	Substrates	% degradation	Incubation time (days)	References
Paracoccus versutus SPNT	Surface mangrove sediment, China	Mixture of phenanthrene, fluoranthene, and pyrene (each concentration at 10 mg L^{-1})	100	10	Guo et al. (2005)
Rhodococcus sp. P14	Oil and PAH- contaminated sediment, China	Phenanthrene (50 mg L^{-1})	43	30	Song et al. (2011)
		Pyrene (50 mg L^{-1})	34		
		Benzo[a]pyrene (50 mg L^{-1})	30		
Streptomyces sp. ERI-CPDA-1	Soil, India	Diesel (1000 mg L^{-1})	98	7	Balachandran et al. (2012)
		Crude oil (1%v/v)	81	30	

Table 2.4 Hydrocarbon-degrading bacteria (continued)

Bacteria	Area	Substrates	% degradation	Incubation time (days)	References
Pseudomonas sp. PH-a	Surface sediment, Argentina	Phenanthrene (150 mg L^{-1})	75	7	Peddeta et al. (2013)
Sphingomonas sp. PH-b			100	7	
Streptomyces sp. (AH4)	Soil, Algeria	Naphthalene (100 mg L^{-1})	85	12	Ferradji et al. (2014)
		Crude oil (1%v/v)	81	30	
		C$_{28}$	92	30	

However, bioaugmentation with pure cultures often fails due to predation or completion with the indigenous microorganisms, the presence of bacteriophages, or the lack of acclimation the environment (Herrero and Stuckey, 2015). Specific consortia may be thus more useful in ensuring successful bioaugmentation, even in the presence of a single contaminant compared with a single strain (He et al., 2013; Jacques et al., 2008). The defined consortium or mixed culture is the use of different single strains and constructed and artificial microbial consortium by mixing several known strains together. Mikesková et al. (2012) explained why consortia achieve the bioaugmentation. The ideal defined consortia should provide the multiple metabolic pathways available for contaminant biodegradation, high diversity in a microbial consortium, ability to degrade a wider substrate and shorter lag period in the degradation than pure cultures, and competition to indigenous bacteria. Therefore, the criteria for selection of single strains are important for formulation of the defined consortia following as

2.5.1 Individual strains having the ability to degrade various substrates

The isolates capable of degrading various substances were selected to reconstitute the effectively mixed culture because they were expected to have a higher ability to degrade chemicals. For example, Jasmine and Mukherji (2015) isolated pure cultures and studied their ability to degrade component of oil including alkane and PAHs. They chose bacteria that were able to degrade both aliphatic and PAHs. The result revealed that the defined consortia composed of 3 to 5 strains could degrade dried oily sludge (15% w/v) up to 42% and 72%, respectively within 30 days. While the individual strains could not degrade dried oily sludge. In such cases, compared to single isolates, these consortia showed high efficacy in degradation of toxic compounds since they are capable of using a larger number of PAHs and have a high degradation and mineralization rate in vitro and soil.

2.5.2 Biosurfactant producing activity

Owing to limitation of bioavailability e.g. low aqueous solubility and high hydrophobicity, biosurfactants, which are surface-active biomolecules produced by microbes, are recognized for enhancement the solubility of hydrocarbon compounds. Moreover, the biosurfactants are considered the utilization including uptake of soluble fraction, production of surfactants for physical modification of substrates and direct interaction of substrates (Cao et al., 2009; Obuekwe et al., 2009).

Bacteria also play a major role in biosurfactant production. The reported microbial communities producing biosurfactant are *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Halomonas*, *Bacillus*, *Rhodococcus*, and *Enterobacter* (Shekhar et al., 2015). Biosurfactants have many advantages such as low toxicity, higher biodegradability, better environmental compatibility, and wide selectivity (Kuyukina et al., 2005). Zhao et al. (2011) found that the addition of rhamnolipids produced by *Pseudomonas aeruginosa* ATCC9027 enhanced the biodegradation of phenanthrene (250 mg L^{-1}) from 82% to 93% in 30 days.

Researchers also found that PAH-degrading bacteria have the ability to produce biosurfactant during PAH degradation. For instance, a new isolated *Brevibacillus* sp. PDM-3 was able to degrade 93% of phenanthrene (250 mg L^{-1}) in 6 days (144 h) whereas a decrease surface tension from 47 to 23 mN m^{-1} in 6 days. It can be suggested that the biosurfactant produced by microbes might enhance the bioavailability of bacteria to toxic compounds (Reddy et al., 2010).

2.5.3 Cell hydrophobicity

In addition, bacteria, which have cell-surface hydrophobicity, directly contact to hydrophobic hydrocarbons. The bacteria with cell-surface hydrophobicity not only results in enhanced interaction between the organisms but also the hydrophobic substrates. Obuekwe et al. (2009) revealed the relationship between cell surface hydrophobicity (CSH) and the ability of such bacteria to degrade hydrocarbons. *Bacillus thuringensis* which exhibited low CSH values (SAT, 2.0 M; MATH, 3.5%; PB, $0.5\pm0.05\times10^{2}$ cells mm^{2}) degraded only 39.6% of the crude oil substrate in 28 days.

While *B. licheniformis* which exhibited higher CSH values (SAT, 1.8 M; MATH, 4.1%; PB, $5.0\pm0.9\times10^{2}$ cell mm^{2}) degraded greater amount (45.4%) of the crude oil as a substrate within 28 days. It suggested that the extent of degradation tended to increase if the CSH of the isolates is increased. Accordingly, cell hydrophobicity can be criteria for selection of bacteria as defined consortia. For example, Chanaim (2015) used the cell hydrophobicity and the biodegradation efficiency of the mixture of PAHs as criteria to select the highest effective bacterial consortium. It was found that two consortia, G1 and G7, had the highest capability of the biodegradation of the PAH mixtures consisting of phenanthrene, anthracene, fluoranthene and pyrene (50 mg L^{-1} of each PAH) at 68% and 63% within 14 days, respectively. The result of biodegradation is correlated to cell surface hydrophobicity by which two consortia had the highest hydrophobicity of bacterial cells at 66% and 68%, respectively.

2.5.4 Genes involved in petroleum degradation

Several aerobic bacteria, which degrade petroleum hydrocarbon, must have specific genes encoding for enzymes in biodegradation pathway. Key genes involved in petroleum degradation are monooxygenase and dioxygenase genes (Abbasian et al., 2015; Cébron et al., 2008; Kohno et al., 2002). Monooxygenase gene is involved in key steps in alkane degradation. Commonly, *n*-alkane are oxidized by an electron carrier dependent monooxygenase system such as alkane hydroxylase. Additionally, monooxygenase is classified into two class base on electron transport system and microorganisms including rubredoxin-dependent enzyme containing 2FeO encoded by the *alkB* gene and alkane hydroxylase-containing cytochrome P450 monooxygenase in *CYP153* gene. Dioxygenase gene is involved in a key step in PAH degradation by which the dioxygenase gene encoded aromatic ring-hydroxylating dioxygenase enzyme. The aromatic ring-hydroxylating dioxygenase enzymes are grouped into four classes: the toluene/biphenyl family, the naphthalene family, the benzoate family, and finally the phthalate family. Based on above data, several oxygenase enzymes are specific for different substrates. Therefore, biodegradation and genetic mechanisms of microbial degradation of petroleum hydrocarbon are necessary to design efficient and predictable remediation procedures.

2.5.5 Metabolic pathway for petroleum hydrocarbon degradation

The petroleum hydrocarbons contaminated in the environment are removed by bacteria because bacteria have the physiological and metabolic capabilities to degrade pollutants. Bacteria play a role in biodegradation of petroleum hydrocarbon, especially aerobic bacteria. The aerobic bacteria use different types of oxygenases to insert one or two atom O_2 into target compounds. Each class of compound was degraded by a specific enzyme because of the high diversity of the molecular structures of hydrocarbons in petroleum oil. Hence, hydrocarbon compounds were mineralized by different metabolic pathways. In the catabolic pathway, hydrocarbons are taken up by specific microbes and then converted into simple organic compounds. A complex mixture of hydrocarbons is the main fraction in fuel oil. Thus, the fundamental fuel oil degradation metabolism can be described by biodegradation pathways of each hydrocarbon type. Mostly, studies have focused on the biodegradation pathway of alkane and PAHs.

2.5.5.1 Aerobic alkane degradation pathway

The alkane compounds are first oxidized into alcohol at the terminal or sub-terminal alkane. A primary alcohol is produced and oxidized into an aldehyde, subsequently transformed via oxidation into a fatty acid which is funnel into β-oxidation. The alkane is oxidized at sub-terminal alkane into a secondary alcohol (Rojo, 2009). Bacterial *n*-alkane degradation pathways are shown in Figure 2.2.
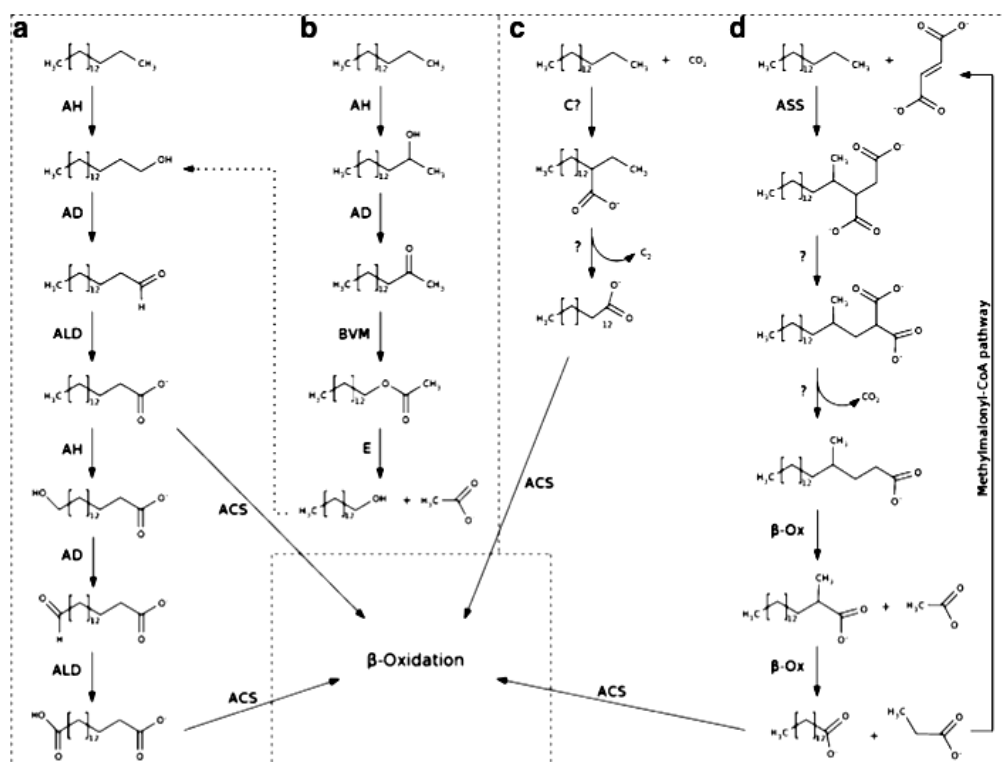
Figure 2.2 Alkane degradation pathways in aerobic and anaerobic bacteria. Aerobic pathways (a, b) are shown in the left panel, while anaerobic pathways (c, d) are shown in the right panel. Aerobic alk-like degradation pathways oxidize alkanes into fatty acids (a). In some cases, ω-hydroxylation generates a dicarboxylic acid. Sub-terminal oxidation of n-alkanes (b) in some *Rhodococcus*, *Mycobacterium*, and *Pseudonocardia* strains yields a primary alcohol two carbons shorter than the original alkane which is further oxidized as shown in a (dotted line). Anaerobic degradation (c) in *D. oleovorans* Hxd3 includes the loss of two terminal carbon atoms via an unknown process. Anaerobic degradation in *D. alkenivorans* AK-01 (d) (Fuentes et al., 2014)

2.5.5.2 Aromatic degradation pathway

For aerobic microbial metabolism, aromatic hydrocarbons (i.e. biphenyl, naphthalene, phenanthrene, and pyrene) are initially oxidized by a ring-hydroxylating dioxygenase (RHD) (Peng et al., 2013; Seo et al., 2012) as shown in Figure 2.3. The ring-hydroxylating dioxygenase oxidizes aromatic molecules into *cis*-dihydrodiol. Then, the *cis*-dihydrodiol are transformed into catechol by a dehydrogenase. The dihydroxylated aromatic ring undergoes fission in *meta*- or *ortho*-position which depends on the catabolic pathway (Peng et al., 2013).
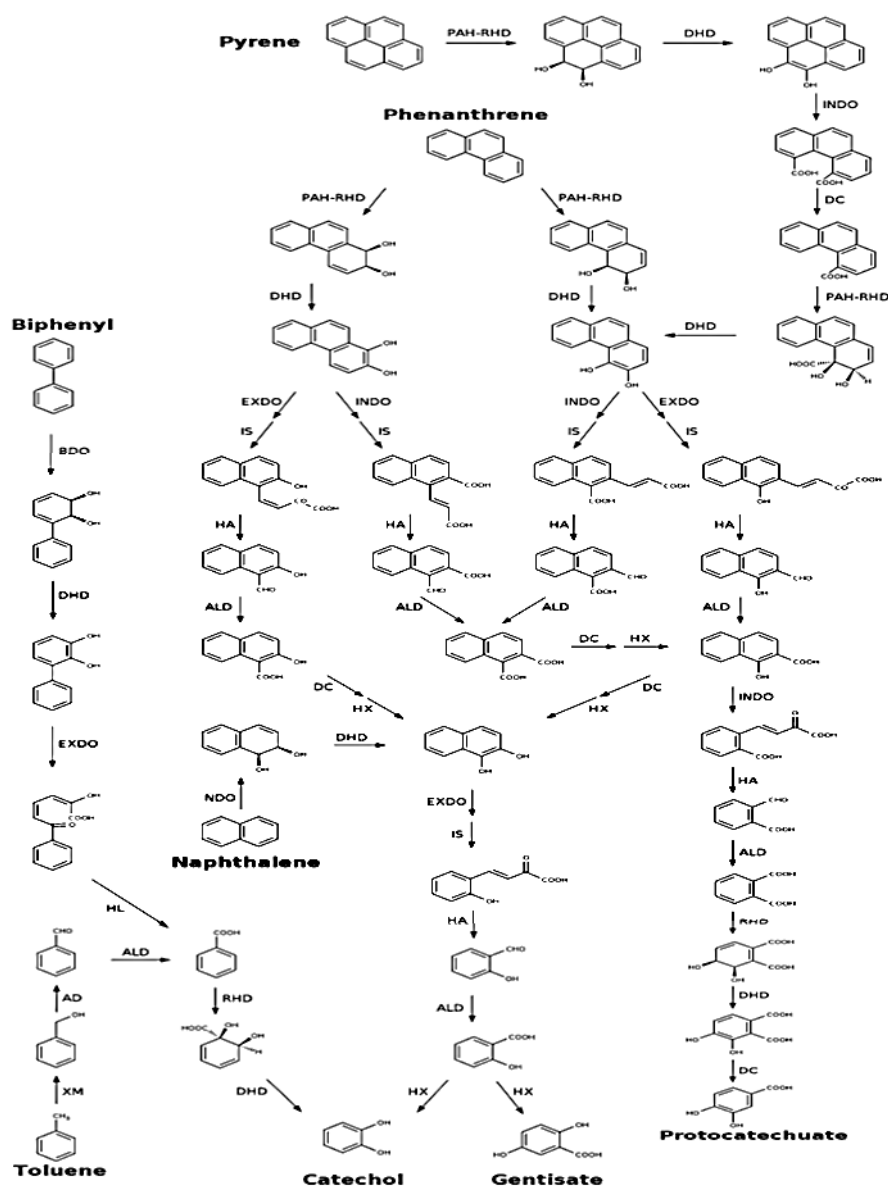
Figure 2.3 Bacterial aerobic aromatic hydrocarbon degradation pathways. In general, initial aromatic ring activation yields a cis-dehydroxylated ring, which is re-aromatized resulting in a catechol-like ortho di-hydroxylated ring. Ring cleavage at *ortho* (intradiol) or *meta* (extradiol) position depends on the catabolic route. NDO naphthalene dioxygenase, DHD dihydrodiol dehydrogenase, EXDO extradiol dioxygenase, INDO intradiol dioxygenase; IS isomerase, HA hydratase aldolase, ALD aldehyde dehydrogenase, HX hydroxylase, PAH-RHD PAH ring-hydroxylating dioxygenase, DC decarboxylase, RHD ring-hydroxylating dioxygenase, BDO biphenyl dioxygenase, XM xylene monooxygenase, HL hydrolase (Fuentes et al., 2014).

2.5.6 Defined consortium

Recently, many studies have employed the individual degradation capacities of different bacterial genera to improve the efficiency of biodegradation of toxic pollutants (Barsing et al., 2011; Cyplik et al., 2011; Gojgic-Cvijovic et al., 2012; Seneviratne et al., 2008; Simarro et al., 2011). Each research studied on the removal of toxic pollutants by the mixed cultures selected by different criteria to reconstitute the effectively defined consortia shown in Table 2.5. Consequently, the capabilities of hydrocarbon-degrading bacteria, biosurfactant producing ability and hydrophobicity of bacterial cell were used as criteria to select effective bacteria for formulation of defined consortia in this study.

Table 2.5 Petroleum oil and its component biodegradation potential of mixed cultures

Mixed cultures	Selection of bacteria	Substrates	% degradation	Incubation time (days)	References
Micrococcus sp. GS2-22, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73, *Bacillus* sp. DS6-86, *Pseudomonas* sp. DS10-129	Chose bacteria that utilized crude oil	Bombay High (BH) crude oil (1%v/v)	78	20	Rahman et al. (2002)
Bacillus sp. IOS1-7, *Corynebacterium* sp. BPS2-6, *Pseudomonas* sp. HPS2-5, and *Pseudomonas* sp. BPS1-8	Chose bacteria that utilized crude oil	Crude oil (1%v/v)	77	25	Sathishkumar et al. (2008)
Pseudomonas aeruginosa. UKMP-8T, *Rhodococcus* sp. UKMP-5T, and *Rhodococcus* sp. UKMP-7T	Chose bacteria that effectively degraded crude oil	Crude oil (1%v/v)	99	7	Hamzah et al. (2013)

Table 2.5 Petroleum oil and its component biodegradation potential of mixed cultures (continued)

Mixed cultures	Selection of bacteria	Substrates	% degradation	Incubation time (days)	References
Pseudomonas sp. XM-01 and *Acinetobacter* sp. XM-02	Chose one bacteria representing as biosurfactant producer and another representing alkane degrader	Crude oil (5%v/v)	87	10	Chen et al. (2014)
Pseudomonas aeruginosa, *Bacillus subtilis*, and *Acinetobacter lwofti*	Chose bacteria that effectively degraded crude oil	Crude oil (0.2% v/v)	89	28	Raed & Shimaa (2014)
Sphingomonas sp. AS1, *Ochrobacterium* sp. BSW, and *Burkholderia* sp. HN1	Chose bacteria that have the ability to degrade aliphatic and aromatic	Extracted oil (0.5% w/v)	72	30	Jasmine & Mukherji. (2015)

Table 2.5 Petroleum oil and its component biodegradation potential of mixed cultures (continued)

Mixed cultures	Selection of bacteria	Substrates	% degradation	Incubation time (days)	References
Ochrobactrum sp. C1 and Bacillus sp. K1	Chose bacteria representing as WEO degrader and another representing as WTO degrader	waste engine oil; WEO (2% v/v)	68	3	Bhattacharya et al. (2015)
		waste transformer oil; WTO (2% v/v)	64		
P. monteilii P26, Pseudomonas sp. N3, Gordonia sp. H19, and Rhodococcus sp. F27	Chose bacteria that degraded LMW and HMW PAHs and had an emulsifying activity	Naphthalene (0.1 mM)	100	7	Isaac et al. (2015)
		Phenanthrene (0.1 mM)	100		
		Pyrene (0.1 mM)	42		

2.6 Application of cell immobilization

For environmental applications, free cell, and immobilized cell have been used as an inoculum in biodegradation in contaminated sites. However, the enhancement of bioaugmentation may also be achieved by delivering suitable microorganisms immobilized on various carriers (Mrozik and Piotrowska-Seget, 2010). There are reported that immobilized cell has a higher efficacy of petroleum degradation than that of a free cell. The immobilized cell provides high biomass, cell recycles, elimination of washout problems at high dilution rate, genetic stability, high resistance, and reduction of maturation time for some products.

In general cell immobilization was defined as "the physical confinement or incapable of movement of intact cells to a certain region of space with preservation of some desired catalytic activity" (Kourkoutas et al., 2004). The immobilization can be divided into four major types (Adsorption, Covalent binding, Entrapment, and Encapsulation) based on the employed physical mechanism shown in Figure 2.4 (Bayat et al., 2015). This study interested a physical adsorption due to quick and simple preparation, low cost, and unnecessary for chemical additives. The adsorption accomplished cell immobilization based on the physical mechanism between the microbe and surface of water-insoluble carriers or matrix. The interaction is weak forces including hydrogen bonds, ionic bonds, hydrophobic bonds, van der Waals forces. Consequently, a better interaction between the substrates and immobilized cells synergistically resulted in developing the degradation rate (Zhen-Yu et al., 2012).

a) Adsorption   b) Covalent Binding   c) Entrapment   d) Encapsulation
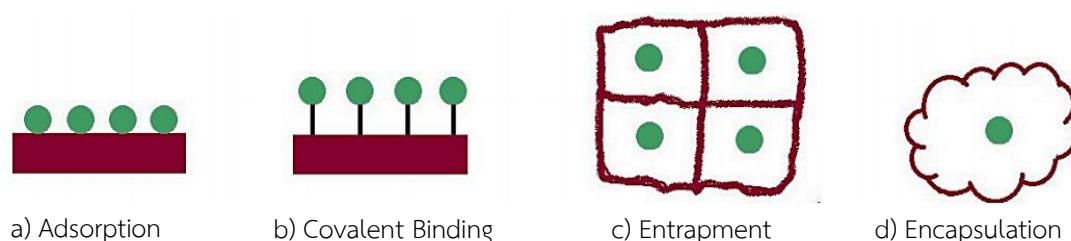
Figure 2.4 Classification of cell immobilization (Bayat et al., 2015)

Tao et al. (2010) investigated the biodegradation of phenanthrene in artificial seawater by using free cell and immobilized cell of *Sphingomonas* sp. GY2B. The results showed that GY2B immobilized on rice straw had a higher degradation rate in both mineral salt medium and 80% artificial seawater than that of the free cell of GY2B. At 32 h, immobilized strains could degrade more than 95% of phenanthrene (100 mg L^{-1}) whereas free cell could degrade around 80% of phenanthrene.

Banerjee and Ghoshal (2011) studied the phenol degradation by *Bacillus cereus* AKG1 and AKG2 in both using free cells and alginate immobilized cells. Various concentrations were investigated the degradation efficiency of free cells and immobilized cells. Compared to free strains, immobilized strains had a higher degradation efficacy at higher phenol concentration (1,500 – 2,000 mg L^{-1}) by 53% – 89% around 26 days for immobilized AKG1 and 36 days for immobilized AKG2. Immobilized cells, thus, have the potential of degrading higher concentrations of toxic pollutants.

Lin et al. (2015) investigated the use of bagasse as a carrier to immobilize *Acinetobacter venetianus*. The degradation of alkane by immobilized *A. venetianus* was observed, and the results revealed that the immobilized cells could remove 93.3% of tetradecane (400 mg L^{-1}) while free cells and bagasse only removed 78% and 24%, respectively after 36 h incubation.

Moreover, Khondee et al. (2012) demonstrated that the oil removal efficiency of chitosan-immobilized *Sphingobium* sp. P2 was higher than that of free cells. The immobilized bacteria could remove 80 – 90% of the 200 mg L^{-1} total petroleum hydrocarbons (TPHs) from both synthetic and car wash wastewater. It was likely due to the number of bacterial cells both on chitosan flasks and in the wastewater, significantly increased 10-fold (1.00×10^8 to 1.67×10^9 CFU g^{-1} chitosan). On the contrary, the efficiency of chitosan-immobilized *Sphingobium* P2 in carwash wastewater was lower than that in synthetic wastewater. It probably was due to the competition for lubricant and another nutrient between the indigenous bacteria and P2 or other toxic contaminants in carwash wastewater.

Above literature reviews, they concluded that the immobilization of bacterial cells has high efficacy on the degradation of petroleum oil. Thus, this study interests

to study the fuel oil removal efficiency by immobilized cells. Polyethylene plastic pellets are interested in this study because this study expected to apply for in situ bioremediation at petroleum oil contaminated-ports in Chao Phraya River. The polyethylene plastic pellet is durable, lightweight, capable of floating, and non-toxic. In addition, few studies are available in the literature on polyethylene plastic pellets as carriers. Nopcharoenkul et al. (2013) immobilized *Pseudoxanthomonas* sp. RN402 on polyethylene plastic pellets as carriers. The immobilized cell not only removed diesel oil by 1,050 mg L^{-1} day^{-1} but also maintained high efficacy and viability throughout 70 cycles of bioremediation treatment of diesel-contaminated water.

Additionally, it is interesting in using immobilized bacterial consortium for enhancement of fuel oil degradation. Recently, there have been reports studying the immobilized bacterial consortium. For example, Shen et al. (2015) showed that the semi-coke immoblized microbial consortium (47%) degraded 3 g L^{-1} of crude oil higher than that of a free microbial consortium (26%) under environmental conditions including 20 g L^{-1} NaCl, temperature of 25°C, and pH at 7.2-7.4 within 5 days. Moreover, SEM and FTIR analysis showed that the structure of semi-coke became more porous resulting in easily adhered to the microbial consortium. It suggested that microbial consortium immobilized might be able to apply in real oil spill bioremediation. This research is the first report that the immobilized defined consortium applied to fuel oil removal in freshwater from Chao Phraya. In conclusion, this research was divided into 3 phases: isolation of hydrocarbon-degrading bacteria from the Chao Phraya and Tha Chin Rivers sediments and their biodegradation capability, formulation of defined consortia for petroleum degradation, and immobilization of a defined consortium after formulation. This study will provide the optimum formulation of defined consortia for petroleum hydrocarbons degradation in water and effective immobilized cells of the defined consortium for petroleum hydrocarbons removal from port water.

2.7 Monitor of bacterial community by 16S rRNA gene amplicon sequencing analysis by next generation sequencing

In reality, in situ bioremediation is a complex circumstance requiring more than one contaminant and different bacterial strains concerning different metabolic pathways. Hence, a monitor of the amount of hydrocarbon-degrading bacteria and petroleum hydrocarbon remaining is necessary for bioremediation. It is important to understand bacterial communities that involved in bioremediation. The use of molecular biology has proven to be powerful tools for analyzing the structure of total bacterial population or exogenous bacteria (added microorganisms) for bioaugmentation such as PCR-DGGE and clone libraries. Nevertheless, these technologies have only provided information on a few bacterial communities. The 16S rRNA gene amplicon sequencing analysis by next generation sequencing has greatly expanded our understanding and provided more comprehensive microbial communities information. According to Techtmann and Hazen (2016) reviewed the metagenomic applications in the environmental monitoring and bioremediation. It was found that 16S rRNA gene amplicon sequencing analysis was applied to monitor bacterial communities in natural attenuation and biostimulation. The 16S rRNA gene amplicon sequencing analysis is interesting to apply with bioaugmentation because it is precise and convenient.

CHAPTER III

METHODOLOGY

3.1 Types of equipment

1. Autoclave from Kakusan, Japan

2. Centrifuge (Model 1920) from Kubota, Japan

3. Controlled environment incubator shaker (model G-27) from New Brunswick Scientific Co. th., USA.

4. Dynamic contact angle meter and tensiometer (Model DCAT21) from Future Digital Scientific Corp., USA.

5. Deep freezer -20°C (model MDF-U332) from Sanyo Electric, Japan

6. Deep freezer -80°C (model ULT1786) from Forma Scientific co.th, USA

7. DNA-Thermal Cycle (model UV-160A) from Gene Technologies co.th, England

8. Flask 250 mL and 500 mL from Pyrex co.th, USA.

9. Gas chromatography (GC) equipped with 320 μm × 30 m HP5 column coated with 5% of phenyl methyl ciloxane (0.25 μm)

10. Gel documentation system (model Gel Doc 2909TM) from Bio-Rad co.th, USA

11. Hot air oven (model D06063) from Memmert, Germany

12. High-Performance Liquid Chromatography (HPLC) equipped with 4.6 × 150 mm Senshu Pak Pegasil ODS column (C18), pump (model LC 10AD*VP*), autosampler (model SIL-10ADVP), and UV-Visible detector (model SPD-10AD*VP*) from Shimadzu Corp., Japan

13. Horiba oil content analyzer from Petro – instrument CROP., LTD.

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

15. Incubator 37°C from New Brunswick Scientific, USA

16. ISSCO laminar flow (model HT-122.5) from International Scientific Supply corp., USA

17. mini agarose gel electrophoresis system (model i-mupid) from Cosmo Bio, Japan

18. Oven from Contherm Scientific, New Zealand

19. pH meter (model 240) from Corning, USA.

3.1 Types of equipment (Continued)

20. Spectrophotometer from Thermo Spectronic, USA.

21. UV-vis spectrophotometer (model nanodrop 2000) from Thermo Scientific corp., USA

3.2 Chemicals

1. Agarose from Research Organics, USA 34.

2. Ammonium nitrate (NH_4NO_3) from Merk, Germany

3. Arabian light crude oil from PPT group co.th., Thailand

4. Bacto agar from Difco, USA

5. Calcium chloride ($CaCl_2 \cdot 2H_2O$) from Merk, Germany

6. Cetyl-trimethyl ammonium bromide (CTAB) from Bio Basic, Canada

7. Chloroform from RCI Labscan, Thailand

8. Diesel oil from PPT group co.th., Thailand

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

10. Di-sodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)

11. Docosane from Sigma, USA

12. Ethanol from Merck, Germany

13. Ethidium bromide from Promega, USA

14. Ethylene Di-amine tetra acetic acid (EDTA) from Sigma, USA

15. Ethyl acetate from Macron, USA

16. Ferric chloride ($FeCl_3 \cdot H_2O$)

17. Fluorene from Kanto, Japan

18. Fluoranthene from Kanto, Japan

19. Fuel oil from PPT group co.th, Thailand

20. Glycerol ($C_3H_8O_3$) from Research Organics, USA

21. GoTaq®qPCR Master Mix from Promega, USA

22. Hexadecane from Sigma, USA 43.

23. Hexane from RCI Labscan, Thailand

24. Hydrochloric acid (HCl) from BDH Chemicals, Australia

25. Isopropanol from Merck, Germany

3.2 Chemicals (Continued)

26. Lysozyme from Bio Basic, Canada

27. Manganese chloride ($MnCl_2$) from Merck, Germany

28. Magnesium chloride ($MgCl_2$) from Merk, Germany

29. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) บริษัท Carlo Erba, France

30. Methanol from Merck, Germany

31. Phenanthrene from Sigma, USA

32. Phenol from Merck, Germany

33. Potassium di-hydrogen phosphate (KH_2PO_4) from

34. Proteinase K from United States Biological, USA

35. Pyrene from Sigma, USA

36. RNase A from Promega, USA

37. Rubidium chloride (RbCl) from Sigma, USA

38. Standard DNA 100 bp DNA ladder from Geneaid, Taiwan

39. Standard DNA Lambda HindIII from New England Biolabs, UK 33.

40. Sodium chloride (NaCl) from Merk, Germany

41. Sodium dodecyl sulfate (SDS) from NacalaiTesque, Japan

42. Sodium hydroxide (NaOH) Merck, Germany

43. Sodium sulfate (Na_2SO_4) Merck, Germany

44. Tetradecane from Fluka, Germany

45. Tryptone from Difco, USA

46. Urea (CH_4N_2O) from Research Organics, USA

47. Yeast extract from Difco, USA

Table 3.1 Primers used in this study

Primers	Nucleotide sequence (5'-3')	Target gene	PCR product size (bp)	Reference
Primers specific for 16S rRNA gene				
27F	AGAGTTTGATCACTGGCTCAG		1465	Lane, 1991
1492R	CGGCTTACCTTGTTACGACTT			
Primers specific for dioxygenase genes involved in PAH degradation				
RHDα-GP-641F	CGGCGCCGACAAYTYGTNGG	Alpha subunit Gram-	292	Cébron et al., 2008
RHDα-GP-933R	GGGGAACACGGTGCCRTGDATRAA	positive RHD		
RHDα-GN-610F	GAGATGCATACCACGTKGGTTGGA	Alpha subunit Gram-	306	Cébron et al., 2008
RHD α-GN-916R	AGCTGTTGTTCGGGAAGAYWGTGCMGTT	negative RHD		
PhnAc-F	CCATTACGGTGATTTCGTGACC	*phnAc*	462	Klankeo et al. 2009
PhnAc-R	ACAAAATTCTCTGACGGCGC			
DbfA1A2-F	CGGCGTCTTCCACGTCTTCG	*dbfA1A2*	392	Nojiri et al. 2002
DbfA1A2-R	CCCGGGGTGGTCATGAGTTC			
NidA-F	ATCTTCGGGCGCGGGCTGGGTGTTTCTCGG	*nidA*	508	Sho et al., 2004
NidA-R	AATTGTCGGGCGGCTGTCTTCCAGTTCG			

Table 3.1 Primers used in this study (continued)

Primers	Nucleotide sequence (5'-3')	Target gene	PCR product size (bp)	Reference
Primers specific for monooxygenase genes involved in aliphatic degradation				
ALK-1F	CATAATAAAGGGCATCACCGT	*alkB*	185	Kohno et al., 2002
ALK-1R	GATTTCATTCTCGAAACTCCAAAC			
ALK-2F	GAGACAAATCGTCTAAAACGTAA	*alkM*	271	Kohno et al., 2002
ALK-2R	TTGTTATTATTCCAACTATGCTC			
ALK-3F	TCGAGCACATCCGCGGCCACCA	*alkB$_1$*	330	Kohno et al., 2002
ALK-3R	CCGTAGTGCTCGACGTAGTT			
AlkB-1F	AAYACNGCNCAYGARCTNGGNCAYAA	*alkB-1*	550	Kloos et al., 2006
AlkB-1R	GCRTGRTGRTCNGARTGNCGYTG			
AlkB1-F	ATCTGGGCGCGTTGGGATTTGAGCG	*alkB1*	629	Whyte et al., 2002
AlkB1-R	CGCATGGTGATCGCTGTGCCGCTGC			
AlkB2-F	ACTCTGGCGCAGTCGTTTACGGCC	*alkB2*	552	Whyte et al., 2002
AlkB2-R	CCCACTGGGCAGGTTGGGCGCACCG			

Table 3.1 Primers used in this study (continued)

Primers	Nucleotide sequence (5'-3')	Target gene	PCR product size (bp)	Reference
Primer specific for monooxygenase genes involved in aliphatic degradation (continued)				
CYP153-F	ATGTTYATYGCNATGGAYCCN	CYP153	820	Wang et al., 2011
CYP153-R	GCGRTTVCCCATRCARCGRTG			
P450F	TGTCGGTTGAAATGTTCATYGCNMTGGAYCC	CYP153	800	Wang et al., 2011
P450R	TGCAGTTCGGCAAGGCGGTTDCCSRYRCAVC K RTG			
AlmAwf	GGNGGNACNTGGGAYCTNTT	almA	1131	Liu et al., 2011
AlmAwr	ATRTCNGCYTTNAGNGTCC			
P450fw1	GTSGGCGGCAACGACACSAC	CYP153	339	Van Beilen et al.
P450rv3	GCASCGGTGGATGCCGAAGCCRAA			(2006)

3.3 Procedure

    3.3.1 Isolation and identification of hydrocarbon-degrading bacteria from sediments

        3.3.1.1 Enrichment and isolation of hydrocarbon-degrading bacteria

    Seven sediments from two rivers, Chao Phraya and Tha Chin Rivers, were collected in the year 2012 following the method from Pollution Control Department (PCD, 2012). The sediment samples were supported by the National Science and Technology Development Agency under the J-RAPID program (P-1201060). The sampling locations of seven sediments were shown in Figure 3.1, and the physiochemical properties of the Chao Phraya and Tha Chin River sediments were shown in Appendix E. To enrich PAH-degrading bacteria, 5 g of sediments were added into 45 mL of carbon free mineral medium (CFMM) (Appendix A) supplemented with mixed PAH solution including fluorene, phenanthrene, fluoranthene, and pyrene (50 mg L^{-1} of each PAH) (Appendix B). The culture flasks were incubated at 30°C with agitation at 200 rpm. After 7 days of incubation, an aliquot of inoculum (5 mL) were transferred to fresh medium containing the same amount of PAHs. After a series of four further enrichments, 0.1 mL of inoculums having broth color change or turbidity were spread on CFMM agar plates supplemented with individual PAH and incubated at 30°C. The colonies surrounded by a clear zone were purified onto Luria-Bertani (LB) agar. Those isolates were cultivated in liquid CFMM supplemented with 100 mg L^{-1} of individual PAH to confirm their activity. The control was CFMM containing PAH without inoculation. All PAH-degrading isolates were identified by 16S rRNA gene sequencing analysis.

(a)
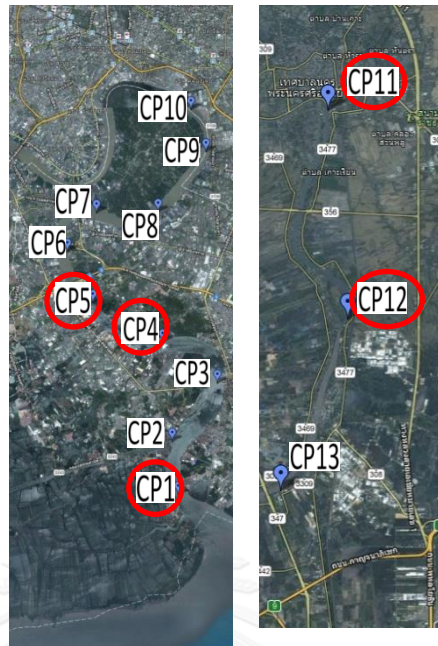
(b)
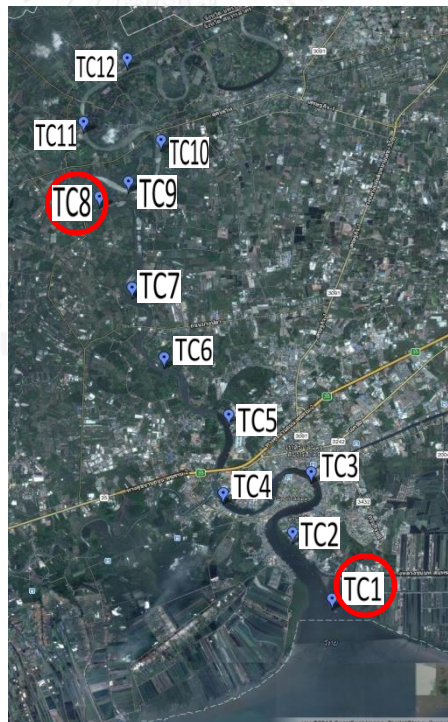
Figure 3.1 Sediment sampling points (a) Chao Phraya and (b) Tha Chin Rivers. The sediments at CP1, CP4, CP5, CP11, CP12, TJ1 and TJ8 were enriched to obtain PAH-degrading bacteria.

3.3.2 Determination of PAH biodegradation efficiency of eight PAH-degrading bacteria

3.3.2.1 Inoculum preparation

Isolates were cultured in 100 mL of 0.25×Luria-Bertani (0.25×LB) broth (Appendix A) and incubated at 30°C with agitation at rpm for 1, 4 and 7 days depending on the type of bacteria. Bacterial cells were harvested by centrifugation at 8,000 rpm, 4°C for 10 min and washed with 0.85% NaCl twice a time. Then, the bacterial cells in 0.85% NaCl solution were measured to obtain OD_{600nm} equal 1 (approx. 10^8 CFU mL^{-1}) and shaken overnight to allow the cells used the acclimated nutrients.

3.3.2.2 Examination of PAH biodegradation of isolated bacteria

The inoculum (0.5 ml) at final concentration 10^6 CFU mL^{-1} was inoculated into 4.5 ml of CFMM supplemented with 100 mg L^{-1} of individual PAH (fluorene, phenanthrene, or pyrene) depending on the substrate used for isolation of PAH-degrading strains in 3.3.1.1. The culture tubes were incubated at 30°C with agitation at 200 rpm. The samples were collected at various time intervals; 3 days for phenanthrene, 7 days for fluorene, and 14 days for pyrene to examine PAH remaining by HPLC and compared with abiotic control (without inoculation). Biodegradation experiments were carried out in triplicate.

3.3.2.3 PAH extraction from CFMM liquid culture

According to the method described in Klankeo et al. (2009), HCl was added to the medium to adjust the acidity-alkalinity of the medium. Ethyl acetate (5 mL) was then added and mixed for 1 min by votex and let stand for separation. The extract was dried using anhydrous sodium sulfate to remove aqueous phase and evaporated. Residual PAHs in the extract were analyzed by HPLC.

3.3.2.4 Analysis of PAH remaining by HPLC

The dried extract was dissolved with 1 mL of methanol and vortex for 1 min. The solution was filtrated with PTFE (0.22 µm). The analysis of PAH remaining was performed by HPLC using the following condition.

Column inertsill® ODS	4.6 diameter × 150 mm length
Column temperature	40°C
Mobile phase	80% methanol
Flow rate	1 mL min^{-1}
UV wavelength	275 nm
Injection volume	10 µL

The peak area from analyzer was calculated for determination of percentage of PAH degradation compared with control as following:

% PAH degradation = $\dfrac{(\text{Peak area of control} - \text{Peak area of sample}) \times 100}{\text{Peak area of control}}$

3.3.3 Analysis of substrate specificity

Eight isolated strains were determined for the substrate specificity against four PAHs (fluorene, phenanthrene, pyrene, and fluoranthene) and two aliphatics (tetradecane and docosane). The experiments were conducted in triplicate by using test tubes containing 4.5 mL of CFMM supplemented with individual substrates. The hydrocarbon substrates were used at different concentrations: 100 mg L^{-1} of fluorene, phenanthrene, pyrene, fluoranthene and docosane, and 500 mg L^{-1} of tetradecane. The abiotic control consisted of CFMM supplemented with hydrocarbons without bacteria. The growth control was the culture without hydrocarbons. The samples were incubated at 200 rpm, 30°C and collected for 14 days. After that, PAH remaining was extracted and analyzed by HPLC according to 3.3.2.3 and 3.3.2.4. Aliphatic remaining was extracted and analyzed by GC-FID as described in 3.3.3.1 and 3.3.3.2.

3.3.3.1 Aliphatic extraction

Residual aliphatic compounds were extracted from the culture with the same amount of hexane. After vortex for 2 min, it was stored at -20°C to allow for the freezing of the aqueous phase and separation of the extract according to previously report (Nopcharoenkul et al., 2013). The extract was then evaporated, dissolved in 1 mL of hexane and filtrated through PTFE filter (0.22 μm).

3.3.3.2 Analysis of aliphatic remaining by GC-FID

According to Nopcharoenkul et al., 2013, the analysis of aliphatic remaining was performed using 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 320°C at 10°C min^{-1}
Carrier gas	He
Flow rate of carrier gas	1.7 mL min^{-1}
Detector temperature	320°C
Mode	Splitless
Injection volume	1 μL

The peak area from analyzer was calculated for determination of percentage of aliphatic degradation compared with control as following:

$$\% \text{ aliphatic degradation} = \frac{\text{(Peak area of control} - \text{Peak area of samples)}}{\text{Peak area of control}} \times 100$$

3.3.4 Surface activities test

Biosurfactant activity of eight isolated strains was investigated by four different approaches: emulsification index (E_{24}), surface tension measurement, oil displacement, and hemolytic activity, as previously described by Thavasi et al. (2011).

3.3.4.1 Pre-culture

Inoculum preparation for surface activities test and cell hydrophobic study was followed by 3.2.1. The inoculum (5 ml) at final concentration 10^{7} CFU mL^{-1} was

inoculated into 45 ml of CFMM supplemented with 2,000 mg L^{-1} of diesel oil. The samples were collected at day 7.

3.3.4.2 Emulsification index (E$_{24}$)

To check emulsification, the culture supernatant was used as biosurfactant source. The culture supernatant (2 mL) was mixed with 2 mL of diesel oil and vortex-shaken for 2 min. The emulsion mixture was allowed to stand for 24 h. Subsequently, the height of emulsion layer was measured and calculated by the following equation.

$$E_{24} = (\text{height}_{\text{emulsion}}/\text{height}_{\text{total}}) \times 100\%$$

3.3.4.3 Surface tension measurement

The surface tension measurement of culture supernatant was determined by tensiometer.

3.3.4.4 Oil displacement

Distilled water (20 mL) was added to a plastic Petri dish, and 20 µL of crude oil was added to the surface of the water. Subsequently, culture supernatant (10 µL) from each bacteria was dropped on the oil surface. If biosurfactant presents in the culture supernatant, the oil is displaced with a clear halo on the oil surface. The diameter of clearing zone was measured and calculated by using the following equation:

% oil displacement = (Diameter sample/Diameter plate) x 100

3.3.4.5 Hemolytic activity

Pure culture was streaked on the sheep blood agar plates and incubated for 48 h at 37°C. The plates were visually investigated for hemolysis around the colony.

3.3.5 Cell hydrophobicity study

The cell pellets were washed and suspended in phosphate urea magnesium sulfate (PUM) buffer. The cell suspension (4 mL) was mixed with 1 mL of hexadecane and vortex-shaken for 1 min. After vortex-shaking, hexadecane and aqueous phases were allowed to separate for 30 min. The OD of the aqueous phase was measured at

400 nm in a spectrophotometer. Percentage of cell hydrophobicity was calculated using the following equation:

$$\% \text{ hydrophobicity} = (1- (OD_{final}/OD_{initial})) \times 100\%$$

3.3.6 Examination of biodegradation of petroleum oil of selected bacteria

After selection of three strains having the ability to degrade various substrates and produce biosurfactants and high cell surface hydrophobicity, they were examined the biodegradation of petroleum oil. The initial concentration of bacterial biomass was 10^6 CFU mL^{-1} for diesel and crude oil biodegradation experiments and 10^7 CFU mL^{-1} for fuel oil biodegradation experiment. The inoculum (0.5 mL) was added to 4.5 mL of CFMM supplemented with 2,000 mg L^{-1} of fuel oil, diesel oil or crude oil. All experiment tubes were incubated at 30°C with agitation at 200 rpm. The abiotic control contained CFMM with petroleum oil (without inoculation). The growth control contained inoculation without petroleum oil. The samples were collected at 3 and 7 days, and the percentage of remaining petroleum oils was analyzed according to the methods in previous reports (Nopcharoenkul et al., 2013). The crude oil remaining was extracted and analyzed by GC-FID according to 3.3.6.1 and 3.3.6.2. The diesel oil remaining was extracted and analyzed by GC-FID according to 3.3.6.1 and 3.3.6.3. The amount of fuel oil remaining was analyzed by oil analyzer according to 3.3.6.4.

3.3.6.1 Crude oil and diesel oil extraction

Residual crude oil and diesel oil were extracted with hexane, vortex for 2 min, and stored at -20°C to allow for the freezing of the aqueous phase and separation of the extract according to previously report (Nopcharoenkul et al., 2013). The extract was then evaporated, dissolved in 1 mL of hexane and filtrated through PTFE filter (0.22 μm).

3.3.6.2 Analysis of crude oil remaining by GC-FID

According to Nopcharoenkul et al., 2013, the analysis of aliphatic remaining 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^{-1}
Carrier gas	He
Flow rate of carrier gas	2.1 mL min^{-1}
Detector temperature	320°C
Mode	Splitless
Injection volume	1 µL

The peak area from analyzer was calculated for determination of percentage of crude oil degradation compared with control as following:

% Crude oil degradation = $\dfrac{\text{(Peak area of control – Peak area of samples)} \times 100}{\text{Peak area of control}}$

3.3.6.3 Analysis of diesel oil remaining by GC-FID

According to Nopcharoenkul et al. (2013), the analysis of aliphatic remaining was performed by GC-FID as following:

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 80°C to 300°C at 10°C min^{-1}
Carrier gas	He
Flow rate of carrier gas	1.7 mL min^{-1}
Detector temperature	320°C
Mode	Split
Injection volume	1 µL

The peak area from analyzer was calculated for determination of percentage of diesel oil degradation compared with control as following:

% Diesel oil degradation = $\dfrac{\text{(Peak area of control – Peak area of samples)} \times 100}{\text{Peak area of control}}$

3.3.6.4 Fuel oil extraction analysis by oil content analyzer

Residual fuel oil was extracted with 25 mL of chloroform, shaken at 200 rpm for 30 min and let stand for separation twice a time. The chloroform layer then was sucked into a new flask. After that, layer with fuel oil and chloroform (500 µL) was transferred into a new tube and evaporated. The extract was dissolved in 10 mL of S316 solution, vortex for 1 min, and filtrated through PTFE filter (0.22 µm). Finally, the fuel oil remaining was analyzed by Horiba oil content analyzer, recorded the results, and compared with control experiment without inoculation.

The value from analyzer was calculated for determination of percentage of fuel oil degradation compared with control as following:

$$\% \text{ Fuel oil degradation} = \frac{(\text{Concentration of control (mg L}^{-1}) - \text{Concentration of sample (mg L}^{-1})) \times 100}{\text{Concentration of control (mg L}^{-1})}$$

3.3.7 Detection of genes involved in petroleum hydrocarbon degradation

Twelve genes involved in petroleum hydrocarbon degradation were detected by using 15 different primers. Alpha subunit Gram-positive RHD (GP), Alpha subunit Gram-negative RHD (GN), *phnAC*, *dbf*A1A2, and *nid*A that involve in PAH degradation were detected. *alk*B, *alk*M, *alk*B1, *alk*B2, *alk*-B, *alm*A, and *CYP*153 that involve in alkane degradation were detected as well. The PCR products and nucleotide sequence of primers were shown in Table 3.1. The reaction was conducted with DNA-Thermal Cycle as following:

RHDα-GP-641F and RHDα-GP-933R

1. initial denaturation   temperature 94°C   for 5 min

2. denaturation   temperature 94°C   for 1 min

3. annealing   temperature 54°C   for 1 min

4. extension   temperature 72°C   for 1 min

5. Go to step 2, 30x

6. final extension   temperature 72°C for 7 min

Note: positive control was *Mycobacterium* sp. PO2 (TRBC 5223).

RHDα-GN-610F and RHD α-GN-916R

1. initial denaturation	temperature 94°C	for 5 min
2. denaturation	temperature 94°C	for 1 min
3. annealing	temperature 57°C	for 1 min
4. extension	temperature 72°C	for 2 min

5. Go to step 2, 29x

6. final extension   temperature 72°C for 7 min

Note: Positive control was *Burkholderia* sp. FP2-1 (Muangchinda et al., 2013)

PhnAc-F and PhnAc-R

1. initial denaturation	temperature 94°C	for 5 min
2. denaturation	temperature 94°C	for 1 min
3. annealing	temperature 57.5°C	for 1 min
4. extension	temperature 72°C	for 1 min

5. Go to step 2, 30x

6. final extension   temperature 72°C for 7 min

Note: no positive control for *phnAC* gene

DbfA1A2-F and DbfA1A2-R

1. initial denaturation	temperature 94°C	for 2 min
2. denaturation	temperature 94°C	for 30 sec
3. annealing	temperature 50°C	for 30 sec
4. extension	temperature 72°C	for 2 min

5. Go to step 2, 25x

6. final extension   temperature 72°C for 7 min

Note: positive control was *Terrabacter* sp. DBF63 (Nojiri et al., 2002).

NidA-F and NidA-R

1. initial denaturation	temperature 94°C	for 5 min
2. denaturation	temperature 94°C	for 1 min
3. annealing	temperature 55°C	for 1.30 min
4. extension	temperature 72°C	for 2 min
5. Go to step 2, 29x		
6. final extension	temperature 72°C for 7 min	

Note: positive control was *Mycobacterium* sp. PO2 (TRBC 5223).

Alk-1F, Alk-1R and Alk2F, Alk-2R and Alk3F, Alk-3R

1. initial denaturation	temperature 94°C	for 5 min
2. denaturation	temperature 94°C	for 1 min
3. annealing	temperature 40°C	for 30 sec
4. extension	temperature 72°C	for 30 sec
5. Go to step 2, 30x		
6. final extension	temperature 72°C for 5 min	

Note: positive control of *alkM* and *alkB1* was consortium G11, which was able to degrade crude oil (Uklam, 2015) but there was no positive control for *alkB* (Alk1 primer).

AlkB-1F, AlkB-1R

1. initial denaturation	temperature 90°C	for 5 min
2. denaturation	temperature 90°C	for 30 sec
3. annealing	temperature 55°C	for 30 sec
4. extension	temperature 72°C	for 1 min
5. Go to step 2, 35x		
6. final extension	temperature 72°C for 5 min	

Note: Positive control was consortium G11, which was able to degrade crude oil (Uklam, 2015).

AlkB1-F, AlkB1-R and AlkB2-F, AlkB2-R

1. initial denaturation	temperature 94°C	for 5 min
2. denaturation	temperature 94°C	for 1 min
3. annealing	temperature 60°C	for 1 min
4. extension	temperature 72°C	for 1 min

5. Go to step 2, 30x

6. final extension temperature 72°C for 3 min

Note: positive control was consortium G11 (Uklum, 2015).

AlmAwf, AlmAwr

1. initial denaturation	temperature 94°C	for 5 min
2. denaturation	temperature 94°C	for 30 sec
3. annealing	temperature 52°C	for 30 sec
4. extension	temperature 72°C	for 1 min

5. Go to step 2, 30x

6. final extension temperature 72°C for 7 min

Note: no positive control

CYP153F1, CYP153R2

1. initial denaturation	temperature 95°C	for 5 min
2. denaturation	temperature 94°C	for 45 sec
3. annealing	temperature 54°C	for 30 sec
4. extension	temperature 72°C	for 1 min

5. Go to step 2, 35x

6. final extension temperature 72°C for 10 min

Note: positive control was consortium G11, which was able to degrade crude oil (Uklum, 2015).

P450F, P450R

1. initial denaturation	temperature 94°C	for 4 min
2. denaturation	temperature 94°C	for 30 sec
3. annealing	temperature 52°C	for 30 sec
4. extension	temperature 72°C	for 1 min

5. Go to step 2, 32x

6. final extension   temperature 72°C for 10 min

Note: positive control was consortium G11, which was able to degrade crude oil (Uklum, 2015).

P450fw, P450rv

1. initial denaturation	temperature 95°C	for 4 min
2. denaturation	temperature 95°C	for 45 sec
3. annealing	temperature 58°C	for 1 min
4. extension	temperature 72°C	for 1 min

5. Go to step 2, 25x

6. final extension   temperature 72°C for 5 min

Note: positive control was consortium G11, which was able to degrade crude oil (Uklum, 2015).

3.3.8 Examination of biodegradation of individual PAH, aliphatic, diesel oil, and fuel oil by defined consortia

Four different defined consortia were formulated by a combination from two up to three strains shown in Table 3.2. The defined consortia consisted the same amount of biomass (10^6 CFU mL^{-1} for PAH, aliphatic and diesel oil and 10^7 CFU mL^{-1} for fuel oil). The defined consortia were examined the biodegradation ability of fuel oil, diesel oil, pyrene, and docosane. The experiments were done in 5 mL of CFMM supplemented with 2,000 mg L^{-1} of fuel oil and diesel oil and 100 mg L^{-1} of pyrene and docosane. All experiment tubes were incubated at 30°C with agitation at 200 rpm. The abiotic control consisted of CFMM supplemented with substrates (without

inoculation). The samples were collected at 7 days. The amount of remaining pyrene, docosane, diesel oil and fuel oil were analyzed according to 3.3.3.2, 3.3.2.4, 3.3.3.2, 3.3.6.3 and 3.3.6.4 respectively. The defined consortium which has the highest efficacy of biodegradation of fuel oil, diesel oil, pyrene, and docosane, was selected for the further experiments.

Table 3.2 Formulation of defined consortia

Defined consortia	bacteria
C1	*Rhodococcus ruber* S103 and *Mycobacterium* sp. Y502
C2	*Rhodococcus ruber* S103 and *Mycobacterium* sp. J101
C3	*Mycobacterium* sp. Y502 and *Mycobacterium* sp. J101
C4	*Rhodococcus ruber* S103, *Mycobacterium* sp. Y502 and *Mycobacterium* sp. J101

3.3.9 Immobilization of individual strains on bio-balls by adsorption

This process was conducted to find the appropriate time of immobilization of each strain on bio-ball. The bio-ball (BCN-009 moving bed media) was available in general and made of high density polyethylene (HDPE) shown in Figure 3.2. The characteristic of bio-ball was shown in Table 3.3. First, the bio-balls (5 g) were added into CFMM media and autoclaved at 121°C, 15 psi for 15 min. Each strain was inoculated into separately flask containing bio-ball and CFMM supplemented with 600 mg L^{-1} fuel oil, and the flasks were incubated on a rotary shaker (120 rpm) at room temperature for 10 days. The bio-ball samples were collected every day to observe the appropriate time that bacteria were able to attach on bio-balls at the most. The immobilized cell (1 g) was added to 9 ml of potassium phosphate buffer at pH 7 to enumerate a number of bacterial cells on bio-balls. Adherence cells were separated by using an ultrasonic bath for 2 min and followed by a vortex mixer for 2

min (this sequence was repeated twice), previously described by Nopcharoenkul et al. (2013). Additionally, the amounts of bacteria in CFMM medium supplemented with 600 mg L^{-1} of fuel oil were analyzed by dilution and viable plate count technique.

Figure 3.2 Bio-balls or (BCN-009 moving bed media)

Table 3.3 Description of bio-ball

Description of bio-ball	
1. Specific gravity	0.95
2. Size	10 mm (wide) × 8 mm (thickness)
3. Color	Black
3. Specific surface	834 $m^2 m^{-3}$
4. Protected surface	494 $m^2 m^{-3}$

3.3.10 Determination of biodegradation of fuel oil by the immobilized defined consortium on bio-balls in CFMM medium

The small-scale experiment was performed to determine the fuel oil removal efficiency of sorbent materials and immobilized defined consortium. Each strain immobilized on bio-balls (at each strain 1 g) was mixed as immobilized defined consortium at a final concentration of immobilized defined consortia 10^7 CFU g^{-1} bio-balls and added into CFMM medium containing 2,000 mg L^{-1} of fuel oil. There were two controls in this experiment. The first control was the sterilized CFMM broth without immobilized cells to study the loss of fuel oil by physicals. The other one war CFMM broth with sterilized bio-ball and supplemented with fuel oil to study the adsorption of fuel oil by bio-balls. The samples were cultured for 7 days at 30°C with agitation at 200 rpm The percentage of remaining fuel oil in CFMM was analyzed by oil analyzer as previously described in 3.3.6.4. In addition, the amount of fuel oil on bio-ball was extracted by adding 10 mL of chloroform and shaken at 200 rpm for 30 min (this process was repeated twice). The chloroform layer having the fuel oil extracted from CFMM medium and bio-balls (500 µL) was sucked into the new tube and evaporated. The extraction was analyzed by oil analyzer according to 3.3.6.4. The value from analyzer was calculated for determination of percentage of fuel oil degradation compared with control as following:

% Fuel oil removal in CFMM $= \dfrac{\text{(Total oil at Day 0 – the amount of oil in CFMM at Day}}{\text{Total oil at Day 0}} \times 100\%$

a) $\dfrac{(\text{Peak area of control} - \text{Peak area of sample}) \times 100}{\text{Peak area of control}}$

% Fuel oil degradation on bio-balls

$= \dfrac{\text{Fuel oil concentration of control at Day a – Fuel oil concentration of samples at}}{\text{Fuel oil concentration of control at Day a}} \times 100\%$

Concentration of fuel oil in CFMM= value from oil analyzer $\times$ (20/100) $\times$ (1000/50) $\times$ dilution

Concentration of fuel oil on bio-balls= value from oil analyzer $\times$ (20/100) $\times$ (1000/20) $\times$ dilution

Note : Total oil = the amount of oil in CFMM + the amount of oil on bio-balls

a : incubation time

Additionally, the bacterial community was monitored by viable plate count technique using 0.1×LB agar, which can observe the color of colony of each strain.

3.3.11 Determination of biodegradation of fuel oil by the immobilized defined consortium on bio-balls in freshwater

To study the possibility of immobilized defined consortia for removal of fuel oil in fresh water from the Chao Phraya River, this experiment was set up. The fresh water collected from Chao Phraya river was analyzed for pH, TOC, COD, BOD, TKN, TP, TPH, heavy metal and total bacteria was used instead of CFMM medium. Each strain immobilized on bio-balls (at each strain 1 g) was mixed as immobilized defined consortium at a final concentration 10^7 CFU g^{-1} bio-balls and added into the freshwater containing 2,000 mg L^{-1} of fuel oil. The non-sterilized freshwater with fuel oil was set up as natural attenuation and compared with abiotic control. The sterilized bio-balls were added into the sterilized freshwater to observe the adsorption of bio-balls. The samples were cultured for 15 days at 30°C with agitation at 200 rpm. The samples were also collected every 5 days. The amount of remaining fuel oil in CFMM was analyzed by oil analyzer previously described in 3.3.6.4. In addition, the amount of fuel oil on bio-ball was extracted by adding 10 mL of chloroform and shaken at 200 rpm for 30 min (this process was repeated twice). The chloroform layer having the fuel oil extracted from CFMM medium and bio-balls (500 µL) was sucked into the new tube and evaporated. The extraction was analyzed by oil analyzer according to 3.3.6.4. The bacterial community was monitored by viable plate count technique using   0.1× LB agar and 16S rRNA gene amplicon sequencing analysis described in 3.3.12.

3.3.12 Monitor of bacterial community by 16S rRNA gene amplicon sequencing analysis

This experiment was performed in order to monitor the bacterial community of defined consortium whether they were able to survive in the system or not. The bacterial cells on bio-balls were separated by using an ultrasonic bath for 2 min and followed by a vortex mixer for 2 min (this sequence was repeated twice). Then, DNA from defined consortium on bio-balls was extracted and amplified 16S rRNA gene by 515F (5'- GTG CCA GCM GCC GCG GTAA – 3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT – 3') (V4 region) primers (Walters et al., 2016). Then, PCR products were purified by AMPure XP beads (Beckman Coulter, USA) to wash buffer primer and primer dimer. The purified DNA was attached to index at the end of the sequencing primer. The reaction of DNA, Index 1 and 2 primers, 2x KAPA HiFi HotStart ReadyMix, and PCR Grade water were set up as following.

	Volume
DNA	5 μL
NexteraXTIndexPrimer 1(N7xx)	5 μL
NexteraXTIndexPrimer 2(S5xx)	5 μL
2xKAPAHiFiHotStartReadyMix	25 μL
PCRGrade water	10 μL
Total	50 μL

After that, PCR clean up 2 was performed again by using AMPure XP beads in order to wash primer-dimer remaining. The concentration of DNA was measured by DeNovix (DeNovix, USA) and calculated using the following equation:

$$\text{Concentration in nM} = \frac{(\text{Concentration in } \frac{ng}{\mu L}) \times 10^6}{660 \frac{g}{\mu L} \times \text{average library size}}$$

Finally, the 16S rRNA gene amplicons were sequenced by Miseq Illumina sequencer (Illumina, USA) using Miseq Reagent Kit V2 (Illumina, USA). Results from sequencer were taxonomically classified by using GreenGenes for the bacterial community and monitored the defined consortium bioaugmented in microcosm.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Bacterial isolates, their identification, and their characteristics

4.1.1 Identification of isolated bacteria and PAH degradation efficacy

The sediments from the Chao Phraya and Tha Chin Rivers were used as sources for isolation of PAH-degrading bacteria because both rivers are the main shipping routes of Thailand. Hence, they are suspected to be polluted with petroleum oil, especially fuel oil which is used in cargo ships. The fuel oil is composed of 15% aliphatic, 62% aromatic hydrocarbon, 15% asphaltene, and 8% resins. There have been reported that PAHs are the main components of fuel oil leading to cause long-term effects on human and environments (Grimwood, 2001; Purcaro et al., 2013). Moreover, several bacteria having ability to degrade PAHs have been isolated from the contaminated sites (Dias et al., 2009). According to (Thoetkiattikul et al., 2017), seven river sediments have different physiochemical characteristics including sediment texture, electric conductivity, and organic carbon content resulting in the variation in groups of inhibiting microbes with different metabolic activities. Therefore, in this study, the collected sediments from the Chao Phraya and Tha Chin Rivers were enriched in liquid CFMM supplemented with mixed PAH solutions including fluorene, phenanthrene, and pyrene (50 mg L^{-1} of each PAH). After 4-5 enrichments, the culture broth having color change or turbidity was spread on CFMM agar containing individual PAH at 100 mg L^{-1} and incubated at 30°C. The colonies with clear zone showing the capability of PAH degradation were found in all types PAHs used. The single colonies were streak on LB agar to check their purity. The pure culture was then inoculated in liquid CFMM containing respective substrates including phenanthrene, fluorene, and pyrene to confirm their activity. It was found that these isolates could degrade PAHs based on the changing the color of cultivation medium which indicates that they utilized PAHs as carbon and energy source and produce some intermediates (Lin et al., 2014; Pinyakong et al., 2012). As a consequence, this study obtained eight PAH-degrading bacteria including four

phenanthrene-, one fluorene-, and three pyrene-degrading bacteria (Table 4.1). These strains were deposited at Microbial Culture Collection Department of Microbiology Faculty of Science, Chulalongkorn University (Appendix C). Four phenanthrene-degrading bacteria strains Y101, Y401, S101, and J801 were identified as *Sphingobium* (Y101 and Y401), *Arthrobacter*, and *Pseudomonas*, respectively, and they degraded phenanthrene more than 90% of initial concentration (100 ppm) after 3 days of incubation. S103 was identified as *Rhodococcus ruber,* and it completely degraded fluorene in 7 days. Three pyrene-degrading bacteria strains J101, Y502, and Y1201 belong to genus *Mycobacterium*. It was noticed that *Mycobacterium* sp. J101 had the highest ability to degrade pyrene at 99% (3 days) followed by Y502 (7 days) and Y1201 (14 days), respectively.

The result indicated that sediment samples from Chao Phraya and Tha Chin Rivers contained various PAH-degrading bacteria in various genus including *Spingobium*, *Arthrobacter*, *Pseudomonas*, *Rhodococcus,* and *Mycobacterium*. There are many reports studying on microbial community and hydrocarbon-degrading bacteria in rivers, estuaries, and sediments. For instance, Pavlova et al. (2008) found that predominant oil-degrading bacteria in the sediment were *Bacillus* spp. (*Firmicutes*). Whereas, dominant oil-degrading microorganisms in the water column were *Rhodococcus* (*Actinobacteria*), *Pseudomonas* (*Gammaproteobacteria*), and *Micrococcus* (*Actinobacteria*). Additionally, Hilyard et al. (2008) obtained bacterial isolates from Elizabeth River sediments by using the selective enrichment grown solely on fluoranthene and pyrene. The bacterial strains were *Pusillimonas* sp., *Alcaligenes* sp., *Mycobacterium* sp., *Pseudomonas* sp., *Parvibaculum* sp., *Stappia* sp., and *Microbacterium* sp. Our study together with several reports showed that various bacteria with ability to degrade petroleum oil and PAHs could be isolated from sediments and these bacteria were interesting to investigate their substrate specificity.

Table 4.1 Bacterial isolates, their identification by 16S rRNA gene sequencing analysis and PAH biodegradation efficacy

Strains	Sediment location	Closest organism (% Identity)	Accession no. of closet strain	Substrates (100 mg L^{-1})	% Degradation	Incubation time (days)
Y101	CP1	Sphingobium quisquiliarum P25 (99%)	NR116446	Phenanthrene	100±0.00	3
Y401	CP4	Sphingobium fuliginis TKP (99%)	NR043537	Phenanthrene	99.62±0.46	3
S101	CP10	Arthrobacter phenanthrenivorans Sphe3 (99%)	CP002379	Phenanthrene	90.59±13.30	3
J801	TJ8	Pseudomonas plecoglossicida FPC951 (99%)	NR024662	Phenanthrene	98.33±1.57	3
S103	CP10	Rhodococcus ruber (100%)	NR026185	Fluorene	99.99±0.02	7
J101	TJ1	Mycobacterium sp. JL852 (99%)	DQ985069	Pyrene	99.25±0.19	3
Y502	CP5	Mycobacterium vanbaalenii PYR-1 (98%)	NR074572	Pyrene	97.46±2.30	7
Y1201	CP12	Mycobacterium gilvum Spyr1 (99%)	CP002385	Pyrene	97.78±1.66	14

4.1.2 The substrate specificity of eight isolated strains

The eight PAH-degrading bacteria were evaluated for their capability to degrade other petroleum hydrocarbons including four PAHs (i.e. fluorene, phenanthrene, pyrene, and fluoranthene) and two aliphatic hydrocarbons (i.e. tetradecane and docosane). These hydrocarbon compounds represent the components of fuel oil. The results of the hydrocarbon biodegradation by eight strains were shown in Table 4.2. Interestingly, *Mycobacterium* sp. J101 had the highest capability to degrade low molecular weight (LMW)-, high molecular weight (HMW)-PAHs and aliphatic compounds. The strain J101 completely degraded phenanthrene and pyrene within 3 days while Y502 and Y1201 completely degraded LMW- and HMW-PAHs in 14 days. On the other hand, *Mycobacterium* sp. Y502 could degrade pyrene at higher rate than Y1201. It was found that *Rhodococcus ruber* S103 degraded tetradecane and docosane better and had a short lag period than four phenanthrene-degrading bacteria (Y101, Y401, S101, and J801). Strain S103 was able to degrade tetradecane and docosane more than 95% within 7 days. *Rhodococcus* and *Mycobacterium* are Actinobacteria, which are the most versatile and efficient petroleum hydrocarbon degraders. They can utilize crude oil, *n*-alkane, phenol, aromatic compounds, and PAHs (Zhang et al., 2012). *Rhodococcus* strains are widely distributed in many environments and capable of degradation of various chemicals including alkane and aromatic compound (Martínková et al., 2009). For example, Peng et al. (2013) isolated *Rhodococcus* sp. p52 from an oil refinery in eastern China. It was found that strain p52 completely removed dibenzofuran within 48 h and metabolized various aromatic compounds such as biphenyl, dibenzo-*p*-dioxin, dibenzothiophene, 2,8-dichlorodibenzofuran, naphthalene, fluorene, phenanthrene, anthracene, carbazole, indole, xanthene, phenoxathiine, xanthone, and 9-fluorenone. In addition, *Rhodococcus* genus also showed the ability to degrade alkane (Sharma and Pant, 2000). *Rhodococcus* sp. NCIM 5126 degraded 500 mg L^{-1} of *n*-tridecane, *n*-tetradecane, *n*-hexadecane, *n*-heptadecane, *n*-ecisonae, kerosene, and pristane at 80, 94, 75, 50, 5, 92, and 30% within 3 days, respectively. Le et al. (2009) also reported that *Rhodococcus ruber* degraded up to 51% of pristane (0.5% v/v).

For genus *Mycobacterium*, it is known as dominant bacteria in the ecosystems such as soil, coal tar contaminated soils, activated sludge from a sewage treatment plant, and estuarine or sediment exposed to petrogenic chemicals (Berekaa and Steinbüchel, 2000; Cheung and Kinkle, 2001; Hormisch et al., 2004). Furthermore, *Mycobacterium* is of specific interest due to their potential to degrade HMW-PAHs which are persistent contaminants and have carcinogenic properties (Kim et al., 2010). HMW-PAHs that *Mycobacterium* was able to degrade are fluoranthene and pyrene (Kanaly and Harayama, 2000; Luan et al., 2006). For example, *Mycobacterium* sp. AP1 (Vila et al., 2001) and *Mycobacterium pyrenivorans* 17A3$^{\mathrm{T}}$ (Derz et al., 2004) could utilize phenanthrene, pyrene, and fluoranthene. Three *Mycobacterium* strains isolated in this study including J101, Y502, and Y1201, had higher efficacy and shorter period degradation of pyrene and fluoranthene than *Mycobacterium gilvum* ASU-06. Strain ASU-06 could degrade to approximately 99% of phenanthrene (100 mg L^{-1}) and 100% of fluoranthene (100 mg L^{-1}) within 20 and 28 days respectively (Darmawan et al., 2015). Tables 4.3 and 4.4 showed the PAH degradation efficiency of *Rhodococcus* and *Mycobacterium* obtained in this study compared to those of other reports, respectively. These results indicated that *Rhodococcus* and *Mycobacterium* isolated in this study had a potential for bioremediation application. .

Moreover, other criteria including the potential of biosurfactant production and cell hydrophobicity of eight strains should be determined for selection and construction of suitably defined consortium.

Table 4.2 Substrate specificity of eight PAH-degrading bacteria

No.	Bacteria	PAHs				Aliphatic	
		Phenanthrene	Fluorene	Pyrene	Fluoranthene	Tetradecane	Docosane
1.	*Sphingobium* sp. Y101	100±0.00 (3d)	83.66±5.21 (14d)	37.45±7.28 (14d)	25.05±5.18 (14d)	85.48±3.66 (7d)	51.68±0.44 (7d)
2.	*Sphingobium* sp. Y401	99.62±0.46 (3d)	98.77±0.93 (14d)	19.07±0.77 (14d)	19.33±0.78 (14d)	94.88±7.24 (7d)	22.44±3.91 (7d)
3.	*Arthrobacter* sp. S101	90.59±13.30 (3d)	2.54±3.60 (14d)	5.87±5.14 (14d)	40.88±11.29 (14d)	100.00±0.00 (14d)	98.20±2.00 (14d)
4.	*Pseudomonas* sp. J801	98.33±1.57 (3d)	99.12±1.02 (14d)	N.D.	N.D.	59.59±7.07 (14d)	32.69±8.06 (14d)
5.	*Rhodococcus ruber* S103	23.09±1.64 (14d)	99.99±0.02 (7d)	2.06±1.52 (14d)	26.93±3.37 (14d)	100.00±0.00 (7d)	95.03±1.24 (7d)
6.	*Mycobacterium* sp. J101	100.00±0.00 (3d)	99.98±0.03 (7d)	99.25±0.19 (3d)	99.63±0.32 (7d)	100.00±0.00 (7d)	100.00±0.00 (7d)
7.	*Mycobacterium* sp. Y502	100.00±0.00 (14d)	73.99±1.74 (14d)	97.46±2.30 (7d)	41.22±11.14 (14d)	98.01±2.81 (7d)	95.03±1.24 (7d)
8.	*Mycobacterium* sp. Y1201	99.99±0.01 (14d)	85.20±10.74 (14d)	34.12±2.23 (7d)	40.75±1.82 (14d)	100.00±0.00 (7d)	69.98±3.75 (7d)

d: day (incubation time); N.D.: Not detected

Table 4.3 Hydrocarbon degradation efficacy of *Rhodococcus*

Rhodococcus	Hydrocarbons	% Degradation	Incubation time (days)	References
Rhodococcus ruber S103	Phenanthrene (100 mg L^{-1})	23	14	This study
	Fluorene (100 mg L^{-1})	100	7	
	Tetradecane (500 mg L^{-1})	100	7	
	Docosane (500 mg L^{-1})	95	7	
Rhodococcus sp. NCIM5126	Tetradecane (500 mg L^{-1})	94	3	Sharma and Pant (2000)
Rhodococcus sp. P14	Phenanthrene (50 mg L^{-1})	43	30	Song et al. (2011)
	Pyrene (50 mg L^{-1})	34	30	
Rhodococcus sp. p52	Tetradecane (200 mg L^{-1})	86	2	Yang et al. (2014)

Table 4.4 Hydrocarbon degradation efficacy of *Mycobacteria*

Mycobacterium	Hydrocarbons	% Degradation	Incubation time (days)	References
Mycobacterium sp. J101	Phenanthrene (100 mg L^{-1})	100	3	This study
	Fluorene (100 mg L^{-1})	100	7	
	Pyrene (100 mg L^{-1})	99	3	
	Fluoranthene (100 mg L^{-1})	100	7	
Mycobacterium sp. NJS-1	Phenanthrene (100 mg L^{-1})	100	14	Zeng et al. (2010)
	Pyrene (100 mg L^{-1})	88	14	
	Fluoranthene (100 mg L^{-1})	62	14	

4.1.3 Biosurfactant activity and hydrophobicity of bacterial cell

Biosurfactant production activity of eight isolated strains was investigated by four different approaches namely emulsification index (E_{24}), surface tension measurement, oil displacement, and hemolytic activity. The hydrophobicity of bacterial cell was also tested. The results showed that eight strains reduced the surface tension ranged from 30 – 46 mN m^{-1} compared to control (55 mN m^{-1}) as shown in Table 4.5. The result of oil displacement revealed that *Rhodococcus ruber* S103, *Mycobacterium* sp. Y502, and *Mycobacterium* sp. Y1201 showed a higher

percentage of oil displacement which ranged from 16 – 20% than others. However, the biosurfactant production activity as detected by an emulsification index was not observed with diesel oil among eight strains. The results further showed that most of the isolated bacteria had high cell surface hydrophobicity and strain S103 had the highest hydrophobicity at 98% (Table 4.5).

It has been indicated that the biosurfactant production activity and cell surface hydrophobicity are critical characteristics in the biodegradation process (Banat et al., 2010; Mukherjee et al., 2006; Pacwa-Płociniczak et al., 2011; Wentzel et al., 2007). Biosurfactants synthesized by several microorganisms including bacteria, yeast and fungi could enhance the hydrocarbon degradation efficiency by facilitating adhesion, decreasing interfacial tension, and dispersing hydrophobic compounds (Martínková et al., 2009; Souza et al., 2014). The cell surface hydrophobicity is important because it is involved in the first step in the process of removing pollutants and it enhances the bioavailability of hydrophobicity compounds to microbes (Obuekwe et al., 2009). Both *Rhodococcus* and *Mycobacterium* have been reported that they produced trehalose lipid biosurfactant (Franzetti et al., 2010). For example, *Rhodococcus ruber* Z25 was used to produce biosurfactant for improving the physical properties of crude oil and oil mobility (Zheng et al., 2012). The results demonstrated that Z25 had a high emulsification and could reduce the interfacial tension approximately to 1.0 mN m^{-1}. *Rhodococcus* sp. CN6 having high cell surface hydrophobicity could degrade *p*-nitrophenol (PNP) up to 300 mg L^{-1} (Zhang et al., 2009). It was suggested that the biodegradation rate of contaminants by the high hydrophobic bacteria was higher than that of the low hydrophobic bacteria.

Consequently, *Rhodococcus ruber* S103, *Mycobacterium* spp. Y502 J101 were selected to formulate the defined consortia because they had the high ability to degrade various chemicals and had a potential to produce biosurfactant and they also had high cell surface hydrophobicity. *Rhodococcus ruber* S103 represented aliphatic-degrading bacteria and had high cell surface hydrophobicity. *Mycobacterium* sp. Y502 served as HMW-PAH-degrading and biosurfactant-producing bacterium due to a shorter lag period in pyrene degradation and lower surface

tension. *Mycobacterium* sp. J101 could be used as LMW- and HMW-PAH and aliphatic-degrading bacteria.

Table 4.5 The ability to produce biosurfactant and hydrophobicity of bacterial cell

No.	Bacteria	E_{24}	% oil displacement	Surface tension (mN/m)	% cell hydrophobicity	Hemolytic activity
1.	*Shingobium* sp. Y101	N.D.	5.04±0.67	41.00±1.29	82.86±2.29	+
2.	*Shingobium* sp. Y401	N.D.	8.33±2.62	32.24±0.62	87.52±0.85	+
3.	*Arthrobacter* sp. S101	N.D.	3.88±0.67	48.58±1.56	31.84±3.39	-
4.	*Pseudomonas* sp. J801	N.D.	5.81±0.00	36.55±1.74	N.D.	+
5.	*Rhodococcus ruber* S103	N.D.	16.28±3.49	39.23±4.28	98.05±0.45	-
6.	*Mycobacterium* sp. J101	N.D.	2.91±0.82	36.14±0.49	75.81±0.74	-
7.	*Mycobacterium* sp. Y502	N.D.	15.89±0.67	33.86±0.95	71.35±6.20	+
8.	*Mycobacterium* sp. Y1201	N.D.	20.35±3.08	30.80±0.12	74.65±0.28	-

Control: Surface tension value of CFMM broth is 54.94±2.98.

N.D.: Not detected; +: positive;-: negative

4.2 Genes involved in petroleum hydrocarbon degradation of the selected strains

The genes involved in petroleum hydrocarbon degradation in three selected strains (S103, J101, and Y502) were detected using the primers shown in Table 3.1. The results revealed the presence of different genes involved in hydrocarbon degradation in each bacterium as shown in Table 4.6. The genes detected was correlated with the hydrocarbon biodegradation efficiency of each strain. For example, *dbfA1A2, alkB1,* and *alkB2* were found in *Rhodococcus ruber* S103 corresponding to fluorene, tetradecane, and docosane degradation, respectively. Similar results were found in previous research that *Rhodococcus ruber* strain SoB contained *alkB* gene (Quatrini et al., 2008). The *alkB* gene is responded for hydroxylation of medium-chain-length alkane (C_{10}-C_{16}) (Nie et al., 2014). Moreover, two dibenzofuran dioxygenase genes, *dbfA* and *dfdA* which are involved in dibenzofuran degradation were found in *Rhodococcus* sp. strain p52 (Peng et al., 2013). The *dbfA* and *dfdA* are genes encoding angular dioxygenase that are distributed among dibenzofuran-degrading actinomycetes strains *Rhodococcus*, *Mycobacterium,* and *Terrabacter* (Iida et al., 2002). Aly et al. (2008) reported that *Rhodococcus* sp. HA01 harbored *dbfA1A2* gene cluster. The *dbfA1* and *dbfA2* have been reported to encode the large and small subunits of terminal oxygenase (Nojiri et al., 2002). Furthermore, alpha subunit of Gram-positive RHD and *nidA* genes were detected in the isolated *Mycobacterium* spp. Y502 and J101 while *alkB1* was found in J101. Alpha subunit of Gram-positive RHD is the PAH-dioxygenase gene (alpha subunit of terminal oxygenase), which was detected in various Gram-positive bacteria including *Mycobacterium vanbaalnii* PYR-1, *Mycobacterium* sp. 6PY1, *Rhodococcus opacus* R7, and *Rhodococcus* sp. NCIMB 12038 (Cébron et al., 2008). The *nidA* gene is a crucial gene for initial hydroxylation of the aromatic ring of pyrene by *Mycobacterium* strains (Brezna et al., 2003; Guo et al., 2010; Hall et al., 2005). Moreover, the *CYP153* gene, alkane hydroxylase gene, was found in *Mycobacterium* sp. Y502. Thus these three strains S103, J101, and Y502 were expected to be able to degrade petroleum oil since they possess the genes involved in the degradation of

petroleum oil components. Their activity on petroleum oil degradation was then examined in a further experiment.

Table 4.6 Genes involved in PAH and aliphatic degradation of three selected strains

Primers	Genes	Bacteria		
		Rhodococcus ruber S103	*Mycobacterium* sp. J101	*Mycobacterium* sp. Y502
GN	Alpha subunit Gram-negative RHD	-	-	-
GP	Alpha subunit Gram-positive RHD	-	+	+
dbfA1A2	*dbf*A1A2	+	-	-
phnAc	*phn*Ac	-	-	-
nidA	*nid*A	-	+	+
Alk1	*alk*B	-	-	-
Alk2	*alk*M	-	-	-
Alk3	*alk*B$_1$	+	+	-
AlkB1	*alk*B1	-	-	-
AlkB2	*alk*B2	+	-	-
AlkB-1	*alk*-B	-	-	-
CYP153	*CYP*153	-	-	-
almAW	*alm*A	-	-	-
P450	*CYP*153	-	-	+
P450fw1&P450rv3	*CYP*153	-	-	+

4.3 Biodegradation of petroleum oils by three selected bacteria

Due to a large variety of petroleum oil contamination in the environments, this study investigated the biodegradation of petroleum oils including fuel oil, crude oil, and diesel oil by a defined consortium consisting of three bacteria having ability to degrade various hydrocarbons and to produce biosurfactants. These oils were mostly used on cargo ships for transportation. The oil degradation activity by individual strain was firstly examined. As a result, the change of oil slick in cultivation medium was different in each experiment as shown in Figures 4.1 – 4.3. Each strain was able to degrade petroleum oils differently as shown in Figure 4.4. After 7 days of incubation, *Rhodococcus ruber* S103 and *Mycobacterium* sp. J101 reached 86% efficiency in crude oil and diesel oil degradation; however, they removed fuel oil around 20%. In contrast, *Mycobacterium* sp. Y502 degraded petroleum oil lower than that of S103 and J101. All of these bacteria were able to degrade crude oil and diesel oil higher than fuel oil because crude oil and diesel oil mainly consist of saturated hydrocarbons more than 70%; while, fuel oil composes of aromatic hydrocarbons such as PAHs that are recalcitrant compounds and are slowly degraded by microorganisms. Additionally, fuel oil has high asphaltene which known to be more resistant to biodegradation by microorganisms (Okerentugba and Ezeronye, 2003). Another reason is that single strain may inefficient to degrade the mixture compounds due to a limited enzyme (Jasmine and Mukherji, 2015; Yuan et al., 2000). There have been reported that bacteria in genera *Rhodococcus* and *Mycobacterium* could degrade crude oil and diesel oil. For example, *Rhodococcus* sp. JZX-01 isolated from oil contaminated soil was able to degrade 65% of crude oil (5% v/v) after 9 days of incubation (Li et al., 2013). *Mycobacterium hyalinum* decomposed 21% of 10,000-ppmv diesel within 5 days (Liu and Liu, 2011). Nevertheless, there have never been reports on the biodegradation of fuel oil. This study is the first report showing the ability of bacteria on fuel oil biodegradation. These strains, thus, were expected to formulate the defined consortia for removal of fuel oil and enhance the efficiency of fuel oil biodegradation potentially.
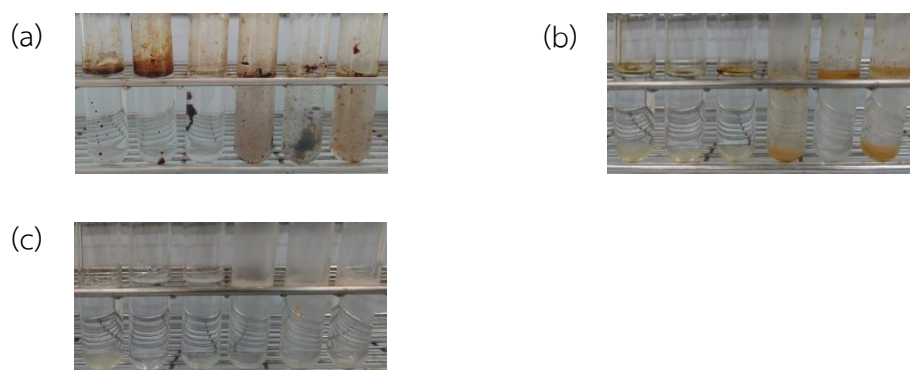
Figure 4.1 The oil slick change in the cultivation medium supplemented with 2,000 mg L^{-1} of fuel oil (a), crude oil (b), and diesel oil (c) of *Rhodococcus ruber* S103. Controls of each experiment were placed in the first three tubes. The samples were incubated for 7 days.
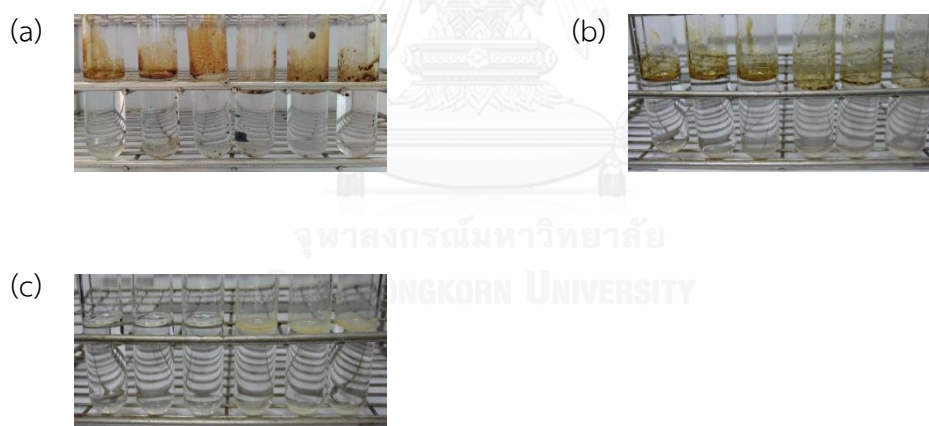
Figure 4.2 The oil slick in the cultivation medium supplemented with 2,000 mg L^{-1} of fuel oil (a), crude oil (b), and diesel oil (c) of *Mycobacterium* sp. J101. Controls of each experiment were placed in the first three tubes. The samples were incubated for 7 days.
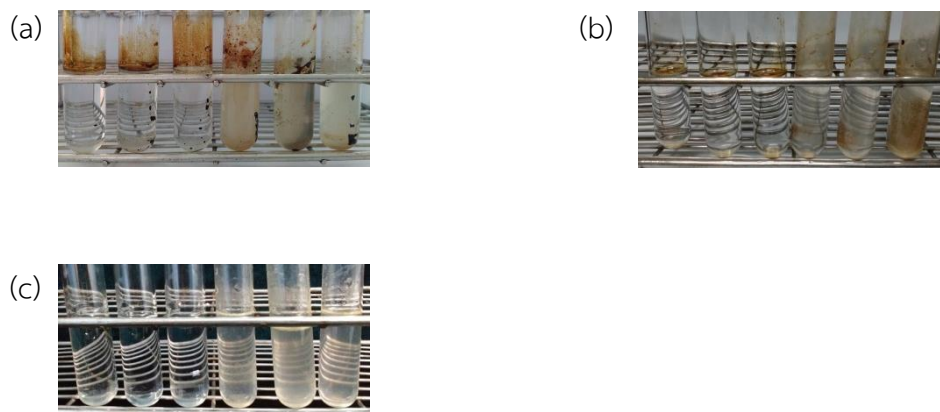
Figure 4.3 The oil slick in the cultivation medium supplemented with 2,000 mg L^{-1} of fuel oil (a), crude oil (b), and diesel oil (c) of *Mycobacterium* sp. Y502. Controls of each experiment were placed in the first three tubes. The samples were incubated for 7 days.
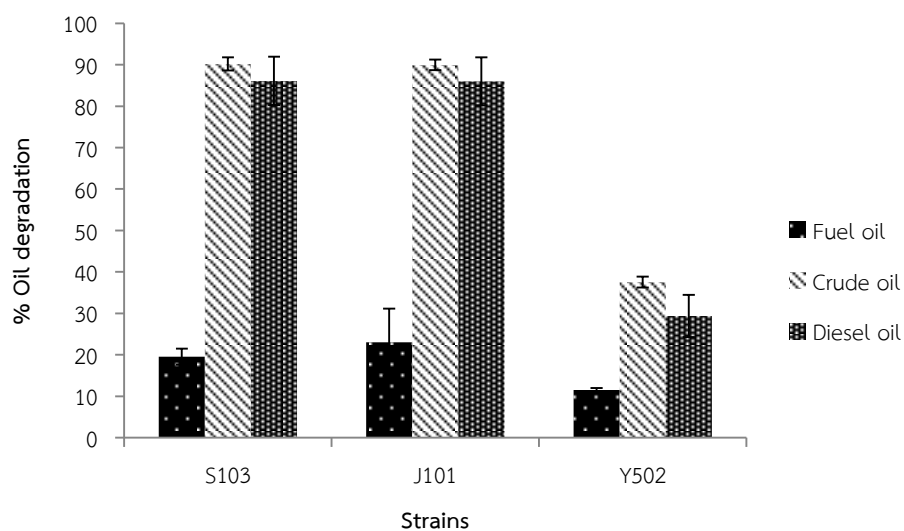
Figure 4.4 The result of petroleum oil biodegradation by three efficient bacteria after 7 days of incubation. The concentration of each petroleum oil type was 2,000 mg L^{-1}

4.4 Biodegradation of fuel oil, diesel oil, pyrene, and docosane by four bacterial consortia

Three hydrocarbon-degrading and biosurfactant-producing bacteria, S103, Y502, and J101, were constituted as four defined consortia. Consortium1 (C1) was comprised of *Rhodococcus ruber* S103 and *Mycobacterium* sp. Y502. Consortium2 (C2) was formed using *Rhodococcus ruber* S103 and *Mycobacterium* sp. J101. Consortium3 (C3) was composed of *Mycobacterium* sp. Y502 and *Mycobacterium* sp. J101. The last consortium was a consortium4 (C4) consisting of *Rhodococcus ruber* S103, *Mycobacterium* sp. Y502, and *Mycobacterium* sp. J101. This study examined the biodegradation of fuel oil (2,000 mg L^{-1}), diesel oil (2,000 mg L^{-1}), pyrene (100 mg L^{-1}), and docosane (100 mg L^{-1}) by four consortia. This examination was conducted to select the highest effective defined consortium for further application use. The results demonstrated that C4 has the most efficiency on fuel oil biodegradation among 4 consortia in which it could degrade fuel oil up to 41% in 7 days (Figure 4.5). In addition, it was noticed that the consortia containing strain Y502 had fuel oil degradation efficiency more than those without Y502 (C2). Oil displacement (with fuel oil) was determined to clarify the tendency on the biosurfactant production of Y502 for enhancing fuel oil bioavailability. The result illustrated that Y502 had higher fuel oil dispersion (11%) than S103 and J101 (2% and 4%). This result indicated that Y502 might produce biosurfactant to enhance the bioavailability of bacterial strains to fuel oil. Furthermore, the results obviously demonstrated that bacterial consortium could degrade fuel oil and diesel oil better and with shorter time than individual strains as shown in Figure 4.5 and 4.6. It is due to a microbial consortium had synergistic metabolisms, and one strain may utilize the intermediates generated by other strains (Jasmine and Mukherji, 2015; Mikesková et al., 2012).
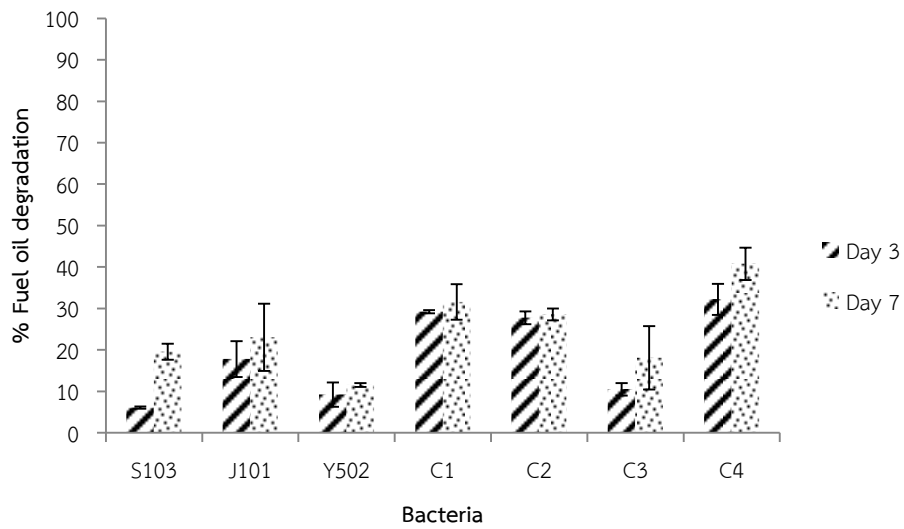
Figure 4.5 Biodegradation efficiency of fuel oil by single strains and bacterial consortia.
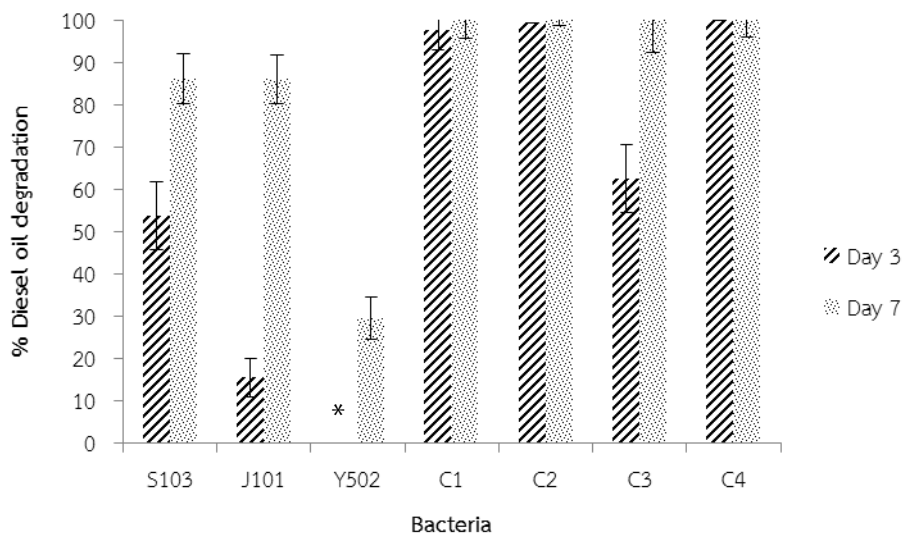
Figure 4.6 Biodegradation efficiency of diesel oil by single strains and bacterial consortia.

Note: * No data of biodegradation of diesel oil by strain Y502 at 3 days.

The result also revealed that the consortia maintained the ability to degrade pyrene and docosane as showed in Figures 4.7 – 4.8. These results demonstrated that a cooperation of different microbes did not inhibit the biodegradation of single chemical. Moreover, bacterial communities of each microbial consortium were monitored by viable plate count based on a different color of three strains as shown in Figure 4.9. The results of viable plate count showed that bacteria still grew and changed in number during degradation of each substance (Tables 4.7 – 4.10). Consequently, C4, defined consortium was chosen for the further experiment because it has higher activity to degrade fuel oil compared with other consortia potentially.
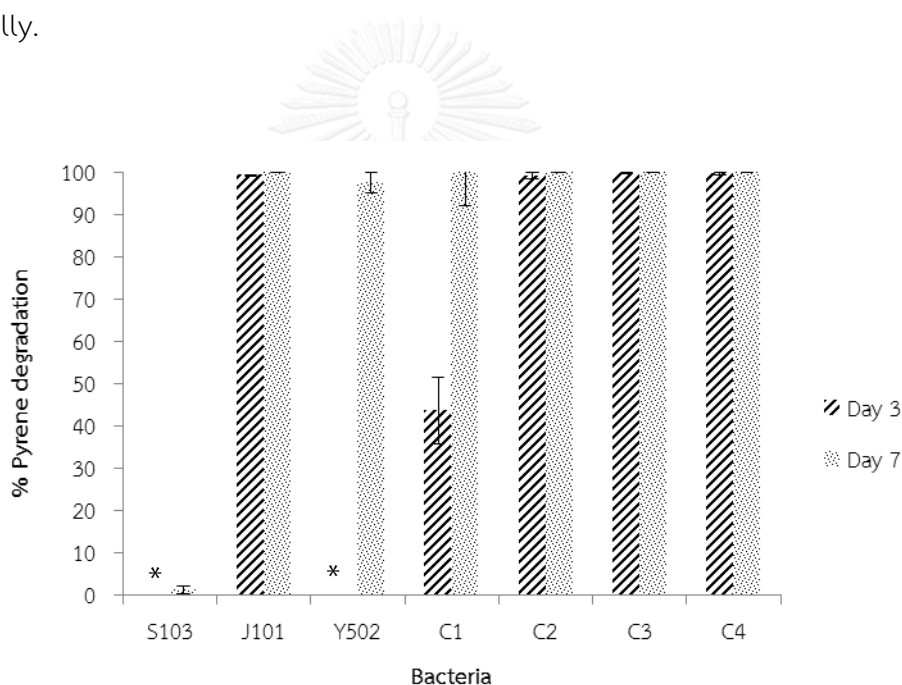
Figure 4.7 Biodegradation efficiency of pyrene by single strains and bacterial consortia.

Note: * No data of biodegradation of pyrene by strains S103 and Y502 at 3 days
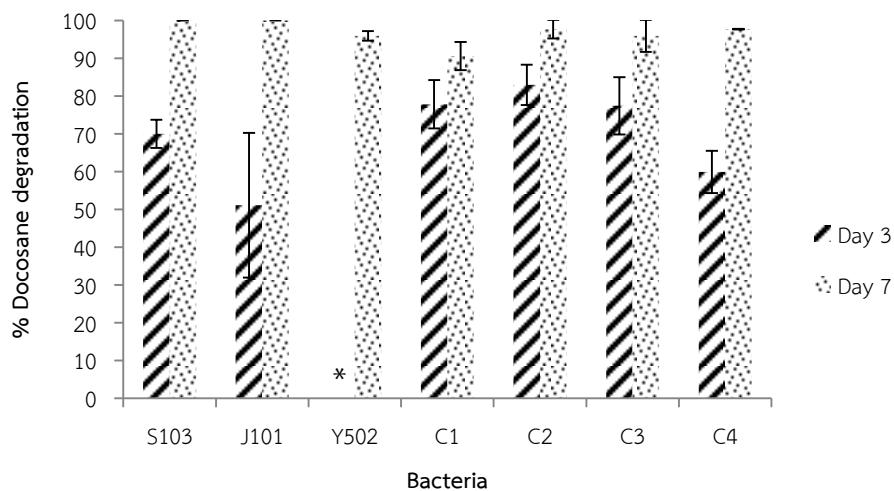
Figure 4.8 Biodegradation efficiency of docosane by single strains and bacterial consortia.

Note: * No data of biodegradation of docosane by Y502 at 3 day.

(a) (b) (c)
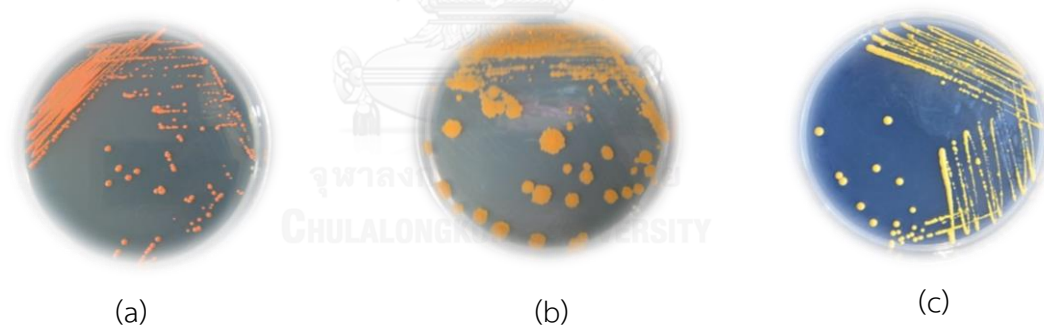
Figure 4.9 The characteristic of *Rhodococcus ruber* S103 (a), *Mycobacterium* sp. J101 (b), and *Mycobacterium* sp. Y502 (c).

Table 4.7 Biodegradation of fuel oil by four consortia and their bacterial growth compared with pure cultures

Bacteria/Consortia		Bacterial number (CFU mL^{-1})		
		Day 0	Day 3	Day 7
C1	*Rhodococcus ruber* S103	$1.27\pm0.24\times10^{7}$	$1.82\pm0.24\times10^{7}$	$1.28\pm0.21\times10^{7}$
	Mycobacterium sp. Y502	$1.56\pm0.49\times10^{7}$	$3.73\pm0.64\times10^{7}$	$5.33\pm0.76\times10^{7}$
C2	*Rhodococcus ruber* S103	$1.29\pm0.06\times10^{7}$	$1.15\pm0.11\times10^{7}$	$3.50\pm0.44\times10^{7}$
	Mycobacterium sp. J101	$1.54\pm0.12\times10^{7}$	$1.44\pm0.18\times10^{7}$	$6.13\pm1.90\times10^{6}$
C3	*Mycobacterium* sp. Y502	$4.33\pm0.45\times10^{7}$	$4.40\pm0.69\times10^{7}$	$3.15\pm0.24\times10^{7}$
	Mycobacterium sp. J101	$1.83\pm0.22\times10^{7}$	$4.87\pm1.95\times10^{6}$	$3.93\pm0.57\times10^{6}$
C4	*Rhodococcus ruber* S103	$1.13\pm0.20\times10^{7}$	$6.53\pm1.27\times10^{6}$	$2.57\pm0.20\times10^{7}$
	Mycobacterium sp. Y502	$1.36\pm0.33\times10^{7}$	$5.00\pm1.66\times10^{6}$	$2.02\pm0.36\times10^{7}$
	Mycobacterium sp. J101	$1.22\pm0.05\times10^{7}$	$4.27\pm1.42\times10^{5}$	$2.70\pm0.10\times10^{6}$
Rhodococcus ruber S103		$1.10\pm0.17\times10^{7}$	$1.27\pm0.06\times10^{7}$	$1.70\pm0.17\times10^{7}$
Mycobacterium sp. J101		$1.83\pm0.06\times10^{7}$	$5.33\pm1.15\times10^{7}$	$3.00\pm0.58\times10^{6}$
Mycobacterium sp. Y502		$1.93\pm0.40\times10^{7}$	$1.00\pm0.00\times10^{8}$	$6.50\pm3.00\times10^{7}$

Table 4.8 Biodegradation of diesel oil by four consortia and their bacterial growth compared with pure cultures

Bacteria/Consortia		Bacterial number (CFU mL^{-1})		
		Day 0	Day 3	Day 7
C1	*Rhodococcus ruber* S103	$7.23\pm0.93\times10^{6}$	$2.40\pm0.40\times10^{7}$	$3.37\pm0.32\times10^{6}$
	Mycobacterium sp. Y502	$4.00\pm0.30\times10^{6}$	$1.07\pm0.06\times10^{6}$	$8.23\pm0.57\times10^{5}$
C2	*Rhodococcus ruber* S103	$5.93\pm0.81\times10^{6}$	$8.55\pm0.64\times10^{6}$	$9.00\pm0.50\times10^{6}$
	Mycobacterium sp. J101	$1.78\pm0.17\times10^{6}$	$4.90\pm1.41\times10^{6}$	$3.57\pm0.15\times10^{6}$
C3	*Mycobacterium* sp. Y502	$4.87\pm0.31\times10^{6}$	$1.38\pm0.07\times10^{7}$	$8.37\pm\pm0.85\times10^{5}$
	Mycobacterium sp. J101	$1.86\pm0.07\times10^{6}$	$4.70\pm0.70\times10^{6}$	$1.27\pm0.09\times10^{6}$
C4	*Rhodococcus ruber* S103	$5.73\pm0.25\times10^{6}$	$8.43\pm0.15\times10^{6}$	$7.70\pm0.36\times10^{6}$
	Mycobacterium sp. Y502	$4.97\pm0.35\times10^{6}$	$3.73\pm1.23\times10^{6}$	$4.00\pm0.56\times10^{6}$
	Mycobacterium sp. J101	$1.79\pm0.06\times10^{6}$	$8.45\pm0.21\times10^{6}$	$4.23\pm0.11\times10^{6}$
Rhodococcus ruber S103		$7.00\pm2.00\times10^{6}$	$3.67\pm0.58\times10^{6}$	$4.67\pm1.53\times10^{6}$
Mycobacterium sp. J101		$9.00\pm0.00\times10^{6}$	$3.00\pm0.00\times10^{6}$	$5.50\pm0.71\times10^{4}$
Mycobacterium sp. Y502		$2.00\pm0.00\times10^{6}$	No data	$1.25\pm0.07\times10^{6}$

Table 4.9 Biodegradation of pyrene by four consortia and their bacterial growth compared with pure cultures

Bacteria/Consortia		Bacterial number (CFU mL^{-1})		
		Day 0	Day 3	Day 7
C1	*Rhodococcus ruber* S103	$7.23\pm0.93\times10^{6}$	$1.21\pm0.23\times10^{7}$	$1.45\pm0.31\times10^{6}$
	Mycobacterium sp. Y502	$4.00\pm0.30\times10^{6}$	$5.03\pm9.24\times10^{6}$	$1.10\pm0.02\times10^{7}$
C2	*Rhodococcus ruber* S103	$5.93\pm0.81\times10^{6}$	$3.90\pm0.81\times10^{7}$	$3.63\pm0.40\times10^{6}$
	Mycobacterium sp. J101	$1.78\pm0.17\times10^{6}$	$7.65\pm0.17\times10^{7}$	$4.80\pm1.44\times10^{6}$
C3	*Mycobacterium* sp. Y502	$4.87\pm0.31\times10^{6}$	$4.87\pm0.31\times10^{6}$	$8.03\pm1.01\times10^{5}$
	Mycobacterium sp. J101	$1.86\pm0.07\times10^{6}$	$1.30\pm0.07\times10^{7}$	$2.46\pm1.02\times10^{6}$
C4	*Rhodococcus ruber* S103	$5.73\pm0.25\times10^{6}$	$9.17\pm1.42\times10^{6}$	$7.57\pm0.15\times10^{5}$
	Mycobacterium sp. Y502	$4.97\pm0.35\times10^{6}$	$1.26\pm0.04\times10^{6}$	$1.62\pm0.21\times10^{5}$
	Mycobacterium sp. J101	$1.79\pm0.06\times10^{6}$	$8.33\pm0.31\times10^{6}$	$5.10\pm0.17\times10^{6}$
Rhodococcus ruber S103		$7.00\pm2.00\times10^{6}$	No data	$1.87\pm0.40\times10^{7}$
Mycobacterium sp. J101		$9.00\pm0.00\times10^{6}$	$1.80\pm0.14\times10^{6}$	$2.00\pm0.45\times10^{7}$
Mycobacterium sp. Y502		$2.00\pm0.00\times10^{6}$	No data	$2.53\pm0.21\times10^{7}$

Table 4.10 Biodegradation of docosane by four consortia and their bacterial growth compared with pure cultures

Bacteria/Consortia		Bacterial number (CFU mL^{-1})		
		Day 0	Day 3	Day 7
C1	*Rhodococcus ruber* S103	$7.23\pm0.93\times10^{6}$	$2.99\pm2.09\times10^{7}$	$2.78\pm0.83\times10^{7}$
	Mycobacterium sp. Y502	$4.00\pm0.30\times10^{6}$	$3.13\pm0.25\times10^{6}$	$5.10\pm0.87\times10^{6}$
C2	*Rhodococcus ruber* S103	$5.93\pm0.81\times10^{6}$	$2.57\pm0.05\times10^{7}$	$4.78\pm2.81\times10^{7}$
	Mycobacterium sp. J101	$1.78\pm0.17\times10^{6}$	$1.04\pm0.10\times10^{6}$	$4.00\pm0.95\times10^{6}$
C3	*Mycobacterium* sp. Y502	$4.87\pm0.31\times10^{6}$	$4.40\pm1.68\times10^{6}$	$4.20\pm0.85\times10^{6}$
	Mycobacterium sp. J101	$1.86\pm0.07\times10^{6}$	$5.80\pm1.73\times10^{5}$	$1.07\pm0.03\times10^{7}$
C4	*Rhodococcus ruber* S103	$5.73\pm0.25\times10^{6}$	$3.70\pm0.61\times10^{6}$	$1.68\pm0.50\times10^{7}$
	Mycobacterium sp. Y502	$4.97\pm0.35\times10^{6}$	$1.24\pm0.04\times10^{5}$	$1.41\pm0.08\times10^{6}$
	Mycobacterium sp. J101	$1.79\pm0.06\times10^{6}$	$4.37\pm0.32\times10^{4}$	$4.20\pm0.79\times10^{6}$
Rhodococcus ruber S103		$7.00\pm2.00\times10^{6}$	$6.33\pm1.15\times10^{6}$	$7.00\pm1.00\times10^{6}$
Mycobacterium sp. J101		$9.00\pm0.00\times10^{6}$	$8.50\pm0.71\times10^{5}$	$4.00\pm1.00\times10^{6}$
Mycobacterium sp. Y502		$2.00\pm0.00\times10^{6}$	No data	$8.00\pm1.00\times10^{6}$

In previous studies, defined consortia or mixed cultures have an ability to degrade organic pollutants including PAHs, polychlorinated biphenyls (PCBs), synthetic dyes, and others and exhibit better performance than monocultures (Bacosa et al., 2010; Ghanem et al., 2011; Hudcova et al., 2011; Li and Li, 2011). It is possible that a microbial consortium has the wider activities and metabolic networks than a single strain. For instance, the mixed bacterial consortium was able to degrade 77% of crude oil (1% v/v) when compared with single strains of *Pseudomonas* sp. BPS1-8, *Bacillus* sp. IOS1-7, *Pseudomonas* sp. HPS2-5, and *Corynebacterium* sp. BPS2-6 which degraded 69, 64, 45, and 41%, respectively (Sathishkumar et al., 2008).

Interestingly, this is the first report using the defined consortia for degrading the fuel oil as shown in Table 4.11. Moreover, this finding showed that these defined consortia had a potential for application use.
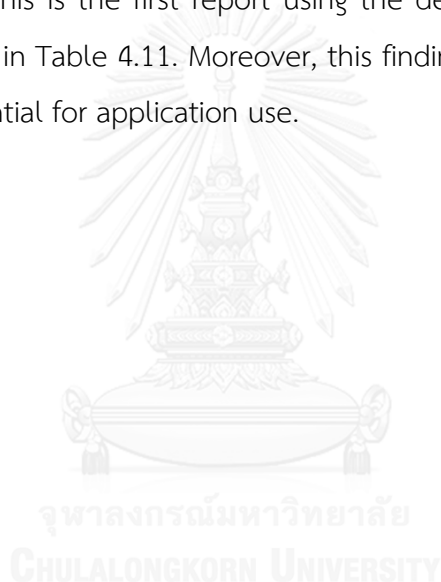
Table 4.11 Comparison of the petroleum oil degradation efficiency of defined consortia in this study and others

	Defined consortia	Oils	Concentration	% Degradation	Incubation time (days)	References
C1	Rhodococcus ruber S103	Diesel oil	0.25% (v/v)	97	3	This study
	Mycobacterium sp. Y502	Fuel oil	3,000 mg L^{-1}	32	7	
C2	Rhodococcus ruber S103	Diesel oil	0.25% (v/v)	99	3	This study
	Mycobacterium sp. J101	Fuel oil	3,000 mg L^{-1}	29	7	
C3	Mycobacterium sp. Y502	Diesel oil	0.25% (v/v)	63	3	This study
	Mycobacterium sp. J101	Fuel oil	3,000 mg L^{-1}	18	7	
C4	Rhodococcus ruber S103, Mycobacterium sp. Y502	Diesel oil	0.25% (v/v)	100	3	This study
	Mycobacterium sp. J101	Fuel oil	3,000 mg L^{-1}	41	7	
	Bacillus sp. APHP6, Pseudomonas sp. APHP9, Pseudomonas sp. APBP1, and Micrococcus sp. APIO4	Diesel oil	1% (v/v)	75	6	Prakash et al. (2014)
	Acinetobacter sp. XM-02 and Pseudomonas sp. XM-01	Crude oil	5% (v/v)	87	10	Chen et al. (2014)
	Lysinibacillus bronitolerans RI18, Bacillus thuringiensis RI16, Bacillus Weihenstephanensis strain RI12, Acinetobacter radioresistens RI7, and Bacillus subtilis SPB1	Diesel	5% (v/v)	49	21	Mnif et al. (2015)

4.5 Fuel oil biodegradation efficacy by immobilized defined consortium

    4.5.1 Immobilization of defined consortium

        To enhance the efficiency of fuel oil removal and for application use, the cell immobilization was set up in this experiment. Based on results of fuel oil biodegradation by defined consortia, C4 composed of *Rhodococcus* sp. S103, *Mycobacterium* sp. J101, and *Mycobacterium* sp. Y502 could be a good candidate for bioremediation of fuel oil contaminated area. Each strain was immobilized on bio-ball and the optimization of immobilization time was investigated. Bacterial immobilization was successfully performed using inoculum 10^7 CFU mL^{-1} in CFMM containing sterilized plastic pellets and 600 mg L^{-1} of fuel oil. Figure 4.10 showed the turbidity of culture medium of immobilized system after 6 days of incubation. Cells of S103, J101, and Y502 attached to the surface of the plastic pellets after incubation on a rotary shaker. As shown in Figure 4.11, the number of bacteria on plastic pellets reached a steady state after 5 days of incubation. Therefore, immobilized cells that reached steady state conditions after 6-day incubations were selected for further determination of the oil removal ability. The preparation method for immobilization in this study was correlated with Nopcharoenkul et al. (2013) that *Pseudoxanthomonas* sp. RN402 was immobilized on plastic pellets within 6 days. In contrast, this study required a shorter incubation period of cell immobilization than *Mycobacterium* sp. Spyr1, which required 10 days for immobilization on glass beads in M9MM medium supplemented with pyrene (Karabika et al., 2009).
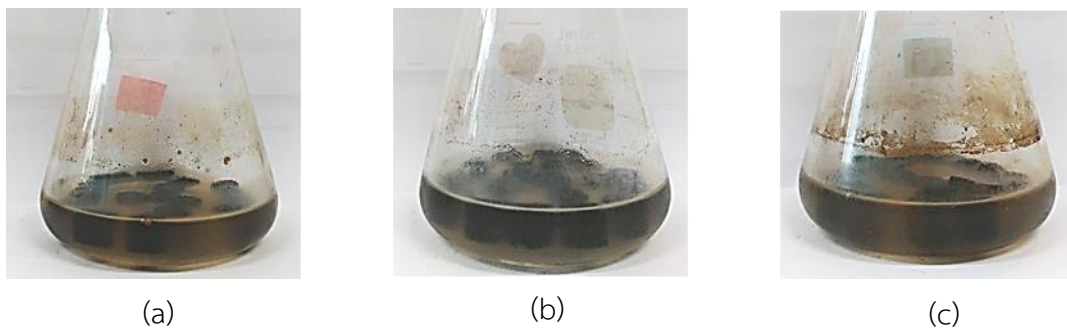
Figure 4.10 The Immobilization of *Rhodococcus ruber* S103 (a), *Mycobacterium* sp. J101 (b), and *Mycobacterium* sp. Y502 (c) after 6 days of incubation time.

(a)

(b)

(c)
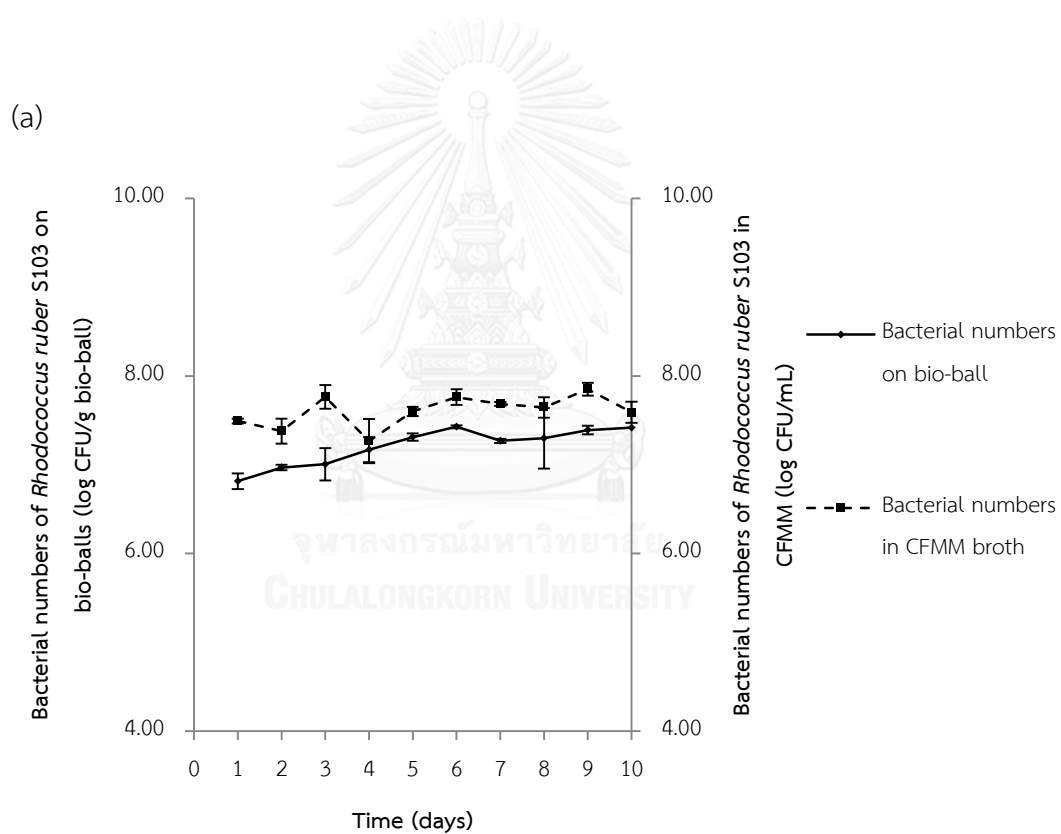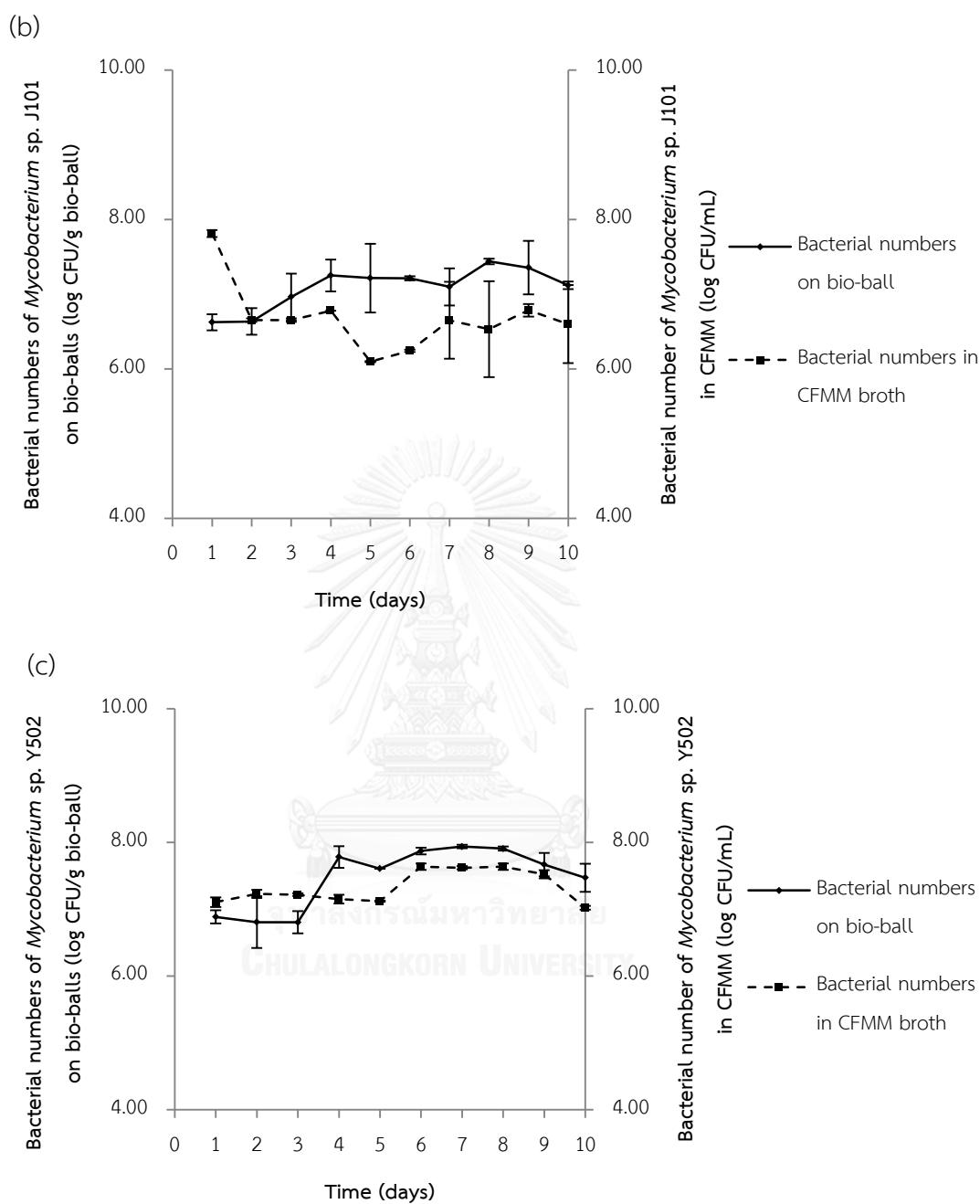
Figure 4.11 Bacterial counted per gram of dried plastic pellets. S103 (a), J101 (b), and Y502 (c) were immobilized with an initial cell concentration of 10^{7} CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

4.5.2 Removal of fuel oil by immobilized defined consortium in CFMM medium

The removal of fuel oil by the immobilized defined consortium in CFMM supplemented with fuel oil (2,000 mg L^{-1}) was investigated. It was observed that there are little the oil slicks in the experiment with bio-balls at day 3 (Figure 4.12b and Figure 4.12c) and day 7 (Figure 4.12e and Figure 4.12f) when compared to control without inoculum as shown in Figure 4.12a and Figure 4.12d.

(a)   (b)

(c)   (d)

(e)   (f)
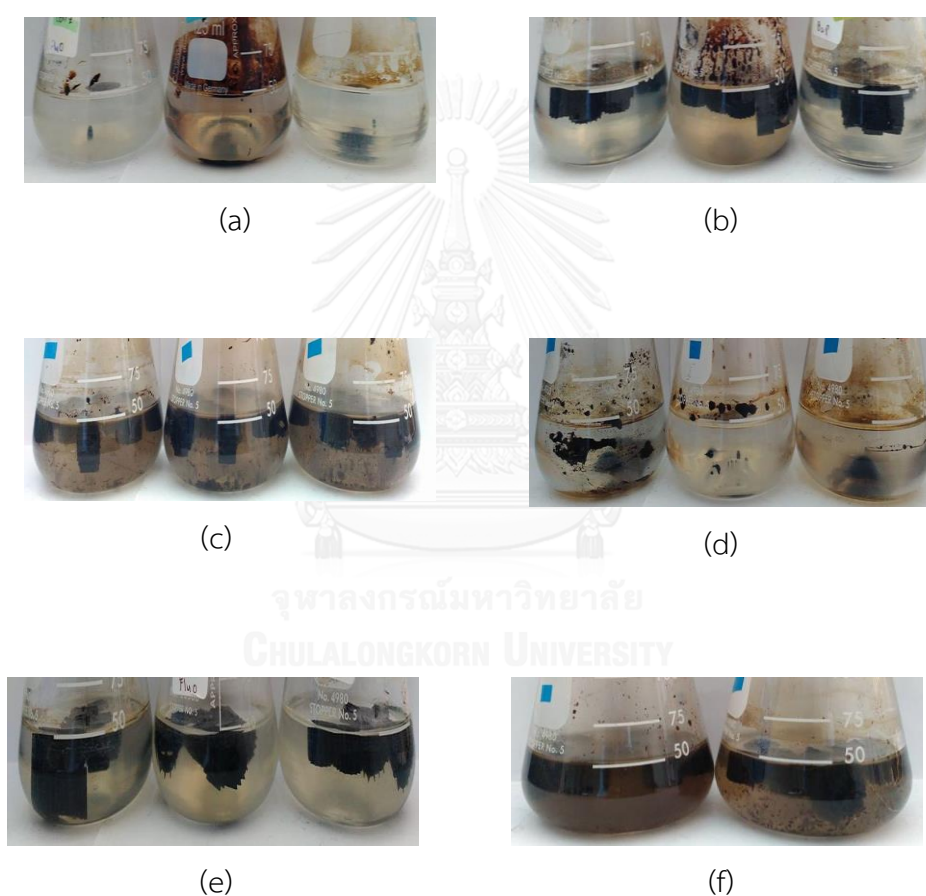
Figure 4.12 The oil slick in the cultivation medium supplemented with 2,000 mg L^{-1} of fuel oil at day 3 (a-c) and day 7 (d-f). (a) and (d) were controls without inoculum at day 3 and day 7, respectively. (b) and (e) were controls with sterilized bio-balls at day 3 and day 7, respectively. (c) and (f) were immobilized defined consortium at day 3 and day 7, respectively.

It was found that the fuel oil in CFMM was removed by bio-balls and immobilized defined consortium at day 0 (Figure 4.13). At day 0, the concentration of fuel oil in CFMM having the bio-balls remained at 1,396.67±23.44 mg L^{-1} and the concentration of fuel oil in CFMM having immobilized defined consortium remained at 1,624.67±110.15 mg L^{-1}. After 7 days of incubation, the concentration of fuel oil in CFMM having the bio-balls remained at 514.00±49.15 mg L^{-1} and the concentration of fuel oil in CFMM having immobilized defined consortium remained at 682.67±12.86 mg L^{-1}. In contrast, the concentration of fuel oil in CFMM having free defined consortium remained at 2,136.67±52.85 mg L^{-1} in 7 days. The result showed that fuel oil in an experiment with bio-balls was removed from CFMM medium higher than the experiment without bio-balls based on adsorption.
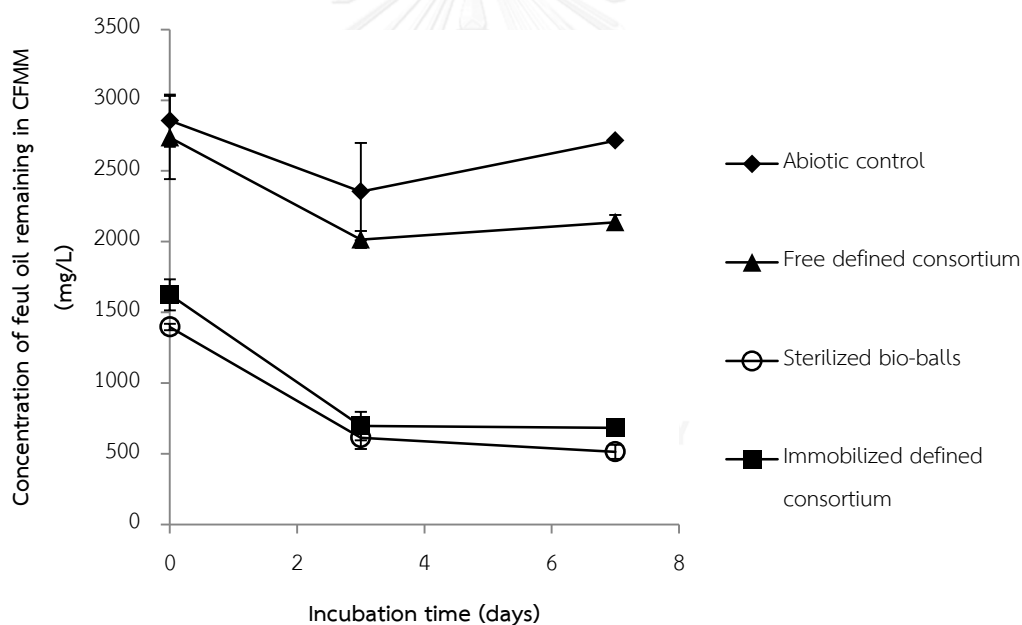
Figure 4.13 The concentration of fuel oil remaining in after 7 days of incubation time and the initial concentration of fuel oil was 2,000 mg L^{-1}. The initial inoculation amounts 10^{7} CFU g^{-1} bio-balls for the immobilized defined consortium and 10^{7} CFU mL^{-1} for the free bacterial consortium.

Due to causing the adsorption of bio-balls, the remaining of fuel oil on bio-balls with and without defined consortium was determined. It was found that the amount of fuel oil on bio-balls with and without defined consortium was different. After 7 days of incubation, the concentration of fuel oil remaining on bio-balls sterilized was 1,330.22±112.07 mg g^{-1} bio-ball, whereas the concentration of fuel oil remaining on bio-ball was 997.33±123.12 mg g^{-1} bio-ball (Figure 4.14). In addition, it was found that the fuel oil on bio-balls was degraded around 25% in 3 and 7 days shown in Figure 4.15.Nonetheless, the immobilized defined consortium had less fuel oil degradation efficiency than free defined consortium which degraded fuel oil at 41% in 7 days. It is due to the presence of fuel oil on bio-ball may decrease uptake of substrates and metabolites (Quek et al., 2006).
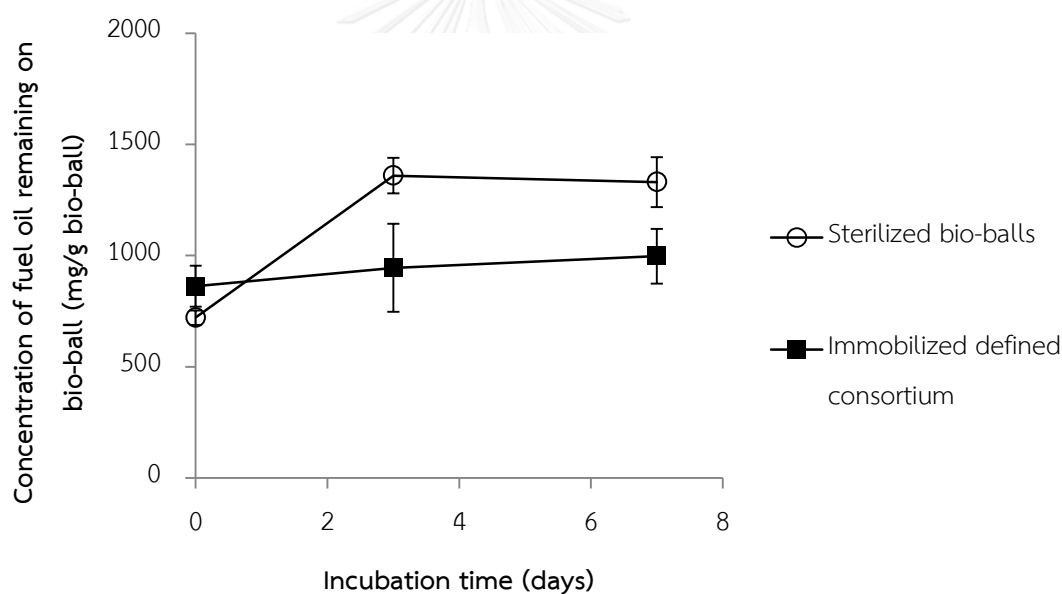
Figure 4.14 The concentration of fuel oil remaining on bio-balls after 7 days of incubation time. The concentration of fuel oil was 2,000 mg L^{-1}. The initial concentration of free cell and immobilized defined consortium was 10^{7} CFU mL^{-1} and 10^{7} CFU g^{-1} bio-balls.
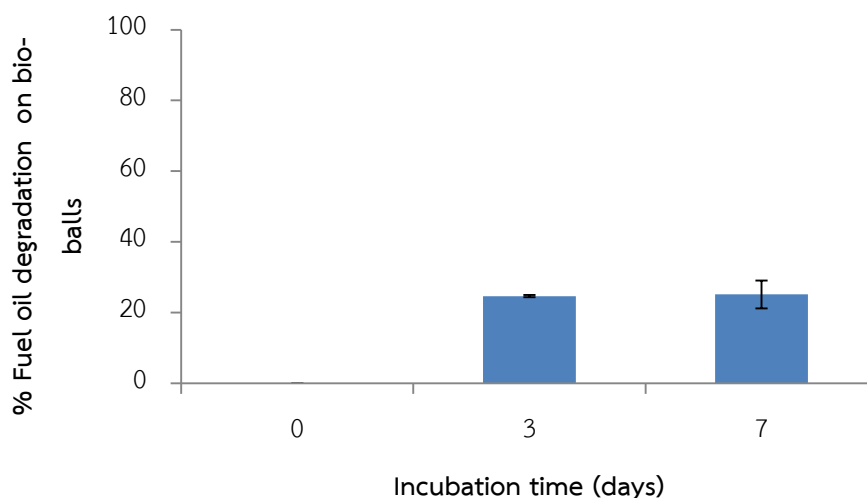
Figure 4.15 % Fuel oil degradation on bio-balls by immobilized defined consortium (CFMM experiment).

On the other hand, there are many types of research showing that the immobilized bacterial consortia have a higher efficacy of xenobiotic compounds biodegradation than free microbial consortia. For example, the immobilized bacterial consortium on sodium alginate-diatomite beads significantly degraded 20 mg L^{-1} of crude oil at 88%. Conversely, the free bacterial consortium composed of *Microbacterium foliorum, Gordonia alkanivorans, and Mesorhizobiu* degraded 78% of crude oil in 14 days (Zhen-Yu et al., 2012). Similarly, Partovinia and Naeimpoor (2014) demonstrated that immobilized mixed culture had a higher phenanthrene degradation efficacy than a free cell at high concentration (500 mg L^{-1}). The immobilized microbial consortium removed 80% of phenanthrene while free cells removed phenanthrene at 62% within 7 days. Our finding showed that the immobilized defined consortium had potential to remove fuel oil; consequently, it was investigated the removal of fuel oil in freshwater.

4.5.3 Removal of fuel oil by immobilized defined consortium in freshwater from Chao Phraya River

This experiment was performed to study the possibility of immobilized defined consortia for removal of fuel oil in freshwater from the Chao Phraya River. The Chao Phraya River was used instead of CFMM, and the immobilized defined consortium was inoculated in freshwater supplemented 2,000 mg L^{-1} of fuel oil. The collected freshwater from the Chao Phraya River was yellow, slightly turbid and odorless. The chemical properties of freshwater are as followings; pH 6.97, 0.7% salinity, COD 64 mg L^{-1}, BOD 5.3 mg L^{-1}, total organic carbon 4.74 mg L^{-1}, total nitrogen 0.9 mg L^{-1}, total phosphorus 1.53 mg L^{-1}, total potassium 106.2 mg L^{-1}, iron 0.76 mg L^{-1}, and mercury 0.0005 mg L^{-1}. From the results, it was found that the cultivation medium having immobilized defined consortium had turbidity at day 5 as shown in Figure 4.16. It was also observed the oil residual and more turbidity in the cultivation medium having immobilized defined consortium at day 10 (Figure 4.17d) and 15 (Figure 4.18d). Whereas, there were oil slicks in cultivation medium of natural attenuation experiment. Additionally, it was observed that there was no oil slick in the controls with sterilized bio-balls.

(a)   (b)

(c)   (d)
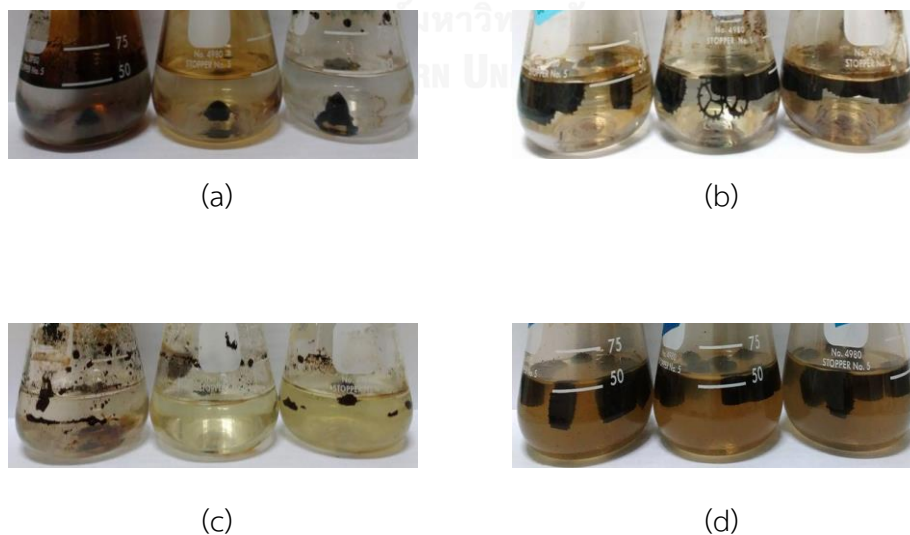
Figure 4.16 The cultivation medium change of control without inoculation (a), control with sterilized bio-balls (b), natural attenuation (c), and the immobilized defined consortium (d) at day 5.

(a) (b)

(c) (d)
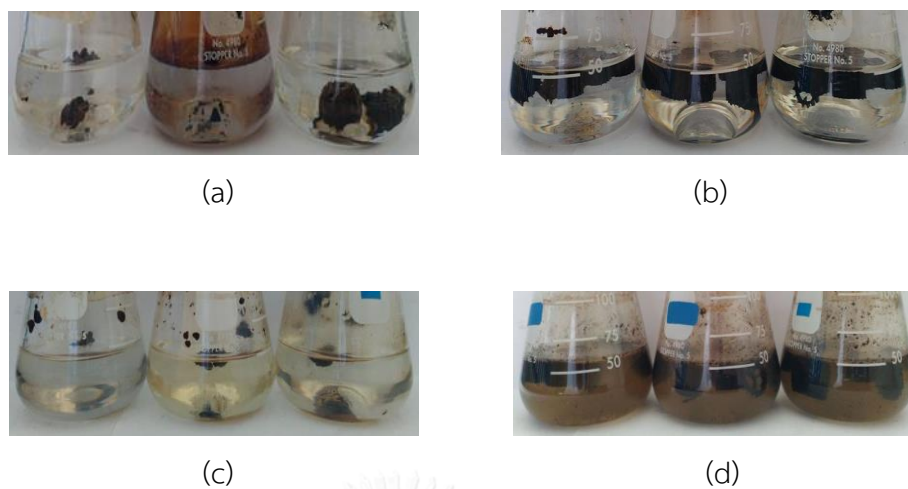
Figure 4.17 The cultivation medium change of control without inoculation (a), control with sterilized bio-balls (b), natural attenuation (c), and the immobilized defined consortium (d) at day 10.

(a) (b)

(c) (d)
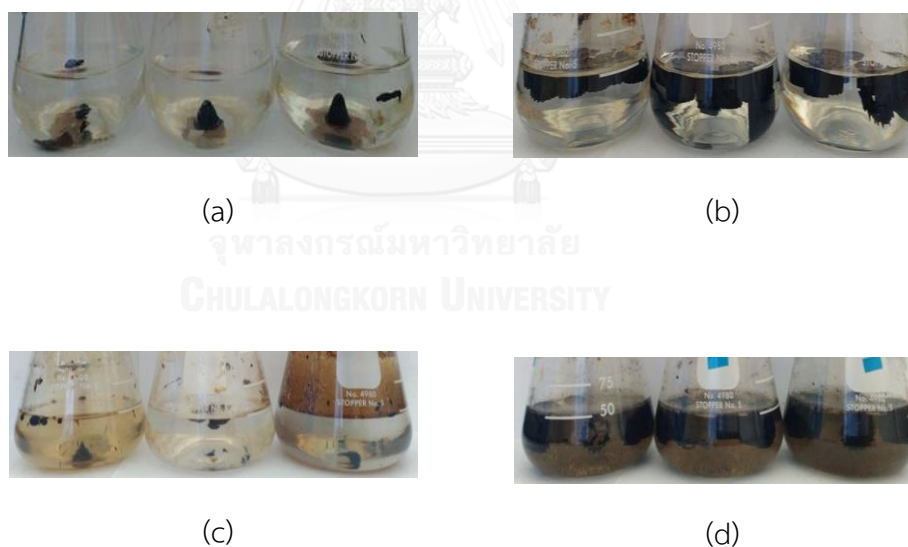
Figure 4.18 The cultivation medium change of control without inoculation (a), control with sterilized bio-balls (b), natural attenuation (c), and the immobilized defined consortium (d) at day 15.

It was found that the concentration of fuel oil in freshwater having bio-balls and immobilized defined consortium was lower than that of freshwater having indigenous microorganisms, which remained at $2,441.33\pm30.55$ mg L^{-1}. In contrast, the concentration of fuel oil in freshwater having immobilized defined consortium remained at 604.00 ± 28.84 mg L^{-1} within 15 days. At the same time, the concentration of fuel oil having sterilized bio-balls remained at 643.33 ± 6.11 mg L^{-1} which was equal to the fuel oil removal efficiency of the immobilized defined consortium as shown in Figure 4.19. Thus, the fuel oil on bio-balls in both control with sterilized bio-balls and the immobilized defined consortium was determined to confirm that the defined consortium could degrade fuel oil. The results demonstrated that the amount of fuel oil extracted from bio-balls with and without bacteria was clearly different. The concentration of fuel oil remaining on bio-ball having the immobilized defined consortium was 816.00 ± 11.39 mg g^{-1} bio-ball, whereas the concentration of fuel oil remaining on sterilized bio-balls was $1,523.11\pm149.25$ mg g^{-1} bio-ball in 15 days as shown in Figure 4.20. Additionally, it was found that the defined consortium slightly degraded fuel oil on bio-balls every 5 days. After 15 days of incubation time, the defined consortium could degrade 46% of fuel oil as shown in Figure 4.21. It was possible that the immobilized defined consortium adapted and mineralized the complex compounds to less complex chemicals promoting other bacteria in freshwater to degrade those intermediate (He et al., 2013).
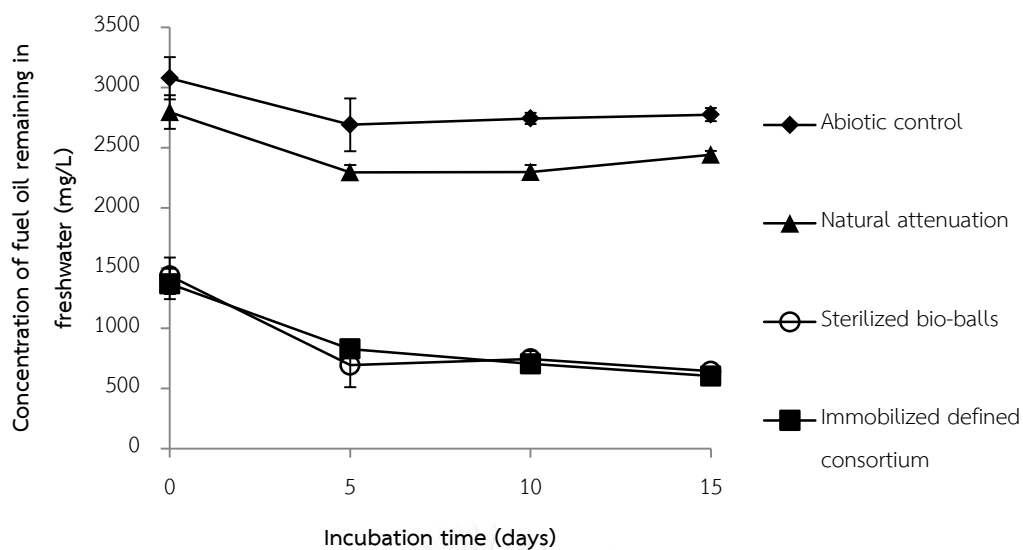
Figure 4.19 The concentration of fuel oil remaining in freshwater from the Chao Phraya River by immobilized defined consortium after 7 days of incubation time. The concentration of fuel oil was 2,000 mg L^{-1}. The initial concentration of free cell and immobilized defined consortium was 10^7 CFU mL^{-1} and 10^7 CFU g^{-1} bio-balls.
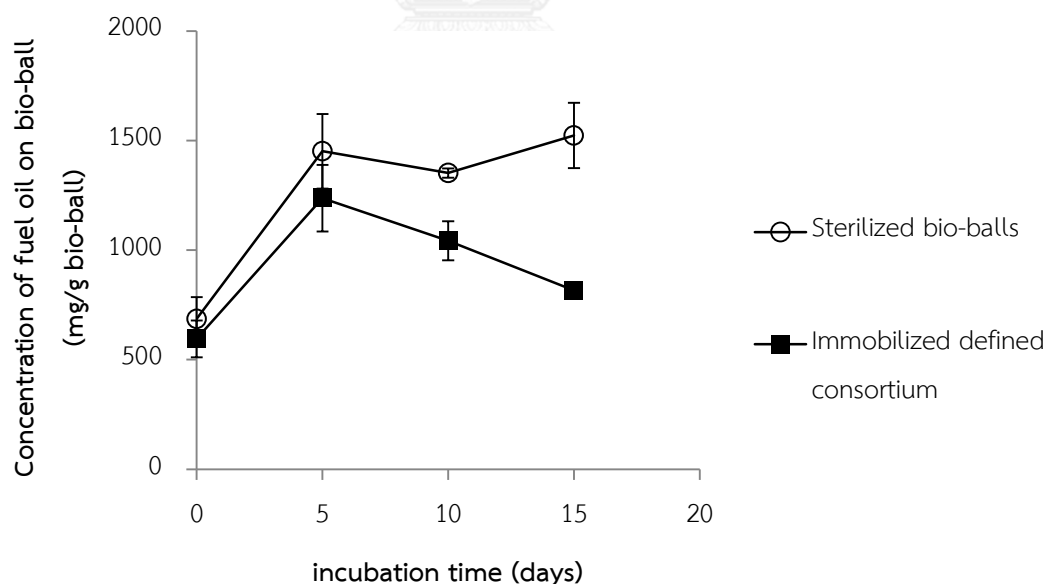
Figure 4.20 The remaining of fuel oil on bio-balls after 7 days of incubation time. The concentration of fuel oil was 2,000 mg L^{-1}. The initial concentration of free cell and immobilized defined consortium was 10^7 CFU mL^{-1} and 10^7 CFU g^{-1} bio-balls.
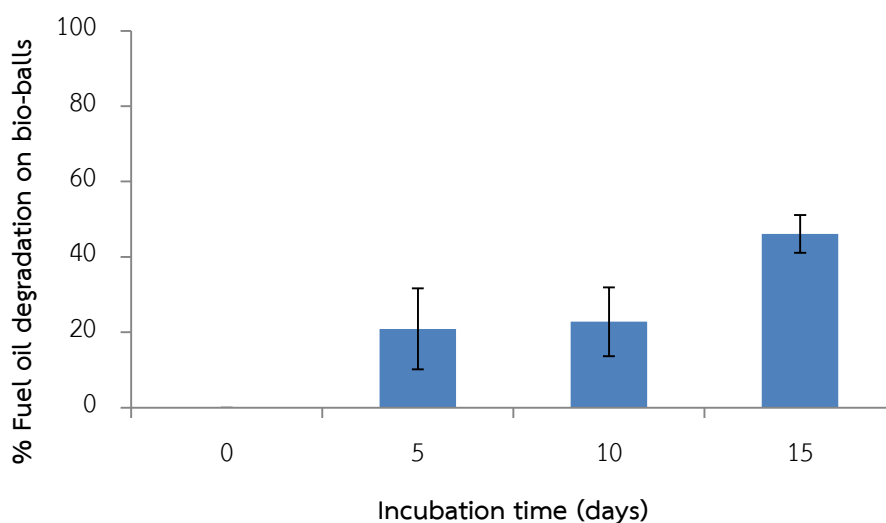
Figure 4.21 % Fuel oil degradation on bio-balls by immobilized defined consortium (freshwater experiment).

Additionally, the amount of immobilized defined consortium could be monitored using the viable plate count technique based on the morphology color of the immobilized strains as shown in Figure 4.22. The result found that the immobilized defined consortium was able to survive throughout the experimental period as shown in Figure 4.23. It indicated that the defined consortium plays a role in the removal of fuel oil in freshwater from the Chao Phraya River. Whereas, bacteria in the natural attenuation experiment grew on the fresh water containing 2,000 mg L^{-1} of fuel oil. The fuel oil was removed only 13% despite an increase of bacteria ranged from 10^5 to 10^6 CFU mL^{-1}. Consequently, the findings demonstrated that the immobilized defined consortium could adapt in the environment and had potential to remove fuel oil. These results are similar to that of Mishra et al. (2001), which evaluated the efficacy of inoculum addition to stimulating in situ bioremediation of oily-sludge-contaminated soil at an oil refinery. The microbial consortium was immobilized on corncob powder, and the initial concentration was 10^{10} CFU g^{-1} material carriers. It was found that the amount of petroleum hydrocarbon was reduced by 92% and the bacterial consortium was able to survive in 1 year demonstrating that the immobilized bacterial consortium was able to adapt and survive in the environment.

(a)   (b)
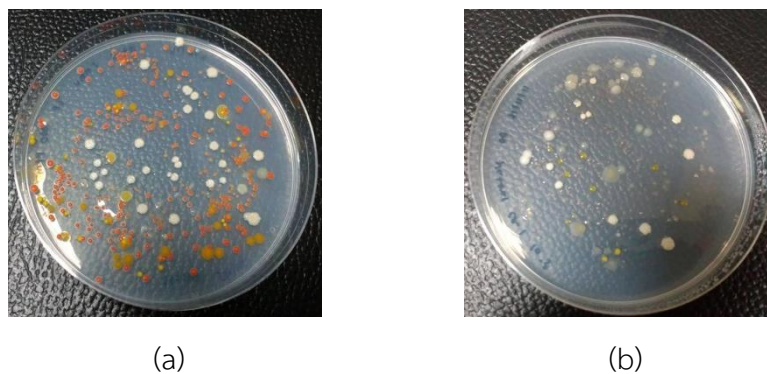
Figure 4.22 The morphology of strains in (a) defined consortium and (b) indigenous bacteria in fresh water from the Chao Phraya River.
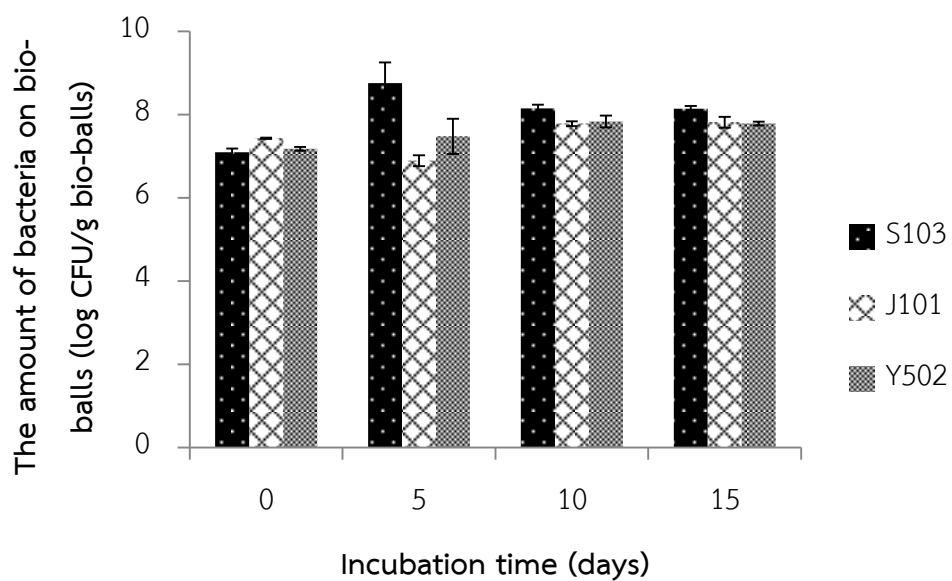
Figure 4.23 The amount of defined consortium on bio-balls in fresh water from the Chao Phraya River

Moreover, this study monitored the immobilized defined consortium by using 16S rRNA gene amplicon sequencing analysis. The results demonstrated that the immobilized defined consortium composed of *Rhodococcus ruber* S103, *Mycobacterium* sp. J101 and *Mycobacterium* sp. Y502 still survived and significantly increased in the system. In addition, it was found that the bacterial community changed during experiment as shown in Figure 4.24. There are few types of research monitoring the augmented bacteria by using 16S rRNA gene amplicon sequencing analysis. For example, *Exiguobacterium* sp. AO-11 capable of degrading of crude oil was augmented into sandy soil microcosm with 4% (w/w) and monitored it by using 16S metagenomic analysis. The finding showed that strain AO-11 was able to degrade 4% (w/w) crude oil at 75% within 100 days and could be monitored by 16S metagenomic analysis. Subsequently, strain AO-11 was detected in the sandy soil contaminated by crude oil only at day 20 (Srisuvanakan, 2016). It was possible that strain AO-11 played role in the biodegradation of medium- and long-chain alkane in first 20 days resulting in the enhancement of other crude oil-degrading bacteria growth.

As a consequence, the immobilized defined consortium achieved the removal of fuel oil in the fresh water from the Chao Phraya River. Moreover, it showed that use of the immobilized defined consortium might help in the failure in bioaugmentation with exogenous bacteria. Interestingly, this is the first report of fuel oil removal by the immobilized defined consortium.
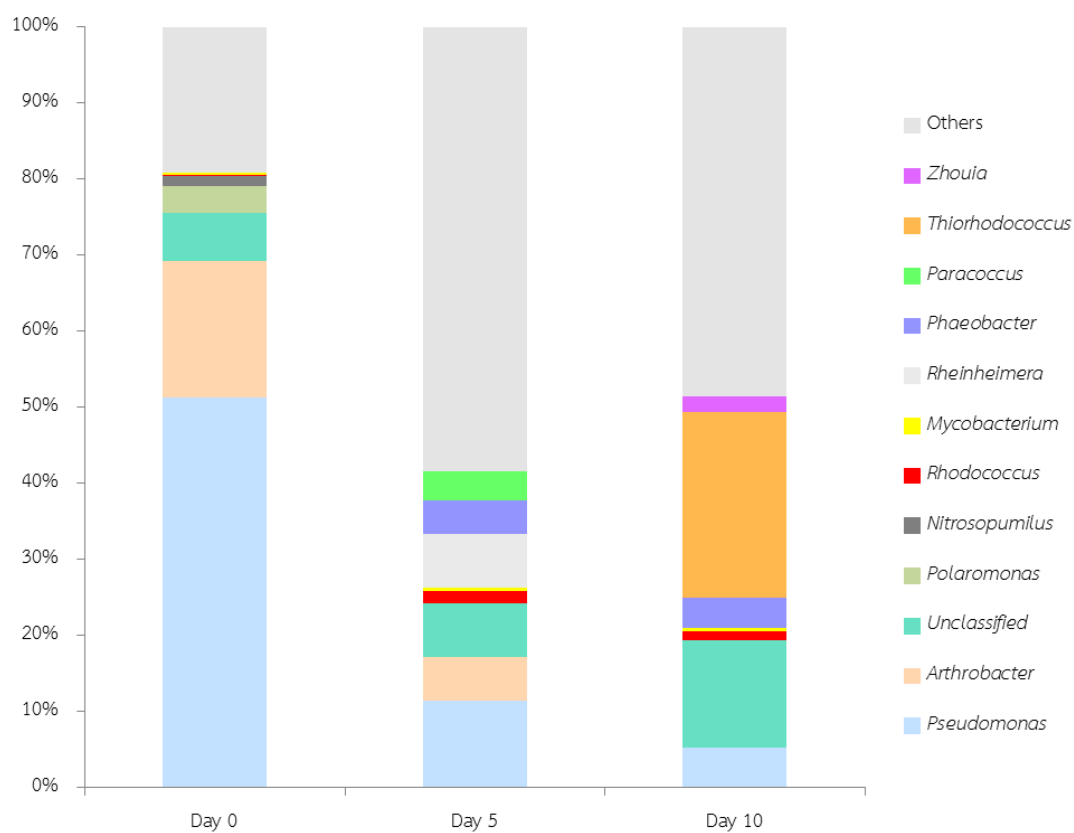
Figure 4.24 The result of 16S rRNA gene amplicon sequencing analysis of immobilized defined consortium composed of strain S103, J101, and Y502 on bio-balls

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Nowadays, there are petroleum oil contamination into the rivers including Chao Phraya and Tha Chin Rivers. It is because of both rivers are the main water transportation. The petroleum oil used for cargo ships is the fuel oil which is the mixtures composed of PAHs, aliphatics, asphaltene, and resin. The Fuel oil is less biodegradation, persists in the aquatic environments and affects the aquatic organisms and human. Therefore, this study aimed to formulate the defined bacterial consortium from the selected effective hydrocarbon-degrading and biosurfactant-producing bacteria having high cell surface hydrophobicity. The hydrocarbon-degrading bacteria were isolated by using the mixture of PAHs consisting of phenanthrene, fluoranthene, and pyrene as a model because PAHs are the main component of fuel oil and are recalcitrant. Then eight strains were obtained and were further examined for the ability to degrade various types of PAHs and aliphatic compounds and to produce biosurfactants. Among these eight strains, three effective hydrocarbon-degrading and biosurfactant-producing bacteria were selected for formulating four defined consortia to degrade petroleum hydrocarbons. *Mycobacterium* sp. J101 was capable of degrading LMW-PAHs, HMW-PAHs, and aliphatics. *Rhodococcus ruber* S103 degraded aliphatic efficiently when compared to other strains. *Mycobacterium* sp. Y502 degraded HMW-PAHs as well as reduced the surface tension of CFMM broth from 55 to 33 mN m^{-1}. The consortium 4 (C4) composed of S103, J101, and Y502 degraded 2,000 mg L^{-1} of fuel oil at 41% within 7 days and completely degraded 2,000 mg L^{-1} of diesel oil within 3 days. Moreover, it obviously showed a higher fuel oil and diesel oil degradation efficacy than a single strain and also maintained the capability to degrade pyrene and docosane. It was possible that C4 had synergistic metabolisms. Subsequently, C4 was chosen for immobilization on bio-balls and removal of fuel oil. The results showed that the experiment with bio-balls had a higher efficiency of fuel oil removal from water than

that of the experiment without bio-balls indicating that bio-balls could adsorb the fuel oil. In CFMM experiment, there was nearly as much fuel oil concentration in CFMM having bio-balls (514.00±49.15 mg L^{-1}) as immobilized defined consortium (682.67±12.86 mg L^{-1}) within 7 days. Similarly, the concentration of fuel oil in freshwater having immobilized defined consortium remained at 604.00±28.84 mg L^{-1} and the concentration of fuel oil having sterilized bio-balls remained at 643.33±6.11 mg L^{-1} within 15 days. Therefore, the fuel oil on bio-balls with and without defined consortium was determined. It was found that the amount of fuel oil on bio-balls was obviously different. In CFMM experiment, the concentration of fuel oil on bio-balls with bacteria remained at 997.23±123.12 mg g^{-1} bio-ball, while the concentration of fuel oil on sterilized bio-balls remained at 1,330.22±112.07 mg g^{-1} bio-ball. In freshwater experiment, the concentration of fuel oil on bio-balls with bacteria remained at 816.00±11.39 mg g^{-1} bio-ball. Meanwhile, the concentration of fuel oil on sterilized bio-balls remained at 1,523.11±149.25 mg g^{-1} bio-ball. The results thus showed that the fuel oil on bio-balls was degraded by defined consortium. In addition, the defined consortium on bio-ball during biodegradation experiments was detected by using viable plate count technique and 16S rRNA gene amplicon sequencing analysis. The results indicated that the immobilized defined consortium could survive throughout the experimental period when it applied to freshwater. This finding demonstrated that the immobilized defined consortium was able to remove the fuel oil from both adsorption and biodegradation activities. Interestingly, this is the first report that uses defined consortium for fuel oil removal. Consequently, the immobilized defined consortium in this study may be applied to remove petroleum oil contaminating around the ports by adding the immobilized defined consortium into the net and tired of around the ports. These results suggested that the immobilized defined consortium had a potential to remediate petroleum contaminated aquatic environments.

5.2 Recommendations for future work

1. Semi-continuous experiment should be performed in order to prove that immobilized cell had higher performance on fuel oil removal than a free cell.

2. The adhesion and viability of the defined consortium biofilm should be monitored by scanning electron microscope (SEM) as well

3. The environmental factors including the concentration of fuel oil, pH, temperature, and salinity should be studied for further application in real environments.

4. The immobilized defined consortium should be studied for its shelf life. The suitable storage condition of immobilized defined consortium, such as temperature, should be investigated.

5. The immobilized defined consortium should be applied to in-situ bioremediation of petroleum oil contaminated in aquatic environments. For example, the immobilized defined consortium will be added into the net and tired of around the ports contaminated by petroleum.

REFERENCES

Abbasian, F., Lockington, R., Mallavarapu, M., and Naidu, R. (2015). A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. *Applied biochemistry and biotechnology, 176*(3), 670-699.

Adams, J., Bornstein, J. M., Munno, K., Hollebone, B., King, T., Brown, R. S., and Hodson, P. V. (2014). Identification of compounds in heavy fuel oil that are chronically toxic to rainbow trout embryos by effects‑driven chemical fractionation. *Environmental toxicology and chemistry, 33*(4), 825-835.

Alonso-Alvarez, C., Pérez, C., and Velando, A. (2007). Effects of acute exposure to heavy fuel oil from the Prestige spill on a seabird. *Aquatic Toxicology, 84*(1), 103-110.

Aly, H. A., Huu, N. B., Wray, V., Junca, H., and Pieper, D. H. (2008). Two angular dioxygenases contribute to the metabolic versatility of dibenzofuran-degrading *Rhodococcus* sp. strain HA01. *Applied and environmental microbiology, 74*(12), 3812-3822.

Asquith, E. A., Geary, P. M., Nolan, A. L., and Evans, C. A. (2012). Comparative bioremediation of petroleum hydrocarbon-contaminated soil by biostimulation, bioaugmentation and surfactant addition. *Journal of Environmental Science and Engineering. A, 1*(5A).

Bacosa, H., Suto, K., and Inoue, C. (2010). Preferential degradation of aromatic hydrocarbons in kerosene by a microbial consortium. *International Biodeterioration & Biodegradation, 64*(8), 702-710.

Balachandran, C., Duraipandiyan, V., Balakrishna, K., and Ignacimuthu, S. (2012). Petroleum and polycyclic aromatic hydrocarbons (PAHs) degradation and naphthalene metabolism in *Streptomyces* sp.(ERI-CPDA-1) isolated from oil contaminated soil. *Bioresource technology, 112*, 83-90.

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, L., Smyth, T. J., and Marchant, R. (2010). Microbial biosurfactants production,

applications and future potential. *Applied microbiology and biotechnology, 87*(2), 427-444.

Banerjee, A., and Ghoshal, A. K. (2011). Phenol degradation performance by isolated *Bacillus cereus* immobilized in alginate. *International Biodeterioration & Biodegradation, 65*(7), 1052-1060.

Barsing, P., Tiwari, A., Joshi, T., and Garg, S. (2011). Application of a novel bacterial consortium for mineralization of sulphonated aromatic amines. *Bioresource technology, 102*(2), 765-771.

Bayat, Z., Hassanshahian, M., and Cappello, S. (2015). Immobilization of microbes for bioremediation of crude oil polluted environments: A mini review. *The open microbiology journal, 9*, 48.

Berekaa, M. M., and Steinbüchel, A. (2000). Microbial degradation of the multiply branched alkane 2, 6, 10, 15, 19, 23-hexamethyltetracosane (squalane) bymycobacterium fortuitum and *Mycobacterium ratisbonense*. *Applied and environmental microbiology, 66*(10), 4462-4467.

Boonyatumanond, R., Wattayakorn, G., Togo, A., and Takada, H. (2006). Distribution and origins of polycyclic aromatic hydrocarbons (PAHs) in riverine, estuarine, and marine sediments in Thailand. *Marine pollution bulletin, 52*(8), 942-956.

Brezna, B., Khan, A. A., and Cerniglia, C. E. (2003). Molecular characterization of dioxygenases from polycyclic aromatic hydrocarbon-degrading Mycobacterium spp. *FEMS microbiology letters, 223*(2), 177-183.

Cao, B., Nagarajan, K., and Loh, K.-C. (2009). Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. *Applied microbiology and biotechnology, 85*(2), 207-228.

Cébron, A., Norini, M.-P., Beguiristain, T., and Leyval, C. (2008). Real-time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHD α) genes from Gram positive and Gram negative bacteria in soil and sediment samples. *Journal of Microbiological Methods, 73*(2), 148-159.

Chao, M., Shen, X., Lun, F., Shen, A., and Yuan, Q. (2012). Toxicity of fuel oil water accommodated fractions on two marine microalgae, *Skeletonema costatum*

and *Chlorela* spp. *Bulletin of environmental contamination and toxicology, 88*(5), 712-716.

Cheung, P.-Y., and Kinkle, B. K. (2001). *Mycobacterium* diversity and pyrene mineralization in petroleum-contaminated soils. *Applied and environmental microbiology, 67*(5), 2222-2229.

Cyplik, P., Schmidt, M., Szulc, A., Marecik, R., Lisiecki, P., Heipieper, H. J., Owsianiak, M., Vainshtein, M., and Chrzanowski, Ł. (2011). Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. *Bioresource technology, 102*(6), 4347-4352.

Darmawan, R., Nakata, H., Ohta, H., Niidome, T., Takikawa, K., and Morimura, S. (2015). Isolation and evaluation of PAH degrading bacteria. *Journal of Bioremediation & Biodegredation, 6*(3), 1.

Das, N., and Chandran, P. (2010). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology research international, 2011*.

Derz, K., Klinner, U., Schuphan, I., Stackebrandt, E., and Kroppenstedt, R. M. (2004). *Mycobacterium pyrenivorans* sp. nov., a novel polycyclic-aromatic-hydrocarbon-degrading species. *International journal of systematic and evolutionary microbiology, 54*(6), 2313-2317.

Franzetti, A., Gandolfi, I., Bestetti, G., Smyth, T. J., and Banat, I. M. (2010). Production and applications of trehalose lipid biosurfactants. *European Journal of Lipid Science and Technology, 112*(6), 617-627.

Fuentes, S., Méndez, V., Aguila, P., and Seeger, M. (2014). Bioremediation of petroleum hydrocarbons: catabolic genes, microbial communities, and applications. *Applied microbiology and biotechnology, 98*(11), 4781-4794.

Ghanem, K. M., Al-Garni, S. M., and Biag, A. K. (2011). Statistical optimization of cultural conditions for decolorization of methylene blue by mono and mixed bacterial culture techniques. *African Journal of Microbiology Research, 5*(15), 2187-2197.

Gojgic-Cvijovic, G., Milic, J., Solevic, T., Beskoski, V., Ilic, M., Djokic, L., Narancic, T., and Vrvic, M. (2012). Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: a laboratory study. *Biodegradation, 23*(1), 1-14.

Grimwood, M. (2001). Crude oil derived petroleum products in the aquatic environment: priorities for control: Environment Agency.

Guo, C., Dang, Z., Wong, Y., and Tam, N. F. (2010). Biodegradation ability and dioxgenase genes of PAH-degrading *Sphingomonas* and *Mycobacterium* strains isolated from mangrove sediments. *International Biodeterioration & Biodegradation, 64*(6), 419-426.

Hall, K., Miller, C. D., Sorensen, D. L., Anderson, A. J., and Sims, R. C. (2005). Development of a catabolically significant genetic probe for polycyclic aromatic hydrocarbon-degrading *Mycobacteria* in soil. *Biodegradation, 16*(5), 475-484.

He, Z., Xiao, H., Tang, L., Min, H., and Lu, Z. (2013). Biodegradation of di-n-butyl phthalate by a stable bacterial consortium, HD-1, enriched from activated sludge. *Bioresource technology, 128*, 526-532.

Herrero, M., and Stuckey, D. (2015). Bioaugmentation and its application in wastewater treatment: a review. *Chemosphere, 140*, 119-128.

Hilyard, E. J., Jones-Meehan, J. M., Spargo, B. J., and Hill, R. T. (2008). Enrichment, isolation, and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth River sediments. *Applied and environmental microbiology, 74*(4), 1176-1182.

Ho, Y., Jackson, M., Yang, Y., Mueller, J., and Pritchard, P. (2000). Characterization of fluoranthene-and pyrene-degrading bacteria isolated from PAH-contaminated soils and sediments. *Journal of industrial microbiology & biotechnology, 24*(2), 100-112.

Hormisch, D., Brost, I., Kohring, G.-W., Giffhorn, F., Kroppenstedt, R., Stackebradt, E., Färber, P., and Holzapfel, W. (2004). *Mycobacterium fluoranthenivorans* sp. nov., a fluoranthene and aflatoxin B 1 degrading bacterium from contaminated soil of a former coal gas plant. *Systematic and applied microbiology, 27*(6), 653-660.

Huang, R.-y., Tian, W.-j., Liu, Q., Yu, H.-b., Jin, X., Zhao, Y.-g., Zhou, Y.-h., and Feng, G. (2016). Enhanced biodegradation of pyrene and indeno (1, 2, 3-cd) pyrene using bacteria immobilized in cinder beads in estuarine wetlands. *Marine pollution bulletin, 102*(1), 128-133.

Hudcova, T., Halecky, M., Kozliak, E., Stiborova, M., and Paca, J. (2011). Aerobic degradation of 2, 4-dinitrotoluene by individual bacterial strains and defined mixed population in submerged cultures. *Journal of hazardous materials, 192*(2), 605-613.

Iida, T., Mukouzaka, Y., Nakamura, K., and Kudo, T. (2002). Plasmid-borne genes code for an angular dioxygenase involved in dibenzofuran degradation by Terrabacter sp. strain YK3. *Applied and environmental microbiology, 68*(8), 3716-3723.

Isaac, P., Sánchez, L. A., Bourguignon, N., Cabral, M. E., and Ferrero, M. A. (2013). Indigenous PAH-degrading bacteria from oil-polluted sediments in Caleta Cordova, Patagonia Argentina. *International Biodeterioration & Biodegradation, 82*, 207-214.

Jacques, R. J., Okeke, B. C., Bento, F. M., Teixeira, A. S., Peralba, M. C., and Camargo, F. A. (2008). Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. *Bioresource technology, 99*(7), 2637-2643.

Jasmine, J., and Mukherji, S. (2015). Characterization of oily sludge from a refinery and biodegradability assessment using various hydrocarbon degrading strains and reconstituted consortia. *Journal of environmental management, 149*, 118-125.

Jonsson, S., Persson, Y., Frankki, S., van Bavel, B., Lundstedt, S., Haglund, P., and Tysklind, M. (2007). Degradation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils by Fenton's reagent: a multivariate evaluation of the importance of soil characteristics and PAH properties. *Journal of hazardous materials, 149*(1), 86-96.

Kanaly, R. A., and Harayama, S. (2000). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *Journal of bacteriology, 182*(8), 2059-2067.

Karabika, E., Kallimanis, A., Dados, A., Pilidis, G., Drainas, C., and Koukkou, A. (2009). Taxonomic identification and use of free and entrapped cells of a new *Mycobacterium* sp., strain Spyr1 for degradation of polycyclic aromatic hydrocarbons (PAHs). *Applied biochemistry and biotechnology, 159*(1), 155-167.

Khalil, M. F., Ghosh, U., and Kreitinger, J. P. (2006). Role of weathered coal tar pitch in the partitioning of polycyclic aromatic hydrocarbons in manufactured gas plant site sediments. *Environmental science & technology, 40*(18), 5681-5687.

Khondee, N., Tathong, S., Pinyakong, O., Powtongsook, S., Chatchupong, T., Ruangchainikom, C., and Luepromchai, E. (2012). Airlift bioreactor containing chitosan-immobilized Sphingobium sp. P2 for treatment of lubricants in wastewater. *Journal of hazardous materials, 213*, 466-473.

Kim, S.-J., Kweon, O., and Cerniglia, C. (2010). Degradation of polycyclic aromatic hydrocarbons by *Mycobacterium* strains *Handbook of hydrocarbon and lipid microbiology* (pp. 1865-1879): Springer.

Klankeo, P., Nopcharoenkul, W., and Pinyakong, O. (2009). Two novel pyrene-degrading *Diaphorobacter* sp. and *Pseudoxanthomonas* sp. isolated from soil. *Journal of bioscience and bioengineering, 108*(6), 488-495.

Kohno, T., Sugimoto, Y., Sei, K., and Mori, K. (2002). Design of PCR primers and gene probes for general detection of alkane-degrading bacteria. *Microbes and Environments, 17*(3), 114-121.

Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., and Koutinas, A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food microbiology, 21*(4), 377-397.

Kuyukina, M. S., Ivshina, I. B., Makarov, S. O., Litvinenko, L. V., Cunningham, C. J., and Philp, J. C. (2005). Effect of biosurfactants on crude oil desorption and mobilization in a soil system. *Environment international, 31*(2), 155-161.

Kvenvolden, K., and Cooper, C. (2003). Natural seepage of crude oil into the marine environment. *Geo-Marine Letters, 23*(3-4), 140-146.

Li, C., Zhou, Z.-X., Jia, X.-Q., Chen, Y., Liu, J., and Wen, J.-P. (2013). Biodegradation of crude oil by a newly isolated strain *Rhodococcus* sp. JZX-01. *Applied biochemistry and biotechnology, 171*(7), 1715-1725.

Li, Y. Y., and Li, B. (2011). *Study on fungi-bacteria consortium bioremediation of petroleum contaminated mangrove sediments amended with mixed biosurfactants.* Paper presented at the Advanced Materials Research.

Lin, C., Gan, L., Chen, Z., Megharaj, M., and Naidu, R. (2014). Biodegradation of naphthalene using a functional biomaterial based on immobilized *Bacillus fusiformis* (BFN). *Biochemical engineering journal, 90*, 1-7.

Lin, J., Gan, L., Chen, Z., and Naidu, R. (2015). Biodegradation of tetradecane using *Acinetobacter venetianus* immobilized on bagasse. *Biochemical engineering journal, 100*, 76-82.

Liu, C.-W., and Liu, H.-S. (2011). *Rhodococcus erythropolis* strain NTU-1 efficiently degrades and traps diesel and crude oil in batch and fed-batch bioreactors. *Process Biochemistry, 46*(1), 202-209.

Luan, T., Keith, S., Zhong, Y., Zhou, H., Lan, C., and Tam, N. F. (2006). Study of metabolites from the degradation of polycyclic aromatic hydrocarbons (PAHs) by bacterial consortium enriched from mangrove sediments. *Chemosphere, 65*(11), 2289-2296.

Lundstedt, S., White, P. A., Lemieux, C. L., Lynes, K. D., Lambert, I. B., Öberg, L., Haglund, P., and Tysklind, M. (2007). Sources, fate, and toxic hazards of oxygenated polycyclic aromatic hydrocarbons (PAHs) at PAH-contaminated sites. *AMBIO: A Journal of the Human Environment, 36*(6), 475-485.

Mao, J., Luo, Y., Teng, Y., and Li, Z. (2012). Bioremediation of polycyclic aromatic hydrocarbon-contaminated soil by a bacterial consortium and associated microbial community changes. *International Biodeterioration & Biodegradation, 70*, 141-147.

Marine Department 2009. Oil spill incidents in the Thai waters. http://www.md.go.th/safety_ environment/04_3pdf/Oil_spill%202552.pdf. (in Thai).

Martin, J. D., Adams, J., Hollebone, B., King, T., Brown, R. S., and Hodson, P. V. (2014). Chronic toxicity of heavy fuel oils to fish embryos using multiple exposure scenarios. *Environmental toxicology and chemistry, 33*(3), 677-687.

Martínková, L., Uhnáková, B., Pátek, M., Nešvera, J., and Křen, V. (2009). Biodegradation potential of the genus Rhodococcus. *Environment international, 35*(1), 162-177.

Matsui, T., Yamamoto, T., Shinzato, N., Mitsuta, T., Nakano, K., and Namihira, T. (2014). Degradation of oil tank sludge using long-chain alkane-degrading bacteria. *Annals of microbiology, 64*(1), 391-395.

Mikesková, H., Novotný, Č., and Svobodová, K. (2012). Interspecific interactions in mixed microbial cultures in a biodegradation perspective. *Applied microbiology and biotechnology, 95*(4), 861-870.

Mishra, S., Jyot, J., Kuhad, R. C., and Lal, B. (2001). Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Applied and environmental microbiology, 67*(4), 1675-1681.

Mrozik, A., and Piotrowska-Seget, Z. (2010). Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiological research, 165*(5), 363-375.

Mukherjee, S., Das, P., and Sen, R. (2006). Towards commercial production of microbial surfactants. *TRENDS in Biotechnology, 24*(11), 509-515.

Nie, Y., Chi, C.-Q., Fang, H., Liang, J.-L., Lu, S.-L., Lai, G.-L., Tang, Y.-Q., and Wu, X.-L. (2014). Diverse alkane hydroxylase genes in microorganisms and environments. *Scientific reports, 4*, 4968.

Nojiri, H., Kamakura, M., Urata, M., Tanaka, T., Chung, J.-S., Takemura, T., Yoshida, T., Habe, H., and Omori, T. (2002). Dioxin catabolic genes are dispersed on the *Terrabacter* sp. DBF63 genome. *Biochemical and biophysical research communications, 296*(2), 233-240.

Nopcharoenkul, W., Netsakulnee, P., and Pinyakong, O. (2013). Diesel oil removal by immobilized *Pseudoxanthomonas* sp. RN402. *Biodegradation, 24*(3), 387-397.

Obuekwe, C. O., Al-Jadi, Z. K., and Al-Saleh, E. S. (2009). Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon

degraders from petroleum-contaminated Kuwait desert environment. *International Biodeterioration & Biodegradation, 63*(3), 273-279.

Oeder, S., Kanashova, T., Sippula, O., Sapcariu, S. C., Streibel, T., Arteaga-Salas, J. M., Passig, J., Dilger, M., Paur, H.-R., and Schlager, C. (2015). Particulate matter from both heavy fuel oil and diesel fuel shipping emissions show strong biological effects on human lung cells at realistic and comparable in vitro exposure conditions. *PloS one, 10*(6), e0126536.

Okerentugba, P., and Ezeronye, O. (2003). Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria. *African Journal of Biotechnology, 2*(9), 288-292.

Ou, S., Zheng, J., Zheng, J., Richardson, B. J., and Lam, P. K. (2004). Petroleum hydrocarbons and polycyclic aromatic hydrocarbons in the surficial sediments of Xiamen Harbour and Yuan Dan Lake, China. *Chemosphere, 56*(2), 107-112.

Pacwa-Płociniczak, M., Płaza, G. A., Piotrowska-Seget, Z., and Cameotra, S. S. (2011). Environmental applications of biosurfactants: recent advances. *International Journal of Molecular Sciences, 12*(1), 633-654.

Padungkwan Srisuwanakarn. Crude oil biodegradation efficiency of *Exiguobacterium* sp. AO-11 isolated from Ao Phrao's sediment Samet island, Thailand (Thesis) Bangkok: Chulalongkorn University, 2015, 172 pages.

Partovinia, A., and Naeimpoor, F. (2014). Comparison of phenanthrene biodegradation by free and immobilized cell systems: formation of hydroxylated compounds. *Environmental Science and Pollution Research, 21*(9), 5889-5898.

Pavlova, O., Zemskaya, T., Gorshkov, A., Parfenova, V., Suslova, M. Y., and Khlystov, O. (2008). Study on the Lake Baikal microbial community in the areas of the natural oil seeps. *Applied Biochemistry and Microbiology, 44*(3), 287-291.

Pedetta, A., Pouyte, K., Seitz, M. K. H., Babay, P. A., Espinosa, M., Costagliola, M., Studdert, C. A., and Peressutti, S. R. (2013). Phenanthrene degradation and strategies to improve its bioavailability to microorganisms isolated from brackish sediments. *International Biodeterioration & Biodegradation, 84*, 161-167.

Peng, P., Yang, H., Jia, R., and Li, L. (2013). Biodegradation of dioxin by a newly isolated *Rhodococcus* sp. with the involvement of self-transmissible plasmids. *Applied microbiology and biotechnology, 97*(12), 5585-5595.

Perelo, L. W. (2010). Review: in situ and bioremediation of organic pollutants in aquatic sediments. *Journal of hazardous materials, 177*(1), 81-89.

Pinyakong, O., Tiangda, K., Iwata, K., and Omori, T. (2012). Isolation of novel phenanthrene-degrading bacteria from seawater and the influence of its physical factors on the degradation of phenanthrene. *Science Asia, 38*(3).

Piyanoot Uklum. Crude oil degradation of bacterial consortia isolated from sediment of the Gulf of Thailand. (Thesis) Bangkok: Chulalongkorn University, 2015. 168 pages.

Purcaro, G., Moret, S., and Conte, L. S. (2013). Overview on polycyclic aromatic hydrocarbons: occurrence, legislation and innovative determination in foods. *Talanta, 105*, 292-305.

Quatrini, P., Scaglione, G., De Pasquale, C., Riela, S., and Puglia, A. (2008). Isolation of Gram-positive n-alkane degraders from a hydrocarbon-contaminated Mediterranean shoreline. *Journal of applied microbiology, 104*(1), 251-259.

Quek, E., Ting, Y.-P., and Tan, H. M. (2006). *Rhodococcus* sp. F92 immobilized on polyurethane foam shows ability to degrade various petroleum products. *Bioresource technology, 97*(1), 32-38.

Ravindra, K., Sokhi, R., and Van Grieken, R. (2008). Atmospheric polycyclic aromatic hydrocarbons: source attribution, emission factors and regulation. *Atmospheric Environment, 42*(13), 2895-2921.

Reddy, M. S., Naresh, B., Leela, T., Prashanthi, M., Madhusudhan, N. C., Dhanasri, G., and Devi, P. (2010). Biodegradation of phenanthrene with biosurfactant production by a new strain of *Brevibacillus* sp. *Bioresource technology, 101*(20), 7980-7983.

Rojo, F. (2009). Degradation of alkanes by bacteria. *Environmental microbiology, 11*(10), 2477-2490.

Ruberto, L., Vazquez, S. C., and Mac Cormack, W. P. (2003). Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the

bioremediation of a hydrocarbon contaminated Antarctic soil. *International Biodeterioration & Biodegradation, 52*(2), 115-125.

Satchanska, G., Topalova, Y., Dimkov, R., Groudeva, V., Petrov, P., Tsvetanov, C., Selenska-Pobell, S., and Golovinsky, E. (2015). Phenol degradation by environmental bacteria entrapped in cryogels. *Biotechnology & Biotechnological Equipment, 29*(3), 514-521.

Sathishkumar, M., Binupriya, A. R., Baik, S. H., and Yun, S. E. (2008). Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. *CLEAN–Soil, Air, Water, 36*(1), 92-96.

Semrany, S., Favier, L., Djelal, H., Taha, S., and Amrane, A. (2012). Bioaugmentation: possible solution in the treatment of bio-refractory organic compounds (Bio-ROCs). *Biochemical engineering journal, 69*, 75-86.

Seneviratne, G., Zavahir, J., Bandara, W., and Weerasekara, M. (2008). Fungal-bacterial biofilms: their development for novel biotechnological applications. *World Journal of Microbiology and Biotechnology, 24*(6), 739.

Seo, J.-S., Keum, Y.-S., and Li, Q. X. (2012). Mycobacterium aromativorans JS19b1 T degrades phenanthrene through C-1, 2, C-3, 4 and C-9, 10 dioxygenation pathways. *International Biodeterioration & Biodegradation, 70*, 96-103.

Sharma, S., and Pant, A. (2000). Biodegradation and conversion of alkanes and crude oil by a marine *Rhodococcus*. *Biodegradation, 11*(5), 289-294.

Shekhar, S., Sundaramanickam, A., and Balasubramanian, T. (2015). Biosurfactant producing microbes and their potential applications: A review. *Critical Reviews in Environmental Science and Technology, 45*(14), 1522-1554.

Shen, T., Pi, Y., Bao, M., Xu, N., Li, Y., and Lu, J. (2015). Biodegradation of different petroleum hydrocarbons by free and immobilized microbial consortia. *Environmental Science: Processes & Impacts, 17*(12), 2022-2033.

Simarro, R., González, N., Bautista, L. F., Sanz, R., and Molina, M. C. (2011). Optimisation of key abiotic factors of PAH (naphthalene, phenanthrene and anthracene) biodegradation process by a bacterial consortium. *Water, Air, & Soil Pollution, 217*(1-4), 365-374.

Song, X., Xu, Y., Li, G., Zhang, Y., Huang, T., and Hu, Z. (2011). Isolation, characterization of *Rhodococcus* sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. *Marine pollution bulletin, 62*(10), 2122-2128.

Souza, E. C., Vessoni-Penna, T. C., and de Souza Oliveira, R. P. (2014). Biosurfactant-enhanced hydrocarbon bioremediation: An overview. *International Biodeterioration & Biodegradation, 89*, 88-94.

Tao, X., Liu, J., Lu, G., Guo, X., Jiang, H., and Sun, G. (2010). Biodegradation of phenanthrene in artificial seawater by using free and immobilized strain of *Sphingomonas* sp. GY2B. *African Journal of Biotechnology, 9*(18), 2654-2660.

Techtmann, S. M., and Hazen, T. C. (2016). Metagenomic applications in environmental monitoring and bioremediation. *Journal of industrial microbiology & biotechnology, 43*(10), 1345-1354.

Thapa, B., Kc, A. K., and Ghimire, A. (2012). A review on bioremediation of petroleum hydrocarbon contaminants in soil. *Kathmandu university journal of science, engineering and technology, 8*(1), 164-170.

Thavasi, R., Sharma, S., and Jayalakshmi, S. (2011). Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. *J. Pet. Environ. Biotechnol. S, 1*, 1-6.

Thoetkiattikul, H., Mhuantong, W., Pinyakong, O., Wisawapipat, W., Yamazoe, A., Fujita, N., Eurwilaichitr, L., and Champreda, V. (2017). Culture-independent study of bacterial communities in tropical river sediment. *Bioscience, Biotechnology, and Biochemistry, 81*(1), 200-209.

Vila, J., López, Z., Sabaté, J., Minguillón, C., Solanas, A. M., and Grifoll, M. (2001). Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two-and three-ring polycyclic aromatic hydrocarbons. *Applied and environmental microbiology, 67*(12), 5497-5505.

Ward, O., Singh, A., and Van Hamme, J. (2003). Accelerated biodegradation of petroleum hydrocarbon waste. *Journal of Industrial Microbiology and Biotechnology, 30*(5), 260-270.

Wattayakorn, G. (2012). Petroleum pollution in the Gulf of Thailand: A historical review.

Wentzel, A., Ellingsen, T. E., Kotlar, H.-K., Zotchev, S. B., and Throne-Holst, M. (2007). Bacterial metabolism of long-chain n-alkanes. *Applied microbiology and biotechnology, 76*(6), 1209-1221.

Wilcke, W., Bandowe, B. A. M., Lueso, M. G., Ruppenthal, M., del Valle, H., and Oelmann, Y. (2014). Polycyclic aromatic hydrocarbons (PAHs) and their polar derivatives (oxygenated PAHs, azaarenes) in soils along a climosequence in Argentina. *Science of the total environment, 473*, 317-325.

Wu, Y., Luo, Y., Zou, D., Ni, J., Liu, W., Teng, Y., and Li, Z. (2008). Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with *Monilinia* sp.: degradation and microbial community analysis. *Biodegradation, 19*(2), 247-257.

Yang, H.-Y., Jia, R.-B., Chen, B., and Li, L. (2014). Degradation of recalcitrant aliphatic and aromatic hydrocarbons by a dioxin-degrader *Rhodococcus* sp. strain p52. *Environmental Science and Pollution Research, 21*(18), 11086-11093.

Yu, K., Wong, A., Yau, K., Wong, Y., and Tam, N. (2005). Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. *Marine pollution bulletin, 51*(8), 1071-1077.

Yuan, S., Wei, S., and Chang, B. (2000). Biodegradation of polycyclic aromatic hydrocarbons by a mixed culture. *Chemosphere, 41*(9), 1463-1468.

Zeng, J., Lin, X., Zhang, J., and Li, X. (2010). Isolation of polycyclic aromatic hydrocarbons (PAHs)-degrading *Mycobacterium* spp. and the degradation in soil. *Journal of hazardous materials, 183*(1), 718-723.

Zhang, D.-C., Mörtelmaier, C., and Margesin, R. (2012). Characterization of the bacterial archaeal diversity in hydrocarbon-contaminated soil. *Science of the total environment, 421*, 184-196.

Zhang, J., Sun, Z., Li, Y., Peng, X., Li, W., and Yan, Y. (2009). Biodegradation of p-nitrophenol by *Rhodococcus* sp. CN6 with high cell surface hydrophobicity. *Journal of hazardous materials, 163*(2), 723-728.

Zhang, Y., and Tao, S. (2009). Global atmospheric emission inventory of polycyclic aromatic hydrocarbons (PAHs) for 2004. *Atmospheric Environment, 43*(4), 812-819.

Zhao, Z., Selvam, A., and Wong, J. W.-C. (2011). Effects of rhamnolipids on cell surface hydrophobicity of PAH degrading bacteria and the biodegradation of phenanthrene. *Bioresource technology, 102*(5), 3999-4007.

Zhen-Yu, W., Ying, X., Hao-Yun, W., Jian, Z., Dong-Mei, G., Feng-Min, L., and Xing, B. (2012). Biodegradation of crude oil in contaminated soils by free and immobilized microorganisms. *Pedosphere, 22*(5), 717-725.

Zheng, C., Yu, L., Huang, L., Xiu, J., and Huang, Z. (2012). Investigation of a hydrocarbon-degrading strain, *Rhodococcus ruber* Z25, for the potential of microbial enhanced oil recovery. *Journal of Petroleum Science and Engineering, 81*, 49-56.

Zinger, L., Amaral-Zettler, L. A., Fuhrman, J. A., Horner-Devine, M. C., Huse, S. M., Welch, D. B. M., Martiny, J. B., Sogin, M., Boetius, A., and Ramette, A. (2011). Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. *PloS one, 6*(9), e24570.

Khaosod. (2015). [online] source: http://www.khaosod.co.th/view newsonline.php?newsid=1449222384 [22 March 2016]

Mthai news (2012). [online] source: http://www.news.mthai.com/hot-news/generalnews/181224.html [22 March 2016]

National News Bureau of Thailand. (2015). [online] source: http://thainews.prd.go.th/website_th/news/news_detail/WNEVN570607001000 1 [8 March 2016]

APPENDIX

Appendix A

Culture medium preparation

Luria-Bertani (LB broth)

Yeast extract	5 g
Tryptone	10 g
NaCl	5 g

Dissolve in 1,000 ml of distilled water, adjust to pH 7.0 with 5M NaCl, and autoclave at 15 pound per square inch, 121°C for 15 min.

Luria-Bertani (LB agar)

Use the chemicals like LB broth, add 20 g of agar, and autoclave at 15 pound per square inch, 121°C for 15 min.

Carbon free mineral medium (CFMM)

Solution 1

NH_4NO_3	3 g
KH_2PO_4	2.2 g
$Na_2HPO_4 \cdot 12H_2O$	0.8 g

Adjust the final volume to 1,000 ml and autoclave at 15 pound per square inch, 121°C for 15 min

Prepare CFMM agar by dissolving the bacto-agar in 100 ml of culture medium and autoclaved

Solution 2

$FeCl_3·6H_2O$	0.05	g/mL
$CaCl_2·H_2O$	0.05	g/mL
$MgSO_4·7H_2O$	0.1	g/mL

Sterilize the solution by using 0.22 µm of CA membrane and added the solution 2 (1 mL each of solution was added into solution 1 (1,000 mL)

Appendix B
Solution Preparation

PAH solution at final concentration 10,000 mg L^{-1} (for addition in CFMM broth)

PAH	500 mg
Dimethylformamide	50 mL

PAH was dissolved in dimethylformamide, filtrated by 0.2 μm PTFE and kept at -20°C.

5M NaOH

NaOH	20 mg
Deionized water	100 mL

0.85% NaCl

NaCl	8.5 g
Distilled water	1,000 mL

The solution was autocalved at 15 pound/square inch, 121°C for 15 min.

Appendix C

Standard curves

C 1 Standard curve of PAHs analysed by HPLC

PAHs including phenanthrene, fluorene, pyrene, and fluoranthene was dissolved in methanol at final concentration (5,000 mg L^{-1}). Then, each PAH was diluted to at final concentration 10, 50, 100, 150, and 200 mg L^{-1}. The solution was filtrated through 0.22 μm PTFE into HPLC vial. Finally, PAHs were analysed by HPLC using 80% methanol as mobile phase. The calculation to determine concentration of PAH in sample is follow:

Peak area = slope × the amount of PAH (mg/L)

Phenanthrene standard curve

y = 55460x
R^2 = 0.9985

Peak area

Phenanthrene concentration (mg/L)
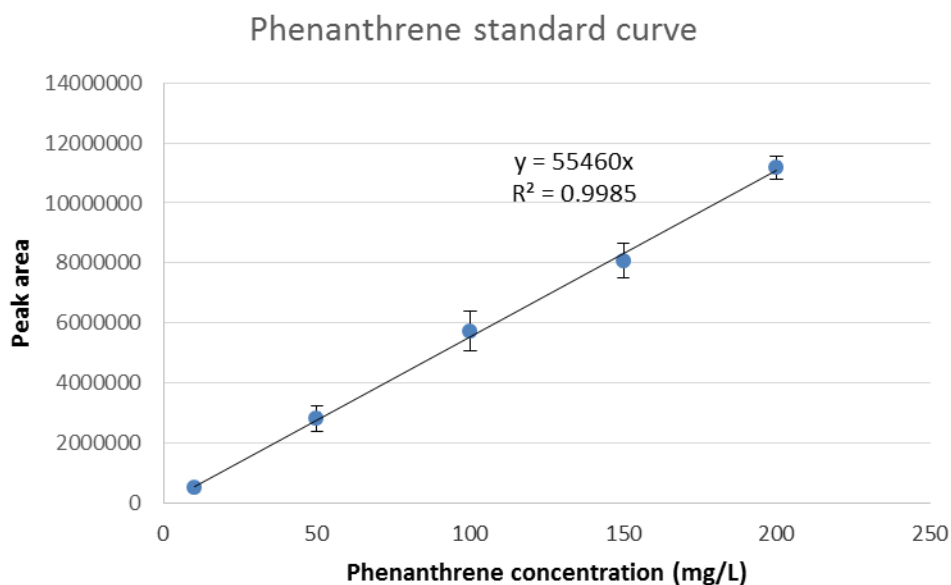
Figure C.1 Standard curve of phenanthrene from HPLC.

Fluorene standard curve

$y = 44539x$
$R^2 = 0.997$

Peak area / Fluorene concentration (mg/L)
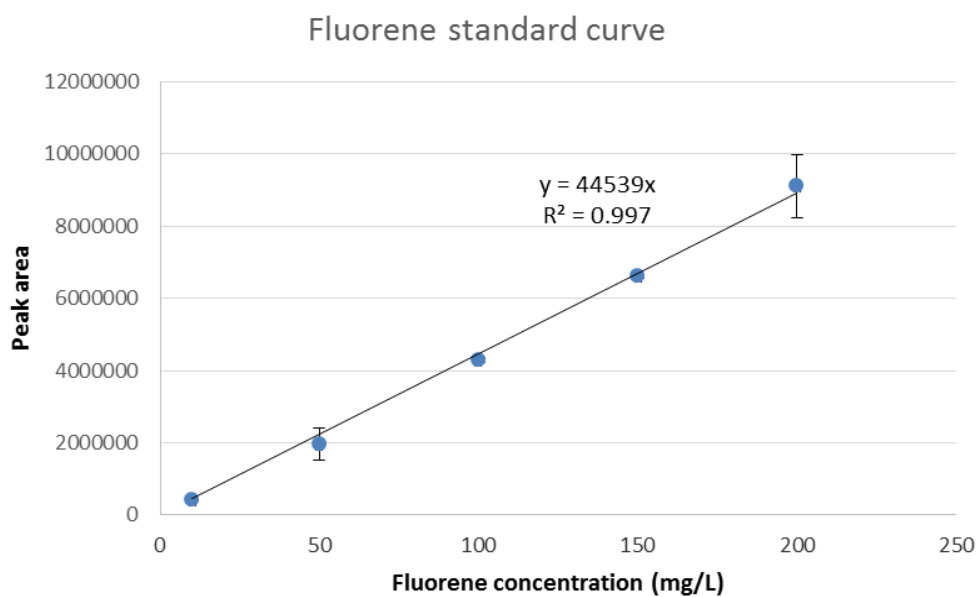
Figure C.2 Standard curve of fluorene from HPLC.

Pyrene standard curve

$y = 101044x$
$R^2 = 0.9984$

Peak area / Pyrene concentration (mg/L)
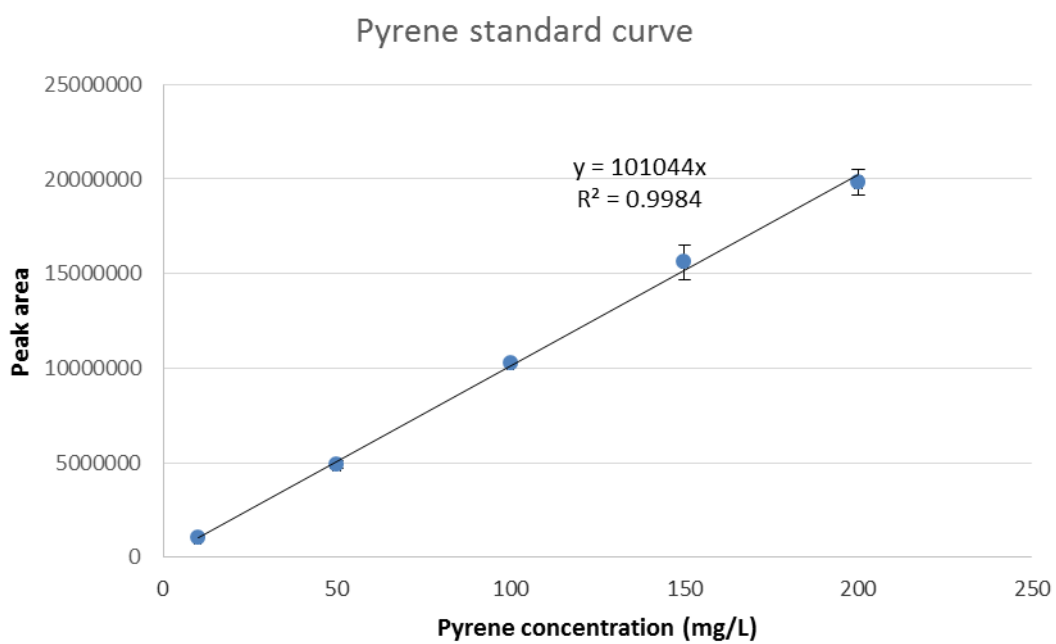
Figure C.3 Standard curve of pyrene from HPLC.
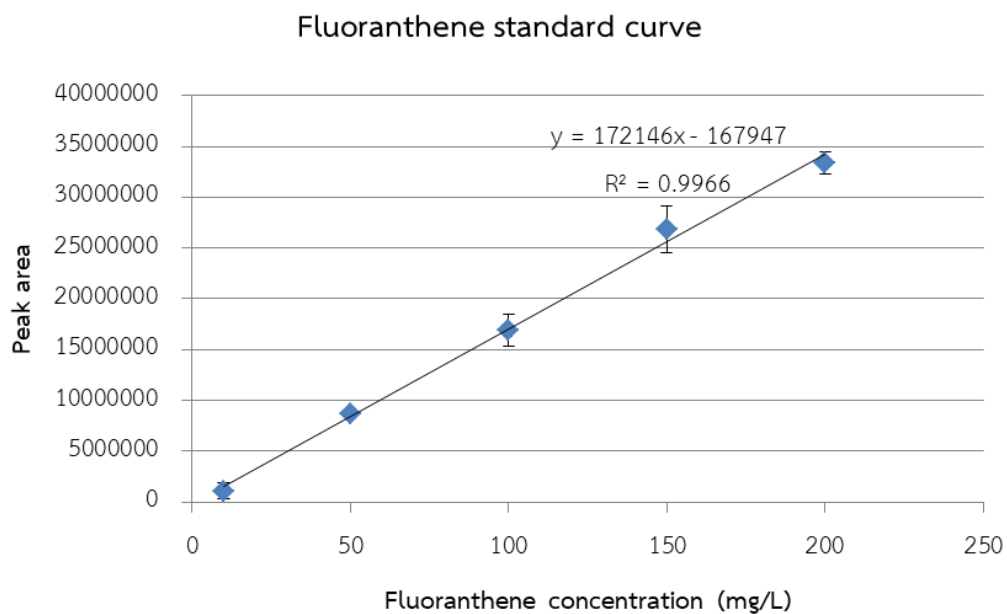
Figure C.4 Standard curve of fluoranthene from HPLC.

C 2 Standard curve of tetradecane and docosane analysed by GC

Tetradecane and docosane was dissolved in hexane at final concentration (5,000 mg L^{-1}). Then, tetradecane was diluted to at final concentration 100, 250, 500, 750, and 1,000 mg L^{-1}. Docosane was diluted to at final concentration 10, 50, 100, 150, and 200 mg L^{-1} The solution was filtrated through 0.22 μm PTFE into GC vial. Finally, aliphatics were analysed by GC using helium as carrier gas. The calculation to determine concentration of aliphatic in sample is follow:

Peak area = slope × the amount of aliphatic (mg/L)

Tetradecane standard curve

y = 2.7732x - 6.5854

R² = 0.9996
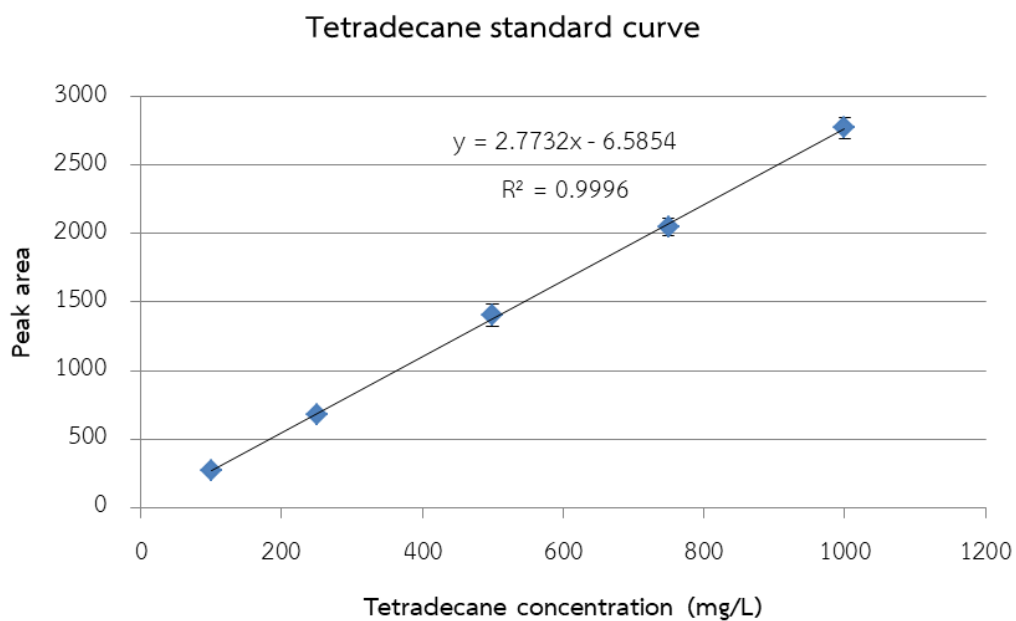
Figure C.5 Standard curve of tetradecane from GC.

Docosane standard curve

y = 6.2916x - 11.34

R² = 0.9993
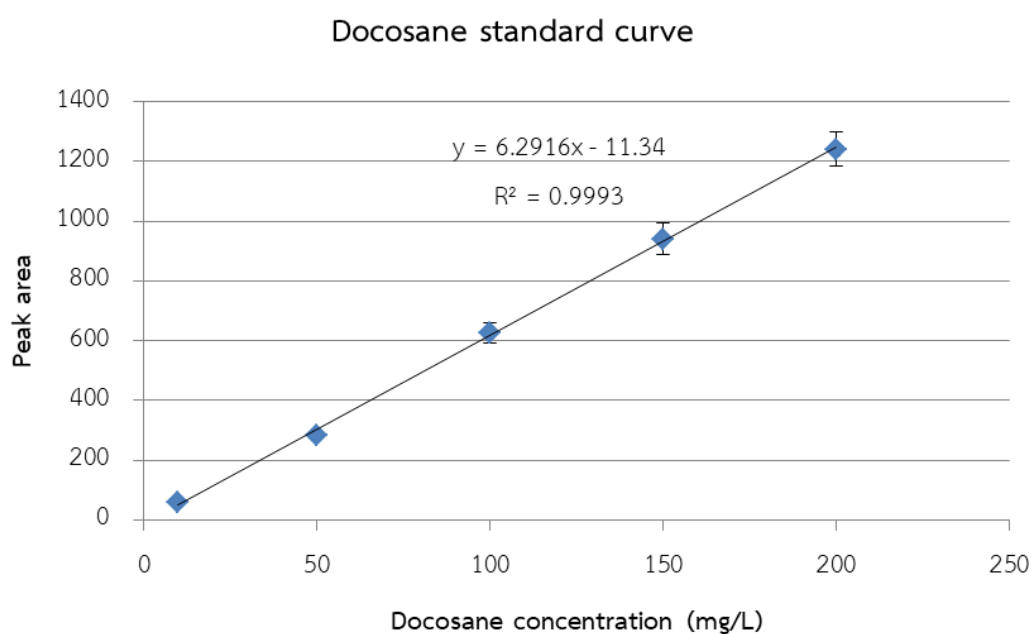
Figure C.6 Standard curve of docosane from GC.

C 3 Standard curve of crude oil and diesel oil analysed by GC

Crude oil and diesel oil was prepared dissolved in hexane at final concentration 500, 1,000, 2,000, 4,000, and 8,000 mg L^{-1}. The solution was filtrated through 0.22 μm PTFE into GC vial. Finally, oils were analysed by GC using helium as carrier gas. The calculation to determine concentration of oil in sample is follow:

Peak area = slope × the amount of oil (mg/L)

Crude oil standard curve

$y = 4.188x + 725.1$

$R^2 = 0.9987$
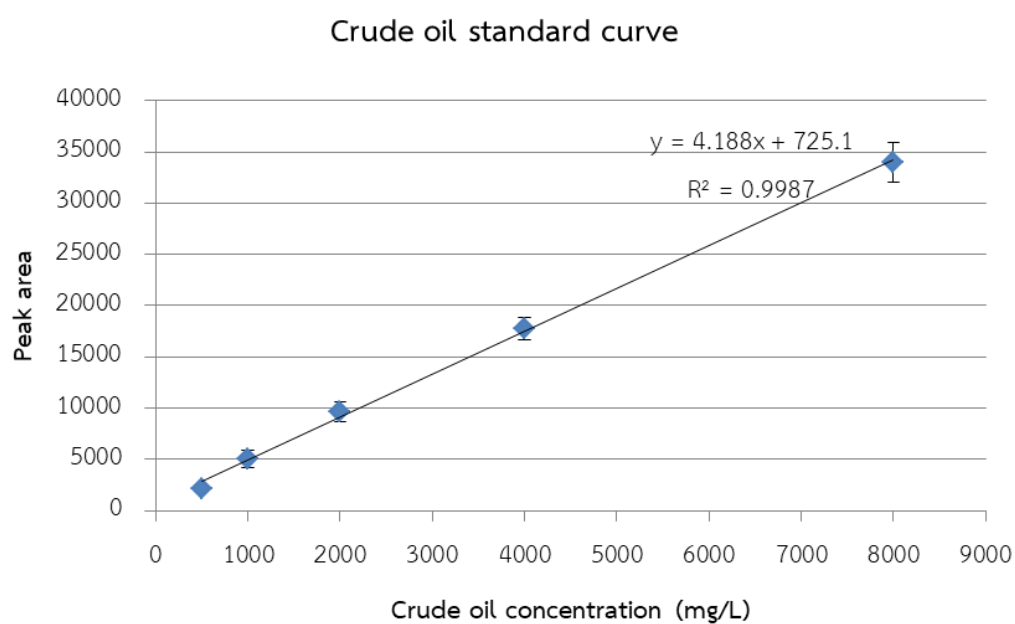
Figure C.7 Standard curve of crude oil from GC.

Diesel oil standard curve
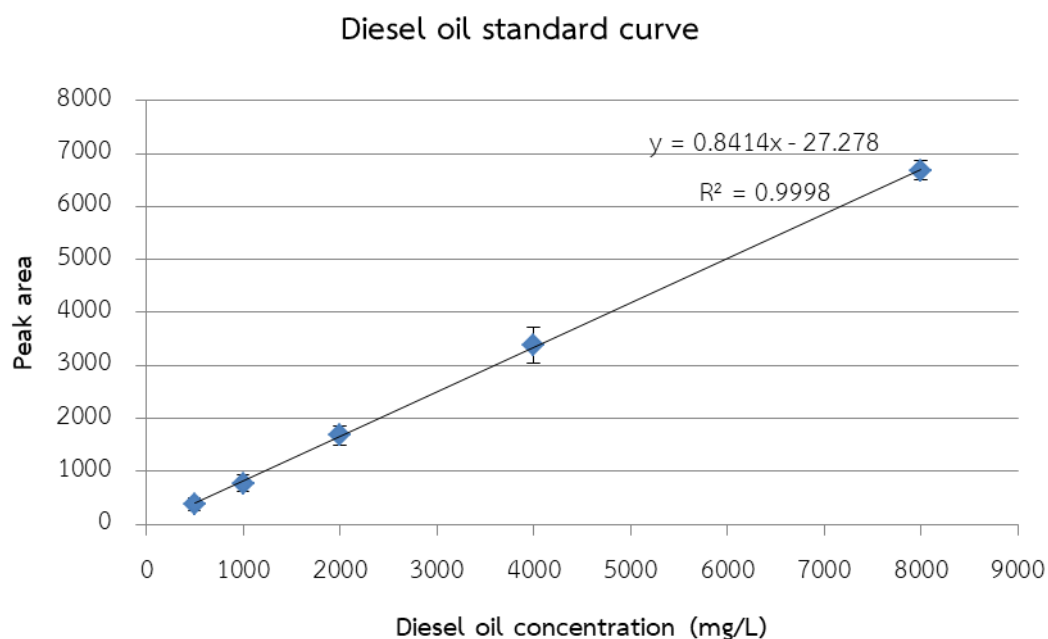
Figure C.8 Standard curve of diesel oil from GC.

C 4 16S rDNA sequencing of 8 strains

16S rDNA sequencing of *Sphingobium* sp. Y101

ACTTCACCCCAGTCGCTAAACCCACTGTGGTCGCCTGCCTCCCTTGCGGGTTAGCTCAACGCCT
TCGAGTGAATCCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCTGGGAACGTATTCACC
GCGGCATGCTGATCCGCGATTACTAGCGATTCCGCCTTCATGCTCTCGAGTTGCAGAGAACAAT
CCGAACTGAGACGACTTTTGGAGATTAGCTTCCACTCGCATGGTCGCTGCCCACTGTAGTCGCC
ATTGTAGCACGTGTGTAGCCCAACGCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCC
TCCGGCTTATCACCGGCGGTTCCTTTAGAGTACCCAACTAAATGATGGCAACTAAAGGCGAGGG
TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCAC
CTGTCACCTATCCAGCCGAACTGAAGGAAAGTGTCTCCACTAACCGCGATAGGGATGTCAAACG
TTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCAGGCCCCCG
TCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGATAACTTAATGCGTTAGCTG
CGCCACTGAAATGCCATGCACCCCAGCAGCTAGTTATCATCGTTTACGGCGTGGACTACCAGGG
TATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAACAATCGTCCAGTGAGCCGC
CTTCGCCACTGGTGTTCTTCCGAATATCTACGAATTTCACCTCTACACTCGGAATTCCACTCACC

TCTCCGATGTTCAAGCAATCCAGTCTCAAAGGCTATTCCGGGGGTTGAGCCCCGGGCTTTCACCT
CTGACTTAAATCGCCGCCTACGTGCGCTTTACGCCCAGTAATTCCGAACAACGCTAGCCCCCTC
CGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTATTCTCCCGGTACTGTCATTATCA
TCCCGGGTAAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGATCAGG
GTTGCCCCCATTGTCCAATATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT
CCCAGTGTGGCTGATCATCCTCTCAGACCAGCTAAGGATCGTCGCCTTGGTCAGCCTTTACCCA
ACCAACTAGCTAATCCTACGCGGGCTCATCCCTGGGCGATAAATCTTTGGACTTACGTCATCAT
CCGGTATTAGCGTCAGTTTCCCGACGTTATTCCGAACCCAAGGGCAGATTCCCACGCGTTACGC
ACCCGTGCGCCACTATCTCCGAAGAGATCGTTCGACTTGCATGTATTAGGCATGCCGCCAGCGT
TCGTTCTG

16S rDNA sequencing of *Sphingobium* sp. Y401

CTTCGGGTGAAACCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCTGGGAACGTATTCA
CCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGCCTTCATGCTCTCGAGTTGCAGAGAACA
ATCCGAACTGAGACGACTTTTGGAGATTAGCTTCCACTCGCATGGTCGCTGCCCACTGTAGTCG
CCATTGTAGCACGTGTGTAGCCCAACGCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTT
CCTCCGGCTTATCACCGGCGGTTCCTTTAGAGTACCCAACTAAATGATGGCAACTAAAGGCGAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGC
ACCTGTCACTCATCCAGCCGAACTGAAGAAATCCATCTCTGGAAATCGCGATGAGGATGTCAAA
CGTTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCAGGCCCC
CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGATAACTTAATGCGTTAG
CTGCGCCACCGAAACTCCATGAGCCCCAGCAGCTAGTTATCATCGTTTACGGCGTGGACTACCA
GGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAACAATCGTCCAGTGAGC
CGCCTTCGCCACTGGTGTTCTTCCGAATATCTACGAATTTCACCTCTACACTCGGAATTCCACTC
ACCTCTCCGATGTTCAAGCAATCCAGTCTCAAAGGCTATTCCGGGGGTTGAGCCCCGGGCTTTCA
CCTCTGACTTAAATCGCCGCCTACGTGCGCTTTACGCCCAGTAATTCCGAACAACGCTAGCCCC
CTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTATTCTCCCGGTACTGTCATTA
TCATCCCGGGTAAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGATC
AGGGTTGCCCCCATTGTCCAATATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTAAGGATCGTCGCCTTGGTGAGCCTTTAC

CTCACCAACTAGCTAATCCTACGCGGGCTCATCCCTGGGCGATAAATCTTTGGACTTTCGTCAT
CATCCGGTATTAGCGTCCGTTTCCAGACGTTATTCCGAACCCAAGGGCAGATTCCCACGCGTTA
CGCACCCGTGCGCCACTAGCCCCGAAAGGCTCGTTCGACTTGCATGTATTAGGCATGCCGCCAG
CGTTCGTTCTGAGC

16S rDNA sequencing of *Arthrobacter* sp. S101

cTTAGTCCCAATCGCCAGTCCCACCTTCGACAGCTCCCTCCCACAAGGGGTTAGGCCACCGGCT
TCGGGTGTTACCAACTTTCGTGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAA
TCCGAACTGAGACCGGCTTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCTTTGTACCGG
CCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCGTCCCCACCTT
CCTCCGAGTTGACCCCGGCAGTCTCCTATGAGTCCCCACCATCACGTGCTGGCAACATAGAACG
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCA
CCACCTGTAAACCGACCGCAAGCGGGGCACCTGTTTCCAGGCGTTACCGGTTCATGTCAAGCCT
TGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGCCCCCGT
CAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTA
CGGCGCGGAAAACGTGGAATGTCCCCCACACCTAGTGCCCAACGTTTACGGCATGGACTACCAG
GGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGTTAATGCCCAGAGACCT
GCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCAGTC
TCCCCTACATCACTCTAGTCTGCCCGTACCCACCGCAGATCCGGAGTTGAGCCCCGGACTTTCA
CGGCAGACGCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGATAACGCTTGCGCC
CTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACTT
TCGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCT
GCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGT
GTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTAGGCC
ATTACCCCACCAACAAGCTGATAGGCCGCGAGTCCATCCAAAACCACAAAAGCTTTCCACCAAC
CACCATGCGATGGAAGGTCATATCCGGTATTAGACCCAGTTTCCCAGGCTTATCCCAGAGTCAA
GGGCAGGTTACTCACGTGTTACTCACCCGTTCGCCACTAATCCACCAGCAAGCTGGCATCATCG
TTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCATCCTGA

16S rDNA sequencing of *Pseudomonas* sp. J801

TTCACCCCAGTCATGAATCACACCGTGGTAACCGTCCTCCCGAAGGTTAGACTAGCTACTTCTG
GTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCG
ACATTCTGATTCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCG
GACTACGATCGGTTTTGTGAGATTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTACCGACCAT
TGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTC
CGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGACAAGGG
TTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCAC
CTGTGTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGCATGTCAAGGCCTGG
TAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAA
TTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGC
CACTAAAATCTCAAGGATTCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTAT
CTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT
CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTC
TACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACATCC
AACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCTG
TATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCA
AGGTATTAGCTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACA
CACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTA
GGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGT
CGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAGCGCAAG
GCCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTTCCTTTCGAAACGTT
GTCCCCCACTACCAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTGAATCAAGGAGCAA
GCTCCCGTCATCCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGA

16S rDNA sequencing of *Rhodococcus ruber* S103

CTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCAGCTTGCTG
GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGC
CTGGGAAACTGGGTCTAATACCGGATAGGACCTCGGGATGCATGTTCCGGGGTGGAAAGGTTTT
CCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAACGGCCCACCAAGGCGAC
GACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTG
AGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTACCGACGAAGCGCAAGTGACGGTAGGT
ACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTG
TCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGTCTGTGAAAACCCGCAG
CTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCC
TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGG
CAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCTAACG
CATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGA
CATACACCGGACCGCCCCAGAGATGGGGTTTCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTG
TTGCCAGCACGTAATGGTGGGGACTCGCAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGG
GACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACA
GAGGGCTGCGATACCGCGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGGT
CTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGT
GGCCTAACCCCCTCGTGGGAAGGG

16S rDNA sequencing of *Mycobacterium* sp. J101

CACCGGCTTCGGGTGTTACCGACTTTCATGACGTGACGGGCGGTGTGTACAAGGCCCGGGAAC
GTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCACGGGGTCGAGTTGC
AGACCCCGATCCGAACTGAGACCGGCTTTGAAAGGATTCGCTCCACCTCACGGCATCGCAGCCC
TTTGTACCGGCCATTGTAGCATGTGTGAAGCCCTGGACATAAGGGGCATGATGACTTGACGTCA
TCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCTCACGAGTCCCCACCATAACGTGCTGGCA
ACATGAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAGCCATGCACCACCTGCACACAGGCCACAAGGGAACCGACATCTCTGCCGGCGTCCTGTGCAT
GTCAAACCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCACATGCTCCGCCGCTTGTGCG
GGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCAAAAAGTAGCTCCCCAGGCGGGGTAC
TTAATGCGTTAGCTACGGCACGGATCCCAAGGAAGGAAACCCACACCTAGTACCCACAGTAGTA
GCGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAG
TTACTGCCCAGAGACCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTCCACCGCTA
CACCAGGAATTCCAGTCTCCCCTGCAGTACTCCAGTCTGCCCGTATCGCCCGCACGCCCACAGT
TAAGCTGTGAGTTTTCACGAACAACGCGACAAACCACCTACGAGCTCTTTACGCCCAGTAATTC
CGGACAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTGGCCGGTGCTTCTTC
TCCAGGTACCGTCACTTGCGCTTCGTCCCTGGCGAAAGAGGTTTACAACCCGAAGGCCGTCATC
CCTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCC
GTAGGAGTCTGGGCCGTATCTCAGTCCCAGTGTGGCCGGACACCCTCTCAGGCCGGCTACCCGT
CGTCGCCTTGGTAGGCCATTACCCCACCAACAAGCTGATAGGCCGCGGGCCCATCCCACACCGC
AAAAGCTTTCCACCACCGACCATGAAGCCGATGATCATATTCGGTATTAGACCCAGTTTCCCAG
GCTTATCCCAAAGTGCAGGGCAGATCA

16S rDNA sequencing of *Mycobacterium* sp. Y502

GCCCCCCCCATCCCCTTCGACGGCTCCCTCCCACAAGGGGTTAGGCCACCGGCTTCGGGTGTTA
CCGACTTTCATGACGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTG
CTGATCTGCGATTACTAGCGACTCCGACTTCACGGGGTCGAGTTGCAGACCCCGATCCGAACTG
AGACCGGCTTTGAAAGGATTCGCTCCACCTCACGGCATCGCAGCCCTTTGTACCGGCCATTGTA
GCATGTGTGAAGCCCTGGACATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTCCGAG
TTGACCCCGGCAGTCTCTCACGAGTCCCCGCCATTACGCGCTGGCAACATAAGATAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCTGC
ACACAGGCCACAAGGGAATACCTATCTCTAGGCACGTCCTGTGCATGTCAAACCCAGGTAAGGT
TCTTCGCGTTGCATCGAATTAATCCACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTTCTT
TGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGTACTTAATGCGTTAGCTACGGCACGGA
TCCCAAGGAAGGAAACCCACACCTAGTACCCACCGTTTACGGCGTGGACTACCAGGGTATCTAA
TCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACTGCCCAGAGACCCGCCTTCGCC
ACCGGTGTTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAGTCTCCCCTGCA
GTACTCCAGTCTGCCCGTATCGCCCGCACGCCCACAGTTGAGCTGTGAGTTTTCACGAACAACG
CGACAAACCACCTACGAGCTCTTTACGCCCAGTAATTCCGGACAACGCTCGGACCCTACGTATT
ACCGCGGCTGCTGGCACGTAGTTGGCCGGTCCTTCTTCTCCAGGTACCGTCACTTGCGCTTCGT
CCCTGGCGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGG
CTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGT
CCCAGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCCTCCCCTTGGTGAACCGTAACCTC
ACCAAAAAGCTGATAGGCCGCGGGCCCATCCCAACCGGAAAAGCTTTCCCCAACAAACCTGCAC
CCAAAGGGGTATTCGGTTTAAACCCGTTTCCCAGGTTATCCAAATGGGGGGGAATACCCCCCGT
GATCACCCGTTCCCCTTAGAACCCAAAGGGCTTTCCTTTACTCTAGGTTAAACCGCCCCTTCCT
TCGGTCCGTGAGAACACTACATAAAAAGGGAGTTATTTTAATTAATTTATATTT

16S rDNA sequencing of *Mycobacterium* sp. Y1201

TGTTACCGACTTTCATGACGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGC
GTTGCTGATCTGCGATTACTAGCGACTCCGACTTCACGGGGTCGAGTTGCAGACCCCGATCCGA
ACTGAGACCGGCTTTGAAAGGATTCGCTCCACCTCACGGCATCGCAGCCCTTTGTACCGGCCAT
TGTAGCATGTGTGAAGCCCTGGACATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTC
CGAGTTGACCCCGGCAGTCTCTCACGAGTCCCCACCATAACGTGCTGGCAACATGAGACAAGGG
TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCAC
CTGCACACAGGCCACAAGGGAACCGACATCTCTGCCGGCGTCCTGTGCATGTCAAACCCAGGTA
AGGTTCTTCGCGTTGCATCGAATTAATCCACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATT
TCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGTACTTAATGCGTTAGCTACGGCA
CGGATCCCAAGGAAGGAAACCCACACCTAGTACCCACCGTTTACGGCGTGGACTACCAGGGTAT
CTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACTGCCCAGAGACCCGCCTT
CGCCACCGGTGTTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAGTCTCCCC
TGCAGTACTCAAGTCTGCCCGTATCGCCCGCACGCCCACAGTTAAGCTGTGAGTTTTCACGAAC
AACGCGACAAACCACCTACGAGCTCTTTACGCCCAGTAATTCCGGACAACGCTCGGACCCTACG
TATTACCGCGGCTGCTGGCACGTAGTTGGCCGGTCCTTCTTCTCCAGGTACCGTCACTTGCGCT
TCGTCCCTGGCGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATC
AGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTC
AGTCCCAGTGTGGCCGGTCACCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTAAGCCATTAC
CTCACCAACAAGCTGATAGGCCGCGGGCCCATCCCACACCGCAAAGCTTTCCAC
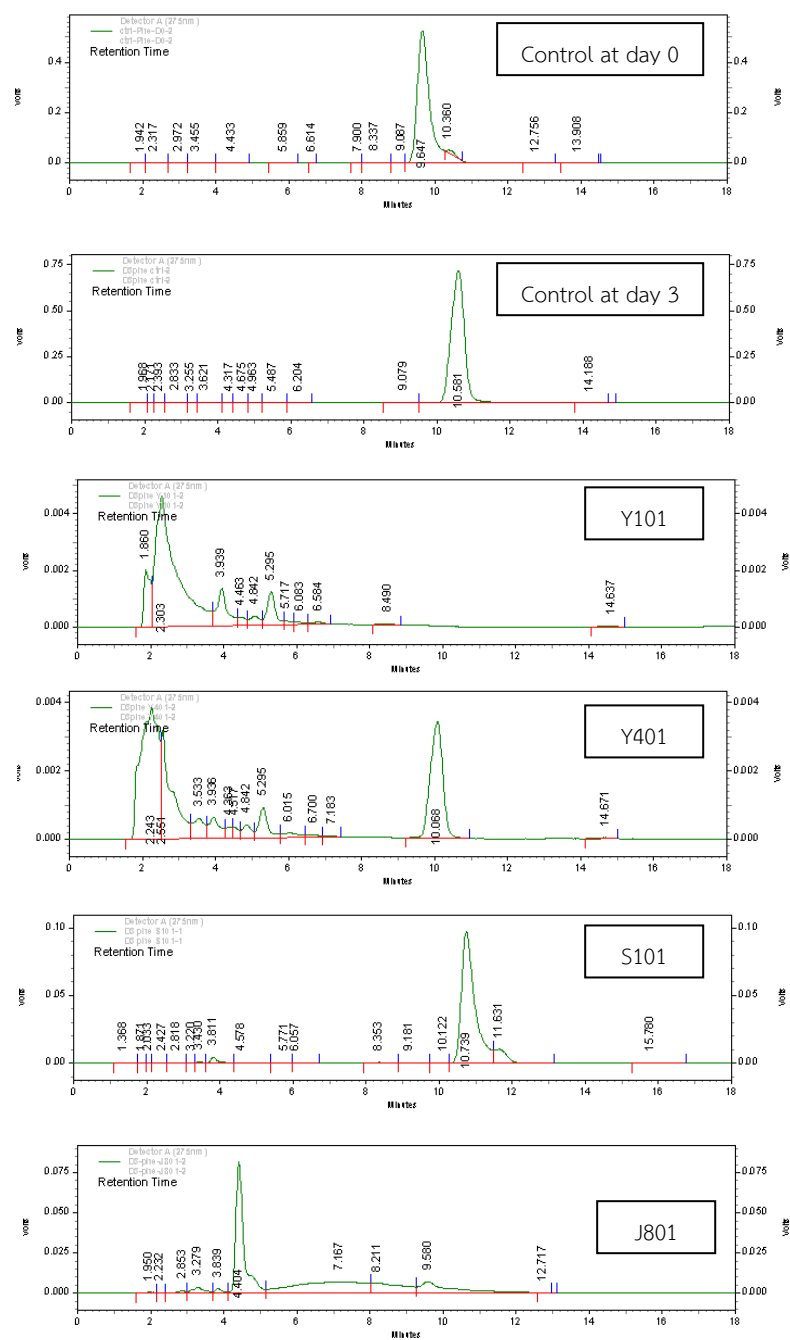
Figure C.9 Chromatograph of phenanthrene degradation by strain Y101, Y401, S101, and J801 at day 3.
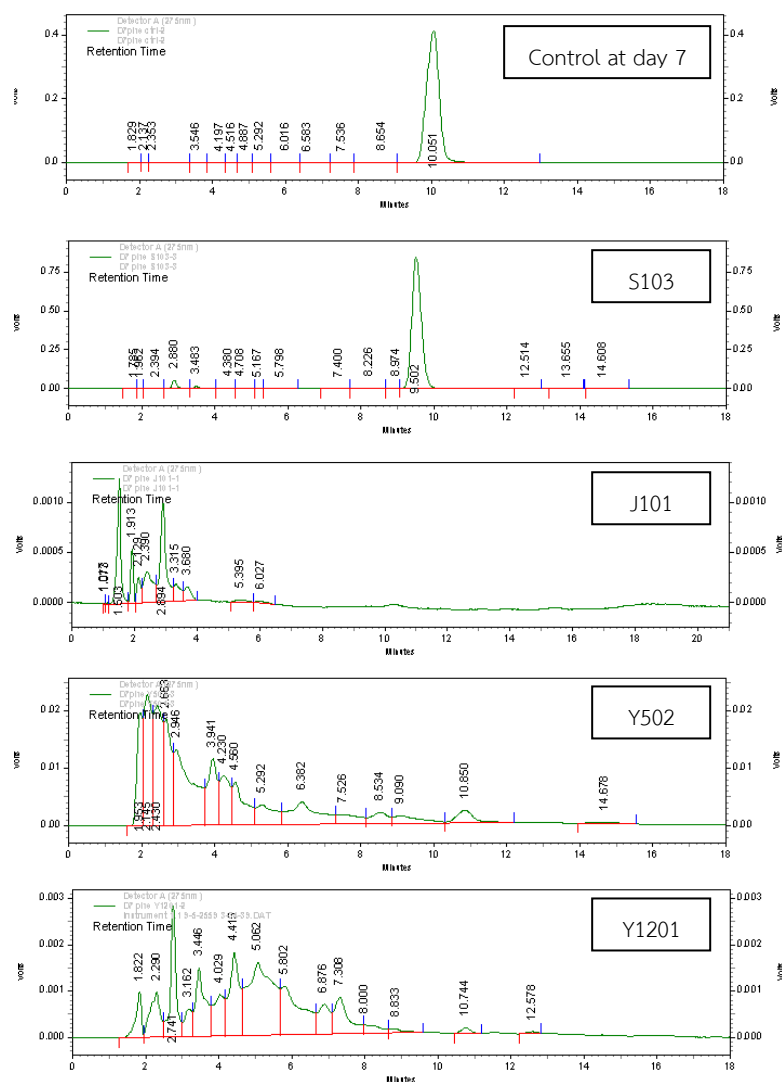
Figure C.10 Chromatograph of phenanthrene degradation by S103, J101, Y502, and Y1201 at day 7.
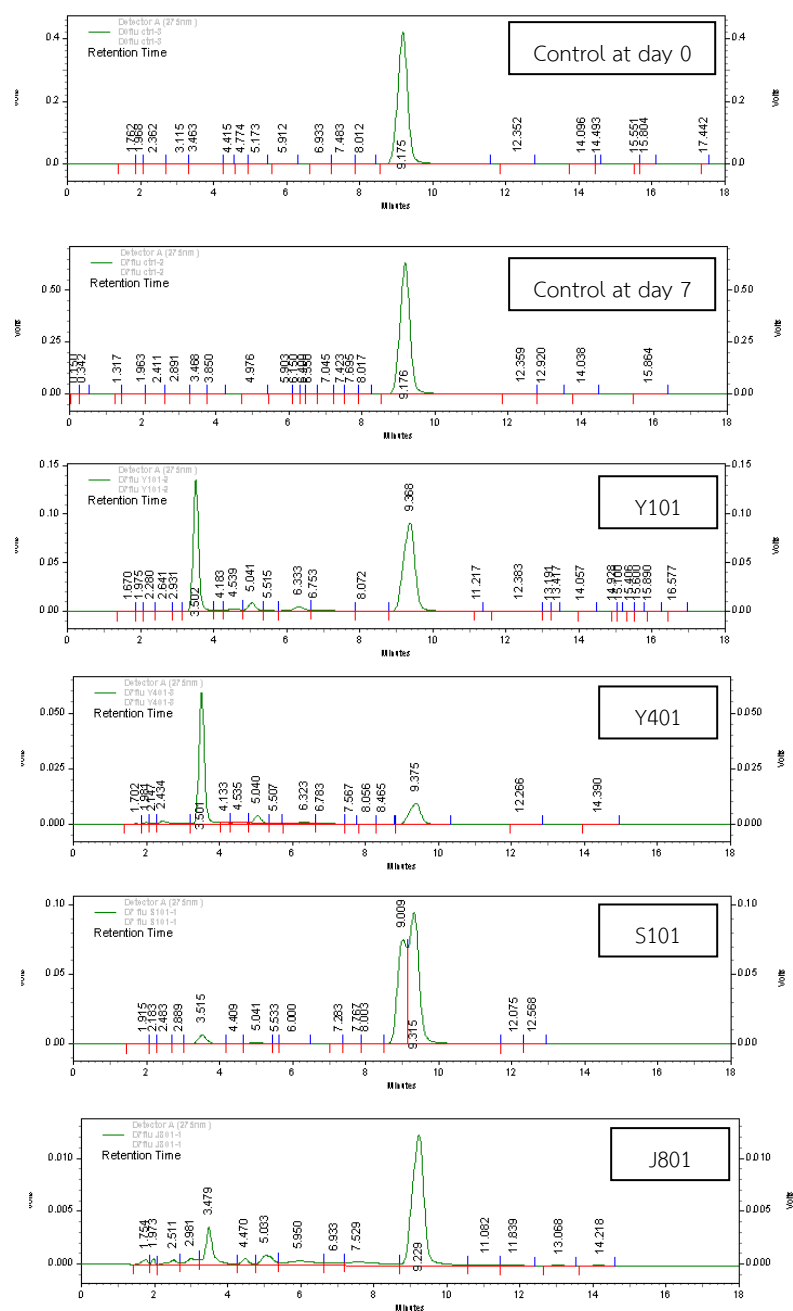
Figure C.11 Chromatograph of fluorene degradation by Y101, Y401, S101, and J801 at day 7.
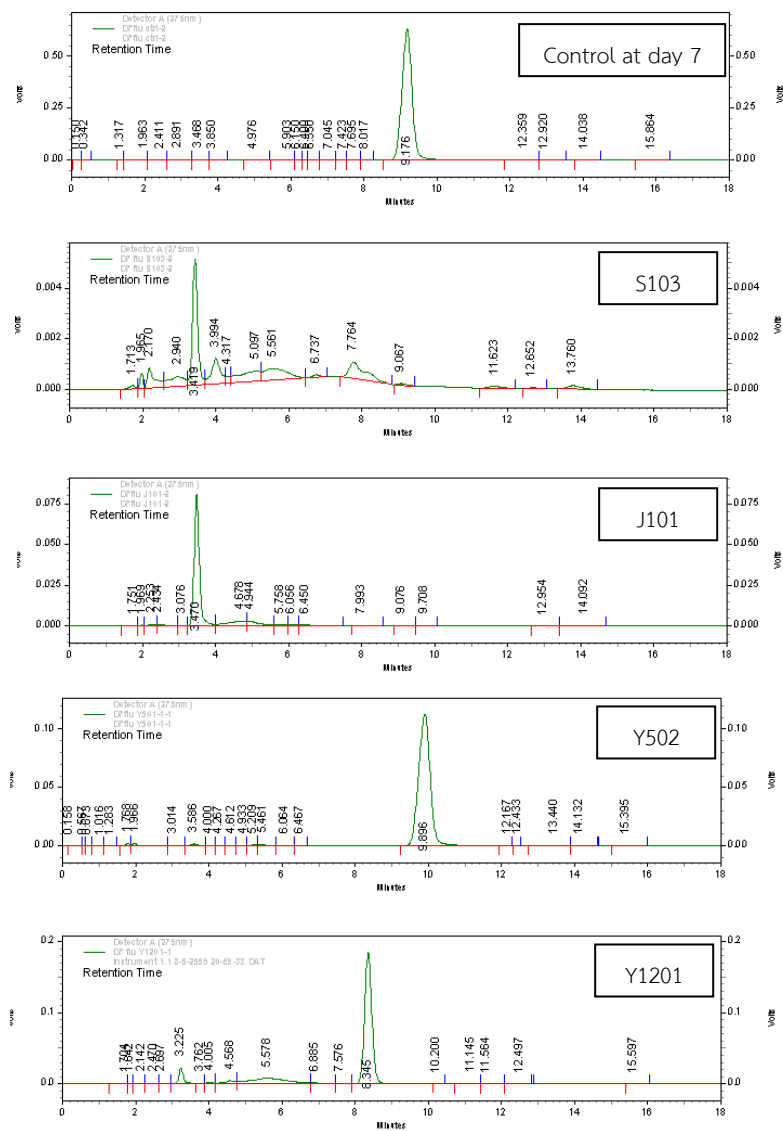
Figure C.12 Chromatograph of fluorene degradation by S103, J101, Y502, and Y1201 at day 7.
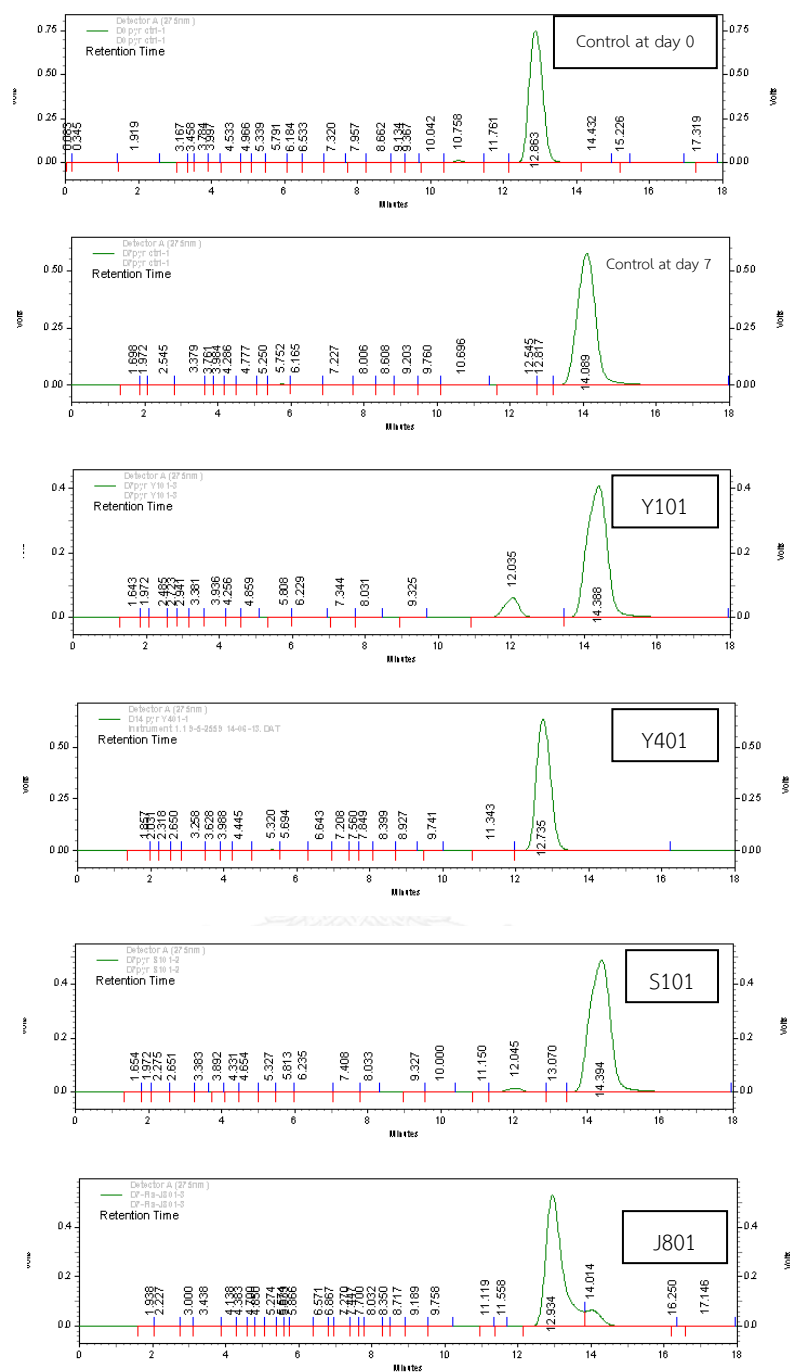
Figure C.13 Chromatograph of pyrene degradation by Y101, Y401, S101, and J801 at day 7.
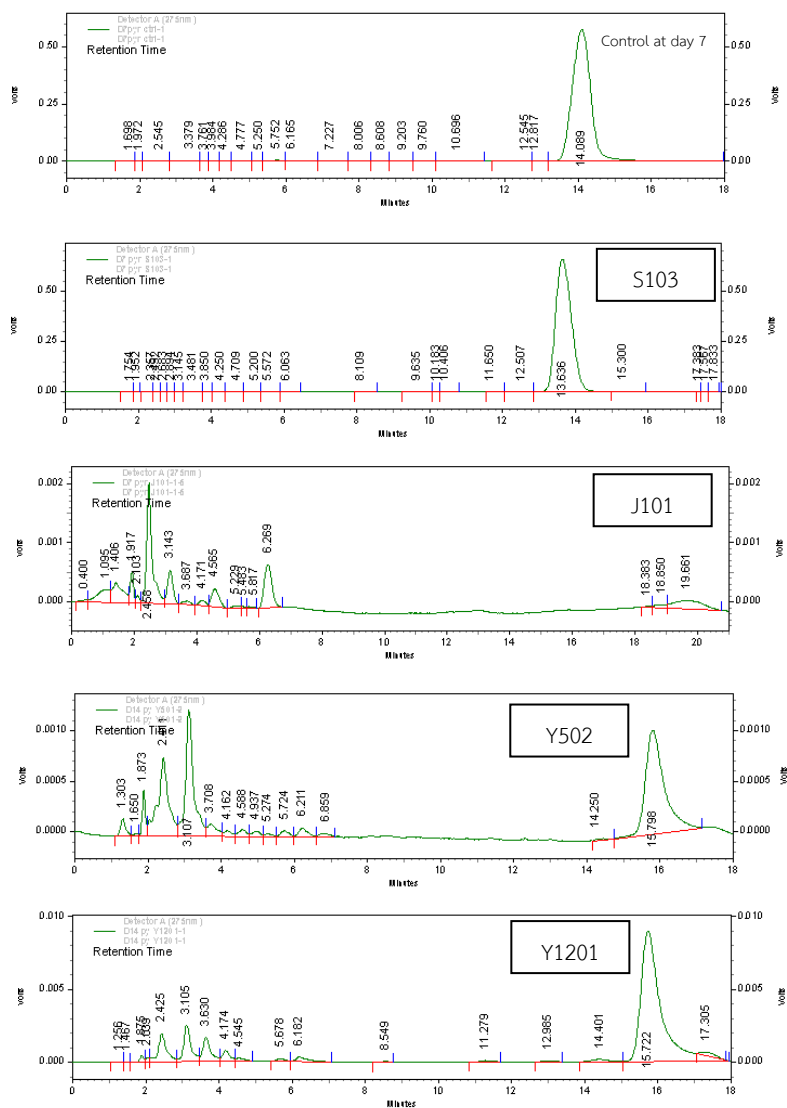
Figure C.14 Chromatograph of pyrene degradation by S103, J101, Y502, and Y1201 at day 7.
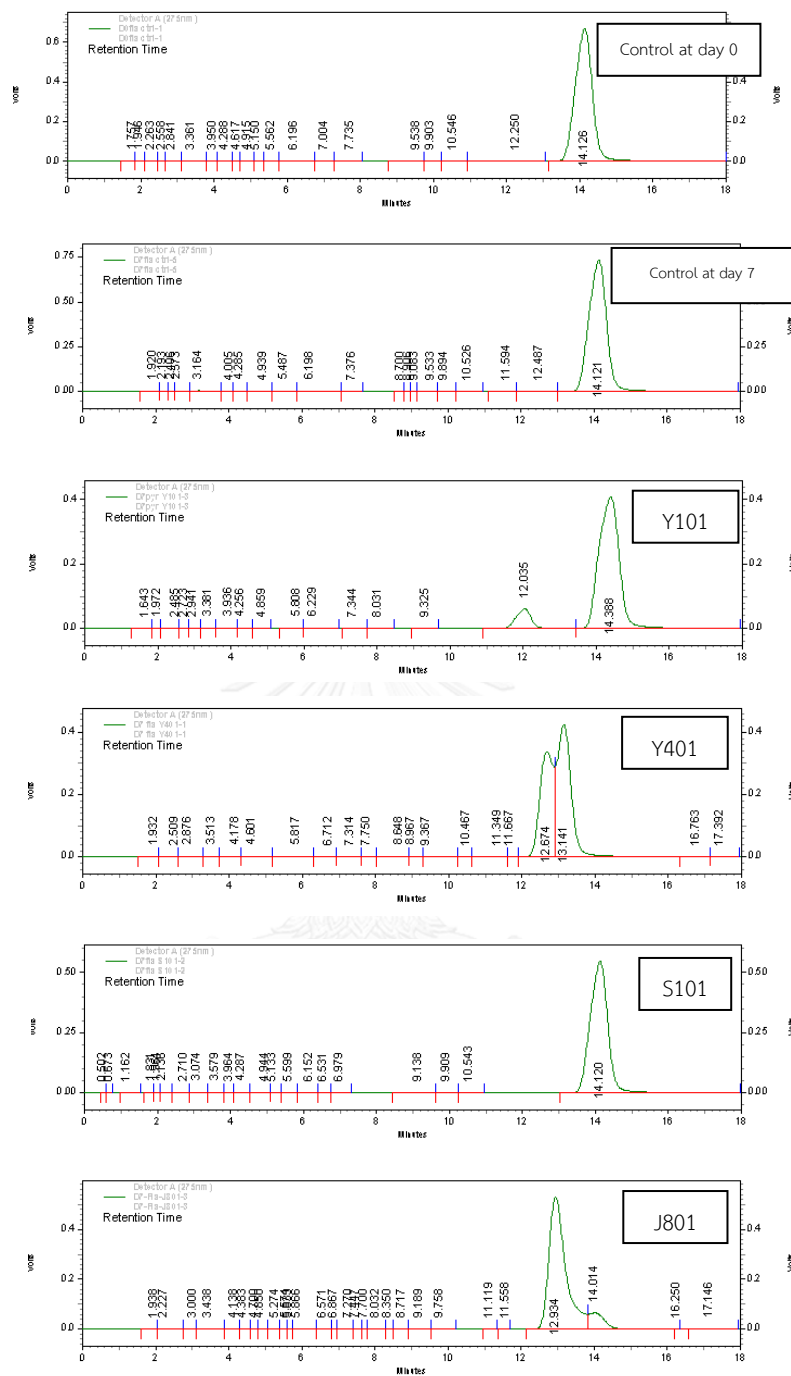
Figure C.15 Chromatograph of fluoranthene degradation by Y101, Y401, S101, and J801 at day 7.
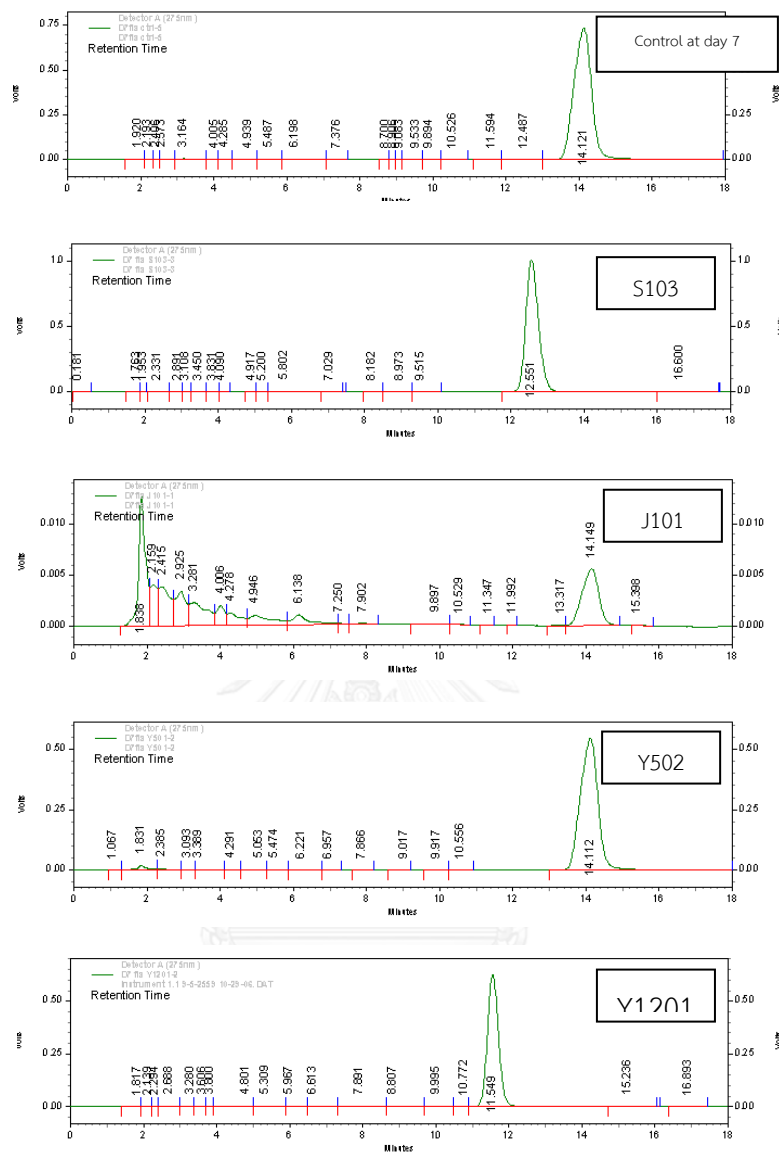
Figure C.16 Chromatograph of fluoranthene degradation by S103, J101, Y502, and Y1201 at day 7.

Table C 1 Deposition of microbial cultures at Microbial Culture Collection Department of Microbiology Faculty of Science, Chulalongkorn University and in the GenBank database

Bacteria	MSCU no.	GenBank accession no.
Sphingobium sp. Y101	MSCU 0799	MF281995
Sphingobium sp. Y401	MSCU 0800	MF281996
Arthrobacter sp. S101	MSCU 0801	KR869686
Pseudomonas sp. J801	MSCU 0802	MF281997
Rhodococcus ruber S103	MSCU 0934	KX375409
Mycobacterium sp. J101	MSCU 0806	MF281998
Mycobacterium sp. Y502	MSCU 0935	MF281999
Mycobacterium sp. Y1201	MSCU 0805	MF282000

Table C.1 PAH biodegradation efficacy of eight strains

Strains	Substrates (100 mg L^{-1})	% PAH degradation			average	Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3		
Y101	Phenanthrene	100.00	100.00	100.00	100.00±0.00	3
Y401	Phenanthrene	99.94	99.29	99.90	99.62±0.46	3
S101	Phenanthrene	81.19	99.99	73.82	90.5913.30	3
J801	Phenanthrene	98.12	96.88	100	98.331.57	3
S103	Fluorene	100.00	100.00	99.97	99.990.02	7

Table C.2 PAH biodegradation efficacy of eight strains (Continued)

Strains	Substrates (100 mg L^{-1})	% PAH degradation				Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3	average	
J101	Pyrene	99.10	99.46	99.20	99.25±0.19	3
Y502	Pyrene	95.36	97.12	99.92	97.46±2.30	7
Y1201	Pyrene	98.39	99.05	95.90	97.78±1.66	14

Table C.3 Substrate specificity of *Sphingobium* sp. Y101

Strains	Substrates (100 mg L^{-1})	% PAH degradation				Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3	average	
Y101	Phenanthrene	100.00	100.00	100.00	100.00±0.00	3
	Fluorene	87.18	77.68	86.13	83.66±5.21	14
	Pyrene	40.27	34.64	*	37.45±7.28	14
	Fluoranthene	19.53	25.80	29.81	25.05±5.18	14
	Tetradecane	*	82.96	88.13	85.48±3.66	7
	Docosane	*	51.99	51.37	51.68±0.44	7

Note: * The high error analysis was not calculated.

Table C.4 Substrate specificity of *Sphingobium* sp. Y401

Strains	Substrates (100 mg L^{-1})	% PAH degradation				Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3	average	
Y401	Phenanthrene	99.94	99.29	99.91	99.62±0.46	3
	Fluorene	99.40	99.21	97.70	98.77±0.93	14
	Pyrene	18.52	19.61	*	19.07±0.77	14
	Fluoranthene	18.43	19.81	19.74	19.33±0.78	14
	Tetradecane	100.00	100.00	89.76	94.88±7.24	7
	Docosane	26.74	19.09	21.47	22.44±3.91	7

Note: * The high error analysis was not calculated.

Table C.5 Substrate specificity of *Arthrobacter* sp. S101

Strains	Substrates (100 mg L^{-1})	% PAH degradation			average	Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3		
S101	Phenanthrene	81.19	99.99	73.82	90.59±13.30	3
	Fluorene	5.08	*	0.00	2.54±3.60	14
	Pyrene	2.20	9.53	*	5.87±5.14	14
	Fluoranthene	34.11	34.62	53.91	40.88±11.29	14
	Tetradecane	100.00	100.00	100.00	100.00±0.00	14
	Docosane	98.56	100.00	96.04	98.20±2.00	14

Note: * The high error analysis was not calculated.

Table C.6 Substrate specificity of *Pseudomonas* sp. J801

| Strains | Substrates (100 mg L^{-1}) | % PAH degradation | | | | Incubation time (days) |
		Triplicate 1	Triplicate 2	Triplicate 3	average	
J801	Phenanthrene	98.12	96.88	100	98.33±1.57	3
	Fluorene	99.89	97.97	99.49	99.12±1.02	14
	Pyrene	0.00	0.00	0.00	0.00±0.00	14
	Fluoranthene	0.00	0.00	0.00	0.00±0.00	14
	Tetradecane	54.59	64.60	0.00	59.59±7.07	14
	Docosane	32.00	41.07	25.00	32.69±8.06	14

Table C.7 *Substrate specificity of Rhodococcus ruber S103*

Strains	Substrates (100 mg L^{-1})	% PAH degradation				Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3	average	
S103	Phenanthrene	21.94	24.25	*	23.09±1.64	14
	Fluorene	100.00	100.00	99.97	99.99±0.02	7
	Pyrene	3.78	1.53	*	2.06±1.52	14
	Fluoranthene	30.55	23.88	26.35	26.93±3.37	14
	Tetradecane	54.59	64.60	0.00	100.00±0.00	14
	Docosane	32.00	41.07	25.00	95.03±1.24	14

Note: * The high error analysis was not calculated.

Table C.8 Substrate specificity of *Mycobacterium* sp. J101

Strains	Substrates (100 mg L^{-1})	% PAH degradation				Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3	average	
J101	Phenanthrene	100.00	100.00	100.00	100.00±0.00	3
	Fluorene	99.94	100.00	99.99	99.98±0.03	7
	Pyrene	99.10	99.46	99.20	99.25±0.19	3
	Fluoranthene	99.27	99.78	99.84	99.63±0.32	7
	Tetradecane	100.00	100.00	100.00	100.00±0.00	7
	Docosane	100.00	100.00	100.00	100.00±0.00	7

Table C.9 Substrate specificity of *Mycobacterium* sp. Y502

Strains	Substrates (100 mg L^{-1})	% PAH degradation				Incubation time (days)
		Triplicate 1	Triplicate 2	Triplicate 3	average	
Y502	Phenanthrene	100.00	100.00	100.00	100.00±0.00	14
	Fluorene	72.76	75.22	*	73.99±1.74	14
	Pyrene	95.36	97.12	99.92	97.46±2.30	7
	Fluoranthene	36.36	53.97	33.35	41.22±11.14	14
	Tetradecane	100.00	100.00	96.03	98.01±2.81	7
	Docosane	*	95.15	95.90	95.03±1.24	7

Note: * The high error analysis was not calculated.

Table C.10 *Substrate specificity of Mycobacterium sp. Y1201*

| Strains | Substrates (100 mg L^{-1}) | % PAH degradation | | | | Incubation time (days) |
		Triplicate 1	Triplicate 2	Triplicate 3	average	
Y1201	Phenanthrene	99.98	100.00	100.00	99.99±0.01	14
	Fluorene	96.30	84.44	74.86	85.20±10.74	14
	Pyrene	98.39	99.05	95.90	34.12±2.23	14
	Fluoranthene	*	42.04	39.46	40.75±1.82	14
	Tetradecane	100.00	100.00	100.00	100.00±0.00	7
	Docosane	67.60	68.04	74.30	69.98±3.75	7

Note: * The high error analysis was not calculated.

Table C.11 Biosurfactant activities and hydrophobicity of bacterial cell of *Sphingobium* sp. Y101 and Y401 and *Arthrobacter* sp. S101

Strains	Methods	Triplicates			Average
		1	2	3	
Y101	% Oil displacement	5.81	4.65	4.65	5.04±0.67
	Surface tension (mN/m)	39.9	40.3	42.81	41.00±1.29
	% Hydrophobicity	80.72	82.59	85.26	82.86±2.29
Y401	% Oil displacement	5.81	8.14	11.05	8.33±2.62
	Surface tension (mN/m)	31.47	33.00	32.24	32.24±0.62
	% Hydrophobicity	86.55	88.13	87.89	87.52±0.85
S101	% Oil displacement	4.65	3.49	3.49	3.88±0.67
	Surface tension (mN/m)	47.58	50.79	47.38	48.58±1.56
	% Hydrophobicity	33.92	33.68	27.93	31.84±3.39

Control: Surface tension value of CFMM broth is 54.94±2.98.

Table C.12 Biosurfactant activities and hydrophobicity of bacterial cell of *Psudomonas* sp. J801, *Rhodococcus ruber* S103, and *Mycobacterium* sp. J101

Strains	Methods	Triplicates			Average
		1	2	3	
J801	% Oil displacement	5.81	5.81	5.81	5.81±0.00
	Surface tension (mN/m)	38.56	35.64	35.46	36.55±1.74
	% Hydrophobicity	0.00	0.00	0.00	0.00±0.00
S103	% Oil displacement	19.78	16.28	12.79	16.28±3.49
	Surface tension (mN/m)	44.59	39.18	34.11	39.23±4.28
	% Hydrophobicity	97.53	98.38	98.23	98.05±0.45
J101	% Oil displacement	2.33	3.49	*	2.91±0.82
	Surface tension (mN/m)	36.83	35.84	35.74	36.14±0.49
	% Hydrophobicity	75.00	75.98	76.45	75.81±0.74

Control: Surface tension value of CFMM broth is 54.94±2.98.

Note: * The high error analysis was not calculated.

Table C.13 Biosurfactant activities and hydrophobicity of bacterial cell of *Mycobacterium* sp. Y502 and Y1201

Strains	Methods	Triplicates			Average
		1	2	3	
Y502	% Oil displacement	16.28	15.28	16.28	15.89±0.67
	Surface tension (mN/m)	35.10	32.81	33.66	33.86±0.95
	% Hydrophobicity	73.14	64.46	76.46	71.35±6.20
Y1201	% Oil displacement	23.84	19.87	18.02	20.35±3.08
	Surface tension (mN/m)	30.69	30.96	30.75	30.80±0.12
	% Hydrophobicity	74.65	74.36	74.39	74.65±0.28

Control: Surface tension value of CFMM broth is 54.94±2.98.

Table C.14 The petroleum oil biodegradation by three efficient bacteria after 7 days of incubation time. The concentration of each petroleum oil type is 2,000 mg L^{-1}

Bacteria	Petroleum oil	%Degradation			Average
		Triplicate 1	Triplicate 2	Triplicate 3	
Rhodococcus ruber S103	Fuel oil	20.88	18.15	*	19.52±1.93
	Crude oil	91.91	88.75	89.90	90.19±1.60
	Diesel	91.66	86.60	80.01	86.09±5.85
Mycobacterium sp. J101	Fuel oil	17.31	*	28.78	23.05±8.11
	Crude oil	91.27	88.75	89.90	89.97±1.26
	Diesel	91.45	86.50	79.97	85.99±5.78
Mycobacterium sp. Y502	Fuel oil	11.84	*	11.22	11.53±0.44
	Crude oil	36.66	38.51	39.09	37.58±1.31
	Diesel	*	33.01	25.80	29.40±5.10

Note: * The high error analysis was not calculated.

Table C.15 Biodegradation efficiency of fuel oil by single strains and bacterial consortia

Bacteria	Day 3				Day 7			
	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
C1	31.06	30.48	26.07	29.20±0.41	36.35	30.29	27.99	31.54±4.28
C2	28.12	26.05	29.08	27.75±1.55	28.36	27.23	30.12	28.57±1.45
C3	11.55	*	9.45	10.50±1.49	10.14	20.97	23.13	18.08±7.66
C4	32.04	28.52	36.03	32.20±3.76	44.78	40.56	36.98	40.77±3.91
Rhodococcus ruber S103	6.25	*	5.87	6.06±0.27	20.88	18.15	*	19.52±1.93
Mycobacterium sp. J101	14.07	16.71	22.54	17.77±4.33	17.31	*	28.22	23.05±8.11
Mycobacterium sp. Y502	*	11.28	7.14	9.21±2.93	11.84	*	11.22	11.53±0.44

Note: * The high error analysis was not calculated.

Table C.16 Bacterial growth of bacterial consortia in CFMM broth supplemented with 2,000 mg L^{-1} of fuel oil.

Bacteria		Day 0 % Degradation				Day 3 % Degradation				Day 7 % Degradation			
		Triplicate 1	Triplicate 2	Triplicate 3	Average	Triplicate 1	Triplicate 2	Triplicate 3	Average	Triplicate 1	Triplicate 2	Triplicate 3	Average
C1	S103	1.00×10^7	1.36×10^7	1.46×10^7	$1.27 \pm 0.24 \times 10^7$	1.54×10^7	2.00×10^7	1.91×10^7	$1.82 \pm 0.24 \times 10^7$	1.04×10^7	1.37×10^7	1.44×10^7	$1.28 \pm 0.21 \times 10^7$
	Y502	1.56×10^7	1.06×10^7	2.05×10^7	$1.56 \pm 0.49 \times 10^7$	3.00×10^7	4.00×10^7	4.20×10^7	$3.73 \pm 0.64 \times 10^7$	5.50×10^7	4.50×10^7	6.00×10^7	$5.33 \pm 0.76 \times 10^7$
C2	S103	1.35×10^7	1.28×10^7	1.23×10^7	$1.29 \pm 0.06 \times 10^7$	1.04×10^7	1.14×10^7	1.26×10^7	$1.15 \pm 0.11 \times 10^7$	3.00×10^7	3.80×10^7	3.70×10^7	$3.50 \pm 0.44 \times 10^7$
	J101	1.44×10^7	1.50×10^7	1.68×10^7	$1.54 \pm 0.12 \times 10^7$	1.27×10^7	1.43×10^7	1.63×10^7	$1.44 \pm 0.18 \times 10^7$	4.20×10^6	6.20×10^6	8.00×10^6	$6.13 \pm 1.90 \times 10^6$
C3	Y502	4.60×10^7	3.90×10^7	4.30×10^7	$4.33 \pm 0.45 \times 10^7$	3.60×10^7	4.80×10^7	4.80×10^7	$4.40 \pm 0.69 \times 10^7$	*	3.30×10^7	3.00×10^7	$3.15 \pm 0.24 \times 10^7$
	J101	1.55×10^7	1.91×10^7	2.00×10^7	$1.83 \pm 0.22 \times 10^7$	7.10×10^6	4.00×10^6	3.50×10^6	$4.87 \pm 1.95 \times 10^6$	3.30×10^6	4.40×10^6	4.10×10^6	$3.93 \pm 0.57 \times 10^6$
C4	S103	1.22×10^7	1.27×10^7	9.10×10^6	$1.13 \pm 0.20 \times 10^7$	7.00×10^6	7.50×10^6	5.10×10^6	$6.53 \pm 1.27 \times 10^6$	2.41×10^7	2.80×10^7	2.40×10^7	$2.57 \pm 0.20 \times 10^7$
	Y502	1.74×10^7	1.13×10^7	1.22×10^7	$1.36 \pm 0.33 \times 10^7$	3.20×10^6	6.50×10^6	5.20×10^6	$5.00 \pm 1.66 \times 10^6$	1.80×10^7	2.44×10^7	1.82×10^7	$2.02 \pm 0.36 \times 10^7$
	J101	1.22×10^7	1.27×10^7	1.18×10^7	$1.22 \pm 0.05 \times 10^7$	4.00×10^5	5.80×10^5	3.00×10^5	$4.27 \pm 1.42 \times 10^5$	2.60×10^6	2.71×10^6	2.79×10^6	$2.70 \pm 0.10 \times 10^6$

Note: * The high error analysis was not calculated.

Table C.17 Bacterial growth of single strains in CFMM broth supplemented with 2,000 mg L^{-1} of fuel oil.

Bacteria	Day 0				Day 3				Day 7			
	% Degradation			Average	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
S103	1.00×10^7	1.30×10^7	1.00×10^7	$1.10 \pm 0.17 \times 10^7$	1.30×10^7	1.30×10^7	1.20×10^7	$1.27 \pm 0.06 \times 10^7$	1.90×10^7	1.60×10^7	1.60×10^7	$1.70 \pm 0.17 \times 10^7$
J101	1.90×10^7	1.80×10^7	1.80×10^7	$1.83 \pm 0.06 \times 10^7$	6.00×10^7	6.00×10^7	4.00×10^7	$5.33 \pm 1.15 \times 10^7$	2.00×10^7	3.00×10^7	3.00×10^6	$3.00 \pm 0.58 \times 10^6$
Y502	1.70×10^7	1.70×10^7	2.54×10^7	$1.93 \pm 0.40 \times 10^7$	1.00×10^8	1.00×10^8	1.00×10^8	$1.00 \pm 0.00 \times 10^8$	2.00×10^7	5.00×10^7	8.00×10^7	$6.50 \pm 3.00 \times 10^7$

Table C.18 Biodegradation efficiency of diesel oil by single strains and bacterial consortia

Bacteria	Day 3				Day 7			
	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
C1	100.00	100.00	92.23	97.49±4.49	100.00	100.00	100.00	100.00±0.00
C2	100.00	99.10	99.08	99.39±0.05	100.00	100.00	100.00	100.00±0.00
C3	53.36	66.43	67.69	62.50±7.93	100.00	100.00	100.00	100.00±0.00
C4	100.00	100.00	100.00	100.00±0.00	100.00	100.00	100.00	100.00±0.00
Rhodococcus ruber S103	58.89	57.68	44.52	53.70±7.97	91.66	86.60	80.01	86.09±5.85
Mycobacterium sp. J101	13.79	11.82	20.32	15.31±4.62	91.45	86.50	79.97	85.99±5.78
Mycobacterium sp. Y502	No data	No data	No data	No data	*	33.01	25.80	29.40±5.10

Note: * The high error analysis was not calculated.

Table C.19 Bacterial growth of bacterial consortia in CFMM broth supplemented with 2,000 mg L^{-1} of diesel oil.

Bacteria		Day 0				Day 3				Day 7			
		% Degradation			Average	% Degradation			Average	% Degradation			Average
		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
C1	S103	8.00×10^6	7.50×10^6	6.20×10^6	$7.23\pm0.93\times10^6$	2.40×10^7	2.00×10^7	2.80×10^7	$2.40\pm0.40\times10^7$	3.00×10^8	3.50×10^8	3.60×10^8	$3.37\pm0.32\times10^8$
	Y502	3.70×10^6	4.30×10^6	4.00×10^6	$1.56\pm0.49\times10^6$	1.00×10^6	1.08×10^6	1.12×10^6	$1.07\pm0.06\times10^6$	8.70×10^8	7.60×10^8	8.40×10^8	$8.23\pm0.57\times10^8$
C2	S103	6.50×10^6	5.00×10^6	6.50×10^6	$1.29\pm0.06\times10^6$	*	8.10×10^6	9.00×10^6	$8.55\pm0.64\times10^6$	9.50×10^8	9.00×10^8	8.50×10^8	$9.00\pm0.50\times10^8$
	J101	1.90×10^6	1.55×10^6	1.85×10^6	$1.54\pm0.12\times10^6$	5.90×10^6	3.90×10^6	*	$4.90\pm1.41\times10^6$	3.70×10^8	3.40×10^8	3.60×10^8	$3.57\pm0.15\times10^8$
C3	Y502	5.20×10^6	4.80×10^6	4.60×10^6	$4.87\pm0.31\times10^6$	1.35×10^7	1.43×10^7	*	$1.38\pm0.07\times10^7$	7.50×10^8	9.20×10^8	8.40×10^8	$8.37\pm0.85\times10^8$
	J101	1.80×10^6	1.93×10^6	1.86×10^6	$1.86\pm0.07\times10^6$	5.20×10^6	4.20×10^6	*	$4.70\pm0.70\times10^6$	1.27×10^8	1.36×10^8	1.19×10^8	$1.27\pm0.09\times10^8$
C4	S103	6.00×10^6	5.50×10^6	5.70×10^6	$5.73\pm0.25\times10^6$	8.30×10^8	8.40×10^8	8.60×10^8	$8.43\pm0.15\times10^8$	7.30×10^8	8.00×10^8	7.80×10^8	$7.70\pm0.36\times10^8$
	Y502	5.30×10^6	5.00×10^6	4.60×10^6	$4.97\pm0.35\times10^6$	5.10×10^6	3.40×10^6	2.70×10^6	$3.73\pm1.23\times10^6$	3.50×10^8	4.60×10^8	3.90×10^8	$4.00\pm0.56\times10^8$
	J101	1.75×10^6	1.85×10^6	1.75×10^6	$1.79\pm0.06\times10^6$	8.30×10^6	8.60×10^6	*	$8.45\pm0.21\times10^6$	4.10×10^8	4.30×10^8	4.30×10^8	$4.23\pm0.11\times10^8$

Note: * The high error analysis was not calculated.

Table C.20 Bacterial growth of single strains in CFMM broth supplemented with 2,000 mg L^{-1} of diesel oil.

Bacteria	Day 0				Day 3				Day 7			
	% Degradation			Average	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
S103	5.00×10^6	7.00×10^6	9.00×10^6	$7.00\pm2.00\times10^6$	3.00×10^7	4.00×10^7	4.00×10^7	$3.67\pm0.58\times10^6$	3.00×10^6	5.00×10^6	6.00×10^6	$4.67\pm1.53\times10^6$
J101	9.00×10^6	9.00×10^6	9.00×10^6	$9.00\pm0.00\times10^6$	3.00×10^7	3.00×10^7	3.00×10^7	$3.00\pm0.00\times10^6$	5.00×10^4	6.00×10^4	*	$5.50\pm0.71\times10^4$
Y502	2.00×10^6	2.00×10^6	2.00×10^6	$2.00\pm0.00\times10^6$	No data	No data	No data	No data	1.20×10^6	1.30×10^6	*	$1.25\pm0.07\times10^6$

Note: *The high error analysis was not calculated.

Table C.21 Biodegradation efficiency of pyrene by single strains and bacterial consortia

Bacteria	Day 3				Day 7			
	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
C1	46.33	34.76	49.81	43.63±7.88	99.99	99.93	99.99	99.97±7.88
C2	99.70	98.20	99.40	99.13±0.75	99.99	100.00	99.98	99.99±0.01
C3	99.49	99.69	99.93	99.69±0.23	99.99	99.99	99.99	99.99±0.00
C4	99.61	99.40	99.79	99.60±0.28	99.99	99.99	99.99	99.98±0.00
Rhodococcus ruber S103	No data	No data	No data	No data	1.61	0.18	1.60	1.13±0.82
Mycobacterium sp. J101	99.10	99.46	99.20	99.25±0.19	99.98	99.91	99.83	99.90±0.07
Mycobacterium sp. Y502	No data	No data	No data	No data	95.36	97.12	99.92	97.46±2.30

Table C.22 Bacterial growth of bacterial consortia in CFMM broth supplemented with 100 mg L^{-1} of pyrene.

Bacteria		Day 0 % Degradation				Day 3 % Degradation				Day 7 % Degradation			
		Triplicate 1	Triplicate 2	Triplicate 3	Average	Triplicate 1	Triplicate 2	Triplicate 3	Average	Triplicate 1	Triplicate 2	Triplicate 3	Average
C1	S103	8.00×10^{6}	7.50×10^{6}	6.20×10^{6}	$7.23\pm0.93\times10^{6}$	1.05×10^{7}	1.11×10^{7}	1.48×10^{7}	$1.21\pm0.23\times10^{7}$	1.70×10^{7}	1.20×10^{7}	*	$1.45\pm0.31\times10^{6}$
	Y502	4.50×10^{6}	6.10×10^{6}	4.50×10^{6}	$1.56\pm0.49\times10^{6}$	4.50×10^{6}	6.10×10^{6}	4.50×10^{6}	$5.03\pm9.24\times10^{6}$	1.12×10^{7}	1.08×10^{7}	1.10×10^{7}	$1.10\pm0.02\times10^{7}$
C2	S103	6.50×10^{6}	5.00×10^{6}	6.30×10^{6}	$1.29\pm0.06\times10^{6}$	4.80×10^{7}	3.00×10^{7}	*	$3.90\pm0.81\times10^{7}$	4.00×10^{6}	3.70×10^{6}	3.20×10^{6}	$3.63\pm0.40\times10^{6}$
	J101	1.90×10^{6}	1.58×10^{6}	1.85×10^{6}	$1.54\pm0.12\times10^{6}$	7.80×10^{7}	7.50×10^{7}	*	$7.65\pm0.17\times10^{7}$	4.40×10^{6}	3.60×10^{6}	6.40×10^{6}	$4.80\pm1.44\times10^{6}$
C3	Y502	5.20×10^{6}	4.80×10^{6}	4.60×10^{6}	$4.87\pm0.31\times10^{6}$	4.20×10^{6}	5.60×10^{6}	4.90×10^{6}	$4.87\pm0.31\times10^{6}$	9.20×10^{3}	7.50×10^{5}	7.40×10^{5}	$8.03\pm1.01\times10^{5}$
	J101	1.80×10^{6}	1.93×10^{6}	1.86×10^{6}	$1.86\pm0.07\times10^{6}$	1.35×10^{7}	1.29×10^{7}	1.27×10^{7}	$1.30\pm0.07\times10^{7}$	3.10×10^{6}	3.00×10^{6}	1.28×10^{6}	$2.46\pm1.02\times10^{6}$
C4	S103	6.00×10^{6}	5.50×10^{6}	5.70×10^{6}	$5.73\pm0.25\times10^{6}$	8.40×10^{6}	1.07×10^{7}	8.60×10^{6}	$8.43\pm0.15\times10^{6}$	7.70×10^{5}	7.40×10^{5}	7.60×10^{5}	$7.57\pm0.15\times10^{5}$
	Y502	5.30×10^{6}	5.00×10^{6}	4.60×10^{6}	$4.97\pm0.35\times10^{6}$	1.23×10^{6}	1.24×10^{6}	1.30×10^{6}	$3.73\pm1.23\times10^{6}$	1.74×10^{5}	1.74×10^{5}	1.37×10^{5}	$1.62\pm0.21\times10^{5}$
	J101	1.78×10^{6}	1.85×10^{6}	1.73×10^{6}	$1.79\pm0.06\times10^{6}$	8.60×10^{6}	8.40×10^{6}	8.00×10^{6}	$8.45\pm0.21\times10^{6}$	5.20×10^{6}	5.20×10^{6}	4.90×10^{6}	$5.10\pm0.17\times10^{6}$

Note: * The high error analysis was not calculated.

Table C.23 Bacterial growth of single strains in CFMM broth supplemented with 100 mg L^{-1} of pyrene.

Bacteria	Day 0				Day 3				Day 7			
	% Degradation			Average	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
S103	5.00×10^6	7.00×10^6	9.00×10^6	$7.00 \pm 2.00 \times 10^6$	No data	No data	No data	No data	2.30×10^7	1.80×10^7	1.50×10^7	$1.87 \pm 0.40 \times 10^7$
J101	9.00×10^6	9.00×10^6	9.00×10^6	$9.00 \pm 0.00 \times 10^6$	1.90×10^6	1.70×10^6	*	$1.80 \pm 0.14 \times 10^6$	2.40×10^7	1.50×10^7	2.10×10^7	$2.00 \pm 0.45 \times 10^7$
Y502	2.00×10^6	2.00×10^6	2.00×10^6	$2.00 \pm 0.00 \times 10^6$	No data	No data	No data	No data	2.30×10^7	2.60×10^7	2.70×10^7	$2.53 \pm 0.21 \times 10^7$

Note: * The high error analysis was not calculated.

Table C.24 Biodegradation efficiency of docosane by single strains and bacterial consortia

Bacteria	Day 3				Day 7			
	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
C1	73.27	82.35	*	77.81±6.42	94.68	89.70	87.32	90.57±3.76
C2	87.27	77.02	84.53	82.94±5.31	95.05	98.87	100	97.65±2.46
C3	72.05	*	82.76	77.40±7.57	91.09	97.82	98.80	95.90±4.20
C4	*	55.95	63.86	59.90±5.60	97.78	97.64	*	97.71±0.10
Rhodococcus ruber S103	67.60	68.40	74.30	69.98±3.75	100.00	100.00	100.00	100.00±0.00
Mycobacterium sp. J101	37.50	64.73	*	51.11±19.26	100.00	100.00	100.00	100.00±0.00
Mycobacterium sp. Y502	No data	No data	No data	No data	97.35	94.15	95.50	95.90±1.24

184

Table C.25 Bacterial growth of bacterial consortia in CFMM broth supplemented with 100 mg L^{-1} of docosane.

Bacteria		Day 0				Day 3				Day 7			
		% Degradation			Average	% Degradation			Average	% Degradation			Average
		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
C1	S103	8.00×10^6	7.50×10^6	6.20×10^6	$7.23 \pm 0.93 \times 10^6$	1.65×10^7	1.93×10^7	5.40×10^7	$2.99 \pm 2.09 \times 10^7$	2.55×10^7	2.09×10^7	3.70×10^7	$2.78 \pm 0.83 \times 10^7$
	Y502	4.50×10^6	6.10×10^6	4.50×10^6	$1.56 \pm 0.49 \times 10^6$	3.10×10^6	2.90×10^6	3.40×10^6	$3.13 \pm 0.25 \times 10^6$	4.60×10^6	6.10×10^6	4.60×10^6	$5.10 \pm 0.87 \times 10^6$
C2	S103	6.50×10^6	5.00×10^6	6.30×10^6	$1.29 \pm 0.06 \times 10^6$	2.56×10^7	2.53×10^7	2.62×10^7	$2.57 \pm 0.05 \times 10^7$	2.83×10^7	3.51×10^7	8.00×10^7	$4.78 \pm 2.81 \times 10^7$
	J101	1.90×10^6	1.58×10^6	1.85×10^6	$1.54 \pm 0.12 \times 10^6$	1.08×10^6	1.11×10^6	9.30×10^5	$1.04 \pm 0.10 \times 10^6$	4.60×10^6	4.50×10^6	2.90×10^6	$4.00 \pm 0.95 \times 10^6$
C3	Y502	5.20×10^6	4.80×10^6	4.60×10^6	$4.87 \pm 0.31 \times 10^6$	5.70×10^6	5.00×10^6	2.50×10^6	$4.40 \pm 1.68 \times 10^6$	4.80×10^6	3.60×10^6	*	$4.20 \pm 0.85 \times 10^6$
	J101	1.80×10^6	1.93×10^6	1.86×10^6	$1.86 \pm 0.07 \times 10^6$	5.40×10^5	7.70×10^5	4.30×10^5	$5.80 \pm 1.73 \times 10^5$	1.09×10^7	1.05×10^7	*	$1.07 \pm 0.03 \times 10^7$
C4	S103	6.00×10^6	5.50×10^6	5.70×10^6	$5.73 \pm 0.25 \times 10^6$	4.10×10^5	4.00×10^5	3.00×10^5	$3.70 \pm 0.61 \times 10^5$	1.18×10^7	1.69×10^7	2.17×10^7	$1.68 \pm 0.50 \times 10^7$
	Y502	5.30×10^6	5.00×10^6	4.60×10^6	$4.97 \pm 0.35 \times 10^6$	1.20×10^4	1.28×10^4	1.25×10^4	$1.24 \pm 0.04 \times 10^4$	1.46×10^6	1.35×10^6	*	$1.41 \pm 0.08 \times 10^6$
	J101	1.78×10^6	1.85×10^6	1.73×10^6	$1.79 \pm 0.06 \times 10^6$	4.00×10^6	4.50×10^6	4.60×10^6	$4.37 \pm 0.32 \times 10^6$	5.10×10^6	3.90×10^6	3.60×10^6	$4.20 \pm 0.79 \times 10^6$

Note: * The high error analysis was not calculated.

Table C.26 Bacterial growth of single strains in CFMM broth supplemented with 100 mg L^{-1} of docosane.

Bacteria	Day 0				Day 3				Day 7			
	% Degradation			Average	% Degradation			Average	% Degradation			Average
	Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3		Triplicate 1	Triplicate 2	Triplicate 3	
S103	5.00x10^6	7.00x10^6	9.00x10^6	7.00±2.00x10^6	5.00x10^6	7.00x10^6	7.00x10^6	7.00±1.00x10^6	6.00x10^6	7.00x10^6	8.00x10^6	7.00±1.00x10^6
J101	9.00x10^6	9.00x10^6	9.00x10^6	9.00±0.00x10^6	9.00x10^3	8.00x10^3	*	8.50±0.71x10^3	3.00x10^6	4.00x10^6	5.00x10^6	4.00±1.00x10^6
Y502	2.00x10^6	2.00x10^6	2.00x10^6	2.00±0.00x10^6	No data	No data	No data	No data	9.00x10^6	7.00x10^6	8.00x10^6	8.00±1.00x10^6

Note: * The high error analysis was not calculated.

Table C.27 Bacterial counted per gram of dried plastic pellets. *Rhodococcus ruber* S103 was immobilized with an initial cell concentration of 10^7 CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

Triplicates	Log CFU g^{-1} bio-balls									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	6.75	6.95	6.88	7.07	7.28	7.44	*	*	7.36	7.39
2	6.88	6.99	7.13	7.27	*	7.42	7.25	7.54	*	7.43
3	6.93	*	*	7.34	7.34	*	7.29	7.06	7.42	*
Average	6.85±0.09	6.97±0.03	7.01±0.18	7.23±0.14	7.31±0.04	7.43±0.02	7.27±0.02	7.30±0.34	7.39±0.05	7.41±0.03

Note: * The high error analysis was not calculated.

Table C.28 Bacterial number in CFMM broth. *Rhodococcus ruber* S103 was immobilized with an initial cell concentration of 10^7 CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

Triplicates	Log CFU mL^{-1}									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	7.48	7.28	*	*	7.64	7.82	7.67	*	7.80	*
2	*	7.48	7.90	7.55	*	7.70	7.70	7.73	*	7.52
3	7.52	*	7.64	7.09	7.56	*	*	7.56	7.90	7.73
Average	7.50±0.03	7.38±0.14	7.77±0.19	7.27±0.25	7.60±0.05	7.76±0.09	7.78±0.02	7.65±0.12	7.85±0.07	7.41±0.03

Note: * The high error analysis was not calculated.

Table C.29 Bacterial counted per gram of dried plastic pellets. *Mycobacterium* sp. J101 was immobilized with an initial cell concentration of 10^7 CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

Triplicates	Log CFU g^{-1} bio-balls									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	*	*	6.74	7.10	*	7.23	*	7.47	*	7.16
2	6.70	6.51	*	7.40	6.89	7.19	6.92	*	7.61	7.08
3	6.55	6.76	7.18	*	7.54	*	7.27	7.41	7.10	*
Average	6.62±0.11	6.64±0.18	7.01±0.18	7.22±0.46	7.31±0.04	7.21±0.03	7.10±0.25	7.44±0.04	7.36±0.36	7.12±0.05

Note: * The high error analysis was not calculated.

Table C.30 Bacterial number in CFMM broth. *Mycobacterium* sp. J101 was immobilized with an initial cell concentration of 10^7 CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

Triplicates	Log CFU mL^{-1}									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	*	*	6.64	6.78	*	6.26	*	6.08	*	6.23
2	7.78	6.67	*	6.78	6.09	6.23	6.29	*	6.73	6.97
3	7.85	6.64	6.67	*	6.10	*	7.01	6.99	6.85	*
Average	7.50±0.03	6.65±0.02	7.65±0.02	6.78±0.00	6.10±0.01	6.24±0.02	7.65±0.51	6.53±0.64	6.79±0.08	6.60±0.52

Note: * The high error analysis was not calculated.

Table C.31 Bacterial counted per gram of dried plastic pellets. *Mycobacterium* sp. Y502 was immobilized with an initial cell concentration of 10^7 CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

Triplicates	Log CFU g^{-1} bio-balls									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	6.95	7.08	6.69	*	7.61	*	7.96	7.88	7.79	*
2	6.82	*	6.92	7.90	7.61	7.90	7.92	*	*	7.32
3	*	6.53	*	7.67	*	7.84	*	7.93	7.54	7.62
Average	6.89±0.10	6.81±0.39	6.80±0.17	7.78±0.16	7.61±0.00	7.87±0.05	7.94±0.03	7.91±0.03	7.67±0.17	7.47±0.21

Note: * The high error analysis was not calculated.

Table C.32 Bacterial number in CFMM broth. *Mycobacterium* sp. Y502 was immobilized with an initial cell concentration of 10^7 CFU mL^{-1} in CFMM with 5 g of plastic pellets and 600 mg L^{-1} of fuel oil and followed by incubation on rotary shaker 120 rpm at room temperature for 10 days.

Triplicates	Log CFU mL^{-1}									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	7.16	7.27	7.23	*	7.11	*	7.64	7.67	7.56	*
2	7.05	*	7.20	7.10	7.12	7.67	7.60	*	*	7.00
3	*	7.19	*	7.19	*	7.60	*	7.60	7.48	7.04
Average	7.11±0.07	7.23±0.06	7.22±0.02	7.15±0.07	7.12±0.05	7.64±0.05	7.62±0.02	7.64±0.05	7.52±0.06	7.02±0.03

Note: * The high error analysis was not calculated.

Table C.33 Fuel oil concentration (mg L^{-1}) in CFMM broth by immobilized defined consortium.

Experiments	Day 0				Day 5				Day 7			
	Triplicates			Average	Triplicates			Average	Triplicates			Average
	1	2	3		1	2	3		1	2	3	
Control without inoculum	2,994	2,980	2,644	2,856±184	1,960	2,512	2,592	2,355±344	2,720	2,712	2,712	2,715±5
Control with sterilized bio-balls	1,370	1,406	1,414	1,397±23	522	654	666	614±80	458	534	550	514±49
Immobilized defined consortium	1,498	1,678	1,698	1625±110	582	736	772	697±101	688	668	692	683±13

Table C.34 The concentration of fuel oil remaining on bio-balls after 7 days of incubation time. The concentration of fuel oil is 2,000 mg L^{-1}.

Experiments	Day 0				Day 3				Day 7			
	Triplicates			Average	Triplicates			Average	Triplicates			Average
	1	2	3		1	2	3		1	2	3	
Control with sterilized bio-balls	684	736	743	721±32	1,268	1,392	1,419	1,360±80	1,207	1,425	1,359	1,330±112
Immobilized defined consortium	968	816	801	862±92	717	1,045	1,072	945±198	856	1,054	1,081	997±123

Table C.35 Biodegradation of fuel oil on bio-balls after 7 days of incubation time. The concentration of fuel oil is 2,000 mg L^{-1}.

Experiments	Day 0				Day 5				Day 7			
	Triplicates			Average	Triplicates			Average	Triplicates			Average
	1	2	3		1	2	3		1	2	3	
Immobilized defined consortium	0.00	0.00	0.00	0.00±0.00	43.43	24.90	24.44	24.67±0.33	29.06	26.01	20.41	25.16±3.96

Table C.36 Fuel oil concentration (mg L^{-1}) in fresh water by immobilized defined consortium.

Experiments	Day 0				Day 5				Day 10				Day 15			
	Triplicates			Average	Triplicates			Average	Triplicates			Average	Triplicates			Average
	1	2	3		1	2	3		1	2	3		1	2	3	
Control without inoculum	3,160	2,876	3,196	3,077±175	2,636	2,932	2,504	2,691±219	2,712	2,796	2,720	2,743±46	2,756	2,732	2,836	2,775±54
Control with sterilized bio-balls	1,266	1,484	1,588	1,436±152	504	710	866	693±182	672	766	798	745±65	638	642	650	643±6
Immobilized defined consortium	1,224	1,436	1,448	1,369±126	876	802	806	828±42	730	720	664	705±36	572	612	628	604±29
Natural attenuation	2,696	2,736	2,956	2,796±140	2,228	2,328	2,332	2,296±59	2,364	2,264	2,268	2,299±57	2,408	2,448	2,468	2,441±31

Table C.37 The concentration of fuel oil remaining (mg L^{-1}) on bio-balls after 7 days of incubation time (fresh water experiment).

The concentration of fuel oil is 2,000 mg L^{-1}

Experiments	Day 0				Day 5				Day 10				Day 15			
	Triplicates			Average	Triplicates			Average	Triplicates			Average	Triplicates			Average
	1	2	3		1	2	3		1	2	3		1	2	3	
Control with sterilized bio-balls	584	689	784	686±100	1,302	1,413	1,637	1,451±170	1,328	1,360	1,366	1,351±21	1,693	1,414	1,461	1,523±149
Immobilized defined consortium	591	513	681	595±84	1,129	1,411	1,171	1,237±152	1,073	1,113	943	1,043±89	815	805	828	816±11

Table C.38 Biodegradation of fuel oil on bio-balls after 15 days of incubation time. The concentration of fuel oil is 2,000 mg L^{-1}.

Experiments	Day 0				Day 5				Day 10				Day 15			
	Triplicates			Average	Triplicates			Average	Triplicates			Average	Triplicates			Average
	1	2	3		1	2	3		1	2	3		1	2	3	
Immobilized defined consortium	0.00	0.00	0.00	0.00±0.00	13.31	0.19	28.50	20.90±10.75	19.18	18.34	31.02	22.78±9.11	51.89	43.07	43.34	46.10±5.02

Appendix D

Gel electrophoresis

M P N 1 2 3

Primer: RHDα-GP-641F, RHDα-GP-933R

PCR product size: 292 bp

M: 100 bp marker

P: Positive control; *Mycobacterium* sp. PO2

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

292 bp
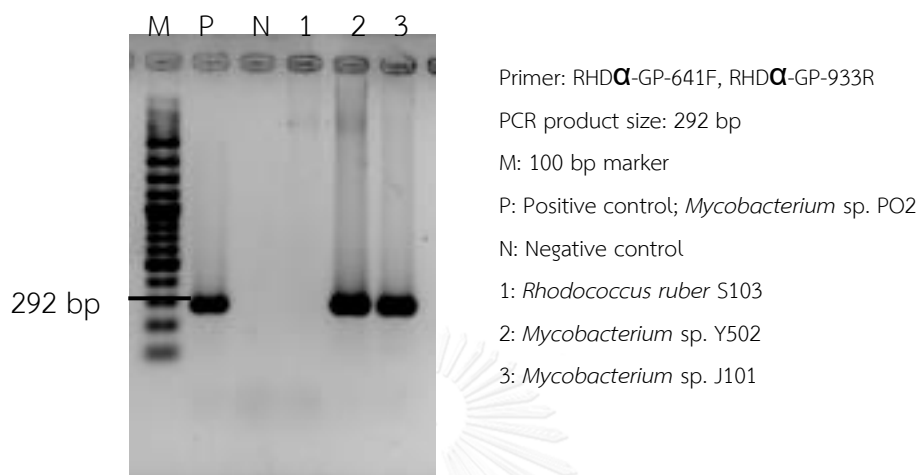
Figure D 1 PCR product by RHDα-GP primer of three isolate strains

M P N 1 2 3

Primer: RHDα-GN-610F, RHD α-GN-916R

PCR product size: 306 bp

M: 100 bp marker

P: Positive control; *Burkholderia* sp. FP2-1

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

306 bp

Figure D 2 PCR product by RHDα-GN primer of three isolate strains

Primer: DbfA1A2-F, DbfA1A2-R

PCR product size: 392 bp

M: 100 bp marker

P: Positive control; *Terrabacter* sp. DBF63

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

Figure D 3 PCR product by DbfA1A2-F, DbfA1A2-R primer of three isolate strains

Primer: NidA-F, NidA-R

PCR product size: 508 bp

M: 100 bp marker

P: Positive control; *Mycobacterium* sp. PO2

N: Negative control

1: *Rhodococcus ruber* S103

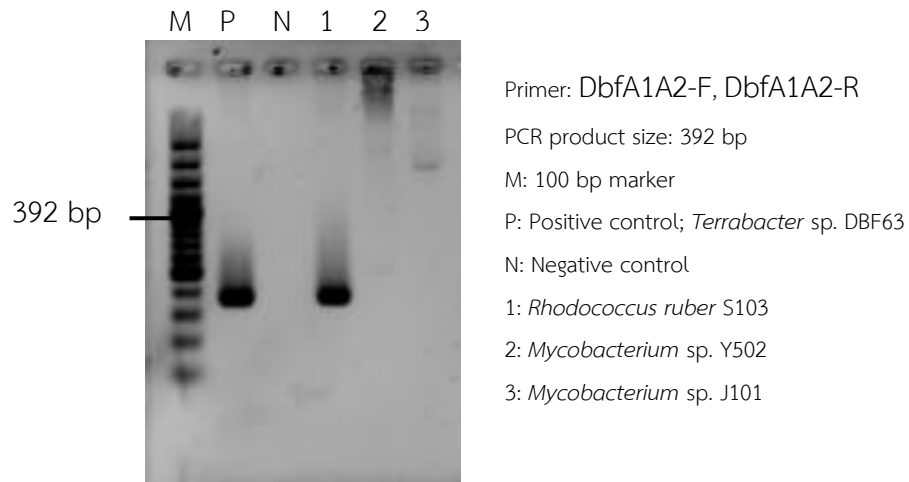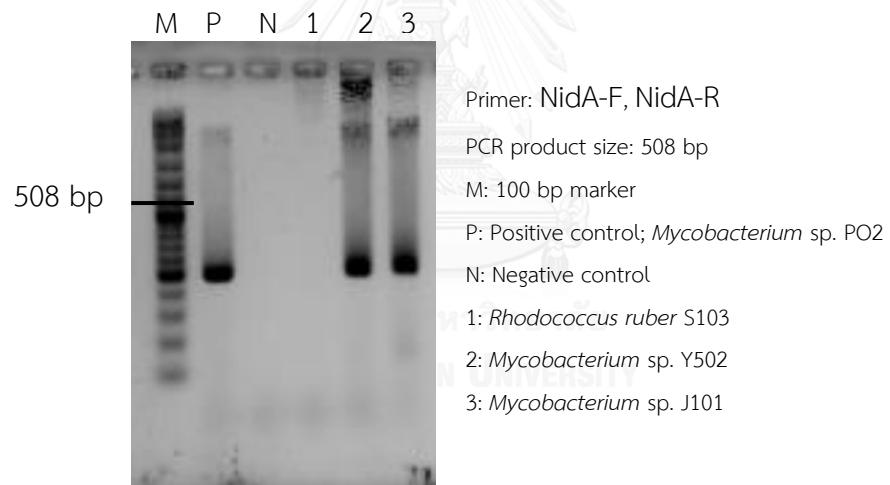2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

Figure D 4 PCR product by NidA-F, NidA-R primer of three isolate strains

Figure D 5 PCR product by ALK-1F, ALK-1R primer of three isolate strains

Primer: ALK-1F, ALK-1R

PCR product size: 185 bp

M: 100 bp marker

P: Positive control; no positive

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

185 bp

Figure D 6 PCR product by ALK-2F, ALK-2R primer of three isolate strains

Primer: ALK-2F, ALK-2R

PCR product size: 271 bp

M: 100 bp marker

P: Positive control; Consortium G1

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

271 bp

Primer: ALK-3F, ALK-3R

PCR product size: 330 bp

M: 100 bp marker

P: Positive control; Consortium G11

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

Figure D 7 PCR product by ALK-2F, ALK-2R primer of three isolate strains

Primer: AlkB-1F, AlkB-1R

PCR product size: 550 bp

M: 100 bp marker

P: Positive control; Consortium G11

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502
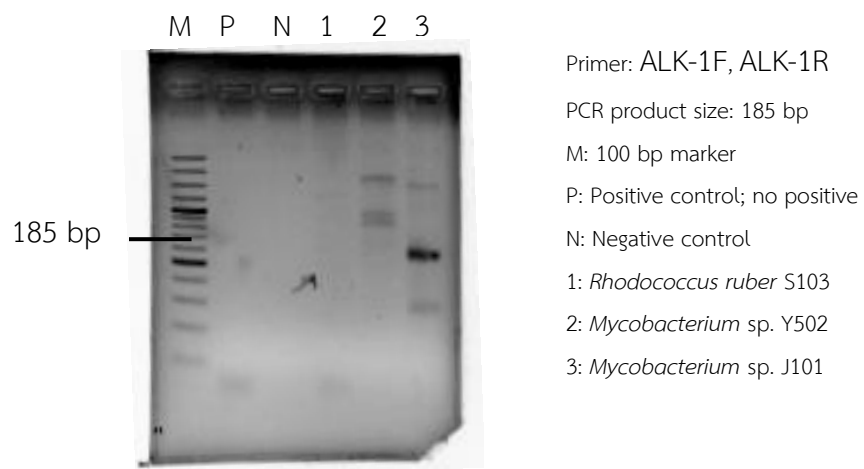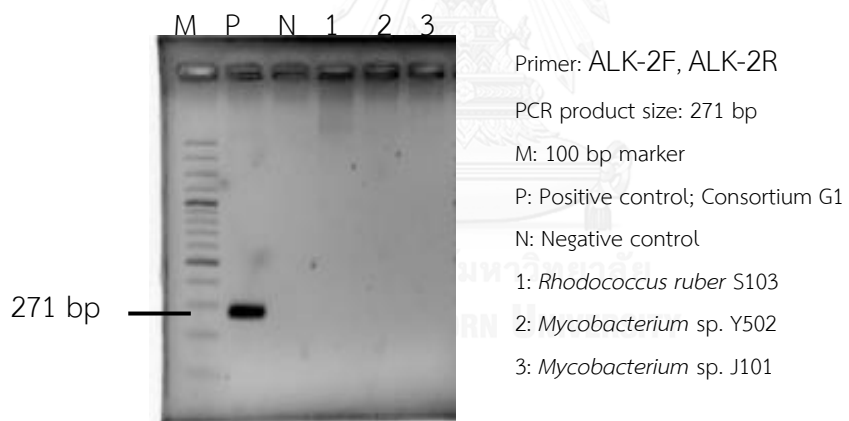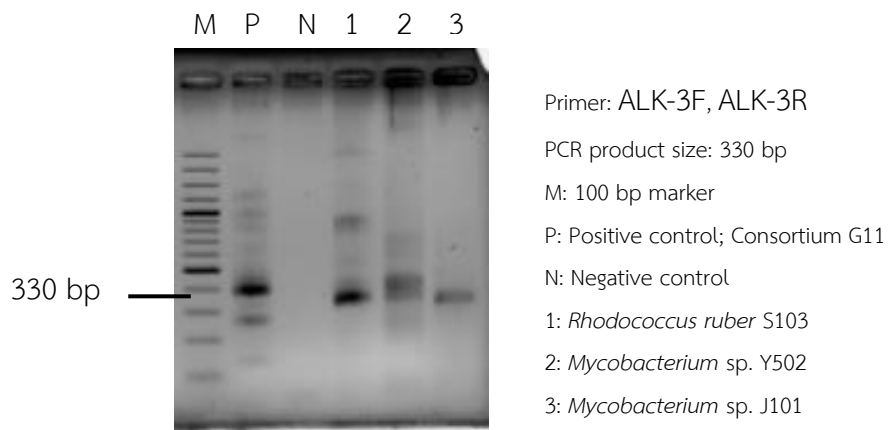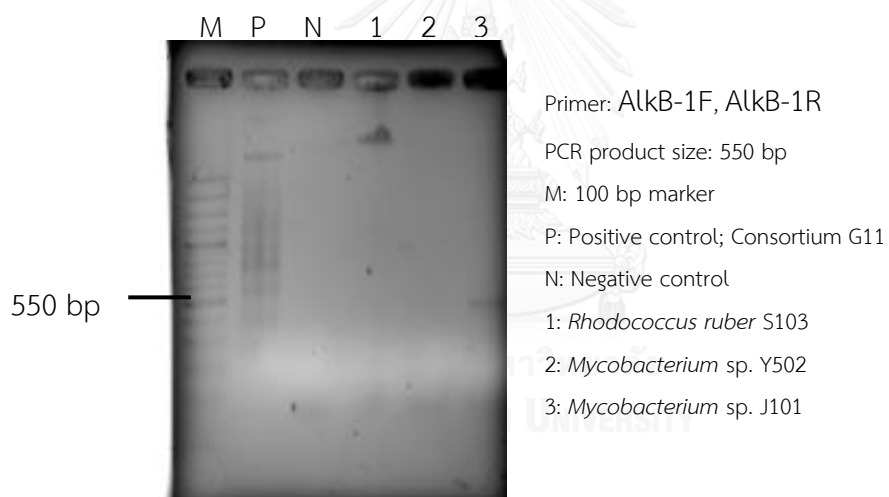
3: *Mycobacterium* sp. J101

Figure D 7 PCR product by AlkB-1F, AlkB-1R primer of three isolate strains
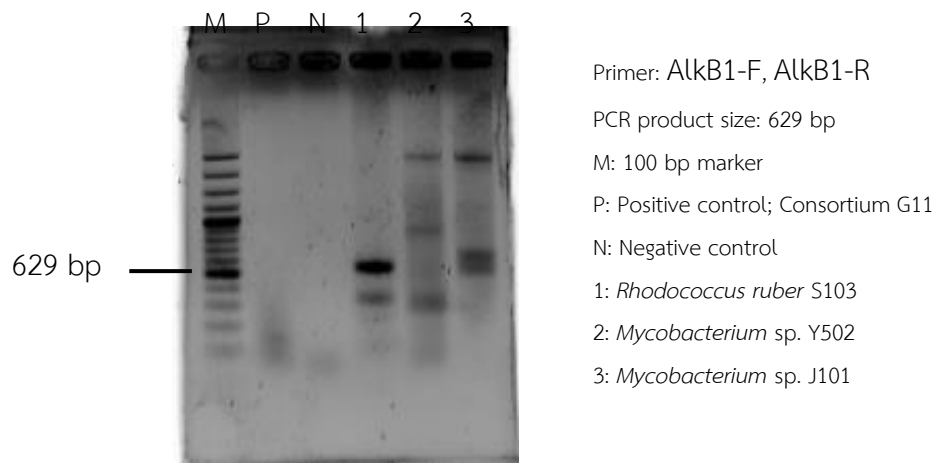
Figure D 8 PCR product by AlkB1-F, AlkB1-R primer of three isolate strains
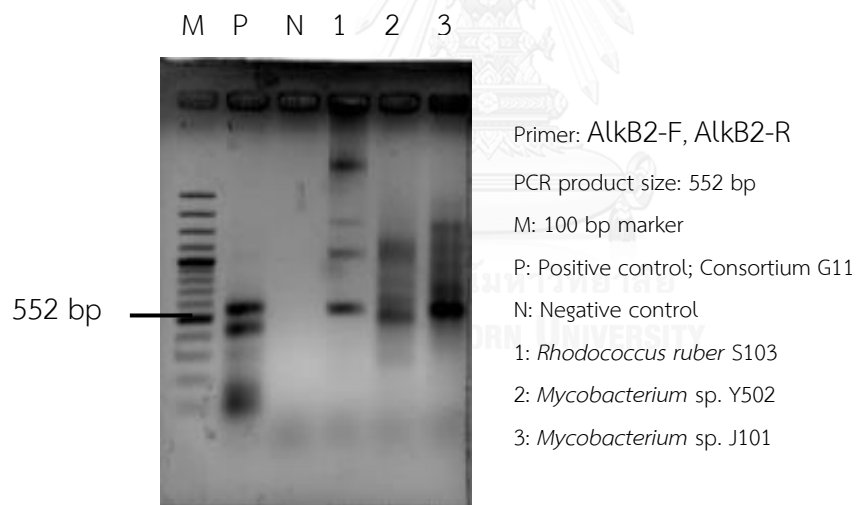
Figure D 9 PCR product by AlkB2-F, AlkB2-R primer of three isolate strains

Primer: CYP153-F, CYP153-R

PCR product size: 552 bp

M: 100 bp marker

P: Positive control; Consortium G11

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

820 bp
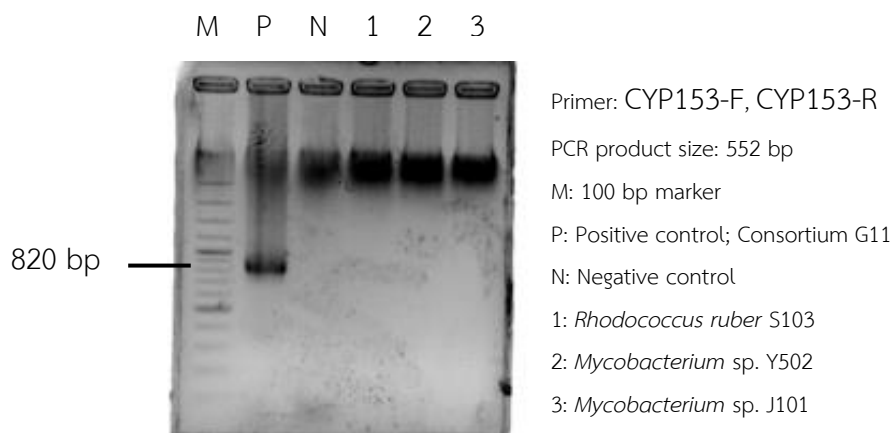
Figure D 10 PCR product by CYP153-F, CYP153-R primer of three isolate strains

Primer: P450F, P450R

PCR product size: 800 bp

M: 100 bp marker

P: Positive control; Consortium G11

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

800 bp
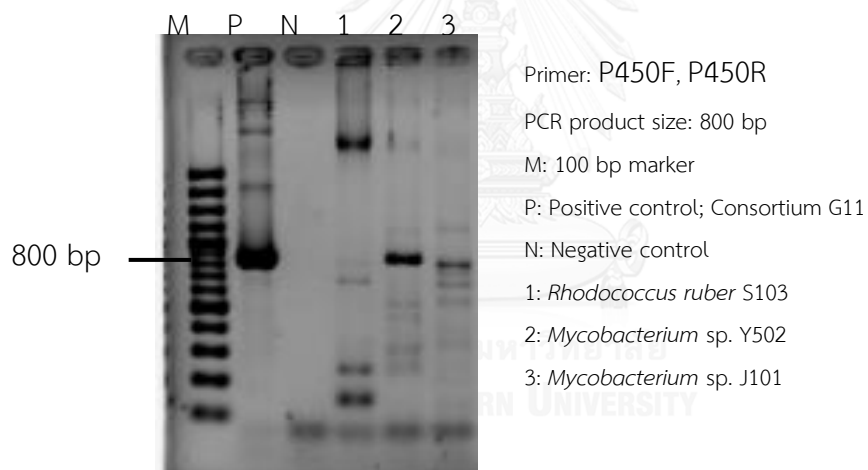
Figure D 11 PCR product by P450F, P450R primer of three isolate strains

Primer: AlmAwf, AlmAwr

PCR product size: 1100 bp

M: 100 bp marker

P: Positive control; No positive

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502

3: *Mycobacterium* sp. J101

1131 bp
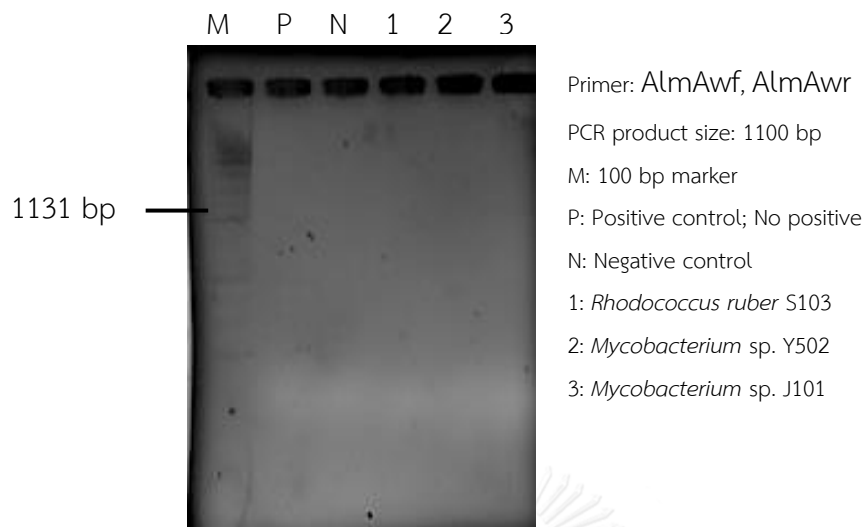
Figure D 12 PCR product by AlmAwf, AlmAwr primer of three isolate strains

Primer: P450fw1, P450rv3

PCR product size: 339 bp

M: 100 bp marker

P: Positive control; Consortium G11

N: Negative control

1: *Rhodococcus ruber* S103

2: *Mycobacterium* sp. Y502
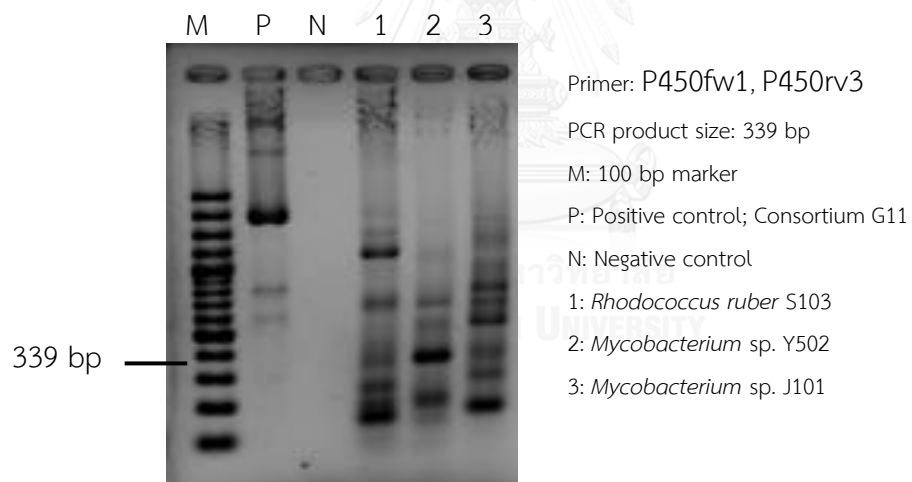
3: *Mycobacterium* sp. J101

339 bp

Figure D 13 PCR product by P450fw1, P450rv3 primer of three isolate strains

Appendix E

SUPPLYMENTARY DATA OF CHAPTER 3

Table E.1 The physiochemical properties of sediments of the Chao Phraya and the Tha Chin Rivers in Thailand

Sample	Sand	Silt	Clay	pH		EC	Total C	OC[1]	IC[1]	Total N	Active P	Active S
	(-------%-------)			Field	(H$_2$O)	(ms/cm)	(---------------%--------------)					
CP1	7.7	37	55	7.26	6.5	1.14	2.02	1.97	0.047	0.18	823	1,343
CP2	0.9	47	52	7.40	5.5	0.13	2.20	2.16	0.045	0.20	1,144	166
CP3	7.2	38	55	6.67	5.8	2.48	2.49	2.45	0.039	0.22	1,298	666
CP4	20	34	46	6.70	6.3	0.53	2.86	2.79	0.074	0.20	965	333
CP5	21	52	27	6.83	6.5	1,927	4.59	4.40	0.191	0.32	1,278	2,431
CP6	19	37	44	7.06	7.2	0.11	1.29	1.26	0.029	0.09	609	544

Table E 1: The physiochemical properties of sediments of the Chao Phraya and the Tha Chin Rivers in Thailand (continued)

Sample	Sand	Silt	Clay	pH		EC	Total C	OC[1]	IC[1]	Total N	Active P	Active S
	(-------%-------)			Field	(H_2O)	(ms/cm)	(----------------%----------------)					
TC1	9.3	57	34	7.15	6.1	17	2.25	2.05	0.199	0.19	712	710
TC2	1.9	60	38	6.97	5.6	10	3.24	3.23	0.015	0.32	513	3,097
TC3	4.9	54	41	6.66	6.9	2.7	3.38	3.28	0.102	0.30	1,144	1,554
TC4	3.4	52	44	6.65	6.7	2.7	3.87	3.78	0.088	0.37	1,152	932
TC5	2.7	56	42	6.64	5.2	1.0	3.73	3.72	0.010	0.27	1,837	1,976
TC6	4.6	58	38	6.44	5.1	1,217	4.30	4.27	0.028	0.35	2,017	1,820

VITA

Miss Duangporn Polrit was born on January 6, 1992 in Bangkok, Thailand. She graduated with a Bachelor of Microbiology department from Faculty of Science of Chulalongkorn University, Thailand. Later, she pursued her master's degree study in International Program in Hazardous Substance and Environmental Management, Graduate School, Chulalongkorn University, Thailand in May 2006.

Conferences/ Publication

Polrit, D., Muangchinda, C., Yamazoe, A., Thoetkiattikul, H., Champreda, V. & Pinyakong, O. (2015). Isolation of PAH-degrading bacteria from Chao Phraya and Tha

Chin river sediments and their biodegradation potential. The 1st International Conference on Environment, Livelihood, and Services: Environment for Life. 25 November 2015, Bangkok, Thailand (Poster presentation, proceeding)

Muangchinda, C., Yamazoe, A., Polrit, D., Thoetkiattikul, H., Mhuantong, W., Champreda, V. & Pinyakong, O. (2017). Biodegradation of high concentrations of mixed polycyclic aromatic hydrocarbons by indigenous bacteria from a river sediment: a microcosm study and bacterial community analysis. Environmental Science and Pollution Research. 24, 4591 – 4602.