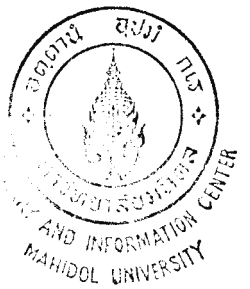


BIOREMEDIATION OF OIL CONTAMINATED GRAVEL

PRAPAIPIT TEPAROT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2001

ISBN 974-665-555-8

Thesis
entitled

BIOREMEDIATION OF OIL COMTAMINATED GRAVEL

Prapaipit Teparot
.....
Miss Prapaipit Teparot
Candidate

Attawut Impoolsup
.....
Dr. Attawut Impoolsup, Ph.D.
Major-Advisor

.....
Prof. Vithaya Meevootisom, Ph.D.
Co-advisor

.....
Asst. Prof. Prayad Pokethitiyook, Ph.D.
Co-advisor

.....
Prof. Liangchai Limlomwongse,
Ph.D.
Dean
Faculty of Graduate Studies

.....
Asst. Prof. Apinya Assavanig, Ph.D.
Chairman
Master of Science Programme
in Biotechnology
Faculty of Science

Thesis
entitled

BIOREMEDIATION OF OIL CONTAMINATED GRAVEL

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Biotechnology)
on
March 30, 2001.

Prapaipit Teparot
.....
Miss Prapaipit Teparot
Candidate

Attawut Impoolsup
.....
Dr. Attawut Impoolsup, Ph.D.
Chairman

.....
Prof. Vithaya Meevootisom, Ph.D.
Member

Charnwit Kositanont
.....
Asst. Prof. Charnwit Kositanont, Ph.D.
Member

.....
Asst. Prof. Prayad Pokethitiyook, Ph.D.
Member

.....
Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof. Amaret Bhumiratana, Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENT

I would like to express my deepest and sincere gratitude to my advisor, Dr. Attawut Impoolsup, for his valuable supervision, guidance, kindness, opportunity and encouragement throughout this study.

My special application is expressed to members of advisory committee, Prof.Dr. Vithaya Meevootisom and Asst. Prof. Dr. Prayad Pokethitiyook, for their valuable advices and comments. I am also very grateful to Asst. Prof. Dr. Charnwit Kositanont. And I wish to sincerely to thank all teachers of my previous studies too.

I am thankful to NSTDA for providing the scholarship to me during the first two years of studying in Mahidol University.

I would like to express my gratitude to the administrator of Bang Sue Diesel Locomotive Depot, which allowed me to study on the contaminated site. Without their kindness, all experiments in this study cannot be occurred.

I sincerely thank all members of BT110 room, all members in the Department of Biotechnology and my friends, who encourage me to perform the smooth experiments and for their helps and friendships.

The very grateful to Prof. Dr. Termchai and Mrs. Vanida Chainuvat, the President of Berlin Pharmaceutical Industry co.ltd., who gave the great opportunity to me for my studying in the University. I am greatly indebted to them.

Finally, my sincere and deepest gratitude is extended to my family for their infinite love, understanding, encouragement and care throughout my life.

PRAPAIPIT TEPAROT

4036476 SCBT/M : MAJOR : BIOTECHNOLOGY ; M.Sc. (BIOTECHNOLOGY)
KEY WORDS : BIOREMEDIATION / BIODEGRADATION / GRAVEL / LOCOMOTIVE DEPOT / MOTOR OIL / DIESEL OIL / USED OIL

PRAPAIPIT TEPAROT : BIOREMEDIATION OF OIL CONTAMINATED GRAVEL. THESIS ADVISORS : ATTAWUT IMPOOLSUP, Ph.D., VITHAYA MEEVOOTISOM, Ph.D., PRAYAD POKETHITIYOOK, Ph.D. 185 P. ISBN 974-665-555-8

Bang Sue Diesel Locomotive Depot area was contaminated with a high amount of used motor oil and diesel oil from cleaning, repairing and oil changing activities for locomotives. These used oils were released on the ground and spread over gravel for more than 10 years. An attempt to do bioremediation of used oil in this area was carried out. In the preliminary study of site assessment, average temperature was 30-32°C, average pH was 6.7-7.2, the amount of phosphate-phosphorous on gravel surface was 0.472 mg/l, the amount of nitrate-nitrogen was 0.068 mg/l, while, ammonia-nitrogen was unable to detect. The amount of used oil per kilogram of gravel was approximately 8.23 g (chloroform extraction method). The majority of bacteria isolated from oil degrading experiments belonged to genus *Pseudomonas*.

From the laboratory study in shake flasks, the enriched soil microorganisms, obtained by the enrichment method of contaminated soil sample were able to reduce the highest amount of used oil (60 % reduction in 30 days incubation). Whereas, the isolated microorganisms (Microorganism group I and group II) from the contaminated site were able to reduce a small amount of used oil (less than 5% reduction in 30 days incubation).

In the study of nutrient compositions for used oil bioremediation (10 g/l), it indicated that NH_4NO_3 (1.0 g/l), KCl (1.9 g/l) and 0.1 M sodium phosphate buffer pH 7 gave the maximum reduction of oil. The ratio of total petroleum hydrocarbon (TPH) : nitrogen (N) : phosphorous (P) was 100:3:1.

Bioremediation of oil contaminated gravel in trays was performed in two ways. Firstly, in solid-phase bioremediation, the degradation of used oil was very low (15% reduction in 6 months). However, the addition of nutrients was able to stimulate viable cell concentration (up to 10^7 CFU/ml). Secondly, in slurry-phase bioremediation, the degradation of used oil was better than solid-phase bioremediation (40% reduction in 6 months). However, the addition of inoculum was not necessary because the amount of indigenous soil microorganisms was sufficient to degrade this used oil. Effect of surfactant (2% w/v Sodium dodecyl sulfate, SDS) on bioremediation was observed in neither shake flask nor tray experiments.

4036476 SCBT/M : สาขาวิชา : เทคโนโลยีชีวภาพ ; วท.ม. (เทคโนโลยีชีวภาพ)

ประไพพิศ เทพารส : การศึกษาการย่อยสลายคราบน้ำมันที่ปนเปื้อนบนก้อนหิน (BIOREMEDIATION OF OIL CONTAMINATED GRAVEL) คณะกรรมการควบคุมวิทยานิพนธ์ : อรรถวุฒิ อิ่มพูลทรัพย์, Ph.D. วิทยา มีวุฒิสม, Ph.D. ประหยัด โภคฐิติยุกต์, Ph.D. 185 หน้า ISBN 974-665-555-8

จากการตรวจสอบแหล่งที่มีการปนเปื้อนน้ำมันบริเวณโรงรถจักรดีเซลบางซื่อ พบว่ามีการปนเปื้อนของน้ำมันเป็นบริเวณกว้าง เนื่องมาจากบริเวณนี้ใช้เป็นที่ในการทำความสะอาด ซ่อมแซม และ เปลี่ยนถ่ายน้ำมัน (น้ำมันดีเซล, น้ำมันหล่อลื่น) ของหัวรถจักร ซึ่งน้ำมันที่ใช้แล้วจะถูกถ่ายทิ้งลงดิน ด้วยสาเหตุนี้เองทำให้มีการปนเปื้อนของน้ำมันบนหิน ในดิน และตามแหล่งน้ำ เป็นบริเวณกว้าง ซึ่งเป็นระยะเวลานานนับสิบปี ดังนั้นจึงมีความพยายามในการที่จะหาวิธีลดน้ำมันที่ปนเปื้อนในบริเวณนี้ โดยการศึกษาเริ่มต้นจากบริเวณที่มีการปนเปื้อน พบว่าอุณหภูมิโดยเฉลี่ยอยู่ในช่วง 30-32°C, pH โดยเฉลี่ยอยู่ในช่วง 6.7-7.2, ปริมาณสารอาหารบนก้อนหินที่ปนเปื้อนด้วยน้ำมัน ในรูปฟอสเฟต-ฟอสฟอรัสคิดเป็น 0.472 มก./ลิตร, ในรูปไนเตรต-ไนโตรเจนคิดเป็น 0.068 มก./ลิตร ส่วนแอมโมเนีย-ไนโตรเจน ไม่สามารถตรวจสอบได้ และปริมาณน้ำมันโดยเฉลี่ยที่ปนเปื้อนต่อก้อนหิน 1 กิโลกรัมคิดเป็น 8.23 กรัม ส่วนการศึกษาเชื้อที่มีความสามารถในการย่อยสลายน้ำมัน ในบริเวณนี้พบว่า เชื้อที่แยกได้ส่วนใหญ่ เป็นกลุ่มเชื้อในสกุล *Pseudomonas*

ผลจากการศึกษาในห้องปฏิบัติการใน shake flask เพื่อหากลุ่มเชื้อที่มีความสามารถในการย่อยสลายน้ำมันที่ปนเปื้อนได้ดีพบว่ากลุ่มเชื้อที่ได้จากการเพิ่มจำนวนเชื้อจากดินจากบริเวณที่มีการปนเปื้อนสามารถย่อยสลายน้ำมันชนิดนี้ได้ดีที่สุด(60 % ในระยะเวลา 30 วัน) และจากความพยายามในการแยกกลุ่มเชื้อเหล่านี้ในห้องปฏิบัติการ และนำมาเลี้ยงกลับเพื่อศึกษาการย่อยสลายน้ำมัน พบว่าความสามารถในการย่อยสลายน้ำมันมีค่าต่ำมาก(น้อยกว่า 5% ในระยะเวลา 30 วัน) และจากการศึกษาผลของส่วนประกอบของอาหารเลี้ยงเชื้อต่อการย่อยสลายน้ำมันที่ปนเปื้อน (10 กรัม/ลิตร) พบว่า แอมโมเนียมไนเตรต (1.0 กรัม/ลิตร), โปแตสเซี่ยมคลอไรด์ (1.9 กรัม/ลิตร) และ 0.1 โมลาร์ โซเดียมฟอสเฟตบัฟเฟอร์ pH 7 สามารถลดปริมาณน้ำมันที่ปนเปื้อนได้มากที่สุด โดยที่อัตราส่วนของน้ำมันที่ย่อยสลายได้ ต่อไนโตรเจน ต่อฟอสฟอรัส คิดเป็น 100:3:1

ส่วนการศึกษาการย่อยสลายน้ำมันที่ปนเปื้อนบริเวณรอบๆ ก้อนหินใน tray โดยวิธี solid-phase พบว่าการย่อยสลายน้ำมันเกิดขึ้นน้อยมาก (ประมาณ 15% ในระยะเวลา 6 เดือน) อย่างไรก็ตามได้พบว่าการเติมสารอาหารที่จำเป็นให้แก่เชื้อ สามารถเพิ่มจำนวนเชื้อได้มากขึ้น ส่วนการศึกษาโดยวิธี slurry-phase พบว่าการย่อยสลายน้ำมันมีประสิทธิภาพมากขึ้น (ประมาณ 40% ในระยะเวลา 6 เดือน) และพบว่าจำนวนเชื้อดั้งเดิมโดยรอบก้อนหินที่ปนเปื้อนมีปริมาณมากเพียงพอ (10^4 CFU/มล.) ดังนั้นการเติมเชื้อเพื่อช่วยเพิ่มประสิทธิภาพการย่อยสลายน้ำมันที่ปนเปื้อนจึงไม่มีผล

ในแง่ของการศึกษาผลของสารลดแรงตึงผิวต่อการย่อยสลายน้ำมัน พบว่า การเติม 2% (w/v) Sodium dodecyl sulfate (SDS) ไม่สามารถช่วยเพิ่มความสามารถในการย่อยสลายน้ำมันชนิดนี้ได้ ทั้งในการศึกษา ใน shake flask และ tray

CONTENTS

CONTENTS (continued)

CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

FIGURE **Page**

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

BH	Bushnell-Hass
C	carbon
CFU	colony forming unit
CRD	complete randomized design
°C	degree celcius
ENS	enriched soil microorganisms
et al.	et alia (Latin), other people
etc.	et cetera (Latin), other things
e.g.	exampli gratia (Latin), for example
Fig.	figure
GC	gas chromatography
g	gram
h	hour
HC	hydrocarbon
i.e.	id est (Latin), that is
IS	internal standard
kg	kilogram
l	liter
mta^{-1}	megaton per area
µl	microliter
mos.	microorganisms

LIST OF ABBREVIATIONS (continued)

µV	microvolt
mg	milligram
ml	milliliter
mm	millimeter
mmHg	millimeter mercury pressure
mV	millivolt
min	minute
M	molar
nm	nanometer
N	nitrogen
N	normal
n-	normal
ND	not determined
no.	number
OD	optical density
ppm	part per million
/	per
%	percent
P	phosphorous
PAHs	polyaromatic hydrocarbons
K	potassium
psi	pounds per square inch

LIST OF ABBREVIATIONS (continued)

rpm	revolution per minute
sec	second
cm^2	square centrimeter
sec^2	square second
TPH	total petroleum hydrocarbon
vs	versus
v/v	volume by volume
w/v	weight by volume

CHAPTER I

INTRODUCTION

Petroleum pollution was concerned as a major environmental problem in the early 1980s (1). In Thailand, which began large scale productions, transportation, storage and use of petroleum products, in addition, with underground storage tanks, service stations, industrial sites, refineries and businesses maintaining the automobile, so the spillage of these petroleum products easily be occurred (2). These problems can cause many adverse effects to human health, limit plant growth, animal and microbial activity (3).

There are three main methods to eliminate petroleum waste contamination, including physical, chemical and biological methods (2). Physical and chemical methods are often successful in removing hydrocarbon from the contaminated site, however, these methods cannot eliminate these pollutants.

Microbial decontamination, referring to "bioremediation", of petroleum waste contaminated site is known to be an efficient, economical and versatile alternative to physical and chemical treatments. Bioremediation involves the use of indigenous or introduced microorganisms to detoxify the contaminant (4,5), moreover, the important factors such as nutrients, pH, temperature etc. also influence the rate of bioremediation (6,7). Thus, it is essential to study these effects clearly before site applications.

Each contaminated site offers a unique set of parameters within, which any potential remediation must operate. Site contamination generally consists of solid (soil and sludges) and liquid (ponds and groundwater) classes of material requiring

treatment. Bioremediation technologies for these contaminated materials fall into three main categories: (1) solid-phase bioremediation, (2) slurry-phase bioremediation and (3) liquid- phase bioremediation.

When considering one of these biological processes for remediation of waste contaminated site, it is important to obtain information indicative of the potential for success prior to undertaking full-scale efforts. Laboratory treatability testing generates data to evaluate the applicability of a process for a particular site and identifies proper parameters for implementation (8). So this study was conducted to evaluate those important data for site bioremediation of oil contaminated gravel at Bang Sue Diesel Locomotive Depot, which heavily polluted with used petroleum products released from maintenance and service processes into soil, and gravel for more than 10 years. The objectives of this study were as follows;

- To evaluate different treatment methods for bioremediation of oil contaminated gravel in laboratory.
- To select for potential microorganisms, which are effective for used oil degradation at the depot.

CHAPTER II

LITERATURE REVIEW

The world demand for oil is enormous, at a level of 3.2×10^9 tons per year, which represents around 39% of world's commercial energy demand. This demand increases every year. The increasing use of the oil has had to an increase in the probability of major spillages. The Middle East, major producer, accounts for 28.6% of world production and 66% of proven reserves. Oil transportation from the points of production to purchasers are performed in tankers, which are frequently of vary considerable size. Over one-third of the present tanker fleet (between 400 and 460 vessels) has a capacity more than 250,000 tons and over 60% has a capacity more than 100,000 tons. These tankers are too large to follow the older trade routes (e.g. through the Suez Cannel). In the case of tankers heading to wards Western Europe, they took routes along the African coast from the Middle East, around the Cape of Good Hope up through the Atlantic. In most cases the tankers follow a minimum depth contour (e.g. 200 m), which frequently takes them relatively close to the coastlines. It is therefore not surprising that most tanker accidents result in widespread coastal pollution, which subsequently received a great deal of public attention (3).

2.1 Hydrocarbon contamination in the environment

2.1.1 Hydrocarbon contamination in marine ecosystem

There are many direct and indirect ways in which oil can reach the marine environments (Table 2-1), the highest amount of oil to the marine environment came from transportation. The largest tanker spill in Europe was probably that from the *Amoco Cadiz* of Brittany, France, in 1978, which lost about 227,000 tons (9,10). In 1989, the *Exxon Valdez* discharged 38,000 tons into Prince William Sound, Alaska (11). Eight years of the oil spill, intensive shoreline clean up had been conducted such as using high-pressure, hot-water washing followed by nutrients addition. In 1990 surface residues were manually removed, and berm relocation implemented at 25 sites. In 1991, there was limited manual removal of surface residues, a large-scale storm berm relocation project along 2 km of gravel beach at Point Helen, and mechanical removal of oil sediments near salmon spawning streams. In 1997, clean up of oil residues were conducted using a shoreline cleaning agent at sites important to the people of Chenega Bay Village. However, the persistence of subsurface oil in some beaches still remained (12,13,14). In 1991, as a result of Gulf War, the marine environment in the Arabian Gulf was subjected to an estimated 1,000,000 tons of crude oil making it the largest oil spill ever record and its effects still remain until now (15).

Table 2-1 Sources of oil to the marine environment (3)

Source	Estimate (mta^{-1})	Total (mta^{-1})
Transportation		1.47
Tanker operations	0.7	
Tanker accidents	0.42	
Bilge and Fuel oils	0.3	
Dry docking	0.03	
Non-tanker accidents	0.02	
Fixed installations		0.17
Coastal refineries	0.1	
Offshore production	0.05	
Marine terminals	0.02	
Other sources		1.38
Manicipal wastes	0.7	
Industrial waste	0.2	
Urban-run-off	0.12	
River run-off	0.04	
Atmospheric fallout of oil-derived	0.3	
Ocean dumping	0.02	
Natural inputs	0.25	
Total		3.27

In Asian region, in the night of 8 November 1973, between 2,000 and 3,000 tons of heavy marine diesel oil, with an aromatic content of about 40% and high sulphur content, were spilled into the East Lumma Channel, Hong Kong (16). The early spillage of oil in Asia occurred in 1997, a Russian tanker, the Nakhodka, broke apart and sank in the Sea of Japan. The bow-half of the ships, which floated for about five days and released approximately 5,000 tons of heavy oil, ran aground on the shelf near the coast of Mikuni-town, Fukui prefecture, while the stern-half fell to the sea floor at the depth of 2,500 m. The tanker carried about 19,000 tons of heavy oil which be continuously leaking (17,18).

Once crude oil is released onto the sea surface a number of different processes immediately begin to act on it. These influence its composition and environmental toxicity i.e. evaporation, dissolution and advaction, dispersion, photochemical oxidation, emulsification, adsorption onto suspended particulate material, biodegradation and sedimentation (Fig. 2-1). Each of which has different effects on oil. In addition, the action of surface waves and currents drive the oil slick away from its point of release and cause many problems to the environment (3,19).

2.1.2 Hydrocarbon contamination in freshwater

Compared to the marine situation little work has been done on the effects of oil in freshwater ecosystems. Nevertheless the chronic pollution of freshwater with hydrocarbons is widespread. Much of it derives from petrol and oil washed from roads together with the illegal discharge of engine oil. Other sources include boats and irrigation pumps, while accidents involving transpotations and spillages from storage tanks are also significants (3).

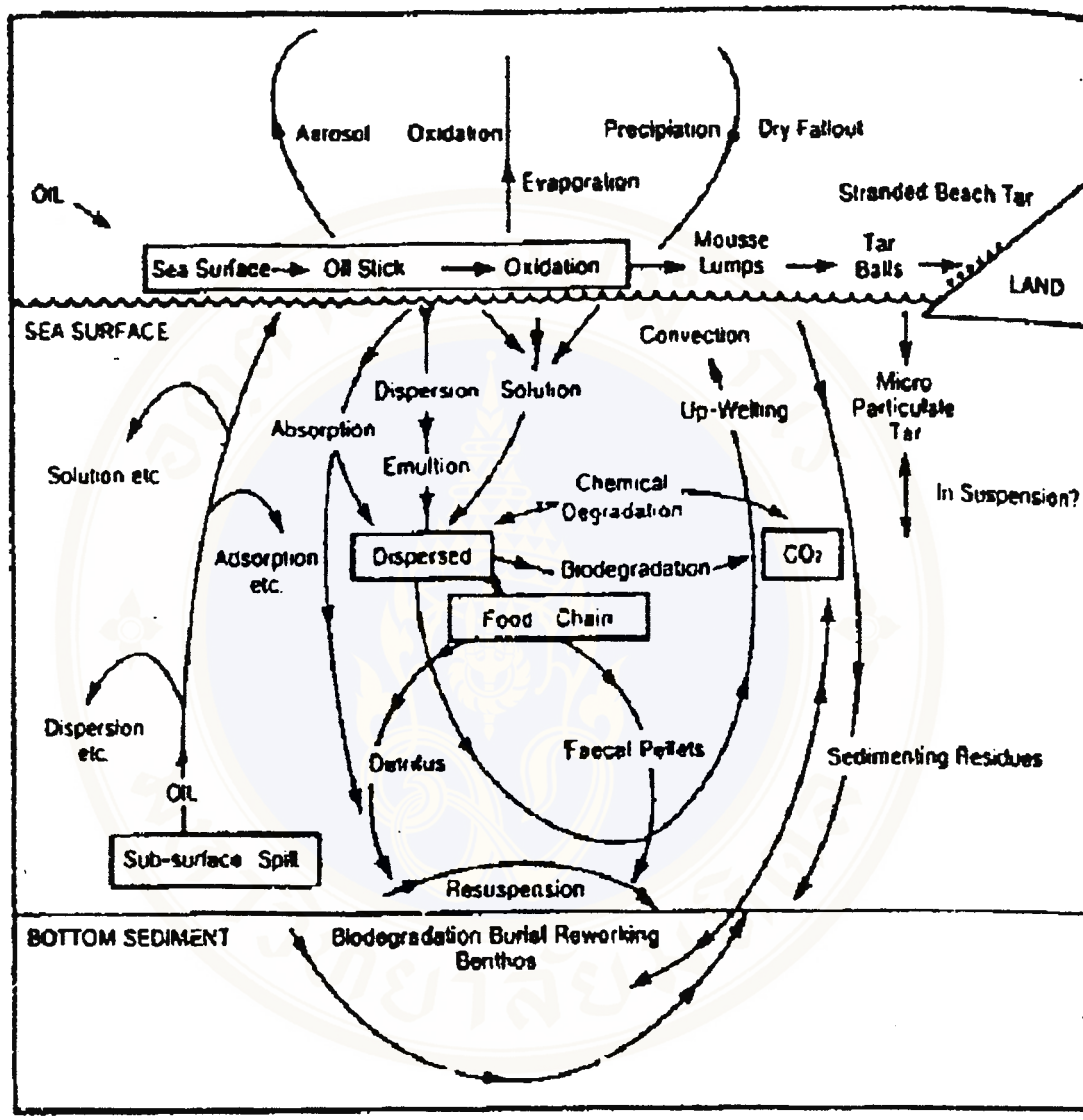


Fig.2-1 Distribution and elimination of crude oil in aquatic environment (19)

Freshwater oil spills have received little attention in the United States, however these are listed as one of the leading causes of fish kills in Missouri. A broken pipeline near Ethel, Missouri, USA caused approximately 451,000 liters of light crude oil to spill into a small tributary, creating an oil slick on the lower 90 km. of the Chariton River (20).

2.1.3 Hydrocarbon contamination in soil and groundwater

Soil is an essential component of terrestrial ecosystems because the growth of plants and biologeochemical cycling of nutrients depend on it. Soil is vitally important for production of food and fiber crops and it is therefore essential that the total productive capability of the world's soil is not impaired. Pollution along with other types of degradation such as erosion and the continuing spread of urbanization, poses a treat to the sustainability of soil resources. Soil pollution can also be a hazard to human health when potentially toxic substances move through the food chain or reach groundwater used for drinking water supplies (3).

Soil and groundwater are often contaminated with petroleum products due to accidental released or improper disposal activities. About 8.8 million metric tons of oil are spilled on land each year (21). Many of the individual hydrocarbon constituents found in petroleum products are considered potential carcinogens (22). Fig. 2-2 gives a representation of the various modes in which hydrocarbons can distributed through a soil and groundwater.

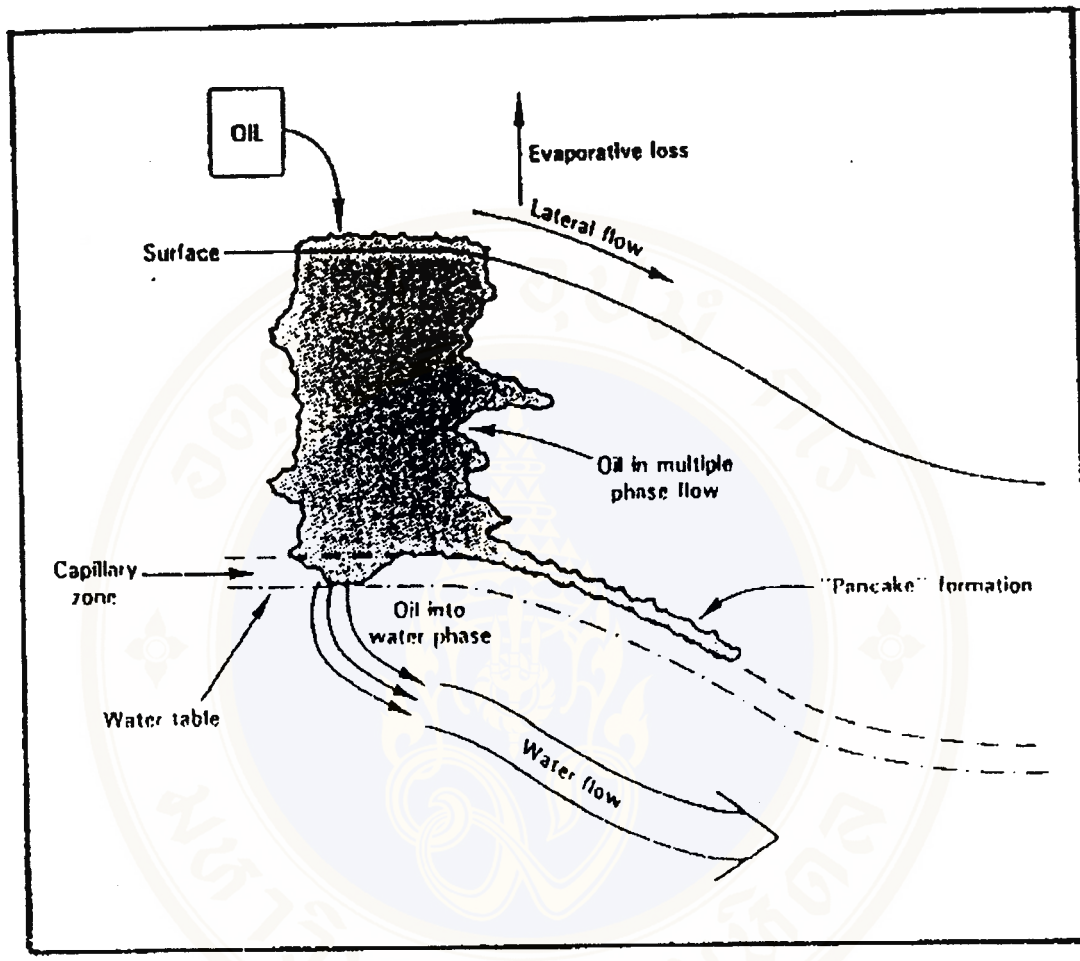


Fig. 2-2 Distribution of crude oil in soil and groundwater (3)

Sources of hydrocarbon contamination to soil and groundwater

A: Fuel storage and distribution; Leaking underground storage tanks and spillages at distribution depots and from road accidents can lead to pollution of soils and aquifers with petrol and diesel fuels, for example oil spill incidents in the North of Sao Paulo coast, Brazil. From here, an important oil terminal (TEBAR: Terminal Almirante Barroso) is located, which is responsible for the storage and distribution of approximately 55% of the oil used in the country. Oil spill incidents caused by tankers as well as pipelines are frequently occurred in this area (23).

B: Disposal of used lubricating oil; In addition to hydrocarbons and abraded particles of metals, old lubricating oil contained polyaromatic hydrocarbons (PAHs) and other products of partial combustion. Some domestic gardens, land around motor repair garages, farm yards and sites of crashed and vandalized cars can often be polluted with this material (3). The production of lubricating oil in the Western countries corresponds to 1-2% of the total amount of refined petroleum. Combustion losses and oil leaks from the engine during use may represent 35% of the oil consumed. In 1980 more than 9 million tons of used motor oil was produced in the United States, and only one third was able to recover. Therefore, it appears that most used motor oil, several million tons, is dispersed to the environment each year (22).

C: Leakage of solvents from industrial sites; Hydrocarbon solvents are used widely in industry for cleaning and decreasing metals and electrical components, and leakages (from storage, distribution and use) frequently result in contamination of soils and aquifers (3). Straube and Deschenes reported that the Southern Maryland Wood Treatment site contains high levels of creosote wastes, which consist primarily (85%) of PAHs. Soils under unlined waste storage lagoons are particularly affected (24,25).

D: Coal stores; Coal is a solid form of hydrocarbon and main hazard associated with it is the risk of fires. Sites of coal stores at former industrial sites and distribution depots are the most likely to contain significant amounts of coal, which could constitute a combustion hazard. An example of soil pollution by coal is the result of bad practices of waste management of residuals from old manufactured gas plants or other similar industries (3). Coal tar is a by-product of coal pyrolysis process, which was used to synthesis main gas until around 1950 or coke for the metal industry. During the operating period of these plants, past of the stored coal tar was spilt (voluntarily or accidentally) on the ground. Due to its low solubility in water and its resistance to biodegradation, coal tar remained in the soil or migrated into the sub-soil and became a potential source of groundwater pollution (26). Moreover, during the incomplete combustion of coal, PAH-bearing particles were transported in the atmosphere and delivered to soil by atmospheric deposition (27,28).

2.1.4 Hydrocarbon contamination in Thailand

Oil spills in Thailand during 1973 to 1998 are catagories in Table 2-2, there were 87 evidences of oil spill. The highest frequency was occurred in 1995 and the highest amount of oil spill was occurred in 1973, more than 2,000 tons of tar, diesel and J.P4 oil released into Chaopraya River (2). More than 50% of the spillages oil were heavy oil including tar oil, lubricating oil and crude oil (Table 2-3).

The serious cases mostly occurred by accidents while the other spillages of oil less than 7 tons mostly occurred by manicipal and industrial wastes. The contaminated area mostly occurred in the river, especially Chaopraya River. In case of marine environment, the Northern of Gulf of Thailand including Chaopraya Delta was frequently recorded for oil spill (Table 2-4) (29).

Table 2-2 Causes and amounts of oil spill in Thailand during 1973-1998 (29)

Causes	< 7 tons	7-700 tons	> 700 tons	unknown	Total
Boat accidents					
Crashed	-	4	1	2	7
Sang	1	1	-	2	4
Fired	4	1	-	2	7
Boat activities					
Oil filling	4	-	-	-	4
Oil replacement	5	1	-	6	12
Illegal oil disposal	4	-	-	15	19
Manicipal and Industrial waste	9	-	-	3	12
Others	1	1	-	20	22
Total	28	8	1	50	87

Table 2-3 Types of spillage oil in Thailand during 1973-1998 (29)

Types	Percent (%)
1.Heavy oil	
1.1 Crude oil	9.20
1.2 Tar oil	50.57
1.3 Lubricating oil	10.35
2. Light oil	
2.1 Diesel oil	12.64
2.2 J.P 4 oil	2.30
3. Others	14.94
Total	100.00

Table 2-4 Sites of oil contamination in Thailand during 1973-1998 (29)

Area	Frequency
1. River	
1.1 Chaopraya River	52
1.2 Bangpakong River	1
1.3 Nan River	1
2. Sea	
2.1 Northern section of Gulf of Thailand	28
2.2 Southern section of Gulf of Thailand	1
2.3 Andaman Sea	4
Total	87

2.2 Effects of hydrocarbon contamination

The effects of hydrocarbon contamination may be acute or chronic in nature. Acute effects on the environment are those that result from a single infusion of oil into the marine environment from an accidental soil. Chronic effects are those that occurred from the release of oils or its derivatives either continuously or sufficiently. The contaminated area does not have time to recover between doses (30). These effects will be discussed in more detail in three main catagories.

A: Physical effects

For example if a coating of oil is fairly thick on the surface of the water, it can interfere the re-aeration of the body of water at the air-water interface. Moreover, photosynthesis may be interfered with (31). Oil trapped in sandy beaches persisted for several months. Sedimentary transport processes can move the oily particles away from the region of initial contact, thus extending them along the shore. This can cause many following problems in biological and economical effects (30).

B: Biological effects

There are various levels of biological effects of oil. At various places in the environment and at various times these will be accorded different priorities in the evaluation of the impact. These effects include the possibility of :

1.Human hazard through inhalation, ingestion and dermal absorbtion of contaminants: (32)

Used motor oil contains carcinogenic substances such as PAHs. Since most of the PAHs dissolved in petrol are not emitted in the combustion gas but accumulate in the oil (22,33). Moreover, the used motor oil also contains a high level of heavy metals. Some metals disrupt biochemical reactions while others block essential biological

processes, including the adsorption of nutrients. Some accumulate in the body, giving rise to toxic concentrations after many years of exposure (3).

2. Damage of wildlife such as seabirds, mammals and fisheries resources; Oil films on the surface of natural bodies of water hinder the respiration of surface-breathing organisms. In addition, the gills of underwater-breathing organisms may become coated with oil. These oil coatings prevent satisfactory transfer of oxygen from the water into the blood of aquatic animal and passage of the carbon dioxide from the blood of animal into the surrounding water (3,30). Joints of rapidly moving aquatic animals may be come clogged and caused difficulties to get food and to avoid predators (34). Unicellular algae are often damaged by oil and will sink to the bottom. Biological decomposition of bottom detritus is hindered by the action of oil on the sludge (31). The diving birds badly affected by oil. Death through drowning, hypothermia or toxic effects of ingested oil (35).

In case of oil pollution in soil, input of oil into soil could limit microbial activity, plant growth and animal activity. The low-boiling components of oil exhibited a high degree of contact toxicity to the tender portion of plant roots and shoots because of the toxicity to lipid membrane, but they have little effect on the woody parts of plants (36). In the vertebrates, the water soluble components of oil were toxic to amphibian. These components entered the gill or skins by passing through cellular membranes and then accumulated in liver and gall bladder (37).

3. The destruction of marine organisms along the shore was enormous. Lobster, crabs, sea urchins, starfish, sea cucumbers, gastropods of all sorts as well as a variety of fishes were killed. These can cause the modification of the ecosystem by elimination of species with an initial decrease in diversity and productivity (30,31).

C: Economical effects

For the spillages of oil into marine ecosystem, the oil was carried ashore by wind and currents. Coral reefs off the shore were blanketed with a thick layer of oil. From the physical point of view, there was heavy erosion of the beaches from a combined action of the waves and oil. Sand was washed away in enormous quantities from the beaches, the sand seemed to form small spherical masses with oil. Some of the oil was carried into mangrove swamps. So economic damage in the area was widespread and severe such as decreasing of aesthetic value, fisheries resources and economical aquatic animal resources. Moreover, lost lots of money and time to decontaminated or reduced these contaminants (31).

2.3 Factors influencing the success of hydrocarbon bioremediation

When bioremediation of hydrocarbon contamination is being considered, it is important to study early about the site characterization such as the characteristic of contaminant, microbial population of the subsurface site, which is vary depend on the type, amount and age of the contaminant. Moreover the prevailing environmental condition such as subsurface temperature, pH, nutrients, percent moisture and dissolved oxygen are very important too (38). Not only site characterization is important for the successful of hydrocarbon degradation but bioprocess optimization of these conditions having the greatest influence on the biodegradation process also (39,40). Bioremediation rate is enhanced by achieving the optimum oxygen level, moisture content, the amount of available nutrients such as nitrogen, phosphorous and some trace elements, contaminant concentration, pH, temperature, and contact

between the appropriate microorganisms and the contaminants (41). The greater detail are described below :

2.3.1 Characteristics of hydrocarbon compounds

The difficulty of establishing the biodegradability of hydrocarbon contaminants depends on the specific chemical compounds, their physical and chemical properties, and the matrix in which they are present (as described in Section 2.4.1). Compounds readily biodegradable under one set of condition may persist under different the conditions (42).

The chemical structure is one of major factors governing the biodegradation rate for mixed molecular weight contaminants, such as petroleum fuels. As a general role, the more complex an organic compound, the more difficult that compound is to biologically degrade (43). Therefore, biodegradation of heavier molecular weight compounds required longer period of times. For example, monoaromatic compounds (e.g. benzene, alkyl benzene) are easily biodegradable due to their high water solubility. The biodegradability of straight-chain (n-alkanes) and branched alkanes (isoalkanes) depends on the carbon chain length and the degree of branching. The biodegradation half-lives of naphthenes, PAHs, and polar compounds increase with an increasing number of rings. Two and three ring compounds are biodegraded relatively fast, while saturates and aromatics with more than four rings may be considered recalcitrant to biodegradation in the time frame of the treatment process (Fig. 2-3) (39,44,45).

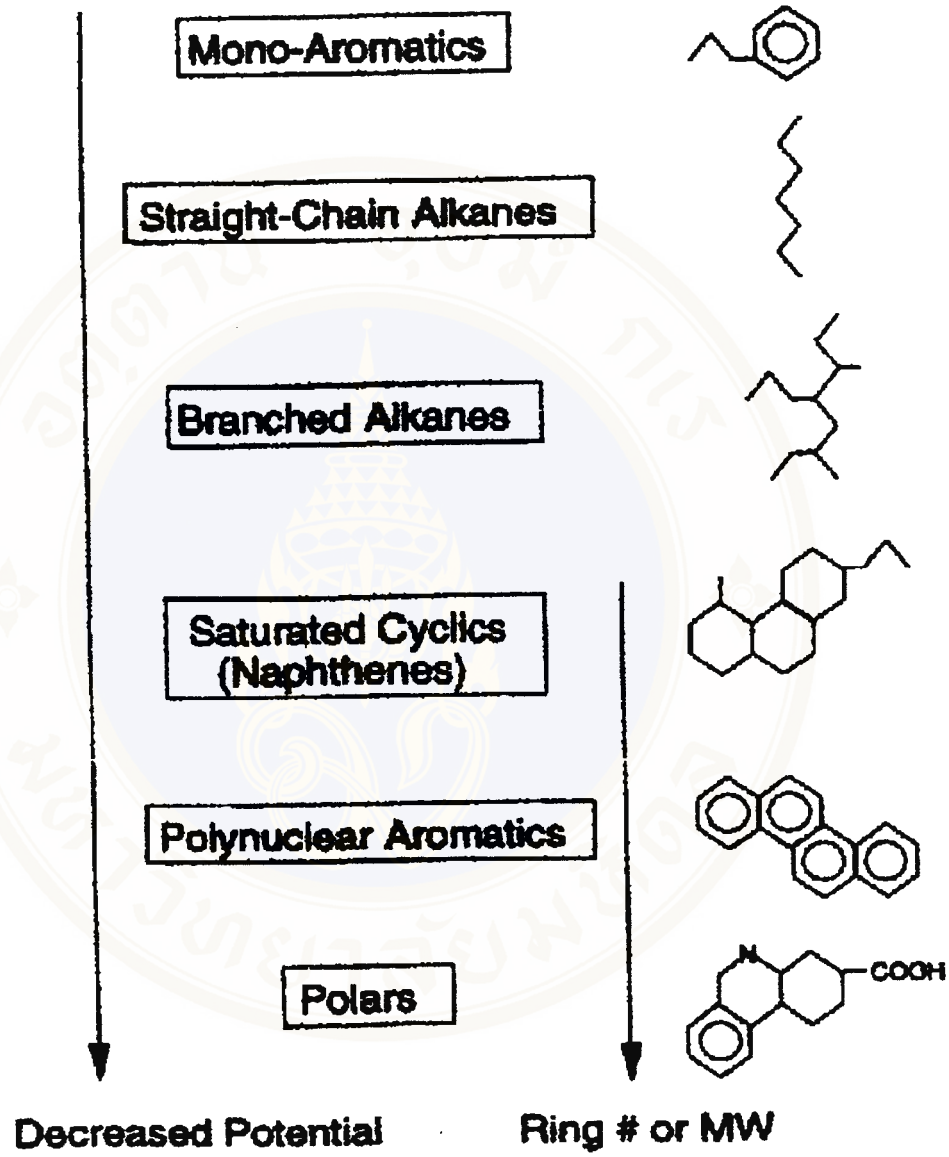


Fig.2-3 Biodegradation potential of hydrocarbon compounds (44)

2.3.2 Microbial type and number ; The microbial decontamination of hydrocarbon pollution is known to be an efficient, economical and versatile alternative to physical and chemical treatment (5). More than 70 microbial genera are known to contain organisms that can degrade petroleum components (19) such as *Acinetobacter* sp., *Artrobacter* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Candida* sp.(46), filamentus fungi (47) and white rot fungi (48) etc. These organisms naturally be occurred in the polluted sites as an indigenous soil microorganisms or are inoculated into the polluted sites to stimulate the degradation of pollutants. Since not every type of microorganism can degrade all type of pollutants. It is essential that the right type of microorganisms are present. It is possible to inoculate the polluted site with specific bacteria strains. However, there are many practices has shown that the right type of bacteria is naturally occurring in the site and that introducing specific environmental conditions, activates these microorganisms so that they will be increase in number and start degrading the contaminants (49).

Rojas-Avelizapa *et al.* (1999) found that naturally occuring aerobic microorganisms in soil have the potential to attract polychlorinated biphenyls (PCBs). The natural microorganisms, which was used in the mixed culture were *Pseudomonas acidovorans*, *Acinetobacter calcoaceticus*, *Achromobacter* sp., *Pseudomonas* sp., *Flavobacterium devorans*, *Bacillus lentus*, *Bacillus mascerans* and *Bacillus thuringiensis* (50). Margesin and Schinner (1997) studied the bioremediation of two diesel oil-contaminated alpine soil. They found that 60-50% of the residual oil could be reduced during 155 days of incubation and the addition of cold adapted diesel oil degrading inoculum enhanced biodegradation rate only slightly and temporarily. They concluded that fertilization with inorganic nutrients significantly enhanced diesel oil

degradation activity of the indigenous soil microorganisms rather than the bioaugumentation with the psychrotrophic inoculum (4,5). As the same study of Hinchee *et al.*(1996), they reported that a soil contaminated with petroleum hydrocarbon and heavy metals had sufficient indigenous microbial activity for hydrocarbon biodegradation under non-limiting conditions (51).

However, there are some studies against these studies. Hozumi *et al.* (2000) studied the bioremediation on the shore after an oil spill from the Nakhoadka in the Sea of Japan by microbial culture (TerraZyme^{TM}). They found that the seeding of microbiological cultures with TerraZyme^{TM} contributed to enhancing biodegradation of asphaltum in the heavy oil samples. TerraZyme^{TM} is made up of various microbiological complexes, which have been collected from many different geographical localities under different environmental conditions, shown extensive clean up impacts on oil pollution under various environmental conditions (17). Webber and Corseuil (1994) also studied the different between the indigenous and the inoculated microorganisms (enrichment from many contaminated site) for the degradation of the sand column experiments, with high contamination with BTEX (benzene, toluene, ethylbenzene and xylene). They found that the degradation of target compounds by the inoculated microorganisms decreased much more rapidly than the indigenous populations. They suggested that if the density of the specific microbe is increased, the degradation rate will be increased also (52). Similar results have been reported from the study of inoculum sizes required for mineralization of *p*-nitrophenol in lake water by Ramadan *et al.* (1990), for which initial density of about 10^5 CFU/ml was needed to prevent microbial elimination by protozoan grazing or failure to survive because of nutrient deficiencies (53). The important of these problems are about

microbial number at the polluted site, soil that has favorable microbial growth conditions, metabolically suitable microorganisms, and a ready supply of biodegradable substrate will typically exhibit viable microbial number of 10^4 CFU/g of dry-weight soil or greater. Soil that is unfavorable to microbial life may have very low viable cell number (less than 10^2 CFU/g of dry-weight soil), in this case addition of inoculum may be necessary (42,54). Another reason is the competition between the indigenous microorganisms and the inoculated microorganisms. Macnaughton *et al.* (1999) found that the inoculated bacteria did not compete favorably with the indigenous bacteria community, even though it was originally derived from a nearby contaminated site. This may be the explanation for the ineffectiveness of the inoculum (55). As the same suggestion of Atlas (1995), he said the biodegradation of oil pollutants in marine environment by seeding will be effective, seed cultures must compete with indigenous hydrocarbon-degrading microorganisms (56).

Another suggestion for using microorganisms for hydrocarbon decontamination is using the mixture of hydrocarbon-degrading population. Korda *et al.* (1997) said petroleum hydrocarbon contamination usually exists as a complex mixture of hydrocarbons. Despite, the huge potential of microorganisms to degrade this compounds under favorable conditions. No single species of microorganisms can degrade all the components of a given oil and no oil degrading 'superbug' has been engineered. Currently, several organisms are known, each capable of degrading usually one or at best, a few petroleum components at a time. Therefore, effective bioremediation of petroleum contamination requires a mixture of populations consisting of different genera each capable of metabolizing the respective compounds (57). Lal and Khanna (1996) also reported that a faster rate of degradation of crude oil

is achieved by the action of a combination of microorganism with different hydrocarbon degrading capabilities rather than by a single versatile organism with the capability to degrade both alkane and aromatic compounds (58). However, bioremediation by microorganisms depend on the 'threshold level' of organisms, reported by Paul *et al.* (1994). That is maximum reduction of contaminant was related to the 'threshold' level for a specific microbial system. Below this level, organisms can not obtain sufficient carbon for growth even though may be available to support the viability of individual cells. If this threshold level is below the cleanup requirements, then bioremediation might be favored. If the threshold level is above the requirements, biological treatment might not be appropriate. The threshold level is highly dependent on a number of organisms-specific and contaminant-specific features (8). Anyway, microbial types and number are not the entry directed indicative of microbial activity or degradation rate.

2.3.3 Nutrients

Bacterial cells require a source of nitrogen (N) and phosphorous (P) in order to carry out petroleum hydrocarbon biodegradation. During the biodegradation process, microorganisms incorporate carbon (C) from the contaminant source together with inorganic N and P from the soil into their cell structures. The C:N:P ratio of bacterial cell is approximately 100:20:1. The amount of N or P necessary for stimulating hydrocarbon biodegradation is less than these theoretical cellular requirements because at all carbon from the contaminants is incorporated into biomass (i.e., fraction is converted into CO_2 and a significant amount of cellular (organic) N is recycle when microbial cell die.

A wide range of C:N and C:P ratio have been reported in the literature. While Frankenberger recommends a C:N:P ratio of 100:10:1, Dibble and Bartha found optimal oil sludge biodegradation with C:N and C:P ratio of 60:1 and 800:1, respectively. By contrast, Brown *et al.* reported that a C:N ratio of 9:1 was optimal for refinery sludge biodegradation, and Huddleston *et al.* suggest that the C:N ratio should be maintained between 25 and 38. Finally, Morgan and Watkinson reviewed numerous biodegradation studies and found that optimal C:N ratios between 9:1 and 200:1 had been reported for waste oil sludges. The apparent discrepancies of optimal C:N:P ratios is most likely the result of differences in waste type, biodegradation kinetics, background concentrations of soil organic N, the rate of nitrogen recycle and other experimental conditions (44). However, there are some confusion in the literatures regarding the limitation of petroleum hydrocarbon biodegradation by available concentration N and P. Several researchers reported that the addition of N and P can increase the hydrocarbon degradation (51,59,60,61,62), but the others, found that it can also have no effect on the biodegradation rate (6,63,64). The greater detail can be described below.

Swindoll *et al.* (1988) found that addition of inorganic nutrients resulted in a more rapid adaptation to the test substrate and a higher rate of metabolism may have been limited by these nutrients. In general, the addition of multiple inorganic nutrients resulted in a greater enhancement the degradation than did the addition of single substrances (61).

Rematad and Sveum (1996) examined the fate and effect of various nitrogen sources in oil-contaminated sediment in a continuous flow seawater column system fed with nutrient enriched sea water. Degradation of oil component is stimulated by a

supply of an enhanced concentration of nitrogen. The most pronounced effect was found with nitrate, compared to ammonium and organic nitrogen. Ammonium was more readily sorbed by the sediment system, either by chemical adsorption or by microbial immobilization (51). However, Walworth (1995) said ammonium is the preferred N source because it is in the reduced form that is biochemically required, whereas nitrate must reduced by organisms prior to incorporation into amino acid and related compounds (62).

Jobson *et al.* (1974) studied the effect of amendments on the microbial utilization oil adapted to soil. They found that application of fertilizer (Urea-phosphate: 27:27:0) resulted in a stimulation of bacterial numbers and in the rate of utilization of n-alkanes components of the substrate fraction (60).

Fedorak and Westlake (1981) found that the N and P addition not only increases the extent of microbial degradation of n-alkanes, but also demonstrates that degradation of many aromatic compounds can be detected (59).

Li *et al.* (1994) studied the bioremediation of a fire-area with the Bunker C oil contaminated soil. They separated the area into 18 plots to test 6 bioremediation methods (a: Aeration with basic nutrients and indigenous organisms, b: Aeration with basic nutrients and inoculation from a refinery waste water treatment facility, c: Aeration with an oleophilic fertilizer and indigenous organisms, d: Aeration with basic nutrients and biosurfactant organisms, e: Aeration with proprietary nutrients and organisms and f: Aeration only for active control). They found that no significant difference for the oil reduction rate among these treatments (6).

Oudot *et al.* (1998) studied the influence of the addition of a slow release fertilizer on the biodegradation rate of crude oil in experimental plots set up in the

mid-tide sediments of an estuarine environment in the Bay of Brest, France. They found that no significant difference in the biodegradation rate between fertilized and non-fertilized plots, which was attributed to the high background level of N and P in the site under study. They suggested that if background level of N in the interstitial pore water of the sediment is higher than 100 μmoles/l then bioremediation though fertilization may be of limited use only (63). Li *et al.* (2000) also found that the amount of N and P contents of the polluted soil typically 0.1% was sufficient to sustain natural or enhanced biodegradation (64).

2.3.4 Temperature

Microbial growth rate is a strong function of temperature. The variation with temperature of hydrocarbon degradation rate in the natural environment depends upon the relative quantities of psychrophilic, mesophilic and thermophilic organisms present (39). The optimal temperature for petroleum biodegradation has been found through the mesophilic temperature 20-35°C (65), although decomposition occures at temperature less than 0°C to as high as 70°C (39). Local degrader may utilize petroleum at a lower optimal temperature. Cold-adapted degraders, psychrophilic and psychrotrophic, are important for biodegradation of low temperature areas such as Arctic, polar zone, deep soil, etc. Margesin and Schinner (1997) found that biodegradation occurred at 10°C in Alpine soil (4,5). However, at low temperature, the viscosity of oil increase, the volatilization of toxic short-chain alkanes reduces and their water solubility increase, the delaying biodegradation (7).

In high temperature areas, there are some thermophilic hydrocarbon degraders, which the optimal growth at 55°C to 65°C. Sorkhoh *et al.* (1993) reported that *B. stearothermophilus* responsed for hydrocarbon degradation in Kuwaiti desert. The

result showed the degradation of narrow range of alkanes with 15, 16, 17 carbon atoms, but long-chain alkanes and aromatics compunds were less degraded. Higher temperatures may increase the toxicity of membrane-toxic components of petroleum, and cause death of the degraders (66).

2.3.5 pH

The subsurface pH may affect the availability and mobolity of contaminants either through sorption, desorption or permanent bonding. In addition, extreames of soil pH can be inhibitory or toxic to microbial life (42). The soil pH will often determine what types of microorganisms can participate in hydrocarbon biodegradation. The pH range that mineralization of hydrocarbon proceeds most rapidly is between 6.0 to 8.0. Adjustment of acidic and alkaline media to with in this range results in increase degradation rate (65,67). The overall rate of hydrocarbon degradation is higher under slightly alkaline than under acidic condition (68).

2.3.6 Oxygen

The metabolism of hydrocarbon normally requires the presence of molecular oxygen, since the initial biochemically step is oxygenase- catalyzed reduction to produced an alcohol or phenol. Following this step, further degradation can normally proceed either aerobically or anaerobically (69,70). However, the higher biodegradation rates observed in aerobic condition, where oxygen is available to serve both as reactant and electron acceptor in metabolism (38).

2.3.7 Soil characteristics

For the soil contamination, there can be a pronounced effect of the soil matrix. The mode and extent of interactions depend on the properties of the compounds and the accompanying matrices (71). Ferdinandy-van Vlerken (1998) said sorption

mechanism is strongly influenced by the characteristics of both pollutants and sediment (49). Loser *et al.* (1999) also said fine particles and organics matter, which cause the increasing of soil matrix, are essentially responsible for the adsorption of PAHs to the soil matrix. For the soil particle itself, it may be interspersed with pores and fissures, which increase the surface of the soil and this is the reason that the pollutants were more strongly bound to the matrix and delaying biodegradation (72). Another important one is soil moisture content. In general microorganisms need water to support their metabolics processes. The percent of moisture content in soil, which appropriate for hydrocarbon degradation, is in the range of 50-80% of water holding capacity (44). So soil characterization such as composition, particle size distribution, percent moisture and percent organics is very important (32,42).

2.3.8 Novel bioprocess design

The evaluation of novel bioprocess designs, such as new reactor design, the immobilization of hydrocarbon degrading cell, the application of surfactants for hydrocarbon biodegradation, etc. may be become the important factors for the success of hydrocarbon degradation.

A: Reactor design

The reactor design is more pronounced in pilot scale remediation, which the main objective is to demonstrate the biological treatment of contaminated material on a practical scale. There are numerous reactor designs were reported for the degradation of hydrocarbon compounds. For instance, Hakstege *et al.* (1998) studied the pilot remediation of sediment from the petroleum harbour in Amsterdam by using a continuous flow reactor consisting of a cascade of 4 bioreactor tanks for treating the fine particle of sediment. It was found that biodegradation in bioreactor on a large

scale is feasible. Furthermore useful information was gathered for the futhre remediation of the petroleum harbour (73). Rockne and Strand (2001) studied the degradation of PAHs by using fluidized bed reactor (FBR). They found that the reduction of PAHs was able to occur under the anaerobic condition (74).

B: Immobilization of hydrocarbon degrading cell

Immobilization of cell is another interested novel bioprocess designs for treating of hydrocarbon contaminants in water to overcome the problem of limited oil/water interfacial area. This study was successfully conducted in laboratory (75).

C: Application of surfactant for hydrocarbon biodegradation

Surfactants are used in the oil remediation process to enhance the solubility and availability of the compounds. The used of surfactants have been demonstrated in several laboratory studies. There are criteria to consider in using surfactant i) they should be biodegradable, ii) in the present of oil, they must not be preferentially utilized a carbon source and iii) they must be non toxic or low toxicity (76).

Surfactant for hydrocarbon decontamination can be used both chemical surfactant and biosurfactant. The influence of them on biodegradation shows diverse results. The presence of surfactant may be beneficial, detrimental or ineffective of biodegradation.

Fu and Alexander (1995) studied various kind of non-ionic and anionic surfactant on the degradation of hydrophobic organic compounds in nonaqueous-phase liquids (NAPLs). They found that the biodegradation of constituents of NAPLs in soil can be increased by the use of TritonX-100, Alconic 810-60, Tergital 5-S-9, Tergital 15-S-40 and Tween-80 (77).

Allen *et al.* (1999) also found that TritonX-100 increased the rate of oxidation of PAHs with *Pseudomonas* sp. Strain 9816/11 with the effect being most noticeable when phenantrene was used as a substrate. However, the surfactant inhibited the biotransformation of both naphthalene and phenanthrene with *Sphingomonas* sp. strain B8/36 under the same condition. They concluded that a non-ionic surfactant could have such contrasting effect on PAHs oxidation by different bacteria, which are known to be important for the degradation of these compounds in the environments (78).

Tiehm (1994) found that non-ionic surfactant and sodium dodecyl sulfate (SDS) increased the concentration of PAHs in the water phase because of solubilization. The degradation of PAHs was inhibited by SDS because this surfactant was preferred as a growth substrate. For non-ionic surfactant, the alkylethoxylate type and alkylphenolethoxylate type with an average ethoxylate chain length of 9 to 12 monomers were toxic to a PAH-degrading *Mycobacterium* sp. and to several PAH-degrading mixed cultures (79).

Bruheim *et al.* (1999) found that the mixture of Tergitol 15-S-7 and the negative charged dioctyl sulfosuccinate (AOT) was able to stimulate the alkane degradation activity to almost zero of *Rhodococcus* sp. even though AOT itself did not affect it. This indicated that there was synergism between the two surfactants. This may be the important thing that has to be considered when we would like to use the surfactants for hydrocarbon degradation (80).

Stelmack *et al.* (1999) studied the adhesion of a *Mycobacterium* strain and *Pseudomonas* strain, isolated from a creosote-contaminated soil, to the surface of highly viscous non-aqueous phase liquids (NAPLs). They found that the presence of

either TritonX-100 or Dowfax 8390 at a concentration of one-half the critical micelle concentration (CMC) inhibited the adhesion of both species of bacteria to the NAPLs. Neither surfactant was toxic to bacteria. If this adhesion of bacteria to hydrophobic surface is important for the biodegradation of virtually insoluble contaminants, then the use of surfactants may be not beneficial (81).

Deschenes, Villeneuve and Samson (1996) studied the biodegradation of 13 of the 16 US. Environment Protection Agency (EPA) priority PAHs in a creosote-contaminated soil, using both biological surfactant from *Pseudomonas aeruginosa* UG2 and chemical surfactant (SDS). They found that biodegradation of three-ring PAHs was rapid and almost complete but was slowed by the addition of 100 μg/g and 500 μg/g SDS. Similarly, at the same concentration, the two surfactants significantly decreased biodegradation rate of the four-ring PAHs. In this case also, the inhibition was more pronounced with SDS. It was suggested that the preferential utilization of surfactants by PAHs degraders was responsible for the inhibition observed in the biodegradation of hydrocarbons (25).

Mercade *et al.*(1996) isolated forty-four strains with the ability to grow on waste lubricating oil and found that four *Rhodococcus* and one *Bacillus* strain were selected for their capacity to lower the surface tension for culture medium. The surface-active compounds from *Rhodococcus* and *Bacillus* were glycolipids and lipopeptide, respectively. The percentage loss of waste lubricating oil after 5 days of incubation ranged from 7.87% to 45.8% depending on the strain selected compared with uninoculated control (82).

2.4 Experimental design for hydrocarbon contamination in laboratory

Bioremediation studies of contaminated oil in the environment consist of laboratory evaluations, pilot studies and field demonstration. Laboratory studies yield information on the expected performance, process control parameters and design criteria. So laboratory studies are often a prerequisite for many bioremediation projects (54).

Normally, laboratory studies have two major purposes.

1. To determine whether a bioremediation process will succeed in treatment of the contaminated hydrocarbon at a specific location.
2. To provide data for proper planing, sizing, and optimization of the bioremediation process or equipment.

If these goals are accomplished, site remediation can usually proceed in an efficient and cost-effective manner (42).

Laboratory treatability tests can be performed in a number of different ways depending on the type of full-scale treatment anticipated. In general, there are three typical types of tests; flask studies, column studies and pan or tray studies

2.4.1 Flask studies; Flask or bottle studies are relatively simple and inexpensive. Depending on the specific of the test, one flask may be sampled repeatedly or replicate flasks may be performed as open or contained systems (8).

For hydrocarbon contamination, flasks are used to study widely for biodegradation of hydrocarbons in water or in soil as soil/water slurries. Proper use of flask studies as indicators for in situ or solid-phase treatment is their inexpensive screen to determine whether bioremediation may or may not work. For example, Aldrett *et al.*(1997) studied the different biological remediation agents for the

degradation of petroleum hydrocarbons in marine environments and their toxicity in shake flasks. They found that three of 13 products was able to enhance the degradation rate. It is believed that this test provides a database for further studies in the field (83). Venosa *et al.*(1992), found that some commercial products were able to enhance the hydrocarbon degradation activity of an indigenous Alaskan microorganisms. This study was performed in closed respirometer flasks and shake flasks in order to determine the oxygen uptake rate by microorganisms (84). Moreover, flask studies provided the rapid and easy experiments for the study of hydrocarbon metabolisms (45,48,85).

Soil sample can also be studied in flasks in form of soil or soil/water slurries. For example, an approximately amount of soil was added in Erlenmeyer flasks and the various treatments were performed. The water content was adjusted to 60% with sterile water. After closed with cotton wool stoppers the flasks were incubated. In order to avoid anaerobic conditions, the contents of the flasks were mixed thoroughly every approximately time or flashed with CO_2-free air (4,65). For soil/water slurries, the supplementary mineral salt medium was added to the contaminated soil and incubated in an incubator shaker (86). However, flask studies do not closely mimic the field condition, they should not be used to decide on progressing directly to full-scale treatment. If the results of flask studies are promising, further studies should be performed, such as the performance of a pilot-scale test in the field or solid phase or soil column treatabilities for solid-phase or in situ treatment, respectively (8).

2.4.2 Column studies; Sealed columns are packed with sites soil, through which water, with or without amendments, is passed (Fig. 2-4).

Loser *et al.*(1999) studied the bioavailability of hydrocarbons during microbial remediation of a sandy soil in a percolator column to obtain kinetics of hydrocarbon degradation for site application (72). Column studies are better simulations of in situ conditions than flask studies. They provide information more consistent with what can be expected in the field, such as soil flushing vs biodegradation, identification of physical constrains of nutrients delivery, and identification of chemical constrains of chemical precipitation and clogging. However, logistics of set up and operation of column studies are more extensive than flask studies, resulting in higher casts for performance. So the studies are not as wide as flask studies (8).

2.4.3 Pan or Tray studies; Solid-phase treatability studies are typically set up in stainless steel or glass pans, utilizing undiluted, solid materials, typically soil. These studies test the feasibility of treating materials in a solid matrix by appropriate amendments and physical mixing using methods similar to those used for landfarming. For example, Margesin *et al.* (1997), used polycarbonate pans to study the biodegradation activities of the indigenous alpine soil microorganisms and the effect of the addition of a cold-adapted diesel-oil degrading inoculum. This is useful for estimating rates and extent of biodegradation of contaminants during full-scale solid-phase treatment (5).

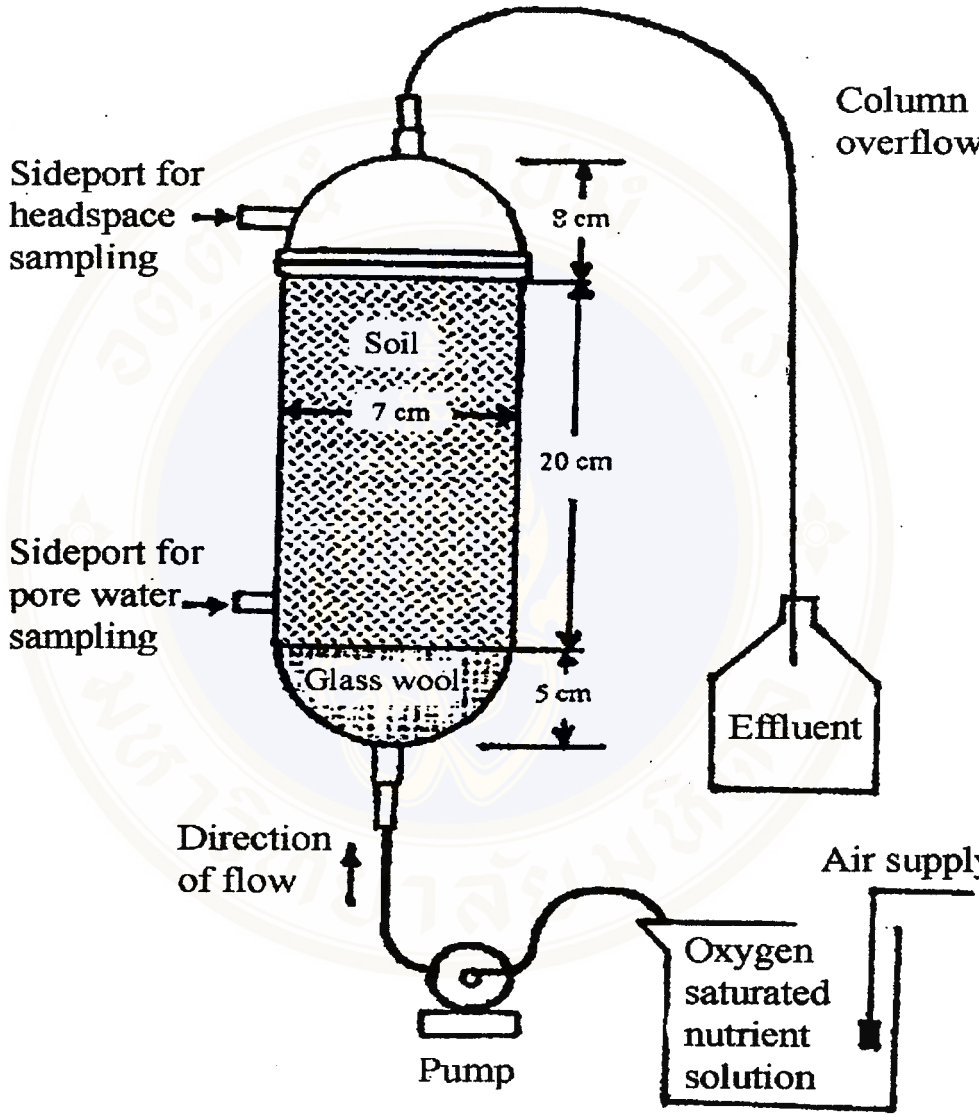


Fig.2-4 Column set up (87)

2.5 Inherent problems with bioremediation studies in laboratory

No laboratory study can ever exactly duplicate all the conditions found at a site. However, by paying close attention to experimental design, laboratory studies can successfully generate much useful data. There are four major areas of difficulty in conducting laboratory biotreatability studies.

2.5.1 Quantitation of contaminants; The progress or sustainability of laboratory biotreatability is usually monitored by chemical measurements. These are generally expensive to perform, complex to monitor the biotreatability. Binding of some contaminants to soil also makes their quantitation difficult. Gas chromatography with or without mass spectrometry and high-performance liquid chromatography are selected analytical methods most widely used to quantitate environmental contaminants (42,59,88,89).

2.5.2 Starting concentrations of the contaminants; Many degradative organisms derive growth and energetic benefit from utilizing waste. Below a certain threshold concentration, this gain is inadequate to drive the process and the responsible enzymes will not be produced; above a higher threshold, the toxic chemical can inhibit the survival of the community or key members of it. So a single biotreatability study at one concentration may be inadequate to supply all the answers needs to make educated decisions about a project (8,90).

2.5.3 Microbial inoculum; A bioaugmentation system developed by Weber and Corseuil (1991) for generation of microorganisms was employed as a source of inoculum. They believed that the rate of contaminant biodegradation in the affected soil or water will be appreciable enhanced because the density of contaminant-specific degraders will have been artificially increased (52).

There are two basic means to achieve the approach. The first one is the site specific strains that exhibit desirable biodegradative qualities. The second is the contaminant specific degraders that have been specifically selected due to their inherent or induced capability to degrade the contaminants of concern (32).

Microbial consortium has been mostly used to degrade a number of target compounds. In some cases, the identification of the consortium has been accomplished, but in many cases, the consortium contains an unknown number of unidentified microbes (91). Moreover, microbes are sensitive to the environmental conditions. So the appropriate culture condition have to be also carefully determined (42).

2.5.4 Laboratory versus site conditions; When biotreatability studies are performed in simulated laboratory systems and run in parallel to pilot or full-scale biotreatment, they can be very effective for aiding the field evaluation. However, care in interpreting these parallel studies is needed because samples displaced from the environment and studied in the laboratory do not necessarily mimic the metabolic activities occurring in the field.

Thus, site samples obtained and studied in the laboratory do not necessarily represent what one might occur in the natural environment and may produce misleading result and poor extraporation from laboratory to field data. So it is evident that careful and exact experimental design is critical to the success of any laboratory endeavor (1,42).

2.6 Future and trend

For a success in bioremediation, the bioremediation methods depend on having the right microbe in the right place with the right environmental factors for degradation (92). Laboratory studies are the most appropriate way to answer these questions by convenience, low cost and expedience. However the results of even carefully planned and executed studies must be interpreted with caution. Furthermore, extending the results from a laboratory-scale system to the design and operation of a field-scale system should be carefully carried out (93). In situ and on site bioremediations of hydrocarbon compounds have been reported (21,94,95,96).

The importance of genetic engineering in hydrocarbon bioremediation is likely to come as a result of further advances in inoculation (88). Most microorganisms have short generation time and reach high population densities in small spaces. Therefore, the rate of evolution of new traits is rapid relative to that of multicellular organism. Bacteria especially are metabolically versatile and can catabolize or resist to toxic effects of a wide variety of environmental chemical contaminants. Within the past three decades, the tools became available for detailed analysis of the genetic basis of the metabolic versatility of bacteria. This resulted in the cloning of a wide variety of enzymatic capabilities in various groups of bacteria and some fungi (40). Moreover, the additional phenotype of tolerance to extreme conditions, such as hyper-saline, high-temperature, can be improved by genetic manipulations (97).

Another way to improve hydrocarbon degradation is utilization of plants to perform rhizosphere bioremediation (phytoremediation), root of plants are colonized by bacteria or mycorrhiza-forming fungi, in which the fungal hyphae are more or less densely colonized by a bacteria biofilm. Plants growing at a contaminated site

themselves improve the appearance of the site. Plant roots prevent erosion, stabilize and increase the microbial population density and diversity of soil. Plant root exudates may also provide co-substrates for bacteria, which can improve degradation on certain contaminating compounds by inducing the right set of genes. Root also can change the porosity of the soil, improving the aeration (10,98). Safonova *et al.* (1999), reported that associations of algae with alcanotrophic bacteria destroyed the black oil more efficiently than pure culture alcanotrophic bacteria *Rhodococcus* sp. 7HX (99). Similarly, Suominen *et al.* (2000) found that *Galga* plants exhibited good growth, nodulation, and nitrogen fixation, and developed a strong rhizosphere in soils contaminated with oil or 2,000 ppm *m*-toluate (100).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

Chemicals used in all experiments are analytical or equivalent grade except chloroform, which is commercial grade, used in the extraction of used oil. All chemicals are purchased from where they are available i.e. E.Merck, Darmstadt, Germany, Difco Laboratories, Michigan, USA, Fluka Chemie AG Industriestrasse, Switzerland and Sigma Chemical, Missouri, USA. These chemicals are listed in Table 3.1. All gases are purchased from Thai Industrial gas (TIG Co.Ltd.), Chachaengsao, Thailand.

3.2 Reagents

3.2.1 Gram's stain reagents (101)

3.2.1.1 Gram's Crystal Violet (Modified Hucker)

Solution A		
Crystal Violet	2	g
95% Ethanol	20	ml
Solution B		
Ammonium Oxalate	0.8	g
Distilled water	80	ml

Gram's Crystal Violet was obtained by mixing solution A and solution B. After solutions A and B were well mixed, it was allowed to stand at room temperature (approximately 25°C) for 24 h before used.

3.2.1.2 Gram's Iodine (Lugol's Iodine) : Mordant

Iodine crystal	1	g
Potassium iodide	2	g
Distilled water	300	ml

Iodine crystal and Potassium iodide were ground to powder in a mortar. Distilled water was added to obtain Gram's Iodine solution.

3.2.1.3 Gram's Safranin

Safranin	0.25	g
95% Ethanol	10	ml
Distilled water	100	ml

Safranin was completely dissolved in ethanol. It was then added with distilled water.

3.2.1.4 Decolorizing agent

95 % Ethanol

3.2.2 Normal Saline solution (0.85%)

NaCl	0.85	g
Distilled water	100	ml

NaCl was dissolved in distilled water. The solution was autoclaved at 121°C 15 psi for 15 min.

Table 3-1 List of chemicals

Chemical name	Chemical formular
Acetone	C_3H_6O
Ammonium nitrate	NH_4NO_3
Ammoniummolybdate	$NH_4Mo_7O_{24}.4H_2O$
Ammoniumoxalate Monohydrate	$(NH_4)_2C_2O_4$
L(+)-Ascorbic acid	$C_6H_8O_6$
Boric acid	H_3BO_3
Bromocresol green	$C_{21}H_{14}Br_4O_5S$
Brucine sulfate hydrate	$(C_{23}H_{26}N_2O_4)_2.H_2SO_4$
Calcium chloride	$CaCl_2$
Chloroform	CH_3Cl
Crystal violet	$C_{25}H_{30}ClN_3$
1-Eicosene	$C_{20}H_{40}$
95% Ethanol	C_2H_5OH
Dipotassium hydrogen phosphate	K_2HPO_4
Disodium hydrogen phosphate	Na_2HPO_4
Disodium tetraborate decahydrate	$B_4Na_2O_7.10H_2O$
Glycerol	$C_3H_8O_3$
Helium (99.999%)	He
n-Hexane	C_6H_{14}
Hydrochloric acid	HCl
Hydrogen (99.99%)	H_2
Iodine crystal	I
Iron (III) chloride	$FeCl_3$
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$
Mercury (II) oxide	HgO
Methyl red	$C_{15}H_{15}N_3O_2$
Nitric acid	HNO_3
Nitrogen (99.999%)	N_2

Table 3-1 List of chemicals (continued)

Chemical name	Chemical formular
Petroleum ether	-
Phenolpthalein	$C_{20}H_{14}O_4$
Potassium antimonyl (III) oxide tartrate hemihydrate	$K(SbO)C_4H_4O_6.0.5H_2O$
Potassium chloride	KCl
Potassium dihydrogen phosphate	KH_2PO_4
Potassium hydrogen phthalate	$C_8H_5KO_4$
Potassium iodide	KI
Potassium nitrate	KNO_3
Potassium sulfate	K_2SO_4
Safranin	$C_{20}H_{19}N_4Cl$
Sodium azide	NaN_3
Sodium chloride	NaCl
Sodium dihydrogen phosphate	NaH_2PO_4
Sodium dodecyl sulfate	$C_{12}H_{25}NaO_4S$
Sodium hydroxide	NaOH
Sodium nitrate	$NaNO_3$
Sodium sulfate anhydrous	Na_2SO_4
Sodiumthiosulphate pentahydrate	$Na_2S_2O_3.5H_2O$
Sulfanilic acid	$C_6H_7NO_3S$
Sulfuric acid (95-97%)	H_2SO_4
TritonX-100	$C_{14}H_{62}O_{12}$
Tween-80	-
Urea	H_2NCONH_2

3.2.3 Buffers

3.2.3.1 0.2 M Sodium phosphate buffer (pH7)

Solution A : NaH_2PO_4 0.4 M

$NaH_2PO_4.H_2O$	55.2	g
Distilled water	1000	ml

Solution B : Na_2HPO_4 0.4 M

Na_2HPO_4	56.86	g
Distilled water	1000	ml

0.2 M Sodium phosphate buffer pH7 could be prepared by mixing 195 ml of Solution A with 305 ml of Solution B. Distilled water was added to make up 1000 ml. The pH of buffer was adjusted to 7 by 0.1 M NaOH.

3.2.3.2 0.2 M Sodium-potassium phosphate buffer (pH7)

Solution A : $NaH_2PO_4.H_2O$ 0.4 M

$NaH_2PO_4.H_2O$	55.2	g
Distilled water	1000	ml

Solution B : K_2HPO_4 0.4 M

$K_2HPO_4.3H_2O$	91.29	g
Distilled water	1000	ml

0.2 M Sodium-potassium phosphate buffer pH7 could be prepared by mixing 195 ml of Solution A with 305 ml of Solution B. Distilled water was added to make up 1000 ml. The pH of buffer was adjusted to 7 by 0.1 M NaOH.

3.2.3.3 0.2 M Potassium phosphate buffer (pH7)

Solution A : KH_2PO_4 0.4 M

KH_2PO_4	54.44	g
Distilled water	1000	ml

Solution B : K_2HPO_4 0.4 M

$K_2HPO_4.3H_2O$	91.29	g
Distilled water	1000	ml

0.2 M Potassium phosphate buffer pH7 could be prepared by mixing 195 ml of Solution A with 305 ml of Solution B. Distilled water was added to make up 1000 ml. The pH of buffer was adjusted to 7 by 0.1 M KOH.

3.2.4 20 % Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate	20	g
Distilled water	100	ml

The solution was autoclaved at 121°C 15 psi for 15 min before used. It was stand on a shelf in a screw cap 250 ml bottle.

3.2.5 20 % Triton X-100

TritonX-100	20	ml
Distilled water	100	ml

The solution was autoclaved at 121°C 15 psi for 15 min before used. It was stand on a shelf in a screw cap 250 ml bottle.

3.2.6 20 % Tween 80

Tween 80	20	ml
Distilled water	100	ml

The solution was autoclaved at 121°C 15 psi for 15 min before used. It was stand on a shelf in a screw cap 250 ml bottle.

3.2.7 1 % w/v 1-Eicosene (Internal standard)

1-Eicosene	1	g
n-Hexane	100	ml

The solution was kept at -20°C in a screw cap 250 ml bottle until used.

3.3 Oils

3.3.1 Tapis crude oil

Tapis crude oil used in this study was obtained by courtesy of Bangchak oil refinery, Sukhumvit Rd. Soi 64, Bangkok, Thailand. It is the Malaysian crude oil.

3.3.2 Diesel and Motor oil

New diesel and new motor oil were obtained as gifts from Bang Sue Diesel Locomotive Depot, Bang Sue, Bangkok, Thailand.

3.3.3 Used oil

Used oil used throughout this study was extracted from the contaminated gravel at Bang Sue Diesel Locomotive Depot. The gravel was heavily and repeatedly contaminated with used diesel oil and used motor oil for years. The extraction procedure is described in Section 3.6.

3.4 Organisms

3.4.1 *Acinetobacter* sp. M1407

Acinetobacter sp. M1407 was isolated from an oil contaminated area in Thailand by Miss Kulwadee Tongpubesra M.Sc. student in the Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok (1998). It has a high ability to degrade 0.5% Tapis crude oil in Mineral salt medium (MSM).

3.4.2 Microorganisms from Bang sue Diesel Locomotive Depot

Two groups (I and II) of microorganism used throughout this study were isolated from Bang Sue Diesel Locomotive Depot. The isolation procedure is described in Section 3.7. Microorganisms group I was isolated by using Tapis crude oil as a sole carbon source in the isolation medium.

Microorganism group II was isolated from the contaminated soil by using used oil as a sole carbon source.

3.5 Media

Nutrient agar (NA) and Nutrient broth (NB) are purchased from Merck, and Difco. Unless otherwise stated, all media in this study were sterilized by autoclaving at 121°C, 15 psi for 15 min (Tomy autoclave, model SS320, Japan or Hirayama autoclave, model HVE-50, Japan). The pH of medium was adjusted to desired value either by 1.0 N hydrochloric acid, 1.0 M sodium hydroxide or 1.0 M potassium hydroxide.

3.5.1 Bushnell-Haas broth (BH)

Bushnell-Haas medium was originally formulated by Bushnell and Haas in 1941. It's composition as followed

KH_2PO_4	1	g
K_2HPO_4	1	g
NH_4NO_3	1	g
$MgSO_4.7H_2O$	0.2	g
$FeCl_3$	0.05	g
$CaCl_2$	0.02	g
Distilled water	1000	ml

For the preparation of BH agar, 15 g of bacteriological agar (Difco) was added into medium 1 l.

3.5.2 BH-oil agar

After warming Tapis crude oil at 60°C for 10 min in a water bath (Thelco,model 10-x-5,USA), the oil was sterilized by filtration through 0.45 µm sterile membrane filter (Gelman, Supor®-450, 25 mm diameter, USA). The filter sterile Tapis crude oil was kept in a refrigerator until used. Sixty microliters of sterile Tapis crude oil was spread onto BH agar plates. This medium was used for studying the ability of hydrocarbon degrading bacteria. In case of used oil degrading bacteria the used oil was used instead of Tapis crude oil.

3.5.3 Phosphate buffer Bushnell-Haas broth

0.1 M phosphate buffer, pH7 was used in order to maintain the culture pH at 7, which is the average value of pH from various areas of Bang Sue Diesel Locomotive Depot. The preparation of the medium is described below

Solution A		
NH_4NO_3	1	g
$MgSO_4.7H_2O$	0.2	g
0.2 M Phosphate buffer	500	ml
Solution B		
$FeCl_3$	0.05	g
$CaCl_2$	0.02	g
Distilled water	500	ml

Solution A and solution B were sterilized by autoclaving separately and were then aseptically mixed together. For the modified BH-medium 1.86 g/l of potassium chloride (KCl) was added in solution A as a K supplemental source.

3.6 Extraction of used oil as a substrate for biodegradation study

For preliminary study, 5 kg of contaminated gravel was collected from Bang Sue Diesel Locomotive Depot and was then added with 1000 ml of chloroform. After gently mixed for 1 h, the chloroform fraction was removed and filtered through the clothes. The mixture of chloroform, used oil and some solid particles was evaporated in a vacuum rotary evaporator (Resona, model Lobo-Rota B300X, Germany) under the pressure of 600 mmHg, 80°C. The oil was kept at room temperature (approximately 25°C).

After preliminary study, 20 kg of contaminated gravel was collected from the site. Chloroform (1000 ml) was added in the container containing 5 kg of contaminated gravel. After gently mixed for 1 h, the chloroform fraction was removed. The extraction was repeated one more time with 1000 ml of chloroform. Another 15 kg of contaminated gravel was extracted by the same procedure. The fractions of chloroform were combined and filtered through filter paper no.40 (Whatman International Ltd., 125 mm diameter, England). The mixture of chloroform, used oil and suspended particles was then evaporated in a vacuum rotary evaporator under the pressure of 600 mmHg, 80°C. The oil was kept at room temperature. It was used as a substrate throughout this study.

3.7 Screening of oil degrading microorganisms from Bang Sue Diesel Locomotive Depot

Soil and water samples from Bang Sue Diesel Locomotive Depot were measured temperature and pH at the site. After that it was collected to the laboratory to determine viable cell concentration. Screening of oil degrading microorganisms was carried out by the enrichment procedure in BH-oil broth as described below.

When Tapis crude oil was used as a carbon(C) source, 1.0 g of soil sample or 1.0 ml of water sample was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of BH-broth supplemented with 1% of sterile Tapis crude oil. After incubation at 30°C, 200 rpm in an incubator shaker (Psychrotherm incubator shaker, New Brunswick model G26, USA) for 3 days, 0.5 ml of culture broth was transferred to a fresh 50 ml of BH-oil broth and incubated further under the same cultivation condition. The samples were then taken at 3 days. After appropriate ten-fold dilution of sample with 0.85% normal saline solution, 0.1 ml of each dilution was spread onto NA plates. After incubation for 48 h at 30°C, the morphologies of individual colonies were observed. Then distinguish colonies were picked and pure isolates obtained by repeated subculturing on NA plates for further studies (Microorganism group I).

For microorganism group II, when used oil was used as a C-source, 0.5 g of contaminated soil (Section 3.8.1) was added into a 250 ml Erlenmeyer flask containing 50 ml of BH-broth. The cultivation was shaken at 30°C, 200 rpm in an incubator shaker for 5 days. The culture broth (1.0 ml) was then transferred to a fresh 50 ml of BH-oil broth and was incubated at 30°C, 200 rpm in an incubator shaker for 45 days. During the cultivation the samples were taken at day 0, 15, 30 and 45. After appropriate ten-

fold dilution, 0.1 ml of each dilution was spread onto NA plates. Every single colony was observed and isolated for further studies.

3.8 Stock culture preparation

3.8.1 Stock culture from the contaminated soil

The contaminated soil was collected from various areas of Bang Sue Diesel Locomotive Depot. These soil samples were mixed thoroughly in an aluminum tray. Then it was divided and kept in sterile vials. Each vial contained 0.5 g of soil and was stored in a deep freezer at -80°C (Sanyo, model 790, Japan) until used.

3.8.2 Stock culture of isolated microorganisms

One loopful of pure culture (incubated 48 h, 30°C on NA plate) was inoculated into a 250 ml Erlenmeyer flasks containing 50 ml Nutrient broth. The culture medium was rotary shaken at 200 rpm, 30°C. After incubation for 48 h, The culture broth (2.0 ml) was added in the sterile vials. Sterile glycerol (45%, 1 ml) as a cryopreservative agent was then added. The vials were kept in a deep freezer at -80°C until used.

3.9 Inoculum preparation

For the inoculum preparation of isolated microorganism group I, group II and group III, one loopful of pure culture was inoculated into a 250 ml Erlenmeyer flask containing 50 ml BH-broth and 0.5 g of used oil. The culture was shaken aerobically in a rotary shaker 200 rpm, 30°C until the stationary phase of growth was reached (5 days).

In case of the inoculum preparation of enriched soil microorganisms, 0.5 g of contaminated soil (Section 3.8.1) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml BH-broth and 0.5 g of used oil. Cultivation condition was carried out as the same procedure as inoculum preparation of isolated microorganisms.

For the inoculum preparation of *Acinetobacter* sp. M1407, one loopful of the organism was inoculated into a 250 ml Erlenmeyer flask containing 50 ml BH-broth and 0.5 g of Tapis crude oil. The culture was shaken aerobically in a 200 rpm rotary shaker at 30°C for 3 days (Tongpubesra, 1998).

3.10 Determination of cell growth by the viable plate count

For the measurement of viable cell concentration in a culture sample, 0.1 ml of each suitable ten-fold dilution in a 0.85% sterile normal saline solution was spread onto NA plate in duplicate. The culture plates were incubated at 30°C until the colonies were clearly observed (2 days). Only plates containing 30-300 colonies were counted. Calculation was performed according to the following equation

$$\text{Viable cell concentration (CFU/ml)} = \frac{\text{Number of colonies}}{\text{Spreading volume (ml)}} \times \text{Dilution factor}$$

3.11 Analysis of residual oil from the culture broth

3.11.1 Extraction of residual oil from the culture medium

3.11.1.1 flask culture

After the addition of 1.0 ml of 1% (w/v) 1-Eicosene as an internal standard (IS) into 50 ml culture broth, the whole broth mixture was transferred into a 1000 ml separatory funnel. The culture flask was rinsed with 100 ml of chloroform, which was then transferred into the separatory funnel. The separatory funnel was shaken for 2 minutes and placed on a stand until both liquid phases were separated from each other. The bottom phase of oil-chloroform mixture was collected in a beaker. After re-extraction with additional 50 ml chloroform, the two collected fractions were combined together. Approximately 20 g of sodium sulfate anhydrous (Na_2SO_4) was added in the chloroform fraction in order to remove residual water. The mixture was filtered through a Whatman filter paper no.40 to remove Na_2SO_4. The chloroform fraction was transferred into a 50 ml evaporating flask and was then evaporated in a vacuum rotary evaporator under the pressure of 600 mmHg, 40°C. After the disappearance of chloroform solution from the evaporating flask, the mixture was further evaporated for 10 minutes to make more completely. The residual oil was analyzed by gravimetric measurement (Section 3.11.2). After re-dissolved by n-hexane (10 ml) and filtered through a Whatman GF/A glass microfiber filter (Whatman International Ltd., 25 mm diameter,England) to remove the undissolved components, the filtrate was kept at -20°C for Gas chromatography analysis (Section 3.11.3).

3.11.1.2 Tray culture

Before the extraction of residual oil, the gravel was dried in a 60°C hot air oven (Memmert, model BM500, Germany) for 24 h. After cooled, dried gravel was added with 2.0 ml of 1% (w/v) 1-Eicosene as an internal standard (IS) and 500 ml chloroform. The tray was rotary shaken at 150 rpm in a controlled environment incubator (Heto Maxi-shake, model SBD 50 BIO, Heto-Hoften, Denmark) for 1.5 h. Chloroform fraction was collected into 2.5 l glass bottle. The gravel was then re-extracted with 500 ml of chloroform for two times under the same condition. The pooled chloroform fraction was filtered through the Whatman filter papers no.4 and no.40 to remove the unsuspended solids. The chloroform fraction was transferred into a 500 ml evaporating flask. It was evaporated in a vacuum rotary evaporator under the pressure of 600 mmHg, 40°C. After reduction to a small volume, the chloroform fraction was transferred into a 50 ml evaporating flask and it was further evaporated at 80°C for 10 minutes. After dried in a desicator, the residual oil was measured by gravimetric measurement. Then it was re-dissolved by n-hexane (250 ml) and filtered through a Whatman GF/A glass microfiber filter (125 mm diameter). The hexane soluble fraction was evaporated in a vacuum rotary evaporator under the pressure of 600 mmHg, 80°C and was then measured by gravimetric measurement.

3.11.2 Determination of residual oil by gravimetric measurement

After the residual oil in an evaporating flask (50 ml) was dried by evaporation, the weight was determined (Denver balance, model 115/230VAC, USA). The amount of the residual oil is calculated as following equation

Weight of residual oil (g) = Weight of flask and oil (g) - Weight of flask (g)

or

$$\% \text{ Residual oil} = \frac{\text{Weight of residual oil (g) at a specific incubation period}}{\text{Weight of residual oil (g) at day 0 (control)}} \times 100$$

3.11.3 Gas chromatography analysis of residual oil

This procedure was conducted by using a GC-9A Shimadzu gas chromatography apparatus (Shimadzu Corporation, Kyoto, Japan), equipped with a flame ionization detector (FID). A chromatogram was analyzed and plotted by Shimadzu C-R3A Integrator (Shimadzu Corporation, Kyoto, Japan). A Petrocol C stainless steel column (Supelco, inc., Bellefonte, USA), with 1/8 inch internal diameter and 20 inch length were used throughout this study. The hexane soluble fractions (0.5 µl) from Section 3.11.1.1 were injected with a 1 µl Hamilton syringe (Hamilton Co.Ltd., USA) into a gas chromatography unit. The injector and detector temperatures were adjusted to 300°C. The column temperature was programmed at between 80°C and 300°C. The increasing rate of column temperature was 15°C/min after 2 min hold interval at 80°C. When the temperature reached 300°C, it was held for 15 min before the run was terminated and cooled down. Nitrogen (99.999%) was used as a carrier gas with a flow rate of 30 ml/min. Air and hydrogen flow rates were set at 0.5 and 0.6 kg/cm^2. GC was

run under splitless mode of operation. The parameters used in GC analysis with integrator are shown in Table 3.2.

Calculation of residual hydrocarbon from GC chromatogram

Residual hydrocarbons from GC chromatogram were presented in percentage. Calculations were performed as following equations.

$$\% \text{ Residual HC} = \frac{\text{Residual HC at a specific incubation period}}{\text{Residual HC at day 0 control}} \times 100$$

where

$$\text{Residual HC} = \frac{\text{Total peak area of HC in a GC chromatogram}}{\text{Peak area of internal standard of the same run}}$$

Residual HC in this study was defined when all components detected by GC except solvent (hexane), impurities and internal standard were used in calculation.

3.11.4 Statistical analysis of residual oil means and density of gravel.

Statistical analysis were performed to test whether the means of residual oil (g or %) and density of gravel in each treatment were significant difference. One-way analysis of variance (a completely randomized design) was used at 95% confidence, in Sigma Statistical Solfware by Jandel Corporation (USA). All data were primarily tested whether they were normally distributed. An example of statistical analysis and interpretation is described in Appendix A.

Table 3-2 Parameters for the analysis of oils by Shimadzu GC-9A and Shimadzu C-R3A Integrator

Parameter	Meaning	Value
Width	Half-height width or slightly narrower of the narrowest peak appearing on chromatogram	3 sec
Drift	Evaluation level for peak and baseline drift	0 μV/min
T.DBL (doubling time)	The value for width and slope were automatically change	0 min
Attenuation	The chromatogram intensity axis scale	1
Method	Calculation process for peak quantitation and qualification (41= normalization method)	41
SPL.WT	Sample quantity	100
Slope	Signal gradient or tangent slope of peak signal varying with time for peak detection sensitivity	100 mV
Minimum area	Minimum peak area	200 $\mu V/sec^2$
Stop time	An estimated end time which was set to stop peak processing	45 min
Speed	Chart speed of plotter	3 mm/min
Format	Report format of quantitative calculation	0
IS.WT	Internal standard substance	1

3.12 Analysis of Nitrogen and Phosphorous in the culture broth

After the used oil was extracted to chloroform fraction and separated from the culture broth, the broth was centrifuged at 6,000 rpm (Sorvall, rotor model SLA-1500, USA), 25°C for 15 min (Sorvall, model RC5Cplus, USA). The supernatant was kept at 4°C for analysis of phosphorous and nitrogen.

3.12.1 Analysis of Phosphorous (phosphate) by the Ascorbic acid Method (102)

Into a 50 ml digestion tube, 10 ml of culture broth was added with 0.2 ml of 97% H_2SO_4 and 1 ml of 70% HNO_3. The mixture was digested at 130°C in a heating box (Boekel, INV 096910) to a volume of 1 ml. After cooled, it was added with approximately 20 ml distilled water, 0.05 ml phenolpthalein indicator and as much as required of 6 N and 1 N NaoH solution to produce a faint pink tinge. Then it was adjusted with 0.02 N H_2SO_4 to return colorless. The neutralized solution was filtered through a Whatman filter paper no.40 into a 50 ml volumetric flask. Distilled water was then added to make up volume to 50 ml. The solution was transferred into a 250 ml Erlenmeyer flask and 8.0 ml of combined reagent (Appendix C) was added and mixed thoroughly. After at least 10 min but no more than 30 min, the sample was measured the absorbance at the wavelength of 880 nm by a spectrophotometer (Spectronic®20, model 3SGA 216040, USA). Distilled water was used as a reagent blank. The amount of phosphorous (P) is calculated by the following equation

$$\text{mg } PO_4\text{-P/l} = \frac{OD_{sample} - OD_{blank} \times \text{factor} \times 1000}{\text{ml.sample}}$$

where

$$\text{factor} = \frac{1}{\text{slope of standard curve of phosphorous}}$$

The preparation of standard curve of phosphorous and reagents is described in Appendix C.

3.12.2 Analysis of Nitrogen (nitrate) by the Brucine sulfanilic acid Method (102)

The culture broth was filtered through a Whatman filter paper no.4 to remove the unsuspended solids. 10 ml of filtrated sample was pipetted into a 50 ml test tube and placed into an ice-bath. After cooled, the sample was consequently mixed with 2 ml of 30% (w/v) NaCl solution, and 10 ml of 25% (v/v) H_2SO_4 solution. The reaction tube was added with 0.5 ml of Brucine sulfanilic acid solution and placed into 95°C water bath for 20 min. The reaction mixture was measured the absorbance at the wavelength of 410 nm by a spectrophotometer after cooled down at room temperature. For the reference solution, 10 ml of distilled water was used instead of filtrated sample. The amount of nitrogen (N) is calculated as follows

$$\text{mg } NO_3\text{-N/l} = \frac{OD_{sample} - OD_{blank} \times \text{factor} \times 1000}{\text{ml.sample}}$$

where

$$\text{factor} = \frac{1}{\text{slope of standard curve of nitrogen}}$$

The preparation of standard curve of nitrogen and reagents is described in Appendix C.

3.12.3 Analysis of Nitrogen (ammonia) by the Titrimetric Method (102)

Fifty ml of sample in the distillation flask was added with 3 ml borate buffer solution. The pH of sample was adjusted to 8.5 with 6 N NaOH solution. The mixture was distilled in the distillation apparatus (Tecator, model 1782, Sweden). At least 100 ml of the distillate was collected in a 250 ml Erlenmeyer flask, which contained 10 ml of 2% indicating boric acid. The distillated was titrated with a standard 0.02 N H_2SO_4 titrant until indicator turned a pale lavender. Distilled water was used as a blank throughout the experiment. The amount of nitrogen (N) is calculated follow this equation

$$\text{mg } NH_3\text{-N/l} = \frac{(\text{ml of sample - ml of blank}) \times \text{N of } H_2SO_4 \times 14 \times 1000}{50}$$

The preparation of reagents is described in Appendix C

3.13 Biodegradation of used oil on agar plates

Fifty-four bacterial colonies (microorganisms group II, section 3.7) were subcultured from the stock culture in 15% glycerol onto nutrient agar plates. After incubation at 30°C for 48 h, individual colonies were picked by a sterile toothpick and spotted 8 spots onto a BH-oil agar plate (Section 3.5.2). After incubation at 30°C during 7 days, colonies and clear zone were observed. Colonies with early appearance, large colony diameter or clear zone were separately cultured in BH-medium with 0.5 g of used oil for further study of oil degradation in liquid culture. This group of microorganism was catagorized as microorganism group III.

3.14 Biodegradation of oils in shake flask (liquid phase bioremediation)

For the degradation of used oil, which was extracted from Bang Sue Diesel Locomotive Depot, various types of microorganism, and medium formulation were performed in order to reduce the amount of used oil in shake flask. Moreover the degradation of diesel and motor oil, which was used in Bang Sue Diesel Locomotive Depot, were also studied. All inoculum preparations are described in Section 3.9. All samples were taken in duplicate throughout this study. Control for abiotic degradation determination was added with 0.5% (w/v) NaN_3.

3.14.1 Determination of used oil degrading microorganisms

3.14.1.1 Biodegradation of oil by *Acinetobacter* sp. M1407, enriched soil microorganisms and microorganism group I

Three groups of oil degrading microorganisms (*Acinetobacter* sp. M1407, enriched soil microorganisms and microorganism group I) were used as an inoculum to find the suitable group for degradation of used oil. Inoculum (2%) of each group was added in a 250 ml Erlenmeyer flask, which contained 50 ml BH-medium and 0.5 g of used oil (for preliminary study). The culture was incubated at 30°C and shaken at 200 rpm in a rotary shaker (made in Thailand) for 45 days. The samples were taken at day 0, 15, 30 and 45 for determination of viable cell concentration, pH, residual oil, nitrogen and phosphorous. Saturated $NaHCO_3$ solution was periodically added to maintain the culture pH at 7.

3.14.1.2 Biodegradation of used oil, diesel oil and motor oil by enriched soil microorganisms and microorganism group II

The degradation of used oil, un-used diesel oil and un-used motor oil of microorganism group II was studied. The degradation activity was compared with that of enriched soil microorganisms. The cultivation condition is described in Section 3.14.1.1, but modified BH-medium (sodium phosphate BH-medium and 1.86 g/l KCl) was used instead of BH-medium. The samples were taken at day 0, 10, 20 and 30 for analysis of residual oil, viable cell concentrations and pH.

3.14.1.3 Biodegradation of used oil by microorganism group III

The degradation of used oil of microorganism group III was studied. For the inoculum preparation of pure culture, one loopful of pure culture was inoculated into a 250 ml Erlenmeyer flask containing 50 ml Nutrient broth. The culture was rotary shaken at 200 rpm, 30°C for 48 h. Either pure culture or mixed culture (2% inoculum) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml modified BH-medium and 0.5 g of used oil. The culture medium was incubated aerobically in a 200 rpm rotary shaker at 30°C for 30 days for analysis of residual oil.

3.14.1.4 Effect of soil on biodegradation of used oil

The soil components, which may have some effects on biodegradation of used oil was studied. Soil from the contaminated area was sterilized by autoclaving for two times, consecutively. The sterile soil (0.5 g) was added in a 250 ml Erlenmeyer flask containing 50 ml modified BH-medium and 0.5 g of used oil. One loopful of microorganism group III were inoculated into the medium and was then rotary shaken at 30°C for 5 days. The culture medium was then used as an inoculum (2% mixed culture) for studying the degradation of used oil as the same procedure as Section

3.14.1.1. The samples were then taken at day 0, 10, 20 and 30 for analysis of residual oil.

3.14.2 Effect of medium composition on biodegradation of used oil.

3.14.2.1 Effect of nitrogen source on used oil degradation

For studying the effect of nitrogen (N)-source, sodium phosphate buffer pH 7 was used and various types of N-sources (NH_4NO_3, KNO_3, $NaNO_3$, NH_4Cl, Urea) were performed. The applications were equivalent to applications of nitrogen in NH_4NO_3. This modified BH-medium was used to study its effect on biodegradation of used oil. Enriched soil microorganisms (2% inoculum) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml modified BH-medium with various nitrogen sources and 0.5 g of used oil. During cultivation in a 200 rpm rotary shaken at 30°C, the samples were taken at day 0, 15 and 30 for analysis of viable cell concentration, pH and residual oil.

However, only modified BH-medium with KNO_3 was found for the potassium (K) supplemental source. So potassium chloride (KCl) were applied in the others modified BH-medium. The applications were equivalent to applications of potassium in KNO_3. The cultivation conditions were similarly with the previous one but only 10 days of incubation was used.

3.14.2.2 Effect of potassium concentration on biodegradation of used oil

For studying the effect of K on used oil degradation, sodium phosphate buffer was used to maintain the pH of the culture medium, NH_4NO_3 was used as a sole N-source. Various K concentrations (0.5,1.0,1.5,2.0 and 3.0 g/l) in the form of KCl were added into this modified BH-medium. 0.5 g of used oil was used as a sole carbon source. The enriched soil microorganism (2% inoculum) was used as an inoculum. The cultivation

condition was 30°C, 200 rpm in a rotary shaker. The samples were taken at day 0, 10 and 20 for analysis of residual oil.

3.14.2.3 Effect of phosphorous source on biodegradation of used oil

For studying the effect of phosphorous in the form of phosphate buffer, NH_4NO_3 was used as N-source. KCl was used as a K supplemental source. Three types of phosphate buffers i.e. sodium, sodium-potassium and potassium phosphate buffer (Section 3.2.3), were studied. Enriched soil microorganisms (2% inoculum) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of various phosphate BH-medium and 0.5 g of used oil. The cultivation condition was 30°C, 200 rpm in a rotary shaker and the samples were taken at day 0, 10, and 20 for analysis of viable cell concentration, pH and residual oil.

3.14.2.4 Effect of surfactant on biodegradation of used oil

Sodium dodecyl sulfate (SDS), TritonX-100 and Tween-80 (they were separately added in the culture medium and found that the amount of cell increased, so it might had no any effect on the microbial growth in this study) were used to study its effect on biodegradation of used oil. Enriched soil microorganisms (2% inoculum) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml modified BH-medium, 0.5 g of used oil and 20% surfactant (5 ml). The cultivation condition was 30°C, 200 rpm in a rotary shaker and the samples were taken at day 0, 10, 20 and 30 for analysis of viable cell concentration, pH and residual oil.

3.14.3 Microbial distribution comparison of enriched soil microorganisms and isolated microorganisms (Microorganism group II)

Either enriched soil microorganisms or microorganism group II were separately used as an inoculum (2%) for biodegradation of used oil (0.5 g) in 50 ml modified BH-medium. During the cultivation periods (day 3, 5, and 7) in a 200 rpm rotary shaker, 30°C, suspensions of both culture medium were taken. After appropriate ten-fold dilution with 0.85% normal saline solution, 0.1 ml of each dilution was spread onto NA plates. The plates were then incubated at 30°C for two days. Colonies, which appeared on the NA plates, were compared with each other. The colonies were then picked and pure isolates obtained by repeated subculturing on NA plates. The isolates were characterized by using Gram's reaction and API kit method. (Appendix B)

3.15 Biodegradation of oil contaminated gravel in a tray

The oil contaminated gravel from Bang Sue Diesel Locomotive Depot (Fig.3-1 and 3-2) was collected to study the biodegradation of used oil in laboratory. The characteristics of gravel, the amount of used oil around the gravel and the amount of nitrogen and phosphorous around the gravel were studied.

3.15.1 Determination of gravel's density

The density of individual gravel was measured by principle of Archimedes's Method. The contaminated gravel (50 pieces) was divided equally into 5 groups. After the contaminated gravel were weighed individually (W), the volume of overflow water replaced by each gravel was measured which is equivalent to the volume of gravel (V). The density of gravel (ρ) is calculated by following equation.

$$\rho_{(g/ml)} = \frac{W\ (g)}{V\ (ml)}$$

The average value of the density was determined in each group. The result was then analyzed by complete randomized design (CRD) for analysis of variance.(appendix A)

Fig. 3-1 Bang Sue Diesel Locomotive Depot

Fig. 3-2 The contaminated gravel at Band Sue Diesel Locomotive Depot

3.15.2 Determination the amount of used oil on gravel

Dried gravel from Section 3.15.1 was extracted by using chloroform solvent. Each gravel was extracted separately in a 100 ml glass bottle. It was added with chloroform (50 ml) and was then shaken vigorously for 5 min. The solvent fraction was collected in a beaker. The gravel was then re-extracted for two times with chloroform (50 ml). After that, the solvent fractions were combined together and filtered through a Whatman filter paper no.40. Na_2SO_4 (approximately 20 g) was added to remove residual water. After the removal of Na_2SO_4 by filtration through a Whatman filter paper no.40, the chloroform fraction was transferred into a 50 ml evaporating flask. Then it was evaporated in a vacuum rotary evaporator under the pressure of 600 mmHg, 40°C. After the disappearance of chloroform solution from the evaporating flask, the mixture was further evaporated for 10 minutes. The residual oil was measured by gravimetric measurement (Section 3.11.2). The amount of used oil on each gravel were reported as the ratio of used oil to unit weight of gravel before extraction as follow

$$\text{Ratio of used oil} = \frac{\text{weight of used oil (g)}}{\text{weight of gravel before extraction (g)}}$$

The same as Section 3.15.1, the average value of ratio used oil in each group were determined and the result was analyzed by complete randomized design (CRD) for analysis of variance (Appendix A).

3.15.3 Selection of suitable solvent for extraction of used oil from the contaminated gravel

Three types of solvent for extraction of oil from the contaminated gravel including chloroform, chloroform : petroleum ether (50:50) and acetone : petroleum ether (50:50) were studied. Each solvent was used to extract 10 pieces of contaminated gravel, which had the same overall characteristics. The experiment was performed in triplicate.

For the extraction step, each gravel was extracted separately as the same procedure as Section 3.15.2 but in different solvents. The average value of ratio used oil from each solvent extraction were determined and analyzed by complete randomized design (CRD) for analysis of variance.

3.15.4 Determination of used oil and nutrients in tray

This experiment was performed in three groups, each group contained three trays and each tray contained 1.5 kg of contaminated gravel. Sterile distilled water (500 ml) was added to cover the gravel in a tray. The trays were shaken at 150 rpm in a controlled environment incubator shaker for 1.5 h. After that the water fraction was separated for analysis of nitrogen and phosphorous (Section 3.12). The contaminated gravel was extracted as the same procedure as Section 3.11.1.2. The amount of used oil (g) in a tray was analyzed by gravimetric measurement. The average value of used oil (g) in each group was determined and analyzed by complete randomized design (CRD) for analysis of variance.

3.15.5 Solid-phase biodegradation of oil contaminated gravel

Solid-phase biodegradation of oil contaminated gravel was studied in laboratory by using open trays (Fig.3-3 and 3-4). Each tray contained approximately 1.5 kg of contaminated gravel. The treatments were performed in 5 groups, which can be described below

Group1 : control group by addition of 6 N H_2SO_4 to control pH at 1-2

Group2 : no addition of nutrients

Group3 : addition of nutrients

Group4 : addition of nutrients and controlled pH at 7 by 1 N NaOH

Group5 : addition of nutrients, enriched soil microorganisms and controlled pH at 7 by 1 N NaOH

During the experiment, the water content was adjusted with sterile water by periodic spraying to the range of 50-80% of the maximum water holding capacity (44). Sometime the acid and base solutions were added in the distilled water to maintain the culture medium around 7. The experiment was carried out in duplicate. The samples were taken at day 0, 7, 15, 30, 60, 90, 135, and 180 for analysis of viable cell concentration, pH, nitrogen, phosphorous and residual oil. (Section 3.15.4)

For the addition of nutrients, the amount of N and P were calculated following the recommendation of Frankenberger (44, C:N:P ratio of 100:10:1). They were applied in the form of NH_4NO_3 and $K_2HPO_4.3H_2O$, respectively. The sterile soil (10 g) was added in every tray as a solid support for the nutrients. In order to avoid leaching or toxicity problems, the total amount of N and P were applied gradually in three fractions and were applied at day 30, 60, and 90.

For the addition of enriched soil microorganisms, 1 l of inoculum solution was prepared (Section 3.9). Spraying of inoculum over the contaminated gravel (10 ml per tray) was carried out for one time at the beginning of experiment. After the incubation time, residual oil, residual nutrients (N,P), and viable cell concentration were determined.

Fig. 3-3 Example of tray with 6 inch diameter and 6 inch height.

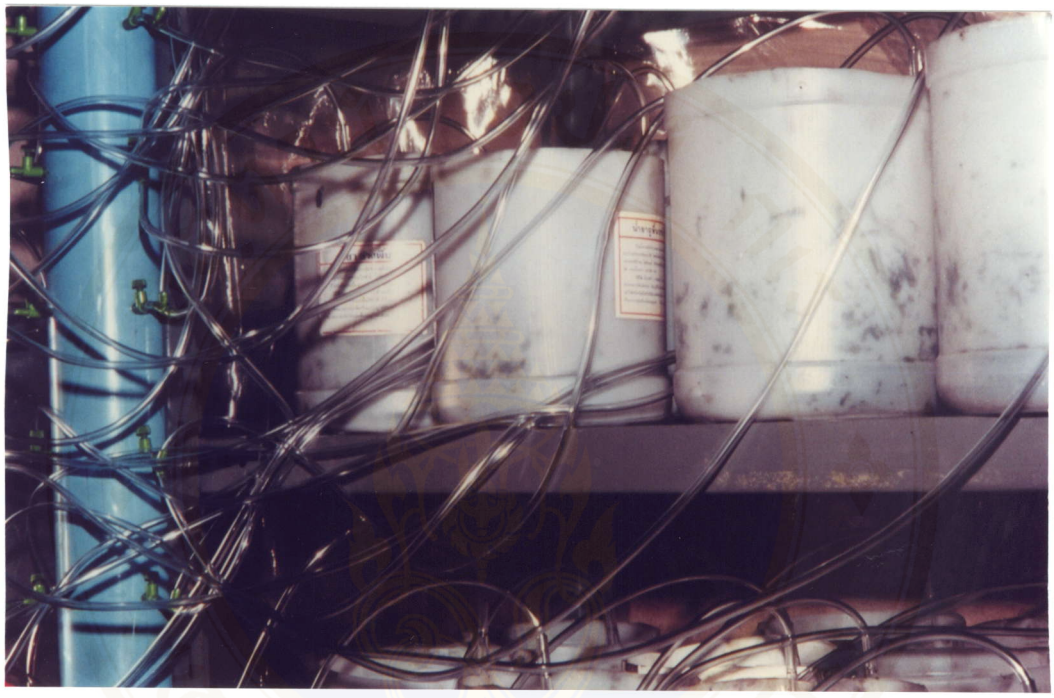


Fig. 3-4 Experimental setting of used oil bioremediation in the tray.

3.15.6 Slurry-phase biodegradation of oil contaminated gravel

Slurry-phase biodegradation of oil contaminated gravel was studied in a tray. Each tray contained 0.5 kg of contaminated gravel. The sterile sodium phosphate BH-medium pH7 (500 ml) was used as the mineral salt medium. The laboratory treatments were performed in 6 groups including

Group1 : control group by addition of NaN_3

Group2 : addition of nutrients

Group3 : addition of nutrients and 2% (v/v) enriched soil microorganisms from Bang sue diesel locomotive depot

Group4 : addition of nutrients and 2% (v/v) microorganism group II

Group5 : addition of nutrients, 2% (w/v) sodium dodecyl sulfate (SDS) and NaN_3

Group6 : addition of nutrients, 2% (w/v) sodium dodecyl sulfate (SDS)

For group 1-4 during the experiment, air was applied into the medium by air bubble at the air flow rate of 3 l/min. For group 5 and 6, the trays were rotary shaken at 100 rpm in a controlled environment incubator shaker (New Brunswick, model G-25KC, USA) instead, to avoid foam problem. NaOH (1 M) was used to adjusted pH of medium around 7. Water losses during incubation were replaced regularly by the addition of sterile water. The trays were covered with plastic sheets, loosely. The experiment was carried out in duplicate. The samples were taken at day 0, 30, 60, 90 and 180 for analysis of viable cell concentration, pH, residual nutrients (N,P) and residual oil (Section 3.15.4).

For the addition of nutrients, phosphorous was added in the form of sodium phosphate buffer, which was also used to maintain the pH of culture medium at 7. KCl was used as a K supplemental source. Ammonium nitrate (NH_4NO_3) was applied as the

nitrogen source. All of them were applied at the beginning of the experiment. For the nitrogen source, it was applied again into the medium when it was unable to detect from the culture sample. The amount of nitrogen is calculated as the same procedure as Section 3.15.5.

For the addition of inoculum in group3 and 4, the preparation of both inoculum are described in Section 3.9.

CHAPTER IV

RESULTS

4.1 Site characterization

4.1.1 Screening of oil degrading microorganisms from Bang Sue Diesel Locomotive Depot

• Using Tapis crude oil as a sole C-source (Microorganism group I)

The samples were collected from 8 sites of Bang Sue Diesel Locomotive Depot for screening of Microorganism group I. Forty-two colonies of oil degrading bacteria were isolated by different colony morphology observation. The sample characteristics, the number of distinguished colonies and the viable cell concentration before and after enrichment method are presented in Table 4-1. Temperature and pH at the site were between 30-32°C and 6.7-7.2, respectively. From preliminary study, most of isolated colonies were Gram's negative bacteria.

• Using used oil as a sole C-source (Microorganism group II)

When used oil, extracted from the contaminated gravel, was used as a sole C-source, fifty-four used oil degrading bacteria were isolated as listed in Table 4-2.

Table 4-1 Sample characteristics for screening of microorganism group I, using Tapis crude oil as a sole C-source at 30°C

Sample code	Sources of sample	Temp (°C)	pH	Number of different colonies	Viable cell before enrichment (CFU/ml or g sample)	Viable cell after enrichment (CFU/ml or g sample)
BC1	Water sample in front of the depot 200 m	32	7	5	1.75×10^6	7.50×10^8
BC2	Water sample in front of the depot 150 m	31	7.2	6	6.20×10^5	8.32×10^8
BC3	Water sample in front of the depot 100 m	31.5	6.7	7	1.01×10^7	1.10×10^9
BC4	Soil sample in front of the depot 50 m	30	7	6	6.40×10^6	5.30×10^8
BC5	Soil sample around the grasses	30	7	5	1.74×10^6	6.20×10^8
BC6	Soil sample inside the depot	32	6.7	3	5.40×10^4	1.20×10^8
BC7	Water sample at the back of the depot 50 m	31.5	7	5	1.06×10^6	7.40×10^8
BC8	Water sample at the back of the depot 100 m	32	7.2	5	5.90×10^6	6.70×10^8

Table 4-2 List of used oil degrading microorganisms (Microorganism group II), using used oil as a sole C-source, incubated at 30°C for 2 days on NA plates.

Microbial code	Colony Morphology					Cell appearance	
	Shape	Surface	Color	Lustrous	Serrate	Gram	Morphology
SS01	Round	Convex	Cream-white	+	-	negative	Rod
SS02	Round	Convex	Cream-orange	+	-	positive	Cocci
SS03	Oval	Convex	Cream-white	+	+/-	positive	Rod
SS04	Round	Convex	Cream-yellow	+	-	negative	Rod
SS05	Round	Flat	Cream-brown	-	+	negative	Rod
SS06	Round	Convex	Cream-white	+	-	negative	Rod
SS07	Oval	Convex	Cream-yellow	+	+/-	negative	Rod
SS08	Round	Convex	Cream-yellow	+	-	positive	Rod
SS09	Round	Convex	Cream-transparent	+	-	positive	Rod
SS10	Round	Convex	Cream-yellow	+	-	negative	Rod

Table 4-2 List of used oil degrading microorganisms (Microorganism group II), using used oil as a sole C-source, incubated at 30°C for 2 days on NA plates. (continued)

Microbial code	Colony Morphology					Cell appearance	
	Shape	Surface	Color	Lustrous	Serrate	Gram	Morphology
SS11	Round	Convex	Cream-yellow	+	-	positive	Cocci
SS12	Oval	Convex	Cream-white	+	+	negative	Rod
SS13	Round	Convex	Yellow-transparent	+	-	negative	Rod
SS14	Round	Convex	Cream-white	+	-	negative	Rod
SS15	Oval	Flat	Yellow-transparent	-	-	positive	Rod
SS16	Round	Convex	Cream-yellow	+	-	positive	Cocci
SS17	Oval	Convex	Cream-yellow	+	+/-	negative	Rod
SS18	Round	Convex	Yellow-transparent	+	-	negative	Rod
SS19	Round	Convex	Yellow-transparent	+	+/-	positive	Rod
SS20	Oval	Convex	Cream-yellow	+	+/-	positive	Rod

Table 4-2 List of used oil degrading microorganisms (Microorganism group II), using used oil as a sole C-source, incubated at 30°C for 2 days on NA plates. (continued)

Microbial code	Colony Morphology					Cell appearance	
	Shape	Surface	Color	Lustrous	Serrate	Gram	Morphology
SS21	Round	Convex	Cream-yellow	+	-	negative	Rod
SS22	Round	Convex	Cream-transparent	+	-	negative	Rod
SS23	Round	Convex	Yellow-transparent	+	+/-	negative	Rod
SS24	Round	Convex	Cream-yellow	+	-	positive	Rod
SS25	Round	Convex	Cream-yellow	+	-	negative	Rod
SS26	Round	Convex	Cream-white	+	-	negative	Rod
SS27	Round	Convex	Cream-white	+	+/-	negative	Rod
SS28	Round	Convex	Cream-orange	+	-	negative	Rod
SS29	Round	Convex	Yellow-transparent	+	-	positive	Rod
SS30	Round	Convex	Cream-yellow	+	+/-	negative	Rod

Table 4-2 List of used oil degrading microorganisms (Microorganism group II), using used oil as a sole C-source, incubated at 30°C for 2 days on NA plates. (continued)

Microbial code	Colony Morphology					Cell appearance	
	Shape	Surface	Color	Lustrous	Serrate	Gram	Morphology
SS31	Round	Convex	Cream-white	+	+/-	negative	Rod
SS31	-	Convex	Yellow-transparent	+	+/-	negative	Rod
SS33	Round	Convex	Cream-brown	+	+/-	negative	Rod
SS34	Round	Convex	Cream-white	+	-	positive	Cocci
SS35	Round	Convex	Cream-transparent	+	-	negative	Cocci
SS36	Round	Convex	Cream-yellow	+	-	negative	Cocci
SS37	-	Convex	Cream-yellow	+	+	positive	Rod
SS38	Round	Convex	Cream-white	+	+/-	positive	Rod
SS39	Round	Convex	Cream-brown	+	+/-	negative	Rod
SS40	Round	Convex	Yellow-transparent	+	+/-	negative	Rod

Table 4-2 List of used oil degrading microorganisms (Microorganism group II), using used oil as a sole C-source, incubated at 30°C for 2 days on NA plates. (continued)

Microbial code	Colony Morphology					Cell appearance	
	Shape	Surface	Color	Lustrous	Serrate	Gram	Morphology
SS41	Round	Convex	Cream-orange	+	+/-	negative	Rod
SS42	Round	Convex	Cream-brown	+	+	negative	Rod
SS43	Round	Convex	Cream-brown	+	+/-	positive	Rod
SS44	Round	Convex	Cream-brown	+	+/-	negative	Rod
SS45	Round	Convex	Cream-transparent	+	-	negative	Rod
SS46	Round	Convex	Cream-white	+	-	positive	Rod
SS47	Round	Convex	Cream-yellow	+	-	negative	Rod
SS48	Round	Convex	Cream-yellow	+	-	negative	Rod
SS49	Round	Convex	Cream-yellow	+	-	negative	Rod
SS50	Round	Convex	Cream-white	+	-	negative	Rod

Table 4-2 List of used oil degrading microorganisms (Microorganism group II), using used oil as a sole C-source, incubated at 30°C for 2 days on NA plates. (continued)

Microbial code	Colony Morphology					Cell appearance	
	Shape	Surface	Color	Lustrous	Serrate	Gram	Morphology
SS51	Round	Convex	Cream-white	+	-	negative	Rod
SS52	Round	Convex	Cream-white	+	-	negative	Rod
SS53	Round	Convex	Yellow-transparent	+	-	negative	Rod
SS54	Round	Convex	Cream-yellow	+	-	negative	Rod

4.1.2 Determination of the density of gravel.

Table 4-3 shows the density of the contaminated gravel from Bang Sue Diesel Locomotive Depot in 5 groups. The average values of density of group 1 to group 5 were 2.74, 2.93, 2.84, 2.69 and 2.60, respectively. The result of statistical analysis comparison by complete randomized design (CRD) for analysis of variance showed that the average values of density of each group was significantly different at 95% confidence.

Table 4-3 The density of the contaminated gravel.

Gravel number	Gravel density				
	Group1	Group2	Group3	Group4	Group5
1	2.86	2.81	2.67	2.58	2.47
2	3.20	2.77	2.96	3.28	2.91
3	2.81	2.77	2.85	2.62	2.81
4	2.80	2.91	2.88	2.45	2.67
5	2.80	3.04	2.72	2.61	2.49
6	2.77	2.75	2.74	2.89	2.71
7	2.83	3.01	3.16	2.59	2.40
8	2.54	2.86	3.14	2.17	2.85
9	2.83	2.72	2.62	3.02	2.69
10	2.83	3.67	2.74	2.69	2.05
Average value	2.74(0.19)	2.93(0.28)	2.84(0.19)	2.69(0.31)	2.60(0.26)

(--), Standard deviation of samples

4.1.3 Determination of the ratios of used oil to unit weight of gravel.

The ratios of used oil on the gravel are shown in Table 4-4. The average values of ratio of used oil from group 1 to group 5 were 0.227, 0.221, 0.176, 0.185 and 0.164, respectively. It was found that the average values of ratio of used oil in each group was not significantly different at 95% confidence.

Table 4-4 The ratios of used oil to unit weight of gravel.

Gravel number	Ratios of used oil to unit weight of gravel				
	Group1	Group2	Group3	Group4	Group5
1	0.22	0.28	0.18	0.33	0.29
2	0.22	0.17	0.39	0.18	0.12
3	0.25	0.15	0.11	0.10	0.19
4	0.13	0.09	0.09	0.20	0.18
5	0.40	0.17	0.11	0.22	0.10
6	0.21	0.34	0.17	0.12	0.06
7	0.33	0.33	0.24	0.20	0.18
8	0.12	0.25	0.13	0.13	0.19
9	0.19	0.21			
10	0.20				
Average value	0.227 (0.084)	0.221 (0.085)	0.176 (0.100)	0.185 (0.073)	0.164 (0.070)

(--), Standard deviation of samples

4.1.4 Determination of the suitable extraction solvent.

Three solvent extraction were evaluated (chloroform, chloroform:petroleum ether, acetone:petroleum ether) for the extraction of used oil. The average values of ratio used oil extracted by various solvents are shown in Table 4-5. The means of average ratio of used oil extracted by chloroform, chloroform:petroleum ether, acetone:petroleum ether solvent were 0.7067, 0.9818 and 0.0915, respectively. The means of average ratio of used oil were significantly different at 95% confidence for acetone and petroleum ether extraction. Chloroform and petroleum ether extraction (50:50) gave the highest amount of extracted oil, whereas acetone and petroleum ether extraction (50:50) gave the lowest. For the chloroform extraction, the average value was 0.7067, which was not significantly different from the chloroform and petroleum ether extraction at 95% confidence.

Table 4-5 The average values of ratio of used oil extracted by various solvents.

Group of sample	The average value of ratio of used oil		
	Chloroform	Chloroform and Petroleum ether	Acetone and Petroleum ether
Group 1	0.7512(0.12)*	0.8924(0.08)	0.0812(0.03)
Group 2	0.6698(0.09)	1.1573(0.15)	0.1050(0.05)
Group 3	0.6992(0.10)	0.8958(0.11)	0.0915(0.07)
Average value	0.7067(0.04)**	0.9817(0.15)	0.0915(0.01)

*(--), Standard deviation of samples : * ; n = 10, ** ; n = 3*

4.1.5 Determination of the used oil and nutrients on gravel.

Table 4-6 shows the amount of used oil, which was extracted from approximately 1500 g of contaminated gravel. The average values of used oil from group 1 to group 3 were 11.777 g, 12.020 g and 13.236 g. The average values of used oil in each group was not significantly different at 95% confidence. For the amount of nitrogen (N) and phosphorous (P) on the gravel, the average values of N concentration in the form of NO_3-N was not significantly different at 95% confidence and also the same as the average values of P concentration in the form of PO_4-P. Very small amount of NO_3-N and PO_4-P were found on the gravel surface. Only approximately 0.068 mg/l of NO_3-N and 0.472 mg/l of PO_4-P were detected. In case of NH_4-N, it was unable to detect from the culture broth.

Table 4-6 The amount of used oil and nutrients (NO_3-N, PO_4-P and NH_4-N), which was extracted from of contaminated gravel.

	Group 1			Group 2			Group 3		
	1	2	3	1	2	3	1	2	3
Gravel weight (g)	1515	1504	1508	1510	1522	1508	1511	1516	1505
Used oil (g)*	13.44	9.75	12.14	12.37	13.03	10.65	13.50	13.44	12.75
NO_3-N (mg/l)**	0.072	0.066	0.072	0.060	0.076	0.064	0.080	0.068	0.056
NH_4-N (mg/l)	-	-	-	-	-	-	-	-	-
PO_4-P (mg/l)***	0.515	0.469	0.479	0.453	0.464	0.469	0.448	0.489	0.464

**The average value of used oil (g) of group 1, 2 and 3 are 11.77, 12.02 and 13.23.*

***The average value of NO_3-N (mg/l) of group 1, 2 and 3 are 0.070, 0.067 and 0.068.*

****The average value of PO_4-P (mg/l) of group 1, 2 and 3 are 0.488, 0.462 and 0.467.*

4.1.6 Composition of used oil, diesel oil and motor by packed column gas chromatography.

Fig 4-1 illustrates the GC chromatograms of hexane soluble fraction of various hydrocarbon compounds, including used oil, diesel oil, motor oil, Tapis crude oil and standard hydrocarbons, from n-dodecane (C12) to n-heneicosane (C21). The retention time of standard hydrocarbons and the major peaks of Tapis crude oil are shown in Appendix D. Motor oil and used oil mostly contained high molecular weight hydrocarbons. Motor oil contained one combined peak of the high molecular weight hydrocarbons at the retention time of 24.11±0.72. Whereas, diesel oil and Tapis crude oil mostly contained the low molecular weight hydrocarbons. Comparing to the standard hydrocarbons, most of them were ranged between C6-C21. The retention time of the internal standard (IS), 1-Eicosene, was 14.31±0.65.

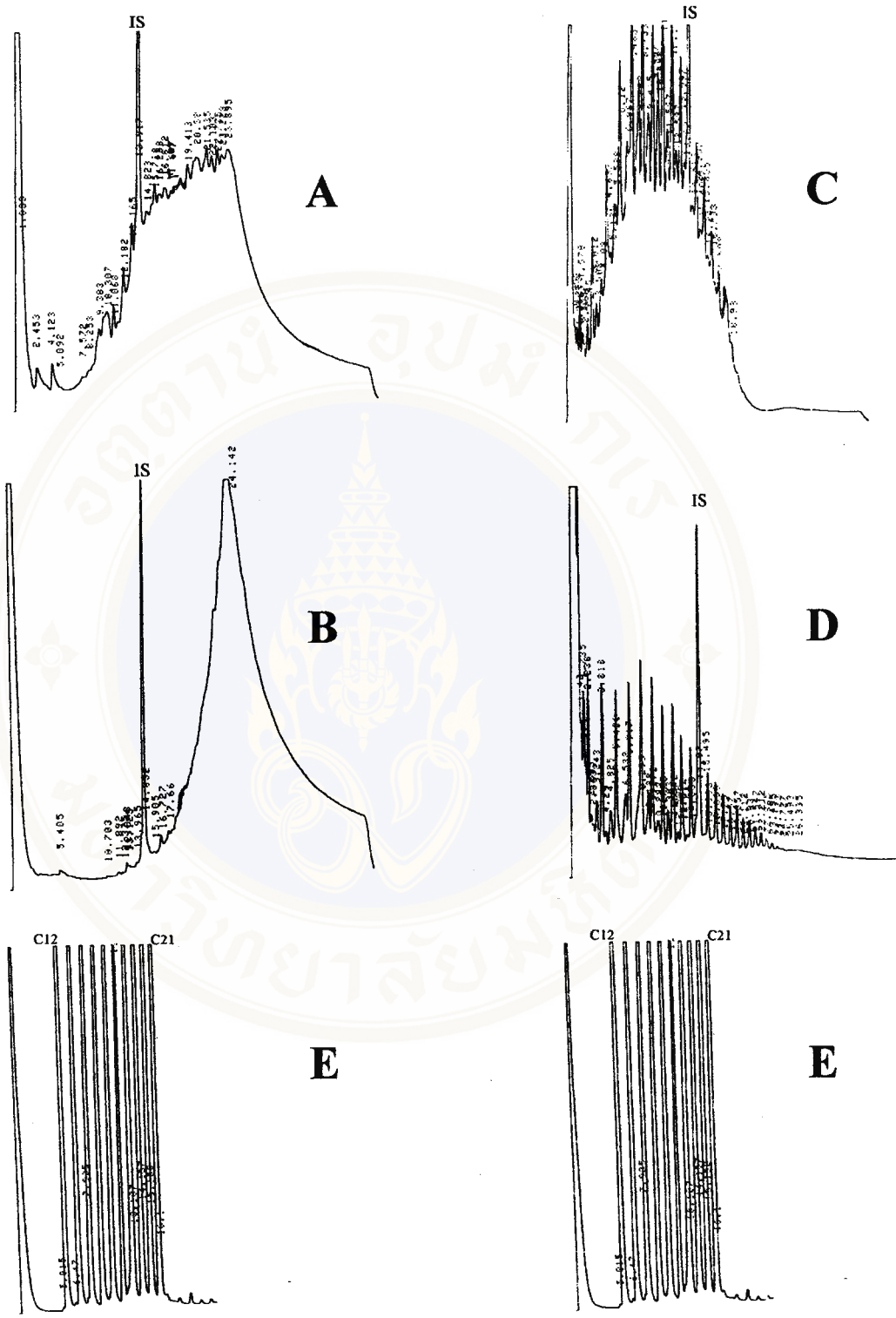


Fig. 4-1 GC chromatogram of used oil (A), motor oil (B), diesel oil (C), Tapis crude oil (D) and standard hydrocarbon n-dodecane to n-heneicosane (E).

4.2 Biodegradation of oil in shake flask (liquid-phase bioremediation)

4.2.1 Abiotic degradation of oil (used, diesel and motor oil) in BH-broth.

The results of abiotic degradation by weight loss of three oils are presented in Table 4-7. After incubation for 30 days, the abiotic degradation of used oil and motor oil were very low (<1%). For diesel oil, the abiotic degradation was highest (16.4%).

GC chromatograms of hexane soluble fraction of these oils are presented in Fig.4-2 and the percentage of residual oil were calculated (Table 4-8). Hexane soluble fraction of the diesel oil decreased continuously (16.8% reduction after incubation for 30 days). Abiotic reduction occurred rapidly during the first ten days. Whereas, the abiotic reduction of motor oil and used oil were very low. As shown in Fig.4-2, the residual amount of short chain alkanes decreased faster than that of longer chain alkanes. Moreover the percentage of reduction mostly occurred in hexane soluble fraction.

Table 4-7 Abiotic degradation of chloroform soluble fraction of used oil, diesel oil and motor oil during 30 days of incubation by gravimatric measurement.

Type of oil	Residual oil* (g)			
	Day 0	Day 10	Day 20	Day 30
Used oil	0.4924±0.0078	0.4905±0.0099	0.4821±0.0015	0.4976±0.0001
	(100%)	(99.6%)	(99.9%)	(100%)
Motor oil	0.5095±0.0070	0.5097±0.0040	0.5044±0.0097	0.5050±0.0043
	(100%)	(100%)	(99.0%)	(99.1%)
Diesel oil	0.5004±0.0039	0.4523±0.0025	0.4448±0.0005	0.4183±0.0070
	(100%)	(90.4%)	(88.9%)	(83.6%)

**, Average of 2 samples ± Standard error of duplication*
(–), The percentage of residual oil were based on the control days 0 as 100%.

Table 4-8 Abiotic degradation of hexane soluble fraction of used oil, diesel oil and motor oil during 30 days of incubation by pack column gas chromatography.

Type of oil	Residual oil (%)			
	Day 0	Day 10	Day 20	Day 30
Used oil	100	92.9	93.9	93.9
Motor oil	100	98.8	96.0	96.9
Diesel oil	100	89.7	86.6	83.2

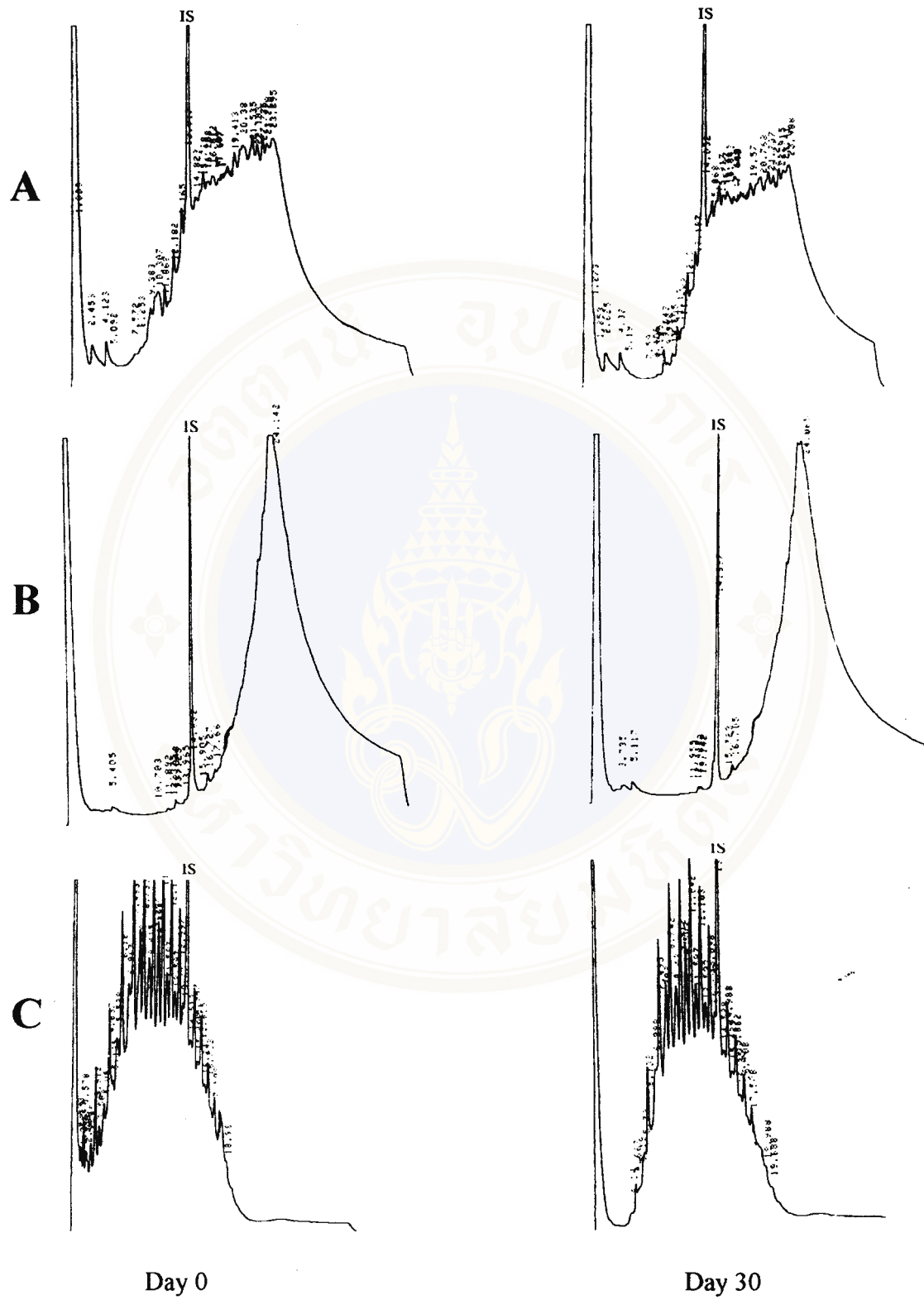


Fig.4-2 GC chromatograms of the residual oil (A: used oil, B: motor oil and C: diesel oil) under abiotic losses (control flasks) at day 0 and day 30 in 50 ml BH-broth + 0.5 g oil+0.5% (w/v) NaN_3, incubated in a rotary shaker (200 rpm) at 30°C.

4.2.2 Determination of used oil degrading microorganisms

4.2.2.1 Biodegradation of used oil by *Acinetobacter* sp. M1407, microorganism group I and enriched soil microorganisms (Preliminary study)

Fig. 4-3 illustrates the growth kinetics of inoculum cultures of the enriched soil microorganisms, microorganism group I and M1407. Viable growth of enriched soil microorganisms increased rapidly within 1 day from 7.1 x 10^6 CFU/ml to 1.6 x 10^8 CFU/ml. However, growth of this mixed culture decreased at the beginning of day 2 and remained constant at 3.01±2.5 x 10^8 CFU/ml from day 3 throughout day 7.

For the growth kinetics of microorganism group I and M1407, the initial viable cell concentration were 4.2 x 10^7 CFU/ml and 5.3 x 10^6 CFU/ml, respectively. Viable growth of them increased rapidly with 2 days and remained in the stationary phase at the level of 3.58±0.52 x 10^9 CFU/ml and 2.70±0.49 x 10^8 CFU/ml, respectively through day 7.

Table 4-9 shows the results of gravimetric analysis of residual used oil over 45 days period of incubation of three groups of microorganism. The degradation of used oil by enriched soil microorganisms increased rapidly (27% weight loss) during the first 15-day period. For the last 30 days, the oil weight loss (2%) decreased slightly. During 45 days of incubation the enriched soil microorganisms was able to reduce approximately 30 % of used oil. For microorganism group I and M1407, similar patterns of degradation were obtained. However, the overall weight loss over 45 days were 19% and 14%, respectively.

Investigation of nitrogen and phosphorous concentrations in the culture broth are illustrated in Fig. 4-4, the amount of ammonia-nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N) and phosphate-phosphorous (PO_4-P) still remained over the 45-days of

incubation. The relationship between the total petroleum hydrocarbon (TPH), nitrogen and phosphorous was observed and the ratio of TPH:N:P was 100:3:1.

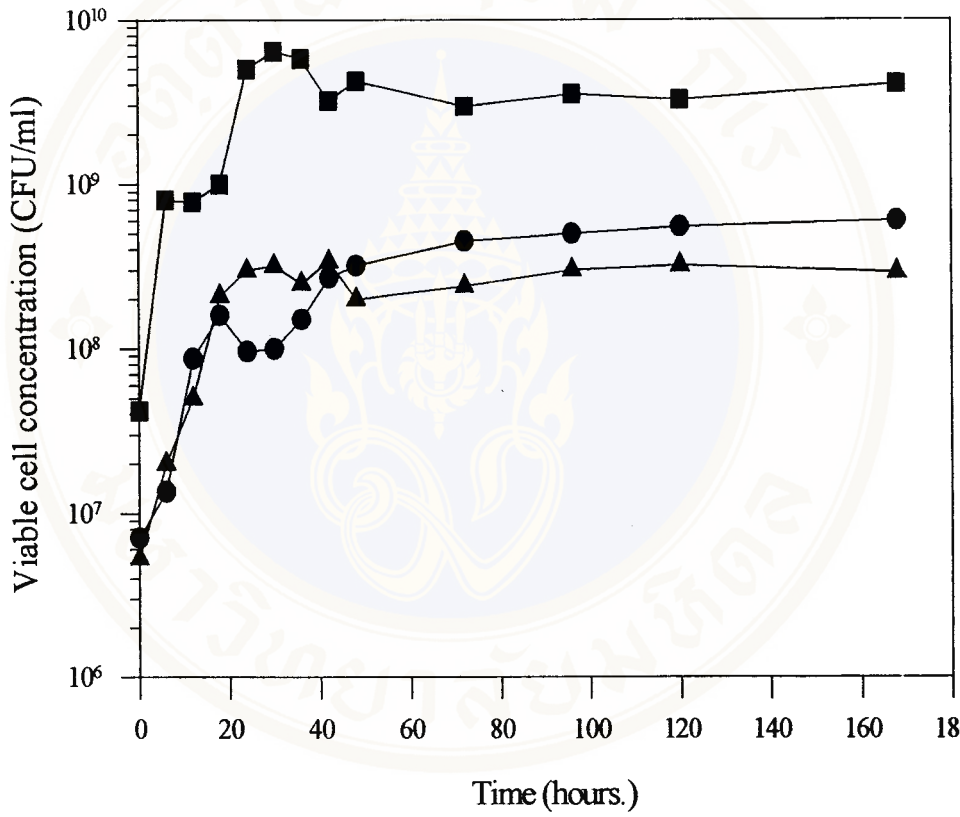


Fig. 4-3 Growth kinetics of enriched soil microorganisms ; (●), microorganism group II ; (■) in 50 ml modified BH-medium with 0.5 g used oil and *Acinetobacter* sp. M1407 ; (▲) in 50 ml modified BH-medium with 0.5 g Tapis crude oil, incubated at 30°C, 200 rpm in a rotary shaker.

Table 4-9 Gravimetric analysis of residual used oil by the degradation activities of enriched soil microorganisms, microorganism group I and *Acinetobacter* sp. M1407 at various days of incubation in 50 ml BH-broth, at 30°C, 200 rpm in a rotary shaker.

Microorganisms	Residual used oil* (g)			
	Day 0	Day 15	Day 30	Day 45
Abiotic losses**	0.4975±0.0024 (100%)	0.4992±0.0112 (100%)	0.4832±0.0053 (97%)	0.4827±0.0042 (97%)
Control without inoculum	0.5010±0.0039 (100%)	0.4379±0.0020 (87%)	0.4532±0.0005 (90%)	0.4419±0.0052 (88%)
Enriched soil microorganisms	0.4940±0.0031 (100%)	0.3664±0.0004 (73%)	0.3428±0.0028 (68%)	0.3511±0.0041 (71%)
Microorganisms group I	0.5049±0.0001 (100%)	0.4369±0.0008 (87%)	0.4255±0.0153 (85%)	0.4056±0.0044 (81%)
Acinetobacter sp. M1407	0.5075±0.0056 (100%)	0.4400±0.0002 (88%)	0.4232±0.0071 (84%)	0.4313±0.0063 (86%)

**, Average of two samples ± Standard error of duplication*
***, Abiotic losses : addition of 0.5 % (w/v) NaN_3*
(--), The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value = 0.5010, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).

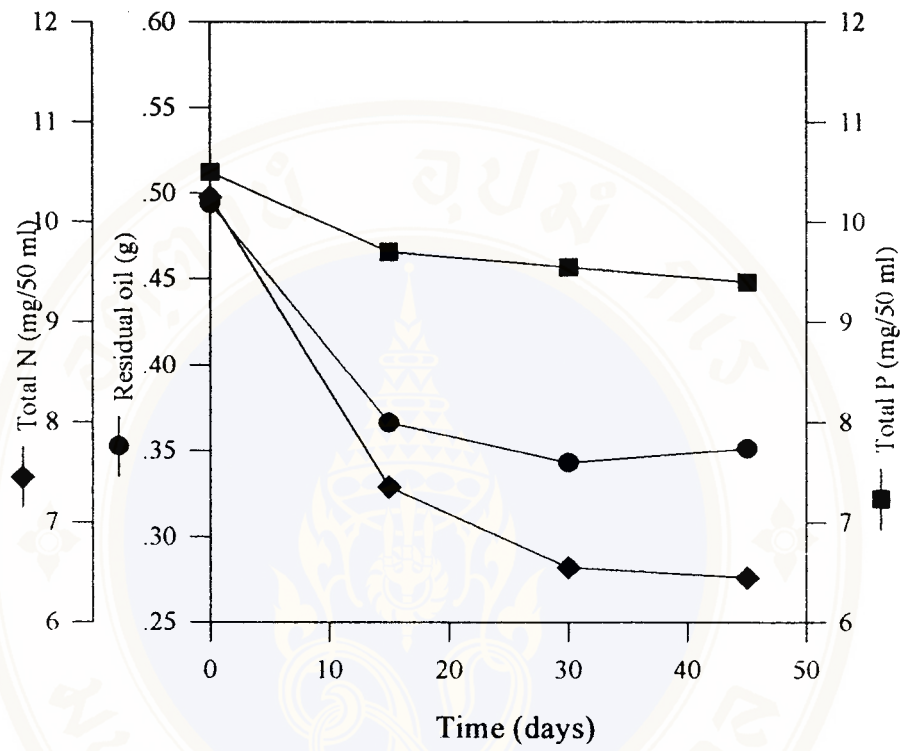


Fig 4-4 Consumption of nitrogen and phosphorous over 45 days period degradation of used oil in 50 ml BH-medium by enriched soil microorganisms, incubated at 30°C, 200 rpm in a rotary shaker.

4.2.2.2 Biodegradation of used oil, diesel oil and motor oil by enriched soil microorganisms and microorganism group II

Table 4-10, 4-11 and 4-12 shows the results of gravimetric analysis during 30 day period of experiment of the enriched soil microorganisms and microorganism group II.

Table 4-10 shows the reduction of used oil. The degradation activity of enriched soil microorganisms was better than microorganism group II (60.8% reduction after incubation for 30 days). The reduction rate was occurred rapidly during the first 10 days (52.3%) after that the reduction rate slightly decreased (8.5%). Whereas, microorganism group II was unable to reduce this oil.

Table 4-11 shows the reduction of motor oil. This also found that, the degradation activity of enriched soil microorganisms was better than that of microorganism group II (51.2% reduction after incubation for 30 days). The reduction rate was occurred rapidly during the first 10 days (19.5%) after that the reduction rate consecutively decreased (31.7%). This is also, microorganism group II was unable to reduce this oil.

Table 4-12 shows the reduction of diesel oil. The enriched soil microorganisms was able to reduce 70% of oil after incubation for 30 days and the decreasing rate was rapidly occurred during the first 10 days also (44.6%) after that the reduction rate consecutively decreased (24.3%). Microorganism group II was unable to use this oil as a sole C-source better than those of two oils. Since it was able to reduce 38.1% of oil after incubation for 30 days.

Table 4-10 Biodegradation of used oil (chloroform soluble fraction) by enriched soil microorganisms and microorganism group II in 50 ml modified BH-medium, buffered with 0.1 M sodium phosphate buffer, incubated at 30°C, rotary shaken at 200 rpm for 30 days.

Incubation time (Days)	Residual used oil* (g)		
	Control (0.5% NaN_3)	Enriched soil microorganisms	Microorganism group II
Day 0	0.4924±0.0046 (100%)	ND	ND
Day 10	0.4905±0.0099 (99.6%)	0.2351±0.0099 (47.7%)	0.4801±0.0009 (97.6%)
Day 20	0.4821±0.0018 (99.9%)	0.2144±0.0018 (43.5%)	0.4848±0.0190 (99.4%)
Day 30	0.4916±0.0001 (99.8%)	0.1928±0.0114 (39.2%)	0.4787±0.0015 (97.2%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual used oil were based on the residual used oil of the control day 0 as 100%.
ND, Not determined

Table 4-11 Biodegradation of motor oil (chloroform soluble fraction) by enriched soil microorganisms and microorganism group II in 50 ml modified BH-medium, buffered with 0.1 M sodium phosphate buffer, incubated at 30°C, rotary shaken at 200 rpm for 30 days.

Incubation time (Days)	Residual motor oil* (g)		
	Control (0.5% NaN_3)	Enriched soil microorganisms	Microorganism group II
Day 0	0.5095±0.007 (100%)	ND	ND
Day 10	0.5097±0.0040 (100%)	0.4100±0.0300 (80.8%)	0.5049±0.0050 (99.6%)
Day 20	0.5044±0.0097 (99.0%)	0.2921±0.0030 (57.6%)	0.4994±0.0036 (98.5%)
Day 30	0.5050±0.0043 (99.1%)	0.2485±0.0181 (49.0%)	0.4950±0.0092 (97.6%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual motor oil were based on the residual motor oil of the control day 0 as 100%.
ND, Not determined

Table 4-12 Biodegradation of diesel oil (chloroform soluble fraction) by enriched soil microorganisms and microorganism group II in 50 ml modified BH-medium, buffered with 0.1 M sodium phosphate buffer, incubated at 30°C, rotary shaken at 200 rpm for 30 days.

Incubation time (Days)	Residual diesel oil* (g)		
	Control (0.5% NaN_3)	Enriched soil microorganisms	Microorganism group II
Day 0	0.5004±0.0039 (100%)	ND	ND
Day 10	0.4523±0.0025 (90.4%)	0.2774±0.0400 (55.4%)	0.3812±0.0055 (76.2%)
Day 20	0.4448±0.0005 (88.9%)	0.2374±0.0111 (47.5%)	0.3631±0.0030 (72.6%)
Day 30	0.4183±0.0070 (83.6%)	0.1555±0.0042 (31.1%)	0.3396±0.0027 (61.9%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual diesel oil were based on the residual diesel oil of the control day 0 as 100%.
ND, Not determined

Fig. 4-5 illustrates the reduction patterns of hexane soluble fractions of used oil (A), motor oil (B) and diesel oil (C) by enriched soil microorganisms, which was analyzed by packed column gas chromatography. The low molecular weight hydrocarbons were reduced rapidly more than the high molecular weight hydrocarbons.

The results of percentage reduction of residual used oil, motor oil and diesel oil (hexane soluble fraction) by enriched soil microorganisms and microorganism group II are shown in Table 4-13, 4-14 and 4-15, respectively.

For the enriched soil microorganisms, used oil and motor oil were degraded rapidly during the first 10 days (57.2% and 35.2% reduction, respectively). In the last 20 days, the utilization of used oil and motor oil decreased slightly (6% and 21% reduction, respectively). In case of microorganism group II, the reduction of used oil and motor oil, after 30 days of incubation, were 18.2% and 25.1%, respectively. For diesel oil, the reduction at the first 10 days was 62.8%, in the last 20 days, the reduction slightly decreased (10.4%). Whereas, microorganism group II was able to use diesel oil as a sole C-source better than used oil and motor oil (39.7% reduction in the first 10 days after that the reduction slightly decreased (14%)).

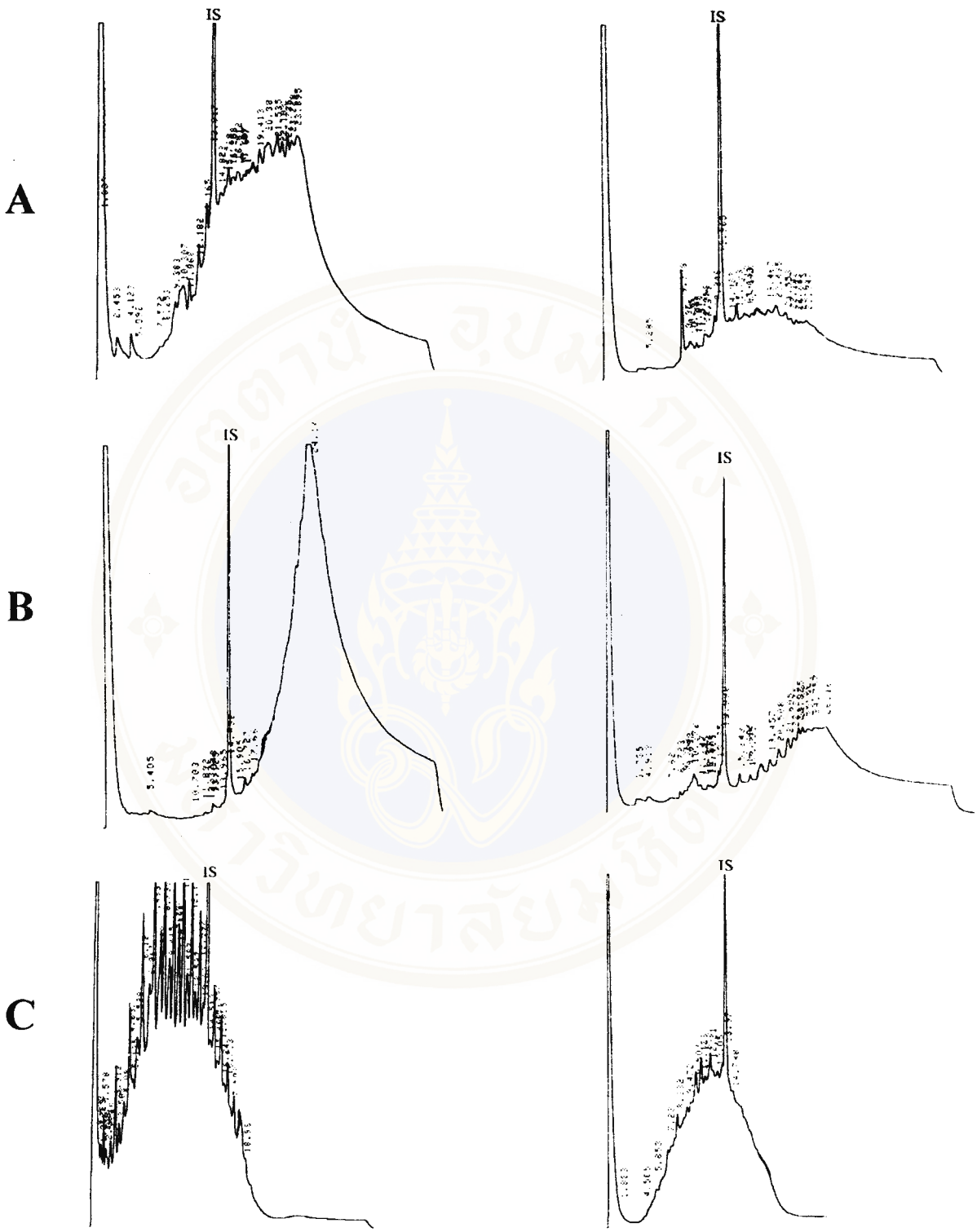


Fig 4-5 GC chromatogram of the hexane soluble fraction with three types of C-source over the 30-days period of incubation by enriched soil microorganisms (A, used oil; B, motor oil and C, diesel oil).

Table 4-13 Biodegradation of used oil (hexane soluble fraction) by enriched soil microorganisms and microorganism group II in 50 ml modified BH-medium, buffered with 0.1 M sodium phosphate buffer, incubated at 30°C, rotary shaken at 200 rpm for 30 days.

Incubation time (Days)	Residual used oil (%)		
	Control (0.5% NaN_3)	Enriched soil microorganisms	Microorganism group II
Day 0	100	ND	ND
Day 10	92.9	42.8	84.4
Day 20	93.9	39.3	80.8
Day 30	93.9	36.8	81.8

Table 4-14 Biodegradation of motor oil (hexane soluble fraction) by enriched soil microorganisms and microorganism group II in 50 ml modified BH-medium, buffered with 0.1 M sodium phosphate buffer, incubated at 30°C, rotary shaken at 200 rpm for 30 days.

Incubation time (Days)	Residual motor oil (%)		
	Control (0.5% NaN_3)	Enriched soil microorganisms	Microorganism group II
Day 0	100	ND	ND
Day 10	98.8	64.8	90.1
Day 20	96.0	51.2	84.5
Day 30	96.9	43.6	74.9

Table 4-15 Biodegradation of diesel oil (hexane soluble fraction) by enriched soil microorganisms and microorganism group II in 50 ml modified BH-medium, buffered with 0.1 M sodium phosphate buffer, incubated at 30°C, rotary shaken at 200 rpm for 30 days.

Incubation time (Days)	Residual diesel oil (%)		
	Control (0.5% NaN_3)	Enriched soil microorganisms	Microorganism group II
Day 0	100	ND	ND
Day 10	89.7	37.2	60.3
Day 20	86.6	31.2	53.9
Day 30	83.2	26.8	46.3

ND, Not determined

Fig.4-6 illustrates the viable cell concentration of enriched soil microorganisms and microorganism group II from experiments with three types of C-source (used oil (a), motor oil (b) and diesel oil (c)).

In Fig. 4-6a, the initial viable cell concentration of enriched soil microorganisms was 5.5 x 10^6 CFU/ml, whereas, the initial viable cell concentration of microorganism group II was 4.3 x 10^7 CFU/ml. After 5 days of incubation viable cell concentration in both groups remained constant at 3.45±1.26 x 10^8 CFU/ml and 6.36±2.08 x 10^8 CFU/ml, respectively.

In Fig. 4-6b, the initial viable cell concentration of enriched soil microorganisms was 5.0 x 10^6 CFU/ml, whereas, the initial viable cell concentration of microorganism group II was 5.2 x 10^7 CFU/ml. After 5 days of incubation viable cell concentration in both groups remained constant at 2.99±1.08 x 10^8 CFU/ml and 2.39±1.12 x 10^8 CFU/ml, respectively.

In Fig. 4-6c, the initial viable cell concentration of enriched soil microorganisms was 5.2 x 10^6 CFU/ml after 5 days of incubation viable cell concentration remained constant at 2.98±1.12 x 10^8 CFU/ml. However, it was slightly decreased at the end of experiment. For the viable cell concentration of microorganism group II, it was started with 6.3 x 10^7 CFU/ml and remained constant at 1.47±0.73 x 10^9 CFU/ml after 5 days of incubation.

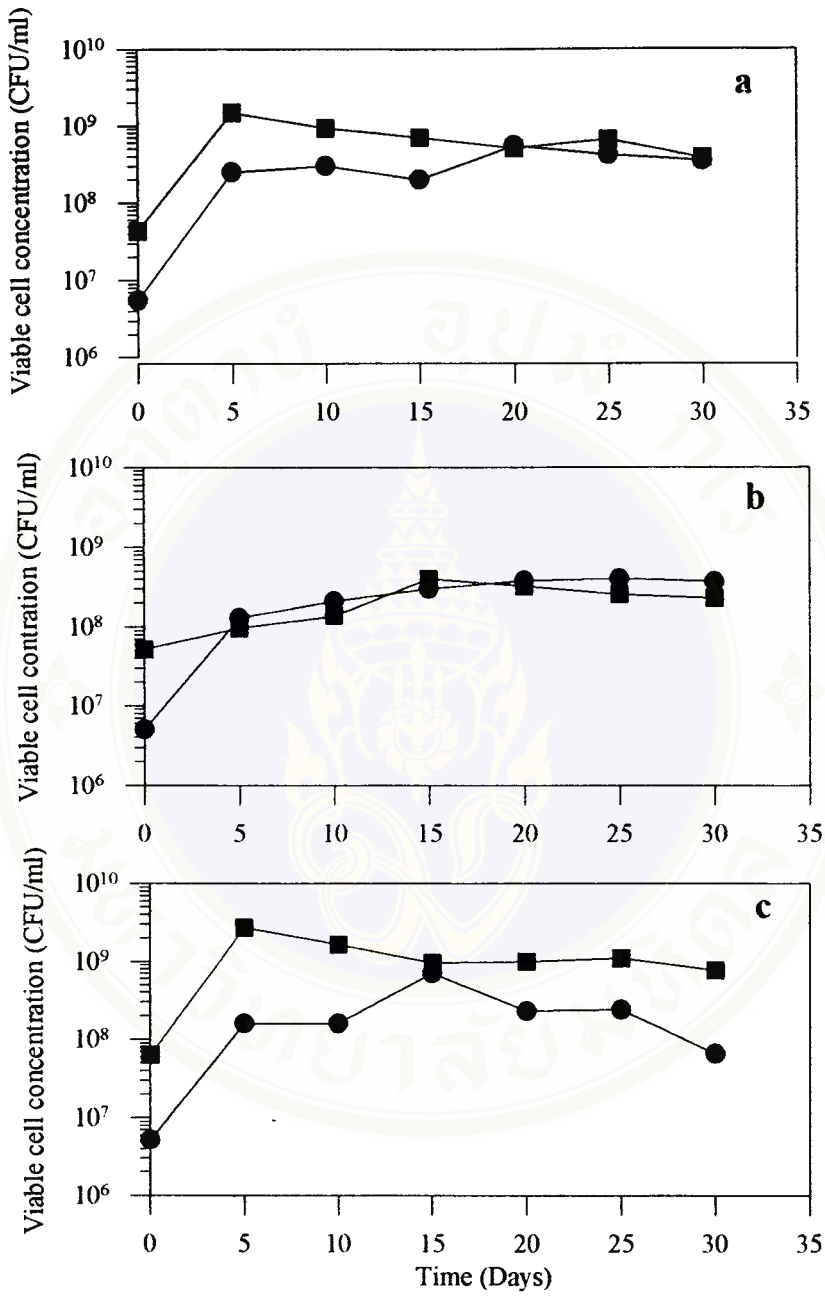
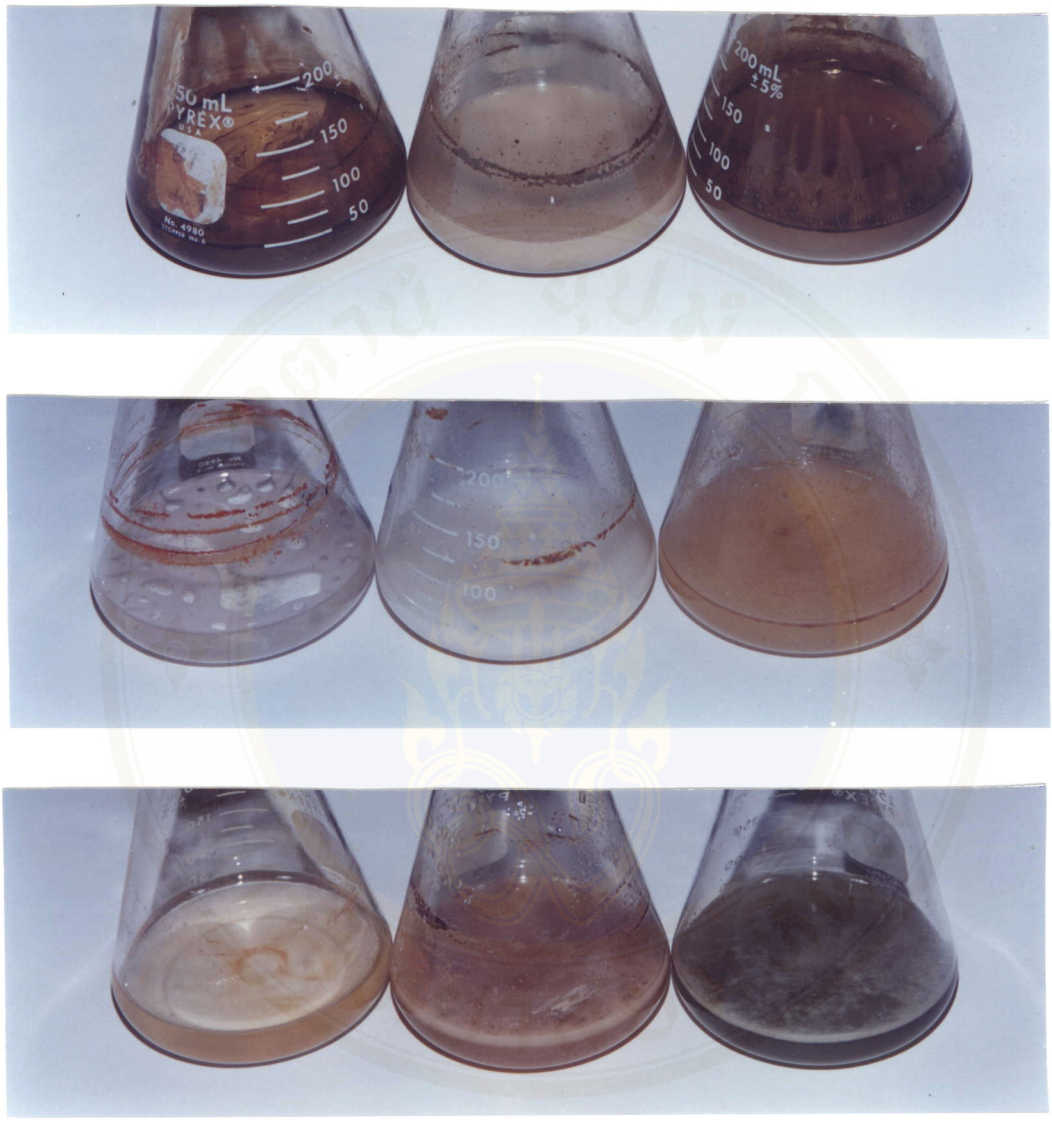


Fig. 4-6 Growth kinetics of the enriched soil microorganisms (●) and microorganism group II (■) with three types of C-source, incubated at 30°C, 200 rpm in a rotary shaker for 30 days (a, used oil ; b, motor oil and c, diesel oil)

The obvious changes of oil in flasks inoculated with enriched soil microorganisms and microorganism group II are illustrated in Fig. 4-7. When enriched soil microorganisms grew in BH-oil broth, some of oil (especially used oil and motor oil) appeared in small droplets, dispersed and forming a suspension of oil. Oil layer disappeared and the whole broth became an emulsion. However, the growth of microorganisms group II in BH-used oil broth was not observed. For BH-motor oil broth, the culture broth was slightly changed but the oil layer still remained. While, the color of BH-diesel oil broth was changed into green color and the oil layer disappeared.



Control Enriched soil microorganisms Microorganism group II

Fig. 4-7 The culture broth of three different substrates (a, used oil; b, motor oil; c, diesel oil) inoculated with enriched soil microorganisms and microorganism group II, incubated at 30°C, 200 rpm in a rotary shaker for 30 days.

4.2.2.3 Biodegradation of used oil on BH-oil agar plates

The degradation activities of 54 pure cultures of microorganisms group II on used oil were studied. During 7 day incubation at 30°C, colonies with early appearance and large colony diameter or clear zone were selected, i.e. SS01, SS05, SS10, SS14, SS17, SS23, SS24, SS34, SS38, SS41, SS42, SS46, SS50, SS53, and SS54. These microorganisms were catagorized as microorganism group III.

4.2.2.4 Biodegradation of used oil by microorganism group III in liquid culture

The degradation activities of the selected microorganisms from microorganism group III on used oil for 30 days are shown in Table 4-17. SS10 and SS42 were able to reduce the used oil better than the others (24.1% and 22.8%, respectively). In case of mixed culture of microorganism group III, the degradation activity was very low (8.9% reduction).

Table 4-17 Biodegradation of used oil by pure cultures and groups of microorganism group III in 50 ml of sodium phosphate BH-medium, incubated at 30°C 200 rpm in a rotary shaker for 30 days.

Organisms	Residual oil*	Residual used oil (%)
Control (NaN_3)	0.5003 ± 0.0010	100
SS01	0.4403 ± 0.0036	87.0
SS05	0.4510 ± 0.0022	89.5
SS10	0.3864 ± 0 .0114	75.9
SS14	0.4738 ± 0.0050	93.1
SS17	0.4539 ± 0.0008	89.7
SS23	0.4786 ± 0.0211	92.3
SS24	0.4110 ± 0.0004	82.2
SS34	0.4253 ± 0.0075	83.3
SS38	0.4482 ± 0.0003	83.3
SS41	0.4406 ± 0.0111	88 0
SS42	0.3933 ± 0.0080	77.2
SS46	0.4902 ± 0.0066	94.7
SS50	0.4213 ± 0.0031	84.5
SS53	0.4159 ± 0.0253	83.3
SS54	0.4591 ± 0.0237	82.4
Mixed microorganism G III		91.1

*, *Average of 2 samples ± Standard error of duplication*

4.2.2.5 Effect of soil on used oil degradation

Soil which, may have an effect on used oil degradation, was studied. The results of gravimetric analysis of residual oil are shown in Table 4-18. During 30 days incubation, weights of residual oil of microorganism group III + sterile soil and control (NaN_3) + sterile soil treatments reduced in similar pattern. While a significant decrease (43.2%) of residual oil in enriched soil microorganism treatment was obtained. No abiotic degradation appeared in the control (NaN_3) treatment.

Table 4-18 Effect of soil on used oil degradation by microorganisms group III (MGIII) and the enriched soil microorganisms in 50 ml of sodium phosphate BH-medium, incubated at 30°C 200 rpm in a rotary shaker for 30 days.

Condition	Residual used oil* (g)			
	Day 0	Day 10	Day 20	Day 30
control(NaN_3)	0.5010±0.0030 (100%)	0.4991±0.0090 (99.3%)	0.4836±0.0280 (96.2%)	0.5022±0.0040 (100%)
control(NaN_3) +sterile soil	0.5011±0.005 (100%)	0.4653±0.0009 (92.8%)	0.4600±0.0324 (91.7%)	0.4468±0.0122 (89.2%)
Enriched soil microorganisms	0.5036±0.0060 (100%)	0.2473±0.0130 (49.2%)	0.2306±0.0080 (45.9%)	0.2173±0.0030 (43.2%)
MGIII+sterile soil	0.5023±0.0001 (100%)	0.4767±0.0024 (94.8%)	0.4611±0.0070 (91.8%)	0.4467±0.0060 (88.9%)

**, Average of 2 samples ± Standard error of duplication*
(–), The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value = 0.5020, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).

4.2.3 Effect of medium composition on used oil degradation.

4.2.3.1 Effect of nitrogen source on used oil degradation

The gravimetric measurement of residual oil during 30 days incubation with effect of various N-sources is shown in Table 4-19. When KNO_3 was used as a sole N-source, 48.7% of residual oil reduced after 30 days incubation. The reduction rate was very rapidly during the first 15 days. For the other N-sources, the reductions were low (3.7-9.2% after 30 days incubation). No abiotic degradation occurred in the control (0.5%NaN_3) treatment. Fig 4-8 illustrates the viable cell concentration in various N-source treatments during 30 days incubation. The initial viable cell concentration was approximately 2.3 x 10^6 CFU/ml. The viable cell concentration between each group did not differ much and the concentration of cell increased rapidly to 10^8 CFU/ml in 5 days in all N-sources. Thereafter, viable cell concentration in all treatments remained stable in the stationary phase throughout day 30.

Table 4-20 shows residual used oil when all other media contained equivalent amount of potassium (as KCl 1.86 g/l) as one with KNO_3. NH_4NO_3 gave the highest percentage reduction of used oil (57.2%). KNO_3 gave lower reduction (40.6%) than NH_4NO_3 did. For $NaNO_3$, NH_4Cl and Urea, the reductions were lower than that of NH_4NO_3 and KNO_3 (20.7%, 22.3% and 30.3% reduction, respectively).

Table 4-19 Effect of nitrogen source on used oil degradation by enriched soil microorganisms in 50 ml of sodium phosphate BH-medium, incubated at 30°C 200 rpm in rotary shaker for 30 days.

Nitrogen-source	Residual used oil* (g)		
	Day 0	Day 15	Day 30
Control (NaN_3)	ND	0.5050±0.0041	0.5031±0.0060
		(100%)	(100%)
NH_4NO_3	0.5089±0.0048	0.4733±0.0008	0.4614±0.0027
	(100%)	(94.6%)	(92.2%)
KNO_3	0.4948±0.0011	0.3083±0.0281	0.2566±0.0011
	(100%)	(61.6%)	(51.3%)
$NaNO_3$	0.5017±0.0050	0.4816±0.0001	0.4634±0.0012
	(100%)	(96.3%)	(92.6%)
NH_4Cl	0.5007±0.0025	0.5051±0.0059	0.4821±0.0008
	(100%)	(100%)	(96.3%)
Urea	0.5036±0.0010	0.4625±0.0004	0.4573±0.0031
	(100%)	(91.8%)	(90.8%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value = 0.5019, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).
ND, Not determined

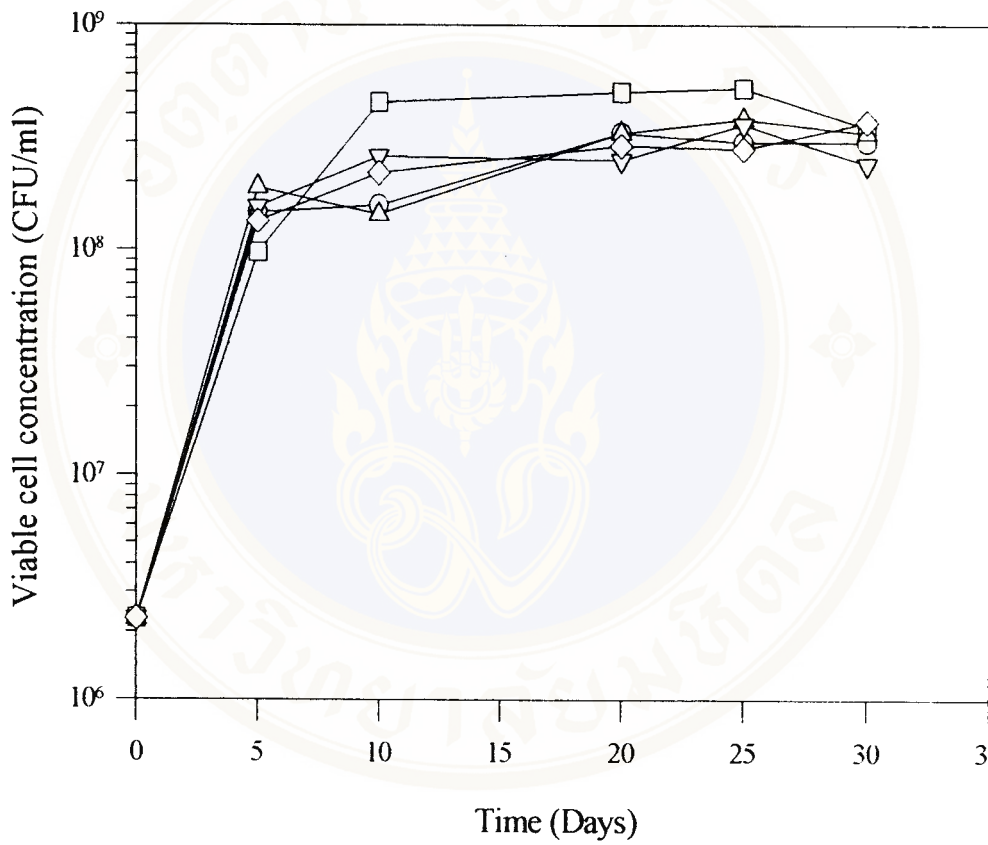


Fig. 4-8 Growth kinetics of the enriched soil microorganisms with five N-sources (NH_4NO_3, ○ ; KNO_3, □ ; NH_4Cl, △ ; Urea , ▽ and $NaNO_3$, ◇) in modified BH-medium with 0.5 g used oil, incubated at 30°C, 200 rpm in a rotary shaker for 30 days

Table 4-20 Effect of nitrogen source on used oil degradation by enriched soil microorganisms in 50 ml of sodium phosphate BH-medium + 1.86 g/l KCl (except one with KNO_3), incubated at 30°C 200 rpm in rotary shaker for 10 days.

Nitrogen source	Residual used oil* (g)	
	Day 0	Day 10
Control (NaN_3)	ND	0.5026±0.0021 (99.1%)
NH_4NO_3	0.5045±0.0041 (100%)	0.2153±0.0006 (42.8%)
KNO_3	0.5078±0.0008 (100%)	0.3008±0.0015 (59.4%)
$NaNO_3$	0.5050±0.0010 (100%)	0.4013±0.0025 (79.3%)
NH_4Cl	0.5077±0.0003 (100%)	0.3936±0.0009 (77.7%)
Urea	0.5102±0.0007 (100%)	0.3528±0.0004 (69.7%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value = 0.5070, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).
ND, Not determined

4.2.3.2 Effect of potassium concentration on used oil degradation

Effect of potassium concentration on used oil degradation is illustrated in Table 4-21. The results of gravimetric analysis showed that the highest oil removal was obtained at potassium concentration of 1.0 g/l (77.6% reduction during 20-days of incubation). The residual oil weights among the other potassium concentrations were not significantly different. The percentage of used oil reduction at potassium concentration of 3.0 g/l was slightly lower than the other potassium concentrations (71.2% reduction during 20 days of incubation). The decrease of used oil was more pronounced in the first 10-days of incubation in all concentrations of potassium.

Table 4-21 Effect of potassium concentration on used oil degradation by enriched soil microorganisms in 50 ml of sodium phosphate BH-medium, incubated at 30°C 200 rpm in a rotary shaker for 20 days.

Potassium concentration (g/l)	Residual used oil* (g)		
	Day 0	Day 10	Day 20
Control (NaN_3)	0.5075±0.0023	0.5011±0.0008	0.5033±0.0012
	(100%)	(98.7%)	(99.2%)
0.5	ND	0.1420±0.0027	0.1285±0.0010
		(28.0%)	(25.3%)
1.0	ND	0.1347±0.0099	0.1137±0.0078
		(26.5%)	(22.4%)
1.5	ND	0.1378±0.0073	0.1202±0.0023
		(28.2%)	(23.6%)
2.0	ND	0.1436±0.0100	0.1307±0.0083
		(28.2%)	(25.7%)
3.0	ND	0.1587±0.0011	0.1460±0.0010
		(31.3%)	(28.8%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual used oil were based on the residual used oil on the control day 0 as 100%.
ND, Not determined

4.2.3.3 Effect of phosphorous source on used oil degradation

In this study phosphorous in the medium was provided in the form of phosphate buffer. Three types of phosphate buffer were studied (sodium phosphate BH-medium, $Na\text{-}PO_4$; sodium-potassium phosphate BH-medium, $NaK\text{-}PO_4$ and potassium phosphate BH-medium, $K\text{-}PO_4$). From the results of gravimetric analysis (Table 4-22), sodium phosphate BH-medium supported the highest level of used oil degradation by enriched soil microorganisms. It was able to remove 76.7% used oil during 20-days of incubation. Lower degradation (67.4%) in sodium-potassium phosphate BH-medium was obtained during 20-days of incubation. Whereas the lowest level of used oil degradation (22.2%) was obtained in potassium phosphate BH-medium. The removal of used oil occurred rapidly during the first 10-days in all experiments. The growth kinetics of the enriched soil microorganisms in three modified BH-media were similar (Fig.4-9).

Table 4-22 Effect of phosphorous source on used oil degradation by enriched soil microorganisms in 50 ml of various phosphate buffer BH-media, incubated at 30°C 200 rpm in a rotary shaker for 20 days.

Buffer type	Residual used oil* (g)		
	Day 0	Day 10	Day 20
Control (NaN_3)	0.5010±0.0030 (100%)	0.4991±0.0090 (99.6%)	0.5036±0.0280 (100%)
Na-PO_4 buffer	ND	0.1389±0.0011 (27.7%)	0.1169±0.0015 (23.3%)
NaK-PO_4 buffer	ND	0.1961±0.0100 (39.1%)	0.1634±0.0034 (32.6%)
K-PO_4 buffer	ND	0.4033±0.0054 (80.5%)	0.3900±0.0030 (77.8%)

**, Average of 2 samples ± Standard error of duplication*
(–), The percentage of residual oil were based on the residual used oil in the control day 0 as 100% (if the amount of residual oil higher than control, the percentage of residual oil was adjusted to 100).
ND, Not determined

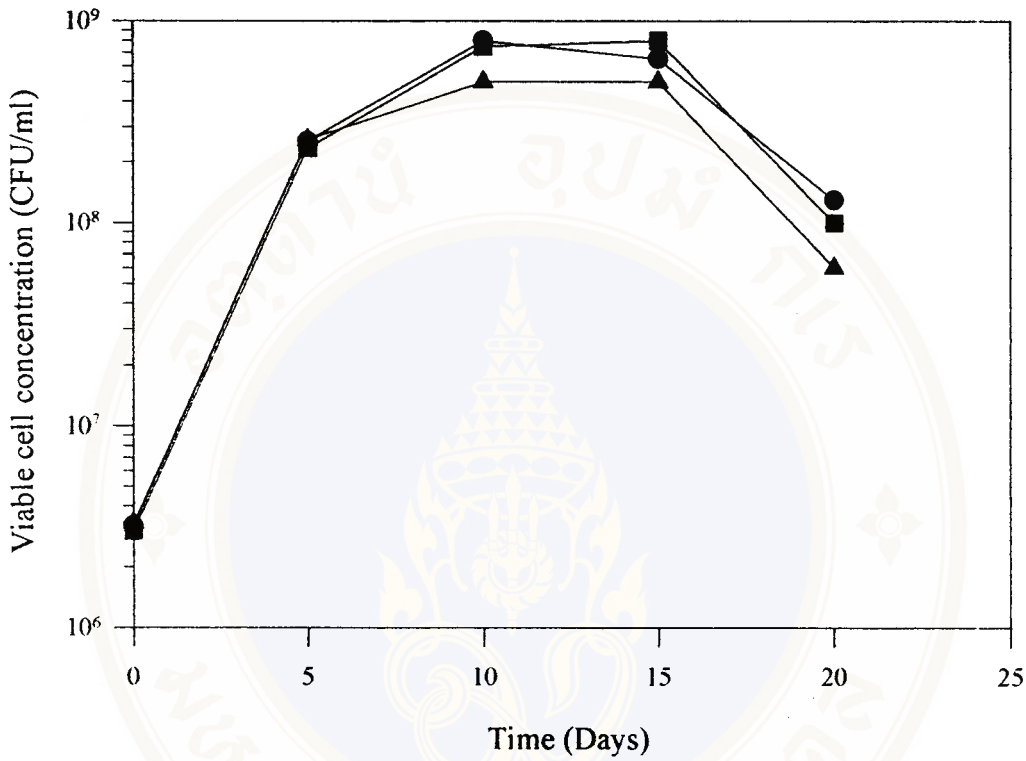


Fig. 4-9 Growth kinetics of enriched soil microorganisms with three types of phosphate BH-medium (Sodium phosphate BH-medium, ● ; Sodium-potassium phosphate BH-medium, ■ and Potassium phosphate BH-medium, ▲) with 0.5 g used oil, incubated at 30°C, 200 rpm in rotary shaker for 20 days.

4.2.3.4 Effect of surfactant on used oil degradation

The effects of Triton X-100 and Tween 80 were not obtained because both surfactants interfered with the extraction of used oil from the culture medium. Therefore analysis of residual used oil could not be carried out.

Effect of SDS on used oil biodegradation is illustrated in Table 4-23. No abiotic loss occurred in the control flask without SDS. While, used oil in the control flasks containing SDS decreased rapidly even on the day 0. In the treatment experiments, residual used oil in the SDS treatment was slightly lower than in without SDS treatment.

From Fig. 4-10, the culture broth containing SDS (10^9 CFU/ml) gave higher viable cell concentration than that with no SDS addition (10^8 CFU/ml) after 10 days incubation.

Table 4-23 Effect SDS on degradation of used oil by enriched soil microorganisms in 50 ml of BH-medium, incubated at 30°C 200 rpm in rotary shaker for 30 days.

Condition	Residual used oil* (g)			
	Day 0	Day 10	Day 20	Day 30
Control(NaN_3)	0.4924±0.0046 (100%)	0.4905±0.0099 (99.6%)	0.4821±0.0018 99.9%)	0.4916±0.0001 99.8%)
Control(NaN_3) + 2% SDS	0.3658±0.0017 (74.3%)	0.2804±0.0100 (56.9%)	0.2830±0.008 (57.4%)	0.2675±0.0045 (54.3%)
Enriched soil mos. (no SDS)	ND	0.2351±0.010 (47.7%)	0.2144±0.002 (43.5%)	0.1928±0.011 (39.2%)
Enriched soil mos. + 2% SDS	ND	0.2607±0.0059 (52.9%)	0.2383±0.0094 (48.3%)	0.1520±0.0050 (30.9%)

**, Average of 2 samples ± Standard error of duplication*
(--), The percentage of residual used oil were based on residual used oil from the control day 0 as 100% (with no addition of SDS).
ND, Not determined

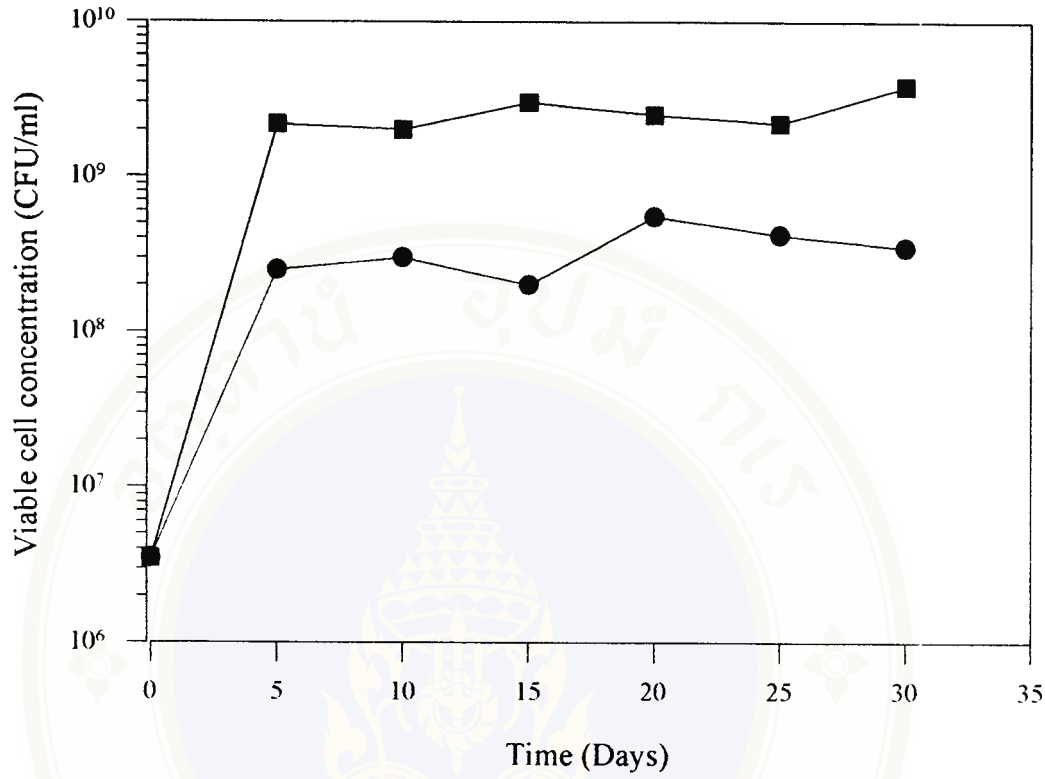


Fig 4-10 Growth kinetics of the enriched soil microorganisms with the addition of SDS (■) comparing with no addition of SDS (●) in sodium phosphate BH-medium with 0.5 g used oil, incubated at 30°C, 200 rpm in a rotary shaker for 30 days.

4.2.4 Microbial distribution comparison of enriched soil microorganisms and isolated microorganisms (Microorganism group II)

The distribution of enriched soil microorganisms and microorganism group II during 7 days of incubation was investigated. The different between these two groups of organism occurred at the beginning of incubation time. The enriched soil microorganisms gave much more variety of microorganisms than those of microorganism group II inoculum.

In enriched soil microorganisms, the dominant microorganisms, identified by API kit method, were *Flavobacterium meningosepticum, Pseudomonas aeroginosa, Pseudomonas putida, Pseudomonas stutzeri* and *Sphingomonas spirivorm.* These microorganisms still remained during 7 days of incubation. *Sphingomonas paucimobilis, Pseudomonas chlororaphis, Oligella urethralis, Pseudomonas alcaligines, Pseudomonas fluorescens, Pseudomonas vesicularis, Flavobacterium breve* and *Xanthomonas maltophilia* were found occasionally.

Whereas, the dominant microorganisms in microorganism group II were *Pseudomonas aeroginosa* and *Pseudomonas* sp. SS016. During incubation for 7 days, *Pseudomonas aeroginosa* increased rapidly and became the dominant one in the culture medium.

Following these results, the dominant microorganism in this oil contaminated area was the *Pseudomonas* sp. The example of the distribution of organisms from enriched soil microorganisms and microorganism group II is illustrated in Fig. 4-11.

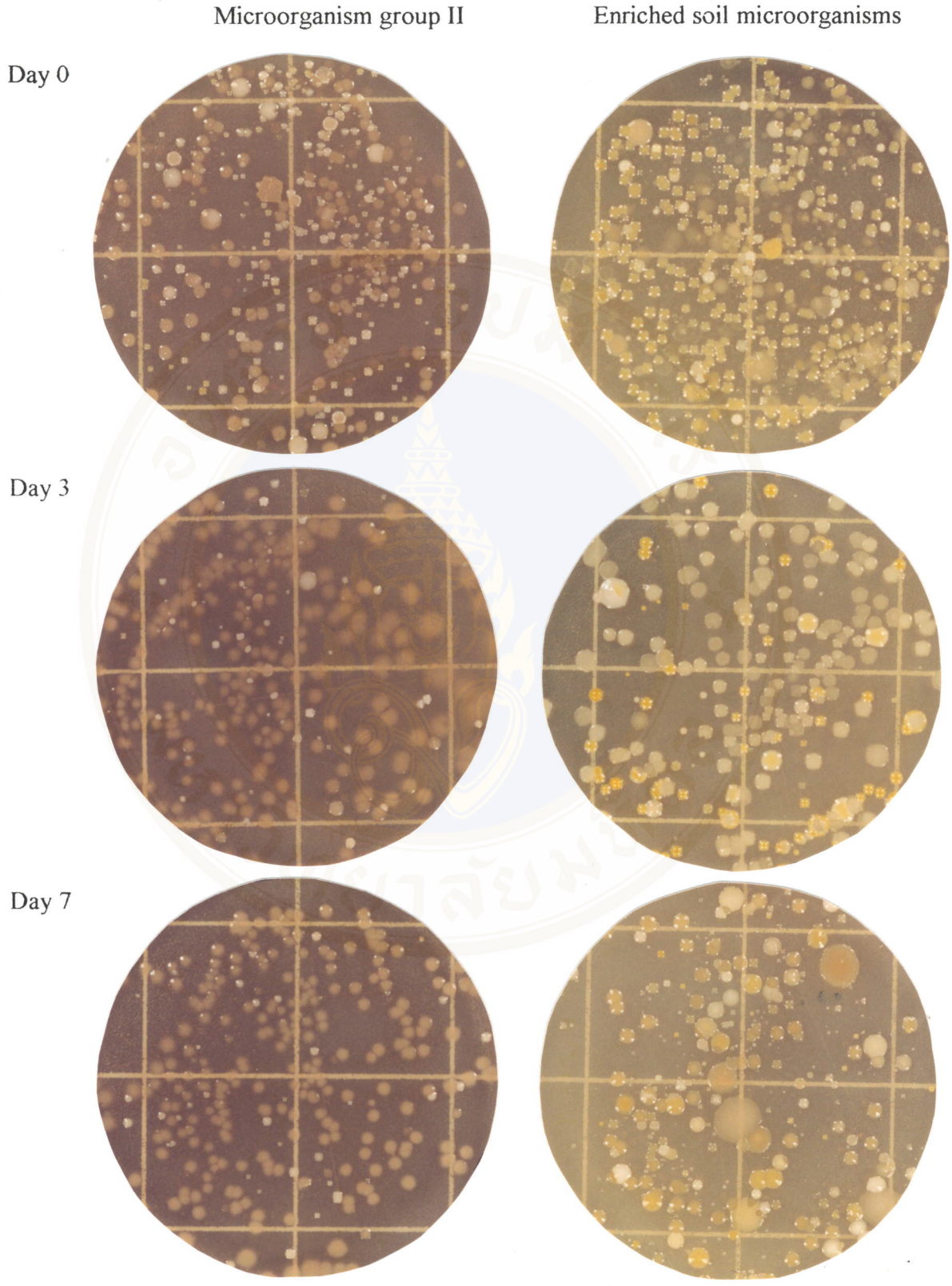


Fig.4-11 Microbial distribution comparison of enriched soil microorganisms and microorganism group II at day 0, 3, and 7, incubated at 30°C on Nutrient agar plate.

4.3 Biodegradation of oil contaminated gravel in a tray

4.3.1 Solid-phase biodegradation of oil contaminated gravel

Viable cell concentrations in solid-phase biodegradation experiments are shown in Fig. 4-12. Initial viable cell concentration in each group was approximately 10^4 CFU/ml except one in group 5 (5.5×10^5 CFU/ml). During the first 10 days of incubation, viable cell concentration in every treatment increased to approximately 10^6 CFU/ml. There were three different patterns of growth observed between day 20 and the end of experiment (day 180). Firstly, in group 2, viable cell increased further to the peak (5.3×10^6 CFU/ml) at day 30. After a decrease between day 30 and day 90, viable cell concentration remained constant at the level of 6×10^5 CFU/ml. Secondly, in group 3, after it showed a plateau between day 10 and day 60, viable cell concentration increased to a constant level (approximately 4×10^7 CFU/ml) at day 90 till the end of experiment. Thirdly, in group 4 and group 5, after a small decrease at day 20 was observed, growth was resumed with slower growth rate to the stable viable cell concentration (approximately 5.5×10^7 CFU/ml) at day 60.

Table 4-24 shows the results of gravimetric analysis of chloroform soluble fraction of residual used oil by solid-phase biodegradation during 180 days of incubation. Residual oil weights of group 3 (addition of nutrients), group 4 (addition of nutrients and pH control), and group 5 (addition of nutrients, enriched soil microorganisms and pH control) were lower than group 1 (control, 6 N H_2SO_4) and group 2 (no addition of nutrients, only H_2O added). Even there was a decrease of residual oil weight in the control. However, in the treatment groups (2-5), residual used oil weights were obtained lower than one in the control in the following order: control < group 2 < group 3 < group 4 < group 5.

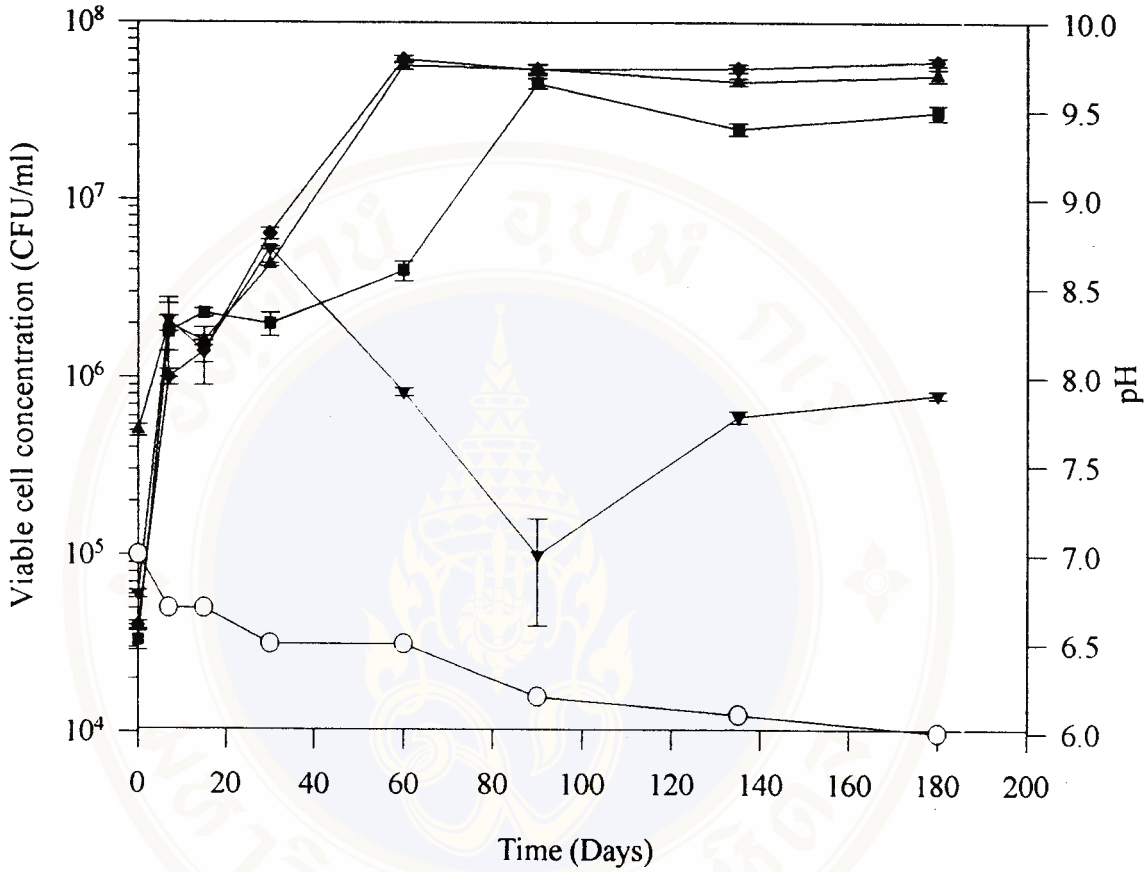


Fig 4-12 Viable cell concentration of solid-phase bioremediation of oil contaminated gravel during 180 days of incubation at 30°C (▼,group 2 no addition of nutrients; ■,group 3 addition of nutrients; ◆,group 4 addition of nutrients and pH control; ▲, group 5 addition of nutrients, enriched soil microorganisms and pH control and O, pH of group 3).

Table 4-24 Residual used oil (Chloroform soluble fraction) by solid-phase bioremediation of oil contaminated gravel in a tray, incubated at 30°C for 180 days.

Incubation time (Days)	Residual used oil*(g/kg gravel)				
	Group 1 Acidic control	Group 2 No nutrient (H_2O)	Group 3 Nutrient	Group 4 Nutrient and pH	Group 5 Nutrient,pH and ENS.+
0	11.61±0.36 (100%)	11.96±0.26 (100%)	11.69±0.25 (100%)	11.91±0.08 (100%)	10.90±0.30 (100%)
7	11.42±0.29 (98..4%)	11.21±0.15 (96.6%)	10.18±0.82 (87.7%)	11.36±0.16 (97.8%)	10.67±0.20 (91.9%)
15	11.55±0.44 (99.5%)	10.41±0.70 (89.7%)	11.80±0.16 (100%)	11.60±0.35 (99.9%)	10.78±0.47 (92.9%)
30	10.46±0.76 (90.1%)	10.76±0.36 (92.7%)	11.12±0.08 (95.8%)	9.99±0.58 (86.0%)	10.70±0.39 (92.2%)
60	10.91±0.60 (94.0%)	10.65±0.50 (91.7%)	10.65±0.36 (91.7%)	9.80±0.39 (84.4%)	9.52±0.35 (82.0%)
90	11.18±0.33 (86.3%)	9.91±0.61 (85.4%)	10.08±0.42 (86.7%)	10.15±0.24 (87.4%)	9.90±0.13 (85.3%)
135	10.31±0.19 (88.8%)	9.28±0.41 (79.9%)	9.50±0.55 (81.8%)	8.61±0.10 (74.2%)	9.01±0.20 (71.6%)
180	9.68±0.14 (83.4%)	9.33±0.25 (80.4%)	9.01±0.88 (77.6%)	8.71±0.07 (75.0%)	8.67±0.44 (74.7%)

**, Average of 2 samples ± Standard error of duplication*

(--), The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value = 11.61±0.34, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).

+, Enriched soil microorganisms (ENS.)

In Fig 4-13a, the initial concentration of NH_4-N of group 2 (no addition of nutrients) was 0.75 mg/l and after 7 days of incubation, it was unable to detect in the culture medium. Whereas, the initial concentrations of NH_4-N of group 3 (addition of nutrients), group 4 (addition of nutrients and pH control) and group 5 (addition of nutrients, enriched soil microorganisms and pH control) were 118.6, 122.1 and 125.1 mg/l, respectively. From the first 30 days, NH_4-N concentration of these groups reduced continuously to 59.97±9.5 mg/l. During periodic addition of nutrients at day 30, 60 and 90, the accumulation of nutrients occurred and remained constant at 71.5±18.7 mg/l through day 135. At 180 days of incubation, very small amount of NH_4-N were detected in the medium (6.78± 3.3 mg/l).

In Fig 4-13b, the initial NO_3-N concentration of group 2 was 19.29 mg/l and it was unable to detect in the medium at day 90. While, the initial concentrations of NO_3-N of group 3 to group 5 were 103.9, 110.3 and 122.6 mg/l, respectively. They were also reduced continuously during the first 30 days (76.8±6.5 mg/l). During the addition of nutrients at day 30, 60 and 90, the accumulation of them increased to 204.3± mg/l at day 90. After that they were reduced continuously to 46.3±13.1 mg/l at the end of experiment.

In Fig 4-13c, which is illustrated in Fig 4-13c, the initial PO_4-P concentration of group 2 was 1.36 mg/l and the PO_4-P concentration at the end of experiment was 0.22 mg/l. The initial concentrations of PO_4-P of group 3 to group 5 were 11.6, 9.7 and 10.4 mg/l, respectively. From the first 30 days, the PO_4-P concentration reduced continuously to 3.64±1.0 mg/l. The residual PO_4-P concentrations were stable at 2.67±0.64 mg/l between day 60 and day 135. However, at the end of experiment the PO_4-P concentrations were 1.10±0.1 mg/l.

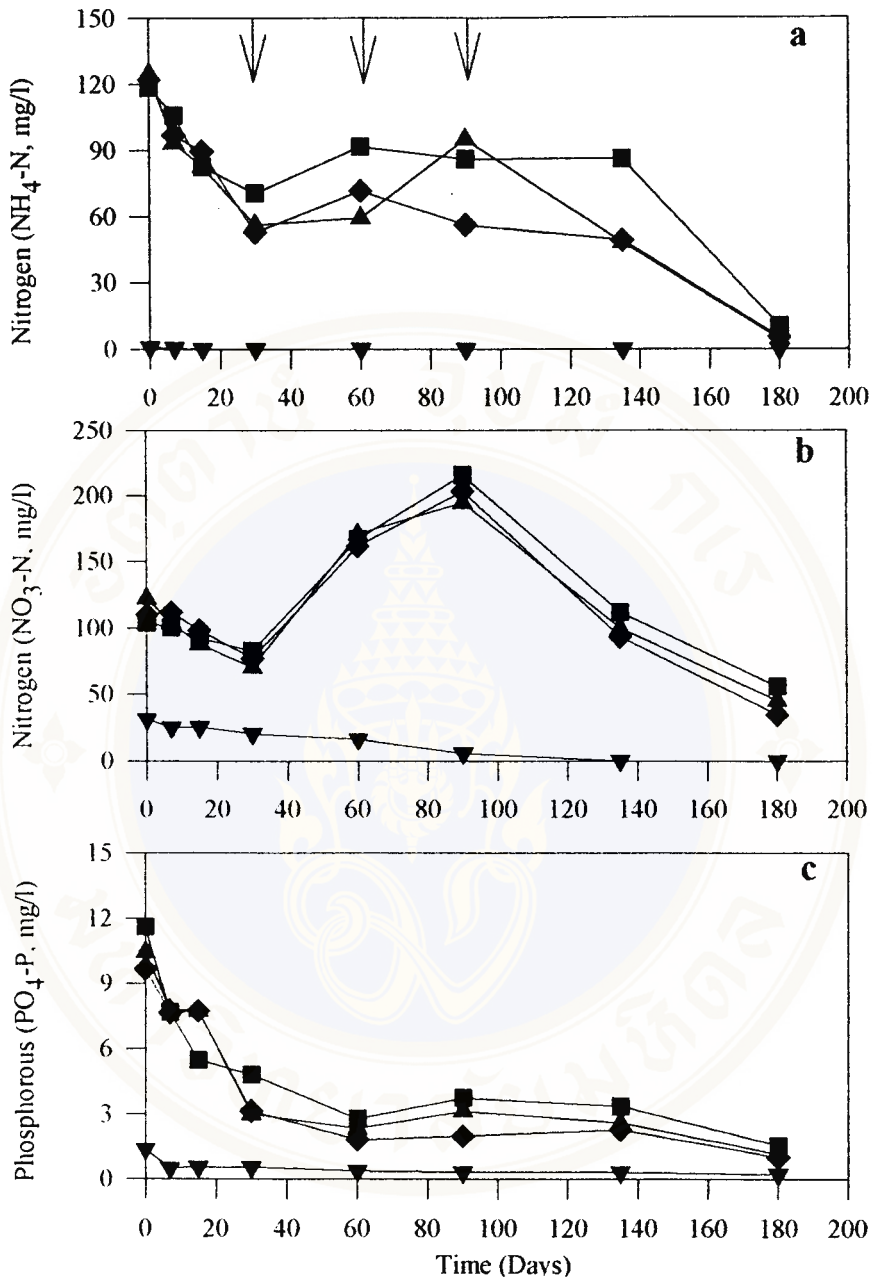


Fig 4-13 The residual nutrients (**a**,NH_4-N; **b**,NO_3-N and **c**,PO_4-P) in the culture media by solid-phase biodegradation of oil contaminated gravel (▼,group 2 no nutrient addition; ■,group 3 nutrients addition; ◆,group 4 addition of nutrients and pH control and ▲,group 5 addition of nutrients, enriched soil microorganisms and pH control, ↓ , Time for addition of NH_4NO_3, $K_2HPO_4.3H_2O$).

4.3.2 Slurry-phase biodegradation of oil contaminated gravel

The viable cell concentrations in all treatments are illustrated in Fig.4-14. Initial viable cell concentration of group 2 (addition of nutrients) was $1x10^4$ CFU/ml. Whereas, group 3 (addition of nutrients and enriched soil microorganisms) and group 4 (addition of nutrients and microorganism group II), initial viable cell concentrations were approximately 10^6 CFU/ml. During the first day, viable cell concentration of group 2 increased rapidly to 9.5×10^7 CFU/ml, while viable cell concentrations of group 3 and group 4 increased to approximately 5.2×10^8 CFU/ml. From this, the increasing rate of cell of group 2 was higher than those of group 3 and group 4. During day 1 to day 4, viable cell concentration of group 2 remained constant at $5.26\pm4.5 \times 10^7$ CFU/ml, while in group 3 and group 4, they remained constant at $3.33\pm2.02 \times 10^8$ CFU/ml. However, viable cell concentrations of group 2 to group 4 were not different at day 5 and they remained constant at $4.44\pm2.3 \times 10^7$ CFU/ml through day 60. After the addition of nutrients at day 60, viable cell concentrations of these groups slightly increased to $1.35\pm0.55 \times 10^8$ CFU/ml and they remained constant at this level.

For the addition of 2%SDS (group 6), initial viable cell concentration was 10^4 CFU/ml. After that it increased rapidly to 5×10^9 CFU/ml in 30 h. During 10 days of incubation, viable cell concentration remained constant at $1.6\pm0.5 \times 10^9$ CFU/ml. Between day 10 and day 20, the viable cell concentration slightly decreased to $7.0 \pm2.2 \times 10^8$ CFU/ml and remained constant throughout the end of experiment.

Table 4-25 shows the results of gravimetric analysis of chloroform soluble fraction of group 1 to group 4 by slurry-phase biodegradation. The average value of residual used oil calculated from the control day 0 in all groups was 10.37 ± 0.17 g/kg gravel. During 180 days of incubation, there was no abiotic reduction (group 1, NaN_3).

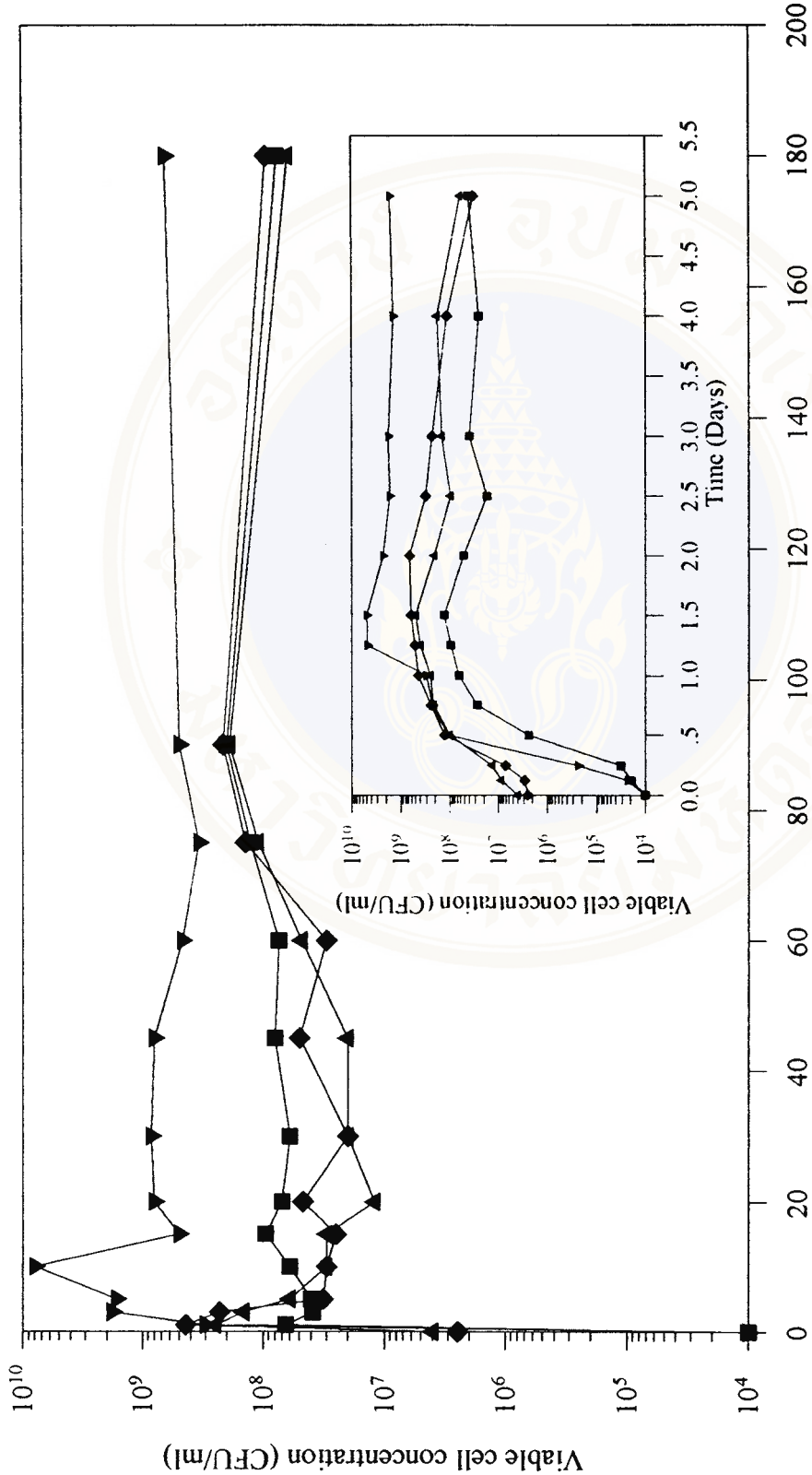


Fig 4-14 Viable cell concentration of slurry phase bioremediation of oil contaminated gravel during 180 days of incubation at 30°C: ■, group 2 nutrients; ▲, group 3 nutrients and enriched soil microorganisms; ◆, group 4 nutrients and microorganism group II and ▼, group 6 nutrients and SDS (The inset illustrates the details during the first 5 days of incubation)

Table 4-25 Residual used oil (Chloroform soluble fraction) by slurry-phase bioremediation of oil contaminated gravel in a tray, incubated at 30°C for 180 days.

Incubation time (Days)	Residual used oil* (g/kg gravel)			
	Group 1 0.5%NaN_3 Control	Group 2 Nutrient	Group 3 Nutrient and ENS[a].	Group 4 Nutrient and MG II[b]
0	10.48±0.01 (100%)	10.25±0.07 (100%)	10.59±0.07 (100%)	10.17±0.21 (100%)
30	9.99±0.08 (96.3%)	8.18±0.38 (78.9%)	8.17±0.35 (78.8%)	7.67±0.35 (74.0%)
60	9.65±0.15 (93.1%)	8.05±0.16 (77.6%)	7.24±0.13 (69.8%)	7.21±0.07 (69.5%)
90	10.05±0.35 (96.9%)	6.59±0.21 (63.5%)	6.52±0.36 (62.9%)	6.53±0.05 (63.0%)
180	10.36±0.15 (99.9%)	6.23±0.12 (60.1%)	6.49±0.04 (62.6%)	6.36±0.16 (61.3%)

**, Average of 2 samples ± Standard error of duplication*

(--), The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value =10.37±0.17, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).

a, Enriched soil microorganisms (ENS.)

b, Microorganism group II (MGII)

The reduction patterns of residual used oil in all treatments were similar. Residual used oil decreased rapidly during 30 days of incubation (approximately 23% reduction). Then it was slightly decreased (approximately 16% reduction at the end of experiment). There was no difference in residual oil reduction between the inoculated (group 3, enriched soil microorganisms and group 4, microorganism group II) and non-inoculated (group 2) treatments during 180 days of incubation (37.4% reduction for group 3, 38.7% reduction for group 4 and 39.9% reduction for group 2).

For the results of gravimetric analysis of hexane soluble fraction (Table 4-26), the average values of residual used oil calculated from the control day 0 in all groups was 9.05± 0.26 g/kg gravel. There was also no abiotic reduction occurred during the experiment. Residual used oil rapidly decreased during 30 days of incubation. At the end of experiment, the residual used oil reductions in all treatments were 35.6% reduction for group 3, 36.5% reduction for group 4 and 40.9% reduction for group 2.

For the effect of surfactant on used oil bioremediation, the results of gravimetric analysis of chloroform soluble fraction is shown in Table 4-27. The residual oil weights of 2%SDS control (group 5, 2%SDS and NaN_3) at day 0 and day 180 were 10.11 and 8.33 g/kg gravel, respectively. The residual used oil rapidly decreased at the first 30 days of incubation. However, in the treatment group 6 containing 2%SDS, reductions in oil weights were more than those in group 5.

The residual oil weight of hexane soluble fraction of 2%SDS treatment is shown in Table 4-28. The residual oil weights of group 5 day 0 and day 180 were 8.44 and 6.40 g/kg gravel, respectively. This also, the reductions of used oil in the treatment group 6 were more than in group 5.

Table 4-26 Residual used oil (Hexane soluble fraction) by slurry-phase bioremediation of oil contaminated gravel in a tray, incubated at 30°C for 180 days.

Incubation time (Days)	Residual used oil*(g/kg gravel)			
	Group 1 0.5%NaN_3 Control	Group 2 Nutrient	Group 3 Nutrient and ENS.[a]	Group 4 Nutrient and MG II[b]
0	8.92±0.34 (100%)	8.81±0.37 (100%)	9.49±0.03 (100%)	8.99±0.17 (100%)
30	8.55±0.07 (94.5%)	7.13±0.17 (78.8%)	6.79±0.21 (75.0%)	6.73±0.15 (74.4%)
60	91.6±0.16 (91.6%)	6.88±0.10 (76.0%)	6.44±0.15 (71.1%)	6.36±0.01 (70.3%)
90	9.51±0.24 (100%)	6.00±0.40 (66.3%)	6.13±0.20 (68.0%)	5.98±0.09 (66.1%)
180	9.03±0.17 (99.8%)	5.35±0.42 (59.1%)	5.84±0.04 (64.4%)	5.75±0.12 (63.5%)

**, Average of 2 samples ± Standard error of duplication*
(--),The percentage of residual oil were based on the average value of residual used oil in each group from the control day 0 as 100% (Average value = 9.05±0.26, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).
a, Enriched soil microorganisms (ENS.)
b, Microorganism group II (MGII)

Table 4-27 Effect of SDS on used oil bioremediation (Chloroform soluble fraction) by slurry-phase bioremediation in a tray, incubated at 30°C for 180 days.

Incubation time (Days)	Residual used oil* (g/kg gravel)		
	Group 1 0.5%NaN_3 Control[a]	Group 5 0.5%NaN_3 and 2%SDS[b]	Group 6 2%SDS[c]
0	10.48±0.01 (100%)	10.11±0.31 (100%)	ND
30	9.99±0.08 (96.3%)	9.01±0.44 (85.1%)	7.32±0.41 (72.4%)
60	9.65±0.15 (93.1%)	9.33±0.09 (92.3%)	4.59±0.12 (45.4%)
90	10.05±0.35 (96.9%)	8.84±0.18 (87.4%)	4.49±0.09 (44.4%)
180	10.36±0.15 (99.9%)	8.33±0.41 (82.4%)	4.97±0.25 (49.2%)

**, Average of 2 samples ± Standard error of duplication*
(--)[a], The percentage of residual oil were based on the average value of residual used oil in group1 to group 4 at the control day 0 as 100% (Average value = 10.37 ±0.17, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).
(--)[b,c], The percentage of residual used oil were based on the residual used oil from the control day 0 of Group 5 as 100% (10.11).

Table 4-28 Effect of SDS on used oil bioremediation (Hexane soluble fraction) by slurry-phase bioremediation in a tray, incubated in an incubation room at 30°C for 180 days.

Incubation time (Days)	Residual used oil* (g/kg gravel)		
	Group 1 0.5%NaN_3 Control[a]	Group 5 0.5%NaN_3 and 2%SDS[b]	Group 6 2%SDS[c]
0	8.92±0.34 (100%)	8.44±0.21 (100%)	ND
30	8.55±0.07 (94.5%)	7.63±0.43 (90.4%)	6.11±0.30 (72.4%)
60	91.6±0.16 (91.6%)	7.27±0.08 (84.8%)	3.63±0.15 (43.0%)
90	9.51±0.24 (100%)	7.50±0.20 (88.9%)	3.90±0.65 (46.2%)
180	9.03±0.17 (99.8%)	6.42±0.33 (76.1%)	4.05±0.19 (47.9%)

**, Average of 2 samples ± Standard error of duplication*
(--)[a], The percentage of residual oil were based on the average value of residual used oil in group1 to group 4 at the control day 0 as 100% (Average value = 9.05 ±0.26, if the amount of residual oil was higher than the average value, the percentage of residual oil was adjusted to 100).
(--)[b,c], The percentage of residual used oil were based on the residual used oil from the control day 0 of Group 5 as 100% (8.44).

Culture pH in all experiments were maintained at pH 7 throughout the experiment except that pH of medium containing SDS decreased to pH 6.5 at the end of experiment.

In Fig.4-15a, the initial NH_4-N concentrations in all treatments were approximately 130 mg/l. During the first 60 days, NH_4-N concentrations reduced continuously and were unable to detect at day 60. After 30 days of the first nutrient addition (day 90), the small amount of NH_4-N were detected (9.93±0.11 mg/l). At the end of experiment (day 180), NH_4-N concentration was 5.0±0.3 mg/l.

In Fig.4-15b, the initial NO_3-N concentration in every treatment was 155 mg/l. During the first 60 days, NO_3-N reduced continuously to 10.1±1.1 mg/l. After the first addition of nutrients, the accumulation of NO_3-N concentration increased to 49±10.1 mg/l at day 90. At day 180, residual NO_3-N concentration was 37.7±6.5 mg/l.

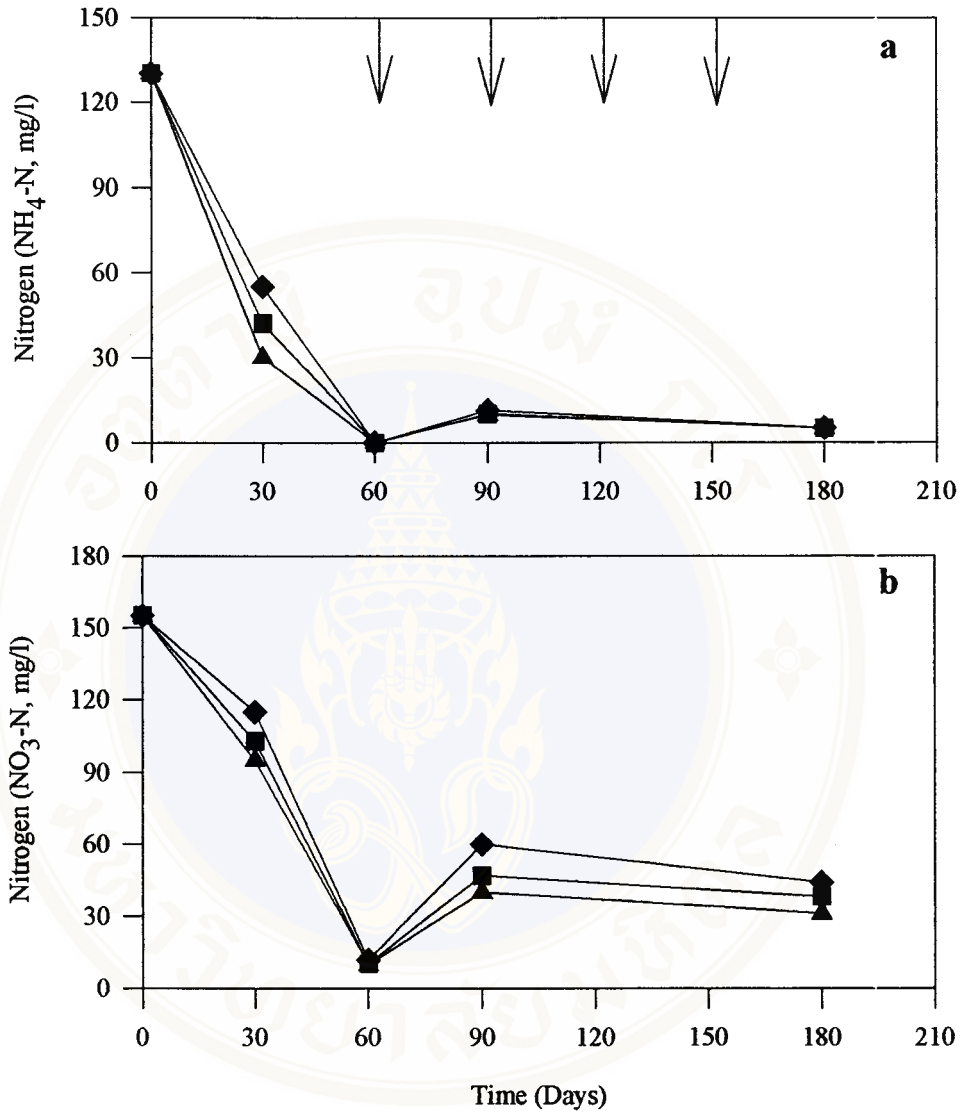


Fig 4-15 Residual **a**, NH_4-N and **b**, NO_3-N in culture media by slurry-phase biodegradation of oil contaminated gravel (■, group 2 addition of nutrients; ▲, group 3 addition of nutrients and enriched soil microorganisms and ◆, group 4 addition of nutrients and microorganisms group II,↓ ; Time for addition of NH_4NO_3).

CHAPTER V

DISCUSSION

5.1 Site characterization

Prior to initiation of any treatment activities for toxic compounds, the extent of overall contaminated site needs to be assessed. Representative samples were collected randomly from Bang Sue Diesel Locomotive Depot for screening of used oil degrading microorganisms. At first, Tapis crude oil, which was used for preliminary screening in our laboratory, was used as a C-source. However, Tapis crude oil degrading microorganisms were found to have very low degradation activity (less than 10%) on used oil. One explanation to this result is that Tapis crude oil is not the oil that contaminated on the site, so it might not be appropriate to use as a substrate for screening of used oil degrading microorganisms. Preliminary extraction of used oil from the site was performed in order to use as a sole C-source instead of Tapis crude oil. Most of isolated microorganisms were Gram-negative bacteria. Sajah *et al.*(1999), reported similar result that the community structures of all oiled samples, measured by using phospholipid fatty acid analysis (PLFA), contained significantly more Gram-negative bacteria than those in the unoiled samples (55).

At the locomotive contaminated site, the averages of temperature and pH from the site were 30-32°C and pH of 6.7- 7.2. In laboratory, pH of BH-oil medium was adjusted to 7 and the incubation temperature was 30°C.

As mentioned earlier, the main objective of this study is bioremediation of oil contaminated gravel. Characterization of contaminated gravel i.e. gravel density, ratio of used oil to unit weight of gravel and nutrient (N and P) on the gravel surface was determined in the preliminary study. Gravel density was needed because it was assumed that the amount of used oil on gravel surface was proportional to the weight of gravel used. However, in this study the density of contaminated gravel was significantly different at 95% confidence. It indicated that gravel from the collection area had different composition. Moreover, there were much gravel covered at some spots with soil and other material particles, which may lead to the difference in the gravel density. So this assumption was invalid.

For the determination of weight of used oil to a unit weight of gravel, the used oil in each group of gravel (10 pieces of gravel/group) was not significantly different at 95% confidence. It concluded that the amount of used oil could be estimated proportionally from the weight of gravel in group.

For, the availability of nutrients (N and P) which is necessary for bacterial growth (44), on the gravel surface, the amounts of N and P were very low. Especially NH_4-N, it was unable to detect in the culture broth. NH_4-N was reported to be consumed easily by microorganisms (103). Moreover, NH_4-N evaporates more easily in the environment than NO_3-N does. Therefore, the addition of nutrients was necessary for the success of bioremediation.

The used oil was exposed in the environment for more than 10 years. It experienced with physical, chemical and biological factors such as O_2, temperature, pH or microorganisms. The residual used oil may be changed to the difficult to degrade form, which was also difficult to be extracted by chloroform typically used in

this study. Therefore, the determination of appropriate solvent for extraction of used oil was conducted. It found that the mixture of chloroform and petroleum ether at the ratio of 50:50 could extract more oil than chloroform alone did. However, the amount of extracted oil from two types of extraction solvent was not significantly different at 95% confidence. Therefore, extraction by sole chloroform was used throughout the study. Moreover, when chloroform was used, it was easy to recover chloroform by distillation. Petroleum ether itself is flammable and very dangerous to use or recover from chloroform by distillation.

5.2 Biodegradation of oil in shake flask (liquid-phase biodegradation)

5.2.1 Abiotic degradation of oils

In our research three types of oils i.e. used oil, new diesel oil and new motor oil, were used. As mentioned before, used diesel oil and used motor oil were released into soil and gravel for more than 10 years. This mixture of oils is so called as the used oil. New diesel oil and new motor oil from the locomotive depot were also studied in order to know about the background of the used oil. Abiotic loss (e.g. evaporation or photooxidation) of motor oil was found to be very low. This may be explained that motor oil mostly contained high molecular weight hydrocarbons (C26 to C38) and some synthetic agents, which were very difficult to remove by abitic loss. In case of diesel oil, GC chromatogram showed that, it contained more low molecular weight hydrocarbons (C12 to C21 or lower). Therefore, some hydrocarbons (i.e. number of carbon atom less than C10) were lost easily by evaporation facilitated by shaking (51,54). For used oil, it was exposed in the contaminated site for many years, so low

molecular weight hydrocarbons were lost via abiotic losses e.g. photooxidation, evaporation by wind action and temperature or seasonal changes and biotic degradation. Moreover, used motor oil itself resulting from the engine operation with high temperature, it contained high amount of PAHs and high molecular weight hydrocarbons, which was very difficult to remove (22,33). In addition, during used oil was extracted under high temperature (80°C) and vacuum (600 mmHg), some of low molecular weight hydrocarbons might evaporate. As the GC chromatogram in Fig.4-1, used oil had a profile with mixed content of motor oil and diesel oil. It contained more motor oil fraction, which led to low abiotic loss. With this result, it agreed that diesel oil was used in locomotive engine cleaning service.

5.2.2 Determination of used oil degrading microorganisms

For used oil bioremediation, when *Acinitobacter* sp. M1407 was used as an inoculum, the degradation activity was very poor. Even it was reported that it could degrade Tapis crude oil quite efficiently (104). However, Tapis crude oil was light crude oil, which contained high amount of low molecular weight hydrocarbons, whereas used oil contained high amount of high molecular weight hydrocarbons. Therefore, M1407 was able to use only small fraction of low molecular weight hydrocarbon in used oil. Moreover, one microorganism may not be appropriate for the degradation of very complex mixture of compounds. It may require co-metabolisms from the other type of microorganisms (51,57,58).

Enriched soil microorganisms, which was obtained by enrichment procedure of soil from the contaminated area, has shown that it had high ability to degrade used oil. This group of microorganism contained various types of microorganism. Most of them were Gram-negative bacteria. Dominant species were *Pseudomonas aeruginosa,*

Pseudomonas putida, Pseudomonas stutzeri, Sphingomonas spiritivorum and *Flavobacterium meningosepticum*. As the result, *Pseudomonas* sp. was a principal genus, which was frequently reported to degrade toxic compounds (C11 to C36 straight chain alkanes, branched alkanes (105,106), phenanthrene, anthracene, naphthalene, benzothiophenes and dibenzothiphenes (107,108,109)). Moreover, it also degraded high-molecular weight resin fraction of crude oil (110). In addition, some *Pseudomonas* spp. were able to produce extracellular biosurfactants, which were effective for hydrocarbon degradation (25,111). Genetics and enzymology of hydrocarbon degradation by this genus has been extensively studied (112). For genus *Flavobacterium* and *Sphingomonas*, they were also reported for the degradation of petroleum products (55,57).

In the attempts to obtain effective microorganisms capable to degrade used oil on gravel, microorganism group I was firstly selected from soil at the contaminated site by using Tapis crude oil as a sole C-source. With the difference in chemical property of Tapis crude oil, used oil degrading activity of microorganism group I was extremely low. In the case of microorganism group II, used oil was used as a sole C-source in the screening medium. However, the degrading activity of microorganism group II on used oil was still insignificant. With the complexity of used oil as shown in Fig. 4-2A, it may be an explanation why microorganism group II activity was so low. On the other hand, these isolated microorganisms had low degradation activity themselves. To elucidate this activity, each selected colony was separately studied for its degradation activity on BH-oil plate and in liquid culture. Microorganism group III, which was mixed culture of 15 different morphology colonies was also study. From this, the

degradation activity was still very low. So the low degradation activities may be resulted from their degradation abilities. In addition, there might be another factor, which affected the selection of microorganisms in the inoculum preparation step i.e. soil component. Thus, effect of soil component on used oil degradation was investigated. However, soil component was found to have no effect on used oil degradation. Finally, the used oil degrading microorganisms could not be obtained. So the distribution of the isolated inoculum (mixed culture of microorganism group II) and the enriched soil inoculum was studied. *Pseudomonas aeruginosa* and *Pseudomonas* sp. SS016 were found mostly in the starter inoculum of microorganism group II. Even it was inoculated with various types of microorganism (54 different colonies) at the beginning of inoculum preparation. During 7 days of incubation, *Pseudomonas aeruginosa* became the dominant one in the culture medium of the pooled microorganism group II. While, the culture medium of enriched soil inoculum contained much more variety (as described earlier). From this result, *Pseudomonas aeruginosa* could compete with the other types of microorganism from the inoculum preparation step. However, its activity on used oil degradation was very low. The used oil is the complex mixtures of hydrocarbon and mostly contained the high molecular weight hydrocarbons. This may be the cause why no single specie of microorganism can degrade used oil. Therefore, the effective bioremediation of this complex mixture requires a population of microorganisms consisting of different genera capable of metabolizing the respective compounds (57). In addition, transformation of contaminants by one organism might give metabolites, which could be used the other organisms, which is called co-metabolism (54,58). In order to search for the right

group of used oil degrading microorganisms, further investigation on the relationship between microorganisms in the contaminated area may be needed.

For *Pseudomonas aeruginosa*, there are many studies reported on its degradation ability of toxic compounds (25,105,106,111). Its degradation activity was mostly focused on low molecular weight compounds. Similarly, it could reduce approximately 40% of diesel oil in this study.

For viable cell concentrations in shake flask culture of the inoculum preparation (Fig. 4-3), microorganism group I gave the highest viable cell concentration in 50 ml BH-medium + 0.5 g used oil. During inoculation, high initial cell concentration was obtained when 42 colonies from microorganism group I were used. Therefore, it gave higher viable cell concentration in the starter inoculum (4.2×10^7 CFU/ml). With high viable cell concentration of 6.42×10^9 CFU/ml in 30 h and low reduction of used oil during bioremediation, it may imply that culture medium might contain other carbon sources, which microorganism group I could utilize more easily than used oil. *Pseudomonas aeruginosa* took this advantage and became dominant. In case of enriched soil microorganisms, it might contain low number of microorganism in contaminated soil sample (0.5 g), which was used as a starter. Therefore the initial viable cell concentration (7×10^6 CFU/ml) was lower than microorganism group I for one log . When these two groups of organism experienced with used oil, they kept the difference for one log throughout day 7. For M1407, Tapis crude oil was used as a carbon source in stead of used oil. Therefore, viable cell concentration was high as to 3×10^8 CFU/ml and remained stable throughout day 7.

For viable cell concentration during bioremediation of various oils (Fig. 4-6), high viable cell concentrations (10^8-10^9 CFU/ml) of enriched soil microorganisms and microorganism group II were obtained in all types of oil used. However, enriched soil microorganisms had lower viable cell concentration in used oil (Fig 4-6a) and new diesel oil (Fig. 4-6c) except one in new motor oil (Fig. 4-6b). Although enriched soil microorganisms consumed all three type of oil better than microorganism group II did (Table 4-10, 4-11 and 4-12).

In Fig. 4-6a, microorganism group II was able to reduce very small portion of used oil (less than 5%) but viable cell concentration was 1.5 x 10^9 CFU/ml and gradually decreased to at 6.36±2.08 x 10^8 CFU/ml. As mention earlier, some nutrients including carbon source might contaminate with used oil. This contaminated C-source supported early growth phase of microorganism group II.

In Fig. 4-6b, even viable cell concentrations of both groups were in similar pattern. However, viable cell concentration of microorganism group II slightly increased from the beginning. It may imply that a small fraction of new motor oil, consumed by microorganism group II, was used for viable cell maintenance.

In Fig. 4-6c, high viable cell concentration but less diesel oil reduction (approximately 40%) may imply that microorganism group II utilized diesel oil for cell production better than enriched soil microorganisms did.

5.2.3 Determination of medium composition for bioremediation of used oil

In the study of the effect of N-source on used oil bioremediation (Table 4-19), KNO_3 gave the maximum reduction in used oil weight in modified BH-medium with 0.1 M sodium phosphate buffer, pH 7. However, medium containing KNO_3 was the

only one, with potassium constituent. To confirm the effect of KNO_3 on used oil bioremediation, KCl as potassium source was added in the modified BH-medium with the other N-sources (Table 4-20). Result indicated that, NH_4NO_3 was the appropriate N-source for used oil degradation. It may be explained by the reason that NH_4NO_3 contains nitrogen atoms in both reduced ammonium (NH_4^+) and oxidized nitrate (NO_3^-) forms, which can be easily consumed by various kinds of microorganisms. Some organisms might prefer to use NH_4^+ as the N-source, while others prefer to use NO_3^-. Swindoll *et al.* (1988) showed that several types of organism, during sequential degradation of some xenobiotic compounds, required their own particular nutrient (61). As a result from the effect of N-source, potassium may play a role in used oil degradation because in the presence of potassium, the biodegradation of used oil was enhanced. The appropriate potassium concentration for used oil degradation in this study was 1 g/l. This incidence was confirmed in the study of the effect of phosphorous source on used oil degradation (Table 4-22). Modified BH-medium containing sodium phosphate buffer and 1.86 g/l KCl, gave maximum reduction in used oil weight. Potassium concentration in this modified BH-medium was approximately 1 g/l. Whereas, the modified BH-medium with sodium-potassium phosphate buffer and potassium phosphate buffer contained 4 and 5.5 g/l of potassium, respectively. Moreover, when modified BH-medium with potassium phosphate buffer was used, the low degradation activity was obtained. Interestingly, at a specific concentration of potassium (1 g/l) enhanced bioremediation of used oil. However, modified BH-medium with potassium phosphate buffer did not contain sodium ion. Therefore, the relationship between sodium and potassium ions may need further

investigation. Zaidi *et al.* (1988) suggested that ion imbalance affected bacterial degradation of organic compounds at low concentrations (113). In the case of phosphorous concentration, it was in excess amount in phosphate buffer solution so no attempt to determine the requirement for phosphorous in this study.

5.2.4 Effect of surfactant

Higher biomass (approximately 3.3 x 10^9 CFU/ml during 30 days of incubation), was obtained in the culture containing SDS compared to the culture with no SDS (approximately 5.2 x 10^8 CFU/ml during 30 days of incubation). However, when the background activity was substracted from the SDS control experiment, the reduction of used oil in the culture with addition of SDS was similar to that in the culture with no SDS. Various explanations can be proposed. The first explanation was that microorganisms prefer SDS to used oil as a substrate for growth (25,78). Whereas, finding of Grimberg *et al.*(1996) on the inhibitory effect of surfactant on biodegradation indicated that, when the partitioning of hydrophobic substances into surfactant micelles occurred, microorganism could not have direct access to utilize compounds inside micelles. Therefore, the occurrence of these hydrophobic substance micelles limited biodegradation (107). Moreover, these oil micelles could not be extracted with chloroform. It may be lead to underestimate the weight of used oil by gravimetric measurement in both control and inoculated cultures (114).

5.3 Bioremediation of oil contaminated gravel in a tray

5.3.1 Solid-phase bioremediation of oil contaminated gravel

The results of solid-phase bioremediation of used oil (Table 4-24), showed that reduction of used oil in all treatments were significantly higher than that of the acid control but not different much from each other. However all biodegradations were very low. Various explanations can be proposed. Firstly, due to mass transfer of oil to water, the used oil did not disperse into water because of its viscous property and high content of high molecular weight hydrocarbons (Fig. 4-1). Therefore it led to the difficulty for microorganisms to degrade used oil. Li *et al.* (1994) suggested that the success of bioremediation could be achieved, when oil dispersed into solutions (6). Moreover, the application of nutrients in the form of solid or powder may be not appropriate due to uneven distribution of nutrients over gravel. Secondly, used oil was a very complex mixture of petroleum waste and mostly contained high molecular weight hydrocarbons, so bioremediation of this toxic contaminant may need microorganism consortium (54,57,58,115). Thirdly due to the characteristics of the contaminated material, gravel surface was full of pores and fissures to form matrices around the gravel surface. These matrices trapped used oil and led to the limitation in biodegradation. Moreover, there were some organic substances and fine particles of soil on gravel surface. These materials also led to the sorption of toxic contaminants on gravel surface, which caused difficulty for microorganisms to degrade used oil. Ferdinandy-van Vlerken (1998) suggested that sorption mechanism was strongly influenced by the characteristics of both pollutants and sediment. This mechanism delayed the biodegradation of the pollutants (49). Loser *et al.*(1999) reported that fine

particles and organic matters on soil surface were essentially responsible for the adsorption of PAHs to the soil matrix (72). Finally, only NH_4NO_3, $K_2HPO_4.3H_2O$, $CaCl_2$ and $FeCl_3$ were added as the nutritional supplementation, other minerals especially sodium should be investigated for their effects on used oil bioremediation in further study.

In case of viable cell concentrations in solid-phase bioremediation (Fig.4-12), the increasing of viable cell concentration of group 2 during the first 10 days may be because of indigenous microorganism was able to use another C-source, which was degraded easier than used oil. After that viable cell concentration decreased since another C-source and nutrients decreased. For group 3 to group 5, similar patterns of growth kinetics were obtained even there was no pH control in group 3. The reduction of pH in group 3 was in the range of 6-6.7, which was not different from the optimum pH (6-8) for general biodegradation (8). Therefore, the reduction of pH in group 3 did not affect the degradation of used oil. However, viable cell concentration in every treatment remained stable throughout 180 days incubation but very low reduction of used oil was observed. Another C-source contaminated on gravel may be an alternative to maintain viable cell.

From the observation of residual nutrients in every tretment (Fig. 4-13), NH_4-N was consumed more rapidly than NO_3-N. This may be resulting from the reason that NH_4-N was reported to be consumed easily by microorganisms (103). Moreover, it evaporated easily. On the other hand, NH_4-N was converted to NO_3-N by microorganisms. Therefore, the accumulation of NO_3-N was observed till day 90. When addition of nutrients was terminated, NH_4-N decreased. Subsequently decrease in NO_3-N was observed.

Although small reduction of used oil was obtained in solid-phase bioremediation in this study, it implied the possibility in using bioremediation to remove used oil at the contaminated site. It may take longer time than 6 months. Cookson (1994) suggested that solid-phase biodegradation was a slow process. It required longer period of time to obtain the success of bioremediation (54). Many investigations on solid-phase bioremediation indicated that, time requirement for petroleum hydrocarbon bioremediation may be last from year to many years (12,13,15,116).

5.3.2 Slurry-phase bioremediation of oil contaminated gravel

For slurry-phase bioremediation of oil contaminated gravel (Table 4-25), the biodegradation rate in all treatments with either nutrient addition or nutrient + microorganism addition were significantly higher than that in the control. However, they were not significantly different between treatments. Indigenous microorganisms may be the reason that they experienced used oil in the contaminated site. Reduction of used oil in both group 3 and group 4 could be the results by the activity of indigenous microorganisms. Cookson (1994) suggested that seeding with the enriched or isolated microorganisms may be effective if background of indigenous microorganisms at the contaminated site was very low (normally less than 10^2 CFU/g of dry-weight soil) (54). While, the indigenous microorganisms in this study was 10^4 CFU/ml. Therefore, seeding was not a proper method of choice under this bioremediation condition.

In all treatments, low levels of N were observed at day 60 (approximately 10 mg/l) (Fig. 4-14). However, viable cell concentrations (Fig. 4-15) were stable at 10^8-10^9 CFU/ml with 4 additions of nutrients at every 30 days interval. Oudol *et al.*

(1998) reported that if background level of N in the sediment was higher than 100 μmoles/l then biodegradation through fertilization (nutrients addition) may be of limited use (63). Moreover, Lai *et al.* (2000) also reported that typical amounts of N and P content of 0.1% in the polluted soil was sufficient to sustain natural or enhanced biodegradation (64). Therefore, 10 mg/l of N was sufficient to support growth of microorganisms for bioremediation of used oil in this study.

However, in every treatment the reduction of used oil was very low. Even high viable cell concentrations were obtained with the additions of nutrients at every 30 days of incubation. Characteristics of used oil and contaminated gravel, mentioned earlier in solid-phase bioremediation (Section 5.3.1), may be one reason. Transport phenomena might be another factors such as "wall" oil, poor aeration and agitation under this investigation condition.

Similarly, the addition of surfactant i.e. SDS did not enhance bioremediation of used oil in the slurry-phase (Table 4-27 and 4-28). Discussion was mentioned in Section 5.2.4.

7. The used oil degradation rate in slurry-phase bioremediation (40% reduction in 6 months) was higher than that of solid-phase bioremediation (15% reduction in 6 months). This may be because of the mixing and transfer rate of used oil and nutrients to microorganisms in slurry is easier than that in solid.
8. The background of indigenous microorganisms at the contaminated site was approximately 10^4 CFU/ml, which was sufficient to degrade the used oil under the appropriate condition..
9. Sodium dodecyl sulfate, SDS (2% w/v) did not promote the degradation of used oil under the condition used in this investigation.
10. An understanding of the benefits and limitations of bioremediation study in laboratory may be the important thing before working in the fields. However, no one product or protocol will serve as a universal solution to remedy the oil pollutions, thus to achieve the most effectiveness, the advance knowledge of this bioremediation should be combined with other available technologies.

REFERENCES

1. Shannon JRM, Unterman R. Evaluating bioremediation: distinguishing fact from fiction. Annu Rev Microbiol 1993;47:715-38.

2. อรรถวุฒิ อิ่มพูลทรัพย์. การศึกษาปัญหาและวิเคราะห์หาเทคโนโลยีที่เหมาะสมเพื่อแก้ปัญหาเกี่ยวกับคราบน้ำมัน ตามแหล่งต่างๆในประเทศไทย. คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล; (2534).

3. Harrison RM. Pollution 3rd ed. England: Cornwall; 1997.

4. Margesin R, Schinner F. Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in alpine soils. Appl Environ Microbiol 1997;63:2660-4.

5. Margesin R, Schinner F. Bioremediation of diesel oil contaminated alpine soil at low temperatures. Appl Microbiol Biotechnol 1997;47:462-8.

6. Li KY, Xu T, Cawley WA, Colapret JA, Bonner JS, Ernest A, Verramachaneni PB. Field test and mathematical modeling of bioremediation of an oil-contaminated soil-Part 1: Field test. Waste manage 1994;14:561-9.

7. Leahy JG, Colwell RR. Microbial degradation of hydrocarbon in the environment. Microbiol Rev 1990;54:305-15.

8. Flathman PE, Jurger DE, Exner JH. Bioremediation field experiment. Michigan: Lewis publishers; 1994.

9. Prince RC. Petroleum spill bioremediation in marine environments. Crit Rev Microbiol 1993;19:217-42.

10. Romantschuk M, Sarand I, Petanen T, Peltola R, Jonsson-Vihanne M, Koivula T, Yrjala K, Haahtela K. Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. Environ Pollut 2000;107:179-85.

11. Swannell RPJ, Lee K, McDonagh M. Field evaluation of marine oil spill bioremediation. Microbiol Rev 1996;62:342-65.

12. Michel J, Hayes MO. Weathering patterns of oil residues eight years after the *Exxon Valdez* oil spill. Mar Pollut Bull 1999;38:855-63.

13. Michel J, Hayes MO. Factor determining the long-term persistence of *Exxon Valdez* oil in gravel beaches. Mar Pollut Bull 1999;38:92-101.

14. Chianelli RR. Bioremediation technology development and application to the Alaskan spill. Oil Spill Conference : 1991.

15. Vazquez MA, Allen KW, Kattan YM. Long-term effects of the 1991 Gulf War on the hydrocarbon levels in clams at selected areas of Saudi Arabian Gulf coasline. Mar Pollut Bull 2000;40:440-8.

16. Sterling HP. Effects of a spill of marine diesel oil on the rocky shore fauna of Lumma Island, Hong Kong. Environ Pollut 1977;12:93-117.

17. Hozumi T, Tsutsumi H, Konos M. Bioremediation on the shore after on oil spill from the Nakhodka the in sea of Japan: I; Chemistry and characteristics of heavy oil loaded on the Nakhodka and biodegradation tests by a bioremediation agent with microbiological cultures in the laboratory. Mar Pollut Bull 2000;40:308-14.

18. Tsutsumi H, Hirota Y, HirashimaA. Bioremediation on the shore after on oil spill from the Nakhodka in sea of Japan: II; Toxicity of a bioremediation agent

with microbiological cultures in aquatic organisms. Mar Pollut Bull 2000 40:315-9.

19. Congress of the United States office of technology assessment. Bioremediation for marine oil spill. Washington DC; US government printing.

20. Poulton BC, Callahan EV, Hurtubise RD, Mueller BG. Effects of an oil spill on leafpack-inhabiting macroinvertebrates in the Chariton River, Missouri. Environ Pollut 1998;99:115-22.

21. Rhykerd RL, Crews B, MeInnes KJ, Weaver RW. Impact of bulking agents, forced aeration and tillage on remediation of oil contaminated soil. Bioresour Technol 1999;67:279-85.

22. Vazquez-Duhalt R. Environmental impact of used motor oil. Sci Total Environ 1989;79:1-23.

23. Lopes CF, Milanell JCC, Prosperi VA, Zanardi E, Truzzi AC. Costal monitoring program of Sao Sebastiao Channel; Assessing the effects of 'TEBAR' oil spill on rocky shore populations. Mar Pollut Bull 1997;34:923-7.

24. Straube WL, Jones-Meehan J, Pritchard PH, Jones WR. Bench - scale optimization of bioaugmentation strategies for treatment of soil contaminated with high molecular weight polyaromatic hydrocarbons. Resour Conserv Recycl 1999;27:27-37.

25. Deschenes L, Lafrance P, Villeneuve JP, Samson R. Adding sodium dodecyl sulfate and *Pseudomonas aeroginosa* UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. Appl Microbiol Biotechnol 1996;46:638-46.

26. Mahjoub B, Jayr E, Bayard R, Gourdon R. Phase partition of organic pollutants

between coal tar and water under variable experimental conditions. Wat Res 2000;34:3551-60.

27. Wild SR, Jones KC. Polynuclear aromatic hydrocarbons in the United Kingdom environment: A preliminary source inventory and budget. Environ Pollut 1995;88:91-108.

28. Wong PR, Christensen ER, Karls JF. Historical PAH fluxes to Lake Michigan sedimenta determined by factor analysis. Wat Res 1998;32:2422-30.

29. ชนะชัย เลิศสุชาติวนิช. สถิติน้ำมันรั่วไหลในประเทศไทย. วารสารกรมเจ้าท่า ; 2542 ; 2(1): หน้า 22-7.

30. National academy of sciences. Petroleum in the marine environment. Washington, D.C: 1975.

31. Niller CG. The biological aspects of water pollution 2nd ed. Illinois: Spring field; 1969.

32. Kostechi PT, Calabrese EJ. Hydrocarbon contaminated soils and groundwater: Michigan: CRC Lewis Publishers; 1991.

33. Vazquez-Duhalt R, Greppin H. Biodegradation of used motor oil by bacteria promotes the solubilization of heavy metals. Sci Total Environ 1986; 52:109-121.

34. Tagatz ME, Plaia GR, Deans CH. Responses of macrobenthos colonizing estuarine sediments contaminated with drilling mud containing diesel oil. Bull Environ Contam Toxicol 1985;35:112-20.

35. Khan RA, Kiceniuk JW. Sublethal effects of crude oil on a cold-water marine leech, *Johanssonia arctica*, following chronic exposure. Bull Environ Contam Toxicol 1989;4:590-6.

36. Zuofa K, Loganathan P, Isirimah NO. Effects of crude oil applications to soil on the growth and yeild of Maize, Okro, and Cassava in Nigeria. Oil Chem Pollut 1988;4:249-59.

37. Duffus JH. Environmental toxicology. London: Edward Arnold; 1980.

38. Young LY, Cerniglia CE. Microbial transformation and degradation of toxic organic chemicals. New York: Wiley-Liss; 1995.

39. Edgehill R. Factors influencing the success of bioremediation. Australas Biotechnol 1992;2:297-301.

40. Ogunseitan OA. Biochemical, genetic and ecological approaches to solving problems during in-situ and off-site bioremediation. Process Eng Pole Cont Waste Minimiz 1994:171-92.

41. Bourquin AW, Bagnod P. Biotreatability of petroleum contaminated soil. Intl Symp Environ Biotechnol 1991:417-9.

42. Baker KH, Herson DS. Bioremediation. New York: McGraw.Hill; 1994.

43. Autry AR, Ellis GM. Bioremediation: An effective remedial alternative for petroleum hydrocarbon contaminated soil. Environ Process 1992;11:13-8.

44. Huesemann MH. Guidelines for land-treating petroleum hydrocarbon-contaminated soil. J Soil Contam 1994;3:1-22.

45. Whyte LG, Hawari J, Zhou E, Bourbonniere L, Inniss WE, Greer CW. biodegradation of variable chain length alkanes at low temperatures by a phychrotrophic *Rhodococcus* sp. Appl Environ Microbiol 1998;64:2578-84.

46. Ijah UJJ. Studies on relative capabilities of bacterial and yeast isolates from tropical soil in degrading crude oil. Waste Manage 1998;18:293-9.

47. April TM, Foght JM, Currah RS. Hydrocarbon degrading filamentous fungi

isolated from flare pit soils in northern and western Canada. Can J Microbiol 2000;46:38-49.

48. Pickard MA, Roman R, Tinoco R, Vazquez-Duhalt R. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAHM 8260 Laccase. Appl Environ Microbiol 1999; 65:3805-9.
49. Ferdinandy-van Vlerken MMA. Changes for biological techniques in sedimant remediation. Wat Sci Technol 1998;37:345-53.
50. Rojas-Avelizapa NG, Rodriguez-Vazquez R, Enriquez-Villanueva F, Martinez-Cruz J, Poggi-Varaldo HM. Transformer oil degradation by an indigenous microflora isolated from a contaminated soil. Resour Conserv recycl 1999;2715-26.
51. Hinchee RE, Douglas GS, Ong SK. Applied bioremediation of petroleum hydrocarbons. Ohio: Battelle Press; 1995.
52. Weber WJ , Corseuil HX. Inoculation of contaminated subsurface soils with enriched indigenous microbes to enhance bioremediation rate . Wat Res 1994;28:1407-14.
53. Ramadan MA, El-Tayeb OM, Alexander M. Inoculum size as a factor limiting Success of inoculation for biodegradation. Appl Environ Microbiol 1990; 56:1392-6.
54. Cookson JT. Bioremediation engineering; Design and application. New York: McGraw.Hill; 1995.
55. Macnaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, White DC. Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 1999;65:3566-74.

56. Atlas RM. Petroleum biodegradation and oil spill bioremediation. Mar Pollut Bull 1995;31:178-82.

57. Korda A, Santas P, Tenente A, Santas R. Petroleum hydrocarbon bioremediation :sampling and analytical techniques, in situ treatments and commercial microorganisms currently used. Appl Microbiol Biotechnol 1997;48:677-86.

58. Lal B, Khanna S. Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. J Appl Bacteriol 1996;81:355-62.

59. Fedorak PM, Westlake DWS. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can J Microbiol 1981;27:432-43.

60. Jobson A, McLaughlin M, Cook FD, Westlake DWS. Effect of amendments on the microbial utilization of oil applied to soil. Appl Microbiol 1974;27: 166-71.

61. Swindoll CM, Aelion CM, Pfaender FK. Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. Appl Environ Microbiol 1988;54:212-7.

62. Walworth JL, Reynolds M. Bioremediation of a petroleum-contaminated cryic soil : Effects of phosphorus, nitrogen, and temperature. J Soil Contam 1995;4:299-310.

63. Oudot J, Merlin FX, Pinvidic P. Weathering rates of oil components in a bioremediation experiment in estuarine sediments. Mar Environ Res 1998;45:113-25.

64. Li G, Huang W, Lerner DN, Zhang X. Enrichment of degrading microbes and bioremediation of petrochemical contaminants in polluted soil. Wat Res

2000;34:3845-53.

65. Dibble JT, Bartha R. Effect of environmental parameters on the biodegradation of oil sludge. Appl Environ Microbiol 1979;37:729-39.

66. Sorkhoh NA, Ibrahim AS, Ghannoum MA, Radwan SS. High-temperature hydrocarbon degradation by *Bacillus stearothermophilus* from oil-polluted Kuwaiti desert. Appl Microbiol Biotechnol 1993;39:123-6.

67. Atlas RM. Effects of temperature and crude oil composition on petroleum biodegradation. Appl Microbiol 1975;30:396-403.

68. Fan Cy, Tafuri AN. Engineering application of biooxidation process for treating petroleum contaminated soil. In Wise DL, Trantolo DJ, editors, Remediation of hazardous waste contaminated soils. New York : 1994.

69. Hutchins SR, Sewell GW, Kovacs DA, Smith GA. Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ Sci Technol 1991;25:68-76.

70. Morga P, Watkinson RJ. Hydrocarbon degradation in soils and methods for soil biotreatment. CRC Crit Rev Biotechnol 1989;8:305-33.

71. Rehm HJ, Reed G, Puhler A, Stadler P. Biotechnology: Environmental Process II 2nd ed. New York: Wiley company; 2000.

72. Loser C, Seidel H, Hoffmann P, Zehnsdorf A. Bioavailability of hydrocarbons during microbial remediation of a sandy soil. Appl Microbiol Biotechnol 1999;51:105-11.

73. Hakstege AL, Geldermalsen-van LA. Pilot remediation of sediment from the petroleum harbour in Amsterdam. Wat Sci Technol 1998;37:403-9.

74. Rockne KJ, Strand SE. Anaerobic biodegradation of naphthalene, phenanthrene,

and biphenyl by denitrifying enrichment culture. Wat Res 2001;35:291-9.

75. Wilson NG, Bradley G. Enhanced degradation of petrol (Slovene diesel) in an aqueous system by immobilized *Pseudomonas fluorescens*. J Appl Biotechnol 1996;80:99-104.

76. Mulkins-Phillips GJ, Stewart JE. Effect of four dispersants on biodegradation and growth of bacteria on crude oil. Appl Microbiol 1974;28:547-52.

77. Fu MH, Alexander M. Use of surfactants and slurrying to enhance the biodegradation in soil of compounds initially dissolved in nonaqueous-phase liquids. Appl Microbiol Biotechnol 1995;43:551-8.

78. Allen CCR, Boyd DR, Hempenstall F, Larkin MJ, Sharma ND. Contrasting effects of a nonionic surfactant on the biotransformation of polycyclic aromatic hydrocarbons to *cis*-Dihydrodiols by soil bacteria. Appl Environ Microbiol 1999;65:1335-9.

79. Tiehm A. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl Environ Microbiol 1994;60:258-63.

80. Bruheim P, Bredholt H, Eimhjellen K. Effects of surfactant mixtures, including Corexit 9527, on bacterial oxidation of acetate and alkanes in crude oil. Appl Environ Microbiol 1999;65:1658-61.

81. Stelmack PL, Gray MR, Pickard MA. Bacterial adhesion to soil contaminants in the presence of surfactants. Appl Environ Microbiol 1999;65:163-8.

82. Mercade ME, Monleon L, Andres CD, Rodon I, Martinez E, Espuny MJ, Manresa A. Screening and selection of surfactant producing bacteria from waste lubricating oil. J Appl Bacteriol 1996;81:161-6.

83. Aldrett S, Bonner JS, Mills MA, Autenrieth RL, Stephens FL. Microbial

degradation of crude oil in marine environments tested in a flask experiment. Wat Res 1997;31:2840-8.

84. Venosa AD, Haines JR, Nisamaneepong W, Govind R, Pradhan S, Siddique B. Efficacy of commercial products in enhancing oil biodegradation in closed laboratory reactors. J Ind Microbiol 1992;10:13-23.

85. Sakai Y, Maeng JH, Kubota S, Tani A, Tani Y, Kato N. A non-conventional dissimilation pathway for long chain n-alkanes in *Acinetobacter* sp. M-1 that starts with a Dioxygenase reaction. J Ferment Bioeng 1996;81:286-91.

86. Cutright TJ, Lee S. Remediation of PAH-contaminated soil using *Achromobacter* sp. Energy Sources 1994;16:279-87.

87. Boopathy R, Widrig DL, Manning JF. In situ bioremediation of explosive-contaminated soil: A soil column study. Bioresour Technol 1997;59:169-76.

88. Hickey RF, Smith G. Biotechnology in industrial waste treatment and bioremediation; Bioremediation research in EPA: An overview of needs, directions and potentials. Michigan: CRC Lewis publishers; 1996.

89. Walker JD, Colwell RR. Microbial petroleum degradation: application of computerized mass spectrometry. Can J Microbiol 1975;21:1760-7.

90. Timmis KN. Designing microorganisms for the treatment of toxic wastes. Annu Rev Microbiol 1994;48:525-57.

91. Levin M, Safety considerations in Biotreatment operations. Genetically Modified Organisms 1995:173-200.

92. Boopathy R. Factoes limiting bioremediation technologies. Bioresour Technol 2000;74:63-7.

93. Hinchee RE, Kittle JA, Reisinger HJ. Applied bioremediation of petroleum

hydrocarbons. Ohio: Battelle Press; 1995.

94. Delille D, Delille B. Field observations on the variability of crude oil impact on indigenous hydrocarbon-degrading bacteria from sub-Antarctic intertidal sediments. Mar Environ Res 2000;49:403-17.

95. Langwaldt JH, Puhakka JA. On-site biological remediation of contaminated groundwater (a review). Environ Pollut 2000;107:187-97.

96. Jorgensen KS, Puustinen J, Suortti AM. Bioremediation of petroleum hydrocarbon contaminated soil composting in biopile. Environ Pollut 2000;107:245-54.

97. Kepley A, Purohit HJ, Chhatre S, Shanker R, Chakrabarti T, Khanna P. Osmotolerance and hydrocarbon degradation by a genetically engineered microbial consortium. Bioresour Technol. 1999;67:241-5.

98. Pletsch M, Santos de Araujo B, Charlwood BV. Novel biotechnological approaches in environmental remediation research. Biotechnol Adv 1999; 17:679-687.

99. Safonova Eth, Dmitrieva IA, Kviko KV. The interaction of algae with alcanotrophic bacteria in black oil decomposition. Resour Conserv Recycl 1999;27:193-201.

100. Suominen L, Jussila MM, Makelainen, Romantschuk M, Lindstrom K Evaluation of the *Galega-Rhizobium galegae* system for the bioremediation of oil contaminated soil. Environ Pollut 2000;107:239-44.

101. มยุรีย์ พันธัย, ชื่นจิตต์ บุญเฉิด, ปราณี สิทธิสาร, เพ็ญจิตร เปรมะบุตรม, จินดา นัยเนตร, วิรุฬห์ ไพธีกุล. จุลชีววิทยา : ปฏิบัติการและหลักเบื้องต้น. กรุงเทพฯ : คณะวิทยาศาสตร์ มหาวิทยาลัย มหิดล ; (2534).

102. Greenberg AE, Clescerl LS, Eaton AD. Standard method for the examination of water and wastewater. 18th edition, Maryland: ESP group; 1992.

103. Chang ZZ, Weaver RW. Nitrification and utilization of ammonium and nitrate during oil bioremediation at different soil water potentials. J Soil Contam 1997;6:149-60.

104. Tongpubesra K. Isolation and chracterization of crude oil degrading bacteria in Thailand [M.S. Thesis in biotechnology]. Bangkok : Faculty of graduate studies, Mahidol University ; 1998.

105. Al-Hadhrami MN, Lappin-Scott HM, Fisher PJ. Bacterial Survival and n-alkane degradation within Omani Crude Oil and a Mousse. Mar Pollut Bull 1995;30:403-8.

106. Van Hamme JD, Odumeru JA, Ward OP. Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture. Can J Microbiol 2000;46:441-50.

107. Grimberg SJ, Stringfellow WT, Aitken MD. Quantifying the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16 in the presence of a non-ionic surfactant. Appl Environ Microbiol 1996;62:2387-92.

108. Hinchee RE, Douglas GS, Ong SK. Bioaugmentation for site remediation. Ohio: Battelle Press; 1995.

109. Radwan SS, Sorkhoh NA, Fardoun F, Al-Hasan RH. Soil management enhancing hydrocarbon biodegradation in the polluted Kuwaiti desert. Appl Microbiol Biotechnol 1995;44:265-70.

110. Atas RM. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 1981;45:180-209.

111. Scheibenbogen K, Zytner RG, Lee H, Trevors JT. Enhanced removal of selected hydrocarbons from soil by *Pseudomonas aeruginosa* UG2 biosurfactants and some chemical surfactants. J Chem Technol Biotechnol 1994;59:53-9.

112. Parekh VR, Traxler RW, Sobek JM. n-Alkane oxidation enzymes of a Pseudomonad. Appl Environ Microbiol 1977;33:881-4.

113. Zaidi BR, Murakami Y, Alexander M. Factors limiting success of inoculation to enhance biodegradation of low concentrations of organic chemicals. Environ Sci Technol 1988;22:1419-25.

114. Thouand G, Bauda P, Oudot J, Kirsch G, Sutton C, Vidalie JF. Laboratory evaluation of crude oil biodegradation with commercial or natural microbial inocula. Can J Microbiol 1999;45:106-15.

115. Venkateswaren K, Harayama S. Sequential enrichment of microbial populations exhibiting enhanced biodegradation of crude oil. Can J Microbiol 1995;41: 767-75.

116. Guerin TF. Long-term performance of land treatment facility for the bioremediation of non-volatile oil wastes. Resour Conserv Recyc 2000; 28:105-20.

APPENDIX A

STATISTICAL COMPARISON OF RESIDUAL CONTAMINATED OIL

The statistical comparison of residual contaminated oil, which was used in Section 4.1 were carried out by the complete randomized design (CRD) for analysis of variance. This method compares the experimentals, which has a single factor. An example of test data is presented in Table A1

Table A1 The gravel density of the contaminated gravel

Gravel number	Gravel density				
	Group1	Group2	Group3	Group4	Group5
1	2.86	2.81	2.67	2.58	2.47
2	3.20	2.77	2.96	3.28	2.91
3	2.81	2.77	2.85	2.62	2.81
4	2.80	2.91	2.88	2.45	2.67
5	2.80	3.04	2.72	2.61	2.49
6	2.77	2.75	2.74	2.89	2.71
7	2.83	3.01	3.16	2.59	2.40
8	2.54	2.86	3.14	2.17	2.85
9	2.83	2.72	2.62	3.02	2.69
10	2.83	3.67	2.74	2.69	2.05
Mean	2.74	2.93	2.84	2.69	2.60

All statistical calculations could be carried out the Sigma Statistical Software of Jandel Corporation (USA).

Calculation of degree of freedom (df)

Amount of data (n) = 50

Amount of treatment (t) = 5 (5 group)

Amount of each group (r) = 10

Total df = n-1 = 49

Treatment df = t-1 = 4

Error df = total df - treatment df = 45

Calculated sum of square (SS)

X = each density gravel

Correction factor (CF) $= ((\sum_{n=1}^{50} X)^2)/n$ = 386.5

Total SS $= \sum_{n=1}^{50}(X)^2 - CF$ = 3.3773

Treatment SS $= (\sum_{i=1}^{5}(\sum_{n=1}^{10} X_i)^2 /r) - CF$ = 0.6592

i = sequence of treatment (1 to 5)

Error = Total SS- Treatment SS = 2.7181

Calculated mean square (MS)

Treatment MS = Treatment SS / Treatment df = 0.1648

Error MS = Error SS / Error df = 0.0604

Calculated value

F- value = Treatment MS / Error MS = 2.73

When F-value was calculated, the analysis was carried out by comparing F-value with a value in the F-distributed table, at treatment df equal to 4 and error df equal to 45, at 95% significance. If the calculated F-value was more than that in the Table, then treatments were significantly different at 95% confidence ($\alpha = 0.05$). The one asterisk could be assigned after the numeric number of the calculated F in the table of analysis of variance (ANOVA).

In Table A2, F-value from the F-table was 2.50, which was less than the calculated one. It indicated that the density of gravel among groups were significantly different at 95% confidence.

Table A2 Analysis of variance of density of gravel

Source of variation	Degree of freedom	Sum of Squares	Mean Squares	F - value	Tabular F ($\alpha = 0.05$)
Treatment	4	0.6592	0.1648	2.73*	2.50
Error	45	2.7181	0.0604		
Total	49	3.3773			

APPENDIX B

API KIT METHOD (API 20 NE)

Identification system for non-enteric Gram-negative rods

The API 20 NE system is a standardized micromethod combining 8 convention tests and 12 assimilation tests for the identification of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae* (e.g. *Pseudomonas, Acinetobacter, Flavobacterium, Moraxella, Vibrio, Aeromonas,* etc.)

PRINCIPLE

An API 20 NE strip consists of 20 microtubes containing dehydrated media and substrates. The conventional tests are inoculated with a saline bacterial suspension, which reconstitutes the media. After incubation, the assimilation tests are inoculated with bacteria in minimal medium. The substrate utilized reactions are read against the Reading Table. The identification is obtained by referring to the Analytical Profile Index or using the identification software, which are provided together with the kit. The compositions of media and reagents are shown in Table B1

Table B1 Compositions of media and reagents

NaCl 0.85% (medium)	Sodium chloride	8.5	g
	Demineralized water	1000	ml
AUX medium	Ammonium sulphate	2	g
	Agar	1.5	g
	Mineral base	82.8	mg
	Amino acids	250	mg
	Vitamins and nutritional substances	35.9	mg
	Phosphate buffer 0.04 M pH 7.1	1000	ml
	Final pH : 7.0-7.2		
James reagent	Compound J 2183 (confidental)	0.5	g
	HCl 1 N	100	ml
NIT 1 reagent	Sulfanilic acid	0.4	g
	Acetic acid	30	g
	H_2O	70	ml
NIT 2 reagent	N,N-dimethyl-1-naphthylamine	0.6	g
	Acetic acid	30	g
	H_2O	70	ml
OX reagent	Tetramethyl-p-phenylenediamine	1	g
	Isoamyl alcohol	100	ml

INSTRUCTION FOR USE

Preparation of speciments

The microorganisms to be identified must first be grown on appropriate culture media according to standard microbiological techniques.

Selection of colonies

API 20 NE should only be used with non-fastidious Gram-negative rods which not belonging to *Enterobacteriaceae.* To select these, note several identical colonies on the isolation medium and perform an oxidase test on one of them as follows:

- Place a piece of filter paper on a glass slide.
- Moisten the paper with 1 drop of water.
- Pick a chosen colony with a wooden or glass applicator and rub it onto the moistened filter paper.
- Added 1 drop of OX reagent.
- A deep purple coloration, which appears within 1 or 2 minutes indicates a positive reaction.

The result of this test should be noted on the result sheet for the identification software (Fig. B1)

Preparation of the strip (The example of the strip is shown in Fig. B2)

- Prepare an incubation box, tray and lid, and distribute about 5 ml of distilled water or demineralized water (or any water without additives or chemicals, which may release gases i.e. Cl_2, CO_2, etc.) into the bottom of the tray to create a humid atmosphere.
- Record the specimen number on the elongated flap of the tray.
- Remove the strip from its individual packaging and place in the incubation box.

Preparation of the inoculum

- Open an ampoule of NaCl 0.85% Medium (2 ml) or use any tube containing 2 ml of 0.85% physicological saline without additives.
- Using a pipette or PSIpette, pick 1-4 colonies of identical morphology from the agar plate either by suction or by successive touches.
- Mix the suspension thoroughly.

Inoculation of the strip

- Inoculate tests NO_3 to PNPG by distributing the saline suspension into the tubes (not including the capsules) using the same pipette. To avoid the formation of bubbles at the base of tubes, tilt the strip slightly forwards and place the tip of the pipette against the side of the capsule.
- Open the ampoule of the AUX Medium and added approximately 200 µl (about 6-8 drops with a PSIpette) of the remaining saline suspension to the ampoule. Homogenize well with the pipette, avoiding the formation of bubbles.
- Fill the tubes and capsules of tests |GLU| to |PAC| with the suspension. Take care to leave a flat or slightly convex, but not concave, meniscus. Capsules under or overfilled may give incorrect results.
- Add mineral oil to the capsules of 3 underlined tests (GLU, ADH, URE) until a convex meniscus is formed.
- Close the incubation box and incubate at 30°C for 24 h.

Reading and interpretation

- After the incubation period, read the strip by referring to the Reading Table

- Record all spontaneous reactions (GLU, ADH, URE, ESC, GEL and PNPG) on the result sheet.
- The reading of two tests NO_3 and TRP should be performed whilst protecting the assimilation tests from airborne contamination. To do this, cover the assimilation tests with the incubation box lid during the reading of the NO_3 and TRP tests.

NO_3 test

- Add 1 drop of NIT 1 and 1 drop of NIT 2 reagents to the NO_3 capsule.
- After 5 minutes, a red color indicates a positive reaction to be recorded on the result sheet.

TRP test

- Add 1 drop of James reagent. The reaction takes place immediately : a pink color, which develops in the whole capsule indicates a positive reaction to record on a result sheet.

Assimilation tests

- Observe the bacterial growth. An opaque capsule indicates a positive reaction. Occasionally, a capsule may show weak growth. In this case, the results should be recorded as ± by comparing the intensity to that of the other tests on the strip.

Identification

- Using the identification software by manually entering the results from the result sheet and reading the result from the computerization.

Disposal of used material

- After use, all ampoules, pipettes, strips and incubation boxes should be autoclaved, incinerated, or immersed in a disinfectant for decontamination prior to disposal.

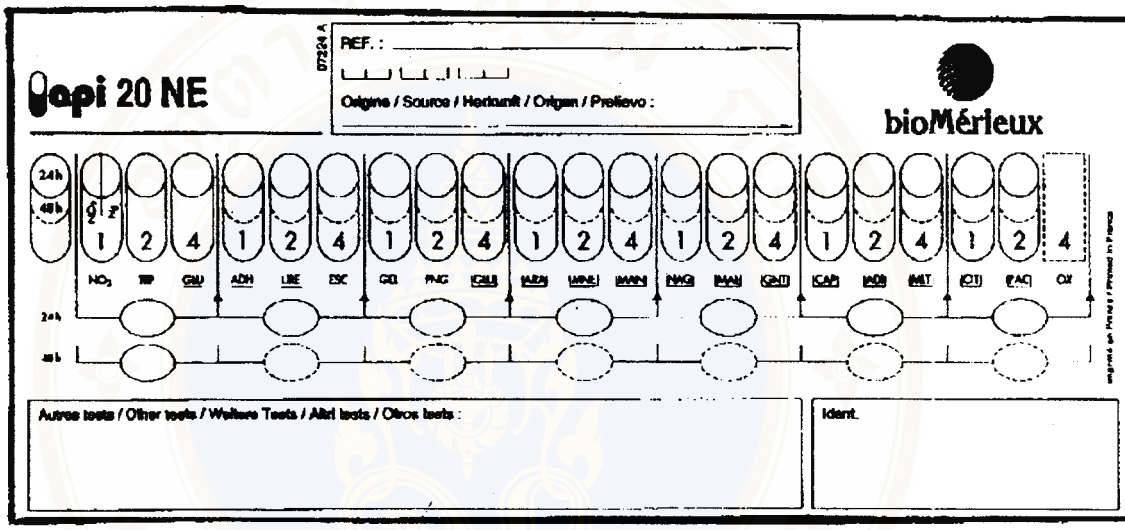


Fig. B1 An example of the result sheet

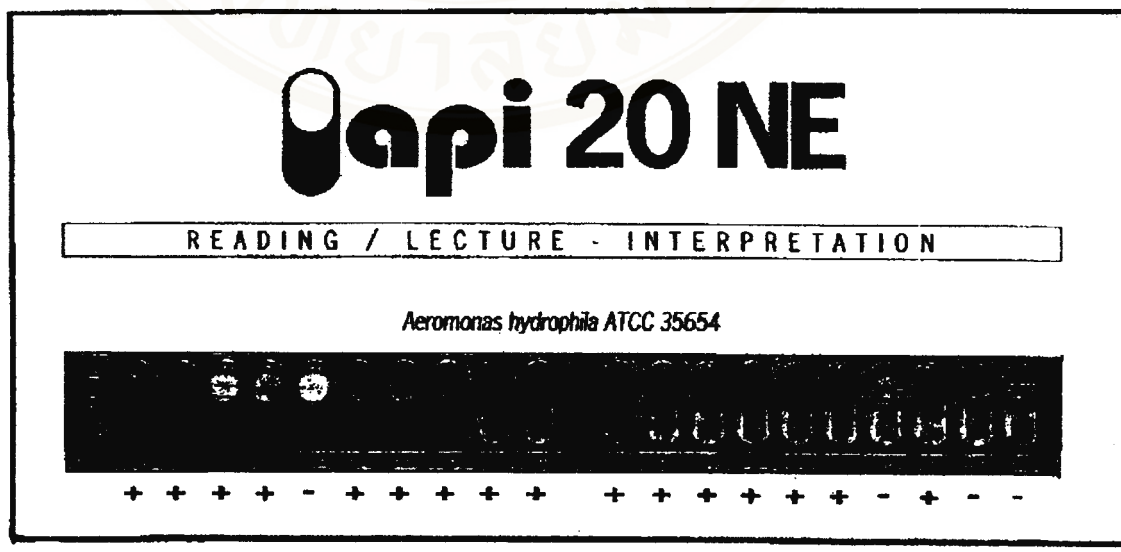


Fig. B2 An example of the strip

APPENDIX C

PREPARATION OF REAGENTS FOR THE DETERMINATION OF NITROGEN AND PHOSPHOROUS CONCENTRATIONS

C1. Preparation of reagents for the determination of Ammonia (nitrogen) concentration by Titrimetric Method

a. Sodium hydroxide 0.1 N : NaOH 4 g were added into the volumetric flask and adjusted to 1000 ml with distilled water. The solution was kept in a plastic bottle.

b. Sodium hydroxide 6 N : NaOH 240 g were added into the volumetric flask and adjusted to 1000 ml with distilled water. The solution was kept in a plastic bottle.

c. Sodium hydroxide 0.02 N : NaOH 0.8 g were added into the volumetric flask and adjusted to 1000 ml with distilled water. The solution was titrated with 0.1 g of anhydrous potassium hydrogen phthalate ($C_8H_5KO_4$, kept in a hot air oven 100°C for 2 h and cooled down in the desicator), which was dissolved in 25 ml of distilled water, in order to make the accurate concentration. The solution was kept in a plastic bottle.

d. Stock solution of sulfuric acid 0.1 N : Conc. H_2SO_4 2.5 ml were added in the volumetric flask and adjusted to 1000 ml by distilled water. The solution was kept in a glass-stopped bottle.

e. Standard sulfuric acid titrant, 0.02 N : 200 ml of stock solution sulfuric acid 0.1 N were pipetted into the volumetric flask and adjusted to 1000 ml with distilled water. It was titrated with 0.02 N NaOH (c.) in order to make the accuracy of concentration.

f. Borate buffer solution : 0.1 N NaOH solution 88 ml were mixed with 500 ml of approximately 0.025 M sodium tetraborate solution (9.5 g of $Na_2B_4O_7.10H_2O$/l). The mixture was adjusted to 1000 ml with distilled water.

g. Mixed indicator solution : Methyl red indicator 200 mg were dissolved in 100 ml of 95% ethanol. Methylene blue 100 mg were dissolved in 50 ml of 95% ehtanol. Then these two solutions were combined and it should be prepared monthly.

h. 2% indicating boric acid solution : H_3BO_3 20 g were dissolved in the distilled water and added with 10 ml of mixed indicator solution and diluted to 1000 ml in a volumetric flask with distilled water. It should be prepared monthly.

C2. Preparation of reagents for the determination of Nitrate (nitrogen) concentration by Brucine-sulfanilic acid Method

a. Stock nitrate solution (100 mg N/l): KNO_3 was kept in a hot air oven at 100°C for 1 h. After cooled down in the desicator, 721.8 mg was added into the volumetric flask and adjusted to 1000 ml with distilled water.

b. Standard nitrate solution (1 µg/ml): 10 ml of stock nitrate solution were transferred into a volumetric flask and adjusted to 1000 ml with distilled water. The final concentration of nitrogen was 1 µg/ml.

c. Brucine-sulfanilic acid solution: 1 g of brucine sulfate and 0.1 g of sulfanilic acid were dissolved into 70 ml of warm distilled water. After 3 ml of 37% HCl were added, the solution was transferred into the volumetric flask and adjusted to 1000 ml with distilled water. The solution was kept at 4°C.

d. Sulfuric acid solution (25% (v/v)): 1000 ml of 97% H_2SO_4 were carefully added into distilled water (250 ml). The solution was kept in a glass-stopped bottle at room temperature.

e. Sodium chloride solution (30%(w/v)): NaCl 300 g was added in a volumetric flask. Then it was dissolved and adjusted to 1000 ml with distilled water.

Preparation of calibration curve

Standard KNO_3 solutions (0, 1.0, 2.0, 4.0, 7.0 and 10.0 ml) were pipetted into 50 ml test tubes separately. Then each tube was adjusted to 10 ml with distilled water and placed in an ice bath. After cooled, the sample was consequently mixed with 2 ml of 30% (w/v) NaCl solution, and 10 ml of 25% (v/v) H_2SO_4 solution. The reaction tube was added with 0.5 ml of Brucine-sulfanilic acid solution and placed into 95°C water bath for 20 min. The absorbances of the reaction mixtures were measured at the

wavelength of 410 nm by a spectrophotometer at room temperature. The absorbances and nitrogen concentrations were plotted to give a straight line passing through the origin. An example of standard curve is illustrated in Fig. C1.

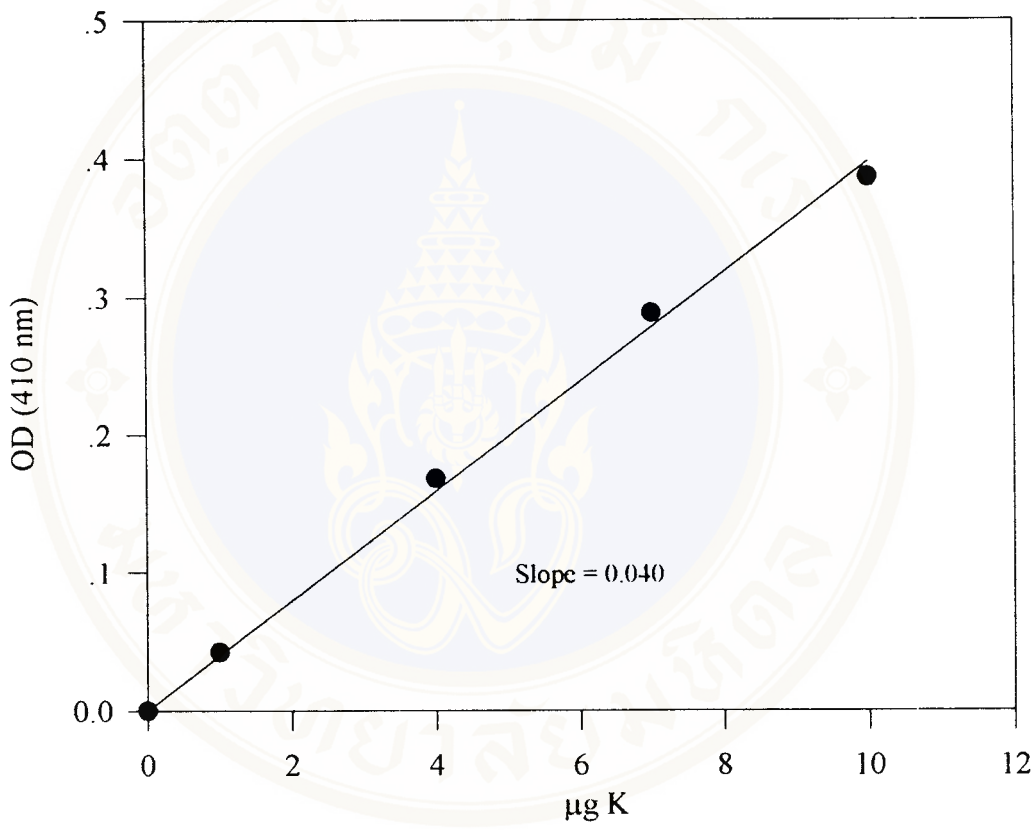


Fig. C1 Example of standard KNO_3 curve

C3. Preparation of reagents for the determination of Phosphate (phosphorous) concentration by Ascorbic acid Method

a. Sulfuric acid 5 N: 97% H_2SO_4 70 ml were diluted to 500 ml of distilled water in a volumetric flask.

b. Potassium antimonyl tartrate solution: $K(SbO)C_4H_2O_6.0.5H_2O$ 1.3715 g were dissolved in 500 ml of distilled water. The solution was kept in a glass-stopped bottle.

c. Ammonium molybodate solution: $(NH_4)_6Mo_7O_{24}.4H_2O$ 20 g were dissolved in 500 ml of distilled water. The solution was kept in a glass-stopped bottle.

d. Ascorbic acid, 0.1 M : Ascorbic acid 1.76 g were dissolved in 100 ml of distilled water. The solution is kept at 4°C and freshly prepared every week.

e. Combined reagent : 50 ml of solution a, 5 ml of solution b, 15 ml of solution c and 30 ml of solution d were mixed to obtain 100 ml of the combined reagent. Combined reagent was stable for 4 h so it was freshly prepared for use every time.

f. Phenolphthalein indicator aqueous solution: 1 g of phenolphthalein was dissolved in 100 ml of 70% ethanol.

g. Stock phosphate solution : 219.5 mg of anhydrous KH_2PO_4 were dissolved in 1000 ml of distilled water.

h. Standard phosphate solution (2.5 µg/ml): 50 ml of stock phosphate solution were diluted to 1000 ml of distilled water. The final concentration of phosphorous in the solution was 2.5 µg/ml.

Preparation of calibration curve

The standard KH_2PO_4 solutions (0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 ml, which were equal to 0, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035 and 0.04 mg P) were pipetted into 50 ml volumetric flasks separately. After that they were adjusted to the volume by distillated water and were then transferred into 250 ml Erlenmeyer flasks. The combined reagent (e.) 8.0 ml was added in each flasks and mixed thoroughly. After at least 10 min but no more than 30 min, the samples were measured the absorbances at the wavelength of 880 nm by a spectrophotometer. The absorbances and phosphorous concentrations was plotted to give a straight line passing through the origin. An example of standard curve is shown in Fig. C2.

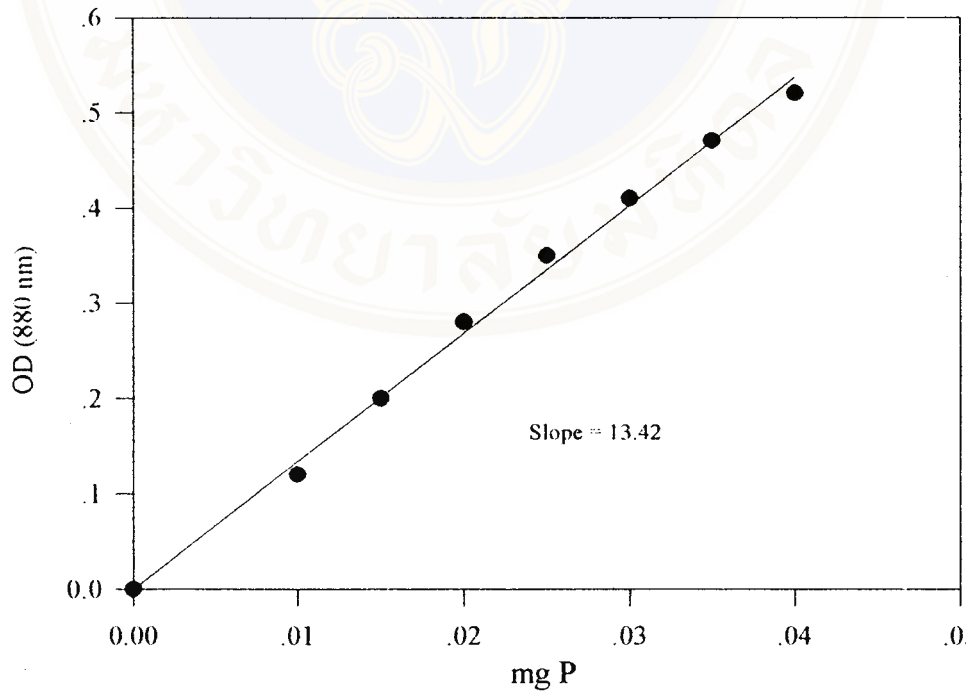


Fig. C2 An example of standard KH_2PO_4 curve

APPENDIX D

THE RETENTION TIME OF HYDROCARBONS

FROM

PACKED COLUMN GAS CHROMATOGRAPHY

Table D1 Retention time of the standard hydrocarbons.

n-alkanes	The retention time (min)
C12 Dodecane	5.472
C13 Tridecane	6.972
C14 Tetradecane	8.347
C15 Pentadecane	9.630
C16 Hexadecane	10.842
C17 Heptadecane	11.955
C18 Octadecane	13.107
C19 Nonadecane	14.157
C20 Eicosane	15.158
C21 Heneicosane	16.100

Table D2 Retention time of the major peak in Tapis crude oil.

n-alkanes	The retention time (min)
C12 Dodecane	5.428
C13 Tridecane	6.917
C14 Tetradecane	8.295
C15 Pentadecane	9.572
C16 Hexadecane	10.775
C17 Heptadecane	11.942
C18 Octadecane	13.017
C19 Nonadecane	14.053
C20 Eicosane	14.970
C21 Heneicosane	15.993
C22 Docosane	16.888
C23 Tricosane	17.750
C24 TeTracosane	18.570
C25 Pentacosane	19.357
C26 Hexacosane	20.112
C27 Heptacosane	20.830
C28 Octacosane	21.547
C29 Nonacosane	22.212
C30 Triacontane	22.865
C31 Untriacontane	23.495
C32 Dotriacontane	24.727

BIOGRAPHY

NAME	Miss Prapaipit Teparot
DATE OF BIRTH	6 June 1974
PLACE OF BIRTH	Prachinburi, Thailand
INSTITUTIONS ATTENDED	Chulalongkorn University, 1993-1997: Bachelor of Science (Microbiology)
	Mahidol University. 1997-2001: Master of Science (Biotechnology)