SURVIVAL AND PAHs DEGRADATIVE ABILITY OF *Sphingomonas sp.*strain P2 IN PAHs CONTAMINATED SOIL AFTER SOIL ACCLIMATIZATION

Miss Prapaporn Deangrueng

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ความสามารถในการอยู่รอดและการย่อยสลายสาร PAHs ของ *Sphingomonas* sp. สายพันธุ์ P2 ในดินที่ปนเปื้อนด้วยสาร PAHs ภายหลังจากการทำให้เคยชินกับสภาพดิน

นางสาว ประภาพร แดงเรือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Prapaporn Deangrueng
Field of study	Environmental Management
Thesis Advisor	Assoc. Prof. Kanchana Juntongjin, Ph.D.
Thesis Co-advisor	Ekawan Luepromchai, Ph.D.

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

…….. Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Manaskorn Rachakornkij, Ph.D.)

…….……. Thesis Advisor
(Assoc. Prof. Kanchana Juntongjin, Ph.D.)

…….. Thesis Co-advisor
(Ekawan Luepromchai, Ph.D.)

…….. Member
(Assistant Professor Suthep Thaniyavarn, Ph.D.)

…….. Member
(Assistant Professor Kobchai Pattaragulwanit, Dr.rer.nat.)

ประภาพร แดงเรือง : ความสามารถในการอยู่รอดและการย่อยสลายสาร PAHs ของ *Sphingomonas* sp. strain P2 ในดินที่ปนเปื้อนด้วยสาร PAHs ภายหลังจากการทำให้เคยชินกับสภาพดิน. (SURVIVAL AND PAHs DEGRADATIVE ABILITY OF *Sphingomonas* sp. STRAIN P2 IN PAHs CONTAMINATED SOIL AFTER SOIL ACCLIMATIZATION) อ. ที่ปรึกษา : รศ. ดร. กาญจณา จันทองจีน, อ. ที่ปรึกษาร่วม : ดร. เอกวัล ลือพร้อมชัย 99 หน้า. ISBN 974-53-1427-7.

Sphingomonas sp. P2 เป็นแบคทีเรียที่คัดแยกจากดินปนเปื้อนน้ำมันเครื่องซึ่งสามารถย่อยสลายฟีแนนทรีนและสาร PAHs อื่นๆ แบคทีเรียสายพันธุ์นี้ยังสามารถโคเมแทโบไลซ์ สาร PAHs ที่มีมวลโมเลกุลสูง เช่น ฟลูออแรนธีน และ ไพรีน ในอาหารเหลวที่มีฟีแนนทรีนอยู่ อย่างไรก็ตามการเจริญและความสามารถในการย่อยสลายสาร PAHs ของแบคทีเรียสายพันธุ์นี้ถูกยับยั้งเมื่อเติมลงในดินไม่ปลอดเชื้อ เพื่อเพิ่มความสามารถในการอยู่รอด และการย่อยสลายของสาร PAHs การทดลองนี้ได้ทำให้ *Sphingomonas* sp. P2 เคยชินกับสภาพดินก่อนเติมลงในระบบนิเวศน์จำลองดินที่ไม่ปลอดเชื้อ ขบวนการทำให้เคยชินทำโดยเลี้ยง *Sphingomonas* sp. P2 ในอาหารเลี้ยงเชื้อที่เตรียมจากสารสกัดจากดินแล้วตามด้วยเลี้ยงในดินปลอดเชื้อ แบคทีเรียที่ถูกทำให้เคยชินกับสภาพดินถูกนำมาใช้เป็นหัวเชื้อในการบำบัดดินที่ปนเปื้อนสาร PAHs เมื่อเปรียบเทียบกับอาหารเลี้ยงเชื้ออื่น *Sphingomonas* sp. P2 ที่เลี้ยงในสารสกัดจากดินผสมน้ำในอัตราส่วน (1: 3) มีการย่อยสลาย PAHs และการอยู่รอดสูงสุดในระบบนิเวศน์จำลองดินที่ปลอดเชื้อขนาด 2 กรัม จึงนำสารสกัดจากดินผสมน้ำในอัตราส่วน (1: 3) มาใช้เป็นอาหารสำหรับเตรียมหัวเชื้อหัวเชื้อแบคทีเรียจากอาหารเหลว หัวเชื้อแบคทีเรียจากดินเตรียมจากการเติมหัวเชื้อหัวเชื้อแบคทีเรียจากอาหารเหลวนี้ในดินปลอดเชื้อที่เติมฟีแนนทรีน การบำบัดดินที่ปนเปื้อนสาร PAHs มีการศึกษาในระบบนิเวศน์จำลองดินไม่ปลอดเชื้อขนาด 20 g เพื่อทดสอบความสามารถในการอยู่รอด และการย่อยสลายสาร PAHs ของแบคทีเรียที่ถูกทำให้เคยชินแล้ว ทั้งนี้ใช้ระบบนิเวศน์จำลองดิน 2 ชนิด ที่มีความเข้มข้นของฟีแนนทรีนต่างกัน คือ (ก) ฟีแนนทรีน 0.1 มก./กรัมดิน ผสมกับไพรีน 0.1 มก./กรัมดิน และ (ข) ฟีแนนทรีน 0.3 มก./กรัมดินผสมกับ ไพรีน 0.1 มก./กรัมดิน เมื่อเปรียบเทียบประสิทธิภาพของหัวเชื้อแบคทีเรียจากดินที่บ่มก่อนเป็นเวลา 4-, 8- และ 12- วัน และหัวเชื้อหัวเชื้อแบคทีเรียจากอาหารเหลว พบว่าหัวเชื้อแบคทีเรียจากดินมีความสามารถในการอยู่รอดและการย่อยสลายสาร PAHs ในดินไม่ปลอดเชื้อได้ นอกจากนี้หัวเชื้อแบคทีเรียจากอาหารเหลวมีประสิทธิภาพในการย่อยสลาย phenanthrene ได้ดีเช่นกัน โดยเฉพาะอย่างยิ่งในช่วงต้นของการทดลอง สำหรับการย่อยสลายของไพรีนพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญ เมื่อเทียบกับชุดทดลองกับชุดควบคุม (ไม่มีการเติมหัวเชื้อแบคทีเรีย) จากการวิเคราะห์16S rDNA-DGGE พบแถบดีเอ็นเอที่ตรงกับ *Sphingomonas* sp. P2 ในทุกๆ เจล ผลการทดลองแสดงว่าแบคทีเรียที่เติมเป็นหนึ่งในประชากรเด่นของระบบนิเวศน์จำลองดิน นอกจากนี้หัวเชื้อแบคทีเรียจากอาหารเหลวและจากดินมีผลต่อประชากรแบคทีเรียในดินแบบเดียวกัน

สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา) ลายมือชื่อนิสิต.....

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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PRAPAPORN DEANGRUENG : SURVIVAL AND PAHs DEGRADATIVE ABILITY OF *Sphingomonas* sp. STRAIN P2 IN PAHs CONTAMINATED SOIL AFTER SOIL ACCLIMATIZATION. THESIS ADVISOR : ASSOC.PROF. KANCHANA JUNTONGJIN, Ph.D., THESIS CO-ADVISOR : EKAWAN LUEPROMCHAI, Ph.D., 99 pp. ISBN 974-53-1427 -7.

Sphingomonas sp. P2 isolated from lubricant contaminated soil is capable of utilizing phenanthrene and several other PAHs. This strain is also co-metabolize high molecular weight PAHs such as fluoranthene and pyrene in liquid medium supplemented with phenanthrene. However, its growth and PAHs degradative abilities were inhibited after added to non-sterile soil. To improve its survival and PAH degrading activities, the study acclimatized *Sphingomonas* sp. to soil conditions before added to non-sterile soil microcosms. Soil acclimatization process was carried out by sequential cultivating of *Sphingomonas* sp. P2 in soil extract media and sterile soil. The acclimatized bacteria were used as inoculum for PAH bioremediation. *Sphingomonas* sp. P2 cultured in soil extract mixed with water (1:3) showed the highest PAHs degradative ability and survival efficiency in 2-g sterile soil microcosm when compared with other media. Soil extract mixed with water (1:3) was therefore selected as media for preparing liquid inoculum. Later, soil inoculum was prepared by adding the liquid inoculum into sterile soil spiked with phenanthrene. PAH bioremediation treatments were conducted in 20-g non-sterile soil microcosms to study the survival and PAHs degradability of acclimatized bacteria. Two types of microcosms with different concentrations of phenanthrene were used; (a) 100 ppm phenanthrene mixed with 100 ppm pyrene and (b) 300 ppm phenanthrene mixed with 100 ppm pyrene. The efficiency of soil inoculums preincubated for 4, 8 and 12 days and liquid inoculum prepared in soil extract mixed with water (1:3) were compared. The result showed that soil inoculum was able to degrade phenanthrene and could survive in non-sterile soil microcosms. Meanwhile, liquid inoculum was also effective in phenanthrene degradation especially at the beginning of the experiment. There was no significant difference in pyrene degradation between the treatments and control (without inoculum). 16S rDNA-DGGE analysis showed DNA band corresponding to *Sphingomonas* sp. P2 in all gels. The results suggested that the inoculated bacteria were one of the dominant populations in soil microcosms. Moreover, liquid and soil inoculum provided similar effects on soil bacterial populations.

Field of study Environmental Management Student's signature .....

Academic year 2004 Advisor's signature .....

Co-advisor's signature .....

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LIST OF ABBREVIATIONS

CFMM	Carbon free mineral medium
CFU	Colony forming unit
EPA	Environmental Protection Agency
min	Minute
ml	Milliliter
mm	Millimeter
MM	CFMM liquid inoculum
MS	Soil extract mixed with CFMM (1:3) liquid inoculum
PAHs	Polycyclic Aromatic Hydrocarbons
ppm	Part per million
O.D.	Obtical density
rpm	Round per minute
RT	Retention time
S	Soil extract mixed with water (1:3) liquid inoculum
S4	4-day soil inoculum
S8	8-day soil inoculum
S12	12-day soil inoculum
µl	Micro litter

CHAPTER I

INTRODUCTION

1.1 Statement of the problem

The increasing of world populations has resulted in a spread of area with polluted soil and water. Population explosion also brings with it a growing pressure on air, water and land resources. In order to meet the demands of people, rapid expansion of industries, food, health care, vehicles, etc. is necessary. It is very difficult to maintain the quality of life with all these new developments. If proper management is not applied, the unfavorable conditions will be happened to the environment (Sasikumar and Papinazath, 2003).

PAHs, polycyclic aromatic hydrocarbons, are a class of hazardous organic chemicals composed of two or more fused benzene rings in linear, angular, or cluster arrangements. Some PAHs are carcinogens and mutagens. These compounds are in the USA Environmental Protection Agency (US-EPA) priority pollutant list. PAHs often persist in the environment because they are hydrophobic and most are practically insoluble in water (Eve and Eve, 1998). Major sources of PAHs contamination in the environment are combustion processes of carbonaceous materials at high temperature, for example emissions from automobiles, industrial processes, and domestic heating systems, waste incineration facilities, tobacco smoking, and several natural sources such as forest fires and volcanic eruptions (Liu et al., 2001).

Bioremediation is a technology that uses microorganisms to clean up contaminated environments. The treatment is usually carried out in non-sterile open environments, which contain a variety of organisms (Watanabe, 2001). Biodegradation of PAHs has gained attention over the last decade. The optimization of the degradation parameters, such as oxygen, nitrogen, phosphorus, temperature and PAHs bioavailability, is very important in order to obtain the effective bioremediation treatment (Eriksson, 2002). There are two general approaches for bioremediation: biostimulation and bioaugmentation (Balba et al., 1998). In general, biological treatment relies upon stimulation and natural selection of indigenous microorganisms in the environment. However, the indigenous organisms may not have metabolic capability to degrade certain compounds. It is therefore necessary to employ bioaugmentation or the addition of specialized bacteria for bioremediation (Eve and Eve, 1998). Bioaugmentation was used in both in situ and ex situ systems (Boopathy, 2000).

The maintenance of bacterial cells to survive and have the required activity in soils is the most important factor for the success of any bioaugmentation protocol. However, population size and activities of the bacteria (inoculum) usually decline more or less rapidly following the inoculation into natural soil. Besides the intrinsic physiological characteristics of the organisms, abiotic and biotic soil factors play an important role on the added bacteria. Abiotic soil factors (e.g., textural type, pH, temperature, and moisture) affect on the dynamics of inoculant population by imposing various stresses on the cells. Biotic soil factors also reduce the inoculants population size due to grazing by predators such as protozoa and other

microorganisms. Another biological factor that affects the survival of inoculants is the competition for available substrate and biological space between the inoculant and indigenous populations (Van Veen et al., 1997).

Bacterial cells face many stresses in soil, which fluctuate in time. The indigenous soil bacteria probably developed survival strategies enabling them to survive in such conditions (Van Elsas, 1993). Since conditions in liquid medium and natural soil are totally different. The survival of bacterial inoculum in soil should be increased by acclimatizing the bacteria to soil conditions before inoculation. For example, Megharaj (1997) cultured *Sphingomonas* sp. strain RW1 in soil extract medium prior to its introduction into soil. The acclimatized bacteria were found to survive better than the bacteria that had not been cultured in soil extract medium. In addition, preincubation of bacteria in sterile soil was showed to enhance the attachment of the bacterial cells to the soil matrix, the microcolony formation, and consequently the increased survival of inoculated bacteria (Van Dyke, 2000). This sterile soil was expected to provide a protective niche to microbial inoculants after apply to non-sterile soil.

1.2 Objectives

The main objective of this research is to develop a soil acclimatization process that could be used to enhance survival and PAHs degradative ability of *Sphingomonas* sp. strain P2 during bioremediation of PAHs contaminated soil. The bacterium was isolated from lubricant-contaminated soil collected from a garage in Prajinburi and has been reported for its high efficiency in degrading PAHs.

Sub-objectives

The sub-objectives of this study were:

(1) To select a medium for preparing liquid inoculum of *Sphingomonas* sp. strain P2 prior to culture in sterile soil.

(2) To prepare the soil inoculum of *Sphingomonas* sp. strain P2 by incubating the liquid inoculum in sterile soil.

(3) To determine the effectiveness of *Sphingomonas* sp. strain P2 soil inoculum for degrading PAHs in non-sterile soil microcosms.

(4) To monitor *Sphingomonas* sp. strain P2 as well as indigenous bacteria in the soil microcosms.

1.3 Hypothesis

Sphingomonas sp. strain P2 will acclimatize to soil condition after sequential cultivating in soil extract and sterile soil, consequently these bacteria will be able to survive and maintain their PAHs degradability after apply to non-sterile PAHs contaminated soil.

1.4 Scope of the Study

This experiment was conducted to evaluate the potential of a soil acclimatization process on enhancing the survival and maintaining PAHs degradability of *Sphingomonas* sp. strain P2 inoculum after apply to non-sterile PAHs contaminated soil. The acclimatization was carried out by cultivating the bacteria in soil extract medium followed by sterile soil. Liquid inoculum and soil inoculum of *Sphingomonas* sp. strain P2 were produced from the soil extract medium and sterile soil, respectively. Later, the soil inoculum was used to bioaugment non-sterile PAHs contaminated soil.

The followings are specific details.

1. Fertile soil collected from a garden in Bangkok was used in this study.
2. *Sphingomonas* sp. strain P2 isolated from lubricant-contaminated soil collected from a garage in Prajinburi by Supaka et al. (2001) was used as PAHs degrader.
3. Phenanthrene and pyrene were used as modeled PAHs pollutants in soil.

4. Three types of liquid media consisted of carbon free mineral medium (CFMM), soil extract mixed with water (1:3), and soil extract mixed with CFMM (1:3) medium were employed to test for their suitability for the acclimatization of *Sphingomonas* sp. strain P2. Medium that gave highest number of *Sphingomonas* sp. strain P2 after incubating in sterile soil contaminated with phenanthrene and pyrene will be selected for the preparation of liquid inoculum *Sphingomonas* sp. strain P2.
5. *Sphingomonas* sp. strain P2 soil inoculum was prepared by adding the liquid inoculum into sterile PAHs contaminated soil and incubating for appropriate period of time.
6. PAHs bioremediation was conducted in non-sterile 20-g soil microcosms pre-spiked with phenanthrene and pyrene. The treatments included the addition of soil inoculum, liquid inoculum, and control (without any addition). The effect of acclimatization (incubation) time was compared using 4-, 8-, and 12- day old soil inoculums. As of degradative ability of the inoculum, phenanthrene was varied to 100 ppm and 300 ppm.
7. The populated *Sphingomonas* sp. strain P2 and indigenous bacterial populations in non-sterile soil microcosms were detected by plate count technique and DGGE method.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Environmental concerns of PAHs

2.1.1 Sources of PAHs

Polycyclic aromatic hydrocarbons are of significant environmental concern as some of them can induce cancer or cause mutations, even at low concentrations. Structure of many PAHs allows them to be oxidized into an active form that is carcinogenic (Roper and Pfaender, 2000). PAHs are composed of fused, aromatic rings which have dense clouds of π-electrons on both sides of the ring structures, making them resistant to nucleophilic attack. Moreover, their physical properties, such as low aqueous solubility and high solid-water distribution ratios prevent them from microbial utilization and promote their accumulation in the solid phases of the terrestrial environment (Johnsen et al., 2005).

Activities associated with the contamination of PAHs to the environment are as follows (Cerniglia, 1992; Wilson and Jones, 1993);

- Gasification / liquefaction of fossil fuels
- Heat and power generation by using fossil fuels
- Coke production, catalytic cracking, asphalt production and their uses
- Carbon-black production and it use
- Coal-tar / coal-tar-pitch production and their used

- Refining / distillation of crude oil and crude-oil-derived products
- Wood-treatment process
- Wood-preservative (e.g. creosote / anthracene-oil) production
- Fuel / oil storage, transportation, processing, usage, and disposal
- Landfill / waste dumps, open and burning (types / refuse / coal etc.), incineration.

Because of their toxicities, mutagenicity, and carcinogenicity, PAHs pose a serious concern for their environmental presence (Yuan et al., 2000). PAHs are harmful and recalcitrant, thus the USA Environmental Protection Agency (US-EPA) classified 16 PAHs compounds as environmental priority pollutants. The list of these PAHs is shown in Figure 2.1.

2.1.2 Physical and chemical properties of PAHs

Physicochemical properties of individual PAH affect its bioavailability and biodegradation by microorganisms (Bauer and Capone, 1988). In general, an increase in the number of fused rings increases chemical stability and hydrophobicity of PAH molecules (Kanaly et al., 2000). Low-molecular-weight PAHs are easier to degrade than high-molecular-weight PAHs. Moreover, high-molecular-weight PAHs (four and more rings) are less bioavailable and more persistent because of their adsorption onto soil organic matter (Potin et al., 2004).

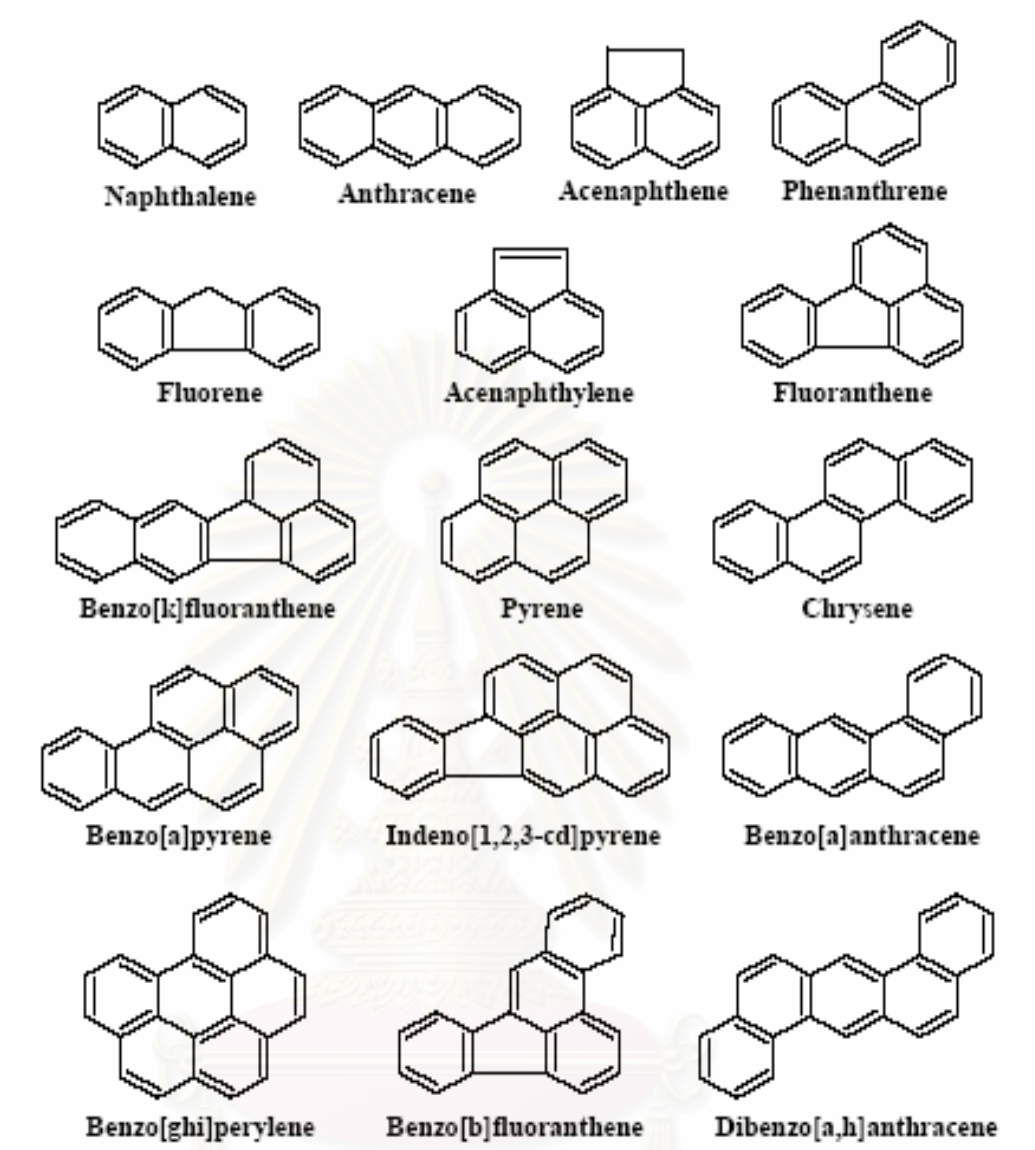
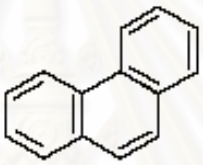
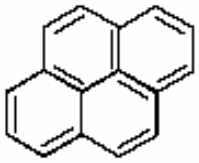


Figure 2.1 Structures and nomenclatures of 16 PAHs on the EPA priority pollutant list (Gong, 2003).

Two PAHs including phenanthrene and pyrene were chosen for the present study because of their abundant in soil. Physical and chemical properties of these two PAH compounds were shown in table 2.1.

Table 2.1 Physical and chemical properties of the selected PAHs (ATSDR, 1995).

Physical and chemical properties	**Phenanthrene**	**Pyrene**
Chemical Structure		
Chemical formula	$C_{14}H_{10}$	$C_{16}H_{10}$
Molecular weight	178.2	202.3
Appearance	colorless crystalline solid	yellow or white crystals and powder
Melting point	100 °C	156°C
Boiling point	340 °C	404°C
Water solubility	1.2 mg/L	0.077 mg/L
Vapor pressure	6.8×10^{-4} mm Hg at 25ºC	2.5×10^{-6} mm Hg at 25ºC

2.1.3 Environmental and health impacts of PAHs

For most populations, the major sources of PAHs exposure are active or passive inhalation of the compounds via tobacco smoke, wood smoke, and contaminated air, and ingestion of the PAHs in foodstuffs. PAHs can bioaccumulate in plants, aquatic organisms, and animals after the intake of contaminant from water, soil, and food (ATSDR, 1995). Effects of PAHs on benthic invertebrates are consisted of inhibited reproduction, delayed emergence, sediment avoidance, and mortality. Fish exposed to PAH contamination will exhibit fin erosion, liver abnormalities, cataracts, and immune system impairments, which will lead to the increased susceptibility to disease. Mammals can absorb PAHs by inhalation, dermal contact, or (more poorly) ingestion. Plants can absorb PAHs from soils through their roots, and translocate them to other plant parts such as developing shoots. Uptake rates are generally governed by PAH concentration, PAH water solubility, soil type, and PAH physicochemical state (vapor or particulate) (ATSDR, 1995).

2.1.4 Environmental contamination and fate of PAHs

PAHs contaminated sites are generally caused by leakage from underground storage tanks and pipelines during the refining, and distribution of oil and residues from gas plant sites, and wood treatment facilities. Moreover, the inappropriate disposal of PAHs contained materials and accidental spillages have given rise to the contaminated sites (Smith et al., 1999). Fates of PAHs after released into the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation and adsorption on soil particles (Yuan et al., 2000).

PAHs released to the atmosphere are subjected to short- and long-range transport and will be removed from the atmosphere by wet and dry deposition onto soil, water, and vegetation (ATSDR, 1995). In surface water, PAHs can volatilize, photolyze, oxidize, biodegrade, bind to suspended particles or sediments, or accumulate in aquatic organisms. Bioconcentration factors of PAHs are often in the range of 10-10,000. In sediments, PAHs can biodegrade or accumulate in aquatic organisms (ATSDR, 1995). PAHs in soil can volatilize, undergo abiotic degradation (photolysis and oxidation), biodegrade, or accumulate in plants. PAHs in soil can also enter groundwater and be transported within an aquifer (ATSDR, 1995).

2.2 Bioremediation and biodegradation of PAH contaminated soil

There are a lot of technologies currently available to treat soil contaminated with hazardous materials. These technologies include excavation and containment in secured landfills, vapor extraction, stabilization and solidification, soilflushing, soilwashing, solvent extraction, thermal desorption, vitrification, and incineration. Many of these technologies, however, are either expensive or failed to completely destroy the contamination (Balba et al., 1998). Bioremediation emerges as the most promising methods for decontaminate wide range of organic contaminant, especially petroleum hydrocarbons (Balba et al., 1998). Moreover, the evolution of soil microbiology has shift from agricultural to environmental applications and environmental clean-up (Verstraete and Top, 1999).

Microbial bioremediation is the biological, especially microorganism process for oxidizing contaminants (Eve and Eve, 1998). Bioremediation consists of two general approaches: environmental biostimulation and bioaugmentation. The technology can be carried out ex situ or in situ (Boopathy, 2000). The selection of a suitable technique primarily depends on degree of saturation and aeration of an area. In situ techniques are applied to soil at the site with minimal disturbance while ex situ techniques are used for the removal of contaminant from the site by excavation (Vidali, 2001). Some example of ex situ and in situ techniques are in Table 2.2.

Table 2.2 Ex situ and in situ bioremediation technologies

Technology	Processes
Land Farming	Solid-phase treatment system (in situ or ex situ)
Composting	Aerobic, thermophilic treatment process
Bioventing	Treat the contaminated soils by supply the oxygen to stimulate microbial activities
Bioaugmentation	Additional of bacterial strain to the contaminated medium (in situ or ex situ)
Biostimulation	Provide the necessary nutrients to stimulation of indigenous microorganism

There are limiting factors in bioremediation for example low temperature, anaerobic conditions, low levels of nutrients and co-substrate bioavailability and

absence of degradation potential. In general, there are four principle routes that resulting in biodegradation of the contaminant (Romantschuk et al., 1999).

- The indigenous microbial strain has been exposed to the xenobiotic contaminant long enough for genetic evolution to create a capacity to degrade that certain compound(s).

- The indigenous microbial strain acquires genes and degradation pathways from bacterial cells immigrating from elsewhere. Transfer of genetic material can take place through conjugation, transduction or transformation. The process of gene transfer has been shown to take place in environmental conditions.

- Indigenous well-adapted microbial strain is artificially supplied with the required degradative capacity. Once the contaminant is known, gene-clusters (e.g. in a conjugative broad host range plasmid) may be supplied. If natural gene clusters are unavailable, these may be constructed. Laboratory strains can be used as donors, either to transfer the capacity to wild type strains freshly isolated from the contaminated site. Moreover, the donors may be introducing into the contaminated site and letting gene transfer to occur.

- A bacterium that is a competitive strain at the contaminated site is chosen, this strain is known to degrade the contaminating compound. If genetic engineering is involved, special considerations should be applied. Thus, if containment of the modified genes is required, suicide function may be inserted into this strain.

There are numerous studies about PAHs degrading bacteria. The examples of these bacteria are in Table 2.3 and 2.4.

Table 2.3 Bacterial strains which can degrade phenanthrene.

Bacterial strains	References
Arthobacter sp.	Savino and Lollini, (1977)
Mycobacterium sp. strain KR2	Rehmann et al., (1998)
Pseudomonas putida	Evan et al., (1965)
Pseudomonas putida NCIB 9816	Yang et al., (1994)
Sphingomonas paucimobilis EPA 505	Ye et al., (1996)

Table 2.4 Bacterial strains which can degrade pyrene.

Bacterial strains	References
Rhodococcus sp. strain UW1	Walter et al., (1991)
Mycobacterium sp. strain KR2	Rehmann et al., (1998)
Mycobacterium sp. strain CH1	Churchill et al., (1999)
Mycobacterium spp.	Cheung and Kinkle, (2000)
Mycobacterium sp. strain PYR-1	Ramirez et al., (2001)

Success in bioremediation methods depends on having the right bacteria in suitable place with optimum condition for effective degradation. Bioremediation has a lot of advantages over conventional methods such as land filling or incineration (Boopathy, 2000). Bioremediation is beneficial for the complete destruction of a broad range of contaminants. These methods can be done on site, without causing a major disruption and less expensive than other technologies. Meanwhile, bioremediation has some limitations. Some hazardous chemicals are not biodegradable, for example: heavy metals, radionuclides, and some chlorinated compounds. There are also some concerns that microbial metabolism may produces more toxic metabolites than parent compound. Moreover, it is difficult to extrapolate from lab-scale studies to full-scale field operation. The other limitation of bioremediation is the longer time operation than other methods, such as excavation and removal of soil or incineration (Vidali, 2001).

Cunningham and Philip (2000) compared the effectiveness of bioaugmentation and biostimulation for treatment of diesel contaminated soil. In this research, biostimulation was conducted by adding inorganic fertilizer (NPK) or manure to provide nutrients and bulking agents to improve aeration. The results showed that rapid remediation occurred in bioaugmented system. The inoculum was isolated from diesel contaminated soil and enriched with diesel in laboratory. The augmentation raised the number of microorganisms about one order of magnitude at the site.

2.2.1 Factors influencing degradation of PAH contaminated soil

Environmental conditions play an important role in determining biological activities, whether of indigenous microorganisms, added microorganisms, or cultured indigenous microorganisms returned to the soil. The conditions are divided into two general categories: the conditions which reduce the microbial activity, such as temperature, humidity and ionic strength and the conditions which restrict the mass transfer of the compound to the microbial cell such as clay and organic-matter content (Vogel, 1996). Environmental parameters which play important roles in biological degradation are in Table 2.5

Table 2.5 Environmental parameters affecting the degradation of PAH contaminated soil.

Parameter	**Optimum values for PAHs degradation***
Soil moisture	30-90 % (1)
Soil pH	7.5-7.8 (1), 7.0 (5), 6-7.5 (6)
Temperature (°C)	30 (2, 4, and 5), 24-30 (3)
Nutrient content	C:N 60:1 (1) C:P 800:1 (1)
Oxygen content	10-40 % O_2 (2)

*References:

(1) Dibble and Bartha (1979) (2) Bauer and Capone (1985)

(3) Heitkamp et al. (1988) (4) Walter et al. (1991)

(5) Strandberg et al. (1985) (6) Hupe et al. (2001)

2.2.2 PAH degradation pathway

Bacterial degradation of PAHs occurs primarily under aerobic condition and resulta in subsequent catabolite formation, ring fission and metabolism (Yuan et al., 2000). The first step of PAHs degradation is the action of intracellular dioxygenases to *cis*-dihydrodiols incorporation of two atoms of oxygen (Samanta et al., 2002). The *cis*-dihydrodiols are further oxidized to catechols and then under through the ortho- or meta-cleavage pathways (Figure 2.2). Anaerobic PAH-degradation may occur in sediments, water-logged soils and aquifers. Furthermore, there is increasing evidence

of anaerobic PAH-degradation with nitrate and sulfate as terminal electron acceptors (Johnsen et al., 2005). Numerous PAH compounds contain a “bay-region” as well as “K-region”, both of which allow metabolic formation of bay- and K-region epoxides, which are highly reactive. Some of these epoxides demonstrated carcinogenicity (Samanta et al., 2002).

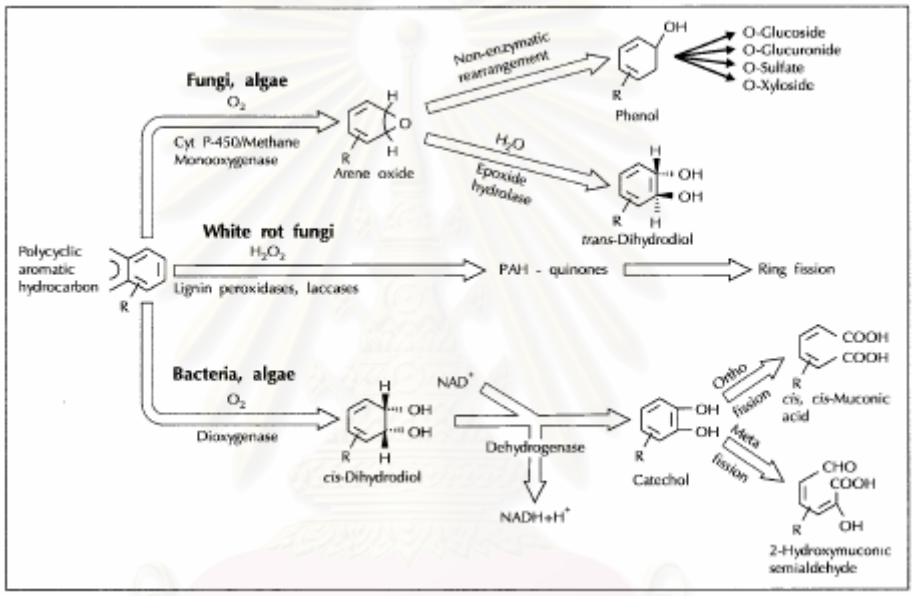


Figure 2.2 Microbial pathways for oxidation of polycyclic aromatic hydrocarbon (Cerniglia, 1993)

2.2.3 Cometabolic requirement for PAHs biodegradation

One metabolic barrier to the microbial degradation of organic compounds is the lack of catabolic enzyme induction. Certain bacteria cannot utilize some PAHs as a carbon and energy source, therefore a growth substrate must be supplied to initiate growth of the organism and to induce the production of catabolic enzymes. Recently, cometabolism plays an important role for the biotreatment of many recalcitrant

compounds. Cometabolism is the fortuitous biotransformation of a non-growth supporting compound by microorganisms that are growing on certain growth substrate. The cometabolism of high molecular weight PAH after application of molecular weight PAH are attractive for PAH biormediation (Juhasz and Naidu, 2000; Wang el at., 2003).

Numerous of bacteria isolated from contaminated sites have been shown to degrade wide range of PAHs when exposed to individual PAH compound. Using single PAHs in a study would not reflect the true complexity of PAH degradation in the natural environment (Dean-Ross, 2002). Some researches therefore studied the degradation of PAH mixtures. For Example:

Yuan et al. (2001) studied the aerobic biodegradation potential of PAHs in river sediment. They found that the presence of phenanthrene enhanced biodegradation of anthracene, fluorene, and pyrene, but did not affect benzo[a]pyrene biodegradation within 12-day incubation.

Dean-Ross et al. (2002) studied the ability of sediment bacteria to utilize PAHs when present as mixtures. They studied 2 bacteria strains. One strain, identified as *Mycobacterium flavescens*, could utilize fluoranthene in the presence of pyrene, although the utilization of pyrene was slower in the presence of fluoranthene than when it is absence. The second strain, a *Rhodococcu*s species, could utilize fluoranthene in the presence of anthracene, although the presence of fluoranthene slowed the rate of anthracene degradation.

2.3 Bioaugmentation

Bioaugmentation, the introduction of exogenous microorganisms into environments has been used in to accelerate bioremediation (Watanabe, 2001). The effects of bioaugmentation should be more than only the addition of a metabolic function. It may influence the bioavailability of pollutants when the application methods involve homogenization, slurrying, or intensive flushing of the system, or when the bacteria added differ from the indigenous populations with respect to their specific affinity for the contaminant, maintenance requirements, ability to co-utilize natural substrates, active or passive mobility, adhesion behavior, or ability to produce biosurfactants and to ingest surfactant solubilized chemicals (Johnsen et al., 2004). The bioavailability of pollutants may also be affected when genetic information responsible for degradation activity of the introduced bacteria is transferred to indigenous recipient bacteria, which deviate with respect to above characteristics (Johnsen et al., 2004).

In PAHs-contaminated site, bioremediation is an available option for PAHs removal, and many PAHs degrading microorganisms have been isolated form contaminated soil. Bioremediation will not have a significant rate, when the population of indigenous microorganisms which capable to degrade the target contaminant is less than 10^5 CFU/g of soil (Frosyth et al., 1995). Therefore, additional inoculum is required. The bacterial degraders are cultured and reintroduced into the soil to enhance the degradation of contaminants (Gentry et al., 2003).

Mishra et al. (2001) studied in situ bioremediation of oily-sludge-contaminated soil after addition of an inoculum to stimulate the degradation. The indigenous population of hydrocarbon-degrading bacteria in the soil was very low (10^3 to 10^4 CFU/g of soil). The addition of bacterial consortium and nutrients resulted in 89.7-92.0 % removal of total petroleum hydrocarbon (TPH) from soil after 1 year, compare to 14.0 % removal of TPH in control plot.

Ruberto et al. (2003) used microcosms systems (250 g soil in 1 l flask) to analyze biodegradation of gas-oil in Antarctic soil under natural conditions. Bioaugmentation with previously isolated psychrotolerant bacterial strain (B-2-2) was improved bioremediation efficiency, in which about 75 % of the hydrocarbon was removed. The conclusion of this study showed that indigenous bacterial flora from Antarctic soil is able to degrade an important fraction of the gas-oil and bioaugmentation can be used as an alternative tool to improve bioremediation.

Supaka (2001) isolated *Sphingomonas* sp. strain P2, which is able to utilize phenanthrene as a sole source of carbon and energy from lubricant contaminated soil sample collected from a garage in Prajinburi province, Thailand. The bacterium can rapidly degrade 100 ppm phenanthrene in liquid medium to undetectable amount within 72 hours. In addition to phenanthrene, *Sphingomonas* sp. strain P2 is able to degrade a wide variety of PAHs, including naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, and dibenzofuran. It is also able to co-metabolize high molecular weight PAHs, fluoranthene and pyrene, in liquid minimal medium supplemented with phenanthrene. The bacterium is able to survive efficiently in sterile soil and can degrade phenanthrene to undetectable amount. However, its

growth and PAHs degradative abilities are inhibited by some factors in non-sterile soil. They found that this strain reduced only 23% of 100 ppm phenanthrene in non-sterile soil. Therefore, a mean to increase the survival and PAH degradative abilities of *Sphingomonas* sp. strain P2 in non-sterile soil is required.

2.3.1 Fate of bacteria in soil

Soil depicts a variety in environment textures, which is composed of solid, liquid, and gaseous phases. The dominance structure in soil system is solid phase, which resist dynamic conditions in the liquid and gaseous phases. The heterogeneous of three difference phases are resulted in difficult distribution between all phases (Van Elas and Van Overbeek, 1993). Pore size distribution strongly determines the fate of introduced bacteria. It was showed that the inoculant has more survival levels in finer-textured (clayey) than in coarser (sandy) soil (Van Veen et al., 1997). Moreover, the condition in non-rhizosphere soil can be unfavorable for the inoculum growth. The inoculation of bacterial suspensions into soil tends to cause a rapidly decrease in population size (Van Veen et al., 1997).

2.3.2 Factors influencing bacterial survival and activities in soil

Although bioaugmentation seems to be a good alternative to clean-up contaminated site, bioaugmentation has its limitations (Forsyth et al., 1995). In general, population sizes of the inoculated bacteria decline more or less rapidly following introduction into a natural soil, and the growth of introduced populations in soils without disturbance is a rare phenomenon (Van Veen et al., 1997). The

microorganism which can grow by using a particular compound as a sole source of carbon in the laboratory does not mean that this inoculum can degrade the compound in the contaminated site (Goldstein et al., 1985). Moreover, the rate of PAHs biodegradation in soil is usually reduced when compare with those observed in laboratory (Amellal et al., 2001).

Besides the intrinsic physiological characteristics of the organisms, abiotic and biotic soil factors play an important role in the reduction of the number and activities of inoculated strain in soil. Abiotic soil factors such as textural type, toxic metals, solvents, pH, temperature, and moisture exert direct effects on inoculant population dynamics by imposing stresses of various natures on the cells (Van Veen et al., 1997). They can also act indirectly by affecting the activity of indigenous soil microflora. In addition to abiotic soil factors, other main causes of inoculant population declines are biotic factors for example the competition from native soil microorganisms and grazing by predators (Van Veen et al., 1997).

To enhance the survival of microorganism in soil, Megharaj et al. (1997) studied the survival and degradation of dibenzo-*p*-dioxin (DD) and dibenzofuran (DF) in soil by soil-adapted *Sphingomonas* sp. strain RW1. The soil-adapted bacteria were obtained by preincubation in soil for 4 weeks in the presence of either DF or DD, followed by culturing in liquid mineral salts medium amended with liquid soil extract. The survival pattern of unadapted and soil-adapted bacteria in sterile and non-sterile soil was studied. Populations of soil adapted bacteria were found to survive better in DF and DD-amended soil and degrade the substrates more efficiently than bacteria that had not been subjected to pre-adaptation.

Table 2.6 Factors influencing bacterial survival in soils (Van Veen, 1997).

Origin	**Factor**	**Effects on the inoculum**
Biotic	Predation	Population size decrease
	Competition	Population size decrease/antagonistic effect on plant pathogens
	Root growth	Release of organic compounds, enhancing survival
Abiotic	Clay minerals	Protection against predation
	Water tension	High tension: water shortage, high osmolarity; low tension: anaerobism, increased nutrient availability by diffusion
	Organic carbon	Selection for copiotrophic or oligotrophic species; limited organic
	Inorganic	carbon results in starvation and reduction in activity
	Nutrients (N, P)	Limitation results in starvation
	pH	Selection for species; release of nutrients (e.g., P) or toxic compounds (e.g., Al^{3+}).
	Temperature	Metabolic activity as well as biotic (predatory) pressure affected
	Chemicals (toxic waste)	Inhibition of sensitive organisms; selection of biodegradative, resistant, or tolerant forms

2.3.3 Utilization of carrier materials for enhancing the survival of inoculum

Carrier materials generally proposed to provide a protective niche for the microbial inoculants after applied to soil. In physical condition, it can provide protective surface or pore space for escape from protozoa. Moreover, it can provide the specific substrate for the inoculum nutrition. A suitable carrier should provide favorable conditions for survival and inoculant function as well as improve survival and activity of inoculant in soil (Van Veen et al., 1997). The carrier should not be toxic and have constant quality. Many of natural materials can be used as carriers e.g. peat, soil, clay, and plant-derived compounds. In addition, defined organic polymers forming porous matrices have been used for immobilize the bacterial cell such as calcium alginate, agarose, and k-carrageenan (Van Veen et al., 1997). Soil was also used as carrier for the introduction of bacteria. The technique is simple, low cost and effective for increasing cell survival (Van Dyke and Prosser, 2000).

Several carrier materials were used to enhance the survival of microorganism in soil. For Example:

Van Dyke et al. (2000) studied the establishment of bacteria in a carrier soil prior to inoculation as a method for enhancing survival in an agricultural soil. The preincubation of *Pseudomonas fluorescens* in a sterile soil carrier led to greater survival in soil microcosms than preincubation in a non-sterile soil carrier or inoculation of a liquid cell suspension.

Cunningham et al. (2004) studied laboratory-scale which examined the potential of immobilised hydrocarbon-degrading microorganisms for clean-up diesel-contaminated soil. They used polyvinyl alcohol (PVA) cryogelation as an entrapment technique and microorganisms indigenous to the site. Laboratory biopiles were constructed to compare the immobilized microorganisms and liquid culture. In terms of percent diesel removal, the immobilised systems were found to be the most successful, with highest removal in a co-immobilisation system containing PVA-entrapped microorganisms and a synthetic oil absorbent after 32 days. Least success treatment was achieved with a commercial liquid bioaugmentation agent containing surfactants and low pH, which also led to a significant phytotoxicity.

2.4 Denaturing Gradient Gel Electrophoresis (DGGE) for monitoring soil bacterial populations

Traditional method for monitoring soil bacteria is culture technique. However, about 99% of the microorganism in nature can not be cultured (Muyzer, 1999). One of techniques that can determine the inoculated as well as indigenous bacteria without cultivation is genetic fringerprinting technique. Step of genetic fingerprinting techniques consist of extraction of nucleic acid, amplification of genes encoding the 16S rRNA, and analysis of PCR product by denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999). DGGE is generally used for the study of complex microbial communities (Sigler et al., 2004). The technique is based on the decreased electrophoretic mobility of partially melted double stranded DNA molecule in polyacrylamide gels, which contain a linear gradient of DNA denaturant (a mixture of urea and formamide). The different DNA molecules with different sequences have a

different melting behavior, which resulting in different mobility in electrophoresis gels.

DGGE technique is widely used for monitoring microorganisms in environmental samples, for instance:

Agnelli et al. (2004) studied the distribution and composition of of indigenous bacterial and fungal communities in a forest soil profile. 16S rDNA-DGGE revealed composite banding patterns reflecting the high bacterial diversity as expected for forest soil, whereas 18S rDNA-DGGE analysis showed a certain stability and lower diversity in the fungal communities. The banding patterns of the different horizons reflected changes in the microbial community structures with increasing depths.

Andreoni et al. (2004) studied physical and chemical characteristics of three different soils, Belgian soil, German soil, and Italian agricultural soil as well as microbiological properties. The three soils were isolated for phenanthrene-degrading cultures. Both biodiversity in soil and enrichment cultures were detected by DGGE profile of the 16S rDNA genes.

CHAPTER III

METHODOLOGY

3.1 Materials and apparatus

3.1.1 Preparation of soil

Soil sample at 2-5 cm depth was collected from a garden in Bangkok (Figure 3.1). All debris was removed and then the sample was air dried overnight. The soil was sieved through a 1.18 mm (No. 16) mesh and kept at 4ºC until use. Sieved soil was analyzed for background PAH concentration by gas chromatography. In this research, we focused on two PAHs, phenanthrene and pyrene.

Soil physical and chemical properties were analyzed by Department of Soil Science, Faculty of Agriculture, Kasetsart University and Soil and Water Group, Agriculture Chemistry Division. The interested properties included soil texture, %organic carbon, %organic matter, total nitrogen (N), available phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), moisture content, EC (electrical conductivity), pH, and maximum water holding capacity. Analysis methods for soil characterization are as follows;.

pH : Dilution soil :water (1:1) measured pH by pH meter

Soil texture : Hydrometer method

Organic matter : Bray II method

Organic carbon : Wet oxidation method

Phosphorus (P) : Walkley-Black method

Potassium (K), calcium (Ca), and magnesium (Mg) : Ammonium acetate extraction

Maximum water holding capacity : Comparison between wet weight and dried weight

Electrical conductivity : EC meter (soil: water; 5:1)

Figure 3.1 Sieved soil sample

3.1.2 Chemicals

1. Phenanthrene and cyclohexamide were obtained from Sigma Chemical, USA.
2. Pyrene was obtained from Kanto Chemical, Japan.
3. Yeast extract, tryptone, and bacto agar were obtained from Difco Laboratories, USA.
4. Sodium chloride (NaCl), sodium hydroxide (NaOH), methanol (CH_3OH), acetone (CH_3COCH_3), and sodiumsulfate anhydrous (anhydrous Na_2SO_4) were purchased from E.Merck, Germany.
5. Ammonium nitrate (NH_4NO_3) was obtained from BDH Chemicals, Australia.
6. Disodiumhydrogen phosphate ($Na_2HPO_4.12H_2O$) and magnesium sulfate ($MgSO_4.7H_2O$) were products of Carlo ERBA, France.
7. Calcium chloride ($CaCl_2.2H_2O$) and potassiumdihydrogen phosphate (KH_2PO_4) were obtained from AJEX Chemicals, Australia.
8. Ferric chloride ($FeCl_3.6H_2O$) was purchased from May & Baker, England.
9. Triton-x 100 was obtained from Amersham Biosciences Co., Ltd.
10. 96 % Multisolvent N-Hexane was obtained from Becthai Bangkok Equipment & Chemical Co.,Ltd.

3.1.3 Equipments

1. Flame ionizing detector (FID) Gas liquid chromatography (Agilent 6890N) equipped with a HP-5 capillary column (0.2 mm inner diameter and 30 m long) was used for PAHs analysis.
2. pH meter Spectronic 21, Bausch & Lomb, USA.
3. Pasteur pipette, Becthai Bangkok Equipment & Chemical Co., Ltd.
4. Ultrasonicator (bath model) FS4000, Decan Ultrasonics, USA.
5. 20, 100, 200, 1000 and 5000 µl micropipettes, Drummond Scientific, USA.
6. 1, 5, and 10 ml of pipettes, Gilson, France.
7. Incubator Hereaus type B 5050 E, Hereaus, Germany.
8. “ISSCO” laminar flow BVT-124, International Sciencetific Supply, USA.
9. Autoclave, Kakusan, Japan.
10. Standard sieve O.S.K. 16 with 1.18 mm of pore size was from Okawa Seiki, Japan.
11. Weighing L2200P and A200S were from Sartorius, USA.
12. Vortex mixer G-560 E was from Scientific Industries, USA.
13. Universal Mutation Detection system, Biorad Dcode™ System
14. FastPrep FD 120, BIO101, Thermo Savant
15. Microcentrifuge, MIKRO 20, Hettich
16. GeneAmp® PCR system 2700, AB Applied Biosystems
17. Digital Filter Fluorometer, Turner® Quantech™
18. UV transilluminater, BioDoc- It ™ System, UVP

3.2 Preparation of inoculums

3.2.1 Preparation of liquid inoculum

Stock culture of *Sphingomonas* sp. P2 from -80 °C was streaked onto carbon free mineral medium (CFMM) agar saturated with phenanthrene vapor then incubated at 30ºC for 5 days. Single colony was transferred to liquid CFMM supplemented with 100 ppm phenanthrene and shaken at 200 rpm for 48 hours. Then, the culture was harvested by centrifuging at 8,000 rpm at 4ºC for 15 min. Cell pellet was washed with 0.85% of sodium chloride solution twice and resuspended in the same solution.

To prepare liquid inoculum, three types of medium were used: carbon free mineral medium (CFMM) (Appendix A), soil extract mixed with water (1:3) and soil extract mixed with CFMM (1:3). Soil extract was prepared according to Megharaj (1997) with details in Appendix A. Two ml of *Sphingomonas* sp. P2 at $OD_{600} \approx 0.5$ was inoculated into 100 ml culture media in 250 ml Erlenmeyer flask. Then, 100 µl of phenanthrene stock solution (in dimethylsulfoxide) was added to give a final concentration of 100 ppm. The culture was incubated on a rotary shaker at 200 rpm, 30ºC, for 48 hours. The cells were harvested and washed twice with 0.85% sodium chloride solution. Finally, cells were resuspended in 5X CFMM to the concentration of 1.0 OD_{600} and used as liquid inoculum.

3.2.2 Preparation of soil inoculum

Soil inoculum was prepared in sterilized soil microcosm. Two g of sieved soil were added to the 22 ml screw-capped with Teflon liner vials then autoclaved at 121^{o}C, 45 min for three successive days. Phenanthrene and pyrene were spiked to the sterilized soil to give a final concentration of 100 ppm each. The stock PAHs were diluted in acetone to the desired concentration and filtered through 0.22 μm PTFE membrane filter right before used. To prevent toxicity from acetone, the spiked soil was kept in a chemical hood for overnight. Then, 200 μl liquid inoculum ($OD_{600} \approx$ 1.0), which prepared in 3.2.1, was added into the vial. This gave the final concentration of 10^8 CFU/gram soil. Control soil microcosms were set up in parallel by adding 200 μl 5X CFMM instead of inoculum. Moisture content of 80 % water holding capacity was adjusted by adding water, and then incubated at 30ºC in the dark. The incubated mixture was used as soil inoculum for bioremediation of PAHs in non-sterile soil microcosms (figure 3.2).

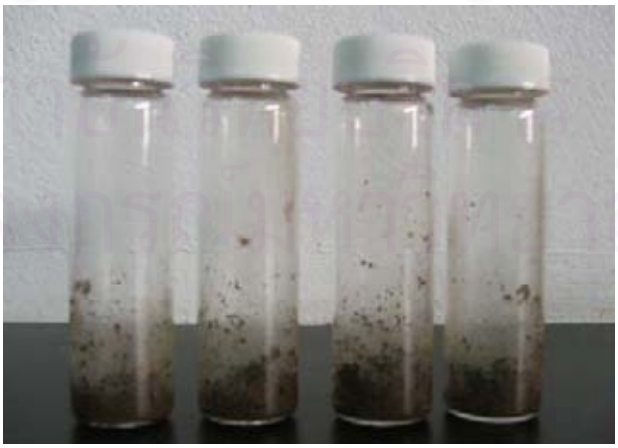


Figure 3.2 Soil inoculum in 22 ml screw-capped with Teflon liner vials

3.2.3 Liquid inoculum calibration curve

Bacteria were cultured as in 3.2.1. Bacterial cells were harvested by centrifugation at 8,000 rpm at 4ºC for 15 min. Cell pellets were washed twice with 0.85% sodium chloride solution and resuspended in the same solution. Bacterial cells were diluted to various OD_{600} by serial dilutions. Diluted solutions were spreaded on the surface of LB agar plate in triplicate. Bacteria colonies were counted after incubation at 30ºC for 2 wks. Standard curve was plotted from OD_{600} and CFU values as shown in Appendix C.

3.3 PAHs bioremediation treatments

3.3.1 Soil microcosm preparation

Twenty g of non-sterile soil was added to 75-ml vial (Figure 3.3). There were 120 vials, in which 40 vials were control treatment set while other 80 vials were liquid inoculum treatment sets. As for the soil inoculum treatment, only eighteen g of non-sterile soil was added to the vial. The treatment required 100 vials for the addition of soil inoculum at 4-, 8-, and 12-day incubation times. 5X CFMM were added into soil inoculum and control microcosms in order to adjust nutrient in soil. Two types of PAHs mixture were spiked to the soil, 100 ppm phenanthrene mixed with 100 ppm pyrene and 300 ppm phenanthrene mixed with 100 ppm pyrene. The stock PAHs were diluted in acetone to the desired concentration and filtered through the 0.22 μm PTFE membrane filter right before used. To prevent toxicity from acetone, the spiked soil was kept in a chemical hood for 16 hrs.

3.3.2 Treatments

PAHs bioremediation treatments were included control (no inoculum), liquid inoculum addition, and soil inoculum addition (Figure 3.4). Two g of soil inoculums (prepared in 3.2.2) were added to 18 g PAHs spiked soil and 2 ml liquid inoculum (prepared in 3.2.1) was added to 20 g PAHs spiked soil in the vial to set up a microcosm. Soil water content of 80% water holding capacity was adjusted by adding water, and then incubated at 30ºC in the dark. Every week, the moisture content was maintained by using the reduced weight of the microcosm as indicator. The experiment was conducted in triplicate for PAHs analysis and another one set for bacterial analysis. The samples were collected at 5-day interval for 20 days.

Figure 3.3 Non-sterile soil microcosms contain 20 g soil in 75 ml vial.

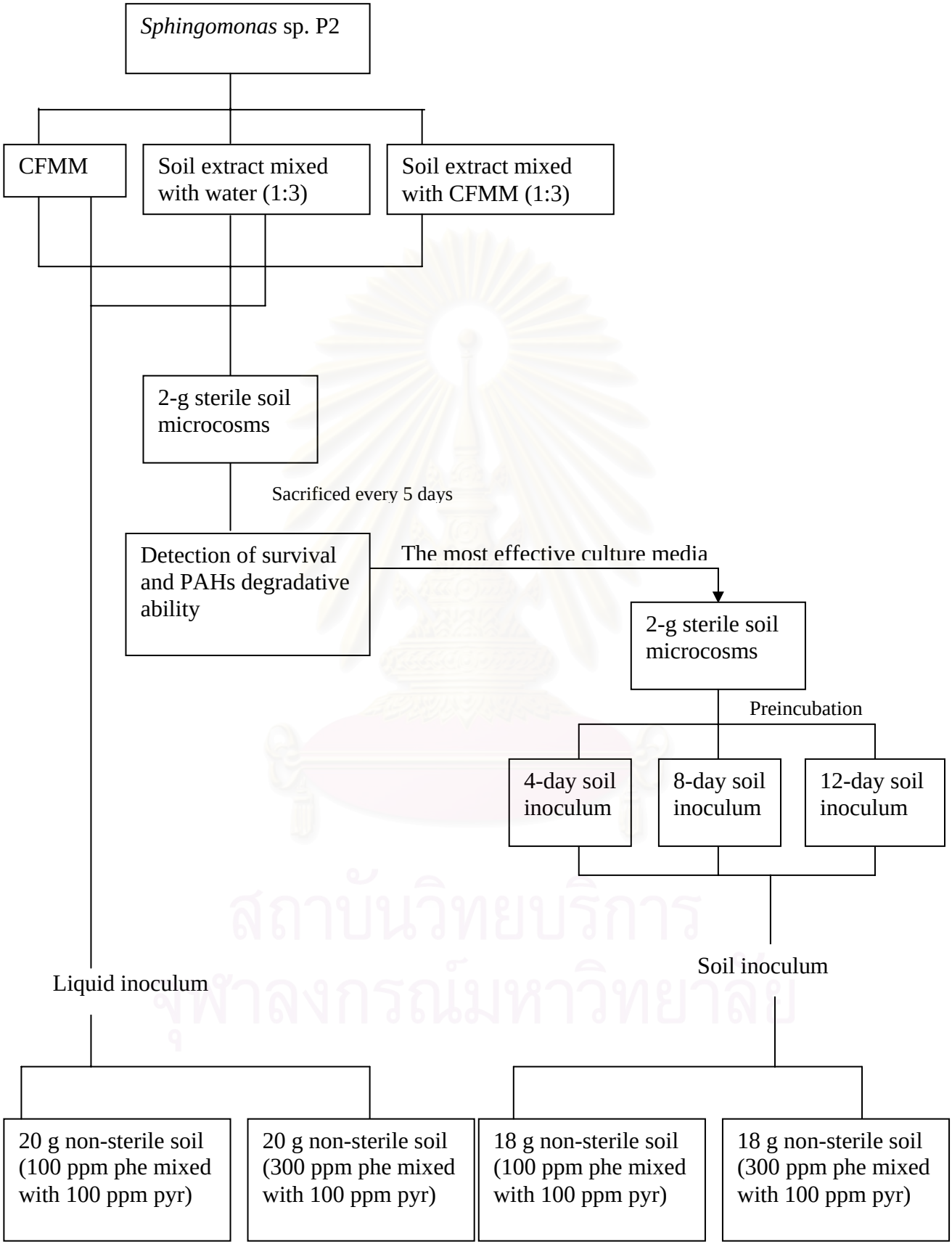


Figure 3.4 Diagram of inoculum preparation and soil treatment experimental design

3.4 Analytical methods

3.4.1 Analysis of PAHs

3.4.1.1 PAHs extraction

PAHs were extracted from soil using a mixture of hexane and surfactant. For 2-g soil microcosm, 4 ml of n-hexane and 1.5 ml of 15 % triton-x 100 solutions (surfactant) was added. Then, the samples were shaken by orbital rotary shaker at 250 rpm for 6 hours. For 20-g soil microcosms, PAHs was extracted using 40 ml of n-hexane and 15 ml of 15 % triton-x 100 solutions. The mixture was then frozen at -4 °C to solidify the lower aqueous layer. Solvent fraction was later transferred to a glass-bottle, where a few gram of anhydrous sodium sulfate was added to eliminate water from the sample. The extracted solvent fraction was transferred to gas chromatography (GC) auto sampler vials for gas chromatography analysis using flame ionization detector (GC-FID).

3.4.1.2 Gas Chromatography analysis

The analysis was performed with external standard using Gas Chromatography model 6890 N equipped with flame ionization detector. One μl of extracted sample was injected under the following conditions; injector temperature: 280ºC, detector temperature: 250ºC, initial column temperature: 80ºC hold for 1 minute then programmed at 80ºC to 160ºC at a rate of 25ºC/min hold for 3 minutes

and 160ºC to 240ºC at a rate of 3ºC/min and hold for 3 minutes and 240ºC to 300ºC at a rate of 40ºC/min and hold for 8 minutes. Carrier gas was helium (average linear volume of 13.3 ml/min) and make up gas was nitrogen at 60 ml/min. Split ratio was kept at 5:1. Retention time of phenanthrene and pyrene are 12.9 ± 0.5 min and 21.8 ± 0.5 min, respectively. Each sample was analyzed for concentration of PAHs by comparing PAHs recovered from soil to a standard curve of PAHs.

3.4.1.3 PAHs calibration curve

A calibration curve was developed for contaminated soil by adding known amounts of PAHs before GC analysis. Stock PAHs standard was diluted in hexane to obtain the desired concentrations (triplicate per each). The calibration standards were analyzed by GC. Calibration curves were shown in Appendix B.

3.4.2 Detection of *Sphingomonas* sp. P2 and indigenous bacteria

3.4.2.1 Total count of bacteria

One gram of soil sample was resuspended in 0.85% sodium chloride solution. The mixture was vigorously shaken on a vortex mixer and then allowed to settle. The supernatant were serial diluted before spreading on LB agar supplemented with 200 ppm cyclohexamide. Bacterial colonies were counted after incubation at 30ºC for 3 days to calculate the number of all soil bacteria.

3.4.2.2 Phenanthrene degraders

Phenanthrene degrading bacteria were quantified as the representative of all PAHs degraders in the soil. Serial dilutions from 3.4.2.1 were spreaded on the surface of CFMM agar supplemented with 200 ppm cyclohexamide. Phenanthrene vapor was supplied by put its crystal on the lid of agar plate. Number of phenanthrene degrading bacteria was counted after incubation at 30ºC for 5 days.

3.4.3 Detection of the bacterial community

3.4.3.1 Extraction of genomic DNA

For soil sample, total DNA was directly extracted from 0.8 g of soil using a bead-beating instrument and FastDNA®SPIN Kit for soil (BIO101, USA) following the manufacture's protocol. For *Sphingomonas* sp. P2, its DNA was directly extracted from 0.1 g cell pellet using DNA-sabai kit (Plant Molecular Biology laboratory, Mahidol University) following commercial direction. The extracted DNA was analyzed by horizontal electrophoresis on 1X TBE (Tris-borate-EDTA buffer) agarose gel (0.8 % w/v) at 110 V. Gels were stained by ethidium bromide and visualized on UV transilluminater.

3.4.3.2 Soil DNA purification

To remove humic acids, the extracted soil DNA was purified using Perfectprep® Gel clean-up Kit (Eppendrof). DNA fragments were excised from agarose gel and extracted following the manufacturing direction. The recovery DNA was used as template for PCR-DGGE analysis. DNA concentration was quantified by Turner Quantech Digital Filter Fluorometer (Barnstead International) after reacted with Hoechst 33258 dye (Sigma-Aldrich) according to the manufacturer.

3.4.3.3 PCR (polymerase chain reaction) of 16S rDNA

Universal bacterial primers targeting 16S rDNA region (i.e. PRBA338f+GC clamp and PRUN518r) from Overeas et al. (1997) were used to amplify DNA fragments. PCR product was about 200 bp long. PCR reaction was run in a GeneAmp® PCR System 2700 thermocycler in 0.2 ml tubes. PCR reaction contained 50 ng DNA, 20 pmol of each primer, 15 μl Taq PCR Master Mix (Qiagen Inc.), and distilled water to a final volume of 30 μl. Initial denaturation step was at 94ºC for 5 min. Amplification was carried out with touchdown program. The first step was 20 cycles of denaturation at 94ºC for 1 min, annealing at 65ºC for 1 min (temperature was reduced 0.5ºC each cycle), and extension at 72ºC for 2 min. The second step was 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 2 min. The final extension was at 72ºC for 10 min. Five μl of the PCR product was run in 0.8 % agarose gels at 120 V to check for a correct

amplification. Gels were stained by ethidium bromide and visualized on UV transilluminater.

3.4.3.4 DGGE (Denaturing gradient electrophoresis)

DGGE was performed with a DCodeTM system (Bio-Rad Laboratories). PCR samples were run on 8% polyacrylamide gels (acrylamide gel stock solution, 37:5:1: Bio-Rad Laboratories, Inc.) with denaturing gradients ranging from 10-60% (where 100% denaturant contains 7 M urea and 40% formamide). After loading PCR products, DGGE was run at constant temperature (60ºC) and voltage (130 V) for 5 hours. DGGE gels were stained using 50 μg/ml ethidium bromide for 20 min. DNA bands were visualized on UV transilluminater.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Soil characteristics

Soil sample used in this research was collected from a garden in Bangkok. The sample was analyzed by GC for detection of background PAHs. We focused on phenanthrene and pyrene, which were used as model PAHs in this experiment. No PAHs was observed in this soil (Appendix B). Physical and chemical properties of the soil sample were shown in Table 4.1. The soil sample was sandy loam, which contained percent sand, silt and clay equal to 76, 11.6 and 12.4, respectively. Percentage of organic matter and organic carbon were 4.18 and 2.43, respectively. C: N: P ratio was 116:10:1. Amount of phosphorus, potassium, calcium and magnesium were 209, 308, 2471 and 316 ppm, respectively. Moreover, water holding capacity of the soil was 43.37 % and % moisture content was 2.25. Electrical conductivity of this soil was 0.292 dS/m. Finally, pH of the soil was 6.4.

Table 4.1 Properties of soil used in the study

Parameters	Analyzed data
Soil texture *	Sandy loam
Sand (%) *	76.0
Silt (%) *	11.6
Clay (%) *	12.4
Organic matter (%) **	4.18
Organic carbon (%) **	2.43
Total – nitrogen (%) **	0.21
Phosphorus (ppm) *	209
C:N:P ratio *, **	116:10:1
Potassium (ppm) *	308
Calcium (ppm) *	2,471
Magnesium (ppm) *	316
Moisture (%) *	2.25
EC (electrical conductivity) (dS/m) *	0.292
pH *	6.4
Water holding capacity (%) *	43.37

* Soil properties were analyzed by Soil and Water group, Agriculture Chemistry Division.

** Soil properties were analyzed by Department of Soil Science, Faculty of Agriculture, Kasetsart University

Environmental conditions play an important role in determining biological activities, whether of indigenous microorganisms, added microorganisms, or cultured indigenous microorganisms returned to the soil. According to Hupe et al. (2001), the optimum soil C: N: P ratio for bioremediation is about 100: 8: 2 (C: N < 20; C: P ≈ 50). The increasing of C: N ratio in soil material has a negative effect on microbial activity. To lower C: N ratio, mineral fertilizer, compost, or nutrient solutions may be added. From the analyzed data, C: N: P ratio of our soil was 116:10:1. The C: N ratio was 11.6, which was suitable for bioremediation. However, the C: P ratio was 116, which shown the deficiency of phosphorus. Nutrient solutions were therefore required. To adjust the amount of nutrients in soil sample, we supplied 5X CFMM at 10 % volume of the soil as described in Supaka et al. (1999). Besides nutrients, the optimum pH value for bioremediation is around 6-7.5 (Hupe et al., 2001). The pH value of our soil sample was in this optimum range. Consequently, soil characterization data suggested that this soil sample had a suitable condition for bioremediation process.

4.2 Comparison of culture media for the acclimatization of *Sphingomonas* sp. strain P2 and preparation of liquid inoculum

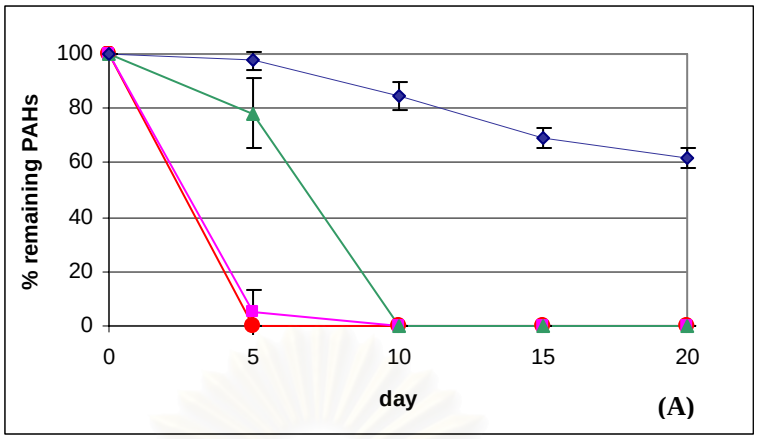
Sphingomonas sp. strain P2 was isolated from a lubricant contaminated soil that collected from a garage in Prajinburi province (Supaka et al., 2001). The bacterium can rapidly degraded phenanthrene in liquid medium from 100 ppm to undetectable amount (by HPLC analysis) within 72 hours. In addition to phenanthrene, *Sphingomonas* sp. strain P2 is able to degrade a wide variety of PAHs, including naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, and

dibenzofuran. It is also able to co-metabolize high molecular weight PAHs, fluoranthene and pyrene, in liquid minimal medium supplemented with phenanthrene. According to Supaka (1999) the bacterium was found to survive efficiently and degrade phenanthrene to undetectable amount in sterile soil. However, its growth and PAHs degradative abilities were affected by some factors in non-sterile soil. The results showed that this strain reduced only 23% of phenanthrene in non-sterile soil within 30 days.

To increase the survival and PAH degradative abilities of *Sphingomonas* sp. strain P2 in non-sterile soil, we therefore screened three culture media for the ability to acclimatize *Sphingomonas* sp. strain P2 to soil conditions. In this experiment, we used three liquid culture media: carbon free mineral medium (CFMM), soil extract mixed with water (1:3) and soil extract mixed with CFMM (1:3). *Sphingomonas* sp. strain P2 was cultured in 100 ml liquid medium and shaken at 200 rpm for 48 hours before using as liquid inoculum ($OD_{600} \approx 1.0$). To monitor the survival and PAHs degradability of the inoculums, population densities and amount of PAHs remaining were monitored in sterile soil microcosms (Figure 4.1 and 4.2). A medium that could maintain *Sphingomonas* sp. strain P2 populations after incubating in sterile soil was selected for the preparation of *Sphingomonas* sp. strain P2 liquid inoculum used in the following experiments.

4.2.1 PAHs degrading activity of the acclimatized *Sphingomonas* sp. strain P2 in sterile soil microcosm

Triplicate sterile soil microcosms were spiked with phenanthrene and pyrene to a final concentration of 100 ppm each. The degradation of phenanthrene and pyrene were in Figure 4.1. Within 5 days, the amount of phenanthrene was drastically decreased to undetectable level in soil microcosms with *Sphingomonas* sp. strain P2 that had been cultured in soil extract mixed with water (1:3). At the same time, there was 5% of phenanthrene remaining in the microcosms inoculated with *Sphingomonas* sp. strain P2 cultured in CFMM. Phenanthrene in this microcosm was decreased to an undetectable level within 10 days of incubation. When compared between soil microcosms with *Sphingomonas* sp. strain P2, the degradation of phenanthrene by *Sphingomonas* sp. strain P2 cultured in soil extract mixed with CFMM (1:3) was slowest. There was 18% of phenanthrene remained in this microcosm after 5-day incubation, however its amount reached an undetectable level at day 10. Meanwhile, phenanthrene remained in the control uninoculated soil microcosm was much higher than in the inoculated soil microcosms. There was 60% of phenanthrene remained in the control soil microcosms after 20-day incubation. The results indicated that the acclimatized *Sphingomonas* sp. strain P2 efficiently degraded phenanthrene in sterile soil.



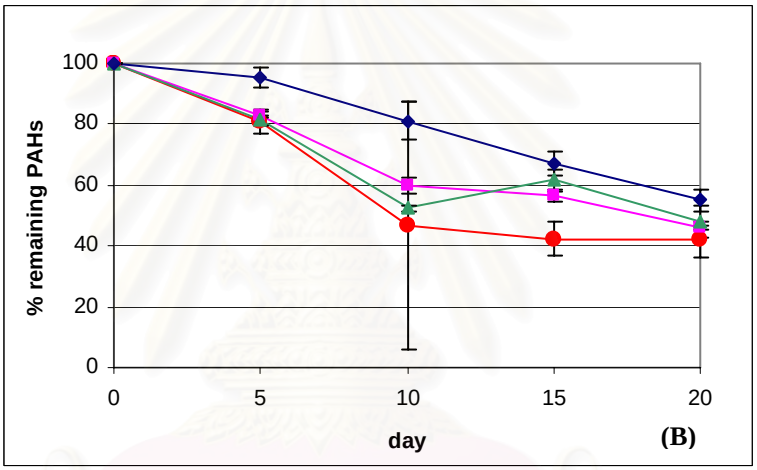


Figure 4.1 Percentage of remaining phenanthrene (A) and pyrene (B) in the sterile soil: (◆) control (no inoculated), (■) carbon free mineral medium (CFMM), (●) soil extract mixed with water (1:3) and (▲) soil extract mixed with CFMM (1:3).

The degradation of pyrene was not significantly different when compared between inoculated and control soil microcosms (Figure 4.1). Percent pyrene degradation within 20 days from soil microcosms with soil extract mixed with water (1:3), carbon free mineral medium (CFMM), soil extract mixed with CFMM (1:3) inoculum were 13, 9 and 7%, respectively. In control soil microcosms, only 45% of pyrene was degraded after 20-day incubation. The results suggested that bacteria inoculum slightly enhanced pyrene degradation in sterile soil microcosms.

4.2.2 Survival of the acclimatized *Sphingomonas* sp. strain P2 in sterile soil microcosm

Survival of the acclimatized *Sphingomonas sp.* strain P2 in sterile soil was studied using spread plate technique on CFMM agar saturated with phenanthrene vapor. The survival of acclimatized *Sphingomonas sp.* strain P2 from three different media were almost similar in sterile soil microcosms. Initial cell density of *Sphingomonas sp.* strain P2 was $1x10^8$ CFU/g soil. The amount of bacteria was rather constant before 15 days; then the bacterial numbers were slightly declined. At day 5, the survival of *Sphingomonas* sp. strain P2 cultured in carbon free mineral medium (CFMM) inoculum(6×10^8 CFU/g soil)was higher than in soil extract mixed with water (1:3) and soil extract mixed with CFMM (1:3) (3×10^8 CFU/g soil and 1×10^8 CFU/g soil), respectively. From day 10, the survival of *Sphingomonas* sp. strain P2 cultured in carbon free mineral medium (CFMM) was reduced to the lowest population density along 20-day incubation. The slight difference of bacterial count was observed from day 25 to 30. Survival of *Sphingomonas* sp. strain P2 inoculum prepared in soil extract mixed with water (1:3) medium was higher than the inoculum prepared from

other two media. The amounts of this inoculum were 4×10^7 CFU/g soil at 25-day and 6×10^7 CFU/g soil at 30-day.

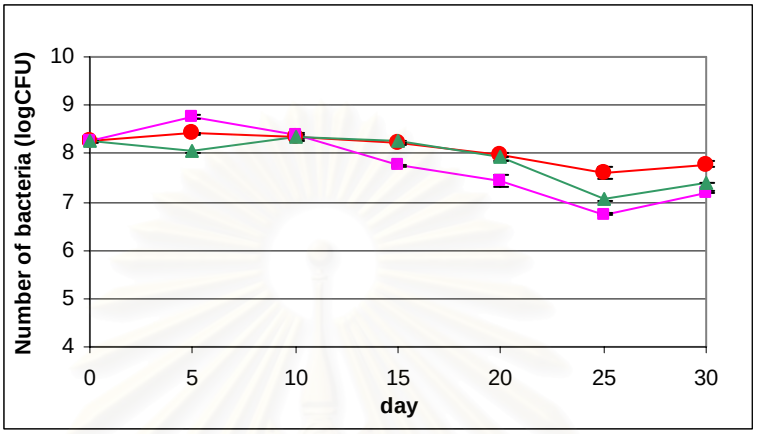


Figure 4.2 Survival of *Sphingomonas* sp. strain P2 in sterile soil microcosm: (■) carbon free mineral medium (CFMM) inoculum, (●) soil extract mixed with water (1:3) inoculum and (▲) soil extract mixed with CFMM (1:3) inoculum.

4.3 Preparation of *Sphingomonas* sp. strain P2 soil inoculum

Carrier materials generally used to provide a protective niche for microbial inoculants after applied to soil. According to Van Dyke and Prosser (2000), sterile soil can be used as carrier material for the introduction of bacteria into soil. The technique is simple, low cost and effective for increasing cell survival. They showed that preincubation of bacteria in sterile soil before adding to non-sterile soil can enhance the survival of bacteria when compared to the addition of a liquid cell suspension. Moreover, Megharaj et al. (1997) stated that pre-adaptation of bacterial

strains to the initial conditions of the target environment prior to their introduction would significantly enhance their density, persistence and activities.

In the study, sterile soil was used as both carrier material and acclimatized condition for *Sphingomonas* sp. strain P2 before applied to PAHs contaminated soil. *Sphingomonas* sp. strain P2 soil inoculum was prepared by sequential cultivating the bacteria in liquid medium followed by sterile soil. From the comparison of culture media in section 4.2, the most effective media for preparing liquid inoculum of *Sphingomonas* sp. strain P2 is soil extract mixed with water (1:3). The media gave higher phenanthrene degradation rate and survival ability of the *Sphingomonas* sp. strain P2 after added into sterile microcosms. Soil extract mixed with water (1:3) was therefore selected for preparing *Sphingomonas* sp. strain P2 liquid inoculum. Soil inoculum was prepared by adding the liquid inoculum into sterile soil spiked with phenanthrene and then incubated for 4, 8, and 12 days. The incubation time was varied to determine the effects of acclimatization (incubation) period on the soil inoculum.

4.4 Bioremediation of PAHs in non-sterile soil microcosms

PAHs bioremediation was conducted in non-sterile 20-g soil microcosms that spiked with phenanthrene and pyrene. The treatments were included addition of soil inoculum, addition of liquid inoculums, and control (without any addition). The effect of acclimatization (incubation) time was compared using 4-, 8-, and 12-day old soil inoculum. To determine the effect of higher phenanthrene concentration on co-metabolism of pyrene**,** non-sterile soil microcosms were set with 2 different

concentrations of phenanthrene: 100 ppm phenanthrene mixed with 100 ppm pyrene microcosm and 300 ppm phenanthrene mixed with 100 ppm pyrene microcosm. In conclusion, the bioremediation treatments can be divided into11 experimental sets as shown in Table 4.2.

Table 4.2 Experimental set of non-sterile microcosm

PAHs concentration	Experiments
100 ppm phenanthrene mixed with 100 ppm pyrene: 6 experimental sets	1. no inoculum added
	2. CFMM liquid inoculum
	3. soil extract mixed with water (1:3) liquid inoculum
	4. 4-day soil inoculum
	5. 8-day soil inoculum
	6. 12-day soil inoculum
300 ppm phenanthrene mixed with 100 ppm pyrene: 5 experimental sets	7. no inoculum added
	8. CFMM liquid inoculum
	9. soil extract mixed with water (1:3) liquid inoculum
	10. 4-day soil inoculum
	11. 8-day soil inoculum

4.4.1 PAHs degradation in non-sterile soil microcosms

Biodegradation of phenanthrene in 100 ppm phenanthrene microcosms with *Sphingomonas* sp. strain P2 could be arranged from high to low degradation as follows: inoculum cultured in soil extract mixed with water (1:3), inoculum culture in CFMM, 4-day soil inoculum, 8-day soil inoculum, and 12-day soil inoculum (Figure 4.3A). When compared between soil inoculum, the most efficient one was soil microcosms with 4-day soil inoculum. This inoculum could degrade 87% phenanthrene within 5 days and reached to undetectable level at 10-day incubation time. Preincubation of *Sphingomonas* sp. strain P2 for 4-day may result in the most active stage of this strain. The degradation of phenanthrene was lowest in the soil microcosm without *Sphingomonas* sp. strain P2 (no inoculum). This microcosm contained more than 85% of the initial phenanthrene after 20-day incubation.

All treatments (with and without inoculum) in the 100 ppm phenanthrene microcosms did not have significant influence on pyrene degradation (Figure 4.3B). According to Supaka et al. (2001), this bacterial strain had ability to co-metabolize pyrene in liquid mineral medium supplemented with phenanthrene. Pyrene could be reduced to 36% of the initial concentration within 7 days. Meanwhile, *Sphingomonas* sp. strain P2 cometabolized only a little of pyrene in our soil microcosms. Herbes and Schwall (1978) suggested that cometabolism of PAHs in soil is less important than in liquid medium because sorption of PAHs to sediment particles will prevent the action of degrading enzymes.

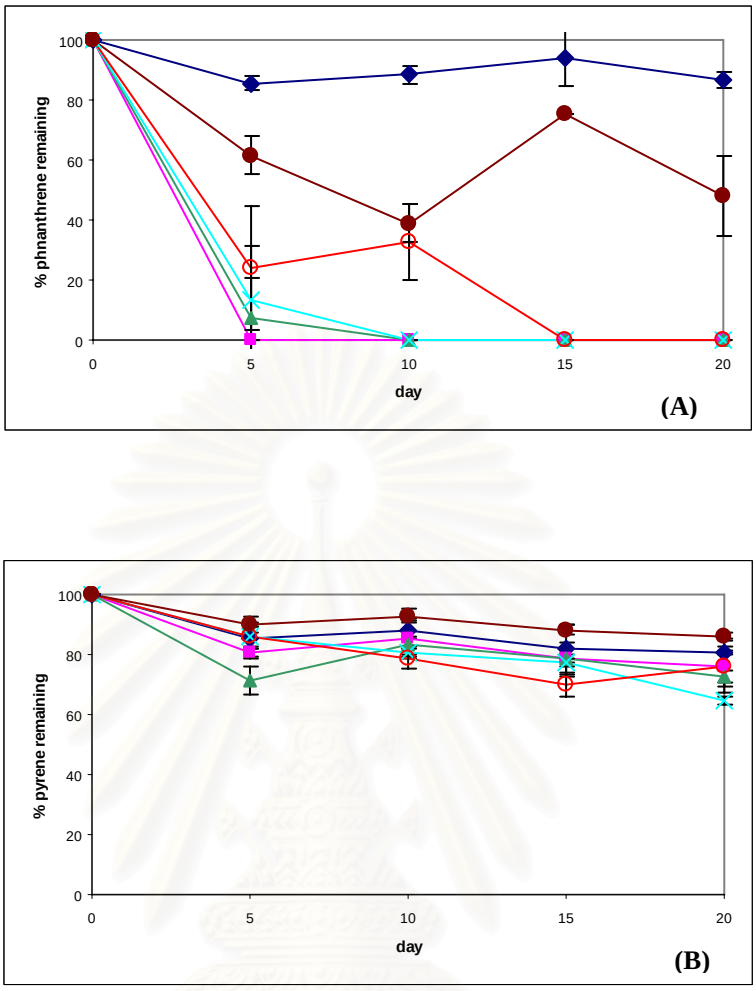


Figure 4.3 Percentage of remaining phenanthrene (A) and pyrene (B) in 100 ppm phenanthrene non-sterile soil microcosms with (◆) no inoculum; inoculum prepared in: (■) soil extract mixed with water (1:3), (▲) CFMM ; Soil inoculum incubated for: (×) 4 days, (O) 8 days, and (●) 12 days.

In 300 ppm phenanthrene microcosms, a rapid decrease of phenanthrene was observed in all inoculated treatments (Figure 4.4A). On the other hand, the uninoculated microcosms had only small amount of phenanthrene degradation. Only 25% of the initial phenanthrene concentration could be degraded. The present result showed that the degradation of phenanthrene was depended on the inoculation of *Sphingomonas* sp. strain P2. Moreover, the results suggested that indigenous soil bacteria had low ability to degrade soil PAHs. To enhance PAH degradation, they may need time to acclimatize to the PAHs in soil microcosm because this soil had no PAHs contamination background. Moreover, microcosm with CFMM liquid inoculum had the highest phenanthrene degradation, in which 94% phenanthrene could be degraded within 5 days and reached undetectable level within 10 days. As same as in 100 ppm phenanthrene microcosms, the degradation of pyrene in 300 ppm phenanthrene inoculated microcosms was not different from soil microcosm without the inoculum (Figure 4.4B). Only small amount of pyrene was decreased after 20-day incubation.

The 100 and 300 ppm phenanthrene microcosm had small amount in pyrene degradation. This result suggested that the inoculated *Sphingomonas* sp. strain P2 may not have enough growth substrate since phenanthrene was decreased rapidly. Higher concentrations of phenanthrene would be required to support pyrene cometabolism. Moreover, the lack of significant pyrene reduction in non-sterile soil microcosms after 20 days may be partly due to the relatively short incubation time of our study. Gentry et al. (2003) suggested that the PAHs degradation affected by incubation time. Roger et al. (1996) demonstrated no significant degradation of pyrene after 4 weeks incubation, after 9 weeks 36% of the initial concentration was degraded.

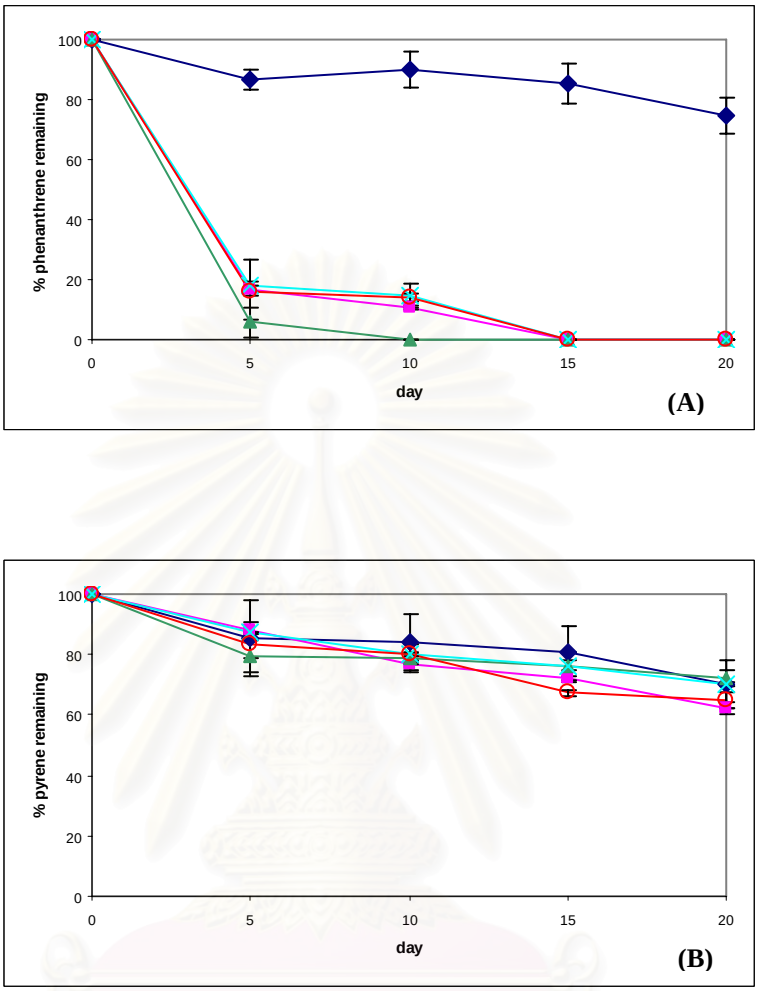


Figure 4.4 Percentage of remaining phenanthrene (A) and pyrene (B) in 300 ppm phenanthrene non-sterile soil microcosms with: (◆) no inoculum; inoculum prepared in (■) soil extract mixed with water (1:3), (▲) CFMM; soil inoculum incubated for: (×) 4 days, and (O) 8 days.

4.4.2 Detection of *Sphingomonas* sp. strain P2 and indigenous bacteria in non-sterile soil microcosms

4.4.2.1 Amounts of total bacteria

The amount of total bacteria in soil microcosms were determined using LB agar with spread plate technique. Initial amounts of total bacteria in soil microcosms were about 10^7 CFU/g soil (Figure 4.5). The result suggested that soil sample was fertile. In 100 ppm phenanthrene soil microcosms, the total count of bacteria increased to 10^8 CFU/g soil within 5 days and remained at this level until 20 days (Figure 4.5A). In 300 ppm phenanthrene soil microcosms, the total bacteria gradually increased to 10^8 CFU/g soil within 10 days and remain constant until 20 days (Figure 4.5B). Consequently, the long lag period in 300 ppm soil microcosms was probably due to higher amount of phenanthrene.

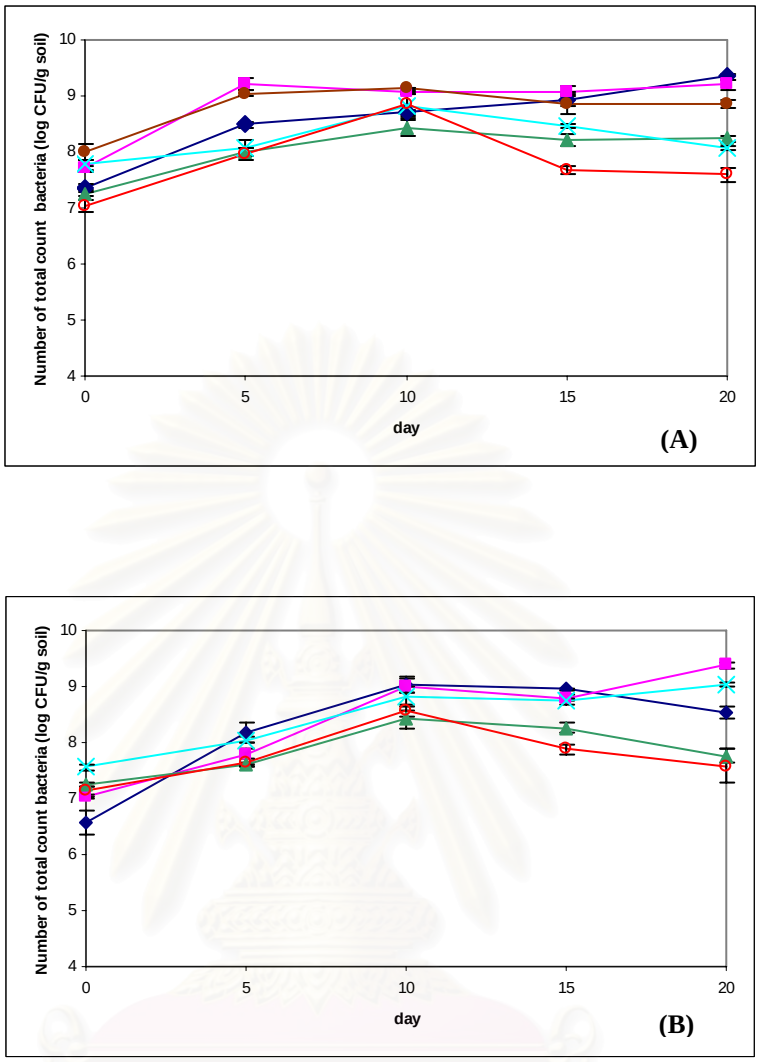


Figure 4.5 The number of total bacteria in 100 ppm phenanthrene microcosm (A) and 300 ppm phenanthrene microcosm (B) in nonsterile soil microcosms: (◆) no inoculum; inoculum prepared in: (■) soil extract mixed with water (1:3), (▲) CFMM; soil inoculum incubated for: (×) 4 days, (O) 8 days, and (●) 12 days.

4.4.2.2 Detection of PAHs degrading bacteria in non-sterile soil microcosms.

PAHs degrading bacteria were detected by plate count technique on CFMM saturated with phenanthrene. The initial amounts of PAHs degraders in non-sterile microcosms were 10^7CFU/g soil (Figure 4.6). In 100 ppm phenanthrene microcosm, number of bacteria increasing 1 order of magnitude within 5 days and constant at ~10^8 CFU/g soil until 20 days (Figure 4.6A). In 300 ppm phenanthrene microcosm, number of bacteria increasing 1 order of magnitude within 10 days and constant at ~10^8 CFU/g soil until 20 days (Figure 4.6B). The number of PAHs degrading bacteria were included both *Sphingomonas* sp. strain P2 and indigenous PAHs degraders. The highest number of PAHs degraders in both 100 ppm and 300 ppm phenanthrene microcosm was found in soil microcosms with soil extract mixed with water (1:3) liquid inoculum and the lowest number of PAHs degraders was found in 8-day soil inoculum. This treatment contained PAHs degraders equal to 3×10^7 CFU/g soil and 2×10^7 CFU/g soil in 100 ppm and 300 ppm phenanthrene microcosm, respectively.

When considering both total bacteria and PAHs degrader, the results showed that 300 ppm phenanthrene microcosms had lower amount of the bacteria count than 100 phenanthrene microcosms (Figure 4.5 and 4.6). According to the Verrhiest et al. (2002), PAHs at 300 ppm can cause a decrease in soil bacterial densities.

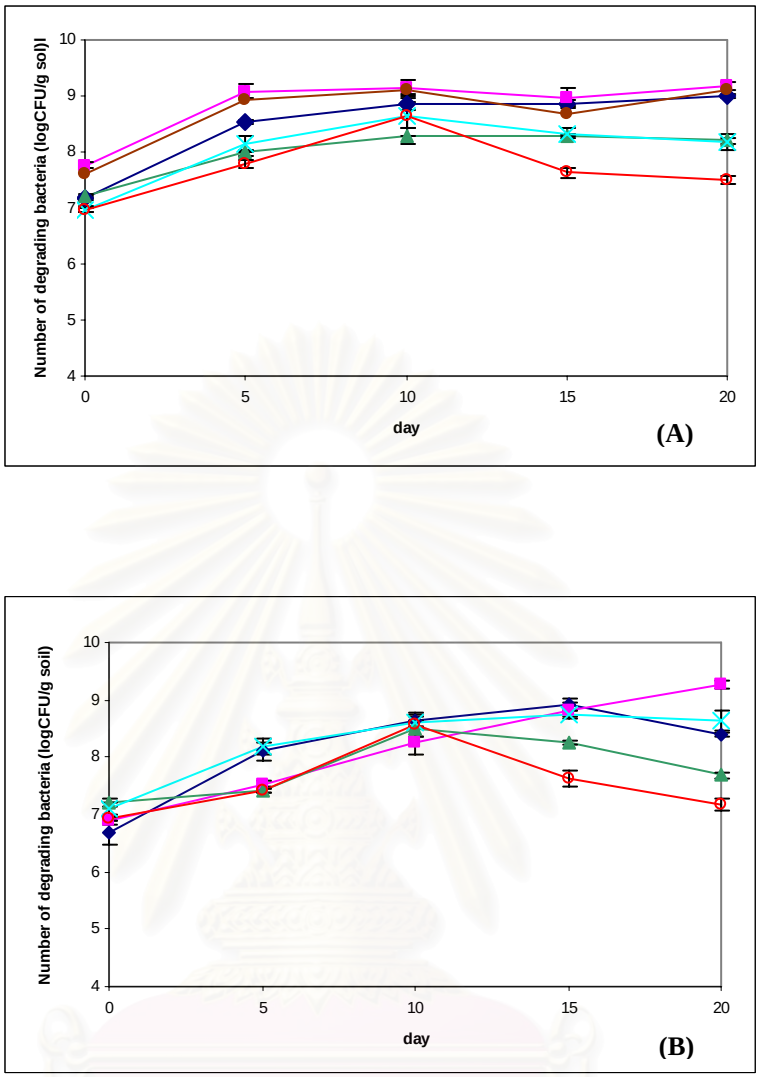


Figure 4.6 The number of degrading bacteria in 100 ppm phenanthrene microcosm (A) and 300 ppm phenanthrene microcosm (B) in nonsterile soil microcosms: (◆) no inoculum; inoculum prepared in: (■) soil extract mixed with water (1:3), (▲) CFMM; soil inoculum incubated for: (×) 4 days, (O) 8 days, and (●) 12 days.

Our results suggested that the increased number of PAHs degraders was come from the addition of *Sphingomonas* sp. strain P2. Moreover, the liquid inoculum contributed to more number of PAHs degraders than soil inoculum. The high number of PAHs bacterial degraders in microcosms with liquid inoculum was probably due to the appropriate soil conditions for bacterial growth. Our soil sample contained rich nutrients, which contributed to low environmental stress in soil. This soil had optimum C: N ratio and was further amended with liquid nutrient to supply other mineral sources. Finally, the soil was dried before used in the experiment. The drying process might reduce some soil organisms, which results in the reduction of some biotic stress such as predation by protozoa and competition with indigenous microorganisms. Consequently, a protective niche (i.e. soil carrier materials) was not necessary for the inoculants after applied to this soil.

4.4.2.3 Bacterial communities in non-sterile soil microcosm

Fate of *Sphingomonas* sp. strain P2 was monitored by genetic fingerprint technique in order to prove its survival and to assess its influence on other bacterial populations over times. 16S rDNA-DGGE was performed using DNA samples from 300 ppm phenanthrene treated microcosms. The samples were from day 0 (Lane 2-6), day 5 (Lane 7-11), day 10 (Lane 13-17) and day 15 (Lane 18-22). DGGE fingerprint profiles in Figure 4.7 illustrated the changes of soil microbial communities after treatment.

The representative lanes of each sample in DGGE fingerprint are as followed:

Lane 1 *Sphingomonas* sp. strain P2

Lane 2 no inoculum (day 0)

Lane 3 CFMM (day 0)

Lane 4 soil extract mixed with water (1:3) (day 0)

Lane 5 4 days soil inoculum (day 0)

Lane 6 8 days soil inoculum (day 0)

Lane 7 no inoculum (day 5)

Lane 8 CFMM (day 5)

Lane 9 soil extract mixed with water (1:3) (day 5)

Lane 10 4 days soil inoculum (day 5)

Lane 11 8 days soil inoculum (day 5)

Lane 12 *Sphingomonas* sp. strain P2

Lane 13 no inoculum (day 10)

Lane 14 CFMM (day 10)

Lane 15 soil extract mixed with water (1:3) (day 10)

Lane 16 4 day soil inoculum (day 10)

Lane 17 8 days soil inoculum (day 10)

Lane 18 no inoculum (day 15)

Lane 19 CFMM (day 15)

Lane 20 soil extract mixed with water (1:3) (day 15)

Lane 21 4 days soil inoculum (day 15)

Lane 22 8 days soil inoculum (day 15)

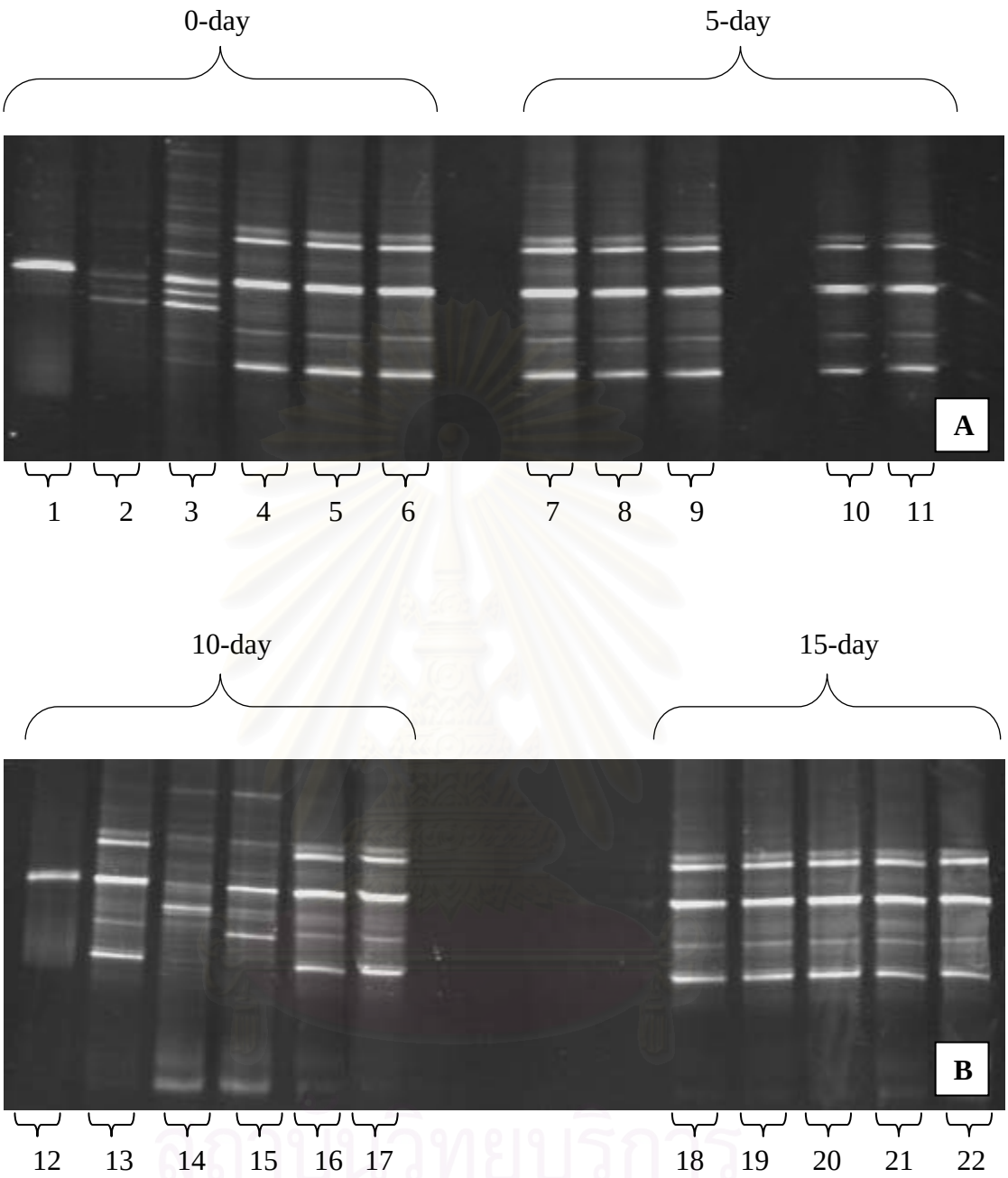


Figure 4.7 DGGE analysis of PCR-amplified 16S rDNA microbial communities of 300 ppm phenanthrene microcosm sampling at day 0, 5, 10, and 15.

The position of DGGE bands on each lane was taken as an indication of species in each sample. Lane 1 and 12 shown *Sphingomonas* sp. strain P2 DNA (Figure4.7). DNA bands corresponding to this inoculum species were seen clearly in soil microcosms with added inoculum. The results showed that inoculated bacteria were one of the dominant populations in soil microcosms. Control uninoculated *Sphingomonas* sp. strain P2 treatment also contained a DNA band at the same position as *Sphingomonas* sp. strain P2. However, this band may be or may be not the *Sphingomonas* sp. strain P2. Since phenanthrene degradation was low in the uninoculated soil microcosms, this indigenous *Sphingomonas* sp. was probably incapable of degrading PAHs. On the other hand, the present of this band at this position may be due to the low resolution of denaturant gradient on acrylamide gel. The DNA fragments may be separated out if we change the denaturing gradient.

When compared between treatments, almost identical fingerprint profiles were seen throughout the study. From the DGGE fingerprint profile, it can be concluded that different types of inoculum (i.e. liquid and soil inoculum) had similar effects on the microbial communities in soil microcosm after 15-day incubation. In addition, differences in preincubation time of the soil inoculum had no effect on microbial composition in soil microcosms. Meanwhile, DGGE fingerprints from uninoculated soil microcosms were similar to inoculated soil microcosms at the end of study. The results suggested that the influences of inoculums on indigenous soil bacteria were probably less than other physical and chemical parameters such as phenanthrene, nutrients, soil texture, and pH.

4.5 Summary of results and discussions

These present results showed the potential of soil and liquid inoculum for enhancing the survival and PAHs degradative ability of *Sphingomonas* sp. strain P2 during PAH bioremediation in non-sterile soil. *Sphingomonas* sp. strain P2 inoculum enhanced phenanthrene degradation in non-sterile soil when compare to the control uninoculated treatment. Moreover, *Sphingomonas* sp. strain P2 was able to survive in non-sterile soil during the experimental period. The results from Supaka et al. (1999) showed that the survival and phenanthrene degradability of *Sphingomonas* sp. strain P2 were decreased in non-sterile clay loam soil. The different in soil texture is probably one of the reasons for these different results.

There were many factors that probably affected the survival and PAHs degradative ability of *Sphingomonas* sp. strain P2 in our experiment. Physical and chemical properties of soil were probably the major factors. This soil was sandy loam with optimum C: N: P ratio for PAHs degradation. Moreover, the soil was fertile with low environmental stress, thus it was suitable for growth of many bacteria. According to Acea and Alexander (1988) predation by protozoa is a major factor causing the decline of bacterial inoculum introduced into soil. The competition between the inoculant and indigenous populations was the other major factor that limits survival and activities (Van Dyke and Prosser, 2000). Our soil sample was dried before used in the experiment. This may reduce some biotic stress i.e. predation by protozoa and competition with indigenous microorganism.

Liquid inoculum had higher ability to degrade phenanthrene than soil inoculum at the beginning of study. This result was probably due to the homogenous distribution of liquid inoculum in soil microcosms. Mixing of soil inoculum with non-sterile soil was rather difficult in the microcosms because soil particles were clumped together after adjusting the moisture content. This would reduce the bioavailability of PAHs to degrading enzymes produced from soil inoculum. In 100 ppm phenanthrene microcosm, the soil extract mixed with water (1:3) liquid inoculum shown the highest phenanthrene degradation. In 300 ppm phenanthrene microcosm, the CFMM liquid inoculum showed the highest phenanthrene degradation. This result suggested that PAH degradability of the inoculum was depended on PAH concentrations.

Although soil inoculum shown lower phenanthrene degradation than liquid inoculum, it can degrade phenanthrene to the undetectable amount within 5 days in 100 ppm phenanthrene microcosm and 10 days in 300 ppm phenanthrene microcosm. Especially, 4-day soil inoculum had almost the same phenanthrene degradation rate as liquid inoculum. Therefore, preincubation time of sterile soil inoculum affected the PAHs degradative ability. From the result, phenanthrene degradability could be arranged in high to low order of 4-day, 8-day and 12-day soil inoculum in 100 ppm phenanthrene microcosm and 4-day, and 8-day in 300 ppm phenanthrene microcosm. The 4-day inoculum was the most effective inoculum for PAH bioremediation.

Sphingomonas sp. strain P2 could co-metabolize pyrene (high molecular weight PAHs) by using phenanthrene as growth substrate (Supaka et al., 2001). The present result illustrated that all inoculum could degrade only small amount of pyrene in 100 and 300 ppm phenanthrene microcosm after 20 days. This result may be due

to the short incubation time. Roger et al. (1996) demonstrated that no significant degradation of pyrene after 4 weeks incubation, but after 9 week bacteria can degrade 36% of the initial concentration. Meanwhile, Herbes and Schwall (1978) suggested that co-metabolism of PAHs in soil is less important than in liquid medium because sorption of PAHs compound to the sediment particles.

The amounts of total bacteria and PAHs degrading bacteria were not much different in bioremediated non-sterile soil microcosms. This result might be from the residence of some bacteria in soil particles. Other reason might be from the disadvantage of culture technique that cannot detect the uncultivable species or inactive form of degraders in soil.

High amounts of PAHs degraders were found in uninoculated soil but only low amount of PAHs was degraded. The results suggested that indigenous soil bacteria had low ability to degrade soil PAHs. This result was supported with DGGE fingerprint profile. The control unioculated soil communities had the same band profile as the inoculated microcosms. This DGGE band was probably come from indigenous *Sphingomonas* sp. which has no degradability or probably need a period of time to acclimatize to the PAHs. The similarity of the DGGE profile from the result implied that the added inoculum had minor effects on bacterial populations in non-sterile soil. This result suggested that other physical and chemical parameters such as phenanthrene, nutrients, soil texture, and pH determined the present of soil bacteria populations.

From our study, this acclimatizing process may be applied to contaminated site which have no or low number of indigenous microorganisms capable of degrading the target contaminant. This strain can use as both liquid and soil inoculum in order to remediate PAHs contaminated site. Contaminated soil should be analyzed for the physical and chemical properties and adjust to optimum condition before start the bioremediation process. Contaminated soil from the real site should be collected to use as both carrier material and acclimatized condition prior introduced to soil. Furthermore, this process is suitable for large contaminated site because it is low cost and can conduct without excavation. After the introduction of inoculum, mixing process is needed in order to enhance bioavailability of the contaminants. This process can be used with other remediation technique such as bioventing and phytoremediation.

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 Conclusions

The main purpose of this research was to develop a soil acclimatization process that can be used for enhancing survival and PAHs degradative ability of *Sphingomonas* sp. strain P2 during bioremediation of PAHs contaminated soil. The results showed that:

1) *Sphingomonas* sp. strain P2 cultured in soil extract mixed with water (1:3) possesed highest PAHs degradative ability and could survive well in 2-g sterile soil microcosm. This culture media was therefore selected as medium for preparing the liquid inoculum of *Sphingomonas* sp. strain P2 prior to culture in sterile soil.

2) Soil acclimatized *Sphingomonas* sp. strain P2 was able to degrade PAHs and could survive in non-sterile soil microcosms. Meanwhile, the liquid inoculum (*Sphingomonas* sp. strain P2 acclimatized in soil extract) was also effective especially at the beginning of the experiment. The results from liquid inoculum were different from Supaka et al. (1999), which may be due to the different of soil sample used in this study.

3) Duration of preincubation period affected the PAHs degradability of *Sphingomonas* sp. strain P2. From the result, 4-day old soil inoculum had highest phenanthrene degradability.

4) There was a little difference in pyrene degradation between treatments with or without *Sphingomonas* sp. strain P2, which was probably due to the rapid decreasing of growth substrate (phenanthrene) and insufficient incubation period.

5) 16S rDNA-DGGE analysis showed DNA band corresponding to *Sphingomonas* sp. strain P2 in all gels. The results suggested that the inoculated bacteria were one of the dominant populations in soil microcosms. Liquid and soil inoculum had similar effects on soil bacterial populations.

5.2 Suggestions for future work

There are many factors that affect the survival and activities of bacteria inoculum when introduce to the soil environment. To enhance PAHs biodegrading activity of the inoculum, we may acclimatize the bacterial strain before introduce into the site. This acclimatizing process may be used for producing other bacterial inoculums as well as for clean-up other contaminants.

The present results illustrated that all inoculum had no significant effect on pyrene degradation rate in 100 and 300 ppm phenanthrene microcosm. This result may be from the short incubation time. Roger et al. (1996) demonstrated no significant degradation of pyrene after 4 weeks incubation, but after 9 week can

degrade 36% of initial concentration. Therefore, further study should extend the incubation time to allow for pyrene degradation. To maintain the amount of *Sphingomonas* sp. strain P2 growth substrate, phenanthrene may be repeatedly added to soil microcosms.

From 16S rDNA-DGGE analysis, there were DGGE bands in control set which identical to *Sphingomonas* sp. strain P2 band. These bands may not be the *Sphingonmonas* sp. P2. To confirm its identity, this DGGE band should be excised from the gel and analyzed for DNA sequences. Moreover, PAHs degradability and survival should be studied in soil with different environmental stress in order to prove the ability of acclimatization process. The success of this acclimatizing process may be more distinct in contaminated sites. Furthermore, the optimum condition (moisture content, temperature, and pH) of the acclimatized inoculum should be studied in order to enhance its PAHs degradability and survival at each site.

Differences in soil characteristics may have different effects to the inoculum. The effect of soil parameters to the acclimatized inoculum should be studied in order to enhance the PAHs degradability and survival. Furthermore, inoculation method may affect the efficiency of the introduced strain in soil. Further experiments should add the inoculum to the sterile soil before adjust moisture content. This would promote homogeneous mixing and consequently enhance PAHs degradability and survival of soil inoculum.

REFERENCES

Acea, M. J., and Alexander, M. 1988. Growth and survival of bacteria introduced into carbon-amended soil. Soil Biol. Biochem. 20: 703-709.

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M. T., Nannipieri, P., and Pietramellara, G. 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. Soil Biol. Biochem. 36: 859-868.

Amellal, N., Portal, J. M., Vogel, T., and Berthelin, J. 2001. Distribution and location of polycyclic aromatic hydrocarbons (PAHs) and PAH-degrading bacteria within polluted soil aggregates. Biodegradation. 12: 49-57.

Andreoni, V., Cavalca, L., Rao, M. A., Nocerino, G., Bernasconi, S., Dell'Amico, E., Colombo, M., and Gianfreda, L. 2004. Bacterial communities and enzyme activities of PAHs polluted soils. Chemosphere. 57: 401-412.

ATSDR (Agency for Toxic Substances and Disease Registry). (1995).Toxicological Profiles for Polycyclic Aromatic Hydrocarbons (PAHs) [online], Available from http://www.atsdr.cdc.gov/toxprofiles/phs69.html. [2005, Feb, 12].

Balba, M.T., Al-Awadhi, N., and Al-Daher, R.1998. Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. Microbiol. Methods. 32: 155-164.

Bauer, J.E., and Capone, D. G. 1985. Degradation and mineralization of polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Appl. Environ. Microbiol. 50: 81-90.

Bauer, J.E., and Capone, D. G. 1988. Effect of co-occurring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. Appl. Environ. Microbiol. 54: 1649-1655.

Boopathy, R. 2000. Factors limiting bioremediation technologies. Bioresour. Technology. 74: 63-67.

Cerniglia, C. E. 1992. Bioremediation of polycyclic aromatic hydrocarbons. Biodegradation. 3: 351-368.

Cerniglia C. E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. Current Opinion in Biotechnology. 4: 331-338.

Cheung, P., and Kinkle, B.K. 2001. *Mycobacterium* diversity and pyrene mineralization in petroleum-contaminated soils. Appl. Environ. Microbiol. 67: 2222-2229.

Cunningham, C. J., Ivshina, I. B., Lozinsky, V. I., Kuyukina, M. S., and Philp, J. C. 2004. Bioremediation of diesel-contaminated soil by microorganisms immobilized in polyvinyl alcohol. Int. biodeterior Biodegradation. 54: 167-174.

Cunningham, C. J., and Philp, J. C. 2000. comparisom of bioaugmentation and biostimulation in ex situ treat of diesel contaminated soil. Land contamination & reclamation. 8: 261-269.

Dean-Ross, D., Moody, J., and Cerniglia, C. E. 2002. Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. FEMS Microbiol. Ecology. 41: 1-7.

Dibble, J. T., and Bartha, R. 1979. Effect of environment parameters on the biodegradation of oil sludge. Appl. Environ. Microbiol. 37: 729-739.

Eriksson, M., Dalhammar, G., and Mohn, W. W. 2002. Bacterial growth and biofilm production on pyrene. FEMS Microbiol. Ecology. 40: 21-27.

Eve, R., Eve, R. 1998. Biological, physical, and chemical processes. Remediation of petroleum contaminated soil. Boca Raton : Lewis Publishers, 115-198.

Gentry, T. J., Wolf, D. C., Reynolds C. M., and Fuhrmann, J. J. 2003. Pyrene and phenanthrene influence on soil microbial population. Bioremediation. 7: 53-68.

Goldstein, R. M., Mollory, L. M., and Alexander, M. 1985. Reasons for possible failure of inovulation to enhance biodegradation. Appl. Environ. Microbiol. 50: 977-983.

Grishchenkov, V. G.,Townsend, R. T., McDonald, T. J., Autenrich, R. L., Bonner, J. S., and Boronin, A. M. 2000. Degradation of petroleum hydrocarbons by facultative anaerobic bacteria under aerobic and anaerobic conditions. Process Biochemistry. 35, 889-896.

Gong, R., Hochmuth, M., and Weatherburn, D. C. 2003. Polycyclic aromatic hydrocarbons and diesel particulates. 26th Australasian Transport Research Forum, Wellington, New Zealand. 1-18.

Heitkamp, M. A., and Cerniglia, C. E. 1988. Mineralization of polycyclic aromatic hydrocarbon by bacterium isolated from sediment below an oil field. Appl. Environ. Microbiol. 54: 1612-1614.

Herbes, S. E., and Schwall, L. R. 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contaminated sediments. Appl. Environ. Microbiol. 35: 306-316.

Hupe, K., Koning, M., Luth, J.–C., Heerenklage, J., Stegmann, R. 2001. Optimisation of microbial soil treatment. In: Stegmann R., Brunner G., Calmono, W. and

Matz, G. (eds.), treatment of contaminated soil. 342-353 New York: Springer-Verlag Ferlin Heidelberg.

Kanaly, R. A., Bartha, R., Watanabe, K., and Harayama, S. 2000. Rapid mineralization of Benzo[a]pyrene by a microbial consortium growing on diesel fuel. Appl. Environ. Microbiol. 66: 4205-4211.

Johnsen, A. R., Wick, L. Y., and Harms, H. 2005. Principle of microbial PAH-degradation in soil. Environ. Pollut. 133: 71-84.

Juhasz, A. L., Naidu, R. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int biodeterior Biodegradation. 45: 57-88.

Leahy, G. J., Colwell, R. R. 1990. Microbial degradation of hycrocarbons in the environment. Microbiol Rev. 54: 305-315.

Liu, Y., Zhu, L., and Shen, X. 2001. Polycyclic aromatic hydrocarbons (PAHs) in indoor and outdoor air of Hangzhou, China. Environ. Sci. Technol. 35: 840-844.

Megharaj, M., Wittich, R.M., Blasco, R., Pieper, D.H., and Timmis, K.N. 1997. Superior survival and degradation of dibenzo-*p*-dioxin and dibenzofuran in soil by soil-adapted *Sphingomonas* sp. strain RW1. Appl Microbiol Biotechnol. 48: 109-114.

Mishra, S., Jyot, J., Kuhad, R. C., and LaL, B. 2001. Evaluation of Inoculum Addition to Stimulate In Situ Bioremediation of Oily-Sludge-Contaminated Soil. Appl. Environ. Microbiol. 67: 1675-1681.

Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology. 2: 317-322.

Potin, O., Rafin, C., and Veignie, E. 2004. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. Int biodeterior. biodegradation. 54: 45-52.

Rehmann, K., Noll, H. P., Steinberg, C. E., Kettrup, A. A. 1998. Pyrene degradation by *Mycobacterium* sp. strain KR2. Chemosphere. 36: 2977-2992.

Romantschuk, M., Sarand, I., Petanen, T., Peltola, R., Jonsson-Vihanne, M., Koivula, T., Yrjala, K., and Haahtela, K. 1999. Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. Environ. pollut. 107: 179-185.

Roper, J. C., and Pfaender, F. K. 2001. Pyrene and chrysene fate in surface soil and sand microcosms. Environ. Toxicol. chem. 20: 223-230.

Ruberto, L., Vazquez, S. C., and Mac Cormack, W. P. 2003. Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hy drocarbon contaminated Antarctic soil. Int. Biodetrior. Biodegradation. 52: 115-125.

Samanta, S. K., Singh, O. V. and Jain, R. K. 2002. polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol. 20: 243-248.

Sasikumar, C. S., and Papinazath, T. 2003. Environmental management: bioremediation of polluted environment. Proceedings of the third international conference on environment and health, Chennai, India. 465-469.

Sigler, W. V., Miniaci, C., and Zeyer, J. 2004. Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure. Microbiol. methods. 57: 17-22.

Smith, J. M., Lethbridge, G., and Burns, R. G. 1999. Fate of phenanthrene, pyrene and benzo[a]pyrene during biodegradation of crude oil added to two soils. FEMS Microbiol. lett. 173: 445-452.

Supaka, N. 1999. Ability of isolated soil bacteria in degradation of phenanthrene and other polycyclic aromatic hydrocarbons. Thesis. Industrial microbiology. Chulalongkorn university.

Supaka, N., Pinphanichakarn, P., Pattaragulwanit, K., Thaniyavarn, S., Omori, T., and Juntongjin, K. 2001. Isolation and characterisation of a phenanthrene-degrading *Sphingomonas* sp. strain P2 and Its ability to degrade fluoranthene and pyrene via cometabolism. Sci. Asia. 27: 21-28.

Van Dyke, M.I., and Prosser, J. I. 2000. Enhanced survival of *Pseudomonas fluorescens* in soil following establishment of inoculum in a sterile soil carrier. Soil Biol. Biochem. 32: 1377-1382.

Van Elsas, J. D., Van Overbeek, L. S. 1993. Bacterial Responses to Soil Stimuli. Starvation in Bacteria. Plemun Press. New York. 55-79.

Van Veen, J. A., Van Overbeek, L. S., and Van Elsas, J. D. 1997. Fate and activity of microorganisms into soil. Microbiol. Mol. Boil. Rev. 61: 121-135.

Verrhiest, G. J., Clement, B., Volat, B., Montuelle, B., and Perrodin, Y. 2002. Interactions between a poly cyclic aromatic hydrocarbon mixture and the microbial communities in a natural freshwater sediment. Chemosphere. 46: 187-196.

Verstraete, W., and Top, E.M. 1999. Soil clean-up: lessons to remember. Int. biodeterior. Biodegradation. 43: 147-153.

Vidali, M., 2001, Bioremediation. An overview. Pure Appl. Chem. 73: 1163-1172.

Vogel, T. M. 1996. Bioaugmentation as a soil bioremediation approach. Current opinion in biotechnology. 7: 311-316.

Walter, U., Beyer, M., Klein, J., and Rehm, H.J. 1991. Degradation of pyrene by *Rhodococcus* sp. UW1. Appl. Microbiol. Biotechnol. 34: 671-676.

Wang, S. J., Loh, K. C., Chua, S. S. 2003. Prediction of critical cell growth behavior of *Pseudomonas putida* to maximize the cometabolism of 4-cholrophenol with phenol and sodium glutamate as carbon sources. Enzyme microb. technol. 32: 422-430.

Watanabe, K. 2001.Microorganism relevant to bioremediation. Current Opinion in Biotechnology. 12: 237-241.

Wilson, S. C., and Jones, K. C. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbon in soil (PAHs) a review. Environ. Pollut. 81: 229-249.

Yuan, S. Y., Wei, S. H., Chang, B. V. 2000. Biodegradation of polycyclic aromatic hydrocarbons by a mixed culture. Chemosphere. 41: 1463-1468.

Yuan, S. Y., Chang, J. S., Chang, B. V. 2001. Biodegradation of phenanthrene in river sediment. Chemosphere. 43: 273-278.

APPENDICES

APPENDIX A

CHEMICAL SOLUTIONS

1. Phenanthrene and Pyrene in acetone solution

Dissolve 0.03 g of each PAHs in acetone 30 ml were mixed vigorously by vortex mixer then filled though filter with 0.20 µm of pore size. This solution should be fresh prepared and keep in 0 °C until use and spiking of PAHs should be hurry performed because acetone solution volatile very fast so the concentration of PAHs may be changed.

2. Phenanthrene in dimethylsulfoxide (DMSO)

Dissolve 0.1 g of phenanthrene in 10 ml of diethyl ether then mix vigorously and filled though 0.22 µm pore size PTFE membrane filter. This solution should be fresh prepared before use.

3. 0.85 % Sodium Chloride

Dissolve 8.5 g of sodium chloride in 1000 ml of distilled water and sterile by autoclave with pressure 15 pound/inch^2 temperature 121 °C 15 minutes.

4. Standard PAHs for Gas chromatography

Dissolve PAHs 1 mg in methanol 1 ml mixed vigorously by vortex mixer then filled though PTFE type membrane filter with 0.20 µm of pore size and sealed with parafilm after that cover with Floyd paper for prevent this solution from photooxidation. Keep it in -20 °C until use.

5. 15 % Triton –x 100

Mixed 15 ml of triton x-100 add water to 100 ml of distilled water keep at room temperature until use.

6. 10X Tris-borate-EDTA (TBE) stock buffer

Tris base	108 g
Boric acid	55 g
EDTA solution	40 ml
Deionized H_2O	to 1000 ml

7. 10X Tris-acetate-EDTA (TAE) stock buffer

Tris base	48.4 g
Acetic acid	11.4 ml
EDTA solution	20.0 ml
Deionized H_2O	to 1000.0 ml

8. Nutrient media

Nutrient media used in this research were Carbon free minimum mineral medium (CFMM medium) and Luria Bertani (LB agar) as shown in table A.1 and A.2, respectively.

Table A.1 Composition of Carbon free minimum mineral medium (CFMM medium).

Constituent	**Concentration (g/l)**
NH_4NO_3	3
KH_2PO_4	0.8
$Na_2HPO_4.12H_2O$	5.5
$MgSO_4.7H_2O$	0.1
$FeCl_3.6H_2O$	0.05
$CaCl_2.2H_2O$	0.05
Distilled water	1000 ml

Adjust pH to 7.5 autoclaved at pressure 15 pound/inch^2 temperature 121ºC 15 minutes.

9. Soil extract

The soil extract was prepared by shaking 200 g of soil with 200 ml sterile distilled water overnight on an orbital shaker followed by centrifugation at 10,000 g and filter sterilization (0.20 µm)

Table A.2 Composition of Luria Bertani (LB agar).

Constituent	**Concentration (g/l)**
Tryptone	10
Yeast extract	5
NaCl	5
Agar	15
Distilled water	1000 ml

Adjust pH to 7.0 autoclaved at pressure 15 pound/inch^2 temperature 121ºC 15 minutes.

APPENDIX B

GAS CHROMATOGRAPHY CHROMATOGRAM

1. Standard curve of phenanthrene

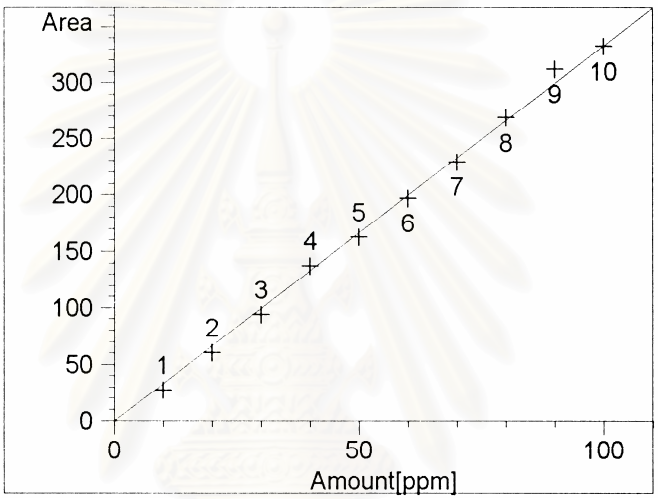


Figure B.1 Standard curve phenanthrene plotted between phenanthrene concentration and peak area analyzed by Gas Chromatography.

The concentration of phenanthrene can be calculated by substitute peak area values in linear equation:

Peak area = slope of standard curve x amount of phenanthrene (ppm)

From this graph slope = 3.33886

2. Standard curve of pyrene

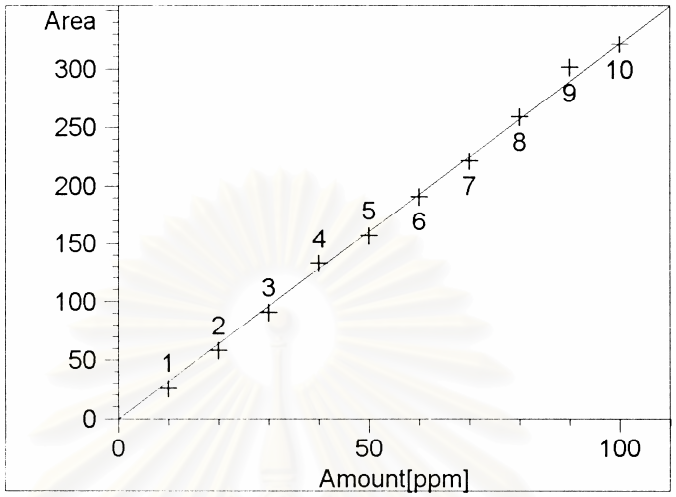


Figure B.2 Standard curve pyrene plotted between pyrene concentration and peak area analyzed by Gas Chromatography.

The concentration of pyrene can be calculated by substitute peak area values in linear equation:

Peak area = slope of standard curve x amount of pyrene (ppm)

From this graph slope = 3.22115

3. Chromatogram PAHs background of soil

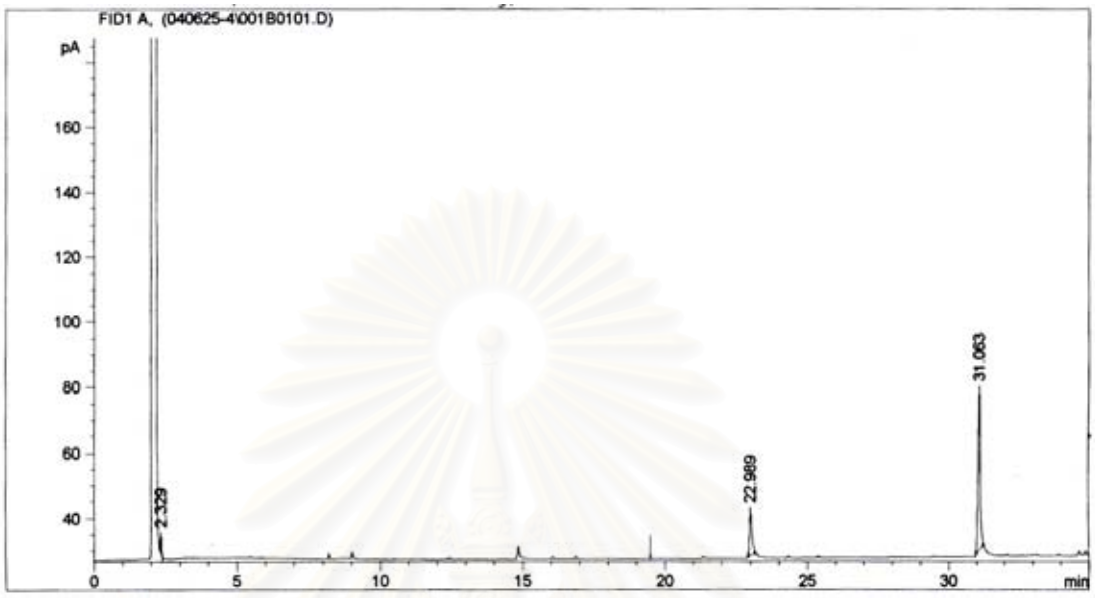


Figure B.3 Gas chromatogram of standard PAHs i.e. phenanthrene (RT = 12.966), and pyrene (RT = 21.818).

APPENDIX C

INOCULUM STANDARD CURVE

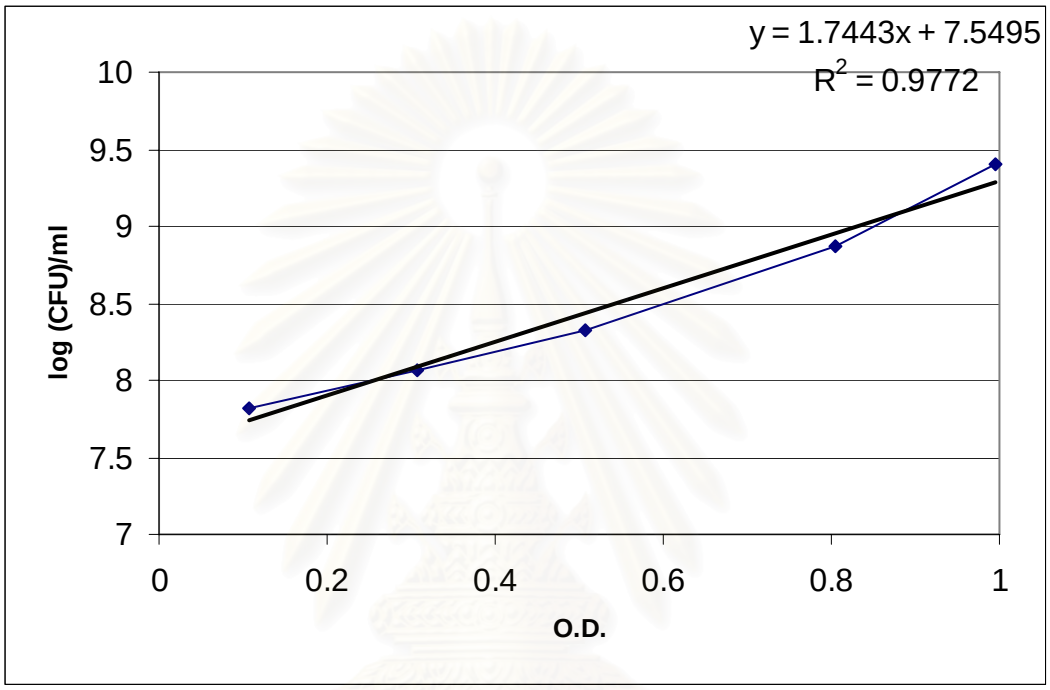


Figure C.1 Standard curve of *Sphingomonas* sp. P2 plotted between log CFU and O.D.

APPENDIX D

RAW DATA

Table D.1 Amount of remaining phenanthrene in 2 g sterile soil microcosm by using GC analysis

day	peak area				% remaining phenanthrene			
	control	s	mm	ms	control	s	mm	ms
0	174.35	171.88	139.71	170.79	100.00	100.00	100.00	100.00
	154.14	163.36	139.16	162.39	100.00	100.00	100.00	100.00
	153.07	151.70	131.42	152.45	100.00	100.00	100.00	100.00
5	163.52	0.00	20.63	149.08	93.79	0.00	14.77	87.29
	153.29	0.00	0.00	112.57	99.45	0.00	0.00	69.32
	152.62	0.00	0.00		99.71	0.00	0.00	
10	142.35	0.00	0.00	0.00	81.65	0.00	0.00	0.00
	139.84	0.00	0.00	0.00	90.72	0.00	0.00	0.00
	124.82		0.00	0.00	81.54	0.00	0.00	0.00
15	118.18	0.00	0.00	0.00	67.79	0.00	0.00	0.00
	113.28	0.00	0.00	0.00	73.49	0.00	0.00	0.00
	101.51	0.00	0.00	0.00	66.31	0.00	0.00	0.00
20	103.32	0.00	0.00	0.00	59.26	0.00	0.00	0.00
	102.18	0.00	0.00	0.00	66.29	0.00	0.00	0.00
	92.63	0.00	0.00	0.00	60.51	0.00	0.00	0.00
day	average				standard deviation			
	control	s	mm	ms	control	s	mm	ms
0	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00
5	97.65	0.00	4.92	78.30	3.34	0.00	8.53	12.70
10	84.64	0.00	0.00	0.00	5.27	0.00	0.00	0.00
15	69.20	0.00	0.00	0.00	3.79	0.00	0.00	0.00
20	62.02	0.00	0.00	0.00	3.75	0.00	0.00	0.00

Table D.2 Amount of remaining pyrene in 2 g sterile soil microcosm by using GC analysis

day	peak area				% remaining pyrene			
	control	s	mm	ms	control	s	mm	ms
0	155.16	156.04	150.88	153.46	100.00	100.00	100.00	100.00
	136.35	148.60	142.73	152.56	100.00	100.00	100.00	100.00
	136.10	148.03	141.24	145.09	100.00	100.00	100.00	100.00
5	141.74	129.52	125.27	129.86	91.35	83.00	83.03	84.62
	132.62	123.04	117.80	123.75	97.27	82.80	82.53	81.11
	131.63	112.71	116.62	115.99	96.71	76.14	82.57	79.95
10	122.31	114.47	94.93	82.55	78.83	73.36	62.92	53.79
	120.13	99.06	84.75	79.05	88.10	66.66	59.38	51.81
	104.41		81.77	75.01	76.72	0.00	57.90	51.70
15	100.62	75.05	86.68	101.08	64.85	48.09	57.45	65.87
	97.45	62.58	83.10	89.80	71.47	42.11	58.22	58.86
	88.12	54.76	77.22	87.77	64.75	36.99	54.67	60.50
20	82.07	68.91	68.61	81.41	52.89	44.16	45.47	53.05
	80.93	68.88	66.21	74.38	59.35	46.35	46.39	48.75
	71.95	52.39	65.26	61.91	52.87	35.39	46.20	42.67
day	average				standard deviation			
	control	s	mm	ms	control	s	mm	ms
0	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00
5	95.11	80.65	82.71	81.89	3.27	3.90	0.28	2.43
10	81.22	46.67	60.07	52.43	6.06	4.73	2.58	1.18
15	67.02	42.40	56.78	61.74	3.85	5.56	1.87	3.66
20	55.04	41.97	46.02	48.16	3.74	5.80	0.48	5.21

Table D.3 Amount of *Sphingomonas* sp.strain P2. in 2 g sterile soil microcosm by using plate count technique

day	CFU/ g soil					
	s	mm	ms	log(s)	log(mm)	log(ms)
0	1.92E+08	1.83E+08	1.83E+08	8.28	8.26	8.26
	1.87E+08	1.80E+08	1.65E+08	8.27	8.26	8.22
	1.95E+08	1.75E+08	1.89E+08	8.29	8.24	8.28
avg	1.91E+08	1.79E+08	1.79E+08	8.28	8.25	8.25
sd	4.04E+06	4.04E+06	1.25E+07	0.01	0.01	0.03
5	2.57E+08	5.80E+08	1.25E+08	8.41	8.76	8.10
	2.49E+08	5.00E+08	1.02E+08	8.40	8.70	8.01
	2.60E+08	5.90E+08	1.20E+08	8.41	8.77	8.08
avg	2.55E+08	5.57E+08	1.16E+08	8.41	8.74	8.06
sd	5.69E+06	4.93E+07	1.21E+07	0.01	0.04	0.05
10	2.20E+08	2.50E+08	1.80E+08	8.34	8.40	8.26
	2.10E+08	2.20E+08	2.40E+08	8.32	8.34	8.38
	2.50E+08	2.50E+08	2.40E+08	8.40	8.40	8.38
avg	2.27E+08	2.40E+08	2.20E+08	8.35	8.38	8.34
sd	2.08E+07	1.73E+07	3.46E+07	0.04	0.03	0.07
15	1.72E+08	5.80E+07	1.64E+08	8.24	7.76	8.21
	1.54E+08	5.40E+07	1.84E+08	8.19	7.73	8.26
	1.70E+08	5.90E+07	1.78E+08	8.23	7.77	8.25
avg	1.65E+08	5.70E+07	1.75E+08	8.22	7.76	8.24
sd	9.87E+06	2.65E+06	1.03E+07	0.03	0.02	0.03
20	9.90E+07	2.50E+07	6.70E+07	8.00	7.40	7.83
	8.70E+07	3.80E+07	8.80E+07	7.94	7.58	7.94
	1.01E+08	2.30E+07	9.20E+07	8.00	7.36	7.96
avg	9.57E+07	2.87E+07	8.23E+07	7.98	7.45	7.91
sd	7.57E+06	8.14E+06	1.34E+07	0.04	0.12	0.07
25	4.50E+07	5.10E+06	1.11E+07	7.65	6.71	7.05
	4.80E+07	5.60E+06	1.18E+07	7.68	6.75	7.07
	3.00E+07	5.80E+06	1.03E+07	7.48	6.76	7.01
avg	4.10E+07	5.50E+06	1.11E+07	7.60	6.74	7.04
sd	9.64E+06	3.61E+05	7.51E+05	0.11	0.03	0.03
30	5.20E+07	1.58E+07	2.82E+07	7.72	7.20	7.45
	6.80E+07	1.50E+07	2.49E+07	7.83	7.18	7.40
	6.20E+07	1.60E+07	2.45E+07	7.79	7.20	7.39
avg	6.07E+07	1.56E+07	2.59E+07	7.78	7.19	7.41
sd	8.08E+06	5.29E+05	2.03E+06	0.06	0.01	0.03

Table D.4 Amount of remaining phenanthrene in 20 g non-sterile soil microcosm (100 ppm phenanthrene microcosm) by using GC analysis

day	peak area					
	control	s	mm	s4	s8	s12
0	62.90	45.13	199.22	139.87	135.69	61.05
	60.74	41.56	104.78	130.53	135.63	58.76
	60.64	39.72	99.15	129.80	130.13	57.80
5	55.29	0.00	44.66	36.58	49.25	41.65
	51.55	0.00	0.00	0.00	47.65	35.55
	50.51	0.00	0.00		0.00	32.10
10	57.68	0.00	0.00	0.00	56.44	28.65
	53.00	0.00	0.00	0.00	32.29	20.40
	52.35	0.00	0.00	0.00	0.00	19.97
15	62.23	0.00	0.00	0.00	0.00	45.86
	60.61	0.00	0.00	0.00	0.00	
	50.45	0.00	0.00	0.00	0.00	
20	54.56	0.00	0.00	0.00	0.00	35.19
	54.09	0.00	0.00	0.00	0.00	22.78
	50.70	0.00	0.00	0.00	0.00	
day	% control	%s	%mm	%s4	%s8	%s12
0	100.00	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00	100.00
5	87.90	0.00	22.42	26.15	36.29	68.21
	84.88	0.00	0.00	0.00	35.13	60.51
	83.30	0.00	0.00		0.00	55.53
10	91.71	0.00	0.00	0.00	41.59	46.93
	87.26	0.00	0.00	0.00	23.81	34.71
	86.34	0.00	0.00	0.00		34.56
15	98.94	0.00	0.00	0.00	0.00	75.11
	99.79	0.00	0.00	0.00	0.00	
	83.19	0.00	0.00	0.00	0.00	
20	86.75	0.00	0.00	0.00	0.00	57.63
	89.06	0.00	0.00	0.00	0.00	38.76
	83.61	0.00	0.00	0.00	0.00	
day	avg-c	avg-s	avg-mm	avg-s4	avg-s8	avg-s12
0	100.00	100.00	100.00	100.00	100.00	100.00
5	85.36	0.00	7.47	13.08	23.81	61.42
10	88.44	0.00	0.00	0.00	32.70	38.73
15	93.98	0.00	0.00	0.00	0.00	75.11
20	86.47	0.00	0.00	0.00	0.00	48.20
day	sd-c	sd-l	sd-mm	sd-s4	sd-s8	sd-s12
0	0.00	0.00	0.00	0.00	0.00	0.00
5	2.34	0.00	12.94	18.49	20.63	6.39
10	2.87	0.00	0.00	0.00	12.57	6.39
15	9.35	0.00	0.00	0.00	0.00	0.00
20	2.74	0.00	0.00	0.00	0.00	13.34

Table D.5 Amount of remaining pyrene in 20 g non-sterile soil microcosm (100 ppm phenanthrene microcosm) by using GC analysis

day	peak area					
0	control	s	mm	s4	s8	s12
	55.52	56.94	122.33	132.57	132.57	59.09
	55.11	54.75	118.01	129.83	130.77	58.17
5	49.82	47.39	89.93	121.54	140.62	54.27
	47.12	45.28	89.96	110.37	120.47	53.31
	45.71	44.98	87.48		105.49	52.31
10	52.42	54.52	108.60	113.47	109.63	58.10
	48.35	47.54	103.69	104.86	101.85	53.87
	47.33	43.66	99.73	104.78	100.69	53.75
15	48.97	46.11	98.09	104.62	98.94	55.11
	48.58	44.49	98.52	102.55	90.96	51.89
	39.84	44.05	97.49	102.08	87.10	50.42
20	45.69	46.38	95.91	90.73	114.82	51.78
	45.21	46.04	91.08	86.36	96.04	51.50
	44.26	37.44	85.95	81.73	91.72	50.48
day	% control	%s	%mm	%s4	%s8	%s12
0	100.00	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00	100.00
5	87.22	80.37	66.09	88.50		89.06
	84.86	79.52	73.54	83.26	90.87	90.21
	82.95	82.15	74.13		80.66	89.94
10	91.78	92.45	79.80	82.62	82.18	95.35
	87.08	83.50	84.77	79.10	76.82	91.15
	85.87	79.74	84.51	80.71	77.00	92.42
15	85.74	78.20	72.08	76.18	74.17	90.45
	87.50	78.14	80.53	77.36	68.61	87.80
	72.29	80.46	82.61	78.63	66.61	86.69
20	80.00	78.65	70.48	66.06	86.07	84.97
	81.42	80.87	74.45	65.14	72.44	87.15
	80.31	68.37	72.83	62.95	70.14	86.78
day	avg-c	avg-s	avg-mm	avg-s4	avg-s8	avg-s12
0	100.00	100.00	100.00	100.00	100.00	100.00
5	85.01	80.68	71.25	85.88	85.77	89.74
10	88.25	85.23	83.03	80.81	78.67	92.97
15	81.84	78.93	78.41	77.39	69.80	88.31
20	80.58	75.96	72.59	64.72	76.22	86.30
day	sd-c	sd-l	sd-mm	sd-s4	sd-s8	sd-s12
0	0.00	0.00	0.00	0.00	0.00	0.00
5	2.14	1.34	4.48	3.71	7.22	0.60
10	3.12	6.53	2.79	1.76	3.04	2.15
15	8.32	1.32	5.57	1.22	3.92	1.93
20	0.75	6.67	2.00	1.60	8.61	1.17

Table D.6 Amount of total bacteria in 20 g non-sterile soil microcosm (100 ppm phenanthrene microcosm) by using plate count technique

day	CFU/ g soil					
	control	s	mm	s4	s8	s12
0	2.20E+07	4.90E+07	1.64E+07	4.80E+07	9.70E+06	6.90E+07
	2.20E+07	5.90E+07	1.88E+07	6.60E+07	1.50E+07	1.20E+08
	2.70E+07	4.30E+07	1.74E+07	6.70E+07	8.80E+06	1.23E+08
5	3.31E+08	1.25E+09	1.05E+08	1.54E+08	7.20E+07	1.00E+09
	2.73E+08	2.07E+09	1.08E+08	8.00E+07	8.50E+07	1.17E+09
	3.43E+08	1.56E+09	8.10E+07	1.25E+08	1.21E+08	1.21E+09
10	4.70E+08	1.16E+09	2.30E+08	1.10E+09	5.10E+08	1.32E+09
	5.30E+08	1.45E+09	2.10E+08	6.70E+08	5.90E+08	1.37E+09
	5.80E+08	1.10E+09	4.20E+08	3.50E+08	1.28E+09	1.40E+09
15	1.05E+09	9.70E+08	1.47E+08	2.80E+08	4.20E+07	4.30E+08
	6.70E+08	1.51E+09	2.21E+08	2.80E+08	5.90E+07	9.20E+08
	8.50E+08	1.25E+09	1.46E+08	3.20E+08	4.50E+07	9.90E+08
20	2.20E+09	1.94E+09	1.89E+08	1.20E+08	4.90E+07	8.20E+08
	1.99E+09	1.26E+09	1.77E+08	1.12E+08	3.20E+07	6.50E+08
	2.42E+09	1.71E+09	1.53E+08	1.30E+08		
day	log(c)	log(s)	log(mm)	log(s4)	log(s8)	log(s12)
0	7.34	7.69	7.21	7.68	6.99	7.84
	7.34	7.77	7.27	7.82	7.18	8.08
	7.43	7.63	7.24	7.83	6.94	8.09
5	8.52	9.10	8.02	8.19	7.86	9.00
	8.44	9.32	8.03	7.90	7.93	9.07
	8.54	9.19	7.91	8.10	8.08	9.08
10	8.67	9.06	8.36	9.04	8.71	9.12
	8.72	9.16	8.32	8.83	8.77	9.14
	8.76	9.04	8.62	8.54	9.11	9.15
15	9.02	8.99	8.17	8.45	7.62	8.63
	8.83	9.18	8.34	8.45	7.77	8.96
	8.93	9.10	8.16	8.51	7.65	9.00
20	9.34	9.29	8.28	8.08	7.69	8.91
	9.30	9.10	8.25	8.05	7.51	8.81
	9.38	9.23	8.18	8.11		
day	avg-log(c)	avg-log(s)	avg-log(mm)	avg-log(s4)	avg-log(s8)	avg-log(s12)
0	7.37	7.70	7.24	7.78	7.04	8.00
5	8.50	9.20	7.99	8.06	7.96	9.05
10	8.72	9.09	8.44	8.80	8.86	9.13
15	8.93	9.09	8.23	8.47	7.68	8.86
20	9.34	9.21	8.24	8.08	7.60	8.86
day	sd-log(c)	sd-log(s)	sd-log(mm)	sd-log(s4)	sd-log(s8)	sd-log(s12)
0	0.05	0.07	0.03	0.08	0.12	0.14
5	0.05	0.11	0.07	0.15	0.12	0.04
10	0.05	0.06	0.16	0.25	0.21	0.01
15	0.10	0.10	0.10	0.03	0.08	0.20
20	0.04	0.10	0.05	0.03	0.13	0.07

Table D.7 Amount of phenanthrene degrader in 20 g non-sterile soil microcosm (100 ppm phenanthrene microcosm) by using plate count technique

day	CFU/ g soil					
0	control	s	mm	s4	s8	s12
	1.48E+07	6.70E+07	1.85E+07	9.90E+06	9.70E+06	5.20E+07
	1.55E+07	5.20E+07	1.45E+07	8.40E+06	1.04E+07	3.50E+07
5	3.84E+08	1.36E+09	9.30E+07	9.40E+07	6.30E+07	7.50E+08
	3.28E+08	8.80E+08	1.09E+08	1.75E+08	5.10E+07	9.80E+08
	3.35E+08	1.53E+09	9.10E+07	1.62E+08	7.00E+07	8.10E+08
10	8.60E+08	1.03E+09	1.33E+08	3.40E+08	2.50E+08	9.00E+08
	5.20E+08	1.61E+09	2.52E+08	2.30E+08	7.50E+08	1.50E+09
	8.10E+08	1.75E+09	2.21E+08	1.15E+09	4.90E+08	1.47E+09
15	6.40E+08	6.10E+08	2.14E+08	1.71E+08	4.30E+07	4.60E+08
	6.40E+08	1.30E+09	1.90E+08	2.20E+08	5.10E+07	4.90E+08
	8.90E+08	1.09E+09	1.92E+08	2.58E+08	3.50E+07	4.80E+08
20	1.01E+09	1.36E+09	1.80E+08	1.10E+08	2.70E+07	8.10E+08
	8.80E+08	1.33E+09	1.65E+08	1.41E+08	3.80E+07	2.97E+09
	1.09E+09	1.79E+09	1.43E+08	2.10E+08	3.20E+07	8.50E+08
day	log(c)	log(s)	log(mm)	log(s4)	log(s8)	log(s12)
0	7.23	7.74	7.19	6.94	6.94	7.53
	7.17	7.83	7.27	7.00	6.99	7.72
	7.19	7.72	7.16	6.92	7.02	7.54
5	8.58	9.13	7.97	7.97	7.80	8.88
	8.52	8.94	8.04	8.24	7.71	8.99
	8.53	9.18	7.96	8.21	7.85	8.91
10	8.93	9.01	8.12	8.53	8.40	8.95
	8.72	9.21	8.40	8.36	8.88	9.18
	8.91	9.24	8.34	9.06	8.69	9.17
15	8.81	8.79	8.33	8.23	7.63	8.66
	8.81	9.11	8.28	8.34	7.71	8.69
	8.95	9.04	8.28	8.41	7.54	8.68
20	9.00	9.13	8.26	8.04	7.43	8.91
	8.94	9.12	8.22	8.15	7.58	9.47
	9.04	9.25	8.16	8.32	7.51	8.93
day	avg-log(c)	avg-log(s)	avg-log(mm)	avg-log(s4)	avg-log(s8)	lavg-log(s12)
0	7.20	7.76	7.21	6.95	6.98	7.60
5	8.54	9.09	7.99	8.14	7.78	8.92
10	8.85	9.15	8.29	8.65	8.65	9.10
15	8.85	8.98	8.30	8.33	7.63	8.68
20	9.00	9.17	8.21	8.17	7.51	9.10
day	sd-log(c)	sd-log(s)	sd-log(mm)	sd-log(s4)	sd-log(s8)	sd-log(s12)
0	0.03	0.06	0.05	0.04	0.04	0.10
5	0.04	0.13	0.04	0.15	0.07	0.06
10	0.12	0.12	0.15	0.36	0.24	0.13
15	0.08	0.17	0.03	0.09	0.08	0.01
20	0.05	0.07	0.05	0.14	0.07	0.32

Table D.8 Amount of remaining phenanthrene in 20 g non-sterile soil microcosm (300 ppm phenanthrene microcosm) by using GC analysis

day	peak area				
0	control	s	mm	s4	s8
	404.63	351.08	373.42	417.21	421.56
	376.43	337.78	353.45	403.93	417.82
5	384.27	99.34	34.00	80.35	75.66
	360.12	50.17	29.99	69.72	70.40
		28.49	0.00		60.16
10	377.34	38.65	0.00	75.83	65.95
	372.84	37.68	0.00	67.44	56.58
	357.55	33.36	0.00	40.85	55.31
15	356.42	34.81	0.00	0.00	0.00
	349.01	0.00	0.00	0.00	0.00
	344.34	0.00	0.00	0.00	0.00
20	311.71	0.00	0.00	0.00	0.00
	304.53	0.00	0.00	0.00	0.00
	302.38	0.00	0.00	0.00	0.00
day	% control	%s	%mm	%s4	%s8
0	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00
5	84.41	28.01	9.08	19.03	17.50
	89.00	14.29	8.03	16.71	16.70
		8.44	0.00		14.40
10	82.89	10.90	0.00	17.96	15.25
	92.14	10.73	0.00	16.17	13.42
	94.98	9.88	0.00	10.11	13.24
15	78.30	0.00	0.00	0.00	0.00
	86.25	0.00	0.00	0.00	0.00
	91.48	0.00	0.00	0.00	0.00
20	68.47	0.00	0.00	0.00	0.00
	75.26	0.00	0.00	0.00	0.00
	80.33	0.00	0.00	0.00	0.00
day	avg-c	avg-s	avg-mm	avg-s4	avg-s8
0	100.00	100.00	100.00	100.00	100.00
5	86.71	16.91	5.70	17.87	16.20
10	90.01	10.50	0.00	14.75	13.97
15	85.34	0.00	0.00	0.00	0.00
20	74.69	0.00	0.00	0.00	0.00
day	sd-c	sd-l	sd-mm	sd-s4	sd-s8
0	0.00	0.00	0.00	0.00	0.00
5	3.24	10.05	4.97	1.64	1.61
10	6.32	0.55	0.00	4.11	1.11
15	6.64	0.00	0.00	0.00	0.00
20	5.95	0.00	0.00	0.00	0.00

Table D.9 Amount of remaining pyrene in 20 g non-sterile soil microcosm (300 ppm phenanthrene microcosm) by using GC analysis

day	peak area				
0	control	s	mm	s4	s8
	116.26	122.38	117.56	130.20	131.86
	108.57	117.52	111.63	128.20	129.58
5	108.05	113.56	100.48	114.41	115.72
	102.40	107.75	90.70	114.01	111.06
		99.84	85.02		101.74
10	102.81	94.88	91.67	107.92	107.30
	102.40	92.72	90.94	107.36	105.39
	98.43	92.15	89.83	98.53	102.54
15	98.85	92.31	93.16	102.90	90.53
	98.46	86.91	92.46	97.66	89.44
	94.33	83.79	78.90	97.63	85.73
	86.35	80.00	87.61	91.81	90.55
	85.61	73.79	81.42	91.11	83.20
20	82.00	73.79	80.80	91.10	82.28
day	% control	%s	%mm	%s4	%s8
0	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00
	100.00	100.00	100.00	100.00	100.00
5	76.48	90.37	85.41	86.82	86.78
	94.32	88.04	77.16	87.57	84.22
		84.96	76.17		78.52
10	72.77	75.50	77.93	81.89	80.46
	88.08	75.76	77.35	82.46	79.92
	90.66	78.41	80.47	76.85	79.13
15	69.97	73.46	79.19	78.08	67.89
	84.69	71.01	78.65	75.01	67.83
	86.88	71.30	70.68	76.15	66.15
20	61.12	63.66	74.47	69.67	67.90
	73.64	60.29	69.26	69.98	63.10
	75.53	62.79	72.38	71.06	63.49
day	avg-c	avg-s	avg-mm	avg-s4	avg-s8
0	100.00	100.00	100.00	100.00	100.00
5	85.40	87.79	79.58	87.19	83.17
10	83.84	76.56	78.58	80.40	79.84
15	80.51	71.92	76.17	76.41	67.29
20	70.10	62.25	72.04	70.23	64.83
day	sd-c	sd-l	sd-mm	sd-s4	sd-s8
0	0.00	0.00	0.00	0.00	0.00
5	12.61	2.71	5.08	0.53	4.23
10	9.67	1.61	1.66	3.09	0.67
15	9.20	1.34	4.76	1.56	0.98
20	7.83	1.75	2.62	0.73	2.67

Table D.10 Amount of total bacteria in 20 g non-sterile soil microcosm (300 ppm phenanthrene microcosm) by using plate count technique

day	CFU/g soil				
0	control	s	mm	s4	s8
	6.50E+06	1.03E+07	1.74E+07	3.60E+07	1.53E+07
	2.70E+06		1.72E+07	4.20E+07	1.60E+07
5	9.70E+07	6.40E+07	3.80E+07	1.05E+08	4.80E+07
	1.76E+08	7.80E+07	4.00E+07	1.33E+08	4.60E+07
	2.07E+08	4.30E+07	4.20E+07	1.01E+08	3.50E+07
10	1.22E+09	1.36E+09	4.10E+08	8.50E+08	3.40E+08
	1.38E+09	7.70E+08	2.10E+08	4.20E+08	4.90E+08
	7.20E+08	1.05E+09	2.10E+08	8.90E+08	3.20E+08
15	9.00E+08	5.60E+08	1.48E+08	5.20E+08	8.00E+07
	9.10E+08	7.30E+08	2.15E+08	4.80E+08	9.20E+07
	8.70E+08	6.00E+08	1.90E+08	6.40E+08	6.00E+07
20	2.50E+08	2.74E+09	7.50E+07	1.06E+09	3.40E+07
	3.90E+08	2.12E+09	5.80E+07	1.01E+09	2.10E+07
	3.80E+08	2.48E+09	4.30E+07	1.20E+09	8.10E+07
day	log(c)	log(s)	log(mm)	log(s4)	log(s8)
0	6.46	7.03	7.30	7.49	7.08
	6.81	7.01	7.24	7.56	7.18
	6.43		7.24	7.62	7.20
5	7.99	7.81	7.58	8.02	7.68
	8.25	7.89	7.60	8.12	7.66
	8.32	7.63	7.62	8.00	7.54
10	9.09	9.13	8.61	8.93	8.53
	9.14	8.89	8.32	8.62	8.69
	8.86	9.02	8.32	8.95	8.51
15	8.95	8.75	8.17	8.72	7.90
	8.96	8.86	8.33	8.68	7.96
	8.94	8.78	8.28	8.81	7.78
20	8.40	9.44	7.88	9.03	7.53
	8.59	9.33	7.76	9.00	7.32
	8.58	9.39	7.63	9.08	7.91
day	avg-log(c)	avg-log(s)	avg-log(mm)	avg-log(s4)	avg-log(s8)
0	6.57	7.02	7.26	7.56	7.16
5	8.18	7.78	7.60	8.05	7.63
10	9.03	9.01	8.42	8.83	8.58
15	8.95	8.80	8.26	8.73	7.88
20	8.52	9.39	7.76	9.04	7.59
day	sd-log(c)	sd-log(s)	sd-log(mm)	sd-log(s4)	sd-log(s8)
0	0.21	0.01	0.04	0.07	0.07
5	0.17	0.13	0.02	0.06	0.07
10	0.15	0.12	0.17	0.18	0.10
15	0.01	0.06	0.08	0.06	0.09
20	0.11	0.06	0.12	0.04	0.30

Table D.11 Amount of phenanthrene degrader in 20 g non-sterile soil microcosm (300 ppm phenanthrene microcosm) by using plate count technique

day	CFU/g soil				
0	control	s	mm	s4	s8
	2.90E+06	9.90E+06	1.78E+07	8.80E+06	7.70E+06
	6.40E+06	7.50E+06	1.86E+07	1.35E+07	8.40E+06
5	8.60E+07	3.80E+07	2.40E+07	1.91E+08	2.70E+07
	1.66E+08	2.90E+07	2.70E+07	1.81E+08	2.70E+07
	1.44E+08		3.10E+07	1.10E+08	2.30E+07
10	5.50E+08	1.01E+08	4.50E+08	3.20E+08	4.30E+08
	3.70E+08	2.19E+08	2.90E+08	4.60E+08	5.00E+08
		2.87E+08	2.40E+08	4.70E+08	2.20E+08
15	8.60E+08	8.40E+08	1.62E+08	4.70E+08	6.00E+07
	6.60E+08	6.90E+08	1.88E+08	6.40E+08	3.00E+07
	1.01E+09	4.90E+08	1.92E+08	5.60E+08	4.20E+07
20	2.60E+08	2.29E+09	4.80E+07	4.20E+08	2.01E+07
	2.80E+08	1.80E+09	5.50E+07	6.60E+08	1.20E+07
	2.30E+08	1.70E+09	4.20E+07	2.80E+08	1.45E+07
day	log(c)	log(s)	log(mm)	log(s4)	log(s8)
0	6.80	6.85	7.13	7.20	6.96
	6.46	7.00	7.25	6.94	6.89
	6.81	6.88	7.27	7.13	6.92
5	7.93	7.58	7.38	8.28	7.43
	8.22	7.46	7.43	8.26	7.43
	8.16		7.49	8.04	7.36
10	8.74	8.00	8.65	8.51	8.63
	8.57	8.34	8.46	8.66	8.70
		8.46	8.38	8.67	8.34
15	8.93	8.92	8.21	8.67	7.78
	8.82	8.84	8.27	8.81	7.48
	9.00	8.69	8.28	8.75	7.62
20	8.41	9.36	7.68	8.62	7.30
	8.45	9.26	7.74	8.82	7.08
	8.36	9.23	7.62	8.45	7.16
day	avg-log(c)	avg-log(s)	avg-log(mm)	avg-log(s4)	avg-log(s8)
0	6.69	6.91	7.22	7.09	6.92
5	8.10	7.52	7.43	8.19	7.41
10	8.65	8.27	8.50	8.61	8.56
15	8.92	8.82	8.26	8.74	7.63
20	8.41	9.28	7.68	8.63	7.18
day	sd-log(c)	sd-log(s)	sd-log(mm)	sd-log(s4)	sd-log(s8)
0	0.20	0.08	0.07	0.13	0.04
5	0.15	0.08	0.06	0.13	0.04
10	0.12	0.24	0.14	0.09	0.19
15	0.09	0.12	0.04	0.07	0.15
20	0.04	0.07	0.06	0.19	0.11

BIOGRAPHY

Miss Prapaporn Deangrueng was born on August 30, 1981 in Bangkok, Thailand. She received Bachelor's Degree in Department of Microbiology from Faculty of Science, Chulalongkorn University in 2003. She pursued her Master Degree study in the Inter-Department of Environmental Management, Chulalongkorn University, Bangkok, Thailand in May 2003. She was awarded Master Degree of Science in Environmental Management in April 2005.