DEGRADATION OF CARBOFURAN BY RHIZOSPHERE SOIL MICROORGANISMS

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การย่อยสลายคาร์โบฟูรานโดยจุลินทรีย์ในดินรอบรากพืช

นางสาวเพ็ญศรี ปลั่งกลาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความสามารถในการย่อยสลายคาร์โบฟูรานโดยจุลินทรีย์ในดินรอบรากพืช โดยได้ทำการคัดเลือกเชื้อที่ความสามารถในการย่อยสลายคาร์โบฟูรานในอาหาร Basal Salt media (BSM) 3 ชนิด ที่มีคาร์โบฟูรานเป็นแหล่งคาร์บอน หรือแหล่งไนโตรเจน หรือเป็นทั้งแหล่งคาร์บอนและแหล่งไนโตรเจน โดยพบว่า แบคทีเรีย PCL3 ซึ่งใช้คาร์โบฟูรานเป็นแหล่งคาร์บอนนั้น สามารถย่อยสลายคาร์โบฟูรานได้เร็วที่สุด โดยค่าครึ่งชีวิตของคาร์โบฟูรานเท่ากับ 3 วัน แบคทีเรีย PCL3 นี้ถูกจำแนกเป็นสายพันธุ์ *Agrobacterium radiobacter* จากนั้นได้ทำการศึกษาการย่อยสลายของคาร์โบฟูรานในดินรอบรากพืช 6 ชนิด คือ กกทราย (*Cyperus iria* Linn) เทียนนา (*Jussiaea linifolia* Vahl) กกสามเหลี่ยมเล็ก (*Cyperus pilosus* Vohl) กกขนาก (*Cyperus difformis* Linn) หนวดปลาดุก (*Fimbristylis miliacea* Vahl) และผักแว่น (*Marsilea crenata* Presl) ผลการทดลองแสดงว่าการย่อยสลายของคาร์โบฟูรานในดินรอบรากกกสามเหลี่ยมเล็กดีที่สุด โดยค่าครึ่งชีวิตของคาร์โบฟูรานในดินนี้ คือ 15 วัน เมื่อทำการเติมแบคทีเรีย PCL3 ลงไปในดินรอบรากกกสามเหลี่ยมเล็ก พบว่า PCL3 ไม่ได้ปรับปรุงการย่อยสลายของคาร์โบฟูรานในดินรอบรากกกสามเหลี่ยมเล็ก โดยสังเกตุได้จากไม่มีความแตกต่างของค่าครึ่งชีวิตของคาร์โบฟูรานในดินทั้งสองชนิดนี้ ซึ่งแสดงให้เห็นว่าการย่อยสลายคาร์โบฟูรานโดยจุลินทรีย์รอบรากพืชก็มีประสิทธิภาพเพียงพอแล้ว การเติม PCL3 ลงไปในดินนาข้าว ทำให้การย่อยสลายของคาร์โบฟูรานดีขึ้น โดยค่าครึ่งชีวิตของคาร์โบฟูรานในดินนาข้าวเมื่อเติม PCL3 คือ 12 วัน ซึ่งสั้นกว่าค่าครึ่งชีวิตในดินนาข้าวที่ไม่มีการเติมคาร์โบฟูราน (58 วัน) ความสามารถในการย่อยสลายคาร์โบฟูรานของ PCL3 เห็นได้ชัดเจนยิ่งขึ้น เมื่อเติม PCL3 ลงไปในดินที่ผ่านการฆ่าเชื้อแล้ว โดยพบว่าค่าครึ่งชีวิตของคาร์โบฟูรานในดินที่ผ่านการฆ่าเชื้อและเติม PCL3 (13-14 วัน) มีค่าสั้นกว่าในดินที่ผ่านการฆ่าเชื้อแต่ไม่เติม PCL3 โดยสรุป การกู้ฟื้นฟูโดยจุลินทรีย์รอบรากพืชเป็นวิธีที่มีประสิทธิภาพวิธีหนึ่งในการย่อยสลายคาร์โบฟูรานที่ตกค้างในดิน การเติมจุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูรานลงไปในดินจะช่วยให้การย่อยสลายคาร์โบฟูรานดีขึ้น

สาขาวิชาการจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....
ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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An ability of rhizosphere soil microorganisms to degrade carbofuran was investigated. An enrichment technique was used to isolate carbofuran degraders from carbofuran phytoremediated rhizosphere soils in C-, N- and C and N-limited Basal Salt Media (BSM) containing 5 mg/L of carbofuran. The shortest half-life of carbofuran, 3 days, was found in C-limited BSM cultured with an isolate named PCL3 indicating that carbofuran was used as a sole C-source. PCL3 was identified as *Agrobacterium radiobacter* and was further used in the bioaugmentaion study. Carbofuran dissipation in rhizosphere soils of 6 weeds i.e. Umbrella sedge (*Cyperus iria* L.), Water primrose (*Jussiaea linifolia* V.), Fuzzy flatsedge (*C. pilosus* V.), Small flower umbrella plant (*C. difformis* L.), Tall-fringe-rush hoorah grass (*Fimbristylis miliacea* V.) and Cover fern (*Marsilea crenata* P.) was conducted. Rhizosphere soil of Fuzzy flatsedge was selected to use in the bioaugmentation experiment because of the shortest half-life of carbofuran in this soil (15 days). Bioaugmentation of carbofuran using PCL3 was conducted to examine its ability to degrade carbofuran in Fuzzy flatsedge rhizosphere soil. The degradation of carbofuran in this soil was not improved by PCL3 suggesting that rhizosphere remediation might be enough for remediating carbofuran contaminated soil. An ability of PCL3 to degrade carbofuran was evident in bulk soil ($t_{1/2}$ of 12 days) and autoclaved soils ($t_{1/2}$ 13-14 days) when compared to soils without an inoculation ($t_{1/2}$ of 58 days). In conclusion, rhizosphere remediation is one of the effective bioremediation techniques to remove or detoxify carbofuran residues in soil. Bioaugmentation of carbofuran in contaminated bulk soil by isolated degraders could improve the degradation of carbofuran.

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

Academic year 2004 Advisor's signature……

Co-advisor's signature……..

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

Page

LIST OF FIGURES

Page

Page

CHAPTER I

INTRODUCTION

1.1 General Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide used to control insects, mites, and nematodes on contact or after ingestion (Extoxnet, 1996). It is widely used in paddy field by spraying directly onto paddy soil and water (EPA, 2002). Carbofuran is of environmental importance because of its high mammalian toxicity through cholinesterase inhibition (LD_{50} = 2 mg/kg) (EPA, 2003). Carbofuran is soluble in water and mobile in soil resulting in a high potential for groundwater contamination (Howard, 1991). It has been detected (1 to 5 μg/L) in water table aquifers beneath sandy soils in New York and Wisconsin (Howard, 1991). Carbofuran is moderately persistent in soil. Its half-life in soil is 30 to 120 days depending on temperature, moisture content, pH and the numbers of microorganisms present (DeVries and Evans, 1999). A primary mechanism of carbofuran degradation in soil and water under neutral to basic conditions is chemical hydrolysis resulting in a metabolite named carbofuran phenol (Getzin, 1973; Sieber et al., 1978). The rate of hydrolysis was reported to increase as pH and temperature increased (Seiber et al., 1978). The degradation of carbofuran in acidic soil is slower than in neutral and alkaline soil (Getzin, 1973; Siddaramappa and Sieber, 1979). Dissipation of carbofuran in water could be influenced by volatilization and photolysis (Sieber et al., 1978; Deuel et al., 1979). Sunlight and high temperature have increased the rate of carbofuran loss from water (Siddaramappa and Sieber, 1979). Johnson and Lavy (1995) reported that the dissipation of carbofuran in paddy water was rapid with a half-life of carbofuran of 3 days, compared to 10 days in paddy soil. Chapman and Cole (1982) found that the carbofuran degradation rate in water was strongly influenced by pH.

Rhizosphere soil is the small volume of soil that attach or directly adjacent to the plant roots where degradation processes take place by interaction between contaminants and rhizosphere microorganisms (Stottmeister et al., 2003). The specialties of rhizosphere soil are the carbon containing compounds such as sugars, alcohols and acids exudated from plant roots stimulating the microbial activities

(Schnoor et al., 1995). Rhizosphere degradation is also known as phytostimulation or plant-assisted bioremediation (Schnoor, 1997). It is defined as the acceleration of organic pollutant breakdown in soil as consequences of enhanced degradation activities of the rhizosphere microorganisms (Siciliano and Germida, 1998). Microorganisms in the root zone, or so-called rhizosphere microorganisms, were reported to be effectively used to remediate organic and inorganic pollutants. The herbicide degradation was increased in the rhizosphere of the herbicide-tolerant plant, suggesting that rhizosphere interactions between the plant and microorganisms have led to the increased degradation of the pesticides (Frazar, 2000; Anderson et al., 1994). There were reports on the rhizosphere remediation of pesticides (Anderson et al. 1994; Singh et al. 2004; Camper, 1999; Yu et al.,2003; Sun, 2004; Parkin and Shelton, 1994) and others organic hydrocarbon (Nakamura et al, 2004; Jordahl et al. 1997). However, there is a very limited information on rhizosphere degradation of carbofuran.

Bioremediation is a promising process using natural biological activity to remediate the environmental contaminants until their concentrations are below detectable limits or less than the maximum contaminant levels (Vidali, 2001). In general, bioremediation uses indigenous microorganisms in the contaminated area as the degraders, which are usually present in very small numbers. In some cases, the indigenous microbes may not have the ability to degrade a particular contaminant at all (United-Tech, 2004). In this instance, there is an interest towards using microorganisms isolated from contaminated media (e.g. soil or water) as degraders to successfully degrade the target contaminants.

Degraders can be isolated from soil with the history of pesticides application. This is because the ability of microorganisms to adapt themselves to use that pesticide as their energy sources, i.e. C- or N- or C and N-sources. Previous research indicated that a repeated application of carbofuran in soil enhanced the degradation of carbofuran resulted from an increased number of carbofuran degraders (Harris et al, 1984; Turco and Konopka, 1990). A study by Read (1983) showed that repeated applications of carbofuran in acid mineral soil reduced the half-life of carbofuran by two times. Morel-Chevillet et al. (1996) reported that previously treated soil with 15 N-methylcarbamate enhanced the degradation rate of carbofuran and increased in the number of carbofuran degraders.

Bioaugmentation is one of the bioremediation treatments conducted by adding microbial cultures to improve contaminant remediation and reduce remediation time and cost (CRA, 2003). The successive bioaugmentation of pesticides (Rousseaux et al., 2002; Peter et al., 2000) and hydrocarbon (Lyle et al., 1999; Ruberto et al., 2003) were reported. Rousseaux et al. (2002) found that an addition of 10^4 cfu/g of atrazine degraders into soil treated with atrazine resulted in a 3-fold increase of atrazine mineralization capacity. The inoculation of strain B-14 (10^6 cells/g) to soil with a low indigenous population of chlorpyrifos-degrading bacteria mixed with 35 mg of chlorpyrifos/kg soil resulted in a higher degradation rate than was observed in non-inoculated soils (Singh et al. 2004). Many reports showed the successful biodegradation of carbofuran by using the isolated degraders (Felsot et al., 1981; Ambrosoli et al., 1996; Chaudhry et al., 2002) and *Pseudomonas* sp. (Felsot et al., 1981). Research by Chaudhry et al. (2002) reported that *Pseudomonas sp.* 50432 isolated from carbofuran-contaminated soil could degrade carbofuran to 4-hydroxycarbofuran. It could also transform carbofuran to 7-phenol by hydrolase. Ambrosoli et al. (1996) reported that *Artheobater, Arthrobacter, Pseudomonas, Bacillus* and *Actinomyces,* isolated from carbofuran treated soil were, able to use carbofuran as a sole carbon source.

As we can see that carbofuran degraders could be a key factor in remediating carbofuran residues in soil. Previous research indicated a promise on rapid degradation of carbofuran in rhizosphere soils. Therefore, the inoculation of carbofuran degraders into rhizosphere soil might improve carbofuran degradation. Thus this study was designed to investigate the ability of rhizosphere soil microorganisms to degrade carbofuran. In order to achieve the main objective, the following sub objectives were 1) to isolate the carbofuran degraders from phytoremediated rhizosphere soil 2) to examine the degradation of carbofuran in rhizosphere soil and 3) to conduct the bioaugmentation treatment of carbofuran by using isolated carbofuran degraders.

1.2 Objectives

1.2.1 Main Objective

To investigate the abilities of rhizosphere soil microorganisms to degrade carbofuran.

1.2.2 Sub Objectives

1.2.2.1 To isolate and identify the carbofuran degraders from carbofuran phytoremediated rhizosphere soil.

1.2.2.2 To examine the degradation of carbofuran in rhizosphere soil.

1.2.2.3 To conduct the bioaugmentation of carbofuran using an isolated carbofuran degrader.

1.3 Hypotheses

1.3.1 Microorganisms around the root zone, rhizosphere microorganisms, of plants grown in soil with a history of carbofuran application can adapt themselves to use carbofuran as a sole C-, N- or C and N-sources.

1.3.2 Bioaugmentation technique can improve carbofuran degradation.

1.4 Scope of the Study

The research was scoped to examine the activities of rhizosphere microorganisms by i) isolating carbofuran degraders from carbofuran phytoremediated rhizosphere soil using an enrichment technique; ii) investigating their abilities to degrade carbofuran as a sole C-, N- and C and N- sources; iii) conducting rhizosphere remediation of carbofuran and iv) applying the isolated carbofuran degrader to rhizosphere soil using bioaugmentation technique.

1.5 Expected Result

An effective carbofuran degrader and its application will be obtained.

CHAPTER II

LITERATURE REVIEW

2.1 Carbofuran

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) (Figure 1) is a broad-spectrum insecticide used to control insects, mites, and nematodes on contact or after ingestion. It is used against soil dwelling and foliar feeding insects of field, fruit, vegetable and forest crops (Extoxnet, 1996). Products containing the active ingredient carbofuran include Furadan, Curaterr, Yaltox, Bay 70143, Carbodan and ENT 27164 (Trotter et al., 1991).

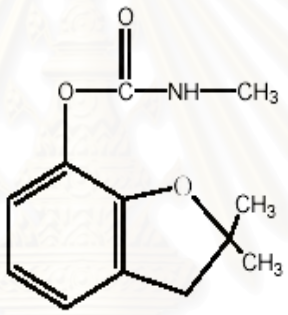


Figure 1 Structure of carbofuran (Evert, 2002)

2.1.1 Properties of carbofuran

Carbofuran is an odorless, crystalline solid varying from colorless to gray depending on the purity of the compound (DeVries and Evans, 1999). Carbofuran is soluble in water (320 mg/L at 25 ^{o}C) (DeVries and Evans, 1999) and has a low adsorption coefficient (K_{oc}= 22, Table 1) resulting in a high potential for groundwater contamination (Howard, 1991). The physicochemical properties of carbofuran were shown in Table 1.

2.1.2 Use of carbofuran

In 2003, Thailand imported carbofuran in liquid and solid form up to 826.2 mt and 45.5 mt, respectively (Chulalongkorn University, 2004). Carbofuran was widely applied in plants and crops growing such as rice, corn, sorghum,

Table 1 Physicochemical properties of carbofuran (IDPID, 1993).

Physicochemical properties	Values
Common name	Carbofuran
Chemical name	2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate
Trade name	Furadan
Empirical formula	$C_{12}H_{15}NO_3$
Molecular formula	$C_8H_6O(CH_3)_2(OOCNHCH_3)$
Molecular weight	221.25
Physical form	Crystalline solid
Melting point	150-153 ^oC
Vapor pressure	8.7 X 10^{-4} mmHg at 25 ^oC
Henry's Law constant	3.9X10^{-9} atm m^3/mol
Octanol/water Partition Coefficient (K_{ow})	17 for 1 ppm at 20 ^oC
	26 for 10 ppm at 20 ^oC
Adsorption Coefficient (K_{oc})	22
Solubility at 25^oC	
- in water	0.07%
- in acetone	15.0%
- in xylene	1.0%
Specific gravity	1.18
Stability-Hydrolysis (half-life at 25 ^oC)	> 20,000 h at pH 3.1
	> 7,000 h at pH 6.2
	13.3-16.4 h at pH 9.1
	2.2 h at pH 9.9

potato, tobacco, banana, cotton, vegetables etc. (Ngampongsai, 1990). In rice fields, Furadan granules 3% (3G) were applied into young plants after 10 days of seeding at the rate of 8 to 10 kg/rai (EZO, 1994). In crop fields, the granular formulations of carbofuran are applied to the soil at the time of seeding. Furadan 5% (5G) could also be applied to potato, onions, turnip and carrot at the rate of 2-5 kg/ha (IDPID, 1993). Carbofuran in liquid form could be applied by ground or aerial equipments. It was registered for use on the same crops as granular formulations (IDPID, 1993).

2.1.3 Sources and environmental fate of carbofuran

The widespread use of carbofuran and other pesticides in order to improve an agricultural productivity provided many possible sources of the pesticide to enter the environment. The pesticides contamination in the environment can be resulted not only from direct use of pesticide in agriculture but also from the runoff of pesticides from croplands and rinsate from cleaning pesticides containers and application equipments (Ferrell, 2003). Because carbofuran was applied directly to the soil, it may be washed off from the soil into nearby bodies of surface water or may percolate through the soil to lower soil layers and groundwater (Extoxnet, 1996). These resulted in adverse effects to human and animals exposed to those contaminated pesticides (Ferrell, 2003).

Carbofuran is moderately persistent in soil. Its half-life in soil is 30 to 120 days depending on temperature, moisture content, pH and numbers of microorganism (DeVries and Evans, 1999). Due to its high water solubility and low adsorption coefficient, carbofuran is relatively mobile in soil and in surface runoff (Cohen, 1996). Carbofuran was sorbed and less mobile in clay soil because of organic matter and clay content (Kumari et at., 1987). The half-lives of carbofuran in water at 25 ^{o}C were 690, 8.2, and 1.0 weeks at pH of 6.0, 7.0, and 8.0, respectively (OHSI, 1991). Carbofuran was detected (1 to 5 µg/L) in water table aquifers beneath sandy soils in New York and Wisconsin (Howard, 1991). Carbofuran has low vapor pressure and low Henry's Law constant (Table 1) resulting in a low tendency to volatilize from water or moist soils (Duel et al., 1979). Shibamoto et al. (1993) reported that 0.3 to 0.66 µg/m^3 of carbofuran were detected after a 44-hour sampling period following an application of 44% active ingredient carbofuran.

Carbofuran and its metabolites have not been observed to accumulate significantly in any biota (Evert, 2002). In the bluegill sunfish, carbofuran and its metabolites all become conjugated and excreted in the urine and bile (Eisler, 1985). Caro et al. (1976) reported that carbofuran was absorbed by roots and transported via plant fluids to other areas such as leaves. Approximately 14% of the applied carbofuran was taken up by the crop (Caro et al., 1976). Carbofuran does not volatilize from water nor does it adsorb to sediment or suspended particles (OHI, 1991). The environmental fate of carbofuran was shown in Figure 2.

2.1.4 Health Effects

Carbofuran is of environmental importance because of its high toxicity through inhalation and ingestion and its moderate toxicity by dermal absorption (Baron, 1991). It causes cholinesterase inhibition in both humans and animals and affects nervous system function (EPA, 2003). Carbofuran has high mammalian toxicity through cholinesterase inhibition (LD_{50} = 2 mg/kg) (EPA, 2003). Rats given very high doses of carbofuran (5 mg/kg·day) for two years showed decreases in weight. Similar tests with mice gave the same results (Baron, 1991). Carbofuran is highly toxic to birds, fish and invertebrates (EPA, 2003). One granule was sufficient to kill a small bird ingested carbofuran granules (EPA, 2003). Smith (1992) reported that red-shouldered hawks were poisoned after eating prey from carbofuran-treated fields. Carbofuran causes highly toxic to fish with the LD_{50} of 0.38 mg/L in rainbow trout and 0.24 mg/L in bluegill sunfish (Kidd and James, 1991). The ecological toxicity of carbofuran was shown in Table 2.

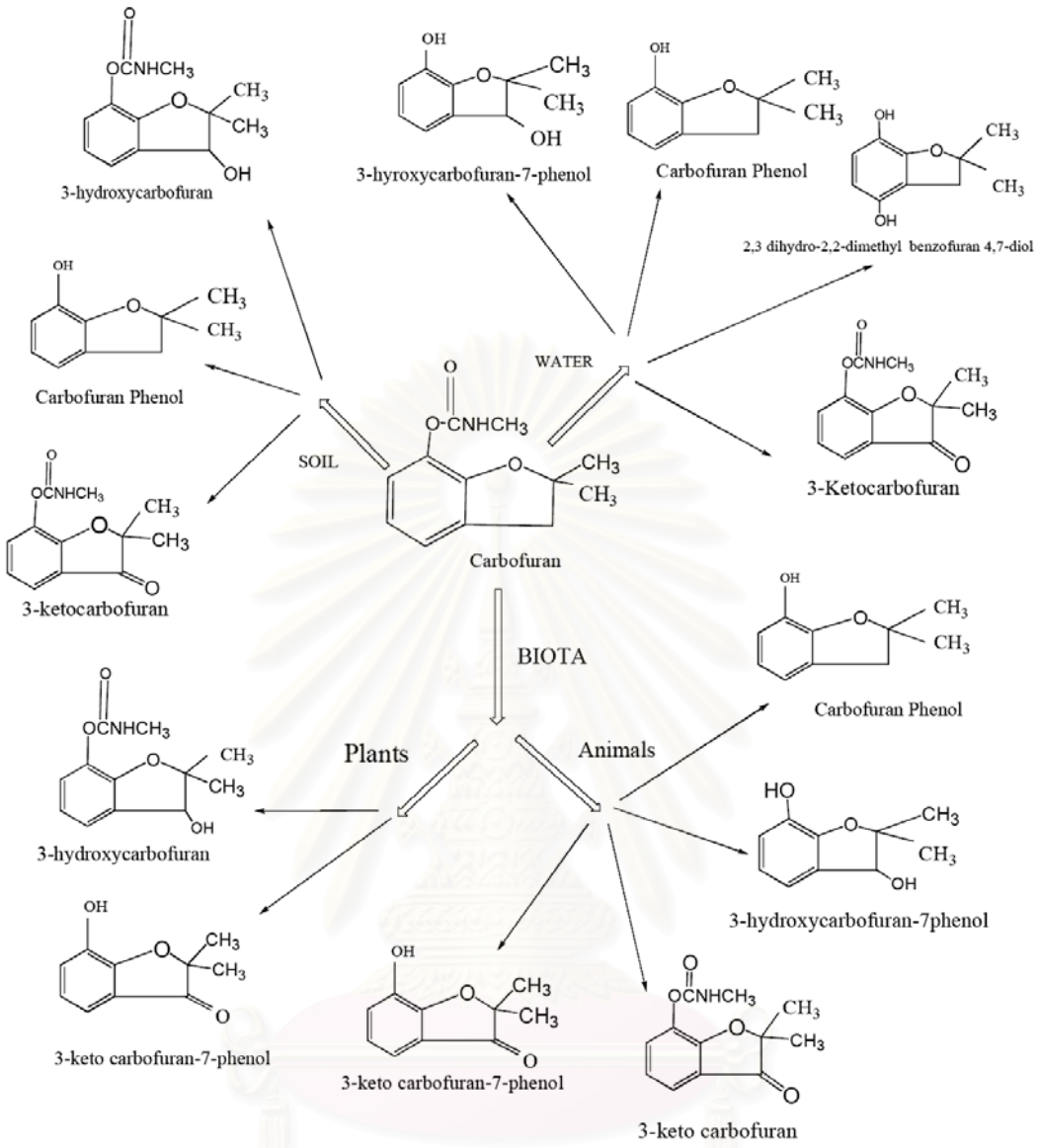


Figure 2 Environmental fate of carbofuran (Evert, 2002)

Table 2 Ecological toxicity of carbofuran (DPR Ecotox database, 2002)

Species	Ecological toxicity	Data
Mallard Duck	LD_{50}	0.4 mg/kg
Mallard Duck	8-Day LC_{50}	190 mg/kg
Bobwhite Quail	LD_{50}	5 mg/kg
Bluegill Sunfish	96-hr LC_{50} (BCF 2-12X)	0.24 mg/kg
Rainbow Trout	96-hr LC_{50}	0.28 mg/kg
Daphnia Magna	48-hr LC_{50}	38.6 µg/kg
Honeybee	48-hr LD_{50}	0.16 µg/bee

2.1.5 Regulatory status

In 1985, the U.S. Environmental Protection Agency (EPA) announced a special review for using of granular carbofuran in the United States because of concerns regarding negative impacts on bird species (IDPID, 1993). A ban on all granular formulation of carbofuran became effective on September 1, 1994. The ban was established to protect birds and was not related to human health concerns (Extoxnet, 1996). There is no ban on liquid formulations of carbofuran. The formulations of carbofuran are in toxicity class I (highly toxic) or class II (moderately toxic). According to the Safe Drinking Water Act set by EPA, the Maximum Contaminant Level Goal (MCLG) and the Maximum Contaminant Level (MCL) for carbofuran were set at 40 parts per billion (ppb). The EPA believes that this level of protection would not cause any of the potential health problems (EPA, 2003).

2.1.6 Degradation of Carbofuran

2.1.6.1 Biological degradation

Biological degradation is the main carbofuran degradation pathway in neutral and acidic conditions. Microorganisms are the key to succeed the bioremediation. Pesticide degraders could be isolated from sites with repeated pesticide application history due to microbial adaptation to use pesticide as the sole C- or N-source (Read, 1983; Morel-Chevillet et al., 1996). There were reports on carbofuran degraders that were isolated from carbofuran-contaminated soil such as *Pseudomonas* sp. (Felsot et al., 1981) and *Pseudomonas* sp. 50432 (Chaudhry et al., 2002), which could degrade carbofuran to 4-hydroxycarbofuran. It could also

transform carbofuran to 7-phenol by hydrolase. Ambrosoli et al. (1996) reported that *Artheobater, Arthrobacter, Pseudomonas, Bacillus* and *Actinomyces* were isolated from carbofuran treated soil. These microorganisms used carbofuran as their sole C-source. A study on the persistence of carbofuran and the effects of carbofuran on microorganisms in soil from paddy fields by Amal et al. (2003) indicated that *Bacillus, Corynebacterium, Aspergillus* and *Phytophthora* could grow in the carbofuran contaminated soil from paddy fields. However, *Pseudomonas, Staphylococcus, Micrococcus, Klepsiella, Humicola* and *Rhizopus* were inhibited. They also reported that carbofuran persistence in soil was only 9 days. The dynamics of carbofuran degraders in soil during three annual applications of carbofuran was studied by Trabue et al. (2001). The result indicated that the carbofuran hydrolysis in treated surface soil after the second application of carbofuran was greater than the first application and that the degradation of carbofuran in soil was a co-metabolic process. Duquenne et al. (1996) studied the effects of inoculum size, microbial distribution, and soil nutrient amendments on the degradation of carbofuran in soil by bacteria strain C28. The study indicated that an increase in the inoculum size and the equal distribution of C28 applied to soil would increase the effectiveness of carbofuran degradation (Duquenne et al., 1996).

Repeated application of carbofuran in soil enhanced the degradation of carbofuran. A study by Read (1983) showed that repeat applications of carbofuran to acid mineral soil reduced the half-life of carbofuran by two times. Morel-Chevillet et al. (1996) reported that previously treated soil with 15 N-methylcabamate enhanced the degradation rate of carbofuran and increased in the number of carbofuran degraders.

2.1.6.2 Chemical degradation

A primary mechanism of carbofuran degradation in soil and water under neutral to basic conditions is chemical hydrolysis resulting in metabolites named carbofuran phenol (Getzin, 1973; Sieber et al., 1978; Yu et al., 1974), hydroxy-7-phenolcarbofuran (Chiron et al. 1996), carbofuran phenol and *N*-methylcarbamic acid via the hydroxylation of the benzofuranyl moiety (Yu et al., 1974). The degradation of carbofuran in acidic soil is slower than in neutral and alkaline soil (Getzin, 1973; Siddaramappa and Sieber, 1979). The study by Mora (1996) indicated that the main process determining the disappearance of carbofuran

form the soil suspension was the abiotic degradation by hydrolysis at the carbamate linkage producing carbofuran phenol as the degradation product. Sieber et al. (1978) reported that as pH and temperature increased the rate of hydrolysis increased. Johnson and Lavy (1995) reported that the dissipation of carbofuran in paddy water was rapid with a half-life of carbofuran of 3 days, compared to 10 days in paddy soil. Chapman and Cole (1982) found that the carbofuran degradation rate in water was strongly influenced by pH. Hydrolysis was also observed to be much more rapid in natural paddy water than deionized (DI) water (Seiber et al., 1978).

2.1.6.3 Physical degradation

The degradation rate of carbofuran in soil could be affected by temperature and moisture content (Ou et al., 1982). The dissipation of carbofuran in water could be influenced by photolysis and volatilization (Sieber et al., 1978; Deuel et al., 1979). Photolysis is a minor route of carbofuran degradation. Photometabolites included 2,3-dihydro-2,2 dimethyl benzofuran-4,7-diol, and 2,3-dihydro-3-keto-2,2-dimethyl benzofuran-7-yl carbamate (or 3-keto carbofuran) (Raha and Das, 1990). Lalah et al. (1996) reported that carbofuran volatilization rates were more rapid in flooded soil than in non-flooded soil because of co-evaporation with the water on the surface of soil. Sunlight and high temperatures have increased the rate of carbofuran loss from water (Siddaramappa and Sieber, 1979). The rate of carbofuran dissipation in soil was also strongly affected by temperature. Yen et al. (1997) found that the half-lives of carbofuran in silty clay loam (pH 6, organic matter 2.9%) were 105 days and 35 days at 15 ^{o}C and 35 ^{o}C, respectively.

2.1.6.4 Degradation pathway of carbofuran

The metabolic degradation pathways of carbofuran were shown in Figure 3.

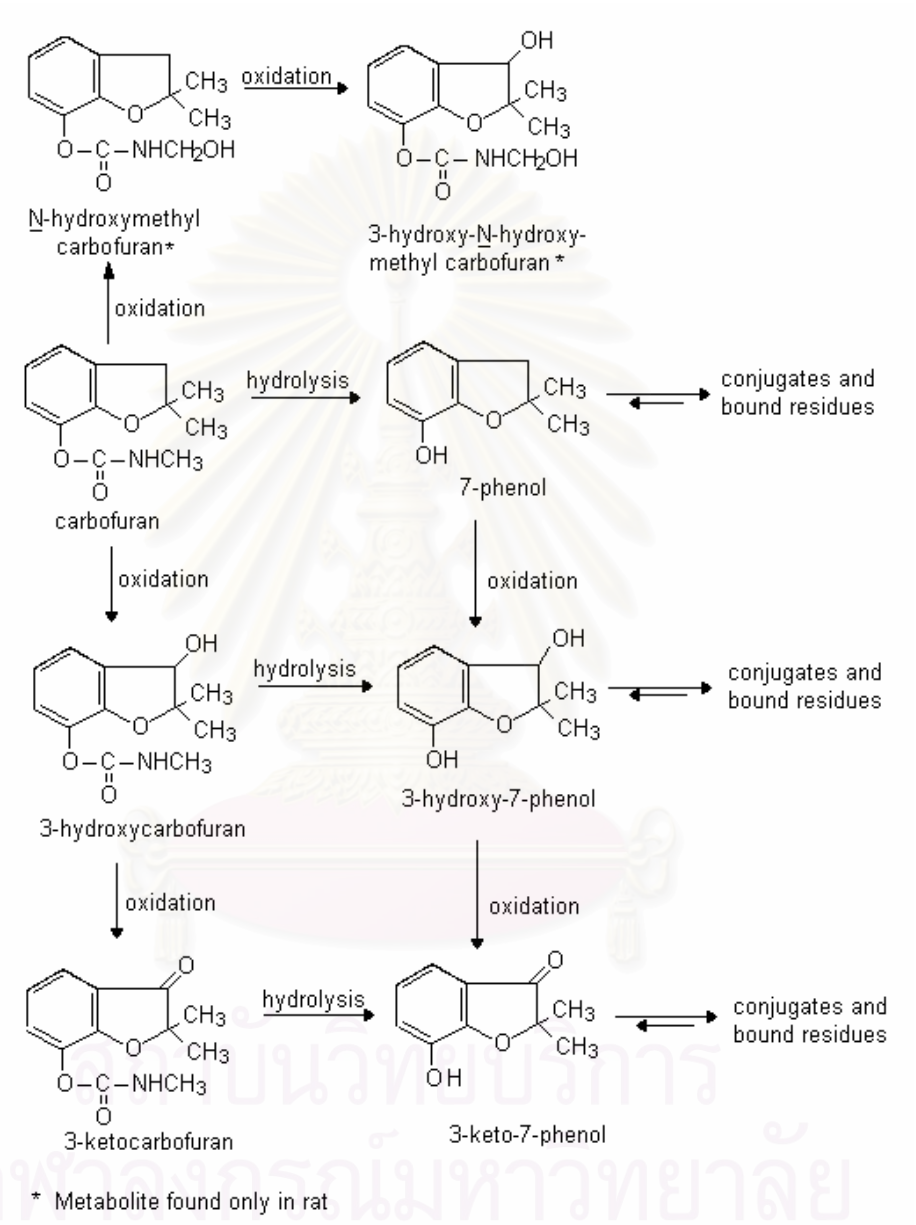
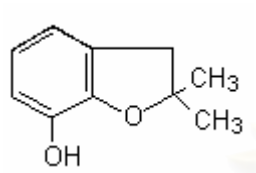


Figure 3 Metabolic degradation pathway of carbofuran (ICPS INCHEM, 2003)

2.1.7 Carbofuran metabolites

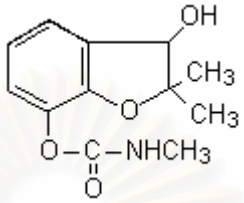
The molecular structures of carbofuran metabolites were shown in Figure 4

CARBOFURAN PHENOL



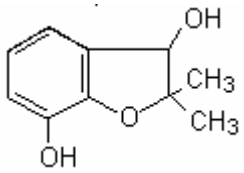
2,3-Dihydro-2,2-dimethyl-7-benzofuranol

3-OH CARBOFURAN



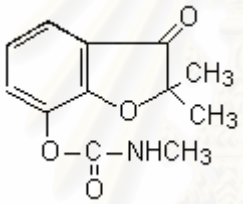
2,3-Dihydro-2,2-dimethyl-3-hydroxy-7-benzofuranol-N-methyl carbamate

3-OH-7-PHENOL



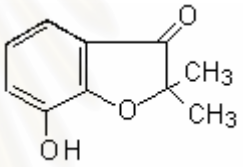
2,3-Dihydro-2,2-dimethyl 3,7-benzofuranol

3-KETO CARBOFURAN



2, 3-Dihydro-2,2-dimethyl-3-oxo-7-benzofuranol-N-methyl carbamate

3-KETO -7-PHENOL



2,3-Dihydro-2,2-dimethyl-3-oxo-7-benzofuranol

Figure 4 Molecular structures of carbofuran metabolites (Mora et al., 1996)

2.2 Phytoremediation

Phytoremediation is a remediation technique using plants and their combinative microorganisms to degrade contaminants in soil and water (Vidali, 2001). Phytoremediation was used for remediating many classes of contaminants including petroleum hydrocarbons, organic solvents, pesticides, explosives, heavy metals, radionuclides and landfill leachates (Susarla et al., 2002). The advantages of phyremediation compared to the other remediation techniques are reducing the amount of waste using low cost, easy to implement and maintain, can be used on variety of organic and inorganic compounds ether in situ or ex situ and aesthetically pleasing to the pupblic (Zynda, 2001). However, the use of phytoremediation is limited by the climatic and geological conditions of the contaminated site,

temperature, altitude, soil type and accessibility by agricultural equipments (Macek et al., 2000).

2.2.1 Processes involved phytoremediation

The US EPA's Phytoremediation Resource Guide classified phytoremediation into 6 types (ITRC, 1999) as followed:

2.2.1.1 Phytoextraction or phytoaccumulation refers to the uptake and translocation of metal contaminants in the soil by plant roots into the aboveground portion of the plants. Certain plants called hyperaccumulator absorb unusually large amounts of metals in comparison to other plants. The contaminants will be accumulated in the stem and leaves of the plants and then will be harvested. The study by Visoottiviseth et al. (2002) showed that ferns were the most proficient plants in accumulating arsenic from soil, attaining concentrations of up to 8350 µg/g (dry mass) in frond.

2.2.1.2 Phytodegradation or phytotransformation is the breakdown of contaminants taken up by plants through metabolic processes within the plant, or the breakdown of contaminants external to the plant through the effect of compounds (such as enzymes) produced by the plants. The examples of pesticides that were phytodegraded were trichloroethylene (TCE) and organophosphorus (OP). TCE in soil and groundwater was transformed by poplar trees (Newman et al., 1997). OP was transformed by plant enzymes of axenically cultivated aquatic plants (Gao et al., 2000b).

2.2.1.3 Phytostabilization is the use of certain plant species to immobilize contaminants in soil and groundwater through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants (rhizosphere). Phytostabilization can result from either physical or chemical effects (Pulford and Watson, 2003). A study by Chaney et al. (1997) showed that Cr and Pb were immobilized by a vegetation cover. Garten (1999) modeled the effects of a forest cover on the loss of ^{90}Sr by leaching from contaminated soil. The results showed that such losses were reduced by approximately 16% under trees relative to grass.

2.2.1.4 Phytovolatilization is the uptake and transpiration of a contaminant by plant, with the release of the contaminant or a modified form of the contaminant to the atmosphere from the plant. Phytovolatilization occurs as growing trees and other plants take up water and the organic contaminants. Some transgenic plants (e.g. *Arabidopsis thaliana*) have converted organic and inorganic mercury salts to the volatile, elemental form (Watanabe, 1997).

2.2.1.5 Rhizodegradation or phytostimulation or rhizosphere biodegradation or enhanced rhizosphere biodegradation or planted-assisted bioremediation/degradation is the breakdown of contaminants in the soil through microbial activity that is enhanced by the presence of the root zone (the rhizosphere) and is a much slower process than phytodegradation. Natural substances released by the plant roots sugars, alcohols, and acids contain organic carbon that provides food for soil microoganisms and additional nutrients enhance their activities. Yu et al. (2003) reported that degradation of butachlor was enhanced greatly in wheat rhizosphere, and especially in rhizosphere inoculated with bacterial community which is capable of degrading butachlor. Enhanced degradation of pentachlorophenol was found in the rhizosphere of wheat grass (Ferro et al., 1994).

2.2.1.6 Rhizofiltration is the adsorption or precipitation of contaminants onto plant roots or the absorption of contaminants into the roots when contaminants are in solution surrounding the root zone. The plants are grown in greenhouses hydroponically (with their roots in water rather than in soil). Once a large root system has been developed, contaminated water is diverted and brought in contact with the plants or the plants are moved and floated in the contaminated water. The plants are harvested and disposed as the roots become saturated with contaminants. A study by Dushenkov et al. (1995) showed roots of terrestrial plant Indian mustard effectively removed heavy metals from aqueous solutions.

2.2.2 The application of phytoremediation

2.2.2.1 Pesticides

Pesticides are common contaminants in soil surface and groundwater (Susarla et al., 2002). Phytoremediation has been effective in treating these types of contaminants. Burken and Schnoor (1997) used poplar trees to uptake and metabolize atrazine. The results showed that poplar trees could take-up, hydrolyze, and dealkylate

atrazine to less toxic metabolites. The transformation of atrazine occurred in roots, stems and leaves. These findings suggested that hybrid poplar trees had the potential for phytoremediation of sites contaminated with atrazine. A study by Gao et al. (2000b) showed that OP compounds were taken up by aquatic plants and subsequently transformed as a function of time. Subsequent plant-mediated transformation of OP compounds was attributed to enzymatic reactions. Wilson et al. (2000) used common cattail (*Typha latifolia*) to uptake metalaxyl and simazine from contaminated water. After 7 days, metalaxyl and simazine activity in solution was reduced by 34 and 65%, respectively, in which activities of both pesticides were detected predominantly in the leaves.

2.2.2.2 Heavy metals

The use of plant as a vegetation cover for the phytoremediation of land contaminated by heavy metals does seem to have considerable potential. Plants can accumulate heavy metals essential to growth and development such as Fe, Mn, Zn, Cu, Mg, Mo, and possibly Ni (Garbisu and Alkorta, 2001). Blaylock et al. (1997) used Indian mustard to demonstrate the capacity of plants to accumulate high concentrations of lead (Pb) when grown in a Pb-contaminated soil. The results showed that the accumulation of Pb in shoots of *Brassica juncea* could be enhanced through the application of synthetic chelates in the soil, facilitating high biomass accumulation as well as Pb uptake. Brown et al. (1994) found that *Thlaspi caerulescens* could tolerate Zn and Cd concentrations up to 18,455 mg/kg Zn and 1,020 mg/kg Cd dry shoots. Ma et al. (2001) found that fern (*Pteris vittata*) could tolerate arsenic concentration of as high as 1,500 μg/g in soil.

2.3 Rhizosphere Degradation

Rhizosphere soil is a small volume of soil that attach or directly adjacent to the plant roots where degradation processes take place by the interaction between contaminants and rhizosphere microorganisms (Stottmeister et al., 2003) (Figure 5). The specialties of rhizosphere soil are the carbon containing compounds such as sugars, alcohols and acids exudated from plant roots stimulating the microbial activities (Schnoor et al., 1995). Garcia et al. (2005) reported that after 6 years of plantation, nutrients, organic matter and microbial activity in the plant rhizosphere

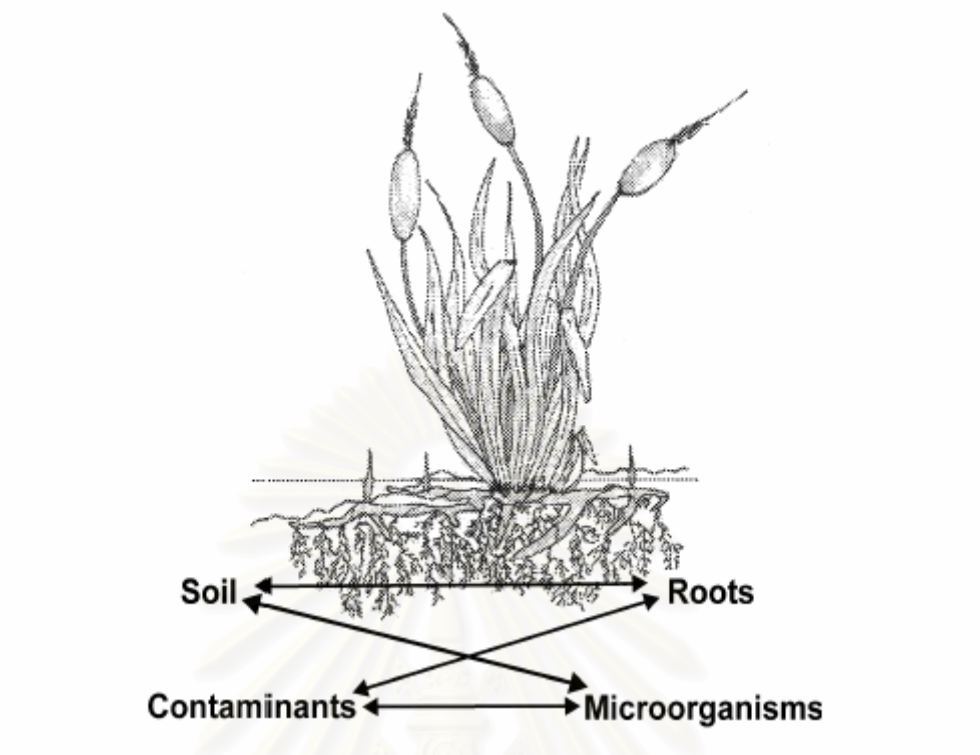


Figure 5 Possible interactions between contaminants and rhizosphere soil microorganisms (Stottmeister et al., 2003)

soil increased compared to non-planted soil. This was because the exudates in the rhizosphere soil supported the growth of microorganisms in which the numbers of microorganisms in rhizosphere soils were 5 to 100 times (approximately 10^8-10^9 cells/g rhizosphere soil) greater than in bulk soil (Gunther et al., 1996).

Rhizosphere degradation is also known as phytostimulation or plant-assisted bioremediation (Schnoor, 1997). It is defined as the acceleration of organic pollutant breakdown in soil as consequences of enhanced degradation activities of the rhizosphere microorganisms (Siciliano and Germida, 1998). Rhizosphere remediation technologies offer potentially cheap and low disturb approaches to remediate the contaminated sites (Anderson et al., 1993). Microorganisms in the root zone, or so-called rhizosphere microorganisms, were reported to be effectively used to remediate organic and inorganic pollutants. The study of Nakamura et al. (2004) found that the Chinese chive rhizosphere microorganism, *Pseudomonas gladioli* M2196, played a significant role in reducing the pentachlorophenol concentration in the soil. Enhanced degradation or mineralization of pesticides, polyaromatic hydrocarbon (PAHs), oil and chlorinated alkanes was demonstrated by Anderson et al. (1993). Jordahl et al.

(1997) reported that the number of benzene, toluene and xylene degraders in the rhizosphere soil of poplar trees were five times more than in bulk soil. Radwan et al. (1998) indicated that hydrocarbon-utilizing bacteria, i.e. *Cellulomonas flavigena, Rhodococcus erythropolis* and *Arthrobacter sp*., were densely adjacent to the roots of Kuwaiti desert plants i.e. *Cyperus conglomeratus, Senecio glaucus, Picris babylonica, Launaea mucronata* and *Salsola imbricata* than in bulk soil. Anderson et al. (1993) demonstrated that, in the rhizosphere soil, the plants exudates built up the organic carbon compounds supporting bacterial activities. This resulted in an increasing of microbial populations improving the degradation of organic contaminants in the rhizosphere zone. The phytoremediation study of Frick et al. (1999) reported that the main mechanism that caused the disappearance of petroleum hydrocarbon was the degradation by rhizosphere microorganisms. Plant root exudated substances stimulated the microbial population at the rhizosphere zone to degrade petroleum hydrocarbon. The study of Gunther et al. (1996) revealed that the number of microorganisms at the rhizosphere zone of ryegrass was higher than that of bulk soil which increased the rate of hydrocarbon degradation. An increase mineralization of tetrachloroethylene from chemical waste dump soil was observed in the rhizosphere soil of *Pinus taeda*, in which the mineralization was doubled in comparison to non-vegetative soil (Anderson and Walton, 1995).

Herbicide degradation was increased in the rhizosphere of the herbicide-tolerant plant, suggesting that rhizosphere interactions between the plant and microorganisms have led to the increased degradation of the pesticides (Frazar, 2000; Anderson et al., 1994). Previous publications have shown that the addition of carbofuran to rhizosphere soil suspension stimulated the oxidation of ammonium up to five times that of untreated soil due to the fact that *Nitorsomonas* sp. associating with rice rhizosphere soil was enriched in the presence of carbofuran (Rmakrishna and Sethunathan, 1982). Sun (2004) indicated that the degradation of aldicarb and oxime carbamate insecticide, in the rhizosphere soil of corn, mung bean and cowpea was more rapid than in unplanted sterile and non-sterile soil. The hizosphere of the selected plant, i.e. *Pennisetum clandestinum*, promoted the degradation of charcoal-fixed atrazine and simazine contaminated in soil. Approximately 45% and 52% of atrazine and simazine, respectively, were degraded in the rhizosphere soil of *P. clandestinum* within 80 days, while only 22% and 20% of the respective herbicide

were degraded in the unplanted soil (Singh et al., 2004). Camper (1999) reported that the pesticides atrazine, mefonoxam and isoxaben contaminated in the rhizosphere system were degraded more rapid than when they were present in bulk soil. Yu et al. (2003) showed that the butachlor degradation could be enhanced in wheat rhizosphere soil especially in the rhizosphere inoculated with the butachlor degrading bacteria.

2.4 Bioaugmentation

Bioremediation is a promising process using natural biological activity to remediate the environmental contaminant until its concentration is below detectable limits or less than the maximum contaminant level (Vidali, 2001). In general, bioremediation uses indigenous microorganisms in the contaminated area as the degraders, which are usually present in very small numbers. In some cases, the indigenous microbes may not have the ability to degrade a particular contaminant at all (United-Tech, 2004). In this instance, there is an interest towards using microorganisms isolated from contaminated media, such as soil or water, as degraders to successfully degrade the target contaminants. The bioremediation technique includes natural attenuation, biostimulation, bioventing, bioaugmentation, landfarming, composting and phytoremediation (Skipper, 1999).

Bioaugmentation is the addition of microbial cultures into the contaminated areas to increase microbial populations and improve a specific biological activity (CRA, 2003). This technique has been practiced intentionally in many areas including wastewater (Rittman and Whiteman, 1994), forestry and agricultural areas (Jasper, 1994). The parameter affecting the effectiveness of bioaugmentation included the contaminants characteristics (e.g. bioavailability, concentration and microbial toxicity), soil physicochemical characteristic (e.g. water, organic mater and clay content), method of inoculation, the present of indigenous activities and capability of the inoculants to degrade the contaminants (Vogel, 1996). The selected strain is the most important parameter for bioaugmentation of a specific contaminant (Singer et al., 2005). Traditional bioaugmentation has achieved its greatest remediation via specific contaminant degrading bacteria isolated from contaminated sites or pollutant repeated application sites (van Veen, 1997; Gilbert and Crowley, 1998).

4.2.1 Bioaugmentation treatment of organic contaminants

There were many reports that presented successful bioaugmentation of pesticides (Rousseaux et al., 2002; Peter et al., 2000) and hydrocarbon (Whyte et al., 1999; Ruberto et al., 2003). The possibility to improve atrazine degradation in soils by bioaugmentation was studied by Rousseaux et al. (2002). The results indicated that an inoculation of the atrazine-mineralizing strain, *Chelatobacter heintzii* Cit1, to the soils that did not present the atrazine mineralization resulted in a 3-fold increase of atrazine mineralization capacity. Peter et al. (2000) demonstrated that oxygenation, coupled with bioaugmentation with enrichments of atrazine-mineralizing bacteria obtained from the contaminated site, *Pseudomonas* sp. strain ADP, decreased the half-life of atrazine mineralization in unamended, anaerobic aquifer material from 730 years to 20 days. However, the oxygenation and bioaugmentation of aquifer material with these strains did not enhance the mineralization of fenamiphos within the time constraints of the experiments. The inoculation of strain B-14 (10^6 cells/g) to soil with a low indigenous population of chlorpyrifos-degrading bacteria mixed with 35 mg of chlorpyrifos/kg soil resulted in a higher degradation rate than what was observed in noninoculated soils (Singh et al. 2004). The study of Whyte et al. (1999) found that the bioaugmentation of the contaminated soils with consortia containing the greatest percentages of hydrocarbon degradative bacteria resulted in the shortest C16 mineralization of hydrocabon acclimation period. Ruberto et al. (2003) reported that bioaugmentation with the B-2-2 strain increased the hydrocarbon bioremediation efficiency (75% of the hydrocarbon was removed). They suggested that autochthonous bacterial flora from Antarctic soils is able to degrade an important fraction of the gas-oil and that bioaugmentation represents a valuable alternative tool to improve bioremediation. Garon et al. (2004) indicated the enhanced biodegradation of fluorine in slurry soil by *A. cylindrospora*, fluorine degrading bacteria, inoculation. The bioaugmentation of the Long Beach soil showed the greatest degradation in the light and heavy total petroleum hydrocarbons compared to the attenuation and biostimulation techniques.

4.2.2 Bioaugmentation in rhizosphere zone

One approach for the enhancement of bioaugmentation is the inoculation of microorganisms, into the contaminated plant rhizosphere zones. The advantage of including plants in the bioaugmentation approach is the supply of the secondary metabolites and root exudates, which stimulate and sustain the inoculants while degrading the pollutants (Singer et al., 2005). Kuiper et al. (2004) selected a strain indigenous to a host plant's rhizosphere, a preferential growth on the plant root exudates, good survival in the rhizosphere and enhanced pollutant degradation ability were observed. A PCB-degrader, *Pseudomonas putida* PML2, was genetic engineered to utilize the predominant root exudates of Arabidopsis for the remediation of polychlorinated biphenyl (PCB) in soil. The results showed that the capability of this engineered strain for degrading PCBs was upon augmentation into the Arabidopsis rhizosphere, while growing on phenylpropanoids, the main root exudates, as a preferential growth substrate (Narasimhan et al. 2003). Yu et al. (2003) reported that the inoculation of wheat rhizosphere soil treated with butachlor by butachlor degrading bacteria enhanced the butachlor degradation in rhizosphere soil.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Chemicals and Reagents

Carbofuran (98% purity) and carbofuran phenol (99% purity) were purchased from Sigma-Aldrich, USA and 3-keto carbofuran (98.5% purity) was purchased from Ehrenstorfer Quality, Germany. Methanol and acetonitrile (HPLC grade) were purchased from Merck, Germany. Dichloromethane (analytical grade) was purchased from BDH, England.

3.2 Isolation of Carbofuran Degraders from Carbofuran Phytoremediated Rhizosphere Soils

3.2.1 Microorganism media

Basal Salt Medium (BSM) (in g/L) contained: 5.57 of $NaHPO_4$, 2.44 of KH_2PO_4, 2.00 of NH_4Cl, 0.20 of $MgCl_2.6H_2O$, 0.0004 of $MnCl_2.4H_2O$, 0.001 of $FeCl_3.6H_2O$ and 0.001 of $CaCl_2$. In order to isolate the carbofuran degraders which possessed the ability to use carbofuran as its sole C-source or sole N-source or a sole C and N-sources, three types of BSM were used. In the first media, C-limited BSM, 5 mg/L of carbofuran was used as a sole C-source. In the second media, N-limited BSM, 5 mg/L of carbofuran was used as a sole N-source instead of NH_4Cl and 10 g/L of glucose was used as C-source. In the last media, C and N-limited BSM, 5 mg/L of carbofuran was used as sole C and N sources. Complete media was BSM containing 5 mg/L of carbofuran and 10 g/L of glucose as carbon sources. pH of the media was adjusted to 7 before autoclaved at 121 °C for 15 min. Carbofuran solution in methanol was added after the media was sterilized. For the BSM agar, 1.5% of bactoagarose was added to BSM before sterilization.

3.2.2 Phytoremediated rhizosphere soil samples

Rhizosphere soil of rice (*Oryza sativa* L.), corn (*Zea mays* L.) and Chinese kale *(Brassica alboglabra* C.) were collected from the previous study of phytoremediation of carbofuran residues in rice field soil by Teerakul (2004). In that

study, soil was mixed with 5 mg/kg soil of carbofuran before being planted with rice, corn and Chinese kale. The soil was taken from Ban Non-reung, A. Muang, Khon Kean Province at the depth of 0-30 cm and classified as loam. Rhizosphere soils from rice and corn were chosen because the half-lives of carbofuran in soils under these two plants were short i.e. 2 and 3 days, respectively, (Teerakul, 2004) suggesting the successful phytoremediation of carbofuran by these two plants. The soil under Chinese kale was chosen to compare with soils under rice and corn because of longer half-life of carbofuran in Chinese kale soil than in the soil under rice and corn i.e. 6 days. Rhizosphere soils were taken after day 120 of carbofuran phytoremediation by shaking out the extra soil around the root zone and then the soils attached to the roots were collected and kept in plastic bag at 4 °C prior to use.

3.2.3 Isolation of carbofuran degraders from carbofuran phytoremediated rhizosphere soils by an enrichment technique

3.2.3.1 Enrichment technique

An enrichment technique was used to isolate carbofuran degraders from carbofuran phytoremediated rhizosphere soil. Five grams of rhizosphere soil from each plant was added to 100-mL C-, N- and C and N-limited BSM and complete media containing 5 mg/L of carbofuran. Flasks were incubated at 30 °C and shaken at 150 rpm. Every 7 days, the media were transferred to a fresh media containing 5 mg/L of carbofuran, incubated at 30 °C and shaken at 150 rpm. This step was repeated four times to obtain soil-free enrichment.

3.2.3.2 Enhancement of carbofuran degradation ability of the isolates carbofuran degraders

The carbofuran degradation ability of carbofuran degraders in soil-free enrichment media was enhanced by inoculating 10% of the soil-free enriched culture from the last step into fresh C-, N- and C and N-limited BSM and complete medium containing 20 mg/L of carbofuran. Flasks were incubated at 30 °C and shaken at 150 rpm. At day 0 and 7, the media were sampled and extracted by the liquid-liquid partitioning method and checked for its carbofuran concentration by HPLC. At day 7, the culture was transferred to a fresh C-, N- and C and N-limited BSM and complete medium containing 40 mg/L of carbofuran, incubated at 30 °C, shaken at 150 rpm and sampled at day 0 and 7 to check for carbofuran concentration. This process was

repeated once again but the concentration of carbofuran in the media was 100 mg/L. After the enhancement at 100 mg/L of carbofuran, all cultured BSM were streaked on their own culture BSM agar coated with 5 mg/L of carbofuran to obtain single colonies.

3.2.3.3 Comparison of carbofuran degradation ability of isolated carbofuran degraders

A one loop-full of each different isolated colony was inoculated into 100-mL of nutrient broth containing 5 mg/L of carbofuran to increase the number of microorganisms. Flasks were incubated at 30 ^{o}C and shaken at 150 rpm for 36 hours. Cells were harvested by centrifugation in HDPE tubes at 10,000 rpm for 10 min. The supernatant was discarded, and the pellets of the isolates were resuspended in sterile 0.85% NaCl with a total volume of 50 mL. Each resuspended culture was adjusted to 10^6 cells/mL and then inoculated into C-, N- and C and N-limited BSM containing average 5.6 mg/L of carbofuran, incubated at 30 ^{o}C and shaken at 150 rpm. The culture media was sampled at every 2 days for 30 days, extracted by the liquid-liquid partitioning method and analyzed for carbofuran and its metabolites concentrations by HPLC. The numbers of the isolates were determined by the spread plate technique (plate count on BSM agar coated with 5 mg/L of carbofuran). Control was C-, N- and C and N-limited BSM containing average 5.6 mg/L of carbofuran without the isolates. The half-lives of carbofuran in the culture media were calculated by fitting with a modified first-order kinetic model; $C = C_0e^{-kt} + Ya$, where C was the mean concentration of carbofuran as a function of time in hours (mg/L), C_0 was the initial carbofuran concentration (mg/L), k was the rate constant (/day), t was time (days) and Ya was an asymptotic estimate of the concentration of carbofuran that degrades very slowly overtime (residual carbofuran) (mg/L). The mean concentrations used in the regression were weighed with inverse of the variance squared, S^{-2}. This method compensated for the non-constant variance and helped to improve the estimation of the parameters. The coefficients of determination, r^2, between 0.80-0.99 would indicate the good fit of the data to the fist-order kinetic model. The isolate with the shortest half-life of carbofuran in BSM was considered as “the best” carbofuran degrader. This isolate was further used in the bioaugmentation study.

3.2.3.4 Identification of isolated carbofuran degraders

The isolates in the culture media that had half-lives of carbofuran less than 10 days were gram stained. The gram negative-rod bacteria were identified using the API 20 NE system (bioMérieux sa, France). The API 20 NE system is a standardized micromethod combining 8 conventional tests and 12 assimilation tests for the identification of non-fastidious Gram-negative rods not belonging to the *Enterobacteraceae* (e.g. *Pseudomonas, Acinetobacter, Flavobacterium, Moraxella, Vibrio, Aeromonas,* etc.). Percentage of identification by API 20 NE system greater than 80% was acceptable. The other microorganisms were sent to be identified at the Thailand Institute of Science and Technology Research, Bangkok.

3.2.3.5 Extraction of carbofuran residues from culture media by the liquid-liquid partitioning method

Two-mL of each culture media was taken and added with 2 mL of methanol and then sonicated for 10 min, 50/60 voltage cycle, for two times. Liquid-liquid partitioning of sonicated culture media to extract carbofuran and its metabolites i.e. carbofuran phenol and 3-keto carbofuran from media was done by extracting the media three times with 4, 2 and 2 mL of dichloromethane, respectively, in separation funnel. The organic fraction of the samples was evaporated to dryness in the fume hood and then redissolved in 4 mL of acetonitrile and passed through a 0.45 µm nylon membrane syringe filter before analyzing by HPLC. The percent recovery of this extraction technique was 98%.

3.2.3.6 An analysis of carbofuran and its metabolites concentrations in the culture media by HPLC

The concentrations of carbofuran, carbofuran phenol and 3-keto carbofuran in extracts were analyzed by Shimadzu 10-A HPLC equipped with 4.6x150 mm-Lunar 0.5 µm C-18 column (Phenomenex, USA), a UV detector operating at 220 nm and a 20 µL injector loop. The operating parameters were: mobile phase, methanol-water (60:40); flow rate, 1 mL/min at the ambient temperature. External standard linear calibration curves of carbofuran, carbofuran phenol and 3-keto carbofuran were used to quantify their concentrations in the aqueous phase. The observed concentration was characterized by its peak areas. The

detection limits of the HPLC for analyzing carbofuran, carbofuran phenol and 3-keto carbofuran were 0.07, 0.01 and 0.01 mg/L, respectively.

3.3 Dissipation of Carbofuran in Rhizosphere Soils by Rhizosphere Microorganisms.

3.3.1 Soils

The rhizosphere soils of six weeds, i.e. Umbrella sedge*; Cyperus iria* Linn. (กกทราย), Water primrose; *Jussiaea linifolia* Vahl. (เทียนนา), Fuzzy flatsedge; *Cyperus pilosus* Vohl. (กกสามเหลี่ยมเล็ก), Small flower umbrella plant; *Cyperus difformis* Linn. (กกขนาก), Tall-fringe-rush hoorah grass; *Fimbristylis miliacea* Vahl. (หนวดปลาดุก) and Cover fern; *Marsilea crenata* Presl. (ผักแว่น), grown in a rice field of Ban Nonmuang, A. Muang, Khon Kean Province, were collected and used throughout this study. These soils were classified as sandy loam. The rice field has a history of carbofuran application. It is hoped that the soil at the root zone of these plants would have rhizosphere microorganisms capable of degrading carbofuran. Rhizosphere soils were collected by digging around and close to the plant roots with the depth to approximately 15 cm. below ground. The extra soil around the root zone was shaken out and the rhizosphere soils which attached to the root were then collected and passed through a 2 mm sieve. Bulk soil was taken, by avoiding the plant roots, from the same site and then passed through 2mm sieve. Both rhizosphere soils and bulk soil were placed in plastic bags and kept at 4 °C before using in the experiment.

3.3.2 Dissipation of carbofuran in rhizosphere soils

A 20 g of each rhizosphere soil was mixed with carbofuran at an average concentration of 6.34 mg/kg dry soil in a 425 cm^3 glass jar capped with a plastic lid. The initial moisture content was adjusted to 15-18% by sterile distilled water. The jars were incubated at room temperature for 100 days. At days 0, 7, 14, 21, 35, 70 and 100 days, samples were sacrificed and extracted by using ASE 100 accelerate solvent extractor and then the concentrations of carbofuran and its metabolites were determined by HPLC. At each sampling date, the numbers of microorganisms in the rhizosphere soils were determined by the spread-plate technique on nutrient agar. The numbers of carbofuran degraders were counted on the

BSM agar coated with 5 mg/L of carbofuran. The half-lives of carbofuran in rhizosphere soils were calculated by fitting with a modified first-order kinetic model: $C = C_0e^{-kt} + Ya$, where C is the mean concentration of carbofuran as a function of days (mg/kg), C_0 is the initial carbofuran concentration (mg/kg), k was the rate constant (/day), t was time (days) and Ya was an asymptotic estimate of the concentration of carbofuran that degrades very slowly over time (residual carbofuran) (mg/kg). Three control sets of this experiment were conducted in similar manners but the soil samples were changed from the rhizosphere soils to autoclaved rhizosphere soils, bulk soil and autoclaved bulk soil. The rhizosphere soil of the plant that possessed the shortest half-life of carbofuran was further used in the bioaugmentation study.

3.3.3 Extraction of carbofuran and its metabolites from rhizosphere soil samples

Carbofuran and its metabolites were extracted from rhizosphere soil samples by using an ASE 100 accelerated solvent extractor (Dionex, Austria) equipped with 11-mL stainless-steel extraction cell. The extraction cell was prepared as follows: the bottom of the extraction cell was cover with a cellulose acetate membrane (16.2 mm I.D., Dionex, Austria) as a filter agent to prevent a frit blockage and fine soil breakthrough into the collection bottle. Afterwards, 13 g of dried rhizosphere soil sample was transferred into the extraction cell and finally, the top of the extraction cell was covered with a cellulose acetate membrane as filter agent before closed. The samples were extracted under the conditions which are 100 ^oC extraction temperature, 5 min static extraction time, methanol (HPLC grade) as the extraction solvent and two extraction cycles. The ASE parameters were used according to the default settings, i.e. the maximum extraction pressure was set not to exceed 1,500 psi, the flush volume was 60% of the extraction cell volume, and the N_2-purge time was set to 1 min. After static extraction, the raw extracts collected in a special 200-mL glass bottle were adjust to the volume of 25 mL in volumetric flask and passed through 0.45 μm nylon membrane syringe filter prior to analyze by HPLC as the method described above. The percent recovery of this extraction method was 96%.

3.4 Bioaugmentation of Carbofuran

A one loop-full of “the best” isolated carbofuran degrader was inoculated into 100-mL of nutrient broth containing 5 mg/L of carbofuran. Flasks were incubated at 30 ^{o}C and shaken at 150 rpm for 36 hours. Cells were harvested by centrifugation in HDPE tubes at 10,000 rpm for 10 min. The supernatant was discarded, and the isolates were resuspended in sterile 0.85% NaCl for a total volume of 50 mL. The resuspended culture, at the cell concentration of 10^5 cells/g soil, was inoculated into 20 g of the rhizosphere soil of the plant that possessed the shortest half-life of the carbofuran in a 425 cm^3 glass jar capped with a plastic lid. Carbofuran solution with an average concentration of 5.4 mg/kg dry soil was added to the soil in the glass jar and well mixed by hand stirring. The initial moisture content of the samples was adjusted to 15-18% before incubating at room temperature avoiding sunlight. The rhizosphere soil samples were sacrificed and collected at days 0, 5, 10, 15, 25, 35 and 50. Soil samples were extracted using an ASE 100 accelerate solvent extractor and then carbofuran and its metabolite concentrations were determined using HPLC. At each sampling date, the numbers of microorganisms in the rhizosphere soils were determined by the spread-plate technique on nutrient agar. The numbers of carbofuran degraders were counted on the BSM agar coated with 5 mg/L of carbofuran. The half-lives of carbofuran in rhizosphere soils were calculated by fitting with a modified first-order kinetic model: $C = C_0e^{-kt} + Ya$, where C is the mean concentration of carbofuran as a function of in days (mg/kg), C_0 is the initial carbofuran concentration (mg/kg), k is the rate constant (/day), t is time (days) and Ya is an asymptotic estimate of the concentration of carbofuran that degrades very slowly overtime (residual carbofuran) (mg/kg). Three control sets of this experiment were conducted in similar manners but the soil samples were changed from the rhizosphere soils to autoclaved rhizosphere soil, bulk soil and autoclaved bulk soil.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Degradation of Carbofuran by Carbofuran Degraders

4.1.1 Isolation of carbofuran degraders and their ability to use carbofuran as their energy sources.

An enrichment technique was used to isolate carbofuran degraders from the carbofuran phytoremediated rhizosphere soil of rice, corn and Chinese kale. The carbofuran degraders were isolated in C-, N- and C and N-limited BSM containing 5 mg/L of carbofuran. Four-times subcultures were done to achieve soil free enrichement C-, N- and C and N-limited BSM. The carbofuran degradation ability of the isolated degraders was enhanced sequentially from 5, 20, 40, up to 100 mg/L of carbofuran. Preliminary results showed that as the initial carbofuran concentration in BSM increased, the percentage of dissipation of carbofuran decreased (Table 3). This may due to the toxicity of carbofuran to the isolates at high concentration. The OD of the enriched culture should be examined to confirm this result.

The percentage of carbofuran dissipation by carbofuran degraders isolated from rhizosphere soil of rice, corn and Chinese kale were not significantly different in all kinds of BSM (Table 3). Carbofuran dissipation in C-, N- and C and N-limited BSM indicated that the enriched cultures used carbofuran as their sole C-, N- or C and N-sources, respectively (Table 3). According to Chaudhry and Ali (1988), there were three groups of carbofuran degrading bacteria. Groups 1 and 2 were capable of hydrolyzing carbofuran and used hydrolysis products as their sole C- or sole N- source, respectively. Group 3 had a capability to hydrolyze carbofuran and its metabolite mainly carbofuran phenol as a sole C- and N- sources.

The percentage of carbofuran dissipation in complete media was low compared to in the other media (Table 3). This may due to the fact that glucose (C-source) and $NHCl_4$ (N-source) in complete media were easier to use than carbofuran. Duquenne et al. (1996) reported that in the presence of glucose, carbofuran degradation rate markedly reduced in soil samples inoculated with the strain C28.

Table 3 Dissipation of carbofuran (%) by enriched culture from phytoremediated rhizosphere soils of rice, corn and Chinese kale at day 7 of incubation.

Initial carbofuran concentration (mg/L)	Carbofuran dissipation (%)[a]											
	C-limited BSM			N-limited BSM			C&N-limited BSM			Complete media		
	rice	corn	Chinese kale	rice	corn	Chinese kale	rice	corn	Chinese kale	rice	corn	Chinese kale
5	98.6	90.4	92.6	91.8	88.0	92.6	87.4	73.9	87.1	9.6	31.0	34.0
20	65.9	55.9	58.8	50.9	41.1	43.3	33.6	24.5	25.8	6.5	14.1	11.5
40	52.3	51.4	57.1	45.5	25.8	32.3	24.4	19.7	27.4	12.0	27.5	11.2
100	20.8	14.5	20.3	20.9	16.2	28.4	15.3	8.8	16.6	6.5	11.5	8.4

[a] Carbofuran dissipation was calculated by $\frac{(\text{conc. at day 0} - \text{conc. at day 7}) \times 100}{\text{conc. at day 0}}$

4.1.2 Biodegradation of carbofuran by isolated carbofuran degraders

After the enhancement of the enriched culture at 100 mg/L of carbofuran in all kinds of media, the culture media from C-, N- and C and N-limited BSM were streaked on their own culture BSM agar coated with 5 mg/L of carbofuran to obtain single colonies. According to the colonies morphology, there were 13-different single colonies growing on the BSM agar (Table 4). Isolates grown on C-, N- and C and N-limited BSM were designated by PCL, PNL and PCNL, respectively (Table 4). There were 7, 4 and 2 isolates growing on C-, N- and C and N-limited BSM, respectively (Table 4).

The carbofuran degradation abilities to degrade the carbofuran of each isolate were examined in its own isolation media containing an average of 5.6 mg/L of carbofuran. The degradation of carbofuran in C-, N- and C and N-limited BSM were shown in Figures 6, 7 and 8, respectively. The corresponding kinetic data fitting to a modified first-order kinetic model was tabulated in Table 5. The coefficients of determination, r^2, ranged between 0.80-0.99 indicating a good fit of the data to the first-order kinetic model (Table 5).

Carbofuran was rapidly degraded by isolated degraders with half-lives of less than 10 days (Table 5) except for the isolates PCL1 and PCL7 in which the half-lives of carbofuran were 33 and 28 days, respectively (Table 5). In control, there was no significant decrease in carbofuran concentration (Figures 6, 7, 8). These results indicated that an inoculation of isolated carbofuran degraders, except for the isolate PCL1 and PCL7, accelerated carbofuran degradation in the BSM. This explanation was supported by the growth of each isolate carbofuran degrader in C-, N- and C and N-limited BSM in which the growth of carbofuran degraders in each kind of BSM increased up to 10-10000 times during incubation (Figures 9, 10, 11) indicating that carbofuran was incorporated as the biomass of the degraders. These findings were similar to the research conducted by Bano and Musarrat (2004) who reported that carbofuran degrader, *Pseudomonas* sp., NJ-101, used carbofuran as its sole C- and N-sources. In addition, Ambrosoli et al. (1996) demonstrated that *Arthrobacter, Pseudomonas, Bacillus* and *Actinomyces* isolated from carbofuran treated soil used carbofuran as a sole C-source and accelerated the carbofuran degradation in the BSM.

Table 4 Morphology of the carbofuran degraders isolated from carbofuran phytoremediated rhizosphere soil on BSM agar

Isolate names	Morphology
PCL1	white, circular , entire, convex, smooth, opalescent, diameter of 0.5 mm
PCL2	cream, circular , entire, convex, smooth, opalescent, punctiform
PCL3	white-pink, circular , entire, convex, smooth, opalescent, punctiform 0.5-1 mm
PCL4	white, lenticular, entire, convex, smooth, opaque, diameter of o.5-1 mm
PCL5	white, circular , entire, convex, smooth, opalescent, diameter of 1 mm
PCL6	pink, circular , entire, convex, smooth, opaque, diameter of 1-1.5 mm
PCL7	white, circular, entire, convex, smooth, opalescent, diameter of 0.5-1 mm
PNL1	white, circular , entire, convex, smooth, opalescent, rubbery, diameter of 2-5 mm
PNL2	white, circular , entire, convex, smooth, translucent, rubbery, diameter of 2-4 mm
PNL3	white, circular , entire, convex, smooth, opalescent, diameter of 1-2 mm
PNL4	orange, circular , entire, convex, smooth, opalescent, diameter of 1-1.5 mm
PCNL1	cream, circular , entire, convex, smooth, opaque, punctiform
PCNL2	white, circular , entire, convex, smooth, opalescent, punctiform

PCL = Isolates cultured from C-limited BSM

PNL = Isolates cultured from N-limited BSM

PCNL = Isolates cultured from C and N-limited BSM

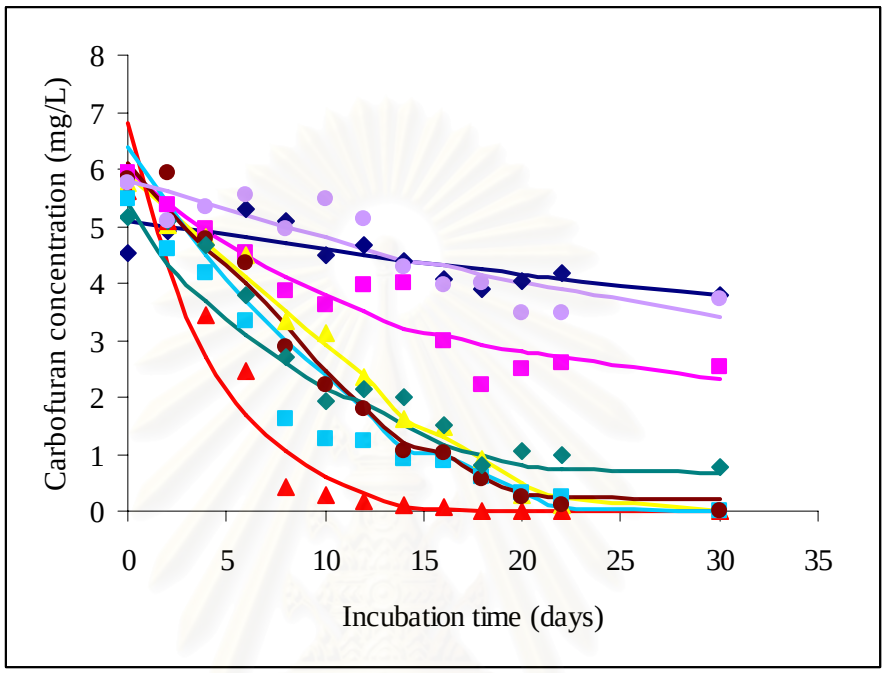


Figure 6 Degradation of carbofuran by isolated carbofuran degraders cultured in C-limited BSM, (◆ = non-inoculate, ■ = isolate PCL1, ▲ = isolate PCL2, ▲ = isolate PCL3, ■ = isolate PCL4, ● = isolate PCL5, ◆ = isolate PCL6, ● = isolate PCL7; lines = carbofuran concentrations in C-limited BSM fitted to the first-order kinetic model)

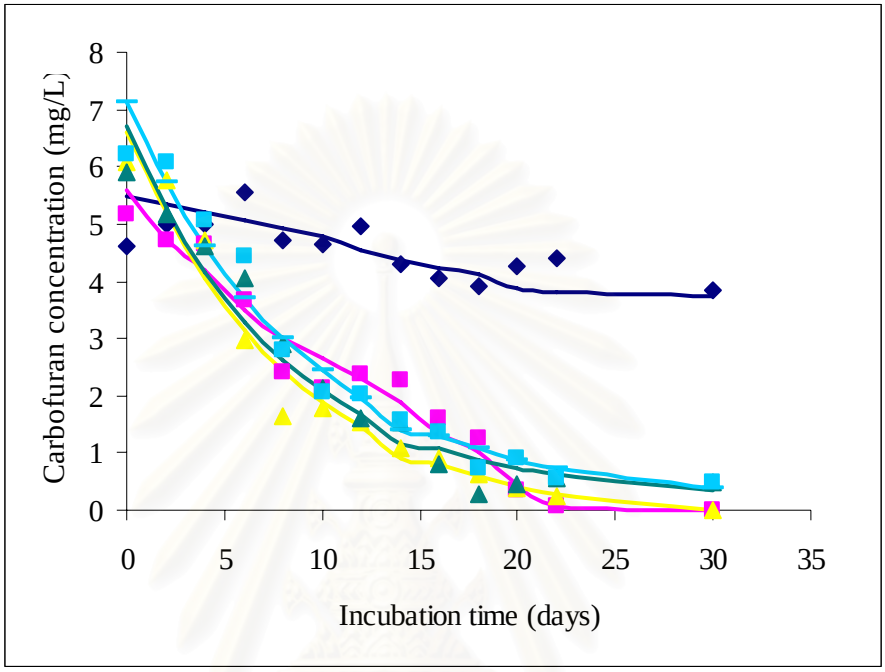


Figure 7 Degradation of carbofuran by isolated carbofuran degraders cultured in N-limited BSM, (◆ = non-inoculate, ■ = isolate PNL1, ▲ = isolate PNL2, ▲ = isolate PNL3, ■ = isolate PNL4; lines = carbofuran concentrations in N-limited BSM fitted to the first-order kinetic model)

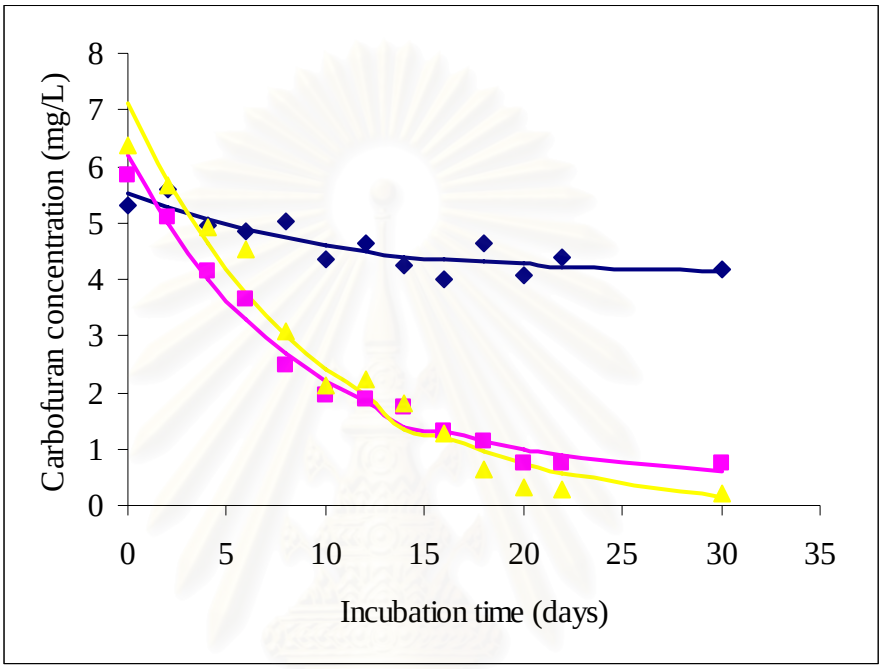


Figure 8 Degradation of carbofuran by isolated carbofuran degraders cultured in C and N-limited BSM, (◆ = non-inoculate, ▲ = isolate PCNL1, ■ = isolate PCNL2; lines = carbofuran concentrations in C and N-limited BSM fitted to the first-order kinetic model)

Table 5 Degradation rate constants (k) and half-lives ($t_{1/2}$) of carbofuran in BSM growing with isolated carbofuran degraders

Isolate names	k_1, day^{-1}	$t_{1/2}$, days	r^2*
PCL1	0.022	33	0.87
PCL2	0.077	9	0.99
PCL3	0.218	3	0.92
PCL4	0.115	6	0.97
PCL5	0.070	10	0.98
PCL6	0.084	8	0.91
PCL7	0.024	28	0.80
PNL1	0.089	8	0.81
PNL2	0.115	6	0.97
PNL3	0.125	6	0.93
PNL4	0.110	6	0.99
PCNL1	0.115	6	0.97
PCNL2	0.103	7	0.96

*Coefficients of determination for non-linear regressions

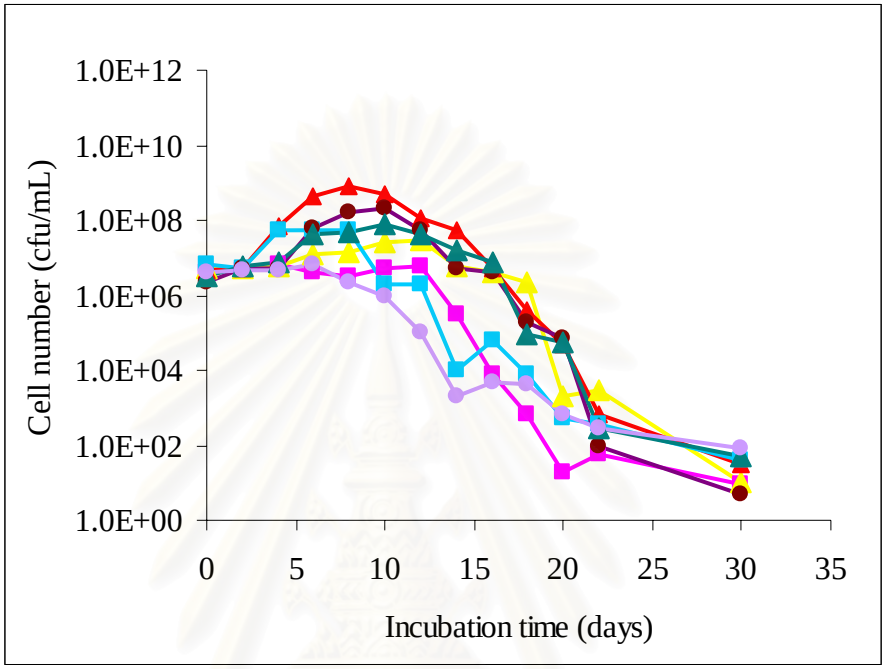


Figure 9 Growth of isolated carbofuran degraders in C-limited BSM during incubation, (■ = isolate PCL1, ▲ = isolate PCL2, ▲ = isolate PCL3, ■ = isolate PCL4, ● = isolate PCL5, ◆ = isolate PCL6, ● = isolate PCL7)

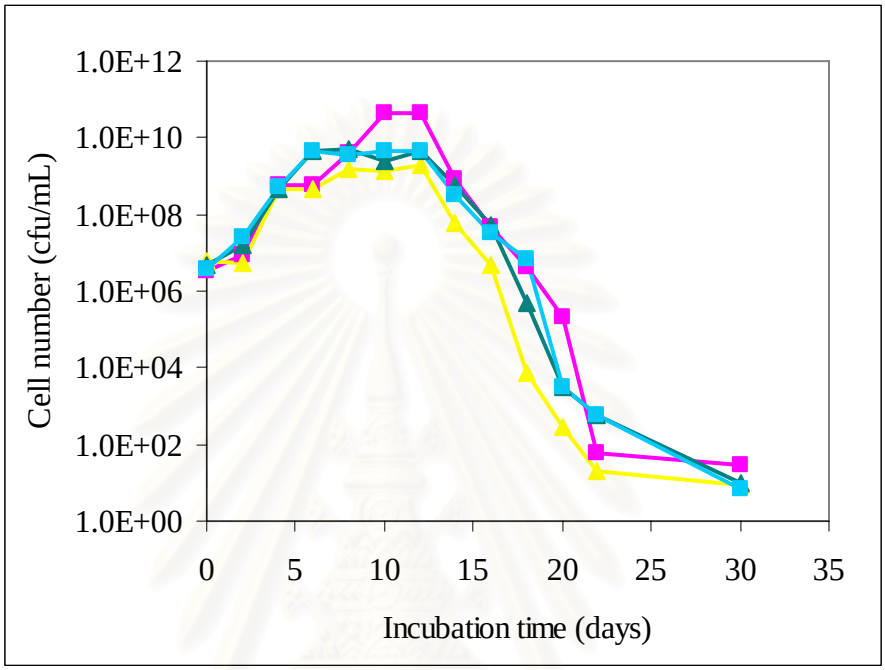


Figure 10 Growth of isolated carbofuran degraders in N-limited BSM during incubation, (■ = isolate PNL1, ▲ = isolate PNL2,▲ = isolate PNL3, ■ = isolate PNL4)

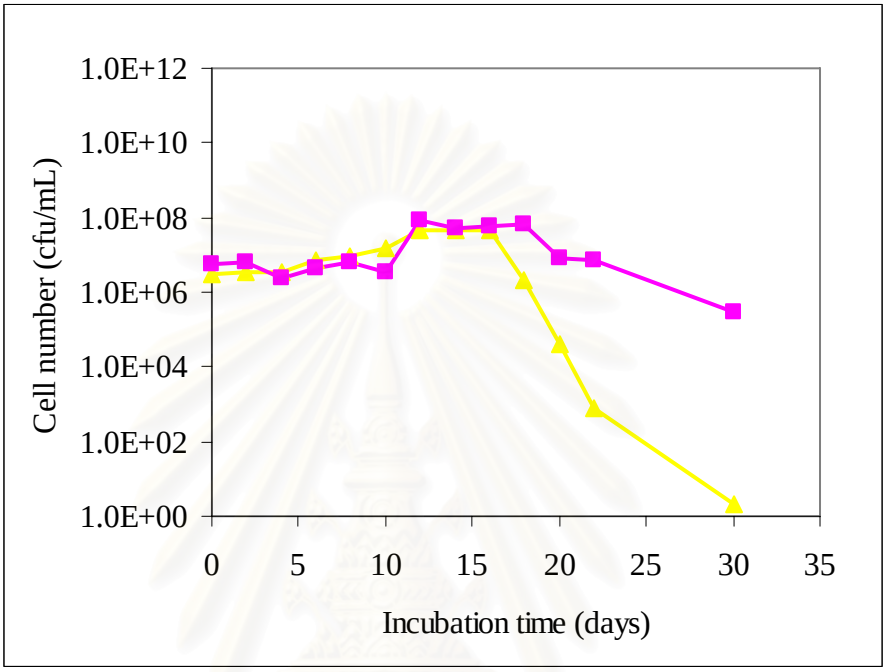


Figure 11 Growth of isolated carbofuran degraders in C and N-limited BSM during incubation, (▲ = isolate PNL1, ■ = isolate PNL2)

From our results, the isolate PCL3, which used carbofuran as a sole C-source, could degrade carbofuran rapidly with the shortest half-life of carbofuran, 3 days, in C-limited BSM (Table 5) and was considered as “the best” carbofuran degrader and urther used in the bioaugmentation study.

The metabolites of the carbofuran observed in this study were carbofuran phenol and 3-keto carbofuran (Tables 6-9). The results were in agreement with Chapalamadugu and Chuadry (1992) which discovered that hydrolysis was the main metabolism of carbofuran yielding carbofuran phenol and methylamine as the degradation products. Methylamine would be further used by microorganisms for their growth. Carbofuran was reported to be metabolized to carbofuran phenol by *Pseudomonas, Arthrobacter* and *Bacillus* sp., which was then degraded to an undetectable compound (Cain and Head, 1991). Carbofuran phenol could be degraded further via ring cleavage yielding CO_2 and H_2O by carbofuran degraders (Trabue et al., 1997). 3-Keto carbofuran concentration in C-, N- and C and N-limited BSM were shown in Tables 8 and 9. This metabolite was observed in both inoculated and non-inoculated BSM (Tables 8, 9). We speculated that photoxidation, abiotic degradation processe, involved the carbofuran degradation in the BSM. Evert (2002) reported that 3-keto carbofuran was found as a degradation product of carbofuran in water. Kale et al. (2001) found that 3-keto carbofuran was the significant metabolite present under flooded conditions. Since carbofuran phenol and 3-keto carbofuran were reported as the metabolites of carbofuran resulted from the activities of hydrolase (Tomasek and Karns, 1989) and oxidase enzyme (Evert, 2002), respectively, therefore, we speculated that our degraders produced enzyme oxidase responsible for degrading carbofuran. The study on the activities of these two enzymes should be conducted to confirm our conclusions.

Table 6 Concentrations of carbofuran phenol in C-limited BSM cultured with isolated carbofuran degraders

Sampling dates	Carbofuran phenol concentrations (µg/L)							
	control CL	PCL1	PCL2	PCL3	PCL4	PCL5	PCL6	PCL7
0	0	0	0	0	0	0	0	0
2	0	0	0	1010	0	0	0	0
4	0	0	0	1,620	80	60	30	0
6	0	0	70	1,610	310	150	110	0
8	0	0	100	1,530	220	120	120	0
10	0	0	140	180	460	220	160	0
12	0	0	160	110	590	450	360	0
14	0	0	170	0	760	460	530	0
16	0	0	150	0	600	390	390	0
18	0	0	100	180	1,030	190	330	0
20	0	0	50	110	610	120	210	0
22	0	0	0	0	130	0	100	0
30	0	0	0	0	60	0	0	0

Table 7 Concentrations of carbofuran phenol in N- and C and N-limited BSM cultured with isolated carbofuran degraders

Sampling dates	Carbofuran phenol concentrations (ug/L)							
	control NL	PNL1	PNL2	PNL3	PNL4	control CNL	PCNL1	PCNL2
0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
4	0	0	0	30	0	0	80	0
6	0	0	0	100	0	0	120	70
8	0	0	0	130	0	0	130	40
10	0	100	90	110	0	0	140	90
12	0	140	120	110	120	0	120	100
14	0	150	140	140	70	0	120	100
16	0	160	140	140	100	0	50	70
18	0	120	100	100	0	0	90	90
20	0	40	0	0	0	0	0	0
22	0	0	120	120	30	0	30	50
30	0	0	70	70	0	0	0	0

Table 8 Concentrations of 3-keto carbofuran in C-limited BSM cultured with isolated carbofuran degraders

Sampling dates	3-keto carbofuran concentrations (µg/L)							
	control CL	PCL1	PCL2	PCL3	PCL4	PCL5	PCL6	PCL7
0	0	0	0	0	0	0	0	0
2	17	0	0	0	0	0	0	0
4	18	19	17	19	15	20	17	8
6	23	23	27	27	19	18	25	18
8	21	20	22	25	18	20	20	26
10	0	21	24	18	20	26	23	24
12	0	23	23	16	18	29	21	22
14	0	21	25	0	20	21	18	17
16	11	8	10	0	10	11	13	12
18	19	20	18	0	0	21	21	18
20	26	22	0	0	0	21	19	22
22	23	27	0	0	17	18	0	19
30	19	24	0	0	0	18	0	18

Table 9 Concentrations of 3-keto carbofuran in N- and C and N-limited BSM cultured with isolated carbofuran degraders

Sampling dates	3-keto carbofuran concentrations (µg/L)							
	control NL	PNL1	PNL2	PNL3	PNL4	Control CNL	PCNL1	PCNL2
0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
4	16	21	17	17	17	17	17	14
6	16	19	24	18	21	21	21	25
8	22	23	20	17	15	23	17	30
10	19	25	18	21	20	24	22	23
12	27	27	21	22	21	25	29	31
14	23	24	23	22	17	25	30	26
16	14	0	11	0	0	10	15	10
18	25	24	22	25	0	27	25	26
20	20	26	17	23	0	24	19	25
22	22	24	18	19	16	27	24	21
30	22	21	0	17	0	24	0	10

4.1.3 Identification of isolated carbofuran degraders

After the enhancement of the enriched culture at 100 mg/L of carbofuran in all kinds of media, the culture media from C-, N- and C and N-limited BSM were streaked on its own culture BSM agar coated with 5 mg/L of carbofuran to obtain single colonies. Then their abilities to degrade carbofuran in their own BSM (C-, N-, or C and N-limited) containing 5.6 mg/L of carbofuran were examined. Only the isolates in the culture media with half-lives of carbofuran less than 10 days were gram stained. The gram stain results indicated that these isolates were gram negative-rod bacteria. In our study, we used the API 20NE system to identify the gram negative-rod bacteria. The strains of the isolates were reported in Table 10. Percentages of all identification by API 20 NE system were greater than 80%, which were acceptable. The isolated carbofuran degraders were identified as *Agrobacterium* sp., *Burkholderia* sp., *Psuedomonas* sp., *Sphingomonas* sp., *Ochrobacterium anthopi* and *Stenotrophomonas maltophilia*. "The best" carbofuran degrader, isolate PCL3, was identified as *Agrobacterium radiobacter*. Isolate PCL1 and PCL7, gram positive bacteria, were not identified because they had a low capability to degrade carbofuran with long half-lives of 33 and 28 days in comparison to the half-lives of the other isolate degraders (Table 10).

Table 10 Strains of the isolates identified by using the API 20NE system

Isolate Names	Strains
PCL1	Non-identified
PCL2	*Agrobacterium* sp.
PCL3	*Agrobacterium radiobactor*
PCL4	*Agrobacterium* sp.
PCL5	*Agrobacterium* sp.
PCL6	*Ochrobacterium anthopi*
PCL7	Non-identified
PNL1	*Stenotrophomonas maltophilia*
PNL2	*Burkholderia* sp.
PNL3	*Burkholderia* sp.
PNL4	*Sphingomonas* sp.
PCNL1	*Pseudomonas* sp.
PCNL2	*Agrobacterium radiobactor*

4.2 Dissipation of carbofuran in rhizosphere soils

The dissipation of carbofuran was studied in rhizosphere soils from six weeds, i.e. Umbrella sedge*; Cyperus iria* Linn. (กกทราย), Water primrose; *Jussiaea linifolia* Vahl. (เทียนนา), Fuzzy flatsedge; *Cyperus pilosus* Vohl. (กกสามเหลี่ยมเล็ก), Small flower umbrella plant; *Cyperus difformis* Linn. (กกขนาก), Tall-fringe-rush hoorah grass; *Fimbristylis miliacea* Vahl. (หนวดปลาดุก) and Cover fern; *Marsilea crenata* Presl.(ผักแว่น). These weeds grew in rice field with a history of carbofuran application. The disappearance profiles of carbofuran in each rhizosphere soil and bulk soil were shown in Figure 12. The degradation of carbofuran in the soils was described by a modified first-order kinetic model. The coefficients of determination, r^2, ranged between 0.95-0.99 indicating a good fit of the data to the first-order kinetic model (Table 11). The results indicated that carbofuran rapidly dissipated in rhizosphere soils with half-lives of 15-19 days, which were much faster than in bulk soil of 58 days (Table 11). This resulted from the fact that the number of carbofuran degraders in the rhizosphere soils were greater than in bulk soil 10 to 100 times (Figure 13) leading to the rapid degradation of carbofuran in rhizosphere soils. The numbers of microorganisms in the rhizosphere soil of plants were reported to be greater than in bulk soil e.g. in ryegrass (Gunther et al., 1996). The degradation of pesticides were more rapid in planted soil compared to non-planted soil (Sun, 2004; Singh et al., 2004). Camper (1999) reported that atrazine, mefonoxam and isoxaben were degraded more rapid in rhizosphere system than in bulk soil. Sun (2004) indicated that the degradation of aldicarb and oxime carbamate insecticide, in the rhizosphere soil of corn, mung bean and cowpea was more rapid than in unplanted sterile and non-sterile soil. The rhizosphere of the selected plants promoted the degradation of charcoal-fixed atrazine and simazine contaminated in soil (Singh et al., 2004). Yu et al. (2003) showed that the butachlor degradation could be enhanced in wheat rhizosphere soil. The incidents of more rapid degradation of pesticides in planted soils than in unplanted soil might due to the fact that the rhizosphere soils have carbon containing compounds such as sugars, alcohols and acids exudated from plant roots that supported the growth of microorganisms (Schnoor et al., 1995) resulting in a larger number of microorganisms and more rapid degradation of pesticides (Camper, 1999; Sun, 2004; Singh et al., 2004) in rhizosphere soil than in bulk soil.

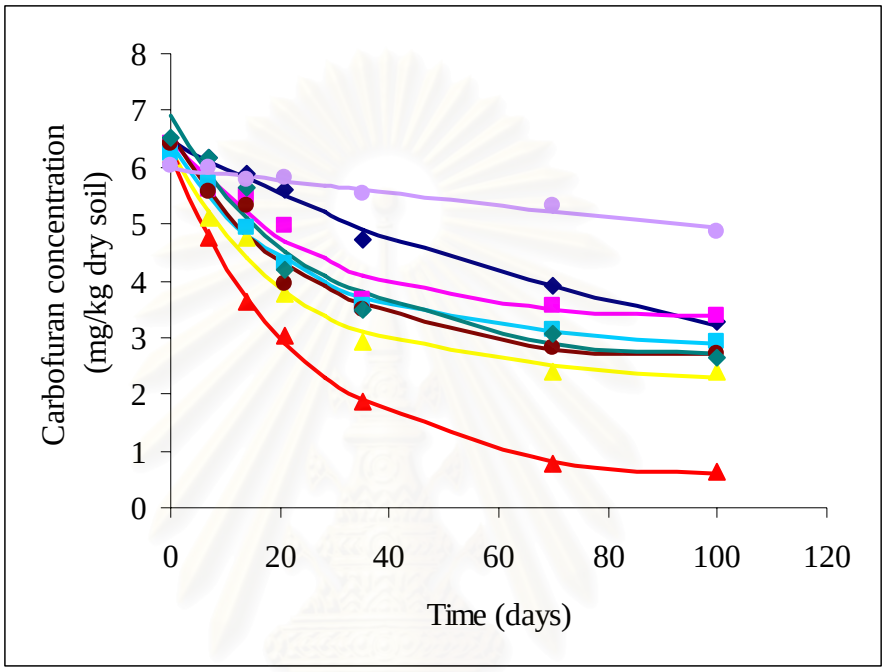


Figure 12 Dissipation of carbofuran in rhizosphere soils of weeds grown in rice field with a history of carbofuran application, (● = autoclaved rhizosphere soil of Fuzzy flatsedge, ◆ = bulk soil ■ = Umbrella sedge, ▲ = Water primrose, ▲ = Fuzzy flatsedge, ■ = Small flower umbrella plant, ● = Tall-fringe-rush hoorah grass, ◆ = Cover fern; lines = carbofuran concentrations in each soil fitted to the first-order kinetic model)

Table11 Degradation rate constants (k) and half-lives ($t_{1/2}$) of carbofuran in the rhizosphere of weeds grown in a rice field with a history of carbofuran application compared to the bulk soil

Weeds	k_1, day^{-1}	$t_{1/2}$, days	r^2*
Bulk soil	0.012	58	0.99
Umbrella sedge	0.038	18	0.95
Water primrose	0.041	17	0.99
Fuzzy flatsedge	0.046	15	0.99
Small flower umbrella plant	0.041	17	0.99
Tall-fringe-rush hoorah grass	0.041	17	0.97
Cover fern	0.037	19	0.96

*Coefficients of determination for non-linear regressions

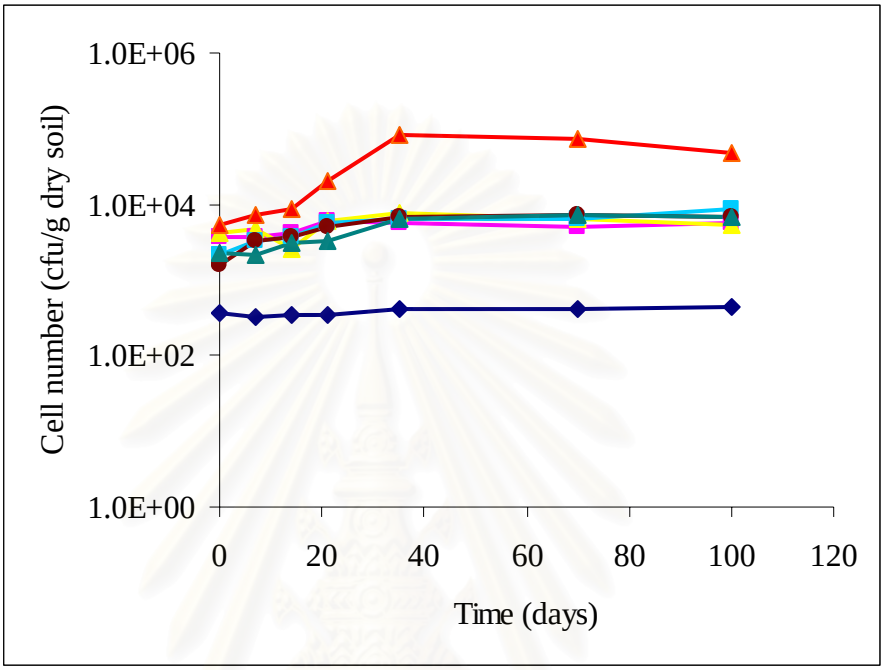


Figure 13 Growth of carbofuran degraders in rhizosphere soils of weeds grown in rice field with a history of carbofuran application, ◆ = bulk soil ■ = Umbrella sedge, ▲ = Water primrose, ▲ = Fuzzy flatsedge, ■ = Small flower umbrella plant, ● = Tall-fringe-rush hoorah grass, ◆ = Cover fern)

Carbofuran phenol was the main degradation product in the rhizosphere soils. The highest concentration of carbofuran phenol was found in the rhizosphere soil of Fuzzy flatsedge at day 35 (Table 12) which was in correlation to the shortest half-life of carbofuran (15 days) (Table 11) in this soil indicating that Fuzzy flatsedge rhizosphere soil was the most effective soil in remediating carbofuran contaminated soil. Therefore, it was chosen to be used in the bioaugmentation experiment. The carbofuran degraders in fuzzy flatsedge might be responsible for this trend, in which the numbers of carbofuran degraders in the rhizosphere soils of Fuzzy flatsedge were highest, 10^5 cells/g dry soil (Figure 13). The rice field where soil samples were collected had a history of carbofuran application, therefore microorganisms in the soils would adapt themselves to use carbofuran as their energy source. Since carbofuran phenol was the major metabolite detected in this study which indicated that rhizosphere microorganisms might contain the hydrolase enzyme (Tomasek and Karns, 1989). 3-Keto carbofuran was not found in this study. Lalah and Wandiga (1996b) showed that after 40 days of treating soil with carbofuran, carbofuran phenol was found to be a major carbofuran metabolite in flooded soil.

Carbofuran was degraded very slowly in the autoclaved rhizosphere soil of Fuzzy flatsedge (Figure 12). This result showed a similar trend to that of the other autoclaved rhizosphere soils and autoclaved bulk soil (data not shown). These suggested that there were the abiotic degradation processes involving carbofuran dissipation in the soils but they did not play a significant role in this study. An abiotic degradation of carbofuran could occur by the hydrolysis of carbofuran at the carbamate linkage (Mora, 1996).

Table 12 Concentrations of carbofuran phenol in the rhizosphere soils of weeds grown in rice field with a history of carbofuran application.

Days	Carbofuran phenol concentrations (µg/kg dry soil)							
	Bulk soil	R1*	R2*	R3*	R4*	R5*	R6*	Autoclaved R1*
0	0	0	0	0	0	0	0	0
7	0	0	210	400	250	0	0	0
11	200	0	740	1,970	200	100	310	0
21	300	600	920	2,300	780	1,000	250	0
35	400	450	1,330	2,860	940	700	460	0
70	0	300	1,200	1,200	430	80	700	0
100	0	0	400	400	420	100	480	0

* R1 = Rhizosphere soil of Umbrella sedge; *Cyperus iria* Linn. (กกทราย)

R2 = Rhizosphere soil of Water primrose; *Jussiaea linifolia* Vahl. (เทียนนา)

R3 = Rhizosphere soil of Fuzzy flatsedge; *Cyperus pilosus* Vohl. (กกสามเหลี่ยมเล็ก)

R4 = Rhizosphere soil of Small flower umbrella plant; *Cyperus difformis* Linn. (กกขนาก)

R5 = Rhizosphere soil of Tall-fringe-rush hoorah grass; *Fimbristylis miliacea* Vahl. (หนวดปลาดุก)

R6 = Rhizosphere soil of Cover fern; *Marsilea crenata* Presl. (ผักแว่น)

4.3 Bioaugmentation of Carbofuran

In order to use an isolated carbofuran degrader in remediating carbofuran contaminated soil, the bioaugmentation of carbofuran by inoculation of the isolate PCL3, *Agrobacterium radiobacter*, into Fuzzy flatsedge rhizosphere soil was conducted. Controls were conducted in bulk soil, autoclaved bulk soil and autoclaved Fuzzy flatsedge rhizosphere soil with and without inoculation of the isolate PCL3. The concentration profiles of carbofuran in the soils were presented in Figure 14. First order kinetics were observed in all carbofuran degradation in all kind of the soils with r^2 ranged between 0.97-0.99 indicating a good fit of the data to the first-order kinetic model (Table 13).

There were no differences in half-lives of carbofuran (12-14 days) (Table 13) in all soils inoculated with the isolate PCL3. This may due to the fact that the numbers of carbofuran degraders (PCL3) (Figure 15) in all soils were not significantly different. In addition, the half-lives of carbofuran in inoculated soils (12-14 days) (Table 13) were not significantly different from the half-life of carbofuran in all kinds of non-inoculated rhizosphere soils (15-19 days) (Table 11). These results indicated that bioaugmentation by adding the isolate PCL3 into the rhizosphere soil of Fuzzy flatsedge did not improve the degradation of carbofuran and only rhizosphere remediation of carbofuran might be enough to remediate carbofuran residues in soil.

The half-life of carbofuran in bulk soil inoculated with isolate PCL3 (12 days) (Table 13) was shorter than in bulk soil without the inoculation (58 days) (Table 13) demonstrating that an inoculation of isolate PCL3 into bulk soil enhanced the degradation of carbofuran. Autoclaved soils with an addition of isolate PCL3 had short half-lives of carbofuran (Table 13), 13-14 days, which was not different from Fuzzy flatsedge rhizosphere soil inoculated with isolate PCL3, confirming the ability of PCL3 to degrade carbofuran. Successful bioaugmentation of pesticides and organic hydrocarbon was discovered in the previous studies. The shorter half-life of butachlor in inoculated wheat rhizosphere soil than in non-inoculated rhizosphere soil and in bulk soil was reported by Yu et al. (2003). The inoculation of atrazine degrading bacteria into the soil treated with atrazine accelerated the atrazine mineralization in soil (Struthers et al., 1998; Topp, 2001; Rousseaux et al., 2003). Strain B-14 (10^6 cells/g) inoculated to soil mixed with 35 mg of chlorpyrifos/kg soil

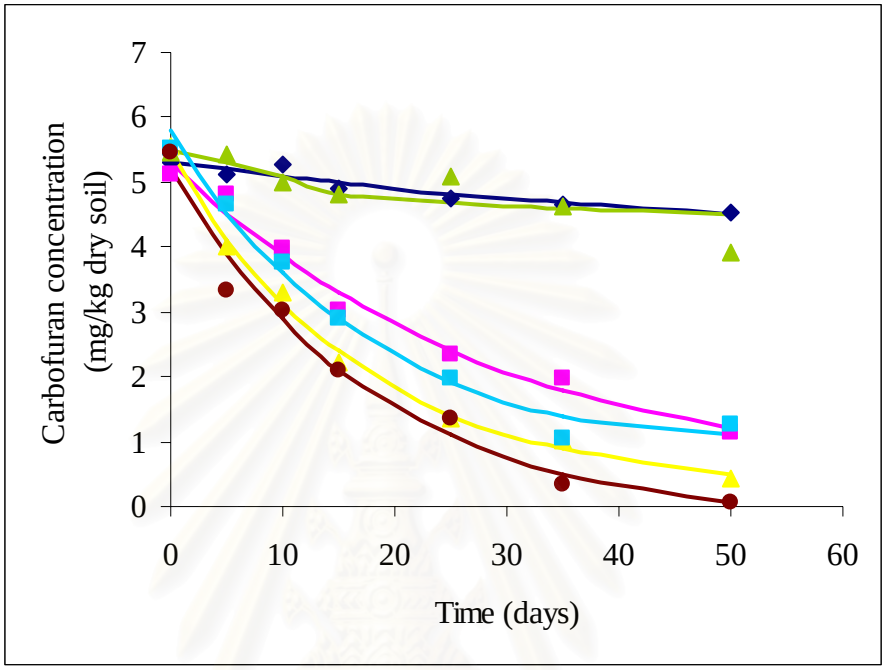


Figure 14 Degradation of carbofuran in the soils inoculated with isolate PCL3, (■ = autoclaved bulk soil, ▲ = bulk soil, ■ = autoclaved Fuzzy flatsedge rhizosphere soil, ● = Fuzzy flatsedge rhizosphere soil) and without isolate PCL3, (◆ = autoclaved bulk soil, ▲ = autoclaved Fuzzy flatsedge rhizosphere soil; lines = carbofuran concentrations in each soil fitted to the first-order kinetic model)

Table 13 Degradation rate constants (k) and half-lives ($t_{1/2}$) of carbofuran in inoculated soils

Samples	k_1, day^{-1}	$t_{1/2}$, days	r^2*
Autoclaved bulk soil + isolate PCL3	0.049	14	0.98
Bulk soil + isolate PCL3	0.059	12	0.99
Autoclaved Fuzzy flatsedge rhizosphere soil + isolate PCL3	0.055	13	0.97
Fuzzy flatsedge rhizosphere soil + isolate PCL3	0.060	12	0 .98
Bulk soil	0.012	58	0.99

*Coefficients of determination for non-linear regressions

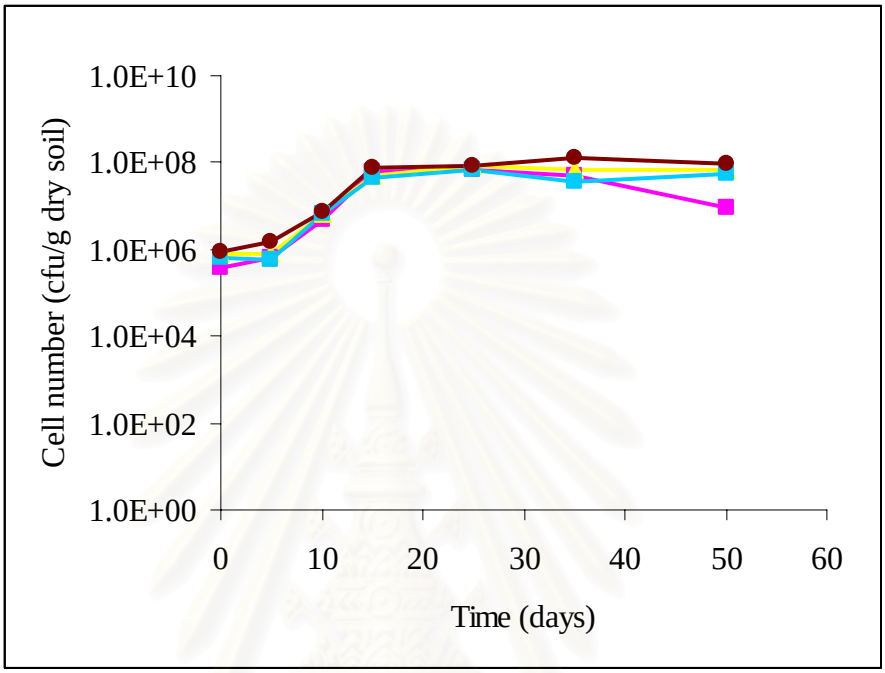


Figure 15 Growth of carbofuran degraders in the soils inoculated with isolate PCL3, (■ = autoclaved bulk soil, ▲ = bulk soil, ■ = autoclaved Fuzzy flatsedge rhizosphere soil, ● = Fuzzy flatsedge rhizosphere soil)

resulted in a higher degradation rate than was observed in non-inoculated soils (Singh et al. 2004). Atrazine mineralization increased 3-fold in soil inoculated with *Chelatobater heintzii* Cit1 (Rousseaux et al., 2003). Not only the bioaugmentation of the pesticides were investigated but also the hydrocarbon. Ruberto et al. (2003) found that bioaugmentation with the B-2-2 strain increased the hydrocarbon bioremediation efficiency (75% of the hydrocarbon was removed). They suggested that autochthonous bacterial flora from Antarctic soils was able to degrade an important fraction of the gas-oil.

Carbofuran phenol was only degradation product in all inoculated soils (Table 14). Lalah and Wandiga (1996b) found that carbofuran phenol was the main degradation product in flooded soil treated with carbofuran.

Table14 Concentrations of carbofuran phenol in the soils with and without inoculation of isolate PCL3

Days	Carbofuran phenol concentrations (µg/kg dry soil)					
	BC	BC+PCL3	B+PCL3	RC	RC+PCL3	R+PCL3
0	0	0	0	0	0	0
5	0	100	220	130	500	570
10	200	1,490	1,320	270	460	1,530
15	450	2,110	2,510	440	1,450	2,950
25	330	2,890	2,940	310	2,110	2,310
35	530	920	790	560	700	1,990
50	440	300	800	220	400	1,040

B = Bulk soil

BC = Autoclaved bulk soil

R = Rhizosphere soil of Fuzzy flatsedge; *Cyperus pilosus* Vohl. (กกสามเหลี่ยมเล็ก)

RC = Autoclaved rhizosphere soil of Fuzzy flatsedge

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Isolation of carbofuran degraders from phytoremediated rhizosphere soils i.e., rice, corn and Chinese kale was a success. Preliminary results showed that isolated carbofuran degraders effectively degraded carbofuran in C-, N- and C and N-limited BSM at a low concentration, 5 mg/L, in which more than 80% of carbofuran was degraded within 7 days. According to the colony morphology, there were 7, 4 and 2 isolates growing on C-, N- and C and N-limited BSM, respectively, suggesting that these isolates used carbofuran as their sole C-, N- and sole C and N- sources, respectively. The carbofuran degradation ability of each isolate was examined in each kind of BSM containing an average of 5.6 mg/L of carbofuran. The results indicated that carbofuran was rapidly degraded in BSM by isolated degraders indicating that carbofuran degradation was improved by the isolated microorganisms due to their capabilities to use carbofuran as sole C-, N- or C- and N-sources for their growth. Carbofuran phenol and 3-keto carbofuran were the metabolites detected in this experiment, suggesting that the isolates might produce hydrolase and oxidase enzyme responsible for degrading carbofuran. The isolates in the culture media that had the half-lives of carbofuran of less than 10 days were chosen to be gram stained. All of these isolates were gram negative-rod bacteria which were identified as *Agrobacterium* sp., *Burkholderia* sp., *Psuedomonas* sp., *Sphingomonas* sp., *Ochrobacterium anthopi* and *Stenotrophomonas maltophilia.* An isolate PCL3 “the best degrader” using carbofuran as a sole C-source was identified as *Agrobacterium radiobacter*. This isolate could effectively degrade carbofuran with the shortest half-life of carbofuran (3 days) in C-limited BSM and was further used in the bioaugmentation study.

Carbofuran degradation was rapid in the rhizosphere soils of weeds grown in a rice field with a history of carbofuran application resulting in carbofuran phenol as metabolite. Carbofuran dissipated in rhizosphere soils (half-lives of 15-19 days) faster than in bulk soil (half-life of 58 days) and autoclaved rhizosphere soil. Carbofuran degraders might be responsible for this trend since the number of carbofuran degraders were 10 to100 times greater in rhizosphere soil than in the bulk soil. The

rice field where soil samples were collected had a history of carbofuran application, therefore microorganisms in the soils might adapt themselves to use carbofuran as their energy sources. In addition, this may due to the fact that the rhizosphere soils have the carbon containing compounds such as sugars, alcohols and acids exudated from plant roots that supported the growth of microorganisms (Schnoor et al., 1995) leading to a larger number of microorganisms in the rhizosphere soil than in bulk soil. The shortest half-life of carbofuran (15 days) was found in the rhizosphere soil of Fuzzy flatsedge which then was selected to use in the bioaugmentation experiment.

In order to use the isolated carbofuran degrader in remediating carbofuran contaminated soil, bioaugmentation of carbofuran by inoculation of the isolate PCL3 into Fuzzy flatsedge rhizosphere soil was conducted. The results revealed that isolate PCL3 did not improve the degradation of carbofuran in rhizosphere soils in which the half-lives of carbofuran in inoculated soils (12-14 days) were not significantly different from the half-life of carbofuran in all kinds of non-inoculated rhizosphere soils (15-19 days) suggesting that only rhizosphere remediation might be effective enough to remediate soil contaminated with carbofuran. However, the half-life of carbofuran in bulk soil inoculated with isolate PCL3 (12 days) was shorter than in bulk soil without the inoculation (58 days), demonstrating that PCL3 accelerated the degradation of carbofuran in bulk soil. Autoclaved soils with an addition of PCL3 had short half-lives of carbofuran, 13-14 days, which was not significantly different from the half-life of carbofuran in Fuzzy flatsedge rhizosphere soil inoculated with isolate PCL3, confirming an ability of the isolate PCL3 to degrade carbofuran. In conclusion, rhizosphere remediation is one of the effective bioremediation techniques to remove or detoxify carbofuran residues in soil. The bioaugmentation of carbofuran in contaminated bulk soil by isolated degraders could improve the degradation of carbofuran.

Recommendations

The results from our study indicated that Fuzzy flatsedge can be used to remediate carbofuran residues in rice field soil. In order to use isolated carbofuran degraders effectively, there is a need to improve their survival in real environment. Immobilization technique, an action of physically confining cells in refined region or space, is one of the options for survival improvement. However, the possibility to retain the carbofuran degradation abilities of the cells, and the proper support materials should be studied. After an application of carbofuran degraders to contaminated sites, we should pay attention to the monitoring of these isolates because we do not know that what are the fate of these isolates after they are released to the environment. Due to the metylcarbamate-degrading gene (*mcd*) coding a carbofuran hydrolase enzyme is evident present and expressed in the carbofuran degraders, the changes of microbial population of carbofuran degraders can be investigated by molecular monitoring of this gene. In addition, the changes of microbial population in the soil can be investigated by cloning the *lux* (lusiferase) gene, gene encoding fluorescent protein, into the inoculants cells. This gene can be detected via the detection of the fluorescent compounds.

Since our experiments conducted using only 5 mg/kg of carbofuran, therefore, the effectiveness of our “best plant - fuzzy flatsedge” and “best degrader – isolate PCL3” should be tested against higher concentrations of carbofuran.

REFERENCES

Anderson, J.A., Kruger, E.L., and Coats, J.R. 1994. Enhanced degradation of a mixture of three herbicides in the rhizosphere of a herbicide-tolerant plant. Chemosphere 28: 1551-1557.

Anderson, T.A., Guthrie, E.A. and Walton, B.T. 1993. Bioremediation in the rhizosphere. Environmental Science and Technology 27: 2630-2636.

Anderson, T.A. and Walton, B.T. 1995. Comparative fate of [14C]trichloroethylene in the root zone of plants from a former solvent disposal site. Environmental Toxicology and Chemistry 14:204-2047.

Amal, C. D. 2003. Influence and persistence of phorate and carbofuran insecticides on microorganisms in rice field. Chemosphere 53: 1033-1037.

Ambrosoli, R., Negre, M. and Gennari, M. 1996. Indication of the occurrence of enhanced biodegradation of carbofuran in some Italian soil. Soil Biology and Biochemistry 28(12): 1749-1752.

Bano, N. and Musarrat, J. 2004. Characterization of a novel carbofuran degrading Pseudomonas sp. with collateral biocontrol and plant growth promoting potential . FEMS Microbiology Letters 231(1): 13-17.

Baron, R.L. 1991. Carbamate insecticides. Handbook of pesticide toxicology. New York. Academic Press: 3–8.

Blaylock, M., Salt, D.E., and Raskin, I. 1997. Enhanced accumulation of Pb in Indian Mustard by soil-applied chelating agents. Environmental Science and Technology 31: 860-865.

Brown, S.L., Chaney, R.L., Angle, J.S. and Baker, AJ.M. 1994 Phytoremediation potential of *Thlaspi caerulescens* and bladder campion for zince- and cadmium-contamunated soil**.** Journal of Environmental Quality 23: 1151-1157.

Burken, J.G., and Schnoor, J.L. 1997. Uptake and metabolism of atrazine by poplar trees. Environmental Science and Technology 31: 1399-1402.

Camper, N.D., and others. 1999. Pesticide/rhizosphere interactions in containerized plants. SNA Research Conference 44: 400.

Cain, R.B. and Head, I.M. 1991. Enhanced degradation of pesticides: its biochemical and molecular biological basis. Pesticide in soil and water. BCPC monography 47: 23-40

Caro, J.H., Taylor, A.W. and Freeman, H.P. 1976. Comparative behavior of dieldrin and carbofuran in the field. Archives of Environmental Contamination 3: 437-447.

Chaney, R.L., Malik, K.M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., et al. 1997. Phytoremediation of soil metals. Current Opinion in Biotechnology 8: 279-284.

Chapalamadugu, S. and Chaudry, G.R. 1992. microbiological and biotechnological aspects of metabolism of carbamates and organophosphates. CRC Critical Review in Biotechnology 12:357-389.

Chapman, R.A. and Cole, C.M. 1982. Observations on the influence of water and soil pH on the persistence of pesticides. Journal of Environmental Science and Health B17: 487-504.

Chaudhry, G. R., and others. 2002. Induction of carbofuran oxidation to 4-hydroxycarbofuranby Pseudomonassp.50432. FEMS Microbiology Letters 214: 171-176.

Chiron, S., Torres, J.A. Fernandez-Alba, A. Alpendurada, M.F. and Barcelo,D. 1996. Identification of carbofuran and methiocarb and their transformation products in estuarine waters by on-line solid phase extraction liquid chromatography- mass spectrometry. Journal of Environmental Analysis Chemistry 65: 37-52.

Chulalongkorn University. Hazardous Chemical Imported Quantity Statistic. [online]. 2004. Available from: http://www.chemtrack.org/Stat-Import/25_38JAN_ DEC02_KGM_ID.pdf

CRA. Bioaugmentation. [online]. (2003). Available from: http://www.craworld.com/resources/inn_2003_aug.pdf

Cohen, S.Z. 1996. Pesticides in ground water in the United States: monitoring, modeling, and risks form the U.S. perspective. Environmental Science and Health. B31(3):345- 352.

Deuel, L.E. JR., and others. 1979. Persistence of carbofuran and its metabolites, 3-keto and 3-hydroxy carbofuran, under flooded rice culture. Journal of Environmental Quality 8(1), 23-26.

Devries, S. and Evans, O. Carbofuran. [online]. (1999). Available from: http://wvlc.uwaterloo.ca/biology447/Assignments/Assignment1Submissions/On-Campus/carbofuran/ Carbofuran.html

Dushenkov, V., Kumar, P. BAN, Motto, H, Raskin, I. 1995. Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. Environmental Science and Technology 29: 1239-1245.

Duquenne, P., and others. 1996. Effect of inoculant density, formulation, dispersion and soil nutrient amendment on the removal of carbofuran residues from contaminated soil. Soil Biology and Biochemistry 28(12): 1805-1811.

Eisler, R. 1985. Carbofuran hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. fish wildl. serv. Biological Republic 85(1.3). 36pp.

Entomology and Zoology office. 1994. Using insecticide and pest. Bangkok: Department of agricultural.

Environmental Monitoring Branch. California Department of Pesticide Regulation (DPR). Pesticide use reporting database. [online]. (2002). Available from: http://www.cdpr.ca.gov/docs/pur/purmain.htm

EPA. Ground water and drinking water [online]. (2003). Available from: http://www.epa.gov/safewater/contaminants/dw_contamfs/carbofur.html.

Evert S. Environmental fate of carbofuran. [online]. (2002). Available from: http://www.cdpr.ca.gov/docs/empm/pubs/fatememo/carbofuran.pdf.

Extension Toxicology Network. Pesticide Information Profiles; Carbofuran. [online]. (1996). Available from: http://extoxnet.orst.edu/pips/carbofur.htm

Felsot, A.S., J Maddox,V., and Bruce, W. 1981. Enhanced microbial degradation of carbofuran in soils with histories of furadan use. Bulletin of Environmental Contamination and Toxicology 26: 781-788.

Ferro, A.M., Sims, R.C., Bugbee, B. 1994. Hycrest crested wheatgrass accelerates the degradation of pentachlorophenol in soil. Journal of Environmental Quality 23: 272-279.

Ferrell, M.A., and Aagard, S.D. Pesticide storage facility, design, and management plan; pesticide education program fact sheet. [online]. (2003). Available

from: http://www.uwyo.edu/CES/PUBS/MP93_Series/mp-93.21_facility .pdfpatein

Frazar, C. The bioremediation and phytoremediation of pesticide-contaminated sites [online]. (2000). Available from: http://clu-in.org/download/studentpapers/ frazar.pdf

Frick, C.M., Farrell, R.E., and Germida, J.J. Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. [online]. (1999). Available from: http://clu-in.org/download/remed/phyassess.pdf.

Gao, J., Garrison, A.W., Hoehamer, C., Mazur, C.S., and Wolfe, N.L. 2000a. Uptake and phytotransformation of o,p'-DDT and p,p'-DDT by axenically cultivated aquatic plants. Journal of Agricultural Food Chemistry 48(12): 6121-6127.

Garbisu, C., and Alkorta, I. 2001. Phytoextraction: a cost-effective plant-based technology for the removal of metals from environment. Bioresearch and Technology 77: 229-236.

Garcia C. Roldan, A., and Hernandez T.H. 2005. Ability of different plant species to promote microbiological processes in semiarid soil. Geoderma 124: 193–202.

Garon, D., Sage, L.,Wouessidjewe, D. and Seigle-Murandi, F. 2004. Enhanced degradation of uorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. Chemosphere 56: 159–166.

Garten, C.T. 1999. Modeling the potential role of a forest ecosystem in phytostabilization and phytoextraction of 90Sr at a contaminated watershed. Journal of Environmental Radioactive 43: 305-323.

Getzin, L.W. 1973. Persistence and degradation of carbofuran in soil. Environmental Entomology 2: 461-467.

Gilbert, E.S. and Crowley, D.E. 1998. Repeated application of carvone-induced bacteria to enhance biodegradation of polychlorinated biphenyls in soil. Applied Microbiological Biotechnology 50: 489–494.

Gunther. T., Dornberger, U. and Fritsche, W. 1996. Effects of ryegrass on biodegradation of hydrocarbons in soil. Chemosphere 33(2): 203-215.

Harris, C.R., Chapman, R.A., Harris, C. and Tu, C.M. 1984. Biodegradation of pesticides in soil: rapid induction of carbamate degrading factors after

carbofuran treatment. Journal of Environmental Science and Health B19: 1-11.

Howard, P. H. 1991.Handbook of Environmental Fate and Exposure Data for Organic Chemicals: Pesticides Chelsea. MI.: Lewis Publishers: 3-15.

ICPS INCHEM. Carbofuran. [online]. (nd). Available from: http://www.inchem.org/documents/jmpr/jmpmono/v96pr03.htm

Information Division of the Plant Industry Directorate (IDPID). Special Review of Carbofuran Insecticide: Effects on Avian Fauna and Value to Agriculture. [online]. (1993). Available from: http://www.hc-sc.gc.ca/pmra-arla/English/ pdf/prdd/ prdd_d9302-e.pdf.

Interstate Technology and Regulatory Cooperation (ITRC). Phytoremediation decision tree. [online] (1999). Available from: http://www.itrcweb.org/ phyto_1.pdf.

Jasper, D.A. 1994. Bioremediation of agricultural and forestry soils with symbiotic micro-organisms. Australian Journol of Soil Research 32:1301-1319.

Johnson, W. G., and Lavy, T. L. 1995. Persistence of carbofuran and molinate in flooded rice culture. Journal of Environmental Quality 24: 487-493.

Jordahl, J.L., Foster, L., Schnoor, J.L. and Alvarez, P.J.J. 1997. Effect of hybrid poplar trees on microbial populations important to hazardous waste bioremediation. Environmental Toxicology and Chemistry 16 (6):1318-1321.

Kamrin, M.A. 1997. Pesticide profiles: toxicity environmental impact and fate**.** 1st ed. New York: Boca Raton: Lewis Publishers.

Kidd, H. and James, D.R. 1991. The Agrochemicals Handbook. Third Edition. Cambridge, UK: Royal Society of Chemistry Information Services: 3-11.

Kuiper, I., and others. 2004. Rhizoremediation: a beneficial plant–microbe interaction. Molecular Plant and Microbe Interaction 17: 6–15.

Kumari, K., Singh R.P., and Saxena, S.K. 1988. Movement of carbofuran (nematicide) in soil columns. Ecotoxicology Environmental Safety 16:36-44.

Lalah, J.O., Wandiga, S.O. and Dauterman, W.C. 1996. Mineralization, volatilization, and degradation of carbofuran in soil samples from kenya. Bulletin of Environmental Contaminant and Toxicology 56:37-41.

Radwan, S.S., Al-Awadhi, H., Sorkhoh, N.A., and El-Nemr, I.M. 1998. Rhizospheric hydrocarbonutilizing microorganisms as potential contributors to phytoremediation for the oily Kuwaiti desert. Microbiological Research 153 (3): 247 – 251.

Macek, T., Mackova, M., Kas, J. 2000. Exploitation of plant for the removal of organics in environmental remediation. Biotechnological Advance 18: 23-34.

Ma, L.Q., Komart, K.M., Tu, C., Zhang, W., Cai, Y., Kennelly, E.D. 2001. A fern that hyperaccumulates arsenic. Nature 409: 579.

Mora, A., Revilla, C.E., Hermosin, M.C. 1996. Persistence and degradation of carbofuran in Spanish soil suspensions. Chemosphere 32(8): 1585-1598.

Morel-Chevillet, C., and others. 1996. Cross-enhancement of carbofuran biodegradation in soil Sample previously treated with carbamate pesticides. Soil Biology and Biochemistry 28(12): 1767-1776.

Nakamura T., Motoyama. T., Suzuki Y., and Yamaguchi I. 2004. Biotransformation of pentachlorophenol by Chinese chive and a recombinant derivative of its rhizosphere-competent microorganism, *Pseudomonas gladioli* M-2196. Soil Biology and Biochemistry 36:787–795.

Narasimhan, K., and others. 2003. Enhancement of plant–microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. Plant Physiology 132: 146–153.

Newman, L.A., Strand, S.E., Choe, N., Duffy, J., Ekuan, G. 1997. Uptake and biotransformation of trichloroethylene by hybrid poplars. Environmental Science and Technology. 31: 1062-1067.

Ngampongsai, A. 1990. Degradation and residues of carbofuran in soil and sweet corn. [Master Thesis in Agricultural]. Bangkok: The Graduate School, Kasetsart University.

Ou, L.T., Gancarz, D.H., Wheeler, W.B., Rao, PS.C., Davidson, J.M. 1982. Influence of soil temperature and soil moisture on degradation and metabolism of carbofuran in soil. Journal of Environmental Quality 11: 293-298.

Parkin, T.B. and Shelton, D.R. 1994. Modeling environmental effects on enhanced carbofuran degradation. Pesticide Science 40:163-168.

Peter, D. F. 2000. Bioaugmentation of atrazine and fenamiphos impacted groundwater: Laboratory Evaluation. Bioremediation Journal 4(3): 237-248.

Pulford, I.D. and Watson, C. 2003. Phytoremediation of heavy metal-contaminated land by trees-a review. Environmental International 29: 529-540.

Raha, P. and Asit, Das. K. 1990. Photodegradation of carbofuran. Chemosphere 21 (1-2): 99-106.

Ramakrishna, C., and Sethunathan, N. 1982. Stimulation of autotrophic ammonium oxidaton in rice rhizosphere soil by the insecticide carbofuran. Applied and Environmental Microbiology 44(1): 1-4.

Read, D.C. 1983. Enhance microbial degradation of carbofuran and fensulfothion after repeated application to acid mineral soil. Agriculture Ecosystem and Environment 10: 37-46.

Rittmann, B.E. and Whiteman, R. 1994 Bioaugmentation: a coming of age. Water Quality International 1:12-16.

Rousseaux, S., Hartmann, A., Lagacherie, B., Piutti, S., Andreux, F., and Soulas G. 2003. Inoculation of an atrazine-degrading strain, Chelatobacter heintzii Cit1, in four di.erent soils:efects of diferent inoculum densities Chemosphere 51:569–576.

Rubertoa, L., Vazqueza, C.S. and Mac Cormackb, W. P. 2003. Effectiveness of the natural bacterialfora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. International Biodeterioration & Biodegradation 52: 115 – 125.

Schnoor, J.L., Licht, L.A., McCutcheon, S.C., Wolfe, N.L. and Carreira, L.H. 1995. Phytoremediation of organic and nutrient contaminants. Environmental Science and Technology 29 (7): 318-323.

Schnoor, J.L. Phytoremediation. [online]. (1997). Available from: http://www .rtdf.org/public/phytolphytobib/bibq_t.html

Scow, K.M., Merica, and R.R. Alexander, M. 1990. Kinetic analysis of enhanced biodegradation of carbofuran. Journal of Agriculture and Food Chemistry 38: 908-912.

Seiber, J.N., Catahan, M.P. and Barril, C.R. 1978. Loss of carbofuran from rice paddy water: chemical and physical factors. Journal of Environmental Science and Health B13: 131-148.

Shibamoto, T., Mourer, C. and Hall, G. 1993. Pilot monitoring of two pesticides in air. Air Resources Board. Report # R-95/581.

Siciliano, S.D., and Germida, J.J. 1998. Mechanisms of phytoremediation: biochemical and ecological interactions between plants and bacteria. Environmental Reviews 6: 65–79.

Siddaramappa R. and others. 1978. The degradation of carbofuran in paddy water and flooded soil of untreated and retreated rice fields. Journal of Environmental Science and Health B13: 369-380.

Siddaramappa, R. and Seiber, J.N. 1979. Persistence of carbofuran in flooded rice soils and water. Protective Water Techology 11(6): 103-110.

Singer, C.A., van der Gast, J.C. and Thompson, P.I. 2005. Perspectives and vision for strain selection in bioaugmentation. TRENDS in Biotechnology 23(2): 74-77.

Singh, M.N., Megharaj, R.S., Kookana, R.N. and Sethunathan, N. 2004. Atrazine and simazine degradation in Pennisetum rhizosphere.Chemosphere 56:257–263.

Skipper, H.D. 1999. Bioremediation of contaminated soils. In: Sylvia, D.M. (Ed.), Principles and Applications of Soil Microbiology Upper Saddle River, NJ. : Prentice Hall: 469–481.

Smith, G. J. 1992. Toxicology and Pesticide Use in Relation to Wildlife: rganophosphorus and Carbamate Compounds. Boca Raton, FL: C. K. Smoley: 3-18.

Stottmeister, U. and others. 2003. Effects of plants and microorganisms in constructed wetlands for wastewater treatment. Biotechnology Advances 22: 93– 117.

Struthers, J.K., Jagachandran, K. and Moorman, T.B. 1998. Biodegradation of atrazine by *agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. Applied and Environmental Microbiology 64(9): 3368-3357.

Sun H, Xu J., Yang S., Liu G. and Dai S. 2004. Plant uptake of aldicarb from contaminated soil and its enhanced degradation in the rhizosphere Chemosphere 54:569–574.

Susarla, S., Medina, V.F., McCutcheon, S.C. 2002. Phytoremediation: An ecological solution to organic chemical contamination. Ecological Engineer 18: 647-658.

Teerakun, M., Reungsang, A., and Virojanakud, W. 2004. Phytoremediation of carbofuran in soil. Songklanakarin Journal of Science and Technology 26(1): 171-176.

Topp, E. 2001. A comparison of three atrazine-degrading bacteria for soil bioremediation. Biology and Fertility of Soils 33: 529-534.

Trabue, S.L., Ogram A.V. and Ou, L.-T. 2001. Dynamics of carbofuran-degrading microbial communities in soil during three successive annual application of carbofuran. Soil Biology and Biochemistry 33, 75-81.

Trotter, D.R., Kent, R., Wong, M. 1991. Aquatic fate and effect of carbofuran. Critical Review in Environmental Contaminant 21(2): 137-176.

Turco, R.F. and Konopka, A. 1990. Biodegradation of carbofuran in enhanced and non-enhanced soils. Soil Biology and Biochemistry 22:195-201.

United-Tech, Bioaugmentation vs indigenous microbial populations for waste water remediation projects. [online]. (2004). Available from: http://www.united-tech.com/bioaugmentation. html.

van Veen, J.A., and others. 1997. Fate and activity of microorganisms introduced into soil. Microbiology and Molecular Biology Reviews 61: 121–135.

Vidali, M. 2001. Boremediation and overview. Pure and Applied Chemistry 73: 1163-1172.

Visoottiviseth, P., Francesconi, K., Sridokchan, W. 2002. The potential of Thai indigenous plant species for the phytoremediation of arsenic contaminated land. Environmental Pollution 118: 453-461.

Vogel, T.M. 1996. Bioaugmentation as a soil bioremediation approach. Environmental Biotechnology 7:311-316.

Watanabe, M.E. 1997. Phytoremediation on the brink of commercialization. Environmental Science and Technology 31: 182A-186A.

Whyte, G.L., Luc Claude, B., and Greer, W. h. 1999 Bioremediation assessment of hydrocarbon-contaminated soils from the high arctic. Bioremediation Journal 3(1): 69-80.

Wilson, P.C., Whitwell, T., and Klaine, S.J. 2000. Metalaxyl and simazine toxicity to and uptake by Typha latifolia. Archives of Environmental Contaminant Toxicology 39(3): 282-288.

Yen, H.J., Fang-Lan, H. and Yei-Shung, W. 1997. Assessment of the insecticide carbofuran's potential to contaminate groundwater through soils in the subtropics. Ecotoxicicology and Environmental Safety 38: 260-265.

Yu, C.C., Booth, G.M., Hansen, D.J., Larsen, J.R. 1974. Fate of carbofuran in model ecosystem. Journal of Agricultural Food Chemistry 22(3): 431-434.

Yu, Y.L., Chen, Y.X., Luo, Y.M., Pan, X.D., He, Y.F., Wong, M.H. 2003. Rapid degradation of butachlor in wheat rhizosphere soil. Chemosphere 50: 771-774.

Zynda, T. Phytoremediation. [online]. 2001. Available from: http://www.envirotools. or/factsheets/phytoremediation.pdf

APPENDICES

APPENDIX A

Raw Data

Table 15 Degradation of carbofuran by isolated carbofuran degraders cultured in C-limited BSM

Incubation time (days)	Carbofuran concentration (mg/L)							
	control CL	PCL1	PCL2	PCL3	PCL4	PCL5	PCL6	PCL7
0	4.53	5.93	5.74	5.62	5.48	5.82	5.15	5.76
2	4.92	5.38	5.01	5.08	4.60	5.91	5.08	5.09
4	4.89	4.94	4.80	3.43	4.18	4.78	4.66	5.35
6	5.28	4.54	4.50	2.44	3.34	4.35	3.80	5.55
8	5.08	3.87	3.34	0.42	1.62	2.88	2.70	4.94
10	4.48	3.61	3.11	0.30	1.27	2.21	1.95	5.47
12	4.68	3.97	2.36	0.19	1.22	1.78	2.15	5.13
14	4.37	4.00	1.61	0.10	0.91	1.05	2.01	4.30
16	4.08	2.99	1.48	0.06	0.87	1.00	1.50	3.98
18	3.91	2.20	0.90	0.00	0.60	0.57	0.80	4.00
20	4.04	2.50	0.28	0.00	0.32	0.23	1.06	3.49
22	4.16	2.58	0.10	0.00	0.24	0.11	0.99	3.48
30	3.80	2.53	0.00	0.00	0.00	0.00	0.76	3.73

Table 16 Carbofuran concentrations in C-limited BSM fitted with the first-order kinetic model

Incubation time (days)	Carbofuran concentration (mg/L)							
	control CL	PCL1	PCL2	PCL3	PCL4	PCL5	PCL6	PCL7
0	5.10	6.00	5.90	6.80	6.40	6.10	5.40	5.80
2	5.00	5.40	5.30	4.30	5.40	5.30	4.30	5.60
4	4.90	4.90	4.70	2.70	4.50	4.60	3.70	5.40
6	4.80	4.50	4.10	1.70	3.70	4.01	3.10	5.20
8	4.70	4.10	3.50	1.04	3.00	3.25	2.60	5.00
10	4.60	3.80	2.90	0.60	2.40	2.44	2.15	4.80
12	4.50	3.50	2.40	0.33	1.80	1.82	1.91	4.60
14	4.40	3.20	1.60	0.08	1.10	1.21	1.51	4.40
16	4.30	3.10	1.30	0.04	0.99	1.00	1.15	4.30
18	4.23	2.90	0.90	0.00	0.65	0.61	1.00	4.14
20	4.15	2.80	0.50	0.00	0.34	0.30	0.81	4.00
22	4.07	2.70	0.25	0.00	0.08	0.25	0.72	3.90
30	3.80	2.30	0.00	0.00	0.00	0.20	0.66	3.40

Table 17 Degradation of carbofuran by isolated carbofuran degraders cultured in N-and C and N-limited BSM

Incubation time (days)	Carbofuran concentration (mg/L)							
	control NL	PNL1	PNL2	PNL3	PNL4	control CNL	PCNL1	PCNL2
0	4.59	5.16	6.07	5.92	6.21	5.31	5.84	6.37
2	5.01	4.73	5.76	5.17	6.09	5.58	5.10	5.67
4	4.98	4.64	4.72	4.61	5.07	4.95	4.14	4.92
6	5.54	3.65	2.98	4.06	4.45	4.85	3.65	4.53
8	4.73	2.41	1.64	2.91	2.81	5.04	2.47	3.06
10	4.65	2.13	1.80	2.13	2.08	4.37	1.95	2.12
12	4.97	2.36	1.54	1.60	2.01	4.62	1.89	2.24
14	4.31	2.27	1.10	1.54	1.58	4.24	1.75	1.81
16	4.06	1.62	0.91	0.81	1.36	4.01	1.32	1.28
18	3.90	1.24	0.63	0.29	0.74	4.64	1.12	0.65
20	4.26	0.35	0.38	0.45	0.91	4.06	0.73	0.31
22	4.40	0.07	0.24	0.56	0.56	4.39	0.75	0.30
30	3.84	0.00	0.00	0.50	0.49	4.17	0.74	0.20

Table 18 Carbofuran concentration in N-and C and N-limited BSM fitted with the first-order kinetic model

Incubation time (days)	Carbofuran concentration (mg/L)							
	control NL	PNL1	PNL2	PNL3	PNL4	control CNL	PCNL1	PCNL2
0	5.50	5.60	6.60	6.70	7.13	5.53	6.20	7.11
2	5.35	4.70	5.20	5.27	5.73	5.27	5.00	5.75
4	5.20	4.20	4.04	4.16	4.61	5.05	4.00	4.65
6	5.05	3.50	3.16	3.29	3.72	4.87	3.30	3.75
8	4.93	3.01	2.45	2.61	3.00	4.73	2.68	3.02
10	4.77	2.64	1.89	2.08	2.43	4.61	2.21	2.42
12	4.53	2.30	1.45	1.66	1.97	4.51	1.84	1.93
14	4.35	1.90	0.90	1.17	1.41	4.39	1.39	1.33
16	4.22	1.36	0.82	1.09	1.31	4.37	1.32	1.21
18	4.13	1.00	0.59	0.89	1.07	4.31	1.14	0.95
20	3.89	0.45	0.42	0.74	0.88	4.27	0.99	0.74
22	3.80	0.08	0.28	0.62	0.73	4.23	0.88	0.56
30	3.75	0.00	0.00	0.35	0.38	4.14	0.61	0.13

Table 19 Growth of isolated carbofuran degraders in C-limited BSM during incubation

Incubation time (days)	Cell number (cfu/mL)						
	PCL1	PCL2	PCL3	PCL4	PCL5	PCL6	PCL7
0	4.20E+06	5.10E+06	4.70E+06	6.70E+06	2.30E+06	3.40E+06	4.20E+06
2	4.50E+06	5.50E+06	5.30E+06	5.10E+06	5.00E+06	5.60E+06	4.50E+06
4	6.40E+06	6.20E+06	6.70E+07	5.13E+07	5.50E+06	8.00E+06	4.80E+06
6	4.30E+06	1.25E+07	4.30E+08	5.22E+07	6.40E+07	4.41E+07	6.70E+06
8	3.40E+06	1.34E+07	7.60E+08	5.13E+07	1.62E+08	4.60E+07	2.30E+06
10	5.50E+06	2.44E+07	4.80E+08	2.00E+06	2.00E+08	8.01E+07	1.00E+06
12	6.00E+06	3.00E+07	1.20E+08	2.00E+06	5.60E+07	4.50E+07	1.00E+05
14	3.00E+05	6.10E+06	5.40E+07	1.00E+04	5.40E+06	1.55E+07	2.00E+03
16	8.00E+03	4.30E+06	5.10E+06	6.00E+04	4.20E+06	7.40E+06	5.00E+03
18	7.00E+02	2.10E+06	4.00E+05	7.60E+03	2.00E+05	9.00E+04	4.00E+03
20	2.00E+01	2.00E+03	5.00E+04	5.00E+02	7.00E+04	5.70E+04	7.00E+02
22	6.00E+01	3.00E+03	7.00E+02	3.80E+02	9.00E+01	3.00E+02	3.00E+02
30	9.00E+00	1.00E+01	3.00E+01	4.00E+01	5.00E+00	5.00E+01	8.00E+01

Table 20 Growth of isolated carbofuran degraders in N- and C and N-limited BSM during incubation

Incubation time (days)	Cell number (cfu/mL)					
	PNL1	PNL2	PNL3	PNL4	PCNL1	PCNL2
0	3.40E+06	6.10E+06	4.50E+06	3.70E+06	3.00E+06	5.40E+06
2	9.00E+06	5.30E+06	1.55E+07	2.50E+07	3.50E+06	6.40E+06
4	5.50E+08	4.60E+08	4.50E+08	5.30E+08	3.60E+06	2.30E+06
6	5.60E+08	4.35E+08	4.68E+09	4.58E+09	7.00E+06	4.50E+06
8	4.20E+09	1.50E+09	4.77E+09	3.61E+09	8.90E+06	6.10E+06
10	4.41E+10	1.43E+09	2.40E+09	4.50E+09	1.55E+07	3.30E+06
12	4.64E+10	2.00E+09	4.70E+09	4.50E+09	4.55E+07	8.87E+07
14	8.43E+08	6.00E+07	5.60E+08	3.30E+08	4.60E+07	4.89E+07
16	4.41E+07	5.00E+06	5.41E+07	3.43E+07	4.33E+07	5.60E+07
18	4.00E+06	7.00E+03	5.00E+05	6.50E+06	2.10E+06	6.78E+07
20	2.00E+05	3.00E+02	3.00E+03	3.00E+03	4.00E+04	7.80E+06
22	6.00E+01	2.00E+01	6.00E+02	5.50E+02	8.00E+02	7.40E+06
30	3.00E+01	9.00E+00	1.00E+01	7.00E+00	2.00E+00	3.00E+05

Table 21 Dissipation of carbofuran in rhizosphere soils of weeds grown in rice field with a history of carbofuran application

Time	Carbofuran concentration (mg/kg dry soil)							
(days)	Bulk soil	R1	R2	R3	R4	R5	R6	autoclaved R3
0	6.37	6.41	6.21	6.21	6.23	6.42	6.53	6.01
7	6.17	5.61	5.11	4.74	5.70	5.57	6.16	5.98
14	5.89	5.51	4.74	3.62	4.92	5.33	5.65	5.77
21	5.59	4.96	3.78	3.04	4.31	3.95	4.20	5.81
35	4.73	3.68	2.94	1.86	3.57	3.48	3.49	5.55
70	3.91	3.57	2.40	0.78	3.13	2.81	3.07	5.32
100	3.28	3.40	2.40	0.63	2.94	2.73	2.63	4.88

Table 22 Carbofuran concentrations in rhizosphere soils of weeds grown in rice field with a history of carbofuran application fitted with the first-order kinetic model

Time	Carbofuran concentration (mg/kg dry soil)							
(days)	Bulk soil	R1	R2	R3	R4	R5	R6	autoclaved R3
0	6.5	6.5	6.3	6.2	6.4	6.6	6.9	6
7	6.1	5.8	5.2	4.8	5.5	5.6	5.9	5.9
14	5.8	5.2	4.4	3.7	4.8	4.8	5.1	5.85
21	5.5	4.7	3.8	2.9	4.4	4.3	4.5	5.76
28	5.2	4.4	3.4	2.3	4	3.9	4.1	5.68
35	4.9	4.1	3.1	1.9	3.7	3.6	3.8	5.6
70	3.9	3.5	2.5	0.8	3.1	2.8	2.9	5.23
100	3.2	3.4	2.3	0.6	2.9	2.7	2.7	4.93

Table 23 Growth of carbofuran degraders in rhizosphere soils of weeds grown in rice field with a history of carbofuran application

Time	Cell number cfu/g dry soil						
(days)	Bulk soil	R1	R2	R3	R4	R5	R6
0	3.60E+02	3.60E+03	4.30E+03	5.40E+03	2.20E+03	1.60E+03	2.30E+03
7	3.30E+02	3.70E+03	4.80E+03	7.10E+03	3.20E+03	3.30E+03	2.10E+03
14	3.50E+02	4.10E+03	2.60E+03	8.90E+03	3.80E+03	3.80E+03	3.10E+03
21	3.50E+02	6.20E+03	6.10E+03	2.00E+04	5.50E+03	5.10E+03	3.30E+03
35	4.20E+02	5.50E+03	7.75E+03	8.20E+04	6.50E+03	6.60E+03	6.40E+03
70	4.10E+02	4.90E+03	6.46E+03	7.30E+04	6.40E+03	7.10E+03	7.12E+03
100	4.50E+02	5.70E+03	5.37E+03	4.70E+04	8.60E+03	6.90E+03	6.70E+03

Table 24 Degradation of carbofuran in the soils with and without isolate PCL3

Time (days)	Carbofuran concentration (mg/kg dry soil)					
	BC	BC+MO	B+MO	RC	RC+MO	R+MO
0	5.30	5.12	5.55	5.46	5.51	5.46
5	5.13	4.80	4.01	5.44	4.66	3.33
10	5.27	3.97	3.29	5.01	3.77	3.01
15	4.90	3.01	2.23	4.80	2.91	2.09
25	4.76	2.33	1.36	5.10	1.96	1.36
35	4.66	1.97	1.02	4.63	1.05	0.33
50	4.53	1.15	0.44	3.91	1.25	0.07

Table 25 Carbofuran concentrations in the soils with and without isolate PCL3 fitted with the first-order kinetic model

Time (days)	Carbofuran concentration (mg/kg dry soil)					
	BC	BC+MO	B+MO	RC	RC+MO	R+MO
0	5.3	5.3	5.5	5.5	5.8	5.2
5	5.2	4.5	4.1	5.3	4.5	3.9
10	5.1	3.9	3.1	5.1	3.6	2.9
15	5	3.3	2.4	4.8	2.9	2.1
25	4.8	2.4	1.4	4.7	1.9	1.1
35	4.7	1.8	0.9	4.6	1.4	0.5
50	4.5	1.2	0.5	4.5	1.1	0.06

Table 26 Growth of carbofuran degraders in the soils inoculated with isolate PCL3

Time (days)	Cell number (cfu/g dry soil)			
	BC+MO	B+MO	RC+MO	R+MO
0	3.60E+05	7.70E+05	6.20E+05	8.50E+05
5	6.50E+05	8.20E+05	5.50E+05	1.53E+06
10	4.85E+06	6.95E+06	6.30E+06	7.60E+06
15	5.93E+07	4.73E+07	4.66E+07	7.66E+07
25	6.68E+07	8.65E+07	6.75E+07	8.14E+07
35	5.13E+07	6.95E+07	3.49E+07	1.30E+08
50	9.20E+06	6.63E+07	5.62E+07	9.85E+07

APPENDIX B

Standard curves of carbofuran, carbofuran phenol and 3-keto carbofuran in acetonitrile and methanol

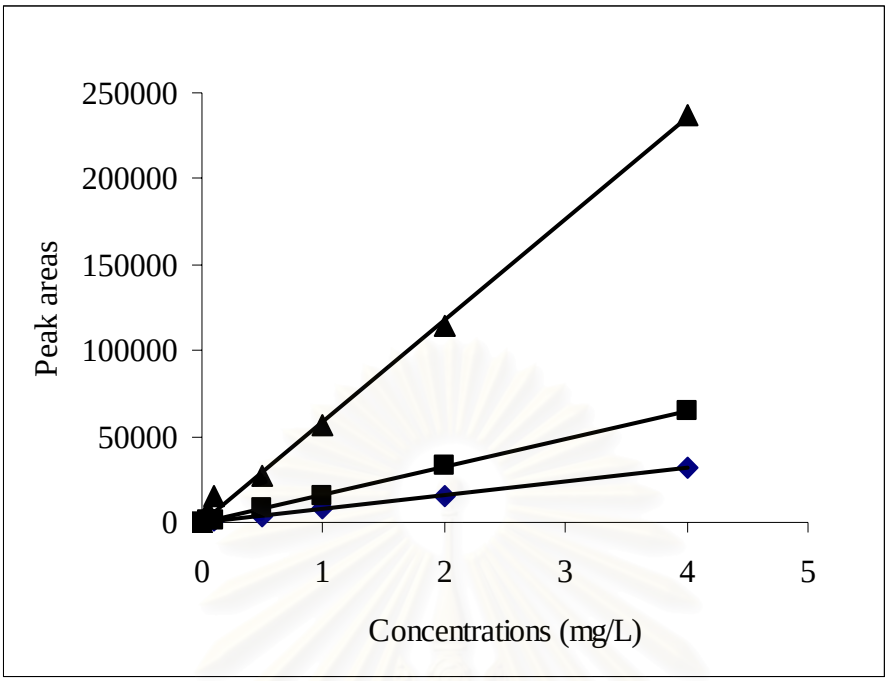


Figure 16 Standard curve of carbofuran (◆), carbofuran phenol (■) and 3-ketocarbofuran (▲) in acetonitrile

Linear equation of carbofuran standard

$y = 7921.1x$ _____(1)

$R^2 = 0.9994$

Linear equation of carbofuran phenol standard

$y = 16315x$ _____(2)

$R^2 = 0.9997$

Linear equation of 3-keto carbofuran standard

$y = 58846x$ _____(3)

$R^2 = 0.9969$

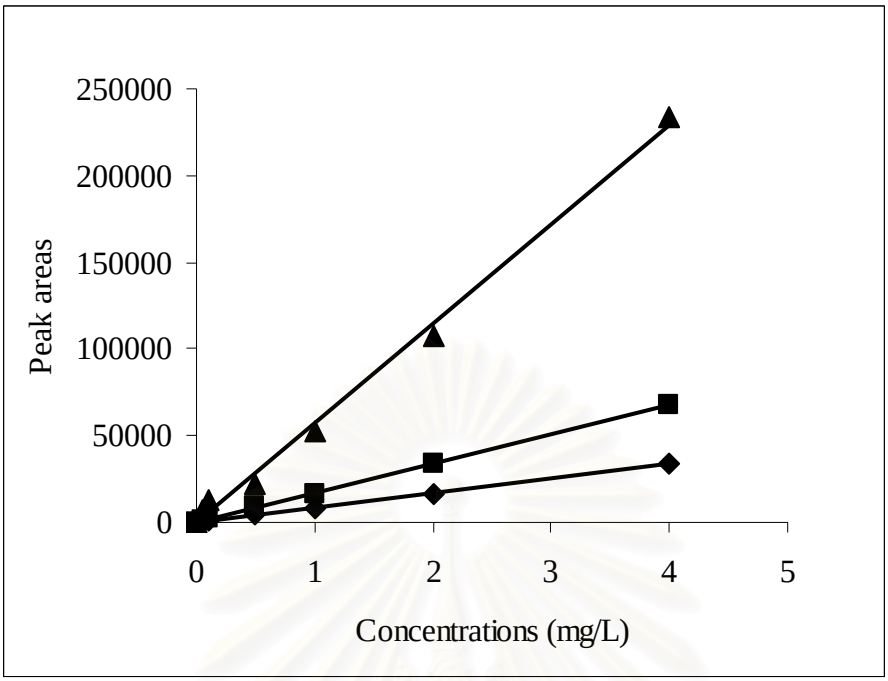


Figure 17 Standard curve of carbofuran (◆), carbofuran phenol (■) and 3-ketocarbofuran (▲) in methanol

Linear equation of carbofuran standard

$$y = 8463.4x \quad (4)$$

$$R^2 = 0.9993$$

Linear equation of carbofuran phenol standard

$$y = 16799x \quad (5)$$

$$R^2 = 0.9997$$

Linear equation of 3-keto carbofuran standard

$$y = 57097x \quad (6)$$

$$R^2 = 0.9952$$

APPENDIX C

Typical high-pressure liquid chromatograms for carbofuran, carbofuran phenol and 3-keto carbofuran

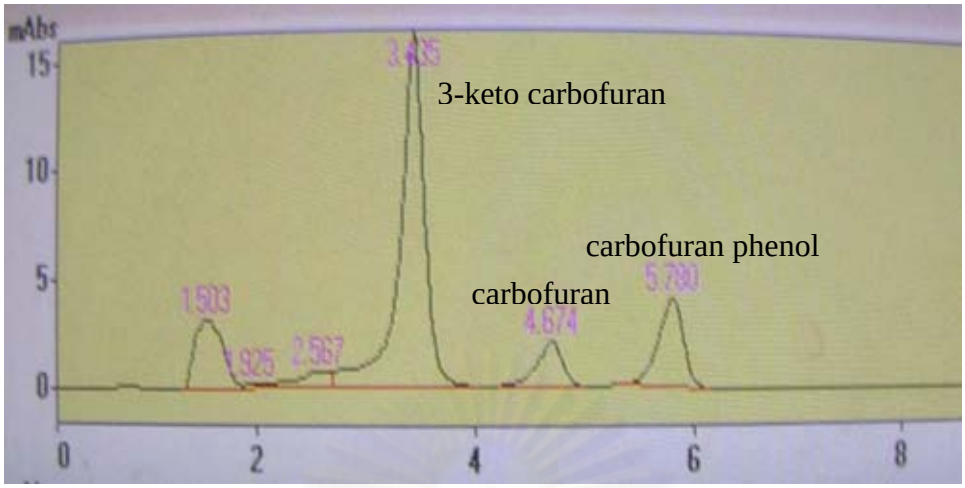


Figure 18 Typical high-pressure liquid chromatogram for standard carbofuran, carbofuran phenol and 3-keto carbofuran

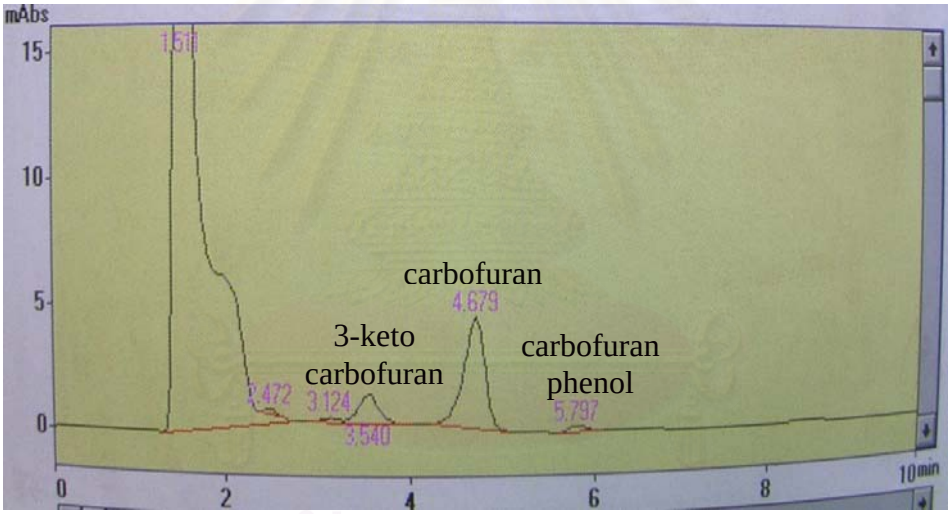


Figure 19 Typical high-pressure liquid chromatogram for carbofuran, carbofuran phenol and 3-keto carbofuran in BSM

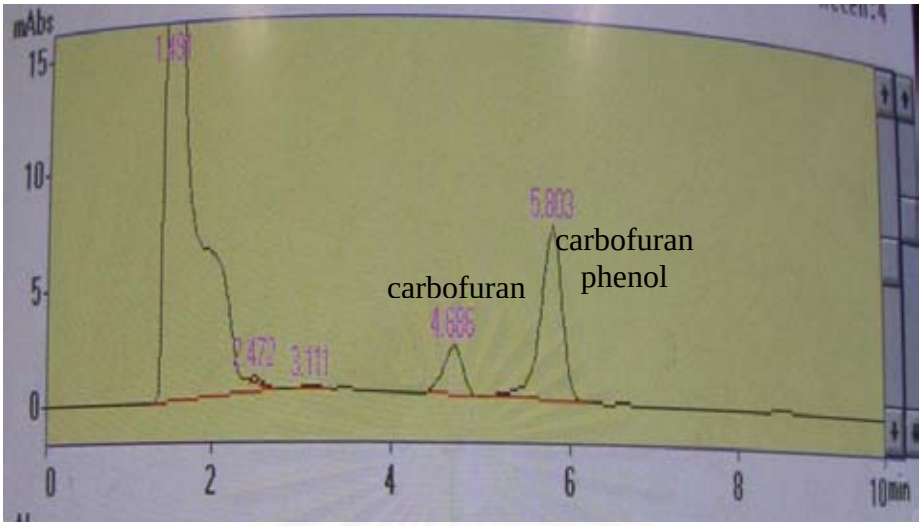
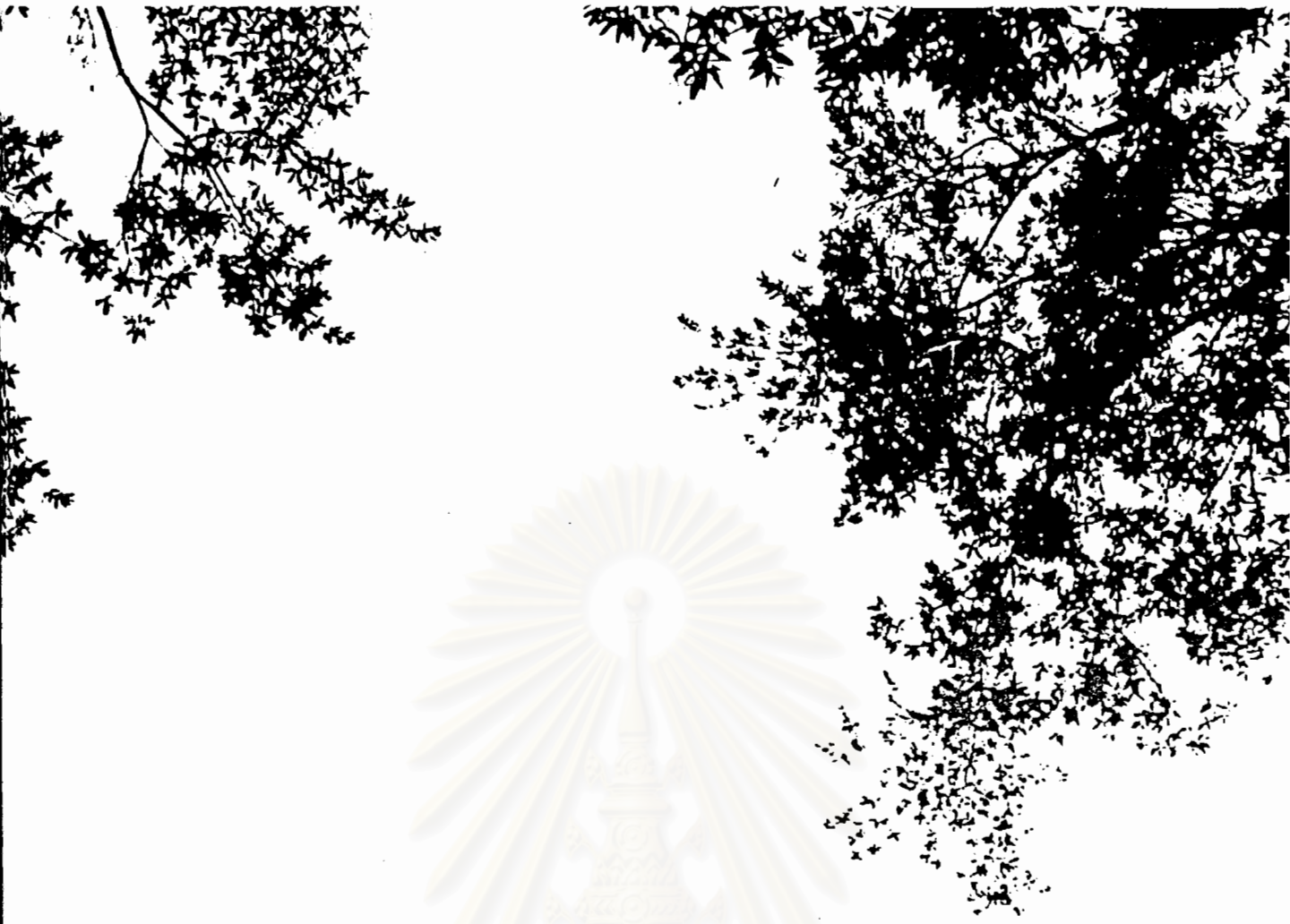


Figure 20 Typical high-pressure liquid chromatogram for carbofuran and carbofuran phenol in soil

APPENDIX D

Isolation of Carbofuran Degraders from Phytoremediated Rhizosphere Soil

A paper published in the proceeding of 4th National Environmental Conference, January 19-21, 2005 at Ambassador City Jomtien Hotel



เอกสารประกอบ

การประชุมวิชาการสิ่งแวดล้อมแห่งชาติ ครั้งที่ 4

19-21 มกราคม 2548 ณ โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน

The Proceeding of 4th National Environmental Conference

JANUARY 19-21, 2005 At Ambassador City Jomtien Hote

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การคัดเลือกจุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูรานจากดินรอบรากพืชที่ใช้ในการฟื้นฟูดินที่ปนเปื้อนคาร์โบฟูราน

Isolation of Carbofuran Degraders from Phytoremediated Rhizosphere Soil

เพ็ญศรี ปลั่งกลาง[1] อลิศรา เรืองแสง[2,4*] และ วันเพ็ญ วิโรจนกูฏ[3,4]

Pensri Plangklang[1] Alissara Reungsang[2,4*] and Wanpen Virojanakud[3,4]

บทคัดย่อ

จุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูรานได้ถูกคัดเลือกจากดินรอบรากพืช 3 ชนิด คือ ข้าว (*Oryza sativa L.*) ข้าวโพด (*Zea mays L.*) และคะน้า (*Brassica alboglabra C.*) โดยคัดเลือกในอาหาร BSM 3 ชนิด ที่มีคาร์โบฟูรานความเข้มข้น 5 มิลลิกรัมต่อลิตร เป็นแหล่งคาร์บอน หรือแหล่งไนโตรเจน หรือเป็นทั้งแหล่งคาร์บอนและแหล่งไนโตรเจน จากนั้นเพิ่มความสามารถของจุลินทรีย์ให้มีความสามารถในการย่อยสลายคาร์โบฟูรานจนถึงความเข้มข้น 100 มิลลิกรัมต่อลิตร ผลการทดลองเบื้องต้นพบว่า ความเข้มข้นของคาร์โบฟูรานลดลงในอาหาร BSM ทั้งสามชนิด ณ วันที่ 7 ของการเลี้ยงเชื้อโดย อยู่ในช่วง 73-98, 25-66, 19-57 และ 8-28 เปอร์เซ็นต์ ที่ความเข้มข้นของคาร์โบฟูรานเริ่มต้นในอาหารเลี้ยงเชื้อเท่ากับ 5, 20, 40 และ 100 มิลลิกรัมต่อลิตร ตามลำดับ การศึกษาลักษณะทางสัญฐานวิทยาของจุลินทรีย์ที่คัดเลือกได้ชนิดแกรมลบรูปร่างแท่งบนอาหาร TSA ที่เคลือบด้วยสารละลายคาร์โบฟูรานความเข้มข้น 5 มิลลิกรัมต่อลิตร โดย API 20 NE system พบว่า แบคทีเรียดังกล่าวเป็นสายพันธุ์ *Burkholderia*, *Pseudomonas* และ *Stenotrophomonas multophilia* โดยจุลินทรีย์เหล่านี้ใช้คาร์โบฟูรานเป็นทั้งแหล่งคาร์บอน แหล่งไนโตรเจน หรือแหล่งคาร์บอนและแหล่งไนโตรเจน

คำสำคัญ : คาร์โบฟูราน; จุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูราน; การคัดเลือก; ดินรอบรากพืช

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Abstract

An enrichment technique was used to isolate carbofuran degraders from phytoremediated rhizosphere soil of rice (*Oryza sativa L.*), corn (*Zea mays L.*) and Chinese kale *(Brassica alboglabra C.)* used in phytoremediation study. Rhizosphere soil from each plants was added to C-, N- and C and N-limited BSM and complete media containing 5 mg/L of carbofuran as a sole C-, N- and C and N-source. The results indicated that carbofuran was dissipated in media. The percentage of dissipation of carbofuran in enriched media was in the range of 73-98%, 25-66%, 19-57% and 8-28% when the initial carbofuran concentrations were 5, 20, 40 and 100 mg/L, respectively. API 20 NE system was used to identify the gram negative-rod single isolates. They were identified as *Burkholderia sp.*, *Pseudomonas sp.* and *Stenotrophomonas maltophilia.* These carbofuran degraders used carbofuran as a sole C-, N- or C and N- sources.

Keywords : Carbofuran; Carbofuran Degraders; Isolation; Rhizosphere Soil

Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad spectrum insecticide used to control rice water weevils. A primary mechanism of carbofuran degradation in soil and water under neutral to basic conditions is chemical hydrolysis resulting in metabolite, carbofuran phenol (1, 2). Sieber et al. (2). reported that as pH and temperature increased the rate of hydrolysis increased. In addition, the dissipation of carbofuran in water also could be influenced by volatilization and photolysis (2, 3). Sunlight and high temperature have increased the rate of carbofuran loss from water siddaramappa and Sieber (4). Siddaramappa and Sieber (4) reported that carbofuran was biologically degraded in soils under flood condition.

Bioremediation is a promising process to remediate the environmental contaminant until its concentration is below detectable limit or less than the maximum contaminant level. Microorganisms are the key to a succeed remediation. In general, bioremediation use indigenous microorganisms in the contaminated area as the degrader. However, there is an interest toward using microorganisms isolated from contaminated media, such as soil or water, as degrader. There were reports on carbofuran degraders isolated from the carbofuran-contaminated soil such as *Pseudomonas sp.* (5) and *Pseudomonas sp.* 50432 (6) which could degrade carbofuran to 4-hydroxycarbofuran. It could also transform carbofuran to 7-phenol by hydrolase. Ambrosoli et al. (7) reported that *Arthrobacter, Pseudomonas, Bacillus, Actinomyces,* were isolated from carbofuran treated soil. These microorganisms used carbofuran as a sole carbon source. A study on persistent of carbofuran and effect of carbofuran on microorganisms in soil from paddy fields by Amal et al. (8) indicated that *Bacillus, Corynebacterium, Aspergillus* and *Phytophthora* could grow in the carbofuran contaminated soil from paddy fields. However, *Pseudomonas, Staphylococcus, Micrococcus, Klebsiella Humicola* and *Rhizopus* were inhibited. They also reported that carbofuran persistence in soil was only 9 days. Dynamic of carbofuran degrader in soil during three annual application of Carbofuran was studied by Trabue et al. (9). The result indicated that the carbofuran hydrolysis in treated surface soil after second application of carbofuran was greater than the first application and degradation of carbofuran in soil was a co-metabolic process. Duquenne et al. (10) studied effect of inoculums size, microbial distribution, and soil nutrient amendment on degradation of carbofuran in soil by bacteria strain C28. The study indicated that increase in inoculums size and equally distribution of C28 applied to soil would increase in

effectiveness of carbofuran degradation. A short lag phase of C28 was found in soil that had low concentration of glucose but growth of C28 could be inhibited by exceeding glucose concentration (10).

Repeated application of carbofuran in soil enhanced the degradation of carbofuran. Study by Read (11) showed that repeated application of carbofuran in acid mineral soil reduced half-life of carbofuran two times. Morel-Chevillet et al. (12) reported that previously treated soil with 15 N-methylcarbamates enhanced degradation rate of carbofuran and increased in the number of carbofuran degrader.

As we can see that carbofuran degrader could be isolated from soils that had repeated application history of carbofuran. However, there is very limited information in isolation of carbofuran degrader from phytoremediated soil. Microorganisms in root zone, or so-called rhizosphere microorganisms, were reported to be effectively used to remediate organic and in organic pollutants e.g. pesticides (13), petroleum hydrocarbon (14), and heavy metal (15, 16). Therefore, in this study we aimed to isolate and identify carbofuran degraders from phytoremediated soil and determine their ability to degrade carbofuran.

Materials and methods

Chemical

Carbofuran (98% purity) was purchased from Sigma-Aldrich, USA.

Microorganism media

Basal Salt Medium (BSM) (in g/L) contained: 5.57 of $NaHPO_4$, 2.44 of KH_2PO_4, 2.00 of NH_4Cl, 0.20 of $MgCl_2.6H_2O$, 0.0004 of $MnCl_2.4H_2O$, 0.001 of $FeCl_3.6H_2O$ and 0.001 of $CaCl_2$. In C-limited BSM, 5 mg/L of carbofuran was used as a sole C-source. In N-limited BSM, 5 mg/L of carbofuran was used as a sole N-source instead of NH_4Cl and 10 g/L of glucose was used as C-source. In C and N-limited BSM, 5 mg/L of carbofuran was used as sole C and N source. Complete media was BSM containing 5 mg/L of carbofuran and 10 g/L of glucose as carbon sources. pH was adjusted to 7 before autoclaved at 121 °C for 15 min. Carbofuran solution was added after the sterilization.

Phytoremediated rhizosphere soil samples

Soils were mixed with 5 mg/kg soil of carbofuran before planting with rice (*Oryza sativa L.*), corn (*Zea mays L.*) and Chinese kale *(Brassica alboglabra C.*). The soils were taken from Ban Non-reung, A. Muang Khon Kean Province at the depth of 0-30 cm and classified as loam. Rhizosphere soils from rice and corn were chosen because half-life of carbofuran in soils under these two plant were short i.e. 2 and 3 days, respectively, (17) suggesting a successful phytoremediation of carbofuran by these plants. Soil under Chinese kale was chosen to compare with soil under rice and corn because of longer half-life of carbofuran in Chinese kale soil than in the soil under rice and corn i.e. 6 days. Rhizosphere soils were taken after day 120 of plantation by shaking out the extra soil around the root zone and then the soil attached to the roots were collected and kept in plastic bag at 4 °C prior to use.

Isolation of carbofuran degraders from phytoremediated rhizosphere soil

An enrichment technique was used to isolate carbofuran degraders from phytoremediated rhizosphere soil. Five grams of rhizosphere soil from each plant was added to 100-ml C-, N- and C and N-limited BSM and complete media containing 5 mg/L of carbofuran. Flasks were incubated at 30 °C and shaken at 150 rpm. Every 7 days, the media were

transferred to a fresh media containing 5 mg/L of carbofuran. This step was repeated until soil-free enrichment was obtained. The carbofuran degradation ability of carbofuran degraders in soil-free enrichment media was enhanced by inoculating 10% of the enriched culture from the last step into fresh C-, N- and C and N-limited BSM and complete media containing 20 mg/L of carbofuran. Flasks were incubated at 30 °C and shaken at 150 rpm. At day 0 and 7, the media were sampled to check for carbofuran concentration by HPLC (18). At day 7, the culture were transferred to a fresh C-, N- and C and N-limited BSM and complete media containing 40 mg/L of carbofuran incubated at 30 °C, shaken at 150 rpm and sampled at day 0 and 7 to check for carbofuran concentration. This process was repeated once again but the concentration of carbofuran in the media was 100 mg/L. After the enhancement at 100 mg/L of carbofuran, the culture was streaked in Tryptic Soy Agar (TSA) coated with 5 mg/L of carbofuran to obtain single colony. The colonies were then identified using the API 20 NE system (bioMérieux sa, France).

Extraction of carbofuran residues from culture media.

Three ml of each culture media was taken and added with 3 ml of methanol and then sonicated for 10 min, 50/60 voltage cycle, for two times. Liquid-liquid partitioning of sonicated culture to extract carbofuran from media was done by extraction three times with 6, 3 and 3 ml of dichloromethane, respectively, in separation funnel. The organic fraction of the samples was evaporated to dryness in the fume hood and then redissolved in 6 ml of acetonitrile and then analyzed by HPLC (18). Percent recovery of this extraction technique was 98%.

Results and Discussions

Enrichment technique was used to isolate carbofuran degraders from phytoremediated rhizosphere soil of rice corn and Chinese kale. As carbofuran concentration increased, percentage of dissipation of carbofuran decreased (Table 1). We speculated that carbofuran at high concentration might be toxic to the enriched culture. The OD of the enriched culture should be examined to explain this result.

Enriched culture from rhizosphere soils of rice corn and Chinese kale showed a similar trend on degrading carbofuran (Table 1). We noticed that the enriched culture from Chinese kale rhizosphere soil also rapidly decreased carbofuran (Table 1). This was contrast from the finding of Teerakun et al. (17). They reported that carbofuran half-life in soil planted with rice and corn were faster than Chinese kale. It could be explained that processes of phytoremediation including not only phytodegradation but also phytoextraction, phytoaccumulation, phytostabilization and rhizofiltration (19) Therefore rhizosphere microorganisms in Chinese kale soil might not play a significant role in degradation of carbofuran, but the other processes did.

Carbofuran dissipation in C-, N- and C and N-limited BSM indicated that the enriched culture used carbofuran as sole C-, N- or C and N-sources, respectively. Ambrosoli et al. (7) reported that *Arthrobacter, Pseudomonas, Bacillus, Actinomyces* isolated from carbofuran treated soil used carbofuran as a sole C-source. Percentage of dissipation of carbofuran in complete media was low compared to in the other media. This may due to the fact that glucose (C-source) and $NHCl_4$ (N-source) in complete media were more accessible than carbofuran. Duquenne et al. (10) reported that in the presence of glucose, carbofuran degradation rate markedly reduced in soil sample inoculated with strain C28.

Table 1 Dissipation of carbofuran (%) by enriched culture from phytoremediated rhizosphere soils of rice, corn, and Chinese kale at day 7 of incubation.

Initial carbofuran concentration (mg/L)	Carbofuran dissipation (%)[a]											
	C-limited BSM			N-limited BSM			C&N-limited BSM			Complete media		
	rice	corn	Chinese kale	rice	corn	Chinese kale	rice	corn	Chinese kale	rice	corn	Chinese kale
5	98.6	90.4	92.6	91.8	88.0	92.6	87.4	73.9	87.1	9.6	31.0	34.0
20	65.9	55.9	58.8	50.9	41.1	43.3	33.6	24.5	25.8	6.5	14.1	11.5
40	52.3	51.4	57.1	45.5	25.8	32.3	24.4	19.7	27.4	12.0	27.5	11.2
100	20.8	14.5	20.3	20.9	16.2	28.4	15.3	8.8	16.6	6.5	11.5	8.4

[a] Carbofuran dissipation was calculated by $\frac{(\text{conc. at day 0} - \text{conc. at day 7}) \times 100}{\text{conc. at day 0}}$

After the acclimation of the enriched culture with 100 mg/L of carbofuran in all kind of media, the colonies were isolated on TSA agar coated with 5 mg/L of carbofuran and then gram stained. The gram negative-rod strains were then identified using API 20 NE system. Percentage of identification by API 20 NE system greater than 80% was acceptable. Figure 1 depicted the morphology and Table 2 reported the strain of the isolates. The carbofuran degradation by *Pseudomonas sp.* has been reported in the study of Ambrosoli et al. (7), Felsot et al., (5) and Chaudhry et al., (6).

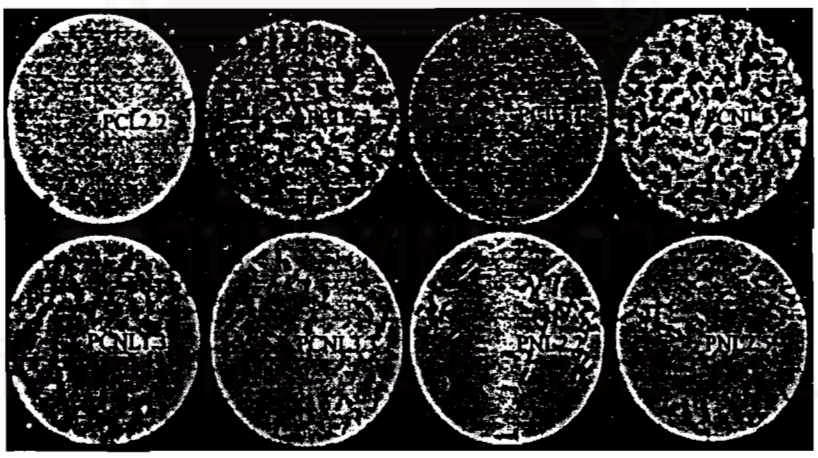

Figure 1 Morphology of carbofuran degraders isolated from phytoremediated soils

Table 2 Morphology and strains of the isolates identified by using API 20 NE system

Isolate Names	Morphology	Strains
PCL2.2	cream, circular , entire, convex, smooth, opalescent, diameter of 1 mm	*Burkholderia sp.*
PCL2.3	cream, circular , entire, convex, smooth, opalescent, punctiform	*Burkholderia sp.*
PCL3.1	white, lenticular , entire, convex, smooth, opalescent, diameter of 0.5-1 mm	*Burkholderia sp.*
PNL2.2	white, circular , entire, convex, smooth, opalescent, rubbery, diameter of 0.5 mm	*Stenotrophomonas maltophilia*
PNL2.5	white, circular , entire, convex, smooth, opalescent, rubbery, diameter of 1 mm	*Burkholderia sp.*
PCNL1.1	cream, circular , entire, convex, smooth, opalescent, diameter of 1-2 mm	*Pseudomonas sp.*
PCNL1.3	cream, circular , entire, convex, smooth, opaque, punctiform	*Burkholderia sp.*
PCNL3.3	white, circular , entire, convex, smooth, opalescent, punctiform	*Burkholderia sp.*

Conclusion

Isolation of carbofuran degraders from phytoremediated rhizosphere soils i.e., rice corn and Chinese kale was a success. These enriched cultures were effectively degrade carbofuran at low concentration, 5 mg/L. A high concentration of carbofuran, 100, mg/L, was toxic to the enriched cultures. The OD of the culture should be examined to explain these findings. Most of single colonies, gram negative-rod, were identified as *Burkholderia sp.*, *Pseudomonas sp.* and *Stenotrophomonas maltophilia.*

Further studies and recommendation

The ability of each isolate to degrade carbofuran will be examined. The best carbofuran degraders will be used in bioaugmentation study.

Acknowledgments

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References

[1] Getzin, L.W., 1973. **Persistence and degradation of carbofuran in soil.** Environ. Entomology, 2, 461-467.

[2] Seiber, J.N., Catahan, M.P. and Barril, C.R., 1978. **Loss of carbofuran from rice paddy water: chemical and physical factors.** J. of Environ. Sci. Health, B13, 131-148.

[3] Deuel, L.E. JR., Price, J.D., Turner, F.T. et al., 1979. **Persistence of carbofuran and its metabolites, 3-keto and 3-hydroxy carbofuran, under flooded rice culture.** J Environ Qual, 8(1), 23-26.

[4] Siddaramappa, R., Tirol, A.C., Seiber, J.N. et al., 1978. **The degradation of carbofuran in paddy water and flooded soil of untreated and retreated rice fields.** J. Environ. Sci. Health, B 13, 369-380.

[5] Felsot, A.S., Maddox, J.V., and Bruce, W., 1981. **Enhanced microbial degradation of carbofuran in soils with histories of furadan use.** Bull. Environ. Contam. Toxicol, 26, 781-788.

[6] Chaudhry, G. R., Mateen, A., Kaskar, B. et al., 2002. **Induction of carbofuran oxidation to 4-hydroxycarbofuranby *Pseudomonassp.* 50432.**, FEMS-Microbiol. Letters, 214, 171-176.

[7] Ambrosoli, R., Negre, M. and Gennari, M., 1996. **Indication of the occurrence of enhanced biodegradation of carbofuran in some Italian soil.** Soil Biol. and Biochem.,. 28(12), 1749-1752.

[8] Amal C.D., 2003. Influence **and persistence of phorate and carbofuran insecticides on microorganisms in rice field.** Chemosphere, 53, 1033-1037.

[9] Trabue, S.L., Ogram A.V. and Ou, L.-T., 2001. **Dynamics of carbofuran-degrading microbial communities in soil during three successive annual application of carbofuran.** Soil Biol. and Biochem., 33, 75-81.

[10] Duquenne, P., Parekh, R.N., Catroux G. et al., 1996. **Effect of inoculant density, formulation, dispersion and soil nutrient amendment on the removal of carbofuran residues from contaminated soil.** Soil Biol. and Biochem., 28(12), 1805-1811.

[11] Read, D.C., 1983. **Enhance microbial degradation of carbofuran and fensulfothion after repeated application to acid mineral soil.** Agr. Ecosystem Environ., 10, 37-46.

[12] Morel-Chevillet, C., Parekh, N.R., Pautrel, D. et al., 1996. **Cross-enhancement of carbofuran biodegradation in soil Sample previously treated with carbamate pesticides.** Soil Biol. and Biochem., 28(12), 1767-1776.

[13] Camper, N.D., Costa, R. and S. Pai. 1999. **Pesticide Interactions in a containerized rhizosphere system. Bull.** SC Acad. Sci., LXI, 71.

[14] Frick, C.M., Farrell, R.E., and Germida, J.J., 1999. **Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites.** Petroleum Technology Alliance of Canada. Calgary, Canada.

[15] Mulligan, C.N., Yong, R.N. and Gibbs, B.F., 2001. **Remediation technologies for metal-contaminated soils and groundwater: an evaluation.** Engr. Geol., 60, 193-207.

[16] Mejáre, M. and Bülow, L., 2001. **Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals.** Trends in Biotechnol, 19, 67-73.

[17] Teerakun, M., 2004. **Phytoremediation of carbofuran residues in soil.** Thesis (Ms.c.), Department of Biotechnology, The Faculty Technology, Khon Kean University.

[18] Teerakun, M., Reungsang, A., and Virojanakud, W., 2004. **Phytoremediation of carbofuran in soil.** Songklanakarin J. Sci. Technol., 26(Suppl. 1), 171-176.

[19] Schnoor, J.L., 1997. **Phytoremediation.** Technology Evaluation Report, The Ground-Water Remediation Technologies Analysis Center (GWRTAC), USA. http://www.gwrtac.org/pdf/phyto_e.pdf [30 June 2004].

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ทำเนียบวิทยากร

ชื่อบทความ	การคัดเลือกจุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูรานจากดินรอบรากพืชที่ใช้ในการฟื้นฟูดินที่ปนเปื้อนคาร์โบฟูราน Isolation of Carbofuran Degraders from Phytoremediated Rhizosphere Soil
ผู้นำเสนอบทความ	นางสาวเพ็ญศรี ปลั่งกลาง
สถานที่ทำงาน	นักศึกษา ภาควิชาเทคโนโลยีชีวภาพ คณะเทคโนโลยี มหาวิทยาลัยขอนแก่น ตำบลในเมือง อำเภอเมือง ขอนแก่น 40002
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ประวัติการศึกษา	- พ.ศ. 2546-ปัจจุบัน นิสิตปริญญาโท หลักสูตรการจัดการสิ่งแวดล้อม (สหสาขา / นานาชาติ) จุฬาลงกรณ์มหาวิทยาลัย - พ.ศ. 2542-2545 วิทยาศาสตรบัณฑิต (เทคโนโลยีชีวภาพ) มหาวิทยาลัยขอนแก่น
ตำแหน่งหน้าที่ปัจจุบัน	นิสิตปริญญาโท หลักสูตรการจัดการสิ่งแวดล้อม (สหสาขา / นานาชาติ) จุฬาลงกรณ์มหาวิทยาลัย
ประวัติการทำงาน	-
ประสบการณ์	-
ผลงาน	บทความเรื่องการศึกษาความเป็นไปได้ในการใช้กากตะกอนจุลินทรีย์จากอุตสาหกรรมผลิตวุ้นเส้น เป็นอาหารปลา (เอกสารประกอบการประชุมวิชาการ เทคโนโลยีสิ่งแวดล้อม ประจำปี ครั้งที่ 15, 24 – 25 กรกฎาคม 2546)

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