การตรวจหา *Sphingobium* sp. สายพันธุ์ P2 ในระบบบำบัดน้ำเสียของสถานีบริการน้ำมันโดย qPCR

นางสาววสุเนตร รัตนสุวรรณศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DETECTION OF *Sphingobium* sp. STRAIN P2 IN WASTEWATER TREATMENT SYSTEM OF PETROL STATION BY qPCR

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วสุเนตร รัตนสุวรรณศรี : การตรวจหา *Sphingobium* sp. สายพันธุ์ P2 ในระบบบำบัดน้ำเสียของสถานีบริการน้ำมันโดย qPCR (Detection of *Sphingobium* sp. strain P2 in wastewater treatment system of petrol station by qPCR) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. อรฤทัย ภิญญาคง, 109 หน้า.

วิธีการบำบัดน้ำเสียทั่วไปยังไม่มีประสิทธิภาพเพียงพอในการบำบัดน้ำเสียจากกิจกรรมล้างรถเนื่องจากไม่สามารถกำจัดน้ำเสียในรูปอิมัลชั่นซึ่งเกิดจากการรวมตัวกันของน้ำ น้ำมัน และสารซักฟอกได้ ดังนั้นการบำบัดทางชีวภาพจึงเป็นทางเลือกหนึ่งในการบำบัดน้ำเสียเหล่านี้ ในงานวิจัยที่ผ่านมาพบว่า *Sphingobium* sp. สายพันธุ์ P2 สามารถย่อยสลายน้ำมันในน้ำอิมัลชั่นได้ งานวิจัยนี้จึงมีวัตถุประสงค์ในการพัฒนาวิธีวิเคราะห์ปริมาณยีนประมวลรหัสอะโรมาติกออกซิจีเนสของแบคทีเรียชนิดนี้ระหว่างการย่อยสลายน้ำมันด้วยเทคนิค real-time PCR การศึกษานี้ได้ออกแบบคู่ไพรเมอร์ที่จำเพาะต่อยีนเฟอรรีดอกซิน (*adhA3*) ซึ่งเกี่ยวข้องกับการย่อยสลายวงอะโรมาติกในสายพันธุ์ P2 เท่านั้น นอกจากนั้นยังทดสอบความสามารถในการย่อยสลายน้ำมันของสายพันธุ์ P2 ตั้งแต่ระดับขวดรูปชมพู่ในห้องปฏิบัติการจนถึงขนาดถังปฏิกรณ์ โดยแต่ละการทดลองได้วิเคราะห์ปริมาณน้ำมันโดยใช้เทคนิค TLC-FID, วิเคราะห์ค่า COD และ หาปริมาณสายพันธุ์ P2 ด้วยเทคนิค real-time PCR การศึกษาเบื้องต้นได้แก่การวิเคราะห์จำนวนสายพันธุ์ P2 ระหว่างการย่อยสลายน้ำมันในภาวะให้อากาศและไม่ให้อากาศ พบว่าสายพันธุ์ P2 ในภาวะให้อากาศสามารถเจริญเติบโตในน้ำมันอิมัลชั่น และสามารถกำจัดน้ำมันได้ถึง 75.16% จากความเข้มข้นน้ำมันเริ่มต้น 200 มก./ล.ภายในระยะเวลา 5 วัน ในขณะที่สายพันธุ์ P2 ในภาวะไม่ให้อากาศมีแนวโน้มลดลงและสามารถย่อยสลายน้ำมันได้เพียง 43.38% ในขณะที่สายพันธุ์ P2 ที่ถูกตรึงบนไคโตซานสามารถย่อยสลายน้ำมัน 59.84% และ 36.76% ในภาวะที่ให้และไม่ให้อากาศตามลำดับ อย่างไรก็ตามปริมาณสายพันธุ์ P2 ตรึงบนไคโตซานไม่ได้ลดจำนวนลง ซึ่งมีจำนวนเท่ากับ 2.13×10^{6} – 2.15×10^{7} ปริมาณยีน *adhA3* /0.2 กรัม ไคโตซาน จึงบ่งชี้ได้ว่าการตรึงเซลล์สามารถช่วยให้แบคทีเรียสามารถอยู่รอดได้ดีกว่าแบคทีเรียในรูปเซลล์อิสระ นอกจากนี้ได้นำสายพันธุ์ P2 ตรึงบนไคโตซานไปประยุกต์ใช้บำบัดน้ำเสียจริงในถังปฏิกรณ์ชนิด Airlift bioreactor แบบต่อเนื่องปริมาตร 3 และ 350 ลิตร (2.5 กรัมต่อลิตร)ในระยะเวลา 30 และ 60 วัน ตามลำดับ ด้วยระยะเวลาเก็บกักน้ำ 2 ชั่วโมง ผลการทดลองแสดงว่าถังปฏิกรณ์ทั้งสองขนาดมีประสิทธิภาพในการกำจัดน้ำมันด้วยประสิทธิภาพเฉลี่ยเท่ากับ 91.50 และ 68.18% ตามลำดับ ยิ่งไปกว่านั้นยังสามารถลดค่า COD เฉลี่ยได้ถึง 26.76 และ 32.96% ตามลำดับ และ พบว่าในถังปฏิกรณ์ปริมาตร 3 ลิตร มีปริมาณสายพันธุ์ P2ในไคโตซานเท่ากับ 1.92×10^{7}-3.87×10^{8}ปริมาณยีน *adhA3* /7.5 กรัม ไคโตซาน และในน้ำเสียเท่ากับ 2.63×10^{6}-3.57×10^{7} ปริมาณยีน *adhA3* /3 ลิตร ในถังปฏิกรณ์ปริมาตร 350 ลิตร มีปริมาณสายพันธุ์ P2ในไคโตซานเท่ากับ 9.08×10^{8}-1.18×10^{10} ปริมาณยีน *adhA3* /875 กรัม ไคโตซาน และในน้ำเสียเท่ากับ 2.87×10^{7}-1.75×10^{9} ปริมาณยีน *adhA3* /350 ลิตร โดยมีสัดส่วนปริมาณยีน *adhA3* ในไคโตซานมากกว่าในน้ำเสียทั้งสองระบบ จากการทดลองจึงสรุปได้ว่าสายพันธุ์ P2 ตรึงบนไคโตซานสามารถย่อยสลายน้ำมันในน้ำอิมัลชั่นและยังคงอยู่รอดได้ทุกภาวะในการทดลองนี้ อีกทั้งวิธีการตรวจวัดที่พัฒนาขึ้นมีความจำเพาะต่อการประเมินการมีอยู่ของแบคทีเรียสายพันธุ์ P2 และยังสามารถนำไปปรับปรุงระบบบำบัดทางชีวภาพสำหรับน้ำเสียในสถานีบริการน้ำมันต่อไป

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต วสุเนตร รัตนสุวรรณศรี

ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก อรฤทัย ภิญญาคง

##5187575420: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS: OIL-IN-WATER EMULSION / BIOREMEDIATION / AROMATIC OXYGENASE GENE / QUANTITATIVE REAL-TIME PCR

WASUNATE RATANASUWANASRI : DETECTION OF *SPHINGOBIUM* SP. STRAIN P2 IN WASTEWATER TREATMENT SYSTEM OF PETROL STATION BY qPCR. THESIS PRINCIPAL ADVISOR : ASST. PROF. ONRUTHAI PINYAKONG, Ph.D., 109 pp.

Conventional techniques are inefficient for car wash wastewater treatment because it cannot remove oil-in-water emulsion formed by admixture of lubricant oil with emulsifier and wash water. Therefore, bioremediation is another choice for treating this problem. In the present work, *Sphingobium* sp. strain P2 was used to degrade lubricant oil emulsion. This study aimed to develop a quantitative real-time PCR method to monitor survival of this strain during oil biodegradation. The PCR primer set specific for ferredoxin gene involved in biodegradation of aromatic compounds in strain P2 was designed. Lubricating oil degradability test by strain P2 was done in both flask-laboratory and reactor scales. These experiments were examined for amount of oil recovery by TLC-FID, COD concentration by COD reagents, and survival of strain P2 by real-time PCR. First, the survival of strain P2 during lubricating oil degradation was determined in air supply and without air supply condition. Strain P2 in air supply was able to grow in lubricant oil emulsion, showing high lubricant oil elimination (75.16% of the initial concentration of lubricant oil of 200 ppm) in 5 days. In contrast, those in the without air supply condition were tend to decrease and could only eliminate 43.38%. While, chitosan-immobilized strain P2 could degrade lubricating oil to 59.84% and 36.76% in air supply and without air supply conditions. However, chitosan-immobilized strain P2 still survived at 2.13×10^{6} to 2.15×10^{7} *adhA3* gene copies number/0.2 g chitosan. These indicated that the immobilization could improve bacterial survival better than in the form of free cells. Moreover, chitosan-immobilized strain P2 was applied to treat real wastewater in 3 l and 350 l airlift continuous bioreactor systems (2.5 g/l) within 30 and 60 days, respectively. Hydraulic retention time was 2 hours. The result showed that both of reactors had high efficiency to remove oil (91.50 and 68.18% average oil removal, respectively). Furthermore, COD concentration was reduced to 26.76 and 32.96% COD removal, respectively. The presence of strain P2 was 1.92×10^{7}-3.87×10^{8} *adhA3* gene copies number/7.5 g chitosan and 2.63×10^{6}-3.57×10^{7} *adhA3* gene copies number/3 l in 3 l aitlift bioreactor, 9.08×10^{8}-1.18×10^{10} *adhA3* gene copies number/875 g chitosan and 2.87×10^{7}-1.75×10^{9} *adhA3* gene copies number/350 l in 350 l reactor. The ratio of number *adhA3* gene copies in chitosan was higher than in wastewater for both systems. In conclusion, chitosan-immobilized strain P2 could degrade lubricant oil emulsion, and it could be maintained in all conditions performed in this study. The monitoring method developed here provided more specific assessment survival of strain P2 and can be further used to develop a biological treatment system for car wash wastewater in petrol station.

Field of Study: Environmental Management Student's signature: Warunate Ratanasuwanasri

Academic Year : 2009 Principal advisor's signature: Onruthai Pinyakong

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CONTENTS

CHAPTER page

CHAPTER page

CHAPTER page

LIST OF TABLES

Table **page**

LIST OF FIGURES

Figure **page**

Figure **page**

NOMENCLATURE

A_{260}	=	Absorbance at wavelength 260 nanometer
A_{280}	=	Absorbance at wavelength 280 nanometer
bp	=	base pairs
COD	=	Chemical oxygen demand
g	=	gram
LB	=	Luria-Bertani
M	=	molar
ml	=	milliliter
µl	=	microliter
N	=	normal
ng	=	nanogram
OD	=	Optical Density
PCR	=	Polymerase Chain Reaction
ppm	=	Parts Per Million

CHAPTER I

INTRODUCTION

1.1 Statement of problem

The oil-water emulsion from car wash wastewater in petrol station contains large volume of hazardous waste. An average of 600 l water containing more than 1% oil per car is generated (Suwit, 1997). Most of them compose of mixed lubricant oil and emulsifier. The used lubricating oil generates toxic substances such as polycyclic aromatic hydrocarbons (PAHs) by the combustion in engine (Vazquez-duhalt, 1989). The conventional techniques can remove free oil in wastewater, but they cannot remove emulsified oil.

Bioremediation is the one alternative method for treating oil-in-water emulsion. The advantage of this method over other chemical and physical methods is that bioremediation is natural, economical, effective and safe process. It is usually conducted using biostimulation and bioaugmentation approaches, by either the addition biostimulator or the supplement effective bacterial strain to enhance biodegradation (Lin *et al.*, 2005). Some studies suggested that oil-degrading strains to be used for bioaugmentation purposes need to be selected on the basic physiological properties (Coppotelli *et al.*, 2010). One of these properties is the bacterial capacity to enhance bioavailability of contaminant. To improve bioavailability, oil-degrading bacteria seem to have developed strategies such as cell surface hydrophobicity, emulsification activity (Lin *et al.*, 2005), and exopolysaccharide production (Obuekwe *et al.*, 2001).

Furthermore, a comprehension of the biochemical and genetic mechanisms of hydrocarbon-degrading bacteria is very important for decision and design efficient and predictable remediation procedures. Several studies described biochemical and genetic analyses of PAH degradation (Peng, 2008). PAH compounds are aerobically degraded where dioxygenases catalyze two critical reactions which are ring hydroxylation and ring cleavage. The ring-hydroxylation yield *cis*-dihydrodiols as the early bioproducts by ring-hydroxylating dioxygenase consists of three components;

terminal dioxygenase, ferredoxin, and ferredoxin reductase (Harayama *et al.*, 1992). These dihydroxylated intermediates are cleaved by two functional enzymes: intradiol aromatic ring-cleavage dioxygenases or extradiol aromatic ring-cleavage dioxygenases, converting to central intermediates that are transformed to tricarboxylic acid (TCA) cycle intermediates (Peng, 2008).

Due to the important of effective microorganisms in bioremediation, the survival to them in the process is of major concern. The effective method to quantify microorganism is Quantitative Real-Time PCR (qPCR) which has ability to measure PCR products in real time. It has been used to monitor the presence of bacteria in environmental samples in several studies. Bladwin *et al.* (2003) used Real-time PCR for enumeration of aromatic oxygenase genes in pollutant-biodegrading microorganisms. Moreover, Cunliffe *et al.* (2006) used qPCR method to monitor expression degradation genes which is *bphC* and *xylE* in *Sphingobium yanoikuyae* B1.

Sphingobium sp. strain P2, which can utilize phenanthrene as a sole carbon source and energy source, was isolated from oil contaminated soil in Thailand (Supaka *et al.*, 2001). In 2003a, Pinyakong *et al.* identified genes for aromatic hydrocarbon degradation in this strain. For example, ferredoxin; is the one of three components of ring-hydroxylating dioxygenase, coded by gene *adhA3*, 2,3-dihydroxybiphenyl 1,2-dioxygenase, and catechol 2,3-dioxygenase; are extradiol aromatic ring-cleavage dioxygenase, coded by gene *bphC* and *xylE*, respectively. In the same year, *Sphingobium* sp. strain P2 has been reported to have a unique group of genes for aromatic degradation with *Sphingobium yanikuyae* strain B1 and *Novosphingobium aromaticivorans* strain F199, which are distantly related to those in pseudomonads and other genera (Pinyakong *et al.*, 2003b). In addition, most of the genes required by the strain for biodegradation of aromatic hydrocarbons have been sequenced and characterized allowing easily study to monitor their activity and survival by qPCR.

According to PAH metabolism, sufficient oxygen plays an important role in the degradation. Therefore, designing aerated tank reactors have to be concerned about air transfer. Many researches considered that the important cost for the systems is mainly determined by energy cost for aeration and maintenance cost for the reactors (Boon, 1996).

Therefore, this study aims to determine lubricant oil degradation ability of *Sphingobium* sp. strain P2 and to detect a gene which act as marker gene for quantify survival of *Sphingobium* sp. strain P2 by qPCR in oil-in-water emulsion which sufficient and insufficient air was supplied for further using in car wash wastewater treatment system in petrol station.

1.2 Objectives

1. To choose marker genes and design specific primers for detection of *Sphingobium* sp. strain P2.

2. To detect marker genes during degradation of lubricating oil by *Sphingobium* sp. strain P2 using real-time PCR in airlift bioreactor.

1.3 Hypothesis

The marker genes and real-time PCR methods presented can be used to assess survival and activity of *Sphingobium* sp. strain P2 in airlift bioreactor system.

1.4 Scope of Study

1. Choosing genes involved in lubricating oil degradation in *Sphingobium* sp. strain P2.

Marker genes was chosen from the following:

- *ahdA3* gene encoding for ferredoxin of dioxygenase
- *ahdA4* gene encoding ferredoxin reductase of dioxygenase
- *bphA1f* gene encoding alpha subunit of ring hydroxylating dioxygenase
- *bphC* gene encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase
- *xlyE* gene encoding catechol 2,3-dioxygenase
- *alkM* gene encoding alkane hydroxylase

2. Detection of genes during lubricating oil degradation with free and immobilized cells in chitosan by Real-Time PCR

The genes were detected in 3 different scales.

2.1 Flask-laboratory scale with artificial wastewater

- using free and immobilized cell with air supply
- using free and immobilized cell without air supply

2.2 Three liters airlift bioreactor system with wastewater from petrol station

2.3 Three-hundred and fifty liters airlift bioreactor system with wastewater from petrol station

CHAPTER II

LITERATURE REVIEW

2.1 Wastewater from car wash station

The waste water produced from car wash stations consists of many impurities such as free oil, oil-in-water emulsion, emulsifier, and clay sludge. This wastewater contains approximately high proportion of more than 1% of oil content (Weber *et al.*, 1997). In commonly, free oil and sludge are separated by existing system of gas stations known as API (American Petroleum Institution) oil-in-water separator, which is considered effective and economical. In contrast, oil-in-water emulsion and emulsifier cannot be treated completely by the existing treatment system. Therefore, the quality of treated wastewater is still lower than standard limit for gas station discharged wastewater.

2.1.1 Source of car wash wastewater

The car washing operation consists of four steps as shown in Figure 2.1 (Panpanit, 2001). Each stage in the process is simply described as follows:

Step 1 Dust cleaning: high pressure water is injected on the car to wash out the dust. The wastewater in this step mostly contains sludge, clay and free oil. Moreover, the mechanical force from high pressure of water makes oil dissolve in water.

Step 2 Foaming: the car wash shampoo is sprayed on the car body. The wastewater in this stage contains highly concentration of emulsifier. Finally, it solves with wastewater in step 1, and becomes to oil-in-water emulsion.

Step 3 Presoak: high amount of fresh water is washed the emulsifier. The wastewater in this step contains low emulsifier concentration because of dilution by water.

Step 4 Rinse water and dryer: high quality car is rinsed to cay body and dried with hot air to polish car color.

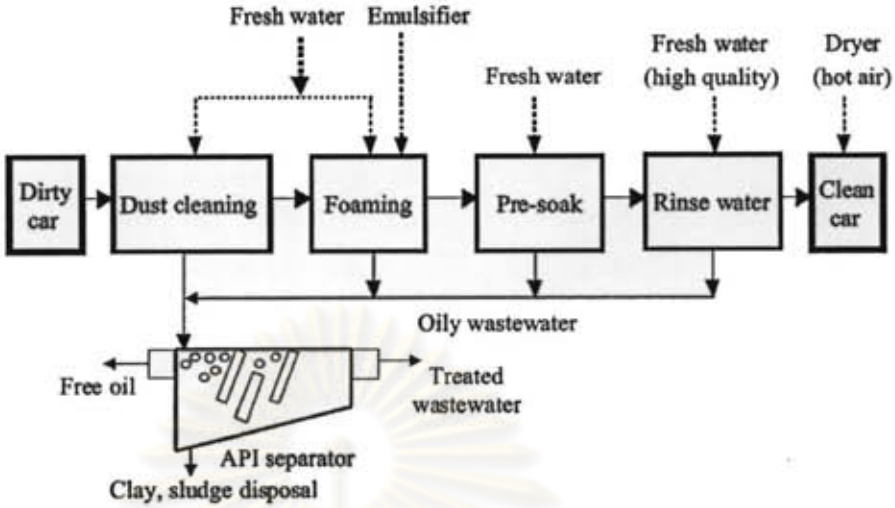


Figure 2.1 Process flow diagram of car wash process (Panpanit, 2001)

2.1.2 Oil-in-water emulsion characteristic

Oil-in-water emulsion is oil dispersed in water phase. It is generally found in the presence of emulsifier, which is called stabilized emulsion. An emulsifier consists of a molecule with hydrophilic and hydrophobic ends. It acts as bridging agent, lowering the interfacial tension of oil, resulting in very small oil droplet size (Alters, 1998). An important factor in emulsion stability is the diameter of the dispersed oil droplets, which is governed by Stoke's law. Oil-in-water emulsions formed in the presence of emulsifier have very small droplets size inferior to 5 μm. The presence of co-surfactant such as fatty alcohol in emulsifier, the emulsion droplet size is in the range of 100-600 Å, thus, their rising velocity is negligible compared to the Brownian movement. Therefore, an emulsion is not settled by gravity force. The classifications of oil-in-water emulsion are summarized in Table 2.1.

Table 2.1 Classification of Oil-in-water emulsions (Aurelle and Verdun, 1997; Alters, 1998)

Classification	Appearance	Diameter of Droplet Size
Fine dispersed, colloidal dispersed	Milky-gray emulsion	Medium/fine 0.1-20 μm
Molecular dispersed	Transparent Micro-emulsion	Micro 100-600 Å

2.1.3 Pollution of oil-in-water emulsion from car washing wastewater

Car wash wastewater is considered as hazardous industrial wastewater because it contains petroleum hydrocarbon. Mixed lubricant oil and emulsifier are always found in car wash wastewater (Panpanit, 2001). According to Table 2.2, there are some reports reveal that car washing wastewater contains many toxic hydrocarbons both of aliphatic and aromatic hydrocarbons. Especially, aromatic hydrocarbons including polycyclic aromatic hydrocarbons (PAHs) are more toxic and persistent than aliphatic fraction. US EPA has identified unsubstituted PAHs as priority pollutants, some of which considered to be possible or probable biological effects: acute toxicity, carcinogenicity, mutagenicity, teratogenicity and endocrine disrupting activity (Kliaugaite, 2008).

Table 2.2 Hydrocarbon quantity considered as toxic chemical in car washing wastewater in Sweden (Paxéus, 1996)

Parameter	substance pollution quantity (ppm)					
	Small-sized car			Large-sized car		
	Average	Mode	Period	Average	Mode	Period
Total oil	291	242	10-1750	550	460	65-1200
COD	1253	1180	120-4200	4600	4500	1700 - 7500
Aliphatic hydrocarbon						
C8-C16	29	22	1-139	103.86	76.72	41-220
C17-C30	0.6	0.4	0-0.001	1.84	1.87	0.9-3.0
Aromatic hydrocarbon						
Benzene	0.01	0.01	<0.01-0.2	0.02	0.02	0.02-0.03
Toluene	0.08	0.05	<0.01-0.6	0.10	0.06	0.03-0.2
Naphthalene	0.17	0.13	<0.001-0.7	1.1	0.75	0.3-3
Biphenyl	0.015	0.005	<0.001-0.1	0.12	0.11	0.04-0.2
Dibenzofuran	0.001	0.002	<0.001-0.03	0.011	0.011	0.009-0.012

Table 2.2 Hydrocarbon quantity considered as toxic chemical in car washing wastewater in Sweden (Paxéus, 1996) (cont.)

Parameter	substance pollution quantity (ppm)					
	Small-sized car			Large-sized car		
	Average	Mode	Period	Average	Mode	Period
Phenanthrene	0.005	<LOD	<0.001-0.03	0.021	<LOD	0.005-0.03
Pyrene	0.003	<LOD	<0.001-0.01	0.009	<LOD	0.01-0.02
Fluoranthene	0.003	<LOD	<0.001-0.0	0.004	<LOD	0.002-0.006
Plasticizers						
Diethyl-phthalate	0.005	0.01	0.002-0.06	0.01	0.01	0.01-0.02
Dihexyl-phthalene	0.05	0.03	<0.001-0.15	0.03	0.21	<0.001-0.7
Bis(2-ethylhexyl) phthalate (DEHP)	0.52	0.38	0.03-4.1	1.50	1.30	0.04-3
Washing agents						
p-nonylphenol	0.60	0.26	0.01-4	0.43	0.41	0.1-0.8
2-Botoxyethanol	25	15	<0.01-270	15	17	<0.001-27

2.2 Lubricating oil

The petroleum composition used as synthetic lubricant generally contains compounds coating 18 or more carbon atoms. The lubricating composition is a complex mixture consisting primary of five characteristics: classes-paraffin naphthenes, condensed naphthene, aromatic naphthenes, naphthalene, and multi-ring aromatics, which appear to be main constituents of the toxic portion in oil. It also contains small amount of heterocyclic compound containing sulfur and nitrogen atoms (e.g. thionaphthene, indole quinoline and carbazone) and various oxygen containing compound, including naphthenic acids, which account for most of the chemically bond oxygen in petroleum compositions (Insuk, 2004). Physical properties of lubricant oil are very low volatility and less water solubility and not

volatile appreciably under normal environmental conditions. Additionally, it has high oxidation stability for chemical and biological treatment.

2.2.1 Types of lubricating oils

Lubricating oils can be classified into two types: refined oil, or mineral oil, and synthetic oil. The mineral oil is refined from crude oil which consists of paraffinic and naphthenic oil groups. In addition, synthetic oils are manufactured which produced from chemical synthesis rather than from the refinement of existing petroleum or vegetable oils (Stachowiak and Batchelor, 2005).

PTT-120 used in this study is lubricating oil motor gasoline products, grade single kind produced by PTT Public Company Limited. It is generated from lubricating base oil and a substance enhances special quality. It is also used well with an automobile that use gasoline general motor such as private car, taxicab, a car using gas fuel, and other agriculture machinery since small-sized to middle-sized.

2.2.2 Toxicity of used lubricating oil

The available information shows that used lubricating oil is a very dangerous polluting product. As a consequence of its chemical composition, world-wide dispersion and effects on the environment, used motor oil must be considered a serious environmental problem. The effect of used lubricating oil on the environment can be concluded as follows:

In Soil

The growth of plants (turnips and beans) has been shown to be inhibited in soils contaminated with used motor oil, and the lead concentrations in surviving turnips and beans were, respectively, 450 and 150% greater than those grown in uncontaminated soil. The lead and zinc concentrations detected in earthworms (331.4 and 670 µg/g, respectively) living in soil near roads are probably fatal for their predators (Vazquez-duhalt, 1989).

In aquatic system

Petroleum hydrocarbons inhibit the growth, photosynthetic activity and respiration of algae, and phytoplankton activity. The damage to crustaceans, molluscs and fish caused by hydrocarbon pollution can also be very important. It has been

found that used lubricating oil is one of the most important mutagenic agents in the aquatic environment (Vazquez-duhalt, 1989).

2.2.3 Environmental consideration of used lubricating oil

The US Environmental Protection Agency's (EPA) regulatory definition of used oil is as follows

"Used oil is any oil that has been refined from crude oil or any synthetic oil that has been used and as a result of such use is contaminated by physical or chemical impurities."

Once oil has been used and drained from the lubricated equipment, it can be collected, recycled, and used again. It can be reprocessed and re-refined for use as new oil or used as an energy source. For example, used motor oil can be re-refined and sold at the store as motor oil or processed for furnace fuel oil. Aluminum rolling oils also can be filtered on site and used over again. Used oil can be recycled via the following processes:

On site reconditioning, involves removing impurities (dirt, heavy deposits, etc.) from the used oil and using it again.

Reprocessing, involves introducing used oil into a petroleum refinery as a feedstock and produce gasoline and coke.

Re-refining, involves removing impurities so that it can be used as base oil for new lubricating oil. Re-refining prolongs the life of the oil resource indefinitely via this regeneration process. This form of recycling is generally the preferred option because it closes the recycling loop by reusing the oil to make the same original product.

Burning for energy recovery, involves removing water and particulates so that used oil can be burned as fuel to generate heat or to power industrial operations.

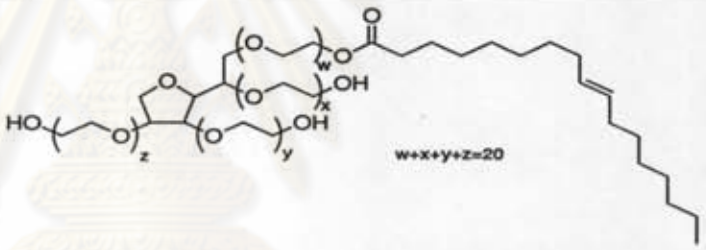
2.3 Emulsifier

Emulsifier is the major constituent in car wash shampoo. Normally, the emulsifier used in industries for cleaning purposes can be divided into two groups, e.g., anionic emulsifier and nonionic emulsifier. In addition, more than 90% of the wastewater in industrial streams contains nonionic emulsifiers (Alters, 1998). The

emulsifier has contaminated surface water with an objectionable foaming property. Moreover, depending on their chemical structure, some emulsifiers are resistant to chemical attack that causes a higher COD in wastewater. In the case of non-ionic emulsifier such as polyethylene glycol, its reaction for chemical oxidation is slow and requires high strong chemical dosage such as ozone and long contacts periods to achieve a significant removal (Langlais *et al.*, 1991).

The emulsifier which is used in this study to produced artificial wastewater is nonionic emulsifiers called Tween-80. It is generally found in car wash shampoo. The physical and chemical properties are shown in Table 2.3.

Table 2.3 Chemical and physical properties of Tween-80 (Merck Index, 13th Edition, 7664)

Properties	
Structure	
Molecular formula	$C_{64}H_{124}O_{26}$
Molar mass	1310 g/mol
Appearance	Amber colored viscous liquid
Density	1.06-1.09 g/ml, oily liquid
Boiling point	> 100°C
Solubility in water	Very soluble
Solubility in other solvents	soluble in ethanol, cottonseed oil, corn oil, ethyl acetate, methanol, toluene
Viscosity	300-500 centistokes (at 25°C)

2.4 Oil-in-water emulsion treatment systems

The oil-in-water emulsion separators can be summarized into three categories: primary or gravity treatment units, secondary process, and tertiary process. According to the previous part, the primary treatment system such as API treats free oil effectively by the gravity concept cannot remove by its techniques (Panpanit, 2001). Many strategies in secondary and tertiary process for counteracting oil-in-water emulsions have been developed and described as follows:

2.4.1 Air Flotation (AF)

This procedure involves separated light sediment from water which can precipitate difficult and take long time. Actually, it uses for free oil and fat separated from wastewater. A skimmer then skims off floating precipitate (Alther, 1998). For maximum efficiency, chemical coagulation and flocculation or emulsion breaking usually accompanies with air floatation such as lime, aluminum sulfate and ferric chloride. On the other hand, the AF with chemical coagulant produces a significant quantity of waste sludge and it is difficult to be treated. The procedure which makes precipitate float can be divided into two types: Dissolved Air Floatation (DAF) and Induced Air Floatation (IAF).

A principle of DAF is dissolved air under pressure which is then released under special conditions to encourage the formation of small bubbles. As the tiny air bubbles rise, they attach and adhere to the oil globules. The resulting bubble and oil complex form rises to the liquid surface due to differential gravity (Panpanit, 2001). In case of IAF, directly blow air at the atmosphere pressure into wastewater to make small bubbles. A tiny bubble then adhere with light sediment and oil, so the sediment rise to the liquid surface. However, this system has lower efficiency than DAF. Due to larger bubble size and lower bubble pressure make floating force of bubble which adhere with light sediment is not high enough (Meyssami and Kasaeian, 2005).

The advantage of AF is high oil removal efficiency. In contrast, the disadvantages are high investment and operating cost, difficult in terms of operation, sludge problem and chemical needed (combined with chemical coagulant) (Panpanit, 2001).

2.4.2 Adsorption

Adsorption is the accumulation of atoms or molecules on the surface of a material. This process creates a film of the adsorbate (the molecules or atoms being accumulated) on the adsorbent's surface. This system is generally used as a post polisher to remove trace of oil hydrocarbon and emulsifier. The adsorbent has been developed widely such as organic polymer, activated carbon, and organophilic clay, etc. Organic polymer such as chitosan is used to adsorb residue oil in wastewater especially in pine oil industry (Ahmed *et al.*, 2005). The research showed that chitosan is cationic biopolymer and has many amino groups for high effective adsorbing oil.

The advantages for using adsorbent in oil wastewater treatment system are good for polishing oil and emulsifier (less than 1 ppm) and low capital cost. However, the disadvantages are low efficiency for high oil and emulsifier concentration, difficult in term of operation, regeneration of spent adsorbent, and sludge from spent adsorbent (Panpanit, 2001).

2.4.3 Sequence batch biological reactor (SBR) process

Sequence batch biological reactor (SBR) process is industrial processing tanks for the treatment of wastewater. SBR reactors treat wastewater such as sewage or output from anaerobic digesters or mechanical biological treatment facilities in batches. Oxygen is bubbled through the wastewater to reduce biochemical oxygen demand (BOD) and chemical oxygen demand (COD) to make suitable for discharge into sewers or for use on land (Dachtanon, 2001). There are five stages to treatment: fill, react, settle, draw and idle. All of these stages are not necessary to treat by chemical. Efficiency of the system depends on appropriate control the period of time in each the stage. The advantages of this process are low investment and operating cost, but the disadvantages are difficult in terms of operation and slow process (Panpanit, 2001).

2.5 Bioremediation

Bioremediation can be defined as any process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition (Boopathy, 2000). There are a number of advantages to bioremediation such as environmental friendly, safe process and economical (Evans and Furlong, 2003). Moreover, the contaminant is metabolized to carbon dioxide and water which is eliminated permanently. Bioremediation may be employed in order to attack specific contaminants such as hydrocarbon compounds.

2.5.1 Biodegradation of petroleum hydrocarbon

Petroleum consists of a complex mixture of hydrocarbons of various molecular weights, while the other organic compounds contain nitrogen, oxygen and sulfur, and trace amounts of metals such as iron, nickel, copper and vanadium (Van Beilen *et al.*, 2003). The hydrocarbons in petroleum are mostly alkanes, cycloalkanes, aromatic and various polycyclic aromatic hydrocarbons (PAH) (Van Beilen and Funhoff, 2007).

2.5.1.1 Aliphatic compounds biodegradation pathway

Many microorganisms can utilize alkane as a sole carbon source and energy source (Table 2.4). Aerobic biodegradation of aliphatic hydrocarbons involves the incorporation of molecular oxygen into the hydrocarbon structure. This is performed by alkane-activating enzymes, which is monooxygenase (or hydroxylase). The most common pathway of alkane degradation is oxidation at the terminal methyl group. Oxidation proceeds as a sequence to a primary alcohol, which is further oxidized by alcohol and aldehyde dehydrogenases. The resulting fatty acids enter the β-oxidation cycle. Short chain alkanes are metabolized via terminal as well as subterminal oxidation, and many methanotrophs co-oxidize short-chain alkanes at terminal as well as subterminal positions. Subterminal oxidation has also been detected for longer alkanes. The secondary alcohols are converted to the corresponding ketone, which is oxidized by a Baeyer-Villiger monooxygenase to an ester. The ester is subsequently hydrolyzed by an esterase to an alcohol and a fatty acid and eventually to carbon dioxide and water (Figure 2.2) (Van Beilen *et al.*, 2003).

Some alkane degraders contain only one alkane hydroxylase which oxidize alkane substrates by oxygen species. However, several alkane degradation systems, being active on alkanes of a certain chain-length or expressed under specific physiological conditions, have been found in many other strains. For example, *alkM* is regulated depending on the alkane present in the medium. Expression of *alkM* is induced by alkanes having a very long chain length (>C_{16}) (Rojo *et al*, 2009).

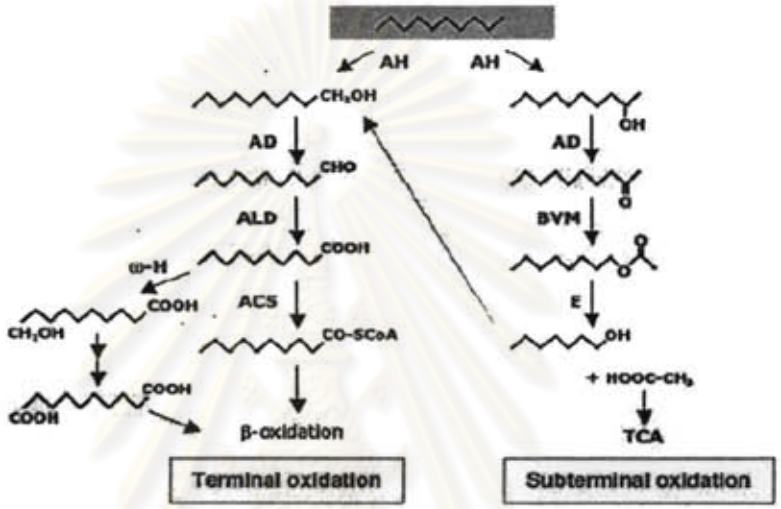


Figure 2.2 Aerobic pathways for the degradation of methane (left), and of larger *n*-alkanes by terminal and subterminal oxidation (right). MMO, methane monooxygenase; MD, methanol dehydrogenase, FLD, formaldehyde dehydrogenase; FMD, formate dehydrogenase. AH, alkane hydroxylase; AD, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; ACS, acyl-CoA synthetase; w-H, w-hydroxylase; BVM, Baeyer–Villiger monooxygenase; E, esterase; TCA, tricarboxylic acids cycle (modified from Rojo *et al, 2009*).

Table 2.4 Examples of aerobically alkane degrading bacteria (Wentzel et al., 2007)

Bacterial species	Strain	n-alkane substrate range
Acinetobacter baylyi	ADP1	$-C_{36}$
Acinetobacter calcoaceticus	EB104	C_9–C_{18}
Acinetobacter calcoaceticus	RR8	C_{10}–C_{34}
Acinetobacter lwoffi		C_{12}–C_{28}
Acinetobacter sp	M-1	C_{13}–C_{44}
Acinetobacter sp.	ODDK71	C_{12}–C_{30}
Acinetobacter sp.	S30	$-C_{33}$
Acinetobacter sp.	DSM17874	C_{10}–C_{40}
Alcaligenes odorans	P20	$-C_{33}$
Alcanivorax borkumensis	AP1	C_{10}–C_{20}
Alcanivorax borkumensis	SK2	C_8–C_{32}
Arthrobacter nicotianae	KCC B35	C_{10}–C_{40}
Bacillus thermoleovorans	B23 and H41	C_9–C_{30}
Bacillus thuringiensis/cereus	A2	C_6–C_{28}
Brachybacterium sp.		C_{10}–C_{20}
Burkholderia cepacia	RR10	C_{12}–C_{34}
Desulfatibacillum aliphaticivorans	CV2803	C_{13}–C_{18}
Dietzia cinnamea P4		C_{11}–C_{24}
Dietzia psychralcaliphila		C_{13}–C_{24}
Geobacillus thermodenitrificans	NG80-2	C_{15}–C_{36}
Gordonia sp.	TY-5	C_3, C_{13}–C_{22}
Marinobacter hydrocarbonoclasticus	617	C_{16}–C_{30}
Marinobacter sp.	BC36, BC38, and BC42	C_{18}
Mycobacterium sp.	HXN600	C_6–C_{24}
Paracoccus sereniphilus/marcusii	A7	C_6–C_{28}
Paracoccus sp.	Ophe1 and Sphe1	C_{10}–C_{28}
Planococcus alkanoclasticus	MAE2	C_{11}–C_{33}
Pseudomonas aeruginosa	PAO1	C_{12}–C_{24}
Pseudomonas aeruginosa	RR1	C_{12}–C_{34}
Pseudomonas aeruginosa	A1, A3, A4, A5, A6	C_6–C_{28}
Pseudomonas fluorescens	CHA0	C_{12}–C_{32}
Pseudomonas sp.	PUP6	C_{12}–C_{28}
Rhodococcus erythropolis	23-D	C_6–C_{36}
Rhodococcus erythropolis	NRRL B-16531	C_6–C_{36}
Rhodococcus erythropolis	42-O and 50-V	C_6–C_{32}

Table 2.4 Examples of aerobically alkane degrading bacteria (Wentzel et al., 2007) (cont.)

Bacterial species	Strain	n-alkane substrate range
Rhodococcus fascians	115-H	C_6–C_{32}
Rhodococcus fascians	154-S	C_6–C_{24}
Rhodococcus sp.	1BN	C_6–C_{28}
Rhodococcus sp.	NCIM5126	C_{13}–C_{20}
Rhodococcus sp.	RR12 and RR14	C_{14}–C_{34}
Rhodococcus sp.	T12 and TMP2	C_9–C_{22}
Thalassolituus oleivorans		C_7–C_{20}
Thermus sp.	C2	C_9–C_{39}
Weeksella sp.	RR7	C_{12}–C_{34}
Xylella fastidiosa	RR15	C_{14}–C_{34}

2.5.1.2 Aromatic compound biodegradation pathway

For aromatic fraction in petroleum product, there are also many bacteria that can utilize aromatic compounds (Table 2.5). PAH compounds are aerobically degraded where dioxygenases catalyze two critical reactions which are ring hydroxylation and ring cleavage. The ring-hydroxylation yield *cis*-dihydrodiols as the early bioproducts by ring-hydroxylating dioxygenase consists of three components; terminal dioxygenase, ferredoxin, and ferredoxin reductase (Harayama *et al.*, 1992). These dihydroxylated intermediates are cleaved by two functional enzymes. Intradiol aromatic ring-cleavage dioxygenases cleave *ortho* to the hydroxyl substituents (between two hydroxyl groups) and typically depend on nonheme Fe(III). In contrast, extradiol aromatic ring-cleavage dioxygenases (edo) cleave *meta* to the hydroxyl substituents (between one hydroxyl carbon and its adjacent nonhydroxylated carbon) and typically depend on nonheme Fe(II) (Eltis & Bolin, 1996)., converting to central intermediates that are transformed to tricarboxylic acid (TCA) cycle intermediates (Peng R.*et al.*, 2008) (Figure 2.3).

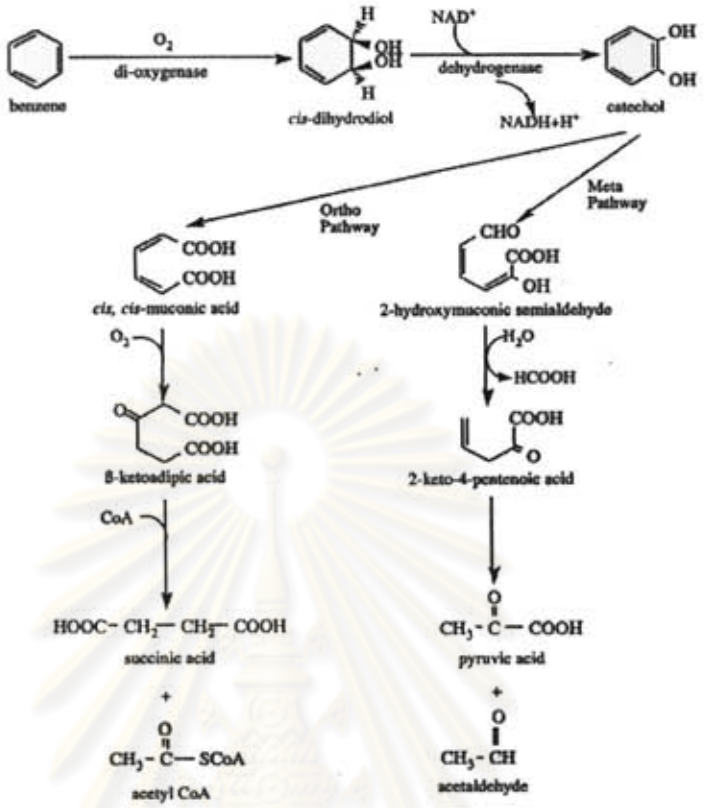


Figure 2.3 Aromatic compound aerobic biodegradation via aromatic dioxygenase (Juhasz and Naidu, 2000).

Table 2.5 Examples of aerobically aromatic compound degrading bacteria (Seo *et al.*, 2009)

Bacterial species	Strains	Aromatics
Achromobacter sp.	NCW	CBZ
Alcaligenes denitrificans		FLA
Arthrobacter sp.	F101	FLE
Arthrobacter sp.	P1-1	DBT, CBZ, PHE
Arthrobacter sulphureus	RKJ4	PHE
Acidovorax delafieldii	P4-1	PHE
Bacillus cereus	P21	PYR
Brevibacterium sp.	HL4	PHE
Burkholderia sp.	S3702, RP007, 2A-12TNFYE-5, BS3770	PHE
Burkholderia sp.	C3	PHE

Table 2.5 Examples of aerobically aromatic compound degrading bacteria (Seo *et al.*, 2009) (cont.)

Bacterial species	Strains	Aromatics
Burkholderia cepacia	BU-3	NAP, PHE, PYR
Burkholderia cocovenenans		PHE
Burkholderia xenovorans	LB400	BZ, BP
Chryseobacterium sp.	NCY	CBZ
Cycloclasticus sp.	P1	PYR
Janibacter sp.	YY-1	DBF, FLE, DBT, PHE, ANT, DD
Marinobacter	NCE312	NAP
Mycobacterium sp.		PYR, BaP
Mycobacterium sp.	JS14	FLA
Mycobacterium sp.	6PY1, KR2, AP1	PYR
Mycobacterium sp.	RJGII-135	PYR,BaA, BaP
Mycobacterium sp.	PYR-1, LB501T	FLA, PYR, PHE, ANT
Mycobacterium sp.	CH1, BG1, BB1, KR20	PHE, FLE, FLA, PYR
Mycobacterium flavescens		PYR, FLA
Mycobacterium vanbaalenii	PYR-1	PHE, PYR, dMBaA
Mycobacterium sp.	KMS	PYR
Nocardioides aromaticivorans	IC177	CBZ
Pasteurella sp.	IFA	FLA
Polaromonas naphthalenivorans	CJ2	NAP
Pseudomonas sp.	C18, PP2, DLC-P11	NAP, PHE
Pseudomonas sp.	BT1d	HFBT
Pseudomonas sp.	B4	BP, CBP
Pseudomonas sp.	HH69	DBF
Pseudomonas sp.	CA10	CBZ, CDD
Pseudomonas sp.	NCIB 9816-4	FLE, DBF, DBT
Pseudomonas sp.	F274	FLE
Pseudomonas paucimobilis		PHE
Pseudomonas vesicularis	OUS82	FLE
Pseudomonas putida	P16, BS3701, BS3750, BS590-P, BS202-P1	NAP, PHE
Pseudomonas putida	CSV86	MNAP
Pseudomonas fluorescens	BS3760	PHE, CHR, BaA

Table 2.5 Examples of aerobically aromatic compound degrading bacteria (Seo *et al.*, 2009) (cont.)

Bacterial species	Strains	Aromatics
Pseudomonas stutzeri	P15	PYR
Pseudomonas saccharophilia		PYR
Pseudomonas aeruginosa		PHE
Ralstonia sp.	SBUG 290 U2	DBF NAP
Rhodanobacter sp.	BPC-1	BaP
Rhodococcus sp.		PYR, FLA
Rhodococcus sp.	WU-K2R	NAT, BT
Rhodococcus erythropolis	I-19	ADBT
Rhodococcus erythropolis	D-1	DBT
Staphylococcus sp.	PN/Y	PHE
Stenotrophomonas maltophilia	VUN 10,010	PYR, FLA, BaP
Stenotrophomonas maltophilia	VUN 10,003	PYR, FLA, BaA, BaP, DBA, COR
Sphingomonas yanoikuyae	R1	PYR
Sphingomonas yanoikuyae	JAR02	BaP
Sphingomonas sp.	P2, LB126	FLE, PHE, FLA, ANT
Sphingomonas sp.		DBF, DBT, CBZ
Sphingomonas paucimobilis	EPA505	FLA, NAP, ANT, PHE
Sphingomonas wittichii	RW1	CDD
Terrabacter sp.	DBF63	DBF, CDBF, CDD, FLE
Xanthamonas sp.		PYR, BaP, CBZ

PYR, pyrene; BaP, Benzo[*a*]pyrene; PHE, phenanthrene; FLA, fluoranthene; FLE, fluorene; ANT, anthracene; NAP, naphthalene; BaA, benz[*a*]anthracene; dMBaA, dimethylbenz[*a*]anthracene; DBA, dibenz[*a,h*]anthracene; COR, coronene; CHR, chrysene; DBF, dibenzofuran; CDBF, chlorinated dibenzothophene; HFBT, 3-hydroxy-2-formylbenzothiophene; BP, biphenyl; CBP, chlorobiphenyl; NAT, naphthothiophene; BT, benzothiophene; BZ, benzoate; ADBT, alkylated dibenzothiophene; CBZ, carbazole; DD, dibenzo-*p*-dioxin; CDD, chlorinated dibenzo-*p*-dioxin; MNAP, methyl naphthalene.

2.5.2 Factors affecting bioremediation of petroleum hydrocarbon

In 1990, Leahy and Colwell have concluded physical and chemicals factors affecting the biodegradation of hydrocarbons as followed.

Chemical composition of oils or hydrocarbons

The structure of hydrocarbons effects to microbial attack. It has been ranked in the order of decreasing susceptibility: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. Microorganisms are the easiest to degrade the saturate, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation. Nevertheless, some case does not follow this pattern. For example, there was study reported greater degradation losses of naphthalene than of hexadecane in water-sediment mixtures from a freshwater lake.

Concentration of oils or hydrocarbon

Biodegradation rates for many hydrocarbons will not display the dependence on concentration observed with more soluble organic substrates. On the other hand, it depends on a function of the hydrocarbon surface area available for emulsification or physical attachment by cell. Heavy, undispersed oil slicks in water can cause to high concentrations of hydrocarbons in environment. Inhibition of biodegradation can be occurred from this situation by nutrient or oxygen limitation or through toxic effects by volatile hydrocarbons. There was a study reported that contamination of seashore sediments with crude oil above maximum concentration prevented biodegradation of the oil because of oxygen and/or nutrient limitation. It likely seems that high concentrations of oil have similarly negative effects on biodegradation rates following oil spills in low-energy environments such as beaches, harbors, and small lakes or ponds, in which the oil is relatively protected from dispersion by wind and wave action. The lowest rates of degradation of crude oil spilled from an oil tanker occurred in protected bays and the highest rates occurred in the areas of greatest wave energy.

Oxygen

The initial steps in the catabolism of aliphatic, cyclic, and aromatic hydrocarbons by bacteria and fungi involve the oxidation of the substrate by

oxygenases, for which molecular oxygen is required. Therefore, aerobic conditions are necessary for the pathway of microbial oxidation of hydrocarbons in the environment. Conditions of oxygen limitation normally do not exist in the upper levels of the water column in marine and freshwater environments. The availability of oxygen is dependent on rates of microbial oxygen consumption, the presence of utilizable substrates which can lead to oxygen depletion. The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in environment.

In 2006, Brambila *et al.* studied five different air modes of supplying oxygen to a membrane-aerated biofilm reactor (MABR), and search for the more efficient ways of treating wastewaters. This research found that all of the modes of oxygen supplied in a MABR were more efficient than the traditional suspended cell process.

Nutrients

The release of hydrocarbons into aquatic environments which contain low concentrations of inorganic nutrients often produces excessively high carbon/nitrogen or carbon/phosphorus ratios, or both, which are unfavorable for microbial growth. Some researches revealed that inorganic salts of nitrogen and phosphorus are effective in enclosed systems.

In recently study, there is comparative between adding and non-adding inorganic nutrient in oil-in-water emulsion. The result showed that adding NH_4NO_3 and K_2HPO_4 to make ratio of C: N: P = 100: 5: 1 can enhance degrading efficiency of immobilized cell in bioreactor and reduce time for storage oil-in-water emulsion in bioreactor.

2.6 Detection and quantification of gene in environmental samples

Due to the important of effective microorganisms in bioremediation, the survival to them in the process is a major concern. Recently, the effective method to quantify microorganism is Quantitative Real-Time PCR (qPCR). qPCR is based on detection of a fluorescent signal produced proportionally during the amplification of PCR product. The DNA binding dyes is the key to the detection system. SYBR green is the most commonly used DNA binding dye that incorporates into double-strand DNA (dsDNA), causing fluorescence to increase which is proportional to the amount

of product generated in each cycle. The detection is determined by identifying the cycle number at which the reporter dye emission intensities rises above ground noise; this cycle number is called the threshold cycle (C_t). The C_t is inversely proportional to the copy number of the target template. If the template concentration is high, the threshold cycle measured is low. A standard curve can be plotted as C_t value and log concentration of known amounts of DNA or plasmid to find out levels of unknown samples.

The advantages of using real-time PCR over other techniques are as follows:

- accurate
- sensitive
- without labor-intensive postamplification analysis
- real-time PCR collects data in the exponential growth phase, while traditional PCR is measured at end-point
- increase dynamic range of detection
- detection is capable down to a 2-fold change

This application has been used to monitor the presence of bacteria in environmental samples in several studies.

- Bladwin *et al.* (2003) developed PCR primers and qPCR protocols for enumeration of aromatic oxygenase genes of pollutant-biodegrading microorganisms. In 2009, Bladwin *et al.* used this technique to elucidate the impact of MPE operation on the aquifer microbial community structure and function at a gasoline-contaminated site. During continuous MPE, aromatic oxygenase genes (ring-hydroxylating toluene monooxygenase (RMO) and naphthalene dioxygenase (NAH)) were not detected in groundwater samples indicating decreased population of BTEX-utilizing bacteria. When MPE was pulsed, total aromatic oxygenase gene copies were not significantly different from pre-start up system. In conclusion, aerobic biodegradation had potential to dissolve BTEX during pulse MPE.

- Cunliffe *et al.* (2006) used qPCR method to study effect of inoculums pretreatment on the survival and PAH-catabolic gene (*bphC* and *xylE*) of *Sphingobium. yanoikuyae* strain B1 in an aged PAH-contaminated soil. The result suggested that using complex media, which had the higest levels of *bphC* and *xylE*

expression, instead of minimal media for cultivating biodegrader may improve efficiency of contaminant biodegradation in soil.

- Cébron *et al.* (2008) developed PCR primers to quantify Gram positive (GP) and Gram negative (GN) bacteria that can degrade PAH in soil and sediment samples with differential contamination levels by using real-time PCR assay. The result revealed that real-time PCR quantification could represent the ratio of the alpha subunit of the PAH-ring hydroxylating dioxygenase (PAH-RHD$_\alpha$) relative to the 16S rRNA gene copy number which was consistent with the PAH-bacterial biodegradation potential and the PAH-contamination level in soil and sediment samples.

2.7 *Sphingobium* sp. strain P2

Sphingobium sp. strain P2, which can utilize phenanthrene as a sole carbon source and energy source, was isolated from oil contaminated soil in Thailand (Supaka *et al.*, 2001). In 2003a, Pinyakong *et al.* identified genes for aromatic hydrocarbon degradation in this strain: ring-hydroxylating dioxygenase and aromatic ring-cleavage dioxygenase. Moreover, *Sphingobium* sp. strain P2 has been reported to have a unique group of genes for aromatic degradation with *Sphingobium yanikuyae* strain B1 and *Novosphingobium aromaticivorans* strain F199, which are distantly related to those in pseudomonads and other genera (Pinyakong *et al.*, 2003b).

The three components of ring-hydroxylating dioxygenase of *Sphingobium* sp. strain P2: ferredoxin, ferredoxin reductase, and five sets of large subunit and small subunit of terminal oxygenase; are coded by gene *ahdA3*, *ahdA4*, *ahdA1*[a–e] and *ahdA2*[a–e] respectively. In addition, Pinyakong *et al.* (2003a) reported that *bphA1f* of *Novosphingobium aromaticivorans* strain F199 is probably the presence of the sixth terminal oxygenase in strain P2. These enzymes have an important role of phenanthrene degradation in the first step in which an iron-sulfur flavoprotein reductase and an iron-sulfur ferredoxin transfer electrons from NAD(P)H to a terminal dioxygenase. The terminal dioxygenase adds both atoms of an oxygenase molecule to the aromatic nucleus of the substrate froming *cis*-dihydrodiol product (Harayama *et al.*, 1992). However, none of the five sets of terminal dioxygenase appear to have any role in the initial ring hydroxylation of phenethrene and biphenyl.

The sixth terminal dioxygenase gene (*bphA1f*) is predicted to encode the terminal dioxygenase component of biphenyl/naphthalene dioxygenase (Romine *et al.*, 1999). Furthermore, both of the electron transport components (*ahdA3A4*) are involved in the upper and lower phenanthrene catabolic pathways in strain P2 (Pinyakong *et al.*, 2003a)

The extradiol aromatic ring-cleavage dioxygenase of *Sphingobium* sp. strain P2 are 2,3-dihydroxybiphenyl 1,2-dioxygenase, and catechol 2,3-dioxygenase coded by gene *bphC* and *xylE*, respectively. In addition, most of the genes required by the strain for biodegradation of aromatic hydrocarbons have been sequenced and characterized allowing easily study to monitor their activity and survival by qPCR. In recent study, our research group studied the activity of 6 types lubricating oil degradation in oil-in-water emulsion by 10 bacterial strains. The initial amount of each bacteria was 10^6 CFU/ml was inoculated into 200 ppm of each type of lubricant oil-in-water emulsion and allowed to grow in incubator shaker (200 rpm) at room temperature for 24 h. The result showed that strain P2 has more ability for PTT V-120 synthetic oil-water emulsion degradation than other types of bacteria and lubricating oil. Moreover, this strain is simple to prepare and use a few time to growth. Therefore, strain P2 and PTT V-120 synthetic oil was selected for doing this research.

2.8 Immobilized cells

Cell Immobilization is one of the optional in incorporating bacterial biomass into a biodegradation process. The advantages of immobilized microorganism over the conventional free cell systems are

- preventing wash out of biomass in continuous flow reactors
- easy separation
- more operational flexibility
- more tolerant too high concentration of toxic chemicals
- higher cell density, resulting in higher rates of biodegradation

2.8.1 Cell immobilization process

Many methods for cell immobilization have been developed. They can be divided into 2 main immobilization processes (Cohen, 2001).

2.8.1.1 Attached growth

Attached growth is the self-attachment of microorganisms to carrier material. A variety of microbial structures which are used for attachment include fimbria (pili), capsules (glycocalyx), various holdfast structures, stalks, cell wall components and slimes. However, the main microbial structure which is believed to involve in attachment is the glycocalyx. The glycocalyx consists of extracellular polysaccharides which have no other special properties beside their participation in the attachment process. In addition, several forces are responsible for the attachment of microorganisms to a surface. Usually, none of the forces could be considered as the dominant force. The strength of the attachment and the composition of the forces are depended on environment conditions, different microbial species, surface properties and with different fluid properties.

The selection of carrier material is relied on many factors including the resistance to microbial degradation, mechanical strength, type of fluid, surface characteristics and the cost of the material.

2.8.1.2 Artificial immobilization of microorganisms to carrier material

There are several methods for this process: carrier-binding, cross-linking, entrapping methods, and encapsulation.

1. Carrier-binding

The carrier binding method is based on binding microbial cells directly to water-insoluble carriers which depends on ionic forces between the microbial cells and the water-insoluble carriers. This method can be classified into 2 types.

1.1 Adsorption

The adsorption method is the simplest method and involves reversible surface interactions between cell and support material by electrostatic forces such as van der Waals forces, ionic and hydrogen bonding interactions. The surface chemistry on cells and support is utilized so no chemical activation or modification is required and little

damage is normally done to cells. The process consists of mixing cell and a support with adsorption properties, under suitable conditions. The immobilized material is then collected, followed by extensive washing to remove non binding cells (Brickerstaff, 1997).

1.2 Covalent binding method

This method involves the formation of a covalent bond between cells and a support material by functional groups that present on the reactive groups of microbial cells and different ligands on the support (Brickerstaff, 1997).

2. Cross-linking

Microorganism can be immobilized by cross-linking each other with bi- or multifunctional reagents such as glutaraldehyde, toluendiisocyanate, or diazotized diamine. Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed microbial cells and also for preventing leakage from polyacrylamide gels (Chibata, 1981).

3. Entrapping method

Immobilization by entrapment, microbial cells are free in solution in polymer matrix. The porosity of matrix is tight enough to prevent leakage of cells, but allow free movement of substrate and product (Brickerstaff, 1997).

4. Encapsulation

Microencapsulation can be achieved by enveloping the biological component with various forms of semipermeable membrane, for example, nylon and cellulose nitrate. The cells are free in solution, but restricted in space as entrapment. Microbial cannot pass through the capsule, except small substrates and products can pass freely across the semipermeable membrane (Brickerstaff, 1997).

Figure 2.4 Principal methods of immobilization (Brickerstaff, 1997).

2.8.2 Cell immobilization for petroleum degradation

Setti *et al.* (1998) showed that adsorbent cooperated with bacterial for heavy oil remediation could enhance efficiency of heavy oil degradation by bacteria within 7 days. In contrast, the free bacterial cell took 40 days to degrade heavy oil in the same condition.

In 2006, Gentili *et al.* examined the removal of clued oil from seawater by microcosm that contained *Rhodococcus corynebacterioides* strain QBTo and concluded that the carrier material used, chitin and chitosan flasks from shrimp wastes. Moreovere, immobilization conditions and the survival of the bacterial strain under different storage temperatures were tested. Percentage removal of crude oil after 15 days, the immobilized strains could treat successfully. This study improved that chitin and chitosan was providing protective niche to bacteria, resulting in a long shelf life and a high crude oil degrading activity in sea water.

The previous research studied efficiency of oil-in-water emulsion by strain P2 immobized with chitosan from crab exoskeleton, shrimp exoskeleton, and squid axis. The research found that immobilized cell with chitosan from squid axis is the most suitable because it had high efficiency than strain P2 immobilized with chitosan from crab exoskeleton, shrimp exoskeleton and easy to use.

2.9 Chitosan

Chitin is natural amino polysaccharide and the supporting material of crustaceans, insect, etc. It is estimated to be produced annually almost as much as cellulose which is well known to consist of 2-acetamido-2-deoxy-β-D-glucose fthrough a β (1→4) linkage. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal area (Kumar, 2000).

Chitosan is the *N*-deacetylated derivative of chitin. The structures of cellulose, chitin and chitosan are shown in Figure 2.5. Chitosan have attractive properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties (Kumar, 2000), chemical stability, high reactivity, excellent chelation behavior and high selectivity toward pollutants (Bhatnagar *et al.*, 2009). For these reasons, chitosan are recommended as suitable functional materials for many applications especially detoxification of water and wastewater. Due to its low cost compared to activated carbon and its high contents of amino and hydroxyl functional groups, chitosan show significant adsorption potential for various aquatic pollutants e.g. metal ions, dyes, radionucildes, phenol and substituted phenols, and different anions and miscellaneous pollutants (Bhatnagar *et al.*, 2009).

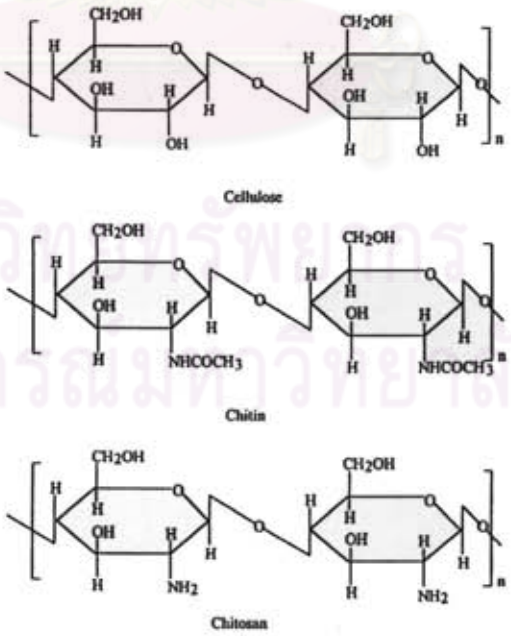


Figure 2.5 Structure of cellulose, chitin and chitosan (Kumar, 2000).

2.10 Airlift bioreactor system

Airlift bioreactor is air introduced into the riser section of the airlift and drives the circulation of the gas, liquid and solid (biofilm) phase throughout the reactor (Nicolella *et al.*, 2000). It can be divided into 2 main types of reactors on the basis of their structure (Figure 2.6): 1) external loop vessels, in which circulation takes place through separate and distinct conduits; and 2) baffled (or internal loop) vessels, in which baffles placed strategically in a single vessel create the channels required for the circulation. The designs of both types can be modified further, leading to variations in the fluid dynamics, in the extent of bubble disengagement from the fluid, and in the flow rates of the various phases.

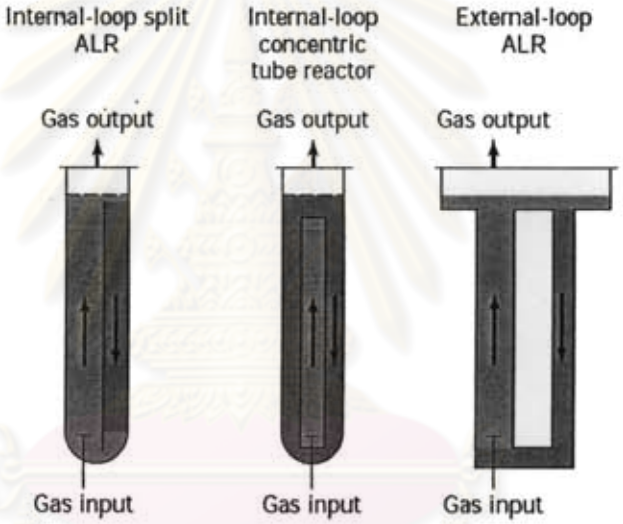


Figure 2.6 Different types of airlift bioreactor (Merchuk *et al.*, 1990)

In recent study, the strain P2 immobilized with chitosan was applied to use in air lift bioreactor which was design according to Wongsuchoto and Pavasant (2003) (Figure 2.7). This bioreactor constructed with the following characteristics: 3 liters working volume, 50 cm height, 15 cm outside diameter. An internal loop has 10 cm height and 10 cm outside diameter while 3 cm height of water above internal loop and 5 cm space between internal loop and bottom column. Furthermore, this reactor is applied to use in field-scale according to Figure 2.8

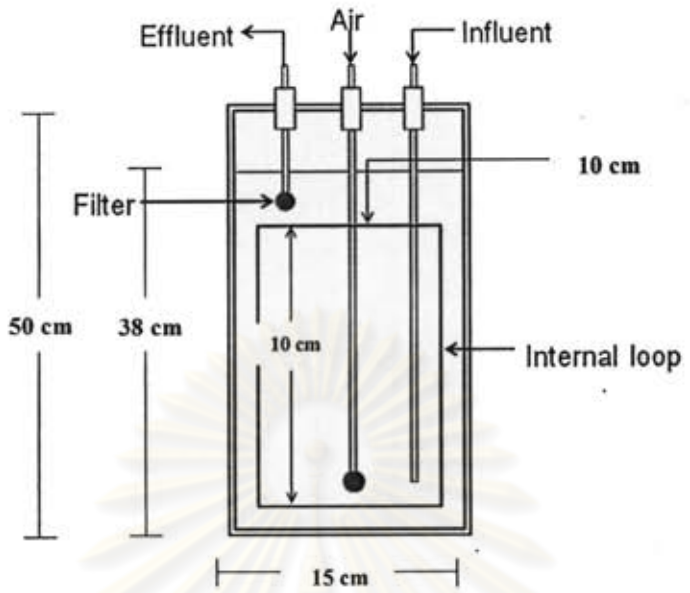


Figure 2.7 The structure of airlift bioreactor used in this study (Bioremediation research unit modified from Wongsuchoto and Pavasant (2003))

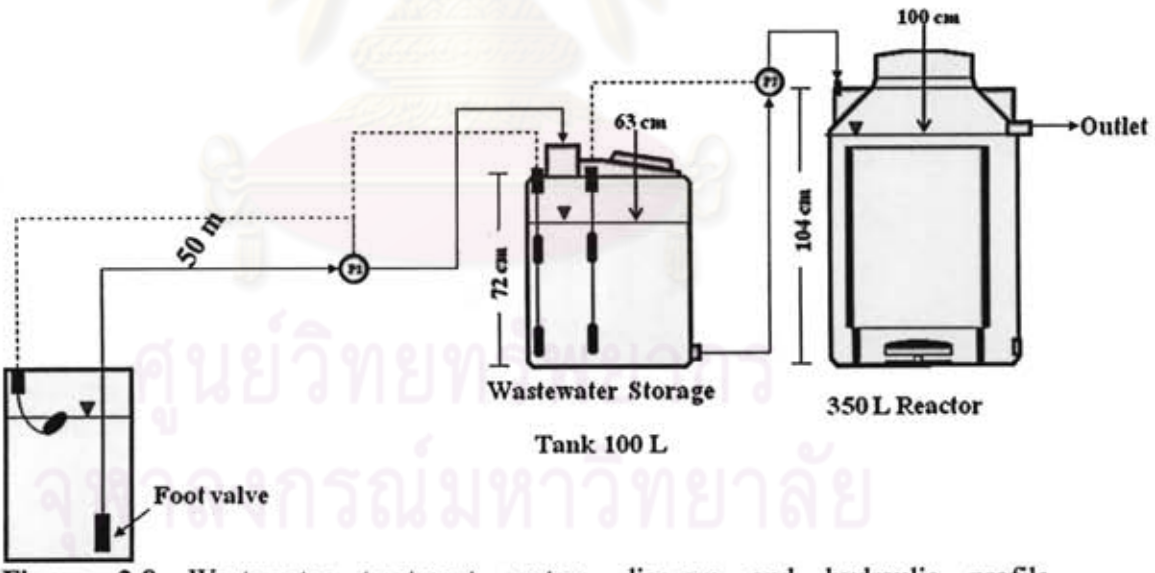


Figure 2.8 Wastewater treatment system diagram and hydraulic profile (Bioremediation research unit)

CHAPTER III

METHODOLOGY

3.1 Research overview

The methodology of this study divided into 3 parts. First, Primer design and specificity test. Second, determination the survival of *Sphingobium* sp. strain P2 during lubricating oil degradation with different air mode in flask-laboratory scale. Third, application immobilized cell to reactor-scale. Both step 2 and 3 was analyzed data by microbial enumeration, lubricating oil extraction and quantification by quantitative real-time PCR assay for determination of gene copy number. Research flow chart was illustrated in Figure 3.1

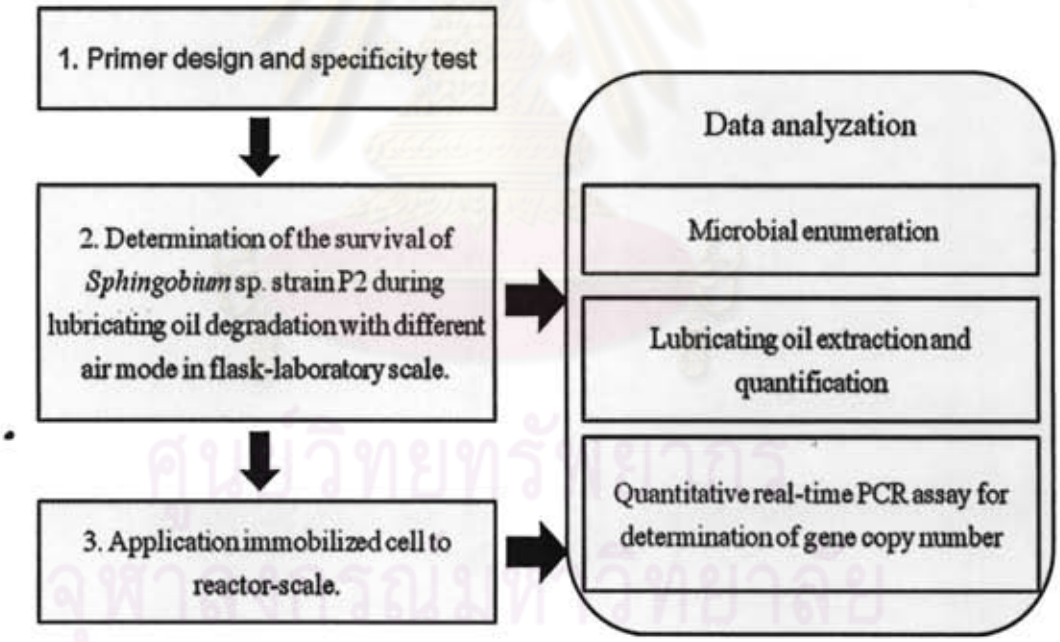


Figure 3.1 Flow chart of the research.

3.2 Chemicals and equipments

3.2.1 Chemicals

1. 100 base pair DNA ladder plus was obtained from New England Biolabs, and Fermentas, USA
2. iScript™ Select cDNA Synthesis Kit, Bio-Rad Laboratories Inc., USA.
3. 1x iQ™ SYBR® Green Supermix, Bio-Rad Laboratories Inc., USA.
4. Agarose gel was obtained from IUAI, Japan
5. Bacto agar was obtained from Difco, USA.
6. Chloroform was obtained from Lab-Scan, Ireland
7. Dichloromethane was obtained from Merck, Germany
8. EDTA (ethylenediaminetetraacetic acid), ($C_{10}H_{14}N_2O_8Na_2 2H_2O$) was obtained from Sigma, USA
9. Flake squid axis chitosan was obtained from E-base company, Thailand
10. Glacial acetic acid (CH_3COOH) was obtained from Merck, Germany
11. Glycerol was obtained from Research organics, Inc., USA
12. Hexane was obtained from J.T.Baker, USA
13. Hydrochloric acid (HCl) was obtained from BDH Chemicals, Australia
14. Isoamylalcohol was obtained from Sigma, USA
15. Lambda *Hin*dIII was obtained from New England Biolabs, USA
16. Lubricating oils (PTT V-120) was obtained from Petroleum Authority of Thailand (PTT), Thailand
17. Magnesium sulfate ($MgSO_4.7H_2O$) was obtained from Carlo ERBA, France
18. Methanol was obtained from Fisher Scientific, UK
19. Phenol was obtained from Merck, Germany
20. Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck, Germany
21. Proteinase K was obtained from US. Biological, USA
22. Quant-iT™ dsDNA BR Assay Kits, Invitrogen, USA
23. Ribonuclease A (RNase A) was obtained from Fermentas, USA
24. SDS (sodium dodecyl sulfate), ($C_{12}H_{25}OSO_3$) was obtained from Nacalai tesque, Japan

25. Sodium chloride (NaCl) was obtained from Merck, Germany
26. Sodium hydroxide (NaOH) was obtained from Merck, Germany
27. *Taq* DNA polymerase was obtained from New England Biolabs, USA
28. Trizma base (tris [hydroxymethyl] aminomethane), ($C_4H_{11}NO_3$) was obtained from Sigma, USA
29. Tryptone was obtained from Difco Laboratories, USA
30. Tween 80 was obtained from Merck, Germany
31. Yeast extract was obtained from Difco Laboratories, USA

3.2.2 Equipments

1. Autoclave, Kakusan, Japan
2. Balance, model P2002-S and AG285, Mettler Toledo, Switzerland
1. Bench-top centrifuge, model Mikro20, Hettich zentrifugen Inc., USA
2. Blender S-600, Philips, Thailand
3. Deep freezer (-20°C), model MDF-U332, Sanyo Electronic, Japan
4. Deep freezer (-70°C), model ULT 1786, Forma Scientific, Japan
5. Digital Dry Bath, model D1100,Labnet International, Inc., USA
6. DNA Thermal Cycler, model 2400, Perkin Elmer, USA and model MJ Mini™ Personal Thermal Cycler, Biorad,USA
7. Gel documentation system, model Gel DOC 2000™, Bio-Rad Laboratories Inc., USA.
8. Hot air oven, model D06063, Memmert, Germany
9. Incubator (30°C), model BE800, Memmert, Germany
10. ISSCO laminar flow, International Scientific Supply, Japan
11. Micropipette (2, 10, 20, 200, 1,000 and 5,000 µl), Gilson, France
12. MiniOpitcon Real-Time PCR detector, Bio-Rad Laboratories Inc., USA.
13. Oven, Contherm Scientific, New Zealand
14. pH meter, model 240, Corning, USA
15. Qubit fluorometer, Invitrogen, USA
16. Rotary vacuum evaporator , EYELA, Japan
17. Spectrophotometer, model UV-160A, Shimadzu, Japan
18. TLC-FID, model IATROSCAN™ MK-6s, Mitsubishi Kaguku Iatron. Inc., Japan

19. Ultrasonicator, model FS4000, Decan Ultrasonics, England
20. UV transilluminater, Fotodyne Co., Inc., USA
21. Vortex mixer, model Genie 2, Scientific Industries, USA

3.3 Primer design and specificity primer test

3.3.1 Bacterial strain and growth condition

Sphingobium sp. strain P2 (Pinyakong *et al.*, 2000) was cultured from freshly streaked two days Luria Bertani or LB agar plates (Sambrook *et al.*, 1989) into 50 ml 0.25 x LB media. After incubation at 30°C for 24 h in an incubation shaker (200 rpm), the culture was spun down at 8,000 rpm for 10 min. the cell pellets were suspended in 0.85% NaCl to wash cell and centrifuged at 8,000 rpm for 10 min in duplicate.

Escherichia coli JM 109 (Sambrook *et al.*, 1989) will be grown in LB media and will be incubated overnight at 37°C, *Pseudoxanthomonas* sp. strain RN402 (Klankeo *et al.*, 2009) will be grown in CFMM medium containing 100 ppm of pyrene as described previously and was incubated at 30 °C for 7 days, *Acinetobacter* sp. strain R2 (Tathong, 2007) was incubate in LB media overnight at 30°C.

3.3.2 DNA preparation

3.3.2.1 Genomic DNA extraction

Genomic DNA was extracted as described by Ausubel et al. (1990) with lightly application. Cell suspension was poured into Eppendrof tubes and centrifuged at 8,000 rpm in microcentrifuge for 6 min. Cell pellets was suspended in 510 µl Tris-EDTA buffer pH8 with vigorous shake, was added 50 µl of lysozyme solution (60 mg/ml in TE buffer) and was incubated at 37°C for 30 min. Thirty microliters of 20% SDS (sodium dodecyl sulphate) and 10 µl of proteinase K solution (10 mg/ml in sterilized distilled water) was added, mixed gently, and incubated at 37°C for 30 min. One hundred and twenty of 5 M NaCl was put in the mixture and inverted 4-5 times, then 250 µl of hexadecyl trimethyl ammoniumbromide/sodium chloride solution (CTAB/NaCl solution), which was warmed at 65°C, added, mixed well, and incubated for 20 min. DNA was separated by putting approximately equal volume of chloroform and centrifuged at 13,000 rpm for 15 min. After that, DNA in aqueous phase (upper phase) was transferred into new tube and put estimate equal volume of

phenaol:chloroform (25:25), centrifuged at 13,000 rpm for 10 min. DNA from upper phase was transfered into new tube and precipitated with 0.6 volume of isopropanol. The solution was gently mixed by inverting for 1 min. The precipitate was collected by centrifugation at 13,000 rpm for 5 min, washed with 70% ethanol and centrifuged at 13,000 rpm for 5 min. After removal supernatant, the pellet was dried in desicator and dissolved with 50 µl of TE buffer containing 0.2 µl of DNA free RNase solution (10 mg/ml in 0.01M sodium acetate pH 5.2). Finally, The DNA solution was incubated at 37°C for 60 min. DNA will be checked by running on 0.9% LE agarose (IUAI, Japan). DNA concentration was measured the absorbance at wavelength of 260 and 280 nm.

3.3.2.2 Gel electrophoresis and DNA concentration measurement

Agarose gel 0.9% was prepared in TAE buffer and poured into gel tray with comb on the tray and let the gel stand until set. The set of agarose gel was put into the chamber and poured TAE buffer until completely cover the whole gel. 2 µl of DNA solution was mixed with 1 µl of 6x loading dye and dropped into the hole of gel. The first hole was dropped by 2 µl of Lambda *Hin*dIII DNA marker that already mixed with loading dye. Run electrophoresis at 100 volts for 27 min. Then, the agarose gel was stained in ethidium bromide for 15 min and destained with water for 10 min. The DNA band can be detected under UV transilluminater at the wavelength of 312 nm.

DNA purity and concentration could be done by measuring the absorbance at wavelength of 260 and 280 nm. Then calculated DNA concentration by using equation below.

Double helix DNA concentration (µg/ml) = A_{260} x 50 x dilution factor

3.3.3 Amplification of genes involved in lubricating oil degradation using polymerase chain reaction (PCR)

3.3.3.1 Primer design

DNA sequences of dioxygenase genes were retrieved from GenBank accession no. AB091692. PCR primers were designed using Primer3 software version 0.4.0 which specific for ferredoxin of dioxygenase, ferredoxin reductase of

dioxygenase, and 2,3-dihydroxybiphenyl 1,2-dioxygenase. The size of design amplicon should be 100-250 bp. Shorter amplicons are typically amplified with higher efficiency. An amplicon should be at least 75 bp to easily distinguish it from any primer-dimers that might form. The amplicon should avoid secondary structure, and maintain a GC content of 50-60%. The other primers were obtained according to Table 3.1.

3.3.3.2 PCR condition for specificity test

Each primer set was tested to observe single amplification product from positive control (*Sphingobium* sp. strain P2). PCR was performed in a total volume of 30 µl containing 20 µM dNTP (Fermentas), 20 pmol of each primer, 2 mM of $MgCl_2$, and 0.5 U *Taq* DNA Polymerase (Biolabs) (or 0.5 U *ExTaq* DNA Polymerase for ahdA3RT and ahdA4RT primers), and 1 µl of template DNA from positive control strain (*Sphingobium* sp. strain P2) and negative control strains (*Acinetobacter* sp. strain R2; *Pseudoxanthomonas* sp. strain RN402; *Escherichia coli* JM 109).

The condition of PCR was described as follow:

1.	Initial denaturation step	at 94°C	for 2 min
2.	Denaturing step	at 94°C	for 30 sec
3.	Annealing step	as each designed primer	for 30 sec
4.	Extension step	at 72°C	for 45 sec
5.	Go to step 2 for 29 times		
6.	Final extension step	at 72°C	for 6 min.

3.4 Determination the survival of *Sphingobium* sp. strain P2 during lubricating oil degradation with different air mode in flask-laboratory scale.

3.4.1 Artificial wastewater preparation

The artificial wastewater was prepared by a Philip's blender which consists of 200 mg/l lubricating oil (PTT V-120), distilled water, and 0.1 wt% a non-ionic emulsifier (Tween-80) for stabilized mixer. The mixer then was stirred with stirrer for 20 minutes. This artificial wastewater concentration is close to found in car wash wastewater in Thailand (Panpanit, 2001).

Table 3.1 PCR primer used in this study

Primers	Target	Sequence 5'→3'	Position	Expected product size (bp)	Annealing temperature (°C)	Reference
alkM-F alkM-R	Gene encoding for alkane hydroxylase	GAGACAAATCGTCTAAAACGTAA TTGTTATTATTCCAACTATGCTC		271	48	Kohno *et al.*, 2002
bphA1f-F bphA1f-R	Gene encoding for alpha subunit of ring hydroxylating dioxygenase	TATTTGGGGGACTTCTGCTG TTGGGAGGATCGACGTATTC	478 901	424	52	In recent study
xylE-F xylE-R	Gene encoding for catechol 2,3-dioxygenase	GGCACTGACCGGTGTACTTCG CGACCTTGAAGGCCATCC	3 217	215	57	Cunliffe *et al.*, 2006
16S-F 16S-R	Gene encoding for 16S rDNA	GTGAGTGATGAAGGCCTTAG CTTTACGCCCAGTAATTCCG	353 499	147/172	55	Kouzuma *et al.*, 2006

3.4.2 *Sphingobium* sp. strain P2 cell preparation

For inoculum preparation, *Sphingobium* sp. strain P2 was inoculated from freshly streaked two days Luria Bertani (Sambrook *et al.*, 1989) or LB agar plates into 50 ml 0.25 x LB media. After incubation at 30°C for 24 h in an incubation shaker (200 rpm), the culture was spun down at 8,000 rpm for 10 min. the cell pellet was suspended in 0.85% NaCl to wash cell and centrifuged at 8,000 rpm for 10 min in duplicate.

3.4.3 Cell immobilization on carrier material

The cell was cultured together with chitosan flakes in CFMM medium with lubricating oil. The cell previously prepared from 3.4.2 was added into 500 ml CFMM medium containing 2.25% (v/v) lubricating oil, and 1.25 g of chitosan. After incubation at room temperature for 24 hr in shaker (150 rpm), the immobilized cell was poured through sterilized paper filter (Whatman No. 4) for free cell separation from immobilized cell. Finally, the immobilized cell was dried in laminar flow for 4 hr.

3.4.4 Degradability test

To study the effect of the mode of aeration air, the following method was tested:

1) with air supplying by testing in 250 ml flask and shaking at 200 rpm, 30 °C.

2) without air supplying by testing in closed bottle and shaking at 200 rpm, 30 °C..

This study was divided into two parts: *Sphingobium* sp. strain P2 in the form of free cell and immobilized cell with chitosan.

3.4.4.1 Degradability test with *Sphingobium* sp. strain P2 in the form of free cell

The cell pellets of *Sphingobium* sp. strain P2 from 3.4.2, which was suspended in 0.85% NaCl, inoculated into 80 ml of artificial wastewater in 250 ml flasks and closed bottles to make final OD_{600} equal to 0.2 and allowed to grow at 30°C. The flasks which contain only artificial wastewater were used as control. The sample and the control was collected every day for five days in triplicate to analyze microbial

enumeration, amount of lubricating oil recovery, and survival of *Sphingobium* sp. strain P2 as method described in 3.6, 3.7, 3.8, respectively.

3.4.4.2 Degradability test with *Sphingobium* sp. strain P2 in the form of immobilized cell with chitosan

The immobilized cell from 3.4.3 (2.5 g/l) was added in 80 ml of artificial wastewater in 250 ml flasks and closed bottles and allowed to grow at 30°C. The flasks which contain only chitosan and artificial wastewater were used as control. The sample and the control was collected every day for five days in triplicate to analyze microbial enumeration, amount of lubricating oil recovery, and survival of *Sphingobium* sp. strain P2 as method described in 3.6, 3.7, 3.8, respectively.

3.5 Application immobilized cell to Reactor-Scale.

3.5.1 Wastewater collection

Wastewater from petrol station was collect from grease-trapping tank.

3.5.2 Cell immobilization on carrier material

Immobilized cell was prepared followed method in 3.4.3

3.5.3 Application of lubricating oil degradation with immobilized cells in 2 different size airlift bioreactors

This experiment was performed in 2.5 g/l of immobilized cell added into the 3 l and 350 l airlift bioreactor continuous system. The hydraulic retention time (HRT) was 2 hr. The control of each sample was influent wastewater to airlift bioreactor. The wastewater and immobilized cell from 3 l airlift bioreactor was collected every 3 days and every week, respectively for 1 month in triplicate. In a part of 350 l airlift bioreactor, the wastewater and immobilized cell was collected every 3 days and every 2 weeks, respectively for 2 month in triplicate. The samples was collected to analyze microbial enumeration, amount of lubricating oil recovery, and survival of *Sphingobium* sp. strain P2 as method described in 3.6, 3.7, 3.8, respectively.

3.6 Microbial enumeration

3.6.1 Total cell count in oil-in-water emulsion

The 10 µl of serial ten-fold dilution (10^{-2}-10^{-7}) of cell suspension in oil-in-water was dropped on LB agar plate in triplicate to determine the total amount of bacteria. The plate was incubated at 30°C for 2 days. The number of strain P2 was calculated in CFU per 1 ml of artificial wastewater.

3.6.2 Total cell count in carrier material

To monitor the survival of bacteria in chitosan, 0.01 g of the dry weight immobilized cell was suspended in 1.50 ml CFMM medium and stand for 2 min. This immobilized cell was sonicated for 3 min and immediately vigorously mixed with vortex mixer for 2 min in duplicate. The 10 µl of serial ten-fold dilution (10^{-2}-10^{-7}) of cell suspension was dropped on LB agar plate in triplicate to determine the total amount of bacteria. The plate was incubated at 30°C for 2 days. The number of strain P2 was calculated in CFU per total weight of chitosan.

3.7 Lubricating oil extraction and quantification

3.7.1 Lubricating oil extraction

The lubricating oil was extracted from the samples by using chloroform as a solvent. For enhance efficiency, NaCl was added with 0.25M final concentration (Rajesh et al., 2005). After adding NaCl, 4 ml of chloroform and 1 ml of 6.25 mg/ml stearyl alcohol (internal standard) was added and shaken at 200 rpm for 30 min in duplicate. Chloroform phase then was taken into new tube and evaporated until final volume was about 1 ml.

3.7.2 Lubricating oil quantification

The 1 µl of the extract was dropped in silica rod (Chromarod) and incubated in solvent tanks including n-hexane for 10 cm (25 min), dichloromethane (DCM) for 6.5 cm (12 min) and 4 cm (5 min), DCM/methanol (95/5, v/v) for 1 cm (1 min), respectively to increasing polarity for separate saturated and aromatic hydrocarbon from resin/asphaltenes. Finally, the silica rods were quantified by using TLC-FID (IATROSCAN MK-6 TLC-FID Analyzer) with scan speed of 30 sec/rod. Flow rate of hydrogen for the FID was 160 ml/min. The retention time of stearyl alcohol,

saturates, aromatics, resin, and asphaltenes was approximately 0.35, 0.13, 0.24, 0.42, and 0.47 min, respectively. Amount of lubricating oil was analyzed by comparing to a standard curve of lubricating oil.

3.8 Quantitative Real-time PCR assay for determination of gene copy number

3.8.1 Recovery of *Sphingobium* sp. strain P2

The samples were recovered *Sphingobium* sp. strain P2 to determine gene copy number by following method.

3.8.1.1 Recovery of *Sphingobium* sp. strain P2 in the form of free cell

The wastewater samples from 3.4.4.1 and 3.5 were centrifuged for 15 min at 12,000 rpm, 4°C to collect cell pellet. The cell pellet was extracted DNA as describe in 3.3.2.1 and quantify gene copy number.

3.8.1.2 Recovery of *Sphingobium* sp. strain P2 in the form of immobilized cell with chitosan

The wastewater samples from 3.4.4.2 and 3.5 were done as well as method in 3.8.1.1. The immobilized cell sample from 3.4.4.2 and 3.5 were suspended in 1.50 ml 0.85% NaCl and stand for 2 min. This immobilized cell was sonicated for 3 min and immediately vigorously mixed with vortex mixer for 2 min in duplicate. The suspension cell was taken into new microtube and centrifuged for 15 min at 12,000 rpm to separate cell and aqueous. The cell pellet was extracted DNA as describe in 3.3.2.1 and quantify gene copy number.

3.8.2 Standard for Real-time PCR calibration

External standards used to determine gene copy number was prepared using plasmid, which had selected gene in Table 2.2. The plasmid concentration was measured by using Quant-iT™ dsDNA BR Assay Kits with Qubit fluorometer. The plasmid copy number was calculated using the following equation (Vaitomaa *et al.*, 2003):

$$\text{Number of copies per microliter} = \frac{(6 \times 10^{23})(\text{DNA concentration})}{\text{molecular weight of one genome}}$$

The molecular weight of 1 bp is 660 g/mol. 6 x 10^{23} was the number of copies per mole. The DNA concentration was given in grams per microliter. The molecular weight of one genome was given in grams per mole. Series of 10-fold dilutions of the plasmid was prepared, and these dilutions of the plasmid were amplified with DNA samples of *Sphingobium* sp. strain P2. Linear regression equation for obtained cycle threshold values (Ct) was calculated as a function of known plasmid copy number.

Table 2.2 Plasmid was be used in this study (Pinyakong *et al.*, 2003a)

Plasmid	Relevant characteristics
pUPA3412d	0.5-kb *Bgl*II-*Bam*HI fragment of pTA2d inserted into *Bam*HI-digested pUPA341d; carries *ahdA3*, *ahdA4*, and *ahdA2d*
pMC4	5-kb fragments containing *xylE* and flanking region of strain P2 in pUC18
pMC3	9.2-kb fragments containing *bphC* and flanking region of strain P2 in pUC18

3.8.3 Real-time PCR for quantification gene

The primer set adhA3RT which act as marker gene was used for quantify amount of *Sphingobium* sp. strain P2 in bioreactor by real-time PCR assay. Real-time PCR experiment was performed in a MiniOpitcon Real-Time PCR detector associated with MJ Opticon Monitor Analysis Software (version 3.1, Bio-Rad). The reaction was conducted in 0.2 ml thin wall, clear PCR strip tubes which had 20 µl reaction volumes containing 1x iQ™ SYBR® Green Supermix, 0.2 µM of selected primers, and 2 µl of template DNA (10 times dilution series of plasmid standard and DNA samples). The amplifications were carried out with following temperature profiles:

1. Initial denaturation step at 95°C for 3 min
2. Denaturing step at 95°C for 15 sec
3. Annealing step at 49.1°C for 30 sec
4. Extension step at 72°C for 30 sec
5. Plate read
6. Go to step 2 for 39 times
7. Melting Curve from 65 °C to 95 °C, read every 0.5 °C, hold 10 sec

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Primer design and specificity primer test

4.1.1 Design primer of gene involved in lubricant oil degradation

In recent study, *Sphingobium* sp. strain P2 had high efficiency to degrade lubricating oil. Therefore, this strain was applied to treat wastewater in petrol station. It is necessary to detect and enumerate this strain in wastewater treatment system for assessment efficiency of the system. Molecular genetic techniques have ability to characterize microbial population in situ by detection and quantification on gene which respects to degradation pathway. In this experiment, PCR primer sets targeting dioxygenase genes involved in aromatic compound degradation of this strain were designed for quantification by real-time PCR.

PCR primers were chosen from DNA sequences of each group of dioxygenase in *Sphingobium* sp. strain P2. The DNA sequences were retrieved from GenBank accession no. AB091692. The PCR primers which specific for ferredoxin of dioxygenase, ferredoxin reductase of dioxygenase, and 2,3-dihydroxybiphenyl 1,2-dioxygenase, respectively, were designed using Primer3 software version 0.4.0. A description of the PCR primers is shown in Table 4.1.

Table 4.1 PCR primer designed in this study.

Primer	Target	Sequence	Start	Expected product size (bp)
ahdA3RT-F ahdA3RT-R	ferredoxin	5'-GACGGGGTATGTCTTGATGG 5'-TTCGTCACCGACAATCTTTG	51 212	162
adhA4RT-F adhA4RT-R	ferredoxin reductase	5'-TCAACAACGGGATCATCGTC 5'-ACATCGGCTTGCAGTAGGAG	764 958	195
bphC-F bphC-R	2,3-dihydroxybiphenyl 1,2-dioxygenase	5'-CACGAACTTTCCGTACATGG 5'-CTCCGACGATCTTGCCTATC	478 727	250

4.1.2 Primer testing for *Sphingobium* sp. strain P2 detection

After PCR primers were designed or obtained from other researches, each primer set was tested and optimized PCR condition with DNA of *Sphingobium* sp. strain P2 by PCR amplification. Primer sets adhA3RT and adhA4RT were specific to *adhA3* and *adhA4* (Ferredoxin and Ferredoxin reductase: components of ring-hydroxylating dioxygenase). Primer sets bphC and xylE were specific to *bphC* and *xylE* (2,3-dihydroxybiphenyl 1,2-dioxygenase and catechol 2,3-dioxygenase: group of extradiol aromatic ring-cleavage dioxygenase). Primer set bphA1F was specific to *bphA1f* (the terminal dioxygenase component of biphenyl/naphthalene dioxygenase). The last primer sets was alkM which specific to *alkM* (catalizing *n*-alkane degradation that has carbon atom more than 12).

For primer sets adhA3RT, bphC, and xylE, the amplification with *Sphingobium* sp. strain P2 DNA and positive-control containing the target yielded amplification products of the expected size (Figure 4.1-4.3). In the meantime, no PCR product was observed with *Sphingobium* sp. strain P2 DNA and primer sets adhA4RT (Figure 4.1), bphA1F, and alkM (Data not shown). As a result, primer sets adhA4RT might not amplify with optimized condition because positive-control and *Sphingobium* sp. strain P2 DNA still had non-specific bands (Figure 4.1). In the part of primer sets bphA1F, *bphA1f* of *Novosphingobium aromaticivorans* strain F199 is probable the presence of the sixth terminal oxygenase in strain P2 (Pinyakong *et al.* 2003a). Moreover, this primer set was designed from *bphA1f* sequence of strain F199, so it might not have complimentary with DNA sequence of strain P2. In the case of primer set alkM, this primer might not specific for bacterial strain *Sphingobium* or *Sphingomonas* (Kohno *et al.*, 2002). For these resons, the primer sets selected in the next experiment were bphC, adhA3RT, and xylE.

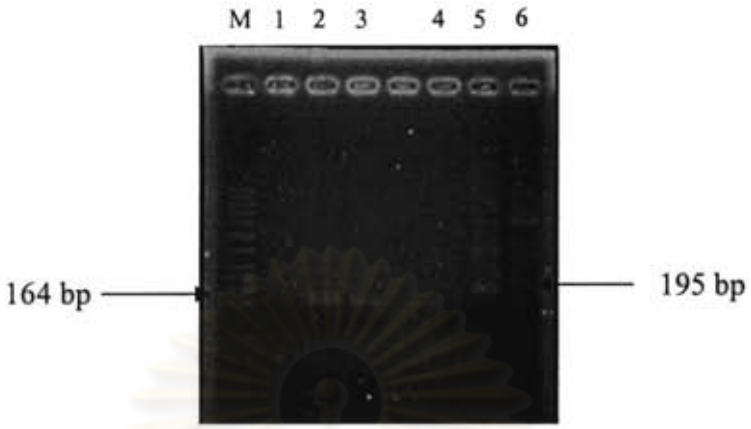


Figure 4.1 PCR products of primers adhA3RTF, adhA3RTR, adhA4RTF and adhA4RTR using plasmid pUPA342c and *Sphingobium* sp. strain P2 as DNA template. The specific products were indicated by arrow. Lane M: 50 bp ladder DNA marker, Lane 1: negative control, Lanes 2-3: PCR product of primers adhA3RTF and adhA3RTR using plasmid pUPA342c (positive control) and DNA of *Sphingobium* sp. strain P2 as template, respectively. PCR product size was 164 bp. Lane 4: negative control, Lanes 5-6: PCR product of primers adhA4RTF and adhA4RTR using plasmid pUPA342c (positive control) and DNA of *Sphingobium* sp. strain P2 as template, respectively. PCR product size was 195 bp.

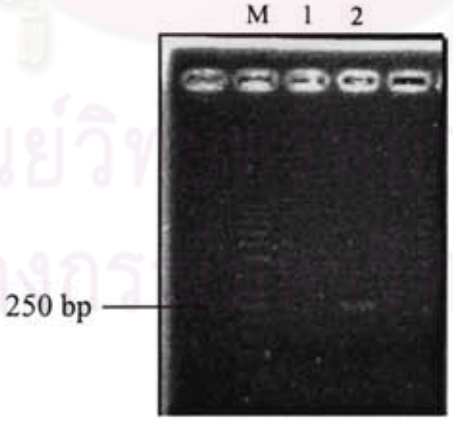


Figure 4.2 PCR products of primer bphC F and bphC R using *Sphingobium* sp. strain P2 as DNA template. The specific product was indicated by arrow. Lane M: 50 bp ladder DNA marker, Lane 1: negative control, Lane 2: PCR product using DNA of *Sphingobium* sp. strain P2 as template. PCR product size was 250 bp.

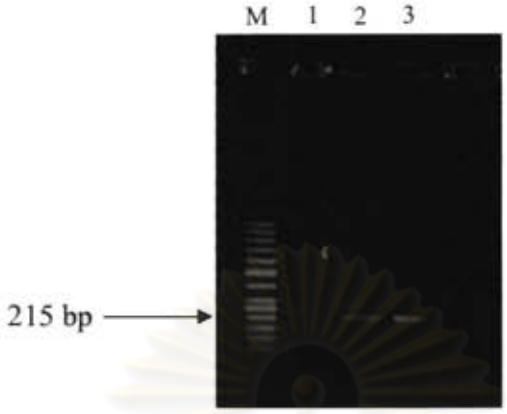


Figure 4.3 PCR products of primers xylE F and xylE R using *Sphingobium yanoikuyae* strain B1 and *Sphingobium* sp. strain P2 as DNA template. The specific product was indicated by arrow. Lane M: 50 bp ladder DNA marker, Lane 1: negative control, Lanes 2-3: PCR products using DNA of *Sphingobium yanoikuyae* strain B1 (positive control) and *Sphingobium* sp. strain P2 as template, respectively. PCR product size was 215 bp.

4.1.3 Primer specificity test

The specificity test of PCR amplification with each primer set from previous experiment was determined with DNAs from positive control strain containing the target gene (strain P2) and negative control strains (*Acinetobacter* sp. strain R2, *Pseudoxanthomonas* sp. strain RN402, and *Escherichia coli* JM 109). PCR amplifications of 16S rDNA were also done with both positive and negative control strains. As a result, PCR products of expected size were obtained from the amplification with all primers and DNA from strain P2 (Figure 4.4). While no PCR product of expected size from negative control strains was observed. Unexpected, the PCR product of primer set bphC with *Pseudoxanthomonas* sp. strain RN402 was observed nearly PCR product of target size. Therefore, PCR primer set adhA3RT was chosen for real-time PCR experiments because this primer set no any nonspecific products were observed when used to amplify based on PCR and newly designed. Furthermore, the primer set was compared to nucleotide sequence in GenBank database using software BLASTn (http://www.ncbi.nlm.nih.gov/), and the result showed no sequence similarity with other strains in this data base.

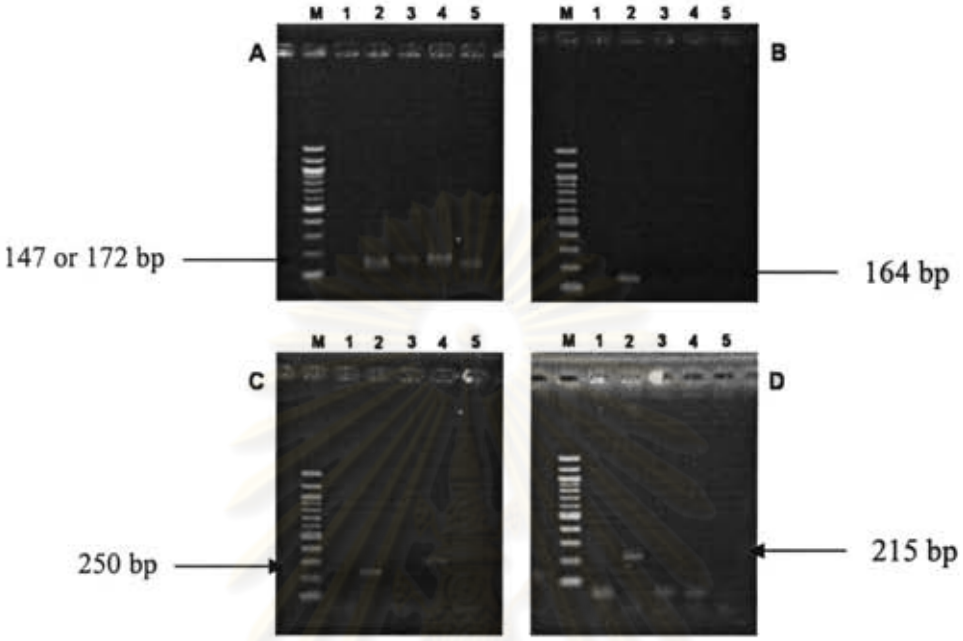


Figure 4.4 PCR products obtained when using primers specific for 16S rDNA (A), *adhA3* gene (B), *bphC* gene (C), *xylE* gene (D). Lane: M, 100 bp ladder DNA markers; Lanes 1-5: PCR product using water, DNA of *Sphingobium* sp. strain P2, DNA of *Acinetobacter* sp. strain R2, DNA of *Pseudoxanthomonas* sp. strain RN402, and DNA of *Escherichia coli* JM 109, respectively.

4.2 Determination the survival of *Sphingobium* sp. strain P2 during lubricating oil degradation with different air mode in flask-laboratory scale.

4.2.1 Lubricating oil degradability test with *Sphingobium* sp. strain P2 in the form of free cells

Lubricating oil degradability test of strain P2 with air supply and without air supply was tested in order to know the effect oxygen to degradability and survival of strain P2 in the form of free cells. This experiment was conducted using 80 ml of 200 ppm lubricating oil-in-water emulsion in 250-ml flasks and closed bottles. All flasks and closed bottles were shaken at 200 rpm, room temperature for 5 days. Then, samples were extracted using chloroform with NaCl addition and analyzed for the amount of oil every day using TLC-FID. The flask with no bacterial addition was used as a control.

Moreover, primer specific for gene encoding for ferredoxin of ring-hydroxylating enzyme (*adhA3*) was used to quantify the survival of strain P2 in oil-in-water emulsion calculated from standard curve equation (Figure 4.7). In addition, number of bacterial survival during the experiment was also determined by total plate count.

According to Figure 4.5, amount of lubricant oil from air supply condition decreased to 78.71% of initial concentration within 1 day and dramatically decreased to 24.84% of initial concentration in day 5. On the other hand, amount of lubricant oil from without air supply condition decreased from 83.42 % of initial concentration in day 1 to 52.62% of initial concentration in day 5. As a result, number of bacteria from air supply condition was no significant change which was ranged from 2.40×10^{10} to 4.00×10^{10} CFU/80 ml (Figure 4.6). Meanwhile, the number of bacteria of without air condition decreased from 4.00×10^{10} to 1.12×10^{9} CFU/80 ml (Figure 4.6). Furthermore, the result of the detection of strain P2 by real-time PCR technique shown in Figure 4.8 revealed that in the first 2 days of incubation, gene copies of *adhA3* gene had similar trend in both conditions. Afterwards, amount of *adhA3* gene copies increased dramatically in air supply condition from 3.14×10^{9} to 4.19×10^{10} *adhA3* gene copies/ 80 ml. On the other hand, amount of *adhA3* gene copies in without air supply condition increased until day 2 from 3.50×10^{9} to 1.57×10^{10} *adhA3* gene copies/ml and decreased to 6.09×10^{8} *adhA3* gene copies/80 ml in day 5. Accordingly, the number of bacteria using total plate count and quantitative real-time PCR also resemble with amount of lubricant oil recovery. It seems probably that growth of strain P2 was limited by air supply, and the activity of oil degradation was inhibited when oxygen was not enough. The requirement of oxygen in enzyme reaction involved in oil degradation might be the reason for this (Peng *et al.*, 2008).

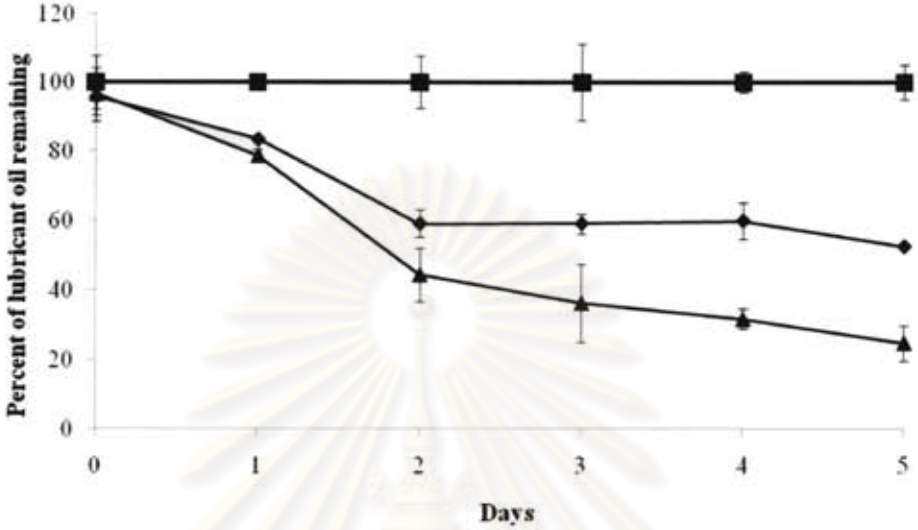


Figure 4.5 Percent of lubricant oil recovery during degradation by *Sphingobium* sp. strain P2 of air supply and without air supply condition within 5 days: control (■), lubricant oil recovery in the present of *Sphingobium* sp. strain P2 from air supply (▲) and without air supply (◆) conditions

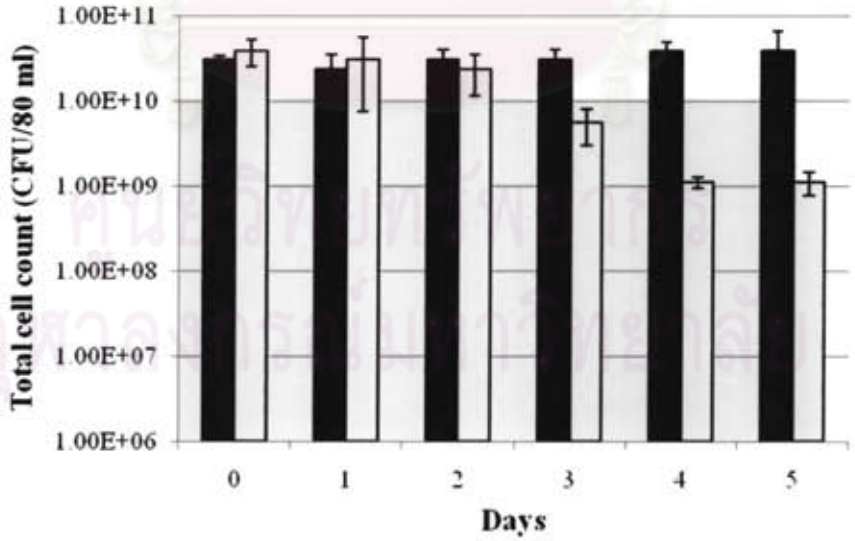


Figure 4.6 Survival of bacteria in oil-in-water emulsion in each different air mode after 5 days of incubation: with air supply condition (■), and without air supply condition (□).

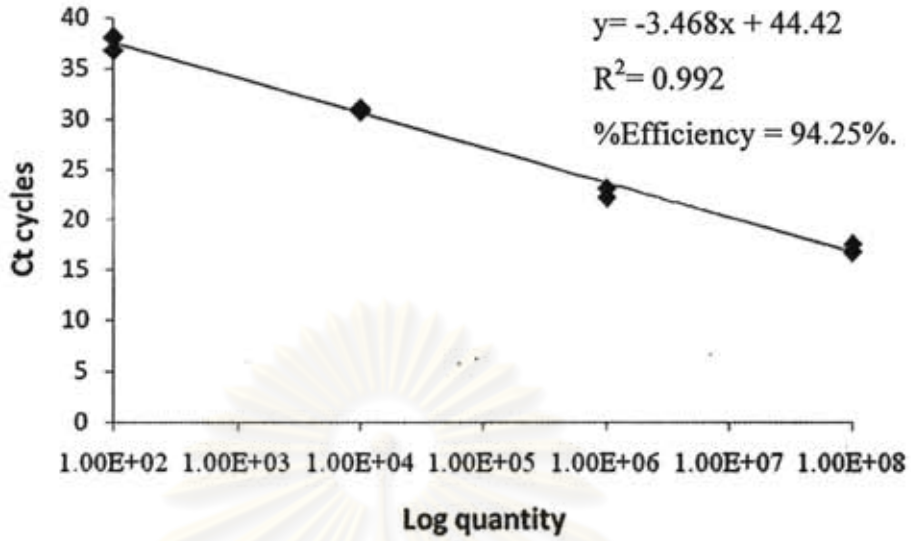


Figure 4.7 Standard curve of the *AdhA3* gene copy numbers from Real-Time PCR amplification assays obtained by plotting the logarithm of the gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value.

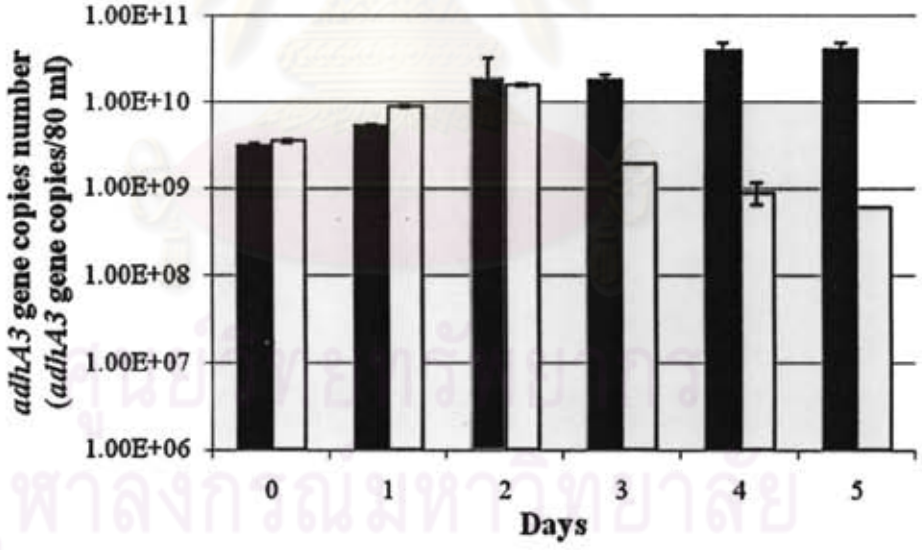


Figure 4.8 *adhA3* gene copy numbers by real-time PCR using oil-in-water emulsion sample after 5 days incubation: with air supply condition (■), and without air supply condition (□).

4.2.2 Lubricating oil degradability test with *Sphingobium* sp. strain P2 in the form of immobilized cells with chitosan

The chitosan-immobilized cells of strain P2 were prepared by cultutivation of the bacteria with chitosan in CFMM media containing 200 ppm lubricating oil. Chitosan-immobilized cells were then separated and dried before using for oil-in-water emulsion treatment. Chitosan-immobilized cells (2.5 g/l) were added to 200 ppm artificial wastewater in 250-ml flasks and closed bottles for determination of lubricating oil degradability and survival of strain P2 in air supply and without air supply conditions. Control treatment was the oil-in-water emulsion that had only 2.5 g/l flake chitosan.

The amount of lubricating oil remained in the control treatment decreased until day 3 (73.87% lubricating oil recovery) and rose again till day 5 (99.87% lubricating oil recovery) (Figure 4.9). It was probably that the adsorbed residue oil clogged in the pores of chitosan (Ahmad *et al.*, 2005). When time goes by, the force of amino site in chitosan was not strong enough to bind residue oil because of centrifugal force of shaker. According to the result, percent of lubricating oil recovery of chitosan-immobilized cells in air supply condition decreased to 40.16% of initial concentration in day 5(Figure 4.9). While percent of lubricating oil recovery of chitosan-immobilized cells in without air supply condition decreased to 55.46% of initial concentration in day 3 and gradually increased to 63.24% of initial concentration. This result might have occurred as the control treatment.

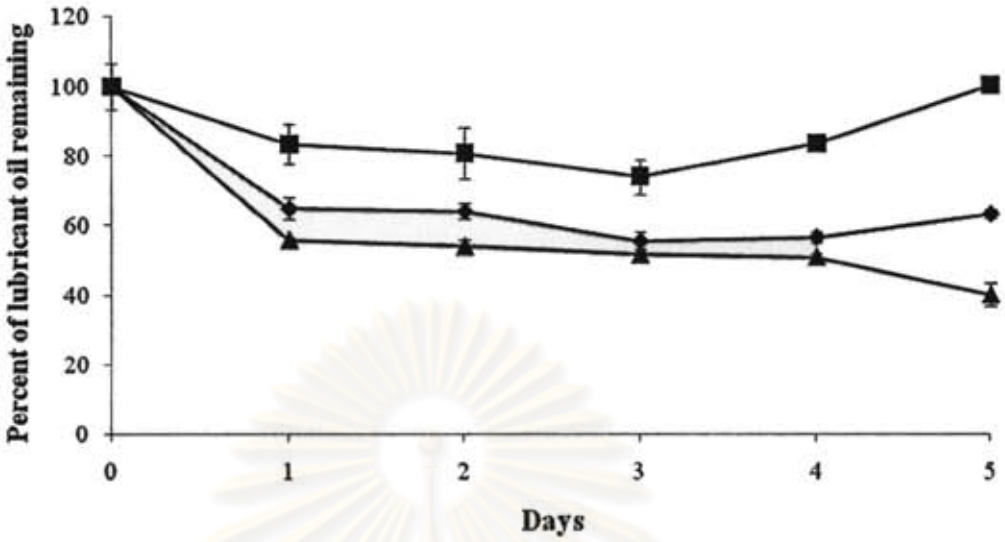


Figure 4.9 Percent of lubricant oil recovery during degradation by *Sphingobium* sp. strain P2 immobilized on chitosan for 5 days of incubation with different air mode: control (■), lubricant oil recovery in the present of *Sphingobium* sp. strain P2 from air supply condition (▲) and without air supply condition (◆) .

The presence of strain P2 in chitosan and emulsion after incubation in oil-in-water emulsion were determined by plate count technique and quantitative real-time PCR. Figure 4.10 showed the results of examination of the bacteria survival in chitosan and oil-in-water emulsion. The initial numbers of bacteria in air supply and without air supply conditions were 9.00×10^6 and 6.00×10^6 CFU/0.2 g chitosan respectively (Figure 4.10A). When the time passed by, the amount of strain P2 in chitosan of air supply condition little dropped to 1.80×10^6 CFU/0.2 g chitosan, then increased to reach maximum at day 3 (1.50×10^7 CFU/0.2 g chitosan). After that, the amount of strain P2 at day 4 and 5 were 3.00×10^6, and 6.00×10^6 CFU/0.2 g chitosan, respectively. Meanwhile, the number of strain P2 of without air supply condition was gradually increased to reach maximum at day 4 (3.00×10^7 CFU/0.2 g chitosan), and a few dropped at day 5 (1.65×10^7 CFU/0.2 g chitosan). According to Figure 4.10B, no bacteria were observed in the emulsion before the addition of chitosan-immobilized cells into the emulsion. When the immobilized cells were incubated in the emulsion of both conditions, there were bacteria populations in the

emulsion phase. This indicated that some bacteria came off from chitosan. In air supply condition, the amount of strain P2 in the emulsion increased rapidly (1.04×10^9 CFU/80 ml at day 1). Afterward, there was no significantly change of number of strain P2 in emulsion in which the number was ranging from 1.60×10^8 to 8.00×10^8 CFU/80 ml. In other word, the number of strain P2 in emulsion from without air supply condition was 8.00×10^5 CFU/80 ml at day 1, after that it increased to range from 8.00×10^7 to 8.80×10^8 CFU/80 ml.

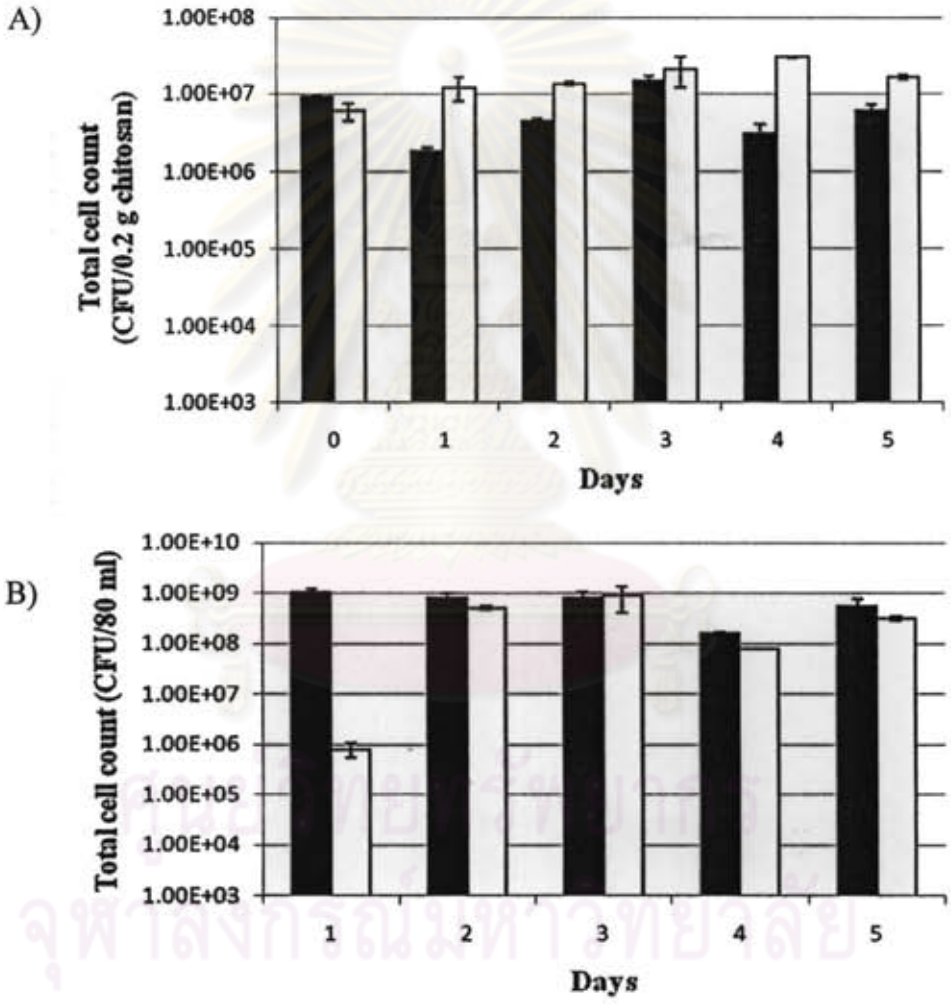


Figure 4.10 Survival of bacteria in chitosan (A) and oil-in-water emulsion (B) in each different air mode after 5 days of incubation: with air supply condition (■), and without air supply condition (□).

As a result of quantitative real-time PCR, the number of *adhA3* gene copies was represented the amount of strain P2 in chitosan and oil-in-water emulsion which calculated from standard curve as shown in Figure 4.11. The initial *adhA3* gene copies number of air supply and without air supply conditions were 1.13×10^5 and 3.00×10^4 *adhA3* gene copies number/0.2 g chitosan (Figure 4.12A). In day 1 to 5, the *adhA3* gene copies of air supply condition was ranged from 2.52×10^5 to 3.12×10^6 *adhA3* gene copies number/0.2 g chitosan which less than the *adhA3* gene copies of without air supply condition (2.13×10^6 to 2.15×10^7 *adhA3* gene copies number/0.2 g chitosan). In oil-in-water emulsion, there was no *adhA3* gene copies were observed at the initial day (Figure 4.12B) as well as Figure 4.10B. Subsequently, chitosan-immobilized cells were added in oil-in-water emulsion for 1 day. There was the number of *adhA3* gene copies in the emulsion which was ranged from 1.27×10^6 to 3.71×10^7 and 1.17×10^6 to 4.80×10^6 *adhA3* gene copies number/80 ml of air supply and without air supply conditions, respectively for 5 days of incubation.

The reason why percent remaining lubricating oil of chitosan-immobilized cell was higher than percent remaining lubricating oil of strain P2 in the form free cell because the initial amount of chitosan-immobilized cell (9.00×10^6 CFU/0.2 g chitosan) was less than the initial amount of strain P2 in the form of free cell (2.40×10^{10} CFU/80 ml). Although the number of strain P2 which determined by both techniques did not equal, both techniques could indicate that the amount of cells did not effect to degrade lubricating oil. In contrast, the factor that effect for lubricating oil degradation was air supply of the treatments. According to Brambili *et al.* (2006), this research studied five different modes of supplying oxygen to a membrane-aerated biofilm reactor (MABR) and concluded that all the mode of oxygen supplied in a MABR were more efficient than the traditional suspended cell process. The reason why the number of strain P2 determined by real-time PCR was less than plate count technique was some problem during recovery and extraction DNA of strain P2 samples. In addition, chitosan-immobilized cells could extend the survival of strain P2 than in the form of free cells when the cells were in without air supply condition for 5 days. These results might be described by the work of

Gentili *et al.* (2006) involving in an examination of the survival of *Rhodococcus corynebacterioides* strain QBTo immobilized on chitosan during storage in polyethylene bags at 25°C and revealed that the inoculants maintained their initial viable cell count for 45 days at 25°C. Therefore, chitosan-immobilized cells incubated 5 days in without air condition was not long enough to effect amount of bacteria in chitosan, while the without air condition effected to survival of strain P2 in the form of free cells.

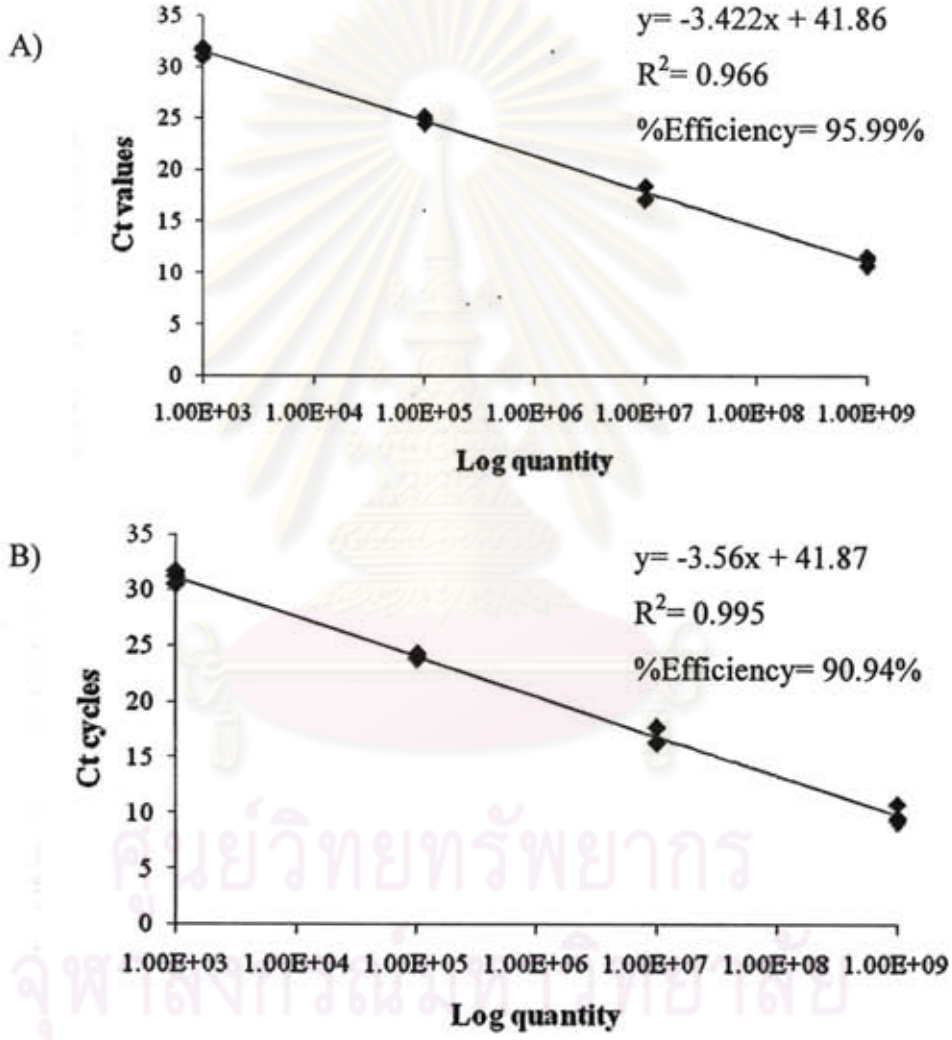


Figure 4.11 Standard curve of the *AdhA3* gene copy numbers from Real-Time PCR amplification assays obtained by plotting the logarithm of the gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value for calculate number of bacteria in chitosan (A) and oil-in-water emulsion (B).

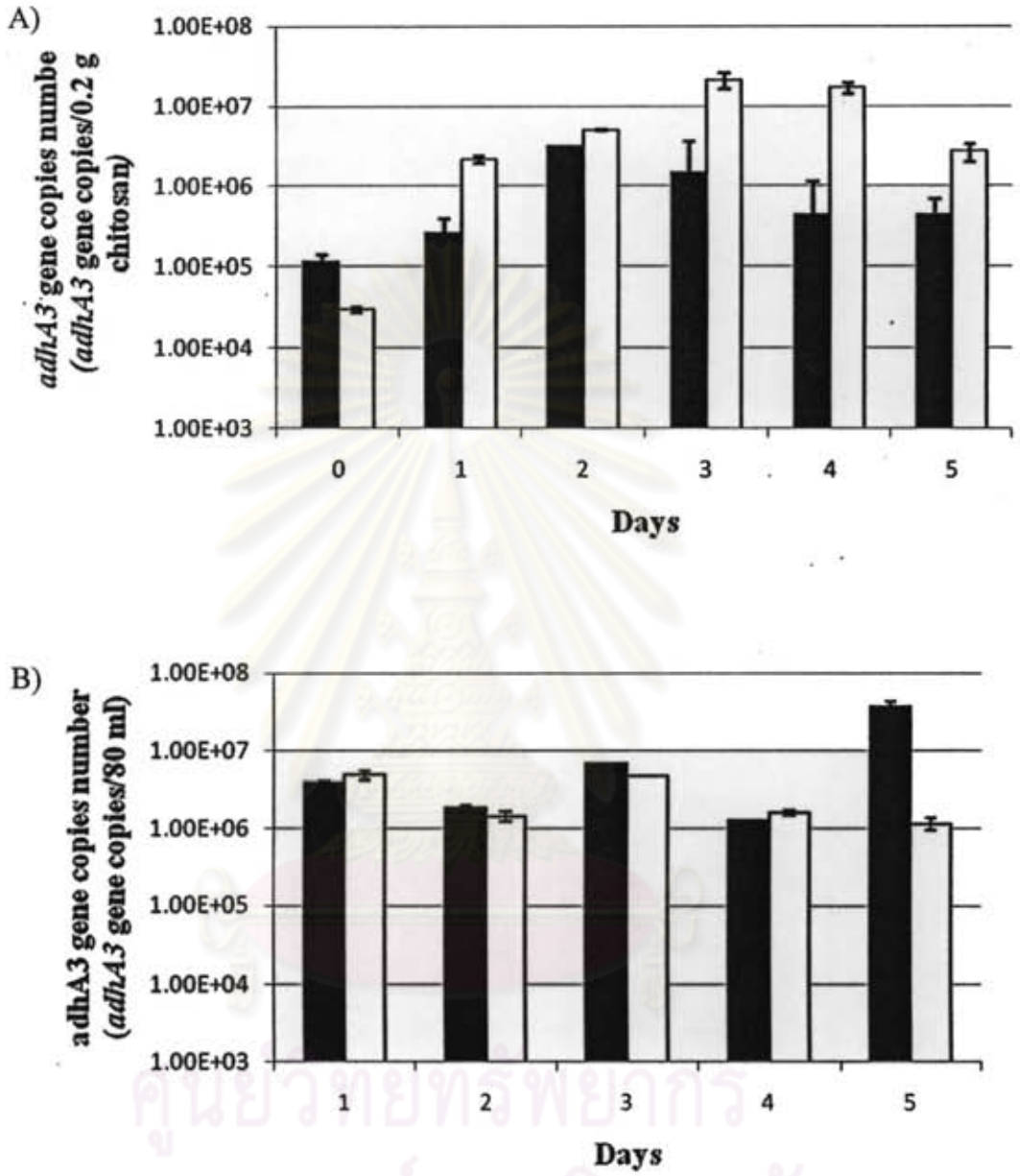


Figure 4.12 *adhA3* gene copy numbers by Real-time PCR using immobilized cells with chitosan (A) and oil-in-water emulsion (B) : air supply condition (■), and without air supply condition (□).

4.3 Application of immobilized cells to Reactor-Scale

4.3.1 Application of lubricating oil degradation with immobilized cells in 3 l airlift bioreactor

According to the previous part of this study, the quantitative real-time PCR could predict the amount of strain P2 in the treatment samples. Therefore, this technique was applied to quantify the number of strain P2 in wastewater treatment system. Recently, research was developed a modified airlift bioreactor for treating wastewater from petrol station (Figure 4.13). The working volume of this study was 3 l for continuous experiment which hydraulic retention time (HRT) was 2 hours. Wastewater used in this study was collected from oil-trapping tank at PPT station, Sanam Pao, Bangkok (Figure 4.14) and blended with 100 ppm lubricating oil and 0.1% Tween-80. The amount of chitosan-immobilized cells added in the reactor was 2.5 g/l. The experiment continuous flew the wastewater in and out the reactor for 1 month. The influent and effluent samples were collected every 3 days for analysis of oil concentration. In addition, the chitosan-immobilized cells and the effluent were determined for amount of bacteria by plate count technique and real-time PCR.

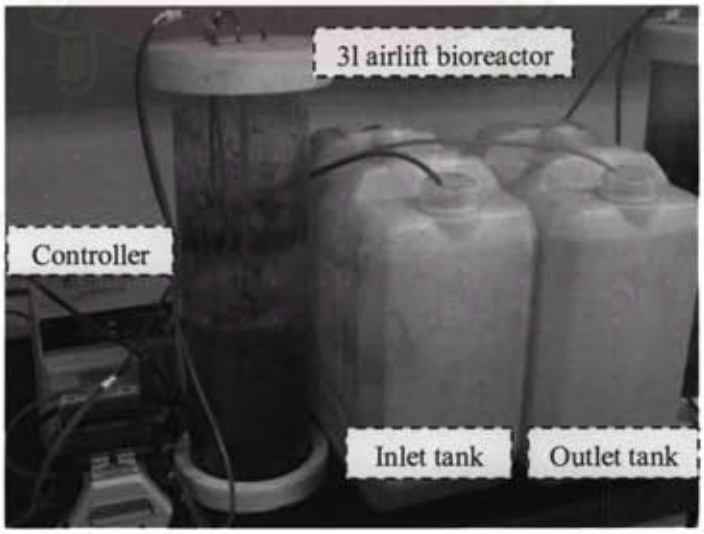


Figure 4.13 Airlift bioreactors containing 3 l wastewater

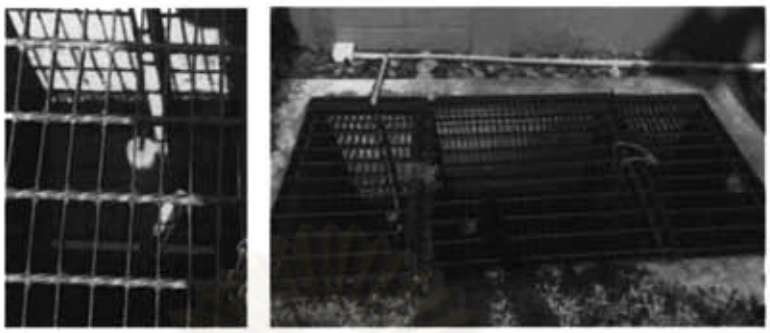


Figure 4.14 Sampling point of wastewater at PPT station, Sanam Pao, Bangkok.

After passing the influent and effluent wastewater, the bioreactor reached a steady state at 8 hours with 83.05% oil removal (Figure 4.15). The steady state condition was assumed to be reached when the percent oil removal almost constant with increasing time. The average of lubricating oil concentration of influent and effluent were 187.53, and 16.83 ppm, which average of percent oil removal was 91.02%. From Figure 4.15, the efficiency was dropped to 55.31 and 52.81% oil removal at day 6 and 12, these might be the effect from chitosan-immobilized cells sampling. For this reason, the system was not into steady-state at day 6 and 12, and return to steady state later within 2 days.

The influent and effluent COD concentration was presented in Figure 4.16. The effect of lubricating oil degradation by chitosan-immobilized cells on COD removal was examined by chemical oxygen demand reagents. From the result, the influent COD concentrations were higher than effluent COD concentrations for 26.67% average COD removal. Therefore, this airlift bioreactor could remove COD concentration. However, COD concentration was stilled higher more than COD standard value as shown in Table 4.2 (less than 200 mg/L), and percent COD removal in last 10 days was very low. This is probably due to chemical structure of Tween-80 that was recently added in wastewater which are resistant to chemical attack (Panpanit, 2001). In addition, after oil was degraded, the compounds might be changed in another form of hydrocarbon that could not be detected by TLC-FID, but could measure by COD analyzation. Thus, efficiency for reduction COD concentration was low. While COD concentration of wastewater before added 100 ppm lubricating oil and 0.1% Tween-80 was under standard value.

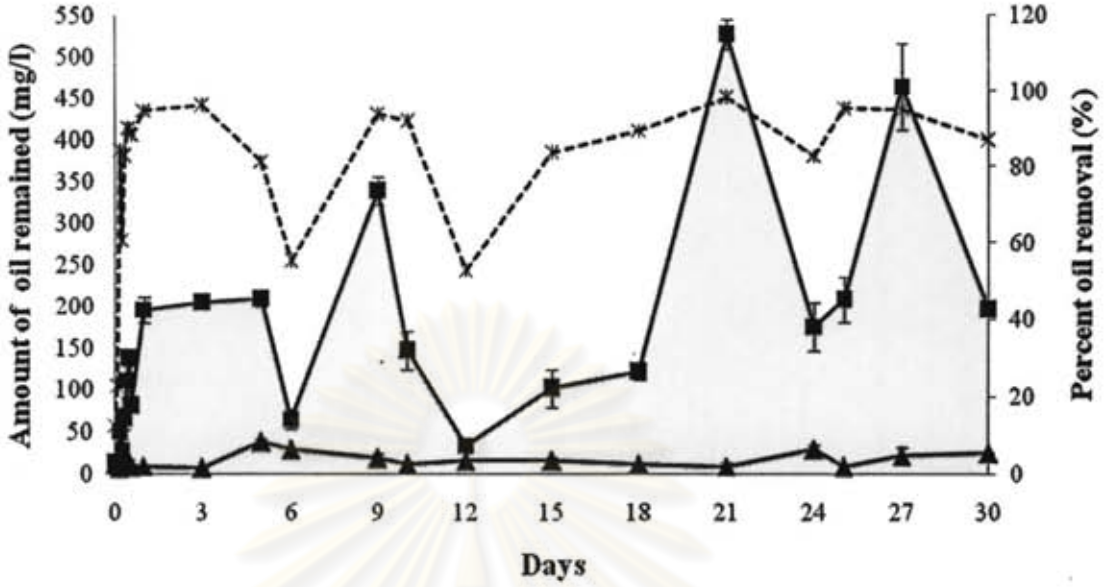


Figure 4.15 Percent of lubricating oil recovery during degradation by chitosan - immobilized *Sphingobium* sp. strain P2 in 3 l airlift bioreactor for 1 month of incubation: amount of oil remained in influent (■), and effluent (▲). The percent oil removal (×).

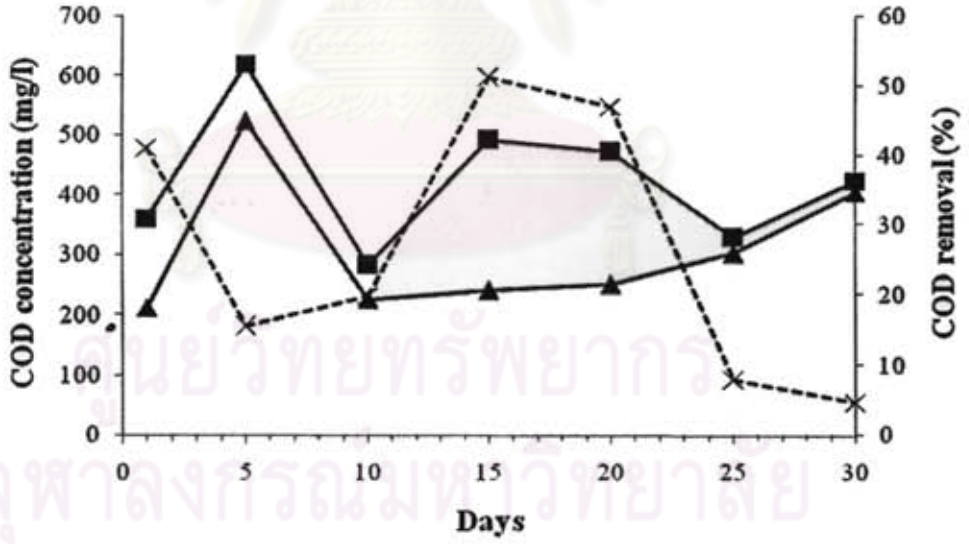


Figure 4.16 COD of wastewater after treatment compare with wastewater before treatment from 3 l airlift bioreactor: amount of oil remained in influent (■), and effluent (▲). The percent oil removal (×).

Figure 4.17 showed the survival of bacteria on chitosan and effluent wastewater during 1 month of incubation which determined by plate count technique. The initial number of bacteria on chitosan was 8.21×10^{10} CFU/7.5 g chitosan. After the bioreactor was reached to steady state, all the inoculants maintained their initial viable counts on chitosan which was ranging from 9.00×10^{9} to 3.04×10^{10} CFU/7.5 g chitosan for 25 days. At 30 days, a decrease about 2 orders of magnitude was detected on chitosan (1.13×10^{9} CFU/7.5 g chitosan). In the effluent wastewater, amount of bacteria was ranged from 6.57×10^{8} to 7.62×10^{10} CFU/3 l for 30 days.

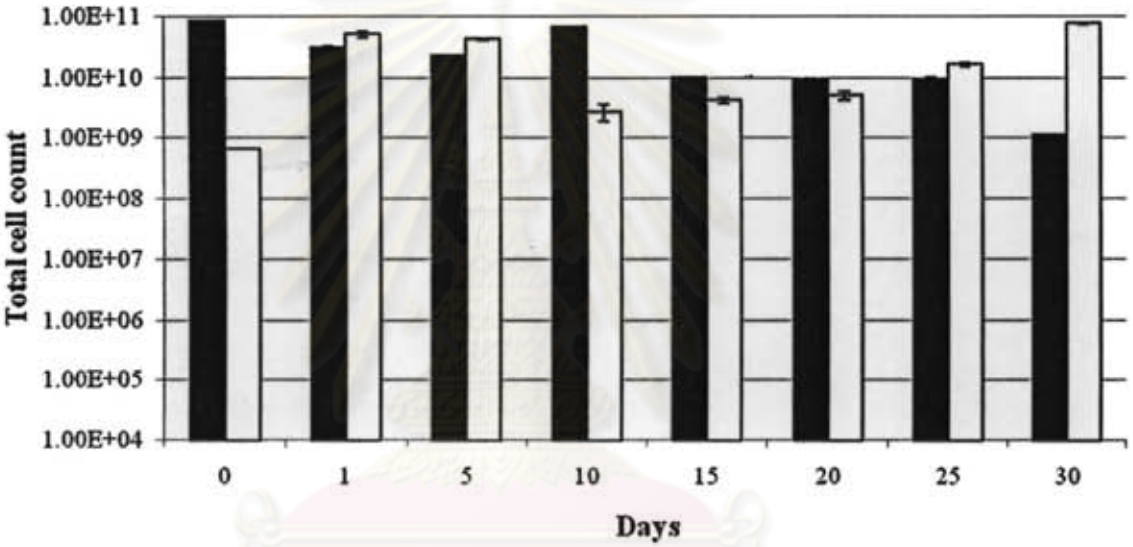


Figure 4.17 Survival of bacteria in chitosan and wastewater in 3 l airlift bioreactor after 1 month of incubation: chitosan; CFU/7.5 g chitosan (■) and wastewater; CFU/3 l (□)

For the results of quantitative detection of strain P2 on chitosan and wastewater, a standard curve was constructed using different plasmid concentration of pUPA3412d plasmid (Figure 4.18). The extracted DNA samples from chitosan and wastewater were quantitatively detected using SYBR green dye based real-time PCR, and the results were shown in Figure 4.19. The initial amount of *adhA3* gene copies number was 1.62×10^{8} *adhA3* gene copies number/7.5 g chitosan. After the system entered to steady state, the amount of *adhA3* gene copies number was ranging from 1.55×10^{8} to 3.87×10^{8} *adhA3* gene copies number/7.5 g chitosan for 25 days. The amount of *adhA3* gene copies

number was then decrease to 1.92×10^7 *adhA3* gene copies number/7.5 g chitosan at day 30. In part of effluent wastewater, the amount of *adhA3* gene copies number was ranged from 2.63×10^6 to 3.57×10^7 *adhA3* gene copies number/3 l for 30 days. According to the results, the amount of bacteria determined by 2 techniques had similar trend, and indicated that the inoculants on chitosan trended to decrease 2 orders after passing time for 1 month. From this experiment, the result was agreeable with another research (unpublished data) which studied the treatment sewerage wastewater in petrol station by 3 l airlift bioreactor. The result showed that chitosan-immobilized cells could treat sewerage wastewater and had efficiency to 98% of average amount of oil 37.28 ppm. Moreover, the number of bacteria on chitosan by plate count technique of previous research tended to decrease 2 orders as well as this experiment. The ratio of *adhA3* gene copies number in chitosan and wastewater were shown in Table 4.2. In most case, the number of *adhA3* gene copies in chitosan was higher than those in wastewater except for *adhA3* gene copies number in day 25 and 30. The ratio values showed that strain P2 was tend to adsorb in chitosan as carrier material than survive in wastewater. Therefore, bacterial cell immobilization allowed the bacterial cells to survive and grow in the system.

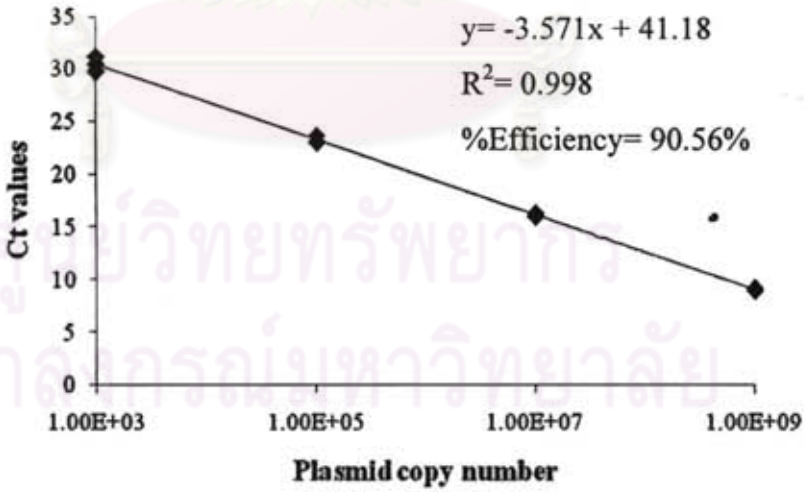


Figure 4.18 Standard curve of the *AdhA3* gene copy numbers from Real-Time PCR amplification assays obtained by plotting the logarithm of the gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value.

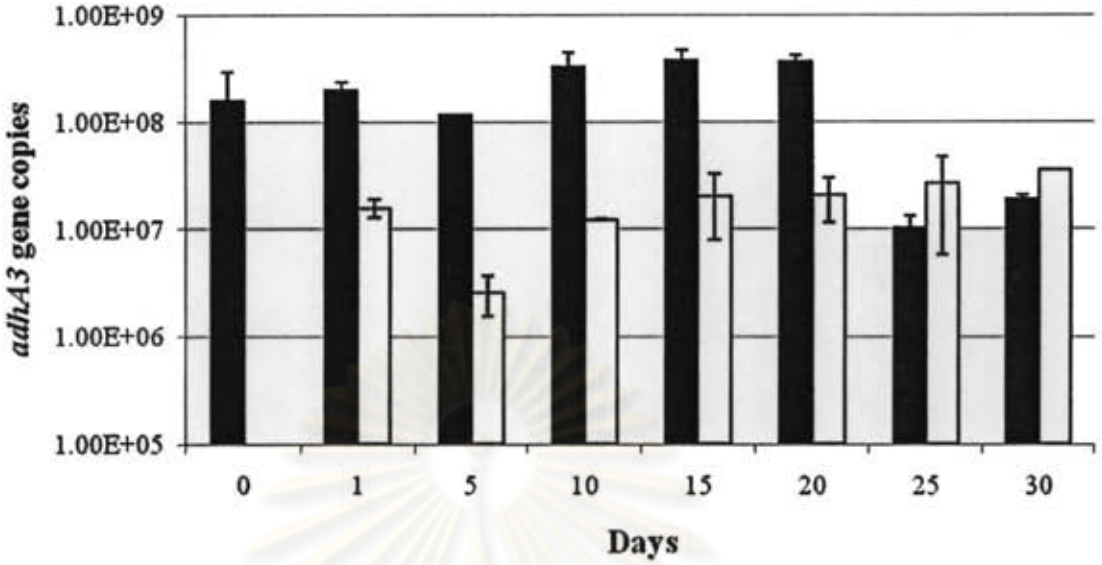


Figure 4.19 *adhA3* gene copy numbers by Real-time PCR using immobilized cells with chitosan and wastewater sample from 3 l airlift bioreactor: chitosan; *adhA3* gene copies number/7.5 g chitosan (■) and wastewater; *adhA3* gene copies number/3 l (□)

Table 4.2 Ratio of *adhA3* gene copy numbers in chitiosan : wastewater in 3 l airlift bioreactor.

Day	Ratio *adhA3* gene copy numbers in chitiosan : wastewater
1	12.7 : 1
5	44.8 : 1
10	27.1 : 1
15	19.0 : 1
20	17.7 : 1
25	1 : 2.6
30	1 : 1.9

4.3.2 Application of lubricating oil degradation with immobilized cells in 350 l airlift bioreactors

Not only 3 l airlift bioreactor was developed by previous research, but also the airlift bioreactor was extended to 350 l working volume in field-scale (Figure 4.20). This bioreactor was continuous-flow experiment. Wastewater from oil trapping tank in petrol station was flew to storage tank, then the wastewater was input to 350 l airlift bioreactor. The treated wastewater was discharged to sewer of petrol station. The hydraulic retention time (HRT) was 2 hours. The input and output wastewater sample was collected almost every day to analyze total petroleum hydrocarbon and COD concentration for 2 months. The chitosan-immobilized cells and output wastewater were collected every 2 weeks to determine amount of bacteria by plate count technique and real-time PCR.

The total petroleum hydrocarbon of influent and effluent wastewater was presented in Figure 4.20. The average of input and output petroleum hydrocarbon concentration was 6.1, 1.9 ppm, respectively. An average of petroleum hydrocarbon removal was 68%. Even though, the lubricating oil were added to 150 ppm final concentration in the input wastewater, the treated wastewater from airlift bioreactor was decreased to 46 ppm petroleum hydrocarbon which oil removal was 69%.

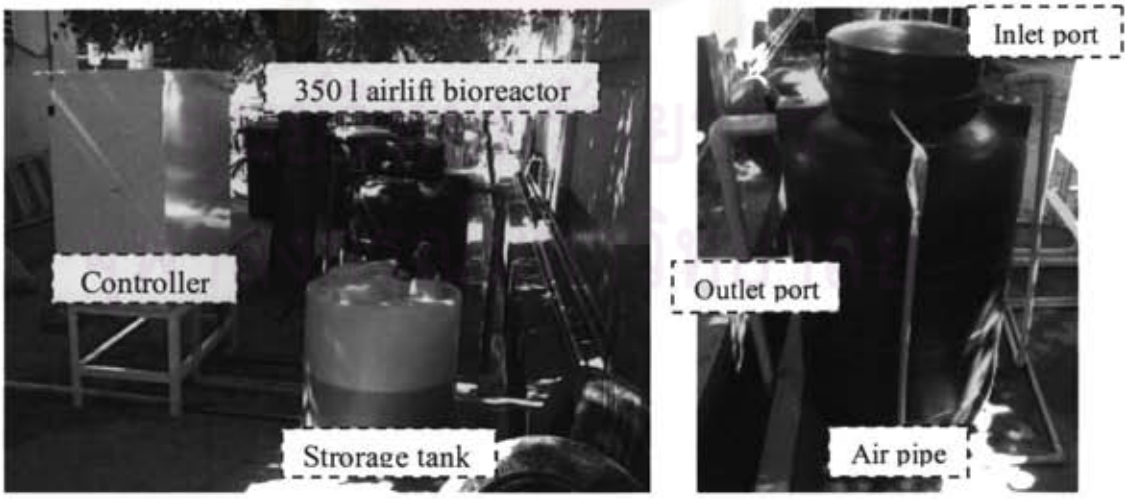


Figure 4.20 350 l airlift bioreactors located at PPT station, Sanam Pao, Bangkok.

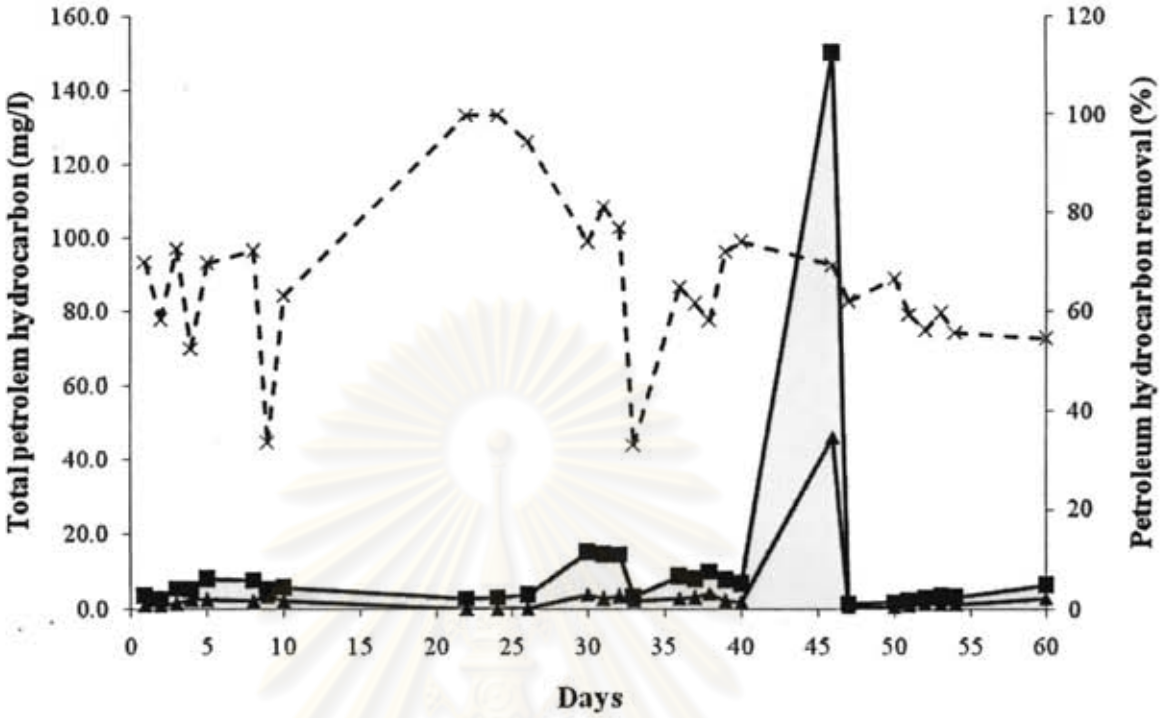


Figure 4.21 Percent of lubricating oil recovery during degradation by chitosan - immobilized *Sphingobium* sp. strain P2 in 350 l airlift bioreactor for 1 month of incubation: amount of oil remained in influent (■), and effluent (▲). The percent oil removal (×).

The COD concentration was analyzed by chemical oxygen demand reagent which was explained in Figure 4.22. The average influent and effluent COD concentration was 89, and 59 ppm, respectively. The average COD concentration removal was 33%. At day 46, the influent COD concentration reached to 328 ppm, but the airlift bioreactor could reduce the COD content of effluent wastewater to 152 ppm. According to the existing standard of discharge wastewater in petrol station as shown in Table 4.3, the COD content of the discharged wastewater within the effluent standard. The effluent oil content also conformed to the standard. Therefore, the airlift bioreactor could remove COD concentration and this wastewater could be discharged to the public sewer system.

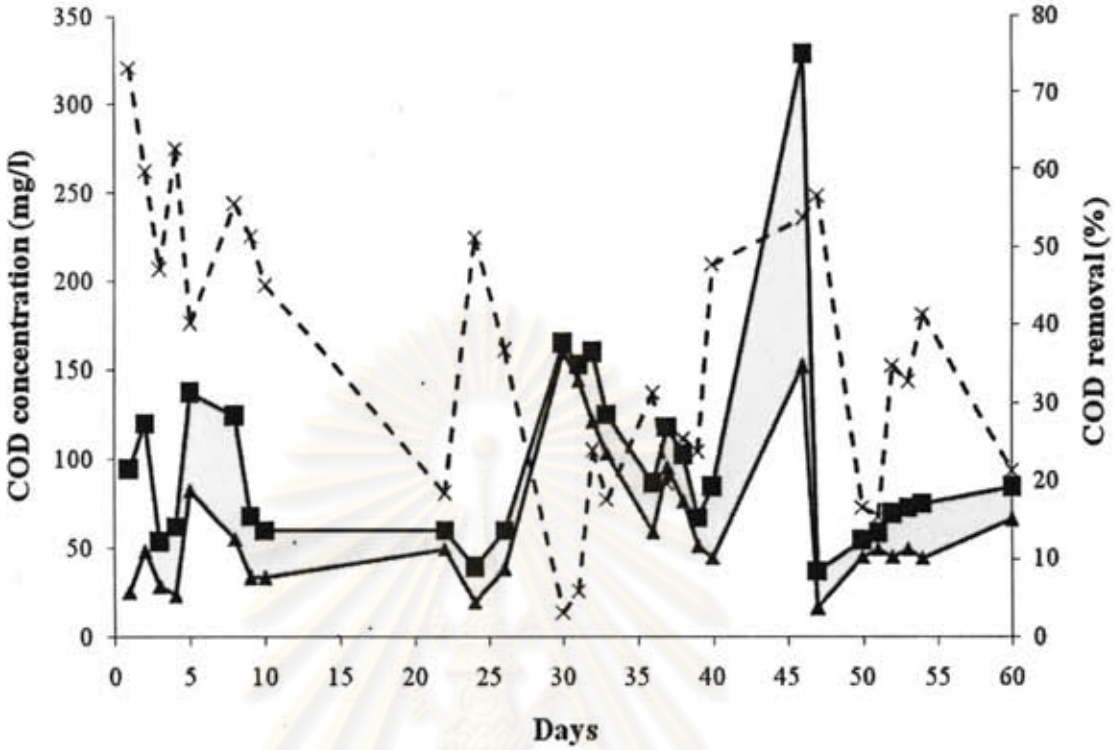


Figure 4.22 COD value of wastewater after treatment compare with wastewater before treatment from 350 l airlift bioreactor: amount of oil remained in influent (■), and effluent (▲). The percent oil removal (×).

Table 4.3 Existing standard of discharged wastewater from petrol station (PCD: Pollution control department, Thailand, 2002)

Parameter	Unit	Standard value	Analytical method
pH	-	5.5-9.0	pH Meter
Chemical Oxygen Demand: COD	mg/l	<200	Potassium dichromate digestion
Suspended Solids	mg/l	<60	Glass fiber filter disc
Fatty oil and Grease	mg/l	<15	Solvent extraction

The bacteria were recovered from chitosan and wastewater in the 350 l airlift bioreactor and determined amount of bacteria by plate count technique and real-time PCR. The chitosan and wastewater samples were taken every 2 weeks during 9 February to 23 March 2010. The amount of bacteria survival in chitosan was ranged from 5.25×10^{11} to 3.54×10^{13} CFU/875 g chitosan, and the amount of bacteria survival in wastewater was ranged from 8.51×10^{11} to 2.52×10^{14} CFU/350 l (Figure 4.23). From the result, there were many kinds of bacteria survived in chitosan and wastewater thus this method could not enumerate lubricating oil-biodegrading microorganism.

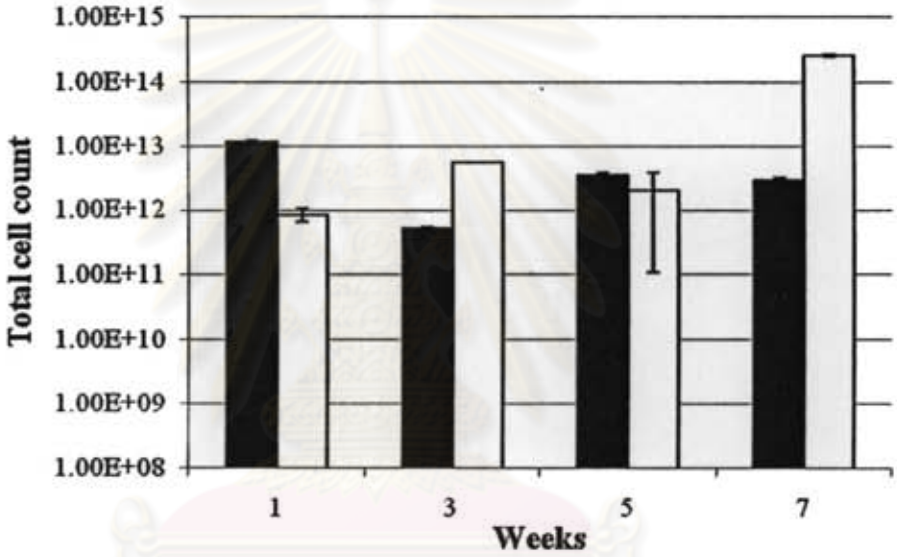


Figure 4.23 Survival of bacteria in chitosan and wastewater in 350 l airlift bioreactor after 1 month of incubation: chitosan; CFU/875 g chitosan (■) and wastewater; CFU/350 l (□).

The real-time PCR could extend ability to enumerate genotypes involved in biodegradation of lubricating oil. This study used primer set AdhA3RT which was specific to ferredoxin of ring-hydroxylating enzyme (*adhA3*) to detect and quantify amount of strain P2 using standard curve as shown in Figure 4.24. The DNA samples extracted from chitosan and wastewater were detected with SYBR green dyes which were described in Figure 4.25. When the system was operated for 1 month, the amount of strain P2 in chitosan was almost constantly which was 1.17×10^{10} *adhA3* gene copies

number/875 g chitosan. On the contrary, the amount of strain P2 in wastewater trended to decrease from 8.16×10^{8} to 2.87×10^{7} *adhA3* gene copies number/350 l because a small amount of petroleum hydrocarbon which was average to 4.9 ppm. This might not enough for cell growth. After the system was run for 39 days, the number of strain P2 was decrease 1 order to 9.08×10^{8} *adhA3* gene copies number/875 g chitosan, but the number of strain P2 in wastewater was increased to 4.62×10^{8} *adhA3* gene copies number/350 l in order that amount of petroleum hydrocarbon was higher than a previous month. In week 7, the amount of strain P2 in chitosan and wastewater were increased to 5.34×10^{9} *adhA3* gene copies number/875 g chitosan, and 1.75×10^{9} *adhA3* gene copies number/350 l, respectively. This might be effect from adding lubricating oil to final concentration was 150 ppm which made the strain P2 could grow more. The ratio of *adhA3* gene copy numbers in chitiosan and wastewater was shown in Table 4.4. According to the result, the number of *adhA3* gene copies in chitosan was higher than those in wastewater. In some week, the ratio was low due to the limited area in chitosan. The excess cells of strain P2 could not be adsorbed in chitosan and grew in wastewater. The results indicated that the strain P2 could survive in this system and still had efficiency to remove lubricating oil at least for 2 months. Moreover, this system tended to operate more than 2 months. The experiment operating this treatment system without adding strain P2 for 18 days showed only 7% oil removal in an average (unpublished data). This indicated that the strain P2 plays an important role in the oil degradation in this wastewater treatment system. For more information, the detection of number of *adhA3* gene copies should be done before the system was run to verify *adhA3* gene copies number in the wastewater.

This study revealed that real-time PCR technique could present lubricating oil-degradation microorganism which was strain P2 as well as other research that used the same technique. According to Raemdonck *et al.* (2006), 1,2-dichloroethane dehalorespiring *Desulfitobacterium dichloroeliminans* strain DCA1 was quantified with SYBR green I, and primer pair targeting unique regions of the 16S rRNA gene in a bioaugmented monitoring well at Tessenderlo, St-Niklaas, and Harbor of Antwerp, Belgium. This paper concluded that real-time PCR assay could be used to survey the

abundance and kinetics of strain DCA1 in *in situ* bioaugmentation filed studies. Moreover, Brett *et al.* (2009) used real-time PCR to enumerate aromatic oxygenase genes for elucidation the impact of muti-phase extraction (MPE) on the function at a gasoline-contaminated site. This study revealed that aromatic oxygenase gene which catabolized further oxidation of hydroxylated BTEX metabolites indicated the potential for aerobic biodegradation of dissolved BTEX during pulsed MPE.

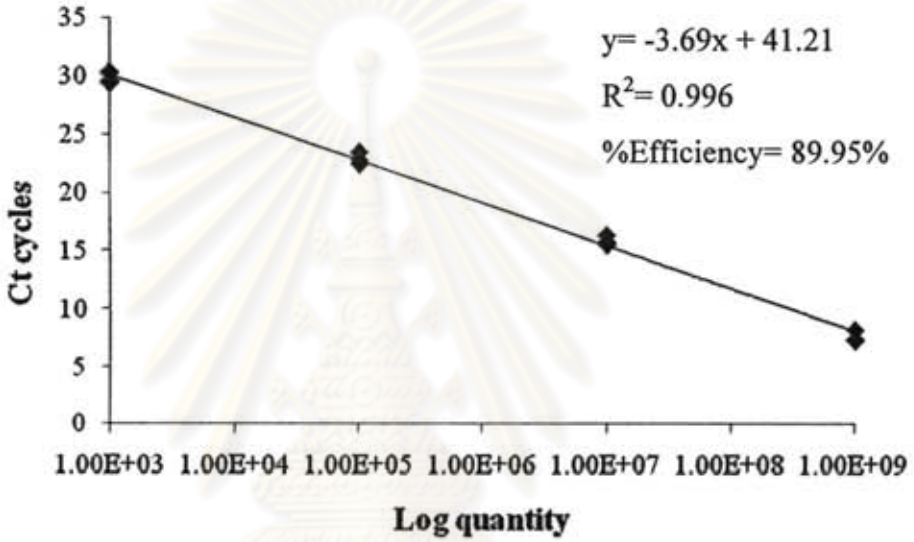


Figure 4.24 Standard curve of the *AdhA3* gene copy numbers from Real-Time PCR amplification assays obtained by plotting the logarithm of the gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value.

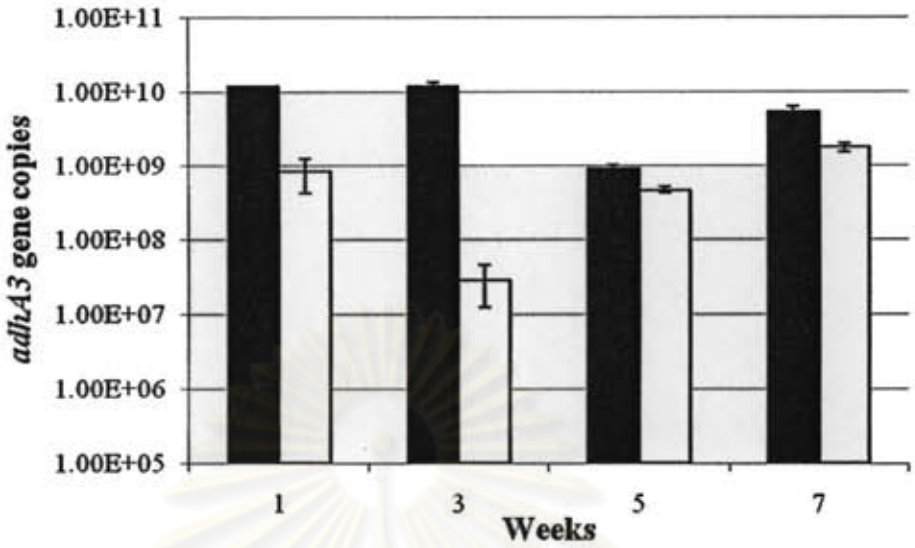


Figure 4.25 *adhA3* gene copy numbers by Real-time PCR using immobilized cellss with chitosan and wastewater sample from 350 l airlift bioreactor: chitosan; *adhA3* gene copies number/875 g chitosan (■) and wastewater; *adhA3* gene copies number/350 l (□).

Table 4.4 Ratio of *adhA3* gene copy numbers in chitiosan : wastewater in 350 l airlift bioreactor.

Week	Ratio *adhA3* gene copy numbers in chitiosan : wastewater
1	14.4 : 1
3	409.0 : 1
5	2.0 : 1
7	3.0 : 1

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Due to the presence of emulsifier and oil in wastewater from petrol station, the oil-in-water emulsion is stable and conventional methods cannot be applied. The biodegradation technique is an alternative to eliminate oil-in-water emulsion. From the previous research, *Sphingobium* sp. strain P2 has highly efficiency to degrade lubricating oil in oil-in-water emulsion. Moreover, this strain was immobilized on chitosan and developed to use in airlift bioreactor for treating wastewater in petrol station.

The main goal of this work were to choose marker gene and design specific primer for detection of strain P2 and detect marker genes during degradation of lubricating oil by strain P2 using real-time PCR in airlift bioreactor.

From this study, PCR primer set adhA3RT which was specific for gene encoding for ferredoxin of ring-hydroxylating enzyme (*adhA3*) of strain P2 was chosen for enumeration strain P2 using real-time PCR. The *ahdA3* gene in this strain is involved in aromatic compound catabolic pathways (Pinyakong *et al.*, 2003a). The primer set adhA3RT was selected because this primer set gave suitable product size, had high specificity with strain P2, and gave no nonspecific amplification product. Afterward, this primer set was examined in flask-laboratory scale to determine the survival of strain P2 during lubricating oil degradation in oil-in-water emulsion with different air mode for 5 days. Firstly, the experiment was lubricating oil degradability test with strain P2 in the form of free cells. As expecting, amount of lubricating oil in air supply condition was decrease to 24.84% of initial concentration in 5 days. In contrast, those in without air supply condition decreased to 52.62% of the initial concentration in 5 days. The number of bacteria of air supply condition tended to increase from 3.14×10^{9} to 4.19×10^{10} *adhA3* gene copies/80 ml, while the number of bacteria of without air supply condition tended to

decrease from 1.57×10^{10} to 6.09×10^{8} *adhA3* gene copies/80 ml. In the form of chitosan-immobilized cell, amount of lubricating oil in day 5 of sufficient air supply condition was decreased to 40.16% of initial concentration, but those in the without air supply condition decreased to 63.24% of initial concentration. Accordingly, the *adhA3* gene copies of air supply and without air supply condition on chitosan was ranged from 1.13×10^{5} to 3.12×10^{6}, and 3.00×10^{4} to 2.15×10^{7} *adhA3* gene copies number/0.2 g chitosan, respectively. Meanwhile, the *adhA3* gene copies of air supply and without air supply condition in oil-in-water emulsion was ranged from 1.27×10^{6} to 3.71×10^{7} and 1.17×10^{6} to 4.80×10^{6} *adhA3* gene copies number/80 ml, respectively for 5 days of incubation. This might be described that without air supply to the treatment was effect to decrease strain P2 in the form of free cells, but chitosan-immobilized strain P2 still survived for 5 days. In addition, an important factor for enhancing of lubricating oil degradation was air supply condition.

After the primer set and real-time PCR was examined to survey the presence of strain P2 in flask-laboratory scale, the next section was the application of immobilized cells to reactor-scale and used real wastewater in the treatment system. The 3 l airlift continuous bioreactor was developed for treating wastewater from petrol station in the laboratory. The wastewater was collected from oil-trapping tank, and 100 ppm lubricating oil and 0.1% Tween-80 were added. Furthermore, samples were collected from 350 l airlift continuous bioreactor in petrol station for indication the survival of bacteria in real situation. The results showed that both of airlift bioreactors could remove lubricating oil and COD concentration as summarized in Table 5.1. In addition, real-time PCR could detect amount of strain P2 which more accurate than those obtained from total plate count technique. Comparison of the number of *adhA3* gene copy numbers revealed that the *adhA3* gene copy numbers in chitiosan was higher than those in wastewater indicating that chitosan could adsorb strain P2 and provided this strain to exist in the system.

This study revealed that *Sphingobium* sp. strain P2 had activity to degrade lubricating oil and could survive in the system. Moreover, *adhA3* specific primers and

real-time PCR could be used for quantify this strain in bioremediation systems. The information obtained could be used to further design effective wastewater treatment system in petrol station. For example, the real-time PCR assay could be used to survey the abundance of strain P2 when the condition of the reactor was changed, and could specified effective long shelf life of this strain.

Table 5.1 Efficiency of 3 l and 350 l airlift bioreactor wastewater treatment system

	3 l Airlift bioreactor	**350 l Airlift bioreactor**
Time	30 days	60 days
Oil content	13.4-526.49 ppm	1.4-150 ppm
Oil removal		
average	91.02%	68.18%
maximum	98.20%	100.00%
minimum	52.82%	33.33%
COD removal		
average	26.76%	32.96%
maximum	51.22%	73.4%
minimum	4.71%	3.05%
Amount of bacteria by total plate count		
in chitosan	1.13×10^{9}-8.21×10^{10} CFU/7.5 g chitosan	5.25×10^{11}-1.16×10^{13} CFU/875g chitosan
in wastewater	2.70×10^{9}-7.62×10^{10} CFU/3 l	8.51×10^{11}-2.52×10^{14} CFU/350 l
Amount of bacteria by real-time PCR		
in chitosan	1.04×10^{7}-3.87×10^{8} *adhA3* gene copies number/7.5 g chitosan	9.08×10^{8}-1.18×10^{10} *adhA3* gene copies number/875 g chitosan
in wastewater	2.63×10^{6}-3.57×10^{7} *adhA3* gene copies number/3 l	2.87×10^{7}-1.75×10^{9} *adhA3* gene copies number/350 l

5.2 Recommendations for future work

Based on this study, some recommendations for further study are proposed as follows: firstly, lubricating oil degradability test with different air mode should extend incubation time for more than 5 days because if the time is longer, it will be more observed the difference of amount of bacteria in sufficient and insufficient air supply condition especially strain P2 in the form of immobilized cell with chitosan. Moreover, the experiment should suppose pulsed and return to continuous system by done insufficient air supply condition for a while and obtain air supply to examine degradability test and amount of bacteria. Secondly, Tween-80 degradability test by strain P2 should be done to find surfactant degradation efficiency. Finally, RNA expression should be done for detect genes involved in lubricating oil degradation to test activity and quantify survival of strain P2 by quantitative RT real-time PCR.

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APPENDICES

APPENDIX A

Media Preparation

Luria Bertani (LB) broth

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

For solid medium, 1.5% agar was added.

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

Carbon free mineral medium (CFMM)

Per liter

$MgSO_4$	0.2	g
$(NH_4)SO_4$	1	g
K_2HPO_4	1.5	g
KH_2PO_4	0.5	g
Trace elememt	5	ml

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

Trace element (Per liter)

H_2SO_4	1	ml
EDTA	24	g
NAOH	4	g
$MgSO_4 \cdot 7H_2O$	2	g
$ZnSO_4 \cdot 7H_2O$	0.8	g
$MnSO_4 \cdot 4H_2O$	0.8	g
$CuSO_4 \cdot 5H_2O$	0.2	g
$Fe_2(SO_4) \cdot 7H_2O$	4	g
Na_2SO_4	20	g
$Na_2MoO_4 \cdot 2H_2O$	0.2	g

APPENDIX B

Chemicals

1N NaOH

NaOH	4	g
Deionized water	100	ml

1 N HCl

12 N HCl	8.33	ml
Deionized water	91.67	ml

70% Ethanol

99% Ethanol	700	ml
Sterilized deionized water	300	ml

10% sodium dodecyl sulfate, SDS

SDS	10	g

Dissolve slowly in 80 ml 0f 60°C-sterilized deionized water. When it completely dissolved, add sterilized deionized water to make final volume of 100 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

10% Proteinase K

Proteinase K	10	mg
sterilized deionized water	1	ml

10 mM Tris-HCl solution, pH 8

Trizma base ($C_4H_{11}NO_3$)	1.2	g

Dissolve in 800 ml of deionized water, and then adjust pH to 8 with HCl. Add deionized water to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

0.5 M EDTA solution

EDTA ($C_{10}H_{14}O_8Na_2.2H_2O$)	186.1	g
NaOH	20	g

Dissolve EDTA in 800 ml deionized water. Add NaOH, mix and wait until the solution cool down to room temperature. Adjust pH to 8 and make volume to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

TE buffer

10 mM Tris-HCl, pH 8	10	ml
0.5 M EDTA solution	0.2	ml

Make volume to 1,000 ml using deionized water. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

CTAB/NaCl

NaCl	4.1	g
CTAB	10	g

Dissolve CTAB in 65°C-deionized water 80 ml. Then, add 0.7 M NaCl. After completely dissolve, add deionized water to 100 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

Phenol/chloroform solution

Mix phenol which has been saturated with Tris-HCl and chloroform in ratio of 1:1 (v/v) by stirring for 15 min. Store at 4°C until being used.

Chloroform/isoamylalcohol solution

Mix chloroform qith isoamylalcohol in ratio of 24:1 (v/v). Store at 4°C until being used.

Phenol/Chloroform/Isoamylalcohol solution (50:49:1)

Phenol saturated with Tris-HCl	50	ml
Chloroform	49	ml

Isoamylalcohol	1	ml

Mix by stirring for 15 min. Store at 4°C until being used.

50X TAE buffer

Tris-HCl	242	g
0.5 M EDTA, pH 8	100	ml
Glacial acetic acid	57.1	ml

Dissolve all chemicals in 800 ml deionized water. After complete dissolve, add deionized water to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

0.9% agarose gel

Agarose gel	0.9	g
1X TAE buffer	100	ml

Melt using microwave oven.

2% agarose gel

Agarose gel	2	g
1X TAE buffer	100	ml

Melt using microwave oven.

10 mg/ml ethidium bromide

Ethidium bromide	0.1	mg
Deionized water	10	mg

Mix well and store in the dark place. When prepare, wearing glove is require since ethidium bromide is proved carcinogen.

0.85% sodium chloride solution

Sodium chloride	8.5	g
Deionized water	1,000	ml

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

APPENDIX C

Lubricating oil calibration curve

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

Amount of lubricating oil = Ratio of concentration x Amount of stearyl alcohol

Ratio of concentration (x) = Ratio of area (y)/1.612

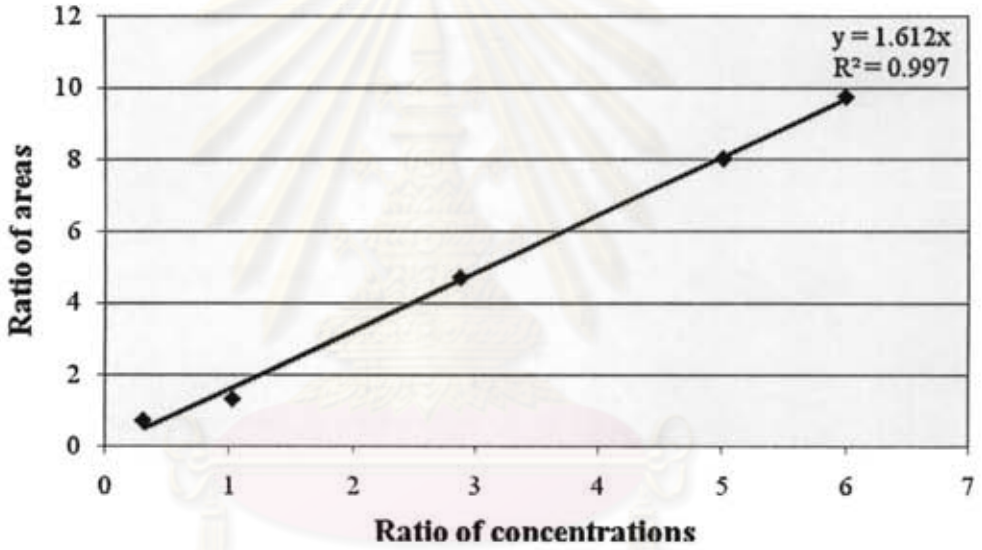

Figure A-1 Standard curve of lubricating oil from TLC-FID. Each data point was averaged from triple spots on chromatorods.

APPENDIX D

Raw data

1. Primer design

```
   1 tcaggcgctctctttcggttgatcgatgcacacccagccgtcttcgatcgtgacggggta
                                                          >>>>>>>>>
                                                          adhA3RT-F
  61 tgtcttgatggggatctggcagggaaaggccttggcagctccggtggcgatgtcgaacga
     >>>>>>>>>>>

 121 accaccgtgaaacgggcactcgataattgtgccgtcctggtagccatcggtcagcatggc


 181 attgccatgggtgcaaagattgtcggtgacgaacacttcgccatcgacgttgtagactgc
                 <<<<<<<<<<<<<<<<<<<<
                                adhA3RT-R
 241 aagcgcaggcatttttttcctggtagaccgcgaccggttcaccgtccttgacggaggctac


 301 ttggcaaaggcgcaatttgttcgacat
```

Figure D-1 The primer results of *adhA3* gene of *Sphingobium* sp. strain P2

```
   1 gtgcgctcgattgcgattgttggtgcgaatctggccggtgggcgcgcagtcgaagccctg

  61 cggcaggcggggttcgacgggcgaatcaccttgatcggcgaagaaccctggcgtccctat

 121 gaacgtccgccgctgtccaaggaagtgctgtgggacccggccaacgtcccggacaatttc

 181 ttcctccaggacgaggcctggtacgatgacaaccgtatcgacatgcggctgggcacccgc

 241 gccgaagcgatcgaccttgcgggcggcggggttcgcctgtcgggcggcgaattggttcag

 301 gcggaccggattctgctcgccactggcggccatgcccgcaagctcaaccttgccgggtcc

 361 aattgcgccaacgtccattatctgcgtacgcgcgacgatgcgacccgcatggccctcgac

 421 ctgcgcgaaggggccagcatcgtgattgtcggcatgggcgtgatcggcgccgaagtcgcc

 481 gccagtgcggtgaaactcggctgcaaggtcaccgtcgtcgagccgatgccggtgccgatg

 541 gagcgagcgctcggccggcgctttgggcaatggctgggcgaggaacatcgcaagcgggga

 601 gtcgcgacccatttcaactgcggcgtgaccggcttcaggttttccgacaaccgtgtcagc

 661 gcggtcgaggcagacgatggcaccgtgatcccgtgcgatgccgtgatcgtgggggtggga

 721 atcgtgccggccacctcgctggcccgcgatgccgggatcgaggtcaacaacgggatcatc
                                                  >>>>>>>>>>>>>>>>
                                                  adhA4RT-F
 781 gtcgaccgtcgctgccagaccagcaacctggcggttttcgcggcgggcgacgtggccgag
     >>>

 841 caggacgggtttttcggcggtcggttccggcaggaaacctaccaaaacgcggcggaccag

 901 gcgcaggcggctgctctggcgatgctggggcaggaggtctcctactgcaagccgatgtgg
                                                  <<<<<<<<<<<<<<<<<<<
                                                                adhA4RT-R
 961 tactggagcgaccagttcgatctcaacatccagttctgcgggcagattccggtggaagcc

1021 gaggtggcgatccgcggcgagatggacagcaacgccttcgtcgccttcttcctggccggc

1081 gaggcgatcgaaggtgtgctgacagtcaaccgcgcgcccgatatgggggtgggaaaacgg

1141 ctcgttgaacgccgggcccgcgccagcgctgcgagcctggccgacgccggtatgtccttg

1201 cgggatctgctcaagcaggcgggctga
```

Figure D-2 The primer results of *adhA4* gene of *Sphingobium* sp. strain P2

```
   1 tcagccgaggggaatatccattccgtagcccgcggcttcgttgccgtgaccgaatatatc

  61 gcgggtgtagtattcctgctggcttggggccttgcgggcaccccagccgaactcccacag

 121 ccagcccgacgggttggcgcagtagaaggtcagcgcctggtcgtttgcatgcttgccgag

 181 ctggagcgcgacgtcgatcttgcgcgcccgcacaatgtcgtgcgcgaggccgagatcgtc

 241 gaggtcggtatattcgaacatcaggtggttgatgcgcttttccatcgggccaagcccgaa

 301 agcgaccgaatgctgccgctcgttgcagtgcatgaagtacggctgcgccaccatcccgtt

 361 gggcagctgcaggtgatactcgaccgacccgcgcagccccagcagtccgtagaacgccgc

 421 cgccgcgggaacatcgtcctgacgcaggatgcaatgcccgattccttccgatccggtcac
                                                              >>>
                                                          bphC-F
 481 gaactttccgtacatggggcgcccaggatggaaaggcttgtgggtgtcgacttgcggccc
     >>>>>>>>>>>>>>>>>

 541 gtaaaaaatttcggtggggttcccacccggatcagccagcttggcaagaccgagcacgcg

 601 ccgctcccgagcttcggcctcgcttgccaccgtcaaggagattccggcggcggtcagctt

 661 tgccaccatggcgtcgaattccaccggatcggcaacgcgccagcccagataggcaagatc
                                                    <<<<<<<<<<<<<

 721 gtcggagtcggaggcatgcaacacgatgcgatgatgccactggtccatgcgcaggtagag
     <<<<<<<
      bphC-R
 781 gcggtcgccttcgccctcgtcgacaatctccatgccggccacttcggcagcataactgcg

 841 ccatgcatcgagatttgttacggtcaacccgaggtaaccgagttccgtgactgctaccat
```

Figure D-3 The primer results of *bphC* gene of *Sphingobium* sp. strain P2

2. Chromatogram from TLC-FID

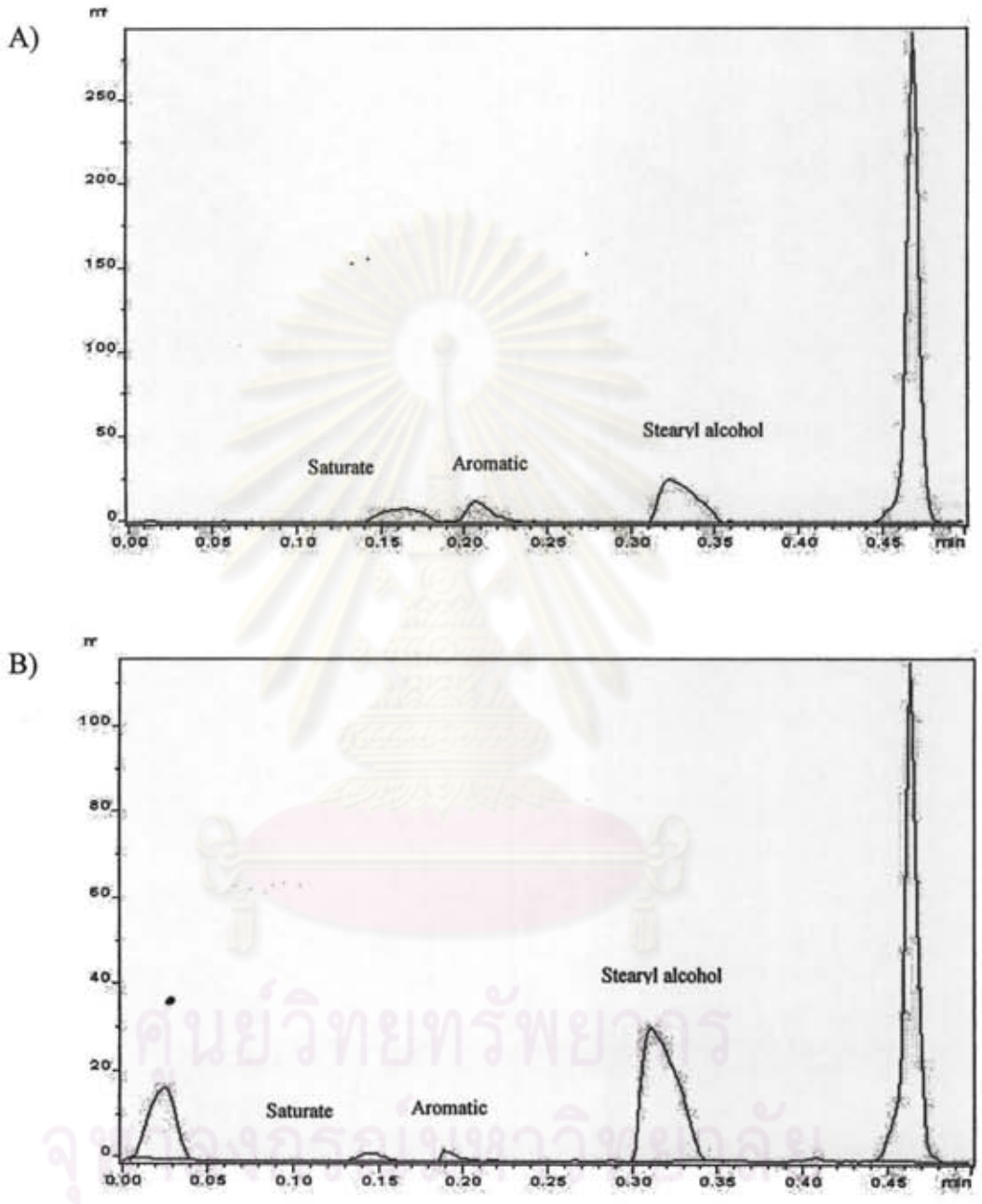

Figure D-4 The sample of chromatogram from TLC-FID: A) Influent of 3 l airlift bioreactor which added 100 ppm lubricating oil and 0.1% Tween-80 B) Effluent of 3 l airlift bioreactor

3. Raw data of each experiment

3.1 Lubricating oil degradability test with *Sphingobium* sp. strain P2 in the form of free cells

Table D-1 Peak area of saturate, aromatic, and total oil remained in oil-in-water emulsion. Each data point was averaged from triple spot on chromarods.

Days	Treatment	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
0	control	25773	16558	42331	20827	2.0325	196.98329	4.33
		24168	23243	48036	23475	2.0463	198.32229	
		27380	15442.333	42822	21816	1.9629	190.23901	
	Air supply	23179	18002	41181	21694	1.8983	183.9733	7.85
		24271	15198	39469	20827	1.8951	183.6638	
		19973	15442.33	35415	17387	2.0369	197.4075	
	Without air supply	20704	15447.67	36152	18250	1.9809	191.9847	5.35
		17213	13114	30327	15909	1.9063	184.7514	
		16356	11935.33	28292	15103	1.8732	181.5501	
1	Air supply	11074	7466.333	18541	11564	1.6034	155.3928	1.76
		10608	7781	18389	11735	1.5671	151.8785	
		17694	13918	31612	19946	1.5848	153.598	
	Without air supply	25640	25233.67	50874	30483	1.6689	161.7499	0.96
		25891	6389.333	32281	19123	1.6881	163.6045	
		19776	14065.67	33842	20110	1.6829	163.0994	
2	Air supply	15952	16734	32686	33535	0.9747	94.46458	7.66
		14849	14184.33	29034	32480	0.8939	86.63372	
		16529	20237.67	36766	29767	1.2352	119.7075	
	Without air supply	18695	18248.67	36944	31902	1.1580	112.2335	3.89
		12141	9738	21879	18587	1.1771	114.0805	
		16529	20237.67	36766	29767	1.2352	119.7075	

Table D-1 Peak area of saturate, aromatic, and total oil remained in oil-in-water emulsion. Each data point was averaged from triple spot on chromarods (cont.).

Days	Treatment	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
3	Air supply	7897	10153	18050	23835	0.7573	73.39477	11.13
		5668	9223.333	14891	24494	0.6080	58.92246	
		7092	7762.667	21637	25952	0.8337	80.80297	
	Without air supply	17404	19141.33	36545	30707	1.1901	115.3431	2.88
		11464	11617.67	23082	19855	1.1625	112.6693	
		14722	9593.333	24315	19900	1.2219	118.4212	
4	Air supply	6481	5544	12025	16609	0.7240	65.05488	2.99
		9061	6030	15091	24760	0.6095	59.0689	
		6398	5065.333	11463	17934	0.6392	61.95023	
	Without air supply	20870	22490.33	43361	34266	1.2654	122.6404	5.32
		21626	23273	44899	37410	1.2002	116.3187	
		18786	19781.33	34641	29958	1.1563	112.0685	
5	Air supply	8893	3260.667	12154	22060	0.5509	53.39448	5.08
		5698	3065	29034	32480	0.8939	86.63372	
		4717	3154	7871	17637	0.4463	43.25303	
	Without air supply	19930	21122.33	41052	38896	1.0554	102.2893	0.51
		15938	16292.33	32230	30464	1.0580	102.5377	
		25475	25316.67	50792	47669	1.0655	103.2668	

Table D-2 Number of total bacteria in oil-in-water emulsion of air supply and without air supply condition after 5 days of incubation by plate count technique

Times	Air supply condition (CFU/80 ml)	Without air supply condition (CFU/80 ml)
Day 0	3.20E+10 ± 3.03E+09	4.00E+10 ± 1.39E+10
Day 1	2.40E+10 ± 1.22E+10	3.20E+10 ± 2.44E+10
Day 2	3.20E+10 ± 9.24E+09	2.40E+10 ± 1.22E+10
Day 3	3.20E+10 ± 9.24E+09	5.60E+09 ± 2.57E+09
Day 4	4.00E+10 ± 9.24E+09	1.12E+09 ± 1.67E+08
Day 5	4.00E+10 ± 2.81E+10	1.12E+09 ± 3.33E+08

Table D-3 The gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value for plotting standard curve of the *adhA3* gene copy numbers

Gene copy number	Ct value
1.00E+08	16.65
	17.37
1.00E+06	22.08
	23.05
1.00E+04	31
	30.74
	30.9
1.00E+02	36.8
	37.98

Standard curve equation: $y = -3.468x + 44.42$

Amplification efficiency $= 10^{(-1/\text{slope})}$

$= 10^{(-1/-3.468)}$

$= 1.9425$

%Efficiency $= (E-1) \times 100\%$

$= (1.9425-1) \times 100\%$

$= 94.25\%$

Table D-4 *adhA3* gene copy numbers by real-time PCR using oil-in-water emulsion sample after 5 days incubation with air supply and without air supply condition

Times	Air supply condition (*adhA3* gene copies/80 ml)	Without air supply condition (*adhA3* gene copies /80 ml)
Day 0	3.14E+09 ± 1.25E+08	3.50E+09 ± 1.77E+08
Day 1	5.35E+09 ± 9.19E+07	8.80E+09 ± 4.02E+08
Day 2	1.95E+10 ± 1.25E+10	1.57E+10 ± 6.02E+08
Day 3	1.81E+10 ± 2.37E+09	1.91E+09 ± 2.29E+07
Day 4	4.00E+10 ± 8.44E+09	8.83E+08 ± 2.42E+08
Day 5	4.19E+10 ± 5.63E+09	6.09E+08 ± 0.00E+00

3.2 Lubricating oil degradability test with *Sphingobium* sp. strain P2 in the form of immobilized cells with chitosan

Table D-5 Peak area of saturate, aromatic, and total oil remained in oil-in-water emulsion. Each data point was averaged from triple spot on chromarods.

Days	Treatment	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
0	control	31541	24037.667	55578	21931	2.5343	245.61495	1.41
		38079	35480.5	72683	29313	2.4796	240.31485	
		28966	27839.667	56806	22476	2.5274	244.94428	
	Air supply	40885	39408.33	80294	31658	2.5363	245.8071	6.67
		74910	0	74910	30705	2.4396	236.4433	
		76697	0	76697	29810	2.5729	249.3547	
	Without air supply	44570	36306	80876	32050	2.5234	244.5606	1.06
		42317	39816.33	82133	32607	2.5189	244.1211	
		47975	42487.67	90463	35619	2.5397	246.1423	
1	control	29514	31892	61406	30219	2.0320	196.9358	5.60
		28415	30878	59293	27637	2.1455	207.932	
		29841	30958.33	60800	28845	2.1078	204.2851	
	Air supply	13994	20044	34038	24256	1.4032	135.999	0.71
		17440	20068.67	37508	26813	1.3989	135.5745	
		32112	17238.33	49350	34920	1.4132	136.966	
	Without air supply	22267	24483.67	46750	28167	1.6598	160.8589	3.09
		19285	19192.67	38478	23978	1.6047	155.5216	
		21308	21238.5	42547	25632	1.6599	160.8727	
2	control	22550	22699	45249	21462	2.1083	204.329	7.32
		25077	26138.67	51216	26157	1.9580	189.7686	
		26302	27387.67	53690	26592	2.0190	195.6775	

Table D-5 Peak area of saturate, aromatic, and total oil remained in oil-in-water emulsion. Each data point was averaged from triple spot on chromarods (cont.).

Days	Treatment	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
2	Air supply	16969	16739.67	33709	24832	1.3575	131.5604	1.69
		20452	19984.33	36333	27001	1.3456	130.4119	
		17872	16946	34818	25232	1.3799	133.7362	
	Without air supply	40683	11087	51770	31479	1.6446	159.3917	2.30
		44989	10878.33	55867	34920	1.5999	155.0532	
		47370	0	47370	29447	1.6087	155.9062	
3	control	34946	36606	71552	38515	1.8578	180.0523	5.01
		38835	36828.33	75664	41925	1.8047	174.9103	
		32353	32936	65289	34218	1.9080	184.9211	
	Air supply	15482	17386	32868	25007	1.3144	127.3834	1.63
		16627	17472	34099	26172	1.3029	126.2698	
		14355	17331	31686	24731	1.2812	124.1729	
	Without air supply	16369	17013.33	33382	24284	1.3746	133.2268	2.52
		20517	16990	37507	26293	1.4265	138.2534	
		18525	17692	34282	24415	1.4041	136.0859	
4	control	25578	31199.67	56777	27061	2.0981	203.3419	1.99
		22034	28306	50340	23808	2.1144	204.9233	
		26087	29896	55983	26998	2.0736	200.9662	
	Air supply	10109	10836.67	20946	19443	1.0773	104.4094	0.85
		14031	20647.33	34678	32708	1.0602	102.7535	
		16433	7395.667	26652	25013	1.0655	103.2652	
	Without air supply	20255	22298	42553	29390	1.4479	140.3239	1.77
		19396	24361.5	43757	30933	1.4146	137.0988	
		31316	12826.5	44142	31126	1.4182	137.4453	

Table D-5 Peak area of saturate, aromatic, and total oil remained in oil-in-water emulsion. Each data point was averaged from triple spot on chromarods (cont.).

Days	Treatment	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
5	control	32789	37591.33	70381	28337	2.4837	240.7164	2.25
		25025	24800	49825	19747	2.5231	244.5328	
		33521	32604	66125	26191	2.5247	244.6873	
	Air supply	11869	12198	24067	24719	0.9736	94.35961	3.37
		12925	12057.67	24982	24610	1.0151	98.38222	
		13655	12677	26332	25257	1.0426	101.0436	
	Without air supply	25153	28081	53234	32986	1.6139	156.4102	1.32
		21433	24651.67	46085	28998	1.5893	154.0262	
		34563	23091	57654	36227	1.5914	154.2381	

Table D-6 Number of total bacteria in chitosan of air supply and without air supply condition after 5 days of incubation by plate count technique

Times	Air supply condition (CFU/0.2 g chitosan)	Without air supply condition (CFU/0.2 g chitosan)
Day 0	9.00E+06 ± 5.77E+05	6.00E+06 ± 1.53E+06
Day 1	1.80E+06 ± 2.08E+05	1.20E+07 ± 4.16E+06
Day 2	4.50E+06 ± 2.08E+05	1.35E+07 ± 5.77E+05
Day 3	1.50E+07 ± 2.00E+06	2.10E+07 ± 8.96E+06
Day 4	3.00E+06 ± 1.00E+06	3.00E+07 ± 5.77E+05
Day 5	6.00E+06 ± 1.15E+06	1.65E+07 ± 1.15E+06

Table D-7 Number of total bacteria in oil-in-water emulsion of air supply and without air supply condition after 5 days of incubation by plate count technique

Times	Air supply condition (CFU/80 ml)	Without air supply condition (CFU/80 ml)
Day 0	0.00E+00 ± 0.00E+00	0.00E+00 ± 0.00E+00
Day 1	1.04E+09 ± 1.60E+08	8.00E+05 ± 2.57E+05
Day 2	8.00E+08 ± 1.60E+08	5.12E+08 ± 3.33E+07
Day 3	8.00E+08 ± 2.12E+08	8.80E+08 ± 4.62E+08
Day 4	1.60E+08 ±8.00E+06	8.00E+07 ± 0.00E+00
Day 5	5.60E+08 ± 2.01E+08	3.20E+08 ± 2.88E+07

Table D-8 The gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value for plotting standard curve of the *AdhA3* gene copy numbers to calculate number of bacteria in chitosan

Gene copy number	Ct value
1.00E+09	11.35
	10.72
	11.65
1.00E+07	18.49
	17.25
	17.14
1.00E+05	25.29
	24.51
	24.9
1.00E+03	31.95
	31.02
	31.65

Standard curve equation: $y = -3.422x + 41.86$

Amplification efficiency $= 10^{(-1/\text{slope})}$

$= 10^{(-1/-3.422)}$

$= 1.9598$

%Efficiency $= (E-1) \times 100\%$

$= (1.9598-1) \times 100\%$

$= 95.98\%$

Table D-9 The gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value for plotting standard curve of the *AdhA3* gene copy numbers to calculate number of bacteria in oil-in-water emulsion

Gene copy number	Ct value
1.00E+09	10.88
	9.28
	9.68
1.00E+07	17.76
	16.44
	16.37
1.00E+05	24.38
	23.97
	23.91
1.00E+03	31.66
	30.54
	31.28

Standard curve equation: y= -3.56x + 41.87

Amplification efficiency $= 10^{(-1/\text{slope})}$

$= 10^{(-1/-3.56)}$

$= 1.9094$

%Efficiency $= (E-1) \times 100\%$

$= (1.9094-1) \times 100\%$

$= 90.94\%$

Table D-10 *adhA3* gene copy numbers by real-time PCR using immobilized cells with chitosan samples after 5 days incubation with air supply and without air supply condition

Times	Air supply condition (*adhA3* gene copies/0.2 g chitosan)	Without air supply condition (*adhA3* gene copies /0.2 g chitosan)
Day 0	1.13E+05 ± 2.70E+04	3.00E+04 ± 2.22E+03
Day 1	2.52E+05 ± 1.38E+05	2.13E+06 ± 2.42E+05
Day 2	3.12E+06 ± 0.00E+00	5.02E+06 ± 5.40E+04
Day 3	1.43E+06 ± 2.21E+06	2.15E+07 ± 4.75E+06
Day 4	4.29E+05 ± 6.89E+05	1.69E+07 ± 2.68E+06
Day 5	4.35E+05 ± 2.42E+05	2.70E+06 ± 7.04E+05

Table D-11 *adhA3* gene copy numbers by real-time PCR using oil-in-water emulsion samples after 5 days incubation with air supply and without air supply condition

Times	Air supply condition (*adhA3* gene copies/80 ml)	Without air supply condition (*adhA3* gene copies /80 ml)
Day 0	0.00E+00 ± 0.00E+00	0.00E+00 ± 0.00E+00
Day 1	3.92E+06 ± 1.96E+05	4.80E+06 ± 5.57E+05
Day 2	1.89E+06 ± 1.24E+05	1.45E+06 ± 2.25E+05
Day 3	6.81E+06 ± 0.00E+00	4.68E+06 ± 0.00E+00
Day 4	1.27E+06 ± 0.00E+00	1.60E+06 ± 1.15E+05
Day 5	3.71E+07 ± 5.53E+06	1.17E+06 ± 2.08E+05

3.3 Application of lubricating oil degradation with immobilized cells in 3 l airlift bioreactor

Table D-12 Peak area of saturate, aromatic, and total oil remained in wastewater. Each data point was averaged from triple spot on chromarods .

Times	Wastewater	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
0 hr.	Influent	1044	1478.333	2522	25216	0.1000	15.50902	2.04
		1093	759.3333	1853	21656	0.0855	13.2658	
		997	1063.333	1728	23428	0.0738	11.43746	
	Effluent	878	870.6667	1748	24055	0.0727	11.27057	2.77
		969	1162.667	2132	23881	0.0893	13.84165	
		569	689.3333	1259	23491	0.0536	8.308647	
2 hr.	Effluent	891	474.6667	1365	22661	0.0602	9.342741	0.99
		1209	540.3333	1750	23966	0.0730	11.3209	
		1142	1069	1677	25161	0.0666	10.3325	

Table D-12 Peak area of saturate, aromatic, and total oil remained in wastewater. Each data point was averaged from triple spot on chromarods (cont.).

Times	Wastewater	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
4 hr.	Influent	2719	4507	7226	22486	0.3214	49.83339	7.43
		3633	2952.333	6585	22898	0.2876	44.59655	
		4988	4137.667	9126	23882	0.3821	59.25364	
	Effluent	441	836.3333	1277	24209	0.0528	8.181906	0.41
		632	403.6667	1036	21786	0.0475	7.371631	
		625	583	1208	24187	0.0499	7.742695	
6 hr.	Influent	1714	1077	2791	23911	0.1167	18.09803	13.58
		2228	1821.667	4049	25537	0.1586	24.58865	
		4024	2654.333	6678	23442	0.2849	44.17683	
	Effluent	887	737.333	1625	23608	0.0688	10.67167	2.37
		1154	1226	2380	26546	0.0897	13.90269	
		783	712.6667	1495	24947	0.0599	9.294691	
8 hr.	Influent	7072	7048.667	14121	26947	0.5240	81.26088	13.26
		5082	6522.667	11605	29864	0.3886	60.25916	
		5492	4942.667	10434	28518	0.3659	56.73705	
	Effluent	1016	1215.667	1893	27253	0.0694	10.76927	1.40
		1211	459.3333	1670	25711	0.0650	10.07194	
		1201	378	1579	19180	0.0823	12.76869	
10 hr.	Influent	8657	8289	16946	26621	0.6366	98.71063	21.67
		11811	11547.67	23359	26583	0.8787	136.2592	
		8385	8524.667	16909	26552	0.6368	98.75301	
	Effluent	849	576.3333	1425	26166	0.0545	8.447063	3.93
		819	525	1344	25581	0.0525	8.145182	
		1705	999	2704	27778	0.0974	15.09682	

Table D-12 Peak area of saturate, aromatic, and total oil remained in wastewater. Each data point was averaged from triple spot on chromarods (cont.).

Times	Wastewater	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
12 hr.	Influent	11261	10903	22164	27287	0.8123	125.9541	9.47
		14545	12587.67	27133	29477	0.9205	142.7333	
		13667	13607	27274	29791	0.9155	141.9646	
	Effluent	1507	1406	2913	30194	0.0965	14.95861	4.13
		2314	1376.333	3691	33949	0.0989	15.34132	
		1091	626	1717	29788	0.0577	8.939835	
14 hr.	Influent	7371	6886	14257	27324	0.5218	80.91339	3.68
		7891	6770	14661	29308	0.5002	77.56952	
		8711	7651.667	16363	29881	0.5476	84.91509	
	Effluent	773	398	1171	27210	0.0430	6.671454	4.02
		821	450	1271	27651	0.0460	7.129674	
		1454	1180.667	2634	29488	0.0893	13.85293	
Day 1	Influent	25483	25800	51283	43373	1.1824	183.3475	15.74
		23607	24570	48177	35037	1.3750	213.2229	
		24152	24421	48573	39706	1.2233	189.6967	
	Effluent	1596	1429.667	3025	43958	0.0688	10.67218	1.25
		10.6722	882.6667	1940	36806	0.0527	8.173501	
		1281	1033.333	2314	37390	0.0619	9.596856	
Day 3	Influent	19244	26832	46076	33320	1.3828	214.4311	2.11
		26937	25501	52438	31739	1.6522	256.2013	
		25915	26981	52896	40026	1.3215	204.9282	
	Effluent	1194	1030.667	2224	45080	0.0493	7.651332	0.40
		1266	940.6667	2206	46170	0.0478	7.410295	
		1459	1185	2644	50077	0.0528	8.187367	

Table D-12 Peak area of saturate, aromatic, and total oil remained in wastewater. Each data point was averaged from triple spot on chromarods (cont.).

Times	Wastewater	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
Day 5	Influent	23867	26003.5	49871	38422	1.2980	201.2727	8.87
		25915	26981	52896	40026	1.3215	204.9282	
		26530	28576.67	55107	39175	1.4067	218.1336	
	Effluent	4386	3687	8073	37989	0.2125	32.95159	4.67
		5814	5644	11458	44016	0.2603	40.36486	
		5679	5825.667	11505	42902	0.2682	41.58349	
Day 6	Influent	10685	9845.5	13687	39006	0.3509	54.41241	11.16
		7111	9608	16719	38768	0.4313	66.87411	
		10114	11467.33	21581	43641	0.4945	76.68457	
	Effluent	4488	3937	8425	43866	0.1921	29.78145	1.70
		4053	3985.667	8039	45056	0.1784	27.66754	
		5069	4170	9239	46166	0.2001	31.03166	
Day 9	Influent	25322	54314	79636	38758	2.0547	318.6172	16.88
		17712	18744.5	36456	16389	2.2245	344.9458	
		18074	18814	36888	16339	2.2577	350.0908	
	Effluent	1939	2771.667	4711	41426	0.1137	17.63331	4.43
		2826	2031.667	4858	45514	0.1067	16.5501	
		4615	3669.667	8285	52007	0.1593	24.70213	
Day10	Influent	13341	26036.67	39378	40276	0.9777	151.6102	23.11
		16035	22888.67	38924	36272	1.0731	166.4068	
		18715	21317.67	40033	51268	0.7809	121.0849	
	Effluent	775	513.3333	1288	27276	0.0472	7.324253	4.40
		1749	1252.667	3001	39960	0.0751	11.64679	
		3043	2601	5644	54257	0.1040	16.13172	

Table D-12 Peak area of saturate, aromatic, and total oil remained in wastewater. Each data point was averaged from triple spot on chromarods (cont.).

Times	Wastewater	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
Day12	Influent	6649	6240.667	12890	46758	0.2757	42.74853	7.50
		2156	1403	3559	16729	0.2127	32.98732	
		2947	2305	5252	29069	0.1807	28.01484	
	Effluent	593	1562	2155	25907	0.0832	12.90085	3.23
		3060	2229.333	5289	49061	0.1078	16.717	
		3321	3454	6775	54346	0.1247	19.33043	
Day14	Influent	9793	13753	23546	43880	0.5366	83.20744	22.66
		8099	9866.333	17966	29433	0.6104	94.65095	
		18048	22264	40312	49258	0.8184	126.9039	
	Effluent	1694	1569.333	3264	40130	0.0813	12.61113	3.47
		4061	1585	5646	46238	0.1221	18.93476	
		3149	3140.333	6290	53483	0.1176	18.23614	
Day18	Influent	7732	7481	15213	20347	0.7477	115.9406	9.74
		8974	8028	17002	22992	0.7395	114.6686	
		9823	9293	19116	22432	0.8522	132.1448	
	Effluent	950	865	1815	23259	0.0780	12.10059	0.48
		1325	607	1932	23810	0.0811	12.58255	
		1424	667	2091	24814	0.0843	13.06707	
Day21	Influent	32815	65690	98505	27897	3.5310	151.6102	18.24
		45281	47592	92873	27927	3.3256	515.6871	
		45556	46751	92307	27727	3.3291	516.2414	
	Effluent	676	136	812	24394	0.0333	5.161716	4.30
		907	650	1557	25841	0.0603	9.343302	
		2243	1793	4036	45456	0.0888	13.76832	

Table D-12 Peak area of saturate, aromatic, and total oil remained in wastewater. Each data point was averaged from triple spot on chromarods (cont.).

Times	Wastewater	Peak area of saturate	Peak area of aromatic	Total peak area	Peak area of Steryl alcohol	Total peak area/Peak area of steryl alcohol	Amount of remaining oil	SD
Day24	Influent	16275	16528	32803	25532	1.2848	199.2277	29.16
		10263	11201	21464	23375	0.9182	142.3902	
		16898	16852	33750	28734	1.1746	182.1373	
	Effluent	3090	2244	5334	30369	0.1756	27.23602	5.85
		3930	3040	6970	29541	0.2359	36.58716	
		4392	1305	5697	34156	0.1668	25.86427	
Day25	Influent	17189	16384	33573	29779	1.1274	174.824	27.33
		19745	18725	38470	26865	1.4320	222.0529	
		16600	28964	45564	31789	1.4333	222.2624	
	Effluent	0	1201	1201	23831	0.0504	7.814871	2.39
		509	1556	2065	26474	0.0780	12.09544	
		0	1281	1281	24500	0.0523	8.107821	
Day27	Influent	37599	14095	51694	19656	2.6299	407.8177	52.18
		34109	34479	68588	20805	3.2967	511.2126	
		45491	16505	61996	20379	3.0422	471.7391	
	Effluent	2200	1432	3632	25087	0.1448	22.4501	10.26
		2038	1897	1968	24969	0.0788	12.21897	
		3006	2652	5658	26804	0.2111	32.7329	
Day30	Influent	15331	14826	30157	24345	1.2387	192.0877	2.41
		15314	16560	31874	25376	1.2561	194.7756	
		15568	16979	32547	25632	1.2698	196.9017	
	Effluent	2359	1729	4088	24895	0.1642	25.4636	1.05
		4430	3287	7717	45038	0.1713	26.56994	
		1378	1553	2931	18579	0.1578	24.46327	

Table D-13 Percent oil removal of 3 l airlift bioreactor.

Times	% oil removal	Times	% oil removal
0 hr.	12.71	Day 6	55.31
2 hr.	22.92	Day 9	94.19
4 hr.	84.84	Day 10	92.01
6 hr.	61.01	Day 12	52.82
8 hr.	83.05	Day 15	83.67
10 hr.	90.50	Day 18	89.59
12 hr.	90.08	Day 21	98.21
14 hr.	88.64	Day 24	82.88
Day 1	95.15	Day 25	95.47
Day 3	96.20	Day 27	95.15
Day 5	81.60	Day 30	86.90

Table D-14 COD concentration of influent and effluent wastewater of 3 l airlift bioreactor.

Day	Influent	Effluent	% COD removal
1	360	212	41.11
5	620	524	15.48
10	282	226	19.86
15	492	240	51.22
20	472	250	47.03
25	328	302	7.93
30	424	404	4.72

Table D-15 Number of total bacteria in chitosan and wastewater in 3 l airlift bioreactor after 1 month of incubation by plate count technique

Days	Number of total bacteria in chitosan (CFU/7.5 g chitosan)	Number of total bacteria in wastewater (CFU/3 l)
0	8.21E+10 ± 4.50E+09	6.57E+08 ± 3.00E+06
1	3.04E+10 ± 1.95E+09	5.10E+10 ± 6.05E+09
5	2.21E+10 ± 2.98E+08	4.29E+10 ± 7.94E+08
10	6.59E+10 ± 1.25E+09	2.70E+09 ± 7.94E+08
15	9.79E+09 ± .98E+08	4.20E+09 ± 5.20E+08
20	9.23E+09 ± 2.25E+08	5.10E+09 ± 7.94E+08
25	9.00E+09 ± 1.13E+09	1.65E+10 ± 1.08E+09
30	1.13E+09 ± 0.00E+00	7.62E+10 ± 1.59E+09

Table D-16 The gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value for plotting standard curve of the *adhA3* gene copy numbers to calculate number of bacteria in chitosan and wastewater in 3 l airlift bioreactor

Gene copy number	Ct value
1.00E+09	8.93
	9.16
	9.24
1.00E+07	16.3
	16.07
	16.03
1.00E+05	23.72
	23.16
	23.03
1.00E+03	31.15
	29.77
	30.53

Standard curve equation: $y = -3.571x + 41.18$

Amplification efficiency $= 10^{(-1/\text{slope})}$

$= 10^{(-1/-3.571)}$

$= 1.9056$

%Efficiency $= (E-1) \times 100\%$

$= (1.9056-1) \times 100\%$

$= 90.56\%$

Table D-17 *adhA3* gene copy numbers by Real-time PCR using immobilized cells with chitosan and wastewater sample from 3 l airlift bioreactor after 1 month of incubation.

Days	*adhA3* gene copy numbers in chitosan (*adhA3* gene copies/7.5 g chitosan)	*adhA3* gene copy numbers in wastwater (*adhA3* gene copies/3 l)
0	1.62E+08 ± 1.33E+08	
1	2.02E+08 ± 3.54E+07	1.60E+07 ± 2.22E+07
5	1.18E+08 ± 0.00E+00	2.63E+06 ± 3.10E+06
10	3.36E+08 ± 1.12E+08	1.24E+07 ± 1.08E+06
15	3.87E+08 ± 8.62E+07	2.04E+07 ± 2.32E+05
20	3.68E+08 ± 6.23E+07	2.08E+07 ± 1.25E+07
25	1.55E+08 ± 3.13E+06	2.68E+07 ± 9.33E+06
30	1.92E+07 ± 2.03E+06	3.57E+07 ± 2.08E+07

3.4 Application of lubricating oil degradation with immobilized cells in 350 l airlift bioreactor

Table D-18 Total petroleum hydrocarbon and percent of oil removal during degradation by chitosan-immobilized *Sphingobium* sp. strain P2 in 350 l airlift bioreactor for 2 month of incubation

Days	Influent	Efluent	% oil removal	Days	Influent	Efluent	% oil removal
1	3.4	1.0	70	33	3.5	2.4	33
2	2.4	1.0	58	36	8.6	3.0	65
3	5.5	1.5	73	37	8.0	3.1	62
4	5.1	2.4	53	38	10.1	4.2	58
5	7.9	2.4	70	39	7.6	2.1	72
8	7.8	2.2	72	40	6.6	1.7	74
9	5.1	3.4	33.33	46	150.0	46.0	69
10	5.7	2.1	63	47	1.7	0.6	62
22	3.0	0.0	100	50	1.9	0.6	67
24	3.3	0.0	100	51	2.6	1.1	59
26	3.7	0.2	95	52	3.3	1.4	56
30	15.1	3.9	74	53	3.5	1.4	60
31	14.4	2.7	81	54	3.2	1.4	56
32	14.6	3.4	77	60	6.3	2.9	54

Table D-19 COD value of wastewater after treatment compare with wastewater before treatment from 350 l airlift bioreactor

Days	Influent	Efluent	% COD removal	Days	Influent	Efluent	% COD removal
1	94	25	73.40	33	125	103	18
2	120	48	60	36	86	59	31
3	53	28	47	37	118	95	19
4	62	23	63	38	102	76	25
5	137	82	40	39	67	51	24
8	124	55	56	40	84	44	48
9	68	33	51	46	328	152	54
10	60	33	45	47	37	16	57
22	60	49	18	50	54	45	17
24	39	19	51	51	58	49	16
26	60	38	37	52	69	45	35
30	164	159	3.049	53	73	49	33
31	153	144	6	54	75	44	41
32	159	121	24	60	84	66	21

Table D-20 Number of total bacteria in chitosan and wastewater in 350 l airlift bioreactor after 2 month of incubation by plate count technique

Days	Number of total bacteria in chitosan (CFU/875 g chitosan)	Number of total bacteria in wastewater (CFU/350 l)
1	1.16E+13 ± 5.25E+11	8.51E+11 ± 1.85E+11
3	5.25E+11 ± 2.63E+10	5.53E+12 ± 5.00E+07
5	3.54E+12 ± 3.47E+11	1.96E+12 ± 1.85E+12
7	2.89E+12 ± 3.47E+11	2.52E+14 ± 1.05E+13

Table D-21 The gene copy number (equivalent to the plasmid copy number) *vs.* the Ct value for plotting standard curve of the *AdhA3* gene copy numbers to calculate number of bacteria in chitosan and wastewater in 350 l airlift bioreactor

Gene copy number	Ct value
1.00E+09	11.35
	10.72
	11.65
1.00E+07	18.49
	17.25
	17.14
1.00E+05	25.29
	24.51
	24.9
1.00E+03	31.95
	31.02
	31.65

Standard curve equation: $y = -3.422x + 41.86$

Amplification efficiency $= 10^{(-1/\text{slope})}$

$= 10^{(-1/-3.422)}$

$= 1.9598$

%Efficiency $= (E-1) \times 100\%$

$= (1.9598-1) \times 100\%$

$= 95.98\%$

Table D-22 *adhA3* gene copy numbers by Real-time PCR using immobilized cells with chitosan and wastewater sample from 350 l airlift bioreactor after 2 month of incubation.

Days	*adhA3* gene copy numbers in chitosan (*adhA3* gene copies/875 g chitosan)	*adhA3* gene copy numbers in wastwater (*adhA3* gene copies/350 l)
1	1.18E+10 ± 0.00E+00	8.16E+08 ± 3.94E+08
3	1.17E+10 ± 1.58E+09	2.87E+07 ± 1.63E+07
5	9.08E+08 ± 1.11E+08	4.62E+08 ± 4.74E+07
7	5.34E+09 ± 1.01E+09	1.75E+09 ± 2.29E+08

BIOGRAPHY

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