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จุฬาลงกรณ์มหาวิทยาลัย

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NATURAL ATTENUATION, BIOSTIMULATION AND BIOAUGMENTATION IN 4-CHLOROANILINE CONTAMINATED SOIL

Miss Roongnapa Tongarun

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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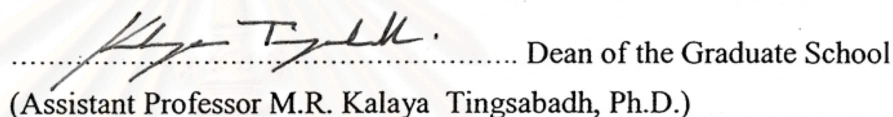
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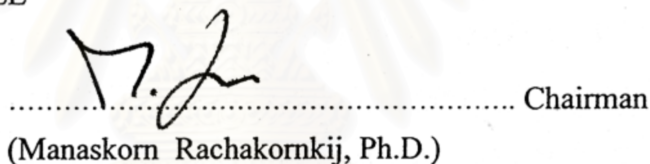
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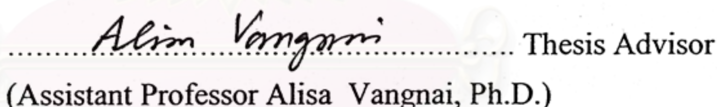
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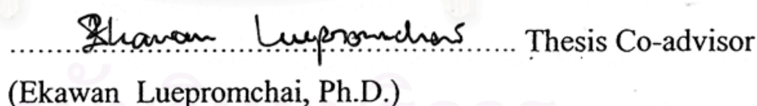
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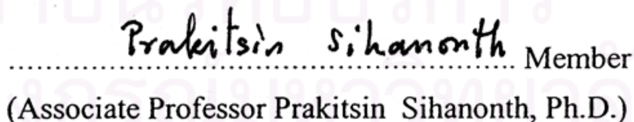
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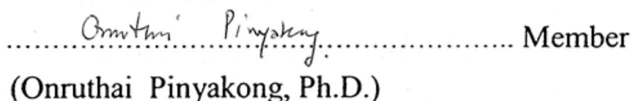
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4-คลอโรอะนิลีนเป็นสารที่มีการใช้กันอย่างกว้างขวางในอุตสาหกรรม 4-คลอโรอะนิลีนยังเป็นสารตัวกลางระหว่างการสลายสารกำจัดวัชพืชด้วยเชื้อจุลินทรีย์ เนื่องจากความเป็นพิษและความทนทานในสิ่งแวดล้อมของ4-คลอโรอะนิลีน จึงจำเป็นต้องมีเทคโนโลยีมาลดและบำบัดสารนี้ วิธีบำบัดทางชีวภาพซึ่งเป็นวิธีที่ประหยัดและสามารถก่อให้เกิดกระบวนการย่อยสลายที่สมบูรณ์ จึงเป็นเทคนิคหนึ่งที่สามารถใช้ในการบำบัด4-คลอโรอะนิลีนได้ จุดมุ่งหมายของการศึกษานี้ได้แก่การหาเทคนิคของการบำบัดด้วยวิธีทางชีวภาพที่ดีที่สุดเพื่อบำบัด4-คลอโรอะนิลีนที่ปนเปื้อน วิธีบำบัดทางชีวภาพประกอบด้วย การสลายสารตามธรรมชาติ (Natural attenuation), การใช้สารเร่งการสลายทางชีวภาพ (Biostimulation) และ การเติมเชื้อจุลินทรีย์ ซึ่งมีทั้งการใช้จุลินทรีย์เดี่ยวหรือเชื้อจุลินทรีย์ (Bioaugmentation) ถูกนำมาใช้เพื่อการศึกษาการย่อยสลาย4-คลอโรอะนิลีนที่มีความเข้มข้น 500 ส่วนในล้านส่วน ซึ่งปนเปื้อนในดิน 2 ชนิด โดยที่ดินชนิดที่หนึ่งคือดินร่วนและดินชนิดที่สองคือดินร่วนปนทราย ผลการทดลองพบว่า ในดินร่วนการสลายของสาร4-คลอโรอะนิลีนเกิดขึ้นมากที่สุดในวิธีการเติมกลุ่มจุลินทรีย์ ซึ่งพบว่าสามารถสลายสารได้ถึง 96% ภายใน 28 วัน การใช้สารเร่งการสลายและการใช้จุลินทรีย์เดี่ยวพบว่าสามารถสลายสาร4-คลอโรอะนิลีนได้ 87%และ84% ตามลำดับ ขณะที่วิธีสลายสารตามธรรมชาติสามารถสลายสารได้เพียง 67% สำหรับดินร่วนปนทรายไม่มีความแตกต่างกันในการสลายสาร4-คลอโรอะนิลีนด้วย 3 วิธีดังกล่าว ซึ่งการสลายมีค่าสูงสุดที่ 58% ภายในสองเดือน เนื่องจากดินร่วนปนทรายมีความเป็นกรด เมื่อมีการปรับความเป็นกรดด่างในดินร่วนปนทรายให้มีความเป็นกลาง พบว่าการสลายของสาร4-คลอโรอะนิลีนเกิดขึ้นมากที่สุดในวิธีการเติมกลุ่มจุลินทรีย์ ซึ่งพบว่าสามารถสลายสารได้ถึง 95% ภายในสองเดือน การใช้สารเร่งการสลายและการใช้จุลินทรีย์เดี่ยวพบว่าสามารถสลายสาร4-คลอโรอะนิลีนได้ 63% และ 64% ตามลำดับ ขณะที่วิธีสลายสารตามธรรมชาติสามารถสลายสารได้เพียง 29% สำหรับการตรวจสอบแอคติวิตี้ของเชื้อจุลินทรีย์ในดิน พบว่ามีการเพิ่มของแอคติวิตี้ของเชื้อจุลินทรีย์มากที่สุดในวิธีการเติมจุลินทรีย์ในดินร่วนแต่ไม่พบแอคติวิตี้ของเชื้อจุลินทรีย์ในดินร่วนปนทราย ส่วนการศึกษาการเปลี่ยนแปลงกลุ่มเชื้อจุลินทรีย์ในดิน โดยผลการทดลองพบว่ามีการเพิ่มของกลุ่มเชื้อจุลินทรีย์ในวิธีการใช้สารเร่งการสลายและการเติมเชื้อจุลินทรีย์เมื่อจุลินทรีย์มีการสลายของสาร4-คลอโรอะนิลีน

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KEY WORD: 4-CHLOROANILINE/ SOIL/ NATURAL ATTENUATION/ BIOSTIMULATION/ BIOAUGMENTATION

ROONGNAPA TONGARUN : NATURAL ATTENUATION, BIOSTIMULATION AND BIOAUGMENTATION IN 4-CHLOROANILINE CONTAMINATED SOIL. THESIS ADVISOR : ASSIST. PROF. ALISA VANGNAI, Ph.D. THISIS CO ADVISOR : EKAWAN LUEPROMCHAI, Ph.D. 108 pp. ISBN 974-14-2280 -6.

4-Chloroaniline has been widely used in various industries. It is also the main intermediates accumulated in the environment as a result of microbial degradation of phenylurea herbicides. Due to its toxicity and persistence in the environment, a remediation technology to reduce and clean up 4-chloroaniline is necessary. Bioremediation which is a natural, safe and complete degradation process is a useful technique to clean up 4-chloroaniline. The objective of this study was to evaluate the best bioremediation technique for 4-chloroaniline treatment. Bioremediation consisting of natural attenuation, biostimulation and bioaugmentation (with bacterial pure culture and bacterial consortium) were carried out to degraded 500 ppm 4-chloroaniline in two soil types; loam soil and sandy clay loam soil. The greatest 4-chloroaniline degradation was observed with bioaugmentation with bacterial consortium (96%) after 28 days of incubation at room temperature in loam soil. The biostimulation (addition of 1 mM aniline) and bioaugmentation with pure bacterial culture showed 87% and 84% of 4-chloroaniline degradation, respectively, while natural attenuation showed 67% of degradation. For sandy clay loam soil, there was no significant difference of 4-chloroaniline degradation in each treatment. The highest percent of 4-chloroaniline degradation was only 58% after 2 months of the incubation. Sandy clay loam soil was acidic soil therefore pH was a factor that should be considered. When pH of sandy clay loam soil was adjusted to neutral pH, bioaugmentation with bacterial consortium showed the greatest 4-chloroaniline degradation (95%) after two months of incubation. The biostimulation and bioaugmentation with pure bacterial culture showed 63% and 64% of 4-chloroaniline degradation, respectively, while natural attenuation showed 29% of degradation. The increasing total microbial activity was detected with bioaugmentation in loam soil while it could not be detected in sandy clay loam soil. The bacterial community shift during each treatment was monitored using the denaturing gradient gel electrophoresis (DGGE). The result showed that the bacterial community in biostimulation and bioaugmentation was changed and some bacteria become dominant when the 4-chloroaniline was utilized.

Field of study Environmental management　　Student's signature.....Roongnapa Tongarun.......
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NOMENCLATURE

CFU = Colony forming unit

EPA = Environmental Protection Agency

ml = Milliliter

μl = Microliter

μm = Micrometer

ppm = Part per million

O.D. = Optical density

rpm = Revolution per minute

min = Minute

HPLC = High performance liquid chromatography

WHC = Water holding capacity

INT = 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride

INF = Iodonitrotetrazolium Violet Formazan

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CHAPTER I

INTRODUCTION

1.1 Statement of problem

Due to high growth rate of the world populations, developments of various industries have been increased. Consequently, hazardous chemical contamination of the environment has been increasing in the last few decades (Evans and Furlong, 2003). 4-chloroaniline is a chemical substance which is extensively used in process of various industries, i.e. synthesis of pesticides, dyes and pharmaceutical products. It is also metabolites of microbial degradation of phenylurea, acyanilide and phenylcarbamate herbicides. It has been contaminated in the environment from industrial productions and extensive use of products involving 4-chloroaniline (Kearney and Kaufmann, 1975 and Bartha, 1968).

Within agricultural practices, several herbicides have been used for weed control. A variety of herbicides such as phenylurea, phenylcarbamate, and acylanilide are derivatives of chloroanilines and has been used extensively in agriculture (Adrian, 1989 and Chang, 1999). The more phenylurea, phenylcarbamate, and acylanilide herbicides have been used, the more chloroaniline derivatives have been contaminated in the environment.

4-chloroaniline is harmful to human and animals. 4-chloroaniline irritates and burns eyes and skin and also causes methemoglobinemia disease. Besides, it may be a potential carcinogen in human (Boehncke et al., 2003). Due to its toxicity and persistence

in the environment, 4-chloroaniline has been classified as one of the priority pollutants. It is on the Hazardous Substance List of the Environmental Protection Agency (EPA). Therefore, it is necessary to clean up contaminated 4-chloroaniline in the environment by appropriate methods.

Various methods have been used to clean up the contaminated 4-chloroaniline. Physical and chemical treatments have been used for 4-chloroaniline removal. However, these methods are more expensive than other methods. It is also possible for 4-chloroaniline to be incompletely treated in some methods of physical and chemical treatment. The techniques are only transferred one form to another form of 4-chloroaniline. Further treatment is required for the complete treatment (Evans and Furlong, 2003).

Alternatively, bioremediation is one of the useful treatments to either reduce or remove 4-chloroaniline from the environment. Bioremediation is a treatment process using microorganism(s) to degrade hazardous substance(s) into less toxic or nontoxic substance(s). Bioremediation is advantageous over other techniques in that it is a natural and a safe process. Moreover, the operating cost is comparatively less expensive (Bento et al., 2004). When the degradation process is occurred completely, the contaminants are broken down by the microorganism(s) into harmless products, mainly carbon dioxide and water.

In addition, bioremediation applications are advantageous in that the contaminants can be treated both *in situ*, i.e. the contaminant can be removed in the location, and *ex situ*, i.e. the excavation of contaminated soil or pumping of groundwater is required for the contaminant removal.

Bioremediation accelerates the natural occurring biodegradation under optimized conditions such as oxygen, temperature, pH, nutrients, moisture and process-related operating conditions such as homogeneity (Hupe, 2001). Three types of bioremediation are predominant in the industry today: natural attenuation, biostimulation, and bioaugmentation. The simplest method of bioremediation is natural attenuation (Kaplan and Kitts, 2004) in which the biodegradation is occurred by natural microorganisms.

Biostimulation is a treatment process in which oxygen and nutrients, such as carbon, nitrogen and phosphorus are added into the soil in order to stimulate the biodegradation (Seklemova and Pavlova, 2001). This process requires site adjustments to provide the indigenous microorganisms with a favorable environment in which they can effectively degrade contaminants (Olaniran, et al., 2005) and improve natural biodegradation rate.

On the other hand, the indigenous microorganisms sometimes do not have ability to degrade the toxic compound or the indigenous microorganisms are present in low numbers or even absent. Therefore it is necessary to add exogenous microorganisms or specialized microorganisms as either a pure culture or a mixed culture for the treatment. This process is called bioaugmentation (Richard and Vogel, 1999). The addition of contaminant- degrading microorganisms leads to the immediate start as well as to speed up the entire degradation process.

Recently, biodegradation of 4-chloroaniline has been efficiently demonstrated in liquid media by a newly isolated bacterium and a consortium isolated in our laboratory. For further application, this study therefore used these bacteria for 4-chloroanilene bioremediation treatments of contaminated soil. The efficiency of 4-chloroaniline

bioremediation treatments was evaluated by comparing three bioremediation processes namely natural attenuation, biostimulation and bioaugmentation. Moreover, the number of 4-chloroaniline degrading bacteria, the total microbial activity and microbial community during bioremediation were monitored.

1.2 Objectives

The main objective of this study was to develop a bioremediation technique to potentially clean up 4-chloroaniline contaminated soil. The sub-objectives were as followed:

1.2.1 To determine the decreasing of 4-chloroanilie in two types of soil which has different physical and chemical properties.

1.2.2 To monitor the number of bacteria, total microbial activity and microbial community during the bioremediation.

1.2.3 To evaluate the efficiency of bioremediation treatments including natural attenuation, biostimulation and bioaugmentation for 4-chloroaniline degradation.

1.3 Hypothesis

1.3.1 Properties of soil affected the efficiency of bioremediation treatment used for clean-up of 4-chloroaniline.

1.3.2 4-chloroaniline was biodegraded more effectively in bioaugmentation (with either pure bacterial culture or bacterial consortium) than biostimulation and natural attenuation.

1.3.3 The 4-chloroaniline degrading bacteria in bioaugmentation treatment were more abundant and active than natural attenuation and biostimulation.

1.4 Scope of study

This research evaluated three bioremediation processes for 4-chloroaniline contaminated soil treatment. The scope of the research includes:

1.4.1 The study of 4-chloroaniline degradation to evaluate the efficiency of bioremediation techniques.

1.4.1.1 Comparison among three biological treatments; natural attenuation, biostimulation and bioaugmentation.

- Natural attenuation: 4-chloroaniline degradation was occurred by natural microorganisms.

- Biostimulation: 4-chloroaniline degradation was occurred by indigenous bacteria after induction by 1 mM aniline.

- Bioaugmentation: 4-chloroaniline degradation was occurred by either the inoculated pure bacterial culture or bacterial consortium.

1.4.1.2 Comparison between two types of soil samples.

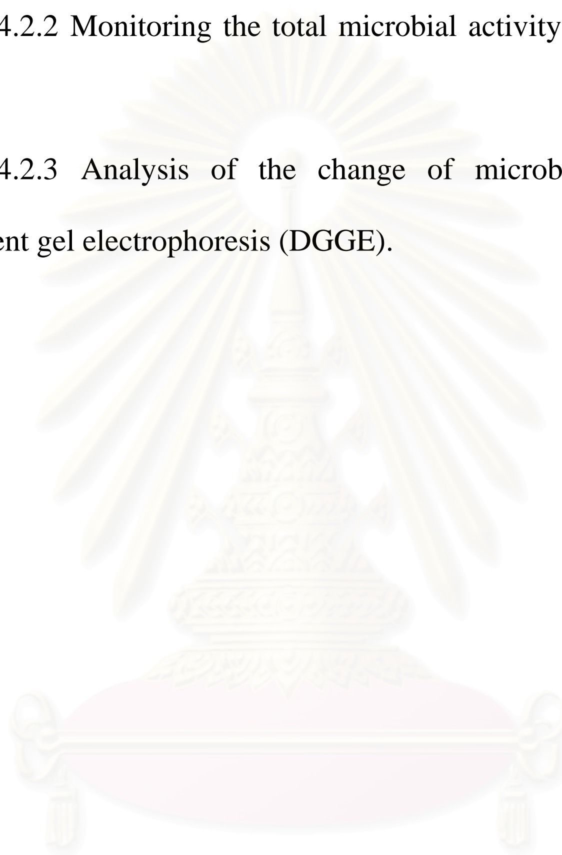
Two types of soil samples which were different in physical and chemical properties were used in the experiment. The samples were collected from agricultural areas in Nakornayok and Chiangmai province.

1.4.2 The study of bacterial populations to support the result of 4-chloroaniline degradation.

1.4.2.1 Determination of the number of 4-chloroaniline degrading bacteria by 10-fold dilution method.

1.4.2.2 Monitoring the total microbial activity by dehydrogenase activity assay.

1.4.2.3 Analysis of the change of microbial community using the denaturing gradient gel electrophoresis (DGGE).



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CHAPTER II

LITERATURE REVIEW

2.1 4-Chloroaniline

2.1.1 The use of 4-chloroaniline

4-chloroaniline is used in various industries including the synthesis of pesticides, dyes, drugs and pharmaceutical products. It is also common metabolites of the microbial degradation of phenylurea, acyanilide and phenylcarbamate herbicides. Phenylurea herbicides such as diuron are widely used for weed control. The more phenylurea herbicides are used in agricultural area; the more 4-chloroanilines are released and accumulated into the soil. Consequently, 4-chloroanilines has been contaminated in the environment both from the industrial productions and the extensive use of the products involving 4-chloroaniline (Brunsbach and Reineke, 1993, Kearney and Kaufmann, 1975 and Bartha, 1968).

In Thailand, phenylurea herbicides such as diuron are widely used in agricultural area. They were at the sixth rank of the imported hazardous chemical substances in 2003 (Table 2.1).

The application of pesticides (mainly phenylureas) may lead to releases of 4-chloroaniline into soils. Monolinuron is reported to contain an average of 0.1% PCA. The insecticide diflubenzuron and the herbicides monolinuron, buturon, propanil, chlorofenprop-methyl, benzoylpropmethyl, chloroaniformmethane, chlorobromuron, neburon, and oxadiazon can release 4-chloroaniline as a degradation product. Besides,

4-chloroaniline can be released from 3,4-dichloroaniline only under anaerobic conditions (Boehncke *et al*., 2003).

Table 2.1 Imported herbicide by value in 2003 (Department of Agriculture, 2003)

Rank	Herbicide name	Quality (kg)	Value (Bath)
1	Glyphosate isopropylammonium	24,812,105	1,824,107,984
2	Paraquat dichloride	8.366,582	1,385,300,727
3	Amethrn	2,374,950	488,492,508
4	2,4-D	5,114,724	392,071,423
5	Atrazine	2,364,450	309,974,446
6	Diuron	984,245	178,858,920
7	Bromacil	304,309	150,816,860
8	Fenoxaprop-P-ethyl	210,658	150,816,860
9	Butachlor	1,309,267	126,341,644
10	Propanil	827,333	118,439,010

2.1.2 Physical and chemical properties

Physical and chemical properties of 4-chloroaniline were presented in the Table 2.2 (Boehncke *et al*., 2003).

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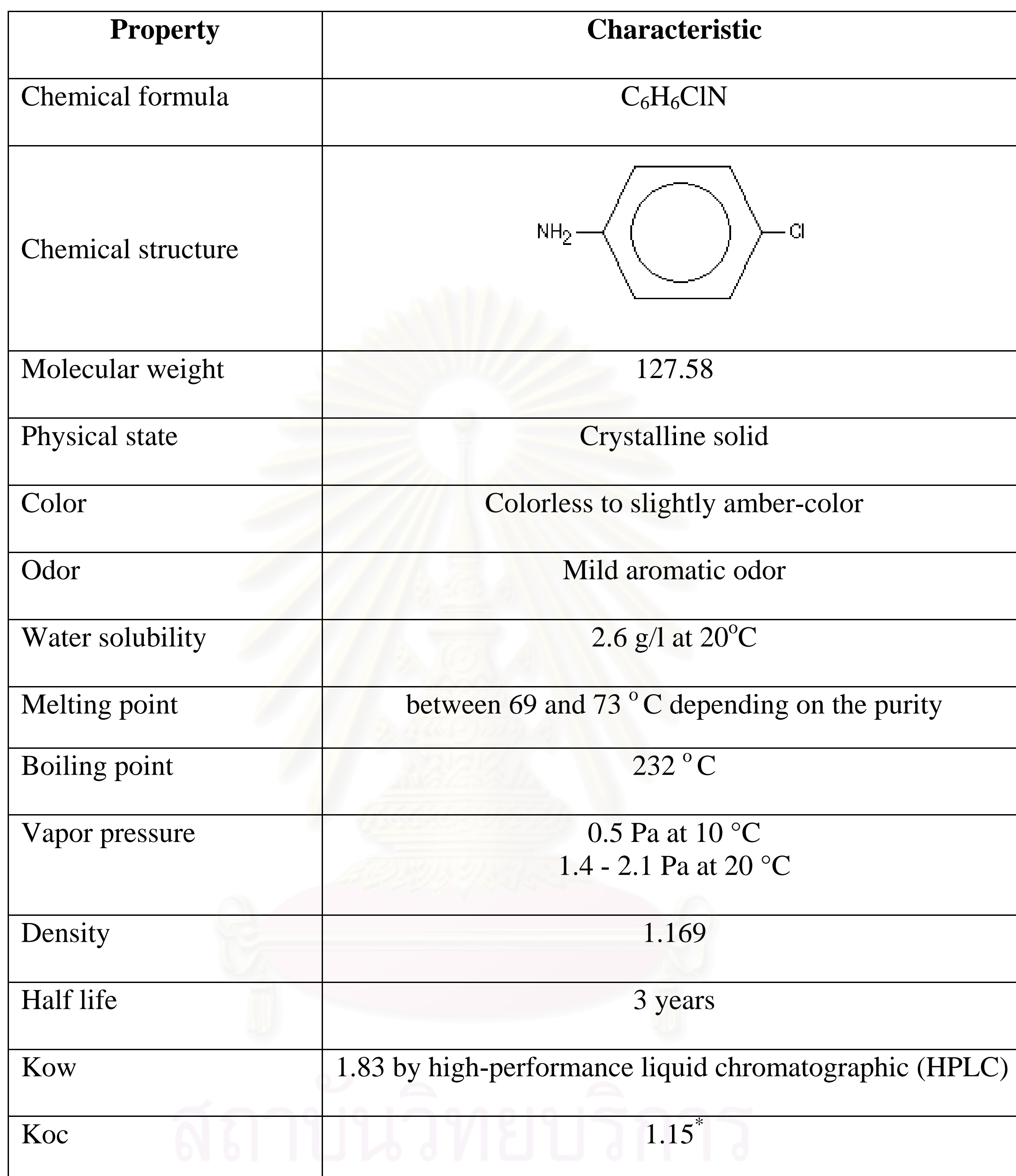
Table 2.2 Physical and chemical properties of 4-chloroaniline



Property	Characteristic
Chemical formula	C_6H_6ClN
Chemical structure	NH_2 Cl
Molecular weight	127.58
Physical state	Crystalline solid
Color	Colorless to slightly amber-color
Odor	Mild aromatic odor
Water solubility	2.6 g/l at 20°C
Melting point	between 69 and 73 °C depending on the purity
Boiling point	232 °C
Vapor pressure	0.5 Pa at 10 °C 1.4 - 2.1 Pa at 20 °C
Density	1.169
Half life	3 years
Kow	1.83 by high-performance liquid chromatographic (HPLC)
Koc	1.15*

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* Koc was calculated from Koc = 0.63Kow (LaGrega et al., 1994)

2.1.3 Toxicity

4-chloroaniline is harmful to human and animals. 4-chloroaniline irritates and burns eyes and skin. High level of 4-chloroaniline can interfere with the ability of

blood to carry oxygen causing headache, dizziness and a blue color to skin and lips (methemoglobinemia disease). Besides, it may be a potential carcinogen in human (Boehncke *et al*., 2003). Because of its hazardous properties and toxicity, it is on the Hazardous Substance List of the Environmental Protection Agency (EPA).

Oral LD_{50} values of 300–420 mg/kg body weight for rats, 228–500 mg/kg body weight for mice, and 350 mg/kg body weight for guinea-pigs were reported (Boehncke *et al*., 2003).

Toxicity for microorganisms, at 3mM 4-chloroaniline inhibited bacterial growth (Radianingtyas et al., 2003a). However, Zeyer and Kearney (1982) reported that 4 mM 4-chloroaniline inhibited bacterial growth.

2.1.4 Environmental fate

4-chloroaniline has been contaminated in the environment both from the industrial productions and the extensive use of products involving 4-chloroaniline (Kearney and Kaufmann, 1975; Bartha, 1968). When 4-chloroaniline was released into the soil, it can combine rapidly with soil components (Bollag *et al*., 1978) and accumulates in soil in the form of non-extractable humic acid-like component (Pillai *et al*., 1982). Only a few percentages of 4-chloroaniline can be volatized from the soil. The sorption of 4-chloroaniline in the soil depends on soil characteristics. The sorption rate increases with increasing of organic matter and decreasing of pH in the soil (Boehncke *et al*., 2003).

If 4-chloroaniline was released to water, it can volatilize and photooxidize in surface layers. It can biodegrade by the microorganisms. It also chemically binds to clays and humus in sediment (Boehncke *et al*., 2003).

If 4-chloroaniline was released to the air, it can be degraded by reacting with photochemically produced hydroxyl radicals. It was possibly degraded by photolysis

in the vapor phase or adsorbed on airborne particulate matter. In addition, 4-chloroaniline can probably be scavenged by rain (Boehncke *et al*., 2003).

The presence of 4-chloroaniline in the environment is caused by the industrial production as it is an intermediate in the production of a number of products, including azo dyes and pigments, cosmetics, and pharmaceutical products. For instance, in Germany in 1990, the release of 4-chloroaniline was reported to be approximately 20 g/ton of its production into air and 13 g/ton released into surface water (Boehncke *et al*., 2003). The annual wastes are estimated to be a maximum of 400 g /ton produced. The discharge of 4-chloroaniline in step of processing was about 25 g/ton released into air at each site and 240 g/ton released into surface water. In USA, total release of 4-chloroaniline in 1995, 1998, and 1999 were reported as 500, 2,814, and 212 kg, respectively (US Toxics Release Inventory, 1999). In addition, 6.1 tons of monochloroanilines (sum of 2-, 3-, and 4-chloroaniline) in 1985, coming completely from industrial processes, were estimated to be released to the river Rhine.

Besides, the release from the industrial production, the source of 4-chloroaniline has been reported from agricultural area. Applications of pesticides such as phenylurea, phenylcarbamate have been reported to lead to the release of 4-chloroaniline into soil. In 54 of 354 agricultural soil samples in Germany, 4-chloroaniline was detected with a maximum concentration of 968 μg/kg (Boehncke et al., 2003). After using pesticide (diflubenzuron) in Finland, in 1984, the concentration of 4-chloroaniline in wild mushroom, blueberries and cranberries was detected, although it was below the detection limit of 10-20 μg/kg (Mutanen et al., 1988). 4-chloroaniline concentrations between 0.9 and 1.3μg/kg were detected in tissue samples of bluegill 19 days after the application of diflubenzuron to an artificial pond (Schaefer et al., 1980). In Thailand, phenylurea herbicides such as diuron are widely

used in agricultural area. A large number of phenylurea herbicides were applied in soil for weed control. As a consequence, phenylurea herbicides and their intermediates were accumulated in the soil. 4-chloroaniline is one of the important intermediates generated by microbial degradation of phenylurea herbicides (Zeyer and Kearney, 1982).

The half-life of 4-chloroaniline in water, measured according to a draft OECD Guideline is 151 days at a water depth of 1 m and a temperature of 20 °C. According to OECD Guideline, 4-chloroaniline has a half-life of about 3 years at 55 °C, pH 3, 7 and 11 (initial concentration 129 mg/l) (Boehncke *et al*., 2003). In experiment with microbial cultures from soil, when non-acclimated inocula were used, the 4-chloroaniline was removed in the range of 0–17% (Bollag *et al*., 1978). Under anaerobic conditions, no significant biodegradation was found in sludge (US EPA, 1981). Under aerobic conditions, 4-chloroaniline released to soil may covalently bind to soil particles, particularly in the presence of high amounts of organic material and/or clay and under low pH levels.

2.2 Regulation of 4-chloroaniline use

Because of its hazardous properties and toxicity, it is legislative control in the Priority Pollutant List of the U.S. Environmental Protection Agency (Federal register, 1979). In addition, the products that containing 4-chloroaniline-based azo dyes were banned by the European Union (EU, 2000 from Boehncke *et al*., 2003). However, 4-chloroaniline could be accumulated in the environment, treatments of 4-chloroaniline are necessary.

2.3 Physical and chemical treatment of 4-chloroaniline

There are several methods which are employed in 4-chloroaniline treatment. Physical and chemical treatment is a method which has been used for 4-chloroaniline removal.

Sanchez, et al. (2002) compared the degradation of 4-chloroaniline by ozonolysis and combined gamma ray-ozone processing. The degradation process was followed by adsorption spectroscopy and HPLC. 4-chloroaniline was degraded more than 90% by ozonolysis. However, the combined gamma ray-ozone processing of 4-chloroaniline is more efficient for 4-chloroaniline degradation.

Mailhot, et al. (2004) studied the iron (III)-photo-induced degradation of 4-chloroaniline in acidic aqueous solution (pH 2-4). The degradation of 4-chloroaniline can be divided to three steps. The first step, 60% of 4-chloroaniline was degraded after 1 hr of irradiation. Following with almost inactive step, 5% of 4-chloroaniline was degraded over a period of 9 hr. Finally, the remaining 4-chloroaniline was degraded within 30 hr.

For photochemical degradation of 4-chloroaniline in aqueous solution, the Photo-Fenton reaction has been studied by monitoring Total Organic Carbon, UV-absorbance at 254 nm and consumption of H_2O_2. Mineralization of 4-chloroaniline reached between 92% and 98% after 5 hours of illumination with a 250 W tungsten lamp (Rupper *et al*., 1993).

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

2.4 Bioremediation of 4-chloroaniline

2.4.1 Bioremediation in general

Bioremediation is one of the useful treatments to either reduce or remove 4-chloroaniline from the contaminated soil. Bioremediation is a treatment process using microorganism(s) to degrade hazardous substance(s) into less toxic or nontoxic substance(s) (Alexander, 1994). Bioremediation is advantageous over other techniques in that it is a natural and safe process. Moreover, the operating cost is comparatively less expensive (Evans and Furlong, 2003 and Alexander, 1994). Bioremediation is the complete degradation process. The contaminant is metabolized to carbon dioxide and water. It eliminates waste permanently. In addition, it has greater public acceptance, with regulatory encouragement (Boopathy, 2000). This process can occur under aerobic and anaerobic conditions. The degradation rate under the aerobic condition is usually faster than the degradation rate under the anaerobic condition (Evans and Furlong, 2003).

2.4.1.1 Basic concept of bioremediation

1. **Mineralization**

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

2. **Cometabolism**

The target contaminant (co-substrate) can not used as the food sources for the microorganisms. The target contaminant (co-substrate) is degraded by the enzyme which reacts with another substrate (primary substrate) (Evans and Furlong, 2003).

3. **Immobilization**

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

2.4.1.2 Factors affecting bioremediation

For bioremediation to be successful, the bioremediation methods depend on having the right microbes in the right place with the right environmental factors for degradation to occur. The right microbes are bacteria or fungi, which have the physiological and metabolic capabilities to degrade the pollutants (Boopathy, 2000).

Boopathy (2000) also summarized factors that affect the success of bioremediation as followed;

1. Energy sources.

Energy source is a factor that affects the success of the bioremediation. Energy source affects the activity of microorganisms that play the important role in the bioremediation processes. The contaminant will be used as an energy source for the activity of microorganism.

The outcome of each degradation process depends on

- Microorganisms (biomass concentration, population diversity, enzyme activities for degradation of the contaminant)
- Substrate (physico-chemical characteristics, molecular structure, and concentration)
- Environmental factors (pH, temperature, moisture content, availability of electron acceptors and carbon and energy sources)

2. Bioavailability of the contaminant.

The rate of biodegradation depends on the rate of contaminant uptake and metabolism and the rate of transfer to the cell. The bioavailability of contaminant is

controlled by the physico-chemical processes such as sorption and desorption, diffusion, and dissolution. The decrease of the bioavailability may result from:

1) The chemical reactions between the contaminants and the natural organic matter,

2) Slow diffusion into very small pores and absorption into organic matter, and

3) The formation of semi-rigid films around non-aqueous-phase liquids (NAPL) with a high resistance toward NAPL-water mass transfer.

Surfactants can be used to increase the availability of contaminants for microbial degradation.

2.4.2 Biostimulation

Biostimulation are the important methods for the bioremediation (Boopathy, 2000). Biostimulation is a treatment process in which oxygen and nutrients, such as carbon, nitrogen and phosphorus are added into the soil in order to stimulate the indigenous microorganisms for degradation (Seklemova and Pavlova, 2001 and Evans *et al*., 2004). It improves natural biodegradation rate.

2.4.3 Bioaugmentation

On the other hand, the indigenous microorganisms sometimes do not have ability to degrade the toxic compound therefore it is necessary to add the exogenous microorganisms or specialized microorganisms as either a pure culture or a mixed culture for the treatment. This process is called bioaugmentation (Richard and Vogel, 1999 and Vogel, 1996). The advantage of bioaugmentation is that the biodegradation can be occurred immediately; therefore the clean up time is reduced (Richard and Vogel, 1999).

Four major concerning conditions for bioaugmentation are:

1) Low of indigenous bacteria

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

2) Time

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

3) Assurance

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

4) Complex waste

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

Vogel (1996) suggested about the important parameters for bioaugmentation.

a. Pollutant characteristic: bioavailability, concentration and microbial toxicity.
b. Soil properties: pH, moisture, organic matter and clay content.
c. Microbial ecology: presence of predators and competition.
d. Microbiology: the presence of co-substrates and enzyme stability and activity
e. Methodology: inoculation concentration and method of inoculation

Determination of the potential success of bioaugmentation requires the three main factors which are the bioavailability of the pollutant, the survival and activity of

the added microorganism(s) and the general environmental conditions that control soil bioremediation rates (Vogel, 1996).

2.4.4 Natural Attenuation

Natural attenuation was an easy method for bioremediation. Natural attenuation was the process that utilizes intrinsic degradation capability of the indigenous microorganisms to degrade contaminants and is a natural degradation process (Yu *et al*., 2005). This strategy was advantageous as low cost (Alexander, 1994). However, natural attenuation often takes a long time to completely degrade the contaminants because of low population size of the indigenous degrading microorganisms. (Forsyth *et al*., 1995).

2.5 4-chloroaniline biodegradation

Zeyer et al. (1985) studied on microbial mineralization of ring-substituted anilines. It showed that *Moraxella* sp. strain G can use aniline, 4-fluoraniline, 4-bromoaniline, 2-chloroaniline, 3-chloroaniline and 4-chloroaniline as sole carbon source and nitrogen source. Besides, the pathway of 4-chloroaniline degradation was investigated by analysis of catabolic intermediates and enzyme activities. It found that 4-chloroaniline was degraded via a modified ortho-cleavage pathway (figure 2.1).

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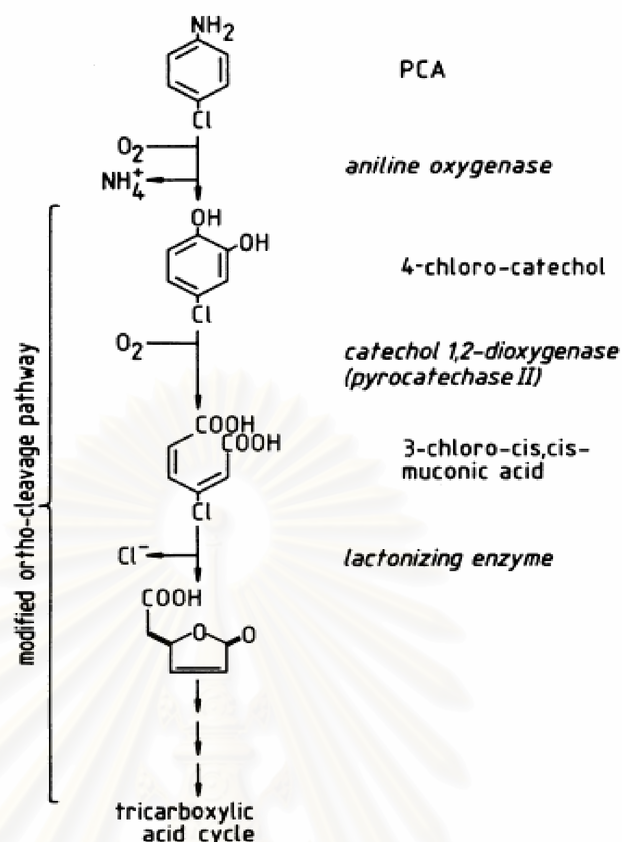

Figure 2.1. A modified ortho-cleavage pathway of 4-chloroaniline degradation by *Moraxella* sp. strain G.

Radianingtyas et al. (2003a) showed that bioremediation could be used for 4-chloroaniline treatment. A bacterial consortium comprising four different species can degrade 4-chloroaniline in presence of aniline. The four species were identified as *Chryseobacterium indologenes* SB1, *Comamonas testosteroni* SB2, *Pseudomonas corrugata* SB4 and *Stenotrophomonas maltophilia* SB5. *Pseudomonas* sp. which isolated from soil also used the 4-chloroaniline as carbon and nitrogen sources. However, the degradation of 4-chloroaniline led to the accumulation of 4-chlorocatechol. The HPLC-UV analysis found that 4-chlorocatechol was further degraded via an ortho-cleavage pathway. The ortho-cleavage pathway was supported by the result from enzyme assay. The study of enzyme essay showed catechol 1,2

dioxygenase activity which occurred in the ortho-cleavage pathway. This enzyme converted catechol and 4-chlorocatechol to cis, cis-muconic acid and 3-chloro-cis, cis-muconic acid respectively.

Zeyer and Kearney (1982) reported that *Pseudomonas* sp. isolated from soil could used 4-chloroaniline as only carbon and nitrogen source with a generation time of 15 hr. After an incubation time of 10 days, 64% of carbon of 4-chloroaniline was released as carbon dioxide, while 60% of the nitrogen and 96% of the chlorine of 4-chloroaniline were accumulated in the medium as ammonium and chloride, respectively. In addition, this strain can grow on aniline and 3-chloroaniline rapidly while it can grow on 2-chloroaniline slowly as sole carbon and nitrogen source.

From the study of Radianingtyas et al. (2003b), was found that 4-chloroaniline can be degraded in the biofilm reactor. The mineralization and detoxification capacity of 4-chloroaniline in the presence of aniline by a microbial community was evaluated in a laboratory-scale biofilm reactor.

2.6 Total microbial activity study

Dehydrogenases wee intracellular enzymes involved in microbial respiratory metabolism. The activities of dehydrogenases were known as a measure for the total oxidative activities of soil microorganisms (Alef, 1995 and Dungan *et al*., 2005). The dehydrogenase activity assay is a sensitive technique that had been used to assess microbial activities in soil amended with organic residues, composted municipal solid wastes, and sewage sludges (Dungan *et al*., 2005).

The 2 (p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) has been used as the substrate for the dehydrogenase activity. The product of this reaction is iodonitrotetrazolium chloride (INF). In some case, the INF concentration

in the autoclaves control soil was higher than the INF concentration in the test condition (Alef, 1995).

Klose et al. (2005) studied about the enzyme activities in a sandy loam soil after fumigation with methyl bromide or alternative biocides. It found that the activities of dehydrogenase were significantly affected by the fumigant treatment and the sampling time.

Kaimi et al. (2006) studied that the biodegradation rate of diesel oil contaminated soil by the Phytoremediation. The result showed that there was significant difference between the measurements of residual total petroleum hydrocarbon (TPH) in planted and unplanted soil. For the study of microbial activity, the number of aerobic bacteria was enumerated by using the plate count technique. It showed that the number of aerobic bacteria in planted and unplanted soil were different. In addition, the soil dehydrogenase activity was also studied for the microbial activity study. Soil dehydrogenase activity was measured with INT reduction. The values of soil dehydrogenase activity in planted soil were significantly different from the soil dehydrogenase activity in unplanted soil. This indicates a significant correlation between the enhanced the decrease of diesel oil in the phytoremediation and the number of aerobic bacteria and amount of dehydrogenase activity.

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2.7 Microbial community analysis

Herbicides, insecticides and pesticides have been used in the agricultural soils. They not only affect the target weed and insect, but also the microbial community in soil (Seghers *et al*., 2003). Therefore, it is important to study about microbial community in soil after the application of herbicides, insecticides and pesticides.

To date, several molecular biology techniques are applied as tools to determine the diversity of microbial community and to monitor population dynamics. One of the techniques is the denaturing gradient gel electrophoresis (DGGE) (Nakatsu *et al*., 2000). The DGGE technique is a separation technique based on differences in melting behavior of double stranded DNA fragment. The general strategies of DGGE method include the extraction of DNA, the amplification of 16s rRNA by polymerase chain reaction (PCR) and analysis of the PCR product by a genetic fingerprinting technique such as DGGE. 16s rRNA is used for the comparison of nucleotide sequence because this sequence is a highly conserved sequence between all organisms (Muyzer et al., 1992).

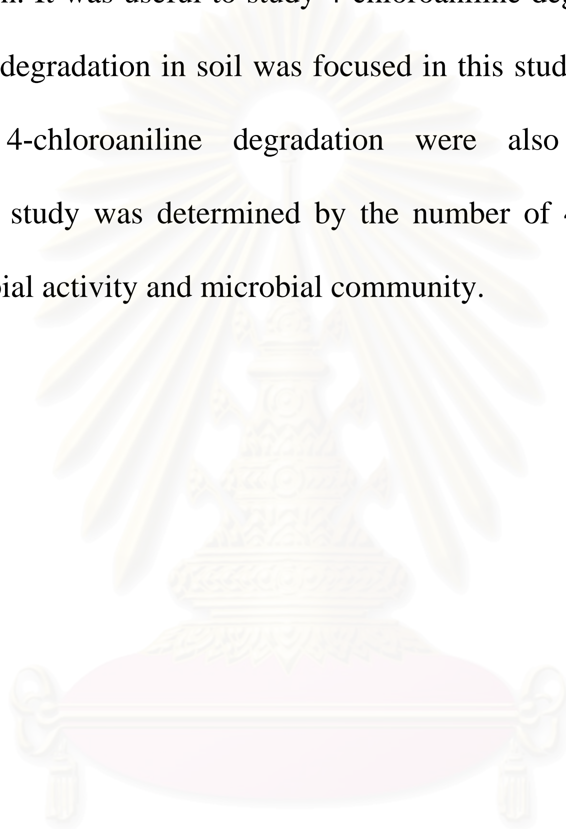
Muyzer *et al*. (1992) showed that DGGE was used to analyze the genetic diversity of complex microbial populations. The polyacrylamide gel showed that there are 10 distinguishable bands, which derived from many different species constituting these populations. Therefore, DGGE is an achievable method for analyzing the complex microbial community.

Agnelli *et al*. (2004) used DGGE to assess the distribution of indigenous bacterial and fungal communities in a forest soil profile. The banding patterns of 16s rDNA-DGGE showed the high bacterial diversity whereas 18s rDNA-DGGE analysis showed a certain stability and lower diversity in the fungal community. The banding pattern was shown to be different when the forest soil depth was increasing. It reflected the change of microbial community with the increasing soil depth.

Said El Fantroussi, *et al*. (1999) studied the effect of phenylurea herbicide (diuron, linuron and chlorotoluron) on soil community with a 10- year history of treatment. Denaturing gradient gel electrophoresis (DGGE) was used for analysis 16s rRNA genes. The results showed that the microbial community was significantly

different between herbicide-treated and nontreated soil. The bacterial community decreased in soils treated with phenylurea herbicide. The sequence determination of several DGGE bands showed that the most affected bacterial species in the diuron and linuron treated soil was an uncultivated bacterial group.

From the literature review study, 4-chloroaniline degradation has been studied in liquid solution. It was useful to study 4-chloroaniline degradation in soil therefore 4-chloroaniline degradation in soil was focused in this study. In addition, the factors that affected 4-chloroaniline degradation were also studied. Besides, the microbiological study was determined by the number of 4-chloroaniline-degrading bacteria, microbial activity and microbial community.



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CHAPTER III

METHODOLOGY

3.1 Chemicals and equipments

3.1.1 Chemicals

1. 4-Chloroaniline (O-363), 99.5% purity was obtained from Chem Service, U.S.A.

2. 2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5-phenyl tetrazolium chloride (INT), iodonitrotetrazolium chloride (INF) and n,n-dimethylformamide were obtained from Sigma Chemical, USA.

3. Ethanol (HPLC grade), methanol (HPLC grade), butanol (HPLC grade) and acetronitrile (HPLC grade) were obtained from Lab-Scan, Ireland.

4. Sodium chloride (NaCl), potassium phosphate (KH_2PO_4) and magnesium sulphate ($MgSO_4.7H_2O$) were obtained from Carlo Brba, France.

5. Agar, peptone, yeast extract and di-sodiumhydrogen phosphate anhydrous (Na_2HPO_4) were obtained from Scharlau, Spain.

6. Aniline and calcium chloride ($CaCl_2.2H_2O$) was obtained from Merck, Germany.

3.1.2 Equipments

1. High liquid performance chromatography LC10ADVP, Shimazu, Japan

2. Microcentrifuge 5804R, Eppendorf, U.S.A.

3. Autoclave MLS-3020, Sanyo Electric, Japan

4. Incubator shaker, Innova4000, New Brunswick Scientific, U.S.A.

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5. Electroporation, Biorad, U.S.A.

6. Gel Document, Syngene, U.S.A.

7. Universal Mutation Detection system, Biorad Dcode™ System, U.S.A.

8. FastPrep FD 120, BIO101, Thermo Savant, U.S.A.

9. GeneAmp® PCR system 2700, AB Applied Biosystems, U.S.A.

10. UV transilluminater, BioDoc-It™ System, UVP, U.S.A.

11. Gel Cleanup Kit, 955152000 (Eppendorf, U.S.A.)

12. FastPlasmid Mini, 0032 007 653 (Eppendorf, U.S.A.)

3.2 Soil preparation

Two types of soil were collected from agricultural areas at the depth of 2-10 cm. The first soil type was collected from a mango orchard in Nakornayok province. Another soil type was collected from a tangerine orchard in Chiangmai province. The soil samples were analyzed for their physical and chemical properties at Department of Soil Science, Faculty of agriculture, Kasetsart University and Soil and Water Group, Agricultural Chemistry Division. The properties including soil texture, pH, organic matter, organic carbon, nitrogen, available phosphorus, C:N ratio, moisture content and water holding capacity were analyzed. Analysis methods for soil properties were in Table 3.1

Table 3.1 The analysis methods for soil properties

Properties	Method
Soil texture	Hydrometer method (Gee and Bauder, 1979)
pH	pH meter with water (1:1)
Organic matter	Wet oxidation method (McLeod, 1973)
Organic carbon	Walkley-Black method (Walkley and Black, 1934)
Phosphorus	Bray II method (Bray and Kurtz, 1945)
Water holding capacity	Comparison between wet weight and dried weight
Total nitrogen	Kjeldahl method (Jackson, 1958)

All debris was removed from soil samples. Then, the soil samples were air dried and sieved through 2 mm mesh to be suitable in the experiment. The sieved soil was kept at room temperature until being used.

The sieved soil was spiked with 500 ppm of 4-chloroaniline. The 4-chloroaniline contaminated soil was used throughout the experiments.

3.3 Recovery of 4-chloroaniline from soil

Organic solvents including methanol and butanol were used to extract 4-chloroaniline from soil. Percent 4-chloroaniline recovery by methanol and butanol was compared to find the highest 4-chloroaniline recovery efficiency.

Five grams of sieved soil were placed in 22-ml screw-capped vial and spiked with 4-chloroaniline to give the final concentration of 500 ppm for triplication. The 4-chloroaniline stock solution was prepared by dissolving 4-chloroaniline in methanol to the desired concentration. Then, 4-chloroaniline was extracted by adding 10 ml of each organic solvent including methanol (50%, 80% and 100%), butanol (50%, 80% and

100%) and the mixture of methanol and butanol (with the ratio of methanol: butanol = 80:20, 50:50 and 20:80). The sample vials were rotated by the rotator overnight at room temperature. The soil suspension sample was allowed to settle before the extracted liquid solution was collected. Then, the amount of 4-chloroaniline recovered from each extraction was analyzed by high performance liquid chromatography (HPLC) which was described in 3.6 (4-chloroaniline analysis).

3.4 Biodegradation of 4-chloroaniline by bacterial consortium in liquid medium

3.4.1 Preparation of bacterial inoculum

Bacterial consortium isolated from agricultural area by our laboratory was consisted of three bacterial strains; C1, C2 and C3. Both bacterial consortium and each single bacteria; C1, C2 and C3 were cultured in liquid mineral medium (Appendix A) with 100 ppm 4-chloroaniline and shaken at 250 rpm, 30°C for 4-5 days. The bacterial cells were harvested by centrifugation at 12,000 rpm for 10 minutes. Cell pellet was washed with 0.85% sodium chloride solution twice. The cell pellet was resuspended with 0.85% sodium chloride solution and diluted to OD_{600} ~ 1.

3.4.2 Study of 4-chloroaniline degradation

10 ml bacterial suspension was added into 90 ml mineral medium containing 100 ppm 4-chloroaniline and shaken at 250 rpm, room temperature. The treatments were as followed;

i) One strain of bacteria; either C1, C2 or C3

ii) Combination of two strains of bacteria; either C1+C2, C1+C3 or C2+C3

iii) Bacterial consortium (C1+C2+C3)

The sample was collected once a week for 9 weeks. Then, the remaining 4-chloroaniline in liquid medium was analyzed by HPLC as described in 3.6.2.

3.5 Biodegradation of 4-chloroaniline in soil

3.5.1 Microcosm description of bioremediation treatments

Three bioremediation processes including natural attenuation, biostimulation and bioaugmentation were studied to evaluate the efficiency of 4-chloroaniline degradation.

(a) Natural attenuation

Five grams 500 ppm 4-chloroaniline spiked soil was placed in 22-ml screw-capped vial (Figure 3.1) for triplication. The spiked 4-chloroaniline was degraded by the ability of the natural microorganisms in soil.

(b) Biostimulation

Five grams of 500 ppm 4-chloroaniline spiked soil were placed in 22-ml screw-capped vial (Figure 3.1) for triplication. Then, aniline which dissolved by methanol was added in the vial with the final concentration of 1 mM. It was used as a nutrient and an inducer to stimulate the 4-chloroaniline degradation ability of the indigenous bacteria. The soil moisture was adjusted by steriled water to 50% water holding capacity (WHC) and incubated at room temperature. The moisture content was maintained by monitoring the using the weight of the microcosm every week.

(c) Bioaugmentation

Two conditions of bacterial inoculum were carried out for the experiment:

(1) Pure bacterial culture; *Klebsiella Planticola*

(2) Bacterial consortium; C1, C2 and C3

Both the pure bacterial culture and bacterial consortium were isolated from the herbicide contaminated soil in the agricultural area by enrichment technique.

- The preparation of bacterial inoculum

Both the pure bacterial culture; *Klebsiella planticola* and bacterial consortium were cultured in the liquid mineral medium with 100 ppm 4-chloroaniline and shaken 250 rpm, 30°C for 4-5 days. The bacteria were harvested, washed and resuspended to give OD_{600} about 1 (approximately of 10^{8} CFU g soil^{-1}) before adding to the bioaugmentation treatment (Appendix B).

- Bioaugmentation treatment

Five grams of 500 ppm spiked soil were placed in 22-ml screw-capped vial. Bacterial pure culture; *Klebsiella planticola* and bacterial consortium were used for bioaugmentation. Each type of the inoculum (500μl) were added into the vials and well mixed with the soil (Figure 3.1) for triplication. The soil moisture was adjusted to approximate 50% water holding capacity (WHC). The vials were incubated at room temperature (~30°C). The moisture content was maintained the reduced weight of the soil microcosm every week and more sterile water was added to compensate.

3.5.1.1 Description of control experiment

Sterilized soil was used for the control condition. The soils were sterilized by autoclaving (3 times at 121°C, 15 min.). Five grams of sterilized soil were placed in 22-ml screw-capped vial and spiked with 4-chloroaniline to give the final concentration of 500 ppm.

3.5.1.2 Sampling time

The soil samples of each treatment were collected at 0, 3, 7, 10, 14, 21 and 28 days for quantitative analysis of 4-chloroaniline and soil microbiological analysis.

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Figure 3.1 Soil microcosms contain 5 g soil in 22-ml screw-capped vial.

3.5.2 Effect of the incubation time on 4-chloroaniline in sandy clay loam soil.

Three bioremediation methods including natural attenuation, biostimulation and bioaugmentation were investigated for 4-chloroaniline degradation in sandy clay loam soil as described in 3.5.1. Meanwhile, the incubation period was extended from one month to two months. The soil samples were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks for 4-chloroaniline quantitative analysis and soil microbiological analysis.

3.5.3 Effect of soil pH on 4-chloroaniline in sandy clay loam soil.

Calcium carbonate ($CaCO_3$) was used to adjust sandy clay loam soil pH from pH 4.3 to pH 7. Five grams of 500 ppm 4-chloroaniline contaminated soil after pH adjustment were placed in 22-ml screw-capped vial.

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The bioremediation methods were focused on the three methods including natural attenuation, biostimulation and bioaugmentation. The description of the microcosm study was as same as the study in 3.5.1 microcosm description of bioremediation treatments. The samples were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks for 4-chloroaniline quantitative analysis and soil microbiological analysis.

3.5.4 Experimental flow chart

The experimental flow chart was outlined in Figure 3.2

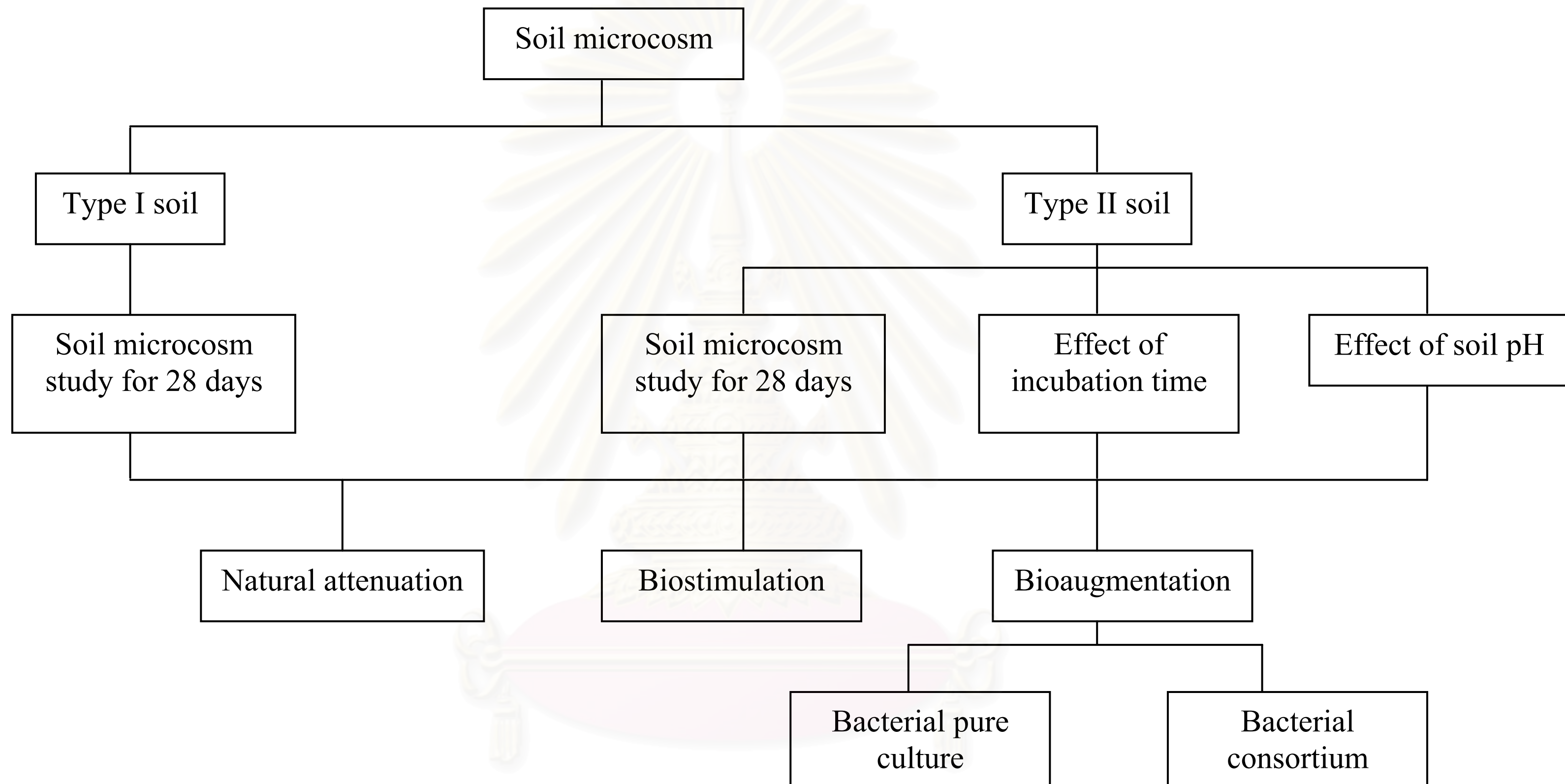

Figure 3.2 The experimental flow chart of soil microcosms.

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3.6 4-chloroaniline analysis

3.6.1 4-chloroaniline extraction

4-chloroaniline was extracted from the soil by liquid extraction. 4-chloroaniline was extracted by adding 10 ml of 80% methanol into the sample vials. The sample vials were rotated by the rotator overnight at room temperature. After that, the sample vials were allowed to settle and then collected the liquid solution.

3.6.2 4-chloroaniline analysis

The liquid solution was centrifuged at 12,000 rpm 10 min. The supernatant was collected and filtered with 0.45 μm diameter filter. 4-chloroaniline was quantitatively analyzed by a reverse phase HPLC with a UV detector at a wavelength of 240 nm. The separation was performed on C18 column (Phenomenex, 250x4.6 mm) with acetronitrile: water: phosphoric acid mixture (70: 29.75: 0.25 %vol/vol) at a flow rate 1 ml/min as a mobile phase.

Detection limit of 4-chloroaniline are in the range of 0.04-100 μg/litre with HPLC method (Boehncke *et al*., 2003).

3.6.3 4-chloroaniline calibration curve

4-Chloroaniline calibration curve was used for 4-chloroaniline analysis in soil. The standard 4-chloroaniline was prepared by dilution to various concentration of 4-chloroaniline. And then, the various concentration of 4-chloroaniline was analyzed in HPLC with a UV detector at a wavelength of 240 nm as described in 3.6.2. The calibration curve was shown in Appendix C.

3.7 Microbiological analysis

3.7.1 Determination of the amount of 4-chloroaniline-degrading bacteria.

Plate count technique was used for determining the amount of 4-chloroaniline degrading bacteria. Ten-fold serial dilution of bacteria was prepared by diluting soil samples with sterile distilled water. The serial dilution of cell suspension was spreaded on mineral medium agar plates which 100 ppm 4-choroanilne as sole carbon source. The plates were then incubated at 30°C for 4 to 5 days. Number of bacteria colony on the agar plate was accounted for the 4-chloroaniline-degrading bacteria. The number of bacteria per milliliter of culture was calculated by

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

3.7.2 Total microbial activity by dehydrogenase activity assay

Total microbial activity in soil was monitored by dehydrogenase activity assay (Alef, 1995). Soil sample (1g) was mixed with 1.5 ml Tris buffer and 2 ml 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution. Next, the suspension was incubated at 40°C in the dark for 2 hr. Then, it was mixed with 10 ml extraction solution and kept in the dark. Finally, it was filtered and measured for enzyme product (Iodonitrotetrazolium Violet Formazan; INF) using a spectrophotometer at 464 nm. The sterile soil was used as a control.

For the calibration curve, INF standard solution (100 μg/ml) was prepared by dissolving 10 mg INF in 80 ml extractant and bringing with the same extractant to 100 ml. INF standard solution was pipetted 0, 1, 2 and 5 ml into test tubes. The 13.5 ml extractant was added to each tube and mixed thoroughly. The calibration

concentrations were 0, 100, 200 and 500 ug INF per test. The INF calibration curve was shown in Appendix D.

3.7.3 Analysis of microbial community

Changes in bacterial populations were studied using denaturing gradient gel electrophoresis (DGGE) of 16S rDNA.

3.7.3.1 Soil DNA extraction

Soil DNA was directly extracted from 0.8 g soil with bead-beating instrument and FastDNA SPIN Kit (BIO 101, USA). The soil DNA was checked by running in agarose gel electrophoresis with 1x TBA (tris-borate-EDTA buffer) at 100 V. Then, the agarose gel was stained in ethidium bromide and destained with water. The soil DNA band can be detected under UV transilluminater.

3.7.3.2 Soil DNA purification

Humic acid was removed from the soil DNA by gel purification technique. The extracted soil DNA was purified using Gel clean up kit (Eppendorf).

3.7.3.3 Polymerase chain reaction (PCR) of 16S rDNA

Polymerase chain reaction (PCR) using PRBA 338F+CG clamp (5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG3') and PRUN518R (5'ATT ACC GCG GCT GCT GG3') primers was carried out to amplify 16s rDNA of soil bacteria. The expected PCR product size was 200 bp long. The PCR reaction contained 50 ng soil DNA, 20 pmol of both primers, 15 µl of Taq PCR Master Mix (Qiagen Inc.) and ultrapure water. The condition was

1. Initial denaturation step at 94°C for 5 min
2. Touchdown program for 20 cycles

    2.1 Denaturation step at 94°C for 1 min

2.2 Annealing step at 55°C for 1 min (temperature was reduced 0.5°C each cycles)

2.3 Extension step at 72°C for 2 min

3. Denaturation step at 94°C for 1 min
4. Annealing step at 55°C for 1 min
5. Extension step at 72°C for 2 min
6. Go to step 3-5 for 30 cycles.
7. The final extension at 72°C for 10 min

5 μl of PCR product was run in 1% agarose gel electrophoresis with 1x TBA (tris-borate-EDTA buffer) at 100 V. for checking the size of PCR product.

3.7.3.4 Denaturing gradient gel electrophoresis (DGGE)

PCR product was run on 8% polyacrylamide gel with a denaturing gradient of urea and formamide denaturant ranging from 25-60% for 5 hr at 130 volts with 1xTAE. DGGE gel was stained in 50 ug/ml ethidium bromide for 20 minutes. DNA band profiles can be detected under the UV transilluminater.

3.7.3.5 DNA sequencing

The interesting DGGE bands were cut from DGGE gel and sequenced to identify the bacterial species. Briefly, the cut DGGE bands were put in eppendorf tube containing 30 μl steriled water for DNA extraction. Then, the DNA solution was amplified by PCR as explained in the topic 3.7.3.3. The PCR product was purified using Gel clean up kit (Eppendorf, U.S.A.). Then, the purified PCR product was ligated through pGem T Easy vector (Promega, U.S.A.) of which the reaction was described as below:

The purified PCR product 3 µl (150 µgDNA)

pGem T easy vector 1 µl (30 ng/µl)

T_4 DNA ligase 1 µl (3 units/µl)

Ligase buffer 5 µl

The ligase reaction (total volume of 10 µl) was incubated at 4°C, overnight.

The ligase product was transformed to the competent *E.coli* DH5α cell by electroporation technique. The transformed solution was incubated at 37°C for 1 hr, shaking at 250 rpm. Then, the transformed solution was spreaded on the LB agar containing 100 µg/ml amplicilin, 30 µg/ml X-gal and 30 µg/ml IPTG.

White colonies were selected to check the insert fragment. The white colonies were grown in the LB broth containing 100 µg/ml amplicilin at 37°C overnight. Then, the plasmid was extracted by miniprep kit (Eppendorf, U.S.A.) and cut by *Eco*RI restriction enzyme (Toyobo, Japan) to confirm the insert fragment. The restriction digestion condition (total volume of 10 µl) was as described below;

Plasmid (pGem T Easy) 2 µl

*Eco*RI enzyme 0.5 µl

Buffer 1 µl

Steriled water 6.5 µl

The insert fragment was examined by running in 1% agarose gel electrophoresis. The correct DNA insert fragment was about 200 base pairs. The plasmids having the correct DNA insert fragment was sent for sequencing at Macrogen, Korea. The sequence results (Appendix G) were analyzed using BlastN program to identify the bacterial species.

CHAPTER IV

RESULTS

4.1. Soil properties

Two types of agricultural soil samples which have been regularly exposed to pesticides and herbicides; Type I and Type II were collected from the agricultural area at the depth of 2-10 cm. Type I soil was collected from mango garden in Nakornnayok province, while Type II soil was collected from tangerine garden in Chiangmai province. The soil samples were sieved through 2 mm mesh. Then, they were analyzed for their physical and chemical properties at Department of Soil Science, Faculty of agriculture, Kasetsart University and Soil and Water Group, Agricultural Chemistry Division.

The soil properties are provided in Table 4.1. Type I soil is classified as loam soil with 40.4% sand, 39.6% silt and 20.0% clay. pH of type I soil was 5.3. Percentage of organic matter, organic carbon and nitrogen were 3.86, 2.24 and 0.19, respectively. C:N ratio was 11.79. Amount of available phosphorus was 81 ppm. Moisture content was 9.18%. Type II soil is sandy clay loam soil with 64% sand, 16% silt and 20.0% clay. pH of type II soil was 4.3. Percentage of organic matter, organic carbon and nitrogen were 1.81, 1.85 and 0.09, respectively. C:N ratio was 20.55. Amount of available phosphorus was 149 ppm. Moisture content was 14.0%.

The properties of soil samples in Table 4.1 showed that the two soil samples had different physical and chemical properties. Therefore, two types of soil samples were used in this study to determine whether soil properties affected 4-chloroaniline degradation during bioremediation.

The background of 4-chloroaniline in loam soil and sandy clay loam soil was 1.5 ppm and 18.91 ppm, respectively. The indigenous bacteria involving 4-chloroaniline degradation in loam soil and sandy clay loam soil was $1.06 \times 10^4 \pm 8.49 \times 10^2$ CFU/g soil and $9.35 \times 10^2 \pm 49.5$ CFU/g soil, respectively.

Table 4.1 Properties of soil samples

Properties	Type I soil (Nakornnayok) (agricultural area)	Type II soil (Chiangmai) (agricultural area)
Soil texture	Loam	Sandy clay loam
% sand	40.4	64.0
% silt	39.6	16.0
% clay	20.0	20.0
pH (1:1) in water	5.3	4.3
Organic matter (%)	3.86	1.81
Organic carbon (%)	2.24	1.85
Nitrogen (%)	0.19	0.09
Available phosphorus (ppm)	81	149
C:N ratio	11.79	20.55
Moisture (%)	9.18	14.0
Water holding capacity (%)	41.92	28.87

4.2 Recovery of 4-chloroaniline from soil

4-chloroaniline contaminated in soil was extracted by organic solvent. Various solvents including methanol (50%, 80% and 100%), butanol (50%, 80% and 100%) and the mixture of methanol and butanol (with the ratio of methanol: butanol = 80:20, 50:50 and 20:80) were used for 4-chloroaniline extraction from soil. The extracted 4-chloroaniline was analyzed by high performance liquid chromatography (HPLC). Recovery of 4-chloroaniline was shown in table 4.2. 80% methanol showed the highest % 4-chloroaniline recovery (84%). Consequently, 80% methanol was used for extract 4-chloroaniline from soil in the following experiments.

Table 4.2 Recovery percentage of 4-chloroaniline from soil

Extracted solvent	% 4-chloroaniline recovery
100% methanol	60±1.26
80% methanol	84±0.68
50% methanol	71±1.25
100% butanol	53±1.02
80% butanol	81±4.17
50% butanol	79±3.51
Methanol:butanol (80:20)	70±3.68
Methanol:butanol (50:50)	74±0.45
Methanol:butanol (20:80)	77±1.03

4.3 Biodegradation of 4-chloroaniline in liquid medium

Bacteria used in bioaugmentation treatment are (i) bacterial pure culture and (ii) bacterial consortium, which were screened from agricultural soil by our laboratory. The bacterial pure culture was *Klebsiella planticola*, a gram negative bacterium. It could degrade 63% of 25 ppm 4-chloroaniline in liquid medium within 12 days (Appendix E). The bacterial consortium consisted of three types of gram negative bacteria; C1, C2 and C3 (Appendix E). Each C1, C2 and C3 bacteria degraded only 30% of 100 ppm 4-chloroaniline in liquid medium after 9 weeks (Figure 4.1). The combination of bacteria; C1+C2, C1+C3 and C2+C3 also degraded 30% of 100 ppm 4-chloroaniline in liquid medium after 9 weeks (Figure 4.2). Meanwhile, the bacterial consortium degraded 99% of 100 ppm 4-chloroaniline in liquid medium within 4 weeks (Figure 4.3). The results indicated that combination of C1, C2, and C3 as bacterial consortium was necessary for 4-chloroaniline degradation.

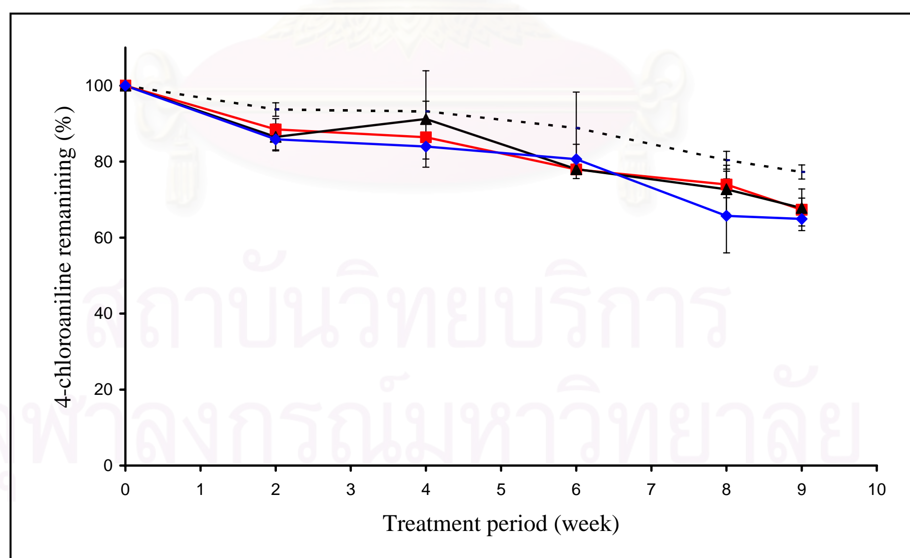

Figure 4.1 % 4-chloroaniline remaining of the single bacteria: C1 (—■—), C2(—▲—) and C3 (—◆—) grown in minimal medium containing 100 ppm 4-chloroaniline. The control was the minimal medium containing 100 ppm 4-chloroaniline (- - - -).

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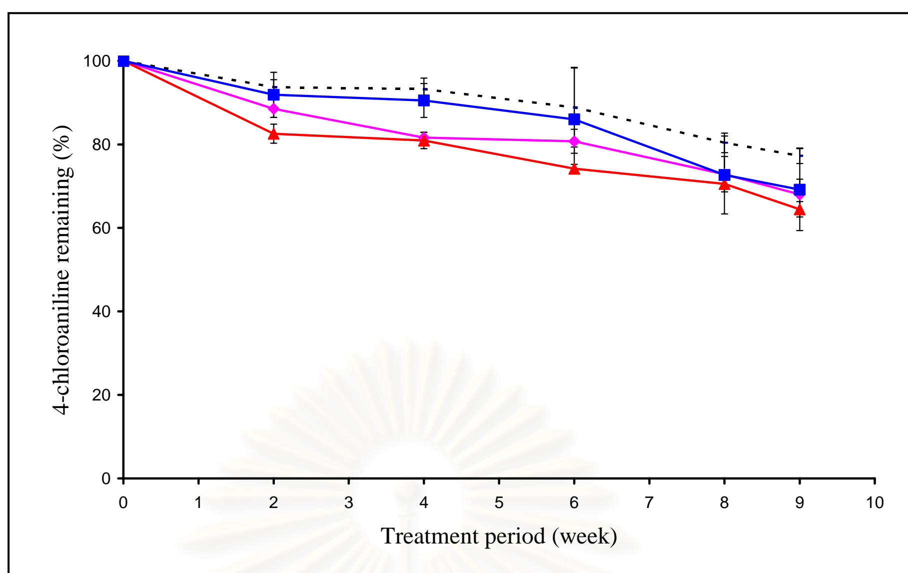

Figure 4.2 % 4-chloroaniline remaining after adding the combination bacteria: C1+C2 (—◆—), C1+C3 (—▲—) and C2+C3 (—■—) grown in minimal medium containing 100 ppm 4-chloroaniline. The control (- - - -) was the minimal medium containing 100 ppm 4-chloroaniline.

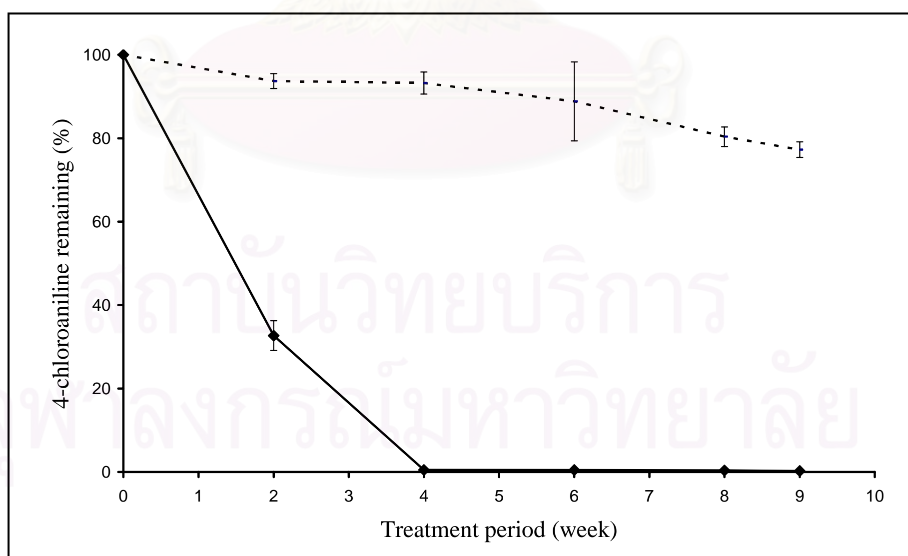

Figure 4.3 % 4-chloroaniline remaining of bacterial consortium (—◆—). The control was the minimal medium containing 100 ppm 4-chloroaniline (- - - -).

4.4 Biodegradation of 4-chloroaniline in soil microcosms

4-chlroaniline biodegradation was conducted at the laboratory scale in which 5 g soil was spiked with 4-chloroaniline before treatment. The bioremediation treatments consisted of natural attenuation, biostimulation and bioaugmentation. Natural attenuation is the process that 4-chloroaniline was reduced due to naturally-occurring process including sorption, chemical reaction as well as degradation by indigenous microorganisms in soil. Biostimulation is the treatment which aniline was added as the inducer of 4-chloroaniline biodegradation. According to previous report, 1 mM aniline is able to stimulate the rate of 4-chloroaniline degradation (Radianingtyas et al., 2003a). Bioaugmentation was carried out in which approximately 4-chloroaniline-degrading bacteria were provided at 10^8 CFU/g soil.

4.4.1 Preliminary test; soil contaminated with various concentrations of 4-chloroaniline

A preliminary test was carried out in loam soil to determine the optimum concentration of 4-chloroaniline for biodegradation. Concentrations of 4-chloroaniline were varied from 100 ppm to 1,000 ppm (Figure 4.4). The treatments were carried out for 14 days at room temperature. At 100 ppm 4-chloroaniline, percent 4-chloroaniline degradation in all biological treatments was insignificantly different. The removal of 100 ppm 4-chloroaniline through natural attenuation, biostimulation and bioaugmentation were shown in the value of 85.78%, 86.28% and 86.55%, respectively. At high concentrations of 4-chloroaniline, the different of 4-chloroaniline degradation efficiency in each treatment became significant. For example, 51.44%, 70.13% and 77.07% of 500 ppm 4-chloroaniline were degraded through natural attenuation, biostimulation and bioaugmentation, respectively. At 750

ppm and 1,000 ppm, percent 4-chloroaniline degradation in all treatments was decreased and the differences between biostimulation and bioaugmentation were insignificant. The results suggested that high concentration of 4-chloroaniline prevented the activity of soil microorganisms. Therefore, 500 ppm 4-chloroaniline was used for further experiment.

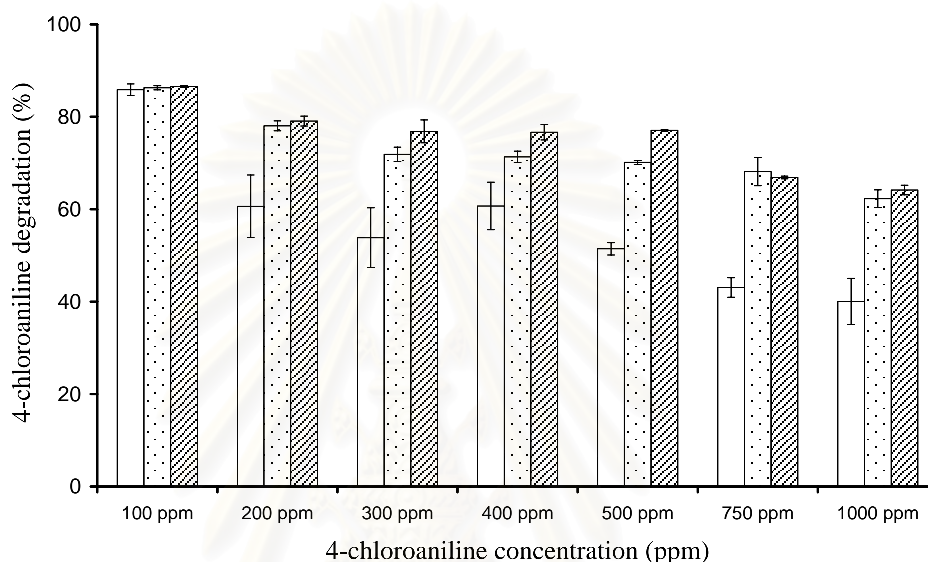

Figure 4.4 Degradation percentage of the various 4-chloroaniline concentrations in each biological treatment; natural attenuation ( ), biostimulation ( ) and bioaugmentation with bacterial pure culture ( ) after 14 days of incubation.

4.4.2 4-chloroaniline degradation in loam soil

4-chloroaniline biodegradation was conducted through three bioremediation treatments: natural attenuation, biostimulation and bioaugmentation using loam soil. The condition of natural attenuation was 5 g soil microcosm with 500 ppm 4-chloroaniline. 1 mM aniline was added into 5 g soil microcosm with 500 ppm 4-chloroaniline for biostimulation. For bioaugmentation, 4-chloroaniline-degrading bacteria were provided at approximately 10^8 CFU/g soil into 5 g soil microcosm with 500 ppm 4-chloroaniline. Both pure culture (*Klebsiella planticola*) and consortium of

4-chloroaniline-degrading bacteria were used in bioaugmentation treatment. 4-chloroaniline remaining in soil microcosm was shown in Figure 4.5.

In loam soil, the highest percentage of 4-chloroaniline degradation (96%) was observed in bioaugmentation with bacterial consortium after 28 days. Bioaugmentation with bacterial pure culture showed 84% degradation of 4-chloroaniline. Aniline at the final concentration of 1 mM promoted 87% of 4-chloroaniline degradation. It was a known inducer for chloroaniline degradation in biostimulation process (Radianingtyas, 2003). Bioaugmentation and biostimulation processes resulted in a high range (87%-96%) of biodegradation, while only 67% of 4-chloroaniline could be reduced via natural attenuation treatment (Figure 4.5).

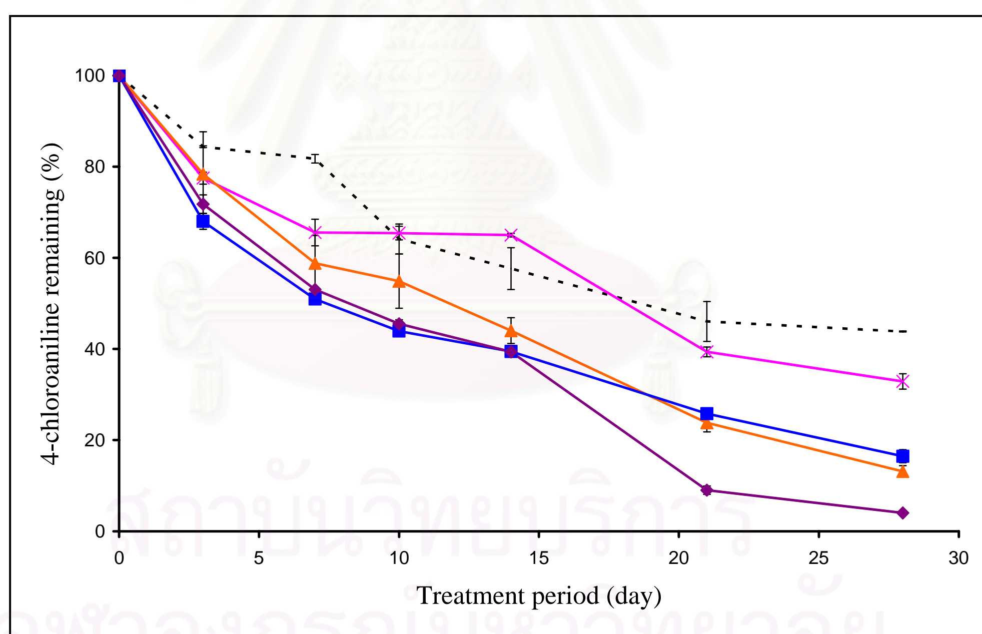

Figure 4.5 Biodegradation of 500-ppm 4-chloroaniline in loam soil. The treatments include bioaugmentation with bacterial consortium (—◆—); pure bacterial culture; *Klebsiella planticola* (—■—), biostimulation (—▲—) and natural attenuation (—×—). The control was steriled soil (- - - - -).

(a) Number of 4-chloroaniline-degrading bacteria in loam soil

The populations of 4-chloroaniline-degrading bacteria were determined by plate count technique with the supplementation of 100-ppm 4-chloroaniline. The number of 4-chloroaniline-degrading bacteria in each treatment was shown in Figure 4.6. 4-chloroaniline-degrading bacterial colony was undetectable in the control treatment. During the bioremediation, the number of 4-chlrooaniline-degrading bacteria increased approximately 10 times through natural attenuation (from $1.03x10^4 \pm 5.65x10^2$CFU/g soil to $3.10x10^5 \pm 4.24x10^4$CFU/g soil). The number of 4-chloroaniline-degrading bacteria increased approximately 1,000 times through biostimulation (from $1.12x10^4 \pm 1.41x10^3$ CFU/g soil to $6.5x10^7 \pm 4.24x10^6$CFU/g soil). When bioaugmented with bacterial pure culture and with bacterial consortium, the number of 4-chloroaniline-degrading bacteria increased approximately 100 times (from $3.00x10^6 \pm 4.2x10^5$ CFU/g soil to $1.32x10^8 \pm 1.41x10^6$ CFU/g soil and from $4.10x10^6 \pm 1.4x10^5$ CFU/g soil to $5.9x10^8 \pm 1.41x10^7$ CFU/g soil, respectively). The increased number of 4-chloroaniline-degrading bacteria in each treatment relatively corresponded to the efficiency of 4-chloroaniline biodegradation.

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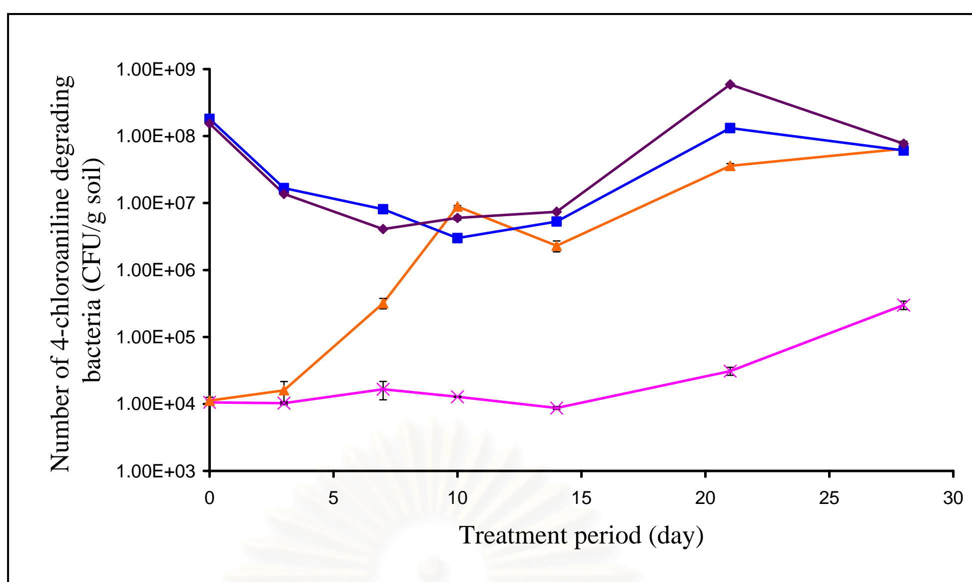

Figure 4.6 Number of 4-chloroaniline-degrading bacteria in loam soil. The number of bacteria was determined, during 28 days of incubation, in each treatment which includes bioaugmentation with bacterial consortium (—◆—); with pure bacterial culture; *Klebsiella planticola*(—■—), biostimulation (—▲—), and natural attenuation (—×—).

(b) Total microbial activity in loam soil

Dehydrogenase activity in soil has been used to monitor total microbial activity (Alef, 1995). Dehydrogenase activity is varied among the treatments and incubation time. Among bioremediation treatments of loam soil, the highest value of dehydrogenase activity was found in bioaugmentation with bacterial consortium (Table 4.3). The addition of bacterial consortium at 10^8 CFU/g soil increased the total microbial activity 352 folds by the end of the incubation time (28 days), while it was increased 219 folds when bacterial pure culture was provided. The addition of 1 mM aniline in biostimulation treatment also significantly increased the dehydrogenase activity 331 folds. However natural attenuation was shown to have the least increasing of microbial activity (18 folds).

Table 4.3 Total microbial activity in each treatment in loam soil

Treatments	Dehydrogenase activity at 0 day (µg INF g^{-1} dwt 2 h^{-1})	Dehydrogenase activity at 28 days (µg INF g^{-1} dwt 2 h^{-1})	Dehydrogenase activity (fold*)
Natural attenuation	0.77	12.31	18±3.26
Biostimulation	1.54	231.54	331±2.72
Bioaugmentation with bacterial pure culture	17.69	153.08	219±4.35
Bioaugmentation with bacterial consortium	16.15	246.15	352±3.81

*The fold of dehydrogenase activity was compared with the control which had the value of 0.70 (µg INF g^{-1} dwt 2 h^{-1}).

(c) Analysis of microbial community in loam soil

DGGE analysis is used to evaluate the effects of bioremediation treatments on microbial community (Muyzer et al., 1992 and Nakatsu, 2000). Soil DNA was isolated from each biological treatment of loam soil. 200 bp fragment of 16s rDNA was amplified with P338f and P518r primers in PCR reaction as described in Method 3.7.3.3. The PCR products were run in DGGE to investigate the bacterial community shift over 4 week period of each biological treatment. The changes of soil bacterial populations were obvious in biostimulation and bioaugmentation, while the change in natural attenuation was not significantly established (Figure 4.7). The results indicated that aniline and bacterial addition influenced the growth of various soil bacteria.

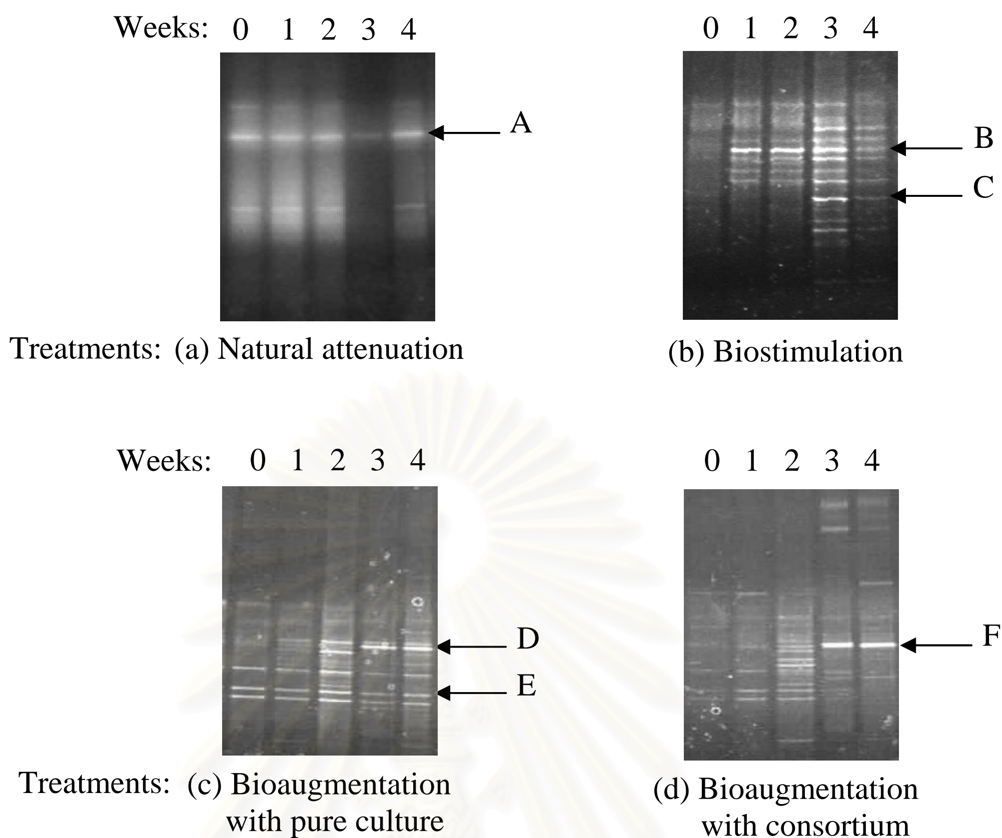

Figure 4.7 DGGE profile of the bioremediation treatments of loam soil; natural attenuation (a), biostimulation (b), bioaugmentation with either bacterial pure culture; *Klebsiella planticola* (c) or bacterial consortium (d) in 4 weeks (28 days) of incubation. Numbers represent the week of incubation. Arrow D and F indicate the dominant bands of bioaugmentation treatment. A, B, C, D, E and F indicate the fragments that were sequenced.

DGGE profiles of biostimulation with 1 mM aniline illustrated the increase number of bacteria populations, which represented by the increasing of DNA bands from the 0-2nd week to the 3rd and 4th week (Figure 4.7b). In bioaugmentation with pure bacterial culture, DGGE profiles were almost the same at all time points; however the intensities of each dominant band were difference between each time

point (Figure 4.7c). Interestingly, DGGE profile from the bacterial consortium bioaugmentation showed a single dominant band at the end of treatment (Figure 4.7d).

Moreover, the dominant bands in each treatment were sequenced (Figure4.7). Fragment A which is the dominant band in natural attenuation treatment, showed high sequence similarity to *Enterobacter* sp. (98%). Fragment B and C detected in biostimulation treatment were matched closely to *Actinomycete* sp. (95%) and *Hyphomicrobium zavarzinii* (100%), respectively. Fragment D and E found in bioaugmentation with pure culture. Fragment D corresponded to *Stenotrophomonas maltophilia* (100%). Fragment E matched closely (96%) with *Sphingomonas* sp. The dominant band in bioaugmentation with consortium is fragment F which corresponded (100%) to *Streptomyces* sp. (Table 4.4).

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Table 4.4 Bacterial strains detected in each biological treatment in loam soil

Treatments	Fragment	Bacterial species	Accession number	Source	% Sequence similarity	Reference
Natural attenuation	A	*Enterobacter* sp.	AM161173	Soil	98	Ripka *et al*. (unpublished)
Biostimulation	B	*Actinomycete* sp.	Z73403	Soil	95	Mcveigh *et al*., 1996
	C	*Hyphomicrobium zavarzinii*	Y14306	Soil	100	Rainey et al., 1998
Bioaugmentation with pure culture	D	*Stenotrophomonas maltophilia*	AY748889	Rhizosphere	100	Barriuso *et al*. (unpublished)
	E	*Sphingomonas* sp.	AB235162	Soil	96	Konno *et al*. (unpublished)
Bioaugmentation with consortium	F	*Streptomyces* sp.	DQ196486	-	100	Tkner *et al*. (unpublished)

4.4.3 4-chloroaniline degradation in sandy clay loam soil

To determine the effect of soil types on bioremediation treatment, three bioremediation treatments; natural attenuation, biostimulation, and bioaugmentation were later used to treat 4-chloroaniline in sandy clay loam soil. The condition of each bioremediation treatment was explained in section 4.4.2 on 4-chloroaniline degradation in loam soil.

4.4.3.1 Effect of the incubation time on 4-chloroaniline degradation

After 28 days of incubation, the removal of 4-chloroaniline in sandy clay loam soil through bioaugmentation with bacterial pure culture, bioaugmentation with bacterial consortium, biostimulation and natural attenuation treatments were 48%, 50%, 41% and 38%, respectively. The degradation of 4-chloroaniline in these soil microcosms was lower than loam soil at the same incubation time. In this study, the incubation time was therefore extended from one month to two months. The biodegradation of 500 ppm 4-chloroaniline in sandy loam clay soil was shown in Figure 4.8.

In sandy clay loam soil, percent 4-chloroaniline removal through bioaugmentation with bacterial pure culture, bioaugmentation with bacterial consortium, biostimulation and natural attenuation treatments were 58%, 57%, 56% and 34% after 2 months of incubation, respectively. The extent of 4-chloroaniline degradation in natural attenuation was significant difference from biostimulation and bioaugmentation. Although, the incubation time was increased from one month to two months, the percentage of degradation 4-chloroaniline through biostimulation, bioaugmentation was slightly increased.

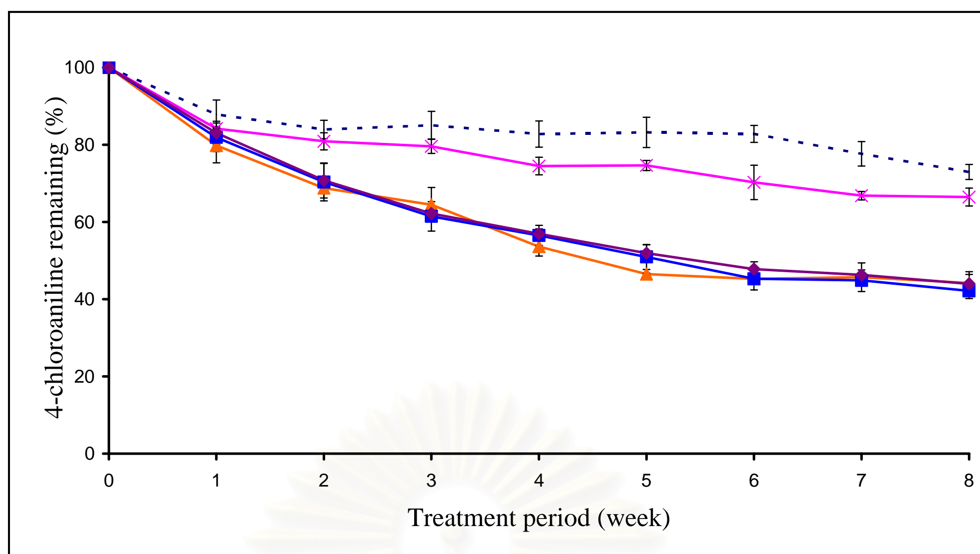

Figure 4.8 Biodegradation of 500-ppm 4-chloroaniline in sandy loam clay soil for 8 weeks incubation time. The treatments include bioaugmentation with bacterial consortium (—◆—); with pure bacterial culture; *Klebsiella planticola*(—■—), biostimulation (—▲—), and natural attenuation (—×—). The control was sterilized soil (- - - - -).

(a) Number of 4-chloroaniline-degrading bacteria for incubation time extension in sandy clay loam soil

The populations of 4-chloroaniline-degrading bacteria were determined by plate count technique with the supplementation of 100-ppm 4-chloroaniline. The number of 4-chloroaniline-degrading bacteria was changed over 8 weeks of incubation period (Figure 4.9). 4-Chloroaniline-degrading bacterial colony was undetectable in the control treatment. There was no significant change for the number of 4-chloroaniline-degrading bacteria in natural attenuation treatment of which the number of 4-chloroaniline degrading bacteria was ranging from $8.85x10^{2}\pm 1.20x10^{2}$CFU/g soil to $3.35x10^{3}\pm 2.12x10^{2}$CFU/g soil within 8 weeks.

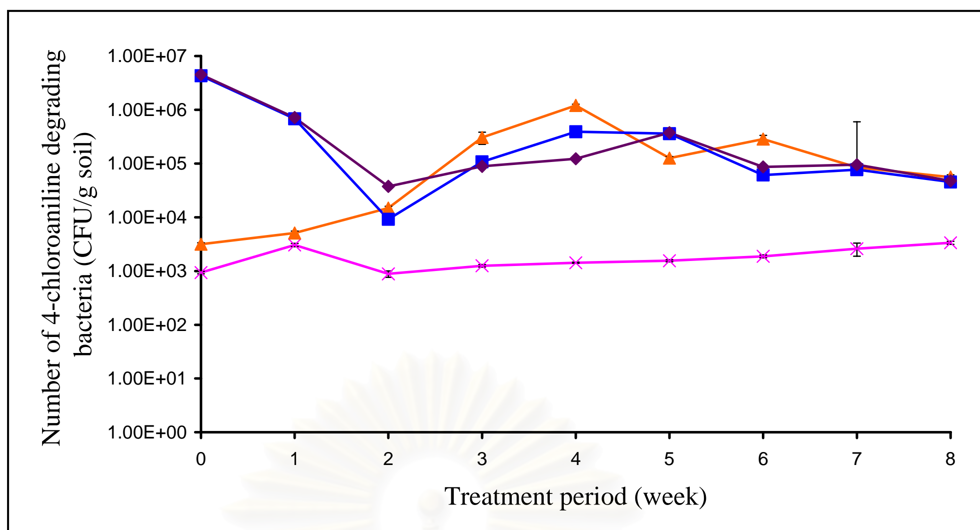

Figure 4.9 The number of 4-chloroaniline-degrading bacteria of sandy loam clay soil in all three bioremediation treatments when the incubation time was extended to 8 weeks. The bioremediation treatment includes bioaugmentation with bacterial consortium (—◆—); with pure bacterial culture; *Klebsiella planticola* (—■—), biostimulation (—▲—), and natural attenuation (—×—).

The number of 4-chloroaniline degrading bacteria increased significantly 1,000 times (from $3.15x10^3 \pm 2.12x10^2$ CFU/g soil to $1.21x10^6 \pm 6.3x10^4$ CFU/g soil) during biostimulation treatment of sandy clay loam soil. When bioaugmented with bacterial pure culture or with bacterial consortium, the number of 4-chloroaniline-degrading bacteria showed similar pattern in that the bacterial number was decreased during the first two weeks of the incubation time and then increased afterwards. For bioaugmentation with bacterial pure culture, the amount of 4-chloroaniline degrading bacteria decreased 1,000 times (from $4.3x10^6 \pm 2.83x10^5$ CFU/g soil to $9.15x10^3 \pm 6.36x10^2$CFU/g soil) in the first two weeks. Then, the amount of 4-chloroaniline-degrading bacteria increased 10 times (from $4.45x10^4 \pm 4.95x10^3$ CFU/g soil to $3.90x10^5 \pm 5.66x10^4$ CFU/g soil) in the six following weeks. For bioaugmentation

with bacterial consortium, the amount of 4-chloroaniline-degrading bacteria decreased 100 times (from $4.50x10^6 \pm 4.24x10^5$ CFU/g soil to $3.75x10^4 \pm 3.54x10^3$ CFU/g soil) in the first two weeks. Then, the amount of 4-chloroaniline degrading bacteria increased 10 times (from $4.75x10^4 \pm 3.54x10^3$ CFU/g soil to $3.75x10^5$ $\pm 4.95x10^4$ CFU/g soil) in the six following weeks.

4.4.3.2 Effect of soil pH for 4-chloroaniline degradation

According to the topic 4.4.3.1, the highest 4-chloroaniline degradation percentage in sandy clay loam soil was only 55-58% even after extended the incubation period to 8 weeks. pH of sandy clay loam soil was 4.3 which was acidic soil. One of the key factors to consider for bioremediation treatment is the soil pH value. From previous studies, most of microorganisms prefer a neutral pH (Hupe, 2001). Consequently, pH of sandy clay loam soil was later adjusted from 4.3 to neutral (~ pH 7) using calcium carbonate ($CaCO_3$). The effect of pH for biodegradation of 500-ppm 4-chloroaniline in sandy loam clay soil was shown in Figure 4.12.

After pH adjustment, the highest percentage of 4-chloroaniline degradation (95%) was observed in bioaugmentation with bacterial consortium after 2 months of incubation. Bioaugmentation with bacterial pure culture showed 64% of 4-chloroaniline was degraded at the same period. Biostimulation treatment with 1 mM aniline promoted 4-chloroaniline degradation to 63%. Bioaugmentation and biostimulation processes showed high range (63%-95%) of 4-chloroaniline biodegradation, while only 29% of 4-chloroaniline was reduced via natural attenuation treatment (Figure 4.10).

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After pH adjustment, soil pH was almost constant in the range of pH 6.6-6.8 through 8 weeks of incubation.

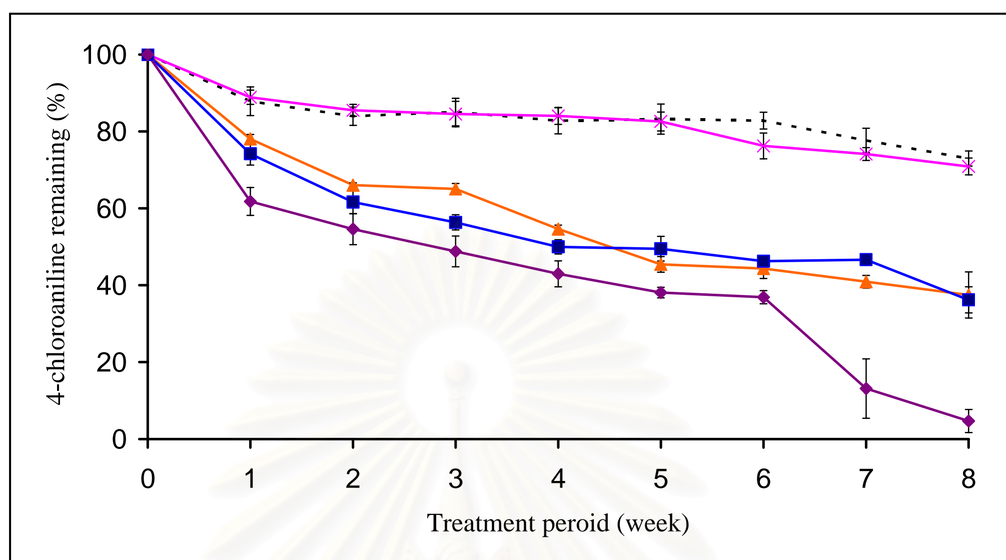

Figure 4.10 The effect of pH for biodegradation of 500-ppm 4-chloroaniline in sandy loam clay soil. The treatments include bioaugmentation with bacterial consortium (—◆—); with pure bacterial culture; *Klebsiella planticola* (—■—), biostimulation (—▲—), and natural attenuation (—×—). The control was sterilized soil (- - - - -).

(a) Number of 4-chloroaniline-degrading bacteria after pH adjustment in sandy clay loam soil

The populations of 4-chloroaniline-degrading bacteria were determined by plate count technique with the supplementation of 100-ppm 4-chloroaniline. The number of 4-chloroaniline-degrading bacteria in each treatment after pH adjustment in sandy clay loam soil (Figure 4.11). 4-chloroaniline-degrading bacterial colony was undetectable in the control treatment. There was no significant change of the number of 4-chloroaniline-degrading bacteria in natural attenuation treatment in which the number was ranging from $9.35 x 10^2 \pm 49.50$ CFU/g soil to $5.80 x 10^3 \pm 4.24 x 10^2$ CFU/g soil. There was significant change in the number of 4-chloroaniline-degrading

bacteria for biostimulation treatment. During the biostimulation treatment in pH adjusted sandy clay loam soil, the number of 4-chloroaniline- degrading bacteria was increased approximately 100 times (from $3.40x10^3 \pm 1.41x10^2$ CFU/g soil to $9.95x10^5 \pm 4.95x10^4$ CFU/g soil).

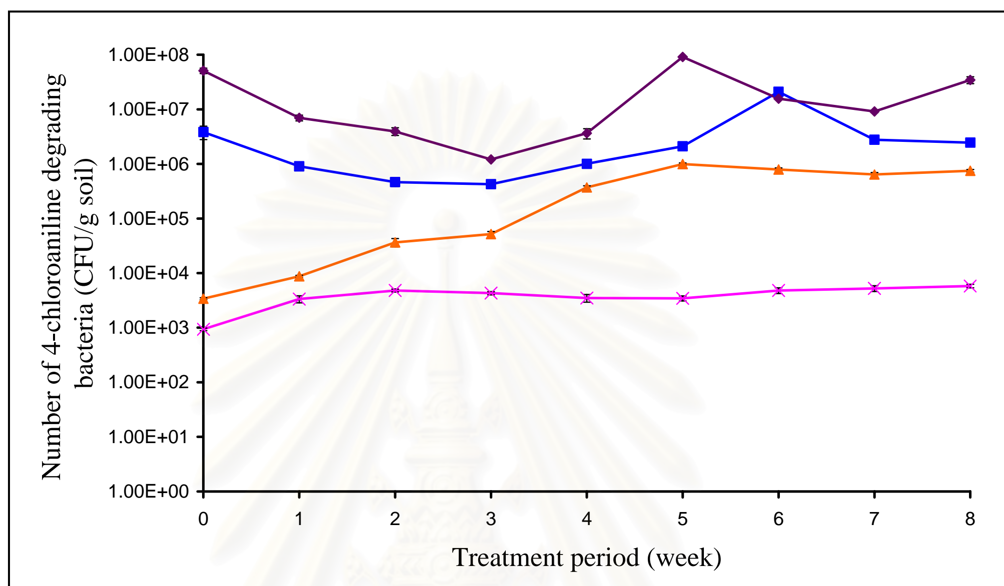

Figure 4.11 The number of 4-chloroaniline-degrading bacteria for effect of pH in sandy loam clay soil. The number of bacteria was determined, during two months of incubation, in each treatment which includes bioaugmentation (with bacterial consortium ; with pure bacterial culture), biostimulation ( ), and natural attenuation ( ).

When bioaugmented with bacterial pure culture or with bacterial consortium, the number of 4-chloroaniline-degrading bacteria in soil showed similar pattern in that the bacterial number was decreased during the first three weeks of incubation time and then incubation afterwards the bacterial number was increased. For bioaugmentation with bacterial pure culture, the amount of 4-chloroaniline degrading bacteria decreased 10 times (from $3.85x10^6 \pm 1.06x10^6$ CFU/g soil to

$4.25x10^5 \pm 4.95x10^4$ CFU/ g soil) in three weeks. Later, they increased 10 times (from $1.01x10^6 \pm 6.36x10^4$ CFU/g soil to $2.10x10^7 \pm 2.83x10^5$ CFU/g soil). For bioaugmentation with bacterial consortium, the amount of 4-chloroaniline-degrading bacteria decreased 10 times (from $5.10x10^7 \pm 2.82x10^5$ CFU/g soil to $1.21x10^6 \pm 4.242x10^4$ CFU/g soil) in three weeks. Later, the number of 4-chloroaniline-degrading bacteria increased 10 times (from $3.65x10^6 \pm 7.77x10^5$ CFU/g soil to $9.10x10^7 \pm 2.83x10^6$ CFU/g soil).

(b) Total microbial activity after pH adjustment in sandy clay loam soil

The total microbial activity of sandy clay loam soil could not be clearly distinguished from that of the control. It has been reported that the total microbial activity tested in control treatment could be higher than that of the treatment; therefore the data interpretation might be unfeasible (Alef, 1995).

(c) Analysis of microbial community after pH adjustment in sandy clay loam soil

DGGE analysis was used to evaluate the microbial community change in each biological treatment after pH adjustment in sandy clay loam soil. The soil DNA in natural attenuation treatment could not be extracted and probably because the number of bacteria in this soil was too low. The changes of soil bacterial populations were obvious in biostimulation and bioaugmentation (Figure 4.12). The results indicated that aniline and bacterial addition influenced the growth of various soil bacteria. DGGE profiles of biostimulation with 1 mM aniline illustrated the drastic changes of soil bacterial populations during the 6th and 8th week (Figure 4.12a).

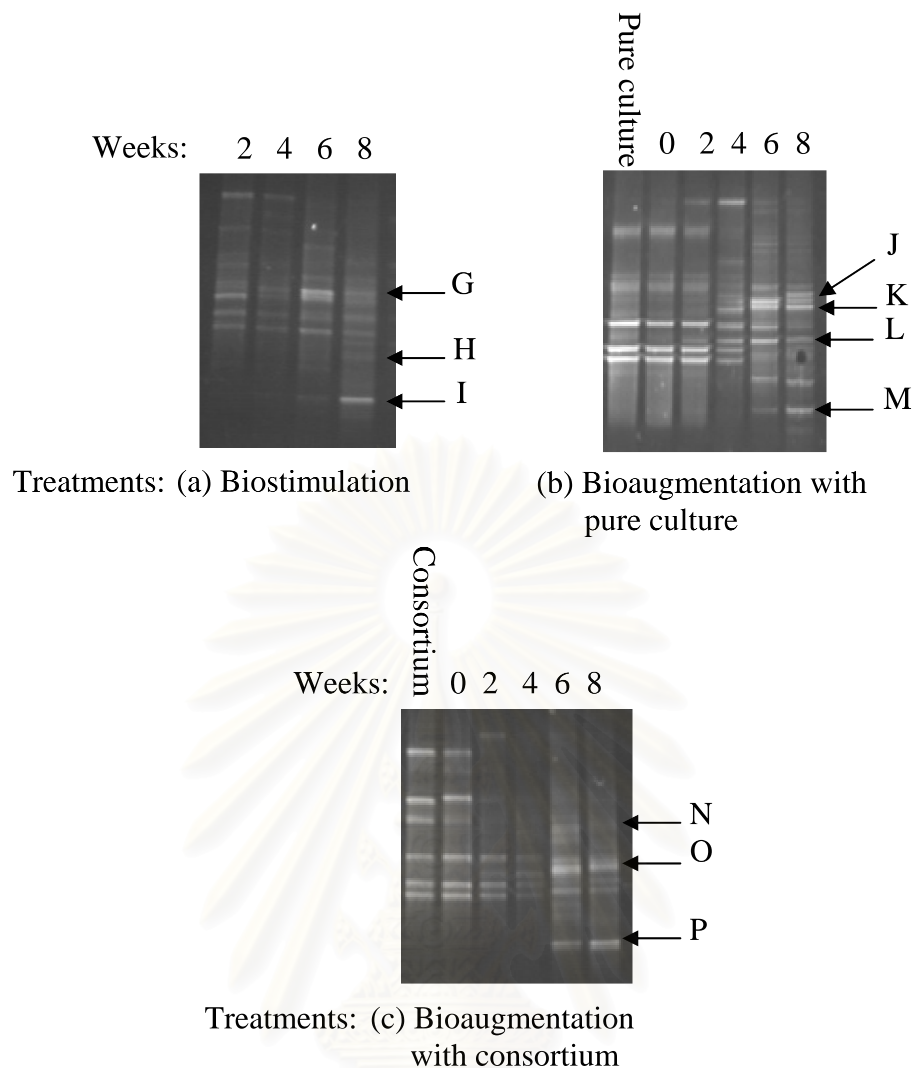

Figure 4.12 DGGE profile of the bioremediation treatments of sandy clay loam soil; biostimulation (a), bioaugmentation with either bacterial pure culture (b), or bacterial consortium (c) during 8 weeks of incubation. Pure culture; *Klebsiella planticola* indicated the 16S rDNA of chromosomal DNA of bacterial pure culture. Consortium indicated 16S rDNA of chromosomal DNA of bacterial consortium. Numbers represent the week of incubation. G, H, I, J, K, l, M, N, O and P indicated the fragments that were sequenced.

Interestingly, DGGE profiles from bioaugmentation with bacterial pure culture illustrated the increase number of bacterial populations, which represented by the increasing of DNA bands from the 0-2nd week to the 4th and 8th week (Figure

4.12b). In bioaugmentation with bacterial consortium, DGGE profiles showed several new dominant bands during the 6th and 8th week (Figure 4.12c).

Moreover, the dominant bands in each treatment were sequenced (Figure 4.12). Fragments G, H and I were found in biostimulation were matched closely to with *Bacillus Ginsenggisoli* (94%), *Bacillus* sp. (99%) and *Micromonospora* sp. (100%), respectively. Fragments J, K, L and M detected in bioaugmentation with bacterial pure culture. Fragments J and K matched closely to *Bacillus ginsenggisoli* (95%) and *Pseudomonas* sp. (95%), respectively. Fragment L matched closely to *Bacillus* sp. and *Bacillus drentensis* (94%). Fragment M corresponded to *Micromonospora* sp. (100%). Fragments N, O and P appeared in bioaugmentation with bacterial consortium. Fragment N corresponded to *Thermomonospora fusca* and *Pantoea endophytica* (100%). Fragment O showed high similarity (98%) to *Microbacterium* sp. Fragment P corresponded (100%) to *Micromonospora* sp. and *Actinoplanes* sp. (Table 4.5).

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Table 4.5 Bacterial strains detected in each biological treatment of sandy clay laom soil

Treatment	Fragment	Bacterial species	Accession number	Source	% Similarity	Reference
Biostimulation	G	*Bacillus ginsenggisoli*	AB245379	Soil	94	Im and Lee (unpublished)
	H	*Bacillus* sp.	DQ314538	Water	99	Lin *et al*. (unpublished)
	I	*Micromonospora* sp.	AF131387	Soil	100	Wang *et al*. (unpublished)
Bioaugmentation with pure culture	J	*Bacillus ginsenggisoli*	AB245379	Soil	95	Im and Lee (unpublished)
	K	*Pseudomonas* sp.	DQ268810	Groundwater	95	Gao *et a*l. (unpublished)
	L	*Bacillus* sp. *Bacillus drentensis*	AY704918 AY466403	Water Soil	94	Zhang *et al*. (unpublished)
	M	*Micromonospora* sp.	AF131387	Soil	100	Wang *et al*. (unpublished)
Bioaugmentation with consortium	N	*Thermomonospora fusca* *Pantoea endophytica*	AM161170 AM161168	- -	100	Ripka *et al*. (unpublished)
	O	*Microbacterium* sp.	AY83801	Biomass	98	Purohit *et al*. (unpublished)
	P	*Micromonospora* sp. *Actinoplanes* sp.	AF131387 AF131344	Soil Soil	100	Wang *et al*. (unpublished) Wang *et al*., 1999

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CHAPTER V

DISCUSSIONS

5.1 Effect of biological treatment

4-Chloroaniline biodegradation has been widely studied in liquid medium. However, there is very little information on the potential for 4-chloroaniline biodegradation in soil. In this study, natural attenuation, biostimulation and bioaugmentation were investigated to determine a suitable bioremediation option for 4-chloroaniline biodegradation in contaminated soil.

The comparison of three biological treatments including natural attenuation, biostimulation and bioaugmentation showed the significant difference between each treatment in loam soil. Bioaugmentation with bacterial consortium showed the greatest 4-chloroaniline degradation, in which 96% of 4-chloroaniline was degraded after one month of incubation. Bioaugmentation treatment showed a rapid and significant biodegradation (0.85 μmole 4-chloroaniline/days) within the first 7 days. This is probably because the numbers of 4-chloroaniline-degrading microorganisms that added into soil in the range of 10^7-10^8 CFU/g soil were enough to start 4-chloroaniline degradation without any adaptation period. In addition, bioaugmentation with bacterial consortium also showed the highest percent of 4-chloroaniline degradation; which was 95% degradation after pH adjustment in sandy clay loam soil after two months of incubation. These bioaugmentation results agree with the previous study when bioaugmentation was used to treat light and heavy petroleum hydrocarbon from Long Beach soil sample. It was shown that bioaugmentation was the best treatment where 75% and 73% of light and heavy

petroleum hydrocarbon were degraded when compared to biostimulation (46% and 45% of light and heavy petroleum hydrocarbon) and natural attenuation (49% and 46% of light and heavy petroleum hydrocarbon) (Bento et al., 2004). These studies supported that bioaugmentation represents a valuable alternative method to treat 4-chloroaniline contaminated soil.

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

The number of 4-chloroaniline-degrading bacteria which was added in bioaugmentation slightly decreased in the first seven days because they adapted themselves to the new environment which had some biotic stress (predators and parasites) and abiotic stress (pH, soil characteristic and environment) (Alexander, 1994). Then, the number of 4-chloroaniline-degrading bacteria increased. The amount of 4-chloroaniline continuously decreased, even the number of 4-chloroaniline-degrading bacteria decreased (Figure 5.1). It is probably because number of the survived 4-chloroaniline-degrading bacteria was enough to degrade 4- chloroaniline.

DGGE results from bioaugmentation microcosms of loam soil showed that the bacterial community was changed after incubation. Meanwhile, the added bacteria were not found in DGGE profile after incubation. It was possible that the proportion of added bacteria was lower than other indigenous soil bacteria, thus we could not extract their DNA out of soil sample. In the 3rd and 4th week, a single dominant band was observed both in bioaugmentation with bacterial pure culture and consortium

(Figure 4.7c and 4.7 d). Interestingly, the number of 4-chloroaniline-degrading bacteria was also increased in the 3rd week. Given that the amounts of 4-chloroaniline were low in this period, this dominant band may represent the indigenous 4-chloroaniline degrader or may be the population that involve in the degradation of 4-chloroaniline intermediates.

From 4-chloroaniline and microbiological analysis, it may be concluded that the decreasing of 4-chloroaniline at the first week was due to the added bacteria. Meanwhile, the number of added bacteria was reduced afterward, which suggested that these bacteria could not adapt to soil conditions. After 3-week incubation, the degradation of 4-chloroaniline was probably occurred by the activity of indigenous bacteria. The increasing of 4-chloroaniline-degrading bacteria at 3rd and 4th week may be the indigenous bacteria which adapted to degrade 4-chloroaniline.

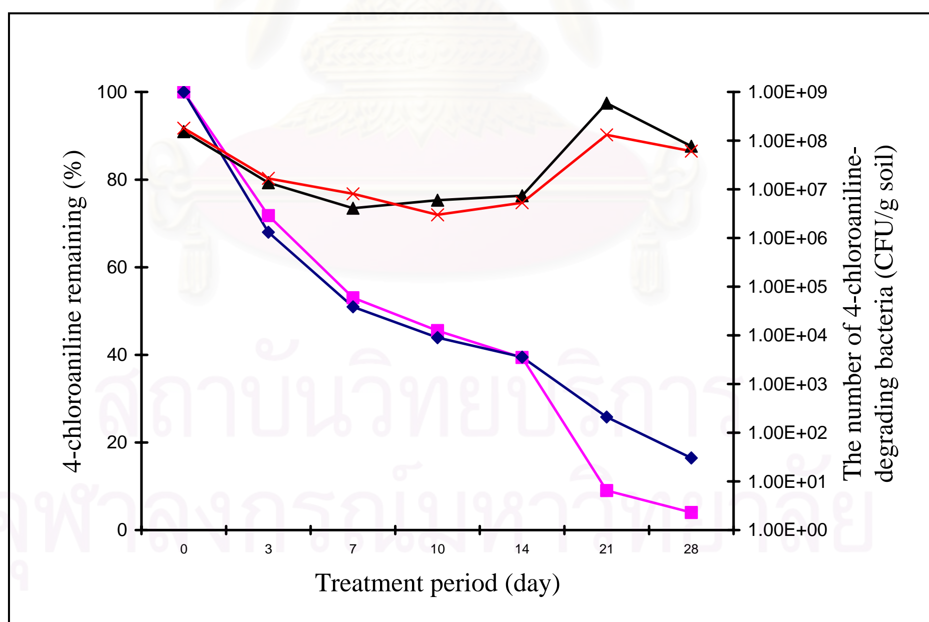

Figure 5.1 Relationship between the amount of 4-chloroaniline; in bioaugmentation with bacterial pure culture (—◆—) and bacterial consortium (—■—) and the number of 4-chloroaniline degrading bacteria; in bioaugmentation with bacterial pure culture (—×—) and bacterial consortium (—▲—) of loam soil.

Biostimulation of loam soil showed that 4-chloroaniline concentration was gradually decreasing when the time passed. The degradation percentages of biostimulation treatments showed the lower value in the first week. Then, the degradation percentages of biostimulation treatment increased when the time passed. In the beginning of biostimulation, the indigenous bacteria used labile carbon sources in the soil for growth (Bento et al., 2004). When the labile carbon source was decreased, the indigenous bacteria adapted themselves to use more complex substances as nutrients. In this study, the indigenous bacteria probably used labile carbon sources such as organic carbon, organic matter in soil and aniline as nutrients. After that the indigenous bacteria might try to use 4-chloroaniline as carbon and nitrogen sources for growth.

The C:N ratio of soil is also an important factor for biodegradation. The optimum value of C:N ratio for enhancing biodegradation generally is less than 20 (Hupe et al., 1995). Loam soil has the C:N ratio of 11.79, suggesting that this soil type is suitable for bacteria growth as well as the biodegradation. When 4-chloroaniline and aniline was added, they could be used as carbon and nitrogen source. The amount of 4-chloroaniline and aniline added into soil was as low as 0.05% and 0.01% (w/w), respectively. Therefore the C:N ratio did not change when 4-choroaniline and aniline was added. It is, therefore, not necessary to add the other carbon and nitrogen into the soil.

Previous studies showed that 1 mM aniline stimulated 4-chloroaniline degradation in liquid media as well as in a laboratory-scale biofilm reactor (Radianingtyas et al., 2003). In this study, aniline at final concentration of 1mM was used as an inducer to stimulate the soil bacteria to degrade 4-chloroaniline. The

results of our investigation illustrated that 1 mM aniline also enhanced 4-chloroaniline degradation in soil.

The number of 4-chloroaniline-degrading bacteria presented in loam soil of biostimulation treatment agreed with the 4-chloroaniline biodegradation results. The number of 4-chloroaniline-degrading bacteria increased when the amount of 4-chloroaniline decreased (Figure 5.2). The amounts of 4-chloroaniline-degrading bacteria in biostimulation treatment were continuously increased because these bacteria adapted themselves step by step to use 4-chloroaniline as nutrients.

The DGGE results in biostimulation of loam soil showed that the bacterial community was changed after incubation. It was observed that the DNA bands obviously increased in the 3rd and 4th week (Figure 4.7b). The results corresponded to the increased number of 4-chloroaniline-degrading bacteria in the same period. Consequently, these DNA bands probably came from the indigenous 4-chloroaniline degraders, which were the dominant populations of biostimulated soil.

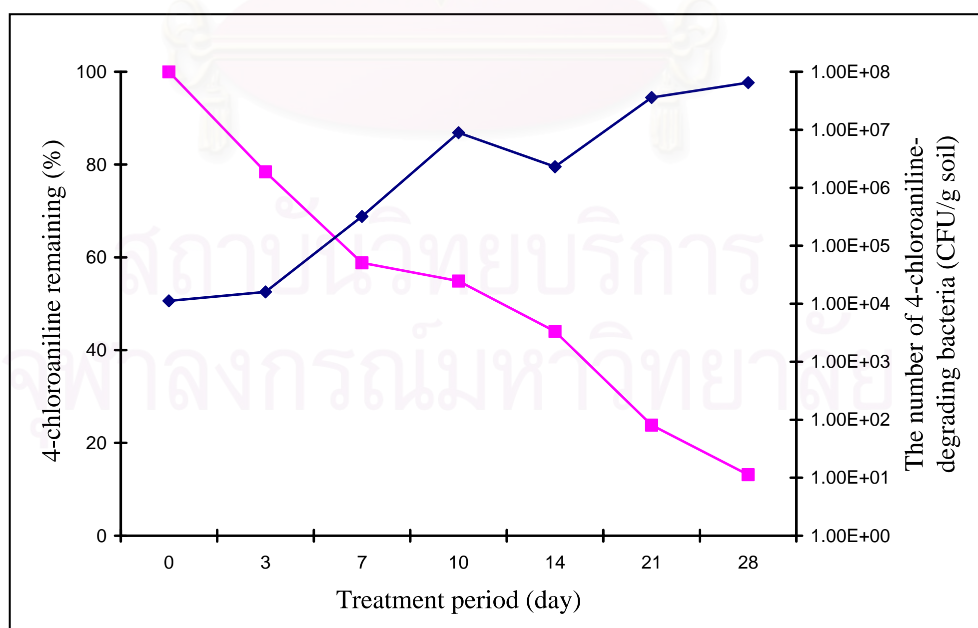

Figure 5.2 The relationship of the amount of 4-chloroaniline (—■—) and the number of 4-chloroaniline degrading bacteria (—◆—) in biostimulation treatment of loam soil.

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Natural attenuation of loam soil showed 67% 4-chloroaniline removal. Besides the loss by biological activities, 4-chloroaniline may be decreased through adsorption because 4-chloroaniline can combine tightly with soil components (Bollag et al., 1978). The number of 4-chloroaniline-degrading bacteria present in the soil of natural attenuation results suggested that some parts of 4-chloroaniline were biodegraded (Figure 5.3). However, it took more time than bioaugmentation and biostimulation. The number of 4-chloroaniline-degrading bacteria began to increase slightly when the amount of 4-chloroaniline decreased in the 3rd and 4th week.

The result of DGGE profile in natural attenuation of loam soil showed that the bacterial community in natural did not change after incubation (Figure 4.7a). This result agreed with the number of 4-chloroaniline-degrading bacteria which was almost stable through out the experiment. DGGE profile in natural attenuation did not change, even though the number of 4-chloroaniline degrading bacteria slightly increased at the end of incubation. The results suggested that 4-chloroaniline degrading bacteria were not the dominant populations in natural attenuation treatment.

When comparison with biostimulation and bioaugmentation, natural attenuation was failure to clean up 4-chloroaniline because the number of the indigenous 4-chloroaniline-degrading bacteria (10^4 CFU/g soil) was too low to degrade 4-chloroaniline. The number of the indigenous 4-chloroaniline-degrading bacteria was more than 10^5 CFU/g soil, they were enough for biodegradation (Providenti, 1993).

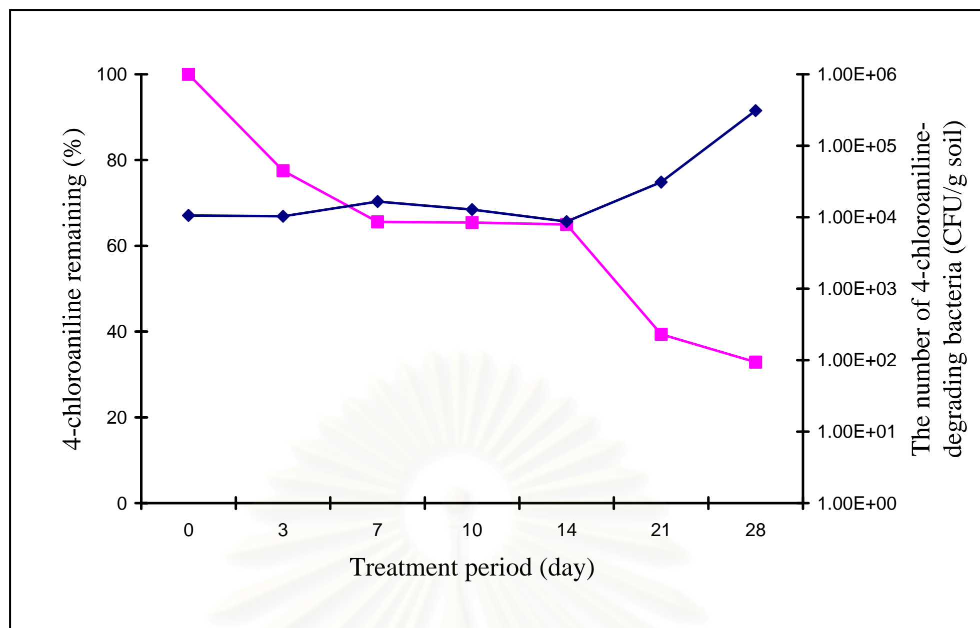

Figure 5.3 Relationship between the amount of 4-chloroaniline (—■—) and the number of 4-chloroaniline degrading bacteria (—◆—) in natural attenuation treatment of loam soil.

5.2 Effect of soil type

The first soil was loam soil with pH 5.3. After 28 days bioremediation, 500 ppm 4-chloroaniline was decreased through natural attenuation, biostimulation and bioaugmentation (with bacterial pure culture and with bacterial consortium) in the value of 67%, 87%, 84% and 96%, respectively.

Another soil was a sandy clay loam soil and moderately acid (pH 4.3). 48%, 50%, 41% and 38% of 4-chloroaniline was removed through natural attenuation, biostimulation and bioaugmentation (with bacterial pure culture and with bacterial consortium), respectively after 28 days. There was no significant difference in each bioremediation treatment in sandy clay loam soil at this period. Although bioaugmentation showed the highest degradation, the difference was not much from other treatments when compared to the result of loam soil where 9% and 29%

differences between bioaugmentation, biostimulation and natural attenuation, respectively.

Although the incubation time was extended to two months, the 4-chloroaniline degradation percentage was slightly increased in the value of 34%, 56%, 57% and 58% through natural attenuation, biostimulation and bioaugmentation (with bacterial pure culture and bacterial consortium), respectively. The extended time slightly increased 4-chloroaniline degradation efficiency. These results indicated that the success of bioremediation treatment may not achieve with different soil types. Other factors that may affect degradation are contaminant concentration and environmental condition (Alexander, 1994). It is possible that the environmental condition was not suitable for the growth of the added 4-chloroaniline degrading bacteria. The environmental condition (such as pH) should be adjusted at beginning of bioremediation to enhance the growth of the added or indigenous 4-chloroaniline degrading bacteria.

Since soil type affects the bioremediation efficiency, in some environmental conditions, bioaugmentation treatment was not completely succeeded. For example, bioaugmentation with bacterial consortium was not effective in enhancing biodegradation of the mixed PAHs in mangrove sediments (Yu et al., 2005). Although, the bacterial consortium was able to degrade three PAHs in liquid medium, the degradation ability of the bacterial consortium was suppressed by other microorganisms in the sediment. The indigenous microorganisms (natural attenuation) had a high potential to degrade the PAHs in mangrove sediments (Yu et al., 2005). Bento et al. (2004) also reported that bioaugmentation was an inappropriate method for degradation of total petroleum hydrocarbons in Hong Kong soil. Bioaugmentation showed the greatest total petroleum hydrocarbons degradation in the Long Beach soil

(pH 6.3 and 15.1 g/kg of organic carbon) while the Hong Kong soil (pH 7.7 and 38.1 g/kg of organic carbon) has the highest degradation upon natural attenuation (Bento et al., 2004). In addition, several literatures showed that soil type is a factor which influences the biodegradation rate (Klier et al., 1999 and Hamby, 1996).

5.3 Effect of soil pH

One factor that plays an important role to enhance biodegradation is soil pH (Hamby, 1996, Vogel, 1996 and Alexander, 1994). In this study, soil pH of sandy clay loam soil was 4.3 (acidic soil). The highest degradation percentage (50%) of 4-chloroaniline was in bioaugmentation after two months of incubation. When pH of sandy clay loam soil was adjusted from 4.3 to the neutral pH (pH ~ 7), the 4-chloroaniline degradation percentage in bioaugmentation was increased to 95 %. Therefore, it could be concluded that the neutral soil pH enhanced 4-chloroaniline biodegradation in sandy clay loam soil. The pH adjustment result agreed with several literatures which showed that most of the microorganisms degrade contaminants at a slightly acid to neutral pH; pH 6-7.5 (Hupe, 2001 and Alexander, 1994).

Bacterial populations of sandy clay loam soil after pH adjustment was investigated by DGGE. DGGE profiles from bioaugmentation with bacterial pure culture and bacterial consortium was changed since the 4th week. This result correlated to the number of 4-chloroaniline-degrading bacteria which increased in the 4th week as well. DGGE profiles of biostimulation illustrated the changes of soil bacterial populations during the 6th and 8th week and the number of 4-chloroaniline-degrading bacteria increased at the same time. It was observed that soil DNA could not extract at the 0 week of biostimulation and natural attenuation, which was probably due to the low numbers of 4-chloroaniline-degrading bacteria as well as

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other bacteria in soil. The number of 4-chloroaniline-degrading bacteria was less than 10^4 CFU/g soil. These results corresponded to 4-chloroaniline degradation. The amount of 4-chloroaniline-degrading bacteria was low, the percentage of 4-chloroaniline degradation was also low.

5.4 Microorganisms involved in 4-chloroaniline degradation

Bioremediation is one of the efficient methods to remove the contaminated 4-chloroaniline from the environment. Therefore, several studies have been performed to screen and isolate microorganisms capable of 4-chloroaniline biodegradation. Previous studies have shown that microorganisms can use 4-chloroaniline as growth substrate, i.e. as sole carbon and nitrogen sources. *Moraxella* sp. strain G can use 4-chloroaniline as sole carbon source and nitrogen source via a modified ortho-cleavage pathway as shown in Figure 2.1 of Literature review. 0.5 mM 4-chloroaniline was completely degraded by *Moraxella* sp. strain G within 9 hours (Zeyer et al., 1985). In addition, *Pseudomonas* sp. was grown on 2.5 mM 4-chloroaniline as only carbon and nitrogen source, 64% of carbon of 2.5 mM 4-chloroaniline was released as carbon dioxide after 10 days of incubation. (Zeyer and Kearney, 1982). Previous study in our laboratory also showed that *Klebsiella planticola* isolated from soil can degrade 63% of 25 ppm (0.2 mM) 4-chloroaniline in liquid medium within 12 days. However, 200 ppm (1.57mM) inhibited the growth of *Klebsiella planticola* in liquid medium.

In addition, 4-chloroaniline can also degrade by a bacterial consortium. A bacterial consortium consisted of three bacteria; C1, C2 and C3 which were gram negative bacteria. The bacterial consortium was isolated in our laboratory and almost completely removed 100 ppm (0.78 mM) 4-chloroaniline after 3 weeks incubation in

liquid medium. This result agrees with Radianingtyas (2003a), who isolated a bacterial consortium from soil comprising of four different species (*Chryseobacterium indologenes* SB1, *Comamonas testosteroni* SB2, *Pseudomonas corrugata* SB4 and *Stenotrophomonas maltophilia* SB5). This bacterial consortium could completely degrade 1 mM 4-chloroaniline in presence of 1 mM aniline after 50 days in liquid medium at 30°C (Radianingtyas et al., 2003a).

In this study, the ability of two bacterial pure cultures, i.e. *Klebsiella planticola,* and bacterial consortium towards 4-chloroaniline degradation was investigated in soil. Biodegradation of 4-chloroaniline was carried out at much higher concentration than that in liquid medium since 4-chloroaniline applied into soil can combine rapidly with soil components (Bollag et al., 1978). The concentration of 4-chloroaniline in this study was varied from 100 ppm (0.78 mM) to 1000 ppm (7.84 mM). The degradation of 4-chloroaniline in soil decreased both in bacterial pure culture and bacterial consortium when the 4-chloroaniline concentration increased. Bioaugmentation with bacterial pure culture showed 86.55% degradation of 100 ppm 4-chloroaniline while 1000 ppm 4-chloroaniline was reduced only 64.14%. The higher concentration became toxic to the microbial culture and inhibited the growth of the microbial culture.

From DGGE analysis, we were able to identify several bacterial populations that dominated in both loam soil and sandy clay loam soil after bioremediation. Some of these bacterial species have been reported as degraders of organic pollutants such as *Enterobacter* sp. *Sphingomonas* sp., *Pseudomonas* sp., *Bacillus* sp. and *Microbacterium* sp. (Table 5.1 and 5.2). A DGGE band from bioaugmentation treatment of sandy clay loam soil was corresponded to *Pseudomonas* sp., which was reported to have 4-chloroaniline degrading ability. Meanwhile, various bacterial

species have never been reported for any degradation abilities. The results suggested that variety of bacteria species may involve in 4-chloroaniline bioremediation. In addition, the present of each bacteria species may influence by soil types and bioremediation treatments.

Table 5.1 Soil bacteria in each treatment of loam soil

Treatments	Bacterial species	Degradation ability	References
Natural attenuation	*Enterobacter* sp.	Hexazinone (herbicide)	Wang *et al*. (2005)
Biostimulation	*Actinomycete* sp.	-	-
	Hyphomicrobium zavarzinii	-	-
Bioaugmentation with pure culture	*Stenotrophomonas maltophilia*	-	-
	Sphingomonas sp.	Atrazine	Smith *et al*. (2005)
Bioaugmentation with consortium	*Streptomyces* sp.	-	-

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Table 5.2 Soil bacteria in each treatment of sandy clay loam soil after pH adjustment

Treatments	Bacterial species	Degradation ability	References
Biostimulation	*Bacillus ginsenggisoli*	-	-
	Bacillus sp.	Azo dyes Petroleum hydrocarbons Polycyclic aromatic hydrocarbons	Khehra *et al*. (2006) Verma *et al*. (2006) Toledo (2006)
	Micromonospora sp.	Natural rubber	Berekaa *et al*. (2000)
Bioaugmentation with pure culture	*Bacillus ginsenggisoli*	-	-
	Pseudomonas sp.	4-chloroaniline Hexazinone	Zeyer and Kearney (1982) Wang *et al*. (2005)
	Bacillus sp. *Bacillus drentensis*	Azo dyes Petroleum hydrocarbons Polycyclic aromatic hydrocarbons -	Khehra *et al*. (2006) Verma *et al*. (2006) Toledo (2006) -
	Micromonospora sp.	Natural rubber	Berekaa *et al*. (2000)
Bioaugmentation with consortium	*Thermomonospora fusca* *Pantoea endophytica*	Aliphatic aromatic copolyester -	Witt *et al*. (2001) -
	Microbacterium sp.	Quinoline-4-carboxylic acid	Röger and Lingens (1989)
	Micromonospora sp. *Actinoplanes* sp.	Natural rubber -	Berekaa *et al*. (2000) -

CHAPTER VI

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

6.1 Conclusions

The comparison of three biological treatments including natural attenuation, biostimulation and bioaugmentation showed the significant difference between each treatment in loam soil. Bioaugmentation with bacterial consortium showed the greatest 4-chloroaniline degradation, in which 96% of 4-chloroaniline was degraded after one month of incubation. Biostimulation (addition of 1 mM aniline) and bioaugmentation with pure bacterial cultural showed 87% and 84% of 4-chloroaniline degradation, respectively. The natural attenuation of this soil resulted in 67% of 4-chloroaniline degradation.

Meanwhile, there was no significant difference in each bioremediation treatment of sandy clay loam soil after one month of incubation. When the incubation time was extended to two months, the 4-chloroaniline degradation was increased but percent 4-chloroaniline degradation was still less than loam soil at the same incubation period. The results suggested that sandy clay loam soil was not suitable for 4-chloroaniline degradation. Sandy clay loam soil was acidic therefore the soil pH was later adjusted to neutral. Bioaugmentation of the pH adjusted soil with bacterial consortium showed the greatest 4-chloroaniline degradation (95%) after two months of incubation. The biostimulation (addition of 1 mM aniline) and bioaugmentation with pure bacterial culture showed 63% and 64% of 4-chloroaniline degradation, respectively. The natural attenuation of this soil resulted in 29% of 4-chloroaniline degradation. It can be concluded that bioaugmentation with bacterial consortium is

the best bioremediation treatment for 4-chloroaniline removal from the contaminated soil both loam soil and sandy clay loam soil.

Comparison of 4-chloroaniline degradation within the same bioremediation treatment, the results indicated that 4-chloroaniline degradation percentage in these two soil types were significantly different. Therefore, the success of bioremediation technique also depended on soil type and characteristic. The suitable soil condition should be optimized to ensure the efficient bioremediation.

The decreased 4-chloroaniline during bioremediation was corresponded with the increased number of 4-chloroaniline degrading bacteria. The 4-chloroaniline degrading bacteria in bioaugmentation treatment are more abundant and active than natural attenuation and biostimulation. However, the number of 4-chloroaniline degrading bacteria in biostimulated soil was gradually increased after incubation. The results indicated that indigenous soil bacteria were able to degrade 4-chloroaniline and their growth was induced by 1 mM aniline. DGGE profiles showed that the bacterial community in biostimulation and bioaugmentation was changed when the 4-chloroaniline decreased. The dominant bacterial populations were probably responsible for the degradation of 4-chloroaniline or its intermediates. On the other hand, the bacterial community in natural attenuation was constant through the experiment.

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6.2 Suggestions for future work

Before application of the bioremediation treatment, the scale up of soil microcosms should be recommended. For example, 5 g soil microcosms should be scaled up to soil mesocosms and field plots to optimize the biodegradation conditions that can provide the highest 4-chloroaniline degradation efficiency.

For bioaugmentation, the inoculation method may affect the survival of the added 4-chloroaniline-degrading bacteria. Preincubation of bacterial inoculum in a sterile soil carrier led to greater survival in soil microcosms than preincubation in a non-sterile soil carrier or inoculation of a liquid cell suspension (Van Dyke and Prosser, 2000). The inoculation method should be studied in order to enhance the survival of the added 4-chloroaniline-degrading bacteria. When the survival of the added 4-chloroaniline-degrading bacteria is maintained in the new environment, it improves the 4-chloroaniline biodegradation.

Sodium pyruvate could be used as an additional carbon source for biostimulation of indigenous bacteria in soil microcosms. It should improve the 4-chloroaniline degradation rate. From previous study, when aniline and 3-chloroaniline were used as sole nitrogen sources and sodium pyruvate was used as an addition carbon source, the degradation rate of aniline and 3-chloroaniline was more rapid than no using sodium pyruvate as an addition carbon source (Boon et al, 2001).

In addition, it is useful to study the behavior of microbial populations responsible for 4-chloroaniline degradation. Changes in populations of 4-chloroaniline-degrading bacteria could be monitored by DGGE using primers for detection of chlorocatechol 1,2-dioxygenase gene. Chlorocatechol 1,2-dioxygenase gene played an important role in 4-chloroaniline degradation (Zeyer,1985 and Radianingtyas, 2003). It helps more understanding in the community change of 4-chloroaniline-degrading bacteria during the bioremediation.

The results from this study showed that bioaugmentation is the best bioremediation for 4-chloroaniline treatment both two types of soil. Therefore, bioremediation technique can be applied to treat 4-chloroaniline in other types of soil which has amount of 4-chloroaniline-degrading bacteria less than 10^5 CFU/g soil and

neutral pH. If 4-chloroaniline degradation percent is low, the other conditions (such as organic matter, C:N ratio) should be optimized to provide the highest ability to degrade 4-chloroaniline. Besides, bioaugmentation can apply to clean up other herbicides. However, the characteristic of soil, the number of the indigenous bacteria and properties of herbicide should be characterized before bioremediation.

For management of 4-chloroaniline in the real site, the site characteristic could be surveyed to determine the soil properties, soil type, pH, moisture content, organic carbon, organic matter. After 4-chloroaniline was treated in the laboratory scale, the scale up should be recommended to provide the optimum condition for the real site. The degradation rate may be slow under the field condition because the different between field and laboratory condition. The environmental factors (such as oxygen, moisture content, pH, and temperature) should be considered to provide the highest efficiency of 4-chloroaniline degradation. For example, oxygen in the real site was not enough for microorganisms growth in the aerobic condition therefore oxygen should be supplied in the system. pH was not suitable for microorganism growth, pH should be adjusted to the optimum mostly neutral pH (Alexander, 1994).

The inhibitors such as the other toxic substances and/or metals in soil should be considered. Besides, the predators or competitors in soil affect the survival of bioaugmented bacteria. Therefore the bacteria have to be added in second addition to survive the adding bacteria and continuously degrade the contaminant (Alexander, 1994).

The bioavailability of 4-chloroaniline depends on the sorption between 4-chloroaniline and organic matter in soil. If the organic matter in soil is high, the 4-chloroaniline sorption rate is also high (Boehncke *et al.*, 2003). The bioavailability of

4-chloroaniline for microorganisms is low. Surfactant can be used to increase the bioavailability of contaminants for microbial degradation (Boopathy, 2000).

Bioremediation can use both *in situ* and *ex situ* treatment. *In situ* treatment, if soil is not removed from the field. The oxygen, nutrient or microorganisms should be added in the real site. *Ex situ* treatment required the removal of contaminated soil to another area to clean up. The system of *Ex situ* treatment is easy to control and adjust the conditions to the optimum conditions (Alexander, 1994).

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APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A

Chemical solutions

1. Mineral medium

Na_2HPO_4	1.4196 g
KH_2PO_4	1.3609 g
$MgSO_4.7H_2O$	0.0985 g
$CaCl_2.2H_2O$	0.0059 g
Deionized water	to 1000 ml

Before using add sterile trace element 1 ml in 1000 ml mineral medium.

2. Trace element

$Fe_3SO_4 .7H_2O$	0.278 g
$ZnSO_4.7H_2O$	0.115 g
$MnSO_4.H_2O$	0.169 g
$CuSO_4.5H_2O$	0.038 g
$CoCl_2.6H_2O$	0.024 g
MoO_3	0.010 g
H_3BO_4	0.116 g
Deionized water	to 100 ml

3. 0.85% Sodium Chloride

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

4. 10X Tris-borate-EDTA (TBE) stock buffer

Tris base	108 g
Boric acid	55 g
EDTA solution	40.0 ml
Deionized water	to 1000.0 ml

5. 10X Tris-acetate-EDTA (TAE) stock buffer

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

6. Luria Bertani (LB nutrient)

Tryptone	1 g
NaCl	1 g
Yeast extract	0.5 g
Deionized water	to 100.0 ml

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APPENDIX B

Inoculum standard curve

Both the pure bacterial culture and bacterial consortium were cultured in the liquid mineral medium with 100 ppm 4-chloroaniline and shaken 250 rpm, 30°C for 4-5 days. The bacterial cell was harvested by centrifugation at 12,000 rpm for 10 minutes. Cell pellet was washed with 0.85% sodium chloride solution for twice time. The cell pellet was resuspended with 0.85% sodium chloride solution and diluted to various OD_{600} by serial dilution. The diluted solution was spreaded on the LB agar plate in triplicate time and incubated at 30°C for 4-5 days. And then, the bacterial colony was counted in the range of 30 to 300 colonies. The standard curve was plotted with the OD_{600} and colony forming unit (CFU). The result was shown in the Figure B.1 and Figure B.2.

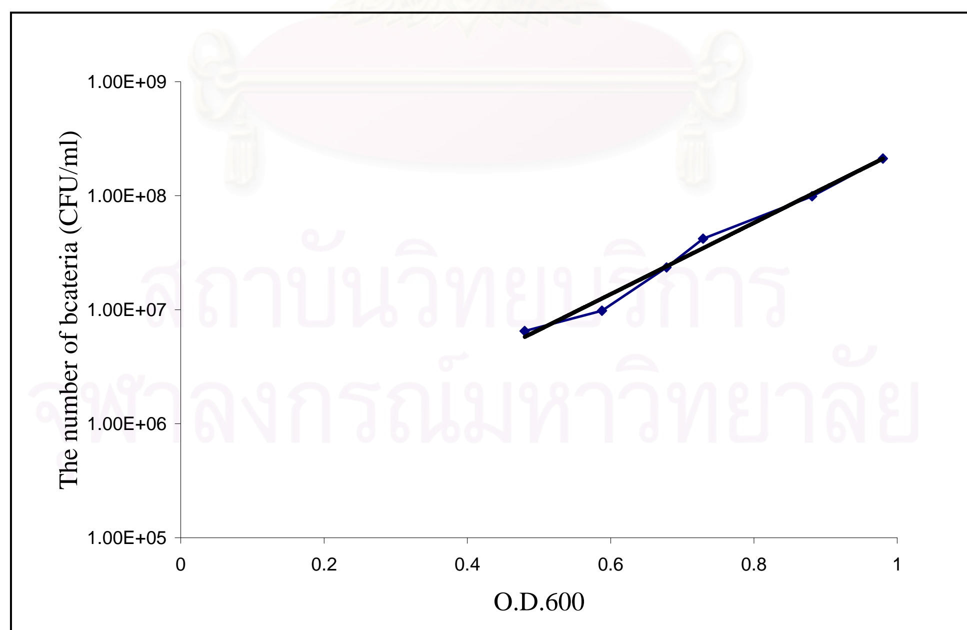

Figure B.1 Standard curve of *Klebsiella Planticola* plotted between the number of bacteria (CFU/ml) and $O.D._{600}$

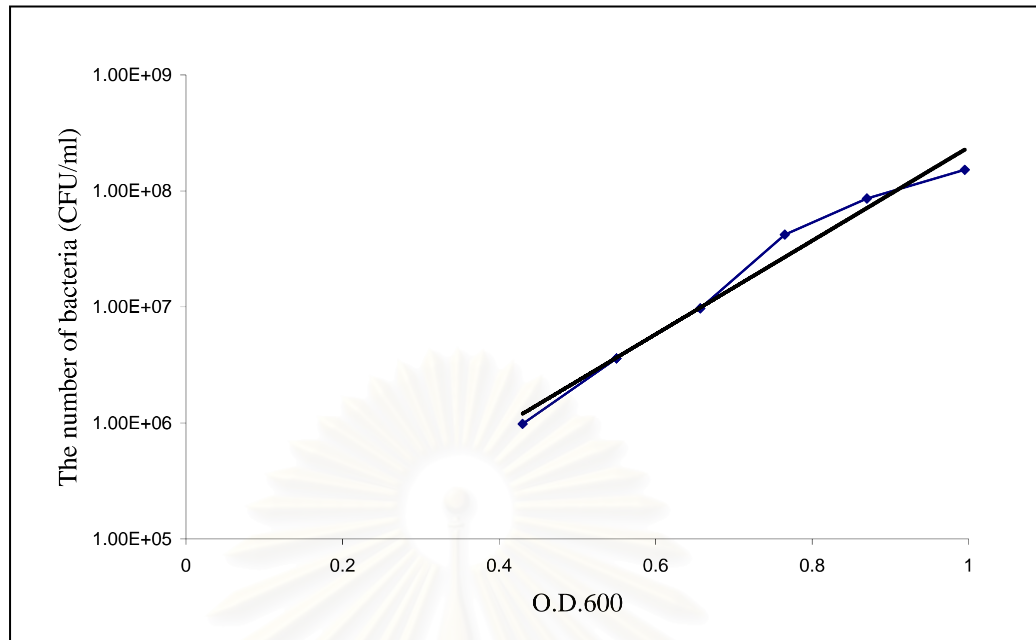

Figure B.2 Standard curve of bacterial consortium plotted between the number of bacteria (CFU/ml) and O.D.$_{600}$

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APPENDIX C

4-Chloroaniline standard curve

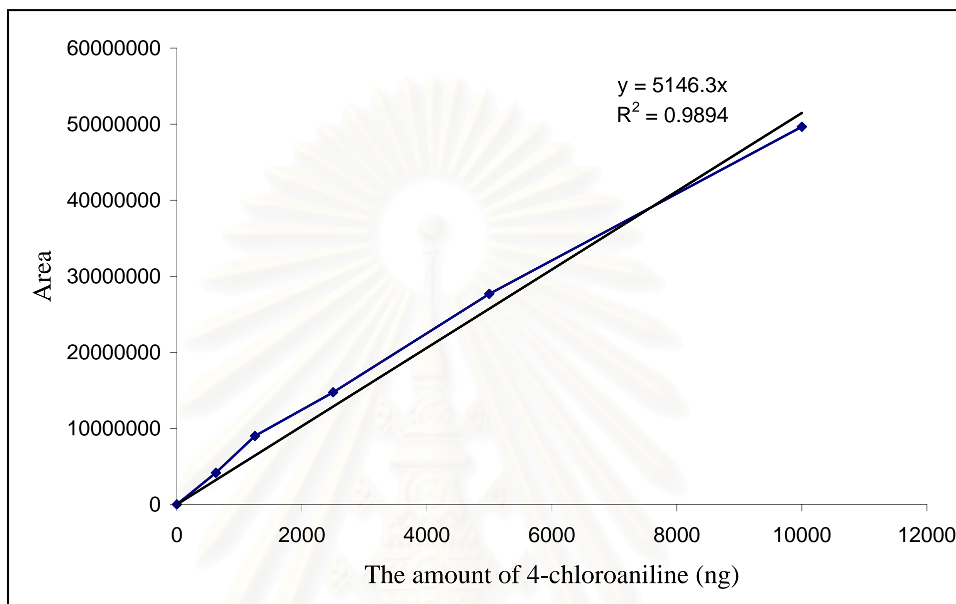

Figure C The standard curve of 4-chloroaniline plotted between the amount of 4-chloroaniline (ng) and peak area analyzed by HPLC.

The slope of standard curve was 5146.3.

The amount of 4-chloroaniline can calculated followed the equation;

The amount of 4-chloroaniline = peak area/5146.3

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APPENDIX D

INF calibration curve

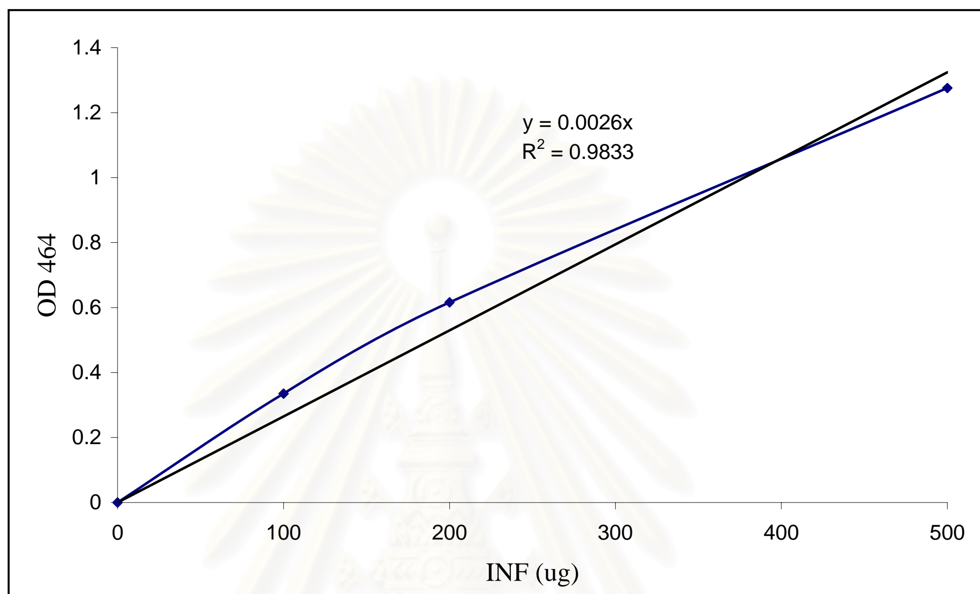


Figure D INF calibration curve for the calculation of total microbial activity (dehydrogenase activity).

INT $+2H^{+} + 2e^{-} \rightleftharpoons$ INF + HCl

The dehydrogenase activity is expressed as µg INF g^{-1} dwt 2 h^{-1} and calculated according the following relationship:

INF (µg INF g^{-1} dwt 2 h^{-1}) = $(S_1\text{-}S_0)$/dwt

S_1 is the INF of the test

S_2 is the INF of the control

dwt is the dry weight of 1 g moist soil

APPENDIX E

Characteristic of 4-chloroaniline degrading bacteria

Bacterial consortium

The bacterial consortium added in bioaugmentation consisted of three bacteria: C1, C2 and C3. The gram staining of three bacteria was showed in Figure E.1.

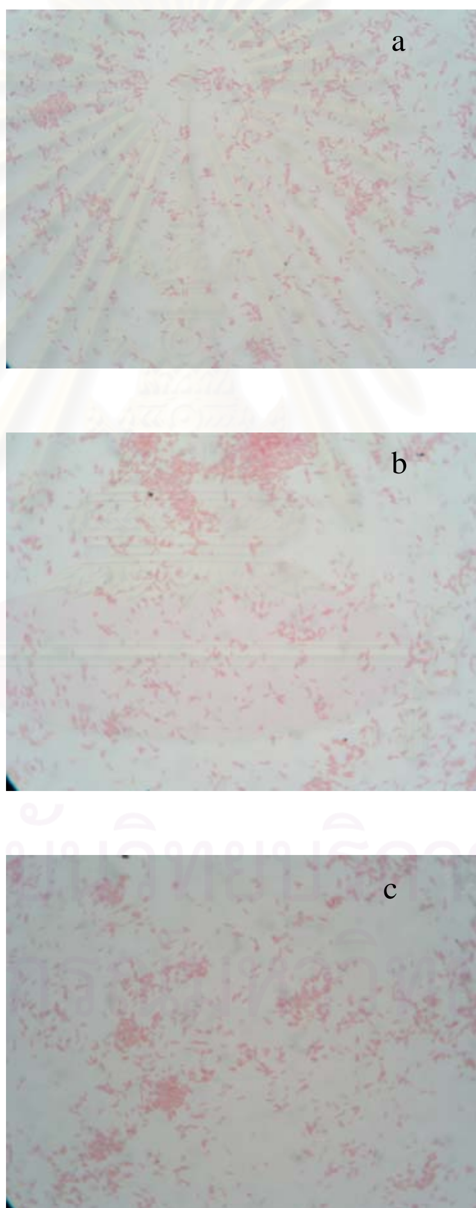


Figure E.1 The gram staining of three single bacteria; C1 (a), C2 (b) and C3 (c) which isolated from the bacterial consortium.

The colony characteristic of C1 was white color, punctiform, smooth surface and entire edge. The colony characteristic of C2 was cream color, circular form, smooth surface and entire edge. The colony characteristic of C3 was white color, circular form, smooth surface and entire edge.

Bacterial pure culture

Bacterial pure culture which was added in bioaugmentation treatment was *Klebsiella planticola*. The gram staining of *Klebsiella planticola* was showed in Figure E.2. The colony characteristic of *Klebsiella planticola* was white color, circular form, smooth surface and entire edge. It can degrade 63% of 4-chloroaniline after 12 days (Figure E.3).

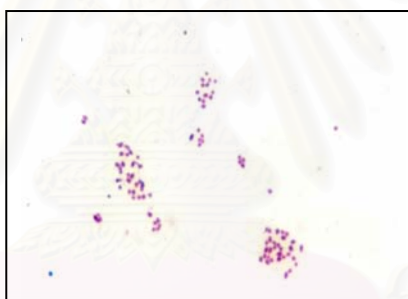


Figure E.2 The gram staining of *Klebsiella planticola*.

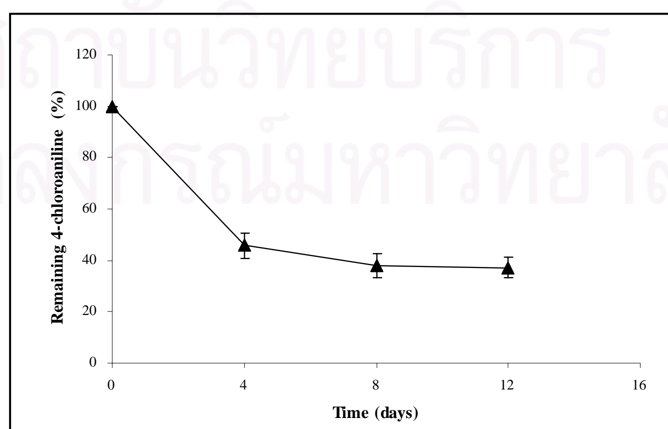


Figure E.3 % 4-chloroaniline remaining of *Klebsiella planticola* (—▲—) in minimal medium containing 25 ppm 4-chloroaniline

APPENDIX F

Raw data

Table F.1 Amount of remaining 4-chloroaniline in 5 g soil microcosm for loam soil by using HPLC analysis

Day	Peak area				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	11755907	10073566	7328496	8421652	8112999
	11770944	10017048	8871446	8287116	8481555
	11663204	10184118	9063846	8263677	8598286
3	9900474	7728186	6526043	5471616	6028687
	9922997	8047254	6554300	5641978	6008958
	9866348	7687668	6550077	5871774	6035067
7	9463356	6230368	4823868	4178197	4547121
	9748450	6867371	4980582	4321663	4533940
	9553236	6755077	4940329	4226161	4283005
10	7956696	6775589	4528637	3652213	3773162
	7123679	6653540	4699956	3786094	3856318
	7492905	6383912	4526516	3533319	3833759
14	6324487	6529487	3176583	3322649	3199356
	6568420	6481550	3693558	3233370	3444130
	7396779	6666719	4272583	3298734	3277013
21	4804710	4025183	1912807	2158004	668444
	5553311	3820271	1974987	2180601	860046
	5847443	4073290	2095719	2104079	745937
28	5096518	3170551	1155459	1282051	340323
	5163255	3545138	1074387	1526123	340022
	5164055	3242832	1066125	1306277	333283
Day	% remaining 4-chloroaniline				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
3	84.37±0.20	77.49±1.32	78.40±9.26	68.01±1.76	71.78±2.03
7	81.74±0.94	65.56±2.92	58.82±6.08	50.96±0.38	53.04±0.36
10	64.14±3.28	65.44±1.50	54.89±5.97	43.93±1.11	45.52±0.87
14	57.65±4.57	65.00±0.41	44.04±2.82	39.46±0.34	39.38±0.71
21	46.04±4.37	39.36±1.06	23.83±2.01	25.80±0.30	9.01±0.90
28	43.83±0.12	32.88±1.69	13.14±1.26	16.47±1.44	4.03±0.08

Table F.2 Amount of remaining 4-chloroaniline in 5 g soil microcosm for sandy clay loam soil by using HPLC analysis

Week	Peak area				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	11728956	10916029	10180889	9179565	9067428
	10365734	11243991	8723510	10226750	9933446
	10627966	11777145	10682448	10855954	10374646
1	7848992	9462239	7418623	7907804	7799966
	7699583	9850102	8252389	8600997	8628734
	8292061	9244525	7856238	8220753	7939006
2	6771877	8634580	6032636	6954239	6947720
	7679266	9374516	7112329	7027569	6899114
	7379285	9442589	7192993	7216859	6870004
3	7148801	9152573	6540843	6028012	5631591
	7042357	8685903	6070673	6333422	6306374
	6951105	9157232	6369320	6169415	6321074
4	6291684	8327098	4886662	5736314	5778189
	6842251	8470300	5205222	5875915	5279095
	6573989	8459798	5738549	5445060	5650480
5	6351330	8185729	4737986	5012054	4992492
	6786850	8620058	4078219	5264637	4926411
	6308164	8514710	4940391	5083324	5313151
6	5846006	8220724	4808667	4178479	4266368
	6585208	8538351	4207614	4933161	5177868
	6092560	7106959	4330352	4593204	4611806
7	5937422	7153257	4356898	4044493	4305611
	5846218	7588324	4640285	4823560	4659479
	6461189	7936690	4444765	4724118	4611467
8	5562101	7539645	4399281	4212873	4236894
	5648763	7489414	4467028	4079649	4288522
	6117651	7502449	4141415	4428745	4358971
Week	% remaining 4-chloroaniline				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
1	72.94±1.96	84.16±0.53	79.82±4.52	81.92±3.71	83.04±3.05
2	66.74±2.32	80.88±2.22	68.78±1.30	70.32±4.84	70.73±4.54
3	64.76±3.32	79.57±1.82	64.46±4.49	61.44±3.79	62.17±1.28
4	60.30±1.79	74.48±2.28	53.62±2.45	56.51±2.62	56.93±1.26
5	59.49±1.51	74.64±1.30	46.51±0.25	50.93±3.23	51.93±2.13
6	56.62±0.62	70.24±4.46	45.21±2.86	45.29±0.33	47.80±1.86
7	55.78±0.66	66.80±1.10	45.68±3.69	44.89±1.14	46.27±1.29
8	53.00±0.76	66.45±2.30	44.17±2.95	42.14±2.00	43.97±2.45

Table F.3 Amount of remaining 4-chloroaniline in 5 g soil microcosm for sandy clay loam soil after pH adjustment by using HPLC analysis

Week	Peak area				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	11728956	11640734	10763204	11670853	10911296
	10365734	11416470	11255371	10657916	10992261
	10627966	10567773	11373601	11265873	10945346
1	9351858	9505618	8264970	8368797	6461148
	9796110	10473137	8986521	8271158	7073009
	9538417	9902013	8821338	8252927	6754321
2	8553748	8863132	7478717	6936942	5643817
	9212630	9966927	7034569	6248389	6315021
	9694119	9926230	7539638	7548843	5972148
3	9069282	9579819	6843829	5762739	5014997
	9492387	9332173	7320120	6496711	5674290
	9216580	9467318	7557993	6685524	5389341
4	8547108	9637841	6000446	5546779	4429067
	9172918	9149920	6077293	5568977	4986677
	9327966	9441905	6155525	5656830	4712864
5	9011817	9027961	4723197	5105581	4051626
	8922226	9430222	5023304	5338042	4295104
	9237984	9285177	5428676	6205333	4156085
6	8496971	8642011	4455399	5179121	4187438
	9517465	8463580	5273191	4923024	3890326
	9063658	8487268	5097112	5436063	4012793
7	8022042	8600217	4530371	4865333	2042028
	8600217	8022042	4868033	5372606	835306
	8749606	8285177	4272912	5436063	1576023
8	7848992	8258054	4738296	3574701	277957
	7699583	7718283	4519960	4671580	747202
	8292061	7843415	3291960	3944884	476934
Week	% remaining 4-chloroaniline				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
1	87.83±3.74	88.88±1.86	78.05±1.14	74.19±2.94	61.78±3.63
2	83.95±2.37	85.48±1.54	66.03±0.59	61.63±3.03	54.59±4.05
3	85.05±3.59	84.51±3.30	65.02±1.43	56.34±1.98	48.79±4.00
4	82.76±3.40	84.03±2.21	54.62±0.98	49.98±1.85	42.98±3.38
5	83.21±3.90	82.59±2.46	45.41±2.04	49.49±3.20	38.10±1.37
6	82.80±2.19	76.22±3.35	44.35±2.61	46.25±0.31	36.87±1.73
7	77.64±3.17	74.12±1.68	40.92±1.65	46.64±1.02	13.12±7.72
8	72.94±1.96	70.89±2.17	37.47±6.01	36.19±3.40	4.67±3.01

Table F.4 The number of 4-chloroaniline-degrading bacteria in loam soil by plate count technique.

Day	The number of 4-chloroaniline-degrading bacteria				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	0±0.00	$1.06x10^4±8.49x10^2$	$1.12x10^4±1.41x10^3$	$1.81x10^8±1.41x10^6$	$1.54x10^8±2.83x10^6$
3	0±0.00	$1.03x10^4±5.66x10^2$	$1.60x10^4±5.66x10^3$	$1.68x10^7±4.24x10^5$	$1.37x10^7±4.24x10^5$
7	0±0.00	$1.66x10^4±5.09x10^3$	$3.20x10^5±5.66x10^4$	$8.10x10^6±2.83x10^5$	$4.10x10^6±1.41x10^5$
10	0±0.00	$1.20x10^4±2.83x10^2$	$8.90x10^6±2.83x10^5$	$3.00x10^6±4.24x10^5$	$6.00x10^6±4.24x10^5$
14	0±0.00	$8.70x10^3±4.24x10^2$	$2.30x10^6±4.24x10^5$	$5.30x10^6±5.66x10^5$	$7.40x10^6±2.83x10^5$
21	0±0.00	$3.10x10^4±4.24x10^3$	$3.60x10^7±2.83x10^6$	$1.32x10^8±1.41x10^6$	$5.90x10^8±1.41x10^7$
28	0±0.00	$3.10x10^5±4.24x10^4$	$6.50x10^7±4.24x10^6$	$6.10x10^7±2.83x10^6$	$7.70x10^7±7.07x10^6$

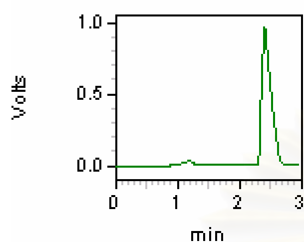
Table F.5 The number of 4-chloroaniline-degrading bacteria in sandy clay loam soil by plate count technique.

Week	The number of 4-chloroaniline-degrading bacteria				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	0±0.00	$9.35x10^2±49.5$	$3.12x10^3±2.12x10^2$	$4.30x10^6±2.83x10^5$	$4.50x10^6±4.24x10^5$
1	0±0.00	$3.05x10^3±2.12x10^2$	$5.10x10^3±4.24x10^2$	$6.80x10^5±4.24x10^4$	$7.17x10^5±4.95x10^4$
2	0±0.00	$8.85x10^2±1.20x10^2$	$1.48x10^4±1.13x10^3$	$9.15x10^3±6.36x10^2$	$3.75x10^4±3.54x10^3$
3	0±0.00	$1.25x10^3±70.70$	$3.05x10^5±7.78x10^4$	$1.08x10^5±6.36x10^3$	$8.85x10^4±2.12x10^3$
4	0±0.00	$1.43x10^3±35.40$	$1.21x10^6±6.36x10^4$	$3.90x10^5±5.66x10^4$	$1.23x10^5±7.78x10^3$
5	0±0.00	$1.55x10^3±70.70$	$1.26x10^5±8.49x10^3$	$3.60x10^5±1.41x10^4$	$3.75x10^5±4.95x10^4$
6	0±0.00	$1.88x10^3±1.1x10^2$	$2.85x10^5±4.95x10^4$	$6.10x10^4±4.24x10^3$	$8.65x10^4±6.36x10^3$
7	0±0.00	$2.60x10^3±7.07x10^2$	$8.45x10^4±3.54x10^3$	$7.70x10^4±5.66x10^3$	$9.50x10^4±4.24x10^3$
8	0±0.00	$3.35x10^3±2.12x10^2$	$5.60x10^4±5.66x10^3$	$4.55x10^4±4.95x10^3$	$4.75x10^4±3.54x10^3$

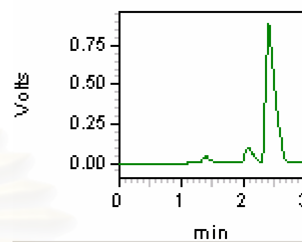
Table F.5 The number of 4-chloroaniline-degrading bacteria in sandy clay loam soil after pH adjustment by plate count technique.

Week	The number of 4-chloroaniline-degrading bacteria				
	Control	Natural attenuation	Biostimulation	Bioaugmentation with pure culture	Bioaugmentation with consortium
0	0±0.00	$9.35x10^2±49.50$	$3.40x10^3±1.41x10^2$	$3.85x10^6±1.06x10^5$	$5.10x10^7±5.65x10^6$
1	0±0.00	$3.35x10^3±4.94x10^2$	$8.75x10^3±3.54x10^2$	$9.05x10^5±6.36x10^4$	$7.00x10^6±8.49x10^5$
2	0±0.00	$4.80x10^3±2.83x10^2$	$3.65x10^4±6.36x10^3$	$4.65x10^5±7.77x10^4$	$3.95x10^6±6.36x10^5$
3	0±0.00	$4.30x10^3±2.83x10^2$	$5.20x10^4±5.66x10^3$	$4.25x10^5±4.95x10^4$	$1.21x10^6±4.24x10^4$
4	0±0.00	$3.50x10^3±5.66x10^2$	$3.70x10^5±2.83x10^4$	$1.01x10^6±6.36x10^5$	$3.65x10^6±7.78x10^5$
5	0±0.00	$3.45x10^3±3.54x10^2$	$9.95x10^5±4.95x10^4$	$2.10x10^6±1.41x10^5$	$9.10x10^7±2.83x10^6$
6	0±0.00	$4.80x10^3±5.65x10^2$	$7.90x10^5±5.65x10^4$	$2.10x10^7±2.83x10^5$	$1.57x10^7±4.24x10^5$
7	0±0.00	$5.25x10^3±6.36x10^2$	$6.40x10^5±5.65x10^4$	$2.78x10^6±3.54x10^4$	$9.15x10^6±4.95x10^5$
8	0±0.00	$5.80x10^3±4.24x10^2$	$7.50x10^5±4.24x10^4$	$2.45x10^6±7.07x10^4$	$3.45x10^7±4.95x10^6$

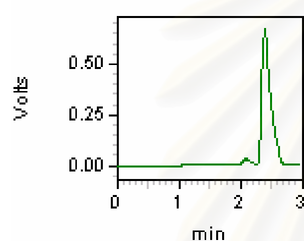
HPLC peak in each bioremediation treatment of loam soil during 4 weeks of the incubation time. Retention time of 4-chloroaniline is 2.4 min determined under the specific HPLC condition as described in Chapter III 3.6.2.



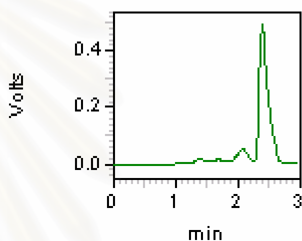
Natural attenuation 0 week



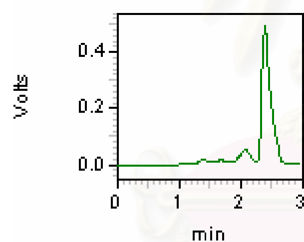
Biostimulation 0 week



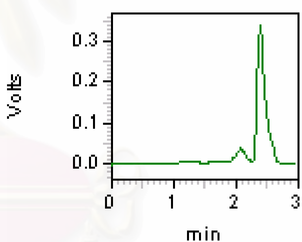
Natural attenuation 1 week



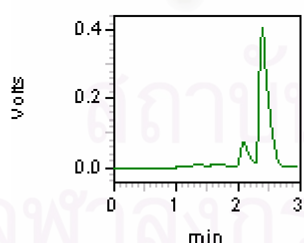
Biostimulation 1 week



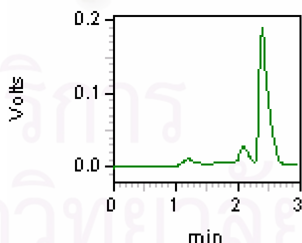
Natural attenuation 2 weeks



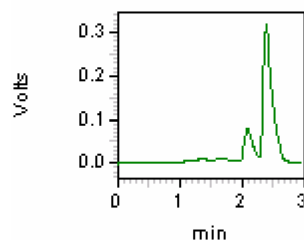
Biostimulation 2 weeks



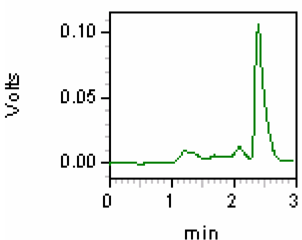
Natural attenuation 3 weeks



Biostimulation 3 weeks



Natural attenuation 4 weeks



Biostimulation 4 weeks

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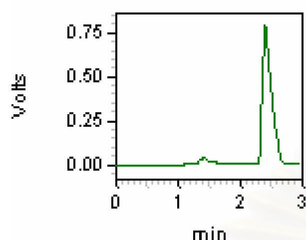
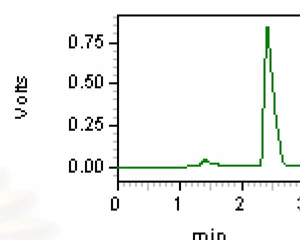
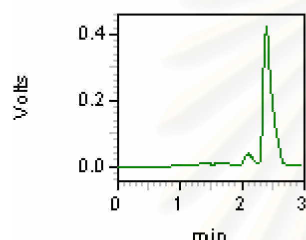
HPLC peak in each bioremediation treatment of loam soil during 4 weeks of the incubation time.



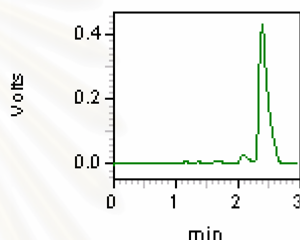
Bioaugmentation (pure culture) 0 week



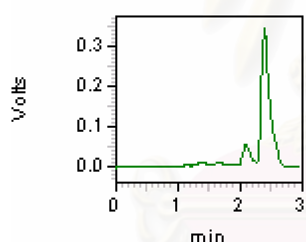
Bioaugmentation (consortium) 0 week



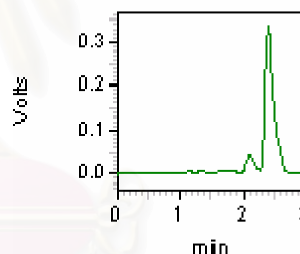
Bioaugmentation (pure culture) 1 week



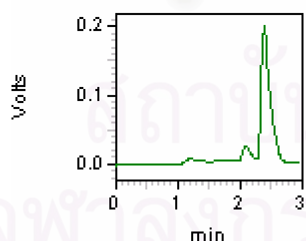
Bioaugmentation (consortium) 1 week



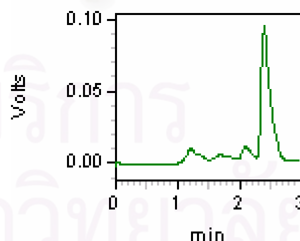
Bioaugmentation (pure culture) 2 weeks



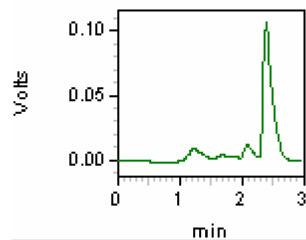
Bioaugmentation (consortium) 2 weeks



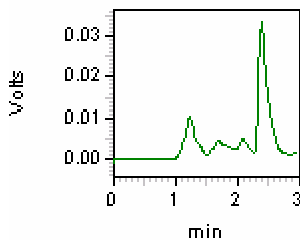
Bioaugmentation (pure culture) 3 weeks



Bioaugmentation (consortium) 3 weeks



Bioaugmentation (pure culture) 4 weeks



Bioaugmentation (consortium) 4 weeks

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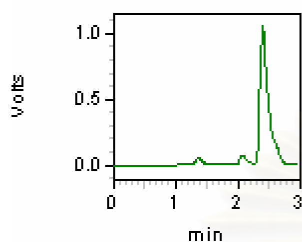
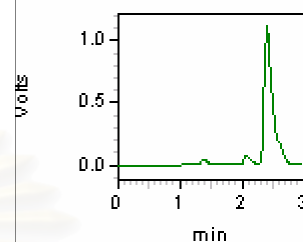
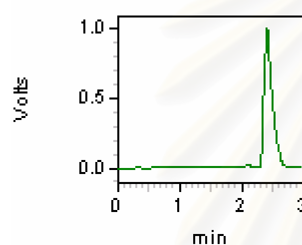
HPLC peak in each bioremediation treatment of sandy clay loam soil for pH adjustment during 8 weeks of the incubation time.



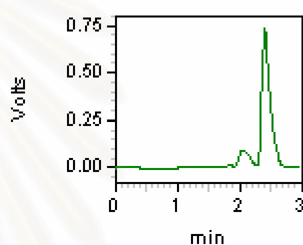
Natural attenuation 0 week



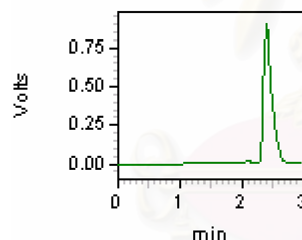
Biostimulation 0 week



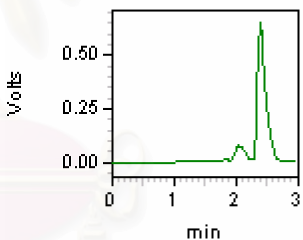
Natural attenuation 2 weeks



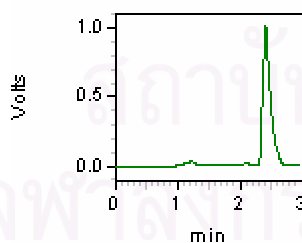
Biostimulation 2 weeks



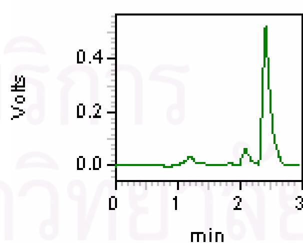
Natural attenuation 4 weeks



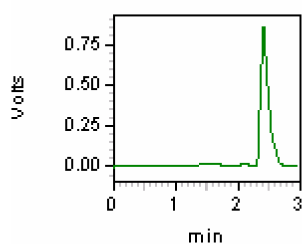
Biostimulation 4 weeks



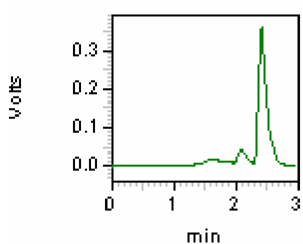
Natural attenuation 6 weeks



Biostimulation 6 weeks



Natural attenuation 8 weeks



Biostimulation 8 weeks

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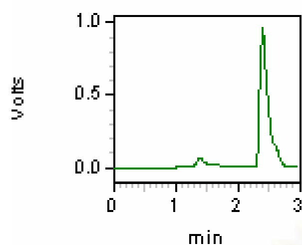
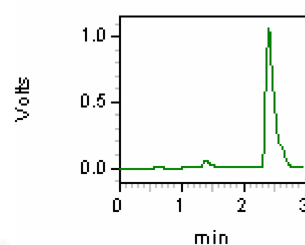
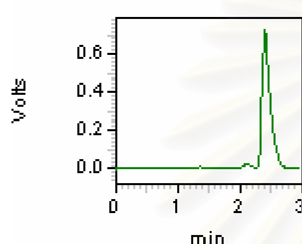
HPLC peak in each bioremediation treatment of sandy clay loam soil for pH adjustment during 8 weeks of the incubation time.



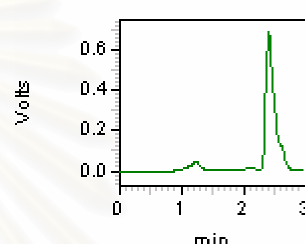
Bioaugmentation (pure culture) 0 week



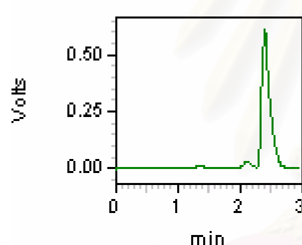
Bioaugmentation (consortium) 0 week



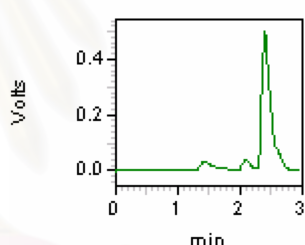
Bioaugmentation (pure culture) 2 weeks



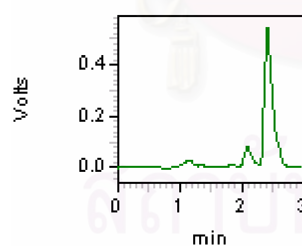
Bioaugmentation (consortium) 2 weeks



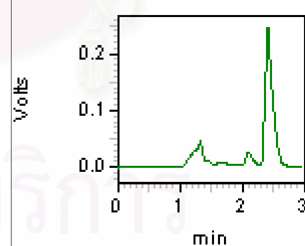
Bioaugmentation (pure culture) 4 weeks



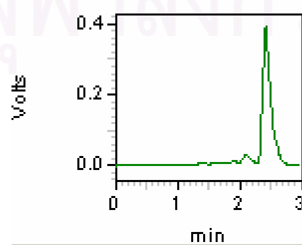
Bioaugmentation (consortium) 4 weeks



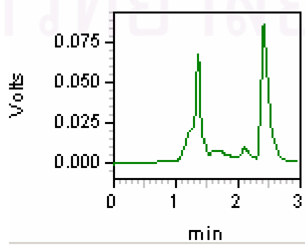
Bioaugmentation (pure culture) 6 weeks



Bioaugmentation (consortium) 6 weeks



Bioaugmentation (pure culture) 6 weeks



Bioaugmentation (consortium) 8 weeks

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APPENDIX G

Sequence results

The sequence results of each treatment in loam soil

Fragment A in natural attenuation

AAAAAAGCAGAACAAGGCGAATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAT
TACCGCGGCTGCTGGATTACCGCGGCTGCTGGATTACCGCGGCTGCTGGATTACCGCGGCTGCTGCCTNNNNNA

Fragment B in biostimulation

AAAAGCGGTACTTGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCGG
GAGGCAGCAGTGGGGAATCTTGCGCTAATGCGCGAAAGCGTGACGCAGCGACGCCGCGTGGGGGATGAAGGCCCTC
GGGTTGTAAACCCCTTTCGGCAGGGACGAAGCGAGAGTGACGGTACCTGCAGAAGAAGTCCCGGCTAACTACGTGC
CAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGT
TGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGT
GAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATG
AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT
TAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGC
TGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGG
GGATAACGCAGGANAGAACATGTGAGNCAAAGGCCAGCAAAAGGCCAGGAAACCGTANAAAGGGCGCGTTGCTGGC
GTTTTTCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGGCGAAACCCGACA
GGACTATAAAGATACC

Fragment C in biostimulation

CGTACATGGGCGATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATTACCGCG
GCTGCTGGCACGTAGTTAGCCGTGGCTTTCTCGCAAGGTACCGTCAAGGTGCCATCAGTTAAATGGCACGTGTTCT
TCCCTTACAACAGAGCTTTACGACCCGAAGGCCTTCTTCGCTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATT
GCGGAAGATTCCCTACTGCTGCCTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCC
CAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTT
TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGT
GCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC
AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACA
GAATCAGGGGATAACGCAGGANAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGT
TGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACANAAATCGACGCTCAAGTCAGAGGTGGCGAAA
CCCGACAGGACTA

Fragment D in bioaugmentation with pure culture

AAAANCCGTAACAAGGCGATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATT
ACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTTTGGGTACCGTCATCCCAACCGGGTATTAACCAGCTG
GATTTCTTTCCCAACAAAAGGGCTTTACAACCCGAAGGCCTTCTTCACCCACGCGGTATGGCTGGATCAGGCTTGC
GCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCCCCCCAATCACTAGTGAATTCGCGGCCGCCTGC
AGGTCGACCATATGGGAGAGCTCCCAACGGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTG
GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTC
CAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCCACTCAAAGG
CGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGANAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAG
GAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGC
TCAAGTCAGAGGTGGCG

Fragment E in bioaugmentation with pure culture

AAAANCCTCTCTAANGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCACAGGATTCTATTAT
TACCCCGGCTGCTATGACGGAAGCCAGCATACCACGTCGGCCCCAATTCGCCCCATAGTGAGTCGTATTACAATTC

ACTGGCCGTCCTTTTCACCCCATACACAACACCCCCAAATCCCACCTCACCTTCCACATAAACTAACATTAATTAG
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CTTACCATCCTCCCTAACTACAAACCACCCCATCCAACAAAAATACACTACCCATACCATACCTTTCACACCTACC
TACCAACCTGTTCGACTCCCCCAAACCTCTCTCACTCCCCTTAAAAATCCTCCCCTAATCCCCACACTTTTTAACC
TACCCACACCTAAATAACCAAACACACCCCTCACTTTTTCCAAAAAAATACCTACCTCCAACTAATATCATATAAC
CCCACCTCCCTCCCTCCACACCCAAAACCTTCTCTTCAATTCATACAATCAACTTTCCTCTTCCCACCTCCAAACC
CCTCAACCCCTCCCTCAAAACAACTCCCCTTCCCTCACCATTTAAACTCACTCCCCCCAAAAACAAACCCCCCTAT
TTTACTCTACTCCACCAAACCCCCAATAAAATTCTCCACCCACAACCTTATCACCCCCCCCCA
CAAACGCCACAAAAACACTACACTACTCAGCTTCTAACCCTCAACACCT

Fragment F in bioaugmentation with consortium

TTACATGGGATGGGCGACTCCAGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATTACCGCGGCTGCTGGC
ACGGAGTCCGTATTACCGCGGCTGCTGGCACGGAGTCCGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCT
TCTTCTGCAGGTACCGTCACTTGCGCTTCTTCCCTGCTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACG
CGGCGTCGCTGCATCAGGCTTCCGCCCATTGTGCAATATTCCCCACTGCTGCCTAATCACTAGTGAATTCGCGGCC
GCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAA
TAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGA
GCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGC
CCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCG
TATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCT
CACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCANGAAAGAACATGTGAGCAAAAGGCCAGC
AAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAA
AAATCG

The sequence results of each treatment in sandy clay loam soil

Fragment G in biostimulation

CGTACATGGGCGATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATTACCGCG
GCTGCTGGCACGTAGTTAGCCGTGGCTTTCTCGCAAGGTACCGTCAAGGTGCCATCAGTTAAATGGCACGTGTTCT
TCCCTTACAACAGAGCTTTACGACCCGAAGGCCTTCTTCGCTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATT
GCGGAAGATTCCCTACTGCTGCCTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCC
CAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTT
CCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG
CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA
GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACT
GACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAG
AATCAGGGGATAACGCAGGANAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACANAAATCGACGCTCAAGTCAGAGGTGGCGAAAC
CCGACAGGACTA

Fragment H in biostimulation

CACATTCGATCTTGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGGG
CACGGGGGGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTACCGGAGTAACTGCCGGTACCTT
GACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGGGCCG
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CCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCC
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ATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTC
ACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAAGCCAGCA
AAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTTCCATAGCTCGCCCCCCTGACGAGCATCACAAAA
TCCACGCTCAAGC

Fragment I in biostimulation

AAAAAACCGTACTTGGGCGATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGG
GCACGGGGGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGC
GTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGTAAGTGACGGTACCTGCAGAAGAA
GCGCCGGCCAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATAT
GGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGG
TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTA
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CCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT

TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATAC
GGTTATCCACAGAATCAGGGGATAACGCANGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAA
AAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
AGGTGGCGAAACCCGACAGGAN

Fragment J in bioaugmentation with pure culture

AAAACCGTATCTTGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGGG
GCACGGGGGGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTAAGGGAAGAACACGTGCCATTTAACTGATGGCACCTT
GACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCC
GCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAA
TAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGA
GCCGGAAGCATAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCC
CGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGT
ATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTC
ACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCA
NAAGGCCAGGAACCGTANAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAA
AATCGACGCTNCAGTCAGA

Fragment K in bioaugmentation with pure culture

TCCCCGATAATTGTGATGGGCCGACGTCGAGCTCCGGCCGTCATGGAGGCCGCGCTTAATTCTATTGGCGGCGGGG
GACTCCTACGGGAGGCAGCAGTAGGGAACCATTCCTCAATGGAGGACAATGTGATGAAGCAGCGCCCCCTGAGGGA
AGAACAACTCCTTCCCGTACAGATCTGTTGTAATAAAAAAAAAAGAGGCCCTTATATGATGACGTCTTGTTGACGC
CCCTGTTGGCGAAAAAAGCGCCTATAAACTTGCCGCCAGCCGCGGCGATAATAACTACCAAAGAATTGCCGGCCTC
CTGCACCGCCCACATATGAAAGCTCCCCCCACCCGGTGGGATGACCTCGTTGATTATACTATAGTGCCATCCACAT
AGGTCGTTCTACTGTGGATCATCTGTCGCCTGCCGGGAACAAGTTGCTATGCTTTTCCATTTCCCACAAACCCTTA
CCCCCCCCTACGTACTCCGGCCTCTACTTCGTCGGGGCCTCATGATTGCGTCTCCTCTAAATTAATCGTATATGGT
ATTCCTGATACCTTTCCCCAGCTCATAATTCTTCTTGCTCCCACACCCCTTTACATCACTTCTCCTCCCGACTGCA
ATATCCCGCTTTACCTTCCTCCTCTCCTTTCCACTCTCTATTCATCTTTTCTCCCATCTCCCCATCCTTAGAATTT
TTTTCTTTGATCCCCCTTCCCTCATCCTCTTGTCTCATCCTTTTCTCCTATCTATTTTCTCCATTTTTTCCCCCCT
TTTTATTATCCCCTTCCTCCCTCTCACCCTTTATCCTATTCCACCGT

Fragment L in bioaugmentation with pure culture

AAAAAACCGTATCTTGGGGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGG
GGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGA
TGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTACCGGAGTAACTGCCGGTACCTTGACGGTA
CCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCA
GGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTG
GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTT
CCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG
CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAA
AGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGC
CAGGAACCGTANAAAGGCGCGTTGCTGGCGTTTTTCATAGGCTCCGCCCCCCTGACGAGCATCACAAAATCGACGCT
TCAGTCAGAGGTGGCG

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Fragment M in bioaugmentation with pure culture

AAAAAGCCAGTACTTAGGGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGG
GGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGG
ATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGTAAGTGACGGTACCTGCAGAAGAAGCGCCGG
CCAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAG
CTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC
TGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTG
GGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCG
TGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGC
TCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATC
CACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCNAAAAGCCAGGAACCGTAAAAAGGCC
GCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCCAGTCAGAGGTGGC
GAAACCC

Fragment N in bioaugmentation with consortium

GGGCAAAAANAANGGATATCATGGCGATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATT
CGATTATTACCGCGGCTGCTGGCACGGAGTCCGTATTACCGCGGCTGCTGGCACGGAGTCCGTATTACCGCGGCTG
CTGGCACGTAGTTGGCCGGCGCTTCTTCTGCAGGTACCGTCACTTACGCTTCGTCCCTGCTGAAAGAGGTTTACAA
CCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTCCGCCCATTGTGCAATATTCCCCACTGCTGCC
TCCCGTAGGAGTCCCCCCGTGCCCAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCC
CAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTT
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GCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC
AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACA
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TGCTGGCGTTTTTCCATAGGCT

Fragment O in bioaugmentation with consortium

AAAAAAAGGGTATCCTATNGGCGAATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCG
ATTGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCC
GGCCAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAG
AGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATA
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GCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTA
TCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGNCAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGG
CCGCGTTGCTGGCGTTTTCCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTG
GCGAAACCCGACGGGACTATA

Fragment P in bioaugmentation with consortium

AAAAAACCCGTACNTTGNGGCGATTGGGCCCGACNTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGAT
TGGGCACGGGGGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGAAAGTGACGGTACCTGCAGAA
GAAGCGCCGGCCAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCA
TATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCA
TGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGT
GTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGG
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GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAA
TACGGTTATCCACAGAATCAGGGGATAACGCANGAAAGAACATGTGAGCAAAAGGCCAGCAAAAAGCCCAGGAACC
GTAAAAAGGCCGCGTTGCTGNCGTTTTTTCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAG
TCAGAGGTGGCGAAACCCGA

BIOGRAPHY

Miss Roongnapa Tongarun was born on July 7, 1982 in Nakornnayok province, Thailand. She received Bachelor's Degree in Biochemistry, faculty of science, Chulalongkorn University in 2004. She pursued her Master degree study in the inter-Department of Environmental Management, Chulalongkorn University, Bangkok, Thailand in May 2004. She finished Master Degree of Science in Environmental Management in May 2006.

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