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ใบไม้และแบคทีเรียที่เกี่ยวข้องสำหรับการย่อยสลายสารพีเอเอชที่สะสมบนผิวใบ

นางคาเรน เวท

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PLANT LEAVES AND THEIR ASSOCIATED BACTERIA FOR THE DEGRADATION OF PAH DEPOSITED ON LEAF SURFACE

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สารโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอน (พีเอเอช) เป็นสารมลพิษหลักซึ่งเกิดขึ้นเสมอจากกระบวนการเผาไหม้ไม่สมบูรณ์ของเชื้อเพลิงจากยานยนต์และอุตสาหกรรม ความสนใจที่จะนำใบไม้มาใช้เป็นกลไกในการย่อยสลายสารพีเอเอชในบรรยากาศ มาจากความรู้ที่ว่าใบไม้สัมผัสกับบรรยากาศและมีจุลินทรีย์อาศัยอยู่บนใบจำนวนมาก สารพีเอเอชบนใบไม้โดยขบวนการตกสะสมของสารในรูปแก๊สและที่จับตัวกับฝุ่นจะมีพอที่จุลินทรีย์สามารถย่อยสลายได้ วัตถุประสงค์ของการศึกษานี้ คือการหาชนิดของไม้ประดับที่มีแบคทีเรียย่อยสลายพีเอเอชจำนวนมาก และการนำพืชและแบคทีเรียนี้ไปใช้เพื่อลดสารพีเอเอชที่ตกสะสม การศึกษาใช้ฟีแนนทรีนเป็นแบบจำลองของสารพีเอเอช พืช 6 ชนิดที่นำมาวิเคราะห์ได้แก่ *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., และ *Hymenocallis littoralis* Salisb ตัวอย่างใบไม้เก็บจากแหล่งที่มีมลพิษทางอากาศต่ำและสูงในกรุงเทพฯ จำนวนแบคทีเรียที่ย่อยสลายฟีแนนทรีนบนใบไม้มีความเข้มข้นระหว่าง $3.5x10^4$ และ $1.95x10^7$ ซีเอฟยู/กรัม ผลการทดลองไม่พบความสัมพันธ์ที่มีนัยสำคัญระหว่างจำนวนแบคทีเรีย และสถานที่ ความชื้น หรือขี้ผึ้งบนใบไม้ การสร้างโคโลนีของแบคทีเรียที่ย่อยสลายฟีแนนทรีนจึงขึ้นอยู่กับลักษณะอื่นๆ ของพืช จากพืชที่นำมาทดสอบ 6 ชนิด *Ixora* sp. มีความเข้มข้นของแบคทีเรียที่ย่อยสลายฟีแนนทรีนมากที่สุด คือเฉลี่ย $9.7x10^6$ ซีเอฟยู/กรัม ต่อมาได้ทำการศึกษาในห้องปฏิบัติการโดยใช้ *Ixora* sp. เพื่อหาอัตราการย่อยสลายฟีแนนทรีนโดยแบคทีเรียท้องถิ่นและ *Sphingomonas* sp. P2 ที่เติม ทั้งนี้ *Sphingomonas* sp. P2 เป็นแบคทีเรียที่สามารถย่อยสลายสารพีเอเอชได้ ผลการทดลองแสดงให้เห็นว่าแบคทีเรียที่เกี่ยวข้องกับใบสามารถลดปริมาณฟีแนนทรีนสะสมบนใบอย่างมีประสิทธิภาพ นอกจากนี้การเติม *Sphingomonas* sp. P2 สามารถเพิ่มอัตราการย่อยสลายฟีแนนทรีน และพบการเพิ่มจำนวนของแบคทีเรียย่อยสลายฟีแนนทรีนอย่างรวดเร็วบนใบที่เติมแบคทีเรียด้วย อย่างไรก็ดีควรศึกษาการอยู่รอดของแบคทีเรียที่เติมต่อไป การศึกษานี้ยังได้แยกแบคทีเรียท้องถิ่นของพืช 4 ชนิด พบว่าแบคทีเรียเหล่านี้สามารถย่อยสลายฟีแนนทรีนในอาหารเลี้ยงเชื้อเหลวที่ความเข้มข้น 100 พีพีเอ็ม อย่างสมบูรณ์ภายในระยะเวลา 9 วัน ท้ายที่สุดยังได้ใช้วิธีวิเคราะห์ลำดับของยีน 16S rDNA จำแนกชนิดของแบคทีเรียท้องถิ่นที่สามารถย่อยสลายฟีแนนทรีนชนิดหนึ่ง พบว่าคือ *Deinococcus* sp การประยุกต์ใช้ *Ixora* sp. และแบคทีเรียที่เกี่ยวข้องเพื่อลดปริมาณสารพีเอเอชในบรรยากาศ เป็นวิธีทางธรรมชาติและเป็นมิตรต่อสิ่งแวดล้อม

สหสาขาวิชา การจัดการสิ่งแวดล้อม (สหสาขาวิชา)
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ลายมือชื่อนิสิต.....Karen Waight.....
ลายชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD : PAH/ BIODEGRADATION/ PLANT/ AIR POLLUTANT

KAREN WAIGHT: PLANT LEAVES AND THEIR ASSOCIATED BACTERIA FOR THE DEGRADATION OF PAH DEPOSITED ON LEAF SURFACE. THESIS ADVISOR: EKAWAN LUEPROMCHAI, Ph.D., THESIS CO-ADVISOR: ONRUTHAI PINYAKONG, Ph.D. 107pp. ISBN 974-14-1956-2

Polycyclic aromatic hydrocarbons (PAHs) are priority pollutants that continuously generated by the incomplete combustion of organic fuels from traffic and industrial processes. Interest in using plant leaves as a mechanism for biodegradation of atmospheric PAHs is motivated by the fact that leaves are exposed to the atmosphere and microbes are the most abundant inhabitants of the phyllosphere. In leaves, PAHs become potentially available to microbial degradation as it accumulates on plants surfaces by gaseous and particle-bound deposition. The objectives of this study were to identify an ornamental plant that contained high number of PAH-degrading bacteria and to apply this plant and its associated bacteria to reduce the deposited PAHs. The study used phenanthrene as a modeled PAH. Six plant species were analyzed; namely *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. Their leaf samples were collected from two locations in Bangkok that had high and low amounts of air pollutants. The concentrations of phenanthrene-degrading bacteria on leaves were ranged from $3.5x10^4$ to $1.95x10^7$ CFU/g. There was no significant relationship between the number of bacteria and location, moisture content, or wax content of the leaves and thus colonization of phenanthrene-degrading bacteria probably depended on other plant characteristics. From the six species of plants screened, the leaves of the *Ixora* sp. showed the highest concentration of phenanthrene-degrading bacteria. Further laboratory studies were carried out using *Ixora* sp. to determine the rate of phenanthrene degradation by indigenous bacteria and added *Sphingomonas* sp. P2, a known PAH-degrader. The results indicated that the leaf associated bacteria effectively reduced the amount of phenanthrene that accumulated on the leaves. In addition, the augmentation with *Sphingomonas* sp. P2 increased the rate of phenanthrene degradation. This was detected along with a rapid increase in number of phenanthrene-degrading bacteria on the augmented leaves. The survival of the augmented bacteria on leaves, however, requires further investigation. The study also isolated four leaf indigenous bacteria and found that they could completely degrade 100 ppm of phenanthrene in liquid medium within 9 days. Finally, using 16S rDNA gene sequencing, one of the indigenous bacteria was identified as *Deinococcus* sp. The application of *Ixora* sp. and their associated bacteria would be a natural and environmentally friendly approach to reduce the amount of atmospheric PAHs.

Field of study Environmental Management (Inter-Department)
Academic Year 2005

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Advisor's signature...Ekawan L.
Co-advisor's signature...Onruthai Pinyakong

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LIST OF ABBREVIATIONS AND SYMBOLS

PAHs	=	Polycyclic aromatic hydrocarbons
LMW	=	Low molecular weight
HMW	=	High Molecular weight
ATSDR	=	Agency for toxic substance and disease registry
WHO	=	World Health Organization
MOSTE	=	Ministry of Science Technology and Environment
OEPP	=	Office of Environmental Policy and Planning
PCD	=	Pollution Control Department
EPA	=	Environmental Protection Agency
PCR	=	Polymerase chain reaction
DNA	=	Dioxyribonucleic acid
rDNA	=	ribosomal Dioxyribonucleic acid
PM	=	particulate matter
HPLC	=	high performance liquid chromatography
GC	=	Gas Chromatography
GC-MS	=	Gas Chromatography Mass Spectrum
ng/m^3	=	nanogram per cubic meter
CFU/g	=	Colony forming units/gram

CHAPTER I

INTRODUCTION

1.1 Statement of problem

There is a growing concern about the quality of air resulting from industrialization. Smoke, haze, dust, odors, corrosive gasses, noise, and toxic compounds are present nearly everywhere. As a country become more industrialized, air pollution is opt to increase bringing along with it obvious environmental damage. According to the Environmental Protection Agency (EPA), total worldwide emissions of pollutants are around two billion metric tons per year (Cunningham and Santiago, 1997).

Air pollutants can be gaseous, liquid or solid in form, and can come from natural as well as human sources. One group of pollutants produced as a result of increase in industrialization is polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons (PAHs) are a group of hazardous organic compounds consisting of two or more benzene rings interlinked into various arrangements. These pollutants are of critical health and environmental concerned due to their chronic health effects, carcinogenicity, microbial agitation and high bioaccumulation potential (Hutzinger, 1980). Although low molecular weight (LMW) PAHs are not carcinogenic in experimental animals, they are genotoxic to mammalian DNA (Mastrangela et al., 1996). On the other hand, high molecular weight (HMW) PAHs has been suggested to be carcinogens (Mastrangela et

al., 1996). Many PAHs are therefore considered to be priority pollutants by the United States Protection Agency (EPA) and by the European Community (Keith and Telliard, 1979).

Atmospheric PAHs are transported over relatively long distances from industrial areas; however, sites nearer urban areas have much higher PAHs deposition rates than rural areas. Emission from human activities such as vehicle exhausts and increase emission from industries are the predominant sources in urban areas (Garivait, 1999). Garivait et al. (2002) studied the average airborne PAHs concentration in Bangkok urban area, and found that total PAHs concentrations were 19.48 ng/m^3 at the Office of Environmental Policy and Planning, which is a residential and commercial area. While at the Ministry of Science Technology and Environment, a site with heavy traffic volume (55,000vehicles/day) located under an expressway, the air was found to contain total PAHs concentrations of 42.95 ng/m^3.

Plant leaves serve as sinks of atmospheric PAHs and sources for long-term air contamination. Airborne compounds in the form of gas and/or particle PAHs with high molecular weight (>218) are mostly found absorbed to dust particles while those with lower molecular weight are in the gaseous phase (Jongeneelen, 1997). PAHs may be transferred from the deposited particles to the cuticle. Karnchanasest and Satayavibul(2005) monitored the amount of atmospheric PAHs in orange jasmine leaves, *Murraya paniculata* (L.) Jack collected from Bangkok roadsides in 2002. They found that the total PAHs in the areas studied ranged from 63.99 mg/kg to 82.46 mg/kg leaves and

concluded that the characteristic of orange jasmine leaves could support the absorption of atmospheric PAHs along the roadsides because they had thick lipid layer. The Pollution Control Department (PCD) of Thailand daily reports of total particulate matter (PM10) in the atmosphere also indicates a high level of pollutants in certain areas of the city. It is thus important, that biodegradation of deposited air pollutants should be investigated and applied for reduction of atmospheric air pollution in a safe and sustainable way.

Biodegradation, which involves the use of microorganisms to degrade and detoxify hazardous organic compounds to harmless substances, is thought to be a major PAHs decomposition process (Cerniglia et al., 1992). PAHs with up to four aromatic rings are biodegradable under aerobic conditions by bacteria and fungi but the biodegradation rate of PAHs with higher number of aromatic rings is very slow (WHO, 1998). Biodegradation could thus be a major pathway for the reduction of PAHs accumulated on leaves. The application of using leaves and associated bacteria for reducing air pollutant was first carried out by De Kempeneer et al. (1998). They studied the removal of airborne toluene by *Azalea indica* augmented with toluene-degrading bacteria, *Pseudomonas putida* TVA8. Meanwhile, the degradation of PAHs deposited on leaves has never been studied.

The interest of plant leaves as a possible method for the reduction of atmospheric PAHs is motivated by the fact that plants cover a significant proportion of the global land area, plant leaves are exposed to the atmosphere, and leaves are inhabited by a qualitative and quantitative diversity of microorganisms, with bacteria being by far the most

numerous inhabitants of the leaves. In this study, phenanthrene was used as a modeled PAHs. Phenanthrene is included in EPA's 16 priority PAHs; and is a good model to use for study on microbial degradation because it has a structure that is shared with several carcinogenic PAHs. The application of plant leaves and bacteria for removal of organic air pollutants deposited on plants could improve environmental quality in an economic, safe and sustainable way.

1.2 Objectives

The main objectives of this study were to identify an ornamental plant that contained high number of PAH degrading bacteria and to apply this plant and its associated bacteria to reduce PAHs deposited on plant leaves.

Sub-Objectives

The sub-objectives of this study were:

(1) To characterize plants in terms of wax content, moisture content and concentration of phenanthrene-degrading bacteria on their leaves.

(2) To investigate phenanthrene-degradation on a selected plant by leaf indigenous bacteria and augmented *Sphingomonas* sp. P2.

(3) To verify the ability of the plant associated bacteria to degrade phenanthrene and identify at least one species of these bacteria found on the ornamental plant used in this study.

1.3 Hypothesis

Bacteria inhabited on leave depend on plant species and may be responsible for the degradation of deposited PAHs on plant leaves.

1.4 Scope of Study

This experiment was conducted in 3 phases to investigate the potential of a selected plant species and associated bacteria to reduce PAHs deposited on leaf surface. Phenanthrene was used as a model PAHs compound since it is not only one of the 16 priority pollutants classified by the US-EPA but it also share the same structural characteristics as other carcinogenic PAHs. In phase 1, six species of ornamental plants common to two locations were selected at random and characterized in terms of wax content, moisture content and concentration of phenanthrene-degrading bacteria on their leaves. One plant species was chosen for further studies in phase 2 and 3. The criteria for selecting the plant species for phase 2 were:

(1) Highest concentration of phenanthrene-degrading bacteria.

(2) Abilities to grow easily, withstand various changes in temperature, and grow all year round.

(3) Inexpensive cost.

In Phase 2, the rate of phenanthrene degradation was monitored using the entire plant and on incubated leaves to confirm that the selected plant along with its associated

bacteria have the potential to degrade phenanthrene. This phase will also investigate the effect of bioaugmentation of a known PAHs degrader on the rate of PAHs degradation.

In phase 3, several phenanthrene-degrading bacteria from phase 2 were isolated and their ability to degrade phenanthrene in liquid medium was investigated. Sequencing analysis of 16S rDNA was used to identify one of the indigenous species.

CHAPTER II

LITERATURE REVIEW

2.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a group of hazardous organic chemicals consisting of two or more benzene rings interlinked in various arrangements. These compounds are of critical public health and environmental concern due to their toxicity, carcinogenicity, mutagenicity, microbial agitation and high bioaccumulation potential (Hutzinger, 1980). The United States EPA has identified 16 PAHs as priority pollutants (Table 2.1). Naturally, PAHs do not occur as a single compound but are found in mixtures. As pure chemicals, PAHs generally exist as colorless, white, or pale yellow-green solid. They are airborne and in the form of gas and/or particle.

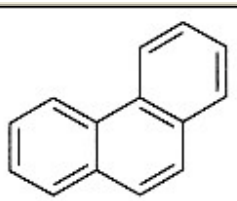
Properties of PAHs are governed by the size (number of carbon atoms) and shape (ring linkage pattern) of the individual molecule (Neff, 1979). All completely unsaturated PAHs are solid at room temperature and have relatively high melting and boiling points. PAHs are soluble in lipid, and are essentially insoluble in aqueous systems. The aqueous solubility decreases with increasing molecular size. Vapor pressures for PAHs are low and decrease with increasing molecular size. The physiochemical properties of phenanthrene – the model PAH compound in this study is provided in Table 2.2.

Table 2.1 United States EPA16 PAH priority pollutants

PAH	**Molecular Weight**	**Formula**
Napthalene	128	$C_{10}H_8$
Acenapthylene	152	$C_{12}H_8$
Acenapthene	154	$C_{12}H_{10}$
Fluorene	167	$C_{13}H_9$
Anthracene	178	$C_{14}H_{10}$
Phenanthrene	178	$C_{14}H_{10}$
Fluoranthene	202	$C_{16}H_{10}$
Pyrene	202	$C_{16}H_{10}$
Benz[a] anthracene	228	$C_{18}H_{12}$
Chrysene	228	$C_{18}H_{12}$
Benzo[b]fluoranthene	252	$C_{20}H_{12}$
Benzo[k]fluoranthene	252	$C_{20}H_{12}$
Benzo[a]pyrene	252	$C_{20}H_{12}$
Dibenz[ah]anthracene	278	$C_{22}H_{14}$
Indeno[1,2,3-cd]pyrene	276	$C_{22}H_{12}$
Benzo[ghi]perylene	276	$C_{22}H_{12}$

Source: ATSDR, 1990

Table 2.2 Relevant properties of phenanthrene, the model PAH used in this study

Properties of phenanthrene	
Molecular Weight (g/mole)	178.22
Melting Point (°C)	100
Boiling Point (°C)	340
Water Solubility	0.423 ppm @ 8.5°C 0.816 ppm @ 21°C 1.227 ppm @ 30°C neutral pH
Vapor Pressure (mmHg)	6.8×10^{-4} @ 20-30°C
Henry's Law Constant (atm*m/mol)	1.59×10^{-4} @ 20-30°C
Log Kow	4.46

Source: http://www.glue.umd.edu/~eseagren/chem.htm

Fate and transport of PAHs in the environment are determined by their characteristics. PAHs, especially those of high molecular weight, enter the environment via the atmosphere as they are absorbed onto particulate matter (WHO, 1998). Within the organic carbon component, PAHs are prevalent constituents of atmospheric pollution. Due to their low natural emission, PAHs are good tracers of anthropogenic sources of

input to the atmosphere. They are mainly formed during incomplete combustion or pyrolysis of organic materials, including oil derivatives, coals, natural gas, biomass and household wastes. In addition, because of their potential carcinogenic effects, PAHs are now regarded as priority pollutants by both the United States EPA and the European Environment Agency (Marchand et al., 2004).

2.2 PAHs deposition on Plants

Plants serve as sinks of atmospheric PAHs leading to its accumulation. Airborne compounds in the form of gas and/or particle PAHs with high molecular weight (>218) are mostly found absorbed to dust particles while those with lower molecular weight are in the gaseous phase (Jongeneelen, 1997). PAHs may be transferred from the deposited particles to the cuticle. Larsson and Sahlberg 1992 cited in Bakker, (2000) studied the deposition of PAHs on leaf cuticle with wash-off experiments. They found that washing of lettuce with water removed a considerable amount of high molecular weight PAHs, but a small amount of the low molecular weight PAHs such as phenanthrene, indicating that that only the gaseous PAHs was absorbed in the leaf cuticle. In contrast, Kaupp (1996) cited in Bakker (2000) found that rinsing maize leaves with aqueous solutions could only extract a minor part of the high molecular weight PAHs, suggesting that the compounds were desorbed from the particles or that the particles were encapsulated in the cuticle.

The deposition of PAHs on plants may occur via several pathways. PAHs compounds bound to atmospheric particles can reach the plant surface by both dry and

wet deposition (Nakajima et al. 1995, Kaupp 1996, cited in Bakker, 2000). This pathway involves transportation from the atmosphere to laminar air boundary layer surrounding the leaf, crossing of the boundary layer and interactions of the molecule with leaf surface (Schreiber et al., 1992). The uptake of gaseous PAHs involves passive diffusion between the atmosphere and plant cuticle. Deposition process of airborne compounds, whether gaseous or particle bound, from the atmosphere to a plant leaf involves three steps as shown in Figure 2.1.

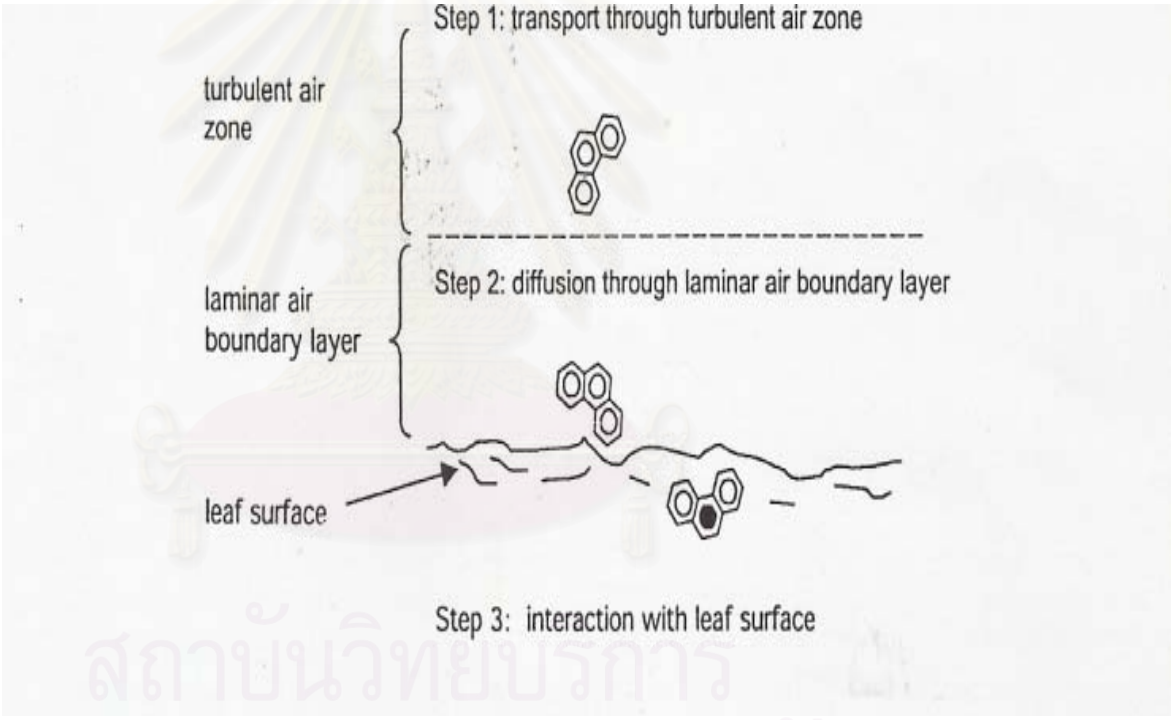


Figure 2.1 Three-step deposition processes of hydrophobic organic compounds from the atmosphere to a leaf.

Source: Schreiber et al., (1992) cited in Bakker, (2000)

2.3 Degradation of PAHs

2.3.1 Photo-degradation

PAHs can be broken down by reacting with sunlight and other chemicals in the air, during a period of days or weeks. PAHs are photo degraded in air by two processes: direct photolysis by light with a wavelength <290 nm and indirect photolysis by at least one oxidizing agent such as OH^-, O_3 and NO_3 in the air. Under environmental conditions, PAHs of higher molecular mass are almost completely absorbed onto fine particles; this reduces the degradation rate markedly (WHO, 1998).

2.3.2 Phytoremediation

Phytoremediation is an emerging technology involving the use of vegetation for in-situ treatment of contaminated soils and sediments. Anderson et al. (1993) demonstrated the importance of bioremediation in the rhizosphere and found out that plants provide habitat for microbial populations and pump oxygen to the roots. Plants remediate organic pollutants via three mechanisms: direct uptake of contaminants and subsequent accumulation of nonphytotoxic metabolites into plant tissue; release of enzymes that stimulate microbial activity and biochemical transformation, and enhancement of mineralization in the rhizophere (Figure 2.2). The combination of plants and microbes proves to be an effective remediation approach. Various types of phytoremediation can be used for soilbased organic contaminants. Phytoextraction for example, could be used

to target moderately hydrophobic organics, such as chlorinated solvents. The contaminants may then be stored in plant biomass or, in some cases, volatilized. One form of phytodegradation involves uptake of organic contaminants and degradation through metabolic processes within the plant. Another form of phytodegradation is rhizodegradation, in which organic contaminants in soil (such as TNT, chlorinated solvents, and petroleum hydrocarbons) are degraded by plant-root and/or soil microbes within the plant's root zone. Some organic contaminants may be degraded because of enzymes, sugars, alcohols, and acids released by plant roots. Other organic contaminants may be affected by soil microbes that are stimulated by various root exudates and/or the oxygen and organic carbon supplied by root systems (Schnoor et al., 1995).

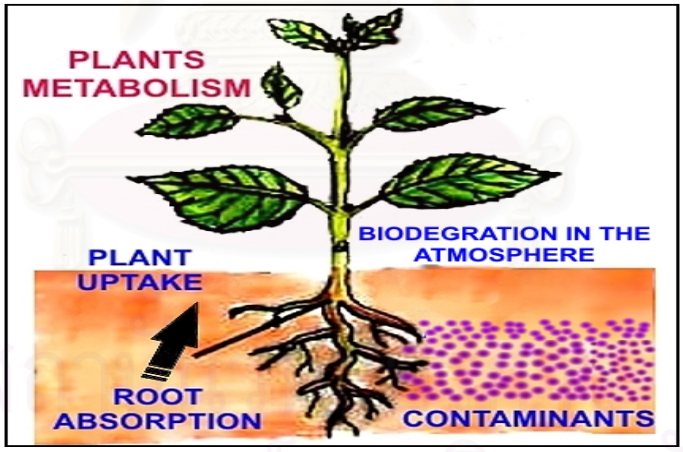


Figure 2.2 Plants mechanisms for the remediation of organic pollutants

Source: http://www.area.pi.cnr.it/ICT/progetti/phyto.htm

2.3.3 Microbial degradation of PAHs

PAHs can be biodegraded or biotransformed by microorganisms such as yeast, fungi and bacteria. Bacterial degradation of PAHs is carried out by a number of strains belonging to a limited number of taxonomy such as *Sphingomonas, Burkolderia, Pseudomonas* and *Mycobacterium* (Kastner et al., 1994). Recently, Supaka et al. (2001) isolated *Sphingomonas* sp. strain P2, which is able to utilize phenanthrene as a sole carbon source and energy from the lubricant contaminated soil sample collected from a garage in Prajinburi province, Thailand. The bacterium could rapidly degrade phenanthrene in liquid medium from 100 ppm to undectectable 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. In this study, the bacterium will be applied on leaves to enhance atmospheric PAHs degradation.

2.3.4 Biodegradation mechanisms for two, three, and four ring PAHs

PAHs with up to four aromatic rings are biodegradable under aerobic conditions. The biodegradation rate of PAHs with higher number of aromatic rings is however, very slow (WHO, 1998). In an aerobic condition, bacteria generally degrade PAHs through two predominant pathways (Figure 2.3). Prior to degradation, PAHs are first taken up by

the bacterial cells. The degradation of PAHs is initiated by the action of intracellular dioxygenase. Bacteria most often oxidize PAHs to *cis*-dihydriols by incorporation of both atoms of an oxygen molecule. The *c*is-dihydriols are futher oxidized to form catechols-like compounds which are then channel through the ortho- or meta- cleavage pathways to form tri-carboxylic cycle intermediates (Cerniglia, 1984; Smith, 1990).

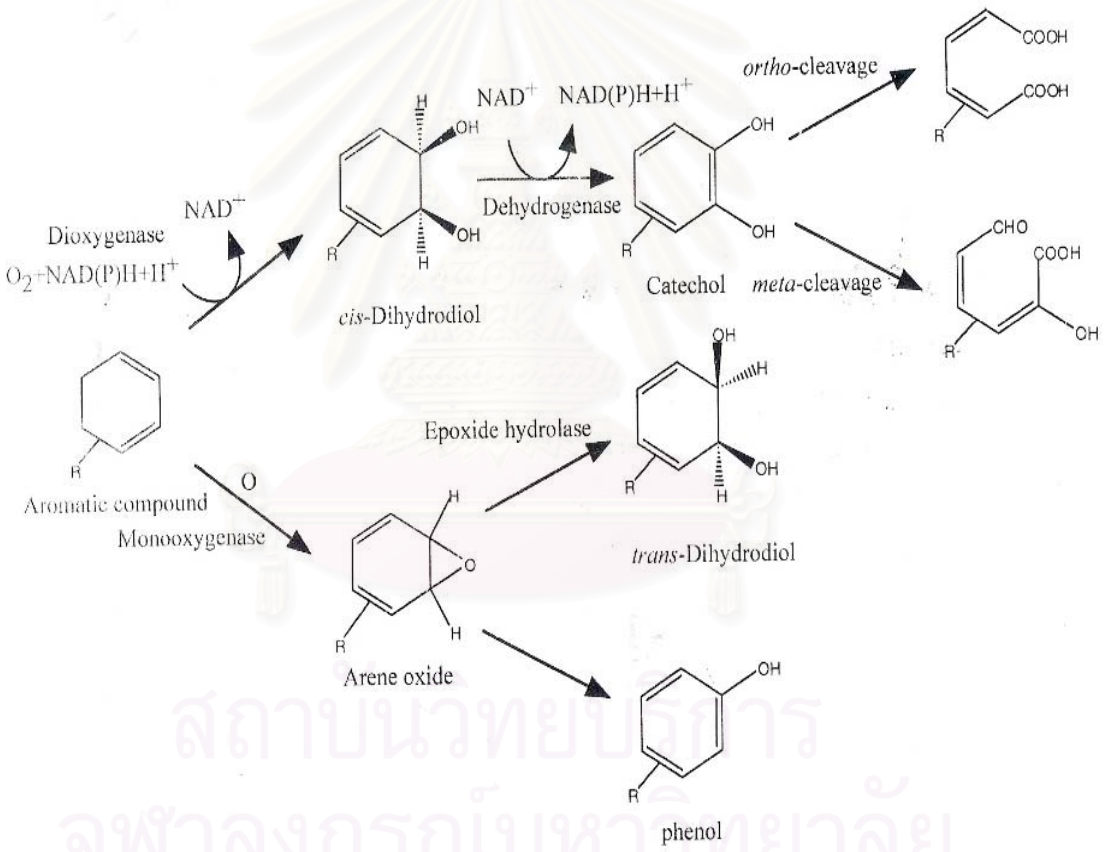


Figure 2.3 General pathways for degradation of PAHs,
Source: Pinyakong (2003)

2.3.5 Metabolism of phenanthrene

Several pathways have been proposed for the degradation of phenanthrene by bacteria. Bacteria such as *Pseudomonads* metabolize phenanthrene via initial dioxygenation at 3,4-position and further metabolize through several steps, which are similar to those of naphthalene (Evan et al., 1965). *Beijerinkia sp.* B-836 [later was reclassified as *Sphingomonas*] and *Pseudomonas putida* 119 can attack phenanthrene by ring-hydroxylating dioxygenase at 1,2 position as a minor pathway(Jerina et al., 1976). Another pathway for the degradation of phenanthrene is by *Mycobacterium sp.* Strain PYR-1, (Figure 2.4). In this pathway, *cis*- and trans-9, 10-dihydrophenanthrenes and *cis*-3, 4-dihydroxy-3, 4-dihydrophenanthrene, 2, 2-diphenic acid, 1-hydroxynaphthoic acid and phthalic acid were identified as products, indicating novel pathways started by the action of both mono and dioxygenation at 9, 10-position (Moody et al., 2001).

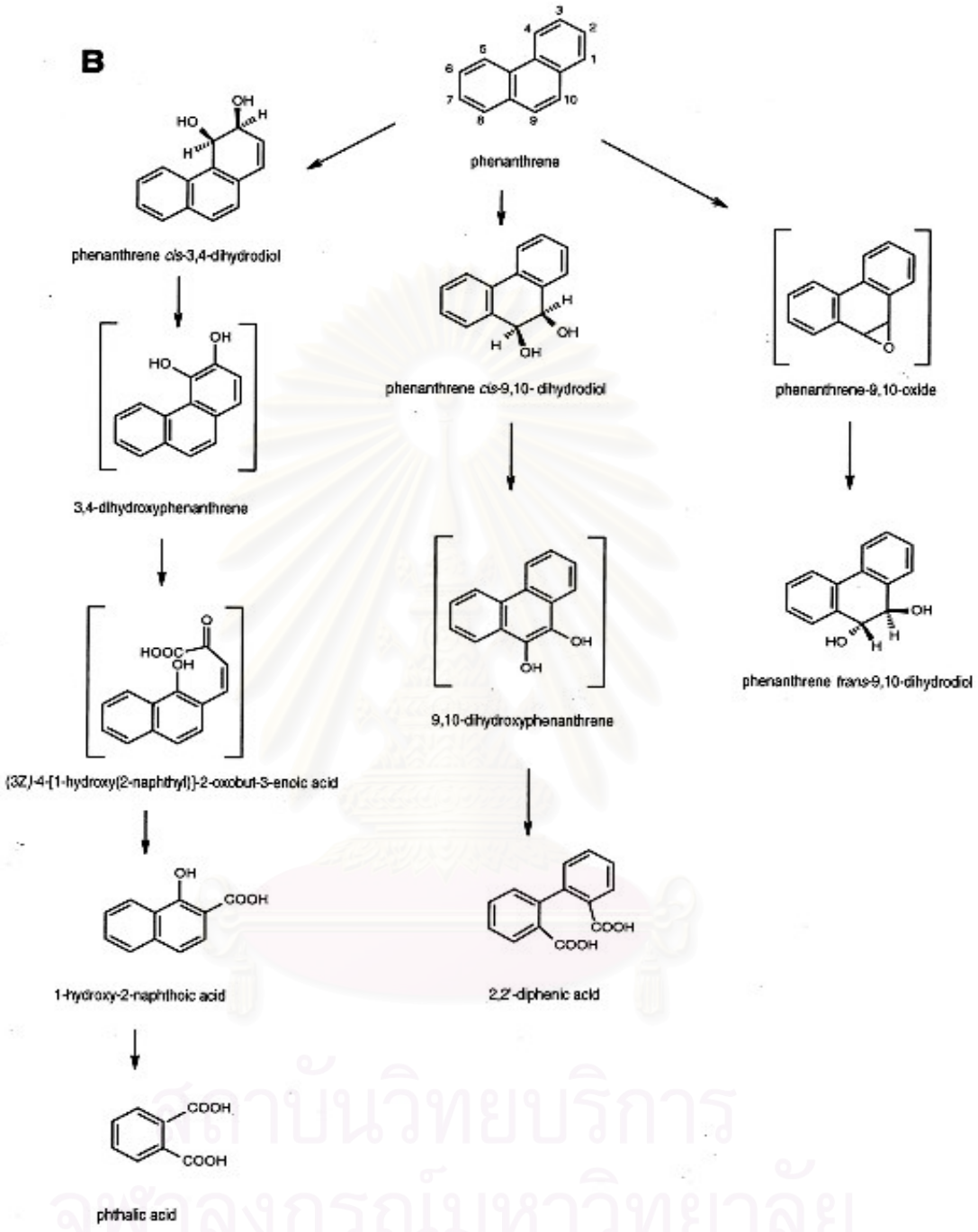


Figure 2.4 Proposed pathways for the degradation of phenanthrene by Mycobacterium sp. strain PYR-1.

Source: Moody et al., (2001)

2.4 Plant Leaves

2.4.1 Structure of leaves

Plant leaves are composed of three layers: epidermis, mesophyll, and veins (Figure 2.5). Epidermis is layer of specialized flattened cells which functions in protection. Situated on top of the epidermis is waxy cuticle which prevents water loss. Mesophyll layer made up of palisade and spongy mesophyll cells is where most photosynthetic cells are found. Palisade layer is the primary site of photosynthesis however, photosynthesis also occurs in the spongy layer. Large spaces between the spongy cells allow diffusion of carbon dioxide. Veins contain vascular tissue: xylem and phloem. Xylem transport water and minerals from roots while phloem moves the products of photosynthesis throughout the plant.

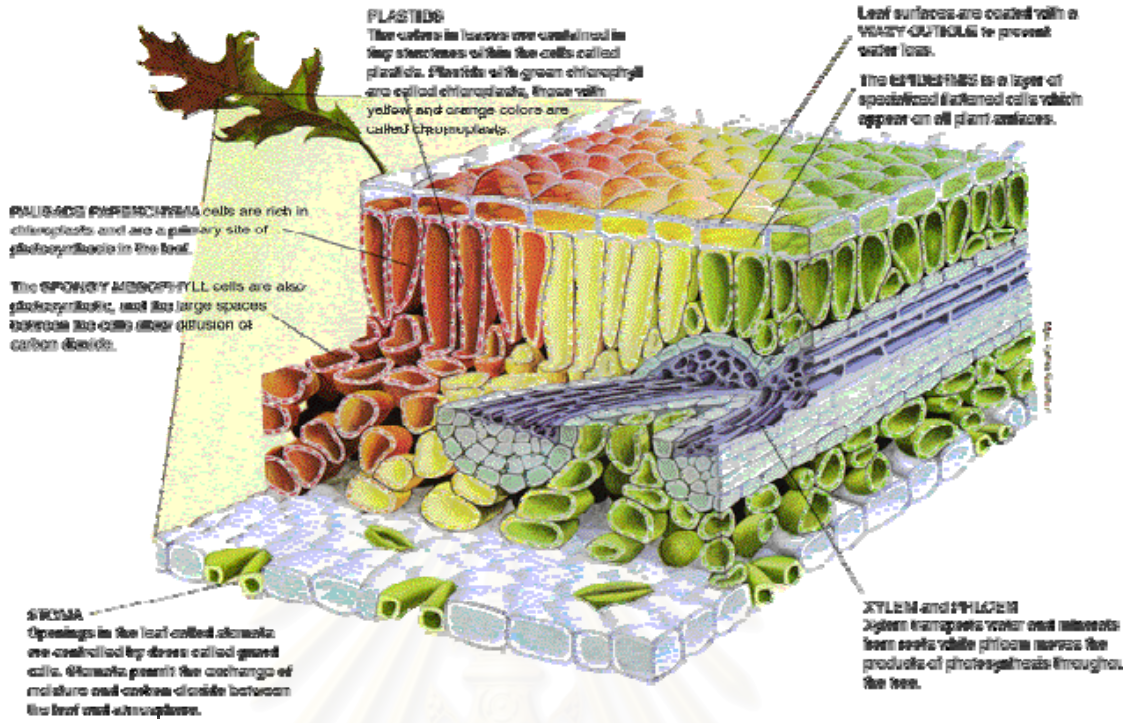


Figure 2.5 Structure of leaf.

Source: Conservation Commission of Missouri;

http://mdc.mo.gov/nathis/seasons/fall/lefstruc/

2.4.2 PAH and plant leaves relationship

Atmospheric Semivolatile organic compounds can be deposited to plants by dry gaseous or wet and dry particle-bound deposition. Once deposited to the leaves or stems of the plant, the compound can be transported through the cuticle layer (Bakker, 2000). Slaski et al. (2000) wrote in their report on the evaluation of PAH accumulated in plants, “Numerous studies have shown that leaves are the main sinks of airborne PAH compounds in plants. The process of accumulation is however affected by several abiotic

and biotic factors, including vapor-particulate portioning in the atmosphere, ambient temperature, leaf surface area and lipid concentration in plant tissues. Compounds of intermediate volatility are mainly subject to dry gaseous deposition while nonvolatile substances are primarily accumulated on the surfaces in the form of dry, particle-bound deposits. The most important anatomical feature of plants that affect the rate of interception and accumulation of airborne PAHs is total leaf area. Volatile PAHs enters plants primarily through gaseous diffusion via open stomata, although absorption by the waxy leaf surface accounts for a portion of total PAHs in tissues. The waxy surface of leaves intercepts both particulate and vapor phase contaminants".

Concentration effects, i.e. higher PAHs concentrations per gram plant weight, occur at lower temperatures when sorption of organic air pollutants on plant surfaces prevails and plant growth is limited. Higher lipid contents of plants in the winter might also favour accumulation of lipophilic air pollutants. At higher temperatures revolatilisation of the low molecular PAH takes place and plant growth leads to a dilution of xenobiotics (Nakajima et al., 1995).

In regards to leaf texture, Bakker, (2000) studied the concentrations of PAHs in leaf wax of three *Plantago* species. They found that sparsely hairy *Plantago lanceolata* leaves were more heavily contaminated with low molecular weight (MW<228) than the densely hairy, partly overlapping *Plantago* leaves. This may be caused by high aerodynamic resistance, high amount of leaf hairs and/or the higher leaf overlap.

It has been shown that plants growing on PAH-contaminated soils may contain PAHs in their tissues (Edwards et al., 1982; Wild and Jones, 1992; Kipopoulou et al., 1999) which may originate from volatile compound absorption by leaves in the surrounding air, deposition of contaminated soil particles (splash), and dust on leaves, followed by retention in cuticle or penetration through it, and soil-to-root transfer followed by subsequent translocation by the transpiration stream. The soil-to-root transfer of PAHs has been demonstrated from hydroponic experiments (Edwards et al., 1982) and spiked soils (Edwards et al., 1982; Edwards, 1988). However, the contribution of root uptake to PAH content in plants remains unclear.

2.4.3 Enzymatic Degradation and Mineralization using plants

Extracellular Enzymes in the Rhizosphere may also contribute to the degradation of PAHs or other pollutants by plants. Studies have shown that the substrate ranges of all the enzymes may be extended with redox mediators enabling potential exploitation in the degradation of xenobiotics. Mn-peroxidase and lignin peroxidase use chelated Mn^{2+} and veratryl alcohol respectively to catalyse the degradation of lignin, and aromatic xenobiotics (phenanthrene, fluorine, pyrene, benzo(a)pyrene) by hydroxylatio n, oxidation and cleavage of aromatic ring structures (Harvey and Thurston, 2001).

Poplar trees for example, have been found to degrade TCE. Research by Newman et al. (1997) suggests that TCE metabolism in poplars may be similar to the mammalian breakdown of TCE. According to a review by Cunningham et al. (1996),

many of the enzyme systems involved in mammalian metabolism of TCE are also found in plants (e.g., cytochrome p- 450 oxygenases and glutathione S-transferases), so this hypothesis seems possible. Another line of research indicates that TCE metabolism in poplars is the result of a dehalogenase enzyme (Schnoor at al. 1995). Dehalogenase is an ethylene degrading enzyme which oxidizes alkanes, alkenes, and methanes and their halogenated analogues. Dehalogenase will ultimately mineralize TCE to CO_2 via an oxidative pathway. In the case of poplars, some species or hybrids can produce more of this enzyme than others, and some may not produce the enzyme at all. As a result, using different hybrids or species at various sites without assaying for the dehalogenase may yield contrasting results. A tree that manufactures large quantities of the enzyme will have the ability to degrade TCE, while plants that produce little or no quantities of the enzyme will tend to volatilize it.

2.4.4 Using plant leaves to monitor atmospheric PAHs

Thomas (1984) studied the accumulation of PAHs, chlorinated hydrocarbons and trace metal in moss (*Hypnum cupressiforme*) in Europe and found that mosses were suitable for monitoring chlorinated hydrocarbon, BHC and PAHs. The results showed a clear PAH concentration gradient in mosses, which was high in the industrial centers in middle Europe and low in northern Europe.

In Thailand, Karnchanasest and Satayavibul (2005) studied the sorption of atmospheric PAHs in Orange Jasmine leaves, *Murraya paniculata* (L.) Jack and the

potential of these leaves as an indicator of atmospheric PAHs. The results indicated that atmospheric and leaf PAHs concentrations were related as indicated by correlation coefficient (r^2)>0.70, particularly with low molecular weight PAHs i.e. acenaphthene, acenapthylene, anthracene, fluorene and phenanthrene. This was because low molecular weight PAHs were mostly present in gas phase, which played a major role in leaf sorption. *Murraya paniculata* (L.) Jack collected from Bangkok roadsides in 2002 showed total PAHs ranging from 63.99 to 82.46 mg/kg leaves.

2.5 Microbial community of plant leaves

Microbial communities of leaves are diverse and include many different genera of bacteria, fungi, yeast, algae, and less frequently, protozoa and nematodes. Bacteria however, are by far the most numerous colonists of leaves, often being found in numbers averaging up to 10^8 cells/g (Andrews et al., 2000). Bacteria colonization however, depends on leaf properties such as nutrient availability, texture, arrangement, leaf area and climatic conditions (Bakker, 2000). Since, the first contact between immigrating bacteria and a leaf normally occurs at the plant cuticle, the quantity of cuticle (i.e. wax) is one factor that determines its colonization by microbes. Bacteria can wet waxy cuticles and decrease the surface tension of water via their outer walls and produce biosurfactants which assists bacteria to establish on leaf surfaces (Schreiber and Schonherr, 1992). Following establishment, bacteria will directly penetrate cuticles to gain access to nutrients.

Bacterial populations associated with aerial plant leaves are composed of populations that differ in the nature of their association with the leaf surface, as well as in their location relative to the leaf surface. For example, some cells may be adhered to the leaf surface, while others are not, and some may be located in endophytic sites, such as in the substomatal cavities or in the spaces between the mesophyll cells. These distinct populations may differ in their ability to grow and survive on leaves due to differences in their exposure to removal forces, such as wind and rain; to environmental stresses, such as desiccation and solar radiation; and to nutrients, which may differ in endophytic versus surface sites (Beattie, 2002).

A study by Kinkel et al. (1997) showed that the total number of culturable bacteria recovered from broad leaf plants such as cucumber and bean was significantly greater than that recovered from grasses or waxy broad leaf plants. This suggested that plant species appeared to influence the microbial carrying capacity of the leaf.

Mercier et al., (2000) studied the relationship between nutrients leached onto leaf surface and bacteria colonization by measuring both the abundance of simple sugars and the growth of *Pseudomonas fluorescens* on individual bean leaves. Under environmentally favorable conditions, population size of epiphytic bacteria on plants is limited by the abundance of carbon sources on the leaf surface. Sugars were depleted during the course of bacterial colonization of the leaf surface. The amounts of sugars on leaves of different plant species were directly correlated with the maximum bacterial population sizes that could be attained on those species.

With regards to climatic conditions, microbial community diversity is lowest during the warmest and driest months highest during the cooler and rainy months (Thompson et al., 1993).

It can thus be concluded that the diverse microbial community of the leaf is dependent on both plants and bacteria characteristics as well as abiotic factors such as ambient air temperature and vapor-particulate portioning in the atmosphere.

2.6 Removal of air pollutants

2.6.1 Air pollution control

The earliest methods employed for controlling air pollution was to move factories and other major pollution sources to remote areas, away from residential areas. Today, a variety of other methods, mostly technological, are also employed.

Depending on the types of pollutant, different emission control technology has been developed. For example, particulates are removed by filters of various types such as electrostatic precipitators and wet scrubbers. For the removal of sulfur oxides several methods including fuel switching, fuel cleaning, fluidized bed combustion, flue gas desulphurization scrubbers, and sulfur recovery are available. Nitrogen oxides may be reduced by fuel switching, staged burners, stratified-charge engines, and catalytic

converters. Finally hydrocarbons may be reduced by catalytic converters, fuel switching or PCV systems (Cunningham and Santiago, 1997).

Air pollutant emissions may also be controlled by legislation and regulations. Environmentalists believes that major reductions in vehicle produced air toxics could be achieved through a wide application of these existing technology, such as cleaner burning gasoline, better emission controls on cars and more effective national inspection and maintenance program to ensure that new cars continue to meet emission standards.

2.6.2 Removal of air pollutants by leaf microbial community

The removal of atmospheric air pollutants by leaf microbial populations is a natural process as the microorganisms inhabiting the leaf surface uses atmospheric pollutants as a carbon source. De Kempeneer et al. (1998) studied the removal of airborne toluene by means of *Azalea indica* augmented with a toluene-degrading bacterium, *Pseudomonas putida* TVA8. This is the first report on bioaugmentation of the leaf surface of plants to remove gaseous pollutants from air. Under the same experimental conditions, inoculated *Azalea* plants removed toluene within 27 hours compared to 75 hours in uninoculated plants. Subsequent additions of toluene further increased the removal efficiency of the bioaugmented system. Hence, inoculation of the leaf surface appeared essential for obtaining rapid removal rates since more bacteria would be necessary to degrade more toluene as bioavailability increases with inoculation.

To date, the utilization of plants for the remediation of hazardous waste contaminants is limited. Phytoremediation, a technology that uses plants for the in-situ treatment of contaminated soils and sediments continues to grow. Some studies have also been done on the use of plant leaves to monitor pollutants in the atmosphere as well as on the possibility of indoor plant having the potential to decrease indoor air pollution. Although research has been done on the microbial population of the leaf, further studies on the various factors influencing colonization is yet to be carried out. This study will investigate the use of the plant leaves and their associated bacteria to remove atmospheric contaminants such as PAH that deposits on leaf surfaces, a known sink for atmospheric PAHs deposition. The results of this study would open the prospect for further research in the area, for using ornamental plants as sinks for atmospheric pollutant deposition, since their leaf associated microbes may degrade the deposited pollutants and for further studies on the use of leaves associated bacteria to degrade other PAHs.

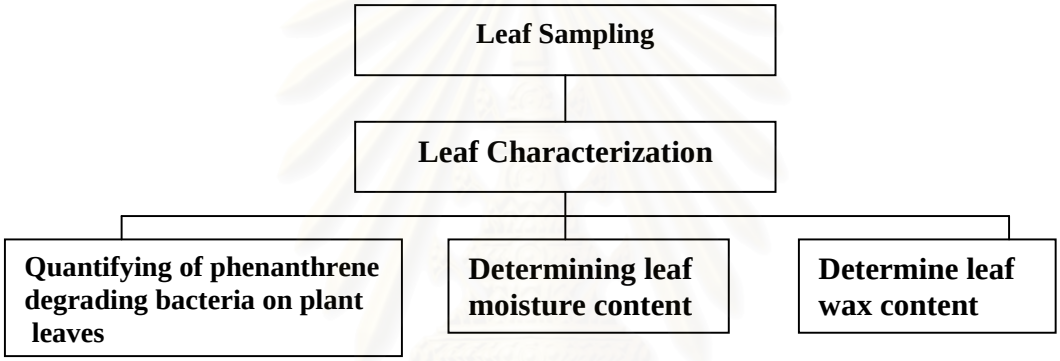
CHAPTER III

MATERIALS AND METHODOLOGY

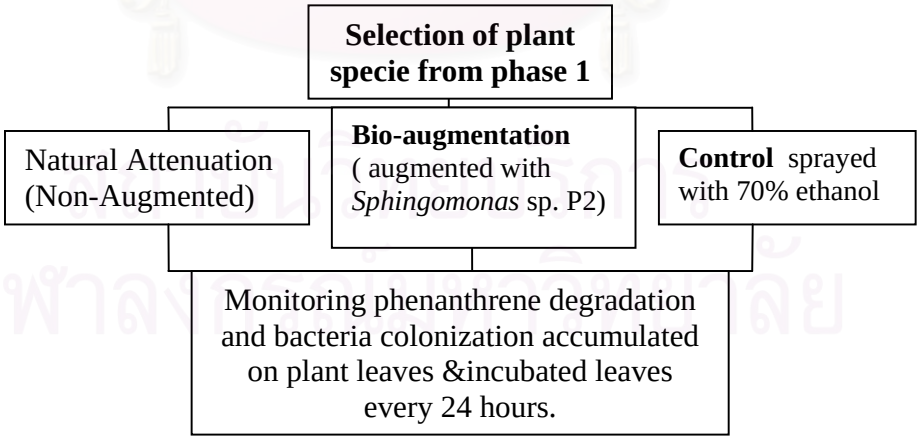
3.1 Flow chart of experimental procedure

The experiment was conducted in 3 phases to investigate the potential of a selected plant species and associated bacteria to reduce PAHs deposited on leaf surface.

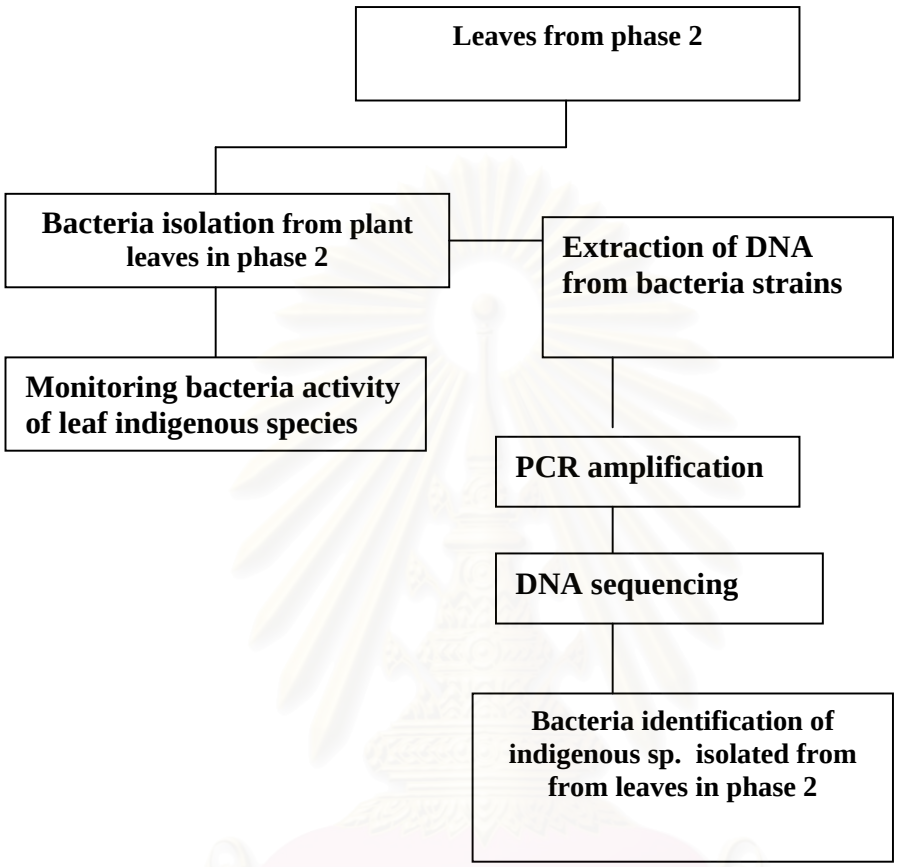
Phase 1 Screening of ornamental plants



Phase 2 Degradation of phenanthrene deposited on leaf surface



Phase 3 Verification of phenanthrene degrading bacteria on leaves



3.2 Materials and apparatus

3.2.1 Chemicals

- Phenanthrene, Sigma Chemical
- 0.1 Potassium Phosphate buffer (pH 7)
- Minimal Salt (MS) medium
- Silica gel 60 particle size 0.063-0.200mm, Merck
- Sodium sulfate anhydrous, Merck
- Hexane (Analytical Grade), Fisher Scientific chemicals
- Dichloromethane (AR grade), Mallinkrodt
- Glass wool
- 1X TAE Buffer, Amersham Biosciences Co., Ltd
- ethidium bromide

3.2.2 Plants

- *Wrightia religiosa* Benth. ex Kurz
- *Pereskia grandiflora* Haw.
- *Hibiscus rosa-sinensis* L.
- *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*
- *Ixora* sp.
- *Hymenocallis littoralis* Salisb.

3.2.3 Glassware

- 250 ml Round bottom flask
- 2x10cm Glass column with a Teflon stop cork
- Separating funnel
- Petri dish
- Beakers
- Cylinders
- Erlenmeyer flask
- 70 mm Glass fiber filter (Whatman (GF/C))
- 50 ml Volumetric flask
- Gas chromatography vials
- 5 ml vials
- desiccators

All glassware were washed, rinsed with deionized water and dried overnight in oven.

3.2.4 Instruments

- Shaker, GFL 3017
- Microwave, Milestone ETHOS SEL
- Rotary Evaporator, Heidolph
- Centrifuge, SORVAL, Biofuge Statos
- Electrophoresis chamber
- Flourometer, Quentech
- Flame ionizing detector (FID) Gas liquid chromatography, Agilent 6890N
- Gas chromatography with flame ionizing detector (GC-FID), Agilent 6890N
- Gas chromatography with mass spectrometry (GC-MS)
- Autoclave, Kakusan
- Vortex mixer G-560 E, Scientific Industries
- FastPrep FD 120, BIO101, Thermo Savant
- Microcentrifuge, MIKRO 20, Hettich
- GeneAmp® PCR system 2700, AB Applied Biosystems
- Digital Filter Fluorometer, Turner® Quantech™
- UV transilluminator, BioDoc- It ™ System, UVP
- Furnace
- 20, 100, 200, 1000 and 5000 µl micropipettes, Drummond Scientific

3.3 Leaf sample collection

Leaf samples were collected during summer 2005 from roadside areas in Bangkok. The leaves of six ornamental plants were collected from two locations, a highly polluted area and a less polluted area. The highly polluted area chosen in this study is in Lad Phrao area along the roadside of Jatujak market and the less polluted area was on Phaya Thai Road in front of Chulalongkorn University. The ornamental plant species were selected randomly and included *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. (Figure 3.1). Samples collected were wrapped in foil paper and stored in plastic bags to be used on the same day.

Ixora sp.

Wrightia religiosa Benth. ex Kurz

Pereakia grandiflora Haw.

Hibiscus rosa-sinensis L.

Excoecaria cochinchinensis Lour. var. *cochinchinensis*

Hymenocallis littoralis Salisb.

Figure 3.1 Plant used in the study

3.4 Leaf characterization

3.4.1 Quantifying of phenanthrene -degrading bacteria on plant leaves

Each morning at 9:00 a.m. four grams of freshly picked leaves were cut into small pieces of about 1 cm^2 and then immersed in 40 ml potassium phosphate buffer and shaken for 30 minutes using a shaker to dislodge the bacteria. The extracted bacteria suspension was diluted with sterile Minimal Salt (MS) medium before spreading on MS agar prepared according to Focht (1994). Triplicate of each dilution were cultured and incubated at room temperature in a glass box supplied with phenanthrene as the sole carbon source for two weeks. After incubation, bacteria colonies were count and the results were averaged.

3.4.2 Determining moisture content

Moisture content was determined by weighing 4 g of leaf sample on a tarred crucible and drying the sample for 8 hours at 105 ^oC. Samples were then cooled in desiccators and weighed. The percentage of moisture was calculated as follows:

% moisture= (weight of sample-weight of dry sample) /weight of sample x100

3.4.3 Determining wax content

Four grams of leaf sample were extracted with 40 ml hexane in a microwave extractor (Milestone ETHOS SEL) with power of 500-1000 watts for 20 minutes (100^{o}C for 10 minutes and 115^{o}C for 10 minutes). The extract was allowed to cool and filtered through a GF/C filter into a pre-weighed drying round-bottom flask. The flask was evaporated by rotary evaporator at 67^{o}C until the solvent dried out. The round bottom flask was reweighed, and the percentage of wax was calculated as follows:

%wax= (reweighed flask-preweighed) flash/weight of sample x 100

Note: % wax is defined as all those dissolve in hexane

3.5 Monitoring the degradation of phenanthrene deposited on leaf

3.5.1 Phenanthrene Degradation using entire plant

Plant samples used in the study were purchased from a florist. All plants were all two months old and approximately 42 cm in height.

The experiment consisted of three bioremediation treatments done in triplicate; including natural attenuation, bio-augmentation and control. Plants were first treated as follows. In non-augmented plant, each plant was sprayed with 30 ml of deionized water. Bioaugmentation was performed by spraying the leaves with 30 ml suspension of *Sphingomonas* sp. P2 (approximately 10^{7} CFU/ml). This was done to investigate the effect of augmentation of a known phenanthrene degrader on the rate of phenanthrene

degradation so as to compare the degradation by augmentation with *Sphingomonas* sp. P2 with that of the indigenous species. The control plants were sprayed with equal volume of 70% ethanol to remove as much phyllosphere bacteria as possible. These plants were left to dry overnight. On the following day, the leaves of each plant was directly sprayed with equal volume of 10^4 ppm phenanthrene dissolved in 20% DCM in hexane (10 ml/plant) using a fine spray glass bottle. Spraying was done approximately 12 inches away from the plant. Plants were then enclosed in plastic bags for 24 hours in order to maintain 100% relative humidity and free moisture on the leaves since the process of PAH accumulation on the leaf is affected by abiotic factors including vapor particulate partitioning in the atmosphere and ambient temperature(Figure 3.2).

Figure 3.2 *Ixora sp.* covered with plastic bags before monitoring of phenanthrene degradation.

After 24 hours, the plants were uncovered. Eight grams of leaves was then removed from each set of plants. Each sample was wrapped in foil paper and placed in plastic bags. These leaves were used to monitor the initial amount of leaf deposited phenanthrene as well as the number of phenanthrene degrading bacteria. Phenanthrene was extracted and analyzed as described in section 3.5. The analysis of bacterial number was described in section 3.3.1. Phenanthrene degradation and bacteria colonization was monitored daily for a period of 72 hours.

3.5.2 Phenanthrene degradation using incubated leaves

Instead of using the entire plant, leaves were removed from *Ixora* sp. and each leaf was cut in four pieces. The leaves were obtained from plants approximately two months old that were purchased from a florist. Approximately 16g of cut leaves were placed in a 500 ml beaker for each treatment (Figure 3.3). There were four treatments done in triplicate; including natural attenuation, bio-augmentation and two control experiments. In natural attenuation, each sample was sprayed with 8 ml of deionized water followed by 3ml of 10^4 ppm phenanthrene dissolved in 20% DCM in hexane. In bio-augmentation, samples were sprayed with 8 ml innoculum of *Sphingomonas* sp. strain P2 followed by 3ml of 10^4 ppm phenanthrene dissolved in 20% DCM in hexane. In treatment 3: control 1, samples were sprayed with 70% ethanol followed by 3ml of 10^4 ppm phenanthrene dissolved in 20% DCM in hexane and in treatment 4: control 2, samples were prepared as in treatment 3 but kept at -4^{o}C throughout the experimental period. After treatment, the beakers were sealed with foiled paper and stored at room

temperature. Forty-eight hours later the initial amount of absorbed phenanthrene and the number of colonized bacteria on the leaves were determined. Phenanthrene degradation and bacteria colonization was thereafter monitored every 24 hours for a period of 48 hours. Triplicate samples were analysed in both cases.

Figure 3.3 Leaves of *Ixora sp.* incubated in 500 ml beakers.

3.6 PAH extraction and analysis

PAH extraction was modified from EPA's standard method and using microwave extractor similar to section 3.3.3. After microwave extraction, the leaf extract was evaporated by rotary evaporator at 67 ^{o}C to a volume of 4 ml. To remove plant wax, the 4 ml samples of leaf extract were passed through silica gel column adapted from Karnchanasest and Satayavibul (2005).

Briefly, silica gel 60 was activated by heating at 500 ^{o}C for 4 hours and left to cool in a desiccator. The leaf extract was loaded onto a column packed with 15 g

activated silica gel, of which 1cm thick anhydrous sodium sulfate was added on top and the column was lined with glass wool at the bottom. Prior to loading, the column was washed with 50 ml of 20% DCM in hexane, until the solvent reached 1 cm above the silica gel level. The analyte was eluted with 100 ml of 20% DCM in hexane and the effluent was collected in a 250 ml round bottom flask and evaporated down to 4 ml by rotary evaporator at 67^{o}C.

GC analysis of the concentrated sample was then performed with a Hewlett-Packard 6890 equipped with a FID detector and a HP-5 fused-silica capillary column (30m x 0.32mmID; thickness, 0.25 µm). The optimal operating conditions shown in Table 3.1 were adapted from Karnchanasest and Satayavibul (2005).

Table 3.1 Optimal operating conditions for Gas Chromatography (GC) Analysis

Injector Type; split less Temperature; 280^{o}C	Detector Type; FID Temperature; 250^{o}C
Split vent Turn of time; 1min.	Temperature program Initial 80^{o}C, 1 min. Rate 1; 25 ^{o}C/min. until 160 ^{o}C for 3 mins. Rate 2; 3 ^{o}C/min. until 300 ^{o}C for 2 mins.
Injection Volume 2 µl	

Source: adapted from Karnchanasest and Satayavibul (2005)

3.7 Verification of phenanthrene degrading bacteria on leaves

3.7.1 Isolation of Bacteria

The bacteria were extracted from the leaves of the *Ixora sp.* as described in section 3.3.1. The extracted solution was spread on MS agar plates and exposed to phenanthrene as a sole carbon source. After growth, bacteria colonies were selected based on abundance and appearance (color). The selected colonies were then isolated by streaking on MS agar. The phenanthrene-utilizing bacteria were then purified by repeated streaking on LB agar medium.

3.7.2 Monitoring the activity of isolated bacteria species

The purified strains was streaked on LB agar and incubated at 30ºC for 3 days. Single colony was then transferred to liquid MS medium supplemented with 100 ppm phenanthrene and shaken at 200 rpm for 4 days. The cultures were then harvested by centrifuging at 6,000 rpm at 4ºC for 5 min. Cell pellet was resuspended in 10 ml MS medium and diluted to give OD_{600} of 0.1. Five ml of the liquid cultures was added to 22-ml vials. Then, phenanthrene was applied to give a final concentration of 100 ppm. The cultures were incubated at 200 rpm, 30ºC, for 9 days. Phenanthrene degradation was monitored after day 4, 7 and 9 by extraction of phenanthrene from the liquid medium. The total amount of phenathrene remaining was determined by GC-FID analysis.

3.7.3 Identification of bacteria

A partial nucleotide sequence of 16S rRNA gene of bacterial strains was determined. The 16S rRNA gene from bacterial strains was amplified by polymerase chain reaction (PCR) and subjected to sequencing. The nucleotide sequences obtained was analyzed with p Draw software. A homology search was done by GenBank databases using a BLAST program.

3.7.3.1 DNA extraction

Extraction of DNA from isolated bacteria was carried out using DNA Sabai Kit. The amount of DNA extracted was measured with a fluorometer. Agarose gel electrophoresis was carried out to examine the intact of extracted DNA by comparing it with 1KB standard. The extracded DNA was photographed with a UVP light transilluminator (Bio Doc-IT system). (Figure 4- 13)

3.7.3.2 PCR amplification

The 27 f (5'-AGAGTTTGATC[A/C]TGGCTCAG-3') and the 1492r (5'-TACGG[A/T/C]TACCTTGTTACGACTT) primers corresponding to the position 8-27 and 1492-1513 of the 16S rRNA gene sequence was used in this study (Pinyakong, 2003). Amplification was performed with 20 pmol of the forward and reverse primers in total volume of 100µl with 200 µM of each dNTP, 2.5mM $MgCl_2$, 2.5U Tag DNA polymerase and template DNA at an estimated concentration.

Polymerase chain reaction was carried out in a Gene Amp PCR system 2400 (Perkin Elmer Cetus). PCR-program was 1 minute at 96 ^{o}C, followed by 30 cycles of 94 ^{o}C for 1 minute, 55 ^{o}C for 1.5 minute, 72 ^{o}C for 2 minutes, maximal ramp rates throughout, with the final 72 ^{o}C for 7 minutes before cooling at 4 ^{o}C (Pinyakong, 2003). The fragment generated was visualized by running on agarose gel and purified using QIAquick PCR Purification Kit Protocol. In case where more than one band was obtained in the PCR product, the target DNA fragment with 1.5 Kb in size is recovered from agarose gel and purified by QIAquick Gel Extraction Kit Protocol. The final concentration of DNA obtained after purification is determined by spectrophotometer and subject to sequencing analysis. The nucleotide sequences obtained was analyzed with p Draw software. A homology search was done by GenBank databases by using a BLAST program.

3.8 Agarose gel electrophoresis

Two (w/v) Agarose gel was prepared by heating in TAE buffer until agarose gel was completely melted. The melted agarose gel was cooled down to approximately 50^{o}C and poured in chamber with comb. DNA sample was mixed with 0.1 volume of gel-loading buffer, containing bromophenolblue and loaded into agarose slot. Submerged gel electrophoresis was carried out at 100V for 30 minutes in electrophoresis chamber with 1X TAE as running buffer. The Gel was stained with ethidium bromide (0.5µl/ml in

distilled water) for 5 minutes and destained with distilled water for 5 minutes. DNA was visualized under UV-light transilluminator (Bio Doc-IT system). 100bp marker was used as a standard DNA marker.

3.9 Preparation of *Sphingomonas* sp. P2 liquid inoculum

Sphingomonas sp. P2 was cultured in MS medium supplemented with 100 ppm phenanthrene and shaken at 200 rpm for 48 hours. The culture was harvested by centrifuging at 6,000 rpm at 4ºC for 5 min. Cell pellet were resuspended in 10 ml of MS medium and centrifuge at 6,000 rpm at 4ºC for 5 min. The pellets harvest was again resuspended in the MS medium and OD was measured with a spectrophotometer. The innoculum of *Sphingomonas* strain P2 applied in the treatment described in section 3.4 was diluted to give OD_{600} of 0.3371 equal to approximately 10^7 CFU/g (Deangrueng, 2005). (Figure 3.2)

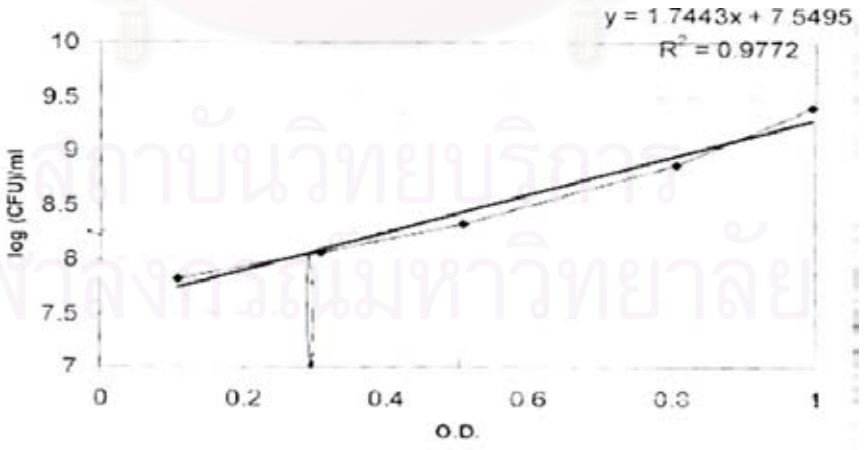


Figure 3.4 Standard curve of *Sphingomonas sp.* P2

Source: Deangrueng, 2005

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Screening of ornamental plants

Six ornamental plants were collected from two locations in Bangkok, a highly polluted area and a less polluted area. These two locations were chosen in order to compare the effect of pollutant concentrations in a given area on the colonization of bacteria on plant leaves. The highly polluted area chosen in this study is along the roadside of Jatujak market located at the end of Paholyothin road in the Lad Phrao area of the city. This is a commercial area with high traffic volume, a known source of atmospheric PAHs. The less polluted area was on Phaya Thai Road in front of Chulalongkorn University where the traffic volume is of much lower intensity. At the time of sample collection in August 2005, the Pollution Control Department, Bangkok daily air quality data reported that the Lad Phrao area had the highest concentration of PM10 in comparison to their other six monitoring stations. Since then the level of PM10 in this area and other areas in Bangkok continues to increase. Although the level of PM10 does not indicate the concentration of PAHs in the atmosphere, it gives an indication of the level of pollutant in the given area. Moreover, in a study to investigate the characteristic of PAH in PM10 collected from Bangkok Urban area Norramit et al. (2005) found that there is a close relation between the concentration of carcinogenic PAHs and the amount of PM10. This is due to their absorptive property on the surface of particles.

The purpose of this study is to identify an ornamental plant for enhancing the degradation of PAHs deposited on plant leaves.

The ornamental plant species were selected randomly and included *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. (Figure 3.1)

General characteristics of the plants shown in Table 4.1 indicated that they were different from one another in terms of leaf arrangement, texture, area, and weight. Studies have shown that the characteristics of leaf play a vital role in PAHs absorption. Slaski et al. (2000) stated that the most important anatomical feature of plants that affect the rate of interception and accumulation of airborne PAHs is the total leaf area, since the surface area of the above-ground parts of plants exceeds by far the area that the plants grows on, with the leaves accounting for the greatest portion. Bakker, (2000) also found that sparsely hair leaves are more heavily contaminated than densely hair since lower canopy roughness increases aerodynamic resistance. Bakker, (2000) also concluded that high leaf over lap decreases PAHs accessibility and thicker laminar boundary decrease absorption.

Simonich and Hites (1994) measured the concentrations of ten PAHs in leaves, bark and seeds of sugar maple and white pine. They concluded tissues containing more lipid accumulates significantly more PAHs. Due to the fact that PAHs are lipophilic

molecules, they are able to pass through leaf cuticle by solubilization in waxes, but they are also strongly kept by Van der Waals or covalent bonds (Fismes et al., 2001). Because of this, low molecular weight PAHs is easier than high molecular weight to penetrate waxy leaf cuticle**.**

Table 4.1 Characteristics of plant leaves collected from location 1 and 2.

Plant	Leaf Arrangement	Leaf Texture	Leaf area[1] (cm^2 /leaf)	Leaf weight[1] (g/leaf)
Ixora sp.	Opposite	Medium, glossy leaves	43	0.80
Wrightia religiosa Benth. Ex Kurz	Opposite	Light, smooth	14	0.16
Hibiscus rosa-sinensis L.	Alternate	Heavy, glossy, fairly moist	37	0.80
Pereskia grandiflora Haw.	Whorl	Rough, hairy	42	1.00
Hymenocallis littoralis Salisb.	Opposite	Heavy, smooth	160	8.00
Excoecaria cochinchinensis	Whorl	Rough, hairy	26	0.36

[1]Each value was averaged from two locations.

From his study, it can be assumed that of the six plant species used in this study, *Wrightia religiosa* Benth. ex Kurz and *Ixora* sp. would have high amounts of PAHs deposited on the leaves since the amount of wax/g of leaf was highest in these two species. The type and concentration of deposited PAHs will however depend on other factors such as the abiotic conditions surrounding the leaf as well as its inhabited microbial community.

Leaf samples were also analyzed for the number of colonized bacteria, percent moisture content, and percent wax content. Plants when grown at different locations had different number of phenanthrene degrading bacteria and wax content but the amount of moisture was not much different (Figure 4.1, and 4.2A).

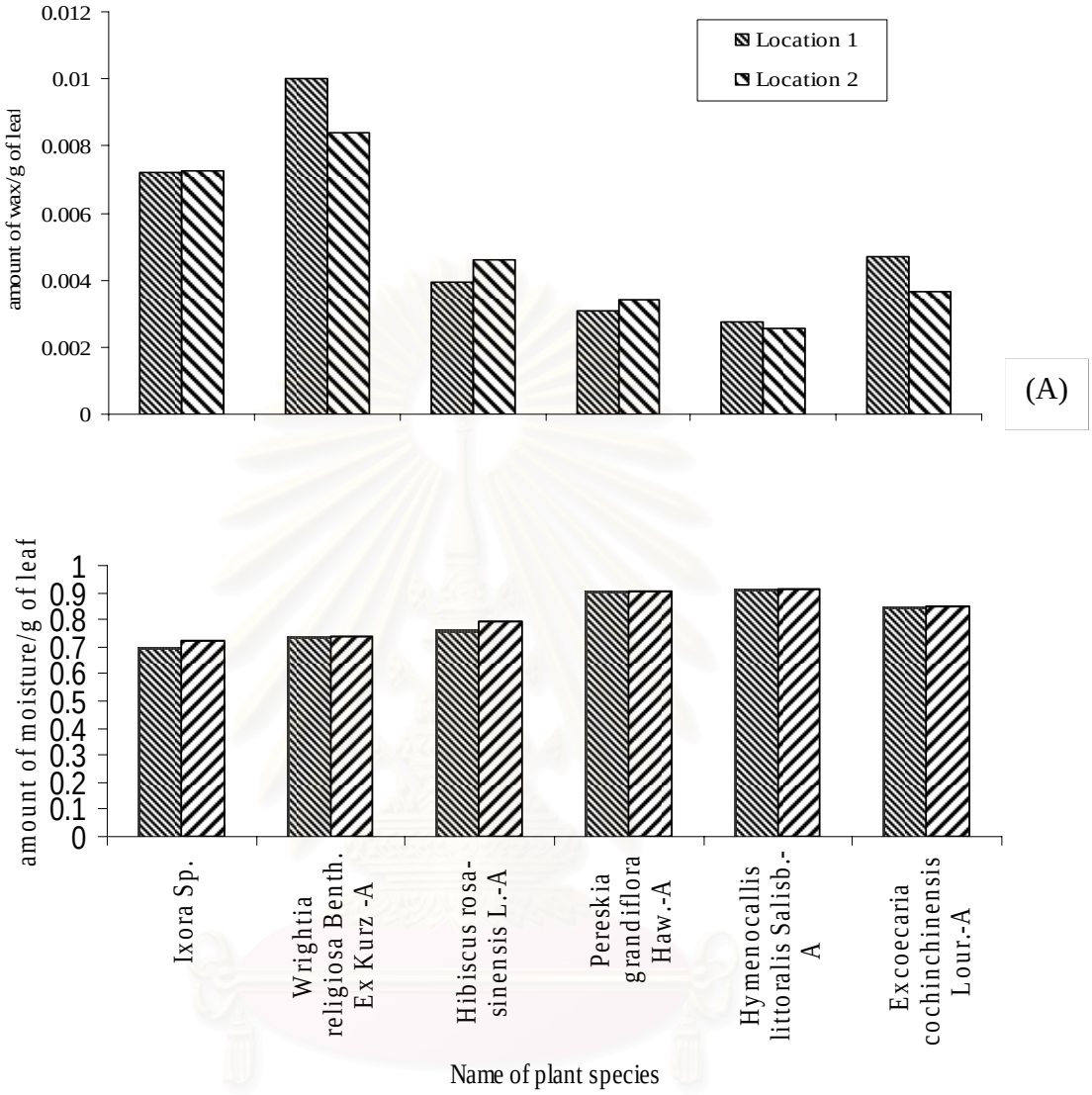


Figure 4.1 Percent wax content (A) and percent moisture content (B) in six ornamental plants. Location 1 and 2 are highly and less polluted area, respectively.

The number of phenanthrene degrading bacteria on leaves was ranged from $3.5x10^4$ to $1.95x10^7$ CFU/g (Figure 4.2). This concentration is similar to a study on the leaf bacterial population on Mediterranean plants carried out by Yadav et al., in 2004. They found that bacteria colonization of the leaves is highly variable, ranging from non-detectable to a maximum of 1.4×10^7 CFU/g. When comparing between each plant species from two locations, *Ixora* sp. showed the highest concentration of phyllosphere bacteria with an average of $9.7x10^6$ CFU/g and *Excoecaria cochinchinensis* Lour. var. *cochinchinensis* contained the least, $3.5x10^4$ CFU/g (Figure 4.2).

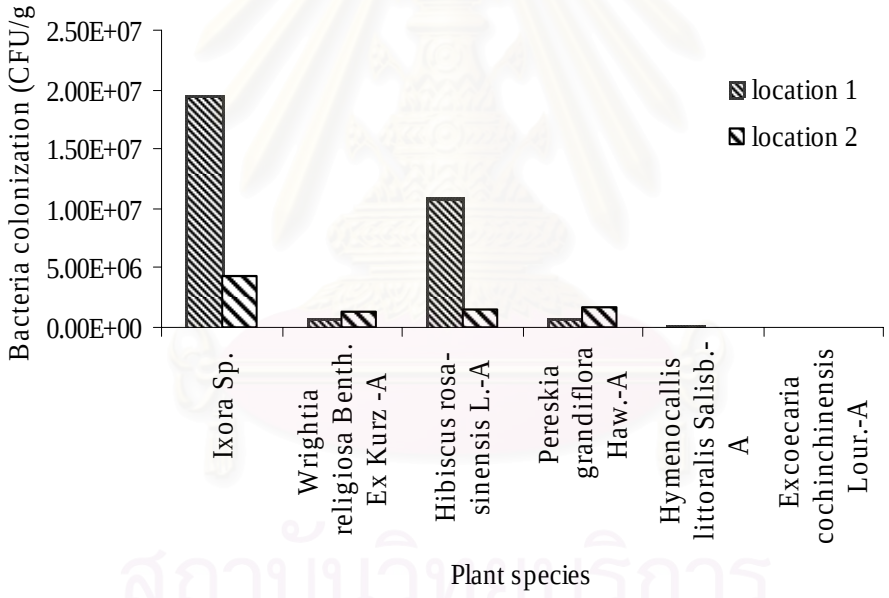


Figure 4.2 Number of phenanthrene degrading bacteria on plant leaves collected from location 1: highly polluted and location 2: less polluted.

In regards to the relationship between bacteria colonization and leaf moisture content, a correlation coefficient (R^2) of 0.54 and 0.47 was obtained in location 1 and 2, respectively (Figure 4.3A). This was done by plotting the number of phenanthrene degrading bacteria and percent moisture obtained from all plant species in each location. This result suggests that there was no significant relationship between the number of bacteria and moisture content of the leaves. The relationship between bacteria colonization and wax content was even more insignificant. When the number of phenanthrene degrading bacteria was plot against percent wax, an R^2 of 0.04 and 0.37 were obtained in location 1 and 2, respectively (Figure 4.3B). Both results suggest that colonization of phenanthrene-degrading bacteria on leaves from both locations was not depended on chemical properties i.e. moisture and wax content of the leaves. Other species specific characteristics may play important role on promoting bacterial colonization, for example, the nutrient composition of the leaf. Mercier et al. (2000) studied the influence of leaf nutrients on bacteria colonization. Their study suggested that the amounts of sugars on leaves of different plant species were directly correlated with the maximum bacterial population sizes that could be attained on those species.

As reported by Andrews (1996), high variability in space and time is a typical feature of epiphytic bacterial population. Population sizes can vary enormously among adjacent, visually identical leaves of the same individual.

From the results obtained in 4.1, the selected plants for further studies in phase 2 was chosen based on its ability to colonized maximum amount of bacteria. This criterion

was most applicable in this study, since no phenanthrene was detected in the leaves, and no significant relationship was shown in regards to moisture content, leaf area, wax content and bacteria colonization. The result indicated maximum bacteria colonization on the leaves of the *Ixora* sp. This was probably because of the ability of this leaf sp. to serve as a sink for deposited PAHs. As shown in Figure 4.1A, the concentration of wax/g of leaf is high, thus these leaves would tend to accumulate high amounts PAHs, which initially would enhance bacteria colonization. The colonized bacteria also must have had the ability to adapt and survive on the Ixora sp. since it has high wax content. This plant was thus chosen for further studies in phase 2 and 3.

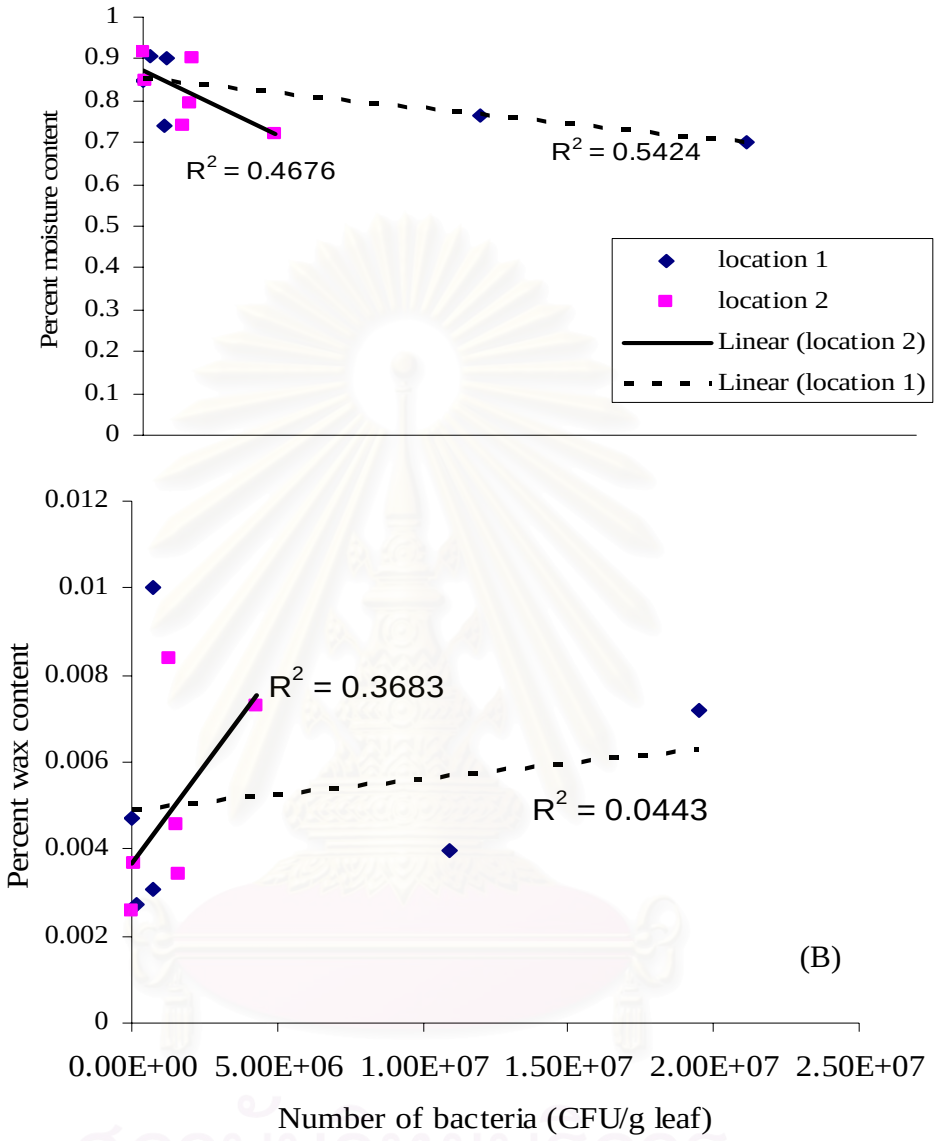


Figure 4.3 Relationship between bacteria colonization and percent moisture content (A) or percent wax content (B) on leaves of the six plant species.

4.2 Monitoring the degradation of phenanthrene using entire plant

The selected ornamental plant from phase 1 is *Ixora* sp. (Figure 4.4). This ornamental plant is a tropical, evergreen shrub belonging to *Rubiaceae* family. It is a medium to tall shrub with largish pointed leaves and clusters of flowers at the end of the branches in red or red-orange colors. It flowers almost continuously in full sun or little shade and grows rapidly. *Ixora* sp. is mostly planted as a hedge and can grow in a wide range of climate.

Figure 4.4 *Ixora* sp.

The experiment consisted of three treatments done in triplicate; natural attenuation, bio-augmentation and control. PAHs degradation was monitored by spraying phenanthrene on the leaves of *Ixora* sp. that were augmented with *Sphingomonas* sp. P2, non-augmented, and cleaned with ethanol (control). The main objective of this phase is to confirm that the selected ornamental plant does contain bacteria that have the potential to

degrade phenanthrene, the model PAHs used in this study. It also investigated the effect of bioaugmentation of a known PAHs degrader on the rate of PAHs degradation. It is expected that the indigenous bacteria will use phenanthrene as a carbon source, thus the concentration of phenanthrene will decrease as time increases and the number of phenanthrene degrading bacteria (CFU/g) will increase. Bioaugmentation of the plants with *Sphingomonas* sp. P2 is expected to increase the rate of degradation.

The initial amount of phenanthrene deposited onto the leaf surface of each plant varied from 480 to 960 ppm. In any case, the initial amount was taken as 100% baseline and time 0. Percent phenanthrene remaining was monitored every 24 hrs thereafter (Figure 4.5).

The amounts of phenanthrene remaining on leaves from control plants decreased slowly to 71.97% after 24 hours. At the end of study, the amount of phenanthrene in control plants was only 18.12%. This significant decrease may have occurred due to physical mechanisms such as volatilization, as well as the action of remaining bacteria. This result thus indicated that another method of removing surface bacteria needs to be investigated. Ethanol was probably ineffective to remove bacteria or vaporized too rapidly and consequently allowed some bacteria to grow back.

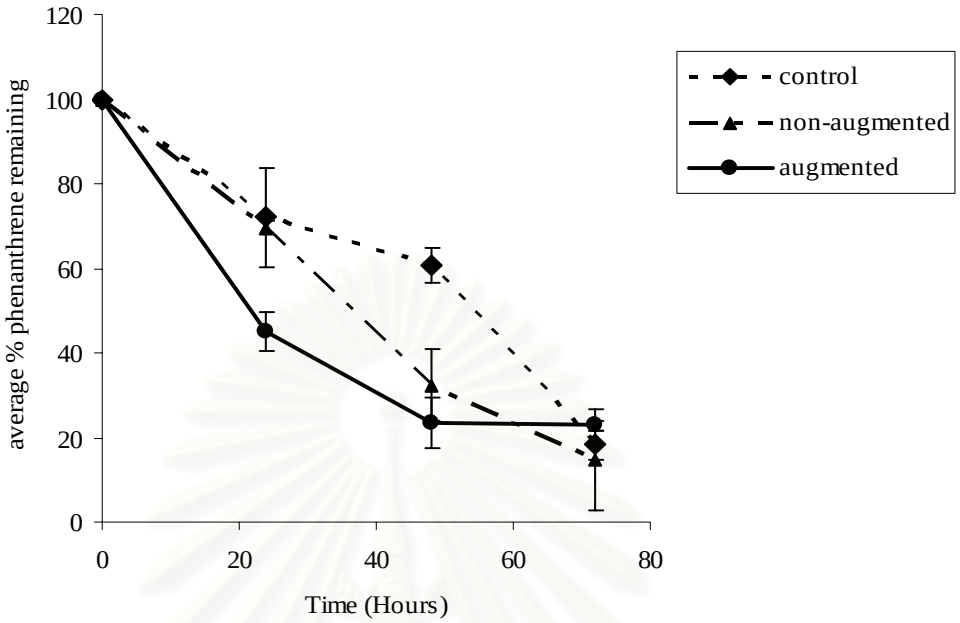


Figure 4.5 Percent phenanthrene remaining on leaves of control, non-augmented and augmented species after each 24 hour period.

In non-augmented plants, the amount of phenanthrene decreased from 69.64% after 24 hours to 32.36% after 48 hours. After 72 hours, only 14.7 % of the initial amount of phenanthrene absorbed into plant leaves remained. This result indicated a gradual decrease in percent phenanthrene remaining after each 24 hour period, suggesting that the indigenous bacteria were indeed degrading phenanthrene. It also suggests that the indigenous bacteria are in fact good PAHs degraders but may require some adjustments before degrading phenanthrene. This is similar to a study by De Kempeneer et al. (1998) who found that uninoculated *Azalea indica* can reduce the amount of airborne toluene. Meanwhile, the inoculation with toluene degrading bacteria is essential for obtaining rapid removal rates.

In plants augmented with *Sphingomonas* sp. P2, there was a sharp decreased in the initial amount of leaf phenanthrene. After 24 hours, only 45.1 % phenanthrene remained in the leaves of these plants indicating and confirming the ability of the augmented bacteria *Sphingomonas* sp. P2 to degrade phenanthrene. The amount of phenanthrene continued to decrease to 23.4% after 48 hours. After 48 hours, however, no significant reduction in phenanthrene was detected and the amount remaining after 72 hours was 22.9%. The augmented *Sphingomonas* sp. P2 probably could not survive or adapt on the leaf surface. Phenanthrene degrading activity of the bacterium on leaves was lower than in liquid medium, in which Supaka et al. (2001) showed that this bacterium could rapidly degrade phenanthrene in liquid medium from 100 ppm to undetectable amount by HPLC analysis within 72 hours. This was probably due to the well mixing of bacteria and phenanthrene in liquid culture.

When comparing the extent of phenanthrene degradation between augmented, non-augmented and control plants, results indicated that degradation in augmented plants increases rapidly within the first 48 hours with a total of 22.9% phenanthrene remaining in comparison to 32.4 % in the non-augmented and 60.8% in the control. This suggested that augmentation of this plant species with *Sphingomonas* sp. P2 increased the amount of PAHs removal as well as the rate of degradation. After 72 hours, the amounts of phenanthrene remaining in all treatments were similar; suggesting that phenanthrene degradation by the augmented bacteria depends on their survival and ability to compete with indigenous phyllosphere microorganisms.

In this study, the number of phenanthrene degrading bacteria was also monitored to find the relationship between rate of phenanthrene degradation and bacteria colonization. Bacteria was extracted from the treated leaves as described in section 3.3.1 and cultured on MS agar exposed to phenanthrene as their sole carbon source. The results indicated that the number of phenanthrene degrading bacteria in augmented plants was significantly higher than the non-augmented and control plants at all time points (Figure 4.6). However, there was a continuous increase in bacteria colonization in the non-augmented species but a slight decrease in the augmented after 48 hours. These results verify the ability of associated plant bacteria to utilize phenanthrene as carbon source.

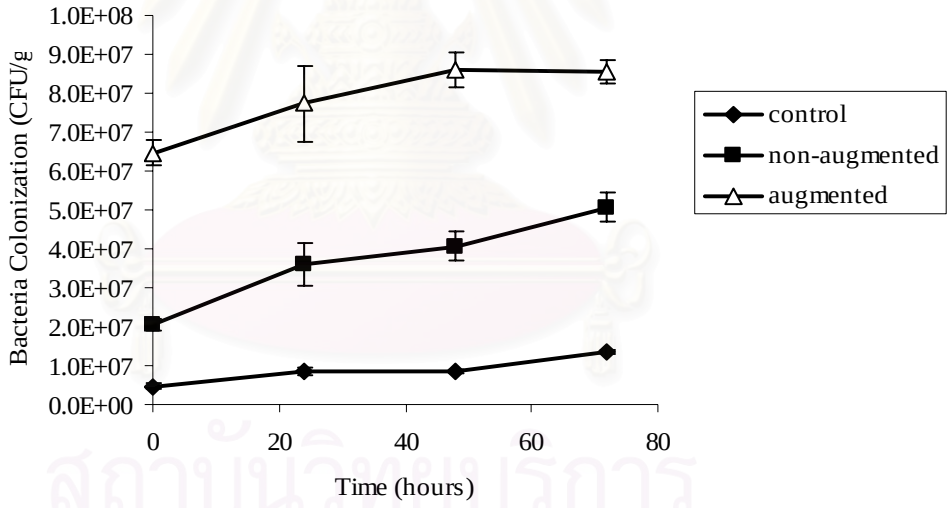


Figure 4.6 Bacteria colonization on leaves of control, non-augmented and augmented species after each 24 hour period.

When comparing the relationship between bacteria colonization and percent phenanthrene remaining within each 24 hour period, the results indicates that bacteria colonization increase as phenanthrene decreases (Figure 4.5 and 4.6). For example, the indigenous bacteria were able to degrade approximately 30.4% of the initially absorbed phenanthrene after 24 hour. At the same period, bacteria colonization on the non-augmented plants was increased from 2.0×10^7 CFU/g to 3.6×10^7 CFU/g. In addition, a decrease in the rate of bacteria colonization in the augmented plants was corresponded to the sudden decrease in phenanthrene degradation after 48 hours.

As predicted, the results suggest that the indigenous phyllosphere bacteria were indeed utilizing phenanthrene as carbon source and that augmentation does increase the rate of degradation. However, it is important to monitor and maintain the survival of the augmented species on the plant leaves.

4.3 Monitoring phenanthrene degradation using incubated leaves

The rational of this study was to compare the difference in phenanthrene degradation when using the entire plant and when using only the leaves. This was necessary, since it is believed that phenanthrene may be lost by volatilization and plant activities such as translocation. It is expected that degradation of phenanthrene will occur in the leaves augmented with *Sphingomonas* sp. P2 as well as in the leaves with the indigenous species of bacteria only (non-augmented).

In this study, plant leaves removed from the *Ixora sp.* were cut into 4 pieces and placed in large beakers where they were treated and sealed. The treatments were similar to the previous experiment, where the entire plant was used. To study the effect of leaf itself, we added another control treatment, in which leaves were cleaned with alcohol and stored in the fridge to inhibit further bacterial activity.

The initial amount of phenanthrene deposited onto each set of leaves varied from 900-1500 ppm. In any case, the initial amount was taken as 100% baseline and time 0. Percent phenanthrene remaining was monitored every 24 hours thereafter (Figure 4.7). In control 1 where leaves were sprayed with ethanol, the initial amount of phenanthrene (100%) increased by 0.8% after 24 hours to 39.6% after 48 hours. This may be due to the ethanol evaporating from the solution formed in the beaker when the leaves were sprayed with10 ml of ethanol and 3 ml of phenanthrene dissolved in 20% dichloromethane in hexane. In control 2 on the other hand, approximately 96% of the phenanthrene remained after 24 and 48 hours. This indicated that the cold temperature inhibited the activity of the bacteria and enhancing the ability of the ethanol to remove as much surface bacteria as possible.

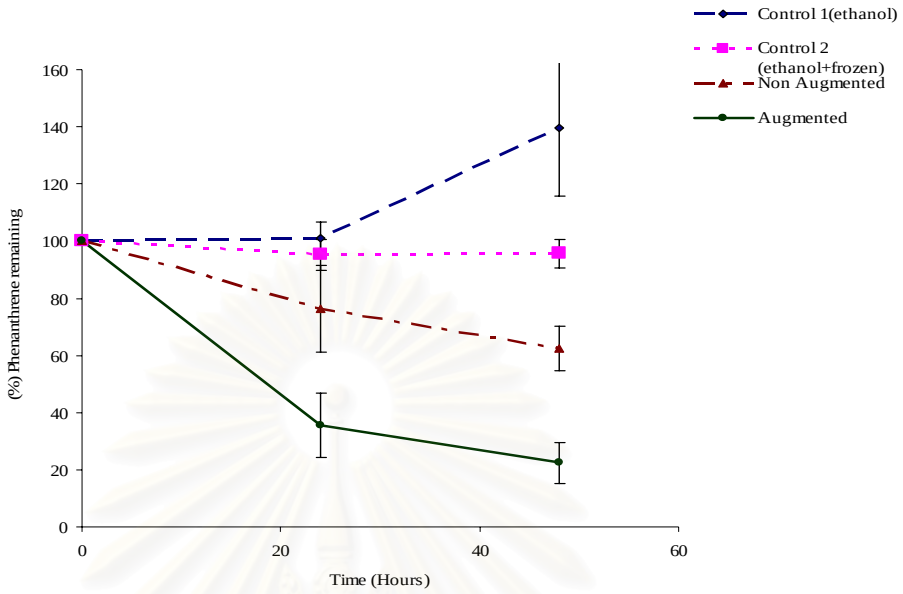


Figure 4.7 Percent phenanthrene remaining on incubated leaves after each 24 hour period.

In the non-augmented leaves, the amount of phenanthrene remaining was decreased from 76.3 % after 24 hours to 62.4 % after 48 hours. And finally, there were 35.3 % and 22.5% of phenanthrene remaining after 24 hours and 48 hours in the augmented plants, respectively. When comparing the extent of phenanthrene degradation between augmented, non-augmented and control plants, results indicated that degradation in augmented plants far exceeds that of the non-augmented and controls after both 24 and 48 hours. This suggested that augmentation of the leaves with *Sphingomonas* sp. P2 increased the amount of PAHs removal as well as the rate of degradation. It also indicates that the indigenous bacteria inhabiting *Ixora* sp. leaves were capable of phenanthrene degradation, but the degradation occurred at a slower rate.

As with the experiment described in section 4.4, the number of phenanthrene degrading bacteria in augmented plants was significantly higher than the non-augmented and controls plants at all time points (Figure 4.8). Bacteria colonization in the non-augmented species ranged from an initial 7.5 x 10^7 CFU/g to 8.1 x 10^7 CFU/g after 24 hours and 1.6 x 10^8 CFU/g after 48 hrs. On the augmented species bacteria colonization was ranged initially 1.1 x 10^8 CFU/g. After 24 hours bacteria colonization increased to 3.1 x 10^8 CFU/g and after 48 hours it was approximately 4.2 x 10^8 CFU/g. Control 2, however, proved to be a better control as bacteria colonization was significantly lower. Initial bacteria colonization detected was approximately 3.5 x 10^4 CFU/g. After 24 hour this number decreased to 2.8 x 10^4 CFU/g and after 48 hours no bacteria were detected on the plates.

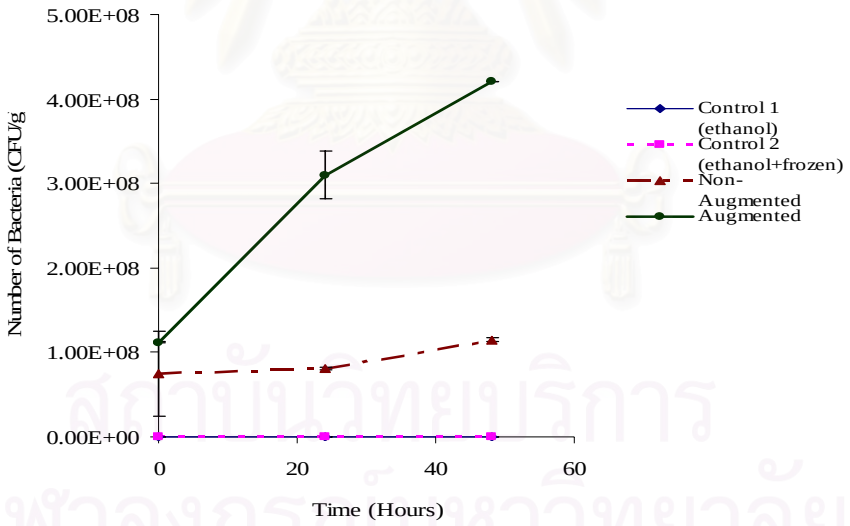


Figure 4.8 Bacteria colonization on *Ixora* sp. leaves of control, non-augmented and augmented species after each 24 hour period.

The results from 4.2 and 4.3 indicated that bacteria associated with leaves of *Ixora* sp. had the potential to degrade phenanthrene, the model PAH used in this study. When the entire plant was used however the rate of degradation occurred faster, which may have been as a result of volatilization or other physical processes carried out by the plants. With regards to the augmented plants, in both experiment it was observed that the rate of degradation increases with bioaugmentaion. The amount of phenanthrene remaining after 48 hours in both studies was generally the same, which is 23%. From this result, the reason for the decrease in degradation in the augmented leaves was the bacteria's inability to adapt well on the leaf surface after a few days.

4.4 Verification of phenanthrene degrading bacteria on leaves

The four most prominent bacteria colonies were selected from agar plates used to culture leaf associated bacteria in phase 2 of the study. The colonies were transferred on MS agar plates and exposed to phenanthrene as a sole carbon source. After growth, the phenanthrene-utilizing bacteria were then purified by repeated streaking on LB agar (Figure 4.9). The rational of this phase was to determine the degradation activity of the leaf associated bacteria and identify their species where possible. This was important in order to compare the activity of the leaf associated bacteria with *Sphingomonas* sp. P2, the augmented bacteria used in this study. It is expected that the rate of degradation of the indigenous bacteria will be slower than that of *Sphingomonas* sp. P2, but yet effective. It is also expected that the indigenous bacteria belongs to different specie, than that of the augmented bacteria.

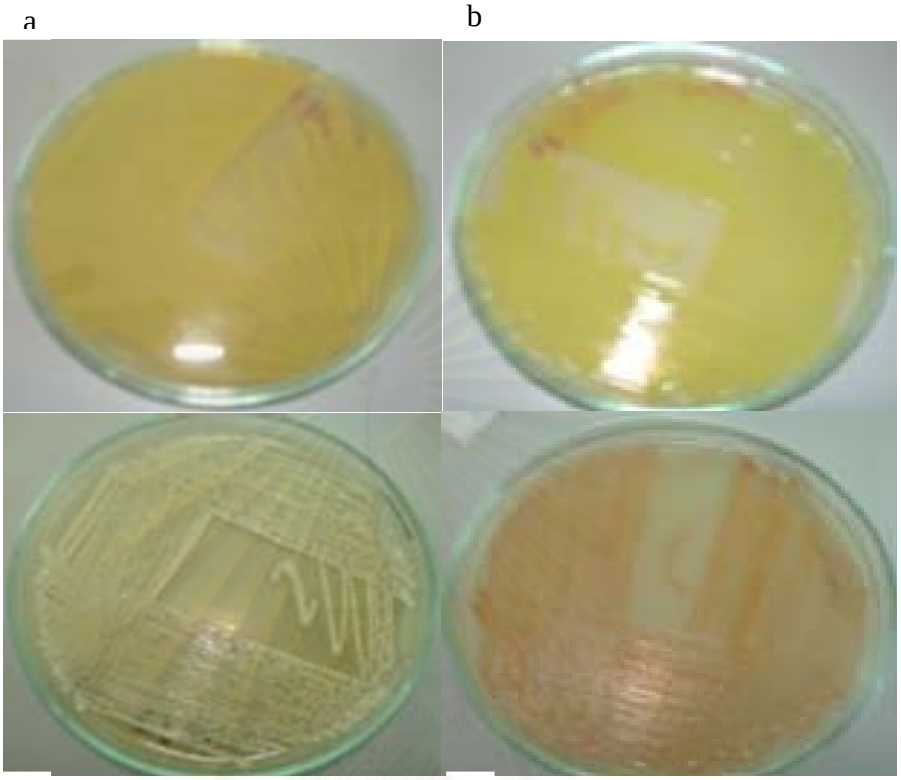


Figure 4.9 Bacteria strains extracted and isolated from leaves of *Ixora* sp.

A: phY B: phLY C: phW D: phP

These bacteria's ability to degrade phenanthrene was investigated by culturing pure strain colonies in 5 ml of the liquid MS medium supplement with phenanthrene to give a total concentration of 100 ppm. Triplicate cultures were incubated on an orbital shaker at 200 rpm, 30ºC, for 9 days. The turbidity of the bacteria was observed after day 2 and day 9 (Figure 4.10) and the amount of phenanthrene remaining after day 4, 7 and 9 was determined by extraction of phenanthrene from liquid medium. The total amount of phenathrene remaining was determined by GC-FID analysis (Figure 4.11)**.** The results on

turbidity indicated that after day 2 the color of the medium changed to a cream color which became darker and more turbid by day 9 as indicated by the brownish color. This color changed signified that a reaction was occurring and that the bacteria were probably degrading the phenanthrene.

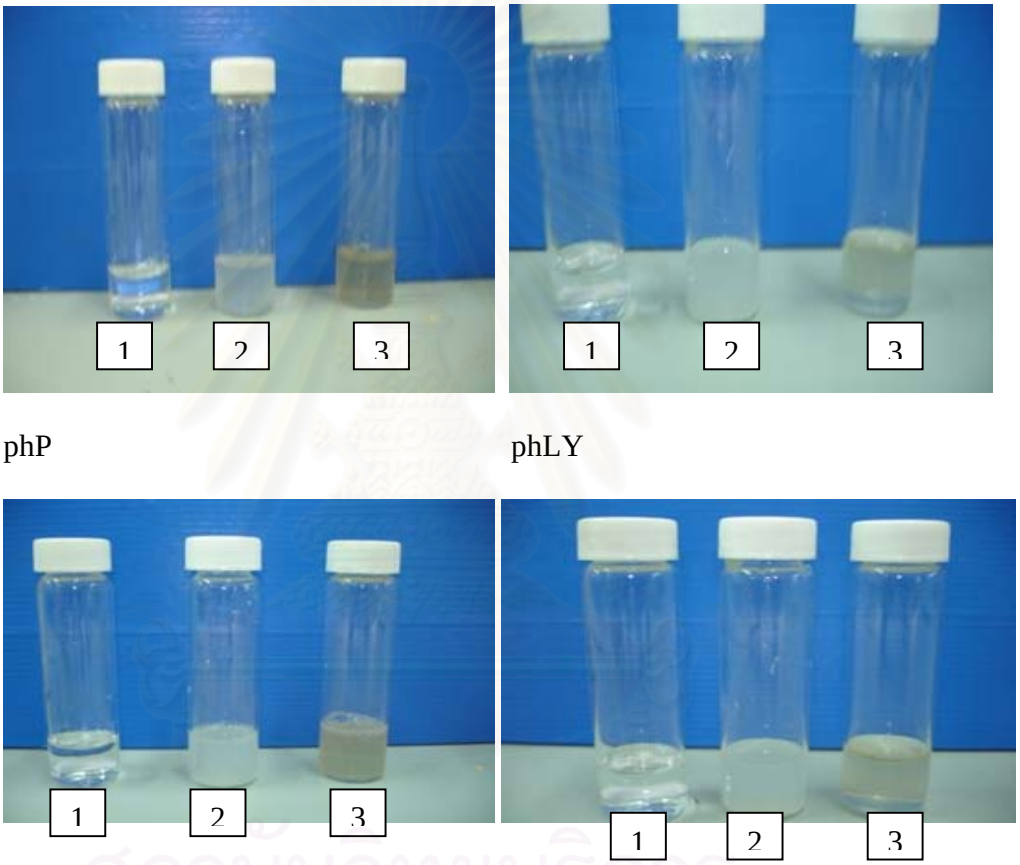


Figure 4.10 Bacteria cultured in MS medium. (1) control-5 ml MSM+phenanthrene (2) bacteria (OD-0.1) in 5ml MSM + phenanthrene after 2 days incubation (3) Bacteria (OD-0.1) in 5ml MSM + phenanthrene after 9 days incubation

The results of the degradation activity indicated that after 4, 7 and 9 days incubation, 100% phenanthrene remained in the all controls. After 4 days incubation, 0.14%, 1%, 1.95% and 0.4% phenanthrene remained in samples labeled, php, phy, phly and phq respectively. At day 7, less than 1% remained in all samples and after day 9 less than 0.5% remained. This result indicates that the indigenous species of bacteria extracted and isolated from the leaves of the *Ixora* sp. all had the ability to degrade phenanthrene in liquid medium. GC chromatogram of control and samples at day 4 are shown in Appendix B.

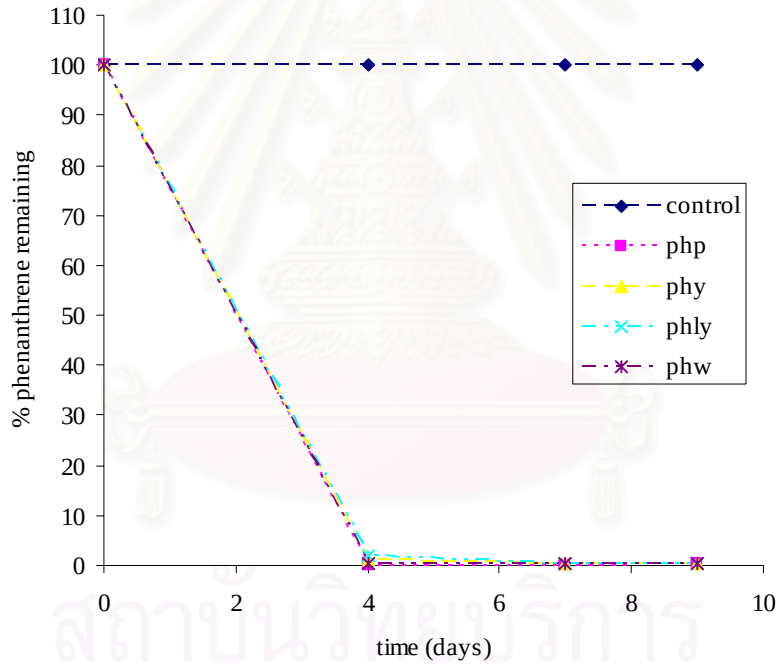
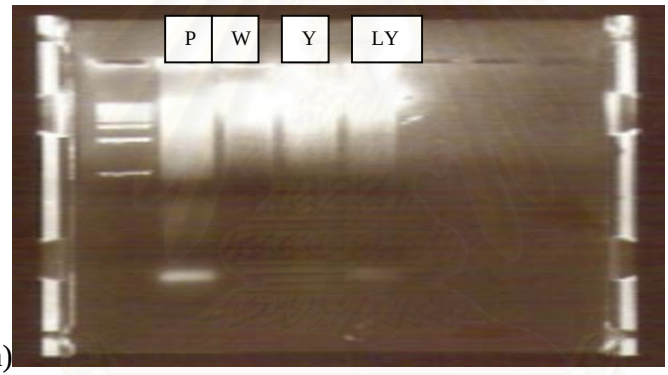


Figure 4.11 Percent phenanthrene remaining when leaf indigenous bacteria species were incubated in liquid medium. The initial concentration of phenanthrene was 100 ppm.

4.5 Bacteria Identification

Extraction of DNA from isolated bacteria was carried out using DNA Sabai Kit. The extracted DNA (Figure 4.12a) concentration was measured with flourometer. Amplification of the 16S rRNA gene from bacterial strains was carried out by PCR. The PCR product (Figure 4.12b) was determined by running on agarose gel. The PCR product were then purified and concentrated to appropriate concentrations required for sequencing analysis.

(a)



(b)

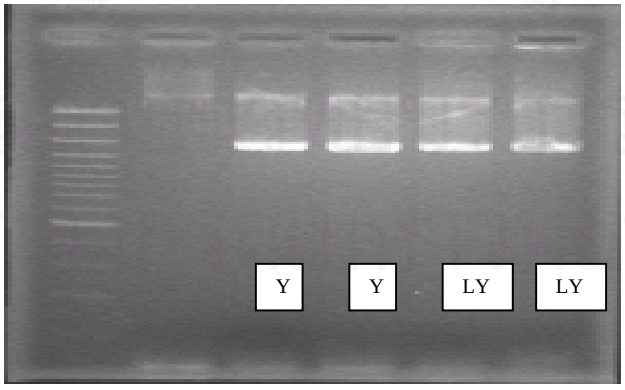


Figure 4.12 (a) DNA extracted from 4 unknown bacteria species and (b) from 2 PCR bacteria species shown in Figure 4.9.

Of the four unknown species of bacteria isolated from the leaves of the ornamental plant, *Ixora* sp., the species of bacteria labeled phP shown in Figure 4.9 could be identified using GenBank databases, BLAST program. The nucleotide sequences obtained were analyzed using GenBank databases, BLAST program. The section of the sequences used for analysis obtained with 27F primer and 1492R primer are shown in (Appendix C). Table 4.2 shows bacteria that have similar sequences to the phP isolate obtained in analysis of sequence from both 27F primer and 1492R primer.

The results reveal the strain phP is closely related to genus *Deinococcus*. *Deinococcus* is a gram-positive bacterium found to form pink or reddish colored colonies. *D*einococcus is known for being the most radiation-resistant vegetative cell. In fact, *Deinococcus radiodurans* can live through blasts of radiation thousands of times greater than the level that would kill a human being, and its Latin name means "strange berry that withstands radiation." This bacterium probably inhabits the leaves of the *Ixora* sp. since this plant can withstand various changes in temperature (high solar radiation) and the *Deinococcus* sp. can survive under these conditions.

Table 4.2 Similarity of phP to bacteria in GenBank Database using BLAST_n program

Bacteria	% Similarity	Accession number	Source	Reference
Deinococcus sp. CRR	485/485 (100%)	DQ003317	Unknown	Food Technology division, Bhanha Atomic research center, India.
Deinococcus sp. *grandias*	480/485 (98%)	Y11329	Soil from Sonoran desert	Int. Journal Syst. Bacteriol. 47(2), 510-514 (1997)
Deinococcus sp.	453/459 (98%)	D78365	Soil from Sonoran desert	Int. Journal Syst. Bacteriol. 179(10), 3350-3353 (1997)
Deinococcus sp. *navajonesis st.* KR33	469/485 (96%)	AY743260	Soil from Sonoran desert	Jour. Appl. Env. Microbio. 71(9), 5225-5235 (2005)
Other: *Deinococcus* sp. 42NP10	280/287	AB242694	Tomato leaf	Unpublished journal. Japan

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Plants have been used in the process of bioremediation known as phytoremediation, as well as for the removal of indoor air pollutants. Studies have also been carried out on the leaves of plants investigating its ability to monitor PAHs in the atmosphere. The accumulation of PAHs on leaves could be a secondary source of air pollution and the degradation of deposited PAHs would reduce the amount of atmospheric PAHs. The main objective of this study was to investigate the possibility of plants and their associated bacteria to degrade PAH deposited on leaf surface.

Phase 1 of this study indicated that the characteristics of leaves were varied with regards to leaf weight, leaf area, position on the plants etc. These differences in characteristics affected the amounts of leaf bacteria, moisture, and wax content. The results showed that there was no significant relationship between the number of bacteria and location, moisture content, or wax content of the leaves and thus colonization of phenanthrene-degrading bacteria probably depended on other plant characteristics. From the six species of plants screened, the leaves of the *Ixora* sp. showed the highest concentration of phenanthrene-degrading bacteria and were therefore used in phase 2 and 3 of this study.

Phase 2 of this study suggested that the indigenous species of bacteria inhabiting the leaves of the *Ixora* sp. were indeed able to degrade phenanthrene. The results of both experiments on monitoring phenanthrene degradation indicated a gradual decrease in percent phenanthrene remaining after each 24 hour period, and a simultaneous increase in bacteria colonization. In the experiment where the entire plants were used the rate of degradation occurred faster than in the experiment using incubated leaves. This is probably because in nature, some of the PAH is volatilizes and some is also used up by plant physical processes such as translocation. Both studies also showed that when the plants were augmented with *Sphingomonas* sp. strain P2 a known phenanthrene-degrading bacteria, the rate of degradation increase rapidly. This was detected along with a rapid increase in number of phenanthrene-degrading bacteria on the augmented leaves. The survival of the augmented bacteria on leaves, however, requires further investigation.

In phase 3, four of the indigenous species of bacteria were isolated from the leaves of the *Ixora* sp. These bacteria showed the ability to degrade phenanthrene in liquid medium. The results indicated that after 9 days the indigenous species could degrade 100 ppm of phenanthrene to undetectable amounts. Of the four indigenous species isolated, we were able to sequence the DNA of phP bacteria and identify its species using GenBank databases and BLAST program as a *Deinococcus* sp. This genus of bacteria has also been found on tomato leaves and studies have indicated their ability to tolerate high radiation.

In conclusion, this study thus suggests the possibility of plant leaf associated bacteria to degrade PAH deposited on leaf surface. Studies by Karnchanasest and Satayavibul (2005) have also showed total PAHs deposition on leaves to be as much as 82.46 mg/kg, thereby decreasing the total concentration of PAHs present in the atmosphere. This procedure of using plants as sinks for atmospheric PAHs deposition thereby decreasing the total amount present in the atmosphere may be applied by planting of ornamental plants such as the *Ixora* sp. or plants with characteristic structures such as thick leaf cuticles and smooth surfaces in area where pollution levels are high or traffic volume is of high intensity. The procedure of using plant leaves for the degradation of PAHs is natural and environmentally friendly and can thus be used as an alternative to conventional technologies.

5.1 RECOMMENDATIONS

Sampling sites should be more than two and perhaps some of these sites should be located in the rural areas. This will justify the effect of pollution in the air in a given location on the characteristics of the leaves, the total amount of PAH deposited on the leaves and thus the effect of PAH deposition on bacteria colonization.

Although, results of both studies to monitor the degradation of phenanthrene using the entire plant or only incubated leaves of *Ixora* sp. showed that leaf associated bacteria of this plant had the ability to degrade phenanthrene, further studies on the effectiveness of these bacteria to degrade PAH needs to be investigated. The actual

percentage of phenanthrene degraded by the bacteria needs to be investigated since this compound is semi-volatile and the plants may also be using phenanthrene in the process of translocation. The amount of phenathrene absorbed into the plant and the amount that volatilize should be further investigated. This can be carried out in chamber experiments, that is spraying the plant enclosed in a chamber with a know concentration of the pollutant and monitoring the amount of pollutant in the chamber as well as the amount on the leaves of the plants.

Further studies should also be carried out to investigate the application of this study in a real situation so as to determine the rate of degradation of phenanthrene, for example applied 100 ppm phenanthrene directly to the leaves on the street. This will assist us in determining the effectiveness of *Ixora* sp. and leaf associated bacteria.

Studies also need to be carried out on the effectiveness of the leaf indigenous bacteria isolated in this study to degrade other PAHs such as pyrene, benzo-pyrene etc.

Finally, other indigenous species should be identified and the degradation rate of these indigenous species should be compared with a known phenanthrene-degrading bacterium such as *Sphingomonas* sp. strain P2 as well as other reported bacteria. These bacteria may be used to augment plant and consequently enhance PAHs degradation.

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APPENDICES

APPENDIX A

Calibration Curve

Calibration curve of the standard was constructed by plotting various concentration of phenanthrene dissolved in 20% dichloromethane in hexane against its corresponding peak area determined by GC analysis.

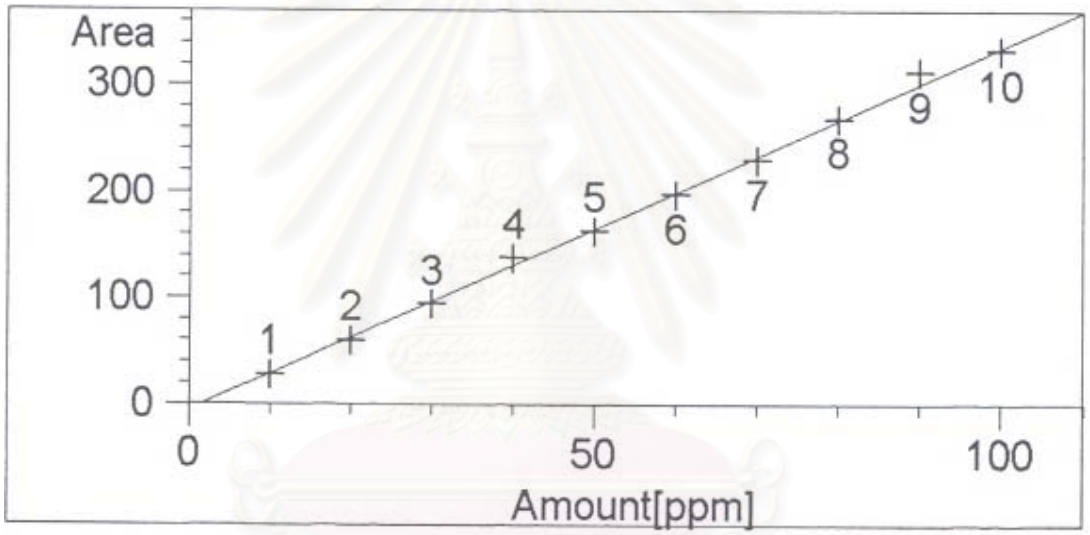


Figure A1 Calibration curve for phenanthrene ; R^2= 0.99

Retention Time

Phenanthrene in 20% dichloromethane was spiked on plant leaves and extracted from leaf sample by microwave. The sample was cleaned by silica gel chromatography and injected into GC/FID column under the conditions described in Table 3.1. A sample

was also injected into GC-MS to verify the presence of phenanthrene in the sample. The The resulting GC chromatograph and GC-MS report is shown below:

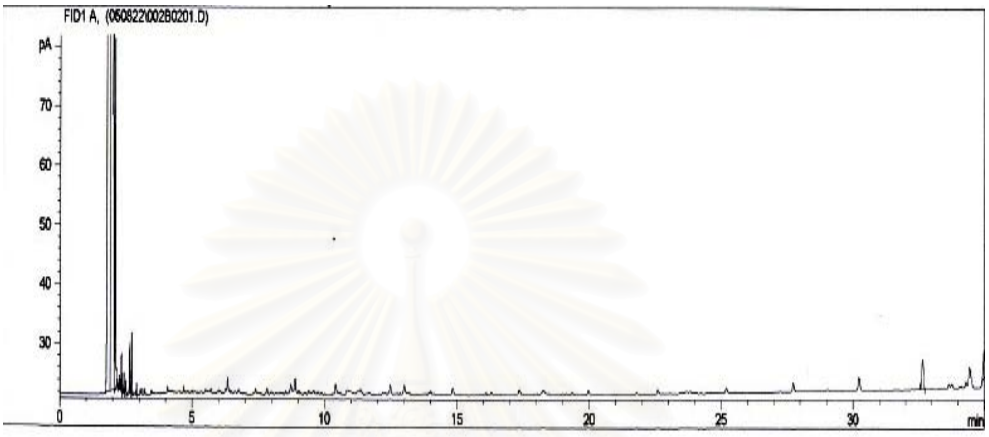


Figure A2 GC Chromatograph of unspiked leaf sample.

Phenanthrene undetected.

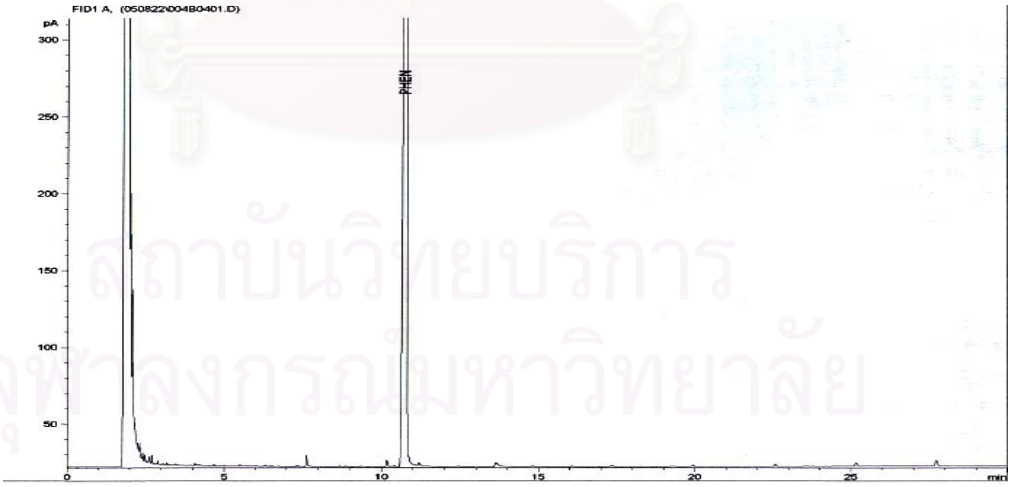


Figure A3 GC Chromatograph of leaves spiked with phenanthrene.

Phenanthrene detected after 10.56 minutes.

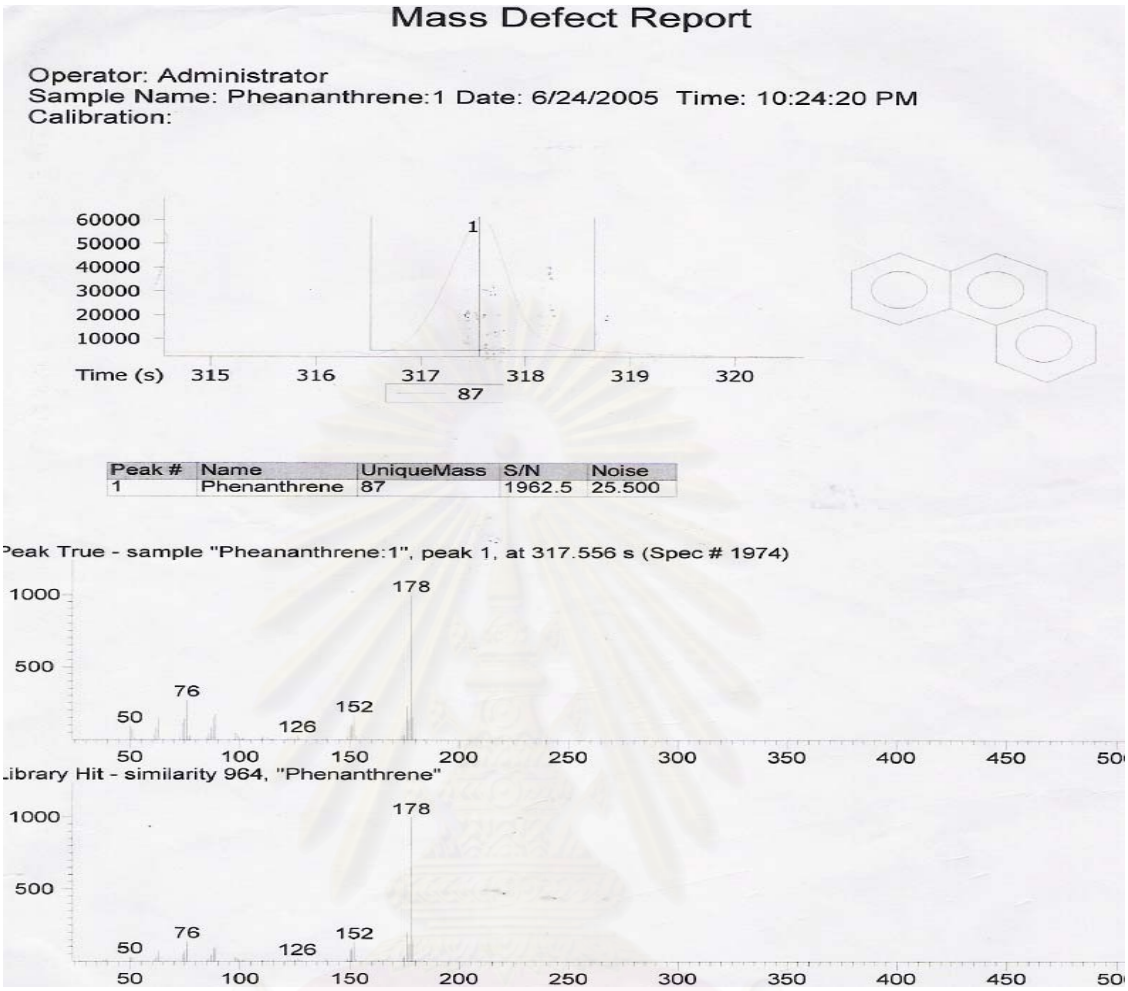


Figure A4 GC-MS Chromatograph of leaves spiked with phenanthrene.

Determining the efficiency of PAH extraction-recovery

The accuracy of the extraction method was determined by investigating the percent phenanthrene recovery in leaves spiked with known concentrations of phenanthrene. This was done in the preliminary investigations and results indicated a recovery of approximately 85%.

The amount of phenanthrene to be recovered by extraction was determined as follows:

Equation 1:

$$\frac{\text{X concentration phenanthrene (ppm) x volume of phenanthrene spiked (µl)}}{\text{Mass of leaves spiked (g)}}$$

Equation 2:

Total amount recovered (ppm) / total amount to be recovered (ppm) x 100%

Eg. 1

8 g of leaves were spiked with 256µl of 10^4ppm phenanthrene. GC results indicated phenanthrene concentration to be 736.518ppm at a retention time of 10.736 minutes.

$$\frac{10^4 \text{ppm} \times 256\ \mu\text{l}}{8\text{g}} = \frac{10^4\ \text{mg} \times 256\mu\text{l}}{10^6\ \mu\text{l} \times 8000\text{mg}}$$

$$= \frac{2.56 \times 10^6}{8 \times 10^9}$$

$$= 3.2 \times 10^{-4} \times 10^6$$

$$= 320\text{ppm}$$

8g leaves evaporated to 3 ml, therefore estimated recovery equal

320ppm x 2.67=854.4 ppm

% recovery = 737.518 ppm / 854.4 ppm x 100 = 86.31%

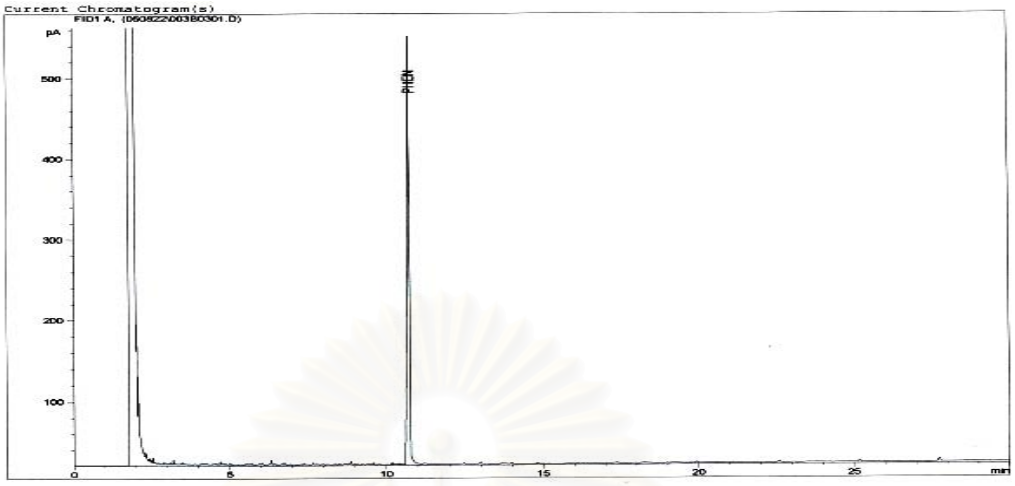


Figure A5 GC Chromatograph showing recovery of phenanthrene when spiked with 256µl of 10^4ppm phenanthrene**.**

Retention time (min): 10.736 Amount phenanthrene (ppm): 737.51855

Appendix B

Table B1 Characteristics of plants collected in phase 1

TABLE SHOWING CHARACTERISTICS OF LEAF SPECIES FROM LOCATION A(JJ-PARK) AND B (ROAD SIDE CHULA)

Leaf species	arrangement of leaves on plant	#leaves/sample(4g)	area(cm^2)/leaf	area(cm^2)/g of leaf	amt. moisture/g	amt. wax/g	avg.CFU/g
Ixora Sp.	opposite	5	43	53.75	0.702	0.00719	19500000
Wrightia religiosa Benth. Ex Kurz -A	opposite	25	14	87.5	0.742	0.01002	710000
Hibiscus rosa-sinensis L.-A	alternate	5	37	46.25	0.765	0.00396	10900000
Pereskia grandiflora Haw.-A	whorl	4	42	42	0.903	0.00307	740000
Hymenocallis littoralis Salisb.-A	opposite	0.5	160	20	0.909	0.00275	193000
Excoecaria cochinchinensis Lour.-A	whorl	11	26	71.25	0.85	0.00471	35000
Ixora Sp.	opposite	5	43	53.75	0.72	0.00728	4300000
Wrightia religiosa Benth. Ex Kurz -B	opposite	25	14	87.5	0.742	0.00838	1300000
Hibiscus rosa-sinensis L.-B	alternate	5	37	46.25	0.795	0.00458	1500000
Pereskia grandiflora Haw.-B	whorl	4	42	42	0.903	0.00341	1630000
Hymenocallis littoralis Salisb.-B	opposite	0.5	160	20	0.915	0.00256	35000
Excoecaria cochinchinensis Lour.-B	whorl	11	26	71.25	0.85	0.003675	53000

Table B2 (i) Results of phenanthrene degradation using *Ixora* sp.

Raw data from GC showing amount of phenanthrene in leaves of *Ixora* sp.

Time (hrs)	Amount of phenantrene present in leaf samples (ppm)			
	control 1	control 2	control 3	avg.
0	165.3	480.8	642.2	429.4333
24	100.5	331.2	495.5	309.0667
48	59.4	292.8	429.3	260.5
72	32.3	99.99	102.1	78.13

Time (hrs)	non-augmented 1	non-augmented 2	non-augmented	avg.
0	552.7	658.3	695.5	635.5
24	376	467.4	484.31	442.57
48	141.3	188	287.78	205.6933
72	19.86	75	186.77	93.87667

Time (hrs)	augmented 1	augmented 2	augmented 3	avg.
0	551.1	963.5	1091.2	868.6
24	280.8	441.4	452.1	391.4333
48	130.9	162.4	317.7	203.6667
72	135	220.9	242.9	199.6

% OF PHENANTHRENE (PPM) REMOVED IN EACH 24 HR. PERIOD

Time (hrs)	% of phenanthrene removed in each 24 hr. period				
	control 1	control 2	control 3	average	sd
0	0	0	0	0	0
24	39.20145	31.11480865	22.843351	28.02919	8.179224
48	64.06534	39.1014975	33.1516661	39.33866	16.40248
72	80.45977	79.20341098	84.101526	81.80626	2.544024

Time (hrs)	non-augmented 1	non-augmented 2	non-augmented 3	average	sd
0	0	0	0	0	0
24	31.97032748	28.99893666	30.36520489	30.35877	1.487295
48	74.43459381	71.44159198	58.62257369	67.63284	8.399457
72	96.4067306	88.60701808	73.14593817	85.2279	11.83881

Time (hrs)	augmented 1	augmented 2	augmented 3	average	sd
0	0	0	0	0	0
24	49.04735983	54.18785677	58.56854839	54.93514	4.765644
48	76.24750499	83.14478464	70.88526393	76.55231	6.145757
72	75.50353838	77.07317073	77.74010264	77.02049	1.148242

* Avg % (P) removed = (avg. amt. of P at time 0)-(avg. amt. of P at time X)/ (avg. amt. of P at time 0) x 100

AVERAGE % PHENANTHRENE REMAINING AFTER EACH 24 HOUR PERIOD

Time(hrs)	control	non-augmented	augmented
0	100	100	100
24	71.97081	69.64123	45.06486
48	60.66134	32.36716	23.44769
72	18.19374	14.7721	22.97951

* Avg. % P remaining = 100% - avg. % P removed

Table B2 (ii) Results of bacteria colonization in *Ixora* sp.

BACTERIA COLONIZATION (CFU/ml) on Ixora species

Time (hrs)	control 1	control 2	control 3	SD
0	4000000	4700000	5400000	700000
24	8600000	7700000	9400000	850490.0548
48	9000000	8100000	8600000	450924.9753
72	14000000	14000000	13000000	577350.2692

Time (hrs)	indigenous 1	indigenous 2	indigenous 3	SD
0	20000000	22000000	19000000	1527525.232
24	30000000	38000000	40000000	5291502.622
48	39000000	38000000	45000000	3785938.897
72	49000000	48000000	55000000	3785938.897

Time (hrs)	augmented 1	augmented 2	augmented 3	SD
0	67000000	66000000	61000000	3214550.254
24	66000000	83000000	83000000	9814954.576
48	82000000	85000000	91000000	4582575.695
72	89000000	85000000	83000000	3055050.463

Average amount of bacteria colonized in each 24 hour period

Time (hrs)	control	indigenous	augmented
0	4700000	20333333.33	64666666.67
24	8566666.667	36000000	77333333.33
48	8566666.667	40666666.67	86000000
72	13666666.67	50666666.67	85666666.67

Calculation:
(sample 1 + sample 2 + sample 3) / 3

Table B3(i) Results of phenanthrene degradation in incubated leaves

Raw data from GC showing amount of phenanthrene in incubated leaves

Time (hrs)	Amount of phenantrene present in leaf samples (ppm)			
	control 1	control 2	control 3	avg.
0	1560.15	1128.07	1352.96	1347.06
24	1633.8	1056.04	1387	1358.947
48	1812.17	1818.14	2064	1898.103

Time (hrs)	non-augmented 1	non-augmented 2	non-augmented 3	avg.
0	2154.39	2221.74	2549	2308.377
24	1891.6	1867.4	1531	1763.333
48	1542.65	1351.19	1431	1441.613

Time (hrs)	augmented 1	augmented 2	augmented 3	avg.
0	997.14	546.61	775	772.9167
24	321.75	273.44	225	273.3967
48	175.48	172.78	173	173.7533

Time (hrs)	frozen 1	frozen 2	frozen 3	avg.
0	901.28	1017.87	1340.25	1086.467
24	903.2	997.38	1202	1034.193
48	915.61	942.8	1108	988.8033

% OF PHENANTHRENE (PPM) REMOVED IN EACH 24 HR. PERIOD

Time (hrs)	% of phenanthrene removed in each 24 hr. period				
	control 1	control 2	control 3	average	sd
0	0	0	0	0	0
24	-4.7207	6.385242051	-2.515964995	-0.88242	5.879828
48	-16.1536	-61.17262227	-52.55439924	-39.6746	23.89564

Time (hrs)	non-augmented 1	non-augmented 2	non-augmented 3	average	sd
0	0	0	0	0	0
24	12.19788432	15.94876088	39.93723029	23.61154	15.04985
48	28.39504454	39.18325277	43.86033739	37.54861	7.931329

Time (hrs)	augmented 1	augmented 2	augmented 3	average	sd
0	0	0	0	0	0
24	67.73271557	49.97530232	70.96774194	64.62792	11.30246
48	82.40166877	68.39062586	77.67741935	77.51978	7.128258

Time (hrs)	frozen 1	frozen 2	frozen 3	average	sd
0	0	0	0	0	0
24	-0.21303	2.013027	10.31524	4.811315	5.548672
48	-1.374	5.472338	7.8203	4.388928	4.777037

* Avg % (P) removed = (avg. amt. of P at time 0)-(avg. amt. of P at time X)/ (avg. amt. of P at time 0) x 100

AVERAGE % PHENANTHRENE REMAINING AFTER EACH 24 HOUR PERIOD

Time(hrs)	control	non-augmented	augmented	frozen
0	100	100	100	100
24	100.8824	76.38845769	35.37207547	95.18869
48	139.6746	62.4513908	22.48021563	95.61107

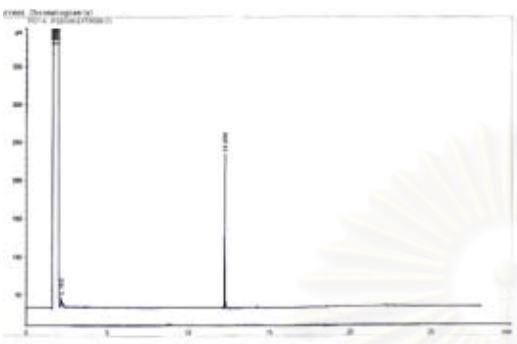
*Avg. % P remaining = 100% - avg. % P removed

Table B2 (ii) Results of bacteria colonization in *Ixora* sp.

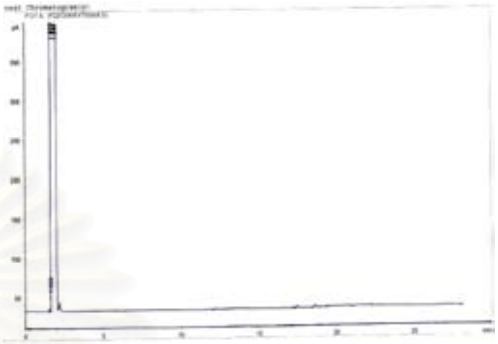
Average amount of bacteria colonized in each 24 hour period

Time (hrs)	control	non-augmneted	augmented	frozen
0	5000	75000000	112000000	35000
24	2000	81000000	310000000	28000
48	0	115000000	420000000	0

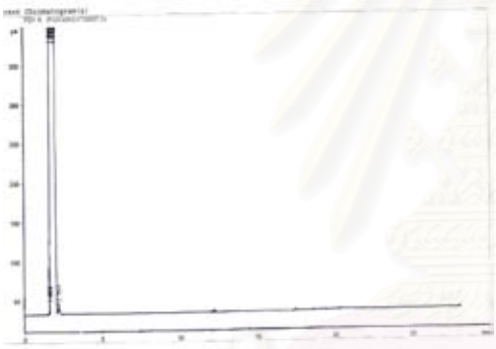
Figure B a,b,c,d,e GC chromatographs showing degradation of 100 ppm phenanthrene after 4 days by indigenous bacteria shown in Figure 4.9



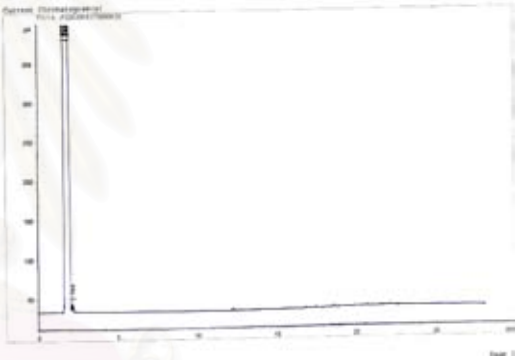
(a) Control 4



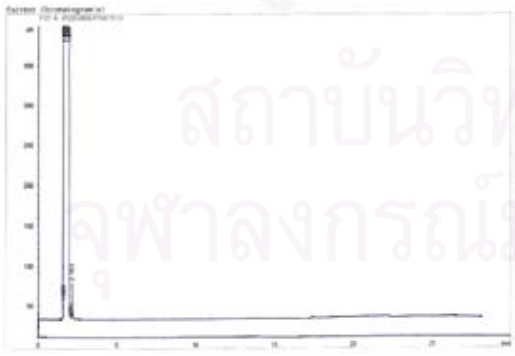
(b) Ly 4



(c)W4



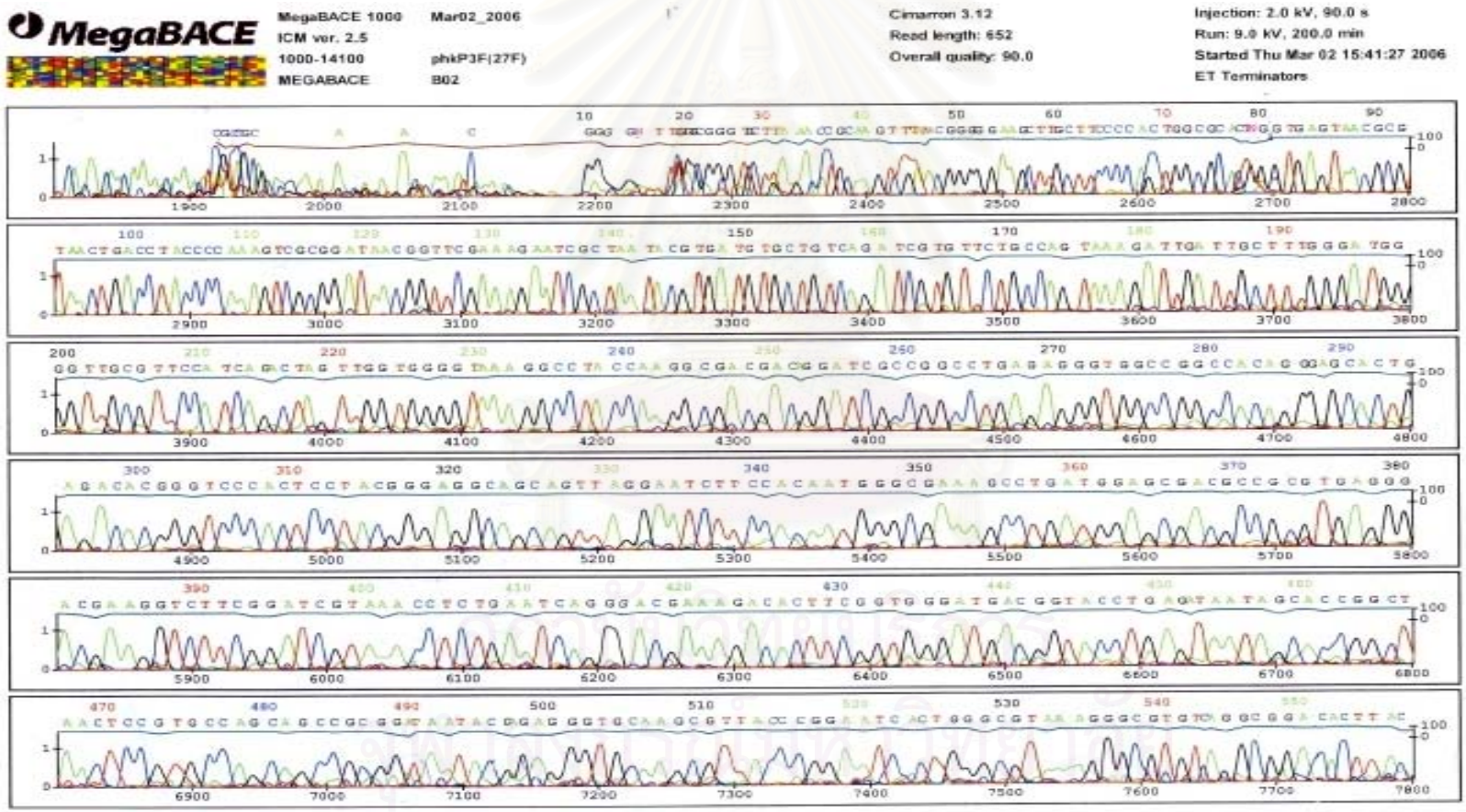
(d) L4

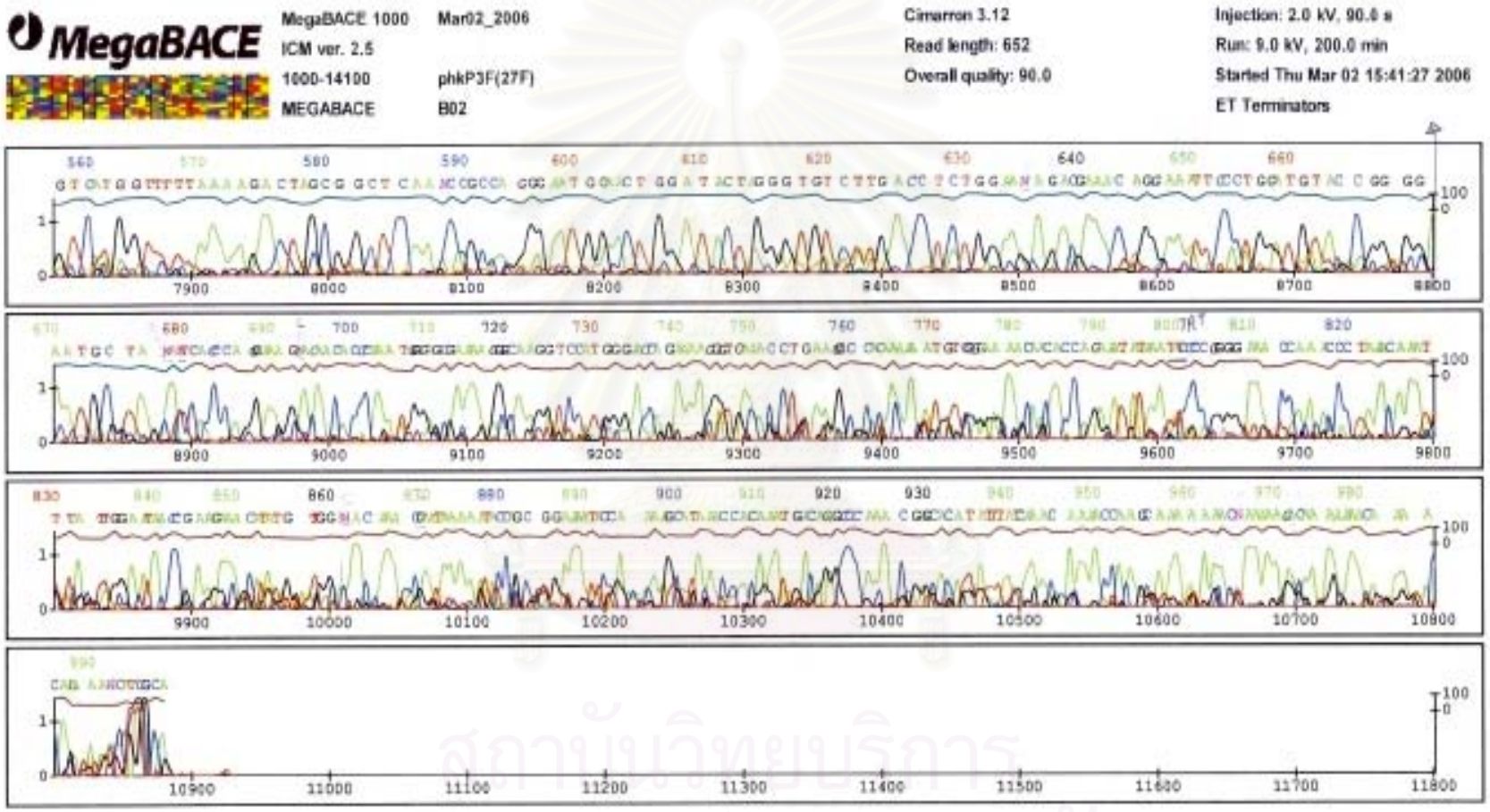


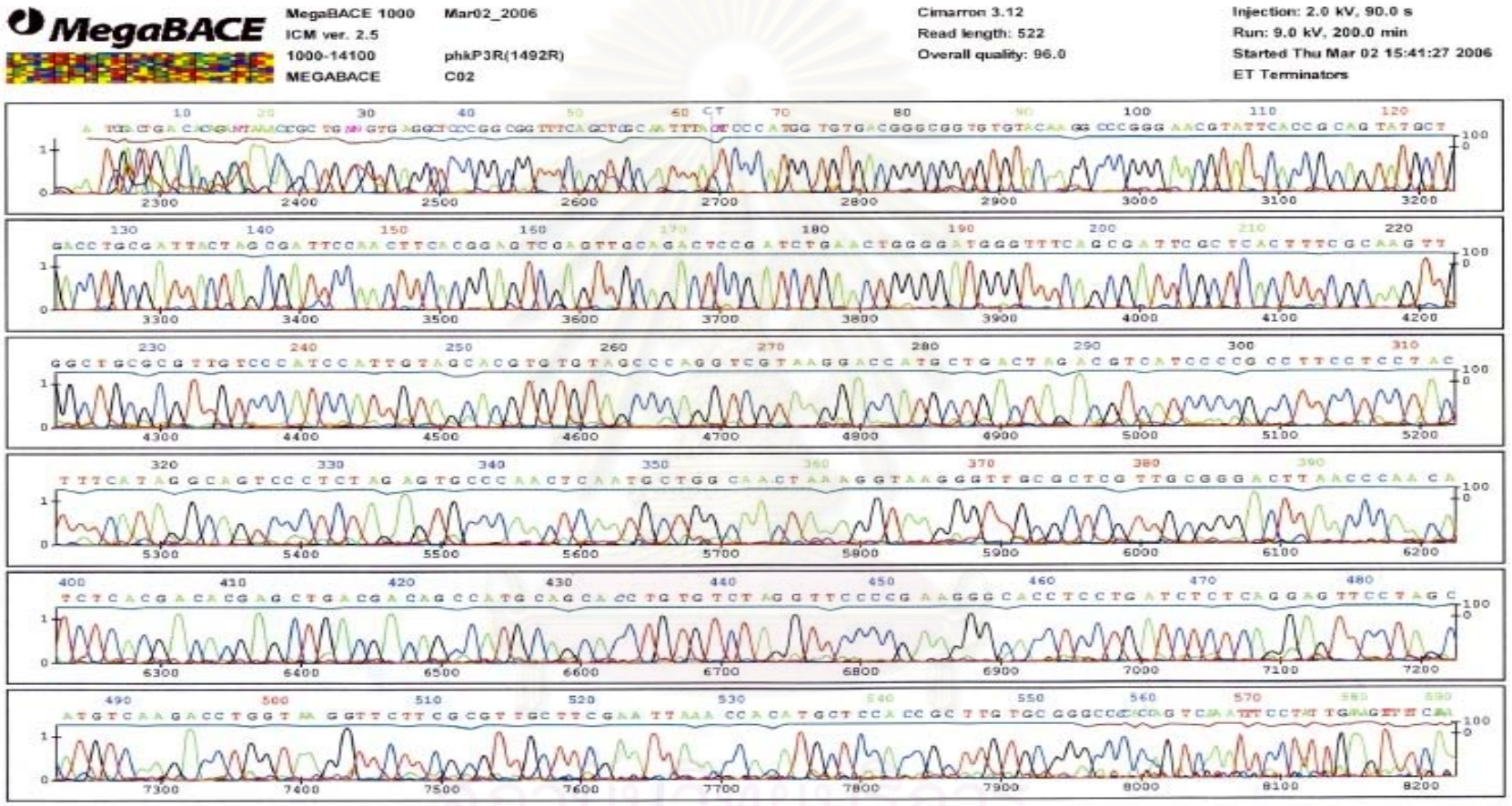
(e) P4

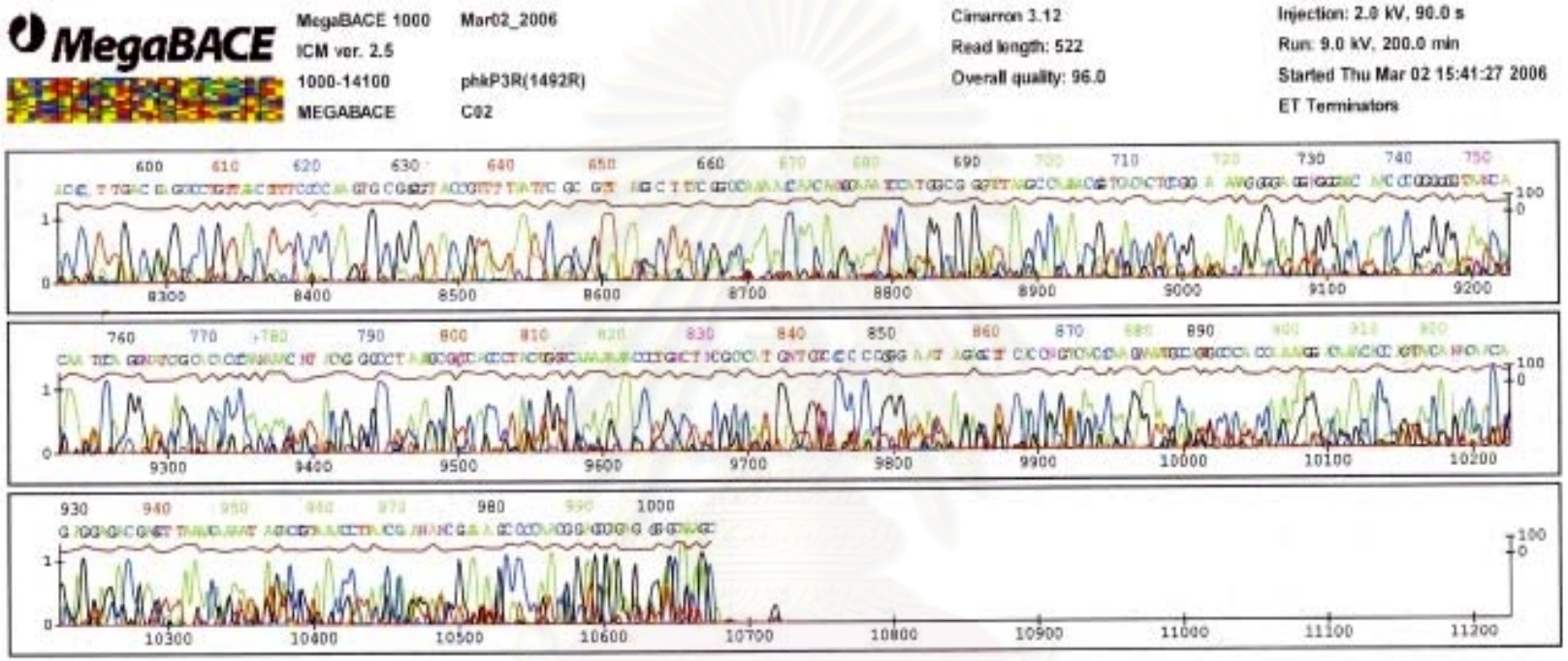
Appendix C

Data from DNA sequencing of Bacteria phP









Data from DNA sequencing used in sequencing analysis

Section from 27F primer used in sequencing analysis

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1 GTGAGTAACG CGTAACTGAC CTACCCCAAA GTCGCGGATA ACGGTTCGAA
51 AGAATCGCTA ATACGTGATG TGCTGTCAGA TCGTGTTCTG CCAGTAAAGA
101 TTGATTGCTT TGGGATGGGG TTGCGTTCCA TCAGACTAGT TGGTGGGGTA
151 AAGGCCTACC AAGGCGACGA CAGGATCGCC GGCCTGAGAG GGTGGCCGGC
201 CACAGGGAGC ACTGAGACAC GGGTCCCACT CCTACGGGAG GCAGCAGTTA
251 GGAATCTTCC ACAATGGGCG AAAGCCTGAT GGAGCGACGC CGCGTGAGGG
301 ACGAAGGTCT TCGGATCGTA AACCTCTGAA TCAGGGACGA AAGACACTTC
351 GGTGGGATGA CGGTACCTGA GATAATAGCA CCGGCTAACT CCGTGCCAGC
401 AGCCGCGGAT AATACGAGAG GGTGCAAGCG TTACCCGGAA TCACTGGGCG
451 TAAAGGGCGT GTCAGGCGGA
```

Section from 1492R primer used in sequencing analysis

```
1  CCCATGGTGT GACGGGCGGT GTGTACAAGG CCCGGGAACG TATTCACCGC
51 AGTATGCTGA CCTGCGATTA CTAGCGATTC CAACTTCACG GAGTCGAGTT
101 GCAGACTCCG ATCTGAACTG GGGATGGGTT TCAGCGATTC GCTCACTTTC
151 GCAAGTTGGC TGCGCGTTGT CCCATCCATT GTAGCACGTG TGTAGCCCAG
201 GTCGTAAGGA CCATGCTGAC TAGACGTCAT CCCCGCCTTC CTCCTACTTT
251 CATAGGCAGT CCCTCTAGAG TGCCCAACTC AATGCTGGCA ACTAAAGGTA
301 AGGGTTGCGC TCGTTGCGGG ACTTAACCCA ACATCTCACG ACACGAGCTG
351 ACGACAGCCA TGCAGCACCT GTGTCTAGGT TCCCCGAAGG GCACCTCCTG
401 ATCTCTCAGG AGTTCCTAGC ATGTCAAGAC CTGGTAAGGT TCTTCGCGTT
451 GCTTCGAATT AAACCACATG CTCCACCGCT TGTGC
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Appendix D

Full paper submitted for conference on Hazardous Waste Management for a Sustainable Future held in Bangkok, Thailand from January 10-12, 2006.

APPLICATION OF PLANTS AND THEIR ASSOCIATED BACTERIA FOR ATMOSPHERIC PAH DEGRADATION

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are continuously generated by the incomplete combustion of organic fuels from traffic and industrial processes. Such pollutants may cause serious health problems due to their toxicity, mutagenicity, and carcinogenicity. Interest in using plant leaves as a mechanism for biodegradation of atmospheric PAH is motivated by the fact that leaves are exposed to the atmosphere and microbes are the most abundant inhabitants of the phyllosphere. In leaves, PAHs becomes potentially available to microbial degradation as it deposits onto plants surfaces by gaseous and particle-bound deposition. The objectives of this study were to identify an ornamental plant that contained high number of PAH degrading bacteria and to apply this plant and its associated bacteria to reduce atmospheric PAH. Six plants were studied; namely *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. Leaf samples were collected from two locations in Bangkok that had high and low amounts of air pollutants. The concentrations of phenanthrene degrading bacteria on leaves were ranged from 3.5×10^4 to 1.95×10^7 CFU/g. No significant relationship was detected between the number of bacteria and locations, moisture content, or wax content of the leaves. Of the six plants investigated, *Ixora* sp. showed highest concentration of phenanthrene degrading bacteria with an average of 9.7×10^6 CFU/g. The different bacterial colonization was probably due to differences in leaf characteristics such as leaf area, texture, arrangement, and nutrient availability. Further laboratory studies were carried out using *Ixora* sp. to determine the rate of PAH degradation by indigenous bacteria and added *Sphingomonas* sp. P2, a known PAH degrader. The results showed that *Ixora* sp. and their associated bacteria effectively reduced the amount of PAH that accumulated on the leaves.

Keywords: PAH; biodegradation; plant; air pollutants

INTRODUCTION

There is a growing concern about the quality of air resulting from industrialization. Polycyclic aromatic hydrocarbons (PAHs), a group of pollutants consisting of two or more benzene rings interlinked in various arrangements is of critical public health and environmental concern due to their chronic health effects, carcinogenicity, microbial agitation and high bioaccumulation potential (Hutzinger, 1980). Emission from human activities such as vehicle exhausts and increase in industries are the predominant sources in urban areas (Garivait, 1999). Consequently, sites nearer urban centers have much higher PAH deposition rates than rural areas.

The interest of plant leaves as a possible method for the degradation of atmospheric PAHs is motivated by the fact that leaves are exposed to the atmosphere and microbes are the most abundant inhabitants of the phyllosphere. The deposition of PAH on plants may occur via several pathways. PAH compounds bound to atmospheric particles can reach the plant surface by both dry and wet deposition (Bakker et al., 2000). This pathway involves the transportation from the atmosphere to the laminar air boundary layer surrounding the leaf, the crossing of the boundary layer and the interaction of the molecule with the leaf surface. For gases, they are sorped onto the surface or diffused into the cuticle (Schreiber et al., 1992).

Biodegradation would be the major pathway for reduction of PAHs that accumulated on leaves. Microbial communities of leaves are diverse and include many different genera of bacteria, fungi, yeast, algae, and less frequently, protozoa and nematodes. Bacteria are often found up to 10^8 cfu/g leaf (Andrews et al., 2000). The application of leaves and phyllosphere bacteria for reducing air pollutant was first carried out by De Kempeneer et al. (1998). They studied the removal of airborne toluene by *Azalea indica* that augmented with toluene-degrading bacteria, *Pseudomonas putida* TVA8. Under the same experimental conditions, inoculated *Azalea* plants removed toluene within 27 hrs compared to 75 hrs in uninoculated plants. Meanwhile, PAHs degradation has never been studied on leaves.

The objectives of this study were 1) to identify an ornamental plant that contained high number of PAH degrading bacteria and 2) to apply this plant along with its associated phyllosphere bacteria for degrading PAHs deposited on leaves. Phenanthrene was used as a modeled PAH and applied directly to plant leaves in biodegradation experiment. The study also investigated effects of bioaugmentation by inoculating a known PAH-degrading bacterium, *Sphingomonas* sp. P2 (Supaka et al., 2001). The application of plant leaves and bacteria for removal of organic air pollutants will improve environmental quality in an economic, safe and sustainable way.

MATERIALS AND METHODS

Plant samples

Six ornamental plants were used in the screening phase. These included *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. Leaf samples were collected during summer 2005 from roadside areas in Bangkok. A plant species was then selected for PAHs biodegradation experiment.

Microorganism and culture maintenance

Sphingomonas sp. P2 generally utilizes phenanthrene as the sole carbon source thus it was cultured on mineral salt (MS) agar and incubated in phenanthrene-equlibrated glass box. Approximated every two weeks, the culture was transferred to a new medium. Mineral salt (MS) medium was used to supply essential nutrients for bacteria and prepared according to Focht (1994). Phenanthrene-induced *Sphingomonas* sp. P2 cells for PAHs degradation experiment were prepared according to Deangrueng (2005).

Quantifying phenanthrene degrading bacteria

Phenanthrene utilizing bacteria were used to represent the bacteria that are capable of PAHs degradation. The number of phenanthrene utilizing bacteria was determined by spread plate technique. To extract bacteria samples: 4 g of freshly picked leaf was immersed in 40 ml potassium phosphate buffer and shake for 30 min to dislodge the bacteria. The extracted solution was diluted with MS medium before spreading on MS agar. Spread plates were incubated at room temperature in a glass box supplied with phenanthrene for 2-3 weeks. Bacteria colonies were then count and the results were averaged.

Moisture content determination

Moisture content was determined by weighing 4 g of leaf sample on a tarred crucible and drying the sample for 8 hrs at 105 °C in oven. Samples are cooled in a desiccator and weighed. The percentage of moisture is calculated as follows:

% moisture = weight of sample-weight of dry sample /weight of sample x 100.

Wax content determination

Percentage wax is determined by cuticle extraction as follows: 4 g leaf sample was mixed with 20 ml hexane and placed in a microwave (Milestone ETHOS SEL) at 100°C for 10 minutes and 115°C for 10 minutes. The extract was allowed to cool and filtered through the GF/C filter into a pre-weighed drying round-bottom flask. The flash was evaporated by rotary evaporator at 35°C until the solvent dried out. The round bottom flask was reweighed, and the percentage of wax was calculated as follows:

%wax = reweighed flask-preweighed flash/weight of sample x 100

Note: % wax is defined as all those dissolve in hexane

PAH extraction and analysis

PAH extraction and analysis was modified from EPA's standard method (as in extraction of wax). The average detection limit was 1.83 mg/Kg. The extracted sample was then

evaporated by rotary evaporator at 69 ° C to a volume of 4 ml. Samples were then cleaned up by silica gel column according to Karnchanasest and Satayavibul (2005). GC analysis was performed with a Hewlett-Packard 6890 equipped with a FID detector and a HP-5 fused-silica capillary column (30m x 0.32mmID; thickness, 0.25 μm). The following operating conditions were used: Injector- splitless, temperature (280^{0}C); Detector- FID, temperature (250^{0}C); temperature program- initial 80^{0}C, 1 min., Rate 1; 25 ^{0}C until 160^{0}C for 3 min and 3^{0}C until 300^{0}C for 2 min; column temperature-325^{0}C; Gasses- Nitrogen (carrier); 100kPa, Nitrogen (make-up); 45 ml/min., Air (detector); 45 ml/min and helium (detector); 40 ml/min.; injection volume: 2 μl.

PAHs biodegradation experiment

To determine PAHs degradation on plant leaves, three treatments consisted of non-augmentation, bioaugmentation and control (bacteria removal by ethanol) were compared. The selected plant species were purchased from a florist. There were approximately two months old and 42 cm in height. In non-augmented plant, each plant was sprayed with 30 ml of deionized water. Bioaugmentation was performed by spraying the leaves with 30 ml suspension of *Sphingomonas* sp. P2 (approximately 10^7CFU/ml). The control plants were sprayed with equal volume of 70% ethanol to remove as much phyllosphere bacteria as possible. PAHs were applied using 10 mg/ml phenanthrene solution at 10 ml per plant. The plants were then covered and sealed in plastic bags. After 24 hrs, the initial amount of phenanthrene absorbed on leaves and the initial concentrations of bacteria were determined by sampling 4 g of leaves randomly. Percent phenanthrene degradation was calculated from the difference between the initial amount of phenanthrene (100% baseline) and the remaining phenanthrene afterward. Number of phenanthrene degrading bacteria on the leaves was also monitored.

RESULTS AND DISCUSSION

Screening of ornamental plants

Six ornamental plants were collected from two locations in Bangkok, a highly polluted area and a less polluted area. The highly polluted area chosen in this study is in Lad Phrao area along the roadside of Jatujak market. This is a commercial area with six lanes of road and there is usually a high traffic volume in the area. The less polluted area was on Phaya Thai Road in front of Chulalongkorn University. The traffic volume in this area is of much lower intensity. These ornamental plant species were selected randomly and included *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. Table 1 showed general characteristics of these plants, which were different from one another in terms of leaf arrangement, texture, area, and weight.

The characteristics of leaf play a vital role in PAH absorption; for example, sparsely hair leaves are more heavily contaminated than densely hair since lower canopy roughness increases aerodynamic resistance (Bakker et al., 2000). High leaf over lap decreases PAH accessibility and thicker laminar boundary decrease absorption. Meanwhile, PAHs were under detection limit in all collected leaves (Table 1). Karnchnasest and Satayavibul (2005) monitored the amount of atmospheric PAHs in orange jasmine leaves, *Murraya paniculata* (L.) Jack collected from Bangkok roadsides in 2002. Amount of total PAHs

was ranged from 63.99 to 82.46 mg/kg leaves. The high amounts of PAHs in their study were probably due to the differences in plant species, sampling location, and sampling period.

Table 1. Characterization of plant leaves in the study.

Plant	Leaf Arrangement	Leaf Texture	Leaf area[1] (cm^2/leaf)	Leaf weight[1] (g/leaf)	Total PAHs (ppm)
Ixora sp.	Opposite	Medium, glossy leaves	43	0.80	n/d[2]
Wrightia religiosa Benth. Ex Kurz	Opposite	Light, smooth	14	0.16	n/d
Hibiscus rosa-sinensis L.	Alternate	Heavy, glossy, fairly moist	37	0.80	n/d
Pereskia grandiflora Haw.	Whorl	Rough, hairy	42	1.00	n/d
Hymenocallis littoralis Salisb.	Opposite	Heavy, smooth	160	8.00	n/d
Excoecaria cochinchinensis	Whorl	Rough, hairy	26	0.36	n/d

[1]Each value was averaged from two locations.
[2]n/d- the concentration is under detection limit (1.83 ppm).

Leaf samples were also analyzed for the number of phenanthrene degrading bacteria, percent moisture content, and percent wax content. Plants when grown at different locations had different number of phenanthere degrading bacteria but similar moisture and wax content (Figure 1). The results showed that concentrations of phenanthrene degrading bacteria on leaves ranged from $3.5x10^4$ to $1.95x10^7$ CFU/g (Figure 1A). When compared between plants in each location, *Ixora* sp. contained the highest concentration of phyllosphere bacteria with an average of $9.7x10^6$ CFU/g. *Ixora* sp. is easily grown, can withstand various changes in temperature, grows all year round, and is inexpensive. The results thus suggest that *Ixora* sp. have the ability to be used in the bioremediation of atmospheric PAHs.

In regards to the relationship between bacteria colonization and leaf moisture content, an R^2 factor of 0.54 and 0.47 was obtained in location 1 and 2, respectively (Figure 2A). This indicates a very low relationship between these two parameters. The relationship between bacteria colonization and wax content was even more insignificant. An R^2 factor of 0.04 and 0.37 were obtained in location 1 and 2, respectively (Figure 2B). The results suggested that there was no significant relationship between the number of bacteria and locations, moisture content, or wax content of the leaves. Colonization of phenanthrene degrading bacteria probably depended on other plant characteristics. A study by Kinkel et

al. (1997) showed that the total number of culturable bacteria recovered from broad leaf plants such as cucumber and bean was significantly greater than that recovered from grasses or waxy broad leaf plants. This suggested that plant species appeared to influence the microbial carrying capacity of the leaf.

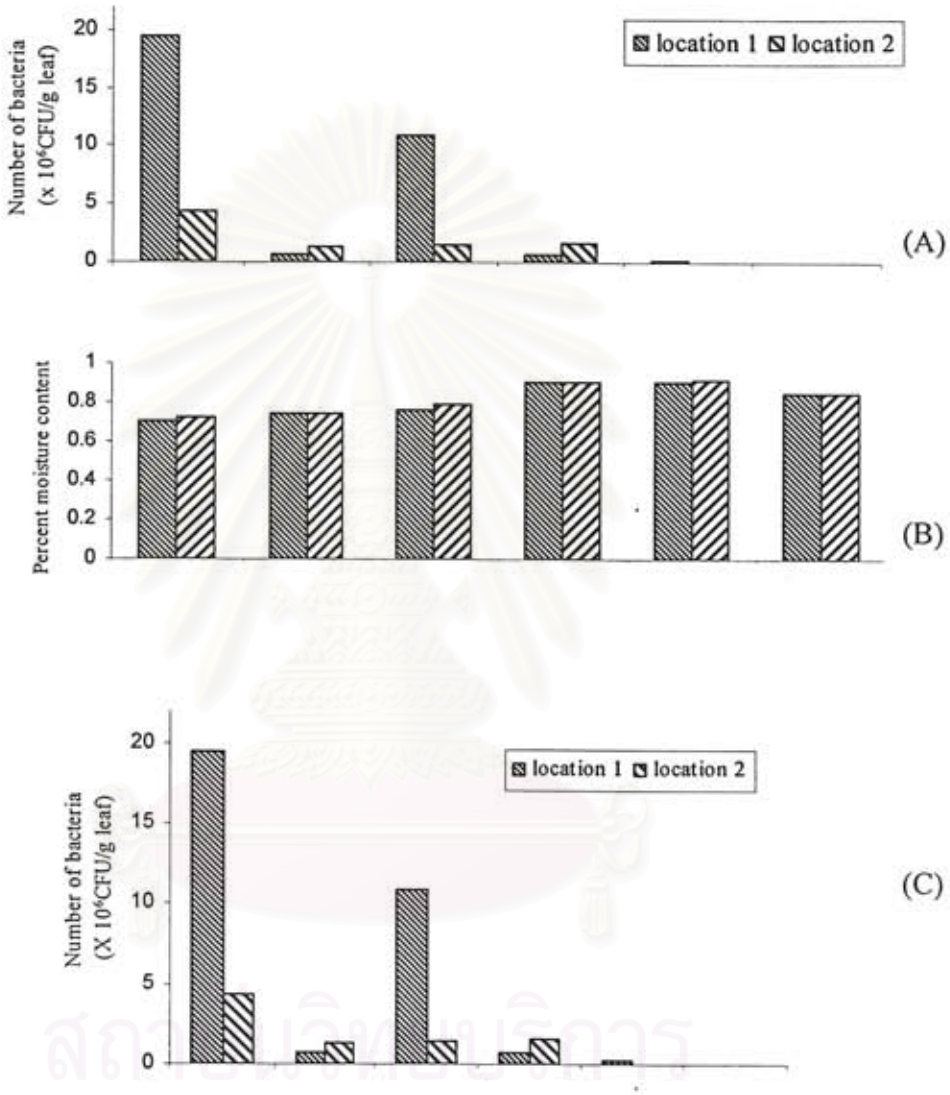

Figure 1. Number of phenanthrene degrading bacteria (A), percent moisture content (B), and percent wax content (C) in six ornamental plants. Location 1 and 2 are highly and less polluted area, respectively.

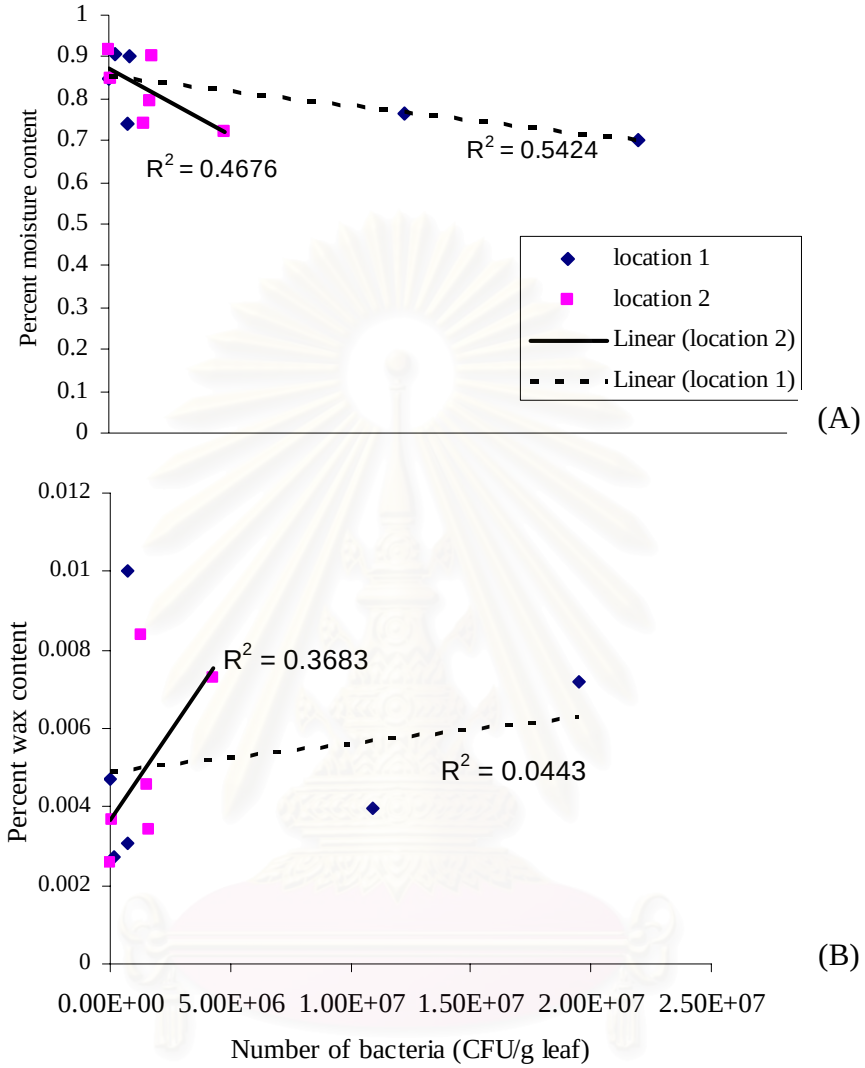

Figure 2. Relationship between number of phenanthrene degrading bacteria and percent moisture content (A) and percent wax content (B). Location 1 and 2 are highly and less polluted area, respectively.

Biodegradation of PAHs by ornamental plants and phyllosphere bacteria

PAHs biodegradation experiments were carried out by spraying phenanthrene on the leaves of *Ixora* sp. that were augmented with *Sphingomonas* sp. P2, non-augmented, or cleaned with ethanol (control). The initial amount of phenanthrene deposited onto each plant varied from 480 to 960 ppm. In any case, the initial amount was taken as 100% baseline and time 0. Percent phenanthrene remaining was monitored every 24 hrs thereafter (Figure 3A). The amount of phenanthrene remaining in control plants ranged from 68.9% after 24 hrs to 18.3% after 72 hrs. In non-augmented plants, the amount of

phenanthrene ranged from 69.55% after 24 hrs to 13.94 % after 72 hrs. There were 46.1 % and 23.2% of phenanthrene after 24 hrs and 72 hrs in the augmented plants, respectively.

When comparing the extent of phenanthrene degradation between augmented, non-augmented and control plants, results indicated that degradation in augmented plants increases rapidly within the first 48 hrs with a total of 23.2% phenanthrene remaining in comparison to 31.8 % in the non-augmented and 63.8% in the control. This suggested that augmentation of this plant species with *Sphingomonas* sp. P2 increased the amount of PAH removal as well as the rate of degradation. Similar results were reported by Supaka et al. (2001), who showed that this bacterium could rapidly degrade phenanthrene in liquid medium from 100 ppm to undetectable amount by HPLC analysis within 72 hrs.

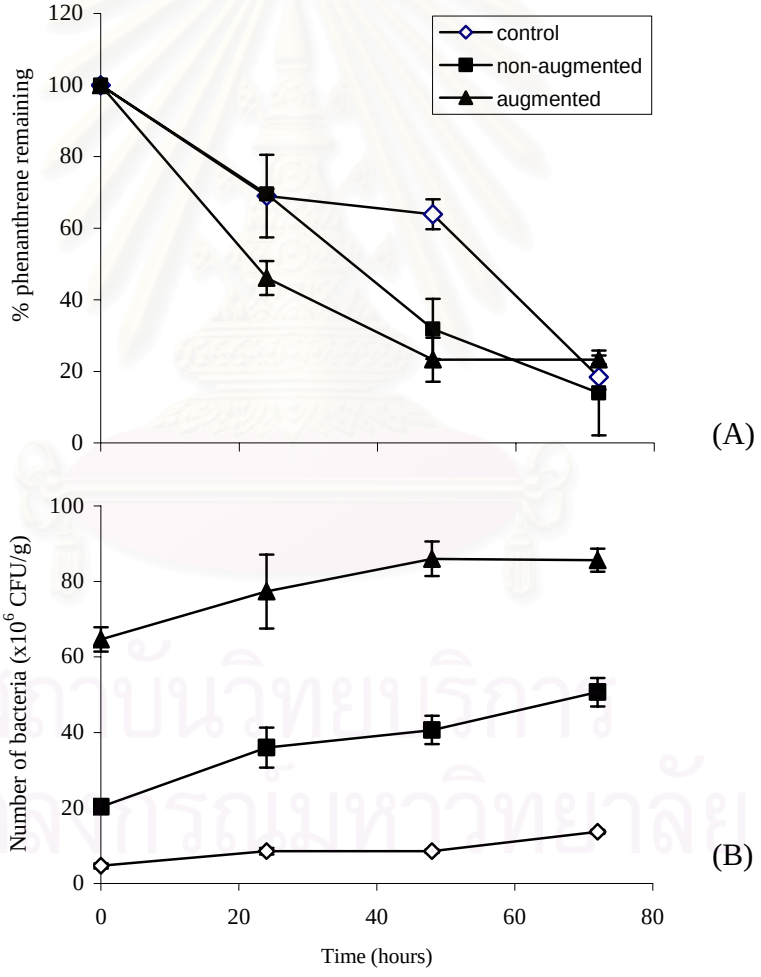

Figure 3. Percent phenanthrene remaining (A) and number of phenanthrene degrading bacteria (B) on leaves after treatment.

After 72 hrs, the percent phenanthrene remaining in all treatments were similar, suggesting that phenanthrene degradation by the augmented bacteria depends on their survival and ability to compete with indigenous phyllosphere microorganisms. It also suggests that the indigenous bacteria are in fact good PAH degraders but may require some adjustments before degrading phenanthrene. This is similar to a study by De Kempeneer et al. (1998) who found that uninoculated *Azalea indica* can reduce the amount of airborne toluene. Meanwhile, the inoculation with toluene degrading bacteria is essential for obtaining rapid removal rates.

The number of phenanthrene degrading bacteria in augmented plants was significantly higher than the non-augmented and control plants at all time points (Figure 3B). However, there was a continuous increase in bacteria colonization in the non-augmented species but a slight decrease in the augmented after 48 hrs. When comparing the relationship between bacteria colonization and percent phenanthrene remaining within each 24 hr period, results indicates that bacteria colonization increase along with percent phenanthrene decreases. This result suggests that indigenous phyllosphere bacteria were indeed utilizing phenanthrene as carbon source. On the other hand, the augmented bacteria could not grow well on leaf surface.

In conclusion, the study clearly indicates that certain ornamental plants such as *Ixora sp.* does contain bacteria capable of PAH degradation and that bio-augmentation with *Sphingomonas* sp. P2 increase the rate of degradation. The application of plant leaves for removal of organic air pollutants is of considerable economic value since it is not only cheaper than conventional technologies which are expensive but it’s also effective. Plants are also a natural and environmental friendly way of air pollutant removal, as they naturally remove excess carbon dioxide from the atmosphere and acts as a collection site for dust and air particles.

BIOGRAPHY

Mrs. Karen Waight nee Bautista was born on May 30th, 1973 in Santa Elena, Cayo District, Belize C.A. She received her Bachelor's Degree from the University College of Belize in 1994 with a major in Biology Education. She then pursued a teaching career as a Biology and Integrated Science teacher at Sacred Heart College in San Ignacio, Belize C.A. from 1994-2003. In May 2004 she decided to pursue her Master Degree study in the International Postgraduate Programs in Environmental Management, Inter-Department of Environmental Management, Chulalongkorn University, Bangkok, Thailand. She completed her Master of Science Degree in Environmental Management in May 2006.