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ปีการศึกษา 2551

FREE AND IMMOBILIZED CELL BIOAUGMENTATION FOR REMOVING ATRAZINE FROM AGRICULTURAL INFILTRATE

Miss Sumana Siripattanakul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Environmental Management
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Thesis Title	FREE AND IMMOBILIZED CELL BIOAUGMENTATION FOR REMOVING ATRAZINE FROM AGRICULTURAL INFILTRATE
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สุมนา สิริพัฒนากุล : การกำจัดอาทราซีนด้วยวิธีการทางชีววิทยาจากน้ำสำหรับเกษตรกรรมซึมผ่านดินด้วยเซลล์อิสระและเซลล์ตรึง. (FREE AND IMMOBILIZED CELL BIOAUGMENTATION FOR REMOVING ATRAZINE FROM AGRICULTURAL INFILTRATE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. วันเพ็ญ วิโรจนกูฏ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ASSOC. PROF. EAKALAK KHAN, Ph.D., 140 หน้า.

งานวิจัยนี้มีจุดประสงค์เพื่อศึกษาการกำจัดอาทราซีนจากน้ำสำหรับเกษตรกรรมซึมผ่านดิน โดยแบคทีเรียเซลล์อิสระและเซลล์ตรึงด้วยสารพอลีไวนิลแอลกอฮอลล์ เพื่อเพิ่มประสิทธิภาพในการกำจัดอาทราซีน การทดลองครอบคลุมถึงผลกระทบจากอัตราส่วนแบคทีเรียต่อสารที่ใช้ในการตรึง ปริมาณแบคทีเรีย และอัตราการซึมของน้ำผ่านดิน แบคทีเรียที่ใช้การทดลองนี้ประกอบด้วยแบคทีเรียชนิดเดียวและกลุ่มแบคทีเรีย โดยแบคทีเรียชนิดเดียวที่ใช้ในงานวิจัยนี้ ได้แก่ *Agrobacterium radiobacter* J14a (J14a) ซึ่งเป็นแบคทีเรียที่ได้คัดเชื้อและทดสอบความสามารถในการย่อยสลายอาทราซีนในการศึกษาอื่น ส่วนกลุ่มแบคทีเรีย (MC) เป็นแบคทีเรียที่คัดเชื้อจากดินที่ปนเปื้อนอาทราซีน จากผลการทดลองพบว่า MC มีความสามารถในการย่อยสลายอาทราซีนร้อยละ 30 ถึง 51 ภายในเวลา 7 วัน *Klebsiella ornithinolytica* ND2 (ND2) และ *Agrobacterium tumefaciens* ND4 (ND4) เป็นแบคทีเรียใน MC และมีสารพันธุกรรม *atzA* สำหรับการย่อยสลายอาทราซีน นอกจากนี้การศึกษานี้ยังได้พัฒนาวิธีการตรึงเซลล์ โดยพบว่าวิธีการนี้ทำให้เซลล์มีความเสถียรและเหมาะสมสำหรับการตรึงเซลล์ และส่งผลให้แบคทีเรียตายน้อย การทดลองการกำจัดอาทราซีนด้วยเซลล์อิสระและเซลล์ตรึงในระบบถังเดี่ยวพบว่า เซลล์ตรึงของ J14a และ MC สามารถกำจัดอาทราซีนได้ดีกว่าเซลล์อิสระ โดยอัตราส่วนแบคทีเรียต่อสารที่ใช้ในการตรึง 3.5 mg/mL มีประสิทธิภาพในการกำจัดอาทราซีนสูงสุด ที่อัตราส่วนนี้สามารถกำจัดอาทราซีนได้ร้อยละ 40 ถึง 50 ภายในเวลา 5 วัน การทดลองในระบบคอลัมน์พบว่า J14a และ MC มีลักษณะการทำงานคล้ายคลึงกัน โดยเซลล์อิสระและเซลล์ตรึงมีประสิทธิภาพในการกำจัดอาทราซีนใกล้เคียงกัน ในการทดลอง ณ 5 ปริมาตรช่องว่าง เซลล์ตรึงของ J14a และ MC สามารถกำจัดอาทราซีนได้ร้อยละ 50 ถึง 100 และ 42 ถึง 80 ตามลำดับ โดยอัตราการซึมของน้ำผ่านดินและปริมาณแบคทีเรียเป็นปัจจัยที่ส่งผลต่อการทดลองอย่างมีนัยสำคัญ ปริมาณแบคทีเรียที่ถูกชะออกจากคอลัมน์ของเซลล์ตรึงน้อยกว่าเซลล์อิสระ 10 ถึง 100 เท่า ในการกำจัดอาทราซีนด้วย MC พบการเปลี่ยนแปลงโครงสร้างของกลุ่มแบคทีเรีย โดยอัตราการซึมของน้ำผ่านดินเป็นปัจจัยสำคัญ ผลการติดตามการกำจัดอาทราซีนระยะยาว ณ 50 ปริมาตรช่องว่าง เซลล์ตรึงสามารถกำจัดอาทราซีนอย่างคงที่ ในขณะที่ประสิทธิภาพการกำจัดอาทราซีนด้วยเซลล์อิสระลดลงเนื่องจากสูญเสียเซลล์

ลายมือชื่อนิสิต.....

สาขาวิชาการจัดการสิ่งแวดล้อม ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4789712720: MAJOR ENVIRONMENTAL MANAGEMENT
KEY WORD: ATRAZINE / AGRICULTURAL INFILTRATE / BIOAUGMENTATION / IMMOBILIZED CELLS / POLYVINYL ALCOHOL

SUMANA SIRIPATTANAKUL: FREE AND IMMOBILIZED CELL BIOAUGMENTATION FOR REMOVING ATRAZINE FROM AGRICULTURAL INFILTRATE. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. WANPEN WIROJANAGUD, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. EAKALAK KHAN, Ph.D., 140 pp.

The remediation of atrazine in agricultural infiltrate by bioaugmented free and phosphorylated-polyvinyl alcohol (PPVA) immobilized cells was studied. The effects of cell-to-matrix ratio, cell loading and infiltration rate on atrazine removal efficiency were examined. Two types of bacterial cultures were used: pure and mixed cultures. The pure culture was a previously isolated atrazine degrader, *Agrobacterium radiobacter* J14a (J14a) while the mixed culture (MC) was enriched from atrazine-contaminated soil. This novel mixed culture degraded 33-51% of atrazine within 7 d. Two isolates, *Klebsiella ornithinolytica* ND2 and *Agrobacterium tumefaciens* ND4, which were purified from the mixed culture, contained an atrazine-degrading gene, *atzA*. In the PPVA immobilization process, an existing cell immobilization procedure was modified. The modified procedure provided a stable and suitable microstructure matrix and had a slight effect on bacterial cell viability. In a batch study, the atrazine removal efficiencies by the immobilized J14a and MC were better than those by the free cells. Higher cell-to-matrix ratios resulted in lower atrazine removal. The cell-to-matrix ratio of 3.5 mg/mL provided the highest atrazine removal efficiency of 40 to 50% in 5 d for both J14a and MC. For atrazine bioremediation tests in a column system, the bioaugmented J14a and MC performed similarly. During the tests at 5 pore volumes (PV), the atrazine removal by the immobilized and free cells was not significantly different. J14a degraded 50 to 100% of atrazine while MC removed 42 to 80% of atrazine. Both infiltration rate and cell loading significantly influenced the atrazine removal. The bacterial loss from the immobilized cell system was 10 to 100 times less than that from the free cell system. In the test by MC, the changes of bacterial community structure after testing for 5 PV were observed. The infiltration rate was a significant factor for the change. For long-term tests at 50 PV, the immobilized cell system provided consistent atrazine removal while the performance of the free cells declined gradually because of the cell loss.

Student's signature.....

Field of study: Environmental management — Principal advisor's signature.....

Academic year: 2008 — Co-advisor's signature.....

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CONTENTS

Page

Page

Page

Page

Page

Page

Page

Table Page

LIST OF FIGURES

Page

Page

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSM	Basal salts medium
CO_2	Carbon dioxide
C147	*Pseudaminobactor* C147
C/C_0	Relative concentration
DEA	Desethylatrazine
DEDIA	Desethyldeisopropylatrazine
DNA	Deoxyribonucleic acid
DI	Deionized
DIA	Deisopropylatrazine
FISH	Fluorescent in situ hybridization
GC-MSD	Gas chromatograph with a mass selective detector
HA	Hydroxyatrazine
HIM	*Arthrobacter nicotinovorans* strain HIM
HPLC	High-performance liquid chromatography
J14a	*Agrobacterium radiobacter* strain J14a
K_H	Henry's constant
K_{oc}	Organic carbon-water partition coefficient
MC	Mixed culture
M91-3	*Ralstonia* M91-3
NA	Nutrient agar
NB	Nutrient broth

NBA	Nutrient broth spiked with atrazine
NH_3	Ammonia
PADP	*Pseudomonas* sp. strain ADP
PATR	*Rhizobium* sp. strain PATR
PCR	Polymerase chain reaction
PPVA	Phosphorylated- polyvinyl alcohol
PV	Pore volume
PVA	Polyvinyl alcohol
rDNA	Ribosomal deoxyribonucleic acid
R_f	Retardation factor
RNA	Ribonucleic acid
SEM	Scanning electron microscope
SP12	*Nocardioides* sp. strain SP12
SSCP	Single stand conformation polymorphism
TC1	*Arthrobacter aurescens* strain TC1

CHAPTER I

INTRODUCTION

1.1 Background

Atrazine (6-chloro-N-ethyl-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine), one of the most widely used herbicides, has been applied to control broad-leaf weeds for corn, sorghum, sugarcane, and other crops. The widespread use has caused a concern about its contamination in different environmental media. Although atrazine is applied to surface soil, off-site movement has been well documented (Gannon, 1992; Koskinen and Clay, 1997). There are several studies, which found atrazine in surface water (Wauchope, 1978; Gannon, 1992) and groundwater (Gannon, 1992; Jaychandran et al., 1994). For instance, in Iowa, United States, maximum atrazine concentrations in surface water and groundwater of 16 and 1,500 μg/L, respectively, were detected (Gannon, 1992). In Australia, atrazine concentrations in surface water and groundwater of up to 53,000 μg/L were reported (Graymore et al., 2001). Infiltrate and contaminated soil leachate are the sources of atrazine contamination in groundwater. The properties of atrazine include organic carbon-water partition coefficient (K_{oc}) of 100 mL/g and water solubility of 33 mg/L at 27°C, which are moderately to highly possible values of soil sorption and water dissolution (Montgomery, 1993). Moreover, a lengthy half-life of atrazine in soil of 60 to 100 days provides high potential for runoff and infiltrate contamination. In this context, the case of agricultural runoff and infiltrate containing atrazine becomes one of the most significant environmental issues.

In the last one to two decades, there has been an increasing interest in developing new techniques for site remediation. Bioremediation is an alternative,

which mainly uses microorganisms for degrading contaminants and leaving behind less toxic end products. In some cases, the traditional bioremediation either cannot cope with all contaminants or takes long time. These drawbacks are often caused by unsuitable strains and/or inadequate numbers of microorganisms at the sites. Adding sufficient numbers of appropriate microorganisms, called bioaugmentation, can be applied to solve the problem. Most studies devoted to bioaugmentation with cells deal with point source pollution control, particularly wastewater treatment (Protzman, 1999; Gentry, 2004). Both pure and mixed cultures were applied.

The success or failure of bioaugmentation depends highly on the survival of the inoculated cells. Their survival can be improved by immobilizing them within a carrier, which can protect them against the natural competition with the soil microflora (McLoughlin, 1994). There are two categories of media for cell immobilization, natural and synthetic materials. In the past, natural polymers including agar, agarose, alginate, and κ-carrageenan were commonly used (Leenen et al., 1996). Their significant disadvantages are their low mechanical strength and durability. On the contrary, most synthetic materials, such as polyacrylamide, and polyvinyl alcohol (PVA) could overcome this problem. This has led to more utilization of synthetic polymers for cell immobilization in recent years.

The aim of this dissertation is to develop a sustainable method, using cell bioaugmentation for removing atrazine, a hydrophilic herbicide, from agricultural infiltrate to prevent or minimize groundwater contamination. This study compares the atrazine remediation efficiencies using bioaugmented pure and mixed cultures in free and immobilized cell forms. PVA is chosen as the cell immobilization matrix for several reasons in addition to its mechanical stability and durability. It is non-toxic to

organisms and environment and can be inexpensively produced. Additionally, the effects of cell-to-matrix ratio, cell loading, and infiltration rate on atrazine removal efficiency are studied. This study expands the use of cell immobilization and bioaugmentation in combination to control non-point source water pollution, which is much more difficult to manage than the point source pollution.

1.2 Objectives

The main objective of this study is to investigate the use of bioaugmentation for treating atrazine-contaminated infiltrate. The research focuses on comparing the bioaugmentation performances of two culture types (pure and mixed cultures) and two cell types (free and immobilized cells). The specific objectives are:

1) to enrich and characterize indigenous atrazine-degrading mixed cultures from atrazine-contaminated soil,
2) to determine the effect of cell-to-matrix ratio of immobilized pure and mixed cultures on atrazine degradation in a batch system for obtaining the optimum condition for preparing immobilized cells, and
3) to determine the effects of cell loading and infiltration rate in a column system on atrazine removal.

1.3 Research Plan

Research plan of this study can be categorized into 3 stages: 1) preparation of bioaugmentation cultures, 2) immobilization of bacteria and investigation of cell-to-matrix ratio effect in a batch system, 3) atrazine remediation in a sand column system. The entire plan is summarized in Figure 1-1.

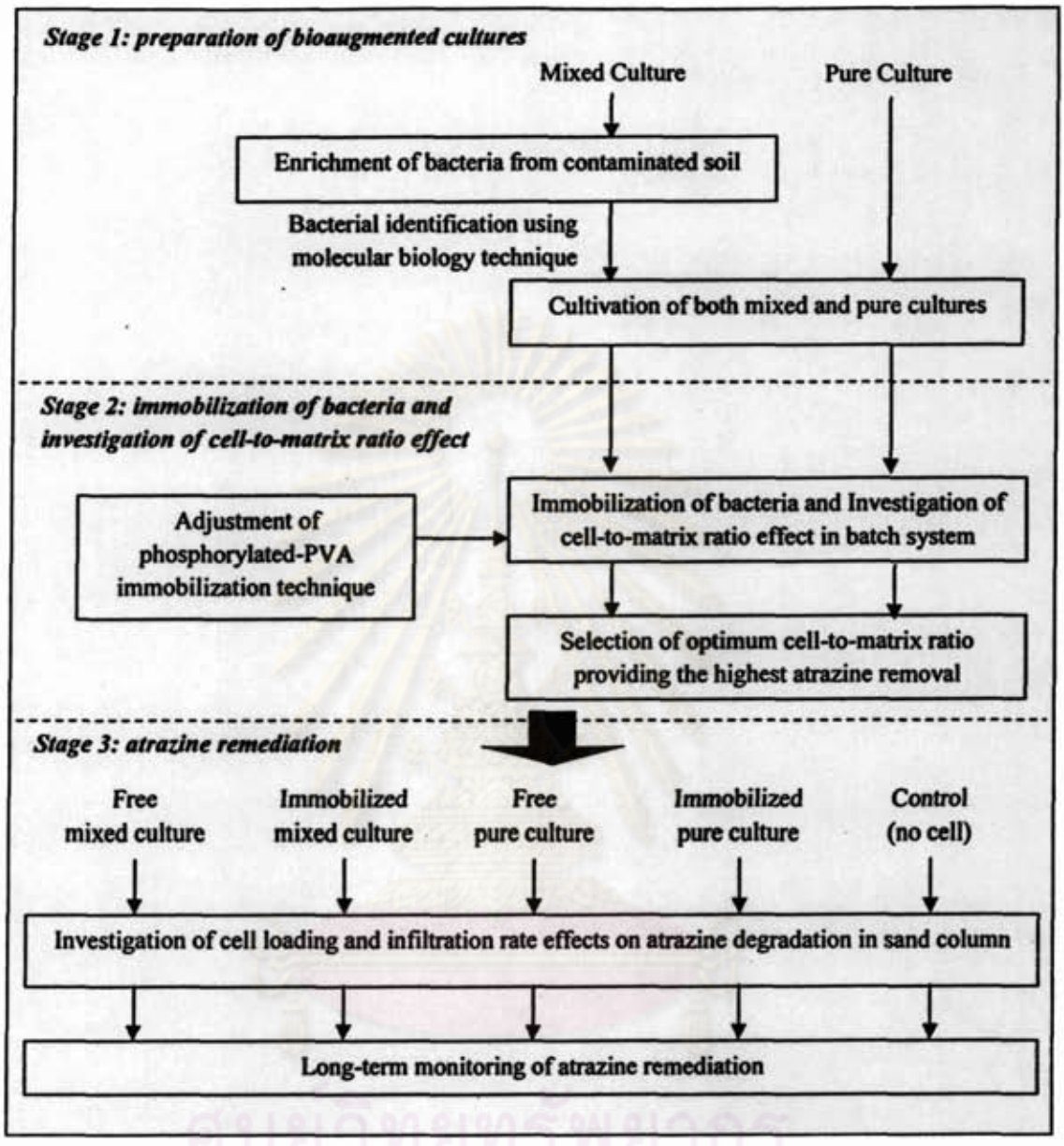


Figure 1-1 Research plan

1.4 Dissertation Organization

This dissertation is divided into 7 chapters. Chapter 1 includes background, objectives, plan, and organization of the dissertation. Chapter 2 contains literature review for the dissertation. Chapters 3, 4, and 5 and 6 are the works from stages 1, 2, and 3 of research plan, respectively (Figure 1-1).

Chapters 3 to 6 were prepared based on manuscripts submitted for publication. Chapter 3 is based on a manuscript entitled "Atrazine Degradation by Stable Mixed Cultures Enriched from Agricultural Soil and Their Characterization". This manuscript has been submitted to Journal of Applied Microbiology. Chapter 4 presents a manuscript entitled "Effect of Cell-to-matrix Ratio in Polyvinyl Alcohol Immobilized Pure and Mixed Cultures on Atrazine Degradation". This manuscript is published in Water, Air, and Soil Pollution: Focus, 2008, volume 8, pages 257-266.

Chapter 5 is based on a manuscript entitled "Atrazine Remediation in Agricultural Infiltrate by Bioaugmented Polyvinyl Alcohol Immobilized and Free *Agrobacterium radiobacter* J14a: A Sand Column Study". This manuscript has been prepared for submission to Chemosphere. Chapter 6 describes the work on atrazine remediation using the mixed culture entitled "Bioaugmentation of Polyvinyl Alcohol Immobilized and Free Mixed Culture for Removing Atrazine from Agricultural Infiltrate and Its Bacterial Community Structural Change". This manuscript will be submitted to Environmental Science and Technology. Lastly, conclusions and recommendations for future work are presented in Chapter 7.

CHAPTER II

LITERATURE REVIEW

2.1 Atrazine

Atrazine, a triazine herbicide, is the common name for 6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine. Atrazine is a heterocyclic nitrogen derivative (Figure 2-1). It is available as dry flowable, flowable liquid, liquid, water dispersible granular, and wettable powder formulations (Montgomery, 1993).

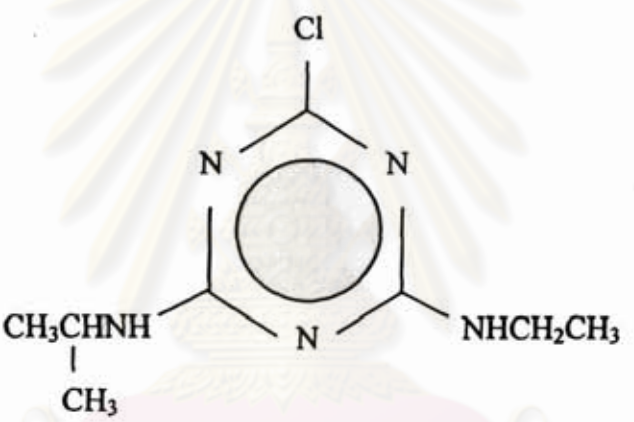


Figure 2-1 Structure of atrazine

2.1.1 Uses of atrazine

Atrazine has been used to control broad-leaf weeds and grasses for corn, macadamia, orchards, pineapples, perennial grasses, sorghum, sugarcane, and other crops (WSSA Herbicide Handbook Committee, 1989; Belluck et al., 1991). Atrazine could be mixed with other herbicides. For example, for corn, atrazine may be mixed with alachor, metolachor, paraquat, propachlor, or simazine. Another example is for sorghum; atrazine may be combined with metolachor, paraquat, propachlor, or terbutryn. Atrazine is also used in some areas for selective weed control on conifer

reforestation, perennial range grasses, and grass-seed fields (WSSA Herbicide Handbook Committee, 1989).

A good illustration of atrazine application is in the United States; US EPA predicted that atrazine was the greatest amount applied herbicide at a rate of 75 to 90 million pounds/year (US EPA, 1991a). Currently, the United States Department of Agriculture recommends an atrazine application rate of 1 lb/acre. Another example of atrazine utilization is in Thailand where agricultural hazardous substances were imported approximately 87,000 tons in 2004. The most significant group of the agricultural hazardous substances including atrazine was herbicides, which were introduced more than 50,000 tons in 2004.

2.1.2 Properties of atrazine

Atrazine is a white crystalline solid. It is stable under normal temperatures and pressures. It may ignite if exposed to heat. Excessive heating of atrazine may cause the production of toxic and corrosive fumes of chloride and of toxic fumes of nitrogen. Atrazine solution is quite stable in neutral condition. It can be hydrolyzed by alkali or mineral acids at high temperatures. Its physicochemical properties are shown in Table 2-1.

2.1.3 Fate of atrazine in the environment

2.1.3.1 Transformation of atrazine

Atrazine transformation occurs via hydrolytic dechlorination, *N*-dealkylation, deamination, and ring cleavage (Redosevich et al., 1995). Main

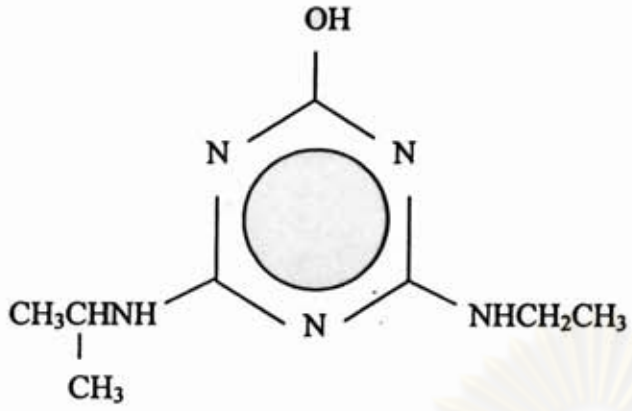
degradation metabolites include hydroxyatrazine (HA), desethylatrazine (DEA), deisopropylatrazine (DIA), and desethyldeisopropylatrazine (DEDIA). The structures of these products are presented in Figure 2-2.

Table 2-1 Physicochemical properties of atrazine

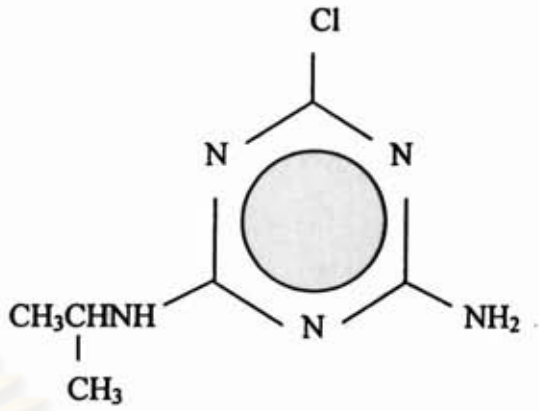
Properties	Description of atrazine
Class	Triazine
Mode of action	Inhibit plant photosynthesis
Chemical name	6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine
Empirical formula	$C_8H_{12}ClN_5$
Molecular weight (g)	215.7
Water solubility (mg/L at 27°C)	33
Vapor pressure (mm Hg at 20°C)	3.0×10^{-7}
Henry's constant (K_H) at 25°C (atm-m^3/mol)	3.04×10^{-9}
K_{oc} (mL/g)	100

Source: Montgomery (1993)

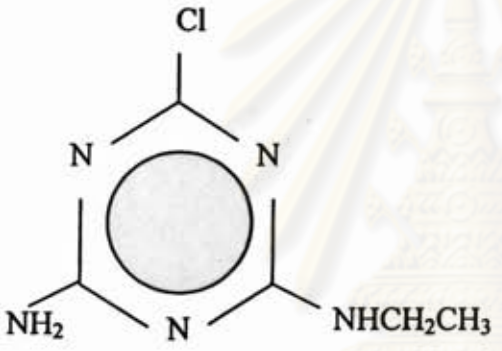
Atrazine transformation pathway is presented in Figure 2-3. Firstly, *N*-dealkylation of ethyl and isopropyl side chains occurs producing DEA and DIA, respectively whereas dechlorination appears at chlorine side chain, which changes atrazine to HA. Then, there are several degradation reactions in other side chains. Although there are many pathways of degradation, all routes produce cyanuric acid, *s*-triazine ring bonded with hydroxide at all side chains. Then *s*-triazine ring of cyanuric acid is cleaved and becomes biuret, which changes to urea later. Eventually, urea is broken down to carbon dioxide (CO_2) and ammonia (NH_3).



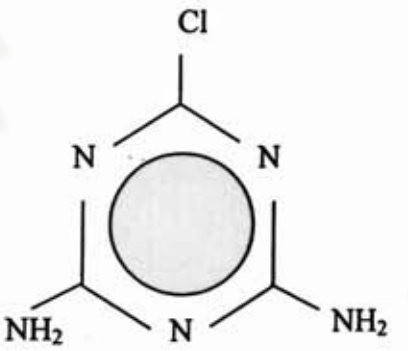
Hydroxyatrazine



Desethylatrazine



Deisopropylatrazine



Desethyldeisopropylatrazine

Figure 2-2 Structures of selected atrazine metabolites

Atrazine degradation can occur through both abiotic and biotic processes. The main abiotic processes are photodegradation (Pelizzetti et al., 1990) and metal oxidation (Cheney et al., 1998). Lanyi and Dinya (2005) reported that the important reaction of atrazine photodegradation was the loss of side chains, particularly chlorine side. Cheney et al. (1998) found that abiotic oxidation by metal oxides, especially manganese oxide, led to atrazine dechlorination and dealkylation. The main metabolites from metal oxidation process are HA, DIA, and DEA. The

significance of abiotic transformation of atrazine varies and depends on several factors including pH, concentration of dissolved organic carbon, and temperature (Koskinen and Clay, 1997; Lanyi and Dinya, 2005). The biotic process consists of various metabolic pathways. The main transformation process is microbial degradation via *N*-dealkylation and dechlorination. The details of biotic process are presented in the next part.

2.1.3.2 Transport of atrazine in environmental media

Atrazine can move into atmosphere, surface water, and groundwater. Volatilization is the major route of atrazine movement into atmosphere. Factors affecting volatilization are vapor pressure of atrazine, Henry's constant, concentration in soil, soil water content, sorption to soil, diffusion rate in soil, air and soil temperature, and air movement. Koskinen and Clay (1997) reported that after 26-day application, 1 to 2% of applied atrazine volatilized. Atrazine in surface water can be either free molecules or absorbed molecules in sediment. Atrazine movement to surface water has been detected ranging from less than 0.1 to 7.2% of applied atrazine.

In soil, factors influencing atrazine movement are moisture, temperature, aeration, and soil chemical properties (Jayachandran et al., 1994). However, microbial degradation and adsorption play an important role in atrazine fate in soil. Microorganisms can degrade atrazine to several metabolites. HA is less water soluble than atrazine, DEA, and DIA; therefore, HA can sorb on soil more than the parent compound and other metabolites. DEA and DIA are more mobile; they then can flow through and contaminate groundwater (Jayachandran et al., 1994).

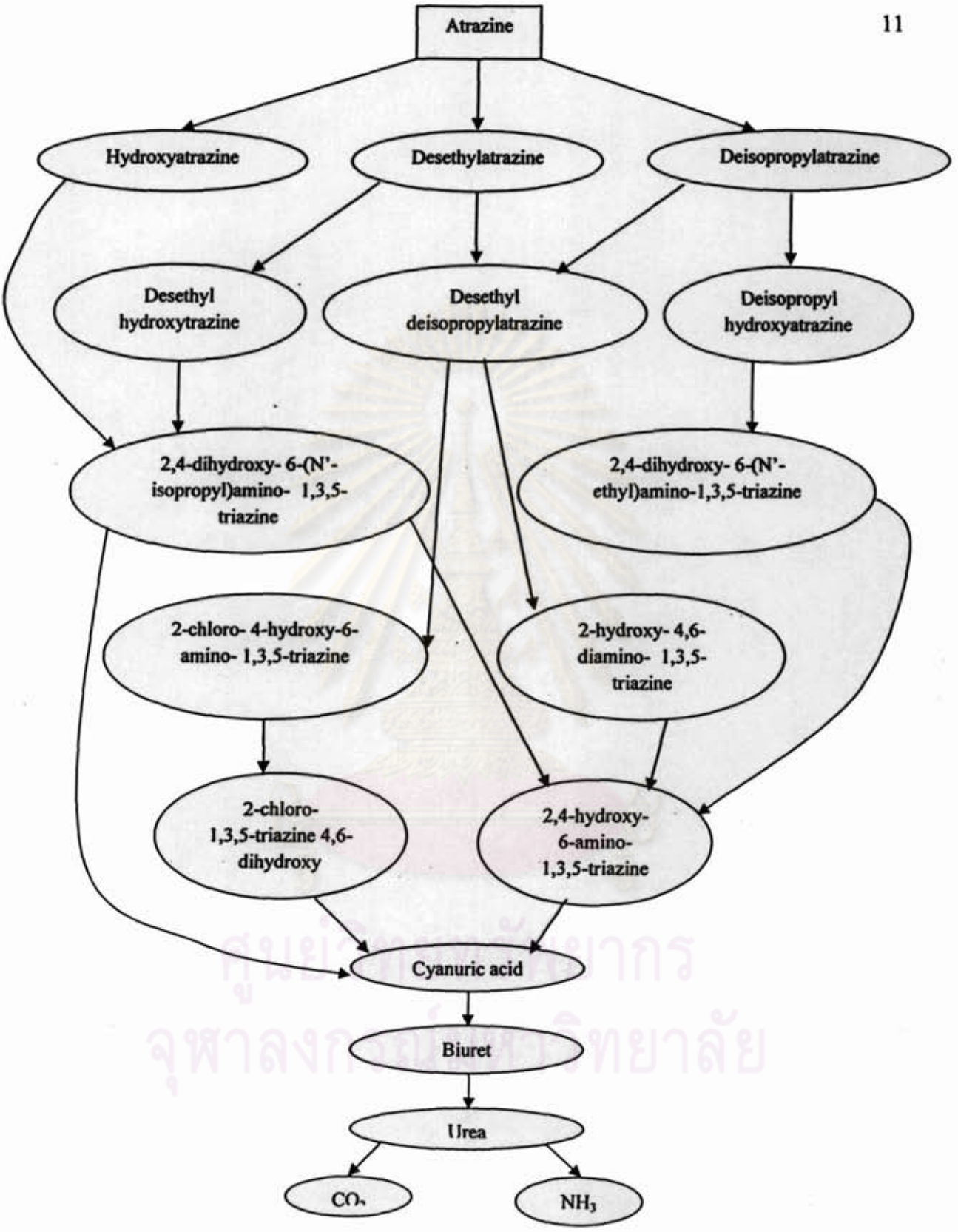


Figure 2-3 Atrazine degradation pathway

(Sadowsky and Wackett, 2001; Carter, 1996)

2.1.3.3 Persistence of atrazine

Atrazine persistence is generally described based on first-order kinetics with half-life values in soil ranging from 14 to 109 days (Koskinen and Clay, 1997). There are numerous factors affecting atrazine persistence, such as, temperature, and soil types and properties (Gu et al., 2003). For instance, in loamy soil, its half-life in aerobic condition is between 60 and 150 days whereas in anaerobic condition the half-life is approximately 660 days (Ribaudo and Bouzaher, 1994).

2.1.4 Biotransformation of atrazine

2.1.4.1 Isolation and characterization of atrazine degraders

Atrazine biodegradation has been studied for more than 30 years (Pick et al., 1992). There are numerous studies reporting atrazine degrader isolation. Table 2-2 presents atrazine degraders and degradation products.

2.1.4.2 Atrazine biodegradation

There have been several studies of atrazine biodegradation, which mainly emphasized on bacterial degradation. Most studies were the isolation of pure atrazine degrading bacteria and their atrazine removal efficiency and mechanism. There were some studies focusing on the use of mixed cultures for atrazine degradation.

Table 2-2 Atrazine-degrading bacteria and degradation products

Microorganisms	Degradation products	References
Pseudomonas ADP	CO_2	Mandelbaum et al., 1995.
Rhodococcus TE1	DIA, DEA	Shao and Behki, 1995.
Ralstonia M91-3	CO_2	Radosevich et al., 1995.
Alcaligenes SG1	CO_2	Boundy et al., 1997.
Rhizobium PATR	CO_2	Bouquard et al., 1997.
Agrobacterium J14A	CO_2	Struthers et al., 1998.
Bacterium 38/38	CO_2	de Souza et al., 1998.
Clavibacter michiganese ATZ1	*N*-ethylammelide	de Souza et al., 1998.
Arthrobacter aurescens TC1	Cyanuric acid	Strong et al., 2002.
Nocardioides sp. SP12	Cyanuric acid	Piutti et al., 2003.
Chelatobacter heintzii Citl	Cyanuric acid	Rousseaux et al., 2003.
Arthrobacter nicotinovorans HIM	Cyanuric acid	Aislabie et al., 2005.

In 1995, Mandelbaum et al. isolated *Pseudomonas* sp. strain ADP (PADP) that metabolized atrazine at high concentrations (> 1,000 ppm) from a herbicide spilled site. PADP used atrazine as its sole nitrogen source. Atrazine was metabolized to HA and CO_2. After this research, there have been studies using PADP for either atrazine biodegradation in different environmental conditions or molecular level investigations of this strain. For example, in 2000, Katz et al. studied atrazine degradation by PADP under anoxic conditions. Results showed that *Pseudomonas* sp. ADP could grow under anoxic conditions although the growth rate under aerobic conditions was higher.

Clausen et al. (2002) examined atrazine degradation by PADP in aquifer sediment under both aerobic and denitrifying conditions. The strain was able to mineralize more than 50% of atrazine within 14 days under both growth conditions. Gonzalez et al. (2003) studied the effect of fertilizer containing nitrogen on atrazine degradation by PADP. They found that nitrogen fertilizer had significant

negative impact on atrazine degradation; however, they also observed that PADP mutant, named MPO102, could overcome this problem. Neumann et al. (2004) reported that PADP was able to simultaneously degrade atrazine of 150 mg/L and phenol of 1,000 mg/L as a sole nitrogen and carbon source, respectively.

Another atrazine degrader, which has been studied by several groups of researchers, is *Agrobacterium radiobacter* strain J14a (J14a). Struthers, et al. (1998) isolated and examined the ability of J14a from soil in agricultural chemical distribution sites. J14a could mineralize 94% of 50 mg/L of [^{14}C-U-*ring*] atrazine in 72 hr with population increasing from 7.9×10^5 to 5.0×10^7 cells/mL. Seffernick et al. (2000) used an enzyme from J14a, and compared with those from PADP and other atrazine degraders to study substrate specificity of enzymes. In 2003, Park et al. evaluated the bioavailability of atrazine sorbed on soil. They used J14a and PADP, as the model atrazine degraders. The biodegradation kinetic of PADP was described by first-order kinetics whereas the biodegradation by J14a was explained by Michelis-Menten kinetics. The CO_2 yield coefficients of PADP and J14a were 0.74 to 0.90 and 0.73 to 0.91, respectively.

Other studied atrazine degraders were mostly the indigenous bacteria. Radosevich et al. (1995) isolated an atrazine degrading culture from an agricultural soil, identified as *Ralstonia* sp. M91-3. This bacterium could use atrazine as the sole source of C and N under aerobic condition. M91-3 had a capability to mineralize side chains of atrazine and cleave triazine ring; approximately 40 to 50% of atrazine-^{14}C was mineralized to $^{14}CO_2$. Bouquard et al. (1997) isolated a *Rhizobium* sp. strain PATR (PATR), from an agricultural soil in France. PATR showed quick dechlorination of atrazine. After 8-day cultivation, HA was detected. In 2000, Topp et

al. investigated atrazine degraders in soil samples from France and Canada. They found an atrazine degrader, named C147, which was in the genus *Pseudaminobactor*. C147 was capable of using atrazine as a sole nitrogen source. Atrazine biodegradation by this strain began with dechlorination and dealkylation followed by triazine ring cleavage.

Kodama et al. (2001) found a novel bacterial strain named N5C. This N5C was identified as *Moraxella (Branhamella) ovis*. It could use atrazine as both C and N sources. Atrazine of 200 mg/L was metabolized to cyanuric acid within 5 days. Another atrazine biodegradation study was conducted by Strong et al. (2002). They isolated *Arthrobacter aurescens* strain TC1 (TC1), which degraded atrazine as the sole sources of both C and N. The strain could handle high concentrations of atrazine (3,000 mg/L) and metabolized it to alkylamines and cyanuric acid. In 2003, Piutti et al. isolated *Nocardioides* sp. SP12 (SP12) from atrazine contaminated rhizosphere soil. SP12 could metabolize atrazine to cyanuric acid. Recently, Aislabie et al. (2005) isolated *Arthrobacter nicotinovorans* HIM (HIM) to degrade atrazine. HIM used atrazine as a sole nitrogen source. Atrazine was degraded to cyanuric acid.

Some studies focused on using mixed cultures for atrazine biodegradation. Alvey and Crowley (1996) studied three bacterial cultures, which were isolated from an atrazine contaminated agricultural soil. From their investigation, this consortium used atrazine as a sole nitrogen source. In inoculated soil, the atrazine degradation rate was higher than that in noninoculated soil whereas planted and nonplanted soils provided similar degradation rate. Smith et al. (2005) examined the degradation pathway of eight bacterial cultures, which had atrazine

degrading genes. Two cooperative catabolic pathways shown in Figure 2-4 were the key results.

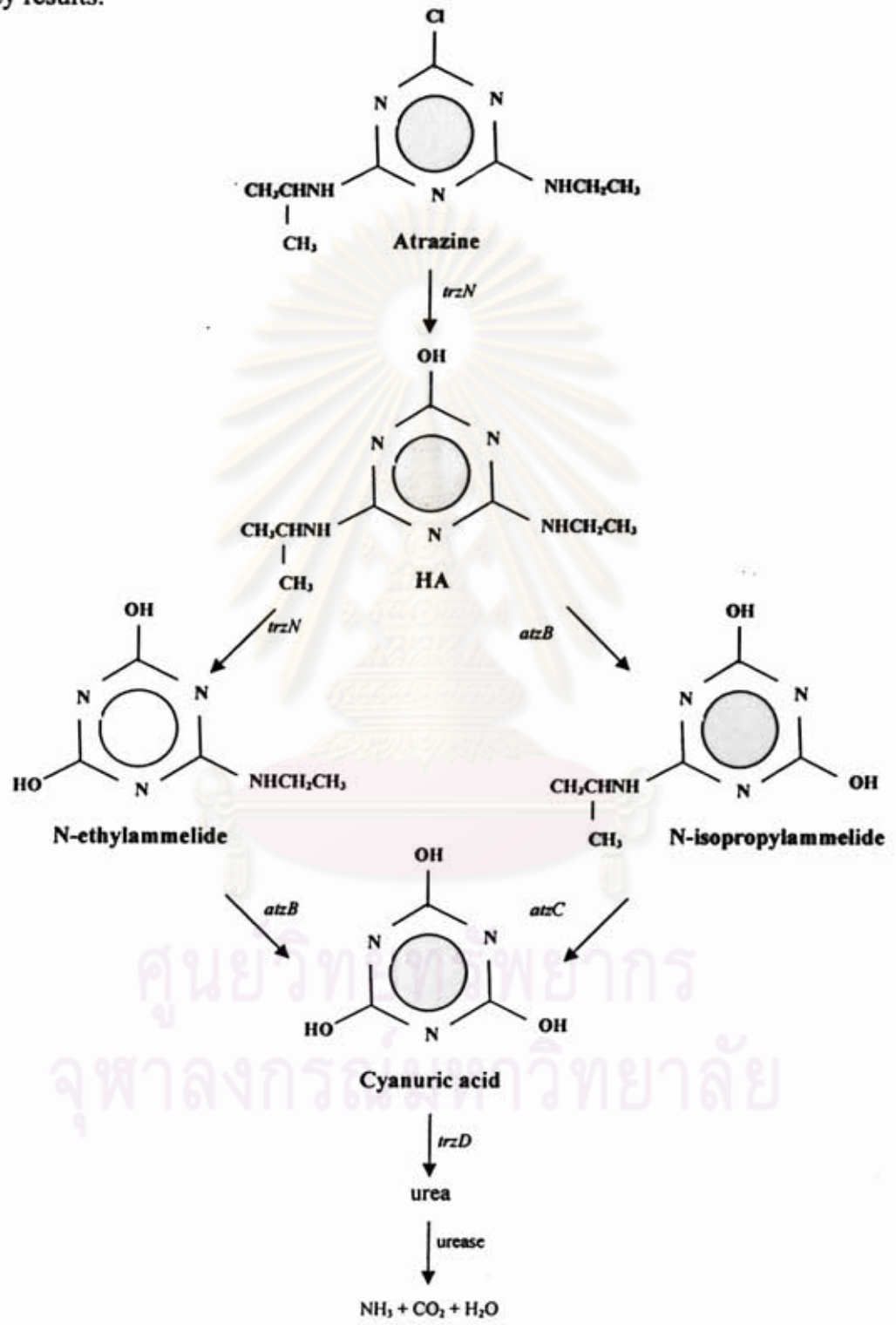


Figure 2-4 Atrazine degradation pathways by consortium

(Smith et al., 2005)

2.1.5 Toxicity of atrazine

Atazine toxicity can be divided into two categories: acute and chronic toxicities. Details of each type of effects are presented as follows.

For acute toxicity, atrazine is slightly to moderately toxic. It can be absorbed into the bloodstream through oral, dermal and inhalation exposure. Atrazine acute toxicity symptoms include abdominal pain, diarrhea and vomiting, eye and mucous membrane irritation, and possible skin reactions. Atrazine is a mild skin irritant. Rashes associated with exposure have been reported. Moderate to severe eye irritation can occur. Exposure to large concentrations of airborne particles or droplets may cause irritation of the mucous membranes (Hayes and Laws, 1990; Meister, 1992).

For chronic toxicity, atrazine has various effects to heart, lung, liver, kidney, spleen, adrenal glands, and brain (US EPA, 1991b). Several studies reported the effect of atrazine on disrupting endocrine hormone metabolism (Moore and Lower, 2001; Friedman, 2002; Hayes et al., 2002) and a cause for cancer (Tchounwou et al., 2001). US EPA has established a Lifetime Health Advisory level for atrazine in drinking water of 3 μg/L. Water containing atrazine at or below this level is acceptable for drinking.

2.2 Bioaugmentation

2.2.1 History of bioaugmentation

Bioaugmentation, the addition of microorganisms, has been implemented in agricultural practices for many years (van Veen et al., 1997; Gentry et al., 2004). There are several applications of bioaugmentation in agriculture, such as, supplying

plant nutrients, stimulating plant growth, and controlling plant pathogen. In the environmental point of view, bioaugmentation is one of the important techniques for increasing waste treatment efficiency. Microorganisms were added for increasing waste decomposition in composting processes and septic tanks (Fang and Wong, 2001). Recently, bioaugmentation has become an important technique for remediating polluted areas (van Veen et al., 1997; Devinny and Chang, 2000; Gentry et al., 2004).

Gentry et al. (2004) reviewed bioaugmentation applications for site remediation. The technique was divided into four categories: cell bioaugmentation, gene bioaugmentation, phytoaugmentation, and rhizosphere bioaugmentation. Cell bioaugmentation is the direct addition of viable microorganisms into soil. Those inoculants are either cell suspension or cells with supporting materials (Pepper et al., 2002). Gene bioaugmentation is the introduction of special gene from contaminant degraders into the indigenous microorganisms. Rhizosphere bioaugmentation is the addition of inoculants at the root zone of plants at polluted sites whereas phytoaugmentation is the supplements of engineered gene into plants. Nowadays, only cell bioaugmentation is practiced since it is simple and provides good remediation efficiency.

2.2.2 Cell bioaugmentation

In bioremediation, sometimes local microorganisms do not have the ability or take unreasonably long time to degrade contaminants. Bioaugmentation could be useful in these situations to enhance the treatability of contaminants. There are various crucial attributes for achieving successful cell bioaugmentation as follows.

2.2.2.1 Sources of microorganisms

Various sources of microorganisms are used for bioaugmentation. These sources comprise previously identified strains, selection and culturing of organisms from the site, commercial preparations, and genetically engineered organisms (Devinny and Chang, 2000). A good illustration of previously identified strains is PADP, a highly effective atrazine degrader. This strain was isolated by Mandelbaum et al. (1995). After that, there were numerous studies using this strain (Katz et al., 2000; Seffernick et al., 2000; Clausen et al., 2002; Gonzalez et al., 2003; Neumann et al., 2004).

Another possible microbial source is the selection and culturing of organisms from the site. These microorganisms are taken from contaminated sites and cultivated in laboratories. The advantage of using these organisms is that they would likely be well grown at the site since they are indigenous species (Devinny and Chang, 2000). Several examples of the selection and culturing of atrazine degraders from the sites are shown in Table 2-2. These microbes could be in the forms of either mixed or pure cultures.

Commercial preparation is an alternative to obtain pollutant degrading microorganisms. This source is convenient since time consuming and expense of contaminant degraders are averted. However, important drawbacks of the commercial cultures are undisclosed information about them (Qasim and Stinehelfer, 1982) and possible inadaptability in environment (Devinny and Chang, 2000).

Recently, genetically engineered organisms have been of interests. These organisms are from either combination or transfer of genes. This novel technique provides high possibility of simultaneous degradation of many

contaminants. For instance, Wackett et al. (1995) produced an engineered halogenated compound degrader, *Pseudomonas putida* by combining seven genes. This strain could degrade fluorinated, chlorinated, and brominated compounds concurrently. Although this technique can cope with multiple contaminants, the drawbacks include high cost, application difficulty, and unexpected harm from engineered cells (Devinny and Chang, 2000).

2.2.2.2 Factors influencing survival of bioaugmented microorganisms

van Veen et al. (1997) reviewed factors affecting the survival of introduced microorganisms in soil and categorized them into 2 types: biotic and abiotic factors as summarized in Table 2-3. The optimum conditions of the above biotic and abiotic factors vary from application to application. These conditions influence the number of active augmented inoculants or their metabolic activity, which are the key attributes for the success of cell bioaugmentation.

2.2.2.3 Methods for assessment of microorganism survival in soil

Microorganism survival can be assessed by monitoring the presence and/or activity of microorganisms. Microbiological and molecular biological techniques can be used for these purposes. Microbiological techniques are more traditional. There are two main microbiological methods for microorganism counting: indirect and direct count methods. The first method is the indirect or viable count including the standard plate count and the most probable number techniques. These two techniques provide the numbers of viable microorganisms. The other method is

the direct count by a microscopic observation using a counting chamber, such as hemacytometer. The direct count method provides total numbers of viable and dead microorganisms (Maier et al., 2000).

Table 2-3 Factors influencing bacterial survival in soils

Process	Factor	Effect
Biotic	Predation	- Population size decrease
	Competition	- Population size decrease/ antagonistic effect on plant pathogens
	Root growth	- Release of organic compounds, enhancing survival
Abiotic	Clay minerals	- Protection against predation
	Water tension	- High tension: water shortage, high osmolarity - Low tension: anaerobism, increased nutrient availability by diffusion
	Organic carbon	- Limited organic carbon results in starvation and reduction in activity
	Inorganic nutrients	- Limitation results in starvation
	pH	- Selection for species - Release of nutrients or toxic compounds
	Temperature	- Metabolic activity
	Chemicals (toxic waste)	- Inhibition of sensitive organism - Selection of biodegradative, resistant, or tolerant chemical compound forms

Source: van Veen et al. (1997)

Molecular biological techniques do not require culturing (Richardson et al., 2002). Gentry et al. (2004) reviewed and summarized molecular biological techniques into 2 groups: polymerase chain reaction (PCR) and hybridization-based, as shown in Table 2-4. The PCR-based approach focuses on amplification of

deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) for detecting the presence and activity of interested organisms. There are numerous sub-approaches; for instance, 16S ribosomal DNA (rDNA), reverse transcription PCR, and real-time PCR. The other approach, hybridization-based methods, uses indicators to detect microorganisms. For example, fluorescent in situ hybridization (FISH) is an in situ measurement using the hybridizing probe tagged with a fluorescent molecule. These oligonucleotide probes are designed based on 16S-rDNA sequences (Aoi, 2002; Gentry et al., 2004).

2.2.3 Cell Immobilization

2.2.3.1 Immobilization methods

There are various methods for immobilizing microorganisms. Several researchers reviewed and classified the types of immobilization techniques in different ways (Dervakos and Webb, 1991; Jen et al, 1996; Kok and Hasirci, 2000). One of the classifications categorized immobilization techniques into 4 groups: 1) physical adsorption and ionic binding, 2) covalent binding, 3) cross-linking, and 4) entrapment (also referred to as encapsulation by some researchers) (Kok and Hasirci, 2000).

Physical adsorption and ionic binding techniques were the oldest and most basic group among the four. These techniques are to adhere or bind microorganisms either onto matrices (Cochet et al., 1990; Jen et al, 1996). Even though the immobilization matrices using these techniques are less durable and stable than the other techniques, they do less damage to microorganisms. There are several

successful applications of immobilized cells through this group of techniques for both wastewater treatment and soil bioremediation.

Table 2-4 Molecular biological methods for detecting survival and activity of introduced microorganisms

Method	Measured parameters	Advantages/disadvantages
16S rDNA PCR	Presence	- Simple, inexpensive and not quantitative - PCR product may be analyzed by other methods, such as terminal restriction fragment length polymorphism analysis, to increase specificity
Reverse transcription PCR	Activity	- Determines gene expression - Can be combined with real-time PCR to be quantitative
Real-time PCR	Presence/activity	- Rapid, quantitative - Very sensitive, possible to detect < 10^4 cells/g soil - Can be combined with reverse transcriptase PCR to measure gene expression
FISH	Presence/activity	- Can visualize, enumerate, and identify microorganisms, directly without culturing - Quantitative - Can analyze complex communities using multiple probes
Microarrays	Presence/activity	- Simultaneous analysis of various genes - Lower sensitivity than other methods - Can be quantitative
Reporter genes	Presence/activity	- Possible to visualize microorganisms in situ - Quantitative - Luminescent reporters enable in situ measurement of gene expression - Multiple different reporters enable simultaneous study of several organisms - Requires genetic modification of host

Source: Gentry et al. (2004)

Covalent binding technique is the immobilization of cells via covalent bond between microorganisms and matrices. This bond is strong leading to firm immobilized cells. Nevertheless, there is a major drawback of this technique; the supporting materials containing hydroxyl, amino, amido, and carboxyl groups have to be activated before the immobilization process (Kok and Hasirci, 2000).

Another immobilization technique is cross-linking. Microorganisms are connected by cross-linkers. This method is not broadly applied since cross-linkers can cause a severe cell damage. Entrapment or encapsulation technique is the stabilization of microorganisms within polymeric supporting materials or semipermeable membrane (Jen et al, 1996; Kok and Hasirci, 2000; Park and Chang, 2000). This method is different from the covalent binding and cross-linking, which are chemical immobilization techniques. Microorganisms are not directly bonded with supporting materials. They are only enclosed in supporting matrices. Therefore, all types of microorganisms can be immobilized by this technique. Many types of natural and synthetic polymers have been used as cell entrapment and encapsulation materials such as PVA, polyurethane foam, alginate, κ-carrageenan, and guar gum (Kok and Hasirci, 2000; Gentry et al., 2004). There are a number of implementations of entrapped and encapsulated cells in environmental, pharmaceutical, and food technology fields.

2.2.3.2 Supporting materials used in immobilization

A variety of types of supporting materials have been used for cell immobilization. Several factors should be considered for the selection of supporting material including chemical properties, physical properties, and cost (Kok and

Hasirci, 2000). Chemical factors consist of hydrophilicity, inertness, and availability of functional groups for modification. Physical factors comprise mechanical strength, porosity, and density. The ideal characteristics of supporting materials are insoluble, nonbiodegradable, high mechanical stability, high diffusivity, simple immobilization procedure, and high biomass retention time (Leenen et al., 1996; van Veen et al., 1997). Supporting materials are categorized into inorganic and organic materials.

Inorganic materials including sand, clay, and glass, have been used as supports for microbial attachment. These materials have appropriate surfaces for immobilizing microbes (Gentry et al., 2004). However, these inorganic materials have some disadvantages including long microbial colonization period, high potential of failure, and increase in suspended solids from eroded materials.

Another group of materials is organic materials, which can be subcategorized to natural and synthetic organic materials. Natural organic materials are biopolymers such as alginate, κ-carrageenan, and guar gum. They are the most extensively used immobilization materials. Although they have easy formulation and no cytotoxicity, they possess a very important disadvantage which is poor mechanical stability (Leenen et al., 1996; Kok and Hasirci, 2000; Gentry et al., 2004). The commonly used synthetic organic materials are nylon, polyurethane, polyacrylamide, PVA, and polyethylene glycol. These polymers exhibit good mechanical and chemical stability and persistence to degradation. However, some materials or preparation chemicals of those materials are toxic substances (Kok and Hasirci, 2000; Gentry et al., 2004).

2.2.3.3 Advantages and drawbacks of cell immobilization

There are several advantages of cell immobilization over freely suspended cells (Cochet et al., 1990; Dervakos and Webb, 1991). Basically, cell immobilization leads to the enhancement of both biological and mechanical stabilities. The immobilization matrix can alleviate physicochemical challenges, such as temperature, pH, solvents, shear, and heavy metals. Other advantages of immobilized cells include high biomass concentration, no need for cell separation, increased product yields and stability, increased reaction selectivity, and versatility in the selection of the reactor. Several studies reported that immobilized cells provided better waste treatment performance or more durable than free cells (Uchiyama et al., 1995; Murakami-Nitta et al., 2003; Cunningham et al., 2004). The main drawbacks of cell immobilization are metabolic changes, cell morphology changes, diffusion limitation, and inconsistence of growth pattern (Dervakos and Webb, 1991; Kourkoutas et al., 2004).

2.2.4 Cell immobilization by PVA hydrogel

2.2.4.1 Properties and structure of PVA

Polyvinyl alcohol is a widely used polymer that can be prepared in the forms of film and hydrogel. It has been used in pharmaceutical, biomedical, industrial, and environmental applications for different purposes including cell immobilization. It is one of the most promising cell immobilization matrices since it is a synthetic material with high mechanical strength and durability (Chang and Tseng, 1998). It is nontoxic (Chen and Lin, 1994; Chang and Tseng, 1998; Gentry et

al., 2004); therefore, it does not negatively affect to both microorganisms and environment.

Raw PVA is a white and free-flowing granule. pH of its aqueous solution is neutral or slightly acidic. The chemical structure of PVA is shown in Figure 2-5. The properties of PVA are based on the polymer chain length (molecular weight) and degree of hydrolysis. The effects of molecular weight and degree of hydrolysis of PVA on its physical stability were investigated (Chang et al., 2005). High molecular weights and degrees of hydrolysis attributed to high mechanical stability and low water solubility.

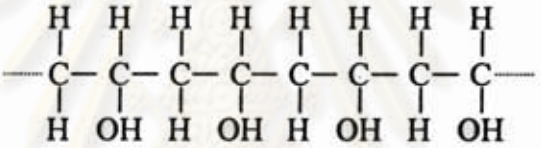


Figure 2-5 Structure of PVA

2.2.4.2 PVA hydrogel preparation techniques

As mentioned earlier, PVA hydrogel is one of the most extensively used cell immobilizing materials. Several preparation techniques were developed for producing PVA gels for cell immobilization including boric acid-PVA, freezing and thawing, and phosphorylated-PVA (PPVA) methods.

The boric acid-PVA technique is simplest and most economical. It was developed by Hashimoto and Furukawa (1987). They immobilized activated sludge by using PVA cross-linking with saturated boric acid and found that their immobilized cell beads were rubberlike and strong while the treatment efficiency was high. The technique has been used in several studies for entrapping different bacteria

(Wu and Wiscarver, 1992; Hanaki et al., 1994; Li et al., 2005b). The hydrogel beads present high mechanical strength and durability. Nonetheless, there are two potential problems associated with the technique: cell damage in the boric acid solution and PVA bead agglomeration (Wu and Wisecarver, 1992). Many researchers modified the procedure to solve these problems. For instance, Wu and Wisecarver (1992) studied *Pseudomonas* sp. immobilized cells, using the boric acid-PVA method with the addition of calcium alginate for preventing bead agglomeration. The PVA-alginate beads showed good performance, and high strength and durability. No bead agglomeration was observed. Li et al. (2005b) examined the degradation of phenanthrene and pyrene using immobilized *Zoogloea* sp. The boric acid-PVA technique supplemented with sodium alginate and powdered activated carbon addition was applied. Successful phenanthrene and pyrene degradation was obtained via optimum immobilization conditions.

The freezing and thawing technique is based on physical cross-linking. There are hydrogen bonds between OH groups of the polymer chain (Lozinsky and Plieva, 1998). This technique does not leave behind toxic agents. It was developed in the beginning of 1970s. The technique is widely used in environmental remediation, such as nitrification of high strength ammonia wastewater (Rostron et al., 2001), and bioremediation of diesel-contaminated soil (Cunningham et al., 2004). The cryogel was strong; however, freezing could affect cell viability (Chen and Lin, 1994). PPVA technique is a well-known effective technique. Details of this technique are presented in the following subsection.

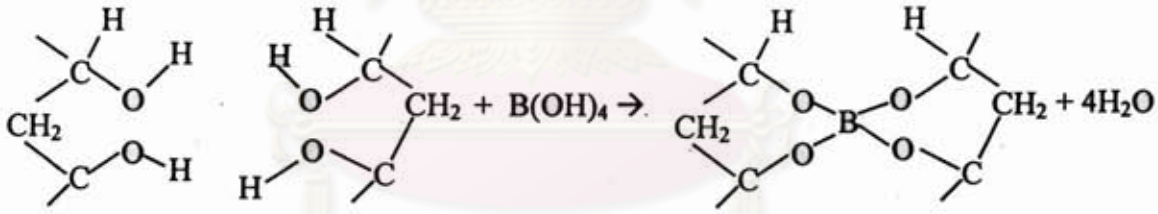
2.2.4.3 Cell immobilization by PPVA technique

Chen and Lin (1994) developed the PPVA method to reduce the boric acid contact time and consequently cell damage associated with the boric acid-PVA method. This modified technique not only decreases the cell damage from boric acid but also increases the strength and durability of immobilized cell beads. The PPVA procedure can be divided into two main steps, spherical bead formation and hardening. In the first step, spherical bead formation, a mixture of PVA and cell suspension was dropped and soaked in saturated boric acid between 10 and 120 min. The PVA-boron cross-linking occurred according to the following reactions:

- Boric acid accepts OH^- from water.

$$H_3BO_3 + 2H_2O \rightarrow B(OH)_4^- + H_3O^+$$

- Boron bonds with two molecules of PVA.



In the second step, bead hardening, spherical beads were left in sodium phosphate solution between 15 and 120 min to increase surface gel strength by PVA phosphorylation. The structure of bonded PVA is presented in Figure 2-6 (Sreenivasan, 2004).

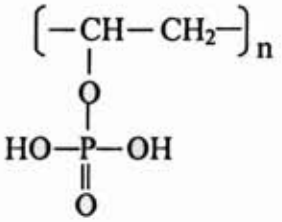


Figure 2-6 Structure of phosphate bonded PVA (Sreenivasan, 2004)

There are numerous environmental applications using the PPVA immobilization method. It was used to immobilize denitrifying bacteria (Chen and Lin, 1994). After that, it was applied to immobilize a mixed culture for simultaneous carbon-nitrogen removal (Chen et al., 1998; Chen et al., 2000). PPVA immobilized cells were used for removing complex chemical substances in wastewater, such as, di-n-butyl phthalate (Jianlong et al., 1997), and azo dye in a batch reactor (Chen et al., 2003a) and a fluidized bed reactor (Chen et al., 2003b). Another example is degradation of 2-methylnaphthalene studied by Sharanagouda and Karegoudar (2002). They used several matrices including polyurethane, alginate, agar, and PVA for immobilizing *Pseudomonas* sp. strain NGK1 and compared their performances with that of free cells. Results indicated that PVA and polyurethane immobilized cells were much better than the other matrices and free cells in term of operational stability and durability. Kim et al. (2001) examined nitrogen removal by using PPVA immobilized denitrifying bacteria. The addition of sodium alginate to the PVA and cell mixture reduced bead floating and produced durable hydrogel beads. High denitrification rates were accomplished via certain immobilization conditions.

CHAPTER III

ATRAZINE DEGRADATION BY STABLE MIXED CULTURES ENRICHED FROM AGRICULTURAL SOIL AND THEIR CHARACTERIZATION

3.1 Introduction

Atrazine, one of the most widely used herbicides, has been applied to control broad-leaf weeds for corn, sorghum, sugarcane, and other crops. The widespread use of this herbicide has led to its contamination in different environmental media (Wauchope 1978; Jayachandran et al., 1994; Koskinen and Clay 1997). Atrazine concentrations above the allowable contaminant levels for drinking water of 0.1 and 3 μg/L in Europe and the United States, respectively, have been frequently detected (Rousseaux et al., 2003).

Biodegradation is one of the key attenuation processes of atrazine in the environment. Prior research on atrazine biodegradation focused on pure atrazine-degrading cultures (Mandelbaum et al., 1995; Radosevich et al., 1995; Struthers et al., 1998; Strong et al., 2002; Piutti et al., 2003; Rousseaux et al., 2003; Aislabie et al., 2005). Several atrazine-degrading microorganisms were isolated from atrazine spilled sites or atrazine applied crop fields including *Pseudomonas*, *Rhizobium*, and *Agrobacterium* strains. Among atrazine-degrading species, two main biodegradation pathways via hydrolytic dechlorination and *N*-dealkylation convert atrazine to HA, and DEA or DIA, respectively. Several genes have been identified in those species that play a role in atrazine biodegradation; for instance, *atzA* and *trzN* are responsible for metabolizing atrazine to HA while *atrA* initiates the biodegradation of atrazine to DEA or DIA (de Souza et al., 1995; Shao et al., 1995; Mulbry et al., 2002).

Mixed cultures have been shown to be more suitable for bioremediation compared to pure cultures. The rationale is that their biodiversity can enhance environmental survival and increase the number of catabolic pathways available for contaminant biodegradation (Alvey and Crowley, 1996; Kontchou and Gschwind, 1999; Smith et al., 2005). There are two main types of mixed cultures used in bioremediation: previously isolated exogenous degraders and indigenous cultures. Previously isolated strains may not be good degraders or do not survive well in environments that are different from their origins (Struthers et al., 1998; Rousseaux et al., 2003). For successful bioremediation, indigenous mixed cultures are a better alternative. However, there is some concern about cultural population dynamics in mixed culture systems resulting in uncertain biodegradation. The cultures need to be acclimated and stabilized before their applications. Only a few studies have been able to enrich or construct stable mixed cultures for degrading atrazine (Mandelbaum et al., 1993; Kontchou and Gschwind, 1999; Smith et al., 2005). These studies involved atrazine concentrations of higher than 30 mg/L that were much higher than typical concentrations in contaminated agricultural soil and infiltrate (Wauchope, 1978).

The ultimate goal of this dissertation research is to use a stable indigenous mixed culture for the bioremediation of atrazine in infiltrate. In the present chapter, indigenous mixed cultures were enriched and then stabilized in bacterial media containing different carbon, nitrogen, and atrazine supplements. Atrazine degradation by the stable mixed cultures was performed in comparison with a known atrazine-degrading strain, J14a. The work also aimed at identifying bacterial species in each mixed culture and detecting atrazine-degrading genes in the species. The isolation and

genc detection process led to the discovery of a new bacterial species containing atrazine-degrading gene.

3.2 Materials and Methods

3.2.1 Chemicals

Atrazine (98% purity) was purchased from Sigma Chemical Co., MO, USA. All other chemicals for bacterial medium preparation and atrazine analysis were analytical grade from VWR International Co., PA, USA.

3.2.2 Stable atrazine-degrading mixed culture enrichment

Atrazine-degrading mixed cultures were isolated from atrazine-contaminated soil from a field site in Oakes, North Dakota, USA. A basal salts medium (BSM) containing (per liter) K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3 \cdot H_2O$ 10 mg, $CaCl_2 \cdot H_2O$ 10 mg, $MnCl_2$ 0.1 mg, and $ZnSO_4$ 0.01 mg was used. The BSM was prepared in a 20 mM sodium phosphate buffer solution (pH 6.8). The BSM solution was modified to obtain four medium formulations that were different in carbon and nitrogen supplements: 1) BSM supplied with 1.0 g of glucose and 0.5 g of $(NH_4)_2SO_4$ (BSMG), 2) BSM supplied with 1.0 g of glucose, 0.5 g of $(NH_4)_2SO_4$, and 20 mg of atrazine (BSMA1), 3) BSM supplied with 1.0 g of glucose, and 20 mg of atrazine (BSMA2), and 4) BSM supplied with 20 mg of atrazine (BSMA3).

Ten grams of soil sample from the field site were mixed with 100 mL of BSMG for one week at 30°C on a rotary shaker at 150 rpm. Following this incubation, 10 mL of BSMG was transferred into BSMA1 and shaken at 150 rpm and

30°C. This step was repeated between BSMA1 and BSMA2 and between BSMA2 and BSMA3, respectively. Atrazine-degrading mixed cultures grown in BSMA1, BSMA2, and BSMA3 were designated MC1, MC2, and MC3, respectively. Before further testing, MC1, MC2, and MC3 were subcultured in BSMA1, BSMA2, and BSMA3, respectively for 3 months (a total of 12 subcultures) to obtain the stable mixed cultures.

3.2.3 Atrazine biodegradation test

Duplicate batch experiments of atrazine biodegradation by the stable mixed cultures and J14a (Struthers et al., 1998) were conducted. The cultures were shaken in 200-mL BSM amended with 1.0 g of glucose, 0.5 g of $(NH_4)_2SO_4$, and 100 μg/L of atrazine at room temperature. Atrazine, DEA, and DIA concentration and cell optical density (at 600 nm) measurements were performed daily for 7 d. A control biodegradation test (no bacterial cultures) was also performed.

3.2.4 Isolate identification

Isolated colonies were obtained by spread plating 100 μL aliquots of mixed cultures grown in BSMA1, BSMA2, and BSMA3 onto their respective agars. All plates were incubated at 30°C for 4 d. Isolated colonies were selected and subcultured to obtain pure cultures.

Gram staining was performed on all pure cultures. The species of isolates was determined by amplifying and sequencing a fragment of the 16S rRNA gene. A single colony was mixed briefly in a 200-μL tube with 40 μL of lysis buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA and 125 ng/μL proteinase K) and heated in a thermocycler at 55

and 80°C for 10 min at each temperature, respectively. The heated samples were combined with 80 µL deionized water and centrifuged at 10,000×g for 30 sec to remove cellular debris. The supernatant was removed and used as a template in PCR reactions. A fragment of the 16S rRNA gene was amplified according to the method described by Piutti et al. (2003) using primers 27f (5'-AGA GTT TGA TCM TGG CTC AG), and 1492r (5'TAC GGH TAC CTT GTT ACG ACT T). PCR products of expected size (approximately 1,400 bp) were purified using ExoSap-IT cleaning kit (GE Healthcare Bio-Sciences, NJ, USA) and sequenced using an ABI 3730 XL DNA analyzer (Applied Biosystems, CA, USA). PCR products were sequenced with forward and reverse primers, 518F (5'-CCA GCA GCC GCG GTA AT) and 800R (5'-CAT CGT TTA CGG CGT GGA C). The sequencing conditions were 2 min at 99°C, 35 cycles of 15 sec at 96°C, 10 sec at 50°C, and 2.5 min at 60°C, and cooling down to 8°C. Sequences were assembled and edited using SeqMan (DNAStar, WI, USA), and compared to non-redundant sequences in GenBank™ using the BLAST algorithm. Sequences from this study and related sequences published in GenBank™ were aligned using ClustalX (Thompson et al., 1997), and a neighbour joining tree was constructed using the Kimura-2-parameter model (Kumar et al., 2004). Bootstrap support for tree topology was calculated from 1,000 replications.

The GenBank accession numbers for the 16S rRNA gene sequences of the four isolates (ND1, ND2, ND3, and ND4) are EU075145, EU075144, EU075147, and EU075146, respectively. The strains and their GenBank accession numbers used in the phylogenetic analysis are: *Klebsiella ornithinolytica* 590681 (Y17662), *Klebsiella ornithinolytica* JCM6096T (AJ251467), *Enterobacter aerogenes* NCTC10006T (AJ251468), *Enterobacter aerogenes* NTG-01 (AY825036), *Klebsiella oxytoca* 566

(AY292871), *Citrobacter freundii* (AF025365), *Klebsiella pneumoniae* ATCC13884T (Y17657), *Alcaligenes faecalis* 5659-H (AJ509012), *Alcaligenes* sp. (ASU80417), *Alcaligenes faecalis* 6818m-E (AJ508999), *Bordetella hinzii* (AF177667), *Bordetella pertussis* CIP 105.894 (BX640420), *Agrobacterium tumefaciens* (DQ468100), *Agrobacterium tumefaciens* CFBP2879 (AJ389892), *Agrobacterium tumefaciens* (ATU16SRDF), *Agrobacterium* sp. ICPPB 60097 (EF687663), *Rhizobium* sp. R-24658 (AM084043), *Bacillus megaterium* M4-2 (EF690405), *Bacillus megaterium* 2-37-4-1 (DQ267829), *Bacillus megaterium* CICCHLJ Q37 (EF528269), *Bacillus circulans* (DQ374636), and *Bacillus senegalensis* RS8 (AF519468).

3.2.5 Atrazine-degrading gene detection

A fragment of the *atzA* gene (500 bp) was targeted in gram-negative isolates using the primers *atzA*f (5'-TGA AGC GTC CAC ATT ACC) and *atzA*r (5'-CCA TGT GAA CCA GAT CCT) as described by de Souza et al. (1995). PADP was used as a positive control (de Souza et al., 1995). Total genomic DNA was used as a PCR template. Each 25-µL PCR reaction consisted of 5.0 µl of template, 1× buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 12.5 pmol each primer, and 0.25 U of Taq Polymerase (Promega, Madison, WI, USA). The PCR conditions were 5 min at 95°C, 35 cycles of 45 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, plus an additional 10-min cycle at 72°C. A fragment of the *trzN* gene (450 bp) was amplified from gram-positive isolates. The primers, *trzN*f 5'-CAC CAG CAC CTG TAC GAA GG and *trzN*r 5'-GAT TCG AAC CAT TCC AAA CG, were used and the PCR conditions were described elsewhere (Mulbry et al., 2002). TC1 was used as a positive control

(Mulbry et al., 2002). PCR products were separated by electrophoresis on a 1.5% agarose gel. PCR products of expected size were purified and sequenced to confirm the presence of homologous atrazine-degrading genes.

3.2.6 Atrazine and metabolite analysis

Atrazine, DEA, and DIA were measured using a solid phase extraction technique and a gas chromatograph with a mass selective detector (GC-MSD) according to the procedures described in Siripattanakul et al. (2008). One-microliter of extracted sample was manually injected to the GC. The GC conditions were: splitless injection and injection port temperature of 220°C. The temperature of GC oven was held at 50°C for 1 min, increased to 280°C at a rate of 18°C/min, and retained for 1 min. Two ions of atrazine, DEA, and DIA at m/z = 200 and 215, 172 and 187, and 158 and 173, respectively, were chosen as the quantification ions under selective ion monitoring mode.

3.3 Results and Discussion

3.3.1 Stable atrazine-degrading culture enrichment

Based on the bacterial medium formulations, MC1 could survive in the presence of atrazine or utilize atrazine whereas MC2 and MC3 were capable of using atrazine as a sole nitrogen source and sole nitrogen and carbon sources, respectively. After the mixed culture acclimatization process (3-month subculturing), all stable mixed cultures were plated. A number of different colony morphologies were present

in MC1, MC2, and MC3 indicating that the soil used in this study was rich in atrazine-degrading cultures.

3.3.2 Atrazine biodegradation test

Atrazine degradation by the stable mixed cultures and J14a was performed twice in bacterial medium containing atrazine of 100 μg/L and additional carbon and nitrogen sources for imitating contaminated agricultural runoff. The growth of stable mixed cultures (MC1, MC2, and MC3) and J14a measured by cell optical density at 600 nm is shown in Figure 3-1A. All cultures (MC1, MC2, MC3, and J14a) showed similar growth patterns with an increase in optical density of 0.05 to between 0.5 and 0.7 within 72 hr. All four cultures reached the stationary phase after 72 hr.

The reduction of atrazine concentration during the biodegradation assay is shown in Figure 3-1B. Atrazine concentration decreased approximately 30 to 40% between 0 and 72 hr, and more gradually thereafter. After 168 hr, MC1, MC2, MC3, and J14a removed atrazine 51%, 49%, 33%, and 38%, respectively. DIA and DEA were not detected during the 7-d atrazine biodegradation assay. The degradation trends in Figure 3-1B agreed well with the first-order kinetics (Table 3-1). The degradation rate coefficients of MC1 and MC2 were close and much higher than those of MC3 and J14a.

From the bacterial growth and atrazine reduction data, it is clear that the stable mixed cultures from this study can degrade atrazine. For the concern on population dynamics in the mixed cultures, reproducible results confirmed that after growing the cultures under the same conditions for a certain period (subculturing 12 times in 3 months), the cultures were acclimated and maintained similar degradation activity.

This indicates that the stable mixed cultures have potential for atrazine bioremediation in terms of removal efficiency and degradation stability.

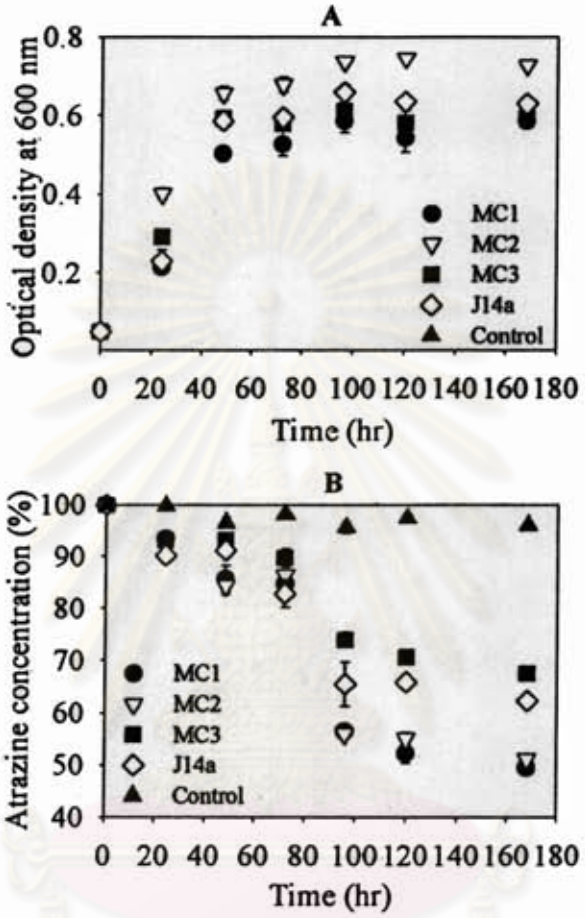


Figure 3-1 Biodegradation of atrazine: A) bacterial growth represented by optical density at 600 nm, and B) atrazine concentration remaining in the medium

Table 3-1 Atrazine degradation kinetic equations and rates

Culture	Atrazine degradation kinetic equation[a]	R^2	Atrazine biodegradation rate (1/d)
MC1	$C = C_0 e^{-0.11t}$	0.88	0.11
MC2	$C = C_0 e^{-0.10t}$	0.86	0.10
MC3	$C = C_0 e^{-0.06t}$	0.90	0.06
J14a	$C = C_0 e^{-0.08t}$	0.89	0.08

[a] C = atrazine concentration at time t (μg /L), C_0 = initial atrazine concentration (μg /L), t = time (d)

Based on the biodegradation test result, MC1 and MC2 removed atrazine more efficiently than MC3 and J14a. MC1 and MC2 comprised three and two predominant species, respectively while only one bacterial species was detected in MC3. It is inconclusive from our study whether MC3 was a pure or mixed culture although only one species was recovered. During the enrichment process, MC3 was maintained in the bacterial medium containing only atrazine as sole carbon and nitrogen sources. This selective pressure could be the reason why MC3 has less biodiversity (only one bacterial species detected) compared to MC1 and MC2. With the comparable growth rates, MC3 and J14a gave similar atrazine degradation result, which was lower than MC1 and MC2. This suggests that mixed cultures are more effective at atrazine biodegradation compared to single isolate cultures. Similarly, de Souza et al. (1998) reported a mixed culture provided higher atrazine biodegradation than a pure atrazine-degrading culture, *Clavibacter michiganese* ATZ1.

3.3.3 Isolate identification

Eight pure cultures were isolated from the stable mixed cultures (MC1, MC2, and MC3). The pure cultures were gram-strained and a phylogenetic analysis of their 16S rRNA gene sequences (1,348 to 1,471 bp) was performed. Four different bacterial species designated ND1, ND2, ND3, and ND4 were identified among the eight pure cultures as *Alcaligenes faecalis*, *Klebsiella ornithinolytica*, *Bacillus megaterium*, and *Agrobacterium tumefaciens*, respectively at 99 to 100% similarity (Figure 3-2). The descriptions of the pure cultures are presented in Table 3-2.

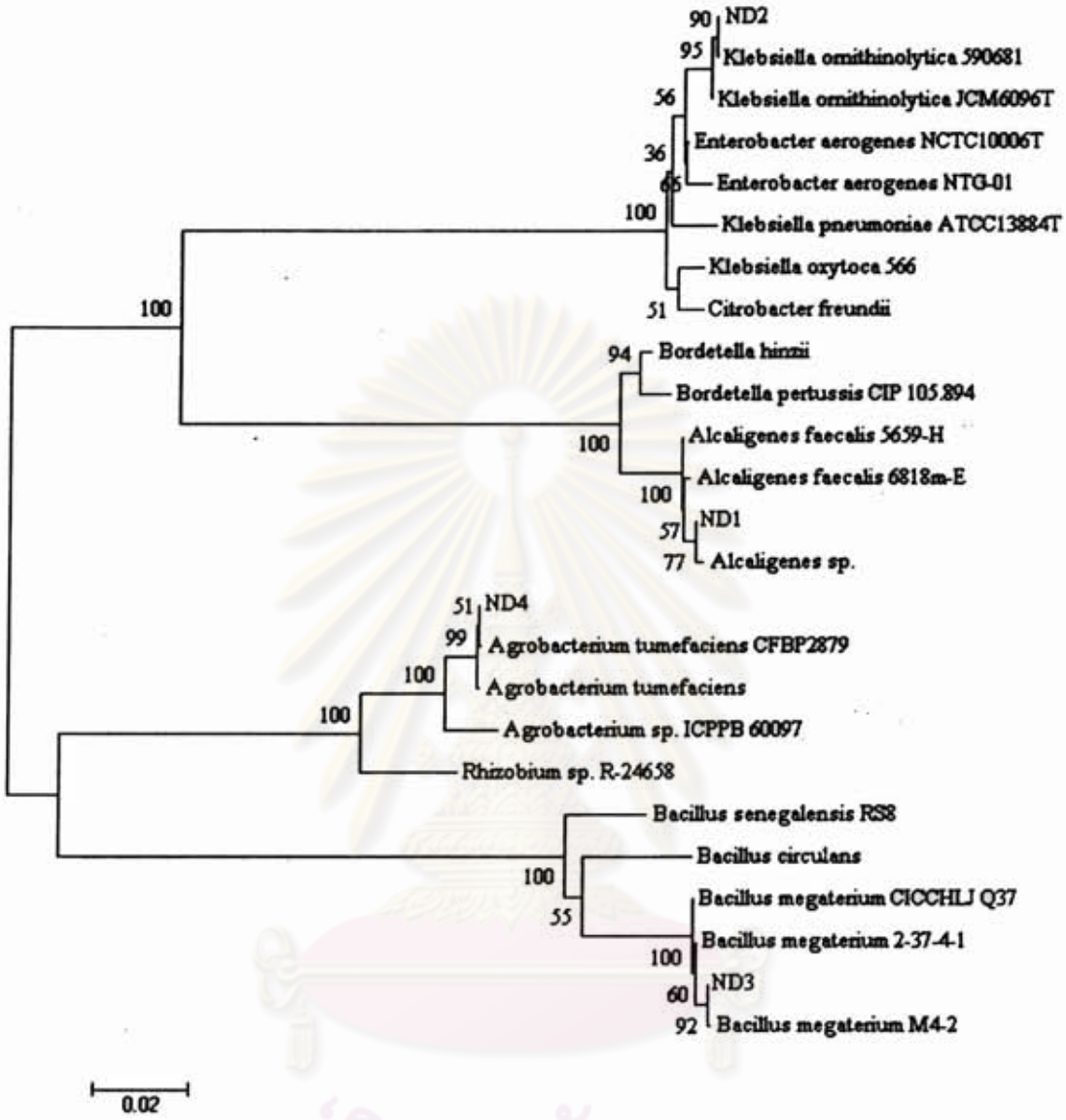


Figure 3-2 Phylogenetic analysis showing the relationship of ND1, ND2, ND3, and ND4 to entries in the GenBank based on 16S rRNA gene sequence. The scale bar indicates 0.02 substitutions per nucleotide position

Table 3-2 Descriptions of pure cultures

Mixed culture	Pure culture	Gram reaction	Designated isolate	Bacterial species
MC1	1	Negative	ND1	*Alcaligenes faecalis*
	2	Negative	ND2	*Klebsiella ornithinolytica*
	3	Positive	ND3	*Bacillus megaterium*
MC2	4	Negative	ND4	*Agrobacterium tumefaciens*
	5	Negative	ND4	*Agrobacterium tumefaciens*
	6	Negative	ND2	*Klebsiella ornithinolytica*
MC3	7	Negative	ND4	*Agrobacterium tumefaciens*
	8	Negative	ND4	*Agrobacterium tumefaciens*

3.3.4 Atrazine-degrading gene detection

The atrazine biodegradation potential of isolated pure cultures (ND1, ND2, ND3, and ND4) was determined by detection of known atrazine-degrading genes. The absence of DEA and DIA as degradation by-products indicated that atrazine was degraded via hydrolytic dechlorination. Based on this data, known atrazine chlorohydrolase genes were targeted. *atzA* has been widely detected in gram-negative species, such as those in the genera of *Pesudomonas*, *Pseudaminobacter*, and *Agrobacterium* whereas *trzN* has been reported in gram-positive species (Strong et al., 2002; Satsuma, 2006). Since the studied mixed cultures comprised gram-positive and gram-negative bacteria, both *atzA* and *trzN* were examined.

The *atzA* gene was detected in ND2 and ND4, while *trzN* was not detected in any isolates from this study (Figure 3-3). Sequencing of amplified products of *atzA* revealed 100% similarity to a published sequence of PADP (GenBank accession number: U55933). Based on these results, *Klebsiella ornithinolytica* ND2 and *Agrobacterium tumefaciens* ND4 contained the atrazine chlorohydrolase gene, which could initiate atrazine biodegradation while *Alcaligenes faecalis* ND1 and *Bacillus megaterium* ND3 did not have either of the known atrazine chlorohydrolase genes. To

the best of our knowledge, an atrazine-degrading gene in *Klebsiella ornithinolytica* has never been reported.

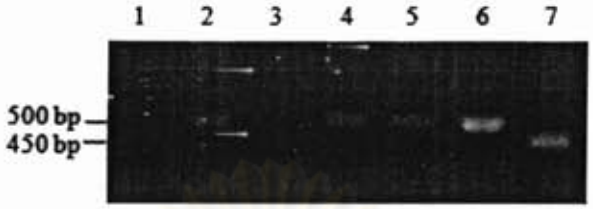


Figure 3-3 Atrazine-degrading gene amplification of isolates

Lane 1: *atzA* of ND1, Lane 2: *atzA* of ND2, Lane 3: *trzN* of ND3, Lane 4: *atzA* of ND4, Lane 5: *atzA* of J14a (positive control), Lane 6: *atzA* of PADP (positive control), Lane 7: *trzN* of TC1 (positive control)

3.4 Summary

The novel stable mixed cultures could be used for bioremediating crop fields contaminated with atrazine. The mixed cultures degraded atrazine approximately 33-51% within 7 d. Four isolates designated ND1, ND2, ND3, and ND4, were purified from the mixed cultures and identified based on sequence analysis of the 16S rRNA gene as *Alcaligenes faecalis*, *Klebsiella ornithinolytica*, *Bacillus megaterium*, and *Agrobacterium tumefaciens*, respectively. An atrazine-degrading gene, *atzA*, was present in ND2 and ND4. This is the first discovery of the *atzA* gene in *Klebsiella ornithinolytica*.

CHAPTER IV

EFFECT OF CELL-TO-MATRIX RATIO IN POLYVINYL ALCOHOL IMMOBILIZED PURE AND MIXED CULTURES ON ATRAZINE DEGRADATION

4.1 Introduction

Atrazine has been applied to control broad-leaf weeds for crops in many countries, such as the United States and Australia (Gianessi, 1987; Vancov et al., 2005). A number of studies found atrazine in surface water, groundwater, and soil (Wauchope, 1978; Jayachandran et al., 1994). Atrazine concentrations above allowable contaminant levels for drinking water of 0.1 and 3 μg/L in Europe and the United States, respectively, have been frequently detected (Rousseaux et al., 2003).

Biodegradation is one of the effective remediation methods of atrazine in environment. Several studies examined atrazine degradation by mixed and pure cultures in either soil or water (Mandelbaum et al., 1995; Alvey and Crowley, 1996; Struthers et al., 1998; Kodama et al., 2001; Strong et al., 2002; Park et al., 2003; Aislabie et al., 2005; Smith et al., 2005). These studies reported different degradation efficiencies. There are many factors influencing the survival of microorganisms and their degradation efficiencies, such as predation and competition from other microorganisms and environmental stresses (van Veen et al., 1997). These issues can be improved by using immobilized cells.

Cell immobilization is a promising technique, which enhances both biological and physical stabilities of bioaugmented microorganisms (Cochet et al., 1990; Dervakos and Webb, 1991). The immobilization matrix can alleviate

physicochemical challenges, such as temperature, pH, and toxic substances. Several studies reported that immobilized cells provide better waste treatment performance than free cells (Uchiyama et al., 1995; Murakami-Nitta et al., 2003; Cunningham et al., 2004). However, in some cases, immobilized cell systems have a limitation on substrate diffusion (Kim et al., 2001; Song et al., 2005).

Gentry et al. (2004) reviewed several applications of immobilized cells using various types of immobilization materials. Based on this review, PVA, a synthetic organic polymer, has been widely used because of its high mechanical strength, durability, and no negative effect to both microorganisms and environment. PPVA technique is a preparation method, which provides durable immobilized cell beads and low damage to microorganisms (Chen and Lin, 1994).

Successful applications of PPVA immobilized cells for removing nitrogen (Chen and Lin, 1994), carbon and nitrogen (Chen et al., 1998; Chen et al., 2000), di-n-butyl phthalate (Jianlong et al., 1997), azo dye (Chen et al., 2003a), and 2-methylnaphthalene (Sharanagouda and Karegoudar, 2002) have been reported. Previous research has focused on contaminant degradation ability, growth conditions (pH, temperature, carbon source, and nitrogen source), and operating conditions (contaminant concentration and hydraulic retention time) (Chen and Lin, 1994; Chen et al., 1998; Chen et al., 2003b; Wu et al., 2005). However, cell-to-matrix (PVA) ratio is another important factor since it could affect substrate diffusivity and degradation ability of immobilized cells (Kim et al., 2001). Currently, there are no published results on the cell-to-matrix ratio effect of PPVA immobilized cells on contaminant removal.

The purpose of the work presented in this chapter was to determine the effect of the cell-to-PPVA matrix ratio of immobilized pure and mixed cultures on atrazine degradation in a batch system. Free (non-immobilized) pure and mixed cultures were included in the atrazine degradation test for comparative purpose. In addition, an existing PPVA cell immobilization procedure was modified to achieve more durable immobilized cell beads. The optimum immobilized cell preparation conditions were determined based on the matrix stability, cell viability, and morphological observation of the cell and matrix.

4.2 Materials and Methods

4.2.1 Chemicals

Atrazine (98% purity) was purchased from Sigma Chemical Co., MO, USA. PVA (99.0-99.8% fully hydrolyzed, molecular weight 77,000-79,000) was obtained from J. T. Baker, NJ, USA. All chemicals for bacterial medium preparation and atrazine analysis were analytical and high-performance liquid chromatography (HPLC) grades, respectively and were purchased from VWR International Co., PA, USA.

4.2.2 Microorganisms and cultural conditions

J14a and a mixed culture (MC) were the atrazine-degrading cultures used in this study. J14a was obtained from the National Soil Tilth Laboratory, Ames, IA, USA (Struthers et al., 1998). The MC was enriched from atrazine contaminated soil collected from a field site in Oakes, North Dakota, USA. The isolation procedure

followed Radosevich et al. (1995) and described in the previous chapter. Note that the mixed culture used in this part was growing in BSMA1 (which was called MC1 in the previous chapter). Both J14a and MC were cultivated in nutrient broth spiked with atrazine of 1.5 mg/L (NBA).

4.2.3 PPVA cell immobilization procedure

Initially, an existing PPVA immobilization process by Chen and Lin (1994) was used for cell immobilization. The PPVA immobilization procedure consisted of two steps: spherical bead formation and hardening. First, the PVA solution was dropped into stirred boric acid leading to PVA-boron cross-linking for spherical bead formation. Second, the beads were taken out of the boric acid solution and placed in a sodium phosphate solution allowing PVA phosphorylation for bead surface hardening. Following the procedure of Chen and Lin (1994) resulted in PVA gel bead agglomeration during the PVA-boron cross-linking in the first step. Stirring the boric acid while dropping the PVA-cell mixture as suggested by Chen and Lin (1994) caused more agglomeration. It was noted that when the PVA solution droplets remained in boric acid without touching one another for 4 to 5 sec, the matrices would harden and no longer stick to each other. To solve the agglomeration problem, a modified PPVA immobilization procedure was developed.

The modified cell immobilization procedure is as follows. The late exponential phase cells were centrifuged at 4,000×g for 10 min. The cell pellet was then resuspended in deionized (DI) water (Kim et al., 2001). The concentration of the cell suspension was approximately 20 mg cells (represented by suspended solids)/mL. PVA was dissolved in DI water by heating at 80°C. The PVA solution was cooled to

room temperature and then mixed with the cell suspension. In the final mixture, the PVA concentration was controlled at 10% (w/v) and the amount of cells was varied as described in section 4.2.7. The mixture was dropped at 2 mL of PVA-cell mixture/min (bead size of 6 mm) into a saturated boric acid solution in a 1-liter cylinder and remained in the solution for 30 to 45 min to form spherical beads. The formed hydrogel beads were then soaked in a 1.0 M sodium orthophosphate solution at pH 7 for 60 min for hardening. The gel beads were washed in DI water, and then stored in NBA at 4°C.

The modified procedure differs from Chen and Lin (1994) procedure in terms of the lack of mixing during the PVA-boron cross-linking as well as the bead contact times with boric acid and phosphate solutions, which were chosen based on the results of the stability tests. The effect of these modifications on the bead characteristics was not known. However, mechanical and chemical stability tests described in section 4.2.4 were conducted to address this issue.

4.2.4 PPVA hydrogel stability test

PPVA beads produced from different combinations of boric acid contact time and phosphate solution contact time were tested for their mechanical and chemical stabilities. The contact times for both boric acid and phosphate solution were varied at 30, 60, and 120 min (3×3 experimental design). In the result and discussion section, abbreviations are used to represent the 9 conditions: B30P30; B30P60; B30P120; B60P30; B60P60; B60P120; B120P30; B120P60; B120P120. For example, B30P60 is an abbreviation for the boric acid contact time of 30 min and the phosphate solution contact time of 60 min.

The durability of the immobilized matrix was examined by adapting mechanical and chemical stability tests from prior research (Wang et al., 2005). The mechanical stability test was performed by shaking the PPVA matrices in DI water containing glass beads for 15 d. For the chemical stability, the matrices were shaken in buffer solutions (pH of 5, 7, and 9) and various salt solutions (1 N NaCl, 1 N KCl, and 1 N $NaHCO_3$). The stability was indicated by the percentage of the bead breakage. The shortest boric acid and phosphate contact times, which provided durable matrices in terms of mechanical and chemical strengths, were chosen as the immobilized cell preparation conditions.

4.2.5 PPVA immobilized cell viability test

The PPVA immobilized cells were examined for their viability using the colony plate count and a commercial fluorescence based assay. Although PVA is not harmful to microorganisms (Chen and Lin, 1994; Chang and Tseng, 1998; Gentry et al., 2004), the other immobilization chemicals, particularly the boric acid, could kill bacteria (Wu and Wisecarver, 1992). The effect of immobilization chemicals on cell viability was determined using the plate count technique. A fluorescence based viability assay was used to confirm the viability of the immobilized cells in PPVA matrices.

J14a and MC were grown in nutrient broth (NB) for 24 hr (the beginning of stationary phase). For the colony plate count, each culture was divided into two 50-mL subsamples: non chemically-exposed and chemically-exposed cells. In the first subsample (the non-chemical exposed cells), cultures were centrifuged at 4,000×g for 5 min and were resuspended in 5-mL DI water. The resuspended culture was serially

diluted and plated onto nutrient agar (NA) plates. A control (sterile DI water) was also tested.

In the second subsample (the chemically-exposed cells), cultures were centrifuged at 4,000×g for 5 min, resuspended in a 5-mL sterile boric acid solution, and allowed to stand at room temperature for 30 min. The bacterial cells were centrifuged at 4,000×g for 5 min and the supernatant containing the boric acid solution was removed. The cells were resuspended in a 5-mL sterile phosphate solution and allowed to stand at room temperature for 60 min. The cells were centrifuged at 4,000×g for 5 min. The supernatant was decanted, the cells were resuspended in 5-mL sterile DI water and serial dilutions were plated onto NA. Plates were incubated at 30°C for 24 hr, after which the numbers of colony forming units were enumerated.

For the fluorescence based viability assay, the PVA-cell mixtures were immobilized as a thin layer onto slides. Five samples (immobilized and free J14a, immobilized and free MC, and no cells) were stained using a LIVE/DEAD® BacLight™ bacterial viability kit (Molecular Probes, OR, USA). This kit was adopted because it has been used successfully in several environmental applications (Boulos et al., 1999; Ramalho et al., 2001; Cunningham et al., 2004). The procedure followed the manufacturer instruction. The samples were washed with 0.85% NaCl. Washed samples were mixed with a combination of 6 μM SYTO9 stain and 30 μM propidium iodide, and incubated at room temperature in the dark for 15 min. The average number of live and dead cells present was determined by counting 20 microscopic fields under 1,000× magnification, using a microscope with an epifluorescence attachment (BX61™, Olympus, PA, USA).

4.2.6 PPVA immobilized cell morphological observation

The PPVA immobilized J14a and MC morphological observation procedure was adapted from Chen et al. (1998). The PPVA beads were fixed for 24 hr using 2.5% glutaraldehyde. The fixed beads were then dehydrated using different ethanol concentrations of 30, 50, 70, 90, and 100% (w/v). The dehydrated beads were then critical point dried using an autosamdri-810 drier with liquid carbon dioxide as a transitional fluid. After that, the beads were cut, attached to aluminum mounts by silver paint, coated with gold using a Balzers SCD 030 sputter coater, and examined using a JEOL JSM-6300 scanning electron microscope (SEM).

4.2.7 Atrazine degradation test in batch system

An atrazine degradation test in a batch system focused on the effect of cell-to-matrix ratio on atrazine degradation efficiency to determine the optimum cell density inside the beads. The ratio was varied at 3.5, 6.7, and 20 mg dry cells/mL matrix. The cell-to-matrix ratio was calculated from the total mass of cell divided by the volume of matrix (Table 4-1). As an example, for the cell-to-matrix ratio of 3.5 mg dry cells/mL matrix, total cell mass of 200 mg (10 mL) was mixed with the PVA solution of 57 mL. It is noted that different amounts of PVA were used to obtain the same final PVA concentration (10% w/v) in the mixture. The cell-to-matrix ratios out of this range make the preparation of immobilized cells difficult. Lower ratios (lower cell density) cause a floatation problem in the PVA-boron cross-linking step. Higher ratios (higher cell density) need more concentrate PVA solutions, which could cause inhomogeneous mixing of the PVA solution and cell suspension. The degradation test

was also conducted on immobilized dead (autoclaved) mixed culture cells at the cell-to-matrix ratio of 3.5 mg/mL and a system without cells as controls.

Table 4-1 Composition of PPVA immobilized cell beads

Cell-to-matrix ratio (mg dry cells/ mL matrix)	Total cell mass (mg dry cells)	Volume of cells (mL)	Volume of PVA solution (mL)	Volume of mixture (mL)	Amount of PVA (g)
3.5	200	10	57	67	6.7
6.7	200	10	30	40	4
20	200	10	10	20	2

Three batch experiments were consecutively carried out in 500-mL Erlenmeyer flasks containing 200 mg bacterial cells (measured as suspended solids) and 100 mL of basal salt media amended with atrazine. One-liter media consisted of K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3 \cdot H_2O$ 10 mg, $CaCl_2 \cdot H_2O$ 10 mg, $MnCl_2$ 0.1 mg, $ZnSO_4$ 0.01 mg, glucose 1.0 g, $(NH_4)_2SO_4$ 0.5 g, and atrazine 1.5 mg in a pH 6.8 buffer solution. Ten batch reactors consisted of four J14a systems (immobilized cells at 3.5, 6.7, and 20 mg dry cells/mL matrix, and free cell), four MC systems (immobilized cells at 3.5, 6.7, and 20 mg dry cells/mL matrix, and free cell) and two control systems (dead immobilized cells and no cell). The flasks were shaken by using a wrist shaker at room temperature. The atrazine concentration was measured daily for 5 d.

4.2.8 Atrazine analysis

A solid phase extraction technique using polymeric sorbent of 200 mg in 6 mL cartridges (StrataX, Phenomenex, CA, USA) was applied for atrazine extraction. The cartridge was prewashed and conditioned using 6 mL of ethyl acetate and 6 mL of methanol, respectively. After loading a sample and drying the cartridge under

vacuum, the cartridge was eluted with 6 mL of ethyl acetate through gravity flow. Then, the extract was concentrated to 1 mL under a stream of dry nitrogen.

The concentrated sample was analyzed on a Hewlett-Packard 5890 gas chromatograph (GC) with a 5973 mass selective detector (MSD). One-microliter sample was injected. The GC conditions were: splitless injection and injection port temperature of 220°C. The temperature of GC oven were held at 50°C for 1 min and increased to 280°C at a rate of 18°C/min then retained for 1 min. The selective ion monitoring mode was used. Two ions of atrazine at m/z = 200 and 215 were chosen as the quantitation ions.

4.3 Results and Discussion

4.3.1 PPVA hydrogel stability

During the mechanical stability test, there was no breakage of the matrices under any of the test conditions. However, the matrices for B30P30 were very soft after soaking in DI water for 1 hr whereas firm matrices were observed for B30P60. This indicates that B30P30 was insufficient for phosphorylation. During the chemical stability test, there was no breakage of the matrices even when the matrices were shaken with glass beads for more than 1 month. All conditions except the B30P30 provided strong matrices in terms of mechanical and chemical resistances. The modified cell immobilization procedure did not affect the matrix stability. Similar to previous research, PPVA immobilized cells were used for 6 months without matrix abrasion problem (Chen et al., 2003).

4.3.2 PPVA immobilized cell viability

The results of the plate count are presented in Table 4-2. Both chemically-exposed J14a and MC counts were slightly less than those of corresponding non chemically-exposed cells. It is possible that some bacteria were killed by the PPVA immobilization chemicals, but the amount was not substantial.

Table 4-2 Enumeration of bacterial colonies

Types of cultures	Viable count (CFU/mL)	
	Subsample without chemical exposure	Subsample with chemical exposure
J14a	3.0×10^{12}	2.6×10^{12}
MC	2.5×10^{12}	2.4×10^{12}

As described in the Materials and Methods section (4.2), the plate count was used to indicate only the effect of the immobilization chemicals on the cells. However, it is possible that the cells experienced physical stresses during the immobilization process. As shown in Figure 4-1, there were both live and dead free and immobilized MC cells. The LIVE/DEAD® assay images of J14a were similar to those of MC (data not shown). Note that the bright green color represented live cells while the bright red color indicated dead ones. The percentages of live cells in free J14a and MC were 54 and 63%, respectively. These proportions of live cells may be expected in a culture that is at the beginning of stationary phase. The percentages of live cells in immobilized J14a and MC were 39 and 41%, respectively. These lower proportions of viable cells under immobilized conditions indicate that immobilization did have a negative effect on cell viability; however, large proportions of live free cells remained viable after immobilization.

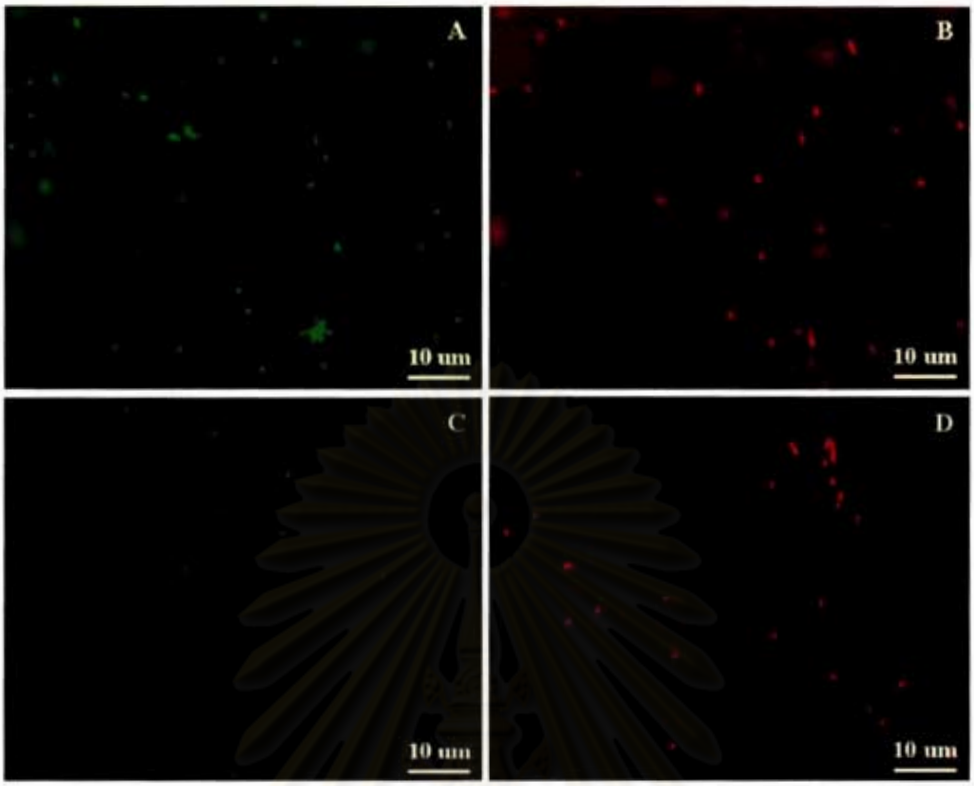


Figure 4-1 Live and dead MC: A) Live immobilized MC in PPVA; B) Dead immobilized MC in PPVA (A and B are from the same frame); C) Live free MC; D) Dead free MC (C and D are from the same frame)

4.3.3 PPVA immobilized cell morphological observation

The diameter of spherical PPVA matrices with cells was around 6-7 mm after soaking in DI water. From visual observation, the matrices appeared dense and lacking permeability. However, based on cross-sectional SEM images presented in Figure 4-2, the PPVA bead microstructure was porous. In addition, the beads appeared to have two layers: interior and exterior. The porous interior layer was formed by the PVA-boron cross-linking whereas the dense exterior shell layer was from PVA phosphorylation. The inside microstructure was similar to those reported for different PVA immobilization processes, such as PVA cryogel structure

(Lozinsky and Plieva, 1998), PVA-boric acid cross-linking (Fang et al., 2004), and PVA-$CaNO_3$ cross-linking (Yujian et al., 2006). The cells were entrapped in both layers (Figures 4-3 and 4-4). The structural configuration was appropriate for cell immobilization. Determined from the SEM results, the exterior layer of the PPVA matrices had encapsulation property while the interior of the bead contained numerous pores for bacteria occupancy. The microstructure of the PPVA matrices with J14a was not shown because it was similar to the MC beads. Therefore, the type of culture did not affect the appearance and microstructure of the beads.

The pores in the bead are necessary for entrapping the cells and transporting substrate and oxygen (Dervakos and Webb, 1991; Lozinsky and Plieva, 1998; Kourkoutas et al., 2004). Figure 4-5 presents the surface of the bead which had a complex mesh structure with pores larger than 2 μm. According to Li et al. (2005a), the beads with 2-μm pores should not have oxygen and substrate transfer problems.

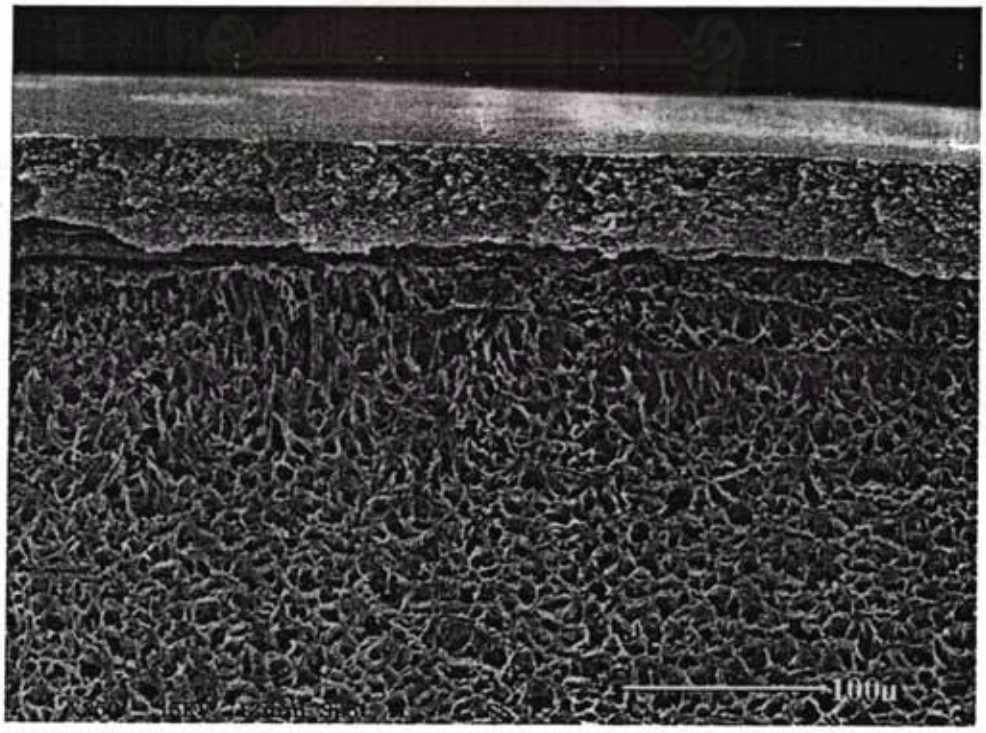


Figure 4-2 PPVA immobilized MC: Cross-section at 250×

Figure 4-3 PPVA immobilized MC: Exterior layer at 3,000×

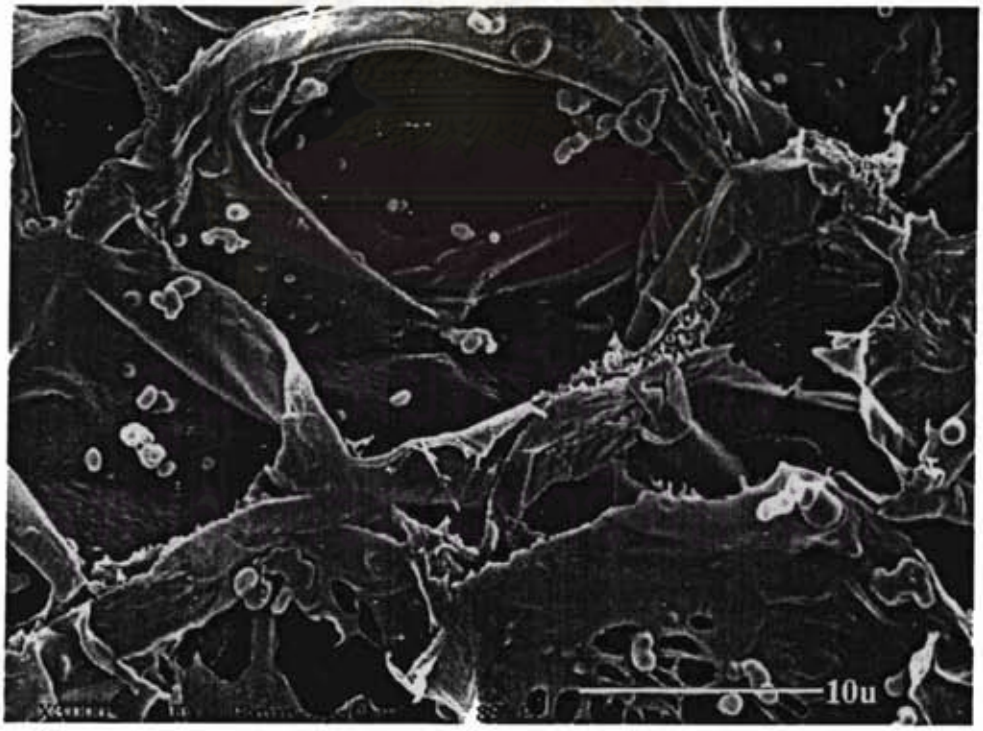


Figure 4-4 PPVA immobilized MC: Interior layer at 3,000×

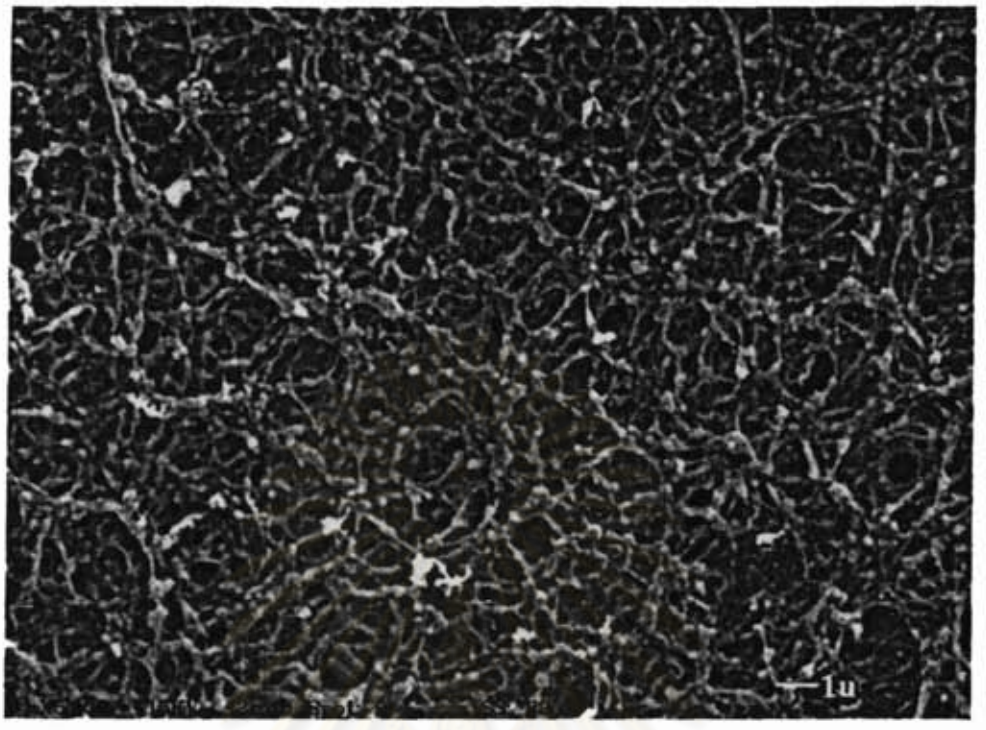


Figure 4-5 PPVA immobilized MC: External surface at 5,000×

4.3.4 Atrazine degradation in batch system

The biodegradation trend and efficiency provided by the immobilized cells in the first batch experiment were different from those in the second and third batch experiments (Figures 4-6 and 4-7). During the first batch experiment, atrazine concentration rapidly decreased within 24 hr and proceeded to reduce gradually there after. At 120 hr, the immobilized J14a degraded the atrazine to between 30 and 80% (Figure 4-6A) of the initial concentration while the immobilized MC degraded atrazine to approximately 30 to 60% (Figure 4-6B) of the initial concentration. It should be noted that the immobilized dead cells (at the cell-to-matrix ratio of 3.5 mg/mL) also removed about 30% of the atrazine mostly in the first 24 hr (Figure 4-6C). This indicates that in addition to biodegradation, adsorption contributed to atrazine removal in the initial 24 hr test period.

In the second and third batch experiments, the degradation results of immobilized cells were similar. The atrazine concentration gradually decreased for both immobilized J14a and MC. At 120 hr, the immobilized J14a degraded between 20 and 50% of the atrazine (Figure 4-7A) while the immobilized MC degraded approximately 20 to 40% of the atrazine (Figure 4-7B). The trends suggested that the degradation by both J14a and MC would continue after 120 hr. There was very low atrazine removal (< 10%) in the immobilized dead cell experiment (Figure 4-7C). This confirms that the atrazine adsorption occurred mostly when the immobilized matrices were first exposed to the compound. For the free cells, both J14a and MC degraded about 30% of the atrazine in all experiments since there was no adsorption in the free cell reactors.

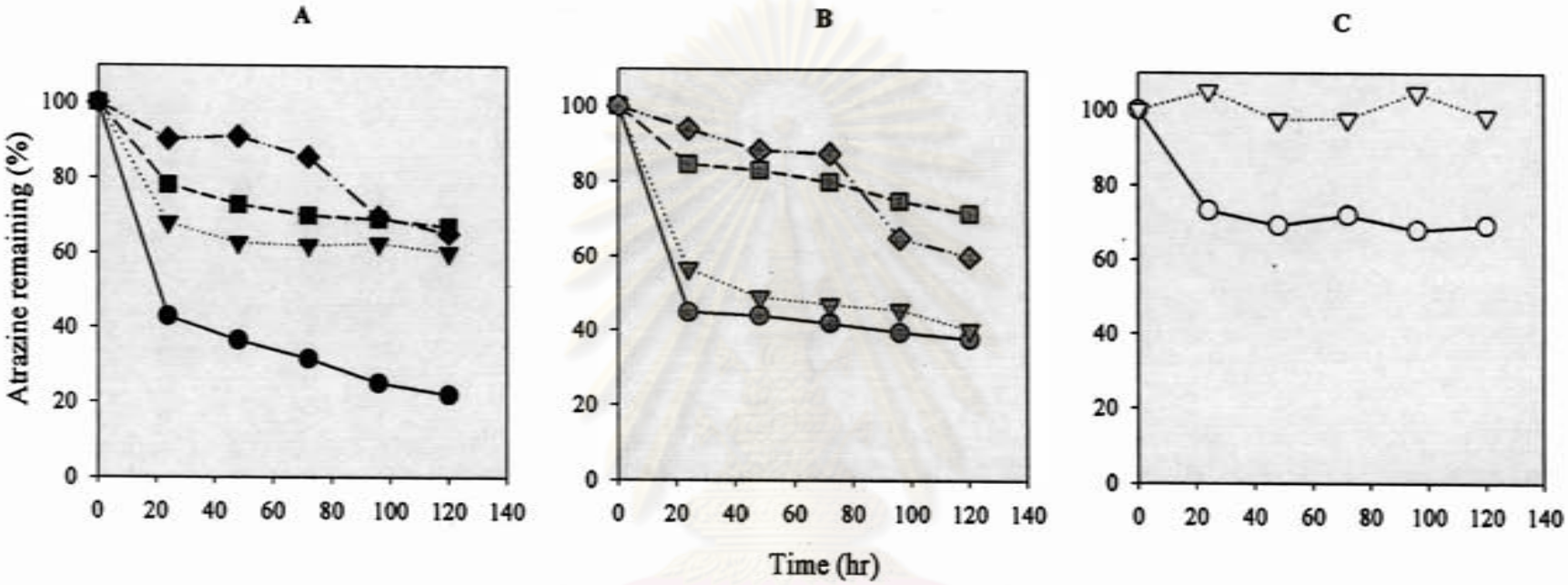


Figure 4-6 Atrazine degradation in the first batch experiment: A) immobilized J14a at 3.5 mg/mL (●), 6.7 mg/mL (▼), 20 mg/mL (■), and free cells (♦); B) immobilized MC at 3.5 mg/mL (●), 6.7 mg/mL (▼), 20 mg/mL (■), and free cells (♦); C) immobilized dead cells (○) and control (▽)

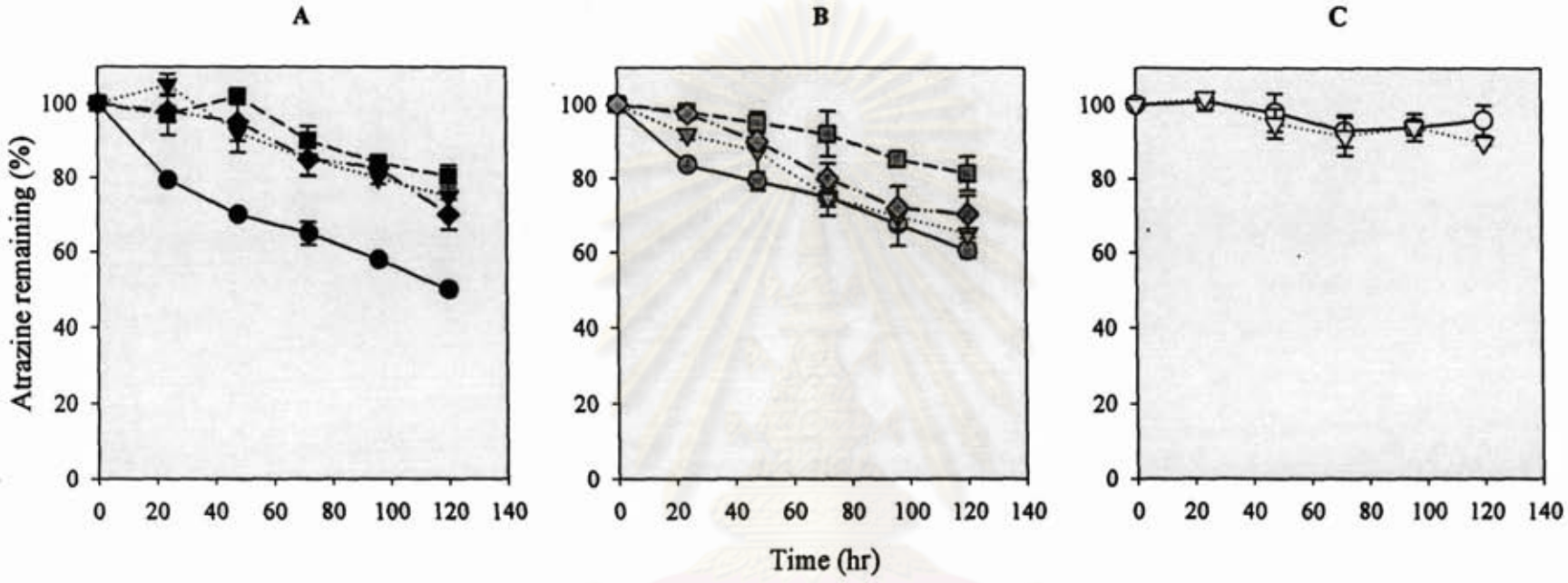


Figure 4-7 Atrazine degradation in the second and third batch experiments: A) immobilized J14a at 3.5 mg/mL (●), 6.7 mg/mL (▼), 20 mg/mL (■), and free cells (♦); B) immobilized MC at 3.5 mg/mL (●), 6.7 mg/mL (▼), 20 mg/mL (■), and free cells (♦); C) immobilized dead cells (○) and control (▽)

If the atrazine adsorption of approximately 30% (Figure 4-6C) is added to the percent atrazine remaining in Figure 4-6A and 4-6B for the data provided by the cell-to-matrix ratio of 3.5 mg/mL, the removal trends of the first experiment are quite linear, similar to the second and third experiments (Figures 4-7A and 4-7B). Although the control experiments were not conducted for the cell-to-matrix ratios of 6.7 and 20 mg/mL, as indicated in the control experiment for the cell-to-matrix ratio of 3.5 mg/mL (Figure 4-6C), the atrazine removal in the first 24 hr was mainly due to adsorption. If the data in the first 24 hr were neglected, the data trends for the cell-to-matrix ratios of 6.7 and 20 mg/mL after 24 hr were similar to those observed in Figures 4-7A and 4-/B. Similar atrazine biodegradation efficiencies from the three batch tests indicated that the immobilized cells were reusable.

The degradation trends in Figures 4-7A and 4-7B agree with zero-order kinetics (Table 4-3). In this study, even though the cell concentration of 2,000 mg/L was inoculated in each batch, different cell-to-matrix ratios provided different biodegradation efficiencies. Lower cell-to-matrix ratios resulted in faster and more biodegradation due to less mass transfer limitation of oxygen and substrates. Previous studies reported lower nitrogen removal efficiencies in systems that had high cell-to-matrix ratios (Kim et al., 2001; Song et al., 2005). Based on the results in Table 4-3, at the most suitable cell-to-matrix ratio (3.5 mg/mL), the immobilized cell systems provided equal or higher atrazine degradation rates than the free cell systems. There are two possible reasons for this observation. Immobilized cells were protected from environmental stresses and/or were metabolically more active compared to free cells. Cassidy et al. (1996) reviewed a number of studies that have reported increased

metabolic activities in immobilized cell systems for different environmental applications.

Table 4-3 Atrazine biodegradation kinetic equations and rates

Culture type	Cell-to-matrix ratio (mg dry cells/mL matrix)	Atrazine biodegradation kinetic equation[a]	R^2	Atrazine biodegradation rate (mg/L/d)
J14a	3.5	y = -0.16x + 1.64	0.94	0.16
	6.7	y = -0.08x + 1.32	0.90	0.08
	20	y = -0.06x + 1.43	0.81	0.06
	Free cell	y = -0.08x + 1.39	0.93	0.08
MC	3.5	y = -0.10x + 1.29	0.95	0.10
	6.7	y = -0.09x + 1.29	0.98	0.09
	20	y = -0.06x + 1.46	0.96	0.06
	Free cell	y = -0.10x + 1.58	0.97	0.10

[a] y = atrazine concentration (mg/L) and x = time (d).

4.4 Summary

The PPVA matrix was proven as a good immobilization material in terms of mechanical and chemical stabilities. The matrices were found unbroken after shaking with glass beads over 15 d under various salt solutions and pH values. The chemicals used in the immobilization process did not substantially affect the viability of the cells. The modified preparation procedure delivered the proper microstructure for cell immobilization. The SEM results revealed the two porous bead layers. The outer layer had less porosity providing an effective structure for cell entrapment. At the proper cell-to-matrix ratio (3.5 mg/mL), the atrazine biodegradation using immobilized J14a and MC was approximately 50 and 40% within 120 hr, respectively.

CHAPTER V

ATRAZINE REMEDIATION IN AGRICULTURAL INFILTRATE BY BIOAUGMENTED POLYVINYL ALCOHOL IMMOBIIZED AND FREE *Agrobacterium radiobacter* J14a: A SAND COLUMN STUDY

5.1 Introduction

Atrazine has been applied to control broad-leaf weeds for crop production. Although atrazine is applied to surface soil, off-site movement has been well documented (Gannon, 1992; Koskinen and Clay, 1997). During irrigations or rain events, applied atrazine transports via infiltrate and contaminates groundwater. Several studies reported that atrazine disrupts endocrine hormone metabolism (Moore and Lower, 2001; Friedman, 2002; Hayes et al, 2002) and causes cancer (Tchounwou et al., 2001). As a result, agricultural infiltrate containing atrazine becomes one of the most problematic environmental issues.

Biodegradation has been known as an effective technique for remediating organic contaminants since it leaves behind less toxic end products. In some cases, biodegradation by indigenous microorganisms cannot cope with all contaminants or takes long time. Microbial bioaugmentation, an addition of sufficient contaminant-degrading microorganisms, can be applied to solve the problems.

Among a number of studies on microbial bioaugmentation for atrazine remediation, different atrazine degradation efficiencies were reported (Alvey and Crowley, 1996; Struthers, et al., 1998). There are many factors influencing the

survival of bioaugmented microorganisms and their contaminant degradation efficiencies, such as predation and competition of indigenous microorganisms, and unsuitable environment. Furthermore, bioaugmented microorganisms could get flushed off the sites at rapid infiltration rates (Madsen and Alexander, 1982; Hekman et al., 1994). Cell immobilization is a potential alternative to minimize these problems. Immobilization matrices can alleviate environmental stresses (Cochet et al., 1990; Dervakos and Webb, 1991). This technique also provides high biomass concentrations and prevention of off-site cell transport.

In the previous chapter, atrazine biodegradation by J14a immobilized in PPVA matrix was performed in a batch experiment. J14a has been known as an effective atrazine-degrading bacterium and successfully applied for the atrazine removal from wastewater and soil in several studies (Struthers et al., 1998; Protzman et al., 1999). PPVA was chosen as an immobilization matrix since it is durable and has no negative effect to microorganisms and environment (Chen and Lin, 1994; Gentry et al., 2004). Results presented in the previous chapter indicated that the PPVA immobilized J14a degrades atrazine well in the batch experiment and has potential for remediating atrazine-contaminated infiltrate.

The main objective of the work described in this chapter was to examine the use of PPVA immobilized J14a for remediating atrazine in agricultural infiltrate in a sand column setup. The free cell experiment was also conducted for comparative purpose. The effects of cell loading and infiltration rate on degradation of atrazine and the loss of bioaugmented atrazine-degrading culture were investigated.

5.2 Materials and Methods

5.2.1 Chemicals

Atrazine, DEA, DIA, and HA were purchased from Sigma Chemical Co., MO, USA. PVA (99.0-99.8% fully hydrolyzed, molecular weight 77,000-79,000, J. T. Baker) and all other chemicals for bacterial medium preparation, synthetic agricultural infiltrate, PPVA immobilization, and atrazine analysis were analytical and HPLC grades from VWR International Co., PA, USA.

5.2.2 Bacterial strain and cultural condition

J14a was obtained from the National Soil Tilth Laboratory, Ames, IA, USA (Struthers et al., 1998). J14a was subcultured every 7 d in a buffered bacterial medium (20 mM sodium phosphate buffer at pH 6.8) containing (per liter): atrazine 20 mg, glucose 1.0 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3 \cdot H_2O$ 10 mg, $CaCl_2 \cdot H_2O$ 10 mg, $MnCl_2$ 0.1 mg, and $ZnSO_4$ 0.01 mg.

5.2.3 PPVA cell immobilization procedure

J14a was immobilized using the PPVA technique similar to the procedure described in Chapter 4, Section 4.2.3. For preparing the immobilized cells, high mass of the concentrated bacterial cells was needed; therefore, J14a was subcultured into nutrient broth spiked with atrazine at 20 mg/L for 2 d. The late exponential phase cells were then taken and centrifuged at 4,500×g for 10 min. The cell pellet was then resuspended in a 20 mM sodium phosphate buffer solution. The concentration of the

initial cell suspension was approximately 20 mg dry cells (represented by suspended solids)/ mL.

PVA was dissolved and mixed with cells to obtain the final PVA concentration of 10% (w/v) and the cell-to-matrix (PVA) ratio of 3.5 mg dry cells/ mL matrix. The mixture was slowly dropped into a saturated boric acid solution in a 1-liter cylinder for 30 min to form spherical beads. The formed hydrogel beads were then soaked in a 1 M sodium phosphate solution (pH 7) for 60 min for hardening. The gel beads were washed in DI water, and then stored in a 20 mM sodium phosphate solution (pH 6.8) at 4°C.

5.2.4 Synthetic agricultural infiltrate, sand, and column preparations

5.2.4.1 Synthetic agricultural infiltrate preparation

Synthetic agricultural infiltrate was prepared in the same manner as the bacterial medium (described in Section 5.2.2) except the addition of 1.5 mg/L atrazine. The infiltrate was sterilized before applying to sand columns.

5.2.4.2 Sand preparation

Industrial silica-quartz sand from Le Sueur, MN (Unimin Corporation, CT, USA) was used. The sand was washed with tap water and dried at 105°C for 24 hr. The cleaned sand was sieved to obtain the grain sizes between 0.25 to 0.42 mm (US standard sieves number 60 and 40). The sieved sand was autoclaved at 121°C for 30 min three times within three consecutive days. The void ratio (v/v) of sieved sand loosely packed in a 400-mL graduate cylinder was 0.30 (a void volume of 120 mL).

5.2.4.3 Column setup

A sand column was set up as shown in Figure 5-1. The entire sand column was modeled a top soil. It was 6.3 cm in diameter and 23 cm in length. It had an effluent sampling port at the bottom. All columns were rinsed with 70% isopropanol and autoclaved de-ionized water, respectively before used. The sterile sand of 400 mL was filled in the control columns (tracers) while the sand of 280 to 400 mL was mixed with the cells and then packed in the bioaugmented columns to obtain the same total empty bed volume (detail described in the next section).

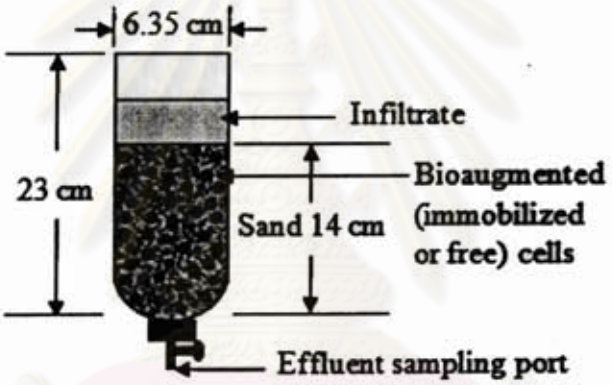


Figure5-1 A sand column setup

5.2.5 Chloride and atrazine tracer tests

Before starting atrazine bioremediation test, chloride and atrazine tracer tests were carried out to determine hydraulic (chloride tracer) and atrazine (atrazine tracer) transports in the sand column setup. All tests were duplicated. The sand columns designated A1 and A2, were packed as described in Table 5-1. The columns were operated as follows.

The sand column was first filled up with a 0.01 M $CaCl_2$ solution. For the chloride tracer test, a 0.05 M $CaCl_2$ solution was then gravity-flowed through the

column at the rates of 30, 90, and 180 mL/d (corresponding to the infiltration rates of 1, 3, and 6 cm/d) to obtain the actual, high, and critical (extremely high) infiltration rates, respectively. The infiltrate was continuously applied as a step input at every 8-hour interval for 10, 30, and 60 mL for the actual, high, and critical flow rates, respectively. The test was run for 5 pore volumes (PV) (total pore water volume of 600 mL). The effluent samples were taken at every 0.25 PV from the columns at the infiltration rates of 1 and 3 cm/d and every 0.50 PV from the column at the infiltration rate of 6 cm/d. The samples were measured for electrical conductivity representing chloride concentration using a conductivity meter (YSI Model 32, YSI Incorporated, OH, USA).

For the atrazine tracer test, the sand column was filled up with a 0.01 M $CaCl_2$ solution. Then, an atrazine solution of 1.5 mg/L was passed through the column in the same manner as the chloride tracer test. The effluent samples were taken at every 0.25 or 0.50 PV for measuring atrazine concentration.

The retardation of atrazine was estimated for characterizing the atrazine transport and sorption. Retardation factor (R_f) of the column tests was calculated by a modified method from the moment analysis as shown in equation 5-1. $PV_{atrazine}$ and $PV_{chloride}$ are pore volumes of atrazine (solute) and chloride (water) at the relative eluted atrazine or chloride concentration (C/C_0) of 0.5.

$$R_f = PV_{atrazine}/PV_{chloride} \qquad (5\text{-}1)$$

Table 5-1 Descriptions of sand columns (A, B, C, and D) and their components

Column set	Column number	Column description (cell type)	Cell loading (mg dry cells/L empty bed volume)	Cell mass (mg dry cells)	Bioaugmented volume* (mL)	Dry sand (mL)	Total empty bed volume (mL)
A	A1	Chloride tracer (no cell)	0	0	0	400	400
	A2	Atrazine tracer (no cell)	0	0	0	400	400
B	B1	Immobilized dead cells	300	120	40	360	400
	B2	Immobilized dead cells	600	240	80	320	400
	B3	Immobilized dead cells	900	360	120	280	400
C	C1	Immobilized cells	300	120	40	360	400
	C2	Immobilized cells	600	240	80	320	400
	C3	Immobilized cells	900	360	120	280	400
D	D1	Free cells	300	120	N/A**	400	400
	D2	Free cells	600	240	N/A**	400	400
	D3	Free cells	900	360	N/A**	400	400

* Volume of J14a and matrices, ** Volume of J14a was negligible.

5.2.6 Atrazine bioremediation test in column system

Duplicate atrazine bioremediation tests in sand columns were performed. The effects of the cell loading and infiltration rate on the atrazine degradation efficiencies and culture losses were studied to determine the optimum application for site remediation. Three sets of sand columns including set B (immobilized dead cells), set C (immobilized cells), and set D (free cells), were packed as described in Table 5-1. Each set of columns comprised 3 columns at the cell loadings of 300, 600, and 900 mg dry cells/L empty bed volume. The columns were operated as follows.

The columns were filled up with a 20 mM sodium phosphate solution (pH of 6.8). The synthetic agricultural infiltrate was then gravity-flowed through the columns at the same rates with the tracer tests (infiltration rates of 1, 3, and 6 cm/d). The effluent samples at every 0.25 or 0.50 PV (the same as the tracer tests) were measured for atrazine and its intermediate metabolite concentrations while the samples at every 1 PV were determined for the number of viable J14a cells using a viable plate count technique. All columns were tested for 5 PV. The operation of selected columns was continued to 50 PV for a long-term performance monitoring.

5.2.7 Analytical methods

5.2.7.1 Atrazine and metabolite analysis

The analytical methods used for atrazine and its metabolites including DEA, DIA, and HA were modified from D'Archivio et al. (2007). A solid phase extraction using polymeric sorbent of 200 mg in 6 mL cartridge (StrataX, Phenomenex, CA, USA) was applied for atrazine and metabolite extraction. The

cartridge was prewashed and conditioned using ethyl acetate of 6 mL and methanol of 6 mL, respectively. The cartridge was then washed with DI water of 6 mL. After loading a sample and drying the cartridge under vacuum condition, the cartridge was eluted with acetonitrile-methanol (1:1, v/v) of 6 mL through gravity flow. Then, the extract was evaporated to dryness under a gentle stream of dry nitrogen. The dry residue was dissolved in water-acetonitrile (1:1, v/v) of 500 μL.

The extract was analyzed for atrazine and its metabolites using a Hewlett Packard 1100 series HPLC equipped with a C18 reverse phase column (Jupiter, Phenomenex, US) at a UV wavelength of 220 nm. The isocratic mobile phase of water-acetonitrile (1:1) at a flow rate of 1 mL/min was used.

5.2.7.2 Viable plate count

The bacterial loss from the column system was determined based on number of viable bacteria in the effluent samples. Each effluent sample was serially diluted and spread onto a selective bacterial medium agar. The agar formulation was the synthetic agricultural infiltrate added with agar of 2% (w/v). Bacterial colonies were counted after a 48-hr incubation at 30°C.

5.2.8 Data analysis

Mass of atrazine in effluent samples was statistically analyzed using JMP IN® 5.1.2 (SAS, NC, USA). The data were analyzed using a multiple regression model and examined for the significance of the atrazine mass difference between test conditions with analysis of variance and *t* test for $p < 0.05$.

5.3 Results and Discussion

5.3.1 Chloride and atrazine tracer tests

Figure 5-2 shows results of chloride and atrazine tracer tests. All breakthrough curves gradually increased just after 0.25 PV and reached a plateau at approximately 3 PV. Theoretically, in one dimensional (advection-dispersion) transport, C/C_0 of a conservative solute (chloride) of 0.5 should be present at 1 PV. In Figure 5-2, C/C_0 of 0.5 at the infiltration rates of 1, 3, and 6 cm/d occurred at 0.7, 0.8, and 1.3 PV, respectively. These deviations from the ideal case of the conservative solute (hydraulic) transport were influenced by the dispersion and diffusion processes governed by step inputs.

The breakthrough curve of the atrazine tracer was compared with the chloride tracer to determine the effects of the diffusion, sorption, and degradation processes. As shown in Figure 5-2, the atrazine breakthrough curves were similar to those of the chloride curves. There were no or low retardation and degradation. In addition, the total masses of both tracers in the effluents (0-5 PV) were statistically compared and found not to be significantly different.

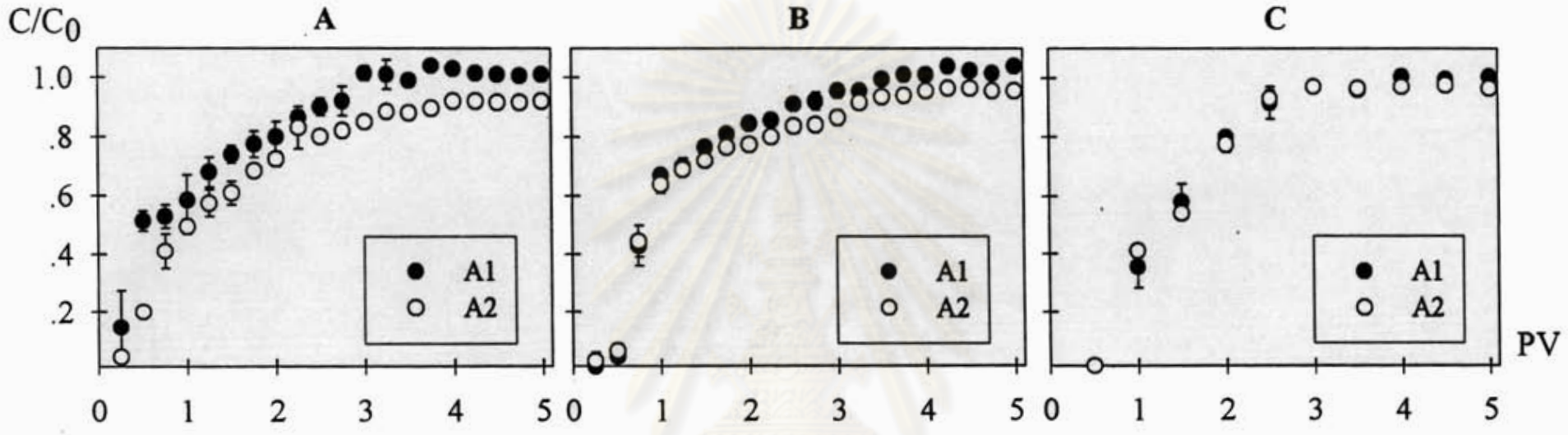


Figure 5-2 Breakthrough curves of chloride (A1) and atrazine (A2) tracer tests at the infiltration rates of A) 1 cm/d, B) 3 cm/d, and C) 6 cm/d

5.3.2 Atrazine transport and removal by the immobilized dead cells

The experiment using immobilized dead cells was conducted to examine the adsorption capacity of the PPVA matrix and dead cells in combination. The atrazine breakthrough curves B1, B2, and B3 (corresponding to the columns at the cell loadings of 300, 600, and 900 mg dry cells/L, respectively) in Figure 5-3 are the results of the immobilized dead cells at the infiltration rates of 1, 3, and 6 cm/d. The trend of these curves was similar to that of the tracer tests; however, a shift in all breakthrough curves was observed. This indicates that the matrix and/or immobilized dead cells were able to adsorb atrazine leading to noticeable retardation. Similarly, the result of the batch experiment described in the previous chapter also reported the atrazine adsorption capacity of the matrix and immobilized dead cells together. The R_f values are presented in Table 5-2. Higher dead cell loadings (also higher amounts of the PPVA matrix) provided higher retardation capacities.

The significance of adsorption was statistically analyzed by comparing total atrazine mass in the effluents (0-5 PV) from the tracer and immobilized dead cell tests at different cell loadings and infiltration rates. The atrazine adsorption by the matrix and immobilized dead cells was significant ($p < 0.0001$). Both infiltration rate ($p = 0.0014$) and cell loading ($p = 0.0001$) were influencing factors.

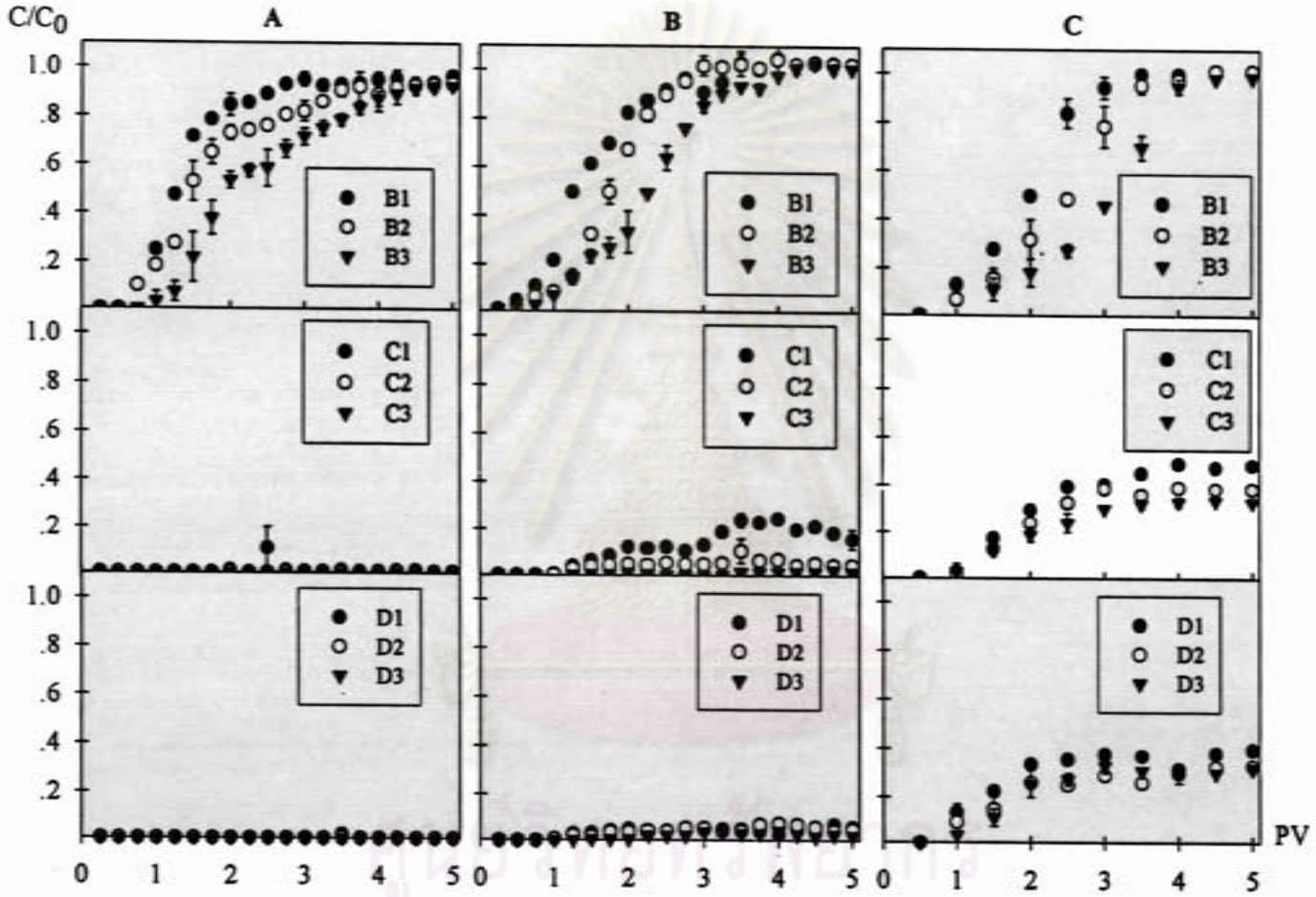


Figure 5-3 Breakthrough curves of atrazine bioremediation using the immobilized dead cells (B1, B2, and B3), immobilized cells (C1, C2, and C3), and free cells (D1, D2, and D3) at the infiltration rates of A) 1 cm/d, B) 3 cm/d, and C) 6 cm/d

Table 5-2 Retardation factors of atrazine transport

Column number	Column description	Retardation factor (R_f) at infiltration rate of		
		1 cm/d	3 cm/d	6 cm/d
A2	Atrazine tracer	1.29	1.00	1.00
B1	Immobilized dead cells at cell loading of 300 mg/L	1.79	1.56	1.54
B2	Immobilized dead cells at cell loading of 600 mg/L	2.14	2.19	1.92
B3	Immobilized dead cells at cell loading of 900 mg/L	2.71	2.81	2.31

5.3.3 Effects of infiltration rate and cell (J14a) loading on atrazine bioremediation test

The breakthrough curves C1 and D1, C2 and D2, and C3 and D3 are the results of atrazine bioremediation from the columns of the immobilized and free cells at the cell loadings of 300, 600, and 900 mg dry cells/L, respectively (Figure 5-3). In all experiments at the infiltration rate of 1 cm/d (Figure 5-3A), atrazine was not substantially detected even at the end of the test (5 PV) while atrazine was detected after 1 PV in the experiments at the infiltration rates of 3 and 6 cm/d (Figures 5-3B and 5-3C). At 5 PV, C/C_o of 0.03 to 0.20 and 0.30 to 0.50 was observed at the infiltration rates of 3 and 6 cm/d, respectively.

The atrazine removal by the free cells was slightly higher than that by the immobilized cells. This is likely because the atrazine transport through the immobilization matrices to the immobilized cells might take longer time than the direct atrazine transport to the free cells. Nevertheless, the statistical analysis showed that the atrazine removal by the immobilized and free cells was not significantly different ($p = 0.8222$). Therefore, J14a in either immobilized or free cell forms was efficient for atrazine remediation.

The statistical result showed that both infiltration rates ($p < 0.0001$) and cell loading ($p = 0.0338$) strongly affected atrazine removal. The atrazine removal efficiency decreased considerably with increasing infiltration rate while the higher cell loading provided more efficient atrazine removal. The cell loading of 300 mg/L gave significantly lower atrazine removal than the cell loadings of 600 and 900 mg/L but the atrazine removals at the cell loadings of 600 to 900 mg/L were not

significantly different to one another. This revealed that the cell loading of 600 mg/L was sufficient for removing atrazine.

During the atrazine bioremediation test, atrazine primary intermediate metabolites (HA, DEA, and DIA) were monitored. A previous study reported that J14a can degrade atrazine via N-dealkylation or dechlorination to HA and DEA, respectively (Struthers et al., 1998). In this study, although atrazine concentration significantly decreased, there were no primary intermediate products detected in all effluent samples. It might be because atrazine was degraded to intermediate products beyond the primary intermediate metabolites. Similarly, no significant amount of intermediate products generated during the test in soil by J14a because of complete biodegradation and mineralization of atrazine (Struthers et al., 1998).

The bioaugmented cell leaching results are presented in Figure 5-4. The bacteria leaching from the immobilized cell columns at all cell loadings (columns C1 to C3) were about 5-7 log CFU/mL while the bacterial losses from all free cell columns (columns D1 to D3) declined from approximately 9 to 6 log CFU/mL. It is believed that the decrease in bacterial leaching in the free cell system with PV was because there were less numbers of cells remaining in the columns with PV. Although it is likely that there was cell growth in the column, it could not keep up with the cell leaching. On the other hand, the bacterial loss from the immobilized cell system was quite consistent and about 10 to 100 times less than that from the free cell system. This indicates that the immobilization material could retain the J14a cells well. Even though the atrazine degradation from the immobilized and free cell systems was not significantly different, the immobilized cells could retain J14a much higher than the free cells.

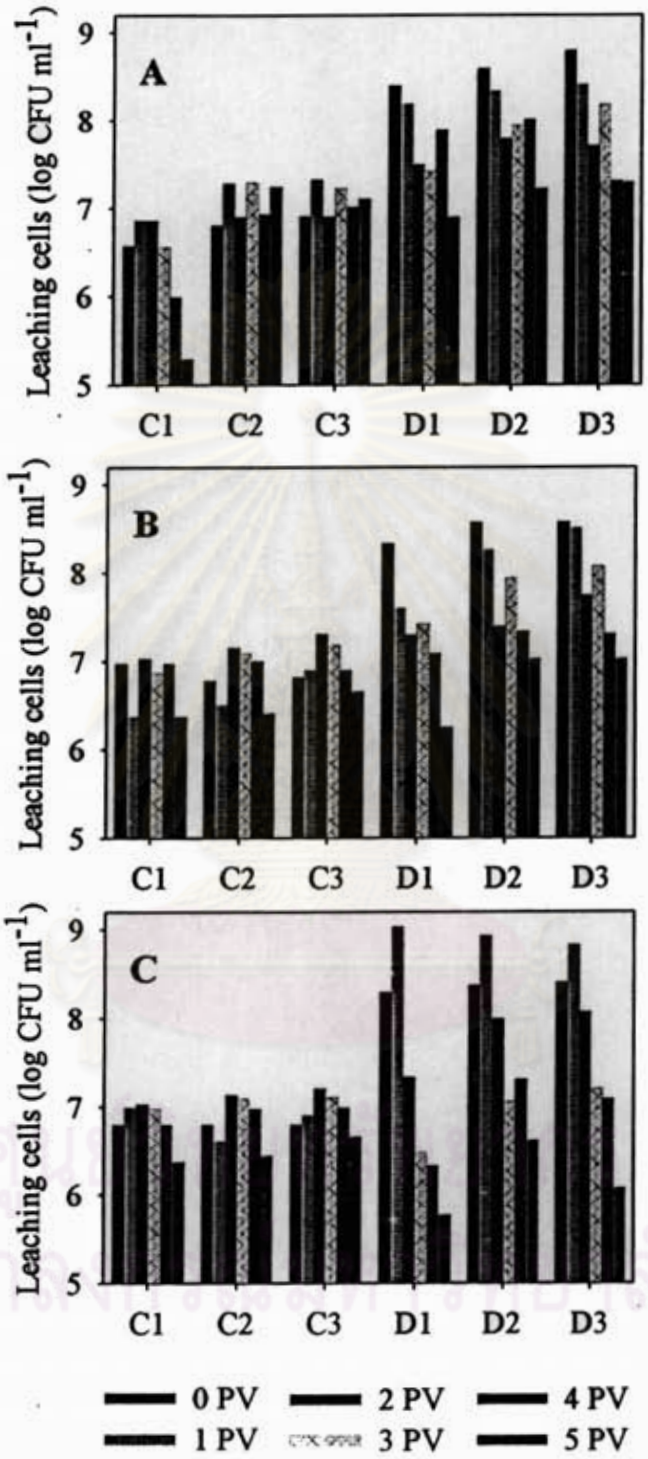


Figure 5-4 Number of viable cells in the effluents from the columns of immobilized and free J14a at the infiltration rates of A) 1 cm/d, B) 3 cm/d, and C) 6 cm/d

5.3.4 Long term atrazine bioremediation performance

The atrazine remediation was conducted for 50 PV to examine the long-term performances of the bioaugmented immobilized and free cells, the condition which is more agreeable with the field-scale application. It is envisioned that these bioaugmented systems will be effective for at least one growing season. The experiment was conducted at the infiltration rate of 6 cm/d, which was the critical infiltration rate. Figure 5-5 shows that all immobilized cell columns at different cell loadings maintained consistent atrazine removal efficiencies at approximately 45-65% for the entire test while the atrazine removal by the free cells started to decrease at 25 PV. At the end of the test, the free cell system removed only 10-15% of atrazine. During the test, atrazine primary intermediate metabolites were not detected in the effluent.

For the cell leaching monitoring, the trend of the cell leaching was similar to the test at 5 PV. The cell leaching from the immobilized cell columns were approximately 5-7 log CFU/mL. For the free cell columns, the cell leaching kept decreasing. At 15 PV, the cell leaching of the free cell columns was less than 4 log CFU/mL, which was much less than the number of initial J14a (approximately 9 log CFU/mL). These long-term monitoring results confirmed the potential of the immobilized cell system for bioremediation. It could protect the cell leaching well leading to stable atrazine removal.

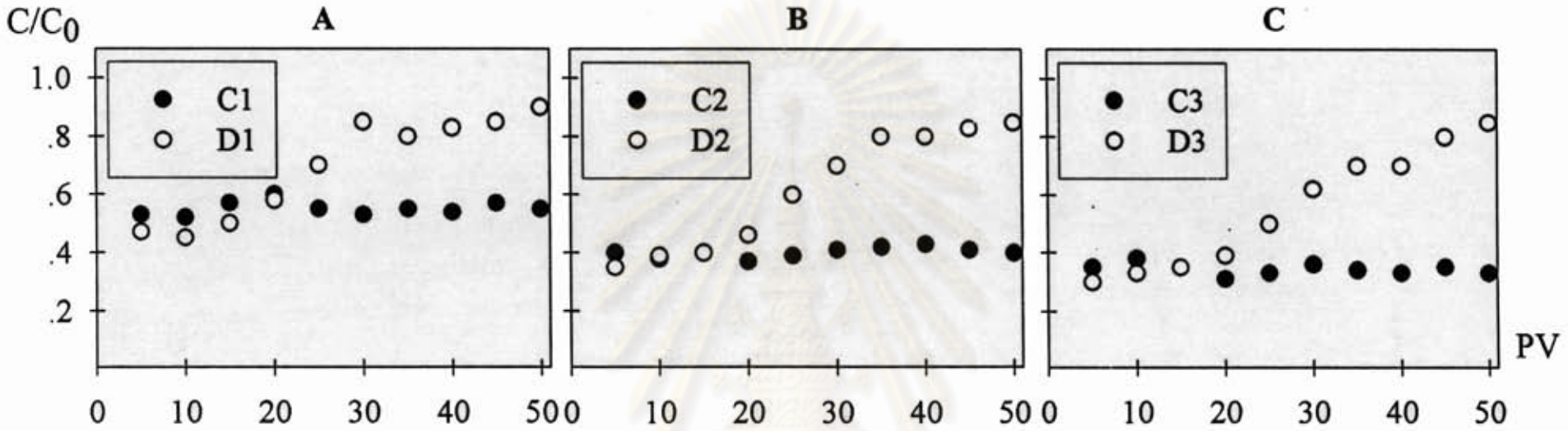


Figure 5-5 Long-term application (50 PV) for remediating atrazine using the immobilized (C1, C2, and C3) and free (D1,D2, and D3) J14a at the infiltration rate of 6 cm d^{-1} and the cell loading of A) 300 mg/L, B) 600 mg/L, and C) 900 mg/L

5.4 Summary

The overall atrazine bioremediation result proved that the bioaugmentation technique has high potential as a remediation method for contaminated agricultural infiltrate. In the short-term study, the immobilized and free cells at the infiltration rates of 1, 3, and 6 cm /d removed 100%, 80-97%, and 50-70% of atrazine, respectively. The matrix and immobilized dead cells significantly retarded atrazine transport. Both infiltration rate and cell loading significantly affected atrazine adsorption (retardation) and removal. The atrazine removal efficiencies by the immobilized and free cell systems were not significantly different; however, there was much less bacterial loss from the immobilized cell system. For the long-term test, the free cells provided much lower atrazine removal efficiency because of a large number of cell leaching out of the test columns. Therefore, the immobilized cell bioaugmentation would be a sustainable alternative for atrazine remediation.

CHAPTER VI

BIOAUGMENTATION OF POLYVINYL ALCOHOL IMMOBILIZED AND FREE MIXED CULTURES FOR REMOVING ATRAZINE FROM AGRILCUTURAL INFILTRATE AND ITS BACTERIAL COMMUNITY STRUCTURAL CHANGE

6.1 Introduction

There has been an increasing interest to develop new on-site remediation techniques. Biodegradation has been known as an effective remediation technique for removing organic contaminants. In some cases, biodegradation by indigenous species cannot cope with all contaminants or takes long time. Cell bioaugmentation, an addition of sufficient contaminant-degrading microorganisms, can potentially be used to solve this problem. There are many factors influencing the survival of bioaugmented microorganisms and their contaminant degradation efficiencies, such as predation and competition of indigenous microorganisms, and unsuitable growth environments (van Veen et al., 1997).

Cell immobilization is a potential alternative to minimize these problems. Immobilization matrices can alleviate environmental stresses (Cochet et al., 1990; Dervakos and Webb, 1991). This technique also prevents cell leaching, which helps to maintain high biomass concentrations. Several studies have reported successful applications of immobilized cell bioaugmentation for point source pollution control, especially wastewater treatment (Chen and Lin, 1994; Chang et al, 2005;

Jittawattanarat et al., 2007a; Jittawattanarat et al., 2007b). Agricultural activities including the use of herbicides are ones of the main contributors of non-point source pollution. Atrazine (6-chloro-N-ethyl-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine) is one of the most widely used herbicides and has been applied to control broad-leaf weeds for crop production. Atrazine detections in groundwater and surface water above the allowable contaminant levels for drinking water of 0.1 and 3.0 μg/L in Europe and the United States, respectively, have been frequently reported (Gannon, 1992; Koskinen and Clay, 1997; Rousseaux et al., 2003).

The main objective of the work presented in this chapter was to examine the possibility of the use of immobilized cell bioaugmentation for remediating non-point agricultural infiltrate contaminated with atrazine. Figure 6-1 presents a possible application scheme of the immobilized cell bioaugmentation for removing the non-point source pollution including atrazine. A first step to validate this potential application is to conduct a bioaugmentation study using bench-scale sand columns. The effects of cell loading and infiltration rate on atrazine remediation were tested using sand column experiments. Bioaugmentation by free cells was evaluated against immobilized cells for their remediation capacity. During the test, the loss of the bioaugmented atrazine-degrading culture and the change of bacterial community were determined. Long-term performance of atrazine remediation was also monitored.

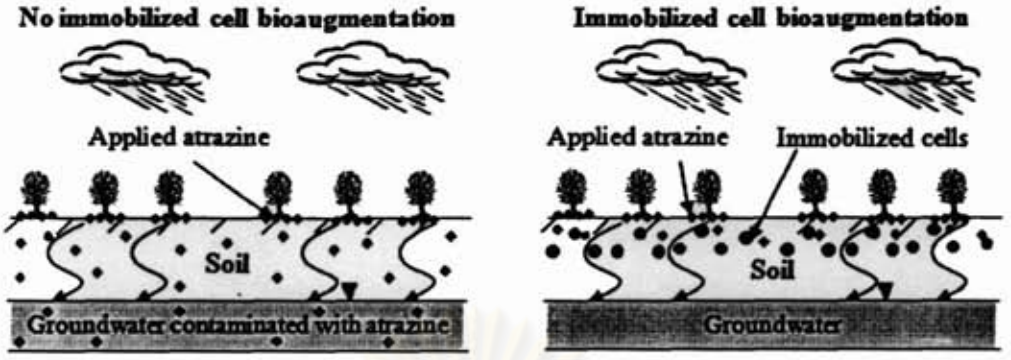


Figure 6-1 A schematic of immobilized cell bioaugmentation for atrazine removal in agricultural infiltrate

6.2 Materials and Methods

6.2.1 Bacteria and cultural condition

A stable atrazine-degrading MC was enriched from atrazine contaminated soil collected from a field site in Oakes, North Dakota, USA following the procedure described in Chapter 4. The MC was subcultured every 7 d in a buffered bacterial medium (20 mM sodium phosphate buffer at pH 6.8), which contained (per liter) 20 mg of atrazine, 1.0 g of glucose, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeCl_3 \cdot H_2O$, 10 mg of $CaCl_2 \cdot H_2O$, 0.1 mg of $MnCl_2$, and 0.01 mg of $ZnSO_4$.

6.2.2 PPVA cell immobilization procedure

The MC was immobilized using the PPVA technique similar to the procedure described in Chapter 4, Section 4.2.3. PPVA was chosen as an immobilization matrix since it is durable and has no negative effect to microorganisms and the environment (Chen and Lin, 1994; Gentry et al., 2004). The concentrated cells (20 mg dry cells/mL) were mixed with a PVA solution. The mixture was slowly dropped into a

saturated boric acid solution in a 1-liter cylinder for 30 min to form spherical beads. The formed hydrogel beads were then submerged in a 1 M sodium phosphate solution (pH 7) for 60 min for hardening. The gel beads were washed in DI water, and then stored in a 20 mM sodium phosphate solution (pH 6.8) at 4°C. The final PVA concentration and cell-to-matrix ratio was 10% (w/v) and 3.5 mg dry cells/mL matrix, respectively.

6.2.3 Synthetic agricultural infiltrate, sand, and column preparations

6.2.3.1 Synthetic agricultural infiltrate preparation

Synthetic agricultural infiltrate was prepared in the same manner as described in Chapter 5, Section 5.2.4.1.

6.2.3.2 Sand preparation

The sand preparation was in the same manner as described in Chapter 5, Section 5.2.4.2.

6.2.3.3 Column preparation

A sand column was 6.3 cm in diameter and 23 cm in length. It had an effluent sampling port at the bottom. All columns were rinsed with 70% isopropanol and autoclaved de-ionized water, respectively before used. The sterile sand was filled in the columns as described in the next section.

6.2.4 Atrazine bioremediation test in column system

Duplicate atrazine bioremediation tests in sand columns were performed. The effects of the cell loadings and infiltration rates on the atrazine degradation efficiencies and culture losses were studied. Three sets of sand columns including set ID (immobilized dead cells), set IC (immobilized cells), and set FC (free cells), were packed as described in Table 6-1. Each set of the columns comprised 3 columns at the cell loadings of 300, 600, and 900 mg dry cells/L empty bed volume.

The columns were operated as follows. The columns were filled up with a 20 mM sodium phosphate solution (pH of 6.8). The synthetic agricultural infiltrate was continuously applied as a step input at 8-hr interval. The flow rates studied were 30, 90, and 180 mL/d corresponding to the infiltration rates of 1, 3, and 6 cm/d for obtaining the actual, high, and critical (extremely high) infiltration rates, respectively. The test was run for 5 PV (total pore water volume of 600 mL).

During the test, the effluent was sampled every 0.25 PV to measure atrazine and intermediate metabolite concentrations for the infiltration rates of 1 and 3 cm/d. However, for the infiltration rate of 6 cm/d, this effluent sampling was conducted at a 0.5 PV frequency. The samples at every 1 PV were determined for the number of viable MC cells using a plate count technique. At 5 PV, the effluent samples from all viable cell bioaugmented columns (column sets IC and FC) were taken for detecting the change of bacterial community structure using a single stand conformation polymorphism (SSCP) technique. After 5 PV, flow was continued for selected columns for long-term monitoring, where the effluent was monitored for atrazine and intermediate metabolite concentrations every 5 PV between 5 and 50 PV.

Table 6-1 Descriptions of sand columns and their components

Column set	Column number	Column description (cell type)	Cell loading (mg dry cells/ mL empty bed volume)	Cell mass (mg dry cells)	Bioaugmented volume* (mL)	Dry sand (mL)	Total empty bed volume (mL)
ID	ID1	Immobilized dead cells	300	120	40	360	400
	ID2	Immobilized dead cells	600	240	80	320	400
	ID3	Immobilized dead cells	900	360	120	280	400
IC	IC1	Immobilized cells	300	120	40	360	400
	IC2	Immobilized cells	600	240	80	320	400
	IC3	Immobilized cells	900	360	120	280	400
FC	FC1	Free cells	300	120	N/A**	400	400
	FC2	Free cells	600	240	N/A**	400	400
	FC3	Free cells	900	360	N/A**	400	400

* Volume of the cells and matrices, ** Volume of the cells was negligible

6.2.5 Analytical methods

6.2.5.1 Atrazine and metabolite analysis

The analytical methods used for atrazine, DEA, DIA, and HA were modified from D'Archivio et al. (2007) as described in Chapter. 5, Section 5.2.7.1.

6.2.5.2 Viable plate count

The bacterial loss from the column system was determined by the number of viable bacteria in the effluent samples using the plate count procedure described in Chapter 5, Section 5.2.7.2.

6.2.5.3 Statistical analysis for atrazine bioremediation test

Mass of atrazine in effluent samples was statistically analyzed in the same manner as described in Chapter 5, Section 5.2.8.

6.2.6 Bacterial community change detection using SSCP technique

6.2.6.1 DNA extraction

Ten beads containing immobilized cells were cut and mixed thoroughly in 10 mL of 20 mM phosphate buffer (pH 6.8) to extract the bacteria from the matrices. The liquid samples (5-10 mL) from this cell extraction procedure, mixed culture prior to immobilization, and column effluent were centrifuged and used for extracting DNA. The genomic DNA extraction procedure followed the instruction

from the DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega, USA).

6.2.6.2 DNA amplification

The PCR-SSCP procedure was modified from Lin et al. (2007). The V3 region of the 16S rDNA (nucleotide positions 334-514 of *Esherichia coli*) was amplified with primers EUB1 (5'-CAG ACT CCT ACG GGA GGC AGC AG 3') and UNV2 (5'-GTA TTA CCG CGG CTG CTG GCA C 3'). A 25-μL PCR reaction contained 1.5 mM of $MgCl_2$, 200 μM of dNTP, 5.0 μl of Taq Polymerase buffer 5× (Promega, CA, USA), 50 μM of each primer, 1.25 U of Taq Polymerase (Promega, CA, USA), and 2 μL of DNA template. Dnase/Rnase-free water was used for making up the volume of samples. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 5 min. The presence of PCR products (approximately 200 bp) was confirmed by 1.5% agarose gel electrophoresis.

6.2.6.3 SSCP gel electrophoresis

The SSCP test was performed in a horizontal electrophoresis setup (Origins, Elchrom Scientific, Switzerland).The SSCP procedure followed the instruction from the manufacturer. Three microliters of PCR products were mixed with 7 μL of a denaturing solution (1 mL of formamide, 10 μL of 1 M NaOH, and 20 μL of 0.02% (w/v) bromphenol blue). The mixtures were heated at 95°C for 5 min and immediately placed into ice until loading to the SSCP gel. The 10 μL denatured PCR products were loaded into a pre-cast polyacrylamide gel (GMA™, Elchrom

Scientific, Switzerland). The gel was run at a constant voltage of 72 V and 9°C for 10 hr. The gel then was visualized with SYBR® Gold staining (Molecular probes, OR, USA).

6.2.6.4 SSCP gel data analysis

The images of DNA profiles were analyzed using Bionumerics version 5 (Applied Maths, TX, USA). The pair-wise similarity among the samples was calculated using Dice index and unweighted pair-group method with arithmetic average.

6.3 Results and Discussion

6.3.1 Atrazine bioremediation within 5 PV

Figure 6-2 shows the breakthrough curves of relative atrazine concentration (C/C_0) of the bioaugmented immobilized dead cells (ID), immobilized cells (IC), and free cells (FC). In all curves, C/C_0 increased after 0.5-1.0 PV and reached a plateau at approximately 2.5-4.0 PV. The immobilized dead cells did not have degradation ability resulting in no atrazine removal (C/C_0 of 1.0) at 3.0 to 4.0 PV while the breakthrough curves of immobilized and free cell columns were stable at C/C_0 of 0.2-0.6. At the same cell loading and infiltration rate, atrazine was detected in the effluent of the free cell columns earlier than the immobilized cell and immobilized dead cell columns, in which atrazine sorption to the immobilization matrix likely occurred. Based on the breakthrough curves of the immobilized dead cells (stable at C/C_0 of 1.0), the PPVA matrix only retarded the atrazine breakthrough but did not permanently remove it.

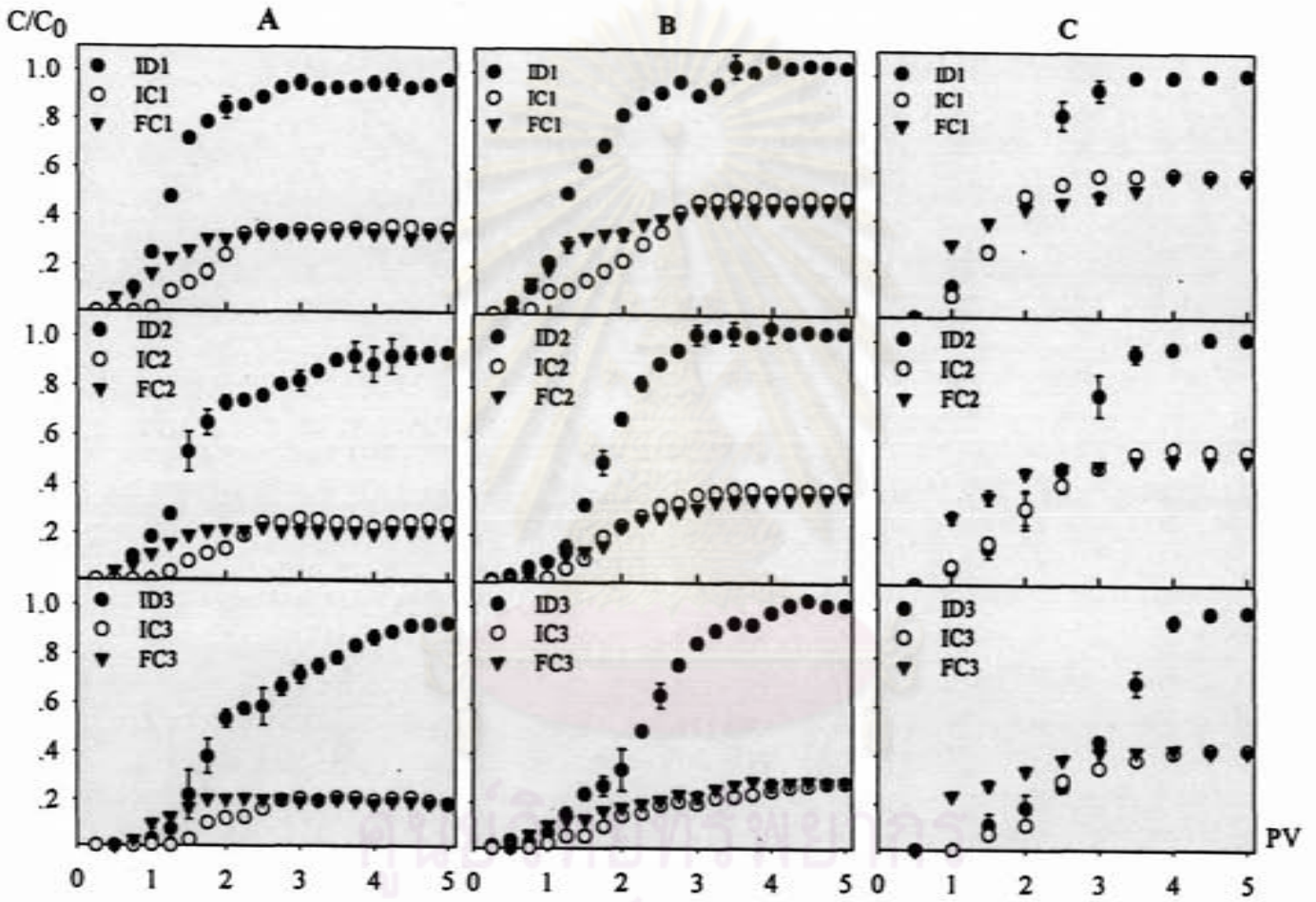


Figure 6-2 Breakthrough curves of atrazine bioremediation at the infiltration rates of: A) 1 cm/d, B) 3 cm/d, and C) 6 cm/d. ID, IC, and FC were the columns of the immobilized dead cells, immobilized cells, and free cells, respectively.

The immobilized and free cell columns at the infiltration rates of 1, 3, and 6 cm/d (Figure 6-2) removed atrazine at 65-80%, 50-73%, and 42-58%, respectively. The atrazine removal efficiency decreased with increasing infiltration rate whereas higher cell loading rates provided greater removal efficiency. The statistical analysis showed that both infiltration rate ($p < 0.0001$) and cell loading ($p = 0.0002$) significantly influenced atrazine removal. However, for all experiments, there were no significant differences between the immobilized and free cell systems for atrazine removal ($p = 0.4493$). Therefore, the atrazine-degrading mixed culture used in this study in either immobilized or free cell forms was efficient for atrazine remediation.

During the atrazine bioremediation test, the atrazine primary intermediate metabolites (HA, DEA, and DIA) were not detected in any effluent samples. The result infers that atrazine was quickly degraded to some other intermediate products beyond the primary intermediate metabolites.

6.3.2 Cell leaching within 5 PV

The results of bioaugmented cell losses are presented in Figure 6-3. The bacteria leaching from the immobilized and free cell columns at all cell loadings was about 6-7 and 6-9 log CFU/mL, respectively. The trends of the cell losses in the immobilized and free cell columns were different. The cell losses from the immobilized cell columns (columns IC1 to IC3) were stable while those from all free cell columns (columns FC1 to FC3) declined with duration of the experiment.

The initial number of cells augmented to all columns was approximately 9 log CFU/mL. At 0 and 1 PV, the numbers of cells leaching from the free cell columns were very close to the initial number of cells. This could be because the sand columns

could not effectively retain the free cells. This led to less numbers of cells left in the columns resulting in less numbers of the leaching cells in later PVs. At 0 and 1 PV, the numbers of cell loss from the immobilized cell columns were about 100 times less compared to those of the free cell columns. This indicates that the immobilization matrix could effectively protect the cells from leaching. Based on the atrazine degradation efficiency and the protection of the cell leaching results, the immobilized cell bioaugmentation should be a better alternative for long-term atrazine bioremediation.

6.3.3 Bacterial community change during atrazine bioremediation

Figure 6-4 presents the SSCP profiles of the 16S rDNA fragment of the atrazine-degrading mixed culture. The samples (initial FC and initial IC) of atrazine-degrading mixed culture before and after immobilization were analyzed for determining the effect that the immobilization process had on the bacterial community structure. The cluster analysis showed that the similarity of the culture before and after the immobilization process was approximately 60%. This suggests that the immobilization process affected the bacterial community structure to some extent. Chemical or physical stresses during the immobilization process might be the cause. As discussed in Chapter 4, the immobilization process affected the bacterial viability. Some bacterial species might be less tolerant than the others and were killed in the immobilization procedure leading to the difference in the bacterial community structure.

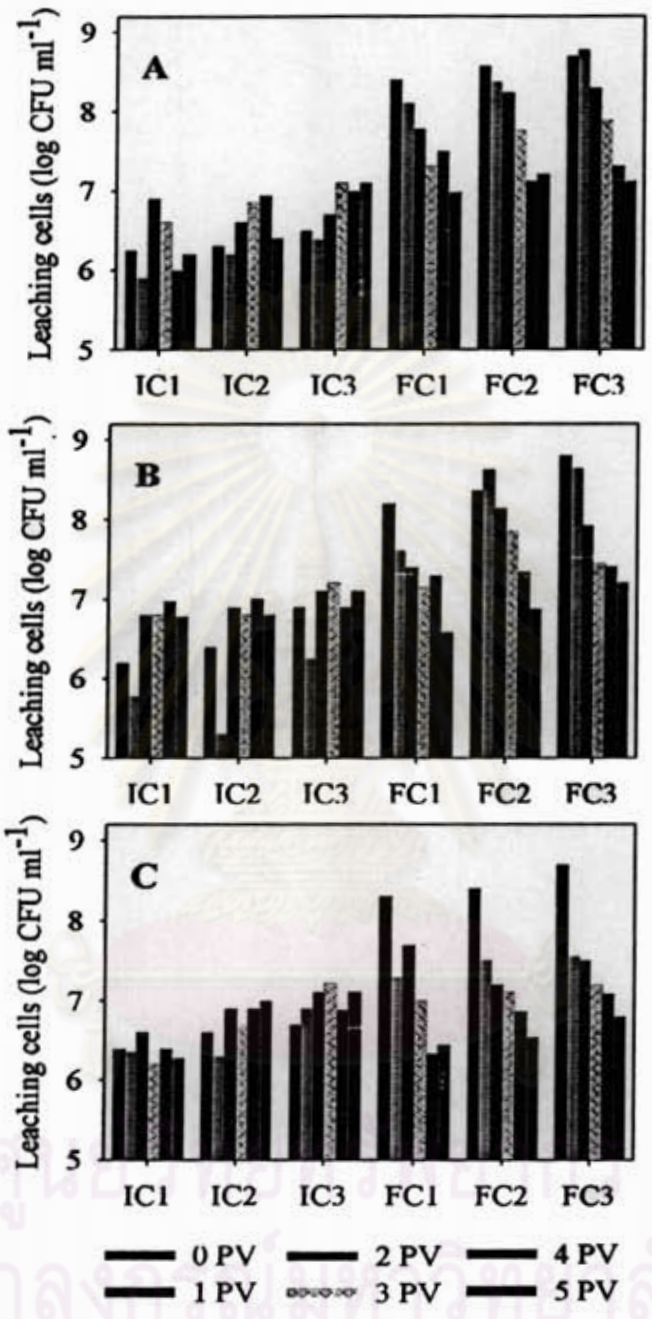


Figure 6-3 Number of viable cells in the effluents from the columns of the immobilized (IC1, IC2, and IC3) and free (FC1, FC2, and FC3) cells at the infiltration rates of: A) 1 cm/d, B) 3 cm/d, and C) 6 cm/d

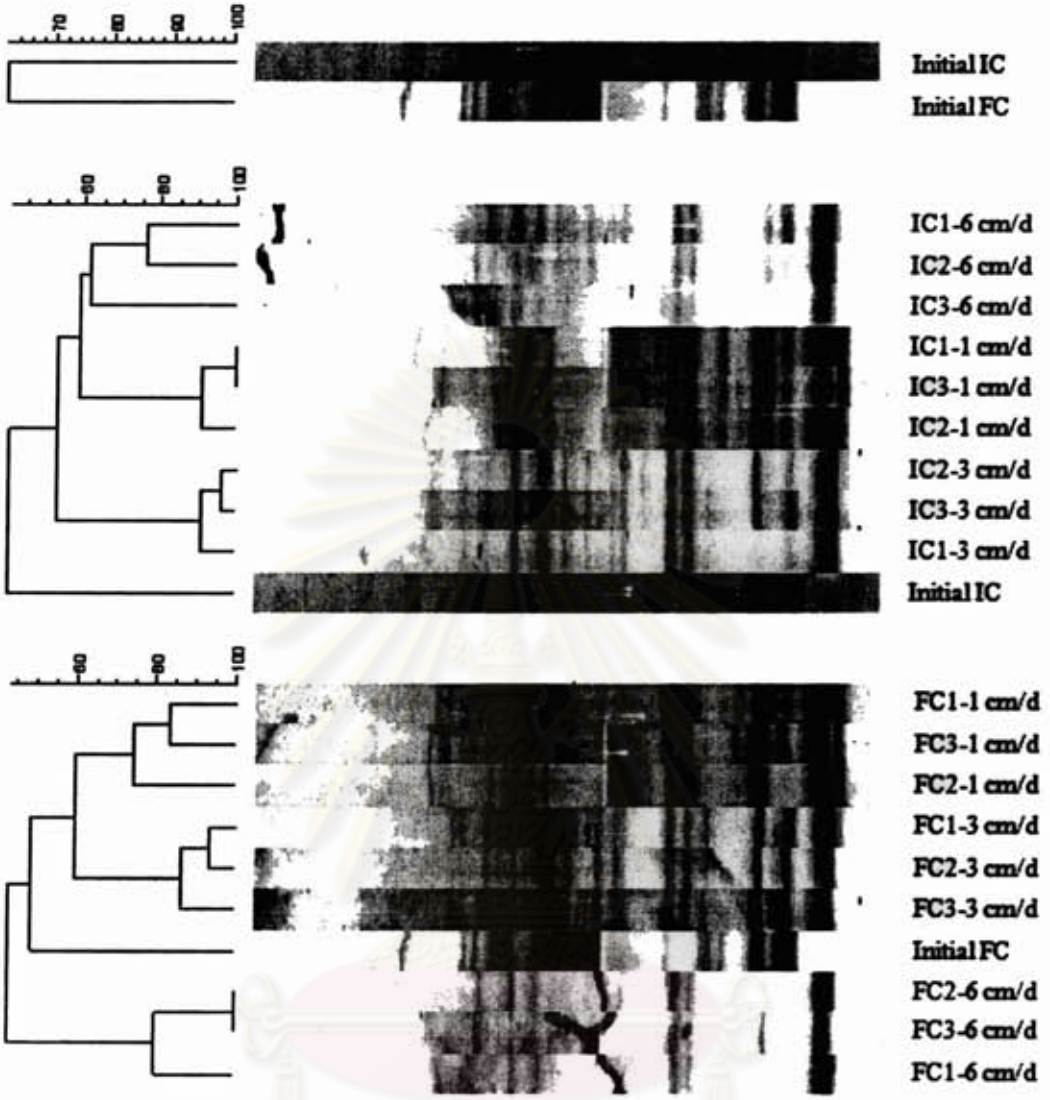


Figure 6-4 SSCP profiles and cluster analysis of PCR-amplified 16S rDNA fragments of the atrazine-degrading mixed culture

Initial IC and FC are the mixed culture before application in immobilized and free cell forms, respectively. IC1 and FC1, IC2 and FC2, and IC3, and FC3 are the effluents of the columns at the cell loadings of 300, 600, and 900 mg/L, respectively

For the pair-wise comparison between before and after the atrazine bioremediation test, the immobilized and free cell systems provided comparable results. The similarity of the bacterial community structure before and after the test was approximately 40-50% (Figure 6-4). The effluent samples at the same infiltration rate but different cell loadings had the similarity of the bacterial community structure of 80-100%. On the contrary, the samples at the same cell loading but different infiltration rates had the similarity of the bacterial community structure of 50-60%. This suggests that the infiltration rate influenced the bacterial community structure more than the cell loading. As shown in the previous section, the infiltration rate also affected the atrazine removal more than the cell loading. This coincident seemed reasonable because intuitively atrazine removal and bacterial community structure should be related.

6.3.4 Atrazine bioremediation and cell leaching within 50 PV (long-term performance)

The long-term performance of the atrazine remediation was conducted to determine the feasibility of the bioaugmentation scheme for the real-world scenario. The experiment at the infiltration rate of 6 cm/d was selected because it was the critical infiltration rate. Figure 6-5 shows that the trend of C/C_0 from the immobilized cell columns was stable whereas the trend from the free cell columns gradually increased after testing for 15 PV. At the end of the test (50 PV), the atrazine removal efficiencies from the immobilized and free cell columns were 40-60% and 10-15%, respectively.

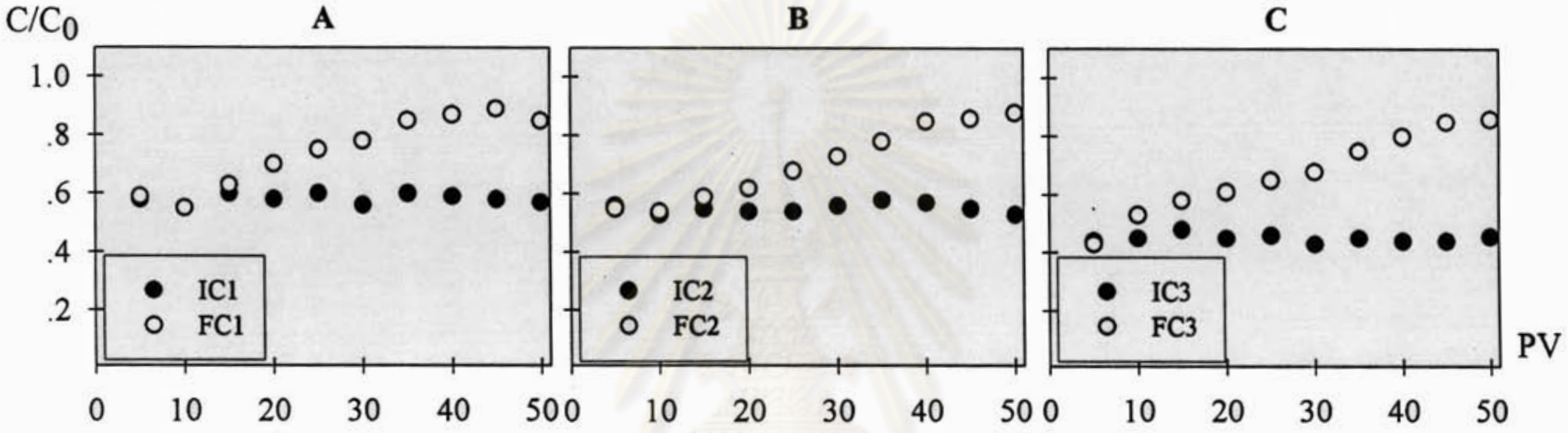


Figure 6-5 Long-term performance of the immobilized (IC1, IC2, and IC3) and free (FC1, FC2, and FC3) cell bioaugmentation at the infiltration rate of 6 cm/d and the cell loading of A) 300 mg/L, B) 600 mg/L, and C) 900 mg/L

Similar to the result of the short-term test (5 PV), the cell leaching from the immobilized cell columns was consistent at about 5-7 log CFU/mL whereas the cell loss from the free cell system decreased with increasing pore water volumes (data not shown). At 10 PV, the cell leaching from the free cell columns was less than 4 log CFU/mL, which was much less than the beginning (9 log CFU/mL). Also, the 10 PV was where atrazine bioremediation by the free cells started to be less efficient than that by the immobilized cells.

6.3.5 Comparison of atrazine bioremediation by bioaugmented J14a and MC

Atrazine bioremediation results of J14a from the previous chapter and MC were similar. The free cell bioaugmentation performed well only at the beginning while the immobilized cell bioaugmentation provided stable atrazine removal for the entire tests. Atrazine removal efficiencies by J14a were better than those by MC for both immobilized and free cell forms. This could be because both J14a and MC were tested with the synthetic agricultural infiltrate having the formulation similar to the bacterial medium. The cultures did not face any environmental stresses such as high or low pH. In addition, the experiment was under a sterile condition; there was no competition of indigenous microorganisms to the cultures. These experimental conditions are suitable for atrazine biodegradation by any atrazine-degrading cultures. Cell masses of MC and J14a used in the test were about the same; however, not all cells in MC were atrazine-degrading cultures. Therefore, atrazine bioremediation by MC was not as effective as J14a.

In the real-world situation, mixed cultures have been shown to be more suitable for bioremediation compared to pure cultures. It is because the biodiversity of mixed cultures can enhance environmental survival and increase the number of catabolic pathways available for contaminant biodegradation (Alvey and Crowley, 1996; Kontchou and Gschwind, 1999; Smith et al., 2005). The results from this study did prove the potential of the immobilized cell bioaugmentation by both pure and mixed cultures over the general (free cell) bioaugmentation. However, the comparison of atrazine bioremediation performances of J14a and MC is inconclusive. In the future, a pilot-scale study at a contaminated site is recommended for testing the environmental effects on each culture type.

6.4 Summary

The bioaugmented MC successfully removed atrazine at 42 to 80% in the short-term test. The effects of the infiltration rate and the cell loading on atrazine bioremediation by MC were similar to J14a. Both infiltration rate and cell loading significantly affected the atrazine remediation. The immobilized and free cells provided similar atrazine removal efficiencies; however, leaching of the free cells was much greater than that of the immobilized cells. For the long-term performance, the immobilized cells provided consistent atrazine removal throughout the test whereas atrazine removal by the free cells substantially decreased after 15 pore volumes of testing. Both immobilized and free cell systems exhibited a significant change in bacterial community structure during the atrazine degradation experiments. The infiltration rate was a significant factor for the change.

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

This research investigated the utilization of free and PPVA immobilized cell bioaugmentation for remediating atrazine in agricultural infiltrate The bioaugmentation performances of two culture types, pure and mixed cultures, were investigated. The pure culture used was a previously isolated atrazine degrader, *Agrobacterium radiobacter* J14a while the mixed culture was enriched from atrazine-contaminted soil. This novel mixed culture repeatedly degraded atrazine as effective as the known pure atrazine degrader. In the mixed cultures, four different bacterial strains designated ND1, ND2, ND3, and ND4 were identified as *Alcaligenes faecalis*, *Klebsiella ornithinolytica*, *Bacillus megaterium*, and *Agrobacterium tumefaciens*, respectively. The strains, which could initiate the atrazine degradation, were *Klebsiella ornithinolytica* ND2 and *Agrobacterium tumefaciens* ND4. This is the first report of atrazine-degrading gene detection in *Klebsiella ornithinolytica*.

During the PPVA immobilization process, the procedure was modified to prevent immobilized cell agglomeration. The procedure provided a durable immobilization matrix and did not affect bacterial viability substantially. The modified procedure also delivered a proper microstructure for cell immobilization. In a batch study, the atrazine removal efficiencies by the immobilized J14a and MC were better than those by the free cells. Higher cell-to-matrix ratios decreased atrazine removal efficiency. The cell-to-matrix ratio of 3.5 mg/mL provided the highest atrazine removal efficiency of 40 to 50% in 120 hr for both J14a and MC.

For the atrazine bioremediation in a column system, both bioaugmented J14a and MC in either free or immobilized cell forms removed 42 to 100% of atrazine in a short-term test. The infiltration rate and cell loading significantly affected the atrazine remediation. The atrazine removal efficiency decreased with increasing infiltration rate whereas higher cell loading rates provided greater removal. During the atrazine bioremediation by MC, both immobilized and free cell systems exhibited a significant change in bacterial community structure. The infiltration rate was a significant factor for the change. For a long-term test, the immobilized cells overcame the cell washout problem and provided consistent atrazine removal throughout the test whereas the atrazine removal by the free cells declined gradually.

It is envisioned that the bioaugmented immobilized cells would remain active for at least one growing season. The application scheme is to bioaugment the immobilized cultures during land preparation (soil tillage) before each growing season. The technique can be applied for some other agricultural contaminants as well.

7.2 Recommendations

This research examined the use of immobilized cell bioaugmentation for removing an organic contaminant in infiltrate compared to free cell bioaugmentation. Even though the atrazine bioremediation by the immobilized pure and mixed cultures was successful, additional work is recommended for future studies as follows.

1) The atrazine bioremediation test in various types of soil should be performed to determine the possibility of applying immobilized cell bioaugmentation at different contaminated sites.

2) The atrazine bioremediation test using an undisturbed soil column system should be conducted for imitating field situation. The soil sample should be taken from contaminated sites.
3) An on-site pilot scale study is suggested for investigating the effects of environmental stresses, such as temperature, pH, and toxic substance, and competition with indigenous microorganisms, on atrazine bioremediation.
4) Determination of the growth of the immobilized atrazine-degrading mixed or pure cultures using a molecular biological technique, such as real-time PCR targeting atrazine-degrading genes, is recommended for supporting atrazine bioremediation activity. The traditional viable plate count is not applicable with immobilized cells because they have to be de-immobilized by heating at 60°C, which could affect cell viability.
5) Examination of atrazine concentration profile during the remediation in the column system is recommended for a better understanding on the working depth of the atrazine-degrading cultures.

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BIOGRAPHY

Miss Sumana Siripattanakul was born on October 15th, 1977 in Bangkok, Thailand. She finished her secondary school from Satri Rachinuthit, Udonthani in 19... After that, she attended Khon Kaen University (KKU), Khon Kaen and received her bachelor degree inEnvironmental Engineering in 1999. In the same year, she started working as a research assistant at the Environmental Management Center, Faculty of Engineering,KKU. One year later, she joined a graduate program in Environmental Engineering at KKU. In 2002, after receiving her master degree, she joined the Department of Chemical Engineering, Ubonratchathani University, Ubonratchathani as a lecturer. She then took a leave of absence in 2004 to pursue her doctoral studyin Environmental Management (international program) at Chulalongkorn University, Bangkok. During her Ph.D. study, she had carried out her doctoral research at North Dakota State University, USA from September 2005 to April 2008. She attended four national and international conferences in environmental and microbiological fields for platform and poster presentations.