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ASSESSMENT OF BIOREMEDIATION POTENTIAL OF WASTEWATER FROM PETROL STATION

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ศูนย์วิทยทรัพยากร

A Thesis Submitted in Partial Fulfillment of the Requirements

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สิทธิ ทาทอง : การประเมินความเป็นไปได้ในการบำบัดน้ำเสียจากสถานีบริการน้ำมันทางชีววิธี (ASSESSMENT OF BIOREMEDIATION POTENTIAL OF WASTEWATER FROM PETROL STATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. อรฤทัย ภิญญาคง, 108 หน้า.

กิจกรรมการล้างรถในสถานีบริการน้ำมัน ได้ก่อให้เกิดน้ำเสียที่ปนเปื้อนน้ำมันออกมาเป็นจำนวนมาก น้ำเสียเหล่านี้หากไม่ได้รับการบำบัดอย่างถูกวิธี จะสามารถก่อให้เกิดอันตรายทั้งต่อมนุษย์และสิ่งแวดล้อมได้ การกำจัดสารพิษโดยชีววิธี (Bioremediation) ถือเป็นอีกวิธีหนึ่งที่สามารถใช้บำบัดน้ำเสียเหล่านี้ได้ โดยในงานวิจัยนี้มีเป้าหมายเพื่อประเมินความเป็นไปได้ในการบำบัดน้ำเสียจากสถานีบริการน้ำมัน และใช้น้ำมันหล่อลื่น (PTT V-120) เป็นสารมลพิษต้นแบบ โดยได้แยกแบคทีเรีย 2 สายพันธุ์ ได้แก่ Tr1 และ R2 ที่มีความสามารถย่อยสลายน้ำมันได้จากน้ำเสียจากสถานซ่อมบำรุงรถ จากการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ 16S rDNA ได้จำแนกแบคทีเรียทั้งสองสายพันธุ์เป็น *Acinetobacter* sp. หลังจากทดสอบความสามารถในการย่อยสลายน้ำมันในรูปของอิมัลชัน ความเข้มข้น 200 ส่วนในล้านส่วนพบว่า แบคทีเรีย R2 มีความสามารถสูงกว่าคือย่อยได้ 78.01 % เปรียบเทียบกับชุดควบคุมที่ระยะเวลา 18 ชั่วโมง นอกจากนี้ยังตรวจพบยีน *alkM* ซึ่งเกี่ยวข้องกับการย่อยสลายน้ำมันในสายพันธุ์ R2 ด้วย การทดสอบการย่อยสลายในระบบนิเวศน์จำลองน้ำนั้น ใช้น้ำเสียจากสถานีบริการน้ำมันปริมาตร 50 มิลลิลิตร ในขวดรูปชมพู่ขนาด 250 มิลลิลิตรซึ่งน้ำเสียมีน้ำมันปนเปื้อนอยู่ 39.80 มก./ล. และได้เติมน้ำมันเพิ่มเป็น 5% (v/v) โดยแบ่งการบำบัดออกเป็น Natural Attenuation, Biostimulation, Bioaugmentation I, Bioaugmentation II และ Abiotic Control โดยเติมแบคทีเรีย R2 (10^7 CFU/มิลลิลิตร) ในชุดทดลอง Bioaugmentation I และ II และเติมไนโตรเจนและฟอสฟอรัสในชุดทดลอง Biostimulation และ Bioaugmentation II ให้มีอัตราส่วน C:N:P สุดท้ายเท่ากับ 100:5:1 ผลการทดลองหลังจากระยะเวลา 12 วัน พบว่า ชุดทดลอง Bioaugmentation II มีปริมาณน้ำมันเหลืออยู่น้อยที่สุด (14.47%) ทั้งนี้เนื่องมาจากการมีเชื้อที่มีความสามารถในการย่อยสลายน้ำมันและปริมาณสารอาหารที่เหมาะสมในชุดทดลอง ผลการทดลอง PCR-DGGE แสดงให้เห็นว่าประชากรแบคทีเรียในระบบนิเวศน์จำลองน้ำ มีการเปลี่ยนแปลงเล็กน้อยและตรวจพบสายพันธุ์ R2 ตลอดการทดลองในระบบที่มีการเติมเชื้อนี้ลงไป นอกจากนี้ยังตรวจพบยีน *alkM* ในทุกชุดการทดลองยกเว้น abiotic control ดังนั้นจึงกล่าวได้ว่ามีความเป็นไปได้ในการบำบัดน้ำเสียจากสถานีบริการน้ำมันโดยวิธีการบำบัดทางชีวภาพ

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....สิทธิ ทาทอง.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....อรฤทัย ภิญญาคง.....

##4989471620: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORD: WASTEWATER / PETROL STATION / BIOREMEDIATION / LUBRICATING OIL

SITTI TATHONG : ASSESSMENT OF BIOREMEDIATION POTENTIAL OF WASTEWATER FROM PETROL STATION. THESIS PRINCIPAL ADVISOR : ASST. PROF. ONRUTHAI PINYAKONG, Ph.D., 108 pp.

Car washing activity has been generating large amount of oil-contaminated wastewater from petrol station that will be released to the environment. Bioremediation is alternative method used to treat oil-contaminated wastewater due to its less expense and public acceptance. In this work, biodegradation potential of oil-contaminated wastewater was investigated using lubricating oil (PTT V-120) as a model contaminant. In order to use in microcosms, 2 oil-degrading bacteria, Tr1 and R2, isolated from oil-contaminated wastewater. Both isolates could be classified as *Acinetobacter* sp. based on 16S rDNA sequence analysis. Oil biodegradation activity of each isolate was determined as well. As a result, the strain R2 showed the higher degradation activity of 78.01% removed oil in culture medium after 18 hrs compared to abiotic control (initial oil was 200 ppm in emulsion form). In addition, *alkM* gene which involved in oil degradation could be detected in strain R2. For microcosms experiment, wastewater collected from petrol station in Bangkok was used. Biostimulation, bioaugmentation I, bioaugmentation II and natural attenuation treatment were conducted with 50 ml of wastewater with initial oil concentration of 39.80 mg/L and spiked with lubricating oil to make the final concentration of 5%(v/v) in 250 ml flasks. Isolate R2 was added to bioaugmentation I and II (approx. 10^7 CFU/ml). Nitrogen and phosphorus were added to give final C:N:P equal to 100:5:1 in both biostimulations and bioaugmentation II. After 12-day cultivation, bioaugmentation II showed the highest degradation efficiency with the value of remaining oil at 14.47 %. This might be due to the presence of both exogenous degrader and appropriate amount of necessary nutrient provided. PCR-DGGE exhibited slightly change in microcosms and strain R2 was detected in treatments that strain was introduced. Moreover, *alkM* gene was detected in all treatments except abiotic control. These findings indicate that there was bioremediation potential of wastewater from petrol station.

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Field of Study: Environmental Management Student's signature: Sitti Tathong

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NOMENCLATURE

A_{260}	=	Absorbance at wavelength 260 nanometer
A_{280}	=	Absorbance at wavelength 280 nanometer
bp	=	base pairs
DGGE	=	Denaturing Gradient Gel Elecrophoresis
g	=	gram
LB	=	Luria-Bertani
M	=	molar
m	=	milli
ml	=	milliliter
µl	=	microliter
N	=	normal
ng	=	nanogram
OD	=	Optical Density
PCR	=	Polymerase Chain Reaction
ppm	=	Parts Per Million
pmol	=	picomole

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CHAPTER I

INTRODUCTION

1.1 Statement of problem

The continuous growth of Thailand's economy during 1998 - 2008 has resulted in an increase of petroleum industries and gas stations. This is especially from1994 to 1997 when the number of gas stations increased from 9,000 to 14,000. These gas stations generate an average of 20 m^3 per day of wastewater. This harmful wastewater and its enormous volume is one major environmental concern. Gas station wastewater is generated from many activities such as car washing, floor cleaning, toilet and cafeteria usage, etc. However, most of the harmful wastewater originates from car washing operations with the average water consumption per car at 300-600 L (Panpanit, 2001). Gas stations generally produce a large volume of wastewater containing various pollutants. Wastewater originating from car washing operations contains suspended solids, emulsifier and oily wastewater.

Mixed lubricant oil and emulsifier is always found in car wash wastewater. Lubricating oils are very complex mixtures of hydrocarbons: linear and branched paraffins; and cyclic alkanes and aromatic hydrocarbons (> C15~ with boiling points between 300 and 600°C) (Vazquez-duhalt, 1989). When lubricating oil is used, other toxic substances, such as polycyclic aromatic hydrocarbon (PAH), will be generated through the combustion in engine, so this can classified the wastewater containing used lubricating oil as toxic (Powell *et al.*, 2005).

In general, oily wastewater from car wash is in oil emulsion form caused by admixture of automotive oil with emulsifier and wash water. Oil water emulsion in the presence of emulsifier is called stabilized emulsion and cannot be treated effectively by the conventional physical treatment methods such as grease trap that always used in petrol station. Therefore, the car wash wastewater is discharged without treatment and creates a crucial problem to the public sewer systems. As a result, it is necessary to clean up wastewater from petrol station in appropriate method.

Physical and, also, chemical treatments have been used to clean up this wastewater. However, these methods cost lots of expenses than other methods. It is also possible for petroleum hydrocarbon to be incompletely treated in some methods of physical and chemical treatment. The techniques are only transfer one form to another form of petroleum hydrocarbon. Further treatment is required for the complete treatment (Evans and Furlong, 2003).

Alternatively, bioremediation is one of the useful treatments to either reduce or remove petroleum hydrocarbon from the environment. Bioremediation is the use of microorganisms or microbial processes to degrade environmental contaminants. Previous studies have reported that bioremediation is less expensive. It eliminates waste permanently, eliminates long-term liability, and has greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods (Boopathy, 2000; Milcic-Terzic *et al.*, 2001).

As a result, many methods of bioremediation have been developed for improving efficiency of bioremediation. The simplest methods is natural attenuation in which bioremediation process occurred by natural microorganism (Kaplan and Kitts, 2004). In some ways, nutrient or oxygen are added to increase degradation rate of intrinsic microorganisms. This method is called biostimulation (Seklemova and Pavlova, 2001). Another way is bioaugmentation. In this method, exogenous degrader might be applied to the contaminated environment to give effective bioremediation (Watanabe, 2001).

However, a successful bioremediation requires monitoring of capable microorganisms to degrade contaminants in that environment since there are many factors that limit bioremediation efficiency; suitable temperature, the presence of oxygen, level of nutrient and co-substrate, bioavailability of contaminants, and the presence of degrading bacteria. If factors in the environment are suitable and achieve microorganisms are present, the process of bioremediation will be occurred (Romantschuk *et al.*, 2000).

Recently, the methodology consisting mainly of direct extraction of DNA from samples and detection of microbial community by monitor 16S rDNA fragment genes by polymerase chain reaction (PCR) with specific primers has been proposed to assess the biodegradation potential of a site. In addition to its specificity, simplicity, and speediness this type of genetic approach has a great advantage over the conventional cultivation (Moon *et al.*, 2006). Not only 16S rDNA fragment, but also

catabolic gene detection by PCR provide useful information on bioremediation potential. If catabolic gene specific to contaminant biodegradation is present, the potential of bioremediation will be available.

Moreover, PCR-DGGE of 16S rDNA is widely used to determine microbial community since only 12% or less of the total microbial population in environment is as yet culturable (Greene and Voordouw, 2004). So, PCR-DGGE of 16S rDNA can give greater information of microbial community.

Therefore, this study have monitored the bioremediation potential of wastewater from petrol station and also found the most effective way of bioremediation including natural attenuation, biostimulation and bioaugmentation. Moreover, PCR was held to detect oil degrading bacteria in wastewater from petrol station to investigate the presence of both indigenous and exogenous oil-degrading bacteria.

1.2 Objectives

The main objectives of this study are to monitor petroleum hydrocarbon-degraders using molecular biology method and to select the most effective method of bioremediation for wastewater from petrol station. Specific objectives are

1.2.1. To isolate petroleum hydrocarbon-degraders for using in bioremediation and to determine biodegradation activity of isolates.

1.2.2. To determine toxicity level of wastewater from petrol station.

1.2.3. To detect petroleum hydrocarbon-degrading bacteria in wastewater.

1.2.4. To develop bioremediation treatments i.e. biostimulation and bioaugmentation for evaluating the most effective way for bioremediation.

1.3 Hypothesis

Bioaugmentation and biostimulation can enhance the petroleum-degradation efficiency of indigenous degrader in wastewater from petrol station.

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1.4 Scope of Study

1.4.1 Microorganism isolation from oil-contaminated wastewater and identification

Wastewater from car maintenance garage was collected to be used for isolation of oil-degrading bacteria. After getting pure culture, identification and degradability test were held to detect gene involve in hydrocarbon degradation.

1.4.2 Samples collection and characterization

Wastewaters from the entrance of oil-trapping tank from 4 petrol stations were collected. Properties of samples, nitrogen, phosphorus, initial oil, TOC and pH, were analyzed. After that, only one water sample was selected to be used in microcosm based on its characteristic and activities in the station.

1.4.3 Lubricating oil degradation in wastewater microcosms

Bioremediation of lubricating oil contaminated water was conducted using 50 ml water microcosms. Lubricating oil was spiked to make the final concentration of oil equal to 5% v/v. The treatments were consisted of:

- Natural attenuation: application of non-sterile water without any isolates and nutrient addition
- Biostimulation: application of nutrient addition (N and P were added to make C:N:P equal to 100:5:1)
- Bioaugmentation I: application of the selected isolate
- Bioaugmentation II: application of the selected isolate with nutrient addition
- Abiotic control: application of sterilized water in order to consider physical degradation.

After 12 days of incubation, amount of lubricating oil in all treatments were quantified. Microbial analyses including enumeration of total and oil-degrading

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bacteria, DGGE of 16S rDNA, catabolic gene involved in oil degradation and toxicity test were analyzed also. Therefore, the most effective way to performed bioremediation of wastewater from petrol station was exhibited.

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CHAPTER II

LITERATURE REVIEW

2.1 Wastewater from petrol station

2.1.1 Wastewater characteristics

Petrol station wastewater is generated from many activities such as car washing, floor cleaning, toilet and cafeteria usage, etc. However, most of the harmful wastewater originates from car washing operations with the average water consumption per car at 300-600 L. 80% of wastewater generated in gas station in Thailand is car wash wastewater (Suwit, 1997). The wastewater generated from car wash stations contains various impurities such as free oil, oil/water emulsion, emulsifier and clay sludge. Table 2.1 exhibits car wash wastewater.

Table 2.1 Car wash wastewater characteristics collecting by time composite sampling method every 1 hour for 4 days (Chawadeth, 1998)

Day	Parameter						
	pH	COD (mg/L)	BOD (mg/L)	Oil & Grease (mg/L)	SS (mg/L)	TDS (mg/L)	$PO_4^{3-}P$ (mg/L)
1	6.91	170	78.8	1,780	83	380	2.75
2	6.74	70	31	283	793	433	2.09
3	7.33	135	62	18,200	986	608	1.11
4	7.22	116	45	2,510	205	705	5.1
Average	7.05	133	54	5,690	517	531	2.8

Typically, automatic car washing operation consists of four consecutive steps. Each step can be described simply as follow (Figure 2.1) (Panpanit, 2001):

Stage (1) Dust cleaning: high-pressure water is injected on the car body and engine in order to wash away the dirty particles. The wastewater in this stage contains a high concentration of sludge, clay and free oil. In addition, the mechanical force from the high pressure of the water breaks oil into oil/water emulsion.

Stage (2) Foaming: The emulsifier solution is sprayed on the car body. The wastewater in this stage consists mainly of the high concentration of emulsifier and later it is combined with oily wastewater from stage 1. Hence, the stabilization of oil/water emulsion takes place.

Stage (3) Presoak: This stage is to remove the emulsifier from the car body by spraying with fresh water. The wastewater generated in this stage mostly contains low emulsifier concentration because it is diluted by fresh water.

Stage (4) Spot free rinse and dryer: The objective of this stage is to polish car color. High quality water is sprayed on the car body, which is later dried by hot air. The fresh water contains high impurity such as hardness and organic matter are avoided in this process because it can creates an unpolished appearance problems such as spot on the vehicle's surface color.

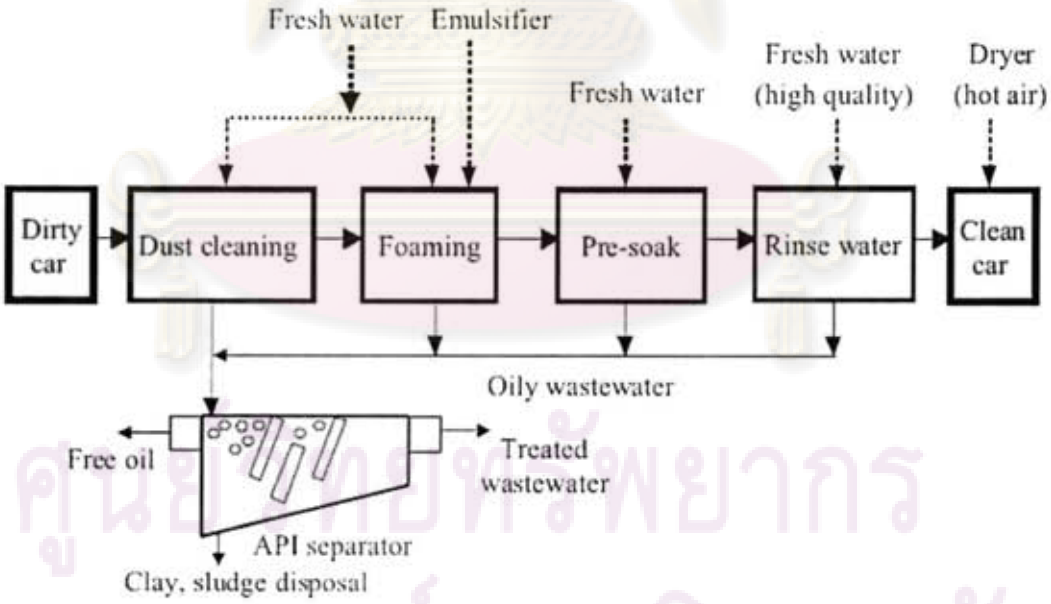

Figure 2.1 Process flow diagram of car wash process (Panpanit, 2001)

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2.1.2 Oil-in-water emulsion

Emulsions are a class of disperse systems consisting of two immiscible liquids. The liquid droplets (the disperse phase) are dispersed in a liquid medium (the continuous phase) (Tadros, 2005). The two main categories of emulsions are oil in water (OW) and water in oil (WO), with water including the most highly polar, hydrophilic (water-loving) liquids. Hydrophobic (water-hating) non-polar liquids are considered "oils." (Alther, 1998). Car wash wastewater is considered to be oil-in-water emulsion since petroleum hydrocarbon is dispersed in water.

Emulsions consist of three phases: The internal, or discontinuous, phase consists of finely divided droplets. The external, or continuous, phase is the matrix that keeps droplets, in suspension. The interphase consists of an emulsifier, or stabilizer, that keeps the emulsion stable, binding the internal and external phases together, and preventing droplets from approaching each other and coalescing (Alther, 1998)

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).

2.1.3 Pollution of oil-in-water car wash emulsion

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).

The lubricating oil is usually a mixture of lubricating base oil and additives. There are essentially two sources of lubricating oil: refining of petroleum crude oil and synthesis of relatively pure compounds which are suitable for a lubricant purpose.

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 characteristic classes-paraffin naphthenes, condensed naphthene, aromatic naphthenes, naphthalene and multi-ring aromatics. 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).

2.2 Lubricating oils (Stachowiak and Batchelor, 2005)

2.2.1 Types of lubricating oils

Oils are generally classified as refined oil, or mineral oil, and synthetic. Paraffinic and naphthenic oils are refined from crude oil while synthetic oils are manufactured. Literature on lubrication frequently makes references to long chain molecules and ring structures in connection with paraffinic and naphthenic oils, respectively. These terms refer to the arrangement of hydrocarbon and carbon atoms that make up the molecular structure of the oils. The distinguishing characteristics between these oils are noted below.

(a) Paraffinic oils.

Paraffinic oils are distinguished by molecular structure composed of long chains of hydrocarbons, i.e., the hydrogen and carbon atoms are linked in a long linear series similar to a chain. Paraffinic oils contain paraffin wax and the most widely used base stock for lubricating oils. In comparison with naphthenic oils, paraffinic oils have:

- Excellent stability (higher resistance to oxidation).
- Higher pour point.
- Higher viscosity index.
- Low volatility and, consequently, high flash points.
- Low specific gravities.

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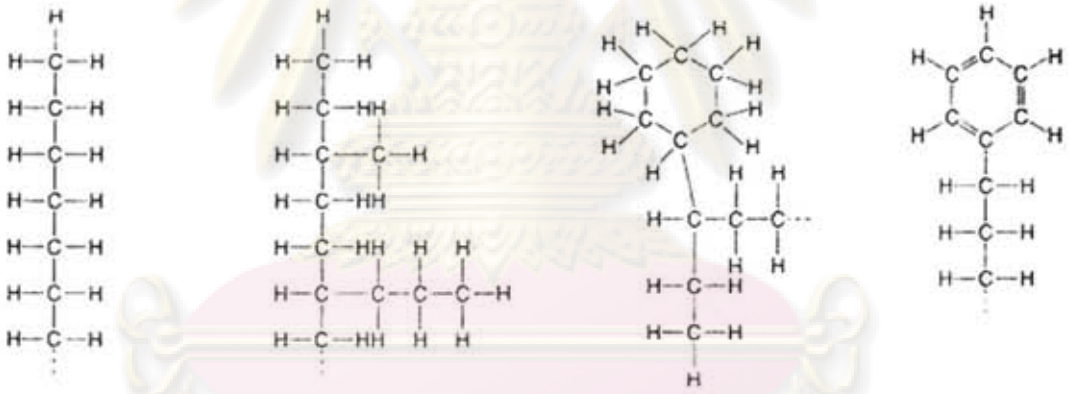
(b) Naphthenic oils

In contrast to paraffinic oils, naphthenic oils are distinguished by a molecular structure composed of "rings" of hydrocarbons, i.e., the hydrogen and carbon atoms are linked in a circular pattern. These oils do not contain wax and behave differently than paraffinic oils. Naphthenic oils have:

- Good stability.
- Lower pour point due to absence of wax.
- Lower viscosity indexes.
- Higher volatility (Lower flash point).
- Higher specific gravities.

Naphthenic oils are generally reserved for applications with narrow temperature ranges and where a low pour point is required.

Figure 2.2 shows chemical structures of mineral or refined oil



(a) (b) (c) (d)

Figure 2.2 Chemical structure of mineral oil; (a) Straight paraffin (b) Branched paraffin (c) Napthene (d) aromatic (Stachowiak and Batchelor, 2005)

(c) Synthetic oils

Synthetic lubricants are produced from chemical synthesis rather than from the refinement of existing petroleum or vegetable oils. These oils are generally superior to petroleum (mineral) lubricants in most circumstances. Synthetic oils perform better than mineral oils in the following respects:

- Better oxidation stability or resistance.
- Better viscosity index.

- Much lower pour point, as low as -46°C (-50°F).
- Lower coefficient of friction.

The advantages offered by synthetic oils are most notable at either very low or very high temperatures. Good oxidation stability and a lower coefficient of friction permit operation at higher temperatures. The better viscosity index and lower pour point permit operation at lower temperatures.

The major disadvantage to synthetic oils is the initial cost, which is approximately three times higher than mineral-based oils. However, the initial premium is usually recovered over the life of the product, which is three times higher than conventional lubricants. The higher cost makes it inadvisable to use synthetics in oil systems experiencing leakage.

Several major categories of synthetic lubricating oils are available including:

- Synthesized hydrocarbons.

Polyalphaolefins and dialkylated benzenes are the most common types. These lubricants provide performance characteristics closest to mineral oils and are compatible with them. Applications include engine and turbine oils, hydraulic fluids, gear and bearing oils, and compressor oils.

- Organic esters.

Diabasic acid and polyol esters are the most common types. The properties of these oils are easily enhanced through additives. Applications include crankcase oils and compressor lubricants.

- Phosphate esters.

These oils are suited for fire-resistance applications.

- Polyglycols.

Applications include gears, bearings, and compressor for hydrocarbon gases.

- Silicones.

These oils are chemically inert, nontoxic, fire-resistant, and water repellant. They also have low pour points and volatility, good low-temperature fluidity, and good oxidation and thermal stability at high temperatures.

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2.2.2 Environmental consideration of used lubricating oil (Mortier and Orszulik, 1992)

There are three basic disposal methods for used lubricating oil;

- Disposal as toxic/hazardous waste
- Refining to produce base oil
- Used fuel

Of the three basic method of disposal or recycling of used oil, the most efficient in term energy conservation, in that is displaces an equipment amount of oil, is the use of the material as a fuel. The fuel gas components of principal environmental concern are PCBs, PAHs, dioxin and heavy metals. Some pretreatment of the oil will be required to ensure that emission standards for these materials are not exceeded when waste oil is used are fuel. Should this is not be possible, the oil must be considered a hazardous waste and treated accordingly.

Disposal waste oil as hazardous waste requires that it be incinerated at high temperature to ensure complete oxidation of PCBs, and PAHs. With regard to refining, the process is able to generate a product of sufficiently high quality; the principal environmental consideration concerns the proposal of the by products of the refinery process.

2.3 Physical and chemical treatment of oily wastewater emulsion

There are several methods which are employed in oily wastewater emulsion. Physical and chemical treatments are methods which have been used for oily wastewater emulsion.

Chang *et al.* (2001) had been applied an ultrafiltration (UF) membrane and ozone treatment to reuse oily wastewater generated from the automobile-components manufacturing. The UF membrane system (capacity, 5 t/d) was employed to treat two kinds of degreasing wastewater and cutting oil wastewater. Flux behavior and organic removal strongly depended on the type of oily wastewater. The UF permeates from the degreasing wastewater could be reused as make-up water. However, the cutting oil formulated with the UF permeates had very different characteristics such as emulsion size and foaming ratio because surfactant existed excessively in the UF permeates.

Partial oxidation of surfactants in UF permeates using ozone makes it unable to change the emulsion size, foaming ratio and refractory index, and thus, possible to reuse the UF permeates as process water.

Oily wastewater cleanup can be also carried out by gas flotation. Moosai and Dawe (2003) had studied and found that properly operated gas flotation units can reduce oil concentrations of wastewater effluents to well below 40 mg/l when gas flotation is particularly valuable for heavy oils (oils having a density close to that of water). The flotation process relies on the attachment of gas bubbles to the dispersed oil droplets. This attachment is heavily dependent on the complex processes involving the surface characteristics of the oil droplets and their interaction with gas, and can only be optimally achieved if the surface science conditions are properly understood. The attachment mechanisms include the oil/bubble contact, the interactions of chemical additives (usually surfactants) in aiding this contact and the spreading of the oil around the gas bubble. Additionally, initial agglomeration of the oil emulsion droplets is needed to increase the droplet size to within the range needed for effective flotation, ~60 μm. This paper examines the essential surface science of the gas flotation process, particularly the gas attachment to oil droplets and the use of surfactants.

Rajakovic *et al.* (2007) had investigated the efficiency of different sorbent materials for oil removal from wastewater; loose natural wool fibers (NWF) recycled wool based non-woven material (RWNM) and sepiolite. Sorption was carried out in continuous tubular contractor (initial oil concentration of 1511 mg/dm3) and batch tank (initial oil concentration of 5066 mg/dm3).Wool-based sorbents showed higher sorption capacity (5.56 g/g for NWF and 5.48 g/g for RWNM) compared to sepiolite (0.19 g/g) in case of sorption in batch tank. The study on sorption in continuous tubular contractor suggested that volume of oily wastewater strongly affected oil removal.

However, disadvantage of the physical and chemical treatment is their expensive cost. In addition, these treatments do not solve the contamination problem. The contaminants are incompletely treated. It transfers the contaminant from one phase to another phase which might become more toxic. Consequently, it needs the addition secondary treatment (Evans and Furlong, 2003).

2.4 Bioremediation

2.4.1 Bioremediation in general

Bioremediation is one of the useful treatment to either reduces or remove petroleum hydrocarbon from contaminated environment. Bioremediation is the use of microorganisms or microbial processes to degrade environmental contaminants (Boopathy, 2000). It is advantageous over other techniques in that it is a natural and safe process. Moreover, the operating cost is comparatively less expensive (Evans and Furlong, 2003 and Alexander, 1994). Bioremediation is the complete degradation process. The contaminant is metabolized to carbon dioxide and water. It eliminates waste permanently. In addition, it also has long-term liability, greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods (Boopathy, 2000).

2.4.1.1 Basic concept of bioremediation

1. Mineralization

The contaminant is taken up by microorganisms as nutrients and metabolized to carbon dioxide and water. Therefore, the contaminant is removed and destroyed by the microorganisms. In some case, it is possible to get the incomplete metabolization. It generates and accumulates the intermediate which maybe further treated by the other microorganisms (Evans and Furlong, 2003).

2. Cometabolism

Primary metabolism of an organic compound has been defined as the use of the substrate as a source of carbon and energy (Boopathy, 2000). When the target contaminant (co-substrate) can not used as the food sources for the microorganisms. The target contaminant (co-substrate) is degraded by the enzyme which reacts with another substrate (primary substrate) (Evans and Furlong, 2003).

3. Immobilization

It refers to the removal of the contaminant, especially metal by the adsorption or bioaccumulation of microorganisms or plants (Evans and Furlong, 2003).

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2.4.1.2 Factors affecting bioremediation

There are many reasons for organic compounds being degraded very slowly or not at all in the environment, even though they are biodegradable. Among those are:

1. Temperature

Inappropriate temperature causes mortality of contaminant degrading bacteria. For example, in soil, particularly in northern industrialized countries in Europe and North America, the soil temperature during a large part of the year is too low for efficient microbial degradation of soil contaminants (Romantschuk *et al*., 2000).

2. Anaerobic condition

Degradation in anaerobic conditions is slow; some compounds are not degraded anaerobically and some are degraded only partly and may give rise to toxic compounds (Romantschuk *et al*., 2000). The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil and of gasoline in groundwater (Leahy and Colwell, 1990).

3. Low levels of nutrients and co-substrates

A contaminated site usually has a sub-optimal nutrient balance. If the contaminant is a hydrocarbon, e.g. oil contamination, there is likely to be a shortage of at least nitrogen, but each site has to be evaluated case by case, taking into account also matters such as solubility of the contaminant in order not to over-fertilize (Romantschuk *et al*., 2000). It is well established that the availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbons in estuarine water and sediment, seawater, marine sediment, freshwater lakes, Arctic ponds, freshwater sediments, and groundwater (Leahy and Colwell, 1990).

4. Bioavailability

The rate at which microbial cells can convert contaminants during bioremediation depends on the rate of contaminant uptake and metabolism and the rate of transfer to the cell (mass transfer). The bioavailability of a contaminant is controlled by a number of physico-chemical processes such as sorption and desorption, diffusion, and dissolution (Boopathy, 2000).

5. Absence of degradation potential

A biological degradation pathway for synthetic, xenobiotic compounds may not exist precluding biodegradation, or genes encoding enzymes that may be active on the compound are not induced by the contaminant. Suitable pathways are, however,

likely to evolve, either naturally or accelerated in laboratory conditions (Romantschuk *et al.*, 2000).

2.4.1.3 Bioremediation approaches

1. Natural attenuation or intrinsic bioremediation

Natural attenuation could be looked upon as doing nothing, it involves in monitoring the natural attenuation process and the self-cleaning potential. The process is monitored and if non-functional abandoned in favor of more direct and extreme measures. The minimum is to monitor the site so that further spread of contaminating compounds is limited (Romasantschuk *et al.*, 2000). A disadvantage of this method is that it will take a long time to completely degrade the contaminants since the size of indigenous degrading microorganisms is low (Forsyth *et al.*, 1995).

2. Biostimulation

Biostimulation are the important methods for the bioremediation (Boopathy, 2000). Biostimulation is a treatment process that stimulate the activity of indigenous microorganisms by addition of nutrient, i.e. carbon; nitrogen; and phosphorus, and oxygen (Seklemova and Pavlova, 2001; Evans *et al.*, 2004).

3. Bioaugmentation

Bioaugmentation is the introduction of exogenous microorganisms into environments in order to accelerate bioremediation (Watanabe, 2001). This method is necessary since the indigenous microorganisms do not have ability to degrade the toxic compound. The great advantage of this method is short-time consuming for degradation of pollutants (Richard and Vogel, 1999).

Four major concerning conditions for bioaugmentation are:

- Low of indigenous bacteria

Amounts of the indigenous bacteria that can degrade the target contaminant are less than 10^5 CFU per gram soil or ml. It is a proper condition for bioaugmentation (Providenti, 1993).

- Time

The rate of decontamination is a main factor. Therefore, adding the degrading bacteria could be used to start the remediation process with little or no lag period in order to shorten the determinant period. It is short time for decontamination (Molnna and Grubbs, 1989).

- Assurance

Bioaugmentation provides a measure of assurance that correct bacteria were present in sufficient number for the degradation (Molnna and Grubbs, 1989).

- Complex waste

When the site is contaminated with high level of non-biodegradable waste types such as heavy metal, the physical or chemical treatment can be used before bioaugmentation (Forsyth *et al.*, 1995).

2.4.1.4 Monitoring of bioremediation potential

Recently, the characterization of genes involved in bacterial organic pollutant degradation has promoted the development and application of molecular techniques to the study of the microbial ecology of contaminated environments (Milcic-Terzic *et al.*, 2001). Even culture-based techniques, e.g., plate counts and most probable number (MPN) assays, are useful for enumerating specific culturable populations and introduced strains, only 12% or less of the total microbial population are as yet culturable (Greene and Voordouw, 2004).

Hence, molecular techniques are developed in order to solve the problems of conventional culture-depending methods such as morphological, metabolic and biochemical assays because of their specificity, simplicity, and speediness (Sanz and Köchling, 2007; Moon *et al.*, 2006). In wastewater treatment technology, Sanz and Köchling (2007) have summarized the widely used techniques which are Polymerase Chain Reaction (PCR) and polymerase chain reaction coupled with denaturant gradient gel electrophoresis (PCR-DGGE).

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Polymerase Chain Reaction (PCR)

This methodology implies the extraction of nucleic acids, amplification by polymerase chain reaction (PCR) and cloning of the 16S rDNA or interested catabolic genes, followed by sequencing and finally identification and affiliation of the isolated clone with gene database. Advantages of this method are as shown below.

- Complete DNA sequencing allows:
    - very precise taxonomic studies and phylogenetic trees of high resolution to be obtained; design of primers (for PCR) and probes.
- This approach covers most microorganisms, including minority groups which hard to detect.
- Identification of newly isolated microorganisms that have not been yet cultured or catabolic genes that have not been identified yet.

Polymerase chain reaction coupled with denaturant gradient gel electrophoresis (PCR-DGGE)

It is based on the differing mobility on a gel of denatured DNA-fragments of the same size but with different nucleic acid sequences, thus generating band patterns that directly reflect the genetic biodiversity of the sample. The number of bands corresponds to the number of dominant species. The most important application of DGGE is monitoring dynamic changes in microbial communities, especially when many samples have to be processed. By PCR-DGGE, monitoring of the community change of microorganisms can be done by amplification of 16S rDNA by PCR and followed by DGGE. Moreover, if catabolic gene of interest is amplified and run into DGGE, we can investigate the diversity of catabolic genes.

As mentioned, molecular biology techniques are widely used in the field bioremediation recently. DGGE is widely used in monitoring the change in microbial population after bioremediation treatment and the result could show better understanding than using conventional method. Muyzer *et al.* (1993) used DGGE of amplified 16S rDNA to analyze genetic diversity of complex microbial populations. The gel profile showed that there were 10 distinguishable bands, which derived from many different species constituting these populations.

For detection of aromatic compounds degraders, Cavalca *et al.* (2000) had selected aromatic hydrocarbon-degrading bacteria from a BTEX (benzene, toluene, ethylbenzene, xylene)-contaminated subsoil obtained from beneath a paint factory. Fifteen isolated strains were studied for their different biodegradative capacities. Among these, further molecular characterization was carried out by randomly amplified polymorphic DNA analysis to ascertain that all the studied strains belonged to different haplotypes. The isolates were characterized for the presence of genes encoding for toluene dioxygenase, xylene monooxygenase by polymerase chain reaction analysis and by Southern analysis.

Kohno *et al.* (2002) have designed 3 sets of primer to detect alkane-degrading bacteria and classified alkane hydroxylase genes into 3 groups. Group I was hydroxylase genes encoding *alkB* which catalyzes medium-chain-length (C_6-C_{12}) *n*-alkane via a terminal oxidation pathway with monooxygenase systems. The genes classified into Group II encode *alkM* which catalyzes long-chain alkanes (C_{12}-) via terminal oxidation with hydroxylase (monooxygenase) systems or with dioxygenase systems. The genes classified into Group III encode *alkB* or *alkB1* which are unknown for substrate specificity, *n*-alkane oxidation pathways, and oxidation systems. The result suggests that the primer system can detect most of alkane-degrading bacteria (59 out of 74) and can be applied to evaluate alkane-degradation potential in the environment.

In 2004, Agnelli *et al.* used DGGE to assess the distribution of indigenous bacterial community in soil. The 16S rDNA-DGGE pattern of bacteria population showed high diversity, moreover, the diversity of population changed according to the increased soil depth.

Moon *et al.* (2006) had determined the biodegradation potential in PAH-contaminated soil using PCR coupled with DNA hybridization.

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2.4.2 Biodegradation of petroleum hydrocarbon

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Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo constituents, most notably complexing vanadium and nickel (Van Hamme *et al.*, 2003). In this mixture, there are many types of hydrocarbon varied from short-chain alkane to heavy hydrocarbon such as

polycyclic aromatic hydrocarbons (PAHs) and long-chain alkane. This makes the fundamental of petroleum hydrocarbon compounds different from group to group (Van Beilen and Funhoff, 2007). Most researches have focused on biodegradation pathways of alkanes and PAHs. As a result, the fundamental of these hydrocarbons is now well understood.

Hydrocarbons in the environment are biodegraded primarily by the bacteria and fungi. Although ubiquitous in terrestrial and aquatic ecosystems, the fraction of the total heterotrophic community represented by the hydrocarbon-utilizing bacteria and fungi is highly variable, with reported frequencies ranging from 6% to 82% for soil fungi, 0.13% (80) to 50% (106) for soil bacteria, and 0.003% to 100% for marine bacteria. Individual organisms can metabolize only a limited range of hydrocarbon substrates, so that assemblages of mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil, freshwater, and marine environments (Leahy and Colwell, 1990).

2.4.2.1 Biodegradation of aliphatic compounds

Many microbial genera include strains that are able to grow on alkanes in order to use them as an energy and carbon source (Belhaj *et al.*, 2002; Vomberg and Klinner *et al.*, 2000). Alkanes are usually activated by terminal oxidation to the corresponding 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, e.g. in *Bacillus*, *Pseudomonas*, and more recently in *Rhodococcus* sp. Q15. 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. In some cases, both ends of the alkane substrate are oxidized, which has been exploited for the production of dicarboxylic acids by yeasts as well as bacteria (Figure 2.3) (Van Beilen *et al.*, 2003).

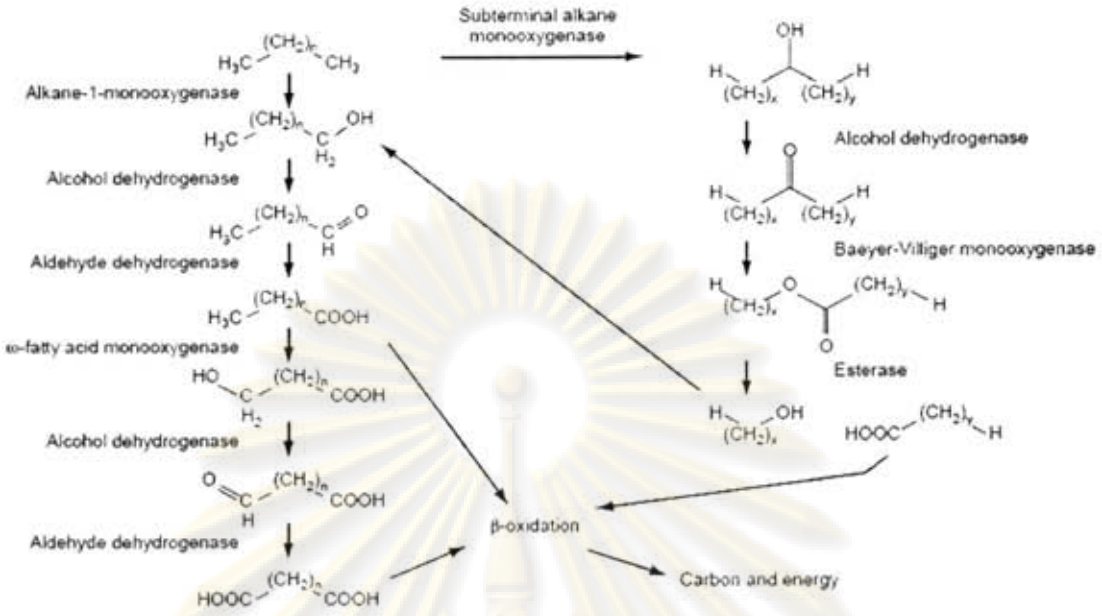

Figure 2.3 Alkane aerobic biodegradation via alkane hydroxylase system (Van Beilen *et al.*, 2003)

The enzyme that plays an important role on aliphatic alkane biodegradation is alkane hydroxylase system since these enzymes introduce oxygen atoms derived from molecular oxygen into the alkane substrate (Van Beilen and Funhoff, 2007; Van Beilen *et al.*, 2003). The first step of this enzymatic system is terminal oxidation with monooxygnase system. For medium-chain-length hydrocarbon ($C_6 - C_{12}$), *alkB* is the gene that encode oxidation systems and are possessed mainly by *Pseudomonas* sp.. For long-chain alkane (C_{12} -), *alkM* will play important role instead of *alkB* for terminal oxidation with monooxygenase systems and are possessed by *Acinetobacter* sp. (Kohno *et al*, 2002).

2.4.2.2 Biodegradation of aromatic compound

For aromatic fraction in petroleum product, there are also many bacteria that can utilize aromatic compounds (Baldwin *et al*., 2003; Habe and Omori, 2003; Sei *et al*., 1999). The first step in the microbial degradation of PAHs is the action of dioxygenase, which incorporates atoms of oxygen at two carbon atoms of a benzene ring of a aromatic resulting in the formation of *cis*-dihydrodiol, which undergoes rearomatization by dehydrogenases to form dihydroxylated intermediates.

Dihydroxylated intermediates subsequently undergo ring cleavage and form TCA-cycle intermediates (Samanta *et al.*, 2002) (Figure 2.4).

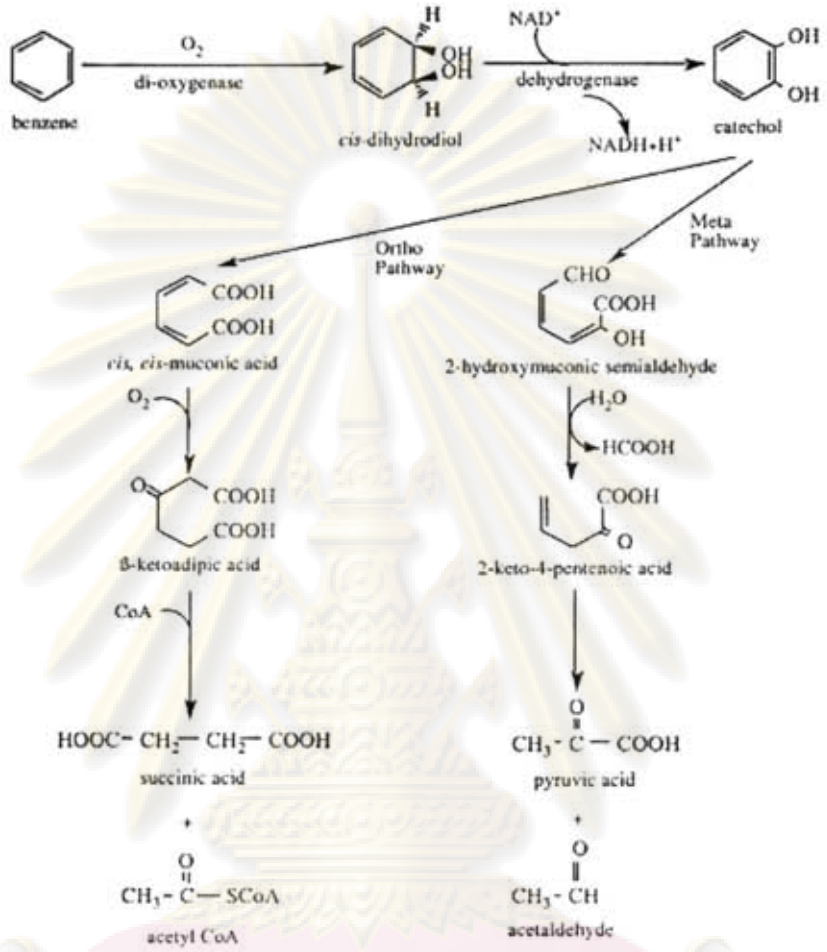

Figure 2.4 Aromatic compound aerobic biodegradation via aromatic dioxygenase (Juhasz and Naidu, 2000)

Tables 2.2 and 2.3 exhibit examples of alkane and aromatic compounds degrading bacteria, respectively.

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Table 2.2 Examples of aerobically alkane degrading bacteria (van Beilene *et al.*, 2003)

Bacterial strains	Degradable *n*-alkane
Acinetobacter sp. ADP1	C_{12}-C_{16}
Acinetobacter calcoceticus 69-V	C_{11}-C_{18}
Acinetobacter calcoceticus EB104	C_6-C_{18}
Acinetobacter calcoceticus NCIB 8250	C_8-C_{16}
Acinetobacter sp. 2796A	C_{10}-C_{16}
Acinetobacter sp. M-1	C_{13}-C_{44}
Alcanivorax borkumensis AP1	C_{10}-C_{16}
Alcanivorax borkumensis SK2	C_{10}-C_{16}
Burkhoderia cepacia ATCC 25416	C_{10}-C_{16}
Burkhoderia cepacia RR10	C_{10}-C_{16}
Pseudomonas aureofaciens RWTH 529	C_{10}
Pseudomonas putida GPo1	C_5-C_{12}
Pseudomonas fluorescens CHA0	C_{12}-C_{28}
Pseudomonas aeruginpsa PAO1	C_{12}-C_{16}
Mycobacterium bovis BCG	C_{12}-C_{16}
Mycobacterium smegmatis	C_9-C_{16}
Nocardiodes sp. *CF8*	C_2-C_{16}
Prauserella rugosa NRRL B-2295	C_8-C_{14}
Rhodococcus sp. 1BN	C_6-C_{28}
Rhodococcus erythropolis Q15	C_8-C_{32}

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Table 2.3 Examples of aerobically aromatic compound degrading bacteria (Juhasz and Naidu, 2000)

Bacterial strains	Substrates
Acinetobacter calcoaceticus	Naphthalene
Alcaligenes denitrificans	
Mycobacterium sp.	
Comamonas testosteroni	
Beijernickia sp.	Acenaphthene
Pseudomonas sp.	
Cycloclasticus sp.	
Neptunomonas naphthovorans	
Aeromonas sp.	Phenanthrene
Arthrobacter polychromogenes	
Micrococcus sp.	
Stenotrophomonas maltophilia	
Agrobacterium sp.	
Sphingomonas sp.	Anthracene
Cycloclasticus pugetii	
Rhodococcus sp.	
Arthrobacter sp.	
Acidovorax delafieldii	Fluoranthene
Pasteurella sp.	
Acinetobacter calcoaceticus	
Bucepacia sp.	

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2.4.2.3 Factors affecting bioremediation of petroleum hydrocarbon

Leahy and Colwell (1990) have summarized factors affecting the biodegradation of hydrocarbons as followed.

- **Physical and chemicals factors**

  Chemical composition of oils or hydrocarbons

  Hydrocarbons differ in their susceptibility to microbial attack and, in the past, have generally been ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. Biodegradation rates have been shown to be highest for the saturates, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation. This pattern is not universal; however, there is study reported greater degradation losses of naphthalene than of hexadecane in water-sediment mixtures from a freshwater lake.

  Physical state of oils or hydrocarbons

  Oil spilled in water tends to spread and form a slick. As a result of wind and wave action, oil-in-water or water-in-oil ("mousse") emulsions may form. Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. However, large masses (or plates) of mousse establish unfavorably low surface-to-volume ratios, inhibiting biodegradation.

  The key differences between petroleum biodegradation in soil and aquatic ecosystems following an oil spill are related to the movement and distribution of the oil and the presence of particulate matter, each of which affects the physical and chemical nature of the oil and hence its susceptibility to microbial degradation. Terrestrial oil spills are characterized primarily by vertical movement of the oil into the soil, rather than the horizontal spreading associated with slick formation. Infiltration of oil into the soil prevents evaporative losses of volatile hydrocarbons, which can be toxic to microorganisms. Particulate matter can reduce, by absorption, the effective toxicity of the components of petroleum, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues.

Concentration of oils or hydrocarbon

High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. There was a study reported that contamination of seashore sediments with crude oil above a threshold concentration prevented biodegradation of the oil because of oxygen and/or nutrient limitation. It is likely that high concentrations of oil have similarly negative effects on biodegradation rates following oil spills in other quiescent, 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.

- **Biological factors**

Adaptation – Effect of prior exposure

Prior exposure of a microbial community to hydrocarbons, either from anthropogenic sources or from natural sources is important in determining how rapidly subsequent hydrocarbon inputs can be biodegraded. This phenomenon, which results from increases in the hydrocarbon-oxidizing potential of the community, is known as adaptation. The three interrelated mechanisms by which adaptation can occur are (i) induction and/or depression of specific enzymes, (ii) genetic changes which result in new metabolic capabilities, and (iii) selective enrichment of organisms able to transform the compound or compounds of interest.

Role of plasmid in adaptation

Plasmid DNA may play a particularly important role in genetic adaptation in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotypes, including hydrocarbon-oxidizing ability, to recipient organisms. Exposure of natural microbial populations to oil or other hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community.

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2.4.2.4 Relevant studies on biodegradation of petroleum hydrocarbons

Many researchers had study on degradation of petroleum product such as crude oil (Meintanis *et al.*, 2006), paraffin (Koma *et al.*, 2001), diesel fuel and gasoline (Viera *et al.*, 2007) and lubricating oil (Lee *et al.*, 2007).

Crude oil could be biodegraded by bacteria isolated from volcanic island as reported by Meintanis *et al.* (2006). They screened bacterial isolate by using degenerated primers developed to amplify genes related to the *Pseudomonas putida* and *Pseudomonas oleovorans* alkane hydroxylases. Isolates were able to grow in liquid cultures with crude oil as the sole carbon source and were found to degrade long chain crude oil alkanes in a range between 46.64% and 87.68% from initial crude oil of 2% w/v.

Microorganisms that degrade long-chain *n*-paraffins from used car engine oil were isolated from soil and the strain mineralized long-chain *n*-paraffins (0.1% w/v) in the minimal medium after cultivation for 96 h and also reduced the weight of the waste oil added (1% w/v) by 20% after 72 h (Koma *et al.*, 2001).

Viera *et al.* (2007) had study the biodegradation of synthetic effluent containing diesel fuel and gasoline. They focused on fuel concentration, nitrogen concentration and culture type. The mixed cultures used in this study were obtained from lake with a history of petroleum contamination. Of the parameters studied, the ones that had the greatest influence on the removal of total petroleum hydrocarbons (TPH) were a nitrogen concentration of 550 mg/L and a fuel concentration of 4% (v/v) in the presence of culture collected from surface sediment. The biodegradability study showed a TPH removal of 90±2% over a process period of 49 days. There was also a report on biodegradation of waste lubricating oil.

Lee *et al.* (2007) had study the limits and extent of lubricants biodegradation at different nutrient conditions in soil. When nutrients were added to contaminated soil with aged lubricant, great stimulation was occurred in fertilized soil for hydrocarbon degradation activity compared to non-fertilized soil. At the end of the experiment (105 days after), the initial level of contamination (9320±343 mg/kg) was reduced by 42–51% in the fertilized soil, whereas, only 18% of the hydrocarbon was eliminated in the non-fertilized soil.

Lu *et al.* (2006) studied effects of culture conditions in vitro and biosurfactant detection were studied on bacterial strains capable of degrading gasoline from contaminated soils near gas station. Three bacteria (strains Q10, Q14 and Q18) were isolated and identified as *Pseudomonas* sp., *Flauobacteriurn* sp. and *Rhodococcus* sp., respectively. The optimal growth conditions of three bacteria including pH, temperature and the concentration of gasoline were similar. The reduction in surface tension was observed with all three bacteria, indicating the production of biosurfactant compounds. Gasoline, diesel oil, benzene, toluene, ethylbenzene and xylene (BTEX) could easily be degraded by the three isolates. The consortium was more effective than the individual cultures in degrading added gasoline, diesel oil, and BTEX. These results indicate that these strains have great potential for in situ remediation of soils contaminated by gas station leaking.

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CHAPTER III

METHODOLOGY

3.1 Chemicals and equipments

3.1.1 Chemicals

1. Lubricating oils (PTT V-120) was obtained from Petroleum Authority of Thailand (PTT), Thailand
2. Yeast extract was obtained from Difco Laboratories, USA
3. Tryptone was obtained from Difco Laboratories, USA
4. Sodium chloride (NaCl) was obtained from Merck, Germany
5. Magnesium sulfate ($MgSO_4.7H_2O$) was obtained from Carlo ERBA, France
6. Disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) was obtained from Merck, Germany
7. Ammonium chloride (NH_4Cl) was obtained from Merck, Germany
8. Potassium chloride (KCl) was obtained from Merck, Germany
9. Calcium chloride ($CaCl_2.2H_2O$) was obtained from Merck, Germany
10. Ammonium nitrate (NH_4NO_3) was obtained from Merck, Germany
11. Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck, Germany
12. Hydrochloric acid (HCl) was obtained from BDH Chemicals, Australia
13. Sodium hydroxide (NaOH) was obtained from Merck, Germany
14. Bacto agar was obtained from Difco, USA.
15. Glycerol was obtained from Research organics, Inc., USA
16. Rubidium chloride (RbCl) was obtained from Sigma, USA
17. Agarose gel was obtained from IUAI, Japan
18. Glacial acetic acid (CH_3COOH) was obtained from Merck, Germany
19. Phenol was obtained from Merck, Germany
20. Chloroform was obtained from Lab-Scan, Ireland

21. Isoamylalcohol was obtained from Sigma, USA

22. Hexane was obtained from J.T.Baker, USA

23. Dichloromethane was obtained from Merck, Germany

24. Methanol was obtained from Fisher Scientific, UK

25. 100 base pair DNA ladder plus was obtained from New England Biolabs, and Fermentas, USA

26. Lambda *Hin*dIII was obtained from New England Biolabs, USA

27. Ampicillin was obtained from Nacalal tesque, Japan

28. Restriction enzymes were obtained from Promega, USA and Fermentas, USA

29. Trizma base (tris [hydroxymethyl] aminomethane), ($C_4H_{11}NO_3$) was obtained from Sigma, USA

30. EDTA (ethylenediaminetetraacetic acid), ($C_{10}H_{14}N_2O_8Na_22H_2O$) was obtained from Sigma, USA

31. SDS (sodium dodecyl sulfate), ($C_{12}H_{25}OSO_3$) was obtained from Nacalal tesque, Japan

32. Ribonuclease A (RNase A) was obtained from Fermentas, USA

33. Proteinase K was obtained from US. Biological, USA

34. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) was obtained from BIO BASIC INC., Canada

35. IPTG (Isopropyl thio-β-D-galactoside) was obtained from BIO BASIC INC., Canada

36. *Taq* DNA polymerase was obtained from New England Biolabs, USA

37. QIAprep Spin Miniprep Kit was obtained from Qiagen, Germany

38. PCR purification kit QIAquick PCR purification kit was obtained from Qiagen, Germany

39. Glass powder for Recovery of DNA EASYTRAP™ Ver.2 was obtained from TAKARA, Japan

40. Chemicals used in DGGE were obtained from Bio-Rad Laboratories Inc., USA

Formamide (Deionized)

40% Acrylamide/Bis solution, 37.5:1 (2.6% C)

Urea

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Ammonium persulfate

TEMED (N,N,N′,N′-Tetra-methyl-ethylenediamine)

50xTAE

Dye solution

Ethidium bromide solution 10 mg/mL

3.1.2 Equipments

1. Rotary vacuum evaporator , EYELA, Japan
2. Mini Gel migration trough, Cosmo Bio, Japan
3. ISSCO laminar flow, International Scientific Supply, Japan
4. Deep freezer (-70°C), model ULT 1786, Forma Scientific, Japan
5. Deep freezer (-20°C), model MDF-U332, Sanyo Electronic, Japan
6. Incubator (30°C), model BE800, Memmert, Germany
7. Incubator (37°C), New Brunswick Scientific, Edison NJ., USA
8. Hot air oven, model D06063, Memmert, Germany
9. Oven, Contherm Scientific, New Zealand
10. Filter paper pour size 0.22 µm, Micropore, USA
11. Water bath, model digital water bath SB-100, EYELA, Japan
12. Ultrasonicator, model FS4000, Decan Ultrasonics, England
13. UV transilluminater, Fotodyne Co., Inc., USA
14. Balance, model P2002-S and AG285, Mettler Toledo, Switzerland
15. Vortex mixer, model Genie 2, Scientific Industries, USA
16. Bench-top centrifuge, model Mikro20, Hettich zentrifugen Inc., USA
17. Autoclave, Kakusan, Japan
18. Spectrophotometer, model UV-160A, Shimadzu, Japan
19. DNA Thermal Cycler, model 2400, Perkin Elmer, USA and model MJ Mini™ Personal Thermal Cycler, Biorad,USA
20. Micropipette (2, 10, 20, 200, 1,000 and 5,000 µl), Gilson, France
21. Digital Dry Bath, model D1100,Labnet International, Inc., USA
22. Gel documentation system, model Gel DOC 2000™, Bio-Rad Laboratories Inc., USA.
23. pH meter, model 240, Corning, USA

24. DGGE equipments, Bio-Rad Laboratories Inc., USA.

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

3.1.3 Nucleotide sequences of primers

Table 3.1 Nucleotide sequences of primers used in detection of catabolic genes

Primer name	Nucleotide sequence (5'-3')	Expected size of product (bp)	References
ALK1-F	CAT AAT AAA GGG CAT CAC CGT	185	Kohno *et al.*, 2002
ALK1-R	GAT TTC ATT CTC GAA ACT CCA AAC		
ALK2-F	GAG ACA AAT CGT CTA AAA CGT AA	271	Kohno *et al.*, 2002
ALK2-R	TTG TTA TTA TTC CAA CTA TGC TC		
ALK3-F	TCG AGC ACA TCC GCG GCC ACC A	330	Kohno *et al.*, 2002
ALK3-R	CCG TAG TGC TCG ACG TAG TT		
RieskeF	TGY MGN CAY MGN GG	78	Chadhain *et al.*, 2006
RieskeR	CCA NCC RTG RTA NSW RCA		

Table 3.2 Nucleotide sequences of primers used in sequencing of 16S rDNA

Primer name	Nucleotide sequence (5'-3')
27F	AGA GTT TGA TCM TGG CTC AG
1492R	TAC GGH TAC CTT GTT ACG ACT T
800R	CTACCAGGGTATCTAAT
1100R	AGG GTT GCG CTC GTT G
341F	CCT ACG GGA GGC AGC AG
534R	ATT ACC GCG GCT GCT GG
M13F	CAC GAC GTT GTA AAC GA
M13R	GGA TAA CAA TTT CAC ACA GG

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3.2 Preliminary detection of gene involved in oil-degradation in wastewater

Wastewaters from car maintenance garage were collected from oil-resting tank and oil-trapping tank.

3.2.1 Wastewater DNA extraction

Bead beating separation and phenol/chloroform/isoamylalcohol extraction method was used according to Lemarchand *et al.*, (2005) with a little modification. Two hundred and fifty ml of raw wastewater was centrifuged at 5,000 rpm for 16 min. at room temperature. The pellet was centrifuged at 13,000 rpm for 10 min at room temperature in an Eppendorf centrifuge and decanted. Pellet was mixed with 0.6 ml of lysis buffer (Appendix B) and transferred in a 2 ml sterile conical screw cap microtube for bead beating containing 1/4 a tube of 0.1 mm diameter glass beads, and 0.6 ml of phenol–chloroform–isoamylalcohol (Appendix B). The tube was beaten at 4,800 rpm for 30 seconds and incubated for 30 min at 65 °C. The sample was centrifuged at 13,000 rpm, room temperature for 15 min. The supernatant fraction was transferred into sterile 1.5 ml microtubes. Then, the nucleic acid was precipitated using 2 volumes of ethanol in the presence of potassium acetate 0.3 M for 1 h at -80 °C and centrifuged at 13,000 rpm, room temperature for 15 min. The nucleic acid was washed with 2 volumes of 70% (v/v) ethanol (Appendix B) and dried before being dissolved in 50 µl of TE buffer containing 0.05 µl RNaseA and incubate for 1 hr at 37°C. DNA solution was kept at -20°C until being used.

3.2.2 Gel electrophoresis and DNA concentration measurement

Agarose gel 0.9% prepared in TAE buffer (Appendix B) was poured into gel tray with comb on the tray and let the gel stand until set. Put set agarose gel into the chamber and poured TAE buffer until completely cover the whole gel. Mixed DNA solution with 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. Then, the agarose gel was stained in ethidium bromide and destained with water. The DNA band can be detected under UV transilluminater at the wavelength of 312 nm.

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

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

3.2.3 Wastewater DNA purification

After gel electrophoresis, band in gel was cut to purified using Glass powder for Recovery of DNA EASYTRAP™ Ver.2 (TAKARA BIO INC, Japan). Put the slice of gel in sterilized microtube and weighted the gel slice with the tube weight subtraction. Added 3 volumes of gel weight of NaI and incubated at 55°C until the gel was completely melted. Added glass powder 5 μl per 1 μg of DNA, mixed well and let it settle down for 5 min. Centrifuged at 10,000 rpm for 5-10 sec, room temperature. Discarded supernatant and then added washing buffer 5 volumes of applied glass powder and mixed well by pipetting. Centrifuged at 10,000 rpm for 5-10 sec, room temperature. Discarded supernatant and air dried washing buffer completely. Added TE buffer 1-2 volumes of applied glass powder, incubated at 55°C for 2-5 min, and centrifuged at 10,000 rpm for 5-10 sec, room temperature. Pipetted DNA solution and transferred to new sterilized microtube. DNA solution was kept at -20°C until being used.

3.2.4 Amplification of catabolic genes involved in oil degradation

PCR with 2 primer sets were used to detect a wide range of genes encoding alkane hydroxylases (ALK1 and ALK2). The details of primers are shown below in table 3.1.

The PCR reaction was contained with about 70 ng DNA, 20 pmol of each primer set, 10x Mg-free buffer, 4 mM of $MgCl_2$, 0.2 mM of each dNTP mix, 2.5 unit of *taq* DNA polymerase and double deionized water to make the final volume of reaction of 30 μl. The condition for primer ALK1 and ALK2 was

1. Initial denaturation step at 94°C for 5 min
2. Denuration step at 94 °C for 1 min
3. Annealing at 40 °C for 30 sec
4. Extension at 72 °C for 30 sec
5. Go to step 2-4 for 30 cycles
6. Final extension at 72 °C for 6 min

PCR product was run in 2% agarose gel with 1X TAE at 100 volts. The gel then was stained in ethidium bromide, destained and checked under UV transilluminater.

3.2.5 Purification of PCR product

QIAquick PCR purification kit (Qiagen, Germany) was applied to purify PCR product using operation method provided in manual. Added 5 volume of PCR product of PB buffer then mixed and transferred to QIAprep spin column. Centrifuged at 13,000 rpm for 1 min in room temperature. Discarded flow-through solution. Added 750 µl to column and centrifuged like in previous step. Discarded flow-through solution and centrifuge again. Transferred column to new sterilized 1.5-ml-microtube. Added deionized water or EB buffer for 30-50 µl to the center of column. Let the column stand for 1 min before centrifuged. Kept purified PCR product at -20°C until being used.

3.2.6 Cloning of PCR product

The purified PCR product was ligated through pDrive cloning vector (Qiagen, Germany) of which the reaction is described as below:

The purified PCR product	3	µl
pDrive cloning vector	1	µl
Deionized water	1	µl
2X ligation master mix	5	µl

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The ligase reaction was incubated at 4 °C, overnight. Then, the ligase product was transformed to the competent *E.coli* JM109 cell which prepared by calcium chloride method (Sambrook and Russell, 2001). The competent cell was prepared by, firstly, streaked *E.coli* JM 109 on Ψb agar (Appendix A) and incubated at 37°C for 16 – 18 hours. Then transferred single colony of the strain to 5-ml-Ψb broth, shaken for 4 hours until OD_{600} was 0.3-0.5. Transferred 5 ml of cell suspension into arm flask that contained 100 ml of Ψb broth, shaken at 37°C until OD_{600} was 0.5. Then, transferred cell suspension into sterilized centrifuged tube and stored in ice for 5 min and centrifuged at 3,000 rpm, 4°C for 5 min. Remarked that all steps below must be done at 4°C. Discarded supernatant and added 40 ml of TfbI solution (Appendix B) and mixed by hand. Stored centrifuge tube contained cell suspension in ice for 5 min and centrifuged. Discarded supernatant and added 4 ml of cold TfbII solution (Appendix B). Re-suspended cell pellet and kept in ice for at least 15 min. Aliquot 100 µl of cell suspension into sterilized microtube. Competent cell was kept at -70°C until being used.

Transformed recombinant plasmid to prepared competent cell by heat shock method (Sambrook and Russell, 2001). Thawed competent cell in ice. Added ligated Recombinant plasmid for 2 µl to 50 µl of competent cell, then mixed and incubated in ice for 20 min. Heat shocked the cell by put into heat box at 42°C for 45-50 sec. After that, put into ice immediately for 2 min. Added 950 µl of SOC broth (Appendix A) and incubated for at least 1 hour at 37°C.

Then, the transformed solution is spreaded on the LB agar containing 100 µg/ml amplicilin (Appendix B), 30 µg/ml X-gal (Appendix B), and 30 µg/ml IPTG (Appendix B). The plate was incubated at 37 °C for 16 – 24 hr. Picked some white colonies to check the insert fragment. The white colonies were grown in the LB broth containing 100 µg/ml amplicilin at 37 °C overnight. The plasmid was extracted using QIAprep Spin Miniprep Kit (Qiagen, Germany) by method provided in manual. Harvested cell by centrifuged cell suspension at 10,000 rpm, room temperature for 2 min. Re-suspended cell in 250 µl of P1 buffer. Added 250 µl of P2 buffer and mixed by inverting. Added N3 solution for 350 µl, inverted until white pellet was observed. Centrifuged at 13,000 rpm for 10 min. Transferred supernatant into QIAprep Spin Column and centrifuged at 13,000 rpm for 1 min. Discarded flow-through solution, then added PB buffer for 500 µl into column and centrifuged at 13,000 rpm for 1 min.

Added 750 µl of PE buffer into column and centrifuged. Discarded flow-through solution and centrifuged again. Transferred column to sterilized microtube and added deionized water or EB buffer for 50-100 µl to the center of the column. Let the column stand for 1 min and centrifuged. Kept plasmid solution at -20°C until being used.

3.2.7 Digestion of recombinant plasmid by restriction enzyme

Digested extracted plasmid by *Eco*RI restriction enzyme to confirm the presence of inserted fragment. The restriction digestion condition is as described below:

Plasmid (pDrive cloning vector)	2	µl
*Eco*RI enzyme	0.5	µl (5 units)
Buffer	1	µl
Steriled water	6.5	µl

The insert fragment is examined by running in 2% agarose gel electrophoresis if the plasmids contain PCR product.

3.2.8 Restriction Fragment Length Polymorphisms (RFLPs)

Recombinant plasmids having the correct DNA insert fragment were analyzed by using Restriction Fragment Length Polymorphisms (RFLPs) to group the plasmids that have the same pattern of DNA arrangement. The order of usage for restriction enzymes for RFLPs is *BSu*RI and then *Hin*FI. The condition of RFLPs was the same as describe for *Eco*RI in 3.2.7.

3.2.9 Sequencing of catabolic genes involve in oil degradation

Selected plasmids were sent for sequencing at 1st Base Co., Ltd., Malaysia Primer T7 promoter (5'-TAA TAC GAC TCA CTA TAG GG-3') was in sequencing. The sequence results were analyzed using and BLASTx programs.

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3.3 Isolation of oil-degrading bacteria from oil-contaminated water

Wastewaters from car maintenance garage were collected from oil-resting tank and oil-trapping tank. One ml of each wastewater was mixed with 5 ml of M9 media (Appendix A) containing 2,000 ppm of lubricating oil in test tube and shaken at 200 rpm, 30°C. Culture was enriched several times by aliquot 1 ml into new oil contained M9 media when the change in lubricating oil was observed. This step was repeated five times.

Isolates were purified by spreading on M9 agar with a drop of lubricating oil on surface (Huy *et al.*, 1999) and incubated at 30°C for 3-5 days until colonies were observed. Purified isolates were re-streaked on LB agar (Appendix 1) in order to confirm the purity of isolates. If isolate gave only one characteristic colony, the isolate was confirmed to be purified. Nevertheless, if there were multiple characteristic colonies, each colony was streaked on M9 agar with a drop of lubricating oil on the surface. Colony that could grow on M9 agar with a drop of lubricating oil on the surface would be picked and re-streaked on LB agar to re-confirm the purity of isolate. Oil degradability of isolates was further checked in M9 broth containing oil.

3.4 Identification of isolated oil-degrading bacteria by sequencing 16S rDNA

3.4.1 Genomic DNA extraction

Genomic DNA of each isolate was extracted according to Ausubel *et al.* (1999). Pick single colony from M9 agar plate and transfer to 5 ml of LB broth. Each tube was shaken at 30°C and 200 rpm for 16 – 18 hours. Transferred 1.5 cell suspensions into sterilized 1.5-ml-microtube then centrifuged at 5,000 rpm for few minutes. Re-suspended cell pellet in 517 µl of TE buffer (Appendix B) using vortex mixer. Added 50 µl 0f 60 mg/ml lyzozyme (Appendix B) and mixed by inverting. After this step, mixing by vortex mixer was not allowed. Then, incubated at 37°C for an hour. After that, 30 µl of 10% SDS (Sodium dodecyl sulfate) (Appendix B) was added, then 10 µl of 10 mg/ml proteinase K (Appendix B) was added and mixed thoroughly before incubating at 37°C for an hour. Added 5 M sodium chloride (NaCl)

(Appendix B) for 120 µl and added 220 µl of CTAB/NaCl solution (Appendix B). Incubated at 65°C for 10 minutes. After that, equal volume of chloroform/isoamylalcohol (Appendix B) was added and mixed. Centrifuged at 13,000 rpm for 5 minutes. Transferred upper part to new sterilized 1.5-ml-microtube. Phenol/chloroform solution (Appendix B) mixed and centrifuged at 13,000 rpm for 5 minutes. Transferred upper part to another sterilized microtube and 0.6 volume of isopropanol was added before inverting until DNA was appeared. Centrifuged at 13,000 rpm for 10 minutes. Discarded supernatant and washed DNA pellet using 450 µl of 70% ethanol (Appendix B), centrifuged for other 10 minutes. Discarded supernatant and dried out ethanol completely. Re-suspended DNA pellet in 100 µl TE buffer containing 0.2 µl of 10 mg/ml RNaseA. Incubated at 37°C for 1 hour. DNA solutions were kept at -20°C until being used.

3.4.2 DNA purification using gel electrophoresis and DNA concentration measurement

Genomic DNA was run in gel electrophoresis and measured for DNA concentration using method described in 3.2.2

3.4.3 Amplification of 16S rDNA using polymerase chain reaction (PCR)

The PCR reaction was contained with about 70 ng DNA, 20 pmol of both forward and reverse primer, 10x Mg-free buffer, 4 mM of $MgCl_2$, 0.2 mM of each dNTP mix, 2.5 unit of *taq* DNA polymerase, and double deionized water to make the final volume of reaction of 30 µl. Primers 27F and 1492R (Table 3.2) were carried out to amplify 16S rDNA. The expected PCR product size was 1,456 bp long. The condition was

1. Initial denaturation step 94°C for 5 min
2. Denaturation step at 94°C for 1 min
3. Annealing step at 55°C for 90 sec
4. Extension step at 72°C for 2 min
5. Repeat step 2 to 4 for 30 cycles
6. Final extension step at 72°C for 7 min

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PCR product was run in 2% agarose gel with 1X TAE at 100 volts using 100 bp ladder as DNA marker. The gel then will be stained in ethidium bromide, destained and checked under UV transilluminater.

3.4.4 Purification of PCR product

PCR product was purified using method described in 3.2.4

3.4.5 Cloning of PCR product

PCR product was cloned followed method in 3.2.5 and digested using method in 3.2.6.

3.4.6 Nucleotide base sequencing

Plasmids were sent for sequencing at 1st Base Co., Ltd., Malaysia. In nucleotide base sequencing, primers M13F, M13R, 800R and 1100R were used. The first two were specific to plasmid vector and others two were specific to 16S rDNA. The sequence results will be analyzed using BLASTn program to identify the bacterial species.

3.5 Degradability test of isolates

3.5.1 Preparation of artificial wastewater

Nine hundred ninety four-ppm-oil/water-emulsion was prepared according to Panpanit (2001). One hundred milliter of 0.1% tween 80 (Appendix B) was pre-mixed with 1 ml of PTT V-120 using blender at maximum speed for 2 min. Added 800 ml of deionized water and mixed by stirring for 10 min. Diluted this synthetic emulsion down to 200 ppm by aliquot 201.2 ml of prepared emulsion and added deionized water to make final volume equal to 1,000 ml.

3.5.2 Inoculum preparation

Isolates were cultured in M9 agar with 2,000 ppm of oil for 5 days. Then colonies were brought to 5 ml of M9 broth containing 5 mg/ml lubricating oil for 5 days. Cells were enriched by bringing into 500 ml flask containing 300 ml M9 broth with concentration of lubricating oil of 5 mg/ml for 5 days. Cells were centrifuged at 8,000 rpm, 4°C for 10 minute and washed using 0.85% NaCl solution (Appendix B), then shaken cells with 0.85% NaCl solution overnight in order to let cells use accumulated nutrients. Cells were washed again with 0.85% NaCl solution and centrifuged.

3.5.3 Degradability test

Cell of each isolate were brought to each 250 ml-flask containing 50 ml of prepared emulsion to make OD_{600} equal to 1 (approx. 10^7CFU/ml). The flask that contains only emulsion was used as control and all flasks were done in triplication. All flasks were shaken at 200 rpm, room temperature for 18 hours. Then, samples were analyzed for the amount of oil at 0, 3, 6, 12, 18 hours.

3.5.4 Oil extraction

For oil extraction, chloroform was used as solvent. 0.75 g of NaCl and 10 ml of chloroform were added and shaken for 30 min. Twelve point five mg of stearyl alcohol was added with chloroform to be used as internal standard. After completely separation of water phase and chloroform phase, chloroform phase was taken out and evaporated until the final volume was around 1 ml.

3.5.5 Quantification of oil

One microlitre of the extract was applied to silica rod (Chromarod) and put rods in solvents of increasing polarity to separate saturated and aromatic hydrocarbon from resin/asphaltenes. Silica rods was be developed in;

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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).

Finally, quantified amount of oil on the silica rods using the FID-TLC (Iatroscan) with scan speed of 30 sec/rod. Flow rate of hydrogen for the FID were 160 ml/min. Retention time of stearyl alcohol, saturates, aromatics, resin, and asphaltenes were approximately 0.35, 0.13, 0.24, 0.42, and 0.47 min, respectively. Amount of lubricating oil will be analyzed by comparing to a standard curve of lubricating oil.

3.6 Wastewater collection and characterization

3.6.1 Wastewater collection

Wastewaters were collected from petro stations around Bangkok. Collected point was entrance of oil-trapping tank.

3.6.2 Wastewater characterization

Wastewaters were analyzed for described parameter; pH, TOC, total P, total N and initial oil concentration. Samples were sent to Department of Environmental Engineering, Faculty of Engineering, Chulalongkorn University for analyzing total N and total P. Methods used for each parameter were shown in table 3.3

Table 3.3 analysis methods for water properties

Property	Method
pH	pH meter
TOC	TOC analyzer
Total N	Macro-Kjeldahl
Total P	Vanadomolybdo phosphoric acid colorimetric method
Initial oil	TLC-FID

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3.7 Biodegradation of lubricating oil in water microcosms

3.7.1 Microcosm description

Five bioremediation processes including natural attenuation, biostimulation, bioaugmentation I, bioaugmentation II and abiotic control were studied to evaluate the efficiency of lubricating oil degradation. All treatments were incubated at room temperature and shaken twice a day for 12 days. Samples were collected every 3 days for quantitative analysis of lubricating oil and microbiological analysis.

(a) Natural attenuation

Fifty milliliters of 5% (v/v) lubricating oil spiked-wastewater sample was placed in 250-ml flask for triplication. The oil was degraded by ability of the indigenous microorganisms.

(b) Biostimulation

Fifty milliliters of 5% (v/v) lubricating oil spiked-wastewater sample was placed in 250-ml flask for triplication. Then, NH_4NO_3, and K_2HPO_4 were added to give final C:N:P of wastewater equal to 100:5:1 (Sei *et al.*, 2003; Depeartment of Industrial Works, 2002).

(c) Bioaugmentation

Fifty milliliters of 5% (v/v) lubricating oil spiked-wastewater sample was placed in 250-ml flask for triplication. Bacterial pure culture which had highest oil degradability that was obtained from 3.4 was used in this treatment. Bacteria was cultured in M9 medium with 200 ppm lubricating oil and shaken at 200 rpm for 5 days. The bacteria was harvested, washed and resuspended in 0.85% NaCl. Approx. 10^7 CFU/ml was added to bioaugmentation treatment.

(d) Bioaugmentation II

Fifty milliliters of 5% (v/v) lubricating oil spiked-wastewater sample was placed in 250-ml flask for triplication. Then, NH_4NO_3, and K_2HPO_4 were added to give final concentration of nitrogen and phosphorus were added to give final C:N:P of

wastewater equal to 100:5:1. Bacterial pure culture was added to the treatment as described in (c).

(e) Abiotic control

Sterilized water sample was used as abiotic control. Sample was sterilized by autoclaving (at 121°C, 15 min). Fifty ml of sterilized water was placed in 250-ml flask for triplication and spiked with lubricating oil for final concentration of 5% (v/v).

3.8 Quantitative lubricating oil analysis

Lubricating oil was extracted and quantified using method described in 3.5.4 and 3.5.5.

3.9 Microbiological analysis

3.9.1 Determination of the amount of lubricating oil-degrading bacteria and total viable bacteria

Plate count technique was used for determining the amount of oil-degrading bacteria and total viable bacteria. The 100 µl of ten-fold serial dilution of wastewater was spread on M9 spread with one drop of lubricating oil to determine amount of oil-degrader and used LB plate to determine the amount of total viable bacteria. Number of bacteria colony on agar plate was accounted for oil-degrader and total viable bacteria. The number of bacteria was calculated by

$$\text{Bacteria per ml of original solution (CFU/ ml water)} = \frac{\text{Number of counted bacteria} \times \text{Dilution factor}}{\text{Volume of added suspension to plate}}$$

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3.9.2 Analysis of microbial community

3.9.2.1 DNA extraction from wastewater

Wastewater DNA was extracted according to method in 3.2.2.

3.9.2.2 Wastewater DNA purification

Wastewater DNA was purified according to method in 3.2.3.

3.9.2.3 Polymerase chain reaction (PCR) of 16S rDNA

Used primer were 341F (Table 3.2) with GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') and 534R (Table 3.2) which specific to V-3 region. The expected size of PCR product was about 200. The PCR reaction was contained with about 70 ng DNA, 20 pmol of both forward and reverse primer, 10x Mg-free buffer, 4 mM of $MgCl_2$, 0.2 mM of each dNTP mix, 2.5 unit of *taq* DNA polymerase, and double deionized water to make the final volume of reaction of 30 µl. The condition of PCR was:

1. Initial denaturation step at 94°C for 5 min
2. Touchdown program for 20 cycles
    2.1 Denaturation step at 94°C for 1 min
    2.2 Annealing step at 60°C for 1 min (temperature was reduced 0.5°C each cycle)
    2.3 Extension step at 72°C for 2 min
3. Denaturation step at 94°C for 1 min
4. Annealing step at 55°C for 1 min
5. Extension step at 72°C for 2 min
6. Go to step 3-5 for 30 cycles
7. The final extension at 72°C for 10 min

Five microlitre of PCR product was run in 2% agarose gel electrophoresis with 1X TAE at 100 volts for checking the size of PCR product.

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3.9.2.4 Denaturing gradient gel electrophoresis (DGGE)

PCR product will be ran on 8% polyacrilamide gel with a denaturing gradient of urea and formamide denaturant ranging from 20 - 70% for 5 hr at 130 volts with 1X TAE. DGGE gel was stained in 50 µg/ml ethidium bromide for 20 minutes. DNA band profiles can be detected under UV transilluminater.

3.9.2.5 Detection of oil-degrading bacteria

PCR with 3 primer sets were used to detect a wide range of genes encoding alkane hydroxylases (ALK1 and ALK2) and Reieske of dioxygenase gene (Rieske). Using method described in 3.4.1. For Rieske primers, the condition of PCR was:

1. Initial denaturation step at 94°C for 5 min
2. Denaturing step at 94°C for 30 sec
3. Annealing step at 48°C for 30 sec
4. Extension step at 72°C for 30 sec
5. Final extension step at 72°C for 5 min.

3.9.2.6 Toxicity test by dehydrogenase assay

Toxicity test was done by method described by Rönnpagel *et al.* (1994). *Bacillus cerues* TISTR 687 was incubated on nutrient agar (NA) (Appendix A) for 24 hours at 37 °C. Then, one-loop-full of *B.cereus* was transferred to 100 ml of nutrient broth (NB) (Appendix A) and incubated at 37 °C, 200 rpm for 18 hours. Transferred 1 ml of bacterial culture into 100 ml of NB and incubated at 37 °C, 200 rpm for 2 hrs 30 min in order to get strain in mid-log phase. Measured the amount of cell by spectrophotometer at 600 nm in order to get optical density of cell at 0.4 (4.5×10^7 CFU/ml). Mix 2 ml of cell and 2 ml of wastewater. Then, incubated at 25 °C for 1 hr. Added 4 ml of resazurin solution (Appendix B), mixed well, and incubated in the dark at 37 °C, 70 rpm for 15 min. Centrifuged solution at 3,400 rpm for 5 min and filtrated supernatant through 0.2 µm sterile membrane. Measured absorbance value of solution at 600 nm and calculated % inhibition of sample by equation below. There were two

sets of control: 1) 100% inhibition control (5 ml of sterilized water), and 2) 0% inhibition (3 ml of sterilized water + 2 ml of cell suspension).

$$\% \text{ inhibition} = 100 - \left\{ \frac{(\text{Abs of 100\% inhibition} - \text{Abs of sample})}{(\text{Abs of 100\% inhibition} - \text{Abs of 0\% inhibition})} \times 100 \right\}$$

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CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Detection of gene involved in oil degradation in wastewater from car maintenance center

Wastewater samples were collected from car maintenance center in Bangkok. Activities in the center were various, i.e. car washing, engine oil changing, and car restoration, so, there was an evidence of availability of oil-degrading bacteria. Two samples were collected from resting tank and oil-trapping tank by grabbing surface water. In oil-trapping tank which is the tank that collects water directly from the center, thick oil film was observed while the thinner oil film was observed in resting tank.

Polymerase chain reaction (PCR) was conducted to detect oil-degrading bacteria in these water samples by detecting *alk* gene involved in alkane biodegradation. Primer sets ALK1, ALK2 specific to *alkB* (group I, catalized C_6-C_{12} *n*-alkane degradation) and *alkM* (group II, catalized *n*-alkane that has carbon atom more than 12) and primer set ALK3 that specific to *alkB* or *alkB1* (group III, that are unknow for substrate specificity, *n*-alkane oxidation pathways, and oxidation system) (Kohno *et a.l*, 2002) were used. The expected product sizes of ALK1, ALK2 and ALK 3 are 185, 271 and 330 bp, orderly.

From the result, *alkM* could be detected in both water samples while the presence of *alkB* and gene in group III could not be observed (Figure 4.1). Since contaminants in the center were heavy petroleum product such as lubricant and engine oil that have carbon atom more than 15 atoms (Vazquez-Duhalt, 1989) while *alkB* gene shows important role for degrading of medium-chain-length *n*-alkane (C_6-C_{12}) (Kohno *et al.*, 2002). Thus, *alkB* might not present in these wastewater since medium-chain-length *n*-alkane was not dominant contaminant in this wastewater sample.

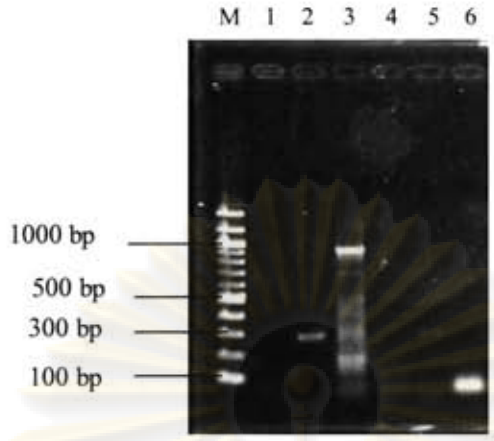

Figure 4.1 Detection of *alk* gene in wastewater from car maintenance center. Lane M: 100 bp ladder DNA marker, Lane 1-3: water from resting tank with primer ALK1, ALK2, and ALK3, respectively, Lane 4-6: water from oil-trapping tank with primer ALK1, ALK2, and ALK3, respectively.

After that, the band in lane 2 (Figure 4.1) was extracted and ligated into pDrive plasmid vector, transformed into *E. coli* JM109. The required colonies were then selected. Six clones were picked. Extracted plasmids were digested by *Eco*RI in order to check the presence of PCR product (Figure 4.2).

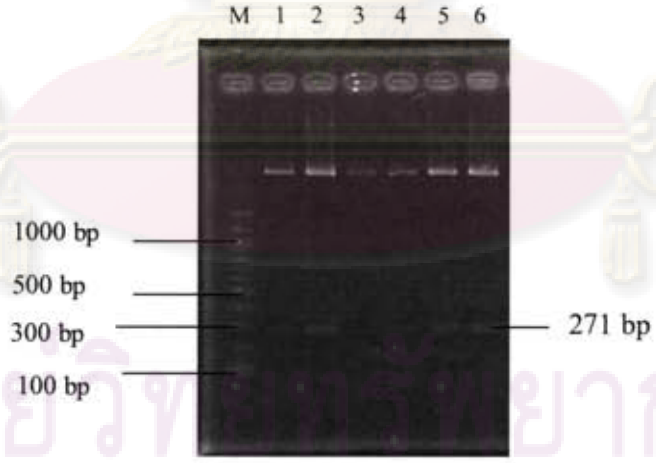

Figure 4.2 Recombinant plasmids after digested by restriction enzyme *Eco*RI. Lane M: 100 bp ladder DNA marker, Lane 1-6: selected colonies REST 1-6, respectively.

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From Figure 4.2, all colonies could be detected the presence of PCR products except sample REST3 in lane 3. Thus, other samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. The results exhibited that all clones shown the same pattern (Figure 4.3).

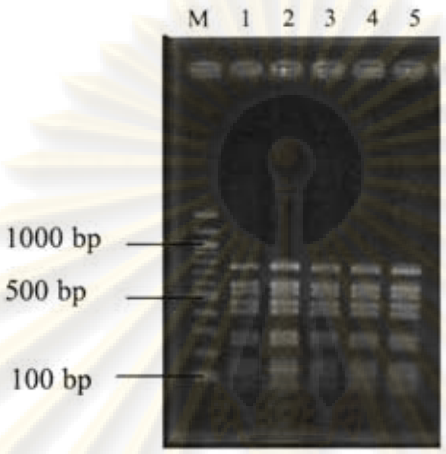

Figure 4.3 Recombinant plasmids after digested by restriction enzyme *Bsu*RI. Lane M: 100 bp ladder DNA marker, Lane 1-2: clones REST 1-2, Lane 3-5: clones REST4-6, respectively.

Therefore, all clones were digested again with restriction enzyme *Hin*fI. Digestion of clones by restriction enzyme *Hin*fI exhibited the difference between 5 clones and sorted these clones into 4 groups as described below (Figure 4.4).

Group 1: clone REST1
Group 2: clone REST2
Group 3: clone REST4
Group 4: clones REST5 and REST6

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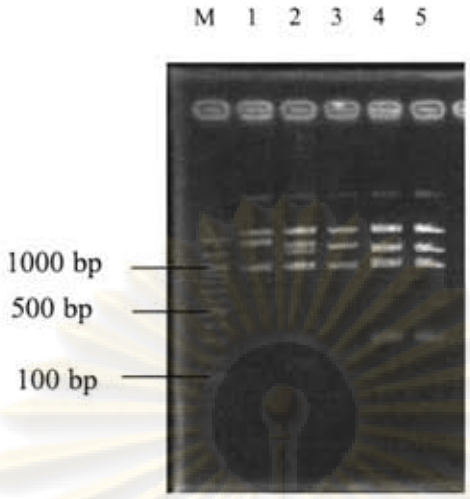

Figure 4.4 Recombinant plasmids after digested by restriction enzyme *Hin*fI. Lane M: 100 bp ladder DNA marker, Lane 1-2: clones REST 1-2, Lane 3-5: clones REST4-6, respectively.

Therefore, clones REST1, REST2, REST4 and REST5 were selected to analyze the sequence of nucleotide base. After compared sequences to GenBank using software BLASTx (http://www.ncbi.nlm.nih.gov/), all clones were similar to alkane hydroxylase and alkane monooxygenase (Tables 4.1-4.4). Gene product of REST1 showed high sequence similarity to alkane hydroxylase A of *Acinetobacter venetianus* (96%). Gene products of REST2, REST4 and REST5 were matched closely to alkane hydroxylase of *Acinetobacter haemolyticus* (80-95%).

Table 4.1 Sequence analysis of gene product of recombinant plasmid REST1

Accession number	Description	Identity (%)	References
AAY26148.1	Alkane hydroxylase A (*Acinetobacter venetianus*)	80/83 (96%)	Throne-Holst *et al.*, 2006
AAS93604.4	Alkane hydroxylase (*Acinetobacter haemolyticus*)	79/83 (95%)	Bihari *et al.* (unpublished)
BAB33284.1	Alkane hydroxylase A (*Acinetobacter* sp. M-1)	77/83 (92%)	Tani *et al.*, 2001
ABR10770.1	Alkane hydroxylase (*Acinetobacter* sp. BUU8)	75/83 (90%)	Phrommanich *et al.* (unpublished)
YP_001084670.1	Alkane 1-monooxygenase (*Acinetobacter baumannii* ATCC 17978)	75/83 (90%)	Smith *et al.*, 2007

Table 4.2 Sequence analysis of gene product of recombinant plasmid REST2

Accession number	Description	Identity (%)	References
AAS93604.4	Alkane hydroxylase (*Acinetobacter haemolyticus*)	76/92 (82%)	Bihari *et al.* (unpublished)
BAB33284.1	Alkane hydroxylase A (*Acinetobacter* sp. M-1)	75/92 (81%)	Tani *et al.*, 2001
AAY26148.1	Alkane hydroxylase A (*Acinetobacter venetianus*)	75/92 (81%)	Throne-Holst *et al.*, 2006
ABO15258.1	Alkane monooxygenase (uncultured organism)	73/92 (79%)	Kuhn and Pellizari (unpublished)
ABO15251.1	Alkane monooxygenase (uncultured organism)	73/92 (79%)	Kuhn and Pellizari (unpublished)

Table 4.3 Sequence analysis of gene product of recombinant plasmid REST4

Accession number	Description	Identity (%)	References
AAS93604.4	alkane hydroxylase (*Acinetobacter haemolyticus*)	77/96 (80%)	Bihari *et al.* (unpublished)
BAB33284.1	Alkane hydroxylase A (*Acinetobacter* sp. M-1)	77/96 (80%)	Tani *et al.*, 2001
ABO15266.1	Alkane monooxygenase (uncultured organism)	78/96 (81%)	Kuhn and Pellizari (unpublished)
ABO15258.1	Alkane monooxygenase (uncultured organism)	78/96 (81%)	Kuhn and Pellizari (unpublished)
ABO15251.1	Alkane monooxygenase (uncultured organism)	78/96 (81%)	Kuhn and Pellizari (unpublished)

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Table 4.4 Sequence analysis of gene product of recombinant plasmid REST5

Accession number	Description	Identity (%)	References
AAS93604.4	alkane hydroxylase (*Acinetobacter haemolyticus*)	76/80 (95%)	Bihari *et al.* (unpublished)
AAY26148.1	Alkane hydroxylase A (*Acinetobacter venetianus*)	76/80 (95%)	Throne-Holst *et al.*, 2006
BAB33284.1	Alkane hydroxylase A (*Acinetobacter* sp. M-1)	74/80 (92%)	Tani *et al.*, 2001
CAH56108.1	Putative alkane hydroxylase (uncultured bacterium)	73/80 (91%)	Heiss *et al.* (unpublished)
ABB40596.1	AlkM (*Acinetobacter venetianus*)	75/80 (93%)	Fani *et al.* (unpublished)

The previous studies reported that the presence of *alk* gene could represent the availability of oil-degrading bacteria since many species of oil-degrading bacteria hold *alk* gene. Van Beilen *et al.* (2003) reviewed that there were many *alk*-harboring bacteria, i.e. *Acinetobacter* sp., *Alcanivorax* sp., *Pseudomonas* sp., and *Mycobacterium* sp.. Thus, these wastewaters from car maintenance center were used to isolate oil-degrading bacteria in following experiments.

4.2 Isolation of oil-degrading bacteria from petroleum contaminated wastewater

4.2.1 Isolation of oil-degrading bacteria

One milliliter of wastewater from car maintenance center were mixed with 5 ml of M9 medium that contain lubricating oil (PTT V-120) with final concentration of 2,000 ppm in test tube. After several days when the changes of medium and oil could be observed in the tube, 1 ml of cell suspension was transferred into new M9 medium with oil. Figure 4.5 represented the observed changed in the tube.

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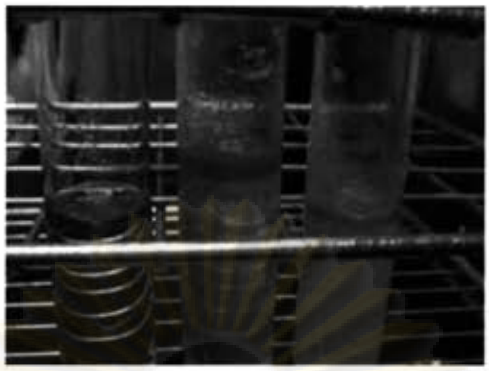

Day 0 Day 5 Day 7

Figure 4.5 The changes medium and oil observed during isolation of oil-degrading bacteria in M9 medium.

After several repeat transferring, cell suspension in the tube was spread on M9 agar with a drop of oil on the surface and incubated at 30°C for 3-5 days. Colonies were picked and streaked on LB agar in order to confirm the purity of isolates. If only one characteristic colony was observed, the isolate was confirmed to be pure. Nevertheless, if there were multiple characteristic colonies, each colony was streaked on M9 agar with a drop of oil on the surface. Colony that could grow on M9 agar with a drop of oil was picked and re-streaked on LB agar. The colony on LB agar was picked and transferred to M9 broth with oil to check the activity of isolate whether the isolate could degrade oil or not.

In this work, 2 pure bacterial strains were isolated from oil-trapping tank and resting tank of car maintenance center; Tr1 and R2 (Figure 4.6). All strains were confirmed to be pure and had ability to degrade lubricating oil.

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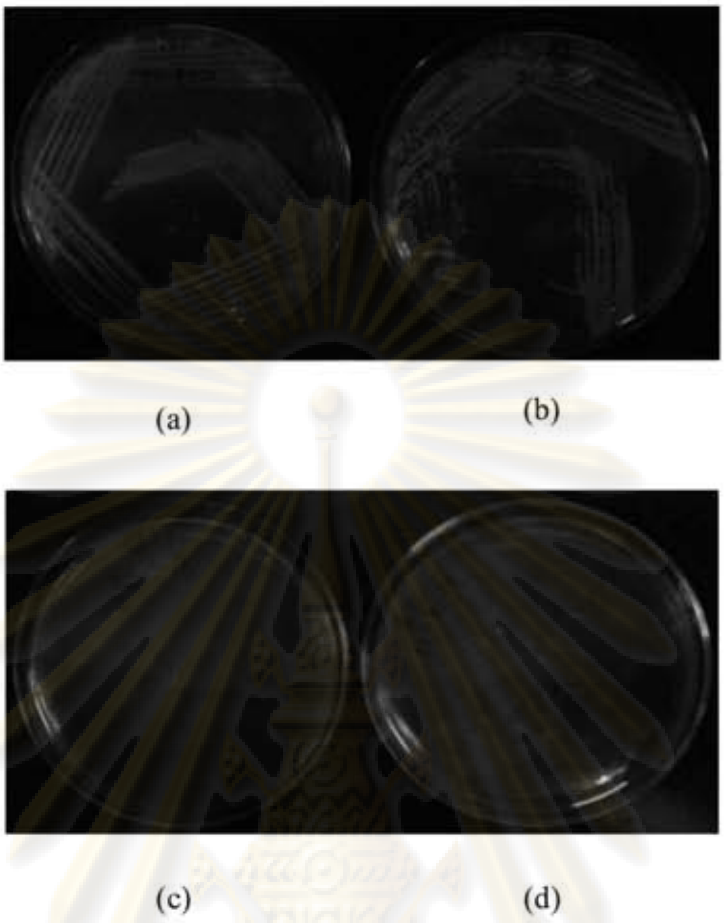

Figure 4.6 Morphology of isolated strains. Strain Tr1: (a) on LB plate, (c) on M9 agar with a drop of oil. Strain R2: (b) on LB plate, (d) on M9 agar with a drop of oil.

4.3 Identification of isolated oil-degrading bacteria by sequencing 16S rDNA

Two strains were cultured in LB broth for 24 hours at 30°C. Cell were harvested and extracted for genomic DNA. PCR of 16S rDNA was conducted using primers 27F and 1492R. PCR products were ligated to pDrive cloning vector and transformed into competent *E.coli* JM109. Required colonies were picked and extracted plasmid that contain inserted PCR products. Extracted plasmids were subjected to sequence analysis and the sequences of PCR products were compared to GenBank database using BLASTn software (http://www.ncbi.nlm.nih.gov/).

In sequence analysis, 1,344 bp and 1,366 bp of 16S rDNA fragments of Tr1 and R2, respectively, were compared to GenBank. Sequences analysis showed that 16S rDNA of strain Tr1 had high similarity to those of *Acinetobacter* spp. (99%) (Liu and Zhang, 2007) (Table 4.5) and strain R2 also belonged to those of *Acinetobacter* sp. (95%) (Shrestha *et al.*, 2007) (Table 4.6).

Table 4.5 The result of comparison 16S rDNA of strain Tr1 to GenBank database using BLASTn software.

	Bacterial strains	**Accession no.**	**Sequence Identity (%)**	**References**
1	*Acinetobacter* sp. w2	EF570077.2	1,338/1,342 (99)	Liu and Zhang, 2007 (unpublished)
2	*Acinetobacter junii*	AF417863.1	1,338/1,342 (99)	Pidiyar *et al.*, 2004
3	*Acinetobacter* sp. 11	AY177359.2	1,338/1,342 (99)	Bodour *et al.*, 2003
4	*Acinetobacter junii* SCH0409	AY881242.1	1,338/1,342 (99)	Bhattarai *et al.*, 2006 (unpublished)
5	*Acinetobacter junii*	AB101444.1	1,338/1,342 (99)	Malik *et al.*, 2003

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Table 4.6 The result of comparison 16S rDNA of strain R2 to GenBank database using BLASTn software.

	Bacterial strains	Accession no.	Sequence Identity (%)	References
1	*Acinetobacter* sp. P-155	AM412159.1	1,301/1,358 (95)	Shrestha *et al.*, 2007
2	*Acinetobacter* sp. P-128	AM412158.1	1,301/1,358 (95)	Shrestha *et al.*, 2007
3	*Acinetobacter* sp. P-108	AM412157.1	1,301/1,358 (95)	Shrestha *et al.*, 2007
4	*Acinetobacter* sp. P-112	AM412156.1	1,301/1,358 (95)	Shrestha *et al.*, 2007
5	*Acinetobacter* sp. P-121	AM412153.1	1,301/1,358 (95)	Shrestha *et al.*, 2007

Acinetobacter sp. is widespread in nature and can be obtained from water, soil and living organisms. *Acinetobacter* sp. is gram-negative bacteria, non-motile, and strictly aerobic. They can use various carbon sources for growth and can be cultured on relatively simple media. Species of *Acinetobacter* have been attractingly growing interest in both environmental and biotechnological applications: they are known to be involved in biodegradation of a number of different pollutants (Barbe *et al.*, 2004).

Acinetobacter sp. was reported that could degrade petroleum hydrocarbon. In the study of Hanson *et al.* (1997), *Acinetobacter* sp. A3 was examined the ability to degrade crude oil (Bombay High Crude Oil (BHCO)). The results showed that strain A3 colud utilized crude oil as sole carbon source. The percentage of degraded crude oil in minimal medium was 60% (120 hr, initial oil was 5% v/v).

Huy *et al.* (1999) could isolate 1 strain of *Acinetobacter* sp. and 3 strains of *Pseudomonas* sp. from soil contaminated site in Vietnam. From their crude oil degradability test, strain of *Acinetobacter* sp. had highest efficiency. In 1 day, it degraded 95% of the crude oil in the culture medium (5% v/v). The researchers also found that isolated *Acinetobacter* sp. could synthesize biosurfactant in order to decrease interfacial tension between crude oil and water, so oil could be easily uptake into cell.

Ron and Rosenberg (2002) also reviewed that many oil-degrading bacteria could produce biosurfactants. These surfactants help to disperse the oil, increase the surface area for growth, and help detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted (Rosenberg, 1993). From the review, *Acinetobacter* RAG-1, *Acinetobacter calcoaceticus* BD4, *Acinetobacter radioresistens* were reported that they could high molecular weight biosurfactants, i.e, polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. Therefore, strains in this work might be able to produce biosurfactant involved in their degradation of oil, but it requires further analysis on biosufactant production of the strains.

4.4 Detection of gene involve in oil degradation in isolated bacteria

PCR with 3 primer sets were used to detect a wide range of genes encoding alkane hydroxylases, using ALK1 and ALK2 primer sets, and Rieske of aromatic dioxygenase gene, using Rieske primer set.

From the result, both strains (Tr1 and R2) showed negative results in detection of *alkB* gene. In detection *alkM* gene, only R2 showed positive results (Figures 4.7). This might because Tr1 belongs to genus that does not have *alkM* gene. Another reason might be that *alkM* gene is on plasmid. Since the strain was cultured in LB medium which is rich medium, plasmid would not be produced (van Beilen and Funhoff, 2007; Kohno *et al.*, 2002).

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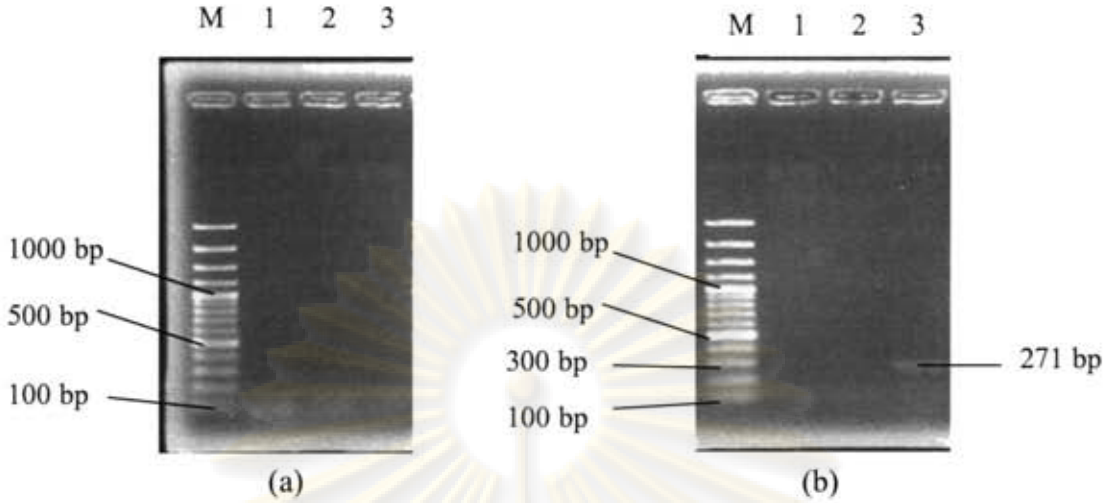

Figure 4.7 Detection of *alkB* (a) and *alkM* (b) in strains Tr1 and R2. Lane M: 100 bp ladder DNA marker, Lane 1: negative control, Lane 2: strain Tr1, Lane 3: strain R2.

The availability of *alkM* gene which encoded enzyme alkane monooxygenase for long-chain *n*-alkane degradation in *Acinetobacter* spp., for example; *Acinetobacter* sp. 2769A, *Acinetobacter* sp. ADP1, *A. calcoaceticus* 69-V, *A. calcoaceticus* EB104, *A. calcoaceticus* NCIB8250 has been reported (Kohno *et al.*, 2002).

Even Rieske primer set was designed to be able to detect various aromatic compounds such as naphthalene and phenanthrene (Chadhain *et al.*, 2006), the detection of Rieske of aromatic dioxygenase showed negative results on both strains (Figure 4.8). This could be hypothesized that genes involved in aromatic degradation of both strains were not in range of Rieske primer set used since dioxygenase gene of aromatic compounds and also Rieske gene are very variety.

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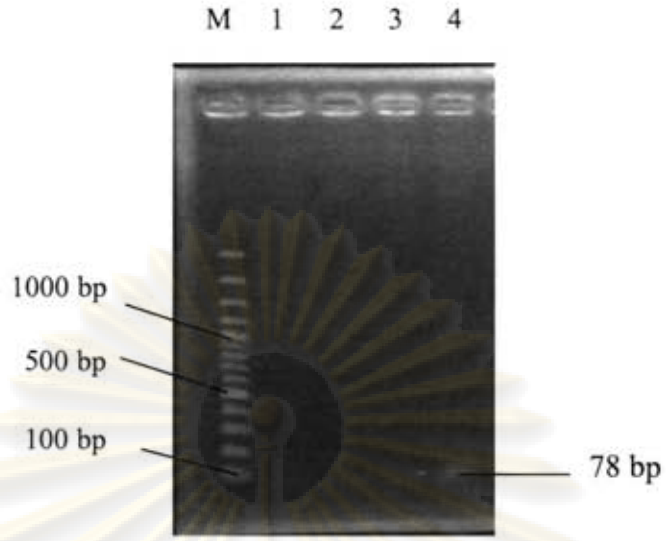

Figure 4.8 Detection of Rieske of aromatic dioxygenase gene in strains Tr1 and R2. Lane M: 100 bp ladder DNA marker, Lane 1: negative control, Lane 2: strain Tr1, Lane 3: strain R2, Lane 4: positive control (recombinant plasmid containing Rieske of *Alteromonas macleodii* BP-PH DNA (Pansri, 2007))

4.5 Degradability test of isolates

Degradability test of isolated was conducted using 50 ml of synthetic 200 ppm-oil-in-water emulsion in 250-ml flask. Initial amount of isolate was 10^7 CFU/ml. All flasks were shaken at 200 rpm, room temperature for 18 hours. Then, samples were extracted using chloroform with NaCl addition and analyzed for the amount of oil at 0, 3, 6, 12 and 18 hours using TLC-FID. The flask with no bacterial addition was used as a control.

Figure 4.9 represented percentage of oil removed by strain Tr1 and R2 normalized by control. From Figure, R2 showed higher oil removal efficiency than strain Tr1. After 18 hours, strain R2 could remove 78.01% compared to control while strain R2 removed only 68.23% compared to control. Therefore, strain R2 was selected to be used in following experiments.

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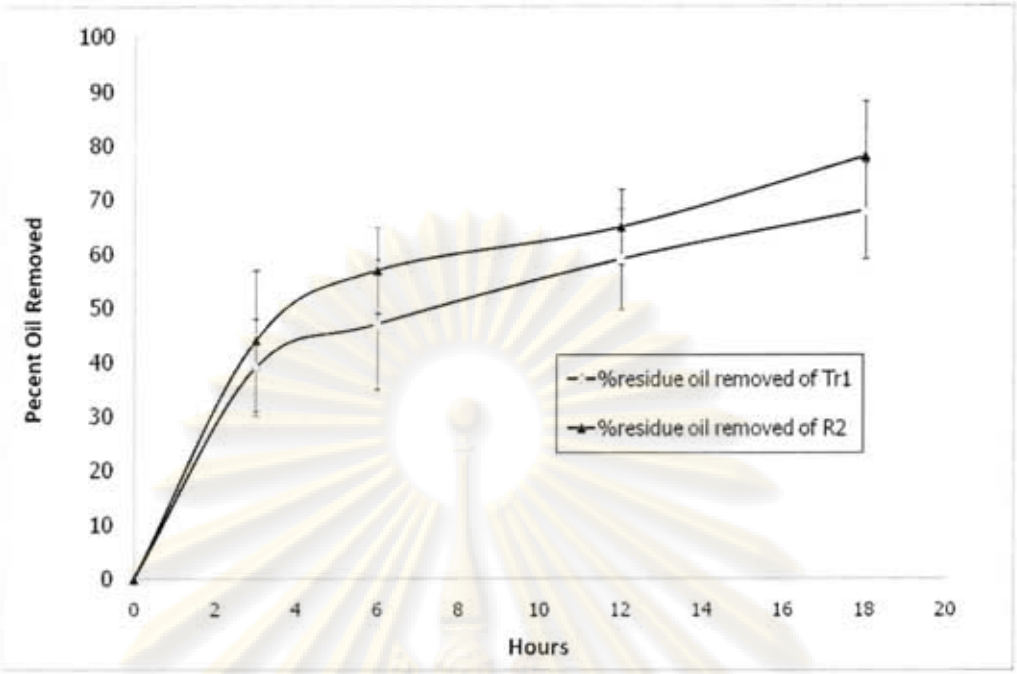

Figure 4.9 Percentage of oil removed by strain Tr1 and R2 normalized by control

4.6 Wastewater collection and characterization

4.6.1 Wastewater collection

Four wastewater samples from 4 petrol stations around Bangkok were collected. The samples were collected from the entrance of oil-trapping tank. Figure 4.10 exhibited wastewater from 4 petrol stations. Name of samples were shown in Table 4.7.

Table 4.7 List of wastewater samples collected from petrol station around Bangkok.

Sample ID	Location	Car washing facilities
TB	Near royal military club	No
SL	Near Lumpini boxing stadium	Yes
JJ	Vipavadee-Rangsit Rd.	Yes
SV-62	Soi Sukhumvit 62	No

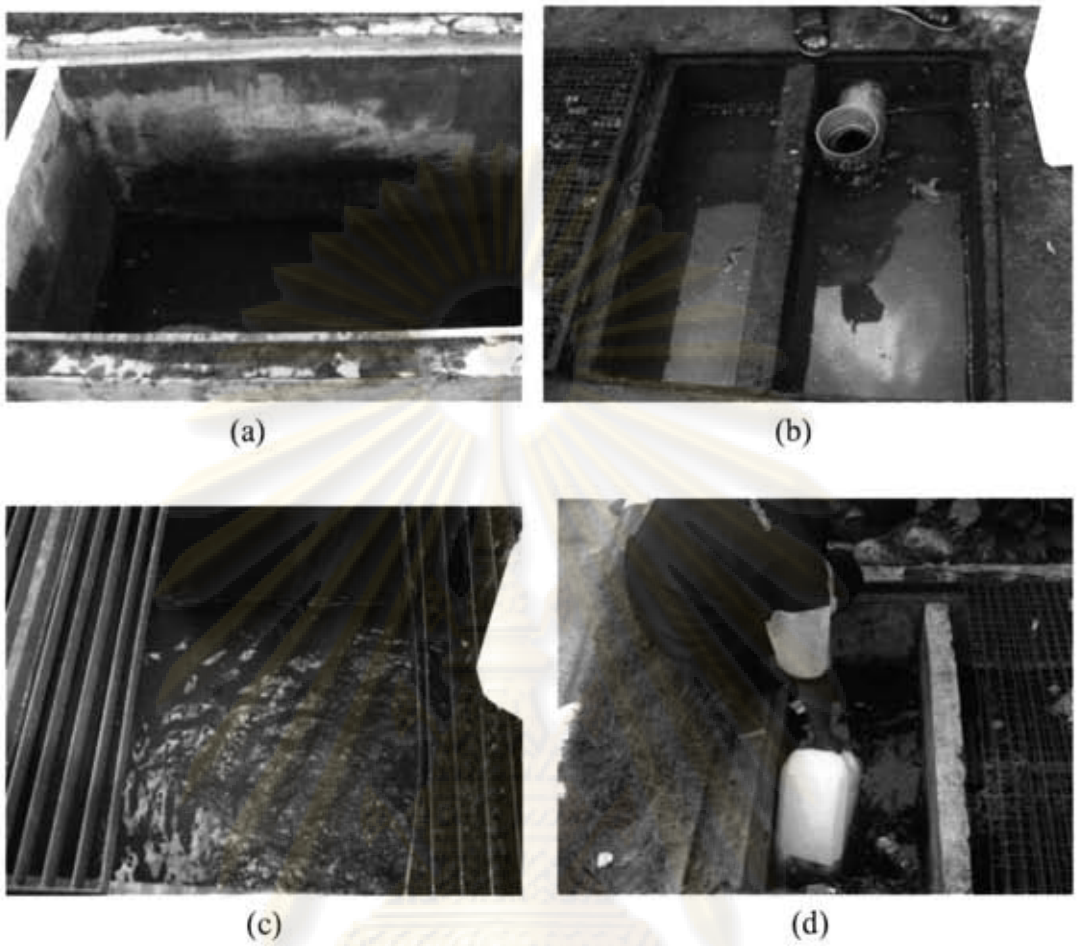

Figure 4.10 Sampling point of each wastewater; (a) TB, (b) SL, (c) JJ, (d) SV-62.

4.6.2 Wastewater characterization

Collected wastewaters were analyzed for pH, TOC, total P, total N and initial oil concentration. For pH, TOC and initial oil concentration, these parameters were analyzed at laboratory. For others parameters, samples were sent to Department of Environmental Engineering, Faculty of Engineering, Chulalongkorn University. Results are described in Table 4.8.

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Table 4.8 Characteristics of wastewater samples.

Sample	pH	TOC (mg/l)	Total N (mg/l as N)	Total P (mg/l as P)	Initial oil (mg/l)	C:N:P
TB	7.12	881	32	2	52.15	100 : 3.6 : 0.22
SL	7.03	781	4	0.6	39.80	100 : 0.51 : 0.08
JJ	7.35	817	4	0.6	19.40	100 : 0.5 : 0.07
SV-62	6.94	1,416	21	2	28.92	100 : 1.48 : 0.14

From Table 4.8, if compared oil concentration of all samples, all samples had exceed the standard for effluent from industry which have to be lower than 15 mg/l (Department of Industrial Work, 2002). Thus, these wastewaters had to be treated properly.

When compared C:N:P value of all samples, all samples had improper value. Department of Industrial Works (2002) reported that suitable value of C:N:P for biological treatment process was 100:5:1 for completely biodegradation. So, this indicated that biostimulation of N and P to all samples was necessary for bioremediation.

From Tables 4.7 and 4.8, sample that had highest amount of oil (52.15 mg/l) was TB. However, activities in the station were not including car washing. The amount of oil in sample might came from floor cleaning and run-off. The absence of emulsifier such as detergent cannot form strong emulsion in wastewater (Alther, 1998). Therefore, oil in this wastewater would be degraded easier than those with the presence of detergent.

Regarded sample SV-62, this sample had the highest TOC value (1,416 mg/l) while oil concentration was only 28.92 mg/l since there was not a separation between wastewater from service area and office. Therefore, TOC might came from office and toilet rather than oil. Moreover, car washing activity was absent, so this sample was not chosen to be used.

For following experiments, sample SL was selected. In the station, there was a separation of wastewater between service area and wastewater from office and toilet. The concentration was relatively high when compared with others (39.38 mg/l). There was a presence of car washing facilities in the station. Thus, this wastewater was selected to be used in following experiments.

4.7 Biodegradation of lubricating oil in water microcosms

4.7.1 Lubricating oil removal efficiency of treatments in water microcosms

Five bioremediation processes including natural attenuation, biostimulation, bioaugmentation I, bioaugmentation II and abiotic control were studied to evaluate the efficiency of lubricating oil degradation. Lubricating oil was spiked to all treatments to give final concentration of 5% (v/v). All treatments were incubated at room temperature and shaken twice a day for 12 days. Samples were collected every 3 days for quantitative analysis of lubricating oil and microbiological analysis.

The result of water microcosms was shown in Figure 4.11. After 12 days, the amount of remaining oil in abiotic control (72.48%) compared to those of biological treatments was significantly different. Thus, it could be concluded that the presence of bacteria had important role in lubricating oil degradation.

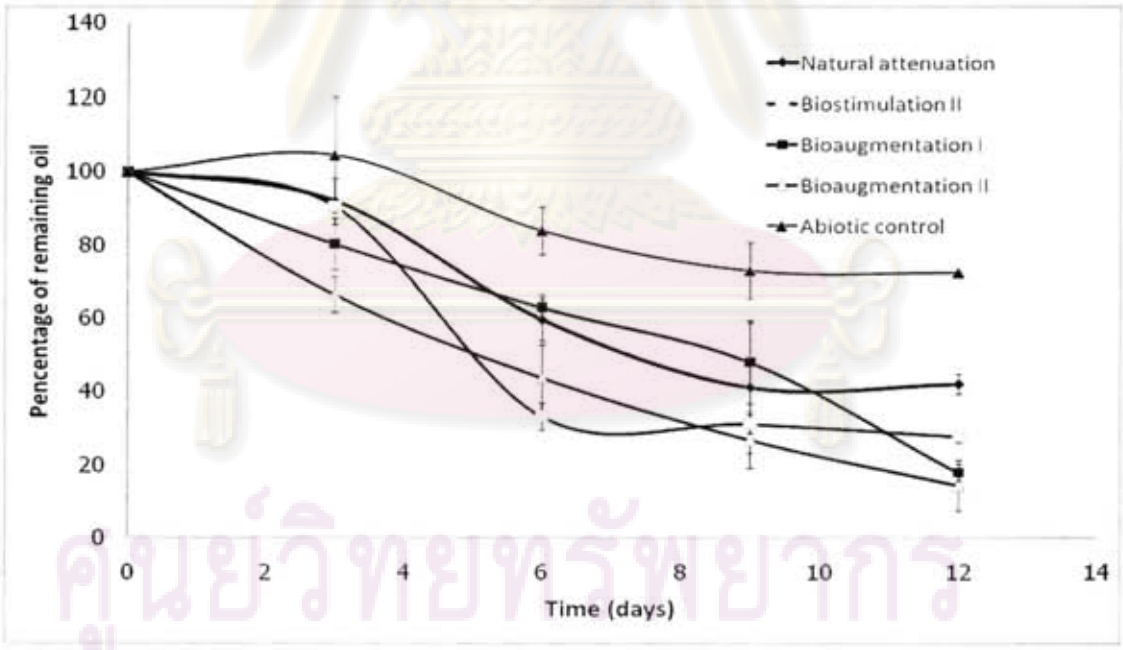

Figure 4.11 Percentage of remaining oil of each treatment in water microcosms after 12 days of incubation.

The comparison of 4 treatments including natural attenuation, biostimulation, bioaugmentation I, and bioaugmentation II showed apparent difference between each

other. Bioaugmentation I and bioaugmentation II showed rapid degradation in the first 3 days. This probably because the number of lubricating oil-degrading bacteria that added into microcosm at about 10^7 CFU/ml was enough to start lubricating oil without any adaptation period. As presented in Figure 4.11, biostimulation, which was added only nutrient, showed small degradation during the first days since indigenous degraders needed time to adapt to the environment. Moreover, in natural attenuation, the carbon input (lubricating oil addition) may result in the depletion of the availability of major inorganic nutrients such as N and P (Lee *et al.*, 2007). So, indigenous tended to need time for adaptation.

These results agreed with the previous study when effective microorganisms are supplemented to the treatment. Bento *et al.* (2004) have studied about biodegradation of light and heavy petroleum hydrocarbon from Long Beach soil sample. It was shown that degrader supplement was the best treatment where 75% and 73% of light and heavy petroleum hydrocarbon were degraded when compared to biostimulation (46% and 45% of light and heavy petroleum hydrocarbon) and natural attenuation (49% and 46% of light and heavy petroleum hydrocarbon).

In addition, Trindade *et al.* (2005) mentioned that bioaugmentation techniques presented biodegradation efficiency approximately twice as higher as natural attenuation in both long-time and recently contaminated environments. It is well known that the addition of exogenous microorganisms in the contaminated environments was extremely important to eliminate the adaptation phase (Alexander, 1994 and Trindade *et al.*, 2005).

When compared only bioaugmentation I (only strain R2 was added) and bioaugmentation II (both R2 and nutrient were added), the results showed that the lubricating removal efficiency of bioaugmentation II was higher. This probably because of nutrient supplement. In bioaugmentation II, N and P were added to get C:N:P ratio equal to 100:5:1 which is the suitable ratio for biological treatment of wastewater (Department of Industrials Works, 2002). Since there was not a nutrient addition in bioaugmentation I treatment, the C:N:P of bioaugmentation I was 100 : 0.51 : 0.08 which was not suitable for bioremediation. Thus, the proper amount of nutrient presented in wastewater is also obviously important for lubricating oil bioremediation.

These findings were consistent with previous study. Wan *et al.* (2002) have studied the effect of organic amendments on diesel oil-contaminated soil. Sewage

sludge or compost was added as an amendment for supplementing organic matter for composting of contaminated soil. The degradation of diesel oil was significantly enhanced by the addition of these organic amendments relative to straight soil.

4.7.2 Enumeration of total bacterial and oil-degrading bacteria in water microcosms

Enumeration of total bacteria and oil-degrading bacteria were conducted using viable plate count method with LB agar plate and M9 agar with a drop of oil on the surface, respectively. Figures 4.12 and 4.13 represented amount of total bacteria and oil-degrading bacteria of water microcosms, respectively. Note that there were no data for abiotic control since it was sterilized.

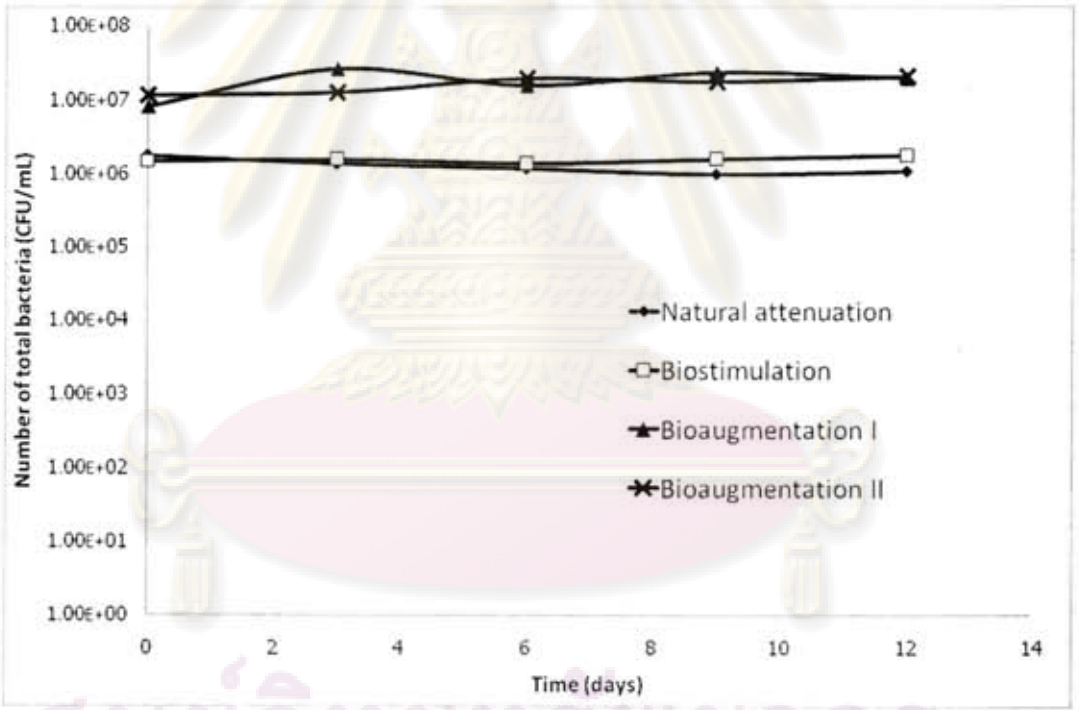

Figure 4.12 Amount of total bacteria in treatments of water microcosms

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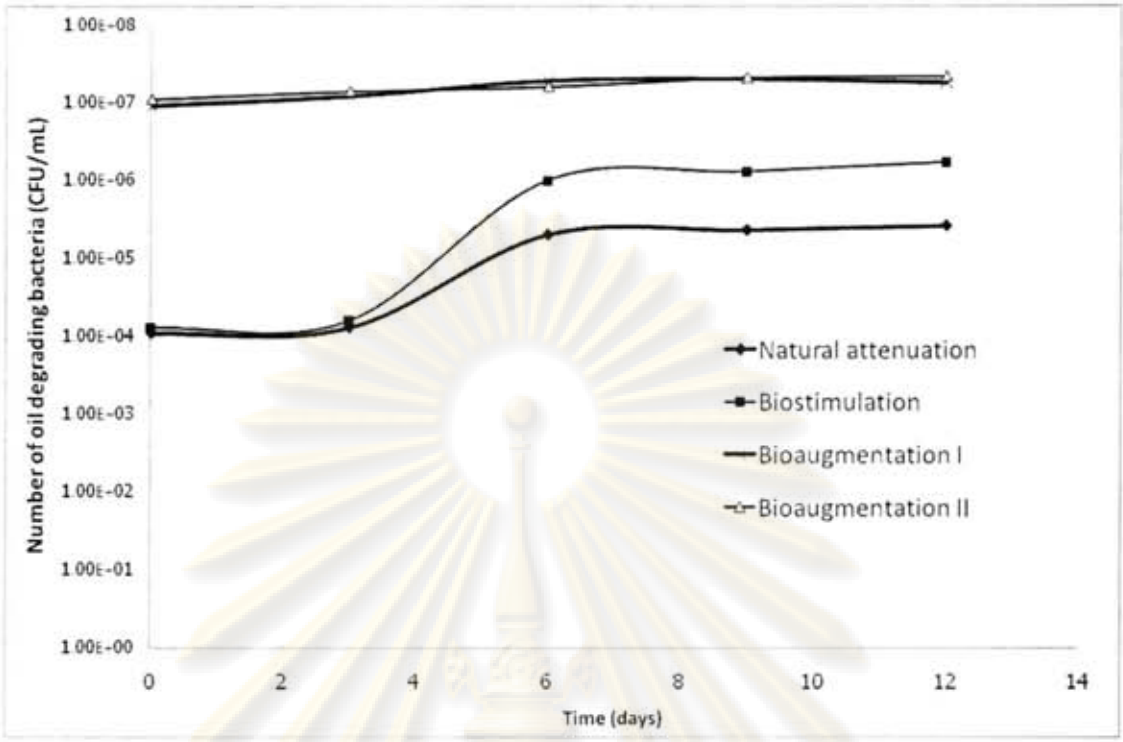

Figure 4.13 Amount of oil-degrading bacteria in treatments of water microcosms

Figures 4.12 and 4.13, bioaugmentation I and bioaugmentation II treatments showed steady line in both total bacteria and oil-degrading bacteria at approx. 10^7 CFU/ml while the amount of total bacteria in biostimulation and natural attenuation were only about 10^6 CFU/ml. The amount of total bacteria in bioaugmentation I and bioaugmentation II were close to the amount of augmented R2. Moreover, the amount of oil-degrading bacteria in these two treatments were also around 10^7 CFU/ml. This may indicated that dominant population of these two treatments was strain R2 since the strain could readily degrade lubricating oil without any adaptation period requirement.

When considered the amount of total bacteria and oil-degrading bacteria of biostimulation and natural attenuation, the amount of total bacteria was steady at around 10^6 CFU/ml. When regarded the amount of oil-degrading bacteria, the amount was steady in the first 3 days at around 10^4 CFU/ml while it increased significantly from day 3 to day 6. From Figure 4.9, both biostimulation and natural attenuation exhibited steeper lines during day 3 to day 6. This may hypothesized that oil-degrading bacteria in these treatments spent the first 3 days to adapt to environment.

As described above, there was a significant increasing of oil-degrading bacteria in biostimulation and natural attenuation. In day 6, the number of oil-degrading bacteria in biostimulation increased from approx. 10^4 CFU/ml to 10^6 CFU/ml while it increased to 10^5 CFU/ml in natural attenuation which 10 times lower. This probably because there was a suitable C:N:P adjusting in biostimulation, so oil-degrading bacteria in biostimulation could increase rather than in natural attenuation.

4.7.3 Analysis of bacterial communities in water microcosms

The analysis of bacterial communities was conducted using DGGE technique. DNA from water microcosms were extracted and were amplified for V3-region in 16S rDNA by PCR using primer 341F with GC clamp and 534R. The amplified fragments were run in DGGE to investigate the bacterial community shift over 12 days of each biological treatment. The changes of bacterial populations were not obvious in all treatments (Figure 4.14).

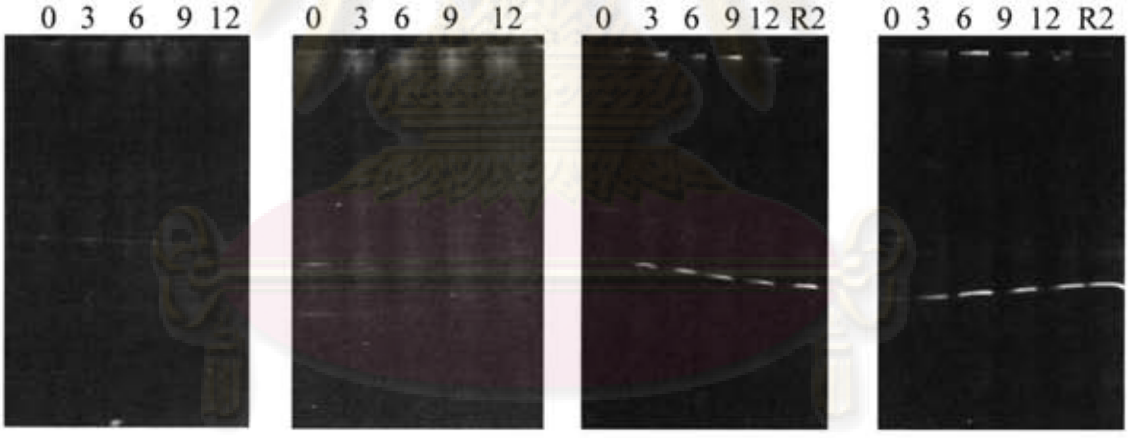

Figure 4.14 DGGE profile of the microcosm treatments; Natural attenuation (a), Biostimulation (b), Bioaugmentation I (c) and Bioaugmentation II (d)

From DGGE profile shown in Figures 4.14 (c) and (d), the band of strain R2 always presented through out 12 days of experiments. Therefore, bacteria strain R2 could survive and tended to be dominant species in bioaugmentation I and

bioaugmentation II like described in 4.7.2. This result agrees with the number of oil-degrading bacteria which was almost stable through out the experiment. DGGE profile in natural attenuation and biostimulation (Figures 4.14 (a) and (b)) also changed a bit. The profiles showed that some bands were disappeared. It might because bacteria that could not utilize were dead and the remaining bands indicated exogenous oil-degrading bacteria.

4.7.4 Detection of gene involved in oil degradation in water microcosms

PCR with 3 primer sets were used to detect a wide range of genes encoding alkane hydroxylases, using ALK1 and ALK2 primer sets, and Rieske of aromatic dioxygenase gene, using Rieske primer set.

From Figure 4.15 – 4.18, all treatments showed positive results only for *alkM* (primer set ALK2). In natural attenuation (Figure 4.15) and biostimulation (Figure 4.16) treatments, the presence of *alkM* might come from indigenous oil-degraders. From literature review, pollutant from car wash wastewater would consist of lubricating oil that has carbon atom more 15 atoms (Vazquez-duhalt, 1989). Therefore, indigenous oil-degraders may have *alkM* themselves.

The presence of *alkM* gene in bioaugmentation I (Figure 4.17) and bioaugmentation II (Figure 4.18) might come from 1) the presence of R2 which carry *alkM* gene, and 2) *alkM* from indigenous oil-degraders.

The detection of Rieske of dioxygenase gene showed negative results for all treatments since Rieske primer used in this study was not cover genes involved in aromatic compounds degradation in microcosms. If using other primers, involving genes might be detected since aromatic dioxygenase genes are very various (Baldwin *et al*., 2004).

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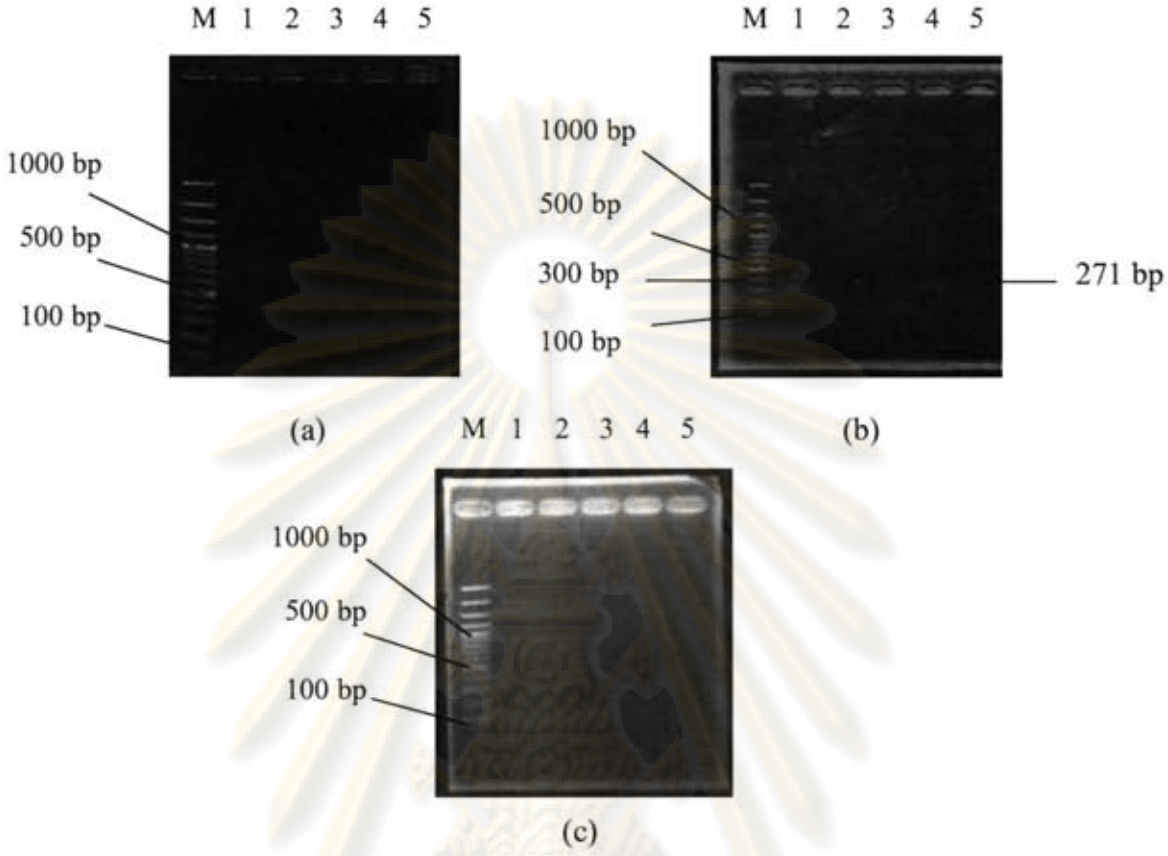

Figure 4.15 Detection of *alkB* (a), *alkM* (b), and Rieske of aromatic dioxygenase (c) in natural attenuation treatment. Lane M: 100 bp ladder DNA marker, Lane 1: Day 3, Lane 2: Day 6, Lane 3: Day 9, Lane 4: Day 12, Lane 5: Negative control.

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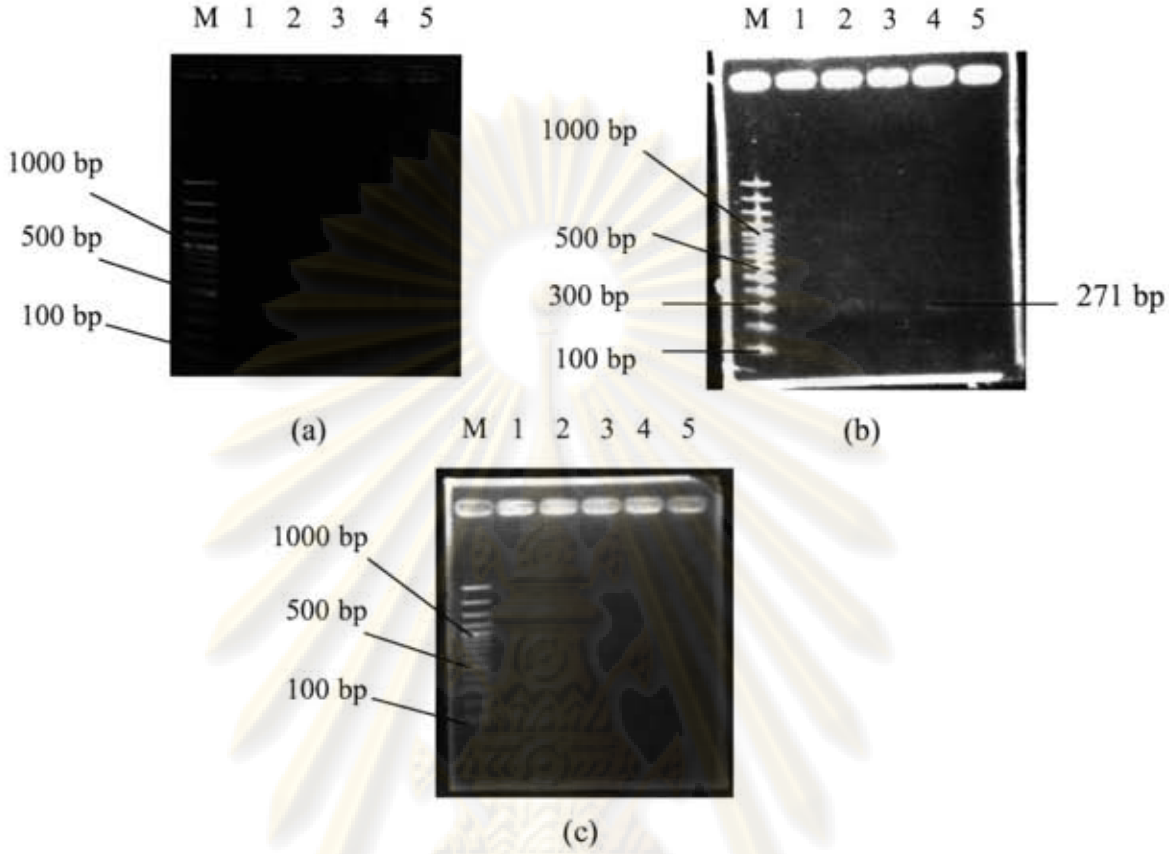

Figure 4.16 Detection of *alkB* (a), *alkM* (b), and Rieske of aromatic dioxygenase (c) in biostimulation treatment. Lane M: 100 bp ladder DNA marker, Lane 1: Day 3, Lane 2: Day 6, Lane 3: Day 9, Lane 4: Day 12, Lane 5: Negative control.

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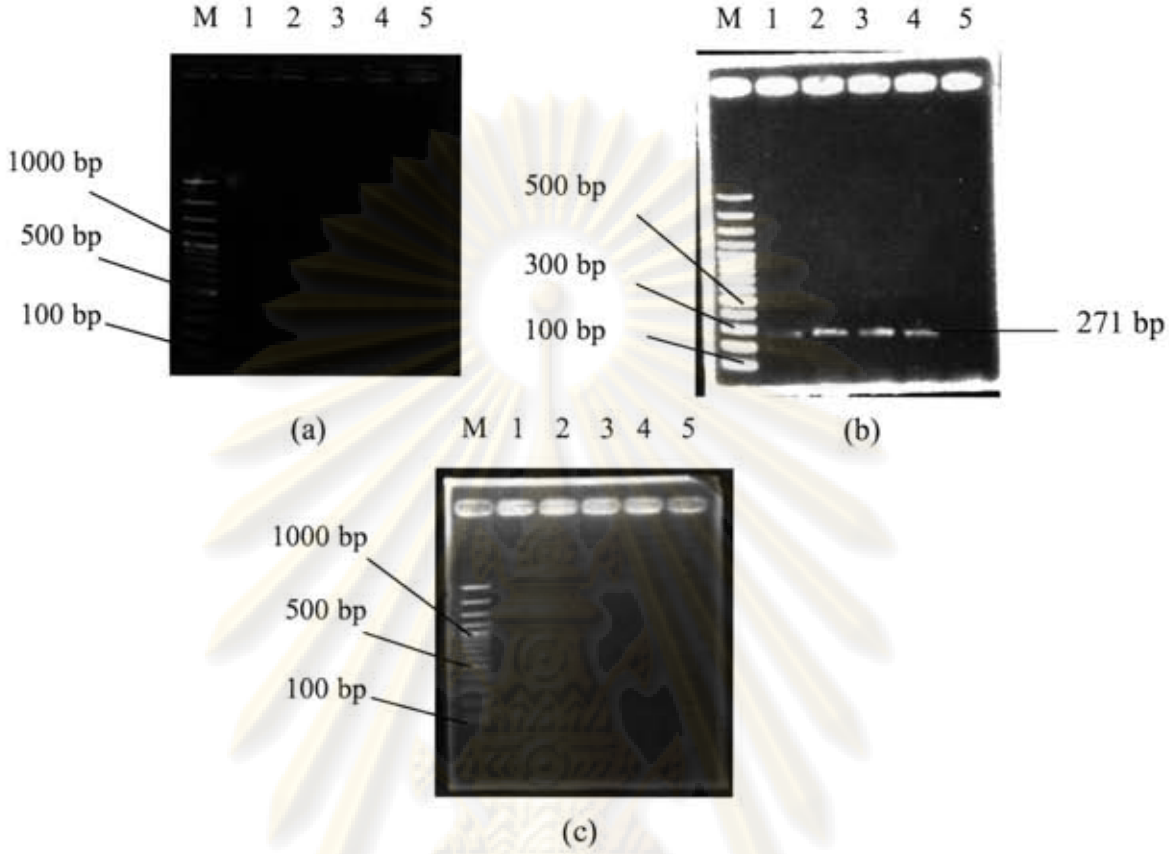

Figure 4.17 Detection of *alkB* (a), *alkM* (b), and Rieske of aromatic dioxygenase (c) in bioaugmentation I treatment. Lane M: 100 bp ladder DNA marker, Lane 1: Day 3, Lane 2: Day 6, Lane 3: Day 9, Lane 4: Day 12, Lane 5: Negative control.

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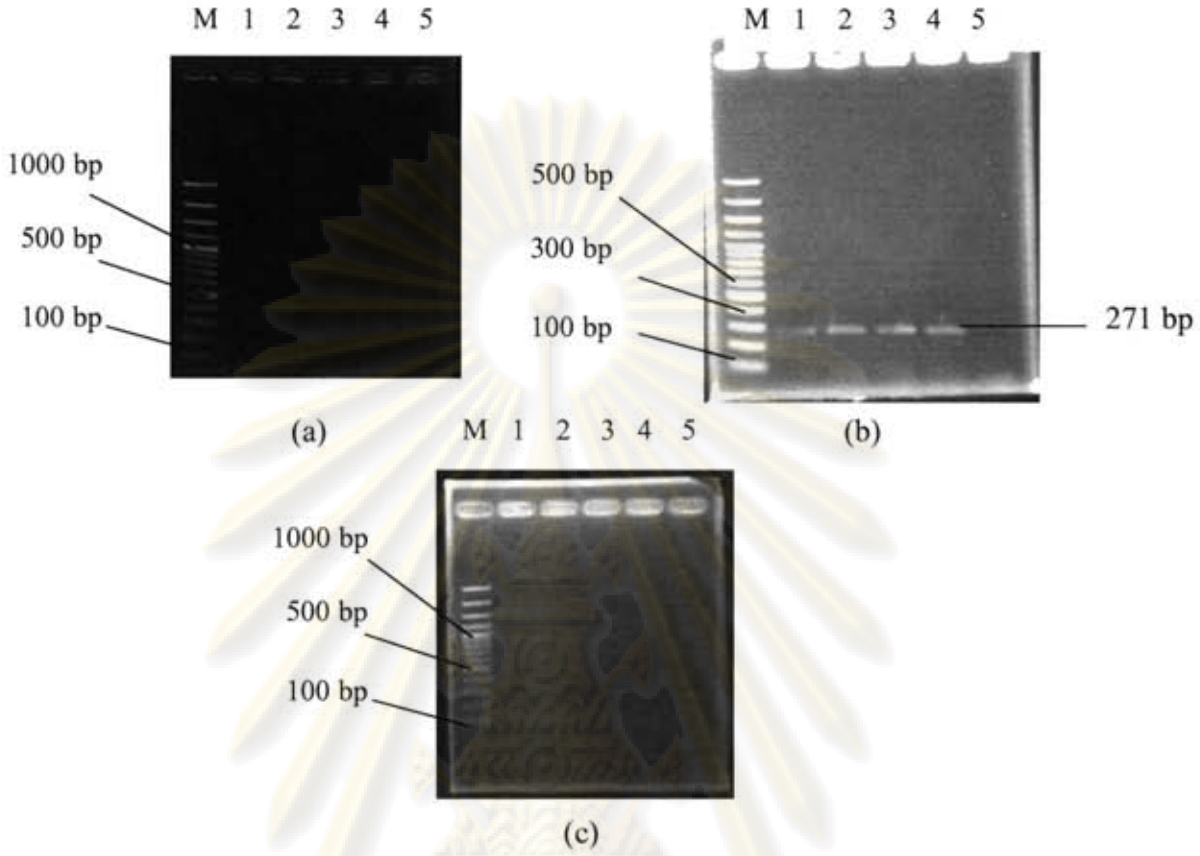

Figure 4.18 Detection of *alkB* (a), *alkM* (b), and Rieske of aromatic dioxygenase (c) in bioaugmentation II treatment. Lane M: 100 bp ladder DNA marker, Lane 1: Day 3, Lane 2: Day 6, Lane 3: Day 9, Lane 4: Day 12, Lane 5: Negative control.

4.7.5 Toxicity test of water microcosms by dehydrogenase assay

Dehydrogenase assay was chosen to test toxicity level of water microcosms since dehydrogenase is enzyme that could indicate activities of bacteria. *Bacillus cereus* TISTR 687 (Rönnpagel *et al*, 1994) was used to evaluate toxicity level of wastewater from microcosms.

The results shown that the toxicity level of each treatments were appapently different. Bioaugmentation II showed the lowest % inhibition at 35.13% after 12 days of incubation while % inhibition of bioaugmentation I, biostimulation, natural attenuation and abiotic control were 36.75, 50.61, 75.63 and 97.51%, respectively (Figure 4.19).

These results agree with percentage of remaining oil after 12 days of incubation (4.7.1). The lowest percentage of remaining oil also exhibited the lowest toxicity level while the toxicity level of abiotic control was nearly stable. Thus, biological treatment could removed lubricating oil and also could reduce toxicity level of wastewater from petrol station.

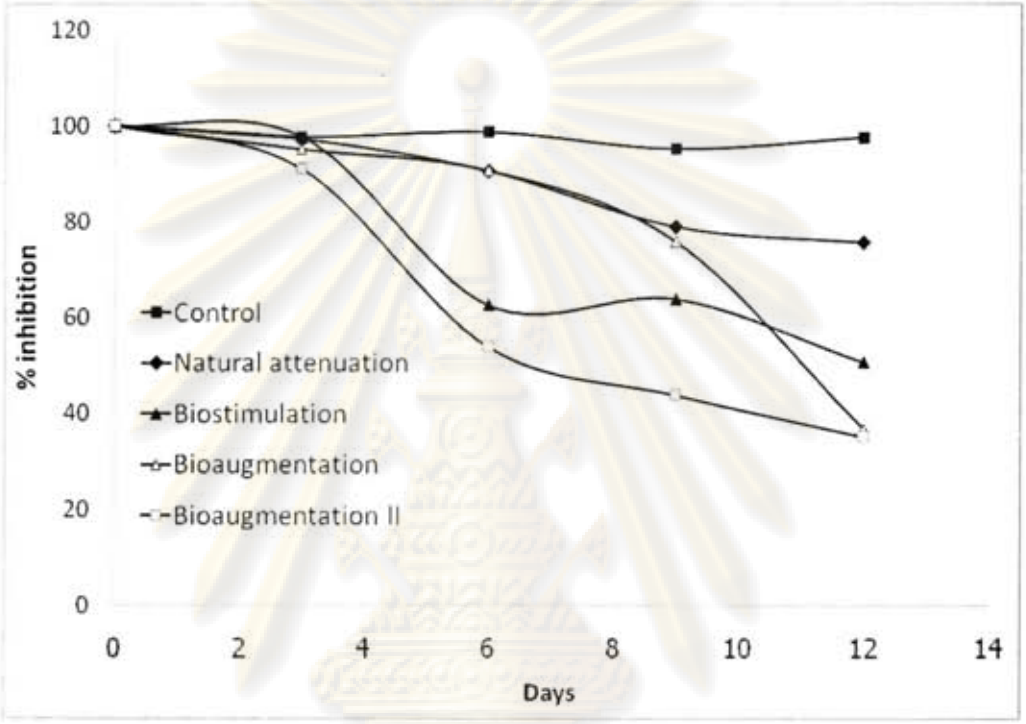

Figure 4.19 Toxicity levels of treatments in water microcosms

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CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Wastewater from petrol station is generated in large amount daily. Most of this wastewater comes from car washing activity in the station. Lubricating oil was always found to be contaminated in car washing wastewater. Interestingly, car washing wastewater is in the form of oil-in-water since it contains detergent used in car washing. Detergent would act as emulsifier, thus, chemically stable oil-in-water emulsion is formed. Oil-in-water requires proper techniques to treat this emulsion. Many physical and chemical treatments, i.e. floatation, ultrafiltertion and ozonization, were used to treat emulsion. The drawbacks of these techniques are expensive cost and incomplete elimination. Therefore, bioremediation was apply since it is less expensive and could eliminate contaminant completely. However, bioremediation requires monitoring of its potential in that environment because there are several factors limiting bioremediation.

Hence, the aim of this study was to assess bioremediation potential of wastewater from petrol station and to investigate the most effective ways for bioremediation using 5 different treatments of microcosms including natural attenuation, biostimulation, bioaugmentation I, bioaugmentation II and abiotic control. Moreover, isolation of oil-degrading bacteria was conducted. DGGE and detection of relevant catabolic genes were applied to monitor the presence of oil-degrading bacteria.

From this study, two isolates, Tr1 and R2, were isolated from oil-contaminated wastewater from car maintenance center. The results of sequence analysis of 16S rDNA exhibited that both strains belonged to *Acinetobacter* sp. which is well-known to be capable to degrade petroleum hydrocarbon. After 200-ppm-oil-in-water emulsion degradability test was done, R2 found to be more effective than Tr1. R2 showed 78.01% of oil-in-water emulsion degradation after 18 hours of incubation

compared to control. Moreover, *alkM* gene could be detected in R2 but it showed negative result for Tr1. R2 was then selected to be applied in following microcosms.

Five treatments of water microcosm were conducted to investigate lubricating oil biodegradation. Real wastewater from petrol station was collected to be used in microcosms. After 12 days of incubation, bioaugmentation II exhibited the lowest percentage of remaining oil at 14.47%. The remaining oil in bioaugmentation I was 18.01%. It could be concluded that addition of nutrient adjustment was important. When compared the results of bioaugmentation I and bioaugmentation II, oil concentration in these 2 treatments were decreased faster than others treatments since the supplement of exogenous degraders could start lubricating oil degradation without any adaptation period. Therefore, addition of exogenous degraders and nutrient addition were important for successful oil biodegradation.

The amount of total bacteria and oil-degrading bacteria in bioaugmentation I and bioaugmentation II were stable at approx. 10^7 CFU/ml which was equal to the amount of augmented bacteria. DGGE profile also exhibited the slightly change during 12 days of experiment. Moreover, the intensity of band of augmented R2 was more intense than others. Therefore, the dominant species in bioaugmentation I and bioaugmentation II might be R2. DGGE profile in natural attenuation and biostimulation did not change, even though the number of oil-degrading bacteria increased during the third and the sixth days. The results suggested that there were no dominant species of oil-degrading bacteria in these treatments. Detection of *alkM* gene was showed positive result for all treatments through out 12 days of experiment which means that the degradation potential was present through out experiment. Moreover, toxicity test indicated the correlation between amount of oil and toxicity level. When amount of oil was decreased, toxicity level of treatment was decreased also.

In conclusion, this study is the first study that applies monitoring of bioremediation potential to the real wastewater. The results indicate that wastewater from petrol station had potential to treat by bioremediation technique. The study on degrader and nutrient availability was important since it would inform whether biostimulation and bioaugmentation were required.

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5.2 Recommendations

1. The amount of augmented degrader at approx. 10^7 CFU/ml might be too large since there was a decreasing of oil concentration but the amount of oil-degrader was stable.

2. Bioaugmentation of bacterial consortium should be studied. Bacterial consortium can enhance degradation efficiency (Mishra *et al.*, 2001).

3. For further application in real situation, other techniques could be coupled with bioremediation. For example, Lyew *et al.* (2007) have immobilized *Mycobacterium austroafricanum* IFP 2012 onto several materials to examine the degradation of MTBE. The result was that all immobilized cell showed higher degradation efficiency than free cell.

4. When require to apply this research to real petrol station site, wastewater have to be analyzed for some necessary characteristics, such as amount of N, P and amount of contaminated oil, since these characteristics are different from time to time. For example, in this research, amount of nutrients (N, P) in each sample were different, so the amount of added nutrients must be different.

5. Sequencing of *alkM* from strain R2 should be conducted.

6. PCR of aromatic dioxygenase gene by other primers should be conducted, for example, PCR using primer for detect single ring aromatic. Since the variety of aromatic dioxydenase is very various, primers used in this study might not cover aromatic dioxygenase gene in this wastewater.

7. The study about plasmid of Tr1 should be done since *alkM* was not presented in extracted genomic DNA. So, the study about plasmid will inform if *alkM* is on the plasmid.

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8. Even bioaugmentation II showed the highest percentage of oil removal in this study, it might not be the most suitable method for applying to real wastewater treatment in the station. The physical and microbiological properties of each wastewater are different from each other. In some cases, bioaugmentation may be necessary, but for some cases, only biostimulation is required. So, this study can be used as a basic guideline for applying bioremediation technique to real wastewater at the station.

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APPENDICES

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APPENDIX A

Media Preparation

M9 broth medium

A. 5X M9 salt

$Na_2HPO_4.7H_2O$	64	g
KH_2PO_4	15	g
NaCl	2.5	g
NH_4Cl	50	g
Deionized water	to 1,000	ml

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

B. 1 M $MgSO_4$

$MgSO_4$	12.324	g
Deionized water	to 50	ml

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

C. 1 M $CaCl_2$

$CaCl_2$	7.351	g
Deionized water	to 50	ml

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

Before using, mix

5X M9 salt	200	ml
1 M $MgSO_4$	2	ml
1 M $CaCl_2$	0.1	ml
Sterilized deionized water	to 1000	ml

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M9 agar

Add 15 g of Bacto Agar to 800 ml of deionized water. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min. Mix as described in M9 broth medium preparation.

Luria Bertani (LB) broth

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

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

LB agar

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

Ψb broth

Yeast extracts	5	g
Tryptone	20	g
$MgSO_4.7H_2O$	5	g

Mix them in deionized water. Adjust pH to 7 with 1 N NaOH (Appendix II). Make volume with deionized water to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

In order to make agar medium, add 15 g of agar to 1,000 ml of Ψb broth before autoclaving.

SOC medium

Solution A

Yeast extracts	5	g
Tryptone	20	g
NaCl	0.58	g
$MgCl_2$	2	g
$MgSO_4.7H_2O$	2.46	g
KCl	0.18	g

Make final volume to 980 ml with deionized water. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

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Solution B

Glucose	3.6	g
Deionized water	20	ml

Sterilize by filter through filter paper pour size 0.22 µm. Mix solutions A and B and store at -20°C until being used.

Nutrient broth (NB)

Beef extract	3	g
Peptone	5	g
Deionized water	1,000	ml

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

Nutrient agar (NA)

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

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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.

20% Proteinase K

Proteinase K	20	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.

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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.

Lysis buffer

10 mM Tris-HCl solution, pH 8	50	ml
0.5 M EDTA	10	ml
10% SDS	30	ml
Deionized water	10	ml

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

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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.

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Amplicilin, Ap

Amplicilin	100	mg
Deioniized water	1	ml

Sterilize by filter through filter paper pour size 0.22 μm.

2% 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)

X-gal	20	mg
Dimethylformamide (DMF)	1	ml

Sterilize by filter through PTFE filter pour size 0.22 μm.

1 M isopropyl-β-D-thiogalactopyranoside (IPTG)

IPTG	238	mg
Deionized water	1	ml

Sterilize by filter through filter paper pour size 0.22 μm.

TfbI solution

Potassium acetate (CH_3COOK)	0.295	g
Rubidium chloride (RbCl)	1.21	g
Calcium chloride ($CaCl_2.2H_2O$)	0.148	g
Manganeses chloride ($MnCl_2$)	0.99	g
Glycerol	15	ml

Dissolve in 70 ml of deionized water. Adjust pH to 5.8 using 0.2 M acetic acid. Add deionized to make volume to 100 ml. Sterilize by filter through filter paper pour size 0.22 μm.

TfbII solution

2-[*N*-morpholino]ethanesulfonic acid (MES)	0.29	g
Rubidium chloride (RbCl)	0.121	g
Calcium chloride ($CaCl_2.2H_2O$)	1.103	g
Glycerol	15	ml

Add deionized water to 100 ml. Sterilize by filter through filter paper pour size 0.22 μm.

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Chemicals used in DGGE

0% denaturing solution in 8% acrylamide gel

40% acrylamide/bis	20	ml
50X TAE buffer	2	ml
Deionized water	78	ml

100% denaturing solution in 8% acrylamide gel

40% acrylamide/bis	20	ml
50X TAE buffer	2	ml
Formamide	40	ml
Urea	42	g
Add deionized water	to 100	ml

10% ammonium persulfate

Ammonium persulfate	0.1	g
Deionized water	1	ml

0.5 mg/ml ethidium bromide solution

10 mg/ml ethdium bromide solution	10	µl
Deionized water	200	ml

Resazurin solution

Dissolving buffer

Solution A

Potassium dihydrogen phosphate ($KH_2PO_4.H_2O$)	13	g
Dipotassium hydrogen phosphate ($K_2HPO_4.H_2O$)	8.2	g
Sodium acetate	2	g
Deionized water	to 1,000	ml

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

Aliquot solution to dissolve 2 g of glucose. Filter glucose solution back to solution A through filter paper pour size 0.45 µm.

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Resazurin dye solution

Resazurin	0.005	g
Dissolving buffer	100	ml

Note that resazurin dye solution has to be prepared freshly before use.

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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

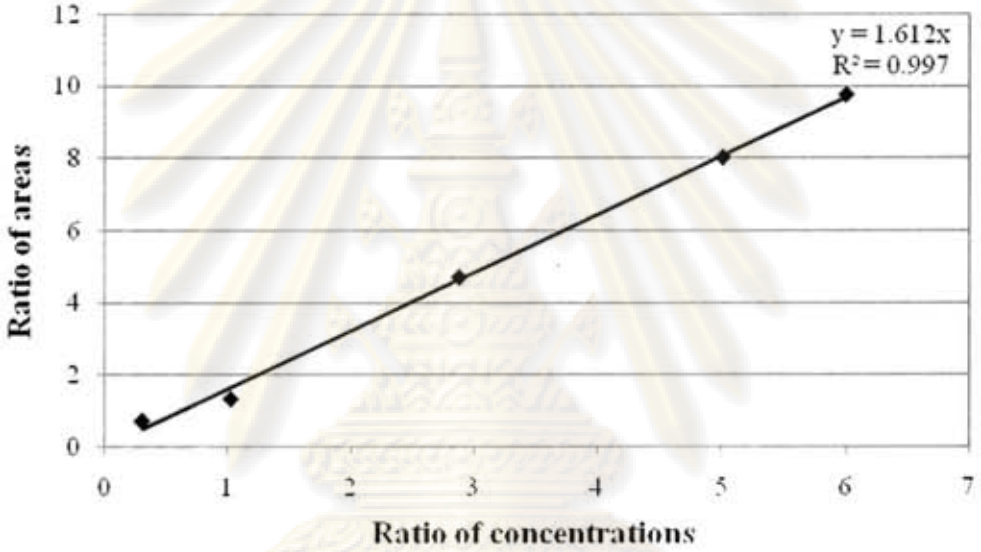

Figure A-1 Standard curve of lubricating oil from TLC-FID. Each data point was averaged from triple spots on chromatorods.

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APPENDIX D

Raw Data

Table D-1 TOC concentration of wastewater samples

Sample	TOC (mg/l)	Average (mg/l)
JJ	776	781
	787	
TB	907	881
	855	
SL	833	817
	800	
SV-62	1,336	1,416
	1,495	

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Table D-2 Amount of remaining oil in water microcosm treatments using TLC-FID (Dilution factor = 200)

Day 3

Treatments	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
Natural attenuation	1451	401	1852	1512	1.2249	9.4969	124.0682
	1991	656	2647	2381	1.1117	8.6196	
Biostimulation I	1392	0	1392	1197	1.1629	9.0165	0.132222
	1476	124	1600	1405	1.1388	8.8295	
Biostimulation II	2849	0	2849	3210	0.8875	6.8814	0.479929
	3376	0	3376	4220	0.8000	6.2027	
Bioaugmentation	1371	899	2270	2097	1.0825	8.3930	0.6837
	1429	636	2065	2156	0.9578	7.4261	
Abiotic control	2132	1274	3406	2870	1.1868	9.2014	1.53329
	1886	1849	3735	2547	1.4664	11.3698	

Day 6

Treatments	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
Natural attenuation	1258	387	1645	2336	0.7042	5.4599	0.57834
	1421	502	1923	2375	0.8097	6.2778	
Biostimulation I	1169	111	1280	2806	0.4562	3.5368	0.365858
	1123	189	1312	3369	0.3894	3.0194	
Biostimulation II	1496	0	1496	3155	0.4742	3.6764	0.895023
	2085	0	2085	3271	0.6374	4.9422	
Bioaugmentation	1276	831	2107	2536	0.8308	6.4418	0.344163
	1145	673	1818	2367	0.7681	5.9551	
Abiotic control	1864	527	2391	2373	1.0076	7.8122	0.637409
	1123	1073	2196	1954	1.1238	8.7136	

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Table D-2 Amount of remaining oil in water microcosm treatments using TLC-FID (Dilution factor = 200) (Cont.)

Day 9

Treatments	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
Natural attenuation	712	0	712	1960	0.3633	2.8165	1.743549
	1306	154	1460	2143	0.6813	5.2823	
Biostimulation I	1456	116	1572	3758	0.4183	3.2433	0.239677
	1144	0	1144	3054	0.3746	2.9043	
Biostimulation II	1573	0	1573	3834	0.4103	3.1810	0.75194
	1004	0	1004	3676	0.2731	2.1176	
Bioaugmentation	1094	852	1946	2732	0.7123	5.5227	1.120925
	1009	448	1457	2869	1457	3.9375	
Abiotic control	1532	200	1732	2019	0.8579	6.6512	0.762251
	1594	646	2240	2247	0.9969	7.7292	

Day 12

Treatments	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
Natural attenuation	1348	0	1348	2415	0.5582	4.3278	0.264178
	1276	0	1276	2502	0.5100	3.9542	
Biostimulation I	1144	120	1264	3727	0.3391	2.6295	0.155808
	1260	143	1403	3817	0.3676	2.8499	
Biostimulation II	350	0	350	2864	0.1222	0.9475	0.67552
	804	0	804	3276	0.2454	1.9028	
Bioaugmentation	427	124	551	2655	0.2075	1.6091	0.2323
	653	0	653	2613	0.2499	1.9376	
Abiotic control	1719	142	1861	2020	0.9213	7.1431	0.009608
	1572	325	1897	2063	0.9195	7.1295	

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Table D-3 Number of total bacteria in water microcosm treatments using plate count technique.

Times	Abiotic control	Natural attenuation	Biostimulation I	Biostimulation II	Bioaugmentation
Day 0	0±0.00	1.80E+06±2.12E+05	1.50E+06±1.80E+05	8.30E+06±4.09E+06	1.20E+07±6.09E+06
Day 3	0±0.00	1.40E+06±1.20E+05	1.60E+06±4.95E+05	2.70E+07±3.56E+06	1.30E+07±3.98E+06
Day 6	0±0.00	1.20E+06±7.0E+04	1.40E+06±2.12E+05	1.60E+07±2.90E+06	2.00E+07±4.76E+06
Day 9	0±0.00	1.00E+06±1.10E+05	1.60E+06±3.54E+05	2.40E+07±7.04E+06	1.80E+07±2.31E+06
Day 12	0±0.00	1.10E+06±5.66E+05	1.80E+06±7.77E+05	2.00E+07±3.87E+06	2.10E+07±4.78E+06

Table D-4 Number of oil-degrading bacteria in water microcosm treatments using plate count technique.

Times	Abiotic control	Natural attenuation	Biostimulation I	Biostimulation II	Bioaugmentation
Day 0	0±0.00	1.10E+04±3.98E+03	1.30E+04±2.12E+03	1.10E+07±3.58E+06	9.00E+06±1.68E+06
Day 3	0±0.00	1.30E+04±4.65E+03	1.60E+04±3.71E+03	1.40E+07±1.96E+06	1.20E+07±7.4E+06
Day 6	0±0.00	2.00E+05±7.74E+03	1.00E+06±5.34E+03	1.60E+07±7.45E+06	1.90E+07±2.64E+06
Day 9	0±0.00	2.30E+05±2.05E+03	1.30E+06±2.07E+03	2.10E+07±3.97E+06	2.00E+07±7.54E+06
Day 12	0±0.00	2.60E+05±5.12E+03	1.70E+06±6.6E+03	2.20E+07±2.15E+06	1.80E+07±6.92E+06

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Table D-5 Toxicity test of natural attenuation treatment in water microcosms

	Abs of %inhibition	Abs of 0% inhibition	Abs of sample	% inhibition of sample
Day 3	2.088	0.146	2.028	96.96032973
	2.077	0.151	2.035	97.32096857
	2.096	0.140	2.038	97.47552808
Average	2.087	0.146	-	97.2523
SD	0.009539392	0.005507571	-	0.26437926
Day 6	2.088	0.146	1.921	91.44770737
	2.077	0.151	1.904	90.57187017
	2.096	0.140	1.889	89.79907264
Average	2.087	0.146	-	90.6062
SD	0.009539392	0.005507571	-	0.82485385
Day 9	2.088	0.146	1.678	78.92838743
	2.077	0.151	1.654	77.69191139
	2.096	0.140	1.703	80.21638331
Average	2.087	0.146	-	78.9456
SD	0.009539392	0.005507571	-	1.26232358
Day 12	2.088	0.146	1.612	75.52807831
	2.077	0.151	1.608	75.32199897
	2.096	0.140	1.622	76.04327666
Average	2.087	0.146	-	75.6311
SD	0.009539392	0.005507571	-	0.37151481

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Table D-6 Toxicity test of biostimulation I treatment in water microcosms

	Abs of %inhibition	Abs of 0% inhibition	Abs of sample	% inhibition of sample
Day 3	2.104	0.139	2.1	99.69387755
	2.196	0.146	2.078	98.57142857
	2.108	0.154	2.002	94.69387755
Average	2.106	0.147	-	97.6531
SD	0.089016852	0.007505553	-	2.62346125
Day 6	2.104	0.139	1.324	60.10204082
	2.196	0.146	1.406	64.28571429
	2.108	0.154	1.387	63.31632653
Average	2.106	0.147	-	62.568
SD	0.089016852	0.007505553	-	2.18991891
Day 9	2.104	0.139	1.2	53.7755102
	2.196	0.146	1.189	53.21428571
	2.108	0.154	1.005	43.82653061
Average	2.106	0.147	-	63.801
SD	0.089016852	0.007505553	-	5.58908331
Day 12	2.104	0.139	1.024	44.79591837
	2.196	0.146	1.205	54.03061224
	2.108	0.154	1.185	53.01020408
Average	2.106	0.147	-	50.6122
SD	0.089016852	0.007505553	-	5.06285976

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Table D-7 Toxicity test of biostimulation II treatment in water microcosms

	Abs of %inhibition	Abs of 0% inhibition	Abs of sample	% inhibition of sample
Day 3	2.106	0.154	1.965	92.86442406
	2.111	0.142	1.899	89.50050968
	2.099	0.132	1.921	90.62181448
Average	2.105	0.143	-	90.9956
SD	0.006027714	0.011015141	-	1.71282136
Day 6	2.106	0.154	1.136	50.6116208
	2.111	0.142	1.267	57.28848114
	2.099	0.132	1.196	53.66972477
Average	2.105	0.143	-	53.8566
SD	0.006027714	0.011015141	-	3.34235101
Day 9	2.106	0.154	1.056	46.53414883
	2.111	0.142	0.967	41.99796126
	2.099	0.132	0.984	42.86442406
Average	2.105	0.143	-	43.7988
SD	0.006027714	0.011015141	-	2.40813328
Day 12	2.106	0.154	0.844	35.72884811
	2.111	0.142	0.812	34.09785933
	2.099	0.132	0.841	35.57594292
Average	2.105	0.143	-	35.1342
SD	0.006027714	0.011015141	-	0.90076223

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Table D-8 Toxicity test of bioaugmentation treatment in water microcosms

	Abs of %inhibition	Abs of 0% inhibition	Abs of sample	% inhibition of sample
Day 3	2.099	0.155	2.005	95.34643226
	2.132	0.166	2.001	95.13960703
	2.055	0.16	1.992	94.67425026
Average	2.095	0.161	-	95.0534
SD	0.03863073	0.005567764	-	0.34427757
Day 6	2.099	0.155	1.927	91.31334023
	2.132	0.166	1.917	90.79627715
	2.055	0.16	1.899	89.8655636
Average	2.095	0.161	-	90.6584
SD	0.03863073	0.005567764	-	0.73367103
Day 9	2.099	0.155	1.655	77.24922441
	2.132	0.166	1.641	76.52533609
	2.055	0.16	1.584	73.57807653
Average	2.095	0.161	-	75.7842
SD	0.03863073	0.005567764	-	1.94455136
Day 12	2.099	0.155	0.824	34.28128232
	2.132	0.166	0.913	38.88314374
	2.055	0.16	0.878	37.07342296
Average	2.095	0.161	-	36.7459
SD	0.03863073	0.005567764	-	2.31834234

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Table D-8 Toxicity test of abiotic in water microcosms

	Abs of %inhibition	Abs of 0% inhibition	Abs of sample	% inhibition of sample
Day 3	2.077	0.046	2.018	96.44513138
	2.088	0.051	2.081	99.69088099
	2.096	0.040	2.028	96.96032973
Average	2.087	0.146	-	97.6987807
SD	0.009539392	0.005507571	-	1.744335121
Day 6	2.077	0.046	2.033	97.2179289
	2.088	0.051	2.088	100.0515198
	2.096	0.040	2.062	98.71200412
Average	2.087	0.146	-	98.66048429
SD	0.009539392	0.005507571	-	1.417497835
Day 9	2.077	0.046	2.005	95.77537352
	2.088	0.051	1.997	95.36321484
	2.096	0.040	1.981	94.53889748
Average	2.087	0.146	-	95.22582861
SD	0.009539392	0.005507571	-	0.629582785
Day 12	2.077	0.046	2.004	95.72385368
	2.088	0.051	2.076	99.43328181
	2.096	0.040	2.036	97.37248841
Average	2.087	0.146	-	97.50987464
SD	0.009539392	0.005507571	-	1.858526431

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APPENDIX E

Sequence results

The sequence results of *alkM* from wastewater from car maintenance center

REST1

TATTCCAACTATGCTCAGGCATAGTACGTTGATACTTACCATTTGCATCTTTTTGACGTTTCAAACCAT
AGTGTTCGATGTAGTTAATCACTTCAAACAAACTAATTCCGTAGAATGCTTGAGTAGCTAAATAAGG
AATCACGCCTTTGCCGAATATACCAACCATAGAACCATGGAAAGCTGCGCTCATTCCCCAACCTTGA
AGCAACTCATTGTCTAATGACCAGAACTCTTTGCCTT TACGTTTTAG

REST2

AATTCGTGATTGAGACAAATCGTCTAAAACGTAAAGGCAAAARTTTYTGGTCRWWRGACAATGAAT
TRYTGCAAGGYTGGGGMATGAGTGCRGCWTTYCAYRGTKCWTTGKTKGSWMKMTTYGGYACWGGS
ATSATTCCKTATTTGGCKACWCARGCTTTYTAYGGMATTAGTTTGTTTGAAATRATTAAYTAYATYG
AACAYTATGGTTTGMWWMGACAAAAAGATGCRCAKGGYAAATAYSAGCGCACYATGCCTGAGCAT
AGTTGGAATAATAACAAAATCTGAATT

REST4

AGAATTCGTGATTTTGTTATTATTCCAACTATGCTCAGGCATGGTGCGCTCGTATTTGCCCTGCGCAT
CTTTTTGTCTAAGCAAACCATAATGTTCAATATAATTAATTATTTCAAACAAACTAATGCCATAAAAA
GCCTGTGTCGCCAAATACGGAATGATCCCAGTACCAAAGCGTGCCAACAATGCACTGTGAAATGCCG
CACTCATGCCCCAGCCTTGCAATAATTCATTGTCCTTCGACCAAAAACTTTTGCCTTTACGTTTTAGAC
GATTTGTCTCAATCTGAA

REST5

AGAATTCGTGATTGAGACAAATCGTCTAAAACGTAAAGGCAAAGAGTTCTGGTCATTAGACAATGAG
TTGCTTCAAGGTTGGGGAATGAGCGCAGCTTTCCATGGTTCTATGGTTGGTATATTCGGCAAAGGCGT
GATTCCTTATTTAGCTACTCAAGCATTCTACGGAATTAGTTTGTTTGAAGTGATTAACTACATCGAAC
ACTATGGTTTGAAACGTCAAAAAGATGCAAATGGTAAGTATCAACGTACTATGCCAGAGCATAGTTG
GAATAATAACAAAATCTGAA

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The sequence results of pure cultures

Strains TR1

GATTGCACCTTATCTTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACA
ACATTCCGAAAGGAATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTG
CGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCT
GTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG
CCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTGAGACTAATACTCTTGGATAGTGGAC
GTTACTCGCASAATAARCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCG
TTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGAG
CTTAACTTGGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATA
CTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA
ACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGC
CTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATACTAGAAACTTTCCAGAG
ATGGATTGGTGCCTTCGGGAATCTAGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCAGCATTTCGGATGGGAACTTT
AAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGG
CCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGATACACAGCGATGTGATGCTAATCT
CAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAA
TCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
AGTCTGTTGC

Strain R2

ATTGCCCTTATCTTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCATTTATTGGGGGACAAC
ATTCCGAAAGGAATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCR
CTWAWTGATGAGCMTAAGTCGGATTAGCTAKTTGGTGGGGTAAAGGCCTACCAAGGCSACGATCTG
TARYGGGTSTGRGAGGATGATCCGCCACMCYGGGACTGAGACACGGCCCAGRMTCCTACGGGAGGC
AGCAGYGGSGAATATKGGACAATGGGMGGAGGCSTGATYCAGCCMYGCCSMRWGTGTGAAGAAGG
CCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGSTACTMTGATTAATACTCAGGGATRGTGGAC
GTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGC
GTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGA
GCTTAACTTGGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAYACCGATGGCGAAGGCAGCCATCTGGCCTAATA
CTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA
ACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGC
CTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTWTAATTSGATGCAACGCGAAGAACCTTACCTGSCCTTGACATACTAGAAACTTTCCAGAG
AKGGATTGGTGCCTTCGGGAATGCAGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCAGCATTTCGGATGGGAACTTT
AAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGG
CCAGGGCTTCACACGTGCTACAATGGTCGGTACAAAGGGTTGATACCGAGCGATCGGATGCTAATCT
CAAAAATCCGATCGTAGTCCGGATTGGAGTATGCAACTCGACTCCTTGAAGTCGGAATCGCTGGTAA

TCGCGGATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
AGTCTGTTGCACCAGAAGTATGTTGTGT

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